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Genotypic Study of Hepatitis B Virus in Al- Diwaniya Governorate

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{قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ}

صدق الله العلي العظيم

سورة البقرة أية (٣٢)

Supervisor's certification

I certify that, this thesis entitled " **Genotypic Study of Hepatitis B Virus in Al-Diwaniya Governorate**" was prepared under my supervision at the Department of Medical Microbiology, College of Medicine /University of Al-Qadisiyah , as a partial requirements for degree of Master of Medical Microbiology.

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Report of the Head of Department of Microbiology

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We, the members of examining committee, certify after reading this thesis **(Genotypic Study of Hepatitis B Virus in Al-Diwaniya Governorate)** and after examining the student in its contents, it was found adequate for degree Master of Medical Microbiology with an excellent degree.

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Dedication

To the expected promise to the anticipated hope, and the Imam of pure people , Imam Mahdi (peace of God upon him).

To the spirit of my dear father

To my mother compassionate not forget me in its links

To my brothers and sisters

To the flowers beautiful in my life for those who gave me all the support and help with all the love, affection and appreciation to my wife and daughters.

Hazem

2018

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Summary

Hepatitis B virus (*HBV*) infection is a public health problem as a cause of liver diseases including hepatocellular carcinoma and liver cirrhosis. Ten *HBV* genotypes (A-J) were documented depending on the virus sequence homogeneity.

This study was carried out to detect *HBV* genotypes among *hepatitis B virus* patients in Al- Diwaniya governorate/ Iraq using nested PCR protocol. To achieve this goal a blood sample were collected from (80) patients which include 59 males , 21 females with age ranged between (12-75 years) confirmed as having viral hepatitis were included in this study.

Sera were tested for HBsAg by ELISA technique Sera positive HBsAg were subjected to polymerase chain reaction (PCR) for confirmation selected numbers of the confirmed positive sera were tested for genotyping by nested- PCR .DNA sequencing that was achieved for selected 8 samples. Data were summarized, presented and analyzed using statistical package for social science (SPSS version 23).

Eighty samples were positive for *HBV* surface antigen (HBsAg) by ELISA screening. Recorded results 33(41.25%) samples were positive for *HBV*- DNA and 47 (58.75%) samples were negative for *HBV*- DNA, by PCR. Survey of DNA positive samples for *HBV* genotypes by nested PCR the results showed that genotype E (3%) was the unique detected in this study, whereas the other samples showed mixed infection of more than one genotype.

The distribution percentage of *HBV* genotypes among the patients were as follows: A+E (3%) , C+D+E (12.12%) , B+C+D+E (15.15%) , A+D (3%) , A+B+C+D+E (18.18%) , A+B+D+ E (18.18), B+D+E (3%) , A+C+D+E (3%) ,B+C+D (6%) , A+B+D (3%) , A+B+C+D (6%) , D+E (6%) , whereas genotype F was not documented in any patient. Statistically there was not significantly different in distribution of genotypes among males and females (P.Value = 0.369), also from the obtained results there was not significantly different in location between rural and urban (P.Value =0.708).

The Phylogenetic tree analysis found that the local *Hepatitis B virus* clones (No.1, No.2, No.3, and No.6) were closely related to National Center for Biotechnology Information NCBI-Blast *Hepatitis B virus* (**KU668447.1)**. The local *Hepatitis B virus* clones (No.4) was closely related to NCBI-Blast *Hepatitis B virus* (**MF618340.1)** .The local *Hepatitis B virus* clones (No.5) was found to be closely related to NCBI-Blast *Hepatitis B virus* **(AY236161.1)**. Whereas, the local *Hepatitis B virus* clones (No.7 and No.8) were closely related to NCBI-Blast *Hepatitis B virus* (**KX196223.1)**.

The discovered sequences were confirmed by NCBI-BLAST Homology Sequence Identity. After that *hepatitis B virus* clones were deposited into of NCBI-Gen Bank to get Genbank accession number for *hepatitis B virus* clones.

This study showed that mixed genotypes *HBV* were rated (97 %) and pure genotype *HBV* was rated (3%).

The presence of mixed infection with about 5 *HBV* genotypes among most of the patients lead us to conclude that these patients are incurred to different sources of infection at different times among the

patients in Al-Diwaniya governorate .The current study showed mixed genotypic infection with 5 genotypes and only one genotype E, whereas genotype F was not found in any patient. This study shown that genotype D (93.94%) was predominant in the mixed genotypes among patients. On the basis of the current study shown that no significant difference males and females and no significant in location between rural and urban areas in occurs of *HBV*.

List of Tables

List of Figures

List of Abbreviations

Abbreviations	Description
ALT	Alanine Amino Transferase
Anti-HBC	Hepatitis B Core Antibody
Anti-HBe	e antibody Hepatitis B
Anti- HBS	Hepatitis B Surface Antibody
APRI	AST to Platelet Ratio Index
AST	Aspartate Amino Transferase
BCP	Basic Core Promoter
bp	Base Pair
CHB	Chronic Hepatitis B
ddH2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxy-Nucleotide Tri-Phosphates
ds DNA	Double Stranded Deoxyribonucleic Acid
CCC DNA	Covalently Closed Circular Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbant Assay
Enh	Enhancers
GRE	Glucocortrcoid Responsive Element
HBC	Hepatitis B Core protein
HBe Ag	Hepatitis B e Antigen
HBs Ag	Hepatitis B Surface Antigen
HBV	hepatitis B virus
HBx	HBV X Protein
HCC	Hepatocellular Carcinoma
HRP	Horseradish Peroxidase
IRF3	Interferon Regulatory Factor 3
IU	International Units
IVDUs	Intravenous Drug Users
LMICS	Low-and Middle- Income Countries
LHB	Lager Hepatitis B –Surface Antigen
(m) RNAs	Messenger Ribonucleic Acid

MHB	Medium Hepatitis B –Surface Antigen
MEGA	Molecular Evolutionary Genetic Analysis
MSA	Multiple Sequence Alignment
NA	Nucles Analogue
NCBI	National Center for Biotechnology Information
NCX	Negative Control axis
NITS	Non Invasive Test
NRE	Negative Regulatory Element
OD	Optical Density
ORFS	Open Reading Frames
pol	Polymerase
PC	Per-Core
PCR	Polymerase Chain Reaction
PgRNA	Pre- genomic Ribonucleic Acid
rc DNA	relaxed- circular Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rpm	Round per minute
SHB	Small Hepatitis B
TBE	Tris-Borate-Ethylene Diaminete Traacidic Acid Buffer
TSP-PCR	Type Specific Primers-Polymeras Chain Rwaction
U	Unit
UV	Ultra-Violet

Chapter One

Introduction

1. Introduction

Infection with *hepatitis B virus* (*HBV*) remains a huge public health issue all over the world (Williams *et al*., 2004; Hutin *et al*., 2018). It is estimated that approximately 1 million people per year die due to acute and chronic infections with *HBV* and eventually 15% to 25% of these individuals will progress to liver cirrhosis and hepatocellular carcinoma. A few viral factors exist which may influence infections with *HBV* including *HBV*- DNA genotype and mutations in surface *HBV* (S), pre core (PC) and basic core promoter (BCP) (Sali *et al*., 2013) . One feature of *HBV* is sequence heterogeneity of the DNA virus (Brown *et al*., 2016) .

The genome of *HBV*. The partially double stranded DNA (dsDNA) with the complete minus strand and the incomplete plus strand. The four open reading frames (ORFs) are shown: pre core/core (pre C/C) that encodes the e antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), PreS1/PreS2/S for surface proteins (three forms of HBsAg, small (S), middle (M) and large (L)) and X for atranscriptional trans-activator protein (Hu and Seeger, 2015).

Analysis of *HBV* sequence divergence enables identification of 10 genotypes of *HBV* (A-J) worldwide (Awan *et al*., 2010; Congly *et al*., 2013). *HBV* genotypes are differently allocated geographically, which may indicate the way of *HBV* spreading. Genotype A is found mainly in North America and North west Europe (Zhang and Cao, 2011) . B and C genotypes are highly prevalent in Australia and Asia (Raimondi *et al*., 2010; Bannister *et al*., 2018). Genotype D is worldwide in distribution with highest prevalence in the Middle East and Southern Europe, genotype E is almost entirely

restricted to West Africa, F genotype in Central and South America (Cheng *et al*., 2012; Gori *et al*., 2018) . Genotype G has been reported in the United States, Germany and France (Liu and Kao, 2013). Genotype H was recently identified in patients from Central America (Nabuco *et al*., 2012) . Genotype I was reported in Italy, Laos and Vietnam, while the newest genotype J has been recognized in Ryukyu island in Japan (Sunbul, 2014) . Hepatitis B virus genotyping is an important technique to explain the way of spread of the virus (Baig *et al*., 2007). In Iraq, several studies on *HBV* prevalence and genotyping were carried out. A study done by, Al.Suraifi, *et al*., 2016, found that all of samples had mixed genotypes, no single genotype were identified and genotype F was not found in any patient .in Duhok, Kurdistan region of Iraq (Abdulla and Goreal, 2016) found that genotype D is the main genotype in Duhok/ Iraq followed by genotype B. High percentage of chronic hepatitis B patients with genotype D are HBeAg positive. Whereas, in Basra (Al-Aboudi and Al-Hmudi, 2015) detected genotype D 92.3% and 7.69% had mixed genotypes D+ E .Also ,in Baghdad (Ahmed, 2013)found that genotype D (80%) was the predominant among CHB patients and mixed genotypes D+F (20%). Whereas In Al-Diwaniya governorate, to our knowledge, there is no previous study dealing with genotyping of *HBV*, so that this study was conducted for molecular detection of *HBV* and determination of this virus genotypes (A-F) using nested PCR protocol.

Enzyme-linked immunosorbent assay use the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibody in a fluid sample. ELISA utilizes enzyme-labeled antigens and

antibodies to detect the biological molecules (Balsam *et al.*, 2013; Canady *et al*., 2013) .

Nested polymerase chain reaction (Nested-PCR) is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites .Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product. This allows amplification for a low number of runs in the first round, limiting non-specific products. The second nested primer set should only amplify the intended product from the first round of amplification and not non-specific product. This allows running more total cycles while minimizing non-specific products. This is useful for very rare templates or PCR with high background (Chevaliez et al., 2017).

The DNA sequence differentiation method was first described by (Kimura 1980) , A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences and applied by Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6.0) software that facilities of comparative analyses of DNA and protein sequences and supposing the molecular evolutionary patterns of genes, genomes, and species over time (Tamura *et al*., 2013).

1.1 Aims of this study

This study aimed to determine the genotypic distribution of *HBV* in patient of Al-Diwaniya in / Iraq. Through the following objectives:

1. Screening of HBsAg in blood sample by using ELISA technique.
2. Detection of HBV Genotypes by nested –PCR technique.
3. DNA sequencing of isolates by phylogenetic tree and ClustalW alignment analysis.

Chapter Two

Literature Review

2.1 Hepatitis

Hepatitis means inflammation of the liver. Viral hepatitis is a major public health problem worldwide. Different viruses including hepatitis A, B, C, D and E viruses cause viral infections of human liver. Other causes include heavy alcohol use, certain medications, toxins, other infections, autoimmune diseases (Schweitzer *et al*., 2015).

Hepatitis B virus (*HBV*) was first discovered by Blumberg *et al* in 1965 (Alodini, 2012), and the relationship between *HBV* and acute hepatitis after blood transfusion was reported by Okochi in 1968 (Okochi and Murakami, 1968). At that time, most studies were based on immunological and serological methods. Molecular-based analyses progressed rapidly after the *HBV* particle was discovered and the *HBV* genome cloned (Hu, 2016). *HBV* infection is major global issue, and is a particular concern in Asia and Africa. Although *HBV* itself is not directly cytotoxic, the immune response to *HBV* infection causes liver damage and eventually leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Ganem and Prince, 2004). More than 350 million people worldwide are thought to be chronically infected with *HBV* and 1-2 million people die every year from *HBV*-related cirrhosis and HCC (Zoulim *et al*., 2013).

2.2 HBV Virology

2.2.1 Taxonomy

The *hepatitis B virus* (*HBV*) is the prototype member of a family the *hepadnaviridae* of hepatotropic DNA virus , genus the *orthohepadnaviruses* that replicate by reverse transcription of an RNA pregenome, *HBV* infects humans , whereas birds the *avihepadnaviruses* (Schaefer, 2007) .

2.2.2 Hepatitis B Genotypes

The *HBV* genome is composed of approximately 3,200 nucleotides (Matsuura *et al*., 2009) . Arauz-Ruiz, Norder, Robertson, & Magnius, (2002) previously classified *HBV* into 8 genotype identified as A-H based on an intergroup divergence of 8% or more in complete nucleotide sequence whose geographical distributions was previously extensively studied by (Sánchez *et al*., 2002) who documented that genotype A is pandemic, B and C are predominant in Asia, D in southern Europe, E in Africa, F in United States of America, G in France while H in Central America. However recent studies by (McMahon, 2009; Cao, 2009; Kurbanov *et al*., 2010) have introduced two new genotypes designated as I and J giving a total of 10 genotypes.

2.2.3 Structure of the Virion

Hepatitis B virus is an enveloped virus, measuring 42-47 nm in diameter, with an icosahedral nucleocapsid that encloses a partially double-stranded relaxed –circular (rc) DNA genome covalently bound to the viral polymerase. The envelope comprises a small amount of lipid of cellular origin and three hepatitis B surface proteins (HBS): large (LHB), medium, (MHB), and small (SHB). The serum of infected individuals contains, in

addition, two types of subviral particles: small spherical particles with a diameter of approximately 20 nm and filamentous particles also with a diameter of about 20 nm but of variable length. These non-infectious subviral particles lacking genomic DNA greatly outnumber the infectious viral particles , and have a composition similar to that of the viral envelope (Hu and Seeger, 2015) .The nucleocapsid is formed by multiple copies of core protein. Of the total 183-185 amino acids (depending upon genotype), the N-terminal 149-151 amino acids are responsible for self – assembly of the nucleocapsid. Although the steps in its assembly remain to be clarified, the first step is the formation of homodimers linked by disulfide bridges. The nucleocapsid contains pores that allow the diffusion of nucleotides during the synthesis of the DNA genome. The C-terminal amino acids of the core protein play a role in the packaging of the pre genome polymerase complex within the nucleocapsid (Bruss, 2007) , as shown in (Figure2.1) .

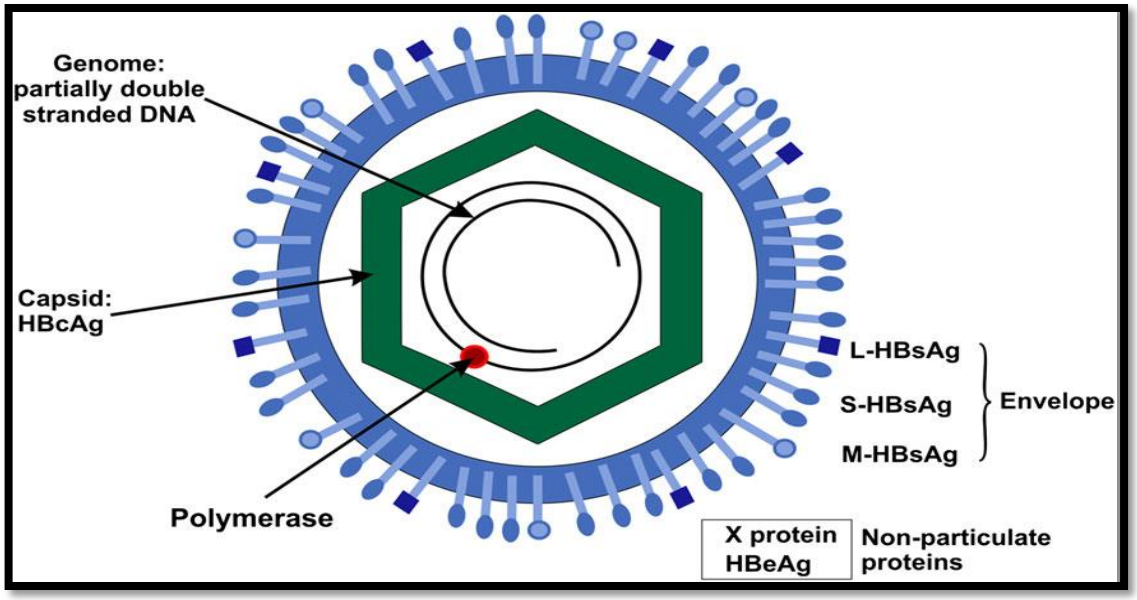
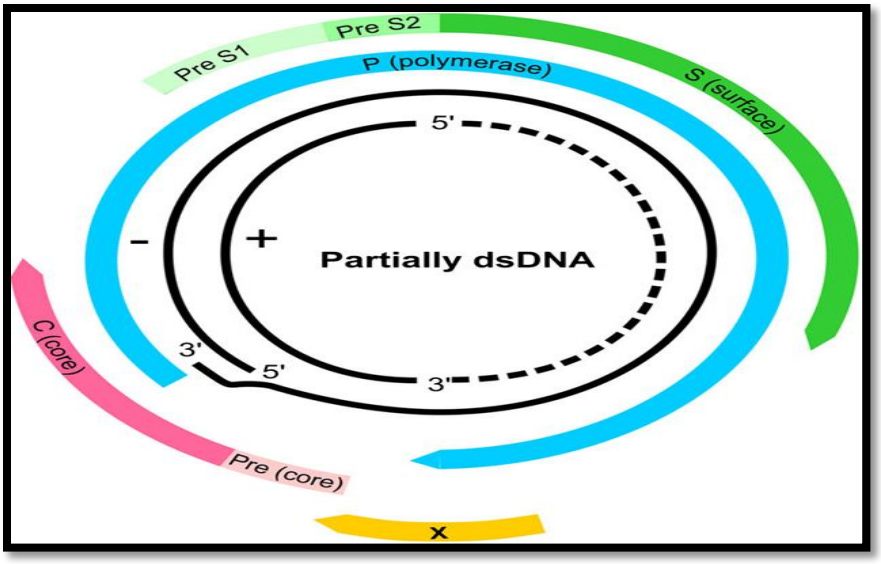


Figure 2.1: Schematic representation of *HBV*, showing the structure of the virion, composed of a partially double stranded DNA genome, enclosed by a capsid, comprised of HBcAg and surrounded by a lipid envelope containing large (L)-HBsAg, middle (M)-HBsAg and small (S)-HBsAg. The virus also expresses two non-particulate proteins X protein and HBeAg (Kim *et al*., 2011).

2.2.4 Structure of the viral genome

*Hepatitis B virus*has a partially double –stranded but not covalently closed circular (CCC) DNA genome composed of between 3182 -3248 nucleotides, depending on the genotype. The genome consists of a complete minus –DNA strand with a short- terminal redundancy , and a shorter plus-DNA strand that leaves a single –stranded gap of variable length in mature nucleocapsids and released viruses (Pairan and Bruss, 2009). Base-pairing of plus-and minus –strands in the cohesive overlap region of the genome maintains the circular configuration. The 5 end of the minus-strand is covalently linked to the N-terminal portion of the viral polymerase. At its 5

end, the plus-strand is linked to a capped RNA oligo-nucleotide that is derived from the 5 end of the RNA pregenome , and serves as the primer for plus-strand –DNA synthesis. The genome consists of four partially overlapping open reading frames (ORFs) that express surface, precore/core, polymerase, and X proteins. Each ORF overlaps at least one other ORF, with the polymerase ORF overlapping all of the others, and every nucleotide is part of at least one ORF. Translation of preS1, preS2 , and S ORFs leads to the expression of the surface proteins, LHB, MHB, and SHB, respectively (Li *et al*., 2017). Four promoters (preC/C, preS1, S, and X) and two enhancers (Enh1 and Enh2) overlap the ORFs. The promoters initiate the transcription of messenger (m) RNAs of 3.5, 2.4, 2.1 and 0.9 kb that allow, by the use of different start codons, the expression of seven proteins. All are of positive orientation, possess a 5 cap, are polyadenylated at their 3end, and serve as mRNA for viral gene products. Enhancer-1, which stimulates the transcription of all viral RNAs , is located between the S and X ORFs , and Enhancer-2, a less potent enhancer , overlaps the pre C/C promoter . In addition to the enhancers , other regulatory elements have been identified : a glucocorticoid responsive element (GRE) is located between Enh1 and Enh2 ; a CCAAT element regulates the transcription of the upstream pre S1 promoter , and activates the transcription of S mRNA; and a negative regulatory element (NRE) appears to inhibit only the pre core/core mRNA (Bömmel *et al*., 2015).Show the structure of the viral genome in (Figure 2.2) .



Figure(2. 2): The genome of *HBV*. The partially double stranded DNA (dsDNA) with the complete minus (_) strand and the incomplete (+) strand. The four open reading frames (ORFs) are shown: pre core/core (pre C/C) that encodes the e antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), PreS1/PreS2/S for surface proteins (three forms of HBsAg, small (S), middle (M) and large (L)) and X for atranscriptional trans-activator protein (Hu and Seeger, 2015).

2.2.4.1 Function of the gene products

(a) Surface proteins

The *HBV* surface protein: small (SHB),medium (MHB) and large(LHB),together with cellular lipid material, form the viral envelope (Yan *et al*., 2012). SHB antigen which represents 85% of hepatitis B surface antigen (HBs Ag), is highly immunogenic and provokes the host is immune response to *HBV*. Excess surface protein circulating in sub viral particles is thought to dilute the host is immunological response to the virus. LHB, in contrast to MHB, is essential for infection and viral morphogenesis. It represents 10-30% of the HBsAg of virions and filaments. LHB plays a

role in viral entry into hepatocytes ,although SHB many also be needed in this process (Ezzikouri *et al*., 2014).

(b) Core protein and e antigen

Core protein (C) is the major structural component of the nucleocapsid. The pre C/C ORF is transcribed into a pre core/core fusion protein. During entry into the endoplasmic reticulum, 19 amino acids are cleaved from the N-terminal end of the pre core protein by a signal peptidase when transported into the Golgi compartment, additional amino acids are removed from the C- terminal end by intra –Golgi proteases to form HBe antigen. This antigen is secreted into the serum. The biological function of HBe remains unsolved (Cheng *et al*., 2017).

(c) Polymerase protein

Polymerase (p) has four domains :a terminal domain, which serves as a protein primer for reverse transcription of pregenomic viral RNA; a spacer region without apparent function , the polymerase domain , which has reverse transcription activity; and the RNase H domain , which is responsible for the degradation of the RNA template during reverse transcription (Ezzikouri *et al*., 2014).

(d) X protein

The X protein (HBx) has been shown to be a promiscuous regulator of transcription that is essential for viral replication. Although not binding itself to DNA, it regulates transcription from HBV enhancers /promoters, and from the promoters of cellular genes, including oncogenes, cytokines, growth factors, and several genes involved in cell-cycle control and

progression, DNA repair, apoptotic cell death, and cellular adhesion. HBx also forms complexes with several signal transduction proteins and regulators of cell growth and survival. It is suspected to play a central role in HBV regulation and pathogenesis (Liu *et al*., 2017).

2.2.5 The viral replicative cycle

In the host, the virus replicates and assembles exclusively in hepatocytes, and virions are released non- cytopathically through the cellular secretory pathway. The *HBV* nucleocapsid is transported to the nucleus to release the rcDNA genome. In the nucleoplasm, the rcDNA is converted into covalently closed circular DNA (cccDNA), which is wrapped by histones to form an episomal chromatinized structure. It then serves as a transcription template for all viral transcripts that are translated into the different viral proteins (Lucifora and Protzer, 2016) . Besides encoding the capsid protein and the viral polymerase, the pregenomic RNA is reverse transcribed into new rcDNA within the viral capsid. The DNA containing nucleocapsids in the cytoplasm are either recycled into the nucleus to maintain cccDNA reservoir, or enveloped and secreted via the endoplasmic reticulum (Seeger and Mason, 2015) . In addition to complete infectious virions (diameter of 42 nm), infected cells produce a large excess of genome-free, non-infectious sub-viral spherical or filamentous particles of 22 nm.(Lampertico *et al*., 2017) . Viral genome integration in the host genome can occur randomly; it is not required for viral replication, but is one of the important mechanisms involved in hepatocyte transformation (Levrero and Zucman-Rossi, 2016) . (Figure 2.3).

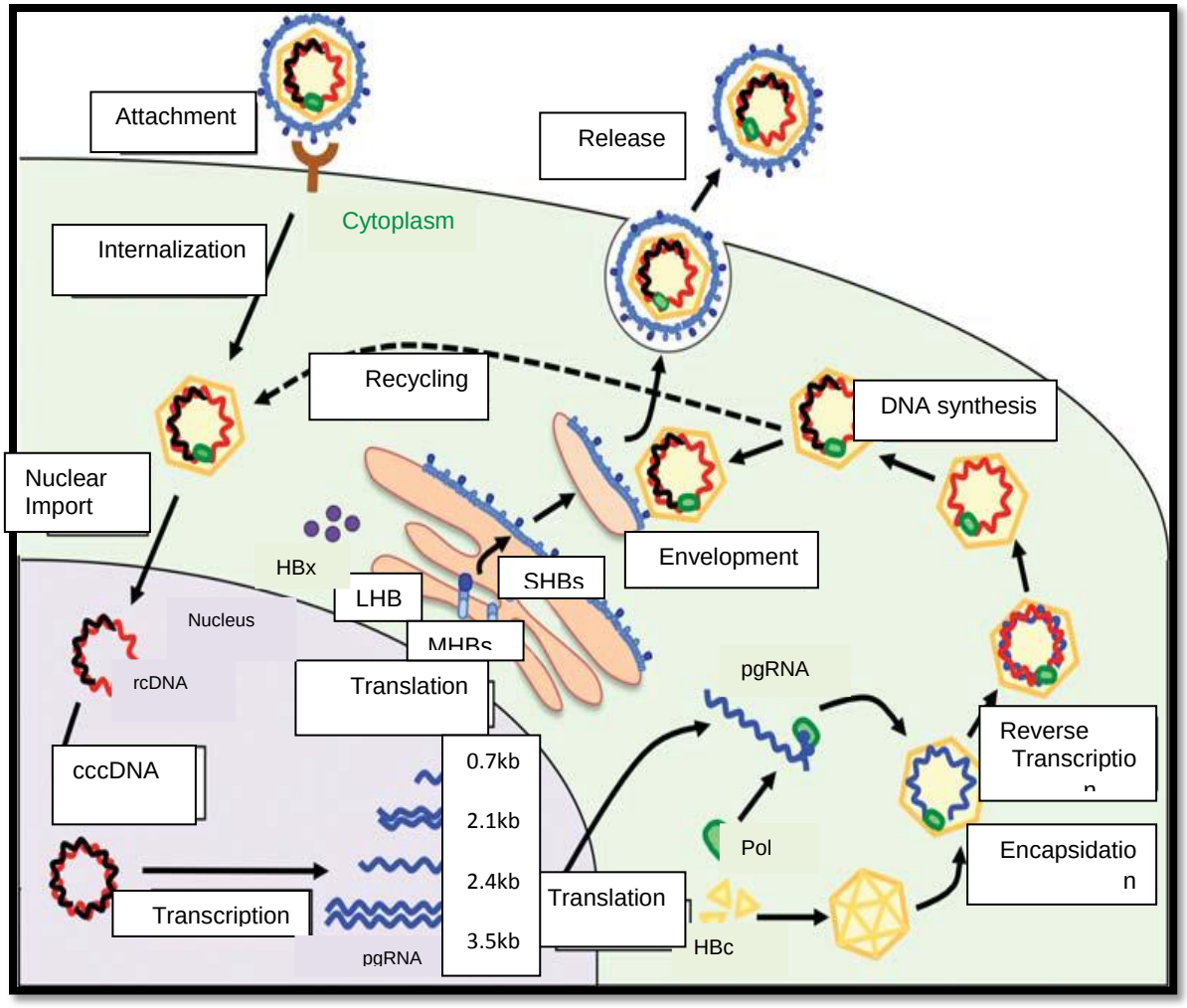


Figure (2.3): The replicative cycle of *HBV*. *HBV*attaches to host hepatocytes, and subsequent entry steps follow, including internalization and membrane fusion. After nuclear import, the relaxed circular *HBV* -DNA genome is converted into covalently closed circular DNA (cccDNA). *HBV* replication proceeds with steps, including transcription, translation, encapsidation, reverse transcription, DNA synthesis, envelopment, release, and recycling. HBc, HBV core proteins; LHBs, large HBV surface proteins; MHBs, medium HBV surface proteins; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; SHBs, small HBV surface proteins (Hu and Liu, 2017).

2.3 Epidemiology of *HBV*

Hepatitis B virus primarily infects humans , although chimpanzees , Chacma baboons ,and tree shrews are also susceptible to infection (Yao, 2017) . *HBV* is one of the most common infectious viruses worldwide. It is estimated that more than two billion people are infected. Approximately 360 million of these are chronically infected (Dienstag, 2008). Approximately one million people die each year from *HBV*-related chronic liver disease ,including liver cirrhosis and hepatocellular carcinoma (HCC) (Rui *et al*., 2017) . HCC is one of the most common cancers in the world ,and chronic *HBV* infection is responsible for 50-90 of HCC in high-risk areas (Chen *et al*., 1997; Thompson *et al*., 2010).

2.3.1 Prevalence and Geographic Distribution

Approximately 45% of the world population lives in areas where chronic HBV infection is highly endemic (>8% of the population are HBsAg-positive); 43% liver in areas where endemicity is intermediate (2-7% HBsAg-positive); and 12% live in areas where endemicity is low (<2% HBsAg-positive) . The prevalence of chronic HBV infection is lowest in North America, Northern and Western Europe, Australia and New Zealand; intermediate in Japan , the Middle East , Eastern and Southern Europe and parts of South America; and highest in sub –Saharan Africa, the Amazon Basin, the people is republic of China, the Republic of Korea, Taiwan (China) and several other countries in South-east Asia (Custer *et al*., 2004) .

The worldwide variation in the endemicity of *HBV* infection is influenced primarily by the predominant age at which infection occurs and the modes of transmission by which it occurs. In areas of high endemicity, the lifetime

risk of *HBV* infection is more than 60% and most infections are acquired from perinatal and child-to-child transmission, when the risk of developing chronic infection is greatest .In these areas, acute *hepatitis B virus* is uncommon because most perinatal and early childhood infections are asymptomatic (Schwestzer *et al* ., 2015). However, rates of liver cancer and cirrhosis in adults are very high. Chronic carriage is thought to result from vertical transmission in China , Taiwan (China), and the Republic of Korea (Chang, 2014) . Of note, *HBV* infection in newborns is less common in Africa. A lower prevalence of HBeAg positivity has been observed in mothers from sub-Saharan Africa compared with mothers in Asia. Child-to-child horizontal transmission accounts for high *hepatitis B virus* infection in this region of Africa (Ott *et al*., 2017).

In areas where endemicity is intermediate, mixed patterns of transmission exist, including infant, early childhood, and adult transmission. In low endemicity areas, most *HBV* infections occur in adolescents and young adults with relatively well defined high-risk groups , including injection drug users, homosexual males, health care workers , and patients who require regular blood transfusion or haemodialysis . in countries where adult horizontal transmission patterns are the principal transmission routes , the incidence of *HBV* infection is highest in adults (Kramvis, 2016) .

Genotype A is widespread in sub-Saharan Africa, Northern Europe, and Western Africa; genotypes B and C are common in Asia; genotype C is primarily observed in Southeast Asia (Liu and Kao, 2013) ; genotype D is dominant in Africa, Europe, Mediterranean countries, and India (Sakamoto *et al*., 2006); genotype G is reported in France, Germany, and the United States (Stanaway *et al* ., 2016) ; and genotype H is commonly encountered

in Central and South America (Allain, 2006). Genotype I has recently been reported in Vietnam and Laos (Revill *et al*., 2016). The newest *HBV* genotype, genotype J, has been identified in the Ryukyu Islands in Japan (Yao, 2017). Geographic distribution of *HBV* genotypes may be related to route of exposure. For example, genotypes B and C are more common in high-endemic regions of perinatal or vertical exposure, which plays an important role in viral transmission. Other genotypes are primarily observed in regions of horizontal exposure. Therefore, genotyping provides an epidemiological clue in the investigation of acquisition, because this lies in the geographical distribution of *HBV* (Schaefer, 2007).

In Iraq ,several studies have been done, in the province Sulaimani mixture the genotype is dominant (Rashid and Salih, 2014) , as well as in the province of wasit (Al-Suraifi *et al*., 2016).

2.3.2 Transmission and risk factors for infection

Hepatitis B virus is highly contagious and is transmitted by percutaneous and permucosal exposure to infected blood and other body fluids (i.e. semen and vaginal fluid). The highest concentrations of the virus occur in blood and wound secretions (WHO, 2001; Ott *et al*., 2012; Ward and Van Damme, 2018). Moderate concentrations of *HBV* are found in semen and vaginal fluid, and lower concentrations occur in saliva.

Hepatitis B virus is not spread by air, food, or water. Common modes of transmission include mother-to-infant, child-to-child, unsafe injection practices and blood transfusions, and sexual contact. *HBV* may be detected in serum 30-60 days following infection , and may persist for widely variable periods of time (Keane *et al*., 2016).

Perinatal transmission from HBsAg-positive mothers to their newborn infants (vertical) or transmission from one child to another (horizontal) is a major source of *HBV* infection is in highly endemic (Watashi *et al*., 2014; Iqbal *et al*., 2015) . The spread of *HBV* from child to child usually happens in household settings but may also occur in child daycare centres and schools (Howell *et al*., 2014) .The most probable pathways of child-to-child spread involve contact of skin sores , small breaks in the skin, or mucous membranes with blood or skin sore secretions (Rossi *et al*., 2012) . *HBV* may also spread because of contact with saliva through bites or other breaks in the skin (Komatsu *et al*., 2016). The virus may spread from inanimate objects such as shared towels or tooth brushes , because it can survive for at least 7 days outside the body , and can be found in high titres on objects ,even in the absence of visible blood (Nelson *et al*., 2011) .

Unsafe injection practices such as the re-use of a syringe or needle from patient to patient without sterilization are a common source of transmission of *HBV* in many developing countries (Lavanchy and Kane, 2016) .Blood transfusion is also a common source of *HBV* transmission in countries where the blood supply is not screened for HBsAg. In addition, the injection of illicit drugs using shared needles is a common mode of *HBV* transmission in many developed countries.

HBV is efficiently transmitted by sexual contact, which accounts for a high proportion of new infections among adolescents and adults in countries with low and intermediate endemicity of chronic *HBV* infection (Terrault *et al*., 2016). Risk factors for sexual transmission include multiple sexual partners, prostitution, and lack of protection in sexual activity (e.g. the use of condoms).

2.3.3 Immunopathogenesis

Hepatitis B virus is primarily a hepatotropic virus, and hepatocytes are the only confirmed site of replication for all members of this virus family. Although the virus has been detected in other cells such as bile duct epithelial cells, peripheral blood mononuclear cells and cells in the pancreas and kidneys , the evidence for viral replication in these cells is controversial (Revill *et al*., 2016).

In acute resolving infections, the response of the innate and adaptive Immune system to *HBV* is efficient and timely. Viral clearance involves the induction of a robust adaptive T cell reaction inducing both a cytolytic dependent and independent antiviral effect via the expression of antiviral cytokines, as well as the induction of B cells producing neutralizing antibodies preventing the spread of the virus (Bertoletti and Ferrari, 2016; Maini and Gehring, 2016). Hepatocyte turnover resulting from infected cell death leads to cccDNA dilution. When the acute infection becomes chronic, there is a progressive impairment in HBV specific T cell function. Chronic *HBV* infection progresses through distinct disease phases that are strongly associated with age. It has been observed that children and young adults with chronic *HBV* infection have an immune profile that is less compromised than that observed in older patients, challenging the concept of 'immune tolerance (Franco *et al*., 2012). Several studies showed that *HBV* persists with virus-specific and global T cell dysfunction mediated by multiple regulatory mechanisms, but without distinct T cell_based immune signatures for clinical phenotypes (or clinical phase of infection) (Hong and Bertoletti, 2017). Genome-wide association studies recently identified the integrator complex subunit 10 (INTS10) gene at 8p21.3 as a novel locus contributing to the susceptibility to persistent HBV infection among Chinese subjects,

and being causative for *HBV* clearance by activation of interferon regulatory factor 3 (IRF3) and then expression of anti-virus interferons here by highlighting the role of innate immunity in viral clearance (Levrero and Zucman-Rossi, 2016) .

2.4 Clinical Features of *HBV* Infection

2.4.1Clinical Phase of Acute *Hepatitis B virus*

Symptoms of acute *HBV* infection are nonspecific and include fatigue, poor appetite, nausea, vomiting, abdominal pain, low-grade fever, jaundice, and dark urine. Clinical signs include liver tenderness, hepatomegaly, and splenomegaly. Acute *HBV* infection typically lasts two to four months (Terrault *et al*., 2016). Approximately 30 to 50 percent of children five years and older and most adults are symptomatic; infants, children younger than five years, and immunosuppressed adults are more likely to be asymptomatic (Bertoletti and Kennedy, 2015). In adults with healthy immune systems, approximately 95 percent of acute infections are self-limited, with patients recovering and developing immunity. Fewer than 5 percent of adults acutely infected with *HBV* progress to chronic infection. A small number (1 percent) develop acute hepatic failure and may die or require emergent liver transplantation (Sagnelli *et al*., 2015).

2.4.2 Clinical Phases of chronic hepatitis B virus

The natural history of CHB is dynamic and complex, and progresses non-linearly through several recognizable phases. The terms “immune-tolerant”, “immune-active”, “immune-control” and “immune-escape” have been commonly used to describe these different phases, but it is increasingly recognized that these descriptions are not fully supported by immunological

data. The phases are of variable duration, are not necessarily sequential, and do not always relate directly to criteria and indications for antiviral therapy (Bertoletti and Kennedy, 2015; Papatheodoridis *et al*., 2016; Varbobitis and Papatheodoridis, 2016).

Phases of chronic hepatitis B virus

1. **The immune-tolerant phase** occurs most commonly in HBsAg-positive children and young adults infected in the perinatal or early childhood period. It usually persists into young adulthood and may last 10–30 years after perinatal infection. Typically, serum HBeAg is detectable, HBV DNA levels are high (usually more than 200 000 IU/mL), and alanine aminotransferase (ALT) levels may be normal or only minimally raised. There is minimal liver inflammation, no or slow progression to fibrosis, and low spontaneous HBe Ag loss (Papatheodoridis *et al*., 2015).

2. This is usually followed by an HBeAg-positive **immune-active phase** of active inflammatory disease. Serum ALT may be abnormal or fluctuate and is accompanied by variable decreases in *HBV*- DNA levels. Symptoms of hepatitis may be present and there is more severe, histologically evident hepatitis and fibrosis. This phase may last from several weeks to years, and may result in successful seroconversion from an HBeAg-positive to an anti-HBe state. Seroconversion rates are higher in those with raised serum aminotransferases (Raffetti *et al*., 2016).

3. The **non-replicative** or **inactive immune-control phase** (previously called the inactive carrier phase) follows successful seroconversion from an HBeAg-positive to anti-HBe state, which occurs in approximately 10–15% of HBeAg-positive persons per year. Once HBeAg is cleared, the

disease may remit, with minimal progression of fibrosis, and serum ALT levels revert to normal with low or undetectable levels of *HBV*- DNA (less than 2000 IU/ mL). HBeAg seroconversion at a young age, prior to the onset of significant liver disease, confers a good prognosis, with a substantially reduced risk of cirrhosis and liver cancer. However, active viral replication can reappear in a proportion of persons (Block *et al*., 2015).

4. In addition to HBeAg-positive chronic hepatitis, HBeAg-negative ("**immune escape-mutant**") active chronic hepatitis occurs in approximately 5–15% of HBeAg-negative, anti-HBe-positive persons in the inactive carrier state (Brouwer *et al*., 2015). HBeAg is undetectable (and anti-HBe detectable) in these persons because mutations in the pre-core or basal core promoter region of the viral genome result in HBV variants that do not express HBeAg. This represents a later phase of disease, generally in older persons, and has a variable course, with abnormal or fluctuating levels of serum ALT and *HBV* DNA, necroinflammatory changes, and more rapid progression to cirrhosis (annual rate of 8–20%) (Yuan *et al*., 2015).

5. **HBV reactivation** may occur spontaneously or may be triggered by cancer chemotherapy and other immunosuppressive therapy, and may lead to fatal acute-on-chronic hepatitis, and pre-emptive nucleoside

analogue (NA) therapy is therefore used. Occult *HBV* infection (defined as persistence of *HBV* DNA in the liver in persons in whom HBsAg is not detectable in the blood) may also be reactivated through prolonged chemo- or immunosuppressive therapy (Papatheodoridis *et al*., 2016). Subjects with occult infection may also represent an important source of new infections in

blood transfusion services in *HBV*-endemic LMICs where HBsAg is used as the sole marker of infection in donor populations. Persons who have cleared HBsAg and who potent immunosuppressive drugs are negative for *HBV* DNA but anti-HBc positive may reactivate if given (İnan and Tabak, 2015).

2.5 Hepatocellular carcinoma

Chronic *HBV* infection leads to an increased risk of death from liver cirrhosis and liver cancer, with an estimated 650 000 annual deaths from HCC (Han *et al*., 2013) . In resource-limited and high *HBV*-burden settings, persons are often diagnosed with HBV only when they present for the first time with HCC. While the majority of these (80–90%) have cirrhosis at the time of diagnosis of HCC, it may sometimes occur without the presence of cirrhosis; this is especially true for HCC due to *HBV*. A further major challenge with HCC is that it is rapidly progressive, and may be asymptomatic until it presents clinically at an advanced stage. Treatment options for advanced HCC are limited and overall survival is extremely poor. The prognosis of HCC is affected by the size and number of tumors, and the underlying liver function (Seo *et al*., 2014). Factors found to be important predictors of HCC development are HBe Ag positivity and high levels of *HBV*- DNA, older age, and male gender (Gounder *et al*., 2013; Chayanupatkul *et al*., 2017).

Hepatitis B virus (*HBV*) contributes to hepatocellular carcinoma (HCC) development through direct and indirect mechanisms. *HBV*- DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces both genomic instability and direct insertional mutagenesis of diverse cancer-related genes. Prolonged expression of the viral regulatory protein HBx and/or altered versions of the pre S/S envelope proteins dysregulates cell transcription and proliferation control and

sensitizes liver cells to carcinogenic factors. Accumulation of preS1 large envelope proteins and/or pre S2/S mutant proteins activates the unfold proteins response, that can contribute to hepatocyte transformation. Epigenetic changes targeting the expression of tumor suppressor genes occur early in the development of HCC (Sze *et al*., 2013). A major role is played by the HBV protein, HBx , which is recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci. Compared with tumors associated with other risk factors, *HBV*-related tumors have a higher rate of chromosomal alterations, p53 inactivation by mutations and overexpression of fetal liver/hepatic progenitor cells genes. The β-catenin pathway is also often activated but *HBV*-related tumors display a low rate of activating β-catenin mutations. HBV-related HCCs may arise on non-cirrhotic livers, further supporting the notion that *HBV* plays a direct role in liver transformation by triggering both common and etiology specific oncogenic pathways in addition to stimulating the host immune response and driving liver chronic necro-inflammation (Yip *et al*., 2011).

2.6. Diagnosis

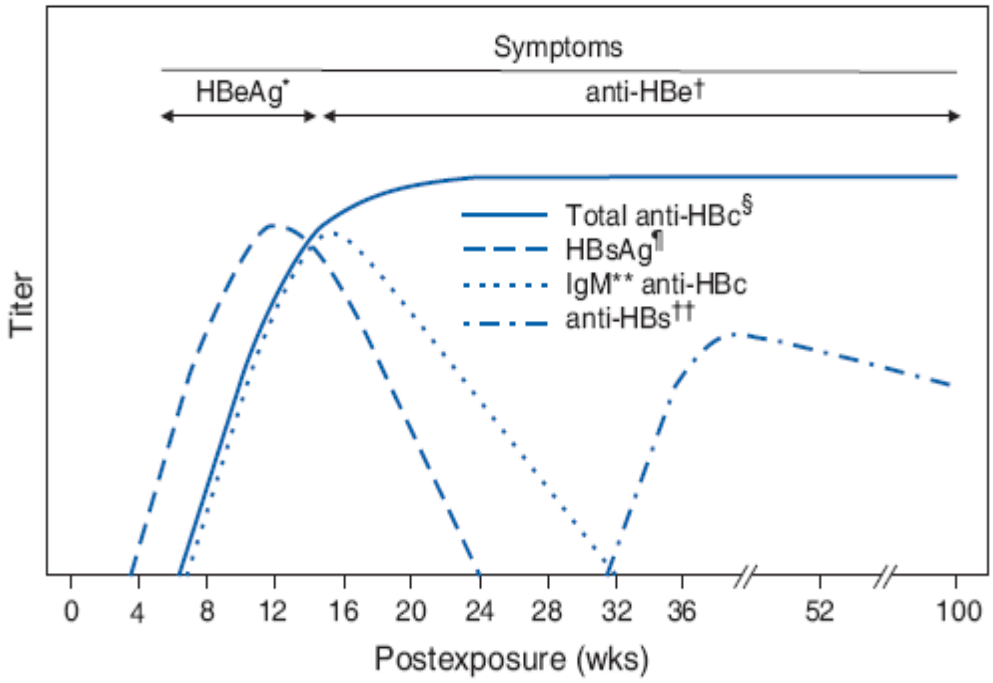
Routine assessment of HBsAg-positive persons is needed to guide management and indicate the need for treatment. This generally includes assessment of: additional serological markers of *HBV* infection (HBeAg); measuring aminotransferase levels to help determine liver inflammation; quantification of *HBV*- DNA levels (Lok and McMahon, 2014) .

2.6.1 *HBV* serological markers

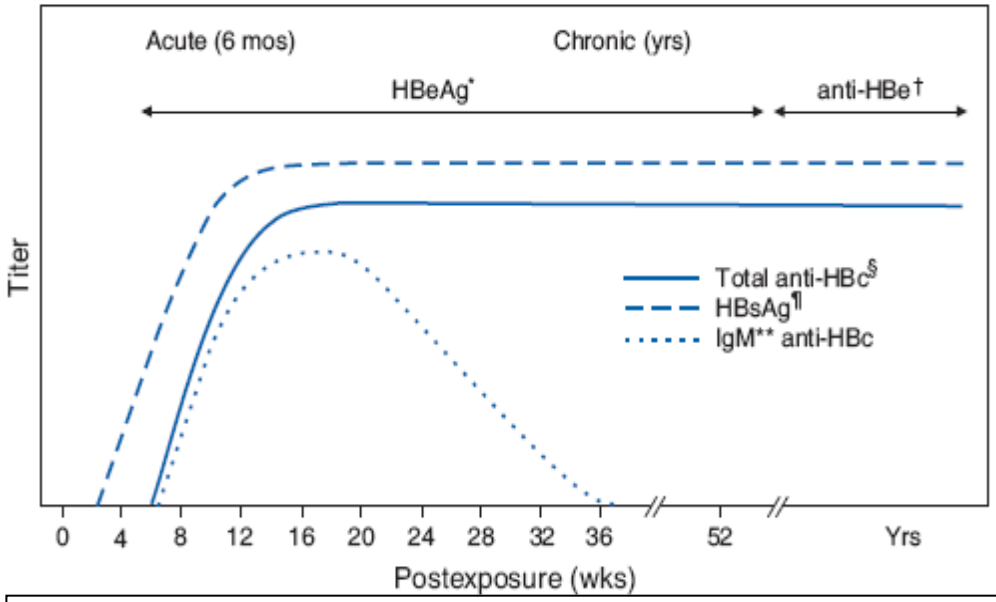
Previous *HBV* infection is characterized by the presence of antibodies (anti-HBs and anti-HBc). Immunity to *HBV* infection after vaccination is characterized by the presence of only anti-HBs. HBsAg is the first serological marker to appear and can be detected 1-2 weeks or as late as 11-12 weeks following exposure figure (2.4). CHB is defined as the persistence of HBsAg for more than 6 months figures (2.5). Recently, quantitative HBsAg level determination has been proposed to differentiate inactive HBsAg carriers from persons with active disease (Jia *et al*., 2015) .

HBeAg: It also needs to be established whether the person is in the HBeAg-positive or HBeAg-negative phase of infection, though both require lifelong monitoring, as the condition may change over time. In persons with CHB, a positive HBeAg result usually indicates the presence of active *HBV* replication and high infectivity. Spontaneous improvement may occur following HBeAg-positive seroconversion (anti-HBe), with a decline in *HBV* replication, and normalization of alanine aminotransferase (ALT) levels. This confers a good prognosis and does not require treatment. HBeAg can also be used to monitor treatment response (Park *et al*., 2011; Oliveri *et al*., 2017) .

Enzyme –linked immune sorbent assay use the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibody in a fluid sample. (Balsam *et al*., 2013; Canady *et al*., 2013) .



Figure(2. 4): Typical serologic course of acute *HBV* infection (Weinbaum *et al*., 2009).



Figure(2. 5): Typical serologic course of chronic *HBV* infection (Weinbaum *et al*., 2009).

2.6.2 Molecular Marker of *HBV* :

The development of host Abs to HBe (anti-HBe) indicates the assessment of immunity and the reduction of viral replication in the infected individual (Kojuri *et al*., 2016) . Carriers of the virus may have chronic hepatitis B virus. PCR and Real time Quantitative PCR tests have been developed to detect and measure the amount of *HBV*- DNA, called the viral load, in clinical specimens**.** Serum *HBV*- DNA concentrations quantified by real-time polymerase chain reaction (PCR) correlate with disease progression (Buti *et al*., 2015) ,and are used to differentiate active HBeAg-negative disease from inactive chronic infection, and for decisions to treat and subsequent monitoring. Serial measures over a few months or longer are preferable, but there remains a lack of consensus regarding the level below which *HBV*- DNA concentrations are indicative of "inactive" disease, or the threshold above which treatment should be initiate (Terrault *et al.*, 2018).*HBV*- DNA concentrations are also used for optimal monitoring of response to antiviral therapy, and a rise may indicate the emergence of resistant variants. WHO standards are now available for expression of *HBV* DNA concentrations (Kania *et al*., 2014) . Serum *HBV*- DNA levels should be expressed in IU/mL to ensure comparability; values given as copies/mL can be converted to IU/mL by dividing by a factor of 5 to approximate the conversion used in the most commonly used assays (i.e. 10 000 copies/mL = 2000 IU/mL) . The same assay should be used in the same patient to evaluate the efficacy of antiviral therapy (Venkatakrishnan *et al*., 2016).

Nested polymerase chain reaction (Nested-PCR) is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites.

Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product. This allows amplification for a low number of runs in the first round, limiting non-specific products. The second nested primer set should only amplify the intended product from the first round of amplification and not non-specific product. This allows running more total cycles while minimizing non-specific products. This is useful for very rare templates or PCR with high background (Chevaliez *et al*., 2017).

The DNA sequence differentiation method was first described by (Kimura 1980) and (Tamura *et al*., 2013) who developed, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences and applied by Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6.0) software that facilities of comparative analyses of DNA and protein sequences and supposing the molecular evolutionary patterns of genes, genomes, and species over time.

2.6.2 .1 Multiple Sequence Alignment

Deoxyribonucleic Acid sequence alignment is considered the 'Holy Grail' problem in computational biology and is of vital importance for molecular function prediction. The widely used databases (Robert *et al.*, 2010; Gardner *et al.*, 2011) are constructed based on multiple sequence alignment (MSA). Molecular function prediction sometimes depends on evolutionary information (Liu *et al.*, 2014). MSA is also required for evolutionary tree reconstruction. Most of the available phylogenetic tree construction software tools require previously aligned sequences as input.

When addressing the evolutionary analysis of bacterial and viral genomes, large-scale similar DNA sequences often prevent these MSA tools from functioning (Wang *et al.*, 2013). The evolution of viruses is rapid, and massive viral DNA sequences often appear in phylogenetic reconstructions. Therefore, it is necessary to improve the scalable capacity of MSA tools when analyzing influenza virus DNA (Chang *et al.*, 2007).

2.6.2.2 Phylogenetic Tree

The goal of phylogenetic analyses is to make inferences of epidemiological processes from viral phylogenies. Thus, most phylogenetic analyses begin with the reconstruction of a phylogenetic tree. Genetic sequences are often sampled at multiple time points, which allow the estimation of substitution rates and the time using a molecular clock model. For viruses, Bayesian phylogenetic methods are popular because of the ability to fit complex demographic scenarios while integrating out phylogenetic uncertainty(Kuhnert *et al.*, 2011).

Chapter Three
Materials and Methods

3. 1 Materials

3.1.1 Equipment and Instruments

Table (3.1): The equipment and instruments that used in this study with their companies and countries of origin:

No.	Equipment & instruments	Company
1	Camera	Nikon/Jaban
2	Deep Freezer	Philips /Holland
3	Disposable container50 ml	Sterile EO. / China
4	ELISA reader	Biotek/U.S.A
5	Eppendorf tubes	Biobasic / Canada
6	Exispin vortex centrifuge	Bioneer/ korea
7	Gel electrophoresis	Biorad /U.S.A
8	High Speed Cold centrifuge	Eppendorf /Germany
9	Microbiological safety cabinet	Labtech/ Korea
10	Micropipettes 5-50, 0.5-10, 100-1000µl	Epprndorf/ Germany
11	Micro – plate washer	Biotek /U.S.A
12	Microwave	Argose/ Germany
13	Nanodrop	Thermo/ USA

14	Plan tube	AFCO-DISPO/Jorden
15	Printer	Epson / U.A.S
16	Refrigerator	Concord /Lebanon
17	Sensitive Balance	Sartorius/Germany
18	T100 thermal cycler	BioRad/ U.S.A
19	U.V. transilluminator	M.N.Lab /U.S.A
20	Vortex Mixer	CYAN/ Belgium
21	Water Bath	Memmert/Germany

3.1.2 Chemicals and biological material

Table (3.2): The chemicals and biological material with their companies and countries of origin used in this study:

No.	Chemical	Company and Origin
1	Absolute Ethanol	BDH (England)
2	Agarose gel	BioBasic/ Canada
3	DNA ladder (100bp)	Bioneer/ Korea
4	Ehidium Bromide	BioBasic/ Canada
5	Free nuclease water	Bioneer/ Korea

6	TBE buffer	BioBasic/ Canada
7	HBs Ag ELISA Test Kit 96 test	Fortress /England

3.1.3 Genomic extraction Kit

Table (3.3): The kits used in this study with their companies and countries of origin in:

No.	Kit	Company	Country
1	**Genomic DNA Extraction Kit**	**Geneaid**	**USA**
	GT buffer		
	GB buffer		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
	Proteinase K 10mg/ml		
2	**AccuPower ™ PCR Premix**	**Bioneer**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl		

	$MgCl_2$		
	Stabilizer and Tracking dye		

3.1.4. ELISA (Enzyme Linked Immune- Sorbent Assay) Kit

This ELISA kit (fortress - U.K) is use for the detection of hepatitis B surface antigen (HBs Ag) in human serum, its content with their quantity are listed in **Table (3.4): The Contents ELISA Kit**

Reagent	**Quantity**
Micro ELISA Plate	8 wells ×12 strips
Reference Standard	2 vials
Positive Control	1vial 1mL
Negative Control	1vial 1mL
Chromogen Solution A	1vial 7mL
Chromogen Solution B	1vial 7mL
HRP Conjugate Diluent	1vial 10mL
Concentrated Wash Buffer (20×)	1vial 30mL
Stop Solution	1vial 7mL

3.1.5 Primers

The HBV detection and Genotyping primers were designed by using the in previous studies (Naito *et al*., 2001) and provided by (Bioneer. Company, Korea) as following table (3.5):

Table (3.5): The PCR primers with their sequence and amplicon size:

Primer	**Sequence (5'-3')**		**Amplicon size**
First PCR -HBV-universal sense primer	P1	TCA CCA TAT TCT TGG GAA CAA GA	1063bp
First PCR -HBV-universal antisense primer	S1-2	CGA ACC ACT GAA CAA ATG GC	
Nested PCR-Mix A sense primers	B2	GGC TCM AGT TCM GGA ACA GT	68bp
Genotype A antisense primer	BA1R	CTC GCG GAG ATT GAC GAG ATG T	
Genotype B antisense primer	BB1R	CAG GTT GGT GAG TGA CTG GAG A	281bp
Genotype C antisense primer	BC1R	GGT CCT AGG AAT CCT GAT GTT G	122bp
Nested PCR-Mix B antisense primers	B2R	GGA GGC GGA TYT GCT GGC AA	119bp
Genotype D sense primer	BD1	GCC AAC AAG GTA GGA GCT	
Genotype E sense primer	BE1	CAC CAG AAA TCC AGA TTG GGA CCA	167bp
Genotype F sense primer	BF1	GYT ACG GTC CAG GGT TAC CA	97bp

'M' represents a nucleotide that could be either an A or a C; a 'Y' represents a nucleotide that could be either a C or a T.

3.2 Methods

3.2.1 Patients and study design

This cross-sectional study was conducted in (80) patients positive with HBsAg (59 males and 21 females) age ranging 12- 75 years. The samples were collected from Al- Diwaniya governorate of Iraq, in the period from 1 October 2017 to 30 February 2018.

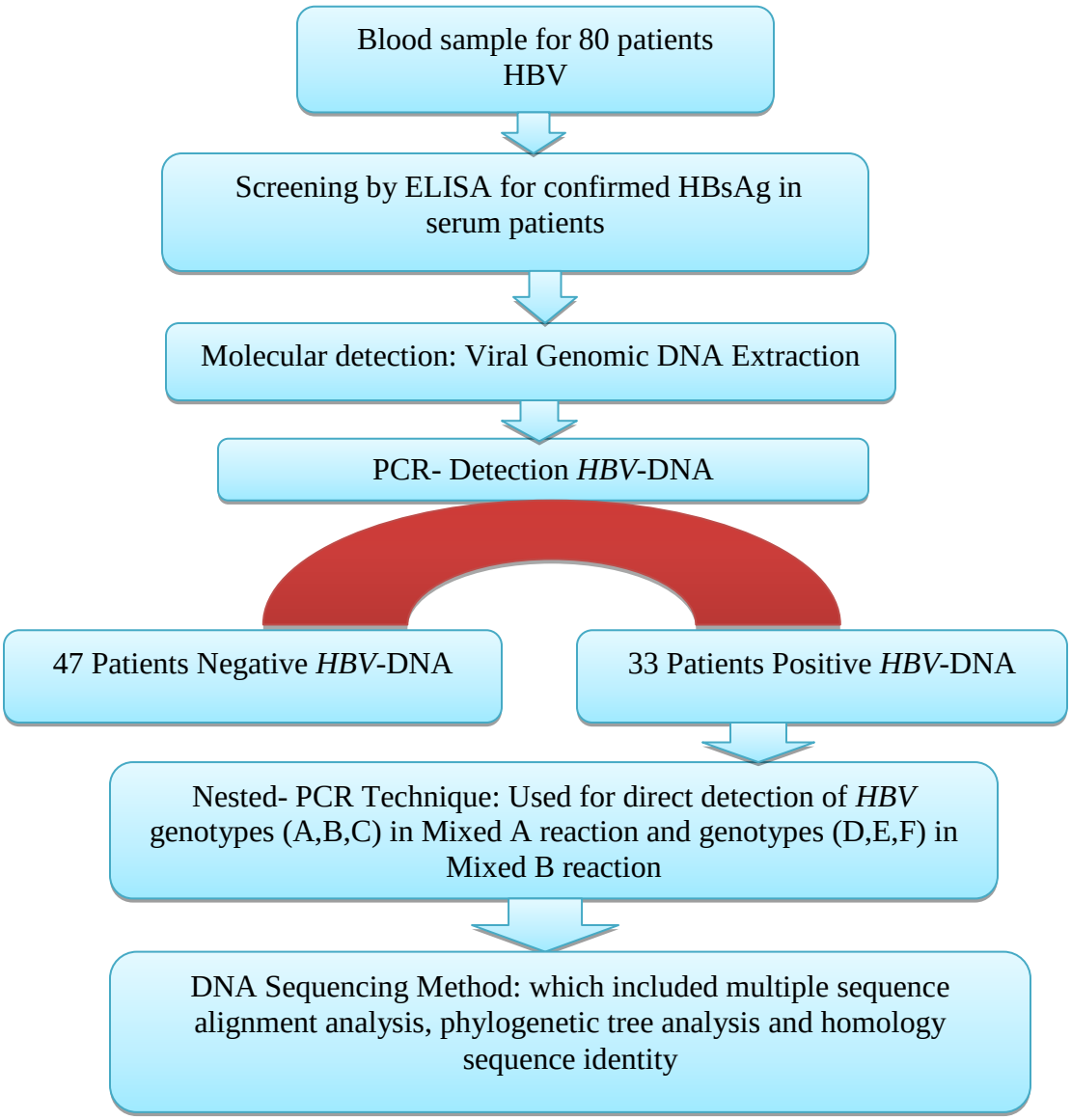


Figure (3.1): Scheme of Work and Study Design

3.2.1 Ethical approvals

Ethical approvals were taken from the Faculty of Medicine, department of microbiology, Hospital managers, Central Blood Bank, hemodialysis, public health laboratory and for blood extraction and hepatitis B testing.

3.2.2 Sample collection

Blood sample were collected by venipuncture from 80 patients (five millimeter of venous blood) were drawing by disposable syringe under a septic technique. Each blood sample was collected directly in a sterile tube and allowed to clot then serum was separated by centrifugation at 3000rpm for 10 minutes. The serum has been collected in Eppendrof tube then stored at -20c to be used for ELISA test, DNA extraction and nested PCR technique.

Been sampling of patients reviewers to institutions health care, it is different ages and from both genders.

3.2.3 *Hepatitis B virus* screening test

Enzyme- linked immune sorbent assay test was used for the detection of hepatitis B surface antigen (HBs Ag) in human serum in clinical laboratories and as a first - line screening assay in blood.

3.2.3.1 Principle of assay

This assay employs the quantitative direct sandwich enzyme immunoassay techniques. The micro ELISA plate has been pre-coated with monoclonal antibody specific to HBs (IgG antibody to HBsAg). This constitutes the solid –phase antibody. The test sample is that contains antigen (HBsAg) incubated in such a well. With the positive reaction this

labeled antibody becomes bound to any solid – phase antibody HBs Ag complex previously formed. Incubation with enzyme substrate produces a blue color in the test-well, which turns yellow when the reaction is stopped with sulphuric acid. If the sample contains no HBs Ag, the labeled antibody cannot be bound specifically and only a low background color develops.

3.2.3.2 Assay procedure

Serum samples were added according to the designation on the ELISA working sheet (fortress, U.K) 2017.

Step 1 Reagents Preparation

Before begging the assay, all kit reagents and samples were brought at room temperature (25c) for at least 30 minutes.

Step2- Numbering Wells

The strips were set in strip-holder, and sufficient number of wells were numbered including three negative control (e.g. B1, C1, D1), two positive controls (e.g.E1, F1) and one blank (e.g.A1, nether samples nor Horseradish Peroxidase -conjugate was added to the blank well).

Step3- Adding Sample and HRP – Conjugate

Fifty µl Specimen, Negative Control and Positive Control were added into each wells and then 50 µl HRP-Conjugate was added to each well except the Blank, and mix by tapping the plate gently.

Step 4- Incubating

The plate was covered with the plate cover and incubated for 60 minutes at 37 °C.

Step 5 – Washing

At the end of the Incubation, The plate cover was removed and discarded. Each well was washed 5 times with 350µl of diluted wash buffer. Each time, the micro well was allowed to soak for 30-60 seconds .After the final washing cycle, the plate was turned down onto blotting paper, and was taped to remove any remainders.

Step 6 – Coloring

Fifty µl of Chromogen A and 50 µl Chromogen B solution were dispensed into each well including the blank, and they were mixed by tapping the plate gently. Covered with plate cover and the plate was incubated at 37°C for 15 minutes avoiding light .The enzymatic reaction between the chromogen solution and the HRP-Conjugate produces blue color in positive control and HBs Ag positive sample wells.

Step 7 – Stopping Reaction

The plate cover was removed. Fifty µl Stop Solution was added into each well and mixed gently. Then a yellow color develops in positive control and HBs Ag positive sample wells.

Step 8- OD Measurement: for determine the optical density (OD value) of each well at once, was read at 450 nm.

3.2.3.3. Calculation of results

Cut off Value was calculated according to equation below for HBs Ag Cut off Value (COV):

Cut- Off Value (C.O.) = NC ×2.1

NC: the mean absorbance value for three negative controls.

S: the individual optical density (OD) of each specimen

Positive Results (S/C.O.≥1): samples giving an absorbance greater than or equal to the Cut-off value.

Negative Results (S/C.O. <1): samples giving an absorbance less than the Cut-off value.

3.2.4 Molecular detection and genotyping assay

3. 2.4.1 Viral Genomic DNA Extraction

Viral genomic DNA was extracted from serum samples by using gSYAN Genomic DNA Mini Kit (Geneaid, USA) and done according to company instructions as following steps:

1. A 200µl of serum samples were transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex. And incubated at 60℃for 5 minutes.

2. After that, 200µl of lysis buffer were added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 60℃for 10 minutes, and inverted every 3 minutes through incubation periods.

3. Two hundred µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000 rpm for 5 minutes. And the 2 ml collection tube containing the flow. Through were discarded and placed the column in a new 2 ml collection tube.

4. Four hundred µl W1 buffer were added to the DNA filter column, then centrifuge at 10000 rpm for 30 seconds. The flowing material was discarded and placed the column back in the 2 ml collection tube.

5. Sex hundred µl Wash Buffer (ethanol) were added to each column. Then centrifuged at 10000 rpm for 30 seconds. The flowing material was discarded and placed the column back in the 2 ml collection tube.

6. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

7. The dried DNA filter column was transferred to a clean 1.5 ml micro centrifuge tube and 50µl of preheated elution buffer were added to the center of the column matrix.

8. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3.2. 4.2 Genomic DNA extraction profiling

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (which measured DNA concentration ng/ µl) that check DNA concentration and estimation of DNA purity through reading the absorbance in at (260 /280 nm) according to the work of the machine as following steps:

After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

1. A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of double distilled water (ddH2O) onto the surface of the lower measurement pedestal.
2. The sampling arm was lowered and clicking OK to blank the Nanodrop, then cleaning off the pedestals.

After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

3.2.4.3 Nested – PCR

Nested-PCR was performed for detection and genotyping of *HBV* from serum samples. This technique was carried out according to (Naito *et al*., (2001) as following steps :

3.2.4.3.1 PCR master mix preparation

PCR master mix was prepared for direct detection of *HBV* by using (AccuPower® PCR PreMix Kit) and this master mix done according to company instructions as following Table (3.6): PCR master mix

PCR Master mix	Volume
DNA template	5µl
P1gene primer (10pmol)	1µl
S1-2 gene primer (10pmol)	1µl
PCR water	13µl
Total volume	20µl

After that, these PCR master mix components that mentioned in table (3.6) above placed in standard AccuPower PCR PreMix Kit that contains all other components which needed to PCR reaction.Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler.

3.2.4.3.2 PCR Thermocycler Conditions

PCR thermocycler conditions for detection of *HBV* were done by using convential PCR thermocycler system according to (Naito *et al*., 2001) **Table (3.7):** PCR thermo cycler.

PCR step	Temp.	Time	repeat
Initial Denaturation	94°C.	5min	1
Denaturation	94°C.	30sec.	40 cycle
Annealing	55°C.	30sec	
Extension	72°C.	1.5min	
Final extension	72°C.	5min	1
Hold	4°C.	Forever	-

3.2.4.3.3 Nested PCR master mix preparation

Nested PCR master mix was prepared for direct detection of HBV genotypes (A,B,C)in Mixed A reaction and genotypes (D,E,F) in mixed B reaction by using (AccuPower® PCR PreMix Kit) and this master mix done according to company instructions **Tables(3.8-A &B):Nested PCR reaction mix.**

Table 3.8-A: Mix A Nested PCR reaction mix:

Nested PCR Master mix	Volume
DNA template	1µl
B2, BA1R, BB1R, BC1Rprimers (10pmol)	1µl
	1µl
	1µl
	1µl
PCR water	15µl
Total volume	20µl

Table 3.8-B: Mix B Nested PCR reaction mix:

Nested PCR Master mix	Volume
DNA template	1µl
BD1, BE1, BF1 bb, B2Rprimers (10pmol)	1µl
	1µl

	1µl
	1µl
PCR water	15µl
Total volume	20µl

After that, these PCR master mix components that mentioned above placed in standard AccuPower PCR PreMix Kit. Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler.

3.2.4.3.4 Nested PCR Thermocycler Conditions

PCR thermocycler conditions for detection of HBV genotypes were done by using convential PCR thermocycler system **Table (3.9): Nested PCR thermo cycler.**

PCR step	Temp.	Time	repeat
Initial Denaturation	94 °C.	5min	1
Denaturation	94°C.	30sec.	20cycle
Annealing	58 °C.	30sec	
Extension	72 °C.	1.5min	
Final extension	72 °C.	40sec	1
Denaturation	94 °C.	20sec.	20cycle
Annealing	60 °C.	30sec	

Extension	72 °C.	40sec	
Final extension	72 °C.	5min	1
Hold	4 °C.	Forever	-

3.2.4.3.5 PCR product analysis

The PCR and Nested PCR products were analyzed by agarose gel electrophoresis following steps:

1- One percent Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- The products were visualized by using UV Transilluminator.

3.2.4.3.6 DNA sequencing method

DNA sequencing method was performed for genetic relationship of local *Hepatitis B virus* of Phylogenetic analysis with NCBI-Genbank *Hepatitis B virus* clones. The sequencing of the PCR product of polymerase gene, where the 1063bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada) (Rashid and Salih , 2915). As the following steps:

1. The specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube.
2. Four hundred µl Binding Buffer II was added to gel fragment. Then, incubated at 60ºC for 10 minutes and shaked until the agarose gel is completely dissolved.
3. The above mixture was added to the EZ EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000 rpm for 2 minutes and discard the flow-through in the tube.
4. Seven hundred and fifty µl Wash Solution was added to each tube and centrifuged at 10000 rpm for one minute. Then, solution discarded.
5. After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.
6. The column was placed in a clean 1.5ml microcentrifuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000 rpm for 2 minutes to elute PCR product and store at -20ºC.

After that, the purified PCR product samples were sent to Macrogen Company in Korea for performed the DNA sequencing by applied biosystemic (AB) DNA sequencing system. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version) (Tamura *et al*., 2013).

3.2.4.3.7 Multiple Sequence Alignment

Multiple sequence alignment analysis of the partial Hepatitis B virus polymerase sequence in local Hepatitis B virus clones (No.1-No.8) and NCBI-Genbank Hepatitis B virus clones based ClustalW alignment analysis

by using (MEGA 6.0, multiple alignment analysis tool) (Alestig et al., 2001).

3.2.4.3.8 Phylogenetic Tree Analysis

Phylogenetic tree analysis based on Hepatitis B polymerase gene partial sequence that used for study of genetic relationship between local Hepatitis B virus clones and NCBI-BLAST Hepatitis B virus clones. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version) (Yu *et al*., 2010).

3.3 Statistics analysis

All the data obtained from the present study were entered and analyzed statistically by Social Science Statistics and the Statistical Package For Social Sciences version 23 for Windows Software and Microsoft Excel 2010. Chi-square test was used for the assessment of association between the variables studied, the level of statistical significance was set at alpha equal to 0.05 (a = 0.05). A value of $P < 0.05$ was considered statistically significant. (Field, 2005).As well as Sensitivity diagnostic of the results that obtained from the data of the present study was calculated according to (Daniel, 1988), applying the following equation:

Sensitivity = $\mathbf{(a / a + b) \times 100}$

a (true positive) ,**b** (false positive)

Chapter Four

Results and Discussion

4.1 *HBV* distribution

In this study (80) patients, having *HBV* infection, confirmed were through detected for HBs Ag by ELISA technique to diagnose their infection with Hepatitis B virus. Recorded results of these patients (100%) were positive for HBs Ag as shown (Table 4.1).

Table 4.1: ELISA screen test for HBs Ag among 80 patients.

Gender	No. of samples	No. of positive samples
Males	59	59
Females	21	21
Total	80	80

Documented data showed that were positive (100%) for HBs Ag which indicated that they are all infected with *HBV* as this antigen is considered the key of *HBV* serological markers in diagnosis and detection of the infection. It is an important envelope protein which appears after short time of infection; HBs Ag can be detected during three to four weeks after the first time of infection and might be reaching to five months in acute infection (Perrillo *et al.*, 2009). It is the initial antigen appears following *HBV* infection. When HBs Ag continues more than six months, this is an indication of CHB infection (Raimondo *et al*., 2008). In other studies (Al-Ameen *et al.*, 2012; Abdullah and Salim, 2014) that were carried out in Iraq it was shown that 49.7% and 87.83% of *HBV* infected patients were positive for HBs Ag, respectively. Also, (Al-Suraifi *et al.*, 2016) reported that 100% of patients were positive for HBs Ag. In Saudi Arabia, (Asaad *et al*., 2015)

studied 160 *HBV* patients and found that all of them were positive for HBs Ag. In Iran, (Yoosefi *et al*., 2016) showed that among 163 of patients and found that 100% were positive for HB Ag. Trepo *et al*. (2014) demonstrated that HBs Ag may be undetectable (negative) in the early phase of infection but in CHB infected patients, HBs Ag marker can be permanently positive .

Regarding the distribution of patients according to gender, there were 59 (73.75%) males and females 21 (26.25%) positive for HBs Ag through used ELISA technique, there was no significant difference (p value = 0.066), as shown in (Table 4.2) .The statistical matching to the group under the study is mandatory to avoid bias in results that may arise from difference in gender distribution.

Table 4.2: Elisa Screen Test for HBs Ag (positive) among (80) patients based on gender.

Hospital or center name	Total	Positive cases and percentage	
		Females	Males
Teaching Hospital of Al-Diwaniya	19	8(42.11)	11(57.89)
Al Shamiya General Hospital	6	1(16.66)	5(83.33)
Afak General Hospital	6	0(0)	6(100)
Al Hamzah General Hospital	10	2(20)	8(80)
Central Blood Bank	13	0(0)	13(100)
Haemodialysis	6	2(33.33)	4(66.66)
Public Health Laboratory of Al-Diwaniya	20	8(40)	12(60)
Total	80	21(26.25)	59(73.75)

$X2 = 11.825^{*}$, P value = 0.066

***non-significant differences at p≤ 0.05**

The present study came in agreement with previous study done by (Al-Rubaye *et al*., 2016), as, they found there was higher in male than female and not statistically significant . Also another study mentioned that the prevalence of HBs Ag was higher among men (0.7%) than women (0.5%) and the difference had no statistical significance (p=0.07). Men to women ratio in regard to HBs Ag positive was 1.2 which goes with the

expected results, though the number of women donors is much less than men (Ataallah *et al*., 2011).

In a study done by (Nile *et al*., 2018) they found the male infected with hepatitis (44.20%) and female infected with hepatitis (16.6%) so the male more infection for hepatitis then female , males donate blood more often than females. In most human populations there is a higher prevalence of chronic carriers of hepatitis virus among males than females. Females are more likely than males to produce anti-hepatitis in response to infection. Diseases associated with increased frequencies of carriers are more prevalent among males (Tanaka *et al*., 2006).

Documented data showed that patients according to location , there were of the positive cases for HBs Ag by using ELISA technique, 50 patients were by a percentage of (62.5%) in the rural areas and 30 patients by a percentage of (37.5%) urban areas, there was no significant difference (p value = 0.755), as shown in (Table 4.3) .

Table 4.3: ELISA Screen Test for HBs Ag (positive) among (80) patients based on location.

Hospital or center name	Total	Positive cases and %	
		Urban	Rural
Teaching Hospital of Al-Diwaniya	19	6(31.57)	13(68.42)
Al Shamiya General Hospital	6	3(50)	3(50)
Afak General Hospital	6	2(33.33)	4(66.66)
Al Hamzah General Hospital	10	4(40)	6(60)
Central Blood Bank	13	5(38.46)	8(61.53)
Haemodialysis	6	4(66.66)	2(33.33)
Public Health Laboratory of Al-Diwaniya	20	6(30)	14(70)
Total	80	30(37.5)	50(62.5)

$X2 = 3.418^{*}$ ، P value = 0.755

***non-significant differences at p≤ 0.05**

The present result agree with previous study done by (Hussein *et al*, 2017) ,as, they demonstrated a no significant difference was observed in the positivity of HBV donors living in the city and rural areas (P= 0.755).

In a study done by (Ataallah *et al*., 2011) they found HBs Ag in both gender has shown a high prevalence of virus in urban areas compared to rural areas.

4.2. Molecular and Genotyping analysis

4.2.1 *HBV*- DNA detection by PCR

This study showed that (80) samples which were tested by PCR technique for *HBV*- DNA by using type specific primers. Thirty three samples (41.25%) were positive for *HBV*- DNA, while fourty seven (58.75%) were negative (Table 4.4) (Figure 4.1).

Table 4.4: Distribution of the detection of HBV DNA by PCR technique among (33) patients.

Gender	No. of positive samples by ELISA	No. of positive samples by PCR	%
Males	59	24	40.67
Females	21	9	42.86
Total	80	33	41.25

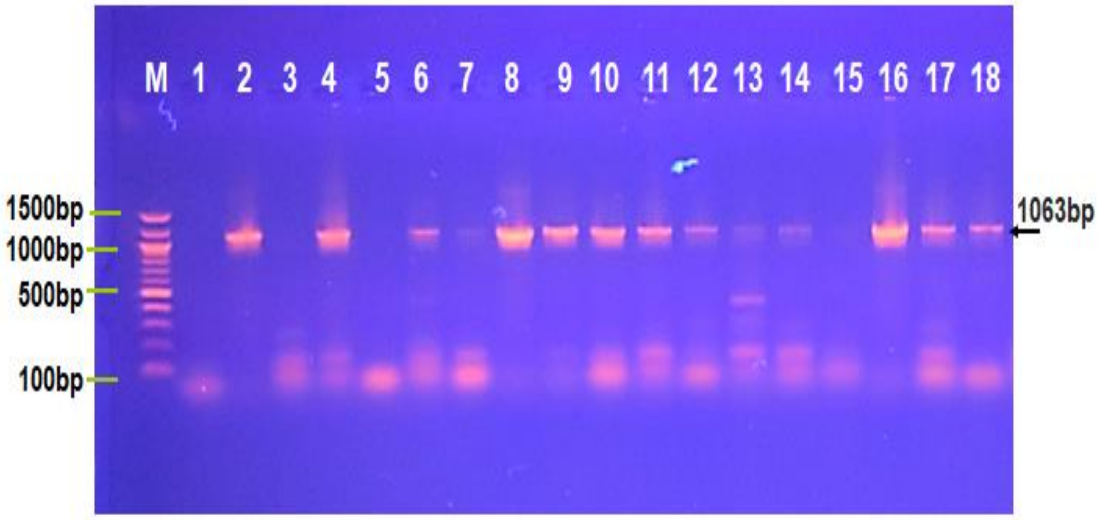


Figure (4.1): Agarose gel electrophoresis image that showed PCR product analysis for Hepatitis B virus polymerase protein gene from serum samples. M (Marker ladder 1500-100bp). Lane (2,4,6,8-12,16-18) positive Hepatitis B virus isolates at 1063bp product size, while lane (1,3,5,7,13-15) negative .

Identification of *HBV* by PCR (33) samples 41.25 % were positive for *HBV*- DNA, while (47) 58.75% were negative .Recorded results this study 47 of them were negative for *HBV*- DNA by PCR technique . This difference did not exclude the presence of infection with *HBV* in these 47 patients as HBs Ag is considered the key of *HBV* serological markers in diagnosis of the infection (Keeffe *et al*., 2006; Perrillo *et al*., 2009). This means that these (47) patients may be chronically infected with *HBV* and are in the non-replicative phase as the natural course of chronic *HBV* infection comprises four phases all of them are HBs Ag (+ve) (Yim and Lok, 2006; Fattovich *et al*., 2008). Predominantly patients in the non-replicative phase are characterized by seroconversion of HBe Ag to anti-HBe , very low or undetectable serum *HBV*- DNA levels (usually <2000 IU/ ml) and normal serum aminotransferases (approximately 40 IU/ml) conferring a favorable

long-term outcome due to immunological control of the infection (McMahon, 2009; Gish *et al.*, 2015; WHO, 2015). Similar results were obtained by (Khaled *et al.*, 2012) who found from 140 *HBV* infected patients (all of them were positive for HBs Ag) only 100 patients were positive for *HBV*- DNA ,also results were obtained by (Al-Suraifi *et al*., 2016) found from 105 patients infected that are 100% positive for HBs Ag only 72 patients were positive for *HBV*- DNA ,while 33 were negative .

Regarding to gender , the present found that there was, no significant difference between males and females patients (p=0.445) as shown in (table 4.5).This results correlates with previous study done by (Abdulla and Goreal, 2016) .They found that the prevalence of *HBV* infection was higher among males (91/135) than the females (43/135). Another study done by , (Ismail *et al.*, 2017) they found that the prevalence of *HBV* infection was higher among males in Egypt.

Table 4.5: Distribution of *HBV*- DNA by PCR technique according to gender

Hospital or center name	**Total**	**Positive cases and percentage**	
		Females	**Males**
Teaching Hospital of Al-Diwaniya	9	3(33.33)	6(66.66)
Al Shamiya General Hospital	2	0(0)	2(100)
Afak General Hospital	3	0(0)	3(100)
Al Hamzah General Hospital	3	1(33.33)	2(66.66)
Central Blood Bank	4	0(0)	4(100)
Haemodialysis	1	0(0)	1(100)

Public Health Laboratory of Al-Diwaniya	11	5(45.45)	6(54.54)
Total	33	9(27.27)	24(72.72)

$X^2 = 5.806$, P value = 0.445

In this study it was found that (21, 63.63%) patients were from the rural and (12, 36.36%) patients were from the center of the city (Table 4.6). Its values were p .value (0.708) no significant difference of location. In studies of numerous in Vietnam found that the prevalence of *HBV* is rural higher than that found in cities (Le Viet *et al*., 2012; Komas *et al*., 2013). In agreement of this, in study from Dohuk the vast majority of our patients were from the rural area. And study done by (Abimiku *et al*.,2017) .They found that the prevalence of *HBV* infection was higher among rural than the urban participants (p> 0.05).Another study done by , (Birku *et al.*, 2015) they found that the prevalence of *HBV* infection was relatively higher among rural dwellers. This might be due to the lack of education programs and vaccination facilities. Any education program should cover those areas with high incidence of the infection.

Table 4.6: Distribution of HBV DNA by PCR technique based on location

Hospital or center name	Total	Positive cases and percentage	
		Urban	Rural
Teaching Hospital of Al-Diwaniya	9	3(33.33)	6(66.66)
Al Shamiya General Hospital	2	1(50)	1(50)
Afak General Hospital	3	1(33.33)	2(66.66)
Al Hamzah General Hospital	3	2(66.66)	1(33.33)
Central Blood Bank	4	1(25)	3(75)
Haemodialysis	1	1(100)	0(0)
Public Health Laboratory of Al-Diwaniya	11	3(27.27)	8(72.72)
Total	33	12(36.36)	21(63.63)

$X^2 = 3.765$, P value = 0.708

4.2.2. *HBV* Genotyping

This study was showed percentage of each genotype is component to the genotype mixed where the ratio was as follows: genotype D (93.94%), E (81.82%), B (69.70%), C (60.61%), A (54.55%) as shown (Figure 4.2).

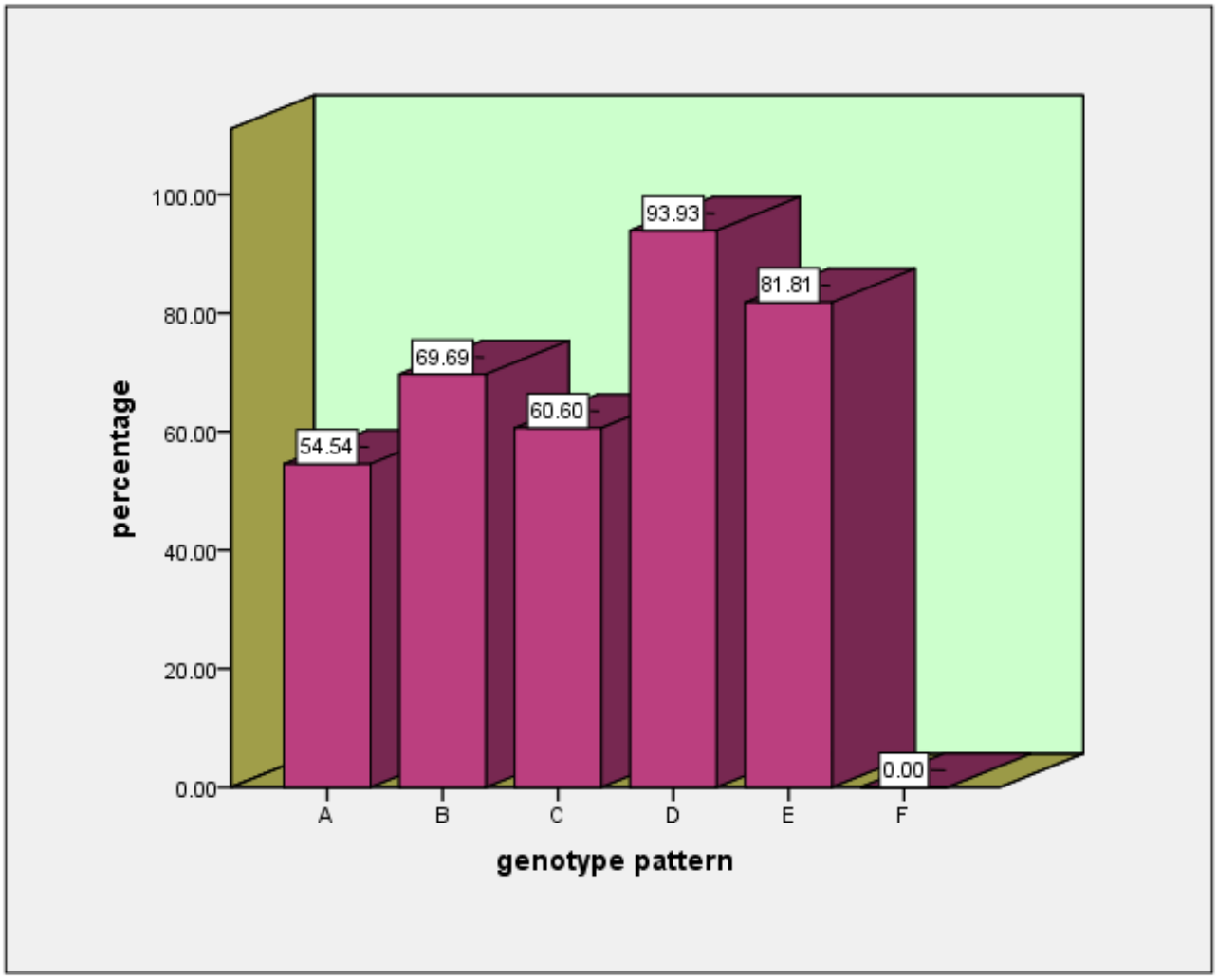


Figure 4.2: percentage of each Genotype found in the genotype mixture of HBV detected by nested PCR .

Results were detected in this study that one single genotype E (3%) was determined and rest of these samples showed mixed infection of genotypes as shown (Table 4.7) (Figure 4.3) (Figure 4.4) (Figure 4.5). The percent distribution of HBV genotypes among patients were as follows: A+E (3%) , C+D+E (12.12%) , B+C+D+E (15.15%) , A+D (3%) , A+B+C+D+E (18.18%) , A+B+D+ E (18.18%), B+D+E (3%) , A+C+D+E (3%) ,B+C+D

(6%) , A+B+D (3%) , A+B+C+D (6%) , D+E (6%) , whereas genotype F was not found in any patient as shown (Figure 4.5).

Table 4.7: Genotypes of *HBV* detected by nested PCR among (33) patients based on gender.

Genotype pattern	Number & (%) of positive samples		
	Males(n=24)	Females(n=9)	Total(n=33)
E	0(0)	1(11.1)	1(3.03)
A+E	1(4.16)	0(0)	1(3.03)
C+D+E	2(8.33)	2(22.2)	4(12.12)
B+C+D+E	4(16.66)	1(11.1)	5(15.15)
A+D	1(4.16)	0(0)	1(3.03)
A+B+C+D+E	5(20.83)	1(11.1)	6(18.18)
A+B+D+E	5(20.83)	1(11.1)	6(18.18)
B+D+E	1(4.16)	0(0)	1(3.03)
A+C+D+E	1(4.16)	0(0)	1(3.03)
B+C+D	2(8.33)	0(0)	2(6.06)
A+B+D	1(4.16)	0(0)	1(3.03)
A+B+C+D	0(0)	2(22.2)	2(6.06)
D+E	1(4.16)	1(11.1)	2(6.06)
total	24(72.72)	9(27.27)	33(100)

$X^{2} = 13.001$, P.Value = 0.369

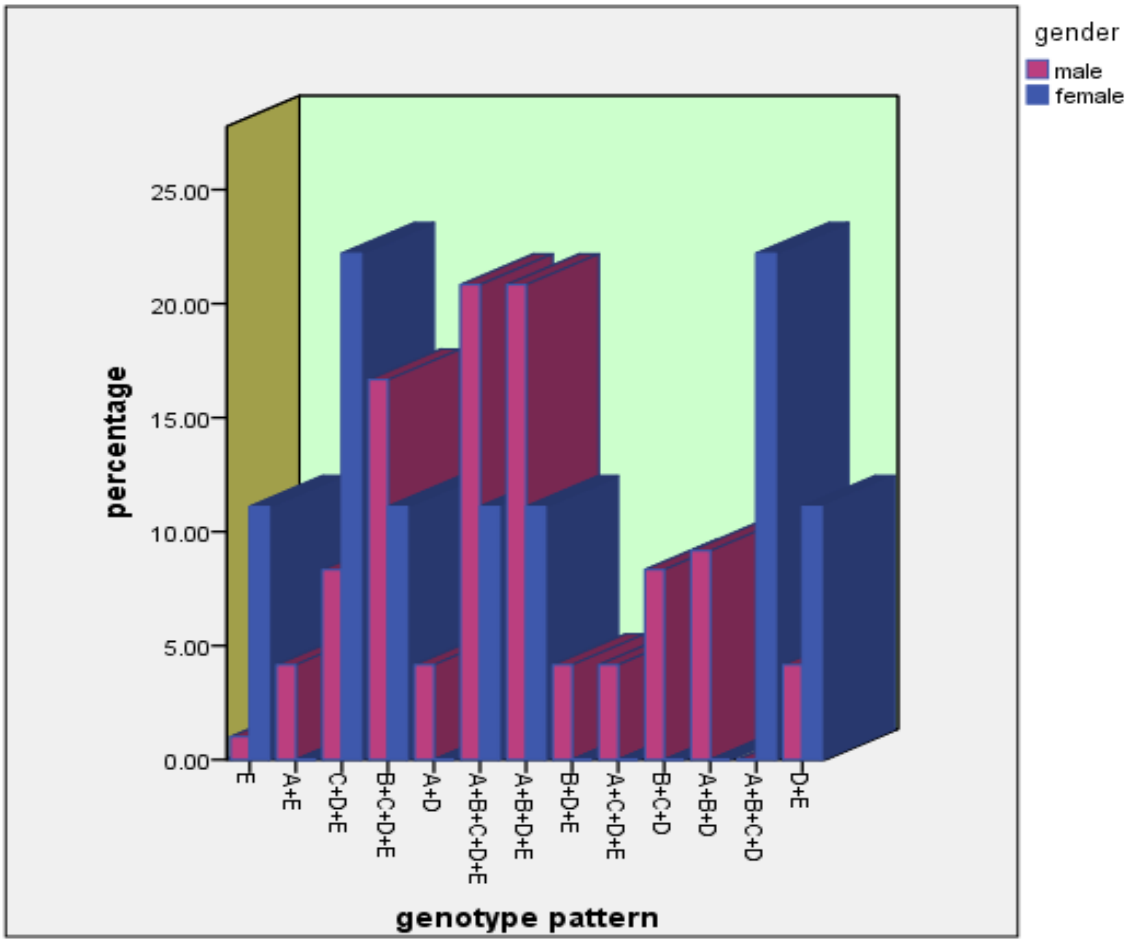


Figure 4.3: Genotypes of *HBV* detected by nested PCR among (33) patients based on gender.

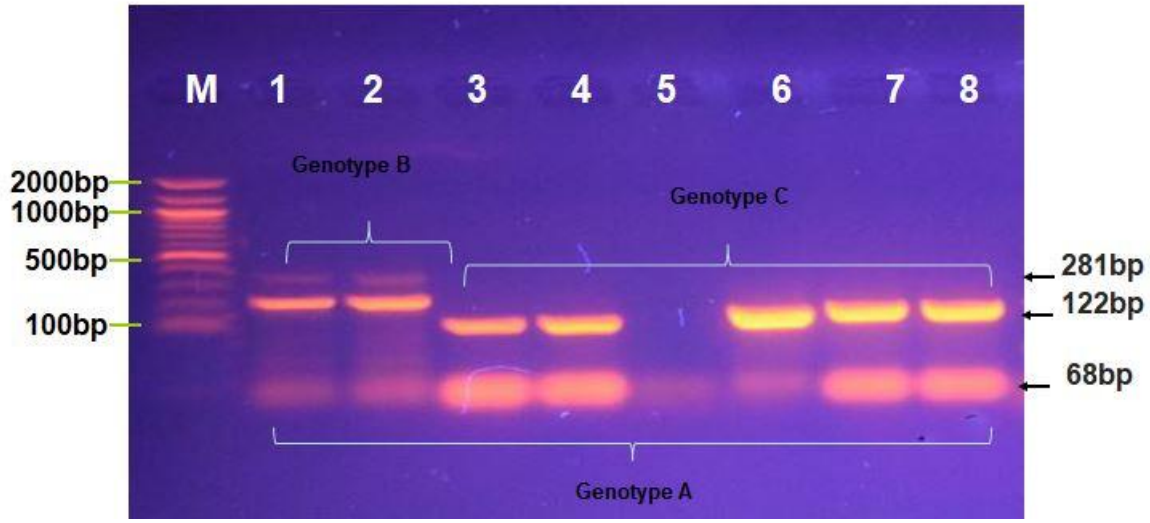


Figure (4.4): Agarose gel electrophoresis image that showed Nested-PCR product analysis for Genotyping *Hepatitis B virus* (A, B, C) from serum samples. M (Marker ladder 1500-100bp). Lane (1 and 2) *Hepatitis B virus* Genotyping B at PCR product 281bp, (2, 4, 6, 7, and 8) *Hepatitis B virus* Genotyping C at PCR product 122bp, and Lane (1 -4 and 6-8) *Hepatitis B virus* Genotyping A at PCR product 68bp.

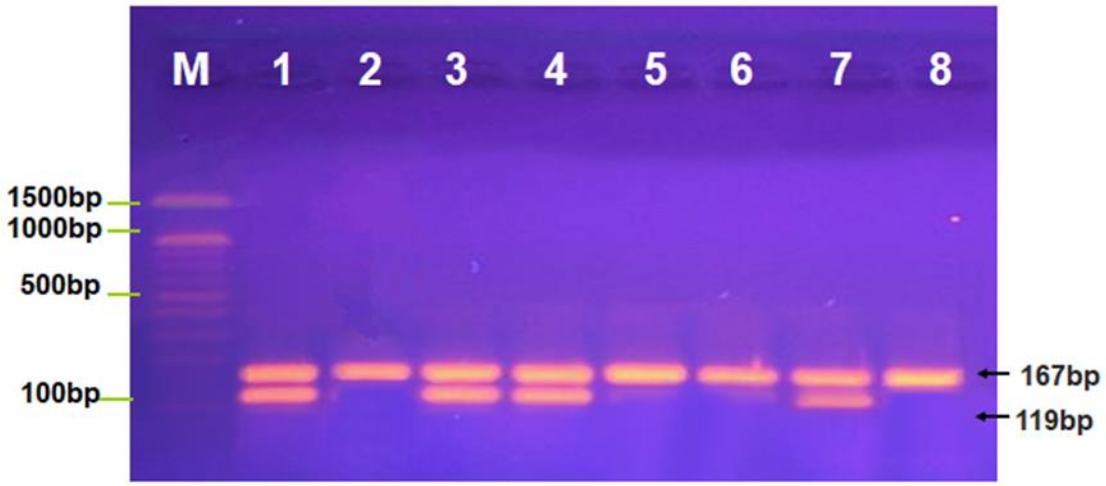


Figure (4.5): Agarose gel electrophoresis image that show Nested- PCR product analysis for Genotyping Hepatitis B virus (D,E,F) from serum samples. M (Marker ladder 1500-100bp). Lane (1 -8) Hepatitis B virus Genotyping E, at PCR product 167bp, and Lane (1, 3, 4, and 7) Hepatitis B virus Genotyping D, at PCR product 119bp.

Hepatitis B virus is characterized by a genetic heterogenecity and 8 genotypes (A to H) can be classified based on comparison of complete HBV genomes (Palumbo *et al*., 2008) .The genotyping of *HBV* is important to clarify the route pathogenesis of the virus because *HBV* variants may differ in their patterns of serologic reactivity, pathogenicity, virulence, and response to therapy and global distribution (Naito *et al*., 2001; Baig *et al*., 2007). New *HBV* genotyping method established based on PCR amplification assay using type specific primers (TSP) which can recognize 6 major genotypes (A-F) of *HBV* (Naito *et al*., 2001). As in Asia A, C and D genotypes were the most predominant (Toan *et al*., 2006), we focused this study on detecting these 6 major genotypes among patients using nested PCR technique developed by Naito *et al*.(2001). In addition, comparison of this method with other techniques of genotyping , this method appears to have a higher sensitivity of recognition and have high ability in distinguishing *HBV* genotypes (Geramizadeh *et al*., 2008).

In this current study ,that single genotype E (3.03%) was determined and that (96.97%) of the patients had mixed infection with (5) genotypes out of (6) detected in this study . This study's results were consistent with those reported from Sulaimania in Iraq (Rashid and Salih, 2015) using the same method, where they found 100% of samples had mixed genotypes 25% had mixed genotypes B+C+D and 75% had mixed genotypes A+B+C+D , as well in wasit province , Al.Suraifi *et al*. (2016) found that all of samples had mixed genotypes , (77.7%) had mixed genotypes A +B+ C+ D+ E , (16.6%) A+ B+ D+ E, (2.7%) A+ B+ C, (1.3%) A+ B+ E, (1.3%) A+ D+ E , also no single genotype were identified and genotype F was not found in any patient .In Duhok, Kurdistan region of Iraq Abdulla and Goreal,(2016) found that genotype D is the main

genotype in Duhok / Iraq followed by genotype B. High percentage of CHB patients with genotype D are HBe Ag positive. Whereas, in Basra Al-Aboudi and Al-Hmudi,(2015) detected genotype D 92.3% and 7.69% had mixed genotypes D+ E .Also ,in Baghdad Ahmed (2013)found that genotype D (80%) was the predominant among CHB patients and mixed genotypes D+F (20%).

Reports about *HBV* genotypes in the neighboring countries illustrated that ; In Iran (Yoosefi *et al*., 2016) found that genotype D in 154/163 specimens (94.5%) and 9/163 (5.5%) had mixture of C/D genotype. In Saudi Arabia (Asaad *et al*., 2015) among 160 *HBV* patients Genotype D was the most common, found in 135 (84.4%) patients, followed by A (18; 11.3%) and E (7; 4.3%). In Oman (Al Baqlani *et al*., 2014) *HBV* genotypes D (130/170; 76.47%) and A (32/170; 18.28%) are predominant in Oman, the *HBV* genotypes C and E were less frequent (each 1.18%). In United Arab Emirates (Alfaresi *et al*., 2010) all 88 patients (100%) *HBV* DNA the genotype D was the most prevalent (79.5%) followed by genotype A (18.2%) and genotype C (2.3%). In Egypt (Khaled *et al*., 2011) showed that genotype D constituted 87% of the total infections (100%) *HBV*- DNA positive, the other 13% showed mixed infections of D/F. In Jordan Genotype D represented the predominant genotype in all Jordanian patients infected with *HBV* (Hamoudi *et al*., 2016).In Pakistan Ten different studies conducted at different regions of Pakistan showed that the most prevalent *HBV* genotype in Pakistan is genotype D with overall prevalence rate of 63.71% followed by genotype A (10.036%), genotype C (7.55%) and genotype B (5.335%) while mixed genotypes were 2.377% and 9.931%, respectively (Ali *et al*., 2011) . That 97% of patients in Syria were infected with genotype D (Antaki *et al*., 2010).

Seven genotypes (A-G) were determined in Asia, of which A, C and D were the most predominant, whereas in Europe and Africa A, C, D and G genotypes were detected. Mixed genotypes A+C and C+D were the predominant in Asia, while C+D in Europe and A+D in Africa (Toan *et al*., 2006) . The high prevalence of mixed infections among this study included patients revealed multiple incurrence of these patients to different *HBV* genotypes, and indicated high endemicity of genotypes A, B, C, D, and E in our community. Worldwide, the most vulnerable individuals to mixed infection with different HBV genotypes are the intravenous drug users (IVDUs) as it was reported by Chen *et al*.(2004). The probability of presence of IVDUs among patients is possible.

In Iraq, drug abuse increased in Iraq after 2003 and still a growing problem. Other possibility of the presence of mixed genotypes was recombination between genotypes as explained by Chen *et al.* (2004) but this possibility was not investigated here. Other Iraqi researchers(Al.Suraifi *et al*., 2016) in Wasit province explained that the high prevalence of mixed infections where was in the rate of 100% patterns Genotype mixed as well as in Sulaimani ,Kurdistan region of Iraq (Rashid and Salih, 2015) . Most likely the cause of the high prevalence of mixed infections in their study may be due to "the migration of a large number of people as refugee to Europe and America and remaining in the refugee camps for long periods, in which communication with people of different countries might cause transmission of infection with different genotypes. In addition, low level of education about the way of disease transmission in negligence of sterilization in the dental clinics and negligence of using disposable materials in makeup and hairdresser salons may be other predisposing factors to *HBV* infection in our community.

4.2.3. Phylogenetic analysis

Deoxyribonucleic acid (DNA) sequencing analysis results were includes *Hepatitis B virus* clones based on phylogenetic tree analysis and ClustalW alignment analysis by using (MEGA 6.0) between local *Hepatitis B virus* clones and NCBI-Blast *Hepatitis B virus***.**

Multiple sequence alignment analysis of the partial *Hepatitis B virus* polymerase sequence in local *Hepatitis B virus* clones (No.1-No.8) and NCBI-Genbank *Hepatitis B virus* clones (No.9-No.18) based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) in polymerase nucleotide sequences between the local *hepatitis B virus* clones and NCBI-Genbanck *hepatitis B virus* clones ,while empty surface polymerase nucleotide sequences indicates a difference (mutation), as shown (Figure 4.6).

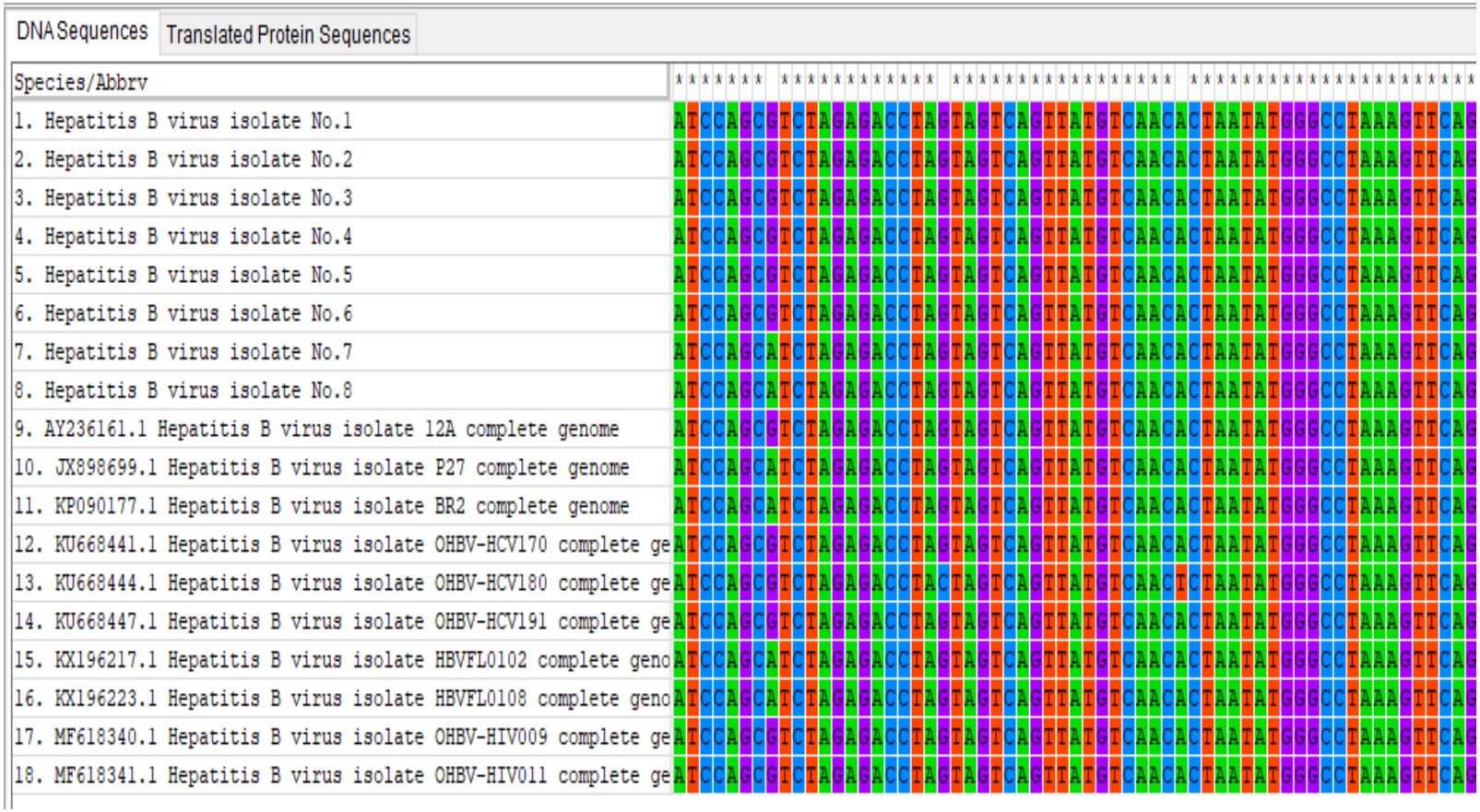


Figure 4.6: Multiple sequence alignment analysis of the partial *Hepatitis B virus* polymerase sequence in local *Hepatitis B virus* clones (No.1-No.8) and NCBI-Genbank *Hepatitis B virus* clones based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) in polymerase nucleotide sequences.

The local *Hepatitis B virus* clones (No.1, No.2, No.3, and No.6) clones were closely related to NCBI-Blast *Hepatitis B virus* (**KU668447.1)**. The local *Hepatitis B virus* clones (No.4) clones was closely related to NCBI-Blast *Hepatitis B virus* (**MF618340.1)** .The local *Hepatitis B virus* clones (No.5) clones was found to be closely related to NCBI-Blast *Hepatitis B virus* **(AY236161.1)**. Whereas, the local *Hepatitis B virus* clones (No.7 and No.8) clones were closely related to NCBI-Blast *Hepatitis B virus* (**KX196223.1)**, as shown (**Figure** 3.7).

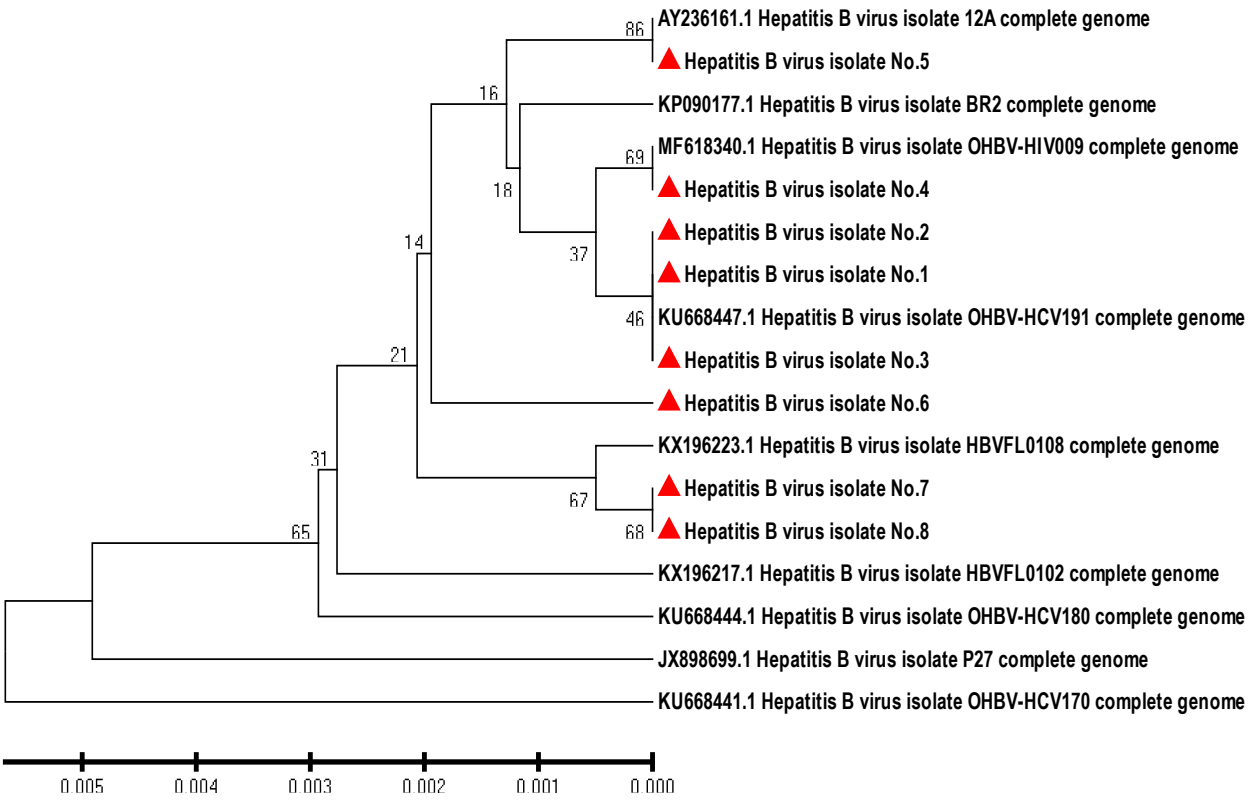


Figure 4.7: Phylogenetic tree analysis based on Hepatitis B polymerase gene partial sequence that used for study of genetic relationship between local *Hepatitis B virus* clones and NCBI-BLAST *Hepatitis B virus* clones. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Hepatitis B virus* clones (No.1, No.2, No.3, and No.6) clones were closely related to NCBI-Blast Hepatitis B (KU668447.1). The local *Hepatitis B virus* clones (No.4) clones was closely related to NCBI-Blast *Hepatitis B virus* (MF618340.1).The local *Hepatitis B virus* clones (No.5) clones were closely related to NCBI-Blast *Hepatitis B virus* (AY236161.1).Whereas, The local *Hepatitis B virus* clones (No.7 and No.8) clones were closely related to NCBI-Blast *Hepatitis B virus* (KX196223.1).

Then confirmed by NCBI-BLAST Homology Sequence Identity (Table 4.8). After that *Hepatitis B virus* clones were deposited into of NCBI-GenBank to get Genbank accession number for *Hepatitis B virus* clones. where the Genbanck accession number was obtained .

Table 4.8: Homology sequence Identity (%) of local Hepatitis B isolates and NCBI-Blast Hepatitis B isolates using NCBI- BLAST alignment tool.

NCBI-Blast Hepatitis B virus clones No.	**Region**	**NCBI-Genbank Accession number**	**Homology sequence Identity (%)**	
			NCBI-BlAST identical clones	**Identity (%)**
Hepatitis B virus clones No.1	India	MH511181.1	**KU668447.1**	**100%**
Hepatitis B virus clones No.2	India	MH511182.1	**KU668447.1**	**100%**
Hepatitis B virus clones No.3	India	MH511183.1	**KU668447.1**	**100%**
Hepatitis B virus clones No.4	India	MH511184.1	**MF618340.1**	**100%**

Hepatitis B virus clones No.5	Italy	MH511185.1	**AY236161.1**	**100%**
Hepatitis B virus clones No.6	India	MH511186.1	**KU668447.1**	**100%**
Hepatitis B virus clones No.7	India	MH511187.1	**KX196223.1**	**100%**
Hepatitis B virus clones No.8	India	MH511188.1	**KX196223.1**	**100%**

Table (4.8) represents the registry of the NCBI-GenBank and the genetic identical between the clones of *hepatitis B viral* local and NCBI-Blast hepatitis B clones ,where the identity 100% for local *hepatitis B virus* clones (No.1,No.2,No.3and No.6) with NCBI-BLAST identical clones (KU668447.1, India) , the identity 100% for local *hepatitis B virus* clones (No.4) with NCBI-BLAST identical clones (MF618340.1, India) , the identity 100% for local *hepatitis B virus* clones (No.5) with NCBI-BLAST identical clones (AY236161.1, Italy) , the identity 100% for local *hepatitis B virus* clones (No.7, and No.8) with NCBI-BLAST identical clones (KX196223.1, India) .

Conclusions

And

Recommendations

Conclusions:

1. The current study showed mixed genotypic infection with 5 genotypes (97%) and only one genotype E (3%) , whereas genotype F was not found in any patient. This study shown that genotype D (93.94%) was predominant in the mixed genotypes among patients.

2. On the basis of the current study shown that no significant difference males and females and no significant in location between rural and urban areas in occurs of HBV.

3. This study the presence of mixed infection with about 5 HBV genotypes among most of the patients lead us to conclude that these patients are incurred to different sources of infection at different times.

Recommendations:

1. Further study based on large sample size is needed to make large data base for determining the effect of mixed infection HBV.
2. Further research is needed to determine the effect of these mutations and mix infection by 5 genotypes related to the course of the disease.
3. Geographic distribution genotype of hepatitis B virus in Iraq requires more studies to assess.

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Appendices

Appendix: patient's datasheet

Case number				
Name				
Age				
Gender	Male		Female	
Location	Rural		Urban	
Blood transfusion history				
Hemodialysis				
History of jaundice in patient				

الخلاصة

ان التهاب الكبد الفيروسي ب هو مشكله صحيه عامه لا نه يسبب امراض للكبد مثل سرطان الكبد و تليف الكبد. على الاقل عشره انماط جينيه لالتهاب الكبد الفيروسي ب (A-J) تم توثيقها اعتمادا على التسلسل المتجانس للفيروس .

وقد اجريت هذه الدراسة للكشف عن الانماط الجينية لالتهاب الكبد الفيروسي ب بين المرضى لالتهاب الكبد الفيروسي ب في محافظه الديوانية / العراق مستخدما بروتوكول تفاعل البلمرة المتسلسل المتداخل. ولإنجاز هذه الهدف ، جمعت عينات الدم من (٨٠) مريض والتي تضمنت ٥٩ ذكر و ٢١ انثى بمعدل اعمار يتراوح ما بين (١٢-٧٥ سنه) لالتهاب الكبد الفيروسي المتضمن في هذه الدراسة .

و تم الاختبار التوكيدي للأمصال لأتأكد من وجود المستضد السطحي لالتهاب الكبد الفيروسي ب بواسطه تقنيه الاليزا .بعد ذللك تم اجراء تفاعل البلمرة المتسلسل للكشف عن وجود المادة الوراثية للفيروس ومن ثم اجراء تفاعل البلمرة المتسلسل المتداخل من اجل معرفه التنميط الجيني للفيروس . وتم اختيار ثمان عينات وارسلت الى البنك الجيني . ومن ثم لخصت البيانات و قدمت وحللت باستخدام التعبئة الإحصائية للعلوم الاجتماعية.

ثمانون عينه كانت ايجابيه للمستضد السطحي لالتهاب الكبد الفيروسي ب بواسطه تقنيه الاليزا . اما نتائج تفاعل البلمرة المتسلسل فكانت ٣٣ (٤١.٢٥%) عينه كانت ايجابيه للحامض النووي لالتهاب الكبد الفيروسي ب و ٤٧(٥٨.٧٥%) عينه كانت سلبيه للحامض النووي لالتهاب الكبد الفيروسي ب. وفيما يتعلق بالأنماط الجينية لالتهاب الكبد الفيروسي ب والتي ظهرت بعد استخدام تفاعل البلمرة المتسلسل المتداخل حيث تم تحديد النمط الوراثي E نقيا اما بقيه العينات فقد كانت خليط لأكثر من نمط جيني .

كان توزيع النسب للأنماط الجينية لالتهاب الكبد الفيروسي ب بين المرضى كالتالي : A+E(٣%))A+B+C+D+E, (١٨.١٨%)، (١٢.١٢%)C+D+E ، (١٥.١٥%)B+C+D+E ، A+D(، (٦%)B+C+D (٣%)A+D+E ، (٣%)B+D+E ، (١٨.١٨%) A+B+D+E، (٣% ، (٦%)D+E، (٦%)A+B+C+D ، (٣%)A+B+D. ،

حيث ان النمط الجيني F لم يوثق لأي مريض . احصائيا، لم يكن هناك اختلاف كبير في توزيع الانماط الجينية بين الذكور و الاناث (القيمة المعنوية=٠.٣٦٩) ، ايضا من النتائج التي تم الحصول عليها لم يكن هنالك اختلاف كبير في الموقع بين الريف والمدينة (القيمة المعنوية=٠.٧٠٨). وجود

عدوى مختلطه بخمسه انماط جينيه لالتهاب الكبد الفيروسي بين اغلب المرضى في محافظه الديوانية .

التحليل الشجيري الوراثي اوجد بان عزلات التهاب الكبد الفيروسي ب المحلية (رقم:١،رقم:٢،رقم:٣،رقم:٦) كانت قريبه الشبه بالتسلسلات البيولوجية لالتهاب الكبد الفيروسي ب (KU668447.1). عزله التهاب الكبد الفيروسي ب المحلية (رقم: ٤) كانت قريبه الشبه بالتسلسلات البيولوجية لالتهاب الكبد الفيروسي ب (MF618340.1). عزله التهاب الكبد الفيروسي ب المحلية (رقم:٥) وجدت قريبه الشبه بالتسلسلات البيولوجية لالتهاب الكبد الفيروسي ب (AY236161.1). بينما، عزلات التهاب الكبد الفيروسي ب المحلية (رقم: ٧، ورقم: ٨) كانت قريبه الشبه بالتسلسلات البيولوجية لالتهاب الكبد الفيروسي ب (KX196223.1).

ان اكتشاف التسلسلات اثبتت بواسطه التناظر المتسلسل . بعد ذلك اودعت عزلات التهاب الكبد الفيروسي ب في البنك الجيني للحصول على رقم انضمام لعزلات التهاب الكبد الفيروسي ب الى البنك الجيني .

هذه الدراسة توضح بان خليط الانماط الجينية لالتهاب الكبد الفيروسي ب بمعدل ٩٧% و الانماط الجينية النقية لالتهاب الكبد الفيروسي ب كان بمعدل ٣% .

الدراسة الحالية اظهرت خليط جيني للإصابة بالأنماط الجينية الخمسة ،والنمط الجيني E هو فقط النقي ، بينما النمط الجيني F غير موجود في اي مريض .هذه الدراسة اظهرت النمط الجيني D(٩٣.٩٤%) هو السائد في الانماط الجينية الخمسة بين المرضى .والدراسة الحالية اظهرت انه لا يوجد فرق كبير بين الذكر والأنثى وكذلك لا فرق كبير بالموقع بين مناطق الريف والمدينة في حدوث التهاب الكبد الفيروسي ب.

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فرع الاحياء المجهرية

التنميط الوراثي لفيروس التهاب الكبد ب في محافظه الديوانية

رسالة مقدمة إلى
مجلس كلية الطب- جامعة القادسية
وهي جزء من متطلبات نيل درجة الماجستير في
ا لأحياء المجهرية الطبية
تقدم بها

حازم سراوه عطية المحنه
بكالوريوس علوم – أحياء مجهرية _ جامعة بابل (٢٠٠٣)

بأشراف

المدرس الدكتور وسام صالح عبود
دكتوراه فيروسات طبيه

١٤٤٠هـ ٢٠١٨م