# Literature Review

## CHAPTER 1

### 1. 1 Overview of HIV/AIDS

Human Immunodeficiency Virus (HIV) is a human pathogenic virus that cause AIDS (Acquired Immunodeficiency Syndrome). HIV/AIDS has been global pandemic for over the last three decades and is depicted as the modern day plague (Quinn, 1996). The United Nations Acquired Immune Deficiency Syndrome (UNAIDS) global report 2012 estimates that by the end of 2011 approximately 34 million people were living with HIV by the end of 2011 (WHO factsheet Number 360 (<http://www.who.int/mediacentre/factsheets/fs360/en/>)) and that over 95% of them are living in low and middle in come countries (Esparza and Bhamarapravati, 2000). There is a significant variation in HIV prevalence among the countries around the globe (Figure 1.1). The UNAIDS 2012 report shows that although the global trend of new HIV infections and HIV-related deaths per year is declining, the current number of HIV infections is the highest since 1990 (Figure 1.2). The sub-Saharan region of Africa is the region most aggravated by the virus with 23.5 million people living with HIV (UNAIDS). UNAIDS estimates that approximately 1 in every 20 adults is HIV infected in this region (UNAIDS). This is 25 or more times the HIV prevalence in any other region of the world. Countries in Sub-Saharan Africa also have varying HIV prevalence with South Africa at the top followed by Nigeria (Esparza and Bhamarapravati). The next severely affected regions, besides African continent, are Asia (China, Thailand, Indonesia), Caribbean and Eastern Europe, North America, western and central Europe (Esparza and Bhamarapravati).

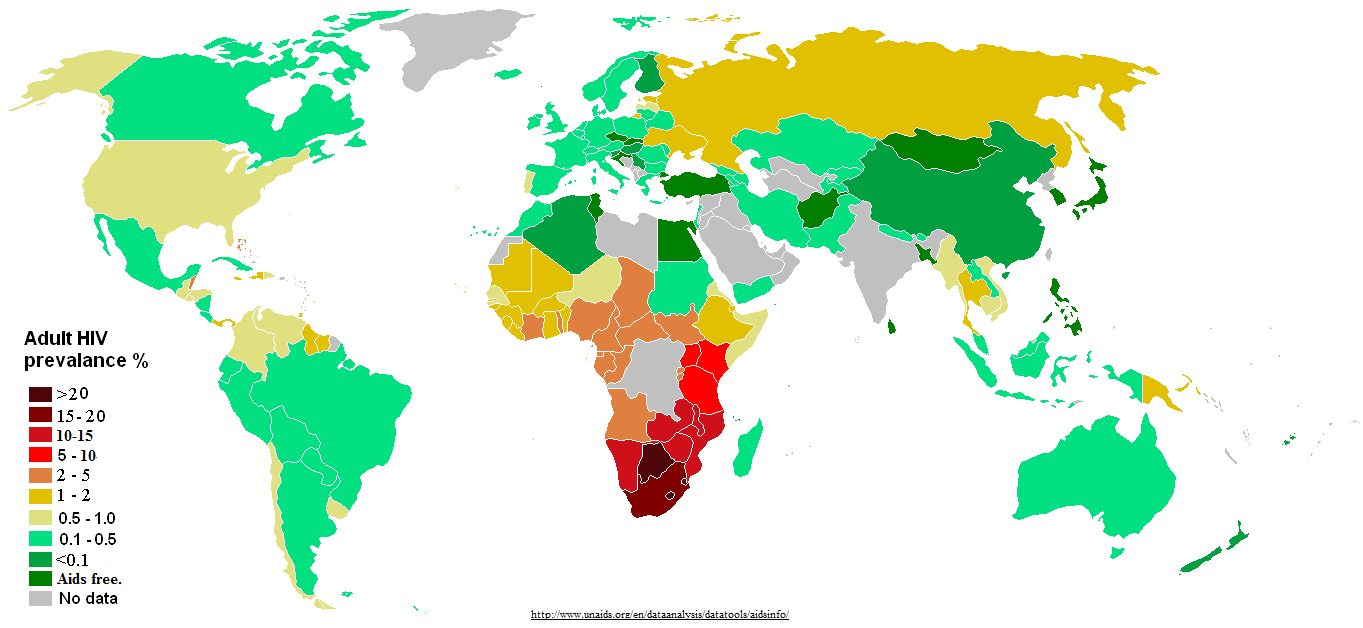


Figure 1.1: Distribution of HIV prevalence in adults around the world.

Source: http://www.unadis.org/en/dataanalysis/datatools/aidsinfo/

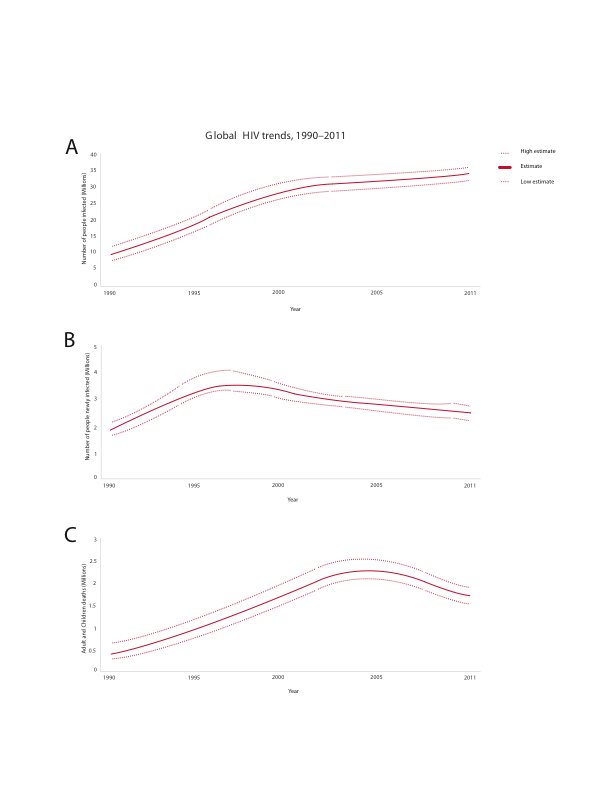


Figure 1.2: Global trend of new HIV infections from 1990 to 2011. The number of people living with HIV globally is increasing (A) while the number of people newly infected with HIV (B) and the number of Adults and child deaths due to HIV are decreasing (C) globally in the time period. This scenario can be attributed to global scale up of drugs while infected people continue transmitting the virus to uninfected people (Zaidi et al.). (Source: modified from UNAIDS 2012)

### 1.2 Discovery and characterization of HIV

As early as 1959, HIV infection cases had been documented but were unreported (Corbitt et al., 1990; Nahmias et al., 1986). Curious doctors at that time collected patient blood samples and kept frozen, which were later shown to have HIV antibodies (Zhu et al., 1998). In June 1981, a case of acute immune depletion associated secondary infection was reported in some homosexuals in the USA (Friedman-Kien, 1981; Friedman-Kien et al., 1981). Their infection was coupled with no lymphocyte proliferation (Gottlieb et al., 1981). Until 1983, the causative agent responsible for the severe immune depletion, named AIDS (Acquired Immunodeficiency Syndrome), was unknown (Francis et al., 1983; Gallo et al., 1983), when Luc Montagnier’s group at “Institut Pasteur” in Paris isolated the virus, which was initially named Human T-cell Leucamia Virus (HTLC) and later named as Human Immunodeficiency Virus (HIV) (Nahmias et al., 1986). Jay Levy’s group in San Francisco, USA also subsequently found the virus confirming the discovery in Paris (Levy et al., 1984). They found HIV was a lentivirus from Group VI retrovirus with two single strand RNA molecules (Baltimore, 1971); unique to any other previously isolated viruses and the virus can transmit from infected to healthy people (Rogers et al., 1987; Wofsy et al., 1986), mother to child through umbilical cord (Gallo et al., 1983; Ziegler et al., 1985).

Very soon, scientists around the world focused on this transmissible retrovirus. Complete sequencing of HIV genome in 1985 (Ratner et al., 1985) led scientists to know more insights of HIV including its origin, genes/proteins and life cycle (Wain-Hobson et al., 1985).

## 1.3 Origin and evolution of HIV

Exploration of the retrovirus led researchers to identify similarities between HIV and a retrovirus in African non-human primates that were then known as Simian Immunodeficiency Virus (SIV) (Gao et al., 1994). About 40 different primates, in Africa, are infected with SIV with some harboring multiple strain of SIVs (Apetrei et al., 2004). Phylogenetic analysis of SIV from African non-human primates and HIV in human provided remarkable understanding of viral transmission as zoonotic (Bailes et al., 2002) and evolution of the virus in human after transmission (Gao et al., 1999).

HIV is divided into two groups – HIV-1 and HIV-2 (Gao et al., 1999). Each group resulted from an independent cross species transmission from different African non-human primates to human (Sharp and Hahn, 2010). HIV-2 is discovered in 1986. This group is transmitted from sooty mangabey monkeys (Cercocebus atys) (Hirsch et al., 1989) and its prevalence is also high in the geographical location of these monkeys in West Africa (Santiago et al., 2005). Sooty mangabey monkeys are naturally infected by a strain of SIV (Hirsch et al., 1989). The phylogenetic analysis of HIV-2 strains shows that they closely group with the SIVsmm strain (Hirsch et al., 1989) that are non-pathogentic to its host monkeys (Gao et al., 1992). SIVsmm evolved in its host to produce multiple strains and subsequent multiple zoonotic transmissions from sooty mangabey monkeys to human (Hahn et al., 2000) gave rise to different subtypes of HIV-2. Although HIV-2 subtypes A to G are identified in human, it is assumed that more subtypes were introduced into human (Gürtler, 2004) but are lost for low adaptation fitness (Damond et al., 2004).

HIV-1 is the result of at least three cross species transmission events from chimpanzees (Pan troglodytes troglodytes (Ptt)) to human (Huet et al., 1990; Peeters et al., 1989). Phylogenetic analysis of HIV-1 sequences has shown that three independent cross transmission of the virus in to the human population, each giving rises to three sub groups: group M (Major), group O (Outlier) and group N (Non M or Non O) (Hahn et al., 2000; Keele et al., 2006). Recently a new HIV-1 strain, classified as group P, distinct from the previous three groups, has been discovered in a patient in Cameroon (Plantier et al., 2009).

Group M is the most prevalent and accounts for 98% of all infections (reviewed in (Sharp and Hahn, 2010)). Its epicenter is thought to be Kinshasha of present day Democratic Republic of Congo (Sharp and Hahn; Sharp and Hahn, 2010). Site stripping for clock detection method shows that group M and its closest simian relative branched out from their common ancestor in 17th century (Salemi et al., 2001) whereas molecular clock analysis of group M shows that the origin of it’s most recent common ancestor dates back to late 1920s (Korber et al., 2000). By 1960, long before human discovered its presence, HIV-1 group M had already diversified substantially (Worobey et al., 2008).

Group O and group N are rare and geographically confined to West African regions such as Cameroon and neighboring countries (Gao et al., 1999). It is still not understood about the non-pandemic characteristics of group O and N HIV-1 virus after the first zoonotic transmission (Ariën et al., 2005). It has been suggested that reduced replication capacity and transmission fitness are keys to their low prevalence(Ariën et al., 2005). Group O strain has at least 50% genetic identity with group M (Gürtler, 2004; VANDEN HAESEVELDE et al., 1996) and the molecular clock model of this group also showed that its origin dates back to 1920s (Lemey et al., 2004a). The date of origin of the introduction of group N into human population has been estimated to be in 1960s (Simon et al., 1998a). Phylogenetic analysis using genetic sequence under evolutionary pressure shows its close grouping with SIV from Chimpanzee (Corbet et al., 2000; Gao et al., 1999). This indicates that group N might be a recombinant strain of SIV and HIV-1 group (Simon et al., 1998a).

Group P is transmitted from gorilla as it is closely related to its SIV (Plantier et al., 2009). A study of HIV infected people in Cameroon shows its low prevalence of 0.06% (Vallari et al., 2011). Although HIV group P is discovery only in Cameroon and confined there, it can still be pandemic as it can adapt in human (Vallari et al., 2011).

### 1.4 HIV-1 Diversity

**1.4.1 HIV-1 subtypes**

HIV-1 group M is highly diversified and it is classified into nine subtypes: A, B, C, D, F, G, H, J and K (Figure 1.3) (Robertson et al., 2000b). The subtype classification is based on the phylogenetic and sequence distance analyses using gene sequence data forming major clades (Robertson et al., 2000b). “At least three epidemiologically unlinked sequences are required for defining a subtype” (Robertson et al., 2000a).

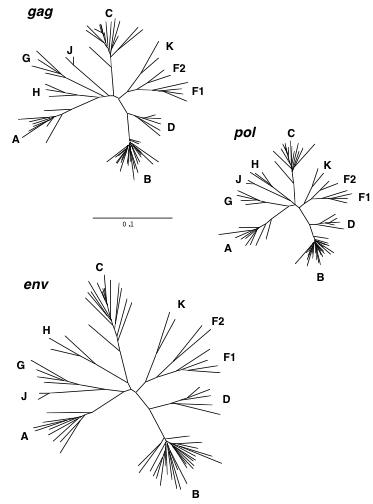


Figure 1.3: Phylogenetic tree showing HIV-1 group M diversification to subtypes A-D, F-H, J and K, inferred from nucleotide sequence alignments of gag, pol and env genes. Source: Robertson et al 2000 (Robertson et al., 2000a)

The classification of new subtype should also follow the same rule as “roughly equidistant from all previously characterized subtypes in all regions of the genome with a distinct pre-subtype branch similar to those of other subtypes” (Robertson et al., 2000b). The predominating group M subtypes are A, B, C and D (reviewed in (McCutchan, 2006)). The range of amino acid variation at gene level within a subtype and between subtypes differs from 15%– 20% and 25% - 35% respectively (Korber et al., 2001). Geographical locations of group M subtypes epidemic are show in Figure 1.4. The analysis from HIV samples collected from 70 countries in 2004 shows that “subtype C accounts for 50% of all infections worldwide” while subtypes A, B, G and D are found in decreasing order 12%, 10%, 6% and 3% respectively (Hemelaar et al., 2006). Subtypes F, H, J and K infections are rare and collectively account for only 0.94% infections (Hemelaar et al., 2006).

Subtypes can be further classified to sub-subtype based on a distinct sister clade formation (Gao et al., 2001) within a clade with the same rule of phylogenetic and distant analyses but not justifiable to call a subtype due to low genetic distance (Robertson et al., 2000b). Only subtypes A and F exhibit distinct sister clades (Gao et al., 2001). Subtype A has sub-subtypes A1 and A2 (A3 and A4 are mentioned by Taylor et al 2008(Taylor et al., 2008)); Subtype F has sub-subtypes F1 and F2 (Figure 1.3); sub-subtype F3 mentioned by Taylor et al 2008 (Taylor et al., 2008).

The lower diversity observed in Group N (Ayouba et al., 2000), O (Lemey et al., 2004b) and P (Vallari et al., 2011) correlate to low prevalence and geographical confinement to Western African countries such as Cameroon. It is still unclear if group O can be sub divided into subtypes (Roques et al., 2002). Group N as well does not show distinct sub clade within itself (Figure 1.5).

**1.4.2 HIV-1 recombination**

Initially, HIV-1 group M subtypes E (Artenstein et al., 1995; Tovanabutra et al., 2002; Wasi et al., 1995) and I (Figure 1.5) were also classified (reviewed in (McCutchan, 2006)). With availability of complete HIV genome sequence and phylogenetic analysis from it, the subtypes E and I were reclassified as circular

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Figure 1.4: HIV diversity around the globe, its level of prevalence in the area and number of genome sequenced. Source: McCutchan 2006

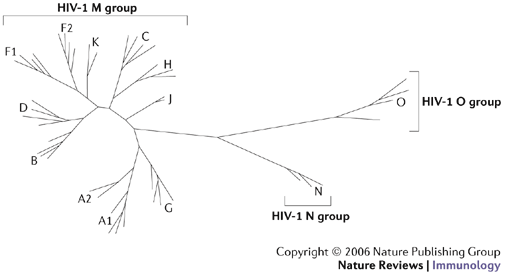


Figure 1.5: Neighboring joining phylogenetic tree showing HIV-1 group M, N and O. Group M shows distinct nine subtypes A-D, F-H, J, K while no specific subtype is observed in group N and O. Source: Letvin 2006 (Letvin, 2006)

recombinant forms CRF01\_AE (recombinant form of subtype A and E) and CRF04\_cpx (recombinant form of more than two subtypes, designated by “cpx”) respectively (reviewed in (McCutchan, 2006)). The same criterion of epidemiological unlinked isolates from three or more people applies for classification as a circular recombinant form (CRF) (Robertson et al., 2000a). A recombinant isolate discovered in single patient is termed as Unique Recombinant Form (URF) (reviewed in (McCutchan, 2006)). There are 55 CRFs listed in Los Alamos National Laboratory database for HIV sequences (<http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html>) as on July 22, 2013. The recombination breakpoints to shuffle HIV genome (Archer et al., 2008; McCutchan et al., 2002; Salminen et al., 1995; Zhang et al., 2010) from different strains of the virus are listed in <http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/breakpoints.html>, each appeared in a publication. CRFs account for at least 20% of all the HIV infections (Robertson et al., 2000a; Robertson et al., 1995; Sharp et al., 1995). CRF02\_AG is the most prevalent circulating recombinant form infecting over 9 million people on the whole (McCutchan, 2000) and geographically epidemic in the West African region (Figure 1.5). CRF01\_AE is the dominant circulating recombinant form in South-East Asia (Figure 1.5) (Taylor et al., 2008).

**1.4.3 Intra-patient HIV diversity**

HIV infection initiates mostly with a single virion (Fischer et al.; Keele et al., 2008). Evidences of multiple HIV variants transmission are also recorded (Long et al., 2000; Ping et al., 2000). Generally, HIV is genetically homogenous for a short post infection time (Delwart et al., 2002; Haase). In the long-term post infection period, virus replicates rapidly to produce genetically heterogeneous population (Long et al., 2000). This heterogeneous viral population consisting of a swarm of highly similar but genetically non-identical HIV viruses is called the HIV quasispecies (reviewed in (McCutchan, 2006)). It is observed that the diversity at a gene (*env*) in viral quasispecies can be approximately 30% (Fouchier et al., 1992). Factors that contribute to high genetic heterogeneity in viral quasispecies are high replication rate and turnover (Ho et al., 1995b), viral genome recombination (Fang et al., 2004; Gu et al., 1995; Lole et al., 1999), higher mutation rate by erroneous reverse transcriptase (Bebenek et al., 1989; Roberts et al., 1988), and host immune selection (Borrow et al., 1997; Price et al., 1997). On the whole, HIV replication (discussed in section 1.6) is the overall source of genetic heterogeneity in the viral population (reviewed in (Smyth et al.)).

Intra patientHIV genome recombination is a common event (Fang et al., 2004; Neher and Leitner). Two genomes from different viral strains from same subtype or different subtypes can be co-packed into single virion during replication (Stuhlmann and Berg, 1992). In the subsequent HIV replication, the ability of reverse transcriptase to switch between the two template genomes produces an intra subtype or inter-subtype recombined viruses at the end of the replication cycle (Ben-Artzi et al., 1996; Kostrikis et al., 2002). Genetic recombination allows rapid and efficient shuffling of advantageous genes and removing deleterious mutations, thus, increasing the viral fitness in the host (reviewed in (Smyth et al.)). Successful transmission of the recombinant forms with high viral fitness to three or more people and circulates in human population establishes Circulating Recombinant Forms (CRFs) (reviewed in (Perrin et al., 2003)), as discussed in section 1.4.2.

### 1.5 HIV genome and proteins – structures and functions

HIV has nine genes and produces 15 proteins (Frankel and Young, 1998). The genes are broadly grouped as accessory (vif, vpr, vpu and nef), structural (*pol*, *gag* and *env*) and regulatory (*tat* and rev). The higher number of proteins than genes is a result of post - transcriptional proteolysis of the products of structural genes (Frankel and Young, 1998).

**1.5.1 Accessory genes:**

Vif promotes the viral infectivity to the host, but has no role in viral production (Jager et al.). Vif is produced in the late stage of viral production (Sheehy et al., 2002; von Schwedler et al., 1993) to suppress the innate antiviral immunity of host (Madani and Kabat, 1998; Simon et al., 1998b). It is observed that vif is expressed only when the virus infects immune cells that express cytidine deaminase APOBEC3G (Navarro and Landau, 2004). The reason is that Vif protein prevents APOBEC3 proteins from hyper mutating HIV reverse transcripts as a mechanism of defense (Conticello et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Marin et al., 2003; Mehle et al., 2004; Schafer et al., 2004; Sheehy et al., 2003; Simon et al., 2005; Stopak et al., 2003; Wiegand et al., 2004; Zhang et al., 2003).

Vpr protein is packed in to nascent virions during budding out (Connor et al., 1995). Vpr is essential for viral core to enter and localize in the host cell nucleus after infecting the cell (Cohen et al., 1996). Vpr arrests the cell cycle during the transfer from G2 to M phase (Jowett et al., 1995; Rogel et al., 1995) by preventing the activation of the human p34cdc2/cyclin B complex (He et al., 1995). Vpr is also important for efficient viral replication in monocyte or macrophage cells, but T-cells (Connor et al., 1995).

Vpu is a protein unique to HIV-1 (Cohen et al., 1988) and is 16 kilo Dalton, 81 amino acids long (Strebel et al., 1988). Biological functions of vpu protein include degradation of CD4 in endoplasmic reticulum (Willey et al., 1992), interference of host immune cell MHC class II antigen presentation on the cell surface allowing the virus for host immune escape (Hussain et al., 2008; Nomaguchi et al., 2008) and viral maturation and release from host cell membrane (Klimkait et al., 1990).

Nef has no role in viral infectivity but plays a role during the biogenesis of viral particles (Laguette et al., 2009) and virulence (Lenassi et al.; Simmons et al., 2001). Nef down regulates the production of major histocompatibility complex type I (MHC type I) in the host cell (Blagoveshchenskaya et al., 2002; Greenberg et al., 1998; Lewis et al., 2012). This impairs the function of cytotoxic T lymphocyte cells to clear the infected cells (Adnan et al., 2006; Baur et al., 1994; Collins et al., 1998; Couillin et al., 1994; Sawai et al., 1994). Nef also down regulates CD4 on host cell surface (Garcia and Miller, 1991; Lama et al., 1999) and modulates cellular activation to evade host immune system (Baur et al., 1994; Sawai et al., 1994).

**1.5.2 Structural genes and proteins**

The Gag gene produces a precursor polyprotein (pr55gag) of ~ 500 amino acids long and weighs 55 kilodalton (Briggs et al., 2004). The Gag precursor has all the building blocks to form a fully infectious virion, even in the absence of other viral products (Wang and Barklis, 1993). A proteolytic cleavage of gag precursor yields the structural proteins – matrix, capsid, nucleocapsid and p6 (Figure 1.6) (Wiegers et al., 1998). The cleavage takes place in the nascent virus after budding out from host cell (Göttlinger et al., 1989). The matrix protein is at the N-terminal and p6 at the C-terminal of gag precursor (Borsetti et al., 1998; Wiegers et al., 1998).

All gag proteins play role at “post assembly and post processing stages in viral infectivity” (Wang and Barklis, 1993). In the HIV replication cycle, matrix domain of gag plays role in targeting gag precursor to the plasma membrane of the host cell and the viral assembly at the site (Dawson and Yu, 1998; Gheysen et al., 1989; Wang and Barklis, 1993; Zhang et al., 1998). The highly basic region in matrix mediates electrostatic association with phospholipids in plasma membrane during assembly (Chukkapalli et al.; Zhou and Resh, 1996). The capsid is a curved and closed shell consisting 250 hexamers and 12 pentamers (reviewed in (Briggs and Krausslich)). Capsid packs viral proteins, nucleocapsid and viral genome during assembly to pass on to new HIV particles (Ganser-Pornillos et al., 2004). Nucleocapsid plays role in efficient viral assembly by making pr55–pr55 inter-protein contacts (Dawson and Yu, 1998; Zhang et al., 1998) and localization of viral proteins (Larsen et al., 2008). P6 protein plays role in detaching and releasing the newly formed HIV particles (Demirov et al., 2002).

The *pol* polyprotein is produced by translational frame shift (Karacostas et al., 1993) (Figure 1.5), such that, as much as 241 nucleotides at 5’ region of the gene overlaps with 3’ region of *gag* gene (Jacks et al., 1988; Ratner et al., 1985). The proteolytic cleavage of *pol* precursor produces essential viral replication enzymes – protease (PR), reverse transcriptase (RT) and integrase (IN). The protease enzyme cleaves the *gag* and *pol* polyprotein to form the viral structure proteins and functional enzymes respectively (Darke et al., 1988b; ERICKSON-VIITANEN et al., 1989; Nutt et al., 1988). The reverse transcriptase enzyme reverse transcribes the viral RNA to produce a cDNA molecule after infecting host cell (Jacobo-Molina and Arnold, 1991; Sarafianos et al., 2009). The RNase H domain in RT degrades the viral RNA molecule following cDNA production (Davies et al., 1991). The integrase enzyme removes two bases from 3’ DNA molecule and functions strand transfer during the process of integrating the proviral DNA into the host genome (Pruss et al., 1994).

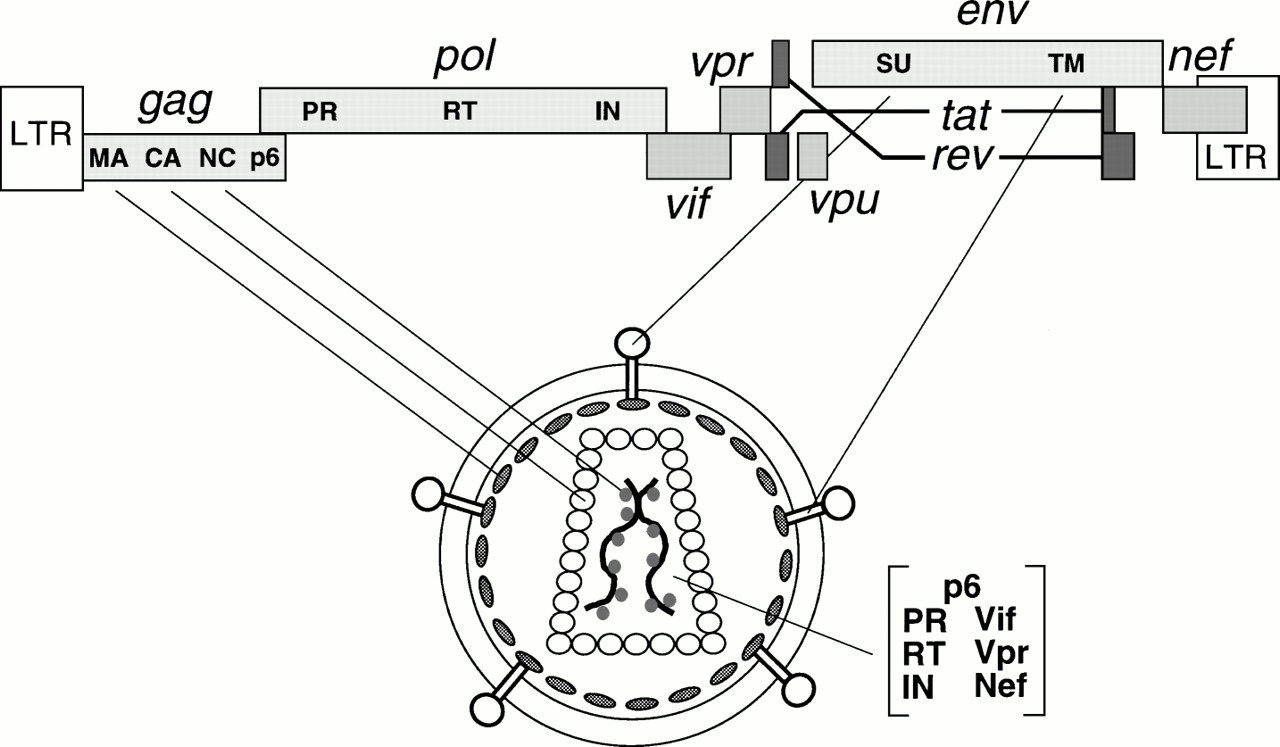


Figure 1.6: HIV genes and proteins positions in the viral genome and their viral parts. Source: Frankel and Young 1998 (Frankel and Young, 1998)

The *env* gene produces a precursor glycopolyprotein (gp160) that is processed at post-translational by human convertase enzymes - PC1 and furin to produce glycoprotein 120 (gp120, HIV-1 SU) and glycoprotein 41 (gp41, HIV-1 TM) (Decroly et al., 1994). Gp120 is a non-covalent complex of external protein and gp41 is a trans-membrane protein; both play vital role for initial steps in viral infection (Chan et al., 1997). Three gp120 molecules bound with three gp41 molecules to form envelop spikes (Pancera et al.). They are organized to form trimeric complexes on the surface of HIV and mediate HIV entry into the host cell (Liu et al., 2008). The exposed external complex gp120 binds to the CD4 receptor on the host immune cell (Rizzuto et al., 1998). This triggers a conformational shift of trimeric complex that enables a conserved gp120 region binding to a chemokine receptor, either CCR5 or CXCR4, to facilitate fusion of the viral and host membranes (Huang et al., 2007; Rizzuto et al., 1998; Wu et al., 1996; Wu et al., 1997). The gp120-CD4 complex also triggers conformational change in gp41 trans-membrane protein from native non-fusion state to fusion state (Chan et al., 1997; Kliger et al., 1997). Gp41 plays role in the viral fusion and release of viral contents in to the host cell (Furuta et al., 1998; Melikyan, 2008),. The gp41 consists of heptad repeats - HR1 and HR2 that play role in fusion process (Furuta et al., 1998; Tan et al., 1997). HR1 is a bundle of three helical motifs and HR2 is trimeric coiled coil structure (Dwyer et al., 2003). During fusion process, HR2 makes numerous contacts with HR1 to form stable six helical bundles (Melikyan et al., 2000).

**1.5.3 Regulator Genes:**

*Tat* is a trans-activating factor localized in the nucleus for HIV gene expression (Rosen and Pavlakis, 1990a; Roy et al., 1990). The HIV proviral genome integrated in to the host genome is regulated by cellular as well as the viral transcription regulatory factors (Cullen, 1991; Gaynor, 1992). Tat is the primary transcriptional regulatory factor (Marcello et al., 2001). An example of Tat action is the control of RNA polymerase II elongation during transcription, which otherwise disengages from the template DNA strand, terminating the transcription prematurely (Bourgeois et al., 2002; Chou et al.; He and Zhou). Sodroski *et al*. (1985) first explained the function of Tat (Sodroski et al., 1985).

Rev is a 19 kilo Dalton phosphoprotein (Malim et al., 1989a) trans-activating factor for HIV gene expression (Rosen and Pavlakis, 1990a). Like Tat, it is also mainly localized in the nucleus of host cell (Rosen and Pavlakis, 1990b), but cycles rapidly between the nucleus and cytoplasm as it promotes nuclear export of the transcriptional products (Fischer et al., 1995; Fischer et al., 1994; Henderson and Percipalle, 1997; Malim et al., 1989b). Rev binds at the Rev Responsive Element (RRE), which is an RNA element encoded within the *env* region of the virus (Daly et al., 1989; Malim and Cullen, 1991).

### 1.6 HIV replication

There are major 11 events in HIV replication cycle (as numbered in Figure 1.7). The initial step of viral entry (Figure 1.7 step 1) in to a host immune cell includes HIV gp120 molecule binding to CD+ receptor followed by binding to a co receptor on the surface of the host cell and fusion of the viral and host cell membranes (see section 1.5.2 for more detail).

Following the fusion, the viral core enters in to the cytoplasm of host cell. The reverse trancriptase enzyme reverse transcribes the RNA molecule to cDNA (Figure 1.7 step 2) in the intact capsid of the viral core (McDonald et al., 2002). The reverse transcriptase enzyme is not perfect at copying mRNA molecule to cDNA and has no error correct capability (Bebenek et al., 1989; Bebenek et al., 1993; Preston et al., 1988; Roberts et al., 1988). The enzyme generates mutations in its genome at the rate of 10-5 per base per replication cycle (Mansky and Temin, 1995). This is a crucial step as it contributes to generation of variations in the viral quasispecies (reviewed in (Goodenow et al., 1989; Nowak et al., 1990)).

Subsequently the capsid is dissembled, termed as uncoating (Figure 1.7 step 3) (McDonald et al., 2002; Shah et al., 2013), releasing ribonucleoprotein complex in to the cytosol (Dismuke and Aiken, 2006). The process can take an hour or less since time of post infection (Hulme et al., 2011). The capsid and nucleocapsid proteins dissociate from cDNA but the reverse transcription complex remains intact along with viral matrix, integrase, vpr and human protein high mobility group I (HMG I (Y)) forming pre-integration complex (PIC) (Bukrinsky et al., 1993; Farnet and Haseltine, 1991; Miller et al., 1997). The PIC protects cDNA from endonuclease degradation (Miller et al., 1997). In an ATP dependent process (Bukrinsky et al., 1992), PIC is transported on host microtubules towards the nuclear membrane (McDonald et al., 2002). Some studies initially suggested that formation of central DNA flap (Zennou et al., 2000), vpr, and matrix mediate nuclear import (Figure 1.7 step 4) of PIC (Popov et al., 1998), however, the no any clear evidence of these factors have been described as such for nuclear import (Riviere et al.). Integrase assists in nuclear import in association with nuclear import machinery like importin (Fassati et al., 2003) and transportin-SR2 (Christ et al., 2008). It is now established that central polypurine tract-central termination sequence (cPPT-CTS) plays role in kinetics of nuclear import (Riviere et al.).

Post-nuclear entry, integrase processes the viral DNA for integration (LaFemina et al., 1992) (Figure 1.7 step 5) into host genome as explained in section 1.5.2. Host transcriptional co-factor LEDGF/p75 and HIV integrase interact to tether to the host chromosome during the integration process (Emiliani et al., 2005).

The integrated HIV provirus hijacks the host cell transcriptional machinery for viral genes to transcribe (Figure 1.7 step 6) (Davey et al., 2011). HIV protein Tat promotes the transcription of the viral DNA (Ott et al., 2011; Razooky and Weinberger, 2011). The viral transcriptome encodes structural proteins, accessory proteins and viral enzymes necessary for a complete functional HIV (reviewed in (Karn and Stoltzfus)).

The viral Rev protein facilitates exporting (Figure 1.7 step 7) of the unprocessed viral transcriptome to cytoplasm for translation (Malim et al., 1989b). HIV has no translation system of its own; the host translational machinery is exploited for translation (Figure 1.7 step 8) of the viral transcriptome to its proteome (Cherry et al., 2005; Thompson and Sarnow, 2000).

Upon translation of all viral proteins, viral *gag* initiates virion assembly (Figure 1.7 step 9) at the cell membrane (Dong et al., 2005; Nermut et al., 1998; Saad et al., 2006). The complete assembled virion particles bud out and are released (Figure 1.7 step 10) from the plasma membrane by the host ESCRT machinery involving Tsg101 and ALIX regulatory proteins (Fujii et al., 2007; Garrus et al., 2001; Saksena et al., 2007). The maturation (Figure 1.7 step 11) of the nascent HIV virions begins concomitantly with budding out (Klimkait et al., 1990; Schubert et al., 2000). *Gag* and *Pol* polyproteins are proteolytically cleaved by protease enzyme in the maturation step (Darke et al., 1988a; Pettit et al., 2005).

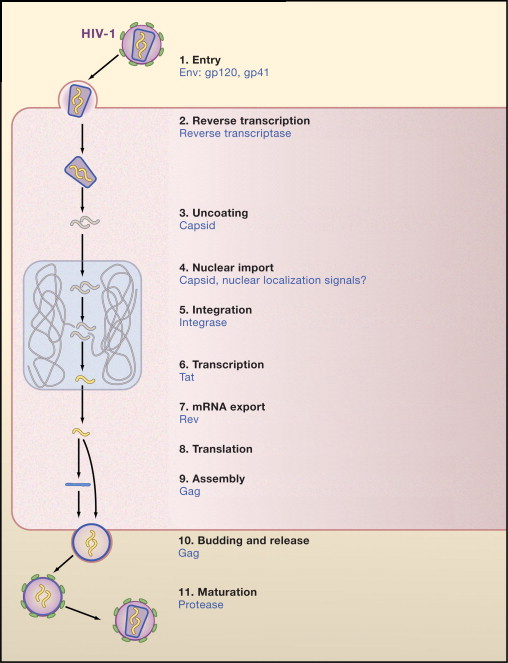


Figure 1.7: The HIV replication cycle showing major stages. Viral proteins that play role in each event are colored blue. (Source: modified from Ho and Bieniasz 2008) (Ho and Bieniasz, 2008)

Each HIV replication cycle releases new infectious virions in the order of 109 per day (Ho et al., 1995a). The number of new infecting HIV determines the replication rate of the virus (Tersmette et al., 1989). A long post infection period shows higher turnover rate associated with CD4+ cell depletion and viral population expansion (Ho et al., 1995a).

### 1.7 Anti Retro Viral Drugs

Currently different inhibitory drugs, each interfering a viral protein normal function at different stages of its replication cycle has been developed clinical practice. Depending upon the targets, drugs used in clinical HIV treatment are grouped as reverse transcriptase inhibitors, protease inhibitors and integrase inhibitors (reviewed in (De Clercq, 2002, 2005a, 2009)).

**1.7.1 Reverse Transcriptase Inhibitors**

1. **Nucleoside Reverse Transcriptase Inhibitors (NRTIs)**

NRTIs are analogs of normal nucleotides but without 3’ hydroxyl group (reviewed in (Sarafianos et al., 2004)). The drug is taken in unphosphorylated form, which cytokinases phosphorylates to form 5’ triophosphates (reviewed in (De Clercq, 2002; Ilina and Parniak, 2008)). It leads to incomplete termination of cDNA synthesis (Sluis-Cremer et al., 2000). There are eight NRTI drugs currently used in ART drug regimen: zidovudine, lamivudine, stavudine, didanosine, zalcitabine, abacavir, emtricitabine and tenofovir (Sarafianos et al., 2009).

1. **Non-nucleoside reverse transcriptase inhibitors (NNRTIs)**

NNRTIs specifically bind at an allosteric site 10 [Å](http://en.wikipedia.org/wiki/%C3%85) from polymerase active site of the HIV-1 reverse transcriptase (Himmel et al., 2006; Sarafianos et al., 2009), close to substrate-binding site. The binding induces conformational change in the enzyme, which distorts the catalytic aspartate triad of active site and inhibits the function of the enzyme (Balzarini, 2004; Esnouf et al., 1995). NNRTIs drug are administered along with NRTI drugs in ART at non-toxic concentration to human cells (De Clercq, 1996). NNRTIs drugs from first generation have inherent disadvantageous characteristics like cross-resistance and low genetic barrier i.e. require only one mutation to select (Adams et al., 2010). There are six NNRTIs drugs used in ART drug regimen are Nevirapine, Delavirdine, Efavirenz, Dapivirine, Etravirine and Rilpivirine (Sarafianos et al., 2009).

**1.7.2 Protease Inhibitors (PR)**

Generally, protease inhibitors are added to second line ART therapy following failure of first line therapy (Pujades-Rodriguez et al., 2008). Protease Inhibitors interfere with cleavage of *gag*-*pol* polypeptide (Seelmeier et al., 1988) as competitive peptidomimetic inhibitors. The hydroxyethylene core in the inhibitors prohibits the cleavage action of the HIV protease enzyme (Vacca et al., 1994; Vacca et al., 1991). However, as an adverse side effect, patients consuming the inhibitors have developed lipodystrophy and hyperlipidemia (Carr et al., 2000; Carr et al., 1998a; Carr et al., 1998b, c; Liang et al., 2001; Miller et al., 2000; Tsiodras et al., 2000). There are nine protease inhibitors approved for clinical use: Saquinavir, Indinavir, Ritonavir, Nelfinavir, Amprenavir, Lopinavir, Atazanavir, Tipranavir and Darunavir (Chow et al., 2009).

**1.7.3 Integrase Inhibitors**

Although the anti retroviral therapy could suppress the viral replication, they are expensive, can lead to multiple drug resistance and, thus, require high levels of adherence (Cohen, 2002). These necessitate alternative drugs that target the third enzyme, the integrase. The feasibility and efficacy of integrase inhibitors have been tested in Rhesus Macaques (Hazuda et al., 2004a). Most of the integrase inhibitors target strand transfer function of the enzyme (Bera et al., 2011; Espeseth et al., 2000; Hazuda et al., 2000; McColl and Chen, 2010; Pannecouque et al., 2002). X-ray structure of the integrase enzyme has revealed the active site model of the enzyme complexes with the DNA (Chen et al., 2008). Diketo acids (DKA) and its derivatives (like L-708,906, S-1360, and V-165 (Fikkert et al 2003)) are the highly potent integrase inhibitors for strand transfer inhibition (Hazuda et al., 2000). Low concentration of DKA is sufficient for effective integrase inhibitor (reviewed in (Pommier et al., 2005)). Integrase inhibitors approved for use are: Raltegravir, MK-2048, Elvitegravir, Diketo Acid derivatives (L-731988, L08841411, 118-D-24), Naphthyridine Carboxamides (L-870810, L-870812) and Quinolinonyl Diketo (RDS 1997, RDS 2197) (Bera et al., 2011).

**1.7.4 Cell entry inhibitors**

The cell entry inhibitors interfere viral binding or fusion of HIV to a host cell. These drugs are added to salvage drug regimen for patients showing multiple high anti viral drug resistance. Following are the two classes of cell entry inhibitors.

1. **CCR5 co receptor antagonist:**

Maraviroc is the only CCR5 antagonist in clinical use (De Clercq, 2005a, b; Fätkenheuer et al., 2005; Rosario et al., 2005; Rosario et al., 2006; Wheeler et al., 2007). It is the only anti retroviral drug that does not target any viral enzyme or protein molecule, but host cell receptor CCR5 (Westby and van der Ryst, 2005). Binding of CCR5 antagonist renders unavailability of the co receptor for HIV gp120 binding, thereby preventing the viral entry in to the cell . However, it is important for HIV tropism test before administrating this drug, as the drug is ineffective against CXCR4 co receptor using viruses.

1. **Fusion Inhibitors**

Fusion inhibitor design is based on targeting the heptad regions HR1 or HR2 of gp41 disabling the virus to make fusion pore (reviewed in (Baldwin et al., 2003)). Enfuvirtide (Duffalo and James, 2003; Poveda et al., 2005) is a synthetic peptide, approved for clinical use in 2003 (Robertson, 2003), which can bind to gp41 HR1 region (Wild et al., 1993). The fusion inhibitor T-1249 also targets HR1 region (Kilby and Eron, 2003). T-1249 is active against Enfuvirtide resistant viral strains but discontinued in 2004 for clinical use (Briz et al., 2006; Melby et al., 2007). Sifuvirtide is another HIV fusion inhibitor peptide under research (Wang et al., 2009).

### 1.8 HIV Treatment

The World Health Organization (WHO) has produced year 2013 clinical guideline (<http://www.who.int/hiv/pub/guidelines/arv2013/art/en/index.html>) for HIV treatment. From the treatment point of view, the guideline has grouped HIV infected people as Adult, pregnant and breast feeding women, children less than 3 years old, children 3 or more years old and HIV people co-infected with other diseases. Regardless of the grouping, the guideline recommends treatment initiation to all confirmed HIV infected people with CD+ cell count less or equal to 350 per mm3. However, in resource poor settings, where laboratory tests for CD+ count may not be feasible, WHO clinical stages should be used as a guide for treatment initiation (Weinberg and Kovarik, 2010).

According to the WHO consolidated anti retroviral guideline recommendation for HIV treatment, a combination of three reverse transcriptase inhibitors (2 NRTIs + 1 NNRTI) are used as first line therapy. A protease inhibitor is recommended for children below 3 years old. On virologic failure to first line, second line drug regimen containing 2 NRTIs and a ritonavir boosted protease inhibitor is recommended. Following failure to second line therapy, a new drug class – integrase inhibitor is introduced in third line therapy along with a reverse transcriptase and a protease inhibitor.

Patients under ART therapy are monitored for effectiveness of the treatment at a defined interval. Laboratory test for viral load should be done for monitoring the treatment response. The viral load of greater than 1000 viral RNA copies/ml blood sample indicates virologic failure to the treatment and the patient is recommended to switch to new drug regimen. If viral load test is not feasible routinely, CD4+ count and clinical monitoring need to be used.

### 1.8 HIV Drug Resistance

Anti retroviral drug therapy has been successful at controlling HIV replication to low viral load within a host (Rosenberg et al., 2000). The therapy has been compromised and threatened for long term sustainability by the emergence of drug resistant HIV viral variants (reviewed in (Clavel and Hance, 2004)). HIV mutates spontaneously in every replication cycle (di Marzo Veronese et al., 1993; Dumonceaux et al., 1998); the error prone reverse transcriptase introduces mutations randomly at the rate of 10-5 per base per replication cycle (Mansky, 1996; Mansky and Temin, 1995). The accumulation of mutations and viral recombination contribute the most for the emergence of drug resistant variants (reviewed in (Menéndez-Arias, 2002; Smyth et al., 2012; Tebit et al., 2007)). In the presence of anti retro viral drugs the HIV variants in the quasispecies get selected (Leslie et al., 2004; Nijhuis et al., 1999). The selected variants proliferate to high number in subsequent replication cycles (Hosseinipour et al., 2013). Transmission of resistant virus also confers drug resistance in drug naïve patients (Boden and Markowitz, 1998; Wensing et al., 2005; Yerly et al., 1999). Although drug resistant mutations escape drug effect, they confer decrease in viral fitness and replication rate at certain level (Lucas, 2005; Mammano et al., 2000). Other mutations counteract the effect of drug resistant mutations to boost up viral fitness and replication rate (Nijhuis et al., 1999).

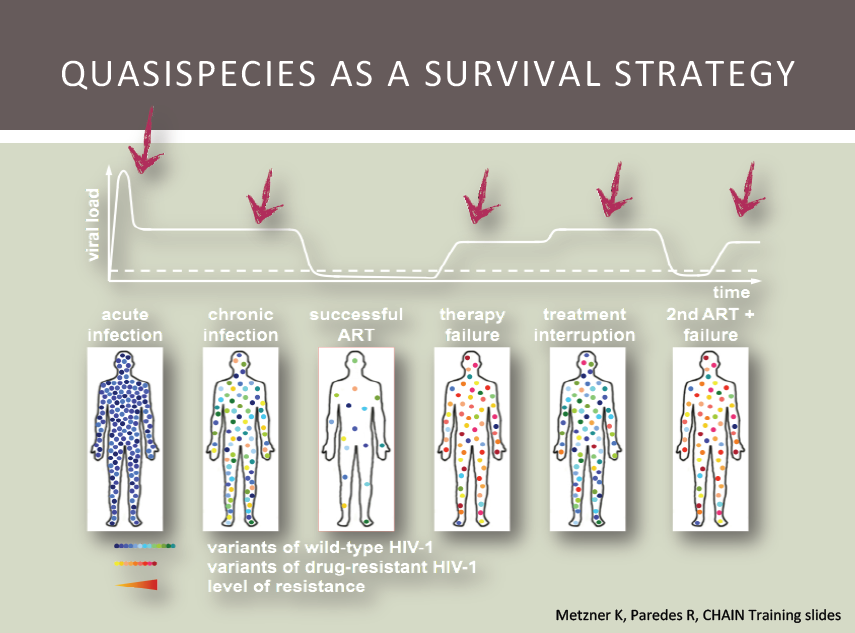
ART drugs are designed based on three dimension structures of the viral proteins to bind at a specific active region of the proteins and interfere with its normal function (reviewed in (Tantillo et al., 1994)). In general, mutations at the active site amino acid residues of HIV proteins change their three-dimensional shape, inactivating the drug functions (Figure 1.12) (Fikkert et al., 2003; Hsiou et al., 2001; Miller and Larder, 2000; Tantillo et al., 1994). Some other modes of resistance development are: hydrolytic removal of bound NRTIs mediated by nucleotide excision mutations (Nikolenko et al., 2005), low pharmacokinetics of protease inhibitors (Kempf et al., 1997), co receptor switch from CCR5 to CXCR4 (Moncunill et al., 2008a; Regoes and Bonhoeffer, 2005)

Depending upon the drug class, the mode of developing resistance may be different. These mutations, attributing resistance to drugs, have been well documented with associated drug and resistance level (Shafer, 2006). The viral quasispecies sequence information can be used to align against the reference sequence and infer the presence of any drug resistant mutation (Kuiken et al., 2003).

**1.8.1 Mechanisms of drug resistance**

HIV gains drug resistance against nucleoside reverse transcriptase inhibitors with substitution mutations (K65R, D67N, T69D, K70R and L74V) close to the nucleotide-binding site. The substituted amino acids either fold over the triphosphate group of the incoming nucleotide analog (Huang et al., 1998) or exerts steric clash with the oxathiolane ring of the inhibitors and interferes with its binding (Sarafianos et al., 1999). Another mechanism of gaining resistance to NRTIs is hydrolytic removal of analog inhibitors mediated by nucleotide excision mutations (Nikolenko et al., 2005). Mutations in RT (M41L, L210W, T215F/Y and K219Q/E) remove NRTIs from the elongating nucleotide chain through phosphorolysis mediated either ATP or pyrophosphate (Arion and Parniak, 1999; Boyer et al., 2002; Mas et al., 2000).

NNRTI drugs interacts with reverse transcriptase enzyme residues and forms electrostatic forces, van der waals forces and hydrogen bonds (reviewed in (Menéndez-Arias, 2002)). Mutations in p66 and p51 subunits of the enzyme hinder drug interactions and bond formation (Tantillo et al., 1994), rendering drug resistance. A single mutation confers viral resistance to NNRTI drugs nevirapine and delavirdine (Hsiou et al., 2001) whereas two mutations are required for resistance against NNRTI drugs efavirenz and capravirine (Ren J. et al., 2001) showing lower and higher genetic barrier respectively.



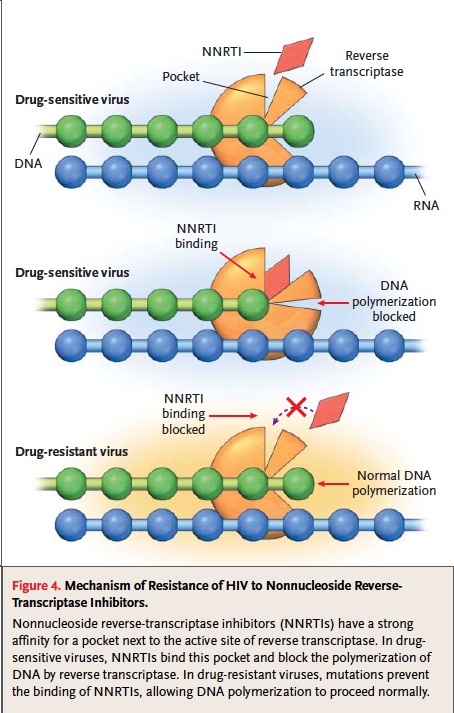


Figure 1.12: Development of HIV drug resistance. Accumulation of Drug resistant mutations changes three-dimensional confirmation of a viral protein, disabling drug binding and carrying out its normal function.

Low pharmacokinetics of protease inhibitors in plasma is responsible for emergence of resistance HIV variants (Kempf et al., 1997). Suboptimal therapy in which a PI is combined with reverse transcriptase inhibitors suffers low pharmacokinetics as protease inhibitors are eliminated by cytochrome P450 from plasma (Dresser et al., 2000). PI doses are kept high in plasma, with adminstration of low dose of ritonavir that suppress cytochrome P450 activity (Danner et al., 1995; Kempf et al., 1997; Sham et al., 1998; van Heeswijk et al., 1999). This delays the emergence of resistant viruses (Molla et al., 1996a; Zhang et al., 1997). HIV also attains protease inhibitor resistance with mutations at cleavage site of *gag* polyprotein (Zhang et al., 1997).

Integrase inhibitors are characterized as low genetic barrier drugs and a mutation can cause high level of resistance (Fikkert et al., 2003). Integrase inhibitors engage divalent metal ions Mg++ and Mn++ at the integrase active site (Grobler et al., 2002). Mutations at the active site of integrase interfere the available space for chelating moieties rendering unsuccessful engagement of the metals (Hazuda et al.). However, some mutations are distal to the metal binding active site and resistance exhibited by these mutations suggests inhibitor-enzyme interactions may extend beyond the active site of integrase enzyme (Miller and Hazuda). HIV is able to switch between the resistance pathways (N155H pathway to Q148H/G140S pathway in case of drug raltegravir) to escape the drug (Mukherjee et al.).

A reason to CCR5 co receptor antagonist resistance development is that HIV switch the use of co receptor to CXCR4 at late post infection (Moncunill et al., 2008a; Regoes and Bonhoeffer, 2005).

The resistance mechanism of HIV against CCR5 mechanism is explained as either competitive or non-competitive (Kuhmann et al., 2004; Moncunill et al., 2008b; Pugach et al., 2007). In competitive resistance mechanism, HIV gp120 changes the confirmation that has high affinity for inhibitor-free CCR5 co receptors as it can only bind to the co receptor at non-saturating concentration of the inhibitor (Gorry et al., 2002; Koning et al., 2003). Increasing the drug concentration might overcome the competitive resistance (Trkola et al., 2002). In non-competitive resistance, HIV gp120 acquires the ability to bind to inhibitor bound co receptor but less efficiently than free co receptor (Pugach et al., 2007). The resistance mechanism of non-competitive resistance is still under cover (Cormier and Dragic, 2002; Huang et al., 2007; Tsamis et al., 2003). The binding of CCR5 antagonist alters the confirmations of binding site in the co receptor that is not recognized by the incoming HIV gp120 (Kondru et al., 2008). Resistant HIV changes gp120 confirmation that recognizes the new confirmation of inhibitor bound co receptor (reviewed in (Briz et al., 2006)).

Mutations at heptad region at N-terminal of gp41 confer resistance to entry inhibitor, Enfuvirtide (Rimsky et al., 1998; Wei et al., 2002). The highly variable regions in gp120/gp41 are also responsible for resistance against fusion inhibitor (Blair et al., 2000; Miller and Hazuda, 2004). Viruses displaying fast fusion kinetics to CD4 and higher affinity to co receptor show reduced susceptibility to fusion inhibitor than the viruses with lower fusion kinetics to CD4 and lower affinity to co receptor (Miller and Hazuda, 2004; Reeves et al., 2002).

### 1.9 Conventional Population Based HIV Drug Resistance Genotyping

The primary cause of virologic failure of anti retro viral therapy is the evolution of HIV variants resistant against the selected drugs regimen (Hirsch MS, 1998). The pattern of drug resistant mutations in HIV has strong relation with virologic failure (Condra et al., 1995; Larder et al., 1989; Larder and Kemp, 1989; Lorenzi et al., 1999; Molla et al., 1996b; van Leeuwen et al., 1995; Zolopa et al., 1999). The application of HIV genotype information on therapy optimization has shown virologic benefits in clinical settings (Baxter et al., 2000; Durant et al., 1999; Van Vaerenbergh, 2001). Conventional HIV genotyping involves Sanger dideoxy termination sequencing method (Ewing et al., 1998; Metzker, 2005; Sanger et al., 1977). The method produces a chromatogram that represents the sequence of the viral population in the host (Struck et al.).

Conventional population based sequencing method has limited sensitivity for detecting viral variants of frequency greater or equal to 20%; low prevalent variants go undetected (Ji et al.; Johnson and Geretti). Thus, sequencing technology underestimates the total variants in the viral population (Palmer et al., 2005). Undetected low frequency HIV variants are clinically significant for designing salvage therapy, as they are usually drug resistant (Paredes et al.; Rowley et al.; Simen et al., 2007; Simen et al., 2009). Those minor variants rebound in the presence of drugs, leading to ultimate virologic failure (Paredes et al.; Rowley et al.). This necessitates improved and highly sensitive sequencing technologies able to detect minor HIV variants in the viral quasispecies (reviewed in (Metzker)) .

### 1.10 Next Generation Sequencing technologies

The newer sequencing technologies developed as an alternative to automated Sanger sequencing is referred as Next Generation Sequencing (NGS) technologies. Commercially available NGS technologies in the market are Roche 454, Illumina, Applied Biosystems SOLiD technology and Ion torrent (reviewed in (Metzker)). All the NGS systems has PCR step for DNA amplification but differ in other features (Shokralla et al.); each one outperforms the others at a particular application (reviewed in (Metzker)).

Roche 454 and Illumina implement ‘Sequencing by synthesis’ (SBS) technique for DNA sequencing (Margulies et al., 2005). Both systems detect fluorescent light emitted from each nucleotide incorporation event during DNA synthesis (Margulies et al., 2005) (reviewd in (Shendure and Ji, 2008)). The intensity of fluorescent light emitted per base addition is too small for detection; this requires amplification of the template DNA to million copies. Simultaneous addition of same nucleotide in million copies produces higher intensity of detectable fluorescent light (reviewed in (Metzker; Shendure and Ji, 2008)). A defined order of free nucleotide molecules are flown in the reaction plate, allow nucleotide to incorporate, detect fluorescent light and wash off any unincorporated nucleotides for next cycle. Roche 454 and Illumina differ at the sequencing step. In Roche 454, polymerase continues nucleotide addition reactions until the base flowing in the reaction plate is complementary to the template sequence. The intensity of fluorescent light emission is detected and the number of bases subsequently added, as a homopolymer run, is calculated based on the light intensity in a particular reaction cycle (Margulies et al., 2005). Illumina, on the other hand, stops the reaction after single nucleotide addition (Bentley et al., 2008). The light emission is detected that determines the base added in that particular cycle of base flow ((Bentley et al., 2008), reviewed in (Shendure and Ji, 2008)). These different sequencing techniques have high impact on sequenced read length. Roche 454 produces high quality sequences of longer read length (Table 1.1). Illumina produces shorter reads in high quantity (Table 1.1).

Applied Biosystems SOLiD implements ‘Sequencing by ligation’ technique for DNA sequencing; thus, bypassing any DNA polymerase related sequencing error (Pandey et al., 2008). The template DNA is amplified in similar way to previous NGS technologies. During sequencing, a universal primer and a library of pre-designed 1,2-probes of 8 nucleotides (or dibase probe) along with DNA ligase enzyme is added. The probes hybridize to the complementary template sequence and the fluorescence of the probe is read. The probe hybridization is repeated for seven cycles extending read length to 35 bases. In the next cycle, a new universal primer is hybridized at offset position of one base (n-1) to the previous primer position followed by ligation sequencing process. The primer resetting cycle is repeated five times providing dual measurement of each base and the final sequence is decoded from color code information using 4 by 4 color code (reviewed in (Mardis, 2008)).

Ion torrent technology implements SBS and electronic sensors connected to CMOS integrated circuit with a microprocessor for signal processing (Jakobson et al., 2002; Milgrew et al., 2004). The sequencing step is similar to Roche 454 homopolymer sequencing but the base detection is completely electronic. During DNA sequencing, a base incorporation

Table 1.1: Comparative analysis of different NGS systems. Source: modified and adapted from Shokralla et al 2012 (Shokralla et al.), Niedringhaus et al 2011 (Niedringhaus et al., 2011) and Glenn 2011 (Glenn, 2011)

| Platform | Read length (bp) | reads/run | Sequencing output/run | Run time | Advantages | Primary applications |
| --- | --- | --- | --- | --- | --- | --- |
| Roche 454 GS FLX | 400–500 | 1 × 106 | ≤500 Mb | 10 h | Longest read lengths among 2nd generation, high throughput compared to 1st generation sequencing | 1\*, 2, 3\*, 4, 7, 8\* |
| Roche 454 GS FLX+ | 600–800 | 1 × 106 | ≤700 Mb | 23 h |
| Roche 454 GS Junior | 400–450 | 1 × 105 | ∼35 Mb | 10 h |
| Illumina HiSeq 2000 | 100–200 | 6 × 109 | ≤540–600 Gb | 11 d | Very high throughput | 1\*, 2, 3\*, 4, 5, 6, 7, 8 |
| Illumina HiSeq 1000 | 100–200 | 3 × 109 | ≤270–300 Gb | 8.5 d |
| Illumina GAIIx | 50–75 | 6.4 × 108 | ≤95 Gb | 7.5–14.5 d |
| Illumina MiSeq | 100–150 | 7 × 106 | ≤1–2 Gb | 19–27 h |
| AB SOLiD 5500 system | 35–75 | 2.4 × 109 | ∼100 Gb | 4 d | Very high throughput; lowest reagent cost needed to reassemble a human genome among the widely accepted 2nd generation platforms, lower error rate | 3\*, 5, 6, 8 |
| AB SOLiD 5500 xl system | 35–75 | 6 × 109 | ∼250 Gb | 7–8 d |
| Ion Torrent -314 chip | 100–200 | 1 × 106 | ≥10 Mb | 3.5 h | Direct measurement of nucleobase incorporation events; DNA synthesis reaction operates under natural conditions (no need for modifed DNA bases) | 1, 2, 3, 4, 8 |
| Ion Torrent -316 chip | 100–200 | 6 × 106 | ≥100 Mb | 4.7 h |
| Ion Torrent -318 chip | 100–200 | 11 × 106 | ≥1 Gb | 5.5 h |

Bold indicates applications that are most often used, economical or growing

1 = de novo BACs, plastids, microbial genomes. 2 = transcriptome characterization.

3 = targeted re-sequencing. 4 = de novo plant and animal genomes.

5 = re-sequencing and transcript counting. 6 = mutation detection.

7 = metagenomics. 8 = other

\*Pooling multiple samples with sequence tags (i.e. MIDs or indexes) is required for efficient use of this application

releases a hydroxyl ion (H+) that shifts pH of the surrounding solution and this correlates directly to the number of cucleotides incorporated in that particular base flow cycle (reviewed in (Niedringhaus et al., 2011)). This change in pH is detected by sensor at the bottom of each well, converted to a voltage and digitalized by semi conductor CMOS integrated circuits (Pennisi). Signal processing software is used to convert the data for measurement of incorporation in that flow using a physical model (Rothberg et al., 2011).

### 1.11 HIV-1 Drug resistance Genotyping in the era of high throughput sequencing

The next generation sequencing technologies are able to sequence millions of DNA fragments simultaneously; hence, called High Throughput Sequencing (HTS). In a sample of highly diverse HIV, HTS increases the chance of sequencing each variant at least once, which is the limitation of conventional population based sequencing. Hence, it is called Ultra Deeping High Throughput Sequencing (UDHTS).

Notes:

Using multiplex identifiers would not only allow for TDR surveillance of mutations within protease and reverse transcriptase but also facilitate recognition of multiple linked DRM within a single specimen (Hoffmann et al., 2007).**Bibliography**

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