CHAPTER IV
RESULTS

1. Preparation of standard HCMV gene fragment control using PCR cloning method

The HCMV IE gene fragment was cloned into the plasmid using the pADV TA cloning kit (Clontech, Saint Quentin en Yvelines, France). The recombinant plasmids were transformed into competent *E. coli* grown on selected Ampicillin medium. The transformed colonies were then tested using the conventional PCR assay. A part of the well-isolated transformed colony was added directly to the PCR mixture for an amplification of the IE gene fragment by using primers CMV IE1 and CMV IE3. The PCR products were detected on 2% agarose gel electrophoresis and the ethidium bromide staining. The successfully transformed cells provided an approximately 74 bp PCR product of HCMV IE gene fragment. Ten colonies were picked up and screened for the presence of HCMV IE gene fragment. All selected colonies contained the expected HCMV IE gene fragment as shown in Figure 8.

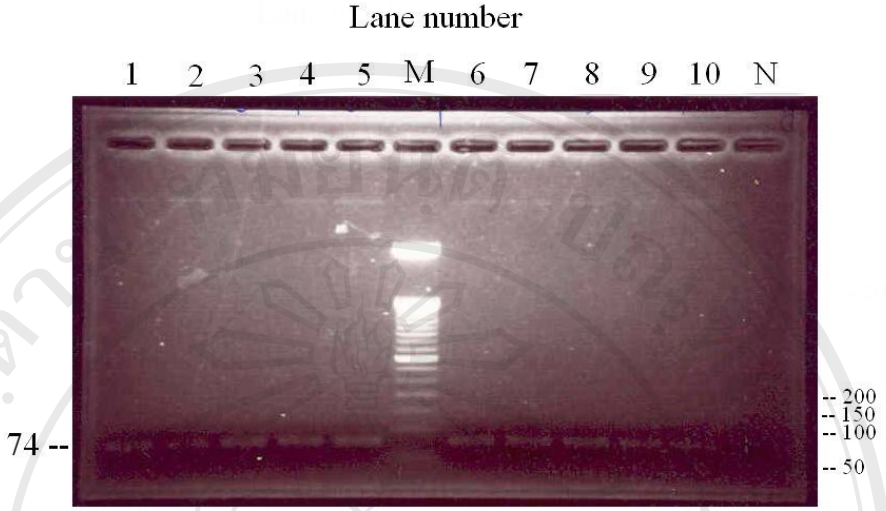

Figure 8. Screening of successfully transformed bacteria. HCMV IE gene fragment from transformed *E. coli* was amplified using conventional PCR. Lane M shows the 50 bp DNA ladder (ranged from 50-800 bp, 16 bands in 50 bp increments), lane 1 to lane 10 show the 74 bp PCR products of HCMV IE gene fragment from different bacterial colonies, and lane N shows the negative control.

Successfully transformed colony no.1 and no.2 were selected. Selected colonies were inoculated to 150 mL of LB medium for a large scale production. Then, plasmid DNA was purified from the transformed *E. coli* by the HiSpeed™ Plasmid Purification Kit. To confirm the presence of HCMV IE gene fragment after large scale production, the purified DNA after 1:100 and 1:10,000 dilution was subjected to the conventional PCR for HCMV IE gene fragment detection. The presence of HCMV IE gene fragment insert in no.1 and no.2 was detected as the band at expected 74 bp size, as shown in Figure 9.

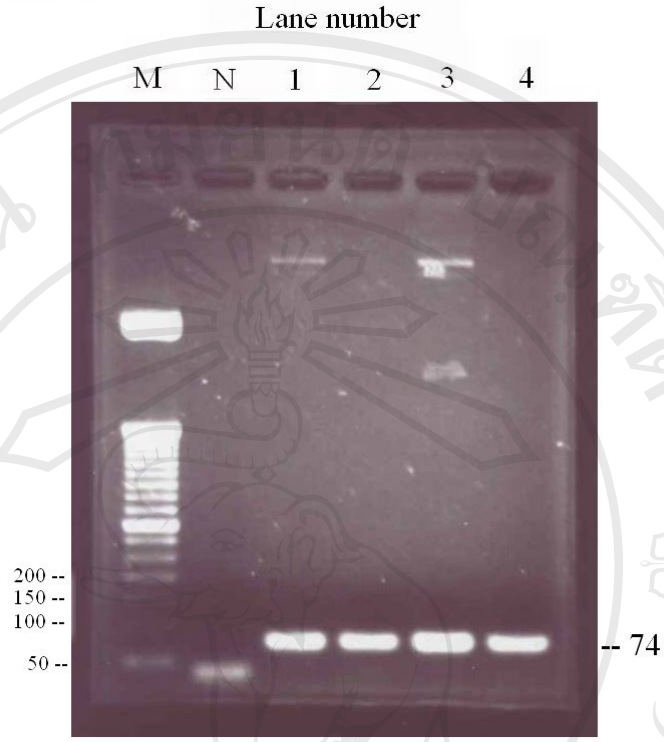

Figure 9. The confirmation of the presence of HCMV IE gene fragment in purified plasmid DNA after large scale production by conventional PCR. Lane M shows the 50 bp DNA ladder (ranged from 50-800 bp, 16 bands in 50 bp increments), lane N shows a negative control. Lane 1 and 2 represent for transformed *E. coli* colony no.1 at concentration of 1:100 and 1:10,000, whereas lane 3 and 4 represent for transformed E. coli colony no.2 at concentration of 1:100 and 1:10,000, respectively. All plasmid DNA in lane 1 to lane 4 show the 74 bps of amplified HCMV IE gene fragment.

Thus, the purified DNA from *E. coli* colony no.1 was used as the standard HCMV IE gene fragment control throughout this study.

The quantity of standard HCMV IE gene fragment control was 890 ng/μL when measured by spectrophotometric method. The calculation was shown as following:

The absorbance at 260 nm = 0.178

The absorbance at 280 nm = 0.097

Therefore,

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

= 0.178 x 100 x 50

= 890 ng/μL

The purity of DNA could be determined by the ratio of the O.D. at 260 nm and 280 nm. The O.D. ratio of DNA with good purity should not be lower than 1.65. Hence, the purity of this DNA preparation was acceptable, as the O.D. ratio was found to be 1.835.

The copy number of the pADV TA vector plasmid with HCMV IE DNA insert was calculated. The amount of this plasmid DNA, at a concentration of 890 ng/μL, was found to be approximately 2 x 10^{11}copies/μL. the following mathematical correlation and formulas were used for amount estimation.

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

MW = number of base pairs x 660 daltons/base pair

1 mol = 6x10^{23} molecules (copies)

Then,

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times 8.9 \times 10^{-7} \text{ (g/μL)}}{2.63 \times 10^{6} \text{ (g/mol)}} = 2.03 \times 10^{11} \text{ copies/μL}$$

$$\approx 2 \times 10^{11} \text{ copies/μL}$$

2. HCMV infection in infants born to HIV-1 infected mothers

2.1. Characteristics of the population

Among the 1,437 HIV-1 positive pregnant women who had been enrolled in PHPT-1 study, 291 women were included in this study. They were from different regions of Thailand, more than 75% living in north and east region. Thirty-eight percent of women were housewife. Among the 62% of women, most of them have difficult working conditions and poorly social-economic. Most of them have lower education level (Table 3)

Table 3. Mother's characteristic descriptions.

	Number of patients
Region	
Central	55 (19 %)
East	83 (28 %)
North	148 (51 %)
South	5 (2 %)
Total	291 (100 %)
Employment	
Farmer	50 (17 %)
Company employee	21 (7 %)
Commerce	28 (10 %)
Labour	77 (26 %)
Hairdresser/tailor	5 (2 %)
Housewife	109 (38 %)
Total	290* (100 %)

(Continued)

Table 3. Mother's characteristic descriptions *(Continued).*

	Number of patients
Education level	
Lower than primary school	75 (26 %)
Primary school	125 (43 %)
Secondary school	52 (18 %)
High school	20 (7 %)
College	11 (4 %)
University	4 (1 %)
Others	1 (0.4 %)
Total	288** (100 %)

*1 missing data ** 3 missing data

Two hundred and ninety-one mothers gave birth with 293 infants, including 2 mothers who gave twin birth. Median maternal HIV viral load was 4.38 $\log_{10}$, with interquartile range (IQR) of 3.97 to 4.81 $\log_{10}$ copies/mL. The mothers received zidovudine prophylaxis for a median of 79 days (IQR: 71 – 87 days).

Among 239 infants, 97 infants were HIV-1 infected and 196 infants were HIV-1 uninfected. Among 97 HIV-1 infected infants, 43% were male, whereas 57% were female. Among 196 HIV-1 uninfected infants, 55% were male, and 45% were female. The median of birth weight in HIV-1 infected infants was 2,800 g, whereas in HIV-1 uninfected infants the median of birth weight was 2,950 g (Table 4.)

Table 4. Infant's characteristic descriptions.

	HIV-1 infected infant	**HIV-1 uninfected infant**
Sex		
Male	42 (43 %)	108 (55%)
Female	55 (57 %)	88 (45%)
Birth weight		
n	77*	157**
Median	2,800 g	2,950 g
IQR***	2,500-3,100 g	2,750-3,200 g

* 20 missing data,** 39 missing data, *** Interquartile Range

2.2. The rate of HCMV infection in infants within 18 months of age

Among 293 infants born to HIV-1 positive mothers, 97 infants were HIV-1 infected, whereas 196 infants were HIV-1 uninfected. Among 97 HIV-1 infected infants, 72 infants (74%) were infected with HCMV. Thirteen infants (13%) were HCMV uninfected within 18 months of age, 9 infants (9%) had negative results until the age of 6 months, and 3 infants (3%) were indeterminate at 18 months.

Among 196 HIV-1 uninfected infants, the rate of HCMV infection was 60% (117 of 196). Seventy infants (36%) were HCMV uninfected within 18 months of age, 3 infants (2%) had negative data until the age of 6 months, and 6 infants (3%) were indeterminate at 18 months. Results are presented in Table 5.

Table 5. Rates of HCMV infection in HIV-1 infected and HIV uninfected infants within 18 months of age.

	HCMV infection within 18 months of age				
	Positive (%)	Negative (%)	Negative until 6 mo. (%)	Indeterminate at 18 mo. (%)	Total (%)
HIV-1 infected	72 (74)	13 (13)	9 (9)	3 (3)	97 (100)
HIV-1 uninfected	117 (60)	70 (36)	3 (2)	6 (3)	196 (100)
Total	189 (65)	83 (28)	12 (4)	9 (3)	293 (100)

Only positive or negative results of HCMV infection within 18 months of age were compared between HIV-1 infected and HIV-1 uninfected infants. Fisher's exact analysis was used because the number of subjects was small.

The rate of HCMV infection in HIV-1 infected infants was significantly higher than HIV-1 negative infants (72/85 (85%) vs. 117/187 (63%), p=0.0003). The risk of being infected with HCMV was approximately 3 times higher when a child was infected with HIV-1, with an exact 95% confidence interval from 1.66 to 6.98 (Odds ratio = 3.31, 95%CI = 1.66-6.98) (Table 6).

Table 6. Comparison of HCMV infection in HIV-1 infected and uninfected infants within 18 months of age.

	HCMV infection within 18 months of age			
	Positive (%)	Negative (%)	Total (%)	*p*-value
HIV-1 infected	72 (85)	13 (15)	85 (100)	0.0003*
HIV-1 uninfected	117 (63)	70 (37)	187 (100)	
Total	189 (69)	83 (31)	272 (100)	

* Fisher's exact analysis was used. *(Computed via http://www.matforsk.no/ola/fisher-.htm)*

2.3. Congenital HCMV infection in infants

Congenital HCMV infection in infant was defined as the detection of viruses or viral DNA in blood or the presence of immunoglobulin (Ig) M within the newborn's first 2 weeks of life. It was found that the prevalence of congenital HCMV infection in the group of HIV-1 infected infants (17%) was statistical significantly higher than the group of HIV-1 uninfected infants (5%), $p = 0.012$ (Table 7).

The risk of being congenital infected with HCMV in HIV-1 infected was estimated to be 4 times higher than HIV-1 uninfected infants (Odds ratio = 4.23, 95%CI = 1.26 – 16.24).

Table 7. Comparison of congenital HCMV infection in HIV-1 infected and HIV-1 uninfected infants.

	Congenital HCMV infection (<2 weeks)			
	Positive (%)	Negative (%)	Total (%)	*p*-value
HIV-1 infected	11 (17)	52 (83)	63 (100)	0.012*
HIV-1 uninfected	5 (5)	100 (95)	105 (100)	
Total	16 (10)	152 (90)	168 (100)	

* Fisher's exact analysis was used. *(Computed via http://www.matforsk.no/ola/fisher-.htm)*

2.4. HCMV infection in twins

Two women gave birth to twins born through vaginal delivery. In one twin, the first newborn was infected with HIV-1 and had a positive IgG HCMV serology at 18 months, whereas the second newborn was uninfected with HIV-1 and showed an

indeterminate result for HCMV serology at 18 months. For the other twins, both newborns were found to be uninfected with HIV-1 and HCMV.

2.5. Evaluation of risk factors associated with HCMV perinatal transmission in infants born to HIV-1 infected mothers

In order to determine the risk factors associated with HCMV infection in HIV-1 infected infants and uninfected infants. Several maternal and infant parameters were analysed. The median of each continuous variable was used to divide 2 groups of subjects.

2.5.1. Maternal risk factors

In the group of HIV-1 infected infants, the rate of HCMV infection in infants born with vaginal delivery was significantly higher than infants born with caesarian section delivery mode (90% vs. 65%, p=0.019). This association with mode of delivery was also observed in the group of HIV-1 uninfected infants (66% vs. 49%, p=0.046).

Moreover, among HIV-1 infected infants, there was a trend toward higher rate of HCMV infection when mother had lower gestational age at delivery (30.3-39.9 weeks) versus higher gestational age (39.4-44.0 weeks), (91% vs. 75%, *p*=0.07).

There was no statistically significant association between HCMV infection and other maternal parameters including the age of mothers, the previous pregnancies, pregnancy complication (such as premature rupture of membrane, premature labor requiring tocolysis, pre-eclampsia, cystitis e.g.), inducing labor, combination with other sexual transmitted diseases (including genital herpes, gonorrhea, trichomonas vaginalis, warts, syphilis, chancroid, vaginal candidiasis, bacterial vaginosis), duration of receiving ZDV prophylaxis, absolute number of lymphocyte, absolute number of CD4, and absolute number of CD8 in both HIV-1 infected and uninfected infants (Table 8, Figure 10, and 11).

Table 8. Maternal risk factors associated with HCMV transmission in HIV-1 infected and HIV-1 uninfected infants.

Risk factors	HCMV infection					
	in HIV-1 infected infants			in HIV-1 uninfected infants		
	Yes	No	*p* value*	Yes	No	*p* value*
Maternal factors						
Age**						
15.5-24.8 years	39 (85%)	7 (15%)	1.00	60 (66%)	31 (34%)	0.37
24.9-43.0 years	33 (85%)	6 (15%)		57 (59%)	39 (41%)	
Previous pregnancies						
0	31 (86%)	5 (14%)	1.00	46 (64%)	26 (36%)	0.88
>1	41 (84%)	8 (16%)		71 (62%)	44 (38%)	
Pregnancy complication						
Yes	12 (85%)	2(15%)	1.00	12 (67%)	5 (33%)	0.80
No	60 (85%)	11 (15%)		105 (62%)	64 (38%)	
Inducing labor						
Yes	10 (83%)	2 (17%)	1.00	8 (62%)	5 (38%)	1.00
No	62 (85%)	11 (15%)		109 (63%)	63 (37%)	
Delivery type						
Vaginal	61 (90%)	7 (10%)	0.019***	97 (66%)	49 (34%)	0.046***
Caesarian	11 (65%)	6 (35%)		20 (49%)	21 (51%)	
Gestational age at delivery**						
30.3-39.3 week	48 (91%)	5 (9%)	0.07	62 (63%)	37 (37%)	1.000
39.4-44.0 week	24 (75%)	8 (25%)		55 (63%)	33 (37%)	
Other STDs						
Yes	6 (86%)	1 (14%)	1.00	9 (75%)	3 (25%)	0.54
No	66 (85%)	12 (15%)		108 (62%)	66 (38%)	
ZDV duration**						
16-79 days	45 (87%)	7 (13%)	0.55	53 (62%)	33 (38%)	0.88
80-113 days	27 (82%)	6 (18%)		64 (63%)	37 (37%)	
Abs.lymphocyte**						
533-1,850 cells/μL	33 (92%)	3 (8%)	0.22	59 (60%)	39 (40%)	0.55
1,851-4,692 cells/μL	39 (80%)	10 (20%)		58 (65%)	31 (35%)	

(Continued)

Table 8. Maternal risk factors associated with HCMV transmission in HIV-1 infected and HIV-1 uninfected infants *(Continued).*

Risk factors	HCMV infection					
	in HIV-1 infected infants			in HIV-1 uninfected infants		
	Yes	No	*p* value*	Yes	No	*p* value*
Abs.CD4**						
10-330 cells/μL	40 (91%)	4 (9%)	0.13	55 (63%)	34 (38%)	1.00
340-1,190 cells/μL	29 (76%)	9 (24%)		58 (62%)	35 (38%)	
Abs.CD8**						
120-910 cells/μL	24 (89%)	3 (11%)	0.73	45 (60%)	31 (41%)	0.60
920-3,940 cells/μL	33 (83%)	7 (17%)		38 (64%)	21 (36%)	

* Fisher's exact analysis was used.

** Continuous variables were dichotomized at the overall sample median.

*** Statistically significant.

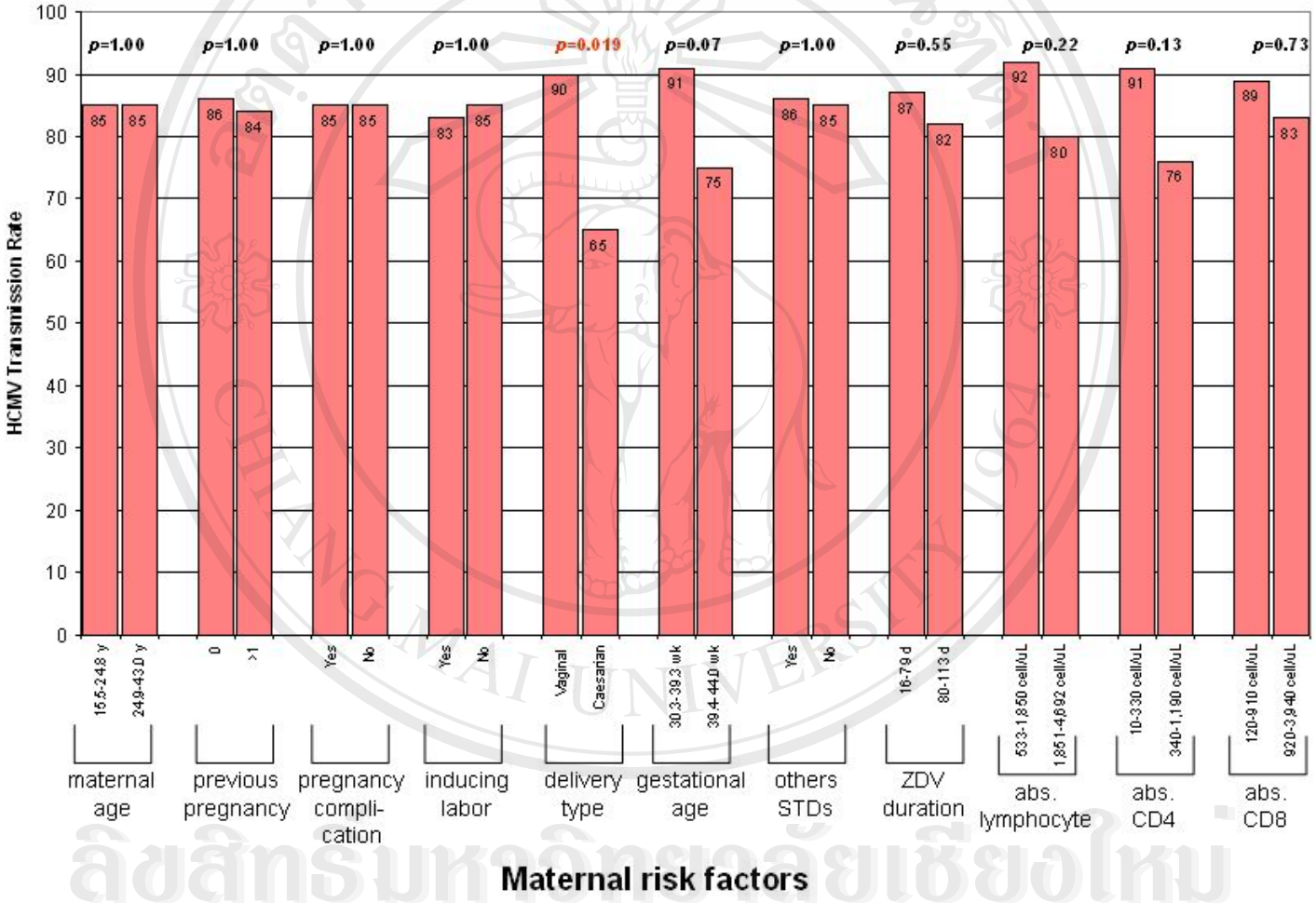

Figure 10. Maternal risk factors associated with HCMV transmission in HIV infected infants.

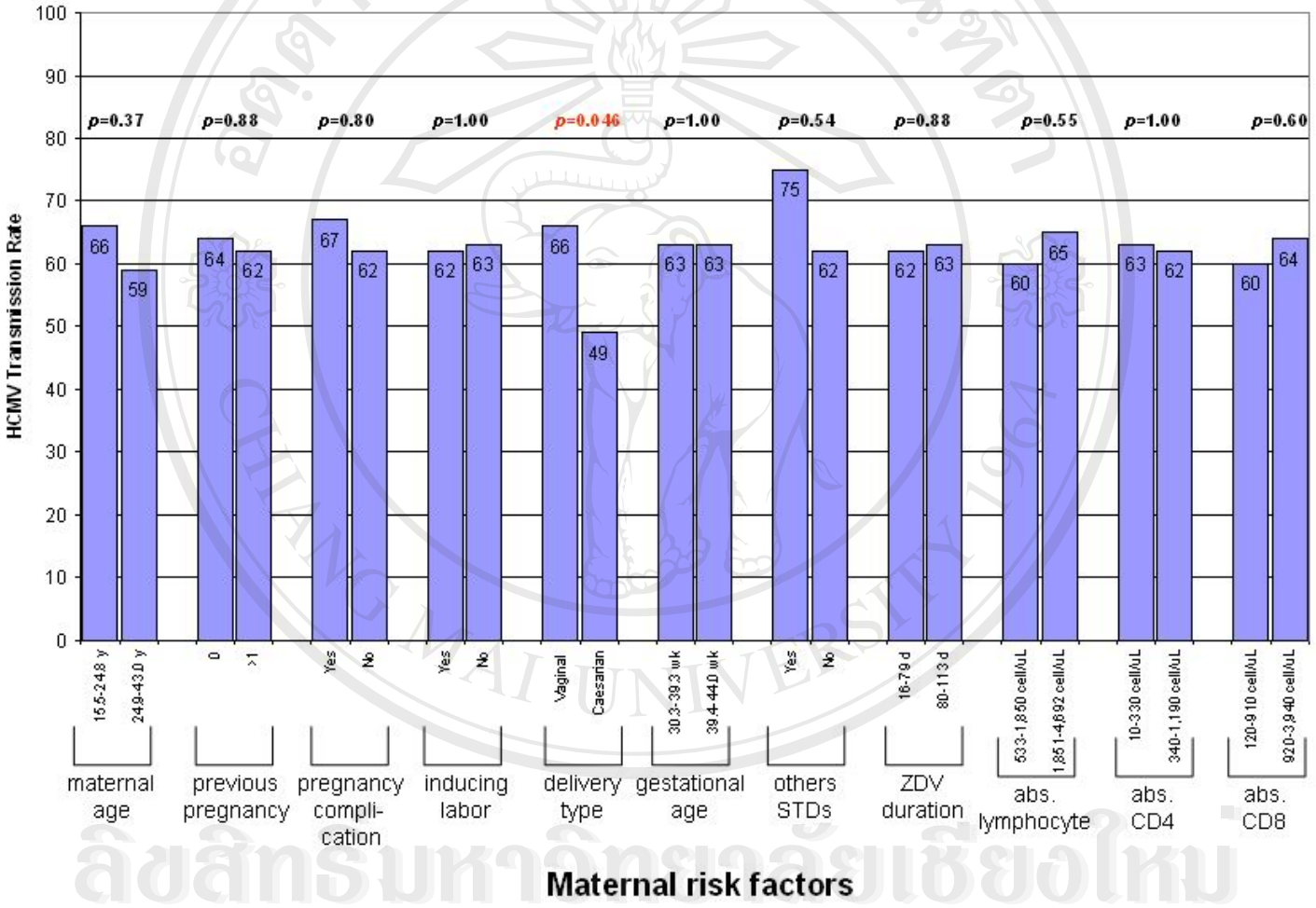

Figure 11. Maternal risk factors associated with HCMV transmission in HIV uninfected infants.

2.5.2. Infant's risk factors

Among HIV-1 infected infants, the rate of HCMV infection is significantly higher in those with low birth weight (lower than median, < 2,910g) than in those with normal birth weight (over the median of 2,910g; 96% vs. 73%, p=0.005). However, in HIV-1 uninfected infants, the rate of HCMV infection was similar in low and normal birth weight groups (62% vs. 63%, p=1.00). The rate of HCMV infection was not significantly different in male and female among HIV-1 infected infants (84% vs. 85%, p = 1.00) and HIV-1 uninfected infants (64% vs. 61%, p= 0.65). Moreover, when HIV-1 infected infants were divided into 2 groups according to the median of absolute CD4+ T cells (1665 cells/μL), there was a trend in higher rate of HCMV infection in the group with lower absolute CD4+ T cells than in those with higher absolute CD4+ T cell number. (91% vs. 72%, *p*=0.06) (Table 9 and Figure 12). The absolute CD4+ T cell number was not available for all HIV-1 uninfected infants.

Table 9. Infant's risk factors associated with HCMV infection in HIV-1 infected and HIV-1 uninfected infants.

Risk factors	**HCMV infection**					
	in HIV-1 infected infants			**in HIV-1 uninfected infants**		
	Yes	**No**	***p* value***	**Yes**	**No**	***p* value***
Sex						
Male	31 (84%)	6 (16%)	1.00	66 (64%)	37 (36%)	0.65
Female	41 (85%)	7 (15%)		51 (61%)	33 (39%)	
Birth weight**						
1,400-2,910 g	45 (96%)	2 (4%)	0.005***	55 (62%)	33 (38%)	1.00
2,920-3,900 g	30 (73%)	11 (27%)		62 (63%)	37 (37%)	
Abs.CD4 before start ARV**						
22-1,626 cells/μL	30 (91%)	3 (9%)	0.06	Not available		
1,704-4,494 cells/μL	23 (72%)	9 (28%)				

* Fisher's exact analysis was used, ** Continuous variables were dichotomized at the overall sample median, *** Statistically significant.

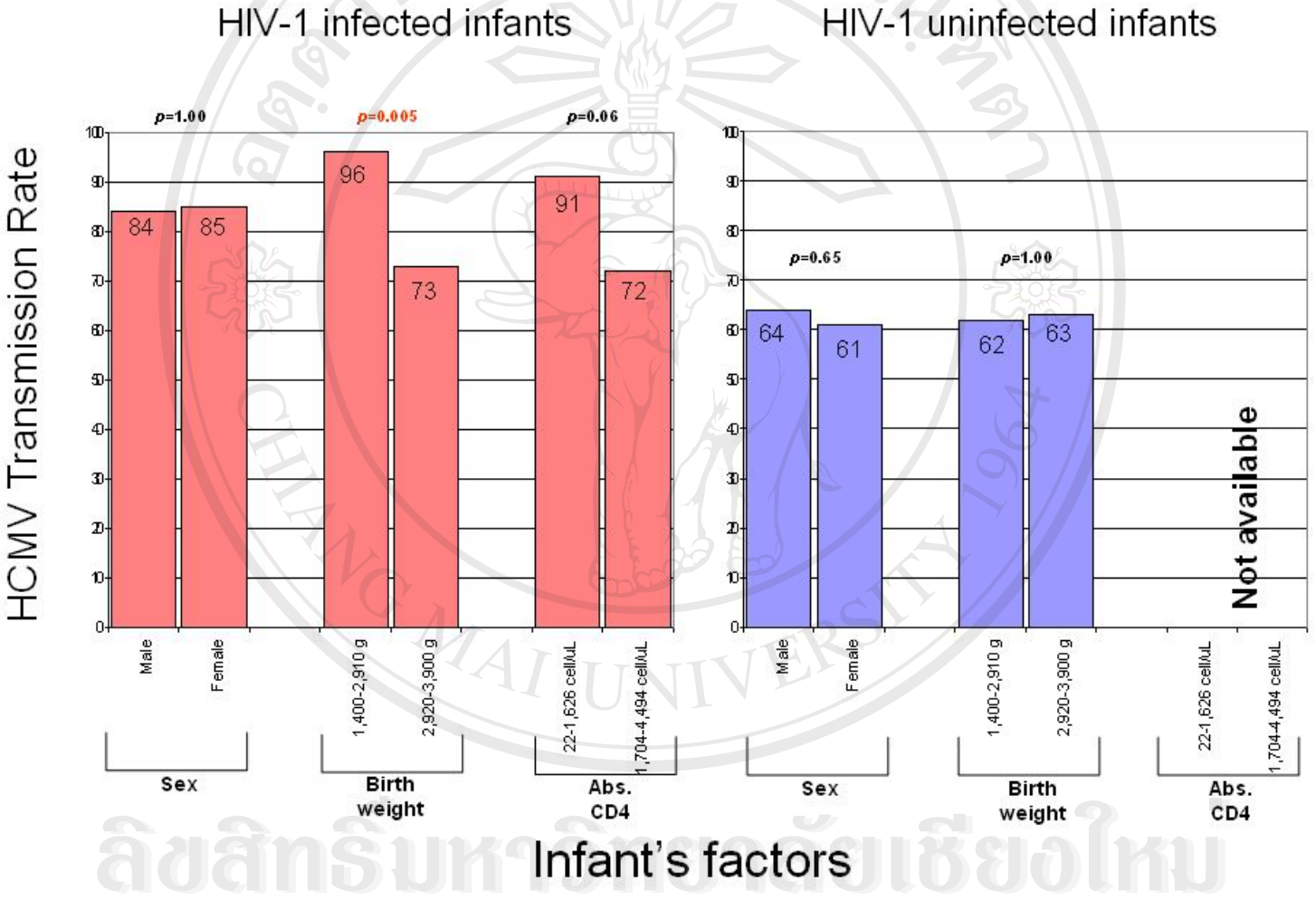

Figure 12. Infant's factors associated with HCMV infection in HIV-1 infected and HIV-1 uninfected infants.

2.6. Time of first diagnosis of HCMV infection

To estimate the survival free of diagnosed HCMV infection, the infant's age at first evidence of HCMV infection (e.g. the first presence of IgM or DNA) was estimated by Kaplan-Meier method. The results showed that HIV-1 infected infants were diagnosed with HCMV infection earlier than HIV uninfected infants (log rank test, $p<0.001$) (Figure 13).

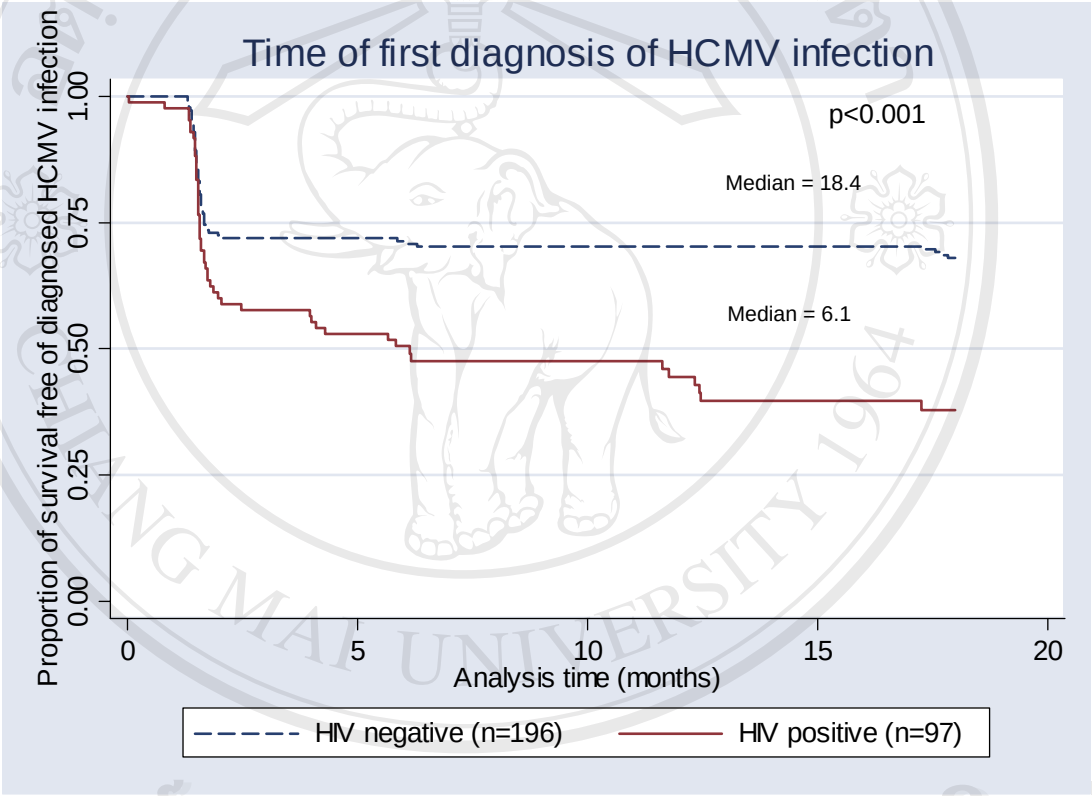

Figure 13. Evaluation of survival free of diagnosed HCMV in HIV-1 infected infants (solid line) and HIV-1 uninfected infants (dash line).

2.7. HCMV infection and HIV-1 disease progression within the first 18 months of life

To analyse the impact of HCMV infection on HIV disease progression in HIV-infected infants, Kaplan-Meier survival estimated was used. Twelve infants, who exhibited no HCMV marker until the age of 6 months and indeterminate results at 18 months, were excluded. Only 85 HIV-1 infected infants were taken into account.

By 18 months of age, 10 infected infants had died, 3 were lost to follow up, 1 was withdrawal of consent, and 1 was protocol violation.

Forty-four of 72 HCMV infected infants and 6 of 13 HCMV uninfected infants died or met the criteria for disease progression before 18 months of age. The Kaplan-Meier survival curve shows that HIV-1/HCMV coinfected infants did not progress to HIV disease more rapidly than those with HIV infected alone (log rank test, p = 0.33) (Figure 14 and 15).

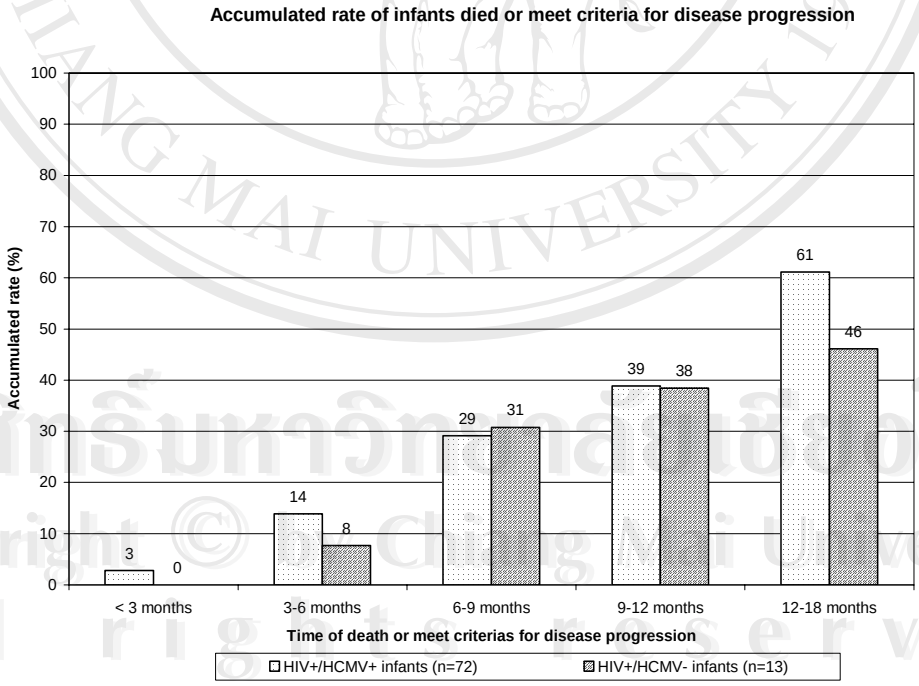

Figure 14. Accumulated rate of infants died or met criteria for HIV disease progression among HIV-1/HCMV coinfected infants and infant with HIV-1 infection alone.

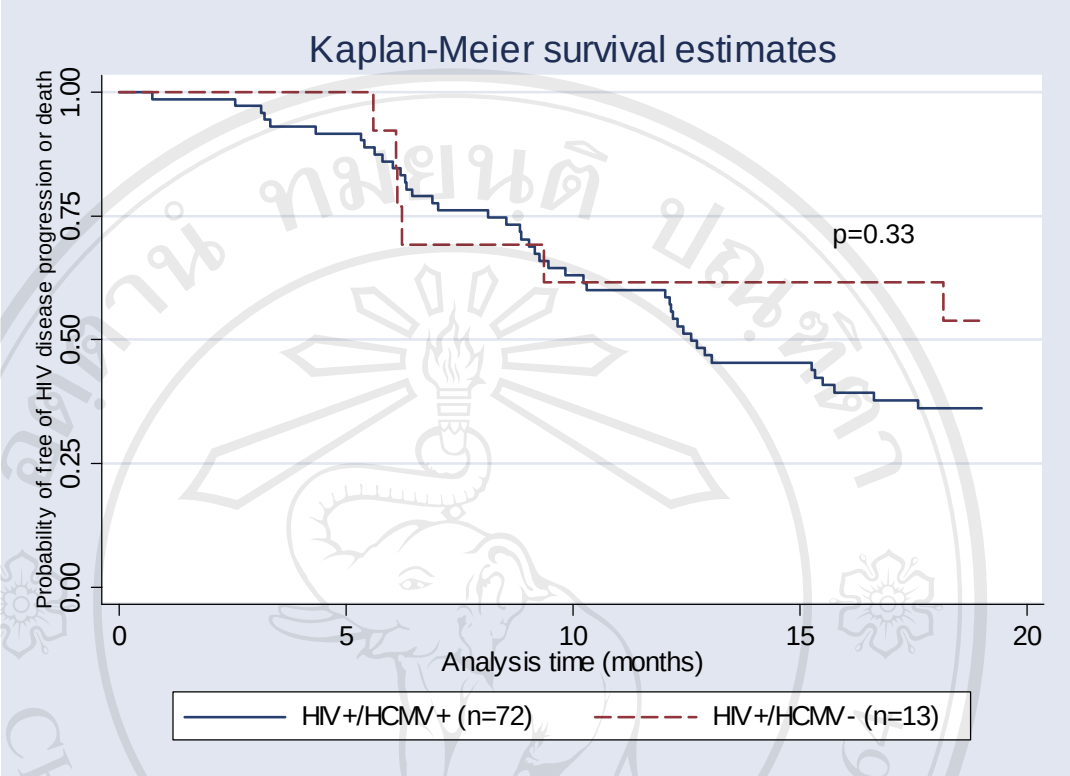

Figure 15. Kaplan-Meier estimates curve showing the probability of HIV disease progression among HIV-1/HCMV coinfected infants (solid line) and infants with HIV-1 infection alone (dash line).