CHAPTER II

LITERATURE REVIEW

2.1 Biology of HIV

Human Immunodeficiency Virus (HIIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). HIV is a member of the genus Lentivirus, part of the family of Retroviridae. Lentiviruses have many morphologies and biological properties in common. Many species are infected by lentiviruses, which are characteristically responsible for long-duration illnesses with a long incubation period (Lévy, 1993). Lentiviruses are single-stranded, positive-sense, enveloped RNA viruses. Upon entry into the target cell, the viral RNA genome is converted (reverse transcribed) into double-stranded DNA by a virally encoded reverse transcriptase that is transported along with the viral genome in the virus particle. Then, the viral DNA is imported into the cell nucleus and integrated into the cellular DNA by a virally encoded integrase and host co-factors (Smith and Daniel, 2006). Once integrated, the virus may become latent, allowing the virus and its host cell to avoid recognition by the immune system. Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles that begin a new replication cycle.

2.1.1 HIV morphology

The morphology of HIV is shown in Figure 1. HIV is roughly spherical with a diameter of about 120 nm (McGovern et al., 2002). It is surrounded by the viral envelope that is composed of two layers of fatty molecules called phospholipids. The viral envelope is composed of proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle (Los Alamos National Laboratory, 2008). This protein, known as Env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle (Chan et al., 1997). Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV (NIAID, 1998).

HIV is also composed of two copies of positive single-stranded RNA that codes for nine viral genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7, and enzymes needed for the development of the virion such as reverse transcriptase, protease, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid to ensuring the integrity of the virion particle (Los Alamos National Laboratory, 2008).

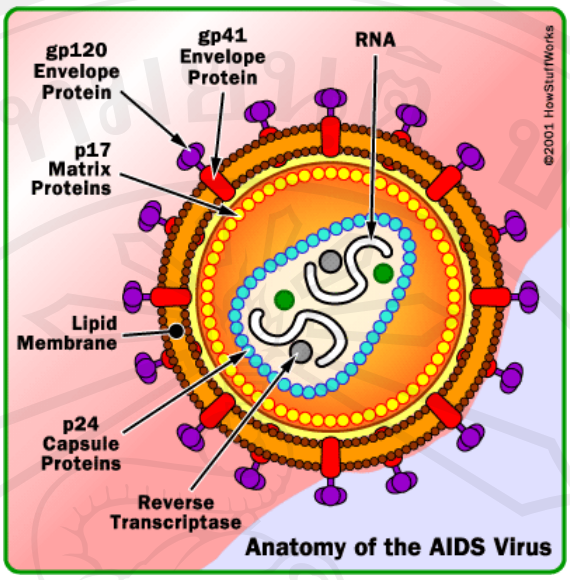

Figure 1 Structure of human immunodeficiency virus (HIV).
(Source: www.static.howstuffworks.com/gif/aids-hiv-anatomy.gif)

2.1.2 HIV genomic structure

The HIV genome is about 9.7 kilobases (kb) in length. There are three structural genes, *gag*, *pol* and *env* located from 5' to 3' which contain information needed to make the structural proteins for new virus particles (Los Alamos National Laboratory, 2008). In addition to the three major genes, the HIV virus also contains several genes including LTR, *vif*, *vpr*, *tat*, *rev*, *vpu* and *nef* that control expression of the viral genome and encoding of HIV viral proteins. The genomic structure of HIV-1 is shown in Figure 2.

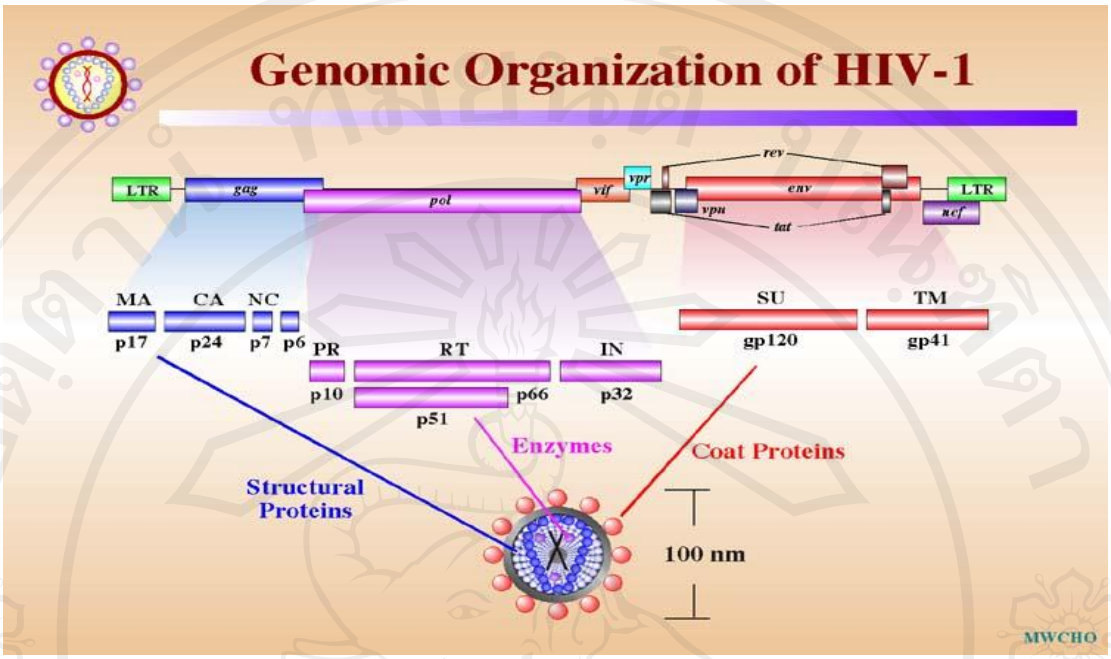

Figure 2 HIV-1 genomic structures.
(Source: www.stanford.edu/group/virus/retro/2005gongishmail/HIV.html)

LTR: Long terminal repeat, this part of the genome is composed of identical, repeating non-coding sequences designated as U3, R and U5. The functions of these units are important for transcription initiation and polyadenylation.

GAG: Group specific antigens, the genomic region encoding the capsid protein. The precursor is the p55 myristoylated protein, which is processed to p17 (MAtrix), p24 (CApsid), p7 (NucleoCapsid), and p6 proteins, by the viral protease. The function of the 55-kDa Gag precursor is called assembling, which refers to its' role in viral assembly.

POL: The genomic region encoding the viral enzymes protease, reverse transcriptase, and integrase. These enzymes are produced as a Gag-Pol precursor polyprotein, which is processed by the viral protease.

ENV: The viral glycoproteins which are responsible to produce the outer envelope. The precursor is *gp*160 which is processed to produce an external glycoprotein *gp*120 and the transmembrane glycoprotein *gp*41.

VIF: Viral infectivity factor which promotes the infectivity of virus. It is likely that Vif increases the efficiency of cell-to-cell transmission of virus.

VPR: Viral protein R, which is incorporated into the virion. The functions of Vpr are to promote the nuclear import of preintegration complexes, inhibit cell growth, and transactivate cellular genes and induce cellular differentiation.

VPU: Viral protein U, which promotes the degradation of CD4 in the endoplasmic reticulum (ER), and enhances viral particle release from the plasma membrane of HIV-1-infected cells.

TAT: Transactivator of transcription protein, which is an essential viral regulatory factor for HIV gene expression and transcription.

REV: The second necessary regulatory factor for HIV expression. Rev is considered the most functionally conserved regulatory protein of lentiviruses.

NEF: One of the first HIV proteins to be produced in infected cells; it is the most immunogenic of the accessory proteins. Nef have been detected in some HIV-1 infected long-term survivors. Nef down-regulates CD4, the primary viral receptor, and major histocompatibility complex (MHC) class I molecules and also increases viral infectivity.

2.1.3 HIV viral replication

The HIV viral replication cycle is shown in Figure 3. For the host cell infection, viral attachment occurs through $CD4^+$-gp120 interaction and also with the

function of co-receptors (generally either CCR5 or CXCR4) on the cell surface to facilitate viral entry (Chan and Kim, 1998; Wyatt and Sodroski, 1998). Once viral entry occurs, the membrane fusion process allows the viral envelope to release the viral core (uncoating) which includes HIV RNA and various enzymes, such as reverse transcriptase, integrase, ribonuclease, and protease (Chan and Kim, 1998). Owing to a classification in family Retroviridae, the replication cycle of HIV-1 requires the conversion of its single-strand RNA genome into complementary DNA (cDNA), so-called reverse transcription using viral reverse transcriptase (RT) enzyme (Zheng et al., 2005). The process of reverse transcription is extremely error-prone, which is the result of the reverse transcriptase's lack of proof reading activity, therefore resulting in viral mutations (which may cause drug resistance or allow the virus to evade the body's immune system). Upon double-stranded DNA (dsDNA) synthesis, it subsequently transported to the nuclease, and intregraded into the host genome by viral intergase enzyme (IN). The intregrated retroviral DNA genome is called the proviral DNA. Next, the proviral DNA is transcribed by the cellular RNA polymerase II into a polyadenylated unspliced mRNA, and consequently translated into viral proteins. Then, the viral genomic RNA and proteins migrate to the host cell membrane, where they assemble with several host cell proteins, like beta-2 microglobulin and human leukocyte antigens (HLA), to form new virions that are still immature (noninfectious form). Finally, the new virions are released by budding from the host cell membrane. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom 1997). The mature virus is then able to infect another cell.

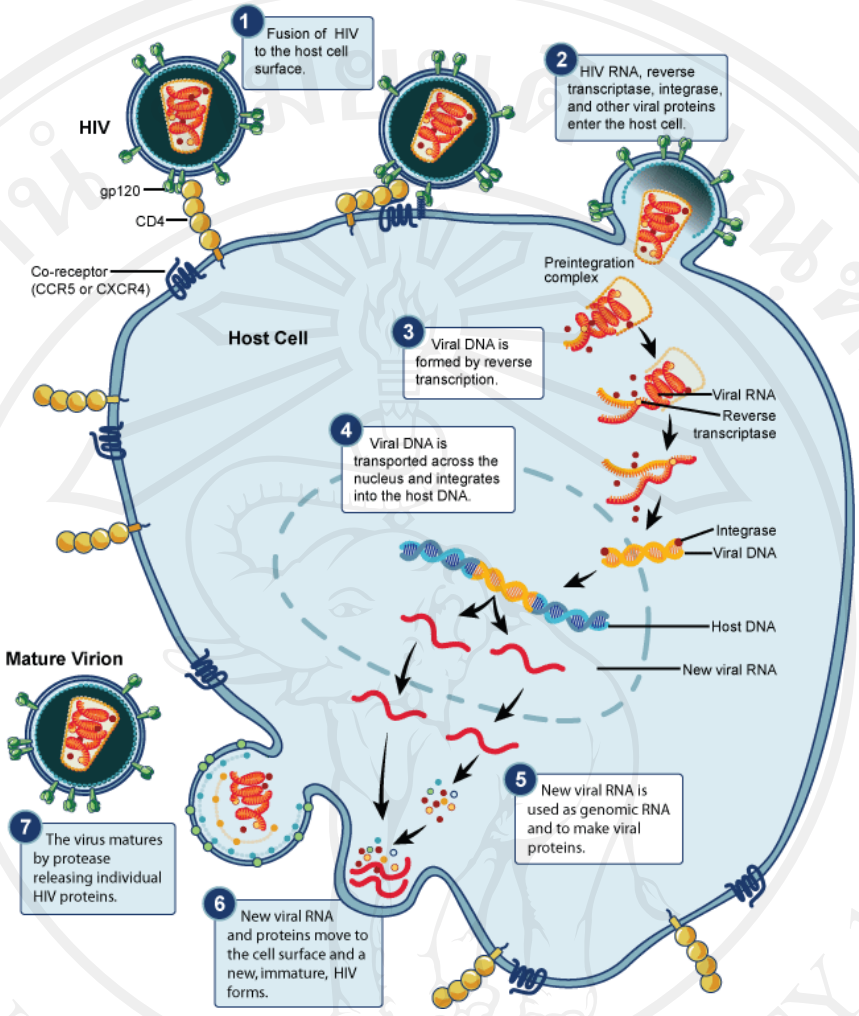

Figure 3 HIV replication.
(Source:www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx)

2.1.4 Classification and global distribution of HIV

Human Immunodeficiency Virus (HIV) is classified into types, groups, subtypes and sub-subtypes according to its genetic diversity (Robertson et al., 2000). Two major types of HIV are currently recognized - HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is the virus that was first discovered. It is more virulent, more infective (Gilbert et al., 2003), and is the causative agent of the majority of HIV

infections worldwide. In contrast, the infectivity of HIV-2 is lower. HIV-2 has a poor capacity for transmission, thus HIV-2 is is restricted to countries in West Africa (Reeves and Doms, 2002).

HIV-1 is divided into three genetic groups: group M (major or main), group O (outlier) and group N (new or non-M, non-O). Recently, a new designed group P was reported to be found in a Cameroonian woman living in France (Plantier et al., 2009). The group P viral sequence, RBF168, forms a distinct HIV-1 linkage that includes SIV sequences from western gorillas (SIVgor; *Gorilla gorilla gorilla*), suggesting that group P originated from gorillas (Van Heuverswyn et al., 2006; Plantier et al., 2009; Takehisa et al., 2009).

While HIV-1 groups O and N are restricted to countries of Central Africa, notably Cameroon. HIV-1 group M is responsible for the AIDS pandemic, accounting for over 90% of HIV infections worldwide (Hemelaar et al., 2006). Nine subtypes of HIV-1 group M are currently known (A–D, F–H, J and K). Some subtypes are further divided into sub-subtypes, such as subtypes A (A1, A2 and A3) and F (F1 and F2). Subtypes and sub-subtypes can form additional mosaic forms through recombination of different strains inside dually- or multiply-infected individuals (Burke, 1997). Some of these recombinant forms may further achieve epidemic relevance, known as circulating recombinant forms (CRFs).

HIV-1 group M, subtype A is typically found in Eastern European countries of the former Soviet Union, and some African countries such as the Democratic Republic of Congo (DRC) and Tanzania. Subtype B is primarily found in the Americas, Western Europe, Japan and Australia. Subtype C dominates most countries of sub-Saharan Africa, Ethiopia and India. Subtype D is found in Libya as well as in

the DRC and Tanzania. Subtype F is present in countries of West Africa and in the DRC. CRFs are sometimes the predominant strains in certain geographic regions, such as CRF01_AE in Southeast Asian countries, CRF02_AG in West African countries, and CRF07_BC and CRF08_BC in China (Hemelaar et al., 2006). In some regions of the world, little information is known about HIV dissemination, particularly in Central Asia. The most prevalent HIV-1 subtypes and their geographic distribution are shown in figure 4.

In South-East Asia, CRF01_AE has dominated the epidemic, and is responsible for 84% of all HIV infections. Other recombinants account for 4%, bringing the combined proportion of CRF and other recombinants to 88%, the highest in the world. In Thailand, Cambodia and Vietnam, CRF01_AE is responsible for more than 95% of infections, with the remaining infections caused by subtype B and other recombinants. Whereas, in Myanmar, CRF01_AE accounts for 52% of infections, along with subtype B (24%), C (12%) and other recombinants (12%) (Hemelaar et al., 2006).

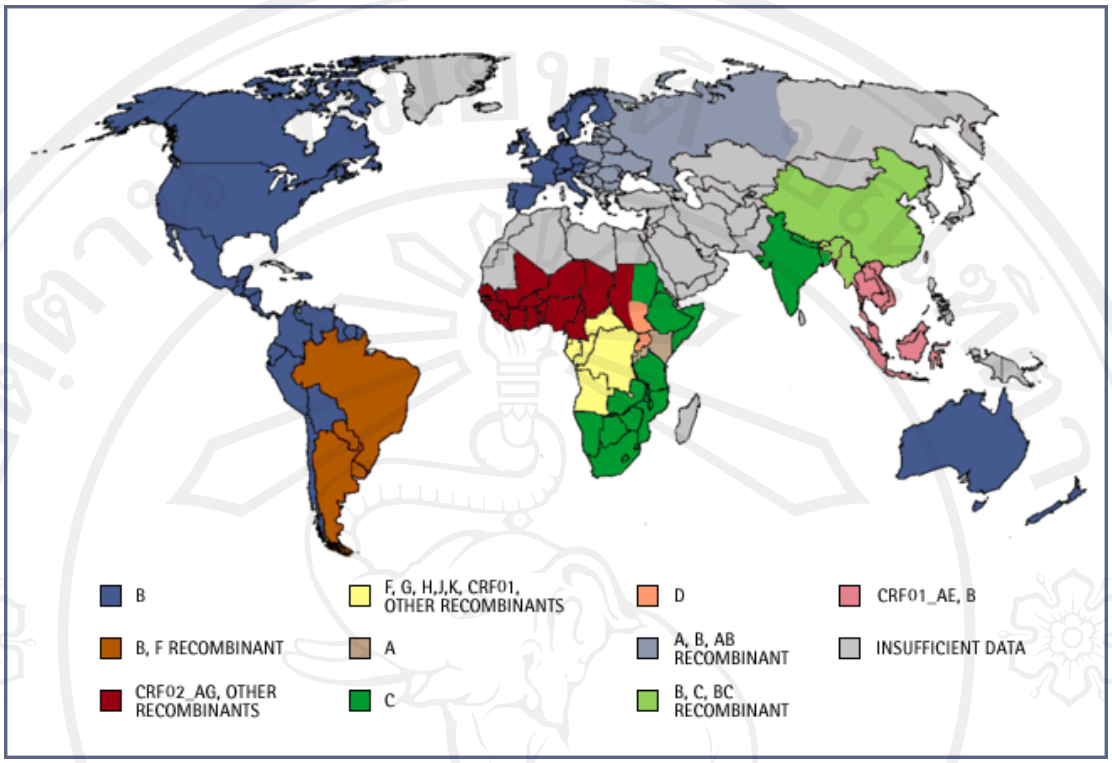

Figure 4 Global distributions of HIV-1 subtypes and recombinants. (Source: www.pbs.org/wgbh/pages/frontline/aids/art/clademap.gif)

2.2 HIV and AIDS

Acquired Immunodeficiency Syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV) (Weiss, 1993). This condition progressively reduces the effectiveness of the immune system and leaves individuals susceptible to opportunistic infections [typically Candida species and *Mycobacterium tuberculosis* (TB)] and tumors. Pneumonia caused by the fungus *Pneumocystis jirovecii* is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus (CMV) or *Mycobacterium avium* complex is more prominent.

HIV is transmitted by direct contact of a mucous membrane or body fluid which contains HIV, such as blood, semen, vaginal fluid and breast milk (CDC, 2003). Routes of transmission include vaginal, anal or oral sex, blood transfusions, contaminated needles, mother-to-child transmission at birth or through breast feeding, or other exposures to one of the above body fluids.

HIV infects cells in the human immune system such as helper T cells (specifically $CD4^+$ T-lymphocyte cells), macrophages, and dendritic cells (Cunningham et al., 2010). HIV-1 infection is associated with a progressive decrease of the $CD4^+$ T-lymphocyte cell count and increase in HIV-1 viral load. The stage of infection can be determined by measuring the patient's $CD4^+$ T-lymphocyte cell count, and the level of HIV-1 viral load in the blood. HIV infection leads to low levels of $CD4^+$ T-lymphocyte cells by three main mechanisms. First, direct viral killing of infected cells, second, increase rates of apoptosis in infected cells, and third, killing of infected $CD4^+$ T-lymphocyte cells by CD8 cytotoxic lymphocytes that recognize infected cells. When $CD4^+$ T-lymphocyte cell numbers decline below a critical level, cell-mediated immunity (CMI) is lost, and the body becomes progressively more susceptible to opportunistic infections. A generalized graph of the relationship between HIV copies (viral load) and $CD4^+$ T-lymphocyte counts is shown in Figure 5.

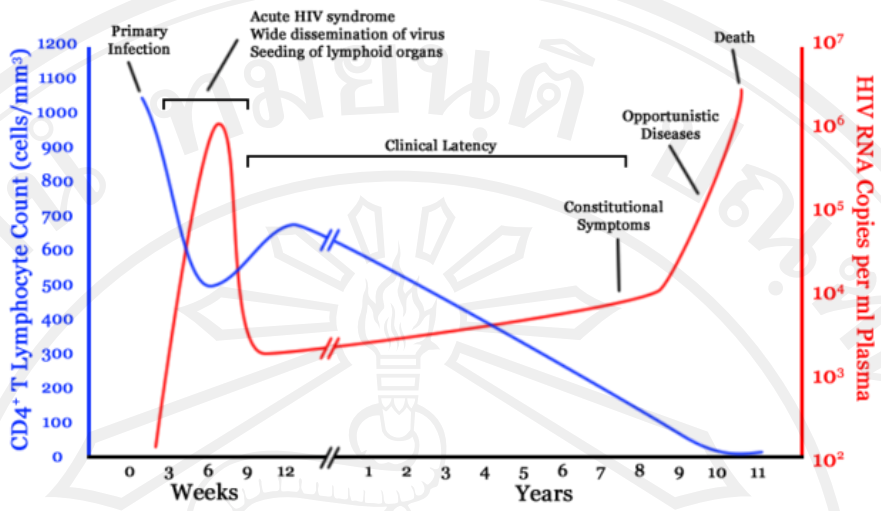

Figure 5 A generalized graph of the relationship between HIV copies number (viral load) and CD4 counts over the average course of untreated HIV infection; any particular individual's disease course may vary considerably. — $CD4^+$ T Lymphocyte count (cells/mm³). — HIV RNA copies per mL of plasma.
(Adopted from Piatak et al., 1993)

2.2.1 Epidemiology of HIV/AIDS

2.2.1.1 Global HIV/AIDS

AIDS was first reported on June 5, 1981, when the U.S. Centers for Disease Control (CDC) recorded a cluster of *Pneumocystis carinii* pneumonia (PCP) in five homosexual men in Los Angeles, USA. Currently, HIV/AIDS remains one of the most serious health problems worldwide. The overall growth of the global AIDS epidemic has stabilized. The annual number of new HIV infections has been steadily declining since the late 1990s and there are fewer AIDS-related deaths due to the significant scale up of antiretroviral therapy over the past few years.

Globally, UNAIDS has estimated that 33.3 million (31.4 million–35.3 million) people were living with HIV in 2009. A global summary report of the AIDS epidemic, ending in 2009, is shown in Figure 6. The global report of the number of

adults and children living with HIV, ending in 2009, is shown Figure 7. The report demonstrates a decline in the number of new HIV infections from a peak of 3.2 million in 1997 to 2.6 million (2.3 million–2.8 million) in 2009. The number of deaths from AIDS has also declined from the peak of 2.1 million deaths (1.9 million–2.3 million) in 2004 to an estimate of 1.8 million deaths (1.6 million–2.1 million) in 2009. The global HIV trend from 1990-2009 is shown in Figure 8. As a whole, the total number of people living with HIV is increasing due to ongoing new infections and fewer people dying from HIV-related illnesses (UNAIDS, 2010).

Overall, at the end of 2009, Sub-Saharan Africa was listed as the worst-affected region, with an estimate of 22.5 million people currently living with HIV (67% of the global total), 1.3 million deaths (72% of the global total) and 1.8 million new infections (69% of the global total). However, the number of new infections declined by 19% across the region between 2001 and 2009, and by more than 25% in 22 sub-Saharan African countries during this period. Asia is the second-worst affected region, with 4.9 million people living with HIV (15% of the global total) (UNAIDS, 2010).

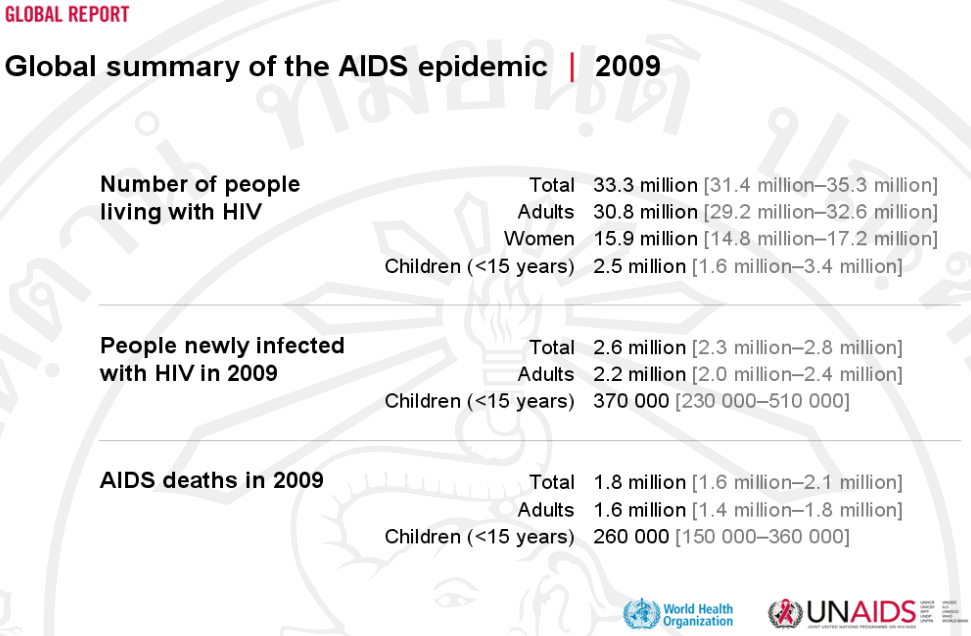

Figure 6 Global summary report of the AIDS epidemic, end 2009. (Source: WHO and UNAIDS, 2010)

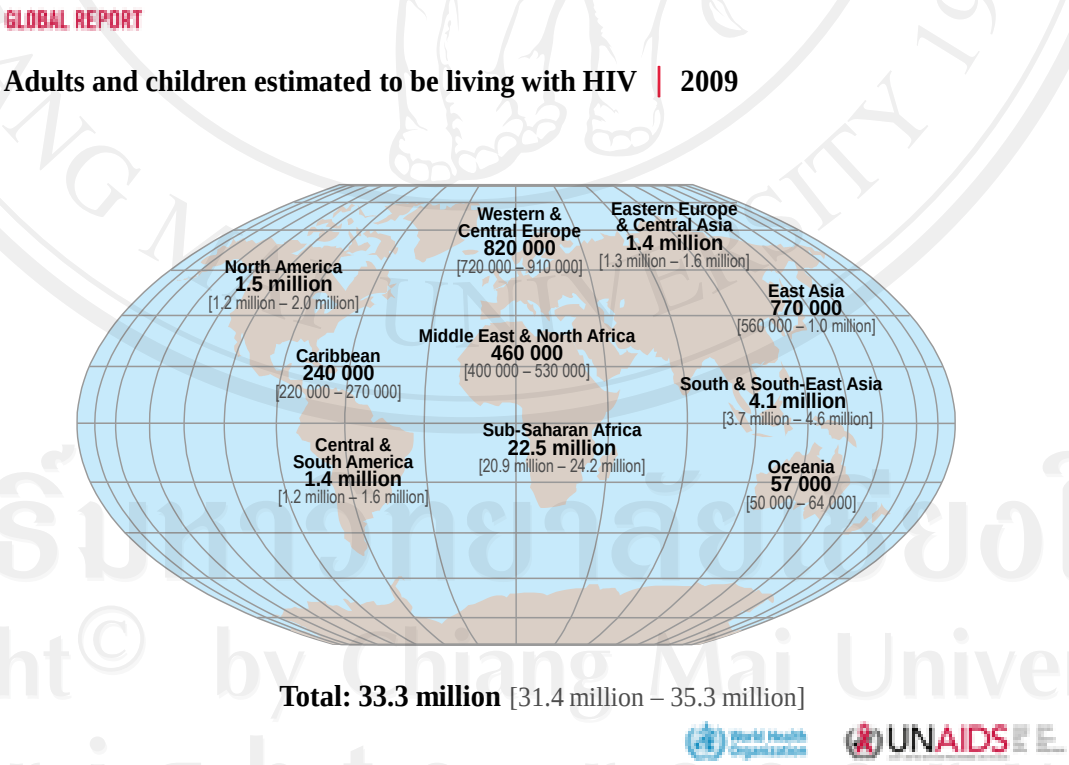

Figure 7 Global report of number of adults and children to be living with HIV, end 2009. (Source: WHO and UNAIDS, 2010)

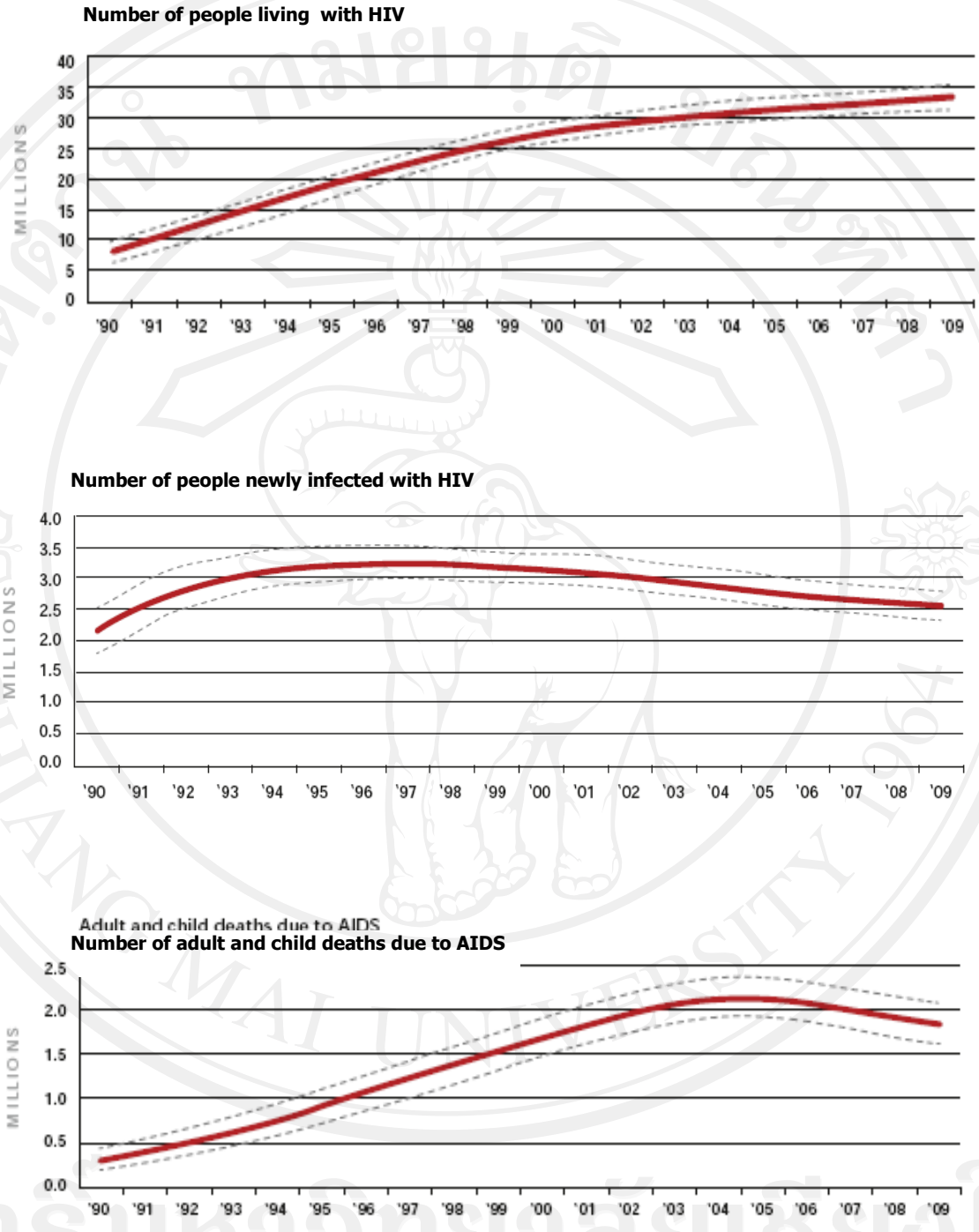

Figure 8 Global HIV trend, 1990-2009. (Dotted lines represent ranges, solid lines represent the best estimate) (Source: UNAIDS, 2010)

2.2.1.2 HIV/AIDS in Asia

In Asia, UNAIDS estimated that 4.9 million (4.5 million–5.5 million) people were living with HIV. They also estimated that 360,000 (300,000-430,000) people were newly infected with HIV and 300,000 (260,000-340,000) people have died due to AIDS. Most nations the HIV epidemics have stabilized. No country in the region has a generalized epidemic (UNAIDS, 2010). The global report of AIDS statistics for Asia in 2001 and 2009 is shown in Figure 9.

Despite the general stabilization of HIV epidemics in Asia, Thailand is the only country in this region with prevalence close to 1%, though its epidemic appears to be stable overall. The adult HIV prevalence was 1.3% (0.8%–1.4%) in 2009, and the HIV incidence was slowed down to 0.1%. In comparison, in Cambodia, the adult HIV prevalence was 0.5% (0.4%–0.8%) in 2009, which declined from 1.2% (0.8%–1.6%) in 2001. The epidemic has remained stable in Malaysia and Sri Lanka during this time (UNAIDS, 2010). The HIV prevalence among adults aged 15–49 years old in Asia, 1990-2009, is shown in Figure 10.

However, the HIV prevalence is increasing in some countries, where drug injecting is the main route of HIV transmission such as Bangladesh, Pakistan and the Philippines. Moreover, in Bangladesh and the Philippines, the HIV incidence has increased by 25% from 2001 to 2009 even as the countries continue to have low epidemic levels (UNAIDS, 2010).

		People living with HIV	People newly infected with HIV	Children living with HIV	AIDS-related deaths
ASIA	2009	4.9 million [4.5–5.5 million]	360 000 [300 000–430 000]	160 000 [110 000–210 000]	300 000 [260 000–340 000]
	2001	4.2 million [3.8–4.6 million]	450 000 [410 000–500 000]	100 000 [69 000–140 000]	250 000 [220 000–300 000]

Figure 9 Global report of AIDS statistics for Asia in 2001 and 2009.
(Source : UNAIDS, 2010)

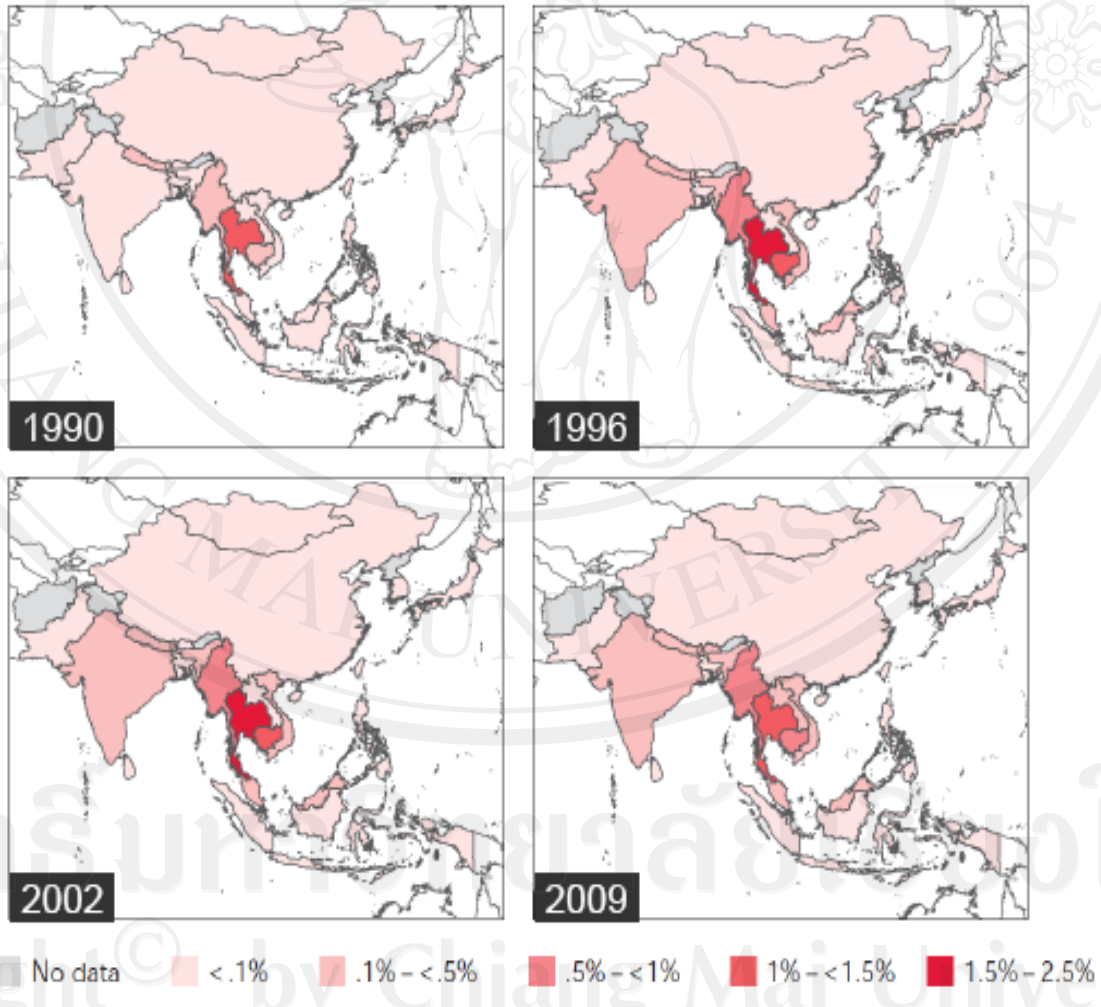

Figure 10 HIV prevalence among adults aged 15–49 years old in Asia, 1990-2009.
(Source: UNAIDS, 2010)

2.2.1.3 HIV/AIDS in Thailand

In Thailand, HIV/AIDS was first reported in 1984. By the end 2009, UNAIDS estimated that 530,000 Thais were living with HIV/AIDS. The current HIV/AIDS situation in Thailand is shown in Table 1. In 2009, the prevalence of HIV in adults was 1.3% (0.8%-1.4%) (UNAIDS, 2010). According to the CIA World Fact book, which lists 2009 statistics for HIV prevalence by country, Thailand has the highest prevalence of HIV in Asia (CIA, 2010). In the past, the HIV/AIDS incidence was increasing steadily in the country. In 1991, however, the government adopted a strategy to combat the disease, and in recent years, the number of new infections has declined. However, the spread of HIV in some provinces is still robust, especially in those regions that receive a large number of tourists and those along the eastern seaboard and Gulf of Thailand.

The early cases of HIV/AIDS in Thailand occurred primarily among men who have sex with men (MSM). The virus then spread rapidly to injecting drug users (IDUs), followed by sex workers and their clients. Currently, HIV prevalence is highest among the IDU population, with estimates ranging from 30% to 50 %. The prevalence among MSM population also remains high. However, prevention activities among this population over the past two years, especially in Bangkok, has resulted in a decline of HIV prevalence from 30.7% in 2007 to 24.7% in 2009. HIV prevalence among direct female sex workers (FSWs) showed steady decline from 33.2 % in 1994 to 5 % in 2007. HIV prevalence among pregnant women has also declined from 3.4% in 1992 to 1% in 2009 (National AIDS Prevention and Alleviation Committee, 2010).

Table 1 Current HIV/AIDS situation in Thailand.
(Source: www.avert.org/thailand-aids-hiv.htm)

Thailand Statistics (UNAIDS, 2010)	
Estimated number of people living with HIV, end 2009	530,000
Adults (15+)	520,000
Women (15+)	210,000
Estimated adult (15-49) HIV prevalence	1.3%
Estimated number of AIDS deaths in 2009	28,000

2.3 HIV/AIDS treatment and antiretroviral therapy (ART)

Presently, there is no publicly available vaccine or cure for HIV/AIDS (Robb, 2008; Gray et al., 2011). In September 2009, a trial conducted vaccine (ALVAC-HIV and AIDSVAX) in Thailand demonstrated 30% efficacy for reducing HIV infection (The Lancet, 2009). Following those results, in July 2010, a vaginal gel containing tenofovir, a reverse transcriptase inhibitor, was shown to reduce HIV infection rates by 39 % in a trial conducted in South Africa (Abdool Karim et al., 2010). Therefore, these reports have shown that no vaccine or other treatments are currently available that can completely cure or prevent HIV infection.

Alternatively, treatment with antiretrovirals (ARVs) increases the life expectancy of people who are infected with HIV. Antiretroviral therapy (ART) has been the major driver in public health in diminishing HIV-related morbidity and mortality over the past decade (Palella et al., 1998). The aim of ART in HIV infection is to reduce the amount of replicating virus to a level as low as possible, thereby preventing infection of new cells and preserving the immune system. Even after HIV has progressed to AIDS, the average survival time with ART is estimated to

be more than 5 years. Without antiretroviral therapy, the median survival time after developing AIDS is only 9.2 months (Morgan et al., 2002).

In 1987, zidovudine (AZT) was the first approved ARV (Davies, 2000). Currently, there are several classes of antiretroviral drugs action on different stages of the HIV life-cycle. The replication cycle of HIV and targets for antiretroviral therapy are shown in Figure 11.

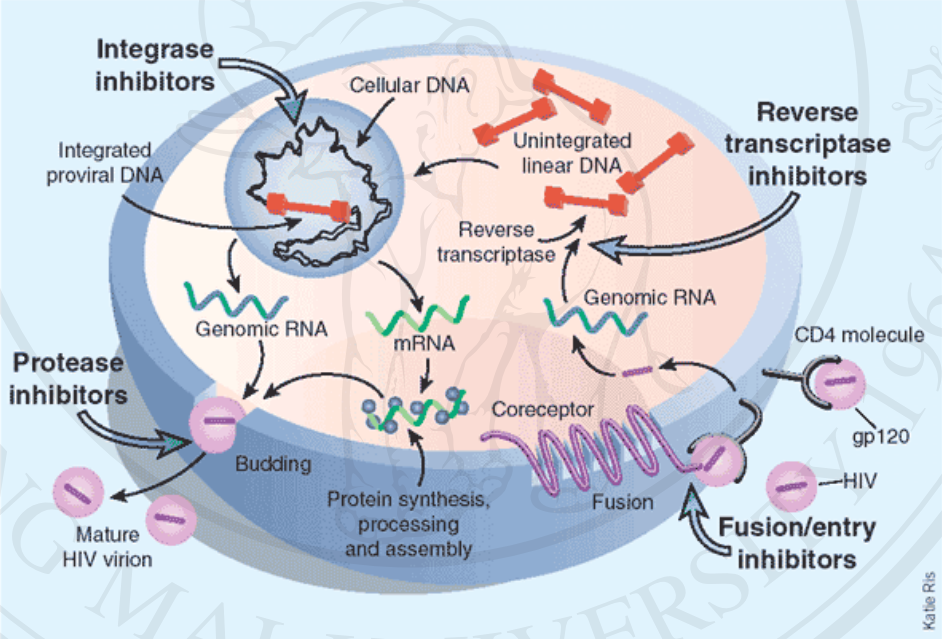

Figure 11 Replication cycle of HIV and targets for antiretroviral therapy. (Source: Anthony S Fauci, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892-2520, USA)

ARV drugs are classified by the phase of the retrovirus life-cycle that are inhibited by ARV. The classes of ARVs are as follows:

- **Nucleoside and nucleotide reverse transcriptase inhibitors (NRTI):** inhibit reverse transcription by incorporating into the newly synthesized viral DNA strand as a faulty nucleotide resulting in DNA chain termination. Eleven such ARVs have been approved such as zidovudine (Retrovir, AZT), stavudine (Zerit, d4T), lamivudine (Epivir, 3TC), tenofovir, a nucleotide analog (Viread, TDF) and Combivir (AZT/3TC combination), etc.
- **Non-nucleoside reverse transcriptase inhibitors (NNRTI):** inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. Five such ARVs have been approved including nevirapine (Viramune, NVP) and efavirenz (Sustiva or Stocrin, EFV).
- **Protease inhibitors (PIs):** target viral assembly by inhibiting the activity of protease which cleaves nascent proteins for the final assembly of new virons. Ten protease inhibitors have been approved such as saquinavir (Invirase, SQV), indinavir (Crixivan, IDV) and ritonavir (Norvir, RTV).
- **Integrase inhibitors (IN):** inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. Raltegravir was the first integrate inhibitor which received FDA approval in October 2007. Several additional INs are currently under investigation through clinical trial.
- **Entry inhibitors or fusion inhibitors:** interfere with binding, fusion, and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and enfuvirtide are the two agents in this class currently available.

- **CCR5 receptor antagonists:** the first ARV drugs which do not target the virus directly. Instead, they bind to the CCR5 receptor on the surface of the T-Cell and block viral attachment to the cell. If HIV cannot attach to the cell, it cannot gain entry to replicate.
- **Maturation inhibitors:** inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein (p24). Because these viral particles have a defective core, the virions released consist of non-infectious particles. Alpha interferon is a currently available agent in this class. Two additional inhibitors under investigation are bevirimat and Vivecon.

In the present, treatment for HIV infection consists of highly active antiretroviral therapy (HAART). It has been highly beneficial to many HIV-infected individuals since its introduction in 1996, when the protease inhibitor-based HAART initially became available (Davies, 2000). Current HAART options are combinations (or "cocktails") consisting of at least three drugs belonging to at least two types, or "classes," of ARV drugs. Typically, these classes are two NRTIs plus either a NNRTI or PI.

Combinations of ARVs create multiple obstacles to HIV replication with the intention of keeping replication low and reducing the possibility of viral mutation. If a mutation that conveys resistance to one of the drugs being taken arises, the other drugs continue to suppress reproduction of that mutation.

Nevertheless, for some patients, HAART activity leads to less than optimal results. This problem is due to medication intolerance/side effects or infection with a

drug-resistant strain of HIV. Non-adherence and non-persistence with therapy are major reasons why some people do not benefit from HAART (Becker et al., 2002). Side effects can also deter people from persisting with HAART, these include lipodystrophy, dyslipidaemia, an increase in cardiovascular risks and birth defects in offspring (Montessori et al., 2004).

The high cost of ARV drugs is also a major barrier to access the medications and treatments available for HIV and AIDS. However, the costs of ARV drugs have fallen recently in low-income countries; therefore the number of patients with HIV/AIDS who received ARVs has increased over the past years. In 2009, UNAIDS reported that an additional 1.2 million people received ART, which increased the total number of people receiving treatment in low- and middle-income countries to 5.2 million, a 30% increase from 2008. Furthermore, in 2010, WHO issued revised treatment guidelines (WHO, 2010) recommending earlier initiation of antiretroviral therapy, at a CD4 count of <350 cells/mm^3. These new criteria increased the total number of people medically eligible for antiretroviral therapy by roughly 50%, from 10 million to 15 million (UNAIDS, 2010).

In Thailand, access to ART has been available since 2001 and has been further increased in 2003 after the initiation of the program called "NAPHA" (National Access to Antiretroviral Programs for People who have AIDS). Through NAPHA, the Ministry of Public Health (Thailand), MoPH, has offered free antiretroviral drugs to HIV-infected patients starting from 2002. The cost of some reverse transcriptase inhibitor antiretroviral drugs (ARV) has been reduced as a result of local production in Thailand. One such drug is the combined drug stavudine (d4T) + lamivudine (3TC) + nevirapine (NVP) called "GPOvir". Therefore, this program should improve

HIV-related morbidity and mortality (Sutthent et al., 2005). By the year 2010, the Joint United Nations Program on HIV/AIDS (UNAIDS) estimate that there are 1,138,020 people living with HIV/AIDS Thailand (UNAIDS, 2010) and more than 150,000 patients are currently treated with ART under the support of the National AIDS Program (NAP) (Sungkanuparph et al., 2010). However, the proper management of antiretroviral-related toxicity and the enhancement of adherence are obviously crucial for the long-term success of ART. However, in the current scheme, due to the inaccessibility of the more costly drug classes that are not NRTI, NNRTI and PI, it is imperative to implement a strategy to prevent the occurrence of HIV-1 drug resistance to available drugs.

2.4 HIV drug resistance

The use of combinations of antiretroviral (ARV) drugs has proven effective in controlling the progression of Human Immunodeficiency Virus (HIV) and prolonging survival (Palella et al., 1998), but these benefits can be compromised by the development of drug resistance (Ledergerber et al., 1999; DeGruttola et al., 2000).

The causes of HIV mutation include high viral replication rate (~10^{10} copies per day) and the error-prone nature of reverse transcriptase (RT), resulting in the generation of a large population of genetically distinct variants so-called quasispecies (Mansky, 1998). Most of these errors are nucleotide substitutions, but duplications, insertions, and recombinations can also occur. It is estimated that every possible single point mutation occurs between 10^4 to 10^5 times per day in an untreated HIV-1 infected individual and that double mutants also occur commonly (Coffin, 1995). Variants with a fitness advantage can rapidly outcompete other variants to replicate

and escape immune responses. Under drugs pressure, if viral replication is not fully suppressed, drug-resistant variants are likely to emerge (Birk et al., 2001; Deeks et al., 2001). Cessation or change of the ART regimen results in the selection of wild-type viruses or alternate mutants due to their greater replicative capacity (Izopet et al., 2000). The drug-resistant variants, once they appear, can persist in the peripheral blood mononuclear cell (PBMC) even when not detected in plasma (Chun et al., 2000; Hermankova et al., 2001). These mutants may be rapidly selected for whenever ART is initiated or resumed (Hirsch et al., 2000).

2.4.1 Nucleoside and nucleotide analogues resistance

Nucleoside analogues and nucleotide analogues inhibit the synthesis of viral DNA. After molecules of the drugs are incorporated into the nascent chain of viral DNA by reverse transcriptase, the synthesis of viral DNA is arrested. These drugs lack the 3' hydroxyl group and therefore no additional nucleotides can be attached to them.

Two mechanisms induce resistance to nucleoside and nucleotide analogues. First, the incorporation of the drug into viral DNA is impaired. Second, the drug is removed from the prematurely terminated DNA chain.

From the first mechanism, several mutations in reverse transcriptase such as M184V, K65R and Q151M complex mutations can promote resistance by impairing the ability of the enzyme to incorporate the drug into viral DNA. This mechanism is shown in Figure 12. As an example of this resistance mechanism, the M184V mutation that results in an amino acid replacement of methionine (M) by valine (V) at position 184 of the reverse transcriptase is the main mutation that confers resistance to

lamivudine (3TC) (Boucher et al., 1993). Methionine 184 is located at the target of the catalytic site of reverse transcriptase. If it is substituted by valine, which has a different side chain, the proper positioning of lamivudine triphosphate within the catalytic site is interfered (Sarafianos et al., 1999). The M184V mutation induces very high levels of resistance to 3TC. When 3TC is used as a single drug therapy, the resistant strains rapidly overtake wild-type virus in a few weeks (Schuurman et al., 1995). When 3TC is used as part of a failing drug combination regimen, the M184V mutation is usually the first mutation to occur (Descamps et al., 2000; Havlir et al., 2000).

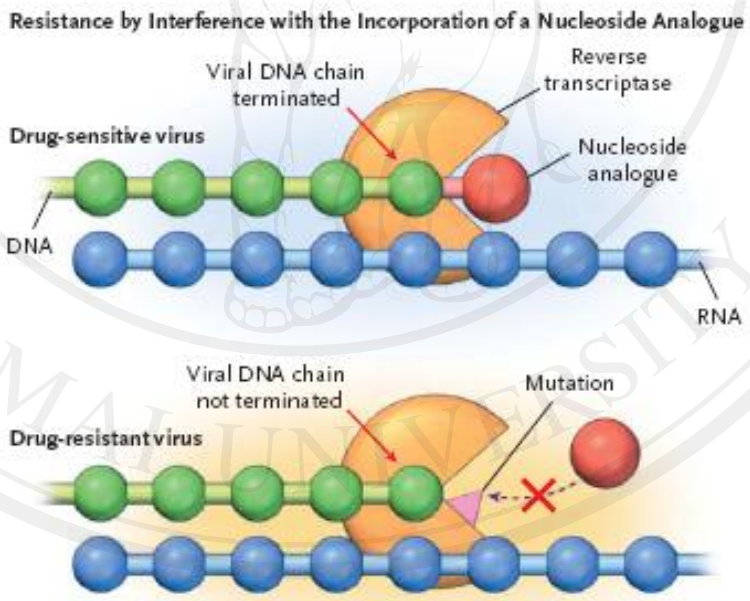

Figure 12 Resistance by interference with the incorporation of a nucleoside analogue (Adopted from Francois and Allan, 2004)

The second mechanism that induces nucleoside and nucleotide analogues resistance is the removal of the nucleoside analogue from the terminated DNA chain. This mechanism is associated with a group of mutations commonly referred to as

"thymidine analogue mutations". Thymidine analogue mutations promote resistance by using ATP or pyrophosphate to remove the nucleoside analogues from the 3' end of the terminated DNA strand (Arion et al., 1998; Meyer et al., 1999). This mechanism is shown in Figure 13. In wild-type HIV virus, ATP and pyrophosphate do not participate in the DNA-polymerization reaction. But if the thymidine analogue mutation has occurred, ATP or pyrophosphate can attack the phosphodiester bond that links the nucleoside analogue to DNA, resulting in removal of the analogue from the terminated DNA chain. Thymidine analogue mutations most frequently occur after the failure of drug combinations that include thymidine analogues such as zidovudine and stavudine. However, thymidine analogue mutations can promote resistance to almost all nucleoside and nucleotide analogues, including tenofovir (Larder and Kemp, 1989; Shafer et al., 1996; Coakley et al., 2000; Picard et al., 2001).

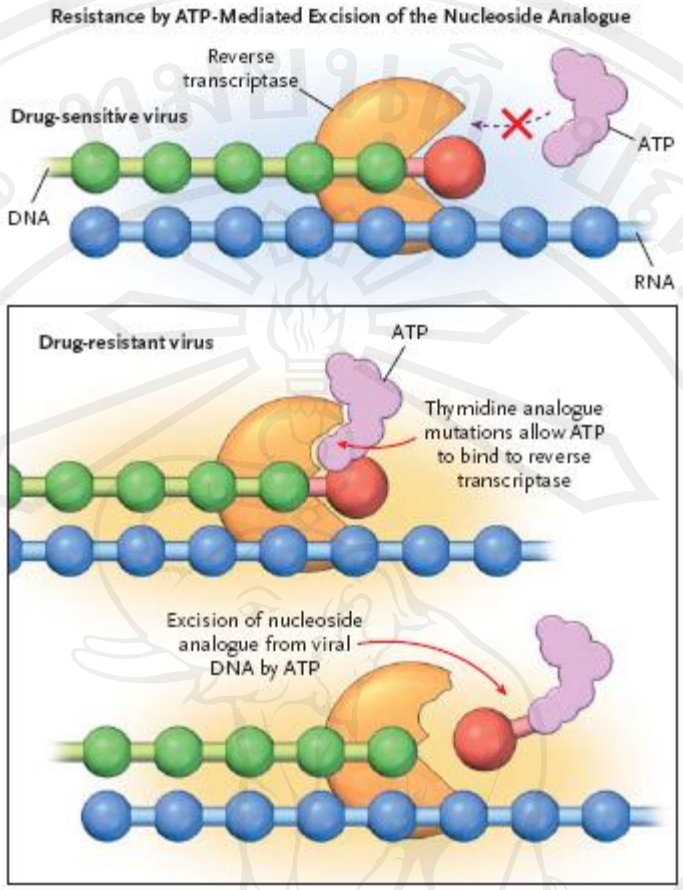

Figure 13 Resistance by ATP-mediated excision of the nucleoside analogue. (Adopted from Francois and Allan, 2004)

2.4.2 Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) resistance

NNRTIs inhibit reverse transcriptase (RT) directly by binding to the enzyme and interfering with its function. The small molecules of NNRTIs have a strong affinity for a hydrophobic pocket located close to the catalytic domain of the RT. This mechanism is shown in Figure 14. The binding of the drug molecule affects the flexibility of the enzyme, thereby blocking its' ability to synthesize DNA. The mutations which occur after treatment failure are all located in the targeted hydrophobic pocket of NNRTIs; therefore, they reduce the affinity of the drug

(Boyer et al., 1993; Esnouf et al., 1997; Richman et al., 1994; Bacheler et al., 2000; Ren et al., 2001; Hsiou et al., 2001).

Some NNRTIs resistance mutations occur more frequently than others, such as nevirapine-resistance associated with the Y181C mutation and efavirenz-resistance associated with the K103N mutation.

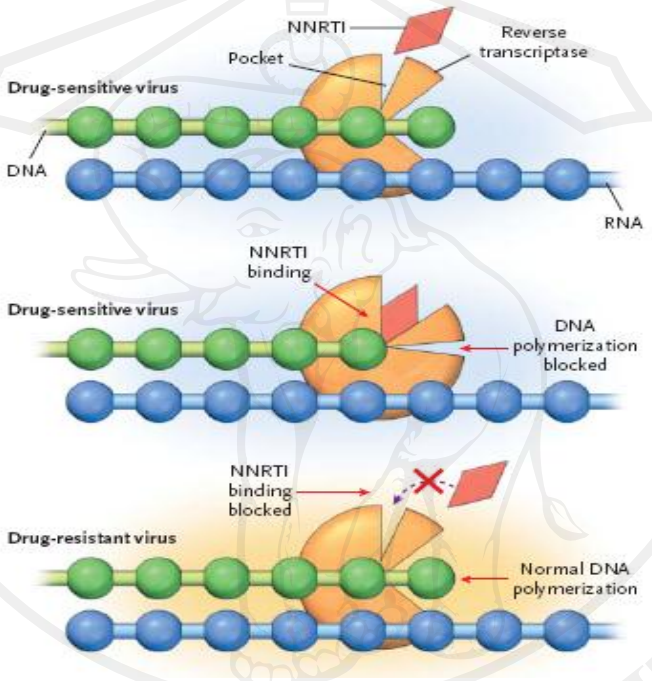

Figure 14 Mechanism of resistance of HIV to non-nucleoside reverse- transcriptase Inhibitors. (Adopted from Francois and Allan, 2004)

2.4.3 Protease inhibitors resistance

According to the HIV life cycle, the HIV protease cleaves the large polyprotein precursors at specific sites - releasing structural proteins and enzymes necessary for the assembly of infectious viral particles. Protease inhibitors inhibit the functional protease. Viral particles are subsequently produced, but they are immature and non-infectious.

Resistance to protease inhibitors is the consequence of amino acid substitutions mutation that occur either inside the substrate-binding domain of the enzyme or at distant sites (Kaplan et al., 1994; Condra et al., 1995; Molla et al., 1996). The amino acid mutations change the structure at the points of contact between the inhibitors and the protease, thereby reducing their affinity for the enzyme (Chen et al., 1995; Ridky et al., 1998; Hong et al., 2000; Prabu-Jeyabalan et al., 2002).

2.4.4 Fusion inhibitors resistance

Fusion inhibitors are antiretroviral drugs that interfere with binding, fusion, and entry of HIV-1 to the host cell thereby blocking the infectivity of HIV-1 to target cells. HIV-1 enters the target cells via interaction between the HIV envelope glycoprotein complex (gp120-gp41) and specific cell surface receptors (Kilby and Eron, 2003). Then, the HIV virus is fused to the host cell membrane. An example of a fusion inhibitor is enfuvirtide, a synthetic 36-amino acid peptide to residue 127-162 of gp41. This drug blocks HIV-1 entry by interacting with the HR1 domain of gp41, thereby preventing formation of the fusion-active conformation. Mutations between amino acids 36-45 in HR1 are associated with reduced susceptibility of the virus to enfuvirtide (Rimsky et al., 1998; Wei et al., 2002).

2.4.5 Cross-resistance

Cross-resistance can occur during antiretroviral therapy. It refers to the resistance of HIV to one drug and the collateral resistance to other drugs in the same class to which it has never been exposed. For example, most HIV that is resistant to

nevirapine (Viramune) is also resistant to efavirenz (Sustiva). This means that nevirapine and efavirenz are cross-resistant.

Cross-resistance is important when deciding to change ARVs. New drugs have to be carefully selected so that they are not cross-resistant to previously used drugs.

2.5 HIV drug resistance testing methods

Viral genotyping and phenotyping are the major principles used in many assays for the detection of antiretroviral drug resistance. The genotyping assays are less labor intensive and cheaper than phenotyping assays for HIV-1 DR testing in a routine clinical setting. The current standard nucleotide sequencing methods either the in house or the commercial assays provide a composite of the HIV genome sequences. In addition, several assays have been developed with less cost alternatively to standard method for resistance testing. Below, methodologies for HIV-1 drug resistance testing were summarized.

2.5.1 Genotypic assays

2.5.1.1 Standard direct sequencing

HIV genotypic assays detect the presence of resistance mutations or nucleotide substitutions mutation in the *gag-pol* region of the HIV-1 genome. This region encodes for the reverse transcriptase and protease enzymes, the targets of current antiretroviral drugs. Specific gene sequences are compared with that of a reference (wild-type) virus, and mutations associated with decreased susceptibility to specific antiretroviral drugs are identified. Genotypic assays can be performed in-house or

using commercial kits such as ViroSeq™ HIV-1 Genotyping System (Applied Biosystems, CA, USA) and TruGene™ HIV Genotyping Kit (Visible Genetics, Inc., GA. USA).

As a limitation, most commercial genotyping tests are designed to detect HIV-1 subtype B viruses whereas sequencing of non-B subtypes can be problematic, and is generally impossible with HIV-1 group O, HIV-1 group N and HIV-2. Besides, these commercial assays are also expensive, therefore being unaffordable for resistance surveillance in resource-limited settings.

2.5.1.2 Single-genome sequencing (SGS)

This technique was developed based on limiting-dilution assays. SGS analyses HIV-1 populations by obtaining cDNA sequences derived from many single viral genomes in plasma sample which containing a minimum of 1,000 copies of HIV-1 RNA. Complementary DNA sequences derived from 20–40 single genomes are typically analyzed by DNA sequencer and compare to the reference HIV-1 sequence. SGS detects minority variants that are present in at least 2% of the viral population (Halvas et al., 2006).

2.5.2 Phenotypic assays

Phenotypic assays evaluate the ability of HIV-1 replication in the presence of increasing concentrations of the antiretroviral agents. It directly measures the amount of drug necessary to inhibit or suppress viral replication in vitro. The drug concentration that inhibits 50% (IC50) or 90% (IC90) of viral replication is calculated from the drug susceptibility curve. The ratio of the IC50 or IC90 of sample and

reference HIV viruses is reported as the “fold” resistance. For example, if the test sample grows twenty times as much as reference virus, it has "20-fold resistance".

As of the limitations due to the time and labor consume and the laboratory infrastructure requirement, the phenotype assay is inappropriate for a routine use in clinical settings.

2.5.3 Virtual phenotype

The virtual phenotypic test is a combination of a genotypic and a phenotypic test. It is an alternative approach to interpreting genotypic drug resistance information. The virtual phenotype correlates the patient genotypic data with a large database of paired phenotypes and genotypes. Then linkage assigns calculated fold-changes in IC50 to query genotypes.

The main limitation of virtual phenotype is its predictive power depends on the number of matched data sets available. Thus, variation is frequently higher in smaller data sets, as well as for newer drugs or complex resistant patterns.

2.5.4 Alternate assays

Several assays have been developed to detect HIV-1 drug resistance alternatively to the aforementioned assays. These methods mostly exploited the fact that the known drug resistant mutations are largely due to the point mutations in the HIV-1 genome, particularly, the *pol* gene. Examples of these assays were listed below including the oligonucleotide ligation assay (OLA) that formed the basis of this dissertation that aimed to apply the OLA for the HIV-1 subtype CRF01_AE in Thai patients.

2.5.4.1 Allele-specific polymerase chain reaction (ASPCR)

Allele-specific PCR (ASPCR) is a highly sensitive and reproducible method for studying point mutations in viral genomes such as the study of minor HIV-1 variants harboring resistance mutations. The method is performed by the amplification of viruses containing mutant and wild-type alleles of a codon of interest and detects amplified product using a real-time PCR. The discrimination and sensitivity of ASPCR are determined by the specific primers used. Wild-type specific primers, mutant-specific primers and nonselective primers are used to amplify the region of interest. The base near the 3′ end of the specific primer is replaced by hypoxanthine in order to enhance the specificity of the primer and the length of primer, which can also affect the discrimination of ASPCR.

ASPCR is significantly less labor-intensive and time-consuming than other techniques used for similar purposes. However, polymorphisms often occur in the sequences of quasispecies in clinical samples and the polymorphisms that occur in the primer binding sites can significantly impair the sensitivity and accuracy of ASPCR.

2.5.4.2 Line probe assay (LiPA)

Line probe assay (LiPA) based on the principle of reverse DNA hybridization. It identifies specific drug resistance specific point mutations by using short oligonucleotide probes immobilized as parallel bands on nitrocellulose strips. These probes contain both wild-type codons and mutant codons known to confer resistance to specific antiretroviral drugs. The final products of probes hybridization to the targets, usually the PCR products, can be visualized by the formation of colored

precipitate bands on the strip. Currently, the LiPA probes development is targeting only a limited number of mutations that encompasses a limited number of drugs.

2.5.4.3 Oligonucleotide ligation assay (OLA)

In 1995, Frankel L, et al. developed the probe-based method, the oligonucleotide ligation assay (OLA) to detect the drug-resistant mutations in HIV-1 subtype B (Frenkel et al., 1995). The OLA has been described as a method to detect known sequence variants or known point mutations in a standardized ELISA plate format, based on the covalent joining of two adjacent, differentially labeled oligonucleotide probes by a DNA ligase that is hybridized to a cDNA template, usually a PCR product. DNA ligases play an essential role in DNA replication, repair and recombination. Ligases carry out the covalent linkage of the 3'-hydroxyl and 5'-phosphate ends of duplex DNA segments generated in several DNA replication processes. This reaction requires the activation of the 5'-phosphate by ligase-mediated conversion to the 5'-AMP intermediate. The ligation of the 3' and 5' ends appears to be mechanically similar to the incorporation of deoxynucleotide triphosphate by DNA polymerase. However, ligases generally do not ligate if a base mispair is on the 3' side of the junction; whereas, ligases appear much more tolerant of the same mispairs when located on the 5' side (Lehman 1974; Timson et al., 2000). Therefore, the specificity of the OLA for mutant versus wild-type virus is conferred by the ligation reaction and it is evident that the 2 bases flanking each side of the oligonucleotide junction be complementary to the target DNA is required for ligation (Landegren et al., 1988). The ligation may not occur if the target has genetic polymorphisms within 2 bases of the ligation site or multiple polymorphisms within

the region complementary to one of the OLA probes (Edenstein et al., 1998; Beck et al., 2002).

To date, the ligation probes can be modified by labeling the probes with a hapten such as digoxigenin or fluorescein. The later version of OLA detector probes for wild-type and mutant have been modified by differently labeling with digoxigenin or fluorescein to the 5' end of probes (Beck et al., 2002; Ellis et al., 2004; Wallis et al., 2005; Beck et al., 2008). The ligated probes can be captured onto a streptavidin-coated plate by adding a biotin moiety to the probe. From this format, the results of the assay are simple to interpret and can be classified as positive or negative for the reporters marking the wild-type or mutant allele. The OLA schemas were shown in Figure 15.

The specificity of the ligation between two oligonucleotide probes of OLA is regulated by three factors: (I) the specificity of hybridization of the oligunucleotide probes to their complementary sequences on the template, (II) the need for these probes to hybridize in a head-to-tail (5'→3') orientation on the template and (III) the fact that the oligonucleotides must have perfect base pairing with the target at their junction. These characteristics allow nonstringent annealing conditions to be used without compromising specificity (Tobe et al., 1996).

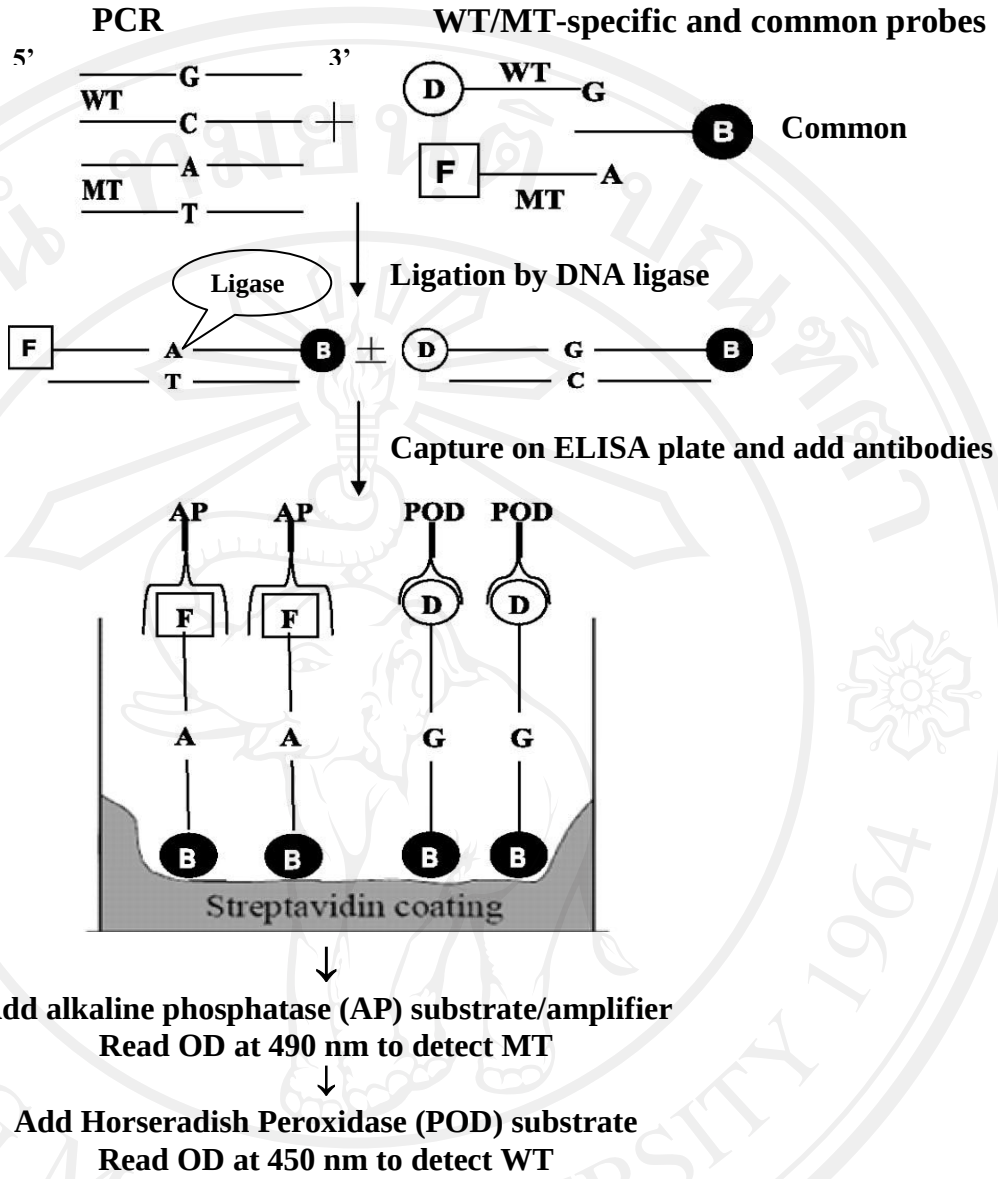

Figure 15 Schematic presentation of OLA. OLA involves the generation of PCR fragments and the use of three labeled probes: digoxigenin (D) for wild-type (WT), fluorescein (F) for mutant (MT), and biotin (B) for the common probe. These probes anneal to the PCR product, and the WT or MT probe is ligated to the common probe by DNA ligase enzyme. Ligated products are captured on streptavidin-coated microtiter wells and detected by ELISA with horseradish peroxidase (POD)-labeled anti-D antibodies and alkaline phosphatase (AP)-labeled anti-F antibodies. After the addition of chromogenic substrates for each reporter enzyme, the appearance of color indicates that ligation has occurred between and allele-specific probe and the common, biotinylated probe (Adopted from Jallow et al., 2007).