HIV Recombination and Pathogenesis – Biological and Epidemiological Implications

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1. Introduction

1.1 The origin, evolution and distribution of HIV: Introduction of HIV into the human population

There are several lines of evidence, which suggest that HIV-1 and HIV-2 originated from primates and were introduced into the human population via cross-species transmission events (Sharp et al., 1995). The viral genome structure of the simian form of the virus, simian immunodeficiency virus (SIV) is very similar to that of HIV (Huet et al., 1990), and phylogenetic relatedness has been established (Gao et al., 1999; Hirsch et al., 1989). SIV has been shown to infect primates that live in geographical areas where HIV is endemic (Gao et al., 1999; Hirsch et al., 1989), and possible routes of transmission, such as the butchering of primates for food and keeping monkeys as pets, have been proposed (Gao et al., 1999).

A natural primate host for HIV-1 has been proposed however there still remains some controversy. Strains of SIV (SIVcpz) from the chimpanzee Pan troglodytes troglodytes, phylogenetically cluster closer to HIV-1 strains than many other characterized SIV strains (Gao et al., 1999). However there is still a moderate amount of diversity between SIVcpz and HIV-1, and the prevalence of SIV infections in wild chimpanzees is low. There are three major groups within HIV-1, M (main), O (outlier) and N (new, or 'non-O non-M'). Each of the three groups of HIV-1 share a common branch with SIVcpz strains, but do not diverge from a common stem with SIVcpz, suggesting that they each arose from different cross-species transmissions (Gao et al., 1999; Thomson et al., 2002b). To support the idea that P.t. troglodytes is the natural reservoir of HIV, the HIV-1 N group was shown to be a recombinant between diverse viral strains within the HIV/SIVcpz group, suggesting an ancestral recombination in this sub-species of chimpanzee (Gao et al., 1999; Garcia et al., 1999).

It is thought that the HIV-1 M group originated in the Democratic Republic of Congo, as an extremely high genetic diversity is seen within the region (Vidal et al., 2000) and the earliest confirmed HIV-1 infection was found there in a stored serum sample from 1959 (Zhu et al.,

1998). Based on sequence analysis of the HIV-1 M group, it was estimated that these strains arose from a common ancestor in about 1931 (Korber et al., 2000).

Unlike HIV-1, the origin of HIV-2 is more definitive. The discovery of a form of SIV in sooty mangabeys (SIVsm) which is nearly identical to HIV-2, and which is found in these primates from the area where HIV-2 circulates in humans, provides very strong evidence that HIV-2 came from these primates (Gao et al., 1992). At present, there are eight designated groups of HIV-2, A-H, which are analogous to the HIV-1 groups (M, N and O) although, groups C-H have only been identified in single individuals (Chen et al., 1997; Damond et al., 2004). All groups of HIV-2 are believed to have arisen from individual cross-species transmission events from sooty mangabeys (Chen et al., 1996). Analysis conducted with HIV-2 strains from subtypes A and B, dated a recent common ancestor to around 1940 and 1945 respectively (Lemey et al., 2003).

Currently, there are 33 million people living with HIV/AIDS globally with 16,000 new infections happening every day (UNAIDS 2010) (**Figure 1**). As the acquired immune deficiency syndrome (AIDS) pandemic enters its third decade, the number of people living with human immunodeficiency virus (HIV) infection continues to increase. Although the HIV/AIDS epidemic was recognized in Southeast Asia later than elsewhere, local risk behaviors have allowed the epidemic to expand rapidly. Today, injecting drug use (IDU) accounts for up to 70% of HIV-1 transmission in many Asian countries, including China, Indonesia, Malaysia, Myanmar, Eastern India and Vietnam (Saksena et al., 2005). Also, there is ample evidence that heterosexual transmission through commercial sex workers has increased over the last few years (Saksena et al., 2005).
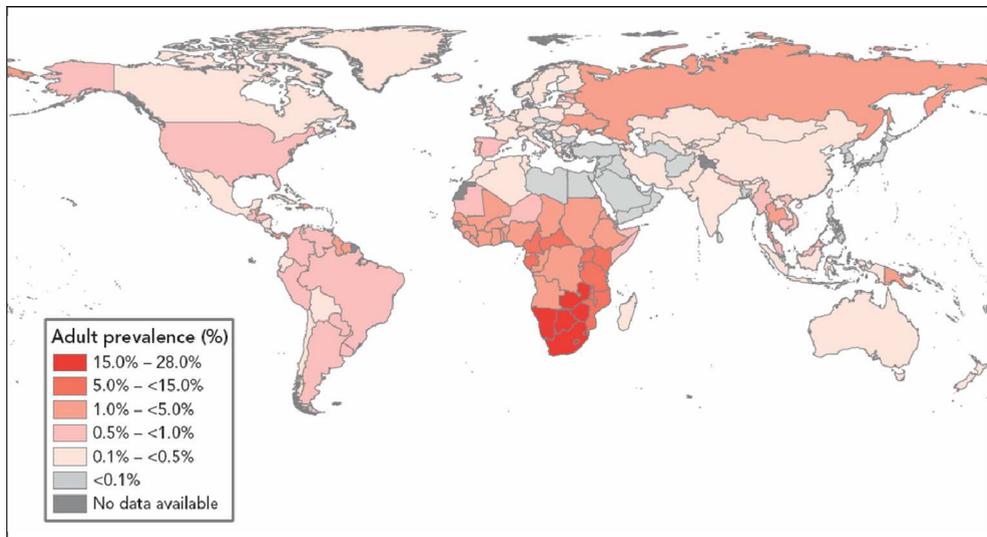

Fig. 1. Estimation of global adult prevalence in 2007, with an approximately 33 million of people living with HIV. Diagram source: UNAIDS, 2008: Report on the global AIDS epidemic.

Fig. 2. Map showing the global dispersal of diverse HIV subtypes (represented by single letter codes) and their replacement by circulating recombinant forms (CRFs).

2. Genetic diversity in HIV: Recombination – A unique trait for the continuation of viral progeny

One of the major hallmarks of HIV infection is high genetic diversity. This high genetic diversity in HIV-1 is attributed to the infidelity of its reverse transcriptase enzyme, which leads to high rates of mutation [Preston et al., 1988] and rapid viral variant turnover in patients-termed quasispecies [Ho et al., 1995 and Wei et al., 1995]. This quasispecies or the swarm of viral variants is a powerful asset of HIV in creating recombinants with superior ability to survive, evade and infect in vivo. In addition, the subtypic diversity within HIV-1 M group also provides the virus with a wider selection of strains to recombine with and disperse effectively by creating creating virulent forms. Currently, major subtypes are being replaced by circulating recombinant forms (CRFs), the evidence of which can be seen in **figure 2**.

The HIV-1 M group can be divided into 9 subtypes (A-D, F-H, J and K), and some subtypes are further broken down into subsubtypes, according to their topologies within phylogenetic trees. There is possibly a 10th subtype, L, for which 2 full-length sequences have been identified (Mokili et al., 2002). All of these subtypes can be found in Africa, where they are thought to have originated (Thomson et al., 2002b). Other continents have a variety of subtypes circulating, resulting in an uneven representation of the subtypes. Based on the amino acid sequence of the env region, each subtype is separated by approximately 25-30% genetic distance (Robertson et al., 1999; Thomson et al., 2002b). Subtypes A and F can be further broken down into subsubtypes, A1-A4 and F1 and F2 (Vidal et al., 2006). Technically

subtypes B and D are closely related and could be classified together as sub-subtypes, however they are generally still mentioned separately for consistency (Lal et al., 2005). In addition, within subtypes there are clusters that represent strains from different geographical locations, such as subtype B strains from Thailand, which are referred to as B′ or Thai B (Kalish et al., 1995). These clusters most likely arose due to the initial introduction of only a small number of strains into a particular region, and their subsequent diversification and spread (known as a founder effect) (Thomson et al., 2002b). In addition to the nine subtypes of HIV-1, there exists circulating recombinant forms (CRFs), which are mosaic viruses of two or more HIV-1 subtypes. To be classified as a CRF, an inter-subtype recombinant virus must be isolated from at least two (preferably three) unlinked individuals and sequenced in full (Carr et al., 1998). Currently 34 CRFs have been recognized, with 26 described in detail (Los Alamos HIV Database) (**Figure 6**). These CRFs now include second generation CRFs such as CRF09_cpx and CRF15_01B, which is contain the primary CRFs CRF02_AG and CRF01_AE respectively. Regions within CRFs that cannot be classified as a known subtype are designated U for unknown, and a CRF comprised of more than two subtypes is denoted by cpx for complex (Peeters, 2000; Robertson et al., 1999). The first CRF identified was CRF01_AE, and was originally classified as subtype E, before it was designated a recombinant (CRF01_AE has subtype A gag and pol regions and a subtype E env) (Gao et al., 1996), and interestingly a full-length subtype E virus has not been identified, possibly indicating that the gag and pol regions were lost through an ancestral recombination event (Gao et al., 1996).

Recombination is common in HIV and it uses recombination in order to acquire viral fitness, virulence and ability to evade the host immune system. Recombination occurs as a result of the low affinity binding of RT, which is necessary for the strand transfers of reverse transcription to occur. As HIV carries two copies of its viral RNA, during reverse transcription both of these can serve as templates to generate the provirus. If the two copies of RNA encapsulated with a virion are distinct, and are both used during reverse transcription, then the resulting provirus will be a recombinant. Thus the first requirement for recombination is a dually infected cell, which can give rise to a heterozygous virion, which carries two distinct copies of RNA. Once a heterozygous virion is formed recombination then occurs upon infection of a new cell (**Figure 3**). The theories behind how recombination occurs at the molecular level can be divided into two groups, recombination that occurs during the synthesis of the minus-strand DNA and that which occurs during the synthesis of the plus-strand DNA. Each theory has its own explanation and some supporting experimental evidence, implying that both mechanisms can occur (Hu and Temin, 1990b).

3. Heterozygous virions and their formation

A requirement for the generation of recombinant HIV genomes is a heterozygous virion, that is, a virion with two non-identical RNA strands (Hu and Temin, 1990a; Weiss et al., 1973). Heterozygous virions are generated in individual cells which are infected with two or more different viral variants, which integrate their proviral genome, and generate new full length viral RNA.

As packaging of the viral RNA is not selective for specific RNA copies (D'Souza and Summers, 2005), a heterozygous virus can be formed, by encapsidation of an RNA copy from each viral variant. Heterozygous virions can then infect other cells and recombination between the two co-packaged viral RNAs can occur during reverse transcription (Duesberg, 1968). (**Figure 3**). The different viral RNAs can come from variants within the viral quasispecies, or from other HIV strains or subtypes, if the patient has a dual infection.

Multiple infection of a single cell with HIV can occur simultaneously or sequentially. There is some debate regarding the sequential infection, as once a cell is infected, HIV downregulates the CD4 and CCR5 receptor molecules (Michel et al., 2005), and therefore simultaneous infection may be the primary mechanism involved. Interestingly, a study by Dang et al. (2004) showed that dual infections of cells occurs at a much higher rate than predicted by chance, in both a T cell line, and primary T cells, regardless of how the virus was transmitted. In a follow up study it was also shown that coreceptor differences were not a barrier to recombination, as viruses using different co-receptors, CCR5 or CXCR4 also exhibited rates of double infection that were higher than predicted from a random distribution (Chen et al., 2005). Likewise, other studies have also shown co-infection of cells to be common, which implies that opportunities for recombination are favoured (Jung et al., 2002).
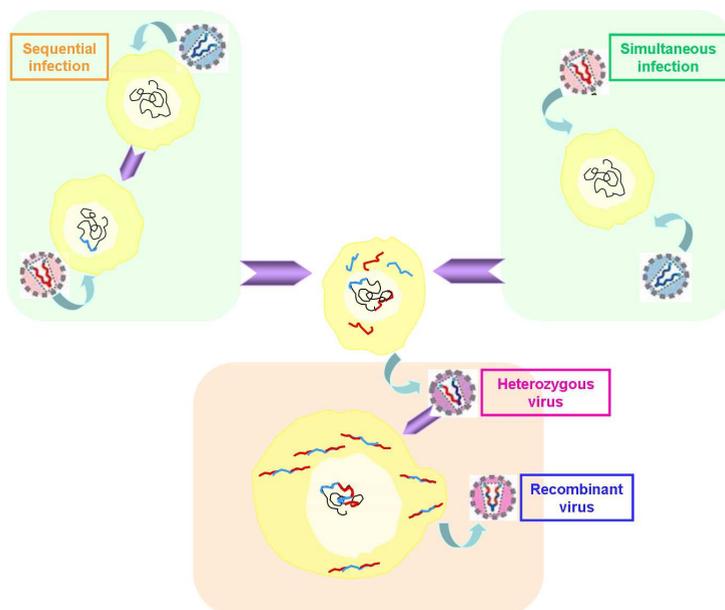

Fig. 3. HIV dual infection of a cell, heterozygous virion formation and recombination. Modified from: Najera et al. (2002).

3.1 Minus-strand recombination
There have been several studies addressing minus-strand recombination, and it has been shown to occur frequently, with an average of three crossovers occurring per replication cycle (Yu et al., 1998).
The first mechanism proposed to account for recombination was the forced copy-choice model (Coffin, 1979), and was based on evidence that suggested the genomic RNA of retroviruses is fragmented. A break in the RNA would halt reverse transcription and consequently force the reverse transcriptase to switch to the second copy of RNA in order to continue synthesis, that is, a strand transfer event would occur (Hu and Temin, 1990b). In order for this to take place, the RT enzyme must be transferred to a homologous region on the second RNA copy. This model assumes therefore that recombination occurs during

minus-strand synthesis, and is comparable to the minus-strand strong stop strand transfer that occurs during reverse transcription (Negroni and Buc, 2001) (**Figure 4**). However, no studies have been able to establish a firm connection between strand switching and the frequency of RNA breaks. Further, experiments have shown that a strand break is not necessary for a template switch (Hu and Temin, 1990b). Consequently, the minus-strand exchange model was proposed which suggests that the low processivity (loose adherence to the RNA template) of RT causes strand transfers (Coffin, 1979; Yu et al., 1998). It has also been shown that obstacles to reverse transcription, causing the enzyme to pause, can trigger a strand transfer (Wu et al., 1995). In addition, studies have also suggested that secondary structures of the RNA templates could also increase template switching without a pause, by bringing the two templates into close proximity (Balakrishnan et al., 2001) (**Figure 4**).

3.2 Plus-strand recombination

Recombination that occurs during the synthesis of the positive strand of DNA is referred to as the strand displacement assimilation model (Hu and Temin, 1990b). This model suggests that both copies of viral RNA are transcribed to produce two minus-strand DNA copies. Synthesis of plus-strand DNA is initially discontinuous, and internally initiated fragments occur eg. at the cPPT (Hsu and Taylor, 1982). Therefore, it has been proposed that an internally initiated fragment can be displaced by an elongating upstream DNA fragment, and this can cause the internally initiated fragment to dissociate and re-anneal to the complementary region of the second minus-strand DNA. The resulting double stranded DNA will have a mismatched region and mismatch DNA repair will then correct the sequence differences (Hu and Temin, 1990b) (**Figure 4**). Two positive strands of RNA, which may contain breaks. During minus-strand synthesis, the RT can switch to the other template at a break.
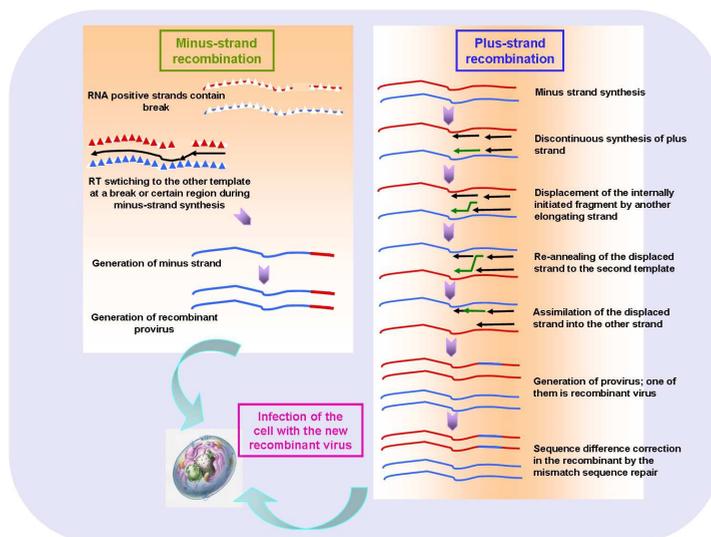

Fig. 4. Model of retrovirus recombination: minus-strand recombination (forced-copy choice) and plus-strand recombination (strand displacement assimilation).

4. The global distribution of HIV-1 subtypes and CRFs

Across the globe there is an uneven representation of the HIV-1 M group subtypes (**Figure 2**), along with the presence of diverse subtypes and its CRFs in Asia (**Figure 5**). This uneven distribution is thought to have arisen partially due to a founder effect, where one subtype is introduced into a region by a single or a few individuals from which the epidemic radiates out (Korber et al., 2000). Subtype A is currently divided in to four sub-subtypes: A1-A4. Subsubtype A1 is one of the more common variants and is found throughout Western, Central and Eastern Africa and Eastern Europe, and two CRF forms containing subtype A1 (CRF02_AG and CRF03_AB) are also widely spread in these regions (Andersson et al., 1999; Bobkov et al., 2004; Dowling et al., 2002; Steain et al., 2005). Subtype A (sub-subtypes 1 and 2) strains are highly predominant in Kenya, with one study showing 93% of all strains found in the region being subtype A or a recombinant containing subtype A (Dowling et al., 2002). In addition, CRF01_AE is one of the major strains found in Thailand and Southeast Asia (McCutchan et al., 1992; Tovanabutra et al., 2004). Subsubtype A2 was first identified from sequences originating in the Democratic Republic of Congo and Cyprus (Gao et al., 2001), and is now also found in Kenya (Visawapoka et al., 2006). In addition, there have been 2 recognised CRF forms, CRF16_A2D and CRF21_A2D, both of which were also identified in Kenya (Visawapoka et al., 2006). CRF16_A2D has also achieved a global spread and has been identified in Korea and Argentina (Gomez-Carrillo et al., 2004). Subsubtype A3 has only been recently described (Meloni et al., 2004a), and has thus far only been identified in areas of West and Central Africa (Meloni et al., 2004a; Meloni et al., 2004b). Similarly subsubtype A4 is a relatively new strain, and has only been identified within the Democratic Republic of Congo (Vidal et al., 2006).

Subtype B is the most common subtype in Australia, as well as the USA, and Western and Central Europe (de Oliveira et al., 2000; Essex, 1999; Herring et al., 2003). It is also found in South America, where CFR012_BF also circulates (Montano et al., 2005), Thailand (B' strains), with CRF15_01B (Tovanabutra et al., 2001), China, with CRF07_BC and CRF08_BC (Saksena et al., 2005), Spain with CRF14_BG (Delgado et al., 2002) and Eastern Europe with CRF03_AB (Liitsola et al., 1998). This subtype was one of the first to spread globally, however generally seems to be on the decline, with the wider spread of other subtypes and intersubtype recombinants (Soares et al., 2005; Tatt et al., 2004).

Subtype C is currently the most prevalent subtype. It is found circulating widely through South Africa, East Africa and India (Bessong et al., 2005) and to a lesser extent in South America, Eastern Europe and Chine (Saksena et al., 2005; Soares et al., 2005). It has been suggested that strains of subtype C possess some selective advantage due to its rapid dispersal across the globe that has been seen in recent years (Walker et al., 2005).

Subtype D is found across most of Africa, particularly East African countries, where in Uganda it has been reported as a predominating strain (Harris et al., 2002). It has also been reported in other continents, though usually as a minor variant (Tatt et al., 2004). In addition, subtype D is frequently a component of unique intersubtype recombinants, from Kenya and other East African countries (Steain et al., 2005), and it has been suggested that recombinants between subtypes A and D are selected for in dually infected patients (Songok et al., 2004). Subsubtype F1 is found in South America and Europe, whereas subtype F strains from Africa (eg Cameroon) more commonly belong to subsubtype F2 (Laukkanen et al., 2000). Subtype F also forms part of CRF12_BF, which has become widespread across South America, and more recently as a part of CRF17_BF, CRF28_BF and CRF29_BF, which have also been identified in South America (De Sa Filho et al., 2006; Hierholzer et al., 2002).

CRF05_DF has also been reported in Europe, although is thought to have arisen in Africa (Casado et al., 2003; Laukkanen et al., 2000).

The spread of HIV-1 subtype C in India to the northern India, and to the neighbouring countries; Nepal, Bangladesh, and Myanmar

The spread of HIV-1 recombinant form; CRF01_AE from Thailand to other neighbouring countries in South-East Asia; Cambodia, Vietnam, Malaysia, Indonesia, the Philippines and Singapore, as well as emergence of other CRF01_AE/B recombinant in the region.

The spread of HIV-1 subtype B and C (and possibly B/C recombinant) from Eastern Myanmar to the Yunnan province of China, followed by dissemination of CRF07_BC to the northwest China (Province of Xinjiang) and CRF08_BC to the southeast China (Province of Guangxi). CRF07_BC was then introduced to southern region; Taiwan and bypassed Hong Kong.
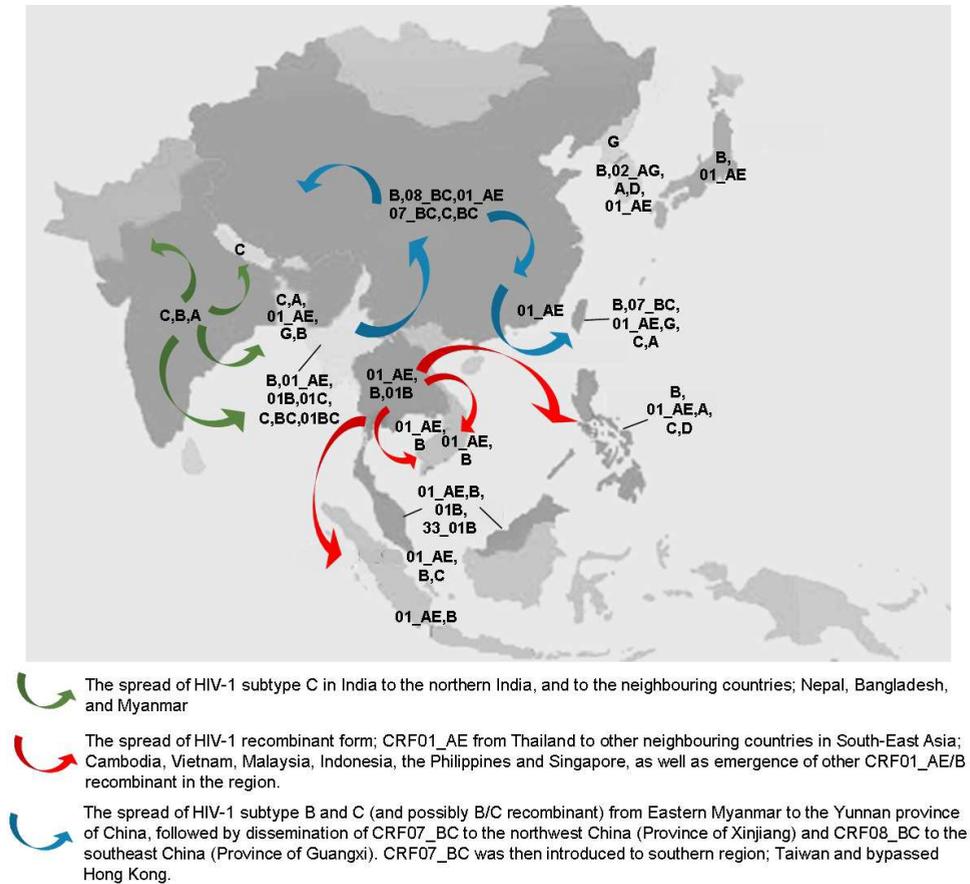
Fig. 5. The complex HIV-1 genetic diversity and the spread of the predominant HIV-1 strains in Asia. Distribution of different HIV-1 subtypes and CRFs (demonstrated in descending order; from the most to the least prevalent strain) in major Asian countries, including subtypes A; B; C; D; G, CRFs such as 01_AE, CRF01_AE; 02_AG, CRF02_AG; 07_BC, CRF07_BC; 08_BC, CRF08_BC; 33_01B, CRF33_01B and other HIV-1 recombinant forms: BC, B/C inter-subtype recombinant; 01B, CRF01_AE/B; 01C, CRF01_AE/C and 01BC, CRF01_AE/B/C. The three different routes of spread for subtype C, CRF01_AE as well as subtype B, C and B/C recombinants are highlighted in green, red and blue, respectively. Diagram source: Lau *et al.* (2007).

Subtype G has been reported across Africa, and parts of Europe (Esteves et al., 2003; Gutierrez et al., 2004; Parreira et al., 2005; Yang et al., 2005a), however it is its CRF02_AG that has had the greater impact. This strain is currently the most prevalent CRF and is found predominantly across West and Central Africa (Mamadou et al., 2003). CRF14_BG, as well as other G

recombinants are also found in Spain and neighbouring regions (Perez-Alvarez et al., 2003). Subtypes H, J and K have had only a minor impact on the HIV epidemic and are generally only found in small numbers across West and Central Africa (Mokili et al., 1999; Thomson et al., 2002b; Vidal et al., 2000; Yang et al., 2005a). The remaining CRFs are also found in varying proportions, usually each within a distinct geographical region. Whole spectrum of emerging and well-established CRFs have been identified, which are listed in **Figure 6**.

5. Dual infections and recombination of HIV

The magnitude of genetic diversity in a host relates to the size of the viral population, the extent of replication, the mutation and recombination rates and the selective pressures placed on the virus (Saksena et al., 2001). Overall the divergence between the circulating HIV strains within a single individual and the original infecting strain/s is thought to increase by around 1% per year in early infection (Shankarappa et al., 1999). Thus, within an individual a heterogeneous viral population exists which has been termed a 'quasispecies'. Within an individual this viral diversity can reach as 15% (Lukashov and Goudsmit, 1997). The reasons behind such viral diversity include a fast turnover of virions, approximately 109 new virions are produced each day (Ho et al., 1995), and the low fidelity of reverse transcriptase. Reverse transcriptase lacks a proof-reading function, meaning that any nucleotides that are mis-incorporated during DNA synthesis are not corrected (Battula and Loeb, 1976). Studies have shown the mutation rate of HIV RT to be 3.4×10^{-5} mutations per base pair per cycle, which is relatively high compared to other retroviruses (Mansky and Temin, 1995). However, as the rate of recombination, 3-9 crossovers per round of replication (Jetzt et al., 2000), exceeds the mutation rate, recombination is the largest contributing factor in viral evolution (Bocharov et al., 2005).

Intra-strain recombination between variants within the viral quasispecies, as well as inter-strain/intersubtype recombination, increases genetic diversity and thereby increases the chance of survival for the virus. Recombination can generate strains capable of evading the host's immune system, strains that are resistant to one or more antiretroviral drugs, or that can replicate faster and more efficiently (Steain et al., 2004). It can also result in the formation of novel genes (Sharp et al., 1996). Recombination can also be a repair mechanism for HIV, allowing viral replication to continue in the presence of a break in a strand of RNA. However, recombination can also result in the emergence of less fit viral strains, as it can break-up favourable combinations of genes and therefore is not always advantageous for the virus (Bretscher et al., 2004).

Studies have shown that multidrug resistant viruses emerge rapidly in the presence of two drugs, due to recombination between strains that were each resistant to a single drug (Moutouh et al., 1996). In vivo, this could allow the emergence of strains that are resistant to many different classes of drugs, and recently intrapatient recombination leading to a multidrug resistant strain has been reported (Weiser et al., 2005). Further, selective pressure placed on the pol region in the presence of anti-retroviral drugs, does not need to be carried across the entire genome as recombination could occur between strains with diverse gag and env regions with an escape mutant in the pol region (Charpentier et al., 2006). Similarly, a study of two patients by van Rij et al. (2003) observed that after the emergence of X4 utilising strains, the R5 and X4 gp120 envelope sequences diverged from each other, whereas the respective gag p17 regions did not. Thus it was proposed that recombination was occurring between the two strains in vivo.
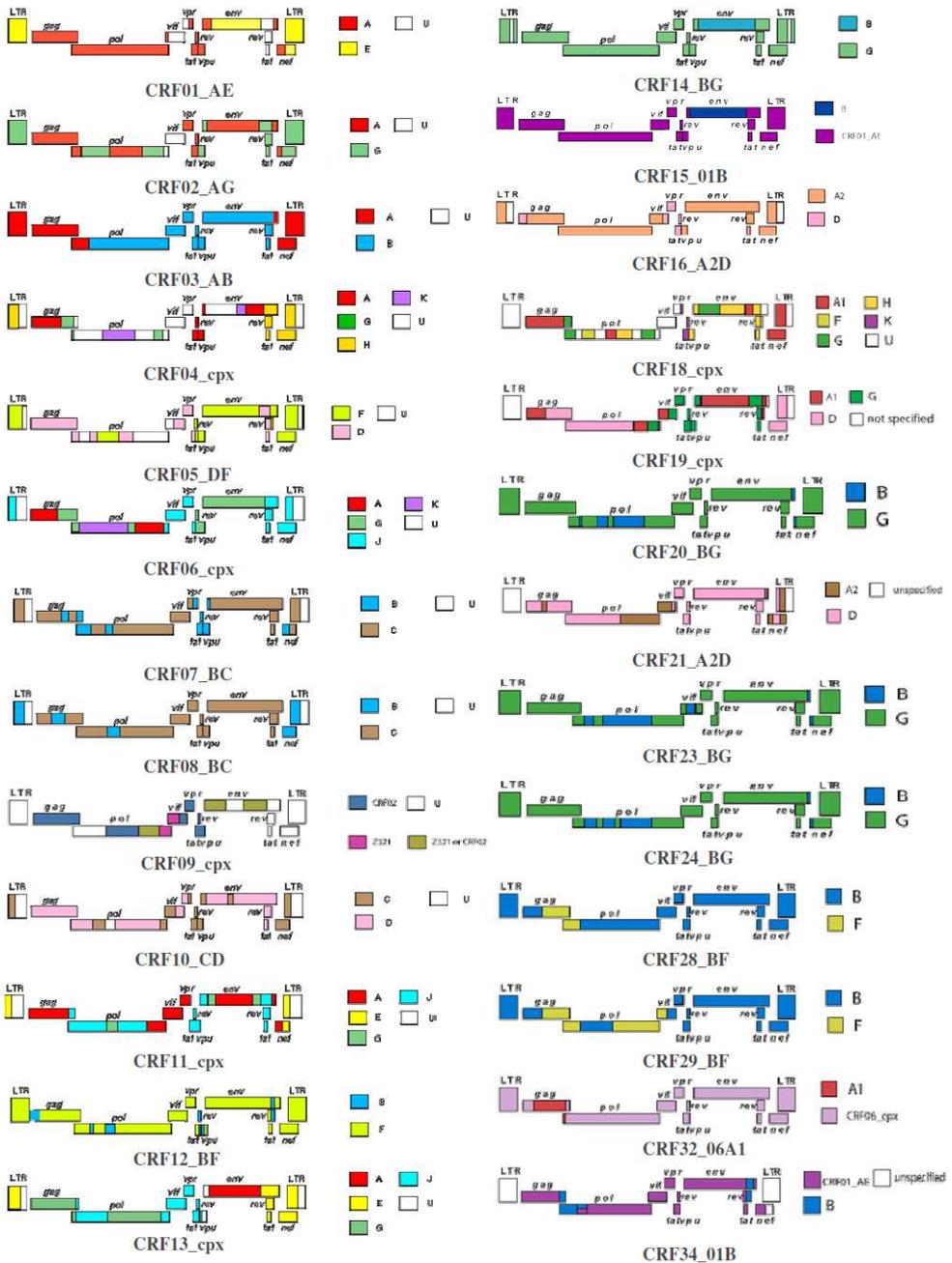
Fig. 6. Schematic representation of the genomic organization of the described CRF genomes. Adapted from the Los Alamos National Laboratory HIV Database (2006b).

Recombination may also produce virus strains that are more successfully transmitted either via sexual contact or perinatal transmission, possibly by increasing the strains affinity for a particular tissue type. A study in Buenos Aires by Thomson et al. (2002b), showed that recombinant viruses predominated in IDUs and heterosexually infected women, whereas subtype B viral strains were more common in men, both heterosexually and homosexually infected (Gao et al., 1996). Studies have also shown that inter-subtype recombinants were more likely to be transmitted via breast milk than subtype C in Tanzania (Koulinska et al., 2006). Further, in Southeast Asia, CRF01_AE was spread much more rapidly than subtype B, which was also present in the area. In addition, no type E gag or pol gene has been found which may suggest that the recombinant CRF01_AE virus was more viable and consequently the pure type E was eliminated by selective pressures

6. HIV co-infection and superinfection: Pathogenic implications

It is widely recognised that a single individual can become infected with more than one HIV-1 subtype or strain i.e. they harbour a dual infection (Gottlieb et al., 2004; Wang et al., 2000). This circumstance is possible via superinfection or coinfection. In superinfection, new infection takes place with a divergent HIV-1 or HIV-2 strain in already infected individuals. In contrast, coinfection is a concomitant exposure to diverse HIV strains prior to seroconversion, and therefore before the immune system has mounted a response (Steain et al., 2004).

For the majority of patients that have been characterized as being infected with two or more HIV strains, it has generally been assumed that the infections occurred within a very short period of time, if not simultaneously. This is because it was originally thought that establishment of infection with HIV would provide some immunity against re-infection, and that the decrease in the expression of CD4 molecules would make superinfection unlikely (Benson et al., 1993). Furthermore, a study by Otten et al. (1999) examined HIV-2 infection in Pig-tailed Macaques and found that a secondary infection could only be established in the first 2-4 weeks after the initial infection.

However, there are to date at least 10 papers examining patients with evidence of superinfection (Chohan et al., 2005; McCutchan et al., 2005; Smith et al., 2005). These include one patient with a triple infection who was thought to have acquired an additional 2 strains through superinfection (van der Kuyl et al., 2005). It was also initially hypothesised that if superinfections were occurring that they would be the result of intravenous inoculation of the virus, with a high dose exposure. However of the reported superinfections, several were acquired via sexual exposures, including heterosexual contact (Chohan et al., 2005).

Many of these papers documenting superinfection note that upon acquisition of a second virus, patients tend to experience a more rapid disease progression, with an increase in plasma viral load and a concomitant decrease in CD4+ T cell count (Smith et al., 2005; van der Kuyl et al., 2005). Superinfection has also been reported to lead to recombination between the two infecting strains (McCutchan et al., 2005). For these reasons HIV-infected individuals should be warned that safe-sex practices are still necessary even between sero-concordant couples, to prevent superinfection and the associated disease progression (Steain et al., 2004).

Now that superinfection has been demonstrated, it is unknown if these are rare cases or if superinfection is a more common event that is not always detected, and may in part provide some explanation for the large number of recombinant strains seen globally. In cases where

the second infecting strain is of the same subtype, the differences between the strains may be attributed to the normal quasispecies variation seen within a patient, and therefore may not be recognized as superinfections. Thus in cases where superinfection is detected it is likely to be intersubtype related and thus any quantification of superinfections may be an underestimation (Steain et al., 2004).

It is important to screen for dual infections, as such patients can provide an ideal setting for examining biological and molecular interactions between two viral strains in vivo. Wang et al. (2000), reported the case of a patient who was co-infected with two divergent forms of subtype B, which appeared to be acting in synergy. The two strains were able to segregate based on a differential tropism for monocytes and macrophages. While one of the strains appeared to dominate when co-cultured in peripheral blood mononuclear cells (PBMCs), it was discovered that this strain was only able to productively infect PBMCs when the second viral strains was present, indicating a potential synergistic effect between the two viral strains. In addition, a greater cytopathic effect was observed when the two strains where co-cultured, further supporting the idea that a synergistic association of these two viral strains resulted in greater pathogenicity. The patient had acquired the infections via intravenous drug use and progressed rapidly to AIDS, dying within 5 years of infection. This case demonstrates that distinct biological differences exist between strains of the same subtype, and that two strains are able to act in synergy.

7. CRFs and pathogenic implications: Asia the "hotbed" of CRFs

The highly unequal geographic distribution of viral variants is the result of the global variation in the HIV-1 strains, the dynamic nature of the HIV-1 epidemic, and the accidental epidemiologic transmissions. The recombinant HIV-1 strains have been reported from almost all geographic regions of the globe where multiple HIV-1 subtypes have been circulating. Despite this, few HIV-1 geographic "recombination hotspots" have been identified around the world, such as central Myanmar [Vidal et al., 2005], Yunnan province of China [Saksena et al., 2005], Argentina [Renjifo et al., 2001], Brazil [Ball et al., 2003], East Africa [Yang et al., 2003,Renjifo et al., 2004] and more recently Cuba [Wu et al., 2001]. While the predominant viral forms in the global HIV epidemic are subtypes A and C [UNAIDS/WHO, 2006], a different and even more complex HIV genetic diversity has been found in Asia. The HIV spread and its epidemiology in Asia are interesting and closely related to the routes of spread of the epidemic. This is evident from the distribution of subtype C (Figure 5), which was found primarily in India and Africa, and is now spreading to Northern India, Myanmar, and Thailand [Eshleman et al., 2005]. Although the biological aspects that explain this high rate of infection remain unclear, subtype C has dominated the HIV-1 epidemic in India and accounts for almost 97% of infections. Apart from the predominant subtype C, the A/C and B/C inter-subtype recombinants have also been recently identified in North-eastern India [Peeters et al., 2000]. Emergence of A/C recombinants is also consistent with the epidemic observed in Bangladesh [Sanders-Nuell et al., 2007], from where triple recombinants between subtypes A, C and G have been recently reported. Also, it is established that the spread of subtypes B and C, as well as B/C recombinants occurred through the drug route from Eastern Myanmar into Yunnan province of China and moving to north and west into Xinjiang province of China (Figure 5). While other recombinants account for only 4% of the total HIV-1 infection in South and Southeast Asia, the CRF01_AE has been found to be responsible for 84% of all HIV-1

infections. CRF01_AE, which was originally identified in Thailand appears to circulate in major parts of Asia, particularly Southeast Asia (Figure 5 and 6). Together, CRF01_AE and other recombinants account for nearly 89%, the highest across the world [Hemelaar et al., 2006]. Since the beginning of HIV pandemic in the last two decades until recently, changes in HIV-1 subtype distribution in Asia have been overwhelming. In Asian countries, the HIV-1 prevalence has been high from the late 1980s to 1990s, with subtype B" (known as the Thai variant of subtype B) being the predominant strain and was most frequently observed amongst IDU [Weniger et al., 1991; Nerurkar et al., 1997]. Concurrently in Thailand and other areas, CRF01_AE was introduced independently in commercial sex workers [Ou et al., 1993]. Interestingly in the last decade, a gradual yet evident spread of the Thai variant of CRF01_AE was witnessed in many countries of Asia [Nerurkar et al., 1996]. It was later observed that CRF01_AE takes over in the HIV-1 epidemic in Southeast Asia, even among IDUs in Thailand, Cambodia, and Vietnam [Nerurkar et al., 1996]. Likewise, countries such as Indonesia and Malaysia demonstrate the predominance of CRF01_AE and subtype B prior to the year 2000.

8. Inter-CRF recombination and its possible epidemiologic implications

Among all the HIV-1 subtypes distributed in Asia, CRF01_AE is reported to play a considerably important role in its epidemic [Hemelaar et al., 2006]. The HIV epidemiology in Asia is bound to be more complex as other recombinant forms are introduced from neighbouring geographic regions, along with the continuing emergence of novel second and third generation recombinant forms of CRF01_AE in this region. Geographic regions known as recombination hotspots in Asia, including Myanmar and Yunnan province of China appear to have varied and complex forms of HIV-1 recombinants, which emerge continually. Between 2002 and 2004, a novel inter-CRF recombinant has been identified in Yangon, Myanmar, which also appears to be a second class of HIV-1 inter-CRF recombinants comprised of CRF01_AE and CRF07_BC [Takebe et al., 2006]. Other Asian region, for instance Macao has first identified the circulation of CRF12_BF (prevalent in Brazil) among the IDUs, although CRF01_AE has always being the major HIV-1 strain [Chan et al., 2007a]. This suggests the epidemiologically associated transmission of the current HIV-1 infection in the region and gives clues to the possible initiation of the emergence of novel inter-CRF recombinants between CRF01_AE and CRF12_BF. Concurrently in Macao, a diverse form of HIV-1 recombinant has been recently full-length characterized and comprised of CRF12_BF, CRF14_BG and subtype G [Chan et al., 2007b].

As the result of the co-circulation of subtypes B and C, two CRFs; CRF07_BC and CRF08_BC have emerged in the Yunnan province of China [Piyasirisilp et al., 2000]. An ongoing evolution and emergence of novel recombinant forms of HIV are anticipated in this region, while these two CRFs continue to co-circulate with "pure" subtypes B and C, along with other URF in Yunnan [Yang et al., 2002]. It is predicted that more new recombinant strains between these two CRFs will continue to emerge [Peeters et al., 2000]. With the extensive variability in recombinant breakpoints and crossover points in China, a possible emergence of second and third generation recombinant CRF will continue to give rise to more HIV-1 variants. A recent study has identified approximately 12% of HIV-1 strains found among the IDUs in Southeast Yunnan to be the diverse forms of inter-CRF recombinants between CRF07_BC and CRF08_BC [Chan et al., 2007]. This further provides a good insight into inter-CRF recombinants, and only time will tell regarding epidemiologic.

9. HIV fitness in vivo and in vitro as a consequence of recombination

Fitness is a parameter defining the replicative adaptation of an organism to its environment [Domingo et al., 1997] as a consequence of the interaction of a multitude of viral and host factors [Quinones-Mateu et al., 2006; Nijhuis et al., 1999]. Within a given viral „quasispecies", each clone possesses a fitness denoting to the selection of the viral properties (e.g. activity and stability) in a particular environment. Under a certain selective pressure in a defined microenvironment, viral replication will take place to encode virus that replicates at high rates [331]. Thus, one or more strains possessing better viral properties within a given quasispecies will be positively selected, while unfit variants will be negatively eliminated [331]. The HIV-1 viral factors that affect viral fitness are mainly the biological processes in the virus life cycle: cell entry, genome replication, protein synthesis and processing, and particle assembly and release from cells. The survival of the fittest form of HIV-1 recombinant leads to further viral evolution in a complex population, suggesting a continuous evolving of HIV-1 dynamics, mainly attributed to an incessant process of growth, competition and selection.

As a result of high mutation rate of HIV-1, wide range of sequence possibilities is created. While sometimes it resulted in non-replicative viruses, others may possess varying degree of fitness. Recombinant viruses may have some advantages over the parental strain and thus, may possess important genetic variability for HIV-1 pathogenesis, transmission, diagnosis, treatment and vaccine development. It is undeniable that different biological properties of diverse subtypes will possibly result in transmissibility and pathogenicity variation. During early infection, most subtypes conform to the non-syncytium-inducing CCR5 receptor usage phenotype. However, towards the late infection, these subtypes will shift to the syncytium-inducing CXCR4 receptor usage phenotype. This is true for most but subtype C and D viruses, which do not follow this pattern [336]. In terms of transmission, several studies of vertical transmission have suggested that the maternal HIV-1 subtype is likely to play a role while others disregard this perception [Yang et al., 2003; Tapia et al., 2003].

What is yet to be known is the consistent role of the subtype-associated differences in the efficiency of transmission via different routes. While some studies have reported that subtype D is associated with faster disease progression compared with subtype A [Condra et al., 1995], others have denied the possibilities that HIV genetic subtype determines the rate of disease progression [Alaeus et al., 1999]. Findings of these studies are inconsistent, due to the difference in the study design (sample size, duration of clinical follow-up and the use of surrogate markers of progression) as well as other virus, host and environmental factors. Previous work has also suggested possible biological differences among the HIV-1 subtypes [Jeeninga et al., 2000]. It was reported that the long terminal repeat (LTR) region of CRF01_AE (the predominant HIV-1 strain in Asia) is much more potent in vitro than the subtype B LTR. When a recombinant CRF01_AE/B virus was constructed in vitro, it exhibited an intense replication advantage compared to the parental subtype B. This indicated that restrained differences in the LTR promoter activity can exert a significant impact on viral replication kinetics. A recent profound analysis was done by Kozaczynska et al. [Kozaczynska et al., 2007] to describe the study over time of HIV-1 isolates in a patient twice superinfected with HIV-1; an initial infection with a subtype B1 strain, followed by first superinfection with a subtype B2 strain and then with CRF01_AE. Again, the LTR of CRF01_AE was found to possess a higher promoter activity, although this was not reflected in the plasma viral load differences. It is remarkable that the later-arriving viruses (strain B2 and CRF01_AE) replicated at much higher

levels in blood compared with the first infecting virus B1. Except for the excessive recombination between both subtype B strains, there was only minimal evidence that the different HIV-1 strains found in the patient appeared to influence the evolution of each other. While HIV-1 has been constantly exposed to host immune system for eradication of the virus, its replication relies profoundly on host cell machinery. Thus, the HIV-1 fitness is said to be closely related to the host environment (e.g. cellular receptors, intracellular factors and host defence mechanism). Viral diversity gives important impact in the determination of viral load, as well as viral diagnosis. Therefore, HIV-1 diagnosis test, which includes HIV-1 immunoassays have to be competent in detecting all known group M subtypes [Koch et al.,]. Other viral load measurements assays have to be reliable too, for instance polymerase chain reaction-based assays for quantification of the HIV-1 RNA from all known genetic variants of HIV-1 [Swanson et al., 2005].

Genotypic or phenotypic variations within different subtypes are somehow related to any differences in ex vivo fitness. Troyer et a.l [Troyer et al., 2005] provided evidence that increased viral fitness in vivo may be related to a concomitant increase in HIV-1 diversity, and thus serves as a crucial factor in determining disease progression. Furthermore, HIV-1 strains that display viral properties that increase their fitness, for instance subtype C isolates appear to have an extra or third nuclear factor-kappa B (NFκB) element in the long-terminal repeat (LTR). This would enhance transcription in the presence or absence of HIV-1 Tat protein [Hunt et al., 2006]. Another study showed that in comparison to subtype B, subtype C possesses an increased protease activity, and thus augmented cleavage of peptide substrates and possibly improved viral fitness [Velazquez et al., 2001]. Therefore, it is proposed that the increased replicative capacity of subtype C over other HIV-1 subtype isolates suggests its dominance in HIV-1 epidemic. However, in another pair-wise competition study by Arien et al. [Arien et al., 2005] to establish the „pathogenic fitness" (or virulence) of HIV in PBMCs, subtype C seems to have lower fitness when competed with other HIV-1 group M (subtypes A, B, D and CRF01_AE). These viruses were classified as using either CCR5 or the CXCR4 co-receptor for entry and were competed against the same phenotype to determine the fitness (based on > 2000 competitions). In the same study, it was reported that all HIV-1 group M viruses have a greater fitness than HIV-2 and HIV-1 group O showed the lowest fitness. Thus, with the exception of subtype C, this fitness order seems to reflect the prevalence of HIV in the human population and also the proposed rates of transmission efficiency. It has been reported in 2003 that the CCR5 HIV-1 subtype C isolates were at least 100-fold less fit than any other group M HIV-1 isolates [Ball et al., 2003]. Throughout the disease, subtype C isolates was predicted as preferentially CCR5-tropic and non-syncytium-inducing (NSI). This has absolute difference in infections with isolates of other HIV-1 subtypes, whereby the viruses switch from CCR5 to CXCR4 co-receptor entry during later stage of the disease. Other ex vivo [Ball et al., 2003] and in vivo study [Walker et al., 2005] has implied that subtype C is efficiently transmitted but is less virulent in comparison with other HIV-1 group M isolates. However, among all HIV strains, HIV-1 group M seems to be more virulent and transmissible. It is believed that its progenitor might have been „fitter" for human infection and more adaptive, even after going through the rapid evolution and passage. By contrast, HIV-2 and HIV-1 groups O and N might have limited expansion in the human population, possibly due to poor host adaptation and transmission efficiencies, although the exact reasons for their poor transmissibility and active establishment in human populations have remained unclear and speculative.

Viral fitness is generally defined as the ability of the virus to replicate within the host and is therefore dependant on host and viral factors [Weber et al., 2003]. Recombination is thought to increase viral fitness. In an HIV-1 recombinant-related fitness study by Njai et al. [Njai et al., 2006], CRF02_AG isolates demonstrated a higher ex vivo replicative fitness compared to subtypes A and G from the same geographic region in Cameroon, irrespective of the level of CD4+ count and co-receptor tropism. A similar study by Konings et al. [Vijay et al., 2008] showed a 1.4 to 1.9 times higher replication rate increase in the CRF02_AG strains, in contrast to its progenitor subtypes A and G; an adaptation which implies its broader spread and predominance in West Central Africa. A computer simulation has been developed that mimic the HIV genomic diversification within an infected individual and elucidate the influence of recombination [Vijay et al., 2008]. This study has shown that recombination increases viral fitness regardless of the size of the effective population. In light of these results, it is likely that HIV-1 recombination events in Asia can also contribute to the emergence of viruses for instance, the widespread CRF01_AE/B inter-subtype recombinants with a biological edge in their host. In vitro studies of the viral fitness and interactions between different viral strains have been assessed with limitation, as only viral replication capacity, defined as "intrinsic capacity of virus to replicate in an ideal environment" [Weber et al., 2003] can be studied in the absence of host selection pressures. As a result, viral replication capacities are compared in vitro between two or more HIV-1 strains in dual infection cultures. This can be achieved by establishing a competition assay, whereby primary strains or recombinant viruses are competed against laboratory strains or parental isolates. In general, two HIV-1 strains are allowed to replicate concurrently for a designated period of time, or as an alternative, HIV-1 recombinant viruses, which are unable to produce new infectious virions are used in a single cycle growth assay, to limit the replication to a single cycle. The experiment is analysed through the measurement of the proportion of each of the initial strains to give a relative replication capacity at the end of the assay. Few studies have taken this approach in order to compare a number of different HIV-1 strains including drug resistance mutants [Weber et al., 2003,Van Maarseveen et al., 2006], isolates from HIV-progressors versus LTNPs [Arien et al., 2005], variable subtypes, as well as different HIV-1 groups or HIV-1 versus HIV-2. To date, none of these studies have been performed on the currently emerging CRF01_AE/B inter-subtype recombinants from Malaysia, particularly CRF33_01B. It is therefore important and urgent to identify and understand the biological advantages of these new HIV-1 forms, which presumably will take over the predominance in HIV-1 epidemic in Malaysia.

10. Anti-HIV therapy, drug resistance and its dissemination: An example of China

In global terms, over the past 15 years the treatment of HIV-1 infection has evolved significantly. In North America and Western Europe, no effective therapy existed until the development and availability of zidovudine (ZDV, AZT) in 1987. In 2005, there are now 26 commercially available antiviral agents (both RT inhibitors [NRTI and NNRTI] and protease inhibitors) to treat HIV-1-infected individuals.

ARV treatment of HIV-1-infected patients in China fell behind that of most developed countries. While highly active antiretroviral therapy (HAART) became widely used in North America and Western Europe in 1996, China was still debating whether or not HIV/AIDS would become a huge epidemic there, despite the large number of IDUs testing positive in

the southwest province of Yunnan and almost all provinces reporting HIV cases. In 1998, facing the rapid upsurge in HIV-1 incidence nation-wide, the Chinese government made a concerted effort to strategize the "Middle and Long-term Programming for the Prevention and Control of AIDS" in China. A year later in 1999, several small clinical trails were initiated in Beijing primarily for safety and efficacy testing, sponsored largely by international pharmaceutical companies. The drug regimen tested then consisted of Combivir plus either Indinavir or Abacavir. This small-scale trial period (1999-2001) can be regarded as the first phase of ARV treatment in China.

The second treatment phase started when the cost of imported drugs used for HAART declined significantly and more patients could afford the medications (2001-2003). The population of Chinese patients undergoing therapy for HIV increased, especially in economically developed areas such as Beijing and Shanghai. However, the number of clinical doctors trained to administer these drugs did not expand. Many patients did not have the opportunity to receive comprehensive care, including standardized immunologic and virologic assessments prior to treatment and regularly scheduled follow-up interviews. Some patients judged the efficacy of the medication by a moderation of their symptoms, and consequently decreased their dosage or stopped taking the medicine altogether, without the consent of a physician. Of the patients who initiated treatment in this period, an estimated 25-30 % stopped taking medicine after only one or two months. Whether or not the other patients were able to persist with treatment and return for follow-up interviews is still to be determined.

The third phase of treatment (2003-present) began with the availability of low-priced domestically manufactured and imported generic anti-HIV drugs. This has been undeniably the most beneficial phase in increasing the number of individuals receiving gratis treatment. Nation-wide free ARV treatment started in 2003, part of the China CARES program, consisting of 51 model sites with plans to further expand to 127 counties. However, the bigger hurdle for this ambitious plan has been again the critical shortage of properly trained doctors, nurses and community care workers. Some patients were so anxious to begin taking medicine for HIV that they obtained the necessary drugs without a doctor's prescription. As a consequence, lacking professional guidance and clinical supervision, they used the medicines improperly, leading to the development of a drug resistant virus. In addition, as generic HIV drugs entered the Chinese market from developing countries, some patients began taking medicine without any medical assessment before treatment, and without choosing to register for interviews during treatment. Furthermore, severe side effects associated with generic ARV produced in China led to a large number of patients stopping medication entirely or becoming unwilling to follow doctors' advice and suggestions. As the incidence of HIV infection rises in China, it is anticipated that problems associated with the abuse of ARV will only escalate. It is therefore expected that drug resistant HIV-1 strains will emerge leading to their high prevalence and transmission over time.

A number of studies in other countries have shown that the prevalence of viruses with drug resistance mutations in acutely or recently infected persons varies between 10 to 20% [Boden et al., 1999; Grant et al., 2002; Little et al., 2002]. Research examining the prevalence and genetic features of drug-resistance strains at national level is lacking in China. Several major institutes in China are combining forces to carry-out genetic studies on viruses collected before and after nation-wide free ARV treatment. Based on preliminary data, it is fairly clear that the prevalence of drug-resistant strains were extremely rare before year 2000.

Between year 2001 and 2003, however, drug-resistant strains began to emerge and in some areas the prevalence is as high as 5%. Beginning in 2004, there was a significant increase in the prevalence of drug-resistant drugs across entire China, coinciding with the nation-wide free ARV treatment. Some areas have reported 20-30% drug-resistant strains specifically against NNRTI (unpublished data), and some areas were reported to have as high as 60% drug-resistant strains. The significant increase in the prevalence of drug-resistance could be due to the selection of the cohort and the time from transmission to resistance testing. However, it has clearly shown that resistance tests should be recommended routinely for patients with new infection.

The widespread use of antiretroviral drugs has led to the development and subsequent transmission of drug-resistant HIV-1 and the transmission of drug-resistant viruses has been documented through vertical, sexual, and parenteral routes [Erice et al., 1993; Masquelier et al., 1993; Veenestra et al., 1995]. Patients who are infected with drug-resistant HIV-1 and initiate antiretroviral therapy show poorer treatment responses than patients who are infected with wild-type (WT) viruses [Grant et al., 2002; Little et al., 2002]. Also, in the absence of selection pressures exerted by drugs, some transmitted drug-resistance mutations may persist for months before reversion to a more replication-competent variant. Even when these drug resistant mutations are no longer detectable by population-based nucleotide-sequence, they can persist in the reservoir of latently infected CD4+ memory T cells and may rapidly reemerge under the selective pressure provided by antiretroviral treatment [Wong et al., 1997; Finzi et al., 1997]. In subjects who acquired drug-resistant virus during primary infection, plasma HIV RNA is not suppressed as readily by potent antiretroviral therapy. The slower response to the treatment and the limited viral suppression may facilitate the selection of variants with greater drug resistance.

Thus, given the current spread and changing trends in HIV epidemiology in China, it is extremely urgent to understand the prevalence of drug-resistant strains in China and its changing patterns over time. Otherwise, we will face insurmountable challenges in tailoring our ARV regimens to elicit optimal therapeutic responses. In addition, the recombination between drug resistance HIV-1 strains in the Asia-pacific can cause epidemiologic shift, which may eventually compromise effective drug treatments currently available in these countries, including China. Since, at present, China and India are the economic hubs of Asia the human trafficking may lead to effective dispersal of such recombinant viruses. Therefore, well-coordinated international approaches are needed for surveillance and monitoring of the emergence of new drug resistance recombinant viruses.

11. Conclusions

It is hard to predict how the future of HIV epidemic will shape-up, since a number of complicating factors appear to unfold, including the possible effects of government intervention and unexpected changes (for the better or for the worse) in the behavior of affected populations. However, it is likely that the number of HIV infections is now on the rise, it is expected that the total number of HIV infections in China and India will surpass rest of the world, if no effective countermeasures are taken. Nonetheless, recombination between HIV-1 strains in geographical areas where multiple subtypes circulate will continue to shape future HIV epidemic through the generation of fitter strains capable of transmitting and dispersing in human populations fatser. China, India and other developing countries provide the right medium for this scenario. These countries stand at a critical juncture to

prevent widespread of HIV transmission from the high-risk groups to the general population. Comprehensive approaches are necessary, integrating prevention and treatment efforts. Government, NGO, and international organizations bear responsibility to stop this epidemic in China. Scientific communities and pharmaceutical companies both inside and outside China need to work jointly to develop more potent anti-HIV drugs and therapeutics to inhibit viral replication and reduce HIV transmission. We have seen clear evidence in favor of evolution of complex second and third generation recombinant viruses. Continued monitoring and surveillance of these viruses is needed, if an HIV vaccine is to be developed. Moreover, concerted efforts by joint ventures between the state and the private sector are highly needed for developing an HIV vaccine for the ultimate control of HIV and its spread in the most populous nation of the world.

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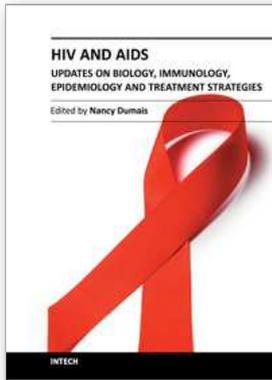
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The continuing AIDS pandemic reminds us that despite the unrelenting quest for knowledge since the early 1980s, we have much to learn about HIV and AIDS. This terrible syndrome represents one of the greatest challenges for science and medicine. The purpose of this book is to aid clinicians, provide a source of inspiration for researchers, and serve as a guide for graduate students in their continued search for a cure of HIV. The first part of this book, "From the laboratory to the clinic," and the second part, "From the clinic to the patients," represent the unique but intertwined mission of this work: to provide basic and clinical knowledge on HIV/AIDS.

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