A Pragmatic Approach to HIV-1 Drug Resistance Determination in Resource-Limited Settings using a Novel RT-only Genotyping Assay

Susan C. Aitken[1#], Michelle Bronze[2], Carole L. Wallis[3], Lieven Stuyver[4], Kim Steegen[2,5], Sheila Balinda[6], Cissy Kityo[6], Wendy Stevens[2,5], Tobias F. Rinke de Wit[7,8] and Rob Schuurman[1].

Running title: Novel HIV-1 RT-only genotyping assay for RLS

[1] University Medical Centre, Utrecht, The Netherlands;

[2] University of the Witwatersrand, Johannesburg, South Africa;

[3] Lancet Laboratories, Johannesburg, South Africa

[4] Janssen Diagnostics BVBA (formerly Virco), Beerse, Belgium;

[5] National Health Laboratory Services, South Africa;

[6] Joint Clinical Research Centre, Kampala, Uganda;

[7] Department of Global Health, Amsterdam Institute for Global Health and Development (AIGHD), Academic Medical Center (AMC), Amsterdam, the Netherlands

[8] PharmAccess International, Amsterdam, the Netherlands

[#]Corresponding author: s.c.aitken@umcutrecht.nl

Abstract

In resource-limited settings (RLS), reverse transcriptase (RT) inhibitors form the backbone of first-line treatment regimens. We have developed a simplified HIV-1 drug resistance genotyping assay targeting the region of RT harboring all major RT inhibitor resistance mutation positions, thus providing all relevant susceptibility data for first-line failures coupled with minimal cost and labour. The assay comprises a one-step RT-PCR amplification reaction, followed by sequencing using one forward and one reverse primer, generating double-stranded coverage of RT aa 41-238. The assay was optimized for all major HIV-1 group-M subtypes in plasma and dried blood spot (DBS) samples using a panel of reference viruses for HIV-1 subtypes-A-D, F-H, and CRF01_AE, and further applied to 212 clinical plasma and 25 DBS samples from HIV-1 infected individuals from Africa and Europe. The assay was subsequently transferred to Uganda and applied locally on clinical plasma samples. All major HIV-1 subtypes could be detected with an analytical sensitivity of 5.00E+03 RNA copies/ml for plasma and DBS. Application on 212 clinical samples from African subjects comprising subtypes-A-D, rare subtypes F-H, CRF01_AE and CRF02_AG, VL range 6.71E+02-1.00E+07 (median 1.48E+05) RNA copies/ml, was 94.8% (n=201) successful. Application on clinical samples in Uganda demonstrated a comparable success rate. Genotyping of clinical DBS samples, all subtype-C with VL range 1.02E+03-4.49E+05 (median 1.42E+04) RNA copies/ml, was 84.0% successful. The described assay greatly reduces hands-on time and costs required for genotyping, and is ideal for use in RLS, as demonstrated in a reference laboratory in Uganda, and its successful application on DBS samples.

Introduction

There are currently more than 3.9 million HIV-1 infected individuals receiving highly active antiretroviral treatment (HAART) in sub-Saharan Africa (1), with recent studies showing an estimated prevalence of 5.6% antiretroviral (ARV) drug resistance prior to treatment initiation, ranging from 1.1% in South Africa to 12.3% in Uganda(2). The use of HIV-1 drug resistance genotyping in resource-limited settings (RLS) is limited to clinical research studies and for pre-authorized private medical care. Limited routine resistance testing is performed due to high cost, infrastructure requirements, and complexity of available commercial assays. The use of HIV-1 drug resistance genotyping is vital in advising policy makers on the status of HIV-1 drug resistance profiles to ensure optimal HAART options are maintained, but should also be considered for individualised treatment management.

Treatment monitoring of individuals receiving therapy primarily includes clinical evaluation with or without CD4 testing. When available and affordable, it is recommended to perform viral load (VL) monitoring. Current guidelines do not recommend HIV-1 drug resistance genotyping be included as part of treatment management in RLS (3), primarily due to the cost and complexity of the assays and limited ARV drug options.

Current ARV regimens for the treatment of HIV-1 in adults and adolescents in RLS, as recommended by WHO guidelines, comprise a first-line regimen consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) (3). There is limited use of protease inhibitor (PI) containing regimens, restricted to second-line therapy, and the occurrence of PI drug resistance is still low (4, 5). According to a systematic review by Barth *et al.* on the

success of ART treatment programs in sub-Saharan Africa, 94% of African adult patients on ART received a combination of NRTI/NNRTI first-line therapy (6). When therapy failure occurs, more than 83% of subjects failing first-line regimens harbour mutations that confer resistance to both NRTIs and NNRTIs. The most common mutation observed is the M184V followed by several NNRTI mutations, such as K103N, Y181C and V106M (7). Complex resistance to NRTIs such as K65R, thymidine analogue mutations (TAMs) and Q151M are also observed, with frequencies increasing when viral load monitoring is not observed (7, 8).

All major mutations that affect efficacy of RTI therapy, as defined by the IAS drug resistance mutation list, are located between RT amino acids (aa) 41 and 238 (9). Furthermore, *in silico* analysis of genotyping profiles of this specific RT region demonstrated that the predicted drug susceptibilities were equally informative compared to sequences that more broadly cover the RT gene (aa1-400) (10). The studies therefore indicate that for failure of current ARV regimens in RLS, an HIV-1 drug resistance genotyping assay that targets the RT region only would be effective for monitoring.

As part of the ART-A initiative to develop affordable resistance testing for Africa (www.arta-africa.org), we have used this knowledge-base to design an RT specific assay for a simplified genotyping screen. For RLS application, the potential use of assay on dried blood spots (DBS) was considered an important addition. This choice, in combination with a smaller fragment for PCR amplification, has enabled the development of a test that fulfils the criteria with an analytical sensitivity of 5.00E+03 RNA copies/ml, at a more affordable cost. We have evaluated the assay on both

plasma and DBS in a WHO reference laboratory in the Netherlands and subsequently shown its application in a reference laboratory in Uganda.

Methods and Materials

Samples

A selection of reference viruses for subtypes A, B, C, D, F, G, H and CRF01_AE (*Table 1*) from the BBI panel (BBI Biotech Research Laboratories Inc., Gaithersburg, USA) was used to evaluate the sensitivity and specificity of the assay. Virus stocks were used for each subtype to prepare dilution series of plasma and spiked whole blood for DBS preparation in the concentrations of 1.00E+04, 5.00E+03, 1.00E+03 and 5.00E+02 RNA copies/ml, which were used to optimize the assay.

The assay was subsequently evaluated on a selection of clinical samples from Africa and The Netherlands that were available at the UMC Utrecht. A total of 212 samples with VL range of 6.71E+02-1.00E+07 (median 1.35E+04) RNA copies/ml, comprising subtypes-A (n=47; 22.1%), B (n=20; 9.4%) C (n=72; 33.9%), D (n=18; 8.4%), CRF01_AE (n=35; 16.5%), CRF02_AG (n=10; 4.7%), and rare subtypes H, G and F (n= 10; 4.7%), were used. For the clinical samples, plasma was separated from EDTA-tube blood and stored at -80°C prior to analysis.

The clinical samples available at the UMC Utrecht were included to assess assay application for various African subtypes. For these samples VL was determined using the COBAS® AmpliPrep/ COBAS® TaqMan® System v2 (Roche, Penzberg, Germany). These samples were obtained from two separate sources: RNA isolates previously genotyped using ViroSeq™ HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA, USA) or with a laboratory developed genotyping assay (n=88) (11); and plasma samples from a study to monitor for the development of drug resistance mutations in children failing therapy in Rwanda (n=67), which were only

156 genotyped with the described method. In addition, clinical samples from South Africa
157 (n= 57) sent for routine VL testing were included. VLs were determined in South
158 Africa using NucliSENSEasyQ® HIV-1 version 1.2 (BioMérieux, Boxtel, The
159 Netherlands). Plasma samples were stored at -80°C and shipped to the Netherlands
160 on dry ice.
161
162 Application of the assay with clinical DBS samples was demonstrated with a
163 selection of 25 clinical DBS samples from South Africa sent for genotyping with a
164 laboratory developed genotyping assay at the UMC Utrecht. Selected samples had a
165 VL range of 1.02E+03 – 4.49E+05 (median 1.42E+04) RNA copies/ml, and all
166 samples were HIV-1 subtype-C.
167
168 Following development and evaluation in the Netherlands, the assay was transferred
169 to and applied in a reference laboratory in Kampala, Uganda. A total of 132 pre-
170 treatment baseline plasma samples from the PASER-M cohort (12) were used for
171 further evaluation. These samples were chosen to represent a distribution of
172 subtypes A (n=60; 45.4%), D (n=43; 32.6%), and unassigned subtype (n=29; 22.0%),
173 with a VL range of 1.05E+03-1.00E+07 (median 6.92E+04) RNA copies/ml. All of
174 these samples were isolated and amplified at the local laboratory. A selection of 50
175 amplicons was subsequently sequenced in order to confirm compatibility with the
176 local sequencing method.
177
178 *Nucleic acid purification*
179 *UMC Utrecht, The Netherlands*
180 Viral RNA was isolated using the manual NucliSens method, the MiniMAG
181 (BioMérieux). For the subtype reference panel plasma dilutions and the clinical

samples, an input volume of 100µl was used. In the case of DBS samples, two 50µl spots were used as input material. Prior to processing, the excised spots were incubated in 2ml NucliSens lysis buffer (BioMérieux) at room temperature for 30 minutes on a gentle shaker, after which filter papers were removed and isolation proceeded according to the manufacturer's instructions. Isolated RNA was eluted in 50µl elution buffer, and either used immediately (n= 67) for RT-PCR or stored at -20°C for 50-120 weeks (n= 145) prior to amplification. For each isolation, a positive and a negative control were used, consisting of subtype-C virus from the subtype panel (2.00E+04 RNA copies/ml) and negative plasma, respectively.

JCRC Kampala, Uganda

Viral RNA was isolated using the QIAamp viral RNA mini kit (Qiagen Gmbh, Germany). For the clinical samples, 100µl of plasma was used as the input volume. Isolated RNA was eluted in 50µl elution buffer, and either used immediately for RT-PCR amplification or stored at -20°C for up to 4 weeks. For each isolation, a positive and a negative control were used, consisting of HIV-1 subtype-C virus from the subtype panel and nuclease-free water, respectively.

Amplification (The Netherlands and Uganda)

Isolated viral RNA was reverse transcribed and amplified using a single-round RT-PCR encompassing the RT gene from aa 41-238. The RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Life technologies, Foster City, CA) as follows. Ten microlitres of isolated RNA was amplified in a reaction mixture consisting of 0.4µM forward primer, 0.4µM reverse primer (*Table 2*), 2xReaction buffer, 0.5µl SuperScript™ III RT/Platinum® *Taq* High Fidelity Enzyme Mix, and nuclease-free water in a final volume of 25µl. The

combined cDNA and amplification reactions were carried out in a GeneAmp 2720 thermal cycler (Life Technologies, Foster City, CA) according to the following conditions: 1 hold at 50°C for 30 minutes, 1 hold at 94°C for 2min, 2 cycles at 94°C for 15 s, 61°C for 30s and 68°C for 1min, 14 cycles at 94°C for 15s, 60°C (decrease of 0.5°C with every cycle) for 30s and 68°C for 1min, 34 cycles at 94°C for 15s, 53°C for 30s and 68°C for 1min, followed by a final extension step at 68° for 7 min.

Amplification products were visualized on an agarose gel, and positive reactions were purified using QIAquick PCR purification columns (Qiagen, Gmbh, Germany) according to the manufacturer's instructions.

Sequence analysis

Applied Biosystems based sequencing, UMC Utrecht, The Netherlands

The cycle sequencing master mix for each primer consisted of 0.2µM primer (*Table 2*), 1xBigDye sequencing buffer, 10-40ng of PCR product, 2µl Big Dye v3.1 and nuclease free water in a final volume of 20µl. The cycle sequencing reactions were performed using a GeneAmp 2720 thermal cycler, and consisted of 25 cycles as follows: 96˚C for 10 seconds, 50˚C for 5 seconds, 60˚C for 4 minutes. Reaction products were purified using an EDTA-ethanol precipitation and subsequently run on an ABI 3730 automated sequencer (Life Technologies), and analyzed using SeqScape data analysis software, v2.6 (Life Technologies).

Beckman Coulter based sequencing, JCRC Kampala, Uganda

The cycle sequencing master mix for each primer consisted of 0.2 µM primer (*Table 2*), GenomeLab DTCS Quick Start Kit mix (Beckman Coulter, Inc., Brea, CA), 10-40ng of PCR product and nuclease-free water in a final volume of 20µl. The cycle

sequencing reactions were performed using a 9800 Fast thermocycler (Life Technologies), and consisted of 30 cycles as follows: 96˚C for 20 seconds, 50˚C for 20 seconds, 60˚C for 4 minutes. Reaction products were purified using an EDTA-ethanol precipitation and subsequently read on a CEQ800 Genetic Analysis System (Beckman Coulter), and analyzed using BioEdit v7.0(13).

Phylogenetic analysis

Consensus sequences where aligned using MEGA 4.1 (http://www.megasoftware.net/) to control for contamination. To determine subtype and HIVDR profile, the NCBI subtyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and GRADE analysis programs (http://www.hiv-grade.de/grade/deployed/grade.pl?program=hivalg) were used, respectively. Consensus sequences obtained as described above, were compared at the nucleotide level to sequences for the same samples generated using both Viroseq and an in-house genotyping assay (11) for quality control purposes.

Results

Assay Design

Based on the optimal RT amplicon to contain all RT resistance mutations that are relevant after an RT based first-line failure, a single-round RT-PCR assay targeting RT aa41-238 was developed. Primers were designed using a database of 267,841 sequences from nine subtypes and seven CRFs in order to have optimal sensitivity and specificity for all major HIV-1 subtypes and CRFs.

Nucleic Acid Amplification (RT- PCR sensitivity)

The assay was optimized for amplification of plasma samples containing ≥5.00E+03 RNA copies/ml. Initial amplification of a dilution series of the subtype panel indicated that amplification from plasma virus was achievable from 5.00E+02 RNA copies/ml, and was reproducibly achievable for samples ≥5.00E+03 RNA copies/ml (*Table 3)*. Results of the dilution series of DBS samples for the subtype panel indicated reliable amplification of samples ≥5.00E+03 RNA copies/ml. All subtypes were amplified and sequenced with equal sensitivity and success rates.

Clinical Samples UMC Utrecht, The Netherlands

A total of 205 of 212 clinical samples (96.7%) were successfully amplified using the single-round RT-PCR. Of the 205 successfully amplified samples, full bi-directional sequencing of RT aa41-238 (*Table 4)* was obtained in 98% of the time *(*201 samples) giving an overall genotyping success rate of 94.8% (201/212). Of the seven samples that did not amplify, five had a VL <5.00E+03 RNA copies/ml, all subtype C, and the remaining two were subtypes C and D, with VLs of 6.30E+03 and 8.78E+03 RNA copies/ml, respectively. Four samples resulted in incomplete sequences for subtypes A (n=1), C (n=2), and D (n=1).

Sequences generated with the described method were compared to sequences previously generated (n=30), using either ViroSeq™ or the in-house assay, and demonstrated an average homology of 98.9% and 99.3% at the nucleotide level, respectively. Sequences from a total of 73 samples were compared to the in-house assay alone and demonstrated a 99.2% homology. Differences observed were almost entirely mixture calling (347/43,362 positions), with only six positions at which nucleotides were different, all being at non-resistance positions.

For the selection of 25 clinical DBS samples genotyped with the described method, amplification success rate was 95.0% above 5.00E+03 RNA copies/ml (19/20), and 88.0% above 1.00E+03 RNA copies/ml (22/25). Sequencing was 95.5% successful for amplified samples (21/22). The overall genotyping success rate was 84.0% (21/25). Generated sequences showed 97.0% nucleotide homology to previously generated sequences from plasma.

Field Implementation Kampala, Uganda

A total of 130 of 132 local plasma samples (98.5%) were amplified using the described method (*Table 5*) with the alternative viral RNA isolation method. Samples that did not provide a positive amplification result were below 5.00E+03 RNA copies/ml, however amplification of lower VL samples, range 1.05E+03-4.92E+03 (median 2.12E+03) RNA copies/ml, was also successful (15/17 samples, 88.2%). Sequencing using the Beckman Coulter sequencing method was successful, with full bi-directional sequences for the 50 amplicons processed.

Costing

The cost of this assay is lower compared to other laboratory developed assays and commercially available genotyping assays due to limiting the amplification and sequencing regions to the minimal region required for sequencing of first-line RTI therapy in RLS. The use of a single-round RT-PCR reaction, reduced amplification and elution volumes, and the need for only two sequencing reactions, equates to a reduction in reagents required. From a reagent perspective, using the described assay would result in a >75% saving compared to using a commercial assay such as Viroseq™ Genotyping System 2.0 (Celera Diagnostics, USA), and approximately a 40% saving compared to using our current inhouse assay(11). Furthermore, the shorter laboratory protocol and sequence to be analysed, results in a lowering of labour compared to currently available methods (11, 14, 15).

Discussion

We have developed a simplified, specific, lower cost assay for determination of HIV-1 drug resistance associated with first-line therapy that can be performed at reference laboratories in RLS and is suitable for use with DBS.

The unique feature of this assay which sets it apart from currently available commercial and laboratory-developed assays is the one-step RT-PCR specifically focusing on the analysis of the most relevant part of RT. The size of the amplicon is roughly half the length of those generated using commercial and in-house HIV-1 drug resistance genotyping assays (11, 15, 16). By amplifying an “as small as possible” part of RT using highly fine-tuned primer combinations, focusing on the region encompassing all relevant HIV-1 drug resistance mutations, it was possible to achieve a genotyping success rate of 94.8% for clinical plasma samples ≥1.00E+03 RNA copies/ml in a single-round RT-PCR. Subsequent sequencing requiring only a single forward and a single reverse primer, compared to four to six primers needed for commercial and in-house assays (11, 14-16), increases throughput for processing and decreases analysis time per sample. Decreasing the number of reactions required in turn decreases the overall cost of the assay as well as minimizes hands-on time, contamination risk, and turnaround time.

The described assay has been specifically designed with several key features for use in RLS. Primers were carefully designed and selected to cover all major HIV-1 group-M subtypes and CRFs, as is demonstrated in Tables 3, 4, and 5. The non-nested

approach to amplification strongly reduces the risk for sample contamination. The reduced number of reactions required for amplification and sequencing ensure efficient use of reagents and allow for greater sample throughput. To demonstrate these principals, the assay was transferred to and applied in a reference laboratory in Kampala, Uganda. Results showed a very high success rate for amplification, with 99% (n=130/132) amplification success for samples with a VL >1.00E+03 RNA copies/ml.

Genotyping from DBS has become a popular sampling method used to overcome the impediments associated with cost and logistics of transport and storage of plasma in RLS, and has been shown to give comparable results to plasma (16-18). Recently, the WHO has identified DBS as the primary sample type for genotyping in RLS (19). The described RT specific genotyping assay using a DBS dilution series and application with clinical DBS samples shows that this method displays adequate sensitivity for use with DBS samples, with 95% and 84% genotyping success for samples with a VL of ≥5.00E+03 and ≥1.00E+03 RNA copies/ml, respectively. Current research is underway in Uganda and South Africa as part of the ART-A project to demonstrate the scalability of HIV-1 drug resistance genotyping using DBS sampling. In this approach, DBS samples are collected from HIV-1 infected individuals and sent to a reference laboratory to screen for virological failure. Samples that test positive for treatment failure, classified as having a VL >5.00E+03 RNA copies/ml (3), are selected for HIV-1 drug resistance genotyping from the same DBS sample using the described assay.

HIV-1 drug resistance testing for individual patient management is currently not recommended in RLS, mostly due to cost limitations. However, targeted monitoring

and surveillance of HIV-1 drug resistance on sentinel populations is increasingly mentioned as a necessity to guide national ART programs (2). The potential use of this test in DBS-supported applications would markedly increase its application in remote-settings. This, in combination with the ability to transport specimens at ambient temperature to a centralized reference laboratory where actual HIV-1 drug resistance genotyping is performed, will contribute to more affordable population-level HIV-1 drug resistance data collection, which is a necessity to keep national ART programs effective in the longer term.

In conclusion, we have designed and tested a simplified method for HIV-1 RT drug resistance genotyping. The sensitivity, broad subtype inclusivity and compact nature of this assay makes it ideal for HIV-1 drug resistance testing in RLS. The described assay generates the most vital information necessary at a lower cost and in a shorter time compared to currently available HIV-1 drug resistance genotyping assays.

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Table 1. Summary of HIV-1 isolates in the subtype reference panel

Subtype	Strain	Country of Origin	Accession Number
A	UG275	Uganda	AB485632
B	BK132	Thailand	AY173951
C	ZB18	Zambia	AB485641
D	SE365	Senegal	AB485648
CRF01_AE	CM240	Thailand	AF067154
F	BZ126	Brazil	AY173957
G	BCF-DIOUM	Zaire	AB485661
H	BCP-KITA	Zaire	AB485665

Table 2. Amplification and sequencing primers

Primer	Position*	Length	Sequence	Application
ARTA_2611_M13-40	2611-2635	42	5'-GTTTTCCCAGTCACGACTTAAACAATGGCCATTGACAGAAGA-3'	Forward Amplification
ARTA_3349_SR	3370-3348	23	5'-ATCCCTGSRTAAATCTGACTTGC -3'	Reverse Amplification/Sequencing
M13-40		17	5'-GTTTTCCCAGTCACGAC-3'	Forward Sequencing

*According to HXB2

Table 3. Assay sensitivity determined using a dilution series, given in RNA copies/ml, performed in duplicate using isolates from the subtype panel.

Subtype	RNA copies/ml			
	10000	5000	1000	500
A	++	++	++	+-
B	++	++	++	++
C	++	++	++	-+
D	++	++	++	-+
F	++	++	++	++
G	++	++	++	++
H	++	++	++	++
CRF01_AE	++	++	++	-+

+: positive amplification; -: negative

Table 4. Amplification and sequencing results of clinical samples tested at UMCU, The Netherlands

VL Range (RNA copies/ml)	n	Subtypes* (n)							Amplification Success (%)	Bidirectional Sequencing Success (%)
		A	B	C	D	AE	AG	Rare		
>125.000	35	10	6	1	2	12	2	2	35/35 (100%)	35/35 (100%)
25.000-125.000	45	12	2	8	7	9	3	4	45/45 (100%)	44/45 (97.8%)
5.000-25.000	89	14	8	47	3	10	5	2	87/89 (97.8%)	84/87 (96.6%)
1.000-5.000	43	11	4	16	6	4	0	4	38/43 (88.4%)	38/38 (100%)
Total	212	47	20	72	18	35	10	12	205/212 (96.7%)	201/205 (98.1%)

VL: Viral load; n: number of samples; * Indicated subtypes as assigned by both NCBI and GRADE.

Table 5. Amplification results of clinical samples tested at JCRC, Uganda

VL Range (RNA copies/ml)	n	Subtypes* (n)			Amplification Success (%)
		A	D	X	
>125.000	48	23	14	11	48/48 (100%)
25.000-125.000	36	14	13	9	36/36 (100%)
5.000-25.000	31	19	11	1	31/31 (100%)
1.000-5.000	17	4	5	8	15/17 (88.2%)
Total	132	60	43	29	130/132 (98.5%)

VL: Viral load; n: number of samples; * Indicated subtypes previously assigned;
X: unassigned subtype, not previously sequenced.