Drug Resistance Mutations from Whole Blood Proviral DNA Among Patients on Antiretroviral Drugs in Zimbabwe

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Abstract: *Introduction*: There are more than 500 000 HIV-infected people on antiretroviral treatment (ART) in Zimbabwe with very limited laboratory monitoring. To ensure effective treatment and prevent transmission of drug resistance, affordable treatment monitoring is needed to guide individual treatment.

Methods: 125 whole blood samples from patients on first-line ART were investigated for drug resistance mutations using an in-house genotypic testing method. Patients had been on HIV reverse transcriptase inhibitors only, with some having been on both HIV and TB treatment. DNA was extracted from whole blood; amplicons were generated by nested PCR and sequenced. Drug resistance mutations were determined using the Stanford HIV drug resistance database. Exact statistics were used to investigate relationships between drug resistance and predisposing factors.

Results: From 125 samples, 108 were successfully analyzed for drug resistance mutations. 11 of the 108 sequences had drug resistance mutations; predominantly M184V and Y181C. For a 100-cell increase in CD4 count, the odds of being resistant were 61% lower than those with the baseline CD4 count (p=0.04, CI: 0.34-0.98). There was no association between concurrent HIV/TB treatment and drug resistance (p=0.41).

Discussion and Conclusion: Although plasma samples are recommended for genotypic testing, the cost of analyzing plasma RNA makes it less feasible in resource limited settings. Lower cost DNA drug resistance testing from whole blood samples was assessed as a treatment-monitoring tool among patients followed by CD4 and clinical monitoring only. The infrequent detection of resistance and higher CD4 is consistent with effective first-line treatment. Further investigation of proviral DNA as a tool to identify drug resistance mutations is warranted.

Keywords: Drug resistance, genotypic testing, HIV-1 subtype C, HIV/TB treatment, proviral DNA, resource-limited settings, treatment monitoring, Zimbabwe.

INTRODUCTION

Antiretroviral therapy was implemented in the public sector in 2004 in Zimbabwe [1] and currently more than 500 000 people are receiving first-line treatment. Although HIV-1 RNA virus load monitoring is recommended, where affordable and feasible [2,3], in resource limited settings, virus load testing has been limited by cost and access, and treatment relies on clinical and immunologic monitoring [4]. In addition Zimbabwe has recently emerged from a series of crises, with the health system nearly collapsing by 2008 [5]. With a crippled economy, antiretroviral (ARV) drugs became scarce, increasing the potential use of counterfeit drugs with questionable efficacy [1]. Drug stock-outs, poor adherence, challenges to access and many other programmatic challenges at individual, clinic and national level may have fostered development of HIV drug resistant mutations (HIVDRMs).

With an estimated 14.7% adult HIV prevalence and about 1.1 million eligible for antiretroviral therapy in 2013, Zimbabwe is one of the countries most affected by HIV/AIDS in Sub-Saharan Africa [6]. Tuberculosis (TB) is the most common opportunistic infection in HIV patients in Zimbabwe [4]. In 2010, 88% (40 714) of TB patients in Zimbabwe were tested for HIV, and of those 77% (31 538) tested positive [7]. We hypothesized that treatment for both infections may lead to more failure and resistance because of increased pill burdens, drug interactions, as well as side effects and toxicities.

The selection and identification of drug resistance mutations among patients on treatment depend on adherence and access to drugs. Studies have demonstrated the rapid reversion to wild-type sequence in plasma RNA, among antiretroviral treatment (ART) patients who interrupt ART, despite the presence of archival drug resistance [8-10]. Among patients failing regimens, a comparative study of sequences obtained from HIV plasma RNA and proviral DNA demonstrated concordance of >98% between nucleic acid sequence and >90% detection of resistance mutations in RNA and DNA [11]. However, some studies have shown differences in mutations detected from proviral DNA

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1570-162X/14 $58.00+.00

compared to RNA [12-14], and therefore further investigation of proviral DNA sequencing for detection of drug resistance mutations is warranted.

Plasma and serum are the preferred specimen types for HIV type 1 (HIV-1) drug resistance testing [15]. Plasma for HIV RNA testing must be separated and frozen within hours of collection and stored at -80°C or tested in real-time. Moreover, robust quality assurance programs are needed to ensure the accuracy of genotypic testing using plasma RNA samples [16]. However, in resource-limited settings the cold chain for sample transport and the laboratory infrastructure for separation, RT-PCR and sequencing are often not available. Analytes with less stringent storage, transport and processing requirements can simplify resistance testing in settings like Zimbabwe. To survey prevalence of archived and fixed drug resistance mutations among patients on treatment regimens without RNA monitoring, we adapted lower cost methods for DNA extraction, amplification and sequencing of provirus DNA from whole blood.

MATERIALS AND METHODS

Study Population and Study Design

In 2010, sequential consenting ART patients at two OI clinics in Zimbabwe were enrolled in a Genomic Medicine study to capture clinical events and drug toxicities. Whole blood was collected for virologic and pharmacogenomic analysis and plasma for levels of EFV and other antiretrovirals. The recruited patients had read and voluntarily signed a consent form upon understanding the purpose, risks and benefits of the study. CD4 counts were analyzed at study enrollment before whole blood samples were frozen. Details of the study design and implementation of the study have been published previously [17]. In 2013, a subset of the samples with complete demographic and clinical data was used for this drug resistance study. Review of medical records provided data on the treatment regimens, CD4 counts, age, sex and the time on ART.

Laboratory Procedures

Whole blood collected in EDTA tubes in 2010 was stored at -20°C within 6 hours of collection, until analysis in 2013. DNA was extracted from 200ul of the whole blood using a QIAamp Mini Kit protocol (QIAGEN GmbH, Germany), according to manufacturer's instructions. The reverse transcriptase (RT) gene was amplified using two rounds of PCR with ThermoScientific Phusion Hot Start II High-Fidelity DNA polymerase (Affibody AB, Sweden). The primers used amplify an 849-bp fragment spanning codons 21 - 304 of the RT gene (Table **1**).

The amplification parameters were as follows: 96°C for 2 minutes, followed by 40 cycles of 96°C for 20 seconds, 55°C for 20 seconds, and 72°C for 2 minutes, then 72°C for 10 minutes and a final hold step at 4°C. The second round PCR was similar to the first round PCR, but the annealing temperature was increased to 56°C with 35 cycles. Amplicons were purified and sequenced on an ABI 3730xl Genetic Analyzer, at Molecular Cloning Laboratory (MCLab) in California.

Drug Resistance Analysis

The RT sequences were assembled using Sequencher v.5.0.1 (Gene Codes Co.). Drug resistance mutations were determined using the Stanford University HIV drug resistance database (HIVdb) [18]. Mutations were analyzed from RT codons with known drug resistance mutations (codons 40 - 230) [19].

Phylogenetic Analysis

Sequences were aligned in Bioedit v7.2.2 using ClustalW [20]. A best-fitting nucleotide substitution model for the aligned sequences was estimated using the jModelTest v2.1.4 [21]. A maximum likelihood phylogenetic tree was constructed based on the inferred model; generalized time reversible with proportion of invariable sites and gamma distribution (GTR + I + G), using the program PhyML v3.1 [22]. Bootstrapping with 1000 replicates was used for internal node support. The bootstrap trees were viewed in FigTree v1.4.0.

Data Analysis and Statistics

Patient characteristics (age, sex, CD4 count), and treatment information (regimen, time on ART) were analyzed for associations with drug resistance mutations. Statistical calculations were done using Stata/MP College Station, Texas (version 10.1) statistical software.

Due to small sample sizes, univariate analyses using exact methods were performed for each descriptive variable

Table 1. Primers used in polymerase chain reaction (PCR) and sequencing.

Stage	Primer	Sequence	Function	HXB2 Position
1st round PCR	Pro1	5'-CAGAGCCAACAGCCCCACCA-3'	Forward	2147-2166
	BC21	5'-CTGTATTTCAGCTATCAAGTCTTTTGATGGG-3'	Reverse	3509-3539
2nd round PCR	M13Pol1	5'-GTTAAACAATGGCCATTGACAG-3'	Forward	2610-2631
	BC20	5'-CTGCCAATTCTAATTCTGCTTC-3'	Reverse	3441-3459
Sequencing	RTC1F	5'-ACCTACACCTGTCAACATAATTG-3'	Forward	2486-2508
	RTC3F	5-CACCAGGGATTAGATATCAATATAATGTGC-3'	Forward	2965-2994
	RTC4R	5'-CTAAATCAGATCCTACATACAAGTCATCC-3'	Reverse	3101-3129

HXB2, nucleotide position of HIV-1 reference sequence.

and relationship to presence of resistance mutations. Exact logistic regression was used to evaluate continuous predictors. The log odds of developing resistance were modeled as a linear combination of the predictor variables. For categorical predictor variables, Fisher's exact test was used to evaluate the relationship between predictors and presence of resistance. Non-parametric Wilcoxon rank-sum (Mann Whitney) test was used for evaluation of CD4 counts relative to categorical predictors. Variables with p-values of 0.05 or less were considered statistically significant.

Ethics Statement

A written informed consent, approved by the Medical Research Council of Zimbabwe (MRCZ), was obtained from each patient under a Genomic Medicine study addressing the diagnosis, prognosis, treatment and monitoring of malaria, TB and HIV/AIDS in Zimbabwe (MRCZ/A/1561).

RESULTS

Patient and Clinical Characteristics

Among 125 samples, 108 (86%) were successfully analyzed for drug resistance mutations. Of the 108 samples analyzed, 70 (65%) were on ART only, and 38 (35%) were receiving both HIV and TB treatment. Table **2** shows patient characteristics of the overall population and by treatment.

All ARV drugs used in this study population fall under first-line regimens in Zimbabwe [4], i.e. stavudine + lamivudine + nevirapine/ efavirenz, zidovudine + lamivudine + nevirapine/ efavirenz and tenofovir + lamivudine + efavirenz. The median time on ART only was 21 months (1-120) compared to 6 months (1-60) for those on HIV/TB treatment ($p<0.01$, Wilcoxon rank-sum (Mann-Whitney) test). The HIV treatment regimens had either NVP (40/108) or EFV (68/108) as the NNRTI-backbone. The median CD4 cell count of ART patients (272 cells/mm^3) was higher than that of HIV/TB patients (202 cells/mm^3) ($p<0.01$, Wilcoxon rank-sum (Mann-Whitney) test) (Table **2**). Individuals on TB treatment were not more or less likely to have resistance (CI: 0.45-7.63), however, for each 100-cell greater CD4 count, the odds of detecting resistance decreased by 38% (CI: 0.05-0.62).

Clinical and demographic characteristics were not significantly different and median CD4 counts were similar between the 17 samples (272 cells/mm^3) that failed to sequence and the 108 samples (235 cells/mm^3) genotyped ($p=0.31$, Wilcoxon rank-sum (Mann-Whitney) test).

HIV Drug Resistance

We investigated drug resistance mutations in a setting where viral loads were not available, with an 86% (108/125) success rate. Of the 108 samples, 11 (10%) had drug resistance mutations. The mutations observed were A62V, K65R, D67N, V75I, K101E, K103N, V108I, F116Y, Q151M, V179D, Y181C/I, M184V, G190A and T215F. NNRTI mutations (V108I, V179D, Y181C/I, G190A) occurred at relatively higher frequencies compared to NRTI mutations (A62V, K65R, V75I, F116Y, Q151M, D67N) (Fig. **1**).

Among the 108 patient samples, 2 (1.9%) had only one NRTI mutation, 4 (3.7%) had two NRTI mutations, and 2 (1.9%) had three or more NRTI mutations. Three (2.8%) samples had only one NNRTI mutation, 7 (6.5%) had two NNRTI mutations and only 1 (0.9%) had three or more NNRTI mutations. Eight of the samples had the M184V mutation and 4 of those had at least one thymidine analogue mutation (TAM), i.e. D67N or T215F. The most common NNRTI mutation was Y181C/I (Table **3**).

Age was not statistically related to presence of resistance mutations, $p=0.15$ (CI: 0.87-1.02). Increased time on ART was also not associated with increased risk of resistance, $p=0.17$ (OR=1.02, CI: 0.99-1.04) (Table **4**). However, for a 100-cell increase in CD4 count, the odds of being resistant were 61% of those with the baseline CD4 count (CI: 34-98%) (Table **4**).

Patients with resistance mutations had a lower median CD4 count (159 cells/mm^3, IQR: 101-286) compared to patients without resistance mutations (240 cells/mm^3, IQR: 149-377) and median CD4 cell counts of patients on ART only were higher than those on HIV/TB treatment, $p<0.05$ (Wilcoxon rank-sum (Mann Whitney) test).

Sex was not significantly associated with presence of resistance mutations ($p=0.36$, Fisher's exact test) (Table **5**). There was no association seen between attending either clinic and development of resistance, $p=0.43$. There was no

Table 2. Patient characteristics of overall population and by treatment.

	Overall	By Treatment		p-Value
Variable	**N=108**	**ART (n=70)**	**HIV/TB (n=38)**	
Females	68 (63%)	49 (70%)	19 (50%)	0.04
Mean age in years (range)	41 (23-58)	42 (24-58)	39 (23-55)	0.14
Median months on ART (range)	13 (1-120)	21 (1-120)	6 (1-60)	<0.01
Median CD4 cell count/mm^3 (range)	235 (210-274)	272 (37-922)	202 (16-518)	<0.01
Drug Regimen				
Nevirapine-based	40 (37%)	40 (57%)	0 (0%)	<0.01
Efavirenz-based	68 (63%)	30 (43%)	38 (100%)	<0.01

ART, antiretroviral treatment; CD4, cluster of differentiation 4; HIV/TB, combined HIV and tuberculosis treatment.

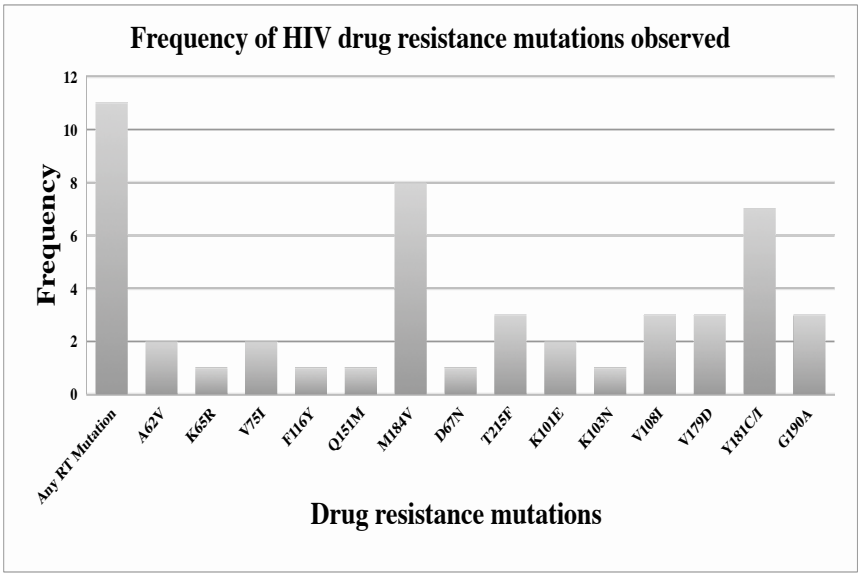

Fig. (1). Frequencies of observed HIV drug resistance mutations.

Table 3. Observed HIV-1 subtype C drug resistance mutations.

Patient ID	CD4 Count (Cells/mm^3)	Drug Regimens	NRTI Mutations	NNRTI Mutations	TAMs
AB007	101	D4T+3TC+NVP	M184V	G190A	D67N
AB010	114	D4T+3TC+NVP	M184V	V108I, Y181C	-
AB026	295	AZT+3TC+NVP	A62V, V75I, M184V	K101E, G190A	-
AB030	331	D4T+3TC+NVP	M184V	V179D, Y181C	-
AB076	220	AZT+3TC+NVP	M184V	K103N, Y181C	T215F
AB097	37	TDF+3TC+EFV	M184V	V108I, Y181C	T215F
AB102	104	AZT+3TC+NVP	-	K101E	-
AB124	236	AZT+3TC+EFV	-	V179D	-
AB136	286	D4T+3TC+EFV+ RHEZ	-	Y181C, H221Y	-
AB156	20	AZT+3TC+EFV+ RHEZ	A62V, K65R, V75I, F116Y, Q151M, M184V	V108I, Y181C, G190A	-
AB157	159	AZT+3TC+EFV+ RHEZ	M184V	V179D, Y181I	T215F

CD4, cluster of differentiation 4; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; TAMs, thymidine analogue mutations.

association detected between concomitant TB treatment and development of resistance mutations, p=0.41. Moreover, the nature of the NNRTI-backbone used was not related to presence of HIVDRMs, p=0.17 (Table **5**).

Quality Analysis

The maximum likelihood phylogenetic tree demonstrated a clear relationship between the study sequences and HIV-1 subtype C reference sequences, without evidence of inter-subtype recombination. There was no geographical clustering of sequences from the two OI clinics. More importantly there was no close clustering of samples, which helped to exclude sample contamination (Fig. **2**). The HIV-1C sequences have been deposited in GenBank under accession numbers KJ412345-KJ412452.

Costing

A cost breakdown of each consumable required was done using pricing from product catalogues. The approximated

Table 4. Exact logistic regression of continuous variables.

Resistance	OR	p-Value	95% Confidence Interval
Age	0.94	0.15	0.87 - 1.02
Time on ART	1.02	0.19	0.99 - 1.04
CD4 count (per 100 units)	0.61	0.04	0.35 - 0.98

ART, antiretroviral treatment; CD4, cluster of differentiation 4; OR, odds ratio; p, probability.

Table 5. Fisher's exact test of categorical variables.

	Variables							
	Sex		Clinic		Treatment		NNRTI-Backbone	
Resistance	M	F	Chitungwiza	Wilkins	HIV/TB	ART	NVP	EFV
No mutations	37	60	43	54	35	62	34	63
Mutations	3	8	4	7	3	8	6	5
Fisher's exact (p-value)	0.36		0.43		0.41		0.17	

ART; antiretroviral treatment, EFV; efavirenz, F; female, M; male, NNRTI; non-nucleoside reverse transcriptase inhibitor, HIV/TB; combined HIV and tuberculosis treatment, p; probability.

cost per sample was US$22.36. An extended cost was added assuming a 50% failure rate, with an approximated total cost of US$33.54 (Appendix 1). Note that the cost of labor and maintaining equipment was not factored in. However, if all factors were considered, the total cost would remain affordable in this setting, compared to currently available genotypic testing assays, which range from about US$250 to US$500 [23,24].

DISCUSSION

Genotypic testing methods have been shown to have greater success when specimens are collected from patients with viral load counts >1000 copies/ml [25]. However, previous studies have shown 78% of samples with <1000 copies/ml and 69% of samples with 50-200 copies/ml being successfully sequenced and concluding that genotypic tests

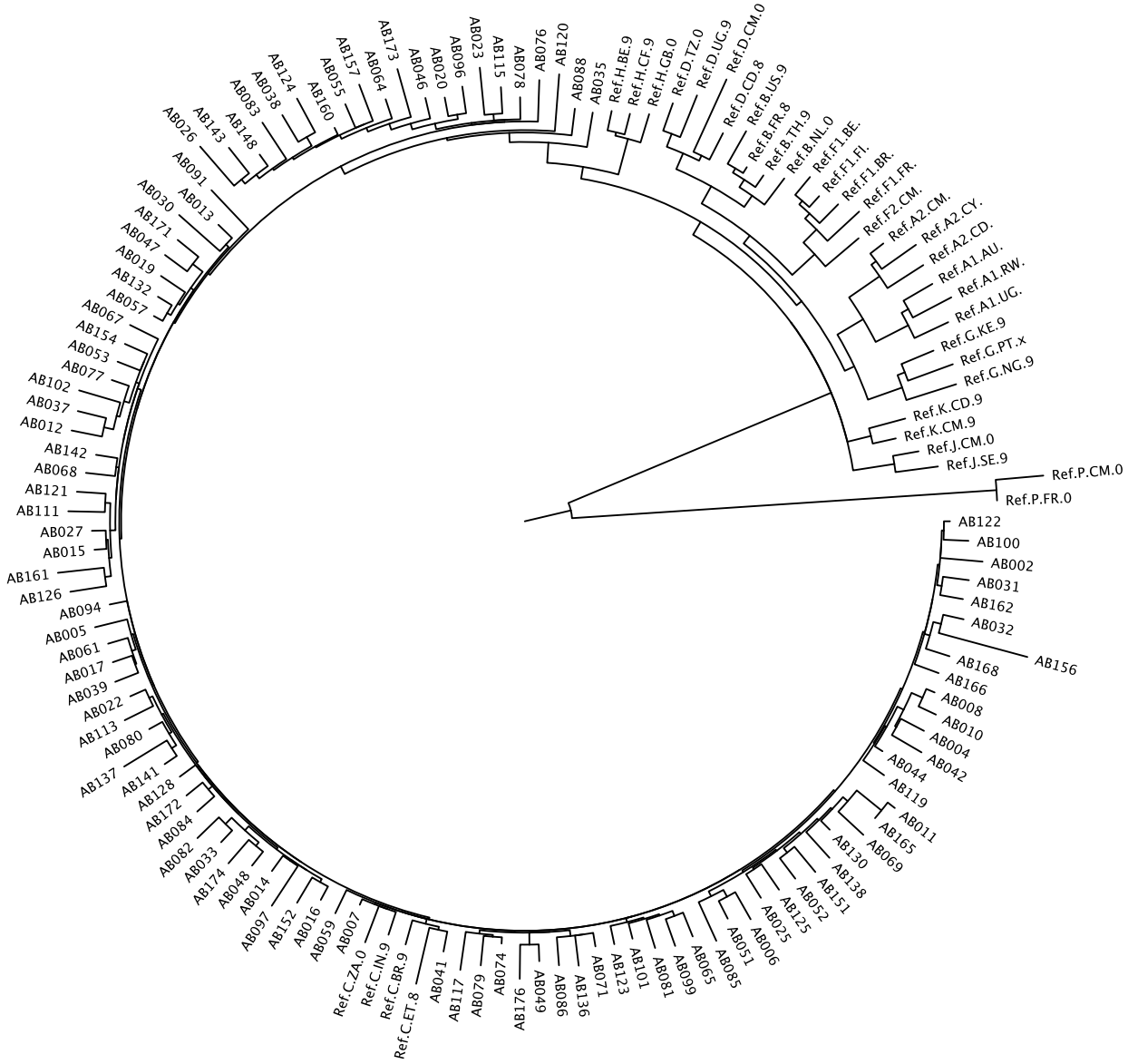

Fig. (2). Phylogenetic analysis of reverse transcriptase gene sequences of the 108 samples.

can still be used in patients with viral loads <1000 copies/ml [26-31]. When samples were collected for this study, viral load counts were not available, reflecting field conditions in this environment.

The cost of HIV drug resistance testing may be reduced by use of specimen types such as whole blood. The Southern African Treatment and Resistance Network (SATuRN) has made efforts to reduce the costs of drug resistance testing by subsidized SATuRN/Life Technologies genotypic drug resistance testing methods [32]. The same efforts to reduce costs were attempted in this in-house method, to make drug resistance testing affordable. Assessing resistance mutations directly from robust analytes may reduce drug costs, by avoiding unnecessary switching of patient regimens to the more expensive second line regimens.

HIVDR mutations were observed in 11 of the 108 sequences. The M184V mutation had the highest frequency, occurring in all the sequences with NRTI DRMs. Though M184V causes a high level of resistance to 3TC, the mutation increases viral susceptibility to AZT, TDF, and d4T. M184V also reverses T215Y-mediated AZT resistance and is associated with clinically reduced replication of HIV-1 [33]. It further prevents or delays emergence of thymidine analogue mutations (TAMs) [34,35]. This provides a rationale for continuing 3TC in second-line HIV drug regimens [4].

TAMs are known to impact resistance to all NRTIs except 3TC, although the level of resistance is based on the number of the TAMs and the drug being considered [36]. The TAMs are M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E [37]. In this study, two TAMs were observed in four patient samples. These are D67N (AB007) and T215F (AB076, AB097, and AB157), with each sample having only one TAM (Table **4**). D67N was observed in one patient who had been on D4T+3TC+NVP for 14 months, whilst the T215F mutation was observed in three female patients of similar age who had been on treatment for over 4 years. These TAMs cause significant levels of resistance to D4T and AZT, though their overall effect could be counteracted by presence of the M184V mutation.

Time on ART had no significant association with development of resistance. Patient AB156 had been on treatment for 3 years and had accumulated up to 9 HIVDRMs. Amongst those mutations was the K65R mutation that is usually associated with intermediate to high-levels of resistance to all NRTIs, although it increases susceptibility of the virus to AZT [38,39]. The patient also had the Q151M mutation that was coupled with A62V and V75I, causing significant levels of resistance to most NRTIs. The same patient had the lowest CD4 count (20 cells/mm^3) and had received concomitant TB treatment (AZT/3TC/EFV and RHEZ).

Concomitant ART and TB treatment involves increased pill burden, potential drug interactions, increased adverse drug reactions and reduced adherence during periods of severe illness all of which increase the risk of developing drug resistance mutations [40]. Overall, there was no association seen between concomitant HIV/TB treatment and the development of HIVDRMs (p=0.19, CI: 0.99-1.04). However, the CD4 counts of patients on HIV/TB treatment were significantly lower than those of patients on ART only ($p<0.05$ (Wilcoxon rank-sum (Mann Whitney) test)).

CONCLUSION

Considering the costs associated with HIV treatment monitoring, methods that are less costly become important where resources are limiting. In such settings, qualitative rather than quantitative viral load assays with high specificity and sensitivity could be optimal in reducing the cost of viral load monitoring [41]. Though the SATuRN has helped to subsidize costs of genotypic resistance tests using RNA, methods that use whole blood samples could be more feasible in resource constrained settings where collection, transport, storage and processing of plasma samples cannot be done effectively in remote sites.

Our major limitations were the lack of viral load counts, a small sample size and the use of whole blood without comparisons to the widely recommended plasma samples. Also, the use of bulk sequencing might have reduced the sensitivity of associating risk factors with development of resistance. Therefore, well-designed clinical trials to investigate use of whole blood proviral DNA for resistance testing in this setting are needed. Such lower-cost monitoring tools in public sector HIV treatment in Zimbabwe could preserve treatment options and improve the quality of patient care.

CONFLICT OF INTEREST

The authors confirm that they do not have any conflict of interest. Funding for this study was received from the International Science Program (ISP), Uppsala University, Sweden and the Stanford University SPARK program, California, USA. Funding was also provided in part by the African Programme for Training in HIV/TB Research, Fogarty International Center U2R TW006878. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGEMENTS

The authors acknowledge the assistance of Dr. E. White and Dr. A. Derache of Stanford University School of Medicine, for their input in developing the method used. Our thanks also go to Dr. J. Manasa for his help with results analysis and to Dr. G. Kadzirange for the indispensible contribution to this write-up. We also extend our thanks to the study participants.

Authors' contributions: Benjamin Chimukangara; performed research, collected data, analyzed data and wrote paper. Collen Masimirembwa; designed research study, contributed important reagents and wrote paper. Lovemore Gwanzura; designed research study and wrote paper. Rebecca Mitchell; analyzed data and wrote paper. David Katzenstein; contributed important reagents, analyzed data and wrote paper.

APPENDIX 1.

Table A1. Approximate cost for genotypic testing from proviral DNA in whole blood.

	Price ($US)	Number of Samples	Price Per Sample ($US)	Total Used	Total Cost ($US) = Price Per Sample x Total Used
DNA extraction					
QIAamp DNA Mini Kit	633	250	2.53	1	2.53
PCR					
dNTP mix 25mM	244	3570	0.07	2	0.14
Phusion HF DNA Polymerase (100U)	149	385	0.39	2	0.78
Primers	15	10000	0.002	4	0.01
Nuclease free water (30ml)	47	600	0.08	1	0.08
Gel electrophoresis					
Agarose	271	1000	0.27	2	0.54
Ethidium bromide	53	1000	0.05	2	0.1
Loading dye (2ml)	44	1500	0.03	2	0.06
Molecular weight marker	130	5000	0.03	2	0.06
Mass ladder	94	350	0.27	1	0.27
10X TBE buffer	279	1200	0.23	1	0.23
Purification					
Qiaquick DNA purification kit	480	250	1.92	1	1.92
Consumables					
1.5 ml centrifuge tubes	43	500	0.09	3	0.27
Pipet barrier tips	125	960	0.13	30	3.9
8-tube Strips (125x8)	103	1000	0.10	2	0.20
8-cap strips	82.5	2400	0.03	2	0.06
96 well plates	10	800	0.01	1	0.01
Sequencing					
Primers	24	9733	0.003	3	0.01
Sequencing	336	96	3.5	3	10.5
Shipping	90	384	0.23	3	0.69
Total					**22.36**
Extended cost (assuming ~50% failure rate)					11.18
Grand Total					**$33.54**

PCR, polymerase chain reaction; $US, United States Dollar.

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Received: April 8, 2014 **Revised: September 19, 2014** **Accepted: October 9, 2014**