RESEARCH ARTICLE

REVISED Use of a mutation-specific genotyping method to assess for HIV-1 drug resistance in antiretroviral-naïve HIV-1 Subtype C-infected patients in Botswana [version 2; peer review: 2 approved]

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V2 First published: 28 Sep 2020, 3:50
<https://doi.org/10.12688/aasopenres.13107.1>

Latest published: 07 May 2021, 3:50
<https://doi.org/10.12688/aasopenres.13107.2>

Abstract

Background: HIV-1 drug resistance poses a major threat to the success of antiretroviral therapy. The high costs of available HIV drug resistance assays prohibit their routine usage in resource-limited settings. Pan-degenerate amplification and adaptation (PANDAA), a focused genotyping approach based on quantitative PCR (qPCR), promises a fast and cost-effective way to detect HIV drug resistance mutations (HIVDRMs). Given the high cost of current genotyping methods, we sought to use PANDAA for screening key HIVDRMs in antiretroviral-naïve individuals at codons 103, 106 and 184 of the HIV-1 reverse transcriptase gene. Mutations selected at these positions have been shown to be the most common driver mutations in treatment failure.

Methods: A total of 103 samples from antiretroviral-naïve individuals previously genotyped by Sanger population sequencing were used to assess and verify the performance of PANDAA. PANDAA samples were run on the ABI 7500 Sequence Detection System to genotype the K103N, V106M and M184V HIVDRMs. In addition, the cost per sample and reaction times were compared.

Results: Sanger population sequencing and PANDAA detected K103N mutation in three (2.9%) out of 103 participants. There was no evidence of baseline V106M and M184V mutations observed in our study. To genotype the six HIVDRMs it costs approximately 40 USD using PANDAA, while the reagents cost per test for Sanger population

Open Peer Review

Approval Status

	1	2
version 2		
(revision)
07 May 2021	view

version 1
28 Sep 2020	view	view

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sequencing is approximately 100 USD per sample. PANDAA was performed quicker compared to Sanger sequencing, 2 hours for PANDAA versus 15 hours for Sanger sequencing.

Conclusion: The performance of PANDAA and Sanger population sequencing demonstrated complete concordance. PANDAA could improve patient management by providing quick and relatively cheap access to drug-resistance information.

Keywords

HIV-1 drug resistance testing, Assay performance, Pan-degenerate amplification and adaptation

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Competing interests: IJM is the CSO and Co-founder of Aldatu Biosciences

Grant information: DM, SM, SG were supported by Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative [grant # DEL-15-006]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust [grant # 107752/Z/15/Z] and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Maruapula D, MacLeod IJ, Moyo S *et al.* **Use of a mutation-specific genotyping method to assess for HIV-1 drug resistance in antiretroviral-naïve HIV-1 Subtype C-infected patients in Botswana [version 2; peer review: 2 approved]** AAS Open Research 2021, 3:50 <https://doi.org/10.12688/aasopenres.13107.2>

First published: 28 Sep 2020, 3:50 <https://doi.org/10.12688/aasopenres.13107.1>

REVISED Amendments from Version 1

We have revised the manuscript with specific suggestions in the same line, as suggested by the reviewers. Some of the recommendations were also re-written into more realistic phrases. We have re-edited the text and added additional explanations in the introduction highlighting recently published data showing that NNRTI resistance are also linked to poor response to first-line dolutegravir based regimen. In the revision, we have revised the statement “sequence detection system” by replacing it with “real-time PCR system”. We have modified Table 4 to clearly state the costing comparison. In the new version of our manuscript, we have included more parameters and clearly indicated the cost inputs for cost comparison in Table 4. We have modified the Discussion to clarify that although most patients are currently initiating on DTG based regimen in Botswana, efavirenz is also currently being used in some patients especially women of child bearing age as DTG was at some point associated with neural tube defects when taken during pregnancy. We have added a new limitation addressing the small samples with HIVDR. We expanded the last sentence in the discussion that future work will build on the findings of this study. We are thankful to the reviewers for their suggestions.

Any further responses from the reviewers can be found at the end of the article

Introduction

HIV remains a major global health problem; currently, 37.9 million adults and children are estimated to be living with HIV with sub-Saharan Africa being the most severely affected region¹. In Botswana, 380 000 people are estimated to be living with HIV of which 310 713 are on treatment². In 2016, Botswana introduced universal HIV treatment to all HIV positive individuals regardless of their immune status³. Combination antiretroviral therapy (cART) has been successful in reducing morbidity and mortality in individuals infected with HIV as well as in prevention of mother-to-child transmission (PMTCT) of HIV⁴. Despite the availability of antiretroviral drugs, which inhibits HIV replication and reducing mortality, one public health concern about the wide scale rollout of cART is the increase in emergence and transmission of HIV drug resistance⁵⁻⁷, which has the potential to reduce the efficacy and compromise the success of ART programmes^{8,9}.

Although first generation NNRTIs, Nevirapine(NVP) and Efavirenz(EFV) have been replaced by DTG as part of the first line cART regimen, presence of baseline NNRTI resistance mutations has been linked to poor response to first line DTG based regimen¹⁰, therefore it is still important to analyze NNRTI mutations that would affect the efficacy of DTG based regimen. HIV-1 reverse transcriptase, protease and integrase mutations introduced into the viral genome contribute to the development of resistance to antiretroviral drugs. Major non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations, such as K103N and V106M, are selected when HIV is exposed to nevirapine (NVP) and efavirenz, which is still used in both low and high resource settings as part of patient management. Also, resistance mutations that develop in patients exposed to the

first generation NNRTIs, NVP and EFV have been shown to confer some cross-resistance to second generation NNRTIs like etravirine and rilpivirine¹¹. M184V is a major NRTI mutation selected for under tenofovir and lamivudine. HIV drug resistance testing is routinely used for clinical care in high-income countries; however, routine HIV drug resistance testing is not available to majority of patients in resource-limited settings due to the high costs of implementation and limited trained manpower. While Sanger sequencing-based methodologies remain the gold standard for mutation detection, the assays are costly and resource-intensive. Thus, it is urgent to use a simple and cheaper detection method for HIV drug resistance. Detecting known specific mutations provides important information that guides patient treatment options. Moreover, utilising point mutation assays could provide a faster crucial information regarding the mutations present in the patient.

In this study, we compare an HIV genotyping method, pan-degenerate amplification and adaptation (PANDAA), a focused point mutation genotyping assay, with Sanger population based sequencing¹². It is anticipated that PANDAA could serve as an alternative method to rapidly detect HIV-1 drug resistance mutations in HIV patients in Botswana.

Methods

Study population

This was a retrospective study utilizing existing data and stored PCR products from 103 specimens previously genotyped by Sanger based population sequencing from a previous completed study conducted at Botswana Harvard AIDS Institute Partnership, Gaborone, Botswana: Novel strategy for HIV drug resistance monitoring in developing countries (BHP063 study)¹³. Briefly, this study enrolled 234 pregnant women diagnosed with HIV and 188 pre-ART from Infectious Diseases Care Clinics (IDCC) between 2012 and 2015. These participants were enrolled to determine the prevalence of HIV transmission at three different geographical locations in Botswana (Gaborone, Molepolole, Mochudi). In samples collected between 2014 and 2015, the following mutations were detected in the main cohort; K103N, G190A and L90M¹³.

For the current study, a convenience sampling method was employed to maximize the number of samples available for analysis and the current study used baseline samples collected between 2014 and 2015 from the main cohort, provided that the stored sample(s) were still available with sufficient remaining volume for PCR products. At the time of the current study, the first-line ART regimen in Botswana consisted of tenofovir + emtricitabine (or lamivudine) + efavirenz (or NVP).

Ethical considerations

Ethical clearance for the BHP063 study was obtained from the Human Research Development Committee (HRDC) at the Botswana Ministry of Health (Ethics permit number: HPDME 13/18/1 Vol (366). All participants consented prior to participation in the study.

The current study was approved by the University of Botswana Institute Review Board (IRB) and the Human Research Development Committee at the Botswana Ministry of Health (Ethics permit number: HPDME 13/18/1 Vol (833)) and the need for informed consent was waived since remnant plasma samples were used for this study.

RNA extraction, reverse transcription and PCR amplification

RNA extraction using EZ1 Advanced XL (Qiagen) automated instrument and PCR were performed in the main cohort as described previously¹³. The primers used were CWF1-LNA2 and CWR1-LNA3 for first round, whereas second-round primers were CWF1-LNA2 and RT20C¹³ (Table 1).

Drug resistance genotyping by population sequencing

Direct population sequencing of the pol gene was previously performed on an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator cycle sequencing kit (Life Technologies, Carlsbad, CA, USA)¹³.

PANDAA qPCR

The stored pol-derived PCR products were diluted prior to PANDAA focused genotyping.

PANDAA qPCR reactions for detecting drug-resistant point mutations K103N, M184V, V106M were performed on an ABI 7500 real-time PCR System (Applied Biosystems).

PANDAA is provided as a 10x mix of primers and probes that are specific for each DRM in three triplex qPCR reactions¹⁴. A single target codon is amplified by the PANDAA primers (proprietary properties of Aldatu Biosciences) and the wild-type variants in each patient is detected using a VIC-labelled TaqMan MGB probe, which is differentiated from the resistant

variant, which is detected by a FAM-labelled probe (Life Technologies, MA, USA). Components of the PANDAA reaction contained 5 µL buffer (kappa Probe Fast, kappa Biosystems), 1 µL PANDAA probes (VIC labelled wild-type and DRM-specific FAM-labelled) and primer mix (forward and reverse primers), 4 µL template to a final volume of 10 µL. Each sample was performed in triplicate under the following thermal cycling conditions: 98°C for 3 minutes followed by 40 cycles of 95°C for 5 seconds then 60°C for 90 seconds during which fluorescence data were acquired. Each sample was run in triplicate for each DRM. PANDAA primers include locked nucleic acids (LNAs) which increase affinity for their target sequences and contain an adaptor region (ADR) that is matched to the probe-binding site and a pan-degenerate region (PDR) that incorporates degenerate bases in the targeted primer-binding site upstream of the ADR. The principle of PANDAA is shown in Figure 1¹².

The different protocols (K103N, V106M and M184V) were performed separately, each with a corresponding set of standards.

PANDAA data analysis

The threshold was set at 0.02 and using the ABI 7500 software, raw qPCR fluorescence data were exported from Applied Biosystems SDS software to excel and Cq values were corrected for differences in probe-binding efficiencies. All reactions were performed in triplicate, and the mean of the three values was used for calculation.

Cycle quantification (Cq) values were recorded for each sample. Samples were considered positive when the amplification of the mutant was statistically significant with respect to control sample. The percent abundance of the DRM was calculated using $E^{\Delta Cq}$, whereby E is the efficiency of probe-binding, and ΔCq is the Cq difference between the wild-type and

Table 1. Detailed sequences of the primers used for PCR and sequencing¹³.

Primer Name	Primer Sequence	HXB2 position
CWF1-LNA2	5'-GAA+G+GACACCAAATGAAAGAYTG-3'	2044-2066
CWR1-LNA3	5'-G+CA+TAC+TTYCCTGTTTCAG-3'	3613-3594
CWF1	5'-GAAGGACACCAAATGAAAGAYTG-3'	2044-2066
CWCS2	5'-AGAACTCAAGA CTTTGGGG-3'	2044-2066
CWCS3	5'-TGCTGGGTGCGGTATTC-3'	3145-3129
CWCS5	5'-TGGTAAA TTTGATATGTCCAT-3'	3577-3557
Seq6	5'-CCATCCCTGTGGAAGCACATTA-3'	3008-2987
Seq2.1-F2	5'-GGCCAGGGAATTTCTTCAGAGC-3'	2120-2142
RT20C	5'-CTGCCAATTCTAATTCTG CTTC-3'	3462-3441

The primers used for Sanger sequencing are those shown in bold.

Figure 1. Overview of PANDAA. Adaptor regions of PANDAA primers that is matched to the probe-binding site and a pan-degenerate region. This figure has been reproduced with permission from MacLeod *et al.*¹².

DRM probes, after correcting for variations in probe-binding efficiency.

Reagent cost comparison

The costs of reagents were estimated according to updated prices. Cost of equipment such as ABI 3130XL and ABI 7500 real-time PCR system were not considered as these items of equipment were already available in the laboratory.

Reaction time

To establish the total time to perform each method, we considered the total time to perform genotyping method and interpretation of results.

Concordance statistics

Agreements between PANDAA and Sanger population sequencing were calculated using Cohen's kappa coefficient. The Mann-Whitney U-test was used to test for differences in CD4 counts and viral loads between the groups with drug resistance mutations and those without drug resistance mutations. Two-sided tests were used and a p-value less than 0.05 implied statistically significant differences. All statistical analysis was carried out using R version 3.5.1¹⁵, other than R^2 , which was calculated using the linear regression function in Microsoft Excel.

Results

Characteristics of participants

All participants were female. The median age was 28 (Q1; Q3: 24; 32) years (Table 2).

Performance of PANDAA

The amplification efficiency was determined by analysing serial dilutions of positive control. A linear standard curve generated from 10-fold dilution was obtained as shown in Figure 2.

PANDAA showed reproducible results when 1:1 mix of wild-type and DRM templates over a range of copy numbers tested in triplicate. The correlation of each mutant detected by PANDAA correlated with expected mutant as shown in Figure 3.

Quantification of drug resistance of patient samples by PANDAA

PANDAA was completed on patient-derived amplicons of 103 ARV naïve individuals for the K103N, V106M and M184V DRMs using PANDAA. PANDAA identified the presence of K103N in three samples. The three samples with K103N were the same samples that Sanger sequencing detected. Only wild-type sequences at codons 106 and 184 of the RT could be identified by both PANDAA and population sequencing. There

Table 2. Characteristics of participants.

Characteristics	Value
Age, median (Q1, Q3) years	28 (24, 32)
CD4+ T cell count, median (Q1,Q3) (cells/uL)	331 (207.5, 495.5)
HIV-1 RNA copies, median (Q1, Q3), log ₁₀ copies/ml (Q1,Q3)	4.1 (3.49, 4.55)

Figure 2. Standard curves generated from ten-fold serial dilutions. Correlation coefficients (r^2) were higher than 99.4%.

Figure 3. Measured mutant correlated with expected mutant. (A) K103N: $R^2=0.99339$. (B) M184V: $R^2=0.99472$.

was a complete concordance between population sequencing and PANDAA assay as PANDAA qPCR confirmed the presence of HIV drug-resistant mutations as identified by population-based sequencing as shown in Table 3.

Differences in CD4 counts and viral loads between the groups with drug resistance mutations and those without drug resistance mutations are shown in Figure 4A and Figure 4B.

Cost and time analysis of each reaction

We calculated the costs for materials and reagents including those associated with the running of samples on the ABI 3130XL sequencer.

The cost of genotyping six drug resistance mutations per patient using PANDAA is 40 USD and Sanger population sequencing

Table 3. Comparison of drug resistance mutations identified by Sanger sequencing and PANDAA.

	Assay		PANDAA		
			Yes	No	Total
K103N	Sanger	Yes	3	0	3
		No	0	100	100
M184V	Sanger	Yes	0	0	0
		No	0	103	103
V106M	Sanger	Yes	0	0	0
		No	0	103	103

Figure 4. Median CD4 (A) and median viral load (B) between samples with detected K103N and samples without K103N mutation. Samples without K103N mutation (n=100). Samples with K103N mutation (n=3)

is estimated at 100 USD per sample. The turnaround time for PANDAA and Sanger sequencing is approximately 2 hours and 24 hours, respectively (Table 4).

Discussion

Here, we show that the HIV drug resistance mutations results of PANDAA are comparable to those produced by Sanger population sequencing. Our study provides baseline data of PANDAA performance and has added an insight that monitoring HIV drug resistance mutations is possible using PANDAA. Having protocols in place for detecting HIV drug resistance mutations using fast and low-cost platforms is important for guiding treatment options and patient management, thereby achieving WHO goal of eliminating HIV by 2030.

When the duration of each method was compared, the results showed that PANDAA required the shortest time for genotyping and had the lowest cost, when compared to Sanger sequencing. It is important to note that PANDAA cost 40 USD for six relevant drug resistance mutations, thus making it much more affordable compared to Sanger sequencing which costs. Sanger sequencing is the widely used and validated method and it is still a relevant platform to use; however, using PANDAA to detect key drug resistance mutations will reduce the cost, especially in this test-and-treat era, thereby enabling quicker results to patients.

Our study had small number of positive samples used to compare the results; however, PANDAA was shown to produce concordant results with sanger sequencing. PANDAA can be considered to rapidly detect drug resistance mutations at a cheaper cost. In addition, PANDAA kit is more cost-effective, and after preparation genotyping results can be obtained in less than two hours.

Botswana has recently introduced universal HIV therapy; however, additional patients are likely to develop drug resistance and transmit these drug-resistant HIV strains to their uninfected partners. As more patients will be receiving ART in Botswana, there is a need to consider investing in fast, low-cost assays to detect mutations associated with drug resistance.

Although most patients are currently initiating on DTG based regimen in Botswana, efavirenz-based regimen is still being used for pregnant women^{16,17} and patients on TB treatment¹⁸. Common drug resistance mutations associated with resistance to efavirenz include K103N (AAA/G to AAC/T) and V106M¹⁹. The key M184V (ATG to GTG) mutation in HIV-1 RT is associated with high-level resistance to the lamivudine (3TC) and emtricitabine (FTC)²⁰; however, M184V has been shown to rapidly decay in the absence of treatment as a result of its impact on viral fitness²¹. HIVDR testing is important to clinicians for patient management, however the cost of reagents and equipment maintenance for resistance testing is the biggest obstacles in resource-limited settings.

In this study, we used PANDAA, to screen for NRTI and NNRTI drug-resistant viruses in 103 newly diagnosed HIV-infected pregnant women from the BHP063 cohort and compared the PANDAA results to those obtained by Sanger based population sequencing. Standard curves generated proved PANDAA to accurately differentiated mutants from wild type. In one hundred and three samples included in our study, the use of PANDAA assay enabled detection of K103N in 3 antiretroviral naïve individuals. Both PANDAA and Sanger sequencing did not detect any mutations at codons 106 and 184 in the HIV strains from this cohort. This study provides insights on the performance of PANDAA, a simple method that utilises primers and probes on any available real-time qPCR platform to detect key HIV drug resistance mutations.

Table 4. Comparison of sequencing cost and time required for PANDAA and Sanger population sequencing.

Sequencing method	Laboratory parameter	Time (Minutes)		Cost /sample US\$
		Estimated Hands on time	Instrument time	
Sanger Sequencing **	RNA extraction	20mins	43min	12
	RT-PCR	10mins	240min	10.68
	Nested PCR	10mins	180min	2
	Gel electrophoresis	10mins	30min	4.45
	PCR product purification	20mins	-	1.45
	Cycle sequencing	10mins	106mins	20.5
	Sequence purification	15mins	-	3.00
	Sequence detection	10mins	420mins (Overnight)	
	Data analysis	20mins		
	Total	1hr 20mins	19hrs 12min	\$54.08
PANDAA *	RNA extraction	10min	86min	12
	One-step RT-PCR	10minutes	120min	24
	Data analysis	20min	20min	
	Total	40min	4hrs 16mins	\$36

These cost do not include costs for gloves, tips and instruments.

** Batch of 13 samples and seven primers

*Batch of 32 samples in triplicate.

The data generated by our study confirm the ability of PANDAA to detect K103N HIV drug resistance mutation as a point mutation assay, and these data correspond to Sanger sequencing data. The results generated from the use of PANDAA provide evidence that this assay represents an alternative strategy for rapid, specific detection of mutations of interest. At the time the samples were collected for this study, 2014–2015, the standard of care for treatment of HIV infection in Botswana was a regimen that included tenofovir, emtricitabine, and efavirenz co-formulated into one pill, Atripla, taken once a day. By using PANDAA, we targeted the most likely mutations to develop to these medications in HIV-1 subtype C, the M184V, K103N and V106M mutations in reverse transcriptase, a targeted and cost-effective approach to genotyping is possible²².

Our study had some limitations. Firstly, we only examined the most common relevant resistant mutations, V106M, K103N and M184V of the reverse transcriptase; therefore, there was a limited number of positive mutations available for analysis. There was no clear correlation between viral load and mutations identified due to small sample size of patients with mutations. Secondly, at the time of the study we utilized

samples from ART naïve patients and not exposed to ART leading to few cases with drug resistant mutations. Another limitation in this study was the lack of samples with M184V and V106M, making it difficult to draw a conclusion on the performance of PANDAA in detecting V106M and M184V. There is a need for further studies utilising samples with more HIV drug mutations. The applicability of this assay can be demonstrated further by testing a larger number of samples with known mutations. Nevertheless, we have shown that it is possible to genotype HIV drug resistance mutations in HIV naïve individuals using PANDAA and future work will build on the findings of this study.

Conclusion

Our findings proved the potential use of PANDAA assay for testing drug resistance mutations in resource-limited settings. This study demonstrates that applying this cost-effective assay to samples from treatment-naïve individuals where background HIV drug resistance may be increasing can provide valuable insight into baseline resistance and allow for decisions to be made to ensure the best prospect of successful HIV treatment. PANDAA holds the same promise for detection of HIV DRM in patients failing ART, although the current study

did not include any participants with known ART exposure. Given the simplicity and cost-effectiveness of PANDAA, it can be performed in any laboratory with real-time PCR capability and its principle could be easily adapted to other clinically relevant point mutations. Overall, the comparative results indicate that PANDAA assay provides similar results with Sanger population sequencing at a much lower cost.

Data availability

Sequence data generated in this study has been deposited with NCBI GenBank under sequential accession numbers [MT908833–MT908846](#) and [MT919428–MT919516](#).

Figshare: Use of a mutation-specific genotyping method to assess for HIV-1 drug resistance in antiretroviral-naïve HIV-1

Subtype C-infected patients in Botswana; <https://doi.org/10.6084/m9.figshare.12644930>²³.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

The authors would like to acknowledge the study participants, principal investigator, and study coordinator from the Novel strategy completed study. We would like to also extend our acknowledgements to University of Botswana and the Botswana Harvard HIV Reference Laboratory for their support and contribution to the success of the study. We thank Aldatu Biosciences for their support throughout the experiment.

References

- UNAIDS: **UNAIDS, Global H. I. V. AIDS statistics**. 2019. (accessed 03 March 2020); [Reference Source](#)
- UNAIDS: **Country factsheets**. Botswana. 2018. (accessed 03 March 2020). [Reference Source](#)
- WHO. World Health organization: **Progress report 2016: prevent HIV test and treat all: WHO support for country impact**. World Health Organization. 2016. (accessed 27/11/2019); [Reference Source](#)
- Braitstein P, Brinkhof MW, Dabis F, *et al.*: **Mortality of HIV-1-infected patients in the first year of antiretroviral therapy: comparison between low-income and high-income countries**. *Lancet*. 2006; **367**(9513): 817–824. [PubMed Abstract](#) | [Publisher Full Text](#)
- Bennett DE, Myatt M, Bertagnolio S, *et al.*: **Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment**. *Antivir Ther*. 2008; **13**(Suppl 2): 25–36. [PubMed Abstract](#)
- Gupta RK, Jordan MR, Sultan BJ, *et al.*: **Global trends in antiretroviral resistance in treatment-naïve individuals with HIV after rollout of antiretroviral treatment in resource-limited settings: a global collaborative study and meta-regression analysis**. *Lancet*. 2012; **380**(9849): 1250–1258. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Pennings PS: **HIV Drug Resistance: Problems and Perspectives**. *Infect Dis Rep*. 2013; **5**(Suppl 1): e5. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hirsch MS, Brun-Vezinet F, Clotet B, *et al.*: **Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA Panel**. *Clin Infect Dis*. 2003; **37**(1): 113–128. [PubMed Abstract](#) | [Publisher Full Text](#)
- Clavel F, Hance AJ: **HIV drug resistance**. *N Engl J Med*. 2004; **350**(10): 1023–35. [PubMed Abstract](#) | [Publisher Full Text](#)
- Siedner MJ, Moorhouse MA, Simmons B, *et al.*: **Reduced efficacy of HIV-1 integrase inhibitors in patients with drug resistance mutations in reverse transcriptase**. *Nat Commun*. 2020; **11**(1): 5922. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Diphoko T, Gaseitsiwe S, Kasvosve I, *et al.*: **Prevalence of Rilpivirine and Etravirine Resistance Mutations in HIV-1 Subtype C-Infected Patients Failing Nevirapine or Efavirenz-Based Combination Antiretroviral Therapy in Botswana**. *AIDS Res Hum Retroviruses*. 2018; **34**(8): 667–671. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- MacLeod IJ, Rowley CF, Essex M: **PANDAA-monium: Intentional violations of conventional qPCR design enables rapid, HIV-1 subtype-independent drug resistance SNP detection**. *bioRxiv*. 2019; 795054. [Publisher Full Text](#)
- Rowley CF, MacLeod IJ, Maruapula D, *et al.*: **Sharp increase in rates of HIV transmitted drug resistance at antenatal clinics in Botswana demonstrates the need for routine surveillance**. *J Antimicrob Chemother*. 2016; **71**(5): 1361–1366. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Denaro HG M, Long JE, Renzette N, *et al.*: **Validation of PANDAA qDx HIVDR RTI a simple and scalable real-time PCR-based HIV drug resistance genotyping kit for the management of NNRTI-based ART failure**. *IAS2019 Conference on HIV science, 21-24 July 2019, Mexico City, Mexico*. Abstract number MOPEB141. 2019.
- Core TR: **R: A language and environment for statistical computing**. (Accessed 28 November 2019). *R foundation for statistical computing*. 2019. [Accessed 28/11/2019]. [Reference Source](#)
- Zash R, Jacobson DL, Diseko M, *et al.*: **Comparative safety of dolutegravir-based or efavirenz-based antiretroviral treatment started during pregnancy in Botswana: an observational study**. *Lancet Glob Health*. 2018; **6**(7): e804–e810. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Zash R, Holmes L, Diseko M, *et al.*: **Neural-tube defects and antiretroviral treatment regimens in Botswana**. *N Engl J Med*. 2019; **381**(9): 827–840. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- MoHW: **Handbook of the Botswana 2016 integrated HIV clinical care guidelines**. 2019; Accessed 27 November 2019. [Reference Source](#)
- Manasa J, Katzenstein D, Cassol S, *et al.*: **Primary drug resistance in South Africa: data from 10 years of surveys**. *AIDS Res Hum Retroviruses*. 2012; **28**(6): 558–65. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Diallo K, Götte M, Wainberg MA: **Molecular impact of the M184V mutation in human immunodeficiency virus type 1 reverse transcriptase**. *Antimicrob Agents Chemother*. 2003; **47**(11): 3377–83. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Cane PA: **Stability of transmitted drug-resistant HIV-1 species**. *Curr Opin Infect Dis*. 2005; **18**(6): 537–42. [PubMed Abstract](#) | [Publisher Full Text](#)
- Nasir IA, Emeribe AU, Ojeamiren I, *et al.*: **Human Immunodeficiency Virus Resistance Testing Technologies and Their Applicability in Resource-Limited Settings of Africa**. *Infect Dis (Auckl)*. 2017; **10**: 1178633717749597. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Maruapula D, MacLeod IJ, Moyo S, *et al.*: **Use of a mutation-specific genotyping method to assess for HIV-1 drug resistance in antiretroviral-naïve HIV-1 Subtype C-infected patients in Botswana**. *Figshare Dataset*. 2020.
- Shafer RW: **Human Immunodeficiency Virus Type 1 Drug Resistance Mutations Update**. *J Infect Dis*. 2017; **216**(suppl_9): S843–S846. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- <http://hivdb.stanford.edu/>.
- Shafer R: **Stanford HIV Drug Resistance Database**. (Accessed 02 June 2017). [Reference Source](#)

Open Peer Review

Current Peer Review Status:

Version 2

Reviewer Report 10 May 2021

<https://doi.org/10.21956/aasopenres.14334.r28573>

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Justen Manasa

Department of Medical Microbiology, College of Health Sciences, Univeristy of Zimbabwe, Harare, Zimbabwe

Vinie Kouamou

Department of Medicine, College of Health Sciences, University of Zimbabwe, Harare, Zimbabwe

The authors have addressed our previous comments adequately.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Virology, Bioinformatics, Molecular Diagnostics, HIV

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 03 November 2020

<https://doi.org/10.21956/aasopenres.14207.r28046>

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Diana B. Dickinson

Independence Surgery, Gaborone, Botswana

This is a very relevant, clearly presented and well done paper, readily reproducible, particularly in

the context of a country, Botswana, that has very strong guidelines such that there is a standard universal first line regimen for both public and private facilities. This study shows us that a cost effective and rapid way is possible to demonstrate any pre-existing drug resistant mutations in all patients pre-initiation of treatment. We would have liked to have been doing that for years, but the cost of universal genotyping using Sanger sequencing was too much for us to do routinely.

Fortunately, as this study shows, our level of transmitted drug resistance in the untreated population is low, however with the PANDAA assay as demonstrated by this study, routine screening for important mutations would be possible, still keeping to a budget, enabling rapid response if relevant mutations are detected.

Of course, now our first line has changed to an integrase inhibitor regimen, the exact point mutations would have to be changed appropriately, but as the investigators point out this is easily done.

It would also be useful to use this modified PANDAA in the rare cases of treatment failure expected, to give a rapid overview on important mutations to craft a salvage regimen while awaiting the full Sanger sequence to fine tune that regimen.

I hope that this study can be used for our national guidelines to focus more easily and effectively on specific mutations expected to be revealed by our local PANDAA modifications.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical HIV specialist with a specific interest in resistance and complicated or failing patients

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 14 October 2020

<https://doi.org/10.21956/aasopenres.14207.r27974>

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Justen Manasa

Department of Medical Microbiology, College of Health Sciences, Univeristy of Zimbabwe, Harare, Zimbabwe

Vinie Kouamou

Department of Medicine, College of Health Sciences, University of Zimbabwe, Harare, Zimbabwe

Abstract:

Background:

1. The combination of mutations evaluated in this study is suboptimal in the context of the current ISTI based regimens. In the context of a TLD regimen, a panel of mutations that includes at least the K65R mutation for TDF in addition to the M184V mutation in the current panel would be much more useful.
2. The statement that, "Mutations selected at these positions have been shown to be the most common driver mutations in treatment failure" is no longer true in the context of the current regimens. This is a historical statement and it should be clearly stated. This will allow the evaluation of mutations that are important to the current regimens.

Methods:

1. The ABI 7500 system is not a sequence detection system BUT a real time PCR system.

Results:

1. The costing comparison is between detecting six mutations using PANDAA vs Sanger sequencing. However, the study evaluated the detection of only 3 mutations?
2. The time comparison only focuses on the real time PCR vs sequencing, it would be more objective to add the first half of the work, RNA extraction to PCR.

Introduction:

1. "HIV-1 reverse transcriptase mutations introduced into the viral genome contributes to the development of resistance to antiretroviral drugs" - Mutations in the PR, Integrase genes also contribute to ART resistance.
2. The statement that, "Major non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations, such as K103N and V106M, are selected when exposed to nevirapine (NVP), which is still used in low resource settings as part of management." omits the role of EFV in the selection of those two mutations and EFV is also used in RLS. It's the drug that was in use at the time of collection of the samples used in this study.

3. "Studies have shown that >98% of patients failing first-line NNRTI regimens would have one or more drug resistance mutations, such as K103N, V106M and M184V" is highly inaccurate. I believe the statement originated from Rhee *et al.* (2015¹) which implied that the set six mutations (K65R, M184V, K103N, V016M, Y181C, and G190A) was 98% sensitive in detecting HIVDR in treatment failure in RLS at that time, taking into consideration the regimens that were is use. Furthermore, if one mentions "studies" it is expected that they provide multiple references or a single systematic review as the reference.
4. "Moreover, utilising point mutation assays reduces the need for several laboratory equipment". This statement may be misleading, especially in reference to the PANDAA assay. The major difference between PANDAA and Sequencing is the method used in the detection of the mutations. One uses real-PCR to detect specific point mutations and the other uses sequencing. The extraction, reverse transcription and PCR are similar. When doing such comparisons, one should be cognoscente of the fact that there are now low cost sequencing platforms that may be even cheaper that some of the real time PCR platforms on the market.

Methods:

1. It's not clear which other samples, other than the Gaborone ANC 2014-2015 (K103N, n=4) samples were used for this study. Please clarify this. This is key to understanding the analysis that was done, the results and their interpretation.
2. The investigators used amplicons that were generated during the sequencing process for the PANDAA evaluation. If this is correct, there is no need to provide details of RNA extraction, reverse transcription, PCR and PCR product clean up. This would have been reported already in the primary paper.
3. When reporting target regions for primers (Table 1), its important to indicate the reference, for example HXB2 if that is the reference you used.
4. Did the investigators do any sequencing for this paper, if not, there is no need to provide details of the method. Reference should be made to the primary paper.
5. "All reactions were performed in triplicate, and the mean of the three values was used for calculation", isn't it better to use median rather than mean?
6. For cost comparison, the investigators should indicate the cost inputs.
7. The analysis of the differences in CD4 counts and viral loads between the groups with drug resistance and those without, seems to be out of place. It's not indicated anyway in the objectives of the study. Nothing about this is even mentioned in the introduction. The power of this analysis is further diminished by the small number of samples with HIVDR mutations (3) in this study.

Results:

1. The investigators mention that they did an analysis to determine the "lower limit of detection" for PANDAA but the result is not indicated.

2. Turnaround time (Table 4). For the statement that the turn around time for PANDAA is 2 hrs and that of Sanger is 24 hrs to be objective, one needs to highlight that this time is just for the real time PCR vs Sequencing, excluding RNA extraction, Reverse transcription, PCR, and PCR clean up. They should also provide the sequencing protocol as well as the sequencing clean up protocol. In general the sequencing PCR should not take more than 1.5 hrs, the clean up 30 minutes and the sequencing electrophoresis, not more than 1.5 hours per injection. If you have a 16 capillary array, your turn around time will be much shorter compared to the 8 capillary array. So, it's important to indicate all this information so as to determine how the 15 hrs came about.

Discussion:

1. This evaluation or comparison would have benefited more from a sample with higher proportions of the different mutations evaluated.
2. The WHO 90-90-90 goals target the elimination of HIV as a public health threat by 2030 NOT 2020.
3. Turn around times need to be assessed in the context of the workflow in patient management. For example, if a patient has to go home and come and collect results after 48hrs, a difference of 13 hrs will not add value for the patient. However, if PANDAA enabled same day delivery of results, then, it would make a significant difference in patient management. Unfortunately the authors only focused on the time post PCR, which gives an illusion that it takes 2 hrs to get results for PANDAA. A proper assessment would have to be from RNA extraction to genotyping result.
4. The statement that, in Botswana, NVP is still being used as prophylaxis for prevention of mother to child transmission of HIV, is mostly likely inaccurate. Please provide a reference like the current HIV management guidelines for Botswana.
5. The authors properly highlight the limitations of this study, some which have been highlighted above. However these are very strong limitations which will significantly reduce the impact of this paper.

References

1. Rhee SY, Jordan MR, Raizes E, Chua A, et al.: HIV-1 Drug Resistance Mutations: Potential Applications for Point-of-Care Genotypic Resistance Testing. *PLoS One*. 2015; **10** (12): e0145772
[PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Virology, Bioinformatics, Molecular Diagnostics, HIV

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 13 Apr 2021

Dorcas Maruapula, Botswana Harvard AIDS Institute Partnership, Gaborone, Botswana

Authors' Response to Reviewers' Comments:

Manuscript Title: Use of a mutation-specific genotyping method to assess for HIV-1 drug resistance in antiretroviral-naïve HIV-1 Subtype C-infected patients in Botswana

We thank the reviewers for their valuable comments, suggestions and insights that have helped us improve our manuscript. We have revised the manuscript by clearly discussing the limitations of the method in the Discussion section. We hope the reviewers will find our revised manuscript improved and suitable for publication.

Please find below a point-by-point response to the reviewers comments.

Sincerely,
D.M

Abstract:

Background:

Reviewer's comments: *The combination of mutations evaluated in this study is suboptimal in the context of the current ISTI based regimens. In the context of a TLD regimen, a panel of mutations that includes at least the K65R mutation for TDF in addition to the M184V mutation in the current panel would be much more useful.*

Authors' response: We thank the reviewer for the suggestions. The mutations used here are important although they are not all inclusive. Future work can focus on other mutations that were not included here. It is most likely that the list of important mutations to be evaluated will always change and more mutations added to the list.

Reviewer's comments: The statement that, "Mutations selected at these positions have been shown to be the most common driver mutations in treatment failure" is no longer true in the context of the current regimens. This is a historical statement and it should be clearly stated. This will allow the evaluation of mutations that are important to the current regimens.

Author's response: We are thankful to the reviewers for the suggestions. We have indicated that the mutations selected were some of the most common driver mutations for treatment failure at the time the study was conducted and for NNRTI based ART regimen. We have also included more literature which shows that baseline NNRTI resistance are linked to poor response to first-line dolutegravir, therefore it is still important to look at NNRTI resistance mutations (MJ Siedner et al, 2020).

Methods:

1. **Reviewer's comment:** The ABI 7500 system is not a sequence detection system BUT a real time PCR system.
2. **Author's response:** We thank the reviewer for the observation. We have corrected the wording in the new manuscript.

Results:

1. **Reviewer's comment:** The costing comparison is between detecting six mutations using PANDAA vs Sanger sequencing. However, the study evaluated the detection of only 3 mutations?

Author's response: We have now clearly stated the costing comparison in the revised manuscript.

1. **Reviewer's comment:** The time comparison only focuses on the real time PCR vs sequencing, it would be more objective to add the first half of the work, RNA extraction to PCR.

Author's response: We thank the reviewer for the suggestion. We have added the first half of the work, RNA extraction to PCR into the manuscript.

Introduction:

1. **Reviewer's comment:** "HIV-1 reverse transcriptase mutations introduced into the viral genome contributes to the development of resistance to antiretroviral drugs" - Mutations in the PR, Integrase genes also contribute to ART resistance.

Author's response: We thank the reviewer for the comment. We have corrected the sentence.

1. **Reviewer's comment:** The statement that, "Major non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations, such as K103N and V106M, are selected when exposed to nevirapine (NVP), which is still used in low resource settings as part of management." omits the role of EFV in the selection of those two mutations and EFV is also used in RLS. It's the drug that was in use at the time of collection of the samples used in this study.

Author's response: We thank the reviewer for the observation. We have improved on the wording of the manuscript.

1. **Reviewer's comment:** "Studies have shown that >98% of patients failing first-line NNRTI regimens would have one or more drug resistance mutations, such as K103N, V106M and M184V" is highly inaccurate. I believe the statement originated from Rhee et al. (2015¹)

) which implied that the set six mutations (K65R, M184V, K103N, V016M, Y181C, and G190A) was 98% sensitive in detecting HIVDR in treatment failure in RLS at that time, taking into consideration the regimens that were is use. Furthermore, if one mentions "studies" it is expected that they provide multiple references or a single systematic review as the reference

Author's response: We thank the reviewer for the suggestions. We have added more references.

1. **Reviewer's comment :** *"Moreover, utilising point mutation assays reduces the need for several laboratory equipment". This statement may be misleading, especially in reference to the PANDAA assay. The major difference between PANDAA and Sequencing is the method used in the detection of the mutations. One uses real-PCR to detect specific point mutations and the other uses sequencing. The extraction, reverse transcription and PCR are similar. When doing such comparisons, one should be cognoscente of the fact that there are now low cost sequencing platforms that may be even cheaper that some of the real time PCR platforms on the market.*

Author's response: We thank the reviewer for the comment. We have corrected the wording of the sentence.

Methods:

1. **Reviewer's comment :** *It's not clear which other samples, other than the Gaborone ANC 2014-2015 (K103N, n=4) samples were used for this study. Please clarify this. This is key to understanding the analysis that was done, the results and their interpretation.*

Author's response: We thank the reviewer for the comment. We have now clearly stated the samples.

1. **Reviewer's comment :** *The investigators used amplicons that were generated during the sequencing process for the PANDAA evaluation. If this is correct, there is no need to provide details of RNA extraction, reverse transcription, PCR and PCR product clean up. This would have been reported already in the primary paper.*

Author's response: We thank the reviewer for the comment. We have highlighted in the manuscript that the amplicons generated were reported elsewhere.

1. **Reviewer's comment :** *When reporting target regions for primers (Table 1), its important to indicate the reference, for example HXB2 if that is the reference you used.*

Author's response: We thank the reviewer for the comment. We indicated the reference in the revised manuscript.

1. **Reviewer's comment :** *Did the investigators do any sequencing for this paper, if not, there is no need to provide details of the method. Reference should be made to the primary paper.*

Author's response: We thank the reviewer for the suggestions. We will update the reference of the primary paper.

1. **Reviewer's comment :** *"All reactions were performed in triplicate, and the mean of the three values was used for calculation", isn't it better to use median rather than mean?*

Author's response: We thank the reviewers for the suggestion. To enable comparisons with previous studies, we also report means.

1. **Reviewer's comment :** *For cost comparison, the investigators should indicate the cost inputs.*

Author's response: We thank the reviewer for the suggestion. We have clearly indicated the cost inputs for cost comparison.

1. **Reviewer's comment :** *The analysis of the differences in CD4 counts and viral loads between the groups with drug resistance and those without, seems to be out of place. It's not indicated anyway in the objectives of the study. Nothing about this is even mentioned in the introduction. The power of this analysis is further diminished by the small number of samples with HIVDR mutations (3) in this study.*

Author's response: We thank the reviewers for the comments. We have included the above point as a limitation in the revised manuscript.

Results:

1. **Reviewer's comment :** *The investigators mention that they did an analysis to determine the "lower limit of detection" for PANDAA but the result is not indicated.*

Author's response: We thank the reviewer for the observation. We have corrected the above text in the manuscript.

1. **Reviewer's comment :** *Turnaround time (Table 4). For the statement that the turn around time for PANDAA is 2 hrs and that of Sanger is 24 hrs to be objective, one needs to highlight that this time is just for the real time PCR vs Sequencing, excluding RNA extraction, Reverse transcription, PCR, and PCR clean up. They should also provide the sequencing protocol as well as the sequencing clean up protocol. In general the sequencing PCR should not take more that 1.5 hrs, the clean up 30 minutes and the sequencing electrophoresis, not more than 1.5 hours per injection. If you have a 16 capillary array, your turn around time will be much shorter compared to the 8 capillary array. So, its important to indicate all this information so as to determine how the 15 hrs came about.*

Author's response: We thank the reviewer for the suggestions. We have highlighted the above comment in the manuscript.

Discussion:

1. **Reviewer's comment :** *This evaluation or comparison would have benefited more from a sample with higher proportions of the different mutations evaluated.*

Author's response: We completely agree with the reviewers and we have clarified this point in the Discussion as a limitation. We had to work with the samples that we had at the time the study was conducted.

1. **Reviewer's comment :** *The WHO 90-90-90 goals target the elimination of HIV as a public health threat by 2030 NOT 2020.*

Author's response: We agree with reviewer, and we have now modified this sentence accordingly.

1. **Reviewer's comment :** *Turn around times need to be assessed in the context of the workflow in patient management. For example, if a patient has to go home and come and collect results after 48hrs, a difference of 13 hrs will not add value for the patient. However, if PANDAA enabled same day delivery of results, then, it would make a significant difference in patient management. Unfortunately the authors only focused on the time post PCR, which gives an illusion that it takes 2 hrs to get results for PANDAA. A proper assessment would have to be from RNA extraction to genotyping result.*

Author's response: A proper assessment was addressed in the revised manuscript.

1. **Reviewer's comment :** *The statement that, in Botswana, NVP is still being used as prophylaxis for prevention of mother to child transmission of HIV, is mostly likely in accurate. Please provide a reference like the current HIV management guidelines for Botswana.*

Author's response: We have modified the manuscript with citations to the statement.

1. **Reviewer's comment :** *The authors properly highlight the limitations of this study, some which have been highlighted above. However these are very strong limitations which will significantly reduce the impact of this paper.*

Author's response: We agree with the reviewer that our study has some limitations that have been highlighted in the manuscript. We however think that this is important work on the use of the groundbreaking PANDAA assay in an HIV-1 subtype C endemic setting. Future work will build on this project as more HDR mutation positions are covered by the assay.

We appreciate your valuable comments and we will address all these in the revised manuscript.

Competing Interests: No competing interests were disclosed.