CHAPTER 1

Introduction

1.1 Historical Background

According to the global estimation in 2015, 36.9 million people were living with HIV infection, out of these, 2.6 million were children, age under 15 years old [1]. In Thailand, at the end of 2014 [2], estimated 445,504 people living with HIV included 6,875 children. The newly infections were estimated 7,816 cases, out of these, 121 cases were children. Antiretroviral (ARV) drug has become a standard of care for HIV infected patient management and for prevention mother-to-child transmission (PMTCT). It has significantly reduced the number of AIDS-related deaths by 20% and the new HIV infections by 50% in children [3-9]. According to current guidelines [10-14], the ART of choice for HIV-1-infected children and adults is three-drug combination consisted of two nucleoside reverse transcriptase inhibitors (NRTIs) with the third agent from a different class, either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). This combination is also known as Highly Active Antiretroviral Therapy (HAART). The use of HAART in HIV infected children and adults has dramatically changed the course of infection, reducing mortality and morbidity events associated with this disease [15, 16].

However, the major constraint to the successful management of HIV infection is the development of resistance against ARV drugs or HIV drug resistance (HIVDR) [17, 18] which lead to complicates further control the viral replication with ART [18] and cause HAART failure. To avoid these problems, HIVDR genotyping which estimates patterns of drug resistance mutations (DRMs) were recommended before initiation of ART in all HIV-infected children and adults in high-income countries [10, 19, 20]

HIVDR in HIV-infected children and infants may result either from acquired HIVDR strains from their mother during pre-partum, intrapartum and post-partum or selected HIVDR strains in children by exposed to ART that used for PMTCT [21, 22]. In addition, HIV-infected children and infants have higher risk to develop HIVDR due to high viral load [23], immature immunological response [24] and poor adherence (non-complete compliance) or use of suboptimal regimens (inadequate dosing) [25, 26]. Emerging of HIVDR to ARV drugs interrupts the expected clinical benefits from HAART in HIV infected children because the loss of ART efficacy of drugs and affected to long-term ART in children from infancy to adulthood.

Although the clinical significance of HIVDR has been well documented in adult populations in Thailand and other countries [27-35], studies on the prevalence and patterns of drug resistance mutations (DRMs) in pediatric population with HIV-infection is remaining limited [36-39], particularly in Thailand. WHO has published a generic protocol for surveillance in order to assess HIVDR in children, age 18 months by using dried blood spots (DBS) in resource-limited countries [40]. This method is very convenience because not only DBS is collected from minimal whole blood but also easy to preserve and transfer.

In Thailand, the Division of clinical microbiology, Department of medical technology, Faculty of associated medical sciences, Chiang Mai University is the reference laboratory center for the early infant diagnosis (EID) for HIV infection. The diagnosis is performed by real-time PCR method [41] use the DBS obtained from children who were born to HIV infected mothers. The children were participated in the National AIDS Program (NAP) of the National Health Security Office (NHSO) of Thailand. More than 15,000 samples from 8,468 children who were participated in EID program at AMS, CMU from year 2007 to 2014 and these samples were sent from 364 hospitals across Thailand. 325 children were diagnosed HIV positive. From the providing DBS, the aim of this study is to estimate the prevalence of HIV drug resistant mutants (DRMs) in newly diagnosed HIV-infected children in Thailand by genotypic testing using DBS specimens. The results that obtained from this study should help to determine regimen selection for pediatric HIV care in Thailand and also help in decision whether to continue doing the resistance test before ART initiation.

1.2 Objective

To study the prevalence of HIV drug resistance mutation in newly diagnosed HIV-infected children aged less than 18 months in Thailand from 2007 to 2014.

1.3 Literature review

1.3.1 Human immunodeficiency virus (HIV) infection and Acquired immune deficiency syndrome (AIDS)

1.3.1.1 HIV structure and replication

HIV is a member of the *Lentivirus* genus of the *Retroviridae* family. The retrovirus genome is composed of two identicalcopies of single-stranded RNA molecules and is characterized by the presence of structural genes *gag*, *pol*, *env*. HIV virus has an icosahedral structure and diameter of 100 nm [42] and is shown in Figure 1.1

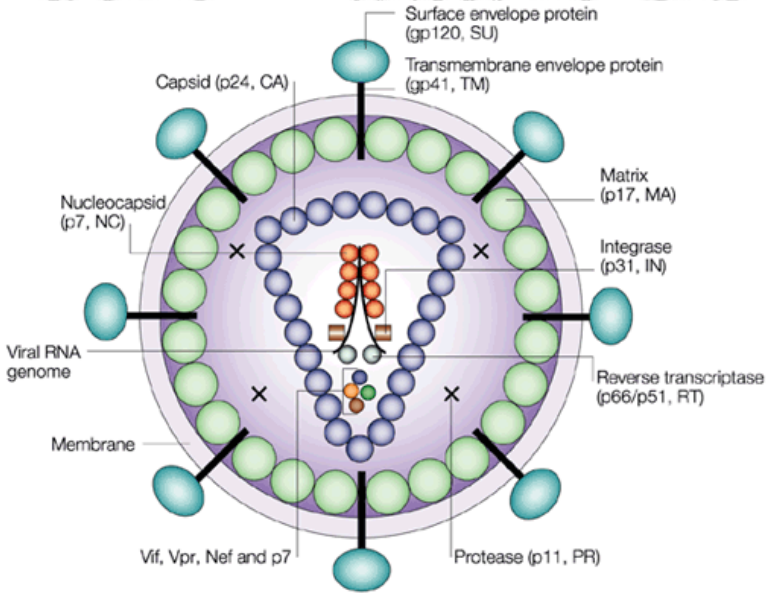

Figure 1.1 Structure of the HIV-1 particle [43].

HIV viral particles are surrounded by a lipoprotein-rich membrane which is acquired from host cell membrane and includes glycoprotein heterodimer complexes of surface glycoprotein (SU, gp120) and transmembrane glycoprotein (TM, gp41).

The major function of these glycoprotein is to mediate recognition of CD4+ cell and chemokine receptor (CCR5 and CXCR4) on host cells. Moreover, the virus may also incorporate into its membrane with various proteins from the host cell membrane, such as HLA class I and II proteins that may facilitate adhesion to other target cells [44]. A matrix protein (p17) is anchored to the inside of the viral membrane. The inner sphere contains the capsid that composed of core antigen (p24) and protease enzyme. The capsid includes two single-stranded copies of HIV RNA combined with nucleocapsid (p7), reverse transcriptase, integrase and the accessary proteins such as, Nef, Vif, Vpr, Rev Tat and Vpu.

The HIV genome is approximately 10 kilobase in length. There are three structural genes *gag, pol, env* like others retrovirus. The HIV-1 and HIV-2 genomes present regulatory genes (*tat*, *rev*), and accessory genes (*vif*, *vpr*, *vpu*, *vpx* and *nef*) with flanking long terminal repeat (LTR) sequences at the left and right edges of genome. The genome organization of HIV-1 is shown in figure 1.2.

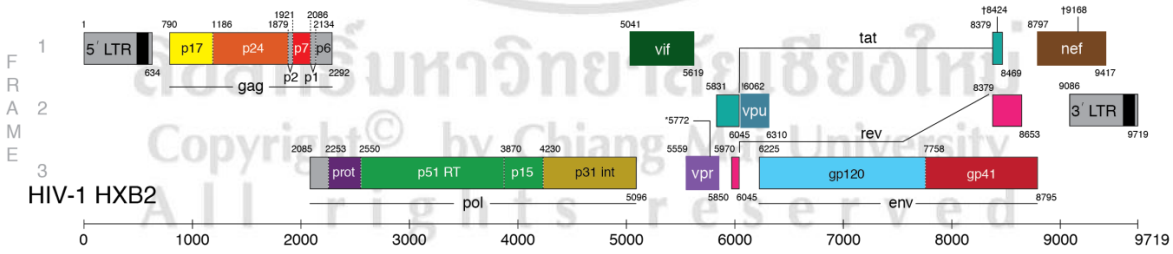

Figure 1.2. Organization and landmarks of the HIV-1 DNA genome [45].

HIV uses the genes to code for the important proteins and enzymes. The *gag* gene encodes gag protein or group-specific antigen. Gag precursor (p55) are proteolytically processed by protease into structural protein including matrix (MA, p17) capsid (CA,

p24) and nucleocapsid (NC, p7). The *pol* gene encodes for the three viral enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR). *Env* gene encodes the envelope glycoproteins precursor (gp160) after processing into surface glycoprotein (gp120) and a transmembrane glycoprotein (gp41) and then gp120-gp41 proteins are formed as a trimer on the membrane surface. The *tat* and *rev* are regulatory genes which modulate transcriptional and posttranscriptional steps of virus gene-expression and propagation. The accessory genes are *vif*, *vpr*, *vpu*, and *nef* and its function is less well understood. The Vpr protein involves in the arrest of the cell cycle and also enables the reverse transcribed DNA to increase access into the nucleus in non-dividing cells such as macrophages. Vpu is a protein necessary for the correct release of virus particle. The *vif*gene codes for a small Vif protein which enhances the infectiveness of newly virus particles. The Nef protein has cellular signal transduction function and could down regulation of the CD4 receptor on the cell surface that allow virus budding in the final of the virus replication cycle [46].

The HIV viral replication cycle model is shown in Figure 1.3. To infecting host cell, HIV attach host cell via CD4+ -gp120 interaction associated with the function of co-receptors, CCR5 or CXCR4, on the cell surface to facilitate viral entry into the host cell by the fusion of viral and cellular membrane. Following fusion, virus uncoats and releases its content including HIV RNA, important enzymes, such as reverse transcriptase, integrase, ribonuclease, protease and various proteins to the cell.

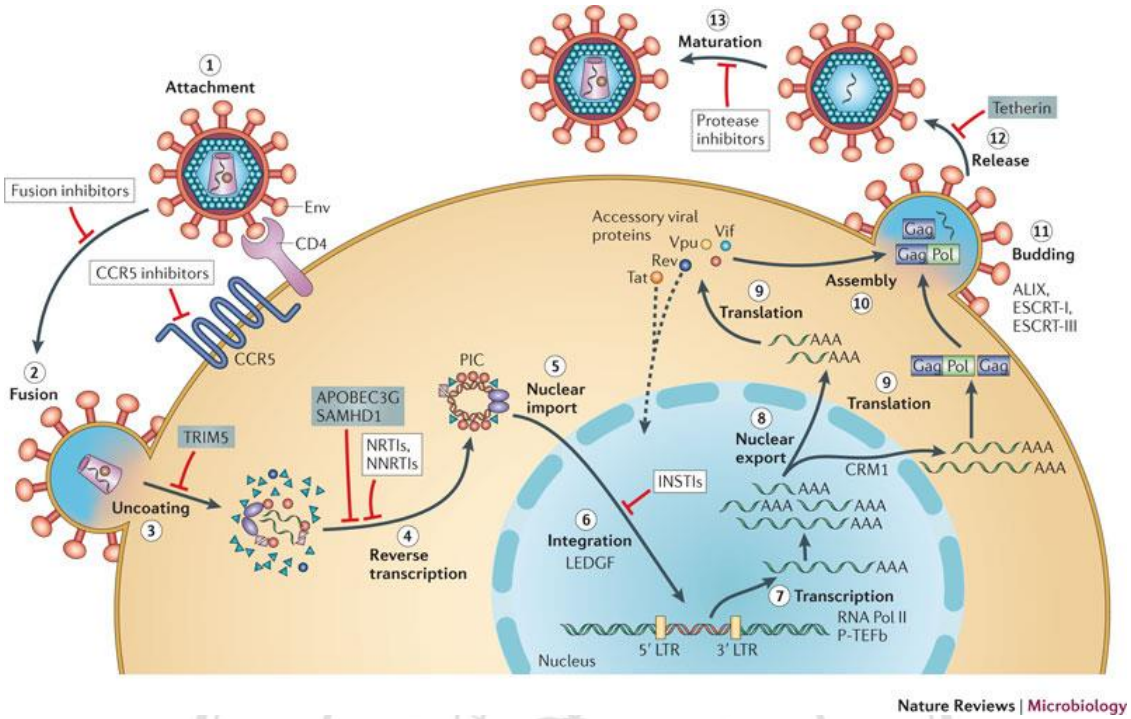

Figure 1.3 Schematic overview of the HIV-1 replication cycle [47].

The reverse transcription process that turn single-stranded RNA genome into complementary DNA and form shape as the pre-integration complex (PIC). PIC was imported into the cell nucleus and intregraded into the host genome by viral integrase enzyme (IN). The integrated retroviral DNA genome or proviral DNA is transcribed using RNA polymerase into viral mRNAs then exported to leave the nucleus. The viral mRNAs serve as a template for viral protein production or as viral particle for assembly with protein components. The viral genomic RNA and proteins budding and release from the host cell membrane as immature virion. Finally, HIV protease develops immature virion into the mature virus that able to infect another cell.

1.3.1.2 HIV classification

HIV are grouped into two types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is responsible for AIDS pandemic, and can be divided into three genetic groups: group M (major or main), group O (outlier) and group N (new or non-M, non-O). In contrast, HIV-2 has remained restricted to West Africa because of their lower transmission rates [48].

Genetic subtypes of HIV-1group M are recognized in nine pure subtypes or non-recombinant forms including A, B, C, D, F, G, H, J, and K. Two subtypes are divided into sub-subtype, subtype A (A1 and A2) and F (F1 and F2). Some intersubtypic mosaic isolates that form through recombination of different strains inside dually- or multiply-infected individuals [49] further achieve epidemic relevance. This form is classified as a circulating recombinant form (CRF). Two of the CRF (CRF01_AE and CRF04_cpx) were classified initially as subtypes (‘‘E’’ and ‘‘I,’’ respectively). Currently, over 40 CRFs have been characterized [50]. Group O infections is limited in Central Africa. Group N infections is identified in only limited numbers of patients from Cameroon [51].

The genotype subtypes have distinct global distribution patterns. Subtype C accounts for most of all HIV-1 infections world-wide [52] . It is typically found in Eastern African countries, in the highly populated India and the southern region of Brazil. Subtype A is prevalent in Central Africa, Iran and Eastern Europe, and in Central Asia. Subtype B is predominates in developed countries, such as the United States, countries in Western Europe, Japan and Australia. The HIV-1 subtypes D, F, G, H, J and K represented together only few amounts in new infections. Some CRFs have great impact in local AIDS epidemics, such as CRF01_AE in Southeast Asia, CRF02_AG in Western Africa, and CRF06_cpx in West Africa, while others CRFs have negligible epidemic relevance. The worldwide distribution of HIV-1 subtypes is shown in Figure 1.4.

CRF01_AE and subtype B' (Thailand variant of subtype B) is widely circulating in Southeast Asia, especially in Thailand. And CRF01_AE became disseminated through-out Southeast Asia, including Cambodia, Vietnam, Malaysia, China, China Taiwan, Korea and Japan [53] . Moreover, CRF15_01B and CRF34_01B have been recently identified in Thailand that is the novel CRFs comprised of CRF01 AE and subtype B (B') [54].

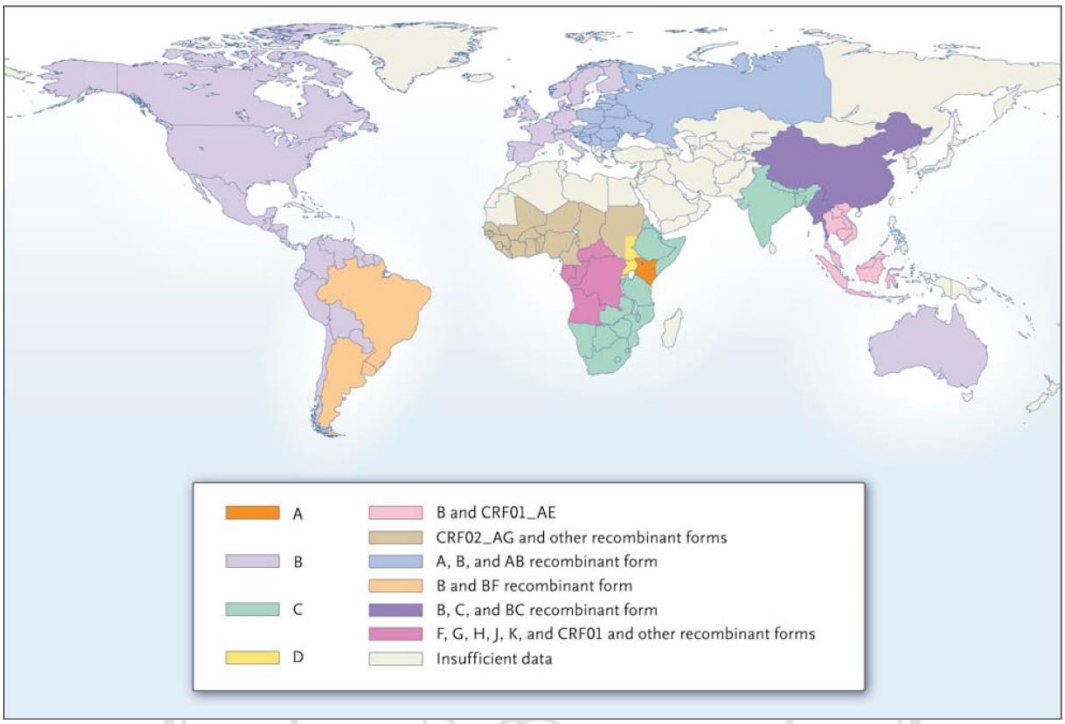

Figure 1.4 Global distributions of HIV-1 subtypes and recombinants [55].

1.3.1.3 Transmission of HIV infection

HIV can be transmitted through the exchanging of a variety of body fluids from infected individuals, such as blood, breast milk, semen and vaginal secretions. The route of transmission can typically occur in three routes, including sexual transmission, blood or blood products and mother-to-child transmission. Sexual transmission can occur when that person exposed sexual secretions of the partner to his/her genital organ, having oral or rectal sex. HIV transmission through blood or blood products is accounted for infections in the intravenous drug users (IVDU), hemophiliacs, and recipient of organs, blood transfusions and receiving blood products. Moreover, Health care workers such as nurses, laboratory technicians and physicians have also been infected by occupational exposure [56] but post exposure prophylaxis (PEP) should be provided.

1.3.1.4 HIV-1 Mother-to-child transmission (MTCT)

HIV-1 MTCT occurs mainly at three stages: prepartum (transplacental passage), intrapartum (exposure of infant skin and mucus membrane to maternal blood and vaginal secretions) and postpartum (breast milk/breast feeding) [57]. In the absence of any intervention, the HIV-positive pregnancy women are having 30 - 45% chance of viral transmission to their infants [58].

During prepartum period, various studies have been reported that HIV-1 has capability to pass through an intact placental barrier maintained *ex vivo* and cause infection in placentas or fetuses [59].

The intrapartum transmission has an evidences more than 50% of the cases that the infants were exposed maternal blood during labor period and the virus passed through birth canal [60]. Moreover, transmission risk of HIV-1 from mother to child increases nearly double if the fetal membranes rupture takes longer than four hours before delivery [61].

During postpartum period, HIV-1 MTCT mainly occurs through breastfeeding, with approximately range 14% to 29% and it is a major problem of MTCT in developing countries [62].

Several maternal factors, including advanced clinical stages of the mother, low antenatal CD4+ lymphocyte counts (<29 % of total lymphocytes), maternal immune response to HIV-1, recent infection, high level of circulating HIV-1, and maternal disease progression have been implicated in an increased risk of MTCT of HIV-1 [63, 64]. Besides, the other factors such as acute infection during pregnancy, the presence of other sexually transmitted diseases or other chronic infections, disruption of placental integrity secondary to chorioamnionitis and smoking have been shown to be associated with MTCT of HIV-1 [65].

1.3.1.5 Paediatric HIV-1 infection

The main cause of HIV-1 infection in children is MTCT. However, the effective of PMTCT process has significantly reduced the number new HIV infection in the infants. In July 2015, UNAIDS and the WHO estimated the number of

children living with HIV was approximately 2.6 million. The new cases of HIV-1 infected children were approximately 220,000 incidents. In Thailand, at the end of year 2014 [2], there were estimated 445,504 persons living with HIV and out of this number 6,875 persons are children. In Thailand, elimination the newly HIV infection cases in children has been dramatically reduced, estimated 121 cases of the newly infected children. This number represents about 41% of HIV reduction comparing to the infection level in year 2010. Therefore, the MTCT rate was estimated 2.1% in year 2014.

Nevertheless, the small proportion of children becomes infected despite PMTCT. HIV Infected children had important features of HIV disease that vary in a different timing starting from the onset of HIV disease and an extraordinarily wide spectrum of clinical manifestations. Young infants may present with signs and symptoms of HIV infection that strongly suggest intrauterine infection, whereas other children may not show visible signs of immunosuppression for many years [66].

Several reports [67-69] have suggested that the progression of AIDS follows two general patterns in children. Firstly, severe immunodeficiency develops in 15 to 20% of infected infants, with serious infections or encephalopathy rapidly intervening within the first year of life. The remaining 80 to 85% have a form of disease that progresses more slowly and is more similar to the disease progression that have seen in adults.

The reasons for rapid disease progression in a subset of infants are not well understood. Some study has suggested that HIV-1 has more target cells available in neonates and infants than adults because neonates and infants have more circulating T-lymphocytes than adults. Furthermore, a higher level of HIV-1 replication in neonatal compared with adult blood T-lymphocytes [70] belong with an infant's immune system is immature and simply is unable to contain the virus.

Early symptoms of HIV disease tend to be predictive of shorter survival [71] and the early symptoms typically cause by opportunistic infections (OIs). *Pneumocystis carinii* pneumonia (PCP), progressive neurologic disease and early onset of growth failure are all associated with rapid disease progression [72]. Furthermore, some clinical manifestations, HIV-1 infection causes a broad spectrum of disease in children include

hepatosplenomegaly, growth failure, oral candidiasis, recurrent diarrhea, parotitis, cardiomyopathy, hepatitis, nephropathy, developmental delay and encephalopathy, lymphoid interstitial pneumonitis, recurrent bacterial infections and specific malignancies. In the endemic area of tuberculosis, primary and reactivated tuberculosis is a major cause of morbidity and mortality among HIV-1-infected children, such as in Africa. Other opportunistic infections during paediatric HIV-1 infection include chronic or disseminated infections with herpesviruses (CMV, HSV and VZV) and atypical mycobacteria. *Cryptococcus neoformans* and *Toxoplasma gondii*. Leiomyosarcomas and lymphomas, including non-Hodgkin' s B-cell Burkitts-type lymphomas, these opportunistic infections caused acute and chronic CNS infections [73].

Virologic and immunologic factors that probably affect the prognosis of an HIV-infected child include the timing of infection, the viral load, and the host immune response. Positive viral diagnostics in the first 48 h of life and early development of CD41 cell lymphocytopenia predict a more rapid rate of disease progression and clinical deterioration. The median survival of pediatric patients with HIV-infection was ranged from 75 to 90 months, with 70% of children surviving to 6 years of age [71, 74].

Early diagnosis of HIV infection in the infants is crucial for interventions such as antiretroviral therapy and prophylaxis against PCP that can be initiated as soon as possible. Early diagnosis of HIV infection is very important because the progress of the disease can be seen and the health care officer will be able to provide regular supportive care in order to improve survival opportunity.

1.3.2 Diagnosis of HIV infection in infants and children

Infant who are infected with HIV during delivery time are having disease progression that occurs suddenly in first month of age and often leading to death. Hence, the infant with HIV-exposure should be identified their infection status then receive antiretroviral (ARV) for prophylaxis and treatment as soon as possible after their infection status have been confirmed.

HIV serological testing is generally used to diagnose HIV infection in adults and in children above 18 months. The early diagnosis in infants (<18 months) who were born to HIV infected mothers will be tested with the assays that detect the virus or its

components (i.e. virological tests: HIV RNA, HIV DNA nucleic acid or p24 Antigen tests), these tests are recommended because of the presence of maternal antibodies [75]. The maternal antibodies can be placental transfer to the fetus and may persist in infant's body until 18 months of age [76]. However, HIV serological testing in infancy can indicate maternal HIV infection and exposure of the infant but cannot be used to confirm HIV infection in the infant.

In 2010, WHO has revised ART and HIV diagnostic recommendations for infants and children [77]. WHO recommends that the children with HIV exposure should receive diagnostic HIV PCR (nucleic acid) test at 4 to 6 weeks or at the earliest opportunity thereafter and if negative at that time, this test should be repeated at approximately six weeks after the complete cessation of breastfeeding (when the child is < 18 months old) or earlier if there is any symptoms suggestive of HIV infection had occurred.

HIV DNA PCR is a sensitive technique used to detect specific integrated HIV viral DNA in peripheral blood mononuclear cells. The specificity of the HIV DNA PCR is 99.8% at birth and 100% at ages 1, 3 and 6 months [78]. The sample collection typically collects in dried blood spot specimens that easily collected at local sites, conveniently transported and good accuracy as same as in whole blood [79].

Following Thailand National Guidelines on HIV/AIDS Treatment and Prevention 2014 [14], the recommendations suggested that HIV-exposed infant should receive an early infant diagnosis (EID) by identification of HIV proviral DNA in white blood cells in dried blood spots using real-time DNA PCR method. The children born to HIV-1 infected mothers are categorized into 2 groups, standard risk and high risk group. The categorization of risk criteria and recommended diagnosis was shown in Table1.1.

Table 1.1 The categorization of risk criteria and recommended diagnosis for early infant diagnosis [14].

Risk to acquire HIV infection from mother	Recommended diagnosis and care
Standard risk ➢ Pregnant woman has antenatal care (ANC) attendance, or ➢ Provision of HAART more than 4 weeks, or ➢ Peripheral viral load ≤ 50 copies/mL during labor.	➢ Receive diagnosis 2 times: at 1 and 2-4 month old, and, if positive at that time, repeated immediately with new collected sample. ➢ Infants receive AZT for 4 weeks as PMTCT.
High risk ➢ Pregnant woman has antenatal care (ANC) attendance, or ➢ Provision of HAART less than 4 weeks, or ➢ Poor adherence of HAART, or ➢ Peripheral viral load > 50 copies/mL during labor.	➢ Receive diagnosis 3 times: at 1, 2 and 4 months old. ➢ Infants receive AZT+3TC+NVP until know first time result, and if positive repeated immediately with new collected sample and change NVP into LPV/r, or ➢ If negative result at first time, provision of AZT+3TC+NVP should stop.

1.3.3 Prevention of mother-to-child transmission (PMTCT)

Before antiretroviral drugs (ARVs) were used as prophylaxis for mother-to-child transmission prevention (PMTCT), mother-to-child transmission rates range from 14 to 32% and 25 to 48% in untreated non-breastfeeding population in high-income countries and breastfeeding population in resource-poor settings (or resource limited settings) respectively [80] . In well-resourced health care systems, HIV testing for pregnant woman, administration of ARVs for prophylaxis or for mother' s health, elective caesarian delivery and avoidance of breastfeeding has reduced MTCT of HIV infection to 1-2% [81-83]. As access to PMTCT service has increased, the number of newly HIV-infected has decreased.

In Thailand, around ninety-six percent of HIV positive pregnant women, both Thai and non-Thai received ARV drugs to reduce the risk of MTCT and approximately 3 percent of the infants born to HIV infected mothers are infected. In addition, the rate of MTCT declined from 2.3% in 2013 to 2.1% in 2014 due to the increment of PMTCT coverage in Thailand [2].

Following WHO recommendations of antiretroviral drugs for pregnant women treatment and prevent HIV infection in the infants in 2010 [84] . The PMTCT recommendations include lifelong ART for HIV-infected women who are required treatment for their own health that is safe and effective in reducing MTCT and ARV prophylaxis to prevent MTCT during pregnancy, delivery and breastfeeding for HIV-infected women. Moreover, maternal ART should be coupled with the ARV drugs for the infants at birth or as soon as feasible, irrespective of the mode of infant feeding [85].

In current Thailand's recommendation (2014) [14], all of HIV-infected pregnant woman should initiate ART regardless of the age of gestation or CD4+ cell count with TDF+3TC+EFV regimen during antepartum period. During intrapartum period, infected pregnant woman should continue with antepartum regimen plus with AZT (with NVP in some case). After labor and delivery, all cases are counseled to avoid breast-feeding and continue ART post- partum in accordance with the treatment for adult cases of HIV infection. The infant should start with AZT (with 3TC and NVP in some case) at birth within 1 hour or as soon as possible and then tested to confirm the infection status. The

ARV drugs detail of Thailand recommendations for treatment in HIV-infected pregnant woman and PMTCT was demonstrated in Table 1.2.

In Thailand, there were 3 main national guidelines of PMTCT including 2006/2007 [86], 2010 [13], and 2014 [14]. The PMTCT recommendations were shown in Table 1.2. The using of ARVs in all guidelines is divided into 3 period including antenatal, labor and delivery, and postpartum period. However, the criteria of when initiate ART and the type of ARV were revised in newer guidelines for enhanced the efficiency and avoided the side effect of drug to development of fetus. As PMTCT program in Thailand achieved high level of prevention efficiency and coverage care for mothers and children during pregnancy and post-partum period, MTCT rate in Thailand is continually declined from 5.5 in 2008 to 2.1% in 2014.

Table 1.2 The prevention guideline of mother-to-child transmission in Thailand.

Thailand Guidelines		**2006/2007**[86]		**2010**[13]		**2014**[14]	
Mother	**Antenatal**	- AZT (250-300 mg/12hr) - AZT + 3TC + NVP - Continue current ART (avoid EFV or d4T+ddI)	Until delivery	- AZT + 3TC + LPV/r - Continue current ART (avoid EFV in first trimester)	Until delivery	- TDF + 3TC + EFV - TDF (AZT) + 3TC + LPV/r - Continue current ART (avoid EFV GA < 6 weeks)	>4 weeks until delivery
	Labor and delivery	- Continue ART - Sd-NVP[d] and AZT/3hr[a]	Before labor	- Continue ART - AZT/3hr[a], or - sd-AZT[b], or - AZT[c] and sd-NVP[d]	Before labor	- Continue ART - AZT/3hr[a] - sd-AZT[b], or - AZT[c] and sd-NVP[d]	Before labor
	Postpartum	- Continue ARTwith adult regimen - AZT(200mg) and 3TC (150mg) / 12hr	7 days	- Continue ART with adult regimen		- Continue ART with adult regimen	
Infant		- Sd-NVP (2mg/kg) and - AZT (2mg/kg/6hr or 4mg/kg/12hr)	After birth 1 or 6 weeks	- Breastfeeding avoidance - AZT/12hr[e] - AZT + 3TC + NVP[e] (No ANC)	4 weeks 6 weeks	- Breastfeeding avoidance - AZT/12hr[e] - AZT + 3TC + NVP[e] (No ANC)	4 weeks 6 weeks

[a] AZT(300mg)/3hr, [b] sd-AZT(600mg), [c] AZT(300mg/3hr or sd-600 mg), [d] sd-NVP(200mg)
[e] AZT(syrup) 4mg/kg/12hr, 3TC(syrup) 2mg/kg/12hr, and NVP(syrup) 4mg/kg/24hr
ANC, antenatal care

1.3.4 HIV treatment

Currently, there is no safe and effective treatment to cure HIV infection but HIV/AIDS can be controlled by antiretroviral therapy (ART) and proper medical care. Treatment for HIV is called antiretroviral therapy or ART. ART can dramatically prolong the lives of many HIV infected people, keep them healthy, having good quality of life and lower the chance of viral transmission to others [15, 16].

1.3.4.1 Antiretroviral (ARV) drugs

Antiretroviral drugs are the synthesis drug used for retroviral infection treatment, especially HIV infection. The most targets of drugs are inhibit the replication and entry to the target cells of HIV cycle. Recently, HIV treatment is typically used the combination regimen that combined three kinds of ARV drugs or more. The approached regimen is known as Highly Active Antiretroviral Therapy (HAART). HAART can reduce HIV-1 levels in plasma to the below detection limit of clinical assays (50 copies of HIV-1 RNA/ml) [87] and rise up the CD4+ count in HIV-positive patients [88].

1.3.4.1.1 Classification of ARV drugs

There were 6 different classes of ARV drugs that affect the various stages of the HIV cycle. ARV drugs that were approved by the FDA (U.S. Department of Health and Human Services, 2015) are shown in Table 1.3.

Table 1.3 The antiretroviral drugs currently approved by FDA [89].

Generic name	Proprietary name	Acronym	FDA approval
NRTIs			
Abacavir	Ziageon	ABC	1998
Didanosine	Videx	ddI	1991
Emtricitabine	Emtriva	FTC	2003
Lamividine	Epivir	3TC	1995
Stavudine	Zerit	d4T	1994
Tenofovir	Viread	TDF	2001
Zalcitabine	Hivid	ddC	1992
Zidovudine	Retrovir	AZT, ZDV	1987
NNRTIs			
Delavirdine	Rescriptor	DLV	1997
Efavirenz	Sustiva	EFV	1998
Etravirrine	Intelence	ETR	2008
Nevirapine	Viramune	NVP	1996
Rilpivirine	Edurant	RPV	2011
PIs			
Amprenavir	Agenerase	APV	1999
Atazanavir	Reyataz	ATV	2003
Darunavir	Prezista	DRV	2006
Fosamprenavir	Lexiva	FPV	2003
Indinavir	Crixivan	IDV	1996
Lopinavir/ritronavir	Kaletra	LPV/r	2000
Nefinavir	Viracept	NFV	1997
Ritronavir	Norvir	RTV	1996
Saquinavir	Invirase	SQV	1995
Tipranavir	Aptivus	TPV	2005
Fusion inhibitors			
Enfuvirtide	Fuzeon	T-20	2003
Entry inhibitors			
Maravioc	Selzentry	MVC	2007
Integrase inhibitors			
Raltegravir	Isentress	RAL	2007
Dolutegravir	Tivicay	DTG	2013

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) are prodrugs that required host cell entry and phosphorylation by cellular kinases before enacting an antiviral effect. The function is inhibits the synthesis of proviral DNA in newly infected cells. The lack of 3' hydroxyl group of the NRTIs prevents the formation of phosphodiester bond between the NRTIs and incoming nucleoside triphosphates, resulting in termination of the growing viral DNA chain. Chain termination can occur during RNA-dependent DNA or DNA-dependent DNA synthesis, inhibiting production of the HIV-1 proviral. Currently, there are eight FDA-approved NRTIs: abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC), zidovudine (AZT or ZDV), and tenofovir (TDF).

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) inhibit HIV-1 reverse transcriptase by binding and inducing the formation of a hydrophobic pocket proximal but not overlapping the active site. The binding of NNRTIs changes the spatial conformation of the substrate-binding site and reduces polymerase activity. There are five approved NNRTIs such as etravirine (ETR), delavirdine (DLV), efavirenz (EFV), nevirapine (NVP) and rilpivirine (RPV).

Protease inhibitors (PIs) bind to the active site of the vial protease enzyme, prevent the processing of the cleavage of the viral gag and gag-pol polyprotein precursors during virion maturation that responsible by protease. To date, ten PIs were approved including amprenavir (APV), atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV).

Fusion inhibitors prevent HIV from entering into the target cells by binding to the HIV envelope protein gp41, which is involved in viral entry. The fusion inhibitors binding interfere the conformational change or folding of the envelope molecule requiredfor fusion with the target cell membrane. The fusion inhibitor is approved by FDA such as enfuvirtide (T-20).

Entry inhibitors or chemokine coreceptor antagonists also prevent the entry of HIV into target cells by binding to coreceptors (either CCR5 or CXCR4) on the surface of CD4 cells that is a main target cell of HIV. Maravioc (MVC) was approved by FDA to be used as entry inhibitors in adults.

Integrase inhibitors prevent HIV from strand development and transfer reaction to host cell chromosome by interfere with the incorporation of reverse-transcribed proviral DNA. Raltegravir (RAL) and Dolutegravir (DTG) were approved to be used in HIV infection treatment [90].

1.3.4.2 Antiretroviral treatment in children.

The using of ART in children can improve their clinical and immunologic outcomes [15]. In order to maintain the healthy immune systems of the infected children. Therefore, they should receive ARV drugs no later than 5 years of age. Following WHO recommendations on the use of antiretroviral drugs for treatment in infant and in children [91], all children age less than 24 months should initiate ART as soon as they are diagnosed HIV-infection regarding to clinical or immunological status. Children who are previously exposed to any NNRTI from their mothers during pregnancy, during delivery or breast-feeding period or directly received by themselves, they should initiate HAART that composed of PI drug. The AZT+3TC+NVP regimen is recommended for children without NNRTIs experience and AZT+3TC+LPV/r for children experienced with NNRTIs.

In Thailand, HIV-1 treatment in children is based on Thailand national guidelines in year 2014[14]. The criteria for ART initiation in children whose age is younger than 1year-old, they should start as soon as possible, regardless signs of infection and CD4 levels. The AZT or ABC+3TC+LPV/r regimen and the alternative regimens; AZT+3TC+NVP, d4T+3TC+LPV/r or d4T+3TC+ NVP regimen are recommended (Table 1.4). In children ages 1-3 years old, the treatment is started when the children are identified either WHO stage 3, 4 or in CDC category B, C or A or the viral load is >100,000 copies/mL or the CD4 level is < 1,000 cell/mm^3 or < 25%. The preferred regimen is similar to the regimens used in 1-year-old children. In case children ages 3-5 years old, the ART should start when children are at WHO stage 3, 4 or in CDC category B, C or if CD4 levels are at < 750 cells/mm^3 or < 25%. The preferred ARV regimen is AZT or 3TC+3TC+EFV and the alternative regimens are AZT or ABC+3TC+NVP, TDF+3TC+EFV or NVP and d4T+3TC+EFV or NVP. ARV initiation in children age 5-15 years-old should be started when they reached WHO stage 3, 4 or in CDC category B, C or have the CD4 level < 350 or 350-500 cells/mm^3

and the preferred regimen is AZT or ABC+3TC +EFVand AZT or ABC+3TC+ NVP, TDF +3TC + EFV or NVP and the alternative regimens are d4T + 3TC + EFV or NVP. In case of children whose age are older than 12 years old, the preferred regimen is TDF + 3TC + EFV and the alternative regimens are AZT + 3TC + EFV, TDF + 3TC + NVP or RPV and AZT or ABC + 3TC + NVP.

Table 1.4 The recommendations of ART treatment regimen in children in Thailand [14].

	< 1 year	**1 to < 3 years**	**3 to 12 years**	**> 12 years**
Preferred regimens	AZT (ABC) + 3TC + LPV/r	AZT (ABC) + 3TC + LPV/r	AZT (ABC) + 3TC + EFV	TDF + 3TC + EFV
Alternative regimens	AZT (d4T) + 3TC + NVP	AZT (ABC) + 3TC + NVP, or d4T + 3TC + LPV/r (NVP)	AZT + 3TC + NVP, or TDF + 3TC + EFV(NVP), or d4T+ 3TC + EFV (NVP)	AZT + 3TC + EFV(NVP), or ABC + 3TC + EFV(NVP)

1.3.5 Antiretroviral drug resistance

The use of HAART has shown the effective in controlling the progression of HIV infection and prolonging survival [92], but these benefits can be lost by the occurrence of drug resistance. The resistance is the results of mutations that emerge in the viral proteins which are targeted by ARV drugs and then the resistance complicates further efforts to control viral replication.

Development of resistance can be divided into two concepts including induced (or acquired) and primary (or transmitted) resistance. The induced (or acquired) resistance is the consequence of developing of resistance in treatment-experienced patients. Actually, the high level of HIV replication and turnover and the lack of a proof reading of HIV reverse transcriptase lead to the spontaneous generation of a large number of genetically distinct viral quasi species coexisting even drug resistant strain in

the same person. In the presence of ARV drugs, drug resistant strain with reduced susceptibility to these drugs become the predominant quasi species in patients lead to virological failure of ART. Moreover, cooperating with poor adherence by the patient and/or to low potency of the regimen lead to the viral replication is incompletely suppressed and consequence with selecting of resistant variants. The primary (or transmitted) resistance is the patient primary infected with the exhibited drug resistance virus [93].

1.3.5.1 Mechanism of resistance

Drug resistance is the consequence of mutations that emerge in the viral proteins targeted by antiretroviral agents. The importance mutations and conferring to affected ARV drugs that used for HIV treatment were shown in Table 1.5.

The substitution of consensus amino acid with another amino acid was name as the codes of consensus amino acid followed by position and another amino acid. For example, The M184V is the substitution of valine (V) against methionine (M) at position 184 of reverse transcriptase. The codes of amino acid were demonstrated in Appendix A. All HIV drug resistance mutations that commented by Stanford HIVDR database were shown in Appendix B.

Table 1.5 Major mutations involved in resistance of HIV to ARV drugs, adapted from [94].

Major NRTI Resistance Mutations

		Discriminatory Mutations					Thymidine Analog Mutations (TAMs)						MDR Mutations		
Positions		**184**	**65**	**70**	**74**	**115**	**41**	**67**	**70**	**210**	**215**	**219**	**69**	**151**	
Cons AA		**M**	**K**	**K**	**L**	**Y**	**M**	**D**	**K**	**L**	**T**	**K**	**T**	**Q**	**Substituted amino acid**
Drugs	**3TC**	**VI**	**R**										**Ins**	**M**	
	FTC	**VI**	**R**										**Ins**	**M**	
	ABC	VI	**R**	**E**	**VI**	**F**	L			W	FY		**Ins**	**M**	
	DDI	VI	**R**	**E**	**VI**		L			W	FY		**Ins**	**M**	
	TDF	***	**R**	**E**		F	L		R	W	FY		**Ins**	**M**	
	D4T	***	**R**	**E**			**L**	**N**	**R**	**W**	**FY**	QE	**Ins**	**M**	
	AZT	***	***	*	*		**L**	**N**	**R**	**W**	**FY**	QE	**Ins**	**M**	

Cons AA, consensus amino acid
Mutations in **bold** are associated with the highest levels of reduced susceptibility or virological response to the relevant NRTI.
Mutations in **bold** reduce NRTI susceptibility or virological response.
Mutations in plain text contribute to reduced susceptibility in combination with other NRTI-resistance mutations.

Major NNRTI Resistance Mutations

		100	**101**	**103**	**106**	**138**	**181**	**188**	**190**	**230**	
Cons AA		**L**	**K**	**K**	**V**	**E**	**Y**	**Y**	**G**	**M**	
Drugs	**NVP**	**I**	**EP**	**NS**	**AM**		**CIV**	**LCH**	**ASE**	**L**	**Substituted amino acid**
	EFV	**I**	E**P**	**NS**	A**M**		CIV	**LC**H	**ASE**	**L**	
	ETR	**I**	E**P**			AGKQ	**CIV**	L	AS**E**	**L**	
	RPV	**I**	**EP**			**AGKQ**	**CIV**	**L**	ASE	**L**	

Cons AA, consensus amino acid
Mutations in **bold** are associated with the highest levels of reduced susceptibility or virological response to the relevant NNRTI.
Mutations in **bold** reduce NNRTI susceptibility or virological response.
Mutations in plain text contribute to reduced susceptibility in combination with other NNRTI-resistance mutations.

Table 1.5 (Continued)

Major PI Resistance Mutations															
Position		**30**	**32**	**33**	**46**	**47**	**48**	**50**	**54**	**76**	**82**	**84**	**88**	**90**	
Cons AA		**D**	**V**	**L**	**M**	**I**	**G**	**I**	**I**	**L**	**V**	**I**	**N**	**L**	
Drugs	**ATV/r**		I	F	IL	V	**VM**	**L**	VTALM		ATFS	**V**	**S**	**M**	**Substituted amino acid**
	DRV/r		**I**	F		**VA**		**V**	**LM**	**V**	F	V			
	FPV/r		**I**	**F**	**IL**	**VA**		**V**	VTA**LM**	**V**	ATS**F**	**V**		**M**	
	IDV/r		**I**		**IL**	V			**VTA**LM	**V**	**AFTS**	**V**	S	**M**	
	LPV/r		**I**	F	IL	V**A**	VM	**V**	**VTALM**	**V**	**AFTS**	**V**		M	
	NFV	**N**		F	**IL**	V	**VM**		**VTALM**		**AFTS**	**V**	**DS**	**M**	
	SQV/r						**VM**		**VTA**LM		AT	**V**	S	**M**	
	TPV/r		I	F	IL	**VA**			**VAM**		**TL**	**V**			

Cons AA, consensus amino acid
Mutations in **bold** are associated with the highest levels of phenotypic resistance and/or with the strongest clinical evidence for interfering with successful PI therapy.
Mutations that are **bold** reduce PI susceptibility or virological response.
Mutations in plain are potential contraindications to the use of the relevant PI.

1.3.5.1.1 NRTIs resistance mechanism

Drug resistance to NRTIs can occur via two mechanisms. The first is impairment of the incorporation of the analogue into DNA by mutations at the end or near the drug binding site of the reverse transcriptase gene. Several mutations in reverse transcriptase can promote resistance including the M184V mutation, the Q151M complex of mutations, and the K65R mutation.

The second mechanism is removal of the nucleoside analogue from the terminated DNA chain that involved with a group of mutations also known as thymidine analogue mutations (TAMs). TAMs promote resistance by fostering ATP- or pyrophosphate-mediated removal of nucleoside analogues from the 3' end of the terminated DNA strand, resulting in removal of the analogue. The TAMs occur in two distinct but overlapping patterns: Type 1, which includes M41L, L210W, and T215Y; and Type 2, which includes D67N, K70R, T215F, and K219Q/E. Type 1 TAMs have a greater negative impact on virological response to an ABC-, ddI-, or TDF-containing regimen than do Type 2 TAMs [18, 94]. The major resistance to NRTIs was shown in Table 1.5.

1.3.5.1.2 NNRTIs resistance mechanism

Drug resistance mechanism of the NNRTI-resistant HIV strains is due to the mutations all located in the pocket targeted by NNRTI compounds, and they reduce the affinity of the drug. Resistance to Nevirapine is often associated with the Y181C mutation, but other mutations, such as Y188C, K103N, G190A, and V106A, also occur. Initial resistance to Efavirenz is generally characterized by the K103N mutation, but the Y188L mutation is also seen [18]. The major NNRTIs DRMs were shown in Table 1.5.

1.3.5.1.3 PIs resistance mechanism

Resistance to protease inhibitors is the consequence of amino acid substitutions that directly or indirectly changes modify the number and the nature of the points of contact between the inhibitors and the protease, thereby reducing their affinity for the enzyme. The emergence of resistance to protease inhibitor likely requires the accumulation of the mutations [90]. The major PI resistance mutations are shown in Table 1.5.

1.3.5.2 Effect of antiretroviral drug resistance to children

Emergence of HVDR variants could be traced to the use of suboptimal antiretroviral drug exposure leading to viral replication under a selective pressure [95]. Sub-optimal drug exposure can be as a result of non-adherence with ARV, changes in drug metabolism and tissue and cellular sanctuaries for HIV [96]. Adherence in children and adolescence is a big challenge due to the issues associated with palatability of some of the pediatric formulations, reliance on caregivers who may be an older sibling almost in the same age group as the patients or other members of the community and the numerous challenges associated with growing adolescents. Infection with drug resistant HIV in infants has important implications for ARV therapy because resistance of HIV to ARVs hampers the expected clinical benefits of ART in HIV-infected children due to the loss of antiviral treatment efficacy of the drugs. Similar to adults with transmitted drug resistance, the resistant forms persisted over time. Several reports demonstrated that significant HIVDR occurs in mothers and in HIV-infected infants treated with

sdNVP and is associated with an increased risk of virologic failure when women or infants are treated with NNRTI-based ART [97-101]. This is a major public health concern because of the effects of resistance on long-term treatment options, taking into cognition the lengthy duration of ART that is expected in HIV-infected children from infancy to adulthood. Hence, the HIV drug resistance testing should be considered in this population.

1.3.5.3 HIV drug resistance testing

1.3.5.3.1 HIV genotypic testing

Genotypic testing was performed in order to identifying a pattern of mutations in nucleotide sequence of viral genome of an individual HIV patient. The genetic patterns that conferred resistance to specific ARV drugs are considered and predicted susceptibility to ARV drugs. The HIV-1 genotypic test is indirect measurement of drug resistance. Although genotypic testing can detect mutations in the relevant HIV-1 genome, the significance of these mutations requires careful interpretation to predict drug susceptibility. There are many genotypic drug resistance algorithms to interpret HIV mutation categories and ARV resistance estimations such as Stanford HIV database (http://hivdb.stanford.edu).

The HIVdb system (Stanford University; Stanford, CA) is a rules-based system that consists of a list of drug penalty scores and comments for each mutation. The total score for a drug is derived by adding the scores for all mutations associated with resistance to that drug to infer 1 of 5 levels of resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance [102].

1.3.5.3.2 HIV phenotypic testing

Phenotypic assays is antiviral drug susceptibility testing. This testing was performed using isolate HIV-1 directly from plasma or peripheral blood mononuclear cells (PBMCs) of infected individuals and test the drug susceptibility of the isolate in vitro. The results demonstrated as the concentration of drug could inhibit virus growth in cell culture by 50% and 90% or the 50% and 90% inhibitory concentration (IC50 and IC90). The wild-type HIV-1 isolates from naïve patients could

determine often in IC50. The isolate virus with less than or equal to IC50 were interpreted as susceptible or sensitive, while the virus with higher drug concentrations are considered resistant.

The susceptibility are typically expressed as a fold change in IC50 of each drug against the virus from patient when compared to the IC50 of the reference wild-type HIV-1 strain [103].

1.3.5.3.3 HIV virtual testing

Virtual testing determined drug resistance by predicting phenotype result from genotype sequence. The genotype sequence were interpreted by using genotypic interpretation database system that accepts a virus sequence and reports the expected level of phenotypic resistance to each of the approved antiretroviral drugs. The procedure involves using database of viruses for both genotype and phenotype testing have been performed to identify viruses with patterns of mutations matching those in the submitted sequence. Only those mutations that are believed to be pertinent to drug resistance are required to match. The phenotypes of matching sequences are then analyzed to determine the median and range in the levels of fold-resistance [102].

1.3.6 The current situation of HIV drug resistance in children.

As mention above, expansion of ART for treatment and prophylaxis has radically changed the face of the HIV/AIDS pandemic. The challenge of successful of ART is emerging of HIVDR. To date, HIVDR prevalence has been well documented in adult worldwide, but in infants and children still remains limited in most countries.

In well-resource country, assessment of the prevalence of genotypic HIVDR in 91 HIV-infected newborns in 1998 and 1999 in New York State, USA was carried out by Parker *et al* [37]. The prevalence of DRMs conferring resistance to NRTI, NNRTI and PI were detected in 7.7%, 3.3% and 3.3%, respectively. The predominant DRMs were M184V and K70R. Moreover, the prevalence of HIV DRMs in 42 HIV-infected infants from 2001-2002 in New York was determined by Karchava *et al* [104]. The DRMs were detected in 7.1% , 11.9% , and 2.4% of NRTI, NNRTI and PI mutations respectively. The frequent DRMs were M184V and K103N.The finding in the United

Kingdom from 1998-2004 of HIV DR in 200 infected children which age varied from <1 to 20 years demonstrated resistance to NRTI, NNRTI and PT in 13.4%, 13.7% and 2.0% , respectively in treated children. The M184I/V and Y181C/I mutations were mostly detectable. Furthermore, the resistance was observed in 6.8% of naïve children and three-class resistance was noted in 42 infants. Interestingly, all researchers strongly suggested that resistance testing should be considered for perinatally infected newborns to avoid use of ARV drugs to which the infant has preexisting resistance. The value of much data from many studies improved and developed world subsequent to low incidence of new infection and often includes old data from children on now outdated regimen.

On the account of the limitation data in children, a systemic review explored the information in HIV-1 drug resistance including transmitted HIVDR (TDR) and DRM prevalence of pediatric HIV-1-infected patients in three main classes of ARVs (NRTI, NNRTI, and PI) in various settings from screening PubMed database until May 2013 [105]. There were 41 studies with showed the HIV DR data from 2,538 patients (558 naive and 1,980 pretreated) from 30 countries in Africa, Asia, America and Europe. Most studies used in-house resistance assays to determine the *pol* coding. The Others studies performed resistance test using commercial assays, such as ViroSeq® HIV-1 Genotypic System (Abbott Molecular) and Trugene® HIV-1 Genotyping Assay (Siemens Health Care Diagnostics) and another used an oligonucleotide ligation assay. For types of sample, the most studies used plasma for HIVDR testing and another used infected cells from DBS.

TDR rates were reported in 14 countries (Cameroon, Uganda, India, Brazil, Argentina, Belize, Panama, El Salvador, Honduras, Cuba, Mexico, USA, UK, and Spain) among 558 naive children with rates from 0% to a maximum rate of 33.3% for NNRTIs, 16.6% for NRTIs and 18.2% for PIs.

The DRM rates were reported in 36 studies from 1980 pretreated patients. DRM prevalence ranged from 0% to 100% for NRTIs, from 3.3% to 100% for NNRTIs and from 0% to 66.7% for PIs. Double resistance to NNRTI+NRTI ranged from 0% to 100%, to NNRTI+PI from 0% to 41.7% and to NRTI+PI from 0% to 58.3%. The most common associated DRMs in RT were K103N, Y181C and K101E/P for NNRTIs and

K70R/ E, M184V and thymidine analogue mutations for NRTIs. For PIs, the most common mutations in PR were M46I/L, D30N, V82A and L90M.

In resource-limited setting, it may not be feasible to perform individual resistance testing for treatment-naïve HIV infected infants and the testing usually restricted to children who failing treatment, since cost and limitation of implementation. WHO has published a generic protocol for surveillance to assess HIVDR among children less than 18 month of age using DBS that is suitable for resource-limited countries [106]. This protocol should help to describe the prevalence of HIVDR in infants and to perform individual testing in infected infant. Currently, the determination of HIV-1 drug mutations in 46 children from northern Tanzania was performed by Shao and colleagues in 2014 [107]. This study was performed follow the WHO generic protocol by used DBS sample. The median of children age was 12 weeks. Genotypic resistance mutations were detected in 28% of children. All major mutations were detected in the RT gene and none in the PR region. The most frequent mutations were Y181C and K103N and conferring resistance to NNRTIs. In Uganda, the HIVDR of 279 children who initiated first-line antiretroviral treatment were determined by Kityo and colleagues in 2010 [108]. HIVDR was present in 10% of all children and 15.2% of children <3 years. NRTIs, NNRTI, and dual-class resistance was present in 5.7% , 7.5% , and 3.2% , respectively.

For Thailand, the data on the prevalence of drug resistance in the newly diagnosed HIV-1 infected infants are limited. Nevertheless, the prevalence of acquired drug resistance mutations in treated children had been reported. In 2005, Lolekha and colleagues [109] determined the occurrence of NRTI mutations among 95 HIV-infected Thai children who were treated with dual NRTI (mostly AZT and ddI). The result showed almost all of the children had at least 1 DRMs. The DRMs patterns were mostly observed including M184V/I, E44D and V118I.

An assessment of the HIV DRMs in 21 children who failed NNRTI-based ART was carried out by Sungkanuparph *et al* in 2009[110]. The DRMs of patients with at least 1 major mutation that confer resistance to NRTI and NNRTI were 52% and 43%, respectively. The TAMs, M184V/ I, and Q151M were most observed. The authors suggested that the PI-base regimen needed for second-line regimen for these patients. In September at the same year, Jinttamala and colleagues [111] determined the rate and

predictors of virologic failure and described pattern of DRMs among 202 Thai children who receiving NNRTI-based ART. The rate of virologic failure was 20% and 16% of these failed in the first year of therapy. The prevalence's of virological failure patients with ≥ 1 major mutations conferring resistance to NRTI and NNRTI were 89% and 97% , respectively. The common patterns were Y181C/I, K103N, M184V/I, K65R, Q151M and TAMs. The patterns of HIV DRMs in 120 Thai children after failure of first-line NNRTI-based ART were studied by Puthanakit and colleagues in 2010 [112]. The NRTI were found in 98.3% and NNRTI were found in 97.5%. The most frequent DRMs were M184V/I, TAMs, Y181C and K103N.