Title

Feasibility and clinical relevance of genotyping samples with low-level viremia in a cohort of HIV infected South African patients on HAART

Principal Investigator: Dr Avania Bangalee[1]

Student number: 1819084

Degree: MMed (Virology)

Supervisor: Dr Lucia Hans[2] MBChB, FCPath (SA) Viro, MMed (UCT), DipObst (SA)

Co-supervisor: Dr Kim Steegen[3] PhD (Medical Science), MSc (Biomedical Science)

1. Registrar, Department of Medical Virology, National Health Laboratory Services, University of the Witwatersrand

2. Clinical Virologist, HIV Molecular Laboratory, Charlotte Maxeke Johannesburg Academic Hospital, Department of Molecular Medicine and Haematology, National Health Laboratory services and University of the Witwatersrand

3. Senior medical scientist, HIV Genotyping Laboratory, Charlotte Maxeke Johannesburg Academic hospital, Department of Molecular Medicine and Haematology, National Health Laboratory services and University of the Witwatersrand

Table of Contents

Declaration

I, Dr Avania Bangalee (student no 1819084) hereby declare that all work submitted as part of this MMed research project has been my own.

All named authors have seen and agreed to the submitted version of the manuscript; that all who are included in the acknowledgements section, or as providers of personal communications, have agreed to those inclusions; and that the material is original, unpublished and has not been submitted elsewhere.

Signed: ----------

Date: 21 February 2020

Dedication

I would like to thank my husband and son for their unwavering support and faith in my work.

In addition, I would like to thank my supervisor, Dr Lucia Hans for her guidance and feedback throughout the project.

I am grateful to my co-supervisor Dr Kim Steegen for her technical advice and expertise in the field of HIV genotyping.

Lastly, I would like to dedicate this work to the millions of people infected and affected by HIV – may we continue to combat this disease through advances in research.

Presentations/Publications from this research project:

This work was presented in part as an oral poster presentation at the 10th IAS Conference on HIV Science, Mexico City, 2019 (Abstract WEPDB0106).

This manuscript will be submitted to the Journal of Antimicrobial Chemotherapy for publication and is therefore in the required format as per journal guidelines.

Abstract

Objectives

The feasibility of HIV genotyping at low-level viraemia (LLV) using an in-house assay in a South African population was assessed and the prevalence as well as the clinical relevance of drug resistance (HIVDR) in this population was determined.

Methods

We conducted an observational, retrospective, cohort study on patient samples with LLV referred for routine HIVDR testing at a public sector Johannesburg laboratory from August 2017 to October 2018. Genotyping was performed using a nested RT-PCR assay. The genotyping success rate was evaluated for different viraemia categories. HIV-1 drug resistance analysis was done using Sanger sequencing and sequences were loaded onto the Stanford HIVdb genotypic resistance tool (v 8.7) for drug resistance interpretation.

Results

Plasma samples from 159 HIV-1 infected, treatment-experienced adults with LLV were analysed. The in-house assay performed well with an overall success rate of 78.6% (125/159, 95% CI 71.6 – 84.3). The prevalence of drug resistance mutations in the LLV cohort was 79.2% (99/125, 95% CI 71.2 – 85.4) with most patients (n = 109, 68.6%) on a PI-based regimen at the time of genotyping. Of 125 sequences obtained, 73.6% (92/125) had ≥1 NRTI mutation while 70.4% (88/125) had ≥1 NNRTI mutation. Importantly, major PI mutations including M46I and V82A were detected in 7.2% (9/125) of patients.

Conclusion

Current South African virological failure guidelines may keep patients on failing regimens for longer than necessary. Our data suggests that genotyping at LLV is feasible and implementation could result in earlier identification and referral of patients requiring third - line regimens.

List of Tables:

List of Figures:

List of Abbreviations

HIV	Human Immunodeficiency Virus
ART	Antiretroviral therapy
WHO	World Health Organization
UNAIDS	The Joint United Nations Programme on HIV/AIDS
DRM	Drug resistance mutation
DR	Drug resistance
NRTI	Nucleos(t)ide reverse transcriptase inhibitor
NNRTI	Non-nucleos(t)ide reverse transcriptase inhibitor
TDF	Tenofovir
3TC	Lamivudine
FTC	Emtricitabine
EFV	Efavirenz
PI	Protease inhibitor
VF	Virological failure
VL	Viral load
LLV	Low-level viraemia
HAART	Highly active antiretroviral therapy
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
cDNA	Complementary DNA
aa	Amino acid
CD4	Cluster of differentiation
pLLV	Persistent low-level viraemia
sLLV	Single-episode low-level viraemia
InSTI	Integrase strand transfer inhibitor

Introduction

The rapid roll-out of antiretroviral therapy (ART) and the test and treat strategy has translated to a significant decline in AIDS related mortality.[1] According to the 2017 South African National HIV Prevalence, Incidence, Behaviour and Communication survey, 71% of adults were on ART. [2] This falls short of the UNAIDS 90-90-90 treatment goals, however it reflects the rapid scale-up of ART as national policy aligned with the test and treat strategy advocated by the WHO in 2016. [3] Thus, as more patients are enrolled in the ART programme, there will be a corresponding need for monitoring tools, including increasing access to HIV-1 drug resistance testing for patients failing therapy.

Current South African guidelines for adults, which were in line with WHO recommendations at the time of the study, advocate a first-line regimen consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI). [4] This is available as a daily fixed dose combination of tenofovir (TDF) with emtricitabine (FTC) plus efavirenz (EFV). Second-line therapy is based on a boosted protease inhibitor (PI) with two NRTIs. A rapid switch to a new regimen is recommended when virological failure (VF) is confirmed after second line therapy i.e. when the viral load (VL) exceeds 1000 copies/mL on two consecutive occasions while addressing adherence issues. [5] Although the measurable aim of successful HAART is a suppressed VL, generally accepted at a lower limit of < 50 copies/mL, guidelines in resource-limited countries recommend genotyping at a much higher VL threshold of ≥ 1000 copies/mL.

However, there is a subset of patients on ART presenting with a VL ranging from 50 – 999 copies/mL, who are not covered by these guidelines, thereby creating uncertainty amongst healthcare providers. This range is generally termed low-level viremia (LLV). [6] South African National Department of Health viral load data (unpublished) for 2018 indicates that

approximately 15% of patients have a VL between 50 – 999 copies/mL. Although the reported prevalence of LLV varies worldwide,[6] a recent observational cohort in South Africa demonstrated that up to 23% of patients failing antiretroviral treatment presented with LLV, defined as a VL between 50-999 copies/mL.[7] Data is sparse regarding the mechanisms for LLV. However, drug resistance mutations (DRMs) in patients having lower viral loads may be caused by continuous viral replication. [8] Moreover, these patients are often retained on a failing regimen resulting in increased transmission, accumulation of mutations and poorer outcomes. [9–12]

There is a concern regarding the inconsistency of successful genotyping at lower viral loads. This is mainly due to lower template concentration in these samples. Many laboratories, in order to provide a sensitive and cost-effective method of testing, have developed in-house genotypic assays, which have achieved good genotyping success rates in samples with a VL<1000 copies/mL. [13–15]

Currently, there is a lack of data regarding HIV drug resistance testing in South African patients with LLV and national policies do not address the management and monitoring of such patients. In order to ensure the continued success of the ART programme in South Africa, patients with LLV on a second-line PI-based regimen may also require genotyping and referral for a third-line regimen, should PI mutations be present.

Thus, the aim of our study was to assess the feasibility of genotyping at LLV using an in-house assay and to determine the prevalence and clinical relevance of drug resistance in this population.

Patients and methods

Ethics

The study was conducted according to the Declaration of Helsinki and the Medical Research council (MRC) guidelines and the study protocol was approved by the ethics committee of the University of Witwatersrand, ethics number M171041.

Study design and collection of patient samples

This was an observational, retrospective, single centre, cohort study conducted at the National Health Laboratory Service (NHLS) HIV Genotyping laboratory, Department of Molecular Medicine and Haematology, Charlotte Maxeke Johannesburg Academic hospital (CMJAH), South Africa. A cohort of 159 EDTA plasma samples with viral loads ranging from 51-999 copies/mL from HIV infected, treatment-experienced adults with LLV sent for routine drug resistance testing at healthcare provider request were genotyped over a 13-month period from 15 August 2017 – 10 September 2018. There were no inclusion or exclusion criteria set for the collection of these samples. Median time calculated from sample collection to centrifugation and storage at -80°C was 2.5 days. A study published in 2016 reported the prevalence of DRMs in South African patients with virological failure (i.e. VL ≥1000 copies/mL) as 72.1% for PI-based regimens and close to 95% for NNRTI-based failures. [27] To calculate the minimum sample size, a power of 80% and an alpha of 5% (1-sided) with a zone of equivalence of -10% was used to ensure that the study was powered to compare the prevalence of resistance in patients with LLV compared to those presenting with VL ≥1000 copies/mL. LLV was defined as a VL ranging between 50-999 copies/mL. Persistent LLV was defined as at least two consecutive VL measurements between 50-999 copies/mL obtained in the last three months prior to genotyping.

HIV-1 drug resistance testing

HIV drug resistance testing was performed using a previously validated in-house protocol applying a nested RT-PCR covering HIV protease (aa 1-99) and reverse transcriptase (aa 1-251) genes. [16] RNA extraction was automated and performed using NucliSENSeasyMAG (bioMérieux, Marcy-l'Étoile, France) with an input of 500µL of plasma. Amplification and sequencing of *pol* fragments from RNA using the Superscript III one-step Platinum Taq HiFi RT-PCR system (Invitrogen, USA) was used to generate cDNA and the first round PCR product. This was followed by a nested PCR using the AmpliTaq Gold DNA Polymerase (Invitrogen, USA). HIV-1 drug resistance analysis was done by sequencing the amplicon using the BigDye^{R} Terminator v3.1 cycle sequencing kit, as per manufacturer's instructions. Extraction and RT-PCR procedures were repeated once for samples that could not be amplified or sequenced after the first attempt. Partial *pol* gene sequences were assembled and manually edited using Sequencher version 4.7 (Genecodes, MI, USA) or RECall (British Columbia's Centre for Excellence in HIV/AIDS Research). MEGA 5.05, version 7 (January 2016) programme was used to align sequences and construct a phylogenetic tree to identify duplicate sampling from the same individual. The HIV drug resistance sequences (FASTA) were loaded onto the Stanford HIVdb v8.7 genotypic resistance system (http://hivdb.stanford.edu/index.html) to generate a resistance report. The prevalence of resistance mutations in samples from adult patients referred over the same period with a VL ≥1000 copies/mL was then determined to provide a comparison with the LLV cohort.

An initial (phase A) study was performed from 15 August to 27 October 2017 to evaluate and test a lower limit of detection for the in-house genotyping protocol. Genotyping success rate was determined for VL categories 51-200, 201-400 and 401-999 copies/mL. During the second study phase (28 October 2017 to 10 September 2018) only those samples that fulfilled the lower limit as determined during the phase A study were genotyped and included in determining the

overall genotyping success rate in patients with LLV. In order to assess the clinical relevance of sequencing data, demographic data (age and gender) and HIV-related information (previous VLs, ART regimen at the time of testing and duration of therapy) were extracted from laboratory request forms and the Laboratory Information Systems (TrakCare, v 2.5, 2015) for further analysis.

Statistical analysis

GraphPad Prism version 8.2.1, August 20, 2019 was used to analyse the data. Frequency tables and percentages were reported for categorical variables while continuous variables were summarised using median and interquartile range (IQR). Associations between VL categories, persistent LLV and resistance were tested using two-sided Fisher's exact test considering a p-value of <0.05 as statistically significant. A relative risk ratio was used to compare the risk of resistance in patients with VLs $\geq$ 1000 copies/mL with those patients experiencing LLV. The modified Wald method was used to calculate confidence intervals at 95%.

Results

Genotyping success

Table 1 indicates the number of samples tested and the relative amplification rates within the specified viral load ranges. In phase A, the initial success rate increased from 45.5% (10/22) for the lowest VL range to 85.7% (12/14) for the middle range and 82.6% (19/23) for samples with a VL between 401 and 999 copies/mL (Figure 1). Based on these results the limit of detection was set at 200 copies/mL. An additional 36 samples with a VL between 201-400 copies/mL and 64 samples with a VL between 401-999 copies/mL were tested in a phase B study, resulting in a success rate of 83.9% (115/137). Combining success rates from phase A and phase B, the assay performed well with a success rate of 78.6% (125/159, 95% CI 71.6 – 84.3).

Study population

Data from 159 patients with low-level viremia referred for genotyping were collected for final analysis. All patients were ART-experienced at the time of genotyping. The median age of the patient cohort was 44 years (IQR 35 - 51), of whom more than half (52.8%) were female. The median viral load at the time of genotyping was 463 copies /mL (IQR 247 - 682). Most patients

(n = 109, 68.6%) were on a PI-based regimen at the time of genotyping consisting of either ritonavir-boosted lopinavir (LPV/r, 89.0%, n=97) or atazanavir (ATZ/r, 11.0%, n=12). Eighteen patients were on a NNRTI-based regimen (16.5%) while 2 patients were on an integrase-based (InSTI), third- line regimen (1.8%). For thirty (27.5%) patients, no data on ART regimen was available.

Of the patients referred for genotypying with LLV, 21.4% (34/159) had persistent LLV (pLLV) while the majority (74.2%, 118/159) experienced a single episode of LLV (sLLV). Viral load history was missing for 4.4% (7/159). When examining the trend in viral load progression, 94.9% (112/118) patients with sLLV demonstrated a declining trend in viraemia levels compared to 44.1% (15/34) with pLLV. Samples that failed amplification (n=32) were excluded when assessing the prevalence of resistance in the two groups. Linking the association of resistance to pLLV, 72.0% (18/25) of patients with pLLV had DRMs compared to 81.1% (77/95) of patients with sLLV; however, this result was not statistically significant (p=0.406).

Overall, 125 sequences were analysed for HIV-1 drug resistance. All sequences were HIV-1 subtype C. The prevalence of DRMs in the LLV cohort was 79.2% (99/125, 95%CI 71.2% – 85.4%) with 70.0% of patients in the VL category 201-400 copies/mL having ≥ 1 DRM (Figure 1). Over the same period the prevalence of resistance in adult patients referred for genotyping with a VL ≥ 1000 copies/mL was 82.9% (1338/1614). The latter group was subdivided by viral load category and the proportion of resistance in each category was analysed (Figure 2).

Using Fisher's exact test when comparing samples with LLV at genotyping and those samples with VL ≥ 1000 copies/mL, there was a statistically significant association between viral load and DRMs (p=0.033). The data was then used to estimate relative risk (RR) of resistance for each category of viral load, using the LLV group (VL ≤ 999 copies/mL) as the reference (Table 2). Compared to the LLV group, all VL categories ≥ 1000 copies/mL had a slightly higher risk

for resistance except for those with the highest levels of viraemia. However, this risk was not statistically significant.

Mutation analysis

At the time of genotyping, 73.6% (92/125) of patients had ≥1 NRTI mutation. By far, the most commonly detected NRTI mutation was M184V (66.4%), followed by K65R (12.8%) (Figure 3). Thymidine analogue mutation (TAM) prevalence was low: 11.2% of patients had ≥1 TAM while 7.2% had ≥3 TAMs. Of note, 2 patients had the Q151M complex mutation coupled with K65R, indicating multinucleoside resistance. Although only 16.5% of patients experiencing LLV were known to be on a NNRTI based regimen at the time of genotyping, 70.4% (88/125) had ≥1 NNRTI mutation, the two most common being K103N (40.8%) and V106M (20.0%) (Figure 4). Major PI mutations were detected in 7.2% (9/125) of patients with LLV. Of these, seven had triple class resistance with a Stanford score of ≥15, while two had dual NRTI/PI resistance. The most common PI mutations were M46I (n=5) and V82A (n=5) (Figure 5). Of the patients with PI resistance, seven were on a LPV/r regimen while two were on an InSTI-based third-line regimen at the time of genotyping. According to current South African guidelines all seven patients would require referral for a third-line regimen. High level resistance to LPV/r was observed in four patients, while three had intermediate resistance. No patient had high level resistance to darunavir, however one patient on a third-line regimen had intermediate darunavir resistance.

Discussion

To our knowledge, this is the first South African study examining the feasibility and clinical relevance of genotyping at LLV. During phase A of the study the lower limit of detection for the genotyping assay was set at 200 copies/mL, achieving an overall success rate of 83.9% for samples with HIV-RNA between 201-999 copies/mL. Thus, although this success rate is lower than the ≥ 95% amplification rate achieved with the previously validated in-house assay for samples ≥ 1000 copies/mL, we achieved a fairly high genotyping success rate from samples with relatively low copy numbers.

Similar success rates have been reported when genotyping samples with LLV, depending on the population, the design of the study and the assay used. [12,18] Some genotypic assays require a high plasma input volume of >1mL to genotype samples < 1000 copies/mL, [19,20] however our study was successful using a plasma input of 500µL.

Genotyping at low level viremia in the South African setting is important as it has been shown that this event occurs frequently [7] and may increase with a maturing ART programme. Data suggests that these viraemic patients even at lower viral loads, are at risk for virological rebound and treatment failure. [11,12,21,22] In addition, an unsuppressed viral load will continue to pose an, albeit smaller, transmission risk.

Although the association between viral load and prevalence of DRMs was significant when comparing patients with LLV (79.2%) to those with VL ≥ 1000 copies/mL (82.9%), this may be attributable to the much larger population size of the latter group (125 versus 1614). However, other studies in higher income settings have reported lower DRM rates in patients with low-level viraemia ranging from 17 – 72%, depending on the population studied and the time of genotyping. [11,17,18] When patients were subdivided into viral load categories and compared to the LLV group, the highest prevalence of DRMs were seen in the 10 000 – 99 999 copies/mL category (87%) which may be explained by the high rate of viral replication coupled with sufficient drug pressure to select for DRMs in this group of patients. Importantly, however, the risk of resistance in patients with VL ≥ 1000 copies/mL did not differ significantly from those with LLV which suggests that genotyping at LLV would be both valuable and informative. [10,12,18,23]

Persistent LLV has been linked with an increased risk of both virological failure [6] and accumulating additional mutations over time. [10,23] Although the prevalence of DRMs were higher in patients with a single episode of LLV (81%) versus patients with persistent LLV (72%), this difference was not statistically significant. Patients with higher VLs preceding the episode of LLV may have been predisposed to developing more DRMs due to uncontrolled viral replication, which could have been impacted by the infrequent VL monitoring and drug resistance testing in the South African public sector. [4,5] The lower prevalence of DRMs in patients with persistent LLV may reflect adherence problems in this group and although the mechanisms of LLV are still uncertain, patients with LLV have been shown to have decreased antiretroviral drug concentrations. [24] Moreover, as this was a retrospective study, the risk of developing new mutations in patients with persistent LLV could not be assessed.

All patients with LLV in this study were HIV-1 subtype C which is the predominant circulating subtype in South Africa and accounts for nearly fifty percent of HIV-1 infections worldwide. [25]

Recently, LLV has been associated with non-B subtypes in HIV-1 infected patients on HAART. [26] However, published literature on resistance mutations associated with LLV is largely from higher income countries where HIV-1 subtype B predominates and where VL monitoring is more frequently performed. Thus, further studies including low to middle-income settings would be informative regarding the development of mutations at LLV in patients with subtype C and the clinical impact thereof.

Drug resistance mutations (DRMs) were prevalent in patients with LLV at the time of genotyping: 73.6% and 70.4% had ≥1 NRTI mutation and ≥1 NNRTI mutation respectively. However, 7.2% of patients had major PI mutations which is lower than the 16.4% prevalence of PI mutations in South African patients failing a PI-based regimen reported in a national survey in 2016. [27] Nevertheless, it is more than the 2.6% of PI resistance found in patients with LLV not responding to a PI-based regimen, reported in a recently published Kenyan study. [28] Because the development of PI resistance is usually a late occurrence, this lower prevalence in patients with LLV may reflect the fact that DRMs in patients with virological failure are accumulating over a longer period. Furthermore, the declining trend in viral loads for patients experiencing a single episode of LLV (94.9%) seems to suggest that many patients with LLV are likely to achieve viral suppression on subsequent testing. However, the clinical significance of identifying these mutations early in patients with LLV needs to be emphasised. Without genotyping, these patients would likely remain on a failing regimen with a risk of accumulating DRMs and a negative impact on morbidity, mortality and future treatment options.

The limitations of this study include its relatively small sample size and the retrospective study design. However, as national guidelines do not recommend genotyping at LLV, clinicians do not routinely refer such patients for HIV drug resistance testing. As this was a retrospective observational analysis, data was often unavailable especially with respect to current and previous ART exposure and duration of treatment. Reproducibility would be desirable for

future investigation especially at lower levels of viremia as variable results were observed on repeat testing of samples in the VL 200-400 copies/mL category (data not shown). Furthermore, due to its descriptive nature, our findings may not be applicable across all settings. However, the concepts illustrated may provide a useful tool in guiding the decision to genotype at LLV even in high income countries. Finally, the Sanger sequencing method was used for genotyping which does not account for minority viral variants with HIV-1 DRMs. However, next generation sequencing is currently not used as a diagnostic tool in the South African public sector.

In conclusion, current national South African virological failure guidelines may actually be keeping patients on failing regimens for longer than clinically advisable. We have demonstrated that HIV drug resistance testing at LLV is feasible and implementation could result in earlier identification and referral of patients requiring third- line regimens.

Acknowledgements

This work was presented in part at the 10th IAS Conference on HIV Science, Mexico City, 2019 (Abstract WEPDB0106). We would like to thank the staff from the HIV genotyping laboratory at the Charlotte Maxeke Academic Hospital and Tonya Esterhuizen for technical assistance.

Funding

The study was conducted as part of the routine laboratory monitoring of HIV-1 infected patients and therefore no specific funding was obtained.

Transparency Declarations

None to declare

References

1. Cohen MS. Successful treatment of HIV eliminates sexual transmission. *Lancet* 2019; **393**: 2366–7.

2. Center for Disease Control and Prevention. The Fifth South African National HIV prevalence, Behaviour and Communication Survey, 2017 (SABSS V). 2018; **2017**: 5–8. Available at: http://www.hsrc.ac.za/uploads/pageContent/9234/SABSSMV_Impact_Assessment_Summary_ZA_ADS_cleared_PDFA4.pdf.

3. UNAIDS. Data 2017. *Program HIV/AIDS* 2017: 1–248. Available at: http://www.unaids.org/sites/default/files/media_asset/20170720_Data_book_2017_en.pdf.

4. South African National Department of Health. National Consolidated Guidelines for the Prevention of Mother-To-Child Transmission of HIV (PMTCT) and the Management of HIV in Children, Adolescents and Adults. *Department of Health Republic of South Africa* 2015: 1–128. Available at: www.doh.gov.za.

5. Meintjies G, Moorhouse M, Carmona S, *et al.* Adult antiretroviral therapy guidelines 2017 as per HIV Medicine SAJ. *South African J HIV Med* 2017: 1–24.

6. Ryscavage P, Kelly S, Li JZ, Richard Harrigan P, Taiwo B. Significance and clinical management of persistent low-level viremia and very-low-level viremia in HIV-1-infected patients. *Antimicrob Agents Chemother* 2014; **58**: 3585–98.

7. Hermans LE, Moorhouse M, Carmona S, *et al.* Effect of HIV-1 low-level viraemia during antiretroviral therapy on treatment outcomes in WHO-guided South African treatment programmes: a multicentre cohort study. *Lancet Infect Dis* 2018; **18**: 188–97.

8. Clutter DS, Jordan MR, Bertagnolio S, Shafer RW. HIV-1 drug resistance and resistance testing. *Infect Genet Evol* 2016; **46**: 292–307.

9. Jordan MR, Winsett J, Tiro A, *et al.* HIV Drug Resistance Profiles and Clinical Outcomes in Patients with Viremia Maintained at Very Low Levels. *World J AIDS* 2013.

10. Li JZ, Gallien S, Do TD, *et al.* Prevalence and significance of HIV-1 drug resistance mutations among patients on antiretroviral therapy with detectable low-level Viremia. *Antimicrob Agents Chemother* 2012; **56**: 5998–6000.

11. Swenson LC, Min JE, Woods CK, *et al.* HIV drug resistance detected during low-level viraemia is associated with subsequent virologic failure. *Aids* 2014; **28**: 1125–34.

12. Santoro MM, Fabeni L, Armenia D, *et al.* Reliability and clinical relevance of the HIV-1 drug resistance test in patients with low viremia levels. *Clin Infect Dis* 2014; **58**: 1156–64.

13. Bruzzone B, Di Biagio A, Sticchi L, *et al.* Feasibility and Reproducibility of HIV-1 Genotype Resistance Test in Very-Low-Level Viremia. *Antimicrob Agents Chemother* 2014; **58**: 7620–1.

14. Gupta S, Taylor T, Patterson A, *et al.* A robust PCR protocol for HIV drug resistance testing on low-level viremia samples. *Biomed Res Int* 2017; **2017**.

15. Santoro MM, Perno CF. Clinical relevance of genotypic resistance testing today. *New Microbiol* 2016; **39**: 91–2.

16. Zhou Z, Wagar N, DeVos JR, *et al.* Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. *PLoS One* 2011; **6**: 1–10.

17. Taiwo B, Gallien S, Aga E, *et al.* Antiretroviral drug resistance in HIV-1-infected patients experiencing persistent low-level viremia during first-line therapy. *J Infect Dis* 2011; **204**: 515–20.

18. Gonzalez-Serna A, Min JE, Woods C, *et al.* Performance of HIV-1 drug resistance testing at low-level viremia and its ability to predict future virologic outcomes and viral evolution in

treatment-naive individuals. *Clin Infect Dis* 2014; **58**: 1165–73.

19. Villahermosa ML, Thomson M, Vazquez de Parga E, *et al.* Improved conditions for extraction and amplification of human immunodeficiency virus type 1 RNA from plasma samples with low viral load. *J Hum Virol* 2000; **3**: 27–34.

20. Mens H, Kearney M, Wiegand A, *et al.* Amplifying and Quantifying HIV-1 RNA in HIV Infected Individuals with Viral Loads Below the Limit of Detection by Standard Clinical Assays. *J Vis Exp* 2011: 1–8.

21. Laprise C, De Pokomandy A, Baril JG, Dufresne S, Trottier H. Virologic failure following persistent low-level viremia in a cohort of HIV-positive patients: Results from 12 years of observation. *Clin Infect Dis* 2013; **57**: 1489–96.

22. Navarro J, Caballero E, Curran A, *et al.* Impact of low-level viraemia on virological failure in HIV-1-infected patients with stable antiretroviral treatment. *Antivir Ther* 2016; **21**: 345–52.

23. Delaugerre C, Gallien S, Flandre P, *et al.* Impact of low-level-viremia on HIV-1 drug-resistance evolution among antiretroviral treated-patients. *PLoS One* 2012; **7**.

24. Leierer G, Grabmeier-Pfistershammer K, Steuer A, *et al.* Factors Associated with Low-Level Viraemia and Virological Failure: Results from the Austrian HIV Cohort Study. *PLoS One* 2015; **10**: e0142923.

25. Buonaguro L, Tornesello ML, Buonaguro FM. Human Immunodeficiency Virus Type 1 Subtype Distribution in the Worldwide Epidemic: Pathogenetic and Therapeutic Implications. *J Virol* 2007; **81**: 10209–19.

26. Saison J, Tardy JC, Scholtes C, *et al.* Low-level viremia is associated with non-B subtypes in patients infected with HIV with virological success following HAART introduction. *J Med Virol* 2013.

27. Steegen K, Carmona S, Bronze M, *et al.* Moderate levels of pre-treatment HIV-1 antiretroviral drug resistance detected in the first South African national survey. *PLoS One* 2016; **11**: 1–12.

28. Kantor R, DeLong A, Schreier L, *et al.* HIV-1 second-line failure and drug resistance at high-level and low-level viremia in Western Kenya. *AIDS* 2018; **32**: 2485–96.

Tables and Figures

Table 1: Success rates of resistance testing at low level viremia

Viral Load Range (RNA copies/mL)		No. of samples tested	No. of samples successful	Success rate (%)	95% CI
51-200	Phase A	22	10	45.5%	26.9 - 65.4
201-400	Phase A	14	12	85.7%	
	Phase B	36	31	86.1%	
	Total	50	43	86.0%	73.5 - 93.4
401-999	Phase A	23	19	82.6%	
	Phase B	64	53	82.8%	
	Total	87	72	82.8%	73.4 - 89.4
TOTAL		**159**	**125**	**78.6%**	**71.6 - 84.3**

Table 2: Relative risk of resistance by HIV viral load category

Risk factor	Risk ratio (95% CI)	p value
HIV viral load category (Reference ≤ 999 copies/mL)		
Total VL ≥ 1000 copies/mL	1.05 (0.95 – 1.15)	0.334
1 000-9 999 copies/mL	1.07 (0.96 – 1.18)	0.223
10 000-99 999 copies/mL	1.09 (0.99 – 1.20)	0.068
100 000-1 000 000 copies/mL	1.02 (0.92 – 1.12)	0.740
> 1 000 000 copies/mL	0.93 (0.80 – 1.07)	0.283

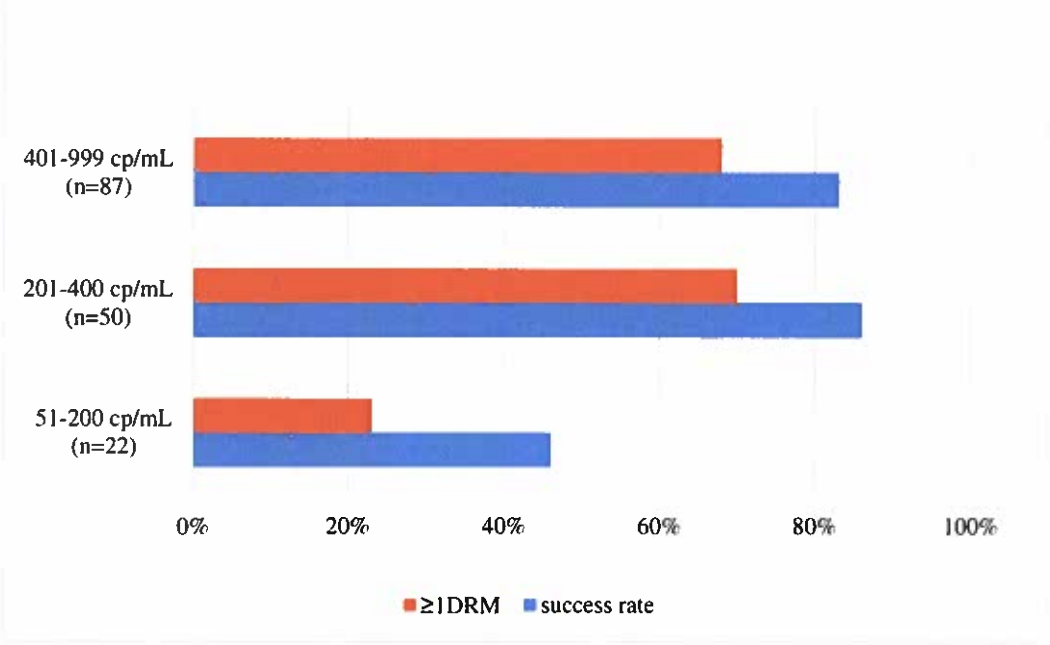

Fig 1: Assay success rate and proportion of LLV patients with ≥ 1 DRM stratified by viral load category

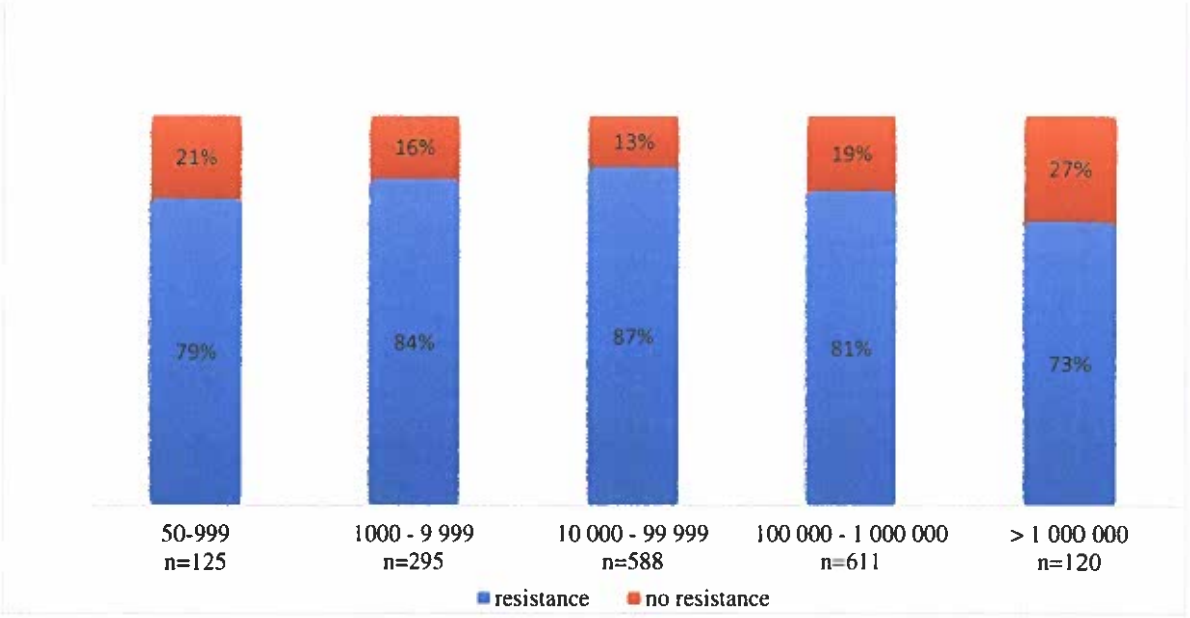

Fig 2: Distribution of patients and proportion of resistance by viral load category

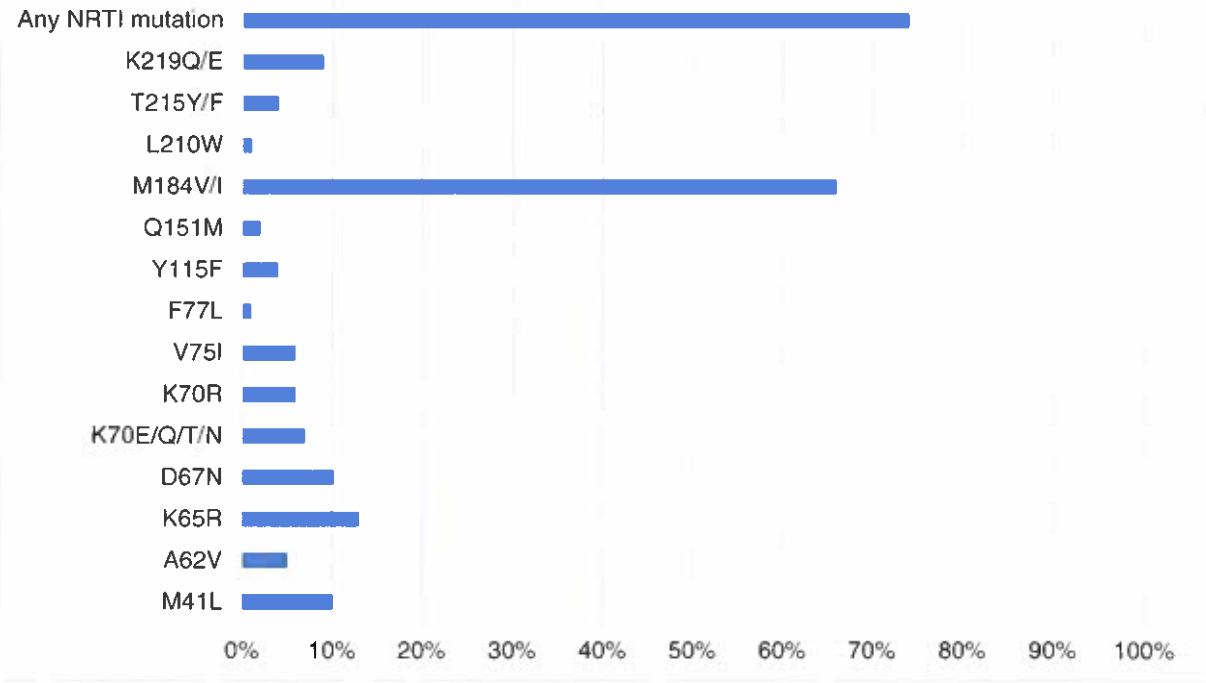

Fig. 3 Prevalence of NRTI mutations in patients with low level viremia

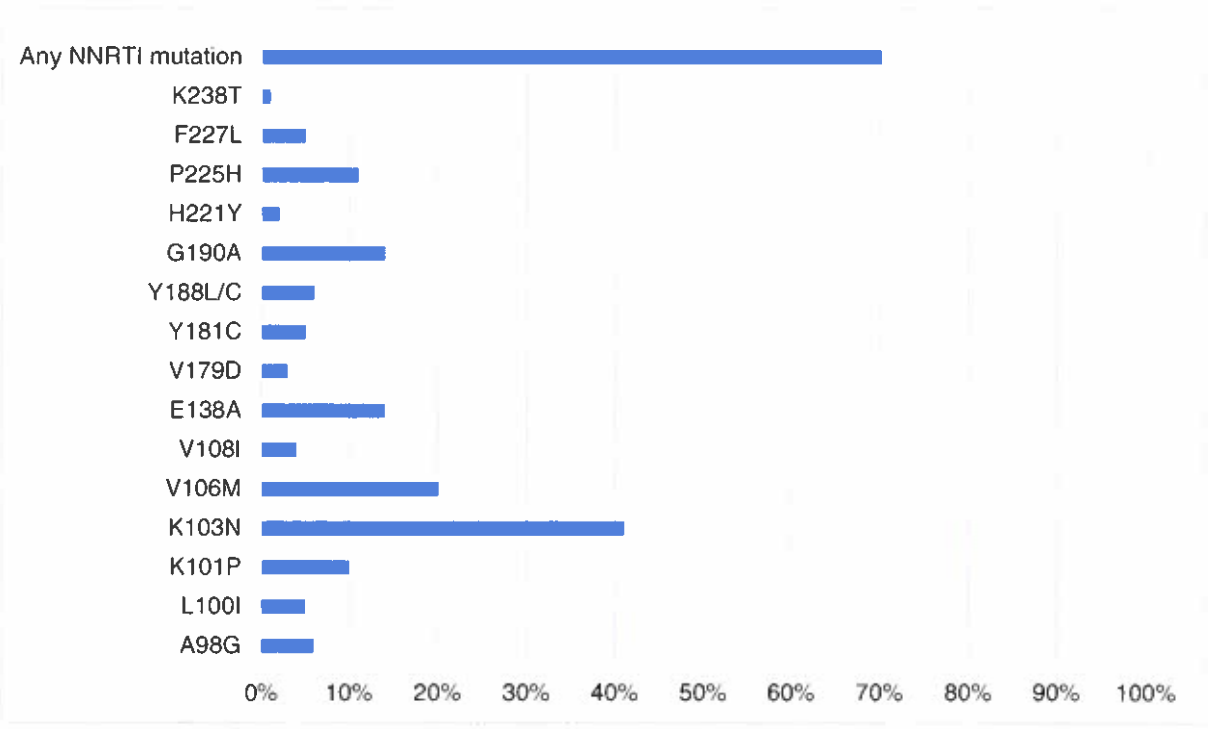

Fig. 4 Prevalence of NNRTI mutations in patients with low level viremia.

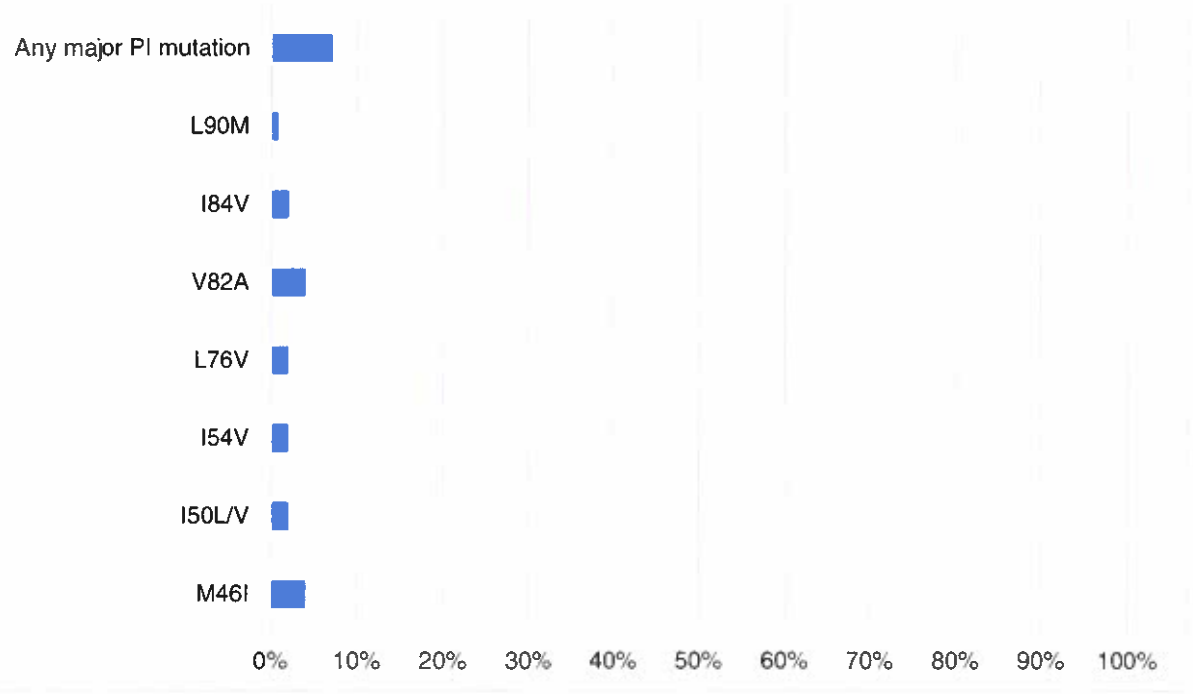

Fig 5. Prevalence of major PI mutations in patients with low level viremia

Feasibility and clinical relevance of genotyping samples with low-level viremia in a cohort of HIV infected South African patients on HAART

Principal Investigator: Dr Avania Bangalee[1]

Student number: 1819084

Degree: MMed (Virology)

Supervisor: Dr Lucia Hans[2] MBChB, FCPath (SA)Viro, MMed (UCT), DipObst(SA)

Co-supervisor: Dr Kim Steegen[3] PhD (Biomedical Science), MSc (Medical Science)

1. Registrar, Department of Medical Virology, National Health Laboratory Services, University of the Witwatersrand

2. Clinical Virologist, HIV Molecular Laboratory, Charlotte Maxeke Johannesburg Academic Hospital, Department of Molecular Medicine and Haematology, National Health Laboratory services and University of the Witwatersrand

3. Senior medical scientist, HIV Genotyping laboratory, Charlotte Maxeke Johannesburg Academic hospital, Department of Molecular Medicine and Haematology, National Health Laboratory services and University of the Witwatersrand

TABLE OF CONTENTS

1. LIST OF ABBREVIATIONS

HIV	Human Immunodeficiency Virus
ART	antiretroviral therapy
WHO	World Health Organisation
UNAIDS	the Joint United Nations Programme on HIV/AIDS
DRM	drug resistance mutation
DR	drug resistance
NRTI	nucleos(t)ide reverse transcriptase inhibitor
NNRTI	non-nucleos(t)ide reverse trancriptase inhibitor
TDF	Tenofovir
3TC	Lamivudine
FTC	Emtricitabine
EFV	Efavirenz
PI	Protease inhibitor
VF	virological failure
VL	viral load
LLV	low-level viremia
HAART	highly active antiretroviral therapy
PCR	polymerase chain reaction
RNA	Ribonucleic acid
EDTA	Ethylene-Diamine-Tetra-Acetic acid
cDNA	complementary DNA
aa	amino acid
CD4	Cluster of differentiation

2. BACKGROUND

There are currently over seven million people that are infected with HIV in South Africa (1). It has been more than 30 years since the discovery of the virus and, still, we do not have a vaccine to prevent infection, nor do the available drugs cure the disease. However, a notable success in the fight to prevent transmission has been the widespread scale-up of antiretroviral therapy (ART) delivery. This has translated to a significant decline in AIDS related mortality in Southern Africa by improving the health of individual patients so that HIV is now a chronic, manageable infection (2). In September 2016, the South African government adopted the Universal Test and Treat Strategy advocated by the World Health Organization (WHO), which aims to provide ART to all adults and children infected with HIV regardless of CD4 count. By 2016, 56% of people living with HIV in South Africa were accessing ART (2). This is a stride towards achieving the UNAIDS 90-90-90 treatment goals which are that 90% of people infected with HIV should be tested, 90% of those tested should be on ART and 90% of those on ART should be virologically suppressed (3).

HIV is a genetically variable virus (4). Should ART not completely suppress viral replication, the emergence of drug resistance mutations (DRMs) is much more likely. Because of this inherent variability, naturally occurring drug-resistant viruses can replicate in untreated patients. However, most variants do not become detectable because they are weaker than wild-type or drug susceptible viruses when there is no selective drug pressure (5).

There are two main facets to drug resistance (DR). The first is transmitted drug resistance, which is drug resistance in a treatment naïve individual. Recent data from a national survey in South Africa from 2013-2014 found a 9% drug mutation prevalence among patients not yet initiated on an ART regimen (6). The second is the much more commonly found acquired drug resistance while on ART (7). During the early era of ART there was a rapid evolution of drug resistance as regimens consisted of only one or two active drugs (8). Since the late 1990's highly active antiretroviral therapy (HAART) consists of three active drugs to target the virus, thereby significantly decreasing the development of resistance (9). However, the risk of acquired drug resistance is related to the time that a patient has been on ART. Thus, the longer a patient has been on treatment and on a failing regimen, the more likely it is that DRMs will develop (10).

Current South African guidelines (11) for adults recommend a first-line regimen based on a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI). This is available as a daily combination of tenofovir (TDF) with lamivudine (3TC) or emtricitabine (FTC) plus efavirenz (EFV). Second-line therapy is based on a boosted protease inhibitor (PI) with two NRTIs. A rapid switch to a new regimen is recommended when virological failure (VF) is detected. Treatment failure is defined as a confirmed viral load (VL) >1000 copies/mL in two measurements taken two to three months apart. The initial VL >1000 copies/mL should be managed by intensive adherence counselling.

The expansion of ART coverage, the use of NNRTI drugs which have a low genetic barrier to resistance and poor adherence to ART regimens, may all contribute to the emergence of early drug resistance mutations, particularly with more patients being kept longer on failing

regimens. For example, in a global assessment of drug resistance in patients failing a TDF-containing first line regimen, the TenoRes study group found a high proportion of drug resistance across low and middle-income regions (12).

In order to ensure the success of the ART programme, it is vital to monitor the emergence of ART resistance. This is important both for the individual patient and from a public health perspective. For example, in 2013, Hosseinipour et al found that in patients who had detectable HIV RNA at 12 months, HIV drug resistance was identified in 60%-72% and in addition, 22% of patients on second-line therapy did not achieve RNA suppression by 6 months (13). This highlights the need for drug resistance testing, especially in resource-limited settings where costlier drugs may not be readily available and advancing disease may be caused by viruses that are resistant to treatment.

The measurable aim of successful ART is suppression of viral replication to below the limit of detection of currently available diagnostic assays. This has been validated previously in various clinical trials and is generally defined as HIV-1 RNA <50 copies/mL, although there is a range of <40-75 copies/mL based on different platforms (14).

Drug resistance testing can be genotypic or phenotypic. Standard genotypic tests have been based on dideoxyterminator Sanger sequencing of the HIV protease, reverse transcriptase and/or the integrase genes against a reference HIV DR mutation database. Currently there is only one genotypic commercial assay, the ViroSeq HIV-1 Genotyping System, which has FDA approval. This assay recommends genotyping at a VL of 2000 copies/mL (15).

However, there is a subset of patients on ART, in whom VL levels range from 50-1000 copies/mL. Sustained low level viremia (LLV) is generally defined as a detectable VL <1000 copies/mL for more than 6 months while on ART (16). Sustained LLV differs from intermittent LLV, known as "blips", which has a low risk of disease progression and accumulation of drug

resistance mutations (17). Data is sparse regarding the mechanisms for LLV; however, drug resistance mutations in patients experiencing VF may be caused by continuous viral replication (5). Patients with persistent LLV appear to be at increased risk for resistance and virological failure compared with patients who have HIV-1 RNA levels <50 copies/mL (18). Similarly, a Canadian study found that drug resistance in patients with LLV is strongly associated with subsequent virological failure (19). Li and colleagues evaluated the emergence of drug resistance and the clinical significance thereof during periods of LLV and found that in patients with >2LLV episodes, 44% accumulated additional resistance mutations (20). Recently, a large South African study, concluded that LLV was associated with an increased risk of ART failure in patients maintained on HAART (21). These studies suggest the need for early genotyping and intervention in order to improve clinical outcomes. However, these findings are in contrast to an earlier study which did not demonstrate adverse virological or immunological outcomes in patients with LLV (22).

Delaguerre and colleagues suggest that sustained LLV episodes are associated with DRMs across drug classes (23). This means that effective ART options for such patients would be limited. This is especially important for low and middle-income countries such as South Africa. In clinical trial subjects on first line ART with LLV, *de novo* DRMs were detected in 37% of cases (24). Analogously, in the UK, major resistance mutations were found in samples with HIV-1 RNA <1000 copies/mL (25). Thus, under selective pressure, mutations may occur early, following treatment failure. In a European multicentre cohort study, genotyping samples with VL<1000 copies/mL identified early antiretroviral drug resistance which may assist in guiding the future management of these patients (26). This highlights a need to develop techniques that allow the detection of DRMs in patients with LLV.

There is a concern however, regarding the inconsistency of successful genotyping at LLV. This is mainly due to a reduced template present in these samples. Even though British HIV

Association guidelines advise antiretroviral drug resistance testing at VL >500 copies/mL, an audit done in 2008 revealed that many laboratories in the UK were performing genotypic resistance testing at VL 50-500 copies/mL, using either the Viroseq assay or an in-house method (27). In 2006, Mitsuya and colleagues were able to sequence virus from 49 of 50 samples with a VL< 75copies/mL, challenging the notion that genotyping in samples with LLV is unreliable (28). Recently, a sensitive and reliable PCR protocol for HIV drug resistance testing was described using samples with low level viremia by means of a nested PCR approach in which all samples were successfully amplified except for those with a VL <100 copies/mL (29).

Many laboratories, in order to provide a sensitive and cost-effective method of testing, have developed in-house genotypic assays. In 2014, two papers were published which described in-house methods to successfully genotype samples with HIV-1 RNA of 50-1000 copies/mL (30)(31). The success rates for amplification and genotyping in these studies were 96.4% and 88% respectively. Both studies showed multiple resistance mutations to drugs across different classes in patients with LLV. Importantly, the study by Gonzalez-Serna et al showed that the detection of acquired drug resistance was predictive of subsequent treatment failure.

According to national guidelines, genotypic testing in South Africa is only recommended for patients failing a PI-based regimen (11). This is in contrast to high income countries such as the United Kingdom where resistance testing is done at entry into care in order to guide treatment selection and later, upon virological failure (32).

Following adherence counselling, patients on any antiretroviral regimen with LLV generate uncertainty among health care professionals. Resistance testing in this instance will assist in guiding the choice of drug regimen and may prevent adverse treatment outcomes.

Therefore, the aim of my study is to assess the genotypic success rate and clinical relevance of samples with LLV from patients on HAART, referred for genotyping, using a nested in-house RT-PCR assay.

An initial validation was performed to evaluate and test a lower limit of detection for the in-house protease/reverse transcriptase genotyping protocol at the Charlotte Maxeke Johannesburg Academic Hospital Genotyping Laboratory. The in-house protease/reverse transcriptase assay performed well for samples with an HIVVL range between 401 and 999 copies/mL with an overall success rate of 80%. Good reproducibility was shown for three samples tested in 5 replicates. In addition, six samples with prior sequencing data showed good correlation between sequences that were obtained when the HIVVL was high compared to the low-level viremia samples. Based on these criteria the new lower limit of detection of this assay is 400 copies/mL.

3. AIM AND OBJECTIVES

Aim: To assess the genotypic success rate and clinical relevance of samples with LLV from patients on HAART referred for HIV drug resistance testing.

Objectives :

I. To establish the overall proportion of samples that can be successfully genotyped at LLV using the new lower limit of detection of the in-house genotyping assay.

II. To calculate the prevalence of DRMs in patients with LLV and to compare the prevalence of DRMs in samples with LLV with samples which had a VL >1000copies/mL over the same period.

III. To analyse the sequence data obtained in order to understand the clinical relevance of LLV in a cohort of South African patients referred for HIV drug resistance testing

4. METHODS

4.1 Study Design

This will be an observational, retrospective, cohort study

4.2 Setting

The study will be conducted at the HIV Genotyping laboratory, Department of Molecular Medicine and Haematology, Charlotte Maxeke Johannesburg Academic hospital

4.3 Study population

The genotypic success rate will be evaluated in samples with LLV sent for routine drug resistance testing at healthcare provider request.

LLV was defined as detectable HIV-1 RNA level between 50-999 copies/mL.

A cohort of samples was selected with LLV that were genotyped over a 14-month period from 15 August 2017 – 10 October 2018 (n = 124). These were plasma samples from HIV-infected, treatment-experienced adults, taken from EDTA whole blood.

4.4 HIV-1 RNA Extraction and resistance analysis

HIV drug resistance testing was performed using a previously validated in- house protocol using a 2-step (nested) RT-PCR. This assay is presently used for routine patient samples at the

HIV genotyping laboratory at CMJAH. Samples were tested according to the standard testing procedure in the laboratory which is as follows:

RT-PCR is used to generate complementary DNA (cDNA) and subsequently amplify and sequence HIV protease (aa 1-99) and reverse transcriptase (aa 1-251) genes. The obtained sequence can identify drug resistance mutations present in these genes.

RNA extraction is automated and is performed using NucliSENSeasyMAG(bioMerieux) with an input of 500ul of plasma. Amplification and sequencing of *pol* fragments from RNA using the Superscript III one-step Platinum Taq HiFi RT-PCR system (Invitrogen, USA) is used to generate cDNA and the first round PCR product. This is followed by a nested PCR using the AmpliTaq Gold DNA Polymerase (Invitrogen, USA). HIV-1 drug resistance analysis is done by sequencing the amplicon using the BigDye^{R} Terminator v3.1 cycle sequencing kit, as per manufacturer's instructions.

Partial *pol* gene sequences are assembled and manually edited using Sequencher version 4.7 (Genecodes, MI, USA) or RECall (British Columbia's Centre for Excellence in HIV/AIDS Research)

The HIV drug resistance sequences (FASTA) will be loaded onto the Stanford HIVdb genotypic resistance system (http://hivdb.stanford.edu/index.html). The Stanford algorithm (version 8.7) will be used to generate a resistance report.

4.5 MEASUREMENTS:

Sample data will be gathered as follows:

- Date of sampling
- Final result of sequencing
- Nucleotide sequences obtained
- Mutations found in each sequence

In addition, DRMs present in samples referred over the same period with VL>1000copies/mL will be analysed to provide a comparison with the LLV cohort.

Samples will be stratified into 3 groups according to the plasma viral load at LLV (51-199, 200-399 and 400-999 copies/mL) and the overall success rate of genotyping will be calculated.

In order to assess clinical relevance of sequencing data, demographic data (age and sex) and HIV-related information (previous VLs, ART regimen at the time of testing and duration of therapy) will be extracted from laboratory request forms and the Laboratory Information Systems (TrakCare) for further analysis.

MEGA 5.05 programme will be used to align sequences and construct a phylogenetic tree to identify duplicate sampling from the same individual.

5. STATISTICAL ANALYSIS

Statistical evaluation will be undertaken together with the Biostatistics Unit of the Faculty of Health Sciences, University of Stellenbosch. Descriptive analysis using frequencies, proportions and 95% confidence intervals will be used to summarise categorical variables, while means and standard deviations will be used to summarise continuous variables. The

prevalence of DRMs in South African patients with virological failure (i.e. VL >1000 copies/mL) was reported as 72.1% for PI-based regimens and close to 95% for NNRTI-based failures (33). To calculate the minimum sample size for LLV, a power of 80% and an alpha of 5% (1-sided) with a zone of equivalence of -10% was used. This will lead to a comparison of the DRM prevalence in the LLV patient cohort with samples with VL >1000 copies/mL followed by an explanatory analysis of genotyping results for samples with LLV. The minimum sample size necessary would thus equal approximately 55 samples.

6. FUNDING

This study will not require any additional funding as it will be a retrospective analysis of data from samples sent for routine testing. There are no major expenses involved.

7. ETHICAL CONSIDERATIONS

The study will be conducted according to the Declaration of Helsinki and the Medical Research council (MRC) guidelines.

Patient privacy will be respected. No personal identifiers will be presented. Personal information will only be available to the primary investigator and study promoter. The study protocol has been approved by the University of Witwatersrand, Faculty of Health Sciences Ethics Committee, number M171041. We aim to publish the resulting manuscript in a peer-reviewed journal. Data will be used for our laboratory to establish a new lower limit of HIV-1 RNA at which drug resistance can be detected and the clinical relevance of genotyping at LLV.

8. TIMING

	Jul **2017**	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Apr	May	Jun	Jul **2018**
Literature review												
Protocol preparation												
Ethics application												
Data collection												
	Aug **2018**	Sept	Oct	Nov	Dec	Jan **2019**	Feb	Mar	Apr			
Data collection												
Data analysis												
Write-up												

9. REFERENCES

1. Release S. Mid-year population estimates 2018. 2019;(July). Available from: www.statssa.gov.zainfo@statssa.gov.za

2. UNAIDS. Data 2017. Program HIV/AIDS [Internet]. 2017;1–248. Available from: http://www.unaids.org/sites/default/files/media_asset/20170720_Data_book_2017_en.pdf

3. 90-90-90: An Ambitious Treatment Target to Help End the AIDS Epidemic. 2014; Available from: http://www.unaids.org/sites/default/files/media_asset/90-90-90_en.pdf

4. Abram ME, Ferris AL, Shao W, Alvord WG, Hughes SH. Nature, Position, and Frequency of Mutations Made in a Single Cycle of HIV-1 Replication. J Virol [Internet]. 2010;84(19):9864–78.

5. Clutter DS, Jordan MR, Bertagnolio S, Shafer RW. HIV-1 drug resistance and resistance testing. Infect Genet Evol [Internet]. 2016; 46:292–307.

6. Steegen K, Carmona S, Bronze M, Papathanasopoulos MA, Van Zyl G, Goedhals D, et al. Moderate levels of pre-treatment HIV-1 antiretroviral drug resistance detected in the first south african national survey. PLoS One. 2016;11(12):1–12.

7. Luca A De, Zazzi M. Interplay Between Transmitted and Acquired HIV Type 1 Drug Resistance: Reasons for a Disconnect. J Infect Dis.2015; 212:8–10.

8. Delaney M. History of HAART – the true story of how effective multi-drug therapy

was developed for treatment of HIV disease. Retrovirology. 2006;3(Suppl 1): S6.

9. Campo JE, Jamjian C, Goulston C. HIV Antiretroviral Drug Resistance. J AIDS Clin Res [Internet]. 2012;01(S5):2–5.

10. Stadeli KM, Richman DD. Rates of emergence of HIV drug resistance in resource-limited settings: a systematic review. Antivir Ther. 2013;18(1):115–23.

11. South African National Department of Health. National Consolidated Guidelines for the Prevention of Mother-To-Child Transmission of HIV (PMTCT) and the Management of HIV in Children, Adolescents and Adults. Dep Health Repub South Africa [Internet]. 2015;(April):1–128. Available from: www.doh.gov.za

12. Gregson J, Tang M, Ndembi N, Hamers RL, Marconi VC, Brooks K, et al. Global epidemiology of drug resistance after failure of WHO recommended first-line regimens for adult HIV-1 infection: A multicentre retrospective cohort study. Lancet Infect Dis [Internet]. 2016;16(5):565–75.

13. Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. J Infect Dis. 2013;207(SUPPL.2):549-556

14. Gilbert PB, DeGruttola V, Hammer SM, Kuritzkes DR. Virologic and regimen termination surrogate end points in AIDS clinical trials. JAMA [Internet]. 2001;285(6):777–84.

15. Eshleman SH, Jr JH, Swanson P, Cunningham SP, Drews B, Brennan C, et al. Performance of the Celera Diagnostics ViroSeq HIV-1 Genotyping System for Sequence-Based Analysis of Diverse Human Immunodeficiency Virus Type 1 Strains Performance of the Celera Diagnostics ViroSeq HIV-1 Genotyping System for

Sequence-Based Analysis of D. J Clin Microbiol. 2004;42(6):2711–7.

16. Laprise C, De Pokomandy A, Baril JG, Dufresne S, Trottier H. Virologic failure following persistent low-level viremia in a cohort of HIV-positive patients: Results from 12 years of observation. Clin Infect Dis. 2013;57(10):1489–96.

17. Lee PK, Kieffer TL, Siliciano RF, Nettles RE. HIV-1 viral load blips are of limited clinical significance. J Antimicrob Chemother. 2006;57(5):803–5.

18. Ryscavage P, Kelly S, Li JZ, Richard Harrigan P, Taiwo B. Significance and clinical management of persistent low-level viremia and very-low-level viremia in HIV-1-infected patients. Antimicrob Agents Chemother. 2014;58(7):3585–98.

19. Swenson LC, Min JE, Woods CK, Cai E, Li JZ, Montaner JSG, et al. HIV drug resistance detected during low-level viraemia is associated with subsequent virologic failure. Aids [Internet]. 2014;28(8):1125–34.

20. Li JZ, Gallien S, Do TD, Martin JN, Deeks S, Kuritzkes DR, et al. Prevalence and significance of HIV-1 drug resistance mutations among patients on antiretroviral therapy with detectable low-level Viremia. Antimicrob Agents Chemother. 2012;56(11):5998–6000.

21. Hermans LE, Moorhouse M, Carmona S, Grobbee DE, Hofstra LM, Richman DD, et al. Effect of HIV-1 low-level viraemia during antiretroviral therapy on treatment outcomes in WHO-guided South African treatment programmes : a multicentre cohort study. Lancet Infect Dis [Internet]. 2018;18(2):188–97.

22. Mira JA, Macias J, Nogales C, Fernández-Rivera J, García-García JA, Ramos A, et al. Transient rebounds of low-level viraemia among HIV-infected patients under HAART are not associated with virological or immunological failure. Antivir Ther.

2002;7(4):251–6.

23. Delaugerre C, Gallien S, Flandre P, Mathez D, Amarsy R, Ferret S, et al. Impact of low-level-viremia on HIV-1 drug-resistance evolution among antiretroviral treated-patients. PLoS One. 2012;7(5).

24. Taiwo B, Gallien S, Aga E, Ribaudo H, Haubrich R, Kuritzkes DR, et al. Antiretroviral drug resistance in HIV-1-infected patients experiencing persistent low-level viremia during first-line therapy. J Infect Dis. 2011;204(4):515–20.

25. Mackie NE, Phillips AN, Kaye S, Booth C, Geretti A. Antiretroviral Drug Resistance in HIV-1Infected Patients with Low-Level Viremia. J Infect Dis [Internet]. 2010;201(9):1303–7.

26. Prosperi MCF, Mackie N, di Giambenedetto S, Zazzi M, Camacho R, Fanti I, et al. Detection of drug resistance mutations at low plasma HIV-1 RNA load in a European multicentre cohort study. J Antimicrob Chemother. 2011;66(8):1886–96.

27. Cane PA, Kaye S, Smit E, Tilston P, Kirk S, Shepherd J, et al. Genotypic antiretroviral drug resistance testing at low viral loads in the UK. HIV Med. 2008;9(8):673–6.

28. Mitsuya Y, Winters MA, Fessel WJ, Rhee S-Y, Slome S, Flamm J, et al. HIV-1 Drug Resistance Genotype Results in Patients With Plasma Samples With HIV-1 RNA Levels Less Than 75 Copies/mL. JAIDS J Acquir Immune Defic Syndr [Internet]. 2006 Sep;43(1):56–9.

29. Gupta S, Taylor T, Patterson A, Liang B, Bullard J, Sandstrom P, et al. A robust PCR protocol for HIV drug resistance testing on low-level viremia samples. Biomed Res Int. 2017; 2017:4979252.

30. Santoro MM, Fabeni L, Armenia D, Alteri C, Di Pinto D, Forbici F, et al. Reliability and clinical relevance of the HIV-1 drug resistance test in patients with low viremia levels. Clin Infect Dis. 2014;58(8):1156–64.

31. Gonzalez-Serna A, Min JE, Woods C, Chan D, Lima VD, Montaner JSG, et al. Performance of HIV-1 drug resistance testing at low-level viremia and its ability to predict future virologic outcomes and viral evolution in treatment-naive individuals. Clin Infect Dis. 2014;58(8):1165–73.

32. Battegy M, Lundgren JD, Ryom L. EACS Guidelines 2017. 2017;(January):97. Available from: http://www.eacsociety.org/files/guidelines_8.2-english.pdf

33. Steegen K, Bronze M, Papathanasopoulos MA, Van Zyl G, Goedhals D, Van Vuuren C, et al. Prevalence of antiretroviral drug resistance in patients who are not responding to protease inhibitor-based treatment: Results from the first national survey in South Africa. J Infect Dis. 2016;214(12):1826–30.

R 14/49 Drs A Bangalee and K Steegen

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M171041

NAME: (Principal Investigator)	Drs A Bangalee and K Steegen
DEPARTMENT:	School of Pathology Department of Virology National Health Laboratory Service
PROJECT TITLE:	Feasability and clinical relevance of HIV-1 drug resistance testing at low viral loads in a South African cohort of patients on HAART
DATE CONSIDERED:	27/10/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr L Hans
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/11/2017

This clearance certificate is valid for 6 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and ONE COPY returned to the Research Office Secretary on 3rd floor Phillip V Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit to the Committee. I agree to submit a yearly progress report. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in October and will therefore be due in the month of October each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

20/11/2017
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

20/09/2018

Drs A Bangalee and K Steegen
School of Pathology
Department of Virology
National Health Laboratory Service

Sent by e-mail to: avana.bangalee@nhls.ac.za

Dear Drs Bangalee and Steegen

Re: **Protocol Ref No: M171041**
Protocol Title: *Feasability and clinical relevance of HIV-1 drug resistance testing at low viral loads in a South African cohort of patients on HAART*
Principal Investigator: Drs A Bangalee and K Steegen

Thank you for your e-mail of 10/09/2018.

I confirm that the amendment you propose to Protocol No. M171041 has been noted and approved.

For the record, the amendment is:

- extension of data collection to include samples collected up to and including 10/09/2018

Thank you for keeping us informed and updated.

Yours Sincerely

J. Burns

Mr I Burns
For the Human Research Ethics Committee (Medical)

W:\s2000\sub000\Acknowledge.doc

L. Harms initials

a0052654:MMed_manuscript_LLV.docx

V. Strezova 2.10.2019

ORIGINALITY REPORT

15%	8%	11%	7%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	3%
2	Wallis, C.L.. "Affordable in-house antiretroviral drug resistance assay with good performance in non-subtype B HIV-1", Journal of Virological Methods, 201002 Publication	1%
3	"Encyclopedia of AIDS", Springer Nature, 2018 Publication	1%
4	pure.uva.nl Internet Source	1%
5	"Oral abstracts of the 10th IAS Conference on HIV Science, 21-24 July 2019, Mexico City, Mexico", Journal of the International AIDS Society, 2019 Publication	1%
6	A. Gonzalez-Serna, J. E. Min, C. Woods, D. Chan, V. D. Lima, J. S. G. Montaner, P. R. Harrigan, L. C. Swenson. "Performance of HIV-1 Drug Resistance Testing at Low-Level Viremia	1%