Published in final edited form as:

J Clin Virol. 2018 June ; 103: 81–87. doi:10.1016/j.jcv.2018.02.008.

Next generation sequencing reveals a high frequency of CXCR4 utilizing viruses in HIV-1 chronically infected drug experienced individuals in South Africa

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Abstract

Background—Entry inhibitors, such as Maraviroc, bind to CCR5 inhibiting entry of CCR5 utilizing viruses (R5 viruses). In the course of HIV infection, CXCR4 utilizing viruses (X4 viruses) may emerge and outgrow R5 viruses, and potentially limit the effectiveness of Maraviroc. The use of Maraviroc is reserved for salvage therapy in South Africa.

Objective—In this study, we examined the frequency of R5 and X4 viruses, using next generation sequencing, in patients under treatment to draw inferences on the utility of Maraviroc in a South African population.

Study design—Proviral DNA was isolated from peripheral blood mononuclear cells (PBMC) of 72 chronically HIV infected patients on antiretroviral treatment. HIV V3 loop gene was amplified and sequenced on an Illumina MiniSeq platform. Viral subtypes were determined by the jumping profile Hidden Markov Model (jpHMM) and REGA genotyping tools. De Novo consensus sequences were derived for the majority and minority populations for each patient using Geneious[®] software version 8.1.5. HIV-1 tropism was inferred using PSSMsinsi, Geno2pheno and Phenoseq-C web-based tools.

Results—Quality V3 loop sequences were obtained from 72 patients, with 5 years (range: 0–16) median duration on treatment. Subtypes A1, B and C viruses were identified at frequencies of 4% (3/72), 4% (3/72) and 92% (66/72) respectively. Fifty four percent (39/72) of patients exclusively

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Authors' contributions

POB, DR and MLH conceptualized and designed the study; NDM performed the laboratory analyses, analyzed the data, and prepared the first draft of the manuscript. LRG, DR and MLH designed some of the sequencing protocols and LRG helped perform the initial sequencing experiments. DMT and POB were involved in the interpretation of data and revised the manuscript for intellectual content. All authors approved the final version of the manuscript.

Competing interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the Research Ethics Committee of the University of Venda, South Africa (SMNS/13/MBY/01/0625); and permission to access public sector health facilities was obtained from the Limpopo Provincial Department of Health, South Africa. Informed consent was obtained from all study participants prior to demographic and clinical data collection, and blood draw.

harboured R5 viral quasiespecies; and 21% (15/72) exclusively harbored X4 viral quasiespecies. Twenty five percent of patients (18/72) harbored dual/mixture of R5X4 quasiespecies. Of these 18 patients, about 28% (5/18) harbored the R5+X4, a mixture with a majority R5 and minority X4 viruses, while about 72% (13/18) harbored the R5X4+ mixture with a majority X4 and minority R5 viruses. The proportion of all patients who harbored X4 viruses either exclusively or dual/mixture was 46% (33/72). Thirty-five percent (23/66) of the patients who were of HIV-1 subtype C harboured X4 viruses ($\chi^2 = 3.58$; $p = .058$), and 57% of these (13/23) harbored X4 viruses exclusively. CD4⁺ cell count less than 350 cell/ μ l was associated with the presence of X4 viruses ($\chi^2 = 4.99$; $p = .008$).

Conclusion—The effectiveness of Maraviroc as a component in salvage therapy may be compromised for a significant number of chronically infected patients harboring CXCR4 utilizing viruses.

Keywords

Next generation sequencing; Chronic HIV infection; Co-receptor usage; Maraviroc; South Africa

1. Background

The entry of human immunodeficiency virus type 1 (HIV-1) into target cells is mediated by the virus' envelope glycoproteins. Specifically, gp120 interacts with the CD4 receptor and co-receptors, either CCR5 or CXCR4 [1]. Viruses with the ability to use CCR5 are classified as R5 viruses, those that use CXCR4 are classified as X4 viruses, while those that use both co-receptors are referred to as dual tropic (R5X4 viruses) [2]. R5X4 viruses are further classified as dual-R (R5+X4, a mixture of R5 and X4 viruses with R5 existing as majority and X4 as minority) or dual-X (R5X4+ a mixture of R5 and X4 viruses with X4 existing as majority and R5 as minority) [3–5].

The majority of initial HIV-1 infections involve the use of CCR5, while CXCR4 predominates at late stages of infection, and this has been strongly associated with low CD4⁺ cell counts in approximately 50% of subtype B infections [6–9]. At the late stages of infection co-receptor switch from R5 to X4 has only been reported in subtypes A, C, D, CRF01_AE, and CRF02_AG infections [10–12].

HIV-1 subtype C accounts for over half of HIV-1 infections worldwide and it is the predominant variant circulating in Southern Africa. Studies have demonstrated an overwhelming predominance of R5 viruses at various stages of HIV-1 subtype C infection, although X4 viruses have also been reported [10,13–18]. In vitro studies have also shown that about 30% of HIV-1 subtype C viruses in individuals with advanced disease proficiently use CXCR4 [19,20].

Maraviroc is an entry inhibitor that binds to CCR5 and inhibits gp120-CCR5 interaction through an allosteric mechanism [21]. The United States Food and Drug Administration first approved Maraviroc in 2007 as salvage therapy. Currently, it is also recommended as a component in first-line antiretroviral therapy (ART) in the United States and other developed countries [22], and is also being considered in HIV prophylaxis [23–26].

In settings such as South Africa, genotypic drug resistance testing is recommended following treatment failure with the first and second line regimens. Additionally, adherence and drug interactions are reviewed to guide the change in drug class. In the case where a third line treatment is required, the decision is reserved for an infectious disease specialist, who may recommend Maraviroc as salvage therapy [27]. However, testing for sensitivity to Maraviroc is not routinely done. Phenotypic and genotypic approaches are used in deciphering HIV coreceptor usage. Monogram Trofile, is a phenotypic based assay that requires cell culture procedures and is time and labour intensive [28]. On the contrary, genotypic based algorithms for co-receptor determination are less time consuming and require the use of web-based algorithms such as Subtype C position-specific scoring matrix (PSSMsinsi), Geno2Pheno and Phenoseq-C [29].

2. Objectives

The objective of this study was to use next generation sequencing to generate HIV-1 V3 loop sequences from treatment-experienced individuals; and analyze the quasiespecies for co-receptor usage; in an effort to draw inferences for the subsequent utility of Maraviroc as salvage therapy in South Africa.

3. Study design

3.1. Study population

The study subjects comprised individuals who presented for routine care at the HIV/AIDS Prevention Group Wellness Clinic (HAPG) in Bela-Bela and Donald Fraser Hospital Hope Clinic (DFHC) in Vhufhuli in Limpopo Province of South Africa. These individuals were recruited from July 2013 to December 2015.

3.2. DNA extraction and Polymerase Chain Reaction (PCR) amplification of V3 loop

DNA was extracted from PBMC of 72 HIV infected individuals under antiretroviral therapy, using Qiagen blood mini kit following the manufacturer's instructions. The C2-V3 region of the envelope gp120 was amplified in a nested PCR using standard PCR reagents including Platinum Taq High fidelity (Invitrogen) and 0.2 μ M of each primer. The first round reaction included primer pair; Env-1F (5'-CAGCACAGTACAATGCACACATGGAAT-3') and Env-1R (5'-TGACGCCGCGCCCATAGTGCTTCCTGCTG-3') to yield an 876 bp product. The nested reaction was performed with the primers; EnVCF2 (5'-ATAATGATTAGATCTGAAAA-3') and EnvV4R (5'-TGATGTATTGCAATAGAAAA-3') to yield a 440 bp product that included the V3 loop. The following cycling conditions were used for both first round and nested PCR; Initial denaturation at 94 °C for 2 min, followed by a 39 cycles of denaturation at 94 °C for 20 s, annealing at 53 °C for 45 s, extension at 72 °C for 1 min, final extension at 72 °C for 5 min and storage at 4 °C.

3.3. Library preparation and MiniSeq sequencing

The HIV V3 loop libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina San Diego, California, USA) following the manufacture's instructions with 20%

Phix as control (Illumina San Diego, California, USA). Agarose electrophoresis (E-gels) was used to verify the size of the libraries, and normalized to 4 nM each to ensure equal library representation in the pool. The prepared libraries were sequenced in an Illumina MiniSeq using a high output kit for 300 cycles (Illumina San Diego, California, USA) and generating paired-end 2×150 bp long sequence/ reads for each.

3.4. De-multiplexing and sequence quality control evaluation

Sequences were de-multiplexed automatically on the MiniSeq as part of the data processing steps and ends pairing. Fastq files were generated for each sample representing the two paired-end reads. Sequence quality was validated using the FastQC programme. The fastq files were imported into Geneious® software version 8.1.5 for filtering and trimming of sequences as well as downstream analysis. All sequences were trimmed at the ends as part of the assembly process using the modified-Mott algorithm and quality scores assigned by the sequencing base caller. The two sequence lists from each sample were paired and mapped to HIV-1 subtype C envelope consensus sequence (Gene-Bank accession number: DQ275658) to identify Indels and mapped to HXB2 for subtyping confirmation. Fig. 1 represents the bioinformatics processes employed for viral subtype and co-receptor determination.

3.5. Intra-patient quasispecies and co-receptor prediction

All reads spanning the HIV envelope V3 region which were mapped to the HIV-1 subtype C envelope consensus were extracted as single reads and De Novo assembled using Geneious® assembler and generating contigs for each quasispecies per sample. Minority variants were called based on the variant read depth, variant frequency and consensus base frequency with > 20 –2% cut-offs using the find variation/SNPs feature from Annotation and prediction menu in Geneious® software version 8.1.5. This parameter also calculates the p-value (maximum variant p-values set to 10^{-6} ; 0.0001% to see variant by chance) for the specified variant frequency call, with lower p-values representing a real variant at the given position (<http://www.geneious.com>). All consensus contigs generated were aligned using Geneious® multiple alignments and translated into amino acids. HIV subtype was inferred with the jumping profile Hidden Markov Model (jpHMM) and REGA subtyping tools. Co-receptor tropism was inferred using PSSMsinsi [30], Geno2pheno [co-receptor] (10% false-positive rate) [31] and PhenoSeq-C [32] web based tools and a consensus co-receptor result amongst the 3 tools were used as final output data. The amino acid net charge, which is the number of positively charged (R/K/H) amino acid residues minus number of negatively charged (D/E) residues was derived from the PSSMsinsi web based tool. Association of co-receptor usage and CD4⁺ cell count, viral load and number of years on treatment were determined using Chi-square computation.

3.6. V3 loop amino acid sequence analysis

Envelope V3 loop amino acid sequence variability at each position of R5 and X4 viruses was determined using the Los Alamos HIV Sequence Database Entropy- one tool (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) and WebLogo (<http://weblogo.threeplusone.com/>). The N-glycosylation sites were determined using the Los Alamos HIV sequence Database N-GlycoSite (<http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html>)

4. Results

4.1. Patients' characteristics

Patients' clinical data including viral loads, CD4⁺ cell counts, number of years on treatment, age and treatment regimen are shown in Table 1. The median viral load log₁₀ copies/mL was 3.9 (range, 0.7–5.4); the median CD4⁺ cell/mm³ was 256 (range, 5–1022); median age was 38, (range, 4–72) median duration on treatment was 5 years (range, 0–16). There were 43 patients on first-line antiretroviral therapy, 15 on second-line regimens, 2 have not started treatment, 1 stopped treatment and 11 patients with unknown treatment regimen.

4.2. Frequency and distribution of X4 and R5 viruses

Quality V3 loop sequences were obtained for 72 individuals. Subtypes A1, B and C viruses were identified at frequencies of 4% (3/72), 4% (3/72) and 92% (66/72) respectively. Fifty four percent (39/72) of patients exclusively harbored R5 viral quasispecies; and 21% (15/72) exclusively harbored X4 quasispecies. Twenty five percent of patients (18/72) harboured R5X4 dual/mixture of R5 and X4 quasispecies. Of these 18 patients, 28% (5/18) harbored the R5+X4, a mixture with majority R5 and minority X4 viruses while 72% (13/18) harbored the R5X4+, a mixture with majority X4 and minority R5 viruses. The proportion of all patients who harbored X4 viruses either exclusively or dual/mixture was 46% (33/72) shown in Table 2. Thirty-five percent (23/66) of the patients who were of HIV-1 subtype C harbored X4 viruses ($\chi^2 = 3.58$; $p = .05$), and 57% of these (13/23) harbored X4 viruses exclusively. The X4 viruses were found to be associated with low CD4⁺ cell counts ($\chi^2 = 6.11$, $P = .0134$), and no association was found with the duration on treatment ($\chi^2 = 0.4213$, $P = .81006$). The three algorithms used were compared to each other to validate the most likely co-receptor prediction. PhenoSeq-C was 76% in concordance with Geno2Pheno and 83% with PSSMsinsi. Geno2Pheno was 81.9% in concordance with C-PSSMsinsi.

4.3. V3 loop amino acid sequence diversity

A high level of amino acid variation was observed in X4 than in R5 viruses. Entropy plots were performed to compare the degree of V3 loop amino acid variability between the R5 and X4 viruses. There were higher entropy levels at 29 amino acid positions in X4 viruses compared to 17 amino acid positions in R5 viruses ($\chi^2 = 9.13$, $P = .002$) (Fig. 2).

The V3 loop sequences of R5 viruses had between 34 and 35 amino acids with deletions but no insertions; whereas sequences of X4 viruses were 31–37 amino acids long with insertions and deletions (Fig. 3). The consensus X4 sequence had insertions of Valine, Isoleucine or Threonine and Glycine or Arginine at positions 14 and 15 respectively. As an indicator of X4 viruses, a significantly higher average net charge was observed in X4 than R5 viruses ($\chi^2 = 35.6$, $P < 0.05$) (Fig. 3). All the R5 viruses had conserved N-glycosylation sites (NXT) and loss of this site was associated with X4 viruses ($\chi^2 = 36.02$, $P < 0.05$). The GPGQ crown motif remained conserved in R5 viruses; while in X4 viruses there were changes to GPGX (where X was usually an Arginine or Histidine). In addition, X4 consensus sequences had an insertion of Arginine and Glycine within the crown motif between position 19 and 20 (Fig. 2).

5. Discussion

Maraviroc binds to CCR5 and prevents its usage by the infecting virus. As a result, X4 viruses will not be sensitive to Maraviroc [21]. It has been shown that viruses in primary infection preferably use CCR5, but as disease progresses viruses switch coreceptor to CXCR4. In this study, we investigated the frequency and distribution of R5 and X4 viruses in HIV chronically infected patients under Maraviroc-free first and second line treatment regimens in South Africa. The aim was to predict Maraviroc utility in the study population, but more importantly in those who are under the second line treatment regimen, and for whom the introduction of a Maraviroc-containing regimen may be the next option, as salvage therapy.

The highest frequency of X4 viruses (30%) thus far reported from South Africa was from treatment-experienced patients in KwaZulu Natal [19]. In the current study, X4 viruses were detected at a higher frequency of 46%. It was interesting to observe that in this study 21% of the patients harboured X4 viruses exclusively and have been on treatment for at least one year. Pure X4 viral populations are frequently detected and often restricted to late stages particularly in subtype C [33]. In our study, about 19% of patients harbouring X4 viruses were in the initial stages of the infection (stage 1 or 2). Our observation is similar to that of [34] in which a relatively high prevalence (15.9%) of predicted X4 viruses was documented during primary infection in a retrospective evaluation of a large number of patients enrolled in the PRIMO cohort in France. It is not known whether the X4 strains detected in early stages were transmitted or evidence that coreceptor switching is not restricted to late stages of the infection. In the current study 22% of patients harboured an R5+X4 mixture, with X4 viruses as a minority population, which could easily be missed when population based sequencing, is applied and might outgrow the R5 viruses and impact negatively on the use of Maraviroc. Also of note is that 53.3% (8/15) of those with X4 viruses were on a second line treatment regimen (AZT+3TC+LPV/r or FTC+TDF+LPV/r or TDF+3TC+NVP), and for whom Maraviroc might be the next option. The predicted X4 viruses were statistically associated with a low CD4⁺ cell count (< 350 cells/μl), but not with viral load. Coreceptor switching has been associated with disease progression, treatment, and viral genotype reviewed in [28]. Overall, subtype C viruses appear to have the least propensity to switch from R5 to X4. However, in the current study, approximately 35% (23/66) of the patients infected with subtype C viruses harbored CXCR4 utilizing viruses and 57% (13/23) of these were X4 exclusively. The mechanism underling the emergence of X4 viruses in treatment-experienced patients is not yet clear; and the question remains whether the identification of co-receptor switching and the emergence of drug resistance mutations are not coincidental events [35,36]. On the other hand, there is data showing that the up regulation of CCR5 is associated with HIV infection, and that this is reversed in the course of treatment [37,38]. Treatment may encourage the flourishing of dual tropic viruses, since down regulation of CCR5 can be perceived as an increase in the utility of CXCR4.

Nevertheless, the high frequency of X4 viruses may not be related to drug exposure but an ongoing evolution of HIV-1 subtype C. Studies have reported a high proportion of X4 viruses in treatment naïve individuals [39,17]. Continuous studies of HIV-1 co-receptor tropism on treatment experienced and naïve individuals will be necessary to unravel the

emergence of high frequency of X4 viruses in subtype C infections. This is necessary since C viruses drive the epidemic in regions with the highest burden of infection. Sequence analysis of the V3 region showed that X4 and dual/mixture R5X4 viruses were associated with an increased number of positively charged amino acids, loss of a potential glycosylation site, as well as variable V3 loop amino acid lengths in contrast to R5 viruses. The significance of Arginine and Glycine insertions observed in the crown motif at positions 19 and 20 respectively in X4 viruses needs to be determined.

Concordance among three algorithms was used to predict the most likely co-receptor preferences. Predictions with PhenoSeq-C had an 83% concordance with Geno2Pheno, and 76% concordance with PSSMsinsi. Geno2Pheno had an 81.9% concordance with PSSMsinsi. Previous studies have demonstrated best concordance (> 85%) between Geno2Pheno and C-PSSMsinsi, [40–42]. In this study we have observed the best concordance with Geno2Pheno and PhenoSeq-C. These tools are used to detect nonrandom distribution of amino acid at adjacent sites associated with empirically determined groupings of sequences; the tools may be limited in dealing with mixed bases.

In this study, the application of De Novo assembly approaches with reads spanning the V3 loop resolved the issue of mixed bases in consensus sequences. And to reduce false positive variants calling, all sequences were trimmed at the ends as part of the assembly process using the modified-Mott algorithm and quality scores assigned by the sequencing base caller. This algorithm trims the ends and improves the error rate by more than the error probability limit threshold set, in this case set to 0.001%, and we have considered only those variants with a read depth of at least 100 and present in at least 50 reads. The cut-offs for depth and read length were selected after a manual inspection of test batches of samples and ensured that at least 5 reads are present to call a variant. Minority variants were called based on the variant read depth, variant frequency and consensus base frequency with > 20–2% cut-offs using the find variation/SNPs feature from Annotation and prediction menu in Geneious® software version 8.1.5 (<http://www.geneious.com>).

In the current study, viral DNA was used from peripheral blood mononuclear cells (PBMCs) to enhance the success of PCR amplification since the study subjects were on treatment and some had very low or undetectable viral loads. HIV-1 tropism can be evaluated in plasma or PBMCs. However, only tropism testing of HIV obtained from plasma RNA has been validated as a tool to predict virological response to CCR5 antagonists in clinical trials. The use of proviral DNA from PBMC for co-receptor determination (an approach that still has to be validated) may be a limitation of the current study. However, a number of studies, employing Sanger sequencing, have shown a strong correlation in co-receptor preference prediction between HIV variants obtained from plasma and PBMC [43–46].

Next Generation Sequencing of HIV-1 from plasma has been shown to predict virologic response to Maraviroc as well as enhanced sensitivity in the Trofile phenotypic assay and also sensitive to minority non-R5 variants than population based sequencing [47–49]. In addition, other studies using Sanger and NGS, have shown that viruses from plasma and PBMC compartments can be used to reliably predict virologic success to Maraviroc, although with an apparent improved outcome with viruses obtained from plasma [50,51]. It

is also known that viruses in plasma are ‘replenished’ from archives such as PBMCs. Hence viruses emanating from PBMCs are clinically relevant.

In conclusion, our findings show that a highly significant number of chronically HIV-1 subtype C infected patients under Maraviroc-free treatment harbor CXCR4 utilizing viruses. The data is useful in the consideration of whether to include entry antagonists such as Maraviroc in alternative forms of treatment for patients failing second line treatment regimen in the study setting. The determination of co-receptor usage prior to initiation of therapy consisting of Maraviroc is suggested.

Acknowledgements

The authors are grateful to the study participants, Mr. Jing Huang at the Myles Thaler Center for Retrovirus Research at the University of Virginia, USA for assisting with NGS protocols, and Elizabeth Mashu Etta of the HIV/AIDS & Global Health Research Programme, University of Venda for assisting with sample collection and processing.

Funding

Research reported in this publication was supported by the South African Medical Research Council (RCDI) through funding received from the South African National Treasury; and the South African National Research Foundation (GUN109312, GUN86037) and funds from the Myles H. Thaler Center for AIDS and Human Retrovirus Research at the University of Virginia. The contents are solely the responsibility of the authors and do not necessarily re-present the official views of the South African Medical Research Council, the National Research Foundation or the University of Virginia.

Nontoko D. Matume was supported by the Research Capacity Development Initiative of the Medical Research Council (RCDI project number: 57009), and the Fogarty International Center/NIH (D43TW006578).

Denis M. Tebit was supported by funds from the Myles H. Thaler Center for AIDS and Human Retrovirus Research at the University of Virginia, and also received partial support through a Carnegie African Diaspora Fellowship Award.

David Rekosh was partially supported by funds from the Myles H. Thaler Professorship at the University of Virginia.

Marie-Louise Hammarskjöld was partially supported by funds from the Charles H. Ross Jr. Professorship at the University of Virginia.

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Fig. 1.

Workflow showing the bioinformatics processes for viral subtype and co-receptor determination.

Fig. 2.

Variation within the V3 region of Subtype C R5 and X4 viruses. (2A) Entropy plots representing the variation at each amino acid position site from amino acid position 1 to 37 for R5 and 1 to 39 for X4 viruses. The N-glycosylation site and crown motif are indicated. (2B) Sequence logos of subtype C V3 loop residues of R5 and X4 consensus sequences. The size of each logo represents the frequency of the amino acid at each site. X4 viruses show more variability than R5.

Note (–) Indicates insertion at position 14, 15, 19 and 20.

Fig. 3. Comparison of (3A) amino acid length and (3B) amino acid net charge of HIV-1 subtype C isolates capable of using CCR5 and CXCR4.

Table 1

Characteristics of individuals harbouring R5 or X4 monotropic and those that harbour dual/mixed R5 + X4 and R5X4+ viruses.

Parameter	Biotype				
	Total	X4	R5	R5 + X4	R5X4+
Number of Patients	72	15 (20.8%)	39 (54.2%)	5 (6.9%)	13 (18.1%)
Median viral load Log10 copies/mL (Range)	3.9 (0.7–5.4)	4.1 (0.7–5.2)	3.8 (1–6.3)	3.6 (1.5–4.6)	4.4 (2.5–5.4)
Median CD4 counts cells/mm ³ (Range)	256 (5–1022)	228.5 (13–381)	347 (43–1022)	64 (41–483)	228.5 (5–362)
Median age (Range)	38 (4–72)	36 (8–60)	38 (4–72)	34 (4–55)	39 (13–60)
Median years on treatment (Range)	5 (0–16)	5 (1–8)	6 (0–16)	5 (2–6)	5 (1–11)
Number of patients on first-line Antiretroviral therapy ^a	43 (59.7%)	10 (23.3%)	22 (51.2%)	3 (7%)	8 (18.5%)
Number of patients on Second-line Antiretroviral therapy ^b	15 (20.8%)	4 (26.6%)	7 (46.6%)	–	4 (26.6%)
Unknown treatment regimen	11 (15.2)	2 (18.1%)	6 (54.5%)	2 (18.1%)	1 (9)
Not started treatment	2 (2.7)	–	2 (100)	–	–
Stopped treatment	1 (1.4)	–	–	–	1 (100)

Note: R5+X4 refers to the quasiespecies of R5 and X4 viruses per patient with majority R5 and minority X4 viruses; and R5 × 4+ refers to the quasiespecies with majority X4 and minority R5 viruses. R5 and X4 refer to viruses that exclusively use CCR5 or CXCR4 respectively.

^aFirst-line regimen includes; TDF + FTC + EFV, ABC + 3TC + EFV, D4T + 3TC + EFV, TDF + 3TC + NVP, TDF + TFC + EFV, AZT + DDI + EFV, D4T + 3TC + EFV.

^bSecond-line regimen; FTC + TDF + LPV/r, ABC + 3TC + LPV/r, AZT + 3TC + LPV/r.

Table 2

Frequency of predicted R5, X4, R5 + X4 and R5X4+ viruses in the study subjects.

<u>Exclusively</u>		<u>Dual/Mixed R5 + X4</u>		<u>Dual/Mixed R5X4+</u>	
R5	X4	R5	X4	R5	X4
54% (39/72)	21% (15/72)	72% (13/18)	28% (5/18)	28% (5/18)	72% (13/18)