CHAPTER 4

Discussion

Of 56 HBV/HIV co-infected pregnant included in the 2 sub-studies, 44 full-genomes and 5 partial genomes of HBV isolated from 49 HBV/HIV co-infected pregnant women could be obtained. HBV DNA was extracted from plasma of pregnant women with HBV DNA levels ranging from 3.46-8.72 $\log_{10}$ IU/mL using the automated Abbott m2000sp instrument (Abbott Molecular Inc., IL, USA). Seven samples could not be amplified, despite HBV DNA levels range between 3.53 - 5.49 $\log_{10}$ IU/mL. The reason of amplification failure could be due to samples stability over time or mutations in the region of primers binding.

Phylogenetic analysis of HBV sequences showed that HBV genotype C (90%) and B (8%) were predominant, which is consistent with another report describing commonly HBV genotypes found in Thailand [115]. This study has also identified one recombinant G/C recombinant rarely found in Thailand [112].

High maternal HBV DNA levels and HBeAg positivity, a marker of high HBV replication, are major risk factors for perinatal transmission of HBV [22, 23]. However, not all women with high viral load transmit HBV to their babies, diversity of HBV may also lead to perinatal HBV transmission [155]. To characterize other virological factors that may be associated with perinatal transmission, this study assessed HBV genetic diversity and mutation patterns in transmitting- and non-transmitting pregnant women [24] matched on maternal HBV viral load and HBeAg.

Genetic diversity of HBV was assessed by three parameters: Shannon entropy, the genetic distance, the ratio of non-synonymous to synonymous mutations (dN/dS). The average overall entropy score was higher in transmitting group than non-transmitting group. Analysis of entropy score according to viral regions showed that virus genetic diversity was higher in transmitting women than in non- transmitting women in *preS/S* and *pol* regions. *PreS/S* regions undergo selective immune pressure to escape host immune response [156] and *pol* region due to an overlap of *pol* and *preS/S* may also undergo selective pressure. However, it is unclear why genetic diversity of *preS/S* and *pol* regions is higher in transmitting women since women in both groups had similar CD4 cell counts and were matched on HBV DNA level and HBeAg. Mean genetic distances between whole genomes were higher in transmitting- than those in non-transmitting women. This may be due to the fact that transmitting women were infected with genotype C or B while non-transmitting women were infected with only genotype C. The average ratio of non-synonymous to synonymous mutations (dN/dS) for all regions was lower than 1, indicating that there was no selective pressure in all regions of HBV and did not differ between transmitting- and non-transmitting women. The absence of pressure may be due to co-infection with HIV. One hypothesis is that HIV-1 infection by increasing the expression of programmed death-1 (PD-1) on CD4+ and CD8+ T-cells leads to exhaustion of CD8+ T-cell activity [119].

According to these results, the genetic diversity of only genotypes C was analyzed to exclude the effect of different HBV genotypes. Shannon entropy score and mean genetic distance results were higher in non-transmitting group than transmitting group, which differ from results when all genotypes were analyzed. Analysis of genetic diversity of HBV genotype B in relationship with perinatal transmission of HBV will require conducting studies in a larger population.

Recently, differences of HBV diversity between genotype B and C during perinatal transmission were demonstrated by Wu, *et al*. They found that HBV genotype C had a higher substitution rate than genotype B [157]. However, the genotype-related differences in the perinatal transmission of HBV remain unknown. Genotype may have a strong effect on mode of transmission and outcome. Livingston, *et al*. suggested that perinatal transmission of HBV is common in Southeast Asia, where genotype C is the

predominant HBV genotype. Thus, genotype C may be responsible for most perinatal transmission [158]. Also, there was a strong association between the presumed mode of infection and HBV genotypes ($P < 0.001$). Genotypes C (49%) and B (31%) were predominant among patients with maternal-infant transmission [159].

Analysis of all mutations to identify mutation patterns potentially associated with perinatal transmission of HBV was conducted in the present study. In BCP/PC region, a mixture of mutations has been found in 8 of 9 transmitting- and all non-transmitting women but no specific mutation pattern was found. Interestingly, the double A1762T/G1764A mutations in basal core promoter known to be associated with decreased production of HBeAg were identified in 3 non-transmitting pregnant women; all were infected with genotype C and had high HBV DNA level (two HBeAg negative- and one HBeAg positive- non-transmitting pregnant women). In precore region, the G1896A mutation, known to completely abolish the production of HBeAg, was detected in one transmitting mother HBeAg positive (genotype B, HBV DNA was 3.61 $\log_{10}$ IU/mL). In preS/S region, there were more mutations in preS1 region, in particular amino acid position at 73, than in preS2 and S regions but no specific mutation pattern was found. Two transmitting pregnant women were infected with HBV genotype B and had multiple mutations in S region. For *pol* region, more RT mutations in non-transmitting pregnant women than in transmitting pregnant women but no specific mutation pattern was found. For X region, there were 30 amino acid position out of 154 at which mutations have been observed but no specific mutation pattern was found. Mutations were observed in 30 amino acids. Mutations at position 30 were found in 3 out of 9 transmitting pregnant women all were I30L. In 9 of 17 non-transmitting pregnant women, I30V was found in 5 and I30L found in 4. Although this study have observed several mutations in all four regions of HBV but were unable to identify general mutation patterns associated with perinatal transmission of HBV. Strikingly, only one transmitting mother had mutations in the "a" determinant and this woman had low HBV viral load of 3.61 $\log_{10}$ IU/mL, confirming previous report by Khamduang [24]. Although no specific patterns could be identified, these results suggest that mutations outside the "a" determinant may be involved in perinatal transmission of HBV. Indeed, preS1 contains functionally important regions involved in the attachment site of human hepatocytes [160, 161] ,and virus-neutralizing epitopes [161]

and mutations in these regions may modify the transmissibility of HBV [155]. In this study, none of the mutations, P110S (*preS1*), P36L (*preS2*) and T473C (*S*), proposed to be related with intrauterine transmission [14] were found. Similarly, this study did not observe the 2 mutations described Cheng *et al*. not to favor infection, A90V (*preS1*) and A184V (*S*) [14].

This study also analyzed the relationship between HBV genetic diversity and absence of plasma HBeAg in high HBV DNA level among HBV/HIV co-infected pregnant women. HBV circulating in HBeAg-positive women had a lower diversity at amino acid level than those in HBeAg-negative women. This may be due to a longer duration of HBV infection and accumulation of more mutations in HBeAg negative women. HBeAg-positive women had lower mean genetic distance among viral whole genome than HBeAg-negative women. Since 1 of 6 HBeAg-negative women infected with a recombinant HBV genotype G/C, this may result in a high genetic distance score in this group. Other studies have shown that high HBV nucleotide diversity in HBeAg negative infections as compared to HBeAg positive infections [150, 162-166]. This may due to more rapid different evolutionary rates in HBeAg negative infections [167]. There was no difference in dN/dS ratio between the viruses in 2 groups. This may related to HIV infection as mentioned above.

The genetic diversity of only genotype C was also analyzed to exclude the effect of different HBV genotypes. Shannon entropy score and mean genetic distance results were higher in HBeAg-positive women than HBeAg-negative women, which differ to the earlier results when all genotypes were analyzed. HBV genotype C circulating in HBeAg-positive women had a greater diversity than those in HBeAg-negative women. This may be due to high viral replication rate results in high diversity. Genetic diversity of HBV genotype B in HBeAg- negative women and HBeAg- positive women should be further studied to clarify the results. Due to HBV genotype may be responsible for difference in natural history of chronic HBV infection. Chu, *et al.* suggested that HBV genotypes may be associated with HBeAg seroconversion. HBV genotype B patients who were HBeAg positive were earlier spontaneous HBeAg seroconversion compared with HBV genotype C patients [168]. Moreover, one study

reported that G1896A mutation, which abrogates HBeAg production, is more frequently found in patients with HBV genotype B than those with genotype C [169].

This study has some limitations. First, amplification of HBV genome in HBeAg negative women was not always successful (40%). Second, this study assessed HBV genetic diversity using the full-length genome sequence for each woman and not clones. Thus, genetic diversity was analyzed between major viral populations of each infected women. Third, this study was unable to identify specific mutation patterns associated perinatal transmission using consensus sequencing data. More sophisticated analysis tools may help identify specific mutation patterns. Also, quasispecies analysis may show specific mutation in minor viral population. Fourth, this was a cross-sectional study, analysis of samples collected longitudinally may provide more information about virus evolution and selection process.