HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 DRUG RESISTANCE AMONG PATIENTS FAILING SECOND-LINE ANTIRETROVIRAL TREATMENT IN BUTHA-BUTHE AND MOKHOTLONG, LESOTHO

by

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Dissertation submitted for fulfilment of the requirements for the Master of Health Sciences in Biomedical Technology

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31th March 2022

Declaration

I (Molisana A Cheleboi) hereby declare that this dissertation for Master of Health Sciences in Biomedical Technology is my original work. It has not been submitted before for any diploma or degree examination at this or any other university. It is my own work at execution and all reference materials contained are therein have been duly acknowledged.

Signature  ……Date…31.03.2022……..

Acknowledgments

Firstly, I would like to thank the almighty God for giving me strength and wisdom to carry out my research project from the beginning until completion.

I would also like to express my gratitude and appreciation to all my supervisors (Prof. Niklaus Labhardt, Prof. Thomas Klimkait, and Dr Dedré Olivier) for giving me guidance to do my research. This dissertation could not have been written well without your support and constructive criticism.

A special thanks goes to Dr Jennifer Brown for your personal and professional guidance. You never got tired of me and you were always there for me when I needed help.

I would also like to thank Dr Talkmore Maruta for all the support you gave throughout my study time.

I extend my appreciation to all my colleagues (Seboche, Butha-Buthe and SolidarMed).

To Kamele Mashaete thank you very much for your encouragement and comforting at time when I wanted to throw the towel.

My family has been a constant source of support during the past years, and for that I also owe a great deal of thanks. Majemina Cheleboi thank you for the love and support.

Finally, I also like to extend my gratitude to the Swiss Tropical and Public Health Institute (Swiss TPH) for the financial assistance.

Abstract

Background: To date, there is no cure for HIV. However, antiretroviral treatment (ART) is used to control the replication of HIV and many people living with HIV take ART. Unfortunately, over time, some patients develop drug resistance which is becoming a major public threat limiting future treatment options for people living with HIV. HIV-1 drug resistance is defined as the ability of HIV-1 to mutate and reproduce itself in the presence of ARV drugs. This occurs as a result of: (a) poor adherence to treatment; (b) inadequate potency of ARVs; (c) suboptimal drug levels and (d) pre-existing resistance. There are two types of resistance-associated mutations (RAMs) namely acquired and transmitted. When an individual is infected with HIV-1 resistant strain, it may become the dominant strain for further transmission leading to increasing number of antiretroviral-naive patients and reducing their therapeutic effectiveness. HIV drug resistance testing is a test used to detect the presence of RAMs to HIV type 1 (HIV-1).

Currently patients are switched empirically in the country. Therefore, it is crucially important to understand the HIV drug resistance pattern in order to find ways how to limit the occurrence. This will helpful in the clinical decision making and selection of regimens upon treatment failure.

Objective: The objective of the study was to access the prevalence and patterns of HIV resistance-associated mutations in patients with an unsuppressed viral load when taking second-line ART in Butha-Buthe and Mokhotlong districts, Lesotho.

Methods: In a retrospective study, we used convenience sampling and sequenced all eligible (patients on second line ART with a viral load ≥ 1000 copies/mL) stored leftover plasma samples taken between January 2016 and October 2020. The study was conducted at Seboche Hospital Laboratory which served all health facilities (hospitals and clinics) in the northern region of Lesotho.

During testing, RNA extraction was done manually using the Purelink Viral/DNA kit. PCR amplification and sequencing of the protease (PR) and reverse transcriptase (RT) region was done using the Amplification and Sequencing module kits developed by Thermo Fisher Scientific. Consensus sequences were derived, aligned, and analysed using the web-based Recall software. The Stanford HIV Drug Resistance Database was used to interpret the presence or absence of drug resistant mutants.

Results and discussion: Out of 55 patients' samples, 30 samples were successfully amplified and sequenced. The median age was 41 years (IQR: 30 to 49) and the majority (62%) of participants were female. The median duration on a second-line regimen at the time of phlebotomy was 1.9 years (IQR: 0.5 to 3.0). The majority of patients (94%) were taking ritonavir-boosted lopinavir-based ART.

Major RAMs were observed in 62% of participants; one patient had major RAMs in the PR while 18 had RAMs in the RT region. All participants had HIV-1 subtype C. The most frequent mutations conferring resistance to nucleoside reverse transcriptase inhibitors were M184V (31%) and K70R/E (16%), while the most frequent mutations conferring resistance to non-nucleoside reverse transcriptase inhibitors were K103N (38%), P225H (19%) and G190A (19%). The observed PR mutations from one sample were M46I, I54L and V82L.

Only 10 (31%) patients had RAMs which conferred resistance to their second-line regimen. Though RAMs were detected, the majority of patients had three active drugs in their second-line regimen and many observed mutations likely conferred resistance to their previous regimens.

Conclusion: Though 62% of patients had RAMs, only 31% had RAMs conferring resistance to their second-line regimen during the first 2 years after switch to second-line ART, indicating that adherence plays a major role in second-line treatment failure. These RAMs reflect the reality of HIV care in resource-limited settings such as Lesotho. Adherence should be strengthened among people with treatment failure while taking second-line ART in order to avoid development of resistance mutations.

Keywords: HIV-1 drug resistance testing, resistance associated mutations, antiretroviral treatment

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List of abbreviations

3TC:	Lamivudine
ABC:	Abacavir;
AIDS:	Acquired immunodeficiency syndrome
ART:	Antiretroviral treatment
ATV/r:	Atazanavir;
AZT:	Zidovudine;
DRV/r:	Darunavir/ritonavir;
DTG:	Dolutegravir;
EFV:	Efavirenz;
FTC:	Emtricitabine;
HIV:	Human immunodeficiency virus
HIV-1:	Human immunodeficiency virus type 1
IQR:	Interquartile ranges
LPV/r:	Lopinavir/ritonavir;
INSTI:	Integrase strand transfer inhibitor
NNRTI:	Non-nucleoside reverse transcriptase inhibitor
NRTI:	Nucleoside reverse transcriptase inhibitor
NVP:	Nevirapine;
PI/r:	Ritonavir-boosted protease inhibitor
PR:	Protease
RAM:	Resistance-associated mutation
RT:	Reverse transcriptase
TAF:	Tenofovir alafenamide fumarate;
TDF:	Tenofovir disoproxil fumarate;

Swiss TPH: Swiss Tropical and Public Health Institute

RAL: raltegravir

RTV: Ritonavir

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CHAPTER 1: Introduction

1.1 Background and Significance

Lesotho is a landlocked country completely surrounded by the Republic of South Africa. The country has 10 administrative districts with Maseru as the capital town. Lesotho has the second-highest HIV prevalence of all countries, with 21.1% of adults living with human immunodeficiency virus (HIV); 82% of whom receive ART (UNAIDS, 2020). Viral load testing is recommended to monitor treatment outcomes (WHO, 2021a). Butha-Buthe district started to implement routine viral load testing in November 2015. Despite all efforts to provide patients with access to ART, some patients still lack access to at least annual viral load monitoring (Glass et al., 2019).

Unfortunately, in the absence of proper monitoring during therapy, a high number of patients fail first- and second-line ART, with very limited options for third-line ART, if any at all (Edessa et al., 2019). In 2018, Lesotho completed its first HIV drug resistance survey among patients on first-line ART and found that 3.2% had acquired treatment failure within 12 months, while 6.9% acquired treatment failure after 48 months (Lesotho HIV Drug Resistance Survey, 2018). To my knowledge, little is known about rates of resistance among patients failing second-line ART in Lesotho.

1.2 **Aim(s):**

This study aims to assess the prevalence and patterns of genotypic resistance in patients with an unsuppressed viral load while taking second-line ART in Butha-Buthe and Mokhotlong, Lesotho.

1.3 Objective(s)

The specific objectives were:

- To assess the prevalence of resistance associated mutations among patients with a viral load ≥1000 copies/mL while taking second-line

CHAPTER 2: Literature review

2.1 HIV/AIDS overview

HIV is a virus that attacks the immune system of human beings. The virus is a lentivirus that belongs to the family retroviruses. (Yan et al., 2019). Retroviruses have an RNA genome that is reverse transcribed to DNA and integrated into host cell genome, then replicate spontaneously (Ryu, 2017). HIV treatment is used to block HIV replication. If HIV is left untreated, it can lead to acquired immunodeficiency syndrome (AIDS). AIDS is a chronic condition of weak immune system (WHO, 2020).

2.3 HIV genome and structure

HIV is a spherical enveloped virus with a lipid bilayer and an RNA genome approximately 9 kilobases in size (Heuvel et al,2022). The lipid bilayer contains two major viral glycoproteins; gp120 and gp41. The major function of gp120 and gp41 is to facilitate recognition of CD4+ cells and enable the virus to attach to and enter CD4+ cells (Li et al., 2021). The viral envelope encapsulates two single-stranded RNA molecules plus many proteins, including three enzymes (reverse transcriptase, integrase, and protease) necessary for HIV replication and maturation (van Heuvel et al., 2022).

HIV uses nine genes to code for the necessary proteins and enzymes. The three main genes are *gag*, *pol*, and *env*. The *gag* gene encodes the structural proteins. The *pol* gene encodes the above-mentioned enzymes. The *env* gene encodes the HIV glycoproteins, which constitute the outer envelope of the virus. The remaining genes (*rev*, *nef*, *vif*, *vpu*, *vpr*, and *tat*) are essential for viral replication and enhancing the infectivity rate (Seitz, 2016). Refer to an upcoming picture.

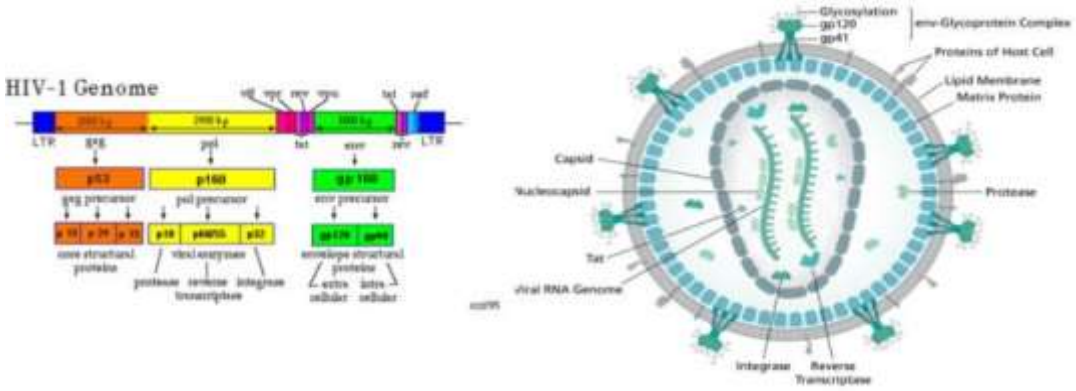

Fig 1: HIV genome and structure (Karki, 2017).

2.4 HIV life cycle

Unlike bacteria that are cells and are able to survive outside host cells, viruses are totally dependent on living cells for replication, utilising the host cell's expression machinery to multiply (Eisenreich et al., 2019). HIV infects certain types of cells: the cells which carry CD4 receptors on their surface such as CD4+ T cells, macrophages, dendritic cells, and monocytes. HIV enters the host cell by first binding with its gp120 surface proteins to the CD4 receptor of the target cell (Vermeire, 2019). After binding, a conformational change occurs in gp41 which brings viral and host cell membranes closer together, leading to membrane fusion. The fusion event is followed by the release of the viral capsid into the cytoplasm of the target cell (Saxena *et al*, 2016).

Once inside target cell, a certain uncoating of the capsid protein occurs; in parallel, the viral enzyme reverse transcriptase reverse transcribes the single-stranded viral RNA into double-stranded DNA. With the completion of this reverse transcription step, a viral preintegration complex forms(Sapp et al., 2022). Once the preintegration complex has entered the nucleus of the host cell, the viral enzyme integrase catalyzes the integration of the viral DNA into chromosomal DNA of the host cell. Transcription of this viral DNA and translation of the viral mRNA is reliant on the host cellular machinery. Thus, the virus hijacks the host cellular machinery for its own replication to produce and release new infectious viral particles to continue into a next cycle of replication (Saxena et al, 2016). Refer to the upcoming picture

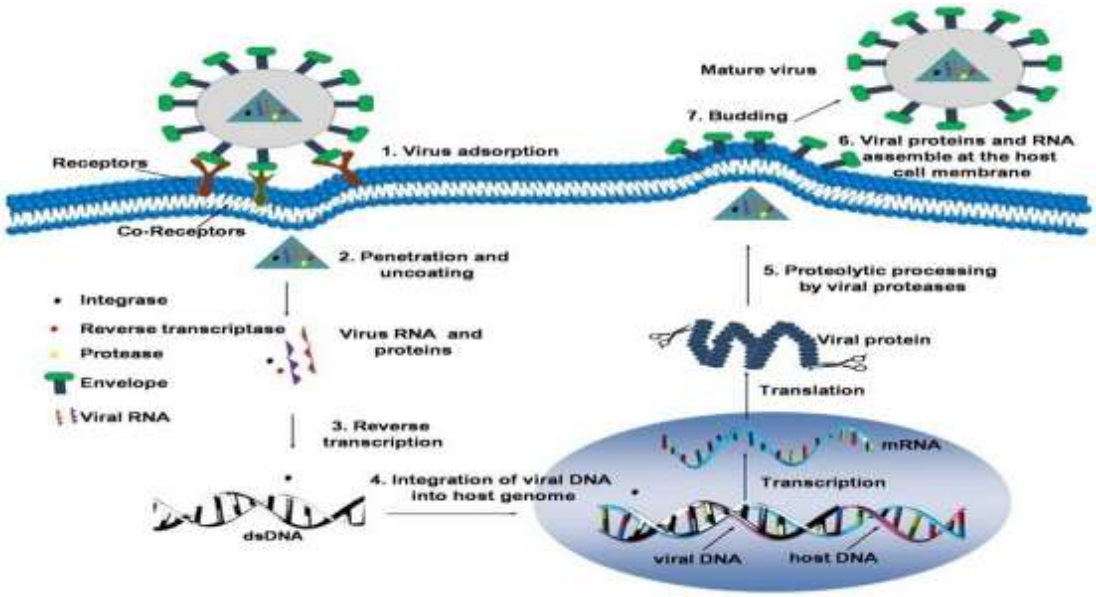

Fig 2: Diagram showing HIV replication (Saxena et al, 2016)

2.5 Molecular evolution of HIV

HIV is characterised by unique genetic diversity. The sources of this high genetic diversity lie in the error-prone nature of the reverse transcriptase, the high replication rate and the ability to undergo recombination (Niama et al., 2017). As the result of a lack of proof-reading activity, the HIV reverse transcriptase enzyme has a mutation rate of approximately 3.4×10^{-5} mutations per base pair per replication cycle. This contributes to the emergence of HIV variants leading to drug resistance (Santoro et al, 2013).

2.6 Mode of transmission

HIV is transmitted by exchange of body fluids (such as blood, breast milk, semen and vaginal secretions) from infected people to another person (WHO, 2020). The virus enters the body via mucous membranes, injured skin or mucosa (Shaw et al, 2012). Sexual intercourse is the most common route of transmission (Maartens et al., 2014). HIV can also be transmitted during blood transfusion or from a mother to her child during pregnancy, delivery and breastfeeding (Brown et al., 2017). In addition, HIV is transmitted during needle sharing among people who use intravenous drugs (Dumchev et al., 2020).

2.7 Epidemiology

There are two main types of HIV, namely HIV-1 and HIV-2. Both can lead to AIDS. HIV-1 accounts for around 90% of all infections worldwide. The strains of HIV-1 can be classified into four groups, namely M, O, N and P. Of the groups, M is the ‘major’ group and is responsible for the majority of the global HIV prevalence (Louten, 2016). HIV-1 group M is further divided into nine genetically distinct subtypes A, B, C, D, F, G, H, J, and K (Maartens et al., 2014) and there can be recombinant forms of the virus combining fragments of several of the above or circulating recombinant forms.

The differences among HIV types and subtypes could have effects on clinical management (Santoro *et al*, 2013). Most current knowledge about antiretroviral drugs, their development and mechanisms of action and the mechanisms of viral resistance to these drugs is based on studies using HIV-1 subtype B virus. The subtype B virus is the predominant variant in North America, Western Europe and Australia (Hemelaar et al., 2019) while subtype C virus is prominent in Southern Africa (Pascal, 2021), including Lesotho.

HIV continues to be a major global public health concern affecting more than 20 million lives in Africa (Giovanetti et al., 2020). Currently, Lesotho has the second-highest HIV

prevalence worldwide; 21.1% of the population is affected (UNAIDS, 2020) . The HIV distribution varies by district with 17.8% Butha–Buthe and 26.1% in Mokhotlong (LePHIA, 2021).

2.8 Antiretroviral Treatment (ART)

2.8.1 Introduction

Antiretroviral treatment (ART) refers to the medication used to suppress HIV and stop disease progression. ART also prevents onward transmission of HIV (Rodger et al., 2019). Clinical experience has shown that HIV is able to escape the antiviral effect of any currently available drug used as mono-therapy through the rapid accumulation of amino acid changes in the targeted proteins (Arts et al, 2012).

2.8.2 National ART Guidelines

In line with WHO consolidated guidelines 2021, the Lesotho National ART guidelines recommend combination of antiretroviral drugs in all HIV-positive patients to minimize the risk of treatment failure. Until recently, most first-line ART was made up of two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), constituting the backbone, and a non-nucleoside/nucleotide reverse transcriptase inhibitor (NNRTI) (National Guidelines, 2016). Guidelines were updated in 2019 to recommend first-line ART consisting of two NRTIs and an integrase strand transfer inhibitor (INSTI) for most people living with HIV. Second-line regimens typically comprise of a ritonavir-boosted protease inhibitor (PI/r) with two NRTIs (National ART Guidelines Addendum, 2019). A switch to second-line ART is recommended in cases where virological treatment failure occurs. In Lesotho, switching to second- and third-line ART is strictly regulated and requires approval by an HIV Expert Committee (National ART Guidelines Addendum, 2019).

2.8.3 Mode of action of HIV treatment

The different antiretroviral drugs act at different stages in the replication cycle of the virus. NRTIs act as DNA chain terminators and inhibit reverse transcription of the viral RNA genome into DNA. The NNRTIs bind in a hydrophobic pocket close to the active site of reverse transcriptase, and restrict the conformational change needed for the catalytic activity of the reverse transcriptase enzyme(Shin et al., 2021) PIs target and inhibit the viral protease, the enzyme required for the cleavage of precursor proteins (gag and gag–pol) into functional proteins, allowing the final assembly of the inner core of viral particle (Aquaro et al., 2020).

The entry or fusion inhibitors block the penetration of HIV into the target cells (Venanzi Rullo et al., 2019). INSTIs inhibit the action of integrase, the enzyme responsible for incorporating HIV DNA into the host cell DNA for transcription of the viral mRNA (Brooks et al., 2019).

Table 1: WHOrecommended first-line ART options (WHO, 2021b)

Population	Preferred first-line regimen	Alternative first-line regimen	Special circumstances
Adults and adolescents	TDF + 3TC (or FTC) + DTG[a]	TDF + 3TC + EFV 400 mg[b]	TDF + 3TC (or FTC) + EFV 600 mg[b] AZT + 3TC + EFV 600 mg[b] TDF + 3TC (or FTC) + PI/r[b] TDF + 3TC (or FTC) + RAL TAF[c] + 3TC (or FTC) + DTG ABC + 3TC + DTG[a]
Children	ABC + 3TC + DTG[d]	ABC + 3TC + LPV/r ABC + 3TC + RAL[e] TAF + 3TC (or FTC) + DTG[f]	ABC + 3TC + EFV (or NVP) AZT + 3TC + EFV[g] (or NVP) AZT + 3TC + LPV/r (or RAL)
Neonates	AZT + 3TC + RAL[h]	AZT + 3TC + NVP	AZT + 3TC + LPV/r[i]

3TC=lamivudine; ABC=abacavir; AZT=zidovudine; DTG=dolutegravir; EFV=efavirenz; FTC=emtricitabine; LPV/r=lopinavir/ritonavir; NVP=nevirapine; TAF=tenofovir alafenamide fumarate; TDF=tenofovir disoproxil fumarate; RAL=raltegravir

Table 2: WHO rrecommended second-line ART options(WHO, 2021b)

Population	Failing first-line regimen	Preferred second-line regimen	Alternative second-line regimens
Adults and adolescents[a]	TDF[b] + 3TC (or FTC) + DTG[c]	AZT + 3TC + ATV/r (or LPV/r)	AZT + 3TC + DRV/r[d]
	TDF + 3TC (or FTC) + EFV (or NVP)	AZT + 3TC + DTG[c]	AZT + 3TC + ATV/r (or LPV/r or DRV/r)[d]
	AZT + 3TC + EFV (or NVP)	TDF[b] + 3TC (or FTC) + DTG[c]	TDF[b] + 3TC (or FTC) + ATV/r (or LPV/r or DRV/r)[d]
Children and infants	ABC + 3TC + DTG[e]	AZT+ 3TC + LPV/r (or ATV/r[f])	AZT + 3TC + DRV/r[g]
	ABC (or AZT) + 3TC + LPV/r	AZT (or ABC) + 3TC + DTG[e]	AZT (or ABC) + 3TC + RAL
	ABC (or AZT) + 3TC + EFV	AZT (or ABC) + 3TC + DTG[e]	AZT (or ABC) + 3TC + LPV/r (or ATV/r[f])
	AZT + 3TC + NVP	ABC + 3TC + DTG[e]	ABC + 3TC + LPV/r (or ATV/r[f] or DRV/r[g])

ATV/r=atazanavir; DRV/r=darunavir/ritonavir; RTV=ritonavir

Note: Lesotho largely aligns with these WHO recommendations (National Guidelines, 2016)

2.8.4 Outcomes of second-line ART

Globally, the increase in the number of people who have access to ART has resulted in a substantial decline in HIV-related morbidity and mortality (Aquaro et al., 2020). For people who do not achieve viral suppression under therapy, switching to the next regimen line must be considered. With more and more persons receiving first-line ART, the number of individuals with virologic failure and the need for a switch to second-line ART is increasing (Nsanzimana et al., 2019).

Roughly 5.5% of the global population has access to second-line treatment. The outcomes of patients switched to second-line ART vary by setting. A study in Asia reported treatment failure rate of 8% to 41% after two years exposure to second-line ART while treatment failure rates of 13% to 40% were reported in an African setting (Thu et al., 2017). In Ethiopia, 2% of patients on HIV treatment are switched to a second-line regimen yearly (Seid et al., 2020). A systemic review and meta-analysis in Sub Saharan African revealed high prevalence of treatment failure during 12 to 18 months period on treatment (Edessa et al., 2019). In South Africa, second line treatment failure was reported as 25% (Shearer et al., 2017)

Viral load suppression rate in on patient on second line was scarce in Lesotho. However, there was a national survey in 2020 (Lesotho Population-based HIV Impact Assessment -LePHIA 2020) and reported viral suppression rate of 81.0% of in the country. The suppression rate vary per district; Butha-Buthe was 74.6% while and Mokhotlong was 83.7% (LePHIA, 2021). Currently the database contains over 28,000 individuals and 611 of them receive second-line ART (Swiss TPH database, 2021).

2.9 Monitoring ART response

As access to ART for HIV is increasingly available in resource-limited settings, there is great concern regarding the development of drug resistance (Edessa et al., 2019). HIV resistance to antiretroviral therapy can be divided into two categories, namely primary and secondary resistance. Primary resistance reflects infection with an HIV strain that is already resistant to one or more drugs, while secondary or acquired resistance develops after a period of HIV treatment in a given person (Khan et al., 2019).

Viral load monitoring is recommended as the gold standard to detect treatment failure in Lesotho and internationally. (National Guidelines, 2016). Since 2016, Lesotho has implemented viral load testing as part of the treatment routine (Lesotho Operational Plan,

2016). Despite all efforts of HIV management, many patients still experience treatment failure. Therefore, resistance testing has become an important diagnostic tool, with genotypic testing being the most commonly used method of detecting resistant HIV-1 strains (Zaki et al., 2020). It is one of the earliest applications of gene sequencing for clinical purposes. Genotypic testing through Sanger sequencing has the ability to detect mutations present at levels of at least 30% of the viral population (Shafer, 2002).

In addition, ART guidelines recommend that National Programmes should develop policies and conduct pilot studies for third-line ART for patients with multi-class resistance after second-line failure (Meintjes et al., 2015). To address this, an agreement on Collaboration in a Pilot Project Introducing Genotypic Resistance Testing for HIV-1 was signed in June 2016 between Seboche Hospital, the District Health Management Team of Butha-Buthe, Swiss Tropical and Public Health Institute, Molecular Virology at the Department of Biomedicine of the University of Basel, Switzerland and the Swiss not-for-profit organization SolidarMed.

CHAPTER 3: Methodology

3.1 Study Location

The research was primarily conducted in Butha-Buthe district involving two hospitals (Butha-Buthe Government Hospital and Seboche Mission Hospital) and 10 health centres supervised by the two hospitals. One health centre from Mokhotlong district was also included in the study.

3.2 Study population and sample collection

The study was a cross-sectional study enrolling patients on second-line ART with a viral load ≥ 1000 copies/mL for whom leftover plasma samples from viral load testing were available in the -80 °C biobank freezer at the Butha-Buthe Laboratory between January 2016 and 26th October 2020.

All laboratory viral load results were imported to a password-protected online database developed at the Swiss TPH. The system allowed for the selection of patients on second-line treatment.

During analysis, each sample was given a unique number linked to the patient identifier, viral load result, and genotyping identity (ID).

3.3 Inclusion and exclusion criteria

Patients receiving viral load monitoring in the Butha-Buthe Government Hospital laboratory, who had at least one sample with a viral load ≥1’000 copies/mL taken while on second-line ART available in the biobank, were included in the study. If patient had multiple samples, second sample (at phlebotomy) was used. Patients for whom resistance testing failed were excluded from further analysis.

3.4 Methods and Materials

3.4.1. Viral load testing

The viral load was monitored at Butha-Buthe Government Hospital Laboratory using the Roche Cobas Ampliprep Taqman system following audited standard operating procedure(s). Plasma samples remaining after viral load testing were stored in a -80 °C biobank until they were used for resistance testing.

3.4.2 Genotypic Resistance Testing (GRT)

Samples meeting study criteria were transferred from Butha-Buthe Laboratory to Seboche Mission Hospital Laboratory for HIV resistance testing. The testing required

RNA extraction, reverse transcription and amplification of viral genes, sequencing, and sequence analysis.

3.4.2.1 RNA extraction

RNA extraction was performed in a designated RNA extraction room. As the first step, stored plasma was removed from the biobank and left at room temperature to completely thaw. Viral RNA extraction was done using Purelink Viral RNA/DNA Mini kit (Invitrogen, Waltham, MA, USA).

3.4.2.2 Polymerase Chain Reaction (PCR)

PCR amplification of the HIV protease (PR) and reverse transcriptase (RT) gene region was done using a commercially prepared HIV-1 genotype Amplification module kit following the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). Amplification was divided into two steps; reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR.

Ten (10) µL of extracted viral RNA was denatured at 65°C for 10 minutes. Then, 39 µL of the commercially prepared master mix (NTP, $MgCl_2$ and buffer solution) with the addition of 1 µL of *Superscript III one-step* enzyme were added. The RT-PCR reaction mix was incubated (GeneAmp PCR system 9700, Applied Biosystems) at 50ºC for 40 minutes (cDNA synthesis) and 94ºC for 2 minutes (initial denaturation), followed by 40 cycles of 94ºC for 15 seconds (denaturation), 50ºC for 20 seconds (annealing) and 72ºC for 2 minutes (extension), and ended with a 10-minute incubation at 72ºC (final extension).

Using 2 µL of this RT-PCR product, a nested PCR was done adding 48 µL of master mix containing 1 µL *AmpliTaq Gold LD DNA Polymerase*. The following thermocycler conditions were used: 94ºC for 2 minutes (initial denaturation), 40 cycles of 94ºC for 15 seconds (denaturation), 55ºC for 20 seconds (annealing) and 72ºC for 2 minutes (extension), followed by a 10-minute hold at 72ºC (final extension).

3.4.2.3 Gel electrophoresis

Amplification of the RT-PR region was confirmed by gel electrophoresis. A 1 % agarose gel was prepared by adding 1 g of agarose powder to 100 mL of 1X TAE buffer which was then heated in a microwave to dissolve the agarose and cooled slightly before adding 5 µL SYBR Safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA) prior to casting the gel.

A total of 2 µL of blue loading dye was mixed with 10 µL of PCR product and loaded onto the gel which was run at 50 V for 40 minutes on an electrophoresis power supply. Ten (10) µL of 1 kb DNA ladder was also run on the gel to assist in determining the size of the PCR product (Figure 3). Since DNA is a negatively charged molecule, it is moved by electric current through a matrix of agarose; smaller DNA fragments travel faster through the gel. The Gel UV Transilluminator (Syngane Bio Imagining) was used to view the gel.

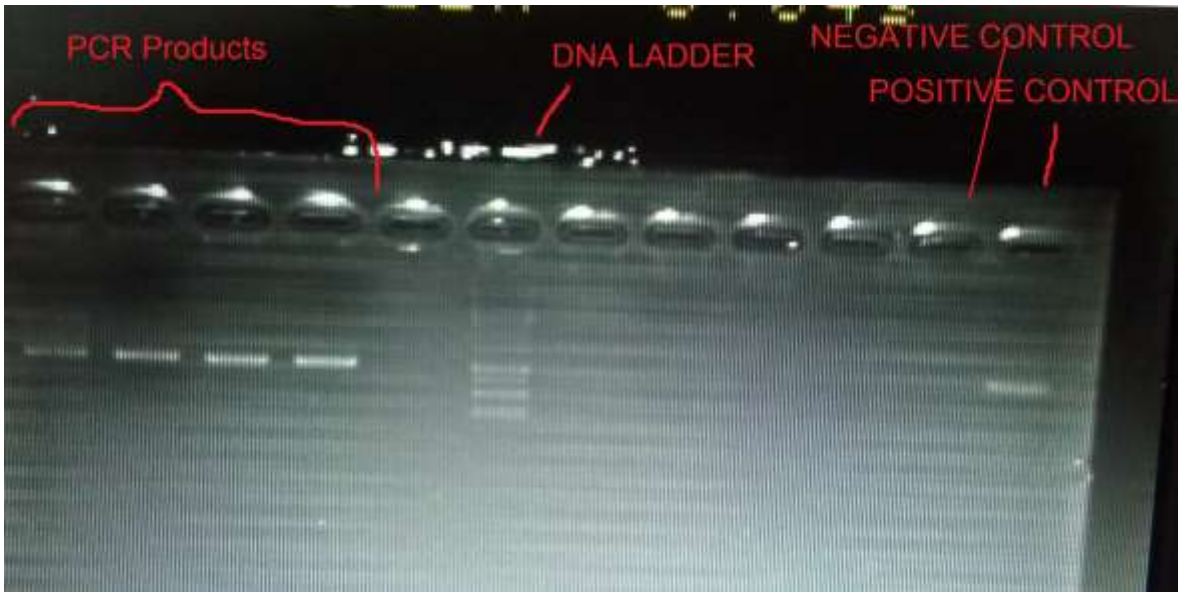

Fig 3: A gel showing patient samples, DNA ladder, negative and positive control. Note: Empty wells were samples that failed to be amplified.

The expected fragment size was around 1100 bp. If the fragment was clearly below or above that size, it was considered an aberrant amplification product of the wrong size and was not purified. Once the presence of the product was confirmed, PCR products were stored at -20ºC until used in Sanger sequencing.

3.4.2.4 Product clean up and sequencing

As a purification step, 10 µL of PCR product was mixed with 4 µL of *ExoSAP-IT* (Thermo Fisher Scientific, Waltham, MA, USA) and incubated on the thermocycler on the following conditions: 37ºC for 15 minutes (digestion), 80 ºC for 15 minutes (heat de-activation) followed by 4ºC on hold.

Sanger sequencing was performed using the commercially prepared HIV-1 genotype sequencing module kit (Thermo Fisher Scientific, Waltham, MA, USA) with set of six specific primers covering PR-RT region (F1, F2, F3, R1, R2, R3). Per sample, all 6 primers were used; for each primer, 2 µL of purified PCR product was mixed with 18 µL

of the specific primer mix provided by the kit and incubated. Thermal cycling conditions were as follows: 25 cycles of 96 ºC for 10 seconds (denaturation), 50 ºC for 5 seconds (annealing) and 60 ºC for 4 minutes (extension).

Sequencing purification was either done using BigDye xTerminator purification kit (Thermo Fisher Scientific, Waltham, MA, USA) or sephadex following an audited SOP (Appendix 1). Individual sequencing reactions per primer were transferred to a 96-well Micro Amp plate (Applied Biosystems, Waltham, MA, USA) and loaded on the SeqStudio genetic analyser for sequencing.

3.4.2.5 Data analysis and results interpretation

Sequences were edited and visualized using the online recall software (beta v3.05) and aligned to an HXB2 reference strain and a subtype C reference strain. For the purpose of quality control, phylogenetic trees; using combination of samples were used for the evaluation of potential contamination (Recall, n.d.).

The FAS files were uploaded to the online “HIVdb Program: Sequence Analysis” program from Stanford University for mutation interpretation. Genotypic sensitivity scores or the prescribed regimen at the time of second-line failure were calculated based on stratified Stanford HIVdb resistance categories: susceptible, potential low-level resistance, intermediate-level resistance, low-level resistance, and high-level resistance (HIVdb Program Database, n.d.). For each drug in the current regimen, a drug-level susceptibility score was assigned according to the Stanford resistance levels (1: susceptible; 0.75: potential low-level resistance; 0.5: low-level resistance; 0.25: intermediate resistance; 0: high-level resistance). The regimen-level susceptibility score corresponds to the sum of the drug-level susceptibility scores of the drugs in the current regimen. This method has been described elsewhere (Boyd et al., 2015).

3.5 Data management and statistical analysis

Patients’ data were extracted from the online database. Data collected was sex, age, ART history, HIV viral load and genotyping. The data was analysed using STATA version 16.1. Descriptive statistics were used to describe the study population, with categorical variables displayed as absolute numbers and percentages, and continuous variables displayed as medians and interquartile ranges (IQRs). Tables 3 and 4 summarize the demographic data and resistance testing outcomes.

3.6 Ethical aspects

The study was part of a protocol entitled “Implementation of routine viral load monitoring in rural Lesotho: A prospective cohort study on virologic outcomes among patients on ART in Lesotho” (ID 134-2016) submitted to National Health Research and Ethics Committee of Lesotho.

Samples were labelled with a unique identifier and not names; names were encrypted in the database and I didn’t have access to the names for the study purpose. The database containing the study variables was also password-protected, and only accessible to key members of the research team and clinicians. The computer capturing patients’ results was also password protected.

CHAPTER 4: Results and Discussion

4.1 Results

4.1.1 Study population

There were 1,345 samples of patients receiving second-line ART available in the database, of which 147 samples had a viral load ≥1’000 copies/mL. Some individuals had multiple samples; if there was such a case, a second consecutive high viral load sample was used.

Eighty (80) samples for 55 patients (some patients had multiple samples) were available in the bio-bank and one sample of all 55 patients’ samples were processed as they were eligible for the study; 20 samples failed to amplify, 35 samples were amplified and sequence results were available for 30 samples. Possible cause of failed PCR amplification and sequence reaction could be technical issues related sample pre-conditions, personnel competency, reagents conditions and hardware problems related to equipment used. The technical issues can be avoided ensuring that RNAs/samples are preserved as far possible, include controls in all steps,

4.1.2 Socio-demographic characteristics

There was one exceptional patient that was included in the study though was on DTG-based regimen. The median age of the participants was 41 years (IQR 30 to 49 years) for both sex and most were female 18/29 (62%). The majority of patients were from two hospitals; Seboche and Butha-Buthe Hospital, accounting for 23/30 (77%), and the remaining 7/30 (23%) were from the clinics (Muela, St Paul, Tsime, Mapholaneng, St Peters health centres). The median time patients spent taking a second-line regimen was 1.9 years (IQR 0.5 to 3.0 years) and the most frequently used regimen was AZT/3TC/LPV/r (n=14, 47%), followed by TDF/3TC/LPV/r (n=9, 30%), ABC/3TC/LPV/r (n=5, 17%), TDF/3TC/ATV/r (n=1, 3%) and AZT/3TC/DTG (n=1, 3%); see table 2.

4.1.3 Resistance associated mutations

Resistance associated mutations (RAMs) were identified in 18/29 (62%) of patients; one had RAMs in the protease region whereas 18 had RAMs in the reverse transcriptase region. There was one patient for whom the protease region failed to be sequenced but mutations were identified in the reverse transcriptase region. All results had subtype C of HIV-1.

Thirty-two (32) NRTI, 26 NNRTI, and 1 PI RAM were identified. Among 32 observed mutations conferring resistance mutation to NRTIs, the most frequent were M184V (n=10, 31%) and K70R/E (n=5, 16%). Among 26 observed NNRTI mutations, the most frequent were K103N (n=10, 38%), P225H (n=5, 19%), and G190A (n=5, 19%). The protease resistance mutations found in one sample were M46I (n=1), I54L (n=1) and V82L (n=1) as shown in table 3.

Table 3: Patient characteristics:

	N=30
Female, n (%)	18 (62)
Male, n (%) [a]	11 (38)
Age in years, median (IQR) [a]	41 (30-49)
Years on regimen at time of phlebotomy, median (IQR)	1.9 (0.5-3.0)
Facility type, n (%)	
Hospital	23 (77)
Health centre	7 (23)
Available data for previous-first line regimen n (%)	
TDF/3TC/EFV	14 (47)
AZT/3TC/NVP	6 (20)
AZT/3TC/EFV	5 (17)
TDF/3TC/NVP	4 (13)
ABC/3TC/EFV	1 (3)
Regimen, n (%)	
ABC/3TC/LPV/r	5 (17)
AZT/3TC/LPV/r	14 (47)
TDF/3TC/LPV/r	9 (30)
TDF/3TC/ATV/r	1 (3)
AZT/3TC/DTG	1 (3)

a. Missing for one participant

Table 4: Resistance testing outcomes.

	N=30
HIV subtype C, n (%)	30 (100)
Major RAMs detected, n (%) [a]	18 (62)
Major RAMs detected in the protease region, n (%) [a]	1 (3)
Major RAMs detected in reverse transcriptase region (NRTI and NNRT), n (%)	18 (62)
NRTI mutations	32
K65R	2 (6)
M184V	10 (31)
K219E/Q	3 (9)
D67E	1 (3)
D67N	2 (6)
K70R/E	5 (16)
Y115F	2 (3)
K215F/Q/V	3 (9)
T69D	1 (3)
L74I	3 (9)
NNRTI mutations	26
K103N	10 (38)
Y181C	2 (8)
P225H	5 (19)
K101E	3 (12)
G190A	5 (19)
V106M	1 (4)
PR mutations	1 (33.3)
M46I	1 (33.3)
I54L	1 (33.3)
V82L	

Number of fully active drugs in current regimen, n (%) [b]	
3	17 (61)
2	5 (18)
1	5 (18)
0	1 (3)
Regimen susceptibility score, n (%) [b]	
3	17 (61)
2.75	1 (4)
2.5	0
2.25	1 (4)
2	3 (11)
1.75	1 (4)
1.5	1 (4)
1.25	0
1	3 (11)
0.75	0
0.5	0
0.25	1 (4)
0	0

a. Including one participant receiving LPV/r-based ART with missing resistance data for the protease region, for whom RAMs were detected in the reverse transcriptase region; including one participant receiving DTG-based ART for whom no RAMs were detected in the protease or reverse transcriptase region, as resistance data for the integrase region is not available.
b. Missing for two participants: one receiving LPV/r-based ART with missing resistance data for the protease region, and one receiving DTG-based ART due to unavailability of resistance data for the integrase region.

4.2 Discussion

In our study, 55 individuals had available samples with a high viral load (≥1000 copies/mL) while taking second-line ART and 30 patients' samples were successfully sequenced. Of these, the majority were female (n=18, 62%). We found that 62% (n=18) participants had RAM while 38% (n=12) participants didn't have. The absence of detectable resistance indicated that those 12 patients were not taking their medication.

From 18 participants that had mutant virus, 60% of them had one or more mutation in the reverse transcriptase region (NNRTI and/or NRTI mutations) and one patient (3%) had major PI resistance mutations. Only ten patients (31%) had RAMs which conferred resistance to the second-line regimen, which is still a major concern since the population is using fixed dosage regimens. From the resistance-associated mutations detected, the majority had three fully active drugs in their second-line regimen (i.e., many observed resistance-associated mutations likely conferred resistance to their previous but not their current regimen).

Many countries including Lesotho are now shifting to DTG-based second-line regimens as per WHO recommendations for patients with treatment failure on NNRTI-based first-line ART (National ART Guidelines Addendum, 2019). According to nucleosides and darunavir/dolutegravir in Africa (NADIA) trial there is encouraging outcome on viral suppression after switch to DTG-based regimen. Despite that there were observed DTG resistance to second-line DTG-based ART regimens (Paton et al., 2022). As such, close monitoring of resistance patterns, including NRTI resistance, remains relevant after the roll-out of DTG

There is also a literature that shows that resistance mutations are more common in the NNRTI group than NRTI (Weng et al., 2019) However, our study found that resistance mutations were higher in the NRTIs. A similar failure characteristic of NRTIs was observed in Thailand (Thao et al., 2016).

There were NNRTI resistance observed but M184V was not present. That was an indicative that the patient did not take the medication for quite some time. M184V has high fitness costs and therefore becomes undetectable to Sanger sequencing over time in the absence of drug pressure, whereas some NNRTI RAMs persist longer. There was presence of K103N mutation that confers high-level resistance to nevirapine and efavirenz, which are NNRTIs and form part of first line regimens. K103N mutations can persist longer in the blood stream even in the absence of treatment (Weng et al., 2019).

Available literature has shown that the outcomes of patients switched to second-line ART vary by setting. In Asia, treatment failure rates of 8% to 41% have been reported, while in African settings failure rates of 13% to 40% have been reported (Thu et al., 2017). In South Africa, second line ART failure was reported as 25%. (Shearer et al., 2017).Our findings (16%) agree with other studies done in Africa that 13-40% patient fail second-line ART within 2 years (Seid et al., 2020). PI resistance was found to be rare because

PIs are known to have high genetic barrier to resistance and the virus needs multiple resistance mutations to become fully resistant (Bulut et al., 2020). The predominant NRTI resistance mutation observed in our study was M184V (n=10), which confers high-level resistance to lamivudine but makes the virus less fit to replicate well (Gagliardini et al., 2018).

4.3 Conclusion

RAMs were observed in Butha-Buthe and Mokhotlong districts. These RAMs reflect the reality of HIV care in resource-limited settings such as Lesotho. Though the sample size of our study is rather small, we believe that these results are important to clinicians and policy makers since resistance data after second-line failure in Lesotho is very scarce. Non-adherence was major driver of treatment failure among individuals receiving second line ART in Lesotho. The non-adherence issue can be address with proper periodic counselling and motivation of patients and their family members.

The prevalence of HIV drug resistance or RAM was 31% among participants on second-line ART during the first 2 years after switching to second-line ART. Only one patient (3%); whom was a female had major PI RAM, which required switching to third-line treatment. The presence of these mutations may compromise future use of second or third-line regimens in the absence of routine HIVDR testing. Switching to second- and third-line treatment is very strictly regulated in Lesotho and requires approval by HIV Expert Committee.

The main limitation was small number of successful sequences. Already from a small sample, there were two samples that the resistance associate mutations could not be sequenced. For future research, the small size problem can be avoided by including a larger geographical area.

CHAPTER 5 : References and Appendices

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5.2 Appendices

5.2.1 Sephadex purification procedure

- Prepare the Sephadex 96-well plate for sequencing reaction purification by filling the black metal plate with Sephadex powder. If you do not need the whole plate, put tape on the neighbouring rows. Fill with spatula and knock on bench to be sure Sephadex is really compact.
- Put the Sephadex plate (with filter at the bottom) on the top of the black metal plate and turn them to make the powder enter in the wells. Knock on bench to be sure all Sephadex enter in the Sephadex plate.
- Add 300 µL of deionised, DNAse-free, water in the well without touching the Sephadex and let swell for 3-4 hours at room temperature (30 min at 37°C).
- With the blue gum adapter, fix the Sephadex plate on the top of the collection plate.
- Calibrate a used Sephadex plate on balance with water.
- Centrifuge 5 minutes at 600 g.
- Discard the water and store the collection plates.
- Fix the Sephadex plate on the top of the sequencing plate.
- Add the sequencing reaction samples on Sephadex without touching it.
- Calibrate a used Sephadex plate on balance with water.
- Centrifuge 5 minutes at 900 g.
- Discard the water (of used plate) and store the Sephadex plates.
- Lay the septa flat on the plate and load the sequencer following procedure or store the plate at +4°C covered with plastic foil. POSSIBLE STOP AT 4°C

5.2.2 Ethical approval

LESOTHO

Ministry of Health
P.O. Box 514
Maseru 100

Category of Review:
[ ] Initial Review
[x] Continuing Annual Review
[ ] Amendment/Modification
[ ] Reactivation
[ ] Serious Adverse Event
[ ] Other _____

REF: ID134-2016-Renew 02

Date: 13th May 2021
To
Josephine Muhairwe
SolidarMed, Lesotho

Dear Dr. Josephine,

Re: Implementation of Routine Viral Load Monitoring in Rural Lesotho: A Prospective Cohort Study on Virologic Outcomes among Patients on ART in Lesotho

This is to inform you that the Ministry of Health Research and Ethics Committee reviewed and **APPROVED** the above named protocol for renewal and hereby authorizes you to continue the study according to the activities and population specified in the protocol. Departure from the approved protocol will constitute a breach of this permission

This approval includes review of the following attachments:
[ ] Study Protocol viral Load monitoring
[ ] English consent forms:
[ ] Sesotho consent forms:
[ ] Data collection forms in Sesotho
[ ] Data collection forms in English modified version
[x] Other materials: Letter of request extension approval and Progress report_13_05_2021

This approval is **VALID** until 14th June 2022.

Please note that an annual report and request for renewal, if applicable, must be submitted at least 6 weeks before the expiry date.

All serious adverse events associated with this study must be reported promptly to the MOH Research and Ethics Committee. Any modifications to the approved protocol or consent forms must be submitted to the committee prior to implementation of any changes.

We look forward to receiving your progress reports and a final report at the end of the study. If you have any questions, please contact the Research and Ethics Committee at rcumoh@gmail.com (or) 59037919/58860246

Sincerely,

Dr. Nyane Letsie
Director General Health Services

Dr. Jill Sanders
Co- Chairperson National Health
Research Ethics Committee (NH-REC)