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Department of Medical Microbiology

Study of TNF- α in Patient with Chronic HBV and HCV and UTR gene in HCV

A Thesis

Submitted to the Council of College of Medicine/University of Babylon in Partial Fulfillment For the Requirements for the Degree of Master in Science / Medical Microbiology

By

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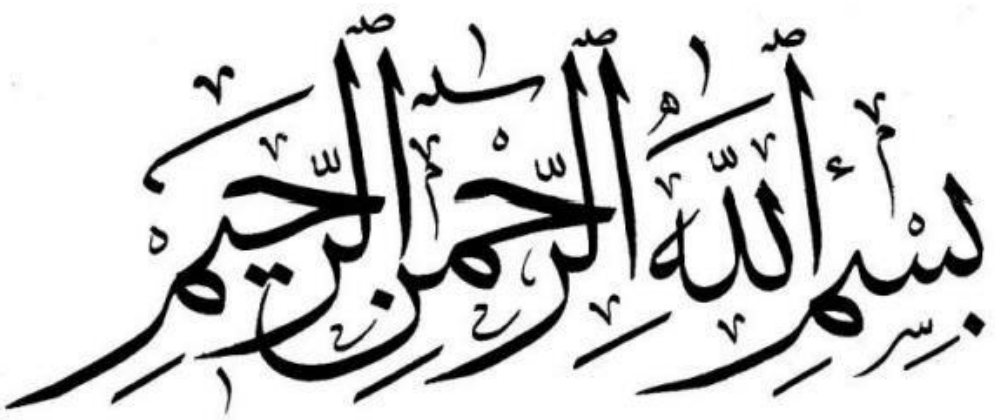

﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا ۝﴾

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Dedication

I dedicate the fruit of my labor to (my soul) who supported me and still supports me, and who supports me in the hardship of the journey and before it.

To the partners of blood and childhood and the companions of the path that ended before completed (my sister **Duha** and my brother **Abdul Aziz**), may God have mercy on them. **And for everyone who acted as a source of light in the midst of darkness.**

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Summary

The hepatitis B virus (HBV) is responsible for all forms of hepatitis endangering the health of the public. The growth, division, and activity of immune cells are governed by chemical mediators called cytokines. Evidence suggests that inadequate immune responses contribute to the persistence of HBV.

Hepatitis C is an inflammation of the liver caused by the hepatitis C virus.

The virus can cause both acute and chronic hepatitis, ranging in severity from a mild illness to a serious, lifelong illness including liver cirrhosis and cancer.

Total 105 samples from patient individual during period From August 2022 to November 2022 ,it is 20 healthy participants and 37 persons with chronic HBV infection were recruited, and 48 persons with chronic HCV infection were recruited, . Healthy controls and research participants ranged in age from 20 to 80, and all of them were analysed using serum samples (3 ml). The levels of HBV, TNF-, and HBeAg in the serum were determined using (ELISA).

affirmative 37a double-antibody sandwich (ELISA), that HBV participants met the inclusion criteria. The findings of the HBsAg ELISA Kit indicated that the prevalence of HBsAg was greatest in those aged 35 to 49 = 12 (32.5%), lowest in those aged 20 to 34 = 8 (21.6%) and 50 to 64 =8 (21.6%) . HBsAg The breakthrough was made possible by using an ELISA Kit. The 37 patients tested positive for HBsAg, 22 were female (59.5%) and 15 were male (40.5%). This suggests that the prevalence of HBsAg infection is higher in females than in males.

The findings 48 of the HCV IgM ELISA Kit indicated that the prevalence of HCV was greatest in those aged 41 to 60 =22 (45.8%), lowest in those aged 21 to 40 =8 (16.7%) and HCV Ag. Of the 48 individuals who tested positive for HCV Ag, 21 (43.8%) were female and 27 (56.2%) were male. This indicates that males are more likely than females to have HCV Ag infection.

The DNA sequencing method was used to genotype identify and analyze the UTR-polyprotein gene of local Hepatitis C virus isolates (IQ.No.1 - IQ.No.5) and related Hepatitis C virus genotype isolates from NCBI-Blast .

The local Hepatitis C virus isolates were revealed to be closely linked to NCBI-BLAST Hepatitis C virus subtype 1a (HQ113638.1) at total genetic alterations (0.3-0.05%), according to the phylogenetic tree genetic relationship study.

The genetic homology sequence identity between the local isolates of the Hepatitis C virus (IQ.No. 1 through IQ.No. 5) and the Hepatitis C virus identified by NCBI BLAST ranged from (99.41% to 99.61%). The local isolates of the Hepatitis C virus (IQ.No.1 to IQ.No.5) were then submitted to the NCBI Genbank and given accession numbers (OQ843893, OQ843894, OQ843895, OQ843896, OQ843897).

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Abbreviations	Full name
µl	Micro liter
Ab	antibody
ADV	Adenovirus
AFIAS	Automated Fluorescent Immunoassay System
ACLF	Acute-on-chronic liver failure
Ag	Antigen
AHB	Acute hepatitis-B infection
AHC	Acute hepatitis-C infection
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
Anti-HBc	Antibody to hepatitis-B core antigen
Anti-HBe	Antibody to hepatitis-B e antigen
Anti-HBs	Antibodies to hepatitis-B surface antigen
Anti-HCV	Antibody to hepatitis –C virus
AST	Aspartate aminotransferase
AVH	Acute viral hepatitis
AIDS	Acquired immunodeficiency syndrome
HBx	Hepatitis B x protein
cccDNA	covalently closed circular DNA
CD+4	Cluster of Differentiation 4
CD+8	Cluster of Differentiation 8
CDC	Centers for Disease Control and Prevention
CHB	chronic hepatitis B
CMV	Cytomegalovirus
DAA	D-Aspartic acid
DNA	Deoxyribonucleic acid
DCs	Distributed Control System
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
cryo-EM	cryo-electron microscopy
HAV	Hepatitis A virus
HDV	Hepatitis D virus
HBc Ab	Hepatitis B core antibody
HBe Ab	Hepatitis B envelope antibody
HBe Ag	Hepatitis B envelope antigen
HBs Ag	Hepatitis B surface antigen

……List of Abbreviations……

HBcAg	Hepatitis B core antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGV	Hepatitis G virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HSV-1	Human herpes virus-1
HSV-1	Human herpes virus-1
IFN-γ	Interferon–gamma
ICOSL	Inducible Costimulator
IG	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-17	Interleukin 17
IL-21	Interleukin 21
INR	International Normalized Ratio
IU	International unit
IVDU	Intravenous drug use
KDa	Kilo Dalton
Kg	Kilogram
mg	Milligram
ml	Milliliter
MOH	Ministry of Health
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NCR	Non-coding regions
NCs	Negative control of the sample
NK	Natural Killer cell
NS	Nonstructural
nm	Nanometer
O.D	Optical Density
OBI	Occult Hepatitis B Infection
^{o}C	Degrees Celsius
PCR	Polymerase chain reaction
Pol	Polymerase
PWID	People who inject drugs
cDNA	Circular DNA
RNA	Ribonucleic acid

……List of Abbreviations……

Ros	Reactive oxygen species
RT	Reverse transcriptase
SS	signal sequence
S.D	Standard deviation
SRP	Signal recognition particle
TBE	Tris borate EDTA buffer
TM	Transmembrane
TNF-α	Tumor necrosis factor alpha
VL	Viral load
VLDLs	Very-low-density lipoproteins
VZV	Varicella-zoster virus
WHO	World Health Organization
WHV	Woodchuck hepatitis virus
YFV	Yellow fever virus

Chapter One

Introduction and Literatures Review

1. Introduction

Hepatitis B virus (HBV) infects the liver causing inflammation, and can have an acute course followed by recovery or develop into a chronic infection. HBV infection remains a global health threat, with 2 billion people worldwide estimated to have past or prior infection and approximately 257 million people in the world estimated to have chronic hepatitis B (CHB) (Lin *et al*., 2020 and Zou *et al.,* 2021). Without treatment, 20–30% of those with CHB develop cirrhosis or hepatocellular carcinoma (HCC), and at least 650,000 people are estimated to die annually from complications of HBV infection (Bogler *et al.,* 2018; Mao *et al*., 2022).

Hepatitis C is an inflammation of the liver caused by the hepatitis C virus. The virus can cause both acute and chronic hepatitis, ranging in severity from a mild illness to a serious, lifelong illness including liver cirrhosis and cancer (WHO).

Hepatitis C virus (HCV) infection is a global health burden. Nearly 185 million subjects (~3 %) of the world's population are affected by this virus. Liver cirrhosis, progressing liver disease, and hepatocellular carcinoma (HCC) are common complications of chronic HCV infection (Yip *et al,*. 2022). Primary infections with HCV are usually asymptomatic (Gholizadeh, *et al*., 2023), and the majority of cases develop chronic infection.

HBV is transmitted from mother to child at the time of birth or through percutaneous or mucosal exposure to infected blood or body fluids, such as through sexual exposure later in life (Chilaka *et al*., 2020). Without post-exposure prophylaxis with HBV vaccination or hepatitis B immune globulin (HBIG), approximately 90% of infants and 30% of children younger than 5 years of age who are infected with HBV will develop CHB. In contrast, the likelihood of developing CHB after

infection as an adult is 5–7% (Joshi *et al.*,2020; Premkumar and Chawla 2021).

A highly effective vaccine against HBV has been widely available since the 1980s, and the World Health Organization (WHO) recommends that the vaccine be administered to every child as soon as possible after birth, ideally with the first dose within 24 hours (Meireles *et al*., 2015).

Aim of study:

The study aims to investigate immunological detection of Hepatitis B virus HBV and HCV in patient and detection TNF- α and UTR gene in HCV

Objectives :

Methodology involved in the study will be the techniques of immunological parameter including:

1- ELISA test for detection HBV and HCV.

2- Measurement the level of TNF in HBV and HCV patients.

3- Detection of HBeAg in chronic HBV patients.

4- Detection Untranslated Region (UTR) gen in HCV

Chapter One …….. Introduction and literature Review

1.1 : Literature review

1.1.1. : History of Hepatitis virus

The term of Hepatitis consists of two sections, the first is the (hepar) which originated from the Greek, meaning the liver, and second the suffix (itis) means inflammation (Dyson *et al*., 2014). Hepatitis is inflammation of the liver. Viral infection is responsible for around half of all cases of acute hepatitis. The term is generally used to refer to the diseases caused by the hepatropic viruses including the diseases hepatitis

The medical history of viral hepatitis is as fascinating as their clinical courses. Epidemic jaundice was mentioned in the Babylonian Talmud in the 5th century AD, and it was also described by Hippocrates in "Of the Epidemics" around year 400 AD (Midgard., 2018).

Hepatitis B virus (HBV) and hepatitis C virus (HCV) constitute a global health issue. Although there are the efective hepatitis B vaccine since 1982 (Ward and Hinman .,2019). WHO reported that approximately 257 million people have been infected with HBV and only 16.7% of the people diagnosed with hepatitis B were on anti-viral treatment as of 2016 (Cheng *et al.,* 2023). Similarly, even there is no vaccine for HCV till now, the direct-acting antiviral drugs (DAAs) for the treatment of HCV were initially developed in 2011 (Dietz and Maasoumy., 2022). and have since yielded high sustained virologic responses (SVRs) (McPhee., 2019).

In 2015, about 71 million people lived with HCV infection; in 2017, 19% (13.1 million) of them knew their infection status and only 15% (2 million) of people already diagnosed with HCV infection received curative treatment in the same year. Overall, between 2014 and 2017, 5 million people received HCV infection eradication treatment, but in the

same year, 1.75 million people had recently developed a chronic HCV infection (Trifan. *et al*.,2023). Therefore, the World Health Organization (WHO) announced in 2016 a target to eliminate HBV as public health threats by 2030 by reducing the hepatitis B surface antigen (HBsAg) prevalence among children to ≤0.1% (Razavi *et al.,* 2021). and to eliminate HCV by 90% reduction in new diagnosis and 65% reduction in HCV related mortality by 2030 (Dore and Cowie., 2021).However, both the expected or unexpected barriers are still existed particularly in remote areas for its less accessibility, high cost and require human and technical resources to eliminate HBV and HCV in the world (Cooke *et al*., 2019;Yamamoto *et al*., 2020).

1.1.2 : Types of Hepatitis B According to Infection

The adults who get the Hepatitis B virus, 95% clear the virus and do not go on to have the chronic form of the disease. Some people have few symptoms or none at all, so many do not realize they have HBV or that they have had it. Many adults will clear the virus completely within six months. The good news is that the protective antibodies produced while fighting the infection mean that people who have had it will never have to worry about HBV again they will be immune. (jerry-kennard .2023).

There are three types of hepatitis B infections:

1. **Healthy chronic carriers of hepatitis B** are not infectious to others and, although they may have a slightly higher risk of cirrhosis and liver cancer than the general population, they mostly live normal lives. The virus can become reactivated if their immune systems become suppressed, such as during a severe illness, during treatment with immunosuppressant drugs for diseases like cancer or AIDS, or with drugs such as steroids.

2. **Chronic infectious hepatitis B** is highly infectious. The person with it may have a very inflamed and damaged liver even when the person has few or no symptoms. People with this type of hepatitis B are more likely to have a progressive disease leading to cirrhosis. Only 5% to 10% have spontaneous remission, become noninfectious to others, and sustain no further or minimal liver damage, although sometimes reactivation of the virus occurs.

3. **Chronic mutant hepatitis B** is a mutated strain of the virus with a permanent alteration of the hepatitis B virus's genetic makeup. Those with it have the potential to be infectious to others and it is thought to be more resistant to treatment than other forms of the disease (jerry-kennard 2023).

1.1.3 : Route of Transmission hepatitis B and C virus :

The most efficient transmission of HCV and HBV are through large or repeated direct percutaneous exposure to infected blood or body fluids containing blood **(**Schillie *et al*., 2014) ,contaminated bodily fluids like (Vanukuri., 2023)

Blood Sweat, Tears, Saliva, Semen, Vaginal secretions, Menstrual blood, Possible forms of transmission include sexual contact, blood transfusions or transplantation from infectious donors and transfusion with other human blood products, dialysis , re-use of contaminated needles and syringes, and vertical transmission from mother to child (MTCT) during childbirth, HCV and HBV not spread by breastfeeding , contact with broken skin on breast sores may be a risk factor for transmission (Blomé., 2016).

The high transmission rate through illicit intravenous drug use (IVDU) explains why the prevalence of HCV among people who acquired human immunodeficiency virus (HIV) through IVDU reaches 90% **(** Obienu *et al*., 2011).In developed settings blood screening and healthcare practices

have virtually eliminated spread, meaning that HCV and HBV are transmission occurs almost exclusively among people who inject drugs (PWID) (Thomas., 2019) .

HBV and HCV can be transmitted between family members within households, possibly by contact of non-intact skin or mucous membrane with secretions or saliva containing viruses , Other sources still include transmission via tattooing/piercing , Barbers (Aliu. *et al.,* 2022).

HBV is survival for 6 months at room temperature and 7 days at 44°C. and The Hepatitis C virus can survive outside the body at room temperature, on environmental surfaces, for up to 3 weeks (Wißmann *et al*., 2021).

1.1.4 : Structure of HBV

The identification of cellular receptor, as well as the development of innovative infection models and molecular techniques, have opened up new possibilities for study into particular stages of the HBV replication cycle as well as the organization and activity of the covalently closed circular DNA (cccDNA),the viral minichromosome that serves as the template for HBV transcription in the nucleus of the infected hepatocytes, enabling maintenance of chronic HBV infection (Glebe *et al*., 2021).

Electron microscopy revealed three distinct viral structures in the serum of HBV-infected patients: Dane particles, circular particles, and filamentous particles (Jiang and Hildt., 2020). A common HBsAg on the surface of all three particles. Circular 20 nm (and filamentous) 22 nm particles are noninfectious because are composed of HBsAg and host-derived lipids, but lack the HBV genome (Umego *et al.,* 2018). On the other hand, Dane particle 42 nm sphere (is a fully infectious HBV virion.

A small circular, partially double-stranded DNA molecule and viral DNA polymerase are found in the core of the Dane particle, which is surrounded by nucleocapsid. Nucleocapsid is built by assembled hepatitis

B core antigen HBcAg and it is covered with a lipid envelope containing HBsAg (Luo *et al*.,2020).

Thus, a nucleocapsid composed of hepatitis B core protein HBc, viral polymerase Pol and viral genome DNA (Tsukuda and Watashi., 2020). HBV's genome is unique since it's not completely double-stranded.

The viral DNA polymerase is attached to one end of the full-length strand. The full-length strand of the genome is 3020–3320 nucleotides long, while the short-length strand is 1700–2800 nucleotides long (Lanini *et al*., 2019). C, X, P, and S are the four recognized genes encoded by the genome. Gene C codes for the core protein (HBcAg) and its start codon is preceded by an upstream in–frame AUG start codon, from which produces the pre-core protein.Proteolytic processing of the pre-core protein produces hepatitis B envelope antigen (HBeAg).Gene P is responsible for encoding DNA polymerase.

The surface antigen is coded for by Gene S.(HBsAg).The HBsAg gene is one long open reading frame with three in frame "start" (ATG) Codons that divide it into three sections: pre-S1, pre-S2, and S. Polypeptides of three sizes are formed as a result of the multiple start codons, they are: large, middle, and small {Pre-S1+Pre-S2+S, Pre -S2 + S, or S}. Although the function of the protein encoded by gene X is not fully known, it is linked to the development of liver cancer, where it stimulates cell growth-promoting genes while inactivating growth-regulating molecules (Zi *et al*., 2022).

The lipid envelope's cholesterol-rich composition is necessary for viral infectivity (Salimi *et al*., 2020). The nucleocapsid induces an orderly and condensed arrangement of the three different surface glycoproteins: L large (M) middle and (S)small on the envelope membrane during budding from the endoplasmic reticulum ER (Chaturvedi *et al*., 2019). The persistence of the S HBsAg in the serum across a period of more than

6 months is usually trusted as a chronic infection and most commercial assays were designed to recognize S HBsAg (Coffin *et al*., 2019).

Other non-infectious particles, such as enveloped particles without a viral genome, those containing viral Ribonucleic Acid RNA, and envelope-less particles)naked nucleocapsids , are currently known to be formed by infection (Bousali *et al.*, 2021).

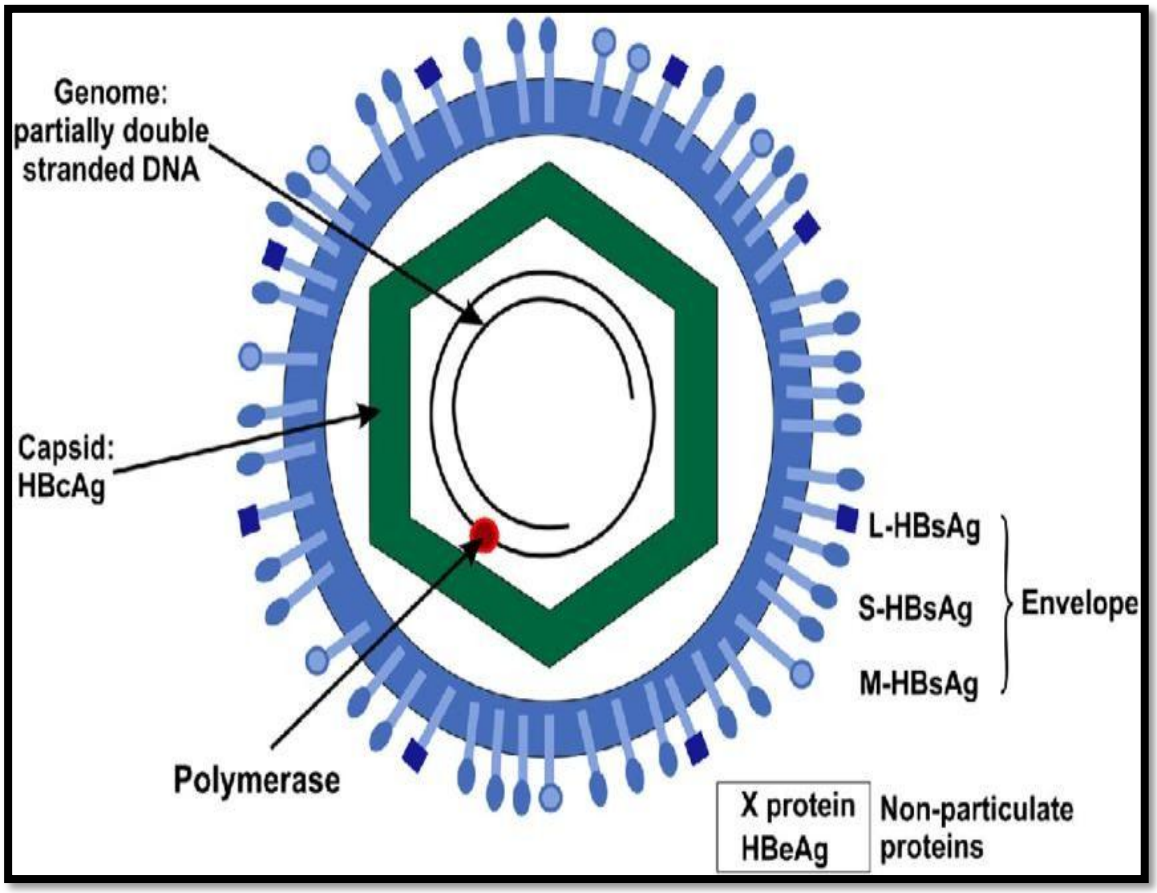


Figure (1-1). Schematic representation of HBV particles. (Tsukuda and Watashi, 2020).

1.1 .5: Structure Of Hepatitis HCV Virus

1.1.5.1: Structure Of The Virion

Once serological identification of HCV and subsequent validation of anti-HCV antibodies was successful, immunogold-labeled HCV was positively identified under the electron microscope. Its smooth lipid-like surface and variable size are consistent with host apolipo particles, leaving little wonder why initial attempts at visual identification failed (Dearborn and Marcotrigiano., 2020). Co-expression of HCV structural proteins in a baculovirus system generated a size- and shape-heterogeneous population of purified particles from which a small set of isometric, 50-nm diameter particles was used to calculate an icosahedral reconstruction. Although limited in resolution (30 Å), the smooth surface was compared favorably to the icosahedrally ordered layer of envelope protein that is tightly associated with a lipid bilayer in related flavivirus structures that have been solved to near-atomic resolution (Dearborn and Marcotrigiano., 2020).

In this model, E1 and E2 together represent the discrete assembly unit, whereas other flaviviruses utilize a single E protein (Dearborn and Marcotrigiano ., 2020; Chmielewski *et al*. 2022).

The core protein was not observed as an icosahedrally ordered structure, suggesting that any nucleocapsid would be randomly oriented relative to the envelope protein as schematically shown in Figure 1-2 A.

This organization is inconsistent with the size and shape heterogeneity observed in that sample, so it might represent a special case within a diverse population. Nonconforming particles may simply represent more random packing of the envelope proteins HCV virions from primary liver cells or Huh-7.5 cells were specifically concentrated onto coated EM

grids via immunocapture (Wang *et al.*,2020; Dearborn and Marcotrigiano 2020).

Extensive size analysis under both negative stain and cryo conditions indicate a continuous distribution of diameters, mostly between 50 and 85 nm, but ranging from 40 to 100 nm. If this size heterogeneity were a result of polymorphism in an icosahedrally ordered system, then the diameter distribution would cluster into discrete modes, each correlated to a T number of quasi-equivalent environments for each assembly unit (Chen *et al.*,2023). As this is not the case, a more flexible model is indicated. The continuously variable size of HCV virions may be a result of variable lipid content

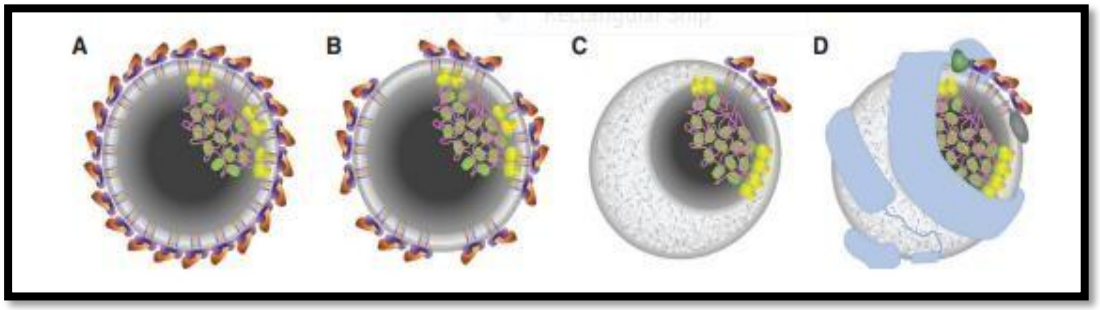


Figure **1.2:** Schematic representations of various models of the hepatitis C virus (HCV) virion.).

(Fig. 1.2 ,C). The density of HCV virions ranges from 1.01 to 1.25 with infectivity associated with particles that have specific gravity of ~1.12 g/mL (Scheel and Rice., 2013).

This further contraindicates an icosahedral envelope lattice as these host proteins would have to displace part of the viral protein lattice and are generally associated with lipoparticles rather than lipid bilayers. Immunocapture of HCV virions from Huh7.5 cells and from patient serum followed by negative stain showed similar internal structures not previously observed (Dearborn and Marcotrigiano., 2020).

Although these particles exhibit a number of staining and desiccation artifacts, absent in previous studies, this desiccation appears to have fortuitously granted stain the access to internal structures. Although particle sizes were not systematically measured, diameters appear to range from 50 to 200 nm in this sample, which may be attributed to RNA and lipid regions (Matlin., 2022). (Fig1.2, D).

The emerging model of the HCV virion suggests that HCV evades the immune system while circulating in the blood by disguising itself as lipoparticles of approximate size and density of very-low-density lipoproteins (VLDLs). This model is consistent with the development of antibodies against apolipoproteins by people chronically infected with HCV (Bridge *et al.*, 2018), the high rate of progression to chronic infection (70%–80%) (Molnar *et al*., 2015), blood contact modes of transmission, and viral use of lipoparticle receptors for hepatocyte targeting(discussed below).

1.1.5.2 : Core

The viral core protein is a 191-amino-acid-long protein (21 kDa) and the first protein in the viral polyprotein. The amino-terminal domain, D1, is ∼120 residues long and condenses with the viral RNA genome to form the nucleocapsid (Fig.(1.3), green and magenta, respectively). Consistent with its RNA-binding function, D1 is nearly 20% arginine, but it is also high in glycine and proline (∼14% each) indicative of an intrinsically unstructured or elastomeric domain (Cheng *et al*., 2010; Dearborn, and Marcotrigiano., 2020). This has been observed experimentally, where recombinant D1 alone is disordered (Moradpour and Penin., 2013).

The carboxy-terminal domain, D2, is ∼50 residues long and associates with lipid droplets and ER membranes. Leucine and alanine account for

30% of the residues in D2, consistent with a hydrophobic domain. The last ∼20 residues serve as a signal sequence (SS) for the next protein, E1, and are cleaved from the core by an intramembrane-cleaving signal peptide peptidase after residue 177, yielding a mature size of ∼19 kDa (Lussignol *et al*., 2016;Dearborn and Marcotrigiano., 2020; Gerold *et al.*, 2020).

The classical model for trafficking a nascent peptide to the ER involves an amino-terminal SS that forms a hydrophobic transmembrane (TM) helix. Briefly, the ribosome translates the SS, which is recognized by the signal recognition particle (SRP), temporarily pausing translation. The SRP binds to its receptor on the ER membrane and threads the SS into the channel of the Sec61 translocon. Translation continues and the SS is proteolytically removed (Ellgaard *et al*., 2016; Karagöz, *et al.*, 2019). The SS for E1 is identified by its hydrophobicity and proximity to the cleavage site, but it is also preceded by the hydrophobic D2 domain, which is predicted to contain another SS between residues 129 and 149. The guided entry of tail-anchored proteins (the GET pathway) was described in yeast (Pécheur *et al.,* 2021).

The Get3 homolog in humans, TRC40, inserts tail-anchored proteins in an ATP-dependent manner, leaving the amino-terminal domain(s) exposed to the cytosol (Guna *et al.,* 2023;Anderson., 2019), and can deliver cargo to the Sec61 translocon (Zheng : Ge., 2022). Although this has not yet been tested for HCV proteins, herpesviruses EBV and HSV-1 have both been shown to exploit TRC40 for localization of their membrane-embedded proteins (Ott *et al*., 2016;Dearborn and Marcotrigiano., 2020).

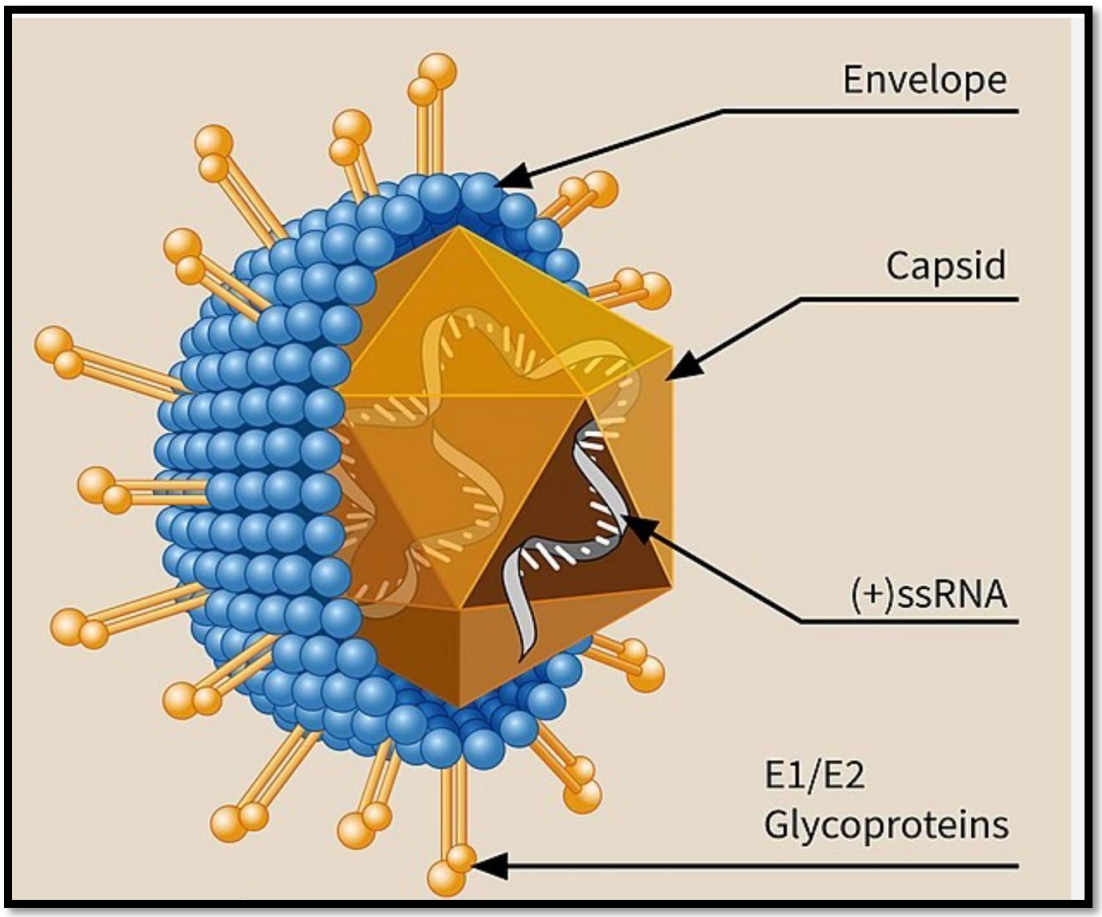


Figure 1.3: Structure and membrane association of core, E1, and E1 proteins (Nouroz et al., 2015).

1.1.5.3 : E1 Envelope Glycoprotein

Viral envelope glycoprotein E1 is 192 amino acids long (21 kDa) after it has been proteolytically cleaved from the viral polyprotein between residues 192 and 383. The carboxy-terminal TM helix of the core protein, residues 178–191, is sufficient to target E1 to the lumen of the ER. The hairpin-helix-sheet of the amino-terminal ectodomain, residues 192–276 , was crystallized as a heavily entwined homohexamerwith two different dimer interfaces. The ectodomain has an amino-terminal hairpin that dimerizes to form one dimer interface, and a helix and three-strandedβ-sheet thatforms (Fung and Liu., 2018; Lavie *et al.*,2018). A carboxy-terminal TM helix serves as the SS for the next protein E2 and then

anchors E1 to the membrane. The intervening half of the protein, residues ∼271–363, is poorly characterized but does contain an absolutely conserved sequence, residues 313–327, which is targeted by a series of broadly neutralizing antibodies (Lu *et al.*, 2018) and a putative fusion loop, residues 265–296 (Banda *et al*., 2019).

1.1.5.4 : E2 Envelope Glycoprotein

The viral envelope glycoprotein E2 is a 363-amino-acid-long (40-kDa) protein, residues 384–746, and is reported to bind to receptors SR-BI and CD81 (discussed below). Like E1, it has an amino-terminal ectodomain (residues 384–661) and a carboxyl-terminal TM helix, although in E2 these features are separated by an amphipathic stem helix, residues 662 on.

The ectodomain includes three hyper variable regions (HVR1, HVR2, and HVR3, corresponding to residues 385–411, 475–481, and 556–580, respectively) to facilitate immune evasion, although HVR1 is disordered in existing structures (Flyak *et al.*, 2018;Deng *et al.*, 2021). Several crystal structures have been determined for most of the E2 ectodomain (Flyak *et al*., 2018), residues 422–591, and all are in good agreement. This portion of the protein adopts an Ig-like β sandwich, stabilized by disulfide bonds, and HVR2 and HVR3 are surface exposed Dearborn and Marcotrigiano., 2020).

1.1.6 : Viral Hepatitis B Replication in the host

The virus enters the cell after binding to a cell surface receptor. The sodium taurocholate cotransporting polypeptide) NTCP (, which is a liverspecific bile acid transporter, has been identified as a cell receptor necessary for HBV entry (Iwamoto *et al.*, 2019;Xu *et al*., 2021). HBV loses its coating and becomes uncoated and being endocytosed in. Since the virus multiplies by RNA made by a host enzyme, the viral genomic

DNA must be transported to the cell nucleus by host proteins called charperones (Ifeanyi., *et al.*, 2018; Payne., 2022).

The viral genome that is partially double-stranded, switched to covalently closed circular doublestranded DNA in the nucleus (cccDNA) All viral transcripts, including a 3.5-kb pregenome RNA, use the cccDNA as a template. The freshly synthesized HBcAg encapsidates the pregenome RNA (Block *et al.*, 2020). The viral polymerase creates a negative-strand DNA copy within the cores by reverse transcription. The polymerase begins the process of synthesising the positive DNA strand, but it does not complete it (Sheraz *et al.*, 2019) Cores bud from the pre-Golgi membranes, acquiring HBsAg-containing envelopes and potentially exiting the cell. Cores may also be reimported into the nucleus and begins a new round of replication in the same cell (Arulselvan., 2015) (Fig.1.4).

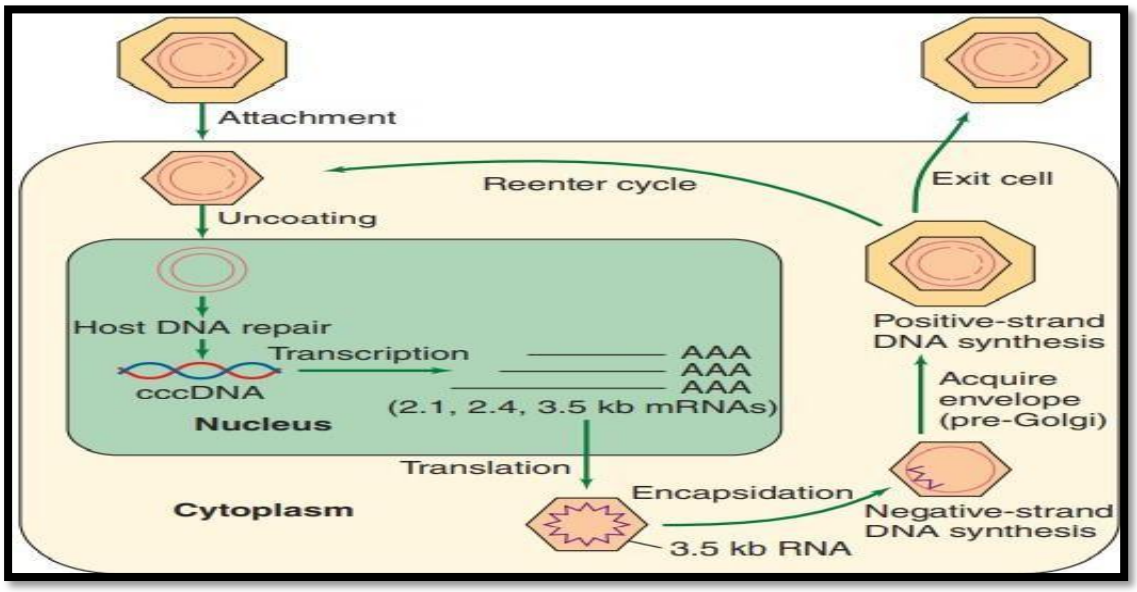


Figure (1.4): Replication cycle of Hepatitis B virus (HBV) (Brooks *et al.*, 2013).

1.1.7 : Viral Hepatitis C Replication in the host

The HCV life cycle is only partly understood; difficulties in establishing an in vitro model of replication and the complex network of cell surface molecules used to 19 mediate viral entry have delayed comprehension of various molecular mechanisms (Hoenen *et al*., 2019;Romero-López and Berzal-Herranz., 2020).

It includes several steps, Firstly, the virus life cycle starts with the entry of the virus particle into the host cell via several membrane proteins, such as the tetraspanin protein CD 81, glycosaminoglycan (GAG), Claudin-1 (CLDN1) and scavenger receptor class B type I (SR-B1), thought to be involved in entry of virus. HCV entry likely involves transit through an endosomal, low pH compartment and fusion with the endosomal membrane (Grassi *et al.,* 2016). After that, the viral genome, of about 9.6 kb, is released from the virion via membrane fusion and uncoating. This is followed by translation of the viral genome using the host cell machinery, producing one large polyprotein of about 3000 amino acids at the endoplasmic reticulum (ER) membrane.

The polyprotein is cleaved by viral and host proteases into at least 10 proteins. The genomic order of the proteins being: Core, E1, E2, p7, NS2, NS3, NS4a, NS4b, NS5a, and NS5b. The next step is viral genome synthesis, called replication, which occurs on ER-derived membranes. The readily produced genome is packaged into a new virion, a process associated with lipid droplets. Subsequent to virus particle assembly, the virion is released from the cell and can infect new hepatocytes (Wahyuni *et al*., 2016). The virus replicates in peripheral blood mononuclear cells (PBMCs) and may be responsible for high levels of immunologic disorders in chronic hepatitis C patients. It is estimated that each infected

cell produces 50 virions daily. But Virus replication occurs mainly in hepatocytes (Mahmud *et al.*, 2018;Shakeri *et al.*, 2013).

1.1.8 : Pathogenesis of Hepatitis B Virus

HBV encodes polypeptides from four ORFs that trigger corresponding immune responses during acute and chronic infections. When these responses are rapid, strong and multi-specific, acute, resolving infection can be achieved (Zaki *et al.*, 2022). When these immune responses are weak and of limited specificity (against few virus epitopes), the carrier state may develop (Michalak., 2020).

Although the pathogenesis of HBV is variable in different hosts, the virus encodes proteins that blunt innate immunity, and as a consequence, adaptive immunity is not triggered at all or to a limited extent (Ochando *et al.*, 2023). The latter causes liver damage over many years without eliminating the virus. Even though available treatments suppress virus replication, none are curative, and the persistence of viral cccDNA sustains infection (Fanning *et al.*, 2019). Production of HBx regulates virus gene expression and replication, but over time, increased integration of HBV DNA fragments encoding HBx results in high levels of HBx expression that epigenetically alter the expression of numerous host genes that up- or down-regulate HBV replication and impact disease activity (Schollmeier *et al*., 2023). For example, HBx activation of AKT decreased HBV replication, but this was accompanied by an inhibition of apoptosis, suggesting that HBx balances HBV replication and cell survival by stimulating signaling that enhance hepatocyte survival at the expense of higher levels of HBV replication (Piracha *et al*., 2018).

The generation of free radicals by immune responses against virus infected cells, combined with HBx mediated alterations in mitochondrial

function, promote HBx activity. These events result in the activation of signaling pathways (Menegazzi *et al.*, 2020). that over-ride apoptosis and/or directly block the activation of critical caspases, so that whether HBx stimulates or block apoptosis depends upon whether the liver is experiencing inflammation and oxidative stress (Feitelson., 2018). It also depends upon whether HBx is being expressed in normal hepatocytes, where apoptotic pathways could be triggered, or whether HBx is expressed at high levels in cells where apoptotic pathways are compromised. In addition to being pro-inflammatory, activated NF-κB protects infected cells against immune elimination (Medhat *et al.*, 2021).

Thus, the dichotomy of HBx activity may be a reflection of the environment wherein HBx is expressed. Importantly, the epigenetic mechanisms whereby HBx regulates virus replication also have an impact on cell growth and survival, and many of these same alterations in host gene expression are also hallmarks of cancer, which may explain why there is such a high risk of HCC among carriers with CLD (Nalesnik and Michalopoulos., 2012). The common denominator is that many of the pathways and molecules that support HBV gene expression and replication also protect infected cells from elimination, and contribute centrally to malignant transformation (Zheng *et al*., 2020).

1.1.9 : Pathogenesis of Hepatitis C Virus

The liver is an extraordinarily resistant organ with a unique regeneration capacity, but the persistent stress induced by chronic inflammation and deregulation of signaling**,** Inflammation is a life-preserving process to maintain cellular homeostasis. It is mostly activated in response to pathogens or tissue injury and is part of a physiological

recovery response. The liver harbors a large spectrum of immune cells distributed within the hepatic compartments (Causey *et al.* 2018).

This organ is constantly exposed to external signaling from commensal molecules and produces a series of neo-antigens derived by its metabolic activities. This leads to the development of a constant and physiological immunotolerance state in the organ (Jenne and Kubes., 2013), which was first recognized by Calne and coworkers in 1969 (Calne *et al,.* 1969). The relative immunotolerance in the liver is necessary to avoid over activation of the immune system but it also facilitates the adaptation and persistence of different liver pathogens, such as malaria, hepatitis B virus (HBV), and HCV (Horst *et al.*, 2016). HCV has developed several strategies to evade the innate and adaptive antiviral responses to infection (Gale and Foy 2005; Rosen 2013). Consequently, failure of viral clearance promotes a chronically inflamed liver that leads to scarification (fibrosis), cirrhosis, and ultimately provokes the development of HCC. According to the World Health Organization most of the HCV-infected patients do not achieve viral clearance and 60%–80% develop chronic hepatic inflammation. In these patients, the risk of developing cirrhosis is ∼15%–35% after 20–30 years of infection (Thrift, 2021). The virus directly accelerates the inflammatory response through a large range of interconnected mechanisms, including pathogen pattern recognition, host–viral protein interactions, activation of inflammasomes, and reactive oxygen species (ROS) production (Gale and Foy 2005; Horner and Gale 2013; Ivanov *et al*., 2015). Liver diseases and fibrosis associated with HCV infection evolve in the context of a strong oxidative microenvironment. HCV core, E1, E2, NS3, NS4B, and NS5A are known to encourage the production of ROS (Bureau *et al,*. 2001; Pal *et al*., 2010; Ivanov *et al*., 2011). The antioxidant defense machine involves different

ROS scavenging enzymes and their synthesis depends on many genes commonly regulated by the transcription factor NF-E2-related factor 2 (Nrf2) (Bureau *et al.*, 2001). Nrf2 expression is inversely correlated with the severity of liver injury in chronic HCV patients and is impaired in end-stage liver disease (Kurzawski *et al.*, 2012; Jiang *et al.*, 2015). In HCV-positive cells, free Nrf2 is trapped at the replicon complexes and is therefore prevented from its entry into the nucleus (Medvedev *et al.*, 2017). This observation is in line with impaired expression levels of antioxidative enzymes like catalase (Lupberger *et al.*, 2019) and superoxide dismutase SOD1 (Levent *et al.*, 2006; Diamond *et al.*, 2012) in infected hepatocytes, which further promote oxidative stress damaging host proteins, lipids, and DNA.

The levels of liver and blood cytokines are associated with HCV microenvironment and liver fibrosis (de Souza-Cruz *et al.*, 2016). In particular, interleukin (IL)-1α is increased in HCV patients and correlates with liver cirrhosis and HCC (Tawfik *et al.*, 2018). Therefore, HCV-induced cytokine signaling increases the oncogenic pressure within the host cell and contributes to a recalibration of hepatocyte functions.

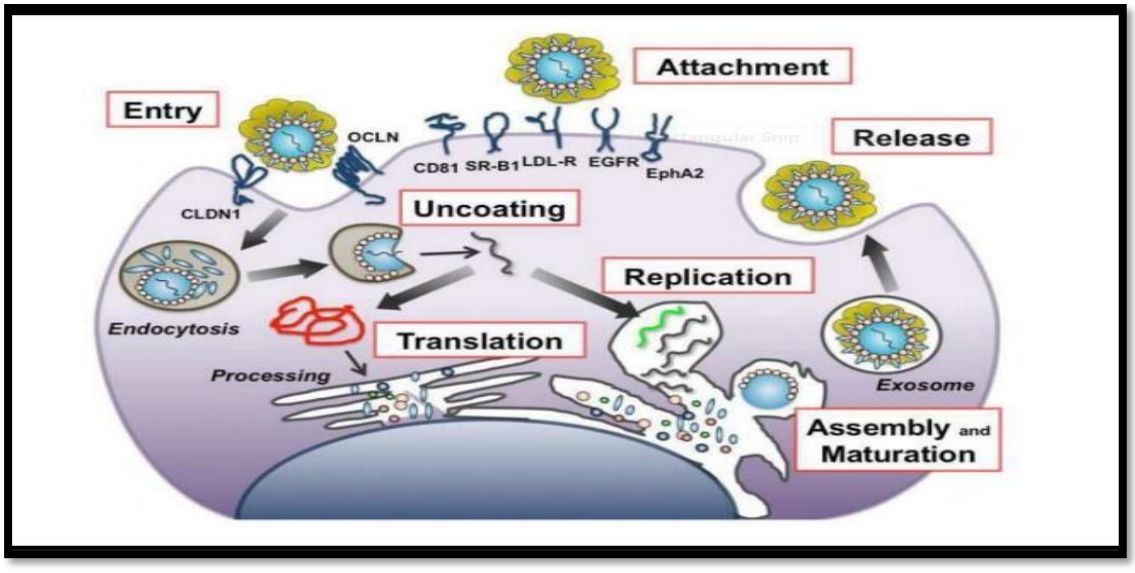


Figure 1.5: **Pathogenesis of Hepatitis C Virus** (Dustin et al.,2016)

1.1.10 : Clinical sign of Acute and Chronic Hepatitis B virus

HBV infection causes chronic hepatitis and increases the risk of liver cirrhosis and hepatocellular carcinoma, which affects the health of people around the world. About 1% of hepatitis B patients progress to liver failure every year. Acute-on-chronic liver failure (ACLF) is a clinical manifestation of acute liver decompensation on the basis of chronic liver disease, which is mainly induced by factors such as infection, alcohol, hepatotoxic drugs . The pathophysiological process of ACLF includes systemic inflammatory response syndrome, inflammation and oxidative stress. Thus, ACLF is a serious threat to human health, and its prognosis is extremely poor, with high mortality (Cheng *et al*.,2022). The most obvious clinical symptoms of hepatitis are nausea, fever, jaundice, vomiting, right upper quadrant discomfort, hepatomegaly, changes in stool colour, dark urine and other unspecific constitutional symptoms (Dimmock *et al.*, 2016;Gupte., 2019).

HBV DNA can usually be identified one to two weeks after exposure, and patients usually have no symptoms and only slightly elevated serum alanine aminotransferase (ALT) levels during this period. HBsAg and HBeAg are the first serological HBV markers that can be detected (Sheena *et al.*, 2022). Fulminant hepatic failure is extremely rare.

The causes and risk factors for fulminant HBV are not quite understood (Gish *et al.*, 2015; Schillie *et al.*, 2018). This may be linked with co-infections with other viruses or substance use. Massive immune-mediated lysis of infected hepatocytes is thought to be the cause of fulminant HBV (Piaserico *et al.*,2019). This is why many fulminant HBV patients have no indication of HBV replication at the presentation (Hyde's., 2015). In adults, the risk of developing fulminant HBV is less than 1%, (Khan *et al.*, 2022). Chronic HBV infections are described as

the presence of HBsAg in the serum of an infected person for at least six months or the presence of HBsAg in a patient who is negative for immunoglobulin (Ig) M antibodies to HBcAg (Wang *et al*., 2019).

Around 90% of babies infected perinatally with HBV and 30% of children horizontally infected with HBV develop chronic infections, on the other hand, only 5-10% of adult infections convert chronic (Kennedy *et al*., 2017). As a result, a physical examination would be normal in the vast majority of cases. Jaundice, peripheral edema, ascites, and encephalopathy, can be present in patients with decompensated cirrhosis (Angeli *et al*., 2018).

There are numerous typical patterns of chronic hepatitis B (CHB) acquired in adult or later childhood: first, infection with a wild type HBV variant, with high HBV DNA, HBeAg positivity, high ALT, active liver disease and there is the classic necroinflammatory state (Mitra and Leonard., 2018). Second, infection with a procure mutant virus, which has recently become much more widespread than wild-type virus (Boyer and Lindor., 2016). Despite significant HBV DNA replication and elevated ALT, HBeAg is negative after infection with a precore mutant virus. Third, there is a low or non-replicative period, in which serum ALT is normal, anti-HBe antibodies are common, HBeAg is negative, and HBV DNA is low or undetectable, this situation is described by the partial immune control of HBV infection (Xi *et al* ., 2021).

1.1.11 : Clinical sign of Acute and chronic of Hepatitis C virus

HCV in a matter of days to eight weeks. Approximately 6–12 weeks after exposure, aminotransferases become elevated (Terrault *et al.*, 2016). A clinical characteristic of acute hepatitis C is jaundice (icteric), which is present in less than 25% of infected people, and the majority of

cases are asymptomatic. Symptoms include fatigue, fever, poor appetite, nausea, weakness, and right upper quadrant discomfort are similar to those seen with other types of acute viral hepatitis (Ferreira-Gonzalez and Shiffman., 2004). Antibodies to HCV are first detected about 8 weeks after exposure, though ELISA testing may take several months in some patients (Boyer and Lindor., 2016).

The infection generally lasts 2–12 weeks in patients that have such acute hepatitis symptoms (Goicoechea., 2018). In around 40% of cases, aminotransferase levels will normalize along with the clinical resolution of symptoms. The loss of HCV RNA, which means hepatitis C cure, occurs in less than 20% of patients, regardless of aminotransferase normalization. The incidence of fulminant hepatic failure as a result of acute HCV infection is extremely rare, possible that it's more common in people who have underlying chronic HBV infection (Papatheodoridis *et al*., 2018).

Genetic diversity of HCV and rapid mutation rate can enable HCV to escape immune detection. HCV infection in childhood appears to be associated with a 50% to 60% lower risk of chronic infection (Dimmock *et al.*, 2016). Eventually, there seem to be ethnic variations with a lower risk of chronicity in special populations, which may be explained in part by differences in host genotypes (Petruzziello *et al*., 2016 a).

1.1.12 : Epidemiology of HBV and HCV

Viral hepatitis is responsible for an estimated 1.4 million deaths per year (Jefferies *et al.*, 2018). HBV and HCV are responsible for about 90% of these mortality, whilst the remaining 10% of mortality are caused by other hepatitis viruses (Soriano *et al.*, 2022). In 2015, an estimated 257 million people globally were diagnosed with chronic HBV infection and

71 million people with chronic HCV infection, in the same year, chronic viral hepatitis caused 1.34 million deaths (Chen., 2018).

HBV and HCV transmission take place mostly through direct blood contact, blood transfusions and/or blood products, through the use of intravenous medications, and through sexual intercourse (particularly in HBV transmission).(Tarky *et al.*, 2018), showed the pattern spread of hepatitis in the urban more than the rural, this may be due to the people of urban more referred to the hospitals and thus exposure to the risk of infection due to contact with contaminated tools.

According to the World Health Organization, Iraq has a low endemicity for HBV and HCV (Kadhem *et al*., 2019), 3674 cases of HBV and 929 cases of HCV were identified out of a total population (37140000) Iraqi people (WHO.,2016;Othman and Abbas 2020) Hepatitis B in Saudi Arabia estimated to be about 1.3% among population (Aljumah *et al.*, 2019). The prevalence rate of anti-HCV among all Saudi nationals was estimated to be 0.7 % (Aljumah *et al.*, 2016).

In Iran, among the general population, the prevalence of HBV infection is around 2.2 % (Salehi-Vaziri *et al*., 2016), while anti-HCV was found in 0.3 % of the low-risk population (Mahmud *et al*., 2018). The prevalence of HBV in the general population in Turkey is differs according to the region) 2–8 %(and rises from west to east (Kahraman *et al*., 2018).

The prevalence of HBV in Pakistan 4.5% (Kumar *et al*., 2020) and HCV 4.8% (Asnake., 2017;Othman and Abbas., 2020). From African countries Sudan, which have the proportion of the population infected with HBV 5%-8.2% (Badawi *et al*., 2017), while HCV is ranging between 2.2% to 4.8% among general population (Yu. *et al*., 2020).

Interestingly, prevalence of HCV in Egypt 15% (Sievert *et al.*, 2011). Mass screening for hepatitis B and C in Southern Upper Egypt were positive for anti-HCV among 14.5 % and for hepatitis B surface antigen (HBsAg) among 4.4 % (Soliman *et al.*, 2019).

Unfortunately, about 65% of patients infected with HBV and 75% infected with HCV are unaware of the infection (Jefferies *et al.,* 2018). About 100 million people in the South-East region of Asia are infected with chronic HBV (Giles-Vernick *et al*., 2016 ;Jefferies *et al.,* 2018), and 10 million with HCV . On the other hand around 6.1 % of the African people have HBV infection and about 18 million souls have chronic HCV infection (Songtanin and Nugent., 2022).

While The prevalence of HBV on the European continent is low, but it's rising eastward (Ishizaki *et al*., 2017). On the other hand , around 7-9 million people in the Caribbean and Latin American countries are HCV-positive and an estimated 0.7% of population were afflicted with hepatitis B (Jefferie *et al.*, 2018). The estimated prevalence of HCV in the whole American continent is 1.3% (Petruzziello *et al.*, 2016). Nationally in United States , hepatitis C prevalence estimated during 2013-2016, 1.3 % among males and 0.6 % among females (Bradley *et al.*, 2020), while the prevalence of all HBV infection during 2015–2018 was 4.3 % (Kruszon-Moran *et al.*, 2020).

1.1.13 : Diagnosis of HBV

There are several methods used to diagnose HBV mainly serological tests that used to detect HBV serological markers; HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and anti-HBc-immunoglobulin M (IgM) are HBV serological markers. HBV infections are indicated by the presence of HBsAg. Anti-HBs is a protective antibody that indicates immunity to HBV (Song., 2016).

It is found in patients who have had their hepatitis B infections resolved and in people who have been inoculated with the hepatitis B vaccine. Patients with hepatitis B chronic reactivation are more likely to have anti-HBc-IgM positivity. An immunoglobulin G (IgG) antibody is the most common antiHBc antibody, it is positive in most cases as long as people are infected with HBV, regardless of whether the virus is eliminated or not (Mulakoli., 2021). Quantitation of HBsAg in the blood can be used to predict disease progression, antiviral effectiveness, and prognosis (Deng *et al.,* 2022).

The real-time quantitative Polymerase chain reaction (RT-PCR) used to detect HBV DNA, genotype, and mutation (Kim *et al.,* 2023). HBV DNA quantification is primarily used to assess the extent of viral replication in chronic HBV infections, it's also used to select antiviral therapy indications and assess efficacy (Mak *et al.,* 2020).

Because of its high sensitivity and accuracy, the real-time quantitative PCR approach is recommended (Hou *et al.,* 2017;Tahamtan and Ardebili., 2020). Several biochemical testes which in majority used to monitoring hepatitis diseases mainly including Serum ALT, AST, and serum bilirubin (Akter *et al.,* 2021). Serum ALT and AST levels are the most widely used markers for show degree of hepatic cell injury (Shibabaw *et al.,* 2019). Hepatic cell damage, intrahepatic and extra hepatic biliary tract obstruction and hemolysis are the most common causes of elevated serum bilirubin (Fargo *et al.,* 2017). Patients with hepatic failure may experience a gradual rise in serum bilirubin levels, with an increase of more or equally 1 time upper limit of normal (ULN) per day, and a divergence phenomenon (i.e. bilirubin elevation and ALT and AST decrease) may occur (Hou *et al.,* 2017;Schneider *et al.,* 2021).

1.1.14 : Diagnosis of HCV

HCV infection can be diagnosed and monitored using a variety of virological tools (Chevaliez and Pawlotsky., 2018). Serologic assay for anti-HCV immunoglobulin is ELISA. Anti-HCV antibodies are currently observed in serum or plasma using thirdgeneration enzyme-linked immunosorbent assays (Qin *et al*., 2020). These tests are specific and sensitive, and they can be completely automated and are inexpensive. The time between infection and seroconversion when anti-HCV antibodies become detectable is variable, varying between 2 and 8 weeks on average "therefore, checking for anti-HCV antibodies alone can miss early infection".

Anti-HCV antibodies remain in chronically infected patients (Chevaliez *et al*., 2012). Additional anti-HCV antibodies will last for years or even decades in patients who have cleared the virus (Pallarés *et al*., 2018).

HCV RNA detection and quantification are useful for diagnosing active infection, HCV genotype and (in the case of genotype 1) subtype determination is required (Saludes *et al.,* 2020), to guide treatment indications (the treatment regimen, the addition of ribavirin, and the duration of therapy), and detecting drug resistance in patients receiving DAAs (Manns *et al*., 2017). HCV RNA detection and quantification are based on real-time PCR or transcription-mediated amplification methods, which are both specific and sensitive (Pawlotsky *et al*., 2015;Manso *et al.*, 2023).

The HCV core antigen, which consists of epitopes expressed on the HCV nucleocapsid protein, is a surrogate marker for HCV replication that can be identified and quantified in the patient's blood (Manns *et al.*,

2017). As a result, HCV core antigen is cost-effective alternative to HCV RNA assays for diagnosing infection and evaluating sustained virological response (SVR) to antiviral therapy. This test, however, is not commonly used in clinical practice (Wang *et al*., 2021). An automated enzyme immunoassay is used to detect and quantify HCV core antigen. The lower limit of detection is 500–3,000 HCV RNA IU per ml, depending on the HCV genotype (Manns *et al*., 2017;Chevaliez., 2019).

1.1.15 : Prevention

Blood screening, avoid transmission from Mother to child , A vaccine is available and is advised for those at risk or in contact with the disease. The vaccine is 80% to 100% effective in preventing hepatitis B and the chronic form of the disease. Seventy-nine percent of World Health Organization members have adopted the universal childhood hepatitis B vaccination policy (Ousmane., 2019) . Unfortunately, the cost of the vaccination and the simple means of transmission of this virus mean that the overall incidence of hepatitis B infections continues to rise. People who know they are infected carriers of the hepatitis B virus can take precautions to prevent infecting others. These include the appropriate disposal of contaminated waste, using separate toothbrushes and scissors, never sharing needles and syringes with others, always using condoms, and avoiding anal intercourse. All people infected with hepatitis B should get a hepatitis A vaccine to prevent potential infection.(Jerry Kennard. 2023).

1.1.16 : Immunity to Hepatitis B virus

1.1.16.1 : Innate immune responses and hepatitis B infection

Chronic hepatitis B virus (CHBV) infection is a significant health problem with a projected 240 million carriers worldwide. Around half a

million people die each year because of the advancement of the infection to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Vyas *al et*., 2021). This is a bigger problem for Asia as the continent contributes to the majority of the global HBV infections.

In the past 2 decades, HBsAg seroconversion is rare, and the pool of cccDNA in the nucleus is a major reason for the persistence of HBV. The CHBV patients showed an impaired immune profile (Vyas *et al*., 2018;Lazarevic *et al.,* 2019), especially dysfunctional HBV-specific CD8+ T-cell responses (Heim *et al.,* 2021). T follicular helper (TFh) cell is a subset of the helper T-cell population and has shown a crucial role in the seroconversion of HBe and HBsAg in CHBV-infected patients (Vyas *et al.*,2018;Islam *et al*., 2023).

The group showed that HBV and TFh cells have also emerged as an essential player in preventing viral expansion within the host and involved in HBeAg (Wang *et al.*,2018).

The fate of HBV infection is a result of a key equilibrium between viral replication and host immunity (Valaydon *et al*., 2016). Most individuals who get HBV infection in adulthood develop icteric acute hepatitis due to vigorous immune response against viruses resulting in self-limiting illness (Kamar *et al.,* 2017). Less than 1% of them develop acute liver failure requiring intensive monitoring and early liver transplantation (Tomescu and Popescu., 2022).

Virological factor and host immune responses govern one of the 3 possible outcomes of the HBV infection: (1) immune tolerance, (2) immune activation, and (3) inactive carrier stage. However, there are abundant similarities and interindividual inconsistencies in these stages. According to (Lampertico *et al.*, 2017) guidelines, "CHBV infection can be classified into 5 phases (I) HBeAg-positive chronic infection, (II)

HBeAg-positive chronic hepatitis, (III) HBeAg-negative chronic infection, (IV) HBeAg-negative chronic hepatitis, and (V) HBsAg-negative phase"((Lampertico *et al.*, 2017). On the basis of the presence and disappearance of HBV antigens (HBsAg, HBeAg, and HBcAg) and antibodies (anti-HBs, anti-HBe, and anti-HBc), different phases of CHBV are described.The immune tolerant phase is characterized by HBeAg positivity (Ghany and Gara., 2017), high HBV DNA levels, normal or low levels of alanine aminotransferases (ALT), and minimal liver necroinflammation hardly leading to any progression to fibrosis (Bousali *et al.*, 2021). However, due to sequential accumulation of HBV virions or mutation in the core promoter, few patients develop "HBeAg-negative CHB infection" which is also characterized by inconsistent levels of ALT and HBV DNA >2,000 IU/mL but with active hepatitis (Elalfy *et al.*, 2020).

The immune tolerant phase is followed by the "immune active" phase, characterized by HBeAg positivity, high serum HBV DNA levels, persistent or intermittent increase in ALT levels, moderate or severe liver necroinflammation, and more progression to fibrosis (Kawanaka *et al.*, 2021). The extent of liver damage is directly related to the duration of this phase. The immune active phase ends with the seroconversion of HBeAg and the formation of anti-HBeAb ,The immune active phase is followed by "immune inactive HBV carrier state" which can be identified by very low or undetectable serum HBV DNA levels (below 2,000 IU/mL) and normal ALT (Vyas *et al.*, 2021). The disease remains stable in this phase due to a very low but ongoing risk of liver-related complications. The spontaneous HBeAg clearance rate varies from 3 to 12% annually, but a rate of HBsAg seroconversion is very rare, only ∼1% (Choi *et al.*, 2022). Even after the disappearance of HBsAg, cccDNA still persists in the liver

but with a very little risk of further progression of liver disease (Durand and Francoz., 2017).

1.1.16.2 : Adaptive Immune Responses and Therapy

In adaptive immunity, CD8+ T cells are considered as one of the most important players in HBV infection, which act through cytolytic or noncytolytic mechanisms via the secretion of IFN-γ and TNF-α (Amin *et al*.,2021). However, defective virus-specific responses by CD8 cells have been observed in CHBV patients (Schurich *et al.,* 2016). Even in the presence of profound DC activation and antigen presentation, HBVspecific CD8+ T-cell exhaustion under continuous exposure to HBV antigens is a major challenge to clear the virus. Regulatory T cells, which are known for downregulation of inflammatory immune responses and maintaining the immune homeostasis, have been shown to fail during chronic HBV infection (Barili *et al*.,2021). Regulatory T cells maintain immunosuppressive functions by secreting the inhibitory cytokines such as TGF-β, IL-10, and IL-35 and suppress the activation of antigen-specific or nonspecific proliferation in most of the function of the immune cells (Ohue and Nishikawa., 2019).

1.1.17 : Mechanisms of HBeAg and HBsAg Seroconversion

B cells do not have critical roles in the elimination of viral particles directly (Hudu *et al*.,2020). However, B- and TFh-cell responses have been shown to play an important role in preventing viral expansion within the host and also the main players in HBe or HBsAg seroconversion. TFh cells reside in the secondary lymphoid organ and help in the B-cell differentiation through the secretion of IL-21(Vyas *et al.,* 2021). TFh cells express distinctive markers, including the transcriptional repressor Bcl6, the chemokine, and the costimulatory molecules such as CXCR5,

ICOS, and CD40L Translocation of TFh cells toward the CXCL13-rich germinal center is mediated by the CXCR5 expression (Stebegg *et al.*, 2018).

TFh cells have been emerged as a crucial player in B-cell differentiation to form antibody-producing plasma cells that offer lifelong protection (Lindgren *et al*., 2017;Elsner and Shlomchik., 2020). It is observed that the absence of TFh cells at the onset of infection reduces the overall humoral response and prevents viral clearance (Milne *et al*., 2021). High-affinity class-switched antibodies are essential in clearing and establishing long-lasting humoral immunity against HBV infection and effective vaccination. It is observed that in response to HBV vaccination, antibody secretion was influenced by genetic polymorphism of CXCR5 and CXCL13 (Chung *et al*.,2020).

1.1.18 : The Role Of TNF In Hepatitis B Virus

TNF is crucial to viral clearance. A low level of TNF is associated with a weak T cell response and subsequently results in failure to clear HBV (Chyuan and Hsu., 2018). However, it is highly likely that TNF alone cannot clear HBV but works in conjunction with other mediators.

In the tree shrew model, TNF could successfully suppress HBV replication but there was persistence of cccDNA (Kuipery., 2022). There are multiple pathways by which TNF exerts antiviral activity. First, as demonstrated in cell culture, TNF decreases viral entry into the hepatocyte (Fanning *et al*.,2019).

1.1.19 :TNF in chronic hepatitis C Virus

TNF-α pathway in chronic HCV infection The role of TNF-α in chronic HCV infection is not well understood. Serum levels of TNF-α and its

soluble receptors (sTNF-R55 and sTNF-R75) are significantly higher in HCV-infected patients than in healthy subjects (Pompili *et al.*,2013).

Serum levels of TNF-α correlate with serum transaminase levels, histological activity and fibrosis, but not with serum HCV RNA levels or viral genotype (Monaghan 2019). Laboratory studies have indicated that the HCV core protein has the potential to inhibit the TNF-α-mediated apoptotic signaling pathway (Chen *et al.*, 2015), providing a selective advantage for HCV replication and avoidance of the host antiviral defense mechanism (Mahajan *et al.*, 2021).Thus, further suppression of TNF-α by biological drugs may pose a potential threat of excessive viral replication and worsening of chronic HCV infection. In contrast, some studies have postulated that the baseline overexpression of TNF-α is associated with reduced cell capability to respond to IFN signaling and, consequently, to reduced viral clearance (Kuipery., 2022).

1.1.20 : The Importance of HBeAg and HBsAg Seroconversion in Hepatitis B Infections

Seroconversion of HBeAg and HBsAg is critical for controlling the pathogenesis of CHBV infection, whereas HBeAg seroconversion is an indicator of inhibition of viral replication, and HBsAg seroconversion is considered the functional cure of the disease. HBeAg is considered the marker of replication and seroconversion of HBeAg, and the formation of anti-HBeAb is a critical incident in the history of CHB infection. Anti-HBeAg formation or seroconversion of HBeAg is associated with the reduction of HBV DNA. While spontaneous HBeAg seroconversion rate has been described to be between 8 and 15% (Li *et al.*,2019).

Earlier seroconversion of HBeAg in CHB patients is found to be associated with an increased chance of sustained remission, as well as

slower pathogenesis of the liver disease (Umemura *et al.*, 2022). There are many factors such as sex, age, and the degree of liver disease that can affect the rate of HBeAg seroconversion. Patients with older history, female carriers, and subjects with the ALT levels higher than 5 times the upper limit of normal are more likely to clear HBeAg. Peg-IFN-α therapy results in sustained HBeAg seroconversion rates of up to 32 and 48%, respec tively, when assessed at weeks 24 and 48 after treatment. In contrast, HBeAg seroconversion rates have been considerably lower (12–15%) with nucleotide analogues (Vyas *et al*., 2021).The clearance of HBsAg is found to be associated with minimal risk of disease progression such as cirrhosis, decompensation, and HCC (Moini and Fung 2022).

1.1.21 :The Role of CD4 Cells in Inducing HBeAg and HBsAg Seroconversion

The presence of HBeAg and HBsAg is considered as a marker of HBV replication and infection. Therefore, the secretion of antibodies against the HBV antigen, specifically HBe and HBsAg, is considered a favorable outcome of CHBV infection. Collected evidence has believed the liver worked like other secondary lymphoid organs, and it supports the priming of naïve T cells for differentiation of effector T cells (Vyas, A. K. *et al*.,2021). studies using animal models of HBV have demonstrated that the immune response depends upon the age, and young age is found to be associated with effective immune stimulation and antiviral immunity (Tsai, K. N *et al*. 2018).

Furthermore, the hepatic lymphoid structures had the ability to take care of the differentiation as well as the maturation of B-cell-mediated responses and the priming of T cells to become TFh cells (Fridman, W. H. . *et al.*,2022).

Other studies also supported indirectly where they showed that the disappearance of HBeAg is linked with higher CD4+ T-cell responses in HBeAg-positive patients after adefovir dipivoxil treatment(Cooksley H *et al*.,2008).

1.1.22 : UTR Gen

HCV is mainly transmitted through transfusion of infected blood and blood products. After transmission, the virus may remain in latent phase due to suppression by host immune system (Kumar *et al*., 2018).

However, during its infectious phase, the replication of HCV is very robust, and around 10 trillion virion particles can be produced per day (Kumar *et al*.,2018). HCV is an RNA virus and its genetic diversity is constantly evolving due to rapid globalization, which ultimately results in varying therapeutic response in different geographical regions (El-Mowafy *et al*., 2021). On the basis of genomic variability, HCV is classified into seven major genotypes and 67 subtypes (Xu *et al*., 2022; Eltayeb *et al.,* 2022). In India, HCV is responsible for acute viral hepatitis in up to 21 per cent (Saba Afzal Shaikh *et al*.,2022). and chronic liver disease in 14-26 percent cases of HCV infections (Jelicic *et al*., 2022). Epidemiological studies have shown that the persistence of HCV infection is a major risk for development of hepatocellular carcinoma (HCC) (Zaki *et al*., 2022). HCC is the fifth most common cancer and a major cause of death in patients with chronic HCV infection and responsible for approximately one million deaths each year (Islam *et al*., 2022).

HCV is a lipoprotein-enveloped ribovirus with a 9.600 nucleotide 5′–3′ UTR (Ullah *et al*,.2021). The RNA genome has an untranslated region (UTR), three structural (core, E1, E2) and seven nonstructural genes (p7, NS2-NS5) (Dash *et al*.,2019). the nonstructural protein (NS5B) is a

moderately variable region and is commonly used for HCV subtyping (Heuss *et al* .,2022). In terms of primary sequence and secondary structures, the 5′ and 3′ UTRs are the most conserved areas of HCV RNA. the 5′ UTR contains reasonably variable areas inclusive of NS5A, which codes for a nonstructural protein (Ullah *et al*,.2021).

The 5′ UTR consists of 341 nucleotides, and due to its 90% sequence identity, it is commonly used for genotype identification. The 5′-UTR stem loop structure contains entry sites (IRES) (Wang *et al*.,2022). Mutations do not usually occur in the 5-UTR, and sometimes compensatory mutations are developed to preserve the base-pairing shape and conserve the structural characteristics associated with translation efficiency (Leppek *et al.*, 2018). Recently, it has been found that the first 145 sequences of the 5′-UTR play a significant role in the replication of HCV RNA (Dai *et al*.,2022). NS5A also has inadequate natural amino acid variability, which conserves its useful characteristics in vivo (Li Petri *et al.*, 2022). Other articles on sequencing substantiate the 5′ UTR (324–341 nucleotides long) as the least mutated region in the HCV genome and describe it remaining conserved in all HCV genotypes (Ullah *et al*,.2021).

Chapter Two

Materials and Methods

Materials and Method

2.1.Patients Specimens

2.1.1.Study Design

The study was approved by the Ministry of Health, Thi-Qar Health Department according to a special model prepared for this purpose. Patients' consent was also taken before starting sampling with a clear explanation for the purpose of the study. The study done in a Al-Hussein Teaching Hospital infectious diseases department the main blood bank of Thi-Qar province, Al-Mousawi Private Laboratory in AL Nasiriya city and Marjan Medical city in Babylon province for sequence .

105 samples were collected, 85 sample of patients were diagnosed with the hepatitis virus (48 patient with hepatitis C and 37 patient with hepatitis B) based on the clinical signs of the disease and serological test result, 20 sample of healthy control for TNF during the period from July 2022 to November 2022 . The ages ranged between 20 and 80 years.

2.1.2. Sample Collection

Three ml of venous blood was taken from patients and the control group collected in gel tubes and left to clot at room temperature, then centrifuged at 3000 rpm for 10 minutes to separate the serum, where 40 µl of serum used for each detection of antibodies of HCV and HBsAg of HBV by ELIS 80ml of serum used for immunological test for estimation of HBeV and TNF-a by ELISA and the remaining serum sample was separated in 1.5 ml eppendorf tubes and frozen at -20˚C until used for PCR .

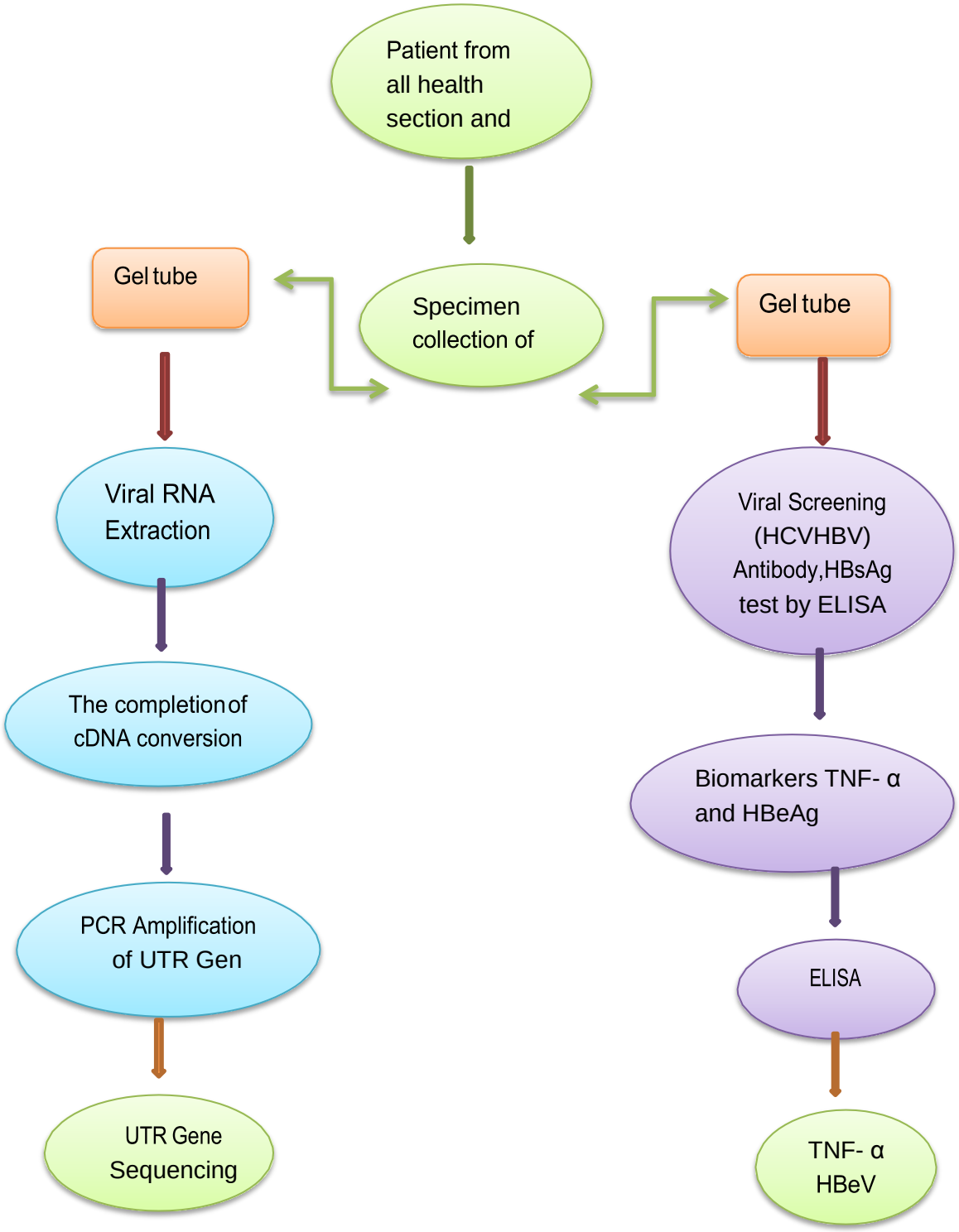


Figure 2.1: The General Design of The Study

2.1.3. Ethical approval:

Verbal agreement was taken from all the patient in the study before taking the specimens. Furthermore, the study design was approved by research Ethical committee at the College of Medicine/Babylon University**.**

2.2. Materials

2.2.1. Equipment and instruments

The equipment and instruments that used in this study with their companies and countries of origin in Table (2-1).

Table (2-1): The equipment and instruments that used in this study.

No.	Equipment and instrument	Company and Countries
1	Cylinder 50-250 ml	Halab/Syria
2	Electrophoresis	Bioneer/ Korea
3	Eppendorf tubes	Bioneer/ korea
4	Exispin vortex centrifuge	Bioneer/ korea
5	Face mask	Broche/Malaysia
6	Gel tube	Afco/Jordan
7	Gloves	Broche/Malaysia
8	High Speed Cold centrifuge	LabNet /USA

9	Ice back	Changzhou jisi/China
10	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
11	Nanodrop	THERMO/ USA
12	PCR T100 Thermal cycler	BioRad /USA
13	Plain tube	Afco/Jordan
14	Refrigerator	Concord /Lebanon
15	Tips	Song bong/China
16	U.V transilluminator	Wised/Korea
17	Urin cup	LoT/P.R.C
18	Vortex	CYAN/ Belgium
19	Water Bath	Plymouth/USA
20	Disposable sterile siring 3 ml size	China
21	ELISASystem micro well system reader micro well system washer	Human / Germany

22	Incubator	Thermoscientific / Germany
23	Filter paper	Chain
24	Flask 500ml	MBL / China
25	Deep freezer	Arcelik / Turkish

2.2.2 Kits

Table (2-2): The kits used in this study with their companies and countries of origin:

Table (2-2): kits used in this study

No.	Kit	Company and Countries
1	anti-HBV ELISA kit	Sun Long / china
2	HCV ELISA Kit	Sun Long / china
3	HBeAg ELISA Kit	Sun Long / china
4	TNF-α ELISA Kit	Sun Long / china
5	Viral RNA extraction AccuZol^{TM} RNA extraction kit	Bioneer/ Korea

	Trizol 100ml	
6	One-Step RT-PCR Premix Kit for cDNA synthesis and PCR	iNtRON/ Korea
	OptiScript™ RT System	
	RT-PCR buffer (10×)	
	dNTPs	
	*i-StarTaq*TM DNA polymerase	
	Stabilizingbuffer	

2.2.3.Primers

The one step RT-PCR primers that used for direct detection and genotyping of Human Hepatitis C virus based UTR region of polyprotein gene were designed according to (Virtanen *et al.*, 2018). These provided by (Scientific Researcher. Co. Ltd, Iraq) as following tables:

Table(2.3): Primers

Primer	**Sequence 5'-3'**		**Amplicon**
UTR-Polyprotein gene primer	F	GTCTAGCCATGGCGTTAGTATGAGTG	374bp
	R	ACAAGTAAACTCCACCAACGATCTG	

2.2.4Chemicals

Table (2-4): The chemicals with their companies and countries of origin used in this study:

Table (2-4) The Chemicals Materials

	Chemical	Company and Origin
1	Absolute ethanol	BDH (England)
2	Chloroform	BDH (England)
3	Isopropanol	BDH (England)
4	DEPC water	Bioneer/ Korea
5	Agarose	iNtRON (Korea)
6	10x TBE buffer	iNtRON (Korea)
7	Ethidium Bromide 10mg/ml	BioBasic (Canada)
8	Nuclease free water	BioLabs/ UK
9	DNA Marker ladder (100bp)	iNtRON (Korea)

2.3 Methods:

2.3.1 Immunological Tests

2.3.1.1. Human anti-Hepatitis B Virus(anti-HBV)

Human anti-Hepatitis B Virus (anti-HBV) ELISA kit is to for the qualitative determination of anti-HBV in Human serum, plasma, culture media or any biological fluid.

A. Test Principle

The ELISA is based on the qualitative enzyme immunoassay technique. The Microplate provided in this kit has been pre-coated with an antigen specific to anti-HBV, make it to solid-phase antigen. Samples are added to the Microplate wells and combined to the specific antigen. Then a Horseradish Peroxidase (HRP)-conjugated antigen specific for anti-HBV is added to each Microplate well and incubated, so the antigen-antibody-Enzyme labeled antigen complex is formed. Following a wash to remove any unbound reagent, then the TMB substrate solution is added to each well. Only those wells that contain anti-HBV and HRP conjugated HBV antigen will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The qualitative determination of anti-HBV is determined by comparing with the cutoff value.

B. Assay Procedure

1. In the Microplate, number the corresponding micro pores of the sample in sequence, leave two wells as negative control, two wells as positive control and one empty well as blank control. (blank control hole don't add samples and HRP-Conjugate reagent, the rest step operation are same).

2. Added 50 ml of Negative and positive control to the negative and positive control wells respectively. For sample wells, 40ml Sample dilution buffer and 10ml sample are added in it. Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubated 30 min at 37℃ after sealed with closure plate membrane.
4. Dilute the concentrated wash buffer with distilled water (30 times for 96T).
5. Wash carefully peel off closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the wash procedure for 5 times.
6. Added 50 ml HRP-Conjugate reagent to each well except the blank control well.
7. Incubation as described in Step 3.
8. Wash as described in Step 5.
9. Add 50 ml Chromogen Solution A and 50 ml Chromogen Solution B to each well, mix with gently shaking and incubated at 37℃ for 15 minutes. avoid light during coloring.
10. Added 50 ml stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow
11. Read absorbance O.D. at 450 nm used a Microtiter Plate Reader. The OD value of the blank control well is set as zero.

C. Calculation of results

Test effectiveness: the average value of positive control ≥1.00; the average value of negative control ≤ 0.10. The critical value (cut off) calculation: critical value = the average value of negative control + 0.15.

Negative judgement: if the OD value < cut off, the sample is Human anti-HBV negative.

Positive judgement: if the OD value ≥ cut off, the sample is Human anti-HBV positive.

2.3.1.2. Human Hepatitis C Virus(HCV) ELISA Kit

Human Hepatitis C Virus(HCV) ELISA kit is to assay HCV levels in Human serum, plasma, culture media or any biological fluid

Test Principle, Assay Procedure , Calculation of results resemble as in 2.3.1.1

2.3.1.3. Human hepatitis B virus e antigen (HBeAg) ELISA Kit

Human Hepatitis B Virus e Antigen (HBeAg) ELISA kit is to assay HBeAg levels in Human serum, plasma, culture media or any biological fluid.

A. Test Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to HBeAg Standards or samples are added to the appropriate Microelisa strip plate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for HBeAg is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain HBeAg and HRP conjugated HBeAg antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of HBeAg. You can calculate the concentration of HBeAg in the samples by comparing the OD of the samples to the standard curve.

B. Assay Procedure

1. Dilution of Standards Dilute the standard by small tubes first, then pipette the volume of 50 ml from each tube to microplate well ,each tube use two wells total ten wells.

2. In the Micro ELISA strip plate, leave a well empty as blank control. In sample wells, 40ml Sample dilution buffer and 10ml sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubated 30 min at 37℃after sealed with Closure plate membrane.

4. Diluted the concentrated washing buffer with distilled water (30 times for 96Tand20 times for 48T).

5. Washed carefully peel off Closure plate membrane, aspirate and refill with the washed solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Added 50 ml HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washed as described in Step 5.

9. Added 50 ml Chromogen Solution A and 50 ml Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 15 minutes. Please avoid light during coloring.

10. Added 50 ml stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450 nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

C. Stander curve

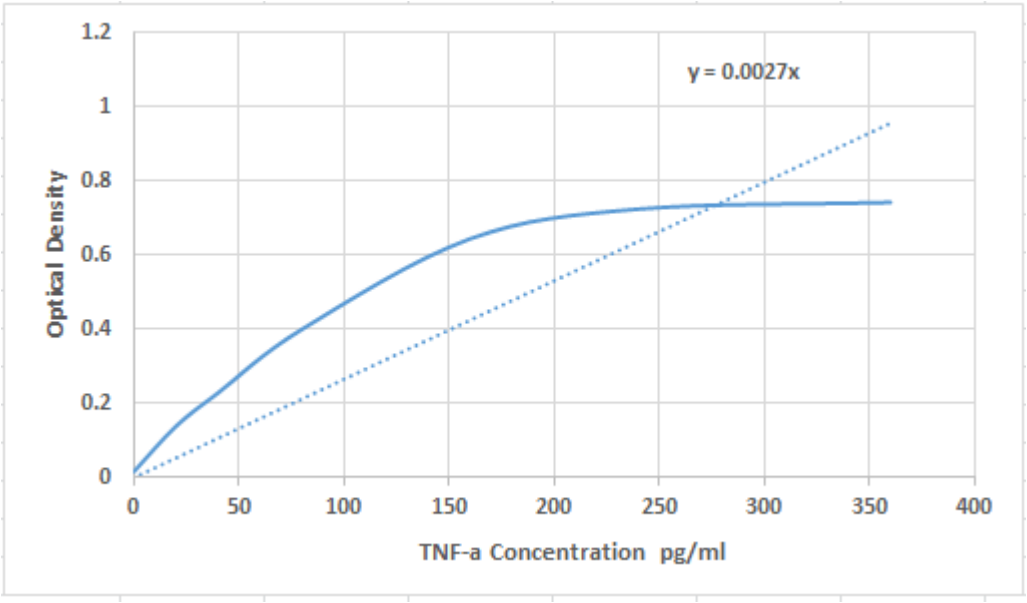


Figure 2.2: Standard Curve of TNF α

2.3.1.4. Human Tumor necrosis factor α (TNF-α)ELISA Kit

human tumor necrosis factor α (TNF-α) ELISA kit is to assay TNF-α levels in serum, plasma, culture media or any biological fluid.

A. Test Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to TNF-α. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for TNF-α is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain TNF-α and HRP conjugated TNF-α antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is

proportional to the concentration of TNF-α. You can calculate the concentration of TNF-α in the samples by comparing the OD of the samples to the standard curve

B. Assay Procedure

1. Dilution of Standards Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100ml Standard solution and 50ml Standard Dilution buffer are added and mixed well. In Well 3and Well 4, 100ml solution from Well 1 and Well 2 are added respectively. Then50ml Standard Dilution buffer are added and mixed well. 50ml solution is discarded from Well 3and Well 4. In Well 5 and Well 6, 50ml solution from Well 3 and Well 4 are added respectively. Then 50ml Standard Dilution buffer are added and mixed well. In Well 7andWell 8, 50ml solution from Well 5 and Well 6 are added respectively. Then 50ml Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50ml solution from Well 7 and Well 8 are added respectively. Then 50ml Standard Dilution buffer are added and mixed well. 50ml solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50ml and the concentrations are 240 pg/ml, 160 pg/ml, 80 pg/ml, 40 pg/ml and 20pg/ml, respectively.

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40ml Sample dilution buffer and 10ml sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. incubated 30 min at 37℃after sealed with Closure plate membrane.

4. Diluted the concentrated washing buffer with distilled water (30 times for 96Tand20 times for 48T).

5. Washed carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Added 50 ml HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washed as described in Step 5.

9. Added 50 ml Chromogen Solution A and 50 ml Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 15 minutes. Please avoid light during coloring.

10Added 50 ml stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

C. Stander curve

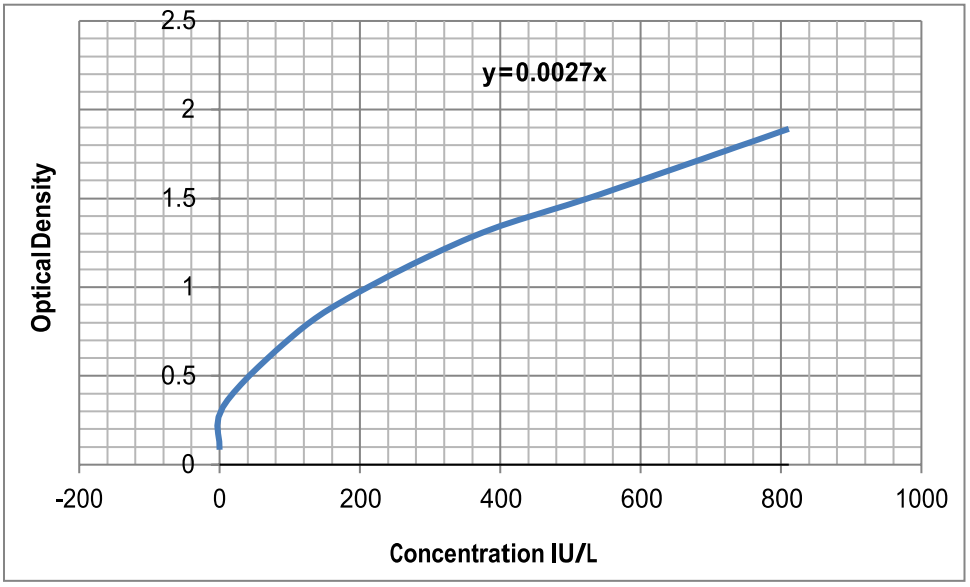


Figure 2.3: Standard Curve of HBeAg

2.3.2. Molecular Methods

2.3.2.1. Viral RNA Extraction

Viral RNA was extracted from serum samples by using AccuZol™ Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as following steps:

1. A 250ml serum samples was transferred into sterile and clean 1.5ml Eppendorf tube, then 750ml AccuZol reagent an mixed by vortex.
2. Chloroform (200 ml) was added to each eppendorf tube and mixed vigorously for 30 seconds.
3. The mixture was incubated on ice for 5 minutes.
4. After that, the mixture was centrifuged at 12,000 rpm, 4C°, for 15 minutes.
5. Supernatant was transferred to a new Eppendorf tube, and 500 ml isopropanol was added.
6. The mixture Mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes.
7. The mixture was centrifuged at 12,000 rpm, 4C°, for 10 minutes.
8. The supernatant was discarded.
9. 80% Ethanol was added into each tube and mixed by vortex, then centrifuged at 12,000 rpm, 4C° for 5 minutes.
10. The supernatant was discarded and the RNA pellet left to dry at room air for 5 minutes.
11. After that, DEPC water (50ml) was added to RNA pellet tubes and mixed by vortex to dissolved the RNA pellet.
12. The extracted RNA sample was kept at -80 deep freezers.

2.3.3.Estimation of extracted RNA

The extracted RNA were estimated by using Nanodrop spectrophotometer that used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio (1.8-2.0) as pure RNA, and done as following steps:

1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

2- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipeted 2 ml of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.

3- After that, the pedestals are cleaned and pipette 1ml of total RNA sample for measurement.

2.3.4. Reverse Transcription PCR (RT-PCR)

RT-PCR was performed for direct detection and genotyping of Human Hepatitis C virus based UTR region of polyprotein gene from serum patients samples. The RT-PCR was done according to following steps:

1- **RT-PCR master mix preparation:**

RT-PCR master mix was prepared by using **(One-Step RT-PCR Premix Kit**) and done according to the company instructions in two step the following tables:

Table 2.5: PCR master mix

PCR master mix	Volume
RNA template 5-100ng	5µL
PCR Forward primer (10pmol)	1µL
PCR Reverse primer (10pmol)	1µL
One step RT-PCR premix	8µL
PCR water	5 µL
Total	20µL

These RT-PCR reaction mixes were placed in sterile 0.2 ml PCR tubes and mixed and centrifuged for 3000 rpm for 3 minutes by Exispin centrifuge, after that transferred into PCR thermocycler.

RT- PCR thermocycler conditions:

PCR thermocycler conditions was set according to primer annealing temperature as in the following tables:

Table 2.6: PCR thermocycler conditions

Step	Condition	Cycle
RT step	42°C 30 min.	1
Initial denaturation	95 °C 5 min.	1
Denaturation	95 °C 30 sec.	35
Annealing	58 °C 30 sec.	
Extension	72 °C 1min.	
Final extension	72 °C 5 min.	1
Hold	4 °C	Forever

2.3.5. RT-PCR product analysis

The RT-PCR products was analyzed by agarose gel electrophoresis following steps:

1- 1.5% 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 3ml 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.
4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.
5- A 10ml of RT PCR product were loaded in to each comb well and 5 ml of (100 bp Ladder) in first well.
6- Then electric current was performed at 90 volt and 80mA for 1.5 hour.
7- RT-PCR products were visualized by using UV transilluminator.

2.3.6. DNA sequencing method

RT-PCR products of positive Human Hepatitis C virus UTR region of polyprotein gene were sent to Macrogen Company in Korea for performed the DNA sequencing by (AB DNA sequencing system). The DNA sequencing analysis was conducted by using phylogenetic tree UPGMA method (MEGA 6.0 version), Multiple alignment analysis based ClustalW alignment analysis, and NCBI-BLAST for homology sequence identity.

2.4. Statistical Analysis

Statistical Analysis was carried out using SPSS version 25. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Pearson' s chi square (X2) , ANOVA and Fisher-exact tests were used to find the association between categorical variables. A p-value of ≤ 0.05 was considered as significant (Daniel and Cross,2018).

Chapter Three

Results and Discussion

3. Result and Discussion

3.1. Distribution of patients with *Hepatitis B* virus in blood samples according to their age

The results of screening blood for presence of anti-HBsAg indicate a history of recent HBV infection. The prevalence of anti-HBsAg trend for a decrease or an increase of prevalence with age shown in table (3-1). There were significant differences between the ages, the distribution of HBV patients according to age is represented in table (3.1) It was found that the age of HBV patients ranged between 20-80 years with a mean of age (48.32**) ,**

Table 3.1: Distribution of patients with *Hepatitis B* virus in blood samples according to their age

The patients of *Hepatitis B*	No.	Mean of age	S.D	S.E	Minimum	Maximum	P-Value
Blood sample	37	48.32	15.813	2.600	20	80	**<0.05**

The result nears of the previous studied where HBV is inactive carriers (53% females, mean age 48.7 ± 13.8 years, range (16-77) year (Magalhães and Pedroto 2015) .

The ages range from 20 to 80. The most affected groups due to this monsoon are the young working class and the weaker immune group, who are the elderly (Tazerji et al., 2022).

3.2 : Frequency of HBV and blood samples according to the age stratum

The results revealed that the highest number of infection rate was in persons (35-49) in the rate of (12) 32.5 % and the lowest number of infected people were at the age of (20-34) and (50-64) (8) in the rate of 21.6%. The result show signification difference at p value <0.05 as in table 3-2 .

Table (3.2.1): Frequency of HBV and blood samples according to the age stratum

Age Stratum	Years	HBV		P value
		No.	%	
	20-34	8	21.6	**<0.05**
	35-49	12	32.5	
	50-64	8	21.6	
	≥ 64	9	24.3	
Total		37	100	

This table show distribution of sample according to sex, female higher than male with percentage 59% and male with percentage 40.5%

The majority were female, a finding consistent with recent studies Indeed, it was noted that the rates of females were higher than that of men at the ages of 20-29 and 30-39 years. These two age ranges correspond to women of childbearing age. This makes sense because individuals in the 20-39 age group represent an active labor force that makes up a large percentage of the total population (Rajkumar *et al*., 2022)

3.2.2 Table show Distribution of sample according sex in HBV

Sample	Male	%	Female	%	Total HBV	Total control
HBV	**15**	**40.5**	**22**	**59.4**	**37**	**20**

The current study included 37 persons who tested positive for HBsAg and ranged from 20 to 80 years old. A total of 37 of the 85 individuals who had their HBVe antigen status examined had positive results.

Females made up more of the sample (n = 22) than males (n = 15) as in (Table 3-2.2). The result of the current study presented distribution of patients with hepatitis according to the age intervals and indicated that majority of hepatitis patients (12) (32.5%) within age group 35-49 years old .This result was agreed with findings of a study conducting in Thi-Qar province, showed that high rate of hepatitis infection occur with age 21-40 years old 48.5% while in the age group 61-70 was 8.5 % in table (3.2.1). (Othman and Abbas 2020).

Mode of transmission for HBV and HCV may be aid the increase of high rate infection in age group less than 30 to 40 years old. Share use of razor blade, Botox injection, tattoo, and sexually activity are the major cause of high incidence of HBV and HCV infection according to (Stevens *et al*., 2016). Despite of availability of HBV vaccine, the results of the previous study showed infection with HBV in age less than 30 to 40 years old was more than infection with HCV. This finding was agreed with results of (Rezaei *et al.*,2020 *)*.

3.3 : Distribution of patients with hepatitis B virus according to the sex with age group

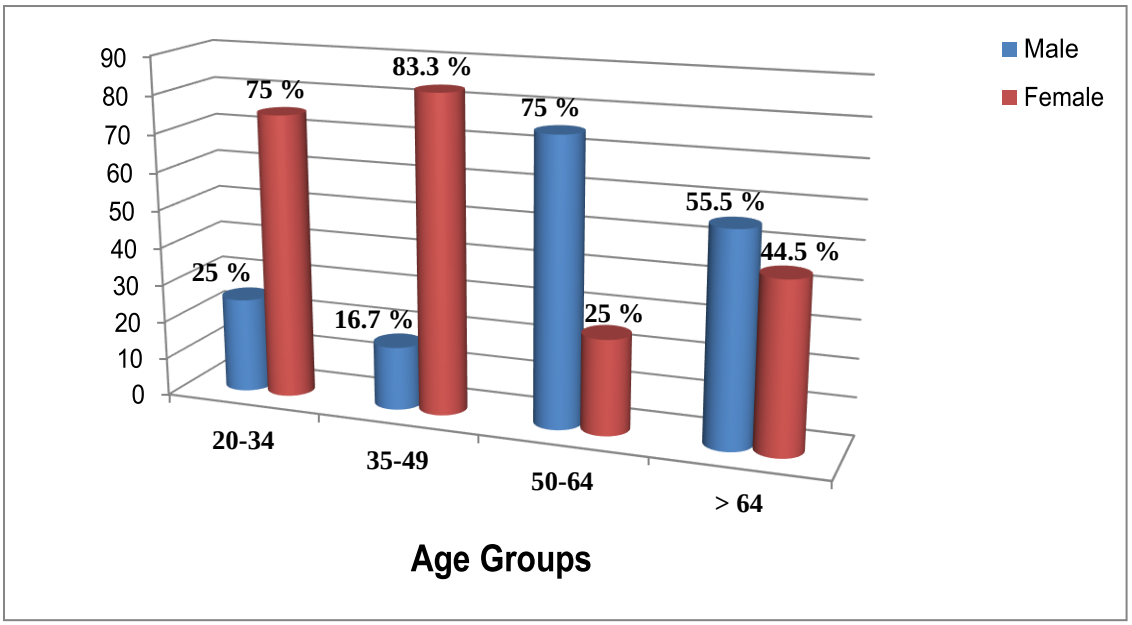


Figure.(3. 1): Distribution of patients with hepatitis B virus according to the sex with age group

Result show the distribution of patient according sex and age group, where was the result high percentage in female in age group 35-49 (83%) and lower percentage in age group 50-64 (25%), in male high percentage in age group 50-64 (75%) and lower percentage in age group 35-49 (16.7).

The high incidence of infection among female of this age is due to the fact that women are in the stage of pregnancy and childbirth, in addition to frequent visits to beauty centers and exposure to contaminated needles and syringes (Teiri et al., 2022).

The increase in infection in men at this age is evidence of a weakening of immunity with advancing age, especially with immunodeficiency patients (Zhang et al., 2023).

3.4. Concentration of TNF α in patients with Hepatitis B virus

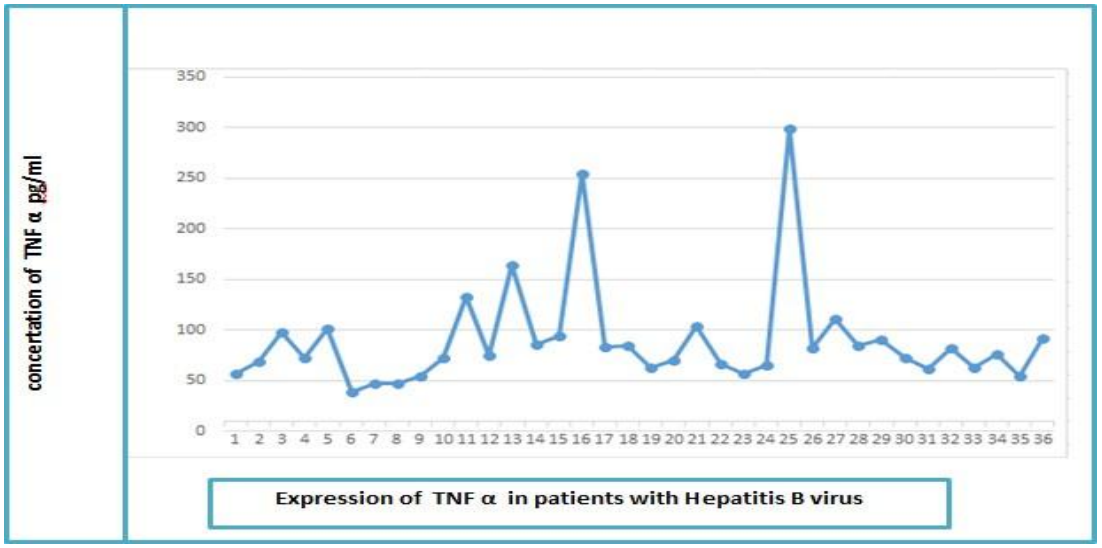


Figure (3.2): Concentration of TNF α in patients with Hepatitis B virus

The figure explain elevation the TNF- α in patient with HBV where was all sample have high level TNF- α compare with normal rang 0-2.2pg/ml.

There are few studies on cytokine production profile in occult hepatitis B patients and the mechanism of liver injury due to OBI is still unclear, but some studies describe that the persistence and transcription of HBV cccDNA in hepatocytes can lead to the production of cytokines, such as TNF-α and INF-γ, which can result in damage for hepatocytes (Yip and Wong., 2019;Germanidis G *et al.*,2012). A reduction in proinfammatory cytokines, such as TNF-α, was observed in patients who resolved HBV infection when compared to healthy individuals (Wang *et al* 2019), and IFN-γ was noticeably decreased, especially in monoinfected patients with HCV genotype 1b (Ribeiro *et al*., 2021).

3.5.1 : The correlation between of HBV and TNF α according to ANOVA

Table:((3.3): The correlation between of HBV and TNF α according to ANOVA

That table represent deference between groups in TNF- α where mean squares 60233 and df 2 with sig 0.000 and deference within groups 97905 and df 54 with sig 0.000

TNF - α	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	60233.079	2	30116.539	16.611	.000
Within Groups	97905.571	54	1813.066		
Total	158138.650	56			

Tumor necrosis factor alpha (TNF-α) plays a special role in the formation of the antiviral immune response (Ruan *et al*.,2010). TNF-α is a multifunctional cytokine with pronounced pleiotropy, takes part in the formation of the body's defense reactions, stimulates the Th-1 cellular immune response, phagocytic and cytotoxic activity of cells, and regulates the processes of immune inflammation. All this contributes to the progression of liver fibrosis with an increase in the cytokine level TNF-α and IL-1βcontrol the balance between cell proliferation and apoptosis (Khayrulla *et al*.,2021).

3.5.2 : Relation between HBV and TNF α according to Description

Table :(3.4) Relation between HBV and TNF α according to Description sample

Table show number male 15 with mean 85.85 , stander deviation 55 , stander error 14.2 , minimum 38.52 and maximum 254.81, number femal 22 with mean 92.54 , stander deviation 50, stander error 10.7 , minimum54.07 and maximum 298.89 with **p-value = ≤ 0.05**

TNF - α	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Male	15	85.8519	55.05118	14.21415	55.3655	116.3382	38.52	254.81
Female	22	92.5421	50.21130	10.70508	70.2796	114.8045	54.07	298.89
Control	20	21.9415	11.54407	2.58133	16.5387	27.3443	7.32	41.07
Total	57	66.0094	53.14042	7.03862	51.9093	80.1094	7.32	298.89

One of the important biological functions of TNF-α is its participation in the regulation of apoptosis, including in cells damaged by the virus (Shapouri *et al.*,2018).TNF-α is secreted by various cells, for example, activated macrophages (Sittisart *et al.*,2016),cytotoxic T-lymphocytes in the liver (Iannacone and Guidotti 2022). There are numerous reports in the literature demonstrating changes in TNF-α production in viral infections. An increased level of TNF-α in blood plasma was found during exacerbation of such chronic infections as viral hepatitis, HIV, herpes type I, Epstein-Barr, influenza, poliomyelitis, tick-borne encephalitis, etc. (Perricone *et al.*,2020). In CHC, there is an increased level of TNFα in the blood serum and in the liver parenchyma in patients (Tawfik *et al.*,2018).

It is believed that the overproduction of this cytokine is one of the main mechanisms of activation of the infectious process during its transition

from the latent state to the phase of clinical manifestations and indicates the progression of the disease . Clinical outcome of HBV infection (Piekut *et al*., 2022). These data may indicate a role for TNF-α in the pathogenesis of hepatitis B, as well as in the development of its complications (Li *et al*,.2016).

3.6 : Concentration of HBeAg in patients with Hepatitis B virus

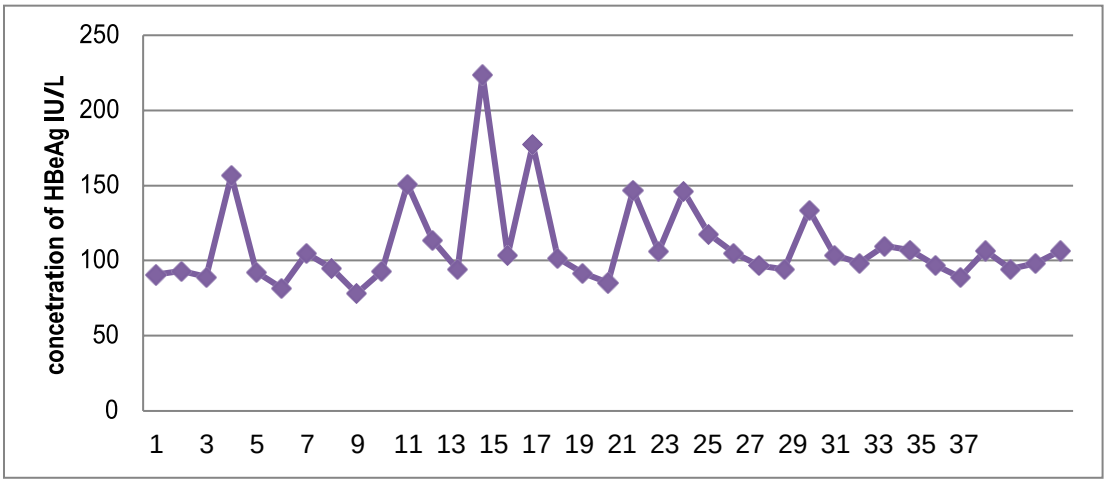


Figure :(3.3) Concentration of HBeAg in patients with Hepatitis B virus

The figure explain elevation the HBeAg in patient with HBV where was all sample have high level HBeAg compare with normal rang less than 5 lU/L.

Among HBsAg-positive persons, the frequency of HBeAg in the province of Thi Qar was 51.38%. This demonstrates a highly infectious population in Thi Qar that promotes viral transmission and evolution, portending a future increase in the prevalence of liver cancer caused by HBV (Figure 3.3). In previous study Among 763 HBeAg+ individuals (56% female), of all ages and HBV genotypes, qHBeAg levels were consistently higher in women. High viral replication and infectiousness are linked to e antigen positivity (HBeAg+) in CHB virus infection. In

addition, HBeAg-positive CHB is linked to a broad range of liver disease severity (Cooper *et al.,* 2021).

Hepatitis B "e" antigen (HBeAg) is a highly conserved protein found in all mammalian orthohepadnaviridae that is essential for viral replication. It is created through the post-translational modification of the pre-core-core protein (Revill, P *et al.,* 2010; Cooper et al., 2019). While its precise function in HBV transmission, replication, assembly, and packing is unclear, it is important to highlight that it is not essential for these processes at present. In HBeAg-positive individuals, liver disease has been seen to span a spectrum from complete absence to cirrhosis and hepatocellular cancer (HCC). Consequently, infected persons who test positive for HBeAg may continue for lengthy periods without experiencing symptoms (with normal serum aminotransferase activity), Only for some of them to develop active CHB, resulting in necroinflammatory liver damage years or even decades later. Those who contract the infection later in life are less likely to exhibit the "immune tolerant" HBeAg+ phenotype, which is characterized by high HBV DNA levels in the blood and normal ALT levels (Abbas *et al.,* 2018). Previous research has shown that one's HBeAg production rate, and not only HBeAg status, is connected with certain biological traits and clinical symptoms (Cooper *et al.,* 2021).

Although hepatitis B e antigen (HBeAg) is not required for HBV replication and its exact function is unclear, it may play a role in chronic HBV infection as HBeAg in the serum generally indicates ongoing HBV replication and liver disease (Yu *et al.,* 2017) The emergence of HBeAg-negative variants correlates with an exacerbation of liver injury and even with viral clearance in some patients (Wang *et al.* 2020).

The presence of HBeAg in the serum of patients with hepatitis B virus is a reflection of active viral replication in hepatocytes and is considered a surrogate marker for the presence of the DNA of hepatitis B virus (Testoni *et al.,* 2019). Testing for the HBeAg can also identify individuals with a high risk of developing liver cancer (Qu *et al.,* 2019). A previous study among pregnant women in Makurdi in north-central Nigeria found that 30.3% were HBV carriers testing positive for HBeAg (Forbi *et al*.,2012). However, no inter gender difference in HBeAg seroprevalence was observed in a study in south-eastern Nigeria (Omeje *et al.*, 2017). This contradiction could be due to the fact that our study was clinic-based while the Enugu study (Ndubuisi e*t al.*,2020). was community-based. Patients in clinical settings are more likely to have medical conditions (e.g. symptoms of hepatitis, presence of HBeAg) than persons living in the communities. So, it remains to be determined whether the gender differences observed in this study is the true reflection of the gender distribution or is associated with the study population or the healthcare-seeking patterns in this region (Shih *et al.*, 2020).

3.7 : Distribution of patients with *Hepatitis C* virus in blood samples according to their age

The results of screening blood for presence of anti-HCV Ag indicate a history of recent HCV infection. The prevalence of (anti-HCV) antibodies and trend for a decrease or an increase of prevalence with age shown in table (3-5). There were significant differences between the ages; the distribution of HCV patients according to age is represented in table (3.5). It was found that the age of HCV patients ranged between 21-80 years with a mean of age (55.52).

Table (3.5): Distribution of patients with *Hepatitis C* virus in blood samples according to their age

The patients of *Hepatitis C*	No.	Mean of age	S.D	S.E	Minimum	Maximum	P-Value
Blood sample	48	55.52	14.500	2.093	21	80	**<0.05**

The table (3.5) shows the Distribution of patients with Hepatitis C virus in blood samples according to their age, where mean of S.D (14.500). S.E at (2.093), the stateside analysis makes at P <0.05.

The study was close to the previous study , where is appeared The mean age of the study population was 54.5 years (SD11.4), 65% were male, Specifically, prevalence ratios for people with HCV were significantly higher for diabetes, renal failure, cancer, asthma, chronic obstructive pulmonary disease, substance use disorder, mood and anxiety disorders and liver failure (Cooper *et al.*, 2019).

Result agrees with Studies estimate that between 55% and 75% of newly infected persons develop chronic HCV infection as determined by detectable HCV RNA in the blood (Chevaliez *et al.*, 2018). Patients of older age at time of infection and impaired immune system are at increased risk of developing chronic HCV infection. (Park *et al*., 2015). A large proportion of chronically HCV-infected persons in the United States are now about 50 to 70 years old and have lived with HCV infection for about 25 to 45 years.

Aging-related mechanisms that have been postulated to increase the risk of liver disease outcomes in the setting of HCV infection include a greater vulnerability to environmental factors, such as oxidative stress, with increasing age; reduction in the rate of hepatic flow; reduced mitochondrial capacity; impaired immunity; and increased carcinogenic potential caused by a reduced ability to repair DNA (de Torres and Poynard.,2003). There are also limited data that HCV infection may be associated with increased markers of immune-senescence, which has been shown to occur in the setting of human immunodeficiency virus (HIV) infection and is thought to play a role in the earlier onset of aging-related comorbidities in HIV infection. HCV infection itself might be associated with loss of early differentiated T cells and progressive accumulation of chronically activate late-differentiated senescent T cells (Reid *et al*,. 2017).

One small study Comparing HCV-infected individuals with healthy control subjects, all of whom were less than 54 years of age, found that the CD4 and CD8 T cells from HCV-infected individuals showed a significant increase in the T-cell immunosenescent phenotype that is more commonly associated with advancing age. Whether or not this increase is associated with the premature onset of not only liver but also non liver clinical outcomes related to aging in HCV-infected persons is unclear (Osuch *et al*,. 2020).

3.8 : Frequency of HCV and blood samples according to the age stratum

The results revealed that the highest number of infection rate was in persons (41-60) (22) in the rate of 45.8. % and the lowest number of infected people were at the age of (21-40) (8) in the rate of 16.7%. The result show signification difference at p value <0.05 in table (3.6.1).

Table 3.6.1: Frequency of HCV in blood samples according to the age stratum

Age Stratum	Years	HCV		P value
		No.	%	
	21-40	8	16.7	**<0.05**
	41-60	22	45.8	
	61-80	18	37.5	
Total		48	100	

The 48 affirmatives out of 48 Using an ELISA (enzyme-linked immunosorbent test) double-antibody sandwich. we determined that HCV participants met the inclusion criteria. The findings of the HCV IgM ELISA Kit indicated that the prevalence of HCV was greatest in those aged 41 to 60 (22) (45.8%), lowest in those aged 21 to 40 (8) (16.7%) and HCV Ag The use of an ELISA kit enabled the discovery. Of the 48 individuals who tested positive for HCV Ag, 21 (56.2%) were female and 27 (43.8%) were male in table (3.6.2). This indicates that males are more likely than females to have HCV Ag infection

Table 3.6.2: Table show Distribution of sample according sex in HCV

Sample	Male	%	Female	%	Total HBV	Total control
HCV	**27**	**56.3**	**21**	**43.7**	**48**	**20**

The reasons for this result are related to Transmission of the virus is linked to lifestyle Males, including frequent visits to barbershops And eat

in popular restaurants that are often Added a condition that lacks health and hygiene control To increase sexual desire during these ages

The result matched the previous study, where it was shown that Age data for HCV prevalence has been typically analyzed and reported as birth cohort prevalence. Recent analysis of birth cohort HCV prevalence estimates in the United States showed the highest rates in persons born during 1945-1965 (1.63%), followed by persons born after 1965 (0.51%), and lowest in persons born prior to 1945 (0.21%) These data are consistent with earlier published data that also show highest HCV prevalence among persons born during 1945 to 1965 (Smith *et al.*, 2012; Castrejon *et al.*, 2017; and Konerman *et al.*, 2017). The relatively high prevalence of HCV infection among persons born between 1945 and 1965 corresponds with the high HCV incidence (new infections) that occurred among young adults using or experimenting with injection drugs in the 1970s and 1980s (Teshale *et al.*, 2022). In recent years, the proportion of cases involving persons born after 1965 has steadily increased.

3.9 : Distribution of patients with hepatitis C virus according Sex and age

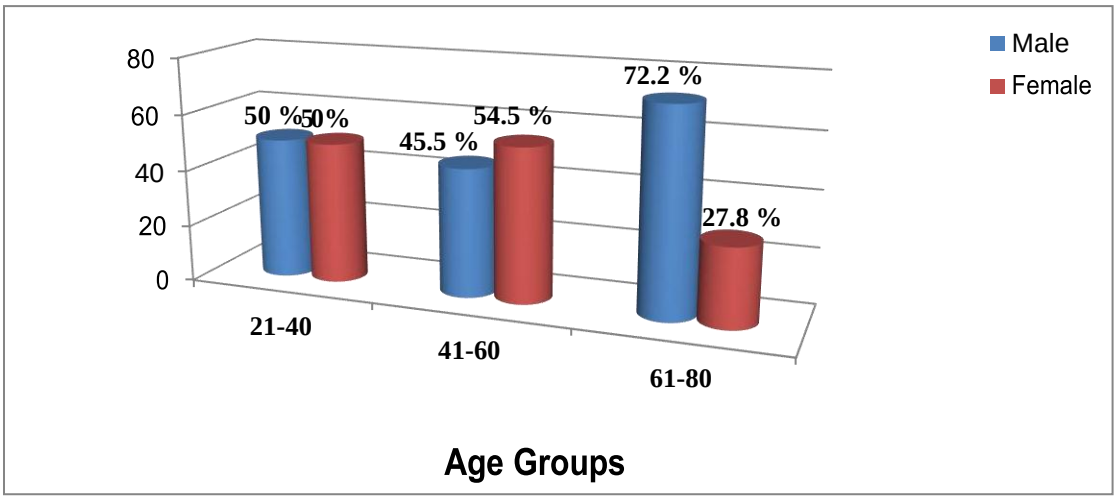


Figure (3.4): Distribution of patients with hepatitis C virus according Gender and age.

Result show the distribution of patient according sex and age group, where was the result high percentage in female in age group 41-60 (54.5%) and lower percentage in age group 61-80 (27.8%), in male high percentage in age group 61-80 (72.2%) and lower percentage in age group 41-60 (45.5).

Other studies The number of reported cases of acute HCV infections in 2019 was higher in males (2,471) than in females (1,653), with males accounting for 60% of the acute HCV infections in 2019 (Zhao *et al*., 2022).

In the United States, the estimated HCV prevalence for adult males is 1.31%, which is 2.3-fold higher than the 0.57% prevalence in adult females; this corresponds with a male to female HCV prevalence ratio of 2.3 (Melikoki *et al.,* 2022).

The result are consistent with previous study the national seroprevalence of HCV in 2008 was 14.7% in people aged 15 - 59 years and was higher in males than in females (Abdel-Gawad *et al* .,2023). Seven hundred seventy-five patients with CHC were included in the study, 434 (56%) were males and 341 (44%) were females (Saif-Al-Islam *et al.*, 2020). Generally males and females are different in their health and disease state. This difference could be explained by the difference in exposure to risk factors, sexual hormones, genetic effect and different corporal structures. In addition, CLD may produce different consequences in both genders (Saif-Al-Islam *et al.*, 2020).

In this prospective study which analyzes the characteristics of HCV infection in males compared to females, we found a preponderance of male gender in patients with chronic HCV which was documented by several previous studies (Naga *et al*., 2019; Al-Zanaty., 2015). Also, there

is variance in sex dependent susceptibility to infectious diseases due to the effect of sexual hormones (Gay *et al.*, 2021).

male patients with chronic HCV carry IL-6 promoter polymorphisms more likely than females (Aboushousha *et al.*, 2021). While certain polymorphisms in Ctla4, an inhibitory T cell receptor which is more common in females exposed to infection, are associated with resolution of HCV infection (Klein.,2012).

The risk of HCV infection from blood transfusion and IV injection of tarter emetic was significantly more in males. This could be explained by that males were overrepresented in trauma and need more units of all blood products in massively transfused traumatized patients (Saif-Al-Islam *et al.*, 2020). Other authors found similar result (Bakr *et al*., 2006). We found the risk of HCV infection from surgical operation was higher in females. This may be due to the role of obstetric procedures, which is increasing nowadays, as a risk for infection (Saif-Al-Islam *et al.*, 2020).

3.10 : Concentration of TNF α in patients with Hepatitis C virus

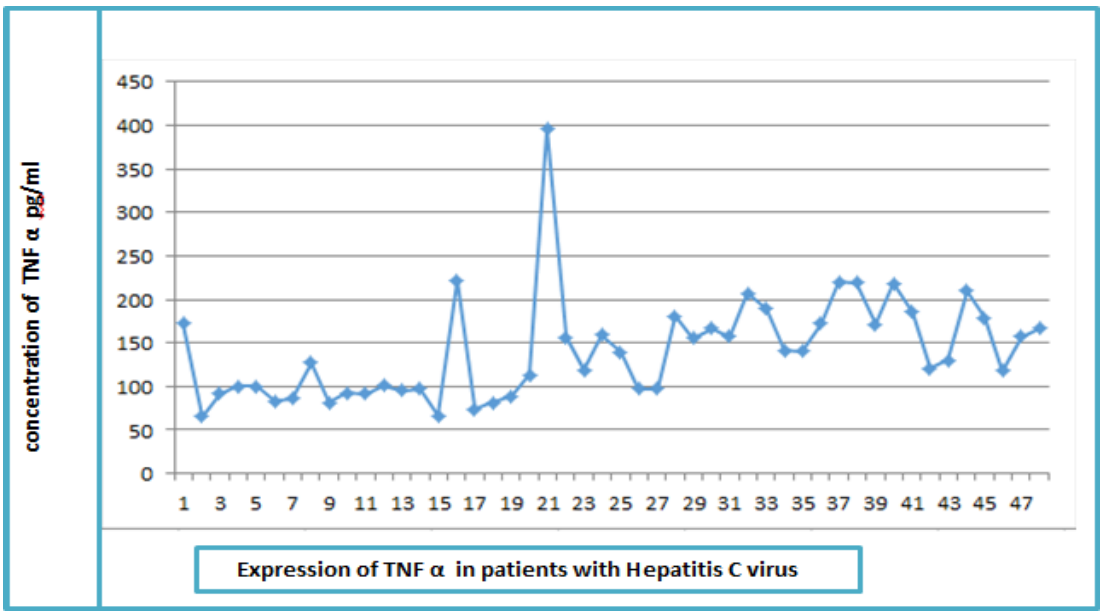


Figure 3.5: Concentration of TNF α in patients with Hepatitis C virus

The figure explain elevation the TNF- α in patient with HCV where was all sample have high level TNF- α compare with normal rang 0-2.2pg/ml.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine produced in response to infectious pathogens. The soluble TNF-α is produced as a result of cleavage from its precursor transmembrane TNF-α by the TNF-α-converting enzyme (TACE). The secreted TNFα binds to its receptors, namely TNFR1 and TNFR2, to exert its biological effects (Zhou *et al*., 2017). Multiple studies indicate that the blood level of TNF-α is increased in HCV patients and its level is positively correlated with HCV pathogenesis and the severity of liver diseases (Li *et al.,* 2018). The major source of TNF-α in response to HCV infection is unclear and thought to be immune cells such as T lymphocytes and macrophages (Usai *et al.*,2020). In this report, we provide evidence to demonstrate that hepatocytes can also produce TNF-α in response to HCV infection. This TNF-α induction is prompt and mediated by TLR7 and TLR8. Furthermore, we also demonstrate that TNF-α, through an autocrine mechanism, prevents the depletion of IFNAR2 by HCV and is required to support interferon signaling in HCV-infected cells (Rudiansyah *et al.*,2022).

HCV patients have an elevated serum level of TNF-α, and this level is positively correlated with the severity of liver diseases (Al-Jiffri., 2017). The source of TNF-α is unclear, but it is generally assumed that it is produced by immune cells such as macrophages (Han *et al*., 2019). In this report, we demonstrated that TNF-α could also be induced in HCV-infected cells. Although the amount of TNF-α produced by HCV-infected hepatocytes might be lower than that produced by professional immune cells such as macrophages (Cheng *et al.,* 2017), it was sufficient to trigger an inhibitory response on HCV replication.

Our finding is consistent with a previous report, which described an increased level of TNF-α in the hepatocytes of HCV patients (Lee *et al.*, 2015).

3.11 : The correlation between of HCV and TNF α according to ANOVA

There were significant difference between HCV and TNF α according to ANOVA That table represent deference between groups in TNF- α where mean squares 192585 and df 2 with sig 0.000 and deference within groups 114800 and df 65 with sig 0.000

Table (3.7): The correlation between of HCV and TNF α according to ANOVA p-value = ≤ 0.05

TNF- α	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	192585.180	2	96292.590	54.521	.000
Within Groups	114800.621	65	1766.163		
Total	307385.800	67			

TNF-α is an immunomodulator with a potent peripheral pro-inflammatory effect. It plays an important role in cell proliferation, differentiation, and apoptosis Involvement of TNF-α in the pathogenesis of viral hepatitis and is associated with inflammation and fibrotic changes in hepatocytes . (Noh *et al.*, 2022)

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that acts both as a mediator of innate immunity and in the cellular immune response. Abnormal TNF-α levels have been associated with chronic HCV infection (Osuji *et al.*, 2018).

HCV stimulates the secretion of TNF-α by human hepatocytes .In particular, it is known that an increase in TNF-α production in chronic

viral hepatitis C at an early stage of the infectious process can mediate increased apoptosis of hepatocytes, which leads to the destruction of the liver tissue, followed by a decrease in apoptotic cell death and, as a consequence, the possible development of malignant neoplasms (Khayrulla *et al*., 2021).

Table(3.8): The correlation between of HCV and TNF α according to Descriptive

TNF- α	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Male	27	140.8230	55.17958	10.61931	118.9947	162.6514	65.56	248.52
Female	21	135.8554	40.68424	8.87803	117.3361	154.3746	74.07	218.15
Control	20	21.9415	11.54407	2.58133	16.5387	27.3443	7.32	41.07
Total	68	104.3238	67.73365	8.21391	87.9287	120.7188	7.32	248.52

Table show number male 27 with mean 140.82 , stander deviation 55 , stander error 10.6 , minimum 65.56 and maximum 248.52, number femal 21 with mean 40.68 , stander deviation 40, stander error 8.87 , minimum74.07 and maximum 218.15 with **p-value = ≤ 0.05**

Depending on the standard manufacturing curve, TNF-α concentration was measured in serum HCV patients, and there was a significant increase in TNF-α concentration .The mean concentration of TNF-α in hepatitis C patients was (2.89212866), While S.D (1.681512959) and S.E (0.242705490) as shown in the table (3.3.2). This result is consistent with a number of studies such as (Sabry *et al.,* 2015) in Egypt, (Aroucha *et al*., 2013) in Brazil and (Elsammak *et al.,* 2005). This may be 95 because TNF- α stimulating a partly overlapping group of antiviral defense mechanisms and level of TNF- α in serum reverses the progression of inflammation. Increased level TNF- α in patients with chronic hepatitis C

may participate to the role of innate immunity in stimulating the adaptive immune responses, so suggest the role of TNF- α in antibody production (Akdis *et al*., 2016) reported that level of TNF- α in the serum of patients was high significantly. They suggested that rise serum level of TNF- α is an important mediator in the pathogenesis of liver necrosis and alterations in microcirculation (Sabr *et al*., 2015).They found elevation significantly in serum level of TNF- α in chronic liver disease and reach its maximum in decompensated cirrhosis. They suggested that this increase in the cytokine concentration may be due to liver dysfunction rather than the inflammatory illness (Ji *et al.,* 2020).

The infected liver display an important rise in gross macrophage numbers, also Chronic HCV is correlated with immune infiltration, high levels of HCV particles could stimulate macrophages to express TNF- α, create a direct mechanism for the HCV to enhance infection (Tanwar *et al.*, 2020). Also (Fletcher *et al.*, 2014) they suggested that hepatitis C stimulates Kupffer Cells (Liver resident macrophages) to express TNF- α.

3.12 : DNA Sequence results:

The DNA sequencing method was carried out to genotyping identification analysis in UTR-polyprotein gene of local Hepatitis C virus isolates (IQ.No.1 - IQ.No.5) and NCBI-Blast related Hepatitis C virus genotypes isolates

The phylogenetic tree genetic relationship analysis was showed that the local Hepatitis C virus isolates were showed closed related to NCBI-BLAST Hepatitis C virus subtype 1a (HQ113638.1) at total genetic changes (0.3-0.05%). As showed in figure (3-6).

The homology sequence identity between local Hepatitis C virus isolate (IQ.No.1 - IQ.No.5) and NCBI BLAST related Hepatitis C virus

were showed genetic homology sequence identity ranged from (99.41%-99.61%). As showed in table (3-9).

Finally, the local Hepatitis C virus isolate (IQ.No.1 - IQK.No.5) were submitted into NCBI Genbank and identified by accession numbers (OQ843893 - OQ843897).

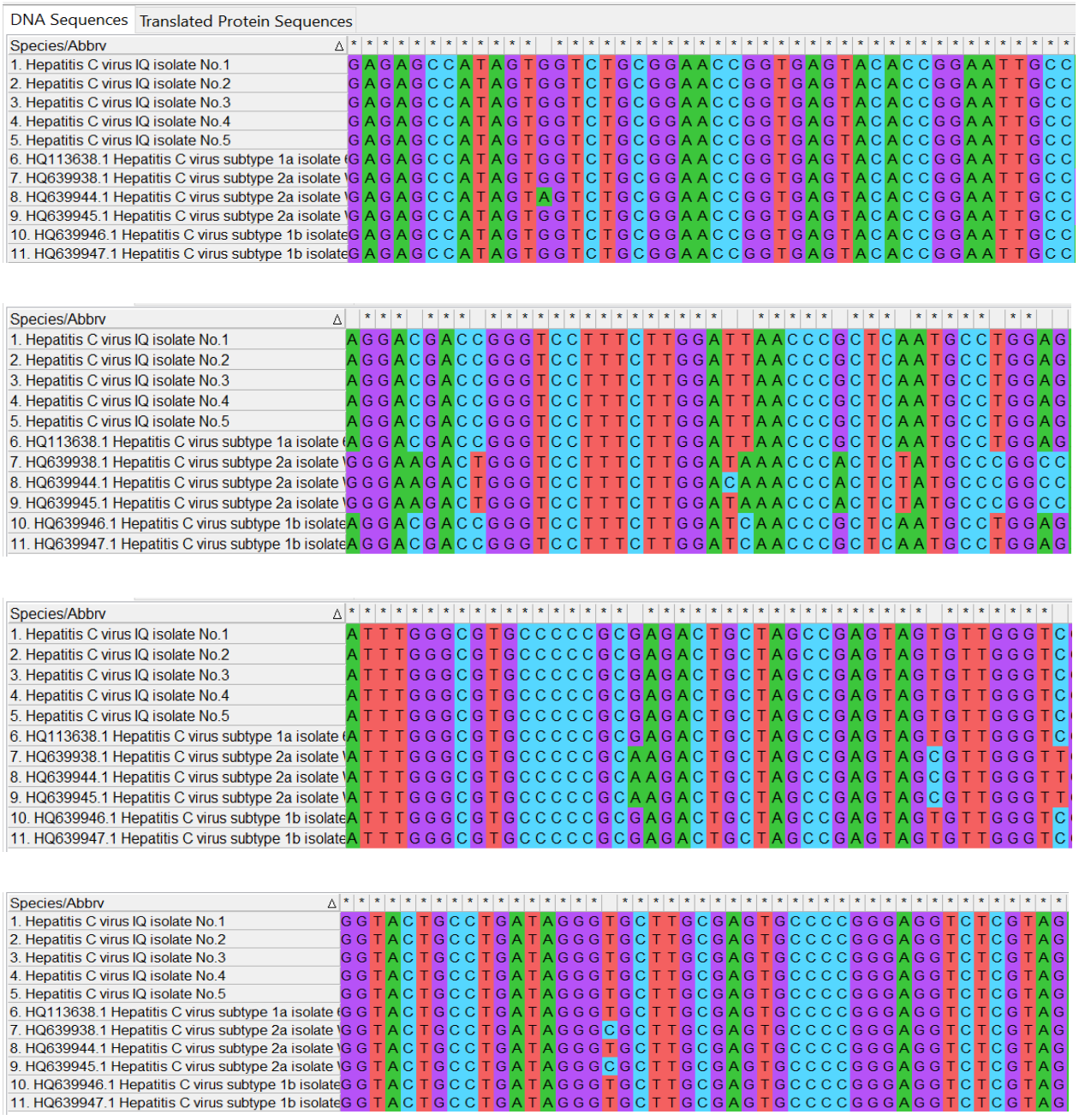


Figure (3.6): Multiple sequence alignment analysis of UTR-polyprotein gene in local Hepatitis C virus isolates and NCBI-Genbank Hepatitis C related genotypes isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0

version). That showed the nucleotide alignment similarity as (*) and substitution mutations in UTR-polyprotein gene between different Hepatitis C virus isolates

Figure (3.7): Phylogenetic tree analysis based on UTR-polyprotein gene partial sequence in local Hepatitis C virus isolates that used for genotyping identification. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Hepatitis C virus isolates were showed closed related to NCBI-BLAST Hepatitis C virus subtype 1a (HQ113638.1) at total genetic changes (0.3-0.05%).

Table (3.9) The NCBI-BLAST Homology Sequence identity (%) between local Hepatitis C virus isolates and NCBI-BLAST submitted related isolates:

Local olate No.	Gen bank Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical isolate	Gen bank Accession number	County	Identy (%)
IQ.No.1	OQ843893	Hepatitis C irus subtype 1a	HQ113638.1	USA	99.4 %
IQ.No.2	OQ843894	Hepatitis C irus subtype 1a	HQ113638.1	USA	99.4 %
IQ.No.3	OQ843895	Hepatitis C irus subtype 1a	HQ113638.1	USA	99.6 %
IQ.No.4	OQ843896	Hepatitis C irus subtype 1a	HQ113638.1	USA	99.6 %
IQ.No.5	OQ843897	Hepatitis C irus subtype 1a	HQ113638.1	USA	99.6 %

New strains of the virus in the gene bank, and therefore we did not find any Iraqi strains registered can compare with them. But when compared with international strains, three of our isolates showed similar sequences to HCV strains from of the United States of America, this may be because Iraq may have imported blood products from the United States or because of the US occupation of Iraq or as a result of the travel of Iraq is to the United States and therefore perhaps the transmission of the virus by one of the known transmission routes

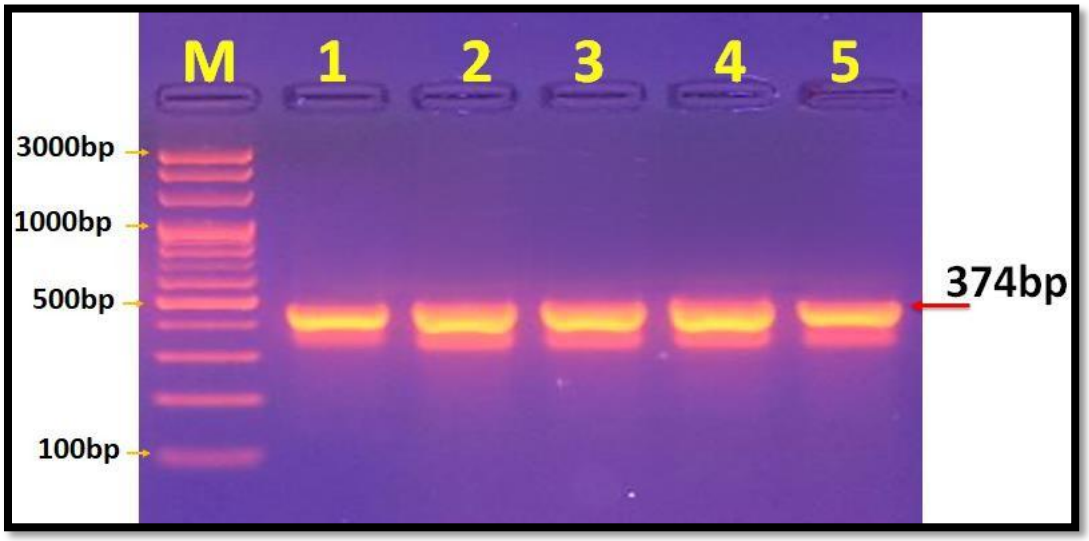


Figure (3-8): Agarose gel electrophoresis image that showed the one step RT-PCR product analysis of UTR-Polyprotein gene for detection Hepatitis C virus. Where Marker ladder (100-3000 bp), Lane (1-5) showed positive Hepatitis C virus at 374bp RT-PCR product size.

The fact of the identity of all investigated samples was determined as they belong to the same viral identity, whether in terms of the viral HCV organism or genotype. However, this notion provided a further indication of the viral identity and genotyping of these locally studied samples. HCV genotyping distribution has an important influence clinically on the morbidity, total costs, and duration of HCV treatment (Cuypers *et al.*, 2015). Therefore, for a better understanding of HCV epidemiology as well as the prevalence of its genotype pattern, performing HCV genotyping studies in Iraq is very important. In the study was conducted in Babylon Province this study gives a different estimation of HCV genotype distribution among infected HCV patients in Babylon from prevalent distribution in Iraq and Middle East Arab countries, but comparable to global distribution (37%) genotype 1 was the most frequent genotype detected followed by 3 (27.3%), 4 (20%), and 2 (2.4%), while mixed genotypes were detected in 13.3% (Dlshad *et*

al.,2018). While, the study conducted in Baghdad by (Karabulut *et al* ., 2018). Reported another result, Genotype HCV-1b showed higher prevalent (52.9%) among the recipients of anti-D Ig therapy while genotype HCV-3a (6.6%) was the lowest.

In Istanbul, Turkey, the HCV genotypes in patients with HCV showed Genotype 1a (82.5%) was the dominant genotype (AL-Kubaisy *et al.*, 2019).

This 5′-UTR -the based comprehensive tree has provided an extremely inclusive tool about the high ability of such 5′-UTR sequences to efficient identification HCV samples using this genetic fragment. This, in turn, gives a further indication of the power of the currently utilized 5′-UTR specific primers to discriminate among the currently investigated isolates.

Conclusions and Recommendations

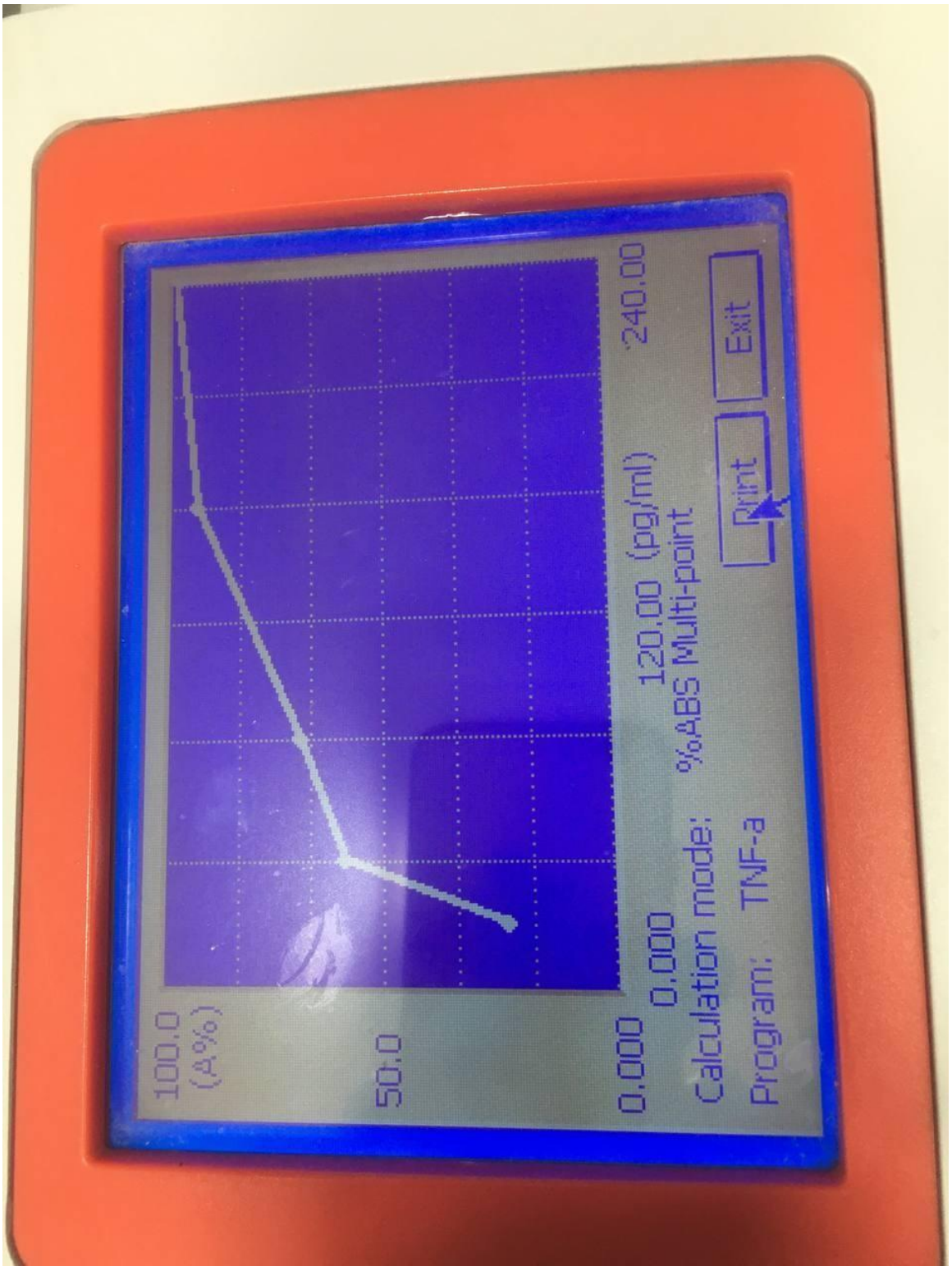
Conclusions:

1. High elevation of HBeAg in patient with HBV
2. High elevation TNF-a in patient with HBV and HCV
3. Female more frequency than male in HBV
4. Male more frequency than female in HCV
5. The risk of HBV reactivation for patients who had coinfection with dialysis no severe cases were recorded compared to the advanced cases of the disease who received immunosuppressive therapy and biological treatment
6. Health procedures, are not commensurate with the dangerous of the disease and the increase in the number of infections.
7. Gene sequencing for UTR is a very effective tool in determining the genotype and subtypes in HCV
8. UTR gene sequence match with USA

Recommendations:

1. The important role of the routine screening program for blood donors as well as pre-operations and pre-marital tests in the detection of people with hepatitis B& C virus
2. Immunization with vaccination to HBV before starting dialysis, thalassemia will reduce infection
3. Imposing a compulsory vaccination program for all and conducting periodic examinations for early detection of the disease and prevention to reduce the development of cases to advanced stages
4. Create a comprehensive database with accurate information about people with infection hepatitis B and C virus.

Appendices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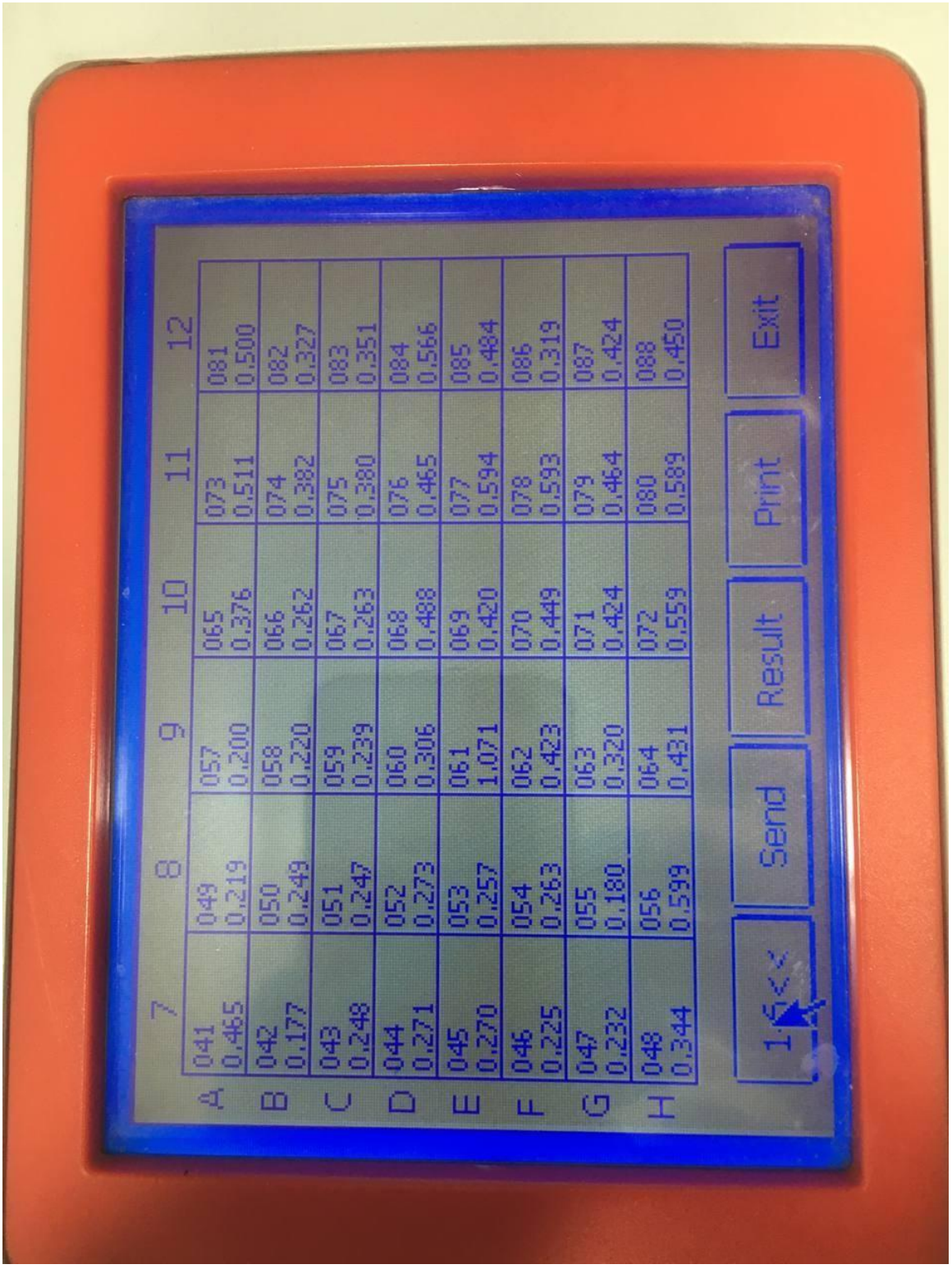
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C 043 0.248 051 0.247 059 0.239 067 0.263 075 0.380 083 0.351
D 044 0.271 052 0.273 060 0.306 068 0.488 076 0.465 084 0.566
E 045 0.270 053 0.257 061 1.071 069 0.420 077 0.594 085 0.484
F 046 0.225 054 0.263 062 0.423 070 0.449 078 0.593 086 0.319
G 047 0.232 055 0.180 063 0.320 071 0.424 079 0.464 087 0.424
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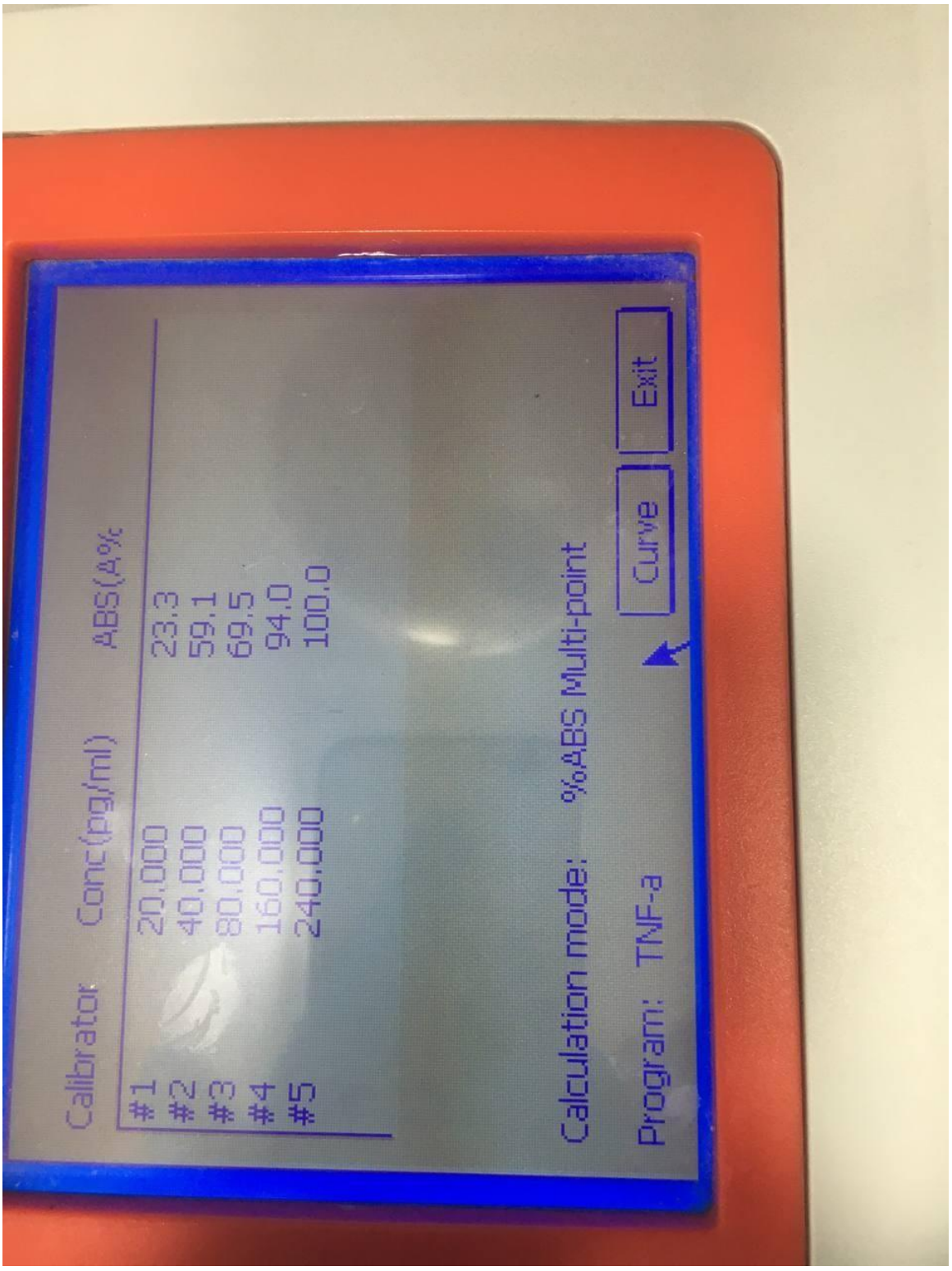
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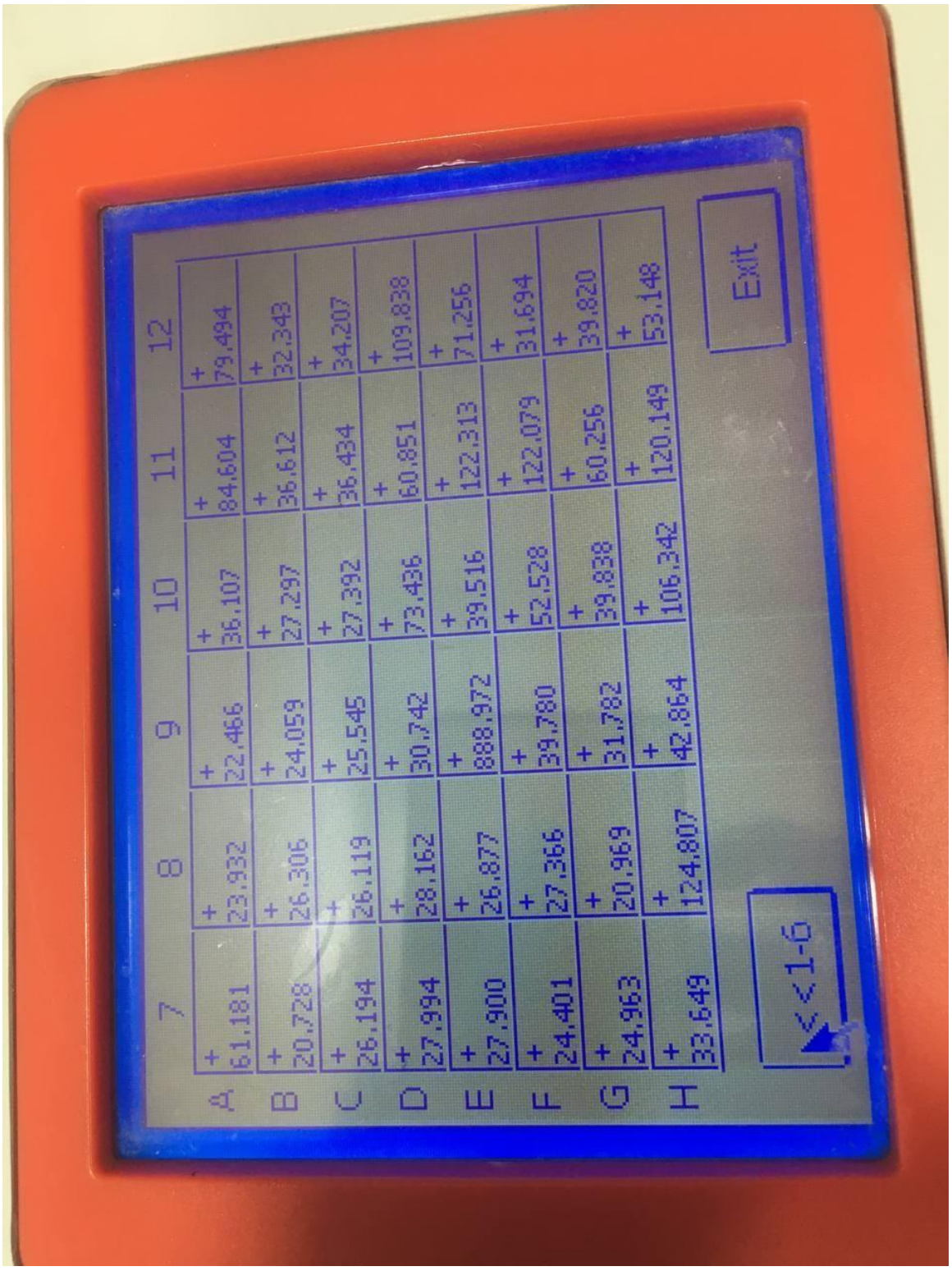
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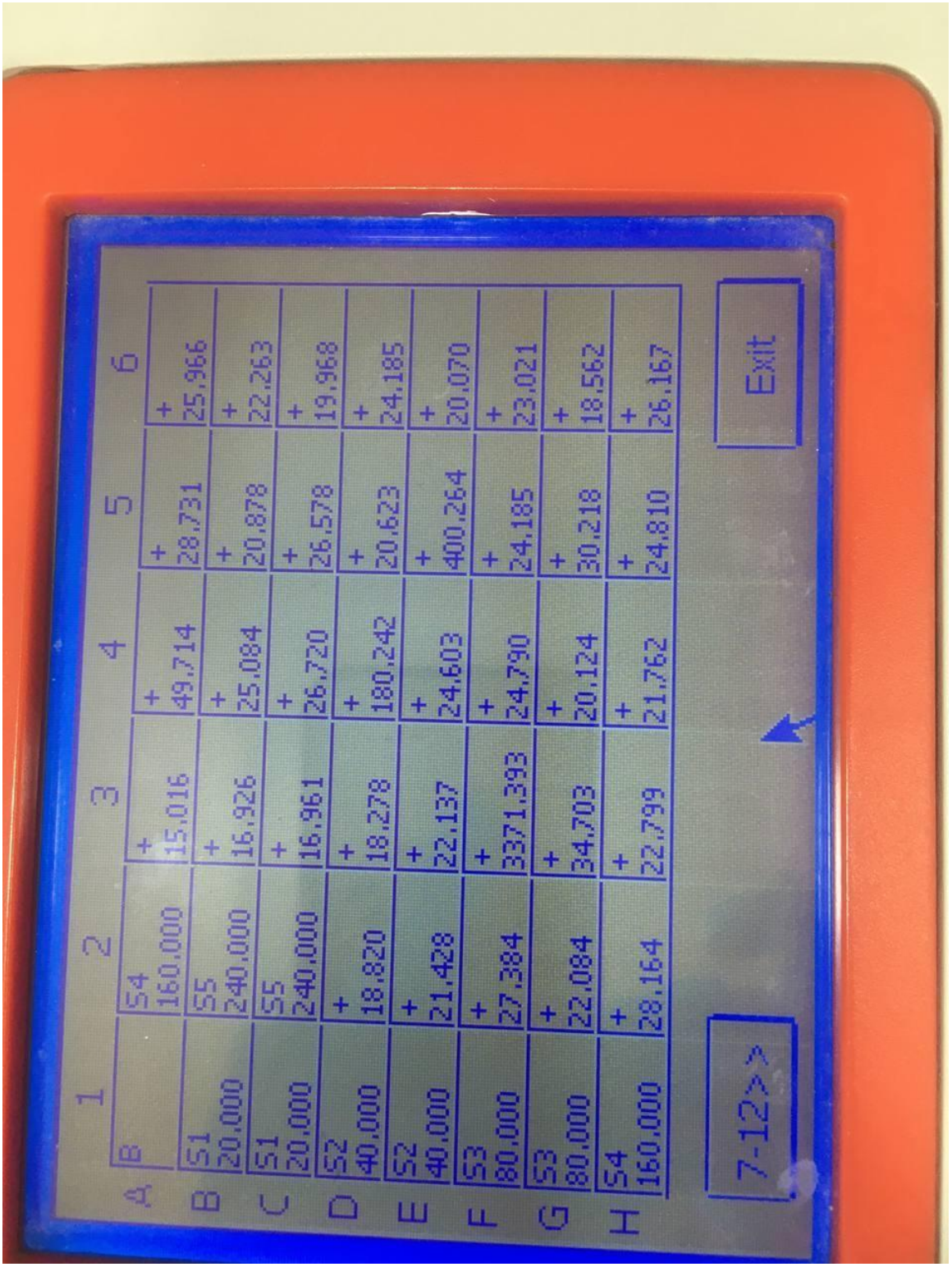
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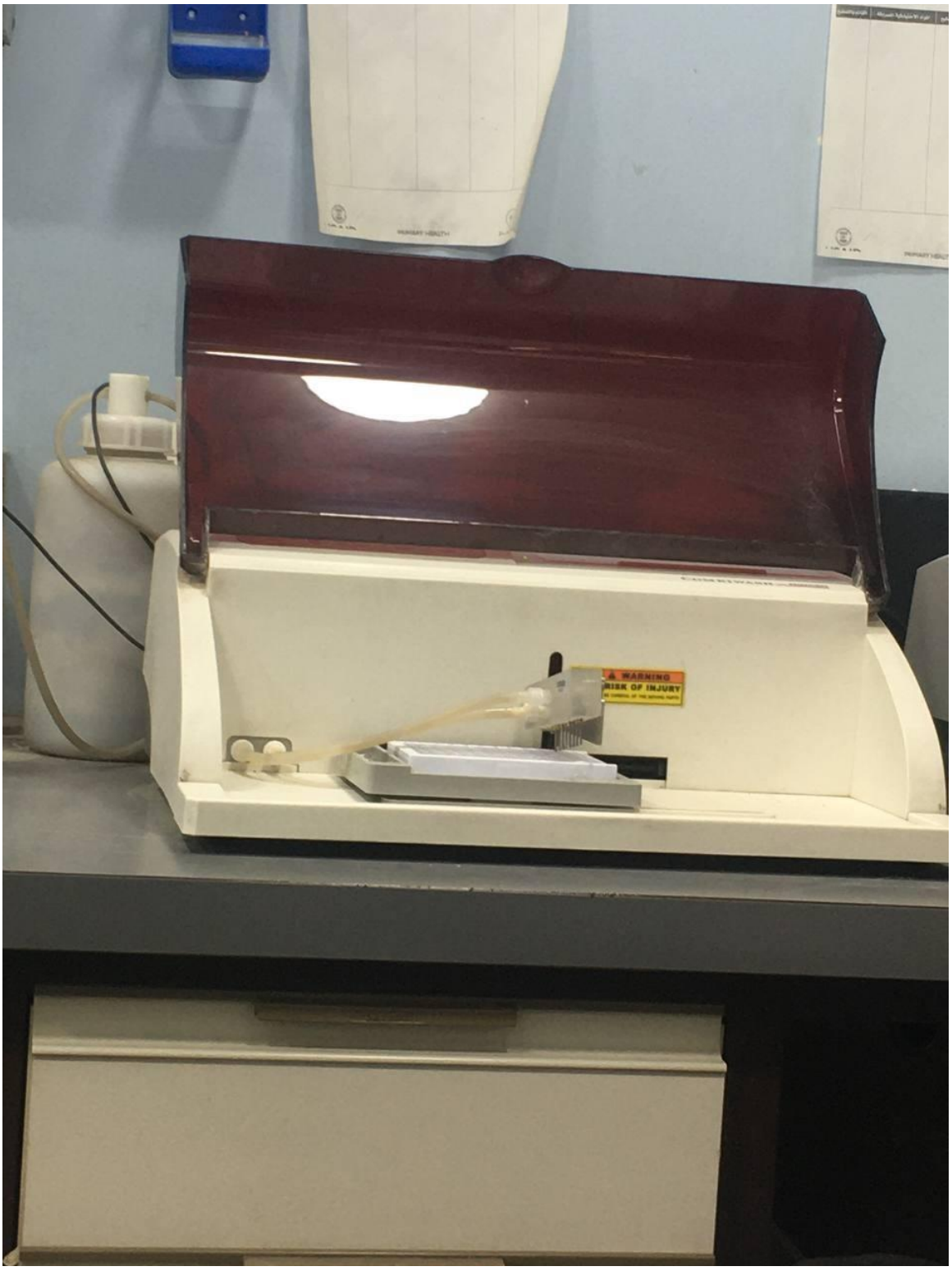
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الخلاصـــــــــــــــــة :-

فيروس التهاب الكبد B (HBV) مسؤول عن جميع أشكال التهاب الكبد التي تهدد صحة المجتمع. يخضع نمو الخلايا المناعية وانقسامها ونشاطها إلى وسطاء كيميائيين يُطلق عليهم السيتوكينات. تشير الدلائل إلى أن الاستجابات المناعية غير الكافية تساهم في استمرار فيروس التهاب الكبد B.

التهاب الكبد الوبائي سي هو التهاب في الكبد يسببه فيروس التهاب الكبد سي.

يمكن للفيروس أن يسبب التهاب الكبد الحاد والمزمن ، وتتراوح شدته من مرض خفيف إلى مرض خطير مدى الحياة بما في ذلك تليف الكبد والسرطان.

إجمالي 105 عينة من الأفراد المرضى خلال الفترة من أغسطس 2022 إلى نوفمبر 2022 ، تم مشاركه 20 مشاركًا سليمًا و 37 شخصًا مصابًا بعدوى التهاب الكبد الوبائي المزمن بي، وتم تحديد 48 شخصًا مصابًا بعدوى التهاب الكبد الوبائي المزمن سي.

تراوحت أعمار المرضى في البحث من 20 إلى 80 ، وتم تحليلهم جميعًا باستخدام عينات مصل (3 مل). تم تحديد مستويات HBV و TNF- و HBeAg في المصل باستخدام بواسطه (ELISA).

إيجابي شخص 37 HBV. أشارت نتائج اختبار HBsAg ELISA Kit إلى أن انتشار HBsAg كان أكبر في أولئك الذين تتراوح أعمارهم بين 35 إلى 49 = 12 (32.5٪) ، والأدنى في أولئك الذين تتراوح أعمارهم بين 20 إلى 34 = 8 (21.6٪) ومن 50 إلى 64 = 8 (21.6٪)) . تم اختبار 37 مريضًا إيجابيًا لـ HBsAg ، وكان 22 من الإناث (59.5 ٪) و 15 من الذكور (40.5 ٪). هذا يشير إلى أن انتشار عدوى HBsAg أعلى في الإناث منه عند الذكور.

أشارت النتائج 48 شخص موجب HCV إلى أن انتشار فيروس التهاب الكبد C كان أكبر في أولئك الذين تتراوح أعمارهم بين 41 إلى 60 = 22 (45.8٪) ، وأدنى في أولئك الذين تتراوح أعمارهم بين 21 إلى 40 = 8 (16.7٪) و HCV Ag. من بين 48 فردًا ثبتت إصابتهم بفيروس HCV Ag ، كان 21 (43.8 ٪) من الإناث و 27 (56.2 ٪) من الذكور. يشير هذا إلى أن الذكور أكثر عرضة من الإناث للإصابة بعدوى HCV Ag.

تم استخدام طريقة تسلسل الحمض النووي لتحديد وتحليل جين UTR-polyprotein لعزلات فيروس التهاب الكبد C المحلي (IQ.No.1 to IQ.No.5) وعزلات النمط الوراثي لفيروس التهاب الكبد الوبائي سي من NCBI-Blast.

تم الكشف عن أن عزلات فيروس التهاب الكبد الوبائي C المحلية مرتبطة ارتباطًا وثيقًا بالنوع الفرعي a1 من فيروس التهاب الكبد الوبائي (HQ113638.1 NCBI-BLAST) عند إجمالي التعديلات الجينية (0.05-0.3 ٪) ، وفقًا لدراسة العلاقة الوراثية لشجرة النشوء والتطور.

تراوحت هوية تسلسل التماثل الوراثي بين العزلات المحلية لفيروس التهاب الكبد الوبائي (IQ.No.1 to IQ.No.5) وفيروس التهاب الكبد الوبائي سي المحدد بواسطة NCBI BLAST من (99.41٪ إلى 99.61٪). تم بعد ذلك تقديم العزلات المحلية لفيروس التهاب الكبد الوبائي (معدل الذكاء رقم 1 إلى IQK.No.5) إلى بنك NCBI Genbank وأعطيت أرقام الدخول (OQ843893، OQ843894، OQ843895، OQ843896، OQ843897

فرع الأحياء المجهرية الطبية

دراسة عامل النخر الورمي الفا في المرضى المصابين بالتهاب الكبد الفايروسي نوع بي و سي المزمن و جين UTR في التهاب الكبد الفايروسي سي

رسالة مقدمة إلى

مجلس كلية الطب / جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم / الاحياء المجهرية الطبية

من قبل

عنيده خريبط عبد

بكالوريوس تحليلات مرضيه ــ كلية العلوم /جامعة ذي قار (2016)

بأشـــــراف

أ د . زيتون عبد الرضا الخفاجي

جامعة بابل / كلية الطب

أ د. علي جابر المعموري

جامعة بابل / كلية الطب

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