Published in final edited form as:

J Virol Methods. 2017 May ; 243: 151–157. doi:10.1016/j.jviromet.2016.11.010.

HIV drug resistance testing among patients failing second line antiretroviral therapy. Comparison of in-house and commercial sequencing

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Abstract

Introduction—HIV genotyping is often unavailable in low and middle-income countries due to infrastructure requirements and cost. We compared genotype resistance testing in patients with virologic failure, by amplification of HIV pol gene, followed by “in-house” sequencing and commercial sequencing.

Methods—Remnant plasma samples from adults and children failing second-line ART were amplified and sequenced using in-house and commercial di-deoxysequencing, and analyzed in Harare, Zimbabwe and at Stanford, U.S.A, respectively. HIV drug resistance mutations were determined using the Stanford HIV drug resistance database.

Results—Twenty-six of 28 samples were amplified and 25 were successfully genotyped. Comparison of average percent nucleotide and amino acid identities between 23 pairs sequenced in both laboratories were 99.51 (± 0.56) and 99.11 (± 0.95), respectively. All pairs clustered together in phylogenetic analysis. Sequencing analysis identified 6/23 pairs with mutation discordances resulting in differences in phenotype, but these did not impact future regimens.

Conclusions—The results demonstrate our ability to produce good quality drug resistance data in-house. Despite discordant mutations in some sequence pairs, the phenotypic predictions were not clinically significant.

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Competing interests

The authors declare that they have no competing interests.

Keywords

HIV drug resistance; treatment failure; genotyping; SATuRN; Zimbabwe

1.0 Introduction

Zimbabwe's national antiretroviral treatment program began in 2004 [1]. As of 2014, antiretroviral treatment (ART) coverage had reached an estimated 77% of those eligible for treatment [2], after adapting World Health Organization (WHO) 2013 consolidated guidelines for ART initiation, i.e. patients with CD4 counts < 500 cells/mm³, WHO clinical stages 3 or 4, or active tuberculosis (TB) disease [3]. Public health and donor funding provide WHO recommended first-line ART to over 780 000 recipients in Zimbabwe [2]. The goal of ART is to suppress HIV to undetectable levels (< 50 RNA copies/ml) using a combination of one non-nucleoside reverse transcriptase inhibitor (NNRTI) and two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) as first-line therapy, and a boosted protease inhibitor (PI) and two NRTIs for second line therapy [4].

The WHO 2015 ART guidelines for public health prevention and individual health benefit “treatment for all”, will expand ART to an additional 500 000 people. The goal of the 90-90-90 program for “Getting to zero new HIV infections” is dependent upon $> 90\%$ suppression of HIV RNA in all patients on treatment [5]. Viral load testing and adherence counseling to attain the “90 percent suppression” goal is critical [5] to the prevention of clinical failure and transmission of drug resistant virus. The WHO 2013 ART guidelines recommend that low and middle-income countries consider HIV drug resistance genotyping for patients failing second-line therapies [6]. However, the high costs of genotypic resistance testing (GRT) and lack of laboratory infrastructure limits the adoption of such policies in Zimbabwe. Strategies to provide GRT services for surveillance and management of patients on ART requires sequencing of reverse transcriptase (RT) and protease (PR) genes to detect drug resistance mutations [7,8]. Genotyping may be performed by commercial systems (such as TruGene and ViroSeq) or more economical “home brew” assays [9].

We sought to develop local HIV drug resistance capacity by adopting a published affordable genotyping method [10]. We performed GRT on plasma from patients failing second-line therapy accessing clinical care at a specialist HIV clinical care center (Newlands Clinic, Harare, Zimbabwe). Amplicons were sequenced at a commercial sequencing facility, at Molecular Cloning Laboratories (MCLAB) in California, and at the African Institute for Biomedical Science and Technology (AiBST) laboratory, in Harare. We compared the “in-house” sequencing to the commercial sequencing laboratory; as a reference-standard. In-house sequencing results were not used for patient management. Sequence agreement was classified by the mutations detected and phenotypic interpretation. Sequencing results with $> 97\%$ agreement were considered high quality [11], and phylogenetics bootstrap values $> 95\%$ were considered good.

While important for ART policy, drug resistance surveillance and patient management costs nearly \$600 per sample in the private sector in Harare. Lower cost high quality GRT may be achieved by local “in-house” sequencing (~\$180/sample), or by sending non-infectious

stable amplicon to regional or international, commercial laboratories (~\$90/sample), such as Inqaba Biotec (<http://www.inqababiotec.co.za>), Macrogen (<http://dna.macrogen.com>) or Molecular Cloning Laboratories (<http://www.mclab.com>). “In-house” dideoxy-sequencing may provide more rapid turn-around-time, but requires laboratory infrastructure, maintenance and high volumes to achieve economies of scale. Sequencing by the mail (SBTM), is a method for sending amplicon to a commercial sequencing laboratory, with a slightly longer turn-around time (~1 week longer). Both methods build capacity in bioinformatics, patient management and public health policy through the translation of genomic information and interpretation of GRT for treatment in the public sector. In-house sequencing capacity fosters research in genomics and phylogenetics, promotes innovative projects by students and researchers, and builds local scientific capacity.

2.0 Methods

Plasma samples (N=28) obtained from patients with virological failure (virus load 1000 copies/ml) exposed to reverse transcriptase and protease inhibitor drugs were assessed using the COBAS AmpliPrep/TaqMan48 HIV-1 Test, v2.0 (Roche Molecular Diagnostics) at Newlands Clinic. The Institutional Review Board of the Biomedical Research and Training Institute (BRTI) reviewed and approved the study protocol, Ref: AP134/16.

2.1 Laboratory Methods

2.1.1 RNA extraction—Plasma samples stored at -80°C were thawed at room temperature prior to extraction. Plasma volumes used for pelleting the virus were based on the sample viral loads. For samples with viral loads $\geq 4.5 \log_{10}$ copies/ml, 500 μl of plasma was used to pellet the virus. We used larger plasma volumes of 1000 μl – 1500 μl to pellet the virus for samples with viral loads $<4.5 \log_{10}$ copies/ml, to increase viral concentration and PCR success. Respective volumes for each sample were centrifuged at $23\,000 \times g$ for 1 hour at 4°C to pellet the virus.

The supernatant was discarded from each sample leaving 200 μl of pelleted plasma sample for extraction. Viral RNA (vRNA) was extracted using a PureLink Viral RNA/DNA Kit (ThermoFisher Scientific, USA) according to manufacturer’s instructions. In summary, 25 μl of proteinase K and 200 μl of lysis buffer were added to 200 μl of pelleted plasma sample, mixed by vortexing and incubated for 15 minutes at 56°C . Absolute ethanol (250 μl) was added to the lysate, mixed by vortexing and incubated for 5 minutes at room temperature. The lysate was transferred to a viral spin column (Invitrogen, California, USA) and centrifuged at $6800 \times g$ for 1 minute. The column was washed twice using 500 μl of wash buffer and spun at $6800 \times g$ for 1 minute discarding flow through. Viral RNA was eluted in 30 μl of buffer and used immediately for reverse transcription.

2.1.2 Complimentary DNA (cDNA) synthesis—A dNTP/primer mix was prepared by mixing 0.5 μl of primer RT21 [5 μM] and 0.5 μl of dNTPs [10mM]. 6 μl of RNA was mixed with the dNTP/primer mix and placed in a PTC-100 programmable thermal controller (MJ Research, USA) at 65°C for 5 minutes, before rapidly cooling to 4°C for 2 minutes. With tubes held at 4°C , 5 μl of enzyme mix containing 1 μl of First Strand Buffer [10 $\times$], 2 μl of MgCl₂ [25mM], 1 μl of DTT [0.1M], 0.5 μl of RNaseOUT [40U/ml], and 0.5 μl of 200U/ml

Superscript III Reverse Transcriptase (ThermoFisher Scientific, USA), was added. The tubes were heated in the thermal cycler at 50°C for 60 minutes then at 85°C for 5 minutes, before cooling to 37°C. The thermal cycler was paused at 37°C and 0.5µl of RNase H was added. The tubes were held at 37°C for 20 minutes and then cooled to 4°C. The cDNA was amplified immediately by nested PCR.

2.1.3 Nested PCR—HIV complete protease (PR) and partial reverse transcriptase (RT) genes were amplified using Southern African Treatment Resistance Network (SATuRN) custom primers shown in Table 1. First round primers were MAW26 and RT21 and second round primers were Pro1 and RT20. 2µl of cDNA was mixed with 23µl of PCR reagent mix containing 18.4µl of DEPC treated water, 2.5µl of buffer [10×], 1.0µl of MgCl₂ [50mM], 0.5µl of dNTPs [10mM], 0.25µl of each primer [5µM], and 0.1µl of 5U/µl Platinum Taq polymerase (ThermoFisher Scientific, USA). Reagent volumes and PCR conditions for the 1st and 2nd rounds were as follows; 94°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 58°C for 20 seconds, 72°C for 2 minutes, and then 72°C for 10 minutes, before a final holding step at 4°C. Second round amplicon was 1.32kb as verified by gel electrophoresis on a 1% agarose gel [10].

2.1.4 Purification of PCR product—Amplicons were purified using a PureLink PCR purification kit (ThermoFisher Scientific, USA) according to manufacturer's instructions. Amplicon was divided into two 15µl volumes for commercial and in-house sequencing. DNA concentrations were determined using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific, USA) and amplicons were stored at -20°C prior to sequencing. An aliquot of each sample was sent for sequencing at Molecular Cloning Laboratory (MC Lab) in California and the data was analyzed at Stanford University School of Medicine in the Division of Infectious Diseases. The duplicate aliquots were sequenced and analyzed in-house.

2.2 In-house Sequencing

2.2.1 Sequencing reaction—Big Dye Terminator v3.1 kit (ThermoFisher Scientific, USA) and four SATuRN custom sequencing primers (provided with the kit) shown in Table 1 were used. 1µl of the amplicon was mixed with 9µl of sequencing reaction mix containing 6.1µl of nuclease free water, 2µl of sequencing buffer (5×), 0.5µl of primer [3.2µM] and 0.4µl of Big Dye terminator sequencing mix. The sequencing reaction conditions were as follows; 96°C for 1 minute, followed by 35 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, and then a final holding step at 4°C.

2.2.2 Sequence reaction purification—The sequencing products were purified prior to capillary electrophoresis. In summary, 10µl of the sample was mixed with 80µl of freshly prepared 75% isopropanol. The tubes were mixed by vortexing and incubated in the dark for 15 minutes at room temperature. They were then centrifuged at $>10\,000 \times g$ for 20 minutes. The isopropanol was aspirated and discarded taking care not to disturb the pellet. 200µl of freshly prepared 70% ethanol was added and mixed by vortexing. The tubes were centrifuged for 5 minutes and the ethanol was aspirated and discarded. This step was repeated followed by a quick spin to remove residual ethanol. The tubes were left open to

dry in the dark for 10 minutes at room temperature. The pellet was re-suspended in 10µl of Hi-Di formamide and vortexed to mix.

2.2.3 Capillary Electrophoresis—Samples were transferred to a 96-well plate and denatured at 96°C for 2 minutes on a thermal cycler, and immediately placed on ice for at least 2 minutes. The samples were loaded on the 3130xl Genetic Analyzer [Applied Biosystems] for capillary electrophoresis.

2.2.4 Sequence editing and analysis—Sequence editing was done in Sequencher v5.4 (Gene Codes Co.). Drug resistance mutations were detected using the Stanford University HIV drug resistance database (HIVdb) [12] and their significance was determined based on the International AIDS Society (IAS) mutation list [13]. Mutations were analyzed from both PR and RT genes spanning codons 1–99 and 1–238, respectively.

2.2.5 Phylogenetics—The qualities of sequences generated and edited in the two laboratories were analyzed by phylogenetic tree reconstruction in Geneious v.8.1.7. Phylogenetics analysis was done for 48 sequences, 23 sequence pairs and 2 sequences from either laboratory. HIV-1 reference sequences obtained from the Los Alamos Database (hiv.lanl.gov) were included. Sequences were aligned in Geneious R8 software [14] using ClustalW. Maximum likelihood tree reconstruction was carried out using the generalized time reversible model with proportion of invariable sites and gamma distribution (GTR+I +G). Internal node support with 1000 bootstraps was done. Bootstrap values >95% were considered good.

3.0 Results

3.1 Patient demographics, social and clinical characteristics

Twenty-eight plasma samples were submitted for genotyping from patients receiving ART for a median 7 years (CI: 5.3 – 8.2 years). Eighteen (64%) received a regimen with tenofovir (TDF), lamivudine (3TC) and atazanavir/ritonavir (ATV/r). Two patients receiving an NRTI or NNRTI-based regimen were included. All 26 patients receiving a boosted PI had failed a first-line regimen including either efavirenz (EFV) or nevirapine (NVP) with either stavudine or zidovudine (AZT), since TDF and abacavir (ABC) have only been recently introduced in Zimbabwe (2010). Table 2 summarizes the demographic, clinical characteristics and current regimen reported at the time of virologic failure.

3.2 Viral load and amplification

Of the 28 samples, 26 (93%) were successfully amplified. The two samples that failed to amplify had relatively low viral loads, at the lower limit of the sensitivity of the assay, i.e., 1247 and 901 copies/ml.

3.3 Sequences and drug resistance mutations

Of the 26 samples amplified, 25 were sequenced, with 23 sequence pairs and 2 with only sequence from a single platform. One of the 2 (the in-house sequence) had no drug resistance mutations and the other (a commercial sequence) had a minor PI mutation A71T

and an NNRTI mutation Y181C. Nineteen of 25 (76%) had drug resistance mutations in either the protease and/or reverse transcriptase. The average percentage nucleotide and amino acid identities between the pairs were 99.51 (SD 0.56) and 99.11 (SD 0.95), respectively.

Of the 23 samples genotyped in both laboratories, four had no drug resistance mutations and 19 had at least one drug resistant mutation. Of the 19 with mutations, 13 were identical and 6 had discordant mutations. Consequently, 5 of the 6 had different phenotypic prediction results for certain drugs and one (NC14) was concordant despite mutation differences (Table 4).

3.4 Differences between in-house and commercial sequence resistance

Among three samples with differences in PI resistance mutations (NC1, NC6 and NC7 as shown in Table 4), in-house sequencing samples demonstrated protease drug resistance mutations that were not seen in the commercial sequences; in-house sequencing detected protease inhibitor mixtures, L24IL and Q58EQ in NC6, and I54VI and an L10FI, mixture in NC7. The mixtures are consistent with early selection of protease resistance and resulted in a modest increase in predicted protease resistance compared to commercial sequencing. The clinical decision to provide Darunavir/r in the third-line regimen was unchanged.

In-house sequencing was also more sensitive to mixtures and mutations at codons associated with NRTI and NNRTI resistance. The NC1 in-house sequence demonstrated a thymidine mutation (TAM) L210W, associated with multi-NRTI resistance, and a I50L PI mutation associated with increased susceptibility to PIs and high level-resistance to Atazanavir [15]. Significantly, the in-house sequencing detected a mixture M184MV in one sample (NC22), not found in the paired commercial sequence. In contrast, commercial sequencing detected NNRTI mutations at Y188L and H221Y in NC28, with only a modest increase in predicted rilpivirine (RPV) resistance.

3.5 Phylogenetics

The phylogenetic tree for the samples showed good concordance between the two laboratories (bootstraps >98%), and evidence of contamination or sample mislabeling was not observed. All samples clustered around the HIV-1C reference sequence. Samples sequenced commercially are labeled with SFD for Stanford, USA, and samples sequenced in-house are labeled with HRE for Harare, Zimbabwe. Sequences have been deposited in GenBank under accession numbers KU508549 – KU508596.

4.0 Discussion

Management of patients failing ART and surveillance for transmitted drug resistance in resource-limited settings (RLS) is constrained by lack of viral load monitoring and resistance testing. Even as virus load monitoring has been recommended for ART, there is very limited capacity to obtain GRT in RLS. Here we identified patient samples with virologic failure from a tertiary treatment centre to assess drug resistance mutations from plasma RNA. We compared in-house (“home-brew”) sequencing to a Virology Quality

Assurance (VQA) accredited reference laboratory that used a commercial facility for sequencing.

Of the 28 samples submitted for genotyping, 26 were successfully amplified. Failure to obtain an amplicon from ultra-centrifuged plasma was associated with lower viral loads. The two samples which failed to amplify had viral loads of 1247 and 901 copies/ml, around the lower recommended levels of plasma virus for genotyping with either ViroSeq [16] or TruGene [17]. Drug resistance mutations in patients with low-level viremia (50 – 1000 copies/ml) may be obtained from proviral DNA [18–20] or by concentrating virus from large volumes of plasma [21], although successful amplification is reduced among samples with lower virus copy numbers [22]. However, it may be important to investigate viral genotypes in such patients, as resistance at low-level viremia is associated with an increased risk of future virologic failure (i.e. VL >1000 copies/ml) [23].

Of the 26 samples amplified, 25 were sequenced, with 23 sequence pairs. Sequencing failure was associated with low amplicon concentration, which produced chromatograms with low signal intensities. Of the 23 paired sequences, nucleotide and amino acid identities demonstrated excellent (>99%) inter-platform concordance similar to a lower cost in-house genotype that was compared to ViroSeq Genotyping System 2.0 [9]. Of the 23 samples sequenced in both laboratories, 19 (83%) had drug resistance mutations, with 6/19 having discordant mutations as shown in Table 4. Of the 6 samples with differences in drug resistance mutations, in-house sequencing detected additional mutations. Five of the 6 demonstrated a modest increase in the estimated resistance (from intermediate to high) to some PI and NNRTI drugs, based on additional within-class mutations [24].

Acquired drug resistance to Lamivudine, Nevirapine and Efavirenz provided as first-line treatment may result in retention of potentially transmissible drug resistance mutations [25]. Interestingly, we found that 18 of the 19 sequence pairs with mutations had one or more NNRTI resistance mutations, although only 2 were receiving NNRTI drugs. This provides further evidence for the persistence of NNRTI mutations, which are estimated to recede to undetectable levels in Sanger sequencing at a rate of about 10–20% annually [15]. However, they may persist in about two thirds of patients despite NNRTI withdrawal through linkage to NRTI and PI resistance mutations, which are maintained under selective drug pressure of ART [26].

Major protease resistance mutations were detected in only 6/24 (25%) of the patients treated with ATV/r (20) or LPV/r (4), suggesting inadequate adherence or pharmacokinetics as a cause for virologic failure. This is consistent with recent studies of boosted PI failure in low and middle income countries which report drug resistance mutations to PI in 10–40% [27,28]. Notably, in this study, none of the samples exhibited resistance to Darunavir. In contrast, with regards to 3TC resistance, the M184V mutation was detected in most samples with resistance mutations (13/19) as is typical in first-line and second-line failure [29,30]. From one patient (NC22) Lamivudine resistance as a mixture M184MV was identified only in the in-house sequence resulting in a difference in phenotype (Table 4). Despite M184V/I mutations, 3TC is usually retained as the M184V/I mutation reduces viral replicative capacity and may reverse AZT resistance caused by T215Y [31–33], delay the emergence of

thymidine analogue mutations (TAMs) [34,35] and increase susceptibility to AZT and TDF [36,37].

Phylogenetic analysis showed good sequence quality between the commercial and in-house sequences, with all sequence pairs clustered on the same operational taxonomic units. All sequences clustered with the HIV-1 Subtype C sequences and there was no evidence of contamination. Detection of drug resistance mutations by Sanger sequencing may depend on drug exposure and adherence [38] and thus we selected patients with confirmed virological failure, (VL> 1000 copies/ml). Differences in phenotypic resistance were due to mixtures detected by the in-house sequencing, suggesting inter-laboratory subjectivity in base-calling, based on cut-offs in peak height ratios.

5.0 Conclusions

Lower cost HIV drug resistance testing in Zimbabwe could be implemented, yielding genotypes comparable to an accredited virology quality assurance certified sequencing and data analysis method at Stanford University. Sequencing amplicon in-house or in a commercial laboratory differed in sensitivity to some mixtures, sample throughput, cost and turn-around time. In-house or commercial sequencing can provide genotypes for surveillance, research and clinical resistance testing in Zimbabwe. However, implementation of such techniques requires a significant investment in laboratory space, acquiring and maintaining laboratory equipment, as well as supporting laboratory personnel, which depend on local and international collaborations.

Acknowledgments

We would like to thank Newlands Clinic for providing plasma samples and clinical data. We also acknowledge the collaboration between the Biomedical Research and Training Institute (BRTI), Newlands Clinic, the African Institute of Biomedical Science and Technology (AIBST) and Stanford University that were involved in setting up the in-house sequencing platform, and the Stanford-SPARK program for their support.

Funding: This work was supported by the International, Clinical Operational and Health Services Research Training Award (ICOHRTA), the African Programme for Training in HIV/TB Research Fogarty International Center/NIH U2R TW006878 and an educational grant to the corresponding author from Stanford-SPARK.

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Highlights

- In-house sequencing can provide reliable genotyping information
- Low-cost sequencing achieved by adopting SATURN protocol
- In-house and commercial sequencing make testing feasible, accessible and affordable
- High quality resistance testing improves patient care

Figure 1.
Flow diagram of study samples processed.

Figure 2. Phylogenetics tree of samples sequenced commercially (SFD) and in-house (HRE)

Maximum likelihood phylogenetics tree, model: GT R+I+G

All sequence pairs had bootstrap values >98%

Key

Ref, Reference sequence; SFD, Stanford; HRE, Harare

Table 1

PCR and sequencing SATuRN custom primers

Primer	Sequence	Direction	HXB2	Stage
MAW-26	5'-TTGGAAATGTGGAAAGGAAGGAC-3'	Forward	2028–2050	1 st round PCR
RT-21	5'-CTGTATTTTCAGCTATCAAGTCCTTTGATGGG-3'	Reverse	3539–3509	
Pro-1	5'-TAGAGCCAACAGCCCCACCA-3'	Forward	2147–2166	2 nd round PCR
RT-20	5'-CTGCCAATTCTAATTCTGCTTC-3'	Reverse	3462–3441	
RTC1F	5'-ACCTACACCTGTCAACATAATTG-3'	Forward	2486–2508	Sequencing
RTC3F	5-CACCAGGGATTAGATATCAATATAATGTGC-3'	Forward	2956–2994	
RTC2R	5'-TGTC AATGGCCATTGTTTAACCTTTGG-3'	Reverse	2630–2604	
RTC4R	5'-CTAAATCAGATCCTACATACAAGTCATCC-3'	Reverse	3129–3101	

HXB2, nucleotide position of HIV-1 reference sequence.

Table 2

Characteristics of the 28 patients with virologic failure

Characteristics	No.	Patients (n=28) %
Sex		
Male	16	57
Female	12	43
Duration on ART		
< 1 year	1	4
1 – 6 years	11	39
> 6 years	16	57
Current Drug regimen		
ABC/3TC/ATV/r	3	11
TDF/3TC/ATV/r	18	64
TDF/3TC/ABC *	1	4
ABC/3TC/LPV/r	4	14
AZT/3TC/ATV/r	1	4
TDF/3TC/NVP *	1	4
Clinical history/Demographics		
	Median	IQR
Age in years	20	18 – 34
CD4 count, cells/mm ³	595	436.5 – 659.3
Log ₁₀ viral load, copies/ml	4.7	3.8 – 4.9
Time on ART, years	7	5.3 – 8.2

ART, antiretroviral treatment; ABC, abacavir; 3TC, lamivudine; ATV/r, atazanavir + ritonavir; TDF, tenofovir; LPV/r, lopinavir + ritonavir; AZT, zidovudine; NVP, nevirapine; IQR, interquartile range;

* patients not on PI regimens.

Table 3

Pairwise sequence identity analysis between the commercial and in-house sequences

	Commercial vs. In-house
Number of samples	23
Percentage nucleotide identity	99.51 (0.56)
Percentage amino acid identity	99.11 (0.95)
Average number of drug resistance mutations	6 vs. 6 (p=0.48)

Table 4
Samples with discordant drug resistance mutations and the consequent differences in predicted phenotypic resistance

Study ID	Protease mutations		Reverse transcriptase mutations		Discordant predictions		Phenotypic
	Commercial	In-house	Commercial	In-house	Commercial	In-house	
NC1	M46L, I54V, V82A, L10V, L33F	M46L, I50L* , I54V, V82A, L10V, L33F	M41L, M184V, T215F, A98G, K103N	M41L, M184V, L210LW* , T215F, A98G, K103N	ABC: I DDI: I TDF: I	ABC: H DDI: H TDF: H	
NC6	M46L, T74P	M46L, L24H* , Q58EQ* , T74P	D67N, K70E, M184V, K101E, V106I, G190A	D67N, K70E, M184V, K101E, V106I, G190A	LPV/r: I SQV/r: L	LPV/r: H SQV/r: I	
NC7	L10F, L33I, Q58E, G73T	I54IV* , L10FT* , L33I, Q58E, G73T	D67N, K70R, M184I, K219Q, V90I, K103N, Y181C	D67N, K70R, M184I, K219Q, V90I, K103N, Y181C	ATV/r: L IDV/r: L LPV/r: P SQV/r: L TPV/r: L	ATV/r: I IDV/r: I LPV/r: L SQV/r: I TPV/r: I	
NC14	None	None	A98G, V106M, Y181C, G190A	A98G, V106M, Y181C, G190A, H221HY*	EFV: H ETR: H NVP: H RPV: H	EFV: H ETR: H NVP: H RPV: H	
NC22	None	None	G190A	M184MV* , G190A	3TC: S ABC: S DDI: S FTC: S	3TC: H ABC: L DDI: P FTC: H	
NC28	None	None	M184V, V90I* , K103N, Y181C, Y188L* , H221Y*	M184V, K103N, Y181C	RPV: H	RPV: I	

Note: Alphanumeric characters in bold and with an asterisk* represent the discordant mutations.

ABC, abacavir; DDI, didanosine; TDF, tenofovir; LPV/r, lopinavir + ritonavir; SQV/r, saquinavir + ritonavir; ATV/r, atazanavir + ritonavir; IDV/r, indinavir + ritonavir; LPV/r, lopinavir + ritonavir; TPV/r, tipranavir + ritonavir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; RPV, rilpivirine; 3TC, lamivudine; FTC, emtricitabine; S, susceptible; P, potential low-level resistance; L, low-level resistance; I, intermediate resistance; H, high-level resistance.