MOLECULAR ANALYSIS OF HIV-1 DRUG RESISTANT MUTATION AND DEFECTIVE CCR 5 ALLELE IN HIV-1 INFECTED NAÏVE PREGNANT WOMEN AND THEIR INFANTS IN THAILAND

SOMSRI AUSWINPORN

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SOMSRI AUSWINPORN: MOLECULAR ANALYSIS OF HIV-1 DRUG RESISTANT MUTATION AND DEFECTIVE CCR5 ALLELE IN HIV-1 INFECTED NAÏVE PREGNANT WOMEN AND THEIR INFANTS IN THAILAND. THESIS ADVISORS: WASUN CHANTRATITA PH.D., PANYU PANBURANA., M.D., SAYOMPORN SIRINAVIN, M.D., ASDA VIBHAGOOL M.D. 116 P. ISBN 974-665-289-3

The purpose of this study was to determine the molecular analysis of HIV-1 drug-resistance mutation, identify the correlation between pairs of infected mothers and their children, and assess the defective CCR 5 allele in naïve HIV-1 infected pregnant women and their infants in Thailand.

HIV-1 drug resistance genotyping was performed on twenty-two infected naïve pregnant women and 4 infected infants born to infected mothers. Blood samples were collected at week 34 of gestation. HIV-1 RNA was reverse transcribed and amplified by RT-PCR. Direct sequencing of the HIV-1 reverse transcriptase and protease was then performed. Changes in nucleotide and amino acid sequences were determined by comparing with the pNL4-3 and CM-240 reference sequence. Data on mutations associated with resistance to antiretroviral drugs were obtained from literature. Apart from HIV-1 drug-resistance genotyping, defective CCR5 allele genotyping was performed by using all mother–child pairs including infected and uninfected subjects. Human CCR5 DNA was carried out by PCR and followed with direct sequencing. Changes in nucleotide and amino acid sequences were determined by comparing with the mutant human CCR5 sequence.

In HIV-1 drug-resistance genotyping, almost all mothers (n=21/95.45%) were infected with HIV-1 clade E, which was similar to 2(50%) of their HIV 1 infected infants. One (4.55%) mother and her 2(50%) infants at birth were all infected with clade B. Mutation in the RT gene associated with NRTIs and NNRTIs had not occurred before among infected mothers and their infants, which included both E and B clades. In the PR gene, M36I occurred in all mothers infected with clade E (100%), but L10I occurred in only one(4.55%). However, no codon mutation associated with protease inhibitor-resistance was found in infected infants born to mothers with clade E or a mother-child pair with clade B. The HIV-1 strain from the naïve group had very few pre-existing mutations to NRTIs, NNRTIs and PIs, which suggested that the transmission of resistant strains was uncommon in HIV-1 infected naïve Thai pregnant women. There was no significant difference in the correlation between the HIV-1 drug-resistance profile and a mother-child pair. In defective human CCR5 allele genotyping, no 32-bp deletion was found in both the homozygote and heterozygote of the CCR5 gene.

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สมศรี อัศวินพร : การวิเคราะห์ลักษณะการดื้อยาของเชื้อ HIV-1 ในระดับโมเลกุลและลักษณะที่บกพร่องของยีน CCR5 ในกลุ่มตัวอย่างหญิงตั้งครรภ์ที่ติดเชื้อซึ่งไม่เคยได้รับการรักษาด้วยยาต้านไวรัสมาก่อนและบุตรที่คลอดในประเทศไทย (MOLECULAR ANALYSIS OF HIV-1 DRUG RESISTANT MUTATION AND DEFECTIVE CCR5 ALLELE IN HIV-1 INFECTED NAÏVE PREGNANT WOMEN AND THEIR INFANTS IN THAILAND.) คณะกรรมการควบคุมวิทยานิพนธ์: วสันต์ จันทราทิตย์, Ph.D., พัญญู พันธ์บูรณะ, M.D., สยมพร ศิรินาวิน , M.D., อัษฎา วิภากุล, M.D. 116 หน้า ISBN 974-665-289-3

จุดประสงค์ในการทำวิทยานิพนธ์ในครั้งนี้เพื่อที่จะศึกษาลักษณะการเกิดการดื้อยาของเชื้อ HIV-1ในระดับโมเลกุลและตรวจหาความสัมพันธ์ของลักษณะการดื้อยาของเชื้อHIV-1ระหว่างแม่และลูกที่ติดเชื้อรวมถึงประเมินลักษณะความบกพร่องของยีน CCR5 ในกลุ่มตัวอย่างหญิงตั้งครรภ์ที่ติดเชื้อซึ่งไม่เคยได้รับการรักษาด้วยยาต้านไวรัสมาก่อนและบุตรที่เกิดในประเทศไทย

ในการศึกษาครั้งนี้ทำการศึกษากลุ่มหญิงตั้งครรภ์ที่ติดเชื้อซึ่งไม่เคยได้รับการรักษาด้วยยาต้านไวรัสมาก่อนจำนวน 22 คนและบุตรที่คลอดแล้วติดเชื้อจำนวน 4 คนเพื่อหาลักษณะการดื้อยาของเชื้อ HIV-1ในด้าน genotyping ตัวอย่างเลือดของหญิงตั้งครรภ์ถูกเก็บที่อายุครรภ์ที่สัปดาห์ที่ 34 HIV-1 RNA ผ่านกระบวนการ RT-PCR ต่อมายีน RT และยีน PR ถูกนำไปทำ direct sequencing การเปลี่ยนแปลงของกรดอะมิโนในลำดับยีนของเชื้อ HIV-1 ถูกเปรียบเทียบกับลำดับยีนอ้างอิงได้แก่ลำดับยีน pNL4-3 และ CM-240 ข้อมูลที่เกี่ยวข้องกับการดื้อยาได้รับจากบททบทวนวรรณกรรม นอกจากการศึกษาลักษณะการดื้อยาของเชื้อ HIV-1ทางด้าน genotypingลักษณะบกพร่องของยีน CCR5 ได้รับการศึกษาในกลุ่มหญิงตั้งครรภ์ที่ติดเชื้อซึ่งไม่เคยได้รับการรักษาด้วยยาต้านไวรัสมาก่อนและบุตรจำนวน 23 คู่ DNAถูกนำไปผ่านกระบวนการ PCR และ direct sequencing การเปลี่ยนแปลงของลำดับเบสถูกเปรียบเทียบกับลำดับเบสของยีนที่กลายพันธ์ซึ่งมีการขาดหายไปจำนวน32 เบสแพร์ภายในยีน CCR5 ลักษณะการดื้อยาของเชื้อ HIV-1เกือบทั้งหมด (n= 21,95.45%) มีการติดเชื้อด้วยสับไทป์อีซึ่งเด็ก 4 คนที่เกิดมาแล้วติดเชื้อมีการติดเชื้อสับไทป์เหมือนแม่โดยมีเด็ก 2 (50%) คนติดสับไทป์อีและเด็ก 2 (50%) คนติดสับไทป์บีลักษณะการกลายพันธ์ในยีน RT ที่มีการเกี่ยวข้องกับการดื้อยาในกลุ่ม NRTIs และ NNRTIsไม่ปรากฏในการศึกษาครั้งนี้ ในยีน PRแม่ที่ติดเชื้อด้วยสับไทป์อีพบการกลายพันธ์ลักษณะ M36I ปรากฏในแม่ทุกคน (100%) และพบลักษณะ L10I เพียง1คน(4.54%) คู่แม่ลูกที่ติดเชื้อด้วยสับไทป์บีไม่พบกลายพันธุ์ในยีน PR ซึ่งเกี่ยวข้องกับการดื้อยาในกลุ่ม PI จากการศึกษาครั้งนี้ไม่พบความแตกต่างของลักษณะการดื้อยาระหว่างแม่และลูกและไม่พบลักษณะความบกพร่องของยีนCCR5ทั้งในลักษณะ homozygote และ heterozygote

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LIST OF ABBREVIATIONS

ACTG	=	AIDS clinical trial group
AZT	=	Azidovudine
bp	=	Base pair
°C	=	Degree of celsius
CCR5	=	Chemokine co-receptor 5
CD	=	Cluster of differentiation
CXCR4	=	Chemokine co-receptor X
delta	=	Deletion
ddNTP	=	Dideoxynucleotide triphosphate
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
D.W.	=	Distilled water
g.	=	Gram
Gag	=	Group antigen gene
gp	=	Glycoprotein
HIV-1	=	Human immunodeficiency virus type 1
Kb	=	Kilobase (1,000)
L	=	Liter
M	=	Molar
MA	=	Matrix
min	=	Minute
MIP	=	Macrophage inflammatory protein
ml	=	Milliliter

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LIST OF ABBREVIATIONS (CONTINUED)

mM	=	Milli-molar
mRNA	=	Messenger ribonucleic acid
M-tropic	=	Macrophage tropic strain
MTCT	=	Mother to child transmission
ng	=	Nanogram
NNRTI	=	Non-nucleoside analogue
NRTI	=	Nucleoside analogue
NSI	=	Non-syncytium inducing
NVP	=	Nevirapine
PACTG	=	Pediatric aids clinical trial group
PHT	=	Perinatal HIV transmission
PCR	=	Polymerase chain reaction
Pol	=	Polymerase gene
PR	=	Protease gene
PI	=	Protease inhibitor
RNA	=	Ribonucleic acid
RT	=	Reverse transcriptase
SAINT	=	South African International Vaccine Trial
sec	=	Second
SI	=	Syncytium inducing
SIV	=	Simian immunodeficiency virus

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LIST OF ABBREVIATIONS (CONTINUED)

T-tropic	=	T lymphocyte tropic strain
u.	=	Micro (10^{-6})
Wt	=	Wild type
ZDV	=	Zidovudine
3TC	=	Lamivudine
List of amino acid abbreviation		
Alanine	=	A
Cysteine	=	C
Aspatate	=	D
Glutamate	=	E
Phenylalanine	=	F
Glycine	=	G
Histidine	=	H
Isoleucine	=	I
Lysine	=	K
Leucine	=	L
Methionine	=	M
Asparagine	=	N
Proline	=	P
Glutamine	=	Q
Arginine	=	R
Serine	=	S

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LIST OF ABBREVIATIONS (CONTINUED)

Threonine	=	T
Valine	=	V
Tryptophan	=	W
Tyrosine	=	Y

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CHAPTER I

INTRODUCTION

1.1 Background

Mother-to-child transmission (MTCT) is by far the largest source of HIV infection in children under the age of 15. In countries where blood products are regularly screened and clean syringes and needles are widely available, it is virtually the only source in young children. In June 2000, an estimated 600,000 infants world-wide were infected with the virus, bringing the total number of young children living with HIV to over 1 million. Of the 5 million infants infected with HIV since the beginning of the pandemic, about 90% have been born in Africa, owing to a combination of high HIV prevalence in pregnant women and high fertility rates. However, the number of cases in India and SouthEast Asia appears to be rising rapidly. AIDS may have already doubled mortality in children under the age of 5 in regions most affected by the virus [1,4,5,8-10,120,122].

The virus may be transmitted during pregnancy, labour, delivery, or after the child's birth during breastfeeding. Among infected infants who are not breast-fed, most MTCT occurs around the time of delivery (that is, just before or during labour and delivery). In populations where breastfeeding is the norm, breastfeeding accounts for more than one-third of all cases of MTCT transmission. In sub-Saharan Africa, MTCT is contributing substantially to rising child mortality rates in many areas. In Harare, Zimbabwe, for example, the death rate among infants in their first year of life increased from 30 to 60 per 1000 between 1990 and 1996. And deaths among one-to-

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five year olds, the age group in which the bulk of child AIDS deaths are concentrated, rose even more sharply -- from 8 to 20 per 1000 -- in the same period. In a growing number of countries, AIDS is now the biggest single cause of child death.

Most studies estimate the probability that an HIV-positive woman's baby will have the virus as ranging from 15% to 25 % in an industrialized country (if there is no antiretroviral treatment) and 25% to 35% in a developing country [4-7,120,121]. These differences are mainly explained by the frequency and duration of breastfeeding. Other factors known to increase the risk of MTCT include advanced stage of disease in the mother, recent maternal infection, and high fetal exposure to infected maternal body fluids during labour and delivery. The risk of HIV-2 transmission is about 20 times less than that for HIV-1. The most important public health measures against MTCT remain the primary prevention of infections in women of childbearing age and the prevention of unwanted pregnancies through adequate family planing.

Until recently there was no means of preventing MTCT during pregnancy, labour and delivery for HIV-positive women who wished to give birth. This situation is now changing as the benefits of various MTCT interventions are being studied and demonstrated. Two used separately or in combination – a regimen of antiretroviral drugs along with replacement feeding -- have already been proven highly effective [1,4-10,120].

Antiretroviral therapy

Until 1998, only one drug regimen had been proven to reduce the risk of HIV transmission from mother to child. A study called ACTG 076 found that zidovudine (ZDV, also known as AZT) given orally starting in the fourth month of pregnancy, intravenously during labour, and for six weeks to the infant in a non-breastfed

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population, reduced mother-to-child transmission of HIV by two-thirds [5-7,11-14]. The ACTG 076 regimen, though now provided routinely in most industrialized countries, is not well suited to widespread use in many developing countries for cost and logistical reasons. Costing an average $1000 per pregnancy in industrialized countries, it requires both oral (i.e., pills) and intravenous administration, and must be started by the mother as early as possible during the second trimester of pregnancy. The early start is a severe disadvantage since many pregnant women in developing countries do not seek or access prenatal care until beginning of labour.

Recently, however, the situation changed dramatically for the better. A CDC-sponsored trial concluded in Thailand in February 1998 showed that a short course of ZDV pills given to women during the last four weeks of pregnancy and labour cuts the rate of vertical transmission during childbirth by half [86,120-122,133]. The average cost of the ZDV in Thailand is US $50 per pregnancy (drug cost only, using a Thai-manufactured generic). Because the women in the study were also given safe alternatives to breast milk, the short treatment reduced MTCT in the study population to 9%, compared with 18% in the control group who did not receive a placebo but did receive replacement feeding (as mentioned, the norm in predominantly breastfeeding populations is 25-35%.)

Results from a number of studies in breastfeeding populations indicate that a short course of antiretrovirals can still reduce the transmission of HIV from the mother to the baby, though not as well as when mothers do not breastfeed. In the pooled analysis of two studies, conducted between 1995 and 1998 in Abidjan, Côte d'Ivoire, and Bobo-Dioulasso, Burkina Faso, [119,120,134] it was found that the rate of HIV infection among breastfed babies whose HIV-infected mothers had a one-month

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course of AZT was about 22% at age 24 months, compared with 30% among babies whose mothers received no antiretrovirals [76-80,88-89,120].

The PETRA trial (coordinated by the UNAIDS secretariat) tested the effectiveness of a number of antiretroviral regimens using two drugs – AZT and lamivudine (3TC) – in combination. When an HIV-positive mother starts taking the two drugs at the time of delivery, and she and her newborn baby continue on the drug regimen for just one week following birth, the risk of the baby becoming infected is reduced to about 12% when measured at 6 weeks of age, as compared with a 19% risk when no antiretroviral drugs at all are given. An even bigger reduction – to 8% -- is seen when the drug regimen is started at 36 weeks of pregnancy, around a month before delivery. At 18 months of age however, the benefits are almost completely washed out because of transmissions that occur during prolonged mixed feeding [120,142-143].

In July 1999, the US National Institutes of Health released the results of a joint Uganda-US study assessing the preventive efficacy of a single dose of the antiretroviral drug nevirapine, given to the mother during labour and to the baby within the first three days of life. Measured at 12 months of age, HIV infection was found in only 16% of the (breastfed) infants who received nevirapine (compared to 24% when a 1 week ZDV regimen was used). Replacement feeding may counterbalance the benefits. It has been suggested that exclusive breastfeeding may lower the risk of transmission when compared to mixed feeding. If confirmed, exclusive breastfeeding associated with early and complete cessation would become an attractive option [10,19,120,134].

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However, drug resistance can occur after a long duration of treatment. Viral variants arise in the course of infection and form quasispecies. These variants are then generated by error of reverse transcriptase at an estimated rate of $3.4x10^{-5}$ [138]. This rate enhances the variant appearance by a high viral population turnover, with a reported production of more than 10^9 HIV-1 varions per day [138,162-163] Once generated, each variant undergoes selective pressure such as antiretroviral therapy. Codon mutation at several mutations often appears in AZT therapy. The prevalence and appearance of codon 215, 41, 70, 210, 219 mutations in the reverse transcriptase gene are associated with AZT resistance in HIV-1 infected pregnant women and their consequence on vertical HIV transmission [139,142-144,146-147,149,151], while 3TC resistance involves only the codon 184 mutation [139-142]. In addition, increasing evidence of Nevirapine resistance has been reported. Nevirapine resistance is associated with the prevalence of codon 101,103, 181, 188, and 190 mutations [134,137,143,145].

From the recent studies suggest that the majority of Perinatal HIV-1 transmission (PHT) occurs during labor and delivery [15,20,24,27]. A substantial proportion of perinatally acquired infections with the human immunodeficiency virus type 1 (HIV-1) occur at or near delivery, which suggests that obstetrical factors may have an important influence on transmission. To evaluate the relation of such factors and other variables to the perinatal transmission of HIV-1. Results from recent studies suggest that elective C-section and the use of anti-HIV drug AZT may further reduce the risk of vertical transmission compared to what has been achieved by using AZT alone [110-111,115]. Results also suggest that elective C-section reduces the risk of vertical transmission independent of the effects of AZT. This procedure protects the

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baby from direct contact with the mother's HIV-containing genital tract secretions and blood.

In addition to an antiretroviral therapy and elective C-section are the prevention of PHT, there are several factors that can lessen PHT such as low level plasma HIV-1 RNA load, high CD4 count, good nutrition in pregnant women ,suppressive HIV-1 replication factors [6,7,68,81], and CCR-5 mutation phenomenon [153,155-157,167]. The presence of a CCR5 mutation may explain the phenomenon of resistance to HIV infection in some cases. Human CD4+ T lymphocytes, macrophages, microglial, dendritic and Langerhans cells, are believed to be targets for HIV-1 infection. The CD4 transmembrane protein along with one of several chemokine receptors is believed to be necessary for viral attachment and fusion to occur. In the initial stage of virus-cell fusion, the HIV surface glycoprotein gp120 attaches the virus to its cell surface receptor. The primary receptor, called "CD4", but a second receptor that loops through the cell membrane 7 times is critical for infection to occur. CCR5 is determined that is the receptor responsible for the fusion of macrophage-tropic (M-tropic) HIV, the most common primary strain of the virus transmitted from one person to another. The 32-nucleotide deletion in CCR 5 chemokine receptor appears to offer very powerful protection against acquiring HIV. The homozygous CCR-5 deletion appears to protect against HIV, while the heterozygous genotype does not. Although people heterozygous for the CCR-5 allele deletion are not resistant to HIV in vitro, the M-tropic isolates seem to replicate less efficiently in their cells than in those of a homozygous wild-type individual [150-152].

Although the treatment of pregnant women and their infants with efficacy of antiretroviral therapy, non-breastfeeding management and mode of delivery

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management has been remarkably effective in preventing the perinatal transmission of HIV-1, a small but significant proportion of mothers who are treated during pregnancy still transmit [144,149,158]. Failure to prevent perinatal transmission among mothers received antiretroviral therapy, specifically ZDV in some cases may be linked to maternal drug resistance [147]. The number of the studies in Europe and United States show that the subjects have been too few assess the impact of drug resistance on perinatal transmission. In several studies, HIV-1 genotypic zidovudine drug resistance occurred in maternal circulation which can be the risk of maternal-infant transmission. The results showed that among maternal isolates had at least one ZDV-associated resistance mutation. This can be the predictive of transmission [147,149]. Apart from the prevalence of codon mutation associated with drug resistance, specifically ZDV in prevention MTCT of HIV-1, the correlation of HIV-1 drug resistance profile between a pair of mother and child is important. This can be the predictive that which major or minor of maternal HIV-1 strain based on pol gene can be selective vertical transmission to the infant [144,147].

To date, in Thailand, there are about 23,000 infected pregnant women who had delivered infants in each year. About 10% of infected infants born to infected mothers [148]. From the recent study which determined in RT gene of infected Thai pregnant women and their children, the result showed that there was no ZDV--associated resistance mutation among infected Thai maternal isolates and no difference of HIV-1 drug resistance profile between mother and her infant in Thailand [148]. However, in several studies, the results showed that there was a different HIV-1 drug resistance profile between mother and her infant [147]. The different HIV-1 antiretroviral drug-resistance profile can be distinguished into 2 classifications. The first, HIV-1

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antiretroviral drug-resistance profile in maternal isolate has a pattern of codon mutation that can cause drug resistance however, there was no mutation associated with drug resistance in infant's circulation [149]. The second, in both mother and infant's isolates have any codon mutation that can cause drug resistance but the profile of HIV-1 antiretroviral drug-resistance may be different [165]. So it is rather important to identify HIV-1 antiretroviral drug-resistance profile among infected pregnant women and the concordance of drug-resistance profile between a pair of mother and child.

Apart from HIV-1 antiretroviral drug-resistance profile, defective CCR5 allele genotyping is interesting. Although a mutant allele bearing a 32-bp deletion is known to occur, primarily in population European heritage, about 1% of who appear to carry two copies of it, that is, they are homozygotes for the deletion mutant. Between 15% and 20% are heterozygotes with one mutant and one wild type allele [159,161,166,167]. In Thailand, there is the evidence of deleting mutation of 32 base pairs (delta 32) in the gene encoding the chemokine receptor CCR5 occurred among Thai population, specifically in Thai injecting drug users. The result demonstrated that the possible occurrence of heterozygous CCR5 deletion of 32 base pairs in gene encoding the chemokine receptor CCR5 [159] was more than the occurrence of homozygous CCR5 deletion of 32 base pairs in gene encoding the chemokine receptor CCR5.

So the identification of CCR5 allele genotyping may be a factor of HIV-1 vertical transmission that can be a predictive of progression of disease.

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1.2 Objective

1.To analyze molecular HIV-1 drug-resistance profile in infected Thai naïve pregnant women and their infected infants.

2. To identify the correlation of molecular HIV-1 drug-resistance profile between infected mothers and their infected infants.

3. To investigate genotypic defective CCR5 allele phenomenon in infected Thai naïve pregnant women and their infants both are infected and uninfected.

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CHAPTER II

LITERATURE REVIEW

2.1 Incidence of Perinatal Transmission

The rate of HIV transmission from mother to infant varies in different areas of the world. In the United States, transmission rates have been in the range of 15% to 30% in the absence of any intervention [8-10]. Several large, prospective, placebo-controlled studies have confirmed an expected transmission rate of approximately 25% among HIV-infected women in the US [11,12]. With approximately 7,000 HIV-infected women giving birth each year, between 1,000 and 2,000 HIV-infected infants would be expected on a yearly basis if no effective intervention occurred [13,14]. In Europe, reported rates of HIV perinatal transmission have been somewhat lower, at approximately 13% to 15% [15,16]. In contrast, perinatal transmission of HIV-1 appears somewhat higher in Africa, with transmission rates of 40% to 50% noted routinely [17,18]. The higher incidence of perinatal transmission in Africa does not reflect an additive effect arising from cases of HIV-2 transmission, since this virus is less likely to be transmitted from mother to infant, resulting in a transmission rate of 1.2%, as opposed to 24.7% for HIV-1 [18].

These geographic disparities may be related to a number of factors, including presence or absence of various cofactors which appear important in predicting perinatal transmission. For example, the differences in transmission rates may be explained in part by variation in the practice of breastfeeding, which is known to be a

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risk factor [19]. Methodologic differences may also be operative [20]. For instance, high infant mortality rates would tend to bias the data towards a lower HIV transmission rate since infants would die before a definitive diagnosis of HIV infection could be made. The proportion of women who first acquired HIV during pregnancy could bias the results, since acute infection is associated with higher HIV viral load [21]. This, in turn, may increase the likelihood of fetal transmission. In developing countries, pregnant women with more advanced HIV disease are more likely to deliver in large government hospitals, which might bias the transmission rates reported from these centers [20]. Specific methods of delivery could alter rates of transmission [22]. As could presence of nutritional deficiencies and/or coexisting infections other than HIV-1, absence of adequate prenatal care, and increased rate of premature deliveries.

2.2 Mechanisms and Timing of Perinatal HIV Transmission mechanisms of Transmission

HIV may be transmitted from mother to infant in three ways:

- First, direct infection may occur *in utero*, via transplacental passage [23,24].
- Second, HIV may be transmitted to the infant during the time of delivery, by breaks in the skin and subsequent direct exposure to infected blood or secretions, or by ingestion of maternal blood or other fluids [25-27]. In this regard, HIV has been isolated from cervicovaginal lavage fluids (CVL) from HIV-infected women [28]. HIV-1 has been isolated from infant gastric aspirates or oropharyngeal secretions immediately following delivery by either

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HIV culture[31] or HIV RNA PCR [32]. In addition, simian studies have demonstrated very effective perinatal transmission of SIV when infant macaques received oral inoculates of virus immediately following birth [33].

- Third, HIV may be transmitted by breastfeeding, since HIV is known to be present in breast milk from HIV-infected mothers[19,34] and transmission of HIV-1 via breastfeeding has been demonstrated in a number of studies [35-37].

2.3 Diagnosis of Perinatally Acquired HIV Infection

A definition of the timing of HIV infection. Early transmission (or *in utero*) is defined as that which may be detected within the first 48 hours of life. Late transmission (or *intrapartum*) is defined as negative virologic evaluations during the first week, with evidence of HIV detection between 7 and 90 days of age in the absence of breastfeeding. Employing viral culture, indicating that HIV infections may have occurred during *in utero* development. Using polymerase chain reaction (PCR) technology, HIV-1 DNA was detected in infected infants studied within 48 hours of birth [42]. The data would indicate that as much as 62% to 85% of perinatal transmission might occur in the *intrapartum* or neonatal periods. Approximately 93% of HIV-infected infants will demonstrate presence of viral markers by 14 days of life with PCR testing, and essentially 100% within 30 to 90 days of birth [39-43]. This excludes infants who are breastfed by HIV-infected mothers, for in this setting HIV transmission may occur throughout the duration of breastfeeding.

2.4 Factors Which Influence the Risk of Perinatal HIV Transmission

2.4.1 Maternal factor

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2.4.1.1 HIV viral load.

Clinically or immunologically advanced HIV disease in the mother has consistently been associated with higher likelihood of transmission to the infant [16,37]. Of note, the subsequent course of HIV disease in the infected children has also been correlated with the severity of maternal HIV disease during pregnancy. In addition, studies have demonstrated that an increased maternal virus load is associated with an increased risk of *in utero* transmission [46,47]. *In utero* transmission, in turn, appears to correlate with an increased rate of disease progression in the infant [48-50]. Multiple studies have confirmed the importance of maternal viral load in predicting the risk of HIV transmission to the infant [51-53]. Maternal HIV-1 viral load is an important determinant of perinatal transmission, and also suggests the possibility of a threshold below which perinatal transmission would probably not occur. However, transmission may occur even with undetectable levels of HIV RNA [55]. The studies would thus indicate that HIV-1 RNA is a powerful predictor of HIV-1 transmission. However, obstetrical factors are also important, such as prolonged intervals between amniotic membrane rupture and delivery, and presence of chorioamnionitis.

2.4.1.2 Obstetrical factors: premature rupture of the membranes.

Premature rupture of the amniotic membranes has been associated with higher risk of perinatal HIV transmission, probably related to increased duration of fetal exposure to infected cervicovaginal secretions. On multivariate analysis, ruptured membranes for over 4 hours nearly doubled the risk of transmission. Premature

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rupture was also shown to be a significant factor in predicting perinatal transmission [59,62].

2.4.1.3 Obstetrical factors: mode of delivery.

Initial studies indicated similar rates of HIV transmission in children born after vaginal or cesarean deliveries [9,25,64]. However, recent data would indicate that elective cesarean section (C-section) might be very effective in reducing perinatal transmission of HIV-1. These discrepancies may be secondary to the fact that elective C-section negates the possibility of premature rupture of the membranes. Differences in results could also have been caused by the possible inclusion of women who underwent nonelective C-section; and by the potential bias that some obstetricians may be more or less likely to perform C-sections on HIV-infected patients with more advanced clinical disease.

More recently, to determine the role of elective C-section in decreasing perinatal transmission [66]. The studied were established, similar results were found when the control population was limited only to those women with rupture of the membranes immediately prior to delivery. When all C-sections were compared with all vaginal deliveries in this meta-analysis, use of C-section remained predictive of decreased risk of transmission, although not to the same degree as that seen with elective C-section alone.

2.4.1.4 Role of breast-feeding.

HIV-1 has been isolated from cell-free breast milk [74], and HIV DNA has been demonstrated in the majority of milk specimens from HIV-infected mothers.

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Evidence of HIV transmission via breastfeeding has come from reports in which children were exposed to HIV-1 only through breastfeeding [76], and from studies in which breastfed infants had an increased risk of perinatal HIV transmission, when compared with children who were bottle-fed [9,16,19,77], infants became infected with HIV through breastfeeding [34]. Plasma viral load is known to be high during primary HIV infection [21], making it difficult to assess the applicability of these data to other stages of HIV infection. Breastfeeding was shown to increase the risk of HIV-1 transmission, with an estimated adjusted odds ratio for 1 day of breastfeeding versus bottle-feeding of 1.19 (95% CI, 1.10-1.28). The odd ratio of transmission increased with the duration of breastfeeding [80].

2.4.2 Factors related to the fetus.

A low birth weight of less than 2500g [51,61,71] and/or gestational age <34 weeks [16] or <38 weeks [51,71] have each been associated with an increased risk of perinatal HIV transmission. This association between prematurity and transmission may simply be a consequence of HIV infection *in utero*, resulting in abnormalities in fetal development and premature birth. Additionally, women with more advanced HIV disease, which is more likely to transmit to their infants, are also more likely to deliver premature infants. It is also possible that infants born prematurely may have less developed immune systems, and thus be more susceptible to infection during labor and delivery. One way to address this issue is to determine the timing of infection. Recent studies of this type in HIV-exposed infants have suggested that the rate of *intrapartum* transmission might be higher among premature babies, implying that

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acquisition of infection occurred because they were born prematurely and not vice versa [72,73].

Aside from prematurity or low birth weight, the birth order of twins has also been associated with differing risks of infection. The majority of twin pairs are concordant in terms of HIV-1 infection. However, a significantly increased risk of HIV-1 infection is also apparent among first-born ("A") as opposed to second-born ("B") twins, independent of mode of delivery [65]. Although the precise mechanisms for this increased risk in first-born twins are unknown, it is certainly consistent with the fact that perinatal infection often occurs at the time of delivery.

2.5 Antiretroviral Interventions to Reduce Perinatal Infection

2.5.1 Abbreviated courses of zidovudine.

Public health recommendations concerning use of zidovudine in pregnancy were based upon the results of PACTG 076 [6,7,84]. As described earlier, this study compared placebo with an intensive three-part regimen of zidovudine, which was administered during pregnancy, at the time of delivery by intravenous infusion, and then to the infant [11,84]. A recent trial performed in Thailand [86,87] studied the use of zidovudine versus placebo, beginning in the last month of pregnancy (week 36). At delivery, intravenous infusions of zidovudine were not employed. The data did indicate that shorter courses of zidovudine might be effective, when compared with placebo. Further, they suggest that shorter courses of zidovudine, even those beginning at the time of delivery, or during the first 48 hours of life, may be effective in decreasing the risk of HIV transmission to the newborn [88,89]. In summary, then,

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prophylaxis of the newborn in the immediate peripartum period (labor and delivery and first 48 hours of life) are extremely important in reducing HIV vertical transmission rates.

2.5.2 Development of drug resistance in pregnant women taking monotherapy during pregnancy.

A potential difficulty of zidovudine monotherapy during pregnancy relates to the risk of development of antiretroviral resistance. Although full data are not yet available, a subset of women enrolled on PACTG 076 underwent genotypic testing for ZDV resistance mutations. The actual incidence of resistance may be higher, however, since participants in PACTG 076 had asymptomatic or early symptomatic HIV disease, and would be expected to have a lower likelihood of developing resistance mutations than individuals with more advanced HIV disease. Most of the currently available antiretroviral agents are associated with the development of particular HIV-1 mutation conferring specific resistance to the individual agent [140,141,144].

2.5.3 Mechanisms of Drug Resistance

The nucleotide sequence that determines a gene is known as the *genotype;* the triplet of nucleotides that determine the amino acid sequence at a specific position in the protein is known as a *codon*. The properties or characteristics of a virus (or viral protein) are known as the *phenotype*; the phenotype is always determined by the genotype. Viral drug susceptibility usually is described in terms of the concentration of drug required to produce a specific amount of growth inhibition *in*

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vitro. For example, the 50% inhibitory concentration (IC_{50}) is the concentration of drug required to reduce the virus yield in cell culture by 50% [170].

Resistance to antiretroviral drugs is determined by mutations in the genes that encode the protease and reverse transcriptase. Primary mutations are those that alter binding of the drug to its target and result in an increase in the amount of drug necessary to inhibit the enzyme. Secondary mutations increase the level of resistance by improving the fitness of viruses carrying primary mutations. Usually secondary mutations have little or no effect on the level of resistance in the absence of primary mutations [170].

2.5.4 Zidovudine.

Zidovudine selects for reverse transcriptase (RT) mutation at codon 41, 67, 70, 210, 215 and 219 both in vivo and vitro and these mutations display widely varying degrees of zidovudine (ZDV) resistance when expressed in isolation or in combination [143,144,146]. Only the codon 70 K to R and codon 215 T to Y mutation appears consistently as primary mutation in vivo. Mutation at the other position appear as compensatory changes, increasing the fitness of the primary mutation under continued selection by prolonged ZDV use. Appearance of antiretroviral resistance has been shown to be associated with worsening clinic course in a number of settings [146,147,149]. Because the development of ZDV resistance correlates with duration of therapy, women with extensive use of ZDV prior to pregnancy may be at higher risk of developing ZDV resistance during pregnancy.

2.5.5 Use of Other Antiretroviral Agents in Pregnancy

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Other nucleoside reverse transcriptase (RT) inhibitors. Of the six currently licensed RT inhibitors, only zidovudine and lamivudine (3TC) have been studied in terms of pharmacokinetics during pregnancy. The PETRA trial of which reduced courses of ZDV alone are underway in Africa, and Haiti (coordinated by the UNAIDS secretariat) tested the effectiveness of a number of antiretroviral regimens using two drugs – AZT and lamivudine (3TC) – in combination. This trial is being undertaken in predominately breastfeeding population. Although combination of ZDV and 3TC can prevent mother to child transmission of HIV, the rapid development of genotypic to 3TC when combined with ZDV can occur [139,140,142,151]. Mutation at codon 184 M to V is the critical mutation, which can decrease susceptibility to lamivudine. It may affect to the protection of mother to child transmission of HIV. However, introduction of the M184V mutation into RT of HIV-1 restores ZDV susceptibility in molecular clones that carry the M41L and T215Y ZDV resistance mutations [172]. In addition, co-administration of ZDV and 3TC results in delayed emergence of resistance to ZDV [173]. Dual resistance to 3TC and ZDV has been selected after prolonged *in vitro* passage of HIV-1 in the presence of both drugs and after prolonged treatment with both drugs [173]. The genetics of ZDV/3TC dual resistance is complex. Polymorphisms in the region around codon 41, as well as in the region between codons 208 and 214, also appear to modulate expression of dual resistance [174-175]. All of these mutations, appear to function by negating the effects of the 184V mutation on ZDV resistance, but do not affect ZDV or 3TC susceptibility on their own.

2.5.6 Efficacy of Nevirapine

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Preliminary data has recently been published regarding the HIVNET 012 trial, in which the efficacy of ZDV was compared to that of nevirapine (NVP) Infants were defined as HIV-infected based on a positive HIV RNA assay, confirmed by either a quantitative HIV RNA assay, or HIV culture. A single oral dose of nevirapine, given to the mother during delivery and to the infant during the first 72 hours of birth, was capable of reducing the risk of vertical HIV transmission by half, when compared with short-course ZDV. Of importance, based on average US wholesale drug costs, the NVP regimen was also approximately 200 times cheaper than ZDV, making this approach economically feasible in these areas. However The recent report from a phase II study (HIVNET006) showing that a single dose of nevirapine led to the selection of NVP-resistant virus [145,146]. The majority or significant fraction of plasma virus containing the K103N mutation. The level of susceptibility to nevirapine can be decrease highly by this mutation [146].

2.5.7 Protease Inhibitor

Protease inhibitors have been used more and more during pregnancy for treatment and prevention of perinatal transmission. They appear to prevent transmission by reducing maternal virus load. Ongoing studies such as PACTG 367 have shown no vertical transmission of HIV in women with undetectable virus loads. Although there is no an appearance of PI resistance in the prevention of the perinatal transmission of HIV, there are several studies report about codon mutation associating PI resistance [178].

Unique classes of PI resistance mutations are those found at cleavage sites in the *gag-pol* polyprotein precursor, the substrate of HIV-1 protease [171]. Cleavage

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site mutations do not produce drug resistance in and of themselves, but compensate for alterations in the protease that result from primary and secondary resistance mutations. Resistance to PIs emerges rapidly when these inhibitors are administered at inadequate doses or as part of suboptimal regimens [176-177]. Studies with ritonavir (RTV) show that the rate at which drug resistance emerges is inversely proportional to the trough concentration of drug [176]. Interpretation of PR mutants is made more difficult by the extensive polymorphism found in PR genes from HIV-1 isolates of PI-naive patients. The significance of these polymorphisms in determining treatment outcome remains uncertain, since most studies have not found any correlation between the presence of these polymorphisms and virologic response, or the rate at which PI resistance emerges.

2.5.7.1 Saquinavir

Resistance to saquinavir (SQV) is associated with acquisition of the G48V and L90M mutations in HIV-1 protease. These mutations confer a 2- to 6-fold reduction in susceptibility to SQV, whereas SQV susceptibility is reduced by 8- to 11-fold in viruses that carry both mutations [178]. The L90M mutation usually emerges first in isolates from patients treated with SQV; [179-180] emergence of G48V has been reported most frequently in patients receiving high-dose SQV [181. Mutations at codons 10, 20, 24, 30, 36, 46, 54, 63, and 71, 77, 81, 84, and 88 also have been noted in isolates from patients failing SQV [182]. No alteration in the mutational pattern associated with SQV has been noted with the change from the hard-gel (Invirase®) to soft-gel (Fortovase®) formulations of SQV.

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2.5.7.2 Ritonavir

Development of high-level resistance to ritonavir (RTV) requires the stepwise accumulation of multiple mutations. The first mutation detected generally appears at codon 82, resulting in a V82A, T, or F substitution [183]. Mutations at this codon reduce RTV binding to HIV-1 protease. The accumulation of the additional mutations at codons 36, 54, and 71 results in higher levels of resistance. The I54V mutation resides in the flap region of protease, and may stabilize the flaps in such a way as to alter the specificity of the enzyme, causing it to favor binding of the natural substrate over RTV. Changes at codons 8, 10, 20, 33, 46, 63, 84, and 90 also have been observed in isolates from RTV-treated patients, but the relative contribution of these mutations to RTV resistance is not known [179,183].

2.5.7.3 Indinavir

As with RTV, high-level resistance to indinavir (IDV) also requires the accumulation of multiple PR mutations. In a regression analysis, mutations at codons 10, 20, 24, 46, 54, 63, 64, 71, 82, 84, and 90 were significantly correlated with reduced susceptibility to IDV. Mutations at codon 82 that result in V82A, T, or F substitutions are correlated most commonly with clinical failure in the setting of IDV monotherapy. Measurable resistance (an increase in IC_{90} of at least 4-fold) requires the presence of three or more mutations. Although many of the same mutations are implicated for RTV and IDV resistance, there does not seem to be any specific order to the accumulation of IDV resistance mutations. No particular amino acid substitution is present in all IDV-resistant isolates [178,184].

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2.5.7.4 Nelfinavir

Passage of HIV-1 in the presence of nelfinavir (NFV) results in selection of a unique mutation (D30N) that confers 7-fold resistance to NFV [184]. The wild-type aspartate residue (D) at codon 30 is important for NFV binding to HIV-1 protease; its substitution by asparagine (N) results in a decrease in affinity of the protease for NFV. Since codon 30 is not involved in binding of other PIs, the D30N mutation is relatively specific for NFV. The D30N mutation is the first to emerge in isolates from patients failing NFV [184]. However, continued treatment with NFV selects for the accumulation of additional resistance mutations, many of, which are common to other PIs, including substitutions at codons 35, 36, 46, 71, 88, and 90. Although the G48V and I84V mutations were observed following prolonged *in vitro* passage of HIV-1 in the presence of NFV, these mutations are rarely seen in clinical isolates from NFV-treated patients.

2.5.7.5 Amprenavir

Amprenavir (APV) is an investigational protease inhibitor that is likely to be approved for clinical use during 1999. Because clinical experience with this agent is still relatively limited, less is known about patterns of resistance and cross-resistance for APV than for the other PIs. *In vitro* passage experiments in the presence of APV selects for mutations at codons 46, 47, and 50. The I50V mutation, which confers 2- to 3-fold resistance, is unique to APV and does not confer cross-resistance to other PIs. Isolates that carry the triple mutation M46I/I47V/I50V have a 20-fold reduction in susceptibility to APV [176]. Clinical isolates from patients treated with APV

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monotherapy confirm the frequent emergence of the I50V mutation, which was accompanied by additional mutations at codons 46 and 47 in several cases [176,178,179].

2.5.8 Cross-resistance among protease inhibitors.

The complex pattern of mutations selected by individual protease inhibitors makes it difficult to predict patterns of cross-resistance with certainty. A number of principles based on structural considerations can be drawn, however. Amino acid residues at positions 82, 84, and 90, which are located at or near the active site, appear to be important for binding of SQV, RTV, IDV, and APV to a greater or lesser extent. Codon 90 appears to be most important for binding of SQV and therefore is selected most commonly following SQV treatment. By contrast, codons 82 and 84 are more important for binding of RTV and IDV, which explains the frequency with which V82A/T/F and I84V mutations are found in isolates from patients receiving those PIs. However, because amino acid residues at these codons are involved in binding to all four drugs, some degree of cross-resistance can be expected due to changes at these positions. By contrast, interactions between amino acid residues at codons 30, 48, and 50 are relatively specific for NFV, SQV, and APV, respectively. However, continued treatment with a failing PI will select additional mutations that confer broader cross-resistance [179,184]. Mutations at these codons do not, therefore, confer broad cross-resistance to other PIs. A survey of plasma virus samples from 1,500 PI-treated patients found cross-resistance in 77% to 95% of viruses that displayed over 10-fold resistance to IDV, RTV, NFV, or SQV [176,178]. The majority of patients had

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received treatment with more than one PI prior to testing, however. The most common mutations included those at codons 10, 36, 46, 54, 71, 77, 82, and 90.

2.6 Use of breastfeeding.

Breastfeeding has been discouraged in HIV-1-infected pregnant women in resource-rich areas, because of the known potential for perinatal transmission by mother's milk. However, in developing areas, the World Health Organization has advised that HIV-infected women continue to breastfeed, since death from infectious diarrhea or dehydration from bottlefeeding with highly concentrated formula has been higher than death rates from AIDS. Nonetheless, the recent data demonstrating a linear relationship between duration of breastfeeding and risk of perinatal transmission provides an alternative option in these settings [81]. With the expected risk of approximately 0.5% per month of breastfeeding [81]. A woman might choose to breastfeed for only 3 to 6 months, for example, which would lead to an added risk of approximately 1.5% to 3%. Another difficulty related to the decision to withhold breastfeeding concerns the lack of privacy and confidentiality that such a decision would mandate. Breastfeeding is expected practice in most resource-poor areas of the world; thus, if a woman chooses not to breastfeed, her HIV-1 status immediately becomes suspect.

2.7 Appropriate nutritional status and avoidance of additional infections.

Relatively nonspecific interventions, such as maintenance of good nutritional status, and avoidance of additional infections, may also be expected to decrease the

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risk of perinatal transmission. Further, such interventions may be less expensive than use of antiretroviral agents.

2.8 Obstetrical Interventions to Reduce Perinatal Transmission

As discussed earlier, elective cesarean section has recently been shown to reduce the risk of perinatal HIV-1 transmission to as low as 2% in pregnant women also taking zidovudine [68]. The importance of membrane rupture beyond 4 hours as a risk factor for perinatal transmission has clearly led to the notion that such prolongation should be avoided. Cesarean section would obviously be one mechanism to avoid such prolongation.

In resource-poor areas, the vast majority of pregnant women deliver at home, and not in hospital settings. It would be extremely difficult to consider the use of elective cesarean section as a method of decreasing the risk of perinatal HIV transmission in these circumstances.

2.9 The Chemokine Receptors: Coreceptors for HIV-1 Infection

In addition to an antiretroviral therapy and elective C-section, there is other PHT prevention factors that can lessen transmission. For example, low level plasma HIV-1 RNA load, high CD4 count, good nutrition in pregnant women [27], suppressive HIV-1 replication factors [28,29], and CCR-5 mutation phenomenon [30]. The presence of a CCR-5 mutation may explain the phenomenon of resistance to HIV infection in some cases. Human CD4+ T lymphocytes, macrophages, and microglial, dendritic and Langerhans cells, are believed to be targets for HIV-1 infection. The CD4

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transmembrane protein together with several chemokine receptors is believed to be necessary for viral attachment and fusion to occur.

The chemokines are a large family of chemoattractant cytokines secreted by tissue cells and macrophages [49]. These molecules mediate inflammation and utilize a variety of related cellular receptors. The chemokine receptors are integral membrane proteins characterized by seven transmembrane segments; the N-terminal extracellular portion of the protein binds the chemokine, while the second cytoplasmic loop interacts with a G-protein and mediates signal transduction. Several of the chemokine receptors can function as obligatory cofactors for HIV entry into cells; the two most important and extensively studied receptors are CXCR4 and CCR5.

2.10 The chemokine co-receptors X

The chemokine co-receptors X (CXCR4) was originally implicated in HIV infection by use of a cell fusion screening technique designed to identify the human cofactor essential for HIV entry into CD4+ cells (50). The natural ligand for this chemokine receptor is stromal derived factor 1 (SDF-1). The CXCR4 chemokine coreceptor was found to be used by T-cell tropic primary isolates of HIV, also known as syncytium-inducing (SI) viruses. While SI viruses are rarely transmitted, they evolve in about half of HIV-infected people within five years of infection. When they do, they are associated with a more rapid disease course. CXCR4 does not function as a coreceptor for macrophage-tropic or non-syncytium-inducing (NSI) isolates.

2.11 The chemokine co-receptors 5

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The chemokine co-receptors 5 (CCR5) The coreceptor for macrophage-tropic (M-tropic) HIV, the most common primary strain of the virus transmitted from one person to another and NSI isolates is a distinct chemokine receptor, CCR5. The natural ligands for this receptor are MIP-1-alpha, MIB-1beta, and RANTES (the beta-chemokines). Indeed, the identification of CCR5 as a coreceptor was facilitated by the observation that these beta-chemokines can block infection by macrophage-tropic and NSI isolates. Interestingly, the isolation of NSI virus has been associated with a relatively slower rate of disease progression; these isolates are restricted to the use of CCR5 as a coreceptor [51]. SI isolates, which have been associated with a more rapid rate of disease progression, can typically use not only CCR5 but also CXCR4 (and rarely, additional receptors such as CCR2b and CCR3). These patient-derived SI isolates display a broadening in their ability to infect cells using multiple chemokine receptors, a characteristic that may explain their relatively greater virulence.

2.12 HIV entry

The mechanism by which the chemokine receptors mediate entry of HIV into cells appears to involve a trimolecular interaction between the viral envelope surface glycoprotein (gp120), the CD4 receptor, and the chemokine coreceptor [50,52]. Mutational analyses have indicated that the function of these receptors as signal-transduction molecules plays no role in their ability to mediate HIV entry. The chemokine receptors alone appear unable to bind directly to gp120. However, in the presence of CD4, gp120 (for example, from the macrophage-tropic clone YU-2) binds with nanomolar affinity to cells that express chemokine receptor (in this example, CCR5) [52]. Similarly, cells that express env will fuse with cells that express CCR5

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only if CD4 is present. Pretreatment of cells that express env with soluble CD4 followed by mixing with cells that express CCR5 results in fusion, but pretreatment of cells that express CCR5 with soluble CD4 followed by mixing with cells that express env does not result in fusion [50]. These results suggest that CD4 activate gp120 for CCR5-mediated fusion and viral entry. This mechanism is substantiated by the observation that in addition to antibodies that recognize the V3 loop of gp120, antibodies that recognize epitopes on gp120 that are induced by CD4 binding can block the binding of gp120 to cells that express CCR5 [52] The portion of the CCR5 molecule that interacts with gp120 is within the N-terminus and appears to overlap that required for chemokine binding; virtually all gp120 molecules derived from NSI isolates across a variety of genetic clades block the binding of chemokines to CCR5 [53]. Although HIV infection does not appear to modulate the expression of chemokine coreceptors, various stimulatory molecules and cytokines can profoundly affect the levels of these molecules on the surface of host cells.

DNA polymorphisms in chemokine receptor genes have been correlated to resistance to HIV-1 infection and to slower progression to AIDS. In particular, a 32-bp deletion mutation in the beta-chemokine receptor CCR5 gene has been associated with relative resistance to HIV-1 infection. That is, individuals who are homozygous for this deletion mutation (delta 32/delta 32) appear to resist HIV-1 infection despite high-risk exposure, whereas those who are [161].

2.13 CCR5 Chemokine Receptor Variant in HIV-1 Mother-to-Child Transmission and Disease Progression in Children

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The recently characterized chemokine receptor gene *CCR5* and its most frequent mutation (*32*) have become the objects of intense interest since their roles in the entry of HIV-1 into target cells were identified. This work reports the first estimation of genotype distribution of the deletion in children born to HIV-1-infected mothers. The genotypes are in Hardy-Weinberg equilibrium, suggesting the absence of deleterious genotypes. The *32* allele frequency in this population is quite similar to that observed in the southern European and Mediterranean populations [26].

Homozygosity for a 32-bp deleted allele in the *CCR5* gene protects adults from HIV-1 infection following blood or sexual exposure [1-4]. So, it is possible that this mutated allele also has a protective effect in children born to HIV-1-seropositive mothers.

A beneficial effect on progression of several studies suggested a lower rate of progression for heterozygous patients in whom the virus was classified as nonsyncytium-inducing i.e. the viral strains that use the CCR5 coreceptor. Once again, study of prospective cohorts of perinatally infected children is advantageous because it is possible to investigate putative predictive factors in homogeneous conditions, the date of infection being known for every case. Although heterozygous children were susceptible to early and severe disease, including encephalopathy, the risks of developing immune deficiency and clinical signs were lower for heterozygotes than for children homozygous for the WT allele. The *CCR532* heterozygous genotype in children born to HIV-1-seropositive mothers does not confer resistance to infection, but it substantially slows development of HIV-related disease. The delta CCR5 allele conferred a significant protective effect against HIV-1 infection and a survival

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advantage against disease progression. Although both progressing and nonprogressing CCR5/delta CCR5 subjects were identified, a distinct survival advantage was shown for those with NSI virus. Thus, the protective effect of delta CCR5 against disease progression is lost when the infecting virus uses CXCR-4 as a coreceptor.

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CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Genotyping

3.1.1 Reagents

3.1.1.1 Reagents for HIV-1 RNA extraction.

1) Absolute alcohol
2) QIAamp mini kit RNA extraction (QIAGEN GmbH, Hilden, Germany)
3) High Pure PCR Template preparation Kit (Boehringer Mannheim, GmbH, Indianapolis, IN, USA)

3.1.1.2 Reagents for Reverse transcription-Polymerase Chain Reaction

1) Superscript-One step RT-PCR
2) RT-Taq enzyme
3) Distill water
4) Primers (stock and working solution)

Oligonucleotide primers, specific for HIV-1 gag-pol gene sequence were synthesized by DNA synthesizer, forward PCR primer 5' TTG GAA ATG TGG AAA GGA AGG AC 3' and reverse PCR primer 5' AAT CTG ACT TGC CCA ATT CAA TTT 3'

3.1.1.3 Reagents for gel electrophoresis

1) 10 X TBE stock solution

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2) 2% Nusieve agarose gel

3) Gel loading buffer

4) 1% Ethidium bromide stock solution

5) Gel marker™ (1 µg/ µl). A standard double stranded DNA size: 100, 200, 300, 400, 500, 800, 1,000, 1,600, 2,000 base pairs for DNA size determination in electrophoresis. (LIFE TECHNOLOGIES)

3.1.1.5 Reagents for Cycle sequencing

1) HIV Sequencing Module (HIV Genotyping System, PE Biosystems)

2) Nanosep® ultrafiltration devices.100 K (PALL Gelman Sciences)

3) Sodium acetate (3 M, pH 4.6)

4) TE Buffer (pH 8.0, 10 mM Tris - HCL, 0.1 mM EDTA)

5) 10X Genetic Analyzer Buffer with EDTA (PE Biosystems)

6) Performance Optimized Polymer 6 (POP 6) with Template Suppression Reagent (TSR) (PE Biosystems)

3.1.1.6 HIV-1 viral load Monitor

1) NASBA (NASBA HIV- RNA QT; Organon , Boxtel, Netherlands.)

CCR 5 defective allele detection

3.1.1 Reagents

3.1.1.1 Reagents for human DNA extraction

1) Isopropanal Alcohol

2) Poly A

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3) HighPure

3.1.1.2 Reagents for PCR

1) 10X PCR buffer minus Mg

2) 50 mM $MgCl_2$

3) 10 mM dNTPs

4) *Taq* DNA polymerase (5 U/ µl) (LIFE TECHNOLOGIES)

5) Distill water

6) Primers (stock and working solution)

Oligonucleotide primers, specific for 32 basepairs deletion of CCR 5 gene sequence were synthesized by DNA synthesizer, forward PCR primer 5' CCAGATCTCAAAAAGGTCT 3' and reverse PCR primer 5' GGTCTTCTCTTTTATTTGTTAGT 3'

3.1.1.3 Reagents for gel electrophoresis

6) 10 X TBE stock solution

7) 2% Nusieve agarose gel

8) Gel loading buffer

9) 1% Ethidium bromide stock solution

10) Gel marker™ (1 µg/ µl). A standard double stranded DNA size: 100, 200, 300, 400, 500, 800, 1,000, 1,600, 2,000 base pairs for DNA size determination in electrophoresis. (LIFE TECHNOLOGIES)

3.1.1.5 Reagents for Cycle sequencing

1) BigDye Terminator Sequencing kit (PE Biosystems)

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2) Nanosep® ultrafiltration devices 30 K (PALL Gelman Sciences

3) Sodium acetate (3 M, pH 4.6)

4) TE Buffer (pH 8.0, 10 mM Tris - HCL, 0.1 mM EDTA)

5) 10X Genetic Analyzer Buffer with EDTA (PE Biosystems

6) Performance Optimized Polymer 6 (POP 6) with Template Suppression Reagent (TSR) (PE Biosystems)

7) Primers (stock and working solution)

Oligonucleotide primers, specific for 32 base-pairs deletion of CCR 5 gene sequence were synthesized by DNA synthesizer, forward PCR primer 5' CTGCATCCTAGGTGCAATGT 3' and reverse PCR primer 5' GGTCTTCTCTTTTATTTGTTAGT 3'

3.1.2 Equipments

1) Biohazard cabinet (Gelaire® ICN Biomedical, Model BSB3A)

2) Centrifuge (Beckman, Model TJ-6)

3) Microcentrifuge (Hettich)

4) Gene Amp® PCR System 9700 Thermocycler (PE Biosystems, Model 9700)

5) Minigel Electrophoresis set (Mupid, Japan)

6) Refrigerator 4 °C, -20 °C, -70 °C

7) Plastic sealer

8) Vortex mixer

9) Microwave oven

10) General glassware

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11) Polypropylene centrifuge tube 15 ml

12) Polypropylene microcentrifuge tube 1.5 ml

13) Polypropylene microcentrifuge tube 0.5 ml

14) Micropipette: 1-10 µl, 5-50 µl, 20-200 µl, 100-1,000 µl with tips

15) HIV Genotyping Systems software version 2.0 (PE Biosystems)

16) Phylogeny Inference Package program (PHYLIP version 3.57c, Joseph Felsentein, University of Washington, Seattle)

17) HIV-1 RT and Protease-A Sequence Analysis Program (HRP-ASAP version 1.0, Robert Shafer, Duane Jung, and Brad Betts, Stanford University)

18) ABI PRISM® 310 DNA Sequencer (PE Biosystems)

19) Capillaries 61 cm x 50 µm (PE Biosystems)

20) Genetic Analyzer Septa and sample tube (0.5 ml)

21) Glass syringe 1.0 ml (PE Biosystems)

22) Thin wall PCR tubes, 0.2 ml

23) PCR strip tubes with attached strip caps, 0.2 ml

24) Rubber glove

3.2 Methods

Genotyping

3.2.1 Study Population

Twenty-two HIV-1 infected pregnant women who enrolled to Dr.Panyu's project were naïve patients and whose viral load was known. The regimen consisted of

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antepartum of one tablet of Combid, which was taken orally early in morning on the day of surgery, and ZDV was given 2 mg/kg intravenously for 1 hour before cesarean section. Lactation was inhibited. Breast-feeding was also forbidden. At 6 weeks' postpartum, routine postpartum care was performed. The infants were also given ZDV syrup (2mg/kg) every 6 hours orally 6 weeks. This should be started immediately after birth. Blood samples for PCR HIV-1 RNA were taken at 48 hours, 1 month, 4 months, 6 months, 12 months, 15-18 months old. When they were proved not to be infected by HIV-1 after 6 weeks, HIV chemoprophylaxis would be ceased. The average viral load in 34 week's gestation of these patients was 15249.16 copies/ml. RNA viral load which is measured by the NASBA (NASBA HIV- RNA QT; Organon , Boxtel, Netherlands.) following the manufacturer's recommendation. Analysis of CD4 lymphocyte subsets was carried out using dual-color fluorescent-activated cell sorter analysis (FACScan, Becton Dickinson, Mount view, California). The patients were recruited in this retrospective study and performed genotyping. They were performed genotyping in the same time of viral load detection.

Four HIV-1 infected infants who were in this retrospective study were in Dr. Sayomporn' s project. They were born in different way. The first two infants were born by natural delivery and the other was born by cesarean section. Both groups of the infants were screened in serology, performed reverse transcriptase-polymerase chain reaction which gave qualitative HIV-1 RNA positive result. The infants who were positive in qualitative HIV-1 RNA were performed genotyping. Analysis of CD4 lymphocyte subsets was carried out using dual-color fluorescent-activated cell sorter analysis (FACScan, Becton Dickinson, Mount view, California).

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3.2.2 Specimen Preparation

EDTA blood of 22 HIV-1 infected patients and 4 HIV-1 infected infants were recruited in this experiment and carried out centrifugation at 12,500 xg at 25 °C for 1 hour and followed by transferring plasma to clean tube which were replaced at –70 °C.

3.2.3 Extraction of HIV-1 RNA

Patients' s plasma was extracted by using QIAGEN and performed as described in QIAamp® instruction. One hundred and forty microliters of blood plasma was added into filter tube-collection tube and 560 ul of lysis buffer and absolute alcohol were added into the previous filter tube, the filter tube-collection tube was taken to centrifuge at 8,000 xg at 25°C for 1 minute. After centrifugation, the filter tube was inserted into a new collection tube and 500 ul of washing buffer1 was added and mixed, centrifuged at 8,000 xg at 25°C for 1 minute. Then 500 milliliter of washing buffer2 was added and mixed, centrifuged at 14,000 xg at 25°C for 3 minutes. After that, the new collection was changed and 50 milliliter of RNA diluent was added. The filter tube-collection tube was centrifuged at 8,000 xg at 25°C for 1 minute. Afterwards RNA solution was stored at –70 °C.

3.2.4 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Twenty microliters of purified RNA was added into 30 ul of Superscript One-Step RT-PCR master mix. Superscript One-Step RT-PCR master mix

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which was ready to use master mix however another components had been added. That was prepared as

Components	Volume
2x Buffer	25 ul
RT/Taq enzyme	1 ul
10 pM sense Specific primers	1 ul
10 pM anti-sense Specific primers	1 ul
Distill water	2 ul
Total volume	30 ul

Afterwards preparing PCR master mix, 20 ul of RNA template was put into them, the components of RT-PCR tube was taken to do RT-PCR. The HIV RNA was reverse transcribed to single - stranded cDNA by using Gene Amp® PCR System 9700 thermal cycler with the profile of cycling parameters were set as

	55 °C	for	30 min	
	94 °C	for	2 min	
	94 °C	for	15 sec	} 40 cycles
	55 °C	for	30 sec	
	68 °C	for	3 min	
hold	72 °C	for	10 min	

The entire protease and about 75% of 5' -end of the reverse transcriptase gene were amplified by two different sequence-specific primers, which were contained in the PCR master mix. After finishing PCR process, we took 5 ul of PCR products to

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run gel electrophoresis, which took time around 30 minutes. Afterwards positive result showed band about 1.6 kb.

3.2.5 Clean-up and Recovery of PCR product

The amplified product was diluted to 500 µl with TE buffer and transferred to a Nanosep® 100 K ultra-filtration device. After centrifugation at 5,000 x g for 10 minutes, the retained sample was recovered with a pipette tip. The retentate cup was rinsed and washed twice with 20 µl TE buffer. The concentration of purified DNA was checked after agarose gel electrophoresis. Ten to 20 ng/µl of purified DNA sample was used in the cycle sequencing step.

3.2.6 Cycle Sequencing Reactions

Fluorescently - labeled, complementary DNA fragments were synthesized by mixing 8 µl of 10 to 20 ng/µl purified DNA with 12 µl of each HIV SEQ MIX A, B, C, D, F, G and H respectively (HIV Sequencing Module). The cycle sequencing reaction was performed as

96 °C	10 sec	}	25 cycles.
50 °C	5 sec		
60 °C	4 min		

3.2.7 Purification of Dye Terminator Sequencing Reactions

The ethanol: sodium acetate solution was prepared by mixing 14 µl of 3.0 M Sodium acetate pH 4.6 with 350 µl of 95% cold ethanol. Fifty-two microliters of the ethanol/sodium acetate solution was added to each sequencing reaction tube and the

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sequencing products were precipitated on ice for 10 minutes. The pellet was obtained after centrifugation at 14,000 x g for 30 min and the supernatant was aspirated carefully. The pellet was then washed once with 250 µl of 70% cold ethanol followed by centrifugation for 5 minutes at 14,000 x g . The 70% ethanol was removed and the pellet was heat-dried at 95 °C for 2 minutes, then fifteen microliter of Template Suppression Reagent (TSR) was added to dried pellet and the reaction was heated at 95 °C for 2 minutes before loading the sample to ABI PRISM 310 Genetic Analyzer (Automate DNA sequencer).

3.2.8 HIV mutation analysis software

Two softwares were used. HIV Genotyping System Software (HGS) version 2.0 was used to assembled the electrophoregram fragments which from the automate DNA sequencer. The HIV text sequence in FASTA format was obtained after the electrophoregram fragments were manually edited by comparing the sample's sequence with pNL4-3 reference sequence. The text sequences were then genotypically analyzed by HRP-ASAP version 1.0 from Stanford University (http://hivdb.stanford.edu/hiv/). Amino acid substitutions (mutation) on both protease and reverse transcriptase genes were carefully interpreted by considering with previous therapeutic histories.

3.2.9 Phylogenetic analysis

Phylogenetic analysis was performed using the PHYLIP (Phylogeny Inference Package, version 3.57c) programs DNADIST, SEQBOOT, NEIGHBOR, CONSENSE

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and DRAWTREE. Tree was outgroup-unrooted by using Simian Immunodeficiency Virus (SIV) in the alignment. Distance matrices were generated with DNADIST using Kimura 2-parameter model or maximum likelihood. Statistical significance was tested by bootstrapping using SEQBOOT to produce bootstrapped datasets (n = 100). Trees were constructed from the distance matrices using the neighbor-joining (NEIGHBOR) method and from the resulting data a consensus tree was calculated using CONSENSE. Trees were visualized using DRAWTREE and TreeView. Nucleotide sequence divergence was calculated for all sequence to rule out laboratory contamination or multiple sampling of the same patients at different testing sites.

3.2.10 Statistical analysis

Pairwise comparisons of genotypic HIV-1 drug-resistance profile between infected mothers and their infected infants by comparing the distribution of substitution of amino acid associated with drug resistance using Fisher Exact test. The P-value reported is for two-tailed comparison. Statistic significance was defined as P-value > 0.05. Estimates of association are given with associated the 95% confidence interval (CI).

CCR-5 Mutation detection

3.2.1 Study Population

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Forty-four pairs of naïve HIV-1 infected pregnant woman and their infant were recruited in this experiment. Their infants who were both HIV-1 infected and uninfected infants were identified that were HIV-1 positive or negative by using PCR.

3.2.2 Specimen Preparation

PBMC of 22 pairs of HIV-1 infected pregnant women and their infants who were both 4 HIV-1 infected infants and 19 HIV-1 uninfected infants are collected in this cohort study. PBMC is stored at –70 °C.

3.2.3 DNA Isolation

Patients' s PBMC is extracted by HighPure method. Two hundred microliters of PBMC was washed with 1000 ul of PBS buffer about 4 times in 1.5 milliliters centrifuged tube. Mixture solution tube was centrifuged at 14,000 xg at room temperature for 1 minute. Supernatant was aspirated and 200 ul of PBMC' s pellet was collected. Afterwards working buffer that contained poly A and binding buffer was added into PBMC suspension in equal ratio, mix briefly. Then 40 ul of proteinase K was filled in mixture and followed by mixing, incubating at 72 °C for 10 minutes. One hundred microliters of isoproyl alcohol was added into the previous tube. Afterward solution was transferred into filter tube-collection tube and centrifuged at 8,000 xg at room temperature for 1 minute. After that suspension in a collection tube was discarded and a filter tube was inserted into new collection tube. Four hundred and fifty microliters of washed buffer was added into filter tube that contained DNA and was centrifuged at 8,000 xg at room temperature for 1 minute. After that

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suspension in a collection tube was discarded and a filter tube was inserted into new collection tube. In the second wash, mixed solution tube was centrifuged at 14,000 xg at room temperature for 3 minutes. After that suspension in a collection tube was discarded and a filter tube was inserted into new collection tube, 50 ul of elution buffer was added into filter tube-collection tube. It was centrifuged at 8,000 xg at room temperature for 1 minute and DNA suspension was collected at -20 °C.

3.2.4 Polymerase Chain Reaction (PCR)

Fifteen microliters of extracted DNA was added in 35 ul of PCR master mix in 500 ul eppendroff tube. PCR master mix was prepared as

Components	Volume
10X PCR buffer minus Mg	5 ul
50 mM $MgCl_2$	2 ul
10 mM dNTP mix	1 ul
10 uM sense specific primer	1 ul
10 uM anti-sense specific primer	1 ul
Taq DNA polymerase	0.5 ul
Distill water	24.5 ul
Total volume	35 ul

After preparing PCR master mix, 15 ul of DNA template was put into them, the components of PCR tube was taken to do PCR. The human DNA was amplified by using Gene Amp® PCR System 9700 thermal cycler with the profile of cycling parameters were set as follow:

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Cycling parameter

	94 °C	for	2 minutes	
	94 °C	for	30 sec	
	55 °C	for	30sec	35 cycles
	68 °C	for	1 minute	
hold	72 °C	for	5 minute	

CCR-5 mutation region was amplified by using sequence specific primers. Afterwards amplified products was detected by gel electrophoresis which took time about 30 minutes. About 200 nucleotides of amplified products could be detected after finishing PCR process.

3.2.5 Clean-up and Recovery of PCR product.

The amplified product was diluted to 500 ul with TE Buffer and transferred to a Nanosep®30 K ultra-filtration device, then centrifuge at 5,000 x g for 10 min. Recover the retained sample with a pipette tip, rinse the retentate cup twice with 20 ul TE Buffer. The concentration of purified DNA is checked after agarose gel electrophoresis by One-D Scan® which compared the intensity of the sample band with 10 ng/ ul of marker band. The sample that contains 10 to 20 ng/ul purified DNA is used in cycle sequencing step.

3.2.6 Nucleotide Cycle Sequencing

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Reaction Fluorescently - labeled, complementary DNA fragments were synthesized by mixing 10 ul of 10 to 20 ng/ul purified DNA with 2 ul of each sense primer and antisense primer respectively and 8 ul of BigDye Terminator. The cycle sequencing parameters were performed as

Cycle sequencing parameter

96°C	for	10 sec	25 cycles
55°C	for	5 sec	
60°C	for	4 min.	

3.2.7 Purification of Dye Terminator Sequencing Reactions

The ethanol: sodium acetate solution was prepared by mixing 14 ul of 3.0 M Sodium acetate pH 4.6 with 350 ul of cold 95% ethanol. Fifty two microliters of the ethanol/sodium acetate solution was added into each sequencing reaction tube and the sequencing products were precipitated on ice for 10 min. The pellet was obtained after centrifugation at 14,000 x g at room temperature for 30 min and aspirating the supernatant carefully. The pellet was washed once with 250 ul of cold 70% ethanol and followed by centrifugation for a further 5 min at 14,000 x g. The 70% ethanol was removed and the pellet was heat - dried at 95°C for 2 min, then 15 ul of Template Suppression Reagent (TSR) was added into dried pellet following heating the reaction at 95°C for 2 min before loading the sample to ABI PRISM 310 Genetic Analyzer.

3.2.8 CCR 5 defective allele analysis software

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Software, which was analyzed, was Autoassemble software. Autoassemble software was used to assemble the electrophoregram fragments which from the automate DNA sequencer. The CCR 5 text sequence in FASTA format was obtained after the electrophoregram fragments was manually edited by pair alignment between the forward and reverse fragments of the sample's sequence. The assembly text sequences was aligned with reference human CCR 5 sequence and 32 basepairs deletion CCR5 sequence. CCR5 defective allele phenomenon (the deletion of 32 nucleotides on CCR 5 gene) on human CCR 5 gene was carefully interpreted by considering with HIV-1 resistance histories.

3.2.9 CCR-5 sequence alignment

CCR5 sequence alignment was performed using the ClustalW. CCR5 sequence of infected naive pregnant women and their infants were aligned with Human CCR5 mutant sequence to investigate defective CCR5 allele resulted in deletion of 32 base-pairs in CCR5 gene sequence.

3.2.10 Statistical analysis

Allele frequencies were calculated as (h+2H)/2N, where H is the number of homozygous mutation genotypes, h is the number of heterozygous mutation genotypes and N is the total of sample population.

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CHAPTER IV

RESULT

4.1 Baseline characteristics of infected naive pregnant women and their infected and uninfected infants.

The characteristics of the infected naïve pregnant women (n=22) and their infected infants (n=4) are shown in Table 1. The median $CD4^+$ and $CD8^+$ cell counts in infected naïve pregnant women at week 34 of gestation were 395.19 cells/ul (range, 137-835) and 934.43 cells/ul (range, 506-1498), respectively, and the median $CD4^+$ and $CD8^+$ cell counts at week 38 were 507.3 cells/ul (range,125-1,083) and 978.7 cells/ul (range,148-1,754), respectively. These results are shown in Table1, 2, 3 and 4.

4.2 HIV-1 drug-resistance Genotyping.

The median plasma HIV-1 RNA level at week 34 and week 38 of gestation was 18,227.86 copies/ml (range, 275-76,000) and 581.5 copies/ml (range,125-4,600), respectively in naïve pregnant women. Qualitative HIV-1 RNA (NASBA) in infected infants was determined during a period after birth (range, from 2 days after delivery to 2 years). Of the 26 plasma RNA samples that were studied in HIV-1 drug resistance genotyping, all could be amplified and analyzed by direct DNA sequencing. The common combination of drugs in this study was zidovudine and lamivudine. Twenty-two maternal blood samples from naïve patients were collected from Ramathibodi hospital after 34 weeks gestation. Blood samples from the 4 infected infants were

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collected after delivery. Two were collected after caesarian section and the others after vaginal delivery.

None of the 22 infected mothers had ever been treated with an antiretroviral drug before joining this study, but they were administered an antiretroviral drug from week 34 of gestation to delivery. Four infants born to infected mothers were investigated for HIV-1 infection by using qualitative HIV-1 RT-PCR (NASBA). Blood samples from the HIV-1 infected patients in this study were analyzed for pol gene sequence. The pol gene sequences of both mothers and children were analyzed for evidence of HIV-1 drug resistance profile, and the correlation of transmitted HIV-1 strain in pairs of mothers and infected infants was identified. The four infected children were tested for HIV-1 infection by assaying for qualitative HIV-1 RT-PCR. For the first child, qualitative HIV-1 RT-PCR was positive after 2 days, 1month, 4 months and 6 months after caesarian section delivery. Qualitative HIV-1 RT-PCR was positive in the second child 1 year after vaginal delivery. The third child was positive for HIV 1 RT-PCR after collecting for 1 month and 4 months after caesarian section delivery. The fourth child was the elder sister of the third one and was recruited in this study at 2 years old. Her result is shown in Table 5.

4.3 HIV-1 Subtype

Of the 26 sequence data, one mother from 22 (4.55%) and two children from 4 (50%) were infected with HIV-1 clade B with no codon mutation in both the protease and reverse transcriptase region. In the HIV-1 clade E group, 21 of 22 (95.45%) maternal pol gene sequences and 2 of 4 (50%) pol gene sequences occurred and were

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found in infected mothers and their infants, respectively. The result is shown in Table 6.

4.4 HIV-1 reverse transcriptase gene mutations

In the reverse transcriptase gene from all HIV-1 infected mothers and infants, there were no substitutions commonly associated with nucleoside reverse transcriptase inhibitors-resistance (NRTIs-resistance) and non-nucleoside reverse transcriptase inhibitors-resistance (NNRTIs-resistance). However, several amino acid changes were found scattered along the reverse transcriptase gene. Cornelissen M. [148] reported that some substitutions could be classified as a specific amino acid subtype, e.g. 43E for subtype A'. This study found that almost all isolates (N=20) among infected mothers and 2 infected infants had this amino acid substitution.

In this study, A98G was associated with Nevirapine resistance at position 98 and P79 was found at A98S.

H208Y was an NRTI mutation at position 208. This occurred with increased frequency in patients receiving multiple NRTI, but was uncommon in untreated patients. This treatment also caused foscarnet resistance. In this study, P146 was found at H208N.

In the pairs of mothers and infants who were infected with HIV subtype B, no amino acid substitution was found in the reverse transcriptase gene.

The occurrence of amino acid substitution associating drug resistance in this study is showed in Table 7.

4.5 HIV-1 protease gene mutations

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All twenty-three pol gene sequences, which included the 21 infected mothers and 2 infected infants, belonged to clade E. In this study, some amino acid substitutions were in a position that conferred protease inhibitor resistance. The replacement of amino acid at position 36 identified M36I. M36I had been found in the entire protease region of all subjects infected with subtype E [100%], including both mothers and children. Apart from M36I codon mutation, 1 of 21 (4.55%) maternal isolates was found to have L10I codon mutation that associated with protease inhibitor resistance. The replacement of amino acid in other positions had been found, but drug resistant properties of a substitution are still unknown.

At position 74, T74A/S occurred only during treatment with protease inhibitors and its effect on resistance has not been studied. P146 was found at A74S.

At position 82, V82A/T/F/S caused resistance to IDV and RTV, with mutations at residues 46 + 82 or 48 + 82 that conferred NFV resistance. P96 was found at V82I.

At position 93, I93L occurred commonly in untreated patients, but even more often in those receiving PIs. In this study, I93L was found in 3 infected naïve mothers.

However, in infant isolates, which were infected with HIV subtype E, there was no amino acid replacement that associated with protease inhibitor resistance.

Regarding pairs of mothers and infants who were infected with HIV subtype B, no amino acid replacement was found in a position that associated with drug resistance in the protease gene. The occurrence of amino acid substitution associating drug resistance in this study is showed in Table 8.

4.6 The nucleotide substitution

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Each pair of mother and child, including those infected with HIV subtype E and B, had a different nucleotide profile. One child in the first mother and child pair had nucleotide substitution in the protease gene that was different to his mother's. This substitution of nucleotide caused an amino acid change at position 12. The child in the second pair also had a nucleotide substitution that was different to his mother's, however, no amino acid change occurred in this case. The third child had nucleotide substitutions that were different to his mother's, and these caused 2 amino acid changes at position 70 and 72. There was a substitution of nucleotide that did not result in an amino acid change at position 68. The fourth child, who was the sister of the third one, had nucleotide substitutions that caused 3 amino acid changes at position 37, 70 and 72.

In the reverse transcriptase gene, there were more nucleotide substitutions than in the protease gene in each mother and child pair. A child from the first pair had a nucleotide substitution that did not result in an amino acid change at position 25 and177. The second child had nucleotide substitutions that caused 6 amino acid changes at position 11, 180, 230, 289, 281 and 307, but no change occurred in amino acid at position 303. The third child had nucleotide substitutions that resulted in an amino acid change at position 13, 178, 272 and 288, but there was no amino acid change at position 103, 141, 148, 152, 155, 198, 250, 256, 259 and 273. The fourth child had nucleotide substitutions that resulted in an amino acid change at position 259, 261 and 273, which caused an amino acid change at position 13, 78, 245, 272 and 288.

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However, all nucleotide substitutions, which resulted in an amino acid change at each position in both the PR and RT gene, had not been associated with drug resistance.

4.7 The phylogenetic analysis

The phylogenetic analysis of sequence among infected pregnant women and 3 infected mother-child pairs were compared to the HIV-1 reference sequences. From the pol clade of HIV-1, the correlation disclosed that 21 maternal and 2 child sequences belonged to HIV-1 clade E, and 1 maternal sequence and 2 child sequences belonged to HIV-1 clade B. Nucleotide genetic distances were calculated from maternal and infant blood samples.

A phylogenetic tree was reconstructed by using the neighbor-joining method for 29 pol gene sequences that included sample, reference and outgroup sequences. Sequences between infected mothers were separated clearly. Sequences from each mother-child pair were clustered together and separated clearly from those of the other mother-child pairs. The mother-child pair in the first and second sequence belonged to subtype E and the last mother-child pair sequence belonged to subtype B. Results concerning the HIV-1 subtype from HIV-1 pol gene sequencing among the patients, and the phylogenetic analysis, are presented in Table 6 and Figure 1 and 2.

4.8 CCR5 defective allele genotyping

Defective CCR5 allele genotyping was studied in infected naïve pregnant women and infected and uninfected infants. Of the 45 peripheral blood mononuclear

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cell DNA samples that were studied in defective CCR5 allele genotyping, all could be amplified and analyzed by direct DNA sequencing.

A total of 45 infected and uninfected subjects, including infected mothers and their infants, were used for this study. Nineteen of the infants were uninfected and 4 were infected by vertical transmission. A total of 22 infected mothers and 22 infected and uninfected infants were placed in a Combid (ZDV/3TC) treated group. One infant had no drug therapy because she was recruited into this study when she was 2 years old. The HIV-1 transmission rate was significantly lower in the Combid treated group than in those taking antiretroviral drugs, as previously reported [160,166]. This study analyzed infected mothers in order to identify the molecular evidence of a CCR5 genotype. It also analyzed both infected and uninfected infants, because the substantial effect of vertical transmission might result from the genetic influence of a CCR5 defective allele phenomenon among pairs of mothers and children in this study. In detecting the 32-bp deletion within the CCR 5 locus, none of the HIV-1 infected mothers or infected and uninfected infants had homozygous or heterozygous delta 32-bp deletion of the CCR5 gene. Results concerning the wild type CCR5 gene are presented in Table 9 and Figure 3.

The absence of this allele was in accordance with previous studies in other Asian populations [159,166,167].

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Table 1. The Characteristic of patients.

Patient	Recruited into project	Ruled out ofproject
P1	/	-
P2	/	-
P11	/	-
P12	/	-
P19	/	-
P26	/	-
P31	/	-
P35	/	-
P45	/	-
P60	/	-
P61	/	-
P70	/	-
P79	/	-
P86	/	-
P96	/	-
P102	/	-
P120	/	-
P123	/	-

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P140	/	-
P141	/	-
P146	/	-
Px	-	/
C1	/	-
C2	-	/
C3	/	-
C4	-	/

P, Mother

C, Infected infant

/, Yes

-, No

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Table 2. Maternal demographic profile

Patient	Age	Personal disease	Family disease	No. of marriages	Risk factor	Anti-HIV status of husband
P1	24	-	-	1	-	+ve
P2	24	-	-	1	-	+ve
P11	22	-	-	1	-	+ve
P12	23	-	-	1	-	+ve
P19	36	-	Her mother is DM	2	Couple anti-HIV +ve	+ve
P26	18	-	-	1	IDU	+ve
P31	27	-	-	1	-	+ve
P35	30	-	-	2	-	+ve
P45	23	-	-	1	-	+ve
P60	27	-	-	2	-	Unknown
P61	28	-	-	1	-	+ve
P70	20	-	-	1	-	+ve
P79	31	-	-	1	-	+ve

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Patient	Age	Personal disease	Family disease	No. of marriages	Risk factor	Anti-HIV status of husband
P86	29	-	-	1	Husband's old wife may be died by HIV	+ve
P96	23	Psoriasis	-	1	-	+ve
P102	26	Syphilis	-	1	-	+ve
P120	27	-	-	1	More than one sexual partner	-ve
P123	32	-	Her mother is DM and Heart disease	3	More than one sexual partner	+ve

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Patient	Age	Personal disease	Family disease	No. of marriages	Risk factor	Anti-HIV status of husband
P140	21	-	-	2	-	+ve
P141	24	-	Her mother is DM	1	-	+ve
P146	27	Anemia	-	1	-	+ve
Px	32	-	-	1	-	+ve

IDU, Intravenous Drug User

+ve, Positive

-ve, Negative

- , None

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Table 3. Characteristics of the maternal sample at week 34.

Patient	Viral load (copies/ml)	Log	CD4 count (cell/ml)	CD8 count (cell/ml)
P1	38,000	4.58	335	858
P2	20,000	4.30	537	506
P11	4,700	3.67	221	995
P12	<350	<2.54	835	754
P19	470	2.67	582	1,123
P26	590	2.77	673	1,322
P31	34,000	4.53	335	904
P35	<275	2.44	339	691
P45	16,000	4.20	261	1,210
P60	25,000	4.40	296	952
P61	37,000	4.57	433	921
P70	20,000	4.30	476	856
P79#	1,300	3.11	494	507
P86	6,500	3.81	207	646
P96	3,400	3.53	481	1,373
P102	8,200	3.91	292	497
P120	8,700	3.94	363	1,310
P123	50,000	4.70	369	1,498
P140	18,300	4.26	333	755

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P141	14,000	4.15	300	885
P146	76,000	4.88	137	1,060
Px*	<400	<2.6	-	-

*** Preterm**

 # Week 36 of gestation

- Not done

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Table 4. Characteristics of the maternal sample at week 38.

Patient	Viral load (copies/ml)	Log	CD4 count (cell/ul)	CD8 count (cell/ul)
P1	<380	<2.58	600	1,304
P2	<700	<2.85	1,083	884
P11	<250	<2.39	343	1,351
P12	<325	<2.51	960	710
P19	<325	<2.51	629	1,055
P26	<325	<2.51	752	1,204
P31	<400	<2.60	581	1,070
P35	<400	<2.60	539	919
P45	<400	<2.60	363	864
P60	<325	<2.51	406	926
P61	<250	<2.39	459	725
P70	<325	<2.51	655	1,106
P79	<300	<2.48	148	148
P86	<375	<2.57	364	828
P96	<300	<2.48	616	1,380
P102	<300	<2.48	380	577
P120	4,600	3.66	363	1,310
P123	<125	<2.09	125	332
P140	ND	ND	ND	ND
P141	<125	<2.09	635	1,754

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P146	1,100	3.04	145	1,127
Px	Preterm	Preterm	Preterm	Preterm

ND, Not done

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Table 5. Characteristics of the infant samples

Characteristic	Infant1(C1)	Infant2(C2)#	Infant3(C3)	Infant4 (C4)
Delivery	**C/S**	**Preterm**	**C/S**	**VD**
Qualitative HIV RNA				
2 days	positive	ND	ND	ND
1 month	positive	ND	positive	ND
4 months	positive*	ND	positive*	ND
6 months	positive	ND	-	ND
1 year	-	positive*	-	ND
2 years	-	-	-	positive*
Drug treatment				
AZT	/	/	no	no
ddI	/	/	/	no
d4T	no	no	/	no
NVP	no	/	no	no

C/S, Cesarean section

VD, Vaginal delivery

ND, Not done

- to be continued

***, Take for genotyping**

#, Symptomatic

/, Drug treatment before genotyping

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Table 6. Characteristics of the HIV-1 subtype among 22 infected pregnant women and their 4 infected infants

Maternal Isolate 22(100)	subtype E 21(95.45)	subtype B 1(4.55)
P1	E	-
P2	E	-
P11	E	-
P12	E	-
P19	E	-
P26	E	-
P31	E	-
P35	E	-
P45	E	-
P60	E	-
P61	E	-
P70	E	-
P79	E	-

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P86	E	-
P96	E	-
P102	E	-
P120	-	B
P123	E	-
P140	E	-
P141	E	-
P146	E	-
Px	E	-

Infant's Isolate	**subtype E 2(50)**	**subtype B 2(50)**
C1	E	-
C2	E	-
C3	-	B
C4	-	B

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Mother and child pair	HIV-1 subtype
P60-C1	E-E
Px-C2	E-E
P120-C3	B-B
P120-C4	B-B

-, Not found

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Table 7. Overall resistance in the reverse transcriptase gene among 22 Thai infected pregnant women and their 4 infected infants

Mutation	No. of individuals (%)	
	infected pregnant women (n=22)	infected infants (n=4)
Primary NRTIs, total		
M41L	wt	wt
K65R	wt	wt
K70R	wt	wt
L74 I	wt	wt
L74V	wt	wt
Q151M	wt	wt
M184V	wt	wt
M184 I	wt	wt
L210W	wt	wt
T215Y	wt	wt
T215F	wt	wt
T215D	wt	wt
T215V	wt	wt
T215S	wt	wt
K219Q	wt	wt
K219E	wt	wt
K219R	wt	wt
K219H	wt	wt
Secondary NRTIs, Total	wt	wt
A62V	wt	wt
D67N	wt	wt
T69N	wt	wt

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T69D	wt	wt
T69A	wt	wt
Primary NNRTIs, Total	wt	wt
K103N	wt	wt
Y181C	wt	wt
Y188L	wt	wt
G190A	wt	wt
Secondary NNRTIs, Total	wt	wt
A98G	wt	wt
V179D	wt	wt
K101E	wt	wt

Wt, Wild type

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Table 8. Overall resistance in the protease gene among 22 Thai infected pregnant women and their 4 infected infants

Mutations	No. of individuals (%)	
	infected pregnant women (n=22)	infected infants (n=4)
Primary PIs, Total	wt	wt
M46I	wt	wt
M46L	wt	wt
G48V	wt	wt
G48M	wt	wt
V82A	wt	wt
V82F	wt	wt
V82 I	wt	wt
I84V	wt	wt
L90M	wt	wt
Secondary PIs, Total	21 (100)	4 (100)
L10I	1 (4.54)	wt
L10V	wt	wt
L10F	wt	wt
L10Y	wt	wt
K20R	wt	wt
K20 I	wt	wt
K20T	wt	wt
L33 I	wt	wt
L33F	wt	wt
L33V	wt	wt
M36I	21(100)	2(50)

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M36V	wt	wt
F53L	wt	wt
F53I	wt	wt
I54V	wt	wt
I54A	wt	wt
I54M	wt	wt
L63P	wt	wt
L63C	wt	wt
L63S	wt	wt
L63A	wt	wt
A71V	wt	wt
A71T	wt	wt
A71 I	wt	wt
G73S	wt	wt
V77 I	wt	wt
V77L	wt	wt
N88D	wt	wt

Wt, Wild type

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Table 9. Characteristics of the defective CCR5 allele

Patient	delta 32/ delta 32	delta 32/ wt	wt/wt
Mothers	-	-	22
Infants			
- Infected	-	-	4
- uninfected	-	-	19

-, Not found

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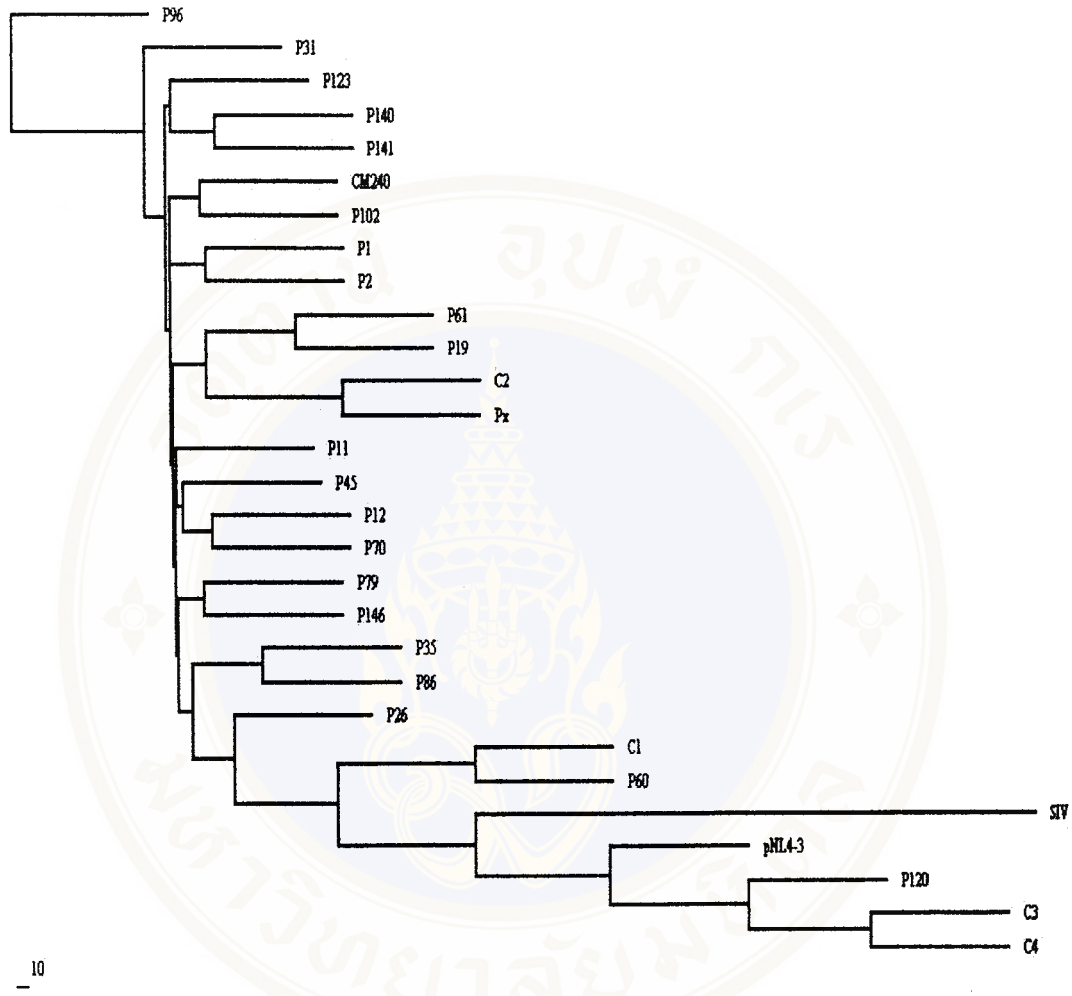

Figure 1. The neighbor-joining tree was constructed using the PHYLIP programs with 100 bootstrap replications. The tree is rooted and branch lengths are proportional to the number of molecular changes. These sequences represent each of the 22 infected Thai naive pregnant women's pol gene sequences. SIV is used as an outgroup. The HIV-1 reference subtype E is represented as CM-240 and reference subtype B represented as pNL4-3.

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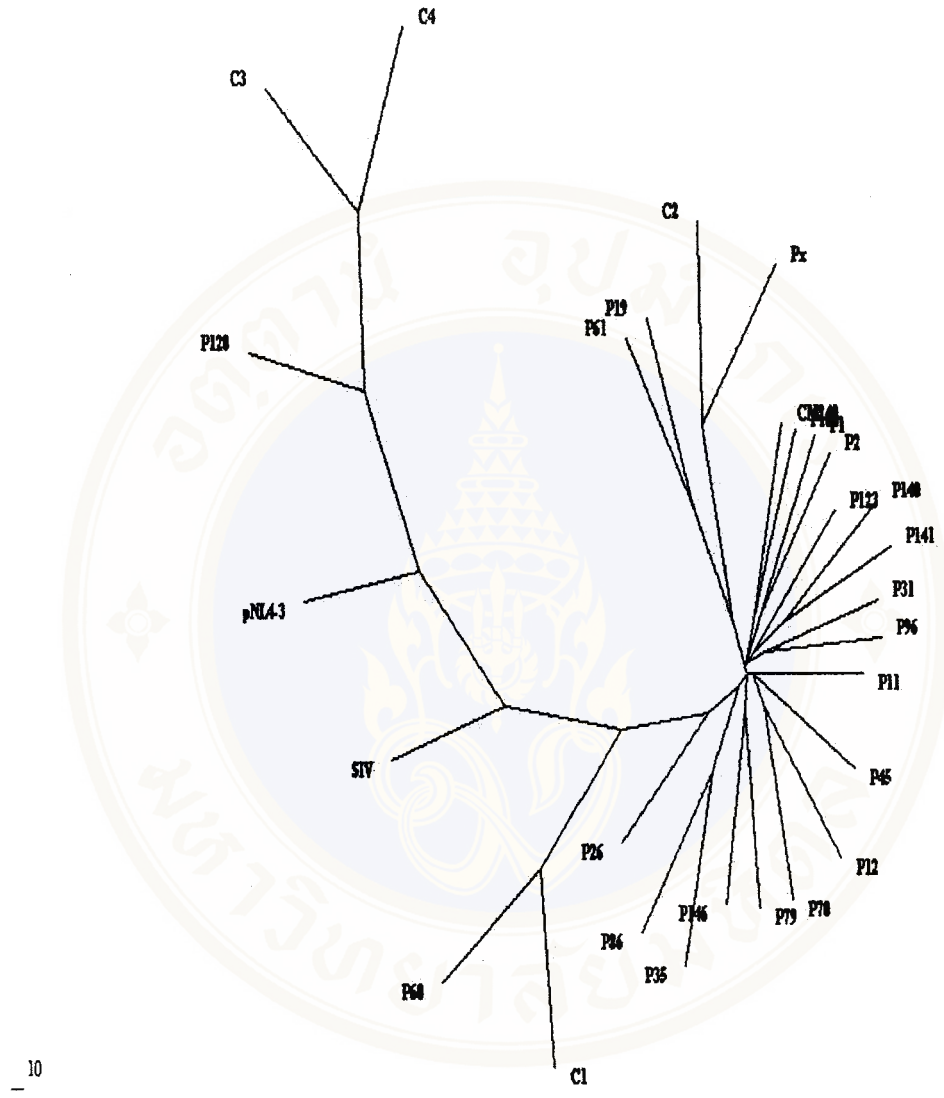

Figure 2. The neighbor-joining tree was constructed using the PHYLIP programs with 100 bootstrap replications. The tree is not rooted and branch lengths are proportional to the number of molecular changes. These sequences represent each of the 22 infected Thai naive pregnant women's pol gene sequences. SIV is used as an outgroup. The HIV-1 reference subtype E is represented as CM-240 and reference subtype B represented as pNL4-3.

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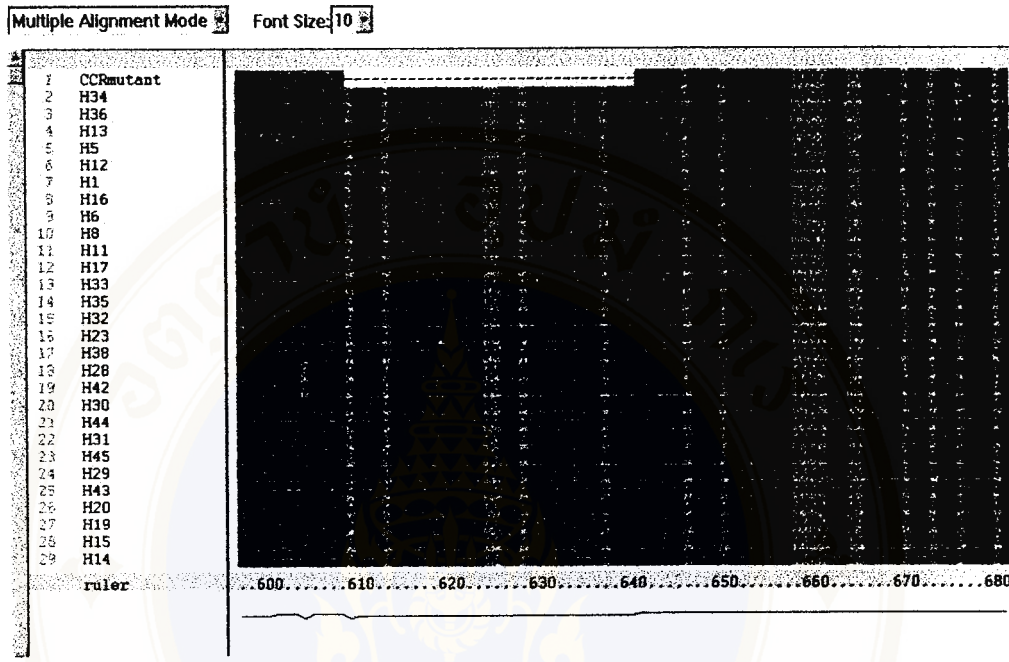

Figure 3. This picture demonstrates about the multiple sequence alignment of between groups of the pair of infected naïve pregnant women and their children and mutant CCR5 sequence. The first sequence represents as mutant CCR5 sequence and the later represents as sequences of mothers and children.

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CHAPTER V

DISCUSSION

Most transmissions of perinatal Human Immunodeficiency Virus type 1 (HIV-1) occur during labor and delivery [8-11]. Most HIV infections among children are caused by perinatal transmission (vertical transmission of the HIV virus from an infected woman to her infant during pregnancy, at the time of birth, or in the week after birth) [20,23-24]. Vertical transmission of HIV-1 by mothers taking antiretroviral drugs during pregnancy has been reported [119,120,134-135]. Many pregnant women have been treated with antiretroviral drugs before pregnancy. A study from Europe and the United States reported that the prevalence of HIV-1 variants in the blood circulation of pregnant women could be an important factor in the mother to child transmission [MTCT] of HIV-1. A maternal drug resistant virus is predictive of transmission [147,149]. This study analyzed the pol gene sequence of infected Thai naïve pregnant women in order to investigate the incidence of HIV-1 drug-resistance profiles and occurrence of codon mutations associating to antiretroviral drug resistance. Apart from providing an HIV-1 profile in naïve Thai pregnant women, this study identified which HIV-1 strains can be transmitted from mother to infant.

The data from this study demonstrated that the rate of vertical transmission is low in Thai naïve pregnant women. This phenomenon might result from receiving antiretroviral drugs before birth and a low HIV viral load level during the period of delivery. However, there is evidence of HIV transmission occurring in this study. In the first pair, P60-C1, the infant was identified as HIV positive by using an immunologic test and qualitative HIV RT-PCR. Qualitative HIV RT-PCR was

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positive after 2 days, 1 month and 4 months, subsequently. This indicated that C1 was infected during the period of ante-partum, and it might have created an impact of high maternal viral load during pregnancy. In this case, cesarean section and non-breastfeeding cannot prevent the transmission of HIV. In the second pair, Px-C2, the infant was born in week 35 of gestation, which is referred to as preterm, and given birth by vaginal delivery. This might be a cause of infection. In the third pair, P120-C3, the infant was identified as infected with HIV by positive qualitative HIV RT-PCR at 1 month and 4 months after cesarean section. This could indicate that C3 was infected during the period of peripartum although, in this case, antiretroviral therapy was performed on the mother before delivery by cesarean section . However, the mother had a rather high viral load level (approximately 5,000 copies/ml), which could be a cause of transmission. In the fourth pair, P120-C4, no information of C4 is available from the period of birth to 2 years old.

Almost all naïve infected pregnant women in this study were HIV-1 clade E, with only one being infected with HIV-1 clade B. No codon mutation associated with nucleoside reverse transcriptase inhibitors-resistance (NRTIs-resistance) and non-nucleoside reverse transcriptase inhibitors-resistance (NNRTIs-resistance) in the reverse transcriptase gene were found in mothers infected with HIV-1 clade E or B. All infected mothers in the HIV-1 clade E group had a virus in the protease gene that carried a protease mutation with PI resistance at codon 36 (M36I), which is in agreement with published data [146]. Mutation at this codon, which can always be found in HIV-1 clade E, has been associated with treatment failure when it combines with other codon mutation [146]. In addition to M36I codon mutation, L10I was the only mutation associated with the protease inhibitor resistance that occurred in this

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study. L10I codon mutation decreased the susceptibility of PIs further [146]. With regard to the pregnant woman who was infected with HIV-1 clade B, no codon mutation was found in either gene. In this study, there were 4 infected children born to mothers who performed genotypic drug resistance. They presented 2 HIV-1 subtypes such as E and B. No amino acid replacement that could cause drug resistance in both the reverse transcriptase and protease gene was found in any of the infected infants. The HIV-1 drug-resistance profile between mother and child in each pair were similar, however, their nucleotides in pol gene sequences were not absolutely identical. This indicates that the strain transmitted to the infant might be an impact of several HIV-1 variants including predominant and minor HIV strains in the maternal circulation.

Prevention of perinatal transmission by a short course of zidovudine and lamivudine

In Thailand, a short course regimen of zidovudine (Bangkok regimen) for HIV 1 infected women reduced transmission from mother to infant by 50% [80,134]. Therefore, the use of zidovudine is recommended for the prevention of perinatal transmission [86-90]. However, it is very important to document whether the use of ZDV-monotherapy during pregnancy presents the development of ZDV resistance, which in these circumstances could associate with codon mutation. A combination of antiretroviral drugs has been recommended to prevent perinatal transmission. In accordance with the PETRA or SAINT trial, they consist of ZDV and 3TC [134,137]. The results of these trials have shown a great reduction of perinatal HIV transmission

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from mother to child, which could affect the synergy between drug combinations [134,137].

This result, from studying infected Thai naïve pregnant women, could indicate that almost all infected pregnant women have not found a codon mutation associated with drug resistance in both the reverse transcriptase and protease gene. This could indicate that the low transmission rate of drug resistance strains could occur commonly in the naïve group and this impact might result in a potent drug combination. This analysis demonstrated that a genotypic HIV-1 drug-resistance profile of infected infants was similar to their mother's, who had not received antiretroviral therapy before. There was no significant difference (P-value > 0.05) in the distribution of a genotypic HIV-1 drug-resistance profile between a mother-child pair with a 95% CI. Potent antiretroviral drugs or other factors such as the inhibition of breast-feeding management could be instrumental in the prevention of HIV-1 transmission from mother to child. In this study, although wild type strains of virus have occurred commonly among infected Thai naïve pregnant women and been transmitted to infants, it is impossible to rule out chance effects given the small number of mutant viral isolates. Chance fluctuations may also play an important role in the transmission of resistance where the inoculum size is small [149].

In addition to antiretroviral therapy, which is an elective C-section, a low-level viral load, high CD4 count, good nutrition and healthy immunity could reduce HIV vertical transmission. A genetic host factor, for example a defective CCR5 allele phenomenon, has been known to influence a low infection rate of Human Immunodeficiency Virus type 1 (HIV-1). A defective CCR5 allele phenomenon or

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genetic mutation in the CCR 5 gene consists of 32-bp deletion results in a non-functional chemokine receptor. This is a major factor, which can reduce HIV vertical transmission and a rapid progress of the disease [153,155,157]. However, several studies reported that the CCR5 32-bp deletion allele rarely occurs outside the Caucasian group. This study found no evidence of genetic mutation of the CCR5 allele, which included both the homozygosity and heterozygosity of the 32- bp deletion in the CCR 5 gene of infected naïve Thai pregnant women and their infected and uninfected infants. This is consistent with previous reports [161,165,167]. This result indicates that the CCR 5 32 bp deletion allele does not contribute significantly to the phenomenon of resistance to HIV infection and long term non-progression to HIV disease (as observed amongst the population of this study).

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CHAPTER VI

CONCLUSION

The results of this study suggested that among infected Thai naïve pregnant women, who had never been treated with an antiretroviral drug before, no amino acid substitution was evident in the reverse transcriptase gene that associated with resistance of the nucleoside and non-nucleoside analogue. However, amino acid replacement occurred in protease gene associated PI resistance. L10I and M36I, which were codon mutations affecting protease resistance, were identified in infected Thai naïve pregnant women. This was interesting because codon mutation associated drug resistance occurred in those women who had never been treated with an antiretroviral drug before. There was no evidence of amino acid replacement that associated with drug resistance. In the comparative pol gene sequence between each mother-child pair, both maternal and infant sequences were not absolutely identical. This could be the transmission result of one from several variants in the maternal circulation [169]. Moreover, the HIV-1 drug-resistance profile involved in the amino acid replacement of infected infants was similar to that of their mother's. However, several considerations limit the conclusion of this study. Firstly, the possibility that confounding variables (such as another chemokine co-receptor apart from CCR5 or either SI- or NSI-viral strains), which can be transmitted during pregnancy, has an impact on the validity of the conclusion. Secondly, the small number of infected infants limited the statistical power of the study to make any detection. Although this study used an effective strategy including antiretroviral therapy before delivery,

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infants were infected despite obstetric and non-breast-feeding management to prevent HIV transmission. This phenomenon might be either an impact of high maternal viral load during pregnancy or other variables such as transmission during intrauterine.

Apart from the HIV-1 drug-resistance genotyping of this study, there was no incidence of a defective CCR5 allele. This associates with several researches that have reported a high prevalence of wild type CCR5 allele, which was in accordance with previous studies of other Asian populations [159,166,167].

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APPENDIX

1. 3 M Sodium acetate (pH 4.6)

Sodium acetate	24.609 g
D.W.	100 ml

Adjust pH with 6 N HCl, autoclave and store at 4 C

2. TE-buffer (pH 8.0)

10 mM Tris-HCl	0.1212 g
0.1 mM EDTA	20 ml
adjust volume with D.W. to	100 ml

Autoclave and store at 4 C

3. 10 X TBE (0.5 M Tris, 0.5 M Boric acid, 10 mM EDTA)

Tris-base	60.5 g
Boric acid	30.85 g
EDTA.Na2.H2O	7.44 g
D.W. to	1 L

4. 2% Nusieve gel

Heat 2 g Nusieve (FMC, ME, USA) dissolved in 100 ml of 1 X TBE with microwave oven or by double boiling, pour the warm gel at below 70 C into the gel casting plate with about 4 mm. thick, and set the comb. After the gel is solidified (15-30 minutes), flood the 1 X TBE to the level just above the surface and gently draw the comb.

5. Gel loading buffer (0.25 % BPB, 0.25 % XC-FF in 30% glycerol)

Bromphenol blue (BPB)	0.025 g
Xylene Cyanol FF (XC-FF)	0.025 g
Glycerol	3 ml

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D.W.	7 ml

Aliquot 1 ml volume and store at 4 C

6. 1% Ethidium bromide stock solution

Ethidium bromide	1 g
D.W.	100 ml

Store at 4 C in brown bottle or wrapped with aluminum foil. 1 ug/ml working Ethidium bromide solution is prepared by dilute 30 ug of stock solution in 300 ml distilled water.

Caution Ethidium bromide is powerful mutagen and moderately toxic. Glove should be worn while handling solution containing ethidium bromide.

7. 75% Ethanol

Absolute Ethanol	75 ml
D.W.	25 ml

8. Working Buffer

DNA binding buffer	250 ul
Poly A	4 ul

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Biography

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