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# Comparing Peripheral Blood Mononuclear Cell DNA and Circulating Plasma viral RNA *pol* Genotypes of Subtype C HIV-1

**Lauren Banks[1], Sharareh Gholamin[1]*, Elizabeth White[1], Lynn Zijenah[2] and David A. Katzenstein[1]**

[1]*Center for AIDS Research, Stanford University Medical Center, Stanford, CA, USA*
[2]*Department of Immunology, University of Zimbabwe, Harare, Zimbabwe*

**Abstract**

**Introduction:** Drug resistance mutations (DRM) in viral RNA are important in defining treatment most likely to provide effective antiretroviral therapy (ART) in HIV-1 infected patients. Detection of DRM in peripheral blood mononuclear cell (PBMC) DNA is another source of information, although the clinical significance of DRMs in proviral DNA is less clear.

**Materials and Methods:** From 25 patients receiving ART at a center in Zimbabwe, 32 blood samples were collected. Dideoxy-sequencing of *gag-pol* identified subtype and resistance mutations from plasma viral RNA and proviral DNA. Drug resistance was estimated using the calibrated population resistance tool on www.hivdb.stanford.edu database. Numerical resistance scores were calculated for all antiretroviral drugs and for the subjects' reported regimen. Phylogenetic analysis as maximum likelihood was performed to determine the evolutionary distance between sequences.

**Results:** Of the 25 patients, 4 patients (2 of which had given 2 blood samples) were not known to be on ART (NA) and had exclusively wild-type virus, 17 had received Protease inhibitors (PI), 18, non-nucleoside reverse transcriptase inhibitors (NNRTI) and 19, two or more nucleoside reverse transcriptase inhibitors (NRTI). Of the 17 with history of PI, 10 had PI mutations, 5 had minor differences between mutations in RNA and DNA. Eighteen samples had NNRTI mutations, six of which demonstrated some discordance between DNA and RNA mutations. Although NRTI resistance mutations were frequently different between analyses, mutations resulted in very similar estimated phenotypes as measured by resistance scores. The numerical resistance scores from RNA and DNA for PIs differed between 2/10, for NNRTIs between 8/18, and for NRTIs between 17/32 pairs. When calculated resistance scores were collapsed, 3 pairs showed discordance between RNA and DNA for at least one PI, 6 were discordant for at least one NNRTI and 11 for at least one NRTI. Regarding phylogenetic evolutionary analysis, all RNA and DNA sequence pairs clustered closely in a maximum likelihood tree.

**Conclusion:** PBMC DNA could be useful for testing drug resistance in conjunction with plasma RNA where the results of each yielded complementary information about drug resistance. Identification of DRM, archived in proviral DNA, could be used to provide for sustainable public health surveillance among subtype C infected patients.

**Keywords:** AIDS; Peripheral blood mononuclear cell; Viral RNA; *pol* sequence; HIV-1 subtype C; Antiretroviral therapy

## Introduction

Drug resistance is a current concern in the provision of effective antiretroviral therapy (ART) for human immunodeficiency virus type-1 (HIV-1) infection [1,2]. The International AIDS Society-USA, the US Department of Health and Human Services, and European guidelines recommend antiretroviral drug resistance testing for those with newly diagnosed HIV infection and treatment failure [1]. Providing drug resistance test results can improve virologic responses through the selection of more effective ART regimens [3-5].

Detection of drug resistance mutations (DRM) and assessment of genotypic susceptibility is dependent on isolation, reverse transcription, amplification and sequencing of Plasma viral RNA (vRNA). An alternate source for detecting DRM is sequencing peripheral blood mononuclear cell (PMBC) DNA [6,7]. Retrieving HIV resistance data from PBMCs may be easier and less expensive, but the clinical significance of DRM in proviral DNA is less clear. The turnover rate of detectable DRMs in cell-associated proviral DNA and vRNA are not the same; DRM may be detected earlier in vRNA as drug resistance develops during treatment. However, detectable DRM in vRNA may be lost as circulating HIV is replaced by wild type, susceptible viruses when drugs are discontinued. In contrast, despite discontinuation or change in regimen, archived DRM in proviral DNA may be retained for 2 years or more [7,8]. Thus the appearance of DRM in PBMCs may be delayed, relative to vRNA, but once drug resistance is established, mutations are retained in archival form in PBMCs for months to years even in the absence of drug pressure [9].

Some studies have found that vRNA demonstrates more DRM compared to PBMC DNA [6,10,11]; some have found specific DRM in PBMC DNA that were not detected in vRNA [12-15] with questions regarding their clinical significance; and some studies have observed no significant difference in DRM between the two sources [16]. Most of these studies have suggested the use of PBMC DNA with vRNA sequencing to increase the sensitivity of drug resistance testing while

***Corresponding author:** Sharareh Gholamin, MD, Center for AIDS Research, Stanford University Medical Center, 300 Pasteur Drive, Room S-146 Grant Bldg, Stanford, CA 94305, USA, Tel: 650-723-8291; Fax: 650-725-2395; E-mail: sharar@stanford.edu

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two studies have suggested PBMC DNA as an alternate to vRNA at lower viral loads [6] or if plasma samples are not available [16]. Thus PBMC sequences may be particularly important as a diagnostic tool in patients on ART with undetectable virus load to plan drug switches for intolerance or toxicity.

More than half of the HIV infections globally are due to subtype C virus, and more than 3 million individuals with subtype C are receiving ART. We appraised the occurrence of resistance in subtype C HIV-1 infected patients with previous extensive ART in vRNA and PBMC to elucidate the differences in resistance profile assessed through sequencing of paired plasma RNA and PBMC DNA from clinical samples.

## Materials and Methods

From 25 patients receiving ART at The Center in Harare, Zimbabwe, in 2001, 2002 and 2004, 32 blood samples were collected. Some patients gave more than one sample. Citrated blood samples were separated and the plasma and cells frozen at -70° C within 6 hours of phlebotomy at the University of Zimbabwe. Citrate tubes of whole blood were separated at the University of Zimbabwe and cells and plasma frozen within 6 hours of collection. Duplicate citrate blood tubes were transported at room temperature from Zimbabwe to Stanford. Upon arrival at Stanford, plasma and PBMC were separated by Ficoll hypaque centrifugation and frozen at -70°C within 48 hours of phlebotomy. Sequencing was equally successful on cells and plasma frozen within 6 hours and 48 hours.

For PBMC Genotyping DNA was extracted from pelleted PBMCs using QIAmp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany). Frozen pelleted cells were re-suspended in 200µl of PBS after thawing. Twenty microliters of Protease K was added to the re-suspended cells along with 200µl of lysis buffer followed by 200µl of 100% ethanol. Lysate was then incubated at 56°C for 10 minutes before being applied to the provided column from the kit. DNA was eluted with 200µl of elution buffer solution. The extraction, isolation, reverse transcription and sequencing of vRNA was performed from thawed plasma as previously described [17].

For both vRNA and PBMC DNA the last 300 base pairs of gag and 760 base pairs of the *pol* gene was amplified using two rounds of PCR with Platinum Taq polymerase. Primers used were at a concentration of 500nM. The thermocycler parameters for the first round were as follows: 95°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, 55°C for 20 seconds, and 72°C for 2 minutes, concluding with one cycle of 72°C for 10 minutes. The thermocycler parameters for the second round PCR were the same as the first round. Five microliters

| Sample code | Sex | Age | CD4 | RNA log | PI treatment history | NNRTI treatment history | NRTI treatment history |
|---|---|---|---|---|---|---|---|
| TC002[a] | Female | 36 | 9 | 5.07 | Saquinavir | - | Lamivudine, Stavudine |
| TC106[a] | Female | 36 | 81 | 3.94 | Saquinavir | - | Lamivudine, Stavudine |
| TC006[b] | Male | 44 | 441 | 2.61 | - | - | Didanosine |
| TC050[b] | Male | 44 | 335 | 2.68 | - | - | Didanosine |
| TC008[c] | Male | 36 | 34 | 3.15 | Saquinavir | - | Lamivudine, Stavudine |
| TC118[c] | Male | 36 | 81 | <2.6 | | Efavirenz | Lamivudine, Stavudine |
| TC215[c] | Male | 36 | 167 | 4.04 | | Nevirapine | Lamivudine, Stavudine |
| TC045[d] | Male | 35 | 155 | 4.19 | Nelfinavir | - | Lamivudine, Zidovudine |
| TC201[d] | Male | 35 | 54 | 5.50 | | Nevirapine | Lamivudine, Stavudine |
| TC114[e] | Male | 32 | 264 | 5.32 | NA | NA | NA |
| TC200[e] | Male | 32 | 249 | 5.99 | NA | NA | NA |
| TC117[f] | Female | 37 | 22 | 5.21 | NA | NA | NA |
| TC242[f] | Female | 37 | 245 | 4.01 | NA | NA | NA |
| TC003 | Male | 46 | 81 | 5.02 | Indinavir | - | Lamivudine, Stavudine |
| TC012 | Female | 36 | 244 | 5.42 | Indinavir | Nevirapine | Lamivudine, Stavudine |
| TC013 | Male | 61 | 3 | 2.58 | Nelfinavir | - | Zidovudine/lamivudine |
| TC041 | Female | 16 | 75 | 2.88 | - | Nevirapine | Zidovudine/lamivudine |
| TC049 | Male | 43 | 15 | 5.07 | Indinavir | Nevirapine | Didanosine, Stavudine |
| TC052 | Female | 42 | 33 | 5.43 | - | Nevirapine | Lamivudine, Stavudine |
| TC056 | Female | 33 | 18 | 4.97 | Saquinavir | Nevirapine | Zidovudine/lamivudine |
| TC059 | Male | N/A | 148 | 4.82 | Amprenavir | Efavirenz | Zidovudine/lamivudine |
| TC060 | Female | 42 | 301 | 3.89 | Indinavir | Efavirenz | Didanosine |
| TC070 | Male | 50 | 25 | 5.47 | Saquinavir | - | Zidovudine, Didanosine |
| TC109 | Female | 45 | 337 | 4.95 | - | Nevirapine | Stavudine, Didanosine |
| TC110 | Male | 33 | 111 | 4.84 | Indinavir | - | Zidovudine, Lamivudine |
| TC111 | Female | 32 | 201 | 4.97 | NA | NA | NA |
| TC113 | Male | 35 | 323 | 3.12 | NA | NA | NA |
| TC203 | Male | 49 | 160 | 5.50 | Saquinavir | - | Zidovudine, Lamivudine |
| TC204 | Female | 45 | 459 | 4.45 | - | Nevirapine | Stavudine, Didanosine |
| TC206 | Female | 41 | 196 | 4.90 | Nelfinavir | Nevirapine | Lamivudine, Zidovudine |
| TC216 | Female | 33 | 16 | 4.92 | Saquinavir | Nevirapine | Zidovudine/lamivudine |
| TC238 | Female | 38 | 290 | 5.35 | Indinavir, Amprenavir | Nevirapine | Stavudine, Lamivudine |

NA, not available

[a-f] Samples with the superscripted letters are from a same patient in different time points.

Zidovudine/lamiuvudine refers to a full dose combination of the two drugs as "Combavir"

**Table 1:** Demographic characteristics, baseline features and drug history of the subjects in Zimbabwe.

of extracted DNA was used in the initial round of PCR, and 5µl of first round product was used in the second round.

The purified PCR product was then sequenced using Rtc-1F, Rtc-2R, Rtc-3F, Rtc-4R, and MAW46 with 125 nM primer and BigDye terminators on a 3010 ABI Sequencer.

Sequences were assembled with AutoAssember. Subtype and individual resistance mutations were identified with "Genotypic Resistance Interpretation Algorithm" found on the HIVdb at Stanford University (hivdb.stanford.edu).

### Numerical resistance score

Each sample was assigned a resistance score for each antiretroviral drug included in the algorithm from 0 to 3: 0 for "susceptible", 0.5 for "potential low resistance", 1 for "low resistance", 2 for "intermediate resistance" and 3 for "high resistance". The initial numerical resistance scores were simplified or collapsed to "resistant", indicating a numerical score of 2 or 3, or "susceptible", indicating a score of less than 2.

### Collapsed resistance score

The initial numerical resistance scores were simplified to "resistant", indicating a numerical score of 2 or 3, or "susceptible", indicating a score of less than 2.

### Treatment-specific resistance score

To evaluate the predictive power of DNA versus RNA sequence, each patient was given a treatment resistance score according to the collapsed resistance scores. Treatment history was available only for 27 of the 32 samples (Table 1). The treatment resistance score is measured on a scale of 0 to 1, and is a fraction of the drugs currently or previously taken for which the sequence shows resistance. For example, if a sample is from a patient on AZT, 3TC, and NVP, and the DNA sequence shows resistance for AZT and NVP but not 3TC, the sample is given a resistance score of 0.67 or 2 out of 3. Current (treatment at the time of sampling) and previous treatment regimens were scored independently.

### Phylogenetic analysis

Assembled sequences were aligned and edited in "BioEdit". The sequences were then converted to a nexus file, a paup file, and a phylip file using the program "DAMBE" (Data Analysis in Molecular Biology and Evolution). A series of commands taken from the program "Modeltest 3.7" was added to the nexus document. The document was then opened in the program "Paup 4.0" which tested 56 evolutionary models to find the model that fit best with the sequences used. The output of this file contained information entered into the commands of the program "PhyML 2.4.4" which produced a maximum likelihood tree. The tree was viewed using "Mega 3.1" and the sequence file was analyzed in "Paup 4.0" to generate genetic distance between the sequences.

## Results

### Baseline characteristics

A total of 32 pairs of vRNA and proviral DNA protease (PR) and reverse transcriptase (RT) sequences from 25 patients were analyzed. Nineteen patients provided a single samples, 5 patients two sequential yearly samples and one patient was sampled three times at one-year intervals. Median age of the patients was 37.5 (range 16-61) years, 12 males and 13 females with a CD4 count median of 148 (range 3-459) and viral load log median of 4.95 (range 2.58-5.50) (Table 1).

### Antiretroviral treatment

Of the 25 subjects enrolled, 21 reported taking prescribed ART regimens within the previous 3 months and 4 patients reported they had not used ART. There were 6 samples obtained from these 4 subjects, with no evidence of drug resistance. The ART regimens of the 21 patients included protease inhibitors (PI) in 19, non-nucleoside reverse transcriptase inhibitors (NNRTI) for 13 and all 21 treated individuals had received NRTIs (see table 1).

### Genotypic analysis

Initially, vRNA and proviral DNA *pol* sequences were compared at the amino acid level. Sixteen patients had a record of taking protease inhibitors (PI) (19 samples). Ten samples had mutations in the protease gene (Table 2). Of these samples, 5 had discrepancies between RNA and DNA mutations. Only one of these 5 (TC049) had a mutation in the DNA *pol* sequence that was not identified in the matching RNA sequence. However, this sample had two mutations identified in RNA sequence that were not identified in DNA. The remaining 4 sequences (TC045, TC002, TC060, and TC216) had at least one mutation in RNA that was not identified in the matching DNA sequence.

Thirteen patients had received non-nucleoside reverse transcriptase inhibitor (NNRTI) drugs. Overall, 15 patients (16 samples) had NNRTI mutations (Table 2). Seven of these 16 pairs had mutations in proviral DNA *pol* gene sequence not found in RNA sequence. Only 3 of these 16 had mutations in RNA that were absent in DNA (TC060, TC109 and TC111). Sample TC060 had a V108VI mutation in RNA not found in DNA as well as an Y181C mutation in DNA that was not observed in RNA.

Similar to the situation for NNRTIs, analysis of nucleoside reverse transcriptase inhibitor (NRTI) resistance mutations revealed genotypes that differed greatly but with identical phenotypes as measured by resistance scores (Table 2). For example, pair TC041 had completely different NRTI resistance mutations identified in RNA and DNA sequences. The RNA sequence identified mutations A62AV, K65R, and K219KQ while the DNA sequence contained L74LV, V75AV, Y115FY and M184MV. Accordingly, while different NRTI resistance mutations were found in DNA versus RNA, the estimated resistances to most NRTIs were similar.

The difference in genotypes between plasma vRNA and PBMC DNA samples were often due to mixtures of wild type and mutant amino acids for a specific resistance position. Of the mutations found only in RNA, 54% were mixtures (14 of 26 mutations) and of those found only in DNA, 47% were mixtures (9 of 19 mutations). In contrast, only 34% of mutations found both in RNA and DNA were mixtures (33 of 96 mutations).

Most samples with different genotypes were similar in predicted phenotype as assessed by the estimates of drug resistance. For example, pair TC012 differed between RNA and DNA in genotype sequence (the mixture of mutation K238KQ at a resistance associated codon was found in DNA but not in RNA). However, once analyzed using the algorithm, both sequences indicated susceptibility to all four NNRTIs.

### Numerical resistance score

In examining drug resistance mutations to PIs, 4 pairs (TC060, TC106, TC110, and TC216) demonstrated differences in resistance scores for at least one drug. Only two pairs (TC106 and TC216) had differences in resistance scores greater than 1. Overall, 28 out of 32 pairs

had the exact same resistance scores for each of the protease inhibitors for both RNA based and DNA based sequence.

Similarly, when examining resistance mutations to NNRTI drugs, a total of 8 pairs contained a difference in resistance scores for at least one drug of the four analyzed in the algorithm. Two samples (TC070 and TC111) had a difference in resistance score of 2 or greater between RNA- and DNA-based sequences for at least one drug. In general, 24 pairs had the exact same resistance scores for all four drugs.

However, 17 pairs had at least one NRTI drug that had a different resistance score in RNA compared to DNA sequence. Of these 17 pairs, 10 had a difference of 2 or greater for at least one drug analyzed by the algorithm. Fifteen pairs had the exact same numerical resistance score for all 7 NRTIs.

| Sample code | PI mutations | | NNRTI mutations | | NRTI mutations | |
|---|---|---|---|---|---|---|
| | Plasma vRNA | PBMC DNA | Plasma vRNA | PBMC DNA | Plasma vRNA | PBMC DNA |
| TC002[a] | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC106[a] | L90M | Wild type | Wild type | Wild type | M184V | M184MV |
| TC006[b] | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC050[b] | Wild type | Wild type | Wild type | Wild type | Wild type | L74V, M184V |
| TC008[c] | Wild type | Wild type | Wild type | Wild type | M184V | M184V |
| TC118[c] | Wild type | Wild type | K103KR, M230LM | K103R, V179D, M230L | L74LV, M184V | M184V |
| TC215[c] | Wild type | Wild type | K103R, V179D, M230L | K103R, V108I, V179D, M230L | M184V, T215F | M184V, T215F |
| TC045[d] | Wild type | Wild type | Y181C, G190A | Y181C, G190AG | M41L, D67N, K70R, Q151M, M184V, T215FY, K219E | M41LM, D67DN, K70R, M184MV, T215Y, K219EK |
| TC201[d] | I154F | Wild type | Y181C, G190A | V108IV, Y181C, G190A | M41L, A62V, K65R, K70R, V75T, Q151M, T215Y, K219E | M41L, K65R, K70R, V75IM, Q151M, M184MV, T215Y, K219E |
| TC012 | Wild type | Wild type | Wild type | K238KQ | Wild type | Wild type |
| TC013 | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC041 | Wild type | Wild type | K103R, Y181CY | K103RT, Y181CY | A62AV, K65R, K219KQ | L74LV, V75AV, Y115FY, M184MV |
| TC045[d] | Wild type | Wild type | Y181C, G190A | Y181C, G190AG | M41L, D67N, K70R, Q151M, M184V, T215FY, K219E | M41LM, D67DN, K70R, M184MV, T215Y, K219EK |
| TC049 | V32VG, M46MI, I47IM, L76V | M46I, L76V, L10I | K103N, Y181C, G190A | K103N, Y181C, G190A | M41L, D67N, T69i, L74V, T215Y | M41L, D67N, L74V, T215Y |
| TC052 | Wild type | Wild type | G190A | G190A, Y188H | M184V | M184V |
| TC056 | L90M, A71V, G73S | L90M, A71V, G73S | Y181C | Y181C | D67N, T69N, K70R, M184V, T215V, K219Q | D67N, T69N, K70R, M184V, T215V, K219Q |
| TC059 | Wild type | Wild type | K103N, Y188*FLY | K103KN, Y188*FLY, P225HP | Wild type | Wild type |
| TC060 | I54IV, V82A, A71AT | V82A | K101E, V106M, V108IV | K101E, V106M, Y181C | K65R | K65R |
| TC070 | L90M | L90M | Wild type | Wild type | D67G, T69i, K70R, K219Q | D67G, T69i, K70R, K219Q |
| TC109 | A71T | A71T | K103KN, V106MV, Y181CY | K103N, Y181CY | Q151KLMQ | Q151KLMQ |
| TC110 | G48V, I54T, V82A, L10I, A71V | G48GV, I54IT, V82VA, L10LI, A71AV | Wild type | Wild type | A62V, K65R, F116Y, Q151M, M184V | A62AV, K65KR, F116FY, Q151KLMQ, M184MV |
| TC111 | Wild type | Wild type | P236LP | Wild type | T215PT | Wild type |
| TC113 | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC114[e] | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC117[f] | Wild type` | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC118[c] | Wild type | Wild type | K103KR, M230LM | K103R, V179D, M230L | L74LV, M184V | M184V |
| TC200[e] | Wild type | Wild type | Wild type | Wild type | V75GV | Wild type |
| | | | | | | |
| TC203 | Wild type | Wild type | Wild type | Wild type | M184MV | M184V |
| TC204 | A71T | A71T | K103N, Y181C | K103N, Y181C | F116Y, Q151M, M184V | K65R, Q151M |
| TC206 | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |
| TC215[c] | Wild type | Wild type | K103R, V179D, M230L | K103R, V108I, V179D, M230L | M184V, T215F | M184V, T215F |
| TC216 | M46I, I84V, L90M, A71V, G73S | L90M, A71V, G73S | K101E, Y181C, G190A | K101E, Y181C, G190A | D67N, T69N, K70R, T215F, K219Q | D67N, T69N, K70R, M184V, T215F, K219Q |
| TC238 | Wild type | Wild type | K103N | K103N | M41L, M184V, T215Y | M41L, M184V, T215Y |
| TC242[f] | Wild type | Wild type | Wild type | Wild type | Wild type | Wild type |

PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; vRNA, viral RNA; PBMC, peripheral blood mononuclear cell; N/A, not available

[a-f] Samples with the superscripted letters are from a same patient in different time points.

* Unique mutations comparing RNA and DNA from the same patient sample are bolded.

**Table 2:** PI, NNRTI and NRTI drug-resistant mutations.

### Collapsed resistance score

When the scores were condensed to indicate whether or not the sequence suggested susceptibility or resistance to a specific drug, 3 of 32 pairs demonstrated disagreement between the RNA and DNA sequence for at least one PI (TC060, TC106, and TC216). In all cases, RNA sequence was resistant and DNA sequence susceptible.

Similarly, 5 of the 32 pairs were discordant between RNA and DNA sequence for at least one NNRTI drug included in the algorithm (TC056, TC060, TC070, TC111 and TC118). All 5 samples differed with respect to resistance scores for only one drug and 4 of them were different because of resistance in DNA sequence and not in RNA.

With respect to resistance to NRTI drugs, 11 of the 32 pairs had differences in the collapsed resistance scores of at least one drug. Of these 11, 5 were a result of ABC, TDF and DDI resistance identified in RNA but not in DNA sequence. However, there were 3 pairs in which resistance to at least one NRTI drug was found in DNA and not in RNA. Resistance mutations to 3TC and FTC were generally found together. Similarly, ABC, TDF, DDI and sometimes D4T resistances were also detected together.

Furthermore, differences in resistance scores between plasma vRNA and PBMC DNA for a specific drug class did not correlate with differences in drug resistance mutations in another drug class. Only 4 out of the 32 samples had discrepancies in more than one drug class.

### Treatment-specific resistance score

The treatment-specific analysis of resistance, in which the efficacy of current treatment was evaluated, indicated the most similarity between RNA and DNA sequence. Through this analysis, only pair TC106 differed in RNA and DNA sequence, where for current treatment the RNA sequence for this sample scored 0.67 out of 1 for resistance, while DNA scored 0.33.

### Phylogenetic evolutionary analysis

Phylogenetic analysis was used to see how closely the sequences in the study were related to one another. Both genetic distances and maximum likelihood trees were generated. The maximum likelihood phylogenic tree is seen in the figure. All of the RNA and DNA sequence pairs were located close to one another on the same branch in the maximum likelihood tree.

## Discussion

Drug resistance profiles were generally similar for PI and NNRTI drugs in plasma vRNA and PBMC DNA. However, there were differences between the vRNA and PBMC DNA in mutations and the estimated PI, NNRTI and NRTI drug resistance scores in 9.4%, 12.5% and 34.4% of the samples, respectively. The increased discordance for NRTI mutations may be due to the greater exposure to multiple NRTIs or the rapid selection of DRMs in vRNA when drug resistance emerges. An increase in susceptible vRNA without mutations may result when drug is withheld or interrupted. Several studies show temporal differences in the appearance of DRMs in vRNA versus PBMC DNA [7,9,18,20] concluding that virus circulating in plasma demonstrate resistance mutations before they are archived in proviral DNA in PBMC. Resistance mutations in PBMC DNA that are not found in vRNA represent archival genetic resistance, which may be transmitted, or re-emerge with treatment.

Consistent with previous studies on drug naïve individuals [6,10,13-

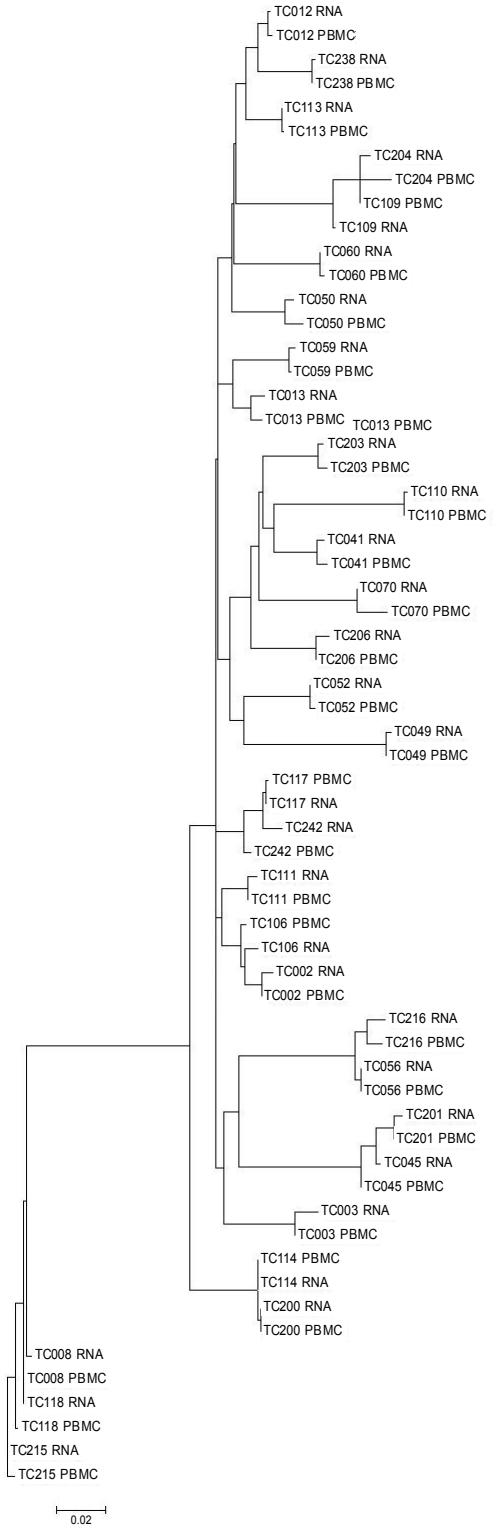


**Figure 1:** Maximum likelihood evolutionary tree showing the evolutionary relationships between all 32 pairs. The tree demonstrating that vRNA sequence and proviral PBMC DNA sequence from the same sample are located in close proximity to one another. Additionally, the tree shows different samples taken at different time points from the same patient and demonstrates that they are also located near one another and thus do not have great genetic distance, as expected.

16,21] and patients with treatment failure [9,11,12,21-24] DRM found in either vRNA or PBMC DNA contain useful and sometimes different information. As an example here, discrepancies in the NRTI resistance scores occurred in both directions; of the 11/34 (34%) of pairs that differed in collapsed resistance score to at least one NRTI, 7/11 (64%) resulted from resistance mutations; M184V, L74V, T69i, Q151M and K65R in vRNA but not in DNA sequences. In contrast, there were 6/11 (55%) samples with mutations in PBMC DNA that were not identified in vRNA; 4 with the M184V and 2 with a K65R mutation. These differences resulted in 3'TC, ABC, TDF, and DDI resistance, which in 7 cases were only found in vRNA and 6 cases in only in PBMC proviral DNA. Four of five pairs differed in estimated NNRTI resistance due to mutations found in PBMC DNA but not in the corresponding vRNA sequence. The discrepancies in DRM between vRNA and PBMC DNA sequences show the potential importance of both when considering which drugs, and drug classes, might be most effective in future therapies.

One of the limitations of this study, apart from small sample size, is the use of blood samples and treatment histories collected 2-5 years before sequencing was performed. However, plasma and PBMC were separated and frozen within 1-2 days and then assayed after storage at -70°C. In resource-limited settings, genotyping is rarely used to guide individual treatment, but the results presented here should be considered in the design of public health surveillance studies, as well as clinical utility. Sequencing proviral DNA has some clear advantages for public health surveillance for drug resistance particularly in resource-limited settings, where subtype C predominates. The durability of PBMC DNA as an analysis allows for collection of whole blood, which can be transported at room temperature for days, with satisfactory recovery of gag-pol sequences. In contrast, vRNA is labile and separation of plasma and storage at -70°C within 6-8 hours of sample collection is advised. These requirements for vRNA collection limit samples and sampling from communities or clinics, which are distant from the laboratory. The long-term stability of proviral DNA allows for collection and transport as whole blood and storage as dried blood spots with little potential loss of signal.

The drug resistance profiles of vRNA and PBMC DNA in this study provide evidence that genotyping from proviral DNA may identify drug resistance in many cases complementary to vRNA mutations. Archival resistance in PBMC DNA indicates transmitted or acquired resistance that is maintained in the absence of drug treatment. Drug resistance in vRNA sequences depends on continuous treatment exposure and adherence. For purposes of public health surveillance for drug resistance, PBMC proviral DNA and vRNA analysis yield broadly similar results. Sustainable monitoring of drug resistance may be more feasible using whole blood samples and sequences from proviral DNA to track the prevalence of drug resistance mutations in communities and clinics.

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