وزارة التعليم العالي والبحث العلمي
جامعة بابل / كلية الطب
فرع الاحياء المجهرية الطبية

توصيف التنميط الجيني لالتهاب الكبد الفيروسي نوع ب وانتشاره لدى مرضى كورونا والعدوى المصاحبة لالتهاب الكبد الفيروسي في العراق

رسالة مقدمة إلى
مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في
العلوم الاحياء المجهرية الطبية

من قبل

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1443هـ

2022م

Ministry of Higher Education and Scientific Research
University of Babylon / College of Medicine
Department of Medical Microbiology

Hepatitis B Virus Genotyping Characterization and Distribution in Patients with SARS-COV-2 and Viral Hepatitis Co-infections in Iraq

A Thesis

Submitted to The Council of The College of Medicine University of Babylon in Partial Fulfillment of The Requirements for The Degree of Doctor of Philosophy in Medical Microbiology

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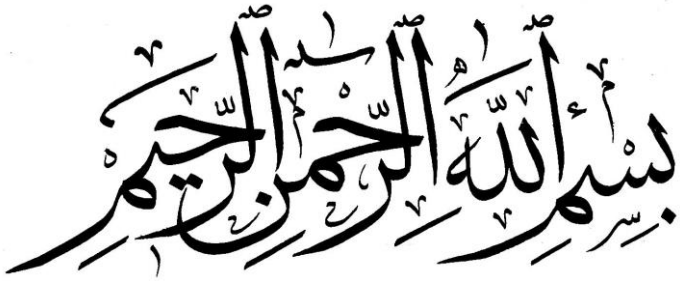

﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا ۝ ﴾

صدق الله العليُّ العظيم

سورة الأسراء (الآية 85)

Dedication

To my support and the pure spring that will continue to narrate my veins... To my tree that does not wither... To the shadow in which I shelter at all times**... My father**

To the wellspring of tenderness and the water of life... To the symbol of love and the healing balm... To God's paradise on earth... To the secret of my success with her prayers**... My mother.**

To the pillars of love and heart beats **... My brothers and My sister.**

Who helped and helped and was intended God's face ...

Give the fruit of my efforts this

I dedicate this work

Laith A.I.K. Al-Kaif

2022

Acknowledgments

Praise be to **Almighty God**, prey, and peace be upon his **Prophet Mohammed** and **his Household** for helping me complete this work .

I would like to express my deep appreciation and my thanks and gratitude to my distinguished supervisors **Prof. Dr. Mohammed A.K. Al-Saadi** and **Prof. Dr. Alaa H.H. Al-Charrakh** for their proposal, invaluable scientific guidance, assistance, cooperation and support through the executing of this work, asking God Almighty to preserve them as an asset for science and its students.

I am extremely thankful to the dean of College of Medicine / Babylon University **Prof. Dr. Safaa S. Naji's** and chairman of the department of microbiology **Prof. Dr. Hayam K. A. Al-masoudi** for their cooperation and notification during the work.

It is pleasure to thank the lab staff in Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City, Center of artificial Kidney, and Center of Hepatology and Gastroenterology Hospital in Marjan Medical City / Babylon for helping me to use the laboratory and to get the study samples.

I would like to thank the volunteered patients who supplied me with samples of the present study.

My special thanks go to the Director of Laboratories of Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City, **Dr. Safaa A. A. Alwaysi**, analytics technician **Alaa Mohammed** and **Dargham** for helping me provide samples and some requirements of the work.

I would like to express my deep appreciation to **Dr. Raheem Tohma Al-Moamory** for his statistical analysis support and some study requirement.

Also, Acknowledgements and gratitudes go to **Prof. Dr. Younis A. Al-Khafaji** and **Dr. Hussain Al-Ameri** for cooperation in providing some requirements and references to complete this study.

I wish to express my thanks and deep gratitude to my best friend Alaa, Zena, Tasahil, Noor, Sarah, Shahad, and Israa for their scientific advices and support during the research period.

Finally, for those I missed mentioning their names, I apologize, thank, and appreciate them all.

Laith A.I.K. Al-Kaif

2022

List of Abbreviations

Abbreviation	Full Name
µl	Micro liter
Ab	Antibody
ADV	Adenovirus
AFIAS	Automated Fluorescent Immunoassay System
Ag	Antigen
AHA	Acute hepatitis-A infection
AHB	Acute hepatitis-B infection
AHC	Acute hepatitis-C infection
AKI	Acute kidney injury
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
Anti-HAV	Antibody to hepatitis-A virus
Anti-HBc	Antibody to hepatitis-B core antigen.
Anti-HBe	Antibody to hepatitis-B e antigen.
Anti-HBs	Antibodies to hepatitis-B surface antigen
Anti-HCV	Antibody to hepatitis –C virus
Anti-HDV	Antibody to hepatitis-D virus
AST	Aspartate aminotransferase
AVH	Acute viral hepatitis
CBC	Complete blood count
cccDNA	covalently closed circular DNA
CD^{+4}	Cluster of Differentiation 4
CD^{+8}	Cluster of Differentiation 8
CD81	Cluster of Differentiation 81
CDC	Centers for Disease Control and Prevention
CHB	chronic hepatitis B
CLDN1	claudin family class 1
CMV	Cytomegalovirus
COVID-19	Coronavirus Disease 2019
CTL	Cytotoxic T Lymphocytes

DHBV	Duck hepatitis B virus
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
EMR	Eastern Mediterranean Region
ETV	Entecavir
GBV-C/HGV	human pegivirus HPgV
HAV	Hepatitis A virus
HBc Ab	Hepatitis B core antibody
HBe Ab	Hepatitis B envelope antibody
HBe Ag	Hepatitis B envelope antigen
HBs Ag	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HSV-1	Human herpes virus-1
IFN-γ	Interferon–gamma
IG	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
INR	International Normalized Ratio
IU	International unit
IVDU	Intravenous drug use
KDa	Kilo Dalton
Kg	Kilogram

mg	Milligram
ml	Milliliter
MOH	Ministry of Health
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NCR	Non-coding regions
NCs	Negative control of the sample
NK	Natural Killer cell
NS	Nonstructural
NS region	Non Structural region
NTR	Non translated region
O.D	Optical Density
^{o}C	Degrees Celsius
ORF	Open Reading Frame
PCR	Polymerase chain reaction
PWIDs	Persons Who Inject Drugs
rcDNA	relaxed circular DNA
RNA	Ribonucleic acid
RT	Reverse transcriptase
S genes	Surface genes
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SR-BI	Scavenger receptor class B type I
ssRNA	Single stranded Ribo Nucleic Acid
TMB	Tetra methyl benzidine
Treg	Regulatory T cell
TTV	Torque teno virus
TDF	Tenofovir Disoproxil Fumarate
VL	Viral load
VZV	Varicella-zoster virus
WHO	World Health Organization
WHV	Woodchuck hepatitis virus

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1: Introduction and Literatures Review

1.1: Introduction

Hepatitis is a worldwide health problem that causes liver damage. Hepatitis is triggered mainly through viral infections, such as 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 causes for heterogeneity in the pattern and clinical result of HBV infection (Abdul Amir, 2018).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has had wide 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).

Concomitant 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).

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 pegivirus 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-infections prevalence, viral interactions, and clinical significance are not fully elucidated (McArdle *et al.*, 2018; Al-Sadeq *et al.*, 2019), particularly in developing countries, including Iraq.

The aim of this study is to investigate the influence of SARS-CoV-2 on the reactivation of Hepatitis B infection and on other types of viral hepatitis infections, and to study the influence HBV genetic mutations on the sensitivity of SARS-CoV-2 infection.

This aim was achieved using the following objectives:

1. Study samples of individuals with confirmed HBV infections.

2. Investigation of the positivity status of viral hepatitis B in all studied groups which included:

a) **Biochemical markers:** which are Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and albumin.
b) **Hematological parameters:** a complete blood count (CBC), coagulation factors (INR), and blood groups (ABO and Rh).

c) Serological diagnosis:

A. Detection of HDV IgG in sera of HBV patients by ELISA.

B. Measurement of IgM and IgG specific to SARS-COV-2 levels in HBV patients by AFIAS.

C. Evaluation of the reactivity of HBV infection by measuring of HBs Ag, Anti-HBc IgM, HBe Ag, and HBe Ab in sera of HBV patients by ELISA.

d) Genetic analysis for viral hepatitis:

A. HBV infection by Genetic analysis

B. Viral load of HBV

C. Viral load of HCV

D. Genotyping of Hepatitis B virus infections

E. Investigation of HBV genetic sequences by analysis of S genes of selected patients for the HBV alone and HBV with SARS-CoV-2.

3. Comparison between HBV patients infected and non-infected with SARS-COV-2.

1.2: Literature Review

1.2.1: Viral hepatitis

Currently, viral hepatitis is a significant health problem worldwide, particularly in Asian countries. Different hepatic viruses cause viral hepatitis and lead to liver-related morbidity (Jefferies *et al.,* 2018). Numerous viruses are known to cause liver inflammation, including but not limited to Epstein-Barr virus, Herpes simplex virus, and cytomegalovirus. However, the hepatotropic viruses, termed A to E, are the most common culprits. Most of the hepatotropic viruses are acute and self-limiting, although forms B, C, and E can become chronic (Zarrin and Akhondi, 2021).

Viral hepatitis is generally reserved for the hepatic infection caused by a small hepatotropic group of the virus. It has been identified to have the liver as its primary target and produce hepatic disease as its main clinical manifestation. The incidence of viral hepatitis varies according to geographical areas and immunization (Abdul-Husin, 2013).

1.2.1.1: Historical Previous for viral hepatitis

The history of viral hepatitis is intriguing and spans thousands of years. When such organisms initially infected humans, a repeated natural cycle began with the ability to infect billions of people, resulting in population extinction; there are tales of jaundice epidemics in China 5,000 years ago and Babylon more than 2,500 years ago. Great jaundice epidemics and pandemics have a long and dreadful history, and they are frequently linked to major wars (Fonseca and Ferrazda, 2010).

Hepatitis was communicable due to outbreaks often occurring in overcrowded and unclean conditions, and it was a significant problem throughout World War I. Furthermore, serum hepatitis was a major problem during World War II, when large numbers of wounded combatants were infected by pooled plasma administered to save lives

threatened by blood loss, and after transfusion of blood or plasma was thought to be transmitted by feces-contaminated water in the 1950s, serum hepatitis had to be distinguished from the more general worldwide infectious hepatitis thought to be transmitted by feces-contaminated water (Feinstone, 2019).

Hepatitis D Virus was discovered in the mid-1970s when a novel nuclear antigen was found in patients with a severe type of chronic hepatitis B. The first report of the delta antigen was published in 1977, and the official designation of hepatitis delta virus was given to the virus in 1983. Even though 'delta' is still used, HDV is now favored. After cloning and sequencing the virus's genome in 1986, it confirmed the virus's uniqueness. After that, HDV was given "the delta virus" as its genus (Pascarella and Negro, 2011; Botelho-Souza *et al .,* 2017).

In the mid-1970s, the world's blood supply was contaminated with an unidentified agent causing post-transfusion non-A, non-B hepatitis. Yet, it was not until 1989 that the first sequences of hepatitis C virus were reported. Hepatitis E was first recognized during an epidemic of hepatitis in Kashmir Valley in 1978. The epidemic involved an estimated 52,000 cases of hepatitis, with 1700 deaths (Khuroo, 2011; Webb and Dalton, 2019).

1.2.1.2: Types of viral hepatitis

Hepatitis, a general term for liver inflammation, can be caused by various infectious (i.e., viral, bacterial, fungal, and parasitic organisms) and non-infectious (e.g., alcohol, drugs, autoimmune diseases, and metabolic diseases).

1.2.1.2.1: Hepatitis by viral infectious

A. Hepatitis A virus (HAV)

Feinstone detected a spherical 27-nanometer particle in the feces of hepatitis A patients using immunological electron microscopy in

1973. Hepatitis A virus (HAV), a member of the picornavirus family, is an RNA virus that causes 1.4 million cases worldwide, with an estimated 7134 deaths in 2016; over half of these cases were recorded in Asia. In 2006, the annual incidence rate in the United States was reported to be 2 cases per 100000 individuals. However, in recent disease outbreaks, infections increased by 294 percent between 2016 and 2018, compared to 2013-2015. (Foster *et al.*, 2019; Castaneda *et al.*, 2021).

Hepatitis A Virus is spread through the fecal-oral pathway, including contaminated food or drinks consumption and person-to-person contact. Blood donors are subjected to polymerase chain reaction testing since transmission through blood transfusion has been observed on rare occasions (Manka *et al.*, 2016). After the virus passes through the mucosa of the small intestine wall, the virus enters the liver via the portal vein. The virus particles then reproduce and are released into the biliary canaliculi. Finally, they go back to the small intestine via the bile ducts, which are re-excreted in the feces. The HAV enterohepatic cycle will continue until the body responds with a suitable immune response in the form of antibodies. CD8+ T lymphocytes and natural killer cells with human leukocyte antigen-restricted, HAV-specific CD8+ T lymphocytes and natural killer cells have been implicated in the damage and death caused by HAV(Castaneda *et al.*, 2021).

The usual HAV incubation period is about 2-4 weeks. Fever, malaise, jaundice have been described as the most common presenting symptoms for HAV infection. Other common symptoms include weakness, fatigue, nausea, vomiting, abdominal pain, arthralgias, myalgias, diarrhea, and anorexia. Patients rarely enter a prolonged cholestatic phase through recovery, while relapsing infections have also been described (Richardson *et al.*, 2001; ECDC, 2020).

Can find Antibodies to the HAV virus (anti-HAV) in the blood. The presence of immunoglobulin (Ig)M anti-HAV confirms the diagnosis. The antibodies can be discovered when the symptoms first appear. Serum IgM levels peak during the acute infection and stay positive for up to 4 months after symptoms appear. HAV total antibody is mainly used to assess whether a person has been exposed to HAV naturally or due to vaccination. Without any clinical signs, IgM antibodies indicate a previous HAV infection with persistent antibodies, silent disease, or a false positive test. A biopsy or imaging investigations of the liver aren't required for a diagnosis. A liver biopsy, if conducted, may reveal severe portal inflammation with typically a lesser degree of necrosis, Kupffer cell proliferation, acidophil bodies, or ballooning when compared to non-HAV viral hepatitis (CDC, 2005; Castaneda *et al.*, 2021).

B. Hepatitis B virus (HBV)

It will be explained in detail in this study according to 1.2.2

C. Hepatitis C virus (HCV)

Hepatitis C virus belongs to the Flaviviridae family but solely to the Hepacivirus genus. Flaviviridae have single-stranded and positive-sense RNA genomes. Harvey Alter first identified HCV in 1978 and named it non-A, non-B hepatitis. HCV, like other Flaviviridae, has an encapsulated nucleocapsid that assembles intracellularly in close collaboration with membranes produced from the infected hepatocyte's endoplasmic reticular Likewise, HCV particles have a genetic material (RNA) core in an icosahedral protective protein shell, which is further encased in a lipid (fatty) envelope of biological origin. The lipid envelope contains two viral envelope glycoproteins, E1 and E2. (Cao *et al.*, 2019; Laugi, 2020).

Core protein, Envelope glycoprotein, and P7 protein are the structural proteins. The core protein is an essential protein that builds up the viral nucleocapsid and is implicated in hepatocarcinogenesis and steatosis hepatitis directly or indirectly. Furthermore, the HCV core protein interacts with various cellular proteins and impacts host cell activities such as gene transcription, lipid metabolism, apoptosis, and multiple signaling pathways (Mahmoud *et al*., 2019).

Hepatitis C Virus is typically transmitted through contact with infected blood. Blood transfusions before 1992, intravenous drug use (IVDU), high-risk sexual activity, solid organ transplantation from an infected donor, occupational exposure, hemodialysis, home exposure, birth to an infected mother, and intranasal cocaine use are all risk factors for transmission (Tsai, 2021).

There are seven major genotypes and seven-sixty subtypes. HCV genotype 1 is the most common globally, accounting for almost half of all HCV infections. HCV genotype 3 is the second most common, accounting for roughly one-third of all HCV infections; it is more prevalent in South Asia, Australia, and some European nations. Genotype 2 is predominant in Asia and West Africa, while genotype 4 is found in Sub-Saharan Africa's central and eastern regions, North Africa, and the Middle East. Genotypes 2 and 4 account for roughly 9% to 13% of all HCV cases. South Africa, Southeast Asia, and the Democratic Republic of Congo are the only places where genotypes 5, 6, and 7 are found, respectively (Gower *et al.,* 2014; Castaneda *et al.,* 2021).

Hepatitis C Virus entrance is the first step in the virus's interaction with the target cell, essential for infection to begin. However, previous research suggests that HCV infection is a multistep, delayed process. Several host cell surface molecules have been discovered as possible HCV receptors or co-receptors, including glycosaminoglycans, CD81,

scavenger receptor class B type I (SR-BI), members of the claudin family (CLDN1, 6, and 9), and several mannose-binding lectins proteins (Irshad *et al.*, 2013).

This interaction is probably mediated by the lipoproteins associated with HCV virions. However, one cannot exclude direct contact between HCV envelope proteins and these cellular proteins. After the initial binding step, the particle likely interacts with SR-BI and CD81. HCV E2 binds with high affinity to the large external loop of CD81, and CLDN1 acts at a late stage of the entry process (Ashfaq *et al.*, 2011).

These receptors have been shown to play an essential role in viral entry. However, despite expressing all known; entry factors, several human cell lines remain non-permissive the entrance of HCV. This finding suggests the requirement of some additional cellular factors that mediate the entry of the virus. Several host restriction factors that protect cells from viral infection have been identified in recent years, such as EW1-2wint. The EW1-2wint is a CD81 associated protein that can inhibit HCV entry into target cells by blocking the interactions between HCV glycoproteins and CD81. It may interfere with activating polymerization during viral entry or block signaling pathways necessary for it (Mohammed, 2018).

The RNA is released into the cytoplasm once inside the cell. The polyprotein translation begins at the internal ribosome entry site, then processed to the preceding ten independent proteins connected with intracellular membranes. HCV RNA replication occurs in the multi-protein complex's area, where NS5B is necessary. In addition, the NS3 NTPase/helicase is required for unwinding stable RNA structures and so enabling RNA synthesis. The HCV RNA is then replicated to minus-stranded RNA, resulting in the production of additional plus-stranded

RNA. The freshly generated plus-stranded RNA can make additional minus-strands or package into viral particles. The virus particles are then transported out from the cell via the Golgi apparatus (Hu *et al.*, 2019; Niepmann and Gerresheim, 2020; Yu *et al.*, 2021).

The diagnosis of HCV infection relies on laboratory tests which include:

1. Anti-HCV, antibody detection assays, relying on third-generation enzyme-linked immunosorbent assays (ELISAs) whose sensitivity and specificity have been demonstrated
2. Viral genome detection assays rely on real-time HCV infection PCR (Gupta *et al.*,2014).

D. Hepatitis D virus (HDV)

Hepatitis delta virus (HDV) is a unique human pathogen and has been the only known species in the genus Deltavirus (Magnius *et al.*, 2018), but was reclassified in a new family Kolmioviridae, genus Deltavirus within one new realm, Ribozyviria (International Committee on Taxonomy of Viruses, 2020). Due to possessing a circular RNA genome and its replication mechanism, similarities exist with viroids, which represent a large family of subviral plant pathogens (Flores et al., 2016; Adkar-Purushothama and Perreault, 2019). But HDV is clearly distinguished from the viroids by its larger genome size and the ability to encode a protein. Furthermore, the recent discovery of delta-like agents in various animal species has broadened the views on the evolutionary history of HDV (Wille *et al.*, 2018; Chang *et al.*, 2019; Hetzel *et al.*, 2019; Paraskevopoulou *et al.*, 2020; Bergner *et al.*, 2021; Iwamoto *et al.*, 2021).

In 1977, Mario Rizzetto and colleagues discovered a novel antigen in the livers of HBV patients. The small HDAg (S-HDAg, 195

amino acids, 24 kDa) and the large HDAg (L-HDAg, 214 amino acids, 27 kDa) are the two HDAg exits. The little HDAg speeds up genome synthesis, while the large HDAg slows down HDV RNA synthesis but is required for morpho-virion formation. The HDV is a satellite virus that relies on HBV for envelope protein production. HBV/HDV co-infection is particularly common in the Mediterranean and South American regions (Lempp *et al.*, 2016; Botelho-Souza *et al.*, 2017).

The HDV infection occurs in two forms: the first form is caused by the co-infection of HBV and HDV. Usually results in more severe acute hepatitis with a higher mortality rate than acute HBV. The HDV is a small 1.7Kb RNA virus contained in a protein envelope consisting of HBsAg of virus-infected cells, and HDAg is located exclusively in the nuclei. The envelope of the HDV consists of all three protein species of HBsAg, and as a result, HDV probably utilizes the same cellular receptor as HBV(Botelho-Souza *et al.*, 2017).

Replication of HDV is restricted liver in the absence of HDV virions, allowing their spread from cell to cell but inhibited by LHDAg. The HBsAg and LHDAg are needed for HDV assembly. Envelope proteins derived from the pre-S and S antigens of HBV encapsidate HDV RNA and HDAg, necessary for viral proliferation. The HBsAg, HDAgs, and HDV RNA is the primary particles of HDV assembly that can only occur in the presence of the helper virus HBV (Netter *et al.*, 2021).

Although there are 8 different described HDV genotypes, including genotype 1, which is the most frequent genotype throughout the world, especially in Europe, the Middle East, North America, and North Africa; in contrast, genotype 2 is mainly prevalent in the Far East, and genotype 3 is only found in the Amazonian area of South America. Distinct clinical courses are associated with different HDV genotypes

with more severe disease in genotypes 1 and 3 and milder disease activity in HDV genotype 2. Several guidelines suggest that all HBsAg-positive patients should be tested for anti-HDVAbs. Testing of anti-HDVAbs shows a high specificity, but at the same time, anti-HDVAbs may not be identified after recovery from infection, so should be confirmed by the detection of HDV RNA. There is no evidence that the HDV RNA levels correlate with any clinical marker of activity or stage of liver disease but rarely result in chronic infection. The second form results from a superinfection of HDV in an HBV carrier. It can manifest as a severe "acute hepatitis in previously asymptomatic HBV carriers or as an exacerbation of underlying chronic HBV(Rizzetto and Smedile, 2015; Le Gal et al., 2017; Netter *et al.*, 2021).

Unlike co-infection, HDV superinfection in HBV carriers almost results in chronic infection with both viruses. More persons with chronic HBV/HDV co-infection develop hepatic decompensation, cirrhosis, and liver cancer than those with chronic HBV infection alone (Abbas *et al.*, 2015).

E. Hepatitis E virus (HEV):

The existence of an epidemic, non-A, non-B hepatitis unrelated to blood transfusion was first recognized in India in the late 1970s. HEV was described as enterically transmitted or water-borne non-A, non-B hepatitis. Its diagnosis was based on clinical and epidemiological features after excluding hepatitis A and B virus infections. In endemic regions, subclinical human infections are two times greater than symptomatic infections among sporadic cases and during outbreaks. The HEV seroprevalence in these countries ranged from 7% to 21%. It has recently been estimated that HEV infection causes approximately 70,000 deaths worldwide. As large as these figures are, this is likely to represent a gross

underestimate of the actual global burden of disease. (Dalton *et al.*, 2018; Webb and Dalton, 2019).

The HEV is a non-enveloped and single-stranded positive-sense RNA genome of around 7.2 kb in length and flanked with short 5′ and 3' non-coding regions (NCR). It consists of three ORF. The ORF-1 is believed to encode non-structural proteins, while the ORF-2 is postulated to encode the capsid protein, and ORF-3 encodes a pleiotropic protein (Kenney and Meng, 2019).

The HEV consists of a single serotype and eight genotypes. Genotypes 1 and 2 have only been isolated from humans and are mainly distributed in developing countries. In this setting, they cause large water-borne outbreaks and sporadic cases and are associated with high mortality among pregnant women and individuals with chronic liver disease. The HEV genotypes 3 and 4 infect human and swine hosts, while genotypes 7 and 8 infect humans and Camel (Raji *et al.*, 2022).

The laboratory diagnosis of acute HEV is based on IgM anti-HEV in serum and/or detection of HEV RNA in serum or stool. To improve the accuracy of serological diagnosis of hepatitis E, several laboratories developed technologies for controlling non-specific IgM binding, for example, supplementing IgM with IgA anti-HEV detection and identifying novel markers such as HEV antigen. However, anti-HEV detection in humans or swine requires assays explicitly designed for specimens from humans or animals. Therefore, the host-independent detection of acute or past HEV infections has been used. The format that allows for such host-independent detection is the double-antigen sandwich ELISA. Antigen attached to the solid phase is used to capture specific antibodies from serum specimens, and the detection of this antibody is achieved using the same antigen labeled with it, for example, horseradish peroxidase. An assay based on this format was recently

developed to detect anti-HEV in human and animal specimens (Wen *et al.*, 2015).

Other less common causes of hepatitis are viruses like Cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and Varicella-zoster virus, but these viruses primarily do not target the liver.

1.2.1.2.2: Hepatitis by non-infectious agents (Non-viral hepatitis)

Non-infectious agents could cause hepatitis, including autoimmune hepatitis, alcoholic hepatitis, ischemic hepatitis, and genetic factors (Galvin *et al.*, 2015; Brantlya *et al.*, 2018; Zachou *et al.*, 2021). In addition, hepatitis can also occur in neonates and is attributable to various causes, some not typically seen in adults. Congenital or perinatal infection with the hepatitis viruses, toxoplasma, rubella, cytomegalovirus, and syphilis can cause neonatal hepatitis. Structural abnormalities such as biliary atresia and choledochal cysts can lead to cholestatic liver injury leading to neonatal hepatitis. Metabolic diseases such as glycogen storage disorders and lysosomal storage disorders are also implicated. Neonatal hepatitis can be idiopathic, and in such cases, biopsy often shows large multinucleated cells in the liver tissue. This disease is termed giant cell hepatitis and may be associated with a viral infection, autoimmune disorders, and drug toxicity (Samyn and Mieli-Vergani, 2015).

1.2.1.3: Immunopathogenesis of viral hepatitis

A viral infection is the most common cause of liver cancer, and HBV and HCV infection are the most common causes of chronic liver disease in several parts of the world. Both viruses induce chronic hepatitis, which can progress to cirrhosis and eventually hepatocellular carcinoma (Zhang *et al.*, 2021).

Immunologically mediated events play an essential role in the pathogenesis and outcome of HBV and HCV infection. The adaptive immune response mediates virtually all liver diseases associated with

viral hepatitis. Chronic hepatitis is characterized by an inadequate T cell response unable to completely clear HBV or HCV from the liver, which sustains continuous cycles of low-level cell destruction. Over long periods recurrent immune-mediated liver damage contributes to cirrhosis and HCC (Tang *et al.*, 2018; Zhang *et al.*, 2021).

Liver damage in chronic HCV and HBV is commonly attributed to immune-mediated mechanisms. Proteins of HBV and HCV interact in several pathways with the host's immune response, disrupt pathogen-associated pattern recognition pathways and interfere with cellular immunoregulation via CD81 binding and subvert the activity of NK (natural killer) cells, CD4+ and CD8+ T-cells. Finally, HBV and HCV-specific T-cells become increasingly unresponsive. They disappear owing to several possible mechanisms such as mutations in critical viral epitopes, lack of sufficient help, clonal anergy, or expansion of regulatory T-cells. The role of neutralizing antibodies remains uncertain, although it is still possible that humoral immunity contributes to bystander damage of virally coated cells via antibody-dependent cellular cytotoxicity. Cytotoxic lymphocytes kill the infected cells via the perforin/granzyme pathway and release Fas ligand and inflammatory cytokines such as IFNγ (interferon γ). The release of soluble effector molecules helps to control HCV infection. Still, it may also destroy uninfected liver cells and attract other lymphocytes without HBV and HCV specificity to invade the liver. Bystander damage of these non-specific inflammatory cells will expand the tissue damage triggered by HCV infection and ultimately activate fibrogenesis (Cella *et al.*, 2014; Belizário *et al.*, 2018; Zhang *et al.*, 2021).

Unlike hepatitis E, there is no clear information on the effects of HAV infection during pregnancy. However, some evidence suggests a higher risk of pregnancy problems and early birth. Furthermore, even

though HAV RNA is negative in blood and stool, the virus might survive in the liver for a long time (Abdul Amir, 2018).

In human hepatocytes, HDV is not immediately harmful. On the other hand, HDV RNA decreased during the chronic period. Furthermore, the late-stage reactivation of HBV was characterized by the development of cirrhosis and HCC due to HDV or HBV replication or remission with clearance of both viruses (Farci and Niro, 2018).

1.2.2: Hepatitis B Virus Description

Hepatitis B Virus (HBV) infection is a lifelong dynamic disease that changes over time. In adults, end-stage liver disease and cancer risk increase with continuing inflammation and HBV viremia. Reversing fibrosis and treatment can decrease progression. Chronic HBV infection may be managed but not healed. Even patients with hepatitis B surface antigen HBsAg can experience reactivation (Peters, 2019; Zhang *et al.*, 2021). The discovery in 1965 of the "Australia antigen," subsequently identified as the hepatitis B virus surface antigen (HBsAg), was such a watershed event in virology that it is often thought to mark the beginning of hepatitis research. Still, it is more accurately seen as a critical breakthrough in a long effort to understand the pathogenesis of infectious hepatitis. The fortuitous detection of an abundant protein in the serum of an Australian aboriginal person provided the long-sought key to the specific diagnosis of hepatitis B. Even though the finding occurred during studies unrelated to viral hepatitis, the investigators pursued the mysterious protein until it was finally identified as the hepatitis B virus (HBV) surface antigen (HBsAg). The discovery of this viral marker solved a dilemma with one stroke that puzzled researchers for decades and was unable, on a basis other than epidemiological circumstances, to differentiate between viral Hepatitis variants and patients' experience (Block *et al.*, 2016).

Hepatitis B Virus consider a significant public health challenge after several years of infection. Approximately 15–40% of chronically infected patients progress serious sequelae such as cirrhosis, liver failure, and hepatocellular carcinoma, and nearly one million people annually die due to HBV-related complications (Nicolini *et al.*, 2019). Although viral hepatitis is the seventh leading cause of death worldwide, historical records have received very little political attention on a global scale (Cox *et al.*, 2020). Furthermore, fewer than 1% of chronic hepatitis B virus infections per year are cured with antiviral treatment, requiring long-term treatment, which poses challenges for patients and health systems. Because the cure is accompanied by recovery of antiviral immunity, a combination of direct-acting antiviral agents and immunotherapy is likely to be required. There is a need for extensive efforts to identify determinants of the failed immune response (Gehring and Protzer, 2019). In addition role of host genetic factors and their interactions with environmental factors lead to chronic HBV infection, and its complications are not well understood. A better understanding of these factors and interactions will lead to more effective diagnostic and therapeutic options. The clinical presentation ranges from subclinical to symptomatic and, in rare instances, fulminant hepatitis (Zeng *et al.*, 2008). Perinatal or childhood infection is associated with few or no symptoms, but it is highly likely to become chronic. A few medications can effectively treat chronic hepatitis B; a safe and effective vaccine is available to prevent hepatitis B infection (Lin and Kao, 2020).

1.2.3: Classification of HBV and Replication Cycle

Although multiple theories of the origins of HBV exist, it appears that the infection of mammals is a much more recent event. The jump into humans may have been only about 40,000 years ago. The present-day *Hepadnaviridae* family consists of small, hepatotropic DNA viruses

divided into two distinct genera based on their divergent genomic sequences and narrow host range of infection. The avihepadnaviruses infect birds, such as duck HBV (DHBV) and heron HBV(Lamontagne *et al.,* 2016).

In contrast, the orthohepadnaviruses infect mammals, including HBV and woodchuck hepatitis virus (WHV). Each member of the *Hepadnaviridae* family is primarily species-specific. For example, the only non-human hosts of HBV are chimpanzees and treeshrew, each of which can be experimentally infected. Additionally, a primate virus similar to HBV, called woolly monkey HBV, has been identified in woolly monkeys and designated the prototype of a new species of hepatitis B-like viruses (Lamontagne *et al.,* 2016). Hepadnaviridae is characterized by a unique viral replication cycle involving a reverse transcriptase (RT) step late in genomic replication. The virus-encoded polymerase has RT, DNA polymerase, and protein priming activities. However, this polymerase lacks proofreading activity, creating genetic variability manifested as viral quasi-species (Revill *et al.,* 2020; Bousali *et al.,* 2021). Members of the Hepadnaviridae family replicate asymmetrically via reverse transcription of an RNA intermediate. According to phylogenetic analyses, HBV can be classified into ten genotypes (A to J) based upon an inter-group divergence of 8 percent or more in the complete nucleotide sequence. There is growing evidence suggesting that HBV genotypes influence clinical outcomes, HBeAg seroconversion rates, mutational patterns in the precore and core promoter regions, and response to interferon therapy (Elizalde *et al.,* 2021).

The hepatitis B viral genome is present in infectious particles in the form of 3.2kb partially double-stranded, relaxed circular DNA (rcDNA), as shown in (Figure 1-1).

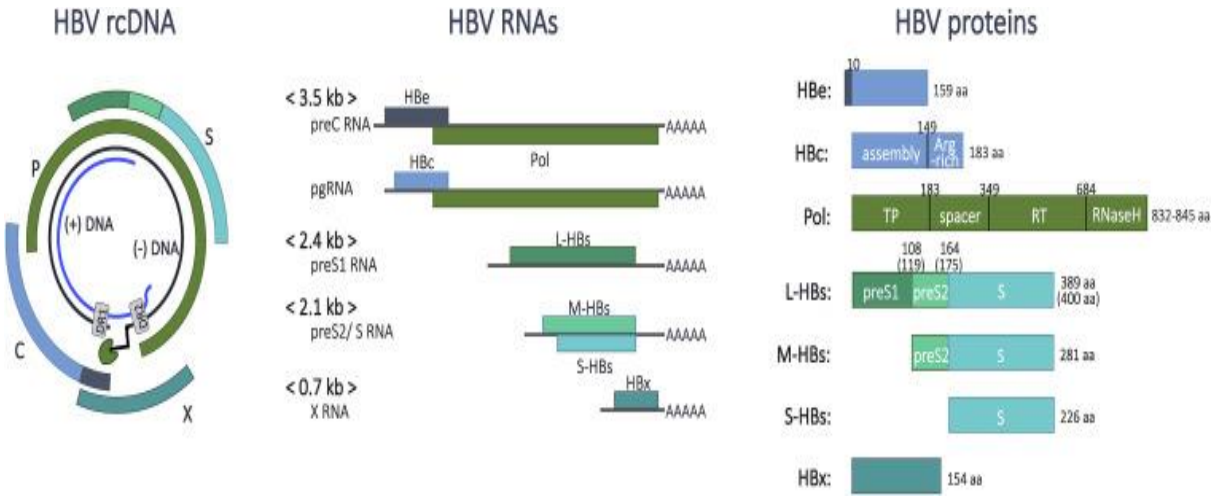


Figure (1-1): Schematic representation of the structure of HBV genomic DNA, RNAs, and proteins. Left, HBV genomic rcDNA and the encoded ORFs (C, P, S, X). Center, HBV RNAs (gray lines) produced by cccDNA transcription and the proteins (boxes) made from the RNAs. The RNA lengths, as well as the names of the RNAs, are shown on the left. Right, HBV proteins and the domain structures. Amino acid numbers and the lengths are shown above the box and on the right, respectively (Tsukuda and Watashi, 2020).

The HBV relaxed-circular DNA genome encodes four overlapping Open Reading Frame (ORF) as shown in (Figure 1-1). The largest ORF encodes the viral polymerase, which has reverse transcriptase (RT) activity that manufactures the first strand of the DNA genome from an RNA intermediate. The second-largest ORF encodes the three viral envelope proteins: large (L-), middle (M-), and small (S-) surface antigen (HBsAg). Another ORF encodes precore, also referred to as HBV e antigen (HBeAg), and the core protein, finally making up the viral capsid. Finally, the smallest ORF encodes the HBV X protein (HBx), a small regulatory protein required for HBV replication both *in vitro* and *in vivo* (Lamontagne *et al.*, 2016; Tsukuda and Watashi, 2020). When HBV DNA is transported into the nucleus of the hepatocyte, then will convert to cccDNA under the action of host enzymes. Unfortunately, the cccDNA is not easily degradable, which is a critical reason chronic hepatitis B is difficult to cure (Zhao *et al.*, 2021).

Following infection, the virus enters hepatocytes via its receptor and the human Na^+-taurocholate co-transporting polypeptide (NTCP). The viral genome is uncoated in the cytoplasm then transported to the nucleus, where the rcDNA is converted to covalently closed circular DNA (cccDNA) (Makokha *et al.*, 2019; Zhao *et al.*, 2021). Nuclear cccDNA formed from the incoming relaxed circular viral DNA serves as the transcriptional template, progeny genomes are created by reverse transcription, which occurs within viral nucleocapsids in the cytoplasm of infected cells, nucleocapsids with mature viral DNA are either assembled into viral envelopes or exported from the infected hepatocyte or if needed, transported to the nucleus to amplify cccDNA copy number, envelope proteins are also secreted as subviral particles, hepatitis B surface antigen (HBsAg), as are large numbers of virus-like particles with empty nucleocapsids (Seeger and Mason, 2016; Tu *et al.*, 2021). The replication cycle of HBV DNA starts with the endonuclease cccDNA transcription of pre-genomic RNA (pgRNA). PgRNA is enveloped in the nucleocapsid during the creation of the virus. HBV DNA polymerase transcribes offspring DNA using pgRNA as the template. The offspring DNAs are then recycled in the nucleus to mediate viral persistence. Some offspring DNAs are assembled into complete virions in the endoplasmic reticulum and secreted from hepatocytes (Liu and Liang, 2018; Inoue and Tanaka, 2019), as shown in (Figure: 1-2).

Hepatotropism is a prominent feature of hepatitis B virus (HBV) infection. The reason viral tropism is restricted to hepatocytes is largely unknown, although it is assumed that infection is limited to the liver because of the tissue-restricted expression of the viral receptor. However, the observation that HBV transgenic mice display viral transcription and replication intermediates primarily in hepatocytes and kidney proximal convoluted tubules indicates that liver-enriched transcription factors

controlling viral RNA synthesis contribute to the hepatocyte-specific tropism of HBV. The dependence of HBV replication on nuclear hormone receptors suggests viral DNA synthesis may be restricted to cells expressing these transcription factors, and this requirement may be a major determinant of viral tropism (Tang and McLachlan, 2001; Hong *et al.*, 2022).

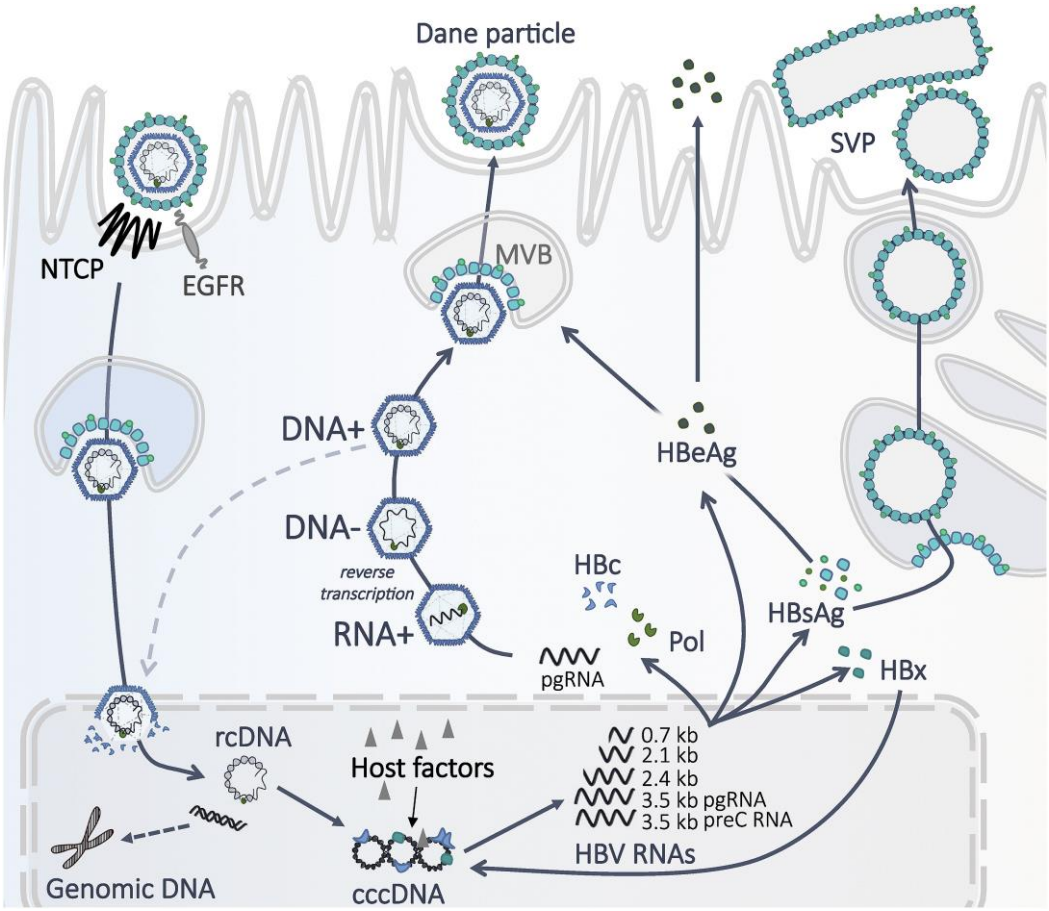


Figure (1-2): Schematic overview of the HBV life cycle (Tsukuda and Watashi, 2020).

The HBV Polymerase is a multifunctional protein with reverse transcriptase activity that has the reverse transcriptase (RT) domain of Polymerase (Hossain *et al.*, 2020). The HBV Pol protein is the main focus of basic and translational research because it is the only current target of any HBV-specific antiviral and the only area frequently sequenced during treatment escape. However, the catalytic activity of HBV Pol happens within the RT and RNase H domains. The TP domain also shows significant functional utility, unique to Hepadnaviridae.

Therefore, any drug cross reactivity would likely be below. HBV Pol is similar in sequence and structure to the polymerase found in the human immunodeficiency virus (HIV). Although also, it contains RT and RNase H domains, the knowledge about HIV Pol is much more, including several high-resolution structures. As a result of their similarity, models of HBV Pol borrow this knowledge about the HIV Pol. Despite similarities, definitive descriptions of the three-dimensional structure of HBV Pol do not yet exist (Buhlig *et al.,* 2020). Although the genome of HBV is made up of DNA, the virus transitions to an unstable RNA state during replication, where it's proofreading deficient reverse transcriptase does not correct errors. Therefore leads to the gathering of mutations. Mutations in the HBV genome of each genotype may affect prevention strategies, diagnosis techniques, the response to treatment, and the course of the disease (Koyaweda *et al.*, 2020).

1.2.4: Etiology, Clinical Features, and Complications for HBV

The incubation period for HBV infection ranges from 6 weeks to 6 months. The clinical manifestations depend on the age at infection, level of HBV replication, and host's immune status, and the age of adult patients at the time of initial presentation with HBeAg positive chronic hepatitis B ranges between 24 and 36 years (mean 31 years) in several reports and men usually outnumber women, the male to female ratio ranging from 1.5 to 4.9. A previous study found approximately 1-3% of healthy adults, 5-10% of immunocompromised adults, and 90% of neonates exposed to HBV develop chronic infection (Al-Jubory, 2008).

Hepatitis B symptoms and signs range from minor to severe. They usually occur one to four months after being infected, though they might appear as early as two weeks after being infected. Some people, tiny toddlers, may not show any signs or symptoms. Abdominal pain, Dark urine, Fever, Joint pain, Loss of appetite, Nausea and vomiting,

Weakness and fatigue, Yellowing of your skin, and the whites of your eyes (jaundice) are all signs and symptoms of HBV (Burns and Thompson, 2014).

Hepatitis B spreads through blood, semen, or other body fluids from an infected person. Your risk of hepatitis B infection increases if you: have unprotected sex with multiple sex partners or with someone who's infected with HBV, share needles during IV drug use, a man who has sex with other men, Live with someone who has a chronic HBV infection, an infant born to an infected mother, have a job that exposes you to human blood, travel to regions with high infection rates of HBV, such as Asia, the Pacific Islands, Africa, and Eastern Europe (WHO, 2018; Pattyn *et al.*, 2021).

A chronic HBV infection can lead to severe complications, such as liver scarring (cirrhosis). The inflammation associated with a hepatitis B infection can lead to extensive liver scarring (cirrhosis), impairing the liver's ability to function. As a result, people with chronic hepatitis B infection have an increased risk of liver cancer. Acute liver failure is when the liver's vital functions shut down. When that occurs, a liver transplant is necessary to sustain life other conditions. In addition, people with chronic hepatitis B may develop kidney disease or inflammation of blood vessels (Tripathi and Mousa, 2022).

1.2.5 Epidemiology for HBV

HBV is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide, resulting in 887,000 deaths per year. An estimated 257 million people have chronic HBV infection globally (CDC, 2020), as shown in (Figure: 1-3). In addition, other studies may reach about 350-400 million infected people worldwide (Sahlan *et al.*, 2019).

In Iraq, many studies were conducted to determine the epidemiological status of the disease, that consider of amongst Eastern Mediterranean Region (EMR) countries which have intermediate endemicity of viral B hepatitis with carrier rates of 2% to 5% in their general population(Al-Asadi and AbdulJalil, 2016). In Basrah, a study on blood donors in 2013 showed that 2.3% of them had serological evidence for hepatitis B virus infection. In 2016 the percentage was 2.1 (Al-Rubaye *et al.*, 2016). Another study on blood donors in Babylon governorate showed a seroprevalence of 0.7%. In a survey on the general Iraqi population, the occurrence was 1.6% (Al–Juboury *et al.*, 2010).

In a study conducted recently in Dhi Qar governorate about HBV, the results showed that (1323) patients (during the period from 2015 to 2019) were infected with hepatitis and distributed as follows: public health laboratory 672(50.8%), central blood bank 515 (38.9%), dialysis center 91 (6.9%) and thalassemia center 45 (3.4%), (Othman and Abbas, 2020).

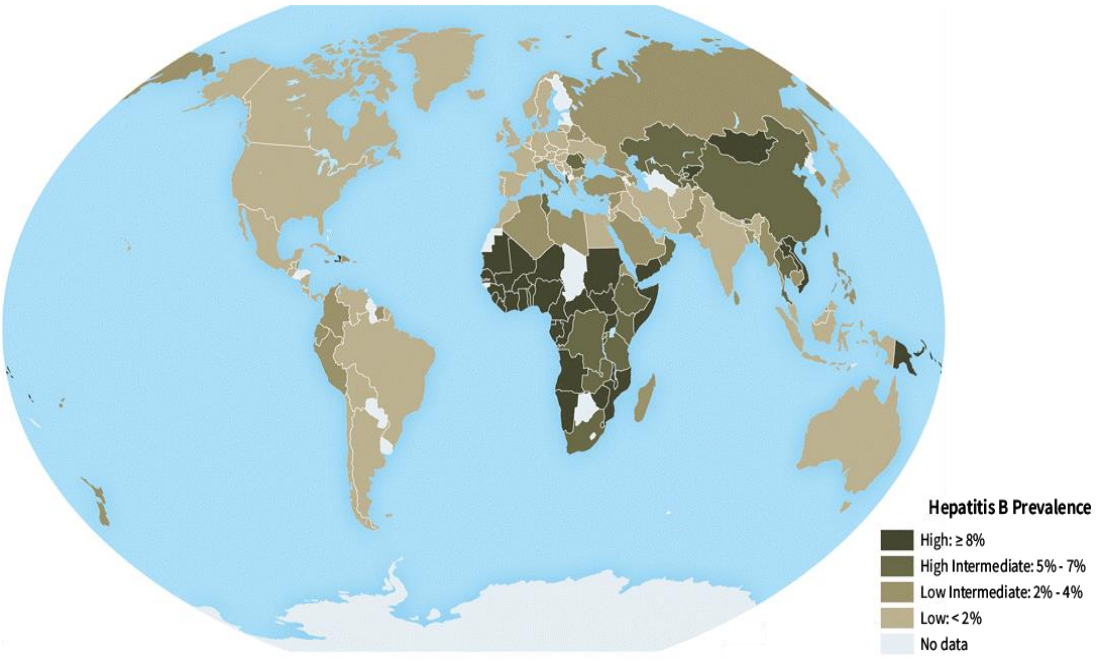


Figure (1-3): Prevalence of hepatitis B virus infection (CDC, 2020).

1.2.6: Predisposing factors for HBV infection

Hepatitis B virus is found in many body fluids, including saliva, blood, semen, menstrual blood, vaginal secretions, a smaller level in breast milk, sweat, urine, and tears of chronically or acutely infected patients (Schillie *et al.*, 2018). Therefore, Hepatitis B virus can be transmitted through contact with infected body fluids (CDC, 2020). It is more infectious, about a hundred times more than HIV and about ten times more than HCV (WHO, 2021). Highly risk groups of HBV includes sexual partners and household contact with infected individuals, sexually active heterosexual women and men, babies born from infected mothers, IVDUs, and individuals with bleeding problems who were receiving unsafe blood or blood yields (for example, leukemic and hemophilic patients), hemodialysis patients, healthcare workers, and international travelers (Daniel, 2008). In addition, The most common factors found to be associated with HBV infection are family size, socioeconomic status, age, educational status, and a history of previous blood transfusion, surgery, or contact with a jaundiced person (Ben-Alaya-Bouafif *et al.*, 2010; Bawazir *et al.*, 2011). As well as, members of low-income families share bedding, eating, and household utensils, which may be sources of infected saliva or serum. Low-income families tend to live in small dwellings. Crowding brings these individuals into close contact, which may facilitate childhood HBV transmission, so the risk of infection was higher in children of low socioeconomic status when compared to children of high socioeconomic status (Qirbi, 2004).

1.2.7: Diagnostic of HBV

The diagnosis of HBV infection and its related disease achieved by a constellation of clinical, biochemical, serological, histological, and genetic analysis, several viral antigens and their respective antibodies can be detected in serum after infection with HBV, proper interpretation of

the results is essential for the precise diagnosis of the various clinical forms of HBV infection.

1.2.7.1: Hepatitis B viral load

The viral load count is the amount of viral DNA or RNA in a blood sample. A high viral load shows that the immune system has failed to fight infections, and it is one of the surrogate biomarkers of hepatitis (Trivedi *et al.*, 2015). Real-time polymerase chain reaction (PCR) and non-invasive measurements of hepatic fibrosis are used to determine the quantitative hepatitis B virus (HBV) DNA viral load (Laing *et al.*, 2019). HBV DNA only originates from mature infectious particles in the natural course of HBV infection, and HBV DNA levels reflect viral replication (Kim, 2017). Although the importance of serum HBV DNA levels as a predictor of the development of cirrhosis and HCC has been broadly reviewed, various hospital-based and community-based case-control and cohort studies have consistently found significant associations between its elevation and risk of liver cirrhosis and HCC (Chen *et al.*, 2009; Liu and Zhang, 2015). Also, the Hepatitis B virus (HBV) viral load (VL) is used as a biomarker to assess the risk of disease progression and to determine eligibility for treatment (Downs *et al.*, 2020).

One study carried out among CHB patients with high HBV DNA viral load (> 6 $\log_{10}$ IU/ml) reported that TDF treatment was associated with a higher incidence of acute kidney injury (AKI) compared to ETV, but this difference was only borderline significant after three years follow-up (Wu *et al.*, 2017; Wang *et al.*, 2021).

1.2.7.2: Serological test

Serological markers for HBV infection consist of HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc IgM and IgG. The identification of serological markers allows:

1. Identify patients with HBV infection.

2. Elucidate the natural course of chronic hepatitis B (CHB).
3. Assess the clinical phases of the disease, and monitor antiviral therapy.
4. HBsAg is the serological hallmark of HBV infection (Song and Kim, 2016)

In other words, serological markers (IgM and IgG) are critical in detecting acute HBV infection and determining its possible evolution towards chronicity (Otero *et al.,* 2019). The first HBV protein discovered was HBsAg; seeing HBsAg in serum is a fundamental diagnostic marker of HBV infection. The antibodies against HBsAg provide protective immunity. The loss of HBsAg and the development of anti-HBs antibodies is the ultimate goal of anti-HBV therapy (Jaroszewicz *et al.*, 2010; Jiang *et al.*, 2021; Vanwolleghem *et al.*, 2022). The presence of anti-HBsAb in anti-HBc Ab witnesses the recovery of chronic HBV infection and life-long immunity against HBV. The anti-HBsAb titer varies over time. After vaccination against HBV, isolated anti-HBs Ab must be present at a titer of $\geq$ 10 IU/mL to confer efficient protection. (Polák *et al*., 2016; Vanwolleghem *et al.,* 2022). The combination of these markers, sometimes extended with the detection of HBeAg or its corresponding antibodies (anti-HBe), are used to establish the patients' status related to the HBV infection and to monitor the progress of disease together with changes in liver enzymes levels (Alberti and Caporaso, 2011).

Occult hepatitis B virus (HBV) infection (OBI) is defined as surface antigen (HBsAg) seronegative, core antibody (HBcAb) seropositive, and HBV DNA positive in serum or liver (de Almeida and de Paula, 2022). OBI may result in HBV reactivation (HBVr), acute exacerbations, cirrhosis, and hepatocellular carcinoma (HCC; Mak *et al.*, 2020). For OBI patients or HBV exposure (HBsAg-negative but HBcAb-

positive; Pattullo, 2015), according to the recent American Association for the Study of Liver Diseases (AASLD) recommendation guideline, HBVr could be defined as (1) HBV DNA is detectable; or (2) reverse HBsAg seroconversion occurs (reappearance of HBsAg; Terrault *et al.*, 2018; Onorato *et al.*, 2021).

The laboratory markers of HBV infection and its interpretation are listed in (Table 1-1).

Table (1-1): Laboratory diagnostic markers for hepatitis B infection (Koffas *et al.*, 2018).

Marker name	Interpretation
HBsAg	Hallmark of infection; positive during early phase of acute infection, persistently positive in chronic infection
Anti-HBs	Recovery from acute infection (or chronic); immunity following vaccination
HBeAg	eAg positivity associated with high replicative state; presence of inflammation and/or fibrosis determines disease phase; eAg negativity reflects a change in disease phase and is usually associated with the emergence of antiHBe; viral mutations in precore and basal core promoter regions result in eAg-negative hepatitis
Anti-HBe	Marker of eAg seroconversion associated with immune control in low viraemic states
Anti-HBc IgM	Positive in acute infection; may be positive during reactivation of HBV
Anti-HBc IgG	Exposure to infection and present in association with HBsAg in chronic infection; HBsAg-negative, anti-HBc-positive serology usually indicative of past exposure to virus; anti-HBs may /may not be positive; if anti-HBs negative, a false positive anti-HBc should be considered (eg after IVIG infusion); HBV DNA must be checked to exclude occult infection

The HBsAg is an intracellular antigen that cannot be detected in serum. Its corresponding antibody (anti-HBc Ab) indicates prior exposure to HBV irrespective of the current HBsAg status. The first antibody detectable in acute HBV infection is IgM anti-HBc which is usually

detected within one month after the appearance of HBsAg. The presence of anti-HBc IgM with a high index value usually indicates a recent HBV infection, and this antibody usually disappears within six months (Florian *et al.,* 2020). In contrast to anti-HBs antibodies, anti-HBc IgG is not neutralizing in vivo. False-negative detection of anti-HBc antibodies may rarely occur in immunosuppressed patients. A rare HBV variant harboring an in-frame deletion of the core gene defective for HBc antigen synthesis named HBV-2 has been reported not to elicit a detectable immune response to HBc Ag in immune-competent patients (Challine *et al.*,2008). Patterns of HBV Markers in Acute and Chronic Infection in figure (1-4).

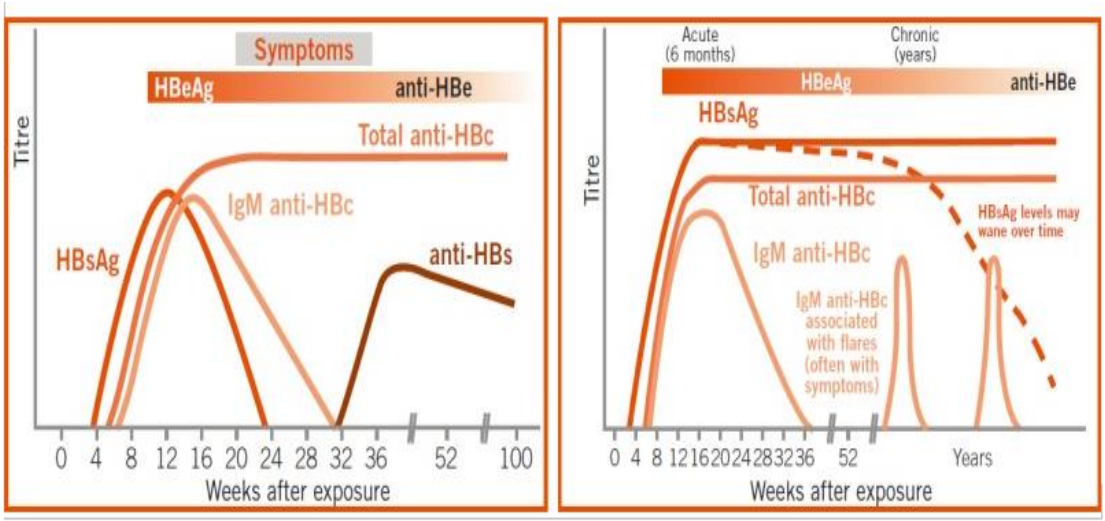


Figure (1- 4): HBV Marker Patterns in Acute and Chronic Infection. Anti HBc = antibodies against HBc; anti-HBe = antibodies against HBe; anti-HBs = antibodies against HBs; HBeAg = HBV envelope antigen; HBsAg = HBV surface antigen; IgM = immunoglobulin M (WHO, 2017).

1.2.7.3: Liver function test

liver function tests are helpful to assess the severity and expect the outcome of certain liver diseases such as viral hepatitis. HBV infection may change the serum levels of certain hepatic enzymes and compounds such as alkaline phosphatase (ALP), Aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and albumin, the elevation of

these enzymes and proteins above their upper reference limits are said to be abnormal except for serum albumin. Marked rise in serum ALT with acute flare-up may be seen in patients with chronic hepatitis AST. ALT is often released into the bloodstream after hepatocellular damage, so ALT serum level elevation correlates more with hepatic damage injury (Abulude et al., 2017; Alhajji, 2021).

1.2.8: Genotyping of HBV

The molecular characteristics of the virus are responsible for the development of acute hepatitis B and various manifestations of chronic liver disease, accounting for an essential percentage of liver diseases. The HBV belongs to the family Hepadnaviridae, a double-stranded circular DNA genome of approximately 3200 base pairs (bps) in size. Unlike other DNA viruses, HBV replication involves a critical reverse transcription step (Datta *et al.,* 2012; Xu *et al.,* 2021). This step involves ribonucleic acid (RNA)-dependent DNA polymerase, lacking proofreading and error-prone viral replication. The error-prone replication is a significant molecular factor for the emergence of genotypes and sub-genotypes. As proof, genotype I is a novel tri-recombinant of genotypes A, C, and G. Genotype J shows high similarity with gibbon genotypes and human genotype C (Tran *et al.,* 2008; Fletcher *et al.,* 2020).

Any evolution of HBV genotypes requires comprehensive molecular investigation to understand its influence on the pathogenesis and outcome of HBV infection (Fletcher *et al.,* 2020).

HBV is classified based on the phylogenetic analyses of the complete viral genome. The degree of nucleotide divergence in the whole genome is a molecular criterion for the designation of genotypes and sub-genotypes. Therefore, HBV genotype prevalence varies geographically. Ten genotypes (A-J) and 46 sub-genotypes have been identified to date.

[A (1-6), B(1-5), C(1-16), D(1-9), E, F(1-4), G, H, I(1-2), and J], (Schaefer, 2007; Toyé *et al.*, 2021)..

Whole-genome sequencing followed by phylogenetic analysis is a gold standard for HBV genotyping, identifying predominant, novel, and recombinant genotypes (Kramvis, 2016).

Identifying HBV genotype is essential for many reasons, including epidemiological studies and its primary distribution in different regions. Genotype distribution shows variations between countries and geographical areas within countries. In addition, associations exist between clinical outcomes and patient treatment efficacy (Liu *et al.*, 2021).

1.2.9: Control and Prevention of HBV by Vaccination

Standard precautions must be followed when handling body mass and blood of humans when handling contaminated or potentially infectious materials (Weinbaum *et al.*, 2008). Also, reducing damage practices for IDUs (injecting drug users) prevents HBV transmission, using disposable tools anywhere possible, and blocking conventional therapy, scarring, and tattooing practices. Including reducing the number of partners and using a protective barrier (condoms), measures have been shown to protect against HBV transmission. Furthermore, checking blood and blood products is an important way to decrease the probability of infection as the blood supply might have HBV, refuse every donor with a history of HBV, IDUs, reserve tattoo, and blood transfusion. In addition to vaccination, which is the primary efficient manner for preventing HBV infection. An effective and safe vaccine to HBV has been available since 1982, and this vaccine can protect individuals from AHB and CHB infection. Hepatitis B vaccine is composed of recombinant HBsAg and at least 20 years is the time of immunity with an efficiency of 95% (WHO, 2011; Al-Suraifi *et al.*, 2016).

Anti-HBs antibodies development titers of more than ten mIU per ml are 100% protective against clinical illness and chronic infection. If their titer in the first dose is higher than 100 mIU /ml, it may protect them from infection risk for more than 30 years, even without giving them the booster dose (Cocchio *et al.*, 2021; Noordeen *et al.*, 2022). The WHO introduces the two-dose vaccine to facilitate adolescent compliance. Other host factors affecting the response to vaccines are age, smoking, obesity, chronic disease, dialysis, and HIV infection. Vaccination against HBV is recommended to several risk groups, preferably before exposure to the virus. A booster immunization dose and doubling of vaccine dose might be required in the second decade after vaccination, especially in immunosuppressed, hemodialysis, and liver transplant patients. The new DNA vaccines provide a vaccination strategy that is less expensive and easier to produce than antigenic proteins. In addition, DNA immunization induces a strong CTL and T-helper and antibody response to the expressed proteins, which is directed against expressed and processed viral epitopes (Gunther, 2006; Chen, 2009; Bajunaid, 2013).

Adverse reaction of HBV vaccination is the most common side effects are usually mild and last 1-2 days. Severe allergic reactions following vaccination are rare but can be life-threatening. A severe allergic reaction can include hives, swelling of the face and throat, difficulty breathing, a fast heartbeat, dizziness, and weakness (Moro *et al.*, 2014; CDC, 2020).

The significance of vaccination against hepatitis B during infancy is recognized worldwide. However, whether booster or revaccination after the primary vaccination is required remains controversial. Recently, cross-sectional epidemiological surveys found that HBsAg prevalence in subjects born after the implementation of mass immunization was increased with age, which was attributed to the waning of anti-HBs over

time. However, a comprehensive analysis of the closely related cross-sectional surveys showed that the age-specific increased HBsAg prevalence was more likely associated with the carry-over of the infection that occurred in early life, probably due to imperfect coverage of hepatitis B vaccination at the beginning of its introduction. Moreover, confirmed breakthrough HBV infection with severe consequences in successfully vaccinated individuals is sporadic. Thus far, no compelling evidence has been acquired to support booster vaccination in adolescence. The uncertainty regarding the duration of protection of hepatitis B vaccination is significantly beyond 30 years after the primary immunization (Zhao and Zhou, 2018).

In terms of revaccination or booster for seropositivity, several studies have recommended there is no need to give booster dose among all the seronegative youths if they stay in immunocompetent status from the public health viewpoint as they might have herd immunity. In addition, there are no increasing hepatitis events happenings observable in this age group. However, among seronegative youths who will work in the medical care systems soon or their family members had been HBV carriers, few studies have been conducted to examine the responses to different numbers of booster doses to monitor the development of seropositive titers in their youth lives (Jan *et al.,* 2020).

1.2.10: Historical review of coronavirus

The term coronavirus was coined to describe certain features of the infective shape (virion) as seen through an electron microscope, including a peculiar bulbous form projection (peplomeric spike), which was later discovered to be proteins molecules anchored on the surface of the lipid bilayer membrane (McIntosh, 1974).

Coronaviruses (CoVs) are a family of RNA viruses that cause disease in humans and other animals. They can infect humans, pets, birds,

bats, mice, and a variety of other wild animals' respiratory, gastrointestinal, hepatic, and central nervous systems (Su *et al.*, 2016). Coronaviruses are enveloped viruses with large single-strand positive sense RNA genomes that belong to the Coronaviridae family (Drosten *et al*., 2003).

At 2003, at least seven forms of coronavirus have been identified as causing disease in humans; the 229E, OC43, NL63, and HKU1 viruses only cause mild cold symptoms. The remaining three viruses, including severe Acute Respiratory Syndrome (SARS-CoV), that caused the SARS epidemic in 2002 and 2003, may cause serious illness (Fouchier *et al*., 2003).

Middle East Respiratory Syndrome (MERS-CoV), which first appeared in 2012 and is still circulating in camels (Dong *et al*., 2020) and SARS-CoV-2, that first reported in Wuhan, China in December 2019, and for which a lot of effort is being made to avoid its spread (Xu *et al*., 2020).

SARS' etiologic agent (an unidentified coronavirus; SARS-CoV) was isolated and its genome sequenced in record time (Homes and Enjuanes, 2003). The mini pandemic was brought under control within 7 months of its onset, thanks to an unprecedented global public health campaign (WHO, 2003).

The first case of Middle East respiratory syndrome (MERS) was discovered in Jeddah, Saudi Arabia, in June 2012, and the majority of cases have occurred in the Arabian Peninsula (Zumla *et al*., 2015). MERS-CoV is a zoonotic virus that has a reservoir host in dromedary camels (Paden *et al*., 2018). Bats are a probable original reservoir; coronaviruses similar to MERS-CoV have been found in bats, although there is no epidemiologic evidence of their function in transmission (Corman *et al*., 2014).

An unknown disease emerged at the end of 2019 and became the subject of attention. Pneumonia-like symptoms and lung fibrosis is caused by the disease (Zhou *et al*., 2020). It all started in Wuhan, Hubei Province, China, a city of 11 million population (Jingchun *et al*., 2020). On December 31, 2019, China was the first country to announce this pneumonia with an unknown cause to the WHO country office. Since then, it has registered thousands of new COV-19 cases.

SARS-CoV-2 is not country-specific virus. It was extremely infectious, spreading to over 100 countries in the last two to three months and affecting over 300000 persons on the world. As of March 24, 2020, the following populations are impacted: China, Republic of Korea, Australia, Malaysia, Japan, Singapore, New Zealand, and others in the Western Pacific Region. A total of 195,511 positive cases were reported in the European Region (Italy, Spain, Germany, the United Kingdom, Norway, and so on), with 24,087 of them occurring in just one day. In a single day, there were 10,189 recorded cases and 1447 deaths (WHO, 2020).

1.2.10.1: Classification of Coronavirus

Coronaviruses are members of Coronaviridae family, and Orthocoronavirinae subfamily of the Nidovirales order. Among RNA viruses, CoVs have the largest genomes, with genome sizes ranging 26 - 32 kb. Depending on genetic and antigenic criteria, coronaviruses are classified into four genera: alphacoronavirus (α-CoV), betacoronavirus (β-CoV), gammacoronavirus (γ-CoV), and deltacoronavirus (δ-CoV) (Lu *et al*., 2020). Bats and mice act as reservoirs for alpha and beta coronaviruses, while birds serve as reservoirs for gamma and delta coronaviruses (Li *et al*., 2020). SARS-CoV-2 belongs to the subgenus

Sarbecovirus of the genus Betacoronavirus (Lorusso *et al*., 2020), according to phylogenetic analysis as shown in (Figure: 1-5).

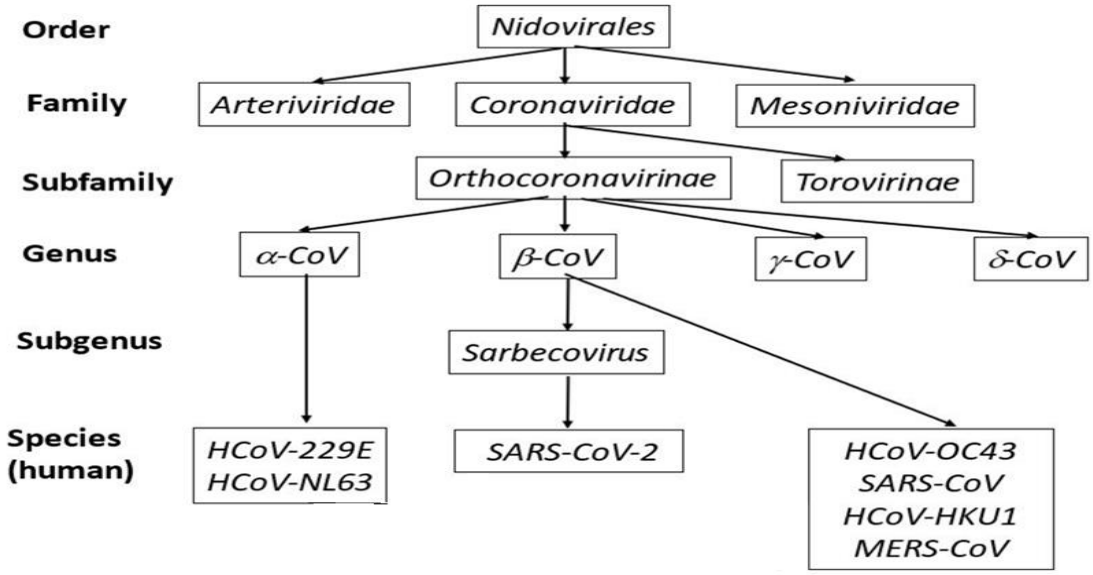


Figure (1-5): Classification of Coronaviruses (Lu *et al*., 2020).

1.2.10.2: The Source and Intermediate Host of Coronaviruses

Coronaviruses are zoonotic, meaning they can be spread from animals to humans. SARS-CoV and MERS-CoV are both thought to have originated in bats and has been passed on to humans through Civet cats and camels (WHO, 2020). Bats were thought to be the original hosts of SARS-CoV-2 based on phylogenetic comparisons with other coronaviruses, as well as the latest virus was 96% linked to two SARS-CoV strains from Bats named Bat-SLCoVZX45 and Bat-SL-CoVZX21 (Lu *et al*., 2020; Xiao *et al*., 2020). However, the virus's intermediate host, which enabled it to cross the species border and infect humans, as well as the transmission route, remain unknown (Kakodkar *et al*., 2020). Snakes were suggested as potential virus carrier from bats to humans, via symmetric recombination within the COVID-19 S protein (Kakodkar *et al*., 2020).

According to a report published in 2006 by Neuman *et al*., scientists in Guangzhou, China, proposed that Pangolins, long-snout and ant-eating mammals widely used in Chinese traditional medicine, may be the possible intermediate host of SARS-CoV-2, dependent on 99% genetic similarities between Coronavirus discovered in Pangolins and SARS-CoV-2. the 1% variance between the two genomes is remain a significant different; as a result, definitive proof findings are expected (Figure 1-6).

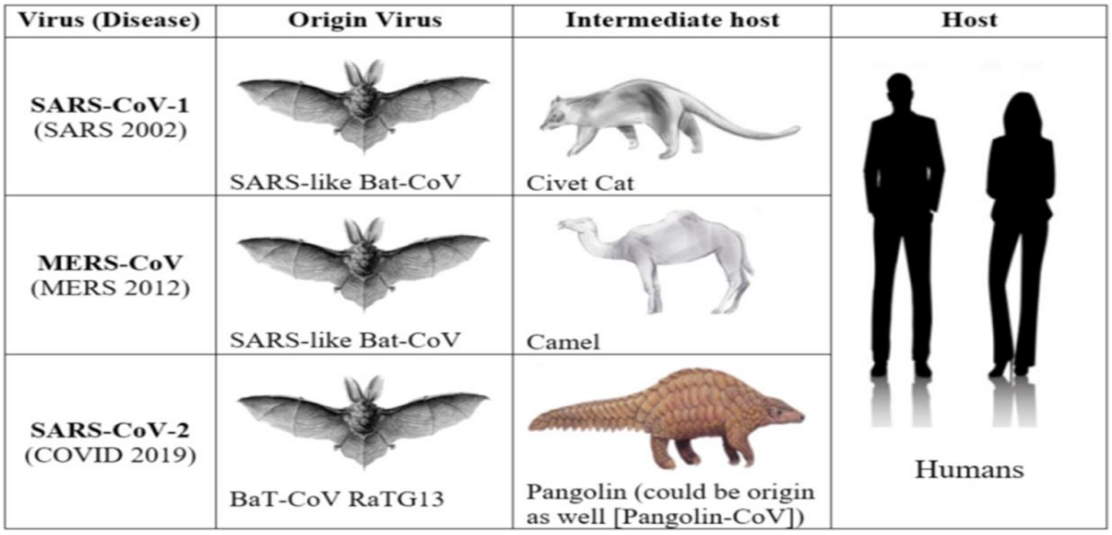


Figure (1-6): The natural reservoir, intermediate host, and target in major Coronaviruses (Kakodkar *et al*.,2020).

1.2.10.3: Structure of coronavirus

Coronaviruses are large particles viruses that are spherical in shape and have spikes that form a surface projection. The particles are about 125 nanometers in diameter. The envelope diameter is 85 nm, and the spikes are 20 nm long; however, SARS-spikes CoV-2's are larger, increasing its pathogenicity (Singer and Blinov, 2014).

The viral envelope, like any other membrane, was made up of a lipid bilayer and a variety of proteins with structural functions, including membrane (M), envelope (E), and spike (S) (Singer and Blinov, 2014) in a ratio of E:S:M 1: 20:300. The particle's total number of spikes is about

74 (Yousif *et al*., 2013). However, as shown in (Figure 1-7), SARS-CoV-2 has another short projection of a proteinous structure known as hemagglutinin esterase (HE) (McIntosh, 1974).

The spikes are folded into homotrimers and are divided into two sections, S1 is the spike's head structure, with receptor-binding domains (RBD) that contain the signal peptide, and S2 is the spike's stem, with heptad repeat regions (HR1 and HR2) and a putative fusion peptide (F). The transmembrane domain and endo-domain are also present. All of these subunits aid in the activation of these subunits to facilitate fusion, which is essential for pathogenesis and maintaining envelope integrity (Cui *et al*., 2019). Finally, the nucleocapsid is made up of nucleic acid (positive-sense single-stranded RNA genome) folded on several copies of proteins (nucleocapsid, N). The organization is organized in a continuous bead-on-a-string pattern (Cui *et al*., 2019). All of these structures are essential for the virus's defense when it is outside of host cells (Decaro *et al*., 2011).

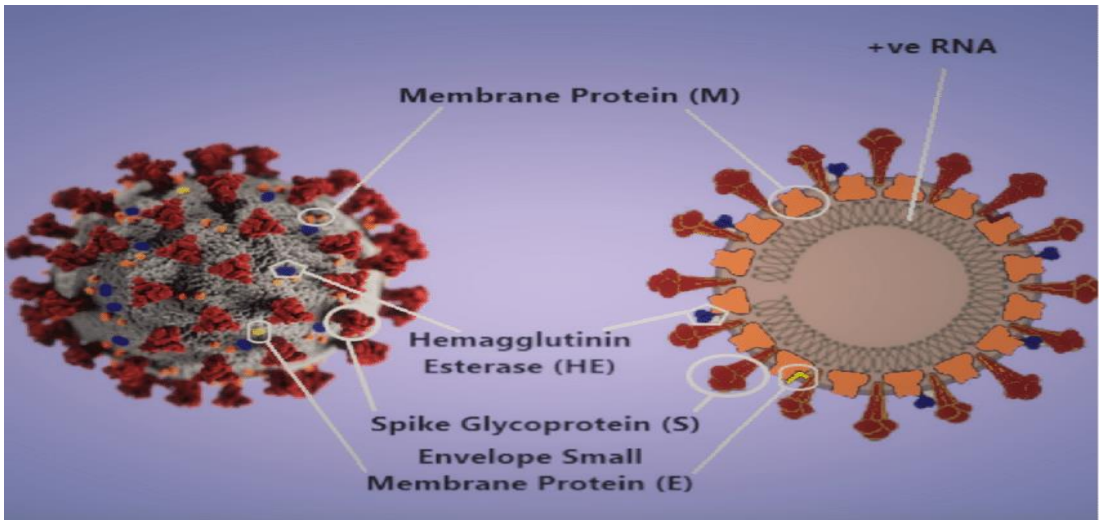


Figure (1-7): Schematic diagram of SARS-CoV-2 (Kakodkar *et al*.,2020).

1.2.10.4: Genome Organization of coronavirus

Coronaviruses are RNA viruses with a genome made up of a single stranded, positive–sense RNA with a size ranging from 26 to 31 kilobases (Knapp, 2020). It is the largest of all RNA viruses in terms of

morphology and genetics. Its RNA has been a 5′ methylated cap and a 3′ polyadenylated tail, related with eukaryotic mRNA (Decaro *et al*., 2011).

The coronavirus genome is sequenced in the following order: 5′-leader-UTR, replicate/transcriptase-spike (S), envelope (E), membrane (M), nucleocapsid (N), and 3′UTR-poly (A) tail (Yuhang *et al*.,2020). It contains several overlapped open reading frames (ORFs), The first were ORFs 1a and 1b, that had been located in the genome's first two-thirds and code the replicase-transcriptase polyprotein (pp1ab), which is then self-cleaved to produce 16 nonstructural proteins (nsp1-nsp16) (Decaro *et al*., 2011). The main structural proteins spike, envelope, membrane, and nucleocapsid were encoded by the other reading frames (Li *et al*., 2005). Other reading frames encoding for accessory proteins were spread among these reading frames. The number of accessory proteins varies, but their functions are distinct among coronaviruses (Decaro *et al*.; 2011). The 5′UTR and 3′UTR are two untranslated regions of unusual composition and structure, the 5′UTR began the genome and the 3′UTR finished it (Yuhang *et al*., 2020). In terms of function, they are in control of viral replication, transcription, and packaging (Yuhang *et al*., 2020).. They may play a part in regulating inter- and intermolecular interactions, especially in relation to RNA-RNA interactions and viral and cellular protein binding (Yuhang *et al*., 2020), as shown in (Figure: 1-8).

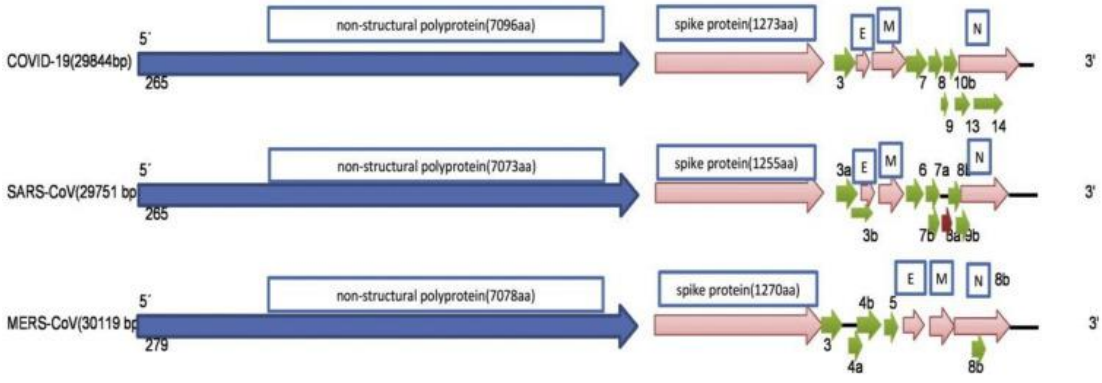


Figure (1-8): The organization of 5′ UTR and 3′ UTR and coding region of COVID-19, SARS-CoV, and MERS-CoV viruses (Yuhang *et al*., 2020)**.**

1.2.10.5: Human receptors of coronavirus

Coronavirus infection (SARS-CoV-2) can be transmitted by bats and humans since it is a member of the Nidovirus order. The Angiotensin-converting enzyme 2 (ACE2) receptors, which can be found in a variety of organs, including the heart, lungs, kidneys, and digestive system, are complementary in form to the spike shape, allowing effective attachment and making it easier for the virus to reach the target cells (Rabi *et al*., 2020).

This binding occurs in the S protein domain of SARS-CoV-2 receptors, which is closely linked to ACE2 of human and bat (Zhang *et al*., 2021). Following the entrance and attachment routes, the membrane of viral and the cell of host fuse (Zhang *et al*., 2021).

Next fusion, the type II membrane serine protease (TMPRSS2) on the cell surface of host removes ACE2 and activates the spike-like S proteins to be attached to the receptor (Rabi *et al*., 2020).

Furthermore, the implanted SARS-CoV-2 later the genomic material will be released into the cytoplasm, and it will become nucleoplasmically localized (Zhang *et al.,* 2021). The genetic material of this virus would be mRNA, which is prepared for translation into a protein (Zhang *et al*., 2021).

This virus's genomic material has been supplemented by around 14 open reading frameworks (ORFs), every one encodes different set of structural and non-structural proteins that aid in the virus's survival and virulence. By contributing to the ribosome frame shifting event, the genetic parts that encode non-structural proteins first convert to ORF1a and ORF1b to create two great superimposed proteins, pp1a and 19 pp1ab, during the transformation stage (Marquardt and Hansler, 2020).

The structural and accessory proteins are then generated from the sub-genomic proteins like M, S, and E, after that they are separated in the endoplasmic reticulum and moved to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). In this time being, an earlier transcribed genomic material program will enter N protein in nucleocapsid form and progress to ERGIC. Nucleocapsid can encounter some other structural proteins in this compartment and create small portfolio vesicles for exocytosis outside the cell (Figure: 1-9) (Fehr and Perlman, 2015).

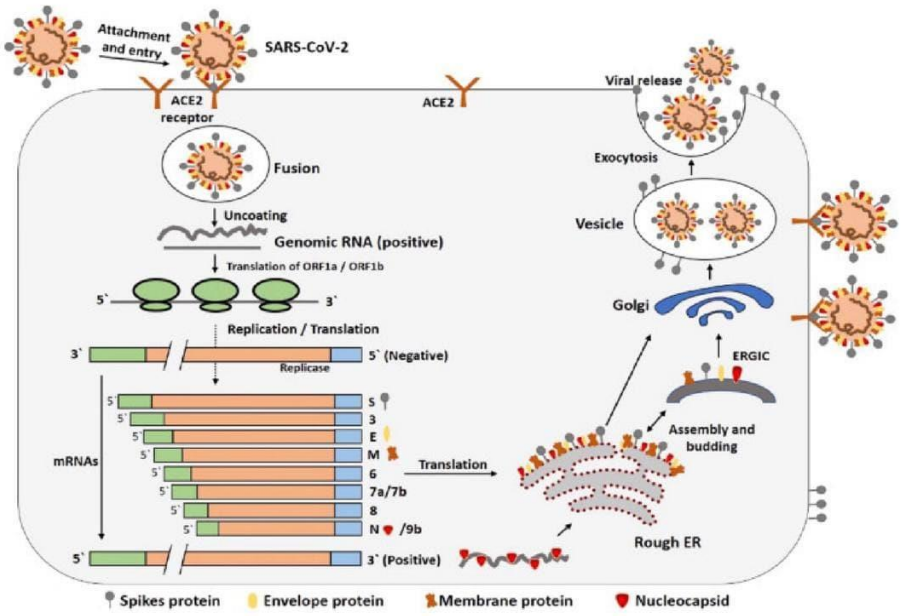


Figure (1-9): The life cycle of SARS-CoV-2 in host cells (Fehr and Perlman, 2015).

1.2.11: SARS-CoV-2 induced HBV reactivation

Since the beginning of January 2022, the number of infections with SARS-CoV-2 began to be limited globally. However, soon the cases rose by 8% during the first two weeks of March 2022, reaching 11 million new infections and 43,000 deaths, bringing the total infections to more than 445 million confirmed cases and more than 6 million deaths universal as of March 13, 2022. Therefore this disease is still considered a pandemic (WHO, 2022).

SARS-CoV-2 has a strong affinity for ACE2 (angiotensin-converting enzyme 2) receptor, expressed on multiple cell types, including hepatocytes and cholangiocytes. In this regard, the upregulation of ACE2 receptors in the liver may contribute to the abnormal activities of liver enzymes seen in patients with COVID-19. The first case of hepatitis B virus reactivation caused by COVID-19 was reported by a study presented by Aldhaleei *et al.* (2020).

Liver workup revealed hepatitis B (HB) surface Ag-positivity, HB core (IgM) Ab-positivity, HB envelops (HBe) Ag-negativity, and HBe Ab positivity, suggestive of reactivation of an HBV infection. His hepatitis B DNA viral load was 2,490 IU/mL (reference: <1000 IU/mL), confirming the diagnosis of an acute HBV infection. His COVID-19 polymerase chain reaction (PCR) test results were also positive (Aldhaleei *et al.,* 2020).

Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has been reported to have multiple clinical manifestations, although primarily they have been pulmonary manifestations. Hepatic manifestations have variably been present in up to 50% of infected individuals. The spectrum ranges from asymptomatic abnormalities in hepatic biochemical tests to the rare case of acute liver failure (Bangash *et al.,* 2020; Zhang *et al.,* 2020; Reddy, 2020).

The cause for hepatic manifestations is unclear at this stage. However, it may be caused by various reasons, such as a manifestation of a systemic illness, ischemic liver injury, immune-mediated liver injury, drug-induced liver injury, or a direct cytopathic effect of the virus (Cano *et al.*, 2017; Xu *et al.,* 2020; Reddy, 2020; Premkumar and Kedarisetty, 2021).

Not uncommonly, patients have concurrent infections, such as hepatitis B virus (HBV), and the impact of SARS-CoV-2 on these infections and associated liver diseases is unknown (Reddy, 2020).

In addition, SARS-CoV may infect livers leading to mild to moderate lobular inflammation and apoptosis. The presence of prominent mitoses among hepatocytes, possibly due to hyperproliferative state and cell cycle arrest, is a cardinal feature of liver pathology in SARS. Therefore, it may target SARS-CoV possible viral replication in hepatocytes, specific therapy to reduce the viral replication and modify the clinical course of the disease (Chau *et al.*, 2004).

1.2.12: SARS-CoV-2 induced HCV reactivation

A study conducted by Lensen *et al.* (2021) on an 82-year-old woman with HCV since 2007 who received the Pfizer COVID-19 vaccine observed HCV reactivation, jaundice, anaphylaxis, and hepatic coma, then death after three weeks of receiving the vaccine. This study also showed a substantial increase in the level of HCV viral load a few days after receiving the vaccine, and the inflammatory response to the vaccine may be sufficient to cause the decay of livers, which leads to the emergence of symptoms and disease severity. Therefore, according to previous studies, the most appropriate explanation for HCV reactivation is that vaccines can cause a reactivation of the virus, especially in patients who are immunosuppressed or recipients of biological therapy. For example, it was seen in a study conducted by Lehmann and Matoba (2018) on a case infected with herpes zoster stromal keratitis. The disease was reactivated due to receiving the herpes zoster subunit vaccine. In addition, SARS-CoV-2 encoded proteins have been found to trigger the reactivation of Kaposi's sarcoma-associated herpesvirus, according to Chen *et al.* (2020). As a result, proteins generated by SARS-CoV-2 could reactivate the hepatitis C virus.

2: Subjects, Materials, and Methods

2.1 Subjects

This study was conducted in the College of Medicine, University of Babylon. A hundred and forty-one (80 males and 61 females) young, middle-aged adults, and elderly were enrolled in this case-control study. After getting all data summarized in appendix 1, this study group was classified by the following:

1. A patient infected with Hepatitis B (non-infected with SARS-COV-2).
2. Patient with Hepatitis B (infected with SARS-COV-2).
3. A patient with Hepatitis B and co-infected with primary viral hepatitis.

All patients in the study were referred and diagnosed in the Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City, Center of artificial Kidney, and Center of Hepatology and Gastroenterology Hospital in Marjan Medical City / Babylon from December 2020 to June 2021.

Table (2-1): The numbers, ages, and gender of subjects involved in the study.

Age (years)	Gender				Total No.	%
	Male	%	Female	%		
Young (< 30)	29	36.25%	17	27.87%	46	32.6 %
Adult (30 - 60)	45	56.25%	39	63.93%	84	59.6 %
Elderly (> 60)	6	7.5%	5	8.2%	11	7.8 %
Total	**80**	**100%**	**61**	**100%**	**141**	**100%**

2.2. Subjects criteria

2.2.1 Inclusion criteria

A. Patients with hepatitis B virus and primary viral hepatitis

B. Patients with hepatitis B virus and SARS-CoV-2.

C. Patients underwent a blood transfusion, hemodialysis, autoimmune diseases, and HBV contact.

3.2.2 Exclusion criteria

Diseases other than viral hepatitis and SARS-CoV-2.

2.3: Ethical Approval

The study was conducted in accordance with the ethical principles in the Declaration of Helsinki. It was carried out with the patients verbal approval before the sample was taken. The study protocol and patient consent forms were reviewed and approved by 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.

2.4: Instruments

According to (Table 2-2), I used the following instruments in this study.

Table (2-2): Instruments, manufacturer companies, and origins.

No.	Instruments	Manufacturer company and Origin
1	Automated Coagulation (CA-600 Series Systems)	Sysmex / Germany
2	Automatic fluorescent immunoassay system (AFIAS)	Boditech Med Inc.
3	CBC Analyzer XP300	Sysmex / Germany
4	Centrifuge tube	Hettich EBA20 / Germany
5	Cobas C311 Biochemistry Analyzer	Roche/ Indian
6	Cylinders	Techico / England
7	Deep freezer	Arcelik / Turkish
8	Digital camera	Sony/Japan
9	EDTA tube	Jordan
10	ELISA Reader	Bio Kit-ELX800 / Germany
11	ELISA Washer	
12	Eppendorf tube rack	China
13	Eppendorf tubes	Eppendorf / Germany
14	Face Mask	China
15	Filter paper (watman No. 1)	Zelpa / China
16	Flask 500ml	MBL / China
17	Gel tube, Gel& clot activator	China
18	Horizontal electrophoresis chamber for	Major Science MP-300V

	agarose gel	power supply, medigene company, china
19	Incubator	Memmert / Germany
20	M32 Automatic Nucleic Acid Extraction Systems	Biocomma / China
21	Micropipette	Slamed / Germany
22	Microwave Oven	GOSONIC, China
23	Mini spin centrifuge	Eppendorf / Germany
24	PCR tips	Eppendorf / Germany
25	PCR tube	Bionear
26	PCR tube rack	Watson Bio Lab
27	PCR tubes	Eppendorf / Germany
28	powder free Gloves/ disposable	Broche / Turkey
29	Real-time cycler	LiNEAR / Spain
30	Refrigerator	Beko / Turkey
31	Sensitive electronic balance	Sartorius / Germany
32	Sodium citrate tube	Jordan
33	Sterile Cotton	Sudican / Turkey
34	Sterile disposable Syringes 10ml, 20ml	Changzhou Kangfulali Medical thing Co. Ltd / China
35	Tips (yellow, blue)	Jordan
36	UV-transilluminator	Major Science, china
37	Veriti™ 96-Well PCR Thermal Cycler	BioRad, USA
38	Vortex mixer	Quality Lab System, England

2.5: Chemicals and Biological Materials

Table (2-3) shows the chemical and biological materials used in this study as follows:

Table (2-3): The chemicals and biological materials, their suppliers, and their origin.

No.	Name of material	Supplier / Origin
1	Agarose	Promega, USA
2	DNA ladder 100bp-1500bp	
3	Ethanol 96%	Crescent / KSA
4	Ethidium Bromide	Promega/ USA
5	G2 Green Master mix	Promega, USA
6	TAE Buffer (Tris-acetate-EDTA), 40X, Molecular Biology Grade	

2.6: Commercial kits:

This study's commercial kits as mentioned in (Tables 2-4).

Table (2-4): The commercial kits and their suppliers and Origin.

No.	Type of kits	Company/country
1	AFIAS COVID-19 Ab (IgM/IgG) test	Boditech Med Inc., Korea
2	HBsAg ELISA Kit	InTec Products, Inc., Xiamen, China
3	Anti-HBc IgM ELISA Kit	
4	HBe Ab ELISA Kit	ACON Laboratories, Inc., San Diego, U.S.A.
5	HBeAg ELISA Kit	
6	HBV genotype A, B, C, D Real-Time PCR Kit	Sacace – Italy
7	HDV IgG ELISA Kit	Diagnostic Automation, Inc., U.S.A.
8	ReliaPrep™ viral Nucleic acid purification kit	Promega-USA
9	Specific primers for *hepatitis B virus*	Macrogen, Korea
10	Viral Nucleic acid purification kit	Bio-comma - China

2.7. Specimens collection:

Blood specimens were taken from all subjects in the study groups and used for biochemical, hematology, immunological, and genetic analysis. Figure (2-1) illustrates the fundamental steps for laboratory study.

2.7.1: Blood Specimens Preparation and Preservation

Blood specimens were collected by drawing 10 ml from each subject included in this study using sterile 20 ml syringes with sterile needle G-22 for persons with hepatitis B virus diagnosed previously clinically and laboratory. In addition, they were collected in sterile 10ml capacity sterile gel, EDTA, and sodium citrate tubes and labeled with number codes and recorded information according to the questionnaire in appendix I.

2.7.2: Whole blood Specimens

Three ml of withdrawn venous blood was taken, placed in each EDTA and sodium citrate container, mixed with the anticoagulant to avoid clot formation, and then placed on Roller Mixer for CBC in Hematology. Next, the EDTA anticoagulated specimens were used in the laboratory for complete blood count (CBC) and blood groups. In contrast, sodium citrate anticoagulated samples were used for coagulation studies, including international normalized ratio (INR) after centrifugation at 2500 r.p.m. for 15 minutes. Finally, plasma specimens were obtained for use in the mentioned tests.

2.7.3: Serum Specimens

After blood clot formation in the gel tube at room temperature within 30 minutes, clot blood specimens were spinning at 3000 r.p.m. for 10 minutes. Next, separated sera specimens were collected, distributed in 1-1.5ml quantities in sterile containers (Eppendorf, size 1.5ml), labeled, and stored at - 20 °C until used. Finally, use sera specimens for biochemical and immunological markers in the laboratory.

2.7.4: Plasma Specimens

After blood collection in the EDTA tube, these specimens were spinning at 2500 r.p.m. for 15 minutes. Plasma specimens were separated and collected, distributed in 1-1.5ml quantities in sterile containers (Eppendorf, size 1.5ml), labeled, and stored at - 20 °C until used. Plasma specimens were used for viral genetic analysis.

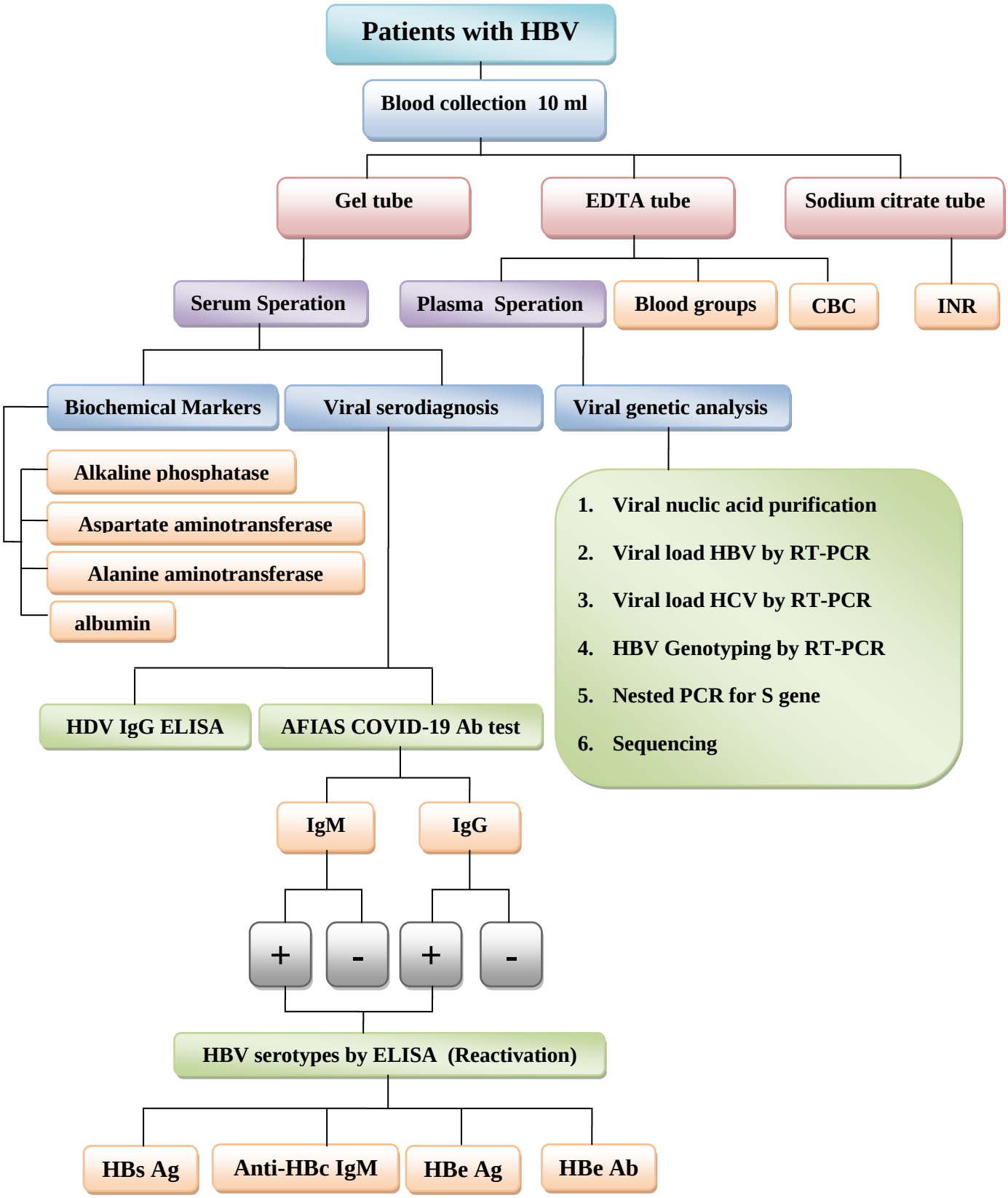


Figure (2-1): Schematic diagram for this study.

2.8: Methods

2.8.1. Hematological Parameter

2.8.1.1. Complete Blood Count (CBC).

Complete blood count was tested by the automated method (Sysmex).

A. Principles of the examination method

Blood specimen collected in EDTA anticoagulant is diluted with a cell pack in a WBC counting container. Then a fixed volume of STROMATOLYSER -WH Solution (1 volume of STROMATOLYSER-WH to 2 volumes of cell pack) was added automatically to obtain a final dilution of 1:500. The addition of STROMATOLYSER-WH lyses the RBC. So the remaining cell stroma is at a level undetectable by the instrument. At the same time, the WBC membrane preserved and stabilized WBC at a level detectable by the device. Therefore, they were counted by the DC method. Hemoglobin was released during RBC lysis and converted to red methemoglobin. A portion of this diluted sample was transferred automatically to the hemoglobin detector, where the absorbance of the red pigment was measured to give blood hemoglobin levels.

B. Examination procedure

a) The STROMATOLYSER-WH was used at a temperature of 15-30'C. Measuring at a temperature above 30°C or below 15°C may give inaccurate WBC count, WBC tri-model size distribution, and hemoglobin level.

b) The bottle's cap was Loosened, removed STROMATOLYSER-WH, and connect to the instrument.

c) Refer to the Operator's Manual of the instrument for detailed information.

2.8.1.2. International Normalized Ratio (INR)

This test was carried out by the manufacturer (Sysmex) (CA-600 Series Systems) using an Automated Coagulation Device. The Clinical and Laboratory Standard Institutes (2017) recommend blood specimens for INR testing in the laboratory be drawn from venous blood and placed straight into a tube with a light blue cap. An anticoagulant is contained in the tube. The sodium citrate

concentration of 3.2 percent is a suitable anticoagulant. The tubes were filled within 90% of the total collection volume. The tube should be flipped a few times for adequate mixing with the anticoagulant, gently and as quickly as feasible. The time between collecting the sample and testing should not be > 24 hours.

2.8.2. Biochemical Markers

This test was performed by the manufacturer (Roche/India) using an Automated Biochemistry Analyzer Device (Cobas C311). Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and albumin were studied in this study.

2.8.3. Viral serodiagnosis

2.8.3.1: HDV IgG test

Diagnostic kits for Hepatitis D virus were detected in all specimens by enzyme-linked immunosorbent assay (HDV IgG ELISA Kit, Diagnostic Automation, Inc., U.S.A.) in human serum or plasma.

A. Test Principles

The DAI HDV IgG ELISA was based on a solid phase, two-step incubation indirect ELISA method.

B. Assay procedure

1. Wells were prepared at three as Negative control (e.g., B1, C1, D1), two as Positive control (e.g., E1, F1), and one as Blank (e.g., A1, neither samples nor HRP Conjugate should be added into the Blank well).
2. 100µl was added of Specimen Diluent into each well except the Blank.
3. 10µl of Positive control, Negative control, and specimen were added into their respective wells except for the Blank. Note: Use a separate disposal pipette tip for each Specimen, Negative Control, and Positive Control to avoid cross-contamination. Mix by tapping the plate gently.

4. Then, the plate was covered with the plate cover and incubated for 30 minutes at 37°C.
5. At the end of the incubation, was removed and discard the plate cover. Wash each well 5 times with diluted Wash Buffer. Each time allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn down the plate onto blotting paper or a clean towel and tap it to remove any remainders.
6. 100µl of HRP-Conjugate was added into each well except the Blank.
7. Then, the plate was covered with the plate cover and incubated for 30 minutes at 37°C.
8. At the end of the incubation, was removed and discard the plate cover. Wash each well 5 times with diluted Wash Buffer. Each time allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn down the plate onto blotting paper or a clean towel and tap it to remove any remainders.
9. 50µl of Chromogen A and 50µl of Chromogen B solutions were added to each well, including the Blank. Incubate the plate at 37°C for 15 minutes, avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and HDV IgG positive sample wells.
10. 50µl of Stop Solution was added into each well and mixed gently. As a result, intensive yellow color develops in Positive control and HDV IgG positive sample wells.
11. Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at 450nm. Suppose a dual filter instrument is used, set the reference wavelength at 630nm. Calculate the Cut-off value and evaluate the results. (Note: read the absorbance within 10 minutes after stopping the reaction).

C. Calculation of results

The cut-off (CO) value was calculated by the mean absorbance value for three negative control and then calculated as follow:

$$\text{Cut-off value} = N_C + 0.21$$

Positive: ratio absorbance is ≥ cut-off value

Negative: ratio absorbance is < cut-off value

2.8.3.2: COVID-19 Ab test

Diagnostic kits for SARS-CoV-2 IgM and IgG were detected in all specimens by an automatic fluorescent immunoassay system (COVID-19 Ab IgM/IgG AFIAS kit, Boditech Med Inc., Korea) in human serum or plasma.

A. Test Principle

This test employs a sandwich immunodetection in which fluorescence-labeled conjugates in dried detection buffer (DB) bind to antibodies in the sample, forming antibody-antigen complexes and migrating onto the nitrocellulose matrix they are caught by immobilized anti-human IgG and anti-human IgM on the test strip. The more antigen-antibody complexes form the other antibodies in a sample, resulting in a more excellent fluorescence signal on the detector antigen, which was processed to reveal anti-CoV IgG and IgM concentrations in the Specimen (Trivedi *et al*., 2019).

B. Contents

Cartridge Box contains (Cartridge, Pipette tip (zipper bag), C- tip (zipper bag), Spare cartridge zipper bag, ID chip, and instruction for use).

C. Test Procedure

1. The general model was selected in the instrument for AFIAS tests.
2. 100 µl of the sample was taken (serum) with a pipette and then dispensed into the cartridge sample well.
3. The cartridge was inserted into the cartridge holder.
4. A tip was inserted into the tip hole of the cartridge.
5. The 'START' icon on the screen was tapped.

6. Test results were displayed on the screen after 10 minutes.

D. Interpretation of test result

- The AFIAS measurement instrument measures the test outcome immediately and shows the results as Positive, Negative, or Indeterminate.
- Ancillary value was served in the form of a cut-off index (COI), as shown in tables (2-5).

Table (2-5): Cut-off index for IgG and IgM of SARS-CoV-2

Cut-off index (COI)	Result	Note
< 0.9	Negative for IgG and IgM	No need to retest
0.9 ≤ Titer < 1.1	Indeterminate	Need to retest
≥ 1.1	Positive for IgG and IgM	Need to a confirmation test

2.8.3.3. HBs Ag test

Diagnostic kits for Hepatitis B virus surface antigen were detected in all specimens by enzyme-linked immunosorbent assay (HBsAg ELISA Kit, InTec Products, Inc., Xiamen, China) in human serum or plasma.

A. Test Principle

This test is a double antibody "sandwich" immunoassay, which employs specific anti-HBsAg antibodies.

B. Assay procedure

1. All Reagents and Specimens were Brought to room temperature (18-25°C) for the assay Swirl gently before use

2. Wells were prepared at one for Blank, two for Negative Control, two for Positive Control, and one for each specimen. Write down the serial numbers for the Controls and Specimens on the datasheet.

3. 20µl specimen Diluent was added to each well.

4. 100µl specimen was added. Negative Control and Positive Control to each appropriate well according to the datasheet (Reserve 1 well for the bank)

5. Tap the plate to mix.

6. The plate was incubated in a 37°C water bath or incubator for 60minutes.

7. 50µl Enzyme Conjugate working solution was added to each well.
8. The plate was incubated in a 37°C water bath or incubator for 30 minutes.
9. Each well was washed five times with wash buffer by wash procedure:
   A. Washing was performed strictly according to the instructions, as incomplete washing will adversely affect the test outcome.
   B. The good contents were completely aspirated into a waste flask. Then the wells were filled with wash buffer (350µl or more). Avoid overflow.
   C. Ensure no fluid remains on the strip holder and strips after the last aspiration (e.g., by blotting with absorbent tissue).
10. 50µl Color A and 50µl Color B were added to each well, and tap the plate to mix.
11. The plate was incubated in a 37°C water bath or incubator for 30 minutes.
12. 50µl Stopping solution was added to each well (maintain the same pipetting sequence and time intervals used for color A/B); tap the plate to mix.
13. Read the absorbance of the solution in each well at 450 and 630nm as reference (dual-wavelength).

C. Calculation of results

The cut-off (CO) value was calculated by the mean absorbance value for two negative control and then calculated as follow:

Cut-off value = NCx* 2.1

Positive: ratio absorbance is ≥ cut-off value

Negative: ratio absorbance is < cut-off value

2.8.3.4. Anti-HBc IgM test

Hepatitis B virus core antibody was detected in all specimens by enzyme-linked immunosorbent assay (Anti-HBc IgM ELISA Kit, InTec Products, Inc., Xiamen, China).

A. Test Principle

The Advanced Anti-HBc IgM Test is a competitive ELISA-based immunoassay.

B. Assay procedure

1. All reagents and specimens were brought to room temperature (18~25°C). Swirl gently before use. Adjust incubator to 37±1 °C.
2. The numbers of specimens and the wells were written on the datasheet. Two wells for blanks, five additional wells for the controls, and one for each sample.
3. 50µl of control (3 negative controls and two positive controls), and each specimen was added into wells (Reserve 2 wells for blanks).
4. 50µl enzyme conjugate was added into each specimen well and controlled well except for the Blank.
5. Gently tap the plate to mix the liquid in the wells thoroughly; do not splash the liquid onto the slip.
6. The plate was incubated at 37°C for 60 minutes in an incubator. Then, balance the plate at room temperature for 5 minutes.
7. Each well was washed five times with a wash buffer.
8. 50µl color A and 50 µl color B were added to each well.
9. The plate was incubated at 37°C for 15 minutes.
10. 50µl stop solution was added to each well, and gently tap the plate.
11. Optical density was measured with an ELISA reader at 450nm (single wavelength) or 450nm and 630nm as reference (dual-wavelength).

C. Calculation of results

Positive control: (C1+C2)/2

Negative control: (C1+C2+C3)/3

The cut-off (CO) value was calculated as follow :

Cut-off value = NCx* 0.2

Positive: ratio absorbance is ≥ cut-off value

Negative: ratio absorbance is < cut-off value

2.8.3.5. HBe Ag test

Hepatitis B virus envelope antigen was detected in all specimens by enzyme-linked immunosorbent assay (HBeAg ELISA Kit, ACON Laboratories, Inc., San Diego, U.S.A.).

A. Test Principle

The HBe Ag EIA Test Kit was a solid phase qualitative enzyme immunoassay based on the sandwich principle for detecting HBe Ag in human serum or plasma.

B. Assay procedure

1. Working wash Buffer was prepared by diluting the Concentrated Wash Buffer at 1:25. Next, pour the bottle containing the concentrated wash buffer into a graduated cylinder and fill it with freshly distilled or deionized water to 1000 mL for 96 wells/plate testing. The Working Wash Buffer is stable for two weeks at 15-30°C.
2. Leave A1 as Blank well.
3. 50 µL of Negative Control was added in wells B1 and C1. (Blue Reagent)
4. 50 µL of Positive Control was added in wells D1 and E1. (Red Reagent)
5. 50 µL of the specimen was added to assigned wells starting at F1.
6. 50 µL of Conjugate was added to each well except for the Blank well. (Red Reagent).
7. 50 µL of Neutralization Solution was added to each well except for the Blank well. (Green Reagent).
8. Mix gently by swirling the microwell plate on a flat bench for 30 seconds.
9. The microwell plate was Covered with the Plate Sealer and incubated in a water bath or an incubator at 37°C ± 2°C for 30 minutes ± 2 minutes.
10. The plate Sealer was removed.
11. Each well was washed five times with 350 pL of Working Wash Buffer per well, removing the liquid .

12. The microwell plate was turned upside down on absorbent tissue for a few seconds. Ensure that all wells have been thoroughly washed and dried.

13. 50 µL of Substrate A was added to each well (Clear Reagent)

14. 50 µL of Substrate B was added to each well. (Clear Reagent)

Note: A clear or light blue color should develop m wells containing Positive specimens.

15. Mix gently; then, the microwell plate was covered with Plate Sealer and incubated in a water bath or incubator at 37°C ± 2cC for 15 min ± 1 min.

16. The Plate Sealer was removed.

17. 50 µL of Stop Solution was added to each well. (Clear Reagent)

Note: A clear or light yellow color should develop m wells containing Positive specimens.

18. Read at 450 / 630-700nm within 30 minutes.

C. Calculation of results

Negative control absorbance = (C1+C2)/2

Blank absorbance = 0.002

NCx= Negative control absorbance – Blank absorbance

Calculated the cut-off (CO) value as follow :

Cut-off value = NCx* 2.1

Positive: ratio absorbance is ≥ cut-off value.

Negative: ratio absorbance is < cut-off value.

2.8.3.6. HBe Ab test

Hepatitis B virus envelope antibody was detected in all specimens by enzyme-linked immunosorbent assay (HBeAb ELISA Kit, ACON Laboratories, Inc., San Diego, U.S.A.).

A. Test Principle

The HBe Ab EIA Test Kit is a solid phase qualitative enzyme immunoassay based on the competitive principle for detecting HBeAb, including IgG, IgM, and IgA antibodies in human serum or plasma.

B. Assay procedure

1. Working wash Buffer was prepared by diluting the Concentrated Wash Buffer at 1:25. Next, pour the bottle containing the concentrated wash buffer into a graduated cylinder and fill it with freshly distilled or deionized water to 1000 mL for 96 wells/plate testing. The Working Wash Buffer is stable for two weeks at 15-30°C.
2. Leave A1 as Blank well.
3. 50 µL of Negative Control was added in wells B1 and C1. (Blue Reagent)
4. 50 µL of Positive Control was added in wells D1 and E1. (Red Reagent)
5. 50 µL of the specimen was added to assigned wells starting at F1.
6. 50 µL of Conjugate was added to each well except for the Blank well. (Red Reagent).
7. 50 µL of Neutralization Solution was added to each well except for the Blank well. (Green Reagent).
8. Mix gently by swirling the microwell plate on a flat bench for 30 seconds.
9. The microwell plate was covered with the Plate Sealer and incubated in a water bath or an incubator at 37°C ± 2°C for 30 minutes ± 2 minutes.
10. The Plate Sealer was removed.
11. Each well was washed five times with 350 pL of Working Wash Buffer per well, removing the liquid .
12. The microwell plate was turned upside down on absorbent tissue for a few seconds. Ensure that all wells have been thoroughly washed and dried.
13. 50 µL of Substrate A was added to each well (Clear Reagent)
14. 50 µL of Substrate B was added to each well. (Clear Reagent)

    Note: A clear or light blue color should develop m wells containing Positive specimens.
15. Mix gently, and then the microwell plate was covered with Plate Sealer and incubated in a water bath or incubator at 37°C ± 2cC for 15 minutes ± 1 minute.

16. The Plate Sealer was removed.

17. 50 µL of Stop Solution was added to each well. (Clear Reagent)

Note: A clear or light yellow color should develop m wells containing Positive specimens.

18. Read at 450 / 630-700nm within 30 minutes.

C. Calculation of results

Negative control absorbance = (C1+C2)/2

Blank absorbance = 0.002

NCx= Negative control absorbance – Blank absorbance

Calculated the cut-off (CO) value as follow :

Cut-off value = NCx* 0.2

Positive: ratio absorbance is ≥ cut-off value

Negative: ratio absorbance is < cut-off value

2.8.4: Viral genetic analysis

2.8.4.1. Viral nucleic acid purification kit (extraction).

This method was made according to the virus DNA/RNA purification Kit supplemented by the manufacturing company (Bio-comma limited, 518118P.R. China).

First: Protocol

Part I: Blood Specimen Preparation

Adequate blood samples (plasma) were Prepared for late use

Part II: Extraction by the nucleic acid extraction system

A. Approximately 100-300µL of preparation specimen was taken and 20µL of Proteinase K (with/without obtaining DNA / RNA specimens, respectively) into well no. 1 of MB32- virus

B. The MB32- virus was placed into the nucleic acid extraction system and performed magnetic adsorption and purification according to the procedure set out in the table below (the nucleic acid extraction system is the Bio-comma M32 nucleic acid extraction system).

C. The solution is well no. 5 is the extracted nucleic acid.

Second: Bio-comma-virus procedure

Run	Well no.	Name	Standby (Min)	Mix (Min)	Volume (µL)	Speed (1-3)	Mag (Sec)	Temp. (40-80°C)
✓	3	Magnetic Beed Transfer	0	1	730	2	15	0
✓	1	Mixture	0	10	1000	3	15	0
✓	2	Wash I	0	2	500	3	15	0
	1	Mixture	0	7	1000	3	15	0
	2	Wash I	0	2	500	3	15	0
✓	3	Wash II	0	1	730	3	15	0
✓	5	Elution	5	3	100	1	30	65
✓	3	Magnetic Beed Transfer	0	1	730	3	0	0

2.8.4.2: Viral load kit

The viral load of patients infected with HBV was determined using real-time PCR for quantitative detection of hepatitis B and C virus in human plasma based on the use of some hospital results and use of the Kit of viral load HBV/HCV Real-Time Amplification test according to the manufacturing company (Sacace 22100-Como-Italy).

2.8.4.2.1: HBV Viral load

The Kit **of HBV Real-TM Quant Dx** was a Real-Time Amplification test for the Quantitative detection of Hepatitis B Virus in human plasma and the simultaneous detection of an HBV-specific Internal Control (IC) by dual-color detection according to the manufacturing company (Sacace 22100-Como-Italy).

A. Principle of Assay

HBV DNA was extracted from plasma, amplified using real-time amplification, and detected using fluorescent reporter dye probes specific for HBV or HBV IC. During each round of thermal cycling, amplification products dissociate into single strands at a high temperature allowing primer annealing and extension as the temperature is lowered.

Exponential amplification of the product was achieved through repeated cycling between high and low temperatures, resulting in a billion-fold or greater amplification of target sequences. Amplification of both targets (HBV and IC) takes place simultaneously in the same reaction. Monitoring the fluorescence intensities in Real-Time allows the detection and quantification of the accumulating product of the reaction tube after the real-time amplification.

Internal Control (IC) serves as extraction and an amplification control for each individually processed specimen and identifies possible inhibition. IC was detected in a channel other than the HBV DNA. HBV-ICL was a lyophilized Internal Control and represented recombinant DNA-containing structure carried through all analysis steps from nucleic acid extraction to PCR amplification-detection. The presence of HBVIC allows not only to monitor the extraction procedure and check possible PCR inhibition but also to verify possible losses of the DNA during the extraction procedure, thus enabling to calculate of precisely the HBV viral load, and results are reported in International Units/mL (IU/mL).

B. Materials Provided

1. **Sacace HBV Real-TM Quant Dx Amplification Reagent Kit, RT-PCR reagent pack (** 96vials (0.2 ml) with lyophilized amplification reagents).
2. **Sacace HBV Real-TM Quant Dx Control Kit[1,2], CONTROL INT** (4 vials with lyophilized reagent HBV-IC-L).

   Control 1 (4 vials with lyophilized reagent HBV-Pos1 –L C+, Sacace HBV Real-TM Quant Dx High Positive Control)

   Control 2 (4 vials with lyophilized reagent HBV -Pos2 -L C+ , Sacace HBV Real-TM Quant Dx Low Positive Control).

   Control – ve (4,0 vials, 4,0 ml per vial with Negative Control).

3. **Sacace HBV Real-TM Quant Dx Calibrator Kit** [1,2]

   CAL 1 (4 vials with lyophilized reagent HBV Quantitative Standard 1)

   CAL 2 (4 vials with lyophilized reagent HBV Quantitative Standard 2)

   [1] Standards' and controls' concentrations are specific for every lot.

   [2] must be used during the sample preparation procedure.

C. Storage Reagent preparation.

All HBV Real-TM Quant Dx PCR kit components must be stored at +2-8ºC when not in use. All HBV Real-TM Quant Dx PCR kit components are stable until the label expires. The shelf life of reagents before and after the first use is the same unless otherwise stated. Current studies refer to EDTA or citrate plasma as the most suitable sample materials for HBV detection.

D. Reagents preparation

Before starting any **HBV Real-TM Quant Dx** protocol, the following reagents were prepared:

1. The required amount of controls, calibrators, and lyophilized centrifuges were briefly selected.
2. Negative Control (CONTROL -ve) was added to the table (2-6) below :

Table (2-6) Preparation of calibration and control for HBV viral load.

Lyophilized reagent l	Control - ve, µl
CAL 1	1100
CAL 2	1100
CONTROL 1	1100
CONTROL 2	1100
CONTROL INT	300

3. Close the tubes and incubate all tubes for 2 min at room temperature. Vortex periodically.
4. Centrifuge the tubes for 5 sec.
5. Dissolved reagents must be stored at 2-8 °C and always protected from light for up to 30 days (do not freeze!).

The manufacturer's instructions carried out DNA extraction. 10µl of CONTROL INT during the DNA isolation procedure was added directly to the sample/lysis mixture in all samples, controls, and calibrators.

E. Assay Calibration.

The quantitative standards CAL1 and CAL2 must be treated the same way as patient specimens. Therefore, before the first use of a new lot of **HBV Real-TM Quant Dx,** 6 calibrators run must be performed beginning from the DNA extraction procedure to generate a calibration curve (two calibrators are run in replicates of three):

1. For each calibration run, three sample preparation tubes were prepared for CAL 1 and 3 for CAL 2.
2. 10 µl of CONTROL INT was added to each tube
3. CAL1 and CAL2 were added to the appropriate tubes in the quantity indicated in the manual of the DNA purification kit.

F. Real-Time PCR Sample Procedure:-

1. The requested quantity of reaction tubes was prepared with lyophilized reagents to perform PCR of extracted samples and controls.
2. **50µl** of eluted samples were added to obtain from the DNA purification step.
   - ❖ Close the tubes and transfer them into the Real-Time PCR instrument.
   - ❖ Create a temperature profile on your Real-time instrument as follows in (Table 2-7):

Table (2-7): Temperature profile of HBV –viral load of RT-PCR.

Stage	Temp.°C	Time	Fluorescence Detection	Cycle Repeats
Hold	95	15min	-	1
Cycling	95	5 s	-	5
	60	20s	-	
	72	15s	-	
Cycling 2	95	5s	-	40
	60	30s	FAM/Green, JOE/ Yellow /HEX	
	72	15s	-	

G. Result Interpretation:-

The **FAM/Green** wavelength monitors the Internal control result, While the Joe/Yellow/HEX refers to the positive viral load of HBV nucleic acid.

2.8.4.2.2: HCV Viral load

The Kit **of HCV Real-TM Quant Dx** was a Real-Time Amplification test for the Quantitative detection of Hepatitis C Virus in human plasma and the simultaneous detection of an HCV-specific Internal Control (IC) by dual-color detection according to the manufacturing company (Sacace 22100-Como-Italy).

A. Principle of Assay

HCV RNA was extracted from plasma, amplified using real-time amplification, and detected using fluorescent reporter dye probes specific for HCV or HCV IC. During each round of thermal cycling, amplification products dissociate into single strands at a high temperature allowing primer annealing and extension as the temperature is lowered.

Exponential amplification of the product was achieved through repeated cycling between high and low temperatures, resulting in a billion-fold or greater amplification of target sequences. Amplification of both targets (HCV and IC) takes place simultaneously in the same reaction. Monitoring the fluorescence intensities in Real-Time allows the detection and quantification of the accumulating product without re-opening the reaction tube after the real-time amplification.

Internal Control (IC) serves as extraction and an amplification control for each individually processed specimen and identifies possible inhibition. IC was detected in a channel other than the HCV RNA. HCVIC-L was a lyophilized Internal Control and represented recombinant RNA-containing structure carried through all analysis steps from nucleic acid extraction to PCR amplification-detection. The presence of HCV Rec IC allows monitoring

of the extraction procedure, checking possible PCR inhibition, and verifying possible losses of the RNA during the extraction procedure, thus calculating the HCV viral load precisely. The assay was standardized against the 4th WHO International Standard for Hepatitis C Virus for Nucleic Acid Amplification Techniques (NIBSC code: 06/102 15), and results are reported in International Units/Ml (IU/mL).

B. Materials Provided

4. **Sacace HCV Real-TM Quant Dx Amplification Reagent Kit, RT-PCR reagent pack (** 96vials (0.2 ml) with lyophilized amplification reagents).
5. **Sacace HCV Real-TM Quant Dx Control Kit[1,2], CONTROL INT** (4 vials with lyophilized reagent HCV-IC-L).

   Control 1 (4 vials with lyophilized reagent HCV-Pos1 –L C+ , Sacace HCV Real-TM Quant Dx High Positive Control)

   Control 2 (4 vials with lyophilized reagent HCV -Pos2 -L C+ , Sacace HCV Real-TM Quant Dx Low Positive Control).

   Control – ve (4,0 vials, 4,0 ml per vial with Negative Control).
6. **Sacace HCV Real-TM Quant Dx Calibrator Kit [1,2]**

   CAL 1 (4 vials with lyophilized reagent HCV Quantitative Standard 1)

   CAL 2 (4 vials with lyophilized reagent HCV Quantitative Standard 2)

   [1] Standards' and controls' concentrations are specific for every lot.

   [2] must be used during the sample preparation procedure.

C. Storage Reagent preparation.

All HCV Real-TM Quant Dx PCR kit components must be stored at +2-8ºC when not in use. All HCV Real-TM Quant Dx PCR kit components are stable until the label expires. The shelf life of reagents before and after the first use is the same unless otherwise stated. Current studies refer to EDTA or citrate plasma as the most suitable sample materials for HCV detection.

D. Reagents preparation

Before starting any **HCV Real-TM Quant Dx** protocol, the following reagents were prepared:

1. The required amount of controls, calibrators, and lyophilized centrifuges were briefly selected .
2. Negative Control (CONTROL -ve) was added to (table 2-8) below :

Table (2-8) Preparation of calibration and control for HCV viral load.

Lyophilized reagent l	Control - ve , µl
CAL 1	1200
CAL 2	1200
CONTROL 1	1200
CONTROL 2	1200
CONTROL INT	300

3. Close the tubes and incubate all tubes for 2 min at room temperature. Vortex periodically.
4. Centrifuge the tubes for 5 sec.
5. Dissolved reagents must be stored at 2-8 °C and always protected from light for up to 30 days (do not freeze!).

The manufacturer's instructions carried out RNA extraction. 10µl of CONTROL INT during the RNA isolation procedure was added directly to the sample/lysis mixture in all samples, controls, calibrators

E. Assay Calibration.

The quantitative standards CAL1 and CAL2 were treated the same way as patients' specimens. Therefore, before the first use of a new lot of **HCV Real-TM Quant Dx,** 6 calibrators run must be performed beginning from the RNA extraction procedure to generate a calibration curve (two calibrators are run in replicates of three):

1. For each calibration run, three sample preparation tubes were prepared for CAL 1 and 3 for CAL 2.

2. 10 µl of CONTROL INT was added to each tube
3. CAL1 and CAL2 were added to the appropriate tubes in quantity indicated in the manual of the RNA purification kit.

F. Real-Time PCR Sample Procedure:-

1. The requested quantity of reaction tubes was prepared with lyophilized reagents to perform PCR of extracted samples and controls.
2. **50µl** of eluted samples were added to obtain from the RNA purification step.
   - ❖ Close the tubes and transfer them into the Real-Time PCR instrument.
   - ❖ Create a temperature profile on your Real-time instrument as follows (table 2-9).

Table (2 -9): Temperature profile of HCV viral load of RT-PCR.

Stage	Temp.°C	Time	Fluorescence Detection	Cycle Repeats
Hold	50	15min	-	1
Hold	95	15min	-	1
Cycling	95	5 s	-	5
	60	20s	-	
	72	15s	-	
Cycling 2	95	5s	-	40
	60	30s	FAM, JOE/HEX/Cy3	
	72	15s	-	

G. Result Interpretation:-

The **FAM/Green** wavelength monitors the Internal control result, While the Joe/Yellow/HEX refers to the positive viral load of HCV nucleic acid.

2.8.4.3: Hepatitis B virus genotyping kit

This method was made according to the HBV Real-Time PCR kit to detect and differentiate hepatitis B virus genotypes A, B, C, and D in HBV-positive clinical material supplemented by the manufacturing company (Sacace 22100 – Como – Italy). HBV Genotype A, B, C, D Real-Time PCR kit is an in

vitro nucleic acid amplification test for detecting and differentiating HBV DNA genotypes A, B, C, and D in HBV-positive clinical material (blood plasma) by using real-time hybridization-fluorescence detection. Therefore, HBV Genotype A, B, C, D Real-Time PCR kit only in HBV-positive clinical material previously tested by the HBV NAT method.

A. Principle assay

HBV genotype detection includes DNA isolation from biological materials and real-time PCR amplification of HBV DNA. HBV detection by the polymerase chain reaction (PCR) amplifies pathogen genome-specific regions using specific HBV genotype primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of the amplified product without re-opening the reaction tubes after the PCR run. HBV Genotype A, B, C, D Real-TM PCR kit uses "hot-start" Taq polymerase, significantly reducing the frequency of nonspecifically primed reactions.

B. Material Provided

Reagent	Volume (ml)	Amount
PCR-mix-1-FRT HBV-G	0.6	1 tube
PCR-mix-2-TM	0.3	1 tube
Hot Start TaqF Polymerase	0.03	1 tube
HBV DNA B/A types (C+)	0.2	1 tube
HBV DNA C/D types (C+)	0.2	1 tube
DNA-buffer	0.07	1 tube
Negative Control (C–)	1.2	2 tubes

C. Protocol

1. Before starting work, thaw, vortex, and quick spin all kit reagents, ensuring no drops on the caps of the tubes.
2. PCR tubes were taken to amplify clinical and control samples (including one negative control of extraction and two amplification controls).

3. The reaction mixture was prepared and mixed in a new sterile tube with the reagents per one reaction:

- ❖ 10 µl of PCR-mix-1-FRT HBV-G,
- ❖ 5 µl of RT-PCR-mix-2-TM,
- ❖ 0,5 µl of Hot Start Taq F Polymerase

Thoroughly vortex and quick spin the mixture, ensuring no drops on the caps of the tubes.

4. 15 µl of the prepared reaction mixture was added to each PCR tube.

5. 10 µl of DNA samples isolated from the clinical samples were added to each PCR tube.

6. Run the **control reactions:**

C -	Add **10 µl of the DNA** sample extracted from the Negative Control to the tube labeled C– (Negative Control of Extraction)
C +	Add **10 µl of Positive Control B/A types (C+)** to the tube labeled C+B/A (Positive Control of Amplification).
	Add **10 µl** of **Positive Control C/D types (C+)** to the tube labeled C+C/D (Positive Control of Amplification)
NCA	Add **10 µl** of **DNA buffer** to the tube labeled NCA (Negative Control of Amplification).

D. Amplification Program

Step	Plate-type instruments		
	Temperature, ° C	Time	Cycles
1	95	15 min	1
2	95	5 s	5
	60	20 s	
	72	15 s	
3	95	5 s	40
	60	30 s Fluorescence acquiring	
	72	15 s	

E. Data Analysis

- ❖ **The HBV genotype A DNA is detected in the Cy5/Red channel**
- ❖ **The HBV genotype B DNA is detected in the Rox/Texas Red/Orange channel.**
- ❖ **The HBV genotype C DNA is detected in the FAM/Green channel**
- ❖ **The HBV genotype D DNA is detected in the JOE/HEX/Yellow channel**

The result of amplification is considered positive if the fluorescence curve was characteristic of real-time PCR (sigmoid-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the below table.

:

The result of amplification was considered negative if the fluorescence curve was not S-shaped and if it did not cross the threshold line (the Ct value is absent). As in (Table 2-10).

Table (2-10) Boundary Value of the cycle threshold of HBV –genotyping.

	FAM/ Green	JOE/ Yellow/Cy3	ROX/ Orange/ TexasRed	Cy5/ Red
	HBV C	HBV D	HBV B	HBV A
Sample	**Ct boundary value**			
NCA	-	-	-	-
C–	-	-	-	-
Pos C+	<28	<28	<28	<28
Clinical samples	<38	<38	<38	<38

F. Result Interpretation

The real-time PCR instrument software interprets the results by crossing or not crossing the threshold line by the fluorescence curve.

- **The sample contains HBV type A** if the Ct value detected in the Cy5 channel is less than 38.
- **The sample contains HBV type B** if the Ct value detected in the ROX channel is less than 38.
- **The sample contains HBV type C** if the Ct value detected in the FAM channel is less than 38.
- **The sample contains HBV type D** if the Ct value detected in the JOE/HEX channel is less than 38.

G. Sensitivity of HBV genotyping

The analytical sensitivity of HBV Genotype A, B, C, D Real-TM PCR kit is specified for samples with 5 x 10^2 copies/ml for any HBV type (A, B, C, D)

2.8.4.4: Sequencing of HBV

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:

2.8.4.4.1: HBV DNA extraction from plasma specimens

The achieved procedure according to the method recommended by the manufacturing company (Promega /USA) as follows:

A. Transferred 200 µl of the HBV plasma sample to a 1.5 mL microcentrifuge tube, 20µl Proteinase K solution was added and briefly mixed.

B. After that, 200µl of Cell Lysis Buffer (CLD) was added to the tube. Next, cap and mix by vortexing for at least 10 seconds. Then was done vortexing step is essential for obtaining a good yield.

C. Then, it was incubated at 56°C for 10 minutes.

D. While incubating the plasma sample, place a ReliaPrep™ Binding Column into an empty Collection Tube.

E. The tube was removed from the heating block. Next, 250µl of Binding Buffer (BBA) cap the tube was added and mixed by vortexing for 10 seconds with a vortex mixer .

F. The tube contents were added to the ReliaPrep™ Binding Column, cap it, and placed in a microcentrifuge.

G. Centrifuge for 1 minute at maximum speed. Check the binding column to ensure the lysate has passed through the membrane. If lysate is still visible on top of the membrane, centrifuge the column for another minute.

H. The collection tube was removed containing flowthrough, and discard the liquid as hazardous waste.

I. The binding column was placed into a fresh collection tube. Add 500µl of Column Wash Solution (CWD) to the column and centrifuge for 3 minutes at maximum speed. Discard the flowthrough.

J. Step 11 was repeated twice for a total of three washes.

K. The column was placed in a new 1.5ml microcentrifuge tube.

L. 50–200µl of Nuclease-Free Water was added to the column. Centrifuge for 1 minute at maximum speed.

M. The ReliaPrep™ Binding Column was discarded and saved eluate. Do not reuse binding columns or collection tubes.

2.8.4.4.2: Primer preparation for nested PCR

Nuclease-free water was used to dissolve all lyophilized primers, as shown in (Table 2-11). Firstly, prepare the primer stock tube to add 300 microliters of nuclease-free water to give a final concentration of 100 picomole/microliter (stock solution). 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 (2-11): Selected primers for 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 N *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	

2.8.4.4.3: Nested-PCR method

DNA from the HBV S gene has amplified using nested PCR depending on two primers called FHBS1, RHBS1, FHBS2, and RHBS2. This method is according to the following steps:

A. Viral DNA was amplified in a final volume of 20 µl reaction mixture as mentioned in table (2-12a) to use primers for the first-round conventional PCR were FHBS1(outer, sense) and RHBS1(outer, antisense).

Table (2-12a): Contents of the Reaction Mixture (Promega)

No.	Contents of the reaction mixture	Volume
1.	G2 Green Master Mix	10 µl
2.	Forward Primer	1 µl
3.	Reverse primer	1 µl
4.	Nuclease free water	-
5.	DNA template	8 µl
Total volume		20 µl

Amplification in first-round conventional PCR condition was subjected to the Applied Biosystems® Veriti® 96-Well Thermal Cycler delivered as shown in table (2-12aa).

Table (2-12aa): Thermal cycling conditions for first-round conventional PCR

Step Type	Temperature	Time	Cycling
Initial Denaturation	95°C	5 min.	1
Denaturation	95°C	30 Sec.	40
Annealing	56°C	30 Sec.	
Extension	72°C	1 min.	
Final Extension	72°C	7 min.	1
Hold	10°C	10 min.	

B. Viral DNA was amplified in a final volume of 25 µl reaction mixture as mentioned in (Table 2-12b) to use primers for the second-round conventional PCR were FHBS2 (inner, sense) and RHBS2 (inner, antisense) were added to the product of the first round.

Table (2-12b): Contents of the Reaction Mixture (Promega)

No.	Contents of the reaction mixture	Volume
1	G2 Green Master Mix	12.5 µl
2	Forward Primer	1 µl
3	Reverse primer	1 µl
4	Nuclease free water	8.5 µl
5	DNA template (from the first round)	2 µl
Total volume		25 µl

Amplification in second-round conventional PCR condition was subjected to the Applied Biosystems® Veriti® 96-Well Thermal Cycler delivered as shown in table (2-12bb).

Table (2-12bb): Thermal cycling conditions for second-round conventional PCR

Step Type	Temperature	Time	Cycling
Initial Denaturation	95°C	5 min.	1
Denaturation	95°C	30 Sec.	40
Annealing	56°C	30 Sec.	
Extension	72°C	1 min.	
Final Extension	72°C	7 min.	1
Hold	10°C	10 min.	

2.8.4.4.4 Agarose Gel Electrophoresis for Amplified Product Detection

A. Preparation of agarose

Confirms successful PCR amplification by agarose gel electrophoresis (Sambrook and Russell, 2001). The prepared agarose gel dissolves 1.5 gm agarose to 100ml of 1x TAE buffer (pH:8) of the previously prepared (added 975 ml dH2O to 25 ml TAE buffer, 40X). First, the solution was heated to boiling (using a Microwave for 2min) until all the gel particles dissolved. Next, a gel was allowed to cool down within 50-60^{o}C and mixed with 10 µg/ml of ethidium bromide (the agarose must be stirred to get mixed and avoid bubbles).

B. The casting of the horizontal agarose gel

The comb was fixed to one end of the tray to make wells to load HBV DNA samples. Next, the agarose solution was poured into the gel tray after sealing the edges with cellophane tapes. The agarose was allowed to solidify at room temperature for 30 minutes. Then, the comb was carefully removed, and the gel was placed in the gel tray. Next, the tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 µl over the surface of the gel at the HBV DNA sample.

C. DNA loading

One microliter of loading dye was applied to each 5µl DNA sample, and samples were added carefully to the individual wells. PCR products were loaded directly. For PCR product, 10µl was directly loaded to well. The electric current (Major Science MP-300V power supply, medigene company, china) was allowed at 100 volts for 10 min, then elevated to 150 volts for 45 min. DNA moves from Cathode to plus Anode poles. UV trans-illuminator 280 nm was used to observe HBV DNA bands (Ethidium bromide-stained bands) in the gel, which were visualized using a digital camera.

2.8.4.4.5: Standard Sequencing

DNA sequencing method was performed for studying the genetic changes analysis of the S gene 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, 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 then centrifuged at 10000 rpm for an additional minute to eliminate any 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. Received the results by email, 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, LC705448, along with the following reference strains for HBV of the S gene.

2.9: Statistical Analysis

Statistical analysis was done by using SPSS (statistical package for social sciences) version 24, in which the researcher used analysis of difference (ANOVA) and independent sample T-test for measuring the data and chi-square (X^2) for categorical data. Set p-value < 0.05 as significant (95%).

3: Results and Discussion

3.1: Infection Rates of HBV Based on Residence.

Among a total of 15 Iraqi governorates (excluding the Kurdistan Region), 141 patients were observed in this study who were admitted to Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City, Center of artificial Kidney, and Center of Hepatology and Gastroenterology in Marjan Medical City, Babylon province during the period extending from December 2020 to June 2021.

The patients observed in this study represented 9 Iraqi governorates as follows: Baghdad 101 (71.63%), Diyala 15 (10.64%), Wasit 12 (8.51%), Anbar 5 (3.55%), Salah Al-Din 3 (2.13%), Babylon 2 (1.42%), Al-Muthanna 1 (0.71%), Qadisiyah1 (0.71%), and Maysan 1 (0.71%).

The highest percentage of HBV infection was identified in Baghdad province, at 71.63%, while the lowest percentage was found in Al-Muthanna, Qadisiyah, and Maysan, at 0.71 % (Figure:3-1).

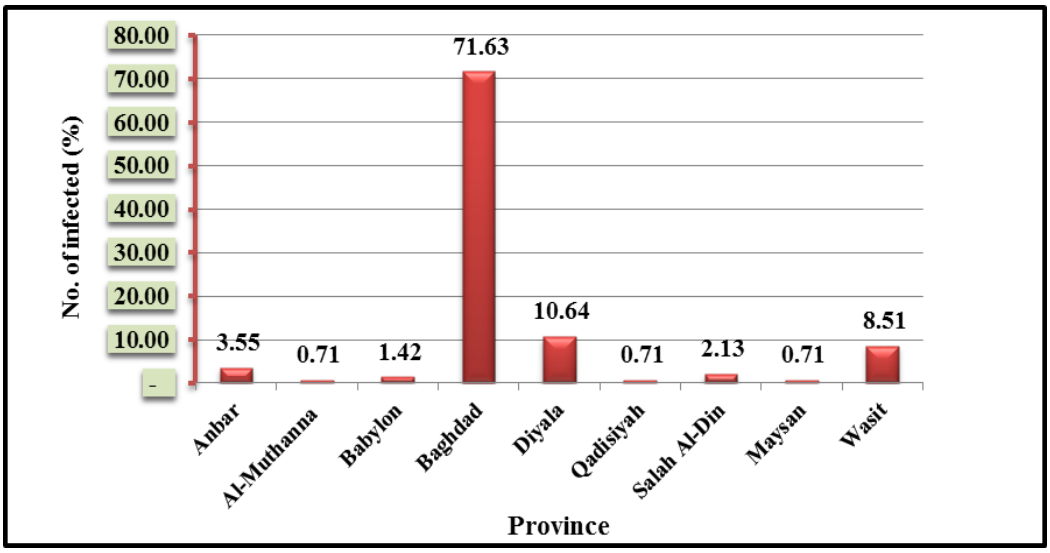


Figure (3-1): The Percentage of HBV Infections in Iraqi Provinces tested in this study

The main reason for the high infection rate in the Baghdad governorate is the high density of the population in Baghdad. In addition, it has the main hospital of Hepatology and Gastroenterology /Baghdad Medical City. Most patients from other governorates come to Baghdad seeking medical consultation and treatment in this Capital's center. As well as most of the health centers and hospitals turning to

COVID-19 epidemiological rooms in Iraq governorates. This finding is substantially compatible with Alhajji's (2021), who revealed that infection rates of HBV were highest in Baghdad (62.4%) compared to the other Iraqi governorates.

Unfortunately, most infection rates throughout Iraq are unavailable due to the lack of epidemiological studies due to the extraordinary political, social, and economic unstable situation from 2003 to 2021. Accordingly, the majority of prior studies in Iraq were focused on HBV prevalence in blood donors, dialysis centers, thalassemia centers, and other disease-related locations compared to the epidemiological studies of HBV among the Iraqi population (Al–Juboury *et al.*, 2010; Al-Rubaye *et al.,* 2016; Othman and Abbas, 2020).

Another study was carried out by AL-Hawaz *et al.* (2014) at Basrah General Hospital, Department of Surgery, between Sept 2012 and Sept 2013 for patients who underwent elective surgery for 1730 patients, with 2.54 % recorded positive infection with HBV.

A 2013 survey of blood donors in Basrah showed that 2.3% of them had hepatitis B virus infection (Al-Rubaye *et al.,* 2016). Another study on blood donors in Babylon governorate showed a seroprevalence of 0.7%. In a survey of the general Iraqi population, the occurrence rate was 1.6% (Al–Juboury *et al.*, 2010). A study in AL-Qadisiyah found that HBV markers among people under low endemicity were 0.38% (Al-Zubaidi and Al-Rubaye, 2016). In a study conducted recently in Dhi Qar province about HBV, the results showed that 634 patients during the period from 2015 to 2019 were infected with viral hepatitis and distributed as follows: public health laboratory (57.3%), central blood bank (40.9%), dialysis center (1.4%) and thalassemia center (0.5%), (Othman and Abbas, 2020).

However, few studies in Iraq have been done to assess the epidemiology of the disease among the Iraqi population. Al-Asadi and AbdulJalil, (2016) assessed the epidemiology of the disease in the Eastern Mediterranean Region, with Iraq being considered to have a moderate endemicity of the hepatitis B virus, with carrier rates ranging from 2% to 5% in the general population

3.2: Immunological Markers

3.2.1: HBV Infection and SARS-CoV-2 Co-infection

To investigate consequences on patients susceptibility to SARS-COV-2 Co-infections. So, through the case information and before conducting the SARS-CoV-2 test for individuals infected with HBV, 39 out of 141 individuals were confirmed positive for SARS-CoV-2 infection by RT-PCR, CT scan, and other tests currently used for diagnosis of this disease.

However, all patients included in the present study were unvaccinated for COVID-19 because the vaccination program in Iraq was started after samples collection and conducting immunological experiments.

The results of the serodiagnosis of SARS-CoV-2 antibody by COVID-19 test among all HBV infected patients. The results showed that only one HBV patient (0.7%) had positive COVID-19-IgM, 80 out of 141 HBV patients (56.7%) gave positive results for COVID-19-IgG antibodies, and 34 out of 141 HBV patients (24.1%) gave positive results for both COVID-19-IgM and IgG antibodies. In contrast, HBV-infected patient's sera showed only 26 out of 141(18.4%) were negative for both COVID-19-IgM and IgG antibodies (HBV alone) (Figures 3-2).

Regarding the 39 patients who were confirmed as SARS-CoV-2 positive (by RT-PCR, CT scan, and other tests currently used for diagnosis of this disease) before conducting the Anti-SARS-CoV-2 test, were represented 48.7% of patients positive for Anti-SARS-CoV-2; IgG (Figures 3-2)

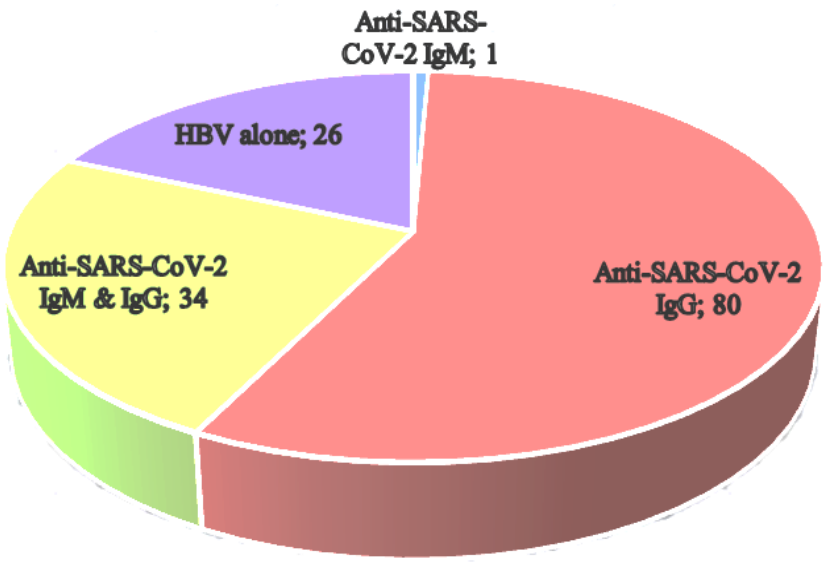


Figure (3-2): The HBV Infected patients and SARS-CoV-2 Co-infected monitored with the Anti-SARS-CoV-2 test.

Since the COVID-19 pandemic, many studies were done on SARS-CoV-2 regarding disease mechanism, ways of spreading, and its impact on co-infection, especially among individuals with transmissible diseases, including HBV. Accordingly, in the current study, the previously HBV infection confirmed specimens by RT-PCR technique were used and recorded in the patient's information case sheet. In addition, IgM and IgG SARS-CoV-2 were tested as a co-infection parameter as some investigators reported that IgM antibodies could be detected three days post-Covid-19-infection as the first line of humoral immunity defense mechanism, followed by IgG antibodies after seven days (Lei *et al.*, 2020; Zhao *et al.*, 2020).

A study by Xiang *et al.* (2020) and Jin *et al.* (2020) indicated that SARS-CoV-2 IgM and IgG positive detection rates gradually increased post-infection and reached more than 80% at 15 days after symptoms onset. In contrast, the IgM-positive detection rate decreased after 20 days. On the other hand, a study carried out by Long *et al.* (2020) showed that both IgM and IgG positive detection rates gradually increased and reached their peak (more than 90% in IgM and 100% in IgG) at 20 days after symptoms onset without a decline in the positive detection rates.

In the meanwhile, no population dealing with these subjects has been found currently, and no similar related research was found in the current publication.

3.2.1.1: HBV Infection Distribution and SARS-CoV-2 Co-infection

3.2.1.1.A: Sex Distribution

A hundred and forty-one HBV patients were enrolled in this study. They included 80 (56.74%) males and 61 (43.26%) females. As shown in (Table 3-1), males had a higher incidence of sex-related co-infection of HBV-infected patients than females, 48.9% and 32.6%, respectively. Thus, the Male: Female ratio in cases of HBV and SARS-CoV-2 was equal to 1:1.5, while in cases of HBV alone was equal to 1:0.73.

Table (3-1): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to sex.

Groups	Sex	Frequency	%	P. value*
HBV and SARS-CoV-2	**Male**	69	48.9	0.024
	Female	46	32.6	
HBV alone	**Ctrl. Male**	11	7.8	0.043
	Ctrl. Female	15	10.6	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

The current results indicated that the sex of the infected person plays an essential role in the predisposition to infection with viral diseases. This study results showed significant differences in infections between males and females. The present study is the first trial to study the correlation between HBV with SARS-CoV-2 and HBV alone. Yet all previous studies had been directed at studying HBV infection. Current results are consistent with the findings of Williams and Wilkins (2005) that infection rates were high in males compared to females. In addition to other studies conducted in Iraq, as the study of Marhoon (2018), which was carried out on 188 and reported that males (115) more than females (66), also the results agreed with Kadham (2018), who found males (130) and females (100) were infected with HBV. In addition to a study by Othman and Abbas, 2020 in Thi-Qar Province -Iraq, from 2015 to 2019, 634 patients with HBV were found to be high in males compared to females. Also, the results agree with Hanash, 2020 who found that HBV infection

was higher in males than females with a ratio of (2.5:1). A study conducted in 2021 by Alhajji mentioned that males were higher infection as 64.5% than females at 35.5% of infections.

The reason is that males represent the most significant proportion of mixing with the external environment and are more socially active due to Arab cultures, especially in Iraq, which point of view equals the number of infections among countries with open cultures such as European countries for example, in addition to some tradition that is allowed by society for males without females, such as cupping (Chinese medicine), which simple people from the general community practice, which is considered one of the important ways to transmit disease, the possibility of narcotics and alcohol consumption, the possibility of males engaging in illegal relationships and other reasons that could be through the everyday use of razors and razors in shop Shaving and males are more than females to travel, so the incidence of males is more than females.

Previous comparative studies on males and females in the last 18 years revealed that females were more resistant to viral infection. This is because they had less infection exposure than males and higher cellular and humoral immune response and innate immune response levels. In addition, females possess more CD4+T-cell and cytotoxic T lymphocytes than males. Thus, it stimulates a more significant number of T cells activated by the interaction of viral antigens with the T cell receptors according to CD4+T-cell, compared to cytotoxic T lymphocytes that were more potent activity combined with over-expression of antiviral and pro-inflammatory genes, many of which contain hormone estrogen response elements in their promoters have been reported in women, so it is unacceptable that females are less susceptible viruses than males due to their more effective antiviral immune defenses (Ruggieri *et al.*, 2018).

3.2.1.1.B: Age groups

The patients were divided into three groups for the subject of HBV with SARS-COV-2 and HBV alone infected according to age. These groups ranged from

lower than 30 years to upper than 60 years. The results revealed that most of the patients in the second group were located 30-60 years with a percentage of 49.6%, and the lowest in the third group with age upper than 60 years as the ratio was 6.4% compared to control (HBV alone) groups (Table 3-2).

Table (3-2): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to age groupings.

Groups	Age	Frequency	%	P. value*
HBV and SARS-CoV-2	**Young (<30 year)**	36	25.5	0.008
	Adult (30 -60 Year)	70	49.6	
	Elderly (> 60 year)	9	6.4	
HBV alone	**Ctrl young (<30 year)**	10	7.1	0.046
	Ctrl adult (30-60 Year)	14	9.9	
	Ctrl elderly (> 60 year)	2	1.4	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

The current studies showed significant differences in the incidence of infection in different age groups studied. For example, (Table 3-2) showed that adults (30-60) have recorded the highest infection rate compared to other age groups.

When comparing the results of this study with other Iraqi studies, it was found that Ataei *et al*. (2019) obtained compatibility with results as they found that HBV infection was significantly higher among (**<60**) older patients. He suggested that due to the length of exposure to the virus and sexual activity.

The present result showed patients less than 20 years that compose the third high group incompatible with Al-Thwani et al., who pointed out that the highest prevalence of HBsAg had been seen in 15-20 years (Al-Thwani *et al.,* 2008). Furthermore, the result of the current study contradicts Marhoon results that found most of the patients were located within (20-30) years (29.83%) and age group of more than 50 years (26.52%). They also observed that the age group (31-40) years and (41-50) years were 13.26% and 19.89%, respectively. Hence, his result

concluded from their result that the age of infection varied according to geographical area and period of sample collection (Marhoon, 2018).

The present results coincide with the previous studies done in Iraq by Al-Waysi and Saleh, who concluded that (30-60y) was the mean age for Chronic Hepatitis B (CHB) patients (Al-Waysi, 2005; Saleh, 2009).

It is well known that protective immunity is not static but varies with age. Although there are distinct features of innate and adaptive immunity from fetal life to adulthood, which may alter the susceptibility of newborn infants to infections compared to adults, increased protection to certain infectious diseases during early life may benefit from a dampened immune response as a result of decreased immune pathology. This concept may offer an alternative interpretation of the different pathological manifestations clinically observed in hepatitis B virus (HBV)-infected patients during the natural history of infection (Hong and Bertoletti, 2017).

3.2.1.1.C: HBV Vaccine Receive

According to the vaccination status of HBV infection, the results are described in (Table 3-3). The results revealed that 5.7% vaccinated for HBV have confirmed cases of HBV with SARS-CoV-2 compared to HBV alone 2.8%. In addition, the results showed that 75.9% of non-vaccinated HBV have confirmed cases of HBV with SARS-CoV-2 compared to HBV alone at 15.6%.

Table (3-3): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to HBV vaccine Received.

HBV vaccine receive	Infection	Frequency	%	P. value*
Vaccinated	**HBV with SARS-CoV-2**	8	5.7	< 0.001
	HBV alone	4	2.8	
Non-vaccinated	**HBV with SARS-CoV-2**	107	75.9	
	HBV alone	22	15.6	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

There are very highly significant differences for people receiving the HBV vaccine through the current study (Table 3-3). For example, some health personnel working in the hospital laboratories found people vaccinated with HBV and became infected. However, they received the vaccine completely (3 doses) and became infected after pricking a needle from an infected person. Therefore, recommended people working in hospitals or centers for the digestive system and liver which are in direct contact with HBV patients to be careful even after receiving three doses of the vaccine.

According to the questionnaire and attributed to appendix I, most of the unvaccinated people (75.9%) may be due to their lack of culture and low education, and they live in villages and work unemployed. Moreover, Iraq's conditions involved embargoes and wars, especially after 2003, and subjected governorates to terrorism, adversely affecting vaccination campaigns and the non-arrival of health teams to some regions because of the wars, which led to the appearance of HBV in many Iraqi areas.

Hepatitis B virus vaccine has been available since 1982 and was given simultaneously with other universally administrated vaccines at birth and after a booster dose that remains highly immunogenic. However, globally the prevalence of the anti-HBsAg carrier state has changed rapidly since the availability and implementation of mass immunization in infants (Dikic *et al.,* 2009).

An important observation that can conclude from this study's results is that the emergence of such a situation in Iraq is contrary to what is expected. The vaccine may limit or restrict infection with the disease, so the possibility of the person receiving the immunization suffers from immunosuppression. Hence, the inability to produce an immune response or the method of administering the vaccine is incorrect (not well preserved), which has lost the efficiency of the vaccine, or that the person suffers from a silent infection without clinical manifestation, this means that the affected person was in an incubation period and consequently when the three doses were given it triggered the action of the pro-virus.

A previous study indicated that the person is still infected if the anti-HBc is positive, suggesting that it is the source of antibodies from the infection and not from the vaccine. Therefore, this study's theoretical results confirm the virus's reactivation in the body, which indicates a sudden increase in the proliferation of HBV (Hoofnagle, 2009).

3.2.1.1.D: HBV Contact

The data in (Tables 3-4) showed the distribution of HBV alone and HBV with SARS-CoV-2 patients according to HBV contact. Among 141 subjects of the study, HBV contact was studied as a demographic distribution, and results presented that the percentage of the study was 26.2% of house contact.

Table (3-4): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to HBV Contact.

Groups	HBV Contact	Frequency	%	P value*
HBV and SARS-CoV-2	**House Contact**	37	26.2	< 0.001
	Work Contact	3	2.1	
	Others	75	53.2	
HBV alone	**House Contact**	7	5.0	
	Work Contact	0	0.0	
	Others	19	13.5	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

There are statistically significant differences in contact with hepatitis B virus through the current study, divided into three groups: House Contact, Work Contact, and Others.

House contact was considered a risk group to infect with HBV since they have direct contact with infected patients, and the risk is increased when the infection is in the asymptomatic stage (Abdul-Husin, 2013).

Clinical laboratory health care workers can become infected through their occupation with blood-borne pathogens by percutaneous injuries and mucocutaneous blood contacts such as cuts, needle sticks, splashes to mucous

membranes, or other body injuries. Otherwise, infected staff can transfer HBV to uninfected patients, further spreading the infection into society (Nejad *et al.*, 2011).

The previous study has proven that body fluids such as saliva, semen, urine, sweat, and tears are also potential sources of HBV transmission, essential factors associated with HBV contact. However, of these body fluids, only serum, saliva, and semen have been demonstrated to be infectious in humans or experimental animal models (Komatsu *et al.*, 2012).

3.2.1.1.E: Disease status.

This study revealed that chronic HBV was more frequent in the status of the diseases (58.2%). While acute, chronic active, and autoimmunity (17%, 3.5%, and 2.8%, respectively) associated with SARS-CoV-2 and HBV patients. Also, the chronic status disease was 12.8% among patients with HBV alone (Table 3-5).

Table (3-5): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to disease status.

Groups	Diseases status	Frequency	%	P. value*
HBV and SARS-CoV-2	**Acute**	24	17.0	< 0.001
	Chronic	82	58.2	
	Chronic Active	5	3.5	
	Autoimmunity	4	2.8	
HBV alone	**Acute**	3	2.1	
	Chronic	18	12.8	
	Chronic Active	3	2.1	
	Autoimmunity	2	1.4	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

The result of the study found there were very high significant differences between the two groups according to the disease status, which was recorded depending on the record of the infected persons (P < 0.001).

The acute case of HBV goes through four stages before beginning with the incubation period, approximately 12 weeks. Then moves to the stage for the onset of symptoms, which develop after the incubation period. First, the high ATL is

observed, lasts 4-12 weeks, and includes anorexia, dark urine, jaundice, and right upper quadrant abdominal discomfort. Acute symptoms are uncommon in infants and children but common in adults. The third phase is a recovery period followed by the normalization of ATL levels. The last phase is the clearance of Hepatitis B surface antigen (HBsAg) in the serum, which follows after a few months of coinciding with the development of anti-HBs. (Villeneuve, 2005).

The chronic case starts in active to last for more than six months, then moves to the chronic stage, which is the most common in humans and the leading cause of death among HBV (Ghadir *et al.*, 2012), which is compatible with the results of our current study in the high incidence of chronic status between the HBV groups.

Chronic hepatitis B is caused by persistent infection with HBV, a unique DNA virus that replicates through an RNA intermediate produced from a stable, covalently closed circular DNA molecule (Lucifora and Protzer,2016; Wei and Ploss, 2021). The course of viral infection and the severity of liver damage are determined by the balance between viral replication and host immune defense mechanisms. Specific cytotoxic CD8 T cells play a critical role in HBV clearance. However, suppressor or regulatory T cells (Treg) inhibit HBV-specific CD8 T cell function in chronic HBV infection, contributing to viral persistence (Fu, 2007).

The immune response to Hepatitis B virus, such as molecular mimicry between HBV antigens and self-proteins, the formation of immune complexes between HBV antigens and antibodies, and apoptosis/tissue damage resulting in the exposure of intracellular antigens to the immune system, all demonstrate this loss of tolerance (Maya *et al.*, 2008). Therefore, Immunosuppressive drugs can reactivate chronic hepatitis B virus infection in persons with autoimmune disorders (Canzoni *et al.*, 2020).

3.2.1.1.F: Liver cirrhosis.

The results of HBV alone and HBV with SARS-CoV-2 are described in (Table 3-6) according to liver cirrhosis. The results revealed that in HBV patients infected with SARS-CoV-2, 5 out of 141(3.5%) with cirrhosis compared with

patients without cirrhosis 110 (78%). In contrast, non-patients (0.00%) only had cirrhosis in the HBV infection group.

Table (3-6): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to liver cirrhosis.

Groups	Liver Cirrhosis	Frequency	%	P. value*
HBV and SARS-CoV-2	**With Cirrhosis**	5	3.5	< 0.001
	Without Cirrhosis	110	78.0	
HBV alone	**With Cirrhosis**	0	0.0	
	Without Cirrhosis	26	18.4	
Total		141	100.0	

***** Different values mean significant differences (P≤0.05).

Clinicians may be concerned about whether HBV-related cirrhosis was associated with poor outcomes in COVID-19. The current study results showed highly significant differences between the two study groups regarding liver cirrhosis. It is worth noting that the present study showed the emergence of five sick cases infected with liver cirrhosis, which does not agree with the results of Xiang and Zheng (2021), who showed significant differences and found only one case. Previous studies on the relationship between liver cirrhosis, HBV, and SARS-CoV-2 are very few or almost non-existent at present, and these results do not agree with the findings of Zhang *et al.* (2020) on the impact of different hepatitis B status (HBV carrier group, hepatitis B/cirrhosis group) on COVID-19. Most HBV carriers do not develop a severe or critical illness, and no significant differences were found in the length of hospital stay, disease severity, and prognosis between the two groups.

In a large cohort study, Marjot *et al.* (2021) enrolled 745 Chronic liver disease patients from 29 countries, of whom 386 had cirrhosis and 359 did not, and mortality was significantly higher in the cirrhotic patients (32% vs. 8%). Mortality increased with Child-Turcotte-Pugh class, which showed for the first time that the stage of liver disease is strongly associated with COVID-19 mortality. The data from other multicenter retrospective studies also supported the conclusion that

patients with liver cirrhosis in COVID-19 had higher mortality and worse prognoses than patients without cirrhosis (Iavarone *et al.*, 2020; Kim *et al.*, 2021). HBV-related cirrhosis only accounted for a small proportion of patients, and most cases of cirrhosis were attributed to nonalcoholic fatty liver disease (24%-32.5%), alcohol-related liver disease (4.6%-24%), and chronic hepatitis C virus infection (24%) (Sarin et al., 2020; Bajaj et al., 2020). More importantly, HBV accounted for the lowest proportion of severe cases and deaths compared with other etiologies. Alcohol-related liver disease rather than HBV was an independent risk factor associated with the outcome of COVID-19. Although the severity of cirrhosis is closely related to mortality and prognosis in COVID-19, the limited data about HBV-related cirrhosis are insufficient to confirm that HBV worsens the clinical outcome (Xiang and Zheng, 2021).

3.2.1.1.G: HBV reactivations

The result in (Table 3-7) showed the distribution of HBV alone and HBV with SARS-CoV-2 patients according to HBV reactivation. Fifty patients did not undergo testing by ELISA selectively as it had nearly similar values to tested patients sera. The current study showed significant differences among HBV with SARS-CoV-2, Reactivation, HBV with SARS-CoV-2, Non- Reactivation, and HBV alone (34.07%, 37.36%, 28.57%, respectively), which a previous description of items 3.2.1 among the diagnosed patients of HBV infected with/without SARS-CoV-2, (81.6%, 18.4%%, respectively), depending on (**3.2.1**).

Table (3-7): Patients HBV Infection Distribution and SARS-CoV-2 Co-infection according to HBV reactivations.

Reactivation	No. of cases	%	% of the total 91 samples tested	P. value*
HBV alone	26	18.4	28.57	0.027
HBV with SARS-CoV-2, Reactivation	31	22.0	34.07	
HBV with SARS-CoV-2, Non- Reactivation	34	24.1	37.36	
HBV with SARS-CoV-2, ND**	50	35.5	-	
Total	141	100.0	100.0	

***** Different values mean significant differences (P≤0.05).

** ND: not determined

Most of the previous studies in the world on hepatitis B virus reactivation were generally focused on and associated with cancer patients and their response to chemotherapy and immunosuppressive drugs, as well as biological therapy. However, the only case regarding Hepatitis B Virus Reactivation Induced by COVID-19 was reported by Aldhaleei *et al.* (2020) from the United Arab Emirates. They reported that the patient with COVID-19 had an acute HBV infection and did not receive any immunosuppressive therapy.

In Iraq, there is no information available on the prevalence of HBV reactivation due to co-infection with other viral pathogens. In addition, there is no information regarding the incidence of HBV with SARS-CoV-2 patients.

This is the first study in Iraq about the incidence of HBV with SARS-CoV-2.

3.2.1.2: Distribution of SARS-CoV-2 with immunological markers according to Sociodemographic and predisposing factors

In the present study, the distribution of SARS-CoV-2 with immunological markers in HBV infected patients according to some Sociodemographic factors (Gender, Age) and predisposing factors (HBV vaccine receive, Diseases Status, HBV Contact, and Liver Cirrhosis) was studied. The results in (Table 3-8) showed significant differences among gender, HBV vaccine received, HBV contact, and liver cirrhosis. On the other hand, there was no significant difference between age and disease status for IgM compared to the control group. However, IgG results showed very significant differences in all demographic distributions compared to the control group.

Table (3-8): Comparison of SARS-CoV-2 demography with Anti-SARS-CoV-2 in HBV-infected patients

Properties		COVID-19			
		IgM mIU/ml		IgG mIU/ml	
		Mean ± SD	P value	Mean ± SD	P value***
Sex	Male*	0.8429 ± 0.86076	0.016	4.8149 ± 2.55139	< 0.001
	Female*	0.7485 ± 0.67222		4.8711 ± 2.49602	
	Con. Male **	0.12484 ± 0.2936		0.37644 ± 0.5036	
	Con. Female **	0.3087 ± 0.23522		0.3313 ± 0.41993	
Age	Young (<30 year) *	0.7972 ± 1.10483	0.072	5.3075 ± 2.86983	0.005
	Adult (30 - 60 Year) *	0.8010 ± 0.61795		4.7981 ± 2.34911	
	Elderly (> 60 year) *	0.8689 ± 0.48113		3.2622 ± 1.72049	
	Con. Young (<30 year) **	0.08589 ± 0.2300		0.44754 ± 0.3450	
	Con. Adult (30 - 60 Year) **	0.24085 ± 0.3629		0.39970 ± 0.4593	
	Con. Elderly (> 60 year) **	0.2400 ± 0.00000		0.3150 ± 0.31820	
HBV vaccine receive	Vaccinated *	0.5912 ± 0.35454	0.011	5.0750 ± 3.09790	< 0.001
	Non-vaccinated *	0.8212 ± 0.80704		4.7824 ± 2.50502	
	Non-vaccinated **	0.2919 ± 0.17145		0.4071 ± 0.40069	
	Vaccinated **	0.2250 ± 0.74285		0.2900 ± 0.46698	
Diseases Status	Acute *	0.7948 ± 0.47828	0.053	4.4356 ± 2.85895	< 0.001
	Chronic *	0.8056 ± 0.89180		4.9437 ± 2.49361	
	Chronic Active *	0.6740 ± 0.31230		3.5560 ± 2.11801	
	Autoimmunity *	0.8675 ± 0.56252		5.5750 ± 2.07285	
	Control **	0.3072 ± 0.19540		0.4184 ± 0.40537	
HBV Contact	House Contact *	0.7130 ± 0.60036	0.012	5.1589 ± 2.46980	< 0.001
	Work Contact *	1.0200 ± 0.49508		5.1467 ± 3.15611	
	Others *	0.8420 ± 0.87721		4.6664 ± 2.53961	
	Control **	0.3023 ± 0.19307		0.4042 ± 0.40369	
Liver Cirrhosis	With Cirrhosis *	0.2880 ± 0.16724	0.02	4.2560 ± 2.40043	< 0.001
	Without Cirrhosis *	0.8286 ± 0.79824		4.8638 ± 2.53105	
	Control **	0.3023 ± 0.19307		0.4042 ± 0.40369	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

Regarding HBV reactivation (Table 3-9) for the two study groups and HBV-infected subjects, the results showed that HBsAg, HBcAb (IgM), and HBeAg in all Sociodemographic factors and predisposing factors have significant differences compared to the control group. As for HBeAb, significant differences were seen in the disease status, HBV contact, and liver cirrhosis. In contrast, compared to the

control group, no significant differences were found among patients regarding gender, age, and vaccine.

Table (3-9): Comparison of SARS-CoV-2 demography with reactivation markers in HBV-infected patients

Properties		Reactivation							
		HBs Ag IU/ml		HBc Ab (IgM) IU/ml		HBe Ag IU/ml		HBe Ab IU/ml	
		Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Sex	Male *	0.5797 ± 0.52597	0.020	0.5942 ± 0.55090	0.019	0.9275 ± 0.89638	0.065	0.6812 ± 0.67503	0.206
	Female *	0.6304 ± 0.60951		0.6522 ± 0.64005		0.9565 ± 0.91788		0.6739 ± 0.66848	
	Control **	0.9231 ± 0.39223		0.9615 ± 0.44549		1.3846 ± 0.69725		0.9231 ± 0.39223	
Age	Young (<30 year) *	0.7500 ± 0.60356	0.008	0.7500 ± 0.60356	0.015	1.1389 ± .89929	0.037	0.7222 ± 0.56625	0.334
	Adult (30-60 Year) *	0.5286 ± 0.53083		0.5571 ± 0.58075		0.8286 ± 0.88418		0.6571 ± 0.72002	
	Elderly (> 60 year) *	0.5556 ± 0.52705		0.5556 ± 0.52705		1.0000 ± 1.00000		0.6667 ± 0.70711	
	Control **	0.9231 ± 0.39223		0.9615 ± 0.44549		1.3846 ± 0.69725		0.9231 ± 0.39223	
HBV vaccine receive	Vaccinated *	1.0000 ± 0.53452	0.003	0.8750 ± 0.35355	0.030	1.5000 ± 0.75593	0.046	1.1250 ± 0.64087	0.064
	Non-vaccinated *	0.5741 ± 0.55034		0.6019 ± 0.59499		0.9074 ± 0.90210		0.6481 ± 0.66014	
	Non-vaccinated **	0.8571 ± 0.35857		0.9524 ± 0.49761		1.3810 ± 0.74001		0.9048 ± 0.43644	
	Vaccinated **	1.2500 ± 0.50000		1.0000 ± 0.00000		1.2500 ± 0.50000		1.0000 ± 0.00000	
Diseases Status	Acute *	0.49329 ± 0.09866	< 0.001	0.53852 ± 0.10770	0.001	0.75719 ± 0.15144	0.004	0.61101 ± 0.12220	0.004
	Chronic *	0.52690 ± 0.05819		0.57185 ± 0.06315		0.88923 ± 0.09820		0.64974 ± 0.07175	
	Chronic Active *	0.83666 ± 0.37417		0.54772 ± 0.24495		1.09545 ± 0.48990		0.54772 ± 0.24495	
	Autoimmunity *	0.50000 ± 0.25000		0.50000 ± 0.25000		0.95743 ± 0.47871		0.81650 ± 0.40825	
	Control **	0.40000 ± 0.08000		0.45461 ± 0.09092		0.70711 ± 0.14142		0.40000 ± 0.08000	
HBV Contact	House Contact *	0.7568 ± 0 .59654	0.002	0.7838 ± 0.62960	0.002	1.1622 ± 0.89795	0.002	0.7838 ± 0.62960	0.034
	Work Contact *	1.0000 ± 0.00000		1.0000 ± 0.00000		2.0000 ± 0.00000		1.3333 ± 0.57735	
	Others *	0.5067 ± 0.52949		0.5200 ± 0.55410		0.7867 ± 0.87446		0.6000 ± 0.67783	
	Control **	0.9231 ± 0.39223		0.9615 ± 0.44549		1.3846 ± 0.69725		0.9231 ± 0.39223	
Liver Cirrhosis	With Cirrhoses *	0.2000 ± 0.44721	0.005	0.2000 ± 0.44721	0.005	0.4000 ± 0.89443	0.024	0.2000 ±0.44721	0.045
	Without Cirrhoses *	0.6182 ± 0.55821		0.6364 ± 0.58619		0.9636 ± 0.89778		0.7000 ± 0.67116	
	Control **	0.9231 ± 0.39223		0.9615 ± 0.44549		1.3846 ± 0.69725		0.9231 ± 0.39223	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

In a retrospective study by Liu *et al.* (2020) on 21 patients with SARS-CoV-2 and HBV co-infection, 19 patients were tested for HBV DNA viral load at least

twice during hospitalization. Of the 19 patients, three patients developed HBV reactivation and manifested as a rapid increase in HBV DNA viral load from undetectable to a high level. These three patients were negative for hepatitis B e antigen and did not receive anti-HBV treatment before admission. Two of the three patients received methylprednisolone during the hospitalization, which may account for the reactivation, and one did not receive any corticosteroids.

The mechanisms of HBV reactivation following infection with SARS-CoV-2 are primarily due to a broken balance between the host's immune state and viral replication. In addition to the host baseline virological indicators, the intensity of glucocorticoids or immunosuppression therapies is a primary risk factor for reactivation of HBV during treatment of COVID-19 (Loomba and Liang, 2017; Shi and Zheng, 2020). Although infection with SARS-CoV-2 has a risk of HBV reactivation, the overall risk is low. One prospective study (Rodríguez-Tajes *et al.*, 2021) evaluated the risk of HBV reactivation in 61 patients with severe COVID-19 and resolved HBV infection (HBsAg-negative, anti-hepatitis B core antibody-positive) undergoing immunosuppressive therapy. After at least 1 month of follow-up, they found no cases developing HBsAg seroconversion, and only two (3%) patients had detectable serum HBV DNA (< 15 IU/mL). Therefore, corticosteroids and immunosuppressants can be selected clinically for patients with severe COVID-19 and coexistent HBV infection. Given the risk of reactivation, the American Association for the Study of Liver Diseases guidelines strongly recommends that anti-HBV treatment be initiated or continued once COVID-19 is diagnosed (Reddy, 2020). At the same time, routine HBV virologic indicators and liver injury-related indicators should be closely monitored during the disease (Xiang and Zheng, 2021).

3.2.2: HBV infection and HDV co-infection

The examination results showed that 92 out of 141 specimens were tested using HDV IgG ELISA (**2.8.3.1)** for diagnosed and confirmed cases with HBV infected by PCR examination, all negative for hepatitis D virus.

The present study found no HBV and HDV co-infection cases among all 92 patients tested. This result was incompatible with Hadi *et al.* (2017), who revealed that 5 cases (5.6%) were positive for HDV IgG in HBV-infected patients.

Dual infections probably depend on aspects such as the endemicity of HDV in the area, the degree of HBV viremia, and the genotypes of HBV and HDV (Husa *et al.*, 2005).

3.3: Result of Hematological Markers

3.3.1: HBV Infection Distribution and SARS-CoV-2 Co-infection According to blood groups

Table (3-10) shows the infection rate for SARS-CoV-2 patients infected with HBV distributed according to blood groups, demonstrating high infection rates among O^{+} and B^{+} (57.4 % and 12.8%, respectively) compared to other blood groups.

Table (3-10): Patient's HBV Infection Distribution and SARS-CoV-2 Co-infection according to blood groups.

Groups	Blood Groups		Frequency	%	P-value*
	Blood type	Rh			
HBV and SARS-CoV-2	O	+ ve	81	57.4	< 0.001
		– ve	5	3.5	
	A	+ ve	6	4.3	
	B	+ ve	18	12.8	
		– ve	2	1.4	
	AB	+ ve	3	2.1	
HBV alone	O	+ ve	22	15.6	
		– ve	0	0.0	
	A	+ ve	0	0.0	
	B	+ ve	3	2.1	
		– ve	0	0.0	
	AB	+ ve	1	0.7	
Total			141	100.0	

*** Different values mean significant differences (P≤0.05).**

The current study aimed to observe the association of the different blood groups on the presenting features and outcomes of HBV alone and SARS-CoV-2 Co-infections. Therefore, significant differences were observed in the presentation and recovery duration among the blood groups.

The results of the relationship between ABO blood groups and HBV infection. The results of the current study suggested that blood group O was higher with HBV infection, giving supportive evidence that statistical association and the biological association between ABO blood groups and HBV infection probably exists. However, this association might be partly attributed to the regional factors due to the high relevance between HBV endemicity and regional health and economic development, which was consistent with some previous studies by Lao *et al.* (2014), Abate and Wolde (2016), and Liu *et al.,* (2018).

This means more measures should be taken to ensure blood safety of the 'universal' blood group O population in high endemic areas because of the large unvaccinated population among the main blood donors in the current era and the window period for detection among the HBV-infected blood donors (Liu *et al.,* 2018).

They reviewed data suggesting that individuals of blood group A were at a higher risk of infection with SARS-CoV-2 and may develop severe COVID-19 outcomes. In contrast, blood group O was considered protective against the infection. However, some of the available studies have been influenced by unaccounted confounders and biases (Shibeeb and Khan, 2022). This concept is inconsistent with our current study.

3.3.2: Distribution of SARS-CoV-2 with a complete blood count according to Sociodemographic and predisposing factors

The total WBC and platelet count results found no significant differences in the two study groups according to Sociodemographic factors (Gender, Age) and predisposing factors (HBV vaccine receive, Diseases Status, HBV Contact, and Liver Cirrhosis) (Table: 3-11).

Table (3-11): Distribution of SARS-CoV-2 with a complete blood count (total WBC and platelet count) according to Sociodemographic and predisposing factors

Properties		Complete blood count			
		Total WBC		Platelets count	
		Mean ± SD	P value	Mean ± SD	P value
Sex	Male*	7.4290 ± 8.54896	0..978	247.5652 ± 97.00038	0.456
	Female*	7.5500 ± 2.88804		229.0000 ± 74.58001	
	Con. Male **	6.6727 ± 2.30785		270.3636 ± 63.30762	
	Con. Female **	7.1400 ± 1.92754		240.2000 ± 59.76765	
Age	Young (<30 year) *	7.2722 ± 2.57012	0.134	232.9722 ± 71.36005	0.800
	Adult (30 - 60 Year) *	6.8671 ± 2.21815		242.3714 ± 98.63271	
	Elderly (> 60 year) *	13.0444 ± 23.63340		251.4444 ± 76.47730	
	Con. Young (<30 year) **	7.5000 ± 2.03470		276.4000 ± 66.32781	
	Con. Adult (30 - 60 Year) **	6.3000 ± 2.02978		235.0000 ± 58.34381	
	Con. Elderly (> 60 year) **	8.6500 ± 1.06066		261.5000 ± 43.13351	
HBV vaccine receive	Vaccinated *	7.5500 ± 3.44840	0.921	260.1250 ± 83.84243	0.709
	Non-vaccinated *	7.4926 ± 7.01609		237.8704 ± 89.33389	
	Non-vaccinated **	6.5524 ± 2.08749		256.2381± 54.90984	
	Vaccinated **	8.3000 ± 0.66833		260.2500 ± 92.24379	
Diseases Status	Acute *	7.2880 ± 2.24320	0.988	229.0000 ± 76.77293	0.164
	Chronic *	7.6049 ± 7.99501		244.7805 ± 92.24605	
	Chronic Active *	6.7600 ± 2.00574		171.6000 ± 48.72166	
	Autoimmunity *	7.3750 ± 3.12130		300.0000 ± 74.67708	
	Control **	6.8520 ± 2.05510		253.5200 ± 63.13354	
HBV Contact	House Contact *	7.0351 ± 2.61855	0.909	235.4595 ± 75.92928	0.364
	Work Contact *	6.4333 ± 1.95533		166.0000 ± 72.38094	
	Others *	7.7373 ± 8.28172		245.4133 ± 94.59831	
	Control **	6.9423 ± 2.06556		252.9615 ± 61.92349	
Liver Cirrhosis	With Cirrhoses *	6.3600 ± 1.45017	0.853	245.8000 ± 31.85436	0.777
	Without Cirrhoses *	7.5282 ± 6.99316		239.8818 ± 90.63494	
	Control **	6.9423± 2.06556		252.9615 ± 61.92349	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

3.3.3: Distribution of SARS-CoV-2 with coagulation factor according to Sociodemographic and predisposing factors

Regarding the coagulation factor in HBV alone and HBV with SARS-CoV-2, results found no significant differences according to sociodemographic factors (sex

and age), while it was found a significant difference according to predisposing factors (disease status and liver cirrhosis) except for HBV vaccine received, and HBV contact compared to control group (Table 3-12).

Table (3-12): Distribution of SARS-CoV-2 with a coagulation factor according to Sociodemographic and predisposing factors

Properties		Coagulation factor	
		INR	
		Mean ± SD	P value***
Gender	Male*	0.9361 ± 0.09472	0.196
	Female*	0.9589 ± 0.18030	
	Con. Male **	0.9927 ± 0.06482	
	Con. Female **	1.0040 ± 0.08773	
Age	Young (<30 year) *	0.17648 ± 0.02941	0.088
	Adult (30 - 60 Year) *	0.10905 ± 0.01303	
	Elderly (> 60 year) *	0.10398 ± 0.03466	
	Con. Young (<30 year) **	0.9940 ± 0.07975	
	Con. Adult (30 - 60 Year) **	0.9971 ± 0.08222	
	Con. Elderly (> 60 year) **	1.0400 ± 0.04243	
HBV vaccine receive	Vaccinated *	0.9225 ± 0.10195	0.346
	Non-vaccinated *	0.9486 ± 0.13819	
	Non-vaccinated **	0.9886 ± 0.06909	
	Vaccinated **	1.0225 ± 0.10563	
Diseases Status	Acute *	0.9524 ± 0.08814	0.015
	Chronic *	0.9341 ± 0.14145	
	Chronic Active *	0.9480 ± 0.08258	
	Autoimmunity *	1.1175 ± 0.20288	
	Control **	1.0024 ± 0.07747	
HBV Contact	House Contact *	0.9759 ± 0.12982	0.070
	Work Contact *	0.9533 ± 0.10693	
	Others *	0.9297 ± 0.13777	
	Control **	0.9992 ± 0.07761	
Liver Cirrhosis	With Cirrhoses *	1.0340 ± 0.18284	0.042
	Without Cirrhoses *	0.9412 ± 0.13246	
	Control **	0.9992 ± 0.07761	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

A study by Aldhaleei *et al.* (2020) reported the first case of hepatitis B virus (HBV) reactivation caused by COVID-19 in a young adult with altered mental status and severe transaminitis. The patient was asymptomatic, hypothermic, his skin was jaundiced with the icteric sclera, with very high levels of aspartate

aminotransferase AST 4,933 U/L (reference: <50 U/L), alanine aminotransferase ALT 4,758 U/L (reference: <40 U/L), alkaline phosphatase (ALP) 212 U/L (reference: 40-129 U/L), and albumin 33 g/L (reference: 35-52 g/L). In addition, his international normalized ratio (INR) was >10 (reference: 0.82-1.20). (AST; 4,933 U/L), and alanine aminotransferase (ALT; 4,758 U/L) levels. It is warranted that patients with abnormal liver functions tend to have an increased risk of COVID-19. Thus, increased attention should be paid to the care of patients with abnormal liver functions, and testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA is warranted in the COVID era.

One clinical result of this case is that SARS-CoV-2 and HBV-infected patients show more severe monocytopenia and thrombocytopenia. In addition, SARS-CoV-2 and HBV-infected patients show more disturbed hepatic function in albumin production and lipid metabolism. Therefore, caution needs to be taken to manage SARS-CoV-2 and HBV-infected patients (Liu *et al.*, 2021).

Dysregulation of immune cells in the blood with SARS-CoV-2 and HBV-infected, where COVID-19 patients showed lower white blood cells (WBC), and reduced lymphocyte counts, mainly causing the low WBC counts. In addition to lower levels of monocyte and higher levels of CD8 T cells noticed in patients with the SARS-CoV-2 and HBV, they showed elevated inflammatory cytokine levels (IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10) between SARS-CoV-2 and HBV-infected or SARS-CoV-2 mono-infected patients (Liu *et al.*, 2021).

In a previous study, lactate dehydrogenase (LDH) and creatine kinase (CK) was noticed as risk factors for severe COVID-19 (Zhang *et al.*, 2020; Zhou *et al.*, 2020), while found Liu *et al.* (2021) that higher levels of creatine kinase indicate a higher risk of disease deterioration for SARS-CoV-2 and HBV patients, (disease severity).

After COVID-19 recovery, the elevation of lymphocyte, monocyte, basophil, eosinophil, T, B, and NK cell counts, AST reduction, and ALB production, could be observed in SARS-CoV-2 and HBV-infected patients. However, the change in red

blood cell and platelet counts was not apparent in both groups (HBV infected with SARS-CoV-2 and SARS-CoV-2 alone) at the time (Liu *et al.*, 2021).

To determine the problem or impacts the SARS-CoV-2 infection patients with HBV through evaluation of different complete blood count parameters, serum biochemistry indicators, and immune responses from people with or without HBV or SARS-CoV-2 and found the following: leukopenia, erythropenia, thrombocytopenia, and moderate liver injury and inflammation are observed in COVID-19 patients. SARS-CoV-2 and HBV-infected did not significantly affect the outcome of COVID-19. However, at the onset of COVID-19, SARS-CoV-2 and HBV coinfected patients presented with more severe monocytopenia and thrombocytopenia and more disturbed hepatic function in albumin production and lipid metabolism. SARS patients with preexisting HBV infections due to acute respiratory distress syndrome development(Liu *et al.*, 2021).

SARS-CoV-2 and chronic HBV-infected patients developed a liver injury, and the proportion of severe COVID-19 was higher in patients with liver injury (Zou *et al.*, 2020). However, they did not observe more severe COVID-19 in patients with HBV infection. Hepatitis B virus reactivation is also a significant concern in COVID-19 patients with chronic HBV infection (Liu *et al.*, 2020). This is mainly associated with COVID-19 management due to immune suppressive corticosteroid therapy or biological therapies such as IL-6 receptor antagonists in patients with current or past HBV exposure (Rodriguez-Tajes *et al.*, 2021). Such patients require HBV DNA load monitoring and treatment with antivirals such as Entecavir or Tenofovir to reduce viral load and hepatitis B flares (Mehta *et al.*, 2020). The fatality rate and recovery rate are comparable between SARS-CoV-2 mono-infected patients and SARS-CoV-2 and HBV coinfected patients, remaining similar between the two groups (Liu *et al.*, 2020). Although liver damage in COVID-19 patients can be caused by drug hepatotoxicity or immune-mediated inflammation, it cannot exclude the possibility of SARS-CoV-2 infection of liver cells (Chai *et al.*, 2019).

Reactivation to patients of SARS with HBV infected resulted in death. Since cytokine dysregulation is essential in the pathogenesis of SARS and COVID-19, steroid or non-steroidal anti-inflammatory drugs have been used as first-line therapy. Although immunosuppression is likely to be beneficial to managing cytokine storms, it may facilitate HBV reactivation. Thus, HBV antivirals such as Entecavir or Tenofovir before immunosuppressive treatment in COVID-19 patients with chronic hepatitis B. In addition, decreasing the viral load may reduce the likelihood of hepatitis B flares (Mehta *et al.,* 2020). COVID-19 patients with HBV may be at an increased risk of morbidity and mortality. Therefore, liver enzyme abnormalities and acute hepatic injuries may be shared among COVID-19 patients with HBV (Mirzaei *et al.,* 2021).

Conditions such as chronic HBV infection could be reactivated, contributing to elevated liver enzyme abnormalities in COVID-19. However, case studies of the interaction between preexisting liver states and COVID-19 require accurate evaluation. In addition, digestive symptoms and abnormal liver enzymes may play an essential role in many patients with COVID-19, particularly those with atypical symptoms. Further studies are warranted to understand better the cause of liver injuries in patients with preexisting liver diseases who have recently contracted COVID-19. Elevated liver-related enzymes are reported in a substantial proportion of patients with COVID-19 (Agarwal *et al.,* 2020; Aldhaleei *et al.,* 2020).

The patient had elevated liver aminotransferases, such as AST, ALT, and gamma-glutamyl transferase (GGT), during the subclinical phase. Contrastingly, increased prevalence of abnormal liver aminotransferase levels, higher AST levels, and liver injury in severe cases of COVID-19. In addition, patients, specifical males with abnormal liver test results, were more likely to have a moderate to a high degree of fever. In contrast, our patient was hypothermic at admission and showed very high levels of liver enzymes, total bilirubin, ammonia, and INR value (Fan *et al.,* 2020; Aldhaleei *et al.,* 2020).

The serum ALP and GGT are the diagnostic biomarkers for cholangiocyte injury. While the COVID-19 cases with HBV co-infection had higher abnormal

GGT than the COVID-19 cases without HBV infection, the majority of COVID-19 instances with or without HBV co-infection had normal results ALP. Moreover, there were no significant differences in serum ALP and GGT levels between the two groups during the entire 3-week period. Thus, these results indicate that direct cytotoxicity to hepatic cholangiocytes is not the primary cause of liver injury. However, inactive HBV carriers with SARS-CoV-2 co-infection are at a higher risk of enhanced liver injury of the hepatocyte type (Cai *et al.,* 2020; Lin *et al.,* 2021).

Mechanisms of immune-mediated liver injury including LDH, D-dimer, and IL-6. The outlier ratios of serum LDH, D-dimer, and IL-6 levels in the COVID-19 cases with HBV who were inactive HBV carriers with COVID-19 were much higher than those in COVID-19 cases without HBV during the three weeks after the onset of symptoms. While there were no significant differences in serum D-dimer and IL-6 levels between the two groups during the entire 3-week period, the mean values of serum LDH levels in the COVID-19 cases with HBV co-infection are significantly higher than those in COVID-19 cases without HBV co-infection at 2-3 week after the onset of symptoms. These results indicated that the immune-mediated liver injury might result from the inflammatory response following SARS-CoV-2 co-infection (Lin *et al.,* 2021).

In-state inactive HBV carriers had abnormal liver function parameters after SARS-CoV-2 co-infection, indicating a high risk of liver injury as hepatocyte type. Therefore, the enhanced liver injury of inactive HBV carriers is likely caused by inflammatory factors (Lin *et al.,* 2021). Inactive HBV carriers with SARS-CoV-2 co-infection are at risk of more significant liver injury. In addition, the inflammatory response may contribute to this injury following SARS-CoV-2 co-infection. Therefore, these are potential threats for patients with chronic HBV infection (Lin *et al.,* 2021).

Besides, drug-induced liver injury is received antibiotic and antiviral drugs, such as arbidol, lopinavir/ritonavir, and interferon, which may cause liver injury (Tillmann and Rockey, 2020).

In patients with HBV, it is essential to be aware of the risk for HBV reactivation related to medications, such as tocilizumab and corticosteroids, used in COVID-19. Reactivation of HBV following the use of tocilizumab and prednisone has been described, and thus prevention against HBV reactivation should be a consideration (Reddy *et al.,* 2015; Chen *et al.,* 2017). In addition, chronic HBV therapy indicated as per guidelines, can be initiated in those newly diagnosed with HBV and continue receiving treatment, regardless of COVID-19 (Terrault *et al.,* 2018).

Risk of HBV reactivation in severe SARS-CoV-2 infected patients. Disruption of the balance between the host's immune status and viral replication contributes to HBV reactivation after SARS-CoV-2 infection. Therefore, the intensity of immunosuppressive therapies is a significant risk factor for HBV reactivation. HBV reactivation in patients infected with SARS-CoV-2 is usually associated with immunosuppressive therapy such as IL-6 or IL-1 receptor antagonists (tocilizumab, anakinra) and high-dose corticosteroids. There are several reports of HBV reactivation in SARS-CoV-2 infected patients. A retrospective study found that three out of 21 patients with SARS-CoV-2 and HBV-infected developed HBV reactivation, of which two received corticosteroid therapy. The most recent prospective study evaluated the risk of HBV reactivation in HBsAg-/anti-HBc+ patients with severe SARS-CoV-2 infection receiving immunosuppressive therapy. At 1-year follow-up, there were no cases of HBsAg seroconversion. Two out of 69 had detectable serum HBV DNA, suggesting a low risk of HBV reactivation in patients with severe SARS-CoV-2 infection and resolved HBV infection (Carroll, 2011; Chen *et al.,* 2017; Rodriguez-Tajes *et al.,* 2021; Chang *et al.,* 2022).

3.4: Result of Biochemical Markers

The results of biochemical markers found there were no significant differences according to sociodemographic and predisposing factors for all biochemical markers such as Albumin, ALT, AST, and Alkaline Phosphatase except for liver cirrhosis with albumin, which showed a highly significant difference

compared to the control group in HBV alone and HBV with SARS-CoV-2 patients (Tables 3-13).

Table (3-13): Distribution of SARS-CoV-2 with biochemical markers according to Sociodemographic and predisposing factors

Properties		Biochemical test							
		Albumin (g/dL)		ALT (IU/L)		AST (IU/L)		Alkaline Phosphatase (IU/L)	
		Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Sex	Male*	3.3319 ± 0.69526	0.325	48.6087 ± 183.71351	0.256	42.6522 ± 56.14027	0.349	98.2754 ± 44.00531	0.350
	Female*	4.0348 ± 3.67134		17.0000 ± 6.81502		33.4565 ± 22.34945		90.6522 ± 31.50465	
	Co. Male **	3.1455 ± 0.70195		117.0909 ± 327.71556		62.7273 ± 102.80670		105.9091 ± 65.06221	
	Co. Female **	3.3267 ± 0.70959		19.9333 ± 8.99577		34.4000 ± 29.31309		82.1333 ± 16.97842	
Age	Young (<30 year) *	3.3750 ± 0.66435	0.902	17.6389 ± 7.34128	0.285	30.1667 ± 17.83175	0.138	86.4444 ± 22.94894	0.448
	Adult (30 - 60 Year) *	3.7729 ± 3.01296		32.6286 ± 130.31825		39.2857 ± 44.60459		98.2857 ± 40.06051	
	Elderly (> 60 year) *	3.3222 ± 0.91074		135.2222 ± 362.19290		71.7778 ± 98.89107		106.5556 ± 74.32549	
	Co. Young (<30 year) **	3.2000 ± 0.85894		19.7000 ± 7.78959		28.1000 ± 23.36403		78.7000 ± 11.97265	
	Co. Adult (30 - 60 Year) **	3.2286 ± 0.60692		97.3571 ± 290.14416		62.5000 ± 91.90610		99.9286 ± 58.47480	
	Co. Elderly (> 60 year) **	3.6500 ± 0.63640		13.5000 ± 2.12132		25.0000 ± 14.14214		105.5000 ± 34.64823	
HBV vaccine receive	Vaccinated *	3.2125 ± 0.59866	0.844	19.3750 ± 8.36553	0.776	31.5000 ± 20.54959	0.831	74.1250 ± 11.75266	0.463
	Non-vaccinated *	3.6324 ± 2.46557		36.9722 ± 147.32769		39.3148 ± 46.98790		96.6111 ± 40.27727	
	Non-vaccinated **	3.3429 ± 0.65998		71.8095 ± 236.88322		49.0000 ± 76.75285		94.4762 ± 49.53546	
	Vaccinated **	2.9500 ± 0.88882		16.7500 ± 4.19325		40.2500 ± 35.48122		84.2500 ± 9.21502	
Diseases Status	Acute *	3.4520 ± 0.49592	0.838	18.5200 ± 6.11773	0.890	33.9600 ± 23.01717	0.634	85.7200 ± 24.33023	0.256
	Chronic *	3.7195 ± 2.81221		42.8415 ± 168.87167		38.3415 ± 51.77783		100.1585 ± 43.79567	
	Chronic Active *	3.2400 ± 0.26077		21.8000 ± 8.25833		43.4000 ± 29.61081		68.4000 ± 4.66905	
	Autoimmunity *	3.0250 ± 0.97767		18.5000 ± 4.79583		72.0000 ± 19.62991		81.5000 ± 7.72442	
	Control **	3.2160 ± 0.69022		62.5600 ± 217.32351		47.6000 ± 71.25482		92.9600 ± 45.44730	
HBV Contact	House Contact *	4.0378 ± 4.10997	0.431	45.8919 ± 179.27304	0.858	43.5946 ± 56.63894	0.826	100.3784 ± 49.35492	0.576
	Work Contact *	2.9667 ± 0.11547		17.6667 ± 6.42910		33.3333 ± 22.67892		70.0000 ± 16.70329	
	Others *	3.4293 ± 0.68236		31.8000 ± 125.29306		36.9200 ± 40.50551		93.6933 ± 34.21513	
	Control **	3.2500 ± 0.69814		61.0385 ± 213.07397		46.3846 ± 70.08970		92.1923 ± 44.70080	
Liver Cirrhosis	With Cirrhoses *	8.7000 ± 10.78981	< 0.001	18.0000± 10.22252	0.742	32.4000 ± 16.96467	0.767	95.8000 ± 47.86126	0.942
	Without Cirrhoses *	3.3818 ± 0.69139		36.7818± 145.97363		39.2727 ± 46.70342		95.2000 ± 39.35065	
	Control **	3.2500 ± 0.69814		61.0385± 213.07397		46.3846 ± 70.08970		92.1923 ± 44.70080	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

Although there were elevated levels of biochemical markers (albumin, ALT, AST, and alkaline phosphatase) above normal values (Upper Normal Level), these levels were not statistically significant between the two groups. These results were

compatible with the results of several authors worldwide (Esmaeelzadeh *et al.*, 2017; Yuen *et al.*, 2005). Yuen *et al.* (2005) studied the ALT level of Chinese CHB patients, and they found that the patients with serum ALT levels of Upper Normal Level (UNL) had a significantly increased risk of complications compared to the patients with serum ALT levels < UNL. Esmaeelzadeh *et al.* (2017) indicated that HBeAg-negative patients with persistently normal ALT levels were not a homogenous group, and those with high to normal ALT shared some associated characteristics with adverse long-term outcomes. The study estimated that the best cutoff values for identifying men at risk of death from liver disease were 31 IU/L for AST and 30 IU/L for ALT and also mentioned that a slightly increased but still normal aminotransferase concentration is associated with an increased risk of death from liver disease in another estimated that 37% of patients with persistently normal ALT had significant fibrosis or inflammation on liver biopsy

One more study found that ALT had a high specificity (89.3%) but low sensitivity (55.3%) to differentiate between mild-moderate and marked-severe inflammation (Xia *et al.*, 2019). The same results were obtained for serum AST, suggesting that nearly half of patients diagnosed with severe liver injury based solely on serum ALT or AST levels may be misdiagnosed.

Wang and his colleagues reported that normal ALT levels do not always indicate the absence of hepatic fibrosis, a combination of ALT levels, sex, and serum HBV DNA load may more effectively identify patients with CHB (Wang *et al.*, 2017).

A low prevalence of significant liver injury in CHB patients with normal ALT levels indicated a possible bias for including blood donors and a high proportion of patients in the immunotolerant phase. Although some studies have detected significant liver injury in CHB patients with normal ALT, there were significant fibrosis and inflammation in 37% of patients with persistently normal ALT. A trend for the normal ALT group to include younger patients, a normal ALT level in an individual patient does not always indicate the absence of significant liver disease

(Marugán and Garzón, 2009). The results came from a Cameroon study that obtained a statistically significant difference between the ALT and AST means of HBV infected cases, and healthy controls justify aminotransferase elevation in circulation during HBV infection following liver damage (Tufon *et al.*, 2016).

3.5: Genetic analysis

3.5.1: Viral load

3.5.1.1: HBV and HCV viral load distribution

Patients' viral load was determined using real-time PCR, where HBV-infected patients were divided based on viral load into patients infected with HBV alone and HCV co-infection.

The genetic diagnosis of HBV and HCV viral load are described in (Figures 3-3). The results revealed that 3 out of 141 (2.13%) HBV had HCV viral load, two female and one male, of different ages (13, 70) and (65) years, respectively. These cases were from different regions; each one of them was from Qadisiyah, Babylon, and Diyala. In addition, they do not receive the HBV vaccine, their disease state is one acute and two chronic, they do not have liver cirrhosis, and their blood group was O^{+}.

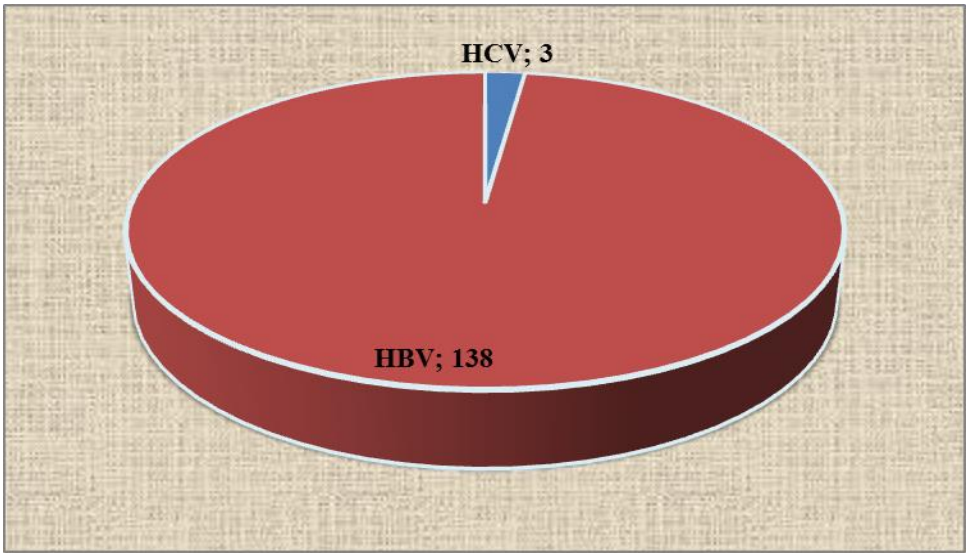


Figure (3-3): viral load distribution of Patient's HBV Infection and HCV Co-infection

3.5.1.2: Distribution of SARS-CoV-2 with HBV and HCV viral load according to Sociodemographic and predisposing factors

In the current study, a significant increase in HBV viral load was seen in liver cirrhosis only from the rest of the demographic distribution, which did not significantly differ from the control group. In contrast, the results of HCV viral load showed a highly significant difference in age from the rest of the demographic distribution that did not show any significant difference compared to the control group, and these results are shown below in (Table 3-14).

Table (3-14): Distribution of SARS-CoV-2 with HBV and HCV viral load according to Sociodemographic and predisposing factors

Properties		Viral load			
		HBV (IU/mL)		HBV (IU/mL)	
		Mean ± SD	P value	Mean ± SD	P value
Sex	Male*	436.7536 ± 552.34419	0.751	0.12039 ± 1.9855	0.621
	Female*	340.9565 ± 335.69179		0.20618 ± 1.9565	
	Con. Male **	388.9091 ± 440.97289		2.0000 ± 0.00000	
	Con. Female **	388.6000 ± 292.04814		2.0000 ± 0.00000	
Age	Young (<30 year) *	337.5556 ± 298.24606	0.901	1.9722 ± 0.16667	0.001
	Adult (30 - 60 Year) *	426.8857 ± 562.61418		2.0000 ± 0.00000	
	Elderly (> 60 year) *	420.6667 ± 339.35674		1.7778 ± 0.44096	
	Con. Young (<30 year) **	449.13947 ± 479.6000		2.0000 ± 0.00000	
	Con. Adult (30 - 60 Year) **	299.78395 ± 322.5714		2.0000 ± 0.00000	
	Con. Elderly (> 60 year) **	397.5000 ± 103.94470		2.0000 ± 0.00000	
HBV vaccine receive	Vaccinated *	434.2500 ± 416.22547	0.884	2.0000 ± 0.00000	0.821
	Non-vaccinated *	393.8241 ± 482.29835		1.9722 ± 0.16510	
	Non-vaccinated **	365.7619 ± 290.57734		2.0000 ± 0.00000	
	Vaccinated **	559.7500 ± 653.50153		2.0000 ± 0.00000	
Diseases Status	Acute *	342.8400 ± 263.23907	0.868	1.9600 ± 0.20000	0.883
	Chronic *	423.6829 ± 543.56189		1.9756 ± 0.15521	
	Chronic Active *	232.2000 ± 98.02908		2.0000 ± 0.00000	
	Autoimmunity *	395.0000 ± 255.31288		2.0000 ± 0.00000	
	Control **	394.9200 ± 360.19450		2.0000 ± 0.00000	
HBV Contact	House Contact *	464.8649 ± 424.48296	0.751	1.9730 ± 0.16440	0.855
	Work Contact *	303.6667 ± 54.99394		2.0000 ± 0.00000	
	Others *	369.4533 ± 511.01458		1.9733 ± 0.16219	
	Control **	388.7308 ± 354.32534		2.0000 ± 0.00000	
Liver Cirrhosis	With Cirrhoses *	916.0000 ± 1120.83853	0.034	2.0000 ± 0.00000	0.654
	Without Cirrhoses *	374.9091 ± 424.53045		1.9727 ± 0.16362	
	Control **	388.7308 ± 354.32534		2.0000 ± 0.00000	

*** HBV and SARS-CoV-2, ** HBV alone, *** Different values mean significant differences (P≤0.05).**

3.5.1.3: HBV patients with HCV co-infection

3.5.1.3.A: Comparison between the type of infection with a complete blood count among HBV-infected patients

The study showed no significant difference between total WBC and platelets count for the two groups of co-infection (B&C) and HBV alone, as in (Table 3-15).

Table (3-15): Comparison of infection type in HBV infected patients according to Total WBC Platelets count

Properties		Complete blood count				
		No.	Total WBC		Platelets count	
			Mean ± SD	P-value	Mean ± SD	P-value
Type of infection	Co-Infection (B&C)	3	5.1333± 0.92916	0.531	302.0000± 25.51470	0.219
	HBV alone	138	7.4275± 6.30204		241.2101± 84.87109	

3.5.1.3.B: Comparison between the type of infection with Coagulation factor among HBV-infected patients

Regarding the coagulation factor (INR), the results revealed no significant difference between co-infection (B&C) and HBV alone. (Table 3-16)

Table (3-16): Comparison of infection type in HBV infected patients according to Coagulation factor

Properties		Coagulation factor		
		No.	INR	
			Mean ± SD	P-value*
Type of infection	Co-Infection (B & C)	3	1.0633± 0.13577	0.140
	HBV alone	138	0.9528± 0.12750	

*** Different values mean significant differences (P≤0.05).**

3.5.1.3.C: Comparison between the type of infection with Biochemical markers among HBV-infected patients

According to (Table 3-17), the current study showed no significant difference between albumin, ALT, AST, and alkaline phosphatase for the two groups of co-infection (B&C) and HBV alone for the biochemical markers.

Table (3-17): Comparison of infection type in HBV infected patients according to Biochemical markers

Properties		Biochemical test								
		No.	Albumin (g/dL)		ALT (IU/L)		AST (IU/L)		Alkaline Phosphatase (IU/L)	
			Mean ± SD	P-value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Type of infection	Co-Infection (B&C)	3	3.7000± 0.45826	0.902	16.6667± 6.65833	0.791	34.0000± 17.32051	0.828	81.0000± 0.00000	0.555
	HBV alone	138	3.5428± 2.20738		41.1087± 159.17697		40.4783± 51.43094		94.9638± 40.75249	

*** Different values mean significant differences (P≤0.05).**

3.5.1.3.D: Comparison between type of infection with immunological markers among HBV infected patients

The results of Anti-SARS-CoV-2 found three patients co-infection (B and C) were infected with COVID-19. However, no significant difference was seen for anti-SARS-CoV-2 for both co-infection (B&C) and HBV alone groups, as in (Table 3-18).

Table (3-18): Comparison of infection type in HBV infected patients according to Anti-SARS-CoV-2

Properties		COVID-19				
		No.	IgM mIU/ml		IgG mIU/ml	
			Mean ± SD	P-value	Mean ± SD	P-value
Type of infection	Co -Infection (B and C)	3	0.7533± 0.41041	0.924	2.3067± 0.82779	0.296
	HBV alone	138	0.7115± 0.74928		4.0572± 2.87648	

Regarding HBV reactivation, no significant difference was seen for the co-infection groups (B&C) and HBV alone (Table 3-19).

Table (3-19): Comparison of infection type in HBV infected patients according to reactivation markers

Properties		HBV reactivation								
		No.	HBs Ag IU/ml		HBc Ab (IgM) IU/ml		HBe Ag IU/ml		HBe Ab IU/ml	
			Mean ± SD	P-value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Type of infection	Co-Infection (BandC)	3	0.6667 ± 0.57735	0.982	0.6667 ± 0.57735	0.966	1.0000 ± 1.00000	0.966	0.6667 ± 0.57735	0.876
	HBV alone	138	0.6594 ± 0.54701		0.6812 ± 0.57927		1.0217 ± 0.88349		0.7246 ± 0.63669	

*** Different values mean significant differences (P≤0.05).**

3.5.1.3.E: Comparison between the type of infection and viral load (HBV and HCV) among HBV-infected patients

Table (3-20) showed no significant difference between HBV and HCV viral load for the co-infection groups (B&C) and HBV alone.

Table (3-20): Comparison of infection type in HBV infected patients according to HBV and HCV viral load

Properties		No.	Viral load			
			HBV (IU/mL)		HCV (IU/mL)	
			Mean ± SD	P-value	Mean ± SD	P-value
Type of infection	Co-Infection (B and C)	3	647.6667± 466.53439	0.338	1.0000± 0.00000	-
	HBV alone	138	391.1884± 456.75957		2.0000± 0.00000	

*** Different values mean significant differences (P≤0.05).**

The results in the current study observed that HCV was dominant over HBV based on the viral load test, where HCV cases were recorded as 28674000 IU/mL, 632000 IU/mL, and 87900 IU/mL, for the three recorded, respectively, compared to HBV viral load which were less than 1000 IU/mL for each case.

In the current study, all the recorded cases of co-infection (HBV and HCV) showed that they were infected with SARS-CoV-2 (3-18), but one of them showed HBV reactivation (3-19). In addition, a high viral load for the co-infection, especially with HCV, and an abnormal result for ALT compared to the rest of the Biochemical and hematological tests, which were in normal values.

Infection with HBV and HCV is a public health problem (Police *et al.*, 2020). Unfortunately, 40-80% of men with chronic hepatitis B or C are unaware of their serostatus and remain infectious to others (Ayele *et al.*, 2020). Late data have shown that about 350 million people are chronically infected with HBV, and around 200 million are infected with HCV (Hussein *et al.*, 2017).

Previous data from 2006 to 2009 suggest a low prevalence of hepatitis B and hepatitis C infection exists in Iraq's capital, Baghdad (Ataallah *et al.*, 2011). It also reported similar results in Basra (Al-Rubaye *et al.*, 2016). In addition, HBV and HVC prevalence are investigated in Erbil (Taher and Saleh, 2020). In a study by

Jamal *et al.* (2019) in Zakho City/Iraq, between January 2019 and October 2019, on 2223 females, 12/2223 (0.54%) samples were positive for HBs Ag. All these 12 patients also showed positivity for HBc IgG. However, only 1/2223 (0.045%) sample was positive for HCV antibodies. The result of HCV-RT-PCR confirmed the positivity for this patient.

Data from developing countries and high/intermediate endemic areas showed that the most common route of infection is still vertical transmission from mother to child, and horizontal transmission between children, particularly siblings (Franco *et al.,* 2012).

The prevalence of hepatitis B surface antigen (HBsAg) and HCV antibody (anti-HCV) was identified in the Turkish population as 2.3% and 0.37%, respectively (Yakaryilmaz *et al.,* 2006). At the national level, the age-standardized prevalence of hepatitis B in Iran fell from 3.02 % (95 % uncertainty interval; 2.26 to 3.96) in 2000 to 1.09 % (95 %uncertainty intervals; 0.85 to 1.37) in 2016, with a cumulative improvement of -64.84 %. In 2016 the prevalence of hepatitis B in males was more than 1.3 times greater than in females (Rezaei *et al.,* 2020).

The present study revealed that HCV was dominant over HBV based on the viral load test. This may be due to the HBV has been suppressing by HCV. Chakravarti *et al.* (2005) reported that in cases of co-infection with HBV and HCV, the replication of either virus can be inhibited, just as either virus can be dominant or the dominance can alternate between the two. It is more common for HBV to appear to be suppressed by HCV. The chronologies of the two infections influence which virus will be dominant.

However, the HBV DNA levels can be lower than those of mono-infected individuals, indicating HCV interference. Therefore, one virus can induce the clearance of another. There are reports of patients infected with HCV with low levels of HBV DNA and reactive anti-HBc. However, non-reactive HBsAg, HBeAg, anti-HBe, and anti-HBs, configure co-infection with HCV with asymptomatic HBV. the data suggest that the evolution of the disease is more severe in co-infected individuals (Crockett *et al.,* 2005; Liu *et al.,* 2006).

Many studies have demonstrated that dually infected patients carry a greater risk of advanced liver disease, cirrhosis, and hepatocellular carcinoma compared with monoinfected patients. Therefore, treatment choice is based on each patient's virological profile, taking into account the dominant virus pattern (Konstantinou and Deutsch, 2015).

In predominant HCV, standard combination treatment with pegylated interferon and ribavirin has proven equally effective in HBV/HCV-coinfected patients as well as in HCV-monoinfected patients. Strikingly, approximately 60% of patients with inactive HBV infection may present HBV reactivation before HCV treatment. In contrast, others experience hepatitis B surface antigen seroconversion after clearing HCV, demonstrating the complexity of the interaction between the two viruses during the follow-up (Konstantinou and Deutsch, 2015).

Interestingly, some reports have shown contradictory results, stating that HBV and HCV replicated independently and that there was no direct evidence of replication interference (Bellecave *et al.*, 2009). Thus, it is likely that HBV and HCV can infect and replicate in the same cells without interference (Yang *et al.*, 2014).

A study by Chen *et al.* (2016) showed that dual infection placed a heavier burden on the host's immune system and weakened the antibody production capacity, leading to lower protective antibodies to each virus. Taken together, our data may contribute to a further understanding the biology of viral infection and immune response in patients with dual infection of HBV and HCV.

Some previous studies have revealed that the HBV DNA levels are associated with the extent of liver damage and liver fibrosis severity. Therefore, it may be an independent factor in predicting antiviral treatment response. In addition, HBV DNA is a virological marker that reflects HBV replication levels (Jia *et al.*, 2019). This is also reflected in HCV co-infection and the severity of diseases.

Gender is a well-known factor associated with acute and chronic HBV infection; it identified a sex difference in HBV viral load in families that had HBsAg-positive siblings, and the finding was that viral load generally higher in

males than females siblings, So the study suggests that sex play important roles on HBV viral load (Hsieh *et al.*, 2017).

A study of HBV infection in transgenic mice demonstrated that the androgen pathway could increase the transcription of HBV genes; androgens bind directly to sites in the viral genome, and higher total serum levels of testosterone with risk of advanced hepatic fibrosis and inflammatory activity in male veterans with chronic HCV infections, it's clear now that estrogen protects against progression of HBV infection and testosterone increase the risk of infections and prognosis of the disease (El-Serag, 2012).

The present study found that two of the three cases with high viral load were elderly patients. This can be explained by the elderly patient's weaker immune system, allowing the virus to replicate efficiently (Oh *et al.*, 2019). Although in 2019, a study of multiple viruses demonstrated a persistent infection by evolving evasion mechanisms of the host immune system, certain viruses can establish latency at low levels of viral replication and also be reactivated to cause devastating symptoms in the absence of appropriate immunity. So aging, a complex biological process, results in profound alterations in the immune system, and these changes can accumulate to produce a progressive deterioration in the ability to respond to pathogens and develop proper and durable immunity after vaccination (Oh *et al.*, 2019).

There is no clear explanation that age will promote immunity. Still, maybe it's just a coincidence that the number of patients was low in the sample collection period, it may be changing in hormones as it's a transition stage in the human life cycle, and everything well changes, including immune system response, as explained earlier by Oh *et al.* (2019) who concluded that aging is a complex biological process. Now that it's clear from the previous results that HBV and the immune system have a complicated relationship established for centuries, which needs more and more effort and age-HBV-specific studies to get a highly accurate complete picture.

3.5.2: HBV genotyping

3.5.2.1: HBV Infection Distribution and SARS-CoV-2 Co-infection According to HBV genotyping

The present study investigated the HBV genotypes among 60 out of 141 patients using RT-RCR (Table: 3-21). The 60 patients were selected for HBV genotypes based on criteria provided by the manufacturer, where only samples having 5 $X10^2$ copies/ml can be detected. So, 81 patients were not tested by RT-PCR genotyping for HBV selectively as it had low viral load values in the plasma of the tested patients. HBV genotypes distributions found a prevalence among HBV and SARS-CoV-2 co-infection patients with genotypes C, A, B, and D (42.9%, 25%, 21.4%, 10.7%, respectively), depending on the outcome in the (Figure 2-4).

Table (3-21): Distribution of patients with HBV and SARS-CoV-2 Co-infection according to HBV genotyping using RT-PCR

HBV genotypes	No.	% of total 141	% of total 60 sample tested	% of positive sample tested	P. value*
Genotype A	7	5.0	11.7	25	< 0.001
Genotype B	6	4.3	10	21.4	
Genotype C	12	8.5	20	42.9	
Genotype D	3	2.1	5	10.7	
Undetermined	32	22.7	53.3	-	

*** Different values mean significant differences (P≤0.05).**

3.5.2.2: Comparison of HBV genotyping with a complete blood count in HBV-infected patients

Table (3-22) shows the total WBC and platelet count between HBV genotyping, where the study showed no significant difference of <0.05.

Table (3-22): Comparison of HBV genotyping with a complete blood count in HBV-infected patients

Properties		Complete Blood count				
		No.	Total WBC		Platelets count	
			Mean ± SD	P value	Mean ± SD	P value
HBV Genotyping	Not detected	81	7.7111 ± 7.98849	0.952	233.0864 ± 90.40467	0.412
	Genotype A	7	5.9000 ± 1.87439		224.2857 ± 71.08613	
	Genotype B	6	7.0667 ± 1.87474		229.3333 ± 54.03579	
	Genotype C	12	6.6083 ± 1.64563		281.7500 ± 62.75367	
	Genotype D	3	5.3000 ± 2.23383		260.6667 ± 79.73916	
	Undetermined	32	7.4031 ± 2.72142		256.3750 ± 81.91370	
	Total	141	7.3787 ± 6.24399		242.5035 ± 84.47220	

*** Different values mean significant differences (P≤0.05).**

3.5.2.3: Comparison of HBV genotyping with Coagulation factor in HBV infected patients

Table (3-23) shows the Coagulation factor (INR) between HBV genotyping, where the results showed a significant difference of 0.018.

Table (3-23): Comparison of HBV genotyping with Coagulation factor in HBV infected patients

Properties		Coagulation factor		
		No.	INR	
			Mean ± SD	P value
HBV Genotyping	Not detected	81	0.9232 ± 0.13618	0.018
	Genotype A	7	1.0257 ± 0.11238	
	Genotype B	6	0.9533 ± 0.11255	
	Genotype C	12	1.0017 ± 0.11862	
	Genotype D	3	0.9567 ± 0.04163	
	Undetermined	32	1.0034 ± 0.09849	
	Total	141	0.9552 ± 0.12817	

*** Different values mean significant differences (P≤0.05).**

3.5.2.4: Comparison of HBV genotyping with biochemical markers in HBV infected patients

Table (3-24) shows no significant difference between HBV genotyping for the biochemical markers.

Table (3-24): Comparison of HBV genotyping with biochemical markers in HBV-infected patients

Properties		Biochemical test							
		No.	Albumin (g/dL)		ALT (IU/L)		AST (IU/L)		Alkaline Phosphatase (IU/L)	
			Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
HBV Genotyping	Not detected	81	3.3901 ± 0.67945	0.092	44.2222 ± 169.73465	0.986	39.3580 ± 52.58762	0.839	97.7037 ± 44.19260	0.845
	Genotype A	7	3.1857 ± 0.66940		21.5714 ± 11.65986		47.7143 ± 24.45209		85.7143 ± 20.73414	
	Genotype B	6	3.4000 ± 0.77460		20.0000 ± 8.00000		28.8333 ± 21.66487		89.3333 ± 21.86931	
	Genotype C	12	5.3833 ± 7.14828		18.2500 ± 7.93296		29.7500 ± 22.73613		99.3333 ± 32.38780	
	Genotype D	3	3.1000 ± 0.43589		17.6667 ± 7.50555		67.6667 ± 22.03028		77.6667 ± 3.51188	
	Undetermined	32	3.4000 ± 0.76538		49.9375 ± 192.59014		44.7813 ± 63.19694		89.7813 ± 40.93798	
	Total	141	3.5461 ± 2.18441		40.5887± 157.50406		40.3404 ± 50.92766		94.6667 ± 40.36418	

*** Different values mean significant differences (P≤0.05).**

3.5.2.5: Comparison of HBV genotyping with immunological markers in HBV-infected patients

In the current study, no significant difference was seen for ant-SARS-CoV-2 between HBV genotyping, as in Table (3-25).

Table (3-25): Comparison of HBV genotyping with anti-SARS-CoV-2 in HBV-infected patients

Properties		COVID-19			
		IgM mIU/ml		IgG mIU/ml	
		Mean ± SD	P value	Mean ± SD	P value
HBV Genotyping	Not detected	0.6506 ± 0.49301	0.657	4.4402 ± 2.76484	0.394
	Genotype A	0.7129 ± 0.37805		2.9400 ± 3.17253	
	Genotype B	0.6317 ± 0.34919		4.4917 ± 2.92304	
	Genotype C	0.6000 ± 0.46018		3.5667 ± 2.77330	
	Genotype D	0.9133 ± 0.35796		3.2500 ± 0.53694	
	Undetermined	0.9072 ± 1.29420		3.3459 ± 3.11860	
	Total	0.7124 ± 0.74285		4.0199 ± 2.85847	

*** Different values mean significant differences (P≤0.05).**

Regarding HBV reactivation and according to (Table: 3-26) for HBV genotyping, the current study results showed that HBsAg, HBcAb, and HBeAg have a significant difference for HBV genotyping compared to HBeAb. However, it did not show any significant difference of <0.05.

Table (3-26): Comparison of HBV genotyping with reactivation markers in HBV-infected patients

Properties		HBV reactivation							
		No.	HBs Ag IU/ml		HBc Ab (IgM) IU/ml		HBe Ag IU/ml		HBe Ab IU/ml	
			Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
HBV Genotyping	Not detected	81	0.5185 ± 0.55025	0.013	0.5432 ± 0.59265	0.018	0.8025 ± 0.88628	0.021	0.5926 ± 0.66667	0.074
	Genotype A	7	0.8571 ± 0.69007		0.7143 ± 0.48795		1.2857 ± 0.95119		1.0000 ± 0.81650	
	Genotype B	6	1.0000 ± 0.00000		1.1667 ± 0.40825		1.6667 ± 0.51640		1.1667 ± 0.40825	
	Genotype C	12	0.7500 ± 0.45227		0.8333 ± 0.57735		1.1667 ± 0.83485		0.8333 ± 0.57735	
	Genotype D	3	0.6667 ± 0.57735		0.6667 ± 0.57735		1.3333 ± 1.15470		0.6667 ± 0.57735	
	Undetermined	32	0.8750 ± 0.49187		0.8750 ± 0.49187		1.3125 ± 0.78030		0.8750 ± 0.49187	
	Total	141	0.6596 ± 0.54550		0.6809 ± 0.57717		1.0213 ± 0.88211		0.7234 ± 0.63366	

*** Different values mean significant differences (P≤0.05).**

3.5.2.6: Comparison of HBV genotyping with HBV and HCV viral load in HBV infected patients

Table (3-27) shows HBV and HCV viral load. The current results showed a very high significant difference for HBV viral load and no significant difference for HCV viral load between HBV genotyping.

Table (3-27): Comparison of HBV genotyping with HBV and HCV viral load in HBV infected patients

Properties		Viral load				
		No.	HBV (IU/mL)		HCV (IU/mL)	
			Mean ± SD	P value	Mean ± SD	P value
HBV Genotyping	Not detected	81	224.3704 ± 99.59335	< 0.001	2.0000 ± 0.00000	0.097
	Genotype A	7	777.1429 ± 100.15892		1.8571 ± 0.37796	
	Genotype B	6	832.8333 ± 115.23440		2.0000 ± 0.00000	
	Genotype C	12	1520.5833 ± 798.91909		1.9167 ± 0.28868	
	Genotype D	3	737.6667 ± 56.95905		2.0000 ± 0.00000	
	Undetermined	32	214.2500 ± 65.20786		1.9688 ± 0.17678	
	Total	141	396.6454 ± 456.77963		1.9787 ± 0.14482	

*** Different values mean significant differences (P≤0.05).**

The results above (Table 3-21) found the predominant C genotype among other genotyping. However, according to previous studies in Iraqi and neighboring countries, HBV genotype D appears to be the only or dominant type in Jordan, Iran, Syria, Saudi Arabia, and Turkey (Ababneh *et al.,* 2019), which were inconsistent with our study in which a difference was observed between the A, B, C, and D genotypes of the diagnosed individuals. In addition, Ahmed (2013) found that genotype D was the predominant genotype among chronic hepatitis B infections (80%) in Baghdad.

In addition, HBV genotypes have a distinct geographic distribution. For example, Genotype D is found worldwide, but it is most common in the Mediterranean, Middle East, and southern Asia (Utama *et al.*, 2009; Jazayeri *et al.*, 2011; Kyaw *et al.,* 2020). Furthermore, host genetic factors, including the Hepatitis B virus genotype, are widely viewed as the everyday basis of the different outcomes of HBV infection (Thio *et al.*, 2003).

Differences in pathogenicity between HBV genotypes are now partially recognized. HBV DNA levels inside cells and HBV DNA and HBeAg levels outside cells were found to be greater in genotypes B and C than in genotypes A and D. HBV DNA and viral antigens accumulated intracellularly may have a role in the development of cellular damage in hepatocytes (Kao *et al.,* 2010; Sunbul, 2014). Furthermore, genotype C was a high replication capability that could cause increased genotype-related severe liver damage. Through in vitro studies, the following was seen (Sunbul, 2014): First, Intracellular HBV core protein expression was raised when a pre-core (PC) or basal core promoter (BCP) region mutation altered HBeAg expression in genotype C. Second, Intracellular HBV surface protein expression was lower in PC wild-type HBV genotype C patients than in HBV genotype B patients. Third, in PC-mutant patients, extracellular HBV DNA was lower. Fourth, HBsAg production was minor in HBV genotype C than in genotype B. Fifth, in HBV genotype B, HBeAg secretion was lower than in genotype C.

The fundamental conclusions shown by previous studies are about the relationship between HBV genotypes, disease severity, and HCC development.

Genotype A is more severe than genotype D and highly prevalent in the asymptomatic group and D in liver cirrhosis. Genotype B is an independent factor for HBeAg seroconversion, associated with higher HBeAg loss. Genotype C is associated with HBe Ag positivity, and the prevalence of genotype C increases from asymptomatic (AS), chronic hepatitis (CH), liver cirrhosis (LC) to hepatocellular carcinoma (HCC), in contrast to genotype B. in addition to it is associated with HCC. In contrast, Genotype D is more related to acute infection than A. Genotype D was associated with higher HBV recurrence and mortality after transplantation than genotypes A and C. Death resulting from liver complications was more common in genotype F patients than in A and D (Guirgis *et al.*, 2010; Lin and Kao, 2021; Khan *et al.*, 2022).

The results of the present study are incompatible with Ni *et al.* (2004), who found no difference in the baseline viral load between HBV genotypes in 460 HBV carrier children, but compatible with both Lindh *et al.* (2000) and Prasad *et al.* (2006), who found a significant correlation between a very high level of viral load and patients with HBV genotype.

3.5.2.7: Distribution of HBV genotyping according to the sociodemographic factors

The results of the distribution of HBV genotyping according to the sociodemographic factors (sex and age) were determined.

Regarding sex, age groups, and their relationship to the distribution of HBV genotyping, according to the current study, the distribution was recorded with respect to sex which is genotype A (2 male, 5 female), genotype B (4 male, 2 female), genotype C (8 male, 4 female), genotype D (3 male). In contrast, the distribution according to age was genotype A above 50 years, genotype B under 30 years, genotype C ranged from 35-50 years, while genotype D focused on the elderly.

The recent findings suggest that the affected person's sex and age group are important factors in their susceptibility to viral infection. Other studies have linked

HBV infection with a considerable rise in HBV seropositivity among males and the elderly and relative genotype distribution. This has been attributed to changes in the infecting genotype as well as mutations in the B-cell and T-cell epitopes in the S-gene of the HBV genome that may contribute to breakthrough infections even among the vaccinated (Vray *et al.,* 2006; Lin *et al.,* 2013; Kiyeng *et al.,* 2018). Differences in circulating genotypes, behavioral factors, host genetic factors, and differences in the application of HBV management methods could explain the differences between our findings and those of the previous study.

Sex is a well-known factor associated with acute and chronic HBV infection; it identified a sex difference in HBV viral load in families that had HBsAg-positive siblings, and the finding was that viral load generally higher in males than females siblings, suggesting that sex plays an important role in HBV viral load (Hsieh *et al.,* 2017).

3.5.3: Sequencing of HBV

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 Figures (3-4).

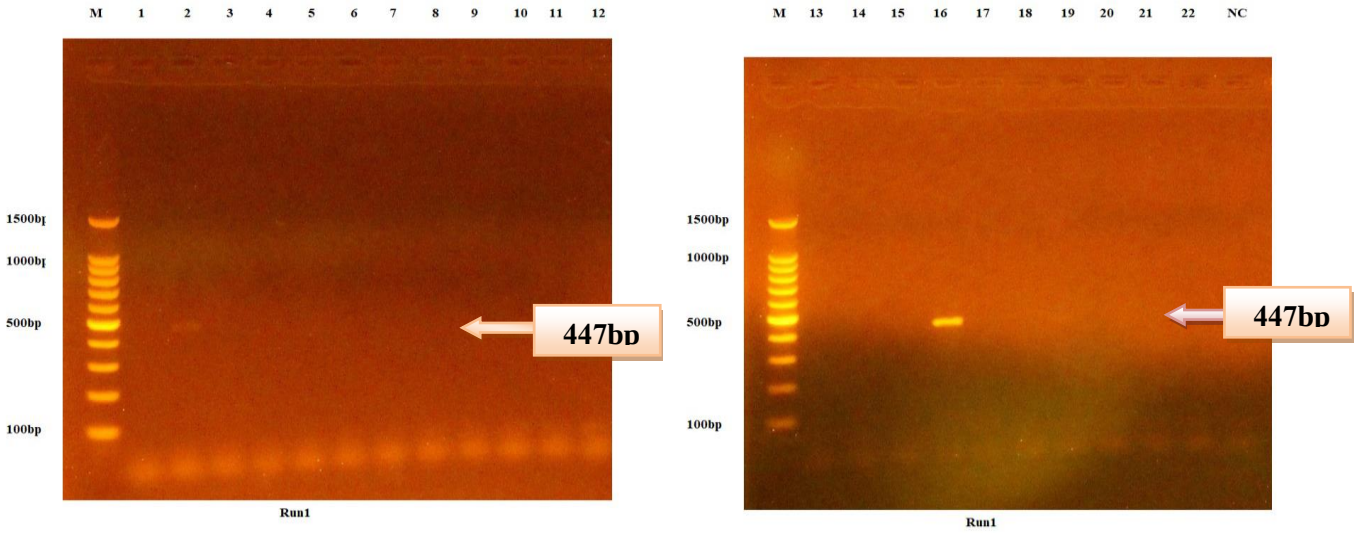


Figure (3-4): The amplification of the Hepatitis B Virus gene fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100-1500bp ladder marker. (2, 16) with positive result for S gene, (1,3,4,5,6,7,8,9,10,11,12,13,14,15,17,18,19,20,21,22) with negative result for S gene. On size 447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

The amplification product size for detecting the S gene after nested PCR 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 Figures (3-5).

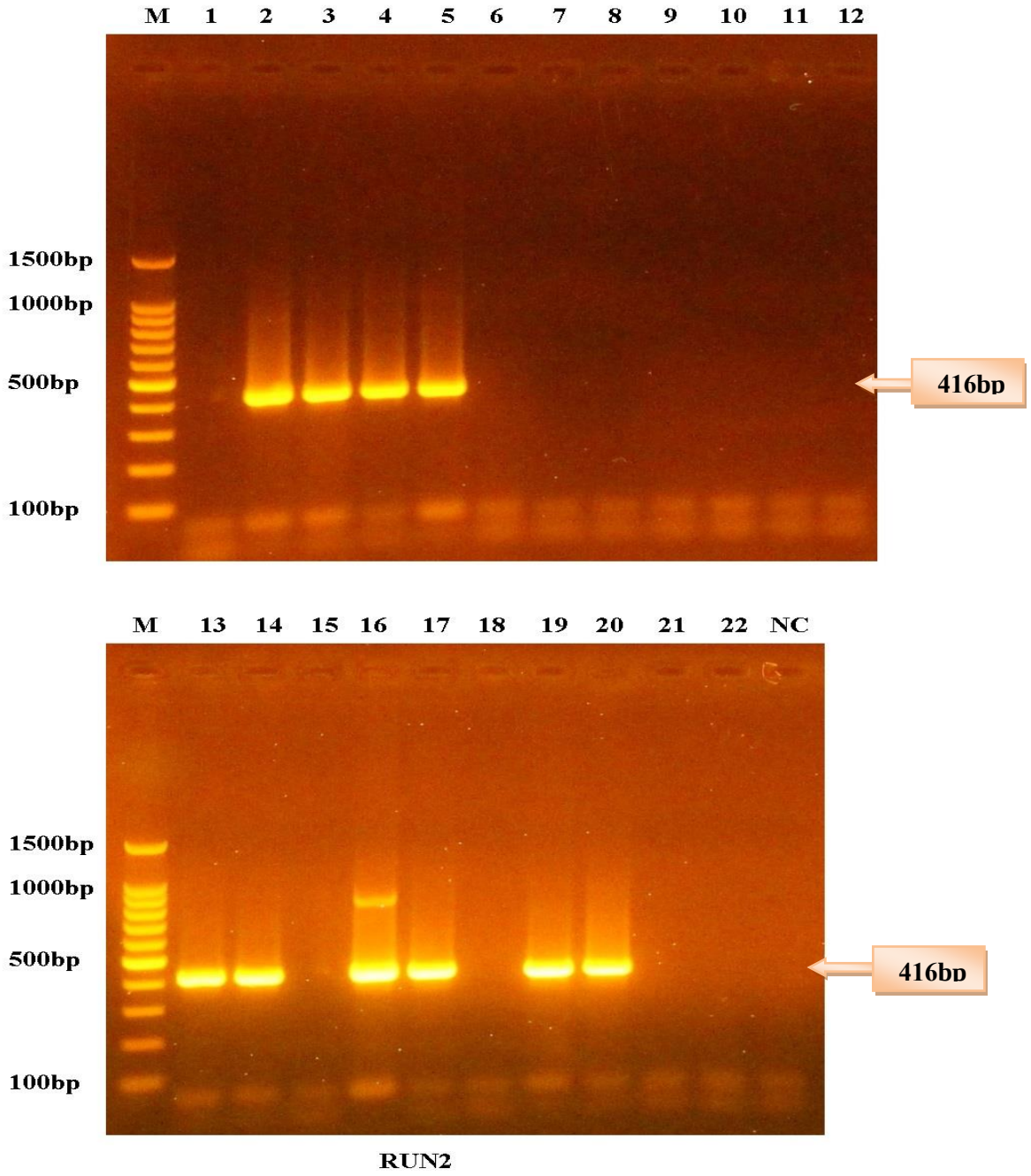


Figure (3-5): The amplification of the Hepatitis B Virus gene after nested PCR fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100-1500bp ladder marker. (2,3,4,5,13,14,16,17,19,20) with positive result for S gene, (1,6,7,8,9,10,11, 12, 15,18,21,22) with negative result for S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

The sequence of nucleotides for the S gene:

For knowledge of 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, as shown in Appendix II.

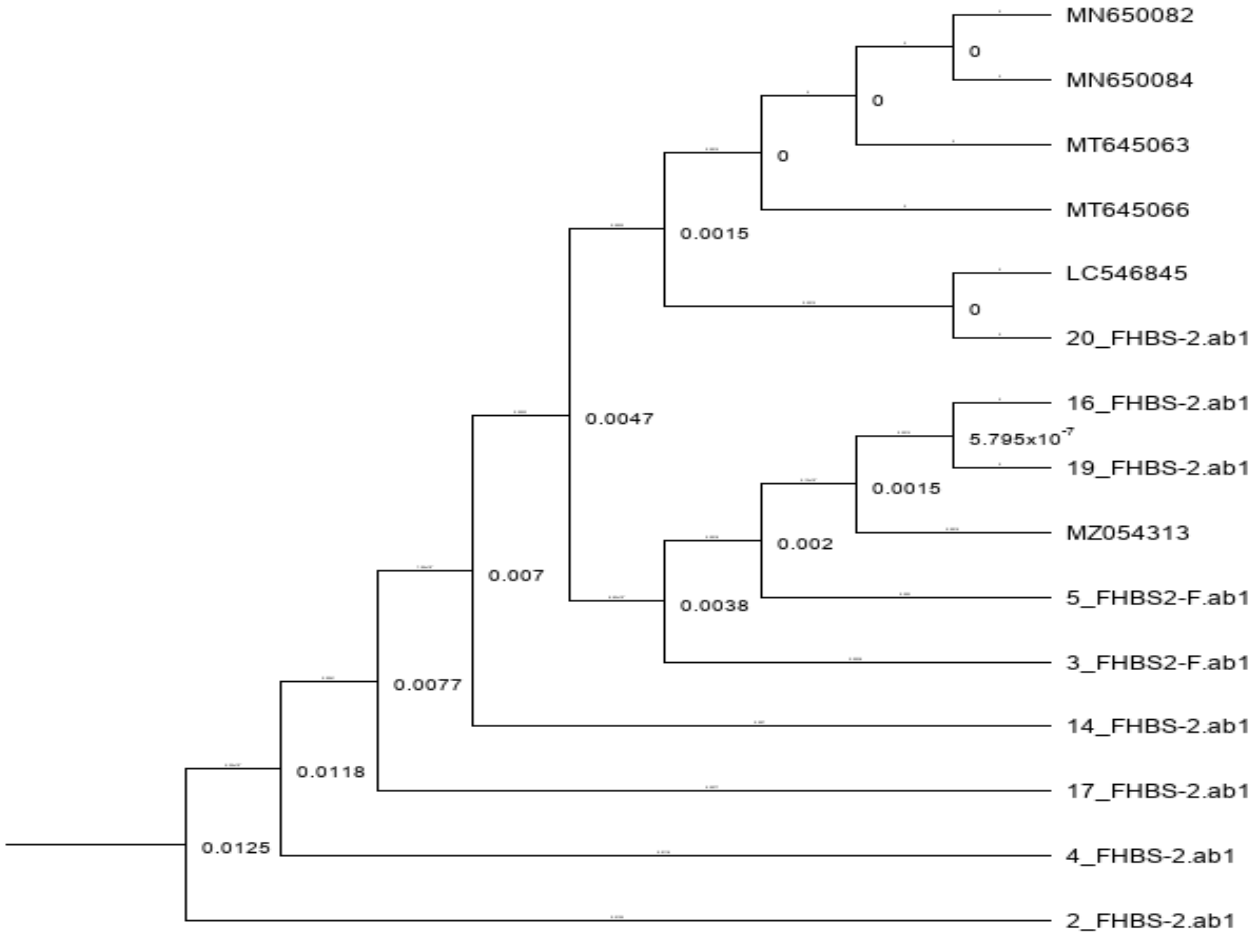
The results of the current study showed the sequence of nested PCR products for ten subjects suffering from HBV (Table: 3-28) depending on appendix II, III, and IV, where the results of the three samples (13, 14, 19) with HBV alone were 1 (rubbish), 1 (100%), and 1 (99.08%), respectively, while the results 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 (3-28): Per. Identities and Accession of Sanger sequencing results for patients infected with HBV alone or HBV and SARS-COV-2.

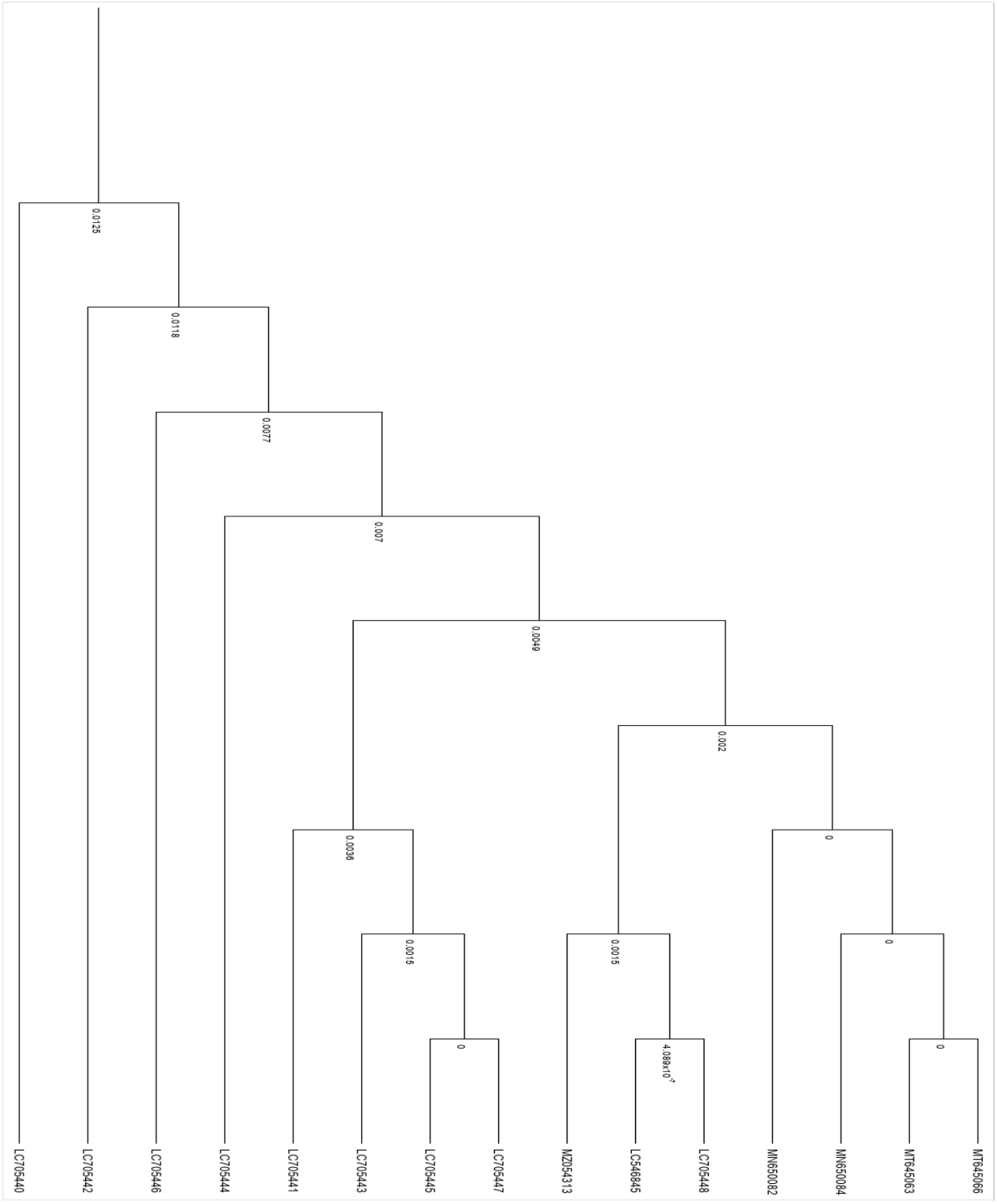
Subject	No. specimen	Description	Per. Identities	Accession	Length	Frequency
HBV alone	13	Rubbish				
	14	**Hepatitis B virus Al-Kaif,etal2022e genes for polymerase, S protein, partial cds**	99.08%	LC705444.1	327	3
		HBV strain s 19 reverse transcriptase gene, partial cds		MK213873.1	1098	
	19	**Hepatitis 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 (Figure 3-6).



A. Before samples in NCBI were confirmed.



B. After samples in NCBI were confirmed.

Figure (3-6): 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.

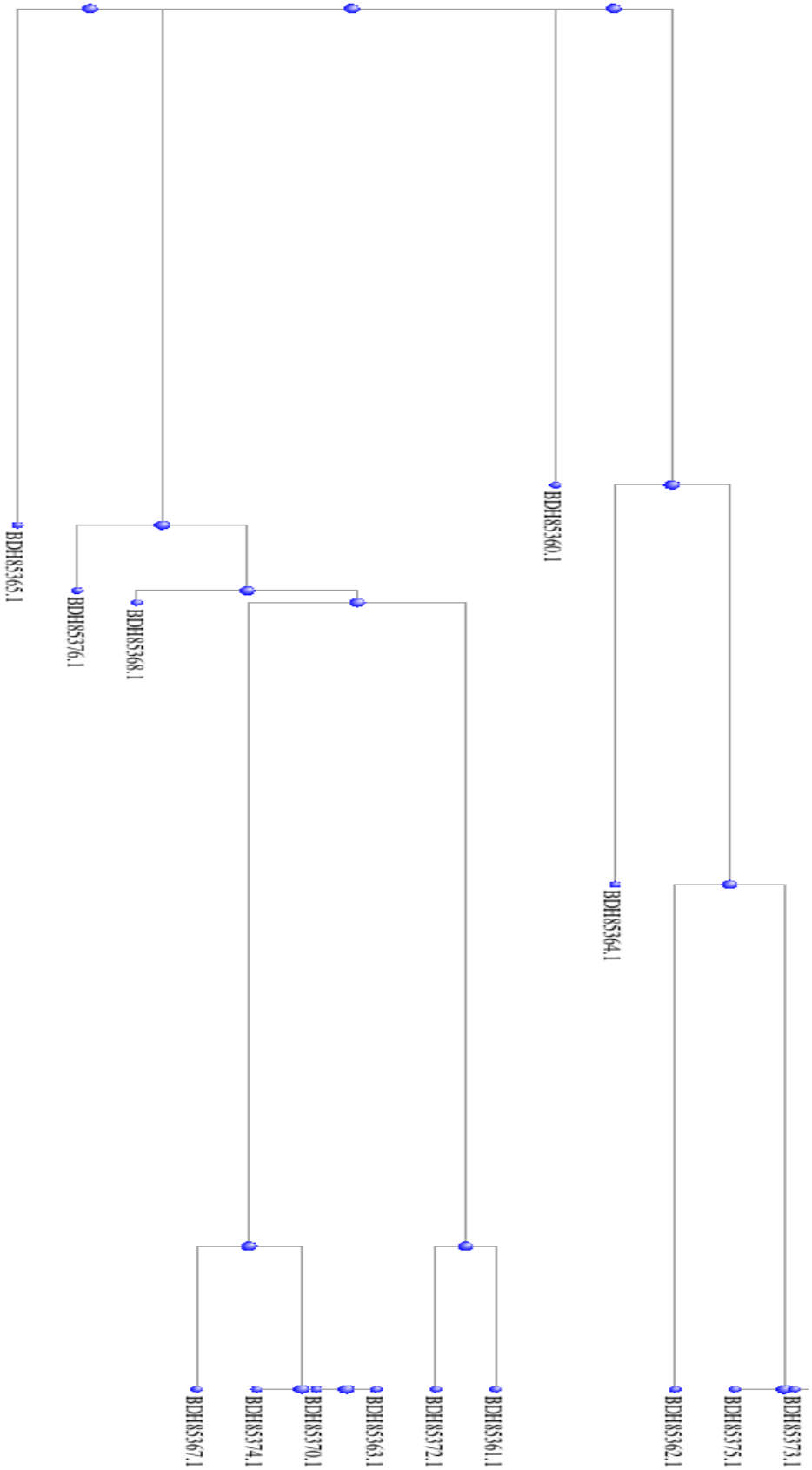


Figure (3-7): Phylogenetic Trees Based on an S protein for viral isolates obtained from the patients infected with HBV alone or HBV and SARS_CoV-2.

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 thus benefit us in the future, especially the Ministry of Health, by following the protocol through the methods of case management, treatment (follow-up), and vaccines. For example, suppose there is an affinity between genetically isolated individuals with hepatitis B virus in Iraq with a country such as China, Japan, Australia, America, England, or any other country. In that case, recommend the Ministry of Health follows the protocol used in the country's genetically related.

Thus, 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 was a perfect program and gave the exact results of identifying percentages with other world strains, and they were ranged from 98.47-100% for 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,etal2022a, No 3: Al-Kaif,etal2022b, No 4: Al-Kaif,etal2022c, No 5: Al-Kaif,etal2022d, No 14: Al-Kaif,etal2022e, No 16: Al-Kaif,etal2022f, No 17: Al-Kaif,etal2022g, No 19: Al-Kaif,etal2022h, No 20: Al-Kaif,etal2022i).

The S gene sequences were submitted to Genbank-bank under submission code No 14: Al-Kaif,etal2022e and No 19: Al-Kaif,etal2022h for infected patients with HBV alone. The result of these sequences was 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,etal2022a, No 3: Al-Kaif,etal2022b, No 4: Al-Kaif,etal2022c, No 5: Al-Kaif,etal2022d, No 16: Al-Kaif,etal2022f, No 17: Al-Kaif,etal2022g, No 20: Al-Kaif,etal2022i for infected patients with HBV with SARS-CoV-2. The result of these sequences was 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 3-8) compared to Norway isolate was MK173200.1 (99.39%) as shown in appendix II.

Hepatitis B virus Al-Kaif,etal2022g genes for polymerase, S protein, partial cds

Sequence ID: LC705446.1 **Length:** 327 **Number of Matches:** 1

Range 1: 1 to 327 GenBank Graphics ▼ Next Match ▲ Previo

Score	Expect	Identities	Gaps	Strand
604 bits(327)	2e-168	327/327(100%)	0/327(0%)	Plus/Plus

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Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCGACTTGTCCTGGTTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCGACTTGTCCTGGTTA  60

Query  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCAT  120

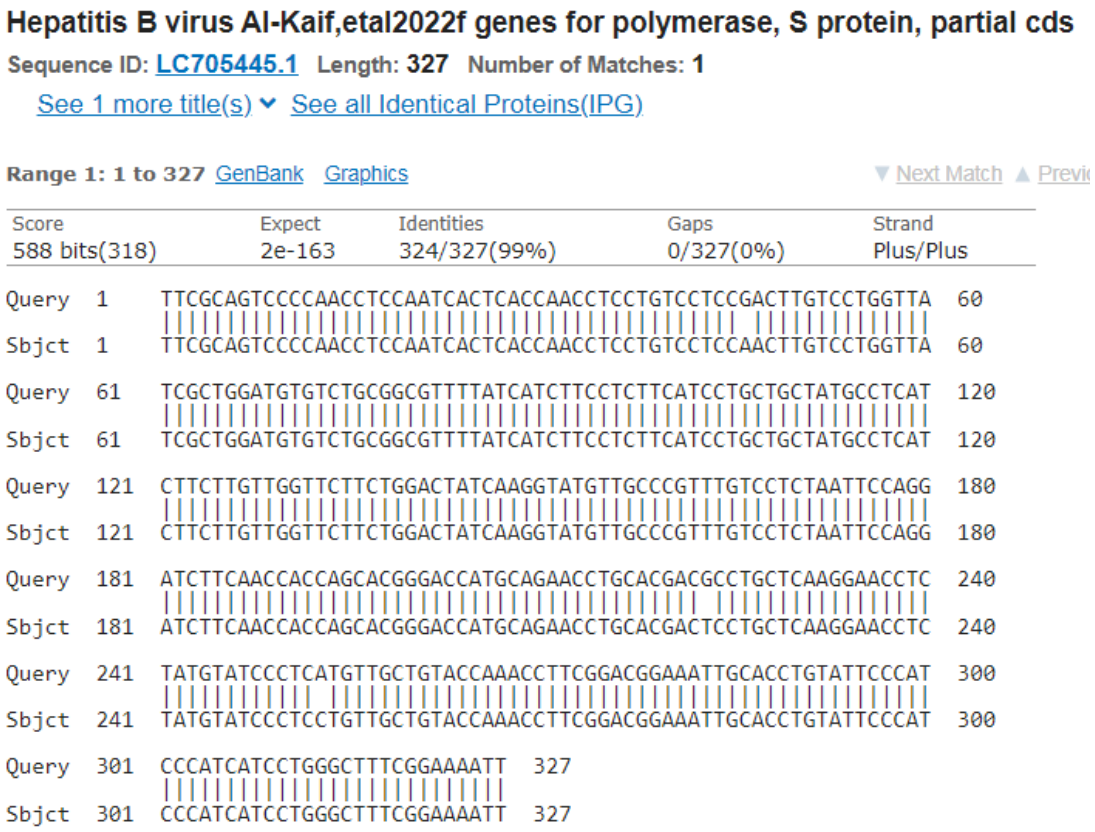
Query  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGG  180

Query  181  ATCTTCAACCACCAGCACGGGACCATGCAGAACCTGCACGACGCCTGCTCAAGGAACCTC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ATCTTCAACCACCAGCACGGGACCATGCAGAACCTGCACGACGCCTGCTCAAGGAACCTC  240

Query  241  TATGTATCCCTCATGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TATGTATCCCTCATGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCAT  300

Query  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
            |||||||||||||||||||||||||||
Sbjct  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
```

(A)



(B)

Figure (3-8): Local Basic Alignment of HBV S gene isolate 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.

While 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 3-7, and the table below:

Table (3-29): 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 3-29) 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 3-9).

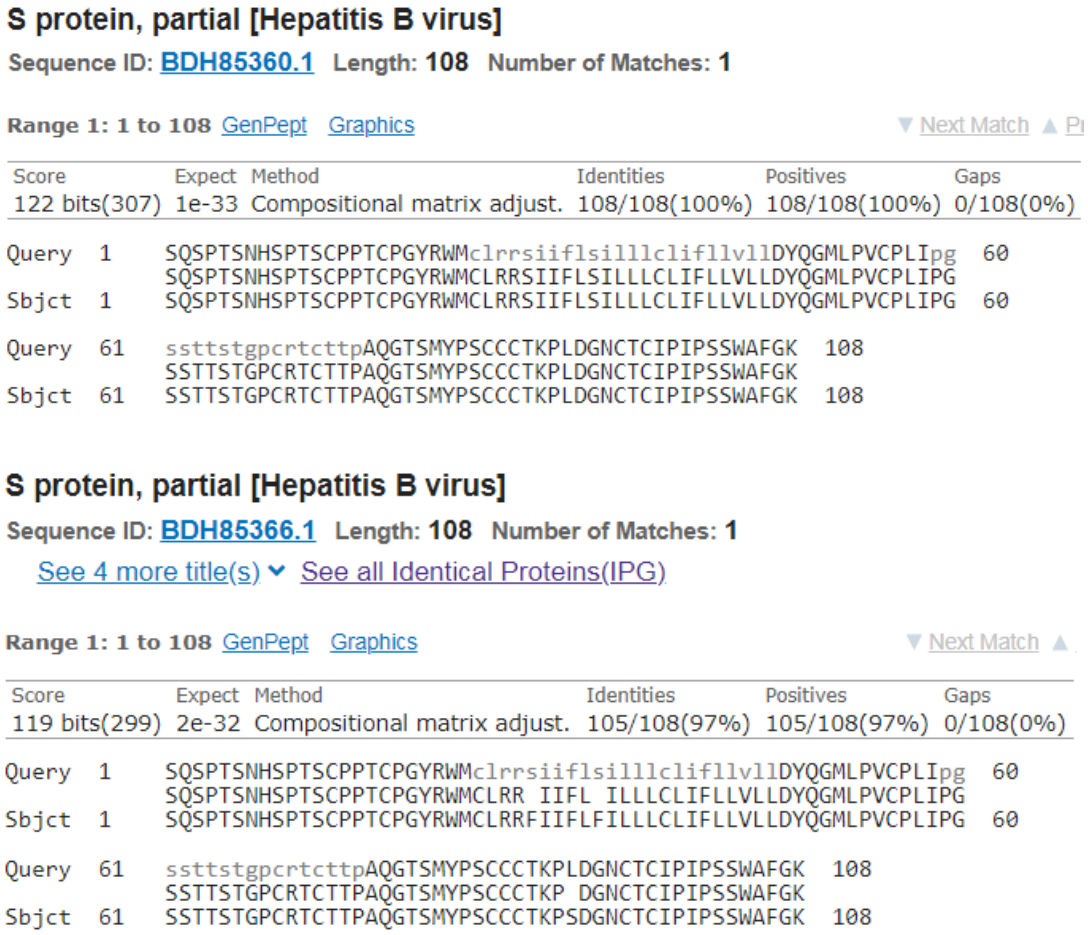


Figure (3-9): Local Basic Alignment of HBV isolates No.2 and 5 with similarity NCBI- blastp (protein-protein BLAST) S protein isolate BDH85360.1 for a patient infected with HBV and SARS-CoV-2.

Therefore, variations or mutations may result from SARS-CoV-2 infection for patients with HBV, whether at the nucleotide or protein level.

Appendix: I
Case information sheet

City :	Patient Number:	Date / /	
Patient Name :	Age:	M:	F:
Address :			
Academic achievement:	Family history for disease:		

Clinical manifestations

Previously infected or not:	Vaccinated or not:	Therapy user or not:
Acute or chronic disease:	Hepatomegaly or not:	Hepatocellular carcinoma:

Risk groups

Hemodialysis	Blood transfusion
Autoimmune diseases	HBV contact
Diabetic:	Other Disease :

Biochemical Markers

AST	ALP	Alkaline phosphatase	Albumin

Hematological Markers

CBC	Total WBCs:	Platelet count:			
Blood groups:		**INR:**			
SARS-CoV-2 induced Hepatitis B Virus Reactivation		HBs Ag	HBc Ab	HBe Ag	HBe Ab

Specimens	**Date of Collection**	**Comment**
	/ /	

Ph.D. Student: Laith Al-Kaif
University of Babylon / College of Medicine
Department of Medical Microbiology

Appendix: II
Sample:2

Hepatitis B virus isolate 178 S protein (S) gene, complete cds

Sequence ID: HM348691.1 **Length:** 681 **Number of Matches:** 1

Range 1: 156 to 482 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
582 bits(315)	7e-162	323/327(99%)	0/327(0%)	Plus/Plus

```
Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCGACTTGTCCTGGTTA  60
Sbjct  156  ............................................................  215

Query  61   TCGCTGGATGTGTCTGCGGCGTTCTATCATCTTCCTCTCCATCCTGCTGCTATGCCTCAT  120
Sbjct  216  .....................................T......................  275

Query  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGG  180
Sbjct  276  ............................................................  335

Query  181  ATCTTCAACCACCAGCACGGGACCATGCAGAACCTGCACGACTCCTGCTCAAGGAACCTC  240
Sbjct  336  ............................................................  395

Query  241  TATGTATCCCTCATGTTGCTGTACCAAACCTTTGGACGGAAATTGCACTTGTATTCCCAT  300
Sbjct  396  ...........C..................C..............C...........  455

Query  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
Sbjct  456  .........................  482
```

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus isolate 178 S protein (S) gene, complete cds	582	582	100%	7e-162	98.78%	HM348691.1
Hepatitis B virus gene for large envelope protein, partial cds, isolate: F4 daughter	579	579	100%	9e-161	98.47%	AB561835.1
Hepatitis B virus isolate NOR496 polymerase (P) gene, partial cds	577	577	100%	3e-160	98.47%	MK173430.1
Hepatitis B virus isolate NOR112 polymerase (P) gene, partial cds	577	577	100%	3e-160	98.47%	MK173200.1
Hepatitis B virus isolate NOR274 polymerase (P) gene, partial cds	577	577	100%	3e-160	98.47%	MK173183.1
Hepatitis B virus isolate NOR473 polymerase (P) gene, partial cds	577	577	100%	3e-160	98.47%	MK173139.1
Hepatitis B virus isolate 525977_NL_2007 S protein (S) gene, partial cds	577	577	100%	3e-160	98.47%	KX659600.1
Hepatitis B virus isolate 304915_NL_2005 S protein (S) gene, partial cds	577	577	100%	3e-160	98.47%	KX659427.1
Hepatitis B virus isolate 282107_NL_2005 S protein (S) gene, partial cds	577	577	100%	3e-160	98.47%	KX659385.1
Hepatitis B virus isolate 259985_NL_2005 S protein (S) gene, partial cds	577	577	100%	3e-160	98.47%	KX659360.1
Hepatitis B virus isolate sn19 S protein gene, partial cds	577	577	100%	3e-160	98.47%	KX649766.1
Hepatitis B virus isolate 13128994-AFIT polymerase, reverse transcriptase region, (P) and S protein (S) genes, partial cds	577	577	100%	3e-160	98.47%	KT201239.1
Hepatitis B virus isolate 48TunSO_12 S protein (S) gene, partial cds	577	577	100%	3e-160	98.47%	KU947266.1
Hepatitis B virus isolate C09 polymerase (P) and large S protein (S) genes, partial cds	577	577	100%	3e-160	98.47%	KT447461.1
Hepatitis B virus isolate Moldova121 S protein and polymerase genes, partial cds	577	577	100%	3e-160	98.47%	KR871262.1
Hepatitis B virus isolate PG-37, complete genome	577	577	100%	3e-160	98.47%	KF471646.1

Sample:3

Hepatitis B virus isolate Bushehr-DM-HBS64 S protein (S) gene, partial cds

Sequence ID: MF419216.1 **Length:** 387 **Number of Matches:** 1

Range 1: 24 to 376 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
641 bits(347)	1e-179	351/353(99%)	0/353(0%)	Plus/Plus

```
Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAACTTGTCCTGGTTA  60
Sbjct  24   ............................................................  83

Query  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCAT  120
Sbjct  84   ............................................................  143

Query  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGG  180
Sbjct  144  ............................................................  203

Query  181  ATCTTCAACCACCAGCACGGGACCATGCAGAACCTGCACGACTCCTGCTCAAGGAACCTC  240
Sbjct  204  ............................................................  263

Query  241  TATGTTTCCCTCCTGTTGCTGTACCAAACCTTCGGACGCAAATTGCACCTGTATTCCCAT  300
Sbjct  264  .....A.............................G.....................  323

Query  301  CCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGTCCGTT  353
Sbjct  324  ....................................................  376
```

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus isolate Bushehr-DM-HBS64 S protein (S) gene, partial cds	641	641	100%	1e-179	99.43%	MF419216.1
Hepatitis B virus isolate AA12-1499-HBB polymerase, reverse transcriptase region, (P) gene, partial cds; and S protein (S) gene, complete cds	641	641	100%	1e-179	99.43%	KT201299.1
Hepatitis B virus isolate 431 polymerase (Pol) gene, partial cds	641	641	100%	1e-179	99.43%	KP997832.1
Hepatitis B virus isolate 1624-11 S protein (S) gene, complete cds	641	641	100%	1e-179	99.43%	KJ416220.1
Hepatitis B virus isolate 6b S protein (S) gene, complete cds	641	641	100%	1e-179	99.43%	HM358309.1
Hepatitis B virus isolate 6a S protein (S) gene, complete cds	641	641	100%	1e-179	99.43%	HM358308.1
Hepatitis B virus isolate 5b S protein (S) gene, complete cds	641	641	100%	1e-179	99.43%	HM358307.1
Hepatitis B virus isolate I_T75, complete genome	641	641	100%	1e-179	99.43%	GU456673.1
Hepatitis B virus isolate GS182 polymerase (pol) gene, partial cds	638	638	100%	2e-178	99.15%	GQ486217.1
Hepatitis B virus Eg51 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.15%	LC542860.1
Hepatitis B virus Eg29 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.15%	LC542838.1
Hepatitis B virus Eg27 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.15%	LC542836.1
Hepatitis B virus Eg18 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.15%	LC542827.1
Hepatitis B virus Eg15 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.15%	LC542824.1
Hepatitis B virus strain s5 reverse transcriptase gene, partial cds	636	636	100%	6e-178	99.15%	MK213859.1
Hepatitis B virus isolate Homeless SDF512 polymerase (P) gene, partial cds; and S protein (S) gene, complete cds	636	636	100%	6e-178	99.15%	MK840532.1
Hepatitis B virus isolate Iq2 polymerase gene, partial cds	636	636	100%	6e-178	99.15%	MK517523.1

Sample: 4

Hepatitis B virus isolate GSK1901, complete genome

Sequence ID: KY629632.1 **Length:** 3215 **Number of Matches:** 1

Range 1: 310 to 636 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
577 bits(312)	3e-160	322/327(98%)	0/327(0%)	Plus/Plus

```
Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCGACTTGTCCTGGTTA  60
Sbjct  310  ............................................................  369

Query  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCAT  120
Sbjct  370  ............................................................  429

Query  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTGTACTTCCAGG  180
Sbjct  430  ...........................................C..........  489

Query  181  ATCTTCAACCTCCAGCACGGGACCCTGCAGAACCTGCACGACCCCTGCTCAAGGAACCTC  240
Sbjct  490  ..........A..............................T.................  549

Query  241  TATGTATCCCTCTTGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCAT  300
Sbjct  550  ............C...........A..................................  609

Query  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
Sbjct  610  ...........................  636
```

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus isolate GSK1901, complete genome	577	577	100%	3e-160	98.47%	KY629632.1
Hepatitis B virus isolate XZ82, complete genome	571	571	100%	2e-158	98.17%	MN683720.1
Hepatitis B virus ADS8376 S and P genes for S protein and polymerase, partial cds	571	571	100%	2e-158	98.17%	LC546845.1
Hepatitis B virus isolate NOR590 polymerase (P) gene, partial cds	571	571	100%	2e-158	98.17%	MK173075.1
Hepatitis B virus isolate 3522_NL_2003 S protein (S) gene, partial cds	571	571	100%	2e-158	98.17%	MH521449.1
Hepatitis B virus isolate 2488_NL_2002 S protein (S) gene, partial cds	571	571	100%	2e-158	98.17%	MH521429.1
Hepatitis B virus isolate 2487_NL_2002 S protein (S) gene, partial cds	571	571	100%	2e-158	98.17%	MH521428.1
Hepatitis B virus isolate 466201_NL_2007 S protein (S) gene, partial cds	571	571	100%	2e-158	98.17%	KX659562.1
Hepatitis B virus isolate B118_2012_CD precore/core protein (C), core protein (C), polymerase (P), large surface protein (S), middle surface protein (S), and small surface protein (S) genes, complete cds	571	571	100%	2e-158	98.17%	KX354997.1
Hepatitis B virus isolate 141_B polymerase (Pol) gene, partial cds	571	571	100%	2e-158	98.17%	KP997385.1
Hepatitis B virus isolate I113-2-5, complete genome	571	571	100%	2e-158	98.17%	KT991427.1
Hepatitis B virus isolate dww1054, complete genome	571	571	100%	2e-158	98.17%	KC774446.1
Hepatitis B virus isolate PG-65, complete genome	571	571	100%	2e-158	98.17%	KF471642.1
Hepatitis B virus strain HBV055, complete genome	571	571	100%	2e-158	98.17%	MW601280.1
Hepatitis B virus isolate IR-65 large S protein (S) gene, complete cds	571	571	100%	2e-158	98.17%	KC339912.1

Sample: 5

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus isolate NOR560 polymerase (P) gene, partial cds	641	641	100%	1e-179	100.00%	MK173170.1
Hepatitis B virus isolate NOR102 polymerase (P) gene, partial cds	641	641	100%	1e-179	100.00%	MK173158.1
Hepatitis B virus isolate NOR584 polymerase (P) gene, partial cds	641	641	100%	1e-179	100.00%	MK173128.1
Hepatitis B virus isolate NOR532 polymerase (P) gene, partial cds	641	641	100%	1e-179	100.00%	MK173097.1
Hepatitis B virus isolate 712HBV2014 polymerase (pol) gene, partial cds	641	641	100%	1e-179	100.00%	MH272715.1
Hepatitis B virus isolate PG-3, complete genome	641	641	100%	1e-179	100.00%	KF471659.1
Hepatitis B virus isolate IR-3 large S protein (S) gene, complete cds	641	641	100%	1e-179	100.00%	KC339907.1
Hepatitis B virus isolate 4-hbv S protein (S) gene, complete cds	641	641	100%	1e-179	100.00%	MW234354.1
Hepatitis B virus isolate Shiraz-486 small surface protein (S) gene, complete cds	641	641	100%	1e-179	100.00%	KC176148.1
Hepatitis B virus isolate Shiraz-479 small surface protein (S) gene, complete cds	641	641	100%	1e-179	100.00%	KC176144.1
Hepatitis B virus isolate 288 S protein (S) gene, complete cds	641	641	100%	1e-179	100.00%	HM358293.1
Hepatitis B virus isolate 161 nonfunctional S protein (S) gene, complete sequence	641	641	100%	1e-179	100.00%	HM348657.1
Hepatitis B virus isolate M-DDRC63-02(48c) S protein (s) gene, complete cds	641	641	100%	1e-179	100.00%	HM229739.1
Hepatitis B virus isolate GS19 polymerase (pol) gene, partial cds	638	638	100%	2e-178	99.71%	GQ486054.1
Hepatitis B virus Eg51 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.71%	LC542860.1
Hepatitis B virus Eg29 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.71%	LC542838.1
Hepatitis B virus Eg27 S and P genes for S protein and polymerase, partial cds	636	636	100%	6e-178	99.71%	LC542836.1

Sample: 16

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus ADS8374 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC546844.1
Hepatitis B virus Eg51 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542860.1
Hepatitis B virus Eg29 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542838.1
Hepatitis B virus Eg27 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542836.1
Hepatitis B virus Eg18 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542827.1
Hepatitis B virus Eg15 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542824.1
Hepatitis B virus isolate Meshaal3 large S protein (S) gene, partial cds	604	604	100%	2e-168	100.00%	MN729574.1
Hepatitis B virus isolate Homeless SDF512 polymerase (P) gene, partial cds; and S protein (S) gene, complete cds	604	604	100%	2e-168	100.00%	MK840532.1
Hepatitis B virus isolate Iq2 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517523.1
Hepatitis B virus isolate Iq4 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517522.1
Hepatitis B virus isolate Iq3 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517520.1
Hepatitis B virus isolate 1DN polymerase (P) gene, partial cds	604	604	100%	2e-168	100.00%	MK410101.1
Hepatitis B virus isolate 12/16/0104 S antigen (S) gene, partial cds	604	604	100%	2e-168	100.00%	MK461100.1

Sample: 14

Hepatitis B virus strain s19 reverse transcriptase gene, partial cds

Sequence ID: MK213873.1 **Length:** 1098 **Number of Matches:** 1

Range 1: 184 to 510 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
588 bits(318)	2e-163	324/327(99%)	0/327(0%)	Plus/Plus

```
Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAACTTGACCTGGTTA  60
Sbjct  184  ..................................................T.........  243

Query  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCAT  120
Sbjct  244  ............................................................  303

Query  121  CTTCTTGTTGGTTCTTCTGGACTATCACGGTATGTTGCCCGTTTGTCCTCAAATTCCAGG  180
Sbjct  304  ............................A.....................T.........  363

Query  181  ATCTTCAACCACCAGCACGGGACCCTGCAGAACCTGCACGACTCCTGCTCAAGGAACCTC  240
Sbjct  364  ............................................................  423

Query  241  TATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCAT  300
Sbjct  424  ............................................................  483

Query  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
Sbjct  484  ...........................  510
```

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus strain s19 reverse transcriptase gene, partial cds	588	588	100%	2e-163	99.08%	MK213873.1
Hepatitis B virus isolate adf28 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	MK886716.1
Hepatitis B virus isolate ada12 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	MK886714.1
Hepatitis B virus isolate NOR93 polymerase (P) gene, partial cds	588	588	100%	2e-163	99.08%	MK173141.1
Hepatitis B virus isolate JO_57 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	MK033373.1
Hepatitis B virus isolate 2385_NL_2002 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	MH521362.1
Hepatitis B virus isolate 2381_NL_2002 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	MH521360.1
Hepatitis B virus isolate 1400027_NL_2013 S protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	KX659672.1
Hepatitis B virus isolate EPA_078 polymerase (P), large S protein (S), middle S protein (S), and S protein (S) genes, partial cds	588	588	100%	2e-163	99.08%	KT288339.1
Hepatitis B virus isolate LS22 large surface protein (S) gene, partial cds; nonfunctional middle surface protein (S) gene, partial sequence; and small surface protein (S) gene, partial cds	588	588	100%	2e-163	99.08%	KT954005.1
Hepatitis B virus isolate IR-SBMU-D74 small HBsAg (S) gene, complete cds	588	588	100%	2e-163	99.08%	KJ174166.1
Hepatitis B virus isolate HBV_NWFD_AHB29 large S protein (S) gene, complete cds; polymerase (P) gene, partial cds; and middle S protein (S) and S protein (S) genes, complete cds	588	588	100%	2e-163	99.08%	MZ054313.1
Hepatitis B virus isolate IR-345 mutant large S protein (S) gene, complete cds	588	588	100%	2e-163	99.08%	KC339793.1
Hepatitis B virus DNA, complete genome, isolate: GaziantepLCC7	588	588	100%	2e-163	99.08%	AB674418.1

Sample: 17

Hepatitis B virus isolate NOR112 polymerase (P) gene, partial cds

Sequence ID: MK173200.1 **Length:** 740 **Number of Matches:** 1

Range 1: 97 to 423 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
593 bits(321)	3e-165	325/327(99%)	0/327(0%)	Plus/Plus

```
Query  1    TTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCGACTTGTCCTGGTTA  60
Sbjct  97   ............................................................  156

Query  61   TCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCAT  120
Sbjct  157  ............................................................  216

Query  121  CTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGG  180
Sbjct  217  ............................................................  276

Query  181  ATCTTCAACCACCAGCACGGGACCATGCAGAACCTGCACGACGCCTGCTCAAGGAACCTC  240
Sbjct  277  ........................................T...................  336

Query  241  TATGTATCCCTCATGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCAT  300
Sbjct  337  ............................................................  396

Query  301  CCCATCATCCTGGGCTTTCGGAAAATT  327
Sbjct  397  .........T..................  423
```

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus isolate NOR112 polymerase (P) gene, partial cds	593	593	100%	3e-165	99.39%	MK173200.1
Hepatitis B virus isolate 2487_NL_2002 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	MH521428.1
Hepatitis B virus isolate 525977_NL_2007 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	KX659600.1
Hepatitis B virus isolate 304915_NL_2005 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	KX659427.1
Hepatitis B virus isolate 282107_NL_2005 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	KX659385.1
Hepatitis B virus isolate 259985_NL_2005 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	KX659360.1
Hepatitis B virus isolate 48TunSO_12 S protein (S) gene, partial cds	593	593	100%	3e-165	99.39%	KU947266.1
Hepatitis B virus isolate Moldova121 S protein and polymerase genes, partial cds	593	593	100%	3e-165	99.39%	KR871262.1
Hepatitis B virus isolate PG-65, complete genome	593	593	100%	3e-165	99.39%	KF471642.1
Hepatitis B virus isolate IR-65 large S protein (S) gene, complete cds	593	593	100%	3e-165	99.39%	KC339912.1
Hepatitis B virus isolate IR-10 large S protein (S) gene, complete cds	593	593	100%	3e-165	99.39%	KC339799.1
Hepatitis B virus isolate SDAC_018 large S protein (S) gene, complete cds; and X protein (X) and precore/core protein (C) genes, partial cds	593	593	100%	3e-165	99.39%	KF170756.1
Hepatitis B virus gene for large envelope protein, partial cds, isolate: F55	593	593	100%	3e-165	99.39%	AB561848.1
Hepatitis B virus isolate MORO-HEP-52 hepatitis B surface antigen (S) gene, partial cds	593	593	100%	3e-165	99.39%	JF271705.1
Hepatitis B virus isolate M-DDRC43_02(28c) S protein (s) gene, complete cds	593	593	100%	3e-165	99.39%	HM229720.1
Hepatitis B virus isolate I_T120, complete genome	593	593	100%	3e-165	99.39%	GU456667.1

Sample: 19

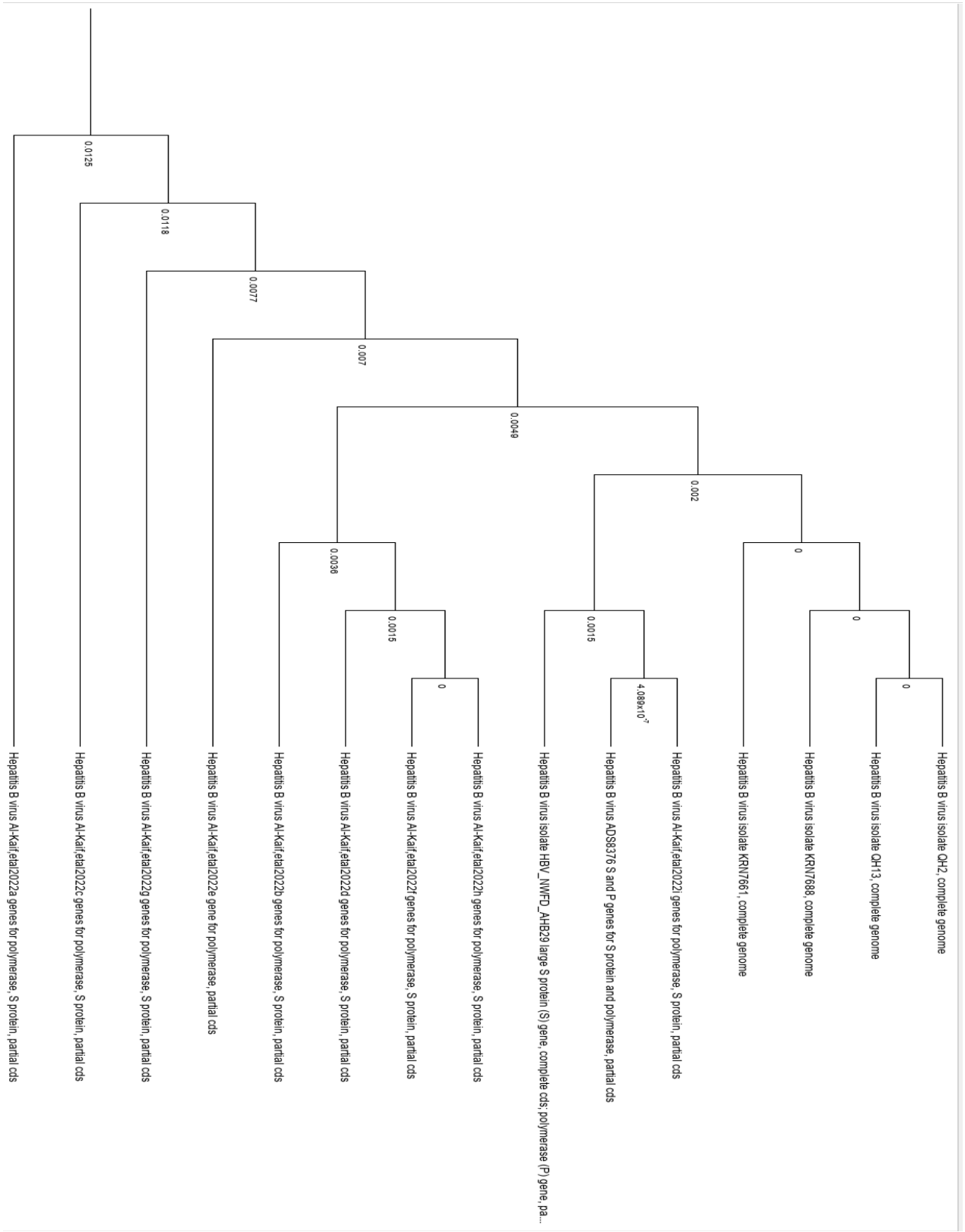
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus ADS8374 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC546844.1
Hepatitis B virus Eg51 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542860.1
Hepatitis B virus Eg29 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542838.1
Hepatitis B virus Eg27 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542836.1
Hepatitis B virus Eg18 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542827.1
Hepatitis B virus Eg15 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC542824.1
Hepatitis B virus isolate Meshaal3 large S protein (S) gene, partial cds	604	604	100%	2e-168	100.00%	MN729574.1
Hepatitis B virus isolate Homeless SDF512 polymerase (P) gene, partial cds; and S protein (S) gene, complete cds	604	604	100%	2e-168	100.00%	MK840532.1
Hepatitis B virus isolate Iq2 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517523.1
Hepatitis B virus isolate Iq4 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517522.1
Hepatitis B virus isolate Iq3 polymerase gene, partial cds	604	604	100%	2e-168	100.00%	MK517520.1
Hepatitis B virus isolate 1DN polymerase (P) gene, partial cds	604	604	100%	2e-168	100.00%	MK410101.1
Hepatitis B virus isolate 12/16/0104 S antigen (S) gene, partial cds	604	604	100%	2e-168	100.00%	MK461100.1
Hepatitis B virus isolate HBV_occult_VNovg46, complete genome	604	604	100%	2e-168	100.00%	MK598645.1
Hepatitis B virus isolate Khab1538/2016 polymerase (P) gene, partial cds	604	604	100%	2e-168	100.00%	MH577839.1
Hepatitis B virus isolate NOR67 polymerase (P) gene, partial cds	604	604	100%	2e-168	100.00%	MK173269.1

Sample: 20

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Hepatitis B virus ADS8376 S and P genes for S protein and polymerase, partial cds	604	604	100%	2e-168	100.00%	LC546845.1
Hepatitis B virus strain HBV055, complete genome	604	604	100%	2e-168	100.00%	MW601280.1
Hepatitis B virus isolate XZDP10, complete genome	599	599	100%	7e-167	99.69%	MN657318.1
Hepatitis B virus isolate XZ241, complete genome	599	599	100%	7e-167	99.69%	MN657316.1
Hepatitis B virus isolate XZ74, complete genome	599	599	100%	7e-167	99.69%	MN657315.1
Hepatitis B virus isolate XZDP5, complete genome	599	599	100%	7e-167	99.69%	MN683725.1
Hepatitis B virus isolate XZ90, complete genome	599	599	100%	7e-167	99.69%	MN683723.1
Hepatitis B virus isolate XZ81, complete genome	599	599	100%	7e-167	99.69%	MN683719.1
Hepatitis B virus isolate XZ76, complete genome	599	599	100%	7e-167	99.69%	MN683716.1
Hepatitis B virus isolate XZ67, complete genome	599	599	100%	7e-167	99.69%	MN683714.1
Hepatitis B virus isolate XZ439, complete genome	599	599	100%	7e-167	99.69%	MN683711.1
Hepatitis B virus isolate XZ438, complete genome	599	599	100%	7e-167	99.69%	MN683710.1
Hepatitis B virus isolate XZ373, complete genome	599	599	100%	7e-167	99.69%	MN683709.1
Hepatitis B virus isolate XZ371, complete genome	599	599	100%	7e-167	99.69%	MN683708.1
Hepatitis B virus isolate XZ310, complete genome	599	599	100%	7e-167	99.69%	MN683705.1
Hepatitis B virus isolate XZ172, complete genome	599	599	100%	7e-167	99.69%	MN683700.1

Appendix: III

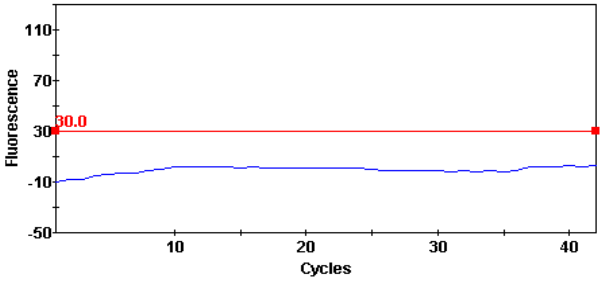
Phylogenetic tree of HBV S gene in NCBI confirmed



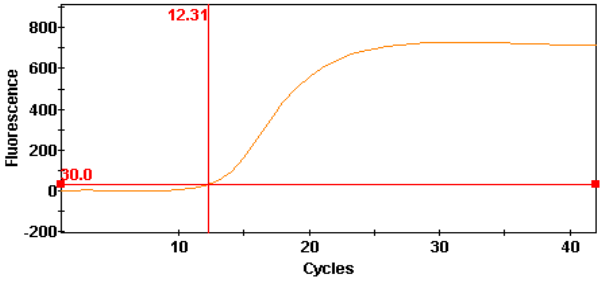
Appendix: IV

HBV viral load of HBV infected patients

1. High level of viral load (FAM curve, but Cy3 is earlier than 25 cycles).

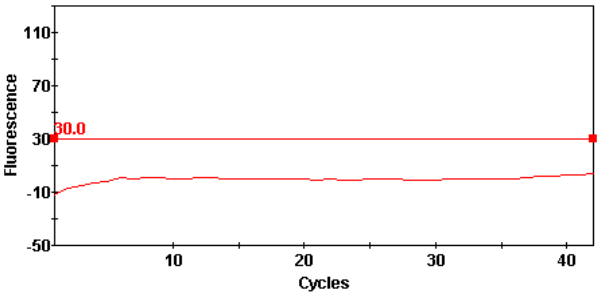


Control

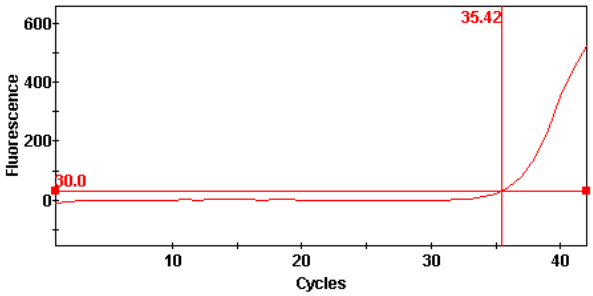


sample

2. Nucleic acid contamination or missing internal control because No FAM (Repeated sample)

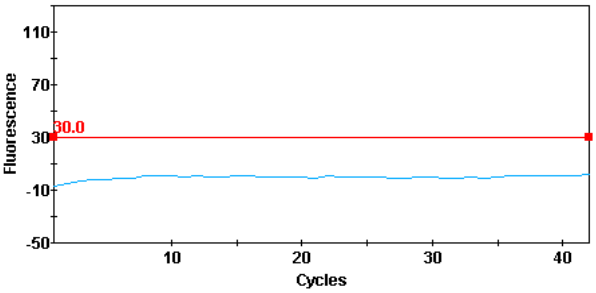


Control

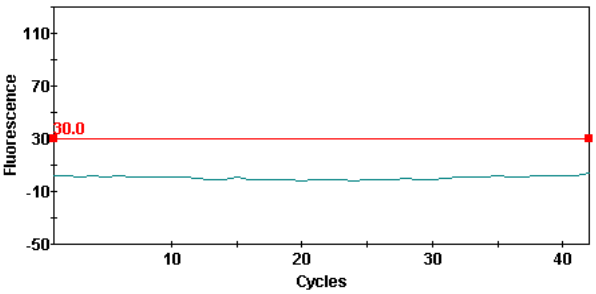


sample

3. Nucleic acid was lost because there was No FAM and no CY3 (Repeated sample).



Control

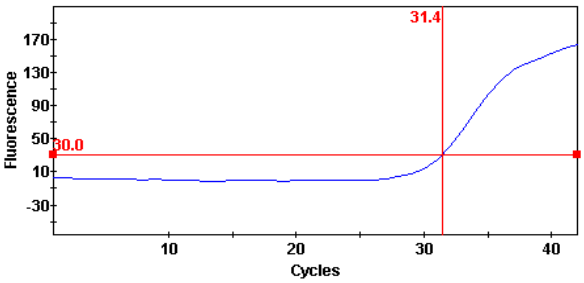


sample

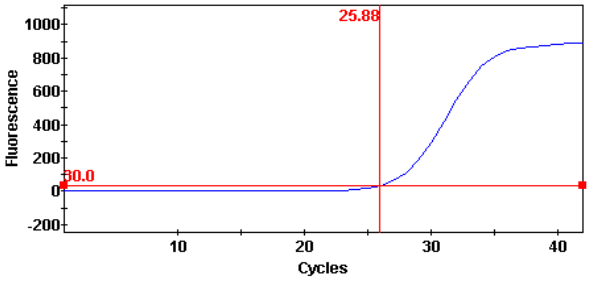
Appendix: V

HCV viral load of HBV-infected patients

1. A positive result (+ve positive FAM and Cy3).

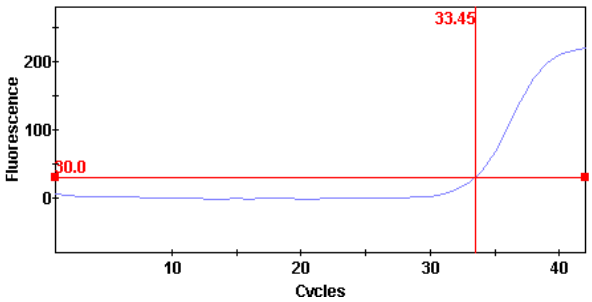


Control

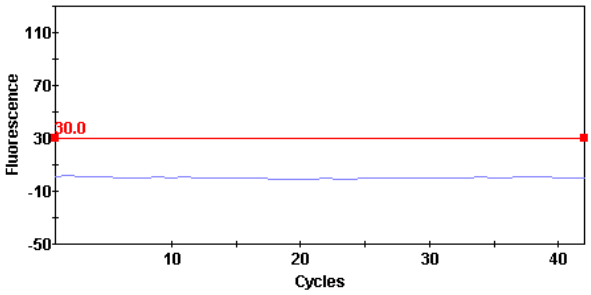


sample

2. A negative result (+ve positive FAM curve, but Cy3 is -ve Negative).



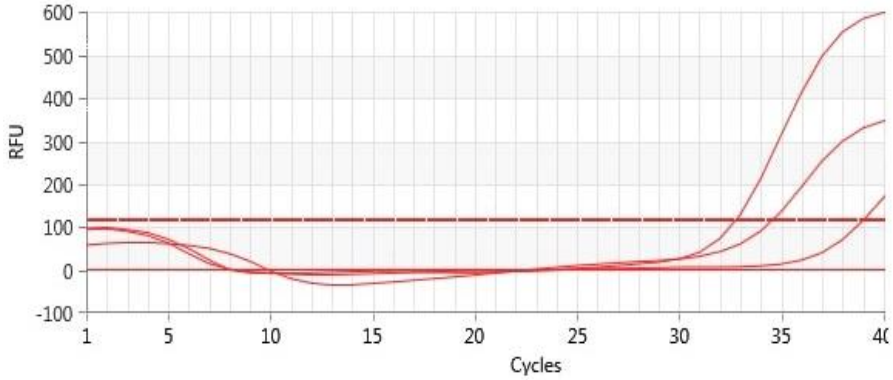
Control



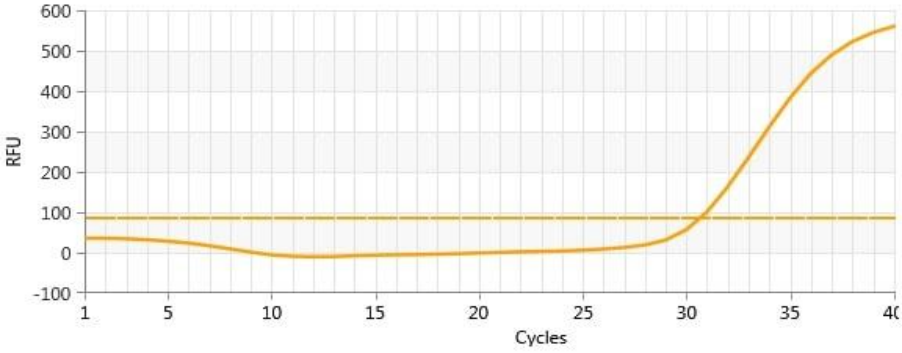
sample

Appendix: VI

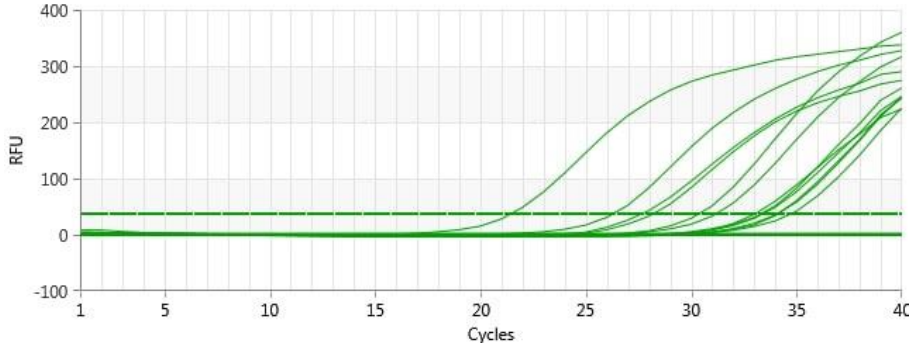
HBV genotyping



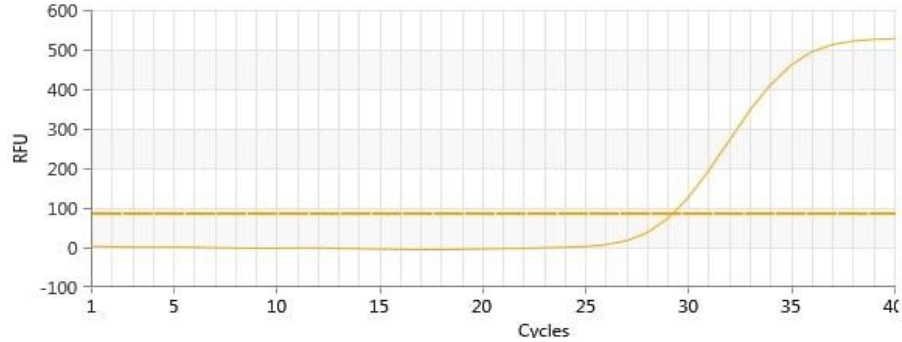
A genotype (Cy5/Red channel)



B genotype (Rox/Texas Red/Orange channel)



C genotype (FAM/Green channel)**.**



D genotype (JOE/HEX/Yellow channel)

Conclusions:

In the light of the previous results, the present study can conclude the following points:

1. Infection by HBV has a higher frequency in the Baghdad patients compared to remaining Iraq patients.
2. High rate of HBV and SARS-CoV-2 co-infection was recorded in this study specially among males .
3. Non-vaccinated cases of HBV alone or HBV with SARS-CoV-2 were predominate in this study
4. Chronic HBV was more frequent HBV alone or HBV with SARS-CoV-2
5. Hepatitis B virus reactivations was recorded in acute and chronic cases.
6. The risk of HBV reactivation for patients who had coinfection with SARS-CoV-2, no severe cases were recorded compared to the advanced cases of the disease who received immunosuppressive therapy and biological treatment
7. Mono-genotypes in patients infected with HBV and genotype C were more prevalent. In addition to the existence of a relationship between viral load and genotyping .
8. It is necessary to consider the special care of persons exposed to infection with SARS-CoV-2 to patients infected with viral hepatitis. In particular, advanced cases of the disease and their stages of treatment as it leads to liver dysfunction and life-threatening complication.
9. Non-successful vaccination coverage of the hepatitis B virus vaccine was performed by MOH.
10. In the case of low viral load samples, sequencing is a practical method to detect HBV infection with high sensitivity and accuracy and enables the detection of mutations in the HBV genome in low viral load samples from low viral load samples HBV-infected patients.
11. Some of the samples in this study were recorded globally for first time genetically in patients infected with HBV and SARS-CO-V-2.

Recommendations:

We recommended that:

1. Hepatitis B virus cases should be studied according to their stage, and periodic follow-up and routine examinations should be conducted on patients infected with SARS-CoV-2 and HBV.
2. Expansion of the study of genotypes in Iraq for affected people to know the prevalence of the most common genotype, management of the clinical case, and appropriate treatments according to each genotype, especially for patients with progressive stages or coinfection or infected with autoimmune diseases.
3. A population-based study is needed to estimate the prevalence of infection.
4. Spreading community awareness and providing high-quality services in the health field, especially for people infected with viral hepatitis in the shadow spread of COVID-19, is the most appropriate way to reduce this disease.
5. Imposing a compulsory vaccination program for all and conducting periodic examinations for early detection of the disease and prevention to reduce the development of cases to advanced stages .
6. Support researchers to advance developed medical preparation to process the global problems of viral hepatitis and SARS-CoV-2 infection.
7. Early detection of HBV drug resistance is crucial for clinicians to decide on the choice of antiviral treatment.
8. Immunization with vaccination to HBV before starting dialysis, thalassemia will reduce infection
9. Individuals working in hospitals or centers for Hepatology and Gastroenterology who are in direct contact with HBV patients to be careful even after receiving the three doses of the vaccine.

Decision of Examination Committee

We, the examination committee, certify that we have read the thesis entitled (**Hepatitis B Virus Genotyping Characterization and Distribution in Patients with SARS-COV-2 and Viral Hepatitis Co-infections in Iraq**) and have examined the student (**Laith Ahmed Imran Al-Kaif**) in its contents and that in our opinion it is accepted as a thesis for Degree of Ph.D. in Medical Microbiology with **excellent** estimation.

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Summary*:*

A hundred and forty-one of Hepatitis B virus blood specimens in this case-control study included a hundred and fifteen HBV with SARS-CoV-2 (case) and twenty-six HBV alone (control) specimens from Hepatology and Gastroenterology Teaching Hospital in Baghdad Medical City, Center of artificial Kidney, and Center of Hepatology and Gastroenterology Hospital in Marjan Medical City / Babylon from December 2020 to June 2021. Information were taken from the patient such as residence, age, sex, HBV vaccine intake, HBV contact, infection status, and liver cirrhosis in the HBV patient. The current study was carried out to investigate the influence of SARS-CoV-2 on the reactivation of Hepatitis B infection and on other types of viral hepatitis infections, and to study the influence HBV genetic mutations on the sensitivity of SARS-CoV-2 infection. Therefore, The tests were conducted on blood groups, hematological markers (WBCs, platelet count, International Normalize Ratio), immunological markers (COVID-19 IgM/IgG, HBs Ag, HBc IgM Ab, HBe Ag, HBe Ab, HDV IgG), biochemical marker(Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, albumin), and genetic analysis (Viral load HBV/HCV, Nested PCR, sequencing) for HBV patients.

The results of demographic data examination among patients with hepatitis B virus showed that the highest rate of HBV infection was identified in Baghdad province, at 71.63%, compared to some Iraqi governorates within the study. The results revealed that 115 (1, 80, and 34) out of 141 HBV infections had positive COVID-19-IgM, IgG, and IgM&IgG, respectively, compared to 26 with HBV alone. Regarding gender as a demographic factor among HBV patients in this study, the results showed that the gender of males had a higher incidence than females. The patients included in this study aged from > 30 to < 60 years old. The results revealed that most of the patients in

the second group were 30-60 years old and recorded the highest infection rate compared to the remaining age groups. As for the HBV vaccination status, the results showed that a very high percentage did not receive the HBV vaccine, 75.9% and 15.6% for the individuals of HBV with SARS-CoV-2 and HBV alone, respectively. The data on HBV contacts in patients with HBV alone and HBV with SARS-CoV-2 showed an incidence of 5% and 26.2%, respectively. This study revealed that chronic HBV was more frequent in the status of the diseases than others, patients infected with HBV alone and HBV with SARS-CoV-2 showed 12.8% and 58.2%, respectively. In addition, the results showed that five out of 141 had liver cirrhosis in patients infected with HBV compared to those without cirrhosis.

The current study results showed significant differences in HBV reactivation in sera patients with HBV and SARS-CoV-2 at $p<0.05$. As 34.07% of patients diagnosed with SARS-CoV-2 showed HBV reactivation, depending on HBs Ag, HBc IgM Ab, HBe Ag, HBe Ab.

The hepatitis D virus examination results showed diagnosed, and confirmed cases with HBV infected, all negative for HDV.

Additionally, subjects with HBV alone and HBV with SARS-CoV-2 showed that high ratio of O^+ (57.4 %, 15.6%) and B^+ (12.8%, 2.1%) blood group, respectively, for having confirmed cases in comparison to another blood group. In this study, it was found that there was no significant difference in total WBCs and Platelet counts between the two study groups for all demographic factors. At the same time, the international normalized ratio (INR) showed significant differences between diseases status and liver cirrhosis compared to the remaining demographic characteristics ($P < 0.05$).

In this study, Albumin, ALT, AST, and Alkaline Phosphatase were studied as biochemical diagnostic parameters for patients with HBV alone and HBV with SARS-CoV-2. The results were not statistically significant for

demographic factors, except that liver cirrhosis with albumin is highly significant compared to the control groups ($P < 0.001$).

The current study from the genetic analysis of individuals with HBV revealed three cases of HCV as coinfection. A significant increase in HBV viral load was seen in liver cirrhosis only from the rest of the demographic distribution, which did not significantly differ from the control group. In contrast, the results of HCV viral load showed a highly significant difference in age from the rest of the demographic distribution that did not show any significant difference compared to the control group.

The study also revealed no significant difference in hematological, biochemical, immunological, and genetic examinations among individuals with HBV and HCV coinfection compared to HBV alone ($P \geq 0.05$).

The study observed a highly significant difference for HBV genotypes compared to the control group ($P < 0.001$). HBV genotypes distributions found a prevalence among HBV and SARS-CoV-2 co-infection patients with genotypes C, A, B, D (42.9%, 25%, 21.4%, and 10.7%, respectively). In the present study of HBV genotyping, total WBCs and platelet count were studied as hematological parameters for HBV patients. The study showed that there was no significant difference of $P \geq 0.05$. While the Coagulation factor (INR) showed between HBV genotyping, where the results showed a significant difference of $P = 0.018$. In contrast, it showed no significant difference between HBV genotyping for the biochemical markers. Regarding HBV reactivation between HBV genotyping, the current study results showed that HBsAg, HBcAb, and HBeAg have a significant difference for HBV genotyping compared to HBeAb. However, it did not show any significant difference of $P \geq 0.05$.

After that, 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. As a result, 10 out of 22 samples were selected for the purpose of 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.

الخلاصـــــــــة :

تضمنت الدراسة الحالية مائة وواحد وأربعون عينة من عينات الدم للأشخاص المصابين بالتهاب الكبد الفيروسي نوع ب، حيث كانت مائة وخمسة عشر عينة من المصابين بالتهاب الكبد الفيروسي نوع ب مع فيروس سارس-2 (حالة) وستة وعشرون عينة من المصابين بفيروس التهاب الكبد نوع ب وحده (مجموعة السيطرة) من مستشفى الجهاز الهضمي والأمراض الكبد التعليمي في مدينة الطب/ بغداد ومركز الكلى الصناعية ومركز الجهاز الهضمي والأمراض الكبد في مدينة مرجان الطبية / بابل من كانون الاول 2020 إلى حزيران 2021. اخذت معلومات من المريض مثل الإقامة والعمر والجنس ولقاح التهاب الكبد الفيروسي نوع ب الذي تم تلقيه والتلامس مع مرضى التهاب الكبد الفيروسي نوع ب وحالة الإصابة وتليف الكبد في مرضى المصابين بالتهاب الكبدي الفيروسي نوع ب. أجريت الدراسة الحالية لتحديد مدى إعادة تنشيط فيروس التهاب الكبد الفيروسي نوع ب اعتمادًا على التأثير المناعي في المرضى المصابين بفيروس سارس-2 وعواقبه على حساسية المضيف والتهابات الكبد الفيروسية. لذلك ، أجريت الاختبارات لفحص مجاميع الدم والمؤشرات الدموية (كريات الدم البيضاء ، عدد الصفائح الدموية ، عوامل التخثر) والمؤشرات المناعية (COVID-19 IgM / IgG، HBs Ag، HBc Ab، HBe Ag، HBe Ab، HDV IgG) والمؤشرات الكيموحيوية (الفوسفاتاز القلوي، ناقلة الأسبارتات، ناقلة امين الالانين، الألبومين) والتحليل الجيني (الحمل الفيروسي لفيروس التهاب الكبد الفيروسي نوع ب ونوع ج، تفاعل البوليميراز المتسلسل المتداخل ، التسلسل الوراثي) لمرضى الالتهاب الكبد الفيروسي نوع ب.

أظهرت نتائج فحص البيانات الديموغرافية بين مرضى التهاب الكبد الفيروسي نوع ب ، أن أعلى معدل للإصابة بفيروس التهاب الكبد الفيروسي نوع ب تم تحديده في محافظة بغداد بنسبة 71.63٪ ، مقارنة ببعض المحافظات العراقية ضمن الدراسة. أظهرت النتائج أن 115 (1، 80، 34) من 141 إصابة بفيروس التهاب الكبد الفيروسي نوع ب كانت إيجابية COVID-19-IgM وIgG وIgM & IgG على التوالي ، مقارنة بـ 26 مع التهاب الكبد الفيروسي نوع ب وحده. فيما يتعلق بالجنس كعامل ديموغرافي بين مرضى الالتهاب الكبد الفيروسي نوع ب في هذه الدراسة، أظهرت النتائج أن جنس الذكور كان أعلى من الإناث. تتراوح أعمار المرضى المشمولين في هذه الدراسة من أقل من 30 إلى أكثر من 60 عامًا. وأظهرت النتائج أن معظم مرضى المجموعة الثانية تتراوح أعمارهم بين 30-60 سنة وسجلوا أعلى معدل إصابة مقارنة مع باقي الفئات العمرية. أما بالنسبة لحالة التطعيم ضد التهاب الكبد الفيروسي نوع ب ، فقد أظهرت النتائج أن نسبة عالية جدًا لم تتلق لقاح فيروس التهاب الكبد الفيروسي نوع ب ، ٪75.9 و 15.6٪ للأفراد المصابين بفيروس التهاب الكبد

الفيروسي نوع ب المصابين بـفيروس سارس-2 و فيروس التهاب الكبد الفيروسي نوع ب فقط على التوالي. أظهرت البيانات الخاصة بالتلامس مع فيروس التهاب الكبد الفيروسي نوع ب في المرضى الذين يعانون من فيروس التهاب الكبد الفيروسي نوع ب وحده و فيروس التهاب الكبد الفيروسي نوع ب مع فيروس سارس-2 حدوث 5 ٪ و 26.2 ٪ على التوالي. كشفت هذه الدراسة أن الالتهاب الكبدي الوبائي المزمن كان أكثر تواتراً في حالة المرض من غيره ، حيث أظهر المرضى المصابون ب فيروس التهاب الكبد الفيروسي نوع ب وحده و فيروس التهاب الكبد الفيروسي نوع ب مع فيروس سارس-2 ٪12.8 و 58.2٪ على التوالي. بالإضافة إلى ذلك ، أظهرت النتائج أن خمسة من أصل 141 أصيبوا بتليف الكبد لدى مرضى مصابين بفيروس التهاب الكبد بي مقارنة بمن لا يعانون من تليف الكبد.

أظهرت نتائج الدراسة الحالية فروقًا ذات دلالة إحصائية في إعادة تنشيط فيروس التهاب الكبد الفيروسي نوع ب في مرضى المصل المصابين بفيروس التهاب الكبد الفيروسي نوع ب و فيروس سارس-2 عند $P < 0.05$. حيث كانت 34.07% مرضى مصابين بفيروس التهاب الكبد الفيروسي نوع ب المصابين بفيروس سارس-2 إعادة تنشيط فيروس التهاب الكبد الفيروسي نوع ب، اعتمادًا على HBs Ag، HBc Ab، HBe Ag، HBe Ab.

وأظهرت نتائج فحص فيروس التهاب الكبد الوبائي (د) حالات تم تشخيصها وتأكيدها على أنها مصابة بفيروس التهاب الكبد B ، وكلها سلبية بالنسبة لفيروس التهاب الكبد الوبائي.

بالإضافة إلى ذلك ، أظهر الأشخاص المصابون بفيروس التهاب الكبد الفيروسي نوع ب وحده و فيروس التهاب الكبد الفيروسي نوع ب مع فيروس سارس-2 أن نسبة عالية من فصيلة الدم O + و B +، ٪57.4 و ٪15.6 و ٪12.8 و ٪2.1 ، على التوالي ، لتأكيد الحالات مقارنة بـ فصيلة دم أخرى. في هذه الدراسة ، وجد أنه لا يوجد فرق كبير في إجمالي عدد كرات الدم البيضاء والصفائح الدموية بين مجموعتي الدراسة لجميع العوامل الديموغرافية. في الوقت نفسه ، أظهرت النسبة الدولية الموحدة (عوامل التخثر) اختلافات معنوية بين حالة المرض وتليف الكبد مقارنة بالخصائص الديموغرافية المتبقية $P<0.05$.

في هذه الدراسة ، تمت دراسة الفوسفاتاز القلوي، ناقلة الأسبارتات، ناقلة امين الالانين، الألبومين كمعلمات تشخيص كيميائي حيوي للمرضى الذين يعانون من فيروس التهاب الكبد الفيروسي نوع ب وحده و فيروس التهاب الكبد الفيروسي نوع ب مع فيروس سارس-2. لم تكن النتائج ذات دلالة إحصائية للعوامل الديموغرافية ، فيما عدا أن تليف الكبد مع الألبومين ذو دلالة إحصائية عالية مقارنة بمجموعة السيطرة $P<0.001$.

كشفت الدراسة الحالية من التحليل الجيني للأفراد المصابين بفيروس التهاب الكبد الفيروسي نوع ب عن ثلاث حالات من التهاب الكبد الفيروسي كعدوى مرافقة. شوهدت زيادة كبيرة في الحمل الفيروسي لفيروس التهاب الكبد الفيروسي نوع ب في تليف الكبد فقط من بقية التوزيع الديموغرافي، والتي لم تختلف بشكل كبير عن المجموعة السيطرة. بالمقابل أظهرت نتائج الحمل الفيروسي فيروس التهاب الكبد الفيروسي نوع ج فرقاً معنوياً في العمر عن باقي التوزيع الديموغرافي لم يظهر أي فرق معنوي مقارنة بمجموعة السيطرة.

كما كشفت الدراسة عن عدم وجود فرق معنوي في الفحوصات الدموية والكيميائية الحيوية والمناعية والوراثية بين الأفراد المصابين بالعدوى المرافقة لـ فيروس التهاب الكبد الفيروسي نوع ب و فيروس التهاب الكبد الفيروسي نوع ج مقارنة بالفحص فيروس التهاب الكبد الفيروسي نوع ب وحده $P \geq 0.05$.

لاحظت الدراسة فرقاً معنوياً عالياً للأنماط الجينية لفيروس التهاب الكبد الفيروسي نوع ب مقارنة بالمجموعة السيطرة ($P<0.001$). وجدت توزيعات الأنماط الجينية لفيروس التهاب الكبد الفيروسي نوع ب انتشارًا بين مرضى التهاب الكبد الفيروسي نوع ب وفيروس سارس-2 المصابين بالأنماط الجينية ا، ب، ج، د (42.9 ٪، 25٪، 21.4٪، 10.7٪، على التوالي). في الدراسة الحالية للتنميط الجيني لفيروس التهاب الكبد الفيروسي نوع ب، تمت دراسة مجموع كرات الدم البيضاء وعدد الصفائح الدموية كمعلمات دموية لمرضى التهاب الكبد الفيروسي نوع ب. أظهرت الدراسة أنه لا يوجد فرق معنوي لـ $P \geq 0.05$. بينما أظهر عامل التخثر بين التنميط الجيني لفيروس التهاب الكبد الفيروسي نوع ب حيث أظهرت النتائج فرق معنوي قدره تساوي 0.018. بالمقابل، لم تظهر أي فرق كبير بين التنميط الجيني لفيروس التهاب الكبد الفيروسي نوع ب للمؤشرات كيموحيوية. فيما يتعلق بإعادة تنشيط فيروس التهاب الكبد الفيروسي نوع ب بين التنميط الجيني لفيروس التهاب الكبد الفيروسي نوع ب ، أظهرت نتائج الدراسة الحالية أن HBsAg و HBcAb و HBeAg لها فرق كبير في التنميط الجيني لفيروس التهاب الكبد الفيروسي نوع ب مقارنة بـ HBeAb. ومع ذلك ، لم تظهر أي فرق كبير من $P \geq 0.05$.

بعد ذلك، تم إجراء التشخيص الجيني للجين S لكل من الافراد المصابين بالتهاب الكبد الفيروسي نوع ب وحده اضافة مع سارس-2 في مركز التفوق العلمي/بغداد باستخدام تقنية تفاعل البلمرة المتداخل عن طريق استخراج الحمض النووي لفيروس التهاب الكبد نوع ب وتضخيمه، لتحديد تسلسل النيوكليوتيد ومقارنتها بالسلالات الدولية في بنك الجيني . نتيجة لذلك ، تم اختيار 10 من 22 عينة لغرض الدراسة الجينوم للجين S وتم تضخيمها بواسطة تقنية تفاعل البلمرة المتداخلة

للجينوم (S) وتم تسجيل 9 عينات في البنك الجيني برقم: LC705440، LC705441، LC705442، LC705443، LC705444، LC705445، LC705446، LC705447، LC705448.