CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

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 HBV infected adults have spontaneously resolved acute infection. However, 5% of infected adults and most of children acquired the virus before 1 year of life 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 to 80% of chronically HBV-infected patients reside in Asia and the western pacific (8, 9). 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) (10, 11). HBV is the tenth leading cause of death worldwide (12) with an estimated 600,000 deaths per year attributed to the acute or consequences of chronic hepatitis B (1, 6, 12). 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 (13). Thus, preventing perinatal HBV transmission is thus the most effective strategy to reduce the global morbidity and

mortality due to hepatitis B infection.

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 2010, 93% (179 of 193) of countries worldwide have reported the implementation of newborn hepatitis B (HB) vaccination (14). With the effective HB active and/or passive immunoprophylaxis, eradication of HBV infection seems possible in the far future but there are still some obstacles. Indeed, some children have been infected with HBV despite adequate HB vaccine and/or immunoglobulin against HBV (HBIg) was provided. These transmissions may be due to 1) ineffective vaccine; HB vaccines are heat sensitive and require a cold chain for transportation and storage, 2) omission of HB vaccine birth dose, 3) the exposition of children to high maternal HBV viral load, or 4) the emergence of HBV mutants or "escape mutants" which can escape the activity of HB vaccine and/or HBIg. 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 an enormous reservoir or a major source of viral spread.

Moreover, co-infection with HBV is a growing problem in human immunodeficiency virus (HIV) infected patient. This accelerates the progression to liver disease which is a major cause of morbidity and mortality in HIV infected patients (15). In Thailand, there are approximately 550,000 individuals living with HIV or acquired immunodeficiency syndrome (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: firstly, the residual risk of perinatal transmission of HBV among HIV-HBV co-infected pregnant women since HIV-related immunosuppression increases the viral replication and viral load of HBV (16) so co-infection with HIV and HBV could theoretically increase perinatal transmission, secondly, the reality and impact of occult HBV infection or undiagnosed HBV infections among HIV-HBV co-infected pregnant women and lastly the long-term efficacy of lamivudine (3TC) -containing highly active antiretroviral treatment (HAART) on HBV virological response in HIV-HBV co-infected patients.

The Thai Ministry of Public Health (MoPH) has integrated HB 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%, irrespective of maternal HBsAg (17). However, despite an adequate immunization, there is a residual risk of perinatal HBV transmission which can be addressed through systematical analysis of the causes of the vaccination failure (18), particularly in infants born to carrier mothers with a high HBV replication (5-10%) (19). Several type of variants can limit action of anti-HBs neutralizing antibodies: vaccine/immunoglobulins escape mutants (20, 21), naturally occurring variants (22) or even polymerase mutants selected during antiviral therapy which can lead to a changes of amino acid residue(s) in HBsAg due to the overlapping reading frames of surface (*S*) and polymerase (*Pol*) genes (23). 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 HBsAg detection assay (24). Individuals infecting with this mutant virus can be further a reservoir of viral transmission whether horizontally or vertically (25).

In term of treatment, lamivudine (3TC), a cytidine analogue, inhibits the reverse transcriptase enzyme of both HIV and HBV. This dual activity of 3TC is potentially beneficial to individuals who are co-infected with HIV and HBV (26). However, the efficacy of 3TC is limited by the emergence of 3TC resistance mutations with an estimated rate of 20% per year (27). 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 that includes 3TC i.e. 3TC and nevirapine plus (stavudine or zidovudine). 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 (28). 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 the three questions for public health concern: what is the residual risk of perinatal transmission of HBV among HIV-HBV infected 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 and its consequence on *S* gene

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 HB vaccine/immunoglobulins and antiviral therapy. Schematic summary of this study is shown in Figure 1.1. We focus 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.

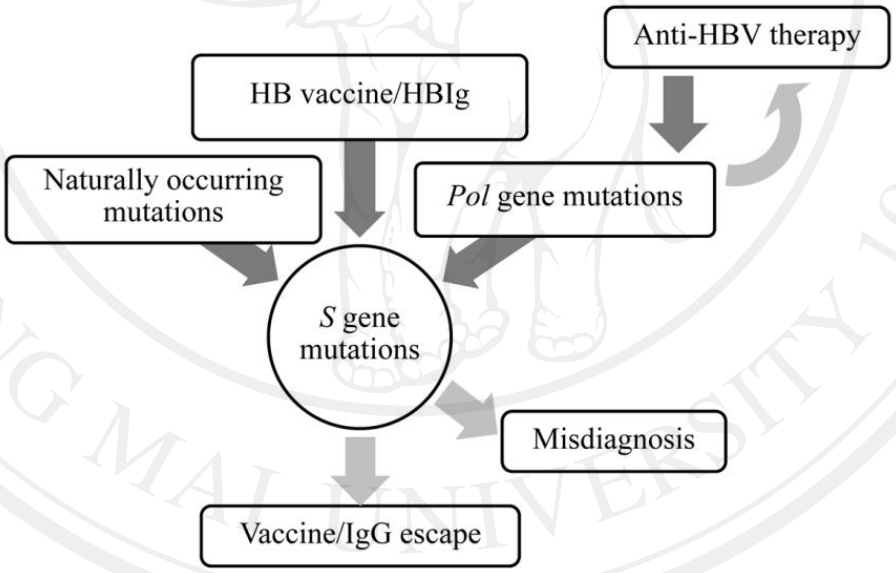


Figure 1.1 Schematic overview of the study. The emergence of hepatitis B surface gene mutations may occur naturally, or be induced by the use of HB vaccination/ immunoglobulins, or anti-HBV drug therapy, reflecting either vaccine escape, misdiagnosis or anti-HBV drug resistance.

1.2 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

1.3 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/immunoprophylaxis 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. The results of this study contribute to a better knowledge of the prevalence and diversity of HBsAg variants. Finally, this study also provides further insights into the emergence of HBV drug resistance mutants and its consequence on *S* gene in HIV-HBV co-infected patients receiving 3TC-containing antiretroviral therapy in Thailand.

This important information gained from the study will help in the decision making, design and plan for public health policy towards HBV prevention and

treatment in Thailand and in other countries with a high HBV endemicity. Therefore, eradication of HBV infection may be possible in the next far future.

1.4 Literature review

1.4.1 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 (29).

The hepatitis B surface protein was discovered accidentally in 1965 by a medical anthropologist, Baruch S. Blumberg, M.D., Ph.D., (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 (30) 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 (31) identified by electronic microscopy some large double-shelled virus-like particles of 42 nm diameter, called thereafter Dane particles (Figure 1.2) 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 was called 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 (10).

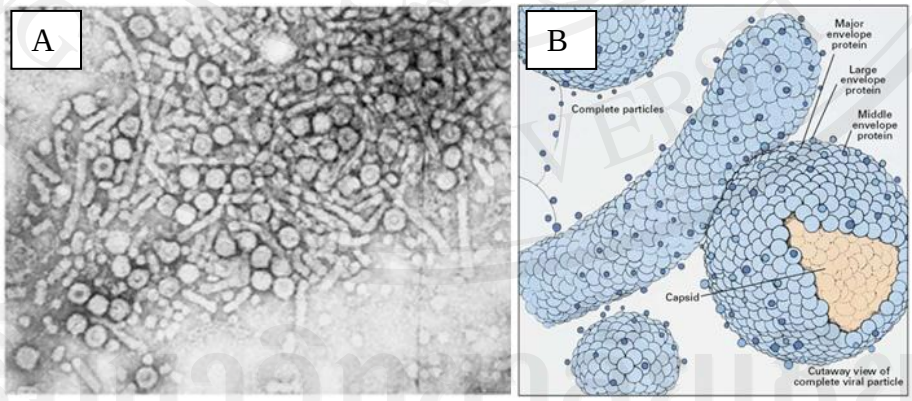


Figure 1.2 The three forms of HBV particles. (A) The electron micrograph shows whole virions (Dane particles)**,** subviral sphere and filamentous forms. (B) The cartoon illustration shows the same features with more details of the surface antigen proteins. [source: modified from *Lee WM, New Eng J Med, 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

1.4.2 Biology of Hepatitis B Virus

1.4.2.1 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 1.3). 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, thus 6 different proteins can be distinguished i.e. 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 1.3). These subviral particles are non-infectious particles because they contain only envelope glycoproteins and host-derived lipid but not viral genome. Their amount usually outnumbers that of virions by 1,000:1 to 10,000:1 (10). 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. (29)

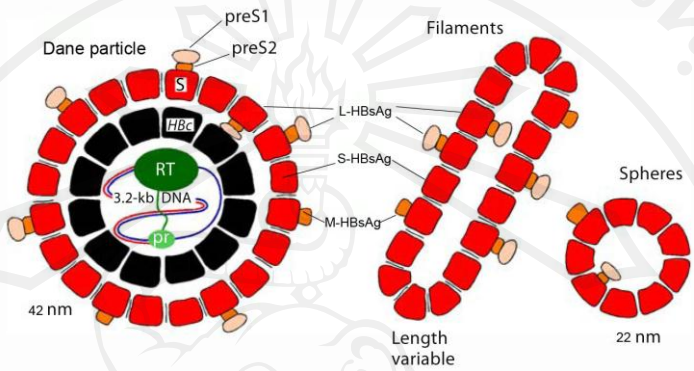


Figure 1.3 Schematic diagrams of the components of 42 nm-Dane particle, 22 nm-sphere and filamentous forms of HBV [source: modified from *Gerlich WH et al., Dig Dis, 2010* (32)]

1.4.2.2 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 (33). The *Hepadnaviruses* are subdivided into two genera according to their host ranges (Figure 1.4), 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). (34)

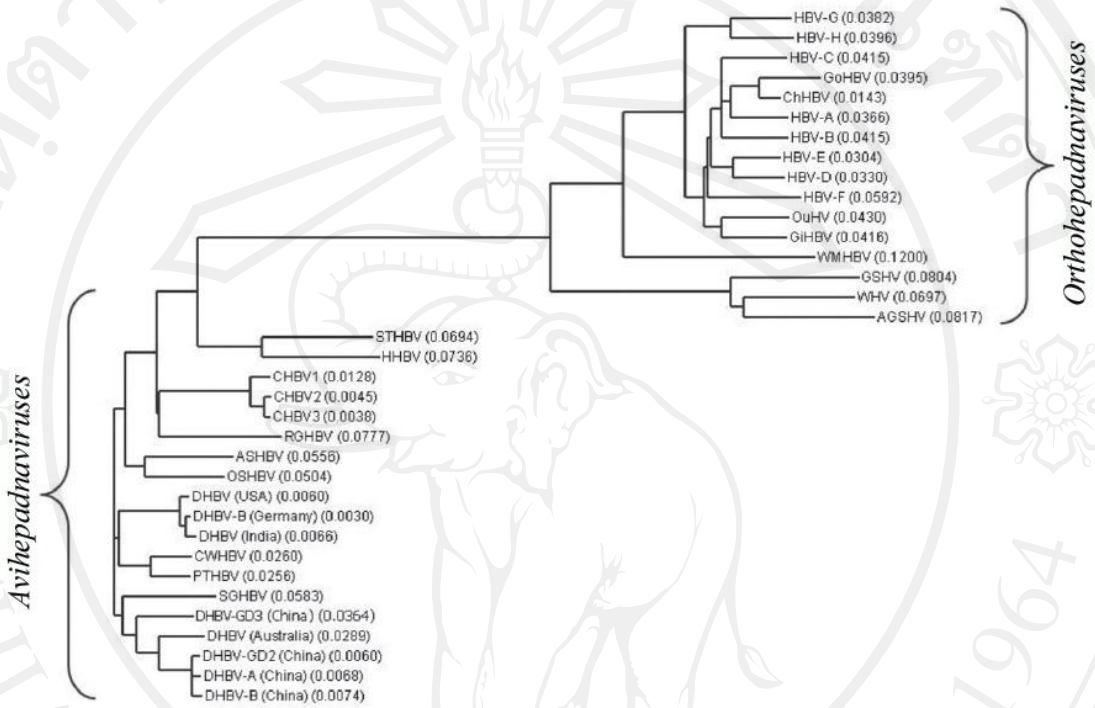


Figure 1.4 The phylogenetic tree of reference strains of *Orthohepadnaviruses* and *Avihepadnaviruses* [source: *Lüsebrink J et al., Hepatology a clinical textbook, 2010* (34)] **Abbreviations:** AGSHV, arctic ground squirrel hepatitis virus; ASHBV, ashy headed sheldgoose HBV: CHBV, crane HBV; ChHBV, 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.

1.4.2.3 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 1.5). Its structure is unusual, the two DNA strands are not perfectly symmetric. The full-length negative strand (blue solid line in Figure 1.5) 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 1.5) whereas the positive strand is shorter and bears a capped oligoribonucleotide at it 5' end (red zigzag in Figure 1.5). 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 (35).

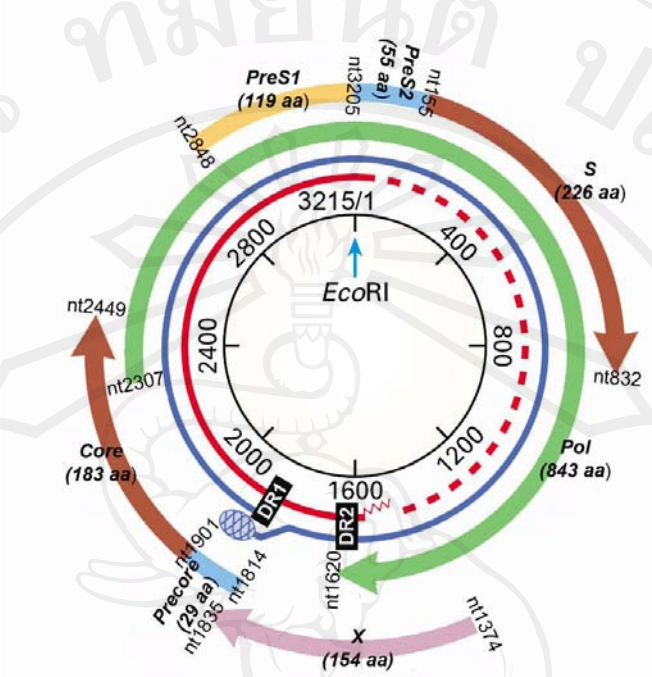


Figure 1.5 Genome organization of HBV genotype B or C, the 2 genotypes predominant in Thailand. [source: modified from *Lee WM, New Eng J Med, 1997* (2)]

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, 36).

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 1.6). 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.

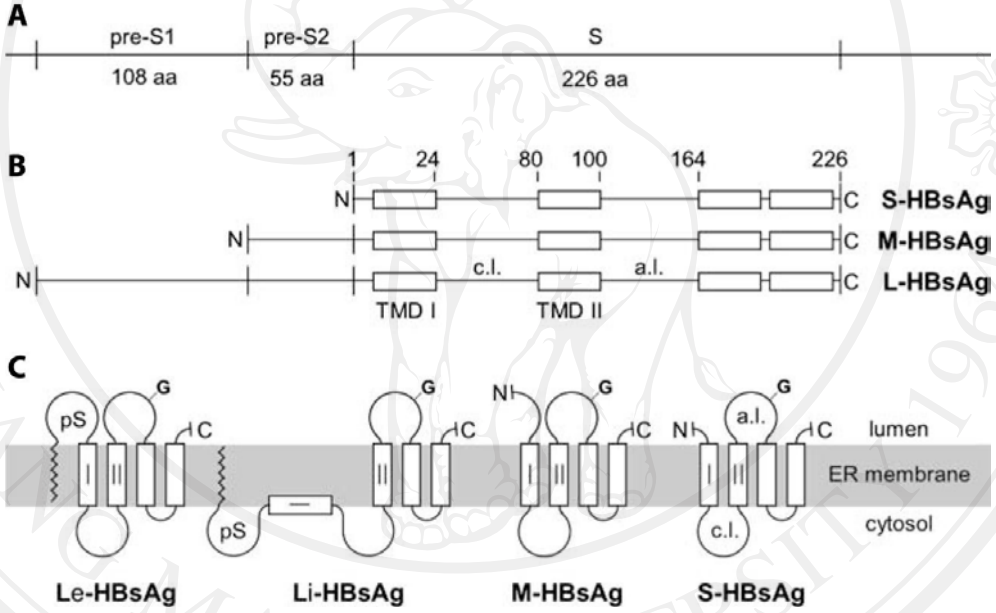


Figure 1.6 (A) Domains of the HBsAg open reading frame. (B) The L-, M-and S-HBsAg are translated from three in-frame initiation sites but sharing common C-terminal S domain. (C) Topology of the L-, M-and S-HBsAg at the endoplasmic reticulum (ER) membrane. The two forms of L-HBsAg are represented: the 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). [*source: modified from Sureau C, Curr Top Microbiol Immunol, 2006* (37)] **Abbreviations**: G denotes Glycosylation site; a.l. denotes antigenic loop; c.l. denotes cytosolic loop; pS denotes pre-S domain

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 (10). The X protein may play a role in the development of hepatocellular carcinoma (HCC) (38).

1.4.2.4 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 (10). 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 1.7) 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 (39). 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 (40). 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 (10, 41, 42).

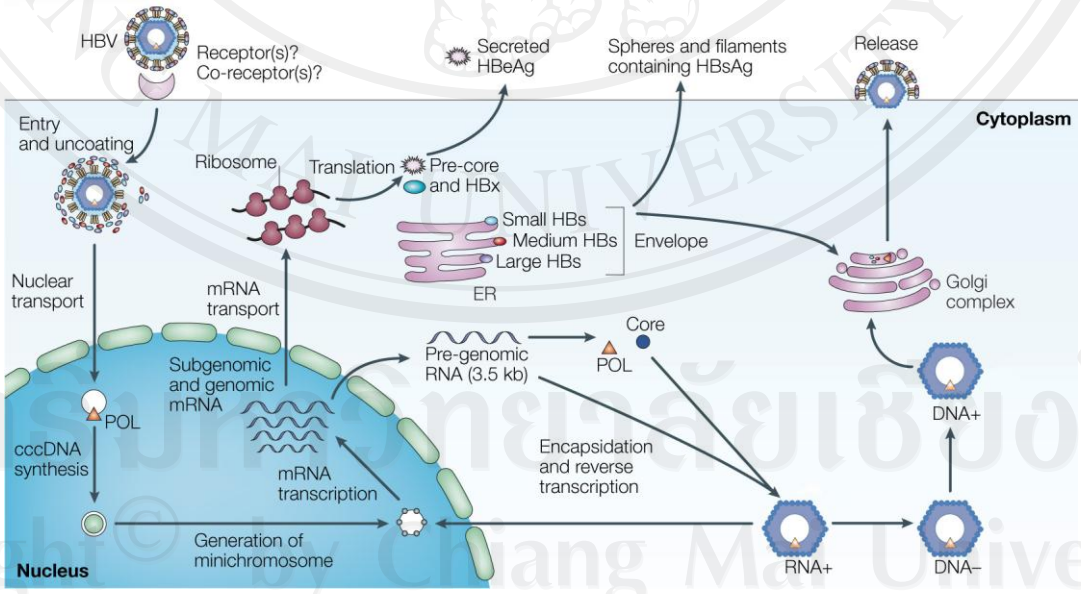


Figure 1.7 Life cycle of HBV [source: modified from *Rehermann B & Nascimbeni M, Nat Rev Immunol, 2005* (43)]

1.4.3 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 (44). The high rate of mutations result in the coexistence of variant viruses genetically linked called "quasispecies (24, 45). According to Carman's proposal, two types of viral diversity can be identified;

1) "Variants" (46, 47) or **"Genotypic diversity"** (48, 49) 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" (46, 47) or **"Phenotypic diversity"** (48, 49) 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 (45, 50). 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**.**

1.4.3.1 Hepatitis B virus variants

1.4.3.1.1 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 (51) with I and J, recently, in Laotian, Vietnamese, and Japanese patients (52-54). Each genotype has different length of viral genome and viral proteins as described in Table 1.1. 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. The word "Clade" is used for divisions within subgenotypes showing less than 4% nucleotide difference (51). 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 (55), and genotype G and C recombinants in Thailand (56).

Table 1.1 Comparison of the length of viral genome and viral proteins between each HBV genotype (48)

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 (57) (Figure 1.8 and Table 1.2). 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 (58). Genotype G has been detected infrequently, and limited to HBV carriers in Europe and USA (59-62). Genotype H is confined to the Amerindian populations of Central America and Mexico (58, 63, 64).

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%. (20, 65-68)

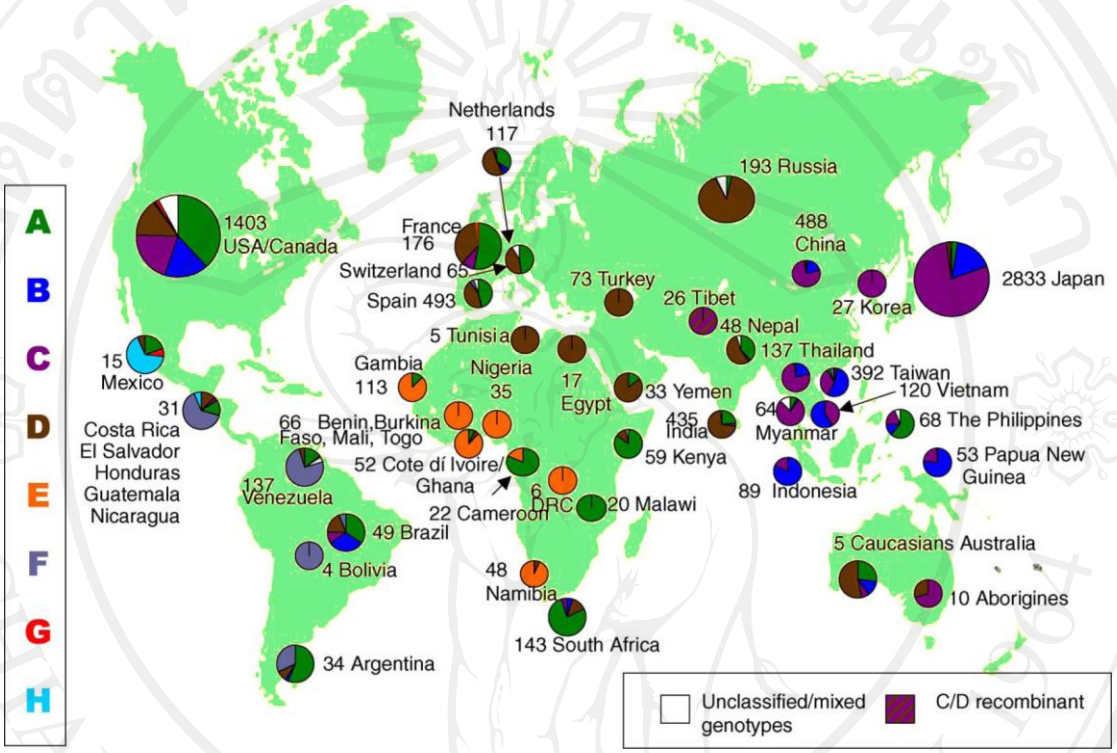


Figure 1.8 Global distribution of 8 genotypes of hepatitis B virus. The numbers next to the pie chart are the number of isolated genotypes [source: *Kramvis et al., 2005*(51)]

Table 1.2 Relationship between genotypes and serotypes, geographical distribution (48, 51)

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 (69). 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 (70). 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 (71). Livingston *et al.* confirmed that genotype F was associated with the occurrence of HCC, compared to A, B, C, or D (72).

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 (68, 73, 74). In Taiwan, Kao *et al.* also reported that genotype C (mostly serotype *adr*) was associated with the development of cirrhosis and HCC (75, 76). However, another study in Thailand found no differences in the risk of developing HCC between patients infected with either of these 2 genotypes (68).

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 (77). 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 (78).

HBV genotype and anti-viral therapy response

Genotypes may also influence the outcome of treatment. In a multicenter trial of pegylated interferon (79), 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 (76) but not in a Japanese study where, genotype B and C carriers responded well to interferon treatment, while genotype A carriers responded poorly (80). 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 (81).

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 (82). Studies in Taiwan (83) and Japan (84) showed that patients with HBV genotype B have a better virological response to lamivudine as compared to genotype C. In contrary, Yeun *et al.* (85) 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 (86-89). Genotypes B and C have a similar risk in developing lamivudine resistance (83-85). In addition, some studies demonstrated

that subgenotype Ba had higher risk to develop lamivudine resistance than subgenotype Bj (90). 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.

1.4.3.1.2 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" (51).

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 (48). 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 (91). 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 1.3).

Table 1.3 Amino acid residues specifying HBV serotypes (92)

Amino acid position on HBsAg protein							Predicted HBsAg subtype
122	**127**	**134**	**159**	**160**	**177**	**178**	
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*

Note: amino acid abbreviations see appendix

1.4.3.2 Hepatitis B virus mutants

1.4.3.2.1 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 (93). These double mutations are often present in patients with advance liver diseases (94-96). Some studies revealed that an association between BCP mutation and HCC, though for HBV genotypes A, C, D but not for genotype F (72). Other less frequent BCP mutants associated with fulminant hepatitis and HCC have been described, such as a mutation at nt1653 (97, 98) and at nt1753-1757 (99, 100). A number of deletions of the BCP have also been reported (45, 101-103).

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 (45). 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 (44, 104, 105).

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 (106). 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 (107). 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 (108).

1.4.3.2.2 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 (109). 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 (110). 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 (44). 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 (111).

1.4.3.2.3 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 (45). 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) (112) and a 20 nucleotide deletion at nt1752–1772

(113), 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 (112), suggesting that these mutations may play a certain role in the pathogenesis of HBV infection.

1.4.3.2.4 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 (45). Some mutations in preS regions appear to be associated with the development of HCC, for example, the preS2 mutation (F141L) of HBV genotype C (114). 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 (115), but its significance need to be confirmed.

1.4.3.2.5 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 (46, 116) (Figure 1.9). 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 (45, 47, 48, 117, 118).

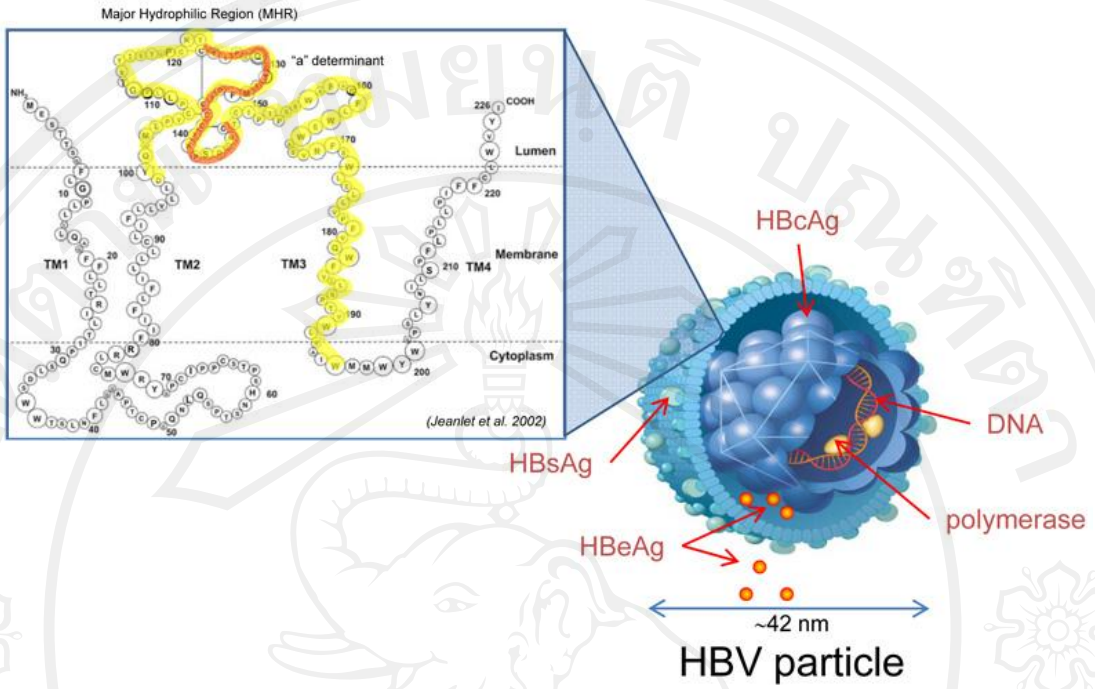


Figure 1.9 Schematic diagram of the secondary structure with amino acids components, location of major hydrophilic region and "a" determinant region of surface antigen of hepatitis B virus genotype C, accession number AF068756.1.

Point substitutions in the S gene are very important because they affect the antigenicity of HBsAg, especially the "a" determinant (117, 118). 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 (119-121) but also in natural isolates. Other mutations at aa116, 120, 123, 124, 126, 129, 130, 133, 141, 142, 143 and 144 occurring alone or in combination have also been reported (47, 122-125). Changes of amino acids in

surface proteins and their impacts that have been previously reported are summarized in Table 1.4.

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 (44). 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 1.4 Changes of amino acids in S proteins and their impacts

HBsAg region	Amino acid position number	Genotype	Wild type residues	Amino acid changes found in association with		
				Vaccine escape (reference)	Escape to HBIg (reference)	Misdiagnosis (reference)
HBs1	105		**A**		**P** (46)	
	114	A, F B, C, D, E	**T** **S**		**R** (46)	
	115		**T**			**S** (46) **A** (126)
	116		**T**	**N** (127)		**S** (46)
	118		**T**		**A** (46) **R** (128)	**S** (46)
HBs2	120		**P**	**E** (129) **S** (130)	**E** (131) **T** (128) **N** (46)	**T** (46, 132) **S** (133, 134) **Q** (133)
	121				**S** (128)	**S** (132)
	122	D, E A, B, C, F	**R** **K**			**N** (126)
	123		**T**		**N** (46)	**A** (126) **N** (132, 134)
HBs3	124		**C**		**R** (46) **Y** (46)	**F** (132) **Y** (132) **I** (132)
	125		**T**	**M** (135) **A** (136)		
	126	A, B, D, E, F C	**T** **I**	**A** (46, 136) **N** (137) **S** (137) **T** (135, 138)	**A** (139) **N** (131)	**I** (46) **N** (126, 132) **S** (140)
	127		**P**	**T** (135, 136)		
	129		**Q**	**H** (46, 136) **R** (21, 141)	**H** (139)	**N** (126)
	130		**G**		**R** (46)	**R** (126)
	131	B, C, D, E, F A	**T** **N**	**I** (136)	**S** (46)	**I** (46, 126, 142)
	133	 D	**M** 	**L** (141)	**I** (46) **L** (139)	**I** (46) **T** (126) **L** (132)
	134	A, B, C, E, F D	**F** **Y**		**Y** (131)	**L** (126) **N** (46)
	135		**P**			**S** (46, 142)
	137		**C**		**Y** (143) **R** (128)	**W** (132)
	138		**C**			**Y** (126)
HBs4	139		**C**		**S** (128)	**S** (132)
	140	E, F A, B, C, D	**S** **T**		**T** (46)	
	141		**K**	**E** (46)	**I** (46)	**E** (142)
	142		**P**	**S** (144)	**S** (142)	**S** (126, 140) **L** (132, 140, 142)
	143	A B, C, D, E, F	**T** **S**	 **W** (136)		**L** (133, 134)

	144	**D**	**A** (123)	**A** (139, 145) **G** (143, 146)	**E** (133, 147) **A** (132, 140, 142)
	145	**G**	**R** (46, 136, 144) **A** (21, 144)	**R** (127, 139, 146)	**R** (134, 140, 142) **K** (126, 142) **A** (132)
	146	**N**	**S** (136)	**S** (46)	**S** (126)
	147	**C**		**S** (46)	**F** (126) **R** (126) **Y** (132)
HBs5	148	**T**	**I** (136)		**H** (142)
	154	**S**			**T** (46) **W** (142)
	155	**S**			**Y** (46) **P** (46)
	156	**W**	**L** (136)		
	157	**A**			**R** (46)
Others	216	**L**			**Stop** (148)
	164+195	**E, I**			**D, M** (148)

1.4.3.2.6 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 1.10). 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 in bind 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 (149).

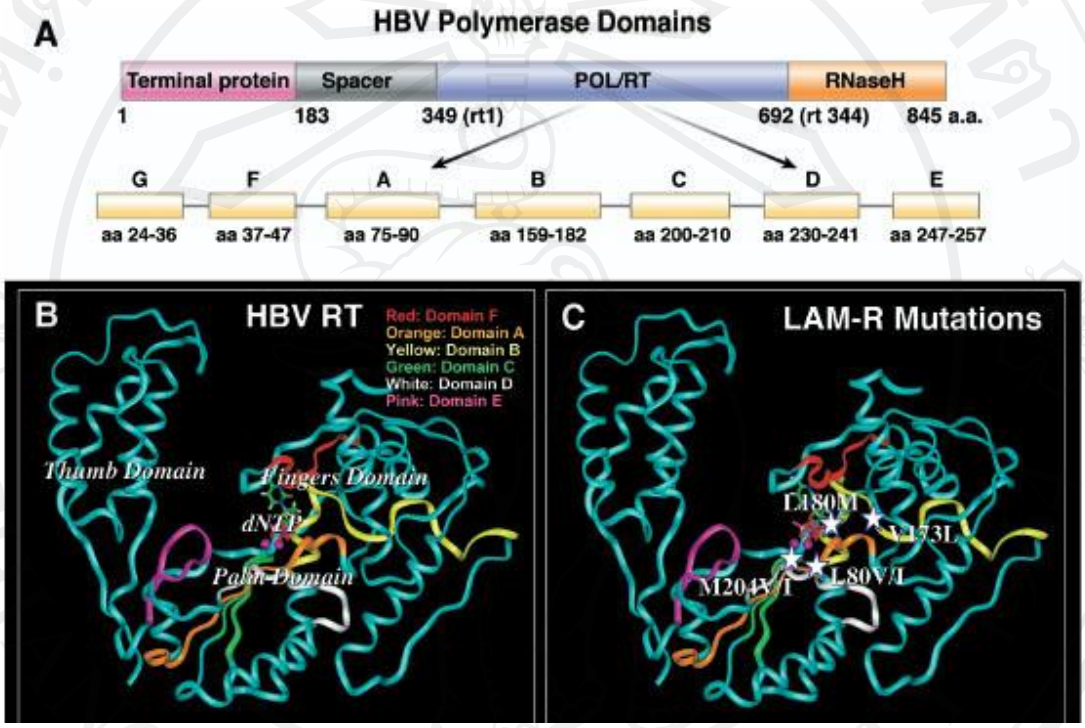


Figure 1.10 (A) Illustration of the HBV polymerase open reading frame with the 4 functional domains and the 7 catalytic subdomains A–G. (B) Proposed structure of the HBV polymerase based on the model of HIV-1 reverse transcriptase. (C) Location of the major lamivudine mutations relative to the conserved domains [source: modified from *Ghany et al. Gastroenterology, 2007* (149)].

The recent development of safe and efficacious antiviral nucleos(t)ide analogues has changed the therapy for chronically HBV infected patients (see more information on anti-HBV drugs in the topic of treatment of HBV infection).

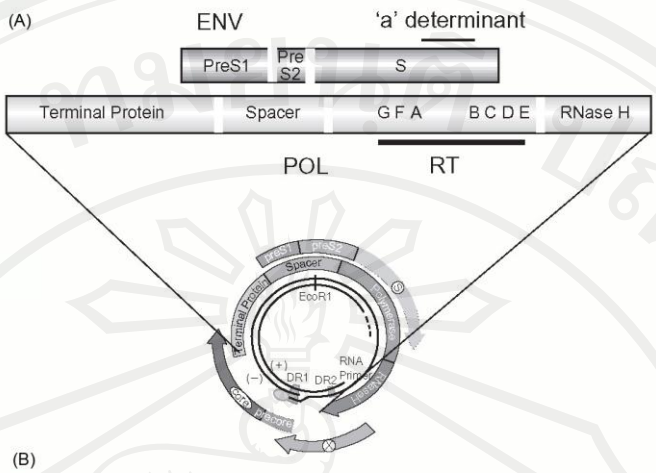
Mutations of the polymerase gene are associated with resistance to nucleos(t)ide analogues and viral persistence. Lamivudine (LAM) is the first licensed anti-HBV drug and most commonly used to treat HBV. LAM is a potent inhibitor of RNA-dependent DNA polymerase of HBV, irreversibly blocking reverse transcription 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 (150) 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 (151). 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 (149). For example, adefovir may select polymerase resistant mutation in the B domain (rtA181T/V) and the D domain (rtN236T) (152).

1.4.3.2.7 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 1.11A). 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 (153). 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 1.11B)(154).



(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 1.11 The overlapping of surface and polymerase genes and its consequence to antigen-antibody binding [source: modified from *Cooley et al., 2003* (155)]

1.4.4 Epidemiology of hepatitis B infection

1.4.4.1 Prevalence of HBV infection

1.4.4.1.1 In general population

As mention earlier, approximately 2 billion people or one-third of world's population have been exposed to the HBV. Although most of HBV-exposed adults 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 (13). 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: it was the fourth most commonly diagnosed cancer (156), 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, 157). 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 (158), although a study in transgenic mice showed that the placenta is an efficient barrier for HBeAg transfer (159).

The prevalence of chronic HBV infection varies worldwide, with the ranging 0.1-20% in different parts of the world (160). 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 1.12)(160). 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% (13)

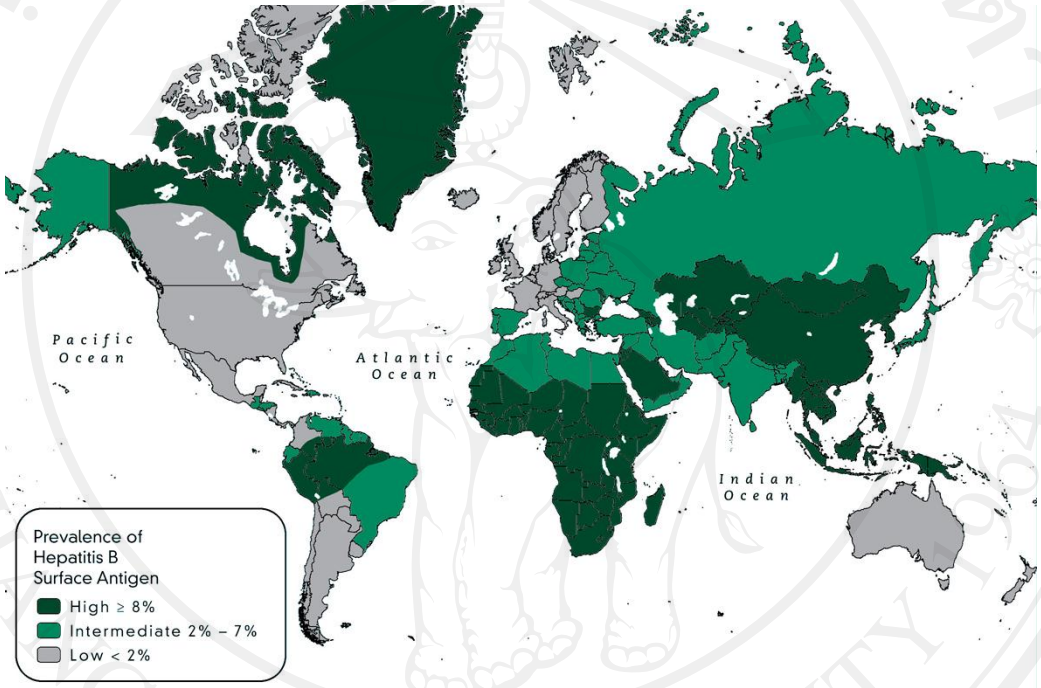


Figure 1.12 Global distribution of chronic hepatitis B infection in 2006 [source: Travelers' health, Centers for Disease Control and Prevention, available at http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-b.htm, accessed July 21, 2011]

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 (161).

In Chiang Mai, the northern city of Thailand, 4.5% of school children (mean age of 12.8 years) were found HBsAg positive during 1998-2000 (18). 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%) (162, 163) or in migrant workers from Laos, Myanmar and Cambodia (7-11%) (164). Prevalence of HBsAg positivity in pregnant women in Thailand was 8-10% (160). This prevalence was lower in the south (3.4%) (165).

The predominant strain is genotype C, in particular subgenotype C1 (164). Prevalence of HBV genotype C and B are 91% and 7%, respectively, while the rest (2%) are recombinant between genotypes B and C (18).

1.4.4.1.2 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 (166, 167). 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 (168). 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)(169). In Asia and Asia-Pacific

regions, the prevalence of HBsAg carriage in HIV-infected patients ranges between 6 to 20%, (Table 1.5).

Table 1.5 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	(170)
Thailand	No	692	8.7	(171)
Thailand	No	529	8.7	(172)
China	No	1,110	6.3	(173)
Japan	No	471	8.9	(174)
India	No	1,178	9.9	(175)
India	No	500	9	(176)
India	No	204	15.2	(177)
Iran	No	391	15	(178)
Australia	No	1,719	4.9	(155)
Taiwan	No (35% of IDUs)	3,164	19.8	(179)
Thailand	Pregnant women	1,437	7.4	(180)
Singapore	Men	47	8.5	(181)
Taiwan	Drug substance users	52	11.5	(182)
India	Co-infected with tuberculosis	951	6.4	(183)

*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. HBV infection is more frequent and more severe in the HIV-infected (15, 184). The presence of HIV prior to HBV infection increases the risk of developing chronic HBV and prolonged ALT elevation (185). HBV-HIV co-infection reduces the rate of spontaneous HBeAg and HBsAg seroconversion, leading

to a higher prevalence of HBeAg-positive patients (168, 186). Also, immunosuppression can cause re-activation of latent HBV infection in individuals who have developed anti-HBs which is frequently found in HIV-infected individuals (60–70%) (187, 188), especially in patients with severe immunodeficiency (189). There is as association between HIV and elevated HBV DNA level, although serum ALT elevation is milder compared to HBV mono-infected patients. Liver damage progresses more rapidly and more severe in patients with co-infection (168, 184, 190). Patients with HIV/HBV co-infection have an increased risk of cirrhosis, hepatocellular carcinoma (HCC), a more rapid progression to end-stage liver disease and death as compared to those not HIV-infected (190). A large multicenter cohort study showed 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 mono-infection and 1.7 per 1000 person-years for HIV mono-infection alone (184). Patients coinfected with HIV-1 and HBV, especially those with low CD4+ nadir counts, are at increased risk for liver-related mortality.

Impact of HBV infection on HIV disease progression

The effect of HBV infection on natural history of HIV disease remains uncertain. The majority of clinical studies that have examined the influence of HBV on HIV disease progression and consider HBsAg a marker of chronic HBV infection have not been able to prove that HBV has any role in HIV disease progression (15, 171, 191). There is, however, an increased risk for liver disease-related morbidity and mortality in HBV/HIV-coinfected patients, as well as more hepatotoxicity under antiretroviral treatment regimens or when active treatment from both HIV and HBV is

interrupted. Meta-analysis performed on data from 12,382 patients enrolled in 11 studies revealed that co-infection with HBV effect on overall mortality of HIV infected patients; pooled effect estimate was 1.36 (95%CI, 1.12-1.64) (191). In addition, 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-infected patients has been described but these is little evidences to supported this (192).

1.4.4.2 Transmission of hepatitis B virus

In infected persons, HBV is found in highest concentrations in blood, saliva, semen, vaginal secretions, and to a lesser extent, perspiration, breast milk, tears, and urine. 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 (193, 194). No infection was demonstrated in persons orally exposed to HBsAg-positive saliva (5), although transmission was demonstrated to animals by subcutaneous inoculation of saliva (195) or tears (196). This infrequent transmission might be due to low concentrations of infectious virus and/or a partial reduction of viral virulence by the innate immune response. Mode of HBV transmission is mainly divided into 2 routes, as follow;

1.4.4.2.1 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 (197) and organ transplantation (198, 199). However, recently, transfusion related hepatitis B is rare because of the establishment of routine screening for hepatitis B virus with highly sensitive methods in most transfusion centers for the last two decade in a lot of worldwide countries. HBV transmission via tattooing and acupuncture has also been reported (200, 201). Unsafe injection drug use remains an important mode of HBV transmission (202).

- ***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 (29).

- ***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 equipments used for therapeutic injections and other health-care related procedures (203-206). 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 (207).

However, the risk of chronicity is low for transmission through sexual contact, intravenous drug use, acupuncture, and transfusion because most of the cases acquired the virus during their adulthood.

1.4.4.2.2 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 (208). The risk of perinatal HBV transmission among infants born to HBV-infected mothers ranges from 10-40% in HBeAg-negative mothers to 70-90% in HBeAg-positive mothers (209, 210). Thus, 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 high viral replication and greater infectivity (211).

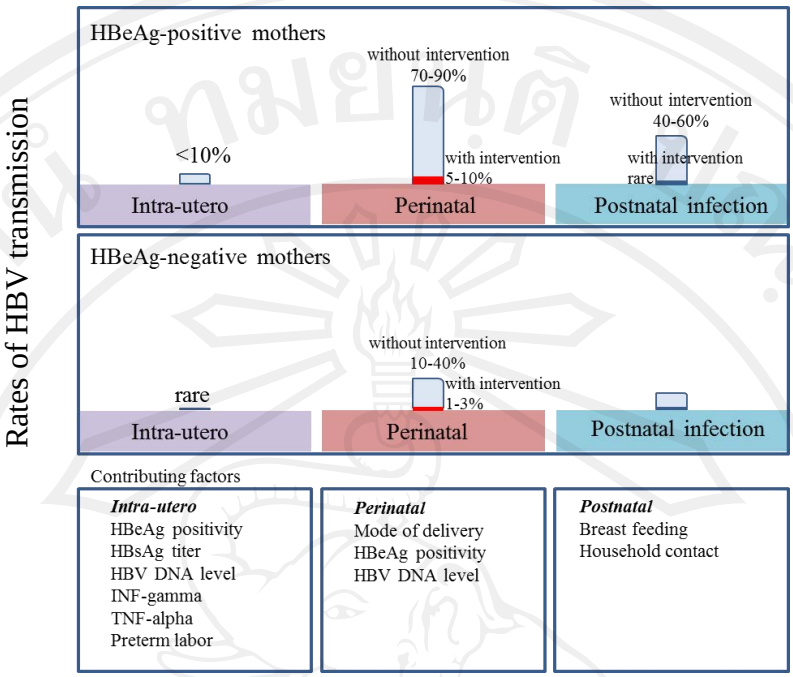


Figure 1.13 Estimated rates of HBV mother-to-child transmission and factors contributing the transmission according to the period of transmissions.

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 1.13).

- ***Prenatal transmission:*** Prenatal or *intrauterine* or *in utero* HBV infection is speculated to occur following passage of HBV from maternal blood through placental leakages, which are not prevented by either passive or active immunoprophylaxis at birth resulting HBV immunoprophylaxis failure. This route of transmission has been estimated to occur in 4-7% in infants born to HBsAg-positive mothers in China (212, 213) but may be rare in regions outside of Asia (214). The rate of intrauterine

transmission increases linearly with maternal serum HBsAg titers and HBV DNA concentration (214). Other factors associated with intrauterine HBV infection include maternal serum HBeAg positivity, history of threatened preterm labor, the presence of HBV in the villous capillary endothelial cells of placenta (212) and of some polymorphisms in the TNF-α and IFN-γ genes (215, 216). Moreover, HBV transmission from father to fetus before birth has been reported (217), this transmission can occur via the spermatid because, beside serum and leukocytes, HBV DNA can be detected in semen and spermatid as well.

- ***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 (209, 210, 218). 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%) (219). 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 (220).

- ***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 (209). Postnatal transmission from mother to newborn can occur through breast-feeding. Another source is that, in

households of chronically infected individuals, HBV infection can occur via person-to-person through nonsexual contact. With appropriate immunoprophylaxis, including HBIg and HB vaccine, breast-feeding of infants of chronic HBV carriers poses no additional risk of transmission of HBV (221). Another source of HBV transmission is represented by HBV carriers' urine which may explain the horizontal transmission of HBV among young children.

1.4.5 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). 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 less than 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 (222). Among infants who acquired HBV before the age of 2 year aged 10% will spontaneously resolve acute infection (Figure 1.14).

Chronic infection may also have serious complications: nearly 25% terminate in serious liver diseases (33). 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. The efficacious immunity may play an important role. Immunosuppressed patients, e.g.

hemodialysis patients or HIV-infected patients, are also at high risk of developing chronic infection (157).

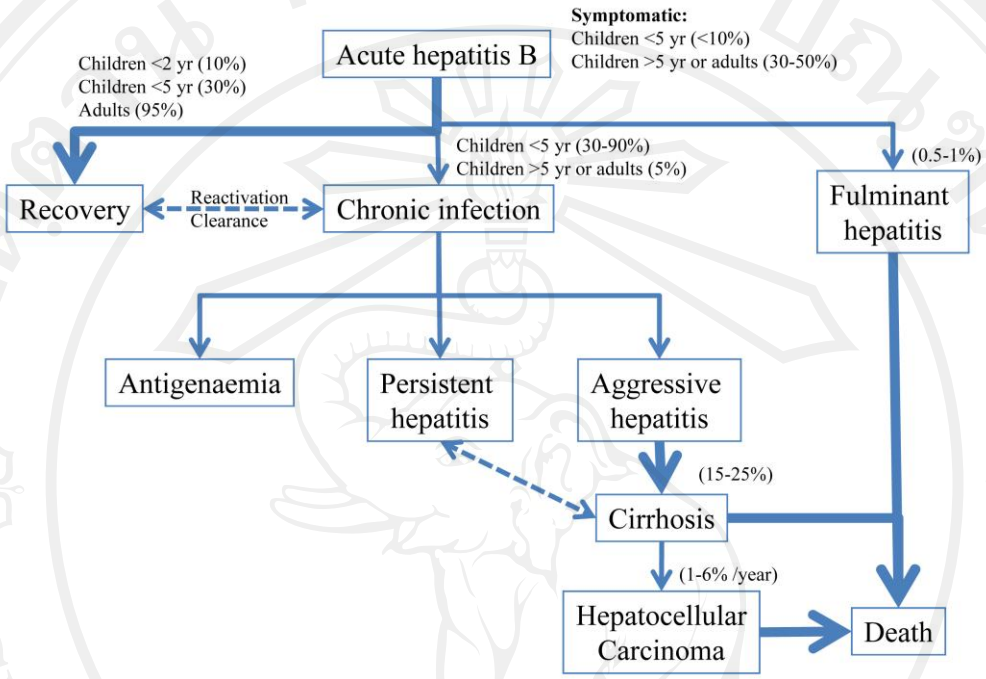


Figure 1.14 Outcomes of acute HBV infection

1.4.5.1 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 efficacious as 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) (223). 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 1.14). 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 aminotransferase 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 1.15A). 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 (10). Immunosuppressed patients can develop reactivation of previously resolved HBV infection (224).

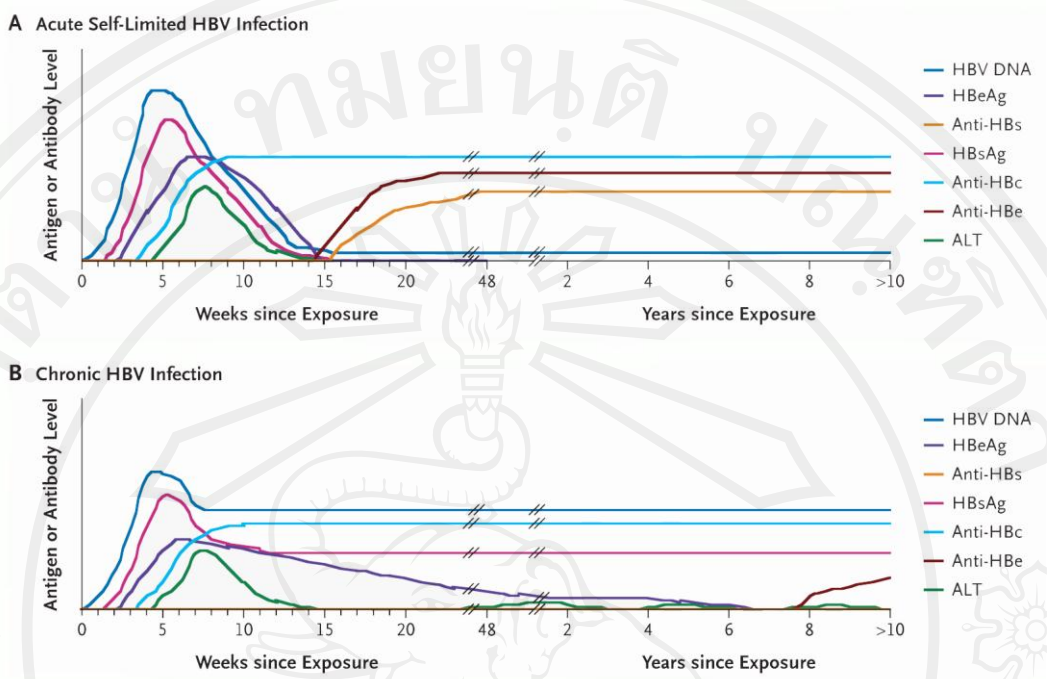


Figure 1.15 Serology and molecular maker patterns during course of acute (A) and chronic HBV infection (B) [*source: modified from Ganem D & Prince AM. N Engl J Med 2004* (10)]

1.4.5.2 Chronic HBV infection

Chronic infection is defined as either the persistence presence of HBsAg in serum of an individual for at least 6 months (33) 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 1.15B)(10). Approximately 0.5% of adults and a lower proportion of children, with chronic HBV infection will clear

HBsAg and develop anti-HBs annually. Without treatment, approximately 15-25% of persons with chronic HBV infection die prematurely from cirrhosis or HCC (225). The median survival time of HCC patients is less than 3 months without appropriate treatment, which includes surgery, percutaneous treatments, hepatic irradiation and chemotherapy (225).

The four phases of chronic hepatitis B infection have been well described, although not all patients go through all phases (Figure 1.16) (13, 152);

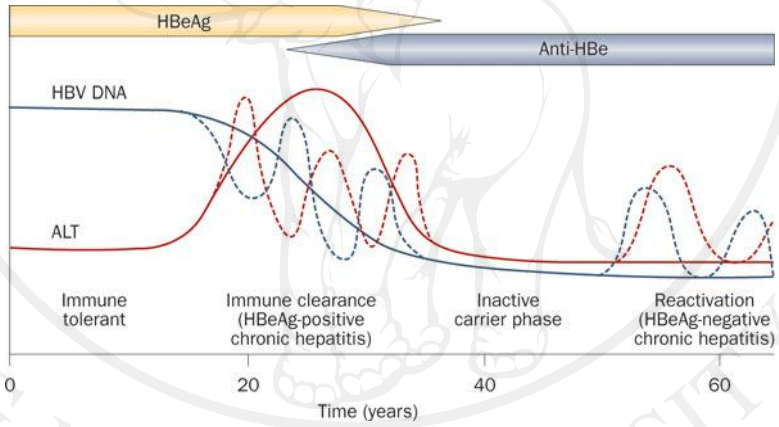


Figure 1.16 Natural history of chronic hepatitis B infection [*source: modified from Kwon, Nat Rev Gastroenterol Hepatol, 2011* (152)]

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 (226).

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 inflammation, 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 (13).

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 (227). 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 (228, 229). 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 (230). In Asia-Pacific, the prevalence of HBeAg-negative

chronic hepatitis B patients among HBsAg-positive individuals is estimated at 15% (231).

1.4.6 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 (10, 232).

1.4.6.1 Innate immune response

Innate immunity is the first line of defense immediately after infection to limit the spread of the virus. Following the infection, infected hepatocytes release interferon (IFN)-α/β cytokines which were triggered directly by virus replication through cellular mechanisms that detect the presence of viral RNA or DNA. Initial recognition of HBV infection may be mediated with toll-like receptors (TLRs). In addition, IFN-α/β cytokines recruit and mediate the activities of antigen presenting cells (APCs), particular Kupffer cells and dendritic cells (DCs). This APCs release interleukin-18 (IL-18) and chemokine CCL3 (232), which induces natural killer (NK) and natural killer T (NKT) cell activity (Figure 1.17). Moreover, the infected hepatocytes can warn neighboring cells of a viral presence by releasing IFN-α/β. The neighboring cells, in response to interferon, produce large amounts of an enzyme known as protein kinase R (PKR). This enzyme phosphorylates a protein known as a eukaryotic translation initiation factor (eIF)-2 in response to new viral infections.

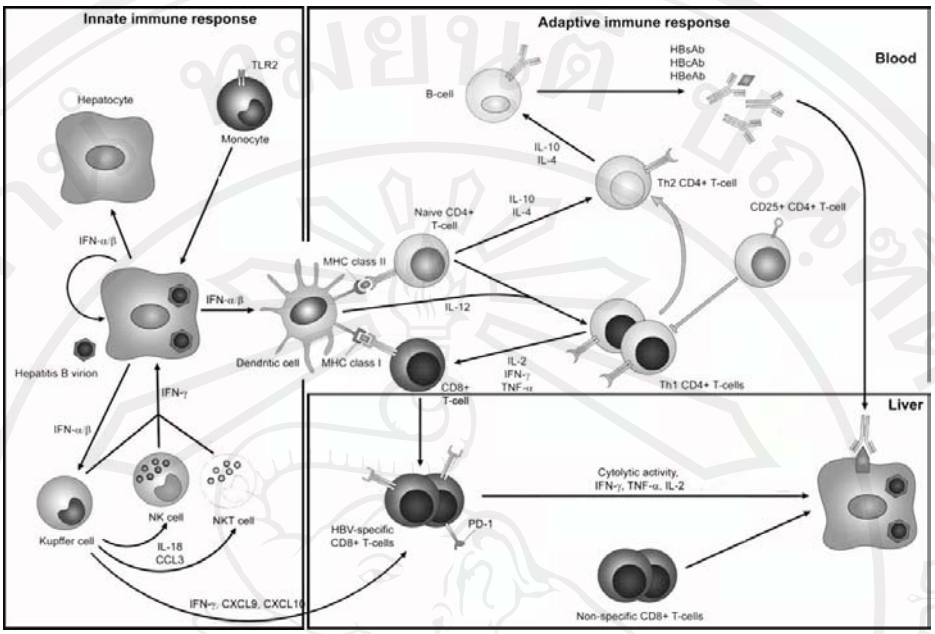


Figure 1.17 Immune responses against HBV infection. Control of HBV infection requires both innate immune response and adaptive immune responses: humoral and cellular arms [*source: modified from Chang JJ & Lewin SR. Immunol Cell Biol, 2007* (232)]

1.4.6.2 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 1.17). 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.

1.4.6.2.1 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 (233). 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.

1.4.6.2.2 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 (41, 234). 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 (232). 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.

1.4.7 Hepatitis B infection and hepatocellular carcinoma

Patients with severe liver disease are at risk of developing hepatocellular carcinoma (HCC). The annual risk of developing HCC in patients with cirrhosis is between 1 and 6 % (235). The incidence of HCC in HBV infected patients seems higher in the Far East. The 5-year probability of HCC in Taiwanese patients with compensated cirrhosis was higher at 20% with an annual HCC incidence of 2.8% while it was 6% with an annual incidence of 1.2% in Caucasian patients (13). In Japan, the cumulative incidence of HCC in patients with cirrhosis after 6 years was 59% (236), almost double the rate of chronic hepatitis B with cirrhosis in Italy, which was 31% in 8 years (237).

In general, 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 (152, 210, 238-240):

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.

1.4.8 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.

1.4.8.1 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 1.6 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.

1.4.8.2 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 (241), HuH-7 (242)) 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 (243).

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 (243). 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.

1.4.8.3 Molecular assays in diagnosis of HBV infection

Several types of molecular assay are available for the diagnosis of HBV infection (244).

1.4.8.3.1 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 1.7 Available commercial hepatitis B virus DNA quantification assays (245, 246)

Assay	Method	Dynamic range of quantification	Conversion factor (IU>copies)
Signal amplification			
HBV Hybrid-Capture II®	Hybrid capture signal in microplates	5.15 – 9.23 $\log_{10}$ copies/mL	
Ultrasensitive HBV Hybrid-Capture II®	Hybrid capture signal in microplates after centrifugation	3.67 – 7.76 $\log_{10}$ copies/mL	
Versant® HBV DNA 3.0 Assay	Semi-automated branched DNA	2.55 – 7.25 $\log_{10}$ IU/mL	5.7
Target amplification			
Amplicor HBV monitor®	Manual quantitative RT-PCR	2.26 – 5.85 $\log_{10}$ IU/mL	
COBAS Amplicor HBV monitor®	Semi-automated quantitative RT-PCR	1.54 – 4.55 $\log_{10}$ IU/mL	5.26
COBAS® Taqman™ HBV monitor	Real-time PCR after automated DNA extraction	1.73 – 8.04 $\log_{10}$ IU/mL	5.82
RealArt™ HBV PCR assay	Real-time PCR	0.60 – 8.00 $\log_{10}$ IU/mL	6
Abbott RealTime™ HBV DNA	Real-time PCR after automated DNA extraction	1.00 – 9.00 $\log_{10}$ IU/mL	3.41

* 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 (247).

1.4.8.3.2 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 (247).

- ***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.

1.4.8.3.3 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 (248).

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 (249). 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 transcriptase 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 (248).

- *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 (40, 248).

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 (250-252), 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 (253).

- 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 (254).

- Ultradeep sequencing or pyrosequencing: Pyrosequencing, an advance technique, has been used to detect minority populations of resistant HBV variants (255), 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.

1.4.9 Treatment of HBV infection

1.4.9.1 HBV treatment 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 pegylated interferon alfa-2a, and oral antiviral agents: i.e. the nucleos(t)ide analogues: nucleoside analogues include lamivudine, telbivudine, clevudine 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 (256). 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 (257).

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 37% of patients with HBeAg-positive chronic hepatitis B achieved HBV DNA suppression, 33% of them had HBeAg seroconversion, and 8% had HBsAg loss (258). However, the side effects of therapy with IFN, e.g. influenza-like symptom myalgia, 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 (10).

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

Two peg-IFNs have been produced: 12-kD linear PEG for IFN-α-2b and 40-kD branched PEG for 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. This new form of IFN reduces the excretion of IFN by the kidney, thus resulting in more stable plasma concentrations of IFN. Peg-IFN-α-2a is given once a week at a dose of 180 μg for at least 6 months. In HBeAg-positive patients, peg-IFN-α-2a is superior to non-pegylated IFN. At 6 months of peg-IFN-α-2a, treatment response, defined as the loss of HBeAg with a serum HBV DNA below 500,000 copies/mL and normal ALT levels, was observed in 28% of patients receiving 180 μg of peg-IFN-α-2a and in 12% of patients who received non-pegylated IFN (259). At 1 year of peg-IFN-α-2a treatment, HBV DNA was suppressed in 25% and 63% in HBeAg-positive and HBeAg-negative chronic hepatitis B, respectively (260). 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 (261).

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 (262). 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 (Figure 1.18). The active form is triphosphorylated and competes with deoxycytidine

5'-triphosphate (dCTP) for incorporation into growing DNA chains, causing chain termination of RNA-dependent HBV polymerase (263). 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 tolerated in both children and adults (10, 264, 265). The oral administration of 100-mg dose of lamivudine can cause complete and sustained suppression or viral replication (266). In addition, 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 (10). About 16-20% HBeAg seroconversion rate can be achieved with 1 year of treatment (266, 267). This rate reached up to 50% at longer duration (3-5 years) of treatment (268-271). However, the major drawback of lamivudine is a high rate of emergence of lamivudine-resistant HBV (272). 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 (10). The viral resistance emerged in 24% of lamivudine-treated patients after just one year of therapy and continued to accumulate with increasing duration of treatment (up to 70% after 5 years of lamivudine treatment) (Figure 1.19)(273). The main mutations responsible for lamivudine resistance include rtM204V and rtM204I (274). The rtM204V mutation usually occurs concomitantly with the rtL180M mutation, whereas rtM204I can occur

alone or together with rtL180M. The rtL180M can restore the replication competency of rtM204V/I mutants and increase drug resistance(275). Another compensatory mutation, rtV173L, occurs with rtM204V/I and rtL180M mutations, and may enhance viral replication. Other mutations associated with lamivudine resistance were rtA181T/V which is a mutation cross-resistance with adefovir (276).

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 (10). Until recently, in developed countries, lamivudine 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 (277). In contrast, due to its well-established safety profile and lower cost, lamivudine is a strong candidate for wide scale use in resource-limited countries where the new anti-HBV agents are not available or at high price and the 2008 Asian Pacific Association for the Study of the Liver (APASL), in its guidelines for HBV management, still recommends lamivudine for treatment of HBV mono-infection in endemic areas (278). Furthermore, if lamivudine resistance occurs, resistant HBV can be treated with other potent nucleos(t)ide analogues (279).

Emtricitabine (FTC)

Emtricitabine is a synthetic cytosine analog with activity against both HIV-1 and HBV (280). It is chemically similar to 3TC, differing only in having a fluoride at

the 5-position of the cytosine ring. FTC is the (-) enantiomer, which is more active than the (+) enantiomer. FTC has a longer half-life than 3TC and similarly induces a rapid and sharp reduction in serum HBV DNA at doses of 200 mg daily. Suppression of HBV replication is maintained over 48 weeks of treatment in more than half of patients (281). Similar to 3TC, FTC is generally well tolerated. Resistance to FTC is correlated with the emergence of the same M184V mutation in reverse transcriptase that mediates resistance to 3TC but this appears to be less frequent in patients failing a regimen containing FTC than in patients failing a 3TC-containing regimen. Like 3TC, FTC should not be used as monotherapy in HBV/HIV co-infected persons due to high risk of selection of the M184V resistance mutation in HIV. However, the use of FTC in combination with tenofovir provides a potent anti-HBV and anti-HIV activities in patients with co-infection (282).

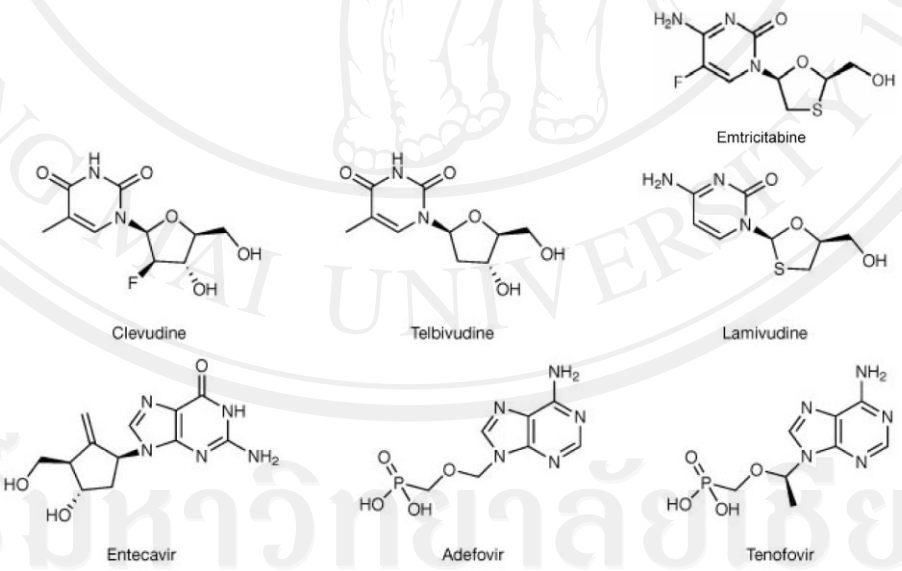


Figure 1.18 Structures of different Nucleoside/nucleotide analogue [*source: modified from Fung et al. J antimicrob chemotherapy 2011*(283)]

Adefovir dipivoxil (ADV)

Adefovir dipivoxil was the second drug licensed for the treatment of chronic hepatitis B (CHB). Indeed, ADV is an antiviral drug active against both HBV and HIV but it is 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 chronic hepatitis B. ADV, a acyclic diphosphonates, is an analogue of adenosine monophosphate that undergo intracellular phosphorylation to its active metabolite, which inhibits the HBV polymerase by competitive inhibition with deoxyadenosine 5'-triphosphate (dATP), resulting in chain termination (284). 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 (285). One of advantageous properties of ADV is its efficacy against lamivudine-resistant mutants (286). 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 (273) (Figure 1.19). The 2 major mutations of HBV polymerase gene responsible for ADV resistance are rtA181V/T and rtN236T (287). Another mutation, rtI233V, has been associated with primary ADV resistance (288).

Entecavir (ETV)

Entecavir 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 (289). ETV (0.5 mg/day) is superior to LAM (100 mg/day) in the treatment of CHB patients (290). 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 of ETV treatment (290). Cumulative rates of HBV DNA undetectability increased to 80% at 2 years (291) and 94% at 5 years of therapy (292). In patients with HBeAg-negative chronic hepatitis B patients, HBV DNA was undetectable in 90% of patients after 1 year of therapy (293). ETV has a high barrier to resistance, it require a combination of three mutations before resistance develops. In addition to the rtM204V and rtL180M mutations, an additional mutation at rtI169T, rtT184G, rtS202I or rtM250V is required for ETV resistance. Thus, very low rates of ETV resistance have been reported in nucleotide analogue naïve patients, only 1.2% after 5 years of therapy (294)(Figure 1.19). However, in patients experienced to lamivudine-resistance, the risk of developing ETV resistance is increased.

Telbivudine (LdT)

Telbivudine 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 (289). LdT is effective at 600 mg daily. Treating with LdT is superior to lamivudine (100mg/day) in HBV DNA reduction and suppression in both HBeAg-positive or -negative patients (295). In HBeAg-positive patients, HBV DNA suppression (<300 copies/mL) was observed in 60% of patients, HBeAg seroconversion was observed in 23% of patients after 1 year of treatment. In HBeAg-negative patients, HBV DNA became undetectable in 88% of patients after 1 year of LdT. Despite the high potency of LdT, virological response rates decrease over time due to the emergence of LdT resistance (Figure 1.19). The overall rate of LdT resistance was 5% in patients with HBeAg-positive CHB and 2.2% in those with HBeAg-negative CHB after 1 year of therapy (295). Resistance rate is still relatively high, at 25% and 11% for HBeAg-positive and –negative patients, respectively, after 2 years of treatment (296). The rtM204I with or without concomitant rtL80I/V and rtL180M mutation, a cross-resistance to LAM, is the main mutation conferring primary resistance to LdTs. Other mutation associated with LdT resistance include mutation at rtA181T/V and rtL229W/V (283).

Tenofovir Disoproxil Fumarate (TDF or PMPA)

Tenofovir Disoproxil Fumarate is an oral prodrug form of tenofovir. Tenofovir is the most recently approved nucleotide analogue for the treatment of CHB in 2008 at a dose of 300 mg/day. TDF is an acyclic nucleoside phosphonate analogue of adenosine monophosphate. After undergoes phosphorylation, 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 (289). 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) (297). At 3 years of therapy, the rate of HBeAg seroconversion increased to 26% and the rate of HBV DNA undetectability reached 78% (298). In HBeAg-negative patients, HBV DNA undetectability was achieved in 93% of patients after 1 year of TDF therapy (297) and 99% after 3 years (299). Furthermore, TDF was also able to suppress the HBV DNA levels to below detectable level in 79% of patients who failed previous nuclos(t)ide analogue treatments after a 2 years follow-up (300). However, the presence of ADV resistance mutations impaired TDF efficacy (52% rate of HBV DNA undetectability) but LAM resistance did not influence the antiviral efficacy of TDF (100% HBV DNA undetectability) (300). However, so far, no TDF resistance has been reported after 3 years of continuous therapy (Figure 1.19)(277), which underlines the high genetic barrier of this drug and its potency.

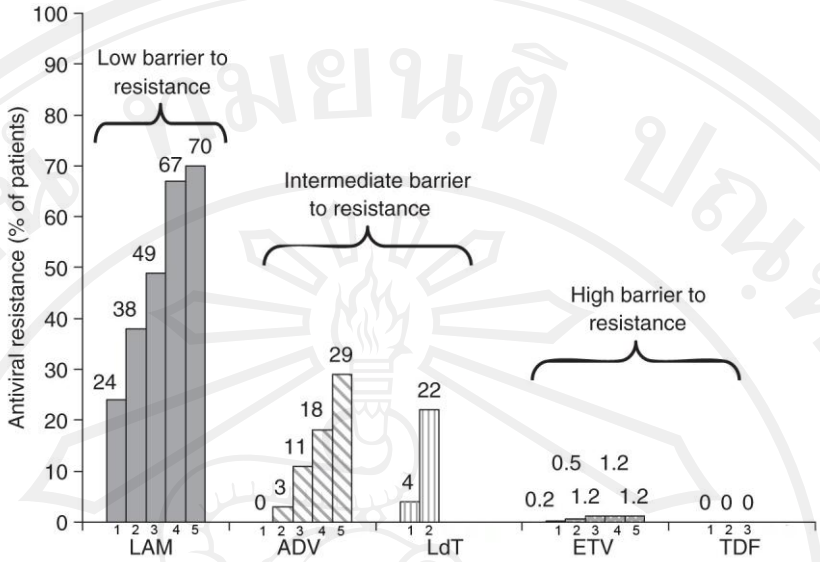


Figure 1.19 Estimated rates of genotypic resistance to anti-HBV treatments in naïve patients. The numbers under the bar indicate years of therapy [*source: modified from EASL Clinical Practice Guidelines, J hepatol 2009* (273)]

Combination therapy

Although currently available agents for treating chronic hepatitis B are licensed for use as monotherapies and also international guidelines recommend to initiate the treatment with monotherapy (260, 273). However, combination therapy could be benefit to chronic hepatitis B patients as it has been demonstrated in HIV treatment. Combination therapy for chronic hepatitis B can be either a combination of immunomodulatory agent with nucleos(t)ide analogs or a combination of more than 2 agents of nucleos(t)ide analogs.

Combining PEG-IFN with nucleos(t)ide analogs is the most appealing approach, but the addition of LAM, ADV to PEG-IFN therapy did not result in higher sustained response rates, whereas a combination of PEG-IFN with ETV or TDF are

not yet available. Studies on the efficacy of combination therapy with nucleos(t)ide analogs are scarce and only combinations of nucleos(t)ide analogs that are no longer considered as first-line treatment were investigated.

A randomized double-blind study conducted in China showed that, after two years of treatment with a combination of more than 2 agents of nucleos(t)ide analogs, LAM plus ADV, was associated with lower rates of resistance to lamivudine, as compared to LAM mono-therapy (15% vs. 43%). Both regimens had the same rates of HBeAg seroconversion and both were well tolerated (301). In another small study, ADV plus Emtricitabine (FTC) (a cytosine analogs structurally related to lamivudine, only one fluorine is added in the cytosine ring for its longer intracellular half-life) therapy have been associated with greater HBV suppression at year 2, as compared to ADV mono-therapy (79% vs 38%) (302). Although it was shown that combination therapy can delay the development of resistance, there are no data to support de novo combination therapy with nucleos(t)ide analogs that have a high barrier to resistance in nucleos(t)ide analogs-naive patients. Moreover, a recent study in patients with incomplete viral suppression during therapy with ADV showed that TDF monotherapy and the combination of TDF and emtricitabine, which is structurally similar to LAM, had similar efficacy.

In overall, 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 (283, 284). Thus, there is insufficiency evidence to recommend combination therapy in CHB patients as a first-

line regimen. Nevertheless, current guidelines recommend that a combination of nucleos(t)ide analogs may be considered in patients at high risk of complications such as in patients with advanced liver disease (260, 273).

Table 1.8 Medication available for the treatment of chronic HBV infection (239)

Agents	Licensed for		Dose active against		Barrier to resistance
	HBV	HIV	HBV	HIV	
Interferon alpha	yes	partial active	5 MU/day or 9-10 MU, 3 times/week	NA	none
Pegylated interferon alpha 2a	yes	active	180 ug/week, 1 time/week	NA	none
Lamivudine	yes	yes	100 mg/day	300 mg/day	low
Emtricitabine	yes	yes	200 mg/day	200 mg/day	
Adefovir dipivoxil	yes	partial active	10 mg/day	high dose	intermediate
Tenofovir disoproxil fumarte	yes	yes	300 mg/day	300 mg/day	high
Entecavir	yes	active	0.5 or 1 mg/day		high
Telbivudine	yes		600 mg/day		intermediate
Clevudine*	yes		30 mg/day		

*Licensed for treat HBV only in Korea and Philippines

1.4.9.2 HBV treatment for HBV/HIV co-infected patients

Nearly 10% of estimated 40 million people having HIV worldwide suffer from chronic hepatitis B virus (166, 167, 303). Treatment of Hepatitis B improves liver disease in HIV/HBV co-infected patients (304) and is usually based on antiviral agents which are active to both viruses as described in Table 1.8.

The data on the use of peg-IFN in patients with HBV-HIV co-infection are scarce at present. Peg-IFN therapy is associated with lower rates of therapeutic success and increased toxicity in HBV/HIV co-infection (305). However, some study reported that the combination therapy of peg-IFN with an oral anti-HBV drug is safe and effective for treating 3TC-resistant HBV in HIV co-infected patients (306).

Given its excellent tolerability, LAM has been widely used as anti-HBV agent in HIV-infected patients (89, 307). 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. Unfortunately, overall HBV resistance mutation can be occurred in 94% of HBV viremic patients with HIV infection who received LAM for over 4 years (308).

Adefovir dipivoxil (10mg/day) suppresses HBV replication, and is associated with a low rate of resistance compared with LAM (309, 310). However, prolong use of ADV in HIV/HBV co-infected patients may theoretically involve a risk of selecting HIV cross-resistance to TDF (239).

Entecavir 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 (290, 293, 303). In a randomized controlled trial of 68 HIV/HBV co-infected patients with LAM-resistant HBV, reduction of HBV DNA after 24 weeks of ETV was similar to that observed in HBV mono-infection (311). To date, resistance to ETV occurred infrequently in patients with wild-type HBV infection but it commonly occurred in of patients with LAM-resistant HBV, up to 39% after 4 years of ETV treatment (239).

Telbivudine has no activity against HIV and has greater anti-HBV efficacy than either LAM or ADV and selects for resistance mutations at intermediate rates (312). Among HIV-infected persons, the antiviral effect of LdT on HIV is still unclear. Some studies reported a decline in HIV RNA load during LdT monotherapy,

indicating that LdT may exert antiviral activity against HIV (313, 314). Later on, other studies could not confirm this finding neither *in vitro* nor *in vivo* (315, 316).

Tenofovir disoproxil fumarate (200 mg/day) has been extensively used with tenofovir in HIV/HBV co-infected patients. It has slightly greater potency and efficacy than lamivudine but cannot be used as monotherapy because of high rates of resistance (303). 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 (279, 317-320). 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 (321) but not superior to TDF alone (322). No resistance was observed after 5-years of therapy. Recently, in Thailand, HAART containing EFV plus TDF/3TC or TDF/FTC has been recommended for treating HIV/HBV co-infected patients as preferred regimen (323, 324).

Recently, the US Department of Health and Human Services guideline for the use of antiretroviral agents in HIV-1 infected adults and adolescents, last updated in January 2011 (http://aidsinfo.nih.gov/guidelines), recommends that;

(1) Prior to initiation of antiretroviral therapy (ART), all HBsAg-positive patients should be tested for HBV DNA using a quantitative assay to determine the level of HBV replication. However, in practically, the accessibility and cost of this test are the major obstacles in resource-limited settings.

(2) As emtricitabine (FTC), lamivudine (3TC), and tenofovir disoproxil fumarate (TDF) have activity against both HIV and HBV, if HBV or HIV treatment is needed, ART should be initiated with the combination of TDF + FTC or TDF + 3TC as the nucleoside reverse transcriptase inhibitor (NRTI) backbone of a fully suppressive ARV regimen.

(3) If HBV treatment is needed but TDF cannot safely be used, entecavir is the alternative recommended HBV therapy to a fully suppressive ARV regimen. Other HBV treatment regimens include peg-IFN α monotherapy or adefovir in combination with 3TC or FTC or telbivudine in addition to a fully suppressive ARV regimen.

(4) Entecavir (ETV) has activity against HIV; its use for HBV treatment without ART in patients with dual infection may result in the selection of the M184V mutation that confers HIV resistance to 3TC and FTC. Therefore, ETV must be used in addition to a fully suppressive ARV regimen when used in HIV/HBV-coinfected patients.

(5) Discontinuation of agents with anti-HBV activity may cause serious hepatocellular damage resulting from reactivation of HBV; patients should be advised against self-discontinuation and carefully monitored during interruptions in HBV treatment.

(6) If ART needs to be modified due to HIV virological failure and the patient has adequate HBV suppression, the ARV drugs active against HBV should be continued for HBV treatment in combination with other suitable ARV agents to achieve HIV suppression.

1.4.10 Pregnant women and hepatitis B virus infection

Pregnant women with hepatitis B virus infection are a major issue of public health problem since the infection or the medications during pregnancy may harm to both the mother and the fetus (325). HBV can transmit from the mother to the fetus *in utero* and to the newborn during labor. Without any medical intervention, in hyperendemic countries, vertical transmission thus occurs to the newborn in 70-90% of HBeAg-positive pregnant mothers and in 10-40% of in those with negative HBeAg, as mentioned above (209, 210, 326). In general, 90% of the infected newborns can ultimately develop chronic hepatitis B. Passive/active immunization of the neonates immediately after birth with HBIg and HB vaccine is used to prevent vertical transmission, especially in mothers with HBeAg positive or with high viral load (327, 328). Intrauterine/transplacental transmission of HBV can occur about 8% of the cases in women with high viral load and/or preterm labor (19). Some studies reported that, in these high viral load pregnant women, monthly administration of HBIg in the third trimester of the pregnancy could prevent the vertical transmission (329, 330). Moreover, in HBsAg-positive pregnant women with high viral load, the use of anti-HBV drug (e.g. lamivudine, telbivudine, tenofovir disoproxil fumarate) in the third trimester of pregnancy, followed by passive and active immunization of the newborns, has been proven to be effective in preventing transmission of HBV to the fetus, is safe for the mother and the fetus, and is also cheaper and more convenient to the mothers than the monthly injections of HBIg (331-334). Lamivudine, adefovir and entecavir are listed by the U.S. Food and Drug Administration (FDA) as pregnancy category C drugs and telbivudine and TDF as category B drugs. This classification is based on the risk of teratogenicity in preclinical evaluations. There

are also many studies reported safety of the use of lamivudine in HBV pregnant women or even in HIV-infected pregnant women who have received TDF and/or LAM or emtricitabine during pregnancy (335). Therefore, based on the existing evidence, TDF, LAM or telbivudine could be considered, especially in the third trimester of pregnancy.

Vaccination against HBV is also completely safe in pregnant women. HBsAg-positive mothers can breastfeed their babies who have been prophylactically immunized. However, in women who take nucleot(s)ide analogs, these compounds are detected in their milk. Until now, it is quite unclear whether the mode of delivery influences the risk of HBV transmission as far as passive and/or active immunization were implied (219, 221, 336).

Another major issue is that, in the majority of pregnant women, serious flares of serum HBV DNA level do not happen during pregnancy and liver enzymes become normal (337). Nevertheless, hepatic flares often occur during the third trimester of pregnancy and even 6 months after labor, reaching rates of 45% (335, 338). Therefore, HBsAg-positive mothers should be closely monitored for many months following labor.

1.4.11 HBV prevention and vaccination

1.4.11.1 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) (339).

Passive immunization or specific HB immunoglobulins (HBIg)

As the discovery that passively acquired anti-HBs soon after exposure can protect individuals from acute clinical hepatitis B and development of chronic HBV infection (340), preparation of HBIg have thus been developed and used for this aspect. 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 (341), percutaneous or mucous membrane exposure to HBsAg-positive blood (342), or sexual exposure to an HBsAg-positive person (343). HBIg are also used to protect patients from severe recurrent HBV infection following liver transplantation (208).

Active Immunization or hepatitis B (HB) vaccination

Hepatitis B can be effectively prevented by vaccination. A vaccine directed against HBsAg (HB vaccine) has been available for more than 30 years. HB vaccine is one of the best human vaccines ever developed; it is safe, cheap, and highly immunogenic, stimulates long lasting protective efficacy, and is the first human cancer vaccine. The first HB vaccine was elaborated from asymptomatic human

HBsAg carriers in 1976 by Maupas P *et al.* (344) and was efficacious in adults, children and newborns (345). The first commercial vaccine against HBV was licensed in 1981 and the first recombinant vaccine appeared in 5 years later.

- ***Plasma-derived HB vaccines:*** The first type of HBV vaccines were initially made of highly purified HBsAg by collecting HBsAg from the non-infectious 22-nm HBV sphere forms in plasma 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 HB 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 (339). The currently used HB vaccines mostly consist of the small surface protein and the middle pre-S2 surface protein assembled into 22-nm particles. Both of small and middle surface proteins contain HBsAg including the common "a" determinant and several subtype determinants (346).

- ***New HB vaccine:*** Recently, the third generation of HB vaccines containing all S, preS1 and preS2 domains of HBV has already been developed (347, 348). 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 (349).

Whatever the production mode, all vaccines are very safe (328). HB 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 (346). However, about 5% of vaccinated individuals do not develop anti-HBs antibody, so called "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 (346). 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 (12, 350-352). Thus, a booster is not recommended in healthy people who are not exposed to a high risk of HBV infection.

In children, a first dose of HB vaccine administered within 12 hours (or 24 hours in some guidelines) after birth, followed by two to three boosts during the first 6 months of life, is 70%-95% effective in preventing transmission to children born to HBV infected mothers (346, 353). Since 1991, the World Health Organization (WHO) has recommended that HBV immunization is incorporated into childhood immunization services, regardless of the mother's HBV status (353), because it is also benefit and effective in preventing infection in children born to HBV-uninfected mothers. The immunization schedule varies slightly from countries to countries

(Table 1.9). Indeed the schedule of immunization proposed by WHO is flexible but adherence to immunization schedule seems important.

Table 1.9 HBV immunization schedule recommendations by WHO [source: modified from *www.who.int/vaccines-documents*]

HBIg	Hepatitis B Vaccine				
	Option	Birth	6 weeks	10 weeks	14 weeks
Recommended if mother HBsAg+	**I**		HepB[2]	HepB[2]	HepB[2]
	II	HepB[1]	HepB[1]		HepB[1]
	III	HepB[1]	DTP-HepB[3]	DTP-HepB[3]	DTP-HepB[3]

[1] monovalent vaccine
[2] monovalent or combination vaccine
[3] combination vaccine

There is a progressive increase of number of countries over the years where HB vaccination has been introduced into their national EPI and 3-vaccine doses coverage (Figure 1.20). In 2010, 179 of 193 countries reported the implementation of newborn HB vaccination in their national Expanded Program for Immunization (EPI) (354). Furthermore, for infants born to HBsAg carriers, the administration of HBIg in addition to the vaccine (passive–active immunoprophylaxis) can further halve the risk of transmission (327, 355). In countries where this program has been implemented, a dramatic reduction in the prevalence of children HBsAg carriers has been observed (17, 356). However, despite passive–active immunoprophylaxis, some infants born to HBV infected mothers are still infected (357).

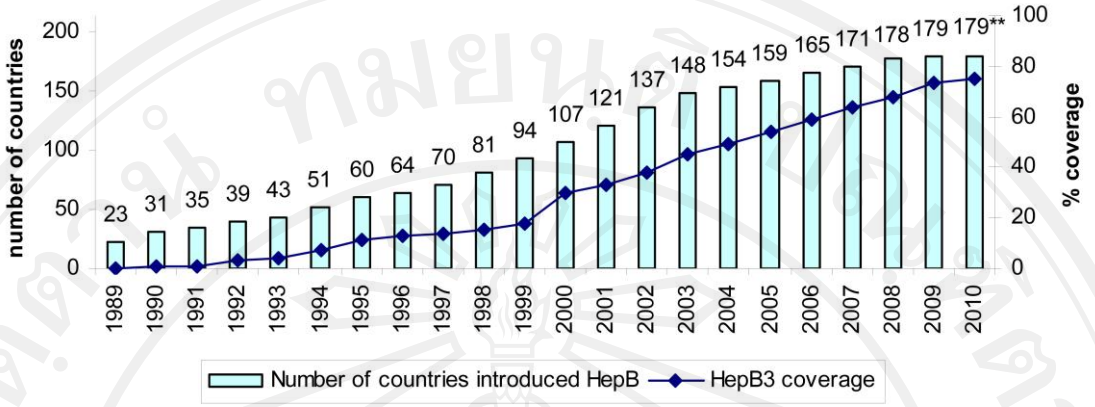


Figure 1.20 An increase of number of countries where HB vaccination has been introduced into their national EPI and 3-vaccine doses coverage from 1989 to 2010 [source: modified from *WHO/UNICEF coverage estimates 2011 revision, July 2012*]

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) (358) and integrated universal HBV vaccination in the nation-wide Expanded Program of Immunization (EPI) in 1992 (359). This has resulted in decreased incidence of acute and chronic HBV infection (17). Currently, HB vaccination for infants is nearly universal, and more than 98% of all newborns in Thailand have been vaccinated (161). 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 (360). 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 (18).

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 (361). Thus, the efficacy of the present vaccine against possible variants needs to be evaluated in order to determine whether vaccine modifications are required.

In Thailand, the recommended immunization schedule was originally within 12 hours of birth (monovalent formulation), 2, 4 and 6 months (polyvalent formulations) but following the observation that infection may have occurred in some infants whose 2-month dose was delayed, particularly in infants born to HBsAg-positive mothers and did not received HBIg. Therefore, an extra dose of monovalent HB vaccine is now recommended exactly at one month of age in infants born to HBsAg positive mothers (Table 1.10) (362-364). Current data show that vaccine induced hepatitis B antibody levels decline with time. However, there is no need to obtain a booster dose of vaccine since immune memory (anamnestic anti-HBs response) will protect immunized individuals if exposed to HBV (352).

Table 1.10 HBV immunization schedule recommendations by Pediatric Infectious Disease Society of Thailand, 2012 (364)

Maternal HBsAg	HBIg	Hepatitis B Vaccine				
		Birth	1 month	2 months	4 months	6 months
Negative or unknown		HepB[1]		HepB[1]		HepB[1]
Positive	Recom-mended	HepB[1]		HepB[1]		HepB[1]
Negative		HepB[1]		DTP-HepB[3]	DTP-HepB[3]	DTP-HepB[3]
Positive	Recom-mended	HepB[1]		DTP-HepB[3]	DTP-HepB[3]	DTP-HepB[3]
Positive	Not available	HepB[1]	HepB[1]	DTP-HepB[3]	DTP-HepB[3]	DTP-HepB[3]

[1] monovalent vaccine
[2] monovalent or combination vaccine
[3] combination vaccine
Abbreviations: HepB, Hepatitis B vaccine (monovalent); DTP-HepB, combined Diphtheria-Tetanus-Pertussis-Hepatitis B vaccine (tetravalent)

Despite use of HBIg and HB vaccine, some infants still suffered from mother-to-child transmission. Transmission rates vary from 3% in Australia (147), 7.4% in China (19) and 12% in South Korea (365). These vaccine failures were significantly associated with maternal HBeAg-seropositivity and high level of HBV DNA. Recent study in Taiwan revealed, despite immunization, a higher rate of children born to HBeAg-positive mothers were positive for antibodies against the hepatitis B core protein (anti-HBc) (16.76%) and HBsAg (9.26%) than children born to HBeAg-negative mothers (1.58% and 0.29%, respectively; $P<0.0001$ and <0.001). Also, among the HBV-infected children, the rate of chronicity was higher among children with HBeAg-positive mothers than those with HBeAg-negative mothers (54% vs. 17%; $P <0.002$) (366). 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 (135).

1.4.11.2 HB vaccination in HIV-infected population

No distinctive adverse clinical reactions to HBV vaccination have been described in the HIV population (303). However, Hepatitis B vaccination is most efficacious before severe immunosuppression. Indeed, both CD4+ cell count nadir <200 cells/μL (367) and current CD4+ cell count <50 cells/μL (368) 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+ T-cell count nadir >200 cells/μL. In patients starting antiretroviral therapy, it may be best to defer this vaccination and other vaccinations until CD4+ T-cell count >200 cells/μL is established (369).

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+ T-cell count were predictors for successful response to hepatitis B vaccination (370). Another study reported a high rate of anti-HBs production failure in HIV-infected children on HAART; only 1% (1/69) had a protective antibody level at 5 years or more of age (371), 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 (372, 373)

1.4.12 Occult HBV infection

The detection of HBsAg in serum remains the mainstay in the diagnosis of HBV infection in the most developing countries. The majority of individuals positive for HBsAg are also positive for HBV DNA in the serum. However, a small proportion of individuals have detectable HBV DNA in the serum or the liver in the absence of circulating HBsAg, so called "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 (374). Seropositive occult HBV infection is characterized by the presence of anti-HBc and/or anti-HBs, while neither anti-HBc nor anti-HBs is detected in seronegative occult HBV infection (375). HBV antibodies, including anti-HBc, anti-HBs, and anti-HBe, are frequently detected in individual with occult HBV infection (376).

The prevalence of occult HBV infection varies greatly across the world, ranging from 0% to 87% in different part of the world. However, these prevalence rates need to be interpreted with caution because several factors could be potentially influence these estimated rates (375), for instance;

1) ***Study population and geographical region:*** prevalence rates vary between high-risk groups and low-risk groups. The groups with high risk for occult HBV infection are patients with chronic HCV or HIV infection, injection drug users, patients with hemodialysis, liver cancer, liver cirrhosis, and liver transplantation. Occult HBV infection is frequently found in individuals with isolated antibodies against core antigen of HBV serological pattern (377). Endemicity of HBV infection is also correlated with prevalence of occult HBV infection.

2) ***Sample size:*** Clearly, if a prevalence rate is calculated from a very large sample it is likely to be more accurate than one calculated from a small sample. Small number of samples tested provides the prevalence rate with a wide confidence interval.

3) ***HBV detection Assay:*** Generally, sensitivity and specificity of each commercial test kit are different. Some test kits are able to detect HBsAg mutants or low amount of HBV DNA while some cannot. These may influence to the precision of the results. Moreover, the change of some amino acid on an "a" determinant of HBsAg may influences to the antigen-antibody binding of some HBsAg test kit and results a false negative result.

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 (25), the formation of HBV surface antigen (HBsAg) and anti-HBs antibodies complexes (378), the presence of mutations in the surface antigen (25), or a reduced production of HBsAg due to either mutations in the surface promoter region (379), or co-infection with hepatitis C virus (HCV) (25, 380).

Several mutations in the HBsAg have been shown to be associated with occult HBV infection: a stop codon mutation at position 216 of HBsAg, the E164D and I195M substitutions in HBsAg, which are associated with LAM-resistance mutations (148), 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 (381). In addition, other 3 amino acid mutations (T123A, M133L, and T143M) in the "a" determinant of HBsAg may involve with HBsAg antigenicity in HBsAg-negative blood donors with DNA viremia (382). Martin *et al.* also described mutations in S gene (Y100F/S, A128T, S136P, G145A/R) in occult HBV patients (383).

For clinical relevance, occult HBV infection may impact in several different clinical contexts, as following;

1) Transmission of occult HBV infection: individuals with occult HBV infection can be a source of HBV transmission via blood transfusion or organ transplantation with the consequent development of hepatitis B in the recipients (384, 385).

2) Reactivation of occult HBV infection: this event is usually found in patients with immunosuppression condition whether induced by therapies or infectious diseases (386, 387).

3) Occult HBV infection is the risk factor of development of chronic liver diseases and liver cancer: in some people who have recovered from self-limited acute hepatitis, HBV genomes and a mild necroinflammation can still be detected in the liver up to 30 years after the resolution of the acute hepatitis (229). In general, although occult HBV infection is usually unable in itself to provoke a clinically relevant liver injury, but if other causes of liver damage co-exist, it might accelerated the progression to liver diseases or HCC, as demonstrated in HCV infected patients (388). Furthermore, occult HBV infection has been shown to reduce response rates to interferon-based treatment in HCV-mono-infected patients (389, 390).

1.4.12.1 Occult HBV infection in HIV-infected patients

Due to common route of transmission, HIV-infected patients are at risk to acquiring HBV either vertically or horizontally. Despite there are conflicting reports on the impact of occult HBV infection on the natural history of HIV disease, so far, occult HBV infection became a major concern in management of HIV-infected patients (391). The prevalence of occult HBV infection in HIV-infected patients remains controversial, with numbers varying between 0 to 89%. 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 (374). In general, Occult HBV infection is frequently found in patients with isolated anti-HBc serological pattern, like HIV uninfected individuals (392). Low CD4 cell count (<200 cell/mm^3) was more commonly found among women with occult HBV infection than among those with no occult HBV infection (393). 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 (394). In HIV-HCV co-infected patients, rate of occult HBV infection varies from 0-40% (395-397) and this co-infection may cause more severe liver disease and lower response to interferon therapy (398). It may contribute to increased plasma HCV RNA and liver transaminase levels (399).

1.4.13 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 (400, 401). Thus at least a proportion of individuals with this serological pattern is HBV carriers and may have potential consequences for themselves, or may be an issue for blood banking and transplantation service.

Several possible underlying mechanisms of having isolated anti-HBc serological pattern were proposed, as described in Table 1.11. The probability that isolated anti-HBc is a sign of 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 (239).

Depending on population studies and technique used, the prevalence of isolated anti-HBc in HIV-uninfected populations varies greatly, as showed in Table 1.12. It relates directly to the prevalence of HBV infection in the population being tested; e.g. 0.4-4% among blood donors in geographic areas with low HBV endemicity, (376, 401-403) and 1-21% in high HBV prevalence countries (382, 404-406). Isolated anti-HBc serology pattern occur frequently in persons engaging in injecting drug use (IDU)(407, 408), generally also infected by HCV (409, 410), among both HIV-infected and HIV-uninfected persons (408, 410-414).

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 (401). There is indirect evidence suggesting that in these individuals the risk of progression to cirrhosis and HCC still exist (415, 416). The risk of transmission in individuals with isolated Anti-HBc is low and, it is further reduced by given prophylaxis (417, 418).

Isolated anti-HBc is very common among HIV-positive patients (414). Prevalence of isolated anti-HBc was 12-26% (Table 1.13). Injecting drug users and anti-HCV seropositive were risk factors of isolated anti-HBc (407, 414). However, the contrast results still reported in Taiwan, an areas of highly endemic chronic HBV infection, the only risk factors of isolated anti-HBc were HIV infection and increasing age but not HCV infection (410). The clinical significance of isolated anti-HBc detection in the HIV-positive population is still unclear. It has been shown that the presence of isolated anti-HBc was not associated with a lower survival rate compared with those with resolved HBV infection in HIV infected patient (419) but rather was associated with immunosuppression condition resulting the shorter duration of survival among HIV-infected patients with isolated anti-HBc than those with anti-HBs persistence (420). Furthermore, lamivudine and emtricitabine are extensively used in HIV infection and these drugs may lead to the emergence of mutations in HBV of immune escape, which are a cause of HBsAg undetectability in HIV infected patients (421).

Table 1.11 The underlying mechanisms of isolated anti-HBc serological profile (422)

Interpretations of an isolated anti-HBc profile	**Possible underlying mechanism and evidence proof**
False positive to anti-HBc	- retested with different ELISA assay
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 a 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

Table 1.12 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		(423)
Ghana	Pregnant women	219		9.1	Real-time	25	(424)
Iran	No	531	2.1				(425)
Iran	Isolated anti-HBc	11		0	Artus real-time		(425)
Germany	No	5,305	1.5				(426)
Germany	No	65		7.7	Nested	100	(426)
USA	Isolated anti-HBc	107		3.7	Branched	50	(427)
UK	Isolated anti-HBc	151		4.0	Nested	100-400	(428)
Mexico	Blood donor	11,240	1.9				(429)
Mexico	Isolated anti-HBc	158		8.2	Nested	30-300	(429)
Germany	Isolated anti-HBc	545		8.1	Real-time	50-100	(430)
India	Isolated anti-HBc	171		22.8	Nested		(431)
Lebanon	Blood donor	5,511	3.7				(432)
Lebanon	Isolated anti-HBc	203		5.4	nested		(432)
Italy	Outpatient	6,544	1.8				(433)
Italy	Isolated anti-HBc	119		4.2	nested	100	(433)
Korea		17,677	8.9				(405)
Korea	Blood donor	12,461	1.2	<0.001	Cobas AmpliScreen	20	(406)
Korea	Isolated anti-HBc	230		1.7	Cobas Ampli/Taqman	4-12 IU	(405)
Korea	Isolated anti-HBc	153		0.65	Cobas AmpliScreen	20	(406)
Hong Kong	Blood donor	13,011		0.12	Cobas Taqscreen	3.7	(434)
India	Blood donor	2,175	7.0	1.4	nested	100	(404)
India	Isolated anti-HBc	153		12.4	nested	100	(404)
Indonesia	Blood donor	309	21.4	8.1	Nested	6	(382)
Indonesia	Isolated anti-HBc	66		28.8	Nested	6	(382)
Turkey	Blood donor	12,858	5.1	0.046	Real-time	19	(435)
Turkey	Isolated anti-HBc	658		0.9	Real-time	19	(435)

Table 1.13 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	No	85		0	Single	200	(436)
Spain	No	176		0	Single	200	(396)
Spain	No	520	27.7				(437)
Spain	Isolated anti-HBc	144		0.7	COBAS Taqman	100	(437)
France	No	160		0.6	Single	200	(438)
Netherlands	No	93		4	Single	200	(378)
Brazil	No	159		5	Nested	100	(439)
USA	No	179		10	TMA	15	(440)
USA	No	40		10	Single	200	(441)
USA	No	909		1.3	10 sample pools Real-time PCR	NA	(442)
USA	Isolated anti-HBc	400		2	COBAS	200 cp	(393)
Brazil	No	101		16	Single	100	(443)
Italy	No	86		20	Single	100	(392)
France	No	30		37	Nested	350	(444)
Switzerland	No	57		89	Nested	100	(445)
Netherlands	No	191		4.7	Nested	50	(374)
France	No	383	12			40	(446)
France	Isolated anti-HBc	48		4.2	Abbott real-time		(446)
France	No	2,185	17				(414)
Iran	No	106	20.8				(447)
Iran	Isolated anti-HBc	22		13.6	Artus real-time		(447)
Brazil	No	43	26				(148)
Brazil	Anti-HBc positive	43		14	Real-time PCR	100 cp	(148)
Thailand	No	140	20				(407)
Côte d'Ivoire	No	495	42				(448)
Côte d'Ivoire	Isolated anti-HBc	209		10	COBAS	35 cp	(448)
Taiwan	No	2,351	17.5				(449)
Taiwan	Isolated anti-HBc	277		8.3	Real-time PCR	1,000 cp	(449)