CHAPTER 2

METHODS AND RESULTS

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

2.1.1 Introduction

More than 370 million people worldwide are infected with hepatitis B virus (HBV) (450) and 75% of the world's HBV carriers reside in Asia, some of whom develop severe liver diseases, e.g. cirrhosis or hepatocellular carcinoma (HCC) (8). HBV infection results in 1 million deaths annually (450). In East-Asia and Pacific, a highly endemic area for chronic HBV infection, HBV mother-to-child transmission (MTCT) remains a major source of chronic infection (278). Without any intervention, the overall prevalence of perinatal HBV transmission is 35-50% (451). This prevalence is ~90% in children born to mothers positive for hepatitis B e antigen (HBeAg) (9). Immunoprophylaxis with hepatitis B (HB) vaccine and/or HB immunoglobulin decreases this prevalence to 10-15% in this high risk group (452).

In Thailand, one of the highly HBV endemic countries, the Ministry of Public Health (MOPH) has integrated HB vaccination of newborns into the national expanded program on immunization (EPI) in 1992. This program has successfully decreased the rate of positive HBV surface antigen (HBsAg) in children from 3.4% to 0.7%, irrespective of maternal HBeAg status (17). In Thailand as well in other Asian

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countries, a percentage of children have acquired HBV infection from their mothers despite administration of HB vaccines. These MTCT could be associated to either the occurrence of mutations on HBsAg (47, 119, 125), high maternal HBV DNA load (453) or the presence of HBeAg (365, 453, 454).

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

2.1.2 Methods

2.1.2.1 Patients

This study included HIV-infected pregnant women and their children who participated in two Perinatal HIV Prevention Trials in Thailand (PHPT-1 NCT00386230 (455) and PHPT-2 NCT00398684 (456)), assessing the efficacy of short duration of zidovudine (ZDV) or single-dose nevirapine plus zidovudine regimens, respectively, to prevent perinatal transmission of HIV. Breast-feeding was not recommended in these two trials. Blood samples were collected from women during pregnancy and children at birth, 6 weeks, 4, 6 and 12 months of age. Informed and written consent were obtained and the study performed according to the World

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Medical Association Declaration of Helsinki and approved by the ethic committees of Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

2.1.2.2 HBV Markers and HBV DNA quantification

HBsAg and HBeAg were tested using an enzyme immune assay (ETI-MAK and ETI-EBK, DiaSorin, Salluggia, Italy) according to the manufacturer's recommendations. HBV DNA was quantified using the Cobas Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, N.J., USA, lower limit of detection: 60 IU/mL) or Abbott real-time HBV DNA™ assay (Abbott laboratories, Rungis, France, lower limit of detection: 15 IU/mL). HBV infection in children was determined by the presence of HBsAg and/or HBV DNA at least once during 2–6 months of age.

2.1.2.3 HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 200 µl of plasma using QIAamp kit (QIAgen, Valencia, CA., USA). Ten µl of extract was used as template for the polymerase chain reaction (PCR) amplification as described by Villeneuve et.al. with slight modifications (457). Briefly, the first-round PCR was performed in a 59 µl volume using Platinum PCR SuperMix High Fidelity primer and primers: Pol1M and Pol2M, yielding fragments of 1,010 bp. PCR conditions included initial 2 min denaturation step at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 48°C, and 3 min at 68°C for. Ten microliters were then used for second-round PCR with Pol3M and Pol4M, which yields an 808 bp amplified fragment. PCR conditions were an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 40 sec at 94°C, 1 min, at

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55°C and 3 min at 68°C. Amplicons were checked on a 1% agarose gelelectrophoresis. These amplicons sequenced using the pol3M and pol4M primers, and the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA), Sequences were analyzed using the Bioedit software (http://www. mbio.ncsu.edu/bioedit/bioedit.html). Mutations on *surface (S)* and *polymerase (Pol)* gene were then analyzed for polymorphisms and mutations known to be associated with vaccine escape through comparison with wild-type reference sequences of similar genotype.

2.1.2.4 HBV cloning and sequencing

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

2.1.2.5 Determination of HBV genotyping and serotyping

HBV genotype was identified by phylogenetic analysis of *S* and *Pol* gene sequences. Briefly, sequences were aligned with published *S* and *Pol* gene sequences of various HBV genotypes available in GenBank using clustalW software.

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Phylogenetic trees were constructed using neighbor-joining method and genetic distances calculated using the Kimura two-parameter method, as implemented in the software MEGA (458). Bootstrap analysis with 100 simulations was used to test the reliability of branching. HBV serotype was deduced from amino acids residuals at codons 122, 126, 127, 160, 168, 177 and 178 of the S gene (48, 459).

Reference sequences used in this study were obtained from GenBank database: X70185, V00866, S50225, X51970, M57663 (genotype A); D00331, M54923, D00329, D00330 (genotype B); M38636, X14193, M12906, D12980, D00630, L08805, X52939, X01587, M38454, V00867, X75665, X75656 (genotype C); M32138, X59795, X02496, X72702, X65257, X65258, X65259, X68292 (genotype D); X75664, X75657 (genotype E); X75663, X75658 (genotype F); AF160501, AB064310, AF405706, AB056513 (genotype G).

2.1.2.6 Statistical analysis

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

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2.1.3 Results

2.1.3.1 Patient characteristics

Among 3,467 HIV-infected pregnant women who participated in 2 clinical trials in Thailand, median age was 25.5 (IQR: 22.4-29.1) years old. Most of them enrolled in eastern, northern, and central part of Thailand (33%, 29%, and 21%, respectively). Median CD4+ and CD8+ T-cell count were 368 (IQR: 240-521) and 904 (IQR: 680-1,190) cells/µL, respectively. Median ALT was 15 (IQR: 10-22), up to 95% of patients had normal ALT level (<40 IU/L). Median HIV RNA level was 3.98 (IQR: 3.35-4.58) copies/mL. Four percent of women had antibodies against hepatitis C virus (anti-HCV) (Table 2.1).

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Table 2.1 Baseline characteristics of HIV-infected pregnant women

Characteristics	N	Median (IQR*) or n (%)
Age at enrollment; years	3,466	25.5 (22.4-29.1)
≤20 years		353 (10)
>20-30 years		2,391 (69)
>30-40 years		690 (20)
>40 years		32 (1)
Missing	1	
Body weight; kgs	1,432	55 (50-60.3)
<40 kgs		10 (1)
40-49 kgs		314 (22)
50 -59 kgs		718 (50)
60-69 kgs		305 (21)
70-79 kgs		66 (5)
>80 kgs		19 (1)
Missing data	2,035	
Region of enrollment	3,467	
Central		712 (21)
Eastern		1,138 (33)
Northern		1,017 (29)
North-eastern		284 (8)
Southern		148 (4)
Western		168 (5)
Region of origin	2,017	
Central		435 (22)
Eastern		303 (15)
Northern		410 (20)
North-eastern		643 (32)
Southern		86 (4)
Western		86 (4)
Immigrant		54 (3)
missing data	1,450	
Project enrollment	3,467	
PHPT-1		1,439 (41.5)
PHPT-2		2,028 (58.5)

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

* IQR: Interquartile range

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2.1.3.2 Prevalence of HBsAg positivity in HIV-1 infected pregnant women

Of 3,312 women with clearly identified HBV status, 245 (7.4%; 95%CI, 6.5-8.3) were HBsAg positive; of whom half were HBeAg positive (Figure 2.1). Median HBV viral load was 4.37 (IQR: 1.83-7.63) IU/mL. Baseline characteristics between HBsAg-positive- and HBsAg-negative pregnant women were not different, except higher ALT level and lower CD4+ T-cells count in HBsAg-positive pregnant women (Table 2.2). No correlation was observed between HBV DNA and HIV RNA levels (P=0.49).

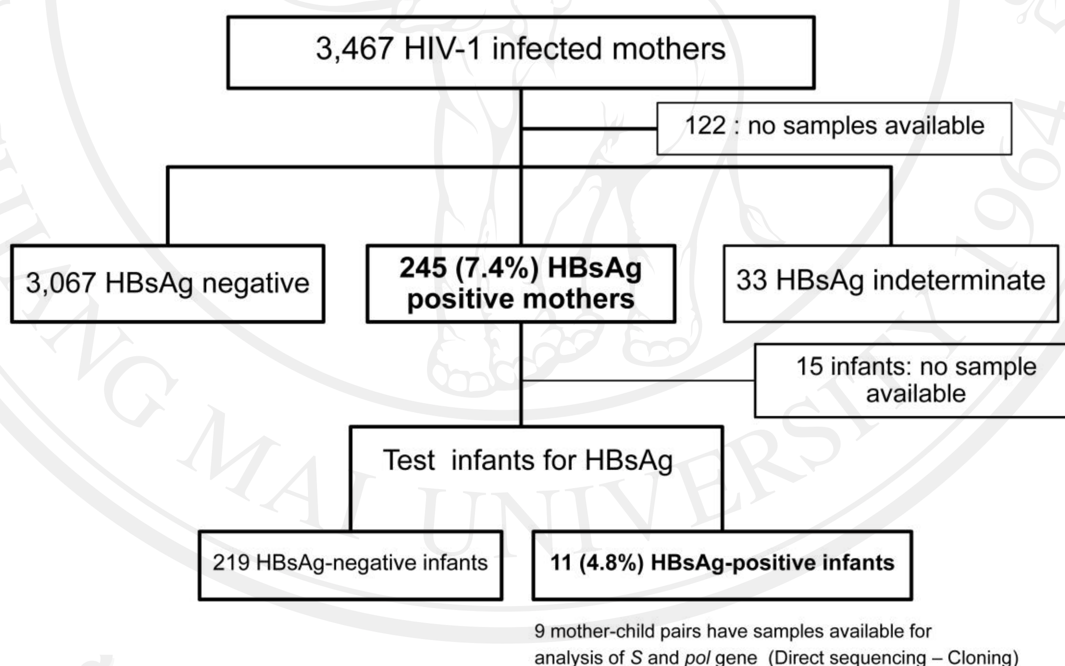

Figure 2.1 Overall study diagram

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Table 2.2 Characteristics of HBsAg-positive- and HBsAg-negative pregnant women

Characteristics	HBsAg positive mothers		HBsAg negative mothers		P-value
	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)	
Age at enrollment; years	245	25.4 (22.0-29.0)	3,067	25.5 (22.4-29.1)	0.62
≤20 years; %		25 (10)		311 (10)	
>20-30 years; %		167 (68)		2,122 (69)	
>30-40 years; %		51 (21)		605 (20)	
>40 years; %		2 (1)		29 (1)	
Body weight; kgs	115	54.5 (50-59)	1,308	55 (50-60.3)	0.52
<40 kgs; %		2 (2)		8 (1)	
40-49.9 kgs; %		25 (22)		286 (22)	
50 -59.9 kgs; %		62 (54)		653 (50)	
60-69.9 kgs; %		21 (18)		281 (21)	
70-79.9 kgs; %		4 (3)		62 (5)	
>80 kgs; %		1 (1)		18 (1)	
Region of enrollment	245		3,067		
Central; %		50 (20)		632 (21)	
Eastern; %		80 (33)		1,007 (33)	
Northern; %		78 (32)		895 (29)	
North-eastern; %		20 (8)		250 (8)	
Southern; %		8 (3)		134 (4)	
Western; %		9 (4)		149 (5)	
Region of origin	130		1,742		
Central; %		25 (19)		378 (22)	
Eastern; %		20 (15)		262 (15)	
Northern; %		28 (22)		355 (20)	
North-eastern; %		43 (33)		555 (32)	
Southern; %		5 (4)		78 (4)	
Western; %		4 (3)		77 (4)	
Immigrant; %		5 (4)		37 (2)	
Project enrollment	245		3,067		0.23
PHPT-1		115 (47)		1,315 (43)	
PHPT-2		130 (53)		1,752 (57)	
CD4 T-cell count; cells/μL	242	343 (219-462)	2,991	370 (243-524)	**0.03**
<200 cells/μL; %		51 (21)		535 (18)	
200-499 cells/μL; %		133 (55)		1,606 (54)	
≥500 cells/μL; %		58 (24)		850 (28)	
CD8 T-cell count; cells/μL	220	855 (626-1,190)	2,674	919 (684-1,186)	0.13
<500 cells/μL; %		30 (14)		236 (9)	
500-1000 cells/μL; %		108 (49)		1,358 (51)	
≥1000 cells/μL; %		82 (27)		1,080 (40)	
ALT; IU/L	240	17 (12-26)	3,004	15 (10-22)	**<0.001**
<40 IU/L; %		216 (90)		2,868 (95)	
40-79 IU/L; %		19 (8)		113 (4)	
≥80 IU/L; %		5 (2)		23 (1)	
HIV viral load; log_{10} copies/mL	242	3.96 (3.36-4.59)	3,044	3.99 (3.37-4.58)	0.90
Undetectable		5 (2)		67 (2)	
log_{10} 1-1.99 copies/mL		5 (2)		37 (1)	
log_{10} 2-2.99 copies/mL		25 (10)		335 (11)	

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$\log_{10}$ 3-3.99 copies/mL		88 (36)		1,091 (39)	
$\log_{10}$ 4-4.99 copies/mL		9 (40)		1,207 (40)	
$\geq \log_{10}$ 5 copies/mL		23 (10)		307 (10)	
anti-HCV positive	129	2 (2)	1,727	77 (4)	0.17
HBV viral load; $\log_{10}$ IU/mL	237	4.37 (1.83-7.63)			
undetectable		35 (15)			
$\log_{10}$ 1-1.99 IU/mL		41 (17)			
$\log_{10}$ 2-2.99 IU/mL		21 (9)			
$\log_{10}$ 3-3.99 IU/mL		18 (8)			
$\log_{10}$ 4-4.99 IU/mL		7 (3)			
$\log_{10}$ 5-5.99 IU/mL		7 (3)			
$\log_{10}$ 6-6.99 IU/mL		18 (8)			
$\log_{10}$ 7-7.99 IU/mL		63 (26)			
$\geq \log_{10}$ 8 IU/mL		27 (11)			
HBeAg positive	169	87 (51)			

2.1.3.3 Prevalence of perinatal HBV transmission

Of 245 infants born to HBV-HIV co-infected women, 230 had samples available. Of these, 11 (4.8%; 95%CI, 2.4-8.4) were found infected with HBV, but not with HIV despite administration of HBV vaccination (Figure 2.1). Complete series of samples were available for 9 mother-child pairs, the other two infant samples could not be amplified because low amount of HBV DNA. Virological assessments of 9 HBV transmitting mother-HBV infected child pairs as well as infant HB immunization are described in Table 2.3. Seven pairs were infected with HBV genotype C, while others two were infected with genotype B (Figure 2.2).

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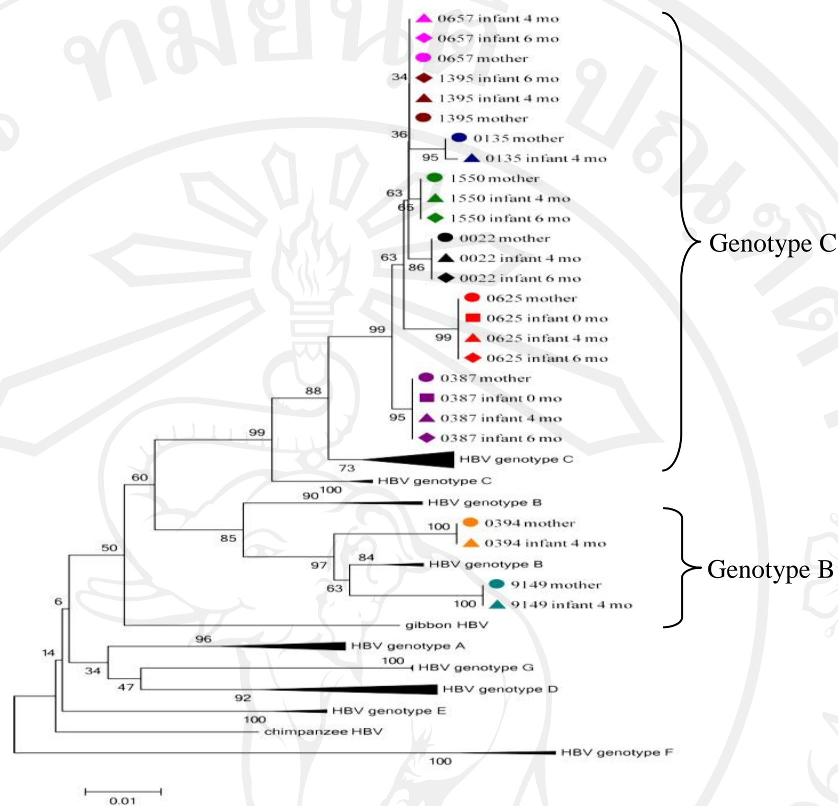
Figure 2.2 Phylogenetic analysis of 9 mother-child pairs



2.1.3.4 Patterns of HBV mother-to-child transmission

Analysis of direct sequences of *Pol* and *S* genes showed no known vaccine escape mutation. Of the 9 infants infected with HBV, 3 were infected with wild-type HBV .0387, 0394 and 0657) and interestingly were born to mothers with high level of HBV DNA (>6.50 log_{10} IU/mL). Three infants had mutations on S gene which was no present in maternal viruses: two infants (0022 and 1395) had lysine substitution by arginine (sK122R) and one (no.0625) had isoleucine substitution by threonine (sI126T). The last 3 infants were infected with HBV variants present in mothers, which may not be the predominant quasispeciestrasI126IT, sI126M+P127S, and sT131N+M133T+T140I+S204R) (Table 2.4). Interestingly, the sS53L and sS210N were found in all mother-child pairs infected with genotype C.

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Table 2.3 HBV DNA load and infant HBV prophylaxis among 11 HBV transmitting mother-child pairs.

Pair	Maternal HBeAg	Maternal HBV load (log_{10} IU/mL)	Infant prophylaxis		Infant HBV load (log_{10} IU/mL)		
		Before delivery	Vaccination (months)	HBIg	Birth – 10 days	4 months	6 months
0387	+	7.84	0, 1, 5, 6	Yes	4.37	8.18	8.93
0625	+	7.92	0, 2, 6	Yes	2.60	5.51	7.90
1550	+	8.04	0, 1, 6	No	1.58	7.12	7.18
0022	+	7.83	2, 4	No	2.48	2.19	7.17
0657	+	7.85	0, 2, 6	No	Und	7.61	8.76
1395	+	8.24	0, 1, 6, 12	No	Und	7.42	7.31
9149	+	3.61	0, 1, 6, 8	No	Und	2.91	Und
0394	-	6.51	0, 1, 6	No	Und	3.08	1.48
0135	-	2.28	Unknown*	Unknown	Und	5.63	NA
0349	+	6.18	0, 1, 6	No	2.52(at 6 wk)	NA	NA
0175	-	<1.58	Unknown	Unknown	2.95 (at 6 wk)	NA	NA

Und: below detectable level; NA: not available due to insufficiency of plasma samples

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

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Table 2.4 Pattern of HBV transmission, genotype, and mutations observed by direct sequencing of S gene among 9 HBV transmitting mother-child pairs.

Pairs	Pattern	Maternal HBeAg	HBV load	HBV genotype	mutations in maternal virus	mutations in infant virus		
						Birth – 10 days	4 months	6 months
0387	1	+	7.84	C	None	None	None	None
0625	2	+	7.92	C	None	None	sI126I/T	sI126T
0657	1	+	7.85	C	None	NA	None	None
0394	1	-	6.51	B	None	NA	None	NA
0022	2	+	7.83	C	None	NA	sK122R	sK122R
1395	2	+	8.24	C	None	NA	sK122R	sK122R
1550	3	+	8.04	C	sI126T	NA	sI126T	sI126T
0135	3	-	2.28	C	sI126I/M, sP127A/S	NA	sI126M, sP127S	NA
9149	3	+	3.61	B	sT131N, sM133M/T, sT140I, sS204S/R	NA	sT131N, sM133T, sT140I, sS204R	NA

NA: not available due to undetectable HBV DNA level or unsuccessful amplification of region of interest

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Analysis of clone sequences showed that in mothers with high HBV DNA level, the predominant HBV was wild-type and this wild type HBV was also predominant in samples of 3 infants born to as well as their mother'samples (Figure 2.3A, 2.3B, 2.3C). In one infant no.0387 (Figure 2.3A), sG145R, a known vaccine-escape HBV mutant, was present in 2 of 15 maternal clones but this mutant was not transmitted to her baby. In the group of children infected with an *S* mutant HBV, analysis of maternal and infant clones showed that a minor mutant HBV quasispecies was transmitted to the child. For pairs no.0022 and 1395 (Figure 2.3D, 2.3E), sK122R mutation was present in 1 of 65 clones and 2 of 67 maternal clones, respectively, suggesting the transmission, despite the administration of vaccine against HBV, of this minor maternal HBV variant which progressively became predominant in infected children. Inference of HBV serotype from sequence data of *S* gene indicate that HBV serotype *adrq+* was predominant in these 2 women, while the serotype *ayr* was rare. Analysis of children HBV clones showed an increase of the serotype *ayr* from 4 months to 6 months, while serotype *adrq+* had declined. For the pair no.0625 (Figure 2.3F), the sI126T variant corresponding to a predicted serotype *adrq+* was identified in 2 of 20 maternal clones and become predominantin infant's samples at 4 and 6 months, accounting for 41% and 76%, respectively.

In the third group of children infected with HBV variants already present in mothers and accounting for 20% or more of all maternal quasispecies, clonal analysis showed that these variants were the predominant viral population or can be detected in children. This for pair no.1550 (Figure 2.3G), HBV serotype *adrq+* (sI126T) was always predominant in both maternal and infant's samples. Interestingly, in the 2

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latter pairs (0625, 1550), women harbored the 2 variants, wild-type sI126I and sI126T, while only the sI126T variant was found in the child. This result indicates that amino acid substitution at position 126 may influence the escape of HBV to vaccine. Again, for no.0135 (Figure 2.3H), the variant sI126M+P127S was selected in infants 4 month-sample. Finally, the multiple-mutations HBV variant, sT131N+M133T+T140I+S204R, was selected in infant no.9149 (Figure 2.3I), though this variant was not observed in maternal samples likely to the low number of clone analyzed.

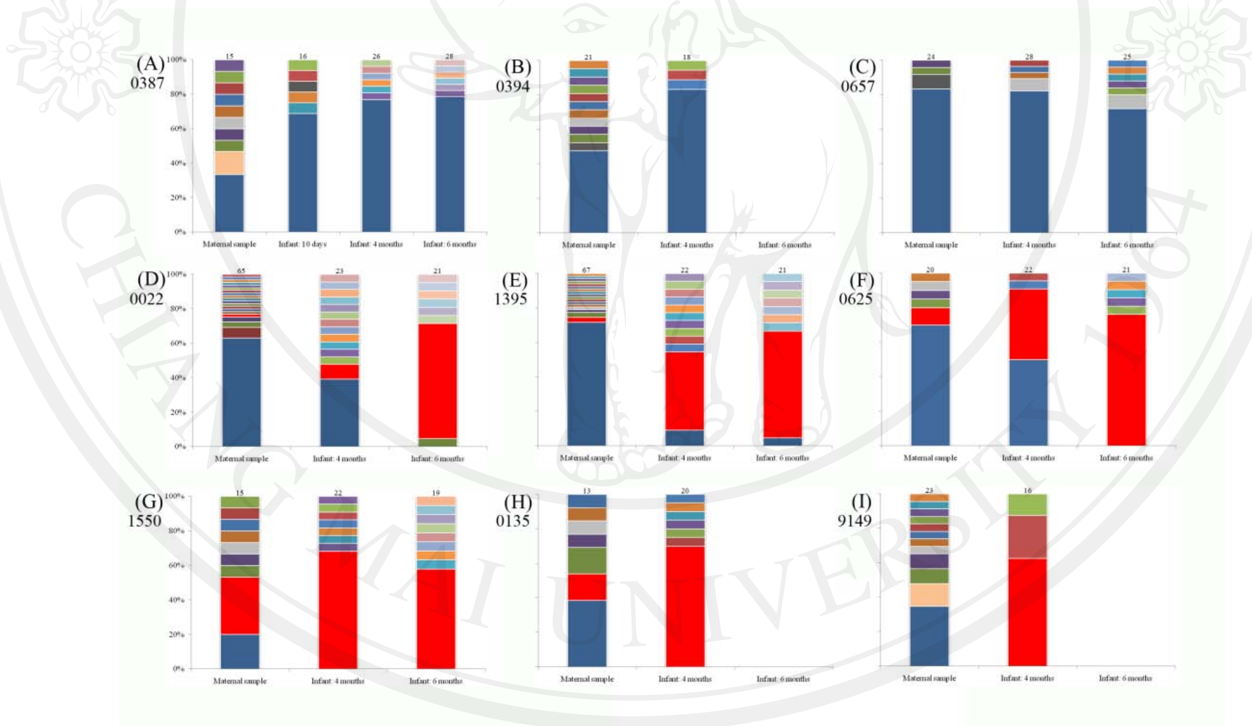

Figure 2.3 Evolution of the HBV quasispecies in 9 representative transmitting mother-child pairs. Numbers above each bar indicate number of clone analyzed. Numbers under the panel label indicate patient's code. For each pair, wild type variant is indicated in blue, the potential mutant escapes are indicated in red, other variants are indicated by other colors. Three possible patterns of HBV mother-to-child transmission are; 1) Transmission of wild-type variants from mothers with high level of HBV DNA (A, B, C), 2) transmission of maternal minor variants to their infants (D, E, F), and 3) the transmission of HBV variants already existing in mothers (G, H, I).

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2.1.4 Discussion and conclusion

We have assessed the prevalence of HBsAg carriers among a large number of HIV-1 infected pregnant women in Thailand and the rate of perinatal transmission of HBV in infants born to those found HBsAg positive. The prevalence of HBsAg positive women was 7.4% (95%CI, 6.5-8.3); of whom 4.8% (11) women transmitted HBV to their offspring despite having received vaccine and/or immununoglobulins. This study indicate that vaccine/immunoglobulin failure may result from transmission of either wild-type variants from mothers with high level of HBV DNA (3 of 9 pairs), maternal HBV minority variants (3 of 9 pairs), or HBV variants present and accounting for 20% or more of maternal viral population (variants detected by direct sequencing). We have also identified several HBsAg mutations, sK122R, sI126IT, sI126M+P127S, and sT131N+M133T+T140I+S204R which significance on vaccine/ immununoglobulins escape is unknown.

We observed a prevalence of positive HBsAg in HIV-infected pregnant women similar to that reported in HIV-infected Thai adults ,7.4% vs. 8.7% (172), or in HIV-uninfected pregnant women between 4-8% in studies conducted in Thailand (460-462) and 9-10% in other South-East Asia countries (463). This is likely due to the fact that in highly endemic areas most HBV infections are acquired very early in life before acquisition of HIV.

The rate of perinatal transmission of HBV found in this study is also consistent with rates observed in HIV-uninfected population worldwide (3-5%) (464) and survey studies showing that the prevalence of HBsAg in schoolchildren aged

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between 6 months to 18 years was 2.3-4.5% (20, 465). Phylogenetic analysis of *S* gene sequences indicated that 78% of 9 Infants were infected with HBV genotype C, while 2 were infected with HBV genotype B; these HBV genotype frequencies are similar to other studies in Thailand (20, 67). Due to the number of HBV transmission, we were unable to assess whether there was a higher risk of perinatal transmission with genotype C.

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

We have identified few mutations in infant samples; i.e. sI126T (2 infants); sI126M1+sP127S (1 infant); sT131N+sM133T+sT140I+sS204R (1 infant) that locate in the "a" determinant of HBsAg. Some mutations have been reported in other

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studies; e.g. sI126T was observed in studies in India (135), Korea (129) and Taiwan (115), the sP120S in Singapore (130), sP120S+P127S in Italy (466), and sM133T in Thailand (20), as well. Predicted 3D structure indicated that amino acid substitution at position 126 involved the largest change in chemical properties, likely to cause structural changes in the HBsAg (467). Change of amino acid in the "a' determinant region may be associated with HB vaccine escape (46). These results suggest that the mutations observed at positions in a well conserved region may favour the virus to escape neutralizing antibodies.

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

The occurrence of *in utero* HBV infection is usually considered as a very rare event as compared to infection at birth and may happen when chronically HBV infected mothers have high maternal HBV DNA (365, 468, 469). In this study, we have demonstrated that 3 mothers with high HBV DNA level (>6.5 log IU/mL) transmitted HBV to their offspring. These results are in favour of the occurrence of transmission during pregnancy before immunoglobulin and vaccine can exert their activity. Immunoprophylaxis, either vaccination administration alone or plus hepatitis B immune globulin, may not be effective to prevent the transmission if infants are infected either in utero or through extremely exposure to blood or contaminated fluids at or around birth (327). Furthermore, although one woman had HBV harboring sG145R mutation, well-known vaccine escape mutant, only wild type virus was

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transmitted. This probably due to the use of recent licensed HBV vaccines able to prevent the infection with sG145R HBV mutant, which had already been demonstrated in chimpanzees (470).

Indeed, HBV infection by vaccine escape mutants does not account for the majority of children who had immunoprophylaxis failure, only 5-39% in 3 previous studies (123, 131, 471). The maternally pre-existed *S* gene mutant seems to be potential predictors of vertical breakthrough infection (469). Other possible causes of unsuccessful neonate vaccination include trans-placental transmission which is related to high level of serum HBV DNA in pregnant women, trans-placental leakage of maternal blood, amniocentesis, and polymorphisms in some cytokine genes or human leukocyte antigen (160). Breast-feeding transmission may influence to the transmission of HBV from mother to child, however, with appropriate immunoprophylaxis, breast-feeding does not pose additional risk for the HBV transmission (221). Anyway, in this study, formula feeding was recommended to all women because they are all infected with HIV.

In conclusion, although HBV vaccine has proved very efficacious in the prevention of mother-to-child transmission of HBV this study confirms that there is still a residual HBV transmission for which different mechanisms may account for. Whether perinatal HBV transmission occurs more frequently in infants born to HIV co-infected women remain to be determined. A systematic virological evaluation of HBV variants selected in HBV infected infants despite active immunization, and their mothers, is needed to further clarify the impact of these mutations on perinatal

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transmission of HBV. Also, the impact of variants identified in this study in the escape to HB vaccine needs further investigation. Understanding the causes of HB vaccine failures will help to develop new HBV vaccine appropriate for the many countries in HBV endemic area such as Thailand and other South-East Asian countries and also develop interventions to decrease perinatal transmission of HBV and accelerate the eradication of HBV infection.

2.1.5 Publications and presentations

- **Khamduang W,** Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Borkird T, Layangool P, Kamonpakorn N, Jitphiankha W, Kwanchaipanich R, Potchalongsin S, Lallemant M, Sirirungsi W, Goudeau A and the Program for HIV Prevention and Treatment (PHPT) group. Hepatitis B vaccine failure in offspring of women co-infected with human immunodeficiency virus and hepatitis B virus in Thailand. *Journal of Clinical Virology*. (Journal Impact factor 2011: 3.969) (Revised version was submitted)

Preliminary results of this works were presented in;

- **Khamduang W,** Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Transmission of Hepatitis B virus (HBV) minor variants in children born to HBV/HIV co-infected mothers. 5th Dominique Dormont International Conference, Mother-to-child transmitted

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viral diseases: from transmission to children care, 26-28th March, 2009, Paris, France, *Retrovirology* 2009; 6 (Suppl.1):O9 ***(O9, Oral presentation)***

- **Khamduang W**, Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Transmission of Hepatitis B virus (HBV) minor variants in children born to HBV/HIV co-infected mothers. 12th National AIDS Conference, 27-29th May, 2009, Bangkok, Thailand. ***(CP08, Poster presentation)***
- **Khamduang W,** Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Sureau C, Lallemant M, Sirirungsi W, Goudeau A and the Program for HIV Prevention and Treatment (PHPT) group. Hepatitis B Escape Mutants in Infants Born to Human Immunodeficiency Virus (HIV)-infected Mothers Co-infected with Hepatitis B Virus (HBV). The 21st Conference of the Asian Pacific Association for the study of the liver (APASL), 17-20th February, 2011, Bangkok, Thailand. ***(PP06.41, Poster presentation)***
- **Khamduang W.** Franco-Thai Highlight: Hepatitis B Vaccine Escape. The International Workshop on "Interdisciplinary Approach to the Management of HIV: A Model for other Infectious Diseases", 16–18th March, 2011, Chiang Mai, Thailand. ***(Oral presentation)***
- **Khamduang W,** Ngo-Giang-Huong N, Sirirungsi W, Chanta C, Karnchanamayul W, Ngampiyaskul C, Sirithadthamrong C, Hongsiriwon S, Kamonpakorn N, Watanayothin S, Jourdain G. Prevalence of hepatitis B virus (HBV) infection in infants born to HIV/HBV co-infected women and factors associated with vertical

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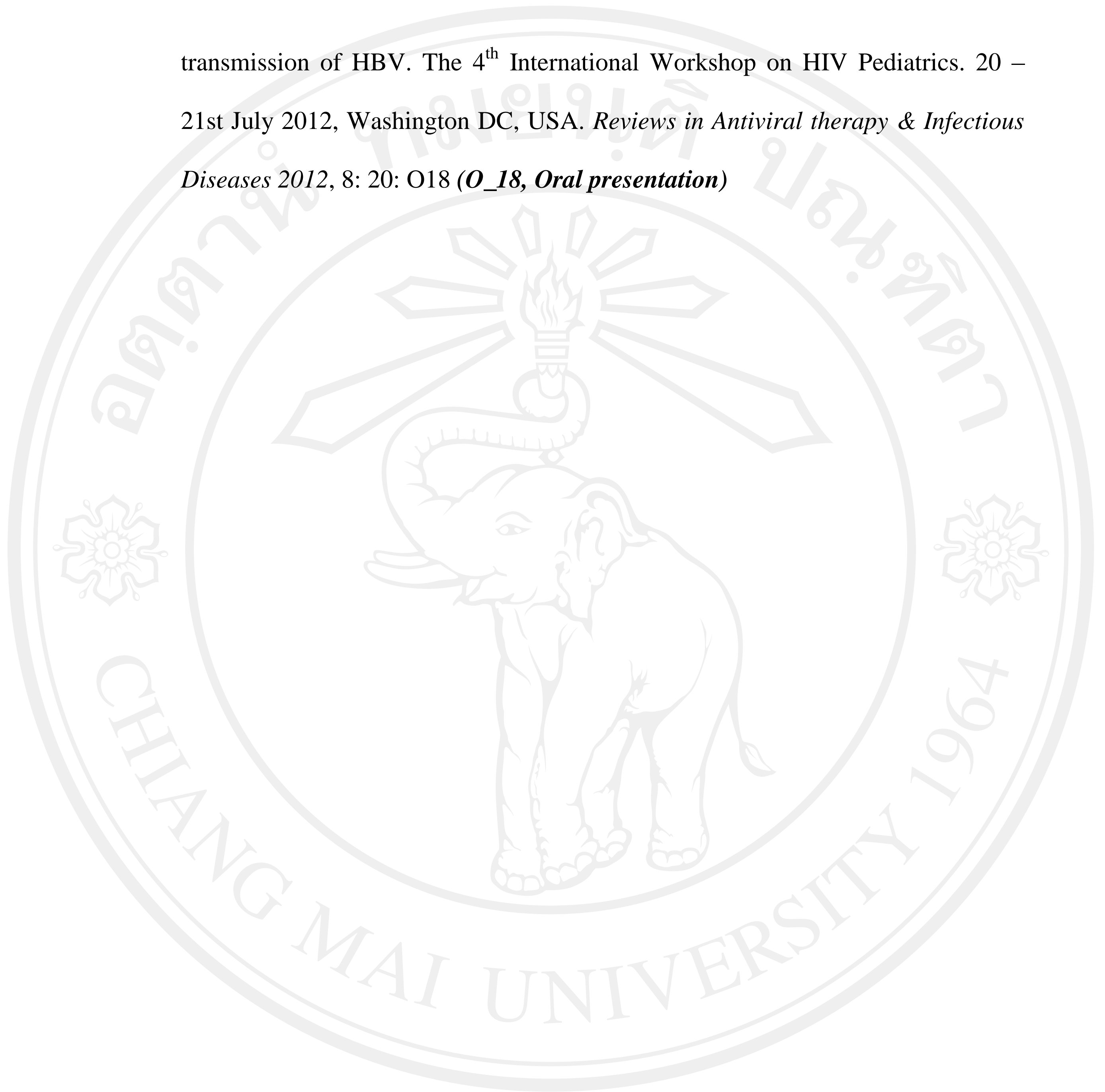
transmission of HBV. The 4th International Workshop on HIV Pediatrics. 20 – 21st July 2012, Washington DC, USA. *Reviews in Antiviral therapy & Infectious Diseases 2012*, 8: 20: O18 ***(O_18, Oral presentation)***



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

2.2.1 Introduction

The diagnosis of hepatitis B virus (HBV) infection is made primarily by detecting HBV surface antigen (HBsAg) in peripheral blood. However, an absence of HBsAg cannot exclude HBV infection. Indeed, antibodies directed against the core of hepatitis B virus (anti-HBc), marker of natural HBV infection, can be found in the absence of other serological markers (401). The clinical significance of isolated anti-HBc is unclear. The majority of individuals with isolated anti-HBc seem to be healthy with normal liver enzyme levels and with no sign of liver disease. Although little is known about its long term outcome, several studies have reported "isolated anti-HBc" serology patterns in patients with cirrhosis and hepatocellular carcinoma (HCC) (25, 376, 472), particularly in those co-infected with hepatitis C virus (HCV) (409, 473). Study in Taiwan showed that HIV-infected patients with isolated anti-HBc at baseline had significantly shorter survival than those with anti-HBs positive at baseline (420).

Whether subjects with isolated anti-HBc require vaccination against HBV remains controversial. Also, there is a growing concern that individuals with isolated anti-HBc are potentially infectious. Indeed, HBV transmission from isolated anti-HBc individuals has been reported following sexual contacts, blood transfusion (474), organ transplantation (475, 476), or during perinatal period (477, 478). Moreover, HBV Transmission of isolate anti-HBc blood has been demonstrated in chimpanzee model (479). The frequency of isolated anti-HBc relates directly to the prevalence of

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HBV infection in the population being tested. Among blood donors in geographic areas with low HBV prevalence, its prevalence is 0.4-4% (376, 401-403). Higher prevalence of isolated anti-HBc is commonly found in persons with chronic hepatitis C virus infection (409), HIV infection (411-413), or injection drug use (IDU) (407).

In HIV-infected patients, the prevalence of isolated anti-HBc has been found consistently higher than in HIV-uninfected patients (43 vs 27%, (408)) (17–81% vs 2–5%, (410-414). The reason of this increased prevalence of anti-HBc is unclear, maybe related to immune suppreesion. Moreover, reactivation of HBV has been observed in HIV-infected patients with isolated anti-HBc (480). Like in general population, ongoing HCV infection (410), history of injection drug use, numerous sex partners, and high HIV RNA levels (408) were factors associated with isolated anti-HBc in HIV-infected population.

Occult Hepatitis B virus infection, is currently defined as the presence of HBV-DNA in serum and/or in liver without detectable hepatitis B surface antigen (HBsAg), irrespective of other HBV serological markers (481). The proportion of occult HBV infection varies depending on the population studied and detection technique used (25); 4-14% in individuals with isolated anti-HBc (401, 482, 483), and 10-20% in endemic areas (484, 485). In HIV infected patients, the prevalence of occult HBV infection ranges between 0–89% and is much higher among individual with isolated anti-HBc (392). However, there is still very limited data available on occult HBV infection in HIV pregnant women and its impact on mother-to-child HBV transmission (25).

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

2.2.2 Materials and methods

2.2.2.1 Study population:

The study population was derived from HIV-infected pregnant women who participated in a clinical trial investigating the efficacy of zidovudine (ZDV) plus single dose nevirapine (NVP) to prevent HIV-1 mother-to-child transmission conducted between 2001 and 2003 in Thailand the NCT00398684 (456). Demographic, clinical and biological data were collected at enrolment in the study.

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

2.2.2.2 Sample collection

Maternal and infant blood samples were collected at entry and during study and plasma/sera were stored frozen at -70° or -20°C until use.

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2.2.2.3 Analysis of HBV infection markers

Women were screened for HBsAg using an enzyme immunoassay (EIA) of 250 pg/mL sensitivity (DiaSorin ETI-MAK-2, Salluggia, Italy). HBsAg-negative women were tested for anti-HBc (MonoLisa® anti-HBc PLUS) and anti-HBs (MonoLisa® anti-HBs PLUS, Bio-Rad laboratories, Marnes La Coquette, France). Women positive for both anti-HBc and anti-HBs antibodies are considered as having resolved HBV infection, those with isolated anti-HBc are considered as having acquired HBV infection, those positive for anti-HBs antibodies only are considered as having received HB vaccine and those negative for both anti-HBc and anti-HBs antibodies are considered as having not acquired HBV infection.

Women with isolated anti-HBc had HBV DNA quantified using Abbott real-time HBV DNA™ assay (Abbott France, Rungis, France; lower limit of detection of 15 IU/mL or 1.18 $\log_{10}$ IU/mL) and HBsAg verified using an HBsAg test kit of 50 pg/mL sensitivity (MonoLisa® HBsAg ultra, Bio-Rad laboratories) and able to detect up to 30 additional mutations on HBsAg proteins.

Infants born to women with isolated anti-HBc and detectable HBV DNA were tested at 4 months of age for HBV DNA using Abbott real-time HBV DNA™ assay.

2.2.2.4 HBV sequencing

HBV sequencing was performed to check for the presence of HBV mutation in patients with occult HBV infection. HBV DNA was extracted from patient's plasma

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using the automatic sample extraction system (Abbott M2000sp, Rungis, France). Ten microliters of HBV DNA extract were used as the template for nested polymerase chain reaction (PCR). Published primers were used to amplify HBV surface/polymerase region (nucleotide position 251 to 1058) (457). Amplicons were sequenced using the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA) and the ABI PRISM 3100 Genetic Analyzer, and sequencing data analyzed using the software Bioedit.

2.2.2.5 Statistical analysis

Characteristics of women including age at enrollment, region of birth, prior pregnancies, alanine transminase enzyme (ALT) level, white blood cells, lymphocytes, CD4+ T-cell and , CD8+ T-cell counts, and the presence of antibodies against syphilis or hepatitis C virus, are described using number and percentage for categorical data and median with interquartile range (IQR) for continuous data. Univariate analyses were performed using logistic regression analysis to identify risk factors for having isolated anti-HBc or occult HBV infection. Continuous variables were transformed into categorical variables using common cut-off values. For multivariate analysis, all factors with p-value <0.20 identified by univariate analysis were then introduced into the forward stepwise logistic regression model, to investigate independent risk factors associated with isolated anti-HBc serology or occult HBV infection. All data analyses were performed using STATA™ version 10.1 software (Statacorp, College Station, TX). Differences were considered statistically significant if the p-value was ≤ 0.05.

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2.2.3 Results

2.2.3.1 Characteristics of women

Of 1,908 HIV-1 infected women who participated in the perinatal HIV prevention trial, PHPT-2, 1,752 were found HBsAg-negative and included in this study (Figure 2.4). Characteristics of women are described in Table 2.5. At enrollment, the median age was 26 years, median ALT level was normal, median of HIV RNA load was 4.03 $\log_{10}$ copies/mL, median of CD4+ and CD8+ T-cell counts were 378 and 915 cells/μL, respectively. One percent of women were anti-syphilis antibody positive and 5% were anti-HCV positive. Women who were not included in this study due to insufficient volume of sample had similar baseline characteristics, i.e. age of enrolment, ALT level, CD4+ and CD8+ T-cell counts, HIV RNA load (data not shown).

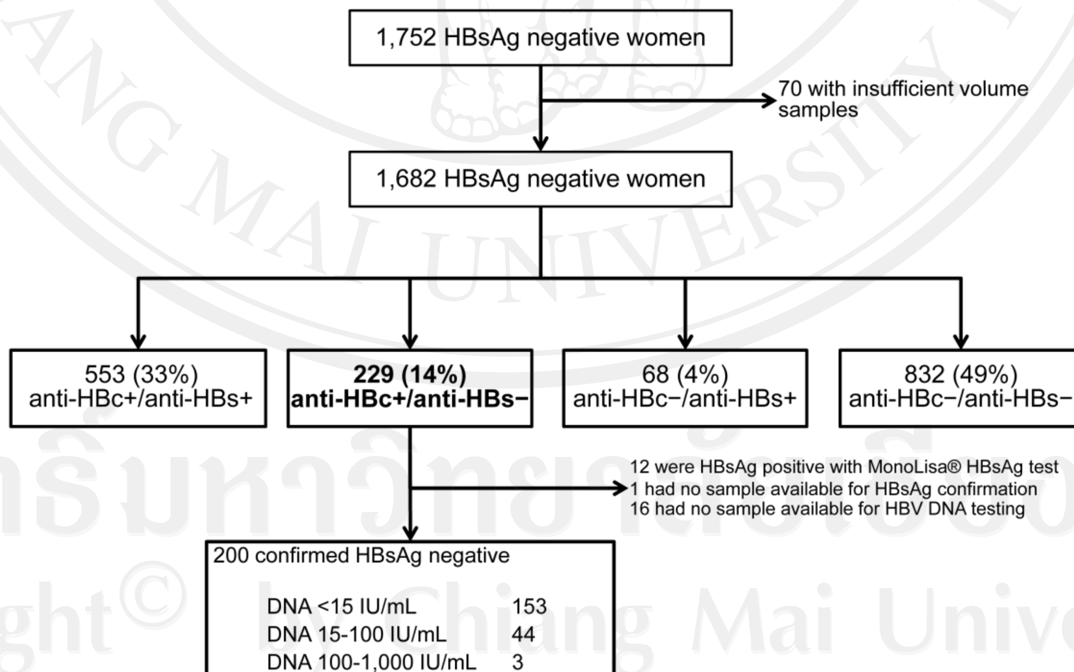

Figure 2.4 Overall study diagram

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Table 2.5 Characteristics of women

Characteristics	N	Categories	Median (IQR[a]) or n (%)
Age at enrollment (years)	1,682		25.9 (22.7-29.7)
Region of birth	1,682	Central	373 (22)
		Eastern	256 (15)
		Northern	348 (21)
		North-eastern	553 (33)
		Southern	76 (5)
		Western	76 (5)
Prior pregnancy	1,678		1,041 (62)
SGPT or ALT (IU/L)	1,638		15 (10-24)
White blood cells (cells/μL)	1,652		8,615 (7,300-10,160)
Absolute lymphocyte (cells/μL)	1,650		1,805 (1,430-2,250)
Absolute CD4 (cells/μL)	1,671		378 (245-531)
Absolute CD8 (cells/μL)	1,634		915 (700-1193)
HIV RNA load (log_{10} copies/mL)	1,660		4.03 (3.37-4.65)
Anti-syphilis antibody positive	1,649		17 (1)
Anti-HCV antibody positive	1,659		75 (5)

2.2.3.2 HBV serology among HBsAg negative HIV-pregnant women

Of 1,682 women with available samples, 832 (49%) were negative for anti-HBs and anti-HBs antibodies and thus considered as having not acquired HBV infection, detected, 553 (33%) were positive for both anti-HBc and anti-HBs antibodies and considered as having resolved HBV infection, 229 (14%) had isolated anti-HBc and considered as having acquired HBV infection, and 68 (4%) had were positive for anti-HBs antibodies and considered as having received HBV vaccine. The prevalence of isolated anti-HBc antibodies differed according to the region of birth. The highest rate, 22%, was found in women born in northern region, while the lowest rate, 4%, was found in southern region (Table 2.6). Median age of women with isolated anti-HBc was 26.6 years old, ranges from 15-46 years old. The prevalence of isolated anti-HBc antibodies increased according to the age of women, 11% in women aged less than 20 years old and increased to 25% in women aged above 40 years (Figure 2.5).

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Table 2.6 HBV serological status of HBsAg negative women according to region of birth.

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

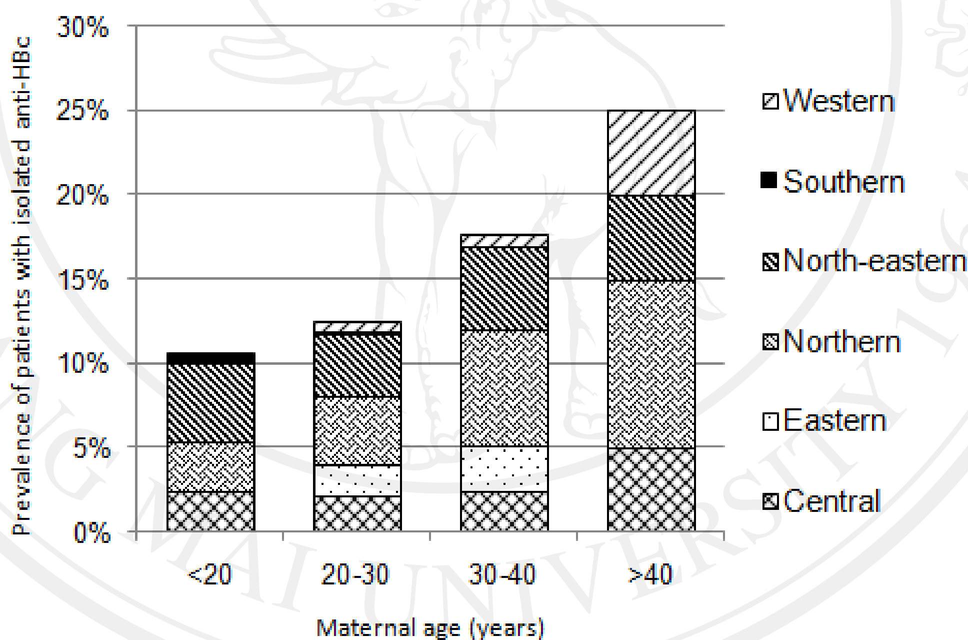

Figure 2.5 Prevalence of HIV-infected pregnant women with isolated anti-HBc serological pattern according to age at enrollment and region of birth

2.2.3.3 Factors associated with isolated anti-HBc

Univariate analysis shows that, among all parameter analyzed, age over 35 years, birth place in northern region, white blood cells counts <7,500 cells/µL, lymphocyte counts <1,000 cells/µL, CD4+ T-cells count <350 cells/µL and HCV

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infection were significantly associated with the presence of isolate anti-HBc antibodies in HIV-1 infected pregnant women.

A multivariate analysis was performed to adjust on all significant parameters associated with the presence of isolate anti-HBc from univariate analysis and is showed in Table 2.7. The results show the same association as in univariate analysis: Age over 35 years (adjusted odds ratio [aOR], 1.8; P=0.03), born in northern region (aOR, 1.8; P<0.001), absolute CD4 count below 350 cells/μL (aOR, 1.5; P=0.02) and much more significantly if CD4 count below 200 cells/μL (aOR, 2.8; P<0.001), and past or present HCV infection (aOR, 2.6; P=0.001), a independently associated with the presence of isolated anti-HBc.

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Table 2.7 Factors associated with isolated anti-HBc among HIV-1 infected pregnant women

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

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

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2.2.3.4 Prevalence of occult HBV infection

We firstly verified the absence of HBsAg in all women with isolated anti-HBc using a different HBsAg-test kit. Of 228 women with available samples, 12 (5%) tested positive with the new test kit. Samples of all but one women showed a low signal to cut-off (S/CO) ratio, ranging from 1.02 to 2.79, median = 1.4, IQR 1.1-2.0). Women with discrepant HBsAg results were then excluded from further analysis.

Among all HIV-1 infected pregnant women with confirmed isolated anti-HBc serology, 200 had a sample available for HBV DNA quantification. All women had HBV DNA below 1000 IU/mL; 153 had HBV DNA below the limit of detection (15 IU/mL), 44 had HBV DNA level between 15 to 100 IU/mL, and 3 had HBV DNA between 101 and 1,000 IU/mL (Table 2.8). The prevalence of occult HBV infection among women with isolated anti-HBc was thus 23.5% (47/200) (95%CI, 18-30). Of 47 women with detectable HBV DNA, only 2 had successful HBV sequencing; one had sS117I, sT118K, and sR160K mutations (GenBank accession number: JX402002) and the other had no S gene mutation (GenBank accession number: JX402003).

Table 2.8 Proportion of occult HBV infection among 200 HIV-1 infected pregnant women carrying isolated anti-HBc

HBV DNA level	N=200	Proportion (95%CI)
DNA 100-1,000 IU/mL	3	1.5 (0.3-4.3)
DNA 15-100 IU/mL	44	22.0 (16.5-28.4)
DNA <15 IU/mL	22	11.0 (7.0-16.2)
Undetectable	131	65.5 (58.5-72.1)

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

Among all parameters analyzed, univariate and multivariate analysis showed that detection of HBV DNA, and thus occult HBV infection, was inversely proportional to HIV RNA level. Rate of occult HBV infection was lowest when HIV RNA level greater than 5 $\log_{10}$ copies/μL (aOR, 0.03; P=0.006) (Table 2.9).

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Table 2.9 Factors associated with HBV DNA positivity among 200 HIV-1 infected pregnant women carrying isolated anti-HBc

Parameters	Categories	N	Occult HBV infection (%)	Univariate analysis OR (95%CI)[a]	P-value[b]	multivariate analysis OR (95%CI)	P-value[c]
Age at enrollment	≤ 25 years	83	20 (24)	1			
	26-30 years	57	12 (21)	0.8 (0.4-1.9)	.67		
	31-35 years	39	8 (21)	0.8 (0.3-2.1)	.66		
	>35 years	21	7 (33)	1.6 (0.6-4.4)	.39		
Region of birth	Central	29	9 (31)	1			
	Eastern	27	4 (14)	0.4 (0.1-1.4)	.16		
	Northern	69	18 (26)	0.8 (0.3-2.0)	.62		
	North-eastern	62	16 (26)	0.8 (0.3-2.0)	.60		
	Southern	3	0 (0)	-	-		
	Western	10	0 (0)	-	-		
Previous pregnancy	No	75	16 (21)	1			
	Yes	124	31 (25)	1.2 (0.6-2.4)	.56		
ALT (IU/L)	≤30	165	42 (25)	1			
	31-60	22	2 (9)	0.3 (0.1-1.3)	.11		
	>60	6	2 (33)	1.5 (0.3-8.3)	.67		
White blood cells (/μL)	>10,000	36	7 (19)	1			
	7,501-10,000	80	17 (21)	1.1 (0.4-3.0)	.82		
	5,001-7,500	70	20 (29)	1.7 (0.6-4.4)	.31		
	≤5,000	8	2 (25)	1.4 (0.2-8.4)	.73		
Absolute lymphocyte (/μL)	>2,000	62	13 (21)	1			
	1,501-2,000	55	16 (29)	1.5 (0.7-3.6)	.31		
	1,001-1,500	45	13 (29)	1.5 (0.6-3.7)	.35		
	≤1,000	31	4 (13)	0.6 (0.2-1.9)	.35		
Absolute CD4+ T-cells (/μL)	>500	40	10 (25)	1			
	351-500	37	8 (22)	0.8 (0.3-2.4)	.73		
	201-350	54	15 (28)	1.2 (0.5-2.9)	.76		
	≤200	68	14 (21)	0.8 (0.3-2.0)	.60		
Absolute CD8+ T-cells (/μL)	>1,500	18	7 (39)	1			
	1,001-1,500	51	9 (18)	0.3 (0.1-1.0)	.07		
	501-1000	104	25 (24)	0.5 (0.2-1.4)	.19		
	≤500	22	5 (23)	0.5 (0.1-1.8)	.27		
HIV RNA load (log_{10} copies/μL)	≤ 3.00	24	10 (42)	1			
	3.01 – 4.00	68	18 (26)	0.5 (0.2-1.3)	.17	0.5 (0.2-1.3)	.17
	4.01 – 5.00	**71**	**15 (21)**	**0.4 (0.1-1.0)**	**.05**	**0.4 (0.1-1.0)**	**.05**
	> 5.00	**34**	**4 (12)**	**0.2 (0.05-0.7)**	**.013**	**0.2 (0.05-0.7)**	**.013**
Anti-syphilis antibody	No	191	45 (24)	1			
	Yes	2	1 (50)	3.2 (0.2-53)	.41		
Anti-HCV antibody	No	174	39 (22)	1			
	Yes	22	8 (36)	2.0 (0.8-5.1)	.15		

NOTE: [a]OR (95%CI): Odds ratio (95% confident interval); [b]Logistic regression analysis was used; [c]Multivariate logistic regression analysis was used

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2.2.3.6 Assessment of HBV infection in infants born to mothers with occult HBV infection

We have assessed HBV infection in infants born to 47 mothers with detectable HBV DNA (>15 IU/mL) at enrollment. No HBV DNA was detected in their infants at 4months of age.

2.2.4 Discussion and conclusion

This is the first detailed analysis of HBV serologic markers among a large number of HIV-pregnant women. We have analysed three variables associated with isolated anti-HBc profile and occult HBV infection: prevalence, risk factors and impact on perinatal transmission of HBV.

Consistent with data from regions where vertical transmission of HBV has significant contribution, about half of HIV-infected pregnant women in this study showed HBV exposure markers. Fourteen percent had isolated anti-HBc. This rate is close to that observed among HIV-infected adults in Bangkok (20%) (407), 13% in Northern areas (S. Thongsawat, unpublished data) and in other countries with high prevalence of chronic HBV infection (382, 404, 405).

About half of HIV-pregnant women had no HBV serological markers, indicating they are HBV susceptible population. Since immunization with HBV vaccine is strongly recommended for all HIV-infected individuals without immunity

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to HBV (486), this finding highlights the need for testing all HIV-infected patients to vaccinate those without HBV markers.

We identified several independent risk factors for isolated anti-HBc serological status in HIV-infected pregnant women i.e. the low CD4 count, age over 35 years, and HCV infection were independent factors. All factors have also been found in other populations either HIV-infected (420, 449, 487) or uninfected (488). The effect of age may be related to the progressive loss of anti-HBs producing capacity over time after resolution of HBV infection, or insufficient level of anti-HBs production (422). We also found that being born in northern region was independently associated with isolated anti-HBc. Other studies have reported higher prevalence of HBsAg positivity in the northern region of Thailand as compared to southern region (163, 165, 359), which may explain the rates of isolated anti-HBc observed in this study. These results are also consistent with other studies describing HCV infection as a main factor for isolated anti-HBc in both HIV-infected and –uninfected population (401, 407, 409, 411-413, 489), possibly as a result of the direct interference of HCV core protein on the synthesis of HBsAg (490, 491).

A wide range (0-89%) of occult HBV infection has been reported in HIV-infected patients with isolated anti-HBc. The heterogeneity of study population and the usage of different sensitivity and specificity of HBV DNA assay may account for these discrepancies. In this study, we used a highly sensitive commercial technique to detect HBV DNA and were thus able to detect HBV DNA in 24% (47 of 200) of HIV-1 infected pregnant women with isolated anti-HBc serological profile. This rate

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of occult HBV infection is within the range found in isolated anti-HBc blood donors (4–24%) of high HBV endemic areas such as India, Taiwan, Japan, and Sardinia (492). When considering the whole population of HIV-infected pregnant women the prevalence of isolated anti-HBc and occult HBV infection was 2.6% (47 of 1,783; 95%CI, 1.9-3.5).

One intriguing observation was the inverse association between the detection of HBV DNA and HIV RNA concentrations. Unlike Lo re et al. (440) who found more frequently occult HBV infection in patients with HIV RNA >1,000 copies/mL (17% in high HIV RNA level patients versus 4.6% in low HIV RNA level patients, n=179), we observed a higher rate of occult HBV infection in patients (n=200) with low HIV RNA concentrations (21% versus 42%, P=0.04, respectively). Possible explanations to this could be that 73% of patients in Lo Re III et al. study were on highly active antiretroviral treatment, while in this study all women were naïve to antiretroviral treatment. Further studies are needed to understand this discrepancy.

Clinical relevance of isolated anti-HBc and impact of low levels of HBV DNA in HIV-pregnant women with isolated anti-HBc are not well known. Walz et al. have recently reported that 7 of 105 infants born to women with isolated anti-HBc were infected with HBV but none were positive for both HBsAg and HBV DNA. Interestingly, only one woman was HBV DNA positive (493). In this study, the level of HBV DNA was below 1000 IU/mL in 47 women with occult HBV infection and none transmitted HBV to their infants. This suggests that there is a very low risk of

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transmission of HBV from women presenting isolated anti-HBc and occult HBV infection, to their offspring.

In conclusion, this study shows that the prevalence of HIV-infected pregnant women presenting isolated anti-HBc/occult HBV infection was low (2.6%) and occult HBV infection was not associated with perinatal transmission of HBV.

2.2.5 Publications and presentations

- **Khamduang W,** Ngo-Giang-Huong N, Gaudy-Graffin C, Jourdain G, Suwankornsakul W, Jarupanich T, Chalermpolprapa V, Nanta S, Puarattana-aroonkorn N, Tonmat S, Lallemant M, Goudeau A, Sirirungsi W and the Program for HIV Prevention and Treatment (PHPT-2) group. Prevalence and factors associated with isolated antibody to hepatitis B core antigen and occult HBV infection in HIV-1 infected pregnant women in Thailand. *Clinical Infectious Diseases.* 2013 (Journal Impact factor 2011: 9.154) (Accepted)

This work was presented in;

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

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

2.3.1 Introduction

Between 350 and 400 million people worldwide are chronically infected by Hepatitis B virus (HBV) (4) and 75 to 80% of these individuals are in Asia and the Western Pacific (8). Annually, around 1 million people worldwide die from the consequences of HBV infection, including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (4). Among HIV-infected populations, the overall prevalence of hepatitis B surface antigen (HBsAg) carriers is estimated to be 8-11% (494); about 10% in Asia-Pacific region (170) and 9% in Thailand (171, 172). In HIV-infected individuals, chronic hepatitis B infection is associated with accelerated liver disease progression, aggressive hepatocellular carcinoma and increased liver-related mortality rate (184, 191). Hepatitis B-related immune reconstitution flares have been observed following initiation of highly active antiretroviral treatment (HAART) (322).

In Thailand, where HBV infection is highly endemic, HBV infection occurs mostly through mother-to-child transmission or during early childhood. HBV genotypes C and B are the most prevalent in the general population, and respectively account for 70-90% and 10-30% of infections (67). More hepatic necro-inflammatory activity and more rapid progression to cirrhosis and HCC have been observed in patients infected with HBV genotype C as compared to genotype B (260). Additional important risk factors associated with the development of cirrhosis and HCC include HBeAg positivity and high HBV DNA viral load (260). Suppression of HBV DNA level is associated with biochemical and histological remission of liver disease (495,

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496). Therefore, suppressing the replication of HBV to undetectable levels is a major goal in HBV treatment.

Lamivudine (3TC) is a cytidine analogue that inhibits the reverse transcriptase of both HIV and HBV (26). The efficacy of 3TC (150 mg twice a day) on HBV replication in HIV-HBV co-infected patients is similar to that of 3TC (100 mg once a day) in HBV mono-infected patients (266, 497). Resistance mutations to 3TC have been observed in HBV-HIV-1 co-infected patients at a rate of 15-20% per year in western countries where HBV genotypes A or D are predominant (27, 307). In Thailand, over 95% of HIV-infected patients receive lamivudine (3TC) as part of highly active antiretroviral therapy (HAART) and 9% are co-infected with HBV. The long-term benefit of 3TC on HBV infection and the incidence of 3TC resistance in these HBV-HIV co-infected patients are not well known.

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

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2.3.2 Methods

2.3.2.1 Study population

Patients were enrolled in the prospective multicenter Program for HIV Prevention and Treatment (PHPT) cohort (ClinicalTrials.gov Identifier: NCT00433030) of HIV-infected adults on antiretroviral therapy in Thailand. This cohort study was approved by the Thai Ministry of Public Health and ethic committees at Chiangrai Prachanukroh, Prapokklao, Chonburi, Bhuddasothorn, Somdej Prapinklao, Nopparat Rajathanee, Bhumibol Adulyadej, Buddhachinaraj, Hat Yai, Samutsakorn, Nakhonpathom, Maharaj Nakornratchasrima, Sanpatong Hospitals. All investigators conducted the study according to the principles expressed in the Declaration of Helsinki. This sub-study was also approved by the ethic committee of the Faculty of Associated Medical Sciences, Chiang Mai University. Prior to starting HAART, all patients were screened for HBsAg and anti-HCV antibodies at each hospital. CD4+ T-cell counts and HIV RNA quantification were performed at start of HAART and every 6 months thereafter. Patients received a quarterly clinical biological follow-up and compliance is assessed at each visit by pill count.

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

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2.3.2.2 HBV and HIV testing

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

2.3.2.3 HBV virological responses

HBV responses to 3TC were categorized according to the Asian Pacific Association for the study of liver recommendations (278). Thus, HBV DNA suppression is defined as undetectable level of HBV DNA (the threshold used was 150 or 2.18 $\log_{10}$ IU/mL since some samples were diluted at 1:10 ratio due to insufficient volume). Virological breakthrough is defined as an initial decline >2 $\log_{10}$ IU/mL followed by an increase of HBV DNA >1 $\log_{10}$ IU. Maintained viral suppression was defined as HBV DNA level persistently <2.18 $\log_{10}$ IU/mL.

2.3.2.4 HBV DNA sequencing

Ten μL of Abbott M2000 HBV DNA extract were used to amplify the *HBV polymerase* region (nucleotide position 251 to 1058) (457). The first-round PCR was

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performed in a 59 µL volume using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) and the primers Pol1M (5'-CCC TGC TCG TGT TAC AGG CGG-3') and Pol2M (5'-GTT GCG TCA GCA AAA ACT TGG CA-3'), which yield an amplicon of 1,010 bp. PCR conditions consisted of an initial 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 68°C for 3 min. The second-round PCR was performed using 10 µL of the first-round product and the following nested primers, Pol3M (5'-GAC TCG TGG TGG ACT TCT CTC A-3') and Pol4M (5'-GGC ATT AAA GCA GGA TAA CCA CAT TG-3') (457), to yield an 808 bp amplified fragment. PCR conditions were an initial denaturation step of 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 1 min, and extension at 68°C for 3 min. Amplicons were visualized under UV light following electrophoresis on a 1% agarose gel stained with ethidium bromide. The second-round PCR products were used directly for bidirectional sequencing with the nested pol3M and pol4M primers. Amplicons were sequenced using the BigDye Terminator Mix V. 1.1 (Applied Biosystems, Foster city, CA), and sequences were analyzed using the Bioedit software (http://www.mbio.ncsu.edu/bioedit). HBV *pol* sequences were analyzed for polymorphisms and mutations known to be associated with 3TC resistance through comparison with wild-type reference sequences of similar genotype (498).

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2.3.2.5 HBV Genotyping

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

2.3.2.6 Statistical analyses

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

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

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2.3.3 Results

2.3.3.1 Baseline characteristics

Of 1,448 HIV infected adults on HAART, 122 (8.4%) tested HBsAg-positive. Of these, 53 were receiving 150 mg twice a day (bid) of 3TC as part of HAART. Among them, 44 were tested for HBV DNA at baseline, 3 and 12 months after treatment initiation. Of 34 patients with detectable HBV DNA at baseline samples, 4 stopped 3TC very early and switched to another regimen. Finally, 30 patients were included in this study (Figure 2.6). Their median age was 31 years [IQR; 27-34], 80% were female. Median CD4+ and CD8+ T-cell counts were 100×10^6 and 562×10^6 cells/L, respectively. Median alanine transaminase (ALT) level was 30 U/L (IQR; 20-39) and median aspartate transaminase (AST) level was 48 U/L (IQR; 38-79). Median HIV RNA was 4.47 $\log_{10}$ copies/mL, and HBV DNA: 7.35 $\log_{10}$ IU/mL. Phylogenetic analysis indicated that 17% of patients were infected with HBV B genotype and 83% with C genotype (Figure 2.7). None had HCV infection. Although 12 (40%) of the patients had received previous antiretroviral treatment, none had been exposed to 3TC except one for a short period. At initiation of 3TC, 19 (63%) of the patients were HBeAg-positive. The baseline characteristics of HBeAg-positive and -negative patients were similar, except for median HBV DNA level which was significantly higher, as expected, in HBeAg-positive patients. (Table 2.10)

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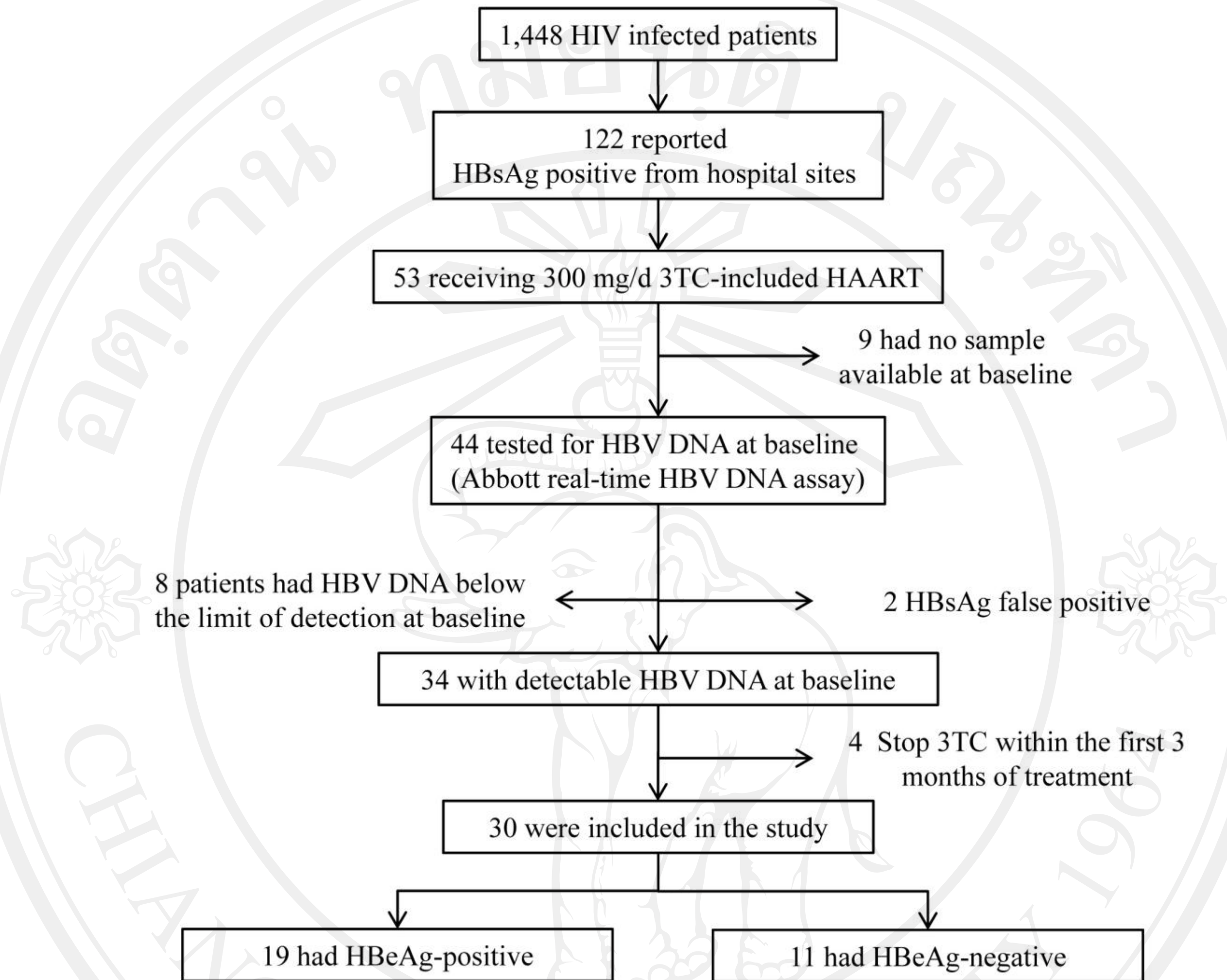

Figure 2.6 Overall study diagram

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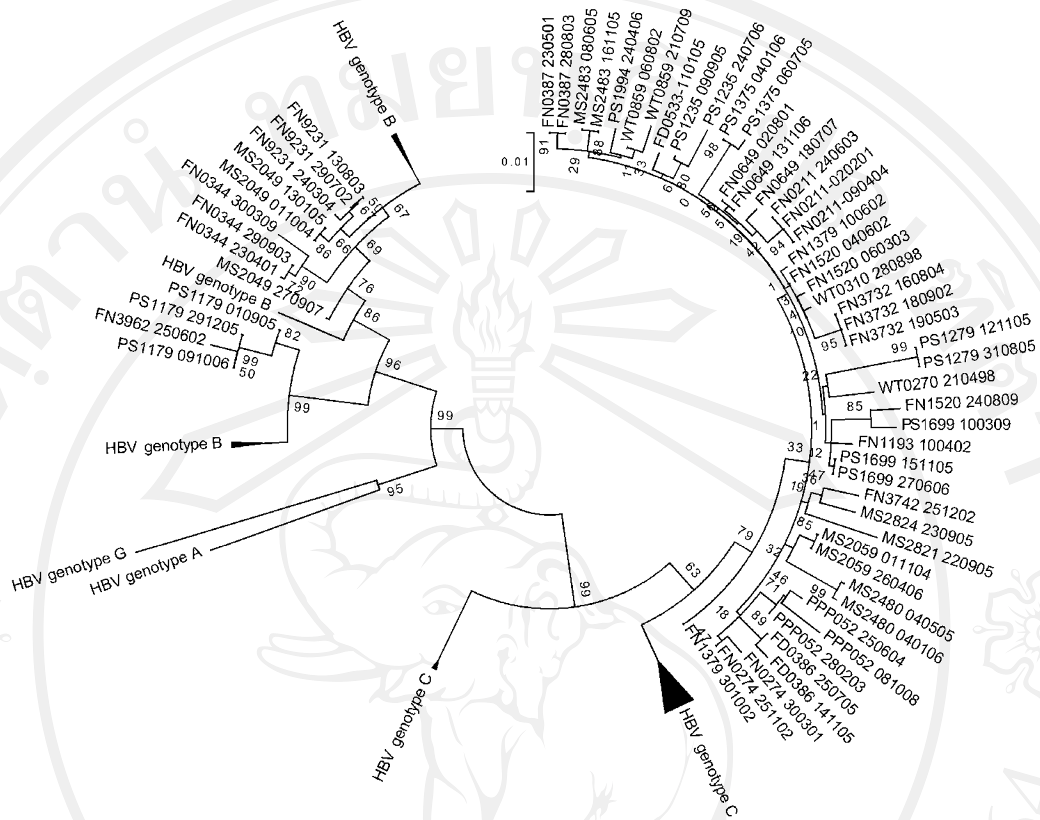

Figure 2.7 Phylogenetic tree analysis for HBV genotyping of 30 HIV/HBV co-infected patients

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Table 2.10 Baseline demographic and clinical characteristics of the study population

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

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

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Table 2.11 HBV and HIV virological response to 3TC in HIV-1/HBV co-infected patients during 12 months of 3TC treatment

Baseline characteristics	Overall (N=30)		HBeAg positive (N=19)		HBeAg negative (N=11)		p-value[a]
	n	%[95%CI] or median [IQR]	n	%[95%CI] or median [IQR]	n	%[95%CI] or median [IQR]	
HBV DNA suppression [b] at 3 months	14	47 [28-66]	6	32 [13-57]	8	73 [39-94]	0.06
Median HBV DNA reduction at 3 months (log_{10} IU/mL)		3.86 [2.56-4.67]		4.26 [3.40-5.48]		1.88 [1.36-3.59]	0.008
HBV DNA suppression [b] at 12 months	20	67 [47-83]	9	47 [24-71]	11	100 [72-100]	0.004
Median HBV DNA reduction at 12 months (log_{10} IU/mL)		4.40 [2.89-5.65]		4.94 [3.97-6.13]		1.88 [1.36-5.30]	0.02
HBV DNA breakthrough	4	13 [4-31]	4	21 [6-46]	0	0 [0-28]	0.27
HIV load ≤50 copies/mL at 3 months	22	73 [54-88]	13	68 [43-87]	9	82 [48-98]	0.67
Median HIV load reduction at 3 months (log_{10} cp/mL)		2.92 [2.54-3.53]		2.93 [2.14-3.48]		2.91 [2.54-4.08]	0.53
HIV load ≤50 copies/mL at 12 months	22	73 [54-88]	14	74 [49-91]	8	73 [39-94]	1.00
Median HIV DNA reduction at 12 months (log_{10} cp/mL)		2.92 [1.52-3.45]		2.93 [1.52-3.32]		2.91 [1.17-4.08]	0.78

[a] Fisher's exact test or Wilcoxon rank-sum test were used
[b] HBV DNA suppression was defined as serum HBV DNA level equal or below 150 or 2.18 log_{10} IU/mL

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2.3.3.2 Efficacy of 3TC on HBV replication

At 3 months, overall median reduction of HBV DNA was 3.86 log_{10} (IQR, 2.56-4.67), and 53% (95%CI, 34-72) of patients achieved HBV DNA suppression. At 12 months, overall median HBV DNA reduction was 4.40 log_{10} (IQR, 2.89-5.65) IU/mL and 67% (95%CI, 47-83) of patients achieved HBV DNA suppression (Table 2.11). Of the 20 patients who achieved HBV DNA suppression at 12 months, 18 were tested for HBsAg and one had lost HBsAg. Of 4 patients who experienced HBV breakthrough during the first 12 months: 2 had HBV DNA suppression at 3 months and 2 had never fully suppressed HBV replication. Six patients had partial HBV DNA suppression. More information on HBV virological response to 3TC-containing HAART is described on Table 2.12.

Twenty-two patients who experienced HBV DNA suppression were included in the analysis of the duration of HBV DNA suppression on 3TC treatment, 2 of them had HBV breakthrough as describe above, one changed drug regimen after 15 months of 3TC treatment, and 19 were followed-up over a median duration 50 months (IQR; 32-65 months). Of these 19 patients, 2 had HBV breakthrough and 17 (89%) maintained HBV DNA suppression until their last medical visit. The estimates cumulative rates of maintained HBV DNA suppression were 91% (95%CI; 68-98), 84% (95%CI; 58-95), and 68% (95%CI; 26-89) at 1, 3, and 5 years, respectively (Figure 2.8). Of those 17 patients with maintained HBV DNA suppression, 3 of 16 (19%) lost HBsAg at their last visit. Among the 8 HBeAg positive patients lost HBeAg at their last visit.

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The rate of HBV DNA suppression at 12 months was significantly higher among HBeAg-negative patients than among HBeAg-positive patients (100% and 47%, respectively; *P*=.004; Table 2.11). Kaplan-Meier analysis showed that HBeAg-negative patients achieved HBV DNA suppression more rapidly than HBeAg-positive patients (Figure 2.9, *P*-value by log-rank test = 0.017). HBV DNA suppression was maintained in all HBeAg-negative patients (Figure 2.8).

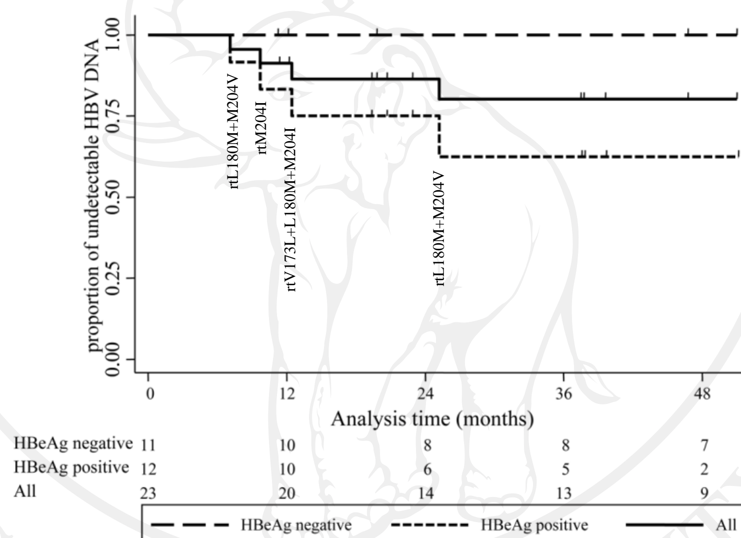

Figure 2.8 Kaplan-Meier curve of time to loss of HBV DNA suppression in 23 HIV-HBV co-infected patients who had achieved HBV DNA suppression within 1 year of 3TC-containing HAART. Lamivudine resistant mutations which were detected at each time points were presented under the lines.

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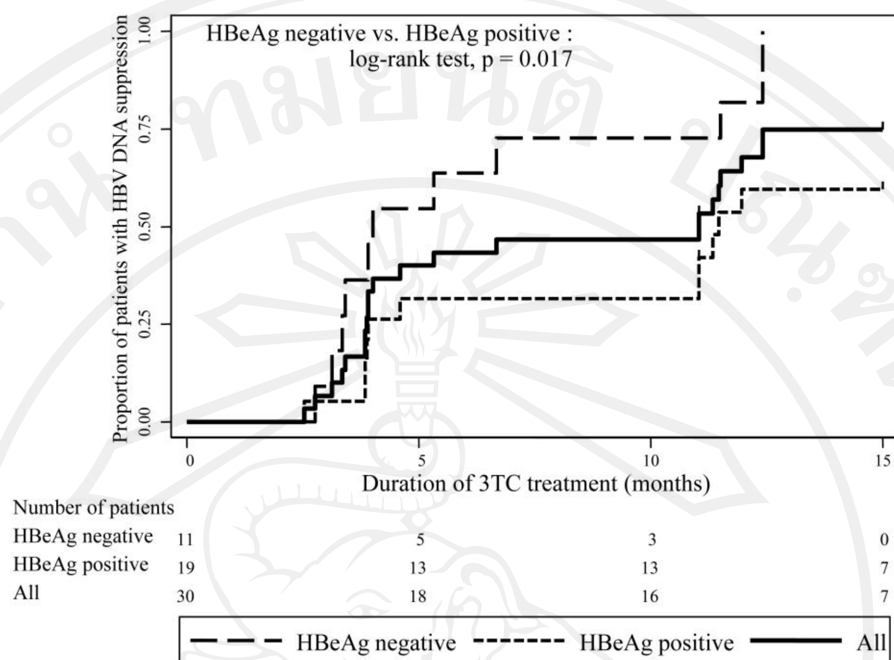

Figure 2.9 Kaplan-Meier curve of time to HBV DNA suppression. HBV DNA suppression is defined as HBV DNA level <2.18 $\log_{10}$ IU/mL, during the first 1 year of 3TC-containing HAART in HBeAg-positive and HBeAg-negative HIV-HBV co-infected patients.

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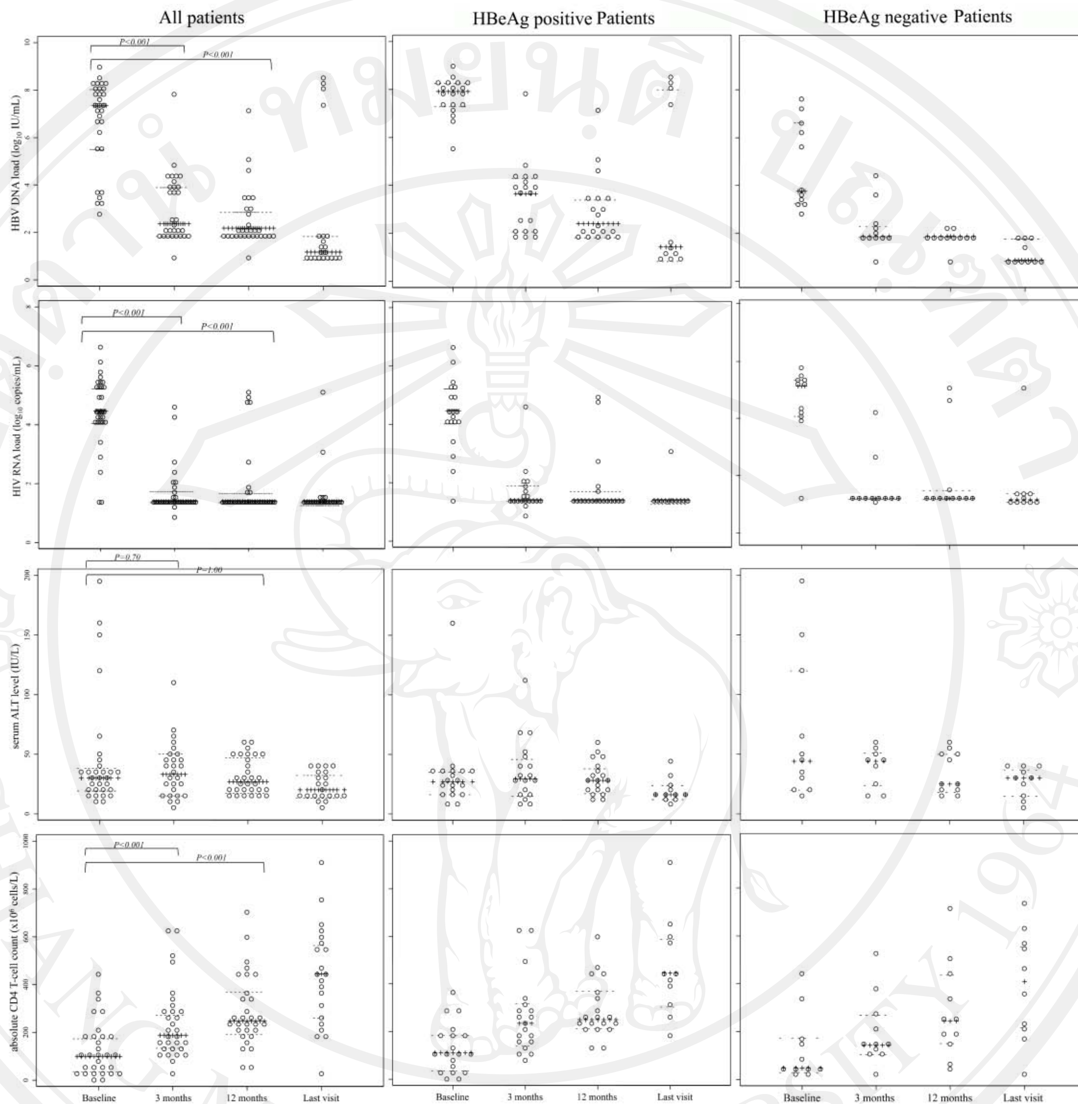

Figure 2.10 Dot plot distribution graphs of HBV DNA load, HIV RNA load, serum ALT level, and CD4+ T-cells count at baseline, 3, 12 months, and last visit in HIV-HBV co-infected patients on 3TC-containing HAART.

2.3.3.3 3TC resistance-associated mutations

Prior to 3TC initiation, all subjects had no 3TC-resistance-associated mutation. At 3 months, among the 16 patients with detectable HBV DNA, HBV sequencing was successful for 14 patients and no 3TC-resistance-associated mutation

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was found. HBV breakthrough was observed in 7 patients, 4 occurred early between 4 and 12 months, and 3 were detected late at 35, 65 and 81 months.

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

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

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

At 3-month, the median reduction of HIV RNA was 2.92 $\log_{10}$ (IQR, 2.54-3.53) and 67% patients achieved undetectable HIV RNA load (<1.7 $\log_{10}$ or 50 copies/mL). At 12-month, the median HIV RNA reduction was 2.92 $\log_{10}$ (IQR, 1.52-3.45) copies/mL and 73% patients achieved undetectable HIV RNA load. More

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information on HIV virological response to 3TC-containing HAART is described on Table 2.13. Reduction of HIV RNA level and proportions of undetectable HIV RNA were similar in HBeAg negative and HBeAg positive groups. Six patients had HIV RNA level above 500 copies/mL and presented the M184I/V mutations associated with HIV resistance to 3TC. CD4+ T-cell counts had risen from 100 (IQR: 38-178) cells/μL at baseline to 247 (IQR: 197-374) and 445 (IQR: 264-568) cells/μL at 12-month and last visit, respectively. ALT levels were normal and did not change during 3TC treatment (baseline: 30 IU/L, IQR 20-39; 12 months: 27, IQR 18-48; last visit: 20, IQR 14-33), as show in Figure 2.10.

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Table 2.12 Summary of HBV DNA and HIV RNA loads of HIV-HBV co-infected patients on lamivudine-containing HAART

Patient ID	Baseline HBeAg	HBV DNA load (log_{10} IU/mL)							HIV RNA load (log_{10} copies/mL)						
		Baseline	3 months	12 months	2 years	3 years	4 years	5 years	Baseline	3 months	12 months	2 years	3 years	4 years	5 years
1	-	2.84	1.88	1.88					4.09	1.40	1.70				
2	+	5.49	2.12	1.88	0.88				4.07	1.40	1.40	1.40			
3	+	8.06	2.18	1.88	0.88				2.30	2.11	1.40	1.40			
4	-	3.28	2.18	2.18	1.88				5.27	2.73	5.17	5.14			
5	+	8.07	1.88	1.88	n/a	1.43			1.40	1.40	1.40	1.40	1.30		
6	+	9.00	7.84	2.18	n/a	1.65			6.05	2.34	1.40	1.40	1.40		
7	-	7.18	3.59	1.88	n/a	0.88			5.25	1.40	1.40	1.40	1.40		
8	-	5.55	2.02	1.88	n/a	n/a	0.88		1.40	1.40	1.40	1.40	1.60	1.30	
9	+	8.16	3.90	1.88	n/a	n/a	1.18		4.46	1.48	1.40	1.40	1.40	1.40	
10	-	3.59	1.88	1.88	n/a	n/a	0.88	0.88	5.48	1.40	1.40	1.40	1.40	1.30	1.30
11	-	3.24	1.88	1.88	n/a	n/a	1.48	0.88	4.25	1.40	1.40	1.30	1.30	1.60	1.30
12	-	6.22	0.88	0.88	n/a	n/a	n/a	1.88	4.31	1.40	1.40	1.40	1.30	1.60	1.60
13	-	7.53	4.38	1.88	n/a	n/a	n/a	0.88	5.50	1.36	1.40	1.40	1.40	1.40	1.60
14	-	6.67	2.32	2.18	n/a	n/a	n/a	1.81	5.86	4.28	4.69	1.40	1.40	1.30	1.30
15	+	7.42	4.86	3.48	3.33	0.88	n/a	0.88	4.49	1.40	1.40	1.40	1.40	1.40	1.40
16	+	6.72	2.05	1.88	n/a	n/a	n/a	1.18	5.25	1.93	1.40	1.40	1.40	1.40	1.40
17	-	3.36	1.88	1.88	n/a	n/a	n/a	0.88	4.45	1.40	1.40	1.40	1.40	1.40	0.88
18	-	3.76	1.88	1.88	n/a	n/a	n/a	0.88	5.59	1.30	1.40	1.40	1.40	1.40	1.40
19	+	7.79	3.65	2.18	n/a	n/a	n/a	1.18	4.85	1.30	1.40	1.40	1.40	1.40	1.40
20	+	7.36	1.88	3.39	3.96	4.14	n/a	7.43	4.11	1.40	1.40	1.40	1.40	1.40	1.40
21	+	7.92	1.88	5.03	4.58	7.65	n/a	8.24	4.33	1.40	1.40	1.40	1.40	1.40	1.40
22	+	8.31	4.11	2.18	8.50	8.59			4.48	1.20	1.40	2.70	3.06		
23	+	8.20	3.81	2.18	0.88	7.89	n/a	8.05	4.06	1.36	1.40	1.40	1.40	1.40	1.40
24	+	7.19	4.40	7.23					3.39	2.04	4.88				
25	+	8.38	2.42	3.44	7.91	7.62			6.67	4.56	2.77	2.10	1.60		
26	+	7.87	4.36	2.68	4.02	3.44	n/a	7.53	2.92	0.78	1.40	1.40	1.40	1.40	1.40
27	+	7.34	3.94	3.04					5.52	1.51	4.78				
28	+	8.32	3.59	2.41	2.71				4.93	1.40	1.73	0.95			
29	+	8.59	4.47	4.60	4.76				5.25	1.77	1.93	1.40			
30	+	6.94	2.49	2.89	3.11	2.42	2.84		4.09	1.40	1.40	1.40	1.40	1.30	

Note: Value under detectable level is indicated in gray
Patients# 1-19: controlled HBV suppression
Patients# 20-23: had experienced HBV suppression and then HBV breakthrough
Patients# 24-26: had HBV breakthrough and never reach HBV undetectable level
Patients# 27-30: never reach HBV undetectable level
Patients# 15, 26-30: had partial HBV virological response at 12 months
Patients# 20, 21, 24, 25: had experienced HBV breakthrough during the first 12 months
Patients# 1-23: were taken into account for analysis of maintaining HBV suppression

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2.3.3.5 Impact of baseline HBV DNA level on HIV response to 3TC-containing HAART

Reduction of HIV RNA levels and proportions of undetectable HIV RNA were similar irrespective of baseline HBV DNA level and HBeAg status (Table 2.13).

Table 2.13 Impact of baseline HBV DNA level on HIV response during 5-years of 3TC-containing HAART

	Duration of 3TC treatment	HBV DNA level			HBeAg		
		<7.35log IU/mL n/N(%)	≥7.35log IU/mL n/N(%)	P value	Positive n/N(%)	Negative n/N(%)	P value
HIV RNA suppression (≤1.70 copies/mL) at:	3 months	11/15 (73)	11/15 (73)	1.00	13/19 (68)	9/11 (82)	0.67
	12 months	10/15 (67)	12/15 (80)	0.68	14/19 (74)	8/11 (73)	1.00
	2 years	11/12 (92)	13/15 (87)	1.00	15/17 (88)	9/10 (90)	1.00
	3 years	10/10 (100)	11/12 (92)	1.00	12/13 (85)	9/9 (89)	1.00
	4 years	9/9 (100)	9/9 (100)	NA	10/10 (100)	8/8 (100)	NA
	5 years	7/7 (100)	8/8 (100)	NA	8/8 (100)	7/7 (100)	NA

2.3.3.6 Impact of baseline HIV RNA load and HBV virological response to 3TC-containing HAART

To analyze the relation between baseline HIV RNA load and HBV virological response to 3TC-containing HAART, patient baseline HIV RNA levels were dichotomized according to the median baseline HIV RNA level (4.47 $\log_{10}$ copies/mL). The baseline HBV DNA levels did not differ between the two baseline HIV RNA groups (7.19 versus 7.42 $\log_{10}$ IU/mL, Fisher's exact p-value = 0.31). At one year, there was no difference in HBV response (67% versus 67%) by baseline HIV RNA group. Furthermore, there was no difference in response during 5-years of 3TC-containing HAART (log-rank p-value = 0.26).

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2.3.4 Discussion and conclusion

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

The rate of early response in this study, 53% of HBV DNA suppression at 3 months, is similar to the 30% reported by the international collaborative (CAESAR) study, conducted in Canada, Australia, Europe and Africa (26), although the median HBV DNA level prior to 3TC initiation was higher in this study, 7.35 vs 6.87 $\log_{10}$ IU/mL. At 12 months, the median HBV DNA decrease was 4.40 log in this study while it was 2.7 log in the CAESAR study likely due to different thresholds of HBV DNA quantification. Another possible cause may be related to the HBV genotypes, highly replicating C and B in this study and likely A or D in the CEASAR study. A recent study conducted in Kenya (499) reported that 89% (17/19) of HIV-HBV co-infected patients achieved HBV DNA suppression (<100 IU/mL) during 18 months of 3TC treatment (baseline HBV DNA level was 3.38 $\log_{10}$ IU/mL). The rates of patient with HBV DNA suppression was 94% (17 of 18) in HBeAg negative patients, while one HBeAg-positive patient was unable the suppress HBV replication under 100 IU/mL. These rates are not different from those found in this study, 47% in HBeAg-positive and 100% in HBeAg-negative patients. Furthermore, we found no relation between the baseline HIV RNA level and HBV response to 3TC-containing HAART

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or between the baseline HBV DNA level and HIV response to 3TC-containing HAART.

Among the 22 patients who had achieved HBV DNA suppression, 17 (77%) had maintained HBV DNA suppression until their last visit (median 50 months). This rate is much higher than the 9% (defined as undetectable by Digene Hybrid Capture assay with the threshold of 4.03 log IU/mL) previously reported by Benhamou et al, in HIV-HBV co-infected patients after 4 years of treatment with the same dosing of 3TC (27). We could hypothesize that the higher response rate in this study is due to a better compliance of patients to their treatment or HBV genotypes B and C are more sensitive to 3TC than genotypes A and D, which require confirmation with in vitro experiments. The estimated cumulative rates of maintained HBV DNA suppression at 1, 3, and 5 years after achieving suppression were 91%, 84, and 68, respectively. HBV DNA suppression was maintained in all HBeAg-negative patients. The higher rate of response to 3TC treatment and duration of HBV DNA suppression among HBeAg negative patients suggest that, in resource-limited countries, HBeAg testing may be valuable to predict the virological response to nucleoside/nucleotide analogs and could be considered when initiating in HIV-HBV co-infected patients the first-line HAART. Indeed in resource-limited countries, 3TC is included in the first-line HAART regimen.

One major limitation of treating HBV with 3TC monotherapy is the rapid emergence of resistance mutations. In HBV-HIV-1 co-infected patients, resistance mutations to 3TC have been shown to occur at a rate of 15-20% per year (27, 500). In

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this study, the incidence of 3TC resistance mutations during the first year of therapy was 3% (1of 30) which is not different to the 7% (2 of 27) in a study conducted in Kenya (p=.60) (499). Over the 6 years of follow-up, 7 patients presented HBV breakthrough and the rtM204I, due to the ntG741A uncommon mutation, and the triple rtV173L+L180M+M204I mutations associated with 3TC resistance were identified in 3 patients. Despite a good compliance to treatment, some patients had experienced HBV breakthrough without any mutations within the *pol* gene. This observation may be explained by the emergence of mutations outside the *rt* domain or other mechanisms that are still unknown.

A nucleotide analogue, tenofovir (TDF), has been shown to be active against both wild-type and 3TC resistant HBV (320, 501). When the PHPT treatment cohort was initiated, TDF was available in Thailand at 38 USD per month, price which exceeded that of the current standard first line HAART (zidovudine/stavudine, 3TC and nevirapine), 30 USD per month (502, 503). Recently, the Thai national (323) and WHO guidelines (504), have recommended to use TDF+3TC or TDF+emtricitabine as the backbone of the HAART combination to treat HIV-HBV co-infected patients. However, this combination may not be provided to all HIV-HBV co-infected patients since less than 50% of Thai HIV-1 infected patients who are on ART had not been assessed for HBV co-infection (505).

This study shows that a significant number of HIV-HBV co-infected patients on 3TC containing HAART, particularly HBeAg negative patients, can achieve long-term HBV DNA and HIV suppression. This study provides further information which

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may be helpful in the management of HIV-HBV co-infected patients in resource-limited countries.

2.3.5 Publications and presentations

- **Khamduang W**, Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Lallemant M, Sirirungsi W, Goudeau A, and the Program for HIV Prevention and Treatment (PHPT) group. Long-term hepatitis B virus (HBV) response to lamivudine-containing highly active antiretroviral therapy in HIV-HBV co-infected patients in Thailand. *PLoS ONE* 7(7), 2012: e42184. doi:10.1371/journal.pone.0042184 (Journal Impact factor 2011: 4.092)

This work has been accepted for presentation at;

- **Khamduang W**, Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Hepatitis B virus (HBV) virological response to combination antiretroviral treatment includes lamivudine (3TC) in HIV/HBV co-infected individuals in Thailand. International Meeting; The molecular biology of hepatitis B viruses, 30th August - 2nd September, 2009, Tours, France. ***(P-20, Poster presentation)***
- **Khamduang W**, Gaudy-Graffin C, Moreau A, Ngo-Giang-Huong N, Jourdain G, Lallemant M, Pipatnakulchai S, Putiyanun C, Kunkongkapan S, Wannarit P, Sirinontakan S, Ardong W, Sirirungsi W, Goudeau A. Hepatitis B virus (HBV)

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virological response to combination antiretroviral treatment includes lamivudine (3TC) in HIV/HBV co-infected individuals in Thailand. 10ème réunion du Réseau National Hépatites de l'ANRS, 21st - 22nd January, 2010, Paris, France. ***(Oral presentation)***

- **Khamduang W**, Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Lallemant M, Sirirungsi W, Goudeau A, and the Program for HIV Prevention and Treatment (PHPT) group. Long-term virological response of Hepatitis B virus to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand. The 13th Thai national AIDS seminar, 29th – 31st March, 2011, Bangkok, Thailand. ***(Poster presentation, AP3)***
- **Khamduang W,** Gaudy-Graffin C, Ngo-Giang-Huong N, Jourdain G, Moreau A, Luekamlung N, Halue G, Buranawanitchakorn Y, Kunkongkapan S, Buranabanjasatean S, Lallemant M, Sirirungsi W, Goudeau A, for the Program for HIV Prevention and Treatment (PHPT) study group. Long-term virological response of Hepatitis B virus (HBV) to lamivudine-containing HAART in patients co-infected with HIV and HBV in Thailand. The XIX international AIDS conference, 22nd - 27th July 2012, Washington DC, USA. ***(Poster presentation, WEPE049)***

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