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Molecular Characterisation and HIV Drug Resistance Patterns of HIV-1 Variants in Plasma and Peripheral Blood Mononuclear Cells Sample Pairs

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Abstract

Objective: Plasma is currently the specimen of choice for routine HIV drug resistance (HIVDR) testing. However, HIV compartmentalization has been well documented in different reservoirs, including peripheral blood mononuclear cells (PBMCs). We molecularly characterized paired plasma and PBMC sample pairs and compared their HIVDR mutation patterns.

Methods: To compare HIVDR mutation patterns in plasma and PBMC sample pairs, whole blood was collected for plasma and PBMC isolation from 43 treatment-naïve and 10 treatment-experienced individuals, and HIVDR profiles determined by sequencing the *pol* gene. Treatment-naïve individuals were initiated on Efavirenz/Emtricitabine/Tenofovir fixed dose combination therapy and follow up HIV-1 viral loads were performed after at least 6 months of treatment.

Results: HIVDR mutation prevalence in the treatment-naïve group was 5.1% in plasma samples compared to 10.3% when both plasma and PBMC sequences were considered. Variable amino acid positions were detected in 21% and 12% of the protease and reverse transcriptase genes, respectively. These were generally not in HIVDR positions and did not form signature patterns. The subset of patients with additional resistance associated sequence variations detected in PBMCs had undetectable HIV-1 viral loads after at least 6 months of anti-retroviral therapy.

Conclusions: The sequencing of paired plasma and PBMC specimens provided additional HIVDR data which were not detected when only plasma samples were tested. However the resistant variants detected in the PBMCs did not seem to negatively affect treatment outcome at 6 months as viral suppression was achieved. These data highlight proviral HIVDR mutations in HIV infected individuals and longer follow up on patients on treatment may be needed to determine their clinical impact.

Keywords: HIV drug resistance; HIV compartmentalization; PBMC reservoir

Introduction

With more than 35 million people currently infected with Human immunodeficiency virus (HIV) worldwide, the AIDS epidemic continues to be one of the greatest challenges of our time. In Sub Saharan Africa, home to more than two thirds of the infected population, the percentage of eligible people who are receiving highly active antiretroviral therapy (HAART) has risen from about 30% in 2009 to above 60% in 2012 (UNAIDS report, 2012). HIV is characterized by high mutation rates and rapid evolution which results in creation of a wide variety of mutants that are present in an individual as viral quasi-species [1,2]. This allows the virus to be highly adaptable to new hosts and selection pressures. The virus is continuously changing to survive in new environments by selecting from this pool of pre-existing variants those variants with better fitness [3]. These variants may play a major role in viral evolution, virulence and evasion of the immune response and also compromise drug and vaccine development efforts. Since the introduction of HAART major successes in the fight against HIV have been achieved. The virus can now be suppressed to levels not detectable by standard viral detection and quantification molecular methods and successful treatment may fully block transmission [4]. However, the virus is only suppressed and not eradicated. Latent but replication-competent HIV genomes which persist in cellular and anatomical reservoirs may lead to treatment failure or viral rebound when treatment is interrupted [5].

Drug resistant variants play a major role in viral persistence and virological failure during HAART. HIV drug resistance testing is therefore a beneficial part of treatment optimization and individualized patient management. The choice of a regimen guided by the baseline resistance profiles would generally lead to improved outcomes compared to the absence of such resistance data and is recommended where feasible [6]. Full-length gene sequencing of the protease (*PR*) and part of the reverse transcriptase (*RT*) genes in the *pol* region of the HIV genome is done to determine whether any relevant mutations are present to provide a complete resistance assessment. Mutations in the genes encoding these enzymes underlie resistance to nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) which are the available drugs in the majority of developing countries. However HIV drug resistance testing is still not routinely carried out

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before treatment initiation and as part of treatment monitoring in developing countries due to limited resources.

Plasma is usually the specimen of choice for HIV resistance testing targeting circulating variants, but in light of virus compartmentalization in different reservoirs, antiretroviral resistance testing of viruses sequenced from plasma may not give a complete picture of an individual's resistance profile. Peripheral blood mononuclear cells (PBMCs) have been described as a latent viral reservoir containing productive HIV DNA [7]. Archived proviral DNA in PBMCs may also retain earlier forms of the virus which may sometimes re-emerge [1]. We characterized HIV variants in plasma and PBMC specimen pairs from treatment-naïve and treatment-experienced individuals and compared them for differences in resistance-associated mutations.

Methods

Participants

Participants were newly diagnosed HIV infected adults who were eligible for HAART initiation based on CD4+ count (<350 cells/μl) and/or World Health Organization (WHO) clinical staging and adults on HAART with symptoms of clinical failure and/or detectable viral load after at least one year on treatment. The participants were sequentially recruited from Tshwane District Hospital HIV clinic and F.F. Ribeiro Clinic in Pretoria Central, South Africa between July 2013 and May 2014 after written informed consent. Treatment-naïve individuals were initiated on a once per day Efavirenz/Emtricitabine/Tenofovir fixed dose combination therapy and follow up HIV-1 viral loads were performed after at least 6 months of treatment. Ethics approval was obtained from the University of Pretoria Faculty of Health Sciences ethics committee (Protocol number 167/2013) and the Gauteng Province Tshwane Health Research Committee (Project number 25/2013).

Sample collection and processing

Plasma was isolated from 10 – 15 ml of whole blood collected in EDTA tubes by centrifugation at 1600g for 10 minutes and stored at -70°C until required for RNA extraction. The volume of plasma isolated from whole blood was replaced with phosphate buffered saline (PBS) and PBMCs isolated on a one layer Ficoll Hypaque gradient. The PBMCs were washed three times in PBS and stored as dry pellets at -70°C until DNA extraction.

HIV genotyping and resistance testing

RNA from plasma and proviral DNA from PBMCs, were extracted using QIAamp Viral RNA and DNA blood Mini kits (Qiagen) respectively as per manufacturer's protocols. Nucleic acid amplification and sequencing were done using previously described methods and primers [8]. Briefly, cDNA was produced from extracted RNA by use of Superscript III reverse transcriptase enzyme (Life Technologies Corporation, California, USA) and polymerase chain reactions (PCRs) were performed to amplify the protease gene and the first 300 codons of the *RT* gene (HXB2 nucleotide 2166 - 3440) using the Platinum Taq enzyme (Life Technologies Corporation, California, USA). PCR amplicons were visualized by ultraviolet illumination and the size validated against appropriate size markers after 1% agarose gel electrophoresis. Amplicons were cleaned and sequenced by BigDye Terminator V3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on a 3100 Automatic capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequencher V 4.5 (Gene Codes Corporation, USA) was used to edit and form contiguous sequences and the sequences were then submitted to the Stanford website (http://hivdb.stanford.edu/) for subtyping, resistance mutations interpretation and quality assessment using the Stanford HIVdb algorithm Version 7.0 [9]. Additional subtyping tools that were used included REGA V. 3 [10] (http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html) and SCUEL [11] (http://www.datamonkey.org/dataupload_scueal.php). NCBI Basic Local Alignment Search Tool (Blast) analysis was used to confirm subtyping results (blast.ncbi.nlm.nih.gov/). For recombinant variants identification and analysis, bootscan analysis plots were constructed using REGA V. 3 [10] and s-distance similarity plots were constructed using the Recombinant Identification Program (RIP) V. 3 (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html).

Phylogenetic analysis

Reference sequences were obtained from the Los Alamos National Laboratory (LANL) database (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Alignment was done using Clustal W [12] (Larkin et al., 2007) in BioEdit Sequence Alignment editor software (Tom Hall, North Carolina State University) and manually edited using BioEdit Sequence Alignment Editor. Maximum likelihood phylogenies were applied to determine evolutionary patterns and divergence rates using Mega 6 [13] with 1000 bootstrap values.

Signature pattern analysis

Viral Epidemiology Signature Pattern Analysis (VESPA) was used to search for PBMC associated signature patterns in the *pol* gene by determining the most common amino acid in each position compared to plasma derived HIV variants (www.hiv.lanl.gov/content/sequence/VESPA/vespa.html). Analysis was done for amino acids in positions 1 – 99 in the *PR* gene and positions 1 – 267 in the *RT* gene.

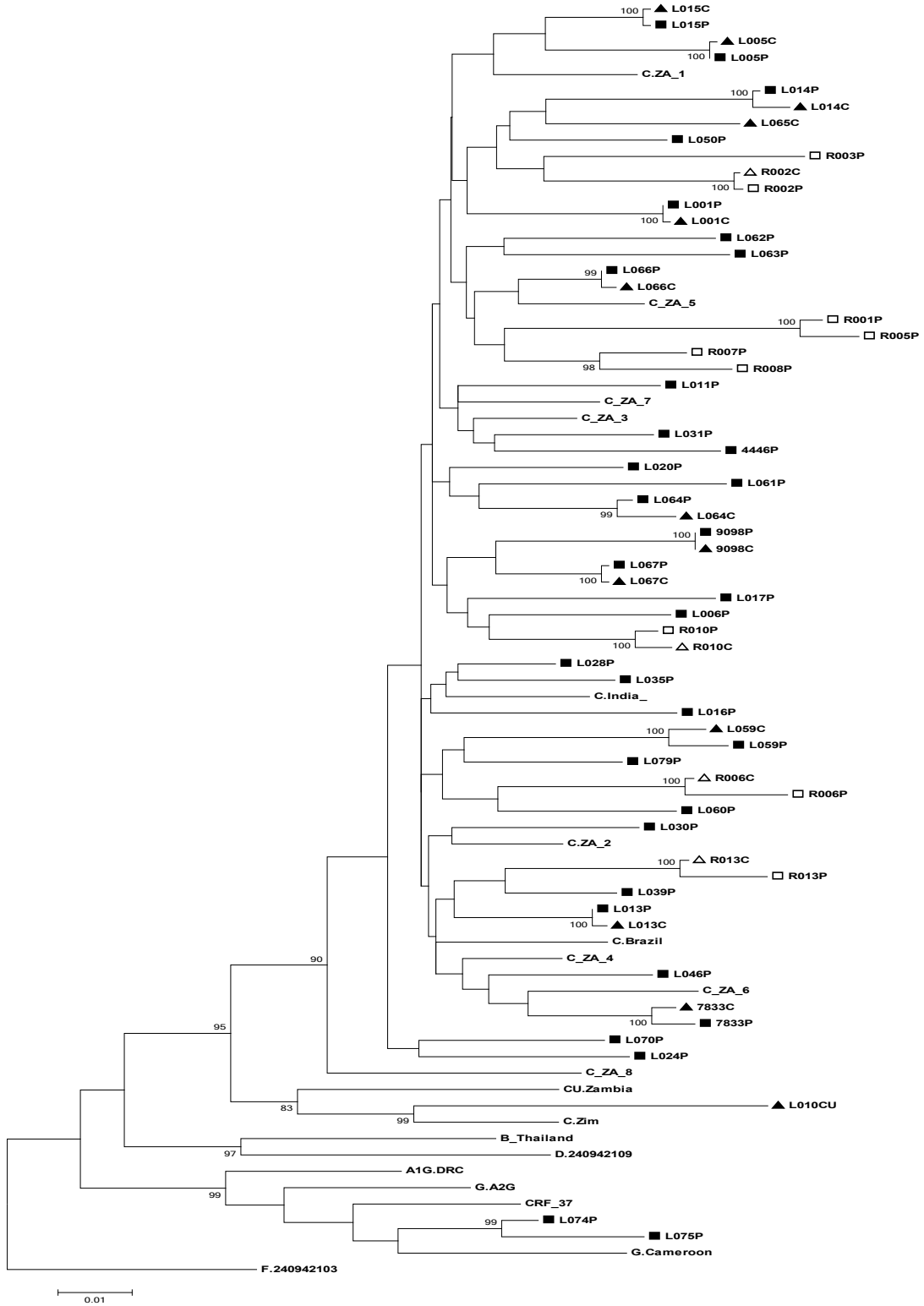
Results

Participant characteristics

A total of 53 participants were tested including 38 females and 11 males. Four additional sequences from samples obtained from the South African National Blood Service were included. The mean age of participants was 34 years (range 23 – 63 years). Thirty nine participants were South African residents with no travel or stay history outside South Africa while 10 participants had travel or stay history in Botswana (1), Congo Brazzaville (1), Democratic Republic of Congo (1), Malawi (2), Mozambique (1) and Zimbabwe (4). There were 43 treatment naïve and 10 treatment experienced participants. Treatment naïve participants had a mean CD4 count of 210 (range 9 – 502).

Phylogenetic analysis

The sequences obtained are presented on the neighbor-joining phylogenetic tree in Figure 1 with bootstrap values of 70% and above indicated. Plasma and PBMC sequences from the same patient clustered together as expected. Fifty patients (94%) were infected with HIV-1 subtype C strains, two were infected with circulating recombinant form 37 complex (CRF 37_cpx) and there was one unclassified subtype C related recombinant. The two sequences classified as CRF 37_cpx subtype came from epidemiologically and sexually related individuals and phylogenetically clustered. As shown in Table 1 both sequences were classified as CRF 37_cpx on the REGA platform but L074 was classified as PR: CRF01_AE, RT: G while L075 was classified as CRF02_AG on the Stanford subtyping platform.



Phylogenetic analysis by Maximum Likelihood method showing sequences from plasma (shaded rectangles = treatment naïve, unshaded rectangles = treatment experienced), PBMCs (shaded rectangles = treatment naïve, unshaded triangles = treatment experienced) and reference sequences. Only sequences covering the whole protease gene and the first 260 amino acids after alignment were used. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 78 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1007 positions in the final dataset. The tree is rooted with HIV-1 subtype F reference sequence from the Los Alamos HIV Database as an out-group.

Figure 1: Phylogenetic analysis by Maximum Likelihood method showing sequences from plasma and PBMCs.

ID	Stanford HIV subtyping	Rega V.3	SCUEL	Blast search/ Phylogenetic analysis	Most likely assignment
L010	PR: B, RT: C	Not classified	U	Subtype C recombinant	Unclassified
L074	CRF02_AG	CRF37_cpx	CRF37_cpx	AG recombinant	CRF37_cpx
L075	PR: CRF01_AE, RT: G	CRF37_cpx	complex	AG recombinant	CRF37_cpx

Table 1: Subtyping results of detected recombinant forms using the HIV-1 pol region.

ID	Gender	Age	CD4 count	Genotype	Resistance associated mutations			
					Plasma PR	PBMC PR	Plasma RT	PBMC RT
L001	M	48	309	C	None Detected	None Detected	None Detected	K219Q
L006	F	26	9	C	T74S	None Detected	None Detected	None Detected
L010	M	54	350	Unclassified	None Detected	None Detected	K103N	M41L K65R V751 F116Y Q151M V90I
L016	F	27	345	C	None Detected	None Detected	None Detected	K103N
L017	F	33	95	C	K20M	None Detected	None Detected	None Detected
L024	M	32	25	C	K20M	None Detected	None Detected	None Detected
L063	F	43	194	C	None Detected	None Detected	K103N	None Detected
L067	F	27	257	C	T74S	T74S	None Detected	None Detected

Table 2: Resistance testing of plasma and PBMC specimen pairs from treatment-naïve participants harbouring drug resistance associated variants.

PR and RT mutations in plasma and PBMC sample pairs in treatment-naïve individuals

Paired plasma and PBMC samples from 43 treatment-naïve individuals were tested for HIV drug resistance associated mutations in the *PR* gene and the first 300 codons of the *RT* gene. Clear readable sequence data were obtained for 39/43 (91%) plasma samples compared to 22/43 (51%) PBMC samples. Table 2 shows participants in which drug resistance associated variants were detected in plasma, PBMCs or both. In the RT gene, HIV drug resistance associated mutations prevalence was 5.1% in plasma samples compared to 10.3% when both plasma and PBMC derived sequences were considered despite the low sequencing success rate in PBMC samples. Of the participants with resistance associated mutations, participant L010 was the only one with at least one drug resistance associated mutation in both plasma and PBMC but the mutation profile was discordant (Table 2). There were two participants, L001 and L016, with resistance associated mutations in PBMCs but with no detectable resistance associated mutations in plasma and one participant, L063, with resistance associated mutations detected in plasma only. The K103N mutation which causes high level resistance to first generation NNRTIs Nevirapine (NVP) and Efavirenz (EFV) was the most prevalent, appearing in 3 participants and also associated with the two participants with plasma derived HIV mutations.

No variants with major drug resistance mutations were identified in the *PR* gene but some *PR* inhibitor associated minor mutations K20M and T74S were detected in 4 patients shown in Table 2. Additionally, common polymorphic mutations V82I and L89M not selected by PIs were encountered in up to 56% of samples and PI inhibitor accessory mutations K20R, T74P and L10M were detected in 22% of samples. All the PI inhibitor associated mutations detected are unlikely to be due to PI exposure nor would they cause any resistance by themselves.

All participants with plasma and/or PBMC resistance associated mutations at baseline had undetectable viral loads (below 50 copies/ml) after at least six months of HAART except for participant L010 who had a viral load of 147 copies/ml. Patient L001 who had a thymidine analogue mutation-2 (TAM-2), K219Q in the PBMC only, also achieved a full VL suppression at 6 months on a Tenofovir-containing regimen.

PR and RT mutations in plasma and PBMC sample pairs in treatment experienced individuals

Paired plasma and PBMCs from 10 individuals with virologic failure were also tested for HIV resistance associated mutations in the PR gene and the first 300 codons of the RT gene. PBMC amplification and sequencing was less successful in this group (30%), compared to the treatment-naïve group (51%) while 80% of plasma samples were successfully amplified and sequenced. Of the 3 successfully sequenced PBMC derived variants two sequences, R002 and R013, had resistance associated mutations and no resistance associated mutations were detected from one sequence, R006 (Table 3).The resistance associated mutation profile in PBMC and plasma was the same in R002 but was discordant in the other 2 participants. The M184V mutation, which causes high level resistance to NRTIs lamivudine (3TC) and FTC was the most prevalent mutation in this group appearing in all cases including plasma and PBMC derived variants, followed by the A98G mutation (57%) and K103N and H221Y both with 43%. The A98G mutation causes resistance to NVP and EFV while the H221Y mutation is an accessory mutation in patients receiving NVP. All treatment-experienced participants had resistance-associated mutations to both NRTIs and NNRTIs.

Resistance mutations discordance between plasma and PBMC sample pairs

Intra-host resistant spectra differences in treatment naïve and treatment experienced individuals were compared for 17 individuals with available sequences for both plasma and PBMCs including 14 from treatment-naïve individuals and 3 from treatment-experienced individuals. Resistance spectra differences between plasma and PBMCs in the RT gene were detected in 6 (35%) of these individuals. These participants are shown in Tables 2 and 3 as L001, L010, L016, L063, R002 and R006. In the Protease gene, there were 5 (29.4%) individuals (L006, L017, L024, R002, and R013) with differences in their resistance spectra between plasma and PBMCs although as previously mentioned

ID	Gender	Age	Plasma viral load (copies/ml)	CD4 count	Treatment	Genotype	Resistance associated mutations				
							Plasma PR	PBMC PR	Plasma RT		PBMC RT
									NRTI	NNRTI	
R001	F	36	15843	304	AZT 3TC NVP	C	L10I	Could not amplify	M41L V75I M184V T215Y	A98G I132L H221Y	Could not amplify
R002	M	31	69606	270	TDF 3TC EFV	C	L10V	L10V T74S	M184V	L100IV K103N	M184V L100I K103N
R003	M	34	5987	105	TDF 3TC LPV/r	C	T74S	Could not amplify	K70E M184V	K103N V108I K238T	Could not amplify
R005	F	29	13723	168	TDF 3TC EFV	C	L10I	Could not amplify	M41L D67N V75I M184V T215Y	A98G Y181C H221Y	Could not amplify
R006	F	31	140875	65	TDF 3TC NVP	C	None detected	None detected	M184V K101H	Y181C G190A	None detected
R007	F	35	109	452	TDF 3TC NVP	C	T74S	Could not amplify	M184V	A98G K103N	Could not amplify
R008	M	44	9387	8	TDF 3TC EFV	C	T74S	Could not amplify	K70E L74V M184V	A98G L100I K103N H221Y	Could not amplify
R013	F	27	1800	438	D4T 3TC NVP	C	I54V L89V	None detected	M184V	K103S G190A	M184V

Table 3: Resistance testing of plasma and PBMC specimen pairs from treatment-experienced individuals.

these were minor PR inhibitor associated resistance mutations not selected by PI exposure.

Comparison of plasma and PBMC sample pairs for signature patterns

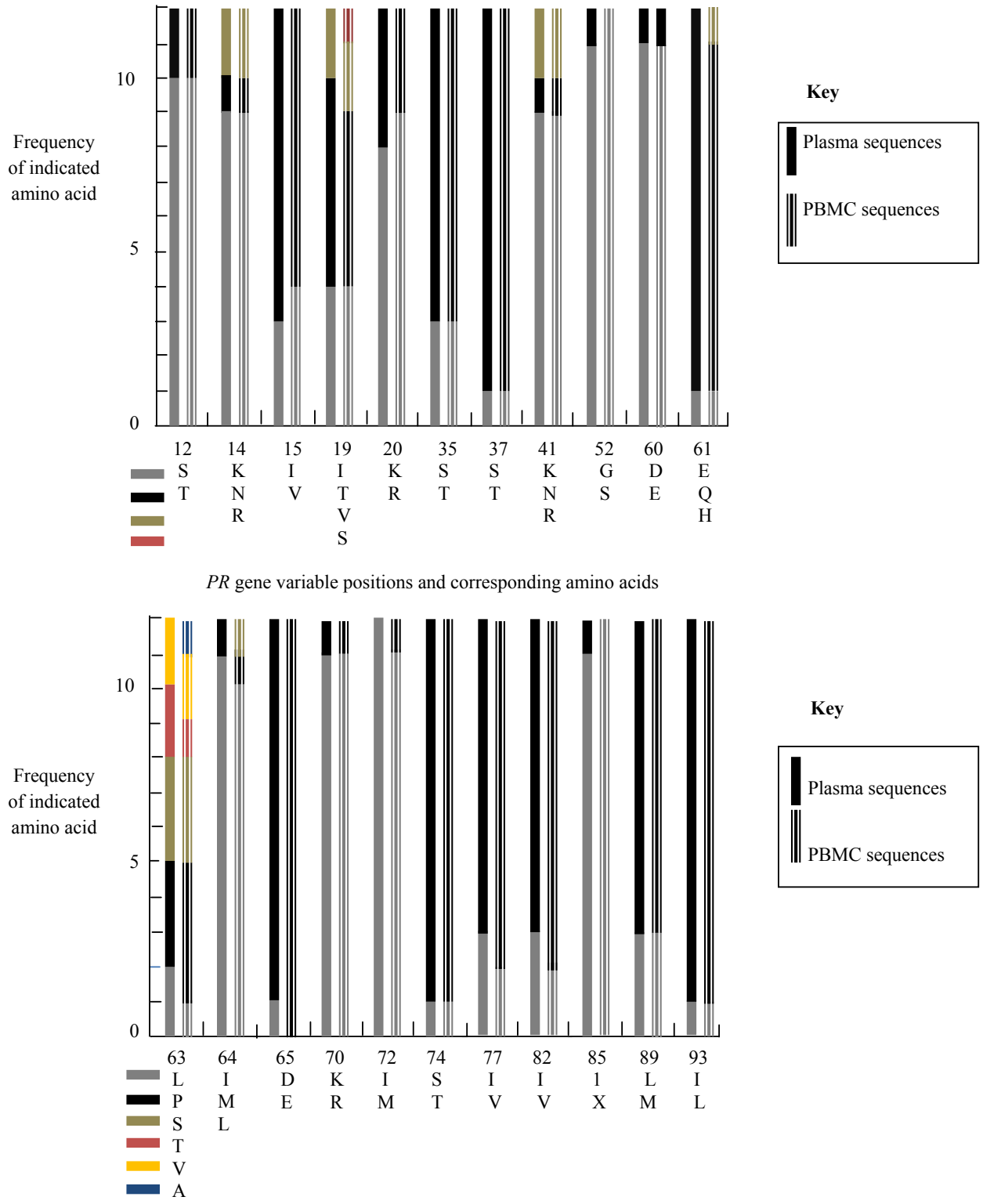
Signature pattern analysis on 12 PBMC and 12 plasma sequences done using VESPA showed no differences between the most common amino acid at each position for the PBMC and plasma sequences studied. A threshold of 70% was considered significant to be an amino acid signature. Although signature markers in PBMCs when compared to plasma were not identified, numerous single nucleotide polymorphisms (SNPs) were detected. Variable amino acid positions in the *PR* and *RT* genes are shown in Figures 2 and 3 respectively. We found 21% of amino acid positions in the PR gene to be variable and 12% of amino acid positions in the RT gene were variable.

Discussion

We compared resistance mutations from plasma and PBMC specimen pairs from treatment-naïve and treatment-experienced individuals from South Africa and also analysed the sequences for signature patterns. Although the 5.1% prevalence figure of plasma HIV drug resistance associated mutations in treatment naïve individuals that we obtained is consistent with figures that have been reported in the region [14, 15] the figure was much higher (10.3%) when drug resistance associated mutations from PBMCs were included. Transmitted drug resistance figures may increase in the near future following the increased roll out of HAART but resistance testing is still not being routinely carried out as part of treatment initiation and monitoring in developing countries because of the associated costs. Prevalence figures of up to 9.3% resistance-associated mutations in *pol* sequences from treatment naïve individuals analysed in a South African study have been reported [16].

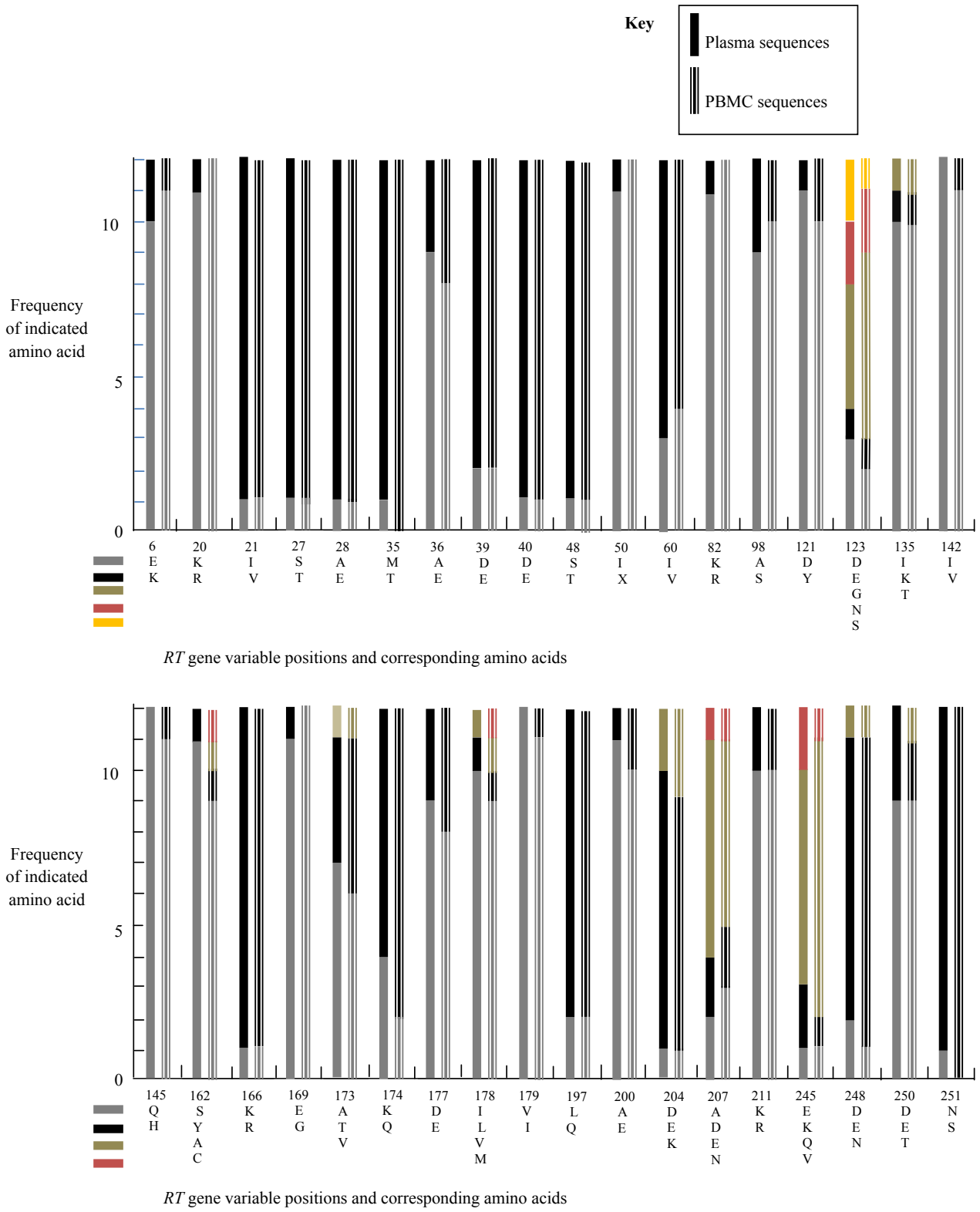
HIV-1 subtype C, which is the dominant subtype in South Africa, was the most prevalent subtype (94%). Other subtypes that were identified were HIV-1 subtype CRF37_cpx and an unclassified subtype C related recombinant form. The CRF 37_cpx subtype has been characterized as a complex strain incorporating subtypes A and G gene segments and in addition has CRF01_AE and CRF02_AG gene segments [17]. Although the recombinants could not be uniformly assigned by the platforms used, all the platforms used were in agreement on detection of the recombination. The HIV-1 CRF 37_cpx subtype strains were detected in individuals from the central African countries of Congo Brazzaville and the Democratic republic of Congo respectively and have not previously been reported in South Africa.

The Sanger sequencing method is still the gold standard sequencing technique but is typically limited to the detection of mutants that have greater than 20% prevalence in clinical samples [18,19] and will miss the minority variants in plasma. These minority variants may still be detectable in PBMCs which can harbour earlier variants but their clinical relevance on treatment outcomes is still unclear as some studies have demonstrated that resistance associated minority variants contributed towards therapy failure [19-23] while others have not found a strong association of minor variants with clinical responses [24,25]. In our study the resistant variants detected in proviral DNA did not seem to negatively affect the treatment outcome as viral suppression was achieved after 6 months of treatment. This could be due to the potent regimen that was used, or that the proviral variants are defective and would not be able to be reactivated to contribute a future circulating pool of drug-resistant virus. A patient with a single TAM-2 K219Q also achieved full viral suppression. Detection of other single TAMs such as M41L at baseline has been shown to have no influence on virological outcome on Tenofovir-based regimens [26] while multiple TAM-1 mutations may affect response to Tenofovir [27,28]. The resistance associated variants in our group which were dominated by the K103N



Variable regions in the *PR* gene for PBMC sequences compared to plasma sequences. VESPA analysis showed no differences between the most common amino acid at each position for the PBMC sequences compared to plasma sequences. There were 19 variable positions (19% of amino acid positions) in PBMC sequences compared to 21 positions (21% of amino acid positions) that were variable in plasma sequences.

Figure 2: Comparison of the PR gene general variability in plasma and PBMC derived HIV variants.



Variable regions in the *RT* gene for PBMC sequences compared to plasma sequences. VESPA analysis showed no differences between the most common amino acid at each position for the PBMC sequences compared to plasma sequences. There were 30 variable positions (11% of amino acid positions) in PBMC sequences compared to 33 positions (12% of amino acid positions) that were variable in plasma sequences.

Figure 3: Comparison of the RT gene general variability in plasma and PBMC derived HIV variants.

mutation were susceptible to 2 out of the 3 drugs that were used at treatment initiation. The activity of these two drugs, Emtricitabine and Tenofovir, could have led to viral suppression. Sequencing of PBMC variants may also provide information on intra-host virus evolution over the near past and in conjunction with plasma variants detect the emergence of new drug resistant variants in the virus quasi-species population.

There was low PBMC amplification success rate in both treatment-naïve and treatment-experienced patients probably due to the low starting volume of whole blood (10-15 ml) used for PBMC isolation and low CD4 T cell count. Other PBMC isolation methods have resulted in comparable or higher PBMC amplification and sequencing rate when compared to plasma and these methods include using cell separation tubes [29], collecting blood in citrated tubes [30] and extracting proviral DNA from quantitated PBMC cells [31].

The *pol* gene is a conserved region of HIV-1 and this was evident in our data as shown by the low intra-host diversity between plasma and PBMC sequences. However there is still some considerable inter-subtype diversity in this gene region. In the *PR* region the 19% - 21% variable amino acid positions that we detected are comparable to the 26 out of the 99 protease amino acid positions that have been reported [32]. These polymorphic positions included positions 20, 63, 82, 85 and 93 which characterize secondary protease mutations in subtype B strains [33]. Position 82 appears on the PR Inhibitor Surveillance Drug Resistance Mutation list [34] but the mutation in our sequences, V82I, is not indicated on the drug resistance associated mutations list. The 11% - 12% variable amino acid positions in the *RT* gene in our data were much lower compared to the reported 23% polymorphic positions in the first 240 *RT* amino acids [32] and are not associated with resistance mutations except for position 179. This position appears on the Non-Nucleoside RT Inhibitor Surveillance Drug Resistance Mutation list [34] but the mutation in our sequence V179I is not indicated as a drug resistance associated mutation.

The limitations of this study include the small sample size, lower amplification and sequencing success observed in PBMCs, and that Sanger sequencing only detects variants with a prevalence of 20% or more. Nonetheless, this study has shown that the sequencing of paired plasma and PBMCs provides additional resistance-associated mutations data which were not detected when only plasma samples are sequenced. We observed a higher prevalence of resistance-associated mutations in treatment-naïve individuals when data from both plasma and PBMCs were considered. These data highlight the presence of proviral HIVDR mutations in HIV infected individuals and longer follow up of patients on treatment may be needed to determine their clinical impact if any, as these variants did not seem to negatively affect treatment outcome at 6 months as viral suppression was achieved. The drug resistance mutations discordance shown between treatment naïve plasma and PBMC sequences may be an indication of inactivity of some of these variants in the short term and in the absence of drug pressure which would otherwise be detected in plasma. To establish what effect resistance-associated HIV variants in proviral DNA might have on treatment outcomes, a bigger sample size as well as more extensive follow up of patients on different types of treatment regimens would be required. The data further showed that differences in resistance spectra may exist between plasma and cellular reservoirs within an individual, highlighting that circulating virus in plasma and provirus in cellular DNA represent distinct compartments that may contribute to the pool of HIV-1 variants that may ultimately emerge in the context of treatment failure.

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