TURKISH REPUBLIC OF NORTHERN CYPRUS

GRADUATE SCHOOL OF HEALTH SCIENCES

MOLECULAR CHARACTERISTICS OF HEPATITIS B VIRUS STRAINS ISOLATED FROM TURKISH PATIENTS IN NORTHERN CYPRUS

ÜNAL SÜMER

PhD THESIS

MEDICAL & CLINICAL MICROBIOLOGY PROGRAMME

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Ünal SÜMER

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"Life cannot have had a random beginning ... The trouble is that there are about 2000 enzymes, and the chance of obtaining them all in a random trial is only one part in 10^{40000}, an outrageously small probability that could not be faced even if the whole universe consisted of organic soup."

Fred Hoyle

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List of abbreviations & symbols

<	**Less than**
>	**More than**
3TC/LAM	**Lamivudine**
aa	**Amino Acid**
ADAPVEM	**Antiviral Drug Associated Potential Vaccine Escape Mutant**
ADV	**Adefovir**
ALT	**Alanine transaminase**
AntiHBc	**Hepatitis B virus core antibody**
AntiHBcIgG	**Hepatitis B virus core antibody Immunoglobulin G**
AntiHBcIgM	**Hepatitis B virus core antibody Immunoglobulin M**
AntiHBe	**Hepatitis B virus envelope antibody**
AntiHBs	**Hepatitis B virus surface antibody**
APC	**Antigen presenting cell**
AST	**Aspartate (amino) transaminase**
bp	**Base pairs**
cccDNA	**Covalently closed circular DNA**
CDC	**Centres for Disease Control**
CHB	**Chronic hepatitis B**
DC	**Dendritic cell**
DNA	**Deoxyribose nucleic acid**
ELISA	**Enzyme linked immunosorbent assay**

ETV	**Entecavir**
FDA	**Food and Drug Administration**
FP	**False positive**
FTC	**Emtricitabine**
GGT	**Gamma glutamyl transferase**
HAV	**Hepatitis A virus**
HBcAg	**Hepatitis B virus core antigen**
HBeAg	**Hepatitis B virus envelope antigen**
HBIg	**HBV immunoglobulin**
HBsAg	**Hepatitis B virus surface antigen**
HBV	**Hepatitis B virus**
HBX	**HBV X protein**
HCC	**Hepatocellularcarcinoma**
HCV	**Hepatitis C virus**
HDV	**Hepatitis D virus**
HEV	**Hepatitis E virus**
HIV	**Human immunodeficiency virus**
IDU	**Injection drug use**
IFN	**Interferon**
IL	**Interleukin**
INN/L FMAU	**Clevudine**
IU/ml	**International units per millilitre**
kb	**Kilobytes**

kDa	**Kilo daltons**
L	**Large**
LC	**Liver cirrhosis/Liver cancer**
LdT/LDT	**Telbivudine**
M	**Middle**
MDR	**Multi drug resistance**
mIU/ml	**Micro international units per millilitre**
MOH	**Ministry of Health**
MOI	**Ministry of Internal Affairs**
mRNA	**Messenger RNA**
MTC	**Mother-to-child**
MTM	**Men to men**
N	**Normal**
NA	**Nucleostide analogue**
NAT	**Nucleic acid test**
NDA	**No data available**
NK	**Natural killer cell**
NKT	**Natural killer T cell**
NRTI	**Nucleoside reverse transcriptase inhibitor**
nt	**Nucleotide**
NTCP	**Sodium taurocholate cotransporting polypeptide**
NtRTI	**Nucleotide reverse transcriptase inhibitor**
OBI	**Occult HBV infection**

ORF	**Open reading frame**
P	**Partly resistant**
PCR	**Polymerase chain reaction**
PEG-IFN	**Pegylated IFN**
pgRNA	**Pre genomic RNA**
PPD	**Pure protein derivative**
R	**Resistant**
rcDNA	**Relaxed circular DNA**
RNA	**Ribonucleic acid**
rt	**Reverse transcriptase (region)**
RT	**Reverse transcription**
rtPCR	**Real time PCR**
S	**Small / susceptible / sensitive**
S/CO	**Sample cut off (value)**
STD	**Sexually transmitted disease**
TDF	**Tenofovir**
TGF	**Transforming growth factor**
TLR	**Toll like receptor**
TNF	**Tumour necrosis factor**
TRNC	**Turkish Republic of Northern Cyprus**
U/L	**Units per litre**
USA	**United States of America**
WHO	**World Health Organisation**

YMDD	**Tyrosine-methionine-aspartate-aspartate**
α	**Alpha**
β	**Beta**
γ	**Gamma**

Özet

BSc. MSc/MRes. Ünal SÜMER

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Hepatit B virüsü (HBV) ile enfekte olan hastalarda aktif bir sürveyans gereksiniminden ötürü, dolaşımdaki HBV suşlarının tanımlanması ve araştırılması önem arz etmektedir. Bu önem, moleküler ve epidemiyolojik özellliklerin bilinmesini kesin kılar. Bu amaç ile yola çıkarak Kuzey Kıbrıs Türk Cumhuriyeti (KKTC)'deki HBV genotip/sübgenotip/serotip dağılımının izole bir topluluk olan KKTCde yeniden belirlenmesi, antiviral tedavi ile ilişkili olan primer, parsiyel ve kompensatuar mutasyonları (*pol* geni) ve HbsAg sübstitisyon mutasyonları (*S* geni); HBIg, aşı, tanı ve bağışık yanıt kaçaklarını analiz etmeyi amaçladık. Yakın Doğu Üniversitesi Hastahanesi Laboratuvarı ve Lancet Tıbbi Tahlil Laboratuvarlarında HbsAg testi yaptıran Türk hastalarda pozitif olarak saptanan numuneler elemeler sonrası calışmaya alındı. Çalışmada, pozitif olan serumların HbsAg düzeyleri HbsAg Qual II (Architect i1000SR/i2000SR, Abbott) kullanılarak teyit edildi. Elde edilen numuneler izolasyon sonrası genotip/sübgenotip tayini için *pol* geni *rt* bölgesi 80-250 aminoasitler arası sekanslama ve amplifikasyon yapıldı (Qiagen Artus HBV RGQ). Yine, ayni aminoasit bölgeleri analiz edilerek antiviral ilaç direnci ve *S* geni mutasyonları tarandı. Aynı sekanslar kullanılarak, numunelerin genotipleri, Geno2pheno ilaç direnci programı (Center of Advanced European Studies and Research, Germany) kullanılarak analiz edildi. 170 numunenin 108'i sekanslanabildi. Bu 108 numuneye yapılan detaylı analizlerde, 7'sinde (%6) HBIg kaçağı, 9'unda (%8) aşı kaçağı, 10'unda (%9) misdiyagnoz ve 9' unda (%8) immün kaçış mutasyonları saptandı. 3 adet numunede (%3) kombine *S* geni mutasyon paterni gözlendi. Yine 108 numunenin 3'ünde (%3) primer rezistans mutasyonları, 2'sinde parsiyel rezistans mutasyonarı ve 29'unda (%27) kompensatuar mutasyonlar gözlendi. 2 numunede (%2) daha önce gözlenmeyen ADAPVEM mutasyon patternleri gözlendi. 108 numunenin, 106'sı (%98) D/D1, 1'i (%1) D/D2 ve 1'i (%1) E genotip/sübgenotip olarak saptandı. Serotipler ise CLC Sequence viewer (CLC bio A/S, Qiagen, Danimarka) kullanılarak 96 numunede (%99) *ayw2* ve 1 numunede

(%1) *ayw3* olarak saptandı. Genotip dağılımı için filogenetik agaç, yine CLC Sequence viewer (CLC bio A/S, Qiagen, Danimarka) kullanılarak oluşturuldu ve geno2pheno sonuçları ile örtüştü. Bulgularımız doğrultusunda söyleyebiliriz ki KKTC'de yaşayan Kıbrıslı Türk ve Türkiye asıllı Türk vatandaşlarında genotip D/D1 baskındır. Dolaşımda az da olsa D/D2 ve E suşlarıda mevcuttur. Bununla birlikte antiviral ve HbsAg kaçış mutasyonlarının epidemiyolojik etkisinin önemli olduğu görülmektedir. Bu bilgilerin dışında yapılan araştırmada KKTC de ikamet eden ve HbsAg pozitif olan Türk vatandaşlarının sadece 3 te 1'i yani %36.4'ü tedavi almaktadır. Ayrıca, konsimatris popülasyonunun HbsAg pozitiflik oranının (%0.58), sivil topluma nazaran daha yüksek olduğu görülmektedir.

Anahtar kelimeler: Kuzey Kıbrıs Türk Cumhuriyeti, Hepatit B virüsü, Epidemiyoloji, *Pol* geni mutasyonları, *S* geni mutasyonları.

Abstract

BSc. MSc/MRes. Ünal SÜMER

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There is a high demand on genotype information and investigation regarding HBV infected people. This importance rules to be informed regarding molecular and epidemiological specifications. With the intention of exploring this important issue, we aimed to analyse dispersion of genotype/subgenotype/serotypes of HBV infected patients together with *pol* gene mutations (primary, partial and compensatory) which are related to antiviral therapy and *S* gene mutations (HBIg, Vaccine, Diagnosis and Immune selected escape) in Turkish Republic of Northern Cyprus (TRNC). HbsAg positive serum samples of Turkish patients were collected from Near East University Hospital Laboratory and Lancet Medical Diagnostic Laboratory and were sorted as accordingly. Samples were analysed using HbsAg Qual II (Architect i1000SR/i2000SR, Abbott). Following isolation and amplification, genotype/subgenotype analysis were performed in *pol* gene *rt* region between 80-250 amino acids (Qiagen Artus HBV RGQ). While analysing the same amino acid regions, antiviral resistances and *S* gene mutations were also screened. Information generated from genotypes/subgenotypes of the samples and antiviral resistances were tabulated using geno2pheno programme (Center of Advanced European Studies and Research, Germany). Only 108 of the 170 samples could be sequenced. *S* gene mutations observed in these 108 sequenced samples were: 7 samples (6%) had HBIg escape, 9 samples (8%) had vaccine escape, 10 samples (9%) had misdiagnosis and 9 samples (8%) had immune escape mutations. 3 of the samples (3%) had a combined mutation pattern. *Pol* gene (*rt*) mutations that were observed in 108 sequenced samples were; 3 samples (3%) primary resistance mutations, 2 samples (2%) partial resistance mutations, and in 29 (27%) samples compensatory mutations. 2 (2%) samples had ADAPVEM mutation patterns which was not observed before. Out of 108 sequenced samples, 106 were (98%) D/D1, 1 was (1%) D/D2 and 1 was (1%) E genotype/subgenotype. Serotypes were detected using CLC Sequence viewer (CLC bio A/S, Qiagen, Denmark); and, 96 samples were (99%) *ayw2* and 1 sample

was (1%) *ayw3*. Phylogenetic tree was also constructed using CLC Sequence viewer (CLC bio A/S, Qiagen, Denmark) for comparison and results were identical with geno2pheno results. From the results we obtained, we can evidently conclude that genotype D/D1 is the dominant strain in TRNC in Turkish Cypriots and Turkish patients. However, there are also other genotypes within the circulation such as D/D2 and E; but, in minor quantities. Along with this information, HbsAg escape mutations and antiviral resistances were observed which indicates the importance of epidemiologic significance. Other than this information, in this study, it was found that only one-third of the Turkish HbsAg positive population (36.4%) are currently being treated. Also, sex worker population has a higher HbsAg positivity (0.58%) when compared to the rest of the patient population.

Keywords: Turkish Republic of Northern Cyprus, Hepatitis B virus, Epidemiology, *Pol* gene mutations, *S* gene mutations.

CHAPTER 1: INTRODUCTION

1.1 History

Despite the effort, the evolution rate and origin of this human pathogen remains unknown. It is known that Hepatitis B virus (HBV) is chronically carried by approximately 350 million people around the globe and one million people die as a result of it (Patterson Ross et al., 2018).

Lack of consensus and research studies make it tough to rebuild a timescale for the origin of HBV and estimate its evolutionary rate. It could be also lamented that the evolutionary rates of HBV are time dependant and also influenced by diverse population dynamics of the genotypes (Zehender et al., 2014).

Origin and evolution of HBV has been a long standing question. There are few conflicting hypotheses concerning this question. It has been suggested that HBV originated in the new world and then span around the globe as a outcome of European colonisations over the past few-hundred years (this conflicts with opinion of HBV widespread in old world apes). Also, it has been proposed that there was a co-divergence of HBV and primate host over periods of 10 to 35 million years. (this demonstrates slow evolution rate, which is incompatible with current molecular approximations, which indicates faster evolution rates). Lastly, it has been proposed that HBV was presented in modern people anatomically and spread as outcome of re locations. All these conflicts raise a common question: how fast is the evolution of HBV? For some, HBV can be viewed as a slowly evolving virus and for some it may be defined as a highly mutable virus which evolves at a faster rate; when compared with other retroviruses (Zehender et al., 2014).

Viral hepatitis dates all the way back to 5^{th} century before Christ. First records of hepatitis, named as yellow jaundice by Pope in the 8^{th} century, and descriptions of the disease can be found in the writings of Hippocrates. Hippocrates described hepatitis as “a disease which was produced by black bile when it flows into the liver” and listed symptoms such as anorexia, fever, vomiting, pale-yellow complexion and pain (Lai and Locarnini, 2002).

The very modern encounter was in year 1963, when Blumberg and his co workers who were researching a serum protein at that time, discovered Hepatitis B virus Surface Antigen (HbsAg) in the sera of an Aboriginal Australian person (Blumberg et al., 1965). Following this discovery, HBV was distinguished from other hepatitis viruses (Blumberg, 1977). Few years after, Dane and his colleagues signalled the occurrence of a virus like particle in the serum of HBV infected patients through electron microscopy. Dane's discovery was then confirmed with the identification of polymerase activity (Alberti et al., 1978). Following these discoveries, subsequently genome and other proteins of HBV was identified and HBV became the first human hepatitis virus (He et al., 1985).

A proposed timeline for HBV research and treatment milestones was created by Thomas et al. (2015). Virologic milestones could be listed as follows:

- 17^{th}-19^{th} centuries: Outbreaks of epidemics of jaundice in civil and military populations during wars.
- 1885: Characterisation of outbreaks of serum hepatitis subsequent to a "vaccination".
- 1908: McDonald hypothesises that the infectious jaundice is caused by a virus.
- 1939 – 1945: World War 2; a chain of outbreaks after immunisation for yellow fever and measles.
- 1947: MacCallum classified viral hepatitis into two categories, where viral hepatitis B was namely classified as "serum hepatitis".
- 1963: Blumberg discovered Australia Antigen (HBsAg).
- 1970: Dane explored the dane particle (whole HBV particle).

Aside from these, therapeutic milestones can be documented as follows:

- 1981: Plasma derived HBV vaccine.
- 1992: Food and Drug Administration (FDA) approved the interferon alpha (α) 2b to treat hepatitis B.
- 1998: FDA accepted Lamivudine to treat hepatitis B.
- 2002 – 2006: Adefovir dipivoxil, peginterferon α 2a, entecavir and telbivudine were respectively approved to treat hepatitis B.
- 2012: FDA approved tenofovir to treat hepatitis B.

1.2 Taxonomy

It could be depicted that all viruses can be divided into different classes, which each have their own approach to transfer genetic information to the next generation. One process which all viruses share can be illuminated that they should perform messenger Ribonucleic acid (mRNA) synthesis. Using mRNA demonstrates that the cellular ribosomes of the host organism are dispensed. By looking at the specific machinery which virus synthesises mRNA, viruses can be segregated with the light of Baltimore classification. This classification categorizes viruses into families by relying on their genomes. It can be articulated that HBV falls into the group VII, deoxyribose nucleic acid (DNA) reverse transcribing of Baltimore classification (https://viralzone.expasy.org/101?outline=all_by_species and Baltimore 1971, Accession date: 22 May 2017).

After cloning and sequencing HBV genome, several related viruses were recognized in woodchucks, ground squirrels and lastly pekin duck. Consequently, new viruses were also explored in mammals and birds which were also cloned. Furthermore, all these viruses are classified in the family of *hepadnaviridae*. This family includes genus *orthohepadnavirus* (mammals) and *avihepadnavirus* (birds). These family of viruses and their hosts are portrayed by the Tables 1.2.1 & 1.2.2 below.

Table1.2.1. *Hepadnaviridae* family, genus *orthohepadnaviridae*. Viruses and their host(s). Table was created using data from Schafer 2007 (Schaefer 2007).

Orthohepadnavirus	**Host (species)**
Hepatitis B Virus	Man (*Homo sapiens sapiens*)
Chimpanzee Hepatitis B Virus	Chimpanzee (*Pan troglodytes*)
Gibbon Hepatitis B Virus	White handed gibbon (*Hylobates lar*)
Orangutan Hepatitis B Virus	Orangutan (*Pongo pygmaeus pygmaeus*)
Gorilla Hepatitis B Virus	Gorilla (*Gorilla gorilla*)
Woolly Monkey Hepatitis B Virus	Woolly monkey (*Lagothrix lagothrica*)
Woodchuck Hepatitis Virus	Woodchuck (*Marmot monax*)
Ground squirrel Hepatitis Virus	Ground Squirrel (*Spermophilus beecheyi*)
Arctic Squirrel Hepatitis Virus	Arctic Squirrel(*Spermophylus parryi kennicotti*)

Table1.2.2. *Hepadnaviridae* family, genus *avihepadnaviridae*. Viruses and their host(s). Table was created using data from Schafer 2007 (Schaefer 2007).

Avihepadnavirus	**Host (species)**
Duck Hepatitis B Virus	Pekin duck (*Anas domesticus*)
Grey Teal Hepatitis B Virus	Grey Teal (*Anas gibberifrons gracilis*)
Heron Hepatitis B Virus	Heron (*Adrea cinerea*)
Maned Duck Hepatitis B Virus	Maned duck (*Chenonetta jubata*)
Ross Goose Hepatitis Virus	Ross Goose (*Anser rossi*)
Snow Goose Hepatitis B Virus	Snow Goose (*Anser caerulescens*)
Stork Hepatitis B Virus	White Stork (*Ciconia cicnonia*) Demoiselle cranes (*Anthropoides virgo*)
Crane Hepatitis B Virus	Grey crowned cranes (*Balearica regulorum*)

1.3 Infection

HBV is a blood-borne pathogen. Once it is in the blood, it reaches the target organ, the liver, through bloodstream. The cell entrance progression comprises a non cell type specific attachment to the cell associated heparan sulfate proteoglycans which is then followed by the irreversible attachment of the virus to a hepatocyte specific receptor. Although the differentiation status and polarisation of the hepatocyte plays a prominent role in viral entry; in 2012, Yan and co-workers identified the sodium taurocholate cotransporting polypeptide (NTCP) as the cellular receptor for HBV to enter the hepatocyte (Yan et al., 2012).

After viral entry, the other steps are poorly understood but investigational evidence shows that HBV move in the hepatocyte via endocytosis, the nucleocapsids are then moved to nuclear periphery through microtubules where they come in contact with nuclear pore complexes (Macovei et al., 2010; Altındiş et al., 2006). It could be mentioned that at this stage, the mature capsids disintegrate which allows core proteins and viral genome to be released into the nucleoplasm. Here, HBV is shown to use cellular DNA repair enzymes such as TDP1 or TDP2 to remove the viral polymerase and start covalently closed circular DNA (cccDNA) biogenesis (Dandri and Petersen, 2016; Koniger et al., 2014).

Furthermore, the cccDNA, a mini-chromosome exploit the cellular transcription machinery to produce all of the viral ribonucleic acid (RNA)'s necessary for the production of proteins and viral replication that takes place in the cytoplasm after the reverse transcription of pre-genomic RNA (pgRNA). It can be stated that viral transcription is regulated by several transcription factors, corepressors, coactivators and lastly by modifying enzymes. In addition to these, pgRNA offers all the components which are required for the production of HBV DNA, containing nucleocapsid and the production of envelope which depends on the transcription of sub genomic HBV RNA (*preS* and *S*) (Levrero et al., 2009).

It can be indicated that both sub-genomic and pregenomic RNA are transported into the cytoplasm; and, at this stage, is either translated or used as a template for the production of progeny genome. The binding of polymerase to pgRNA, with core proteins starts the packaging process. Within the nucleocapsid, the reverse transcription takes place which occurs away and is protected from innate immune mechanisms. The first product is a single strand DNA which has negative charge, and remains linked to the polymerase enzyme. The pgRNA is degraded, only few nucleotides are not degraded and these nucleotides serve as primers for positive stranded DNA synthesis (Nassal, 2008).

The final replication process, assembly and release of relaxed circiular DNA (rcDNA), is not completely understood; however, some studies have demonstrated that release of viral particles occur via multivesicular structures whereas sub-viral

particles are released through general secretion (Figure 1.3.1) (Hoffmann et al., 2013).

In recent studies, it has been found that HBV-miR3, which is a micro RNA encoded by the virus itself, targets its own transcripts to reduce the HBV infection. This contributes to creation of a better understanding about how HBV leads to minor damage in liver cells and how it establishes and maintains a persistent infection (Yang et al., 2017).

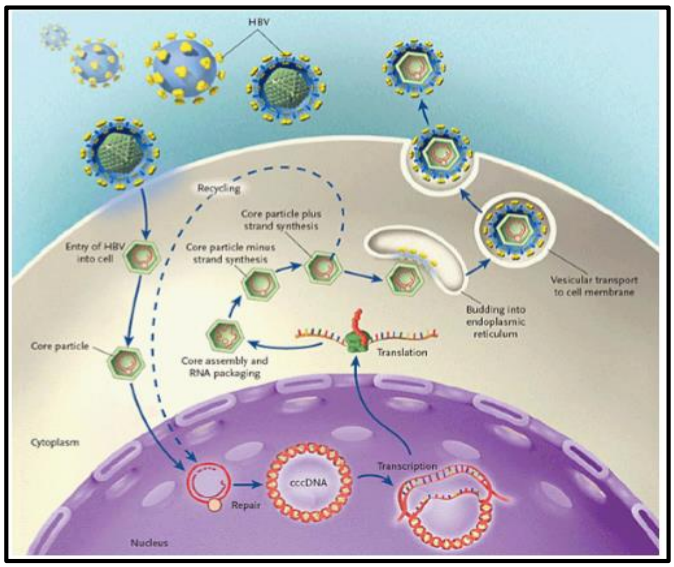

Figure 1.3.1. Life cycle of HBV. HBV virus particles bind to receptors on the hepatocyte surface and get internalised, followed by nucleocapsids that being released into the cytoplasm and they migrate to nucleus where the partially double stranded DNA genome gets converted to cccDNA. The cccDNA aids as a template for transcription of pgRNA and is translated in the cytoplasm into viral proteins. Viral capsids are assembled by the use of pgRNA, polymerase and core proteins. The pgRNA is then reverse transcribed into viral DNA within the capsid and mature nucleocapsids either gets recycled into the nucleus to be converted to cccDNA or they bud into the golgi complex where envelop eventually forms and they get exported from the cell via endoplasmic reticulum. (Ganem and Prince, 2004)

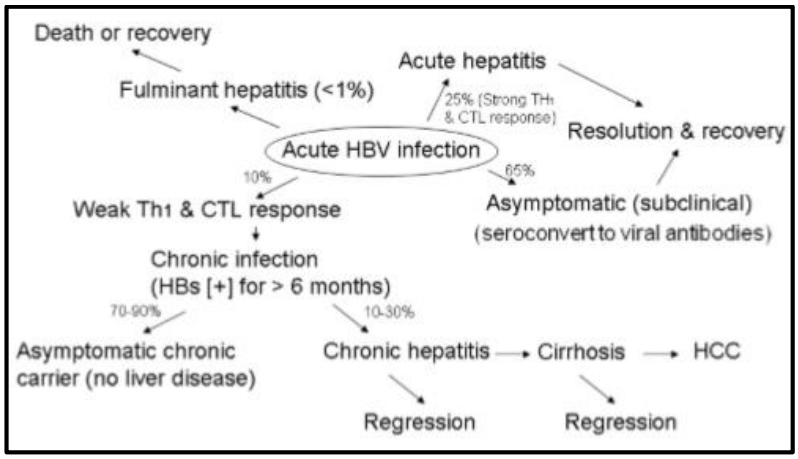

Figure 1.3.2. Outcomes of HBV infection. Upon acute infection, around 10% of the cases will become chronic infections (out of these, 70-90% will be asymptomatic carriers, whereas 10-30% becomes chronic hepatitis patients followed by cirrhosis and eventually hepatocellularcarcinoma), 65% of the patients will show no symptoms and become subclinical and 25% of acute hepatitis patients will have resolved infection and recover; only less than 1% of the patients will have fulminant hepatitis where recovery is rare (Fieltson and Larkin, 2001).

It could be illuminated that other than hepatic infection (demonstrated by Figure 1.3.2), HBV also has extrahepatic manifestations. These occur in 1-10% of the individuals and are believed to be caused by immune complex mediated damage related to high levels of HBV persistence in blood. These manifestations are explained below.

Serum sickness like syndrome occurs during acute HBV infection and usually manifests with jaundice. Clinical features are rash, fever and polyarteritis. These symptoms may persist throughout the infection but usually are parallel to the virus load, in other terms quick clearance of virus leads to quick resolution. The reason for this illness could be identified as the immune complexes formed during infection activating the complement pathways which ends up as complement-mediated injury (Liang, 2009).

Necrotizing vasculitis, which is also named as polyarteritis nodosa is the vascular injury of blood vessels due to immune mediated vascular damage and may affect large, medium or small vessels. Clinical symptoms include high fever, anaemia and leucocytosis. Multisystem involvement such as renal disease, heart disease and neurological disorders are common (Liang, 2009).

Membranous glomerulonephritis is a form of nephropathy and children are more likely to suffer from membranous glomerulonephritis when compared with adults. In HBV envelope antigen (HBeAg) seroconversion, children usually suffers from less symptoms but for minority of adults, renal failure is inevitable.

Papular acrodermatitis which is also called as giannotti crosti syndrome, can be expressed as a skin manifestation of HBV which occurs during childhood. Macopapular, erythematous and nonpruritic skin lesions could occur on face and extremities. The disease is seen together with lymphadenopathy and hepatomegaly (Liang, 2009).

1.3.1 Acute or transient infection

When HBV infection occurs and HBsAg gets eradicated from serum, alanine transaminase (ALT) levels return to normal in less than 6 months. Traces of HBV DNA can still be found by using sensitive polymerase chain reaction (PCR) techniques. During acute infection, strong cell mediated immune response occurs at the surface, core and polymerase protein of the HBV. Also, humoral immune response is seen for surface, core, envelope antigens and polymerase proteins (portrayed by Figure 1.3.1.1) (Kara et al., 2004).

Anti Hepatitis B surface antibodies (AntiHBs) produced by humoral immunity are able to bind HBV surface proteins which serves for 2 purposes. The first purpose could be declared as to facilitate the elimination of virus from blood by opsonisation. The second purpose which is considered as the most crucial one is to block receptors for virus attachment to the other hepatocytes (Alkbar et al., 1999).

CD8+ T cells (cytotoxic T cells) cause infected cells to undergo apoptosis and release cytokines which leads to non-cytotoxic clearance of the virus. Early innate immune response; in other terms, production of inteferon (IFN) α, beta (β), and interleukin (IL) 2 occur. Noordeen (2015) indicates that immune cells such as natural killer cells (NK), macrophages, granulocytes, natural killer T cells (NKT) and

lastly dendritic cells (DC) have viral clearence features however their roles are poorly understood.

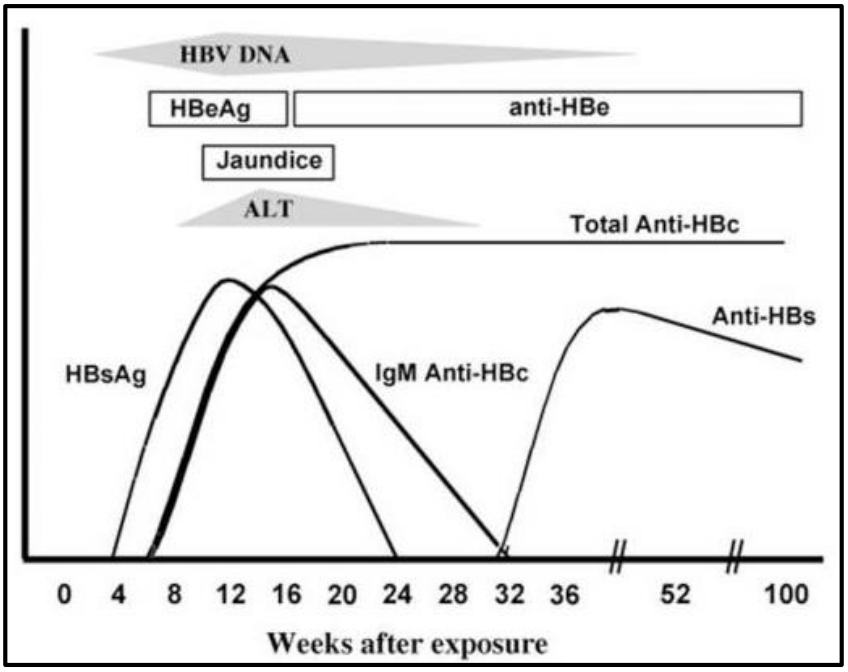

Figure 1.3.1.1. Acute HBV infection. 66.7% of the acute HBV infection patients have mild and symptomatic illness. 33.3% of the adults with acute infection will develop clinical symptoms wheresome are mild and some are severe symptoms. The clinical incubation period averages 2-3 months and can range 1-6 months following exposure. The incubation period is followed by preicteric period which during this phase ALT levels peak and high level of HBV DNA and HBsAg are detectable. This phase lasts 2-7 days and is followed by jaundice. The icteric phase lasts 1-2 weeks where viral levels are decreased. In convalescence phase, jaundice resolves, symptoms may last for months. At this phase, HBsAg is cleared and HBV DNA levels fall below detectable levels. HBsAg can be detectable 0-3 weeks followed by anti-core IgM/IgG. The typical AntiHBs production starts around 32^{nd} week and time between HBsAg disappearance and AntiHbs appearance; 24^{th} – 32^{nd} weeks is called as the window period (Liang, 2009).

1.3.2 Chronic infection

It could be indicated that when there is a failure to clear HBsAg from serum for more than 6 months, chronicity occurs (illustrated by Figure 1.3.2.1). It can be lamented that 4 phases of chronic HBV infection are present. These phases could be signified as immune tolerance, immune clearance, residual/inactive/immune control, reactivation/immune escape. Explanations of these phases are as follows;

Immune tolerance phase: Occurs when the infection is contracted during childhood and persists for around 1-2 decades. At immune tolerance phase, high levels of HBV DNA (usually 2 x 10^{6-7} international units/milliliter (IU/ml)) with normal ALT levels and minimal inflammation and/or fibrosis are encountered. HBeAg is present in the serum. In immune tolerance phase, HBeAg is the protein which induces T cell tolerance. In addition to this, liver histology in this phase exhibits non-specific histologic changes. During this phase treatment is not recommended since there is a lack of proven efficacy (Noordeen, 2015; Croagh and Lubel, 2014).

Immune clearance phase: Is the e antigen positive chronic hepatitis B (CHB). Beside of this, increased levels of HBV DNA and Hepatitis B virus core antigen (HBcAg) within the hepatocytes yields to immune mediated death of hepatocytes and therefore results in raised ALT levels in the serum. But the immune response is not enough to clear the virus, therefore constant liver damage occurs. Based on the nature of this phase, patients may develop cirrhosis or fibrosis. After repeated attempts to clear the virus, viral replication would be suppressed and immune system would have reduced amount of infected hepatocytes; and, this causes transition to Hepatitis B virus envelope antibody (AntiHBe) positivity. Alpha feto protein (AFP) levels may also rise at this very specific phase. The duration of this phase has shown that it fuels development of complications. It could be elicited that patients who are at least 40 years old and experienced with seroconvertions are more likely to have a higher risk of advance of hepatocellularcarcinoma (HCC) and liver cancer (LC) when compared to those who have encountered with seroconversion before the age of 30 (Rehermann, 2000; Croagh and Lubel, 2014).

Residual/inactive/immune control phase: Once the patients are seroconverted to AntiHBe, they are considered to shift to the inactive carrier stage. It can be mentioned that at residual/inactive/immune control phase; HBV DNA levels are lowered and ALT levels are identified as insistent. Moreover, it could be stated that 0.2 – 2% of the chronic HBV patients manage to clear HBsAg and seroconvert to Anti HBs (Alkbar et al., 1999).

Reactivation/Immune escape: It can be mentioned that almost 30% of patients will undergo re-activation with reversion to HBeAg positivity and majority of these patients acquired the virus during childhood. Noordeen (2015) had solicitated that

patients after seroconversion to AntiHbe may reactivate without having any symptoms of HCC or LC.

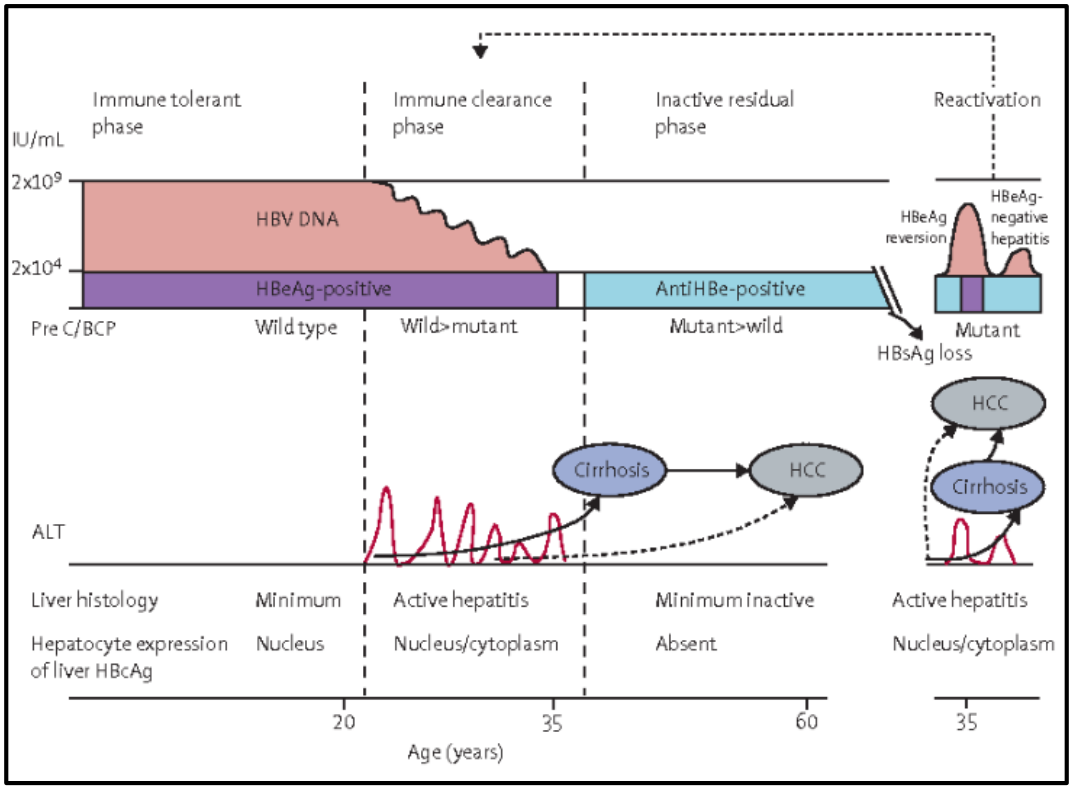

Figure 1.3.2.1. Chronic HBV infection. Natural course and occurrence of HBV infection which is acquired during infancy and/or perinatally. The reactivation phase is similar in every characteristic to immune clearance phase with an exception of HBeAg status. Infections that are acquired during adulthood are presented in immune clearance or reactivation phase. The pathophysiology during immune clearance and reactivation phases may lead to HCC and cirrhosis (Liaw and Chu, 2009; Croagh and Lubel, 2014).

Resolved HBV infection: Apart from the stages which above-mentioned, there is resolved HBV infection stage which is considered HBsAg negative phase after loss of HBsAg. It can be signified that low level HBV duplication persist with HBV DNA which could be detected in the liver however it can not be monitored within the serum. Futhermore, it could be elicited that HBsAg clearance only occurs after HBeAg seroconversion. Loss of HBsAg is the closest point to cure HBV infection, but the viral DNA can be "stored" in host genome in the form of cccDNA. This creates a problem that in case of immunosuppression, HBV reactivation may occur. Some of these patients still have detectable amounts of HBV DNA (occult infection)

although HBsAg loss has occurred. These patients are susceptible for reactivation following chemotherapy regimens (Croagh and Lubel, 2014).

1.3.3 Occult HBV infection (OBI)

OBI is recognised as the constancy of HBV DNA in *S* antigen negative patient's liver with or without other indications of past HBV infection. Few mechanisms of OBI occurrence have been proposed. Some authors discussed that host immune and epigenetic systems are involved whereas numerous scholars suggested that a modification in the steric configuration of HBsAg molecule by *a* determinant mutations takes place. These modified HBsAg molecules are either not detected by commercial assays or they are weakly exposed in the hepatocyte surface (poor recognition by immune system) (Cento et al., 2013b).

1.3.4 Infection during pregnancy

CHB infection which occurs during pregnancy could be considered as one of the crucial worldwide problems. It may be postulated that approximately over the half of the total CHB carriers obtain their infection perinatally. Moreover, without immunoprophylaxis, new-borns born to HBeAg positive mothers have 40-90% risk of transmission (Piratvisuth, 2013; Stevens et al., 1975).

Furthermore, it can be depicted that HBV infection which occurs during pregnancy does not rise the fetal or maternal morbidity and mortality. Parallel to this argument Wong et al (1999) had conducted a research to explore difference in pre-term delivery, neonatal jaundice, birth weight or congenital anomalies. Results revealed that no significant difference existed in terms of preterm delivery, birth weight, neonatal jaundice, congenital anomalies or perinatal mortality. In addition to

this, Tse et al (2005) reported that mothers who are carrying HBsAg had increased risk of type 3 diabetes, antepartum haemorrhage and pre-term labour.

A normal gestation period is linked with an increased levels of corticosteroids and hormones (oestrogen), which caused an increased HBV viremia. These cytokine and hormone changes may lead to minimal variations at liver function tests. Peripartum hepatitis flares may lead to liver decompensation (Piratvisuth, 2013).

1.3.5 Immune response

HBV engages different immune components over the infection period. IFN gamma (γ) and CD8+ T cells target the infected hepatocyte, in acute phase. Interferons have critical role at acute infection as HBV infected hepatocytes secrete IFN α/β which hinders viral packing (postulated by Figure 1.3.5.1) (Chang and Lewin, 2007).

Moreover, several writers pointed that chronic infection occurs at immune tolerant phase where the patient is asymptomatic and HBV DNA, HBsAg and HBeAg are measureable in serum, particularly for perinatally infected patients (Sandhu et al., 2017).

Some of the immune cells and their functions during the infection can be summarised as follows;

NK cells: NK cells play a significant role in acute HBV infection, as their reduced activation exhibit reduced cytolytic activity which agrees with peak viremia. Also, CHB patients have shown to have decreased numbers of NK cell activation markers and reduced IFN γ and tumor necrosis factor (TNF) α production (Tjwa et al., 2011).

NKT cells: Are natural killer cells with toll like receptors (TLR) attached that are against lipids; they get activated early during HBV infection and contribute in priming of T and B lymphocytes (Zeissig et al., 2012).

Kupffer cells: These cells could be identified as resident macrophages of the liver. They act as a first line of defence against any pathogen. They trigger IL-6 production and hinder HBV replication and transcription. Moreover, these cells produce transforming growh factor (TGF) β. Since these immune cells are tolerogenic in their nature, high expression of programmed death ligand 1 and production of other anti-inflammatory proteins suggest that they might be accountable for reduced T lymphocyte activity (Liet al., 2012).

DC's: It may be lamented that DC's circulate through the liver and are important for stimulating adaptive immune response. It was detected in CHB patients that they impair cytokine production in myeloid DC's and plasmacytoid DC's when compared to healthy patients. Besides of these, the total number of myeloid DC's elevate the response towards to adefovir (ADV) treatment (van der Molen et al., 2006).

T Lymphocytes: CD8+ T lymphocytes recognise for polymerase, envelope and nucleocapsid antigens, CD4+ T lymphocytes follow similar recognition but are more specific to core proteins (Boni et al., 2007).

B lymphocyte: B cell response whereas play the most crucial role for HBV detection and resolution. Antibody response against HBV is crucial in different phases of the infection as antibody titres are used to categorise the disease extent. Specific antibodies against all proteins of HBV occurs early, but the most important antibody is AntiHBs which provides protective immunity against infections in the later phases (Szmuness et al., 1980).

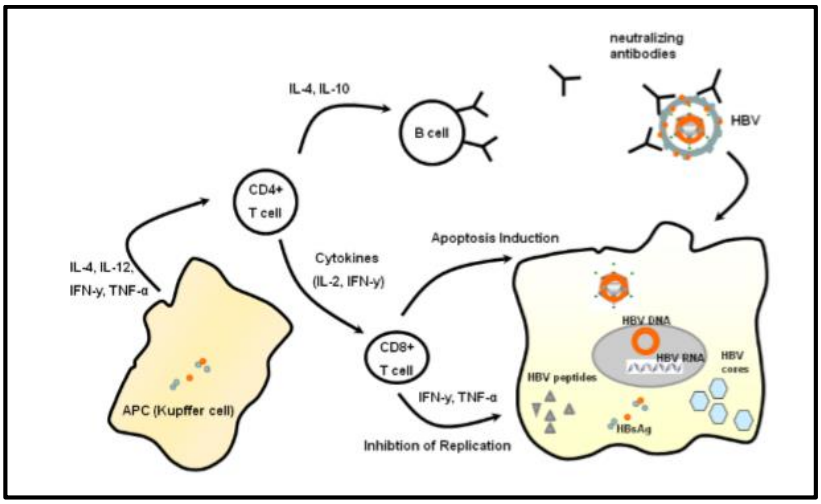

Figure 1.3.5.1. Cellular immune response against HBV. Antigen presenting cells (APC) recognise and take up HBsAg and virions after replication process. After degradation of viral proteins, these peptides are presented on the surfaces of APCs via both major histocompatibility complex classes 1 and 2. CD4/8+ T cells recognise these peptides and CD4+ T cells activate B cells to produce immunglobulins. Both CD4/8 + T cells recognise these viral peptides on infected hepatocytes. Recognition of these either lead to direct induction of apoptosis or inhibit viral replication by the production of IFN-γ and TNF-α (Ganem and Prince, 2004).

1.3.6 Avoiding immune system

Sub-viral particles which outnumber virions by 1 000-10 000 fold, are believed to limit the efficiency of immune responses by prompting the development of circulating immune complexes which neutralises the circulating antibodies (Ganem and Prince, 2004).

Other than empty sub-viral particles, HBV produces and secretes HBeAg which is not needed for viral replication nor infection, but evidence has showed that presence of HBeAg contributes for viral persistence by its immunomodulatory functions (Dandri and Petersen, 2016).

Pathogen recognition receptors, for example TLR, plays a vital role in early immune response and serve as a link between adaptive and innate immune responses. Surprisingly, it has been found that 20-30% of mature HBeAg retains in the cytoplasm, and there it antagonises the TLR signalling pathways (Land et al., 2011).

The HBV X (HBX) is defined as a non-structural, multifunctioning and regulatory protein with a trans- activating potential, It can inhibit innate immunity by downregulating mitochondrial antiviral signalling proteins through supressing RIG-I-MDA5 pathways and also by means of interactions with cellular epigenetic family members (Wei et al., 2010).

Moreover, it could be illuminated that HBX protein possesses tumour promoter activity. Numerous research have shown that over-expression of HBX causes transactivation of many viral elements and cellular promoters. Studies which were conducted in-vitro have demonstrated that various cytoplasmic signal pathways are also effected including Src kinase, MAP kinase, Jak1/STAT and few others (Zhang et al., 2004).

HBX is enlisted to the cccDNA, where it is believed to elaborate in regulation of HBV replication and in a study, it is suggested that HBX can act as an effective epigenetic transforming factor in the human liver, by modulating transcription of DNA methyl transferase enzymes which are required for hypo methylation of tumour suppressor genes. Unlike human immunodeficiency virus (HIV), HBV doesn't need to integrate into the host genome as a part of its replication, but integration occurs, predominantly in presence of DNA damage. At this point, HBV can cause modification of human genome; genomic variability and direct insertional mutagenesis, which plays an important role in initiation of hepatocellular carcinogenesis (Park et al., 2007; Dandri et al., 2002).

1.4 Characteristics and structure

HBV could be described as a member of *hepadnaviridae* viruses and is a circular, double stranded, enveloped DNA virus. It is one of the major agents causing chronic liver disease (Orito et al., 2001) and infects hepatocytes of wide range of animals (Zehender et al., 2014). In addition to these, HBV is a hepatotrophic and non-cytopathic virus which can cause acute and/or chronic hepatitis (Sünbül, 2014). Apart from these, HBV could be considered as one of the main denominators of liver

cancer and is believed that it infected 240 million people around the world. The virus is categorized by high degree of genetic heterogeneity due to the usage of a reverse transcriptase (*RT*) enzyme during viral replication. HBV genotypes have a characteristic ethnic and geographic distribution (Zehender et al., 2014).

Furthermore, Babanejad et al. (2016) in their study, outlined that HBV is a communal health problem and could be considered as a major cause of morbidity and mortality in the developing world. Around one-third of the world population is estimated to be infected with HBV and more than half a million people die each year, because of chronic or acute HBV infection.

Hepatitis B virus which also named as Dane particle, is a 42nm particle (as portrayed by Figure 1.4.1) which is composed of 27nm nucleocapsid core (HBcAg), enclosed by outer lipoprotein coat that is called envelope and it contains surface antigen (HBsAg) (Dane et al., 1970).

The virion's nucleocapsid comprises of the genomic DNA and a DNA polymerase enzyme with *RT* activity. Primase, which is called as terminal protein is also present in the nucleocapsid. It can be articulated that the outer surface of the virion contains three proteins namely Small (S) Middle (M) and Large (L) and a lipid layer which originates from membranes of the host cell. Apart from the virion itself, few of the non-infectious particles are also found in the serum of infected people in acute phase or chronic non-replicative stage. The surface antigen (HBsAg) is produced in excess amounts by the infected hepatocyte and is secreted on the basis of empty spheres of 22nm particles and filamentous or tubular structures with 22nm diameters (as postulated by Figure 1.4.1). It may be indicated that the spherical forms are the most abundant ones which are present whereas other filamentous and tubular structures are presented in less amounts (Gerlich et al., 1993).

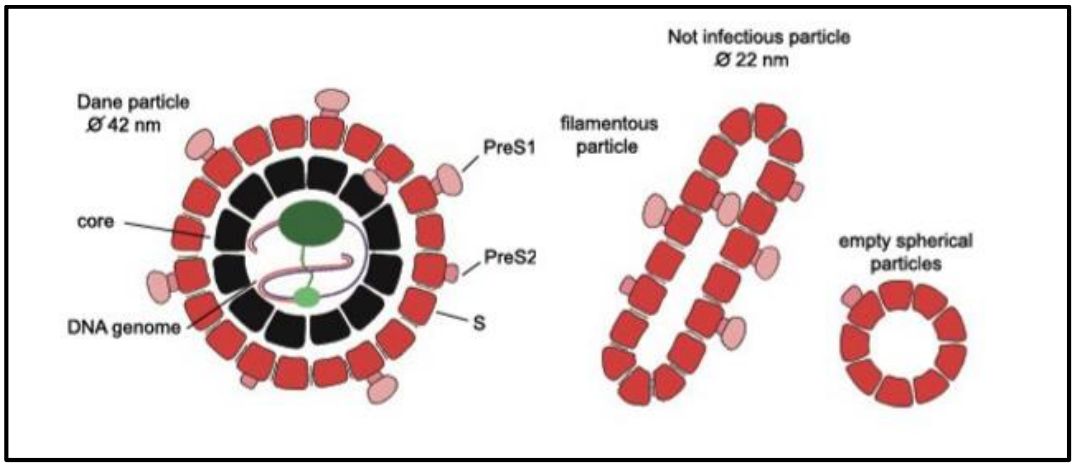

Figure 1.4.1. Dane particle and other virus like particles. On the left: Infectious Dane particle with *PreS1*, *PreS2* and *S* proteins. The particle is 42nm. HBV genome is capsulated with core proteins. On the Right: Non-infectious particles measuring 22nm; spherical particles are organised as an octahedral sphere and filamentous particles consists of same diameter but differ in length **(**Alexandra S, 2014).

Inside the nucleocapsid, the genome arranges into a relaxed circular and partially double stranded DNA which is around 3.2 kilobytes (kb)- 3200 basepairs (bp). This genome is covalently attached to the viral polymerase. The complete HBV genome is organised in a condensed matter, which all the genes are coded within open reading frames (ORF) which overlap with each other (Dandri and Petersen, 2016).

As previously mentioned; the viral membrane is assembled from host derived lipids, and 3 envelope proteins are named according to their sizes; *pseS1*(L), *pseS2* (M) and *S*(S). All these proteins commonly share same C-terminal domain which contains the surface antigen HBsAg. *PreS1* and *preS2* proteins have N-terminal extensions that are important for receptor recognition (Dandri and Petersen, 2016) (figure 1.4.3).

Essentially, 3 types of viral particles can be seen in the sera of infected patients by electron microscopy (Figure 1.4.2); which contains the infectious virion and sub-viral particles. These sub-viral particles are presented as spheres or filaments (mentioned above) that are composed of lipids and envelope proteins. The purpose of these non-infectious particles are not completely known but it has been proposed that, they may engage with neutralising antibodies produced by host; which are

produced in higher numbers in comparison to the virions (Glebe and Urban, 2007; Ganem and Prince, 2004).

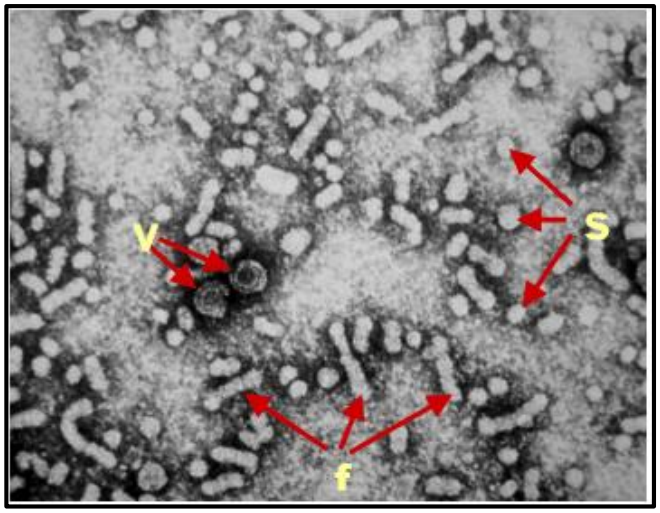

Figure 1.4.2. HBsAg L, M and S particles. Electron Micrograph of HBV virion. Complete virions are shown as "v", spherical particles as "s" and filamentous particles are as "f" (Lai and Locarnini, 2002).

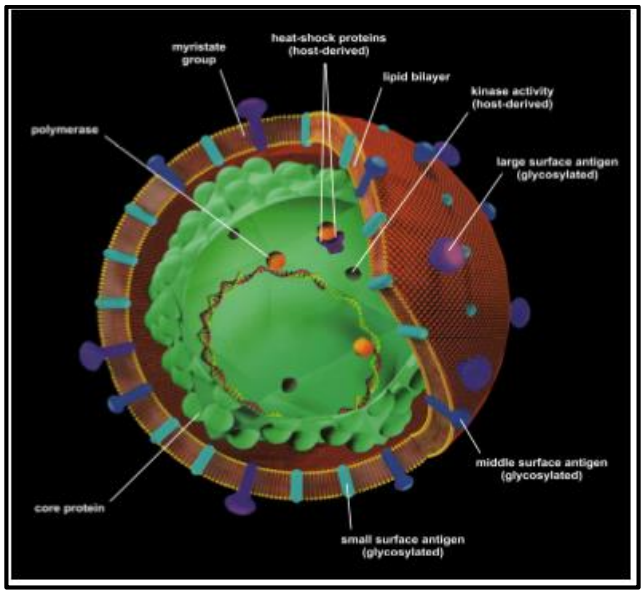

Figure 1.4.3. HBV structure. Structure and enzymatic proteins of the Dane particle (Lai and Locarnini 2002)**.**

1.5 Genetics and genomic organisation

The virus is categorized by great degree of genetic heterogeneity as a result of the usage of an *RT* enzyme during the viral replication, as mentioned earlier. 10 genotypes have been described so far for HBV, which further divide into sub-genotypes and serotypes. These have shown an ethnic and geographic distribution (Zehender et al., 2014).

The viral genome is a circular, partly double-stranded DNA which stores information about 3.2 kb. The minus strand is incomplete within the virion. The information encrypts for four partially overlapping genes which are named as *PreS/S*, *PreC/C*, *P* and *X* . These genes translate to seven different proteins (Zehender et al., 2014).

- *PreS/S* → 3 surface proteins (S, L and M *S* protein)
- *PreC/C* → 2 core antigens (HBcAg and HBeAg)
- *P* → Polymerase

  X → Small regulatory X protein (Zehender et al., 2014; Bhattacharya et al., 2015).

Both minus and positive strands of the genome have cohesive ends which stretch over 200nt, which compasses two 11nt direct repeat sequences where that facilitates formation of the circular DNA shape. Several regulatory proteins such as enhancer regions, U-5 like sequences, a polyadenylation signal and putative glucocorticoid-responsive element are also present (Tur-Kaspa et al., 1988).

As mentioned above, the ORFs overlap. Most importantly, the *RT* and HBsAg ORF overlap at *RT* amino acid (aa) 8-236, with HBsAg ORF shift downstream by 1 nucleotide (nt). Certainly, the 3rd nt at *RT* codon corresponds to the 2nd nt at the *S* codon. Likewise, the 2nd nt of *P* codon matches the 1st nt of *S* codon. The 1st nt of *P* codon corresponds to the 3rd nt of *S* codon at position 1. Therefore, nt substitution at *P* codon's 2nd nt and *S* codon's 1st nt would affect aa in both *RT* and HBsAg ORFs, which indicates this can not only be a therapeutic agent target; but also that mutations at these specific areas may result in resistance (Figure 1.5.1) (Cento et al., 2013a).

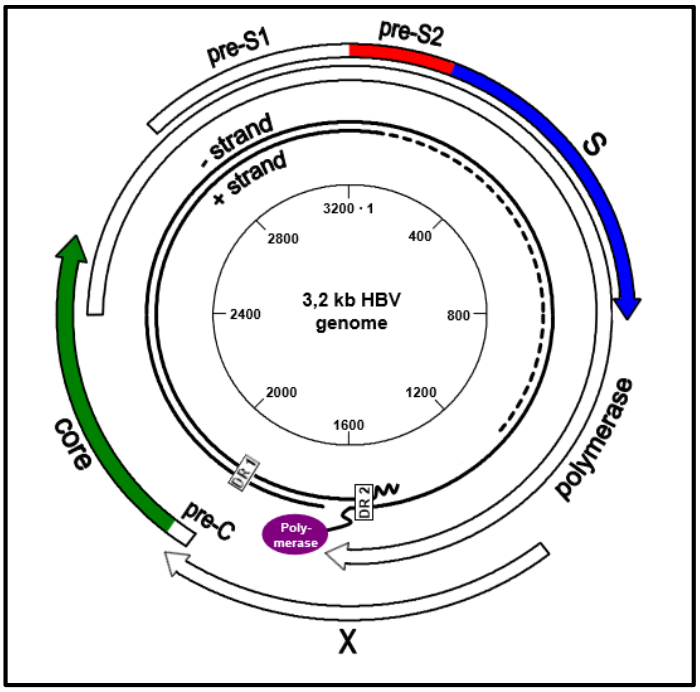

Figure 1.5.1. HBV genome organisation. 3.2 kb HBV genome has 4 overlapping ORFs (arrows) which these encode seven different transcripts. The negative strand has 7-9 nt terminal redundancy and viral polymerase is linked to its 5' end. The positive strand length is variable (dotted line). 5' end of positive strand is capped with an RNA primer. Two 11 nt direct repeats are shown (DR1 and DR2). (Kann, 2002)

1.5.1 Genomic products

The four ORFs explained above encode for both structural and non-structural proteins of HBV.

The *pre S/S* ORF: encodes three envelope proteins S, M and L and has 3 genomic regions *preS1*, *PreS2* and *S*. These regions all have specific start codons and a mutual stop codon. The first start codon generates L protein which covers all *preS1*, *pres2* and *S* regions. The second start codon generates M protein which covers *preS2* and *S* regions. The third and innermost start codon encodes for main HBsAg protein (Neurath et al., 1986).

The *C* ORF: encodes for two products. HBcAg (capsid protein) and HBeAg (envelope protein). There are 2 in frame transitional start codons which divide this gene into *preC* and *C* sections. The first start codon generates HBeAg and the second

start codon generates HBcAg from *C* gene, involved in capsid formation (Bruss and Gerlich, 1988; Koschel et al., 1999).

The *P* ORF: covers nearly 80% of the genome and partially overlaps with other three ORFs, and it codes for the viral polymerase. This produces a 90 kilodaltons (kDa) protein which is multifunctional and has 4 domains, which are important for the replication (Ganem and Schneider 2001). The first domain encodes for a terminal protein (Weber *et al.*, 1994), the second domain is a spacer, the third domain encodes for DNA dependent DNA polymerase (*RT* activity) (Bavand *et al.*, 1989) and lastly the fourth domain has ribonuclease H activity which cleaves the RNA in DNA-RNA hybrids during reverse transcription (Radziwill et al., 1990).

The *X* ORF: encodes for a 154 aa polypeptide protein with a mass of 17.5 kDa. *X* acts as a transcriptional trans-activator for many viral and cellular promoters (Kann and Gerlich, 1998).

Some modulatory functions of *X* are; involved in signal transduction pathways, protein degradation and cell cycle control (Bouchard and Schneider, 2004). Some studies suggest that this protein can induce and block cell apoptosis (Kanda et al., 2004; Huo et al., 2001). The *X* protein is linked to angiogenesis and metastasis during aggressive progression of HCC and is associated with oncogenesis.

HBV may have genetic information of 3215 nt; such as genotypes B, C, F and H. Because of deletions and insertions within the genome, other HBV genotypes differ in terms of nt numbers and genome length; for example genotype G has 3248nt and genotype D has 3182 nt (Table 1.5.1) (Schaefer, 2007; Zhang et al., 2006).

Table 1.5.1. Table of Genotypes and their genome length. 8 genotypes (A-H) and their genome lengths. The genome length of B, C, F and H are same whereas due to ORF differences (insertions and deletions of amino acids) A, D, E and G have shorter or longer genome lengths respectively. (aa: Amino acid) Table was created using data from Schafer 2007 (Schaefer, 2007).

Genotype	Genome lenght (nt)	ORF differences
A	3221	İnsertion aa 153 and 154 in HBeAg
B	3215	-
C	3215	-
D	3182	Deletion of aa 1-11 in PreS1
E	3212	Deletion of aa 11 in PreS1
F	3215	-
G	3248	İnsertion of 12 aa in HBcAg Deletion of aa 11 in PreS1
H	3215	-

After going into hepatocyte nucleus, the rcDNA genome is transformed to cccDNA through sequences of molecular changes (completion of positive DNA strand, removal of RNA primer, removal of *pol,* ligation of gaps and supercoiling of DNA). These cccDNA molecules act as a reservoir for HBV infection. Immature nucleocapsids are formed by packaging of pgRNA inside the nucleocapsids in the cytoplasm. After packaging, viral polymerase converts pgRNA into rcDNA. The envelope formation of HBV results from highly coordinated interactions between nucleocapsid and the exposed *preS* region of the L protein. The *S* protein plays a key role in secretion and budding of virus particles. Sub-viral particles occur as are result of surface protein budding without nucleocapsid in the endoplasmic reticulum lumen (Noordeen, 2015).

1.5.2 Mutations and their implications

Unlike other DNA viruses, HBV has a high mutation rate (10^5 change/base/replication) and high replicative capability (>10^{12} virion/day) increases genetic variability. HBV mutations have been observed in both acute and CHB patients, in all four ORFs. Understanding of the link among mutations and disease development is important for an efficient clinical management in HBV patients with other resistances to antiviral drugs, HBsAg escape mutants, occult HBV and HCC (Caliguri et al., 2016).

HBV *Pol* gene completely overlap with *S* gene. Nucleostide analogue (NA) resistance mutations in the *pol* gene usually result in variations in the overlapping *S* gene. These *Pol/S* gene overlap mutants are named as Antiviral drug associated potential vaccine escape mutant (ADAPVEM) (Sayan and Bugdaci, 2013).

1.5.2.1 *S* OFR and *S* gene mutations

As discussed above, *PreS/S* ORF codes for 3 distinctive surface antigens. HBsAg is the main form which is recognised by the immune system and is accountable for coupling of the virus to the hepatocyte. Point mutations, deletions and genomic recombinations have been found in this region, which is known as the highest heterogenic part of HBV genome (Caliguri et al., 2016).

The aa positions 99 - 169 are major hydrophilic regions, where the *a* determinant is located, which this is the key target for neutralising B cell response. Mutations cause conformational changes within the *a* determinant which effect antigenicity of surface antigen and is responsible for avoiding vaccine induced immunity, escaping Hepatitis B immunglobulin (HBIg) therapy and causes false negative outcomes in diagnostic assays (Zehender et al., 2014).

sG145R (glycine to arginine substitution in position 145), is the main vaccine induced immune escape mutant and increase in this mutation is reported in the last years. Other mutations of the *a* determinant are: sT116N, sP120S/E, sI/T126A/N/I/S, sQ129H/R, sM133L and sD144A/E. These are also considered as immune escape mutants. In recent studies, it has been shown that 3/4 of HBV reactivated patients were carriers for more than one HBsAg mutation (Caliguri et al., 2016; Salpini et al., 2015).

Mutations in this region might lead to hepatocarcinogenesis. The patophysiology of this process is thought to be as; deletions source a reduction in synthesis and release of surface antigen which gather in the hepatocyte endoplasmic reticulum and cause endoplasmic reticulum to stress and oxidative DNA damage occurs which triggers mutagenesis and HCC (Caliguri et al., 2016).

1.5.2.2 *P* ORF and *P* gene mutations

As explained above, *pol* ORF codes for *RT* domain of HBV *pol* which is the main target for antiviral drugs. Use of NAs cause selective pressure, mutations and resistance. Other than high mutation rate, other factors such as viral genetic barrier, potency and fitness are associated with resistance. Due to the overlap of *S* reading frame, *RT* domain causes the appearance of escape mutants. The absence of proofreading activity leads to random mutations in this region. HBV polymerase error rate is approximately $1x10^{5-7}$ base synthesis (Caliguri et al., 2016). Earlier research showed that lamivudine is the main cause of tyrosine-methionine-aspartate-aspartate (YMDD) mutations rtM204I/V in the *C* domain of HBV *P* ORF. rtL180M and rtA181T/V also confers to lamivudine (LAM) and tenofovir (TDF) resistances (Yuan et al., 2009). Other *pol* gene mutations caused by NA usage are explained in more detail below in the text.

1.5.2.3 *C* ORF and *C* gene mutations

As stated above, *PreCore/Core* ORF encodes for HBcAg and HBeAg. Mutations in these regions cause e antigen negative hepatitis. A1762T and G1764A is responsible for reduced synthesis. G1896A mutation is the most prevalent mutation which inhibits the HBeAg synthesis (producing of a stop codon) and causes a worse prognosis of hepatitis. To be more precise, these mutations decrease HBeAg synthesis, enhance viral replication and is often related with a more severe liver disease. This is mostly seen in genotypes B-F and these mutations were first found in Mediterranean area where majority of patients are Genotype D (Besharat et al., 2015).

1.5.2.4 *X* ORF and *X* gene mutations

As mentioned above, *X* ORF encodes multifunctional non-structural protein. It is named as *X*, as the functions are unknown and not clear. It has been proposed that it may be involved in viral replication and establishment of infection. Furthermore, it is hypothesised that it plays a role in HBV carcinogenesis (Caliguri et al., 2016).

The *X* gene overlays with core promoter region and mutations at this site usually modify the functions of *X* protein. HBV *X* mutants related to core promoter mutations may control p53, stimulating or avoiding proliferation and transformation. 12 mutations have been found and have been associated with hepatocarcinogenesis, suppression of HBeAg release and viral DNA synthesis upregulation (Yan et al., 2015).

1.6 Genotypes/subgenotypes and serotypes

HBV has diverse genotypic differences between sub-genotypes. In previous decades, classification was made according to the change of subtype that may result from one-point mutation at the *S* gene. With advances of technology and molecular evolutionary analysis in our day; we can divide HBV into genotypes and sub-genotypes. In 2001, there were only 7 defined genotypes, whereas in 2019, there are 10 defined genotypes with various sub-genotypes (Orito et al., 2001).

The genotype/sub-genotype characterisation of HBV assists understanding the natural history of this viral infection. Genetic heterogeneity of the virus implies biological properties which effects the clinical outcome of the infection and the response to antiviral agent's treatment (Chacha et al., 2017).

Area specific localisation of HBV sub(genotypes) are associated with anthropologic history; and, in many studies it has been reported that there are

remarkable differences in both virologic and clinical outcomes of the disease, which will be discussed in outcomes of the disease section (Orito et al., 2001).

Despite its obliged genetic evolution, the HBV genome is categorized by variability because of the use of an RNA intermediate and a *RT* during replication cycle. As a result of this, HBV has been classified into 10 genotypes and some sub-genotypes as previously explained. The mean nucleotide difference between genotypes is more than or equal to 8%, whereas 4-7% difference is seen between sub-genotypes (Zehender et al., 2014).

Initially HBV isolates were classified according to their serological subtype or serotype, but these serotypes did not correspond with the geographical distribution of HBV. Currently, classification of genotypes is used, which displays geographical distribution (Magnius and Norder, 1995).

The viral types resemble to the formerly described serotypes which is based on the occurrence of two pairs of genomic mutually exclusive antigenic determinants in the HBV surface antigen (*d/y* and *w/r*) (Norder et al., 2004).

The three surface glycoproteins contain the "*a*" determinant epitope which is located at the aa positions 127–147, as stated before. The serological subtype of HBV is determined with the addition of 2 other determinants. The first one is at aa position 122; lysine residue represents subtype *d* and arginine residue represents subtype *y*. The second one is at aa position 160; lysine residue represents subtype *w* and arginine residue represents subtype *r*. The residue at aa 127 further differentiates the *w* subtype into 4. The *adr* subtypes are further divided into q^- and q^+. By the combination of these determinants, a total of 9 different serological subtypes have been identified (Yokosuka and Arai, 2006).

Clinically, observations show that antiviral therapy selects for HBV mutants that code shortened HBsAg and therefore accelerate the progression to HCC. Treatment of HBV with antivirals can result in the selection of HBV variants with point mutations in *pol* that not only cause antiviral resistance but also effect changes to HBsAg structure. As an example, specifically the point mutation that causes the rtA181T change in *pol* also codes for a stop codon (sW172*) in the overlapping

surface protein, causing in shortening of the last 55 amino acids of the C-terminal hydrophilic region of the *S* protein molecule (Figure 1.6.1). Therefore, it is important to monitor these mutations and to know the serotype of a specific mutant strain before starting therapy (Locarnini and Yuen 2010).

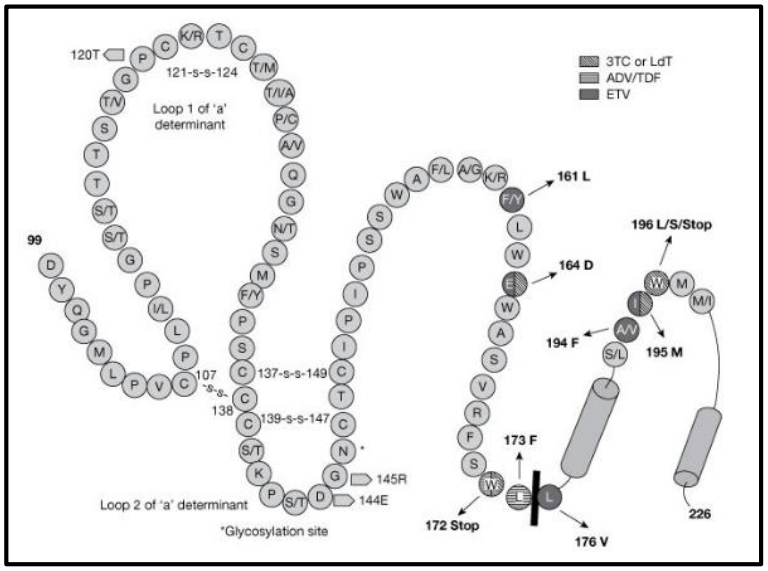

Figure 1.6.1. HBsAg mutations selected during antiviral therapy (Locarnini and Yuen 2010).

Phylogenetic investigation of HBV genome aids understanding the epidemiology of this infection and virus movement in a given geographical region. These genotypes/sub-genotypes are thought to share the same ancestor, over time there have been diverse evolutionary histories for the spread of HBV in geographic areas. It is supposed that the migration process have effected this distribution of HBV genotypes/sub-genotypes throughout the globe which was discussed in the history section of this thesis (Chacha et al., 2017).

Prevalence of some genotypes are widespread. For example, HBV-A is present in North-Western Europe (mainly D1, D2, D3 and A2 sub-genotypes), North America and Central Africa; HBV-D is found throughout the world although highest rates are observed in Mediterranean region, Middle East and South Asia. HBV B and HBV-C are only present in Asia. HBV-E is found in sub-Saharan Africa. HBV-F is prevalent in South and Central America. HBV-G is observed in France and United

States of America (USA). HBV-H and HBV-F are epidemic in Northern Latin America and Alaska. A newer genotype, HBV-I, was first found in North-West China, Eastern India, Laos and Vietnam between 2008-2010. Another newer genotype, HBV-J, was first isolated in Japan in 2009 (Zehender et al., 2014).

The 2 genotypes accountable for the majority of HBV infection in Europe are HBV-A in North-West (mostly sub-genotype A2) and HBV-D in North-East and Mediterranean Area (mainly D1, D2 and D3 sub-genotypes) (Zehender et al., 2014).

Turkish Republic of Northern Cyprus (TRNC) is a country of heterogeneous population in relation to its strategic position in Mediterranean region. Studies have previously described circulating HBV genotypes/sub-genotypes amongst the population. The most frequent was HBV-D (Arikan et al., 2016). This information is consistent with the literature and previous research studies.

Genotype differences and clinical outcomes are believed to be linked (Table 1.7.1). For explaining this phenomenon further, few examples can be given as;

In studies, it was found that ALP levels of HBV-B patients were lower than HBV-C. The positivity of HBeAg in HBV-B patients were lower compared to HBV-C patients. HCC was higher in HBV-C compared to HBV-B (Orito et al., 2001).

Some other differences observed were;

HBeAg positive patients were more reactive to IFN in genotype C compared to others. HBV-A patients were more responsive to IFN compared to HBV-D patients. Pegylated (PEG) -IFN monotherapy or PEG-IFN + LAM was influenced by HBV genotype, and HBV-A and HBV-B were better responders compared to HBV-C and HBV-D; and, resistance against LAM appeared to occur earlier in HBV-A than HBV-D (Guirgis et al., 2010).

With no doubt, genotype and/or antiviral induced genomic resistance(s) and differences are important. This resistance and related topics will be explained later on in the text.

1.7 Pathological consequences

HBV infects up to 2 billion individuals around the globe and around 248 million will develop chronic infection. Out of these 248 million, 648 000 are expected to die as a result of life threatening problems such as hepatic decompensation, HCC and/or LC. These figures make HBV the 10th leading cause of death globally (Jaffe and Brown 2017). Nearly 25% of childhood-infected adults die from either LC or HCC (Irshad et al., 2016; Amiri et al., 2016).

HBV infection affects liver and results in a wide spectrum of disease outcomes (Figure 1.3.2.). The infection can resolve and end up with protective immunity, or the infection can become chronic and cause liver failure (Villiano et al., 2015).

HBV infection in immunocompetent adults, usually results in a self-limited liver sickness in which viral control is accomplished in >95% of adults. Whereas >90% of people which were exposed to HBV at birth or in early ages, becomes persistently infected (Dandri and Petersen, 2016). Experiments shown that not only age but also size and route of the inoculum affect the kinetics of viral spread and therefore affect immunological priming as well as the infection outcome (Asabe et al., 2009).

It is interesting that patients infected with Hepatitits A virus (HAV) and Hepatitis E virus (HEV), who possessed pre-existing HBV and Hepatitis C virus (HCV) infections, experiences development of a serious disease with high morbidity and mortality; whereas HAV and HEV infections alone resolve without becoming chronic (Irshad et al., 2016).

As the virus itself is non cytopathic, the continuous and inappropriate inflammatory reactions against it causes liver damage. This inflammation of the liver triggers activation of hepatic stellate cells and produces deposition of extracellular matrix which causes what is known as fibrosis. In approximately 10% of HBV infected patients, the ongoing fibrosis leads to cirrhosis and if left untreated, the 5-year survival rate drops by 50%. Additionally, 2% of cirrhosis patients develop HCC

and account for around half of all LC are due to HBV worldwide (Katrinli et al., 2017).

In the sub-Saharan region of Africa, HBV is hyper endemic and there is a high occurrence of HCC (Mammas et al., 2017). The risk of becoming chronic HBV carrier is age associated; neonates 90%, infants 25-30% and immunocompetent less than 5% (Broderick and Jonas, 2003).

HBV infection may lead to some clinical presentations which range from asymptomatic carrier to acute hepatitis, chronic hepatitis, fulminant hepatitis, LC and HCC. Progression of chronic hepatitis B to LC and/or HCC is mainly regulated by the genetics of the host, also viral factors and environmental factors have impact on the progression. There is a clear genotype related link existence between clinical outcome and treatment for chronic hepatitis patients as discussed above (Sünbül, 2014).

A good example for this is; investigation of reasons for; longer immuneclearence periods in HBV genotypes B and C patients, higher levels of viral replication, recurring or high ALT levels, IFN and NA, low response to treatment respectively. To find the possible association; peripheral blood follicular helper T cells of chronic hepatitis B patients under treatment were observed. These cells have important functions such as signal spreading and affecting cellular division, with help of B cell activation; to regulate humoral immune response against HBV. Also they secrete specific IL-21 (cytotoxic T lymphocyte interleukin) to sustain an effective and long lasting antiviral immunity in chronic scenarios. Therefore, we can conclude that higher ALT and HBV DNA levels in HBV-C might be related to lower numbers of peripheral blood follicular helper T cells which causes low IL-21 levels when compared to HBV-B (Sünbül, 2014).

Development of chronic infection remained increased in patients who have acute infection with HBV-A. However, in China; chronic infection was established more in patients with sub-genotype C2 compared to sub-genotype B2. Other than genotype effect, there are factors which affect this phenomenon such as the quantity of viral inoculum, the way of acquiring and the interactions of virus and the host (Table 1.7.1) (Sünbül, 2014).

In acute HBV, CD8+ and CD4+ T cell reponses to HBV proteins are strong and specific. Whereas in chronic HBV, immune responses are weak and hardly focused (Guidotti and Chisari, 2001; Bertoletti and Ferrari, 2012).

HBeAg seroconversion & HBsAg seroclearence are important for natural progression of HBV infection. E antigen seroconversion is usually accepted as a positive result. But a late seroconversion or lack of the seroconversion may indicate development of chronic HBV infection to LC. It has been reported that, HBeAg seroconversion rate of HBV-C was lower than HBV-B in patients which indicated longer persistence of HBV replication, and enlightens why LC and/or HCC developed in patients with this genotype (Sünbül, 2014).

Table 1.7.1. Table of genotypes and their characteristics. Table showing all 10 genotypes and their clinical characteristics (ND: No data available) (Sünbül, 2014).

Genotype	B	C	A	D	E-J
Clinical characteristics					
Modes of transmission	Perinatal/vertical	Perinatal/vertical	Horizontal	Horizontal	Horizontal
Tendency of chronicity	Lower	Higher	Higher	Lower	ND
Positivity of HBeAg	Lower	Higher	Higher	Lower	ND
HBeAg Seroconversion	Earlier	Later	Earlier	Later	ND
HBsAg seroclearance	More	Less	More	Less	ND
Histological activity	Lower	Higher	Lower	Higher	ND
Clinical outcomes (LC, HCC)	Better	Worse	Better	Worse	Worse in genotype F
Response to INF-α	Higher	Lower	Higher	Lower	Lower in genotype G
Response to nucleos(t)ide analogs	No significant differences among genotypes A to D				ND
Virological characteristics					
Serum HBV DNA level	Lower	Higher	ND	ND	ND
Frequency of PC A1896 mutation	Higher	Lower	Lower	Higher	ND
Frequency of basal core promoterT1762/A1764 mutation	Lower	Higher	Higher	Lower	ND
Frequency of preS deletion mutation	Lower	Higher	ND	ND	ND

1.7.1 Risk factors for HCC

Host factors include: cirrhosis, male sex, older age, family history of HCC, smoking, diabetes, alcohol use/abuse, obesity and exposure to aflatoxins. Viral factors include: high HBV DNA levels, HBV-B and HBV-C, HBeAg positivity, HBV mutations, HBsAg levels and co-infections with other viruses such as HCV, Hepatitis D virus (HDV) or HIV (Dandri and Petersen, 2016).

Chronic HBV infection is classically acquired at birth, or in early childhood especially in African and Asian nations where HBV is endemic. The risk of developing chronic infection after exposure is 90% in neonates and 1-5% in adults. Most infections in adults resolve within few months with the loss of HBsAg, failure to do so is the beginning of chronicity as stated previously (Kennedy et al., 2017).

Chronic infections acquired during childhood or prenatally involves prolonged stages such as immune tolerance, immune active phase, immune control and re-activation in some patients (Kennedy et al., 2017). Globally, over 50% of HCC cases are related to chronic HBV. Each year, HBV accounts for 749 000 new HCC cases and 692 000 HCC associated deaths. Each year non-cirrhotic HCC incidence is lower than 1% and cirrhotic HCC is 2-3% in HBV infected patients (Zamor et al., 2017).

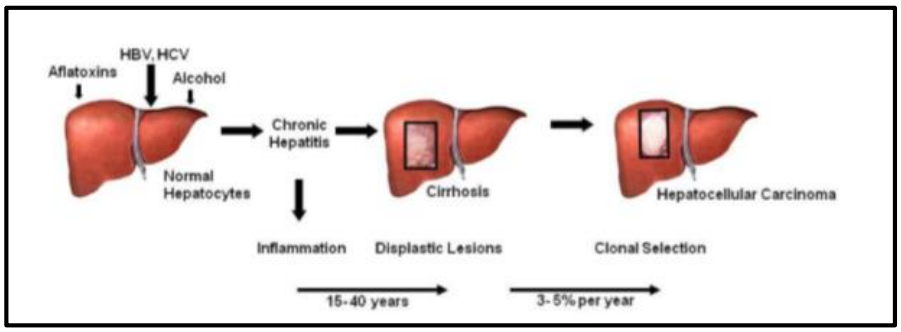

Figure 1.7.1.1. Development of HCC. Aflatoxins, HBV, HCV, alcohol abuse, cirrhosis and other metabolic liver diseases signifies major risk factors for occurence of HCC. The development of HCC is a multistep process which may go on for several years. These include genetic alterations, malignant transformation, chronic inflammation, subsequent injury and generation and others (Levrero, 2006).

Male patients have a higher risk compared to females. Male gender together with older age, carry a higher risk for HCC. This gender difference is due to protective effects of oestrogen which interacts with hepatocyte nuclear factor α and IL-6 signalling. (Fattovich et al., 2004; Wang et al., 2012)

HCC risk also increases with co-infection with other liver virus infections such as HCV, HDV and even HIV. HDV can only exist in presence of HBV, as it

relies on HBV proliferation. There is heterogeneity that HCC risk increases with HBV-HDV co-infection (Zamor et al., 2017).

Persistent hepatocyte inflammation leads to malignant and premalignant characteristics that are related with oxidative stress, which involves recurring cycles of apoptosis and necrosis (Figure 1.7.1.1) (Zamor et al., 2017).

In a study performed by TRNC Ministry of Health (MOH) in 2017, in the year 2012; 2.4 % of the population had liver cancer, however number of CHB patients for that year is not known to comment percentage of CHB patients develop into cancer (http://saglik.gov.ct.tr/, Accession date 20 December 2018).

1.8 Diagnosis

Since 1956, when HBsAg was originally discovered, many other antigenic and nucleic acid components of HBV have been identified. Simultaneously with the in-line discoveries, diagnostic assays were also set up for the detection of them. Detection of these in clinical specimens led to a better understanding of HBV infection. In our day, application of both molecular biology and immunometric tests allowed us to characterise the flow of HBV infection. New diagnostic tools are being discovered and applied together with higher sensitivity and specificity. Commercially available chemiluminescent microparticle immunoassays and radio immunoassays are used for primary diagnosis of HBV. HBV DNA PCR is the golden standard for diagnosis (Bonino et al., 2010).

Frequency and timing of testing is important in means of before, during and after therapy. The American Association for the study of liver disease guideline recommends; during therapy, HBV DNA levels to be tested every 3-6 months and assessment of liver tests every 3 months. Also; HBeAg and AntiHBe should be tested twice a year for HBeAg positive chronic patients and HBsAg testing is advised every 6-12 months with HBeAg negative patients with continuous undetectable levels of HBV DNA. Patients receiving immunomodulators, such as PEG-IFN are

advised to be tested for a full blood count every month together with thyroid stimulating hormone every quarter year. Due to the side effect issues regarding use of TDF and ADV, serum creatinine and phosphate levels may also be measured (Andersson and Chung, 2009).

The European Association for the study of the liver guideline recommends; following therapy, serum liver function tests, HBV DNA levels, HBeAg, and AntiHBe to be analysed every 1-3 months for the first year after treatment and 6-12 month follow-ups after one year (Andersson and Chung, 2009).

1.8.1 Conventional and serologic tests

Qualitative and/or quantitative antibody detection tests are available for human serum or plasma and they aid in the diagnosis and monitoring of HBV infection. These test assays are used to detect persons infected with HBV, and to stop transmission of the virus by blood and blood products as well as to follow patients with HBV serological markers (Abbott USA, HBsAg Qualitative II kit insert).

Many of the virological HBV markers are measured in sample cut-off (S/CO), a commercial measurement value with exception of AntiHBs microinternational units per milliliter (mIU/ml) and some others (indicates actual titre of the given antibody). Liver function tests can be measured in units per liter (U/L). HBV DNA PCR results are whereas are measured in IU/ml, as World Health Organisation (WHO) has standardized the unit of measure for this assay to standardize the heterogeneity of assays (Andersson and Chung, 2009).

1.8.1.1 HBsAg and Anti Hbs

During infection, HBV produces excess amounts of HBsAg. HBsAg as stated before, is mainly responsible for binding virus to the liver and is the main target of neutralising antibodies. HBsAg is the very first serological marker after infection which can be detected 1-10 weeks after exposure and 2-8 weeks before clinical symptoms occur. It persists during acute phase which is followed by clearance in late convalescence phase. Failure to clear HBsAg within 6 months indicates presence of carrier state. In most nations, testing of HBsAg is a part of the prenatal screening programme for identification of HBV positive mothers and to prevent perinatal infections (Abbott USA, HBsAg Qualitative II kit insert).

AntiHBs test is often performed to monitor the success of vaccination. Presence of AntiHBs in serum signifies protection against HBV infection. It can also be used to screen convalescence and recovery of HBV infected persons. Presence of AntiHBs after acute HBV infection and disappearance of HBsAg can be useful indicator of resolution. Detection of AntiHBs in asymptomatic patients may designate previous exposure. Based on the WHO recommendation; more than 10 mIU/ml is accepted as protected against infection, following the vaccination (Abbott USA, Anti HBs kit insert).

1.8.1.2 HBcAg, Anti HBc IgM and Anti HBc IgG/Total

HBcAg is one of the most significant markers for HBV infection. HBcAg is released into the blood after process of envelopment in the form of dane particles. The quantity of HBcAg demonstrates viral load, thus correlates with HBV DNA. This means that HBcAg could be a indicator for viral load. Phage enzyme linked immunosorbent assay (ELISA) is a technique, where a modified ELISA with primary antibody of fusion phage can be used to measure. There are other detection methods such as dot blot and immunoprecipitation assays (Hashimoni et al., 2005).

Phage display mediated immune PCR method is also used, this method can detect HBcAg as low as 10 nanograms, which is 10 000 folds more sensitive than phage ELISA method (Monjezi et al., 2013).

Anti hepatitis B core antibody (Anti HBc) tests (IgG/IgM) can be used for indication of current or past HBV infection. Anti HBc can be found in serum soon after appearance of HBsAg in acute infection. It will persist after the loss of HBsAg and before appearance of Anti Hbs. In the absence of other HBV markers, must be considered that a patient with AntiHBc may be actively infected or the infection may be resolved leading to immunity. AntiHBc may be the only serological marker of infection and potentially infectious blood. Occurrence of AntiHBc does not distinguish between acute or chronic infection unless IgM or IgG is measured specifically (Abbott USA, Anti HBc II kit insert).

Viral specific Anti HBc IgM is detected in most of the acute viral infections and is the reliable marker of acute disease. Concentrations increase rapidly in acute infection, and can be detected in acute HBV infected patients. At the same time, HBsAg will generally be present during acute infection, but there are reports of HBsAg being not detectable at this stage. In convalescence phase, AntiHBc IgM will persist after loss of HBsAg, and decline gradually over time. In the absence of other HBV markers, patients with positive Anti HBc IgM may indicate active infection or that infection may have resolved. It is also present in chronic infections (Abbott USA, Anti HBc IgM kit insert).

Differentiation of acute and chronic HBV with use of viral markers such as HBsAg, AntiHBs, AntiHBe and Anti HBc is challenging as most of these markers are measurable at both acute and chronic disease. Ever since there is a high association between Anti HBc IgM concentration and acute HBV infection, Anti HBc IgM may assist to differentiate acute HBV causes, due to HBV versus superimposed infections by other agents such as HAV, HCV or HDV (Abbott USA, Anti HBc IgM kit insert).

1.8.1.3 HBeAg and Anti HBe

HBeAg measurements can be used to monitor progress of HBV infection. HBeAg is first detectable in the early phase, after the appearance of HBsAg. Levels for both antigens rise quickly during the viral replication period in acute infection. The levels of HBeAg correlates with raised numbers of infectious viral particles, occurrence of core proteins in the hepatocyte nucleus, HBV DNA and HBV DNA *Pol*. HBeAg may persist in the serum with HBsAg in chronic infection as well. However; some chronically infected patients have no HBeAg, and are not positive for AntiHBe. These patients may also be positive for HBV DNA (Abbott USA, HBeAg kit insert).

HBeAg and antibody for it AntiHBe, can be found in association with HBV infection. Seroconversion from HBeAg to AntiHBe during acute phase usually indicates resolution of the infection and reduced level of infectivity. Negative HBeAg indicates either early acute infection before viral replication peak or early convalescence; afterwards HBeAg decreases to below detectable levels in the serum. The presence of AntiHBe helps to distinguish between these two. HBeAg to AntiHBe seroconversion can be used as an indicator or virological response during treatment of chronic patients (Abbott USA, Anti Hbe kit insert).

1.8.1.4 Liver Function Tests (AST, ALT, GGT)

Aspartate aminotransferase (AST), also called glutamate oxaloacetate transaminaseis one of the groups on enzyme which catalyses the interconversion of amino acids and alpha keto acids. The greatest amounts of AST can be found in heart, liver, muscle and kidneys. Damage to these organs can elevate serum AST levels. Serum levels can increase to 10-15 fold normal levels, and increase is proportional to the degree of tissue damage (Abbott USA, AST kit insert).

Alanine Aminotransferase (ALT), also called glutamate pyruvate transaminase is an enzyme which is involved in amino acid metabolism. It is found in various tissues around the body but highest levels are present in liver and kidney. Tissue damage and destruction leads to release of this intracellular enzyme into the bloodstream. Elevated levels of ALT are found in variety of diseases which usually involves the liver such as hepatitis, mononucleosis and cirrhosis. ALT is regarded as a specific indicator of liver disease as high ALT levels are not seen in other diseases such as myocardial infarction (Abbott USA, ALT kit insert).

Gamma-glutamyl transferase (GGT) can be found at highest levels in kidneys; the measurable enzyme in the serum originates mainly from hepatobiliary system. GGT is elevated in many forms of liver disease. 5-30-fold increase can be seen in hepatic biliary obstruction, but moderate elevations such as 2-5 fold are seen with hepatitis infection (Abbott USA, GGT kit insert).

1.8.2 Molecular techniques

As discussed above, usual diagnosis regime of HBV infection is done with serological markers. But there are circumstances where serological diagnosis is not the preferred way. As an example, some serological markers cannot distinguish between present and past infections, also these serological markers cannot address the antigenic variations together with infections with different genotypes, silent carriers and in early phase of the infection (absence of related antibodies). Although the full hepatitis panel should be analysed for each patient, some of the tests are not readily available everywhere. Another problem is with new-borns where maternal antibodies make it difficult to identify infections. To overcome these problems, nucleic acid tests (NATs) have been developed to detect the viral genome in patients' blood for diagnosis. They benefit from direct investigation of infectious agent’s genome in the serum (Irshad et al., 2016).

Such NAT is; PCR, which is used in practice in many laboratories around the world. Conventional PCR is a long and timely technique which limits its use. Also,

each marker needs to be examined separately and this usually is time consuming. Because of these problems, real time PCR (rtPCR) is a better option for viral diagnosis as it can detect pathogen related nucleic acid in shorter period of time. It can also be used for molecular detection of forms and variants of species, not just for viruses but also for bacteria and parasites. rtPCR uses specific and sensitive probes and primers to detect targeted sequences in genome of interest. As technology advances, automation of the system resulted in reduced post-PCR procedures and this decreased the cross contamination between samples. Recent discoveries of PCR coupled with oligonucleotide microarray technology have shown noteworthy increase in sensitivity of detection. Taking into account these advantages which are shorter time spent for diagnostic tests, lowered costs and seeing results on a screen; these newer techniques are replacing others used previously (Irshad et al., 2016).

HBV DNA level measurements represent the direct product and hallmark of viral replication. The availability of highly standardised quantitative rtPCR for HBV DNA has enhanced the investigation of viral replication and natural HBV history. Currently, HBV DNA is an important tool for management of HBV; to identify disease progression, select candidates for therapy and guide treatment regimes. However, HBV DNA only describes the viral status at the time of sample collection, but does not represent overall balance of host immunity and virus replication. In situ hybridisation techniques may also be used, for example for cccDNA. But they do not provide diagnostic advantages (Bonino et al., 2010).

1.8.3 Interpretation of test results

Tests and their corresponding HBV infection stages are explained previously in the text in detail. By performing the so called “HBV panel”, it is possible to conclude at which stage the infection is at and this helps for accurate diagnosis and treatment (Table 1.8.3.1.).

Table 1.8.3.1. Interpretation of results. Each test result resembles a specific information in each of the phases of HBV infection. By evaluating these parameters, we can conclude the diagnosis. (+: positive, -: negative, N: normal, >: above 2000IU/mL, <: below 2000 IU/mL, FP: false positive, MTC: mother-to-child. Table was created using data from Akhan et al., 2014 and Schillie et al., 2018. (Akhan et al., 2014; Schillie et al., 2018).

Infection Type	HBsAg	AntiHBs	HbeAg	AntiHBe	AntiHBc IgG	AntiHBc IgM	HBV DNA	ALT
Acute	+	-	+	-	-	+	++	+++
Acute, window period	-	-	-	-	-	+	+	+++
Acute, resolving	-	+/-	-	+	+	+	+/-	++
Chronic	+	-	+/-	+/-	+	-	>	+
Chonic, Immune Tolerant	+	-	+	-	+	-	+++	**N**
Chronic, Reactivaton	+	-	+/-	+/-	+	+/-	+	++
Inactive Carrier	+	-	-	+	+	-	+/<	**N**
Occult HBV	-	+/-	-	-	+/-	-	+	**N**
Vaccine	-	+	-	-	-	-	-	**N**
Never Infected	-	-	-	-	-	-	-	**N**
Resolved Infection	-	+	-	+	+	-	-	**N**
FP/ Past Infection/ MTC	+/-	-	+/-	+/-	+	-	+/-	**N/+**

1.9 Treatment

The primary goal of HBV treatment is to eliminate the virus or to sustain suppression of replication. More importantly, it is crucial to choose an effective drug with low risk of resistance to achieve constant virological response (You et al., 2014).

Once a patient is infected, antiviral agents can only reduce the complications but complete eradication of HBV is not possible. Only loss of HBsAg and development of AntiHBs can be defined as the viral cure of HBV, which is rare. Therefore, prevention of transmission remains as the most effective way (Jaffe and Brown, 2017).

A reduction in cccDNA levels (1 log reduction) (Figure1.9.1) is usually achieved after 1 year of treatment. This reduction is obtained as a result of the lack of

arriving viruses from blood and inadequate recycling of viral nucleocapsids to the nucleus as a result of the inhibition of viral DNA synthesis in the cytoplasm. Long period antiviral therapy is required to achieve more reduction of cccDNA. (Lutgehetmann et al., 2008; Wursthorn et al., 2006)

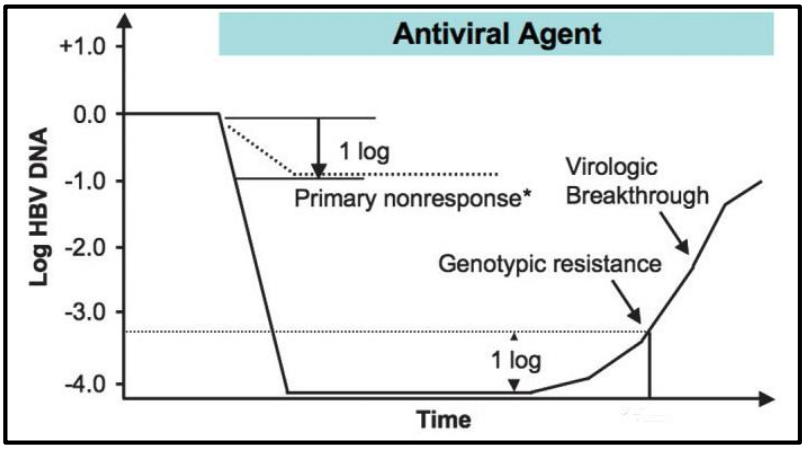

Figure1.9.1. Definitions of antiviral treatment failure during therapy. (*Defined as 1 log decrease in serum HBV DNA after 6 months of treatment.) Primary nonresponse, inability of the given drug to reduce serum HBV DNA by 1 log 10 IU/mL within the first 6 months of treatment. Virological breakthrough, the first clinical sign of the progress of antiviral drug resistance; defined as rise in serum HBV DNA by 1log 10 IU/mL on two or more times at least 1 month apart while on treatment after accomplishing a primary response. Genotypic resistance, the detection of viral amino acid substitutions in the *RT* region of the HBV genome that have been shown to confer resistance to antiviral drugs (Ghany and Doo, 2009).

cccDNA persistence in the hepatocyte is the reason for the failure to clear the virus and for the relapse of viral activity (Dandri and Petersen, 2016). A durable cccDNA reduction (2 log) can be achieved in patients with 1 year use of combination therapy (polymerase inhibitors and IFN α) which shows direct antiviral effects and immunomodulatory effects. By targeting cccDNA transcription, IFN α can straight cause lowered HBeAg and HBsAg, also IFN α administration was shown to promote cccDNA degradation partially (Lutgehetmann et al., 2008; Wursthorn et al., 2006).

Regarding 5 to 10 years' risk for HCC advance in patients above the age of 30, antiviral therapy is required to slow HBV replication and decrease its oncogenic progression. Therapy with PEG IFN α is typically effective only in immune active phase patients. And even conversion to immune control phase of infection is rare, about 30% for HBeAg positive disease. In these situations, PEG IFN α works by

enhancing the host immune response against infected hepatocytes, therefore controlling the disease. HBsAg loss is considered to be the endpoint of treatment, and is achieved in around 10% of HBeAg negative patients with 3 years of PEG IFN α treatment. When this occurs, the risk of HCC is reduced considerably (Liaw, 2010).

Anti-viral therapy with nucleostide analogues will suppress virus replication at any stage of the HBV infection. However, there are some concerns regarding this such as lifelong treatment with these agents may have adverse side-effects. Also, withdrawal or termination of the therapy may lead to more severe liver disease, careful monitoring is required post treatment (Kennedy et al., 2017).

The treatment responses for LAM, ADV, telbivudine (LDT) and entecavir (ETV) are similar in different HBV genotypes. So there is no variance in the response between HBV genotype and these nucleostide analogues (Sünbül, 2014).

The main difference between NAs and PEG-IFN/IFN, the PEG-IFN has the benefit of finite period of use, whereas NAs' use is indefinite. The major downside of PEG-IFN is the high frequency of side effects; in the other hand, NAs have the risk of experiencing drug resistance (You et al., 2014).

Other than pathological effects of HBV, there is also an impact in economics regarding the treatment of this vital disease. In the year 2004 in Turkey, it was suggested that the economic impact of HBV antiviral therapy was 120 million Turkish liras (TL) and treatment of complications was 930 million TL which sums up to 1.05 billon TL for CHB (data of approximately 50 000 patients) (Akhan et al., 2014).

Later in the text, agents used for treatment in TRNC, their amounts and treatment-receiving patient numbers are explained in detail.

1.9.1 Agents and strategies

Currently, 2 sets of treatments are available for chronic HBV infection. First one is NAs, second is PEG IFN therapy. The goals for HBV treatment are; loss of HBV DNA, loss of HBeAg, production of Anti HBeAg, improved liver histology and loss of HBsAg together with seroconversion to AntiHBs. The ideal end point to stop treatment in 2 groups of patients (HBeAg negative and positive) is HBsAg loss with or without seroconversion to AntiHBs. With current treatment regimes, HBsAg loss is uncommon, approximately 4% of patients treated with PEF IFN and 2% of patients treated with NAs lose HBsAg (Ashgar et al., 2017).

1.9.1.1 Telbivudine (LdT)

LDT is a L reverse transcriptase inhibitor which has shown to have no effect on human nt and DNA synthesis. LDT is also a category B drug for pregnancy. Only concern regarding LDT use is that only a single substitution is required (rtM204I) to induce resistance, which is a lower genetical barrier to resistance when compared to TDF and ETV. One concern is that rtM204I mutation also reduces the susceptibility to the other L nucleoside analogues, such as LAM and ETV which later on will limit treatment options (Jaffe and Brown, 2017).

In some studies, it has been shown that LDT has no viral resistance when used for 24 weeks, whereas some studies found that after 2 years of use, resistance was observed. Therefore, resistance remains as concern for LDT when taken in account with newer agents like TDF. This concludes that LDT can be used for short duration therapy (Seifer et al., 2009; Kim et al., 2015). It is not suggested as a first line therapy alone, but it may be used together with other agents such as ADV or TDF (You et al., 2014).

1.9.1.2 Lamivudine (3TC/LAM)

A cytidine analogue, is the first effective nucleoside reverse transcriptase inhibitor (NRTI) against HBV and is the longest & most used antiviral drug for treatment of chronic HBV. LAM is a category C drug for pregnancy (animal studies revealed adverse effects, but no human studies have been performed). Use of it results in reduced HBV DNA and ALT levels, and most importantly HBeAg seroconversion. Nevertheless, the biggest disadvantage of this drug is occurrence of drug resistance and drug resistant mutants (Jaffe and Brown, 2017; Lai et al., 1998).

More than 70% of the patients will become resistant over time, especially it is reported that treatment in 3rd trimester can lead to mutations (rtM204I/V or rtA181T) in 19% of pregnant women (Ayres et al., 2014). The emergence of YMDD mutants has been a key problem with use of LAM. Therefore, it is not suggested as the first line treatment for CHB, but it may be recommended in combination therapies with other agents such as ADV (You et al., 2014).

1.9.1.3 Adefovir (Adefovir Dipivoxil/ADV)

ADV has therapeutic efficiency against both wild type and LAM resistant YMDD mutant (You et al., 2014). Similarly, to LAM; it has developed resistance problems and also showed adverse effects such as nephrotoxicity. ETV, on the other hand, is more refractory for development of resistance and also is the second line agent in case of LAM resistance (Tanji et al., 2001; Tenney et al., 2009).

ADV use have proven virological enhancement in patients with LAM resistance, nevertheless of being used alone or in combination therapy. It is not suggested to be used as first line agent for HBeAg positive/negative CHB patients as it is less effective compared to other NAs such as ETV or TDF (You et al., 2014).

1.9.1.4 Tenofovir (Tenofovir Disoproxil Fumerate/TDF)

TDF is the most recent nucleotide reverse trasncriptase inhibitor (NtRTI) which acts as a chain terminator by competing with adenosine during HBV replication. There is no documentation regarding tenofovir resistance but has adverse effects of being nephrotoxic (Tenney et al., 2009 and Karras et al., 2003).

TDF is the first line therapy agent for HBV infection during pregnancy due to its safety and resistance profiles as well as efficacy and potency. It is classified a category B drug for pregnancy. Studies have shown that no resistance was observed for up to 3 years of use as a monotherapy. Also less than 1% or resistance or virological breakthrough was observed when used for 6 years as a monotherapy. Therefore, monotherapy with TDF may be as effective as combination therapy (Heathcote et al., 2011; Tenney et al., 2009).

No genotypic resistance is observed, the results of its use provided solid confirmation that it was harmless and effective in long term use in the cure of CHB patients. Current guidelines suggest TDF and ETV as the two main effective agents as first line treatment (You et al., 2014).

1.9.1.5 Entecavir (ETV)

ETV is a deoxyguanosine nucleoside analogue which has a high antiviral activity and a great genetic barrier against resistance. In long duration use, it has shown to be safe, well tolerated and impose high degree of HBV DNA suppression. Also, long term use has shown that it is responsible for reversal of fibrosis or cirrhosis as well as histological improvements. Due to its low resistance profile, it is suggested as first line therapy for treatment of both compensated and decompensated CHB patients (You et al., 2014).

1.9.1.6 Clevudine (INN)

INN is a pyrimidine analogue with constant antiviral activity. Previous research conducted in Korea has shown that it has effective antiviral activity during therapy and has post treatment antiviral effect up to half a year after being used for 4 to 6 months. The activity is later hampered due to resistance. A single rtM024I mutation confers resistance to INN. Also, high amounts of myopathy are observed due to mitochondrial damage in the muscle cells. Therefore, it is not suggested as a first line treatment (You et al., 2014).

1.9.1.7 Cytokine mediated treatment (Support therapy)

Support therapy includes use of IFN (IFN α 2b) or PEG-IFN α which helps to boost immune system and improve formation of IFN simulated genes. IFN α use results in a lower viral replication and reduction of HBV DNA in serum as HBV transcription is reduced. It is administered intramuscularly or subcutaneously. It not only reduces HBV DNA, but also DNA *pol* and HBcAg as well. Other than these, it has been sown to upregulate APOBEC3A & APOBEC3B which results in cytidine deamination and degradation of cccDNA without effecting the host genome (Sandhu et al., 2017; Lucifora et al., 2014).

NAs have no effect on clearing cccDNA. Whereas IFN, through its immunomodulatory effect, can induce cytotoxic T cells' activity that will lead to clearance of infected hepatocytes which in the end reduces numbers of cccDNA levels. In terms of high HBV DNA levels, this reduces the T cell response to the HBV related antigens; NA treatment suppresses HBV DNA; and, as a result of this, the immune system restores CD4+ & CD8+ T cell response against HBV. Addition of IFN to the treatment at this stage enhances the clearance of cccDNA, as explained previously (Ashgar et al., 2017).

Common side effects of PEG-IFN include; injection related problems, neutropenia, flu like symptoms, thrombocytopenia, lowered appetite, abdominal discomfort, pruritus, rash, thyroid problems, depression and high costs (You et al., 2014).

In IFN based therapy, the response can be measured with the following parameters; HBV DNA levels, ALT levels, HBeAg status and genotype. Many research have concluded that genotype plays a significant role in IFN treatment. Response rate is higher with HBV-A and HBV-B when compared with HBV-C and HBV-D. Also it was indicated that HBV-C patients, reach the earliest HBV DNA negativity with use of IFN treatment. Whereas, HBV-E is the hardest genotype to treat (Sünbül, 2014).

1.9.1.8 Treatment in pregnancy

For preventing mother-to-child transmission, WHO and Centers for Disease Control and Prevention (CDC) have recommendations of both passive and active immunoprophylaxis; using HBIg and HBV vaccine. However, 10-30% of infants still develop HBV infection when their mothers have a high viral load (Jaffe and Brown, 2017).

Management of CHB during pregnancy and preventation of mother-to-child transmission is a crucial step in eliminating or reducing global burden of HBV. Therefore, management of the infection during pregnancy is an important opportunity to interrupt perinatal transmission. HBV infection during pregnancy represents clinical challenge due to the complex link between physiological changes during gestation period and pathophysiological response to HBV. In the absence of immunoprophylaxis, mother-to-child transmission of HBV is as high as 70 to 90% in infants with HBeAg positive mothers and 10 to 40% in infants with HBeAg negative mothers (Piratvisuth, 2013).

Early study by Beasley et al. (1975) have shown that HBIg administration to new-borns can reduce the rate of transmission by more than 90%. When combined with the vaccine, the rates of transmission fell to 3-7%. However, ineffective immunoprophylaxis leading to mother-to-child transmission has been reported in 9-32% of infants with HBeAg positive mothers, with high HBV DNA levels (Beasley et al., 1975).

All current anti HBV drugs are FDA pregnancy category C, excluding LDT and TDF which are category B drugs. Use of LAM to prevent transmission in HBeAg positive, highly viremic mothers have shown significantly lowered rates of mother-to-child transmission (Piratvisuth, 2013).

In use of LDT; however, an important reduction in HBV DNA (7.38 log10 copies/ml to 4.08 log10 copies/ml) was seen. All newborns also received HBIg and vaccine at birth. As a result, none of the infants acquired HBV infection (Han et al., 2011).

Use of TDF, because of its efficacy; is expected to be as effective as other drugs such as LAM and LDT. It has been used by HIV infected mothers as well, with no evidence of congenital malformations, renal problems and other side effects (Piratvisuth, 2013).

Use of any drugs during pregnancy is a risk factor for the infant, but it has been found that prevalence of birth defects for all antiviral agents are statistically similar to the prevalence of general population reports. No difference in birth defect rates, based on time of use during pregnancy, was observed. These information could be merged to conclude that these medications lack teratogenicity (Jaffe and Brown, 2017). When comparing percentage proportions of birth defects compared to live births; LAM has 3.1% in first trimester and 2.8% in the second trimester. TDF has 2.2% in first trimester and 2.1% in second trimester. LDT, ADV and ETV however has no birth defect risk in either trimester. Therefore, we could generalize that LDT, ADV and ETV can be used during pregnancy, they do not affect the baby but they may have other side effects (Piratvisuth, 2013)

1.9.1.9 Future of treatment with novel agents

The treatment logic has shifted since mutations and resistances became more common. The HBV genome is prone to mutations due to lack of correction mechanisms. High rates of nt mismatch does occur during treatment with NAs especially in *RT* region of the HBV *pol* gene (Liu et al., 2014).

There are novel and possibly effective treatment options such as siRNAs and antisenseRNA. Both agents promise to target viral mRNAs from both cccDNA and HBV genome itself. Another agent is Myrcludex B which is an NTCP inhibitor, prevents HBV entry into the hepatocytes (Sandhu et al., 2017; Volz et al., 2013).

1.9.2 Treatment for HCC

Liver transplantation remains as the favoured surgical choice for HCC, but long waiting times are problematic. Excision of cirrhotic liver leads to lower risk of recurrence and long-time survival (Desai et al., 2017).

There are 2 approved therapies for HCC to date. Trans catheter arterial chemoembolization (to restrict tumours blood supply) and multi kinase inhibitor, Sorafenib (suppresses tumour growth) both extend survival of patients, (especially trans catheter arterial chemoembolization up to 2 years) (Lammer et al., 2010).

Sorafenib was shown to improve survival and is accepted as the standard care for advanced HCC. Sorafenib related adverse effects such as diarrhoea, hand foot skin reactions, hypertension and abdominal pain could be observed (Kudo et al., 2011).

There are also other agents currently being used. Their genetic alterations in HCC and molecular targets are listed below, together with adverse effects they may cause.

Brivanib, VEGFR and FGFR inhibitor. Grade 3/4 adverse effects could be seen. Sunitinib, VEGFR, PDGFR, C-KIT and RET inhibitor. Toxic and unsuccessful. Linifanib, VEGFR and PDGFR inhibitor. Poorly tolerated and grade 3/4 adverse effect seen. Ramucirumab, recombinant Immunoglobulin G1 monoclonal antibody. Binds to VEGFR-2 with high affinity. Inhibits angiogenesis. Grade 3 or higher adverse effects could be seen. Everolimus and Erlotinib, cell proliferation inhibitors, both have serious side effects.

Newer drugs such as angiogenesis inhibitors, anti-metabolites and immune checkpoint inhibitors exist, but Sorafenib monotreatment remains as standard treatment for HCC (Desai et al., 2017).

1.10 Epidemiology

Eastern Mediterranean region is the site of one of the 6 regional offices of WHO globally, consisting of 22 members dealing with an estimated total population of 605 million. In this region, it is also estimated that 4.3 million people are infected with HBV. The reason for this is unsafe blood transfusions, poor public health and decreased awareness. WHO has defined prevalence of this disease as low (<2%), intermediate (2-8%) and high (>8%). In these regions, some are marked as low-intermediate and some are marked as high prevalence (Figure 1.10.2.) (Babanejad et al., 2016).

Southern and Eastern Europe Countries, Southern and Central America, Central Asia and Middle East has a medium endemicity profile for hepatitis B virus infections. Turkey, therefore TRNC can also be classified as a country of low-intermediate endemicity as Northern Cyprus population shares many characteristics with Turkey population (Karabulut, 2015).

Worldwide, there are around 240 million chronic HBV carriers, around 65 million of these people live in Africa (Mammas et al., 2017). In Europe, HBV rates are variable between different regions; Southeastern Europe is still at high level

endemicity whereas Western Europe is low endemicity. 7 000 - 8 000 new diagnosis of HBV are made annually in Europe (Villiano et al., 2015).

Geographic distribution of HBV genotypes are also associated to their route of exposure. For example, HBV-C and HBV-B are more mutual in high-endemic areas of perinatal and/or vertical exposure, which is important for viral transmission. Other genotypes are seen in regions where horizontal exposure occurs (Sünbül, 2014).

Due to global and geographical variations of HBV incidence, HCC burden also varies. Asian-Pacific and sub-Saharan Africa has the highest incidence of HCC, wheras lower risk of HCC is seen in USA. Due to diversity, Europe has different area distributions for risk. Low risk in West and North Europe, high risk in East and South (Figure 1.10.1.) (Di Bisceglie, 2009).

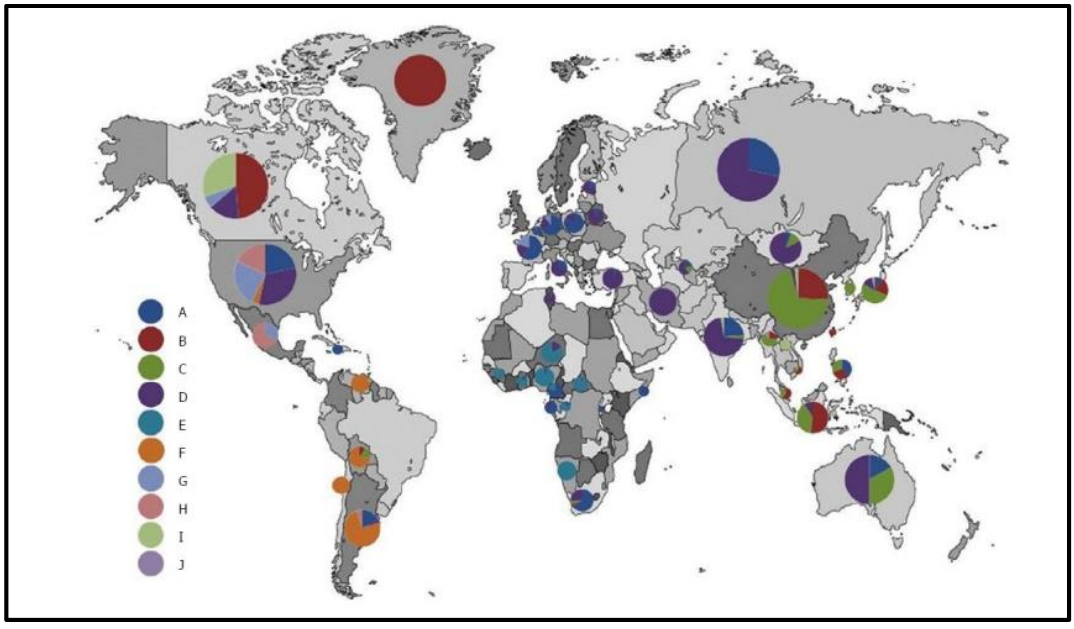

Figure 1.10.1. Distribution of genotypes around the globe. The genotypes and their respective colours are shown in pie charts on each continent/country (Sünbül, 2014).

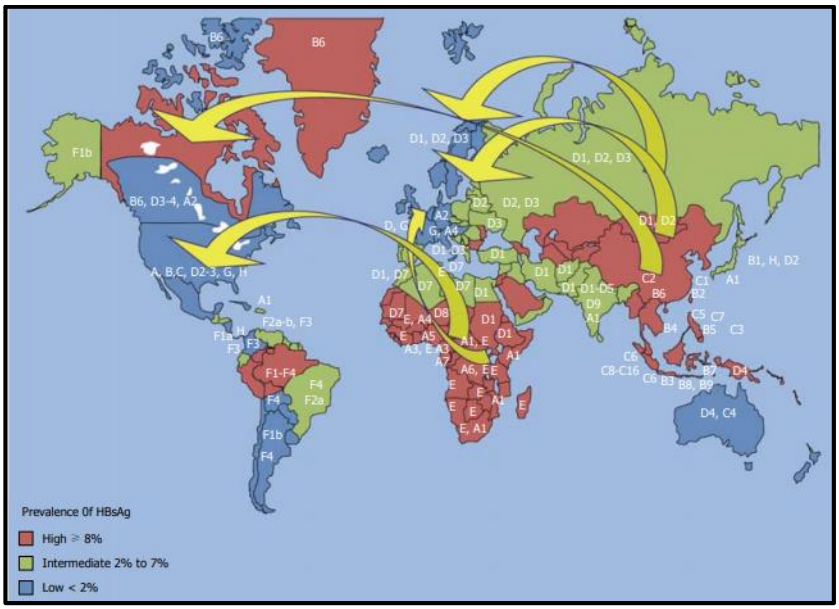

Figure 1.10.2. Prevalence and geographical dispersal of HBV geno/subgenotypes and HBsAg prevalence. Yellow arrows represent direction of subgenotype through immigration from high and intermediate endemic areas to low endemic areas (Pourkarim et al., 2014).

1.11 Anti-viral resistances

HBV drug resistance is a great challenge as multidrug, cross- and various other resistance issues are encountered at high rates (Table 1.11.1.) (Liu et al., 2014).

Normally, NAs are administered until the patient seroconverts to AntiHBs or AntiHBe, in many cases this takes more than a year and HBV may become resistant to the given specific NA. This causes NA resistant HBV mutants. Also this causes the 'mutant' to replicate relatively faster compared to the 'wild type' due to growth advantage (Noordeen, 2015).

Table 1.11.1. Table of single and multi-base mutations and their outcomes in viral resistance to NAs in the RT region (R: resistant, S: sensitive, I: intermediate, LAM: lamivudine, LDT: telbivudine, ETV: entecavir, ADV: adefovir, TDF: tenofovir). Table was creating using data from Tacke and Kroy, 2016 & He et al., 2015 (Tacke and Kroy, 2016; He et al., 2015). (continued on the next page)

	LAM	LDT	ETV	ADV	TDF
Wild Type HBV	S	S	S	S	S
Single Base Mutations	LAM	LDT	ETV	ADV	TDF
rtM204I	R	R	R/S	S	S
rtL180M	R	R	S	S	S
rtM204V	R	R	R	S	S
rtV207M	R	S	S	S	S
rtS213T	R	S	S	S	S
rtN/H238T	S	S	S	R	S
rtT184A	S	S	R	S	S
rtV214A	S	S	S	R	S
rtN236A	S	S	S	R	S
rtN236T	S	S	S	R	I
rtV173L	R	S	S	S	S
rtL180M/I	R	R	S	S	S
rtA181T/V	I	S	S	R	S
rtN/H238T/A	S	S	S	R	S
rtA194T	R	S	S	S	I
Muti Base Mutations	LAM	LDT	ETV	ADV	TDF
rtL180M+rtM204I	R	R	S	S	S
rtL180M+rtM204V	R	R	S	S	S
rtV173L+rtM240I	R	R	S	S	S
rtL180M+rtM240V/I	R	R	S	S	S
rtL180M+rtM240V/S	R	R	I	S	S
rtL180M+rtM240I/L	R	R	S	S	S
rtV241A+rtN/H237T	S	S	S	R	S
rtM214I+rtN236A	R	R	R	R	S
rtV173L+rtL180M+rtM204V	R	R	R	S	S
rtL180M+rtT184A+rtM240V	R	R	R	S	S
rtL180M+rtM240I+rtV207M	R	R	R	S	S
rtL180M+rtM204V/I±rtT184G±rtS201I/G	R	R	R	S	S
rtL180M+rtM240V/I±rtI169T±rtV173L ±rt3250V	R	R	R	S	S
rtL180M+rtM204V+rtS213T+rtN/H238T +rtT184A	R	R	R	R	S
rtV173L+rtL180M/I+rtM204I+rtA181T +rtN236T	R	R	R	R	S

Resistance mutations, which are related to NAs, are usually found at the polymerase gene of the HBV (*P* region). The aa from upstream to downstream in *P* region is named ranging from rt1 to rt344. *P* region is also classified as seven sub regions: *A*-*G*. Mutations usually occur at *A*-*D* regions (Zoulim, 2004).

LAM; the first FDA approved nucleoside analogue, inhibitor of HBV replication, is effective as it has shown to reduce the risk of HCC in 5 year follow-up in cirrhotic patients. However, over the time, appearance of LAM resistant mutants became a major matter. Viral breakthrough due to this mutation can lead to immune activation with hepatic flares and causes immune mediated liver injury (Kennedy et al., 2017; Tsebe et al., 2001).

To date, TDF and ETV are the only nucleostide analogues with little or no viral resistance. These drugs are considered to be the first line treatment options and is the best hope for HCC prevention (Table 1.11.1) (Kennedy et al., 2017).

The long term and wide application of these agents results in HBV resistances. HBV patients with NA resistances usually develop a rise in HBV DNA, elevation of ALT, persistent HBeAg positivity and clinical deterioration (Mast et al., 2005).

1.11.1 Lamivudine resistance

LAM resistance effects the YMDD motif on the active site of the polymerase, where rtM204I/V mutations occur. The rtM204V mutation usually occurs along with rtL180M/S (Tacke and Kroy, 2016).

Although it is very safe and well tolerated in HBV liver disease, it has inadequate clinical use as a result of high rate of drug resistance. Formerly, ADV was used as alone or in combination with LAM to treat rtM204V/I mutants. Nevertheless, the rate of ADV resistance following substituting to LAM compared to ADV treatment in LAM resistant patients is as great as 18%. The rescue therapy of switching to ETV for LAM resistant cases was used but genotypic ETV resistance

and virological breakthrough established in half of the cases. This can be explained as cross resistance is shared by LAM and ETV. Therefore, ETV therapy alone is no more recommended as a rescue therapy. For LAM resistant patients, changing to TDF and ADV add on therapy is suggested. IFN based treatment is only advised in some guidelines (You et al., 2014).

1.11.2 Adefovir resistance

ADV resistance is caused by either rtN236T mutation at the *D* domain or rtA181T/V at the *B* domain. Studies have shown that long period use of ADV might trigger mutants. TDF has shown to be effective in patients with LAM and ADV resistances. As TDF has some grade of cross resistance to ADV, the combination therapy of TDF + LDT/LAM or ETV for patients with ADV resistance may be extra beneficial than TDF monotreatment in order to avoid further mutants from emerging (You et al., 2014).

1.11.3 Telbivudine resistance

Similar to LAM, LDT causes YMDD (mostly rtM204I) in addition to rtA181T/V and rtL229W/V (Tacke and Kroy, 2016). LDT has a relatively higher potency when compared to LAM. But, continued treatment with LDT is restricted by high rates of mutations due to its low genetic barrier. Studies have discovered that LDT and ADV therapy together leads to a substantial decrease in HBV DNA levels in patients with virological breakthrough to LDT (You et al., 2014).

1.11.4 Clevudine resistance

rtM204I mutation plays a crucial role in INN resistance. ADV and TDF were the most effective drugs to use in INN resistant mutants (You et al., 2014).

1.11.5 Entecavir resistance

ETV resistance arises following a two step mutation development. Firstly, a pre-existing rtM204V and rtL180M by LAM. Secondly, the primary ETV resistance by rtT184G and rtS202I or rtM250V. These mutations decrease susceptibility of ETV and lead to treatment failure by virological rebound. It is recommended to add on ADV or TDF or switch to TDF in patients with ETV resistance (You et al., 2014). Also, additional changes in the *B* domain (rtI169T or rtS184G), *C* domain rtS202G/I or *E* domain rtM250V have also been observed (Tacke and Kroy, 2016).

1.11.6 Tenofovir resistance

TDF is known as the safest and most effective antiviral NA for NA treatment naïve or NA treatment failure patients. Some studies revealed that TDF monotherapy maintains its effect for up to 6 years during treatment, with no resistance. As a result of this, TDF is suggested as an important drug for liver disease regardless of resistance to other agents (You et al., 2014).

TDF shares few structural similarities with ADV, which raises concerns of possibility cross resistance. Only resistance mutation rtA194T has been seen in HIV-HBV co infected patients during TDF treatment. This mutation is related with partial drug resistance against TDF. Although, this mutation impairs the replication of HBV

in vivo. This perhaps explains why this resistance occurs in low numbers (Tacke and Kroy, 2016).

1.11.7 Multi drug resistance (MDR)

Generally, progressive NA monotherapy against HBV can cause MDR. Especially, it occurs frequently with continuous treatment using NAs with related features such as LAM followed by ADV or ETV (Figure 1.11.7.1)**.** To name a multi drug resistance, antivirals used together for treatment should be in different calsses, drugs within the same class causing resistance together is not MDR. A combination therapy of TDF + ETV has shown to be efficient in MDR patients including LAM, ADV and ETV resistances. Combination therapy including TDF is better than other NA monotherapies or sequential therapy (You et al., 2014).

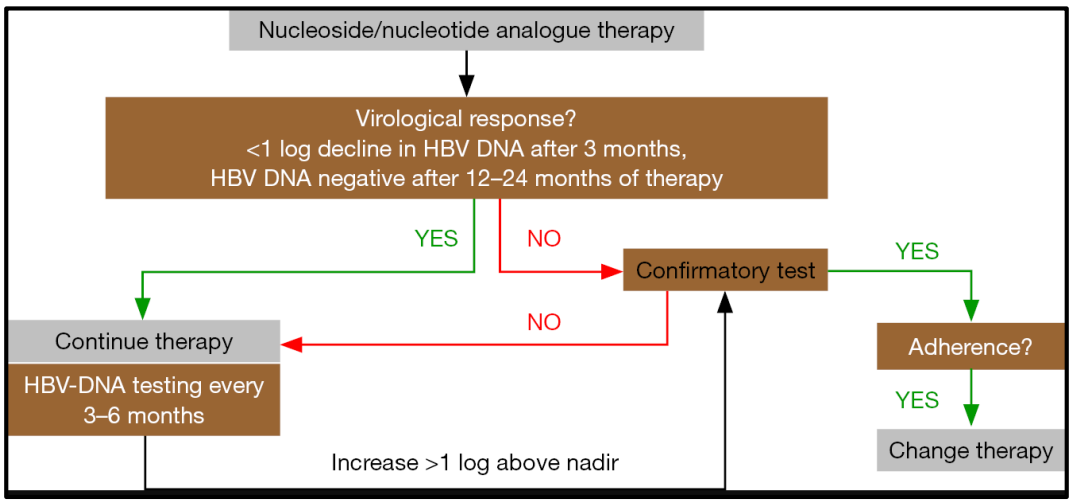

Figure 1.11.7.1. Management of treatment failure. After 3 months, 1 log decline and 1-2 years of being HBV DNA negativity; therapy is accepted as a success. If the course of treatment is uninterrupted the same patients should be tested every 3 to 6 months for HBV DNA. In case of treatment failure, confirmatory tests always should be performed such as HBV DNA and Nucleostide resistance profiles. If after all, the treatment failure or the suspicion of it continuous; treatment need to be modified (Tacke and Kroy, 2016; Ghany and Doo, 2009).

Ghany and Doo, in their work in 2009, explained the preferred management strategies for resistances. LAM resistance with mutations rtM204V/I and rtA181V/T; add or switch to TDF. LDT resistance with rtM204I; add ADV or switch to TDF. ADV resistances with rtA181V/T; add LAM or LDT, with rtN236T; switch to either TDF or ETV. ETV resistances with rtL180M and rtM204V plus rtI169T and rtM250Vor rtT184G and rtS202I; add ADV or switch to TDF (Ghany and Doo, 2009).

1.12 Vaccine(s)

The most effective way in avoiding HBV infection is immunization. The HBV vaccine is the first vaccine to act against 2 viruses; HBV and HDV; and, therefore it acts against HCC. The risk of chronic HBV infection increases with age (from the age which HBV was acquired). Hence, it is really vital to avoid HBV as early as possible. WHO recommends birth dose vaccination but this is not implemented in many countries including most African countries (Mammas et al., 2017).

While there is a safe vaccine against HBV, the infection still stands as a public health concern particularly in some countries such as Africa, Asia and South America (Sünbül 2014). Typical monovalent and multivalent vaccines are administered usually soon after birth, with a schedule of 6-10-14 weeks of age (Mammas et al., 2017).

In 1989, Global Advisory Group of the Expanded Programme of Immunisation have suggested hepatitis B vaccine to be included in their programme. In year 1991, WHO recommended HBV vaccine to be integrated in to the national immunization programme for countries with HBV carrier rate of 8% or higher by 1995 and in all countries by 1997 (Komatsu 2014). In 1992, WHO have endorsed this recommendation. Both plasma derived and recombinant forms of the vaccine are shown to be highly effective in preventing infection with HBV (Figure 1.12.1) (Bhattacharya et al., 2015).

The efficiency of mass vaccination programmes, with no doubt, had a positive effect on HBV infections. A good example is in Gambia in 90s where prevalence of chronic HBV reduced from 12.4% to 0.8% (Peto et al., 2014). Another example is the vaccination programme in South Africa, where HBV vaccination at 6th, 10th and 14th weeks of life lead to protection of 87% of the immunised infants from 1996 to 2001 (Tsebe et al., 2001).

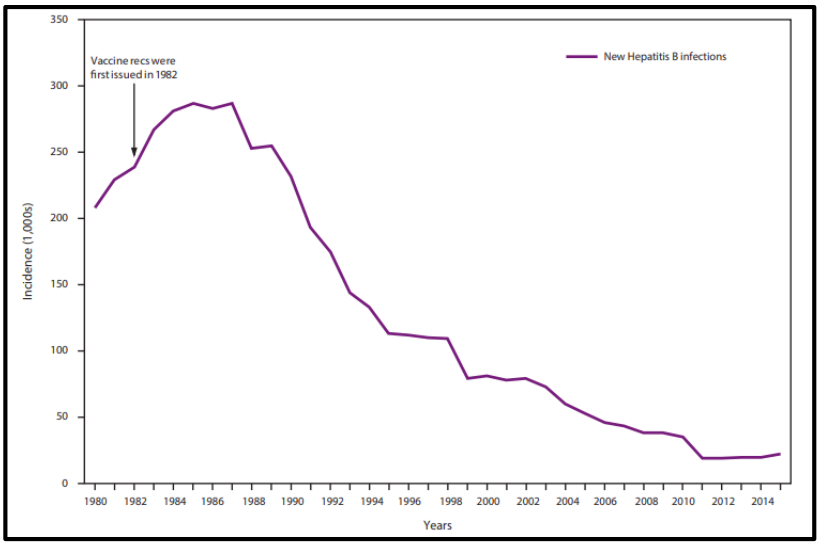

Figure 1.12.1. Effects of the HBV vaccine. Pre vaccine era of 1980 with discovery of diagnostic tools increase is seen in the amount in cases. After the 1990, a decrease is seen which shows that vaccine effects were started to be seen. Following mid-90s, new infection numbers declined (Schillie et al., 2018).

In general practice; 2 forms of vaccines are used commonly for prophylaxis of HBV infection:

1) Plasma derived vaccine: composed of *S* antigen particles purified from the sera of CHB patients.

2) Genetically engineered recombinant vaccine: in which the *S* gene is being expressed in a yeast via a plasmid vector to produce HBsAg virus like particles.

Both vaccine types are greatly immunogenic and result in AntiHBs production. 3 intramuscular doses of the vaccine protect 95 to 99% of infants, children and young adults (Mast et al., 2005).

6 to 8 weeks after the last shot of the vaccine, immune response can be assessed by measuring AntiHBs levels. AntiHBs levels of >10 mIU/ml is accepted to be protective. Some individuals may be non-responsive (AntiHBs <10mIU/ml) or hyper responsive (AntiHBs levels >100 mIU/ml) (Noordeen, 2015).

In TRNC, immunisation was rare since 1985. In 1990s, immunisation was started to be more appreciated. North Cyprus introduced vaccination programme for HBV in July 1998. The vaccination programme was schedulded as the first dose at 2 to 3 months, second dose around 3 to 4 months and the last dose at 6 to 9 months. From mid 80s to early 90s, 2 vaccines were mainly used; Heptavax and Genhevac B Pasteur vaccines. These were usually legally obtained/self-bought from Turkey or illegally obtained from Greek Republic of Southern Cyrus. In the last decade, Genhevac B and Engerix B were the 2 main types of vaccines used. In the past few years, apparently Turkey stopped importing Genhevac B; therefore, the main vaccine in use is Engerix B (Interview with Ahmet Kirisoglu, MD, 2018; Kurugol et al., 2009). Both of these recombinant vaccines and their effects on society can be seen in Table 1.17.1.

1.13 Risk populations

Nowadays, blood donation is a vital and lifesaving intervention. WHO indorses that all blood donation samples to be screened for infections such as; HIV, HBV, HCV and syphilis. Information shared by 164 countries in the WHO global database on blood safety shows that more than 92 million donations occur each year. Out of these 92 million samples, 1.6 million are excluded as they contain infectious markers, which includes HBsAg. However, the transmission risk still exists in several developing countries (Babanejad et al., 2016).

There is evidence that mentally ill patients are at increased risk for HIV, HBV and HCV infections compared to the general public. The main reasons for this include; low quality of life, less knowledge about transmission of infectious diseases,

less knowledge about protective measures, risky sexual behaviour and low standard of living or hospitalisation conditions (Karabulut, 2015).

In most of the developed countries such as Western Europe and USA; the endemicity of HBV is low but most infections still occur amongst high risk population groups such as homosexuals and injection drug users (IDUs) (Alter, 2006).

Risk factors for viral hepatitis include; substance use such as IDU, having multiple sex partners, high risk sexual activity such as anal intercourse, people coming or travelling to high endemic areas, needle stick injury, tattoo and/or piercing with non-sterile equipment, household contact and/or sharing personal items with an infected person, having multiple sex partners and a sex partner with hepatitis B (Krajden et al., 2005). More detailed explanations are provided below.

Injection drug use

Drug use was reported in 30% of the 1657 new HBV cases in 2017 by CDC in case reports. Chronic HBV infection can be seen in 3.5-20% of IDUs and nearly 22% of IDUs have evidence of past infection (Schillie et al., 2018).

Sexual exposure (heterosexual and men to men (MTM))

Among case reports, 26.4% of people have been reported to have 2 or more sexual partners, 3.3% reported to have sexual intercourse with a HBV infected person and 11.8% males reported that they had sex with another male. Around 10-40% of adults who seek medical treatment in sexually transmitted disease (STD) clinics had evidence of current or past HBV infection (Schillie et al., 2018).

Household contact

Estimated value of 45% of people sharing the same household with HBV infected people had proof of past HBV infection and 16% have recent HBV infection (Schillie et al., 2018).

Disabled peoples in long term care facilities

This patient group historically have had HBV prevalence as high as 20%. The introduction of routine vaccination has dramatically declined the infection in these settings (Schillie et al., 2018).

Correctional facilities

Prevalence of HBV infection is higher among prisoners when compared to general population. People entering these facilities would acquire HBV from IDU or by having sex with multiple partners and MTM intercourse (Schillie et al., 2018).

Occupational exposure of healthcare workers

Before vaccination, being healthcare worker was recognised as a common occupational risk. Vaccination and use of precautions have decreased infection numbers from 1983 to 2010 by 98%. Occupational health and safety administration mandates that employers should offer HBV vaccine to all employees together with availability of post-exposure prophylaxis (Schillie et al., 2018).

Hemodialysis patients

2001 data shows that HBV infection in dialysis patients have decreased 95% since introduction of vaccination and implementation of additional infection control protocols. Since 1995, the annual incidence has been stable around 1% (Schillie et al., 2018).

People with chronic liver disease

People with other liver illnesses such as fatty liver disease, cirrhosis, alcoholic liver disease or autoimmune hepatitis are not at risk for HBV unless they get exposed to it (Bell, 2000).

Travelling

Travelling to countries where HBV is endemic has shown that short term travellers are at risk if they travel to high or intermediate endemic countries. They may be exposed to HBV by exposure to blood in medical or disaster relief activities,

by sexual activity or drug use. Monthly incidence for long term travellers is 0.025-0.42% (Johnson et al., 2013).

People with HIV

Around 10% of HIV patients are coinfected with HBV. Chronic HBV is seen in 6-14% of HIV positive patients, including 9-17% of MTM sex and 7-10% of intervenous drug users (Schillie et al., 2018).

Diabetic people

Diabetic people have 60% higher incidence of previous or current HBV infection and double the chance of acquiring HBV compared to non-diabetic people. Use of blood glucose monitoring devices together with finger prick pens and assumable sharing of these items have led to diabetics being under contentious risk (Reilly et al., 2012).

1.14 Transmission

HBV is transmitted via percutaneous membrane and mucous membrane exposure to infectious body liquids and blood (Alter, 2003). Percutaneous exposures such as IDU, blood transfusions, contaminated injection equipment and such health care procedures. Perinatal, mother-to-child (MTC) transmission and sexual transmission are also highly efficient in spreading of HBV. Person to person spread may also occur amongst chronically infected people and household contacts (Alter, 2006).

1.14.1 Vertical transmission:

Most common route for HBV acquisition in developing world is via MTC transmission. However, this transmission can also be seen in low endemic regions as

well. For pregnant mothers, high HBV viral load and HBeAg positivity increases the likelihood of transmission to the baby. This leads to 90% chance of transmission to the baby. Out of these infants, around 90% will develop chronic infection when compared to 50% of children infected at a younger age than 3 years and less than 5% who were infected at adulthood years (Jaffe and Brown, 2017).

Vertical transmission can occur in 3 possible ways

1- Intrauterine transmission: HBV can spread to fetus via placental barrier. But influence of this style of transmission is not clear. Detection of HBsAg in cord blood of newborns has been reported in 37% of those who was born from an HBsAg positive mother. HBsAg positivity at delivery does not predict consequent infection. A Chinese study found that 3.7% of neonates who are HBsAg positive at birth were caused by intrauterine infection. This indicates that intrauterine infection is not the dominant mode of MTC transmission (Piratvisuth, 2013; Xu et al., 2002).
2- Transmission during delivery: is the most frequent mode of transmission. The administration of HBIg and vaccine will prevent this in >85% of the infants who are born from HBsAg positive mothers. During delivery, micro transfusions from mother's blood, rupture of membranes, direct contact of infant's mucosal membranes with mother's infective secretions or blood could cause transmission (Piratvisuth, 2013).
3- Post-partum transmission: 34% of the infants who did not acquire infection during delivery will get HBV within the next 6 months. This may be due to close contact of the baby with mother, breast feeding which is a major worry through ingestion of HBV or by contacting with skin lesions on the mother's breast (this could take place despite the fact that breast milk has immunoglobulins and proteins such as lactoferrins which has antiviral properties) (Piratvisuth, 2013).

1.14.2 Horizontal transmission:

HBV transmission may occur via parenteral routes such as needlestick injury, organ transplantation, blood transfusion and sexual interaction. Horizontal transmission occurs from infected mothers to the neonate; and is a significant way in hyper endemic areas. Patient groups such as haemophiliacs who go through blood transfusion regularly, professions such as surgery and dentistry as well as the healthcare workers are at risk. Living in the same household and using same toothbrush and razor with a carrier is also a risk. IDU also increases risk of HBV transmission as stated before (Noordeen, 2015).

HBV is sustainable for long periods in the surroundings. This was shown in an experiment conducted by CDC, a highly infectious sample was placed on a glass slide and was left in the surroundings for a week; and, was found to be still infectious to a chimpanzee. This proposes that any kind of accidental contact or exposure to blood or body fluids of HBV carriers might spread infection (Noordeen 2015). It is also stated that saliva, urine, bites, broken skin and even tears can also be accepted as possible ways for transmission (Pourkarim et al., 2014).

1.15 Preventation

HBV transmission via blood and plasma derived product donation has been eradicated in most of the countries due to the donor screening procedures. But transmission still occurs with inadequately sterilised medical instruments, reusing of single use disposable needles and contamination of multiple dose medications (Alter, 2006).

Condom use is recommended to avoid sexual spreading of infection to or from infected individuals. Immune prophylaxis with HBIg has shown good defence against infection in HBV exposed persons (Szmunes et al., 1974).

Education on HBV, infection preventation and transmission; delivered especially to high risk groups such as IDU's, is theoretically the most cost effective way of control (Alavian et al., 2007).

One of the most important preventation measures is passive immunisation using HBIg. Previous research has shown that HBIg at postpartum period could induce 2 log drop in mothers' HBV DNA levels and decrease in MTC transfer rates (Piratvisuth, 2013).

1.16 Phylodynamics

Phylodynamics and phylogeography

Both of the terms were first introduced to the subject in 2004 by Greenfell, and is described as how microbial genetic variations are controlled by the host immunity and transmission together with epidemic dynamics which these factors regulate the diversity of pathogen phylogenies seen at a scale from an individual to a population (Greenfell et al., 2004).

As with previous work, in Mediterranean region and in TRNC; main genotypes in the society are known to be D, A and E. In this section, we explain the phylodynamics of these genotypes in detail.

1.16.1 Genotype D

HBV-D is the most dominant genotype. It is found in North-Eastern Europe, Central and Eastern Mediterranean, Middle East and North Africa, Indian sub-continent, group of islands in Indian Ocean and Oceania. Nine sub-genotypes of HBV-D have been described so far. D1 is mostly present in Turkey, Greece and North Africa. D2 is generally observed in North-Eastern Europe (Belarus, Russia,

Estonia) and Albania. D3 is observed in Serbia and Italy. D4 is encountered in Oceania (main strain). D5 is seen in primitive tribes in India. D6 is observed in Indonesia and Papua. D7 is seen in Morocco and Tunisia. D8 and D9 are encountered in India and Nigeria (accepted as recombinant forms of HBV-D, HBV-E and HBV-C) (Zehender et al., 2014).

An important question to answer is "how did genotype D reach Mediterranean from all the way from India?". Reconstruction done by Zehender et al, (2014) explains that it is believed that world war II plays a vital role in spread from India to the rest of the globe; together with further spreading due to unsafe medical use of vaccinations in medical exercise. The first D genotype originated in India in 19th century. D5 sub genotype was probably the first one to diverge. It was followed by D1 and D3 in Central Asia (between 1930s to 1940s) and move from there to Europe and Mediterranean region by either of 2 routes.

South western route: crossed Middle East and stretched to North Africa and South-East Mediterranean.

North western route: crossed former Soviet Union and stretched to Eastern Europe and Mediterranean via Albania (Zehender et al., 2014).

1.16.2 Genotype A

HBV-A is the second most distributed and isolated HBV genotype and is mainly seen in Europe, Asia, Africa and USA. For genotype A, 7 sub-genotypes have been described so far. A1 spreads over South and East Africa, South Asia and South America (South Africa, Malawi, Uganda, Tanzania, Somalia, Congo, India, Bangladesh, Nepal, Philippines and Brazil). A2 is widespread in Europe, North America and South Africa. A3 is observed in Cameroon and Gabon. A4 is seen in Mali. A5 is encountered in mainly Nigeria and Haiti. A6 is only reported in African-Belgian patients. Newly suggested A7 is isolated in Cameroon. It has been proposed

that A3, suggested A4, A5 and suggested A7 must be classified as quashi sub-genotype A3 (Zehender et al., 2014).

How did Genotype A reach these countries from Africa? Hannoun and collegues suggested that A2 sub genotype originated from Africa, and reached Europe by Portuguese sailors in 16th and 17th century; and, A1 to Asia via trade and travel between Africa and South Asia (Hannoun et al., 2005).

It has also been suggested that for sub-genotype A2, the spread is mainly via the result of sexual transmission between late 1960s and beginning of 1980s (Zehender et al., 2014).

1.16.3 Genotype E

HBV-E is widespread in Central and West Africa and is only seen in people who were born in Africa; so we can describe genotype E as an African genotype. It is interesting that, isolates do not have any genotypic differences; therefore, E genotype has no sub-genotypes. E genotype was identified in Colombia in 2010, and it was suggested that it was a new introduction to the community (Alvarado et al., 2010). The reason Genotype E is widespread within Africa is probably due to the use of needles during several mass immunization programmes against yaws and few others. These programmes were seen in West and Central Africa between the years of 1920s and 1960s (Zehender et al., 2014).

1.17 Hepatitis B virus and North Cyprus

Going through the timeline of HBV in TRNC, there isn't many research and/or studies on this subject. There are few but some are missing information such as genotype/sub-genotype analysis and some do not have enough positive samples to commerce such research outcomes regarding molecular analysis as genotyping of

HBV is essential for epidemiological and molecular studies. The clinical significance of different HBV genotypes has become increasingly documented in patients with chronic infection. One and only important research regarding this molecular epidemiology is published by Arikan and her colleagues. The outcomes are explained below.

Within studies performed in Turkey, genotype D is the most dominant genotype of HBV; although others such as Genotypes A, E, G and H is seen as well. Regarding these findings, there were no genotypic data available for TRNC until the year 2016. The study, which was done by Arikan and her colleagues have enlightened the TRNC's HBV molecular epidemiology. As a conclusion in their work, out of 13 892 samples, 160 were HBsAg positive and only 68 of these were eligible for phylogenetically analysis and they have found that genotypes and their respective percentages were as follows: D1 (70.6%), D2 (5.9%), D3 (1.5%), A1 (7.4%), A2 (2.9%) and E (11.8%) which demonstrates the dominance of Genotype D in TRNC (Arikan et al., 2016).

Whereas in this study, we focused of randomly selected 170 samples of HBsAg positivity with high results of sample cut-off values to increase the likelyhood of obtaining more HBV DNA, to have a look at wider range of samples, wider genotype chances and to investigate the antiviral resistance profiles of these samples as well as serotypes as there is no current work or data regarding this subject.

Another study was published in 2006 in which Altindis and his colleagues screened 17 545 people; and, concluded that HBsAg positivity was 2.46% and out of these, HBV DNA positivity was 2.25%. Another study published in 2009 by Kurugol and his colleagues indicated that TRNC should be considered as low endemic for HBV as they concluded that HBsAg positivity was 0.85%. In 2012, Suer and his colleagues screened 1 500 blood donors and found out that total positivity for HBsAg was 0.6%. In 2014 Guler and his colleagues conducted a similar study, and out of 16 372 people, 1.4% was found to be HBsAg positive. The study mentioned previously, Arikan and her colleagues concluded that HBsAg positivity was 1.2%. These data are summarised below in Table 1.17.1.

Table 1.17.1. HBV and North Cyprus. 6 studies performed and their summaries.

Study	Total samples	Patient group(s)	HBsAg positivity	HBV DNA	Genotype detected	Techniques/ Tests used	AntiHBs positivity
Papaev-angelou et al., 1988*	4892	Blood donors (795) Army rec.(388) Hospital staff (1872) Hemodialysis patients(135) Thalassemia patients(559) Instutionalised adults(722) Mentally retarded(98) Family contacts (323)	0.77% 1.01% 2.94% - - 5.40% 6.12% 18.27%	N/A	N/A	RIA for; HBsAg AntiHBs	- - 22.5% 43.7% 82.9% 33.2% 12.2% 28.5%
Altındiş et al., 2006	17545	Turkish Soldiers(11234) TRNC Civil donors(5057) TRNC soldiers (1254)	2.16% 3.0% 2.71%	1.98% 2.90% 1.83%	N/A	HBsAg HBV DNA PCR	N/A
Kurugol et al., 2009	600	TRNC Civilians	0.85%	N/A	N/A	HBsAg AntiHBs HBcAg	>90%
Suer et al., 2012	1500	Turkish Citizens TRNC Citizens Other Citizens	0.6%	NA	N/A	HBsAg	N/A
Guler et al., 2014	16372	TRNC Citizens	1.4%	N/A	N/A	HBsAg	N/A
Arikan et al., 2016	13892	Civil population Blood donors	1.2%	5.9%	D/D1 70.6% D/D2 5.9% D/D3 1.5% A/A1 7.4% A/A2 2.9% E 11.8%	HBsAg HBV DNA PCR AntiHBcIgG AntiHBe HBeAg	N/A

*This is the only study performed in South Cyprus and is the very first research done regarding HBV in the history of Cyprus island.

Cyprus, is an island located in the east of the Mediterranean sea and at the South of Turkey. There are 2 communities living on the island. These are Turkish Cypriots and Greek Cypriots. Since 1974, the island is divided into two due to political disagreements and war. This isolation has caused lack of international educational activities as 43.21% of the male students in a survey done in 2013 said that internet is their source of information and medical authorities were only used by 7.57% of the students (Kaptanoglu et al., 2013).

TRNC is one of the destinations for casino tourism and entertainment. During the last one and a half decade; it also became a target country for sex workers from Eastern Europe and former Soviet Union. This created a problem as sex workers with STDs to come to the island and work without a comprehensive control mechanism

(these premises and workers are regularly and continuously controlled and investigated by government). These diseases can spread really fast as the clients of night clubs are primarily young and middle-aged Turkish Cypriots and Turkish Citizens. Although Turkish Cypriots are Muslims, pre-martial sex of women is judged; however, for males, it is encouraged or excused. This results in a high demand for premises offering sexual experience services (Kaptanoglu et al., 2013).

In 2013, there were 27 night clubs/brothels (premises offering sexual experience) and 774 sex workers (Kaptanoglu et al., 2013). These numbers have increased since 2013. The total number of sex workers, and number of night clubs are listed in more detail in table 1.17.2 below.

Table 1.17.2. Total numbers of night clubs, pubs and sex workers by year in TRNC. (MOI: Turkish Republic of Northern Cyprus Ministry of Internal Affairs database, USA:Trafficking of persons report 2014-2018)

	Night Club, n		Pub, n		Sex worker, n	
Year*	TRNC MOI	USA	TRNC MOI	USA	TRNC MOI	USA
2014	39	40	2	-	1168	1168
2015	38	36	2	-	1131	1481
2016	38	-	2	-	970	1314
2017	38	-	2	2	1080	1084
2018	31	-**	2	-**	1102	-**

*For TRNC MOI data, the date range for given data are as follows; 2014;04/2014-01/2015, 2015;04/2015-12/2015, 2016;04/2016-12/2016, 2017;04/2017-12/2017, 2018;04/2018-01/2019.
** 2018 data is not published yet.

In Trafficking in Persons Report 2018, human trafficking profile of South Cyprus can be summarised as; mainly people from Latvia, Bangladesh, China, India, Bulgaria, Dominican Republic, Romania, Cameroon, Cote d'Ivoire, Slovakia, Philippines, Togo, Paraguay, Czech Republic and Moldova were victims of human trafficking. Women mainly from Vietnam, India, Eastern Europe and Africa were subjected to sex trading. These trafficking activities occurred in isolated apartments and hotels, sometimes on the streets and in some bars and pubs, also mainly in cabarets. Most of the sex trafficking victims were apparently recruited to the island

with false assurances of marriage or worker as barmaids. South and South East Asian migrant workers were being forced to work in agriculture (Trafficking of Persons Report 2018).

In North Cyprus, the situation is different. Apparently, Turkish authorities did not entirely meet the slightest standards for eliminating human trafficking and are not making any effort to do so. Also it was stated that the North Cyprus authorities did not keep data on law enforcement efforts against human trafficking. Only data was from nightclubs and similar places of entertainment. As of January 2018, there were 400 females working in these premises. This number was 342 in January 2017. Out of these women, majority were from Moldova, Belarus, Russia, Armenia, Kazakhstan, Ukraine, Kenya, Morocco, Kyrgyzstan, Tajikistan, Tanzania, Uzbekistan and Paraguay. In 2016, 445 women were deported, in 2018 this number fell down to 331 (deportation cause not stated). Also in TRNC, women mainly from Central Asia, Eastern Europe and Africa were exposed to forced prostitution in night clubs controlled by the government. In the previous years, spectators have stated that some number of women arrived to the TRNC on three-month tourist or with student visa and have been involved in prostitution in flats and private homes in Nicosia, Kyrenia and Famagusta. Some are believed to be trafficking targets (Trafficking of Persons Report 2018).

This data is not agreeable. In our study, we have obtained different and additional data from TRNC MOI. The numbers they have supplied us were different that the Trafficking 2018 report and are stated in Table 1.17.2. The numbers and nationalities of sex workers are listed in table E1 in Enclosure 3 section (Except 2016 as there was data loss for this period) (Turkish Republic of Norhthern Cyprus,Ministry of Internal Affairs database).

Despite this situation, Turkish Cypriots are informed about STDs; a study performed in 2013 revealed the following information: 91.25% of the students had knowledge about STDs. 32.7% of male students had full knowledge about STDs. 1.65% did not know the relationship between STDs and drug use. Unprotected sexual intercourse, blood transmission, oral sex and mouth to mouth kissing were recognised as modes of infection by 92.67%, 72.10%, 39.72% and 28.13%

respectively. Hepatitis B was known by 28.61% of the students (Kaptanoglu et al., 2013).

Alongside with proper diagnosis and treatment, most important tool is knowledge about Hepatitis B. Education and awareness are powerful ways of fighting HBV in TRNC.

As North Cyprus is an isolated country, restriction of entry/residence of people with infectious diseases is implemented. This is regulated by the law.

Section 105, article 6c (1982, 1989, 2006, 2007, 2008, 2011, 2014, 2016) which states that;

> "Any person who risks the society's health with an infectious disease/agent should be deported."

Again in Section 284, article 2 states that (1990);

> "Any person with the risk of an infectious disease should be hold under custody until the government officials do the compulsory testing to proof that the person will do harm to the society."

These laws exclude Turkish Cypriots. Any other 3rd party nation citizens, including Turkey Republic citizens should get their immigration testing done. This process only includes people who are between the ages of 18 – 65 and who wish to stay, work or live in TRNC for more than 30 or 90 days depending on the visa stamp given at the day of entry (http://www.mahkemeler.net/cgi-bin/default.aspx, Accession date 14 November 2018).

The required tests are listed below:

HBsAg, Anti-HCV, HIV, Syphilis, Pure Protein Derivative (PPD) Tuberculin test or Chest X ray for Tuberculosis.

For employees working in the food industry, care takers and some healthcare employees, additional Salmonella and Shigella stool testing must be performed. The above tests are performed by both government healthcare facilities (Hospitals and branches) and private laboratories or private hospitals.

For night club (sex) workers, the situation is different.

Their stay and control is stated in Section 7/2000-30/2007, articles 15 and 17 which states that (1992, 1993, 2007)

"Upon arrival to the country they should undergo custody, their blood sample is taken by a government official and tested for above tests together with a vaginal swab. These sex workers are only tested by the government laboratory. If any of the performed tests are positive, these people will be deported immediately."

Working or residential people undergo these tests every 6 to 12 months, including students whereas sex works get tested every week. Possible results of immigration testing (positive results) and their outcomes can be summarised as;

- If the person tests positive for Hepatitis B, they can stay and work with no problem, except healthcare or food related workers.
- If the person tests positive for Anti-HCV, the person gets deported. With the new improvements, if the person accepts to pay for their treatment, they may be treated and they will be able to stay, under control of infectious disease experts.
- If the person tests positive for HIV, the person gets deported immediately.
- If the person tests positive for Syphilis, the person will get treated.
- Same implies for Tuberculosis. A person with positive PPD test or risky Chest X-ray will get treated and re-vaccinated if possible.
- If a food related worker tests positive for Salmonella or Shigella, the person gets treated and cannot work in food industry as they might be silent carriers.
- As mentioned earlier, sex workers get tested every week. If any of the above test are positive, they will be deported immediately.

Any person which tests positive for any of the parameters would be always re-tested by the government employed infectious disease experts, by more sensitive tests such as HBV DNA PCR, HCV RNA PCR, HIV PCR, Chest X-ray, TPHA and other diagnostic tools, to confirm the results (http://www.mahkemeler.net/cgi-bin/default.aspx, Accession date 14 November 2018). 5 years' data on HBV infections in TRNC are shown in table 1.17.3.

Table 1.17.3. HBV Infection numbers of different populations in North Cyprus. Last 5 years' data. (http://saglik.gov.ct.tr/, Accession date 2 February 2019).

HbsAg + population	2014	2015	2016	2017	2018
Turkish (Cypriot)	23	18	10	2	5
Turkish (Turkey)	645	652	650	650	494
Other origin	271	267	308	308	278
Sex workers	8	6	14	2	2
Total	947	943	982	962	779

Cyprus has an predictable population of 1.19 million in 2018, more populated than the 2011 census of 839 000 and the 2011 estimation of 1.11 million. Of the 1 193 635 people in Cyprus, about 352 000 live in the North of island, although it is supposed that this number has climbed up to 500 000, half of whom are Turkish immigrants or Cypriot-born children of immigrants. 230 000 are classed as native born TRNC citizens. The exact total population still remains unknown as the North Cyprus has a dynamic society of students and tourists who regularly visit the island (http://worldpopulationreview.com/countries/cyprus-population/, Accession date 28 December 2018 and https://cyprus-mail.com/2018/04/03/foreign-students-propel-sharp-population-increase-north/, Accession date 28 December 2018).

Table 1.17.4. HBsAg positivity percentages per of given population in TRNC. (http://saglik.gov.ct.tr/, Accession date 2 January 2019).

	2014	2015	2016	2017	2018
Turkish (Cypriot)	0.001%	0.0007%	0.004%	0.0008%	0.002%
Turkish (Turkey)	0.23 %	0.24 %	0.24%	0.24%	0.18%
Sex workers	0.68 %	0.53%	1.44%	0.18%	0.18%
Other origin*	N/A	N/A	N/A	N/A	N/A

* N/A (Not available) as the given population numbers are not exact/known.

Total known sex worker numbers between years 2014 – 2018 were 5 451, and total known sex workers whowere HBsAg positive was 32. Therefore, we can

estimate that between the years 2014 – 2018, sex worker HBsAg positivity rate was 0.58%. This is quite high when compared to the native population.

Antiviral NAs and support therapy agents bought by the government (MOH) are listed below in table 1.17.5.

Table 1.17.5. Names, amounts and numbers of patients using drugs for HBV treatment. NDA: No data available. (original numbers are as units of each drug bought by the government). (http://saglik.gov.ct.tr/BAKANLIK/%C4%B0la%C3%A7-ve-Eczac%C4%B1l%C4%B1k-Dairesi, Accession date 5 January 2019; http://www.mik.gov.ct.tr/maliye/f?p=477:1:0, Accession date 6 January 2019).

Agents	2014	2015	2016	2017	2018
HBIg 500IU (Inj)	50	50	40	50	30
Lamivudine 150mg (Box 100s)	300	300	350	350	350
Lamivudine 100mg (Box 30s)	33	33	33	33	1330
Tenofovir 245mg (Box 30s)	200	200	133	500	600
Entecavir 1mg (Box 10s)	800	1500	1500	12000	800
Adefovir	NDA	NDA	NDA	NDA	NDA
Telbivudine	NDA	NDA	NDA	NDA	NDA
Peginterferon alpha - 2b (recombinant) 100mcg (Inj)	300	300	400	300	600
Total number of patients receiving treatment*	150	170	173	474	190

* Peginterferon was not included as it is used in HCV treatment too.

By calculating above numbers for treatment periods, we can derive how many treatment receiving patients are present.

For HBIg, 44 (30-50) 500 IU units were used each year. As we know HBIg is mainly used as prophylaxis if accidental exposure to a non-vaccinated person occurs, or mostly for preventing horizontal exposure. This indicates that for the past 5 years, each year around 44 babies were born from HBV positive mothers.

LAM, is being used in 2 forms. 150mg or 100mg depending on the treatment (combo) that the infectious disease specialist chooses. As HBV patients must take their medications every day, we can calculate how many Lamivudine receiving patients are out there.

As an example, for 150mg medication, 1 box of 100 pills will be enough for 3 months, and 4 boxes of 100 pills; 400 pills would be needed for a whole year for a patient. Therefore, we can calculate that there were 75 patients (years 2014 and 2015) and 88 patients (2016 - 2018) who received this treatment.

For 100 mg medication, 1 box of 30 pills will be enough for a month, therefore 12boxes are needed for a year for a patient. 3 patients (2014-2017) and 111 patients (2018) were receiving this treatment strategy.

TDF is used in 245mg doses. 1 box of 30 pills will be enough for a month, therefore 12boxes are needed for a year for a patient. 17 patients (2014-2015), 11 patients (2016), 42 patients (2017) and 50 patients (2018) were receiving this treatment strategy.

ETV is used in 1mg doses. 1 box of 10 pills; 3 boxes of 30 pills will be enough for a month, therefore 36 boxes are needed for a year for a patient. 22 patients (2014), 42 patients (2015-2016), 333 patients (2017) and 22 patients (2018) were receiving this treatment strategy.

For recombinant PEG-IFN α 2b, 380 (300-600) 100mcg doses were used. IFN treatment is used as a support therapy. It is impossible to calculate how many HBV patients received these as other diseases such as HCV patients also use these support agents.

ADV and LDT however are not being used.

Recently as of the 6th month of 2019, the TRNC Ministry of Health opened a tender for general medicines, the required numbers (not bought yet) of antiviral agents are listed below, with amounts of patients expected to use these medicines in their treatment.

- HBIg, 60 units (60 patients)
- LAM 100mg, (2667 boxes; 222 patients)
- TDF 245mg, (1000boxes; 83 patients)
- ETV 1mg, (1200boxes; 33 patients)
- New addition ETV 0.5mg, (300boxes; 8 patients)

- PEG-IFN α2b, 100 units (http://www.mik.gov.ct.tr/maliye/f?p=477:1:0, Accession date 23 February 2019).

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample selection

HBsAg reactive serum samples were collected and stored in -80℃ at the Near East University Laboratory, Nicosia and Lancet Medical Diagnostic Laboratory, Famagusta.

These samples were obtained from patients who attended to either of the health centres for residential permit screening, pre/post-operation screening, blood bank screening or privately requested tests. These samples were all screened for HBsAg, HIV Ag/Ab, Anti-HCV and Syphilis TP using Abbott Architect i1000SR/i2000SR automated analysers, using chemiluminescencent microparticle immunoassay technique.

Samples were collected between the dates of January 2015 to August 2018 in both Laboratories.

HBsAg positive samples with defined data titles (such as age at time of blood draw, sex, HBsAg S/CO result and Nationality) were all collected and tabulated. Total samples were then separated according to criteria selections which is explained in detail below.

Out of the samples, only Turkish and Turkish Cypriot samples were selected. These samples were then further filtered in means of; S/CO reading above 500 (to increase chance of obtaining genomic product for sequencing) followed by discharging of haemolytic, microbial contaminated, icteric and dense lipemic samples (as these will interfere with assays).

Serum quantities above 1.0 ml were chosen, if a sample was less than 1.0 ml but fits to the above criteria, were topped up with sterile saline solution to make up to 1.0 ml. At the end of the selection process, 170 samples were isolated to be used in this study.

The samples were then re-labelled from 1-170 and transferred aseptically into 2.0 ml vials with an o ring cap to prevent spillage. The necks of the vials were also

wrapped with parafilm for further protection, and stored accordingly until analysis. The data for samples were then tabulated in a new spreadsheet.

2.2 Sanger Sequencing

HBV gene is liable to mutations as a result of the absence of correction mechanisms during transcription step. High rates of nt mismatches do arise during the treatment with both nucleotide and nucleostide analogues. These mostly occur at the *RT* region of the HBV *pol* gene. These mismatches lead to antiviral drug resistance as they are genotyping mutations. It is essential to detect, monitor and reduce the incidence of *RT* mutations in patients during treatment (Liu et al., 2014).

Current approaches for detecting HBV mutation include; nt sequencing, reverse hybridisation, restriction fragment length polymorphism, ultradeep pyrosequencing, DNA microarrays and few others. Direct PCR sequencing (Sanger sequencing) is accepted as the golden standard for genotypic resistance mutation detection. It can detect both numerous mutation sites during assay and identify both known and unknown mutations at the same time. It also has a very low false positive rate (Keeffe et al., 2008).

HBsAg positive samples, HBV genotypes and sub-genotypes were analysed by HBV *pol* gene (Reverse Transcriptase; *rt* region, between amino acids 80-250). HBV *pol* gene amplification (742 bp), forward (F: 5'-TCGTGGTGGAC TTCTCTCAATT-3') and backward (R: 5'-CGTTGACAGACTTTCCAATCAAT-3') primers were designed and used.

For PCR conditions, 10 minutes pre-denaturation at 95°C, followed by 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 45 seconds heat/time cycles were used. PCR products were purified by using High Pure PCR Product Purification Kit (Roche Diagnostics, Germany). PCR was only performed to obtain genomic product for further analysis.

In sequencing protocol, Phire Hot Start DNA polymerase (Finnzymes Oy, Finland) enzyme was used. BigDye Terminator v3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., USA), 36 cm capillary and POP-7 TM polymer were used as stated by the supplier at ABI PRISM 3130 platform (Applied Biosystems Inc., Foster City, USA) (Sayan et al., 2010).

2.3 Determination of genotypes/sub genotypes, serotypes and analysis for anti-viral resistances

Sequences obtained were further analysed by using a special software, Geno2pheno (Centre of Advanced European Studies and Research, Bonn, Germany) drug resistance platform. Here, the fasta formatted unknown sequences (input) of our samples were reverse inserted into the system. The system, as the name suggests, is a HBV database which is specially and specifically designed for virtual phenotyping of HBV. The system searches and matches the homology between our input and sequences which are stored on the system database. The system also stores significant information for HBV genotype, *S* gene mutations and drug resistance on the *rt* domain of the *pol* gene. The system searches for specific amino acid positions for both targets (28 for *S* gene and 12 for *rt* gene). Further manual search for other amino acid positions was performed manually.

Serotype analysis was performed by using CLC sequence viewer (phylogenetic tree) as geno2pheno system was not able to detect this information. Set parameters and more information is explained later in the text.

Target region and amino acid positions of *RT* gene mutations, *S* and *RT* gene overlapping gene mutations are listed in Tables 2.3.1, 2.3.2 and 2.3.3 (Shaw et al., 2006; Avellon and Echevarria, 2006; Sayan et al., 2012).

Table 2.3.1. Target regions and amino acid positions in the determination of HBV surface gene mutation.

HBsAg protein target regions	Amino acid position
ADAPVEM region	161, 164, 172, 173, 175, 176, 182, 193-196
HBIg selected escape mutation region	118, 120, 123, 124, 129, 133, 134, 144, 145
Vaccine escape mutation region	120, 126, 133, 143-145, 193
Hepatits B misdiagnosis mutation region	120, 131, 133, 143
Immune selected mutation region	100, 101, 105, 109, 110, 114, 117, 119, 120, 123, 127, 128, 130, 131-134, 140, 143, 144, 145

Table 2.3.2 Target region and amino acid positions in the determination of HBV polymerase gene mutation (overlapping).

Polymerase gene overlapping *S* gene target region	Amino acid position
Overlapping surface gene segment	100, 101,105, 109, 110, 114, 117, 118-121, 123, 124, 126 -135, 137, 139, 140-142, 144-149, 151-153, 155-157, 161, 172, 173, 175, 176, 193- 196

Table 2.3.3 Target region and amino acid positions in the determination of HBV polymerase gene mutation.

Pol gene target region	Amino acid positions
Reverse transcriptase gene segment	74, 80, 82, 84, 85, 139, 149, 156, 169, 173, 180, 181, 184, 194, 200, 202, 204, 214, 215, 233, 236, 237, 238, 250

2.4 Construction of phylogenetic tree(s)

HBV genotype/sub-genotypes were also phylogenetically analysed. Primarily, sample sequences and reference sequences were all aligned for 495 bp. Short sequences (less than 495 bp) were removed from the aligment as data was not sufficient. Genotype phylogenetic tree was created using CLC Sequence Viewer 8.0 (CLC bio A/S, Qiagen, Denmark). Phylogenetic parameters of Neighbour Joining/Jukes-Cantor were used and boostrap value was used as 1000. The final cladogram was converted into circular cladogram in increasing order. Only 98 of the 108 sequences were used as the rest of the sequences were too short.

Serotype analysis was performed using CLC sequence viewer (CLC bio A/S, Qiagen, Denmark) for sequenced samples as geno2pheno system eas not able to

detect this information. After aligning the sequences with reference sequences, phylogenetic parameters of UPGMA/Jukes-Cantor were used and boostrap value was again used as 1000. The final phylogram was converted into circular phylogram in increasing order. Only 97 of the 108 sequences were used as the rest of the sequences were too short.

Although genotypes and subgenotypes were already determined by using geno2pheno system as explained above, the phylogenetic analysis of HBV sequences (495bp) were analysed. The nucleotide sequences were compared to those from GenBank (Sayan et al., 2012). This will offer us information regarding how closely related these samples are.

The reference sequences that were used for constructing the phylogenetic tree were all obtained from GenBank and are listed below in table 2.4.1 (www.ncbi.nlm.nih.gov, via CLC sequence viewer Accession dates 5-6-7 March 2019).

Table 2.4.1. Codes of reference sequences and their corresponding information.

Type of Reference	Code of reference
Genotype A	KY886219.1
Genotype B	FJ562300.1
Genotype C	FJ023667
Genotype D	KP997995
Genotype D/D1	LC365689.1
Genotype D/D2	GU456635.1
Genotype E	KF922438.1
Genotype F	KY458062
Genotype G	KX264500.1
Genotype H	KX264501.1
Genotype I	GU357844.1
Genotype D ayw1	AY576433
Genotype D ayw2	KT749854.1
Genotype D ayw3	FJ349218.1
Genotype D ayw4	FJ349207.1

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Sequenced Samples Details

The analysis of 170 samples were performed. It was calculated that male:female ratio was 1.65:1, which indicates more males than females were positive for HBsAg. This phenomenon was observed in many studies regarding HBV. Mean HBsAg S/CO reading was 3 882.5 which may show that our samples were from HBV carrier population. 29% of the samples were from Turkish Cypriot native residents and 71% were Turkish residents from Turkey. Out of 170 samples, only 108 were sequenced as rest didn't yield any genomic material following PCR procedure. The clinical demographics of the samples are given below in Table 3.1.1.

Table 3.1.1. Demographic details of primary and sequenced samples.

Characteristic	Patient group	Study group
Patient, n	170	108
Gender, M/F, n (%)	106 (63) / 64 (37)	68 (63) / 40 (37)
Age, years (mean ± SD)	49 ± 31	41.5 ± 23.5
Nationality		
Turkish	122 (71)	83 (77)
Turkish Cypriot	48 (29)	25 (23)
HBsAg value, S/Co* (mean ± SD)	3882.5 ± 3712.5	3882.5 ± 3712.5

Abbreviations; M: male F: female, *S/Co: Sample/Cut-off
HBsAg value was obtained using Abbott Architect i1000SR/i2000SR systems (Abbott, USA)

3.2 Sequence Results

Analysing the HBV *S* gene, it can be seen that total of 17 (16%) samples yielded such escape mutations for HBIg selected escape, vaccine escape, hepatitis B misdiagnosis and immune selected amino acid substitution respectively 7(6%), 9(8%), 10(9%), 9 (8%).

10(9%) of the *S* gene mutations were hepatitis B misdiagnosis mutations, followed by 9(8%) vaccine escape and immune selected amino acid substitution,

with the least amount of mutations 7(6%) of HBIg selected escape. The *S* gene mutation patterns are summarised below in Table 3.2.1.

3 (3%) of the total sequenced samples had more than one mutation (mutation combination of HBIg selected escape, Vaccine escape and immune selected amino acid substitution separately). These samples are also listed below in Table 3.2.1.

Table 3.2.1. HBV *S* gene mutations detected. Some samples may have more than one mutation.

HBsAg escape mutation category	Mutation pattern	Patient n, (%)	Combined pattern	Patient n, (%)
HBIg selected escape	sP120T, sQ129H, sM133I, sY134N, sD144E, sC147S,	7(6)	sM133I + sD144E	1 (0.9)
Vaccine escape	sP120S, sQ129H, sS143L, sD144E, sC147S, sS193L	9(8)	sT126S + sS193L	1 (0.9)
Hepatitis B misdiagnosis	sP120T, sP120S, sR122K, sT131I, sM133I, sC147S	10(9)	-	-
Immune selected amino acid substitution	sQ101H, sG119R, sP120T, sT123N, sT131N, sY134F, sD144E	9 (8)	sG119R + sT123N	1 (0.9)
TOTAL*	-	17 (16)	-	3 (3)

*Total number of patients in who HbsAg amino acid substitution was detected.

3.3 Genotypes, subgenotypes and serotypes

Following HBV DNA sequence analysis, as stated above; genotypes and sub genotypes of only 108 samples were detected. 106 (98%) of the samples were D/D1, only 1 (1%) was D/D2 and only 1(1%) was E. Genotypes and sub genotypes & their origins are summarised below in Table 3.3.1. Data from phylogenetic tree for serotypes is summarised in Table 3.3.2.

Table 3.3.1. HBV Genotypes/sub genotypes detected.

Genotype	Subgenotype	n, (%)	Nationality*
D	D1	106 (98)	TR, TRNC
	D2	1 (1)	TRNC
E	-	1 (1)	TR
Total	-	108 (100)	-

*TR: Turkey, TRNC: Turkish Republic of Northern Cyprus.

Table 3.3.2. HBsAg serotypes for genotype D detected.

HBsAg Serotype*	n, %
ayw2	96 (99)
ayw3	1 (1)
Total	97(100)

*Genotype E strain and short sequences (n=11) were not included in serotype analysis

3.4 Antiviral resistances

Following sequencing, certain *rt* gene mutations were observed. Out of 108 sequenced samples, 3(3%) yielded primary resistance mutations, 2 (2%) yielded partial resistance mutations and 29 (27%) yielded compensatory mutations. For primary resistance mutations, 2 (2%) of the samples had rtM204I which corresponds to LAM, LDT, L-FMAU, Emtricitabine (FTC) resistances and 1 (0.9%) of the samples had rtI233V mutation which corresponds to ADV resistance. For partial resistance mutations, rtL80I was detected in 1 (0.9%) of the samples which corresponds to LAM, LDT resistances and rtL180M mutation was detected in 2 (2%) of the samples which corresponds to LAM, LDT, L-FMAU, FTC resistances. For compensatory mutations, 18 (17%) yielded rtQ215H/P/S mutation which confers to LAM, L-FMAU, FTC, TDF resistances. rtQ149K and rtL91I was seen separately in 7 (6%) of the patients which confers to ADV and LDT resistances respectively. Least amount of mutations were observed in 2 (2%) of the samples; rtV214A for LAM, L-FMAU, FTC, TDF resistances. These mutation patterns and their corresponding antiviral resistances are summarised in table 3.4.1.

ADAPVEM was detected in 2 (%2) of the samples with rtM204I/sW196L mutations which is due to LAM/LDT resistance. These mutation patterns are summarised in table 3.4.2.

Table 3.4.1. Antiviral resistances detected. Some samples may have more than one mutation.

Mutation characteristic	Mutation pattern	Nucleos(t)ide analogue	Patient, n(%)
Primary resistance mutation(s)	rtM204I	LAM, LDT, L-FMAU, FTC	2 (2)
	rtI233V	ADV	1 (0.9)
TOTAL*	-	-	3 (3)
Partial resistance Mutations	rtL80I	LAM, LDT	1 (0.9)
	rtL180M	LAM, LDT, L-FMAU, FTC	2 (2)
TOTAL*	-	-	2(2)
Compensatory mutation(s)	rtL91I	LDT	7 (6)
	rtQ149K	ADV	7 (6)
	rtV214A	LAM, L-FMAU, FTC, TDF	2 (2)
	rtQ215H/P/S	LAM, L-FMAU, FTC, TDF	18 (17)
	rtN238D	ADV	6 (6)
TOTAL*	-	-	29(27)

*Total rt gene mutations in 108 sequenced samples. The aa position 250 is where we expect a specific mutation to occur, 2 mutations rtM250G/H detected were ETV related amino acid substitutions; these are not mutations which cause nucleostide resistance. (LAM, Lamivudine; LDT, Telbivudine; L-FMAU, Clevudine; FTC, Emtricitabine; TDF, Tenofovir; ADV, Adefovir; ETV, Entecavir)

Table 3.4.2. ADAPVEM mutations and their corresponding nucleostide analogue resistance.

Mutation characteristic	Mutation pattern	Nucleos(t)ide analogue	Patient, n(%)
ADAPVEM	rtM204I/sW196L	LAM/LDT	2 (2)

(LAM, Lamivudine; LDT, Telbivudine)

3.5 Phylogenetic trees

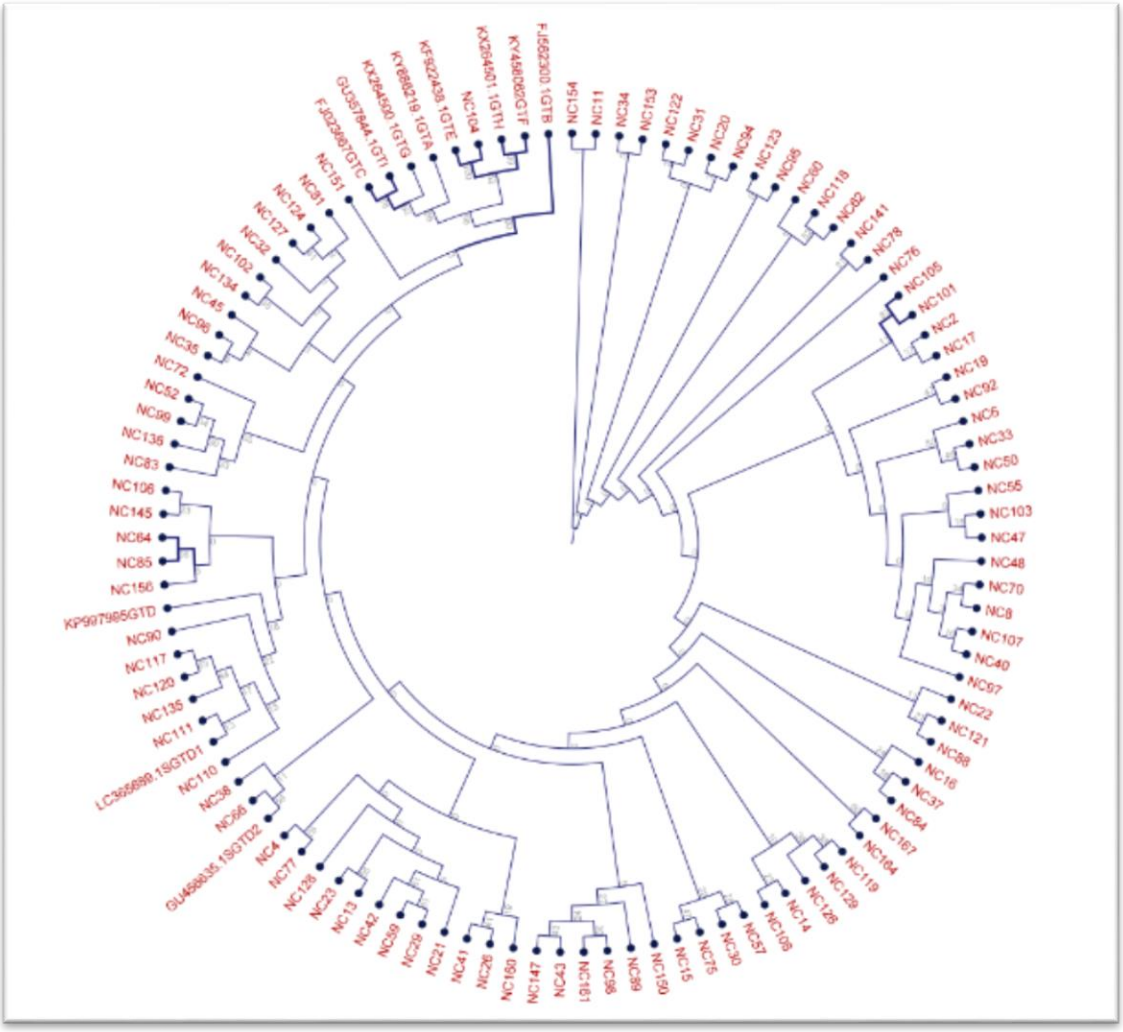

Figure 3.5.1. Increasing order circular cladogram of HBV genotypes and subgenotypes. Phylogenetic tree was constructed using CLC sequence viewer (CLC bio A/S, Qiagen, Denmark). The HBV reverse transcriptase region length was 495 base pairs in the alignment. Neighbour-Joining and Jukes-Cantor methods were used. Bootstrap value was chosen as 1000. HBV genotype A: KY886219.1, B: FJ562300.1, C: FJ023667 D: KP997995, D/D1: LC365689.1, D/D2:GU456635.1, E: KF922438.1, F: KY458062, G: KX264500.1, H: KX264501.1, I: GU357844.1 reference sequences were obtained from GenBank.

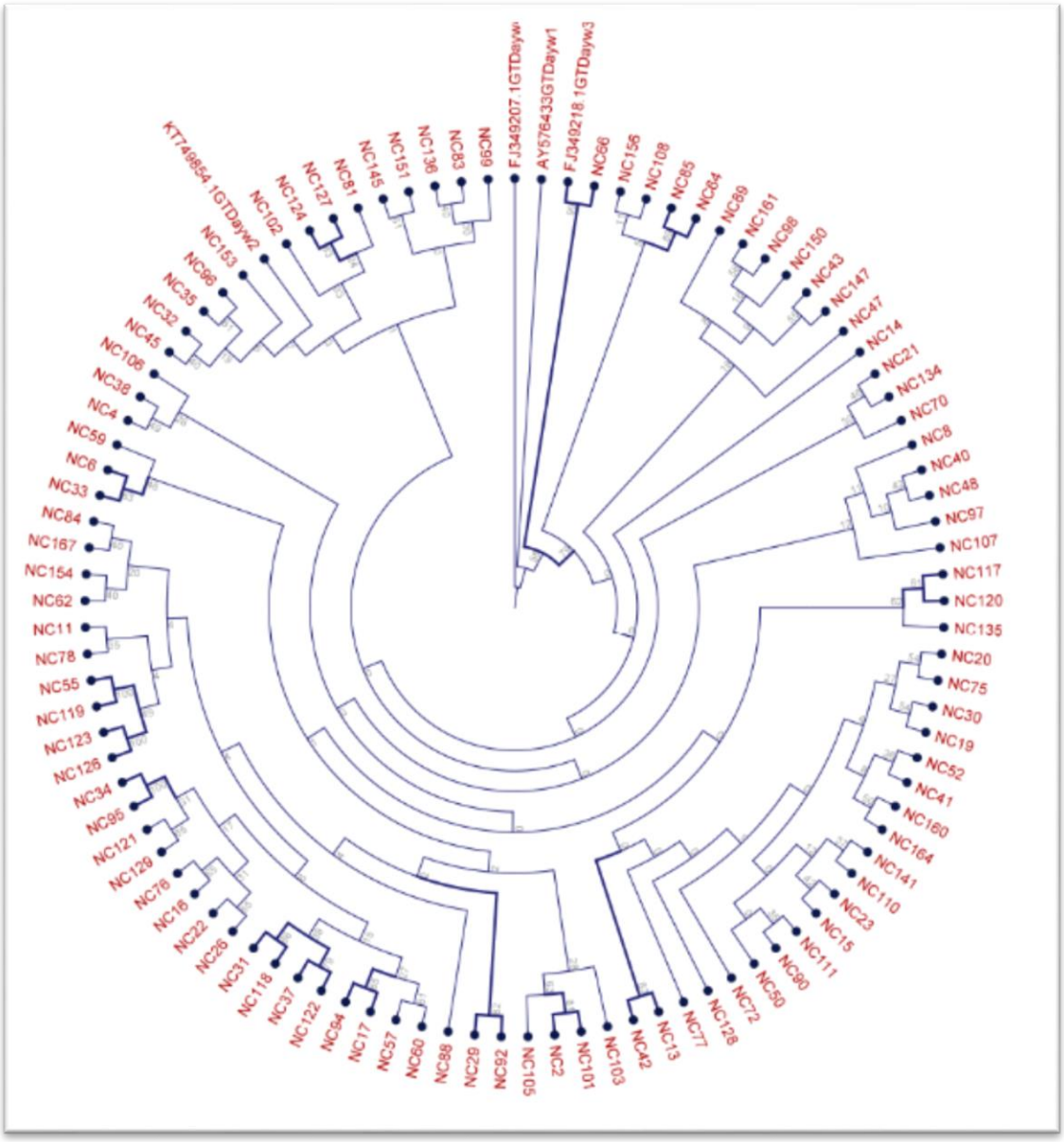

Figure 3.5.2. Increasing order circular cladogram of HBsAg serotypes. Phylogenetic tree was constructed using CLC sequence viewer (CLC bio A/S, Qiagen, Denmark). The HBV reverse transcriptase region length was 495 base pairs in the alignment. UPGMA and Jukes-Cantor methods were used. Bootstrap value was chosen as 1000. HBV D Serotype *ayw1*: AY576433, *ayw2*: KT749854.1, *ayw3*: FJ349218.1, *ayw4*: FJ349207.1 reference sequences were obtained from GenBank.

CHAPTER 4: CONCLUSIONS

4.1 Concluding remarks

The HBV genotypes/subgenotypes found were similar to previous research. In their work, Arikan and colleagues found as D/D1; 70.6%, D/D2; 5.9%, D/D3; 1.5%, A/A1; 7.4%, A/A2; 2.9% and E; 11.8%. Where as in this study, D/D1 was found in 98%, D/D2 was found only in 1% and E was also found in only 1%. The most detected D/D1 is from both Turkish and Turkish Cypriot patients, similar to previous work. We can therefore conclude that genotype D/D1 is the dominant strain of HBV in TRNC. The main dissimilarity we observed in this study is that D/D2 was found in a Turkish Cypriot; this was not observed before in TRNC. Similarly, a Turkish person was found to have genotype E, which again was not observed previously in TRNC (Arikan et al., 2016).

Genotype E was first identified and detected in Turkey in 2014. That patient was a Nigerian male who was working in Turkey for 5 years. And yet in our study, the patient with genotype E was a 28 years old Turkish (Turkey origin) male, and unfortunately we could not track him to obtain more information. Doubtless, he probably lived or worked in West or Middle Africa for some period, travelled there for a period of time or he had sexual intercourse with a person from this region (Sayan et al., 2014).

As mentinoned earlier, genotype D is the most widespread genotype in the Mediterranean region. Our results match with this. As majority of the students, sex workers and labour workers mostly travel to TRNC from Europe, Turkey and Africa; we can substantiate that D genotype was introduced into the society by migration of people from these regions. Also it is important to mention that TRNC is a low endemic region; however, people from some parts of Europe and Africa where endemicity is high travel or live in (Zehender et al., 2014).

In 2018, an article published the global genotype distribution of HBV, assessing 125 countries and over 900 publications. Their findings indicated that genotype D was dominant in Eastern Europe, majority of Asia and North Africa.

HBV genotype distribution shows similar pattern among the countries in the same region but varies amongst different parts of the world (Velkov et al. 2018).

Large population migrations can modify public dynamics. High frequency of genotypes A - D was observed in North America following migrations from Asia and Europe. A similar situation was observed in the Caribbean where genotypes A and D were found as a result of migrations from African continent (Al-Sadeq et al. 2019; Velkov et al. 2018). We can clearly see that migrations mainly from Turkey for working and living, and other parts of the Middle East for other purposes such as studying, have caused genotype D to be dominant and the introduction of new genotypes such as E to occur in TRNC.

In this study; total of 3% primary resistance mutations, 2% partial resistance mutations and 27% compensatory mutations were observed in the *rt* gene. In previous work, primary/partial resistance mutations were identified in 1% of the patients and compensatory mutations was detected in 37% of the patients. Looking in more detail, only rtV173M (primary/partial resistance mutation) was observed previously whereas in this study; rtM204I, rtI233V, rtL80I and rtL180M mutations were detected. Compensatory mutations of rtL91I, rtQ149K, rtQ215H/P/S and rtN238D were observed previously. In this study, all compensatory mutations stated above were observed together with an additional mutation of rtV214A (Arikan A, 2015).

When analysed in greater detail, rtM204I, rtI233V, rtL80I and rtL180M mutations were not detected before, and these mutations, particularly rtL80I and rtL180M, restore the viral polymerase to near wild type levels, which helps to promote the replication of mutants. This indicates that treatment naïve population is prone to such mutations, which has a significant impact on treatment procedures and costs, also primary/compensatory mutations alone may increase HBV DNA levels and cause failure in future treatment (Sayan et al. 2010; Sayan and bugdaci 2013).

S gene mutations, indicate different patterns compared to earlier work. In our study, total *S* gene mutations were 16% and combined *S* mutations were 3%. Previous work showed that total *S* gene mutations were 29% and combined *S* gene mutations were 9% (Arikan A, 2015).

Immune selected amino acid substitution mutation patterns in previous work was 24%; sY100C, sI110L, sP120L/R, sT123N, sT127L, sP127T, sA128V, sT131N, sS132P, sY134F/H, sT140I/S, sD144E, sS143T, sD144E, sS144T and sP210S. Where as in this study 8% and only sQ101H, sG119R, sP120T, sT123N, sT131N, sY134F and sD144E mutations were observed. sQ101H, sG119R and sP120T mutations were detected in TRNC for the first time in this study (Arikan A, 2015).

HBV vaccine escape mutation patterns in prior work was 10%; sT126I, sD144A/E, sG145A/R, sS193L and sP210T. In this study 8%; sP120S, sQ129H, sS143L, sD144E, sC147S, sS193L were observed. sP120S, sQ129H, sS143L and sC147S mutations were observed in TRNC for the first time (Arikan A, 2015).

HBIg selected escape mutation patterns in former work was 6%; sT118A, sP120T, sD144A/E and sG145A/E/R. In this study, different patterns are seen with same percentage of 6%; sP120T, sQ129H, sM133I, sY134N, sD144E, sC147S. Only 2 mutual mutations sP120T and sD144E were observed in both studies, but sQ129H, sM133I, sY134N and sC147S mutations were observed for the first time in TRNC (Arikan A, 2015).

Hepatitis B misdiagnosis mutation patterns in earlier work was only 4%; sT118A, sT131I, sP120T, sC121Y, sD144A and sG145R. Where as in this study it was 9%; sP120T, sP120S, sR122K, sT131I, sM133I and sC147S. sP120S, sR122K, sM133I and sC147S mutations were newly detected in TRNC in this study (Arikan A, 2015).

Combined HbsAg amino acid substitution mutations previously described were as follows. sI110L + sS193L mutations for immune selected amino acid substitution and vaccine escape categories, sP120L + sT123N + sT126I + sA128V + sY134H + sD144E + sG145A mutations for vaccine escape and HBIg categories, sT118A + sP127T mutations for immune selected amino acid substitutions and HBIg categories, sT131I + sS132P mutations for immune selected amino acid substitutions and hepatitis B misdiagnosis categories, sI110L + sP120T + sD144A + sG145R mutations for all HbsAg amino acid substitution categories and sP120R + sC121Y mutations for immune selected amino acid substitutions and hepatitis B misdiagnosis categories. In this study, the situation is unlike as only **s**M133I + sD144E mutations

for HBIg category, sT126S + sS193L mutations for vaccine escape category and sG119R + sT123N mutations for immune selected amino acid substitution category was observed (Arikan A, 2015).

A study performed in the Middle East and North Africa region, *S* gene mutations were detected in Egyptian, Saudi, Palestinian and Tunisian patients where B, D/D1, D/D3 and D/D7 were genotypes identified; we have only detected 5 common mutations when compared, furthermore all of the *S* gene mutations we observed were from D/D1 patients (Al-Sadeq et al. 2019). This indicates that different geographical regions may have different *S* gene mutation profiles, even though the genotypes of the patients are same or not.

There was no data on the topic of ADAPVEM and HBsAg subtypes in TRNC. In this work, 2% of the samples yielded ADAPVEM mutations. These were rtM204I/sW196L mutations which corresponds to resistances and substitutions due to LAM/LDT. Majority of subtypes were *ayw2* with minority of *ayw3*.

ADAPVEM analysis revealed mutations. In Turkey, the same mutation pattern was observed in 8.7% of the patients together with other ADAPVEMs. Previously ADAPVEM status was not known in the TRNC, these results are initial data for monitoring of such mutations in the future (Ozguler and Sayan 2018).

Quantities of antiviral drugs bought by the government and the total number of treatment receiving patients do not correlate with number of HbsAg+ patients. By calculating the fractions of how many HBsAg+ patients were there and how many of these patients were receiving treatment (from tables 1.17.3 and 1.17.5), we could calculate that for years; 2014 only 22%, 2015 only 25%, 2016 only 25%, 2017 only 72% and 2018 only 38% of the HbsAg+ patients received treatment. Therefore, we can conclude that merely 36.4% of the HbsAg+ patients received treatment between dates of 2014-2018.

Data from Table 1.17.5 indicates that the treatment receiving patient numbers are not persistent. For example in years 2014, 2015, 2016 and 2018 the number of treatment receiving patients did not pass 200; where as in years 2017 and 2019 the numbers have increased to numbers above 400. This shows that the amounts of

patients who receive HBV treatment constantly change as result of immigration. The more migrants with HBV travel for living, studying or working, the more diagnoses could be made; therefore, more applications are made to the government for receiving treatment. As a new government regulation has been passed that forces every student (including Turkish students) to get tested every year for immigration (before, it was only once when they enter to TRNC), we do expect these numbers to increase in the following years.

Phylogenetic analysis of sequenced D/D2 sample is in a close cluster with D/D1 samples which indicates that these saples are all localised in a tight geographical area. Whereas, sequenced E sample is in a far cluster. This indicates that E sequence is located far from D sequences phylogenetically and geographically. However sequenced serotype analysis have revealed information which was not analysed before. The detected serotypes *ayw2* and *ayw3* were closely clustered; whereas, altought a far distance was not observed, *ayw1* and *ayw4* were also closely related to these serotypes as they are all subtypes of the same genotype D.

In terms of quality assurance of routine diagnosis, the TRNC government has been using cutting-edge technology instruments and reagents for blood banking, routine control and immigration testing since the beginning of laboratory automation era in the country. This is important as we have observed possible misdiagnosis patients (9%) and without these technologies which can detect mutants, these patients would have been misdiagnosed as HbsAg negative.

As stated before, North Cyprus has introduced a vaccination programme for HBV in July 1998. Although vaccinations were done prior to this date, it could clearly be seen that the impact of the mass vaccination has been efficient since the implementation date (Kurugol et al., 2009).

Genotype E was detected in a patient with Turkish origin. This indicates that genotype E is in the HBV pool of North Cyprus and could also be detected in the future. Due to the law of TRNC for infectious diseases, the dynamics will change in the future as new genotypes are expected to be seen as HbsAg carriers/positive people are not being deported from the country; and, in the following decades, these people may even become citizens of TRNC and add their genotypes to the gene pool

of TRNC. Also, as stated in Trafficking in persons report 2018, members of many countries have been residing, working or have been forced to work for sex industry in North Cyprus, and this increases the probability of new genotype introduction to the country.

Also, as stated before, health of the sex workers are being constantly monitored by the government; however, a ratio of women have arrived on 3-month tourist visa or with student visa and have been involved in prostitution in apartments and private homes in large towns. Few of these women are believed to be trafficking victims (Trafficking of Persons Report, 2018). This signifies a problem where uncontrolled sexual activities conducted by people with an unknown health status increases the spreading probability of STD and serious viral infections such as HBV, HCV and HIV. It would be impossible to control non-government regulated sexual activities, and at this step, the importance of immigration testing comes into account. In order to decrease the probability of disease spreading, any person entering TRNC should be tested immediately (or upon visa application/when arriving to the island, a government verified laboratory result should be asked). In order to expain this in a scenario, a person with 1-month tourist visa may enter the island, get involved in sexual activity (personal or forced-trafficked) and leave the island, leaving behind the possible infection which he/she contracted to a person in the country. Then, this individual with transmitted disease might get involved in other sexual activities with other individuals including sex workers who will transmit the infection to other individuals. Although sex workers get tested every week, a newly transmitted infection won't be identified until 2-3 weeks (incubation period followed by detectable levels of viral makers); and in the time being, many people who do not use sexual protective methods such as condoms could be infected.

Sex worker HBsAg positivity rate was found as 0.58% between the years 2014 to 2018. This is quite high when compared to civilian population rates (0.0007-0.001% Turkish Cypriots, 0.18-0.24% Turkish).

Examining numbers of the sex workers, (Figure E1) it can be ellicited that majority of sex trafficking occurs from Moldova (33.64%), Ukraine (15.20%) and Morocco (11.20%). As it could also be seen from the map (Figures E1 and E2)

mainly genotype D is seen to be transferring from these countries. It is important to note that most of these sex workers come from Europe (64.59%), Asia (17.95%) and Africa (15.07%) which may indicate the reason of why we detected genotype E in the circulation.

There are minority groups of sex trafficking occcuring from countries such as Russia (8.43%), Belarus (6.20%), Kenya (3.43%), Uzbekistan (4.90%), Kyrgyzstan (7.53%) and Kazakhistan (4.73%). The least number of sex workers are trafficked from South America (1.12%) and North America (0.35%). Other than these countries and continents, there remains a very small percentage of other countries for which percentages are below 1. This information does correlate with Northwestern route which can be explained as: Genotype D crossed former Soviet Union and reached Eastern Europe and Mediterranean (Zehender et al., 2014).

The very first vaccine to be used was available in early 80s and was composed of HBsAg extracted from blood of HBV infected donors and genotype A2, *adw2* was used. As stated before, the main vaccine we use in North Cyprus is Engerix B. This vaccine is a recombinant type which is obtained by DNA recombination from yeast cells. (Cheah et al., 2018; Raymer et al., 2015).

These second generation recombinant vaccines are based on the *S* polypeptide of HBV. This region contains the *a* deteminant, common to all HBV subtypes and the variable parts which are *d/y*, *w/r* determines the subtype of the given strain. As stated above, the *a* determinant is the main region which is recognised by the B cells, therefore regardless of which subtype is used in the vaccine, it should confer protection (cross-protection) against all subtypes. Other than this, manufacturing processes, adjuvant type, technological differences, amounts of antigen used are all different in various types of vaccines produced (Raymer et al., 2015).

The importance of the subtype used and its effects on the immunity arises from many complex ideas in a vaccine. Heijtink et al. (2002) debated the differences of *adw*, *ayw* and *adr* subtypes in vaccines and their effects (ability and efficiency of produced antibody to bind other HBV subtypes other than the one used in the given vaccine). In their study, they have stated that HbsAg/*adw2* vaccine does deliver a

good but not optimum immunologic response with other virus strains. They investigated HBsAg in three different antigenic forms (*adw2*, *ayw3* and *adr*) which allowed them to explore the impact of variant amino acids in the binding of immune AntiHBs after vaccination. They concluded that *adr* related antibodies (*adr* vaccine) had 2–3 fold lower binding capacity when compared to *adw2* and *ayw3* vaccines (Heijtink et al., 2002).

Regardless of these observations; Avazova et al. (2008) confirmed the efficiency of vaccination regardless of the type of subtype used in the vaccine. In more detail, they analysed 333 immunisations with *adw2* type based Engerix-B and 48 with *adr* type based Hepavax-Gene. As stated previously, no major differences were detected in this study amongst groups which received other vaccine formulations (Avazova et al., 2008).

This observation of obtaining different subtypes than the vaccine induced is calledas subtype mismatch, where non-A2 subgenotype HBV infection was induced despite the vaccination. Unfortunately, we do not know how many of our patients received their vaccination or even if they did, and which type of vaccines they have received (Cheah et al., 2018). Our dominant *ayw2* subtype does not match with vaccine induced *adw2* subtype. If we knew the AntiHbs titers of the samples we could have concluded if the *adw2* subtype vaccine actually mistached or not.

Before passing away in 2011, Professor Blumberg underlined the significance of elimination of HBV. He believed that complete eradication of HBV could be achieved by 3 important steps. Universal vaccination, precise diagnosis and effective treatment of carriers (Pourkarim et al., 2014).

In September 2015, the First World Hepatitis Summit was in Glasgow, Scotland (UK). There is was resolved that “a concerted effort should be taken to eliminate HBV infection globally by the year 2030” (WHO, 2015).

For many years, HBV have been silenced by other infections such as malaria, AIDS and tuberculosis (TB), although international burden of HBV is equivalent to that of TB and malaria. Due to the Arab spring, conflicts and violence in Middle East and Africa; shifting of large sections of people occured and this carried HBV from

areas of high endemicity to other low or moderate endemicity areas. These, definitely slowed down efforts for global eradication of HBV (Mammas et al., 2017).

Some HBV genotypes are prevalent in/and between regions and have epidemic spreading patterns. This is believed to be due to parenteral transmission which leads to higher occurrence in general population, selectively for genotypes D and E; or, as a result of sexual transmission in highly endemic areas, some individuals are still liable to such infections like subgenotype A2. Some strains like Genotype F have endemic distribution patterns. They are being found in more geographically limited areas and have been circulating for longer periods in the specific populations and this mainly spread as outcome of vertical transmission (Zehender et al., 2014).

By analyzing the demographic data of patients from Table 3.1.1, we can tell that the patients of this study are inactive carriers of HBV. Significance of ADAPVEM in TRNC was stated for the first time in this study. No dual infection of HBV was seen; neither with HCV or HIV.

As majority of the positive samples were from the immigrant population, constant screening for migrants must be a priority. Both *rt* and *S* gene mutations emphasized that treatment of naive patients had increased amounts of mutations, which still remains as a severe problem for treatment and diagnosis.

Discovery of new antiviral agents, such as core inhibitors which eventually inhibit cccDNA activity, will make it possible to achieve a better treatment in the future. However, there are still many gaps in the knowledge about HBV disease and the virus; therefore, further studies are necessary (Sünbül, 2014).

Large scale and long lasting use of NAs for clinical use leads to increased resistance mutations over time. So, it is necessary to continuously monitor resistance mutations of HBV (He et al., 2015). As stated before, alongside with proper diagnosis and treatment, most important tool to fight HBV is knowledge and education.

In conclusion, HBV-D/D1 is the dominant strain, and *ayw2* is the most detected HBsAg serotype among Turkish Cypriots. Cyprus is an island located in the

Eastern Mediterranean region, a strategic location for human trafficking and immigration, and as a result of this reputation it is necessary to analyse HBV phylogenetically for international and local importance. However, data from Greek Cypriot is necessary, as it would enable a complete island survey to be performed. With this work, I believe that, I have set the ground for this topic for further research.

4.2 Reliability of Experiments

The experiment performed were as reliable as previous study, similar pattern of HBV genotypes/subgenotypes were observed, although there were some differences in terms of *S* gene mutations, new *S* gene mutations were observed and this was also observed in the *rt* gene mutations.

One of the limitations of this study is sample size, as larger samples will generate more significant results. The lack of prior work is another limitation as there is only one previous study, and additional work will uncover significant results in the future. Another limitation is that information is not available about the HBV infection status or phase of the patients as they were not follow-up patients.

4.3 Future work

As there is a very limited data on this topic in TRNC, this study stands as a reference guide and a major literature source for both public health databases and future studies. However, there were some limitations encountered that could be further improved in future studies. Increase in sample size, preferably at least 150 sequences can be obtained by analysing around 250 HBsAg positive samples. Follow-up patients can be chosen for the study, especially from the treatment patients. More parameters can be studied such as, full hepatitis panel for observing in which stage of HBV they are in. A specific patient group can also be studied such as

just Turkish Cypriots, to provide the data regarding accurate HBV dynamics of Turkish Cypriots. Even, maybe samples/patients from South Cyprus can be added to the study as well, then the research will take a complete new level as therefore can specify HBV dynamics of Cyprus. Finally, in future work, it should be important to note that where in TRNC/Cyprus HBsAg positive carriers live/work; a map of HbsAg positivity can be created (for example Kyrenia is a tourist destination, Famagusta is a student destination).

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Enclosure 1

This project took Ethical Committee approval on 29 March 2018 from Near East University Ethical Committee.

Approval Number YDU/2018/56-539.

Project Number 539

Committee number 2018/56

As this is a retrospective study, no informed consent forms were required.

Enclosure 2

Sumer and Sayan, 2019

Sumer U, Sayan M. Molecular characteristics of Hepatitis B Virus strains isolated from Turkish patients in Northern Cyprus. *Polish Journal of Microbiology*. 2019;xx(x): xx-xx. (Article in press)

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Complete article at the back of thesis.

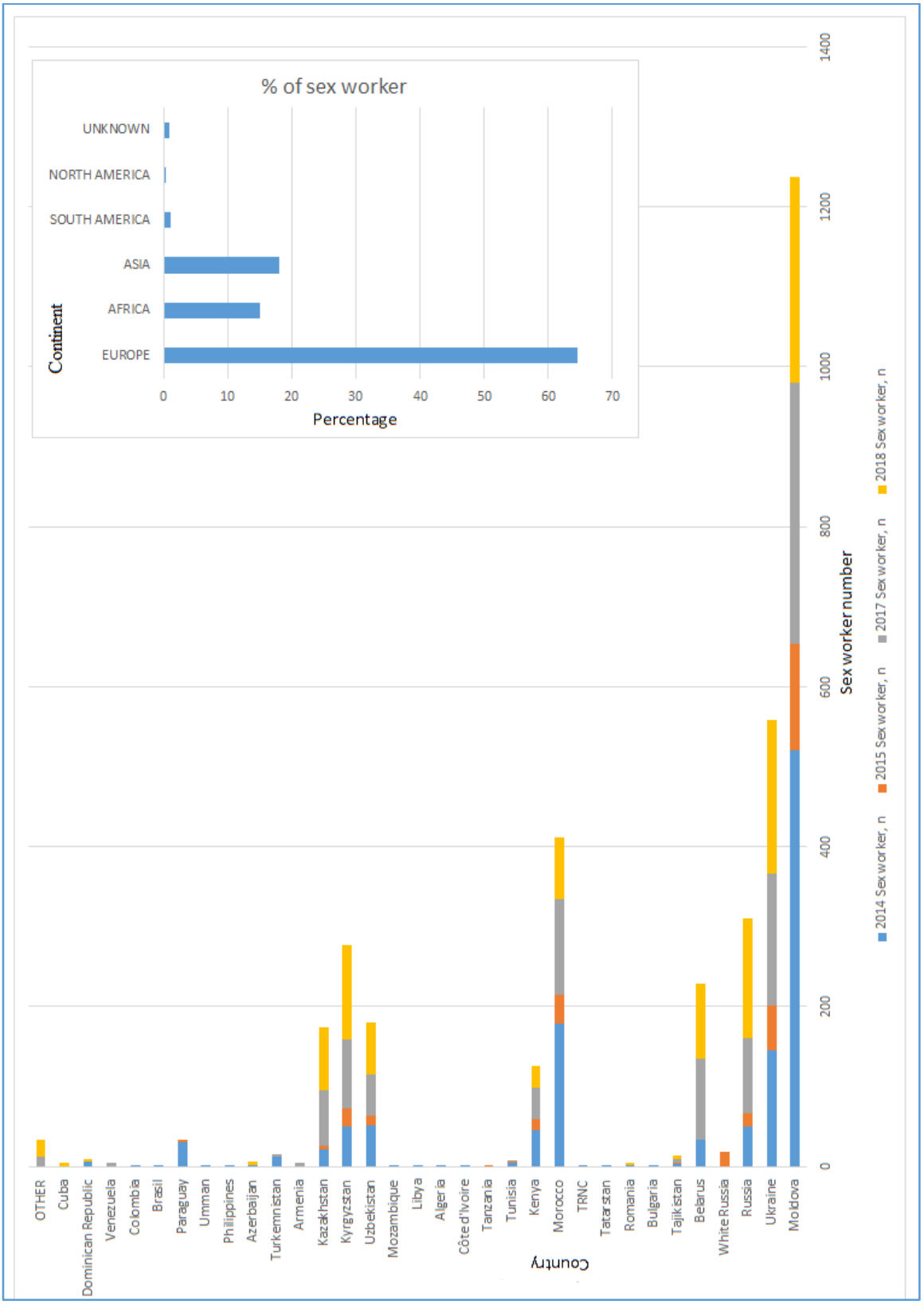
Enclosure 3

Table E1. Continents, nationalities and total number of sex workers in TRNC between 2014-2015 and 2017-2018. (Continued next page)

Continent	Nationality	Sex worker, n				Total	
		2014	2015	2017	2018	n,	%,
Europe	Moldova	520	133	328	256	1237	33,64
	Ukraine	145	56	165	193	559	15,20
	Russia	50	17	94	149	310	8,43
	White Russia	0	19	0	0	19	0,52
	Belarus	33	0	102	93	228	6,20
	Tajikistan	3	2	4	5	14	0,38
	Bulgaria	1	0	0	0	1	0,03
	Romania	1	1	0	3	5	0,14
	Tatarstan	1	0	0	0	1	0,03
	TRNC	1	0	0	0	1	0,03
	Total	755	228	693	699	2375	64,59
Africa	Morocco	179	36	119	78	412	11,20
	Kenya	46	13	39	28	126	3,43
	Tunisia	5	1	2	0	8	0,22
	Tanzania	0	2	0	0	2	0,05
	Côte d'Ivoire	2	0	0	0	2	0,05
	Algeria	2	0	0	0	2	0,05
	Libya	1	0	0	0	1	0,03
	Mozambique	1	0	0	0	1	0,03
	Total	236	52	160	106	554	15,07
Asia	Uzbekistan	51	13	51	65	180	4,90
	Kyrgyzstan	50	23	86	118	277	7,53
	Kazakhstan	22	4	69	79	174	4,73
	Armenia	0	0	5	0	5	0,14
	Turkemnistan	12	1	3	0	16	0,44
	Azerbaijan	1	0	0	5	6	0,16
	Philippines	1	0	0	0	1	0,03
	Umman	1	0	0	0	1	0,03
	Total	138	41	214	267	660	17,95
South America	Paraguay	31	3	0	0	34	0,92

	Brasil	1	0	0	0	1	0,03
	Colombia	1	0	0	0	1	0,03
	Venezuela	0	0	5	0	5	0,14
	Total	33	3	5	0	41	1,12
North America	Dominican Republic	6	0	0	3	9	0,24
	Cuba	0	0	0	4	4	0,11
	Total	6	0	0	7	13	0,35
Unknown	Other	0	0	12	22	34	0,92
	Total	0	0	12	22	34	0,92
Total*		1168	324	1101	1101	3677	100.00

*Total of all data



FigureE1. Graph of sex workers and their origin 2014-2015, 2017-2018.

Figure E2. Continents which sex workers come to TRNC from. Note that Genotype D is present in all arrows. Genotypes are stated in alphabetical order. World map was created using data from Sumbul 2014. Orange arrows represent border crossing points. NDA: No data available. T.R.N.C.: Turkish Republic of Northern Cyprus. G.R.S.C.:Greek Republic of Southern Cyprus. World & Cyprus map was created using map creator online https://www.scribblemaps.com/.

CV

1. KİŞİSEL BİLGİLER

ADI, SOYADI: DOĞUM TARİHİ ve YERİ:	ÜNAL SÜMER 20/12/1990 MAĞUSA/KKTC
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5. SON BEŞ YILDAKİ ÖNEMLİ YAYINLAR

Sumer U, Sayan M. Molecular characteristics of Hepatitis B Virus strains isolated from Turkish patients in Northern Cyprus. *Polish Journal of Microbiology*. 2019;xx(x): xx-xx. (Article in press)

MOLECULAR CHARACTERISTICS OF HEPATITIS B VIRUS STRAINS ISOLATED FROM TURKISH PATIENTS IN NORTHERN CYPRUS

By ünal sümer

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ORIGINALITY REPORT

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SIMILARITY INDEX