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Genetic diversity and phenotypic characterization of HIV-1 Circulating Recombinant Forms (CRFs) among drug-naïve and -exposed patients in Burkina Faso

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Summary

The genotypic and phenotypic characteristics of HIV-1 CRF02_AG strains from infected patients in Burkina Faso were determined in order to understand about resistance development and susceptibility to antiretroviral drugs of HIV-1 non-subtype B. Drug resistance studies among Nevirapine (NVP) naïve and exposed women from Nouna, and HAART-exposed HIV patients attending the University Hospital in Ouagadougou, Burkina Faso were performed. The diversity and evolution of HIV-1 reverse transcriptase (RT) quasispecies in paired plasma, breast milk whey and/or breast milk cells from seventeen HIV-infected NVP-naïve and exposed women were determined by direct sequencing and clonal analysis. NVP resistance mutations were detected in 8% of infected mothers by direct sequencing. By using clonal analysis, the detection rate increased to 46% which indicates the high sensitivity of clonal analysis in detecting low proportion of drug resistance variants. To analyze compartmentalization of virus population in different anatomic compartments, phylogenetic analysis comparing virus populations of plasma, breast milk and/or breast milk cells from individual patients at both single and multiple time points was performed and the circulation of both compartmentalized (50%) and non-compartmentalized (45.5%) variants were found. These results suggest that in some individuals, viruses in breast milk whey may be evolving separately from plasma viruses. In most cases, breast milk whey variants were completely different from those of breast milk cells suggesting that free HIV virions in breast milk do not originate from infected breast milk cells. Different resistance mutation patterns were observed between viruses in plasma, breast milk and breast milk cells which suggest that transmission of HIV-1 from mother-to-child via breastfeeding may involve variants harbouring resistance mutations which cannot be predicted from variants present in plasma.

To determine possible influences of genetic background on drug susceptibility of virus, a new CRF02_AG proviral plasmid (pBD6TB9RI) and the derivative CRF02_AG/subtype B chimera containing PR-RT fragment of pNL4-3 were generated. By testing drug susceptibility against 5 protease inhibitors (PIs), 6 nucleoside reverse transcriptase inhibitors (NRTIs), and 3 non-nucleoside reverse transcriptase inhibitors (NNRTIs), the CRF02_AG virus showed similar phenotypic results like the CRF02_AG/subtype B chimera. To further evaluate the CRF02_AG plasmid backbone, PR-RT amplified fragments derived from HAART-experienced HIV patients were cloned into the pBD6TB9RI plasmid and transfection derived viruses were tested against the panel of antiretroviral drugs. The phenotypic results for both NRTIs and NNRTIs strongly correlated with the predicted genotypic resistance patterns. However, there were minor discordances with some PIs. This suggests that the novel recombinant viral assay should be useful in assessing the drug susceptibility of CRF02_AG and other non-B strains which are widely distributed in West and Central Africa.

Zusammenfassung

Die genotypische und phänotypische Eigenschaften von HIV-1 CRF02_AG Subtyp von infizierten Patienten aus Burkina Faso wurden bestimmen um die Entwicklung der Resistenz und die Suszeptibilität für antivirale Medikamenten dieses HIV-1 Subtypes zu verstehen. In dieser Arbeit wurde die Resistenz von HIV gegen antivirale Medikamenten in Nevirapin (NVP)-naiven bzw. –therapierten Patientinnen aus Nouna und HIV-Patienten unter hochaktiver antiretroviraler Therapie (HAART) am Universitätsklinikum in Ouagadougou, Burkina Faso untersucht. Die Diversität und die Evolution der HIV-1 Reverse Transkriptase (RT) Quasispezies im Blutplasma Muttermilch und/oder Muttermilchzellen von siebzehn HIV-infizierten, NVP-naiven bzw. -therapierten Patientinnen wurde durch direkte Sequenzierung und Klonalanalyse ermittelt. Die Ergebnisse zeigen, dass die klonale Analyse eine höhere Sensititvität bei dem Nachweis von Resistenzvarianten aufweist, die in der Viruspopulation nur in einem kleinen Anteil vorliegen. Während die direkte Sequnzierung nur bei 8% der HIV-infizierten Mütter NVP-Resistenzmutationen nachwies, wurden durch Klonalanalyse bei 46% dieser Frauen Mutationen detektiert.

Die Kompartimentierung von Viruspopulationen in Blutplasma gegenüber Muttermilch und/oder Zellen aus Muttermilch wurde mittels phylogenetischer Methoden analysiert. In 50% der Fälle zeigte sich eine Kompartimentalisierung der Viruspopulationen. Dies weist darauf hin, dass zumindest in einem Teil der Patientinnen eine unabhängige Entwicklung der Viren in Muttermilch stattfindet. Da sich die Viren in Muttermilch in den meisten Fällen von den Viren in Muttermilchzellen unterschieden, kann man annehmen, dass die freie HIV-Virionen in Muttermilch nicht von der Mehrzahl der infizierten Muttermilchzellen stammten. Aufgrund des Unterschieds von HIV-Varianten in Blutplasma, Muttermilch und Muttermilchzellen ist anzunehmen, dass Resistenzmutationen enthaltende Viren außerhalb von Blutplasma bei der Mutter-Kind Übertragung durch das Stillen eine wichtige Rolle spielen. Dies muss berücksichtigt werden, weil die meisten HIV-infizierten Mütter in Afrika ihre Kinder noch stillen.

Um mögliche Einflüsse des viralen Genotyps auf die Suszeptibilität von HIV gegnüber antiretroviralen Medikamenten zu untersuchen, wurde ein prototypisches provirales Plasmid des in Westafrika verbreiteten AG rekombinanen Subtyps (pBD6TB9RI) hergestellt, und die Sensitivität dieses Virus gegenüber 5 Protease-Inhibitoren (PI), 6 nukleosidischen Reverse-Transkriptase-Inhibitoren (NRTI) und 3 nichtnukleosidischen Reverse-Transkriptase-Inhibitoren (NNRTI) bestimmt. Ein Austausch der PR-RT kodierenden Region mit entsprechenden Sequenzen aus dem Subtyp B Isolat NL4-3 bewirkte keine Veränderung des Sensitivitätsprofils. Anschließend wurde die PR-RT Region aus Viren aus dem Plasma von Patienten unter HAART in pBD6TB9RI überführt und die Suszeptibilität der rekombinanten Viren gegen die verschiedenen Inhibitoren getestet. Überwiegend ergab sich eine Übereinstimmung mit der genotypischen Vorhersage. Im Fall der PI ergaben sich zum Teil jedoch Abweichungen zwischen den Ergebnissen der genotypischen und der phnotypischen Bestimmung. Dies zeigt den Nutzen des hier etablierten phänotypischen Resistenztests für Viren des AG rekombinanten Subtyps.

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1 Introduction

1.1 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) causes a chronic infectious disease known as the acquired immunodeficiency syndrome (AIDS), which progressively damages the host's immune system. HIV belongs to the lentivirus subfamily of retroviruses (family Retroviridae). HIV genome is encoded as a positive sense RNA which is transcribed into double stranded DNA by the reverse transcriptase enzyme, the most noticeable feature of retroviruses (Vogt, 1997).

Since HIV was discovered in 1983, it has rapidly emerged as one of the most devastating infectious pathogens of this century (Gallo et al., 1983). By the end of 2007, an estimated 30 million people worldwide were living with HIV of whom 2.1 million were children. Approximately 2.5 million new infections occurred in 2007 with 1.7 million (68%) of these in sub-Saharan Africa (UNAIDS/WHO, 2008).

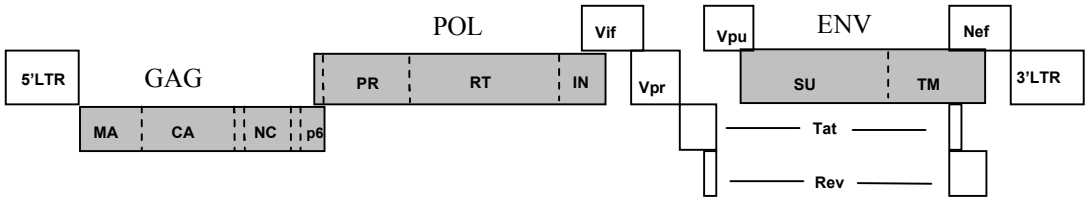
1.1.1 HIV-1 structure and genomic organization

The HIV-1 virion has a spherical structure with an approximate size of 145 nm in diameter (Briggs et al., 2003). The virion contains two copies of single stranded HIV-1 genomic RNA encoding the structural protein (Gag), enzymatic protein (Pol), glycoprotein (Env) and accessory proteins (e.g. Vif, Vpr, Vpu, Nef, Rev, Tat) that regulate gene expression and modulate pathology (Fig. 1).

The *gag* gene synthesizes the structural polyprotein precursor Gag ($Pr55^{Gag}$) consisting of the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6 domains as well as two spacer peptides (SP1 and SP2). The Pol proteins consist of the viral enzymes protease (p11), reverse transcriptase/Rnase H (p51/p66) and integrase (p32) which are generated by subsequently cleavage of the Gag-Pol polyprotein precursor (Pr160). The Env protein is expressed as a precursor glycoprotein gp160 that is subsequently cleaved by the cellular protease furin into the surface protein gp120 (SU)

and the transmembrane protein gp41 (TM). The long terminal repeats (LTRs) flank the coding region and are necessary for reverse transcription and integration (Fig. 1).

A.



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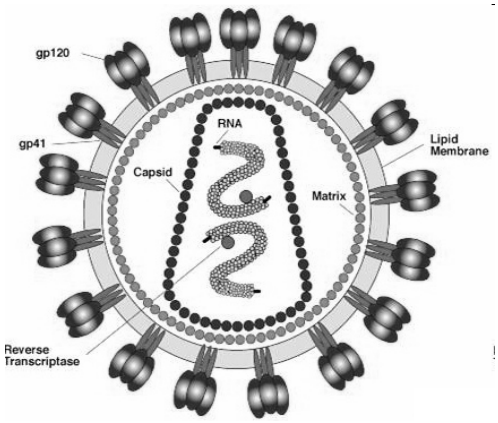

Fig. 1 Genomic organization of HIV-1. (**A**) The open reading frames of the viral proteins are depicted. The virus structural genes are shaded and the accessory genes and the LTR are shown as open boxes. LTR, Long terminal repeat; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; TM, transmembrane glycoprotein; SU, surface glycoprotein. (**B**) Typical representation of a mature HIV particle: spherical, approximate 100 nm in diameter and consisting of a lipid bilayer membrane surrounding a conical nucleocapsid. Adapted from Knipe et al.,2001.

1.1.2 Replication cycle

HIV-1 infection begins with the interaction of gp120 with the cellular CD4 receptor on the surface of the target cells (Dalgleish et al., 1984). Subsequently, an interaction between gp120 and the chemokine receptor CCR5 or CXCR4 occurs leading to a conformational change exposing gp41, which induces fusion of the virus and cell membrane (Stein et al., 1987). After fusion, HIV releases the core into the cytoplasm of the target cell. In the cytosol, viral reverse transcriptase (RT) synthesizes a double stranded DNA from single stranded viral RNA. Inside the nucleus, the viral DNA is integrated into the host genome by HIV-1 integrase (IN) enzyme, resulting in the so called provirus which can remain latent or lead to active synthesis of the viral progeny (Bushman et al., 1990). Viral transcription is achieved by cellular RNA polymerase II

which transcribes viral DNA into mRNA. Subsequently, viral mRNAs are exported to the cytoplasm for translation as polyproteins and associate with other viral components to form the immature, non-infectious particles. The immature virions are release from host cell membrane where they acquire viral envelope proteins. Finally, the viral polyprotein precursors within the immature particles are proteolytically processed by the viral protease (PR) enzyme leading to the development of mature infectious HIV virions. The free mature HIV-1 virions can further infect other cells and initiate their life cycle (Coffin, 1997) (Fig. 2).

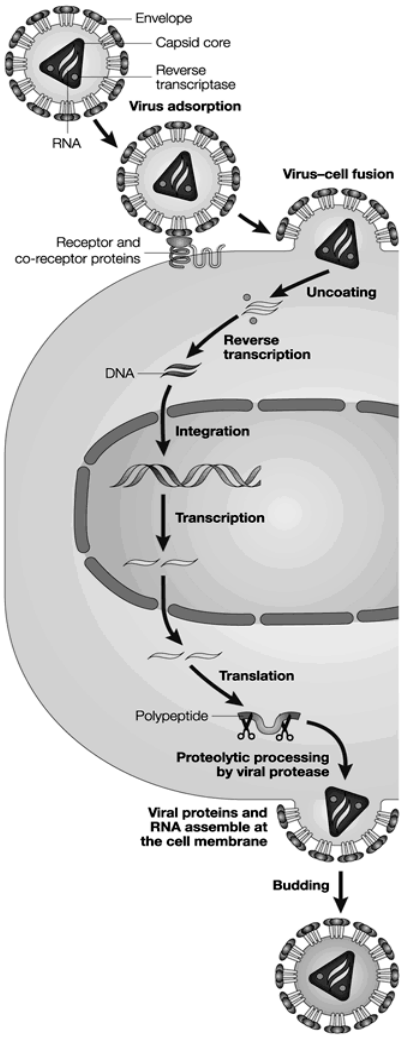

Fig. 2 HIV-1 replication cycle. This figure is adapted from De Clercq, 2002 and the replication cycle is described in the text.

1.1.3 Classification of immunodeficiency viruses

HIV belongs to the primate lentivirus subfamily of retroviruses and can be divided into 2 groups; HIV-1, HIV-2. HIV-1 and HIV-2 are highly divergent with their envelope protein sequences differing by as much as 60%, reflecting their distinct origin (Sharp, 2002).

Presently, HIV-1 is subdivided into 3 groups: Major (M), Outlier (O) and non-M/non-O (N). However, very recently, a new strain of HIV-1 similar to SIVs gorilla was isolated from a Cameroonian living in France and was tentatively called HIV-1 group P (Plantier et al. 2009). Group M is responsible for the majority of infections worldwide and can be divided into 9 subtypes (A-D, F-H, J and K), 5 sub-subtypes (A1-A3 and F1-F2) and 43 circulating recombinant forms (CRFs) (Robertson et al., 2000; Tebit et al., 2007; Kuiken et al. 2009). Comparing the protein coding sequences between HIV-1 subtypes a nucleotide diversity of up to 30% is observed in *env*, 20% in *gag* and 15% in *pol* (Gao et al., 1997).

Group O viruses show high variability between isolates. Partial *gag*, *pol* and *env* sequences have been used to classify group O viruses into putative subtypes (Mas et al., 1999; Roques et al., 2002). These studies indicate a high diversity of group O and do not show the same subtype formation like group M viruses (Fig. 3) (Quinones-Mateu et al., 1998; Yamaguchi et al., 2003). The group N viruses form an independent clade related to group M, whereas sequences from the 3' end cluster more closely with SIVcpzUS chimpanzee virus, suggesting a possible ancient recombination within humans or prior to cross-species transmission (Fig. 3) (Gao et al., 1999; Corbet et al., 2000).

HIV-2 subtypes are mainly restricted to West Africa and can be categorized as epidemic subtypes (A-B) and non-epidemic subtypes (C-G) (Lemey et al., 2003). Individuals infected with HIV-2 develop AIDS as well, but with a longer incubation period and lower morbidity (Gao et al., 1994).

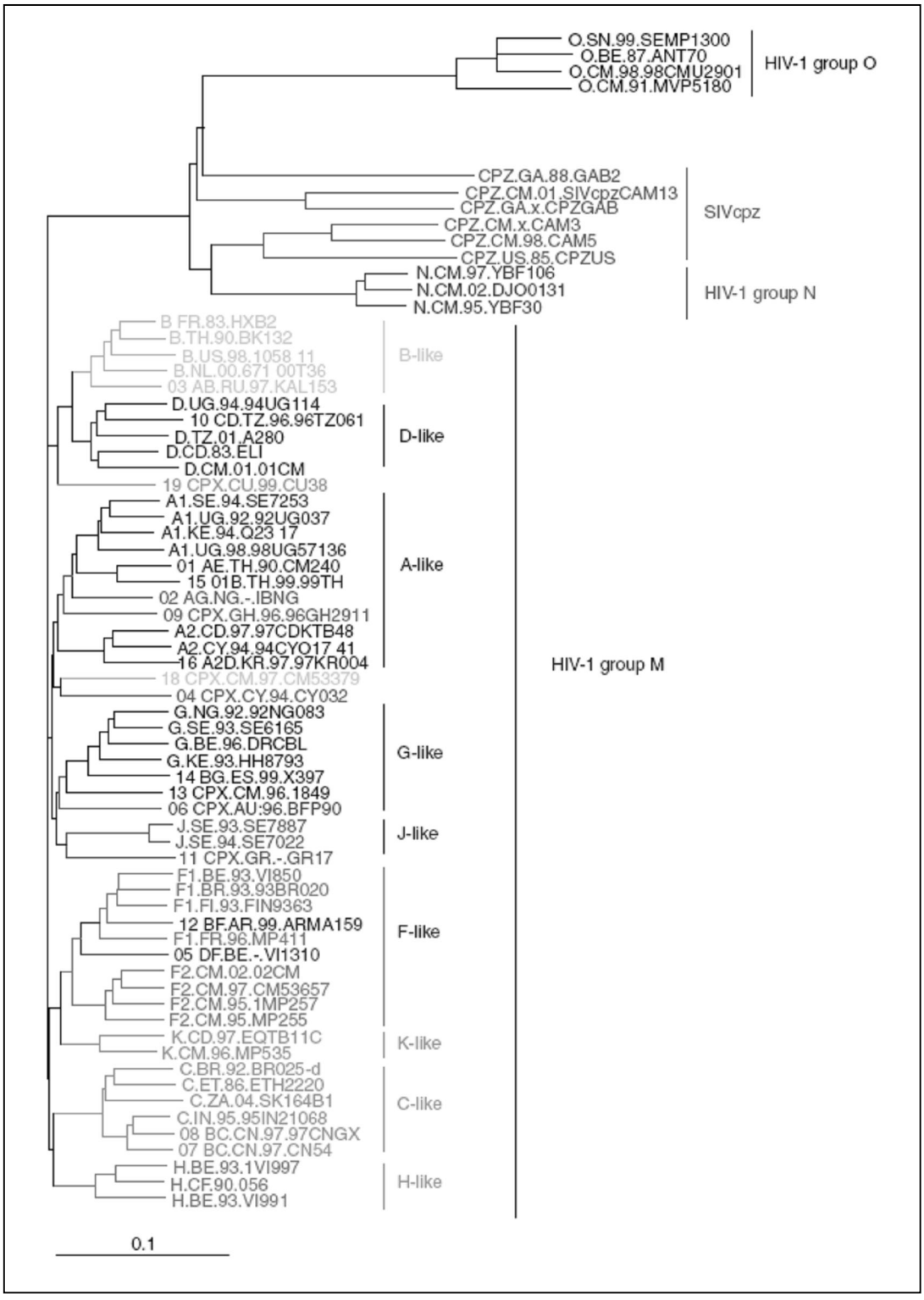

Fig. 3 Phylogenetic tree representing the genetic diversity in HIV-1 group M, N, O and SIVcpz. HIV-2 was use as the out group. Subtype and CRFs are represented in black and different shades of gray. Within the HIV-1 group M the term "x-like" is used to denote clusters which are similar to the different group M subtypes. Adapted from Tebit et al., 2007.

1.2 Circulating recombinant forms (CRFs)

HIV recombination occurs when a single cell is infected by two different viruses to generate a heterozygous or heterodiploid virion. Due to the switching of the reverse transcriptase between RNA genomes during minus (-) strand DNA synthesis (Preston and Dougherty, 1996; Temin, 1993; Wain-Hobson, 1992), *de novo* infection by this heterodiploid virus yields a recombinant retroviral DNA sequence. HIV-1 can recombine not only within subtypes (intra-subtype recombination), but also between subtypes (inter-subtype recombination) and groups (inter-group recombination) (Morris et al., 1999; Peeters, 2000; Takehisa et al., 1999).

In general, circulating recombinant forms (CRFs) are recombinants which are comprised of two different subtypes (Table 1). However, some CRFs are composed of more than two different subtypes, so called complex CRF (CRFcpx) (Table 1). These recombinants may occur due to recombination events between CRFs and other pure subtypes (Table 1). CRFs are responsible for about 10-20% of all new infections (Robertson et al., 2000; Peeters and Sharp, 2000). The most dominant forms in the epidemic are the CRF01_AE, CRF02_AG, CRF07, 08_BC and CRF12_BF viruses found in Asia, West Africa, China and South America, respectively (Table 1).

Table 1. Characteristics and geographical distribution of circulating recombinant forms (CRFs) (adapted from Kuiken et al., 2009; Tebit et al., 2007).

Name	Reference strain	Subtypes	Distribution	Fitness	Identified sequences[a]
CRF01_AE	CM240	A, E	Thailand, central Africa	group M-like	6332
CRF02_AG	IbNG	A, G	West and central Africa	AG>A=G	3769
CRF03_AB	Kal153	A, B	Eastern Europe	unknown	149
CRF04_cpx	94CY032	A, G, H, K, U	Cyprus, Greece	unknown	22
CRF05_DF	VI1310	D, F	Belgium	unknown	29
CRF06_cpx	BFP90	A, G, J, K	West Africa	unknown	845
CRF07_BC	CN54	B', C	China, Taiwan	unknown	136
CRF08_BC	GX-6F	B', C	China	unknown	187
CRF09_cpx	96GH2911	CRF02, A, U	West and central Africa	unknown	39
CRF10_CD	TZBF061	C, D	East Africa	unknown	202
CRF11_cpx	GR17	A, CRF01, G, J	Central Africa	unknown	529
CRF12_BF	ARMA159	B, F	South America	F>B=BF[b]	348
CRF13_cpx	96CM-1849	A, CRF01, G, J, U	Central Africa	unknown	78
CRF14_BG	X397	B, G	Europe, Asia	unknown	90
CRF15_01B	99TH.MU2079	CRF01, B	Thailand	unknown	18

Table 1. Characteristics and geographical distribution of circulating recombinant forms (CRFs) (adapted from Kuiken et al., 2009; Tebit et al., 2007) *(continued)*

Name	Reference strain	Subtypes	Distribution	Fitness	Identified sequences[a]
CRF16_A2D	KISII5009	A2, D	Kenya, Korea, Argentina	unknown	6
CRF17_BF	ARMA038	B, F	South America	F>B=BF[b]	7
CRF18_cpx	CU76	A1, F, G, H, K, U	Cuba, central Africa	unknown	36
CRF19_cpx	CU7	A1, D, G	Cuba	unknown	10
CRF20_BG	CB228	B, G	Cuba	unknown	5
CRF21_A2D	99KE_KER2003	A2, D	Kenya	unknown	2
CRF22_01A1	CM53122	CRF01, A1	Cameroon	unknown	3
CRF23_BG	CB118	B, G	Cuba	unknown	2
CRF24_BG	CB378	B, G	Cuba	unknown	3
CRF25_cpx	02CM_1918LE	A, G, U	Cameroon	unknown	2
CRF26_AU	02CD_MBTB047	A, U	Pending	unknown	NA
CRF27_cpx	97CDKTB49	A, E, G, H, J, K	D.R.C	unknown	9
CRF28_BF	BREPM12609	B, F	South America	F>B=BF[b]	2
CRF29_BF	BREPM16704	B, F	South America	F>B=BF[b]	3
CRF30_0206	00NE36	CRF02,CRF06	Niger	unknown	1
CRF31_BC	04BR142	B, C	Brazil	unknown	17
CRF32_06A1	EE0369	CRF06, A1	Eastern Europe (Estonia)	unknown	23
CRF33_01B	05MYKL007_1	CRF01, B	Asia (Malaysia)	unknown	4
CRF34_01B	OUR2478P	CRF01, B	Thailand	unknown	3
CRF35_AD	AF095	A, D	Afghanistan	unknown	4
CRF36_cpx	NYU830	CRF01, CRF02, A, D	Cameroon	unknown	2
CRF37_cpx	NYU926	CRF01, CRF02, A, G, U	Cameroon	unknown	2
CRF38_BF1	UY05_4752	Pending	Uruguay	unknown	3
CRF39_BF	03BRRJ103	B, F	Brazil	unknown	3
CRF40_BF	04BRRJ115	B, F	Brazil	unknown	4
CRF41_CD	CO6650V1	C, D	Pending	unknown	3
CRF42_BF	luBF_13_05	B, F1	Luxembourg	unknown	21
CRF43_02G	J11223	CRF02, G	Saudi Arabia	unknown	4

[a] = global estimate based on information from the HIV database and published data; [b] = preliminary results, analysis in progress; [c] = all BF-like viruses combined, NA= data not available; pending = sequences is still to be determined

1.3 Routes of HIV-1 transmission

HIV-1 can be transmitted by both homosexual and heterosexual contact with infected partners (Clumeck et al., 1985; Kingsley et al., 1989). HIV-1 can be isolated from both semen and female genital secretions (Ho et al., 1984; Wofsy et al., 1986).

Transmission also occurs by transfusion of whole blood, cellular blood components, plasma and clotting from HIV-1 infected subjects to the recipients (Curran et al., 1984; Ward et al., 1987). HIV is transmitted among injecting drug users (IVDU) via contaminated needles. Risk factors of IVDU also include frequency of needle sharing, injections and prevalence of HIV infection in the area (Des Jarlais et al., 1989; Schwenbaum et al., 1989).

Transmission from an HIV infected mother to the newborn has been shown to occur across the placenta (Lapointe et al., 1985), at the time of delivery by exposure to maternal secretion or post-delivery via breast feeding (Van de Perre et al., 1992; Ziegler et al., 1985). Breast feeding has been identified as an important route for vertical transmission of HIV-1 (Dunn et al., 1992; John et al., 2001), particularly in sub-Saharan Africa where antiviral therapy and infant formula are not widely available (Nduati et al., 2000). Transmission of HIV from mother-to-child through breast milk (BM) is associated with several factors such as maternal viral load, cell-free and cell-associated HIV in breast milk, low maternal CD4 cell count and mastitis (Coovadia et al., 2007; Rousseau et al., 2004). Transmission can take place at any point during lactation and the cumulative probability of acquisition of infection increases with duration of breast feeding (Miotti et al., 1999).

1.4 Antiretroviral therapy and drug resistance mutations

There are currently 5 classes of drugs approved by the United States Food and Drug Administration (US FDA) for HIV-1 treatment (Table 2). Most of the antiretroviral drugs target viral enzymes such as the protease inhibitors (PIs), reverse transcriptase inhibitors (RTIs) consisting of nucleoside/nucleotide RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs), and integrase inhibitor (INI). Two entry-inhibitors, enfuvirtide and maraviroc are also available for HIV-1 therapy.

Table 2. Antiretroviral drugs for HIV-1 treatment

Class	Drugs
NRTIs	Abacavir (ABC)
	Didanosine (ddI)
	Emtricitabine (FTC)
	Lamivudine (3TC)
	Stavudine (d4T)
	Tenofovir (TDF)
	Zidovudine (ZDV,AZT)
NNRTIs	Efavirenz (EFV)
	Etravirine[a] (ETR)
	Nevirapine (NVP)
PIs	Atazanavir[b] (ATV)
	Darunavir[b] (DRV)
	Fosamprenavir[b] (FPV)
	Indinavir[b] (IDV)
	Lopinavir[b] (LPV)
	Nelfinavir (NFV)
	Saquinavir[b] (SQV)
	Tipranavir[b] (TPV)
INI	Raltegravir (RAL)
Entry inhibitors	Enfuvirtide (T-20)
	Maraviroc (MVC)

[a] = ETR shows activity against HIV strains that are resistant to previously approved NNRTIs.
[b] = PIs are currently used in combination with Ritronavir (RTV) which acts as a pharmacologic booster.

HIV-1 mutations occur due to the rapid turnover and high error rate of reverse transcriptase which lacks 3'- 5' exonuclease proof reading activity (Preston et al., 1988). These mutations facilitate HIV-1 to escape from the immune system (Ho et al., 1995; Piatak et al., 1993) and also facilitate the escape from antiretroviral drug pressure resulting in the emergence of drug resistant viruses which account for a large portion of treatment failures (Coffin, 1996).

In general, primary or major mutations decrease drug susceptibility by themselves, whereas secondary or minor mutations reduce drug susceptibility in combination with

primary mutations or improve the replicative fitness of virus isolates with primary mutations.

1.4.1 Protease inhibitors (PIs)

PIs bind to the active site of the viral protease enzyme and prevent the processing of viral proteins into functional forms. In this way, the new virions are unable to mature or become infectious. Mutations in the protease substrate cleft cause resistance by reducing the binding affinity between inhibitor and protease enzyme. Mutations elsewhere in the enzyme either cause resistance by altering enzyme catalysis, dimer stability, re-shaping the active site or compensate for the decreased kinetics of enzyme (Barbour et al., 2002; Erickson et al., 1999; Muzammil et al., 2003).

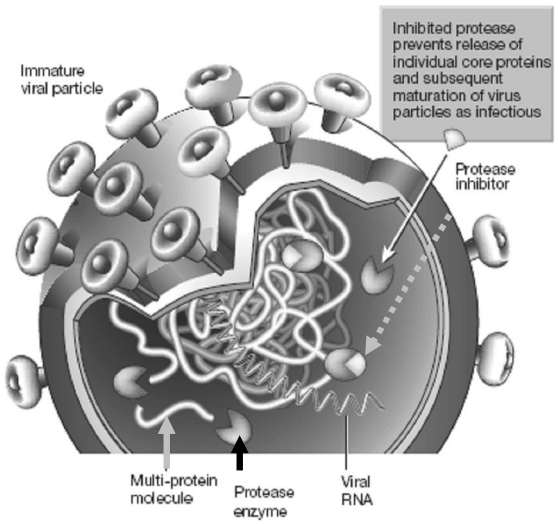

Fig. 4 Mechanism of action of protease inhibitors. Protease inhibitors (indicated by dash gray arrow) bind to protease enzyme (indicated by solid black arrow) and prevent the polyprotein clevage (indicated by solid gray arrow). Adaped from Richman, 2001.

1.4.2 Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)

NRTIs have a similar molecular structure to the natural building blocks, dNTP. Both nucleoside and nucleotide analogs are prodrugs that must be phosphorylated by host cellular enzymes to become the active form. Phosphorylated NRTIs compete with natural dNTP for incorporation into a newly synthesized DNA strand, therefore they terminate the ongoing viral DNA synthesis (Richman, 2001; Shafer, 2002) (Fig. 5A). There are two biochemical mechanisms of NRTI drug resistance. The first mechanism is mediated by

mutations that allow the reverse transcriptase enzyme to distinguish between NRTIs and dNTPs during polymerization, thereby preventing the incorporation of NRTI to the DNA chain (Huang et al., 1998; Sarafianos et al., 1999). The second mechanism is mediated by mutations that promote the hydrolytic removal of the NRTI and permit DNA elongation (Arion et al., 2000; Mayer et al., 2003).

1.4.3 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs are noncompetitive inhibitors which interact with an allosteric non-substrate binding site of HIV-1 reverse transcriptase (De Clercq, 2002; Hsiou et al., 2001). This interaction affects the activity of reverse transcriptase by restricted enzyme mobility and function (Richman, 2001) (Fig. 5B). NNRTIs do not require metabolic activation like NRTIs resulting as a high potency drug. However, NNRTI resistance usually emerges rapidly. A single mutation in the NNRTIs binding pocket can result in high-level resistance that can also confer cross-resistance to other drugs in the NNRTI-group (Havlir et al., 1996; Jackson et al., 2000).

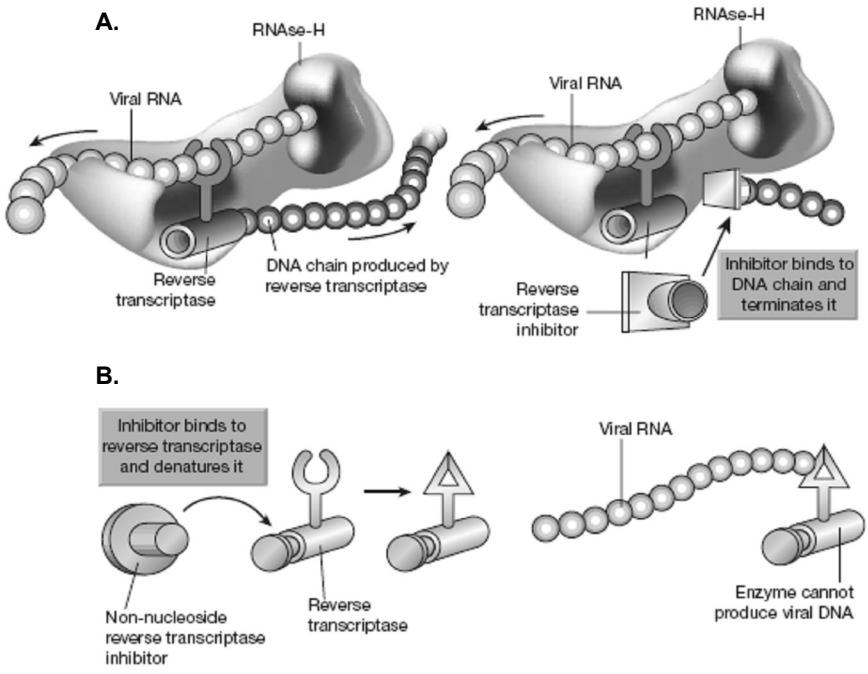

Fig. 5 Mechanism of action of reverse transcriptase inhibitors. (A) NRTI is incorporated into viral DNA and terminates viral DNA synthesis. (B) NNRTI binds to RT enzyme and inhibits RT enzyme activity. Adapted from Richman, 2001.

1.4.4 Integrase inhibitor (INI)

The HIV-1 integrase enzyme is responsible for the integration of viral DNA into the host cell genome. Integrase possesses two major catalytic activities: an endonucleolytic cleavage at each 3'-OH extremity of the viral genome, named 3'-processing and a strand transfer reaction leading to the insertion of the processed viral DNA into the target DNA by a one-step trans-esterification (Ellison et al., 1990; Miller et al., 1997). Raltegravir is the first approved integrase inhibitor for clinical use. This inhibitor binds to integrase- viral DNA complex and thereby selectively blocks the strand transfer step (Delelis et al., 2008). However, resistance mutations to raltegravir have been reported. These mutations affect function of intergrase by altered both 3' processing and strand transfer activities (Malet et al., 2008).

1.4.5 Entry inhibitors

The first FDA approved entry inhibitor, enfuvirtide (T-20) is a 36–amino acid peptide derived from the heptad repeat 2 (HR2) region of the gp41 transmembrane domain of HIV-1 subtype B. T-20 is predicted to bind to a highly conserved hydrophobic groove located on the trimeric coiled coils of HR1, thereby preventing the formation of the six-helix bundle and inhibiting membrane fusion (Fig. 6). The amino acid position 36-43 (**GIV**QQQNN) of heptad repeat 1 (HR1) primarily determines T-20 responsiveness. Therefore, changes within the GIV motif are sufficient to cause drug resistance to T-20 (Reeves et al., 2005).

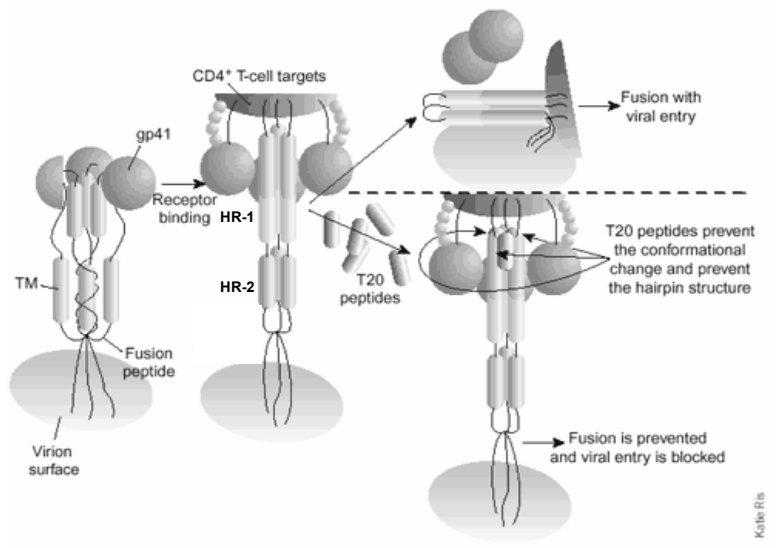

Fig. 6 Mechanisms of action of fusion inhibitor. After gp120 binds to a CD4+ cell, the transmembrane (TM) domain undergoes a conformational change that includes unfolding which results in the 'spring-loaded' formation of coiled-coil helices in preparation for viral entry. Enfuvirtide (T-20) binds to the TM domain and prevents fusion to the host cell and viral entry. Adapted from Pomerantz & Horn, 2003.

The second entry inhibitor, maraviroc, is a co-receptor antagonist which blocks entry of CCR5 (R5) tropic viruses to target cells (De Clercq, 2007). Viruses that can use both CCR5 and CXCR4 (termed dual/mixed or D/M) or only CXCR4 (X4) do not respond to maraviroc treatment (Westby et al., 2006; Wilkin et al., 2007). A virologic failure with maraviroc therapy is frequently associated with outgrowth of X4 virus that preexisted as a minor population below the level of assay detection (Johnson et al., 2008).

1.4.6 HIV-1 treatment strategies and drug resistance mutations

Current treatment strategies for HIV-1 infected individuals include the use of combination antiretroviral drug therapy also termed highly active antiretroviral therapy or HAART. The use of antiviral drugs has been shown to suppress HIV-1 replication and lead to a significant decrease in disease progression with improved clinical status (Pilcher et al., 1999). However, these combination strategies do not completely eliminate viruses and the viruses may rebound when the antiviral treatment is interrupted (Steingrover et al., 2008). One limitation of complex therapeutic regimens is that they may induce emergence of more complex resistance patterns. Several studies performed in both developed and developing worlds have reported the presence of resistance mutations among treatment-naïve HIV patients, suggesting transmission of resistant viruses (Little et al., 2002; Oette et al., 2006). Approximately 30 million people worldwide are infected with HIV and 68% of these infected people are living in sub-Sahara Africa (UNAIDS/WHO, 2008). The availability of HAART for HIV-infected people in sub-Sahara Africa has also rapidly increased (Koening et al., 2006). Several studies reported the emergence of drug resistance in individuals undergoing treatment in African countries (Kantor et al., 2002; Petrella et al., 2001; Richard et al, 2004). Due to the extensive use of single-dose nevirapine (SD-NVP) to prevent mother-to-child transmission (PMTCT) in sub-Sahara Africa, NVP- associated mutations have been found most commonly (Arrive et al., 2007).

1.5 Effects of single dose nevirapine (SD-NVP) and viral compartmentalization

Single dose Nevirapine (SD-NVP) given to women during labor and to infants within 72 hours of birth has been shown to be an effective and low cost intervention for

the reduction of mother-to-child transmission (MTCT) in resource limited countries (Jackson et al., 2003). However, administering SD-NVP can lead to rapid selection of resistant variants. The most common mutations among them are the K103N and/or Y181C mutations in reverse transcriptase which confer cross-resistance to other approved antiviral drugs in the NNRTI group (Deeks 2001; Richman et al., 1994). The K103N mutation appears to have little effect on the replication capacity of HIV-1 allowing this variant to persist long after NVP therapy is stopped (Lecossier et al., 2005; Palmer et al., 2006a). Several studies have demonstrated that the frequency of NVP-resistance mutations decreases over time, but they can persist for more than 1 year in plasma after the exposure to SD-NVP (Flys et al., 2005; Palmer et al., 2006b). NVP resistant variants have been also detected in the breast milk of some SD-NVP exposed women (Kassaye et al., 2007; Lee et al., 2005) and transmission of NVP-resistant variants via breast feeding has been reported (Eshleman et al., 2001).

Viral compartmentalization occurs due to constraints on viral entry and replication, target cell differences and differing immune responses in distinct anatomical sites that result in the independent selection of subsets of the virus population for continuous replication (Henderson et al., 2004). Moreover, the emergence of drug resistant strains may vary in diverse anatomical compartments and this variation can be attributed to the different pharmacokinetic properties of a particular drug (Kepler and Perelson, 1998; Wong et al., 1997). Several compartments such as cerebrospinal fluid (Lafeuillade et al., 2002), genital tract secretions (Overbaugh et al., 1996) and lymphoid tissue (Omrani and Pillay, 2000) have been shown to be poorly accessible to different antiretroviral drugs.

In recent years, different anatomical compartments have been analyzed for the evolution of HIV-1 as well as for the emergence of drug resistance variants. Different compositions of viruses have been recovered from plasma and peripheral blood mononuclear cells (PBMC) during HIV-1 infection (Livingstone et al., 1996). Several studies have reported compartmentalization of HIV-1 between PBMC and mucosal compartment (Poss et al., 1995), PBMC and renal epithelial cells (Marras et al., 2002), blood and different anatomical compartments such as lung (Singh et al., 1999), brain, spleen and lymph node (Wong et al., 1997) and genital tract (Diem et al., 2008; Kemal et al., 2003) as well as body fluid (Delwart et al., 1998; Ellerbrock et al., 2001; Overbaugh et al., 1996) within an individual. However, information on the viral population and NVP associated drug resistance mutations in breast milk is still unclear (Becquart et al., 2002,

2007; Henderson et al., 2004; Lee et al., 2005). Such knowledge is important especially in Africa where breastfeeding is still highly practiced even by HIV infected mothers.

1.6 Drug resistance assays

As the detection of resistance becomes an integral part of patient management, resistance tests have been recommended to clinicians to select the optimal drug regimens after the first or multiple treatment failures (Hirsch et al., 2003). Moreover, because of the emergence of drug resistance variants, resistance tests are also recommended in primary HIV infection (Little et al., 2002). Evaluation of drug resistance can be monitored by either genotypic or phenotypic assays.

1.6.1 Genotypic assays

Genotypic assays are mostly based on DNA sequencing which can be performed by different methods. The dideoxynucleotide (Sanger) sequencing or chain termination method is carried out by using dye labeled dideoxynucleotides (ddNTPs) which are incorporated into the PCR product and lead to termination of elongation. The lengths of sequencing products are determined by the fluorescence spectra of the dye-label on an automated sequencing machine (Schuurman et al., 1999) (Fig. 7A). The Sanger sequencing method is widely used to detect drug resistance mutations from patient-derived PCR products, so called direct PCR sequencing. However, direct PCR sequencing is unable to detect low proportions of drug-resistance variants in the heterogeneous virus population existing in a patient's plasma sample (Palmer et al. 2005).

Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis (Ronagi et al., 1998). The nucleotide composition of a growing DNA-strand is determined by an enzyme cascade system (O'Meara et al., 2001; Ronaghi, 2001) (Fig. 7B). The ultra-deep pyrosequencing could detect minority variant harbouring drug-resistance mutations in antiretroviral-experienced patients in whom mutations were no longer detectable by standard direct PCR sequencing (Le et al., 2009; Wang et al., 2007).

The other approaches to analyze viral genotype are based on the hybridization technique such as line probe assay (LiPA) (Stuyver et al., 1997), oligonucleotide ligation

assays (OLAs) (Beck et al., 2002; Troyer et al., 2008), and the high density oligonucleotide arrays (GeneChip) (Kozal et al., 1996; Wilson et al., 2000).

In general, genotypic assays are convenient to perform and relatively rapid but provide indirect evidence of resistance. Furthermore, it is difficult to interpret the sequences with unusual amino acid substitutions (Paolucci et al., 2003) or complex mutation patterns (Ross et al., 2001).

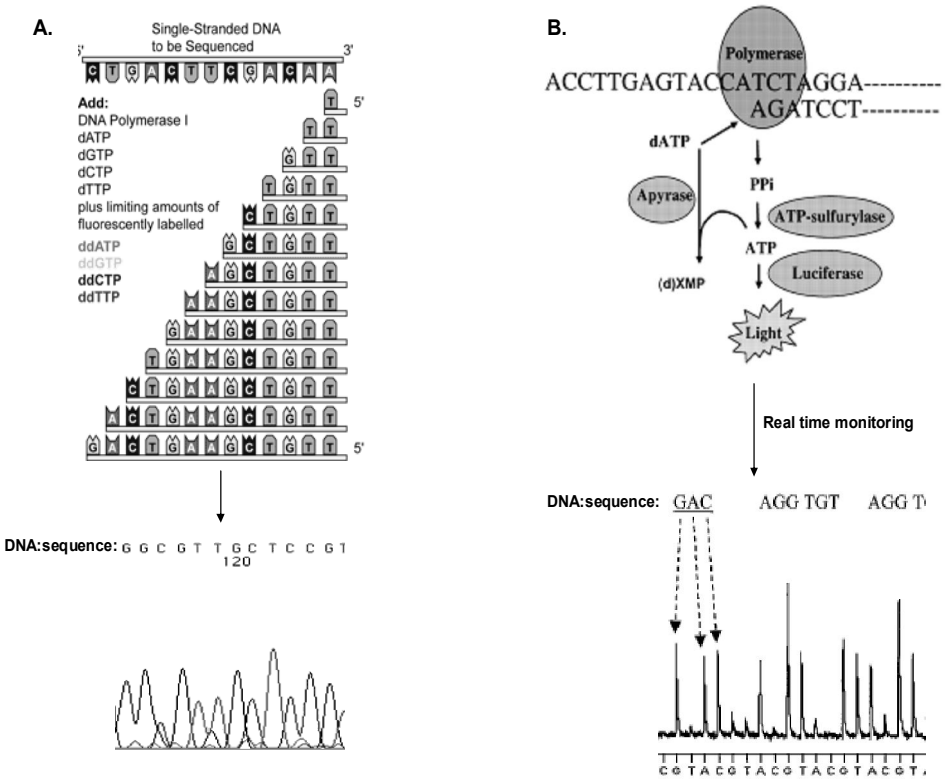

Fig. 7 Schematic representation of the DNA sequencing techniques. (A) Chain termination method (Sanger sequencing). The DNA template is added to a mixture containing dNTPs, a primer, a DNA polymerase and a limited amount of four dideoxynucleotides (ddATP, ddGTP, ddCTP, ddTTP) labelled with different fluorescence dyes that emits different colors upon laser excitation (above). An electropherogram showing the nucleotide composition of a particular sequence as determined by fluorescence (below). (B) Pyrosequencing method. The reaction mixture consists of single stranded DNA with an annealed primer and four enzymes. The four different nucleotides are added stepwise and the cascade starts with a nucleic acid polymerization reaction in which pyrophospate (PPi) is released as a result of nucleotide incorporation by DNA polymerase. The released PPi is subsequently converted to ATP by ATP sulfurylase which provides the energy to luciferase to oxidize luciferin and generate detectable light. The exceeded nucleotides are continuously degraded by enzyme apyrase allowing addition of subsequent nucleotide. dXTP indicates one of the four nucleotides (above). The pyrogram showing the nucleotide sequence in a specific section of DNA (below). This figure is adapted from O'Meara et al., 2001; Ronaghi, 2001 and http://clinical-virology.org/

1.6.2 Phenotypic assays

These assays determine the *in vitro* ability of an HIV-1 isolate to replicate in the presence of antiviral drugs and thus provide a direct quantitative resistance measurement. Previously, phenotypic drug susceptibility was performed by culturing viruses derived from patients' peripheral blood mononuclear cells (PBMCs) and measuring the effects of different concentrations of a drug on viral replication in cell culture which was both labor intensive and time consuming (Japour et al., 1993). The novel methods based on the development of recombinant viruses have developed and these assays have been termed Recombinant Viral Assays (Kellam and Larder, 1994). In general, recombinant viruses are generated by amplification of patient derived protease-reverse transcriptase (PR-RT) sequences which are then inserted into a PR-RT-deleted provirus backbone, generally derived from HIV subtype B strains either by homologous recombination (Hertogs et al., 1998) or direct cloning (Petropoulos et al., 2000; Klimkait, 2002; Paolucci et al., 2004; Garcia-Perez et al., 2007). The recombinant viruses therefore retain the drug susceptibility of the PR and RT of the patient sample.

1.7 Global HIV distribution

HIV-1 subtype C comprises about 52% of all HIV infections in the world (Ghys et al., 2003). The highest prevalence of HIV-1 is found in Southern Africa which also has the lowest HIV-1 diversity due to the dominance of subtype C (Tebit et al., 2007) (Fig. 8). In East Africa (Fig. 8), subtypes A, D and C are the predominant strains (Zhu et al., 1998). West and Central Africa has been described as an "HIV diversity hotspot", because it carries a mixture of nearly all HIV strains. However, CRF02_AG is responsible for the majority of the HIV-1 infection in this part of Africa (Montavon et al., 2000; Tebit et al., 2002) (Fig. 8).

Asia and Eastern Europe (Fig. 8) are currently observing a dynamic HIV epidemic. Although subtype B was the first strain introduced into many Asian countries, it has not been spread widely in comparison to other subtypes. In China, subtype C and B as well as the CRF07_BC and CRF08_BC are highly prevalent. Subtype C is the most common subtype found in India (Shankarappa et al., 2001) and CRF01_AE in South-East Asia (Carr et al., 1996) (Fig. 8).

In Eastern Europe, sub-subtype A1 is dominant, but subtype B and CRF03_AB also co-circulate (Liitsola et al., 1998). In North America, Western Europe and Australia, subtype B is the most prevalent form of HIV-1 (Fig. 8). However, there has been a rapid increase in non-B subtypes and CRFs in Western Europe (Hemelaar et al., 2004). South America has HIV epidemics with considerable subtype and CRF diversity including subtypes B, C, F and BF recombinant (Tebit et al., 2007) (Fig. 8).

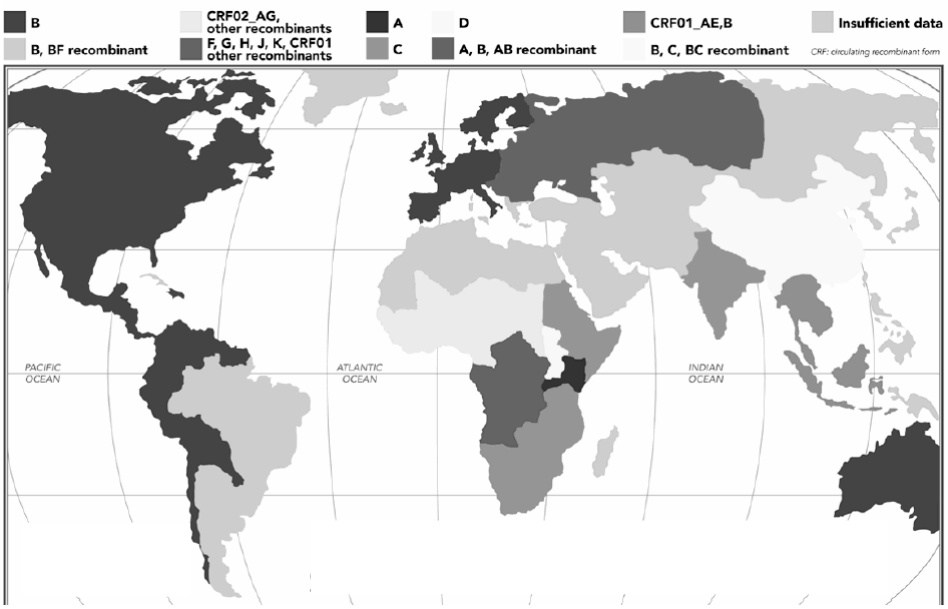

Fig. 8 Global distribution of HIV-1 subtypes and recombinants. Adapted from http://iavireport.org

1.8 Brief background of HIV in Burkina Faso

Burkina Faso is a sub-Saharan country in West Africa with little information about HIV. HIV seroprevalence rates of 5-7% have been reported for the urban regions of this country (Meda et al., 2001; Simpore et al., 2004). HIV prevalence in Burkina Faso was estimated at 2% in 2005 (UNAIDS/WHO, 2007). An HIV prevalence of 3.6% has been reported for a rural area (Collenberg et al., 2006). The first study on HIV genetic variability in this country by Ouedraogo-Traore et al., (2003) indicated CRF06_cpx as the dominant HIV strain in Ouagadougou, the capital city. However, recently we reported that the co-circulating dominant viruses in rural areas are CRF02_AG and CRF06.cpx (Tebit et al., 2006).

The recent introduction of antiretroviral therapy in Burkina Faso necessitated two different types of studies. The first was to define the prevalence and resistance patterns in exposed patients failing therapy in Ouagadougou, Burkina Faso. NRTIs and NNRTIs constitute the first line of therapy in sub-Saharan Africa as well as in Burkina Faso. Accordingly, the prevalence of resistance-associated mutations in subjects failing HAART was 85% for NRTIs, 76% for NNRTIs, and 40% for PIs, corresponding to their respective usage (Tebit et al., 2008). The second study was aimed at screening drug naïve patients for the presence of drug resistance mutations. A high prevalence of NRTI and NNRTI resistance mutations of 10.6% and 6.1% respectively was observed (Tebit et al., 2009). A similar prevalence of NRTIs (6%) and NNRTIs (11%) resistance mutations was observed among 17 naïve patients from another study in Ouagadougo, Burkina Faso (Nadembega et al., 2006). Drug resistance mutations occurred at similar frequencies among CRF02_AG (12.8%) and CRF06_cpx (10.8%) infected naïve subjects (Tebit et al., 2009).

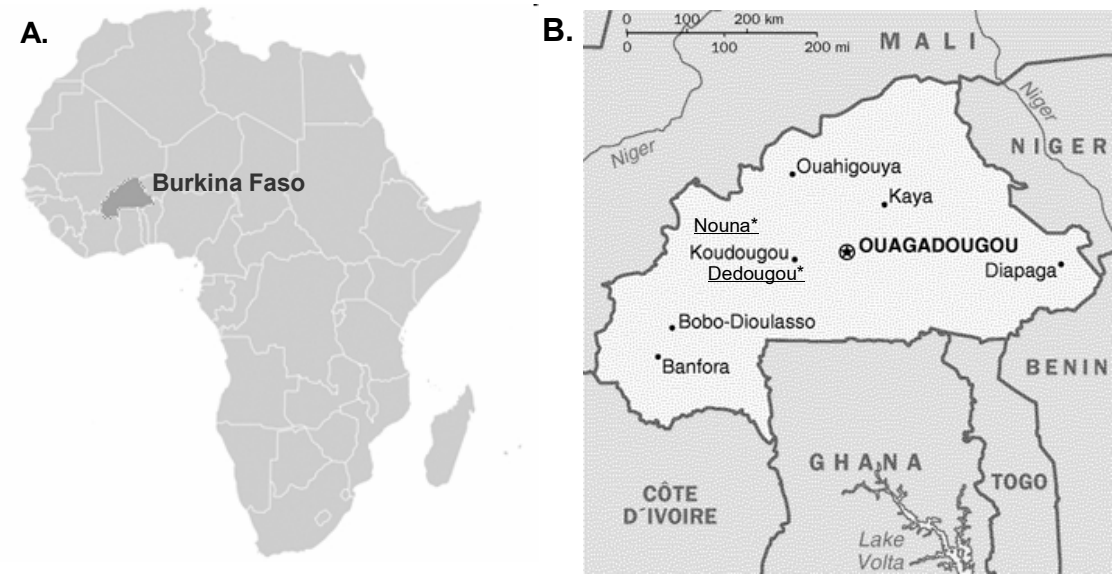

Fig. 9 **(A)** Location of Burkina Faso in the Africa continent. **(B)** An enlarged view of Burkina Faso and its neighboring countries. The sampling areas (Nouna and Dedougou) are underlined. Adapted from http://100prozent-fuer-afrika.com and http://geography-site.co.uk

1.9 Objectives of this study

Burkina Faso is one of the African countries where the current epidemic of HIV- 1 is dominated by non-subtype B, particularly CRF02_AG and more than 50% of HIV-1

infected patients are women (UNAIDS/WHO, 2008). Since, most of HIV-1 infected mothers in this country still breast feed their infants and the extensively used of SD-NVP to prevent mother to child transmission, drug resistance variants might be possibly transmitted from infected mothers to their infants via breast feeding. Moreover, the availability of HAART for HIV-infected patients in Burkina Faso is becoming more accessible which may increase the emergence of drug resistant viruses. Therefore, the knowledge about the molecular genetics and phenotypic characteristics of HIV-1 CRF02_AG from infected drug naïve individuals in Burkina Faso would provide important information prior to the introduction of large scale antiretroviral therapy or new classes of antiretroviral drugs. Moreover, development of a drug resistance assay would provide useful information for non-subtype B HIV-1 infected people in Africa where the number of individuals receiving antiretroviral therapy is increasing.

The overall aim of this study was to determine the genotypic and phenotypic characteristics of HIV-1 CRF02_AG strains from HIV-1 infected patients in Burkina Faso. The specific aims were:

i) To determine the polymorphisms and drug resistance mutations to the entry inhibitor enfuvirtide (T-20) among drug naïve subjects in rural Burkina Faso.

ii) To determine HIV resistance mutations in paired plasma and breast milk from HIV infected NVP-naïve and -exposed women by population sequencing and clonal analysis. Further, to analyze the diversity and evolution of HIV resistance using phylogenetic methods to compare the virus populations between plasma and breast milk from individual patients at both single and multiple time points. These analyses will establish whether HIV populations in plasma and breast milk in these women are compartmentalized.

iii) To investigate the phenotypic drug susceptibility of non-B subtypes based on a RVA using a similar genetic backbone like the viruses circulating in this region.

2. Materials and Methods

2.1 Materials and instruments

Material	Manufacturer
For biological methods	
Cell culture plate	Orange Scientific, Belgium
Dulbecco's Modification of Eagle's Minimum Essential Medium (DMEM)	GIBCO® Invitrogen Cell Culture, Karlsruhe, Germany
ELISA plates	Maxisorb, Nunc, Wiesbaden, Germany
Fetal calf serum (FCS)	Biowest, Nuaillé, France
Luciferase lysis buffer/substrate	Steady-Glo®, #E2520, Promega, Mannheim Germany
Luciferase plates	Corning Costar #3912, 96 well plate, Fisher Scientific
Roswell Park Memorial Institute 1640 Medium (RPMI 1640)	GIBCO® Invitrogen Cell Culture, Karlsruhe, Germany
Trypsin	10x Trypsin/EDTA (0,5% / 0,2%), Biochrom AG, Berlin, Germany
For molecular methods	
Agarose	Agarose Serva, Heidelberg, Germany
Cloning kit	TOPO TA Cloning ® kit, Invitrogen, Karlsruhe, Germany
DNA gel extraction kit	Nucleospin® Extraction II, Macherey-Nagel, Düren, Germany
Mutagenesis kit	QuickChange II XL Site-Direct Mutagenesis Kit, Stratagene, La Jolla, CA.
Plasmid purification kit	QIAprep® Miniprep kit, QIAGEN, Hilden, Germany
	NucleoBond MaxiPrep Kit, Macherey-Nagel, Düren, Germany
PCR amplification kit	SuperScript™ III One-Step RT-PCR with Platinum® Taq DNA polymerase (RT-PCR), Invitrogen, Karlsruhe, Germany
	Expand High Fidelity^{Plus} PCR system (PCR for cloning), Roche, Mannheim, Germany
PCR purification kit	Qiaquick PCR purification kit (Qiagen, Hilden, Germany
	Nucleospin Extract II kit, Macherey-Nagel, Düren, Germany
Restriction enzymes	MBI Fermentas (St. Leon-Rot, Germany)
	New England BioLabs (Frankfurt a.M., Germany)
Sequence analysis kit	GenomeLab® Methods Development Kit, Beckman Coulter
Ultrafiltration columns	Vivaspin centrifugal concetrators (Vivaspin 6 and Vivaspin 20;

	3 or 10 kDa), Sartorius AG, Göttingen, Germany
viral DNA extraction kit	DNeasy® Blood & Tissue kit, QIAGEN, Hilden, Germany
	Qiagen DNA extraction kit, QIAGEN, Hilden, Germany
viral RNA extraction kit	QIAmp® Viral RNA mini kit, QIAGEN, Hilden, Germany

Instruments	**Manufacturer**
Centrifuges	J2HC or J2HS with rotors JA-10, JA-17, JA-20, Beckman Coulter, Fullerton, CA, USA
	Eppendorf 5415D, Eppendorf Deutschland, Hamburg, Germany
	SIGMA 2K15, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany
	Omnifuge 2.0 RS, Heraeus Sepatech
DNA electrophoresis	Mini-sub or wide mini-sub cell GT cell, Bio-Rad Laboratories GmbH, München, Germany
ELISA reader	spectrophotometric microplate reader, Dynatech, Enbrach, Switzerland
Incubator	Infors, Einsbach GmbH, Germany
Luminescence reader	Luminoskan Ascent, Thermo Labsystems, USA
PCR thermocycler	PTC-200 Peltier Thermalcycler, BioZyme, Oldendorf, Germany
Sequencer machine	CEQ-2000 sequencer, Beckman Coulter, CA, USA
Spectrophotometer	DU 640, Beckman Coulter, Fullerton, CA, USA
Ultracentrifuge	L8M or Optima XL-70, rotor SW-41 or SW-60 Ti, Beckman Coulter, Fullerton, CA, USA

2.2 Media, buffers and reagents

Name	**Concentrations**	**Recipe**
LB medium for bacteria culture (1000 ml)	1% peptone 0.5% yeast extract 171 mM NaCl	10 g tryptone 5 g yeast extract 5 g NaCl 0.5 ml 10 N NaOH pH 7.0; autoclaved
LB agar for bacteria culture	1.5% agar in LB medium	1000 ml autoclaved LB medium 15 g agar pour in sterile 10 cm dishes; store at 4°C
LB-ampicillin or kanamycin agar for bacteria culture	100 µg/ml ampicillin or kanamycin	1000 ml autoclaved LB medium 15 g agar 100 mg ampicillin or kanamycin pour in sterile 10 cm dishes; store at 4°C
50x TAE buffer (1000 ml)	2 M Tris-acetate 50 mM EDTA	242 g Tris 57 ml acetic acid 100 ml 0.5 M EDTA pH 8.0

10x DNA loading buffer (10 ml)	100% glycerol 2.5% bromphenol blue 2.5% xylene cyanol	10 ml glycerol 0.25 g bromphenol blue 0.25 g xylene cyanol
10x Phosphate buffered saline (PBS) (1000 ml)	1.37 M NaCl 27 mM KCl 80 mM Na_2HPO_4 18 mM KH_2PO_4	80 g NaCl 2 g KCl 14.4 g $Na_2HPO_4.2H_2O$ 2.4 g KH_2PO_4
10x PBST (1000 ml)	10x PBS 0.5% Tween	1000 ml PBS 5 ml Tween
2x HeBS transfection buffer (1000 ml)	280 mM NaCl 50 mM HEPES 1.5 mM Na_2HPO_4 pH 7.09-7.12	16.4 g NaCl 11.9 g HEPES 0.267 g $Na_2HPO_4.2H_2O$ filter sterilize through 0.45 µm pore size filters; store at 4°C
2x $CaCl_2$ transfection buffer (1000 ml)	250 mM $CaCl_2$	36.8 g $CaCl_2 \cdot 2H_2O$; filter sterilize through 0.45 µm pore size filters; store at 4°C

2.3 Antiretroviral drugs

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID.

PIs: Lopinavir (LPV), Indinavir (IDV), Ritronavir (RTV), Tipranavir (TPV), Darunavir (DRV).

NRTIs: Zidovudine (AZT, ZDV), Dideoxyinosine (ddI), Stavudine (d4T), Lamivudine (3TC), Emtricitabine (FTC), Tenofovir (TDF).

NNRTIs : Nevirapine (NVP), Efavirenz (EFV), Etravirine (ETR).

2.4 Methods of biology

2.4.1 Ethical clearance for the study

In order to collect blood samples from HIV-infected individuals, an ethical clearance was obtained from the University of Heidelberg Ethics Committee, the Nouna Ethics Committee, and the Ministry of Health in Burkina Faso.

2.4.2 Sample collection

Three distinct sample sets for three different experiments were collected in this study. For screening of T-20 resistance related mutations, whole blood samples were obtained from HIV-positive pregnant women enrolled in the prevention of mother-to-child transmission (PMTCT) program at the Nouna District Hospital, Nouna as well as from HIV-positive individuals presenting at the Dedougou Regional Hospital (DRH) in Dedougou, Burkina Faso between July 2003 and October 2004. At the time of sampling, all subjects were reported to be drug naïve (Tebit et al., 2006). The samples derived from HAART-exposed patients attending the University Teaching Hospital, Ouagadougou, Burkina Faso were used for phenotypic drug susceptibility assays. Samples for analyzing NVP resistance and compartmentalization consisted of plasma, breast milk and breast milk cells and were collected from NVP-exposed and –naïve women attending the PMTCT site in Nouna, Burkina Faso at different post delivery stages from 2004-2008.

The whole blood samples were collected in vacutainer tubes containing anti-coagulants (EDTA). Afterwards, plasma and buffy coat were separated from the whole blood by centrifugation at 4,000 rpm for 15 minutes. Fifteen milliliters of breast milk was collected from lactating women in Falcon tubes and centrifuged at 1,500 rpm for 10 minutes at 4^{o}C to obtain the cellular, milk whey and lipid fractions. The samples were stored in aliquots at -80^{o} C and shipped to Heidelberg later on dry ice.

2.4.3 CD4 count and viral load determination

The CD4 cell count was determined for whole blood up to 6 hours after collection using a FACScount fluorescence cytometer (Becton Dickinson, San Jose, CA) in Nouna, Burkina Faso. Plasma viral load was determined at the Virology Unit of the Center Hospital Universitaire Yalgado Ouedraogo (CHUYO) in Ouagadougou by using the Abbott RealTi*me* HIV-1 assay with automated *m*2000 System (Abbott Molecular Diagnostics). Breast milk viral load was determined at the Department of Virology, University of Heidelberg after shipment by using the Amplicor HIV-1 monitor assay (Roche, Mannheim, Germany).

2.4.4 Cell culture

Two adherent cell lines (293T and TZM) and one suspension cell line (C8166) were used for phenotypic drug susceptibility assays in this study.

293T is a highly transfectable human embryonic kidney cell line which was used for transfection experiments (Charneau et al., 1992). TZM (JC53BL) cells are derived from the HeLaP4 cell line. This cell type expresses CD4, CCR5 on their cell surface and carries genes for β-galactosidase and firefly luciferase under the control of the HIV-1 LTR (Wei et al., 2002). This allows the measurent of HIV-1 infection by β-galactosidase or luciferase assays. The C8166 cell-line is a HTLV-I transformed human leukemia cell line highly susceptible to HIV-1 infection which forms syncytia after being infected (Salahuddin et al., 1983).

Adherent cells were maintained in DMEM (Dulbecco's modified Eagle medium) high glucose (Gibco) containing 10% FCS (Biowest), 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES, pH 7.4. Cells were passaged after detachment with 0.05% trypsin in PBS and then further cultivated in fresh medium. Suspension cells were cultivated in RPMI 1640 including the same supplements. All cells were incubated at 37°C and 5% CO_2 in a humid atmosphere.

2.4.5 Transfection of adherent cells with plasmid DNA

Transfection was performed by using the standard calcium phosphate precipitation method (Chen and Okayama, 1987). 1 x 10^6 293T cells were seeded in a 10 cm culture dish and incubated overnight at 37°C, 5% CO_2. On the next day, 10 µg of the plasmid DNA was mixed with 500 µl of 0.25 M $CaCl_2$ and the same volume of 2x HeBS buffer (pH 7.09-7.12) was added during vortexing. After 15 minutes incubation at room temperature, this mixture was introduced drop-wise onto cells with gently swirling. Medium was changed about 6 or 16 hours post-transfection depending on the experiment. Virus particles in culture supernatant were harvested 48 hours after transfection and clarified by centrifugation at 1500 rpm for 5 minutes. The virus stocks were aliquoted and then stored at -80^{o}C.

2.4.6 Virus infectivity and replication kinetics

To determine the viral infectivity, 5 x 10^3 TZM cells were infected in triplicate with the serial dilution of cleared viral supernatants in 96 well plates. TZM cells were lysed 48 hours after infection with the Steady-Glo® lysis buffer including luciferase substrate (#E2520; Promega, Mannheim Germany). The luciferase activity was measured with the Luminoskan Ascent luminometer (Thermo Labsystems).

To determine the replication kinetics of viruses, 5 x 10^5 C8166 cells were infected overnight with 50 ng of HIV-1 p24 antigen from the cleared viral supernatants. On the next day, cells were washed twice with PBS, re-suspended in 2 ml RPMI and maintained for 2 weeks. Culture supernatants were collected every 2-3 days for quantitative p24 ELISA (described below in section 2.4.8) and an equal volume of fresh RPMI medium was added to the cultures (Garcia-Perez et al., 2008).

2.4.7 Drug susceptibility assays

Drug susceptibility assay is adapted from Garcia-Perez et al. (2007). To determine the susceptibility to protease inhibitors (PIs), 293T cells were trypsinized at approximately 16 hours post-transfection and 5 x 10^4 cells were distributed into 48 well plates in the presence of serial PI dilutions in 500 µl total volumes. Viral stocks were harvested 48 hours after transfection and were used to infect 5 x 10^3 TZM cells in triplicate in 96 well plates in the absence of PIs.

To measure the susceptibility to reverse transcriptase inhibitors (RTIs), viral stocks generated in the absence of drug were harvested 48 hours after transfection and were used to infect 5 x 10^3 TZM cells in triplicate using 96 well plates in the presence of increasing concentration of RTIs.

Replication capacity of viruses in the presence or absence of antiviral drugs was monitored by measuring luciferase expression in infected TZM cells with the same procedure as described in section 2.4.6.

2.4.8 Enzyme-linked immunosorbent assay (ELISA)

This ELISA is modified from Konvalinka et al. (1995). Ninety six well plates (Maxisorb, Nunc, Wiesbaden, Germany) were coated over night at room temperature in a moist chamber with 100 µl/well of monoclonal mouse-anti-CA antibody (183-H12-5C; diluted 1:1000 in PBS). Plates were washed and blocked with 10% FCS/PBS which prevents unspecific binding for 2 hours at 37°C. After washing, 100 µl of different diluted culture supernatants pre-treated with 0.1%Triton X-100 as well as dilution series of purified CA protein (6.25 – 0.1 ng/ml) were added to the plate and incubated overnight in a moist chamber at room temperature. On the following day, these plates were washed and incubated with rabbit-anti-CA antiserum (1:1000 in PBST/10% FCS) and subsequently with peroxidase-coupled goat-anti-rabbit antibody (1:2000 in PBST/10%

FCS) for 1 hour at 37°C each. The amount of bound antibody corresponding to p24 was revealed by adding the chromogenic enzyme substrate tetramethyl benzidine (TMB) for 5 minutes. The reaction was stopped by adding sulfuric acid and the colored reaction product was quantified by measuring absorbance at 450 nm with a spectrophotometric microplate reader (Dynatech, Enbrach, Switzerland).

2.5 Methods of molecular biology

2.5.1 Nucleic acid extraction from patient samples

Viral DNA was extracted from buffy coats and cellular fractions of breast milk by using the Qiagen DNA extraction kit (QAIGEN, Hilden, Germany) and DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), respectively, following the manufacturer's recommendations. Before the extraction, the breast milk cells were washed twice with PBS.

Viral RNA was extracted from 140 µl of plasma and 280 µl of breast milk using QIAmp® Viral RNA mini kit (QAIGEN, Hilden, Germany) according to the manufacturer's instructions. If viral RNA from 280 µl of breast milk was not successfully extracted, the volume of breast milk was increased to 1 ml and centrifuged at 44,000 rpm at 4^{o}C for 1 hour to precipitate the virus particles before the extraction procedure.

2.5.2 Polymerase Chain Reaction (PCR)

PCR allows the selective amplification of DNA sequences in presence of two specific oligonucleotide primers complementary to the 5'- and 3'- regions of the DNA molecule. All the primers used in this study are listed in Appendix 3. The amplification reagents and thermal cycling protocols used for PCR were chosen according to template and length of the fragment to be amplified (Appendix 4 and 5).

To obtain the PCR fragment from plasma sample, extracted RNA (section 2.5.1) was amplified with the SuperScript™ III One-Step RT-PCR with Platinum® Taq DNA polymerase (Invitrogen) as instructed by the manufacturer. For amplification of fragments less than 1 kb from DNA samples (section 2.5.1), PCR was performed with Taq polymerase, while long PCR fragments greater than 1 kb were generated with Expand High Fidelity^{Plus} PCR system (Roche, Mannheim, Germany) following the manufacturer's instructions.

All PCR in this study were carried out in the PTC-200 Peltier Thermalcycler (BioZyme, Oldendorf, Germany). The PCR products were purified either with Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) or Qiaquick PCR purification kit (QAIGEN, Hilden, Germany).

2.5.3 Construction of CRF02_AG provirus backbone

To construct the CRF02_AG provirus plasmid backbone for phenotypic assay, the original pBD6-15 plasmid previously described in Tebit et al. (2003) was adapted to generate pBD6TB9 which was used for further modification as the parental plasmid. The initial step of plasmid modification was performed by knocking out an *EcoRI* restriction site at nucleotide (nt) position 4660 of the parental pBD6TB9 with primers listed in Appendix 3 by using the QuickChange II XL Site-Direct Mutagenesis Kit (Stratagene, La Jolla, CA), as instructed by the manufacturer.

A new *EcoRI* site was introduced at the beginning of the protease gene (nt 2242 of pBD6TB9) by PCR mutagenesis. In the first round PCR, the 5' fragment (nt 1-2260 of pBD6TB9) and 3' fragment (nt 2231-3555 of pBD6TB9) were amplified with Expand High Fidelity^{Plus} PCR system (Roche, Mannheim, Germany). In the second round, the purified 5' fragments (2.2 kb) and 3' fragments (1.3 kb) from first round PCR were used as the template. In this step, the 5' and 3' fragments were ligated via the overlapping region and generated the 3.5 kb final PCR products.

The constituents of the PCR reaction and the cycling conditions are listed in Appendix 4 and 5.

2.5.4 Re-amplification of protease and reverse transcriptase (PR-RT) fragments for phenotypic assays

Determination of drug resistance mutations (genotypic test) was performed by amplifying the PR-RT region with the ViroSeq HIV-1 Genotyping System (Applied Biosystems) as recommended by manufacturer. This amplification step was performed by the diagnostic section, Department of Virology, Heidelberg University. PCR products from these analyses were kept at -20 ^{o}C and were re-amplified with Expand High Fidelity^{Plus} PCR system (Roche, Mannheim, Germany) to obtain the 1.3 kb of patient derived PR-RT coding sequences with the primers, reaction components and cycling

conditions listed in Appendix 3-5. The 1.3 kb PR-RT fragment from HIV-1 subtype B, pNL4-3 was also amplified by the same procedure.

2.5.5 Restriction digest and ligation

2.5.5.1 DNA cleavage with restriction enzymes

All enzymes and buffers for DNA cloning and restriction digest were from MBI Fermentas (St. Leon-Rot, Germany) or from New England BioLabs (Frankfurt a.M., Germany). For a control restriction digest, approximately 1-2 µg DNA was incubated with 10 U restriction enzyme in a volume of 20 µl at the recommended temperature. For further cloning purposes, 2-20 µg of plasmid or 30 µl purified PCR product was digested with 20 U of specific restriction enzyme in a volume of 50-100 µl**.**

2.5.5.2 TA cloning vector

Taq polymerase has a non-template dependent terminal transferase activity which adds a single deoxyadenosine (A) to the 3'end of PCR product. This activity facilitates the cloning of Taq amplified fragments into a commercially available linearized vector pCR®2.1 TOPO supplied with the TOPO TA Cloning ® kit (Invitrogen, Karlsruhe, Germany). This vector has single overhanging 3' deoxythymidine (T) residues together with topoisomerase activity which allows PCR fragments to efficiently ligate with the vector. The ligation was performed following the manufacturer's instruction and subsequently used to transform competent bacteria provided with the kit. Fifteen clones per sample were selected for sequencing analysis.

2.5.5.3 Ligation of PR-RT fragments into circularised vector

To generate the new vector backbone for phenotypic assay, the 3.5 kb PCR fragments (section 2.5.3) were digested with *ApaI* and *Stu I* (MBI Fermentas, St. Leon-Rot, Germany) and were subsequently ligated to pBD6TB9 parental plasmid digested with the same enzymes, providing the final plasmid "pBD6TB9RI". To generate the patients-derived recombinant plasmids and chimeric CRF02_AG/subtype B plasmid, the 1.3 kb PR-RT fragments (section 2.5.4) were digested with *EcoRI* and *StuI* and ligated into the *EcoRI – StuI* digested pBD6TB9RI backbone. About 5 to 50 ng of vector was ligated with DNA fragments in a 1:5 molar ratio and in a total volume of 20 µl with 2 units of T4 DNA ligase and incubated at 16^{o}C overnight. In all ligation experiments, a

control reaction composed of only vector was included. Competent bacteria were transformed with 5-10 µl ligation reaction.

2.5.6 DNA electrophoresis and isolation of DNA fragments

DNA electrophoresis was used for the analysis of PCR products, or purified restriction digested DNA fragments. Depending on the size of the DNA fragment to be purified, 1–1.5% (w/v) agarose in TAE buffer was melted by heating, 2 µg/ml ethidiumbromide was added and the gel was poured into the gel tray and cooled down. DNA samples mixed with DNA loading buffer were loaded onto the gel and resolved for 40 minutes at 90 volt. DNA bands were detected with UV light of 312 nm or of 366 nm to avoid DNA damage for further cloning steps. The fragment size was estimated by comparison with the DNA 1 kb ladder marker on the same gel. Bands of the correct size were cut out and purified with the NucleoSpin® Extract kit (Macherey-Nagel, Düren, Germany).

2.5.7 Sequencing analyses

Sequence analysis of RT fragments and plasmid DNA was performed in-house based on the dye terminator method by using GenomeLab® Methods Development Kit on a CEQ-2000 sequencer (Beckman Coulter, CA, USA). For sequencing reactions,50 fmol of plasmid DNA, 1.6 µM primers (Appendix 3) and manufacturer's premix solution (containing DNA polymerase, dNTPs, labeled ddNTPs, buffer) were used to amplify the DNA fragment of interest in a total volume 20µl. After that, the reaction was stopped and DNA fragments generated by the reaction were precipitated by adding 5 µl stop-solution (3 M NaAc pH 5.2, 100 mM EDTA pH 8.0 and glycogen). The pellet was washed once with absolute ethanol and then with 70% ethanol, air-dried and re-dissolved in 40 µl of sample loading solution providing with the kit (Beckman Coulter, CA, USA). The dissolved DNA was transferred to a 96-well plate and overlaid with mineral oil to prevent evaporation.

All DNA samples after March 2008 were commercially sequenced by GATC Biotech (Konstanz, Germany).

2.5.8 Bacteria transformation and plasmid DNA preparation

2.5.8.1 Bacteria strains

For transformation experiment with the pCR®2.1-TOPO both *E. coli* strain DH5α (Invitrogen) with the genotype F$^-$ θ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk$^-$, mk$^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ$^-$ and TOP10 competent bacteria with the genotype F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G, supplied with the TOPO TA cloning kit ((Invitrogen), were used. In the case of proviral plasmid preparation, MAX Efficiency® Stbl2^{TM} Competent Cells (Invitrogen) were used since this *E. coli* strain shows particularly few recombination events. Stbl2^{TM} bacteria are derived from JM109 strain and the genotype is: F– *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *lon gyr*A96 *thi sup*E44 *rel*A1 λ– Δ(*lac-pro*AB).

2.5.8.2 Transformation

Bacteria were transformed using the standard heat-shock procedure. Approximately 150 ng (1-2 µl) of plasmids or 10 ligation reactions were added to 50 µl of chemically competent bacteria (DH5α, TOP10 or Stbl2). The mixture was incubated for 20 minutes on ice, heated for 30-45 seconds at 42°C and then cooled on ice for 2 minutes and 500µl of antibiotic-free LB was added. The transformed bacteria were incubated in a shaker incubator (200 rpm) at 37°C for 1 hour. The bacteria was spread on an LB plate containing ampicillin or kanamycin and incubated over night at 37°C. Single colonies were randomly picked to inoculate 2 ml and 200 ml of LB medium containing ampicillin or kanamycin for small and large amounts plasmid DNA preparations, respectively.

2.5.8.3 Preparation of small amounts of plasmid DNA (mini-preparation)

Mini-preparation of plasmid DNA was performed using the QIAprep® Miniprep kit (QAIGEN, Hilden, Germany). Bacteria culture was pelleted for 3 minutes at 5,000 rpm and resuspended in 250 µl P1 buffer. Bacteria were lysed by addition of 250 µl P2 buffer, and the reaction was stopped by neutralization with 350 µl N3 buffer. The cell debris was pelleted for 10 minutes at 13,000 rpm and supernatant was subsequently applied to spin column and centrifuged 13,000 rpm for 30 seconds. The column was washed twice with 750 µl of PE buffer and plasmid DNA was eluted with 50 µl of EB buffer. All preparation steps were carried out at room temperature.

2.5.8.4 Preparation of large amounts of plasmid DNA (maxi-preparation)

Maxi-preparation of plasmid was performed by using NucleoBond® Maxiprep kit (Macherey-Nagel, Düren, Germany). The bacteria was resuspended, lysed and neutralized by using buffer S1, S3 and S3, respectively. The supernatant containing plasmid DNA was loaded onto Nucleobond® column pre-wet with buffer N3, subsequently washed with buffer N4 and plasmid DNA was eluted from the column with buffer N5. Purified plasmid DNA was then precipitated with isopropanol and washed with 70% ethanol. Plasmid DNA was dried at room temperature and re-dissolved in water.

2.6 Bioinformatical methods and analysis

2.6.1 Identifying drug resistance mutations

Sequence editing was performed using the program Vector NTI Advance™ 10 (Invitrogen, Karlsruhe, Germany). The resistance mutations in PR (codon 1-99), RT (codons 1-250) and gp41 were determined by using the Drug Resistance Algorithm from the Stanford HIV drug Resistance Database (http://hivdb6.stanford.edu). This tool compares the input sequences with sequences of HIV-1 subtype B in the database which confer resistance to anti-HIV drugs.

2.6.2 Phylogenetic analyses, viral diversity and divergence

Sequences of HIV-1 RT fragments were aligned with Clustal X 2.0.6 (Larkin et al., 2007) with the reference sequences of major HIV-1 subtypes available from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov) with minor manual adjustments. All alignments were gap-stripped for the generation of trees. Phylogenetic analyses were performed with MEGA 4 (Tamura et al., 2007) by using neighbor joining with the Kimura's two parameter (K2P) method and 1,000 bootstrap resamplings of the data. Maximum likelihood trees were also constructed using the PHYLIP 3.67 (Felsenstein, 1989). Evolutionary distances between sequences were determined by calculating the mean pair wise genetic distances among all sequences from individual patients at each time point with MEGA 4 using the K2P model with transition/tranversion ratio of 2.0 (Leitner et al., 1996; Becquart et al., 2007; Gottlieb et al., 2008).

Viral divergences were measured as the pair wise genetic distances of all clones at a given time point to the bulk sequence at the first time point (most recent common ancestor, MRCA) for each patient (Troyer et al., 2005).

2.7 Statistical analyses

To determine possible differences in viral load between plasma and breast milk and to verify whether the time after SD-NVP exposure influenced compartmentalization, statistical analyses were performed using the Mann-Whitney test. Differences in the genetic diversity and divergence of viruses between plasma, breast milk and/or breast milk cells were performed using the Wilcoxon signed rank test or Kruskal-Wallis test with Dunn's Multiple Comparison post-test.

To measure virus susceptibility to various antiretroviral drugs, the inhibition percentage was calculated using the following formula (Garcia-Perez et al., 2007);

$$\%I = \left[1 - \left(\frac{RLUp}{RLUa}\right)\right] \times 100,$$

where *%I* is an inhibitory percentage, RLU*p* is a luciferase activity in the presence of drug and RLU*a* is a luciferase activity in the absence of drug.

The dose-response curves were obtained by plotting the percent inhibition versus $\log_{10}$ of drug concentration. The 50% inhibitory concentration (IC_{50}) of each drug was computed by nonlinear regression curve using a sigmoidal dose-response equation with the GraphPad Prism5 software (version 5.02). The fold changes in susceptibility were calculated by dividing the IC_{50} of the mutant virus by IC_{50} of wild type virus. Results were expressed as the mean value, standard error of mean (SEM), and coefficient of variation (CV) of 3 to 4 independent experiments. To determine possible differences in IC_{50}, statistical analysis was performed by using the Friedman test with Dunn's multiple comparison post-test. The p-values below 0.05 were considered to be statistically significant.

3 Results

3.1 Genotypic resistance to enfuvirtide (T-20)

T-20 drug resistance–associated polymorphisms were determined in the HR1 and HR2 sequences of gp41 from 38 naïve HIV infected patients consisting of HIV-infected individuals from Dedougou regional hospital (DRH) and pregnant women who enrolled in the PMTCT program in Nouna which was implemented in 2003.

The CD4 values among pregnant women ranged from 93-747 cells/µl, whereas CD4 counts of patients from DRH ranged from 1-718 cells/µl. The viral load was in range from 10^3 to 10^6 copies/ml which is generally high as expected from HIV-drug naïve patients (Tebit et al., 2006). The genotypes obtained were as follows: CRF02_AG (55%), CRF06_cpx (45%) and other subtypes e.g. CRF09_cpx and A3 (5%) (Tebit et al., 2006).

As demonstrated in figure 10, in the HR1 region, the **GIV** motif which is primarily responsible for T-20 resistance was conserved among all sequences. The most common polymorphism observed within this region was N42S (n = 36) which was dominant among CRF02_AG and CRF06_cpx viruses (Fig. 10). The Q56K/R polymorphism was found among sub-subtype A3, CRF02_AG, and CRF09_cpx viruses, but not in CRF06_cpx viruses (Fig 10). The combination L54M/Q56K was observed in three sequences of CRF02_AG and one of CRF06_cpx viruses (Fig. 10).

Ten amino acids in HR2 at position 117, 120, 123, 127, 131, 134, 138, 141, 145 and 148 are known to be involved in the sensitivity to T-20. Six of the ten amino acids at positions 120, 127, 131, 134, 141 and 145 were totally conserved in all strains (Fig. 10). Meanwhile, S138A (n = 4) was the most common polymorphism found among the remaining four non-conserved amino acid positions. The polymorphisms N126K (n = 3), N126S (n = 1) and N140I (n =3) were also detected in HR2 sequences. Generally, the amino acid sequence of the HR2 region was more heterogeneous compared with that of the HR1 region (Fig. 10).

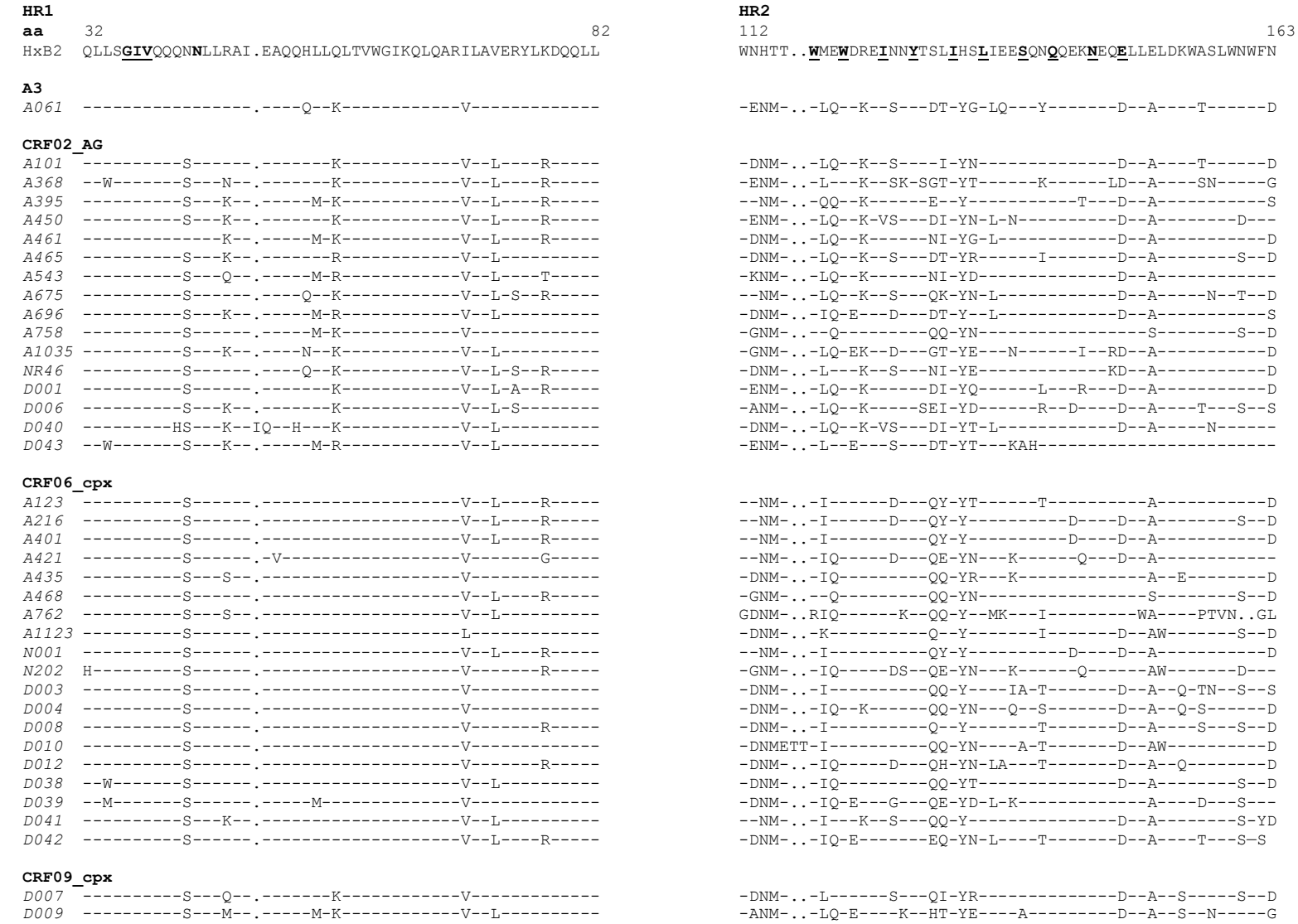

Fig 10. Alignment of T-20 resistance mutations in HR1 and HR2 regions of gp41 of 38 HIV-1 infected-patients (*Italic*). HXB2 is the HIV-1 subtype B strain. The amino acid (aa) positions associated with T-20 resistance were underlined. dot (.) = deletion; dash (-) = amino acid identity.

3.2 NVP resistance mutations in paired plasma-breast milk

3.2.1 Patients and Clinical data

Fifty three selected HIV infected breast feeding women, who have enrolled in PMTCT programe in Nouna, provided paired plasma and breast milk samples for this study. However, RNA or DNA could be derived from both plasma and breast milk or breast milk cells of 17 women (Table 3). Five women provided two samples, while one woman provided three samples at different time points respectively (Table 3).

Of the 17 women, 10 received SD-NVP at labor and only one received a drug combination comprising AZT at week 28 of pregnancy, single dose of NVP+AZT+3TC at labor and AZT+3TC at 7 days post delivery, which is the new regimen for PMTCT recommended by the Ministry of Health in Burkina Faso. Six women were drug naïve because they did not receive SD-NVP at labor (Table 3). The average $CD4^+$ cell count pre and post delivery was 355 and 438 cells/mm^3 respectively. The plasma viral load ranged between 500 to 500,000 copies/ml (average = 165,025 copies/ml). The viral load in breast milk was significantly lower than in plasma (Mann-Whitney test, p = 0.0001) with a mean of 4,498 copies/ml ranging between <40 to 44,603 copies/ml (Table 3).

Table 3. Clinical characteristics of patients who provided plasma and breast milk samples

ID	Age at baseline	Drug regimen	Subtype	CD4 pre-delivery (cells/mm^3)	CD4 post-delivery (cells/mm^3)	Time of sampling (mo/yr)	Viral load (copies/ml)	
							PL	BM
A368	30	SD-NVP	02_AG	280	157	-/04	786,242	760
						-/05	344,000	ND
A401	23	SD-NVP	06_cpx	277	216	-/04	261,999	4,000
						-/05	72,997	<40
A435	28	SD-NVP	02_AG	377	217	-/04	586,635	85
A465	29	SD-NVP	02_AG	615	ND	-/04	141,000	893
ABa249	32	new regimen[a]	02_AG	487	654	11/07	557	ND
ABk162	34	SD-NVP	06_cpx	260	341	06/07	4,493	440
ADb169	23	SD-NVP	02_AG	ND	473	11/07	ND	216
					559	04/08	ND	69
AGn031	28	SD-NVP	06_cpx	287	523	04/07	163,996	<40
						10/07	ND	ND[c]
ASn079	33	SD-NVP	02_AG	425	325	05/08	78,267	15,068

Table 3. Clinical characteristics of patients who provided plasma and breast milk samples *(continued)*

ID	Age at baseline	Drug regimen	Subtype	CD4 pre-delivery (cells/mm^3)	CD4 post-delivery (cells/mm^3)	Time of sampling (mo/yr)	Viral load (copies/ml)	
							PL	BM
AY113	23	SD-NVP	06_cpx	ND	418	02/08	1,946	ND[c]
AY134	31	SD-NVP	02_AG	ND	802	02/07	2,069	ND[c]
					621	07/07	20,726	ND[c]
					560	02/08	4,461	120
ABm107	33	naïve[b]	02_AG	ND	432	01/08	5,993	119
ADb365	30	naïve[b]	02_AG	425	379	01/08	4,457	44,603
ADr304	25	naïve[b]	06_cpx	290	633	04/08	1,667	ND[c]
AGg078	32	naïve[b]	06_cpx	117	359	11/07	89,754	ND[c]
AGn006	22	naïve[b]	02_AG	ND	432	03/07	35,924	975
AY172	28	naïve[b]	02_AG	417	441	06/07	1,810	<40
					369	08/07	1,268	ND[c]

[a] = combination regimen (AZT at week 28 of pregnancy, single dose of NVP+AZT+3TC at labor and AZT+3TC at 7 days post delivery); [b] = did not receive SD-NVP at labor; [c] = PCR positive in breast milk cells; SD-NVP = single dose Nevirapine; PL = plasma; BM = breast milk; ND = not done; mo = month; yr = year.

3.2.2 Genotypic drug resistance in NVP –naïve and exposed women

3.2.2.1 Subtype classification

To determine the distribution of HIV-1 subtypes that are circulating among HIV-infected pregnant women in Nouna, rural Burkina Faso, the BM and plasma samples were PCR amplified and sequenced. The bulk sequences of the 5' RT region spanning about 950 bp from 24 samples of 17 HIV infected women were aligned with reference subtype sequences from the Los Alamos Database and a phylogenetic tree was constructed using maximum likelihood method as implemented in the Phylip 3.67 program. HIV sequences from eleven women (64.7%) clustered with HIV-1 CRF02_AG (Fig. 11), HIV isolates from six women (35.3%) were closely related with CRF06_cpx (Fig. 11). Plasma, breast milk and/or breast milk cells sequences from the same patients clustered together (Fig. 11).

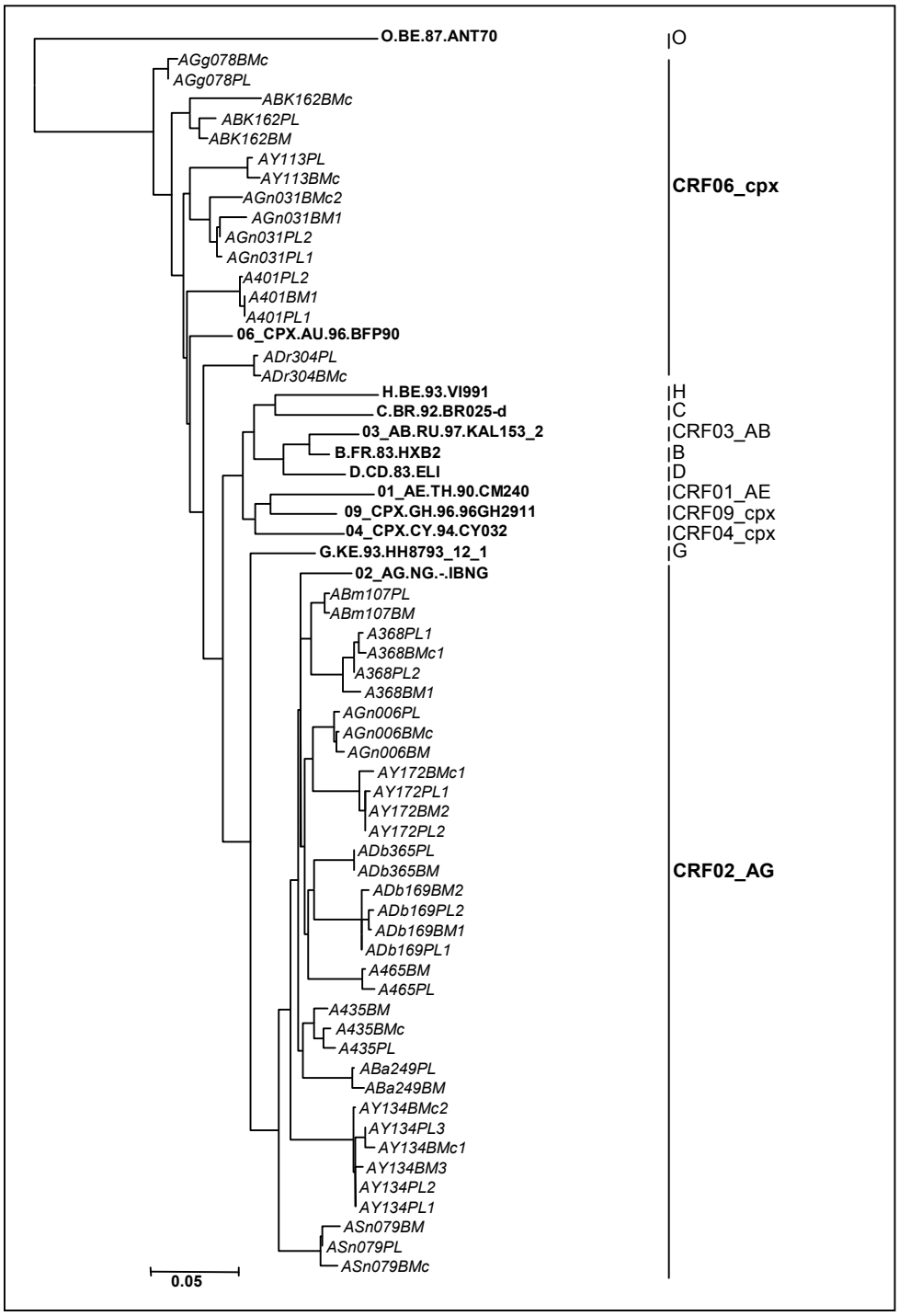

Fig. 11 Phylogenetic tree of *pol* region of the 17 HIV-1 infected women from Nouna, Burkina Faso (*Italic*). The tree was generated using the maximum likelihood method by PHYLIP 3.67 program. Reference sequences were obtained from the Los Alamos HIV database. The tree was rooted using HIV-1 subtype O as an outgroup. Subtypes are indicated to the right.

3.2.2.2 NVP-resistance mutations detected by direct /clonal sequencing

3.2.2.2.1 Direct sequencing

A genotypic drug resistance test based on direct PCR-product sequencing has been widely used to detect drug resistance mutations in HIV-1 infected patients. However, a major limitation of direct sequencing is that it does not detect variants which comprise less than 20% of sequences in the virus population (Palmer et al. 2005). As demonstrated in figure 12, direct sequencing of PCR products of the RT-region from patient-ABK162 indicates the presence of two different populations as observed from double peaks at the nucleotide position 541 in the electropherogram (top panel and arrow). However, the nucleotide G is the higher peak and so is detected as the dominant population. In contrast, when individual clones of this RT-fragment were analyzed, both distinct nucleotides were detected in this position which shows that the sample contains two groups of viruses (Fig. 12; middle and bottom panels).

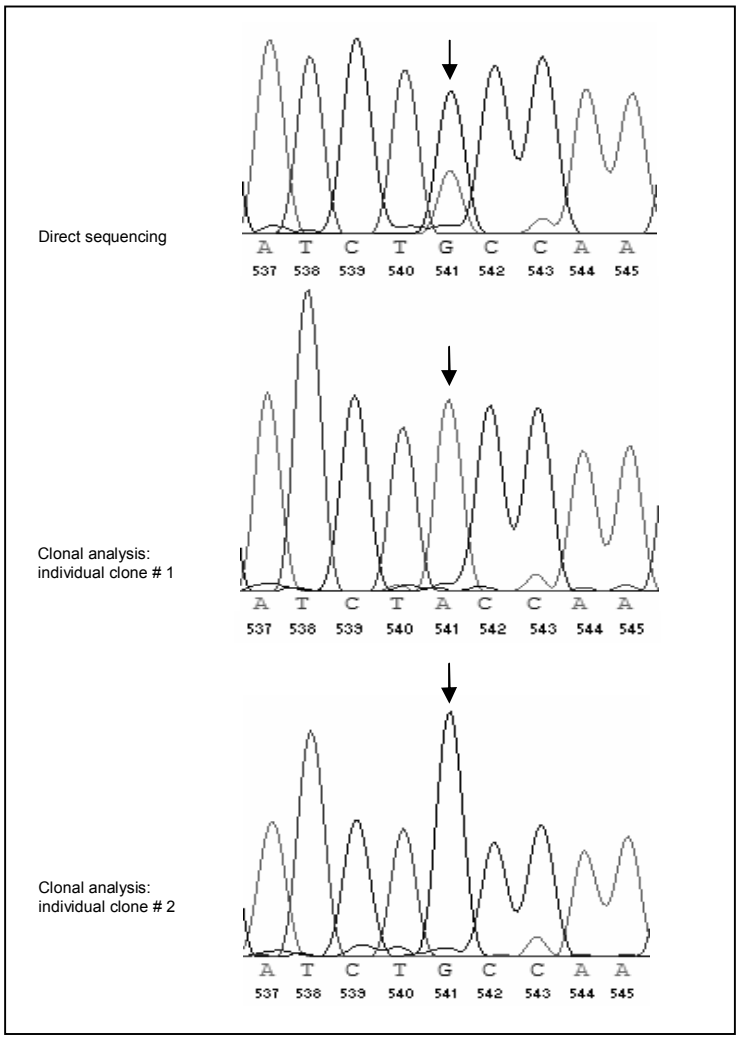

Fig. 12 Electropherograms of RT fragment from patient ABK162. Top panel is a sequence derived from direct sequencing representing two different nucleotides at the same position (double peak and arrow). Middle and bottom panels demonstrate the sequences from individual clonal analysis. The two different nucleotides are indicated by arrows.

Two major NVP-resistance mutations, **K103N** and **Y181C**, were detected in paired plasma-breast milk samples from two NVP-exposed women by direct sequencing (Table 4). Amino acid substitution at position 179 (V179E) which slightly reduces susceptibility to all drugs in NNRTI group was detected in paired samples from one NVP-exposed woman at both sampling time points (Table 4).

Some NRTI resistance mutations were detected among NVP-naïve and exposed women with no NRTI history. The M184I mutation which decreases 3TC, ABC and FTC susceptibility was observed from breast milk cells of one NVP-exposed woman (Table 4). Moreover, another NRTI resistance mutation, E44D, was found in one NVP-naïve woman (Table 4). Although, E44D mutation alone does not substantially alter NRTIs susceptibility, this mutation could correspondingly increase cross-resistance to other NRTIs in the presence of Thymidine Analogue-associated mutations (TAMs) (Walter et al., 2002).

Table 4. Drug resistance mutations detected by direct sequencing and clonal analysis

ID	Day post-delivery	Direct sequencing			Clonal analysis		
		PL	BM	BMc	PL (n)	BM (n)	BMc (n)
A368/1**	180	**K103N**	WT	**K103N**	WT (2) **K103N (11)** **K103N** + M230V(1) K238Q (1)	WT (13) **K103N (1)** F227L **(1)**	**K103N (15)** **K103N+Y181C (1)**
A368/2#	360	WT	NA	NA	WT (14) **K103N (1)**	NA	NA
A401/1***	210	WT	WT	NA	WT (13) P225S + **F227C** (1) **F227C** (1)	WT (13) L100W (1) **G190E (1)**	NA
A401/2#	390	WT	NA	NA	WT (15)	NA	NA
A435***	150	WT	WT	WT	WT (13) **K103N (2)**	WT (8) **K103N (6)** **K103E (1)**	WT (14) ***K65R*** (1) ***K65R***+ K219E (1)
A465***	-	WT	WT	NA	WT (15)	WT (15)	NA
ABa249***	0	WT	WT	NA	WT (15)	WT (15)	NA
ABk162*	10	**Y181C**	**Y181C**	G190R + (*M41I*+***M184I***)	WT (5) **Y181C (6)** **Y188C (3)** K103R + **Y181C(1)** **M230L (1)**	WT (9) **Y181C (7)**	G190R+ (*M41I* + ***M184I***) (15) **V106A**+G190R + (*M41I* + ***M184I***) (1)

Table 4. Drug resistance mutations detected by direct sequencing and clonal sequencing *(continued)*.

ID	Day post-delivery	Direct sequencing			Clonal analysis		
		PL	BM	BMc	PL (n)	BM (n)	BMc (n)
ADb169/1***	180	V179E	V179E	NA	V179E (13)	V179E (15)	NA
ADb169/2*	330	V179E	V179E	NA	V179E (14)	V179E (15)	NA
						V179G (1)	
AGn031/1*	3	WT	WT	NA	WT (13)	WT (13)	NA
					V108A (2)	**K101E (1)**	
						K103R (1)	
AGn031/2*	180	WT	NA	WT	WT (14)	NA	WT (16)
					G190A (1)		
ASn079*	2	WT	WT	WT	WT (15)	WT (16)	WT (15)
					G190R (1)		
AY113*	360	WT	NA	A98T	WT (15)	NA	A98T (15)
							A98T + K219R (1)
AY134/1*	7	WT	NA	WT	WT (14)	NA	WT (14)
					V179L (1)		**Y188H (1)**
AY134/2***	210	WT	NA	WT	WT (14)	NA	WT (9)
					K130N (1)		V179T (6)
					L100S (1)		K103R +V179T(1)
AY134/3***	360	WT	WT	NA	WT (16)	WT (15)	NA
ABm107***	30	WT	WT	NA	WT (16)	WT (14)	NA
						V106A (1)	
						A98T (1)	
ADb365***	21	WT	WT	NA	WT (15)	WT (16)	NA
ADr304*	180	WT	NA	WT	WT (13)	NA	WT (16)
					V118I (1)		
AGg078*	2	*E44D*	NA	*E44D*	WT (2)	NA	*E44D* (12)
					E44D (13)		
					E44D + P225L (1)		
AGn006*	435	WT	WT	WT	WT (15)	WT (14)	WT (14)
						V108I (1)	F227L **(1)**
AY172/1*	120	WT	NA	WT	WT (15)	NA	WT (15)
AY172/2***	180	WT	WT	NA	WT (14)	WT (13)	NA
						V179A (1)	

PL = Plasma; BM = Breast milk; BMc = Breast milk cells; WT = wild type; NA = not amplified; **Bold** = high level resistance to NVP (NNRTI); **Bold-underline** = cross resistance mutation to ETR (NNRTI); underline = polymorphism or mutations associated with modest NNRTIs resistance; **Bold-Italic** = high level resistance to NRTIs; *Italic = polymorphism or mutations associated with modest NRTIs resistance*; * = compartmentalization; ** = partial-compartmentalization; *** = non-compartmentilzation; # = not analyzed for compartmentalization.

3.2.2.2.2 Clonal analyses

Using the same PCR products as for the direct sequencing, the RT fragments were cloned in a TA vector and more than 10 clones per sample were sequenced. The mutations associated with high level resistance to NVP were detected in 11 of 24 cases (46%) as major or minor variants by clonal analyses (Table 4). Besides the major mutations, other mutations which slightly reduce NVP susceptibility as well as some polymorphisms were also found mostly as minor population (Table 4). The most common mutations among NVP-exposed women were at positions 103 and 179 (Fig. 13; Table 4). Mutations at position 103 included K103N, K103E, K103T and K103R with **N** being the most common, constituting about 70% of all 103 mutants. Sequences carrying both the **K103N**/R and **Y181C** mutations were extremely rare. Only two patients carried this double mutation; one had **K103N+Y181C**, while the K103R+**Y181C** mutations were observed in the other (Table 4). Some other mutations such as **K101E, G190A** and **Y188C/H** were found as minor variants (Table 4).

NRTI resistance mutations were also detected by clonal analysis. The K65R mutant was detected only in breast milk cells of patient-A435 as a small proportion (2 of 15 clones; Table 4). However, it was absent in plasma and breast milk. In patient-ABK162, the M184I mutation was again found in breast milk cells as the dominant population (16 of 16 clones; Table 4).

From NVP-naïve women, NVP resistance mutations such as **V106A, V108I** were found in low proportions which were undetectable by direct sequencing of the PCR population products (Table 4). Moreover, the NRTI mutation, E44D which could be detected by direct sequencing was also present as the dominant population in plasma (13 of 15 clones) and breast milk cells (12 of 12 clones) in one patient (Table 4).

In summary, mutations associated with high level resistance to NVP were detected in only 8% of patients by direct sequencing, while the detection rate increased to 46% by clonal analysis (Table 4). This finding suggests that the clonal analysis reveals a higher prevalence of NVP resistance mutations than direct sequencing.

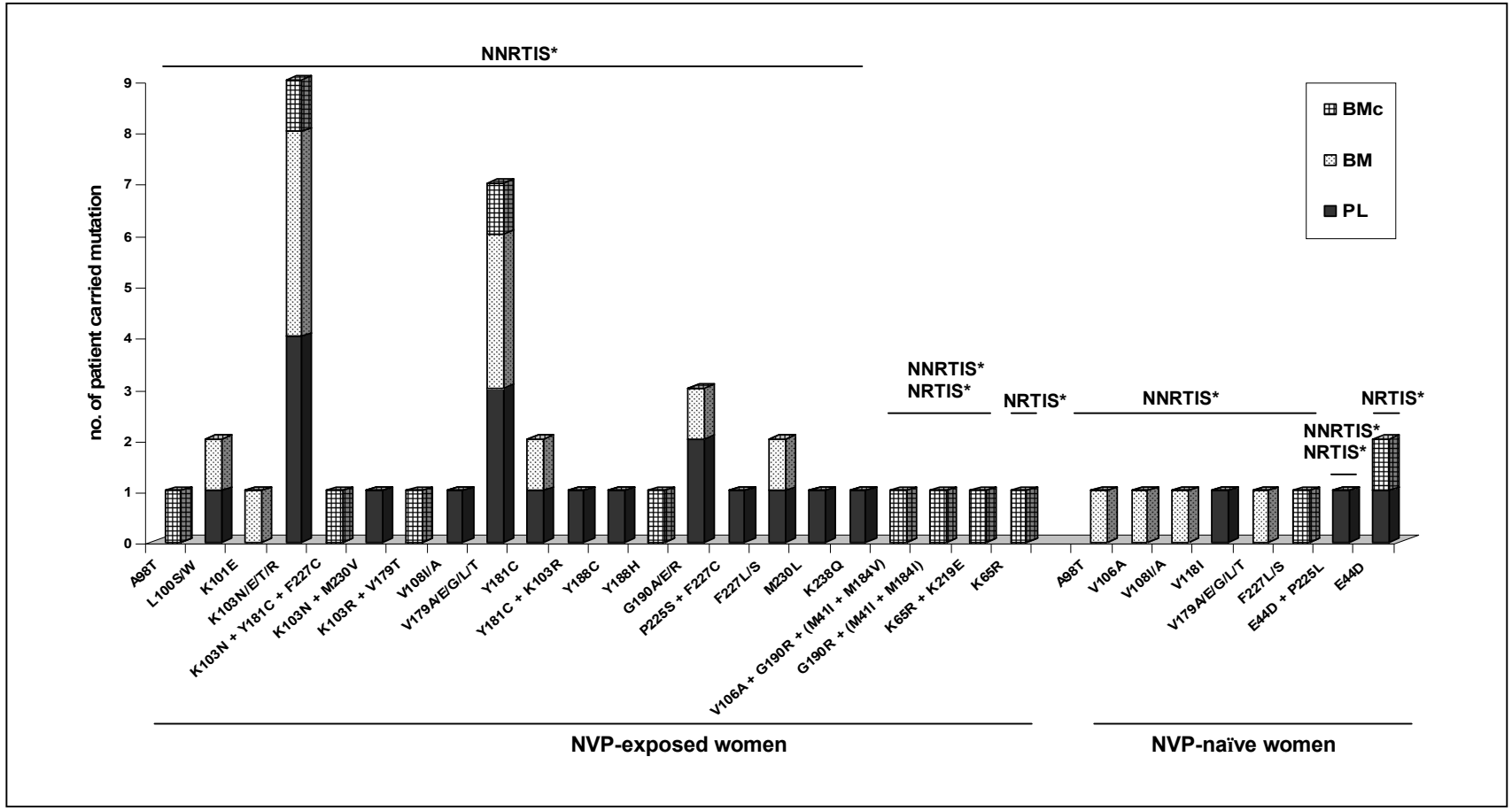

Fig. 13 NVP resistance mutations detected in plasma (PL), breast milk (BM) and breast milk cells (BMc) among patients by clonal analysis.
NRTIs* = mutations to nucleoside reverse transcriptase inhibitors, NNRTIs* = mutations to non- nucleoside reverse transcriptase inhibitors.

3.2.2.3 Persistence of NVP resistance associated mutations

To determine the persistence of resistance variants over time, clonal analysis was performed from two paired plasma-breast milk samples obtained from two subjects (A368, and ADb169) and two paired plasma-breast milk cells samples from one subject (AY134) at different time points after delivery (Table 3). In A368, sampling was done at respectively, six and twelve months post delivery (Table 3). As expected, in this patient, the proportion of viruses containing NVP resistance mutation **K103N** declined over time. This mutation which represented the dominant population (detected in 11 of 15 plasma clones) at six months post delivery nearly disappeared from the virus population (detected in 1 of 15 plasma clones) after twelve months post delivery (Table 4). However, the V179E mutation detected from patient ADb169 which causes low level reduction in NNRTI susceptibility persisted as a dominant population in plasma and breast milk, although the patient received NVP almost eleven months prior to sampling (Table 4). It is, however, possible that this mutation occurred as wild type in this subject even pre-SD NVP administration. In patient AY134, NVP resistance mutations such as **K103N** as well as other polymorphisms which have been detected at seven months post-NVP exposure completely disappeared at twelve months (Table 4).

3.2.3 Phylogenetic analyses

3.2.3.1 Viral distribution and compartmentalization

To determine the distribution of the variants from the various compartments, the clonal and population sequences were aligned and phylogenetic trees constructed. To obtain phylogenetic trees from individual patients, both Neighbor-Joining (NJ) and Maximum-likelihood (ML) trees were constructed by programs MEGA4.0 and PHYLIP 3.67, respectively. The obtained trees were grouped into three categories, i.e. compartmentalized, partially-compartmentalized and non-compartmentalized trees. A tree is defined as compartmentalized, if the sequences between plasma and breast milk or breast milk cells sampled at the same time point are completely segregated. If a cluster of sequences sampled at the same time point either from breast milk or breast milk cells is not completely distinguished from plasma, this type of clustering is defined as partial-compartmentalization. Finally, if individual sequences from samples collected at the same time point from plasma, breast milk and/or breast milk cells mix with each other, this tree is classified as non-compartmentalized. From 24 samples, only 22 paired samples could

be used for sequence analysis. In two samples namely A368/2 and A401/2, no PCR products could be obtained from either breast milk or breast milk cells (Table 4).

Compartmentalization was observed in eleven cases, seven of these were from NVP-exposed and four from NVP-naïve patients (Table 4; Fig. 14A - 14K). Partial-compartmentalization was found in only one NVP-exposed woman (Table 4; Fig. 15A) and non-compartmentalization presented in ten cases, seven and three cases from NVP-exposed and NVP-naïve patients respectively (Table 4; Fig. 15B - 15K).

Most of the samples which were used for phylogenetic analysis were either paired plasma-breast milk or plasma-breast milk cells. However, a complete set of sequences including plasma, breast milk and breast milk cells at a single time point could be obtained from four NVP-exposed and one NVP-naïve women. Viral populations between breast milk and breast milk cells were segregated in 4 cases: ABK162 (Fig. 14A), ASn079 (Fig. 14B), AGn006 (Fig. 14C) and A368/1 (Fig. 15A), but not for A435 (Fig. 15B). Statistical analysis showed that time after receiving SD-NVP did not have any significant effect on compartmentalization [Mann-Whitney test, $p = 0.6746$, n (compartment) = 11, n (non-compartment) = 10].

3.2.3.2 Viral heterogeneity

The diversity of HIV variants may be influenced by the environment in which they evolve. Thus, the paired samples from two patients, ADb169 and AY134 were analyzed for their viral heterogeneity in plasma and breast milk or breast milk cells at two different time points. The time interval between the first and second sampling was approximately five to seven months.

The phylogenetic tree of patient-ADb169 at the first time point showed that the virus population in breast milk was separated into 2 clusters (Fig. 16A). Almost all of the breast milk clones (10 of 15 clones) were genetically distinct from the plasma clones, whereas a small proportion of breast milk variants (5 of 15 clones) were genetically closely relate to the major virus population in plasma. The tree of patient-ADb169 at the second time point showed that the virus population in breast milk was completely separate from the virus population in plasma (Fig. 16A). For patient-AY134, virus population in breast milk cells from this patient at the first time point was completely separate from virus population in plasma, whereas at the second time point viruses in breast milk cells were inter-mix with the plasma viruses (Fig. 16B).

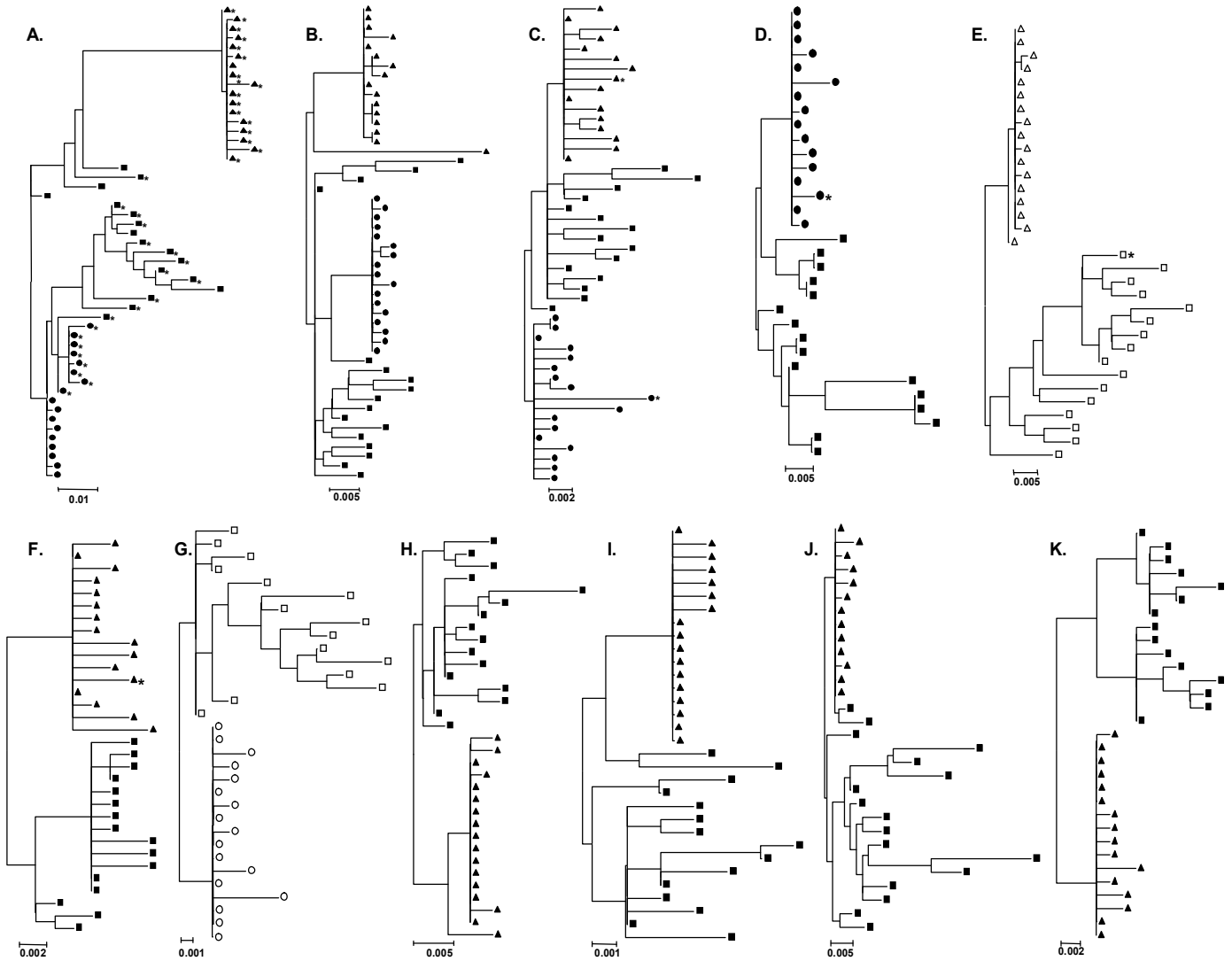

Fig. 14 Maximum-likelihood (ML) phylogenetic trees of *pol* sequences from plasma (PL), breast milk (BM), and breast milk cells (BMc) at different time points representing compartmentalization of A) ABK162 (NVP-exposed), B) ASn079(NVP-exposed), C) *AGn006 (NVP-naïve)*, D)AGn031/1(NVP-exposed), E) AGn031/2 (NVP-exposed), F) AY134/1 (NVP-exposed), G) ADb169/2 (NVP-exposed), H) AY113 (NVP-exposed), (I) *ADr304 (NVP-naïve)*, J) *AGg078 (NVP-naïve)*, K) *AY172/1 (NVP-naïve)*. Close squares, close circles and close triangles represent sequences from PL, BM and BMc respectively at first time point. Open squares, open circles and open triangles represent sequences from PL, BM and BMc respectively at second time point. Asterisks represent sequences harboring resistance mutation.

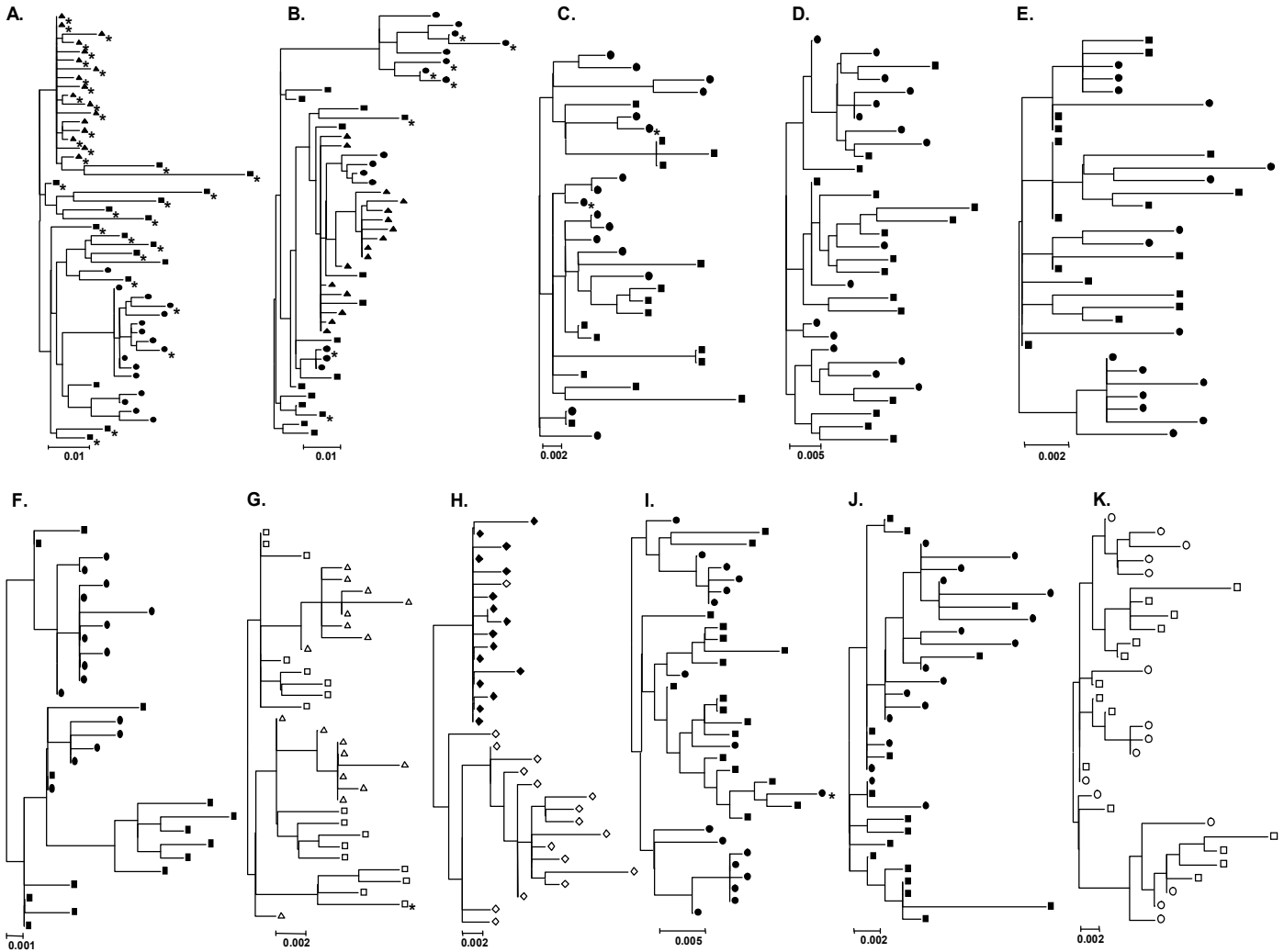

Fig. 15 Maximum-likelihood (ML) phylogenetic trees of *pol* sequences from plasma (PL), breast milk (BM), and breast milk cells (BMc) at different time points representing of partial-compartmentalization of A) A368 (NVP-exposed) and non-compartmentalization of B) A435 (NVP-exposed), C) A401/1 (NVP-exposed), D) A465 (NVP-exposed), E) Aba249 (NVP-exposed), F) ADb169/1 (NVP-exposed), G) AY134/2 (NVP-exposed), H) AY134/3 (NVP-exposed), (I) *ABm107 (NVP-naïve)*, J) *ADb365 (NVP-naïve)*, K) *AY172/2 (NVP-naïve)*. Close squares, close circles and close triangles represent sequences from PL, BM and BMc respectively at first time point. Open squares, open circles and open triangles represent sequences from PL, BM and BMc respectively at second time point. Open and close diamonds represent sequences from PL and BM respectively at third time point. Asterisks represent sequences harboring resistance mutation.

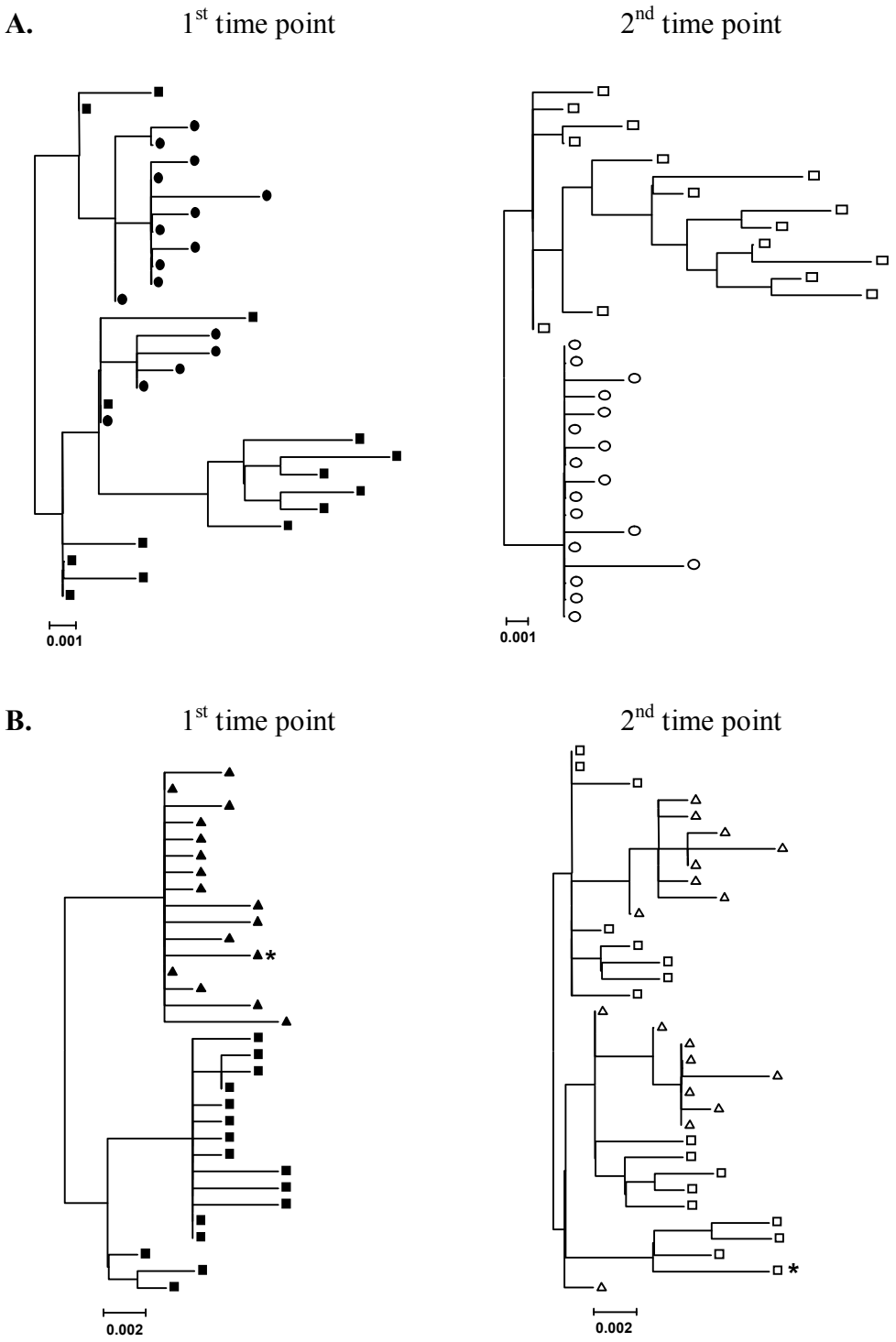

Fig. 16 Maximum-likelihood phylogenetic trees of *pol* sequences from plasma, and breast milk or breast milk cells representing **(A)** patient ADb169 sequences at the first and second time point and **(B)** patient AY134 sequences at the first and second time point. Close squares, close circles and close triangles represent respectively sequences from plasma, breast milk and breast milk cells at the first time point. Open squares, open circles and open triangles represent respectively sequences from plasma, breast milk and breast milk cells at the second time point. Asterisks represent sequences harboring drug resistance.

3.2.4 Evolution of HIV-1 in different anatomic compartments

3.2.4.1 Genetic diversity

To determine the genetic diversity of the virus in plasma, breast milk and breast milk cells, evolutionary distances were calculated as the mean pair wise distance among all clones from individual patients. The difference in evolutionary distances of sequences between the two groups e.g. plasma-breast milk, plasma-breast milk cells or breast milk-breast milk cells was statistically tested by Wilcoxon signed rank test, and by Kruskal-Wallis test with Dunn's Multiple Comparison post-test for three types of sequences e.g. plasma-breast milk-breast milk cells.

The results showed that the genetic diversity of plasma variants was significantly higher than the breast milk variants in nine of fifteen (60%) paired samples (Table 5). This observation was also found in paired plasma and breast milk cells samples where nine of twelve (75%) plasma sequences showed significantly higher genetic diversity than those from breast milk cells. In comparison to variants in breast milk and breast milk cells, only two of five (40%) of breast milk sequences showed a significantly higher genetic diversity than the variants from breast milk cells (Table 5).

The level of genetic diversity of variants derived from the complete sample set consisting of plasma, breast milk and breast milk cells within individual patients was calculated. From four NVP-exposed patients (A368, ABK162, Asn079 and A435) and one NVP-naïve patient (Agn006), the genetic distances of plasma variants was the highest compared to variants obtained from breast milk or breast milk cells (Fig. 17A - 17D). There was a wide distribution of breast milk variants in one case, A435 forming two main clusters (Fig. 17E). In general, variants from breast milk cells were much more homogenous than those in breast milk and plasma.

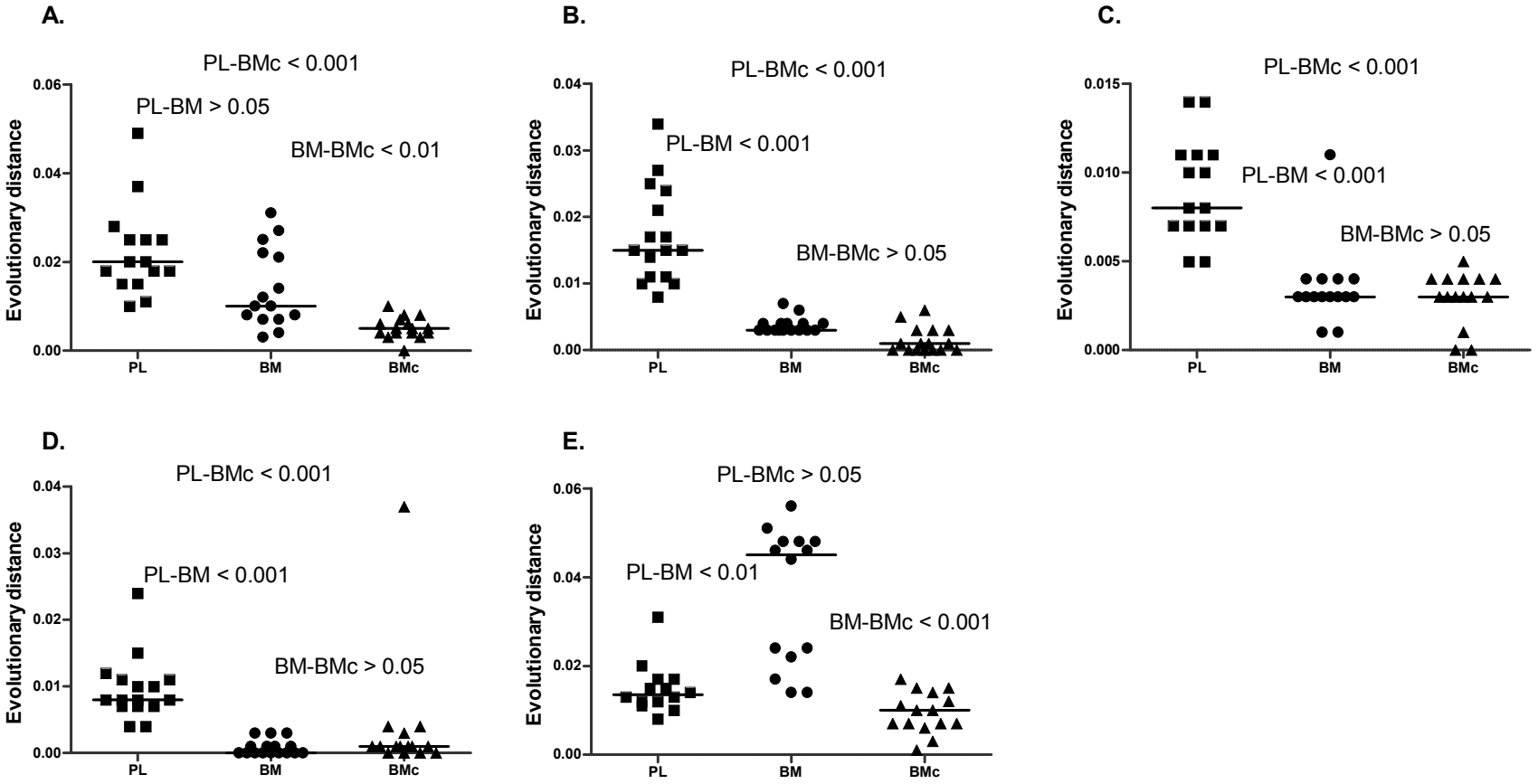

Fig. 17 Evolutionary distance between variants in plasma (PL), breast milk (BM) and breast milk cells (BMc) from individual patients at single time point representing the genetic diversity of **(A)** A368, **(B)** ABK162, **(C)** Agn006, **(D)** Asn079 and **(E)** A435. Close squares, close circles and close triangles represent mean distance in PL, BM and BMc sequences respectively. Scatter plots show median, p-values are from nonparametric, Kruskal-Wallis test with Dunn's Multiple Comparison post-test.

Table 5. Genetic diversity of HIV variants from PL, BM, and BMc represented as mean pair wise distances

ID	Mean pair wise distance at 1st time point				Mean pair wise distance at 2nd time point				Mean pair wise distance at 3rd time point			
	PL	BM	BMc	p-value	PL	BM	BMc	p-value	PL	BM	BMc	p-value
A368	0.0223	0.0139	0.0051	PL-BM > 0.05[b] **PL-BMc < 0.001[b]** **BM-BMc < 0.05[b]**	0.0129	-	-	ND	-	-	-	ND
A401	0.0132	0.0106	-	0.2483[a]	0.0059	-	-	ND	-	-	-	ND
A435	0.0149	0.0359	0.0095	**PL-BM < 0.01[b]** PL-BMc > 0.05[b] **BM-BMc < 0.001[b]**	-	-	-	ND	-	-	-	ND
A465	0.0148	0.0121	-	**0.0184[a]**	-	-	-	ND	-	-	-	ND
ABa249	0.0051	0.0133	-	**0.0007[a]**	-	-	-	ND	-	-	-	ND
ABK162	0.0171	0.0038	0.0015	**PL-BM < 0.001[b]** **PL-BMc < 0.001[b]** BM-BMc > 0.05[b]	-	-	-	ND	-	-	-	ND
ADb169	0.0057	0.0039	-	0.3659[a]	0.0083	0.0010	-	**0.0011[a]**	-	-	-	ND
AGn031	0.0139	0.0081	-	**0.0041[a]**	0.0175	-	0.0006	**0.0007[a]**	-	-	-	ND
ASn079	0.0096	0.0008	0.0037	**PL-BM < 0.001[b]** **PL-BMc < 0.001[b]** BM-BMc > 0.05[b]	-	-	-	ND	-	-	-	ND

Table 5. Genetic diversity of HIV variants from PL, BM, and BMc represented as mean pair wise distances *(continued)*

ID	Mean pair wise distance at 1st time point				Mean pair wise distance at 2nd time point				Mean pair wise distance at 3rd time point			
	PL	BM	BMc	p-value	PL	BM	BMc	p-value	PL	BM	BMc	p-value
AY113	0.0065	-	0.0011	**0.0007**[a]	-	-	-	ND	-	-	-	ND
AY134	0.0031	-	0.0025	0.2575[a]	0.0053	-	0.0059	0.2296[a]	0.0063	0.0015	-	**0.0011**[a]
ABm107	0.0086	0.0092	-	0.2053[a]	-	-	-	ND	-	-	-	ND
ADb365	0.0061	0.0070	-	0.4540[a]	-	-	-	ND	-	-	-	ND
ADr304	0.0044	-	0.0004	**0.0024**[a]	-	-	-	ND	-	-	-	ND
AGg078	0.0136	-	0.0011	**0.0024**[a]	-	-	-	ND	-	-	-	ND
AGn006	0.0090	0.0035	0.0029	**PL-BM < 0.001**[b] **PL-BMc < 0.001**[b] BM-BMc > 0.05[b]	-	-	-	ND	-	-	-	ND
AY172	0.0076	-	0.0014	**0.0010**[a]	0.0071	0.0076	-	0.8011[a]	-	-	-	ND

[a] = nonparametric (Wilcoxon signed rank test); [b] = nonparametric (Kruskal-Wallis test with Dunn's Multiple Comparison post-test).
Bold = statistically significant; ND = not determined; PL = Plasma; BM = Breast milk; BMc = Breast milk cells.

3.2.4.2 Genetic divergence

Longitudinal sampling of four NVP-exposed patients (A368, ADb169, AGn031, and AY134) and one NVP-naïve patient (AY172) were available for determining the genetic divergence of viruses. Viral divergences were measured as the pair wise genetic distances of all clones at a given time point compared to the bulk sequence at the first time point (most recent common ancestor- MRCA) for each patient.

In plasma, five of six cases had no significant difference ($p > 0.05$) in genetic divergence between the first and second time points, with an exception of sequences from patient A401 which showed a significant difference in genetic divergence ($p = 0.0378$) between the first and second time points (Table 6). However, in subject AY134, there were no significant differences in the divergence of plasma variants between the first and second, or the second and third time points, but there was a significant difference between the first and third time points (Fig. 18A; Table 6). In breast milk (ADb169; Table 6) and breast milk cells (AY134, Fig. 18B; Table 6) between the first and second time points, significant differences in genetic divergence were observed in both cases (Table 6).

Overall, the genetic divergence in plasma increased with time in four of six women, but only two of them were statistically significant (Table 6), whereas in breast milk and breast milk cells, the genetic divergence significantly increased with time in both cases (Table 6).

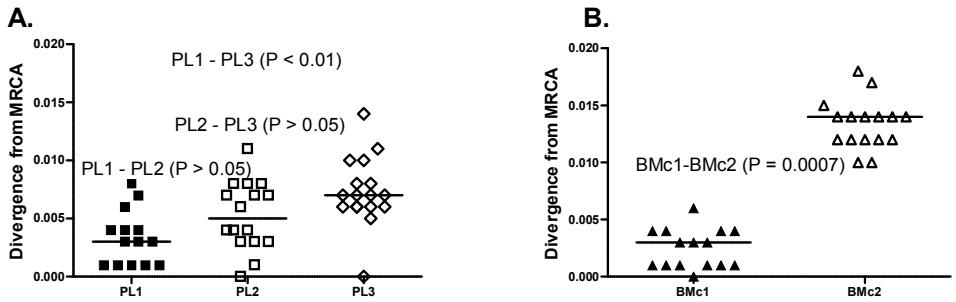

Fig. 18 Level of genetic divergence of plasma (PL) and breast milk cells (BMc) sequences at different time points represented by scatter plots. (A) Divergence of PL sequences at 3 time points from patient AY134, **(B)** divergence of BMc sequences at 2 time points from patient AY134. Close squares, open squares and open diamonds represent distance in plasma sequences at first, second and third time points respectively. Close triangles and open triangles represent mean distance in breast milk cells sequences at first and second time points respectively. MRCA = most recent common ancestor (bulk sequence at first time point). Scatter plots show median, p-values are from nonparametric, Kruskal-Wallis test with Dunn's Multiple Comparison post-test or Wilcoxon signed rank test.

Table 6. Genetic divergence of HIV variants from plasma (PL), breast milk (BM), and breast milk cells (BMc) at different time points

	Genetic divergence from MRCA (mean)				**Genetic divergence from MRCA (mean)**			**Genetic divergence from MRCA (mean)**		
	PL1	PL2	PL3	p-value	BM1	BM2	p-value	BMc1	BMc2	p-value
A368	0.0218	0.0167	-	0.0554[a]	-	-	-	-	-	-
A401	0.0132	0.0093	-	**0.0378[a]**	-	-	-	-	-	-
ADb169	0.0057	0.0066	-	0.5325[a]	0.0039	0.0108	**0.0011[a]**	-	-	-
AGn031	0.0139	0.0195	-	0.0545[a]	-	-	-	-		-
AY134	0.0034	0.0053	0.0073	PL1-2 > 0.05[b]				0.0025	0.0134	**0.0007[a]**
				PL1-3 < 0.01[b]						
				PL2-3 > 0.05[b]	-	-	-			
AY172	0.0076	0.0093	-	0.2708[a]	-	-	-	-	-	-

PL1, PL2, PL3 = plasma at the first, second and third time points respectively;
BM1, BM2= breast milk at the first and second time points respectively;
BMc1, BMc2 = breast milk cells at the first and second time points respectively;
[a] = nonparametric (Wilcoxon signed rank test); [b] = nonparametric (Kruskal-Wallis test with Dunn's Multiple Comparison post-test);
Bold = statistically significant; MRCA = most recent common ancestor.

3.3 Phenotypic characterization of a CRF02_AG based plasmid backbone

3.3.1. Construction and validation of a CRF02_AG based plasmid backbone

To better understand the influence of a non-subtype B plasmid backbone on recombinant viral assays, the infectious molecular clone pBD6TB9 was modified by knocking out an *EcoRI* restriction site (nucleotide position 4460). After that a new *EcoRI* site was introduced at the beginning of the protease gene (nucleotide position 2242) to generate pBD6TB9RI (Fig. 19). To clone the patient-derived PR-RT fragments into pBD6TB9RI, the newly introduced *EcoRI* site and a *StuI* restriction site present in the reverse transcriptase gene were used. The modifed and parental plasmids were transfected into 293T cells and their supernatants were used to infect C8166 cells for measuring replication kinetics or TZM cells for testing viral infectivity.

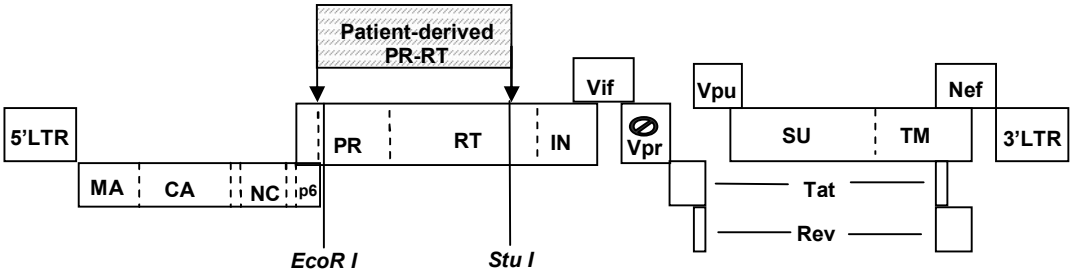

Fig. 19 Schematic representation of pBD6TB9RI. Amplified PR and RT fragment from patients are inserted into an indicator vector by using *EcoRI* and *StuI* restriction sites.

The replication kinetics of the viruses generated from the new plasmid backbone pBD6TB9RI was measured by comparing the level of p24 production from C8166 infected cells to the parental molecular clone pBD6TB9. The virus produced from the newly constructed pBD6TB9RI displayed similar replication kinetics to its parental virus shown by a comparable level of p24 production as observed in the figure 20A.

Luciferase activity produced from the TZM-infected cells was measured to evaluate the viral infectivity of viruses produced from pBD6TB9RI and parental pBD6TB9. As demonstrated in the figure 20B, the infectivity of both viruses was similar.

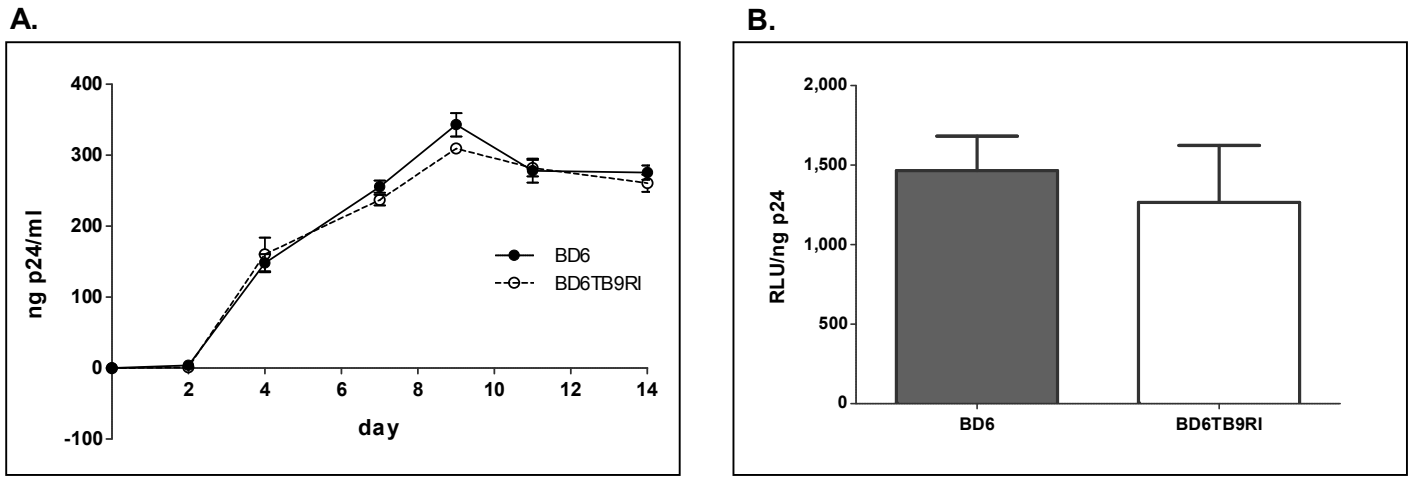

Fig. 20 (A) Replication kinetics of parental BD6TB9 virus (solid line) compared to the modified BD6TB9RI virus (dash line). C8166 cells were infected with equal amounts of p24 and virus replication measured by quantifying HIV-1 p24 (ng/ml) production in culture supernatant during 2 weeks. **(B)** Viral infectivity between parental BD6TB9 virus (dark gray bar) and modified BD6TB9RI virus (white bar) were measured. TZM cells were infected with BD6TB9 or BD6TB9RI viruses and luciferase activity was detected in cell lysates 48 hours after infection.

3.3.2 Effect of the CRF02_AG derived plasmid backbone on susceptibility to antiretroviral drugs

To evaluate the drug susceptibility profile of the CRF02_AG recombinant plasmid pBD6TB9RI backbone, and its effect on the antiretroviral drug susceptibility, a chimeric virus was generated by cloning the PR-RT fragment derived from the pNL4-3 into pBD6TB9RI, hereafter named BD6TB9RINL. The susceptibility profiles of the viruses were determined by adding serial dilutions of PIs to transfected cells or RTIs to infected cells. Drugs that inhibit virus replication should reduce luciferase activity in dose-dependent manner, and this provides a quantitative measure of drug susceptibility. Inhibition of luciferase activity was plotted against drug concentration (log_{10}) for each tested drug. Drug susceptibility fold changes were determined by comparing IC_{50} of tested virus with the IC_{50} of the control virus.

The phenotypic drug susceptibility profiles for BD6TB9RI (wild type CRF02_AG), BD6TB9RINL chimera and NL4-3 (wild type B) are shown in the figure 21 and Table 7. These three viruses revealed similar drug susceptibility profiles to all RTIs. The susceptibility profiles to PIs among these three viruses were also similar. However, they exhibited differences in susceptibility against two PIs: ritonavir (RTV) and tipranavir (TPV). There was a difference in the drug concentration fold change for RTV (>2.5-fold) for chimeric BD6TB9RINL and NL4-3 viruses respectively compared to wild type BD6TB9RI virus (Table 7). For TPV, there was also a >2.5-fold change difference for NL4-3 compared to wild type BD6TB9RI (Table 7).

The IC_{50} of RTV and TPV of the BD6TB9RI virus was lower than for the chimeric BD6TBRINL and NL4-3 viruses shown by a shift to the left of the dose-response curves (Fig. 21). The dose-response curves of RTV and TPV of theBD6TB9RINL chimera which contained the PR-RT fragment from NL4-3, subtype B virus, was closely similar to NL4-3 (Fig. 21). To confirm the observed differences, a statistical analysis was performed to determine differences in IC_{50} values of RTV and TPV between these three viruses by using the Friedman test with Dunn's multiple comparison post-test. The BD6TB9RI virus produced from the CRF02_AG plasmid showed no significant difference in IC_{50} to both PIs compared to chimeric BD6TB9RINL and NL4-3 viruses ($p>0.05$).

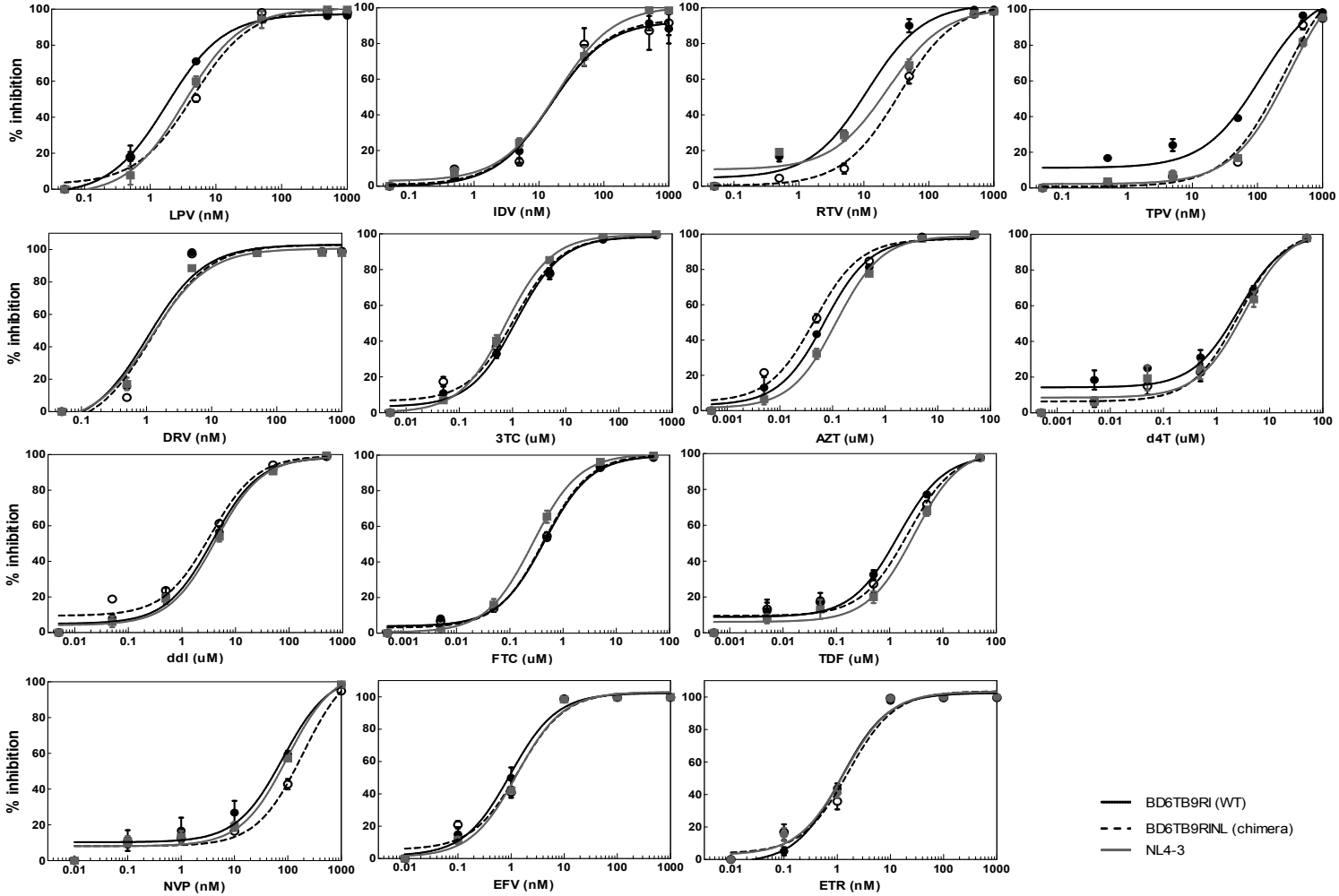

Fig. 21 Phenotypic drug susceptibility to the PR and RT inhibitors representing CRF02_AG wild type virus (BD6TB9RI, black solid line), CRF02_AG/subtype B chimera (BD6TB9RINL, dash line) and subtype B virus (NL4-3, gray solid line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated by nonlinear regression curve. The error bars indicated means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of test virus by the IC_{50} of the wild type (WT).

Table 7. Drug susceptibility fold change of the CRF02_AG/subtype B chimera (BD6TB9RINL), subtype B (NL4-3) viruses in comparison to CRF02_AG wild type virus (BD6TB9RI)

				Fold change in susceptibility (IC_{50} for test virus/IC_{50} for control virus) [a]													
	Resistance mutations detected by genotypic test			PIs					NRTIs						NNRTIs		
Sample	PIs	NRTIs	NNRTIs	LPV	IDV	DRV	RTV	TPV	AZT	d4T	3TC	ddI	FTC	TDF	NVP	EFV	ETR
BD6TB9RINL[b]	none	none	none	2.5	1.1	1.1	3.2	2.2	0.7	1.1	1.1	1.0	1.0	1.6	2.4	1.6	1.3
NL4-3[c]	none	none	none	1.9	1.4	1.1	2.7	2.9	1.7	1.5	0.7	1.2	0.6	2.0	1.2	1.5	1.2

[a] = fold change was calculated as the IC_{50} ratio between the sample and the control virus (BD6TB9RI) that was tested in the same experiment; [b] = Chimeric virus [plasmid contained PR and RT region from pNL4-3 (subtype B) and backbone from pBD6 (CRF02_AG)]; [c] = Subtype B virus.

3.3.3 Phenotypic drug susceptibility testing with patient-derived recombinant viruses

3.3.3.1 General information of patient-derived recombinant viruses

For phenotypic drug susceptibility assays, the sample set was collected from 75 HAART-exposed HIV-infected patients presenting at CHUYO in Ouagadougou from 2004-2006 (Tebit et al., 2008). The range of CD4 counts was between 2-469 cell/mm^3 and viral load ranged from 1 x 10^3 to 4 x 10^6 copies/ml (Tebit et al., 2008). Recombinant viruses in this present study were generated by cloning a 1.3 kb PR-RT fragment re-amplified from five patient-derived PCR products used previously for genotypic assays (Tebit et al., 2008) into the pBD6TB9RI plasmid backbone. These five selected patients were infected with HIV-1 CRF02_AG, received different antiretroviral drugs regimens and showed distinct resistance mutation patterns (Table 8).

Individual clones carrying different drug resistance mutations were randomly picked from recombinant libraries. Sequence analyses confirmed that there were no differences in the PR and RT mutations between the original patient samples and the corresponding recombinant viruses (Table 9).

Table 8. Clinical data and drug resistance mutation patterns derived from genotypic analysis

									Drug resistance mutations by sequence analysis (genotypic assays)		
ID	Sex	Age	VL[a]	CD4[b]	Current regimen	Past regimen	Subtype	Resistance status	PIs	NRTIs	NNRTIs
120	F	53	61,996	5	3TC-AZT-IDV	none	CRF02.AG	+/-PI, +/-NRTI	**M46I, L76V, I84V**, *L10I, E35G, Q58E*	**M41L**, E44D, **D67N**, V118I**, M184V, L210W, T215Y**, K219N	K101Q, E138K
127	M	47	333,000	173	AZT- ddI	none	CRF02.AG	+/-NRTI	none	**M41L, T215Y**	none
228	M	9	25,000	59	3TC-d4T-NVP	none	CRF02.AG	+/-NRTI,+/-NNRTI	none	**K65R,** T69D, K219R	K101Q**, Y181C, Y188CY, G190A**
273	M	-	43,000	-	-	none	CRF02.AG	+/-NRTI	none	**M41L, M184V, L210W, T215Y,** K219W	A98G
41	F	41	1,120,000	22	3TC-d4T-IDV	none	CRF02.AG	+/-PI, +/-NRTI,+/-NNRTI	**M46I, I84V,** *L10I*	M41l, **D67**H**N,** T69NT**, M184V, T215Y**	**K103**K**N**

[a] = viral load (copies/ml); [b] = cells/mm^3; PI = protease inhibitors; NRTI = nucleoside reverse transcriptase inhibitors; NNRTIs = non-nucleoside reverse transcriptase inhibitors; **Bold** = mutations at position associated with drug resistance; *Italic* = minor resistance mutations to protease inhibitors.

Table 9. Comparison of amino acid substitutions in PR and RT between original patient samples and the corresponding recombinant viruses

Sample	Source	Amino acid at the following positions																							
		PIs							NRTIs										NNRTIs						
		L10	*E35*	**M46**	*Q58*	**L76**	**I84**	*L89*	**M41**	E44	**K65**	**D67**	**T69**	V118	**M184**	**L210**	**T215**	**K219**	**A98**	**K101**	**K103**	**E138**	**Y181**	**Y188**	**G190**
120	Original[a]	*I*	*G*	**I**	*E*	**V**	**V**		**L**	D		**N**		I	**V**	**W**	**Y**	N		Q		K			
	Recombinant[b]	*I*	*G*	**I**	*E*	**V**	**V**		**L**	D		**N**		I	**V**	**W**	**Y**	N		Q		K			
127	Original[a]								**L**								**Y**								
	Recombinant[b]								**L**								**Y**								
228	Original[a]										**R**		D					R		Q			**C**	**C**/Y	**A**
	Recombinant[b]										**R**		D					R		**H**			**C**		**A**
273	Original[a]							*V*	**L**						**V**	**W**	**F**	W	G						
	Recombinant[b]							*V*	**L**						**V**	**W**	**F**	W	G						
41	Original[a]	*I*		**I**		**V**	**V**		**L**			H/**N**	N/T		**V**		**Y**				K/**N**				
	Recombinant[b]	*I*		**I**		**V**	**V**		**L**			**N**			**V**		**Y**				**N**				

Bold = mutations in positions associated with resistance to protease (PIs) and reverse transcriptase (NRTI, NNRTI) inhibitors; *Italic* = minor resistance mutations to protease inhibitors; [a] = original patient plasma samples used for genotypic assay (direct sequencing); [b] = individual clones randomly selected from recombinant libraries (clonal analyses).

3.3.3.2 Phenotypic drug susceptibility profiles of patients-derived recombinant viruses

The phenotypic drug susceptibilities of these recombinant viruses carrying patient-derived PR-RT coding sequences were analyzed as outlined in section 3.3.3.1. Representative results of susceptibility folds change are presented in Table 10 and are explained below.

Table 10. Drug susceptibility fold change of the patient-derived recombinant viruses in comparison to CRF02_AG wild type virus (BD6TB9RI)

	Fold change in susceptibility (IC_{50} for test virus/IC_{50} for control virus)[a]													
	PIs					NRTIs						NNRTIs		
Sample	LPV	IDV	DRV	RTV	TPV	AZT	d4T	3TC	ddI	FTC	TDF	NVP	EFV	ETR
BD6TB9RI120[b]	31.4	29.8	1.8	14.3	1.8	72.5	5.0	>500	4.5	>100	7.3	1.7	0.9	1.4
BD6TB9RI127[b]	5.3	1.1	1.1	2.3	1.2	9.6	2.3	2.0	1.6	1.7	2.9	0.7	0.3	0.2
BD6TB9RI228[b]	0.4	0.4	0.4	0.3	0.1	0.3	3.1	29.8	4.9	24.6	4.6	>125	416.9	12.8
BD6TB9RI273[b]	0.9	0.5	0.9	0.6	0.3	62.6	2.0	>150	1.6	>100	2.3	0.2	0.2	0.5
BD6TB9RI41[b]	33.2	7.3	0.9	6.0	0.2	2.7	2.1	>300	3.3	>100	2.1	48.5	12.0	0.2

[a] = fold change was calculated as the IC_{50} ratio between the sample and the control virus (BD6TB9RI) that was tested in the same experiment; [b] = patient-derived recombinant viruses.

(i) BD6TB9RI120; the patient who provided this sample had been treated with the combination of 3TC, AZT and IDV and harbored the mutations with highly decreased susceptibility to PIs and NRTIs (Table 8). The susceptibility profile obtained from this recombinant virus is in agreement with the therapeutic history (Fig. 22). This recombinant virus was resistant to three protease inhibitors; LPV (31-fold), IDV (30-fold) and RTV (14.3-fold) (Fig. 22; Table 10) but was susceptible to protease inhibitors; DRV (1.8-fold) and TPV (1.8-fold) (Table 10). Due to the accumulation of multi-nRTI resistance mainly with thymidine analogue-associated mutations (TAMs) such as M41L, D67N, L210W, and T215Y which affect all currently approved NRTIs, this recombinant virus was highly resistant to all tested NRTIs in this experiment (Fig. 22; Table 10). The amino acid

substitutions at positions K101Q and E138K in RT gene which were earlier reported to cause low level resistance to NNRTIs by genotypic testing were observed although this patient was NNRTIs naïve (Table 10). However, this recombinant virus was shown to be susceptible to NNRTIs by phenotypic testing (Fig. 22; Table 10).

(ii) BD6TB9RI127; this sample was from a patient who received a combination of AZT and ddI and harbored mutations to NRTIs (Table 8). Low level resistance to AZT accompanied by d4T cross resistance and ddI accompanied by TDF cross resistance was detected by the phynotypic assay (Fig. 23; Table 10).

(iii) BD6TB9RI228; was obtained from a patient treated with 3TC, d4T and NVP combination regimen (Table 8). An increase in fold resistance was detected in all tested NRTIs (Fig. 24; Table 10). However, this recombinant virus was susceptible to AZT due to the presence of the K65R mutation which causes AZT hypersusceptibility (Fig. 24; Table 10). Several NNRTIs resistance mutations e.g. K101Q, Y181C, Y188C/Y, and G190A were observed due to the NVP-exposure. These mutations induced cross resistance to all NNRTIs including ETR, the newly approved NNRTI (Fig. 24; Table 10).

(iv) BD6TB9RI273; this sample was from a patient with unavailable treatment history (Table 8). This recombinant virus carried several mutations to NRTIs such as M184V and three TAMs: M41L, L210W, and T215Y when analyzed genotypically. As expected, the intermediate to high level resistance to NRTIs was detected (Fig. 25; Table 10). This recombinant virus remained susceptible to NNRTIs, although the substitution at position A98G which has been shown to possibly affect ETR susceptibility (Johnson et al., 2008) was detected (Fig. 25; Table 10). One minor resistance mutation to PIs, L98V, was found but did not alter the susceptible to PIs used in this experiment (Fig. 25; Table 10).

(v) BD6TB9RI41; this sample was derived from a patient with documented use of the combination therapy of 3TC, d4T and IDV (Table 8). The phenotypic assay demonstrated its resistance to three PIs; LPV (33-fold), IDV (7-fold) and RTV (6-fold) (Fig. 26; Table 10). In contrast, this recombinant virus was susceptible and hypersusceptible to the PIs, DRV (0.9-fold) and TPV (0.2-fold) respectively (Fig. 26; Table 10). High level resistance as well as cross resistance to NRTIs was detected which is in agreement with the patient's therapeutic history (Fig. 26; Table 10). The mutation

K103N, which confers high level resistance to EFV and NVP but not ETR (Jonnson et al., 2008) was detected in this recombinant virus despite the patient's non exposure to NNRTI. The resistance profile showed an intermediate to high level resistance to EFV (12-fold) and NVP (49-fold), but remained susceptible for ETR (0.2-fold) (Fig. 26; Table 10).

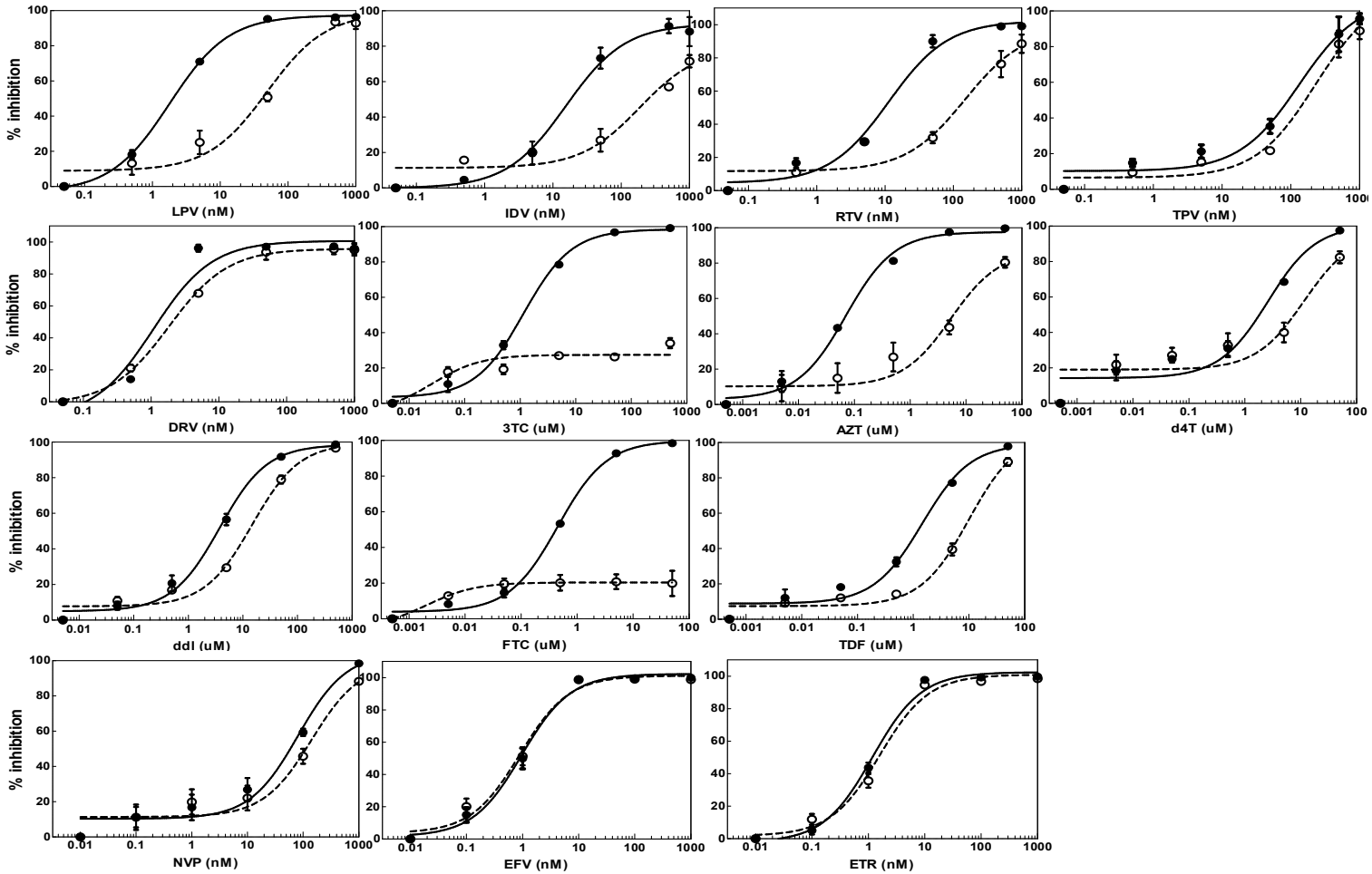

Fig. 22 Phenotypic drug susceptibility to PR and RT inhibitors representing the CRF02_AG wild type virus (BD6TB9RI, solid line) and the patient-derived PR-RT recombinant virus (**BD6TB9RI120**, dash line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated and is illustrated by a nonlinear regression curve. The error bars indicate means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of the test sample by the IC_{50} of the wild type (WT).

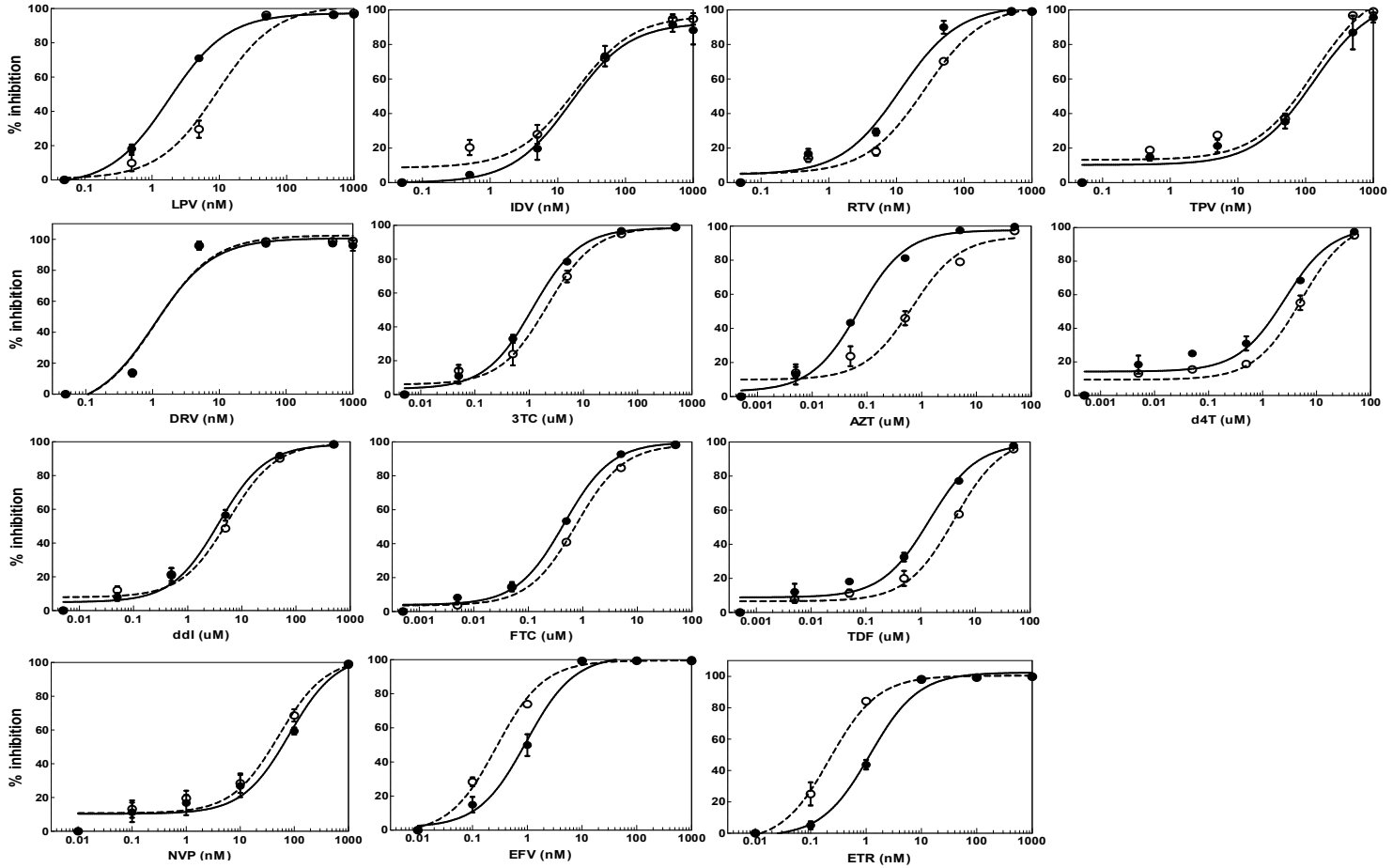

Fig. 23 Phenotypic drug susceptibility to PR and RT inhibitors representing the CRF02_AG wild type virus (BD6TB9RI, solid line) and the patient-derived PR-RT recombinant virus (**BD6TB9RI127**, dash line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated and is illustrated by a nonlinear regression curve. The error bars indicate means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of the test sample by the IC_{50} of the wild type (WT).

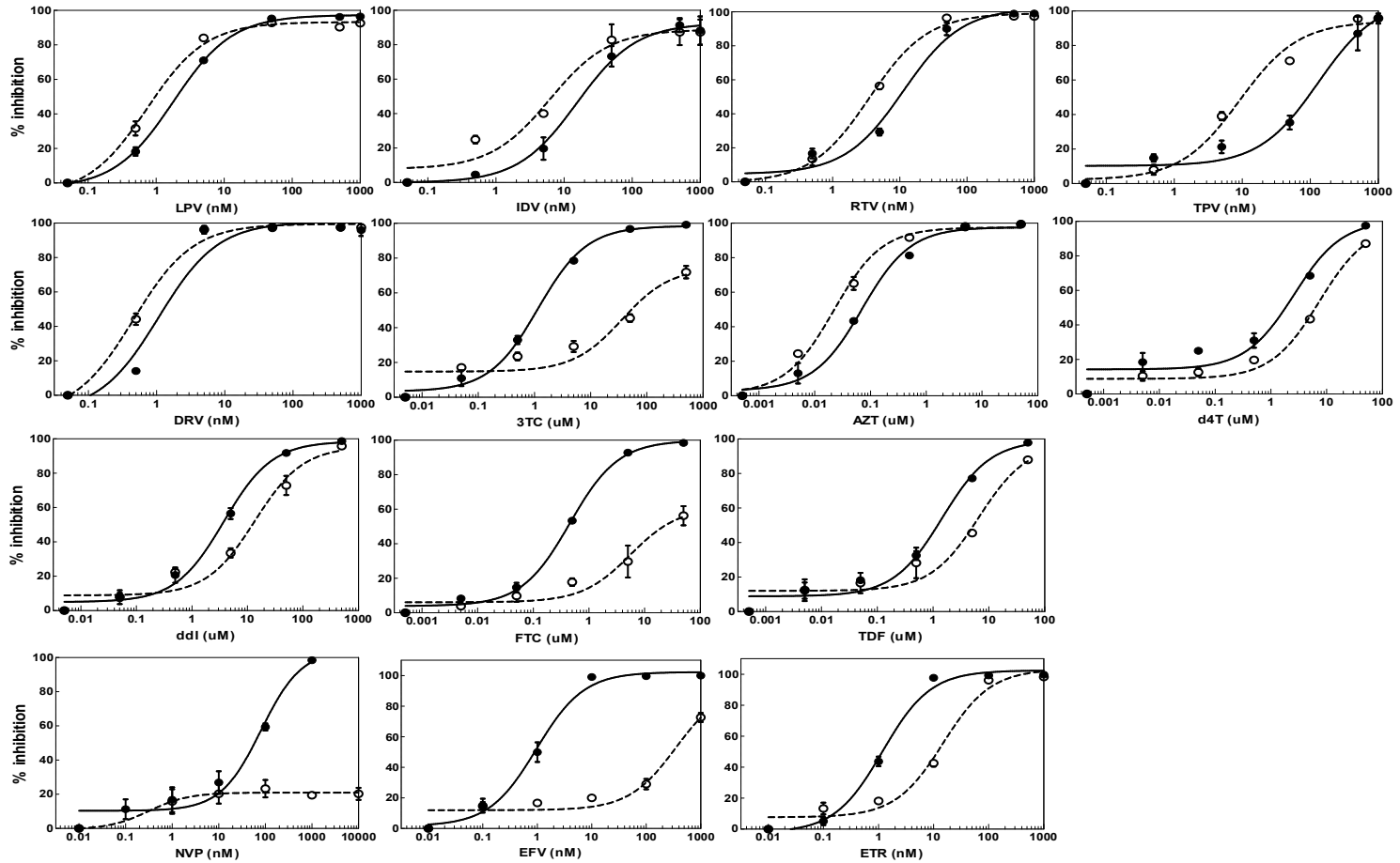

Fig. 24 Phenotypic drug susceptibility to PR and RT inhibitors representing the CRF02_AG wild type virus (BD6TB9RI, solid line) and the patient-derived PR-RT recombinant virus (**BD6TB9RI228**, dash line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated and is illustrated by a nonlinear regression curve. The error bars indicate means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of the test sample by the IC_{50} of the wild type (WT).

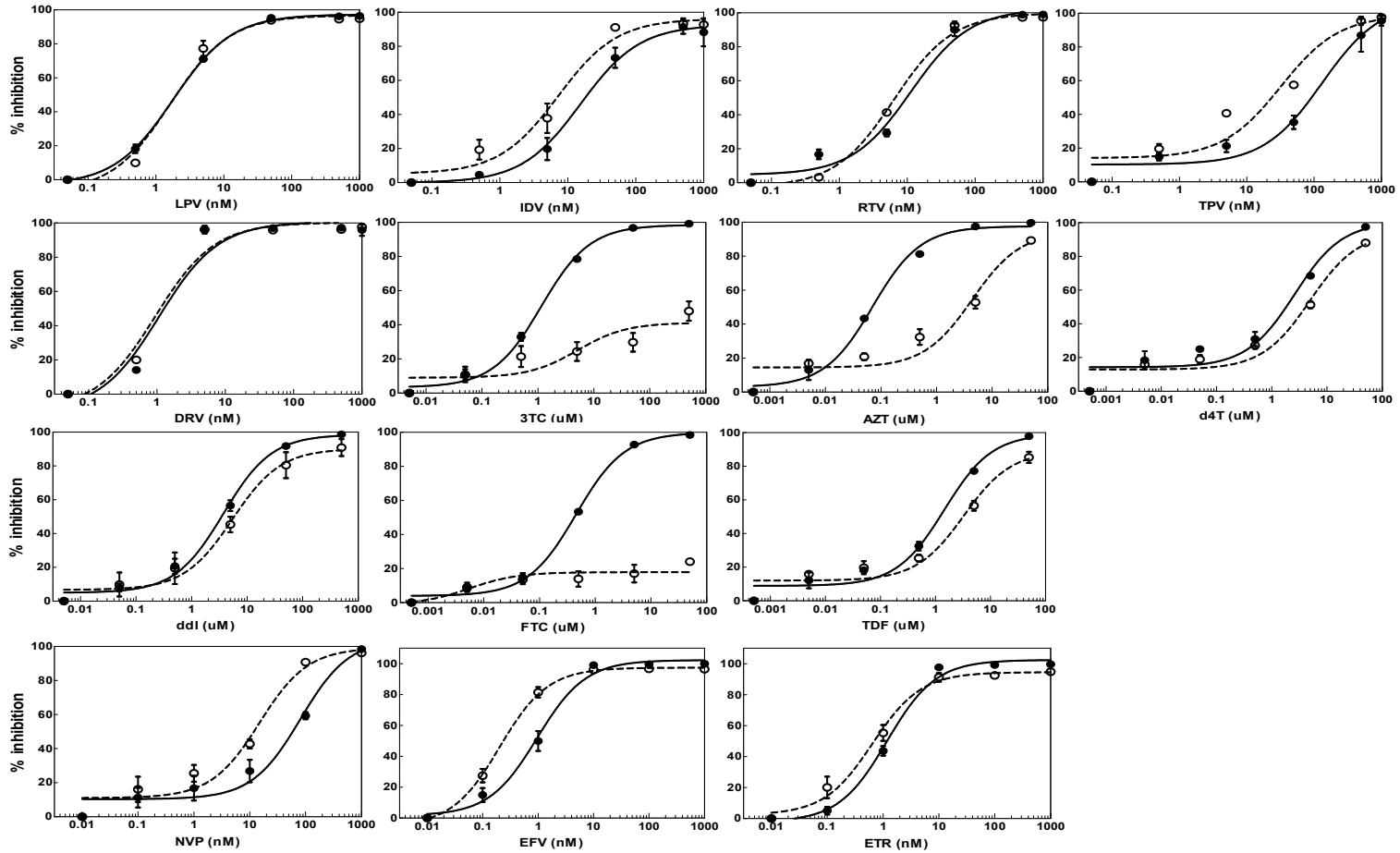

Fig. 25 Phenotypic drug susceptibility to PR and RT inhibitors representing the CRF02_AG wild type virus (BD6TB9RI, solid line) and the patient-derived PR-RT recombinant virus (**BD6TB9RI273**, dash line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated and is illustrated by a nonlinear regression curve. The error bars indicate means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of the test sample by the IC_{50} of the wild type (WT).

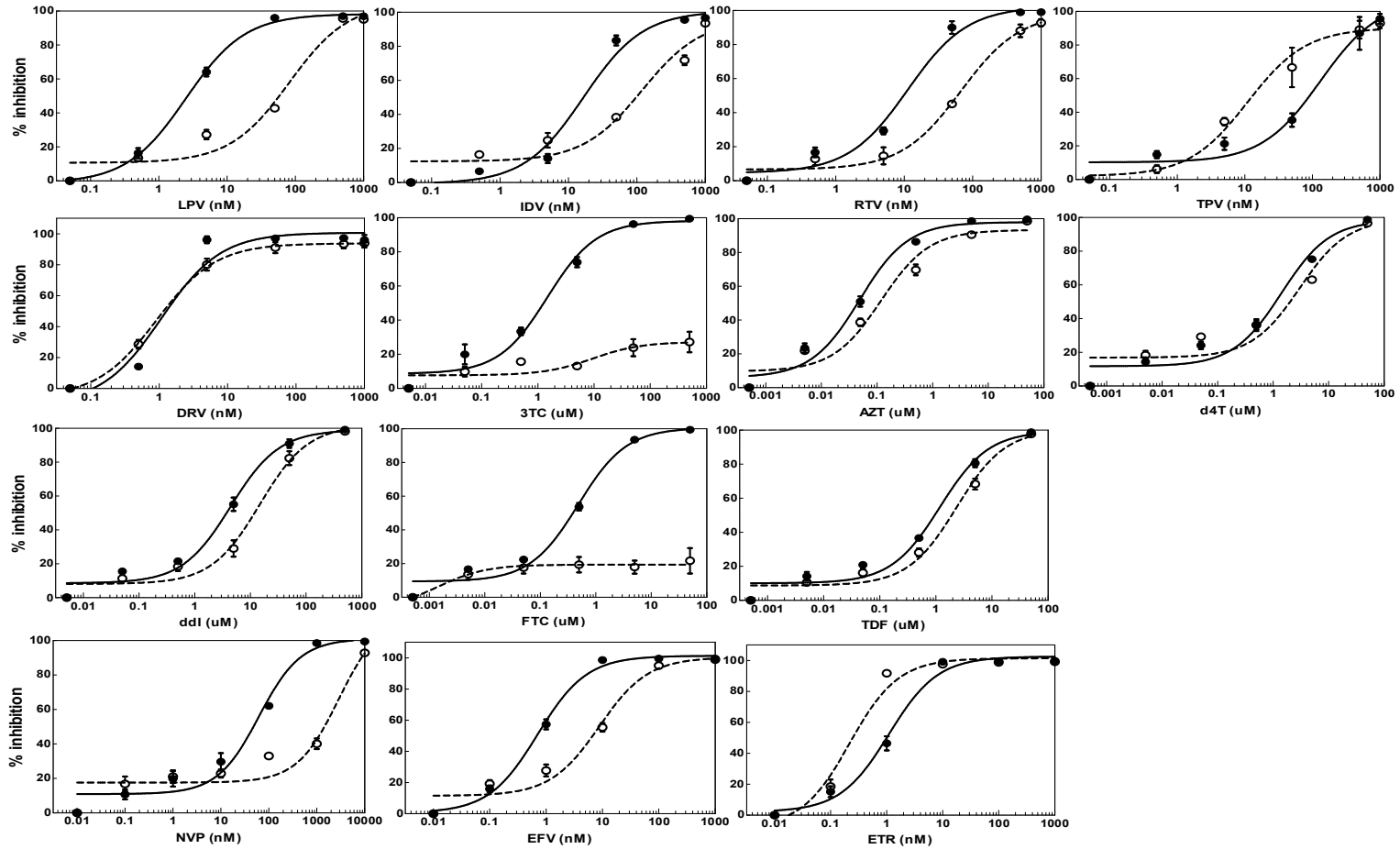

Fig. 26 Phenotypic drug susceptibility to PR and RT inhibitors representing the CRF02_AG wild type virus (BD6TB9RI, solid line) and the patient-derived PR-RT recombinant virus (**BD6TB9RI41**, dash line). The dose-response curves were obtained by plotting the inhibition percentage versus the $\log_{10}$ of drug concentration. The IC_{50} was calculated and is illustrated by a nonlinear regression curve. The error bars indicate means ± SEM. Changes in drug susceptibility were determined by dividing the IC_{50} of the test sample by the IC_{50} of the wild type (WT).

3.3.4 Reproducibility of the phenotypic drug susceptibility assay

Drug susceptibility experiments were repeated three to four times to evaluate the reproducibility of the IC_{50} for each drug in a control virus (BD6TB9RI) and recombinant viruses (five patient-derived recombinant viruses and one chimeric BD6TB9RINL virus). Each assay was performed in triplicate. The mean of IC_{50}s, standard error of mean (SEM) and coefficient of variation (CV) was calculated. As demonstrated in Table 11, the respective phenotypic resistance profiles can be reproducibly generated in these recombinant viruses.

Table 11. Reproducibility of drug susceptibility assays with CRF02_AG based plasmid backbone

Sample and Statistics[a]		PIs (nM)					NRTIs (μM)						NNRTIs (nM)		
		LPV	IDV	DRV	RTV	TPV	AZT	d4T	3TC	ddI	FTC	TDF	NVP	EFV	ETR
BD6TB9RI[b]	Mean IC50	1.78	15.30	1.07	11.37	137.97	0.07	2.54	1.16	3.75	0.45	1.44	80.42	1.00	1.18
	SEM	0.13	2.03	0.02	0.65	27.36	0.004	0.25	0.12	0.63	0.04	0.12	1.83	0.24	0.11
	CV	0.07	0.13	0.02	0.06	0.02	0.05	0.10	0.10	0.17	0.09	0.08	0.02	0.24	0.10
BD6TB9RI120[c]	Mean IC50	55.75	450.47	1.86	164.87	221.10	5.18	12.25	>500	15.08	>50	10.73	141.00	0.90	1.62
	SEM	3.77	52.66	0.26	33.90	12.71	0.80	2.89	ND	1.78	ND	2.82	19.67	0.22	0.23
	CV	0.07	0.12	0.14	0.21	0.06	0.15	0.24	ND	0.12	ND	0.26	0.14	0.24	0.14
BD6TB9RI127[c]	Mean IC50	9.06	17.32	1.14	26.36	136.70	0.67	5.44	2.16	5.61	0.75	4.14	52.55	0.28	0.23
	SEM	1.22	2.41	0.09	0.62	23.20	0.15	0.97	0.56	0.15	0.06	0.32	13.12	0.04	0.05
	CV	0.13	0.14	0.08	0.02	0.17	0.23	0.18	0.26	0.03	0.08	0.08	0.25	0.14	0.23
BD6TB9RI228[c]	Mean IC50	0.73	6.05	0.47	3.52	9.26	0.02	7.53	34.47	16.39	11.33	6.54	>10,000	384.30	15.10
	SEM	0.09	0.61	0.06	0.32	1.04	0.005	0.94	6.67	4.24	4.13	0.32	ND	64.94	1.74
	CV	0.12	0.10	0.14	0.09	0.11	0.21	0.13	0.19	0.26	0.36	0.05	ND	0.17	0.12
BD6TB9RI273[c]	Mean IC50	1.58	7.83	0.95	6.38	30.80	4.52	4.99	>150	5.53	>50	3.43	14.93	0.21	0.63
	SEM	0.09	2.81	0.02	0.38	10.32	1.22	0.72	ND	1.53	ND	0.98	0.50	0.04	0.22
	CV	0.06	0.36	0.02	0.06	0.33	0.27	0.14	ND	0.28	ND	0.28	0.03	0.20	0.35
BD6TB9RI41[c]	Mean IC50	82.19	119.03	0.95	67.81	30.74	0.14	2.95	>500	15.67	>50	2.41	2,982.67	8.86	0.22
	SEM	7.28	3.26	0.13	1.62	21.53	0.03	0.59	ND	4.20	ND	0.44	457.40	1.74	0.02
	CV	0.09	0.03	0.13	0.02	0.7	0.25	0.20	ND	0.27	ND	0.18	0.15	0.20	0.09

Table 11. Reproducibility of drug susceptibility assays with CRF02_AG based plasmid backbone *(continued)*

Sample and Statistics [a]		PIs (nM)					NRTIs (μM)						NNRTIs (nM)		
		LPV	IDV	DRV	RTV	TPV	AZT	d4T	3TC	ddI	FTC	TDF	NVP	EFV	ETR
BD6TB9RINL[d]	Mean IC50	4.34	16.03	1.21	35.78	248.00	0.05	2.60	1.18	3.38	0.44	2.21	193.33	1.40	1.59
	SEM	0.66	0.54	0.03	6.31	7.98	0.007	0.60	0.37	0.12	0.04	0.42	16.69	0.16	0.19
	CV	0.15	0.03	0.03	0.18	0.03	0.14	0.23	0.31	0.04	0.10	0.19	0.09	0.11	0.12
NL4-3[e]	Mean IC50	3.40	19.68	1.18	30.77	313.80	0.12	3.57	0.76	4.25	0.28	2.92	98.32	1.28	1.35
	SEM	0.48	3.32	0.04	4.51	44.94	0.02	0.65	0.10	0.48	0.03	0.24	2.06	0.04	0.13
	CV	0.14	0.17	0.03	0.15	0.14	0.16	0.18	0.13	0.11	0.12	0.08	0.02	0.03	0.10

[a] = Drug susceptibility experiments were repeated at least 3 times to evaluate the IC_{50} for each drug from control and sample viruses. The mean IC_{50}s, standard error of mean (SEM) and coefficient of variation (CV) were calculated; [b] = control virus (CRF02_AG); [c] = patient-derived recombinant viruses; [d] = chimeric virus [plasmid contained PR and RT region from pNL4-3 (subtype B) and backbone from pBD6 (CRF02_AG)]; [e] = subtype B virus; ND = not done.

4 Discussion

The present study provides a comprehensive analysis of drug resistance mutations of non-subtype B HIV-1, particularly CRF02_AG strain which is circulating in Burkina Faso. The importance of this study relates to the genotypic and phenotypic characterization of drug resistance mutation variants. The genotypic assay was performed by two different methods, namely; direct sequencing and clonal analysis, to determine the development of resistance mutations as well as the persistence and distribution of resistance variants in HIV-1 infected individuals. For the phenotypic assay, the plasmid backbone from the CRF02_AG subtype was generated. This tool would greatly facilitate the study of the antiretroviral drugs response of CRF02_AG variant. Overall, this study gives an insight into genetic characteristics of CRF02_AG strain which provides useful information and better understanding about development of drug resistance mutations and antiretroviral drug response in HIV-1 non-subtype B.

4.1 Absence of enfuvirtide (T-20) -associated resistance mutations among drug naïve Burkinabes

Resistance mutations to T-20 are mediated by amino acid substitutions within HR1 at positions 36-45 including G36D/S, G36V/E, V38A/E/M, Q40H, N42T, and N43D (Marcelin et al., 2004; Jonhson et al., 2008). Among 38 drug naïve patients in Burkina Faso, no amino acid mutation was found in the highly conserved three amino acid motif at codons 36-38 (**GIV**) that are important determinants of viral susceptibility to T-20 (Aghokeng et al., 2005). This finding confirms a previous study that natural T-20 genotypic resistance is rare in drug naive individuals (Roman et al., 2003). The L54M and Q56K polymorphisms which were recently found to cause approximately 5-fold reduced sensitivity to inhibition by T-20 (Chinnadurai et al., 2005; Sen et al., 2009) were observed in 38% and 75% of all CRF02_AG samples, respectively. The N42S polymorphism

which was found in HR1 sequences from 36 patients in this study is subtype-specific and polymorphism at this position is reported to be associated with T-20 hypersusceptibility (Hudelson et al., 2009; Roman et al., 2003). In addition, the N126K and S138A mutations in HR2 which contribute to the reduction of T-20 susceptibility (Lu et al., 2006; Xu et al., 2005) were also detected from the study samples. Other mutations, such as N140I, which provide immunologic gain (Sen et al., 2009) were found in some samples in this study.There were some polymorphisms observed in the HR1 and HR2 regions among patient sequences whose phenotypic implications are still unknown. It needs to be further determined whether these polymorphisms lead to phenotypic changes in these CRFs.

In summary, the lack of any known T-20 resistance mutations among the HIV subtypes and CRFs circulating in Burkina Faso implies that this drug has not been introduced in this area and it could be used in patients failing other drug regimens. Detailed phenotypic studies using primary isolates from this country will be useful.

4.2 NVP associated resistance mutations are prevalent and persist in plasma and breast milk

This study demonstrates the presence and persistence of NVP-associated resistance mutations as well as the compartmentalization of HIV-1 population in paired plasma and breast milk or breast milk cells from 17 NVP-naïve and -exposed women from Nouna, Burkina Faso. Genetic analyses of these patients' sequences by direct sequencing and clonal analysis provide an insight into the distribution pattern of HIV variants in these compartments.

In this present study, HIV infected breast feeding women in Nouna, a rural area of Burkina Faso, were infected mostly with CRF02_AG (64%) but also included CRF06_cpx (35%). This observation confirms the co-dominant distribution of CRF02_AG and CRF06_cpx in this area (Tebit et al., 2006). The co-dominant circulating between CRF02_AG and CRF06_cpx was also previously reported in Bobo-Dioulasso in Burkina Faso (Manigart et al., 2004) and in Niger, the neighboring country of Burkina Faso (Mamadou et al., 2002). In contrast to rural area, the CRF06_cpx was reported as the predominant strain in Ouagadougou, the capital city of Burkina Faso (Ouedraogo-Traore et al., 2003).

4.2.1 Detection of NVP-associated resistance mutations by direct sequencing and clonal analysis

Direct PCR sequencing or population-based sequencing can only identify variants that constitute more than 20% of the virus population (Palmer et al., 2005, 2006a). This method is therefore not sensitive enough to detect minor variants that harbor resistance mutations. Several studies have shown that minor drug-resistant variants that are not detected by direct sequencing are clinically relevant and often responsible for the virological failure of a new antiretroviral treatment regimen (Jourdain et al. 2004; Palmer et al. 2006b). By using direct sequencing, NVP resistance mutations were detected in 8% of infected mothers in the study, similar to the range reported from previous studies (Jourdain et al., 2004; Cunningham et al., 2002; Eshleman et al., 2004). This, however, increased to 46% by clonal analysis, in accordance with earlier studies confirming that a higher prevalence of resistance mutations could be detected by clonal analysis (Becquart et al., 2002, 2007; Kassaye et al., 2007).

4.2.2 Development and persistence of drug resistance mutations among NVP-exposed women

Several studies have reported the development of NVP resistance mutations within several weeks after SD-NVP exposure in patients infected with different HIV-1 subtypes (Eshleman et al., 2001; Jackson et al., 2000; Lee et al., 2005). In this study, the development of NVP resistance mutations was observed in some patients within three to ten days after SD-NVP exposure (Table 4). However, because the pre SD-NVP exposure samples were not sequenced, the possibility that some of the mutations existed due to the transmission of resistant variants before NVP exposure cannot be excluded. Such rapid development of resistance mutations was also previously detected within ten days postpartum from patients infected with CRF01_AE in Thailand (Jourdain et al., 2004).

The development of NVP resistance mutations could be due to several factors and has been extensively reported in prevention of HIV mother-to-child transmission programs in Africa (Eshleman et al., 2001; Jackson et al., 2000). Firstly, the long half-life of nevirapine which allows a prolonged sub-therapeutic level providing long term selective pressure for the emergence of resistance strains (Mirochnick et al., 1998). Secondly, a single mutation **K103N** (from AA**A** to AA**T** or AA**C**) or **Y181C** (from T**A**T/T**A**C to T**G**T/T**G**C) can cause a high level resistance to NVP. Finally, the

possibility that minor variants harboring these mutations are likely to be present in undetectable levels in infected women prior to NVP administration (Havlir et al., 1996).

In this study, the **K103N** mutation was more frequently selected compared to the **Y181C** mutation (Table 4). This selection may reflect the fitness advantage of **K103N** over **Y181C** (Richman et al., 1994; Eshleman et al., 2001). Moreover, because of the slight reduction of the replicative capacity of **K103N**, this variant is not completely overgrown by the wild type variant in the absence of NVP selective pressure (Hance et al., 2001) and may replicate at low frequencies (Nicastri et al., 2003) or preserved latently in reservoirs (Briones et al., 2006). At twelve months post SD-NVP exposure, the **K103N** variant was still detectable by clonal analysis but not by direct sequencing (1 of 15 plasma clones). This is consistent with previous studies which reported the persistence of **K103N** variants more than one year post NVP administration (Flys et al., 2005; Palmer et al., 2006b). Early reports on the effects of SD-NVP raised concerns that the persistence of NVP-related mutations could affect future treatment with NNRTIs or even a subsequent administration of SD-NVP in a second pregnancy. However, studies of Jourdain et al. (2004) and Lockman et al. (2007) suggest that given enough time between the initial NVP exposure and the subsequent administration, these resistance variants may fade out.

Beside the NVP resistance mutations, NRTIs resistance mutations were also detected in this study. The observation of two NRTI resistance mutations, K65R and M184I, from two NVP-exposed women confirms our previous findings that these patients might have been exposed to other antiretroviral drugs or misreported their therapeutic histories, a common phenomenon in Burkina Faso (Tebit et al., 2009).

4.2.3 Prevalence of NVP-associated resistance mutations among naïve patients

The prevalence of resistance mutations to all classes of antiretroviral drugs in HIV-1 naïve patients varies between 8-20% (Little et al., 2002; Oette et al., 2006). In a previous study among drug naïve patients in Burkina Faso, we found a high prevalence of NRTIs and NNRTIs resistance mutations of 10.6% and 6.1% respectively (Tebit et al., 2009). Although no **K103N** or **Y181C** resistance mutations were detected among drug naïve patients in the present study, other NVP resistance mutations such as V106A, V108I as well as E44D mutation which causes low level resistance to NRTIs were also

detected in naïve patients (Table 4). This finding suggests that resistance variants are circulating and may be transmitted to drug naïve patients in this area.

4.2.4 Compartmentalization of virus populations in plasma and breast milk

Compartmentalization occurs when a small population of variants is partially restricted from entering or exiting an anatomical site (Nickle et al., 2003). Moreover, difference of target cells, tissue specific and immune pressure in distinct anatomical sites may contribute to the independent selection of a variant population for further replication in such anatomical compartments (Becquart et al., 1999; Kemal et al., 2003; McKeating et al., 1989). Previous studies demonstrated contradictory results of HIV-1 compartmentalization in plasma and breast milk (Becquart et al., 2002, 2007; Henderson 2004). In this study, three different distribution patterns of viral populations between plasma and breast milk or breast milk cells were observed from 22 paired samples namely; compartmentalized (11 of 22 cases), partial-compartmentalized (1 of 22 cases), and non-compartmentalized (10 of 22 cases) (Fig. 14 and 15).

Compartmentalization in breast milk can be influenced by breast infections such as mastitis or abscesses. Inflammatory chemokine secretion in cases of mastitis or abscess may impair the blood-mammary gland permeability and lead to the migration of virus populations from plasma into the breast milk compartment or vice versa (Becquart et al., 2007; Pillay et al., 2000). Furthermore, mastitis has been reported to increase the virus load of breast milk (Semba et al., 1999). Unfortunately, patient clinical data about the breast pathology at the sampling time were not available; therefore, it cannot be stated categorically, if compartmentalization was influenced by mastitis or other breast infections.

The viral load in breast milk was significantly lower than in plasma (Table 3), a finding which is in agreement with previous studies which have shown that some secretions in breast milk, such as lactoferrin or the local humoral immune response, may inhibit virus replication or affect the viability of free virus particles and, therefore, contribute to the low virus copies in breast milk (Becquart et al., 1999; Koulinska et al., 2006; Pillay et al., 2000; Viani et al., 1999)

Breast milk from HIV infected women contains both cell-free virus particles and cell-associated virus. Transmission of HIV-1 during early stage of lactation is frequently

associated with cell-associated virus whereas transmission of cell-free virus frequently occurs at later stages of lactation mostly after nine months postpartum (Koulinska et al., 2006). To characterize the difference of virus population within breast milk, the viral RNA reflecting the cell-free virus and viral DNA reflecting the cell-associated virus were amplified from five patients. The viral RNA in breast milk revealed a closer genetic relationship with virus in plasma, but clearly distinct from viral DNA in breast milk in four of five patients. As observed previously by Becquart and his colleagues (2002, 2007), genetic difference between viral RNA and DNA in breast milk indicates that cell-free virus was not produced by the majority of infected cells in breast milk. This may be caused by differences of cellular composition in blood and breast milk in which epithelial cells are the predominant cell population (Becquart et al., 2007). These cells are not highly permissive to HIV infection and produce low copies of virus in *vitro* (Toniolo et al., 1995). Furthermore, most of infected cells in milk are found to be latently infected with transiently silent viruses (Boulerice et al., 1990). Thus, it is possible that free virus in breast milk originated from two major cell populations, macrophages and lymphocytes which could be infected locally at sub-mucosal sites within the mammary gland (Becquart et al., 2002, 2007) or migrated from other peripheral mucosal sites (Kourtis et al., 2003). Moreover, a study by Lee et al. (2005) showed that viruses from the left and right breasts differed in the resistance mutations which they carried suggesting independent evolution and compartmentalization. This difference in drug resistance mutations might be influenced by several factors such as cytokine secretion or pharmacokinetics of a particular drug in individual compartment (Kepler and Perelson, 1998).

In conclusion, compartmentalization of virus populations occurs but is not a dominant phenomenon between plasma and breast milk.

4.2.5 HIV diversity and divergence during viral evolution in plasma and breast milk

Previous studies have reported a lower viral diversity of genital tract variants compared to those from blood plasma (De Pasquale et al., 2003; Ellerbrock et al., 2001). In this study, diversity of the HIV-1 population was significantly less in breast milk and breast milk cells than in plasma (Fig. 17; Table 5). Taken together, these findings indicate the restriction of the pool of HIV-1 quasispecies undergoing active replication within the mammary compartment. In contrast, HIV-1 quasispecies circulating in plasma originate

from virus replication occurring at numerous sites of lymphoid tissues throughout the body (Cohen et al., 1995; Erice et al., 2001).

The viral divergence in plasma originating from six patients at multiple time points post NVP exposure were mostly similar, and showed no significant difference (Table 6). In contrast, there was a significantly divergent virus population in breast milk (1 case, Table 6) and breast milk cells (1 case, Table 6). Such differences could be due to local factors such as cell composition (Becquart et al., 2007), anti-viral substances (Swart et al., 1996) or local specific immunity (Becquart et al., 1999) as well as pharmacokinetics of antiretroviral drug concentration in mammary gland which may differ markedly from the systematic compartment (Bennetto-Hood et al., 2007). Alternatively, this could be due to changes in the cellular and immune environment overtime in maternal milk (Koulinska et al., 2006). Thus, these different factors in individual compartments could have acted as a driving force influencing the viral divergence within such compartments.

4.3 Phenotypic characterization of resistance mutations in CRF02_AG plasmid backbone

Treatment with highly active antiretroviral therapy (HAART) has significantly reduced the rate of mortality and disease progression of HIV-1 infected patients. Selection for HIV-1 variants with decreasing drug susceptibility caused by mutations in the viral protease and reverse transcriptase represents the major reason of treatment failure (Perrin and Telenti, 1998). Therefore, resistance testing is recommended to guide the choice of more efficacious drug regimens after the first or multiple treatment failure (Hirsch et al., 2003) or at the initiation of therapy in drug naïve patients at risk of infection with resistant virus (Little et al., 2002). Current methods for the detection of drug resistance include genotypic and phenotypic assays. Although both methods are of clinical utility, genotypic and phenotypic assays have multiple advantages and disadvantages. Genotypic assays are relatively rapid but difficult to interpret when complex mutational patterns, cross-resistance, or resistance reversal are identified (Gingeras et al., 1996, Hertogs et al., 1998). On the other hand, phenotypic test measure directly the ability of HIV to grow in the presence of each antiviral drug. Thus, phenotypic testing can provide direct evidence of resistance including information on

cross-resistance, multidrug resistance, or resistance reversal (Paolucci et al., 2003; Ross et al., 2001). However, phenotypic assay which used the viruses derived directly from the patient by cocultivation methods were both difficult to perform and time consuming (Japour et al., 1993). The development of the recombinant virus assay (Kellam and Larder, 1994) demonstrated the way for rapid, reproducible, and large scale phenotypic analysis of drug resistance. Recombinant viruses are generated by amplification of patient derived protease-reverse transcriptase (PR-RT) sequences which are then cloned or introduced into a PR-RT-deleted provirus backbone either by homologous recombination (Hertogs et al., 1998) or direct cloning (Garcia-Perez et al., 2007; Petropoulos et al., 2000).

4.3.1 Effect of CRF02_AG plasmid backbone on susceptibility to antiretroviral drugs

Previously, the provirus backbone used for recombinant viral system was constructed based on HIV-1 subtype B (Garcia-Perez et al., 2007; Hertogs et al., 1998; Petropoulos et al., 2000) which dominates in developed countries, although this subtype represents only a small fraction of global HIV-1 infection (Osomanov et al., 2002). The current epidemic of HIV-1 infection in African countries is dominated by non-B subtype (Janssens et al., 2000; Spira et al., 2003). This study outlines the phenotypic aspects of a recombinant viral assay developed by generating a CRF02_AG proviral plasmid backbone (pBD6TB9RI) based on the CRF02_AG subtype which has been previously reported as one of the dominant circulating HIV-1 strains in Burkina Faso (Tebit et al., 2006). CRF02_AG had also been previously reported to have a higher replicative fitness compared to parental subtypes A and G (Njai et al., 2006; Konings et al., 2006). Phenotypic assays using a backbone with similar genetic background may reveal useful information about antiviral drug susceptibility in non-subtype B strains. To investigate the effect of proviral backbone on antiviral drug response, a chimeric virus carrying the PR-RT fragment from subtype B, pNL4-3 was generated. The result showed that similar drug susceptibility profiles to RTIs (Fig. 21; Table 7) between control CRF02_AG virus derived from proviral backbone (pBD6TB9RI), chimeric CRF02_AG/subtype B virus (BD6TB9RINL), and subtype B (NL4-3) virus was observed as reported by Fleury et al. (2006). However, minor differences in drug susceptibility between control CRF02_AG virus, chimera, and subtype B virus were observed for some PIs (Fig. 21; Table 7), i.e. the

control CRF02_AG virus was shown to be more susceptible to RTV and TPV than subtype B virus (NL4-3) without statistical significance (Fig. 21; Table 7). This observation is in agreement with a previous study which reported a higher susceptibility to RTV and TPV in drug naïve patients infected with CRF02_AG than subtype B (Abecasis et al., 2006).

4.3.2 Phenotypic drug susceptibility testing with patient-derived recombinant viruses

In the present study, patient-derived sequences were re-amplified from frozen PCR products previously used for genotypic testing and were then incorporated into the pol-deleted CRF02_AG proviral backbone by direct cloning (Petropoulos et al., 2000). This method is highly efficient and reproducible compared to the relatively inefficient and random process of homologous recombination. Therefore, the recombinant viral system described here could provide both genotypic and phenotypic information from the same patient sample. Moreover, the comparison of the amino acid substitutions between original plasma samples previously used for genotypic assays and corresponding recombinant viruses demonstrated a nearly identical substitution patterns (Table 9). This observation suggests that *in vitro* phenotypic resistance pattern could reflect the resistance pattern of circulating viruses tested by genotypic assay (Hertogs et al., 1998).

Several studies have demonstrated the comparable *in vitro* susceptibility of non-B subtype viruses to most antiretroviral drugs (Shafer et al., 1999; Toni et al., 2002; Weidle et al., 2001). A good correlation was observed between resistance mutations patterns and the drug susceptibility profiles in cases of RT inhibitors in this study as well (Table 8 and 10). In addition, most of the patient-derived recombinant viruses exhibited concordant PI susceptibility profiles and genotype (Table 8 and 10). However, some discordance between drug susceptibility and resistance mutations to some PIs was found in two patient-derived recombinant viruses (BD6TB9RI 120 and BD6TB9RI 41). These two recombinant viruses which harbor major resistance mutations to PIs were susceptible to DRV and TPV (Fig. 22 and 26; Table 8 and 10). Moreover, other two recombinant viruses (BD6TB9RI 228 and BD6TB9RI 273) with no major mutations to PIs were found to be hypersusceptible to TPV (Fig. 24 and 25; Table 8 and 10). Previous studies have shown that the resistance mutations to PI may differ between subtypes (Cane et al., 2001; Caride et al., 2001). Furthermore, some polymorphisms occurring at positions which are

not associated with subtype B resistance or do not present in subtype B, may alter the PI susceptibility in non-B subtypes (Abecasis et al., 2006; Fleury et al., 2006; Kantor and Katzenstein, 2003). Therefore the resistance mutations which are associated with PI resistance in subtype B as well as polymorphisms found in subtype B may not be applicable for some non-B subtypes.

4.3.3 Reproducibility of phenotypic drug susceptibility testing

In order to obtain better sensitivity and reproducibility of this phenotypic assay, the recombinant virus stocks generated from transfection were used to infect TZM target cells in presence or absence of antiviral drugs and viral replication was measured through luciferase activity 48 hours after infection. This strategy is more sensitive than HIV-gag p24 detection and less time is required to perform the recombinant viral assay compared to standard virus isolation from peripheral blood mononuclear cells which typically takes from 1 to 4 weeks (Tebit et al. 2002). This method also avoids selection of minority virus population (Garcia-Perez et al., 2007; Kusumi et al., 1992). Furthermore, the high level of reproducibility obtained from the present study (Table 11) suggests that this assay is able to distinguish minor differences in susceptibility to antiretroviral drugs.

4.4 Future perspectives

Although clonal analysis provides a sensitive means of detecting NVP-resistance variants, other more sensitive methods such as the oligonucleotide ligation assay-OLA (Troyer et al., 2008) and pyrosequencing have been applied (Le et al., 2009). All the paired breast milk and plasma samples used for this study are presently being analyzed by OLA and pyrosequencing to compare these viral quasispecies to those observed by normal sequencing. The point of interest will be the detection of low level K103N and Y181C mutations in plasma, breast milk and breast milk cells. Also, pyrosequencing is being used to analyze the C2-V3 of the envelope gene from these samples. These analyses will provide a clearer picture of the quasispecies population present in these compartments. Screening of minority populations in envelope is important for co-receptor based treatment regimens (CCR5 antagonists) which will definitely be introduced in Africa with time.

The influx of antiretroviral therapy in Africa within the last few years has been substantial. This increase has been met with a rise in prevalence of drug resistance mutations among drug naïve HIV infected individuals. Unfortunately, the rise in treatment has not been equally met by an increase in infrastructure for resistance diagnosis. The future however, does not look completely bleak with the noticeable rise in the number of Sequencers in African countries following scale up in ART. Although genotypic analysis through sequencing will be easier to perform in this set up, the development of phenotypic methods as well as cheaper methods to monitor resistance in Africa is needed. Phenotypic assays most often referred to as recombinant viral assays have been made based mostly on subtype B. By modifying a previously described CRF02_AG infectious molecular clone, we have been able to generate a CRF02_AG based vector which was used to create chimeric viruses by introducing PR and RT amplified directly from patient RNA. This method will be improved in the near future by modifying the CRF02_AG clone to suit the yeast recombination cloning method which is actually more efficient and represents the quasispecies present in a patient's sample. We hope to extend this cloning strategy to include the envelope gene in order to facilitate detection of viral phenotype (CCR5 or CXCR4 using isolates) in patients who would be treated with receptor based regimens such as Enfuvirtide (T-20) or Maraviroc. Adapting the yeast cloning strategy will enable us to test several drug naïve sequences from both CRF02_AG and CRF06_cpx strains for their susceptibility against PR and RT inhibitors. Such studies will help explain the preliminary reports from our previous study which suggested that CRF06_cpx strains develop NRTIs drug resistance mutations especially TAMs faster than CRF02_AG strains. In brief, the CRF02_AG based recombinant assay will be useful in screening different new drugs and also testing for viral fitness.

Appendix 1 List of abbreviations

3TC	Lamivudine
AZT	Zidovudine
AIDS	Acquired Immunodeficiency Syndrome
AMP	ampicillin
bp	base pair
BM	breast milk
BMc	breast milk cells
d4T	Stavudine
ddI	Dideoxyinosine
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTPs	deoxy-nucleoside-triphosphates
ddNTPs	dideoxy-nucleoside-triphosphate
DRV	Darunavir
E. coli	Escherichia coli
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EFV	Efavirenz
env	gene coding viral envelope glycoprotein
ETR	Etravirine
FCS	fetal calf serum
FTC	Emtricitabine
Fig.	figure
gp41	glycoprotein 41
h	hour
HAART	highly active anti-retroviral therapy
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human Immunodeficiency Virus
HR1	heptad repeat 1
HR2	heptad repeat 2
IC_{50}	inhibition constant; the concentration of substance that provides 50% inhibition to certain reaction
IDV	Indinavir
kb	kilo-base pair
LB	Luria-Bertani broth
LPV	Lopinavir

M	molar concentration
min	minute
NRTIs	nucleoside reverse transcriptase inhibitors
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
nt	nucleotide
OD	optical density
p24	alternative designation of the HIV-1 CA protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIs	protease inhibitors
PL	plasma
PMTCT	prevention mother-to-child transmission
pol	gene coding viral enzymes
PR	protease
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute 1640 Medium
RT	reverse transcriptase
RTIs	reverse transcriptase inhibitors
RTV	Ritonavir
SD-NVP	single dose Nevirapine
TAE	Tris-acetate-EDTA buffer
TBS	Tris buffered saline
TDF	Tenofovir
T_m	melting temperature
TPV	Tipranavir
Tris	Tris(hydroxymethyl)aminomethane
u	unit
UV	ultraviolet light
wt	wild-type

Appendix 2 List of plasmids

name	Prepared by	restriction	vector	resistance
pBD6-15 (Tebit et al., 2003)	D. M. Tebit		pCR-XL-TOPO	Kana
pBD6TB9 (unpublished)	D. M. Tebit	ApaI/StuI	pGEM-T-easy	AMP
pBD6TB9RI[a]	K. Sathiandee	EcoRI/StuI	pGEM-T-easy	AMP
pBD6TB9RINL[a]	K. Sathiandee	EcoRI/StuI	pGEM-T-easy	AMP
pLucBD6TB9 (unpublished)	D. M. Tebit	EcoRI/StuI	pGEM-T-easy	AMP
pLucBD6TB9RI (unpublished)	K. Sathiandee	EcoRI/StuI	pGEM-T-easy	AMP
pBD6TB9A (unpublished)	K. Sathiandee	ApaI/StuI	pGEM-T-easy	AMP
All plasmids which contained patients-derived RT fragment (to determine NVP resistance mutation)[a]	K. Sathiandee	EcoRI	pCR2.1-TOPO	Kana

[a] = manuscript in preparation.

Appendix 3 List of oligonucleotides

List of oligonucleotides used for amplification of different parts of HIV-1. The oligonucleotides are listed according to their number in the list of the department of Virology. The sequences are always oriented from 5'- to 3'-end. The presence of restriction sites is underlined.

#	name	sequence	location	used for
[a]	Mlubase1	GGGCCCACGCGTGATGGGTTAATTTACTCCAAGA AAAGACAAGA	4-40 of HXB2	introduction of new *EcoRI* site
[a]	Env5^{+}	TCAGACCTGGAGGAGGAGATATGA	7627-7650 of HXB2	amplify gp41 domain
1013	GP40F1	TCTTAGGAGCAGCAGGAAGCACTATGGG	7789-7816 of HXB2	amplify gp41 domain
1014	GP41R1	AACGACAAAGGTGAGTATCCCTGCCTAA	8347-8375 of HXB2	amplify gp41 domain
1016	GP47R2	TTAAACCTATCAAGCCTCCTACTATCATTA	8265-8294 of HXB2	amplify gp41 domain
1669	RT-rev-StuI	TTTCTGCTACTAGGCCTTTGCTGGGTCATAATAG ACTCCATGTACAGGTTCTTTT	3550-3555 of pBD6	introduce new *EcoRI* site or amplify RT region
1725	RT1-for	AGTAGGACCTACACCTGTCAACATAATTGG	2491-2520 of pBD6	amplify RT region
1726	RT3-for	AATATGTTGACTCAGATTGGTTGTACTTTAAAT T	2525-2558 of pBD6	amplify RT region
1727	RT4-rev	CTTTTAGAATTTCCCTGTTCTCTGCCAATTC	3474-3504 of pBD6	amplify RT region
2118	Pro-for-EcoRI	GAGGGACAAGGAATTCTACCCTCCTTTAGCTTCCC TCAAA	2231-2270 of pBD6	introduce new *EcoRI* restriction site
2119	Pro-rev-EcoRI	GCTAAAGGAGGGTAGAATTCCTTGTCCCTCGGTTC CTGCT	2221-2260 of pBD6	introduce new *EcoRI* site
2190	KnockRI-F	ATGTCACACAAGAATTTGGGATTCCCTACAATCCC CAAAGTC	4641-4682 of pBD6	knock out *EcoRI* site
2191	KnockRI-R	TGGGGATTGTAGGGAATCCCAAATTCTTGTGTGAC ATTTG	4638-4677 of pBD6	knock out *EcoRI* site
2261	7Stu-R-BD6	CTGTATTTCTGCTATTAGGCCTTTGATGGGTCAT AATAC	3500-3539 of pNL4-3	amplify PR/RT region from pNL4-3
2287	7EcoRI-F-BD6	GAGCCGATAGACAAGGAATTCTATCCTTTAGCTTC	2218-2252 of pNL4-3	amplify PR/RT region from pNL4-3

[a] = Tebit 2001

Appendix 4 PCR reaction components

The RT-PCR reaction contained:

25 µl	2x Reaction mix (a buffer contain 0.4 mM of each dNTP, 3.2 mM $MgSO^4$)
2 µl	SuperScript™ III RT/Platinum® Taq mix
1 µl each	forward/reverse -Primer (10 µM)
0.01 pg - 1 ng	RNA template
Sterile distilled water to 50 µl total volume	

The PRC reaction for fragment less than 1 kb contained:

5 µl	10x PCR buffer (1.5 mM $MgCl_2$)
1 µl	dNTP (200 µM)
1 µl	Taq polymerase
1 µl each	forward/reverse -primer (10 µM)
20-200 ng	DNA template
Sterile distilled water to 50 µl total volume	

The PRC reaction for fragment greater than 1 kb contained:

10 µl	5x Expand High Fidelity^{Plus} buffer (1.5 mM $MgCl_2$)
1 µl	dNTP (200 µM)
0.5 µl	Expand High Fidelity^{Plus} Enzyme (2.5 U/reaction)
1 µl each	forward/reverse -primer (10 µM)
2 - 500 ng 100 pg - 10 ng	genomic DNA plasmid DNA
Sterile distilled water to 50 µl total volume	

Appendix 5 Thermal cycling

The following temperature cycling protocol was used for RT-PCR:

step	temperature	duration
1: cDNA synthesis	45-60°C	15-30 min
2: initial denaturation	94°C	2 min
3: denaturation	94°C	15 sec
4: annealing	55 – 65°C*	30 sec
5: elongation	68°C	1 min/kb**
repeat steps (3-5) 39 cycles		
6: final elongation	68°C	5 min

The following temperature cycling protocol was used for short fragment PCR:

step	temperature	duration
1: initial denaturation	94°C	2 min
2: denaturation	94°C	10-30 sec
3: annealing	55 – 68°C*	30 sec
4: elongation	72°C	1 min/kb**
repeat steps (2-4) 25-35 cycles		
5: final elongation	72°C	7 min

The following temperature cycling protocol was used for long fragment PCR:

step	temperature	duration
1: initial denaturation	94°C	2 min
2: denaturation	94°C	10-30 sec
3: annealing	55 – 68°C*	30 sec
4: elongation	68-72°C	1 min/kb**
repeat steps (2-4) 10 cycles		
5: denaturation	94°C	10-30 sec
6: annealing	55 – 68°C*	30 sec
7: elongation	68-72°C	1 min/kb** + 10 sec. cycle elongation for each successive cycle
repeat steps (5-7) 15-25 cycles		
8: final elongation	72°C	7 min

* depending on the melting temperature of the primers

** depending on the product length (~1 kb/min)

Appendix 6 List of antiretroviral drug concentrations

Antiviral drugs	Serial concentration (final concentration)	% DMSO (final concentration)[d]
PIs:		
LPV[a]	0.5, 5, 50, 500 and 1000 nM	0.1% in every dilution
IDV[c]	0.5, 5, 50, 500 and 1000 nM	0.1% in every dilution [d]
RTV[a]	0.5, 5, 50, 500 and 1000 nM	0.1% in every dilution
DRV[a]	0.5, 5, 50, 500 and 1000 nM	0.1% in every dilution
TPV[a]	0.5, 5, 50, 500 and 1000 nM	0.1% in every dilution
NRTIs:		
AZT[b]	0.005, 0.05, 0.5, 5 and 50 uM	0.1% in every dilution [d]
d4T[c]	0.005, 0.05, 0.5, 5 and 50 uM	0.5% in every dilution [d]
3TC[a]	0.05, 0.5, 5, 50 and 500 uM	0.5% in every dilution
ddI[a]	0.05, 0.5, 5, 50 and 500 uM	0.5% in every dilution [d]
FTC[c]	0.005, 0.05, 0.5, 5 and 50 uM	0.5% in every dilution [d]
TDF[c]	0.005, 0.05, 0.5, 5 and 50 uM	0.5% in every dilution [d]
NNRTIs:		
NVP[a]	0.1, 1, 10, 100, 1000 and 10000 nM	0.1% in every dilution
EFV[a]	0.1, 1, 10, 100 and 1000 nM	0.1% in every dilution
ETR[a]	0.1, 1, 10, 100 and 1000 nM	0.1% in every dilution

[a] = stock solution in DMSO; [b] = stock solution in Tris buffer; [c] = stock solution in water
[d] = DMSO was added into drugs which dissolved in Tris and water.

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List of Publications

Journal papers:

Tebit, D.M., Ganame, J., Sathiandee, K., Nagabila, Y., Coulibaly, B., Kraeusslich, H.G., 2006. Diversity of HIV in rural Burkina Faso. J. Acquir. Immune Defic. Syndr. 43 (2): 144-152.

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Tebit, D.M., Sangaré, L., Tiba, F., Saydou, Y., Makamtse, A., Somlare, H., Bado, G., Kouldiaty, B.G., Zabsonre, I., Yameogo, S.L., Sathiandee, K., Drabo, J.Y., Kräusslich, H-G. 2009. Analysis of HIV-1 pol gene diversity and drug resistance associated changes among drug naïve individuals in Burkina Faso. J. Med. Virol. 81(10): 1691-1701.

Manuscripts in preparation:

Sathiandee, K., Tebit, D.M., Tiba, F., Ouedraogo, T., Kräusslich, H-G. HIV-1 pol gene quasispecies and drug resistance in paired plasma and breast milk from Nevirapine-exposed and naïve women.

Sathiandee, K., Tebit, D.M., Tiba, F., Ouedraogo, T., Kräusslich, H-G. Phenotypic characterization of HIV-1 drug resistance mutations with CRF02_AG plasmid backbone.

Poster presentations:

Sathiandee, K., Sarker, M., Tiba, F., Babic, D., Ouedraogo, T., Böhler, T., and Kräusslich, H-G. Viral, immunological and public-health associated factors in transmission and therapy of human immunodeficiency virus infection in Burkina Faso. April 2008. DFG Evaluation, SFB544, Heidelberg, Germany.

Sathiandee, K., Tebit, D.M., Tiba, F., Ouedraogo, T., Kräusslich, H-G. Genetic diversity and phenotypic characterization of HIV-1 Circulating Recombinant Forms (CRFs) among drug-naïve and -exposed patients in Burkina Faso. November 2009. 5th Joint PhD Students Meeting „New Trends in InfectiousDisease Research“, Heidelberg, Germany.