Hepatitis B virus is a serious global public health problem. Severely cases of HBV can lead to chronic liver disease, including cirrhosis and hepatocellular carcinoma. Previously, HBV genotypes have been classified into eight genotypes (A-H) and because of genome diversity is a hallmark of HBV virus allowed its classification into 10 genotypes (A–J). The major classification of HBV subtype is classified into 4 subtypes or serotypes (adr, adw, ayr, and ayw). The clinical relevance of such genotype is yet unclear. However, because the HBV-induced disease is the resultant of virus-host interaction, the disease characteristics may be influenced by the genotypes of the virus. Varieties of methods have been used to detect HBV genotype including whole or partial genome sequencing, genotype-specific PCR amplification, line probe assay, enzyme-linked immunoassay as well as serological methods.

The author has finished his Bachelor degree in Microbiology from University of Mosul in 1989. His Master degree completed in Biotechnology from University Technology Malaysia in 2011. Finally, His PH.D. degree in Microbiology was completed from University of Mosul in 2015. He is teaching Medical biology and Virology for medical students.

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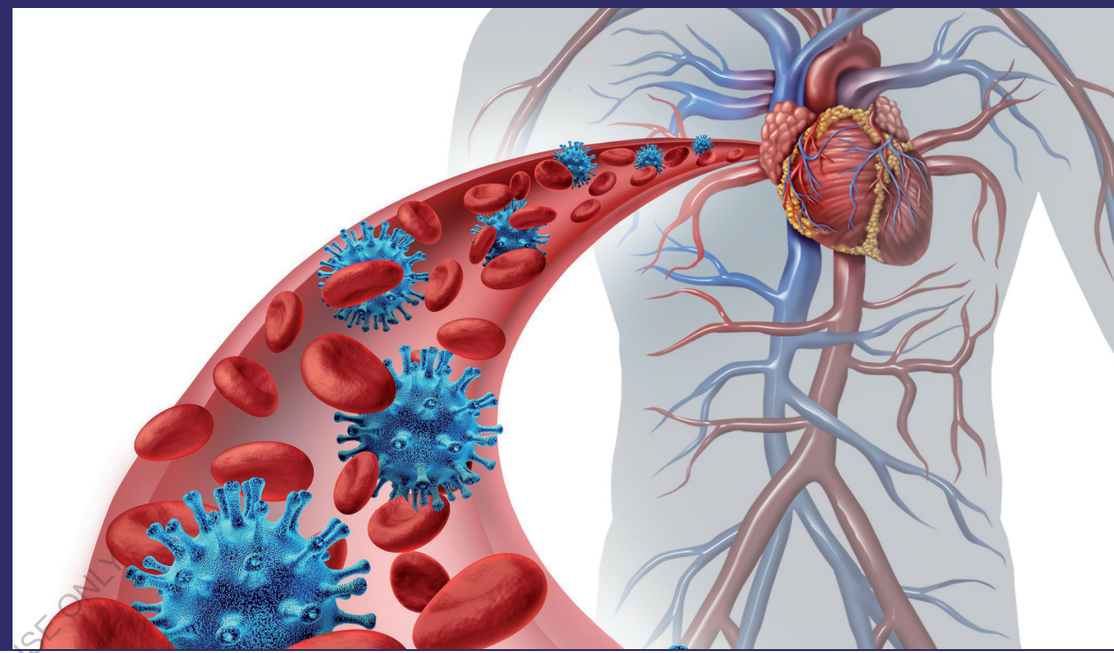
HBV-Geno-subtype



Ali Dawood

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Hepatitis B virus, Overview, Novel Genotypes & Subtypes

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2019

Preface

The current (First Edition), Hepatitis B virus, Overview & Novel Genotypes & Subtypes consists of 7 parts and 94 pages. This is a manifestation of not only the wealth of new knowledge acquired on this virus infection, but also the discovery of newly-recognized emerging infections, classical and new genotypes. This edition reexamined the overview of the virus like types of infection cases, life cycle, structure, and had explained the ways for treatment and prevention of infections.

This edition emphasizes the rapid accumulation of new information in such fields of immunity, vaccination and types of serotypes of HBV. Moreover, this edition not only offered a new aspects of laboratory diagnostic of the virus and detection of genotypes, but also was designed to explain some mutations within the virus genome.

July, 2019

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Part One

Introduction
Types of Infections

Introduction

Hepatitis simply means inflammation of liver. This word came from heap: the Latin for liver and "titis" means inflammation. In addition to viruses, many varieties of agents can cause hepatitis such as bacteria, parasites, fungi and chemical agents including drugs, toxins and alcohol.

Currently, 11 types of viruses are recognize causing hepatitis, Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and 9 of hepatotropic viruses. Only 3 out of these 9 viruses are well characterized from A-E. Hepatitis A (HAV) sometimes called infectious hepatitis. Hepatitis B (HBV) is called serum hepatitis. Hepatitis C (formerly none A non B hepatitis NABA). Hepatitis D (HDV) which is formerly enteric transmitted hepatitis. Newly discovered forms of viral hepatitis including hepatitis F (HFV), hepatitis G (HGV), and transfusion transmitted virus (TTV) and SEN virus. They all predominantly affect and infect liver cells. Despite significant overlap in the clinical manifestation caused by them, these types of viruses differ widely in their morphology, genomic organization, taxonomic classification and mode of replication.

History of Hepatitis B Virus

Previously, around the 5th century BC, Babylonian Talmud had been recorded history as an ailment characterized by the distinctive yellow discoloration of its victims, then, in the 4th century BC writings of Hippocrates and Chinese literature from the 10th century AD. Viral hepatitis was first described by Lurman in 1885, who reported the development of jaundice in 15% of 1,289 shipyard workers in Bremen after their vaccination with human derived smallpox vaccine.

While Baruch Blumberg was studying hemophilia at the National Institute of Health (NIH), he discovered common antibodies (Abs) in two American hemophilia patients (1963). These antibodies neutralized with antigens in serum from Australian aborigine which referred to as (Au-Ag). Later in 1965, it was recognized that Au-Ag was the viral antigen HBsAg. Dane *et al* (1970) visualized the HBV virion (42 nm particles) by electron microscope in sera of American hemophilia patients then called Dane particles. Since then, considerable progress has been made regarding the epidemiology, virology, natural history, and treatment of this hepatotropic virus.

Hepatitis B virus is classified as an Orthohepadna virus (Genera) within the family Hepadnaviridae. This family Includes the wood chuck hepatitis virus WHV, the duck hepatitis virus DHBV, and several other avian and mammalian variants. The human HBV has been shown to infect chimpanzees, Barbary macaques and tree shrews. All hepadnaviridae have similar to hepatotropism and life cycles in their hosts.

HBV infection is a global health problem which is 50 -100 times more infectious than HIV. Approximately 400 million people are carriers of chronic liver disease every year due to consequences of the disease. Not only HBV can infect hepatocytes but also infects in extrahepatic sites including lymph nodes, bone marrow, circulating lymphocytes, spleen and pancreas. Hepatitis B virus can occur as an acute or chronic disease.

People at high risk of infection including those requiring frequent transfusions or hemodialysis, physicians, dentists, nurses, and other health care workers, intravenous drug users, police, firemen and others who are likely to come into contact with potentially infected blood products, as well as, sexual contacts with an acute or chronically infected persons. In the US, homosexually

active men consist of 6%, whereas heterosexually with multiple partners consist of 0.5% from all risk factors.

Approximately 5 % of the infected world`s population may lead to cirrhosis and HCC worldwide. It is approximated that (500, 000 to 1000, 000) persons die annually from HBV related liver disease. Most infections occur at birth or during early childhood. Infections usually cluster in households of chronically infected patients.

Acute Hepatitis B

Acute disease typically occurs in the infected adolescents or adult who have not been vaccinated. This acute presentation can be life threatening due to massive liver damage from the host immune reactions. Most people with HBV experience few or no symptoms; in fact, a 65% are unaware that they carry the virus. Although, a 30% of people with acute hepatitis B have no symptoms and most people with chronic HBV also have few or no symptoms, most symptoms may include fatigue (unusual, prolonged tiredness), fever, malaise (a flu-like feeling), nausea, vomiting, yellowing of skin and eyes (Jaundice), loss of appetite (anorexia), abdominal pain or bloating, indigestion, headache, itching (pruritus) and muscles or joints aches. Acute hepatitis may in some cases progress to fulminant hepatitis leading to liver failure, which is a state with high mortality.

The weak immune response generated by young children acutely infected hepatocytes. For this reason, clinical symptoms suggestive of acute HBV infection are frequently absent in this patients population. For those patients who resolve their infection, HBsAg disappears at about 3-6 month, often just prior to the detection of antibodies to hepatitis B surface antigen (anti-HBs), while some

patients with self-limited infection, and however may still have low levels of HBV DNA in blood; whether the HBV DNA is a part of intact virions remains unknown.

The clinical features of acute infection resemble those of the other viral hepatitides. Acute hepatitis B is frequently anicteric and asymptomatic, although a severe illness with jaundice can occur and occasionally acute liver failure may develop.

In some people, the hepatitis B virus can also cause a chronic liver infection that can later develop into cirrhosis (a scarring of the liver) or liver cancer.

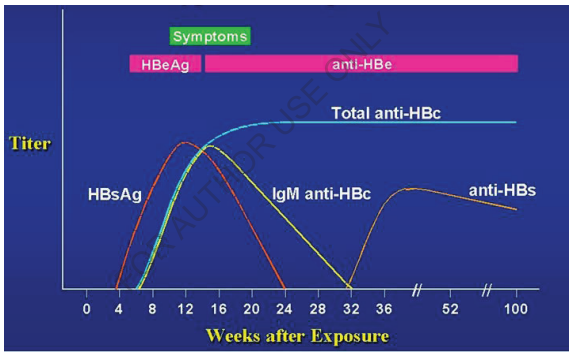

Figure (1): Acute Hepatitis B virus infection with recovery typical serological course.

Chronic Hepatitis B

Chronic HBV (CHB) infection can be define as the presence of hepatitis B surface antigen (HBsAg) in the serum of an infected individuals for at least six months or as the presence of HBsAg in a patient who is negative for immunoglobulin M antibodies to the hepatitis B core antigen (anti-HBc).

Chronic hepatitis can be divided into four stages. The first stage, the immune tolerance phase which is characterized by active viral replication and immune system tolerance. In this initial phase, HBV DNA replicates at a high levels and the HBsAg and HBeAg can be detectable while the Aminotransferase (ALT) levels are normal or low, mild or no liver necroinflamation and no or slow progression of fibrosis. In this phase, more prolonged in subjects infected prenatally or in the first years of life. Next, the immune clearance phase: The immunologic response is causing inflammation and hepatic injury as a result of viral clearance. Here, the ALT levels are elevated and moderate/severe necroinflammation in liver biopsy is observed. The third phase, inactive carrier state: The viral clearance is accompanied by seroconversion of HBsAg, resulting in relatively low HBV DNA level and normalized ALT levels. Few patients reach the final stage, when the HBsAg is completely cleared and anti-HBs becomes detectable as a sign of immunity.

The risk of developing chronic hepatitis B infection that depends on the age at which infection is acquired. The risk is the lowest in adults and >90% in neonates whose mothers are HBeAg-positive. Chronic infection is less frequent in those infected as the children. The risk of becoming chronically infected with hepatitis B is increased in those whose immunity is impaired.

Clinically, the e-antigen HBeAg is important in chronic infection as it is regarded a marker for replication and indicative of ongoing infection. When seroconversion occurs, it normally reflects remission of liver disease and viral clearance. Persons with chronic HBV may have HBeAg or anti-HBe in their sera. In persons who are HBeAg positive, spontaneous seroconversion from HBeAg to anti-HBe commonly occurs with often accompanied by a flare in aminotransferase ALT levels. After conversion of HBeAg to anti-HBe, most

persons have normal ALT levels and lower levels of HBV DNA which is usually <10^3 copies / ml.

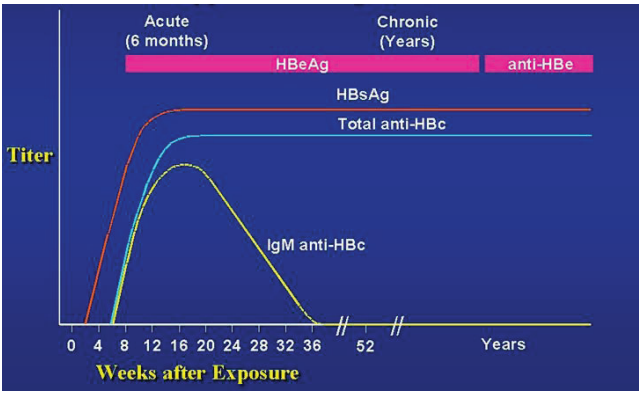

Figure (2): Progression of chronic Hepatitis B virus infection typical serological course.

Occult Hepatitis B infection (OBI)

In this stage of infection, HBV DNA in the serum or in the liver may in some cases still be detectable in the absence of HBsAg which is termed occult hepatitis B. The clinical importance of this is not completely understood, but occult hepatitis B has been associated with reactivation in the setting of immunosuppression and enhancing risk for liver cancer. This type of infection represents a potential transmission source of HBV via blood transfusion or organ transplantation. In addition, occult HBV infection has been associated with cryptogenic CH and HCC. Furthermore, some studies suggested that occult hepatitis B might affect responsiveness of chronic HCV to interferon therapy and disease progress.

Different clinical situations have been associated with OBI. OBI may follow recovery from infection, displaying anti-HBs and persistent low-level viraemia, escape mutants undetected by the HBsAg assays, or healthy carriage

with anti-HBe and anti-HBc. Overtime, in the latter situation, the anti-HBe and anti-HBc may become undetectable, but in the most cases, the explanation is probably due to the low level of viral replication. Currently, there are no consensus guidelines for diagnosis of occult HBV. Testing has been advocated for a variety of settings, including cryptogenic liver disease, impending immuno-suppression patients with HBV risk factors and isolated positivity for core antibody. From the perspective of diagnostic yield, liver biopsy samples may be better specimens than serum, as HBV DNA positivity rates have been found to be higher in liver than in serum in studies of paired samples. Nowadays, there are currently no available reports on treatment of occult HBV.

Cirrhosis and Hepatocellular carcinoma (HCC)

Some reports have been estimated that up to 40% of individuals with chronic hepatitis B (CHB) will progress to cirrhosis and may lead to hepatocellular carcinoma (HCC). Worldwide, more than 50% of HCC cases, and in highly endemic areas 70-80% of HCC cases are attributable to HBV and 20% of the 400 million people with chronic hepatitis B infection will develop to HCC. It has been showed that the presence of HBeAg and higher levels of HBV DNA have been found to be strong risk factors for HCC in patients with chronic HBV infection and mainly develops in patients with liver cirrhosis. The mechanisms of oncogenesis by HBV remain obscure. HBV may stimulate active regeneration and cirrhosis which may be associated with long-term chronicity. However, HBV associates tumors occasionally arise in the absence of cirrhosis, and such hypotheses do not explain the frequent finding of integrated viral DNA in tumors. Although insertional mutagenesis of HBV remains an attractive hypothesis to explain its oncogenicity. Like many other cancers, there is insufficient supportive evidence development of hepatocellular carcinoma likely

to be a multifactorial process. The incidence of HCC may also be affected by factors other than HBV infection such as HCV co-infection, alcohol intake and aflatoxin B1 in the food supply. In the Amazonian basin, the genotype F infections are associated with fulminant hepatitis, but this occurs in the context of co-infection or super infection with Hepatitis Delta Virus (HDV) genotype III.

Many other risk factors have been implicated in the progression of liver disease and the development of HCC. In such co-infections have been reported that HBV may carriers with more than one genotype. Some common co-infection occurs between genotype B and C, A and D, which is presumably due to the coexistence of these genotypes in the same regions. Recombination between genotypes has been reported as genotypes A with D. The clinical impact of co-infections is unclear, but the viral loads have been reported to be higher in the co-infected patients. The frequency of co-infection may be associated with genotyping method as the reported frequency varies widely. Persons who are co-infected with both HBV and HCV also have an increased risk of developing HCC, as compared with those who are infected with either virus alone. Even though, co-infection with HDV has not been shown to increase the risk of HCC. One study demonstrated that HCC appears at younger ages in co-infected persons than it does in those infected solely with HBV. Using chronic alcohol also appears to increase the risk of cirrhosis.

Part Two

Viral Structure

Viral Structure

HBV is the smallest DNA virus with (3200) base pairs, which contains four overlapping genes (Open Reading Frames or ORFs) encoding the viral envelope (S and Pre-S), necleocapsid (Precore and Core), polymerase with reverse transcriptase enzyme and X proteins. HBV is a (42 nm) viral particle, comprises an icosahedral protein capsid surrounding the viral DNA with an envelope lipoprotein**.** The HBV genome is a relaxed circular, partially double stranded DNA. The long L (-) strand is a linear and of fixed length of about 3.2kb. The short S (+) strand is of variable length, ranging from 50 to 100% of that of the L (-) strand. The circularity is maintained by cohesive ends. The L (-) strand transcript of HBV contains the four ORFs that are conserved in all strains. The open reading frames (ORFs) are combined with four promoters; S1, S2, Core promoter, and X-promoter and two enhancer elements; Enhancer I and II. Hepatitis B virus is a very efficient virus with every nucleotide in at least 1 coding region and many times (2) coding regions. Consequently, a relatively small genome generates (7) distinct proteins. These proteins are the polymerase protein (Pol gene), which is the largest HBV protein; core antigen and early e antigen (both are from the C gene); large, medium, and small surface-antigen proteins (S gene); and the X protein (X gene).

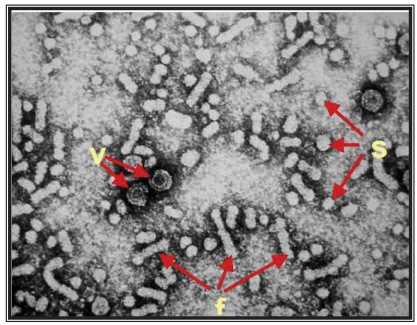

Figure (3): Electron micrograph of HBV virions. This electron micrograph shows complete virions (v) as well as spherical (s) and filamentous (f) subviral particles which can be detected in the blood of chronic carriers.

Figure (4): EM of HBV particles, https://www.shutterstock.com/search/hepatitis+b

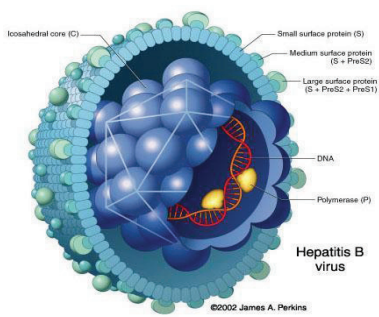

Figure (5): The architecture of a Dane particle.

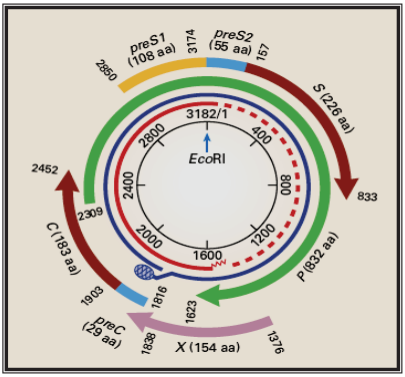

Figure (6): Schematic representation of HBV genome.

The above figure represents the viral DNA which is partially double-stranded (red circle). The long strand of fixed length (blue circle) encodes 7 proteins from 4 overlapping reading frames (surface S, core C, polymerase P, and X gene), shown as long arrows and 3 upstream regions (Pre C, Pre-S1, and Pre-S2). A protein is covalently linked to the 5´end of the long strand (hatched blue oval) and a short oligoribonucleotide at the 5´end of the short strand (red zigzag). The *EcoR*I restriction-enzyme binding site is included as a reference point.

In comparison to mammalian HBV viruses, avian hepadnaviruses have the major structural differences. For example, the DHBV (duck hepatitis B virus) DNA is almost fully double stranded, only has two surface proteins, lacks X-gene and has several hundred bases shorter.

Structural Proteins

Hepatitis B Surface (S) protein

HBsAg is the first virologic marker detectable in serum within (1–12) weeks usually between week 4 and week 10 in acute infection while in chronic HBV infection, the HBsAg persistence for six months and remains detectable beyond (6) months. Anti-HBc is primarily of the IgG class, and anti-HBs is either undetectable or detectable with low levels.

The S ORF (related to the press gene) encodes the three proteins S gene, Pre-S1 region and Pre-S2 region. The S gene and Pre-S2 region are of constant length in all the genotypes. In general, there is about twice as much amino acids variability in the Pre-S1 and Pre-S2 regions as in the S gene, therefore, the Pre-S1 and Pre-S2 regions could be more strongly involved in the host immune response and less in the virus assembly than the S gene.

Moreover, the Pre-S domain (as mentioned composed of Pre-S1 and Pre-S2) overlaps with the nonessential spacer domain of P protein. In keeping of view, the sequence of S-gene, which is more conserved rather than Pre-S region, as the former overlaps the active site in the P gene, which is encoded in a different frame.

The small Hepatitis B surface protein (HBs or SHBs) is encoded solely by the S gene. This protein is the prime constituent of all hepatitis B particle forms. It is manufactured by the virus in high quantities. It also contains a highly antigenic epitope, called Hepatitis B surface antigen (HBsAg), which is a conformational antigen that the serotype classification of the virus depending on it. According to the antibody recognition of the HBsAg determinants, recognizes one constant area, the "*a*" determinant (amino acids 124-127), which is flanked by two mutually exclusive *d/y*

determinants at the position (122) and *w*/*r* determinants at the position (160). The major hydrophilic region ("*a*" determinant) has an assumed two-loop structure and is located on the surface of the viral particles. This two-loop structure encompassing conserved residues (124-147). SHBs also constitutes the basis of current HB vaccines.

Middle Hepatitis B Surface protein (MHBs) is encoded by the Pre-S2 region (an additional 55 amino-acid domain) shared with the S gene. It has been proposed that this protein is involved in HBV attachment and entry into the liver cells (hepatocytes). MHBs are in general hydrophilic and contain a dominant epitope located at the surface of the envelope. This epitope is a di-sulphide bond independent and apparently more immunogenic than are the epitopes of SHBs.

Large Hepatitis B Surface protein (LHBs) is encoded by the Pre-S1 domain as well as the Pre-S2 and S domains. The LHBs may also be involved in the attachment of HBV to hepatocytes. Overexpression of the LHBs alone results in the retention of the protein in the ER, which may lead to the development of hepatocellular carcinoma (HCC). In general, the surface or envelope proteins play a vital role in the host specificity of the virus. During the viral life cycle, the virus binds to the surface of the hepatocyte and fuses with it.

Dimer structure of Hepatitis B c protein (HBc)

The C ORF encodes two distinct proteins, the core protein (referred to as HBc) and the early protein (HBe) where the two methionines (Met) at positions (1814) and (1901) are candidates for the initiation of their translation. The basic soluble unit of HBcAg is a dimer (Figure 8a). A HBcAg monomer is 183 residues long (for in vitro studies we refer to it as core protein 183 or Cp183). The first 149 residues form the predominantly

α-helical assembly domain (Cp149). The remaining 34 residues are the arginine-rich RNA-binding, C-Terminal Domain (CTD). This domain is located inside the capsid and has been identified to be intrinsically disordered based on sequence and Cryo-EM studies. The assembly domain alone is sufficient for assembly of morphologically regular empty capsids. The assembly domain has 5 α-helices connected by loops (Figure 8a). Helices 1, 2 and 5 are part the chassis of the capsid. A tight proline-rich loop connects helix 5 to the CTD. Holding together a dimer, helices 3 and 4 from one half-dimer form a 4-helix motif with corresponding helices from the other half-dimer. A total of about 3200 Å2 of hydrophobic surface is buried by this interaction. This motif is flanked by salt bridges and hydrogen bonds that stabilize the hydrophobic interface. Helix 4 is kinked in the middle and is sometimes referred to as helices 4a and 4b. A disulfide bond can form over time between highly conserved C61 residues in helix 3, which contributes to the dimer interface.

The core structural protein is (183 – 185) amino acids in length depending on the genotype of the virus and, compared to the envelope gene is relatively well conserved. The primary sequence of HBcAg can be divided into two parts: The N-terminal (149) or (151) aa (depending on the genotype) is sufficient for the self-assembly of capsids. This part of the HBcAg is called the assembly domain. The C-terminal (34) aa, designated the protamine domain, is rich with arginine residues that confer a positive charge to this domain. This domain is essential for the packaging of the pregenome / HBV Pol complex. HBcAg can be overproduced in prokaryotic and eukaryotic expression systems and assembles in these systems to capsid particles. Capsid assembly starts with the formation of HBcAg dimmers that are cross linked by a disulfide bond.

However the C gene has two initiation codons, a precore and a core region, so if translation is initiated at the precore region, the protein product is HBeAg, which has a signal peptide that binds it to the smooth endoplasmic reticulum SER as a result leads to its secretion into the circulation. If translation begins with the core region. HBcAg is the protein product which has no signal peptide and does not secreted, but it assembles into nucleocapsid particles. DNA polymerase is also packaged within the nucleocapsid core in which directs replication and repair of HBV DNA. The HBc is the major component of the nucleocapsid shell packaging of the HBV genome. More generally, the HBc protein is the major target of the host immune response in chronic hepatitis B and several immunodominant epitopes have been identified. Moreover, HBc contributes to the emergence of escape mutants. HBcAg particles remain in the hepatocytes which can be readily detectable by immunohistochemical staining, and are exported after encapsidation by an envelope of HBsAg.

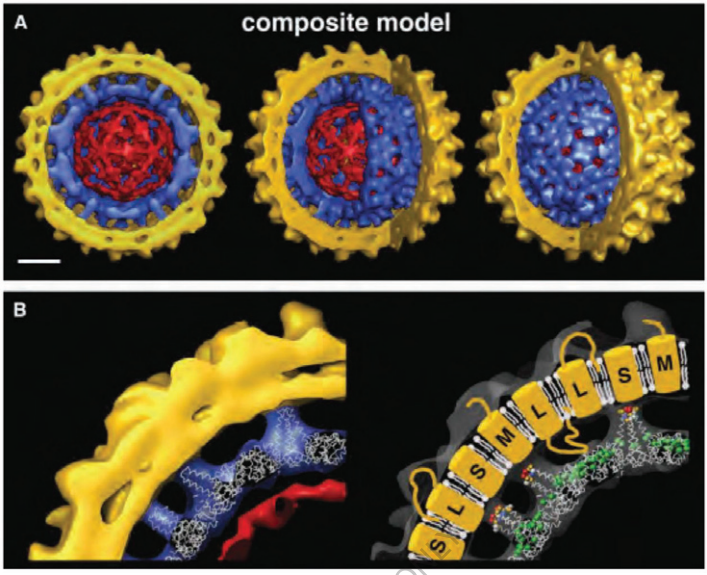

Figure (7): A composite cryo-EM reconstruction of a dane particle. (a) Cut away views of a composite model of the HBV virion comprised of an icosahedral capsid (blue) containing packaged DNA (red) and an outer envelope (gold) with protein projections spaced 60Å apart. Views are cross-sections (left), and two cut away (b) X-ray crystal structure of recombinant capsid (36) docked into the cryo-EM density map of the virion capsid (left). The tips of the core spikes are in close apposition but do not penetrate the envelope. Additional details and cartoon of interpretation (right). The surface protein projections are ascribed to HBsAg and are designated as large (L), medium (M), and small (S) arbitrarily. These figures reproduced with permission from Dryden *et al*.

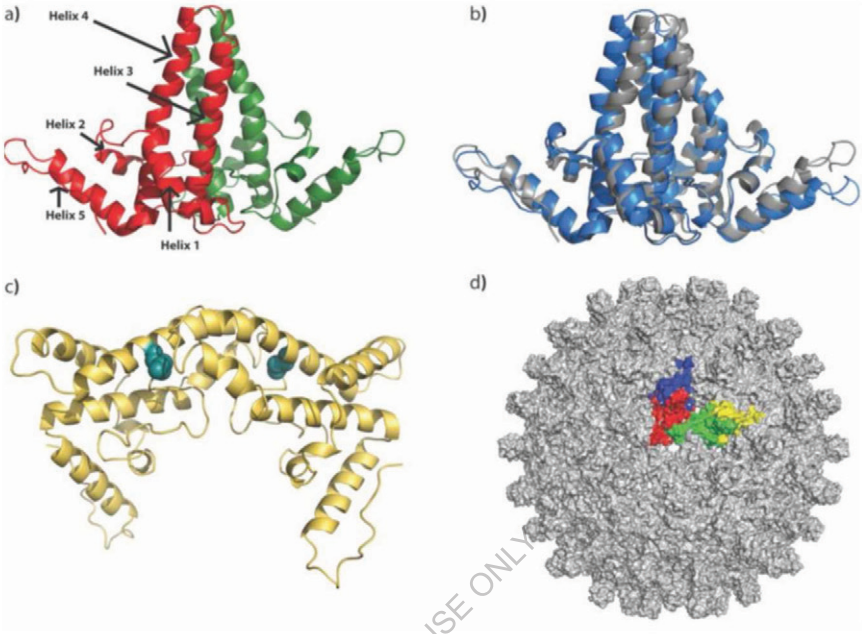

Figure (8): X-ray crystal structures of the assembly domain. (a) HBcAg dimer in the context of a capsid. The helices in HBcAg are named 1–5 from N to C-terminus. The dimer interface comprises of a four-helix bundle created by two helices from each monomer. (b) a superposition of an HBcAg dimer in the context of a capsid (grey) on a free dimer (Y132A mutant) (blue). The free dimer is less compact than the dimer in the context of a capsid (c) An HBeAg dimer. The dimeric interface is drastically altered and stabilized by disulfide bonds (d) An HBcAg T=4 capsid with the asymmetric unit in color. The individual subunits are A (blue), B (red), C (green) and D (yellow) or AB and CD dimers.

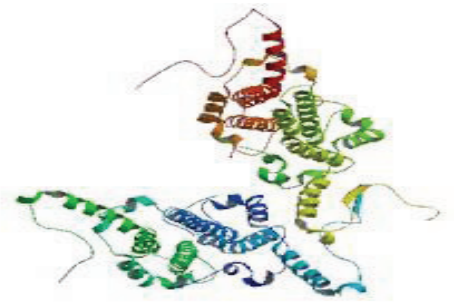

Figure (9): Crystal 3d structure of HBcAg (PDB data bank).

http://www.rcsb.org/pdb/results/results.do?tabtoshow=Current&qrid=1E9A0C9A

Nonstructural Proteins

Hepatitis B early protein (HBe)

HBe was named due to its "early" appearance during the acute HBV infection. This protein is a non-structural protein (16 and 20 kDa) that shares about 90% of its amino acids with the nucleocapsid protein HBc. The HBe protein contains two epitopes: conformational (e1), and linear (e2). The e1 epitope sequence overlaps that of the conformational HBc epitope therefore, e1 is presented at the surface of HBV nucleocapsids and expressed together with HBc at the surface of infected cells. Although HBe protein does not appear to be essential for HBV lifecycle, its' presence is associated with immune tolerance, high-level HBV replication and high transmissibility. The precore sequence encodes a hydrophobic transmembrane domain, resulting in the translation/translocation of HBe into the lumen of the Endoplasmic Reticulum (ER) and the release of HBe into the circulation of the infected patient. This fundamental changes in the location of protein expression and alters the antigenicity of HBe such that

it does not share antigenic homology with HBc, despite having nearly identical amino acid sequences.

The HBeAg is a soluble, secreted, non-particulate form of the viral nucleocapsid that is not necessary for either viral replication or infection. The role of HBe protein in the viral life cycle is not well understood although production of this antigen is thought to be required for the establishment of persistent infections in acutely infected individuals.

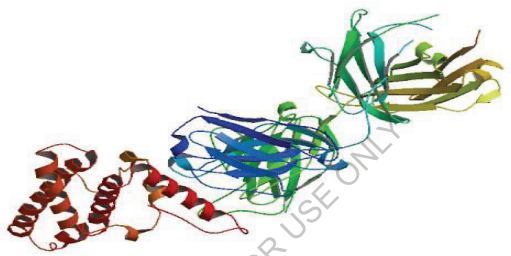

Figure (10): Crystal 3d structure of HBcAg (PDB data bank).

http://www.rcsb.org/pdb/results/results.do?tabtoshow=Current&qrid=1E9A0C9A

Hepatitis B x Protein (HBx)

The smallest ORF in the HBV genome comprises (154) amino acids and encodes the HBx protein which is a non-structural protein. The function of HBx is still partially unknown, but it seems to be involved in inhibiting the cellular processes of protein degradation. The ζ-stem loop which at the end of the X ORF plays a key role in HBV DNA encapsidation. Sequence analysis shows that the ζ-stem loop is a series of inverted repeats that are predicted to fold into a three-dimensional stem-loop structure. This stem-loop is conserved among all hepadnaviruses,

despite differences in the primary sequence. It is believed that the polymerase protein recognizes and interacts directly with the stem-loop structure. Interaction of the hepatitis B polymerase and the ζ - stem loop initiates both encapsidation as well as reverse transcription of the HBV pregenomic RNA (pgRNA), within the core particles. HBxAg is capable of transactivating the transcription of both viral and cellular genes. In the cytoplasm, HBxAg effects calcium release (possibly from mitochondria), which activates signal-transduction pathways that lead to stimulation of HBV reverse transcription and HBV DNA replication. The transactivating activity can enhance the transcription and replication of other viruses besides HBV, such as HIV.

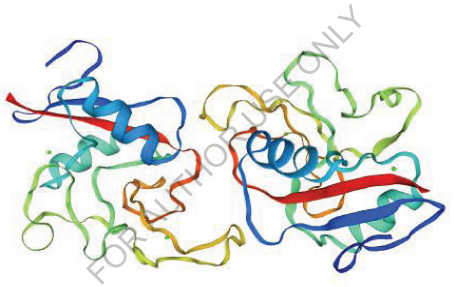

Figure (11): Crystal 3d structure of HBxAg (Swiss Model)
https://swissmodel.expasy.org/repository/uniprot/Q7Z4G9

Hepatitis B Polymerase (P) protein

The P ORF which is the largest gene has several functions in virus replication, such as reverse transcription, RNA pregenome encapsidation, priming of DNA synthesis, and (+) strand DNA-polymerization. The multi-functional nature of HBV polymerase gene is unique to hepadnaviruses due to the composed of four distinct domains: an N-terminal protein (tp) that serves as the primer for the reverse transcription,

a spacer region of unknown function, the reverse transcriptase (RT) polymerase domain and ribonuclease H (RNase H). The RT/polymerase domain shares some sequence homology with the other retroviruses such as Human Immunodeficiency virus (HIV).

The polymerase domain has been further divided into A-E subdomains and the RT domains into F and G subdomains. Subdomains A, C and D are mainly involved in deoxyribonucleotide triphosphate (dNTP) binding and catalysis and correspond to the palm domain of the polymerase protein. Subdomains B and E interact with the RNA template and primer regions respectively. The C subdomain contains the "YMDD" (Tyrosine-Methionine- Aspartic acid-Aspartic acid) motif, which has a fundamental role in the treatment of hepatitis.

Transmission

HBV is transmitted by contact with contaminated blood, percutaneous or mucosal exposure to the blood, blood products and other body fluids such as semen. HBV can also transmit from an infected mother to her newborn during childbirth. Childhood infections with HBV are usually asymptomatic and these infections may not be recognized for many years. HBV can also transmit through close contact within households, through exposures to unscreened blood transfusion or unsafe injections in health care settings, through injection-drug use or from sexual contact with an infected person. Travelers may be at increased risk due to engaging in casual and unprotected sex or even through obtaining tattoos or having acupuncture. Adults with diabetes mellitus are at an increased risk of acquiring HBV infection. It has been reported that genotype A has been associated with sexual contact, while genotype D has been associated with blood transmission.

Epidemiology

The World Health Organization (WHO) has estimated that over (350) million people worldwide are chronically infected with HBV. The WHO has categorized countries based upon the prevalence of HBsAg into high (more than 8%), intermediate (2 to 8%) and low (less than 2%) endemicity countries. In many high-prevalence countries, 10% or more of the population have chronic hepatitis B infection. High-prevalence regions were include sub-Saharan Africa, most of Asia and the Pacific islands. Intermediate-prevalence regions include the Amazon, southern parts of Eastern and Central Europe, the Middle East and the Indian sub-continent. Low-prevalence regions include most of Western Europe and North America.

In endemic areas of Africa and Asia, the epidemiological patterns differ from those seen in North America and Western Europe. In these regions, most infections occur in infants and children as a result of maternal-neonatal transmission or close childhood contact with family, although percutaneous exposure with contaminated needles or following unsafe injections is always a possibility in these countries. The chronic liver disease and HCC associated with HBV infections are among the most important human health problems in high-prevalence regions.

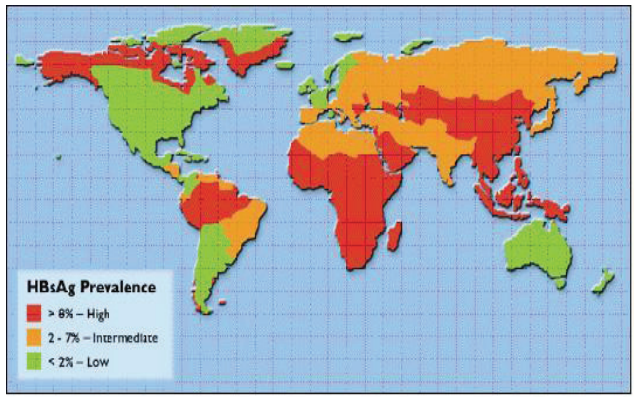

Figure (12): Prevalence of HBV in the world.

Age distribution of HBV infection

An important determinant of epidemiology of HBV is the age of acquisition of the virus. In areas with high endemicity, most infections occur in children (4-8 years), and in area with intermediate endemicity, the infections occur in adolescents and adults, while in areas with low endemicity, the most infections also occur in adolescents and young adults (15-29 years).

Gender distribution of HBV infection

An epidemiological study done by Sobeslavsky in (1980) demonstrated a relatively higher prevalence of HBsAg among males than females in most countries. Once infected with HBV males are more likely to remain persistently infected than females, who are more likely to be infected transiently and to develop anti-HBs.

Part Three

Immunity, Viral activation

Immune responses in HBV infections

Humoral immunity

The `*a*` determinant epitopes of HBsAg trigger protective neutralizing antibodies that prevent viral spread and subsequently eliminate circulating virus. The presence of anti-envelope antibodies denotes the resolution of acute hepatitis B. The excessive production of HBsAg and the high viral replication induce immunological anergy or tolerance by the exhaustion of antiviral antibodies and cytotoxic T lymphocytes CTLs. The virus also affects the immune system directly by infecting the lymphocytes.

Cell Mediated Immunity (CMI)

Cell-mediated immunity (CMI) is important in terminating the infection and under certain circumstances, in promoting immune-mediated liver damage and in the genesis of autoimmunity. Also, evidence suggests that progressive liver damage may result from an autoimmune reaction directed against hepatocyte membrane antigens, initiated in many cases by infection with hepatitis B virus. CMI is triggered by acute HBV infection yielding polyclonal specific cellular response. The core protein HBc contains also helper T cell epitopes and its' gene eliminates replicating HBV in chronic infection which is known as cytotoxicity that enhance viral clearance and inhibit HBV gene expression through the secretion of antiviral cytokines.

HBeAg is strictly T-cell dependent and may partially or totally block the cellular immune responses against HBcAg and promote virus persistence because HBcAg and HBeAg have cross-reactive T-cell epitopes. Persistent HBV infection is characterized by a weak adaptive immune response be due to inefficient $CD4^+$ T cell priming early in the infection and subsequent development of a quantitatively and qualitatively ineffective $CD8^+$ T cell response. Moreover,

factors contribute viral persistence are immunological tolerance, mutational epitope inactivation, T cell receptor antagonism, incomplete down-regulation of viral replication. Persistent infection is characterized by chronic liver cell injury, regeneration, inflammation, widespread DNA damage and insertional deregulation of cellular growth control genes, which collectively lead to cirrhosis of the liver and hepatocellular carcinoma.

Mutant HBV induces more severe immune disorders in host, resulting in the activation of lymphocyte and release of cytokines such as TNF-alpha, IFN-gamma, IL-6 and IL-8 were higher in patients with severe than those in acute hepatitis B. The ratio of $CD4^+/CD8^+$ was higher in mutant than in wild-type HBV. The cellular over expression of the immunogenic HBcAg in precore mutants during virus replication may have direct cytopathic effect and trigger vigorous T cell-mediated immune response to core that contributes to the development of fulminant hepatitis.

Some studies showed that HBV does not exert any direct cytopathic effects in immune competent patients. This is supported by the fact that during the first phase of infection, viral replication is very high without signs of liver damage. Hepatocellular injury is generally accepted to be the result of attacks from the immune system. The immune response to HBV infection is both B- and T-cell derived. Antibodies (anti-HBs, anti-HBc, and anti-HBe) are produced and targeted towards their antigens. After the HBV infection, the anti-HBc develops first while anti-HBs antibody is detected later. The delay detection of anti- HBs is likely due to the rapid binding of the antibody to the HBsAg present at high levels in infected patients. Anti-HBe is detected only during acute HBV infections. It does not directly neutralize the HBV virion because intact virus particles do not contain HBeAg. However, it has been reported that anti-HBe levels decline when viremia declines, indicating this immune response may have a protective nature. The immune response is coordinated by the human leukocyte antigen (HLA) class

I and class II molecules which present foreign antigens to $CD8^+$ cytolytic T cells and to $CD4^+$ helper T cells respectively.

It has been estimated that HBV induces proliferation of human mesangial cells (HMCs), and extracellular matrix expression through the deposition of immune complexes in renal tissue. Immune complexes have been identified in variable proportions of patients with virtually all the recognized chronic sequelae of acute hepatitis. Deposits of such immune complexes have also been demonstrated in the cytoplasm and plasma membrane of hepatocytes and on or in the nuclei. Perhaps complexes are critical pathogenic factors only if they are of a particular size and of a certain antigen/antibody ratio.

Interferon (IFN) plays an important role for enhancing human leukocyte antigen (HLA) class I expression on hepatocyte cell membranes. The epitopes are presented on the cells evoke a T-cell response. T-cell response is also mediated by MHC class II expression on antigen-presenting cells (APCs) activating T-helper cells (Th). It has been shown that cytotoxic T-cell response is dependent on HLA (MHC class I) type and that the Th response is genetically restricted.

Virus reactivation

There are three stages in the pathogenesis of HBV reactivation. Reactivation starts with an increase in viral replication caused by chemotherapy-induced immune suppression and this is the stage (1). Here, the ALT level is elevated by up to (3) weeks. Also in this stage, the HBeAg and HBsAg reappearance as well as decrease of anti-HBs titer. After chemotherapy is discontinued, the function of the immune system is restored and hepatocytes infected with HBV are destroyed which means here as stage (2). In this stage, the ALT level is increased while the HBV DNA level is decreased as well as appearance of jaundice owing to hepatocellular injury. In the recovery stage stage (3), clinical hepatitis which is indicated by an increase in ALT levels, resolves

and markers of HBV infection return to baseline levels. Occasionally, the second stage is more severe and can be lethal. The risk of HBV reactivation is influenced by both types of malignancy and the particular immunosuppressive therapy that is administered and the lymphoma patients may be a particular risk. The other risk factor of HBV reactivation is the elevation of HBV DNA in serum. The HBV reactivation can be observed in up to 36% of patients with hepatocellular carcinoma whom received systemic chemotherapy.

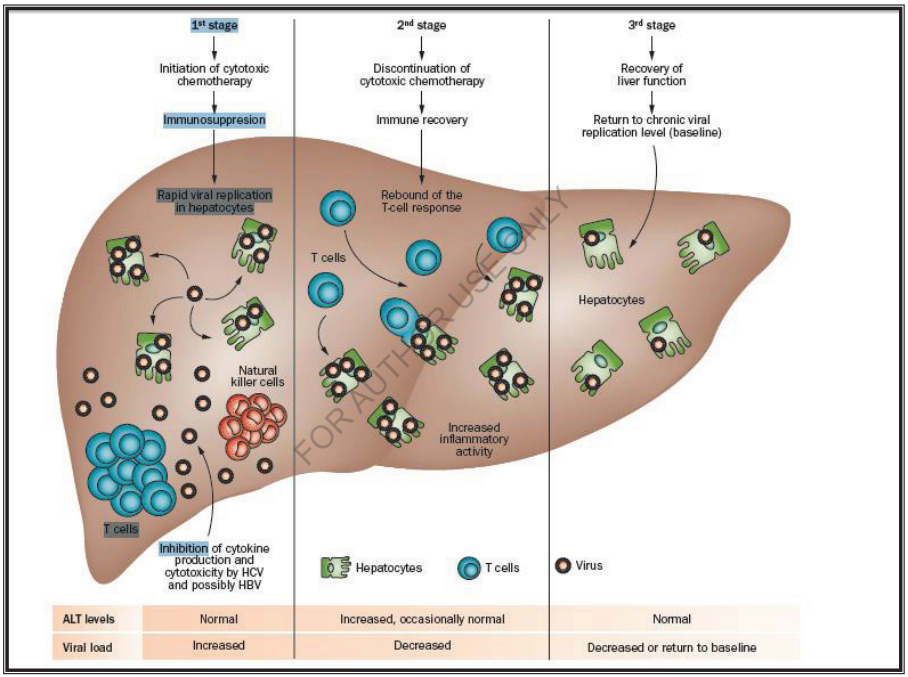

Figure (13): HBV reactivation.

Virus Inactivation

The immunogenicity and antigenicity of HBsAg are retained after exposure to ether, acid, and heat, while the virus is inactivated by boiling for two minutes, autoclaving at (121ºC) for (20) minutes, or dry heat at (160ºC) for one hour as

well as sodium hypochlorite 0.5% for 10 minutes, 0.1 to 2% gluteraldehyde and 70% isopropyl alcohol for (2) minutes. HBV retains its infectivity when stored at 40ºC for six months and when frozen at (–20ºC) for (15) years.

Part Four

Treatment, Prevention & Life Cycle

Treatment

The goal of chronic HBV therapy is to minimize the risk for complications and to prevent progression of liver disease to its life-threatening complications; cirrhosis and HCC. Taking a decision to treat chronic HBV is based on the assessment of multiple parameters including clinical, biological and histologic parameters. Antiviral treatment is currently recommended in patients with an HBV DNA titer above (2,000 IU/ml), elevated serum alanine aminotransferase ALT activity, and or evidence of chronic hepatitis with or without cirrhosis.

There are two types of drug treatments for chronic hepatitis B: antiviral compounds: pegylated interferon alpha *(peginterferon alfa-2a, Pegasys)* and nucleoside analogues that inhibit the reverse transcriptase domain of viral polymerase. Five of these analogues are approved in Europe and in US for the treatment of chronic HBV, including lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil fumarate. Nowadays, three registered treatment regimens are used against HBV: interferon-alpha (IFN- α), lamivudine and adefovir. These treatment options can suppress wild-type HBV to undetectable levels, allowing a mutant HBV strain to emerge as the predominant form, so it can be easily to detect mutants include immunotherapy (vaccination, administration of HBV immune globulin) and nucleoside analogs.

Some studies estimated that HBV subtype *ayw* respond better to lamivudine monotherapy than HBV subtype *adw*. The treatment is beneficial if patients have elevated liver enzymes (ALT) and low viral load, while treatment for patients with very low HBV DNA levels and normal ALT levels are not usually recommended. The HBV DNA level should be recommended on treatment with a sensitive real-time PCR assay (lower

limit of detection: 10 to 15 IU/ml). If the HBV DNA level remains detectable after (48) weeks of infection, a second antiviral compound with no cross-resistance with the first one must be added in order to prevent subsequent resistance.

Some researchers reported that lamivudine is more efficient in the treatment of patients with HBV genotype D as compared to those with other types of HBV. Health care workers should note that lamivudine may be useful in the treatment of a large number of HBV infected patients.

Although HBV genotypes A and B globally appear to better respond to interferon-based therapy than genotypes C and D, HBV genotype determination is not yet recommended to guide the therapeutic decision in the absence of strong individual predictive value.

Prevention

The hepatitis B vaccine is the mainstay of hepatitis B prevention. WHO recommends that all infants receive the hepatitis B vaccine as soon as possible after birth, preferably within 24 hours. The low incidence of chronic HBV infection in children under 5 years of age at present can be attributed to the widespread use of hepatitis B vaccine. Worldwide, in 2015, the estimated prevalence of HBV infection in this age group was about 1.3%, compared with about 4.7% in the pre-vaccination era. The birth dose should be followed by 2 or 3 doses to complete the primary series. In most cases, 1 of the following 2 options is considered appropriate:

- A 3-dose schedule of hepatitis B vaccine, with the first dose (monovalent) being given at birth and the second and third (monovalent or combined vaccine) given at the same time as the first and third doses of diphtheria, pertussis (whooping cough), and tetanus – (DTP) vaccine; or

- A 4-dose schedule, where a monovalent birth dose is followed by three monovalent or combined vaccine doses, usually given with other routine infant vaccines.

The complete vaccine series induces protective antibody levels in more than 95% of infants, children and young adults. Protection lasts at least 20 years and is probably lifelong. Thus, WHO does not recommend booster vaccination for persons who have completed the 3 dose vaccination schedule.

All children and adolescents younger than 18 years-old and not previously vaccinated should receive the vaccine if they live in countries where there is low or intermediate endemicity. In those settings it is possible that more people in high-risk groups may acquire the infection and they should also be vaccinated. They include:

- People who frequently require blood or blood products, dialysis patients, recipients of solid organ transplantations;
- People interned in prisons;
- Persons who inject drugs;
- Household and sexual contacts of people with chronic HBV infection;
- People with multiple sexual partners;
- Healthcare workers and others who may be exposed to blood and blood products through their work; and
- Travelers who have not completed their hepatitis B vaccination series, who should be offered the vaccine before leaving for endemic areas.

The vaccine has an excellent record of safety and effectiveness. Since 1982, over 1 billion doses of hepatitis B vaccine have been used worldwide. In many countries where between 8–15% of children used to become chronically infected with the hepatitis B virus, vaccination has

reduced the rate of chronic infection to less than 1% among immunized children.

In 2015, global coverage with the third dose of hepatitis B vaccine reached 84%, and global coverage with the birth dose of hepatitis B vaccine was 39%. The WHO Region of the Americas and WHO Western Pacific Region were the only regions that have wide coverage. In addition, implementation of blood safety strategies, including quality-assured screening of all donated blood and blood components used for transfusion, can prevent transmission of HBV. Worldwide, in 2013, 97% of blood donations were screened and quality assured, but gaps persist. Safe injection practices, eliminating unnecessary and unsafe injections, can be effective strategies to protect against HBV transmission. Unsafe injections decreased from 39% in 2000 to 5% in 2010 worldwide. Furthermore, safer sex practices, including minimizing the number of partners and using barrier protective measures (condoms), also protect against transmission.

WHO response

In March 2015, WHO launched its first *"Guidelines for the prevention, care and treatment of persons living with chronic hepatitis B infection"*. The recommendations:

- Promote the use of simple, non-invasive diagnostic tests to assess the stage of liver disease and eligibility for treatment;
- Prioritize treatment for those with most advanced liver disease and at greatest risk of mortality; and
- Recommend the preferred use of the nucleos(t)ide analogues with a high barrier to drug resistance (tenofovir and entecavir, and entecavir in children aged between 2–11 years) for first- and second-line treatment.

These guidelines also recommend lifelong treatment in those with cirrhosis; and regular monitoring for disease progression, toxicity of drugs and early detection of liver cancer.

In May 2016, The World Health Assembly adopted the first *"Global Health Sector Strategy on Viral Hepatitis, 2016-2020"*. The strategy highlights the critical role of Universal Health Coverage and the targets of the strategy are aligned with those of the Sustainable Development Goals. The strategy has a vision of eliminating viral hepatitis as a public health problem and this is encapsulated in the global targets of reducing new viral hepatitis infections by 90% and reducing deaths due to viral hepatitis by 65% by 2030. Actions to be taken by countries and WHO Secretariat to reach these targets are outlined in the strategy.

To support countries in moving towards achieving the global hepatitis goals under the Sustainable Development Agenda 2030, WHO is working in the following areas:

- Raising awareness, promoting partnerships and mobilizing resources;
- Formulating evidence-based policy and data for action;
- Preventing transmission; and
- Scaling up screening, care and treatment services.

Hepatitis B Vaccine

The first hepatitis B vaccine, a plasma-derived vaccine, was licensed by the US Food and Drug Administration (FDA) in 1981. After that, the plasma-derived vaccine was replaced with a recombinant version, which expresses the hepatitis B surface antigen (HBsAg) and is produced in *Saccharomyces cerevisiae*. This vaccine is purified HBsAg particles from

human plasma donors. It was replaced by DNA-recombinant HBV vaccines when they became available and licensed by FDA in 1986 currently used in the United States. Vaccine efficacy studies reported 90-100% protection and the vaccine also proved to be effective in young children and infants, despite the fact that young children can be poorly responsive to vaccines due to their immature immune system. Currently, the second generation DNA recombinant vaccine is used which has been available for using since 1991. This recombinant vaccine is an anticancer vaccine which prevents hepatocellular carcinoma. Nowadays, tow single antigen vaccines are used, Recombivax HB (Comvax Merck) and Energix-B (GlaxoSmithKline, Belgium). The first combination vaccine contains a Haemophilus b conjugate antigen with the recombinant HBsAg. It is given as a series beginning at (2) months of age with boosters given at (4) months and between the ages of (12 to 15) months. The CDC recommends that newborns receive the monovalent HBV vaccine at birth followed by (3) additional doses of the combined Haemophilus b and HBsAg vaccine. The second combination vaccine consists of recombinant HBsAg, diphtheria, tetanus, and acellular pertussis (DTaP), and inactivated poliovirus (IPV). This vaccine has not been approved for children younger than (6) weeks of age or over the age of (7) years. The other combination vaccine includes inactivated hepatitis A virus and recombinant HBsAg (Twinrix, GlaxoSmithKline). This vaccine is recommended for individuals 18 years of age and up who are at an increased risk for HAV and HBV infections. The vaccine is also effective when given to immunocompromised patients. All vaccines come from the *adw* subtype of HBsAg and does not appear to be significantly different.

Currently, hepatitis B vaccines confer protection against both homologous and heterologous subtypes of HBV presumably by the

development of anti-*a* antibody. The development of monospecific anti-*d* in the absence of anti-*a* response after HBV vaccination has been documented which finally reappear the re-infection of patient with HBV despite HBV vaccination.

It has been demonstrated that the vaccine is not effective in patients with acute hepatitis B, and is not necessary for individuals known to have markers of current (HBsAg) or past (anti-HB) infection. However, immunization should not be delayed while awaiting any test results. Vaccines for mutants in S region are sG145R and sT126A/S, which are estimated to account for almost half of breakthrough infections. In 1990, hepatitis B vaccine was introduced in Iraq as part of the expanded program on immunization.

Life cycle of HBV

During infection, the HBV virion in the blood moves to the liver as well as found in pancreas and the kidney. The mechanism of entry into the hepatocyte is not well unknown. The HBV replication begins with the attachment of virion by its envelope to hepatocytes through surface receptors via the Pre-S1which is a part of the HBsAg. This binding depends on the interaction of the viral envelope glycoproteins with cellular receptors on the surface of the hepatocyte. The surface or envelope proteins play a vital role in the host specificity of the virus. After penetrates, the virus is transported to the nucleus where the partially circular DNA is converted to covalently close circular DNA (cccDNA) by the host cell enzymes. HBV cccDNA serves as a template for transcription of mRNAs and pregenomic RNA (pgRNA) which is then encapsidated into core proteins and reverse-transcribed. At this time the reverse transcriptase (RT) enzyme (HBV polymerase) synthesizes a new viral DNA genome. By the

way, the core particles with the newly synthesized partially-circular are finally packaged into viral envelopes in the Endoplasmic reticulum, and then transported out of the cell. There are two sources of cccDNA: entry of new virus particles or translocation of newly synthesized HBV DNA from the hepatocyte cytoplasm. The messenger RNAs (mRNA) synthesized from cccDNA are (3.5), (2.4), (2.1) and (0.9) kb lengths which are transported into the cytoplasm. The large (3.5) kb mRNA encodes HBc and HBe antigens and the HBc mRNA also codes for the polymerase and serves as template for replication of the whole genome. HBcAg and HBeAg share 90% of their protein sequence but are translated differently. HBeAg is secreted into serum and does not self-assemble like a capsid antigen. The (2.4) and (2.1) kb mRNAs encode for the surface proteins (HBs, MHBs and LHBs). The last (0.9) kb mRNA encodes for the X protein which probably is involved in transcription regulation and acts as a proteinkinase. The pgRNA is transported to the cytoplasm incorporated in the nucleocapside and retro transcripted by reverse transcriptase into minus strand L (-) DNA. When the HBV polymerase reaches the 5' end of the L (-) DNA strand, a small stretch of pgRNA is left undigested by the RNAseH. This segment of RNA is composed of a small sequence including the Direct Repeat 1 (DR1) region. The RNA oligomer is then translocated and annealed to the direct repeat 2 (DR2) region at the 5' end of the L (-) DNA. This RNA oligomer is used as a primer for the S (+) DNA synthesis. Then, the S (+) DNA is completed by the polymerase elongation mechanism using the L (-) DNA strand. It appears that the reverse transcription, as well as S (+) strand synthesis, may occur in the completed core particle. This different number of free nucleotides within the core particle may cause the variation in length of (+) DNA strands of HBV genomes in completed virions.

HBV is also known to replicate through RNA intermediates on which reverse-transcriptase enzyme acts to produce complementary DNA (c-DNA) for further replication through DNA-polymerase enzyme. Reverse transcriptase is a proofreader so the nuclear substitution during HBV replication is a spontaneous event.

Two different pathways lead to the formation and secretion of new virions after assembly of the envelop proteins. The nucleocapsids reach the ER, where they are associated with the envelope proteins and bud into the lumen of the ER, from which they are secreted via the Golgi apparatus out of the cell while, the envelope proteins have previously inserted themselves into the lipid membrane of the ER.

Additional processing goes on to form progeny virions which are released from the cell by exocytosis or returned to the nucleus and re-cycled to produce even more copies.

However, a large excess of the HBsAg is made during this replication process, it is also released from infected cells as empty spherical and filamentous particles. These HBsAg particles can be detected in serum as a diagnostic marker of active HBV replication. The number of viral particles generated in infected persons can be as high as (1011) viral particles/ml per day.

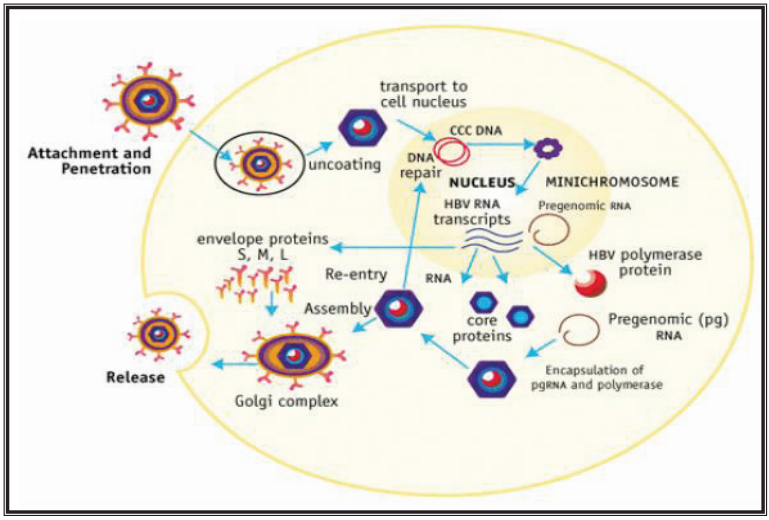

Figure (14): The life cycle of HBV.

Part Five

HBV Genotypes, Subtypes & Serotypes

HBV genotypes

Previously, HBV genotypes have been classified into eight genotypes (A-H) and because of genome diversity is a hallmark of HBV virus allowed its classification into (10) genotypes (A–J). The major classification of HBV subtype is sorted into 4 subtypes or serotypes (*adr*, *adw*, *ayr*, and *ayw*).

The clinical relevance of such genotype is yet unclear. However, because the HBV induced disease is the resultant of virus-host interaction, the disease characteristics may be influenced by the genotypes of the virus. HBV genotype and subgenotype are strong factors in predicting outcomes of chronic HBV infections.

Traditionally, HBV genotypes has been based on one of the following criteria: an intergroup divergence of 8% (similarity in 92%) or greater over the complete genome sequence, or 4±1% or greater divergence of the surface antigen HBsAg. Detection of HBV genotype is very important to clarify the pathogenesis, rout of infection and virulence of the virus. In the context of the findings described, there might be a need to further differentiate between genetic variants versus genotypes. Since the HBV genotype is due to the entire nucleotide sequence, the HBV genotype is more appropriate for investigation of geographic distribution and epidemiologic connections.

Previously, HBV genotypes have been classified into eight genotypes (A-H) and because of genome diversity is a hallmark of HBV virus allowed its classification into (10) genotypes (A–J). Genotypes A-D were identified in 1988 under the sequence divergence in the entire genome exceeding 8%, and designated by capital letters of the alphabet. Genotype E-F were identified in 1993 and genotype G was identified in 2000.

Genotype H which is phylogenetically closely related to genotype F was proposed in 2002. Genotype I has been described and isolated from Hanoi in the northern part of Vietnam , Laos, a primitive tribe from northeast India as well as in the northwest of China. Finally, the newest genotype J was identified in the Ryukyu Islands in Japan and this genotype has a close relationship with gibbon/orangutan genotypes, and human genotype C.

Zekri and coworkers found that HBV mixed genotype infections could probably be of clinical significance in HBV-induced liver diseases. He established that prevalence of mixed A/D genotype infections related to induce chronic liver diseases and evaluation of therapy.

Relationship between HBV genotypes

There are structural, functional, infectivity and clinical differences between HBV genotypes. Such differences include prognosis, progression of disease, complications as cirrhosis and hepatocellular carcinoma, as well as response to antiviral therapy. Structurally, HBV genotypes differ in the length of their genomes. The numbering of HBV genome from the *Eco*RI site leads to difficulties in comparing nucleotide positions between genotypes. Functionally, the Pre-S region that is important for virus attachment and cell entry, shows momentous differences between genotypes. Genotype A differs mainly in sequence of the Pre-S2 region, and has insertion of six nucleotides in the terminal protein portion of the polymerase gene overlapping the core gene, and shares some structural features with genotype F. Genotype D genome is most similar to Genotype E, especially in the X-gene. Differences in RNA splicing folding between genotypes could be predicted.

A recent study by Chan et al. indicated that genotype C was associated with more severe liver fibrosis than genotype B probably

because of delayed HBeAg seroconversion and prolonged active disease. The major structural differences between HBV genotypes are shown in table (1) below depending on nucleotide numbering, length and characteristic Insertion/Depletion of HBV genotypes:

Table (1): Differences between the main HBV genotypes.

Genotype	Length	Insertion/Depletion
A	3221	Insertion core: 6 bp
B	3215	
C	3215	
D	3182	Depletion Pre-S1: 33 bp
E	3212	Depletion Pre-S1: 3 bp
F	3215	
G	3248	Insertion core: 36 bp , Depletion Pre-S1: 3 bp
H	3215	

Prevalence and epidemiology of genotypes and subgenotypes

Humans are only reservoir for HBV, which is 50-100 times more infectious than HIV. The prevalence of HBV infection varies in different parts of the world, with most of the disease burden occurring in Asia and Africa.

Genotype A

Genotype A derived mainly from Europe, India, Africa, and North America. The existence of subgenotypes within genotype A has been reported (A1/Aa) from South Africa and South Asia. Subgenotype Ae/A2 is mainly endemic in Europe and United States. Ac/A3 is mostly found among populations of West and Central Africa. These subgenotypes were significantly distinguished by bootstrap at phylogenetic analyses complete genomes. The differences between European and Afro-Asian of genotype A strains that the subgenotype A1 strains encoding Asparagine (Asn-207) and Leucine (Leu-209), while the A2 strains had Serine (Ser-207) and Valine (Val-209). All strains specifying *ayw1* serotype belonged to A1, and most of them were from Africa. Genotype A is corresponding to subtype.

Genotype A and Its Subgenotypes Genotype A is characterized by a 6- nucleotide insert at the carboxyl end of the core gene. Comprehensive analysis of genotype A has led to the classification of this genotype into subgenotypes A1, A2, A4, and quasi-subgenotype A3 because the latter group of sequences does not meet the criteria for a subgenotype classification Subgenotypes A1, A4, and quasi-subgenotype A3 are found mainly in Africa, whereas A2 prevails in northern and central Europe and North America.

Genotype B

Genotype B is originated mainly from China, Japan, and Southeast Asia (Vietnam, Thailand, and Indonesia). Four subgenotypes, designated B1–B4, were confirmed by significant bootstrap when comparing complete genomes. Other classification of genotype B isolates into two

groups: Bj ("j" stands for Japan), mostly found in Japan, and Ba ("a" stands for Asia). All strains specified *adw2* serotype with the exception of the strains in B4 which specified *ayw1* or *adw3* serotype according to the strain. Subgenotype B1 was formed mainly by 18 of the 25 S genes of genotype B strains from Japan, corresponding to the described group Bj while the most genotype B strains from China belonged to subgenotype B2 which also comprised strains from Vietnam. Subgenotype B3 was formed by four strains from Indonesia. Subgenotype B4 comprised only strains from Vietnam. Apart from the Arginine (Arg-122) in B4, there were no amino acid substitutions in HBsAg characteristic of individual subgenotypes within B.

Novel Subgenotypes of genotype B

According to the novel classification using phylogenetic and sequence divergence of >4%, the subgenotypes of B have been reclassified into 6 subgenotypes: B1, B2, B4–B6, and quasi-subgenotype B3. Subgenotype B1 (previously Bj), found mainly in Japan, and B5 (previously B6) from a Canadian Inuit population represent genotype B without recombination with genotype C in the precore/core region, as opposed to the remaining subgenotypes of B that have this recombination. Subgenotype B1 was probably the ancestor of B5, possibly carried by indigenous peoples during migration from Siberia and Alaska to North America and Greenland.

Genotype C

Genotype C genome shows four subgenotypes, C1–C4 supported by 96–100% bootstrap with clear geographical clustering. The *ayr* subtype is widespread in genotype C. Australian strains specified *ayw3*. In the subgenotypes C1–C3, there were an intermixture within the *adr* strains of

strains specifying *adw2* or *ayr*. The constraints against substitutions of subtype specifying residues122 and 160 thus seem less pronounced for genotype C than for the other genotypes. C1 was formed by the majority of the strains from the Far East (Japan, Korea, and China). Sakamoto found a novel subgenotypes of HBV/C5 and HBV/B5 among chronic liver disease patients in the Philippines.

Novel Subgenotypes of genotype C

According to Paraskevis *et al*., genotype C is the oldest HBV genotype. It has the highest number of subgenotypes, C1–C16, reflecting the long duration of its endemicity in humans. A large number of subgenotypes circulate in Indonesia. Subgenotype C4 is exclusively found in indigenous people of northern Australia, who are descended from a founder group that emigrated from Africa at least 50,000 years ago.

Genotype D

Genotype D is the most widespread genotype and predominated in the Mediterranean area, the Near East, and as far as India. It was also found in Aboriginal populations in Asia from Indonesia and Papua. The strains specified *ayw2*, *ayw3*, or *ayw4* serotype with the exception of two European strain specifying Lysine (Lys-122), Theronine (Thr-127), and Lysine (Lys-160) corresponding to the putative subtype *adw3*. Phylogenetic analysis of complete genomes have been distinguishing four subgenotypes D1–D4. Strains specifying *ayw2* were found in D1, D3, and D4. The geographical distribution of the subgenotypes within D was less restricted than that of genotypes A, B, and C. Although, the strains from Middle East mainly belonged to D1 those from South Africa and Alaska mainly to D3, while those from Oceania and Somalia were only found in D4. Genotype D is currently segregated into eight subgenotypes (D1–D8).

A novel D9 isolates do not possess any unique motif in the Pre-S/S ORF that can distinguish them from the other eight subgenotypes of D. D9 subgenotype is originated from discrete recombination events between genotypes D and C as evident from the fact that both genotype C and D9 sequences are monophyletic in the core region. Genotype D is characterized by a 33 nucleotide deletion at the N-terminus of the Pre-S1 region, therefore it has the shortest genome of the eight HBV genotypes (3182 nucleotides).

Novel information of genotype D

In a recent systematic and comparative analysis of the subgenotypes of D, it was concluded that there are 6, not 8, subgenotypes. Subgenotypes D1–D6 can be differentiated by distinct clustering with high bootstrap support and signature amino acids. Subgenotypes D3 and 'D6' were reclassified as a single subgenotype D3, and 'D8' was shown to be a genotype D/E recombinant rather than a subgenotype. Subgenotype D4, which is found in aboriginal populations in Papua New Guinea and Australia and in a small percentage of the Canadian Inuit population, may be an early subgenotype dating from the time of early human intercontinental migrations. Moreover, a recombinant of subgenotype D4 has been identified in Sudan. It is possible that D4 originated in Africa but has subsequently been replaced by other subgenotypes of D and the recombinant is a remnant of the original strain/s. Subgenotype D4 carriers were significantly older than both D3 and B6 carriers. Although it has been suggested that subgenotype D5 is the most ancient of the subgenotypes of D, this conclusion is neither supported by the relatively low intragroup divergenceof this subgenotype, nor by the dated HBV phylogeny of the subgenotypes of D. Additional sequences of subgenotype D5 from

geographical regions besides the Paharia tribe of India may resolve these discrepancies.

Genotype E

Genotype E is definitely the dominant genotype in West Africa and has very low intra-genotypic diversity suggesting that this genotype has spread only recently. Genotype E strains specified of subtype *ayw4,* and all derived from West Africa apart from one strain which was derived from Madagascar. There were no subdivisions or specific amino acid substitutions distinguishing the strains from each other. All strains expressed Sereine (Ser-140) also present in the genotype F. Study analysis of the complete genome of genotype E strains showed that the chimpanzee strain was not ancestral as compared with the human strains. This chimpanzee has probably also been inoculated with human serum at capture, since the majority of indigenous HBV strains from chimpanzees cluster separately from human strains.

Genotype E has the unique serological subtype *ayw* 4 and can be differentiated from genotypes A–D, F, H, and I by a 3-nucleotide deletion in the preS1 region. This genotype is endemic in western, central Africa, with a low genetic diversity, intimating a recent emergence of 200 years or less. As opposed to subgenotype A1, which was dispersed by the slave trade, genotype E is rarely found outside Africa, except in individuals of African descent, further supporting its recent emergence after the forced migrations of slavery. Using Bayesian inference, a median time of evolution from a most recent common ancestor (tMRCA) of 130 years has been calculated. This differs from a tMRCA of 6,000 years estimated by others. However, as suggested previously, it is possible that genotype E existed in indigenous African populations and has recently been

reintroduced. Genotype E has been isolated from Pygmies and the Khoi San and in Colombia and India in individuals with no history of travel to or from Africa. Without an accurate determination of the nucleotide substitution rate of HBV, the variance of the estimated age of genotype E will be difficult to resolve.

Genotype F

Genotype F has been isolated from Amerindian population in different countries. Genotype F strains are subdivided into four subgenotypes. Subgenotype F1 particular F1a have been found in Alaska, El -Salvador, Guatemala, Costa Rica and Nicaragua; whereas F1b has been reported in Peru and Argentina. Strains of subgenotype F2 has been found in Costa Rica, Nicaragua, Venezuela and Brazil. Subgenoype F3 is found in Colombia and Venezuela and F4 in Bolivia and Argentina. F1 and F2, each characterized by specific substitutions in the S gene product, Leucine (Leu-44) and Theronine (Thr-45), respectively. Subgenotype F1 was mainly formed by strains from Central America. F2 mainly containing strains from South America encompassed all strains from Venezuela and Colombia and the few strains from Polynesia and were characterized by an Aspartic acid (Asp-2) Glutamic acid (Glu) substitution. Subtype *adw4q–* is alongside with *adrq–* the dominating subtypes in Polynesia. This supports a dual origin of its population, and the close relation of the Polynesian strain to strains from Colombia. Most of genotype F strains specified *adw4* subtype. All had the Proline (Pro-178) Glutamine (Gln) substitution assumed to abolish the expression of *q*. Some strains lacked the Proline (Pro-127) Leucine (Leu) substitution characteristic of genotype F and specified the putative subtype *adw2q*. All genotype F isolates belong to serological subtype *adw* 4 and cluster into 4 subgenotypes, F1–F4. This

genotype is found in the Amerindian populations of Central and South America, as well as Alaska.

Genotype G

Genotype G is mostly detected in co-infection with other HBV genotypes with mostly genotype A. Genotype G strains are originating from the USA, Mexico, and Europe which are all specifying *adw2* subtype. The genotype G strains shared two unique substitutions, Glutamine (Gln-51) Leucine (Leu) and Threonine (Thr-63) Isoleucine (Ile), were not found in any other genotype. The S gene products of the strains showed the highest similarity to those of genotype A. However, these strains showed a high divergence from the other HBV strains, when complete genomes were compared. Genotype G strains have a 36 bp insertion immediately after the initiation codon of the C gene, increasing the size of HBcAg by (12 aa). This does not effect on Polymerase but a one codon deletion in the Pre-S1 region reduces both Pre-S1 and Pol by one amino acid.

Genotype G is characterized by a 36-nucleotide insert, 3′ of position 1905, and two translational stop codons at positions 2 and 28 of the precore/core region, abrogating HBeAg expression. This genotype can establish chronic infection only in the presence of other genotypes, most frequently genotype A, that can supply HBeAg in *trans*. A major risk factor is sexual transmission by men who have sex with men. Genotype G is least divergent from genotype E, with which it shares the 3-nucleotide deletion in the core region and a unique sequence in the preS. Although not yet detected in Africa, an African origin of genotype G has been postulated.

Genotype H

All strains belonging to the genotype H derived from Nicaragua, Mexico, and California. These strains differ from genotype F strains by

two unique substitutions, Valine (Val-l44) and Proline (Pro-45), as well as Isoleucine (Ile-57), Threonine (Thr-140), Phenylalanine (Phe-158), and Alanine (Ala-224). Genotype H prevails in Mexico in both the indigenous populations and mestizos (mixed descent), suggesting this genotype has a long history among the descendants of the Aztecs, before the arrival of Europeans. It is most closely related to genotype F.

Genotype I and Its Subgenotypes

In 2008, sequence analysis of the complete genome of a single isolate (AB231908) from a Vietnamese male found it to be closely related to 3 previously described 'aberrant' Vietnamese strains and a 9th genotype, I, was proposed. This proposal was not accepted because the mean genetic divergence of these 4 strains from genotype C was 7% and the recombination analysis was not robust. Subsequently, sequences derived from Laos, the Idu Mishmi tribe in northeast India, a Canadian of Vietnamese descent, and China have expanded the number of sequences. The nucleotide divergence of most of these sequences relative to genotype C was at least 7.5%, with good bootstrap support for the group, thus meeting the criteria for genotype assignment. Two subgenotypes, I1 and I2, with serological subtypes *adw* 2 and *ayw* 2, respectively, were described. This separation into subgenotypes was questioned when additional strains from India clustering within subgenotype I2 were sequenced and the intersubgenotype divergence was calculated to be <4%. By analyzing all 19 complete genotype I genomes, without indels, available in the GenBank, the intergroup divergence between subgenotype I1 and I2 was found to be 3.40 ± 0.30% (mean ± SD), below the 4% cutoff. However, as for subgenotype D1 and D2, an exception can be made because of the different serological subtypes. The highest intergroup divergence of 4.1% was between the Laotian strain FJ023663 and the

Indian strain EU835242. The wide geographical distribution suggests this genotype has been endemic in a wide area of Asia for a long time. Genotype I is a recombinant of genotypes A/C/G and an indeterminate genotype, which clusters close to genotype C when the complete genome is analyzed, and with genotype A in the polymerase. The genotype A and C regions are closely related to subgenotypes A3 and C3, respectively. Genotype I has been functionally characterized in both Huh7 cells and by acute hydrodynamic infection of a mouse. In both systems, genotype I secreted HBsAg at levels comparable to genotypes A, B, and C, and higher than D, but HBeAg at similar levels to genotype A but lower than B, C, and D.

Genotype J

This strain was isolated from a single Japanese man with hepatocellular carcinoma (HCC), who had lived in Borneo for a prolonged period of time. The complete genome clusters with nonhuman HBV, including isolates from gibbons, orangutans, chimpanzees, and gorillas. When compared with 1,440 human and nonhuman HBV strains, its sequence diverged by 10.7–15.7% from other genotypes and did not show any evidence of recombination. In a later analysis, using additional gibbon/ orangutan HBV sequences for comparison, Locarnini *et al*. concluded that genotype 'J' is in fact a recombinant of genotype C and gibbon HBV in the S region. Thus, although the high intergroup divergence of genotype 'J' meets the criterion for classification into a separate genotype, it may represent a cross-species transmission, and identification and analysis of additional sequences will be required before the existence of this 10th genotype can be confirmed.

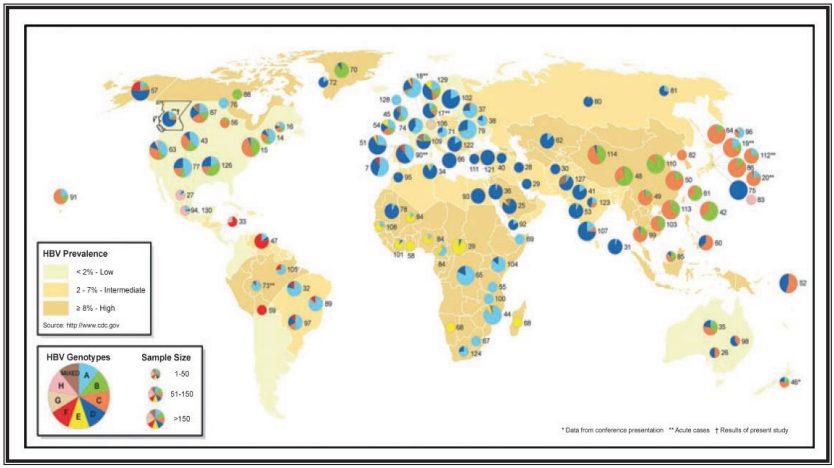

Figure (15): Global geographical distribution of HBV genotypes.

Some studies have shown that different HBV genotypes and subgenotypes may cause differences in disease progression, response to antiviral treatment regimens and in clinical outcomes. Therefore, the accurate classification of HBV is important for clinical and etiological investigations.

HBV Serotypes

The major classification of HBV subtype is sorted into 4 subtypes or serotypes (*adr*, *adw*, *ayr*, and *ayw*). The molecular basis for this classification was variation at few sites in the S region. The '*a'* determinant (aa 124-148) is the major antigenic determinant common and confers protection against all serotypes, while the *d/y* and *w/r* variations depend on Lys/Arg substitutions at residue (122) and (160) respectively. If the amino acid at position (122) is Arg (122R) then the subtype is *y*, and if it is Lys (122K) then the subtype is *d*. In the same way, (160R) defines the *r* subtype and (160K) defines the *w* subtype. The four possible combinations define the major subtypes and additional amino acids contribute to

immunogenicity. These subtypes can be further classified into (9) serotypes (adw2*, adw4q-, adrq+, adrq-, ayw1, ayw2, ayw3, ayw4* and *ayr*). Epidemiologic studies found that the prevalence of these serotypes varies in different parts of the world. To date, there has been very little data on the clinical significance of HBV serotypes. While the ability to detect HBsAg was of obvious importance for the safety of the blood supply, serotyping was useful for widely employed in clinical, virological, epidemiological studies, including studies of nosocomial and iatrogenic infections and intra-familial transmission.

Determinants *w1/w2*, *w3* and *w4* are classified by Pro, Thr or Leu substitutions at residue (127) respectively. *w1* variation is distinguished by Arg122, Phe134 and/or Ala159. It has been found that the epitope in *adw* contains (18Val-19Pro), whereas these amino acids are replaced by hydrophilic residues Thr-Ser in the *ayw1*, *2*, and *3* subtypes. As a consequence of these substitutions, the conformation of the epitopes, as predicted by 3D modelling and analysis of crystal structures, was drastically changed. Cui and coworkers found that the serotype *adw* is based on Lys (120) and Lys (160). To a large extent, genotypes and subgenotypes have replaced the usage of serotypes. Most *ayw* serotypes are grouped in genotypes B and D. The serotype *ayw* occurs in all genotypes except in 'C'. Serotype *adw* is associated with all genotypes except D and E, whereas *adr* and *ayr* subtypes are encountered with genotype C. There is no stringent correlation between phenotypic HBsAg markers and sequence variation outside the S gene but such a correlation between genetic and phenotypic markers is required for epidemiological studies.

Part Six

Laboratory Diagnosis

Overview of diagnostic methods

There are several assays used to detect genotype, subgenotype, and serotype of HBV such as ELISA for detecting HBsAg which is the common assay used in diagnostic laboratory. This assay is also used to determine HBV subtype. PCR, DNA sequencing and other commercial technique are used to detect HBV genotype.

Diagnostic tests can be grouped into 3 classes: direct detection, indirect examination (virus isolation), and serological tests. In general, the majority of common viral infections can be diagnosed by serology. The specimen used for direction detection and virus isolation is very important. A positive result from the site of disease would be of much greater diagnostic significance than those from other sites.

Direct Examination of Specimen

The clinical specimen is examined directly for the presence of virus particles, virus antigen or viral nucleic acids such called rapid diagnostic methods because they can usually give a result either within the same or the next day. This is extremely useful in cases when the clinical management of the patient depends greatly on the rapid availability of laboratory results. It is important to know that not all direct examination methods are rapid and conversely, virus isolation and serological methods may sometimes give a rapid result. Direct examination includes:

1. Electron Microscopy morphology / immune electron microscopy
2. Light microscopy histological appearance (e.g. inclusion bodies).
3. Antigen detection immunofluorescence, ELISA etc.
4. Molecular techniques for the direct detection of viral genomes.

Viral Genome Detection

Methods based on the detection of viral genome are also commonly known as molecular methods. Although, using these methods are indeed increasing, the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods. Classical molecular techniques such as dot-blot and Southern-blot depend on the use of specific DNA/RNA probes for hybridization. The specificity of the reaction depends on the conditions used for hybridization which may allow for the quantification of DNA/RNA present in the specimen. It is often found that the sensitivity of these techniques is not better than conventional viral diagnostic methods.

Newer molecular techniques such as the polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid based amplification (NASBA) and branched DNA (bDNA) depend on some form of amplification, either the target nucleic acid or the signal itself. PCR is an extremely sensitive technique which is possible to achieve a sensitivity of down to (1) DNA molecule in a clinical specimen.

Indirect Examination

The specimen into cell culture, eggs or animals in an attempt to grow the virus: this is called virus isolation and includes:

1. Cell Culture and show the conformational changes of the cells which includes cytopathic effect, haemadsorption, and confirmation by neutralization, interference, immunofluorescence etc.
2. Eggs pocks on CAM - haemagglutination, inclusion bodies.
3. Animal's disease or death confirmation by neutralization.

Serological methods

Serological diagnosis can be made by the detection of rising titers of antibody between acute and convalescent stages of infection, or the detection of IgM in primary infection. Serological tests divide into two types: Classical techniques which include Complement fixation test, Haemagglutination Inhibition test, Immunofluorescence technique, Neutralization test, and Single Radial Haemolysis. Newer techniques which include: Radioimmunoassay (RIA), Enzyme Linked immunosorbent assay (ELISA), Particle Agglutination, and Western Blot (WB), Recombinant Immunoblot assay (RIBA) and Line immune assay (Liatek). Recently, ELISA is the main assay used for detecting HBV antigens or antibodies and other HBV markers.

Biochemical Assays

The biochemical assessment of liver function include: total and direct bilirubin (TSB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, globulin and coagulation profile.

Detection of Alanine aminotransferase (ALT)

ALT enzyme (previously named SGPT) is an enzyme produced by hepatocytes. The level of ALT in the blood increases when liver cells are damaged or die. The elevation of ALT due to taking drugs, alcohol use, some over-the-counter painkillers, toxins, viruses, and other substances can also cause ALT levels to rise. ALT is the main biochemical marker used in viral hepatitis in the serum. ALT level is used as a surrogate marker for necroinflammation of the liver. Some HBV infected adults and children with normal ALT levels have been known to develop liver damage. An elevated ALT is also associated with better serological response to antiviral

treatment. Some data have been showed that between 12% and 43% of patients with chronic HBV and normal ALT levels have significant hepatic fibrosis. If there is a significantly higher ALT level for more than 6 months, then a liver biopsy would be indicated. The normal ALT levels are commonly reported as 0 to 40 IU/L and up to 40 U/L is generally considered as healthy for men and about 30 U/L is healthy for women ,while repeated ALT levels of (80) to (100 U/L) may considered as normal for someone who consistently tests within this range.

Detection of Aspartate aminotransferase (AST)

AST previously called SGOT, is an enzyme similar to ALT but is less specific for liver disease. In many cases of liver inflammation, the ALT and AST levels are elevated. Normal ranges for AST are (0) to (37 IU/L). The exact levels of AST and ALT cannot be used to determine the degree of liver disease or predict the future prognosis for liver function but they are only used to detect inflammation due to injury or damage to the liver from any source. Even in conditions when AST and ALT are very elevated, the liver still may function properly.

Detection of Bilirubin

Bilirubin is the major breakdown product of old red blood cells. Hemoglobin is released from the red blood cells then the “heme” portion is further broken down to bilirubin. When liver function is impaired, as with acute hepatitis or end stage liver disease, bilirubin accumulates in the blood and causes yellowing of the skin and eyes and this is called jaundice. The bilirubin levels in hepatitis B are usually normal until the significant amount of liver damage has occurred. Bilirubin is often reported as total, indirect the amount of “unconjugated” bilirubin or free bilirubin that has not been attached to a glucuronide molecule. Direct (the amount of

"conjugated" bilirubin or bilirubin that has been chemically attached to a glucuronide in the liver and then excreted from liver cells into the bile and stored in the gallbladder or transferred to the duodenum). The normal range of total bilirubin is (0.1 to 1.2 mg/dL).

Molecular Diagnosis

Previously, several assays were used to determine the HBV-DNA level in serum and performed using non-amplified hybridization, such as (Dot blot hybridization, Liquid hybridization, North blot and branched DNA assays). These assays have a limit of quantification of (10^5-10^6copies / ml) and should no longer be used for routine management of patients with CHB infection.

Detection of surface antigen HBsAg

Hepatitis B surface antigen HBsAg is an antigen of three proteins that makes up the envelope of the HBV virion. It is secreted as lipoprotein particles in excess of virions by a ratio of greater than (1000:1). In addition, the ability of HBsAg assays to detect HBV variants bearing nucleotide substitutions in the S gene leading to modifications of the 3D structure of HBsAg. It is recommended that the first detection of HBsAg be confirmed by neutralization in order to eliminate a false-positive result. HBsAg may not be detectable during chronic hepatitis B: in the low replication asymptomatic HBV carriers, in the case of HBV variants bearing nucleotide substitutions in the S gene leading to the synthesis of an HBsAg that is not recognized by commercial assays. Although, HBsAg can be detected before six months, infrequently, in ≤1–5% of patients with acute HBV infection, the levels of HBsAg are too low to be detected. Chronic hepatitis B may be inactive and cause no significant health problems, but

may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma when undetectable of HBsAg.

ELISA is a common assay where used to detect HBsAg. The enzymes are linked to antibodies such that the complexes have both immunological and enzymatic activities. The enzymes degrade chromogenic or fluorogenic substrates, yielding accurate and sensitive detection of the presence of enzyme. First, a polyclonal or monoclonal antibody is adsorbed to the surface of a plate. Then, a solution containing the antigen of interest is added, followed by a series of washing and blocking steps.

The Vidas HBsAg an ultra assay is performed on a Vidas instrument which is an automated immunoassay system. After a preliminary washing step, the HBsAg present in the sample will bind simultaneously to the monoclonal antibodies (two) coating the interior of the solid-phase receptacle and to a polyclonal antibody conjugated with biotin and the unbound sample components are washed away.

Immunochromatographic assay ICA: A rapid diagnostic test is widely used in most of developing countries. This method is considerably cheaper than EIA methods less time consuming when compared to EIA but till now , it is unknown if this method accurate enough to detect acute or chronic HBV infections.

Elecsys HBsAg is a two-step sandwich assay for the qualitative detection of HBsAg in human serum or plasma. All the reaction steps of Elecsys HBsAg are performed automatically from the patient by the Elecsys 2010 system. This system has better sensitivity than those of well-established HBsAg screening assays. Furthermore, Elecsys HBsAg

combines a high sensitivity (100%) and specificity (100%), whereas usually these criteria are mutually exclusive.

Detection antibody of surface antigen (anti-HBs)

This protective antibody develops with the resolution of acute infection or following the successful vaccination against HBV. Very occasionally, anti-HBs and HBsAg can be found together with unknown clinical significance. Anti-HBs becomes detectable in serum after HBsAg disappears and remains detectable indefinitely thereafter. The association between the appearance of anti-HBs and resolution of HBV infection as well as the observation that persons with anti-HBs in serum are protected against reinfection with HBV suggests that anti-HBs is the protective antibody, therefore, strategies for prevention of HBV infection are based on providing susceptible persons with circulating anti-HBs. The anti-HBs titer often varies over time, and these antibodies may become undetectable several years after the acute episode. The assessment of anti-HBs antibody levels by different assays is not accurate and consistent, yielding incomparable quantitative results in spite of the calibration with international standards. After vaccination against HBV, the titer of anti-HBs must be present at more than 10 IU/ml to confer efficient protection.

Detection of core antigen (HBcAg)

In the serum, HBcAg is an intracellular and is sequestered within an HBsAg coat. The naked core particles do not circulate in serum, therefore, HBcAg is not detectable routinely in the serum of patients with HBV infection. HBcAg is produced in the cytosol of hepatocyte during HBV replication, surrounding the viral genome and the associated polymerase and then packaged within the envelope before secretion from the hepatocyte. HBcAg particles remain in the hepatocyte, where they are

readily detectable by immunohistochemical staining, and are exported after encapsidation by an envelope of HBsAg.

Detection antibody of core antigen (anti-HBc)

This antibody is a peptide of the core protein, which has been processed within an antigen presenting cell. Anti-HBc immunoglobulin M (IgM) predominates during the first (6) months after acute infection, and found in a high concentrations which gradually decline, complementing the corresponding increase of anti-HBc IgG over the three to six month period. Elevation of anti-HBc IgM usually signifies acute infection, but low elevations may also occur during the reactivation of chronic HBV. Anti-HBc IgG remains positive for life following exposure to HBV. Most serological assays do not directly measure anti- HBc IgG, but test for total anti-HBc antibody. Anti-HBc is readily demonstrable in serum, beginning within the first (1–2) weeks after the appearance of HBsAg and preceding detectable levels of anti-HBs by weeks to months. False-negative detection of anti-HBc antibodies may rarely occur in immunosuppressed patients. Anti-HBc antibodies may be the only marker present in chronically infected patients. There are several significant differences in the genetic variability of HBV between chronic carriers with anti-HBc as the only serological marker and HBsAg-positives. These differences affect functionally important regions of both the HBsAg and the polymerase.

Detection of early antigen (HBeAg)

HBeAg is a nonstructural an accessory, soluble, non-particulate, nucleocapsid protein, which is from intact the precore region of the HBV genome. This protein is produced during active viral replication and may act as an immunogen or a tolerogen, leading to persistent infection. HBeAg, appears concurrently with or shortly after HBsAg. Its' appearance

coincides temporally with high levels of virus replication and reflects the presence of circulating intact virions and detectable HBV DNA. The loss of HBeAg and the development of anti-HBe is termed as HBeAg seroconversion. It has been used as an end-point for treatment of HBeAg-positive people. It has been shown that HBeAg seroconversion is associated with a lower risk of disease progression. Persistence of HBeAg in serum beyond the first (3) months of acute infection may be predictive of the development of chronic infection. HBeAg can be detected on average (6 to 12) weeks after exposure. It is followed by the appearance of anti-HBe antibodies during the HBe seroconversion phase. Despite, persistence of HBeAg is generally observed in the patients who develop chronic infection, HBeAg-negative variants are generally selected during the immune clearance of chronic HBV infection. An important event in the natural history of chronic hepatitis B is the loss of HBeAg and seroconversion to anti-HBe. This HBeAg seroconversion is usually followed by normalization of serum transaminases and improvement of liver histology. Thus, HBeAg seroconversion usually represents a transition from chronic hepatitis B to an inactive HBsAg carrier state.

Detection antibody of e antigen (anti-HBe)

Anti-HBe is not a protective antibody. It appears usually coincides with a significant immune change associated with lower HBV DNA replication $<10^5$ copies/ml or 20,000 IU/ml.

Detection of HBV DNA

The HBV DNA level and aminotransferase activity provide valuable prognostic information. During early chronic HBV infection, HBV DNA can be detected both in serum and in hepatocyte nuclei. It has become possible to directly quantify the level of HBV replication with the advent

of molecular amplification technology, such as the polymerase chain reaction (PCR). PCR target amplification assays involve a process of lysing the virion and purifying the DNA, which is then amplified and quantified HBV DNA from serum. Currently, the unit of measurement HBV DNA was copies/ml, which is now being standardized to international units (IU)/ml, using a conversion factor of between (5–6) genome copies/ml=1 IU/ml which depends on the type assay. For example, the level of (20,000 IU/mL) (around 10^5 copies/ml) has been arbitrarily selected as the level below which there is a relatively low likelihood of hepatic damage. The level of HBV DNA is a dynamic parameter in chronic HBV, while the level of circulating HBV has been shown to be the strongest predictor of the development of cirrhosis and hepatocellular carcinoma (HCC). HBV DNA detection and quantification is useful in clinical practice to diagnose chronic HBV replication, to establish the prognosis of liver disease and assess the risk of progression towards cirrhosis and hepatocellular carcinoma, to identify patients who need antiviral therapy and offer them the most adapted treatment, to monitor the virological response to therapy and identify amino acid substitutions responsible for resistance to nucleotide analogues.

HBV DNA level is unstable over time and this depends on the infection phase such the immunotolerance phase is characterized by high levels of viral replication; the immuno-elimination phase is characterized by generally lower. Some reports estimated that who were positive for HBsAg, were tested at baseline for viral load and followed for (11) years showed that the relative risk of mortality associated with a high viral load (HBV DNA $\geq 10^5$ copies/ml) while low viral load (HBV DNA $< 10^5$ copies/ml) had no significant association with mortality. The detection of HBV DNA in peripheral blood (plasma or serum) is also important to

establish for the diagnosis of occult HBV in peripheral blood or liver in the absence of HBsAg.

The presence of HBV-DNA in serum or plasma denotes active HBV infection and, depending on the viral load, correlates with the infectivity of the patient.

Real-time quantitative PCR assay is the major assay used to detect HBV DNA for all genotypes in clinical laboratories with specific advantages including, the good clinical sensitivity, excellent clinical specificity (100%), broad linear dynamic range (56 to $5.0X10^8$ IU/ml) and equal detection quantification of HBV genotypes A to G b**y** using semi-nested and one-round PCR can be used to detect HBV DNA from oral fluid samples suggesting that this combination is the best choice for HBV DNA detection.

Real-time detection PCR (RTD-PCR) based on Taq Man chemistry is an efficacy assay was evaluated by quantitatively measuring sequential levels of synthetic DNA and DNA in clinical serum samples. The detection limit of this system was as few as 10 DNA copies/reaction. TaqMan HBV is a sensitivity assay to improve the clinician's ability to reliably detect the presence of HBV DNA in serum and plasma and monitor changes in HBV viral load in patients particularly those with very low HBV DNA titers.

Hybridization Methods: When HBV-DNA is hybridized with a HBV RNA probe. The DNA-RNA hybrids are immobilized onto a microtiter plate using anti-DNA-RNA antibodies. Antihybrid antibodies conjugated to an enzyme are used for detection in combination with appropriate chemiluminescent substrates. Hybrid Capture Technology and the Branched DNA Assay For the branched DNA (bDNA) assay, HBV-specific probes are used to capture HBV DNA in the sample on the

microtiter plates. Subsequently, extender probes, bDNA and dioxetane substrate, are used for detection and the bDNA molecules serve as signal amplification molecules.

Recently, DNA detection has been done in the liver tissue in patients with chronic hepatitis. HBV DNA was found in the liver tissue of some patients with negative viremia and positive HBsAg which so-called "hepatitis B *in situ*" It has been approximated that the HBV DNA measurements in serum, saliva and urine, had non-linear correlation between the levels of HBV DNA in serum, saliva or urine while the CDC report states that saliva is not to be considered potentially infectious unless it contains blood.

COBAS AMPLICOR Monitor test (COBAS-AM assay), with special emphasis on anti-HBVe antigen (HBeAg)-positive patients with low HBV DNA levels. The COBAS-AM assay was much more sensitive than the Digene HC II assay and was suitable for the determination of low levels of HBV viremia.

Piezoelectric is a magnetic particles methods for DNA detection. piezoelectricity means 'pressure-driven electricity' which uses the piezoelectric effect to measure acceleration, pressure, strain, or force by generating electrical charge.

Table (2): Serological profiles of patients with HBV infection.

Serological Tests	HBV Immunisation	Acute HBV	HBV Recovery	Chronic HBV	Inactive or asymptomatic carrier	Occult HBV
Anti-HBs	+	-	+	-	-	- / +
Anti-HBc	-	+	+	+	+	- / +
Anti-HBe	-	-	+	-	+	- / +
HBsAg	-	+	-	+	+	-
HBeAg	-	+	-	+	-	- / +
HBV DNA	-	+	-	+, $>10^5$ copies	+, $<10^5$ copies	+, $<10^3$ copies

Detect HBV genotype

Detection of HBV genotype is very important to clarify the pathogenesis, rout of infection and virulence of the virus. The HBV genotypes are variable that could potentially influence the outcome of chronic HBV and the success of antiviral therapy. HBV genotype testing has not yet been widely adopted in clinical laboratories. A variety of methods have been used, including whole or partial genome sequencing, PCR based restriction fragment length polymorphism (RFLP), genotype-specific PCR amplification, PCR plus hybridization, line probe assay, enzyme-linked immunoassay and serology. Whole-genome sequencing is the "gold standard," and it is particularly accurate for detecting recombinant viruses. The common assays are:

INNO-LiPA: This reverse hybridization method has been developed by Innogenetics and is commercially available as INNO-LiPA. This method is easy to perform, very convenient, rapid, and suitable for detecting mixed

genotype infections. First, HBV DNA is amplified by PCR using biotinylated primers complementary to a conserved sequence in the S/Pre-S ORF. The amplified biotinylated PCR products are then hybridized to probes immobilized onto membrane strips that detect genotype specific differences in the HBV polymerase gene domains B to C. After washing, alkaline phosphatase (ALP)-labeled streptavidin is added, followed by substrate (BCIP/NBT chromogen) that gives a purple/brown precipitate in the presence of ALP. The overall success rate is 98%. These methods may fail to type all isolates and interpretation of results may be difficult particularly in the case of mixed genotype infections. In addition, this assay is not suitable for large-scale surveys nor accurate to identify mixed infection.

HBV DNA-Chip assay: The whole HBV genome is amplified by a duplex PCR. The labeled PCR products were purified using a purification kit. Samples were hybridized on the HBV DNA-Chip prototype and stained with streptavidin-phycoerythrin conjugate on a GeneChip fluidics station 400. Finally, the HBV DNA-Chips were scanned on an HP Gene Array scanner and were analyzed by using DNA-Chip evaluation software. DNA-Chip technology is currently not used routinely in a clinical laboratory.

Nested-Multiplex qPCR: A detection assay is used specific primers. This assay is greater accuracy in genotyping and greater sensitivity to identify mixed genotypes when compared to sequencing reactions. This method can be useful with large clinical scale and epidemiological studies, especially in regions with high prevalence of HBV infection.

Oligonucleotide microarray: Can determine genotypes A-G. The amplified products are heat-denatured and added to silylated slides, to which genotype-specific probes are immobilized.

Enzyme Immunoassay (EIA): This commercially assay is used with monoclonal antibodies raised against genotype specific epitopes in the Pre-S2. Although this assay may fail to type the HBV DNA in clinical samples due to the presence of mixtures of genotypes or low levels of HBsAg in the sample, it offers a convenient, serologically based assay. EIAs were less sensitive than rapid assays. ELISAs for HBsAg are generally considered more sensitive. It has been showed that the samples with low HBsAg/HBcAg ratios were much more likely to have undetectable Pre-S2 epitopes by the genotyping ELISA that used.

TaqMan-MGB probe: This assay has several advantages. On the one hand, conjugated MGB can improve the melting temperature of probe, thus increasing probe specificity. In addition, it permits shorter probes to be used (usually 13 to 18 nt). On the other hand, shorter probes make fluorescence and quencher closer A type-specific nested PCR assay established and applied for investigation of HBV genotype. The TaqMan technique is suitable for typing.

Line probe assay: This assay is detected sequence specific oligomers for each genotype are immortalized on a paper strip, to which PCR amplified test samples are hybridized (reverse hybridization).

Limitations of using in-house assays

Many of limitations emerge when using in-house assays depending on the type of assay. It has often been suggested that in-house PCR assays suffer from problems with standardization, false positivity, or contamination, making them unsuitable for routine clinical diagnostic use. The lack of an internal control does not allow to rule out false-negative results due to the presence of inhibitors to PCR amplification. The limit of detection and the upper limit of the dynamic range are approximates, as a

lot more replicates and lot-to-lot testing would be necessary to verify these values.

One disadvantage of ELISA is that not all antibodies can be used monoclonal antibodies must be qualified as matched pairs, meaning they must recognize separate epitopes on the antigen so they do not hinder each other's binding. Also, there is a limit to its sensitivity since the amplification is restricted by the amount of enzyme that can be conjugated to antibodies. Immunoreactivity of the antibody may be reduced by enzyme labeling, which in itself is an expensive and time-consuming process.

HBV genotyping based on complete genome sequences is an ideal methods, but sequencing is still expensive and not easy to carry out for large scale study. The developed precise PCR genotyping system using type-specific primers, allowing the identification of types A through F. This assay system may be useful for rapid and sensitive genotyping of the HBV genome either epidemiological, pathological, transmission studies and can be carried out in large scale. Mixed-genotype infection is very difficult to detect by direct sequencing. Since direct sequencing or Sanger sequencing can pick up mixed populations only at ratios above 20:80 simultaneous.

PCR-RFLP

RFLP depends usually on PCR amplification of the S gene, restriction enzyme digestion, and separation of digested fragments by electrophoresis. A combination of different restriction enzymes has been used for RFLP, the choice of which has been determined according to the different HBV genotype sequences in GenBank. This method has been used to determine genotypes A–F. In 2004, Zeng *et al*. developed a

modified RFLP technique based on the S gene allowing the detection of HBV genotypes A–H. In this method, two PCR rounds were undertaken prior to restriction enzyme digestion by five enzymes, namely *Sty*I, *Bsr*I, *Dpn*I, *Hpa*II, and *Eae*I. The method was compared with another RFLP method targeting the Pre-S1 region and the results were concordant in 96.8%.

Nested PCR-RFLP method for HBV genotyping is simple and inexpensive for clinical diagnostic in large scale. PCR-RFLP assay is more sensitive to identifying HBV viral populations. This method can detect mixed genotypes and can determine subgenotypes in large population studies. Toan *et al*. used the restriction enzyme *Tsp*509I to restrict patterns and predicted fragment sizes determined HBV genotypes, while Zeng *et al*. used five restriction enzymes, *Sty*I, *Bsr*I, *Dpn*I, *Hpa*II and *Eae*I were deemed to be suitable for yielding restriction patterns. These enzymes restrict at Per-S region (other study used *EcoR*1. This novel method would identify several relative advantages. Firstly, it can identify all eight HBV genotypes. Secondly, it is more accurate because it was based on analyzing many of the sequences deposited in GeneBank. Thirdly, a simple and inexpensive strategy can be adopted according to the most prevalent HBV genotypes in a particular geographical region. Moreover, this method can be useful in evaluating clinical, epidemiological and virological differences between genotypes. Venegas used restriction enzymes *Sau*3A I, *Bsr* I or *Hpa* II to cut the DNA at S region. Vivekanandan and coworkers were used *Hinf*I and *Tsp* 509I restriction enzymes but he found that genotypes could not be assigned for a small proportion of strains and this may be due to the presence of infection with multiple genotypes or with strains that have altered and or additional recognition sites for the restriction enzymes used in testing. Neisi *et al*. used *Ava*II and *mbo*I

restriction enzymes but he resulted that the RFLP method cannot type mixed genotype infections. Other study used *Ava*II and *Dpn*II.

Badar used five restriction enzymes *Alw*I, *Ear*I, *Hph*I, *Nci*I and *Nla*IV. He explained that the genotyping system can help to evaluate the etiological or clinical relevance of HBV genotypes and to predict the progression of liver disease or to investigate routes of infection. Allen and coworkers described that the RFLP assay method has been commonly used for identifying known polymorphisms in DNA from many organisms or tissues and for detecting YMDD motif variations associated with in vitro lamivudine resistance patients. Moreover, although the RFLP assay was more sensitive in identifying HBV viral populations, one advantage of DNA sequencing over the RFLP that the DNA sequence provides information at sites other than at specific codons and continues to be useful in the detection of sequence variations at other sites for detecting quasi species. PCR-RFLP has some limitations. These include its retrospective nature and small sample size. Also, using a method is based on only a part of and not the entire of HBV genome.

Restriction fragment mass polymorphism (RFMP) is another method for detecting genotypes. Lee *et al.* utilized RFMP for HBV genotyping based on genotypic variations in the S gene, which is similar to RFLP. This method depends on restriction enzyme digestion of PCR products to produce genotype specific oligonucleotide fragments. The mass of the produced fragments is then determined using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry. Other studies have reported the use of MALDI-TOF mass spectrometry for determination of YMDD (tyrosine–methionine–aspartate–aspartate motif) mutations, which are linked to lamivudine (LAM) drug resistance.

Future trends in molecular diagnostic testing for chronic hepatitis B:

The adoption of novel technologies and the identification of new virological or host markers could potentially provide opportunities for growth and evolution of molecular testing in chronic HBV. Emerging technologies that have not yet penetrated significantly into diagnostic laboratories may become useful in the future such as a high-density array that can complete with sequencing for the identification of mutations and genotypes has been reported. This system has a number of advantages including flexibility and throughput. Another method, Microarrays currently pose numerous technical, regulatory and cost challenges that will have to be overcome widely adopted in clinical laboratories. Emerging more sensitive technologies have facilitated diagnostics and therapeutics, particularly for HBeAg CHB and occult hepatitis B. They have also helped to better define the natural history of chronic HBV and how the host responds to therapy. Moreover, these new methods have also raised questions and spurred debate on the most useful virological markers for documenting the response to antivirals which can only improve the application of molecular methods to this field. Exploration new assay, DNA nanomachines where DNA is used to build synthetic molecular machinery. This technology aims to take advantage of systems already in place within biology. DNA nanomachines are made by self-assembly, using techniques that rely on sequence specific interactions that combine complementary oligonucleotides together in a double helix.

Mutations of HBV genome

Mutation rate of HBV is commonly estimated around ($4.2\text{-}10^{-5}$) substitutions per site per year among the non-overlapping part of the four ORFs. This rate, which is high for DNA viruses (around 10^4 times) and is closer to that observed for RNA viruses. This can be explained by the

replication mechanism, via a reverse transcription step, which makes the virus prone to mutation. Many types of assays have been used to detect the HBV mutations such as, restriction fragment length polymorphism analysis (RFLP), line-probe assay, colorimetric point mutation assay, ligase chain reaction assay (LCR), and enzyme-linked immunosorbent assay (ELISA) for genotype-specific epitopes. Direct sequencing is the most accurate assay. McMahon and coworkers used line probe assay and he compared with INNO-LIPA and RFLP-PCR assays.

HBV variants with point mutations in the viral polymerase gene have been detected in patients on prolonged lamivudine therapy which are the most common variations mutations which are located in or near the active site of the polymerase within a conserved region of the gene known as YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain, causing the methionine (M) residue (amino acid 204) to be replaced with either isoleucine (rtM204I) or valine (rtM204V). Other has been detect at codon 528 (L528M) and 552 (M552V/I). Elevate of ALT levels are thought to be related to more rapid selection of YMDD mutations.

Other reports have indicated that HBV-drug resistant mutants (YIDD and YVDD) can be detected in infected patients that none of them took lamivudine therapy. These mutants potentially produce false-negative test results in susceptible HBsAg immunoassays.

The emergence of a vaccine escape mutant with an altered "*a*" determinant was first described in Italy around middle of 1980s for example glycine residue at position (145) by arginine (G145R) was caused by a mutation in the viral genome (guanine to adenine at nucleotide position 587). This mutation allowed the variant virus to emerge as the

dominant viral form despite the presence of neutralizing anti-HBs. Mutations of amino acids outside the "*a*" determinant region could alter the antigenicity of HBsAg. Alteration of residues in the "*a*" determinant can result in reduced antigenicity and reduced levels of protein expression with significant 3 dimensional changes in the antigenic epitope.

Some mutations, especially in the core HBc (A1762T, G1764A) and precore (G1896A) regions have been associated with the outcome of the infection or in the progression of the disease.

Several studies have revealed that the site of a mutation and not the number of mutations appear to be important in determining non-reactivity in an assay. Other mutations are associated with occult HBV infection. These mutations usually occur in the Pre-S regions of the HBV genome. The peptides coded in the Pre-S1 region contain the hepatocyte binding site (amino acids 21–47). This peptide section is essential for virion assembly and the transport of intact virions out of the host cell. These mutations result in the inactivation of a promoter within the Pre-S2/S region resulting in the interference with HBsAg secretion.

The other common of several mutations that can cause HBeAg negativity is a guanine to adenine transition at nucleotide position 1,896 (G1896A), which creates a TAG stop codon at codon 28 of the precore (PC) protein.

HBsAg "vaccine escape" mutants is the main target for viral neutralization, either by natural or vaccine-induced anti-HBs. However, structural and genetic constraints limit major alterations of the protein. The complex secondary and tertiary structure of HBsAg is not yet fully understood. The basic working model is that of a protein with (4) transmembrane helices and with several residues at the N- and C-termini

and a central major hydrophilic region (approximately residues 103-173) exposed at the surface of viral particles. The immunodominant "*a*" determinant (residues 124-147), against which most neutralizing antibodies are directed and which is the major target of HBsAg detection tests and is formed by loops (2) and (3). In addition, the region coding HBsAg also codes part of the reverse transcriptase/DNA polymerase domain of the viral polymerase. Some deletion mutants have been reported, but they were isolated from long-term HBV carriers and may represent dead-end products. Small insertions in loop (1) of HBsAg have also been reported. They were isolated from patients serologically negative for HBsAg, at least when monoclonal antibody based tests were used, indicating that although these insertions do not occur within the "*a*" determinant itself they can affect its structure. These vaccine escape mutants can be found in non-vaccinated people where they exist as minor viral populations. They only emerge to become the major viral population in patients in the face of immune pressure, usually vaccine-induced or prophylactic treatment of liver transplant patients with human anti-HBs immunoglobulins (HBIg).

Part Seven

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