CHAPTER II

LITERATURE REVIEW

2.1. Discovery of Human Immunodefiency Virus

In 1981, the Centre for Disease Control and Prevention (CDC) published a report on 5 cases of confirmed *Pneumocystis carnii pneumonia* (PCP), cytomegalovirus (CMV), and Candida mucosal infections in young homosexuals in Los Angeles, California [62]. The occurrence of PCP is very rare and closely related to severely immunosuppressed condition. Gradually, the reported cases having the common immunodeficiency syndrome shown by cellular immune dysfunction with the decreased CD4+ T cells were observed in not only homosexual men but also haemophiliacs [63]. Then in 1982, the new disease was named Acquired Immune Deficiency Syndrome (AIDS) [64].

In 1983, Barre-Sinoussi and Montagnier *et al* at the Pasteur Institute in France isolated a virus from the lymph node of a man with persistent lymphadenopathy syndrome and named lymphadenopathy-associated virus (LAV) [1]. Later, in early 1984, Gallo's group also isolated a virus from the PBMC of AIDS patients and called human T cell leukemia virus type III (HTLV-III) [2]. The virus was also identified by another group and named AIDS-associated retroviruses [65]. In 1989 the International Committee on the Taxonomy of Viruses gave the AIDS virus a common name, the Human Immunodeficiency Virus (HIV) [66].

2.2. Natural progression of HIV infection

In the absence of HAART, the clinical progression of HIV infection undergoes 3 distinct phases: acute seroconversion, asymptomatic infection, and AIDS (Figure 1) [67].

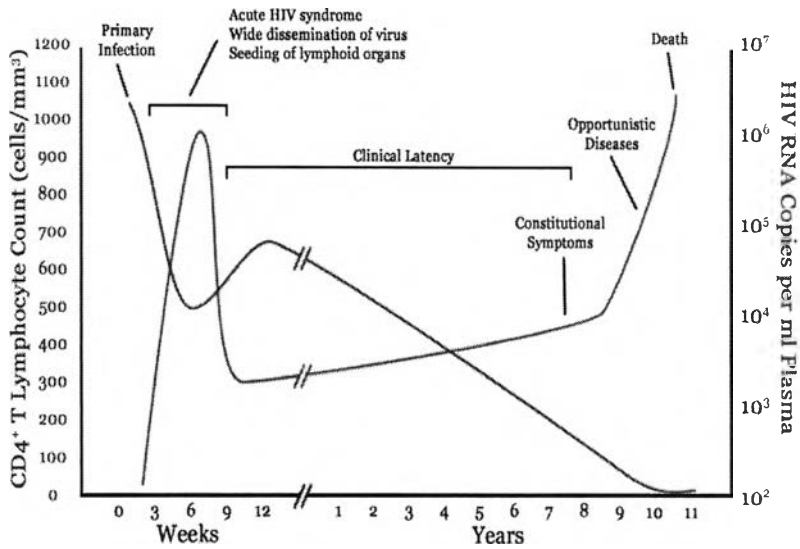

Figure 1. Natural progression of HIV infection.

Source: *http://en.wikipedia.org/wiki/HIV*

2.2.1. Acute seroconversion

This phase is identified as the time of initial HIV infection to the development of an antibody response. This can be within days to weeks. During this time, patient may not show any symptom or has a flu-like syndrome including fever and sore throat, lymphadenopathy, skin rash, and nausea. Normally, viremia is elevated to a high level while the number of CD4+ T cells declines. Lately, when anti-HIV antibodies and CD8+ T cell responses are generated, HIV viral load decreases to a steady state and CD4+ T cell count increases to a level which is slightly lower than before infection.

2.2.2. Asymtomatic infection

In this phase, the balance between viral replication and host immune response is established. Usually, patient exhibits few or no clinical signs and symptoms for years. However, during this time, CD4+ T cells are still being gradually destroyed and persistently decreased.

2.2.3. AIDS

In this stage, CD4+T cells are seriously killed and the number declines below the critical level of 200 cells/mm^3, the equilibrium is broken leading to damage of host immune system shown by various opportunistic infections and cancers.

2.3. HIV genotype classification and global distribution of HIV epidemic

There are two major types of related human retroviruses causing AIDS: HIV-1 and HIV-2. HIV-1 is the cause of most HIV infections worldwide whereas HIV-2 is prevalent in West Africa and some parts of India [68]. Based on the phylogenetic analysis of the full length of HIV sequences, four distinct groups of HIV-1 have been identified (M for Main, O for Outliner, N for non M, non O, and group P for "putative or pending the identification of further human cases") (Figure 2).

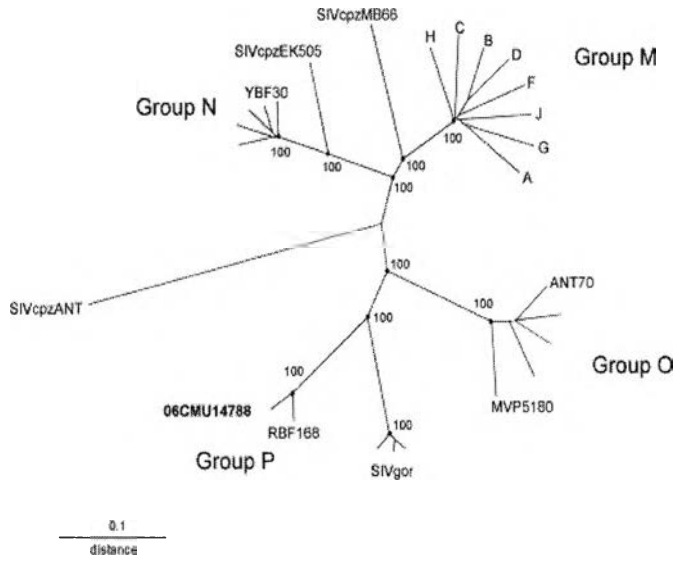

Figure 2. Phylogenetic tree of HIV-1 groups and subtypes.

Source: Vallari A, Holzmayer V, Harris B, Yamaguchi J, Ngansop C, Makamche F, Mbanya D, Kaptue L, Ndembi N, Gurtler L, Devare S, Brennan CA. 2011. Confirmation of putative HIV-1 group P in Cameroon. J Virol 85(3):1403-1407.

Group M accounts for the majority of the HIV-1 strains globally; group O is only found in sporadic infections in central Africa; group N is identified in some cases in Cameroon; and group P is newly discovered in 2 cases from Cameroon [69, 70]. Within group M, nine genetic subtypes have been classified (from A to D, from F to H, J, and K [71]. Some subtypes have been recognized for group O but limited isolates has been found for group N [72]. Among nine discrete genetic subtypes, some sub-clusters were distinguished within subtypes, creating sub-subtype such as sub-subtype A1, A2 for subtype A and F1, F2 for subtype F (Figure 3) [71].

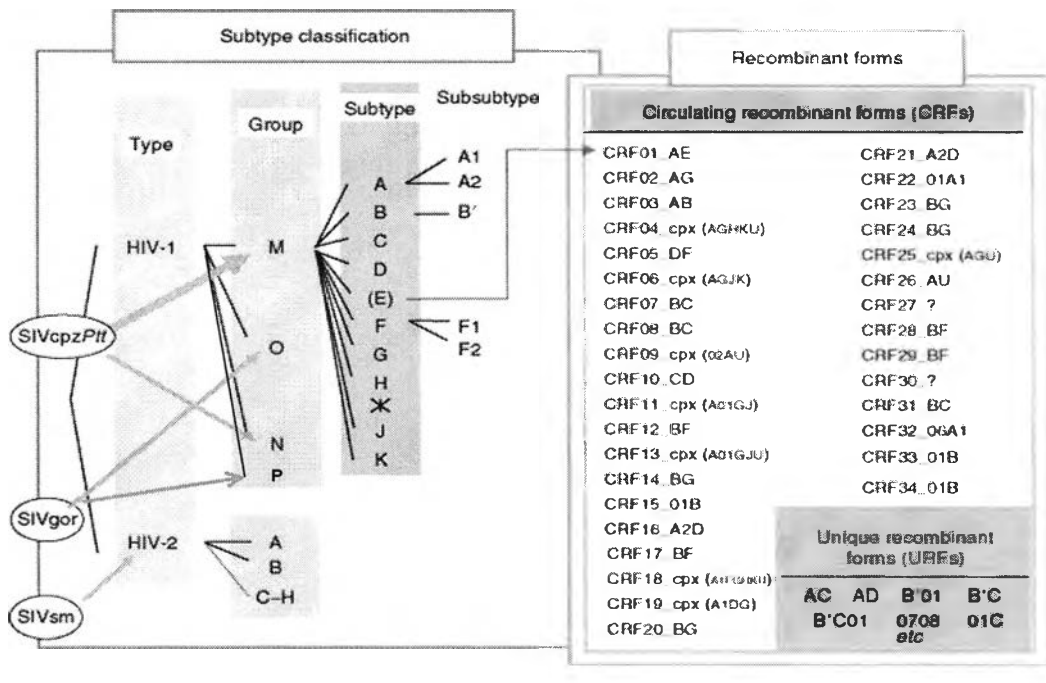

Figure 3. Classification of HIV genotypes and their origins.

Source: Adapted from Takebe Y, Uenishi R, Li X. 2008. Global molecular epidemiology of HIV: understanding the genesis of AIDS pandemic. Adv Pharmacol 56:1-25.

Certain HIV-1 strains with genome which is a recombination of different subtypes were also found. This mosaic genome, if identified in at least three epidemiologically unlinked persons by analysis of full-length sequencing is identified as circulating recombinant forms (CRFs). CRFs are designated by sequential numbers corresponding to the order of their

discovery followed by the letters displaying involved subtypes (U for unknown parental strain), or cpx (complex) for the recombination of more than 3 parental subtypes. Currently, 34 CRFs have been identified (Figure 3) [72]. Besides CRF, unique recombinant forms (URFs) have been also found in the region with different subtypes circulating. URFs is a diverse recombination of different subtypes in an unique mosaic genome found in only an individual or in few epidemically connected people (Figure 3) [72].

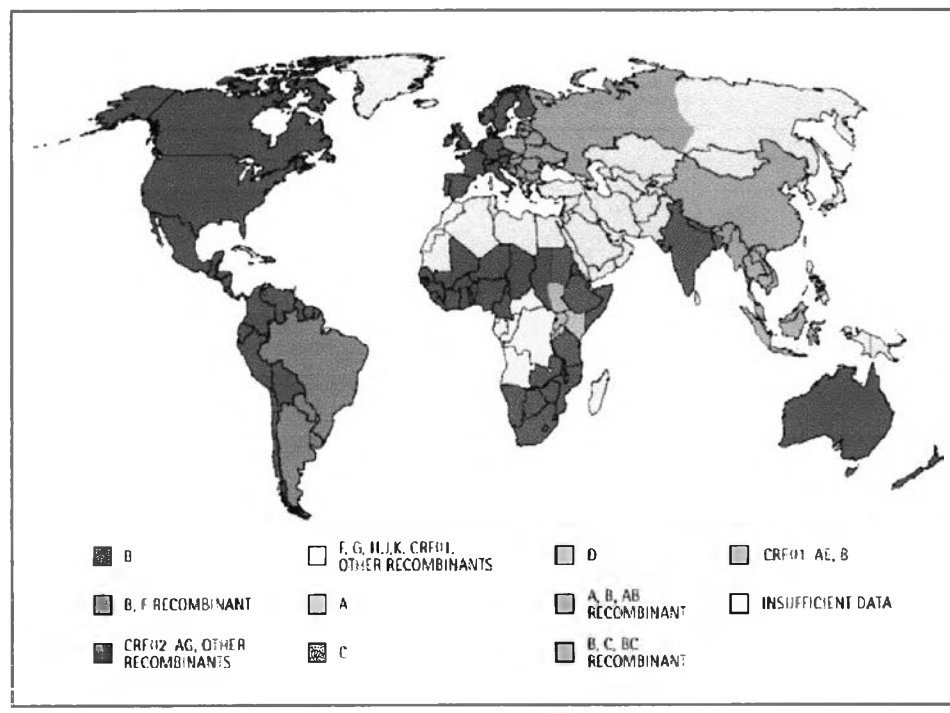

Figure 4. Global distribution of HIV-1 subtypes.

Source: http://www.pbs.org/wgbh/pages/frontline/aids/atlas/clade.html

Globally, some HIV-1 subtypes represent the major prevalence of all HIV-1 infection (subtypes C (56%), A (23%), B (8%)) [73] (Figure 4). Subtype C predominates in southern Africa, India, and Ethiopia. Infection of subtype A is prevalent in countries of central and east Africa such as Kenya, Uganda, Tanzania, and Rwanda, and in Eastern Europe including former Soviet Union mostly in injecting drug users. In west and in parts of central Africa, the

most frequently circulating subtype is the recombination clade CRF02_AG. Subtype B is the prevalent isolate in the Americas, western and central Europe, Australia and several countries in Asia (Japan, Korea, Taiwan), Middle East and among South African and Russian homosexuals. The CRF01_AE, a central African ancestry, is highly predominant in Southeast Asia. The epidemic began in the late 1980s among female sex workers in Thailand and their clients, gradually transmitted to injecting drug users and propagated to neighboring countries including Cambodia, Vietnam, China, Taiwan, Korea [72, 74].

2.4. HIV structure

HIV is a member of the group of *Lentivirures* in the family of *Retroviridae.* HIV particles are spherically shaped with a diameter of 100 nm surrounded by a lipid membrane as envelope which is spiked with 72 glycoprotein complexes. Each spike is a trimer of a highly glycosylated protein including a surface unit (SU) gp120 connected with a transmembrane protein (TM) gp41 by a loose non-covalent link (Figure 5). Thus, gp120 could be easily free from the viral particle and found in serum and lymphatic tissue. Gp41 plays a major role in the fusion step between virus and cell membrane whereas gp120 carries determinant molecules used to bind the CD4 receptor and CCR5/CXCR4 coreceptors on the surface of target cells. During the budding step of the viral cycle, the virus incorporates molecules of host cell membrane into its envelope such as human leukocyte antigen (HLA), intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function-associated antigen 1 (LFA-1) [67].

The inner portion of the envelope is a scaffold of matrix protein (MA) p17 for viral structure. Inside MA is a cone-shaped capsid protein (CA) p24 which contains the nucleocapsid (NC) p7 and two single-stranded RNA copies with the reverse transcriptase (RT) molecules (p66). It also contains other enzymes: protease (PR) p11 and IN p32 and some accessory and regulatory viral proteins (vpr, nef, vif) [67].

The HIV genome is two identical copies of a 9.2 kb single stranded RNA flanked by 2 long terminal repeats (LTR) which contain transcription promoter and terminator, a polyadenylation signal, and regulatory elements. These two LTRs, in the proviral phase of the viral life cycle, connect to the cellular DNA of the host cell after integration by the viral IN [75].

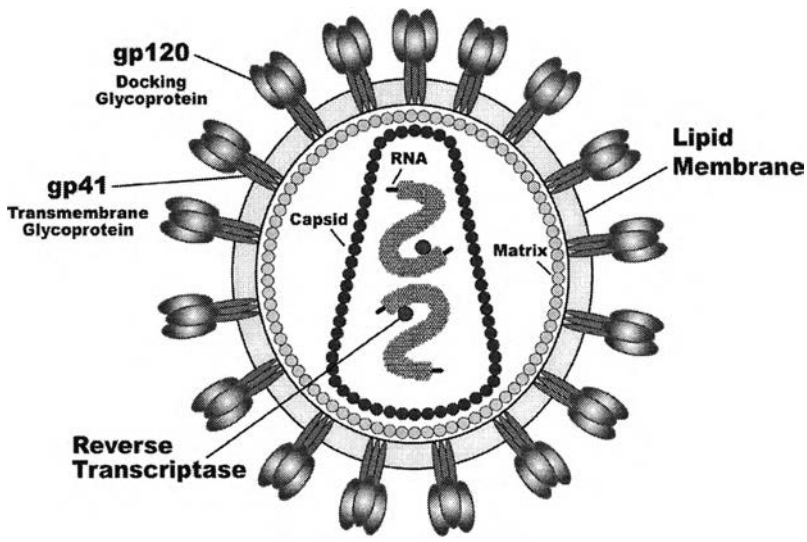

Figure 5. Structure of an HIV virion.

Source: *http://commons.wikimedia.org/wiki/File:HIV_Virion-en-2.png*

HIV genes include 3 types: structure genes (gag, pol, env), regulatory genes (tat, rev) and accessory genes (vif, vpr, vpu, nef) (Figure 6). Gag and pol genes are transcribed as a unit and translated into two polyproteins: gag precursor (p55) and gag-pol precursor (p160). Gag precursor is eventually processed by the viral protease into matrix proteins MA (p17), capsid proteins CA (p24), nucleocapsid proteins NC (p7), and p6; gag-pol precursor is also cleaved by viral protease into MA; p17, CA; p24, viral enzymes: protease (PR, p10), reverse transcriptase and RNaseH (RT, p66/51) and integrase (IN, p32). Env gene encodes for the precursor of viral envelope, gp160 which is processed by cellular protease into surface glycoprotein (SU, gp120) and transmembrane glycoprotein (TM, gp41). Viral regulatory and

accessory genes are not processed. Tat gene encodes for trans-activating protein which regulates transcription of integrated DNA of HIV within the host nucleus. Rev gene codes for regulator of viral protein expression that allows the nuclear export of RNA transcripts. Nef gene codes for negative regulator factor needed to maintain the high virulence of HIV by helping virus to escape the attack of the host immune system. Vif gene encodes for the viral infectivity protein that plays a role in stabilizing newly formed viral particles. Vpu encodes a protein that is important for the budding phase of viral life cycle. Vpr encodes a protein which assists the transport of the viral pre-integration complex into host nucleus (Figure 6) [67, 75, 76].

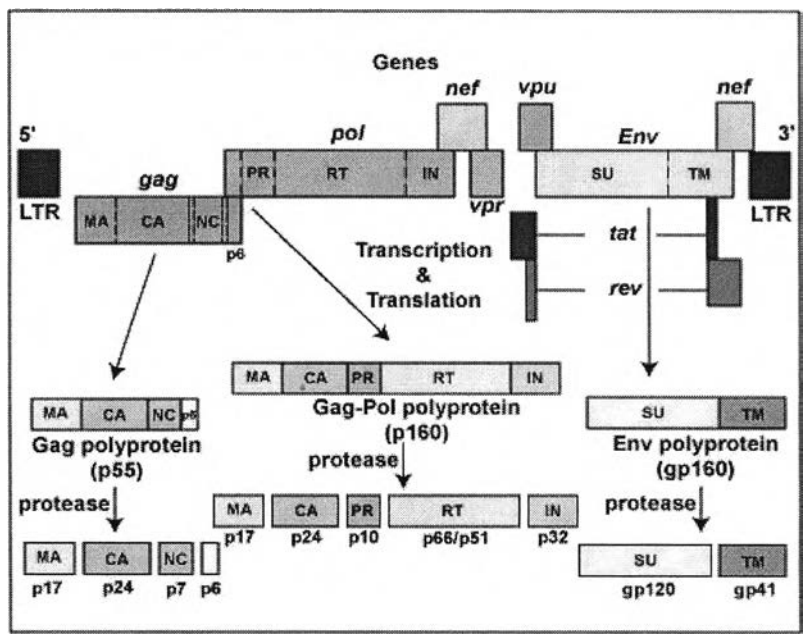

Figure 6. HIV genome.

Source: http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit3/viruses/images/hivgenes.jpg

2.5. HIV replication cycle

The HIV replication includes several steps (Figure 7).

2.5.1. HIV entry

The infection of a target cell by HIV is initiated by the entry of virions processed by the binding of SU gp120 to the CD4 receptor on the cell surface of T-lymphocytes, macrophages and dendritic cells. This process is followed by sequential binding to either the CCR5 or CXCR4 chemokine co-receptor located on the cell surface. These bindings induce the conformational changes in the TM subunit (gp41), facilitating the fusion of viral and cellular membranes leading to the injection of virion core into the cytoplasm of the target cell [67, 76].

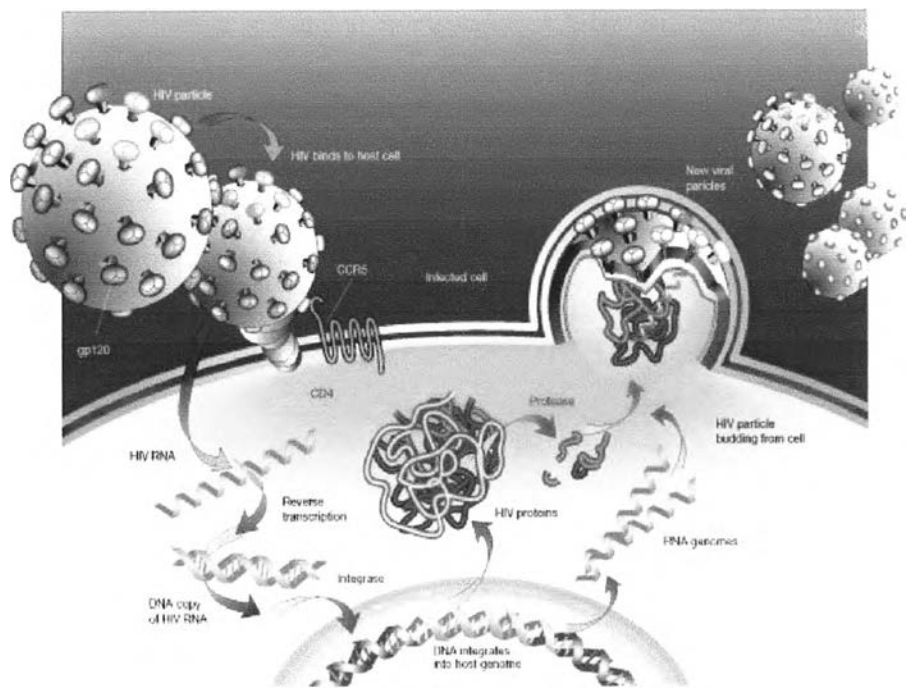

Figure 7. HIV replication cycle.
Source: Weiss RA. 2001. Gulliver's travels in HIVland. Nature 410(6831):963-967.

2.5.2. Reverse transcription

In the cytoplasm, the virus core is uncoated to form a nucleoprotein reverse transcription complex (RTC) containing the MA and CA structural proteins, accessory vpr

protein, viral IN, and double-stranded RNA bound to the viral RT. The viral RT processes reverse transcription resulting in the synthesis of double stranded DNA with LTR at each end inside the RTC. This leads to the formation of pre-integration complex (PIC) which also consists of several cytoplasmic proteins such as the Barrier-to-Autointegration factor (BAF) and the lens-epitheliumderived growth factor (LEGDF/p75).

2.5.3. Integration

The PIC is carried to the cellular nucleus via an active transport signaled by viral proteins: MA, vpr, and IN. After entering to the nucleus, PIC gains access to the chromatin and the newly generated viral DNA is inserted into host genome by the viral IN.

2.5.4. Transcription and protein synthesis

When integrated into the cell genome, the provirus may remain in inactive or latent form or express its genes in different levels. Because the cell cannot differentiate proviral sequences from host sequences, the virus uses cellular machinery for transcription. The viral transcription is mediated by cellular RNA polymerase to produce cellular as well as viral RNA molecules. The newly synthesized RNAs have two fates: i) they are transported to the cell plasma to become viral RNA in viral particles; ii) they are spliced to form a template for the translation of Gag, Gag-Pol and Env precursors. The Env precursor is subsequently cleaved by a cellular enzyme into functional units gp120 and gp41 and they are transported from the endoplasmic reticulum to the cell surface [74].

2.5.5. Viral budding and maturation

The unspliced viral RNA, the Gag and Gag-Pol polyproteins, and the vif, vpr and nef proteins are packaged as a new viral particle. This new particle spiked with envelope gp160 is extruded from the cell membrane to form a new virion. This new virion is not mature and is

not able to infect new cells until the viral protease is active and processes Gag and Gag-Pol precursors.

2.6. Antiretroviral therapy

By the end of 2011, there have been 25 ARV drugs approved for clinical practice from 5 different classes: NRTI, NNRTI, PI, EI, and INI (Table 1). Each class of ARV attacks each critical steps of the viral replication, slowing down the speed of viral replication. By combination of at least 3 ARVs, although the virus cannot be eradicated completely, viremia is suppressed to undetectable level (50 copies/ml) and CD4+ T cell count increases, allowing immune function restore.

2.6.1. Mechanisms of actions

2.6.1.1. Nucleoside/nucleotide reverse transcriptase inhibitors

NRTIs terminate the production of viral DNA by reverse transcriptase. After phosphorylation by cellular kinases, these agents are attached into extending DNA by RT. However, because these compounds do not have a 3' hydroxyl group, no nucleotide can be further bound to the DNA, ending the synthesis process. The nucleotide analogues can bind directly to DNA since they are already phosphorylated [32].

2.6.1.2. Non-nucleoside reverse transcriptase inhibitors

Non-nucleoside analogues are small molecules which display a strong affinity for hydrophobic pocket near the catalytic domain of reverse transcriptase [32]. This binding has an effect on RT, making it work incorrectly and the synthesis of DNA stops.

2.6.1.3. Protease

HIV-1 produces its proteins in the form of large polyproteins Gal and Gal-Pol precusors. These polyproteins are lysed by the HIV protease at different cleavage sites in

sequential order to structural proteins and enzymes necessary for the formation of mature infectious viral particles [77]. If protease does not work correctly, viral virions are assembled but not infectious [78].

Table 1. Antiretroviral drugs used in treatment of HIV infection approved by U.S. Food and Drug Administration by 2011

Drug class	Drug name	Brand name
Nucleoside Reverse Transcriptase Inhibitors		
	Abacavir (ABC)	Ziagen
	Zidovudine (AZT)	Retrovir
	Stavudine (d4T)	Zerit
	Emtricitabine (FTC)	Emtriva
	Didanosine (ddI)	Videx
	Lamivudine (3TC)	Epivir
Nucleotide Reverse Transcriptase Inhibitors		
	Tenofovir (TDF)	Viread
Non-nucleoside Reverse Transcriptase Inhibitors		
	Delavirdine (DLV)	Rescriptor
	Efavirenz (EFV)	Sustiva
	Nevirapine (NVP)	Viramune
	Etravirin (ETV)	Intelence
	Rilpivirine	Edurant
Protease inhibitors		
	Amprenavir (APV)	Agenerase
	Atazanavir (ATV)	Reyataz
	Fosamprenavir (FAPV)	Telzir
	Indinavir (IDV)	Crixivan
	Lopinavir (LPV)/ritonavir	Kaletra
	Ritonavir (RTV)	Norvir
	Saquinavir (SQV)	Invirase
	Nelfinavir (NFV)	Viracept
	Darunavir (DRV)	Prezista
	Tipranavir (TPV)	Aptivus
Entry Inhibitors		
CCR5 co-receptor antagonist		
	Maraviroc (MVC)	Celsentri
Fusion inhibitors		
	Enfuvirtide (ENF)	Fuzeon
Integrase inhibitors		
	Raltegravir (RAL)	Isentress

HIV-1 protease is a homodimer enzyme including 2 symmetrical subunits which form a symmetric, substrate-binding cavity in the central. Based on the knowledge of this domain and substrates of the enzyme, protease inhibitors have been designed to mimic the natural viral peptides processed by the viral protease. Protease inhibitors have a strong affinity for active sites of the enzyme, preventing viral polyproteins from being cleaved by viral protease [79].

2.6.1.4. Entry inhibitors

2.6.1.4.1. CCR5 antagonist

In the second step of viral entry, the binding of the viral gp120 to CD4 receptor on cell surface makes a conformational change in gp120 allowing it to interact with CCR5 or CXCR4 chemokine co-receptor. CCR5 antagonists are small molecules which bind to a hydrophobic pocket formed by the transmembrance helices of the CCR5 receptor, altering the conformation of the receptor and the CCR5 cannot interact with gp120 [80]. The compound is designed for CCR5 tropic HIV-1 isolate.

2.6.1.4.2. Fusion inhibitors

In the third step of viral entry, following the interaction between gp120-CD4 complex with chemokine co-receptors, gp41 comprising of the repeat region or Heptad region 1 (HR1) and 2 (HR2) undergoes another conformational change resulting in viral and cell membrane fusion [81, 82]. Enfuvirtide is a synthesis peptide which mimics the HR2 region of viral gp41. The binding of this compound to HR1 region blocks the forming of a structure needed for the fusion process and that prevents virus entry [83].

2.6.1.5. Integrase inhibitors

Following reverse transcription, the IN catalytic activity takes place with the cleavage of the terminal dinucleotides GT from the 3′ ends of the newly synthesized double stranded

HIV-1 DNA, leaving 2 CA overhangs. This process so called the 3′-processing reaction takes place in the cytoplasm of infected cell in the pre-integration complex (PIC). Remaining attached to each of the 3'ends of the processed DNA, IN binds to the host protein (LEDGF/p75). PIC then migrates to the nucleus along a microtube and through a nuclear pore. In the nucleus, IN catalyzes the join of the viral 3′-hydroxy ends to the host genomic DNA (the strand-transfer reaction) [47, 84]. RAL is a strand transfer inhibitor. This compound is a molecule which has motif interact with the Mg++ in the active site formed by IN and the 3' HIV-1 DNA ends, resulting in functional changes and that inhibits the integration of viral DNA into the host chromosomal DNA [85].

2.6.2. HIV drug resistance

2.6.2.1. General concepts of drug resistance

HIV has a dynamic of rapid replication with approximately 10^{10} new virions generated every day [86]. Viral RT is prone to make errors with a rate of 1 error per 10,000-30,000 incorporated nucleotides. With 10,000 base-pair long, a new HIV genome produced in one life cycle includes at least one random mutation. In HIV infected patients with normal viral load of 30,000 copies/ml, amino acid substitutions can occur at all positions of the entire HIV genome on the daily basis [87], resulting in a highly diverse viral population with genetically related isolates called viral quasispecies. In addition, with its high level of replication and the ability of recombination, the virus can generate multiple quasispecies in viral population in the body.

Generation of drug-resistant mutants is an intrinsic aspect of HIV-1 replication. Under conditions that allow ongoing viral replication in the presence of drug pressure, drug-resistant mutants acquire a selective advantage over WT virus and become dominant within the quasispecies. The resistant mutants can be detected in genotypic resistance assays and the effect of resistance mutations can be determined in phenotypic resistance assays. Although

having recognized limitations, resistance tests in current routine use provide an important guide to patient management. Many factors determine the relative rate of resistance selection with different drugs and drug combinations, reflecting in the genetic barrier to resistance such as interactions between mutations, effects of individual resistance mutations on viral replication, fitness influence on mutational pathways, and the overall impact of resistance mutations on viral phenotype. Certain mutations may be antagonistic, whereas others may confer resistance to some drugs while conferring hypersusceptibility to others.

2.6.2.2. Mechanisms of ARV resistance

Normally, viral resistant mutations are substitutions of amino acids and sometimes one or more insertions or deletions in the specific peptides, causing conformational changes in protein, resulting in a reduction of susceptibility to ARV.

2.6.2.2.1. Resistance to NRTIs

There are two mechanisms of HIV to develop resistance to NRTI: discrimination of analogue NRTIs and excision of analogue NRTIs from the terminated viral DNA (Figure 8).

2.6.2.2.1.1. Diminished affinity of NRTIs to viral RT

Mutations M184V, K65R, L74V, and the Q151M complex (Figure 8) allow viral RT to reduce affinity to NRTIs while maintaining affinity to the natural dNTP substrates. As a consequence, NRTIs cannot incorporate into growing DNA chain.

Mutation M184V/I

Mutations M184V and M184I located in a conserved part of the HIV-1 RT near the active site confer a high level of resistance to 3TC [88], emtricitabine (FTC) [89], and lower to abacavir (ABC) [90] and ddI [91]. These mutations, the first and the most likely resistance-associated mutations, develop early in patients receiving incompletely suppressive 3TC

containing regimens [92-94]. In the continuous presence of drug, the mutation M184I usually appears first and frequently is replaced by M184V due to a higher fitness of viruses harboring M184V [95]. However, without selective pressure, this mutation displays a lower replicative advantage and is not detected in the absence of drug. These mutations increase the susceptibility to AZT and TDF [96] by impairing the viral rescue of terminated DNA synthesis [97]. 3TC containing regimens still display a beneficial outcome in the presence of M184V mutation due to the ability to maintain drug pressure with the increased susceptibility of the virus to AZT and TDF caused by the mutation [98, 99].

Mutations K65R and L74V

The mutations K65R and L74V situated in the loop of the "finger" region of RT make an important contact to incoming NTPs in the polymerization [100]. These are seen in patients with NRTIs failure, especially when the regimen includes TDF, ABC. These mutations plus other secondary mutations T69N/S/A and V75T appear to confer resistance to most NRTI analogues, with the exception of AZT [32]. Both mutations increase the sensitivity of HIV-1 to AZT and the K65R does not appear in patients receiving AZT containing regimen [101, 102]. The mutated substitutions at position 69 are the most frequent NRTI mutations after thymidine-analogue resistance mutations (TAMs), and M184V. Insertion at this position together with T215Y/F and other TAMs displays a high level of resistance to all of licensed NRTIs [103, 104]. V75T usually occurs in isolates with multiple nucleoside resistance mutations.

The Q151M complex

The mutation Q151M is usually followed by mutations at positions 62, 75, 77, and 116 that enhance resistance and increase the activity of the enzyme. Strains with A62V, V75I, F77L, F116Y, and Q151M confer high level resistance to each of these NRTIs, and low-level

resistance to 3TC and TDF [105, 106]. The complex is not common, accounting for less than 5% of all cases with resistance to NRTIs.

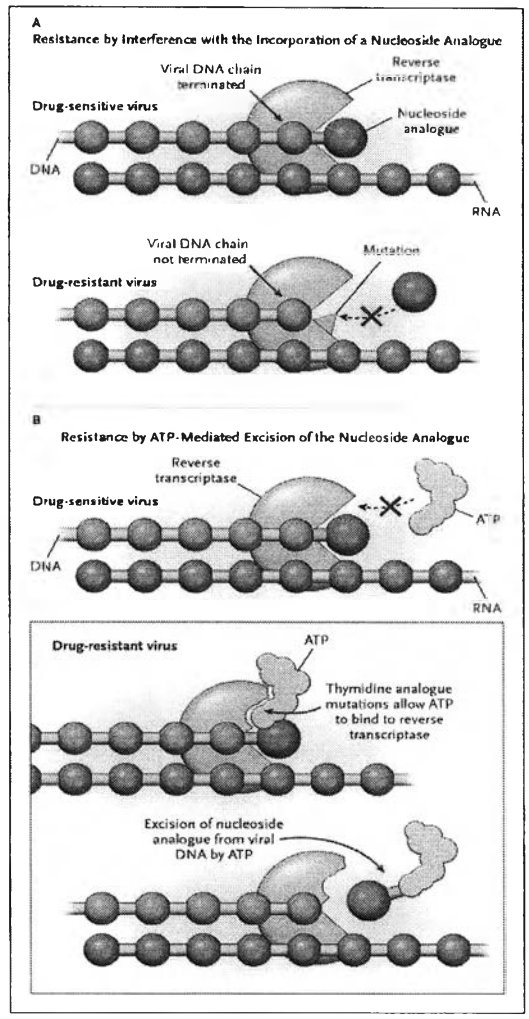

Figure 8. The two principal mechanisms of resistance of HIV to nucleoside analogues.

In Panel A, the incorporation of a nucleoside analogue into drug-sensitive viruses results in the termination of the viral DNA chain. Mutations in drug-resistant viruses prevent the incorporation of the nucleoside analogue into the growing viral DNA chain.
In Panel B, ATP in drug-sensitive viruses does not have access to a reverse transcriptase that has formed a complex with a nucleoside analogue. Mutations that cause resistance to nucleoside analogues, referred to as thymidine analogue mutations, allow ATP to bind reverse transcriptase near the 3' end of viral DNA terminated by the incorporation of a nucleoside analogue. ATP then excises the analogue from viral DNA, allowing reverse transcription to proceed normally.
Source: Clavel F, Hance AJ. N Engl J Med 2004;350:1023-1035.

2.6.2.2.1.2. Removal of the NRTIs from the terminated DNA chain

A group of mutations at positions 41, 67, 70, 210, 215, and 219 called thymidine-analogues mutations (TAMs) promote the process of removing nucleoside analogue from the 3' end of the terminated cDNA, producing an unblock cDNA chain (Figure 9). TAMs, a combination of M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, is the most frequent mutations selected during the failure of regimens containing thymidine-analogue (ZDV and d4T), but it can confer cross-resistance to all NRTIs including TDF but the degree of cross-

resistance depends on the analogues used and the number of TAMs accumulated in the isolates (Figure 10) [107]. There are two distinct TAMs profiles: TAM 1 includes the mutations M41L, L210W, T215Y and TAM 2 contains the mutations D67N, K70R, T215F and K219Q/E which are mutually exclusive in the early accumulation. These mutations appear gradually and in various orders under incompletely suppressive thymidine-analogue-containing regimens. However, the presence of other mutations such as M184V/I can delay the selection of TAMs [108] and slightly increase the susceptibility viral RT to other NRTIs despite some TAMs mutations accumulated in the isolates [32].

2.6.2.2.2. Resistance to NNRTIs

There are currently 2 classes of NNRTIs: the first generation including EFV, NVP and the second generation with ETV and Rilpivirine (RPV).

2.6.2.2.2.1. Resistance to EFV and NVP

Although the first generation of NNRTIs is generally well-tolerated, its main limitation is the low genetic barrier for the virus to develop resistance. Mutations selected for EFV and NVP after failure of treatment are all located within or surrounding the drug binding pocket of the enzyme. These mutations induce the conformational changes of the hydrophobic pocket directly interacted with the inhibitor, resulting in a diminished affinity to the drug [109] (Figure 9). A single mutation in the NNRTI binding pocket may reduce significant susceptibility to both NNRTIs. Due to different interactions between hydrophobic pocket and EFV or NVP [110], the earliest and most frequent mutations are somewhat different. The initial resistant mutation to NVP is often Y181C, but other mutations, such as Y188C, K103N, G190A, and V106A, also occur whereas the virus usually develops resistance to EFV with the K103N mutation, but the Y188L mutation is also selected [32]. The mutation K103N occurs in about 30% of failing patients with NVP-containing regimen and about 50% of

patients failing EFV. It causes 20 to 50-fold resistance to EFV and NVP and by itself, K103N is sufficient to cause virological failure for both NNRTIs. NVP usually selects for Y181C in the failing regimen not containing thymidine analogue. Y181C has a high level resistance to NVP (50 to 100-fold). However, because it increases the susceptibility of the virus to ZDV, the K103N mutation is emerged preferably when ZDV or d4T are included in the regimen. The mutation Y181C develops in less than 5% of patients with EFV failure and causes two to three fold reduced susceptibility to EFV. Nevertheless, in patients failing NVP-containing regimens with isolates harboring Y181C, only a transient virological response to EFV-containing salvage regimens has been observed.

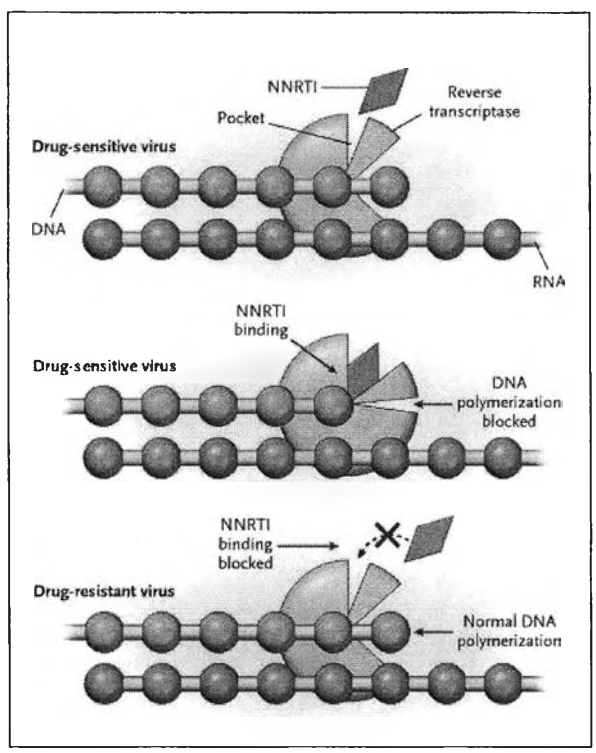

Figure 9. Mechanism of resistance of HIV to non-nucleoside reverse transcriptase inhibitors.

Source: Clavel F, Hance AJ. N Engl J Med 2004;350:1023-1035.

As mentioned above, the first generation of NNRTIs has low genetic barrier and cross-resistance patterns. This cross-resistance is partly due to the fact that most NNRTI

resistance mutations conferring resistance to both drugs and either EFV or NVP can select for multiple mutations despite only one or two predominant mutations detected by genotyping. Mutations could be selected in mothers after receiving single dose of NVP to prevent mother to child transmission and there is evidence that these mutations can have a deleterious impact on the future response to NNRTI containing regimen [111, 112].

NVP and EFV have a natural of long half life in plasma which helps to maintain a sufficient drug concentration during continuous treatment. However, because of their slow decay, the termination of these drugs will lead to a suboptimal level of drugs in plasma for a period of time as administering monotherapy which easily selects cross-resistance mutations.

2.6.2.2.2.2. Resistance to ETV and RPV

Etravirine (ETV; formerly TMC125 approved by FDA in 2008) and rilpivirine (RPV; formerly TMC278 approved by FDA in 2011) are potent second-generation NNRTIs that remain the antiviral activity to viruses harboring EFV-associated mutation K103N [113, 114]. In addition, as developed later RPV appears to be effective against ETV-resistant strains [115].

Both drugs are diarylpyrimidine molecules with flexible structures that can bind to RT in different conformations, allowing the compounds interact with viral RT strongly despite the changes of binding pocket induced by common NNRTI resistance mutations. ETV and RPV are considered to have a high genetic barrier. Researches *in vitro* suggested that the development of resistance to ETV and RPV takes longer than the first-generation NNRTIs. However, in general, the more mutations related to the first generation NNRTIs are selected, the less chance for ETV and RPV containing regimens to achieve suppress viral load.

There have been 17 ETV resistance-associated mutations identified: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S and M230L. Of these

mutations, Y181I/V confer highest reduction in virological response, followed by L100I, K101P, Y181C and M230L.The common mutation Y181C has a modest decrease in susceptibility to ETV. Single DRAM could not display a significantly reduced response to ETV, but accumulative DRAMs do [116]. Clinically, the mutations frequently seen in 93 patients experiencing failure with ETV-containing regimens in the phase III DUET studies include those at position V179 (n=35), E138 (n=17), Y181 (n=15), and K101 (n=13) [117]. The mutations at position E138 have been studied recently following the findings of clinical trials and the results showed that although the mutation E138K is usually the first mutation to emerge [118], this mutation and others at the same position E138A/G/Q/R or S demonstrate a low fold-change decrease in ETV susceptibility and a modestly reduced replication capacity compared with WT virus *in vitro* [118]. Single E138 mutation display a low resistance, not complete loss of sensitivity to ETV [119].

2.6.2.2.3. Resistance to PIs

The development of PI resistance both *in vitro* and *in vivo* is a stepwise process [120, 121]. The more mutations are accumulated, the higher degree of resistance is obtained [121]. The initially selected mutations (primary mutations) could be different between PIs, but usually they occurs in the substrate-binding cleft of the enzyme first, and sequentially additional mutations away from the active site (secondary mutations) are also emerged [122]. Primary mutations often have a limited effect on resistance [121]. To display a significant resistance, they must accumulate with additional secondary mutations. Because most PIs differ slightly in chemical structure, variants containing multiple mutations not only confer high level of resistance to a PI but also demonstrate cross-resistance to other PIs [121].

Primary resistance mutations inducing changes of the substrate binding cleft also lead to a less efficiency of the enzyme to cleave the Gag and Gag-Pol precursor proteins, causing fitness reduction. However, under continuous PI pressure, the virus generates compensatory

mutations to improve its replication capacity, resulting in a larger decrease in susceptibility to PIs. Compensatory amino acid substitutions could also occur in protease-cleavage sites Gag or Gal-Pol. These mutations change the structure of the substrates, allowing the changed viral protease induced by primary mutations bind and cleave its nature substrates.

A PI usually is administered with a low dose of ritonavir (RTV) which inhibits the cytochrome P450-mediated metabolism of PIs to increase the PI plasma level. Therefore, the virus must generate multiple mutations to develop a full resistance. PIs requiring the virus to accumulate several mutations to achieve resistance are considered to have a high genetic barrier. All mutations related to 8 approved PIs are listed in Figure 10 with primary mutations in bold.

2.6.2.2.4. Resistance to Entry Inhibitors

2.6.2.2.4.1. Resistance to CCR5 antagonist

The CCR5 antagonist can only be useful for patients with the virus using CCR5 as a co-receptor. For the viruses that use CXCR4 and mixture of CXCR4 and CCR5, the drug is not prescribed. *In vivo*, CCR5 antagonist failure is usually associated with an outgrowth of CXCR4 or mixed viruses emerged from a pre-existing minority viral population present at levels below the limit detection of conventional assay [123]. In patients failing CCR5 antagonist without a shift to CXCR4 use, mutations in gp120 were observed. These mutations usually located in V3 loop which is the determinant of viral tropism allow the virus adapt and bind to the drug-bound CCR5 complex.

2.6.2.2.4.2. Resistance to fusion inhibitors

Resistance to enfuvirtide (ENF) usually involves the selection of mutations in the first heptad repeat (HR1) region, from codons 36 to 45 [124] (Figure 10). Various changes of codons from 36-45 have been observed [125] and they confer different levels of resistance to

the drug; a single or double mutations could display a significantly reduced susceptibility to ENF [126]. Thus, ENF is considered to be a low genetic barrier compound. Together with its limited administration, twice daily subcutaneous injection and distinct resistance pattern anticipating no cross-resistance with other classes of ARV, ENF should only be used in deep salvage therapy when no other treatment options are available. Amino acid changes in HR2 region or in gp120 may affect on the susceptibility of the virus to ENF [127], but no consensus has been made.

2.6.2.2.5. Resistance to integrase inhibitors

RAL is the first approved INI. Two other INIs including elvitegravir (EVG) and dolutegravir (DTG, S/GSK1349572) are in advanced stages of clinical development. In this group, resistance to RAL and EVG emerges quickly *in vitro* and in monotherapy *in vivo.* DTG appears to have a higher genetic barrier to resistance than RAL or EVG and it displays a potential antiviral activities against viruses conferring resistance to both RAL and EVG [47].

As with other ARV classes NRTI, NNRTI, and PI, the INI resistance begins with primary mutations followed by secondary mutations that could play a role in elevating resistance and/or compensating for reduced viral fitness caused by the primary mutations [50].

Resistance to RAL usually evolves one of the 3 exclusively mutational pathways: Q148H/R/K, N155H, and Y143C/R [128]. These positions are located within the catalytic domain of the enzyme; therefore amino acid changes usually are associated with the defect of viral replication [38]. Although each of these signature mutations demonstrates a significantly reduced susceptibility to RAL (Table 2) *in vitro*, most resistant viruses harboring two or more mutations including secondary mutations to recover the defect of the enzyme caused by primary mutations have been observed in RAL failing patients [38]. Generally, *in vitro* all secondary mutations by themselves demonstrate a modest effect on drug susceptibility [50].

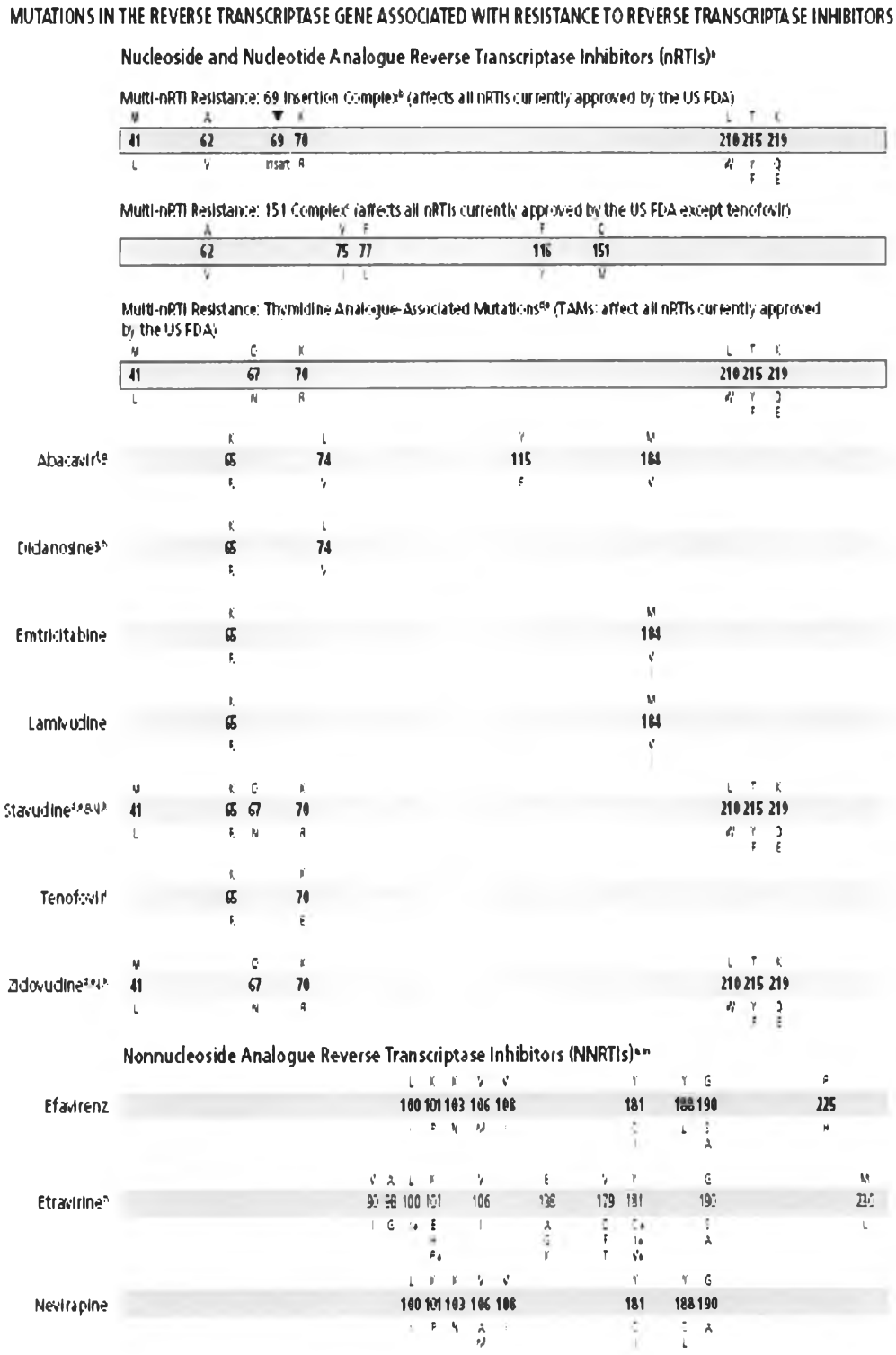

Figure 10. Mutations associated with all available ARV drugs

Source: Johnson VA, Brun-Vezinet F, Clotet B, Gunthard HF, Kuritzkes DR, Pillay D, Schapiro JM, Richman DD. 2010. Update of the drug resistance mutations in HIV-1: December 2010. Top HIV Med 18(5):156-163.

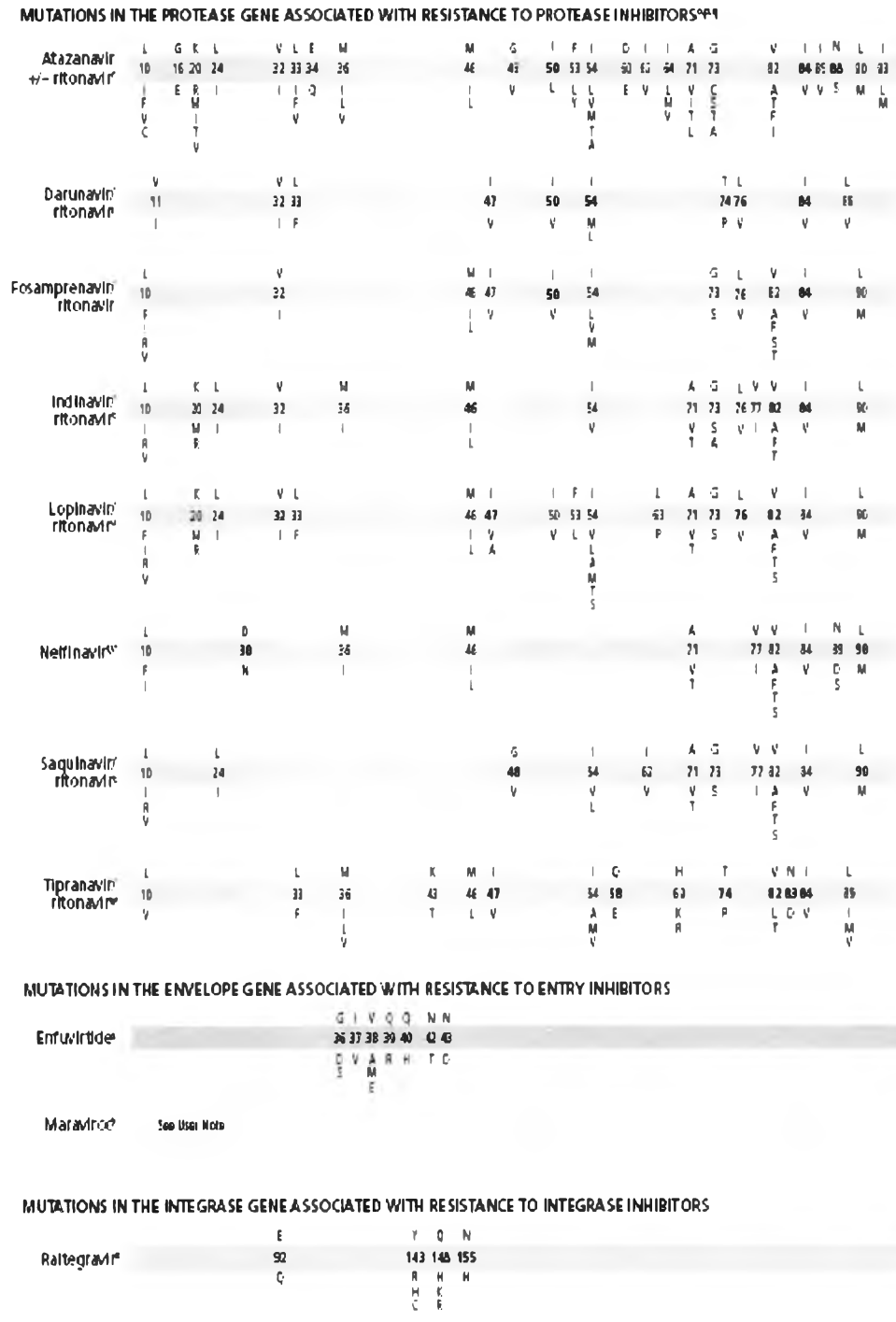

Figure 10 (continued). Mutations associated with all available ARV drugs

Source: Johnson VA, Brun-Vezinet F, Clotet B, Gunthard HF, Kuritzkes DR, Pillay D, Schapiro JM, Richman DD. 2010. Update of the drug resistance mutations in HIV-1: December 2010. Top HIV Med 18(5):156-163.

Q148H/R/K remarkably reduce susceptibility to RAL particularly Q148H and also display a reduction in replication capacity. Secondary mutations related to the Q148H/R/K pathway include L74M, E138A/K, or G140A/S [50]. Of these mutations, the G140 mutants, and to a lesser extent the E138 mutants are the most frequent. In clinical studies, isolates carrying Q148H plus G140S confer >1000 fold reduced susceptibility to RAL and EVG (Table 2).

Secondary mutations frequently encountered in the N155H pathway consist of L74M, E92A/Q, T97A, Y143H/C, V151I, G163K/R or D232N [50].

The third common pathway of RAL resistance is Y143C/R associated with T97A as a secondary mutation which largely elevates Y143C/R-mediated RAL resistance [38].

The development of RAL resistance seems to follow the stepwise process with the initial selection of N155H and eventually under the continuous RAL pressure this mutation is replaced by Q148H mutant usually together with G140S to restore the fitness cost [41, 129]. The mechanism of this phenomenon is still under investigation so far. However, *in vitro* studies have showed that viruses with N155H mutant alone reduce replication capacity less than viruses with Q148 mutant alone [46]. The addition of secondary mutation E92Q to the N155H strains decreases susceptibility only and does not compensate replication loss [38]. Since Q148H + G140S mutations demonstrate the greatest defect of RAL susceptibility (Table 2) and high replication capacity [45], viruses with N155H ± E92Q are gradually replaced by predominant isolates with G140 + Q148 mutations [38, 45].

For EVG resistance, primary mutations include T66I, E92Q, S147G, Q148R/H/K, and N155H together with secondary mutations H51Y, T66A/K, L68I/V, S119R/G, E138K, G140S/C, E157Q, K160N, R166S, E170A, S230R, and D232N [50]. Resistance patterns to RAL and EVG share some common mutations (Table 2), indicating a cross- resistance

between the two drugs. The 2 most frequent mutation-patterns associated with RAL resistance Q148H/R/K+G140S/A/C and N155H+E92Q also confer a high level of resistance to EVG. However, the third often seen pathway Y143C/R ± T97A does not seem display EVG resistance [47].

Table 2. Cross resistance between RAL and EVG

	Fold change in IC50 of single IN mutations									Fold change in IC50 of double, triple IN mutations				
	T66I	E92Q	E138K	G140S	S147G	Q148R	Q148H	Q148K	N155H	T66I+S14G	T66I+E92Q	E92Q+N155H	G140S+Q14H	E138K+S147G+Q 148R
RAL	1.4	6.0	0.9	2.0	1.0	20	34	30	23	2.5	33	135	>1,000	34
EVG	15	33	0.7	5.0	8.0	6.4	6	118	38	46	145	166	>1,000	175

Source: Adapted from Daar ES: Emerging resistance profiles of newly approved antiretroviral drugs. Top HIV Med 2008;16:110-116.

Resistance to DTG has been limited in results of studies *in vitro* so far. Mutations related to DTG resistance consist of E92Q, L101I, T124A, Q148H/R, S153F/Y, G193E [51]. DTG retains its full antiviral activity against isolates containing N155H ± E92Q and Y143C/R ± T97A. However, the pattern Q148H/R/K ± G140S/A/C ± E138K/A reduces 10-20 folds susceptibility to DTG [47].

2.7. Genetic barrier to develop drug resistance

The genetic barrier can be defined as the accumulative number of DRAMs required for the virus to escape drug-selective pressure. It is one of the crucial factors contributing to the development of drug resistance [53, 54]. Each ARV compound displays different genetic barrier to resistance. In general, 3TC/ FTC and first generation of NNRTIs EFV, NVP are consider to have low genetic barriers as one mutation to those drugs can confer a high level of resistance. An intermediate genetic barrier is for NRTIs as AZT and TDF. In contrast, RTV

boosted PIs have a high genetic barrier as multiple mutations are required [130]. In the new class of INI, the first generation RAL and EVG appear to have lower genetic barrier than the second generation DTG [47].

The evolution of mutations is influenced by the type and number of nucleotide changes from WT to mutated codon. A transition of nucleotide defined as a replacement of a purine by another purine (A ↔ G) or of a pyrimidine by another pyrimidine (C ↔ T) occurs 2.5 times more likely than a transversion defined as a replacement of a purine by a pyrimidine and vice versa (A ↔ C, A↔ T, G↔ T, G ↔ C) [54]. For example, the M184V mutation is caused by a transition from ATG → GTG, the L74V mutation is as a result of a transversion of GTA → TTA. Some mutations need more nucleotide substitutions as T215Y is due to changes in the first two bases ACC → TAC [130] . Thus all the mutations are not selected with a similar frequency. Given the HIV's huge genetic variability at nucleotide level, different HIV subtypes might have an impact on genetic barrier of resistance. An example is the high frequency of the V106M mutation to EFV in subtype C. Although EFV failure occurs frequently in patients with subtype B, this mutation is hardly seen in this subtype due to the requirement of 2 nucleotide changes (GTA to ATG) to acquire this mutation in subtype B compared to only 1 nucleotide substitution to achieve that in subtype C (GTG to ATG) [131]. The genetic barrier of INI is investigated in the manuscript 3 of our study in this thesis.

2.8. Minority drug resistance

Minority drug resistance can be the remains of dominant resistant variants previously selected in patients exposed to ARV or a result of primary (transmitted) drug resistance which is fading gradually. In general, the viral population is almost homogeneous in the phase of acute infection and it becomes heterogonous in the chronic phase due to its natural diversity. Under HAART, the number of viruses together with various viral quasispecies is reduced.

The incomplete suppression of viral replication caused by lack of adherence and suboptimal drug concentration in certain compartments (not all drugs penetrate equally well) [132] leads to the emergence of drug resistance variants called acquired or secondary resistance, resulting in drug failure. If treatment interrupts, WT variants overgrow rapidly becoming dominant as a result of a fitness advantage. These dominant variants were detected by conventional sequencing [133]. However the drug resistant isolates do not vanish completely, rather they exist as a minority quasispecies in plasma RNA detected by more sensitive methods such as AS-PCR [134] or as are archived as proviral DNA in latent reservoirs. HIV-1-infected resting CD4 cells present a well-characterised latent reservoir [19]. Besides, dendritic cell [135], natural killer cells [136], and macrophages [137] have also been identified as latent reservoirs of the virus. Thus, in drug failure patients, the dominant variants in plasma are not always represent the complexity of viral quasispecises in the viral population.

Even though in the presence of a prolonged effective HAART, the viral load in plasma goes undetectable by all currently commercial assays, residual viral replication still occurs resulting in a continuous evolution of viral drug resistance [15]. If drug resistant variants are once developed in a patient, they can persist at low level or be archived in genome of latently infected cells despite the fact that the subsequent ARV demonstrates a complete suppression of plasma viremia [16, 134, 138, 139]. If the same drug pressure is later resumed, archived resistant isolates will re-emerge quickly [140] as the cells containing these viruses become activated. In clinical practice, repeated resistance testing is not useful if the previous test results documented the extensive drug resistance because once selected, drug resistant variants do not disappear completely but hide for years, possibly permanently.

Primary resistance or transmitted drug resistance occurs when a person is infected with HIV-1 viruses already resistant to one or more drugs. Although resistant strains are not well transmitted as WT ones, given the widespread use of ARV, drug resistant incidence is

increasing leading to a large pool of quasispecies of resistant viruses to generate new infections and transmission of drug resistant variants occurs irrespectively of the route of infection [141, 142]. Transmitted mutants persist for years as dominant quasispecies or can revert to WT variants and may gradually no longer exist as dominant quasispecies, only persisting as minority strains in plasma RNA or as archived resistance within proviral DNA [143-145]. Once selective drug pressure is introduced, transmitted resistant variants presenting as minority quasispecies can quickly become dominant (Figure 11).

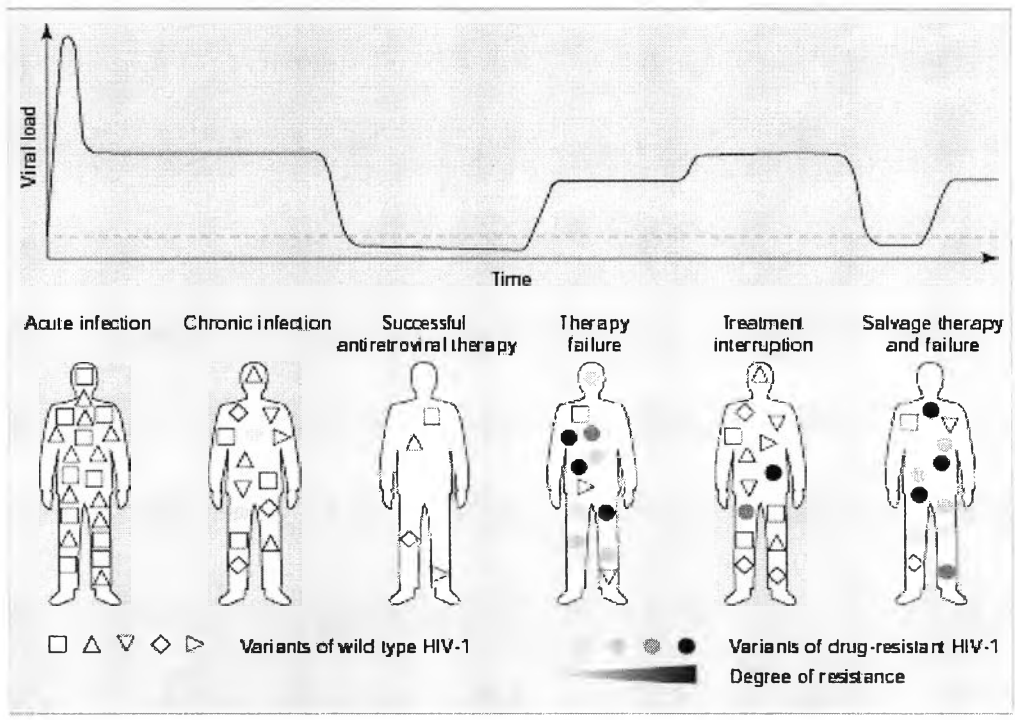

Figure 11. HIV-1 infection and a model of the distribution of viral quasispecies under HAART.

Source: Antiretroviral Resistance in Clinical Practice. Geretti AM, editor.London: Mediscript; 2006.

Current commercial tests for HIVDR including genotypic and phenotypic methods are limited in detecting minority variants in the virus population, leading to an underestimation of the rates of primary HIVDR. In one study using technique to detect low level of resistance variants of three common resistance mutations to NRTI, NNRTI, and PI

(L90M, K103N and M184V), 10 out of 49 patients were identified harboring primary resistance mutants compared with only five of those 10 patients being found by conventional sequencing [146]. In another study using AS-PCR to detect common protease mutation L90M and reverse transcriptase mutations M41L, K70R, K103N, Y181C, M184V, and T215F/Y Johnson *et al* found further mutations present at low levels in the population of newly diagnosed ART-naive persons with no detectable resistance-related mutations by conventional sequencing [17].

In the prevention of mother to child transmission (PMTCT) of HIV-1, drug resistance mutants can emerge rapidly in mothers receiving a single dose of NVP and these were transmitted to their infants. In one trial, the mutation K103N was detected in 8 of 9 women after 6-8 weeks of administration of single-dose of NVP and in 4 of 5 their infants and persists as a minority variant for more than 1 year [18]. The same results were also found in different studies [147]. However, the role of minority quasispecies of DRAM in clinical practice remains to be a topic of debating. Although minority DRAMs found in mothers and infants after administration of single dose NVP, up to now, the available data on their impact on clinical outcome have been limited. One study showed that women receiving single dose of NVP had higher risk leading to a failure with sequential NVP-based regimen than did women without previously exposed to NVP. However, this negative impact was no longer present after 6 months after receipt of a single peripartum dose of NVP. Unfortunately, this study did not identify minority DRAMs in the studied population [148]. In treatment naïve persons, some studies showed a association between the presence of DRAMs at low-frequency and virological failure of the first-line NNRTI-based HAART [14, 17, 149, 150] but others did not [151]. For the INI minority DRAM, although there was a high frequency of Q148R minority variant its relevance to treatment failure was not established [152].

2.9. Standard assays to identify drug resistance

Currently, there two methods to assess drug resistance applied in clinical practice: phenotyping and gentotyping (Table 3)

Table 3. Advantages and disadvantages of phenotyping and genotyping

	Advantages	Disadvantages
Phenotypic assays	✓ Direct measure of resistance ✓ Results simpler to interpret ✓ Does not require prior knowledge of the effect of mutations ✓ Assesses impact of interactions between mutations ✓ Assays available to test ARV drugs in new classes ✓ Potential to measure replication capacity	✓ Less rapid results ✓ More expensive ✓ Potential for lack of good correlation between FC and clinical outcome ✓ Lack of standardization of cutoff values between assays ✓ Limited sensitivity to minor subpopulations of HIV ✓ Potential for misleading results in case mixtures are present in the sample
Genotypic assays	✓ Simple to perform ✓ Rapid results ✓ Less expensive ✓ Early detection of mutants that may not yet cause phenotypic resistance particularly valuable in testing prior to therapy ✓ Results potentially applicable to future drugs targeting the sequenced region	✓ Indirect measure of viral susceptibility to ARV medications ✓ Can only predict the effect of previously described mutations ✓ Unable to detect linage interactions of multiple mutations ✓ Require expert interpretation; lack of standardization between interpretation algorithms ✓ Limited sensitivity to minor subpopulations of HIV

Source: Virco's training package, 2008

2.9.1. Phenotypic assay

Phenotypic assay measures the level of susceptibility of viruses harboring mutations to drug by assessing the ability of these viruses growing in different concentrations of ARV drug compared to viruses with WT (laboratory strain) sequences. In general, phenotyping includes the initial step of amplification of interested regions of the HIV genome by PCR. Generation of a recombinant virus by inserting amplified fragment into the backbone of a WT clone of HIV in which this region is deleted. This replication-competent recombinant virus containing region of interest is produced and transfected into susceptible cells. The ability of the recombinant virus to grow in various drug concentrations shown by viral antigens produced or by the expression of a reporter gene such as green fluorescent protein or luciferase is compared to that of a HIV reference strain. The drug concentration inhibiting 50% of replication is calculated and the ratio of these of the test and reference viruses is reported as the fold-change (FC) in IC50 (Figure 12).

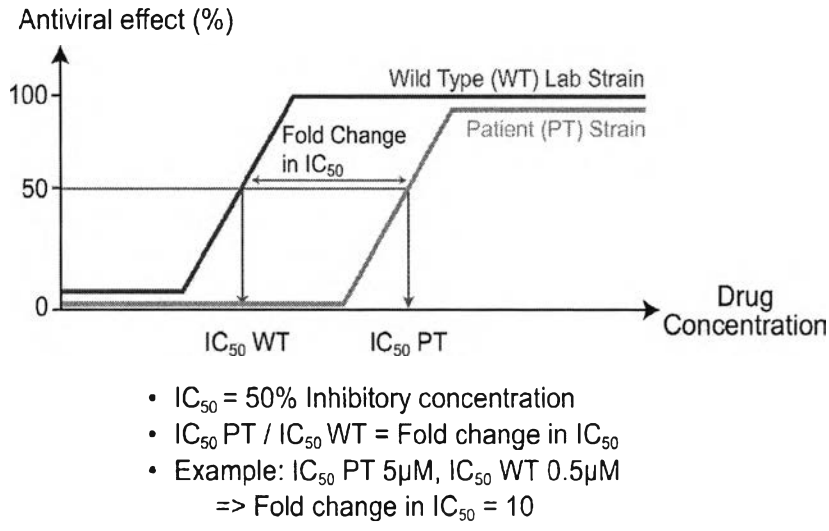

Figure 12. Calculation of fold change.

Source: Viro training package, 2008

2.9.2. Genotypic assay

The interested region of HIV genome harboring expected mutations is amplified and the sequence of this region is identified by population-based Sanger sequencing. The nucleotide sequence is translated into amino acid. Aligned and comparing with the WT sequences, the amino acid changes which are previously reported to be associated with changes in susceptibility or reduced clinical response to ARV are determined. The process of analyzing the sequence to interpret drug resistance following algorithms is known as genotype interpretation. This process is important to have correct results for a good therapeutic regimen.

Genotyping can be performed in "in-house" or commercial assays. In- house population- based genotypic assay is less expensive, more flexible. It can be designed to target different regions of interest in HIV genome for sequencing and to identify mutations in different genes of different subtypes. However, because the development and calibration are carried out in individual laboratories, these assays seem to be various between laboratories and subject to be inter-subtype dependent. Although there are a number of available commercial genotypic assays for HIV-1 drug resistance, only two to date were approved by the US FDA TrueGene™ (Visible Genetics Inc., Toronto, Canada) and ViroSeq™ (Abbott Laboratories, Chicago, IL, USA)) for genotypic resistance test.

Both methods are similar in principal for detecting mutations in protease, RT genes and currently integrase. For TruGene™, amino acids 1-99 of protease gene and 1-247 of RT gene are sequenced. For ViroSeq™, the same amino acids of protease gene are sequenced but longer fragment of RT gene (1-335) are determined. The primers are designed to target the conserved regions, allowing anneal to most HIV-1 group M variants. The minimum viral load for the assays to obtain optimal performance is 1000 copies/ml. However, by applying highly sensitive protocols, the tests can be done and give reliable results at viral load below 500

copies/ml. These ultrasensitive protocols require more volumes of plasma to yield higher concentration of viral RNA and extra amplifying PCR step before proceeding normal protocol. The interpretation algorithm is integrated into both assays' softwares to assess the mutations in relation to level of drug resistance. However, the sequences are also assessed using the online database of drug resistance such as the Stanford University database (http://hivdb.stanford.edu) or the French Agence Nationale de Recherches sur le SIDA (ANRS) (http://www.hivfrenchresistance.org).

2.10. Laboratory technology to identify minority drug resistant variants

The current conventional genotypic tests are limitedly able to detect the resistant mutants that represent at least 20–25% of the viral population [19, 20]. Thus, minority variants go undetectable by this technique. Different technologies have been applied to develop assays for minority strains quantification (Table 4).

2.10.1. Standard cloning and direct sequencing of multiple clones

It is a classical, easy technique, available in most laboratories [153, 154]. Fragment of interested region of HIV genome amplified by PCR is incorporated into a plasmid vector containing an antibiotic resistance gene. This vector also includes blue and white screening makers on X-gal medium allowing a colour selection for successfully inserted vectors. Recombinant vector is transfected into bacterial cells and then plated in solid medium containing antibiotic which allows only cells with recombinant constructs grow. Each colony containing one recombinant DNA is selected according to its colour. Interested DNA is purified and subject to be conventionally sequenced. Although this technique allows the study of unknown mutations and the linkage of mutations in sequences, it is very laborious and not feasible to identify very low abundant variants in multiple samples. According to Poisson distribution, in order to identify a mutation present at 10% of total population with 95%

confidence, at least 30 colonies must be selected and sequenced and if the cut off is 1%, 300 colonies must be analyzed [155]. If multiple samples are studied, it is very laborious and time consuming to achieve the detection of low level of minority variants.

2.10.2. Single genome sequencing

Based on limiting-dilution assays, this technique allows comprehensive analyses of viral populations by assessing DNA sequences obtained from many single viral genomes in plasma sample [156].

Two hundred filthy to one thousand eight hundred microliter of plasma containing at least 1,000 copies of RNA molecules are used to extract RNA. The entire viral RNA extraction was used for cDNA synthesis by random hexamers. The amplified cDNA is diluted following a series of dilutions from 1:3 to 1:2,187 to have PCR produces containing a single cDNA molecule. Each cDNA dilution is subject for ten separate real-time PCR amplifications. Following the Poisson's distribution, each cDNA dilution having PCR product in 3/10 real-time PCRs includes one copy of cDNA per positive PCR approximately 80% of the time. Therefore, 70 nested PCRs were performed, yielding ~30% positive reactions. Positive nested PCRs were detected by agarose gel electrophoresis and sequenced by direct sequencing [156]. Normally, sequences from 20-40 single genomes are assessed in one sample, but this number of genomes can be increased to obtain lower sensitivity of 2%. Although this technique provides analysis of a large region and the linkage of mutations in one genome, it is time consuming and expensive.

Table 4. Techniques to detect and quantify minority quasispecies of HIV-1 drug resistance

Method	Advantages	Disadvantages	Reference
Standard cloning and direct sequencing of multiple clones	Complete genotype; Detection of unknown mutations	Labor and time-consuming; Many clones must be analyzed if variant present in low level of the total viral population	*[153, 154]*
Single genome sequencing	Complete genotype	Time-consuming; Expensive; Quantification of minority quasispecies depends on amount of clones analyzed	*[156]*
Heteroduplex tracking assay (HTA)	Quantification to levels ≥3–5%; Simple handling	Inaccurate quantification of minority quasispecies; Sensitive to polymorphisms; Limited fragment size; Difficult interpretation	*[13, 157]*
Line probe assay (LiPA)	Quantification to levels ≥4–8%; Simple handling	Inaccurate quantification of minority quasispecies; Sensitive to polymorphisms; No detection of unknown mutations	*[158-160]*
Oligonucleotide ligase-based assay (OLA)	Quantification to levels ≥5%	Each single mutation requires a separate assay; Sensitive to polymorphisms; No detection of unknown mutations; Expensive; Labor intensive	*[161, 162]*
Allele-specific real-time PCR (AS-PCR)	Accurate quantification to levels ≥0.01%	Each single mutation requires a separate assay; Sensitive to polymorphisms; No detection of unknown mutations;	*[134, 146, 147, 163]*
Ultra deep sequencing (UDS)	> 0.5-1%, high throughput	Require strong bioinformatics support. Very expensive	*[164-166]*
Pyrosequencing	≥1- 5%,high throughput, less labor intensity	Expensive; No detection of unknown mutations	*[167-169]*

Source: Adapted from Antiretroviral Resistance in Clinical Practice.London: Mediscript; 2006.

2.10.3. Heteroduplex tracking assay (HTA)

HTA is based on the detection of probe-PCR product heteroduplexes separated by gel electrophoresis. These heteroduplexes are products when a radioactively labelled probe is bound to a PCR product [13]. By designing probe specific for predefined interested point mutations by introducing nucleotides changes close to the sites of interest, HTA allows the detection of multiple specific mutations. The mobility and intensity of the probe-mutation and probe-WT heteroduplex bands are compared. The presence of multiple mutations usually makes a change affecting the mobility of the heteroduplex in comparison with single mutations and with WT. With the step of isolating RNA/DNA heteroduplexes and DNA sequencing, minority variant frequency and the linkage of mutations are analysed. Even though this assay is simple for handling, it is difficult to interpret the results of gel electrophoresis leading to a possible inaccurate quantification of minority variants [157].

2.10.4. Line probe assay (LiPA)

Line probe assay (LiPA) is based on the principle of reverse hybridization carried out on a nitrocellulose strip containing oligonucleotide probes for WT and mutants or polymorphisms. It is developed to commercial assays: VERSANT HIV-1 Protease Resistance Assay (LiPA) detect mutations and polymorphisms at codons: 30, 46, 48, 50, 54, 82, 84 and 90 of the protease gene and VERSANT® HIV-1 RT Resistance Assay (LiPA) detects WT and mutants at codons 41, 69, 70, 74, 75, 103, 106, 151, 181, 184 and 215, within the RT gene [158, 160]. Despite of being a very simple assay, like other assays based on hybridization, LiPA is sensitive to polymorphism in the oligonucleotide binding sites and is limited to detect known mutations only.

2.10.5. Oligonucleotide ligase-based assay (OLA)

OLA is a method based on the covalent link between two oligonucleotide primers when they anneal adjacently onto a DNA amplifying template. It is highly specific because a single nucleotide mismatch between primers and the template occurred in two nucleotides in each direction at the site of the junction completely precludes the ligation. The oligonuclotides specific for WT or mutated codon can be modified at the 5' terminus to have a reporter. They are also captured and immobilized on wells of a streptavidin-coated plate. Thus, the results of WT or mutant sequences could be analyzed visually or by a spectrophotometer [161, 162] . OLAs is rapid, specific, and sensitive reactions for the identification of known point mutations in large scale with many samples with the detection limit of minority variants approximately down to 5% of viral population (Table 4).

2.10.6. Allele specific real time PCR (AS-PCR)

The assay applies two separate real-time PCRs for quantification of viruses containing mutation and WT alleles of interested codon using mutant and WT specific primers. Alternatively, mutant codon is amplified and quantitated using mutant specific primer and the similar primer except 3' end stop just one nucleotide before the target nucleotide of mutant specific primer is designed to detect all sequences in the viral population. To increase the specificity of the assay, an intentional base mismatch is usually introduced at the -1 position of the 3' terminus of mutant specific primer following the method of Kwok S *et al* [170]. The real-time PCR for quantification of the copy number of viruses using mutant specific and WT (non-specific) specific primers is performed delicately in separate wells in one plate (usually in two separate halves of a plate) together with standards as known-copy number plasmids. The percentage of mutated sequences is determined as the following formula:

Percentage of mutated sequences = (copy number of mutated sequences/ copy number of total sequences) x 100 [134]

AS-PCR is inexpensive, less labour intensive and fast assay. It is the most sensitive method to detect minority variants with the limited detection level down to 0.001% [163]. We applied this technique to detect the IN mutation N155H in a longitudinal study on 5 heavily treated patients failing RAL containing regimen reported in paper 2 of this thesis. However, like other hybridization assays, polymorphisms in the primer binding sites, particularly those located near 3' end of the primer, could affect on the efficiency of the test, leading to an underestimation of the real proportion of minority mutants. As with others in the group of point mutation assay in group of point mutation assay, AS-PCR can detect only known critical resistant mutations and only one allele is analyzed in each experiment. Therefore, given the multitude of mutations and their interactions that contribute to drug resistance, it is not feasible to develop AS-PCR to detect all mutations and the linkage of mutations is not analyzable by the assay.

2.10.7. Ultra-deep sequencing

Ultra-deep pyrosequencing (454 Life Sciences-Roche) is a new sensitive method that allows not only the detection and quantitation of low-level resistant mutants, providing both proportional and absolute number of sequencing reads with a mutation but also analysis of the linkage of mutations. Using microfabricated high-density picoliter reactors and pyrosequencing protocols, this technique creates a massively parallel pyrosequencing of sequences generated from hundreds of thousands of independent molecules amplified from RT-PCR of unique HIV strains. After reverse transcription and amplification of interested region of the HIV-1 genome from extracted HIV RNA using primers with adapters in the 5' terminus, each cDNA is attached to DNA captured bead. Clonal amplification of bead-bound amplicons on beads is performed using sequencing kits in an emulsion environment. Beads

with clonally amplified copies of a single DNA molecule attached are loaded onto a picoliter plate with a diameter restricted for only one bead per well for pyrosequencing. Pyrosequencing is performed on a Genome Sequencer (GS) FLX instrument [166]. The assays can archive thousand of bead reads at each nucleotide position, providing accurate detection and quantitation of minority variants down to a frequency of 1% [164]. Because the data created from each run is quite large, it require sophisticated software to analyze with expertise scientists to interpret the result. Although this technology is shown to be a potential and powerful tool to analyze minority variants, its high cost (although it is reducing) and difficulty to interpret correct results restrict the assay to a limited number of laboratories.

2.10.8. Pyrosequencing

Pyrosequencing (PSQ) is a robust and simple sequencing method well suited for the analysis of low level of known point mutations. PSQ provides less information than ultra-deep pyrosequencing particularly no information on genetic linkage of mutations is given. However, it is much cheaper, faster and easier to perform as well as to interpret the results than massive parallel pyrosequencing.

PSQ technology is a real time sequencing method based on the detection of PPi as it is released when a nucleotide that is complementary to a base in a DNA template strand is incorporated into an extending cDNA by polymerase with the initiation of sequencing primer. Biotinylated PCR products bound to beads are denatured to become single stranded templates for sequencing primer to anneal on to begin the sequencing process (Figure 13). A single PSQ cycle includes 5 steps involving the cooperative actions of enzymes, substrates, nucleotides (A). The first step is the dispensation of a new nucleotide triphosphate. If the nucleotide is complementary to the base in the template, it is added and PPi is released. This PPi is used to convert AMP to ATP that helps to produce visible light through the action of luciferase. Thus, the light is generated only if the nucleotide is matched to the base in the

template and incorporated into the growing cDNA. The cycle is finished when unused dNTPs and ATP are degenerated by apyrase. (B) is an example of a theoretical pyrogram as a result of cyclic nucleotide dispensation order. The generated light is converted to peak with the high correlated to the number of added nucleotides. The sequence result is displayed at the bottom of the panel. (C) is an example of a theoretical program obtained using specific nucleotide dispensation order[171, 172]. Using the allele frequency quantification function of the SNP software (Biotage AB, Uppsala, Sweden), the proportion of mutant in the total sequences present in the sample can be quantified [167] . We applied this technology to develop assays to detect minority DRAMs Y181C and M184V in studied population in paper 1.

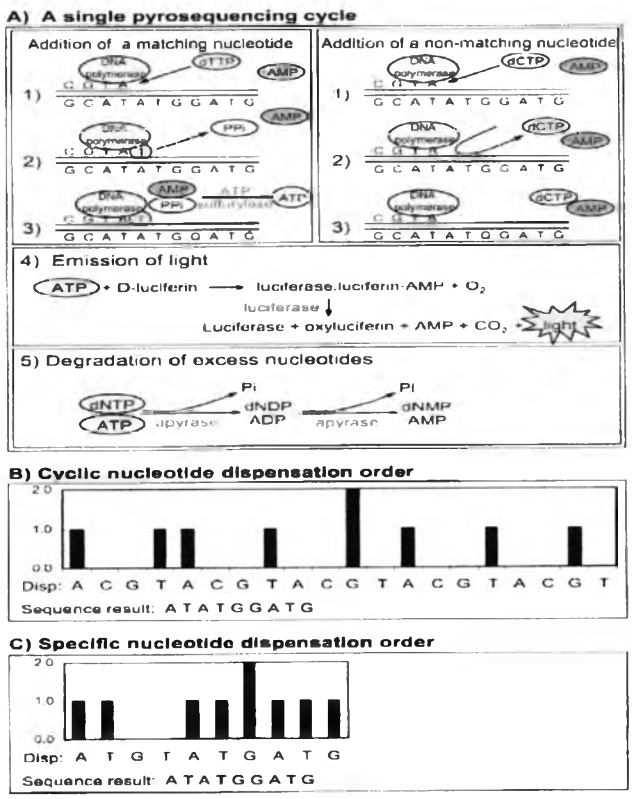

Figure 13. Principle and chemistry of pyrosequencing.

Source: Lindstrom A, Odeberg J, Albert J. 2004. Pyrosequencing for detection of lamivudine-resistant hepatitis B virus. J Clin Microbiol 42(10):4788-4795.