CHAPTER 2

Materials and Methods

2.1 Research design

A retrospective study was performed in HIV-1-exposed infants whose age less than 18 months and participated in the National Health Security Office (NHSO) of Thailand to receive early HIV-1infant diagnosis at the Faculty of Associated Medical Sciences, Chiang Mai University, during 2007 to 2014. The remnants of positive HIV-DNA dried blood spots (DBS) from infants were selected. The HIV-DNA was extracted from the DBS by NucleoSpin® Tissue (Macherey-Nagel, Germany) and amplified the *pol* gene using Nested-PCR assay with specific primers to amplify reverse transcriptase and protease region. The amplified product of protease and reverse transcriptase region of *pol* gene were amplified to 507 and 798 base pairs (bps), respectively. Then, the amplified products were purified by Nucleospin®Gel&PCR Clean-up (Macherey-Nagel, Germany) and sequenced using cycle sequencing method. The nucleotide sequences were analyzed with Geneious R8 genetic analyzer (Biomatters, NZ) and interpreted the HIV drug resistance mutation by submitting the nucleotide sequences to the Stanford HIV Drug Resistance Database (http://sierra2.stanford.edu/sierra/servlet/JSierra). Moreover, the HIV-1 subtypes were determined using the Stanford HIV Drug Resistance Database and REGA HIV-1 subtyping tool version 3.0. Finally, the results of drug resistance mutation and HIV-1 subtype were analyzed in descriptive statistic between infant factors (Age, birth year/month, ARV regimen and HIV subtype) and maternal factors (ARV regimen) by using STATA 10 (Stata Corporation, USA). The flow chart of study design is represented in Figure 2.1.

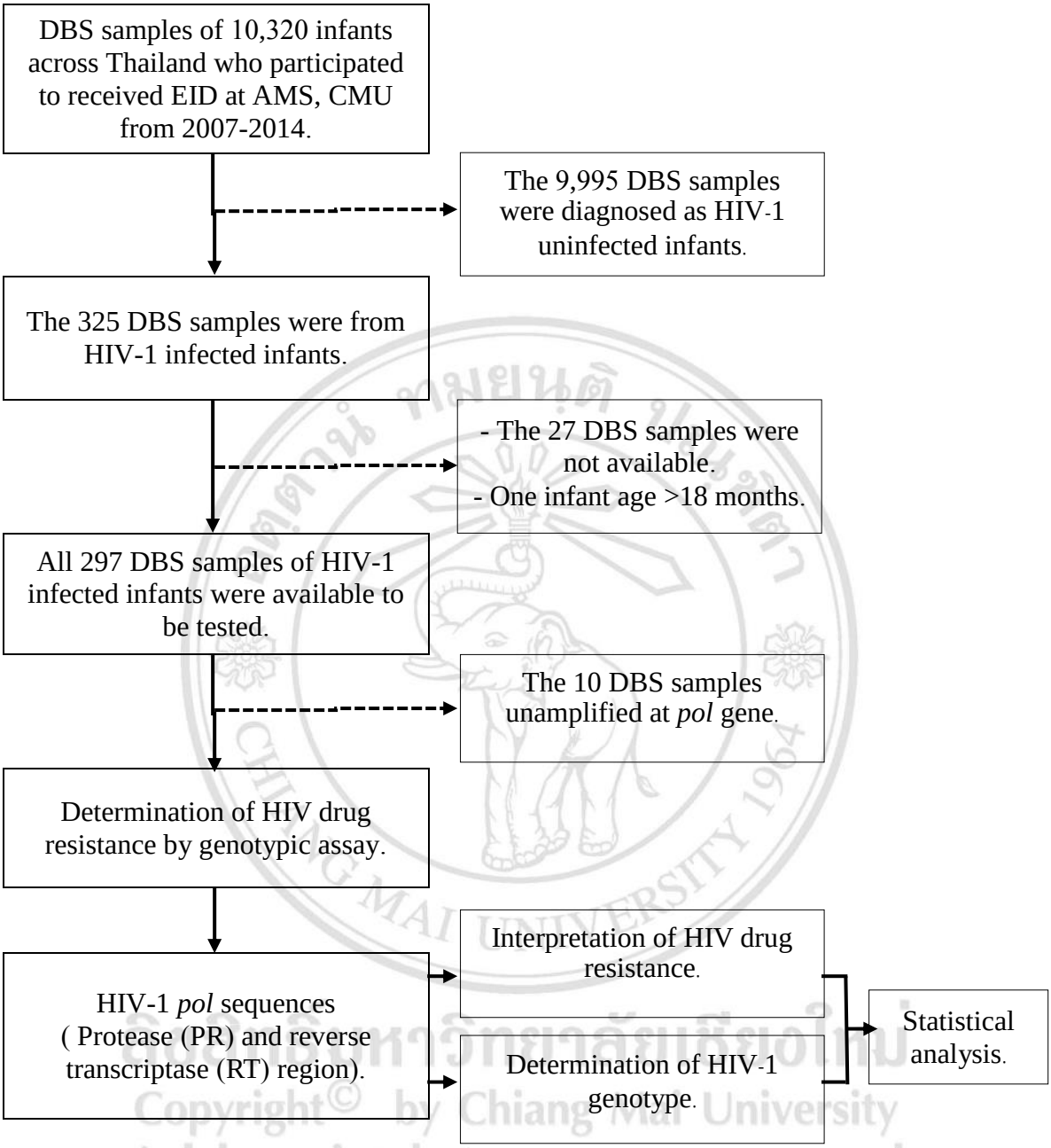

Figure 2.1 The flow chart of research study design.

2.2 Materials and methods

2.2.1 Study population

Dried blood spots (DBS) samples of HIV-1 infected infants were selected from the infants who participated in the National Health Office (NHSO) program [14] and received an early infant diagnosis (EID) at the Faculty of Associated Medical Sciences (AMS), Chiang Mai University from year 2007 to year 2014. The infants were diagnosed the HIV infection by real-time DNA PCR method using dried blood spots (DBS) specimens [41]. These DBS samples were sent from 364 different hospitals all over Thailand (Table 2.1). In DBS collection for EID, the first DBS sample was collected at 1-2 months of age, and second DBS sample was collected immediately for confirmatory HIV-1 infection status, if the first DBS test was positive. In this study, the initial DBS was given priority in this study, while the second DBS was used instead if first DBS was unavailable or unamplified. The DBS samples were obtained from whole-blood of HIV-exposed infants which collected in Whatman no. 903 Protein Saver Card (Whatman, UK) and stored at -20 °C. A total of 325 HIV-1 infected infants were included in this study, but only 297 infants had available DBS specimens. These DBS samples are remnant DBS from early infant HIV-infected diagnosis. All available databases include age of infant at diagnosis, gender, signs of infection, infant prophylaxis, MTCT prophylaxis and maternal treatment were recorded and used as anonymous.

Table 2.1 The number of children, who were born from HIV-1 infected mothers and attended for Early HIV-1 Infant Diagnosis at the Division of Clinical Microbiology, Chiang Mai University, from 2007 to 2014, and registered hospital.

Region	Number of hospitals	Number of registered children	Number of HIV-1 infected children	Number of HIV-1 uninfected children
Northern	84	1,903	46	1,857
North Eastern	90	2,106	73	2,033
Central	87	2,662	86	2,576
Eastern	22	1,178	33	1,145
Western	21	615	20	595
Southern	63	1,850	63	1,787
Unidentified origin	-	6	4	2
Total	**367**	**10,320**	**325**	**9,995**

2.2.2 DNA extraction

HIV-1 genomic DNA was extracted from one circle spot of DBS (approximately 50 µl per each spot) using NucleoSpin® Tissue (Macherey-Nagel, Germany) according to the manufacturer's instruction. The extracted DNA concentration was measured using Eon™ Microplate Spectrometer (BioTek Instruments, USA) and the concentration should have at least 2-3 µg/ml. After that, the extracted DNA was stored at -20°C until used.

2.2.3 HIV-1 drug resistance determination

2.2.3.1 Amplification of polymerase gene

The protease and reverse transcriptase region were amplified by a nested-PCR with specific primers (Table 2.2) that follow the Agence Nationale de Recherche sur le Sida (ANRS) in-house HIV drug-resistance testing protocol [113]. Binding position of these primers and protease and reverse transcriptase region were shown in Figure 2.2.

The Nested-PCR was performed using 5U Platinum *Taq* DNA polymerase High Fidelity (Invitrogen™, USA) on a GeneAmp®2700 PCR system. Fifty microliters of PCR mix consist of 1 µl of 20 µM of each primer, 8 µl of 1.25 mM dNTPs, 22.8 µl of sterile water, 2 µl of 50 mM $MgSO_4$, 5 µl of 10x buffer, 0.2 µl of 5U *Taq* and 10 µl of DNA template.

In the first round of amplification step, outer primers that use for PR region were 5' prot 1 and 3' prot 1 which bind at position 2082 and 2734 bps of polymerase gene. The outer primer pairs for RT region were MJ3 and MJ4 which bind at position 2480 and 3420 bps (Alternative sets, RT18 and RT21 which bind at 1951 and 3113 bps) were used. This step was started from the initial denaturation at 94 °C for 3 minutes, 35 cycles were performed consisting of the denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and the extension at 68°C for 1 minute. The final extension was performed at 68°C for 10 minutes. The amplified products were approximately 652 and 940 (1,163 from alternative sets) bps of PR region and RT region, respectively. And the first round amplified products were used as templates for the second round of amplification.

The second step used 5' prot 2 and 3' prot 2 as inner primer pairs for PR region, which bind at position 2136 and 2650 bps of polymerase gene. A35 and NE135, which bind at position 2530 and 3334 bps (Alternative sets, RT1 and RT4 which bind at position 2178 and 2824 bps) were used as inner primers for RT region. The second round condition started with initial denaturation at 94 °C for 3 minutes, 35 cycles were performed consisting of the denaturation at 94°C for 15 seconds, annealing at 58°C for

30 seconds, and the extension at 68°C for 1 minute. And the final extension was performed at 68°C for 10 minutes.

The second round amplified products were approximately 507 and 798 (647 from alternative sets) bp of PR region and RT region, respectively. Next, the amplified products were analyzed by 1% agarose gel electrophoresis. Finally, the PCR products were purified using Nucleospin®Gel&PCR Clean-up (Macherey-Nagel, Germany) according to the manufacturer's instructions and kept at -20°C until use.

Table 2.2 The sequence of oligonucleotide primers [113].

Primers	**Sequences**
5' prot 1	5'-TAA TTT TTT AGG GAA GAT CTG GCC TTC C-3'
3' prot 1	5'-GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG-3'
5' prot 2	5'-TCA GAG CAG ACC AGA GCC AAC AGC CCC A 3'
3' prot 2	5'-AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC 3'
MJ3	5'-AGT AGG ACC TAC ACC TGT CA-3'
MJ4	5'-CTG TTA GTG CTT TGG TTC CTC T-3'
A(35)	5'-TTG GTT GCA CTT TAA ATT TTC CCA TTA GTC CTA TT-3'
NE1(35)	5'-CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT-3'
Alternative sets	
RT18	5'-GGA AAC CAA AAA TGA TAG GGG GAA TTG GAG G-3'
RT21	5'-CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG G-3'
RT1	5'- CCA AAA GTT AAA CAA TGG CCA TTG ACA GA -3'
RT4	5'- AGT TCA TAA CCC ATC CAA AG -3'

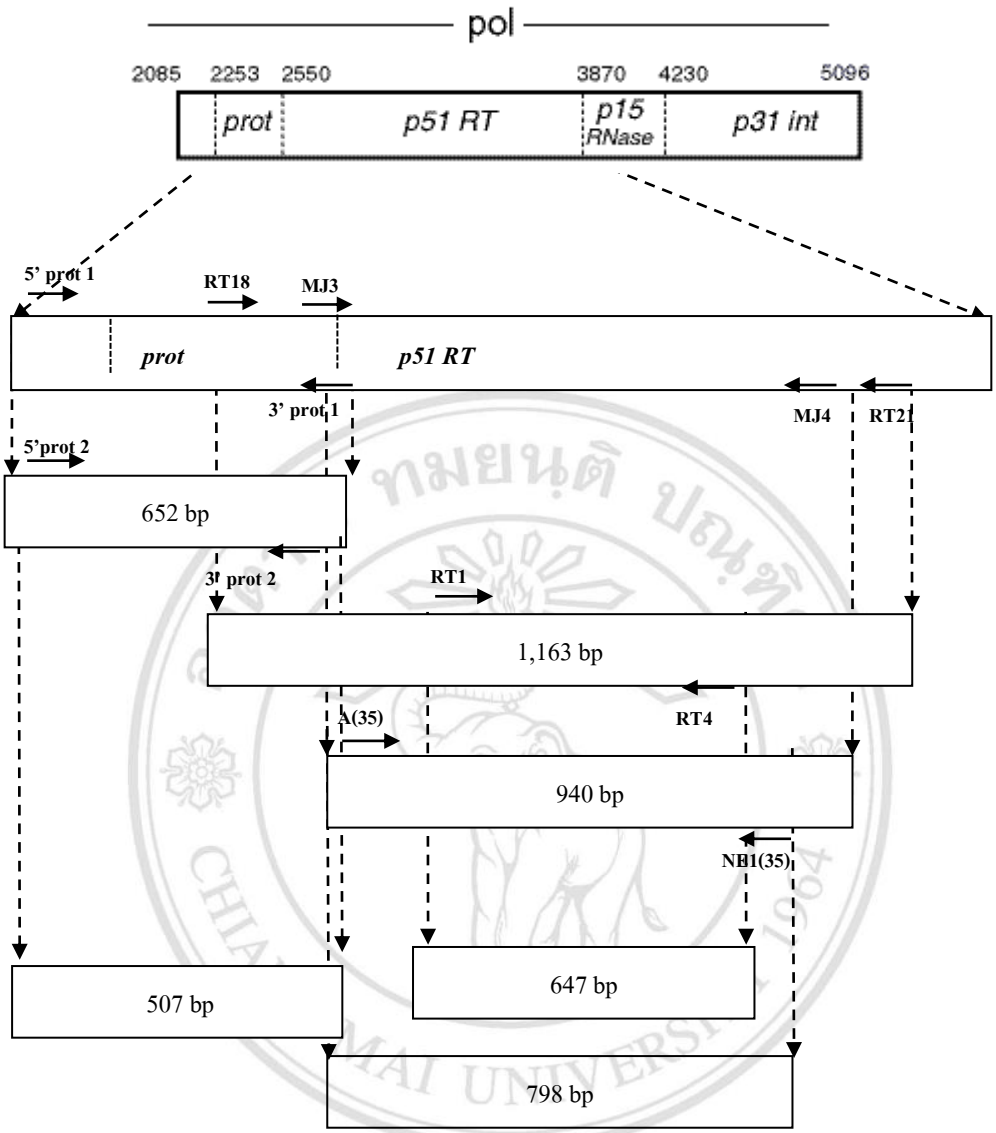

Figure 2.2 The diagram of binding of specific primer on protease and reverse transcriptase region in polymerase gene.

2.2.3.2. Protease and Reverse transcriptase region sequencing

The purified PCR products of were sequenced using cycle sequencing assay with BigDye terminater V.3.1 cycle sequencing kit (Applied Biosystem, USA) with inner primers similar as Nested-PCR step. The 5' prot2/3' prot2 were used for analyzing the nucleotide sequence on PR gene and A (35)/NE1(35) primers (Alternative sets, RT1/RT4) were used for RT gene. The cycle sequencing was performed both bidirectional and overlapping sequences, which covered all protease and reverse transcriptase inhibitor resistance-associated mutations on *pol* gene, were provided from these primers.

Ten microliters of cycle sequencing mix were performed using BigDye™ terminator V3.1 cycle sequencing kit (Applied Biosystems, USA) which consist of 2.8 µl purified PCR product, 3.2 µl of 2 µM of primer, 1 µl of BigDye reagent and 3 µl of sterile water per 1 well of 96-well plates. The cycle sequencing was performed using the 9700 PCR system (Applied Biosystems®, USA) at 96°C for 1 minute, 30 cycles of denaturing at 96°C for 15 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes.

After that, the excess dye terminators were removed by purification step using precipitation method. Eighty microliters of 75% isopropanol was added to the sequencing mix and incubated at 4°C overnight. The 96-well plate was centrifuged at 2,000 g for 45 minutes for precipitated DNA. Next, the 96-well plate was inverted and centrifuge again for 1 minute to discard the supernatant. The precipitated sequencing DNA was reconstituted by adding 10 µl of formamide solution and covered by 96-well plate septa. Then, the nucleotide sequences were analyzed using the automatic ABI Prism 3100/3130 Genetic Analyzer (Applied Biosystems, USA).

2.2.3.3. Analyzing and editing of nucleotide sequences

The nucleotide sequences were assembled and edited to correct the bases using Geneious R8 genetic analyzer (Biomatters, NZ) and compared with a HIV-1 reference sequence strain CM240.

2.2.3.4 Interpretation of drug resistance mutation

The drug resistance mutation was interpreted using the analyzed nucleotide sequences altogether with Stanford HIVdb Program Genotypic Resistance Interpretation Algorithm (Version 7.0 (last updated 02/27/14)) [106]. The FASTA formats of individual protease and reverse transcriptase sequences were submitted to a web service (http://sierra2.stanford.edu/ sierra/servlet/JSierra). After that, the program identified the mutations and returns inferred levels of resistance to protease and reverse transcriptase inhibitors. The levels of resistance are susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance to inhibitors.

2.2.4 HIV-1 genotyping

The analyzed nucleotide sequences of reverse transcriptase and protease region were determined the HIV-1 subtype by 2 different methods.

2.2.4.1 The Stanford HIVdb Program Genotypic Resistance Interpretation Algorithm

The drug resistance interpreted results from the program also provided the subtype results. This subtype results were collected and compared with REGA HIV-1 subtyping results.

2.2.4.2 REGA HIV-1 subtyping tool

All edited sequences were used for HIV-1 subtyping using the REGA HIV-1 automated subtyping tool version 3.0 (http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/). In REGA HIV-1 subtyping tool, phylogenetic method was used to identify the subtype of a specific sequence. And the recombination was analyzed using boot scanning methods.

2.2.5 Statistic analysis

The data were recorded in an electronic spreadsheet (Microsoft Excel, USA). The 95% confidence intervals (CI) including continuity correction were calculated

using programs STATA 10 (Stata Corporation, USA). Chi-square analysis was use in the comparison between groups including infant factors (Age, birth year/month, ARV regimen and HIV subtype) and maternal factors (ARV regimen) versus occurrence of drug resistance mutations were performed by using STATA 10 (Stata Corporation, USA). P-values of <0.05 were considered for determine the significant of association.

2.2.6 Ethical approval

This thesis was ethically approved by the Research Ethic Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Reference No. AMSEC-59EM-001)