CHAPTER 1

Introduction

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family, a family of enveloped hepatotropic DNA viruses. Its genome, a partially circular double stranded DNA, includes 4 coding regions: polymerase (*pol*), precore/core (*preC/C*), presurface/surface (*preS/S*) and X regions. HBV replicates via reverse transcription of an RNA intermediate using its error-prone polymerase leading to high genetic diversity. Thus, individuals with chronic HBV infection harbor multiple closely related variants or quasispecies. The estimated mutation rate of the hepadnavirus genome is about 2×10^{-4} base substitutions/site/year, about 100 times higher than that of other DNA viruses [1]. Other factors influences on its genetic diversity are the host immune pressure and/or insufficiently efficacious antiviral drugs. The genetic diversity of HBV play an important role in the natural course of infection [2], liver disease progression [3], and response or resistance to antiviral therapy [4, 5].

HBV genetic diversity results from the lack of proofreading activity of the viral RNA polymerase during the replication cycle and from immune and/or antiviral drug pressure [6]. Several studies have shown that specific HBV genetic mutation patterns are associated with progression of liver disease in HBV mono-infected patients [7, 8]. The well-known naturally occurring mutations include a precore stop codon mutation and the double basal core promoter (BCP) mutations, inhibit or diminish production of Hepatitis B e antigen (HBeAg), respectively [9, 10]. These mutations can be found in chronically HBV infected individuals negative for HBeAg and have been associated with more aggressive liver disease and development of hepatocellular carcinoma (HCC) in HBV mono-infected patients [11, 12]. These mutations are related to the development of acute

liver failure in infants born to anti-HBe-positive women with high HBV viremia levels [13]. An association between specific HBV mutations in Hepatitis B surface antigen (HBsAg) and intrauterine HBV infection has been reported [14].

During pregnancy, women undergo various physiological modifications in order to achieve immune tolerance toward paternal antigens expressed on fetal cells and prevent rejection of the fetus. The mechanisms of immune tolerance involved suppression of cell-mediated immunity while normal humoral immunity is retained [15]. As a consequence of this unique immune condition, pregnant women are thought to be more susceptible and more severely affected by infectious diseases [16-18]. Effect of pregnancy on HBV replication is unclear. A study in chronic HBV-infected pregnant women has found that HBV DNA levels were increased during pregnancy and declined after delivery [19]. On the contrary, Soderstrom *et al*. reported that there was no significant viremia change during pregnancy [20]. Pregnancy is also associated with high levels of hormones but their impact on HBV replication is still controversial. Adrenal corticosteroids might increase levels of viraemia, and estrogens have been demonstrated to decrease HBV replication in animal models [21]. Women with HBV viral load, greater than 10^8 International Units per milliliter (IU/mL) and/or HBeAg positivity have a high risk to transmit HBV to their offspring [22, 23]. Also, HBV transmission has been reported from HBeAg-negative mother with high HBV viral load to their offspring [24]. The absence HBeAg in transmitting mother may be resulted from HBV genetic diversity [25].

Hepatitis B virus co-infection with human immunodeficiency virus (HIV) has a significant impact on the course of HBV infection by increasing liver disease progression [26-28] and on the genetic diversity of HBV [29, 30]. However, impact of HIV infection on genetic diversity of HBV is still controversial. Indeed, in a study conducted in Argentina, HBV genetic diversity was significantly higher among HBV mono-infected (n=8) than among HBV/HIV co-infected patients (n=9) suggesting that HBV may develop more mutations to evade immune system control among people with fairly well-preserved immune function [30]. Since HBV/HIV co-infected individuals usually have lower CD4 cell counts than those with HBV mono-infection [26]. One hypothesis is that low immune pressure may lead to higher HBV replication and thus more variants [31]. However, Tangkijvanich *et al*. reported no differences in the frequencies of common

HBV mutations between HBV/HIV co-infected (n=24) and HBV mono-infected patients (n=31) [32].

Despite effective vaccines against HBV are available in Asia-Pacific, perinatal HBV transmission still continues to occur [33]. High maternal HBV viral load is a major risk factor for perinatal transmission of HBV however impact of HBV genetic diversity on perinatal transmission is unclear. Since HIV infection may modify natural history of HBV infection and may increase HBV replication, analysis of HBV genetic diversity among HBV/HIV co-infected pregnant women may help to assess its impact on perinatal transmission of HBV.

1.1. Literature review

1.1.1. Discovery of Hepatitis B Virus

Hepatitis B virus was discovered by Dr. Baruch Blumberg and his colleagues in 1963. Originally, they were actually studying the human genetic markers for disease susceptibility. They planned to look for genetic differences that may be associated with a disease using micro-Ouchterlony gel diffusion, a classical immunological technique to study antigen/antibody interactions. The serum samples were collected from a wide variety of native populations around the world during the 1950s to 1960s. They detected that sera of two hemophiliac patients who had received multiple blood transfusions formed an immunodiffusion precipitin line with the serum of Australian Aborigine. At that time, they assumed that these hemophiliac patients would have developed antibodies against serum proteins of Australian Aborigine. Dr. Blumberg and his team then identified an unusual antigen in several samples from Australian Aborigine, which they tentatively called Australia antigen (AuAg). At the same time, Alfred Prince reported a serum hepatitis (SH) antigen in the blood of Hepatitis B patients was identical to AuAg [34]. Then, AuAg was confirmed to be the antigen that caused Hepatitis B by several researchers [35-37]. By the end of 1970, nomenclature for AuAg was changed to hepatitis-associated antigen (HAA); it is now officially called Hepatitis B surface antigen (HBsAg).

1.1.2. Biology of Hepatitis B virus

Hepatitis B virus belongs to the *Hepadnaviridae* family which is divided into two genera, the *Orthohepadnaviruses* and *Avihepadnaviruses*. HBV is the prototype of the *Orthohepadnaviruses* that have been isolated from mammals, including humans, woodchucks, squirrels, and woolly monkeys. Human HBV is closely related to HBV in woodchucks and ground squirrels with 70 % sequence homology [38] . *Avihepadnaviruses* have been isolated from duck, heron, snow goose, crane and stork [39].

The infectious HBV virion (or Dane particle) is roughly spherical, with a diameter of 42-47 nm, consisting of a lipid envelope with surface glycoprotein and an icosahedral nucleocapsid with a diameter of about 30 nm, in that contains the viral genomic DNA. The envelope glycoprotein of HBV contains three protein forms, designated as large (L), medium (M) and small (S) domains. These proteins made any of Hepatitis B surface antigens (HBsAg). In natural infections with HBV, there is an excess of empty subviral particles (SVP) that not contains the nucleocapsid and viral genome. SVPs are non-infectious [40] and exist in two main forms: spherical particles of 25 nm and filamentous particles of 22 nm, in diameter [41]. (Figure 1.1)

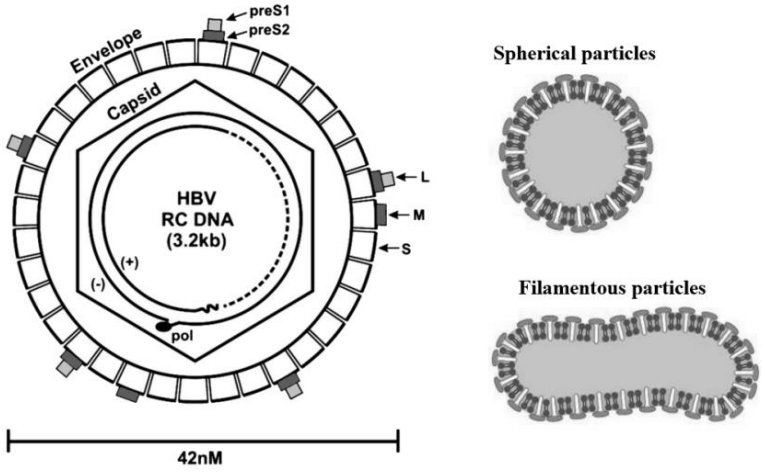

Figure 1.1 Schematic diagram of HBV structures [42]

1.1.3. HBV Genome organization

The genome of HBV is a circular partially double stranded DNA with the size of approximately 3,200 base pairs (bp) in length. HBV genome contains a completed negative (-) sense strand about 3.2 kb and an incompleted positive (+) sense strand with a variable length (1.7 to 2.8 kb) that varies between different particles [43, 44]. The 5'end of negative strand is linked to the viral DNA polymerase, while 5'end of positive strand is attached to a short, capped oligonucleotide. HBV has a compact genome encoding four major overlapping open reading frames (ORFs) [40] as following: (Figure 1.2)

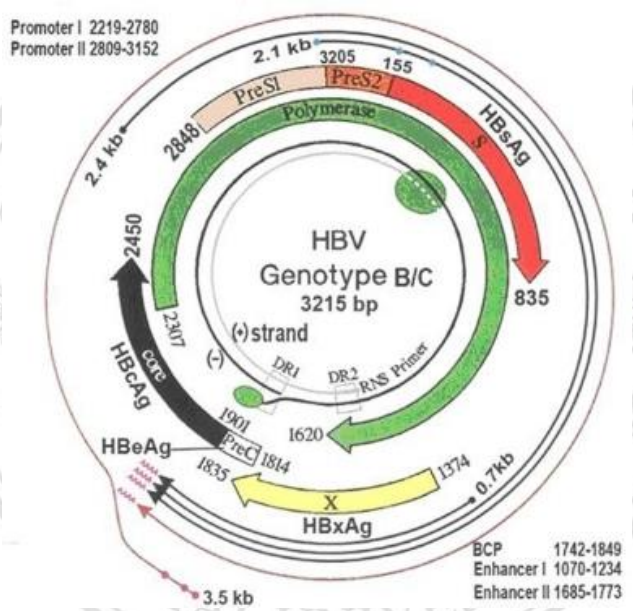

Figure 1.2 Organization of HBV genome genotype B or C [45]. The inner circle represents negative (-) and positive (+) sense DNA strands. HBV polymerase is shown as a green oval bound to 5'end of negative strand. The different ORFs encoded by genome, designated as preS1, preS2, S, preC, core, polymerase and X, are indicated by the arrows. The outer circle represents pregenomic RNA (pgRNA), an RNA intermediate (3.5 kb in length) for viral DNA replication, and other sub-genomic RNAs (0.7, 2.1, and 2.4 kb in length). The genome nucleotide numbering is based on the unique *Eco*RI cleavage site. Nucleotide numbers designate the boundaries of each ORFs with position "1" mapped at *Eco*RI cleavage site. Abbreviations: HBsAg, Hepatitis B surface antigens; HBcAg, Hepatitis B core antigen; HBeAg, Hepatitis B e antigen; BCP, basal core promoter.

1) Polymerase region

Polymerase region (*pol*) spans almost 80 percent of the HBV genome and overlaps the core and X regions and the entire surface region. *Pol* encodes HBV reverse transcriptase which is composed of four domains that include an N-terminal protein (TP) domain, a spacer region, a reverse transcriptase (RT) domain and a C-terminal ribonuclease H (RNase H) domain. The TP domain attaches to the 5′ end of negative (-) sense strand DNA and plays a critical role in the RNA packaging, and protein priming. The spacer has a function unknown. The RT domain was reverse transcriptase which similar activity and amino-acid sequence homology to the retroviral polymerases [46]. In this region, the conserved tyrosine (Y), methionine (M), aspartate (D), aspartate (D) motif is required to catalyze DNA synthesis. Amino acid substitutions in YMDD motif have been reported frequently and are associated with anti-HBV drug resistance [47]. The C-terminal RNase H is responsible for degrading RNA from RNA-DNA intermediate following negative-strand DNA synthesis [48].

2) Precore/core region

The entire *precore/core* region (*preC/C*) contains two in-frame initiating start codons which translate into both the Hepatitis B e antigen (HBeAg) and the Hepatitis B core antigen (HBcAg), a viral capsid protein. The 5'ends preC region located 87 nucleotides upstream of the core (nucleotides, nt, position 1814 to 1900, numbering from the *Eco*RI cleavage site). Translation initiating from the preC region leads to synthesis of preC protein that is further proteolytically modified in the endoplasmic reticulum into HBeAg. HBeAg is a non-structural protein, but is secreted from infected cells and detected in the blood circulation. In general, the level of HBeAg correlates with HBV DNA levels in the blood circulation. Therefore, HBeAg can be used as a marker of active viral infection. The second initiation is located in core region (nt 1901 to 2452) encodes the 21 kDa protein consists of 183 or 185 amino acids (aa) depending on the genotype. This protein assembles to form 27 nm particles designated HBcAg, which contains the viral DNA. Transcription of *preC/C* is controlled by core promoter (nt 1591 to 1849), which overlaps the 3' end of the *X* region and the 5' end of the *preC/C* region. The core promoter (CP) consists of the basal core promoter (BCP) and the upper regulatory region (URR). CP has conserved sequences, which is essential for maintaining active viral

replication, and variation may contribute to the viral persistence, leading to chronic infection [49].

3) Presurface/surface region

The three HBV surface proteins (L-, M-, and S-HBsAg) are encoded by presurface/surface ORF (nt 2848 to 835) that contains three in-frame start codons divided into preS1, preS2 and S. These ORFs are translated from different in-frame start codons to a common stop codon, result in production of three surface proteins that share the same C-terminal portion but have different N-terminal extensions (Figure 1.3). The large HBs (L-HBs) or preS1 protein is the 389 aa (or 400 aa depending on the genotype) translational product of PreS1, preS2 and S regions. The L-HBsAg contains the N-terminal preS1 (108 or 119 aa), the central preS2 (55 aa), and the C-terminal S domains (226 aa). This protein is thought to play key roles in the binding of virus to the host cell receptors and the assembly of the virion and its release from the cell. The middle surface protein (M-HBs) contains preS2 and S domains and the small (S-HBs) consists of the S domain only, but it is the most abundant portion in the virus particles [50]. All HBV surface proteins have the major epitopes that localized in the region known as the "a" determinant, amino acid residues from 124 to 147 within the major hydrophilic loop of HBsAg (Figure 1.3) [51], is the target of neutralizing antibody produced during natural infection or following vaccination.

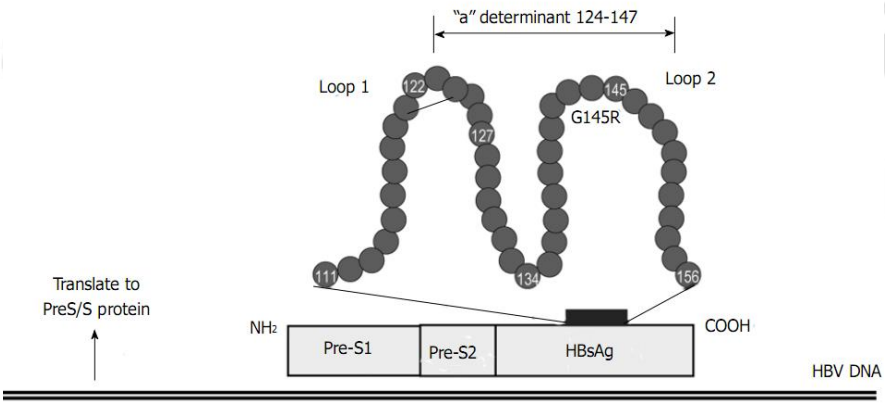

Figure 1.3 Schematic structure of the HBV surface proteins [52].

4) X region

The smallest HBV ORFs (nt 1374 to 1838), X region encodes for the 146 to 154 amino acids X-protein (HBx)[43]. The activity of HBx is absolutely required for replication and spread of the virus [53, 54]. HBx is a regulatory protein which modulates host-cell signal transduction and can affect host and viral gene expression [43]. Moreover, this protein may play a role in the development of hepatocellular carcinoma (HCC) via interrupt the apoptotic process of liver cells, inhibit the ability of cells to repair damaged DNA, and modulate transcriptional activation of cellular growth regulating genes [55, 56].

1.1.4. HBV Replication

The main characteristics of *Hepadnavirus* family are replication through a pre-genomic RNA intermediate and using RNA- dependent DNA polymerase (reverse transcriptase) enzyme. The initial phase of HBV replication, preS1 and S domains of HBV surface proteins bind to HBV- specific cellular receptors of a hepatocyte [57]. Recently, sodium taurocholate cotransporting polypeptide (NTCP) was identified as a functional receptor for viral entry [58]. After attachment, the two entry pathways have been proposed. First pathway is endocytosis followed by release of nucleocapsids from endocytic vesicles. The other pathway is fusion of the viral envelope with plasma membrane. After entry, the viral nucleocapsid containing the relaxed circular partially double stranded DNA (rcDNA) with its covalently linked polymerase reach to the nucleus, where the viral genome is delivered. Within the nucleus, the single-stranded gap region in the DNA plus strand of HBV genome is repaired by the viral polymerase. Following by a short RNA-primer and the polymerase from the 5'end of the minus strand DNA that used for the DNA plus strand synthesis were removed. The HBV DNA is circularized to the covalently closed circular form (cccDNA) that serves as a template for viral gene expression and replication.

The cccDNA is transcribed by host transcriptional machinery to produce the pregenomic RNA (pgRNA), an RNA intermediate (3.5 kb in length) for viral DNA replication, and other sub-genomic RNAs (0.7, 2.1, and 2.4 kb in length), which have the common 3'end (Figure 1.4). First, the pgRNA serves as a transcript for the translation of

the core protein, the secreted HBeAg and the viral polymerase. In addition to the pgRNA, the 2.1 kb RNA encodes the middle and major surface glycoproteins, the 2.4 kb RNA encodes the large envelope protein HBV surface antigen and the 0.7 kb RNA encodes HBx protein. For DNA synthesis, pgRNA-polymerase complex is packed in assembling nucleocapsids. The pgRNA is then reverse transcribed to double-stranded DNA (dsDNA) within the capsid. After the DNA genome is synthesized, a nucleocapsid can migrate to the nucleus and build up a cccDNA pool within the nucleus [59] or interact with envelope proteins to form an infectious virion[60].

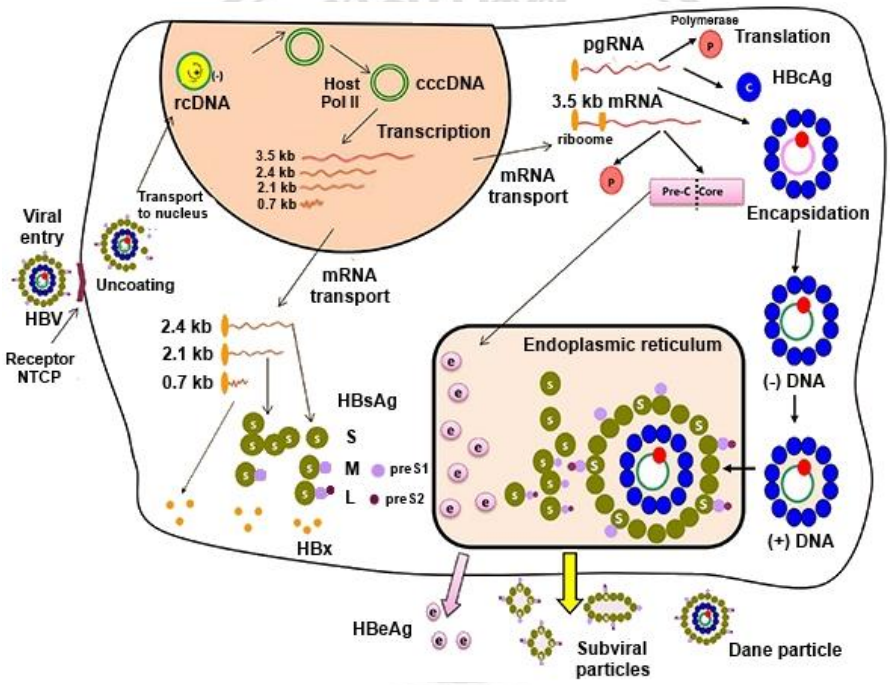

Figure 1.4 Schematic of the HBV replication cycle (Adapted from Germer, J. J. *et al.*, J Clin Microbiol**,** 2006) [61]. Abbreviations: NTCP, sodium taurocholate cotransporting polypeptide; host pol II, host cell RNA polymerase II; L, large HBV surface protein; M, middle HBV surface protein; S, small HBV surface protein.

1.1.5. Natural history of HBV infection

Hepatitis B virus infection leads to a wide spectrum of liver disease ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The clinical course of HBV infection varies with host immune response, viral strains, and host-viral interactions. HBV infection can be self-limited with clearance of virus and development of protective immunity against HBV. Progression from acute to chronic infection results from a failure of the immune system to combat and eliminate the virus. The risk of developing a chronic HBV infection after acute exposure is directly related to the age of HBV acquisition. Approximately 90% of children infected at birth will develop chronic infection [62, 63]. Children who acquired the infection after the perinatal period (<1 year of age) and during childhood period (1-5 years of age) chronic infection will develop in 90-95% and 25-30%, respectively [62, 63]. In contrast, the risk of developing chronic infection is less than 5% in healthy adults [64]. In addition, immunosuppressed persons such as patients undergoing chemotherapy, or infected with HIV, are more likely to develop chronic HBV infection [65].

1) Acute Hepatitis B

After acquisition of HBV, the average clinical incubation period (time from exposure to onset of jaundice) of acute Hepatitis B is 2–3 months and can range from 1–6 months [66]. Signs and symptoms include nausea, abdominal pain, vomiting, fever, jaundice, dark urine, hepatomegaly and splenomegaly. In some cases, clinical symptoms cannot be observed. After incubation period, HBsAg are then detected in blood circulation within 2-12 weeks. Antibodies against the core antigen, anti-HBc IgM, are usually detected 2 weeks after the detection of HBsAg then disappear within 4-12 months after infection [67]. During this phase, serum alanine aminotransferase enzyme (ALT) levels rise and high levels of HBsAg and HBV DNA are detectable. In self-limited HBV infection, HBsAg and HBeAg disappear at about 3 to 6 months and follows with development of antibodies to HBsAg (anti-HBs) and antibodies to HBeAg (anti-HBe) [66].

2) Chronic Hepatitis B

Chronic infection is defined as the persistence of present HBsAg in serum for at least 6 months. Chronic HBV infection is characterized by four distinct phases (Figure 1.5).

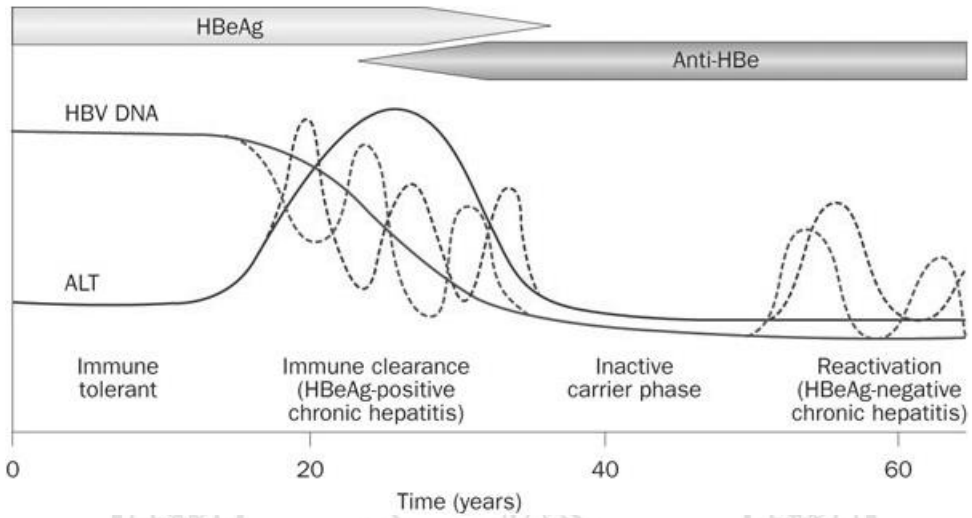

Figure 1.5 Natural course of chronic HBV infection [68]

Phase 1: Immune tolerance phase is characterized by the presence of HBeAg, high HBV DNA levels (greater than 20,000 IU/mL), normal or minimally elevated ALT levels, indicating that the host immune response against the infected hepatocytes is lacking or minimal. In adult patients, this phase lasts about 2-4 weeks or shorter depending on the age at acquisition of HBV infection. This phase may last for decades when infection is acquired perinatally or during childhood [69].

Phase 2: Immune clearance, also called the immune active phase, is characterized by elevated ALT levels and elevated HBV DNA. This phase may last several months or years depending on the efficiency of immune system. Spontaneous seroconversion HBeAg to anti-HBe occurs at the annual rate of 10-20% [70] and is associated with a decrease of HBV DNA level. Clearances of HBeAg, whether spontaneous or after antiviral therapy, reduce the risk of liver disease progression and improve survival [70].

Phase 3: Immune control phase or inactive carrier phase were characterized by the absence of HBeAg and the presence of anti-HBe, normal liver function tests and undetectable or low level of HBV DNA (less than 2000 IU/mL). Long-term follow-up studies have shown that patients in this phase are at low risk of developing advanced liver disease and its related complications [71]. Most patients (70%-80%) will remain inactive carrier state for a lifetime. However, about 20% of inactive carriers may reverse back to HBeAg positivity with hepatitis flares [72].

Phase 4: Immune escape (reactivation) phase, also called HBeAg negative chronic Hepatitis B, is characterized by absence of HBeAg, presence of anti-HBe and high levels of HBV DNA (greater than 2000 IU/mL) [70]. Most patients with HBeAg negative chronic hepatitis harbor a precore mutant that cannot produce HBeAg. The most common precore mutation, guanosine (G) to adenine (A) substitution at nucleotide 1896 (G1896A) producing a stop codon, results in the lack of HBeAg production but the virus is still able to replicate, and is called precore HBV mutant. Reactivation of HBV replication can occur either spontaneously or in case of immunosuppression [73]. The prevalence of HBeAg negative chronic HBV patients was 33% in the Mediterranean, 15% in Asia Pacific, and 14% in the USA and Northern Europe [74]. In addition, chronic hepatitis B patients who harbor HBeAg-negative mutants have been associated with more aggressive liver disease and development of HCC [11, 12].

1.1.6. The immune response to HBV infection

The immune responses that terminate viral infection typically begin with early activation of innate immunity followed by development of adaptive immunity that can limit the spread of virus and / or cause pathological damage of the infected cells. The mechanisms of immune responses that control HBV infection comprise both innate immunity and adaptive immunity.

1) Innate immunity

Innate immune response plays a role immediately after infection to limit viral replication. The intracellular receptors of infected hepatocytes, including endosomal Toll-like receptors (TLRs) and cytoplasmic receptors, recognize viral components like

DNA, RNA and glycoprotein and subsequently trigger the production of type I interferon (IFN)-α/β cytokines. The produced IFN initiates adaptive immune response and the activation of natural killer (NK) cells and natural killer T-(NKT) cells.

2) Adaptive immunity

The mechanism of viral elimination by the adaptive immune response is comprised of many effector cell types. Infected cells, phagocytic cells and antigen presenting cells, such as dendritic cells, can play a direct role in contribute to the initiation of the adaptive immune response. The process of viral clearance relies upon the antiviral functions of cytotoxic T lymphocytes (CTLs), helper T cells (Th), and B cells.

2.1) Cellular immune response

After the exponential increase in HBV replication phase, HBV-specific CD4- and CD8-mediated responses become usually detectable immediately. CD4+ T cells, classically referred to Th cells, are vigorous producers of cytokines and are required for the efficient development of effector cytotoxic CD8+ T cells and B cell antibody production. Base on the different cytokine production, Th cells can be divided into two types; Th1 and Th2 cells. Th1 cells produce IL-2, TNF-α, and IFN-γ, which regulate the cellular immune response, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are recognized to stimulate antibody production. HBV-specific CD8+ T-cells directly kill infected hepatocytes, and the recruited host-derived inflammatory cells, such as polymorphonuclear neutrophil (PMNs), antigen-nonspecific T cells, NK cells, and macrophages, also cause damage to infected hepatocytes. In patients self-limited infected decrease of HBV DNA levels occur within 2-3 weeks after the peak of viral replication and before detection of liver damage, indicating that virus is eliminated without liver cell destruction by non-cytopathic mechanisms sustained by CD8+ T cell production cytokines, including IFN-α and TNF-γ [75].

2.2) Humoral immune response

Although the cellular immune response is a major contributor to HBV clearance, humoral responses also play a role in controlling HBV infection. B-cells produce antibodies against HBsAg, HBcAg and HBeAg via T-cell-dependent process [76]. Anti-HBs serve as neutralizing antibodies to block viral attachment and entry into the cells. In contrast to anti-HBs, anti-HBc and anti-HBe are not protective. Anti-HBc is detected in virtually all HBV infected patients. The presence of anti-HBc IgM alone is used to distinguish acute from chronic infection. The presence of anti-HBe is seen as marker of viral replication stop.

1.1.7. HBV transmission

HBV is a blood-borne transmissible infectious pathogen. This virus infects the liver and circulates in blood circulation of infected individuals. HBV is very durable, can remain stable and infectious in dried blood up to 7 days [77]. For this reason, HBV is transmitted by exposure to infected blood, either directly or through contact with contaminated environmental surfaces. In addition, the virus has been found in semen and cervical secretions, and, as a result, HBV is transmitted also by sexual and vertical routes. Depending on the epidemiological pattern, the modes of transmission significantly differ across regions. Two major modes of transmission are recognized:

1) Horizontal transmission

Transmission occurs via percutaneous or mucosal exposure to infective blood or body fluids. First, HBV can be transmitted by transfusion of HBV contaminated blood or blood products. [78]. The discovery of HBV led to the development of blood-screening method that significantly reduced the incidence of post-transfusion hepatitis from HBV. The introduction of the nucleic acid amplification test (NAT) for HBV in donor screening reduces the window period and residual risk of HBV transmission by infected donors who have not seroconverted[79]. However, occult HBV infection (OBI), defined as having low HBV DNA level (usually <100 IU/mL) in serum of individuals who test negative for the HBsAg [80], has shown to be the risk of Hepatitis B transmission through blood transfusion[81]. Second, HBV infection among intravenous drug users usually occurs via the reuse of needles and syringes [82]. Third, nosocomial transmission has been

associated with inadequate infection control practices, including reuse of contaminated equipment and improper disinfection and sterilization practices for equipment and environmental surfaces [83]. HBV transmission to health care workers seems a rare event due to implementation of HB vaccination and/or HBV prophylaxis [33, 74]. Finally, sexual intercourse with HBV infected partners. HBV transmission usually occurs from mucous membrane exposures to infectious blood [33, 84].

2) Vertical transmission

Vertical or mother-to-child transmission (MTCT) of HBV is defined as HBsAg or HBV DNA positivity within the first 12 months of life in an infant born to an HBV infected mother [85]. Transmission can occur during pregnancy (intrauterine transmission), at the time of delivery (perinatal transmission) and after delivery (post-natal transmission). The mechanism of intrauterine transmission has been described in various ways including: first, HBV can cross the placental barrier in early stage of pregnancy due to the layer of trophoblastic cells becomes thinner and turns into chorion vessel membrane, which makes it easier for HBV to pass the placental barrier [86, 87]. Then the viruses infect and replicate in all types of placental cells, therefore, HBV can reach the fetus. Second, membrane rupture or transplacental leakage of the maternal blood into fetal circulation due to prolonged threatened preterm labor or threatened abortion [88]. Furthermore, the presence of HBV DNA in oocytes of infected females and sperms of HBV-infected males may be possible for the fetus to become infected with HBV [89]. Perinatal transmission, which is the most frequent mode of vertical transmission [88], can occur when newborns swallow infective secretions or blood during delivery. Vaginal delivery from mothers with high viremia increases the risk of transmission as compared to elective cesarean section [90]. Although HBsAg and HBV DNA are present in the breast milk of HBsAg carrier mothers [91], breastfeeding does not pose a significant risk for the HBV transmission among infants who had appropriate immunoprophylaxis at birth and continued as scheduled [92]. Nevertheless, immunoprophylaxis for newborns with HBsAg-positive mothers is not 100% successful in preventing transmission [93]. Immunization failures in newborns may resulted from either a very high level of HBV replication in infected mother during pregnancy/delivery or mutations in the *S* gene of

HBV that cause conformational changes in the “a” determinant of HBsAg, the target region for neutralizing antibody [94].

1.1.8. Epidemiology of HBV

Hepatitis B is one of the major diseases that continue to be a public health problem worldwide despite the implementation of effective vaccination programs and potent antiviral treatments. Approximately 2 billion people have serological markers of previous exposure to HBV, and 248 million individuals in the general population are chronically infected [95] [96]. Estimated 800,000 people die from complications of chronic HBV infection, mostly from cirrhosis and hepatocellular carcinoma (HCC) every year [96].

Level of endemicity of chronic HBV infection is defined by the prevalence of HBsAg-positive in general population [33]. On a global level, average HBsAg prevalence was 3.61%, ranged from 0.1 to more than 20% [95]. The prevalence of HBV infection varies geographically (Figure 1.6).

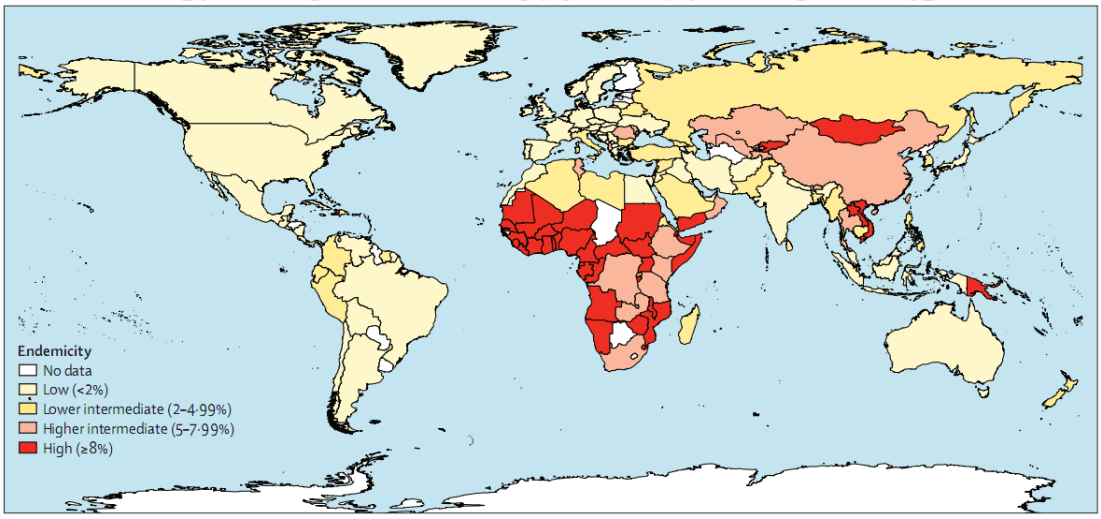

Figure 1.6 Worldwide distribution of chronic HBV infection [95]

The high HBV endemicity was classified by the prevalence of HBsAg positive greater than 8% in general population. Most countries in Africa, except for Algeria, Eritrea, and the Seychelles, were classified as high HBV endemicity [95]. Intermediate endemicity areas are divided into high (5-7.99%) and low (2-4.99% of HBsAg prevalence) intermediate endemicity. Western Pacific region, China, and Thailand were high intermediate endemicity regions whereas the Eastern Mediterranean and Russia were

low intermediate endemicity. Low endemic areas, where HBsAg prevalence was less than 2%, include countries in the Americas, Europe, and the USA.

HBV epidemiology in Thailand

Currently, Thailand is classified as a high intermediate endemicity country which has the prevalence of HBsAg positive 6.42% (6.37-6.47) [95]. The decline prevalence of HBV is mostly the result of the expanded program on immunization (EPI) in 1992 [95]. }. In the northern province of Chiang Mai, where the HB vaccination program started in 1988, the prevalence rate of HBsAg in schoolchildren during 1998-2000 was 4.5% [97]. Another study reported that HBsAg seroprevalence among vaccinated 6 month to 18-year-olds children was decreased from 4.3% to 0.7% [98]. Similarly, the prevalence of HBV infection among first-time blood donors has decreased from 7.1% in 1988 to 2.6% in 2009 [99]. In high risk group, HBV prevalence was 8.7% in Thai HIV-infected patients [100], 11.9% in highly active antiretroviral therapy (HAART)-naïve HIV-infected patients [101], 3.3% in perinatally HIV-infected adolescents (n=521) [102] and was 7.4% in HIV-1 infected pregnant women (n=3,315) [24].

1.1.9. Genetic diversity

Unlike other DNA viruses, HBV DNA replicates using a reverse transcriptase (HBV polymerase) which is an error-prone polymerase enzyme. The HBV polymerase lacks 3'- 5' proofreading activity to repair incorrectly incorporated nucleotides. Therefore, this enzyme produces a high rate of nucleotide substitutions. The error rate of the HBV polymerase has been estimated as 2 nucleotide substitutions per 100,000 bases and year of infection ($1.4\text{-}3.2 \times 10^{-5}$ nucleotide substitutions/site/year) [1, 103]. This mutation rate is 100 times higher than other DNA viruses but lower than that of RNA viruses. HBV also has high daily production rate which is up to 10^{11} virions per day. Thus, high rate of mutations and production result in the coexistence of multiple viruses genetically linked called "quasispecies". The diversity of HBV genome can be viewed from two perspectives [104];

1.1.9.1. Genotypic diversity

Genotypic diversity describes the natural variability that occurs without selective pressure. Based on nucleotide sequence divergence over the whole genome of more than 8%, HBV has been classified into ten genotypes designated as A to J [105]. HBV genotypes and sub-genotypes have distinct geographical distributions (Figure 1.7). In summary, genotype A has been found in European Union, South America and Sub-Saharan Africa. Genotype B and C are mostly found in Eastern Asia and Oceania, D in the Mediterranean and Middle East regions, whereas genotype E is restricted in Western Africa and F and H in Pacific and Latin America. Until now, the genotype G has been localized only in Europe, USA and Japan. Genotypes I and J, 2 new genotypes, have been discovered in 2008 in Laotian and Japanese patients [105-110]. In Thailand, HBV genotype C and B are the 2 predominant and represents about 54-91% and 7-25%, respectively [97, 111, 112].

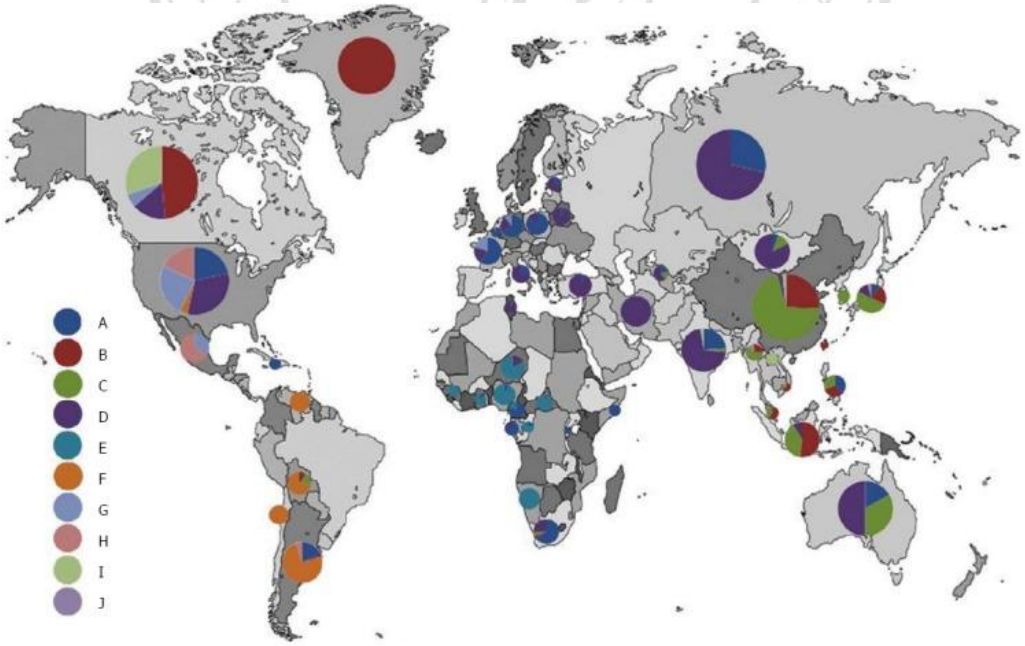

Figure 1.7 Geographic distribution of hepatitis B virus genotypes worldwide [113]

Notably, the inter-genotype recombination has also been reported previously, which may play an important role in the evolutionary history of HBV. Recombination is favored in particular geographical regions. In recent study conducted in China, B/C inter-genotype recombinant HBV with a novel genome mosaic structure has been reported [114]. In Thailand, a recombinant HBV genotype G and C were isolated from a Thai patient with hepatocellular carcinoma [115].

1.1.9.2. Phenotypic diversity

As a result of host immune responses against HBV infection, immunoprophylaxis and antiviral therapies, HBV mutants or variants can emerge within the same patient over the chronic course of infection [104]. Several studies have shown that the HBV variants play critical roles in the pathogenesis in HBV infection [116-118].

a) Precore/core mutations

The *precore/core* region contains two in-frame start codons which translate into the core protein and the protein HBeAg. Translation from the first start codon leads to synthesis of HBeAg which is a soluble protein, acts as a strong immunogen and the important target of host immune clearance mechanisms [119]. Mutations within basal core promoter (BCP), precore and core regions result in diminish or inhibit HBeAg production. These variants result from HBV attempt to escape host immune control and emerge at later stages of chronic infection. In addition, these mutants naturally imply disease of longer standing and a higher risk of cirrhosis [3].

The most naturally occurring mutation is the substitution of guanine (G)-to-adenine (A) at nucleotide 1896 (G1896A or A1896) [9, 120] , which converted TGG to TAG stop codon at the end of precore open reading frame. This stop codon leads to prevent HBeAg production whereas core protein is normally produced. This variant retains replicative capacity [121] and has been associated with development of HCC [122]. Other mutations have also been found in the precore region including mutation at the precore start codon (ATG to ACG or CUG) and mutation of the second codon to a stop codon [123].

In addition to precore mutation, the common HBeAg variants are the core promoter mutants. The double A1762T and G1764A substitutions within BCP region is the most frequently found and has been associated with a decrease of mRNA synthesis and thus decreased expression of HBeAg [124-126]. In the USA/Australia collaboration study of 81 HBV/HIV co-infected patients, the double mutations (A1762T/G1764A) and the triple mutations (T1753C/A1762T/ G1764A) were present in 15% and 6% of sequenced strains, respectively [127].

b) Pol gene mutations

HBV polymerase mutations can result from selective pressure exerted by anti-HBV nucleos(t)ide analogs (NAs). Complex mixtures of *pol* mutant quasispecies undergo significant genetic evolution over time. During long-term use of NAs against HBV, mutants can emerge leading to viral breakthrough and HBV drug resistance. In retrospective cohort study of HBV/HIV co-infected patients who were exposed to lamivudine (LAM) for 4 years, more than 90% of them had the amino acid mutations rtV173L, rtL180M and rtM204I/V/S, which are known LAM resistance position in the reverse transcriptase region of the pol gene [4]. Furthermore, a cross-resistance is common during NAs therapy with, LAM, emtricitabine, entecavir and telbivudine. LAM-resistant strains increase the risk of developing entecavir resistance [128].

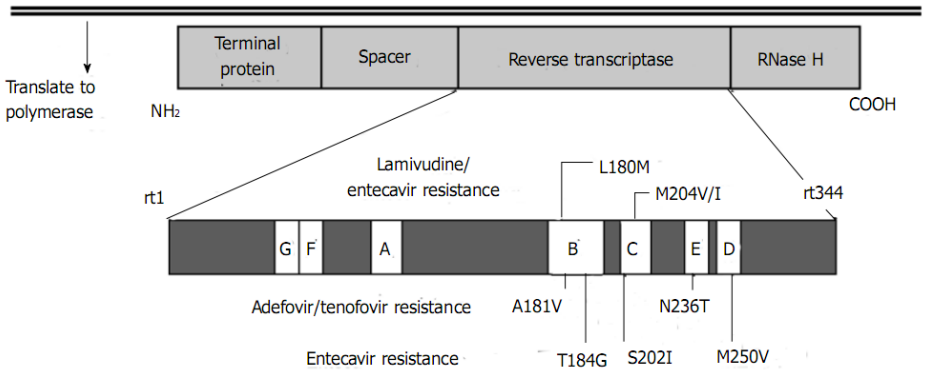

Figure 1.8 Polymerase gene mutations conferring drug resistance [52]

c) Surface gene mutations

PreS and *S* mutations can emerge as a result of escape to host-immune response [104], namely "immune-escape mutants", after exposure to anti-HBV antibodies, namely "vaccine-escape mutants", [129] or by pressure on the *pol* gene that induces mutations on the overlapping portion of the S gene. The aberrant encoding of the *preS/S* ORF was common found in the two main *preS* mutations (preS1 and preS2). The "a" determinant of HBsAg is the most important target for protective antibody response, diagnosis and immunoprophylaxis. Mutations within this region can produce mutants that can be undetectable by routinely used immunoassays. In cohort study, the occurrence of this misdiagnosis was found in 23% of HBV/HIV co-infected who started antiretroviral treatment in South Africa and might be associated with profound HIV-related immunosuppression [130]. Several amino acid mutations such as sP120T/S and sG145R/K/A have been reported to involve in diagnostic failure and escape from neutralizing antibodies eliciting by HBV vaccines as well as resulting in HBIg therapy failure [131]. The *preS/S* ORF completely overlaps with the *pol* ORF, thus S gene mutations patterns during exposure to NAs, particularly the sE164D, sW196S, sI195M, sM198I and sE164D/I195M, can be emerged in connection with LAM resistance and are responsible for reduced binding to anti-HBs antibodies [132]. Furthermore, a combination of three mutations, rtV173L, rtL180M and rtM204V on pol gene which are the LAM-resistance-associated mutations, caused sE164D and sI195M mutations in the S gene and resulted in conformation changes of HBsAg. These mutant patterns could lead to HBV infection in vaccinated chimpanzees despite the presence of high anti-HBs in their blood [133]. Such mutations have been found in 17% of HBV/HIV infected patients from the USA/Australia study [127].

d) X gene mutations

During the natural history of HBV infection, mutations tend to accumulate in the HBV genome. Several HBx mutants have been identified from sera and/or liver tissues from patients with HCC. Double mutations K130M and V131I are more frequent in sera of patients with liver cirrhosis than in patients with chronic hepatitis B [134]. An insert mutation at nucleotide position 204 (insert AGGCCC) always accompanied with G260A and G/C/T264A was found to be the most frequent mutant

pattern in tissues and sera samples from HCC patients [135]. Moreover, C-terminal deletion mutant of HBx (deleted at nucleotides 382-400) may lead to alteration of HBx functional domains in regulating cell proliferation, viability, and transformation [136].

1.1.10. Impact of HIV on HBV infection

HIV has significant impacts on the natural history of chronic HBV infection [26-28]. Higher HBV DNA levels, low CD4+ cell counts and the high rate of HBV reactivation were observed in co-infected patients compare to HBV monoinfection [137-139]. In addition, cohort study conducted in 2002 demonstrated the risk of liver-related mortality was increased in people with HBV/HIV co-infection, mostly those with the severe immunosuppression [28]. The difference rate of HBV variability genome between HBV monoinfection and HBV/HIV co-infection was observed, the higher rate of *preS2* deletion was previously found among the HBV/HIV co-infected population [29].

1.1.11. HBV infection in pregnancy

During pregnancy, the maternal immune system may tolerate fetal antigens by suppressing cell-mediated immunity while retaining normal humoral immunity [15]. The immunologic changes may induce a state of increased susceptibility to certain intracellular pathogens, including HBV. Moreover, these changes result in an increase of HBV DNA and a reduction of ALT levels. After delivery the immune system is restored thereby causing opposite consequences; an increase in ALT and a reduction of HBV DNA in postpartum period [19, 140].

Apart from immunologic changes, increased levels of adrenal corticosteroids and estrogen hormones during pregnancy have effects to HBV viremia [21]. Another retrospective study of 38 chronic HBV infected women has found that HBV DNA levels were increased during pregnancy and declined after delivery [19]. Moreover, these changes can lead to ALT fluctuations which tend to increase in late pregnancy and the postpartum period [19]. However, a retrospectively analysis in HBV DNA levels change during and after pregnancies found no statistically significant viremia changes [20].

The consequences of chronic HBV infection on perinatal outcome have not been clearly defined. A study conducted in 1999 found no differences in gestational age at

delivery among HBsAg-positive women when compared with HBsAg-negative women. They suggested that HBV infection in pregnant women does not pose additional risk for the pregnancy [141]. However, a recent study showed that HBsAg-positive pregnant women have a higher frequent of gestational diabetes mellitus and ante partum hemorrhage than HBsAg-negative pregnant women [142]. These clinical complications were reported to association with preterm delivery [143, 144].

The impact of pregnancy on HBV genetic diversity has never been investigated. The alteration of immune response during pregnancy may have an effect on HBV replication, which may have an effect on viral genetic diversity.

The most important risk factors for perinatal HBV infection, are a high maternal viral load (greater than 10^8 copies/mL) [22] and the presence of HBeAg [23]. Without prophylaxis, the rates of perinatal transmission are 70-90% when mothers are HBeAg-positive [145], 25% when mothers are HBeAg-negative/anti-HBe-negative and 12% when mothers are HBeAg-negative/anti-HBe-positive [23, 146, 147].

1.2. Objectives

1) To assess the relationship between HBV genetic diversity and perinatal HBV transmission among HBV/HIV co-infected pregnant women

2) To assess the relationship between HBV genetic diversity and absence of plasma HBeAg in high HBV DNA level pregnant women