TRANSMITTED DRUG RESISTANCE AND PHYLOGENETIC ANALYSIS OF HIV-1 VIRUS FROM KELANTAN, MALAYSIA BASED ON REVERSE TRANSCRIPTASE (RT) GENE

by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

AIDS	Acquired immunodeficiency deficiency syndrome
ARV	Antiretroviral
AZT	Zidovudine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
$CD4^+$	Cluster of differentiation 4
$CD8^+$	Cluster of differentiation 8
Cdna	Complementary DNA
°C	Degree Celsius
d4T	Stavudine
dNTP	deoxynucleoside Triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
EFV	Efavirenz
env	Envelope
Et-Br	Ethidium bromide
g	Gravity
gag	Group-specific antigen
gp	Glycoprotein
H_2O	Water
HIVDR	HIV drug resistance
IDU	Injecting drug user
kDa	Kilodalton
3TC	Lamivudine
$MgCl_2$	Magnesium Chloride
Min	Minute
M_w	Molecular weight
M	Molar
mM	Millimolar
µL	Microlitre
mL	Milliliter
mg	Milligram
µg	Microgram
ng	Nanogram
NCBI	Center for Biotechnology Information
NFW	Nuclease free water
NRTI	Nucleoside reverse-transcriptase inhibitors
NNRTI	Non-nucleoside reverse-transcriptase inhibitors
NVP	Nevirapine
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PLHIV	People living with HIV
PCR	Polymerase chain reaction
pol	Polymerase
pM	picomolar
p	protein
rev	Regulator of Expression of Virion Proteins

rpm	Revolution per minute
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
U	Unit
UV	Ultraviolet
Tat	Trans-Activator of Transcription
TDF	Tenofovir
TBE	Tris borate EDTA
TDR	Transmitted drug resistance
THIVDR	Transmitted HIV drug resistance
WHO	World Health Organisation
UNGASS	United Nations General Assembly Special Session
vpu	Viral Protein Unique
vpr	Viral Protein R
vif	Viral infectivity factor
nef	Negative Regulatory Factor

ABSTRAK

Walaupun penggunaan antiretroviral (ARV) telah dikaitkan dengan penurunan yang ketara dalam morbiditi dan kematian yang berkaitan dengan jangkitan HIV, keberkesanannya terhad kerana kemunculan virus yang rintang terhadap dadah ini. Tujuan kajian ini dijalankan adalah untuk mengenal pasti kehadiran HIV virus dari Kelantan yang rintang terhadap dadah ARV secara transmisi (THIVDR) sebelum menjalani terapi ARV. Kajian ini juga bertujuan untuk menjalankan analisa filogenetik terhadap virus HIV ini berdasarkan gen 'reverse-transcriptase (RT)'. Fokus utama kami adalah ke atas THIVDR kerana virus ini menyebabkan rawatan ARV lebih rumit dan mahal di samping maklumat tentang kehadiran THIVDR ini di Malaysia adalah sangat terhad. Dalam kajian ini, gen RT virus HIV telah diamplifikasi dan dibaca jujukan nukleotida dan asid aminonya. Selepas itu, mutasi terhadap gen RT yang selalu dikaitkan dengan kerintangan dadah ARV telah dianalisa dengan menggunakan software Standford HIVdb: Rintangan genotip Tafsiran Algoritma dan LANL ADRA: Antiviral Drug Resistance Analysis. Daripada keputusan ini, mutasi K101Q dan K103N telah dikenal pasti masing-masing pada 3 dan 1 virus HIV dari Kelantan. Kesemua 4 virus ini disyaki rintang kepada dadah Efavirenz NNRTI. Di samping itu, analisa filogenetik untuk penentuan rangkaian dan subtip virus HIV Kelantan juga telah dijalankan. Analisa ini menunjukkan bahawa 9 daripada virus HIV ini telah dikelasifikasikan sebagai subtip rekombinan CRF01_AE dan hanya 1 virus dikenal pasti sebagai subtip CRF01_AE / B. Kajian ini juga mendapati virus HIV Kelantan mempunyai persamaan gen RT yang tinggi (97%-99%) dengan virus HIV Thailand kerana faktor perkongsian sempadan antara kedua-dua negara ini. Kesimpulan dari kajian ini, kehadiran THIVDR telah berjaya dikenal pasti di Kelantan, Malaysia. Kajian lanjut yang

melibatkan saiz sampel yang lebih besar perlu dijalankan untuk mendapatkan gambaran lebih jelas berkenaan dengan prevalen THIVDR di Kelantan.

ABSTRACT

Although the introduction of antiretroviral (ARV) treatment has been associated with marked decreases in HIV-related morbidity and mortality, its effectiveness is limited by the emergence of drug resistance. This study was conducted to determine the presence of transmitted HIV drug resistance (THIVDR) viruses and phylogenetic analysis of HIV-1 virus from naive ARV therapy patients from Kelantan, Malaysia based on RT gene. Our interest was on THIVDR as it makes ARV treatment more complicated and costly and there is limited information on transmitted HIVDR available in Malaysia. In this study, reverse transcriptase (RT) gene of the HIV viruses were amplified and sequenced. After that, specific mutations of RT gene associated with drug resistance were analyzed using Standford HIVdb: Genotypic Resistance Interpretation Algorithm and HIV LANL ADRA: Antiviral Drug Resistance Analysis. From the result, mutations K101Q and K103N were identified in 3 and 1 of the studied HIV viruses respectively. These viruses are expected to have resistance to Nucleoside reverse-transcriptase inhibitors (NNRTI) Efavirenz drugs. Subsequently, these sequences were phylogenetically analyzed to determine the source and subtype of the viruses. Phylogenetic result showed that out of 10 HIV-1 viruses used in this study, 9 were of the CRF01_AE subtype and only one was under CRF01_AE/B subtype. This study also found that Kelantan HIV-1 viruses had high similarity (97-99%) to the Thailand HIV-1 viruses because these countries share a border with each other. In conclusion, transmitted HIVDRs were successfully identified in Kelantan, Malaysia. However, further study involving larger samples to determine the actual prevalence of THIVDR in Kelantan need to be carried out.

CHAPTER 1 INTRODUCTION

1.1 Background of study

The HIV/AIDS epidemic in Malaysia has emerged as an important health problem since the first HIV case detected in 1986 (Reid *et al.*, 2005). According to Global AIDS Response Progress Report 2012, Malaysia had a cumulative figure of 94,841 HIV, 17,686 AIDS and 14,986 deaths, thus giving reported PLHIV of 79,8552 by the end of 2011 as demonstrated in Figure 1.1. It has been estimated that by end of 2015, Malaysia will have an estimated 81,317 people living with HIV.

Indicator	Number/ percentage
Cumulative no. of reported HIV infections since first detection in 1986	94,841
Cumulative no. of reported AIDS since 1986	17,686
Cumulative no. of reported deaths related to HIV/AIDS since 1986	14,986
Estimated no. PLHIV [EPP 2011]	81,000
Total number of PLHIV [surveillance data]	79,855
New HIV infections detected in 2011	3,479
Notification rate of HIV (per 100,000) in 2011	12.18
Women reported with HIV in 2011	735
Cumulative no. of women reported with HIV as of December 2011	9,494
Children aged below 13 with HIV in 2011	65
Cumulative no. of children under 13 with HIV as of December 2011	974
Estimated no. PLHIV eligible for treatment [EPP 2011]	37,306
No. PLHIV receiving ART (surveillance data) as of December 2011	14,002
Estimated adult (15 – 49 years) with HIV & prevalence [EPP 2011]	64, 562 [0.41%]

Figure 1.1: Overview of the HIV epidemic in Malaysia 2011 (Modified from: Ministry of Health)

The use of combination ARV drugs are successful in reducing morbidity and mortality among HIV patients (Manosuthi *et al.*, 2006; Kumarasamy *et al.*, 2005; Jongwutiwes *et al.*, 2007; Sungkanuparph *et al.*, 2008; Zhou *et al.*, 2007). ARV treatment in Malaysia has been available since 1989 (UNGASS Country Progress Report – Malaysia, 2008). In 2010, the revision of ARV treatment initiation threshold from $CD4^+$ level of 200 cells/mm^3 to 350 cells/mm^3 by WHO has been adopted (Global AIDS Response Progress Report 2012). The standard ARV regimen of choice currently practiced in Malaysia includes the combination of two nucleoside reverse-transcriptase inhibitors (NRTIs), typically either zidovudine (AZT) + lamivudine (3TC) or stavudine (d4T) + 3TC with a non-nucleoside reverse-transcriptase inhibitor (NNRTI), typically either nevirapine (NVP) or efavirenz (EFV). There is also a generic triple-drug combination which is low cost and widely available called SLN (stavudine + lamivudine + nevirapine). However, the 2009 WHO guidelines recommended the phasing out of stavudine (d4T) as first-line therapy because of its long term irreversible side effects which include severe peripheral neuropathy, disfiguring lipodystrophy and risk of fatal lactic acidosis. Instead, WHO recommended that AZT or tenofovir (TDF) to be used as they are less toxic and are equally effective alternatives (Koh, 2010).

Although the introduction of ARV treatment has been associated with marked decrease in HIV-related morbidity and mortality (Buchacz *et al.*, 2010; Ray *et al.*, 2010), the main problem in using ARV to suppress HIV is the development of drug resistance (Little *et al.*, 2002; Grant *et al.*, 2002; Conway *et al.*, 2001; Ross *et al.*, 2000; DeGruttola *et al.*, 2000). The first HIV drug resistance reported was to zidovudine (ZDV) in 1989 (Larder *et al.*, 1989). HIV drug resistance refers to the

ability of HIV to replicate in the presence of drugs that usually suppress its replication. HIV drug resistance is caused by mutations in the virus's genetic structure. Mutations are very common in HIV-1 because the virus replicates very fast and does not have the proteins needed to correct the mistakes it makes (Bertagnolio *et al.*, in press). According to World Health Organization (WHO), HIV drug resistance may be transmitted or acquired. Acquired drug resistance could develop if patients interrupt their treatment or do not follow the prescription whereas transmitted drug resistance occurs if people are infected by others who had HIV drug resistance.

Resistance can be interpreted using numerous methods and the most common being the International AIDS Society-USA mutation list (IAS-USA) (Johnson *et al.*, 2011), the Stanford University HIV Drug Resistance Database programme (HIVdb) (Standard University) and the WHO drug mutation list for transmitted resistance (Bennett *et al.*, 2009). The list and database has reported that common major mutations of HIVDR virus on reverse transcriptase (RT) gene occurred at amino acid 41, 184 for NRTI and 101, 103, 181 and 188 for NNRTI while in protease gene was found at amino acid 82 and 90. In this study, we focused only on NRTIs and NNRTIs drug resistance involving RT gene since ARV in Malaysia is using combination NRTIs (Zidovudine, Stavudine, Tenofovir and Lamivudine) and NNRTIs (Nevirapine, Efavirenz) (Koh, 2010).

1.2 Problems and rationale of the study

The presence of transmitted drug resistance mutations (TDRM) in patient unexposed to antiretroviral (ARV) treatment is the main issue in the management of HIV-1 infection. Several studies had demonstrated a high risk of virological failure to first therapy in patients who have resistance mutations conferring resistance to any of the drugs received (Wikttkop *et al.*, 2011; Bannister *et al.*, 2009; Oette *et al.*, 2006; Shet *et al.*, 2006). Transmitted drug resistance (TDR) makes ARV treatment more complicated such as limit therapeutic options for new patients and affect virological and immunological response to first line antiretroviral treatment and also potentially more costly (Wikttkop *et al.*, 2011; Bhaskaran *et al.*, 2004; Little *et al.*, 2008; Menzo *et al.*, 2003; Pillay *et al.*, 2006; Zaccarelli *et al.*, 2004; Zaccarelli *et al.*, 2005; Grant *et al.*, 2002). Other studies stated that transmitted HIVDR needs longer time to reach viral suppression after starting treatment (Little at al., 2002; Grant *et al.*, 2002; Harzic *et al.*, 2002) and shorter period of relapse of viremia after viral suppression (Little at al., 2002). Currently there is limited information available on the transmitted HIVDR in Kelantan, Malaysia. This study needed to conduct for a more efficient treatment and reduce transmission of HIV through optimal delivery of ARV. Therefore, the purpose of this study was to screen the presence of TDR mutation of HIV-1 virus from Kelantan and to perform phylogenetic analysis for the epidemiology transmission pattern of HIV-1 viruses in Kelantan.

1.3 Objectives of the study

The objectives of this study were,

General

To screen the presence of the transmitted HIVDR and perform phylogenetic analysis of HIV-1 viruses from Kelantan, Malaysia.

Specific

- To confirm the reverse transcriptase (RT) gene of the HIV viruses by sequencing.
- To analyze the specific mutations of RT gene associated with transmitted drug resistance virus by Standford HIVdb: Genotypic Resistance Interpretation Algorithm and HIV LANL ADRA: Antiviral Drug Resistance Analysis.
- To study molecular epidemiology of HIV-1 virus on transmitted drug resistance gene by phylogenetic analysis.

CHAPTER 2 LITERATURE REVIEW

2.1 HIV-1

HIV-1 is the abbreviation used for the Human Immunodeficiency Virus. It is an enveloped virus. It is spherical in shape and has a diameter of 1/10,000 of a millimeter. It contains RT and two identical copies of a positive sense, linear RNA genome (Figure 2.1). The outer surface of the viral particle is comprised of a bilayered lipid envelope that is derived from the host cell membrane. The transmembrane gp41 protein is non-covalently linked to the matrix protein on the inside and to the gp120 glycoprotein on the outside of the virus (Luciw, 1996; Veronese *et al.*, 1985; Poignard *et al.*, 2001). Matrix protein forms a shell underneath the lipid bilayer. Inside this shell are two genomic RNA molecules surrounded by a core of capsid proteins.

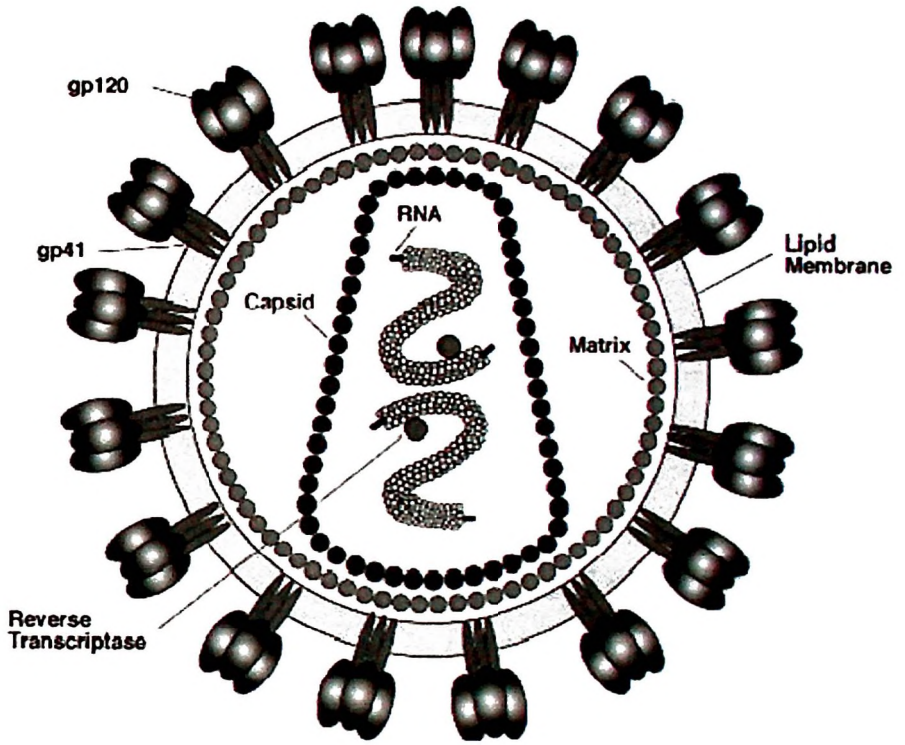

Figure 2.1: HIV-1 viral structure (Modified from: Stanford.edu).

2.1.1 HIV-1 genome

HIV-1 has a complex genome containing several major gene encoding major structure of protein. In between the *pol* and *env* regions, there is *vpu* that separates these two genes. There are 9 genes present that can be classified into 3 functional groups which are structural, regulatory and accessory genes. Structural genes comprise *gag, pol, and env.* The *gag* and *env* genes code for the nucleocapsid and the glycoproteins of the viral membrane; the *pol* gene codes for the reverse transcriptase and other enzymes. Regulatory genes involve *tat and rev* and accessory genes consist of *vpu, vpr, vif, and nef. Tat* and *rev* stimulate the transcription of proviral HIV-1 DNA into RNA, promote RNA elongation, enhance the transportation of HIV RNA from the nucleus to the cytoplasm and are essential for translation. *Vpr* is important for the transport of the viral pre-integration complex to the nucleus and may arrest cells in the G2 phase of the cell cycle. *Vpu* is helping in virus budding process and *vif* function as supporting viral replication. *Nef* has been proven to have a number of functions which include induce downregulation of CD4 and HLA class I molecules from the surface of HIV-1-infected cells and interfere with T cell activation by binding to various proteins that are involved in intracellular signal transduction pathways. In addition, HIV-1 also has a long terminal repeat gene (LTR) (Figure 2.2) represent the two end parts of the viral genome, that are connected to the cellular DNA of the host cell after integration and do not encode for viral proteins (Levey, 1993; Strebel *et al.*, 1998; Hoffmann and Rockstroh, 2011).

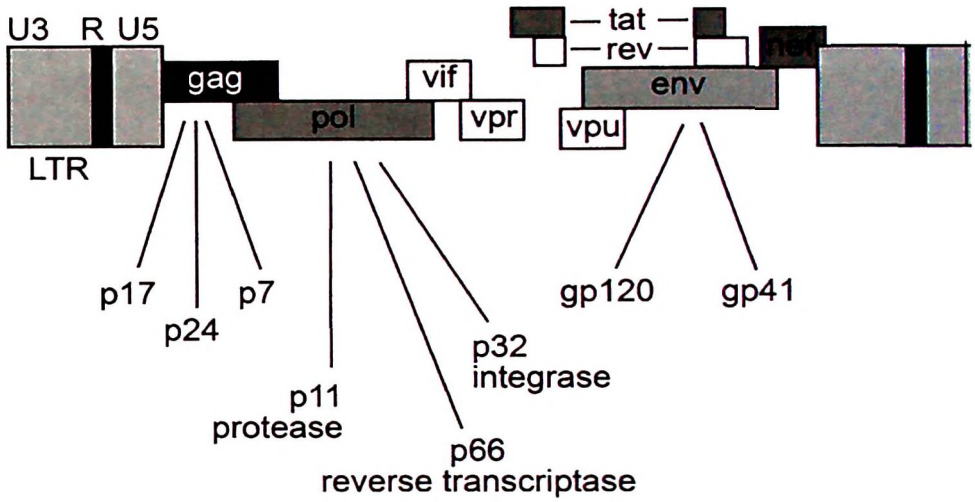

Figure 2.2: HIV-1 genome organization (Modified from: Hoffmann and Rockstroh, 2011).

2.1.2 HIV-1 replication cycle

A general overview of the replication cycle in a single cell is presented in Figure 2.3. The process begins when a virus particle bumps into a cell that carries on its surface a special protein called $CD4^+$. The spikes on the surface of the virus particle stick to the $CD4^+$ and allow the viral envelope to fuse with the cell membrane. After direct fusion of the virion and cellular lipid membranes, the viral core is released into the cytoplasm where it uncoats and releases the RNA genome. The viral genome is then reverse transcribed and transported to the nucleus where it integrates into host DNA by using integrase enzyme. The early gene products, *tat, rev, and nef* are first transcribed, followed by the rest of the HIV genome. Assembly and budding of progeny virions takes place at the plasma membrane. The newly matured HIV particles are ready to infect another cell and begin the replication process all over again (Costin, 2007).

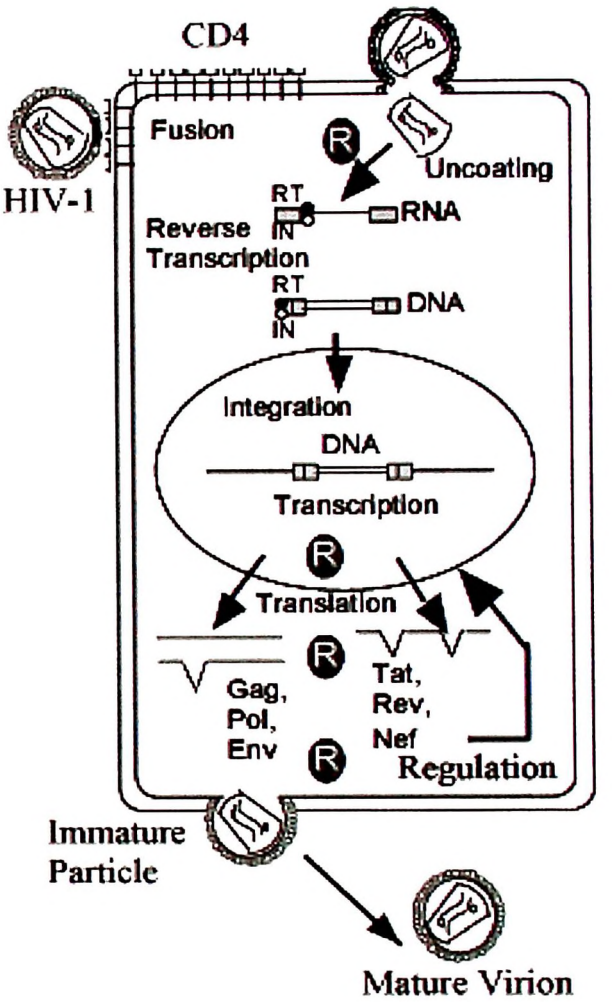

Figure 2.3: Overview of the replication cycle of HIV-1 (Adapted from: Macpherson *et al.*, 1999)

2.1.3 HIV-1 pathogenesis

Infection with HIV is categorized into three clinical stages which are an acute viremia, latency phase of variable duration, and clinical AIDS as shown in Figure 2.4. During the initial infection, virus can be detected in the blood of patients (Daar *et al.*, 1991; Zhu *et al.*, 1993). It lasts a few weeks and the patient has short flu-like illness. After the initial viremia peaks, the level of virus in the blood falls off and a phase of "latency" occurs. During the latency phase, HIV load is generally very low to non-detectable, though there is a high turnover of CD4+ T cells and HIV virion production (Ho *et al*, 1995; Coffin, 1995; Perelson *et al*, 1996, Piatak *et al.*, 1993, Wei *et al.*, 1995). Before the introduction of highly active antiretroviral therapy (HAART), it was stated that the lower the level of virus in the blood at this stage, the better the prognosis and shorter time course of progression to AIDS (Arnaout *et al.*, 1999; Fahey, 1998; Piatak, 1993). During the latency phase, $CD4^+$ T cell counts also begin to decline and an inversion of the $CD4^+$ /$CD8^+$ T cell ratio occurs. A $CD4^+$ T cell count <200 cells/mm^3 and infection with at least one opportunistic infection, such as Pneumocystis Carinii defines clinical AIDS. This last stage involves the impairment of patients' immune system and patients eventually have secondary infections.

2.1.4 HIV-1 transmission

HIV-1 can be transmitted through unprotected sexual intercourse, vertical transmission (mother to child transmission during pregnancy, birth delivery or breast feeding and exchange of infected blood (such as sharing infected needles among drug addicts, blood transfusion and organ transplant) (Malaysian AIDS Council 2008). Newly infected persons with transmitted drug resistance (TDR) also act as a source for the onward transmission of resistant variants (Clare *et al.*, 2007).

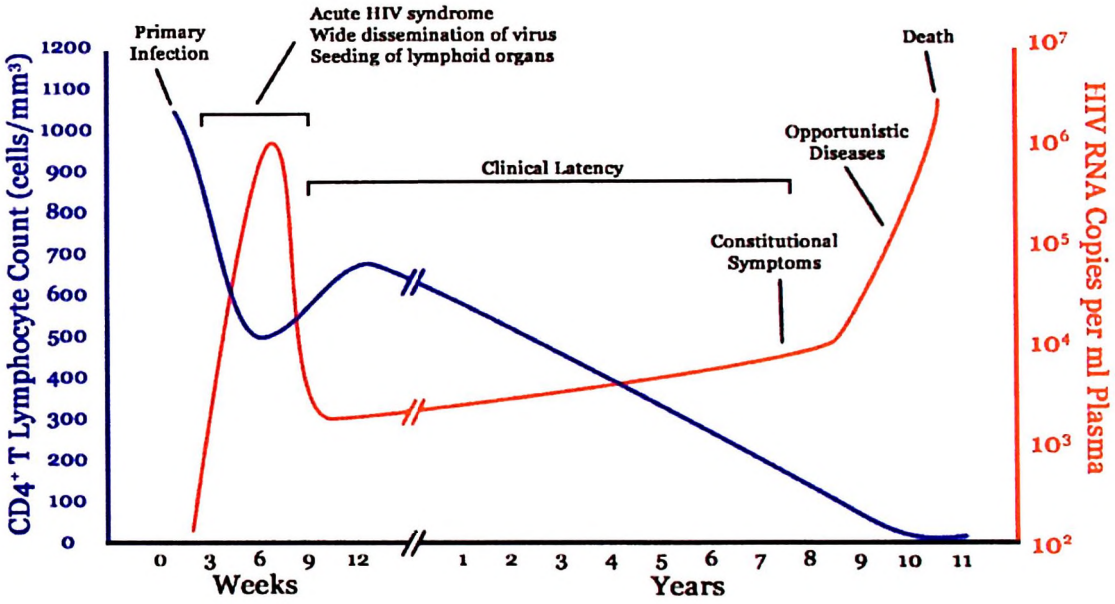

Figure 2.4: Time course of HIV infection showing correlation of viral load, $CD4^+$ T cell and $CD8^+$ T cell counts (Modified from: The Pathogenesis of AIDS, 2009).

2.2 Incidence and distribution of HIV-1 infection

2.2.1 HIV-1 case in worldwide

The WHO estimates that around 2.7 million people become infected with HIV every year and about 2 million die of AIDS every year. In 2010, it was estimated that 34 million people are living with HIV worldwide (UNAIDS, 2011). HIV is spreading most rapidly in Eastern Europe and Central Asia, where the number of people living with HIV increased by 150% between 2001 and 2007 (World Health Statistics, 2008).

2.2.2 HIV-1 case in Malaysia

In the earlier phase of the pandemic, injection drug user (IDU) was the driven factor for HIV in Malaysia. However, in 2011 sexual transmission had superseded IDU as the main driven factor for the epidemic (UNGASS Country Progress Report – Malaysia, 2008). About 26% of the reported cases were among young people aged between 13-29 years old and around 1% were less than 13 years old (Figure 2.5). Overall, the country has a decreasing proportion of new reported HIV among young people aged 13-29 years and increasing trend among older adult of 30-39 years (UNGASS Country Progress Report – Malaysia, 2008).

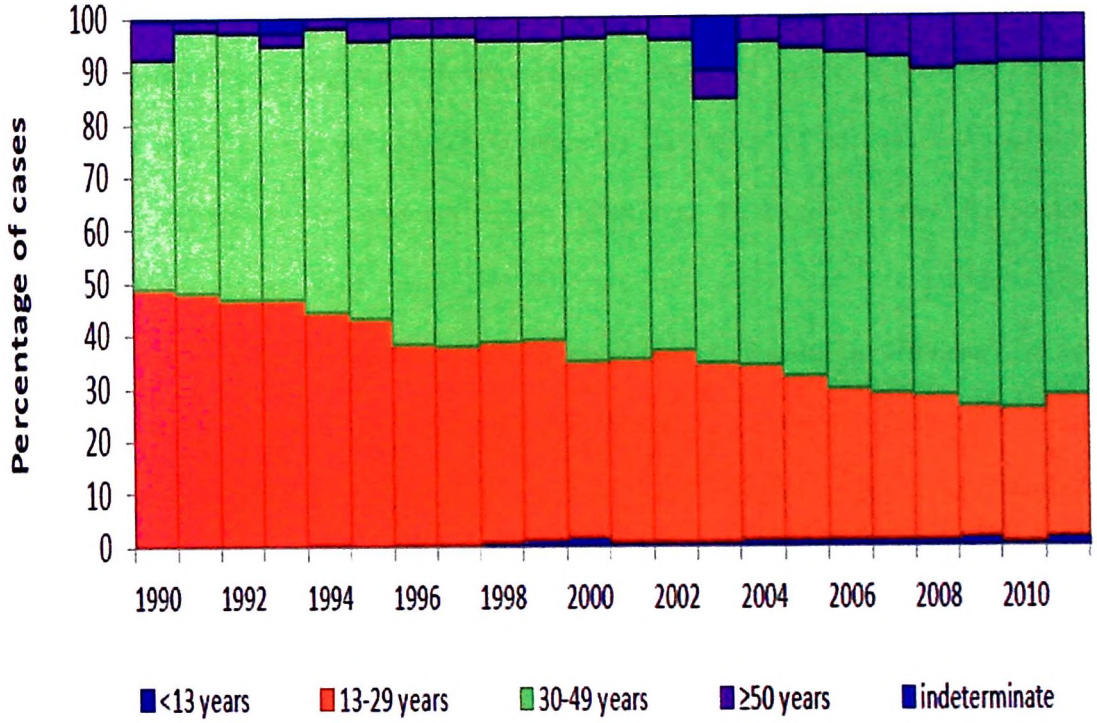

Figure 2.5: Distribution of reported new HIV cases by age group, 1990–2011 (UNGASS Country Progress Report – Malaysia, 2008).

In Malaysia, most of the cumulative HIV cases were reported in men but starting from 2003, infection among male shows a significant decrease, which is in contradictory to the female (Figure 2.6). Among men, 48% acquired infection via injecting drug use and 47% through sexual mode. Most HIV infections among women occurred mainly through heterosexual transmission (87%) (UNGASS Country Progress Report – Malaysia, 2008).

Regarding to geographical distribution, there is quite a notable trend in transmission mode over a period of 10 years in the states of Malaysia. It seems that some states especially in eastern region (Pahang, Terengganu and Kelantan) of Peninsular Malaysia are still caused by IDU whereas some states in northern (Perlis, Kedah, Penang and Perak) and southern region (Negeri Sembilan, Melaka and Johor) more to sexually driven. The central region (Selangor and Kuala Lumpur) had shift from IDU driven to sexually driven whereas east Malaysia (Sarawak, Sabah and Labuan) have been mainly sexually driven since the beginning of the epidemic (UNGASS Country Progress Report – Malaysia, 2008).

2.2.3 HIV-1 case in Kelantan

The state of Kelantan is situated on the northeastern part of peninsular Malaysia and shares a border with Thailand on the north. Kelantan has among the highest number of newly reported HIV cases in Malaysia in 2010 (Mohamad *et al.*, 2012). In addition, according to World Health Statistics in 2008, the state of Kelantan, with a cumulative total of 970 reported female HIV cases, has the highest number of women found to be infected since 1986, compared to all other states in Malaysia.

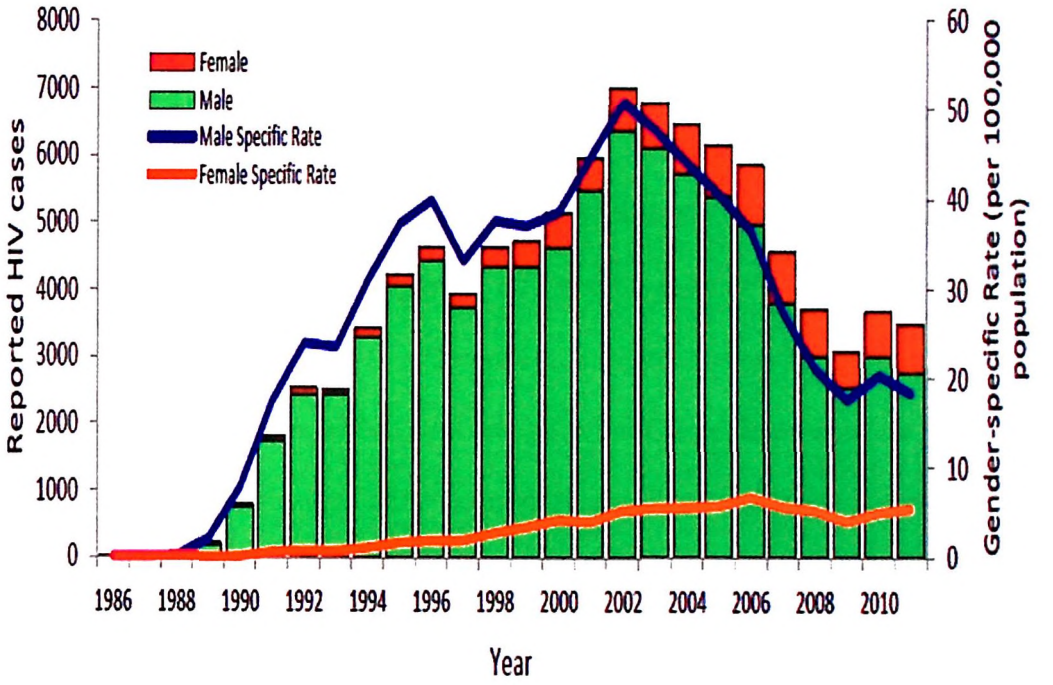

Figure 2.6: Gender-specific HIV Prevalence, Malaysia 1986–2011 (UNGASS Country Progress Report – Malaysia, 2008).

2.3 Reverse transcriptase

The most important enzyme in HIV-1 is RT which is encoded in *pol* gene. RT converts the RNA to DNA in the cytoplasm and can be integrated into the host DNA for virus latency (Jonckheer *et al.*, 2000). RT was the first target in drug therapy and is important in HIV treatment (Jonge *et al.*, 2005). The two main types of drugs used to inhibit RT are nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

RT consists of two subunits; p66 and p51. Subunit p66 is larger than p51 and resembles the human right hand (Figure 2.7). The p66 subunit is split into sub domains including the thumb, palm, fingers and connection subdomains. In the palm of the p66 subdomain is the polymerase active site, and near the finger domain is the RNaseH sub domain. The two main activities of RT are polymerization and ribonuclease H activity. The p51 subunit is the smaller of the two subunits and contains the thumb, palm and connection subdomains. The p51 subunit is a product of the same gene as the p66 subunit, however, the RNase H domain is absent in the p51 subunit as a result of proteolytic cleavage (Kohlstaedt *et al.*, 1992).

Unlike the DNA polymerase, RT is lacked of proof reading activity (Sebastian and Franki, 2004). This leads to 10-100 fold increase in errors. This errors process generates a new variant virus that can invade the immune system.

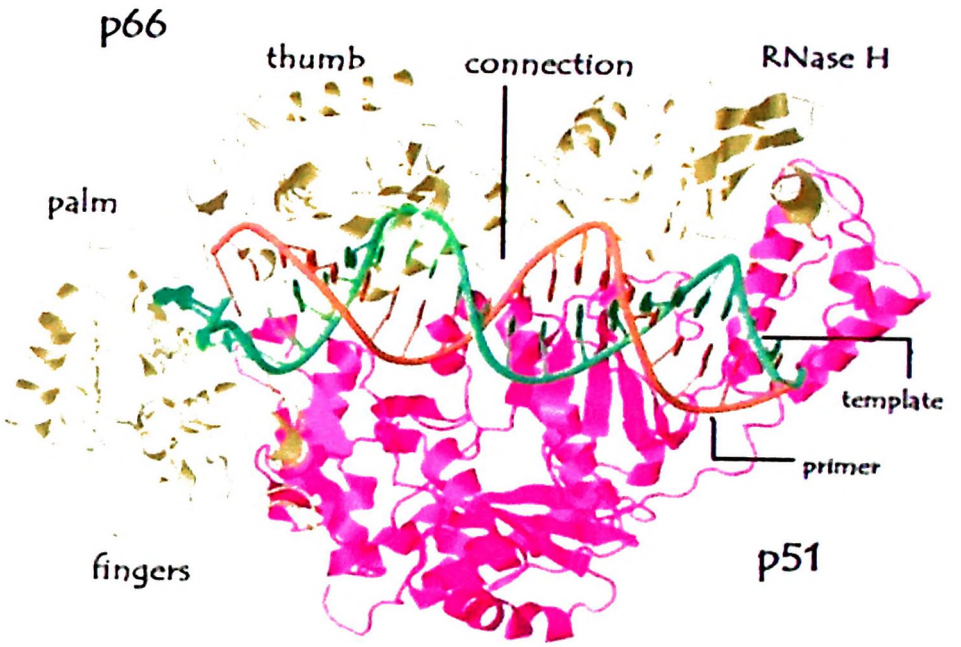

Figure 2.7: RT Structure. The p66 subunit is shown in yellow and p51 subunit in purple (Adapted from: Warren K *et al.*, 2009).

2.4 Reverse transcriptase inhibitors

2.4.1. NRTIs

The NRTIs is the first class of antiretroviral drugs developed. This class contains 7 drugs that have been Food and Drug Administration (FDA) approved (lamivudine, abacavir, zidovudine, stavudine, didanosine, emtricitabine, tenofovir, and zalcitabine) (www.fda.gov). NRTIs are nucleoside analogues that act as competitive inhibitors of HIV-1 RT. They are activated through phosphorylation by cellular enzymes, recognized by RT and thus bind into viral DNA. The addition of cellular nucleosides is then prevented and causing chain termination and thereby suppressing replication of the virus (Hoffman and Landovitz, 2004).

2.4.2. NNRTIs

In the NNRTI drug class, 5 drugs have been FDA approved (delavirdine, efavirenz, etravirine, nevirapine, and rilpivirine) (www.fda.gov). These compounds bind to the RT and inhibit it noncompetitively. This binding causes RT unable to interact properly with the viral RNA to produce viral DNA (Stolk and Luers, 2004).

2.5 HIV drug resistance (HIVDR)

2.5.1 Factors of HIV drug resistance (HIVDR) development

One of the reason for resistance development is HIV has a high genetic diversity. Second, HIV has a very high rate of nucleotide sequence evolution which is due to the high error rate of the viral RT. This contributes to the generation of many variants of HIV in a single infected patient (Robertson *et al.*, 1995). Besides, factors such as unsuitable choice of antiretroviral agents and lack of patient compliance could cause development of HIV drug resistance. In the term of pharmacokinetic, poor oral

absorption, alteration of drug metabolizing enzyme by other agent and various drug and drug interactions affect the levels of antiretroviral agent. Drug resistance mostly develops due to exposure to antiretroviral drug while some variants may exhibit intrinsic resistance to these agents (Preston *et al.*, 1988; Perelson *et al.*, 1996; Marinez-Picardo *et al.*, 2000; Martin, 2006).

2.5.2 Mechanism of HIV drug resistance (HIVDR)

Mechanism 1: Resistance to NRTIs

Resistance to NRTIs occurs through 2 mechanisms: the first is mutation (e.g., M184V) of the drug binding site of the RT gene that results in reduced incorporation of the NRTIs into the growing DNA chain (Figure 2.8A). This is the main mechanism of resistance to most of the NRTIs. The second mechanism of NRTIs resistance is through the increase removal of drug from its site of attachment at the end of the DNA chain. These RT mutations allow ATP or pyrophosphate to bind and attack the bond that binds the drug to DNA. Thus, the drug is released and the effect of drug is stopped (Martin, 2006).

Mechanism 2: Resistance to NNRTIs

Resistance to NNRTIs occurs mainly through mutation of hydrophobic RT residues within the binding pocket for the NNRTIs. Mutations in this area will affect binding of all of the NNRTIs as they bind to the same region of RT (Figure 2.8B) (Martin, 2006). Various mutations such as L100I, Y181C that can confer NNRTI resistance involve amino acids that form the hydrophobic binding pocket. The K103N mutation, which causes high level resistance to all the available NNRTIs, has a different mechanism. Position 103 is not part of the hydrophobic NNRTI binding pocket.

However, it is near to the entrance of the pocket. K103 creates a hydrogen bond in unliganded reverse transcriptase and this hydrogen bond aids by closing the entrance of the pocket, making it harder for NNRTI to enter into the pocket (Clavel, 2004).

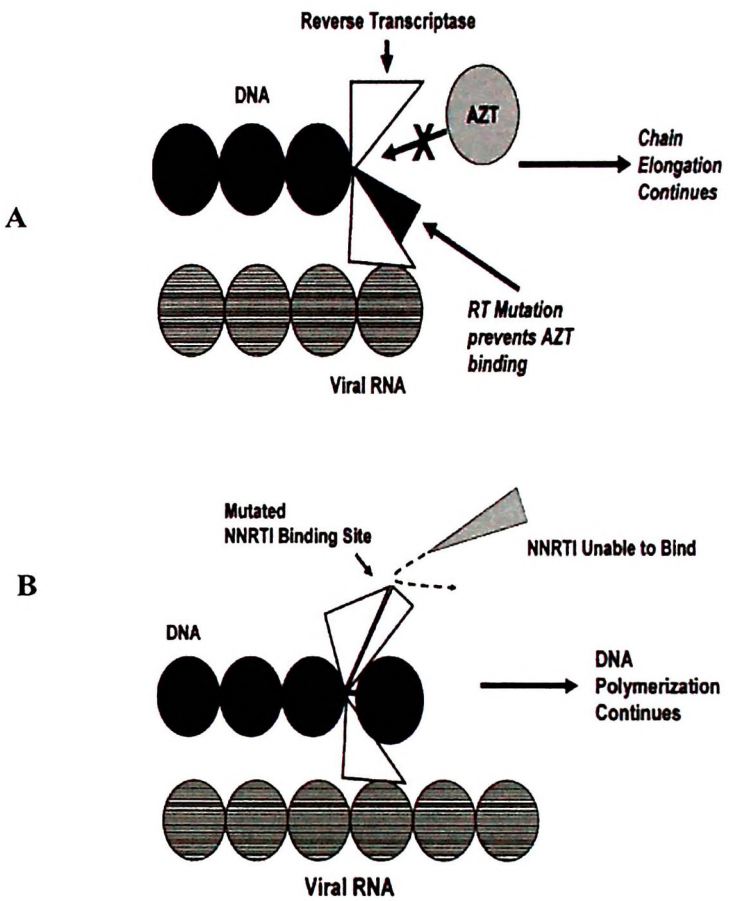

Figure 2.8 (A) RT mutation prevents AZT binding. (B) RT mutation prevents NNRTI binding (Martin, 2006).

2.6 Evolution pathways of transmitted drug resistance

Viruses with resistance mutations can be transmitted to other peoples. There are 3 possible evolutionary pathways for transmitted drug-resistant variants. First, when there is an intense effect on the replication rate of the virus, the resistant variant may return back to wild-type. Second, atypical variants (a novel amino acid that is neither the wild-type amino acid nor an intermediate towards wild-type) may be noticed when it results in higher replication rate than the original transmitted resistant variant. Finally, the resistant variant can persist. After transmission to a new host, evolution may be expected to occur but if all possible nucleotide changes would initially decrease the replicative capacity, reversion to wild-type will be blocked (Pingen *et al.*, 2011).

2.7 Drug resistance mutation panel

Various lists of drug-resistance mutations are used in different interpretation algorithms such as those from the International AIDS Society-USA (IAS-USA) (Johnson, 2008), The French National Agency for AIDS Research (ANRS) (www.hivfrenchresistance.org.), The Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) and The Rega Institute (www.rega.kuleuven.be/cev/). These are lists of amino acid mutations that are related to drug resistance, derived mostly from research in subtype-B HIV-1-infected individuals. These lists include mutations that were identified by *in vitro* experiments, drug susceptibility testing, clinical experience in patients failing therapy and genotypic studies.