



Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Short communication

Affordable in-house antiretroviral drug resistance assay with good performance in non-subtype B HIV-1[☆]

Carole L. Wallis^{a,*}, Maria A. Papathanasopoulos^a, Shabir Lakhi^b, Etienne Karita^c, Anatoli Kamali^d, Pontiano Kaleebu^d, Eduard Sanders^e, Omu Anzala^f, Linda-Gail Bekker^g, Gwynn Stevens^h, Tobias F. Rinke de Wit^{i,j}, Wendy Stevens^{a,k}

^a Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, South Africa

^b Zambia Emory HIV Research Project (ZEHRP), Lusaka, Zambia

^c Project San Francisco (PSF), Kigali, Rwanda

^d MRC/UUVRI Uganda Virus Research Unit on AIDS, Uganda

^e Centre for Geographic Medicine Research-Coast (CGMRC), Kenya Medical Research Institute (KEMRI), Kilifi, Kenya

^f Kenya AIDS Vaccine Initiative, Nairobi, Kenya

^g Desmond Tutu HIV Foundation, Cape Town, South Africa

^h International AIDS Vaccine Initiative Johannesburg, South Africa

ⁱ Linking African and Asian Societies for an Enhanced Response to HIV/AIDS (LAASER)

^j PharmAccess Foundation, Amsterdam, The Netherlands

^k National Health Laboratory Services (NHLS), Johannesburg, South Africa

ABSTRACT

Article history:

Received 3 April 2009

Received in revised form 20 October 2009

Accepted 5 November 2009

Available online xxx

Keywords:

HIV-1 subtype C

Antiretroviral drug resistance

Mutation profile

Affordable

The introduction of antiretroviral (ARV) therapy in resource-poor settings is effective in suppressing HIV-1 replication and prolonging life of infected individuals. This has led to a demand for affordable HIV-1 drug resistance assays, since treatment failure due to development of drug resistance is common. This study developed and evaluated an affordable “in-house” genotyping assay to monitor HIV-1 drug resistance in Africa, particularly South Africa. An “in-house” assay using automated RNA extraction, and subtype C specific PCR and sequencing primers was developed and successfully evaluated 396 patient samples (viral load ranges 1000–1.6 million RNA copies/ml). The “in-house” assay was validated by comparing sequence data and drug resistance profiles from 90 patient and 10 external quality control samples to data from the ViroSeqTM HIV-1 Genotyping kit. The “in-house” assay was more efficient, amplifying all 100 samples, compared to 91 samples using Viroseq. The “in house” sequences were 99.2% homologous to the ViroSeq sequences, and identical drug resistance mutation profiles were observed in 96 samples. Furthermore, the “in-house” assay genotyped 260 of 295 samples from seven African sites, where 47% were non-subtype C. Overall, the newly validated “in-house” drug resistance assay is suited for use in Africa as it overcomes the obstacle of subtype diversity.

© 2009 Published by Elsevier B.V.

[☆] *Support for the work:* The majority of the work was supported by the CIPRA-SA “Safeguard the Household” award (1U19 AI53217-01). Partial funding was received from the PharmAccess Studies to Evaluate Resistance (PASER) project, which is part of the Linking Asian and African Societies for an Enhanced Response to HIV/AIDS (LAASER) program. LAASER is a collaboration of the Dutch Aids Fonds, The Foundation for AIDS Research/TREAT Asia, PharmAccess Foundation and the International Civil Society Support group. LAASER is funded by a 65% grant of the Dutch Ministry of Foreign Affairs (grant 12454) and the Netherlands Organization for Scientific Research/Netherlands Foundation for the Advancement of Tropical Research (NWO/WOTRO), under the Netherlands African Partnership for Capacity Development and Clinical Interventions against Poverty related Diseases (NACCAP) for the Affordable Resistance Test for Africa (ARTA) project (grant: W.07.05.204.00). Evaluation of different subtypes performance on this assay was made possible with funding from the International AIDS Vaccine Initiative, including funding from USAID Grant Number: GPH-G-00-01-0004-00. The contents of this manuscript are the respon-

The introduction of highly active antiretroviral therapy (HAART) in patients with AIDS is effective in suppressing HIV viral replication and prolonging life (Fauci et al., 1996). However, failure of antiretroviral (ARV) treatment may arise as a result of drug toxicity, lack of adherence to a drug regimen and the development of ARV drug resistance. Knowledge of the ARV drug resistance profile is important for the clinical management of patients. The U.S. Department

sibility of the authors and do not necessarily reflect the views of USAID or the US government.

* Corresponding author at: B22, 3rd Floor Wits Medical School, Department of Molecular Medicine & Hematology, University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa. Tel.: +27 11 489 8567; fax: +27 11 484 5812.

E-mail address: cwallis@mweb.co.za (C.L. Wallis).

of Health and Human Services and other international agencies in industrialized countries recommend evaluation and monitoring of ARV drug resistance in patients prior to and during ARV therapy (Hirsch et al., 2008). In contrast, the World Health Organization (WHO) recommends that resistance testing should be performed for drug resistance surveillance purposes rather than for individual patient monitoring in resource constrained settings (Bennett, 2006).

Drug resistance testing can be performed using either phenotypic or genotypic assays. Generally, population-based genotyping assays are favored since they identify both novel and known mutations. Most genotyping methods detect HIV drug resistance by sequencing the protease (PR) and reverse transcriptase (RT) regions of the *pol* gene. There are currently two FDA approved, commercially available genotyping methods, ViroSeq™ HIV-1 Genotyping Version 2.7 (Applied Biosystems, Foster City, CA, USA) and TruGene (Siemens, Deerfield, IL USA). Commercial and “in-house” sequencing methods are characterized by manual viral RNA extraction steps (Eshleman et al., 2004; Kuritzkes et al., 2003; Lee et al., 1999; Loubsher et al., 2004) and often have a nested amplification step (Lee et al., 1999; Loubsher et al., 2004), which are ill-suited practices for large volumes of routine diagnostic work. Several laboratories have reported improved sensitivity using ‘in-house’ assays, compared to the ViroSeq assay in non-B HIV-1 subtypes (Engelbrecht et al., 2007). Thus, a number of research groups have developed “in-house” genotyping assays (Lee et al., 1999; Loubsher et al., 2004) to overcome cost and subtype-specific constraints.

HIV-1 subtype C is the predominant subtype circulating in the heterosexual population in South Africa, and worldwide. However, the ViroSeq™ HIV-1 Genotyping System does not genotype this subtype as well as subtype B. Two studies found that this genotyping assay only successfully sequenced 69% (Eshleman et al., 2004) and 66.2% (Engelbrecht et al., 2007) of HIV-1 subtype C samples. To date, over 560,000 patients with AIDS have enrolled on the South African National Treatment program (International Treatment Preparedness Coalition, 2007). However, this program makes no provision for HIV-1 drug resistance monitoring. Since studies have shown that 80% of patients failing therapy have HIV-1 drug resistance mutations (Marconi et al., 2008; Wallis et al., in press), continued surveillance and/or individual HIV-1 drug resistance monitoring will need to be used to help clinical management of patients. This is only possible if a robust, simple, affordable HIV-1 subtype C-specific genotyping method can be developed and implemented. This study focuses on the development and validation of an HIV-1 subtype C specific genotypic drug resistance assay for use in South African patients failing HAART.

Three hundred and ninety-five samples were used in the analysis of the “in-house” assay. Ninety plasma samples were obtained from patients samples sent for routine HIV-1 drug resistance testing (viral load >1000 RNA copies/ml) and were used to validate the “in-house” drug resistance assay. In addition, 10 samples from external quality assurance panels obtained from the National Institutes of Health Virology Quality Assurance (VQA) Laboratory, USA were used to validate the “in-house” method. The 10 samples had viral loads ranging from 3557 to 57,005 copies/ml, and were of known subtype (four subtype B, three subtype C, one subtype A1, one subtype A2 and one subtype F) and resistance profiles. Results from these panels were forwarded to the VQA for independent analysis of the “in-house” drug resistance assay. Lastly, 295 recently infected patient samples from seven African sites in Kilifi and Kangemi in Kenya, Kigali in Rwanda, Cape Town in South Africa, Masaka and Entebbe in Uganda and Lusaka and Copperbelt in Zambia, where patients are known to be infected with C and non-C subtypes, were used to determine the impact of HIV-1 subtype on the “in-house” drug resistance assay.

Ethical clearance for the use of patient sample material was obtained through the human ethics committee of the University of the Witwatersrand (ethics clearance number: M061025) and ethical clearance was obtained from the participating African sites.

Viral RNA from the 90 patients and 10 VQA plasma samples was extracted, amplified and sequenced using the ViroSeq™ HIV-1 Genotyping System Version 2.0 (Celera Diagnostics, Alameda, CA, USA; for purposes of this study considered the reference method), as per the manufacturer’s instructions. The sequences obtained were assembled and analyzed on the ViroSeq™ HIV-1 Genotyping System Software Version 2.7 (Applied Biosystems, Foster City, CA, USA). The *fasta* sequences created from ViroSeq were submitted to the Stanford Database (<http://hivdb.stanford.edu/index.html>) to generate an HIV-1 drug resistance report. The Rega HIV-1 subtyping tool (<http://hivdb.stanford.edu/>) on the Stanford database was used to designate the HIV-1 subtype of each patient sample.

Optimal extraction and PCR amplification protocols for the “in-house” drug resistance assay were developed. Viral RNA was extracted from all the 395 plasma samples using the automated Roche MagNa Pure LC analyzer (Roche, Germany) and the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche, Germany), according to manufacturer’s instructions. PCR and sequencing primers were designed from the 2004 consensus HIV-1 subtype C sequence available on the Los Alamos Database (www.hiv.lanl.gov/). Extracted viral RNA was synthesized into cDNA using the reverse primer CWR1 (5′-GCA TAC TTY CCT GTT TTC AG-3′; HXB2 nucleotide position 3610–3594) and the Expand Reverse Transcriptase (RT) kit (Roche Diagnostics, Germany), as per manufacturer’s instructions. Amplification was performed using primers CWF1 (5′-GAA GGA CAC CAA ATG AAA GAY TG-3′; HXB2 nucleotide position 2047–2066) and CWR1 and 20 μl of synthesized cDNA with the Expand High Fidelity^{PLUS} kit (Roche, Germany), as per manufacturer’s instructions. The cycling conditions consisted of an initial denaturation step of 94 °C for 2 m, followed by 10 cycles of 94 °C for 30 s, 54.5 °C for 30 s and 72 °C for 2 m and then 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 m increasing by 10 s each cycle, and a final elongation step of 72 °C for 10 m. The resulting amplicon (approximately 1.5 kb in length) was purified using Microcon-100 microconcentrators (Celera Diagnostics, CA, USA), and analyzed and quantified on a 2% agarose gel.

Samples were diluted according to their concentration and dideoxy (chain termination) sequencing performed using 3.2 pmol each of five sequencing primers (CWCS1: 5′-CCTCAAA-TCACTCTTTGGC-3′; HXB2 nucleotide position 2253–2271; CWCS2: 5′-AGAATCAAGACTTTTGGG-3′; HXB2 nucleotide position 2796–2814; CWCS3: 5′-TGCTGGGTGTGGTATTC-3′; HXB2 nucleotide position 2846–2830; CWCS4: 5′-TCCTGTCTCTG-CCAATTC-3′; HXB2 nucleotide position 3472–3453; CWCS5: 5′-TGGTAAATTTGATATGTCCATTG-3′; HXB2 nucleotide position 3577–3555), 13 μl of PCR product, 0.5× BigDye[®] Terminator v1.1/3.1 Sequencing Buffer and 0.5× Ready Reaction Premix from the ABI PRISM[®] BigDye[®] Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl, with standard cycle sequencing conditions as per manufacturer’s instructions. Unincorporated ddNTPs were then removed by precipitation with 80% isopropanol, and the dried pellet was resuspended in 20 μl of Hi-Di[™] Formamide (Applied Biosystems, UK). Sequence data were obtained using an ABI 3100 genetic analyzer and edited using the Sequencing analysis 3.3 program (Applied Biosystems, Foster City, CA, USA). Complete sequences encompassing the *pol* region of interest were assembled and manually edited using Sequencher version 4.7 (Genecodes, MI, USA). The ViroSeq and “in-house” sequences were generated at different time points and phylogenetic tree analysis was performed to ensure there was no cross-contamination of samples. The “in-house” sequence data were submitted to the Stanford Database

to generate an HIV-1 drug resistance report and to determine subtype, as described previously.

Ninety (ViroSeq) patient samples genotyped previously, with viral loads ranging from 1300 to 1.6 million RNA copies/ml, were used to successfully validate the “in-house” HIV-1 drug resistance assay. No samples below 1000 RNA copies/ml were tested in either assay. This is not a limitation to the study as clinical management of patients in resource constraint countries is not affected below this point. All 90 samples were amplified successfully using the “in-house” HIV-1 drug resistance amplification step, whereas nine of the 90 samples (10%) could not be amplified using the ViroSeq method. The subtype distribution was as expected with 96.7% (n=87) of the samples being classified as HIV-1 subtype C. Two of the three remaining samples were classified as HIV-1 subtype B and one as subtype A. The nine samples that failed to amplify on ViroSeq had viral loads ranging from 2300 to 627,000 RNA copies/ml, indicating that primer mismatch in the amplification step rather than a low viral load were likely responsible for lack of amplification. Subtype analysis of these nine sequences generated using the “in-house” assay revealed they were all subtype C. Furthermore, the drug resistance profiles generated for these nine patient samples indicated that 78% (n=7) had mutations that would result in resistance to both nucleoside and non-nucleoside reverse transcriptase inhibitors, indicating the importance of analyzing successfully these samples.

The nucleotide sequences of the 81 samples that were amplified successfully using both HIV-1 drug resistance assays were compared and found to have an average sequence homology of 99.20%, using the Hamming distance algorithm. Seventy-six of the 81 samples were found to have identical mutation profiles generated by the two HIV-1 drug resistance assays. Of the five that were different all had additional mutations that were present in mixtures, indicating the presence of minority quasispecies harboring drug resistance mutations. Phylogenetic tree analysis revealed the high level of homology was a result of unique sequences.

Of the 100 patient samples sequenced with the “in house” drug resistance assay, 14 did not have complete bidirectional sequenc-

ing data. Sequence primer mismatch analysis was performed on these 14 samples by aligning the single sequence fragment with the specific sequencing primer that failed to amplify using Clustal W (Fig. 1(a-c)). Primer CWCS1 was found to successfully sequence 100 out of 100 (100%) of the patient samples, CWCS2 was unable to sequence 12 of the 100 samples (12%), CWCS3 was unable to sequence seven of the 100 samples (7%), CWCS4 was unable to sequence three of the 100 sequences (3%) and CWCS5 was unable to sequence two of the 100 samples (2%). Alternative sequencing primers, including degenerate primers to overcome HIV-1 diversity, are being investigated currently to ensure full bidirectional coverage of the region of interest.

The “in-house” assay amplified successfully all samples in the VQA panels and the external analysis revealed a high sequence homology compared to sites partaking in the panels. Most importantly, the mutation patterns were comparable to the other laboratories partaking in the panels, resulting in the “in-house” assay described here being certified by the NIH VQA.

The “in-house” drug resistance assay was evaluated further for its suitability in amplifying and sequencing non C HIV-1 subtypes from other African sites. The “in-house” assay was performed on 295 samples from seven African sites to determine the impact of subtype sequence variation. Two hundred and sixty of the 295 (88%) samples were successfully amplified and sequenced using the “in-house” method. Subtype analysis revealed that the subtype distribution was 53% subtype C (n=138), 29% subtype A1 (n=76), 9% subtype D (n=23), 1% subtype B (n=3) and 8% were unassigned (n=20). Thirty-five of the 295 samples (12%), failed to amplify using the “in-house” drug resistance assay. Of these 35, 12 were amplified successfully using the ViroSeq assay. No further attempts to amplify the remaining 23 samples were undertaken. Subtype analysis of the 12 sequences revealed that six were subtype A1, two were subtype C, one was subtype D and three were unable to have their subtype assigned. This indicates that the newly developed “in-house” drug resistance assay can be used for HIV-1 subtypes other than subtype C and can be used in countries with a high prevalence of subtype A1 and D.

```

a) Primer CWCS2 -----AGAACTCAAGACTTTTGGG-----
TJC2394970  AT TTCAGGGA ACTCAATAAAAAGAACTCAAGACTTTTGTGAGGTTCAATTAGG Subtype C
GT00253238  AT TTCAGGGA ACTCAATAAAAAGAACTCAAGACTTTTGGGAGGTTCAATTAGG Subtype C
TJC2407731  AT TTCAGGGA ACTTAATAAAAAGAACTCAAGACTTTTGTGAAGTCCAATTAGG Subtype C
GT00317006  AT TTCAGGGA ACTTAATAAAAAGAACTCAAGACTTTTGTGAGTCCAATTAGG Subtype C
TJC2402982  AT TTCAGGGA ACTCAATAAAAAGAACTCAAGACTTTTGTGAAGTCCAATTAGG Subtype C
TJC2440440  AT TTCAGGGA ACTCAATAAGAGAACTCAGGACTTTTGGGAAGTTCAGTTAGG Subtype C
TJC2355930  AT TTCAGGGA ACTCAATAAAAAGAACTCAGGACTTTTGGGAAGTTCAGTTAGG Subtype C
GT00352161  AT TTCAGGGA ACTTAATAAAAAGAACTCAGGACTTTTGGGAAGTTCAGTTAGG Subtype C

```

Fig. 1. Alignment of sequencing primers CWCS2 (a), CWCS3 (b) and CWCS4 (c) and patient samples sequences to show primer/sample sequence mismatches. Patient nucleotide sequences that are different from the primer sequences are highlighted. The majority of the samples had mismatches with the primers which would influence primer binding, and subsequent sequencing. Two of the samples which had mismatches were subtype B (TJC2410296) and A1 (TJC2427332).

Overall, results demonstrate the “in-house” approach used in this study amplifies successfully more HIV-1 subtype C samples than ViroSeq, which can be attributed to the use of subtype C specific primers. Moreover, the newly developed and validated “in-house” drug resistance assay performs well on HIV-1 subtypes A1, D and unique and circulating recombinant forms present throughout Africa. It should be noted that all currently available assays (commercial and “in-house” assays) are unable to successfully amplify 100% of patient samples tested, mainly due to the high genetic variability of HIV-1. Alternative strategies to genotype samples that cannot be amplified need to be investigated. The use of an automated extraction method may result in more efficient isolation of minority quasispecies, which were detected as mixtures in the “in-house” assay in some patients. Detection of these extra mutations/polymorphisms did not alter the patient’s antiretroviral drug resistance profiles; however their presence may influence future treatment outcome. The automated extraction method allows for increased sample throughput (30 test samples and two controls to be isolated in 90 m) and reduced risk of cross-over contamination. The use of only 5 primers to generate a fully bidirectional sequence with coverage of PR 1-99 and RT 1-240 increases the number of samples that can be sequenced on one 96 well plate (19 samples as opposed to 13 using ViroSeq), thereby improving assay cost-effectiveness. However, it should be noted that although the “in-house” drug resistance assay costs a third of the commercially available genotyping methods in South Africa, the initial cost of expensive instrumentation and subsequent maintenance costs may limit its widespread use.

Overall, we have developed and validated a more affordable “in-house” drug resistance assay designed specifically for HIV-1 subtype C that can be used to effectively monitor patients failing ARV therapy, as well as to collect surveillance data on the emergence and transmission of HIV-1 drug resistance isolates within the South and southern African population where subtype C predominates. Future work should focus on improving the performance of this assay in non HIV-1 subtype C samples. If necessary, subtype-specific primers can be designed for implementation of this assay format for different geographic regions.

Uncited reference

Johnson et al. (2008)

References

- Bennett, D.E., 2006. The requirement for surveillance of HIV drug resistance within antiretroviral rollout in the developing world. *Curr. Opin. Infect. Dis.* 19, 607–614.
- Engelbrecht, S., Van Zyl, G., Claassen, M., Laten, A., Jacobs, G., Preiser, W., 2007. A sensitive in-house RT-PCR assay for the assessment of HIV-1 Antiretroviral drug resistance. *SA-AIDS*, 528.
- Eshleman, S.H., Hackett Jr., J., Swanson, P., Cunningham, S.P., Drews, B., Brennan, C., Devare, S.G., Zekeng, L., Kaptue, L., Marlowe, N., 2004. Performance of the Celera Diagnostics ViroSeq HIV-1 genotyping system for sequence-based analysis of diverse human immunodeficiency virus type 1 strains. *J. Clin. Microbiol.* 42, 2711–2717.
- Fauci, A.S., Pantaleo, G., Stanley, S., Weissman, D., 1996. Immunopathogenic mechanisms of HIV infection. *Ann. Intern. Med.* 124, 654–663.
- Hirsch, M.S., Gunthard, H.F., Schapiro, J.M., Brun-Vezinet, F., Clotet, B., Hammer, S.M., Johnson, V.A., Kuritzkes, D.R., Mellors, J.W., Pillay, D., Yeni, P.G., Jacobsen, D.M., Richman, D.D., 2008. Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an International AIDS Society-USA panel. *Clin. Infect. Dis.* 47, 266–285.
- International Treatment Preparedness Coalition, 2007. Missing the target number 4: time is running out to end AIDS-treatment and prevention for all!
- Johnson, V.A., Brun-Vezinet, F., Clotet, B., Gunthard, H.F., Kuritzkes, D.R., Pillay, D., Schapiro, J.M., Richman, D.D., 2008. Update of the drug resistance mutations in HIV-1: spring 2008. *Top. HIV Med.* 16, 62–68.
- Kuritzkes, D.R., Grant, R.M., Feorino, P., Griswold, M., Hoover, M., Young, R., Day, S., Lloyd Jr., R.M., Reid, C., Morgan, G.F., Winslow, D.L., 2003. Performance characteristics of the TRUGENE HIV-1 genotyping kit and the Opengene DNA sequencing system. *J. Clin. Microbiol.* 41, 1594–1599.
- Lee, S., Espin, F., Turner, J., Griswold, M., Kahn, D., 1999. Genotyping of HIV-1 drug-resistance: comparison between in-house procedure and visible genetics Trugene[Tm] HIV-1 assay. *Abstr. Intersci. Conf. Antimicrob. Agents Chemother.* 39, 462.
- Loubsher, S., Sherman, G., Chezzi, C., Jones, J., Cohen, S., Puren, A., Morris, L., 2004. Characterization of Nevirapine resistance mutations using RT-PCR and DNA sequencing methods in a mother-infant cohort following single dose Nevirapine. *Antivir. Ther.* 9, S145.
- Marconi, V.C., Sunpath, H., Lu, Z., Gordon, M., Koranteng-Apeagyei, K., Hampton, J., Carpenter, S., Giddy, J., Ross, D., Holst, H., Losina, E., Walker, B.D., Kuritzkes, D.R., 2008. Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal. *S. Afr. Clin. Infect. Dis.* 46, 1589–1597.
- Wallis, C.L., Mellors, J.W., Venter, W.D.F., Sanne, I., Stevens, W., 2009. Varied drug resistance profiles following first-line regimen failure in the South Africa Antiretroviral Program. *J. AIDS*, in press.