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Genetic analysis of hepatitis B Virus (HBV) mutants in HBV/HIV-1 co-infected patients in Thailand

Analyse des mutants du virus de l'hépatite B (VHB) chez des patients co-infectés par le VIH et le VHB en Thaïlande

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Woottichai KHAMDUANG

Summary

Genetic analysis of hepatitis B virus (HBV) mutants in HBV/HIV-1 co-infected patients in Thailand

Thailand has been considered as an endemic area for chronic HBV infection (prevalence HB surface antigen ≥8%). Despite effectiveness of HBV vaccine, perinatal HBV transmission remains a major cause of chronic infection. This study aimed at identifying HBV mutants that may be associated with vaccine failure, misdiagnosis of chronic HBV infection and antiviral treatment failure.

In the first part, we analyzed the prevalence of perinatal HBV transmission in a large HIV prevention cohort in Thailand and characterized the HBV vaccine escape mutants. Among 3,349 HIV-infected pregnant women, 237 (7%) were found HBsAg positive (ETI-MAK-4, Diasorin). Eleven of 229 (5%) children born to HBsAg-positive mother were found HBsAg and/or DNA-positive at 2–6 months of age. Complete series of samples were available for 9 mother-child pairs. Based on direct sequencing and cloning analysis, 3 patterns of transmission were observed; 1) transmission of wild-type variants from mothers with high HBV DNA level (>6.5 log10IU/mL), 2) transmission of maternal minor variants (e.g. sK122R, sI126T), and 3) transmission of variants already present (>20% of viral population) in maternal blood samples (e.g. sI126M+P127S, sT131N+M133T+T140I+S204R).The capacity of mutant HBV variants to escape anti-HBs neutralization response in vitro will be further studied using HBV-pseudoviral particles harboring the mutations identified.

In the second part, we assessed the prevalence of isolated anti-HBc in HIV-infected pregnant women and the prevalence of occult HBV infection among those carrying isolated anti-HBc. We also analyzed the risk factors associated with isolated anti-HBc and occult HBV infection.1,752 HBsAg-negative (ETI-MAK-4, Diasorin) women were included in this study. Of 1,682 available samples, 553 (33%) had seropositive for both anti-HBc(MonoLisa® anti-HBc PLUS) and anti-HBs (MonoLisa® anti-HBs PLUS), 229 (14%) had isolated anti-HBc, 68 (4%) had anti-HBs seropositive alone, while 832 (49%) had no HBV infection markers detected. Of the 210 women with isolated anti-HBc and a sample available for HBV DNA quantification (limit of

detection was 15 or 1.18 log10 IU/mL), 160 had HBV DNA below the limit of detection, 47 had HBV DNA level between 15 to 100 IU/mL, and only 3 had HBV DNA above 100 IU/mL but below 1,000 IU/mL. One patient had virus with sS117I+T118K+R160K mutations. In addition, 177 isolated anti-HBc women were retested for HBsAg with another test kit (MonoLisa®HBsAg ultra) and 12 (7%) had discrepant results. The multivariate analysis showed that women over 35 years old (adjusted odds ratio [aOR], 1.8; P=0.03), born in northern region (aOR, 1.8; P>0.001), absolute CD4 count below 200 cells/μL (aOR, 2.8; P>0.001), and past or present HCV infection (aOR, 2.6; P=0.001), were independently associated with the presence of isolated anti-HBc antibodies. Whereas HIV RNA level was a factor associated with occult HBV infection in isolated anti-HBc women, rate of occult HBV infection was lowest when HIV RNA level greater than 5 log10 copies/μL (aOR, 0.03; P=0.006).

In the last part, we evaluated the 1-year and long-term effect of lamivudine (3TC) on HBV replication in 30 HIV/HBV co-infected patients receiving 3TC-based highly active antiretroviral therapy (300 mg/day of 3TC). HBV DNA, HIV RNA, absolute CD4 and CD8 T-cells counts, and liver enzymes were measured at baseline, 3 and 12 months and long-term last visit (median: 50 months; IQR: 32-65). The median baseline HBV DNA level was 7.35 $\log_{10}$IU/mL (IQR: 5.55-8.07). At 3 and 12 months of treatment, the median HBV DNA decrease was 3.86 and 4.40 $\log_{10}$IU/mL with 53% and 67% of patient achieving HBV DNA suppression to ≤150 or 2.18 $\log_{10}$IU/mL, respectively. Of 19 patients with a long-term follow-up, 17 (89%) had sustained HBV DNA suppression. In patients who achieved HBV DNA suppression, the estimated cumulative rate of sustained HBV DNA suppression at 1, 2, 3, 5, and 7 years were 95%, 91%, 84%, 84%, and 64%, respectively. Sustained HBV DNA suppression was observed in all HBeAg-negative patients. Of 7 patients with HBV breakthrough, 2 harbored virus with triple mutation (rtV173L+rtL180M+rtM204I) and 1 with single mutation (rtM204I). Our results suggested that long-term suppression of HBV replication is an additional benefit provided by 3TC-containing HAART for a significant number of HIV/HBV co-infected patients in resource-limited countries.

Key words: Hepatitis B, HIV co-infection, vaccine failure, lamivudine resistance, isolated anti-HBc

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Introduction

1. Introduction

Hepatitis B virus (HBV) infection is one of the most common infections in the world. According to World Health Organization (WHO) report in August 2008, 2 billion people or one-third of the world's population have been infected with HBV [1]. Most have spontaneously resolved acute infection. However, 10% of adults and most of children under age 5 years become chronically infected. An estimated 350 to 400 million people (about 6% of the world population) are chronically infected with HBV [2-7]. About 75-80% of chronically HBV-infected patients reside in Asia and the western pacific [8, 9]. Southeast Asia is a highly endemic area for chronic HBV infection as defined by the prevalence of hepatitis B surface antigen (HBsAg) carriers of 8% or more. Perinatal HBV transmission remains a major cause of chronic infection in this region since most HBsAg carriers have been infected at birth or in early childhood [10].

Individuals with chronic hepatitis B infection are at high risk of developing severe liver diseases and complications, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [11, 12]. HBV is the tenth leading cause of death worldwide [13] with an estimated 600,000 deaths per year attributed to the acute or consequences of chronic hepatitis B [1, 6, 13]. Preventing perinatal HBV transmission is thus the most effective strategy to reduce the global morbidity and mortality due to hepatitis B infection.

Fortunately, an effective vaccine against HBV has been available for nearly 30 years. The vaccine is very safe and there is no convincing evidence of any long-term undesirable sequelae. In 2006, 84% (of 193) countries worldwide have reported the implementation of newborn hepatitis B (HB) vaccination [14]. Even though eradication of HBV infection seems possible in the near future there are still some obstacles. Indeed, only 27% of newborns worldwide have received a HB vaccine birth dose [14]. Also, some children have been infected with HBV despite adequate vaccine and/or immunoglobulin against HBV was provided. These perinatal transmissions may be due to 1) ineffective vaccine; HB vaccines are heat sensitive and require a cold chain for transportation and storage, 2) the exposition of children to high maternal HBV viral load, or 3) the emergence of HBV mutants or "escape mutants" which can escape the activity of vaccine and/or immunoglobulin. Another obstacle to HBV eradication is the high number of chronically HBV infected subjects, who are not yet treated because of the limited access to anti-HBV treatment or are not aware of their HBV infection. These chronically HBV infected subjects may thus represent a major source of viral spread.

In Thailand there are approximately 550,000 individuals living with HIV/AIDS, of whom 9% are co-infected with HBV. Due to the size of this co-infected population and the public health consequences of these infections, it is important to address the following concerns related to HBV infection: the residual risk of perinatal transmission of HBV among HIV-HBV pregnant women, the reality and impact of occult HBV infection or undiagnosed HBV infections among HIV-HBV pregnant women and lastly the long term efficacy of 3TC-containing HAART on HBV infection in HIV-HBV co-infected patients.

The Thai Ministry of Public Health (MOPH) has integrated HBV vaccination of all newborns into the national expanded program on immunization (EPI) since 1992, successfully decreasing the rate of HBsAg in children from 3.4% to 0.7% [15]. However, despite an adequate immunization, there is a residual risk of perinatal HBV transmission [16], particularly in infants born to HBeAg-positive carrier mothers (5-10%) [17], which can be addressed through systematical analysis of the causes of vaccination failure.

Several type of variants can limit action of anti-HBs neutralizing antibodies: vaccine/Immunoglobulins escape mutants [18, 19], naturally occurring variants [20]. or polymerase mutants selected during antiviral therapy which can lead to viruses with changes in HBsAg due to the overlapping reading frames of surface (*S*) and polymerase (*Pol*) gene [21]. Moreover, it has been shown that mutations within the *S* gene, either caused by selection or natural variation, can lead to false-negative results in assay for HBsAg [22]. Individuals infecting with this mutant virus can be a reservoir of viral transmission whether horizontally or vertically [23].

Lamivudine (3TC) is a cytidine analogue which inhibits the reverse transcriptase of both HIV and HBV. This dual activity of 3TC is potentially beneficial to individuals who are co-infected with HIV/HBV [24]. However, the efficacy of 3TC is limited by the emergence of 3TC resistance mutations with an estimated rate of 20% per year [25]. Most of data come from western countries where prevalent HBV genotypes are different from those circulating in Asia. In Thailand, since 2002 the first line antiretroviral regimen for treating HIV-infected patients is a

fixed dose combination (stavudine or zidovudine + 3TC + nevirapine) that includes 3TC. Long term use of this combination in HIV-HBV co-infected patients may lead to the emergence of 3TC resistance mutations which in turn may lead to the occurrence of HBsAg mutations. It is thus necessary to evaluate the HBV virological response in HIV/HBV co-infected patients receiving 3TC-containing HAART and the possible consequence on *S* gene mutation in patients in Thailand.

Therefore, it is a good opportunity to address in the HIV-HBV co-infected population three questions of public health: what is the residual risk of perinatal transmission of HBV among HIV-HBV pregnant women in the context of EPI, what is the prevalence and impact of occult HBV infection among these women and lastly what is the long term efficacy of 3TC-containing HAART on HBV infection. The common point to these 3 questions relates to the possible occurrence of mutations of the *pol* or *S* genes of HBV and their potential negative impact on diagnosis, response to vaccine/immunoglobulins and antiviral therapy.

In summary, the overall objective of this study, showed in Figure 1.1, is to investigate in the context of HIV-HBV co-infection, the HBV *Pol* and *S* variants emerging under different types of pressure, HB vaccine, specific HBV antibodies or 3TC antiviral drug, which may impact on the perinatal transmission of HBV, misdiagnosis or the response to antiviral therapy.

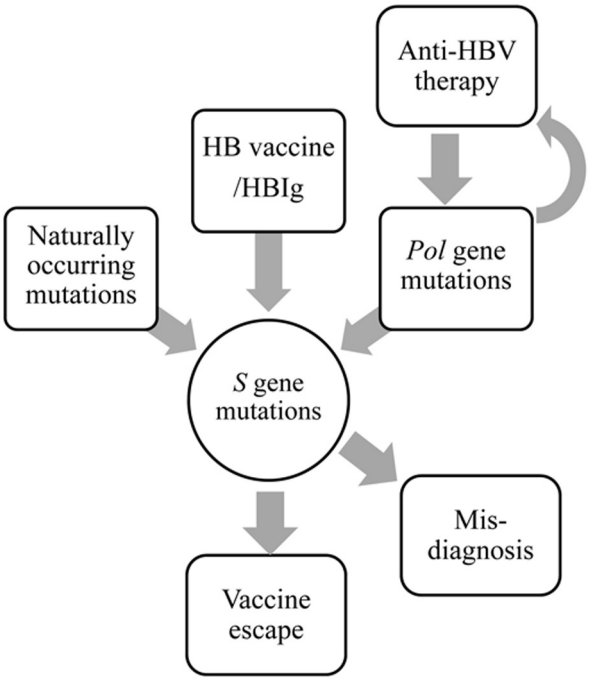


Figure 1.1. Schematic overview of the study. The emergence of hepatitis B surface gene mutations may occur naturally, or be induced by hepatitis B vaccination/ Immunoglobulins, or anti-HBV drug therapy, reflecting either vaccine escape, misdiagnosis or anti-HBV drug resistance.

1.1. Objectives of this study:

1. To determine the rate of perinatal HBV transmission in infants born to HIV-1 mothers co-infected with HBV, characterize HBV vaccine escape mutants, and describe viral diversity in mothers and infants.
2. To determine the prevalence and identify the factors associated with isolated antibody to hepatitis B core antigen and occult hepatitis B infection in HIV-1 infected pregnant women in Thailand.
3. To determine HBV virological response to combination antiretroviral treatment includes lamivudine (3TC) in HIV/HBV co-infected individuals in Thailand

1.2. Education/Application advantages of this study

This study provides an assessment of the efficacy of HB vaccine in infants born to HIV-HBV co-infected mothers in Thailand, a better understanding of HB vaccine/seroprophylaxis failure and the rate of misdiagnosis of HBV infection due to HBsAg mutants or the prevalence of occult HBV infection in HIV-HBV co-infected pregnant women. Finally, this study also provides further insights into the emergence of HBV resistance mutants in HIV-HBV co-infected patients receiving 3TC-antiretroviral therapy in Thailand.

The results of this study will contribute to a better knowledge of the prevalence and diversity of HBsAg variants and help in the decision making, design and plan of public health policy towards HBV prevention in Thailand and in other South-East Asian countries.

2. Literature review

2.1. History of hepatitis B virus

In the past, it was believed that living in bad conditions is the cause of catarrhal jaundice. In 1883, Lürmann observed an outbreak of jaundice (15%) in 1,289 shipyard workers within 2-8 months after receiving a smallpox vaccine prepared from human lymph nodes. Later on, in 1937, a larger outbreak of hepatitis occurred in a military camp where soldiers developed severe jaundice after receiving a yellow fever vaccine derived from human serum. It is only in the decade after World War II (1945) that clinical and epidemiologic studies for hepatitis began. Based on epidemiological studies, 2 types of agents were suggested to cause jaundice: 1) **type A** mainly transmitted via the faecal-oral route and 2) **type B** mainly transmitted via human serum and was called serum hepatitis and is now referred to as hepatitis B [26].

The hepatitis B surface protein was discovered accidentally in 1965 by a medical anthropologist, Baruch Blumberg (Nobel Prize in 1976 in Physiology or Medicine), and colleagues during their search for polymorphic serum proteins as genetic markers in the blood of an Australian aborigine [27] and was called Australia antigen (termed Au). They also identified this antigen Au in serum of patients with leukemia, leprosy, and hepatitis, although its relationship with hepatitis was initially unclear. At the same time, Prince et al. independently identified an antigen, termed SH (serum hepatitis related antigen), that appeared in the blood of patients during the incubation period of hepatitis, and which was later found identical to Au. Two year later the association between the occurrence of Australia antigen and hepatitis

infection was established. In the serum of patients suffering from type B hepatitis, Dane and colleagues [28] identified by electronic microscopy some large double-shelled virus-like particles of 42 nm diameter, called thereafter Dane particles (Figure 2.1) and showed they cross-reacted with antibodies against Australia antigen. Their significance as potential viral agent of hepatitis B was confirmed by the detection of antibodies against the inner shell (termed core or nucleocapsid) of the Dane particle in patients with acute hepatitis B. The core antigen was called HBcAg and the Australia antigen hepatitis B surface antigen (HBsAg); inducing corresponding anti-HBc and anti-HBs antibodies, respectively. These seminal studies made possible the serologic diagnosis of hepatitis B and opened up the field to rigorous epidemiologic and virological investigation [11].

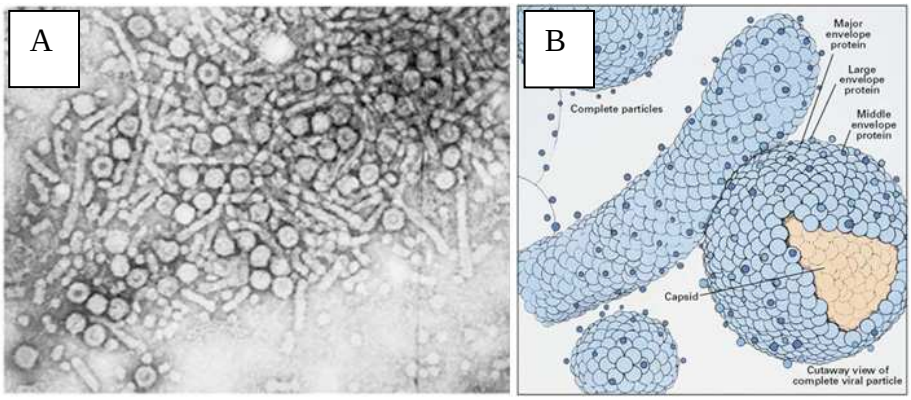


Figure 2.1. The three forms of HBV particles. (A) The electron micrograph shows whole virions (Dane particles), subviral sphere and filamentous forms. (B) The cartoon illustration shows the same features with more details of the surface antigen proteins. [source: modified from *Lee WM, New Eng J Med, 1997* [2]]

In May 2010, the World Health Organization had designated the 28 July, Blumberg's birthday, as "World Hepatitis Day" in order to provide an opportunity for education and greater understanding of viral hepatitis and diseases that it causes as a

global public health problem, to strengthen preventive and control measures in member countries and coordinate a global response to hepatitis

2.2. Biology of Hepatitis B Virus

2.2.1. Structure of the hepatitis B virus

Hepatitis B virions are double-shelled particles of 40 to 42 nm diameter. The envelope or surface consists of approximately 240 subunits comprising 3 different membrane-spanning proteins, termed large (L), middle (M), and small (S) surface proteins (HBsAg) (Figure 2.2). L-HBsAg consists of an S domain, preS1, and preS2 domains. The preS1 and preS2 domains of L-HBsAg are localized either at the viral surface or inside the virion. M-HBsAg contains only the S and preS2 domains and S-HBsAg consists only of the S domain. Because all proteins can be glycosylated at one or 2 positions, 6 different proteins can be distinguished, glycoprotein (GP) 42, protein (P) 39, GP36, GP33, GP27 and P24 kDa. These HBsAg proteins are overexpressed and assemble either in subviral sphere of 20-22 nm diameters or in filamentous form (Figure 2.2). These subviral particles are non-infectious particles because they contain only envelope glycoproteins and host-derived lipid. Their amount usually outnumbers that of virions by 1,000:1 to 10,000:1 [11]. Within the envelope is the viral nucleocapsid or core which encapsidates the viral genome. The core particles probably interact with the internally localized preS domain of HBsAg. The HBcAg consist of 185 amino acid localized inside the lumen of the particles. The core molecules form dimers in the cytosol. Ninety or 120 dimers assemble spontaneously, so that 2 populations of core particles appear, forming an icosahedral structure. [26]

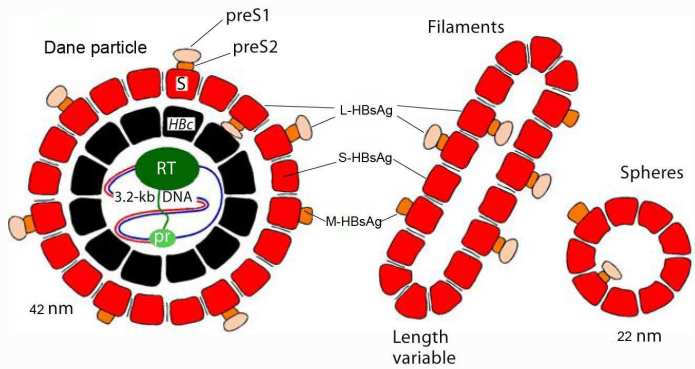


Figure 2.2. Schematic diagrams of the components of 42 nm-Dane particle, 22 nm-sphere and filamentous forms of HBV [source: modified from *Gerlich WH et. al., Dig Dis, 2010* [29]]

2.2.2. Classification of Hepadnavirus family

Hepatitis B virus belongs to the family of *Hepadnaviridae* (hepatotropic DNA virus). Hepadnaviruses preferentially infect liver cells, but small amounts of hepadnaviral DNA can be found in kidney, pancreas, and mononuclear cells. Infection at these sites, however, is not linked to extrahepatic disease [30]. The *Hepadnaviruses* are subdivided into two genera according to their host ranges (Figure 2.3), 1) the ***Orthohepadnaviruses*** found in mammals and 2) the ***Avihepadnaviruses*** found in birds. HBV is the prototype virus of the *Orthohepadnaviruses* and is genetically related to other species in this genus, such as woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and woolly monkey hepatitis B virus (WMHBV). The prototype virus of *Avihepadnavirus* is duck hepatitis B virus (DHBV), and other members of this genus are heron hepatitis virus (HHBV), snow goose hepatitis B virus (SGHBV), crane hepatitis B virus (CHBV), and stork hepatitis B virus (STHBV). [31]

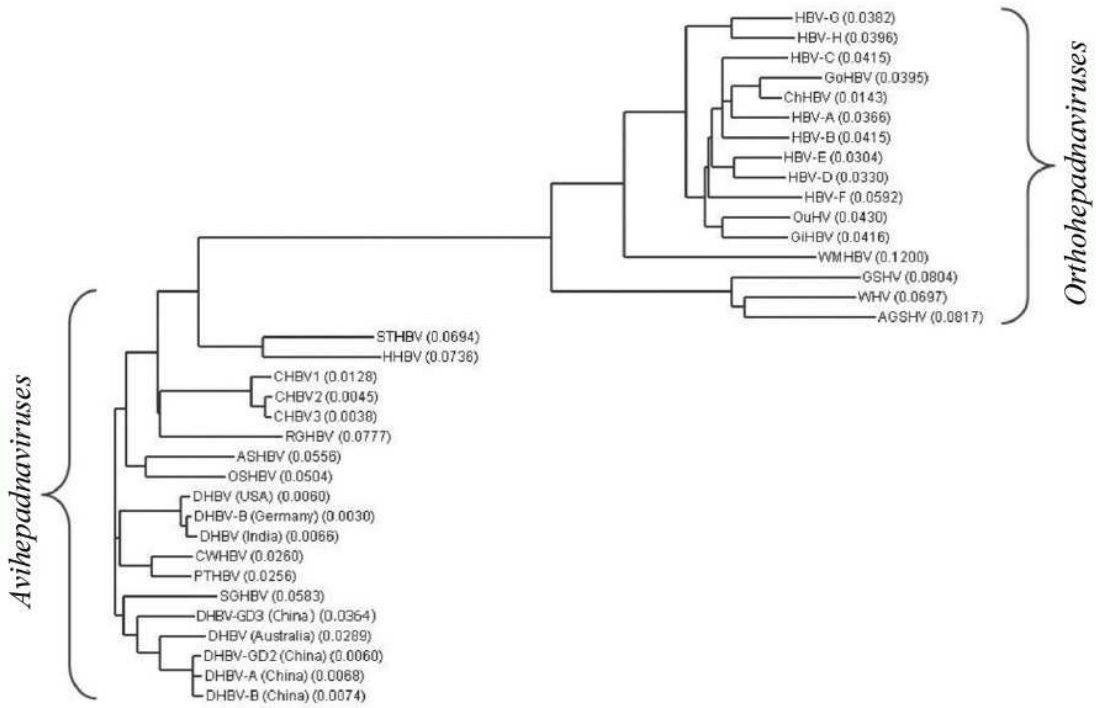


Figure 2.3. The phylogenetic tree of reference strains of *Orthohepadnaviruses* and *Avihepadnaviruses* [source: *Lüsebrink J et. al., Hepatology a clinical textbook, 2010* [31]] **Abbreviations:** AGSHV, arctic ground squirrel hepatitis virus; ASHBV, ashy headed sheldgoose HBV: CHBV, crane HBV; ChHBV, chimpanzee HBV; GiHBV, gibbon HBV; GoHBV, gorilla HBV; GSHV, ground squirrel hepatitis virus; CWHBV, chileo wigeon HBV; HHBV, heron HBV; OSHBV, orinoco sheldgoose HBV; OuHV, orangutan hepadnavirus; PTHBV, puna teal HBV; RGHBV, ross' goose HBV; SGHBV, snow goose HBV; STHBV, stork HBV; WHV, woodchuck hepatitis virus; WMHBV, woolly monkey HBV.

2.2.3. Genome of the hepatitis B virus

The genome of HBV is a 3.2 kb relaxed-circular, partially double-stranded DNA whose circularity is maintained by 5'-cohesive ends (Figure 2.4). Its structure is unusual, the two DNA strands are not perfectly symmetric. The full-length negative strand (blue solid line in Figure 2.4) is the template for the synthesis of the viral mRNA transcripts and its 5' end is covalently linked with the P-protein (hatched blue oval in Figure 2.4) whereas the positive strand is shorter and bears a capped

oligoribonucleotide at it 5’ end (red zigzag in Figure 2.4). Importantly, the 5’ ends of both strands DNA have short direct repeats (DRs) regions composed of 11 nucleotides. The 5’end of negative-stranded DNA maps within the repeat termed DR1, whereas positive-stranded DNA maps within DR2. These repeats are important for priming the synthesis of their respective DNA strands [32].

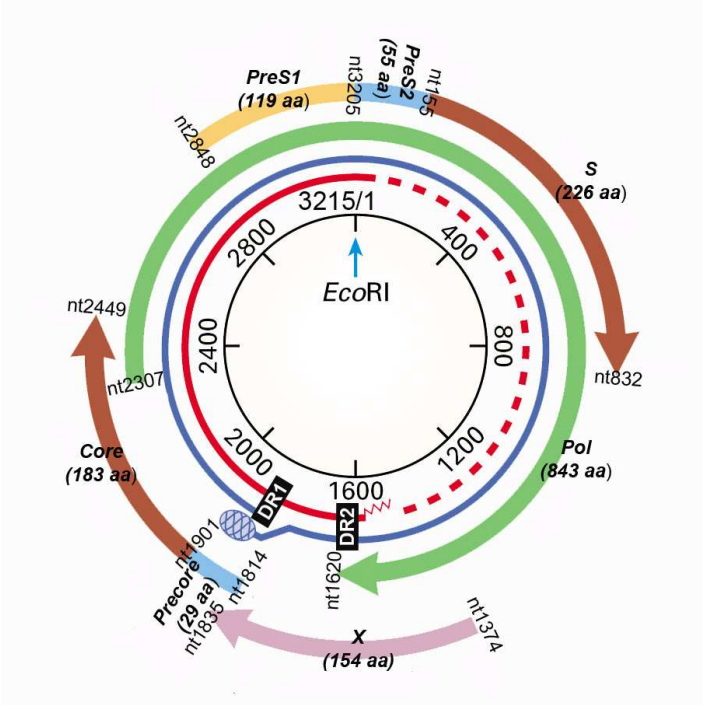


Figure 2.4. Genome organization of HBV genotype B or C, the 2 genotypes predominant in Thailand. [source: modified from *Lee WM, New Eng J Med, 1997* [2]]
Abbreviations: nt denotes nucleotide position; aa denotes amino acid

The coding organization of HBV DNA is highly compact: every nucleotide in the genome is within a coding region, and more than half of the sequence is translated in more than one frame. HBV DNA has the following four open reading frames (ORFs);

1) **The ORF-P (P region)** encodes the viral polymerase, and the terminal protein found on negative-stranded DNA. The viral polymerase consists of 4 domains: the priming domain, a so-called spacer, the reverse transcriptase domain (which

catalyses RNA-dependent DNA synthesis) and the RNase H domain (which degrades the RNA form the DNA-RNA hybrid). This multifunctional enzyme is involved in DNA synthesis and RNA encapsidation.

2) **The ORF-precore/core (preC/C region)** has 2 in-frame AUG codons. The internal initiation ORF, core region, encodes the 21-kDa C protein, the structural protein of nucleocapsid (HBcAg). Initiation at the upstream AUG encodes a 24-kDa C-related protein that is not incorporated into the viral membrane but instead is secreted from infected-cells, called HBeAg. Formerly, the preC region encodes a signal sequence, which directs the chain into the secretory pathway. As the chains traverse the Golgi complex, cleavage by cellular proteases generates HBeAg, a 16 kDa fragment that is secreted into the blood stream. The HBeAg contains a signal peptide that targets it to the endoplasmic reticulum for secretion into serum, while HBcAg does not contains a signal peptide and is incorporated into the virion. HBeAg function is still not clear: it plays no role in viral assembly, and is not required for the viral replication; mutants bearing chain-terminating lesions within the preC region arise frequently during natural infection and replicate well in culture. Unlike HBV particle, HBeAg can cross the placenta and may function as an immune tolerogen to HBV, possibly predisposing the fetus to the establishment of chronic HBV infection [2, 33].

3) **The ORF-pre-S/S (pre-S/S region)** encodes the viral surface glycoproteins (HBsAg). Pre-S region could be divided into 2 subregions (pre-S1 and pre-S2). The largest of HBsAg is the 39-kDa L-HBsAg protein, which is the product of initiation at the first AUG of the ORF. L-HBsAg is thought to play key roles in the binding of the virus to the host-cell receptor and the assembly of the virion and its release from the cell. Initiation at the second AUG generates the 31-kDa M-HBsAg

which function is unknown. The small HBsAg contains only the S domain, commonly called 24-kDa S-HBsAg and is the most abundant protein on viral surface. All HBsAg proteins share the common C-terminal S domain and differ principally by the length and structure of their N-terminal end (Figure 2.5). M-HBsAg accounts for 5-15% of the total circulating pool of S-related antigens, the L-HBsAg representing only 1-2%, the rest is S-HBsAg. Dane particles are substantially enriched for L-HBsAg.

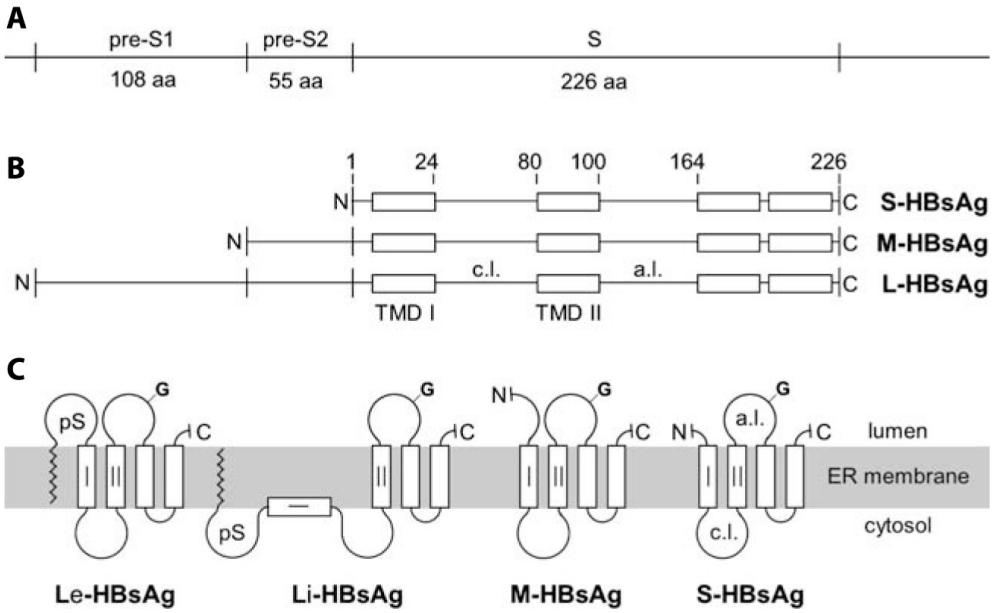


Figure 2.5. (A) Domains of the HBsAg open reading frame. (B) The L-, M-and S-HBsAg are translated from three in-frame initiation sites but sharing common C-terminal S domain. (C) Topology of the L-, M-and S-HBsAg at the endoplasmic reticulum (ER) membrane. The two forms of L-HBsAg are represented: the pre-S1 plus preS2 domains can reside on the cytoplasmic side of the ER membrane (Li-HBsAg), or it can be translocated through the membrane as found on the secreted particles (Le- HBsAg). The broken line indicates the myristate group linked to the amino terminus of L-HBsAg. Open rectangles represent trans-membrane domains (TMDs). Abbreviations: G denotes Glycosylation site; a.l. denotes antigenic loop; c.l. denotes cytosolic loop; pS denotes pre-S domain *(source: modified from Sureau C, Curr Top Microbiol Immunol, 2006 [34])*

4) **The ORF-X** encodes for the X-protein. The product of ORF-X is a complex regulatory protein which modulates host-cell signal transduction and can directly and indirectly affect host and viral gene expression. The activity of X-protein is absolutely required for replication and spread of the virus [11]. The X protein may play a role in the development of hepatocellular carcinoma (HCC) [35].

2.2.4. Replication cycle of the hepatitis B virus

Progress in understanding the molecular basis of viral replication became possible only in the late 1970s due to the advance of techniques for molecular cloning and the discovery of natural animal models of viral infection [11]. The cardinal feature of the *Hepadnavirus* replication is the replication of the DNA genome by reverse transcription of an RNA intermediate. Incoming HBV virions are bound by cell-surface receptors, the identity of which remains unknown (Figure 2.6) though. many protein candidates have been proposed such as. human squamous cell carcinoma antigen 1, immunoglobulin A receptor, asialoglycoprotein receptor, transferrin receptor, annexin V, fibronectin, and an 80-kDa membrane protein, reviewed in Xie's paper [36]. After membrane fusion, cores are presented to the cytosol and transported to the nuclease. There, their DNA genomes are converted to an episomal covalently closed circular form -called cccDNA, which serves as the transcriptional template for the host RNA polymerase II. This enzyme generates a series of genomic and subgenomic RNA transcript. The amount of cccDNA is maintained at about 5–50 copies per hepatocytes [37]. All viral RNA is transported to the cytoplasm, where its translation yields the viral envelope, core, and polymerase proteins, as well as the X and preC polypeptides. Next, nucleocapsids are assembled in the cytosol, and during this process a single molecule of progenomic RNA

(pgRNA) is selectively incorporated into the assembling viral core. Once the viral RNA is encapsidated, reverse transcription by the co-packaged P protein begins to generate new relaxed circular DNA (rcDNA) genomes. The first DNA strand is made from the encapsidated RNA template. During or after the synthesis of first strand, the RNA template is degraded and the synthesis of the second DNA strand proceeds with the use of the newly made first DNA strand as a template. Some cores bearing the mature genome are transported back to the nucleus, where their newly generated DNA genomes can be converted to cccDNA to maintain a stable intranuclear pool of transcriptional templates. Most cores, however, bud into regions of intracellular membranes bearing the viral envelope proteins and during that process acquire lipoprotein envelopes containing the L-, M-, and S-HBsAg and are then exported from the cell as progeny virions [11, 38, 39].

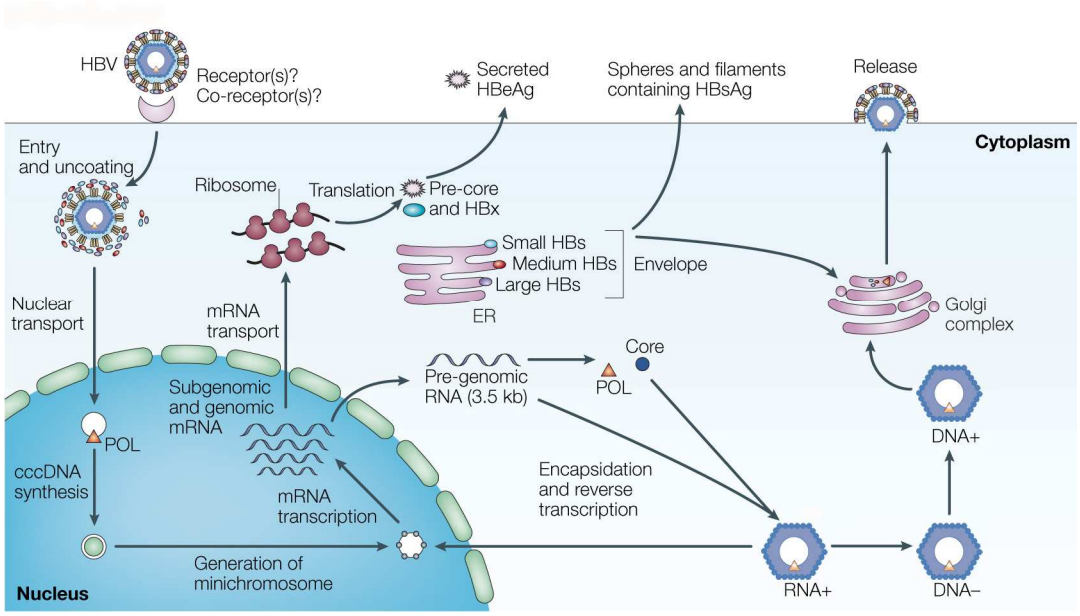


Figure 2.6. Life cycle of HBV (source: modified from *Rehermann B & Nascimbeni M, Nat Rev Immunol, 2005* [40])

2.3. Viral quasispecies of hepatitis B virus

As described earlier, the step of HBV replication cycle that provides high rates of mutation is the reverse transcription from pgRNA to single stranded DNA. Indeed, the reverse transcriptase lacks 3'-5' proofreading activity, which allows mutations to occur make. HBV exhibits a mutation rate approximately >2 x 10^4 base substitutions/site/year, 100-fold higher or more than other DNA viruses; but about 1000 times lower than that for RNA viruses [8]. Furthermore, accuracy of replication by the reverse transcriptase has been shown to vary with intracellular deoxynucleotide triphosphate concentrations [41]. The high rate of mutations result in the coexistence of variant viruses genetically linked called "quasispecies [22, 42]. According to Carman's proposal, two types of viral diversity can be identified;

1) "Variants" [43, 44] or **"Genotypic diversity"** [45, 46] is used to describe natural subserotypes that occur without selection pressure and have geographical differentiation. Identification is based on monoclonal antibodies and corresponds to replacement of one or only few amino acids. Viral fitness is the most important factors.

2) "Mutants" [43, 44] or **"Phenotypic diversity"** [45, 46] is restricted for variant viruses that emerge under selection pressure, as is the case with human intervention such as vaccination or antiviral therapy. Mutations are usually observed in four principle groups: vaccines recipients, patients infected with serologically non-reactive virus, patients on treatment with Hepatitis B immunoglobulin (HBIg) therapy, and during chronic infection with or without immunosuppression.

Although this distinction may be valuable in a working hypothesis, it is considered somewhat artificial by some researchers [42, 47]. The reason is that it easily leads to confusion, since the expression "genetic variation" has other meanings

and is usually reserved for the phenotypic differences among individuals in a population. Moreover, the origin of the changes is often unknown and makes the definitions difficult to apply. In some cases it remains unclear whether “variants” are selected from pre-existing minority species or arise as a result of mutational events, for example, immune pressure. Thus, they proposed the terms “mutant” and “variant” can both be better used for describing all genetically heterogeneous viruses, irrespective of the underlying causal mechanisms. However, in this thesis, the HBV quasispecies will be classified into two classes; 1) the variants and 2) mutants**.**

2.3.1. Hepatitis B virus variants

2.3.1.1. Genotypes and subgenotypes of hepatitis B virus

The genotypes of HBV are defined by a divergence between groups of 8% or more in the complete genome sequence and 4% or more in the S gene. HBV is classified into 10 genotypes, from A through J [48] with I and J, recently, in Laotian, Vietnamese, and Japanese patients [49-51]. Each genotype has different length of viral genome and viral proteins as described in Table 2.1. Some genotypes are further divided in subgenotypes. The subgenotype is used to identify subgroups of HBV genotypes with inter-group nucleotide differences between 4% and 8% across the complete genome. “Clade” is used for divisions within subgenotypes showing less than 4% nucleotide difference [48]. Moreover, genetic recombination between genotypes occurs in geographical regions where different genotypes co-circulate and provides a mechanism of variation within individuals and at the population level. Genotype A and D recombinants have been found in India [52], and genotype G and C recombinants in Thailand [53].

Table 2.1. Comparison of the length of viral genome and viral proteins between each HBV genotype [45]

Genotype	Number of amino acid of						
	Genome (nt)	Pol	HBcAg	PreS1	PreS2	HBsAg	HBxAg
A	3221	845	185	119	55	226	154
B	3215	843	183	119	55	226	154
C	3215	843	183	119	55	226	154
D	3182	832	183	108	55	226	154
E	3212	842	183	118	55	226	154
F	3215	843	183	119	55	226	154
G	3248	842	195	118	55	226	154
H	3215	843	183	119	55	226	154
I	3215	843	183	119	55	226	154
J	3182	832	183	108	55	226	154

Geographical distribution of HBV genotype

The distribution of HBV genotypes varies across regions and with population migration [54] (Figure 2.7 and Table 2.2). Genotype D is ubiquitous, scattered worldwide, but predominates in the Mediterranean area, whereas genotype A is prevalent in sub-Saharan Africa, North America, and Europe. Genotypes B and C are common in Asia and Oceania. Genotype E is mainly restricted to western Africa, and genotype F is found in aboriginal populations in Central and South America [55]. Genotype G has been detected infrequently, and limited to HBV carriers in Europe and USA [56-59]. Genotype H is confined to the Amerindian populations of Central America and Mexico [55, 60, 61].

In Thailand, several studies have reported the high prevalence of HBV genotype C (54-94%) over genotype B (4-24%),. Genotype A has also been found though with lower frequencies between 1-22%. [18, 62-65]

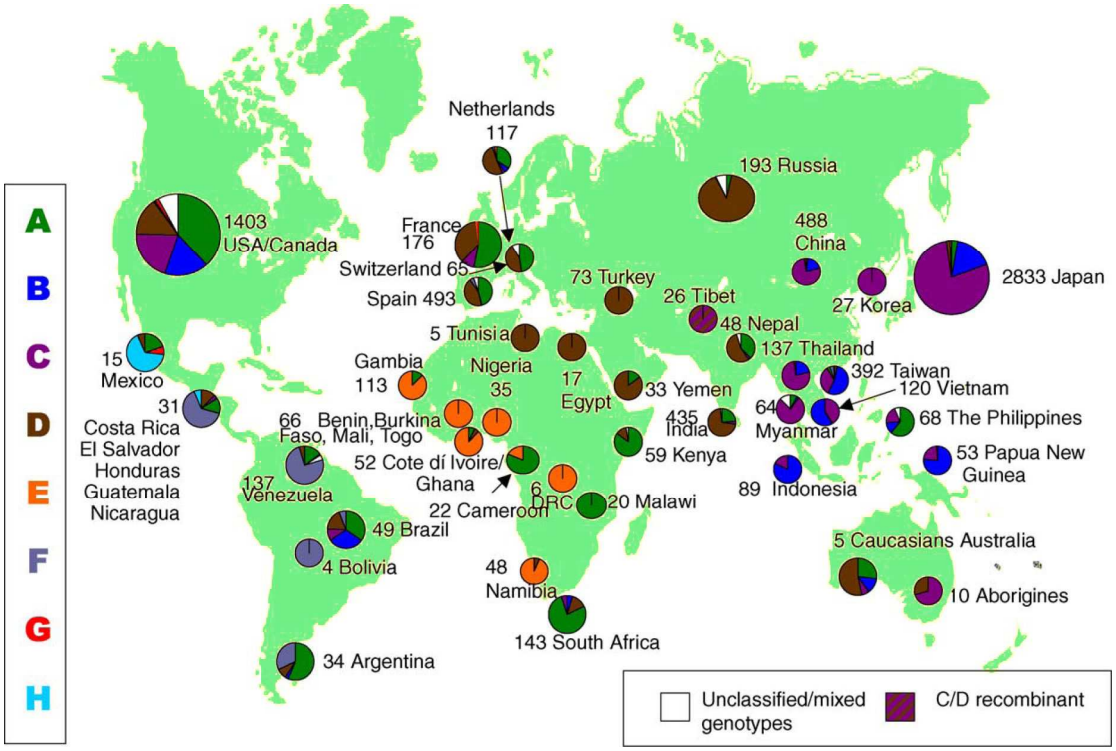


Figure 2.7. Global distribution of 8 genotypes of hepatitis B virus. The numbers next to the pie chart are the number of isolated genotypes (source: Kramvis et al., 2005[48])

Table 2.2. Relationship between genotypes and serotypes, geographical distribution [45, 48]

Genotype	Subgenotype	Serotype	Geographical distribution
A	A1 (Aa)	*adw2*, *ayw1*	Africa, Asia
	A2 (Ae)	*adw2*, *ayw1*	Northern Europe, North America
B	B1 (Bj)	*adw2*	Japan
	B2 (Ba)	*adw2*, *adw3*	Rest of Asia, Thailand
	B3	*adw2*, *ayw1*	Indonesia, China
	B4	*ayw1*, *adw2*	Vietnam, Cambodia
C	C1	*adrq+*, *ayr*, *adw2*, *ayw1*	Far-East, Thailand, Vietnam, Myanmar
	C2	*adrq+*, *ayr*	Far-East Japan, Korea, China
	C3	*adrq−*, *adrq+*	Pacific Islands
D	D1	*ayw2*, *adw1*, *ayw1*	Europe, Middle-East, Egypt, India, Asia
	D2	*ayw3*, *ayw1*	Europe, Japan
	D3	*ayw3*, *ayw2*, *ayw4*	Europe, Asia, South Africa, USA
	D4	*ayw2*, *ayw3*	Australia, Japan, Papua New Guinea
E		*ayw4*, *ayw2*	Sub-Saharan Africa, UK, France
F	F1.1 (FIa)	*adw4*, *ayw4*	Central America
	F1.2 (FIb)	*adw4*	Argentina, Japan, Venezuela, USA
	F2 (FII)	*adw4*	Brazil, Venezuela, Nicaragua
	F3 (FIII)	*adw4*	Venezuela, Panama, Columbia
	F4 (FIV)	*adw4*	Argentina, Bolivia, France
G		*adw2*	USA, Germany, Japan, France
H		*adw4*	USA, Japan, Nicaragua
I			Laos, Vietnam
J		*ayw*	Japan

HBV genotype and disease severity

Several studies have attempted to link a particular genotype to severity of the liver disease, but the results are controversial. In India, where genotypes A and D co-exist, genotype A, as compared to genotype D, is more often associated with ALT elevation, HBeAg positivity in patients aged ≥25 years, and cirrhosis [66]. A Swiss study also demonstrated that progression from acute to chronic hepatitis is more likely to occur in patients infected with genotype A than in those with genotype D [67]. In contrast, the study conducted in Spain among patients infected with genotype A, D, and F showed the rates of sustained biochemical remission and clearance of HBV DNA and HBsAg were higher in patients infected with genotype A than in those with genotype D or F though HBeAg seroconversion rates were similar in patients infected with genotypes A and D. Moreover. Sanchez-Tapias et al., reported higher rate of liver disease-related deaths with genotype F than with genotype A or D [68]. Livingston et al. confirmed that genotype F was associated with the occurrence of HCC, compared to A, B, C, or D [69].

In Asian countries where genotypes B and C are predominant, patients infected with genotype C were more often HBeAg positive, experienced delayed HBeAg seroconversion, had more severe liver disease and exhibited earlier progression of cirrhosis and HCC than those infected with genotype B [65, 70, 71]. In Taiwan, Kao et al. also reported that genotype C (mostly serotype *adr*) was associated with the development of cirrhosis and HCC [72, 73]. However, another study in Thailand found no differences in the risk of developing HCC between patients infected with either of these 2 genotypes [65].

Impact of HBV genotype on HBeAg seroconversion was assessed in several studies. The first one conducted in China showed that patients infected with genotype B had a higher cumulative rate of spontaneous HBeAg seroconversion (HBeAg to anti-HBe) than those infected with genotype C [74]. Other studies conducted among children and adults in Taiwan showed that 50% of anti-HBe seroconversion seem to occur before age of 10 years when children are infected with genotype E, later before age 20 when they are infected with genotypes A and D, at around 30 years when they are infected genotype B and at older age 40 years with genotype C [75].

HBV genotype and anti-viral therapy response

Genotypes may also influence the outcome of treatment. In a multicenter trial of pegylated interferon [76], patients infected with genotypes A and B had a higher rate of HBeAg loss as compared to patients with genotype C or D, 45% vs. 26%. This finding was confirmed in Taiwan [73] but not in a Japanese study where, genotype B and C carriers responded well to interferon treatment, while genotype A carriers responded poorly [77]. This discrepancy may be due to the type of interferon used, the HBV serotypes, or to the small number of treated patients in the Japanese study [78].

In India, after 12 months-treatment with lamivudine, patients with genotype D achieved higher sustained viral response rate (HBV DNA negativity at 18 months) than those with genotype A [79]. Studies in Taiwan [80] and Japan [81] showed that patients with HBV genotype B have a better virological response to lamivudine as compared to genotype C. In contrary, Yeun et al. [82] in Hong Kong, found no

differences in the virological response to the 12 months of lamivudine therapy between the patients with genotypes B or C.

In Europe, HBV genotype A (serotype *adw*) is associated with a higher risk of lamivudine resistance and more rapidly resistance than genotype D (serotype *ayw*) in both HBV mono-infected patients or patients co-infected with HIV [83-86]. Genotypes B and C have a similar risk in developing lamivudine resistance [80-82]. In addition, some studies demonstrated that subgenotype Ba had higher risk to develop lamivudine resistance than subgenotype Bj [87].

At present, HBV genotyping is not a standard test for management of HBV infected patients. However, if more evidence that HBV genotype can affect disease progression or treatment prognosis accumulates, it may become so in the future.

2.3.1.2. Serotypes and subserotypes

To avoid confusion and ensure uniformity in the terms “serotype” and “serological subtypes” should be used synonymously to define the antigenic determinants of HBsAg instead of the term “subtype” [48].

After discovery of the determinant *“a”* of Australian antigen by Blumberg et al., in 1965, further research revealed the immunological heterogeneity of this antigen. Indeed, sera of patients who had seroconverted to anti-HBs did not react in the same way to HBsAg from different chronic carriers. This different reactivity was due to the viral variability and HBV isolates were therefore classified into serotypes based on the reactivity of the patient isolate HBsAg with standard panels of antisera

[45]. Two pairs of allelic variations, *"d/y"* and *"w/r"*, were discovered in 1971 and 1972, respectively, leading to the 4 serotypes adr, adw, ayr, ayw. The *"a"* determinant was further refined with 4 sub-determinants of *"w" (w1-w4)* into 8 serotypes; *ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4* and *adr*. Following the discovery of the *"q"* determinant in 1975, *adr* was subdivided into *"q+"* and *"q-"*. The tenth serotype, *adw3*, was described in 2002 [88]. Serotyping is useful for epidemiological studies, including those on nosocomial and iatrogenic infections and intra-familial transmission.

Relationship between HBV genotypes and serotypes

The development of DNA sequencing methodologies has facilitated the identification of amino acids in HBsAg responsible for the different reactivity patterns to monoclonal antibodies. There is a certain correlation between serotype and genotype, but it is far from perfect (Table 1.2). The combination of amino acids present at 7 positions on HBsAg seems to determine the serotype. The two major serotype epitopes are the *"d/y"* and *"r/w"* determinants. Both determinants are comprised of two mutually exclusive epitopes that depend on the amino acid at positions 122 and 160 of HBsAg. If the amino acid at position 122 is Arg (R122) then the serotype is *"y"*, and if it is Lys (K122) then the serotype is *"d"*. Similarly, R160 defines the *"r"* serotype and K160 defines the *"w"* serotype. The amino acids at position 127 were responsible for *w2-w4* reactivities. Phe134, Ala159 or both, are involved in *w1* reactivity and recent research suggests that position 140 may be more important in resolving *w1* reactivity than position 134. Position 177 is involved in *"q"* reactivity of *adr* specimens and position 178 is involved in *"q"* reactivity of *adw4* specimens (Table 2.3).

Table 2.3. Amino acid residues specifying HBV serotypes [89]

Amino acid position on HBsAg protein							Predicted
122	**127**	**134**	**159**	**160**	**177**	**178**	**HBsAg subtype**
K	P	F	A	K	V	P	*adw2*
K	T	F	A	K	V	P	*adw3*
K	L	F	G	K	V	Q	*adw4q-*
K	P	F	A	R	V	P	*adr*
K	P	F	V	R	A	P	*adrq-*
R	P	F	A	K	V	P	*ayw1*
R	P	Y	G	K	V	P	*ayw2*
R	T	F	G	K	V	P	*ayw3*
R	L/I	F	G	K	V	P	*ayw4*
R	P	F	A	R	V	P	*ayr*

* Amino acid abbreviations see appendix

2.3.2. Hepatitis B virus mutants

2.3.2.1. Basal core promoter and Pre-core mutants

The core promoter plays a central role in HBV replication and morphogenesis, directing the transcription of both pregenomic RNA and precore mRNA. The double mutations in the basal core promoter (BCP) region at nucleotide 1762 and 1764 (ntA1762T and ntG1764A) result in diminished production of HBeAg because the mutated BCP cannot longer bind a liver-enriched transcription factors and that the transcription of only precore RNA and accompanied by an increase in progeny virus production [90]. These double mutations are often present in patients with advance liver diseases [91-93]. Some studies revealed that an association between BCP mutation and HCC, though for HBV genotypes. A, C, D but not for genotype F [69]. Other less frequent BCP mutants associated with fuminant hepatitis and HCC have

been described, such as a mutation at nt1653 [94, 95] and at nt1753-1757 [96, 97]. A number of deletions of the BCP have also been reported [42, 98-100].

The most frequently observed pre-core mutation is a G to A transversion at nucleotide 1896 (ntG1896A) resulting in the cessation of HBeAg expression, so-called pre-core stop codon mutants. The less common pre-core mutants resulting HBeAg negativity include initiation codon mutations (nt1814 or 1815), a nonsence mutation at nt1874, a missence mutation at nt1862, frame shift mutations [42]. The presence of the G1896A mutation is restricted to specific viral genotypes which harboring a T nucleotide at position 1858 (B, C, D, and E). These HBV genotypes are not uniformly distributed around the world. This mutation is more prevalent in geographic regions where genotypes B, C, and D are predominant, such as Asia and the Mediterranean area, where it can be detected in more than 50% of individuals with chronic hepatitis B. It is less prevalent in North America and Europe (12-27%), where genotype A is more common [41, 101, 102].

In Asian countries, BCP and pre-core mutants are commonly found. In a recent study from China, 38% of the HBeAg-negative patients harbored the pre-core stop codon, 42% had the double BCP mutations and 12% had both mutations [103]. In Thailand, the rate of double BCP mutations was 76% (19/25) and of pre-core stop codon mutation was 24% (6/25) in HBeAg-negative chronic hepatitis patients [104]. Similar results were reported by Tangkijvanich et al; of the 24 PCR-positive HBeAg-negative patients, 18 (75%) had mutations in the BCP region and 8 (33.3%) had pre-core stop codon and one (4.2%) displayed a deletion between nucleotides 1758-1772 [105].

2.3.2.2. Core mutants

The core gene contains both humoral and cytotoxic T-cell epitopes. Mutations within immunodominant cytotoxic T-cell epitopes may be exploited by viruses to evade protective immune responses. HBV core gene deletions may alter core protein, thereby decreasing immune recognition by cytotoxic T cell and contributing to HBV immune escape [106]. Furthermore, Ehata et. al. observed that all patients with fulminant or severe hepatitis exhibited core mutations, but not all exhibited pre-core mutations, suggesting that core mutations may be more virulent than pre-core mutations and thus play an important role in the pathogenesis of hepatitis B viral disease [107]. HBV with extensive core gene deletion mutants (resulting in the absence of core nucleocapsid protein) would be unable to produce viable virus and they probably replicated in the presence of low levels of wild-type HBV [41]. In Taiwan, core gene deletions were detected in 5% of 365 HBV infected children which can appear as early as the age of 5 years. The duration of their appearance ranges from 0.5 to 5 years. Horizontal rather than perinatal transmission of HBV was a favorable factor for these mutants to develop [108].

2.3.2.3. X gene mutants

The HBx protein exhibits numerous activities affecting intracellular signal transmission, gene transcription, cell proliferation, DNA repair, and apoptosis. HBx may play a role in persistence of HBV infection and in the development of HCC [42]. A number of deletions in the X gene have been described, e.g. an eight-nucleotide deletion at the 3’ end of the X gene and within the core promoter/enhancer II (CP/ENII) region (nt1770-1777) [109] and a 20 nucleotide deletion at nt1752–1772

[110], in HBV PCR-positive/ HBsAg and HBeAg-negative patients. These deletions have been shown to suppress HBV DNA replication and expression of HBV proteins, resulting in HBsAg negativity. These two mutations were frequently found in patients with the severity of liver diseases [109], suggesting that these mutations may play a certain role in the pathogenesis of HBV infection.

2.3.2.4. PreS1 and PreS2 mutants

Numerous deletions or mutations in the preS regions have been described: deletions of up to one-half of the entire preS1 region, deletion of the preS2 translation stop codon and other codons (entirely preventing the expression of the preS2 protein), numerous point mutations, and a series of small deletions and insertions. Some deletions not only eliminated the preS2 promoter region, but also sites of B and T cell recognition. In contrast, the hepatocyte-binding site located in the preS1 region was conserved. Deletions in this region would lead to impaired virus clearance without affecting HBV attachment to the hepatocytes and their subsequent penetration, and therefore could contribute to the development of chronic hepatitis [42]. Some mutations in preS regions appear to be associated with the development of HCC, for example, the preS2 mutation (F141L) of HBV genotype C [111]. Other mutations such as P110S in preS1 region, P36L in preS2 region and C107R in S region have been proposed to be associated with intrauterine infection, permitting the infection in fetuses more readily [112], but its significance need to be confirmed.

2.3.2.5. S mutants

HBsAg is the major envelope lipoprotein and the main target for viral neutralization, either by vaccine-induced antibodies or passive anti-HBV

immunoglobulin. HBsAg (S-HBsAg) is composed of 226 amino acids (aa) but its three-dimensional structure is not fully elucidated. Its central core, composed of amino acids 99–169, is referred to as the major hydrophilic region (MHR), is exposed at the surface of the virus and is involved in binding to antibodies directed against HBsAg. Carman W.F. has proposed the cysteine web model to explain the structure of the MHR of HBsAg. In this model, potential disulphide bridges between eight highly conserved cysteines at position 107, 121, 124, 137, 138, 139, 147 and 149 forming 2 loops (aa107-138 and aa139-147) external to the virion and probably in opposition, and another tight loop between aa121 and aa124 [43, 113] (Figure 2.8). The MHR can be separated into at least five functional areas corresponding to antigenic epitope clusters, indicated as HBs1 (upstream of aa120), HBs2 (aa120-123), HBs3 (aa124-137), HBs4 (aa139-147) and HBs5 (aa149-169). Antibodies found in vaccinated people and those used in monoclonal antibody based-immunoassays for HBsAg, are directed against these regions; in particular, to a cluster of B-cell epitopes called the "*a*" determinant, which comprises two loops of amino acids 124-147 [42, 44, 45, 114, 115].

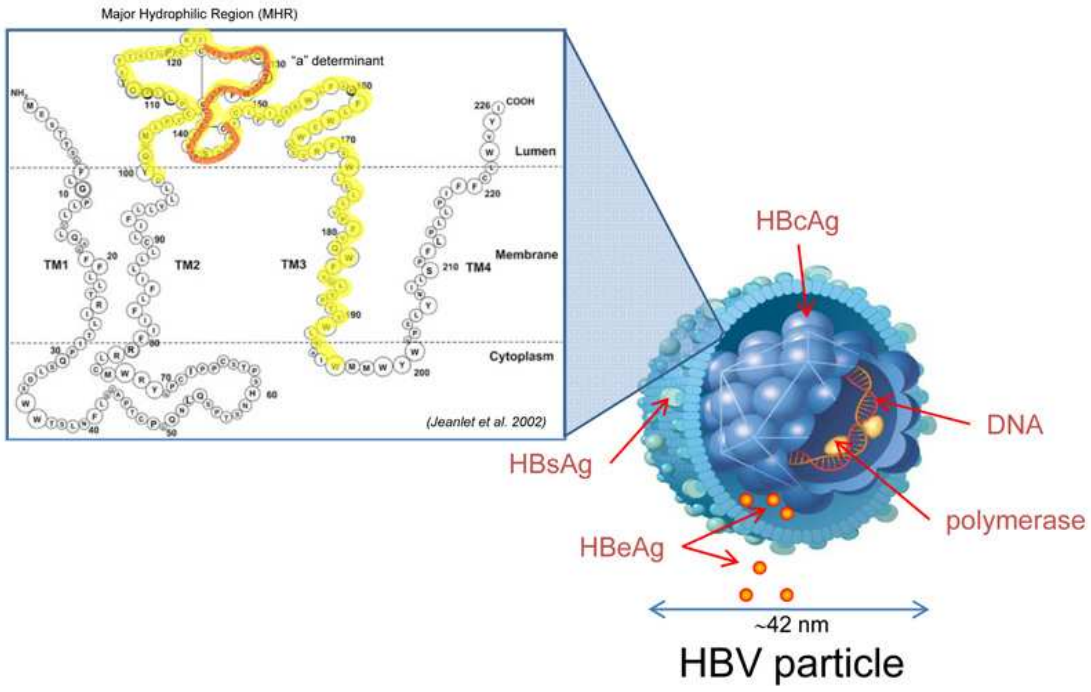


Figure 2.8. Schematic diagram of the secondary structure with amino acids components, location of major hydrophilic region and "a" determinant region of surface antigen of hepatitis B virus genotype C, accession number AF068756.1.

Point substitutions in the S gene are very important because they affect the antigenicity of HBsAg, especially the "a" determinant [114, 115]. This "a" determinant is the major immune target of polyclonal antibodies directed to HBsAg. The most common escape mutation is a glycine to arginine substitution at amino acid 145 (sG145R), caused by a guanosine to adenosine substitution at nucleotide position 587 (nt587), that was identified from HBsAg vaccinated persons and patients with liver transplantation [116-118] but also in natural isolates. Other mutations at aa116, 120, 123, 124, 126, 129, 130, 133, 141, 142, 143 and 144 occuring alone or in combination have also been reported [44, 119-122]. Changes of amino acids in surface proteins and their impacts that have been previously repoted are summarized in Table 2.4.

Consequences of mutations in HBsAg are clinically important in both HBV prevention (through vaccination) and diagnosis. Efficacy of HBV vaccine may decrease in the long term if vaccine escape mutants were to spread. Evaluation of large scale HBV vaccination programs in endemic regions has revealed a 2-3% incidence of vaccine escape mutants resulting from mutations in the HBsAg protein, particularly the sG145R. Patients infected with HBV harboring surface mutations in "a" determinant region may not be found positive for HBsAg since HBsAg mutants are not detectable by many HBsAg diagnostic assays, especially in the context of the *y* serotype. These mutations are thus of great public health significance because patients harboring HBV with these surface mutants do not exhibit quantifiable HBsAg, but remain infectious and remain detectable by HBV-DNA and/or HBeAg testing [41]. New generations of diagnostic kits have been developed to overcome this problem. However, monitoring of the capacity of the diagnostics kits to detect HBsAg mutants may be needed over time.

Table 2.4. Changes of amino acids in S proteins and their impacts

HBsAg region	Amino acid position number	Wild type residues	Genotype	Amino acid changes found in association with		
				Vaccine escape [reference]	Escape to HBIg [reference]	Misdiagnosis [reference]
HBs1	105	**A**			**P** [43]	
	114	**T** **S**	A, F B, C, D, E		**R** [43]	
	115	**T**				**S** [43] **A** [123]
	116	**T**		**N** [124]		**S** [43]
	118	**T**			**A** [43] **R** [125]	**S** [43]
HBs2	120	**P**		**E** [126] **S** [127]	**E** [128] **T** [125] **N** [43]	**T** [43, 129] **S** [130, 131] **Q** [130]
	121				**S** [125]	**S** [129]
	122	**R** **K**	D, E A, B, C, F			**N** [123]
	123	**T**			**N** [43]	**A** [123] **N** [129, 131]
HBs3	124	**C**			**R** [43] **Y** [43]	**F** [129] **Y** [129] **I** [129]
	125	**T**		**M** [132] **A** [133]		
	126	**T** **I**	A, B, D, E, F C	**A** [43, 133] **N** [134] **S** [134] **T** [132, 135]	**A** [136] **N** [128]	**I** [43] **N** [123, 129] **S** [137]
	127	**P**		**T** [132, 133]		
	129	**Q**		**H** [43, 133] **R** [19, 138]	**H** [136]	**N** [123]
	130	**G**			**R** [43]	**R** [123]
	131	**T** **N**	B, C, D, E, F A	**I** [133]	**S** [43]	**I** [43, 123, 139]
	133	**M**	 D	**L** [138]	**I** [43] **L** [136]	**I** [43] **T** [123] **L** [129]
	134	**F** **Y**	A, B, C, E, F D		**Y** [128]	**L** [123] **N** [43]
	135	**P**				**S** [43, 139]
	137	**C**			**Y** [140] **R** [125]	**W** [129]
	138	**C**				**Y** [123]

HBs4	139	**C**			**S** [125]	**S** [129]
	140	**S** **T**	E, F A, B, C, D		**T** [43]	
	141	**K**		**E** [43]	**I** [43]	**E** [139]
	142	**P**		**S** [141]	**S** [139]	**S** [123, 137] **L** [129, 137, 139]
	143	**T** **S**	A B, C, D, E, F	 **W** [133]		**L** [130, 131]
	144	**D**		**A** [120]	**A** [136, 142] **G** [140, 143]	**E** [130, 144] **A** [129, 137, 139]
	145	**G**		**R** [43, 133, 141] **A** [19, 141]	**R** [124, 136, 143]	**R** [131, 137, 139] **K** [123, 139] **A** [129]
	146	**N**		**S** [133]	**S** [43]	**S** [123]
	147	**C**			**S** [43]	**F** [123] **R** [123] **Y** [129]
HBs5	148	**T**		**I** [133]		**H** [139]
	154	**S**				**T** [43] **W** [139]
	155	**S**				**Y** [43] **P** [43]
	156	**W**		**L** [133]		
	157	**A**				**R** [43]
Others	216	**L**				**Stop** [145]
	164+195	**E, I**				**D, M** [145]

2.3.2.6. Pol gene mutants

The Polymerase (P) protein is translated from the pgRNA and is essential for viral replication. The P gene has at least four domains; N-terminal domain, spacer, polymerase, and C-terminal domain (Figure 2.9). The terminal protein encoded in the N-terminal domain is linked to the 5'-end of the minus strand of virion DNA and is necessary for priming of minus strand synthesis. The polymerase domain encodes the reverse transcriptase enzyme. The C-terminal domain encodes RNase H. HBV polymerase is functionally and structurally similar to HIV reverse transcriptase, it has a right-handed configuration with thumb, palm, and fingers domains. This enzyme has

7 subdomains (A-G). The Domains A and D, involved inbind to deoxynucleoside triphosphate (dNTP), correspond to the fingers structure. The domain C includes the tyrosine-methionine-aspartate-aspartate (YMDD) motif at the active site, which participate directly in catalysis. The triphosphates of the nucleotide substrates are catalysed at the active site of polymerase. The domains B and E, involved in binding to the template or primer, correspond to the palm structure and thumb structure. Domains F and G are upstream of domain A. This region may be involved in interactions with the incoming dNTP and also with the template nucleotide [146].

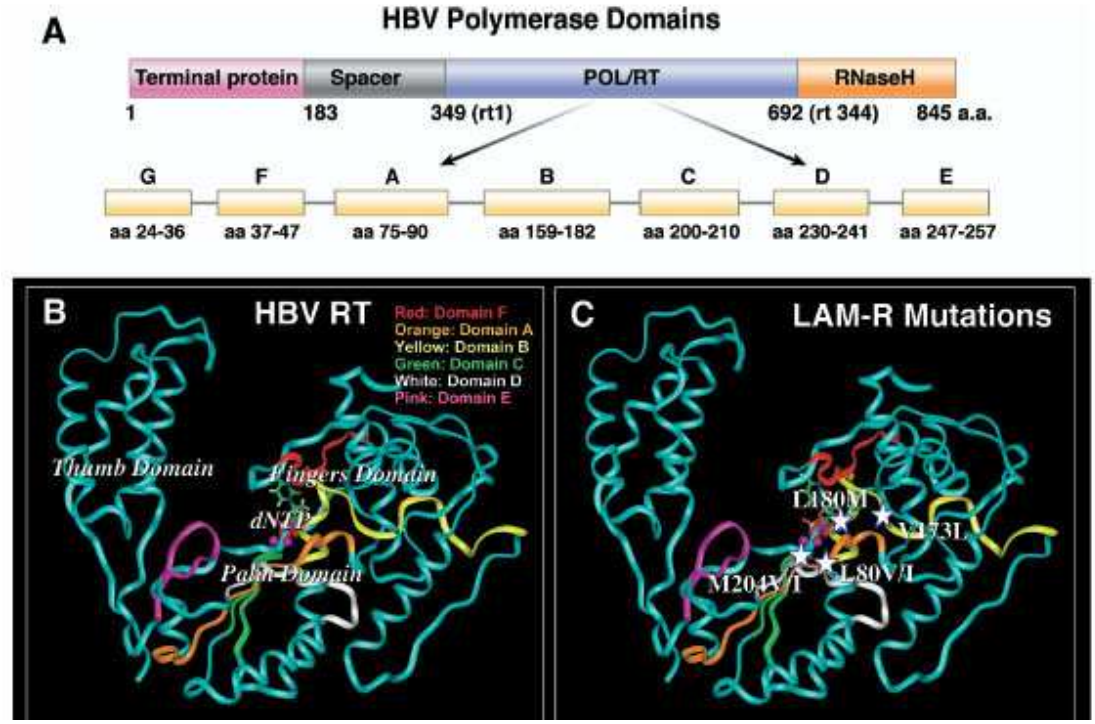


Figure 2.9. (A) Illustration of the HBV polymerase open reading frame with the 4 functional domains and the 7 catalytic subdomains A–G. (B) Proposed structure of the HBV polymerase based on the model of HIV-1 reverse transcriptase. (C) Location of the major lamivudine mutations relative to the conserved domains (source: modified from *Ghany et al. Gastroenterology, 2007 [146]).*

The recent development of safe and efficacious antiviral nucleos(t)ide analogues has changed the therapy for chronically HBV infected patients. Mutations of the

polymerase gene may be associated with resistance to nucleos(t)ide analogues and viral persistence. Lamivudine (LAM) is the first licensed anti-HBV drug and most commonly used to treat HBV. LAM is a potent inhibitor of RNA-dependent DNA polymerase of HBV, irreversibly blocking reverse transcription and inhibiting viral replication. However, long-term treatment with LAM may lead to resistance mutations that disrupt the YMDD catalytic site of the polymerase gene. Mutations leading to lamivudine resistance have been reported, e.g. the mutation consists of methionine either to valine (rtM204V) or isoleucine (rtM204I) substitution [147] or leucine to methionine (rtL180M) change in the B domain, which occurs often in association with the M204V mutation. The replicative capacity of the rtM204V, rtL180M+M204V, or rtM204I mutants is markedly decreased compared to that of wide-type HBV [148]. Besides these mutations can cause failure of lamivudine-treatment, they can also result in cross-resistance to other anti-HBV drugs to which virus has never been exposed to (e.g. Telbivudine, Entecavir). In addition, not only lamivudine-induced mutations, the HBV mutations associated with other nuclos(t)ide analogues have been reported [146]. For example, adefovir may select polymerase resistant mutation in the B domain (rtA181T/V) and the D domain (rtN236T) [149].

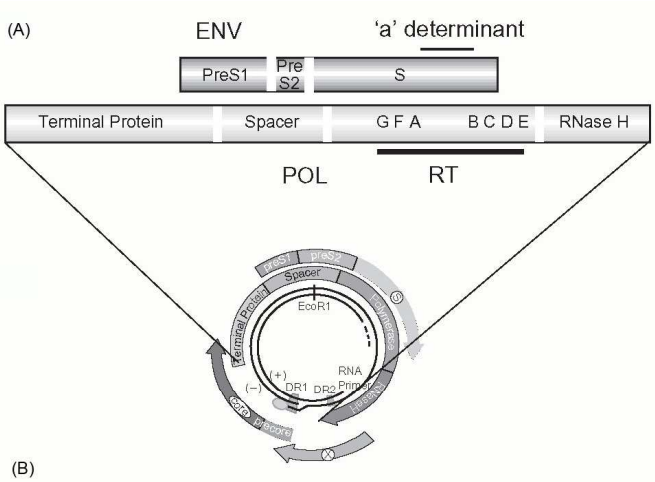
2.3.2.7. Overlapping of surface gene and polymerase gene

Due to the overlapping of the polymerase gene and envelope gene of HBV, some mutations selected during antiviral resistance cause concomitant changes in overlapping reading frame, and consequently altering the C-terminal region of HBsAg (Figure 2.10A). For example, the rtM204V mutations, induced by LAM, is associated with a change at sI195M in the HBsAg, while the rtM204I mutation is associated with three possible changes, sW196S, sW196L, or a termination codon [150]. The

rtN236T, an ADV-resistance mutation, does not affect the envelope gene and overlaps with the stop codon at the end of the envelope gene, while the mutation at rtA181T and rtA181V corresponds to stop mutation (sW172stop) and sL173F in the envelope, respectively. In fact, mutations that result in a stop codon in the envelope gene such as those for LAM and ADV would be present in association with a low percentage of wild-type for viral packaging.

Mutations triggering resistance to ETV (rtI169T, rtS184G, and rtS202I) also affect HBsAg and result in concomitant changes sF161L, sL/V176G, and sV194F while the rtM250V is located after the end of HBsAg. The mutation sF161L is located adjacent to the “a” determinant region. Its effect on the envelope structure and significance for diagnostics and vaccine escape needs further investigation.

More generally, the effect of C-terminal mutations on diagnostic assays, vaccine escape, replication fitness, and pathogenicity needs further investigation. One of the most common HBV mutations selected during lamivudine treatment, is the triple rtV173L+L180M+M204V mutations which result in the mutations sE164D+I195M in HBsAg in approximately 25% of HIV-HBV co-infected individuals and in 10% of HBV mono-infected patients failing LAM treatment. The HBsAg containing these mutated residues have reduced -antibody binding, (Figure 2.10B)[151].



(B)

Envelope Changes	Polymerase Changes*	Ag-Ab Binding [IC_{50} (µg/ml)]**
Wild type	Wild type	1.09
HBIG-Escape		
sG145R	rtW153Q	>55.0
Antiviral Drug Resistant		
sE164D	rtV 173L	14.86
sW196S	rtM204I	8.29
sI195M	rtM204V	5.26
sM198I	rtV207I	12.5
sE164D/I195M	rtV173L/rtL180/rtM204V	54.53

Figure 2.10. The overlapping of surface and polymerase genes and its consequence to antigen-antibody binding (source: modified from *Cooley et. al., 2003* [152])

2.4. Epidemiology of hepatitis B infection

2.4.1. Prevalence of HBV infection

2.4.1.1. In general population

Approximately 2 billion people or one-third of world's population have been exposed to the HBV. Although most of HBV-exposed individuals spontaneously recover, more than 350 million develop chronic infection and 75% of them reside in Asia [2-7]. An estimated 50 million new cases are being diagnosed annually [10]. Each year over 1 million people die from HBV-related liver disease, mainly from cirrhosis and HCC. HCC is one of the most common cancers worldwide, and HBV is responsible for at least 75% of these cancers [5].

One of the features of HBV infection is that the risk of chronicity varies greatly with the age at which the infection is acquired. The risk of chronicity is 90% for neonates and infants who acquire the infection before age of 1 year, about 30%. for children aged 1-5 years, and around 2% for children older than 5 years and adults, [3, 153]. The reason for the high risk of chronicity in neonates and in children younger than 1 year is still uncertain. The transplacental passage of the HBeAg from an infected mother to the fetus might induce immunological tolerance to the virus [154], although a study in transgenic mice showed that the placenta is an efficient barrier for HBeAg transfer [155].

The prevalence of chronic HBV infection varies worldwide, with the ranging 0.1-20% in different parts of the world [156]. High prevalence (HBsAg positivity rates >8%) regions include East-Asia and Pacific (except Japan), sub-Saharan Africa, the Amazon brasin, and also the Arctic. Intermediate prevalence (2-8% HBsAg positive) regions include India, Japan, part of Central Asia and the Middle East, Eastern and southern Europe, and parts of South America. Low prevalence (<2% HBsAg positive) regions include the USA, Northern Europe, Australia, and parts of South America (Figure 2.11)[156]. The age at primary infection is perhaps the best-established determinant of chronicity in highly endemic areas, the majority of infections occur through perinatal transmission at birth or during early childhood. As the majority of these infections are asymptomatic, the infected children remain undetected and unwittingly serve as a reservoir of HBV. In contract, in most developed countries, where HBV is primarily a disease of adolescents and adults resulting from behavioral, lifestyle, sexual contact, or occupational exposure, the rate of HBsAg positivity is less than 2% [10]

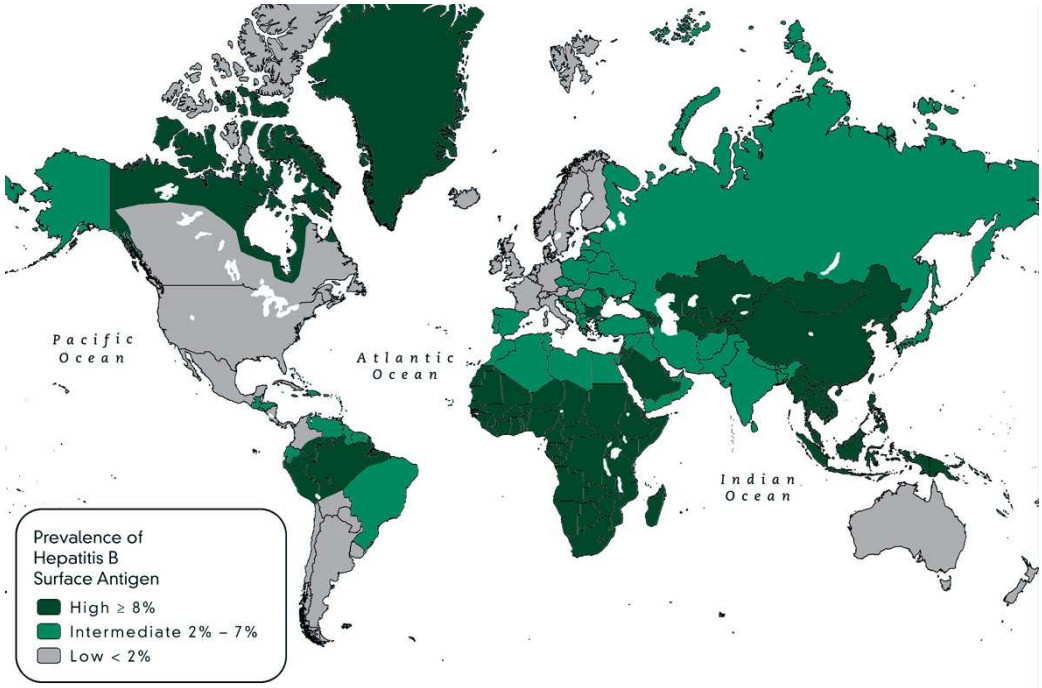


Figure 2.11. Global distribution of chronic hepatitis B infection in 2006 (source: Travelers' health, Centers for Disease Control and Prevention, available at http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-b.htm, accessed July 21, 2011)

In Thailand, the prevalence of HBV infection in new blood donors had dramatically declined from 7.1% in 1988 to 2.6% in 2009, this probably is the result of an effective expanded program on immunization (EPI) against HBV and the current HBV vaccine coverage rate in newborns is more than 98% nationwide [157]. In Chiang Mai, northern city of Thailand, 4.5% of school children (mean age of 12.8 years) were found HBsAg positive during 1998-2000 [16]. There is however disparity in the prevalence distribution across the regions HBV infection is widely spread in some rural ethnic populations of northern region (10-14%) [158, 159] or in migrant workers from Laos, Myanmar and Cambodia (7-11%) [160]. Prevalence of HBsAg positivity in pregnant women in Thailand was 8-10% [156]. This prevalence was lower in the south (3.4%) [161].

The predominant strain is genotype C, in particular subgenotype C1 [160]. Prevalence of HBV genotype C and B are 91% and 7%, respectively, while the rest (2%) are recombinant between genotypes B and C [16].

2.4.1.2. In HBV/HIV co-infected population

Approximately 40 million people worldwide are infected with HIV. Due to shared modes of transmission, co-infection is common, and an estimated 4 million people worldwide are infected with HIV/HBV [162, 163]. Several factors influence these co-infection estimates, including geographic differences in the prevalence of chronic infection by age, the efficiency of exposures that account for most transmission, and the prevalence of persons at high risk for infection. The prevalence of HBV in HIV-infected individuals varies with the population studied. In USA, up to 10% of all HIV-infected individuals have HBV infection [164]. In sub-Saharan Africa, 9-17% of HIV-infected individuals are HBsAg positive and more than 80% have been exposed to HBV (anti-HBc positive)[165]. In Asia and Asia-Pacific regions, the prevalence of HBsAg carriage in HIV-infected patients ranges between 6 to 20%, (Table 2.5).

Table 2.5. Prevalence of HBsAg carriage in HIV-infected patients in Asia and Asia-Pacific

Countries	Selected population	Number of subjects	Prevalence (%)	References
International observation cohort in Asia and Pacific region	No	1,641	10.4	[166]
Thailand	No	692	8.7	[167]
Thailand	No	529	8.7	[168]
China	No	1,110	6.3	[169]
Japan	No	471	8.9	[170]
India	No	1,178	9.9	[171]
India	No	500	9	[172]
India	No	204	15.2	[173]
Iran	No	391	15	[174]
Australia	No	1,719	4.9	[152]
Taiwan	No (35% of IDUs)	3,164	19.8	[175]
Thailand	Pregnant women	1,437	7.4	[176]
Singapore	Men	47	8.5	[177]
Taiwan	Drug substance users	52	11.5	[178]
India	Co-infected with tuberculosis	951	6.4	[179]

*IDUs: Injection drug users

Impact of HIV infection on HBV disease progression

It is widely accepted that HIV has a significant impact on the natural history of HBV infection. The presence of HIV prior to HBV infection increases the risk of developing chronic HBV and prolonged ALT elevation. HBV HIV co-infection

reduces the rate of spontaneous HBeAg and HBsAg seroconversion, leading to a higher prevalence of HBeAg-positive disease [164, 180]. Also, immunosuppression can cause re-activation of latent HBV infection in individuals with previous immunity markers [181, 182], especially in patients with severe immunodeficiency [183]. There is as association between HIV and elevated HBV DNA level, although serum ALT elevation is milder compared to HBV monoinfected patients. Liver demage progesses more rapidly and more severe in patients with co-infection [164, 184, 185]. Patients with HIV/HBV co-infection have an increased risk of liver-related complication and death. A large multicenter cohort study show that liver-related mortality rate in co-infected patients was 14.2 per 1000 person-years, compared to 0.8 per 1000 person-years for HBV nomo-infection and 1.7 per 1000 person-years for HIV mono-infection alone [184].

Impact of HBV infection on HIV disease progression

The majority of clinical studies that have examined the influence of HBV on HIV disease progression have not been able to prove that HBV has any role in HIV disease progression [167, 186]. However, a theoretical effect of HBV on HIV transcription that might enhance HIV replication and lead to more rapid reduction of CD4+ T cells counts in HBV HIV co-infectited patients has been described but these is little evidence to supported this [187, 188].

2.4.2. Transmission of hepatitis B virus

In infected persons, HBV is found in highest concentrations in the blood, and lower concentrations in saliva, semen, vaginal secretions, and wound exudates. HBV can remain viable for more than 7 days on environmental surfaces at room

temperature. The average incubation period is 90 days from time of exposure to onset of symptoms, but may vary from 6 weeks to 6 months [5]. Although HBV DNA or HBsAg were detected in a variety of body fluids of people infected with HBV only serum and semen may be infectious [189, 190]. No infection was demonstrated in persons orally exposed to HBsAg-positive saliva [5], although transmission was demonstrated to animals by subcutaneous inoculation of saliva [191]. This infrequent transmission might be due to low concentrations of infectious virus in saliva and/or a partial reduction of viral virulence by the innate immune response inherent in saliva. Mode of HBV transmission is mainly divided into 2 routes, as follow;

1) Horizontal transmission

- ***Percutaneous or parenteral transmission***: parenteral transmission means transmission via needle-puncture of the skin. This mode includes transfusion of blood or blood products [192], contaminated equipment used for therapeutic injections and other health-care related procedures [193-195]. However, transfusion related hepatitis B is rare. The establishment of routine screening for hepatitis B virus with highly sensitive methods in most transfusion centers for the last two decade in at lot of developed countries. HBV transmission via tattooing and acupuncture has also been reported [196, 197]. Unsafe injection drug use remains an important mode of HBV transmission [198].

- ***Sexual contact-associated transmission:*** Promiscuous sexual activity is probably the most important mode of HBV transmission in low HBV prevalence areas. Transmission of HBV through heterosexual contacts is not always (100%) happen but still relatively efficient way of transmission. Transmission is very frequent between homosexual males, depending on their sexual practice. The HBV transmission by

kissing is never been reported whereas bites transmit very efficiency. The rate of transmission from HBV carriers to contacts is proportional to the HBV DNA levels, frequency and intensity of exposure [26]. However, the risk of chronicity is low for transmission through sexual contact, intravenous drug use, acupuncture, and transfusion.

- ***Nosocomial transmission:*** Nosocomial spread of HBV infection in hospital does occur when apparently adequate practices to control infection are not followed. Transmission also occurs from contaminated environmental surfaces, inadequately sterilized needles and medical instruments [199]. HBV infection is an occupational hazard among people who work in laboratories or exposed to infected blood, however occurs rarely since hepatitis B vaccination of health-care workers has been implemented [200].

Moreover, HBV transmission from father to fetus before birth has been reported. This transmission can occur via the spermatid because, beside serum and leukocytes, HBV DNA can be detected in semen and spermatid, as well [201].

2) Vertical transmission

In high-incidence areas, such as south-east Asia, vertical or perinatal transmission of HBV from chronically infected mothers to newborns appears to be the most important factor for the high prevalence of HBV infection. Without prophylaxis, 48% of infants born to HBsAg positive women become infected [202]. HBeAg is one of the main maternal factors determining whether infection of newborns will occur. The presence of HBeAg in the mother's serum is associated with greater infectivity [203]. The risk of perinatal HBV transmission among infants born to HBV-infected mothers ranges

from 30-50% in HBeAg-negative mothers to 70-90% in HBeAg-positive mothers (Figure 2.12) [204, 205].

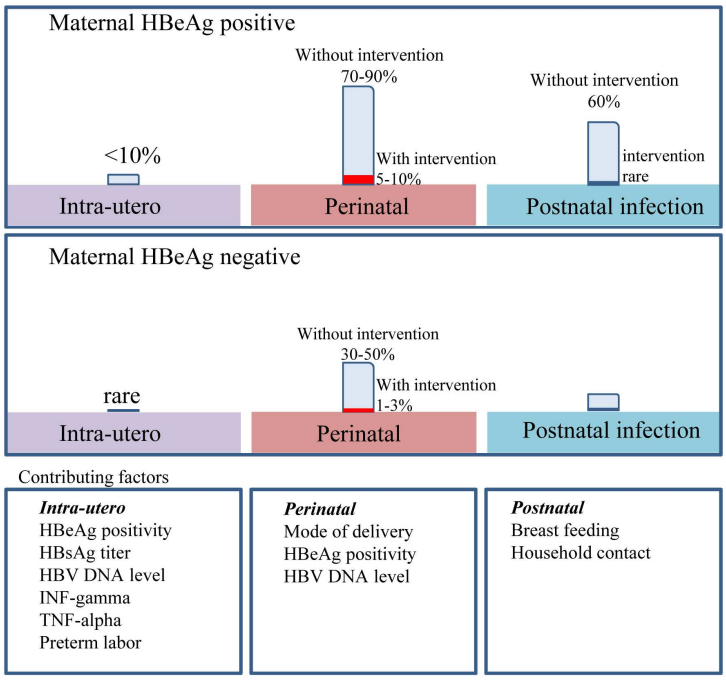


Figure 2.12. Estimated rates of HBV mother-to-child transmission and factors contributing the transmission according to the period of transmiisions.

There are 3 possible routes of transmission of HBV from infected mothers to infants: i) prenatal transmission in the first or second trimester of pregnancy, ii) perinatal/natal transmission (Perinatal period begins from 28 weeks of gestation until delivery), and iii) postnatal transmission (during child care or through breast milk) (Figure 2.12).

- ***Prenatal transmission:*** Prenatal or intra-uterine or in-utero HBV infection is speculated to occur following passage of HBV from maternal blood through placental leakages. This type of transmission may explain the failure of passive and active immunoprophylaxis and largely depends on maternal HBV load [206]. This route of

transmission has been estimated to occur in 4-7% in infants born to HBsAg positive mothers in China [207, 208] but may be rare in regions outside of Asia [206]. Factors associated with intrauterine HBV infection are maternal serum HBeAg positivity, history of threatened preterm labor, and detectable HBV in the villous capillary endothelial cells of placenta [207] and high level of HBV DNA [209].

- ***Perinatal/natal transmission:*** This is considered as the main mode of mother-to-child transmission of HBV. In developing countries of high chronic hepatitis endemicity, children born to mothers with positive HBsAg/HBeAg are at 70-90% risk of acquiring HBV infection in delivery period, [204, 205, 210]. A meta-analysis showed strong evidence of the reduction in the risk of perinatal/natal HBV transmission when elective caesarian section was performed, as compared to vaginal delivery (10% vs. 28%) [211]. However since there is no data from well-designed randomized controlled trial, most obstetric guidelines do not endorse routine use of caesarian section to prevent perinatal transmission of HBV [212].

- ***Postnatal transmission:*** Children of HBsAg-positive mothers who do not become infected perinatally remain at high risk of infection during early childhood. Before the availability of neonatal immunization, 60% of those born to HBsAg-positive mothers became infected by the age of 5 years [204]. Postnatal transmission from mother to newborn can occur through breast-feeding. In households of chronically infected individuals, HBV infection can occur via person-to-person through nonsexual contact. With appropriate immunoprophylaxis, including hepatitis B immune globulin and hepatitis B vaccine, breast-feeding of infants of chronic HBV carriers poses no additional risk of transmission of HBV [213]. Another source of HBV transmission is represented by HBV carriers' urine which may explain the horizontal transmission of HBV among young children.

Despite use of HB immunoglobulin and vaccine, some infants still suffered from mother-to-child transmission. Transmission rates vary from 3% in Australia [144], 7.4% in China [17] and 12% in South Korea [214]. These vaccine failures were significantly associated with maternal HBeAg-seropositivity and high level of HBV DNA. In addition, a study in India showed that 3% of babies aged 2 years old were found infected with HBV variants presenting point mutations in the “a” determinant region, although they had preexisting anti-HBs antibodies at 24 months post immunization, presence [132].

2.5. Natural history and clinical manifestations of hepatitis B infection

HBV has no direct cytopathic effect. The spectrum of disease in HBV is determined by the host immune response, CTLs mediate hepatocytes injury leading to acute and chronic hepatitis [2] and the age of acquisition. The risk of developing chronic HBV infection is closely related to the age at time of infection. Among children infected with hepatitis B, about 90% of infected infants and 30% of infected children aged under 5 years of age develop chronic infection. In adults, 95% of acute infection resolve spontaneously, approximately 0.5-1 % lead to fetal fulminant hepatitis and only 5% or fewer develop chronic infection [215]. Among infants who acquired HBV before the age of 2 year aged 10% will spontaneously resolve acute infection (Figure 2.13).

Chronic infection may also have serious complications: nearly 25% terminate in serious liver diseases [30]. It remains unclear why, after exposure to HBV, some individual develop an acute infection and spontaneously recover, while others develop a chronic infection or spontaneously clear virus after years of viral production.

Immunosuppressed patients, e.g. hemodialysis patients, HIV-infected patients, are also at high risk of developing chronic infection [153].

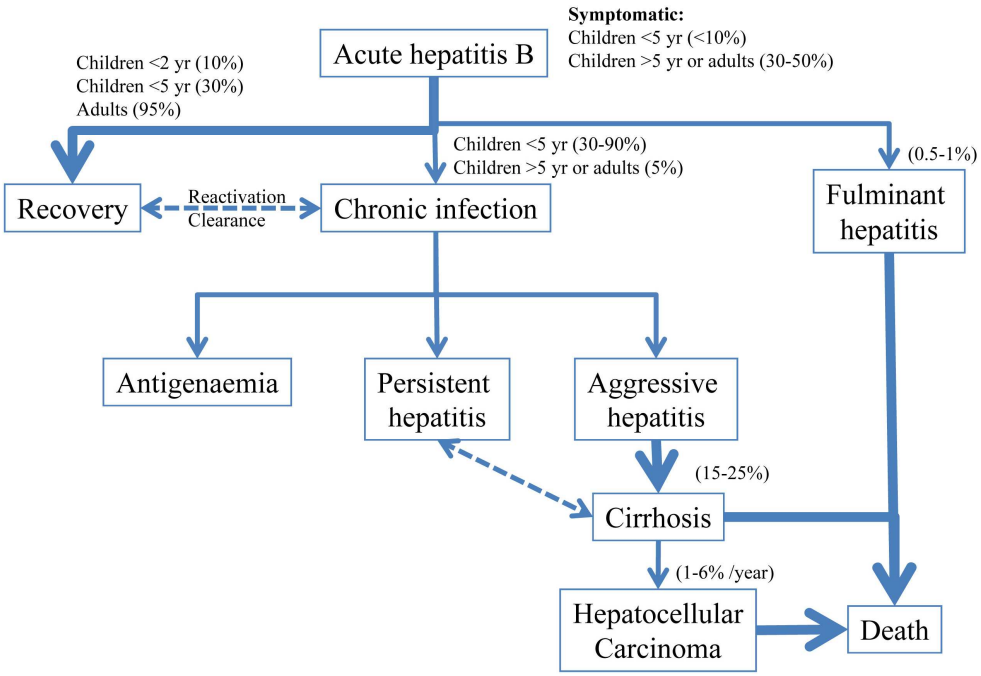


Figure 2.13. Outcomes of acute HBV infection

Acute HBV infection

As mentioned above, the majority of HBV infections (95%) in adult are acute while only 10% infections are acute in neonates. The reason is that the immunoregulation of viral infection in adult is much more efficaciousas compared to that in neonates. For newly infected individuals who develop acute hepatitis, the average incubation period (time from exposure to onset of jaundice) is 90 days (range 60-150 days) [216]. The likelihood of developing symptoms of hepatitis is age-dependent. Over 90% of perinatal HBV infections are asymptomatic, while the typical manifestations of acute hepatitis are found in 10% of newly infected children age 1-5 years old and in 30-50% of older children, adolescent, and adults [6] (Figure

2.13). Signs and symptoms of acute hepatitis B include nausea, abdominal pain, vomiting, fever, jaundice, dark urine, change in stool color, and hepatomegaly or splenomegaly.

After an incubation period of 4-10 weeks, the first serological markers to become detectable in individuals with acute HBV infection are HBsAg and antibodies to HBcAg (anti-HBc), mainly IgM isotype in the early stage. Viremia with very high viral titer is well established, when HBsAg is detected. HBeAg become than detectable in most cases. Alanine aminotranferase levels increase after liver injury triggered by T-cell mediated immune response. Then, the titers of virus in blood and liver begin to drop. In the 4-12 months after infection, IgM anti-HBc becomes undetectable. Total anti-HBc immunoglobulins persist for life and are found in individuals who recover from infection. In individuals who recover from HBV infection, HBsAg and HBeAg are eliminated from blood stream and anti-HBs as well anti-HBe develop during convalescence, (Figure 2.14A). The presence of anti-HBs indicates immunity to HBV infection. Most individuals who recover from natural infection (resolved infection) will be positive for both anti-HBs and anti-HBc, but anti-HBs may become undetectable in some individuals over time. Resolved acute infection is not a risk factor for subsequent cirrhosis or HCC. Surprisingly, in some cases, despite acute infection was resolved, low levels of HBV DNA in blood may persist for many years [11]. Immunosuppressed patients can develop reactivation of previously resolved HBV infection.

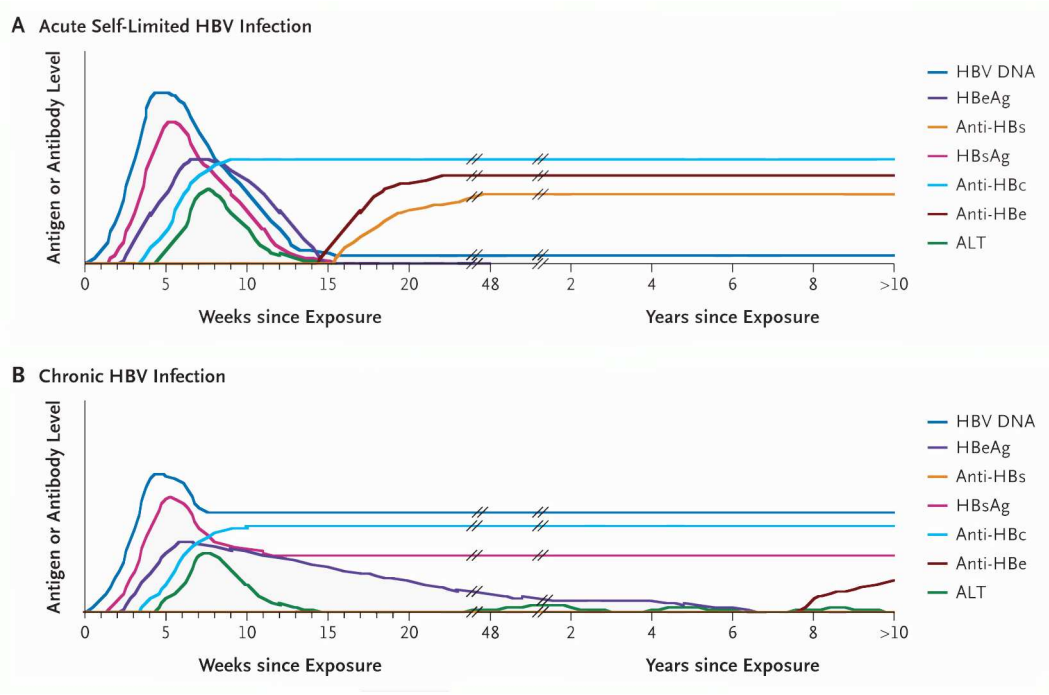


Figure 2.14. Serology and molecular maker patterns during course of acute (A) and chronic HBV infection (B) [11]

Chronic HBV infection

Chronic infection is defined as either the persistence presence of HBsAg in serum of an individual for at least 6 months [30] or the presence of HBsAg in an individual who tests negative for anti-HBc IgM. Unlike individuals who recover from acute HBV infection, individuals with chronic infection do not develop anti-HBs, while HBsAg typically persists for decades or often for life. Titers of viral DNA tend to decline over time. HBeAg is also usually found in the early phase of illness. In many individuals with chronic infection, HBeAg becomes undetectable usually a decade or more after acute infection (Figure 2.14B)[11]. Approximately 0.5% of adults and a lower proportion of children, with chronic HBV infection will clear HBsAg and develop anti-HBs annually. Approximately 15-25% of persons with chronic HBV infection die prematurely from cirrhosis or HCC.

The four phases of chronic hepatitis B infection have been well described (Figure 2.15), although not all patients go though all phases [10, 149];

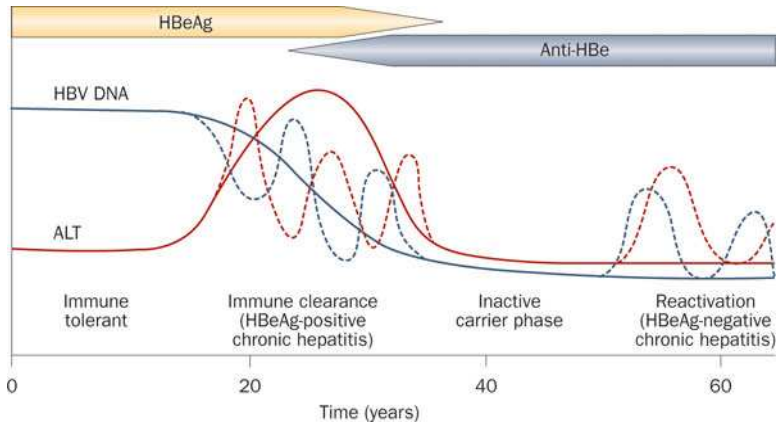


Figure 2.15. Natural history of chronic hepatitis B infection *(source: modified from Kwon, Nat Rev Gastroenterol Hepatol, 2011 [149])*

Phase 1: Immune tolerance phase is characterized by the presence of HBeAg, elevated levels of serum HBV DNA (>20,000 IU/mL), mild or no symptoms, persistently normal ALT levels and minimal histological activity in the liver. The age of acquiring the infection affects the course of the disease. This phase is typical of infection in children and adolescents. It lasts about 2 - 4 weeks or shorter in healthy adults but may last for decades in children who acquire the infection during the perinatal period. Subjects in this group are highly infectious and transmit the virus easily.

Phase 2: Immune active or immune clearance phase is characterized by the markedly reduction of HBV replication levels, spontaneous seroconversion from HBeAg to anti-HBe at a rate of 10-20% per year, and is usually accompanied by the increase in the serum ALT levels due to the immune-mediated lysis of infected hepatocytes. This phase may last from months to years depending on the efficiency

of immune system. If the immune system is efficient, this stage will be self-limiting, lasting for only 3-4 weeks and the host eventually being cleared of the virus. If inefficient, this phase may persist much longer, probably for 10 years or more. Histologically, there is severe chronic hepatitis and the ultimate outcome for the patient depends on the duration and severity of the liver injury during this stage. This phase usually occurs when the patients is between 15 and 35 years of age. The rate and average age of seroconversion from HBeAg to anti-HBe varies by HBV genotype, because persons infected with genotype C remain HBeAg-positive for many years longer than those infected with genotypes A, B, D, or F. Patient who undergo spontaneous HBeAg seroconversion before the age of 40 have a good prognosis. Clearance of HBeAg reduces the risk of hepatic decompensation and improves survival [217].

Phase 3: Inactive phase or low replicative stage is characterized by the absence of HBeAg, development of anti-HBe, low (<2,000 IU/mL) or undetectable level of serum HBV DNA, persistently normal ALT levels, improvement in liver fibrosis and inflamation, although serum HBsAg still persists. Most of the HBV-infected hepatocytes are cleared by the host immune response. Patients in this phase have a favorable prognosis. HBsAg clearance is unusual in Asian patients, but may occur in Caucasians at the rate of 1–2% per year, increasing with time [10].

Phase 4: Reactivation phase (also called HBeAg-negative chronic hepatitis B) is characterized by the presence of HBsAg positive and anti-HBeAg, absence of HBeAg, transiently or persistently elevated serum HBV DNA and ALT levels, and active inflammatory in the liver. Some authors classified this phase into the immune tolerant phase because viruses are just not able to secrete HBeAg. Mutations in precore or basal core promoter region are the causes of this absence [218]. As

supercoiled HBV DNA persists in the liver, some of inactive HBsAg carriers may develop HBV reactivation with recrudescence of liver disease. Reactivation of HBV replication can occur either spontaneously or after immunosuppression due to reactivation of the wild type virus with reversion back to the HBeAg positive state, or much more frequently with precore or core promoter HBV variants that prevent or decrease the production of HBeAg. In addition, HBV DNA can be detected by PCR in serum, liver and peripheral blood mononuclear cells more than decade after recovery from HBV infection [219, 220]. This suggest that recovery from HBV infection may not result in complete virus elimination, but rather the immune system keeps the virus at very low level. However, these patients have no risk to develop progressive liver diseases [221]. In Asia-Pacific, the prevalence of HBeAg-negative chronic hepatitis B patients among HBsAg-positive individuals is estimated at 15% [222].

2.6. Immune response to hepatitis B virus

Following HBV infection, there is an initial hepatitis that may or may not be symptomatic. Successful clearance and resolution of infection depends on the age at which HBV is acquired and the immune status of individuals. The immune determinants of clearance of HBV are not fully understood but both innate and adaptive immune responses are important in the control of HBV infection [11, 223].

2.6.1. Innate immune response

Innate immunity is the first line of defense immediately after infection to limit the spread of the virus. The components of innate host responses are: production of interferon (IFN)-α/β cytokines from infected hepatocytes triggered directly by virus

replication through cellular mechanisms that detect the presence of viral RNA or DNA,

In the liver, natural killer (NK) cells and natural killer T (NKT) are activated via interleukin-18 (IL-18) and chemokine CCL3 released from Kupffer cells and dendritic cells (DCs) following the recognition of stress-induced molecules and/or the modulation of the quantity of major histocompatibility complex (MHC)-class I molecules on the surface of infected cells [223] (Figure 2.16).

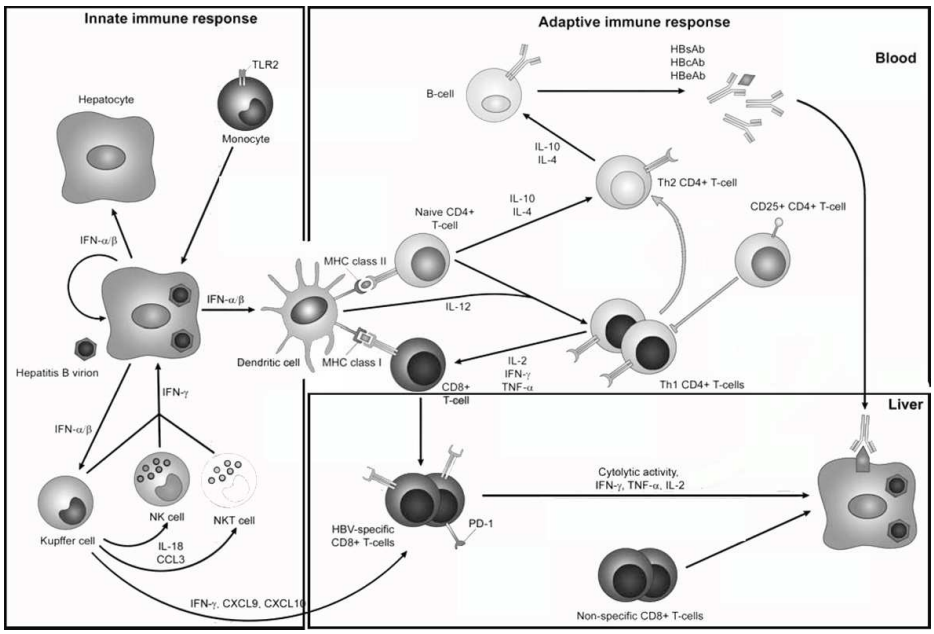


Figure 2.16. Immune responses against HBV infection. Control of HBV infection requires both innate immune response and adaptive immune responses: humoral and cellular arms

2.6.2. Adaptive immune response

Many effector cell types participate in the development of adaptive immune responses against hepatitis B proteins either humoral or cellular immune responses (Figure 2.16). Antigen presenting cells (APCs), such as Kupffer and dendritic cells,

are important for the presentation and maturation of HBV-specific T-cells, the main effectors of HBV clearance. APCs present viral antigens to $CD4^+$ and $CD8^+$ T-cells and produce cytokines, interleukin (IL)-12 and tumor necrosis factor (TNF)-α, which induce the production of IFN-γ and proliferation of $CD8^+$ T-cells. IL-12 also induces $CD4^+$ T_h cell differentiation into the T-helper cell type 1 ($CD4^+$ T_h1 cells). $CD4^+$ T_h1 cells are robust producers of Th1 cytokines and are required for the efficient development of effector CTLs and B-cell antibody production. T cell-derived cytokines and chemokines also participate in the stimulation of antiviral antibody responses that contribute to viral clearance mainly by blocking virus entry into susceptible cells and by removing infectious virions from the circulation.

Cellular immune response

A virus-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) and $CD4^+$ T helper cells ($CD4^+$ T_h cells) play key effector and regulatory roles, respectively. In acute HBV infection, HBV-specific CD4+ T-cells can be detected at the time of elevated HBV DNA and persist long after recovery from HBV infection. CD4+ T-cell responses specific for HBcAg or HBeAg (peptides c50–69) have been more often detected than those specific for HBV envelope and polymerase proteins. An HLA class-II-restricted T cell response to HBcAg is vigorous in patients who clear the virus spontaneously but weak and defective in chronically infected patients, suggesting that the outcome of chronic HBV infection may depend on variations in the host immune response against the virus and infected hepatocytes. CTLs are believed to play a major role in both virus clearance and the pathogenesis of liver cell injury. An HLA class-I-restricted T cell response against HBV peptides expressed on the surface of liver cells plays a major role in the pathogenesis of liver damage. The assumption of

protective role is based on the observation that a vigorous CTL response specific for HBV-encoded proteins was observed in patients with acute hepatitis B, who ultimately cleared the virus while it was weak or undetectable in patients with chronic infection. Another observation in favor of the protective role of CTLs is the development of a CTL response in chronic HBV-infected patients who experienced a spontaneous or interferon-induced remission that was similar in strength and specificity to that of patients who recovered from acute hepatitis B [224]. These observations suggest that vigorous CTL responses to HBV are essential for viral clearance. HBV-specific CTLs exert both cytolytic and non-cytolytic activities against HBV-infected hepatocytes. Non-lytic mechanisms induced by IFN-γ and TNF-α participate in the clearance of acute HBV infection. IFN-γ is mainly produced by HBV-specific CTL but can also be produced by natural killer (NK), natural killer T (NKT) cells and $CD4^+$ T_h1 cells. TNF-α and IFN-γ clear HBV through several mechanisms including destabilization of the viral capsid via the NF-κB pathway, degradation of viral proteins via nitric oxide and proteosome activity and post-transcriptional degradation of HBV RNA.

Humoral immune response

The humoral response is also critical to long-term clearance of HBV and protection from infection with HBV. Neutralizing and non-neutralizing antibodies can also promote antiviral and pathogenetic events by activating the complement system, which can lyse antibody-coated viruses or virus-infected cells, and can also prevent re-infection [38, 225]. In patients who recover from acute HBV infection, activated CD4+ T-helper cells type 2 ($CD4^+$ T_h2 cells) induce B-cell production of antibodies against HBsAg, HBcAg and HBeAg. Antibodies against HBs (anti-HBs)

are synthesized early in infection but are not detectable because they are complexed with the excess of HBsAg produced during virus replication. Neutralizing anti-HBs antibodies provide protective immunity against subsequent HBV infections and are the basis of protection in vaccinated individuals. The pathogenic role of antibodies to non-envelope protein remains controversial. It is generally accepted that anti-HBc antibodies do not exert-neutralizing activity [223]. Recovery from hepatitis B results in lasting protective immunity that is mediated by neutralizing HBsAg specific antibodies and by HBV-specific $CD4^+$ and $CD8^+$ T cells.

2.7. Hepatitis B infection and hepatocellular carcinoma

Patients with severe liver disease are at risk of developing hepatocellular carcinoma (HCC). In the Far East, the annual incidence of HCC is 0.8% in patients with chronic hepatitis B. During a mean follow-up of 6 years or more, 9% of 349 Caucasian patients with compensated cirrhosis developed HCC. The 5-year probability of HCC in Taiwanese patients with compensated cirrhosis was higher at 20%, with an annual HCC incidence of 2.8%. The annual risk of developing HCC in patients with cirrhosis is between 1 and 6 % [226]. In Japan, the cumulative incidence of HCC in patients with cirrhosis after 6 years was 59% [227], almost double the rate of chronic hepatitis B with cirrhosis in Italy, which was 31% in 8 years [228].

The rate of progression to cirrhosis and/or HCC depends on the age of the patient at infection, and on several additional host, viral, and external factors [149, 205, 229-231]:

1. ***Age at infection:*** HBeAg seroconversion rates are low in younger individuals

2. ***Host factors:*** increasing age, male gender, immunosuppression, host genetic factors e.g. HLA, TNF-alpha, Mannose binding protein (MBP), and vitamin D receptor, recurrent ALT flare, persistently increased ALT levels, diabetes
3. ***Viral factors:*** Persistent high level of HBV DNA, persistent presence of HBeAg, HBV genotype C rather than genotype B, basal core promotor mutations (e.g. 1762T/1764A mutations)
4. ***Exogenous factors:*** co-infection with HIV or other hepatotropic virus (e.g. HCV, HDV), heavy alcohol consumption, cigarette smoking, aflatoxin exposure.

2.8. HBV virological assessment

Virological assessment of HBV infection relies on a series of assays that are essential for diagnostic purposes and to adopt therapeutic decisions.

2.8.1. Serological testing for HBV status determination

Serological testing for HBV infection can be done using the routine enzyme-linked immunosorbent assay (ELISA). The test measures several hepatitis B viral specific antigens and antibodies. Different serological “markers” or combinations of markers are used to identify different phases of HBV infection and to determine whether a patient has acute or chronic HBV infection, or he/she is immune to HBV as a result of prior infection or vaccination, or is susceptible to infection. Based on knowledge of the natural history of chronic HBV infection, patients can be classified according to their serological status as shown in table 1.6.

Table 2.6. Determination of HBV status according to serological testing

	HBsAg	Total anti-HBc	IgM anti-HBc	Anti-HBs
Susceptible	neg	neg	neg	neg
Immune due to natural infection	neg	POS	neg	POS
Immune due to hepatitis B vaccination	neg	neg	neg	POS
Early infection before anti-HBc response	POS	neg	neg	neg
Early infection	POS	POS	POS	neg
Chronically infected	POS	POS	neg	neg
Four interpretations possible*	neg	POS	-	neg

*This probably results from either 1) recovering from acute HBV infection, 2) distantly immune and insufficient sensitivity of the test to detect very low level of anti-HBs in serum, 3) susceptible with a false positive anti-HBc, 4) undetectable level of HBsAg present in the serum although the person is actually a HBV carrier.

2.8.2. Cell culture and animal models for HBV

A major obstacle to the research on the development of drug and gene-based therapies for HBV infections is the lack of an efficient cell culture system or a readily available small-animal model, permissive for viral infection and replication. Recently, in vivo models of HBV infection based on cell culture have been developed and generally involve primary hepatocytes or cell lines derived from hepatocytes (e.g. HepaRG [232], HuH-7 [233]) or surrogate models such as woodchuck hepatocytes for WHV infection, duck hepatocytes for DHBV infection. However, infection of these cells with HBV has produced poor viral replication, low viral yields and poor reproducibility. Thus, these cell culture systems are suitable for the viral infectivity

and may be useful for some drug studies but not for studying the viral life cycle. Although HBV can be generated from integrated HBV genome into host cell chromosomes the mode of viral replication is different from that in natural infection [234].

For in vivo models, chimpanzees are natural hosts for HBV. Chimpanzees develop acute hepatitis after HBV infection and mount immune responses, but they do not develop chronic liver disease. Recently, researchers have shown that a strain of tree shrews (*T. belangeri sinensis*) develops acute and, in some cases, chronic hepatitis after infection with HBV. Some researchers have thoroughly studied surrogate animals (e.g., woodchuck, duck, and ground squirrel) that host hepadna viruses specific to their species. Unfortunately, these animals do not develop cirrhosis and thus are not useful for testing anti-HBV vaccines. Transgenic mouse models expressing open reading frames of HBV have allowed investigators to study the replication, gene expression, and immunopathogenesis of HBV. However transgenic mice do not naturally mount an immune response to HBV or develop hepatitis. To mimic natural infection, researchers have generated human-mouse chimeric liver models by transplanting human hepatocytes into immunodeficient mice and then reconstitute its immune system by transplanting human and/or mouse bone marrow cells. Another animal model has been developed immune-competent rats made tolerant to human hepatocytes by injecting human cells into the fetal peritoneal cavity. These rats can accept transplanted human hepatocytes that can be subsequently infected in vivo with HBV [234]. Thus, although no single cell culture system or animal model is ideal for studying all features of HBV hepatitis, researchers are developing imaginative and novel animal models that are designed to investigate specific aspects of pathobiology, prevention, and therapy of HBV.

2.8.3. Molecular assays in diagnosis and management of HBV infection

Several types of molecular assay are available for the diagnosis and management of HBV infection [235].

2.8.3.1. Quantitative HBV DNA assay

HBV DNA quantification is considered to be most advanced method currently available for monitoring HBV replication. Techniques for HBV DNA quantification are based on amplification either of signal or target (Table 1.7). **Signal amplification** techniques require the use of a specific "capture" oligonucleotide probe that hybridizes to denatured DNA. The signal from the probe-DNA hybrid is then amplified for detection and quantification. **Target amplification** requires amplification of the viral genome (amplicon) which is then detected. A drawback of signal amplification techniques is their inability to detect very low levels of HBV DNA. Target amplification techniques such as PCR based assays have a remarkably high sensitivity (detection limit as low as 4 IU/ml of HBV DNA using Taqman-based real-time PCR). These assays use specific primers that attach to each strand of target double stranded DNA. The introduction and development of real-time PCR techniques, that increase sensitivity of HBV DNA, is a major progress in the field. Indeed, simultaneous amplification and quantification of the viral genomes can be achieved with real time PCR, thereby obviating the need for post-PCR manipulations. Real-time PCR assays can detect a wide range of HBV DNA levels and are more rapid and sensitive than conventional PCR techniques and therefore provide a better assessment of HBV replication. Taqman technology uses a fluorescent probe annealed to target DNA sequences for the quantification of DNA. Several other "in-

house" real time PCR techniques have been developed which also exhibit remarkable sensitivity and reproducibility.

Table 2.7. available commercial hepatitis B virus DNA quantification assays [236]

Assay	Method	Lower limit of detection	Dynamic range of quantification
Signal amplification			
HBV Hybrid-Capture II®	Hybrid capture signal in microplates	142,000 copies/mL	5.15 – 9.23 log10 copies/mL
Ultrasensitive HBV Hybrid-Capture II®	Hybrid capture signal in microplates after centrifugation	4,700 copies/mL	3.67 – 7.76 log10 copies/mL
Versant® HBV DNA 3.0 Assay	Semi-automated branched DNA	375 IU/mL	2.55 – 7.25 log10 IU/mL
Target amplification			
Amplicor HBV monitor®	Manual quantitative RT-PCR	180 IU/mL	2.26 – 5.85 log10 IU/mL
COBAS Amplicor HBV monitor®	Semi-automated quantitative RT-PCR	35 IU/mL	1.54 – 4.55 log10 IU/mL
COBAS® Taqman™ HBV monitor	Real-time PCR after automated DNA extraction	12 IU/mL	1.73 – 8.04 log10 IU/mL
RealArt™ HBV PCR assay	Real-time PCR	4 IU/mL	0.60 – 8.00 log10 IU/mL
Abbott RealTime™ HBV DNA	Real-time PCR after automated DNA extraction	10 IU/mL	1.00 – 9.00 log10 IU/mL

* Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction; IU/mL, International Units/milliliter

As the results of quantitative assays are usually expressed in different units, which make the comparisons between different assays difficult. In order to interpret the same way in the different assays currently available clinical practice, standardization of HBV DNA assays has been established through standardization of the quantification units (titer of WHO international standard is set arbitrarily to

1,000,000 IU/ml), and the reporting of HBV DNA levels using a logarithmic rather than a linear scale. The international standard for HBV DNA assays and serum HBV DNA levels are now expressed in international unit (IU)/ml in all available assays, allowing direct comparison of HBV DNA assay results. The implementation of this standard is essential for defining clinically appropriate treatment guidelines based on serum HBV DNA levels [237].

2.8.3.2. Genotyping assays

It is possible to classify HBV genotype without determining the entire whole sequence of the viral genome because the genotypic variation of HBV is reflected in partial sequence of the HBV genome. Since the sequence of the S gene is more conserved than the pre-S region, it is much more suitable for genotyping [8]. After amplification by polymerase chain reaction (PCR) of the target of interest, entire genome or a partial sequence of the HBV genome, different methods for HBV genotyping can be used [237].

- ***Direct sequencing:*** The amplified products are directly sequenced and derived sequences are compared with published sequences to determine homology with known genotypes or using phylogenetic tree analysis. This technique is a reference method and suitable for analysis of new genotypes or recombination between genotypes but it is labor intensive and time consuming.

- ***Restriction fragment length polymorphism:*** The amplified products containing genotype-specific regions are digested by restriction enzymes and HBV genotypes are differentiated on the size of the digested fragments. This method is cheap and easy to perform but it is limited for only known genotypes.

- Line probe hybridization assay: Amplified products of the S gene are hybridized to strips pre-coated with genotype-specific oligonucleotide probes. Determination of HBV genotypes is based on the pattern of reactive bands. This commercial assay (INNO-LIPA) is easy to perform and can identify mixed genotypes but it is also limited for only known genotypes and costly.

Non molecular assays have also been used

- Enzyme-linked immunosorbent assay: The principle of this assay is based on the binding of monoclonal antibodies to genotype-specific epitopes of the pre-S2 protein in specimens. The advantages of this method are easy to perform, can identify mixed genotypes, and suitable for subject negative for viremia. This assay can detect only known genotypes.

2.8.3.3. Drug resistance mutation tests

When a mutation occurs during replication, it results in a nucleotide substitution that may be synonymous (not associated with an amino acid change) or non-synonymous (associated with an amino acid change). Some of the mutations inducing an amino acid change are associated with a decrease of the sensitivity to an antiviral drug [238].

Genotypic antiviral resistance designates the presence of unique nucleotide and corresponding deduced amino acid mutations in the drug target gene, e.g. the HBV polymerase gene, that have been previously demonstrated to be associated with antiviral resistance. Ideally, to identify potential genotypic resistance, the nucleotide and deduced amino acid sequence of HBV isolated from the patients during virologic breakthrough should be compared to the sequence of HBV isolated from pre-treatment sample from the same patients. When pre-treatment samples are not

available for analysis, sequence data at the time of virologic breakthrough should be compared to consensus published sequences of the same HBV genotype. Primary drug resistance mutations cause an amino acid substitution that result in reduced susceptibility to an antiviral agent while secondary compensatory mutations cause amino acid substitutions that restore functional defects in viral polymerase activity, replication fitness, associated with primary drug resistance.

Initially identification of drug resistant mutations was based on individual definition of the mutation location on the HBV genome and was thus confusing as the HBV genotypes vary in the genomic length. In 2001, Stuyver and colleague overcome this problem by dividing the HBV polymerase into four different functional units and re-numbering each functional unit [239]. The reverse transcriptase (rt) region of the polymerase gene is common for all genotypes. Mutations within this region are prefixed with the letters rt followed by the consensus deduced amino acid, the codon number relative to the start of the rt region, followed by the deduced amino acid derived by the mutation. For example, the primary LAM resistance changes are defined as rtM204L (substitution of the methionine at codon 204 in the reverse transecriptase region of the HBV polymerase gene for leucine)

HBV drug resistance assays include;

- In vitro phenotypic assays: These assays are based on the comparison of in vitro susceptibility of replication-competent mutant clone and wild type clone to antiviral drugs. In vitro phenotype testing is based on the determination of changes to the effective concentration of the drug required to inhibit 50% of the target (EC50 or IC50) relative to the “wild-type” reference HBV. This assay is the “gold standard” to confirm genotypic antiviral resistance but is time consuming and labor intensive due to the lack of convenient cell culture system and the need for specific HBV replication

competent clones. In addition, multiple substitutions or sequences elsewhere in the HBV genome may influence the results [238].

- Virtual phenotypic assay or genotypic assays: In these assays, single or multiple mutations are identified and then analyzed for correlation with the patient treatment and response data, for example, the mutation M204I in the RT gene is associated with lamivudine resistance. The method relies on relational databases containing clinical, virological, and HBV sequence information that are integrated and analyzed statistically via linkage and require large numbers of patients with virological breakthrough during treatment [37, 238].

Assays available to identify resistant mutations include:

- Direct PCR sequencing: Direct PCR sequencing can detect the resistance mutations if they represent approximately 20% of the total HBV quasispecies pool. It also allows the identification of all mutations occurring, including additional potential compensatory mutations and new undefined mutations associated with resistance to existing therapies.

- Restriction fragment length polymorphism (RFLP) analyses: RFLP analyses can detect viral mutants that constitute as little as 5% of the total viral population. However, separate sets of endonuclease reactions must be designed specifically for each mutant of interest. Some mutations result in a new restriction site and RFLP is therefore as easy method; some other mutations destroy a restriction site and in this case RFLP analysis should be used with caution as lack of enzyme digestion may be due to loss of a restriction site or technical problems with the assay. RFLP analysis may not be possible for all resistant mutations as specific endonucleases may not exist for such sequence.

- ***Reverse hybridization Assay:*** The commercially available reverse hybridization assay (LiPA DR, Innogenetics, Belgium) contains a series of short membrane-bound oligonucleotide probes [240-242]. which can detect single nucleotide mismatches thus emerging viral resistance when HBV encoding the resistance mutations constitute 5% or more of the total viral population. Their major limitation is that new set of specific probes are required for every mutant and a number of probes may be required to detect a single nucleotide change.

- ***Sequencing with Microchip-based Technology:*** Sequencing with microchip-based technology using oligonucleotide microarrays may be used to detect new mutations. This technology is expensive and not widely available [243].

- ***Matrix assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS):*** MALDI-TOF MS is based on mass spectrometric analysis of small DNA fragments containing the site of variation. This assay has been shown to be very sensitive and can detect mutants that constitute only 0.1% of the viral population. However, it is costly (mass spectrometer) and can be used only for known mutations [244].

- ***Ultradeep sequencing or pyrosequencing:*** Pyrosequencing has been used to detect minority populations of -resistant HBV variants [245], but this method is labour intensive and requires highly skilled personnel. Furthermore, given the high rate of spontaneous mutations during HBV replication, the clinical significance of mutants that may be present in <0.1% of the viral population is uncertain.

2.9. Treatment of HBV infection

2.9.1. HBV management for HBV mono-infected patients

Seven drugs are currently approved by the US Food and Drug Administration (US-FDA) for the treatment of chronic hepatitis B, including immunomodulatory agents: i.e. interferon alfa-2b and peginterferon alfa-2a, and oral antiviral agents: i.e. the nucleos(t)ide analogues. Nucleoside analogues include lamivudine, telbivudine and entecavir, while nucleotide analogues include adefovir dipivoxil and tenofovir disoproxil fumarate. Immunomodulatory agents display both antiviral and immunomodulatory activity. Nucleos(t)ide analogues act primarily by inhibiting the reverse transcription of the pregenomic RNA to the first strand of HBV DNA. Viral relapse is common when treatment is stopped. Immunomodulatory agents have been limited by its poor tolerability and significant side effect profile while the efficacy of nucleos(t)ide analogues have been hampered by the necessity of prolonged use and emergence of resistance [246]. Many novel anti-HBV agents are currently under investigation in pre-clinical and clinical trials. Several viral targets are the focus for development of new and more potent drugs to help enhance viral clearance and prevent resistance, for example, inhibition of viral entry using preS1 peptides, inhibition of capsid information by phenylpropenamide derivatives and heteroaryl-pyrimidines (HAP), or blocking viral morphogenesis and egress by an inhibitor of protein folding and trafficking [247].

Immunological treatments with immunomodulatory agents

Interferon alfa-2b (IFN-α-2b)

IFN-α-2b enhances the innate immune response by binding to the type 1 interferon receptor, resulting in activation of the Jak-Stat pathway and up-regulation of multiple interferon-stimulated genes, which limit viral dissemination. The

recommended dose for adults is 5 million units (MU) daily or 10 MU three times a week for 4-6 months. About 30% of patients had successful response, defined as a HBeAg seroconversion, and a decline of serum ALT [248]. However, the side effects of therapy with IFN, e.g. influenza-like symptoms myalgias, thrombocytopenia, and depression, make it difficult to treat for many patients. Also, in many patients a flare of liver injury occurs during administration of IFN, reflecting the immunomodulation activity of IFN [11].

Pegylated interferon alfa-2a (peg-IFN-α-2a)

Peg-IFN-α-2a is an immunomodulatory agent with the same activity as a IFN-α-2b but has a longer half-life than IFN-α-2b due to the addition of the polyethylene glycol. Pegylated alfa-2b is given once a week at a dose of 0.5 or 1.0 micrograms for at least six months. In HBeAg-positive patients, peg-IFN-α-2a is superior to non-pegylated IFN [249]. At 48 weeks of peg-IFN-α-2a treatment, HBV DNA was suppressed in 25% and 63% in HBeAg-positive and HBeAg-negative chronic hepatitis B, respectively [250]. Incidence of influenza-like symptoms and depression was lower in the groups receiving Peg-IFN-α-2a than in the group receiving IFN-α-2b [251].

Antiviral treatment based on Nucleoside/nucleotide analogue

Lamivudine (LAM or LMV or 3TC)

In 1998, lamivudine became the first commercially available oral agent for the treatment of chronic hepatitis B [252]. This was a landmark in the management of HBV infection. Lamivudine is the negative enantiomer of 3'thiacytidine, a 2'3'-dideoxynucleoside and contains a sulphur atom in the 3' portion of the sugar ring. The

active form is triphosphorylated and competes with dCTP for incorporation into growing DNA chains, causing chain termination of RNA-dependent HBV polymerase [253]. This may occur during reverse transcription of the first strand as well as during synthesis of the second-strand HBV DNA, resulting in decreased synthesis of HBV DNA, of export of new virions and intracellular replenishment of cccDNA. LAM treatment is safe and well toleratedt in both children and adults [11, 254, 255]. The oral administration of 100-mg dose of lamivudine can cause complete and sustained suppression or viral replication [256]. Treatment with lamivudine results in a reduction of 3-4 $\log_{10}$ serum HBV DNA levels in the first three months of therapy; this decline is associated with a more rapid loss of HBeAg, seroconversion to anti-HBe positive status, and improvement in serum aminotransferase levels [11]. About 16-20% HBeAg seroconversion rate can be achieved with 1 year of treatment [256, 257]. This rate reached up to 50% at longer duration (3-5 years) of treatment [258-261]. However, its prolong use has been associated with the emergence of lamivudine-resistant HBV. The rate of emergence was around 15-20% per year [25]. Until recently in developed countries, LAM has not been considered a first-line agent for chronic HBV patients because of its low barrier to resistance resulting in a high rate of drug-resistant mutations [262]. The emergence of viral variants results from one or more mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of the HBV polymerase gene that is the nucleotide-binding domain (catalytic site) of viral DNA polymerase. The resulting mutants are slightly less fit than wild-type HBV in the absence of the drug, but they are strongly selected for in its presence [11]. LAM resistance occurred in up to 70% of patients after 5 years of therapy (Figure 2.17)[263]. The clinical significance of the development of resistance is still being debated. Clearly, in many patients, resistance presages a return to higher level

viremia, and in some of these patients further liver injury develops. However, although the level of viremia rises, in many patients it may still remain below pretreatment levels, perhaps as a result of the reduced fitness of the variants [11]. Despite high resistance rates, many patients experience continued virological suppression during LAM monotherapy. Also, with its well-established safety profile and low cost, LAM is a strong candidate for wide scale use in Asian developing countries. Furthermore, LAM-resistant HBV can be treated with other potent nucleos(t)ide analogues [264].

Adefovir dipivoxil (ADV)

ADV was the second drug licensed for the treatment of chronic hepatitis B (CHB). ADV was not approved by the FDA for treatment of HIV due to toxicity issues, but a lower dose (10 mg/day) is approved for the treatment of CHB. ADV, a acyclic diphosphonates, is an analogues of adenosine monophosphate that undergo intracellular phosphorylation to its active metabolite, which inhibits the HBV polymerase by competitive inhibition with deoxyadenosine 5'-triphosphate, resulting in chain termination [265]. One-year treatment with ADV (10 mg daily) resulted in HBeAg seroconversion in 12% of HBeAg-positive patients and 21% achieved HBV DNA negativity by PCR (<400 copies/mL). At 5 years on ADV therapy, HBeAg seroconversion was observed in 48% of patients and 39% had an HBV DNA level <1000 copies/mL. In HBeAg negative CHB, after 1 year of ADV therapy, 51% of patients became negative for HBV DNA. At 5 years therapy, 67% of patients had HBV DNA <1000 copies/mL and >70% improved in liver histology. The rate of selection for ADV-resistant HBV is lower than the rated with LAM. ADV-resistant

mutations are not present within 1 year but, at 5 years, resistant mutations were detected in up to 29% of patients (Figure 2.17)[262].

Entecavir (ETV)

ETV is a carbocylic analogue of 2'-deoxyguanosine with selective activity against HBV. It is phosphorylated to the active triphosphate form and competes with the substrate dGTP to inhibit HBV polymerase. It can inhibit both the priming and elongation of viral minus strand DNA [266]. ETV (0.5 mg/day) is superior to LAM (100 mg/day) in the treatment of CHB patients. In HBeAg-positive patients, HBV DNA was suppressed to below the limit of detection (300 copies/mL) in 67% of patients and HBeAg seroconversion occurred in 21% of patients after 1 year. Cumulative rates of HBV DNA undetectability increased to 80% and 94% at 2 and 5 years of ETV therapy, respectively. In patients with HBeAg-negative CHB, HBV DNA was undetectable in >90% of patients after 1 year of therapy. Very low rates of ETV resistance have been reported in nucleotide analogue naïve patients (Figure 2.17)[262].

Telbivudine (LdT)

LdT is a synthetic thymidine nucleoside analogue with activity against HBV DNA polymerase. It is phosphorylated by cellular kinases to the active triphosphate form, and then inhibits HBV DNA polymerase by competing with the natural substrate, dTTP. Incorporation of LdT 5'-triphosphate into viral DNA causes DNA chain termination, resulting in inhibition of HBV replication. LdT is an inhibitor of both HBV first strand and second strand synthesis [266]. LdT is effective at 600 mg daily. Although it has been demonstrated that LdT produces improved reductions in

HBV DNA level compared to treatment with LAM (100mg/day) for the CHB treatment but there is no difference in normalization of ALT level, HBeAg loss, or anti-HBe seroconversion [267]. In HBeAg-positive patients, HBeAg seroconversion was observed in 23% of patients, HBV DNA suppression (<300 copies/mL) was observed in 60% of patients after 1 year of treatment. In HBeAg-negative patients, HBV DNA became undetectable in 88% of patients after 1 year of LdT. The overall rate of LdT resistance was 5% in patients with HBeAg-positive CHB and 2.2% in those with HBeAg-negative CHB [267]. Despite the high potency of LdT, virological response rates decrease over time due to the emergence of LdT resistance. LdT resistance mutations are responsible for cross-resistance to LAM and have been found in up to 22% of patients after 2 years of therapy (Figure 2.17)[262].

Tenofovir Disoproxil Fumarate (TDF)

Tenofovir is the most recently approved nucleotide analogue for the treatment of CHB. TDF is an acyclic nucleoside phosphonate analogue of adenosine monophosphate. Tenofovir diphosphate inhibits HBV DNA polymerase by competing with dATP for incorporation into nascent DNA, resulting in premature chain termination. Tenofovir has activity against HBV and LAM resistant HBV [266]. TDF demonstrated superior anti-viral efficacy compared to ADV for both HBeAg-positive and -negative CHB patients. In HBeAg-positive CHB patients, at 1 year treatment, HBeAg seroconversion was observed in 21% of patients and HBV DNA undetectability was achieved in 76% (<69 IU/mL) [268]. At 3 years of therapy, the rate of HBeAg seroconversion increased to 26% and the rate of HBV DNA undetectability reached 78% [262]. In HBeAg-negative patients, HBV DNA undetectability was achieved in 93% of patients after 1 year of TDF therapy [268] and

99% after 3 years [262]. Furthermore, TDF was also able to suppress the HBV DNA levels to below 69 IU/mL in 79% of patients who failed previous nuclot(s)ide analogue treatment after a 2 years follow-up. However, the presence of ADV, but not LAM, resistance mutations impaired TDF efficacy. However, so far, no TDF resistance has been reported after 5 years of continuous therapy (Figure 2.17)[262], which underlines the high genetic barrier of this drug and its potency.

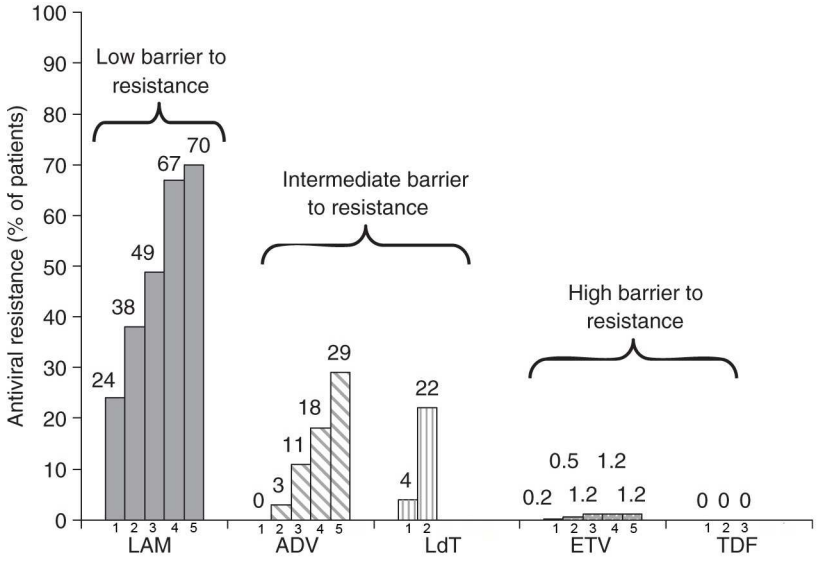


Figure 2.17. Estimated rates of genotypic resistance to anti-HBV treatments in naïve patients. The numbers under the bar indicate years of therapy (source: modified from *EASL Clinical Practice Guidelines, J hepatol 2009 [263]*)

Combination therapy

Use of combination therapy in HBV mono-infected patients has been not consistently associated with increased rate of virological suppression, but has been associated with decreased resistance rates [265]. However, LAM plus ADV therapy was associated higher HBV suppression and ALT normalization rates, and resistance to lamivudine, as compared to LAM mono-therapy [269]. ADV plus FTC therapy

have been associated with greater HBV suppression, as compared to ADV mono-therapy [270]. In HIV/HBV co-infected patients naïve to therapy, the TDF plud LAM combination was superior to LAM monotherapy, but it was not superior to TDF monotherapy [271].

2.9.2. ***HBV treatment for HBV/HIV co-infected patients***

Nearly 10% of estimated 36 million people having HIV worldwide sffer from chronic hepatitis B virus [272]. In HBV/HIV co-infection, peg-IFN therapy is associated with lower rates of therapeutic success and increased toxicity [273]. The dose of LAM is 300 mg/day is recommended for treating HIV/HBV co-infection and the drug should always be given with at least two other anti-HIV agents. Given its excellent tolerability, LAM has been widely used as anti-HBV agent in HIV-infected patients [86, 274]. Unfortunately, overall HBV resistance mutation can be occurred in 94% of HBV viremic patients with HIV infection who received LAM for over 4 years [275]. ADV (10mg/day) suppresses HBV replication, and is associated with a low rate of resistance compared with LAM [276, 277]. ETV is more potent in suppressing serum HBV DNA than LAM and ADV and is effective against wild type and LAM-resistant and ADV-resistant HBV [272, 278, 279]. LdT has no activity against HIV and has greater anti-HBV efficacy than either LAM or ADV and selects for resistance mutations at intermediate rates [280]. Emtricitabine or FTC (200 mg/day) has been extensively used with tenofovir in HIV/HBV co-infected patients. HAART containing EFV plus TDF/3TC or TDF/FTC has been recommended for treating HIV/HBV co-infected patients as preferred regimen in Thailand [281]. It has slightly greater potency and efficacy than lamivudine but cannot be used as monotherapy because of high rates of resistance [272].

Several studies have demonstrated that TDF was associated with a significant reduction of HBV DNA levels and well tolerated in HIV patients co-infected with or without LAM-resistant HBV.[264, 282-285]. Moreover, there was a trend toward greater suppression of HBV DNA in patients receiving HAART regimen which included LAM and TDF as compared to regimen including LAM only [286] but not superior to TDF alone [271].

2.10. HBV prevention and vaccination

2.10.1. HBV prevention in general population

Prevention of HBV transmission include the avoidance of high-risk behaviors, prevention of exposure to blood and body fluids, highly sensitive screening test for blood units, screening for HBV in pregnant women, and passive or active immunization before or after exposure. There are active immunization method using vaccines against HBV (HB vaccine) and passive immunization with specific immunoglobulins containing high titer of anti-HBs (HBIg) [287].

Passive immunization or specific HB immunoglobulins÷

As the discovery that passively acquired anti-HBs soon after exposure can protect individuals from acute clinical hepatitis B and chronic HBV infection [288], preparation of HBIg have been developed. HBIg are prepared from serum containing high titer of anti-HBs using the Cohn fractionation procedure and their concentration is standardized to 100,000 IU/mL. HBIg are effective, and generally use in combination with hepatitis B vaccine, as post-exposure prophylaxis following perinatal exposure in newborns born to HBsAg-positive mothers [289], percutaneous

or mucous membrane exposure to HBsAg-positive blood [290], or sexual exposure to an HBsAg-positive person [291]. HBIg are also used to protect patients from severe recurrent HBV infection following liver transplantation [202].

Active Immunization

Hepatitis B can be effectively prevented by vaccination. The first HBV vaccine was elaborated from asymptomatic human HBsAg carriers in 1976 by Maupas P et. al. [292] and was efficacious in adults, children and newborns [293]. The first commercial vaccine against HBV was licensed in 1981 and the first recombinant vaccine appeared 5 years later.

- ***Plasma-derived HBV vaccines:*** The first type of HBV vaccines were initially made of highly purified HBsAg by collecting HBsAg from the plasmatic 22 nm spheres of chronic HBV infected subjects. However, their production has progressively stopped over past few years due to the unavailability of plasma of HBsAg carriers, the concerns of the safety of blood product, and the development of new technologies.

- ***Recombinant HBV vaccines:*** these vaccines are produced by introducing HBsAg gene into yeast cells (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster ovary cell, CHO). Antibodies conversion rates and the titers of antibodies generated by recombinant vaccines are similar to those obtained with plasma-derived HBV vaccines [287]. The currently used HBV vaccines mostly consist of the small surface (S) protein and the middle pre-S2 surface (M) protein assembled into 22-nm particles. Both of S and M proteins contain HBsAg containg, the common “a” determinant and several subtype determinants [294].

- ***New HBV vaccine:*** Recently, third generation of hepatitis B vaccines containing all S, preS1 and preS2 domains of HBV have been developed [295, 296]. In addition, the

“edible” vaccine based on transgenic plants (such as banana, tobacco, potato and tomato) is one of the most promising directions in novel types of vaccines and are under investigation [297].

Whatever the production mode, all vaccines are very safe [298]. HBV vaccines are administered by intramuscular route in the deltoid muscle and are highly immunogenic, inducing protective anti-HBs antibody titers (>10 IU/mL) in more than 95% of healthy children or young adults [294]. However, about 5% of vaccinated individuals do not develop anti-HBs antibody and are vaccine non-responders. Several factors associated with the non-response have been identified, including genetically determined non-responsiveness, age older than 40 years, high body mass index, and immunosuppression [294]. In vaccine responders, the anti-HBs titers may decline to undetectable levels several years after vaccination though immunity against clinical disease can persist longer up to 20 years, suggesting the existence of an immunologic memory [13, 299-301]. Thus, a booster is not recommended in healthy people who are not exposed to a high risk of HBV infection.

Vaccination remains the best prevention against acquisition of HBV infection. In Thailand, in 1988, the MOPH has initiated a pilot project on hepatitis B vaccination in 2 provinces (Chiangmai and Chonburi) and integrated universal HBV vaccination in the nation-wide Expanded Program of Immunization (EPI) in 1992. This has resulted in decreased incidence of acute and chronic HBV infection [15]. Currently, HBV vaccination for infants is nearly universal, and more than 98% of all newborns in Thailand have been vaccinated [157]. This program has proved highly efficient in protecting newborns from HBV infection. The overall HBsAg positive carriage was only 0.55% among 180 randomly selected children (aged 2 months to 15 years) attending in Hat Yai hospital which is located in the south of Thailand [302].

However, among children aged 4-9 years, this rate of HBsAg positivity was 1.2% in Chiangmai, a big city in north of Thailand and about 7% of them still had anti-HBc antibodies suggesting HBV infection despite adequate immunization [16]. In another study, 12.4% (12/97) of infants born to HBsAg/HBeAg positive mothers were found HBsAg positive at 13 months of age despite having been vaccinated [303]. Thus, the efficacy of the present vaccine against possible variants needs to be evaluated in order to determine whether vaccine modifications are required.

2.10.2. HB vaccination in HIV-infected population

No distinctive adverse clinical reactions to HBV vaccination have been described in the HIV population [272]. However, Hepatitis B vaccination is most efficacious before severe immunosuppression. Indeed, both CD4+ cell count nadir <200 cells/μL [304] and current CD4+ cell count <50 cells/ μL [305] have been associated with a poor vaccine response. Therefore, hepatitis B vaccine should be offered to HIV-infected patients who do not demonstrate serologic evidence of infection (i.e. HBsAg negative, anti-HBc negative) and have CD4+ cell count nadir >200 cells/ μL. In patients starting antiretroviral therapy, it may be best to defer this vaccination and other vaccinations until CD4+ cell count >200 cells/mm3 is established.

In Thailand, among HIV-1 infected patients, only half of (46%) those HB-vaccinated had good response to vaccination (anti-HBs >10 mIU/ml). Younger age and higher CD4 cell count were predictors for successful response to hepatitis B vaccination [306]. Another study reported a high rate of HBV immunoprophylaxis failure in HIV-infected children on HAART; only 1% (1/69) had a protective antibody level at 5 years or more of age [307], while 64–97% had protective antibody

in healthy children [299-301], indicating that most HIV-infected children are still susceptible to HBV infection. Currently, booster vaccination after priming against hepatitis B during infancy was found to benefit HIV-infected children with immune recovery after HAART [308, 309]

2.11. Occult HBV infection

Occult HBV infection is usually defined by the absence of HBsAg and the presence of HBV DNA in plasma and/or the liver. [310]. The mechanism responsible for the absence of HBsAg in the presence of HBV DNA remained to be elucidated. Several hypotheses have been put forward, such as a very low-level of HBV replication [23], the formation of HBV surface antigen (HBsAg) and anti-HBs antibodies complexes [311], the presence of mutations in the surface antigen [23], or a reduced production of HBsAg due to either mutations in the surface promoter region [312], or co-infection with hepatitis C virus (HCV) [23, 313]. Occult HBV infection is frequently found in individuals with isolated antibodies against core antigen of HBV serological pattern [314].

Isolated anti-HBc

The serological pattern of isolated antibodies against core antigen of HBV (isolated anti-HBc) or anti-HBc alone is characterized by the presence of anti-HBc as the only marker for hepatitis B, irrespectively of HBV DNA. Accumulated data strongly imply that isolated anti-HBc is not compatible with acute and resolved infection but it is associated with chronic HBV infection [315]. Thus at least a proportion of individuals with this serological pattern is HBV carriers and may have potential consequences for themselves, the contacts and for blood banking and

transplantation service. Currently, there are limited data on clinical aspects of individuals with isolated anti-HBc. Most of them seem to be healthy with the normal liver enzyme levels and with no signs of liver diseases. However, some individuals do present signs of chronic hepatitis [315]. There is indirect evidence suggesting that in these individuals the risk of progression to cirrhosis and HCC still exist [316, 317]. Isolated anti-HBc serology occur frequently in persons engaging in injecting drug use (IDU), generally also infected by HCV, among both HIV-infected and HIV-uninfected persons [318]. The probability that isolated anti-HBc signs an HBV infection, rather than a false positive reaction, depends on the prevalence of HBV infection in the population studied and the anti-HBc titers [230]. The risk of transmission in individuals with isolated Anti-HBc is low and, it is further reduced by given prophylaxis [319, 320]. Several possible underlying mechanisms of having isolated anti-HBc serological pattern were proposed, as described in Table 1.8.

Table 1.1. The underlying mechanisms of isolated anti-HBc serological profile [321]

Interpretations of an isolated anti-HBc profile	**Possible underlying mechanism and evidence proof**
False positive to anti-HBc	- retested with different ELISA assay format REF?
Chronic infection or "occult HBV infection"	- chronic carrier with low level of HBV DNA or HBsAg? - infection by HBV mutants in pre-S, S, and Pol genes - co-infection with other hepatotropic viruses - formation of HBsAg/anti-HBs immune complexes - occult infection has been confirmed by detection of HBV DNA using PCR. However, in some cases, DNA PCR may be negative with an unique measurement because of the fluctuation of HBV levels in peripheral blood. HBV DNA may be found positive temporarily.
Window phase of a resolving acute HBV infection	- HBsAg disappears followed by anti-HBs a few weeks later - confirmed by anti-HBc IgM
Late immunity, low level of anti-HBs under detection limit	- occurring most often decades after resolution of infection - confirmed by review of medical history or re-vaccinate

Depending on population studies and technique used the prevalence of isolated anti-HBc and HBV DNA positivity in HIV-uninfected populations varies greatly, as showed in Table 1.9.

In HIV-infected population, prevalence of isolated anti-HBc was 12-26% (Table 1.10). IDU and anti-HCV seropositive were risk factors of isolated anti-HBc [322]. Liang et al. reported, in areas of highly endemic chronic HBV infection as in Taiwan, the only risk factors of isolated anti-HBc were HIV infection and age but not HCV infection [323]. In study in USA found no differences in patient demographic between chronic and occult HBV infected patients, except the median of HBV DNA

was lower in occult HBV infected patients [324]. Although the clinical significance of HIV-infected patients with isolated anti-HBc is still unclear, however there was a report showed that these patients had shorter duration of survival than those with anti-HBs persistence [325].

The prevalence of occult HBV infection in HIV-infected remains controversial, with numbers varying between 0 to 89%, as shown in Table 1.10. The cause of this variation is unclear, but may be related to lack of standardization in the HBV DNA isolation and variation in the sensitivity of the quantification assays, or differences in studied populations and regional difference [310]. Low CD4 cell count (<200 cell/mm3) was more commonly found among women with occult HBV infection than among those with no occult HBV infection [326]. The occurrence of late-onset chronic HBV infection in HBsAg negative HIV/HBV co-infected adults with isolated anti-HBc and positive HBV DNA have been reported, almost exclusively in those with low CD4+ T-cell count [327].

HBsAg negativity in HBV/HIV co-infected patients may be explained by different mutations of HBsAg: a stop codon mutation at position 216 of HBsAg, the E164D and I195M substitutions in HBsAg, which are associated with LAM-resistance mutations [145], the rtV191I mutation, induced by lamivudine treatment which can create a stop-codon in the overlapping surface antigen (sW182stop) and thus deletion of the last 44 amino acids of the HBsAg, resulting in HBsAg negativity in routine diagnostic tests [328]. In addition, other 3 amino acid mutations (T123A, M133L, and T143M) in the “a” determinant of HBsAg may involved with HBsAg antigenicity in HBsAg-negative blood donors with DNA viremia [329]. Martin et al. also described mutations in S gene (Y100F/S, A128T, S136P, G145A/R) in occult HBV patients [330].

Table 2.8. Prevalence of isolated anti-HBc and HBV DNA positivity in HIV-uninfected populations

Country	Population Selected	n	% isolated anti-HBc	% occult HBV infection	HBV DNA PCR method	Lower detection limit	Reference
South Africa	Blood donor	109		49.6	Real-time PCR		[331]
Ghana	Pregnant women	219		9.1	Real-time	25	[332]
Iran	No	531	2.1				[333]
Iran	Isolated anti-HBc	11		0	Artus real-time		[333]
Germany	No	5,305	1.5				[334]
Germany	No	65		7.7	Nested	100	[334]
USA	Isolated anti-HBc	107		3.7	Branched	50	[335]
UK	Isolated anti-HBc	151		4.0	Nested	100-400	[336]
Mexico	Blood donor	11,240	1.9				[337]
Mexico	Isolated anti-HBc	158		8.2	Nested	30-300	[337]
Germany	Isolated anti-HBc	545		8.1	Real-time	50-100	[338]
India	Isolated anti-HBc	171		22.8	Nested		[339]
Lebanon	Blood donor	5,511	3.7				[340]
Lebanon	Isolated anti-HBc	203		5.4	nested		[340]
Italy	Outpatient	6,544	1.8				[341]
Italy	Isolated anti-HBc	119		4.2	nested	100	[341]
Korea		17,677	8.9				[342]
Korea	Isolated anti-HBc	230		1.7	Cobas Ampli/Taqman	4-12 IU	[342]
Hong Kong	Blood donor	13,011		0.12	Cobas Taqscreen	3.7	[343]
India	Blood donor	2,175	7.0	1.4	nested	100	[344]
India	Isolated anti-HBc	153		12.4	nested	100	[344]
Indonesia	Blood donor	309	21.4	8.1	Nested	6	[329]
Indonesia	Isolated anti-HBc	66		28.8	Nested	6	[329]
Turkey	Blood donor	12,858	5.1	0.046	Real-time	19	[345]
Turkey	Isolated anti-HBc	658		0.9	Real-time	19	[345]

Table 2.9. Prevalence of isolated anti-HBc and HBV DNA positivity in HIV populations

Country	Population Selected	n	% isolated anti-HBc	% occult HBV infection	HBV DNA PCR method	Lower detection limit	Reference
Spain		85		0	Single	200	[346]
Spain		176		0	Single	200	[347]
France		160		0.6	Single	200	[348]
Netherlands		93		4	Single	200	[311]
Brazil		159		5	Nested	100	[349]
USA		179		10	TMA	15	[350]
USA		40		10	Single	200	[324]
USA	Isolated anti-HBc	400		2	COBAS	200 cp	[326]
Brazil		101		16	Single	100	[351]
Italy		86		20	Single	100	[352]
France		30		37	Nested	350	[353]
Switzerland		57		89	Nested	100	[354]
Netherlands		191		4.7	Nested	50	[310]
France	No	383	12		Abbott real-time	40	[355]
France	Isolated anti-HBc	48		4.2			[355]
Iran	No	106	20.8				[356]
Iran	Isolated anti-HBc	22		13.6	Artus real-time		[356]
Brazil		43	26				[145]
Brazil	Anti-HBc positive	43		14	Real-time PCR	100 cp	[145]
Thailand		140	20				[322]

STUDY WORKS

PART I

Hepatitis B vaccine failure in offspring of women co-infected with human immunodeficiency virus and hepatitis B virus

3. Hepatitis B vaccine failure in offspring of women co-infected with human immunodeficiency virus and hepatitis B virus

3.1. Introduction:

More than 370 million people worldwide are infected with hepatitis B virus (HBV) [357] and 75% of the world's HBV carriers reside in Asia, some of whom develop severe liver diseases, e.g. cirrhosis or hepatocellular carcinoma (HCC) [8]. HBV infection results in 1 million deaths annually [357]. In East-Asia and Pacific, a highly endemic area for chronic HBV infection, HBV mother-to-child transmission (MTCT) remains a major source of chronic infection [358]. Without any intervention, the overall prevalence of perinatal HBV transmission is 35-50% [359]. This prevalence is ~90% in children born to mothers positive for hepatitis B e antigen (HBeAg) [9]. Immunoprophylaxis with hepatitis B (HB) vaccine and/or HB immunoglobulin decreases this prevalence to 10-15% in this high risk group [360].

In Thailand, one of the highly HBV endemic countries, the Ministry of Public Health (MOPH) has integrated HB vaccination of newborns into the national expanded program on immunization (EPI) in 1992. This program has successfully decreased the rate of positive HBV surface antigen (HBsAg) in children from 3.4% to 0.7%, irrespective of maternal HBeAg status [15]. In Thailand as well in other Asian countries, a percentage of children have acquired HBV infection from their mothers despite administration of HB vaccines. These MTCT could be associated to either the occurrence of mutations on HBsAg [44, 116, 122], high maternal HBV DNA load [361] or the presence of HBeAg [214, 361, 362].

There are limited data on the prevalence of perinatal transmission of HBV in HIV/HBV co-infected women and the rate of HB vaccine failure in children born to these women. The study was aimed to assess, among a large number of HIV-infected pregnant women, the prevalence of chronic HBV infection, the prevalence of HBV mother-to-child transmission in children born to HBsAg-positive women, and characterize the transmitted virus.

3.2. Methods

3.2.1. Patients

This study included HIV-infected pregnant women and their children who participated in two Perinatal HIV Prevention Trials in Thailand (PHPT-1 NCT00386230 [363] and -2 NCT00398684 [364]), assessing the efficacy of short duration of zidovudine (ZDV) or single-dose nevirapine plus zidovudine regimens, respectively, to prevent perinatal transmission of HIV. Breast-feeding was not recommended in these two trials. Blood samples were collected from women during pregnancy and children at birth, 6 weeks, 4, 6 and 12 months of age. Informed and written consent were obtained and the study performed according to the World Medical Association Declaration of Helsinki and approved by the ethic committees of Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

3.2.2. HBV Markers and HBV DNA quantification

HBsAg and HBeAg were tested using an enzyme immune assay (ETI-MAK and ETI-EBK, DiaSorin, Salluggia, Italy) according to the manufacturer's recommendations. HBV DNA was quantified using the Cobas Amplicor HBV

Monitor test (Roche Diagnostics, Branchburg, N.J., USA, lower limit of detection: 60 IU/mL) or Abbott real-time HBV DNA™ assay (Abbott laboratories, Rungis, France, lower limit of detection: 15 IU/mL). HBV infection in children was determined by the presence of HBsAg and/or HBV DNA at least once during 2–6 months of age.

3.2.3. HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 200 µl of plasma using QIAamp kit (QIAgen, Valencia, CA., USA). Ten µl of extract was used as template for the polymerase chain reaction (PCR) amplification as described by Villeneuve et.al. with slight modifications [365]. Briefly, the first-round PCR was performed in a 59 µl volume using Platinum PCR SuperMix High Fidelity primer and primers: Pol1M and Pol2M, yielding fragments of 1,010 bp. PCR conditions included initial 2 min denaturation step at 94^{o}C, followed by 40 cycles of 1 min at 94^{o}C, 1 min at 48^{o}C, and 3 min at 68^{o}C for. Ten microliters were then used for second-round PCR with Pol3M and Pol4M, which yields an 808 bp amplified fragment. PCR conditions were an initial denaturation step of 94^{o}C for 2 min, followed by 30 cycles of 40 sec at 94^{o}C, 1 min, at 55^{o}C and 3 min at 68^{o}C. Amplicons were checked on a 1% agarose gelelectrophoresis. These amplicons sequenced using the pol3M and pol4M primers, and the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA), Sequences were analyzed using the Bioedit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Mutations on *surface (S)* and *polymerase (Pol)* gene were then analyzed for polymorphisms and mutations known to be associated with vaccine escape through comparison with wild-type reference sequences of similar genotype.

3.2.4. HBV cloning and sequencing

The second round PCR products were cloned into a TA Cloning Kit (Promega, Medison, WI) using standard cloning technique. Briefly, amplicons were purified using the NucleoSpin Extract II kit then directly ligated into the pGEM-T vector, and transformed into competent cells and plated on Amp/X-gal plates. At least 24 white colonies were picked and grown in LB medium with ampicillin. The correct insert size was confirmed using EcoRI enzyme digestion. The recombinant plasmid DNA was isolated with the NucleoSpin Plasmid kit. Sequencing reactions were performed using primer pol3M and pol4M and analyzed as mentioned above.

3.2.5. Determination of HBV genotyping and serotyping

HBV genotype was identified by phylogenetic analysis of *S* and *Pol* gene sequences. Briefly, sequences were aligned with published *S* and *Pol* gene sequences of various HBV genotypes available in GenBank using clustalW software. Phylogenetic trees were constructed using neighbor-joining method and genetic distances calculated using the Kimura two-parameter method, as implemented in the software MEGA [366]. Bootstrap analysis with 100 simulations was used to test the reliability of branching. HBV serotype was deduced from amino acids residuals at codons 122, 126, 127, 160, 168, 177 and 178 of the S gene [45, 367].

Reference sequences used in this study were obtained from Genbank database: X70185, V00866, S50225, X51970, M57663 (genotype A); D00331, M54923, D00329, D00330 (genotype B); M38636, X14193, M12906, D12980, D00630, L08805, X52939, X01587, M38454, V00867, X75665, X75656 (genotype C); M32138, X59795, X02496, X72702, X65257, X65258, X65259, X68292 (genotype

D); X75664, X75657 (genotype E); X75663, X75658 (genotype F); AF160501, AB064310, AF405706, AB056513 (genotype G).

3.2.6. Statistical analysis

Baseline characteristics of study population, including maternal age at enrollment, mother's body weight, region of origin, alanine transminase enzyme (ALT) level, CD4+ T-cells and CD8+ T-cells count, HIV RNA load, and the presence of hepatitis C virus antibodies, are described using number and percentage for categorical data and median with interquartile range (IQR) for continuous data. Women's characteristics were compared according to the HBsAg status using Wilcoxon rank-sum (Mann-Whitney) test or chi-square. All data analyses were performed using STATA™ version 10.1 software (Statacorp, College Station, TX). Differences were considered statistically significant if the p-value was <0.05.

3.3. Results:

3.3.1. Patient characteristics

Among 3,467 HIV-infected pregnant women who participated in 2 clinical trials in Thailand, median age was 25.5 (IQR: 22.4-29.1) years old. Most of them enrolled in eastern, northern, and central part of Thailand (33%, 29%, and 21%, respectively). Median CD4+ and CD8+ T-cell count were 368 (IQR: 240-521) and 904 (IQR: 680-1,190) cells/µL, respectively. Median ALT was 15 (IQR: 10-22), up to 95% of patients had normal ALT level (<40 IU/L). Median HIV RNA level was 3.98 (IQR: 3.35-4.58) copies/mL. Four percent of women had antibodies against hepatitis C virus (anti-HCV) (Table 3.1).

Table 3.1. Baseline characteristics of study population

Characteristics	Total N	Median (IQR) or n (%)
Age at enrollment; years	3,466	25.5 (22.4-29.1)
≤20 years; %		353 (10)
>20-30 years; %		2,391 (69)
>30-40 years; %		690 (20)
>40 years; %		32 (1)
missing	1	
Body weight; kgs	1,432	55 (50-60.3)
<40 kgs; %		10 (1)
40-49.9 kgs; %		314 (22)
50 -59.9 kgs; %		718 (50)
60-69.9 kgs; %		305 (21)
70-79.9 kgs; %		66 (5)
>80 kgs; %		19 (1)
Missing data	2,035	
Region of enrollment	3,467	
Central; %		712 (21)
Eastern; %		1,138 (33)
Northern; %		1,017 (29)
North-eastern; %		284 (8)
Southern; %		148 (4)
Western; %		168 (5)
Region of origin	2,017	
Central; %		435 (22)
Eastern; %		303 (15)
Northern; %		410 (20)
North-eastern; %		643 (32)
Southern; %		86 (4)
Western; %		86 (4)
Immigrant; %		54 (3)
missing data	1,450	
Project enrollment	3,467	
PHPT-1		1,439 (41.5)
PHPT-2		2,028 (58.5)

Characteristics	Total N	Median (IQR) or n (%)
CD4 T-cell count; cells/µL	3,378	368 (240-521)
<200 cells/µL; %		615 (18)
200-499 cells/µL; %		1,815 (54)
≥500 cells/µL; %		948 (28)
missing data	89	
CD8 T-cell count; cells/µL	3,035	904 (680-1,190)
<500 cells/µL; %		275 (9)
500-1000 cells/µL; %		1,533 (51)
≥1000 cells/µL; %		1,227 (40)
missing data	432	
ALT; IU/L	3,383	15 (10-22)
<40 IU/L; %		3,216 (95)
40-79 IU/L; %		137 (4)
≥80 IU/L; %		30 (1)
missing data	84	
HIV viral load; copies/mL	3,397	3.98 (3.35-4.58)
undetectable		77 (2)
log 1-1.99 copies/mL		44 (2)
log 2-2.99 copies/mL		379 (11)
log 3-3.99 copies/mL		1,213 (36)
log 4-4.99 copies/mL		1,340 (39)
≥log 5 copies/mL		344 (10)
Missing data	70	
anti-HCV positive	1,988	85 (4)
Missing data	1,479	

3.3.2. Prevalence of HBsAg positivity in HIV-1 infected pregnant women

Of 3,312 women with clearly identified HBV status, 245 (7.4%; 95%CI, 6.5-8.3) were HBsAg positive; of whom half were HBeAg positive (Figure 3.1). Median HBV viral load was 4.37 (IQR: 1.83-7.63) IU/mL. Baseline characteristics between HBsAg-positive- and HBsAg-negative pregnant women were not different, except higher ALT level and lower CD4+ T-cells count in HBsAg-positive pregnant women (Table 3.2). No correlation was observed between HBV DNA and HIV RNA levels (P=0.49).

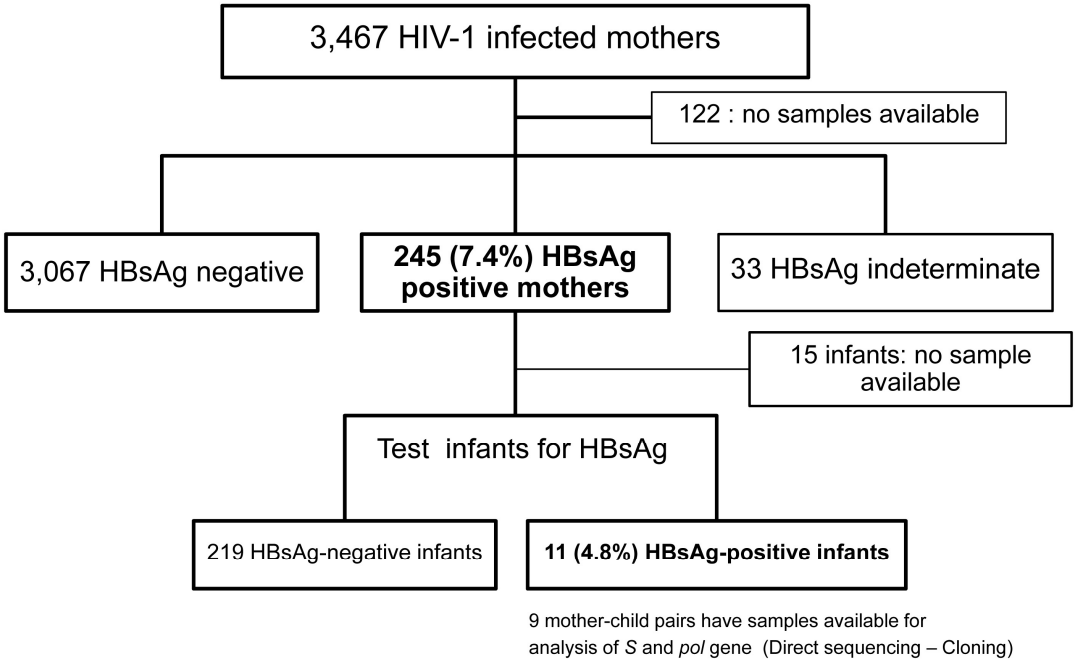


Figure 3.1. Overall study diagram

Table 3.2. Characteristics of HBsAg-positive- and HBsAg-negative pregnant women

Characteristics	HBsAg positive mothers		HBsAg negative mothers		P-value
	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)	
Age at enrollment; years	245	25.4 (22.0-29.0)	3,067	25.5 (22.4-29.1)	0.62
≤20 years; %		25 (10)		311 (10)	
>20-30 years; %		167 (68)		2,122 (69)	
>30-40 years; %		51 (21)		605 (20)	
>40 years; %		2 (1)		29 (1)	
Body weight; kgs	115	54.5 (50-59)	1,308	55 (50-60.3)	0.52
<40 kgs; %		2 (2)		8 (1)	
40-49.9 kgs; %		25 (22)		286 (22)	
50 -59.9 kgs; %		62 (54)		653 (50)	
60-69.9 kgs; %		21 (18)		281 (21)	
70-79.9 kgs; %		4 (3)		62 (5)	
>80 kgs; %		1 (1)		18 (1)	
Region of enrollment	245		3,067		
Central; %		50 (20)		632 (21)	
Eastern; %		80 (33)		1,007 (33)	
Northern; %		78 (32)		895 (29)	
North-eastern; %		20 (8)		250 (8)	
Southern; %		8 (3)		134 (4)	
Western; %		9 (4)		149 (5)	
Region of origin	130		1,742		
Central; %		25 (19)		378 (22)	
Eastern; %		20 (15)		262 (15)	
Northern; %		28 (22)		355 (20)	
North-eastern; %		43 (33)		555 (32)	
Southern; %		5 (4)		78 (4)	
Western; %		4 (3)		77 (4)	
Immigrant; %		5 (4)		37 (2)	
Project enrollment	245		3,067		0.23
PHPT-1		115 (47)		1,315 (43)	
PHPT-2		130 (53)		1,752 (57)	
CD4 T-cell count; cells/µL	242	343 (219-462)	2,991	370 (243-524)	0.03
<200 cells/µL; %		51 (21)		535 (18)	
200-499 cells/µL; %		133 (55)		1,606 (54)	
≥500 cells/µL; %		58 (24)		850 (28)	

CD8 T-cell count; cells/µL	220	855 (626-1,190)	2,674	919 (684-1,186)	0.13
<500 cells/µL; %		30 (14)		236 (9)	
500-1000 cells/µL; %		108 (49)		1,358 (51)	
≥1000 cells/µL; %		82 (27)		1,080 (40)	
ALT; IU/L	240	17 (12-26)	3,004	15 (10-22)	<0.001
<40 IU/L; %		216 (90)		2,868 (95)	
40-79 IU/L; %		19 (8)		113 (4)	
≥80 IU/L; %		5 (2)		23 (1)	
HIV viral load; copies/mL	242	3.96 (3.36-4.59)	3,044	3.99 (3.37-4.58)	0.90
undetectable		5 (2)		67 (2)	
log 1-1.99 copies/mL		5 (2)		37 (1)	
log 2-2.99 copies/mL		25 (10)		335 (11)	
log 3-3.99 copies/mL		88 (36)		1,091 (39)	
log 4-4.99 copies/mL		9 (40)		1,207 (40)	
≥log 5 copies/mL		23 (10)		307 (10)	
anti-HCV positive	129	2 (2)	1,727	77 (4)	0.17
HBV viral load; IU/µL	237	4.37 (1.83-7.63)			
undetectable		35 (15)			
log 1-1.99 IU/mL		41 (17)			
log 2-2.99 IU/mL		21 (9)			
log 3-3.99 IU/mL		18 (8)			
log 4-4.99 IU/mL		7 (3)			
log 5-5.99 IU/mL		7 (3)			
log 6-6.99 IU/mL		18 (8)			
log 7-7.99 IU/mL		63 (26)			
≥log 8 IU/mL		27 (11)			
HBeAg positive	169	87 (51)			

3.3.3. Prevalence of perinatal HBV transmission

Of 245 infants born to HBV-HIV co-infected women, 230 had samples available. Of these, 11 (4.8%; 95%CI, 2.4-8.4) were found infected with HBV, but not with HIV despite administration of HBV vaccination (Figure 3.1). Complete series of samples were available for 9 mother-child pairs, the other two infant samples could not be amplified because low amount of HBV DNA. Virological assessments of 9 HBV transmitting mother-HBV infected child pairs as well as infant HB

immunization are described in table 3. Seven pairs were infected with HBV genotype C, while others two were infected with genotype B.

3.3.4. Patterns of HBV mother-to-child transmission

Analysis of direct sequences of *Pol* and *S* genes showed no known vaccine escape mutation. Of the 9 infants infected with HBV, 3 were infected with wild-type HBV .0387, 0394 and 0657) and interestingly were born to mothers with high level of HBV DNA (>6.50 $\log_{10}$ IU/mL). Three infants had mutations on S gene which was no present in maternal viruses: two infants (0022 and 1395) had lysine substitution by arginine (sK122R) and one (no.0625) had isoleucine substitution by threonine (sI126T). The last 3 infants were infected with HBV variants present in mothers, which may not be the predominant quasispeciestrasI126IT, sI126M+P127S, and sT131N+M133T+T140I+S204R) (Table 3.3). Interestingly, the sS53L and sS210N were found in all mother-child pairs infected with genotype C.

Table 3. 3. HBV genotype, HBV DNA load, mutation observed by direct sequencing among 9 HBV transmitting mother-child pairs.

Pair ID	Geno-type	Maternal sample		Infant samples						Prophylaxis	
		Before delivery		Birth – 10 days		4 months		6 months			
		HBV load (log IU/mL)	S gene mutation	HBV load (log IU/mL)	S gene mutation	HBV load (log IU/mL)	S gene mutation	HBV load (log IU/mL)	S gene mutation	Vaccination (months)	HBIg
0387	C	7.84	Ø	4.37	Ø	8.18	Ø	8.93	Ø	0, 1, 5, 6	Yes
0394	B	6.51	Ø	Und	NA	3.08	Ø	1.48	NA	0, 1, 6	No
0657	C	7.85	Ø	Und	NA	7.61	Ø	8.76	Ø	0, 2, 6	No
0022	C	7.83	Ø	2.48	NA		sK122R		sK122R	2, 4	No
1395	C	8.24	Ø	Und	NA		sK122R		sK122R	0, 1, 6, 12	No
0625	C	7.92	Ø	2.60	Ø	5.51	sI126I/T	7.90	sI126T	0, 2, 6	Yes
1550	C	8.04	sI126T	1.58	NA		sI126T		sI126T	0, 1, 6	No
0135	C	2.28	sI126I/M, sP127A/S	Und	NA	5.63	sI126M, sP127S	NA	NA	Unknown*	Unknown
9149	B	3.61	sT131N, sM133M/T, sT140I, sS204S/R	Und	NA	2.91	sT131N, sM133T, sT140I, sS204R	Und	NA	0, 1, 6, 8	No

Ø: no mutation observed; Und: below undetectable level; NA: not available

* No record of vaccination, however this child was born in a provincial hospital of the northeastern region of Thailand where the standard of care was to provide HB Immunoglobulin and HB vaccine or at least HB vaccine to all infants born to positive HBsAg mothers.

Analysis of clone sequences showed that in mothers with high HBV DNA level, the predominant HBV was wild-type and this wild type HBV was also predominant in samples of 3 infants born to as well as their mother'samples (Figure 3.2A, 3.2B, 3.2C). In one infant no.0387 (Figure 3.2A), sG145R, a known vaccine-escape HBV mutant, was present in 2 of 15 maternal clones but this mutant was not transmitted to her baby. In the group of children infected with an *S* mutant HBV, analysis of maternal and infant clones showed that a minor mutant HBV quasispecies was transmitted to the child. For pairs no.0022 and 1395 (Figure 3.2D, 3.2E), sK122R mutation was present in 1 of 65 clones and 2 of 67 maternal clones, respectively, suggesting the transmission, despite the administration of vaccine against HBV, of this minor maternal HBV variant which progressively became predominant in infected children. Inference of HBV serotype from sequence data of *S* gene indicate that HBV serotype *adrq+* was predominant in these 2 women, while the serotype *ayr* was rare. Analysis of children HBV clones showed an increase of the serotype *ayr* from 4 months to 6 months, while serotype *adrq+* had declined. For the pair no.0625 (Figure 3.2F), the sI126T variant corresponding to a predicted serotype *adrq+* was identified in 2 of 20 maternal clones and become predominantin infant's samples at 4 and 6 months, accounting for 41% and 76%, respectively.

In the third group of children infected with HBV variants already present in mothers and accounting for 20% or more of all maternal quasispecies, clonal analysis showed that these variants were the predominant viral population or can be detected in children. This for pair no.1550 (Figure 3.2G), HBV serotype *adrq+* (sI126T) was always predominant in both maternal and infant's samples. Interestingly, in the 2 latter pairs (0625, 1550), women harbored the 2 variants, wild-type sI126I and sI126T, while only the sI126T variant was found in the child. This result indicates that amino acid substitution at position 126 may influence the escape of HBV to vaccine. Again, for no.0135 (Figure 3.2H), the variant sI126M+P127S was selected in infants 4 month-sample. Finally, the multiple-mutations HBV variant, sT131N+M133T+T140I+S204R, was selected in infant no.9149 (Figure 3.2I), though this variant was not observed in maternal samples likely to the low number of clone analyzed.

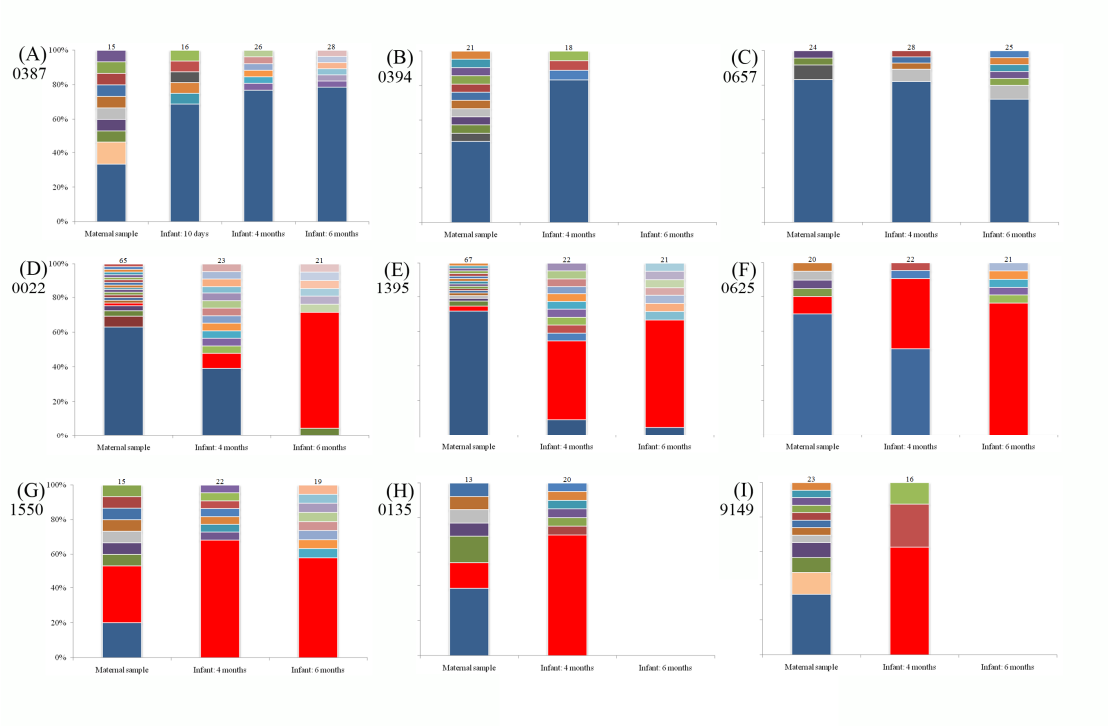


Figure 3.2. Evolution of the HBV quasispecies in 9 representative transmitting mother-child pairs. Numbers above bar indicate number of clone analyzed. Numbers under panel label indicate patient's identification number. The wild type variant found in each patient is indicated in blue, the potential mutant escapes are indicated in red, other variants are indicated by additional colors. The same color indicates identity between viral variants detected at different samples within each mother-child pairs and not between different mother-child pairs. Three potential fashions of HBV perinatal mother-to-child transmission can be concluded; 1) Transmission of wild-type variants from mothers with high level of HBV DNA (A, B, C), 2) transmission of maternal minor variants to their babies (D, E, F), and 3) the transmission of HBV variants that already existing in maternal blood circulation (G, H, I)

3.4. Discussion

We have assessed the prevalence of HBsAg carriers among a large number of HIV-1 infected pregnant women in Thailand and the rate of perinatal transmission of HBV in infants born to those found HBsAg positive. The prevalence of HBsAg positive women was 7.4% (95%CI, 6.5-8.3); of whom 4.8% (11) women transmitted HBV to their offspring despite having received vaccine and/or immununoglobulins.

Our study indicate that vaccine/immunoglobulin failure may result from transmission of either wild-type variants from mothers with high level of HBV DNA (3 of 9 pairs), maternal HBV minority variants (3 of 9 pairs), or HBV variants present and accounting for 20% or more of maternal viral population (variants detected by direct sequencing). We have also identified several HBsAg mutations, sK122R, sI126IT, sI126M+P127S, and sT131N+M133T+T140I+S204R which significance on vaccine/ immununoglobulins escape is unknown.

We observed a prevalence of positive HBsAg in HIV-infected pregnant women similar to that reported in HIV-infected Thai adults ,7.4% vs. 8.7% [168], or in HIV-uninfected pregnant women between 4-8% in studies conducted in Thailand [368-370] and 9-10% in other South-East Asia countries [371]. This is likely due to the fact that in highly endemic areas most HBV infections are acquired very early in life before acquisition of HIV.

The rate of perinatal transmission of HBV found in our study is also consistent with rates observed in HIV-uninfected population worldwide (3-5%) [372] and survey studies showing that the prevalence of HBsAg in schoolchildren aged between 6 months to 18 years was 2.3-4.5% [18, 373]. Phylogenetic analysis of *S* gene sequences indicated that 78% of 9 Infants were infected with HBV genotype C, while 2 were infected with HBV genotype B; these HBV genotype frequencies are similar to other studies in Thailand [18, 64]. Due to the number of HBV transmission, we were unable to assess whether there was a higher risk of perinatal transmission with genotype C.

Our study shows that vaccine failure can occur in different circumstances: high maternal HBV DNA or transmission of variants which may escape neutralization by passive immunoglobulins or active immunization. Maternal minority HBV variants can be transmitted to children who had been administered HB vaccine. Indeed 2 mothers had a predominant HBV population of serotype *adrq+* but it was the HBV minority variant with the sK122R mutation that was selected in the infants. The predicted serotype of this variant was *ayr*. Although the impact of the sK122R mutation on HBV vaccine escape is unknown, we can hypothesize that the change of serotype may have allowed the virus to escape the vaccine-induced neutralizing antibodies. Unfortunately, we were unable to verify the vaccine serotype used for these 2 children. External source of contamination from other person in the family could be excluded since the phylogenetic analysis with other genotype C sequences shows that infant and mother's sequences grouped within the same cluster.

We have identified few mutations in infant samples; i.e. sI126T (2 infants); sI126M1+sP127S (1 infant); sT131N+sM133T+sT140I+sS204R (1 infant) that locate in the "a" determinant of HBsAg. Some mutations have been reported in other studies; e.g. sI126T was observed in studies in India [132], Korea [126] and Taiwan [112], the sP120S in Singapore [127], sP120S+P127S in Italy [374], and sM133T in Thailand [18], as well. Predicted 3D structure indicated that amino acid substitution at position 126 involved the largest change in chemical properties, likely to cause structural changes in the HBsAg [375]. Change of amino acid in the "a' determinant region may be associated with HB vaccine escape [43]. Our results suggest that the mutations observed at positions in a well conserved region may favour the virus to escape neutralizing antibodies.

We observed two mutations, sS53L and sS210N , present in all mother-child pairs infected with genotype C, which may represent polymorphisms specific to HBV variants circulating in Thailand.

The occurrence of in utero HBV infection is usually considered as a very rare event as compared to infection at birth and may happen when chronically HBV infected mothers have high maternal HBV DNA [214, 376, 377]. In our study, we have demonstrated that 3 mothers with high HBV DNA level (>6.5 log IU/mL) transmitted HBV. Our results are in favour of the occurrence of transmission during pregnancy before immnunoglobulins and vaccine can exert their activity. Furthermore, although one woman had HBV harboring sG145R mutation, well-known vaccine escape mutant, only wild type virus was transmitted. This probably due to the use of recent licensed HBV vaccines able to prevent HBV infection with sG145R mutant, which had already been demonstrated in chimpanzees [378].

Immunoprophylaxis, either vaccination administration alone or plus hepatitis B immune globulin, may not be efficative to prevent the transmission if infants are infected either in utero or through extremely exposure to blood or contaminated fluids at or around birth [379].

Indeed, HBV infection by vaccine escape mutants does not account for the majority of children who had immunoprophylaxis failure, only 5-39% in 3 previous studies [120, 128, 380]. The maternally pre-existed *S* gene mutant seems to be potential predictors of vertical breakthrough infection [377]. Other possible causes of

unsuccessful neonate vaccination include trans-placental transmission which is related to high level of serum HBV DNA in pregnant women, trans-placental leakage of maternal blood, amniocentesis, and polymorphisms in some cytokine genes or human leukocyte antigen [156]. Formula feeding was recommended to all women in this study because they are all infected with HIV. Although postnatal HBV transmission, breast-feeding transmission may influence to the transmission of HBV from mother to child, however, with appropriate immunoprophylaxis, breast-feeding does not pose additional risk for the HBV transmission [213].

In conclusion, although HBV vaccine has proved very efficacious in the prevention of mother-to-child transmission of HBV our study confirms that there is still a residual HBV transmission for which different mechanisms may account for. Whether perinatal HBV transmission occurs more frequently in infants born to HIV co-infected women remain to be determined. Also, the impact of variants identified in our study in the escape to HB vaccine needs further investigation. A systematic virological evaluation of HBV variants selected in infected infants despite active immunization, and their mothers, is needed to further clarify the impact of these mutations on perinatal transmission of HBV. Understanding the causes of HB vaccine failures will help to develop new HBV vaccine appropriate for the many countries in HBV endemic area such as Thailand and other South-East Asian countries and also develop interventions to decrease perinatal transmission of HBV and accelerate the eradication of HBV infection.

3.5. Publications

Preliminary results of this works were presented in;

- **Khamduang W,** Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Transmission of Hepatitis B virus (HBV) minor variants in children born to HBV/HIV co-infected mothers. 5th Dominique Dormont International Conference, Mother-to-child transmitted viral diseases: from transmission to children care, 26-28th March, 2009, Paris, France, *Retrovirology* 2009; 6 (Suppl.1):O9 ***(O9, Oral presentation)***
- **Khamduang W,** Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Transmission of Hepatitis B virus (HBV) minor variants in children born to HBV/HIV co-infected mothers. 12th National AIDS Conference, 27-29th May, 2009, Bangkok, Thailand. ***(CP08, Poster presentation)***
- **Khamduang W,** Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Sureau C, Lallemant M, Sirirungsi W, Goudeau A and the Program for HIV Prevention and Treatment (PHPT) group. Hepatitis B Escape Mutants in Infants Born to Human Immunodeficiency Virus (HIV)-infected Mothers Co-infected with Hepatitis B Virus (HBV). The 21st Conference of the Asian Pacific Association for the study of the liver (APASL), 17-20th February, 2011, Bangkok, Thailand. ***(PP06.41, Poster presentation)***

- **Khamduang W.** Franco-Thai Highlight: Hepatitis B Vaccine Escape. The International Workshop on “Interdisciplinary Approach to the Management of HIV: A Model for other Infectious Diseases”, 16–18th March, 2011, Chiang Mai, Thailand. ***(Oral presentation)***

STUDY WORKS

PART II

Prevalence and factors associated with isolated antibody to hepatitis B core antigen and occult HBV infection in HIV-1 infected pregnant women in Thailand

4. Prevalence and factors associated with isolated antibody to hepatitis B core antigen and occult HBV infection in HIV-1 infected pregnant women in Thailand

4.1. Introduction

The diagnosis of hepatitis B virus (HBV) infection is made primarily by detecting HBV surface antigen (HBsAg) in peripheral blood. However, an absence of HBsAg cannot exclude HBV infection. Indeed, antibodies directed against the core of hepatitis B virus (Anti-HBc), marker of natural HBV infection, can be found in the absence of other serological markers [315]. The clinical significance of isolated anti-HBc is unclear. The majority of individuals with isolated anti-HBc seem to be healthy with normal liver enzyme levels and with no sign of liver disease. Although little is known about its long term outcome, several studies have reported “isolated anti-HBc” serology patterns in patients with cirrhosis and hepatocellular carcinoma (HCC) [23, 381, 382], particularly in those co-infected with hepatitis C virus (HCV) [383, 384]. Study in Taiwan showed that HIV-infected patients with isolated anti-HBc at baseline had significantly shorter survival than those with anti-HBs positive at baseline [325].

Whether subjects with isolated anti-HBc require vaccination against HBV remains controversial. Also, there is a growing concern that individuals with isolated anti-HBc are potentially infectious. Indeed, HBV transmission from isolated anti-HBc individuals has been reported following sexual contacts, blood transfusion [385], organ transplantation [386, 387], or during perinatal period [388, 389). Moreover, HBV Transmission of isolate anti-HBc blood has been demonstrated in chimpanzee model {Thiers, 1988 #450]. The frequency of isolated anti-HBc relates directly to the

prevalence of HBV infection in the population being tested. Among blood donors in geographic areas with low HBV prevalence, its prevalence is 0.4-4% [315, 381, 390, 391]. Higher prevalence of isolated anti-HBc is commonly found in persons with chronic hepatitis C virus infection [383], HIV infection [318, 392, 393], or injection drug use (IDU) [322].

In HIV-infected patients, the prevalence of isolated anti-HBc has been found consistently higher than in HIV-uninfected patients (43 vs 27%, [394]) (17–81% vs 2–5%, [318, 323, 395] [318, 392, 393]. The reason of this increased prevalence of anti-HBc is unclear, maybe related to immune suppreesion. Moreover, reactivation of HBV has been observed in HIV-infected patients with isolated anti-HBc [396]. Like in general population, ongoing HCV infection [323], history of injection drug use, numerous sex partners, and high HIV RNA levels [394] were factors associated with isolated anti-HBc in HIV-infected population.

Occult Hepatitis B virus infection, is currently defined as the presence of HBV-DNA in serum and/or in liver without detectable hepatitis B surface antigen (HBsAg), irrespective of other HBV serological markers [397]. The proportion of occult HBV infection varies depending on the population studied and detection technique used [23]; 4-14% in individuals with isolated anti-HBc [315, 398, 399], and 10-20% in endemic areas [400, 401]. In HIV infected patients, the prevalence of occult HBV infection ranges between 0–89% and is much higher among individual with isolated anti-HBc [352]. However, there is still very limited data available on occult HBV infection in HIV pregnant women and its impact on mother-to-child HBV transmission.[23].

In this study, we aimed to assess, among HIV-infected pregnant women in Thailand, the prevalence of isolated anti-HBc, the prevalence of occult HBV infection among those with isolated anti-HBc, and analyze the risk factors associated with isolated anti-HBc and occult HBV infection.

4.2. Materials and methods

4.2.1. Study population:

The study population was derived from HIV-infected pregnant women who participated in a clinical trial investigating the efficacy of zidovudine (ZDV) plus single dose nevirapine (NVP) to prevent HIV-1 mother-to-child transmission conducted between 2001 and 2003 in Thailand the NCT00398684 [364]. Demographic, clinical and biological data were collected at enrolment in the study.

Only HBsAg-negative women were included in this study. Informed and written consent has been obtained and the study has been performed according to the World Medical Association Declaration of Helsinki and procedures have been approved by the Ethic Committee of Faculty of Associated Medical Sciences, Chiang Mai University.

4.2.2. Sample collection

Maternal blood samples collected at entry, prior to ZDV prophylaxis, were centrifuged, and plasma or serum were frozen at -70^{o} or -20^{o}C

4.2.3. Analysis of HBV infection markers

HBsAg was screened using an EIA assay (DiaSorin ETI-MAK-2, Salluggia, Italy). anti-HBc was detected using MonoLisa® anti-HBc PLUS (Bio-Rad laboratories, Marnes La Coquette, France) and anti-HBs using MonoLisa® anti-HBs PLUS (Bio-Rad laboratories, Marnes La Coquette, France). For those presenting isolated anti-HBc serology pattern, HBV DNA was quantified using Abbott real-time HBV DNA™ assay (Abbott France, Rungis, France)(lower limit of detection of 1.18 $\log_{10}$ or 15 IU/mL) and HBsAg was verified with another HBsAg test kit (MonoLisa® HBsAg ultra, Bio-Rad laboratories)

4.2.4. HBV sequencing

HBV sequencing was performed to check for the presence of HBV mutation in patients with occult HBV infection. HBV DNA was extracted from patient's plasma using the automatic sample extraction system (Abbott M2000sp, Rungis, France). Ten microliters of HBV DNA extract were used as the template for nested polymerase chain reaction (PCR). Published primers were used to amplify HBV surface/polymerase region (nucleotide position 251 to 1058) [365]. Amplicons were sequenced using the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA) and the ABI PRISM 3100 Genetic Analyzer, and sequencing data analyzed using the software Bioedit.

4.2.5. Statistic analysis

Characteristics of women including age at enrollment, region of birth, alanine transminase enzyme (ALT) level, white blood cells, lymphocytes, CD4+ T-cells,

CD8+ T-cells count and the presence of antibodies against syphilis or hepatitis C virus, are described using number and percentage for categorical data and median with interquartile range (IQR) for continuous data.

Univariate analyses were performed using logistic regression analysis to identify potential risk factors for having isolated anti-HBc. Continuous variables were transformed into categorical variables using common cut-off values. All factors with p-value <0.20 identified by univariate analysis were then introduced into multivariate logistic regression analysis to investigate independent risk factors associated with the isolated anti-HBc serology pattern. All data analyses were performed using STATA™ version 10.1 software (Statacorp, College Station, TX). Differences were considered statistically significant if the p-value was <0.05.

4.3. Results

4.3.1. Characteristics of women

Of 2,028 HIV-1 infected women who participated in the perinatal HIV prevention trial, PHPT-2, 1,752 were found HBsAg-negative and included in this study (Figure 4.1). Characteristics of women are described in Table 4.1.

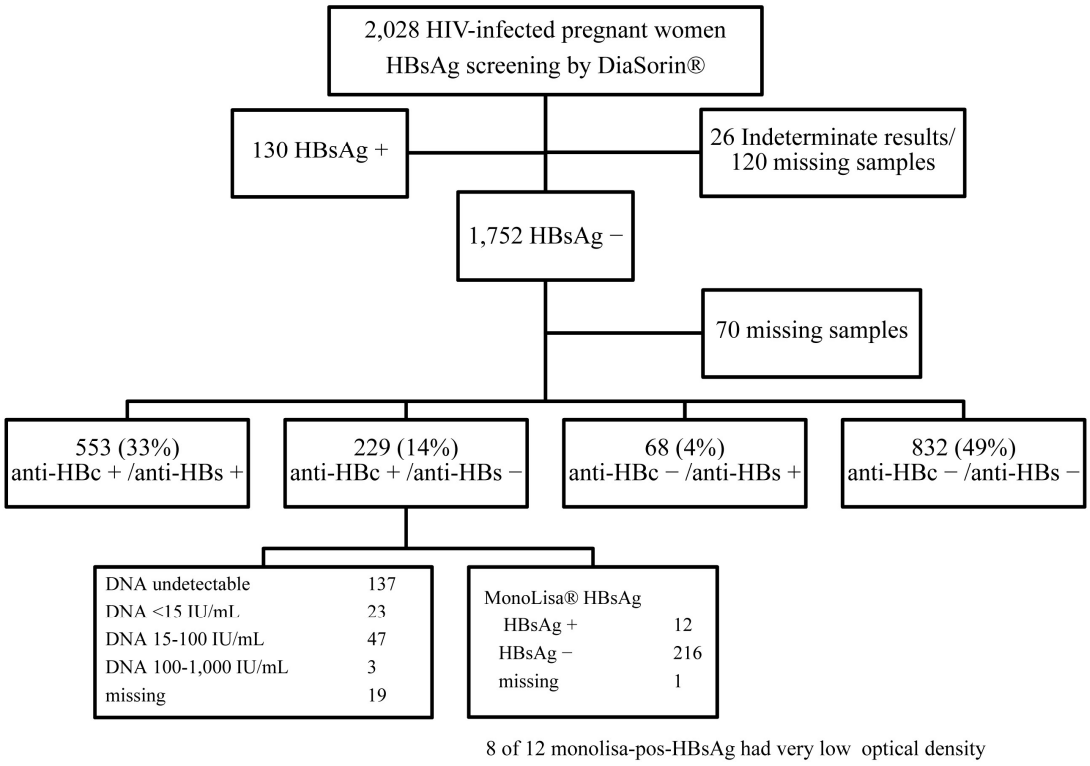


Figure 4.1. Overall study diagram

Table 4.1. Characteristics of women

Characteristics	N	Categories	Median (IQR) or proportion
Age at enrollment (years)	1,752		26.0 (22.8-29.7)
Region of birth (%)	1,689	Central	375 (22)
		Eastern	258 (15)
		Northern	349 (21)
		North-eastern	555 (33)
		Southern	76 (5)
		Western	76 (5)
Region of enrollment (%)	1,689	Central	390 (23)
		Eastern	459 (27)
		Northern	362 (21)
		North-eastern	248 (15)
		Southern	84 (5)
		Western	146 (9)
Prior pregnancy (%)	1,748		1,084 (62)
SGPT or ALT (IU/L)	1,706		14 (10-24)
White blood cells (cells/μL)	1,718		8,600 (7,260-10,100)
Absolute lymphocyte (cells/μL)	1,716		1,800 (1,425-2,240)
Absolute CD4 (cells/μL)	1,739		376 (244-529)
Absolute CD8 (cells/μL)	1,702		913 (697-1193)
HIV RNA load (log_{10} copies/mL)	1,729		4.05 (3.37-4.65)
Anti-syphilis antibody positive (%)	1,717		17 (1)
Anti-HCV antibody positive (%)	1,727		77 (4)

HBV status among HIV-pregnant women HBsAg negative

Of 1682 women with available samples, 832 (49%) were negative for anti-HBs and anti-HBs antibodies and thus considered as having not acquired HBV infection, detected, 553 (33%) were positive for both anti-HBc and anti-HBs antibodies and considered as having resolved HBV infection, 229 (14%) had isolated anti-HBc and considered as having acquired HBV infection, and 68 (4%) had were positive for anti-HBs antibodies and considered as having received HBV vaccine.

The prevalence of isolated anti-HBc antibodies differed according to the region of birth. The highest rate, 22%, was found in women born in northern region, while the lowest rate, 4%, was found in southern region (Table 4.2). Median age of women with isolated anti-HBc was 26.6 years old, ranges from 15-46 years old.

Table 4.2. HBV serological status of HBsAg negative women according to region of birth.

	N (%)						
	Central	**Eastern**	**Northern**	**North-eastern**	**Southern**	**Western**	**Total**
anti-HBc + /anti-HBs +	128 (34)	74 (29)	148 (43)	161 (29)	20 (26)	22 (29)	553 (33)
anti-HBc + /anti-HBs –	38 (10)	31 (12)	77 (22)	69 (12)	3 (4)	11 (14)	229 (14)
anti-HBc – /anti-HBs +	26 (7)	10 (5)	16 (5)	10 (2)	1 (1)	5 (7)	68 (4)
anti-HBc – /anti-HBs –	181 (49)	141 (55)	107 (31)	313 (57)	52 (68)	38 (50)	832 (49)

Prevalence of occult HBV infection

Among 229 HIV-1 infected pregnant women with isolated anti-HBc, 210 had a sample available for HBV DNA quantification. Of these, 160 had HBV DNA below the limit of detection (15 IU/mL), 47 had HBV DNA level between 15 to 100 IU/mL, and only 3 had HBV DNA above 100 IU/mL but below 1,000 IU/mL (Table 4.3). The prevalence of occult HBV infection was thus 24% (IC95%, 18-30). Of all women with detectable HBV DNA, HBV sequencing was successful only for 2 cases, one had sS117I, sT118K, and sR160K mutations, and the other had no S gene mutation.

Table 4.3. Proportion of occult HBV infection among 210 HIV-1 infected pregnant women carrying isolated anti-HBc

HBV DNA level	**N=210**	**Proportion (95%CI)**
DNA 100-1,000 IU/mL	3	1.4 (0.3-4.1)
DNA 15-100 IU/mL	47	22.4 (16.9-28.6)
DNA <15 IU/mL	23	11.0 (7.1-16.0)
Undetectable	137	65.2 (58.4-71.7)

We also verified the absence of HBsAg in all women with isolated anti-HBc using a different test kit. Of 228 women with available samples, 12 (5%) showed discrepant HBsAg results, that is negative became positive, while 8 of this 12 had low level of optical density (signal to cut-off (S/CO) ratio ranges from 1.02 to 6.10) (Table 4.4).

Table 4.4 Relationship of HBV DNA load and second HBsAg results

HBV DNA load	Second HBsAg results			Total
	Positive	Negative	not done	
Undetectable	6	131	0	137
<15 IU/mL	1	22	0	23
15-100 IU/mL	3	44	0	47
100-1,000 IU/mL	0	3	0	3
Missing data	2	16	1	19

Factors associated with isolated anti-HBc

Univariate analysis shows that, among all parameter analyzed, age over 35 years, birth place in northern region, white blood cells counts <7,500 cells/µL, lymphocyte counts <1,000 cells/µL, CD4+ T-cells count <350 cells/µL and HCV infection were significantly associated with the presence of isolate anti-HBc antibodies in HIV-1 infected pregnant women.

A multivariate analysis was performed to adjust on all significant parameters associated with the presence of isolate anti-HBc from univariate analysis and is showed in Table 4.5. The results show the same association as in univariate analysis: Age over 35 years (adjusted odds ratio [aOR], 1.8; P=0.03), born in northern region (aOR, 1.8; P<0.001), absolute CD4 count below 350 cells/µL (aOR, 1.5; P=0.02) and much more significantly if CD4 count below 200 cells/µL (aOR, 2.8; P<0.001), and past or present HCV infection (aOR, 2.6; P=0.001), a independently associated with the presence of isolated anti-HBc.

Table 4.5. Factors associated with isolated anti-HBc among HIV-1 infected pregnant women

Parameters	Categories	N	univariate analysis			multivariate analysis	
			anti-HBc alone (%)	OR (95%CI)[a]	P-value[b]	OR (95%CI)	P-value
Age at enrollment	≤ 25 years	725	90 (12)	1.0			
	>25-30 years	569	69 (12)	1.0 (0.7-1.4)	0.88		
	>30-35 years	284	48 (17)	1.4 (1.0-2.1)	0.06		
	>35 years	104	22 (21)	**1.9 (1.1-3.2)**	**0.016**	**1.8 (1.1-2.9)**	**0.029**
Region of birth	Central	373	38 (10)	1.0			
	Eastern	256	31 (12)	1.2 (0.7-2.0)	0.45		
	Northern	348	77 (22)	**2.5 (1.6-3.8)**	**<0.001**	**1.8 (1.3-2.5)**	**<0.001**
	North-eastern	553	69 (12)	1.3 (0.8-1.9)	0.29		
	Southern	76	3 (4)	0.4 (0.1-1.2)	0.098	0.4 (0.1-1.1)	NS
	Western	76	11 (14)	1.5 (0.7-3.1)	0.28		
Previous pregnancy	No	637	83 (13)	1.0			
	Yes	1,041	145 (14)	1.1 (0.8-1.4)	0.60		
ALT (IU/L)	≤40	1561	207 (13)	1.0			
	>40-80	60	11 (18)	1.5 (0.8-2.9)	0.26		
	>80	17	4 (24)	2.0 (0.7-6.2)	0.23		
White blood cells (cells/µL)	>10,000	440	44 (10)	1.0			
	7,501-10,000	723	91 (13)	1.3 (0.9-1.9)	0.18		
	5,001-7,500	442	78 (18)	**1.9 (1.3-2.9)**	**0.001**		
	≤5,000	47	10 (21)	**2.4 (1.1-5.2)**	**0.02**		
Absolute lymphocyte (cells/µL)	>2,000	624	74 (12)	1.0			
	1,501-2,000	533	63 (12)	1.0 (0.7-1.4)	0.98		
	1,001-1,500	349	52 (15)	1.3 (0.9-1.9)	0.18		
	≤1,000	144	33 (23)	**2.2 (1.4-3.5)**	**0.001**		
Absolute CD4 (cells/µL)	>500	489	44 (9)	1.0			
	351-500	423	43 (10)	1.1 (0.7-1.8)	0.55		
	201-350	446	65 (15)	**1.7 (1.1-2.6)**	**0.009**	**1.5 (1.1-2.2)**	**0.02**
	≤200	313	76 (24)	**3.2 (2.2-4.9)**	**<0.001**	**2.8 (2.0-4.0)**	**<0.001**
Absolute CD8 (cells/µL)	>1,500	179	21 (12)	1.0			
	1,001 – 1,500	489	58 (12)	1.0 (0.6-1.7)	0.96		
	501 – 1000	835	119 (14)	1.3 (0.8-2.1)	0.38		
	≤500	131	24 (18)	1.7 (0.9-3.2)	0.11		
HIV RNA load (copies/µL)	undetectable	42	5 (12)				
	log 1.18 – 3.00	216	23 (11)	0.9 (0.3-2.5)	0.81		
	log 3.01 – 4.00	551	75 (14)	1.2 (0.4-3.1)	0.76		
	log 4.01 – 5.00	660	85 (13)	1.1 (0.4-2.9)	0.86		
	> log 5	191	38 (20)	1.8 (0.7-5.0)	0.23		
Anti-syphilis antibody	No	1632	220 (13)	1.0			
	Yes	17	2 (12)	0.9 (0.2-3.8)	0.84		
Anti-HCV antibody	No	1584	202 (13)	1.0			
	Yes	75	23 (31)	**3.0 (1.8-5.1)**	**<0.001**	**2.6 (1.5-4.3)**	**0.001**

[a]OR (95%CI): Odds ratio (95% confident interval); [b]Logistic regression analysis was used; NS: Not significant

Detection of HBV DNA is inversely correlated with HIV RNA concentration in HIV-1 infected pregnant women with isolated anti-HBc

Among all parameters analyzed, univariate and multivariate analysis showed that detection of HBV DNA, and thus occult HBV infection,was inversely proportional to HIV RNA level. Rate of occult HBV infection was lowest when HIV RNA level greater than 5 $\log_{10}$ copies/μL (aOR, 0.03; P=0.006) (Table 4.6).

Table 4.6. Factors associated with HBV DNA positivity among 210 HIV-1 infected pregnant women carrying isolated anti-HBc

Parameters	Categories	N	Univariate analysis			multivariate analysis	
			HBV DNA positive (%)	OR (95%CI)[a]	P-value [b]	OR (95%CI)	P-value
Age at enrollment	≤ 25 years	86	20 (23)	1			
	>25-30 years	60	13 (22)	0.9 (0.4-2.0)	0.82		
	>30-35 years	43	10 (23)	1.0 (0.4-2.4)	1.00		
	>35 years	21	7 (33)	1.7 (0.6-4.6)	0.34		
Region of birth	Central	33	10 (30)	1			
	Eastern	28	4 (14)	0.4 (0.1-1.4)	0.15		
	Northern	71	19 (27)	0.8 (0.3-2.1)	0.71		
	North-eastern	65	17 (26)	0.8 (0.3-2.1)	0.66		
	Southern	3	0 (0)	-	-		
	Western	10	0 (0)	-	-		
Previous pregnancy	No	78	16 (21)	1			
	Yes	131	34 (26)	1.4 (0.7-2.7)	0.37		
ALT (IU/L)	≤40	189	45 (24)	1			
	>40-80	10	3 (30)	1.4 (0.3-5.5)	0.66		
	>80	4	1 (25)	1.1 (0.1-10.5)	0.96		
White blood cells (cells/µL)	>10,000	41	9 (22)	1			
	7,501-10,000	82	17 (21)	0.9 (0.4-2.3)	0.88		
	5,001-7,500	73	21 (29)	1.4 (0.6-3.5)	0.43		
	≤5,000	8	2 (25)	1.2 (0.2-6.9)	0.85		
Absolute lymphocyte (cells/µL)	>2,000	65	14 (22)	1			
	1,501-2,000	59	17 (29)	1.5 (0.7-3.3)	0.35		
	1,001-1,500	48	4 (13)	1.5 (0.6-3.5)	0.36		
	≤1,000	31	4 (13)	0.5 (0.2-1.8)	0.32		
Absolute CD4 (cells/µL)	>500	41	11 (27)	1			
	351-500	40	9 (23)	0.8 (0.3-2.2)	0.65		
	201-350	58	16 (28)	1.0 (0.4-2.6)	0.93		
	≤200	70	14 (20)	0.7 (0.3-1.7)	0.41		
Absolute CD8 (cells/µL)	>1,500	20	7 (35)	1			
	1,001 – 1,500	51	9 (18)	0.4 (0.1-1.3)	0.12		
	501 – 1000	110	27 (25)	0.6 (0.2-1.7)	0.33		
	≤500	24	6 (25)	0.6 (0.2-2.3)	0.47		
HIV RNA load (copies/µL)	undetectable	5	4 (80)	1		1	
	log 1.18 – 3.00	20	7 (35)	0.1 (0.01-1.4)	0.098	0.1 (0.01-1.4)	0.098
	log 3.01 – 4.00	71	19 (27)	**0.1 (0.01-0.87)**	**0.04**	**0.1 (0.01-0.87)**	**0.04**
	log 4.01 – 5.00	76	16 (21)	**0.07 (0.007-0.64)**	**0.02**	**0.07 (0.007-0.64)**	**0.02**
	> log 5	35	4 (11)	**0.03 (0.003-0.36)**	**0.006**	**0.03 (0.003-0.36)**	**0.006**
Anti-syphilis antibody	No	201	48 (24)	1			
	Yes	2	1 (50)	3.2 (0.2-51.2)	0.42		
Anti-HCV antibody	No	184	42 (23)	1			
	Yes	22	8 (36)	1.9 (0.8-4.9)	0.17		

[a]OR (95%CI): Odds ratio (95% confident interval); [b]Logistic regression analysis was used; NS: Not significant

4.4. Discussion and conclusion

This is the first detailed study of HBV serologic markers among a large number of HIV-pregnant women and the evaluation of occult HBV infection in those presenting a serologic profile with isolate anti-HBc. Our results showed that between 2001 and 2003, the prevalence of isolate anti-HBc in HIV pregnant women throughout Thailand was 14%. This figure is consistent to that observed among HIV-infected adults in Bangkok (20%)[322] and 13% in Chiang Mai areas (Thongsawat S., personal communication). Using a highly sensitive technique to detect HBV DNA, it was possible to show that occult HBV infection, as defined by positive HBV DNA, was detected in 24% (50 of 210) of HIV-1 infected pregnant women with isolate anti-HBc serologic profile or 3% of all HIV-infected women tested. Interestingly, the level of HBV DNA was below 1000 IU/mL in all women and below 100 IU/mL in 47 of them raising the question about the clinical significance of occult HBV infection. One intriguing observation was that detection of HBV DNA was inversely correlated to the HIV RNA. In contrast to Lo re et al. [350] who found occult HBV infection more frequently in patients with HIV RNA >1,000 copies/mL, we observed higher rate of occult HBV infection in patients with low HIV RNA concentrations. One possible explanation to this could be the definition of occult HBV infection used and how it is assessed. Indeed, occult HBV infection may involve a combination of viral, host-dependent factors [382], and its prevalence may differ according to the efficiency of kit to detect HBsAg [47]. Viral factors include low replication rate of HBV with mutation in the S gene REF. Host factors include profile of cytokines, age at the time of infection, titer of neutralizing anti-HBs antibodies, chronic alcohol consumption, and HCV co-infection. Use of monoclonal antibody-based diagnostic assays for the detection of HBsAg may be unreliable in populations where the circulating

subtype/genotypes or variants are distinct from the virus strain used for the production of monoclonal antibody [402]. Polyclonal based diagnostic assays may thus be favored. In our study, HBV DNA levels were very low and when retesting all 210 samples with another kit the results were similar in 98% (4 of 210), suggesting that the absence of HBsAg detection may not be due to a problem of kit. Furthermore, amplification and sequencing of S gene was possible in 2 of 50 women with HBV DNA detectable and we observed the sS117I and sT118K mutations that may affect the detection of HBsAg, which have been previously identified in other studies [403-405].

One hypothesis is that isolated anti-HBc was more often seen in women with advanced disease. Indeed, we found that isolate anti-HBc serological profile was associated with low CD4 counts in HIV pregnant women. The association of isolate anti-HBc profile with immunosuppression has been also reported in 2 studies conducted among HIV-infected patients in Taiwan [325, 406] . The low CD4+ T-cells count may reflect the low levels of T-helper cells type 2-derived cytokines (e.g., Interleukin-4 and -10), resulting in decreased production of antibodies against HBV from B-cell. Analysis of HBV serological markers in HIV-uninfected pregnant women would help to clarify the impact of immune suppression. This rate compared to that found in isolated anti-HBc blood donors (4–24%) in a high HBV endemic areas such as India, Taiwan, Japan, and Sardinia [407].

Clinical relevance of isolate anti-HBc and impact of the low levels of HBV DBA in HIV-women with isolate anti-HBc are still unclear. Walz et al. have reported

perinatal transmission of HBV from women with isolated anti-HBc serological patterns [408].

Using multivariate analysis, we identified other factors independently associated with isolate anti-HBc : age over 35 years, birth in northern region, and HCV infection. Some of those factors have also been found in other studies. Thus, age was directly correlated to the presence of isolate anti-HBc in Italian donors, with an exponential fit [409] and HIV infected patients in Taiwan [406]. The effect of age may be related to the loss of anti-HBs-producing capacity decades after resolution of HBV infection, or insufficient level of production [321]. Prevalence of HBV infection and its natural course vary depending on demography and geography as well on viral and host factors. Several studies have reported that the prevalence of HBsAg positivity is usually high in northern region of Thailand as compared to southern region [15, 159, 161] which may explain the parallel rates of isolate anti-HBc we observed in our study.

Several studies have described that infection with HCV is a main factor related to isolate anti-HBc in both HIV-infected or –uninfected population [315, 318, 322, 383, 392, 393, 410] likely as a result of the direct interference of HCV core protein on the synthesis of HBsAg [321].

Surprisingly, in this study, about half of HIV-pregnant women had no HBV serological markers, indicating they are HBV susceptible population. This finding highlights the need of vaccination of all HIV-infected patients without HBV markers

and also the importance of counseling and panel testing against HBV infection in Thailand.

The first limitation of our study is we did not perform HBsAg testing at 2 different time points to confirm chronic HBV status. However, in Thailand, the majority of chronically HBV-infected patients acquired HBV at birth or during early childhood [10]. The second limitation is HBV DNA level was measured on a single sample. Since HBV DNA levels fluctuate over the natural course of HBV infection [408], we may have underestimated the prevalence of occult HBV infection. However, the contrary may be also true.

In conclusion, our study showed that 14%. HIV-infected pregnant women in Thailand had isolate anti-HBc serologic profile; of whom 24% had occult HBV infection. Independent risk factors associated with anti-HBc were older age, low CD4 count and HCV infection. Whether this serologic profile is a reflect of the general immune status of HIV pregnant women or a real marker of natural HBV infection remain unclear. Also since most of women with occult HBV infection had HBV DNA levels below 100 IU/mL, the clinical impact of occult HBV needs to be elucidated.

4.5. Publications

This work was presented in;

- **Khamduang W,** Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. The low prevalence of occult Hepatitis B infection in HIV-1 infected pregnant women with antibody to hepatitis B core antigen alone in Thailand. 18th international AIDS conference, 18-23th July, 2010, Vienna, Austria. ***(THPE0205, Poster presentation)***

STUDY WORKS

PART III

Long-term virological response of Hepatitis B virus to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand

5. Long-term virological response of Hepatitis B virus to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand

5.1. Introduction

Between 350 and 400 million people worldwide are chronically infected by Hepatitis B virus (HBV) [4] and 75 to 80% of these individuals are in Asia and the Western Pacific [8]. Annually, around 1 million people worldwide die from the consequences of HBV infection, including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) [4]. Among HIV-infected populations, the overall prevalence of hepatitis B surface antigen (HBsAg) carriers is estimated to be 8-11% [187]; about 10% in Asia-Pacific region [166] and 9% in Thailand [167, 168]. In HIV-infected individuals, chronic hepatitis B infection is associated with accelerated liver disease progression, aggressive hepatocellular carcinoma and increased liver-related mortality rate [184, 411]. Hepatitis B-related immune reconstitution flares have been observed following initiation of highly active antiretroviral treatment (HAART) [271].

In Thailand, where HBV infection is highly endemic, HBV infection occurs mostly through mother-to-child transmission or during early childhood. HBV genotypes C and B are the most prevalent in the general population, and respectively account for 70-90% and 10-30% of infections [64]. More hepatic necro-inflammatory activity and more rapid progression to cirrhosis and HCC have been observed in patients infected with HBV genotype C as compared to genotype B [250]. Additional important risk factors associated with the development of cirrhosis and HCC include HBeAg positivity and high HBV DNA viral load [250]. Suppression of HBV DNA

level is associated with biochemical and histological remission of liver disease [412, 413]. Therefore, suppressing the replication of HBV to undetectable levels is a major goal in HBV treatment.

Lamivudine (3TC) is a cytidine analogue that inhibits the reverse transcriptase of both HIV and HBV [24]. The efficacy of 3TC (150 mg twice a day) on HBV replication in HIV-HBV co-infected patients is similar to that of 3TC (100 mg once a day) in HBV mono-infected patients [256, 414]. Resistance mutations to 3TC have been observed in HBV-HIV-1 co-infected patients at a rate of 15-20% per year in western countries where HBV genotypes A or D are predominant [25, 274]. In Thailand, over 95% of HIV-infected patients receive lamivudine (3TC) as part of highly active antiretroviral therapy (HAART) and 9% are co-infected with HBV. The long-term benefit of 3TC on HBV infection and the incidence of 3TC resistance in these HBV-HIV co-infected patients are not well known.

The aims of our study were thus to analyze the effect of 3TC-containing HAART regimens on HBV replication among HIV-HBV co-infected Thai patients and determine the rate of maintained HBV DNA suppression over 12 months and more of treatment and characterize the 3TC resistance HBV variants that have emerged on treatment.

5.2. Methods

5.2.1. Study population

Patients were enrolled in the prospective multicenter Program for HIV Prevention and Treatment (PHPT) cohort (ClinicalTrials.gov Identifier:

NCT00433030) of HIV-infected adults on antiretroviral therapy in Thailand. Informed and written consent were obtained and the study performed according to the World Medical Association Declaration of Helsinki and approved by the local ethic committees. Prior to starting HAART, all patients were screened for HBsAg and anti-HCV antibodies at each hospital. CD4+ T-cell counts and HIV RNA quantification were performed at start of HAART and every 6 months thereafter. Patients received a quarterly clinical biological follow-up and compliance is assessed at each visit by pill count.

Patient were included in this analysis if 1) HBsAg seropositive, 2) receiving HAART regimens which included 3TC (150 mg twice a day), 3) stored blood samples collected prior to 3TC use (baseline), and at least 3 and 12 months after HAART initiation were available, and 4) HBV DNA was detectable at baseline. The "3-month" sample range from 2-6 months and "12-month" sample range from 10-18 months.

5.2.2. HBV and HIV testing

HBsAg positive patients had HBV viral load quantified at baseline, 3, 12 months, and the last visit using the Abbott real-time HBV DNA™ assay, Abbott France, Rungis, France (linear range 1.18 $\log_{10}$ to 9 $\log_{10}$ IU/mL). If HBV DNA was found negative at baseline HBsAg was re-tested using an EIA assay (DiaSorin ETI-MAK-4, Salluggia, Italy). If HBV DNA was detectable at baseline, HBeAg was tested using DiaSorin ETI-EBK PLUS (Salluggia, Italy). HIV RNA was quantified using the COBAS Amplicor HIV-1 Monitor Test v.1.5. (Roche Molecular Systems, Branchburg, NJ) (lower limit of detection: 50 copies/mL) and the Abbott real-time HIV RNA™ assay, Abbott, (lower limit of detection: 40 copies/mL).

5.2.3. HBV virological responses

HBV responses to 3TC were categorized according to the Asian Pacific Association for the study of liver recommendations [358]. Thus, HBV DNA suppression is defined as undetectable level of HBV DNA (the threshold used was 150 or 2.18 $\log_{10}$ IU/mL since some samples were diluted at 1:10 ratio due to insufficient volume). Virological breakthrough is defined as an initial decline >2 $\log_{10}$ IU/mL followed by an increase of HBV DNA >1 $\log_{10}$ IU. Maintained viral suppression was defined as HBV DNA level persistently <2.18 $\log_{10}$ IU/mL.

5.2.3.1. HBV DNA sequencing

Ten μL of Abbott M2000 HBV DNA extract were used to amplify the *HBV polymerase* region (nucleotide position 251 to 1058) [365]. The first-round PCR was performed in a 59 μL volume using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) and the primers Pol1M (5'-CCC TGC TCG TGT TAC AGG CGG-3') and Pol2M (5'-GTT GCG TCA GCA AAA ACT TGG CA-3'), which yield an amplicon of 1,010 bp. PCR conditions consisted of an initial 2 min denaturation step at 94^{o}C, followed by 40 cycles of denaturation at 94^{o}C for 1 min, annealing at 48^{o}C for 1 min, and extension at 68^{o}C for 3 min. The second-round PCR was performed using 10 μL of the first-round product and the following nested primers, Pol3M (5'-GAC TCG TGG TGG ACT TCT CTC A-3') and Pol4M (5'-GGC ATT AAA GCA GGA TAA CCA CAT TG-3') [365], to yield an 808 bp amplified fragment. PCR conditions were an initial denaturation step of 94^{o}C for 2 min, followed by 30 cycles of denaturation at 94^{o}C for 40 sec, annealing at 55^{o}C for 1 min,

and extension at 68^{o}C for 3 min. Amplicons were visualized under UV light following electrophoresis on a 1% agarose gel stained with ethidium bromide. The second-round PCR products were used directly for bidirectional sequencing with the nested pol3M and pol4M primers. Amplicons were sequenced using the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA), and sequences were analyzed using the Bioedit software (http://www.mbio.ncsu.edu/bioedit). HBV *pol* sequences were analyzed for polymorphisms and mutations known to be associated with 3TC resistance through comparison with wild-type reference sequences of similar genotype [415].

5.2.4. HBV Genotyping

HBV genotype was identified by phylogenetic analysis. Briefly, *pol* gene sequences were aligned with published *pol* sequences of various HBV genotypes available in GenBank using the software clustalW. Phylogenetic trees were constructed using neighbor-joining method. Genetic distances were calculated using the Kimura two-parameter method, as implemented in the software MEGA. Bootstrap analysis with 100 simulations was used to test the reliability of branching.

5.2.5. Statistical analyses

STATA™ version 10.1 software (Statacorp, College Station, TX) was used to compare baseline characteristic data according to HBeAg status. Fisher's exact test was used for categorical variables and Wilcoxon rank-sum test was used for continuous variables. Results are reported as percentage with 95% confidence interval (95%CI) or medians with interquartile ranges (IQR).

Kaplan-Meier analysis was used to estimate the rate of HBV DNA suppression and time to achieving serum HBV DNA suppression. In patients who achieved HBV DNA suppression within the first 12 months of 3TC therapy, Kaplan-Meier analysis was used to estimate the rate and time of maintaining such suppression. The log-rank test was used to compare the cumulative rate of virological responses between HBeAg-positive and -negative patients. Statistical significance was defined as $p<0.05$.

5.3. Results

5.3.1. Baseline characteristics

Of 1,448 HIV infected adults on HAART, 122 (8.4%) tested HBsAg-positive. Of these, 53 were receiving 150 mg twice a day (bid) of 3TC as part of HAART. Among them, 44 were tested for HBV DNA at baseline, 3 and 12 months after treatment initiation. Of 34 patients with detectable HBV DNA at baseline samples, 4 stopped 3TC very early and switched to another regimen. Finally, 30 patients were included in this study (Figure 5.1). Their median age was 31 years [IQR; 27-34], 80% were female. Median CD4+ and CD8+ T-cell counts were 100×10^6 and 562×10^6 cells/L, respectively. Median alanine transaminase (ALT) level was 30 U/L (IQR; 20-39) and median aspartate transaminase (AST) level was 48 U/L (IQR; 38-79). Median HIV RNA was 4.47 $\log_{10}$ copies/mL, and HBV DNA: 7.35 $\log_{10}$ IU/mL. Phylogenetic analysis indicated that 17% of patients were infected with HBV B genotype and 83% with C genotype. None had HCV infection. Although 12 (40%) of the patients had received previous antiretroviral treatment, none had been exposed to 3TC except one for a short period. At initiation of 3TC, 19 (63%) of the patients were HBeAg-positive. The baseline characteristics of HBeAg-positive and -negative

patients were similar, except for median HBV DNA level which was significantly higher, as expected, in HBeAg-positive patients. (Table 5.1)

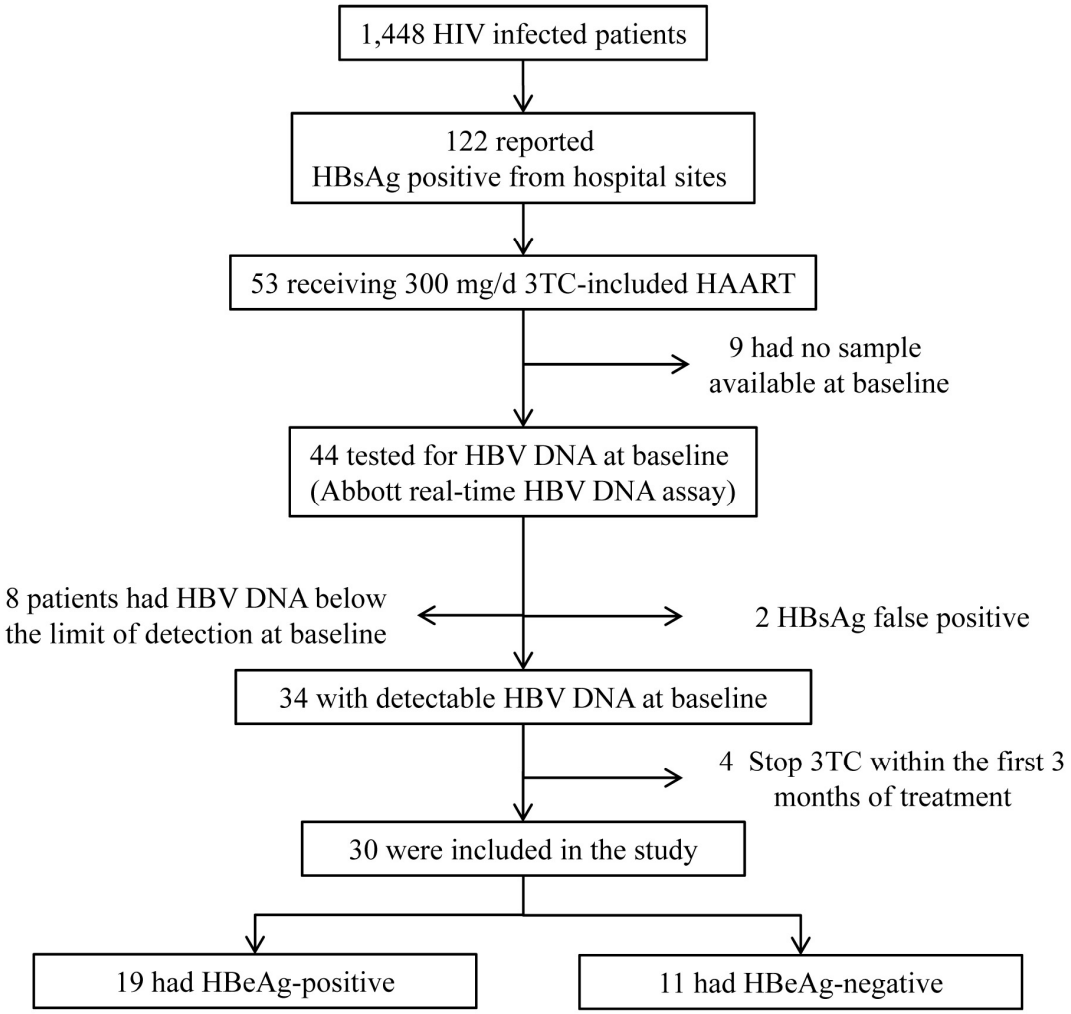


Figure 5.1. Overall study diagram

Table 5.1. Baseline demographic and clinical characteristics of the study population

Baseline characteristics	**Overall**		**HBeAg positive (N=19)**		**HBeAg negative (N=11)**		**p-value[a]**
	n	**Value**	**n**	**Value**	**n**	**Value**	
Age (year) [median (IQR)]	30	31 (27-34)	19	29 (27-33)	11	33 (27-35)	0.59
Female [n (%)]	30	24 (80)	19	17 (89)	11	7 (64)	0.16
Treatment-experienced [n (%)]	30	12	19	7 (37)	11	5 (45)	0.71
CD4+ T-cell count ($x10^6$/L) [median (IQR)]	30	100 (38-178)	19	110 (38-188)	11	48 (33-178)	0.78
CD8+ T-cell count ($x10^6$/L) [median (IQR)]	19	562 (396-912)	13	679 (421-938)	6	506 (353-792)	0.33
HIV RNA (log_{10}copies/mL) [median (IQR)]	30	4.47 (4.09-5.27)	19	4.46 (4.06-5.25)	11	5.25 (4.25-5.50)	0.29
Alanine transaminase (IU/L) [median (IQR)]	30	30 (20-39)	19	27 (17-36)	11	44 (21-121)	0.06
HBV DNA (log_{10}IU/mL) [median (IQR)]	30	7.35 (5.55-8.07)	19	7.92 (7.34-8.31)	11	3.76 (3.28-6.67)	<0.001
HBV Genotype B : C [n (%)]	30	5:25 (17:83)	19	4:15 (21:79)	11	1:10 (9:91)	0.63

[a] Fisher's exact test or Wilcoxon rank-sum test were used

Table 5.2. HBV and HIV virological response to 3TC in HIV-1/HBV co-infected patients during 12 months of 3TC treatment

Baseline characteristics	**Overall (N=30)**		**HBeAg positive (N=19)**		**HBeAg negative (N=11)**		**p-value[a]**
	n	**%[95%CI] or median [IQR]**	**n**	**%[95%CI] or median [IQR]**	**n**	**%[95%CI] or median [IQR]**	
HBV DNA suppression[b] at 3 months	14	53 [34-72]	6	32 [13-57]	8	73 [39-94]	0.06
Median HBV DNA reduction at 3 months (log_{10} IU/mL)		3.86 [2.56-4.67]		4.26 [3.40-5.48]		1.88 [1.36-3.59]	0.008
HBV DNA suppression[b] at 12 months	20	67 [47-83]	9	47 [24-71]	11	100 [72-100]	0.004
Median HBV DNA reduction at 12 months (log_{10} IU/mL)		4.40 [2.89-5.65]		4.94 [3.97-6.13]		1.88 [1.36-5.30]	0.02
HBV DNA breakthrough	4	13 [4-31]	4	21 [6-46]	0	0 [0-28]	0.27
HIV load ≤50 cp/mL at 3 months	20	67 [47-83]	11	58 [33-80]	9	82 [48-98]	0.25
Median HIV load reduction at 3 months (log_{10} cp/mL)		2.92 [2.54-3.53]		2.93 [2.14-3.48]		2.91 [2.54-4.08]	0.53
HIV load ≤50 cp/mL at 12 months	22	73 [54-88]	14	74 [49-91]	8	73 [39-94]	1.00
Median HIV DNA reduction at 12 months (log_{10} cp/mL)		2.92 [1.52-3.45]		2.93 [1.52-3.32]		2.91 [1.17-4.08]	0.78

[a] Fisher's exact test or Wilcoxon rank-sum test were used

[b] HBV DNA suppression was defined as serum HBV DNA level equal or below 150 or 2.18 log_{10} IU/mL

5.3.2. Efficacy of 3TC on HBV replication

At 3 months, overall median reduction of HBV DNA was 3.86 $\log_{10}$ (IQR, 2.56-4.67), and 53% (95%CI, 34-72) of patients achieved HBV DNA suppression. At 12 months, overall median HBV DNA reduction was 4.40 $\log_{10}$ (IQR, 2.89-5.65) IU/mL and 67% (95%CI, 47-83) of patients achieved HBV DNA suppression. Of the 20 patients who achieved HBV DNA suppression at 12 months, 18 were tested for HBsAg and one had lost HBsAg. Of 4 patients who experienced HBV breakthrough during the first 12 months: 2 had HBV DNA suppression at 3 months and 2 had never fully suppressed HBV replication. Six patients had partial HBV DNA suppression.

Twenty-two patients who experienced HBV DNA suppression were included in the analysis of the duration of HBV DNA suppression on 3TC treatment, 2 of them had HBV breakthrough as describe above, one changed drug regimen after 15 months of 3TC treatment, and 19 were followed-up over a median duration 50 months (IQR; 32-65 months). Of these 19 patients, 2 had HBV breakthrough and 17 (89%) maintained HBV DNA suppression until their last medical visit. The estimates cumulative rates of maintained HBV DNA suppression were 91% (95%CI; 68-98), 84% (95%CI; 58-95), and 68% (95%CI; 26-89) at 1, 3, and 5 years, respectively (Figure 5.2). Of those 17 patients with maintained HBV DNA suppression, 3 of 16 (19%) lost HBsAg at their last visit. Among the 8 HBeAg positive patients lost HBeAg at their last visit.

The rate of HBV DNA suppression at 12 months was significantly higher among HBeAg-negative patients than among HBeAg-positive patients (100% and 47%, respectively; P=.004; Table 5.2). Kaplan-Meier analysis showed that HBeAg-negative patients achieved HBV DNA suppression more rapidly than HBeAg-positive patients (Figure 5.3, P-value by log-rank test = 0.017). HBV DNA suppression was maintained in all HBeAg-negative patients (Figure 5.2).

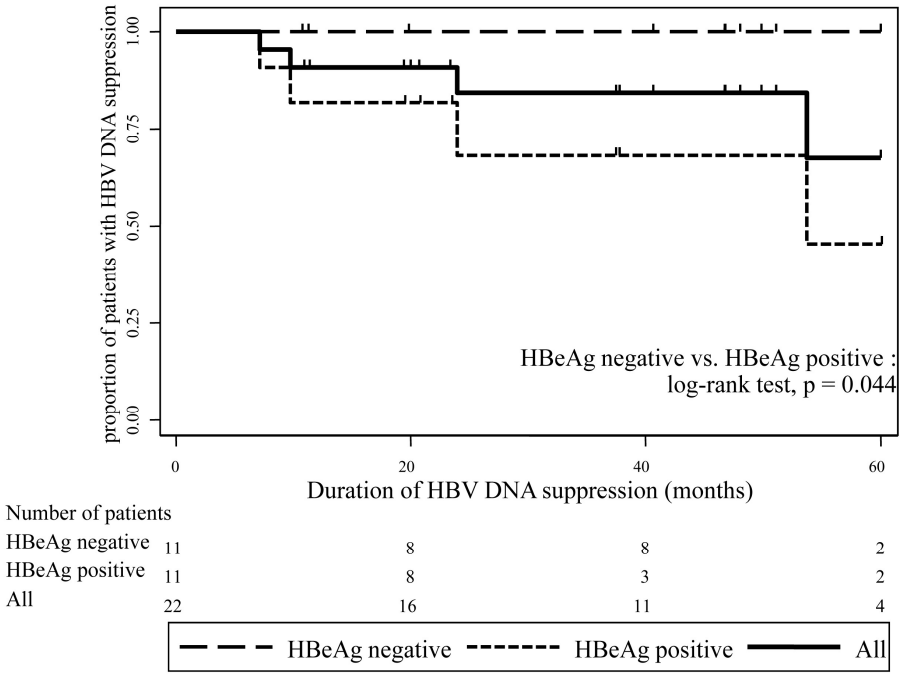


Figure 5.2. Kaplan-Meier curve of time to loss of HBV DNA suppression in 22 HIV-HBV co-infected patients who had achieved HBV DNA suppression within 1 year of 3TC-containing HAART

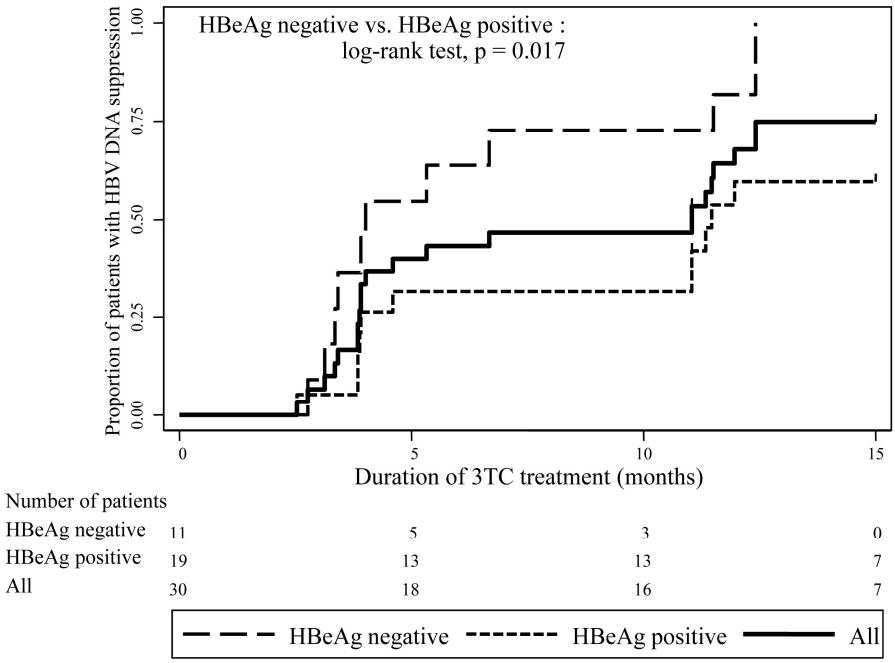


Figure 5.3. Kaplan-Meier curve of time to HBV DNA suppression. HBV DNA suppression is defined as HBV DNA level <2.18 $\log_{10}$ IU/mL, during the first 1 year of 3TC-containing HAART in HBeAg-positive and HBeAg-negative HIV-HBV co-infected patients.

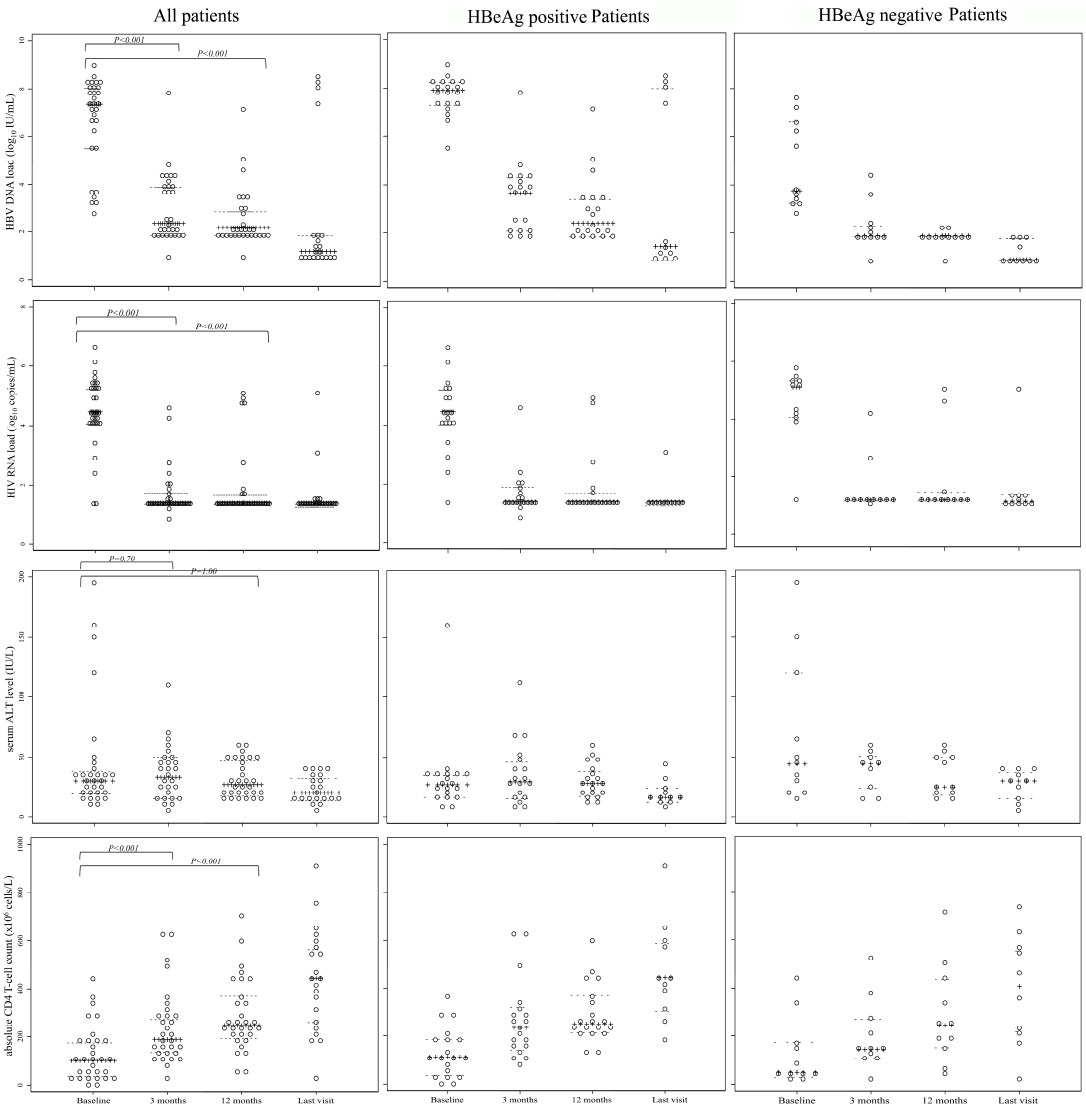


Figure 5.4. Dot plot distribution graphs of HBV DNA load, HIV RNA load, serum ALT level, and CD4+ T-cells count at baseline, 3, 12 months, and last visit in HIV-HBV co-infected patients on 3TC-containing HAART.

5.3.3. 3TC resistance-associated mutations

Prior to 3TC initiation, all subjects had no 3TC-resistance-associated mutation. At 3 months, among the 16 patients with detectable HBV DNA, HBV sequencing was successful for 14 patients and no 3TC-resistance-associated mutation was found. HBV breakthrough was observed in 7 patients, 4 occurred early between 4 and 12 months, and 3 were detected late at 35, 65 and 81 months.

Of the 4 patients with early breakthrough, one developed the 3TC resistance-associated mutation ntG741A, resulting in the known rtM204I mutation and also in a concomitant substitution in the *s* protein of a tryptophan at codon 196 to a stop codon (sW196stop) as a result of overlapping reading frames of the envelope and polymerase genes. Another mutation also emerged, ntT843G, which resulted in a change from asparagine to lysine in the reverse transcriptase protein (rtN238K) and whose significance is unknown. In the 3 other patients, no 3TC resistance-associated mutation was observed. However, during their long-term follow-up (i), the emergence of rtV173L+L180M+M204I, well-known 3TC resistance mutations was observed in one patient at 42 months (ii) HBV DNA could not be amplified for one patient, (iii) and one patient stopped 3TC after 18 months of treatment.

Of the 3 patients with late HBV breakthrough, one had the 3TC-resistance mutation pattern, rtV173L+L180M+M204I, detected at 65 months of treatment.

5.3.4. Efficacy of HAART on HIV replication, CD4 cell count and alanine transaminase level

At 3-month, the median reduction of HIV RNA was 2.92 $\log_{10}$ (IQR, 2.54-3.53) and 67% patients achieved undetectable HIV RNA load (<1.7 $\log_{10}$ or 50 copies/mL). At 12-month, the median HIV RNA reduction was 2.92 $\log_{10}$ (IQR, 1.52-3.45) IU/mL and 73% patients achieved undetectable HIV RNA load. Reduction of HIV RNA level and proportions of undetectable HIV RNA were similar in HBeAg negative and HBeAg positive groups. Six patients had HIV RNA level above 500 copies/mL and presented the M184I/V mutations associated with HIV resistance to 3TC. CD4+ T-cell counts had risen from 100 (IQR: 38-178) cells/µL at baseline to 247 (IQR: 197-374) and 445 (IQR: 264-568) cells/µL at 12-month and last visit, respectively. ALT levels were normal and did not change during 3TC treatment (baseline: 30 IU/L, IQR 20-39; 12 months: 27, IQR 18-48; last visit: 20, IQR 14-33), as show in Figure 5.4.

5.4. Discussion

We analyzed the long term HBV virological response in a group of 30 HIV-HBV co-infected patients, 63% HBeAg positive, who received 3TC for the first time as part of HAART regimen in Thailand. At initiation of 3TC, median HBV DNA level was 7.35 $\log_{10}$IU/mL. After 12 months of HAART, the overall HBV DNA suppression rate was 67%; 47% in HBeAg positive patients and 100% in HBeAg negative patients.

The rate of early response in our study, 53% of HBV DNA suppression at 3 months, is similar to the 30% reported by the international collaborative (CAESAR) study, conducted in Canada, Australia, Europe and Africa [24], although the median HBV DNA level prior to 3TC initiation was higher in our study, 7.35 vs 6.87 $\log_{10}$ IU/mL. At 12 months, the median HBV DNA decrease was 4.40 log in our study while it was 2.7 log in the CAESAR study likely due to different thresholds of HBV DNA quantification. Another possible cause may be related to the HBV genotypes, highly replicating C and B in our study and likely A or D in the CEASAR study. A recent study conducted in Kenya [416] reported that 89% (17/19) of HIV-HBV co-infected patients achieved HBV DNA suppression (<100 IU/mL) during 18 months of 3TC treatment (baseline HBV DNA level was 3.38 $\log_{10}$ IU/mL). The rates of patient with HBV DNA suppression was 94% (17 of 18) in HBeAg negative patients, while one HBeAg-positive patient was unable the suppress HBV replication under 100 IU/mL. These rates are not different from those found in our study, 47% in HBeAg-positive and 100% in HBeAg-negative patients.

Among the 22 patients who had achieved HBV DNA suppression, 17 (77%) had maintained HBV DNA suppression until their last visit (median 50 months). This rate is much higher than the 9% (defined as undetectable by Digene Hybrid Capture assay with the threshold of 4.03 log IU/mL) previously reported by Benhamou et al, in HIV-HBV co-infected patients after 4 years of treatment with the same dosing of 3TC [25]. We could hypothesize that the higher response rate in our study is due to a better compliance of patients to their treatment or HBV genotypes B and C are more sensitive to 3TC than genotypes A and D, which require confirmation with in vitro experiments. The estimated cumulative rates of maintained HBV DNA suppression

at 1, 3, and 5 years after achieving suppression were 91%, 84, and 68, respectively. HBV DNA suppression was maintained in all HBeAg-negative patients. The higher rate of response to 3TC treatment and duration of HBV DNA suppression among HBeAg negative patients suggest that, in resource-limited countries, HBeAg testing may be valuable to predict the virological response to nucleoside/nucleotide analogs and could be considered when initiating in HIV-HBV co-infected patients the first-line HAART. Indeed in resource-limited countries, 3TC is included in the first-line HAART regimen.

One major limitation of treating HBV with 3TC monotherapy is the rapid emergence of resistance mutations. In HBV-HIV-1 co-infected patients, resistance mutations to 3TC have been shown to occur at a rate of 15-20% per year [25, 417]. In our study, the incidence of 3TC resistance mutations during the first year of therapy was 3% (1of 30) which is not different to the 7% (2 of 27) in a study conducted in Kenya (p=.60) [416]. Over the 6 years of follow-up, 7 patients presented HBV breakthrough and the rtM204I, due to the ntG741A uncommon mutation, and the triple rtV173L+L180M+M204I mutations associated with 3TC resistance were identified in 3 patients. Despite a good compliance to treatment, some patients had experienced HBV breakthrough without any mutations within the *pol* gene. This observation may be explained by the emergence of mutations outside the *rt* domain or other mechanisms that are still unknown.

A nucleotide analogue, tenofovir (TDF), has been shown to be active against both wild-type and 3TC resistant HBV [285, 418]. When the PHPT treatment cohort was initiated, TDF was available in Thailand at 38 USD per month, price which exceeded that of the current standard first line HAART (zidovudine/stavudine, 3TC and nevirapine), 30 USD per month [419, 420]. Recently, the Thai national [281] and WHO guidelines [421], have recommended to use TDF+3TC or TDF+emtricitabine as the backbone of the HAART combination to treat HIV-HBV co-infected patients. However, this combination may not be provided to all HIV-HBV co-infected patients since less than 50% of Thai HIV-1 infected patients who are on ART had not been assessed for HBV co-infection [422].

Our study shows that a significant number of HIV-HBV co-infected patients on 3TC containing HAART, particularly HBeAg negative patients, can achieve long-term HBV DNA and HIV suppression. This study provides further information which may be helpful in the management of HIV-HBV co-infected patients in resource-limited countries.

5.5. Publications

This study has been submitted to the "Antiviral Therapy" Journal in August, 2011.

- **Khamduang W**, Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Lallemant M, Sirirungsi W, Goudeau A, and the Program for HIV Prevention and Treatment (PHPT) group. *Long-term virological response of Hepatitis B virus to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand.* ***(Submitted)***

This work has been accepted for presentation at;

- **Khamduang W**, Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Hepatitis B virus (HBV) virological response to combination antiretroviral treatment includes lamivudine (3TC) in HIV/HBV co-infected individuals in Thailand. International Meeting; The molecular biology of hepatitis B viruses, 30th August - 2nd September, 2009, Tours, France. ***(P-20, Poster presentation)***
- **Khamduang W**, Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Hepatitis B virus (HBV) virological response to combination antiretroviral treatment includes lamivudine (3TC) in HIV/HBV co-infected individuals in Thailand. 10ème réunion du Réseau National Hépatites de l'ANRS, 21st - 22nd January, 2010, Paris, France. ***(Oral presentation)***
- **Khamduang W**, Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Lallemant M, Sirirungsi W, Goudeau A, and the Program for HIV Prevention and Treatment (PHPT) group. Long-term virological response of

Hepatitis B virus to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand. The 13th Thai national AIDS seminar, 29th – 31st March, 2011, Bangkok, Thailand. ***(AP3, Poster presentation)***

Conclusion

6. Conclusions

Despite the availability of hepatitis B vaccines and antiviral drugs, HBV infection remains the most common infection and a major health problem throughout the world. It is estimated that 350 to 400 million people are chronically infected with HBV [2-7]; of whom three quarters reside in Asia and the western pacific [8, 9]. Perinatal HBV transmission remains a major cause of chronic infection in this region since most HBsAg carriers have been infected at birth or in early childhood [10].

Thailand is considered as a country with high prevalence of chronic HBV infection i.e prevalence of HBsAg >8%. Furthermore, Thailand has been one of the countries the hardest hit by the HIV-1 pandemic; it is estimated that over half a million people are currently living with HIV; of whom 9% are co-infected with HBV. We have addressed in this HIV-HBV co-infected population 3 questions of public health: what is the residual risk of perinatal transmission of HBV among HIV-HBV pregnant women in the context of EPI, what is the prevalence and impact of occult HBV infection among these women and lastly what is the long term efficacy of 3TC-containing HAART on HBV infection. The common point to these 3 questions relates to the possible occurrence of mutations of the *pol* or *S* genes of HBV and their potential negative impact on diagnosis, response to vaccine/immunoglobulins and antiviral therapy.

The most effective means to decrease HBV burden and HBV disease complications is to prevent mother-to-child transmission of HBV. Passive and active immunoprophylaxis have led to 90-95% decrease of perinatal infection indicating that a percentage of children had acquired HBV infection despite adequate vaccine and/or immunoglobulin against HBV were provided. It is thus important to investigate the reasons of vaccine failure. In the first part of our work, we have assessed the prevalence of HBV mother-to-child transmission (MTCT) and characterized virus which had been transmitted to children born to HBV/HIV-1 co-infected women. We have found a residual HBV transmission of 5% for which different mechanisms may

account for. Indeed, we have demonstrated that vaccine/ immunoglobulin failure may result from transmission of either wild-type variants from mothers with high level of HBV DNA, maternal HBV minority variants, or HBV variants present and accounting for 20% or more of maternal viral population. We have also identified several HBsAg mutations, sK122R, sI126IT, sI126M+P127S, and sT131N+M133T+T140I+S204R. The impact of variants identified in our study on the escape to HB vaccine is unknown. A systematic virological evaluation of HBV variants selected in infected infants despite active immunization, and in their mothers, is needed to further clarify the impact of these mutations on perinatal transmission of HBV. Understanding the causes of HB vaccine failures will help to develop new HBV vaccines appropriate for the many countries in HBV endemic area such as Thailand and other South-East Asian countries or develop other interventions to decrease perinatal transmission of HBV and accelerate the eradication of HBV infection.

Another obstacle to HBV eradication is the high number of chronically HBV infected subjects, who are not yet treated because of the limited access to anti-HBV treatment or are not aware of their HBV infection. These chronically HBV infected subjects may thus represent a major source of viral spread. However, not all hepatitis B virus co-infections are symptomatic and even routine serological markers can miss the diagnosis of HBV disease. Thus occult hepatitis B virus (HBV) infections have been recently described and are defined by positive HBV-DNA in the absence of serum HBsAg [381]. Occult HBV infection has been a major concern for blood banks or organ transplantation. One consequence of occult HBV infection in HIV-infected patient, is the possible re-activation of HBV infection, particularly after immune suppression [221]. Occult HBV infection has been frequently found in individuals with isolated anti-HBc serologic profile [314, 352]. In the second part of this study, we have first reported that the prevalence of isolated anti-HBc in HIV pregnant women throughout Thailand was 14% and the prevalence of occult HBV infection among those with isolated anti-HBc was 24%; 94% of women with HBV occult infection had HBV DNA level <100 IU/mL and 6 % had HBV DNA <1000 IU/mL. We have also showed that older age, birth in northern region, low CD4 count and HCV infection were independent factors associated with isolated anti-HBc serologic profile. Further studies are needed to ascertain whether mothers with occult HBV infection transmit HBV to their infant.

In Thailand, the first line recommended antiretroviral regimen for treating HIV-infected patients in Thailand was until recently a fixed dose combination including 3TC. It is usually admitted that treatment of HBV in HIV-HBV co-infected patients leads to the emergence of HBV mutations associated with resistance to 3TC at a frequency of 15-20% per year. As the consequence of overlapping genes of HBV, this resistance may lead to the occurrence of HBsAg mutations. In the last part of our study, we have evaluated the HBV virological response in HIV/HBV co-infected patients receiving 3TC-containing HAART and analyzed in patients with HBV breakthrough the selection of virus with 3TC resistance mutations and their possible consequence on mutation *of S* gene. We have demonstrated that a significant number of HIV-HBV co-infected patients on 3TC containing HAART can achieve long-term HBV DNA suppression, particularly HBeAg negative patients. The cumulative rates of HBV DNA suppression were 91%, 84%, and 68% at 1, 3, and 5 years, respectively. The rate of HBeAg and HBsAg loss at the last visit were 87% and, 19% respectively. Surprisingly the rate of resistance mutations to 3TC was lower than expected from the data published in the literature. Indeed mutations were found in 3 patients: 2 had the rtV173L+L180M+M204I triple mutation pattern and 1 had the rtM204I mutation. None of the 3TC induced-mutations identified resulted in mutation of the virus *S* gene. Our results show that 3TC exert an activity on HBV longer than what was reported in studies conducted in Europe. This study provides further information which may be helpful for the management of HIV-HBV co-infected patients in resource-limited countries.

7. Perspectives

Our study brings additional knowledge on HB vaccine/immunoglublin failure in infants born to HIV-HBV co-infected women in the context of EPI, on occult HBV infection and long term response to 3TC in HIV-HBV co-infected adults on HAART. Furthermore, it also provides information about the HB variants of genotype C and B identified during vaccine or antiviral failure.

However, there are some missing pieces to the puzzle e.g. assessing the impact of variants identified in our study in the escape to HB vaccine. This work as already been initiated in collaboration with Dr. Camille Sureau, Molecular virology laboratory, French institute of blood transfusion, Paris, France. We have produced HBV pseudoviral particles using Huh-7 cell derived HDV-like particles which harbored the mutations of interest. The next step is to analyze the susceptibility of produced HBV pseudoviral particles to neutralization by antibodies from HBV-vaccinated or naturally induced-HBV infected individuals.

Another perspective is to ascertain whether occult HBV mothers had transmitted HBV to their infants. All the knowledge gained may be useful for researchers and public health specialists to develop new strategies or interventions to decrease HBV burden in Thailand but also in other Southeast Asian countries.

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Annexes

Annexe 1: Table of standard amino acid abbreviations and properties

Amino Acid	3-Letter	1-Letter	Side-chain polarity	Side-chain charge (pH 7.4)	Hydropathy index
Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	−4.5
Asparagine	Asn	N	polar	neutral	−3.5
Aspartic acid	Asp	D	polar	negative	−3.5
Cysteine	Cys	C	polar	neutral	2.5
Glutamic acid	Glu	E	polar	negative	−3.5
Glutamine	Gln	Q	polar	neutral	−3.5
Glycine	Gly	G	nonpolar	neutral	−0.4
Histidine	His	H	polar	positive (10%), neutral (90%)	−3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	−3.9
Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	−1.6
Serine	Ser	S	polar	neutral	−0.8
Threonine	Thr	T	polar	neutral	−0.7
Tryptophan	Trp	W	nonpolar	neutral	−0.9
Tyrosine	Tyr	Y	polar	neutral	−1.3
Valine	Val	V	nonpolar	neutral	4.2

[Source: http://en.wikipedia.org/wiki/Amino_acid]

Annexe 2: Abbreviations

%	Percent
α	Alpha
β	Beta
γ	Gamma
μ	Micro
μg	Microgram
μL	Microliter
3TC	2',3'-dideoxy-3'-thiacytidine or Lamivudine
95% CI	95 percent confidence interval
A	Adenine
ACPs	Antigen presenting cells
ADV	Adefovir dipivoxil
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase or anine aminotransferase
Anti-HBc	Antibodies against hepatitis B core antigen
Anti-HBs	Antibodies against hepatitis B surface antigen
AST	Aspartate transaminase or Aspartate aminotransferase
bp	Basepair
C	Cytosine
cccDNA	Covalently closed circular deoxyribonucleic acid
CD	Cluster of differentiation
CD4+ T-cells	Mature T helper cells expressing the surface protein CD4
CD8+ T-cells	Killer T cells expressing the surface protein CD8
CTL	Cytotoxic T cell
dATP	Deoxyriboadenosine triphosphate
DBS	Dried blood spot
DCs	Dendritic cells
dCTP	Deoxyribocytosine triphosphate
dGTP	Deoxyriboguanine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

dsDNA	Double stranded deoxyribonucleotide triphosphate
dTTP	Deoxyribothymine triphosphate
dUTP	Deoxyribouracil triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme liked immunosorbent assay
ETV	Entecavir
FTC	Emtricitabine
G	Guanine
g	Gram
GP	Glycoprotein
HB vaccine	Hepatitis B vaccine
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIg	Hepatitis B Immunoglobulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type-1
HLA	Human leukocyte antigens
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile range
Kbp	Kilobasepair
kDa	Kilodalton
LB medium	Luria-Bertani medium
LdT	Telbivudine
L-HBsAg	Large form of hepatitis B surface antigen
M	Molarity

mg	Miligram
M-HBsAg	Medium form of hepatitis B surface antigen
MHC	Major histocompatibility complex
min	Minute
mL	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural killer cells
NKT	Natural killer T-cells
nm	Nanometer
no.	Number
O.D.	Optical density
^{o}C	Degree Celsius
ORF	Open reading frame
P	Protein
p24	Phosphoprotein 24, typical protein of lentiviruses
PBL	Peripheral blood leukocyte
PCR	Polymerase chain reaction
Peg-IFN	Pegylated interferon
pgRNA	Progenomic ribonucleic acid
pmol	Picomole
rcDNA	Relaxed circular deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Rounds per minute
RT-PCR	Reverse transcription polymerase chain reaction
S-HBsAg	Small form of hepatitis B surface antigen
ssDNA	Single stranded deoxyribonucleic acid
T	Thymine
TDF	Tenofovir Disoproxil Fumarate
TGF-β	Tumors growth factor – beta
TNF	Tumor necrosis factors

TNF-α	Tumors necrosis factor – alpha
UV	Ultraviolet light
vs.	Versus
w/v	Weight by volume
YMDD	tyrosine-methionine-aspartate-aspartate motif of HBV polymerase gene
ZDV	Zidovudine

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- **Khamduang W,** Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Transmission of Hepatitis B virus (HBV) minor variants in children born to HBV/HIV co-infected mothers. 12th National AIDS Conference, 27-29th May, 2009, Bangkok, Thailand. (CP08)
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Analyse des mutants du virus de l'hépatite B (VHB) chez des patients co-infectés par le VIH et le VHB en Thaïlande

RESUME

L'infection par le VHB est endémique en Thaïlande. Malgré l'introduction des programmes de vaccination contre le VHB, la transmission périnatale reste une cause majeure d'infection chronique. Les objectifs de ce travail étaient d'identifier les mutants du VHB pouvant être associés à des échecs de vaccination, de diagnostic et de thérapeutique. Le travail présenté ici est divisé en trois parties.

Dans une première partie, nous avons analysé la prévalence de la transmission périnatale du VHB dans une cohorte issue d'un protocole thérapeutique de prévention de la transmission materno-fœtale du VIH. Nous avons cherché à caractériser les mutants d'échappement à la vaccination contre le VHB. Parmi 3349 femmes enceintes séropositives pour le VIH, l'antigène (Ag) HBs était positif dans 7% des cas. L'Ag HBs était détectable à l'âge de 2 et 18 mois chez 11 enfants nés de mères porteuses chroniques. Les variants du VHB présents au sein de 9 de ces paires mère-enfant ont pu être étudiés après séquençage et clonage. Trois types de transmission du VHB ont pu être décrites ; i) transmission de variants non mutés par les mères présentant une charge virale VHB élevée ii) transmission d'un virus mutant minoritaire isolé chez la mère, et iii) transmission de mutants déjà présents à plus de 20% chez la mère. La capacité *in vitro* de ces mutants à échapper à la réponse neutralisante anti-HBs sera étudiée en utilisant un modèle de pseudo-particules portant les mutations identifiées.

Dans une seconde partie, nous avons sélectionné une cohorte de femmes enceintes séropositives pour le VIH pour lesquelles la présence isolée d'Ac anti-HBc a été détectée. Dans cette population, la présence de marqueurs d'infection occulte par le VHB a été recherchée. Parmi 1682 femmes AgHBs négatif, 229 (14%) avaient des Ac anti-HBc isolés et l'ADN du VHB était détectable par PCR chez 50 d'entre-elles. L'analyse multivariée a montré que l'âge (>35 ans), le lieu de naissance (région Nord de la Thaïlande), le nombre de CD4 >350 cellules/mL et plus significativement le nombre de CD4 >200 cellules/mL et la trace d'un contact avec le VHC étaient indépendamment associés à la présence isolée d'Ac anti-HBc.

Dans la dernière partie, nous avons évalué l'efficacité à un an et à long terme de la lamivudine (3TC) sur la réplication du VHB chez 30 patients co-infectés par le VIH et le VHB, recevant une thérapie antirétrovirale hautement active. La quantification de l'ADN du VHB a été réalisée à l'introduction du traitement, à 3 mois, à 12 mois et à l'issu du suivi à long terme. La virémie VHB médiane était de 7.35 log10 IU/mL. Après 3 et 12 mois, la virémie avait diminué de 3.86 et de 4.40 log10 IU/mL. Elle était négative chez tous les patients présentant initialement un AgHBe négatif. A l'issu du suivi à long terme, la virémie était négative chez 17 des 19 patients. Le taux cumulé d'obtention d'une virémie négative après 1, 2, 3, 5 et 7 ans était respectivement de 95%, 91%, 84%, 84% et 64%. Sept patients étaient rechuteurs. Deux patients étaient infectés par des variants avec une triple mutation et un patient par une seule mutation de résistance à la lamivudine. Nous avons donc montré que l'administration de multithérapie antirétrovirale contenant de la lamivudine induisait la suppression à long terme de la réplication du VHB. Chez les patients co-infectés par le VIH et le VHB, cette stratégie thérapeutique apporte donc un bénéfice dans les pays en voie de développement.

Mots-clés : **Hepatite B, co-infection par le VIH, échec de vaccination, résistance à la lamivudine, infection occulte par le VHB**

SUMMARY

Thailand is an endemic area for chronic HBV infection. Despite implementation of HBV vaccination, perinatal HBV transmission remains a major cause of chronic infection. This study aimed at identifying HBV mutants that may be associated with vaccine failure, misdiagnosis of chronic HBV infection and antiviral treatment failure. The dissertation is divided in three parts.

In the first part, we analyzed the prevalence of perinatal HBV transmission in a large HIV prevention cohort in Thailand and characterized the HBV vaccine escape mutants. Among 3,349 HIV-infected pregnant women, 7% were found HBsAg positive. Eleven children born to HBsAg-positive mother were found HBsAg-positive at 2–18 months of age. Complete series of samples were available for 9 mother-child pairs. Based on direct sequencing and cloning analysis, 3 patterns of transmission were observed : *i*) transmission of wild-type variants from mothers with high HBV DNA level, *ii*) transmission of maternal minor variant and *iii*) transmission of variants already present in maternal blood samples. The capacity of HBV variants to escape from anti-HBs neutralization *in vitro* will be further studied using HBV-pseudoviral particles harboring the characterized mutations.

In the second part, we selected a cohort of HIV-infected pregnant women who had isolated anti-HBc antibody and screened them for markers of occult HBV infection. Of 1,682 women who were AgHBs negative, 229 (14%) had isolated anti-HBc and 50 of them had detectable HBV DNA (>15 IU/ml) when tested by PCR. The multivariate analysis showed that age over 35 years old, birth in the northern region, CD4 count below 350 cells/µL and, more significantly, CD4 count below 200 cells/µL as well as past or present HCV infection, were independently associated with the presence of isolated anti-HBc antibody.

In the last part, we evaluated the 1-year and long-term effect of lamivudine (3TC) on HBV replication in 30 HIV/HBV co-infected patients receiving 3TC-based highly active antiretroviral therapy. HBV DNA, was measured at baseline, 3 and 12 months and at long-term visits. The median baseline HBV DNA level was 7.35 log10 IU/mL. At 3 and 12 months, HBV DNA had decreased to 3.86 and 4.40 log10 IU/mL, respectively with 53% and 67% of patient becoming HBV DNA negative. HBV DNA suppression was observed in all HBeAg-negative patients. 17 of 19 patients with a long-term follow-up remained HBV DNA negative. The estimated cumulative rate of sustained HBV DNA suppression at 1, 2, 3, 5, and 7 years were 95%, 91%, 84%, 84%, and 64%. 7 patients experienced a HBV breakthrough. Two were infected by variants with a triple mutation and one by viruses with a single mutation. Our results suggested that long-term suppression of HBV replication is an additional benefit provided by 3TC-containing HAART for a significant number of HIV/HBV co-infected patients in resource-limited countries.

Key words : **Hepatitis B, HIV co-infection, vaccine failure, lamivudine resistance, occult HBV infection**