CHAPTER III
RESEARCH DESIGN, MATERIALS AND METHODS

1. Study population

Origin of the subjects

The children who participated in the present CMV study were selected among infants born to HIV-1 infected mothers who participated in PHPT-1[91], a clinical trial testing four regimens of different zidovudine (ZDV) prophylaxis durations to prevent mother-to-child transmission of HIV-1 in Thailand. Briefly, PHPT-1 was a randomized, four-arm, double-blind study, comparing safety and efficacy of maternal and infant zidovudine regimens of various lengths. One thousand four hundred thirty seven women were randomized to receive 300 mg tablet bid ZDV from either 28 or 35 weeks gestation. All mother received one 300 mg tablet every 3 hours during labor and infants were randomized to receive ZDV orally for either 3 days or 6 weeks. All infants were formula fed. Infant blood draw visits were scheduled at birth, 6 weeks, 4 months, 6 months, 12 months, and 18 months. More information is available in the paper reporting the results of the clinical trial [91]

HIV-1 infected pregnant women and their infants were enrolled in 27 hospitals, located in 12 provinces in the northern, central, eastern, and southern regions of Thailand (Figure 5) from June 1997 to December 1999. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, the Ethic Committee of the Faculty of Medicine of Chiang Mai University, and the Institutional Review Board of Harvard School of Public Health. Informed consent was obtained from each woman.
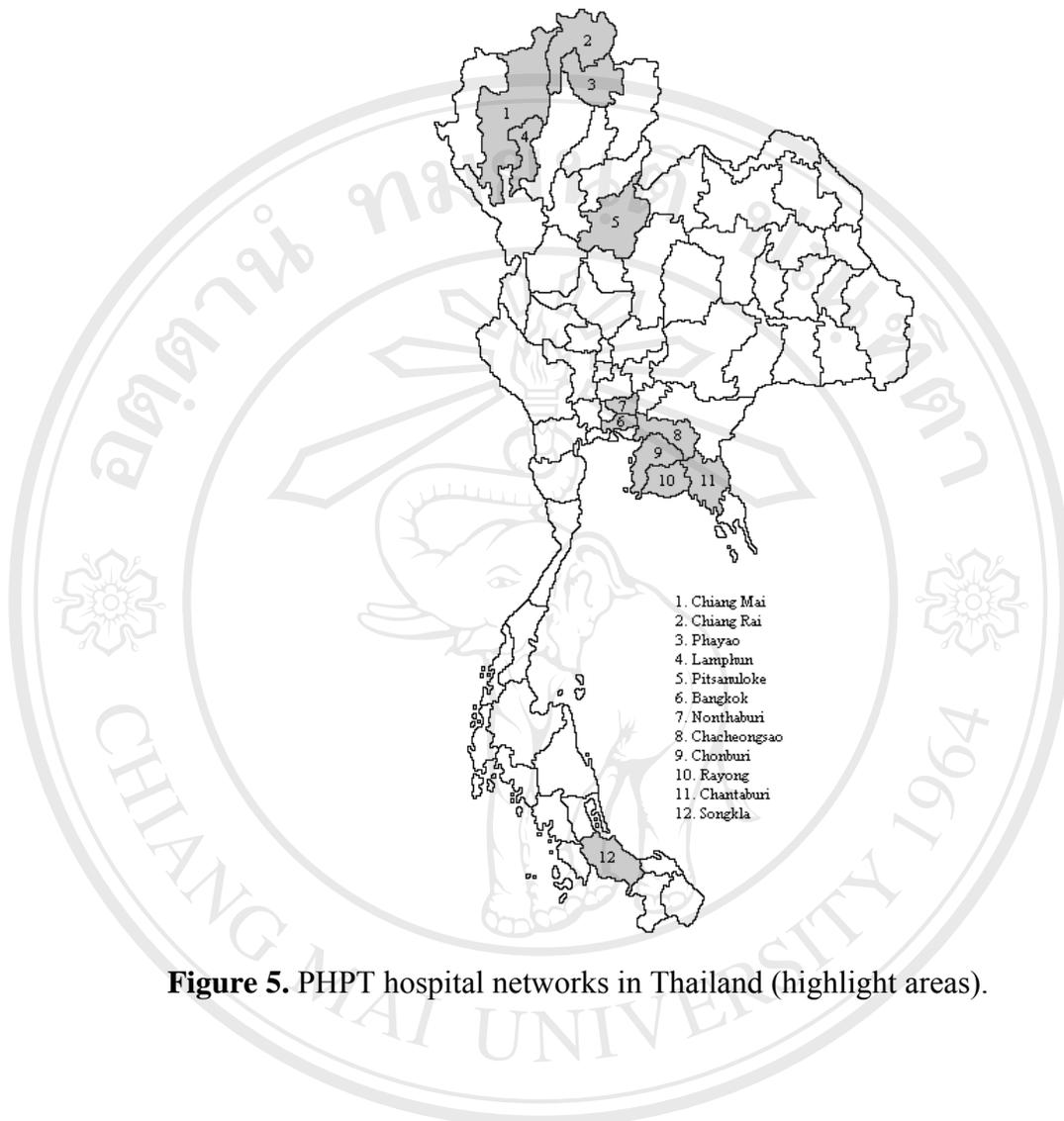
Figure 5. PHPT hospital networks in Thailand (highlight areas).

2. The study design

To compare the rate of HCMV infection in perinatally HIV infected and HIV uninfected children, a sample of children infected and uninfected was selected among the children born to HIV infected mothers who participated in PHPT-1.

The present study made use of the samples from all 97 infected children and those of 196 uninfected children matched (ratio of 1:2) to the infected children on the baseline maternal viral load measured before starting zidovudine prophylaxis.

The 293 infants selected for the present CMV study were:

- all 98 infants born to the 97 transmitting mothers, including a pair of twins discordant for their HIV status

- and 195 uninfected infants born to 194 non transmitting mothers matched on the maternal baseline viral load, including a pair of twins.

In summary, the total number of HIV infected children was 97 and the number of uninfected children was 196.

The HIV status of all infants born to HIV-1 infected mothers was determined using HIV DNA PCR testing (Roche Amplicor® HIV-1 DNA Test, Version 1.5) on blood draws performed at birth, 6 weeks, 4 months, 6 months, and a confirmation anti-HIV IgG antibody serology was performed at 18 months.

All mothers and infants's plasma samples were analysed for HIV-1 RNA quantitation using a commercially available RT-PCR assay with a detection limit of 400 copies/ml (Cobas Amplicor® HIV-1 monitor, Roche Diagnostic Systems, NJ). They also underwent serial immunologic evaluations for CD4+/CD8+ T cells by standard flow-cytometric techniques at local hospitals.

To determine HCMV infection in infants, all infants were tested at 18 months of age for HCMV IgG antibodies using an ELISA technique (Vironostika anti CMV II, BioMerieux, France). If a blood sample was not available at 18 months of age, anti-CMV IgM testing and/or HCMV DNA PCR testing were performed on any sample drawn at birth, 6 weeks, 4 months, or 6 months. All IgG antibodies positive cases were tested for IgM antibodies by ELISA technique (Vironostika anti CMV IgM II, BioMerieux, France) at birth, 6 weeks, 4 months and 6 months as well. If cell pellets were available, genomic DNA was extracted using the QIAamp kit (QIAgen, Germany) and then quantitated using the Hoefer DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). HCMV DNA detection was then performed using real time PCR technique (ABI PRISM 7000 Analyzer, Perkin-Elmer Biosystems). The schematic diagram is shown in Figure 6.

3. Sample collection

The samples were routinely collected at PHPT hospital networks (Figure 5) during the study. All samples were shipped to the Study Coordination Center laboratory (IRD URI 174/PHPT laboratory) at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University and stored at $-20^{o}C$ or $-70^{o}C$ until testing. Mother's blood samples were collected at the pre-entry visit of the HIV infected women in the clinical trial, subsequent study visits, and delivery visit. Infant's samples were collected at birth, 6 weeks, 4 months, 6 months, 12 months and 18 months of age. Plasma/serum samples were used for serology testing. Detection of HCMV DNA was performed from white blood cells contained EDTA pellets after plasma removal or on dried blood spot (DBS).
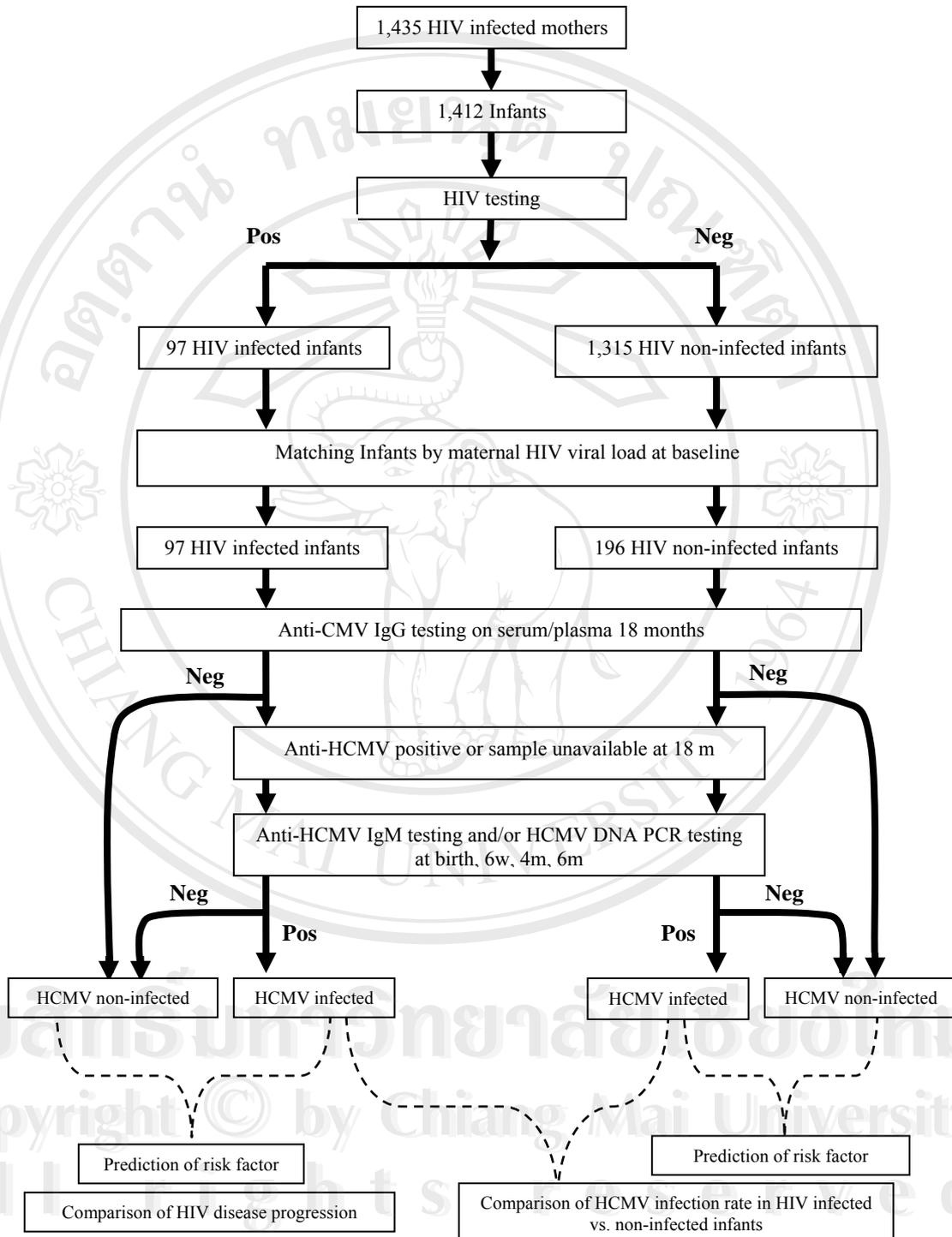
Figure 6. The schematic diagram of the research design in this study.

4. Determination of HCMV Infection

4.1. Detection of HCMV IgG antibodies by ELISA technique

Detection of HCMV IgG antibodies is based on a "sandwich ELISA" principle (Vironostika® anti CMV II, bioMerieux, France). Specifically, microwells coated with murine monoclonal anti-CMV constitute the solid phase. Inactivated HCMV antigen is added to the wells and a solid-phase antibody/antigen is formed. With the addition of a test sample (or control) containing anti-CMV, immune complexes are formed as the antibody binds to the solid phase antibody/antigen. After wash, sheep anti-human immunoglobulin conjugate labeled with horseradish peroxidase (HRP) is added. The conjugated antibody binds to the antibody/antigen/antibody complex during this incubation. Following incubation with tetramethylbenzidine (TMB) substrate, a blue color develops. The color reaction is stopped with sulfuric acid. Then, the color turns yellow, and its absorbance is measured at 450 nm. The intensity of the color is proportionate to the concentration of anti-CMV in the sample. The cutoff value could be calculated from a following formula:

$$\text{Cutoff value (COV)} = \frac{\text{Mean of negative control's absorbance} + \text{Mean of positive control's absorbance}}{2}$$

A test sample is reactive for total antibodies to CMV if the sample absorbance is higher than COV. In contrast, a test sample is nonreactive for total antibodies to CMV if the sample absorbance is lower than COV.

The manufacturer shows 99.5% of sensitivity and 99.7% of specificity.

4.2. Detection of HCMV IgM antibodies by ELISA technique

Detection of HCMV IgM antibodies is based on an antibody-capture "sandwich ELISA" principle (Vironostika®anti CMV IgM II, bioMerieux, France). Specifically, microwells coated with sheep antibody to human IgM constitute the

solid phase. The sample is added to a well and incubated. Any IgM antibody in the sample will bind to the solid phase antibody. Following, HCMV antigen and conjugate of sheep anti-CMV labeled with horseradish peroxidase (HRP) are added to the well and incubated. The antigen and the labeled antibody will bind to any of the solid phase anti-IgM/anti-CMV IgM complex previously formed. Following wash and incubation with tetramethylbenzidine (TMB) substrate, a blue color develops that turns yellow when the reaction is stopped with sulfuric acid. Then, the absorbance is measured at 450 nm. The intensity of the color is proportionate to the concentration of anti CMV IgM in the sample. The cutoff value could be calculated from a following formula:

$$\text{Cutoff value (COV)} = \frac{\text{Mean of negative control's absorbance + Mean of positive control's absorbance}}{2}$$

A test sample is reactive for anti CMV IgM if the sample absorbance is higher than COV. In contrast, a test sample is nonreactive for anti CMV IgM if the sample absorbance is lower than COV.

The manufacturer shows 87 % of sensitivity and 99 % of specificity.

4.3. HCMV DNA detection by real time PCR

4.3.1. DNA extraction from white blood cell pellets

After blood draw, EDTA-anticoagulated peripheral blood samples were separated into plasma and cell pellets, and stored at -20°C. Cell pellets were used for CMV DNA detection. DNA was extracted from cell pellets with the QIAamp DNA blood midi kit as recommended by the manufacturer (QIAGEN, Inc., Valencia, CA, USA). Briefly, 200 μL protease and 2.4 mL lysis buffer (AL buffer) were combined with 2.0 mL blood cell pellets (if the cells are clumped, add the appropriate volume of RPMI) and incubated at 70°C for 10 min. After incubation, 2.0 mL ethanol (96-100%) was added, and the entire mixture was applied to a column of silica gel filter

tube (QIAamp spin column). Then, QIAamp spin columns were centrifuged and were washed with washing buffer (AW buffer) twice to remove any PCR inhibitory residuals and other cellular proteins. The purified DNA was eluted from the column with 300 μL of provided elution buffer, and DNA concentration was determined by Hoefer DyNA Quant 200 Fluorometer. Before the real time PCR assay, extracted DNA was adjusted to the concentration of 50 μg/mL. A 20 μL volume representing 1 μg extracted DNA was used in each PCR mixture.

4.3.2. Real time PCR probe and primer

Primers and probe targeting *UL123 exon 4* gene [92] were designed by using the Primer Express 1.0 Software (Applied Biosystems). *UL123* gene encodes for HHV5gp109 IE1, immediate-early transcriptional regulator protein (Figure 7). The sequences of the forward and reverse primers, which amplified a 74 bp product, are 5'-2791 AGC GCC GCA TTG AGG A 2806-3' and 5'-2833 CAG ACT CTC AGA GGA TCG GCC2853-3', respectively. Sequence of the probe is 5'-2808 ATC TGC ATG AAG GTC TTT GCC CAG TAC ATT 2827-3' (Table 2). The Taqman probe was labeled at the 5'end with 6-carboxyfluorescin (FAM) as the reporter dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher dye (Table 2). PCR mixture consisted of 25 μL of 2X TaqMan Universal PCR master mixture (Perkin-Elmer Biosystems), each of the primer at a concentration of 25 nM, (0.5 μL), and 20 nM TaqMan probe (0.5 μL), in a total volume of 30 μL/reaction. Then, 20 μL of extracted DNA of sample (1 μg) was added. PCR was performed in 96-well microtiter plates under the following condition: after 2 minutes at 50°C and 10 minutes at 95°C, the samples were submitted to 50 cycles, with each cycle consisting of a step at 95°C for 15 seconds, followed by a step at 60°C for 1 minute. PCR product was detected as an increase in fluorescence using ABI PRISM 7000 instrument (Perkin-Elmer Biosystems).

A plasmid containing the amplified sequence of AD169 strain was constructed with the pADV TA cloning kit (Clontech, Saint Quentin en Yvelines, France). This

plasmid was used as a HCMV positive control and a part of PCR inhibition control. The results were reported as positive or negative result.

Controls of PCR assay included positive control, negative control, and inhibition control.

For positive control, 20 μL of extracted HCMV DNA at the concentration of 2,000 copies/mL were tested in duplicate for each CMV PCR assay

To exclude false-positive PCR results, 20 μL of water instead of DNA was tested in duplicate for each CMV PCR assay (negative control).

To exclude false-negative PCR results, the following inhibitor control procedure was applied. The CMV PCR assay of each sample consisted of 2 PCR experiments which contained: i) 20 μL of sample DNA; ii) 15 μL of sample DNA, and 5 μL of HCMV plasmid concentration of at 2,000 copies/mL (inhibition control). The positive result of PCR experiment containing HCMV plasmid confirmed an absence of PCR inhibitor.

The test was valid only if the negative control was negative and the positive control revealed a clear positive signal whereas no PCR inhibition was shown.
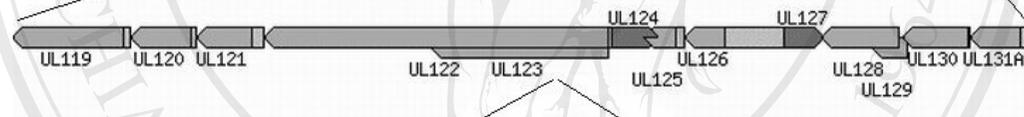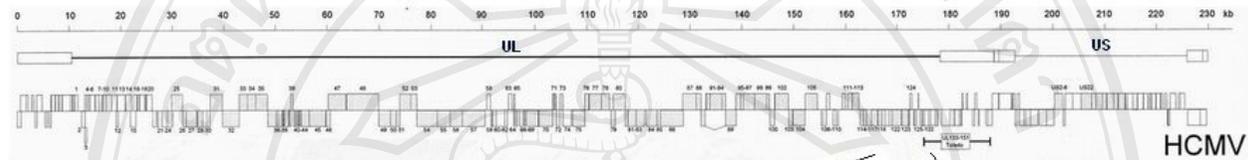
Figure 7. The location of HCMV DNA target for amplification.

Table 2. HCMV PCR primers and Taqman® probe information.

Name	Sequence (5' to 3')	Length (bp)	Product length (bp)
Primer CMV IE1	**5'**-AGC GCC GCA TTG AGG A-**3'**	16	74
Primer CMV IE3	**5'**-CAG ACT CTC AGA GGA TCG GCC-**3'**	21	
CMV probe	**5'**-**FAM**-ATC TGC ATG AAG GTC TTT GCC CAG TAC ATT-**TAMRA-3'**	30	-

4.3.3. Preparation of HCMV positive control

A known amount of HCMV DNA (AD169 laboratory strain) was prepared for use as controls in all experiment. HCMV recombinant plasmid was kindly provided by Dr. Marianne Leruez-Ville of Laboratoire de Virologie, CHU Necker-Enfants Malades, Paris, France. This plasmid was amplified by the gene cloning technique.

4.3.3.1. Transformation of the recombinant plasmid DNA

The recombinant plasmid was transformed into the high efficiency competent *E. coli* cells (JM109 strain, Promega, USA). The transformation was carried out in accordance with the protocol recommended by manufacturer. Briefly, 6 μL of plasmid CMV (10^8 copies) was added into 30 μL of the competent cells in a sterile 1.5 mL microcentrifuge tube on ice. The tube was flicked to mix the content and place on ice for 20 minutes. Then, the cells were heat shocked at exactly 42 ^{o}C in the waterbath for 50 seconds, and the tubes were returned immediately to the ice bath for 2 minutes. Nine hundred and fifty microlitres of SOC medium, a rich media used primarily in the recovery step of *E. coli* competent cell transformations, were added into the reaction tube containing cells transformed with ligation reaction, and incubated for 3 hours at 37 ^{o}C while slowly shaking (~150 rpm). One hundred microlitres of transformation

culture medium were placed onto a Luria-Bertani (LB) plate, a nutritionally rich medium, containing 100 μg/mL of ampicillin and incubated at 37 ^{o}C overnight. After incubation, the bacterial colonies were screened for the presence of recombinant plasmid. Only transformed colonies had grown as it was containing of ampicillin resistant gene.

4.3.3.2. Screening for the recombinant plasmid DNA in the transformed bacteria

The randomly selected colonies were directly subjected to the conventional PCR assay (see appendix) for detecting of HCMV DNA fragment. Then, HCMV amplified product was demonstrated in 2% agarose gel electrophoresis (see appendix). Only colonies that showed PCR products specific to the HCMV gene were confirmed as successfully transformed.

4.3.3.3. Purification of HCMV recombinant plasmid

To obtain HCMV DNA standard, the HCMV gene fragment was prepared from transformed colonies by using the HiSpeed Plasmid Purification Kit (QIAgen, Germany). The purification procedure was performed as recommended in the manufacturer's instruction manual. Briefly, the confirmed colony was picked from a freshly streaked selective plate and inoculated into a starter culture of 2.0 mL LB medium containing 0.1 mg/mL of ampicillin, then incubated for 8 hours at 37 ^{o}C with vigorous shaking (~150 rpm) The 0.3 mL of starter culture was diluted into 150 mL of selective LB medium, and growth at 37 ^{o}C for overnight with vigorous shaking (~150 rpm). The bacterial cells were harvested by centrifugation at 6,000X g for 15 minutes at 4 ^{o}C. The bacterial pellet was resuspended in 90 mL of buffer P1 (resuspension buffer: 50 mM Tris-Cl, pH8.0; 10mM EDTA; 100 μg/mL RNase A). The mixture was added to 10 mL of buffer P2 (lysis buffer: 200 mM NaOH; 1% SDS (w/v)), mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 minutes. The lysate was added to 10 mL of chilled buffer P3 (neutralization buffer: 3.0 M potassium acetate, pH 5.5), and mixed immediately but gently by inverting 4-6

times. The lysate was poured into the barrel of the QIAfilter Maxi Cartridge, and incubated at room temperature for 10 minutes. The Hispeed Maxi tip was equilibrated by applying 10 mL of buffer QBT (equilibration buffer: 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)) and column was allowed to empty by gravity flow. The cap was removed from the QIAfilter outlet nozzle, the plunger was gently inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into the Hispeed Maxi tip previously equilibrated. The cleared lysate was allowed to enter the resin by gravity flow. The Hispeed Maxi tip was rinsed with 60 mL of buffer QC (wash buffer: 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)). The DNA was eluted with 15 mL of buffer QF (elution buffer: 1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol (v/v)). The eluted DNA was precipitated by adding 10.5 mL of isopropanol, mixed and incubated at room temperature for 5 minutes. The eluate/isopropanol mixture was transferred into QIAprecipitator Maxi syringe, then mixture was washed with 2 mL of 70% ethanol. The membrane was air-dried, and the DNA was redissolved in 1 ml of TE buffer. Finally, the purified DNA containing the HCMV gene fragment was confirmed by PCR.

4.3.3.4. Quantitation of the purified HCMV plasmid DNA by spectro-photometry

The concentration of the purified HCMV plasmid DNA was determined using the spectrophotometry method. The DNA was diluted in distilled water at an appropriate dilution and the optical density (O.D.) was then measured for nucleic acid and protein at a wavelength of 260 and 280 nm, respectively, using a UV spectrophotometer (Shimadz model 1101, Japan). The quantity of DNA was calculated using the following equation:

Quantity of DNA (ng/μL) = O.D.$_{260}$ x dilution factor x 1 O.D.$_{260}$ unit of dsDNA conc.

= O.D.$_{260}$ x dilution factor x 50

1 O.D.$_{260}$ unit of double stranded DNA concentration (dsDNA conc.) = 50 ng/μL

The purity of HCMV plasmid DNA preparation could be determined by the O.D. 260/280 ratio, which fell between 1.7-1.9 and constituted a purified DNA. If the value had been lower, the preparation would have contained contaminants (e.g., protein or phenol).

The purified HCMV plasmid DNA concentration 50 ng/μL was calculated to convert into a number of HCMV copies by using the mathematical correlation and formulas as follows:

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/μl)}}{\text{MW (g/mol)}} = \text{amount (copies/μL)}$$

MW = number of base pairs x 660 daltons/base pair
1 mol = 6×10^{23} molecules (copies)

4.3.3.5. Quantitation of extracted DNA by the fluorescence assay

The concentration of extracted DNA was measured by DNA fluorescence assay using the Hoefer DyNA Quant 200 fluorometer. Bisbenzimide, commonly know as Hoechst 33258 (H 33258) dye, exhibits changes in fluorescence characteristics in the presence of DNA that allow accurate DNA quantitation. In the absence of DNA, the excitation spectrum of H 33258 peaks at 356 nm and the emission spectrum peaks weakly at 492 nm. When H 33258 binds to DNA, these peaks shift to 365 nm and 458 nm. In the cuvette well, the sample is exposed to filtered light (365 ± 7 nm) from a mercury lamp. This light excites the DNA-dye

complex, causing light that peaks at 458 nm to be emitted. An emission filter in front of the photodetector allows only fluorescence at 460 nm, ±15 nm, to register. Thus, the measured fluorescence is a direct indicator of the DNA concentration. (H 33258 binds to the minor groove of DNA). When 365 nm light (long UV) excites this bound dye, its fluorescence at 458 nm can be measured.)

Two microlitres of Calf thymus DNA standard (fluorometry standard at concentration of 100 ng/µL) and sample DNA was diluted in 2 ml of assay solution that had H33258 dye in the component. Then, the light (458 nm) emitted from the DNA-H33258 dye complex in the mixture was measured by the Hoefer DyNA Quant 200 fluorometer. The quantity of DNA was calculated by the Hoefer DyNA Quant 200 fluorometer using the following equation:

$$\text{Quantity of DNA (ng/µL)} = \frac{\text{O.D.}_{458} \text{ of sample x standard concentration (100 ng/µL)}}{\text{O.D.}_{458} \text{ of standard}}$$

5. Categorization of HCMV infection

Four categories were considered with regarding to HCMV infection in infants born to HIV-1 infected mothers.

i) Infants were defined as HCMV infected when a positive HCMV IgG result at 18 months of age, and/or a positive for HCMV IgM or HCMV DNA testing at any age.

ii) HCMV uninfected infants were defined as a negative HCMV IgG result at 18 months of age or later.

iii) Infants without IgG serology available at 18 months, and without a HCMV IgM or HCMV DNA result available after 6 months of age, were categorised into the group of HCMV uninfected infants until 6 months. The reasons were due to the lack of samples, infants died or lost to follow up after 6 months.

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iv) Infants who exhibited an indeterminate result for IgG at 18 months were categorised into the group of infants with indeterminate HCMV status, they were assumed to have HCMV late infection.

6. Determination of HIV disease progression

The 1994 revised classification system for HIV infection in infants less than 13 years of age from the Center for Disease Control and Prevention (CDC), USA [93] was used to classify infants according to the immunologic and clinical stage of HIV-1 disease (see appendix). HIV-1 disease progression is defined as the presence of class C symptoms or CD4+T cell counts less than 750 cells/μL by 1 year of age and less than 500 cells/μL by 18 months of age, or starting anti-retroviral drug treatment, or death by 18 months of age,
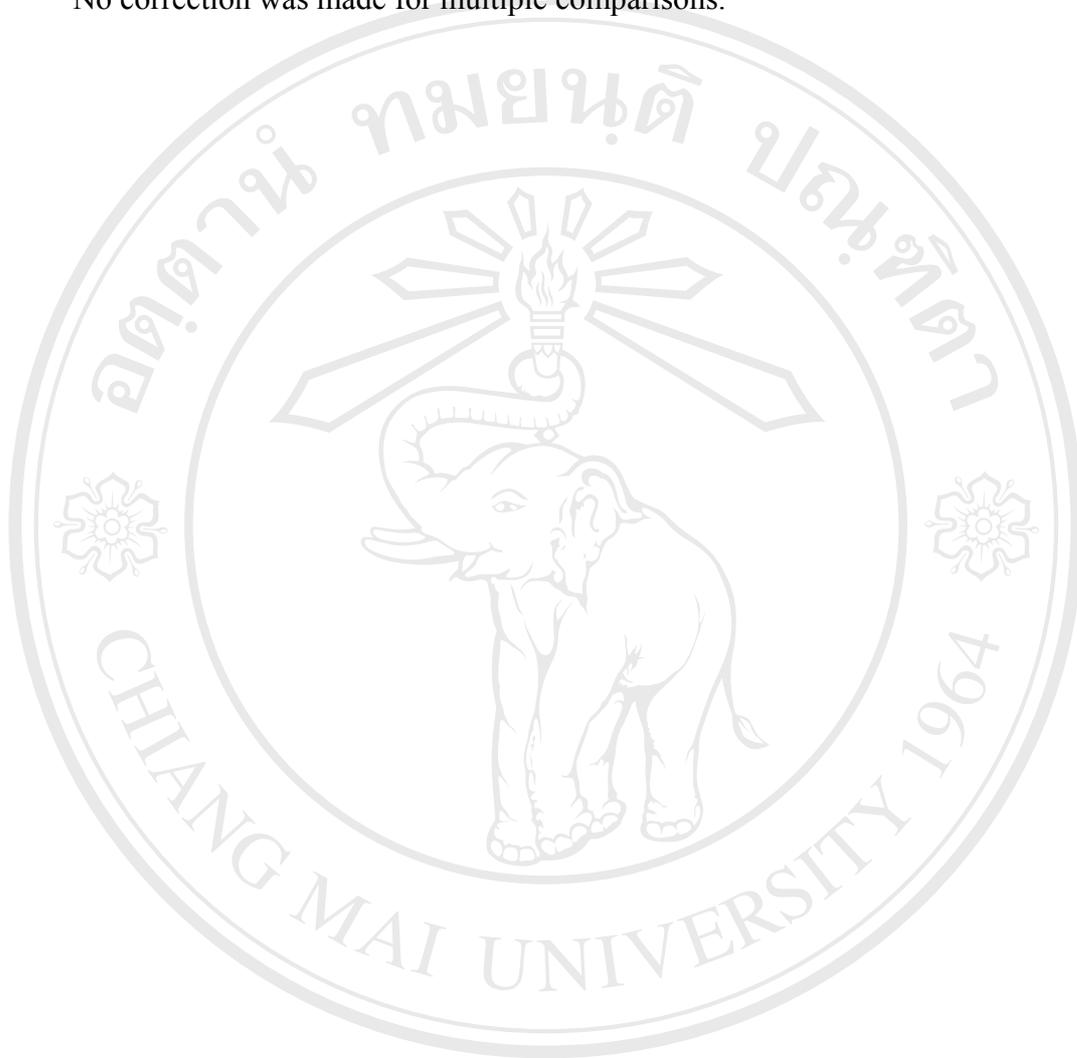
7. Statistical analysis

The association between congenital HCMV infection and perinatal HIV infection was tested using Fisher's exact test. Odds ratios and 95% CI were computed as a measure of the association.

The analysis of the association between potential risk factors and HCMV transmission to infants made use of Fisher's exact test. Continuous variables, including maternal age, gestational age at delivery, duration time of ZDV prophylaxis, total lymphocyte count, CD4, CD8, infant birth weight were dichotomized using the overall sample median and frequency in uninfected and infected children were compared using Fisher's exact test.

To study the age at acquisition of HCMV infection, Kaplan-Meier estimates of the infant's maximal age at first diagnosis of HCMV IgM or DNA were computed. Comparisons were made using the log rank test.

The relationship between HIV disease progression and HCMV co-infection was studied using Kaplan-Meier estimates of HIV disease progression-free survival in HCMV infected and uninfected infant at 6 weeks, 4 months, 6 months and 18 months of age and the log rank test.

All statistical analysis was performed by using STATA™ 8.0 software (Texas, USA). Difference were considered statistically significant if the P-value was <0.05. No correction was made for multiple comparisons.