



Faculty of Sciences

Department of Biological Sciences and Biotechnology

**Evaluation of the Potential Impact of Multi-Class
Antiretroviral Drug Resistant Variants on Newly Approved
Antiretroviral Therapy (ART) Among People Living with HIV
in Botswana**

by

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
ART	Antiretroviral therapy
BCPP	Botswana combination prevention program
CA	Capsid
CCR5	C-C chemokine receptor 5
CDC	Centers for Disease Control and Prevention
CRF	Circulating recombinant forms
CT	Cytoplasmic tail
CXCR4	C-X-C motif chemokine receptor 4
DC-SIGN	Dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
DOR	Doravirine
DRM	Drug resistance mutations
DTG	Dolutegravir
ESCRT	Endosomal sorting complex required for transport
ETR	Etravirine
FDA	Food and drug administration
FPR	False positive rate
FTR	Fostemsavir
gp41	Glycoprotein 41
gp120	Glycoprotein 120
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HR1	Heptad repeat 1
HR2	Heptad repeat 2
HTLV III	Human T-Cell lymphotropic virus 3
HXB2	Human Immunodeficiency Virus type 1
IAS	International AIDS Society
INSTI	Integrase strand transfer inhibitor

ICTV	International committee on taxonomy of viruses
LAV	Lymphadenopathy associated virus
MA	Matrix
MDR	Multi drug resistant
MVC	Maraviroc
NC	Nucleocapsid
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitor
PCP	Pneumocystis pneumonia
PCR	Polymerase chain reaction
PIs	Protease inhibitors
PLHIV	People living with HIV
PSSM	Position specific scoring matrices
RNA	Ribonucleic acid
RPV	Rilpivirine
R5	Chemokine receptor 5 using HIV variant
RT	Reverse transcriptase
RT PCR	Reverse transcriptase polymerase chain reaction
SIV	Simian immunodeficiency virus
TAM	Thymidine analogue mutation
T20	Enfuvirtide
TMD	Transmembrane domain
VL	Viral load
X4	Chemokine receptor 4 using HIV variant

DECLARATION REGARDING THE WORK AND COPYRIGHT

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Thesis Titled: Evaluation of the Potential Impact of Multi-Class Antiretroviral Drug Resistant Variants on Newly Approved Antiretroviral Therapy (ART) Among People Living with HIV in Botswana

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I, **David Onnalena Nkwe**, hereby confirm that I have inspected the above titled thesis and, to the best of my knowledge, it is based on the original work of the candidate.

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ABSTRACT

Botswana has over the years reported a rise in cases of HIV-associated drug resistance to all four available drug classes. This multi-class drug resistance is associated with virologic failure and increased disease progression, which significantly reduces antiretroviral drug (ARV) choices, especially in settings with limited treatment options. This highlights the need to monitor the emergence and spread of drug resistance, to avoid selection of ineffective ART regimens. This study therefore sought to determine the prevalence of multi-class drug resistance and determine if second generation non-nucleoside reverse transcriptase inhibitors (NNRTIs) [Doravirine (DOR), Etravirine (ETR) and Rilpivirine (RPV)] and entry inhibitors [Maraviroc (MVC), Enfuvirtide (T20) and Fostemsavir (FTR)] can be used as alternative therapy for individuals with multi-class drug resistance. Four approaches were used to analyze a total of 7473 HIV sequences: (i) the Stanford HIV drug resistance database to evaluate multi-class drug resistance and resistance to DOR, ETR and RPV; (ii) computational evaluation of MVC resistance by determining CXCR4 coreceptor usage using geno2pheno, webPSSM and the 11/25 rule; (iii) using the 2023 IAS mutations update to determine T20 resistance; and (iv) determining FTR resistance by probing previous studies that published FTR-resistance associated mutations. The proportions of drug resistance mutations (DRMs) were estimated using binomial exact methods. Predictors of multi-drug resistance were determined using the univariate and multivariate logistic regression hazard models. The prevalence of multi-class drug resistance (MDR) was 682/7473 (9.1%; 95 % CI; 8.3-9.6). High prevalence of resistance to second generation NNRTIs was observed within the MDR group at the following rates: 79.3%, 63.6 % and 67.1% for RPV, ETR, and DOR, respectively. For entry inhibitors, 7.9% (31/391) CXCR4 coreceptor usage was observed, indicating low prevalence of MVC resistance. A total of 113/626 (18.1%) MDR variants presented FTR resistance. T20 resistance was observed in 313/623 (50.2%) of MDR variants. ART experience, virologic failure at viral load of >400 copies/mL and being male were significantly associated with developing MDR. This study reports high resistance to second generation NNRTIs and T20 which reduces their potential use as alternative therapy for people living with HIV who harbor MDR variants in Botswana. In contrast, low prevalence of FTR and MVC resistance allows for their potential use as alternative therapy for MDR individuals, although genotypic testing prior to use of these drugs would be necessary in order to avoid selection of ineffective ARV regimens.

CHAPTER 1: INTRODUCTION

1.1 Background

The human immunodeficiency virus (HIV) remains a significant global public health concern, having caused an estimated 85.6 million infections and 40.4 million deaths since its start. HIV has affected all global regions, with Sub-Saharan Africa being the most burdened, shouldering about 50% of global infections (UNAIDS, 2023a). Development of an HIV cure has proven to be futile, but the successful management of HIV has been inevitable through the use of antiretroviral drugs (ARVs), that can suppress viral replication and maintain suppressed viral loads over long periods of time (Weichseldorfer, Reitz and Latinovic, 2021; Arentoft *et al.*, 2022). As a measure to reduce high HIV prevalence rates globally, free combination antiretroviral therapy (cART) was introduced, and Botswana was one of the first countries to implement the HIV national treatment program in 2002 (Glassman and Temin, 2016; Kelepile, 2019). Over the years, Botswana increased access to ART along with other countries globally (Wester *et al.*, 2009; Miles *et al.*, 2007), adopting the Test-and-Treat policy in 2016 (Lebelonyane *et al.*, 2021). Botswana has outdone herself, surpassing the 95-95-95 UNAIDS 2030 targets aimed at eliminating HIV. Currently, 95.1% of people living with HIV (PLHIV) in Botswana know their HIV status. Of those aware, 98.0% are receiving ART. Moreover, 97.9% of those on ART are virally suppressed (Mine *et al.*, 2022).

The increased access to antiretroviral therapy (ART) has led to a positive outcome on life expectancy and health, through significant reduction of HIV-related deaths and new infection rates (Zhou *et al.*, 2016). However, prevailing incidence rates are still very high due to the persistence of drug resistance, which is a major drawback in the management of long-term HIV-1-infected individuals with extensive ART experience (Choi *et al.*, 2014; Sierra and Walter, 2012). Development of drug resistance mutations (DRMs) is associated with decreased antiretroviral drug (ARV) drug efficacy, increased risk of clinical disease progression, causes death among PLHIV and also increases chances of transmitting resistant variants (Ma *et al.*, 2021; Bereda, 2021). Drug resistance is driven by multiple factors, including treatment interruptions, lack of adherence to medication, poor health service quality and stock-outs of drugs (Seatla *et al.*, 2018). Cases of HIV variants resistant to more than three drug classes have been reported in Sub-Saharan Africa, including Botswana (Seatla *et al.*, 2018). Treatment of individuals with multi-class drug resistant (MDR) HIV infections is challenging especially in settings with limited resources, where ARV options are limited (Joseph Davey *et al.*, 2018). Therefore, alternative treatment options other than the first generation non-nucleoside reverse

transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) are required for these individuals.

There are currently five classes of ARV used for the management of HIV, namely the NNRTIs, NRTIs, PIs, integrase strand transfer inhibitors (INSTIs) and entry inhibitors. The HIV treatment guidelines in Botswana are inclusive of four classes, the NNRTIs, NRTIs, PIs and INSTIs as shown in **Tables 1.1A and 1.1B** (Botswana Ministry of Health, 2016). Treatment regimens that contain Dolutegravir (DTG) are used as salvage or first-line therapy for adults and adolescents. Genotypic resistance testing is used to determine cART to be used as second-line therapy.

Table 0.1: Guidelines implemented in 2016 for the treatment of adults and infants living with HIV in Botswana

Table 1.1A: Pediatrics treatment guidelines

Age/weight	1 st Line	1 st Line Modifications For toxicities	2 nd Line
< 1month	AZT/ ₃ TC/NVP (Switch infants to ABC/ ₃ TC/ALU at 3months)	<u>ABC or NVP Rash</u> CBV/ALU	Based on resistance testing results and consultation with HIV specialist
>1 month to 3 years	ABC/ ₃ TC/ALU	CBV/ALU	Based on resistance testing results and consultation with HIV specialist
> 3 years (<40kg)	ABC/ ₃ TC/EFV	<u>ABC Rash</u> CBV/EFV	Based on resistance testing results and consultation with HIV specialist
		<u>CNS Toxicities</u> ABC/ ₃ TC/ALU	

Table 1.1B: Adults and adolescents treatment guidelines (Including pregnant women)

Age/Weight	1 st Line	1 st Line Modifications	2 nd Line
All adults and adolescents (> 40kg)	Truvada and Dolutegravir	TDF renal toxicity w/o CDV risk: ABC/ ₃ TC/DTG TDF renal toxicity and insufficiency with CDV risk or DTG toxicity: Discuss with specialist	Based on resistance testing results and consultation with HIV specialist

Source: (Botswana Ministry of Health, 2016)

Botswana introduced the use of DTG, a second generation-INSTI as first line therapy in 2016 along the initiation of the Test-and-Treat policy. DTG is preferred for initiating HIV treatment due to its effectiveness, minimal drug interactions, tolerability and high genetic barrier to resistance (Günthard *et al.*, 2016; Gibert, 2016), and has been reported to have higher suppression rates of >80% within 48 weeks of use (Punekar *et al.*, 2021; Nabitaka *et al.*, 2020; Mondli *et al.*, 2019). However, the presence of HIV DRMs has been associated with reduced susceptibility to DTG, leading to increase in HIV viral load. Therefore, patients with resistance to multiple drug classes are likely to fail DTG with low-level viral loads (Achieng and Riedel, 2019). Similarly, there has been data on pre-treatment HIV-1 drug resistance to NNRTIs impairing efficiency of first line DTG-based regimens (Siedner *et al.*, 2020). A study done among PLHIV in Botswana and taking INSTI-based therapy, reported 32% of INSTI-associated DRM among heavily treatment-experienced individuals (Seatla *et al.*, 2021). A case study done in Botswana on a 51 year old male patient who was highly ARV treatment experienced, and taking DTG as salvage therapy, showed that his viral load increased from 2888 to 3690 copies/ml after developing accessory mutation T97A on the integrase (IN) region (Seatla *et al.*, 2018). Similar results were reported in two patients in USA within 24 weeks of DTG use (George *et al.*, 2018). Based on this evidence, the presence of multi-drug resistance may reduce the efficacy of DTG leading to virologic failure. This, along with cross resistance that has been observed between first- and second-generation NNRTIs leaves a requirement to monitor the transmission and spread of DRM associated with DTG, first-generation and second-generation ARVs (Seatla *et al.*, 2018).

The development of more potent ARVs with high efficacy rates, such as the entry inhibitors (coreceptor antagonists, fusion inhibitors, attachment and post attachment inhibitors), and second-generation NNRTIs that lack cross-resistance with first-generation ARTs gives new therapeutic options for individuals with MDR HIV-1 infections (Ndashimye, Reyes and Arts, 2022). Inclusion of these ARVs in treatment programs would potentially aid in the management of HIV in individuals that have run out of treatment options from available ARVs. Therefore, I aimed to determine the prevalence of HIV multi-drug resistance among both ART-experienced and -naïve PLHIV, and identify the predictors associated with HIV multi-class drug resistance. The study also aimed to determine if circulating MDR HIV-1 strains are possibly susceptible to newer NNRTIs (Doravirine, Etravirine and Rilpivirine) and entry inhibitors (Maraviroc, Enfuvirtide and Fostemsavir) that have never been used in

Botswana. This sought to determine if these ARVs can potentially be used as alternative therapy for individuals with multi-class drug resistance.

1.2 HIV History

Initial clinical observations of acquired immuno-deficiency syndrome (AIDS) were in America, in 1981 (Pinching, 1986; Silverman, 1987). This was noted when a number of young homosexual men with no known **immunocompromise** were presenting with *Pneumocystis pneumonia* (PCP), an uncommon opportunistic infection that usually affects individuals with a weakened immune system. Although PCP is normally asymptomatic in immunocompetent individuals, it presented a very high mortality rate (Chavan, 2011; Adler *et al.*, 2012). It was later discovered that the opportunistic infections occurred due to an immuno-deficiency of unknown cause. Homosexual men were assumed to be the only group susceptible to the disease until cases of the disease were observed in heterosexual individuals and transfusion patients (Silverman, 1987). The Centers for Disease Control and prevention (CDC), in 1982, defined the cause of infection as diminished cell-mediated immunity and they called it AIDS (Roberts *et al.*, 1984).

In 1983, an unknown retrovirus was isolated from a patient with swollen lymph nodes (Lymphadenopathy) by Luc Montagnier and team, and it was named the Lymphadenopathy Associated Virus (LAV) (Osborn, 1986). In 1984, Gallo *et al* published a paper on a novel virus HTLV-III isolated from CD4 T-cells of AIDS patients. It was suggested that both LAV and HTLV-III were identical viruses and causative agents of AIDS (Popovic *et al.*, 1984; Harada, Koyanagi and Yamamoto, 1985). The virus was eventually renamed Human Immuno-Deficiency Virus (HIV) by the International Committee on Taxonomy of Viruses (ICTV) in 1986 (Curtis, Johnson and Owen, 2016).

A retrovirus related to HIV was isolated from AIDS patients in West Africa, in 1985 (Clavel *et al.*, 1986). This virus was named HIV-2, while the one that emerged from America was named HIV-1 (Essex and Kanki, 1988; Clavel *et al.*, 1986). HIV is thought to have originated as Simian Immunodeficiency Virus (SIV) from primates of non-human nature in Central Africa, and evolved into HIV as it infected humans (Essex and Kanki, 1988). HIV-1 ancestry has been traced back to SIV strains common in chimpanzees while that of HIV-2 has been traced back to sootey mangabeys (Tebit and Arts, 2011; Sharp and Hahn, 2011; Peeters,

D’Arc and Delaporte, 2014; Barin, 2022). The traditional practice of hunting and consumption of bush-meat in Central Africa is believed to have resulted in the cross species transmission of the virus (Cook and Karesh, 2012).

1.3 HIV Burden

The global burden of HIV remains a formidable challenge, impacting millions worldwide and posing significant health, social, and economic implications. In 2022 alone, there were 39 million PLHIV globally, of which 37.5 million were adults over 15 years and 1.5 million were children aged 0 – 14 years. New infections totaled 1.3 million and there were 630,000 deaths caused by HIV-related infections (UNAIDS, 2023b). The continuous increased access to ART led to a 38% reduction in HIV incidence rates from 2010 where 2.1 million people were newly infected with HIV (UNAIDS, 2023b; WHO, 2023). This has also been accompanied by reduction in annual AIDS-related deaths from 2.0 million in 2004. Sub-Saharan Africa bears the highest burden of HIV globally, with three of its countries being the most affected. Botswana stands as the third most infected at 18.6%, after Eswatini (27.9%) and Lesotho (20.9%), among individuals aged 15-49 years (Mine *et al.*, 2022).

1.4 Molecular Epidemiology

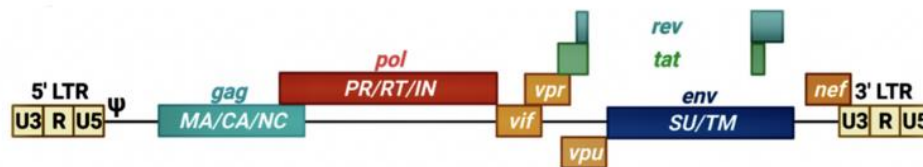
HIV-1 is categorized into four different known groups (M, N, O and P), and it is the most prevalent type of HIV. Group M predominates in most parts of the world and has given rise to various subtypes including A1 and 2, B, C, F1 and 2, G, H, J, K and circulating recombinant forms (CRFs) (Taylor *et al.*, 2008). Nearly 90% of HIV-1 infections originate from group M. The groups N, O and P are primarily confined to the African region (Ndung’u and Weiss, 2012). The HIV-1C strain, also known as HIV-1 subtype C predominates worldwide and accounts for half of global infections. It is predominantly present in the regions of Southern and Eastern Africa, as well as India and Nepal (Gartner *et al.*, 2020; Wang *et al.*, 2019). Strain B is most common in America, Europe, Australia and Japan (Ndung’u and Weiss, 2012). Although HIV has multiple subtypes, the most diverse range of subtypes are established in Western and Central Africa where it evolved, and smaller HIV groups (N, O, and P) can hardly be found outside of this region (Appah *et al.*, 2022; O’Hara, 2022).

1.5 HIV Virology

1.5.1 The HIV genome and structure

Human immunodeficiency virus, a member of the lentivirus family, is a retrovirus capable of performing reverse transcription, which involves production of double stranded DNA. HIV genomes consist of two 9.7 kilobase single stranded ribonucleic acid (RNA) (Geronikaki, Eleftheriou and Poroikov, 2018; Reddy, 2014). Each RNA has nine genes which are *tat*, *vpu*, *rev*, *vpr*, *vif*, *nef*, *gag*, *pol* and *env*, as shown in **Figure 1.1A**. HIV genes code for production of fifteen proteins which can be classified into different groups shown in **Figure 1.1B**. Structural proteins are encoded by *env* [transmembrane protein gp41 and surface protein gp120] and *gag* [nucleocapsid (NC), capsid (CA), budding protein (p6) and matrix (MA)]. Catalytic proteins are encoded by *pol* [protease (PR), integrase (IN) and reverse transcriptase (RT)]. Regulatory proteins, on the other hand are encoded by *rev* (regulator of virion protein expression) and *tat* (trans-activator of transcription). Lastly, the accessory proteins are encoded by *nef* (negative regulatory factor), *vpu* (viral protein U), *vif* (virion infectivity factor), and *vpr* (viral protein R) (Swanson and Malim, 2008; Carr *et al.*, 1996).

A. HIV-1 Genome



B. HIV-1 mature virion

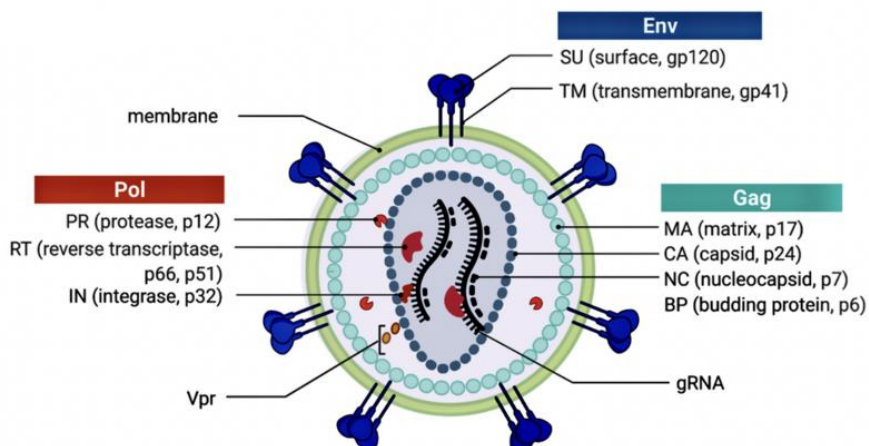


Figure 0.1: Diagrammatic portrayal of the HIV genome and fully formed virion

1.5.2 HIV Gene Products and Function

HIV gene products play various roles in the life cycle of the virus. The *gag* gene encodes precursors of the structural proteins, the outer core membrane MA, CA, NC and the p6 (Freed, 1998). The MA forms the inner membrane layer of HIV, the CA forms the conical capsid and NA forms the nucleocapsid and RNA complex (Blood, 2016). Gag proteins are crucial for assembly, release and maturation of HIV virions (Waheed and Freed, 2012). During the late phase of HIV replication, MA propels Gag towards the plasma membrane and incorporates envelope proteins into viral particles (Freed, 1998). The CA is responsible for recruiting viral RNA into virions, and p6 is involved in virus particle release (Freed, 2015; Waheed and Freed, 2012; Freed, 1998).

Proteins of the *pol* region are the most significant with regards to infectivity of the virus. The RT is essential for reverse transcribing viral RNA to DNA. Viral DNA is then integrated into host cell genome by the integrase enzyme prior to transcription and translation into viral proteins. The protease cleaves gag into different subunits, leading to maturation of the virus (Hill, Tachedjian and Mak, 2005). *Env* genes generate viral subunits that consist of two membrane proteins that are non-covalently linked: gp120 an outer envelope protein, and transmembrane protein gp41 responsible for anchoring the glycoproteins to virion surface membrane. Envelope glycoproteins, gp120 and gp41 are crucial for attachment and entry of HIV into host cells (Checkley, Luttge and Freed, 2011; Arrildt, Joseph and Swanstrom, 2012; Cenci *et al.*, 2012). Structurally, gp120 is made up of five variable (V) regions interspaced between five constant (C) regions (**Figure 1.2**). The variable regions consist of **region 1 to 5** whose primary responsibilities are infectivity, transmission and confers antibody neutralizing resistance (Arrildt, Joseph and Swanstrom, 2012). The role of the variable 1 (V1) region is to protect CD4 binding sites from neutralization by antibodies. The role of the V2 region is to interact with $\beta 7$ host cell receptor which is important for virus capture (van Gils *et al.*, 2011). The V3 binds the host cell CCR5 coreceptor to initiate entry of the virus into the host cell. Acquiring specific mutations allows the V3 loop to utilize the CXCR4 coreceptor. The roles of V4 and V5 are not yet fully understood but they are assumed to play a role in envelope conformation and glycan packaging (Cenci *et al.*, 2012; Zhang *et al.*, 2010; Wilen, Tilton and Doms, 2012; Le *et al.*, 2015).

The function of gp41 is to facilitate coalescing of viral and host cell membranes, creating a pore that will allow deposition of the viral capsid into the host cell. Structurally,

gp41 consists of several components: an extracellular domain that contains the fusion peptide (FP), two sets of seven amino acid residues (heptad-repeats 1 and 2, or HR1 and HR2), a region outside of the membrane (membrane proximal external region or MPER), a transmembrane domain (TMD), and a cytoplasmic tail (CT). A magnified view of the gp41 CT shows several features, including an internalization signal (YSPL), a kennedy sequence, three amphipathic alpha helices known as LLP one to three, and the C-terminal dileucine motif (LL) that plays a role in envelope intracellular trafficking and endocytosis (Checkley, Luttge and Freed, 2011).

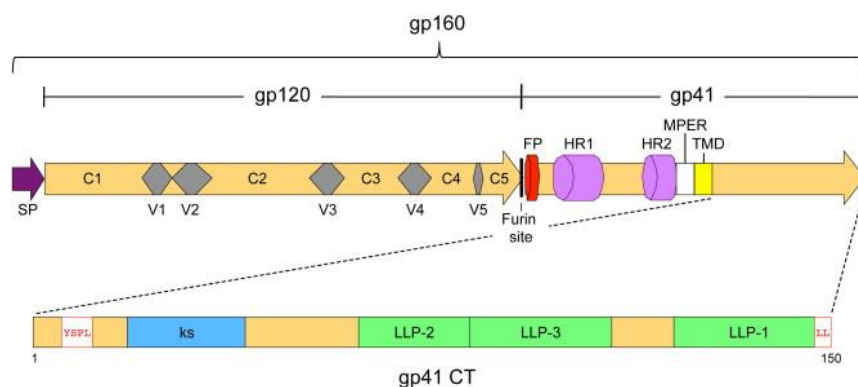


Figure 0.2: Genomic structure of gp160

Source: (Checkley, Luttge and Freed, 2011)

Accessory proteins act as adapter molecules that alter the function of host factors through protein-to-protein interactions to increase virus infectivity (**Table 1.2**) (Strebel, 2013; Richter, Frasson and Palu, 2009). The role of regulatory protein rev is to transport incompletely spliced or unspliced RNA from the nucleus to the cytoplasm. The *tat* gene products regulate the transcription of the HIV genome and cellular gene expression, creates an environment that permits the virus to replicate by modifying immune responses and facilitating the spread of the virus to numerous tissue (Clark, Nava and Caputi, 2017; Richter, Frasson and Palu, 2009).

Table 0.2: Summary of HIV-1 genes and their functions

Gene	Protein name (size)		Protein function
	Precursor	Product	
<i>Gag</i>	Gag (p55)	CA (p24)	Encodes precursors of the structural proteins
		MA(p17)	Pilots the multimerization of Gag
		NC (p7)	Propels Gag towards the plasma membrane
		p6	Recruits the viral RNA into virions
			Contains motifs that recruit ESCRT to facilitate budding
<i>Pol</i>	Pol (pr 160 ^{gag-pol})	RT	Encodes viral enzymes
		(p65/55)	Reverse transcribes viral RNA to DNA
		PR (p15)	Cleaves Gag precursor protein to mature virions
		IN (p11)	Integrates viral DNA into host cell genome
<i>Env</i>	Env (gp160)	SU	Encodes viral membrane glycoproteins
		(gp120)	Envelope surface protein
		TM(GP41)	Envelope transmembrane protein
<i>Nef</i>		Nef (p27)	Collapses the self-defense of infected cell
<i>Rev</i>		Rev (p19)	Regulates the splicing and transport of viral mRNA
<i>Tat</i>		Tat (p14)	Transactivates the expression of viral genes
<i>vif</i>		Vif (p23)	Deactivates host cell defense proteins
<i>Vpr</i>		Vpr (p18)	Guides viral genome to the host cells' nucleus
<i>Vpu</i>		Vpu (p15)	Allows the virus to escape host cell restriction factors

1.6 Transmission and Pathogenesis of HIV-1

Most cases of HIV-1 infection are transmitted through sexual intercourse, with 80% of individuals becoming infected after exposure to HIV through mucosal surfaces in the genital area (Sharp and Hahn, 2011). HIV-1 also spreads by incidents of percutaneous exposure, and perinatal pathways. HIV infections can be transmitted through a limited number of body fluids including breast milk, vaginal secretions, semen, and blood (Siberry, 2014; Sharp and Hahn, 2011). Normal day to day interactions like hugging, kissing, handshakes or sharing of personal items, water, or food do not pass HIV infections between individuals (Hasan *et al.*, 2022;

McGee, 2020). According to previous research, a single virus-infected cell or a single virus causes about 80% of productive infections (Woodman and Williamson, 2009; Parrish *et al.*, 2013; Cohen *et al.*, 2011). It has also been demonstrated that apart from having a large number of closely related variants in the donor, only two or three variants are transmitted to the recipient due to a genetic bottleneck. Also, PLHIV who are on ART and with suppressed viral loads are at a reduced risk of infecting their sexual partners. Early access to ART as well as encouragement to continue ART are critical not only for improving the health of PLHIV but also to eliminate the spread of HIV (WHO, 2022).

HIV initially infects mucosal cells (epithelial, dendritic, CD4⁺ T- cells and microphages) by attaching itself to the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) found on mucosal cell surfaces (Wu, 2008). The attached virus migrates through the mucosa, towards CD4⁺ T- cell where it can cause infection. The virus infects T cells expressing the CCR5 or CXCR4 coreceptor, which then migrate to the lymph nodes. These infected CD4⁺ T-cells remain in lymph nodes until they replicate enough to cause plasma viremia (Weber, 2001; Hernandez-Vargas and Middleton, 2013). The virus will thereafter spread broadly throughout the **body, this** marks the initial phase of infection called primary infection. Primary infection is associated with development of symptoms that include lymphadenopathy, rash, headache, sore throat, and fever within days to weeks after infection (Naif, 2013). Sero-conversion occurs during this period as the immune system produces antibodies in response to the virus (Hirschel, 2019). Additionally, the infection is followed by a long asymptomatic phase, characterized by a gradual decline in CD4⁺ T cells. There are no visible symptoms or indicators associated with this stage of HIV infection. Despite a person's appearance and apparent health, HIV nonetheless compromises their immune system. Without an HIV test, many people would not be aware that they are infected during this stage, which can extend for a number of years (on average, 8 to 10 years) (**Figure 1.3**) (Hirschel, 2019; Naif, 2013).

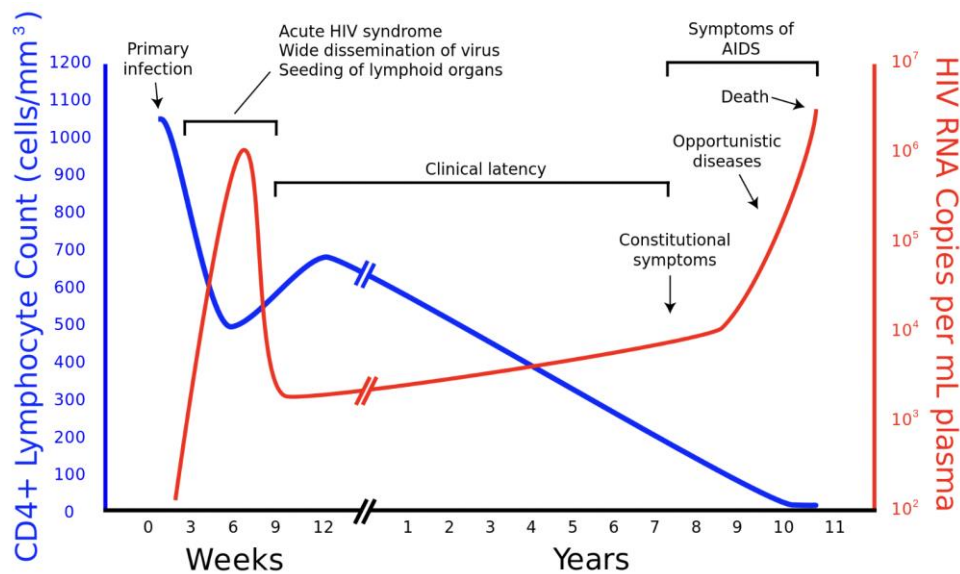


Figure 0.3: Progression of HIV infection

The progression of HIV infections initiates with primary infection, followed by acute infection about three to nine weeks after initial infection, where flu like symptoms might be experienced following high viral loads and spread of the virus. Clinical latency follows at about nine weeks to seven years after infection, associated with mild to no symptoms due to low rates of HIV replication. After about seven to eight years severe immune-suppression occurs as CD4⁺ T-Cells significantly drop, leading to acquisition of opportunistic infections.

Source: (Hirschel, 2019)

When the cellular defense against HIV is depleted, and the CD4 count is $<200 \text{ cell/mm}^3$, the illness is in its late stages. HIV gradually weakens and damages the immune system, causing the emergence of symptoms like fatigue, mouth ulcers, weight loss, diarrhea and thrush that can start off modest but get worse over time. The symptoms occur due to emerging opportunistic infections, which prey on a person's weakened immune system, for example: Kaposi's sarcoma, *Pneumocystis pneumonia*, and *toxoplasmosis*. The last stage of the infection is AIDS which is diagnosed by CD4 counts of $<200 \text{ cells/mm}^3$, high viral load and presence of opportunistic infections (Hirschel, 2019; Naif, 2013).

1.7 HIV Life Cycle and Drug targets

1.7.1 The life cycle of HIV

HIV infects white blood cells that express CD4 receptors and CCR5 or CXCR4 coreceptors. Viruses that utilize CCR5 coreceptors are called R5 variants while those that utilize CXCR4 coreceptors are known as X4 variants. A variant with the ability to utilize both CCR5 and CXCR4 coreceptors is dual tropic, also known as R5X4 (Naif, 2013). The HIV life

cycle exhibits early and late stages of replication (**Figure1.4**). Early stages involve the HIV entry process through interaction and binding of the viral envelope proteins to the host cell surface receptors until the viral genome is integrated into the host cell's genome. The late phase occurs from gene expression until mature viral particles bud off from the host cell (Freed, 2015). HIV entry involves attaching of gp120 to the CD4 receptor binding site and further interaction of third variable loop of gp120 (gp120 V3) with the CCR5 or CXCR4 coreceptor of the host cell. After attaching of gp120 V3 loop to a coreceptor, the structure of gp41 changes to form six helix bundle that juxtapose the viral and host cell membranes, allowing them to fuse and create a pore for deposition of the capsid into the host cell (Zhang *et al.*, 2010; Le *et al.*, 2015; Naif, 2013). The viral capsid uncoats to allow reverse transcription of viral RNA to DNA (Engelman and Cherepanov, 2012). Viral DNA is imported into the nucleus where it is integrated to the host cell DNA by the integrase enzyme (Freed, 2015; Arhel, 2010).

During the late replication phase, in the host cells' nucleus, viral DNA is transcribed to messenger RNA (mRNA) along with the DNA of the host cells (Waheed and Freed, 2012). Viral mRNA is transported to the cytosol and translated to Gag polyprotein precursors, GagPol polyprotein precursors, viral envelope glycoproteins (gp120 and gp41), regulatory and accessory viral proteins. Gag polyprotein precursors contains the MA, NC, CA and the p6 domain. The GagPol polyprotein precursors are synthesized through a programmed frameshift during translation of Gag encoding viral RNA and contain MA, NC, CA, RT, PR and IN domains (Waheed and Freed, 2012; Freed, 2001). **Gag is trafficked to the plasma membrane through electrostatic interactions that occur at the highly basic region of the MA, which is close to the myristoylated (Myr) N-terminus** (Monje-Galvan and Voth, 2020). Afterwards, Gag attaches to the lipid microdomains of the plasma membrane by interacting with the membranes phospholipid phosphatidylinositol 4,5-bisphosphate and by inserting its amino-terminal myristate into the lipid bilayer (Freed, 2015). During the assembly process, CA pilots the multimerization of Gag. Viral RNA is recruited into virions by the NC which aids during the assembly process. Viral envelope proteins are transported from the rough endoplasmic reticulum to the Golgi apparatus through vesicles, via the secretory pathway, to the plasma membrane where they are incorporated into the assembling particle (Yang *et al.*, 2022; Freed, 2015). The process of completing budding involves recruiting endosomal sorting complex required for transport (ESCRT) proteins, which facilitate membrane fission. The recruitment is facilitated by interactions of the p6 PTAP motif and the ESCRT-I tumour susceptibility gene 101, as well as binding of the ESCRT associated factor ALG2-interacting protein X to the p6

YPXnL motif (Freed, 2015). Further recruiting of the ESCRT III complexes and the vacuolar protein sorting 4 drive the scission reaction of the membrane, leading to the release of immature virus particles. After immature virions are released, virion maturation occurs when protease cleaves GagPol and Pol precursor polyproteins, triggering morphological transformation that leads to formation of the capsid as can be seen in **Figure 1.4** (Freed, 2015; Sutton, Dotson and Dong, 2013).

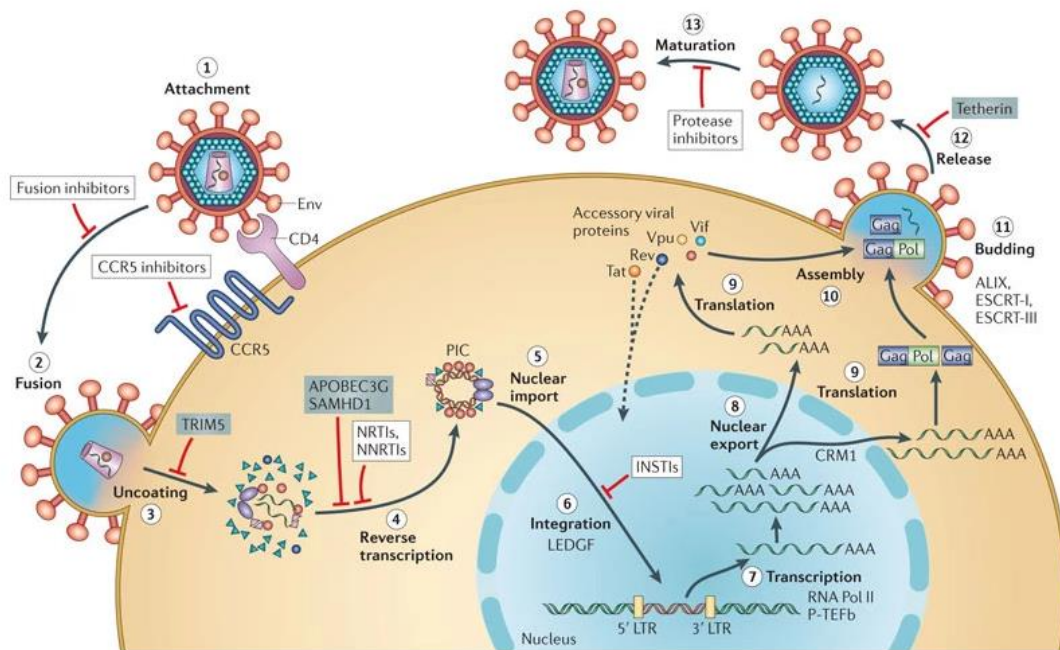


Figure 0.4: Stages of the HIV replication cycle and ARV targets of inhibition

Schematic presentation of the HIV replication cycle and the stages targeted by ARV drugs for viral suppression. HIV infects the host cell in multiple stages: 1- HIV enters the host cell through interaction and attaching of gp120 to the CD4 receptor and the coreceptors CCR5 and/or CXCR4; 2- Host cell and viral membranes fuse and allow deposition of the viral capsid into the host cell; 3- Capsid partially uncoats; 4- Viral RNA is reverse transcribed to DNA; 5- Viral DNA is imported to the nucleus; 6- Viral DNA is integrated to the host cell genome forming a provirus; 7- Proviral DNA is transcribed mRNA; 8- mRNA is exported from the nucleus to the cytoplasm; 9- mRNA is translated to viral proteins; 10- Viral proteins assemble and ; 11- bud off from the host cell; 12- Viral proteins are cleaved by protease to mature and infectious virus particles. Different ARV target different steps of the replication cycle for inhibition, sites of action are indicated in white boxes. Fusion inhibitors, CCR5 inhibitors, NNRTIs- non-nucleoside reverse transcriptase inhibitors, NRTIs- nucleoside reverse transcriptase inhibitors, INSTI- integrase strand transfer inhibitors and PIs- protease inhibitors.

Source: (Engelman and Cherepanov, 2012)

1.7.2 ARV Drug Targets and Mechanism of Action

The Food and Drug Administration has approved over twenty-five HIV ARVs, **listed in Table 1.3**, belonging to different drug classes: the entry inhibitors, NNRTIs, NRTIs, INSTI and PIs. Although ARVs do not cure HIV, they help manage HIV by inhibiting viral replication through targeting various stages of its life cycle as shown in **Figure 1.4**.

Entry inhibitors are a group of ARV drugs that inhibit the viral entry pathway, these are inclusive of coreceptor antagonists (CCR5 or CXCR4 antagonists), fusion inhibitors, attachment inhibitors and post attachment inhibitors (Chesna and Fellner, 2017; Lai, 2021). The function of CCR5 antagonists is to inhibit interaction of the coreceptor with gp120 V3 loop through binding to the CCR5 coreceptor. This action effectively prevents virus entry into the host cell (Koma *et al.*, 2021). Fusion inhibitors bind to gp41 preventing conformational changes that allow virus and host cell membranes to fuse. This prevents the virus from infecting the host cell (Monteiro, Karl and Hicar, 2021). Attachment inhibitors bind to the gp120 subunit disrupting its interaction with the host cells CD4 receptors. As a result, the initial attachment required for viral entry is prevented. Additionally, studies have shown that attachment inhibitors can also impede post-attachment stages required for viral entry, which are dependent on gp120 (Lai, 2021; Cihlar and Fordyce, 2016). Post-attachment inhibitors bind to CD4 receptors expressed on surfaces of host cells. **This prevents interaction of the CD4 receptors with viral gp120, hence preventing the virus from gaining access into the host cell** (Cihlar and Fordyce, 2016).

NNRTIs and NRTIs inhibit viral DNA synthesis through halting reverse transcription of viral RNA (Patel and Zulfiqar, 2022). NNRTIs inhibit viral replication through allosteric inhibition of RT. NNRTIs bind to the highly hydrophobic pocket situated near the RT active site, thus altering the RT active site, disrupting it from carrying out its normal function, hence inhibiting viral replication (Schauer *et al.*, 2014). NRTIs are cellular nucleotide base analogues that are utilized by RT to synthesize viral DNA. NRTIs unlike natural nucleosides lack a 3' hydroxyl (OH) group required for addition of next nucleotide. Once incorporated into the growing cDNA strand they halt further addition of nucleotides, preventing synthesis of viral DNA (Patel and Zulfiqar, 2022).

INSTI's bind to and inhibit enzyme integrase, preventing it from integrating viral DNA to host cell DNA. Unintegrated viral DNA cannot be transcribed and translated to viral proteins

required to establish an infection (Jóźwik, Passos and Lyumkis, 2020). Protease inhibitors simulate HIV Gag-polyproteins that are usually cleaved by protease, but contain chemical modifications that prevent them from being cleaved by the enzyme. PIs therefore binds to and inhibits protease, preventing cleavage of GagPol precursors into mature viral proteins required for viral replication (Fun *et al.*, 2012).

Table 0.3: Clinically Approved ARVs

ARV drugs [name, abbreviation (*Brand name*)] listed according to different drug classes.

Entry Inhibitors	
CCR5 Antagonist	Fusion Inhibitors
Maraviroc, MVC (<i>Selzentry</i>)	Enfuvirtide, ENF/T-20 (<i>Fuzeon</i>)
Attachment Inhibitors	Post attachment Inhibitors
Fostemsavir (<i>Rukobia</i>)	Ibalizumab (<i>Trogarzo</i>)
NNRTIs	NRTIs
Delavirdine, DLV (<i>Rescriptor</i>) Doravirine, DOR (<i>Pifeltro</i>) Efavirenz, EFV (<i>Sustiva</i>) Etravirine, ETR (<i>Intelence</i>) Nevirapine, NVP (<i>Viramune</i>) Rilpivirine, RPV (<i>Edurant</i>)	Abacavair, ABC (<i>Ziagen</i>) Didanosine, ddl (<i>Videx</i>) Emtricitabine, FTC (<i>Emtriva</i>) Lamuvudine, 3TC (<i>Epivir</i>) Stavudine, d4T (<i>Zerit</i>) Tenofovir alafenamide, TAF (<i>Descovy</i>) Tenofovir disoproxil fumarate, TDF (<i>Viread</i>) Zidivudine, AZT/ZDV (<i>Retrovir</i>)
PIs	INSTIs
Atazanavir, ATV (<i>Reyataz</i>) Darunavir, DRV (<i>Prezista</i>) Fosamprenavir, FPV (<i>Lexiva</i>) Indinavir, IDV (<i>Crixivan</i>) Nelfinavir, NFV (<i>Viracept</i>) Ritonavir, RTV/r (<i>Norvir</i>) Saquinavir, SQV (<i>Invirase</i>) Tipranavir, TPV (<i>Aptivus</i>)	Bictegravir, BIC (<i>Bictarvy</i>) Dolutegravir, DTG (<i>Trivicay</i>) Elvitegravir, EVG (<i>Vitekta</i>) Raltegravir, RAL (<i>Isentress</i>)

1.8: Drug Resistance

1.8.1 Principles of drug resistance

Antiretroviral drug resistance occurs due to accumulation of mutations that decrease ARVs ability to inhibit viral replication effectively (WHO, 2022). Reverse transcriptase's extremely fast replication rate, together with its inadequate capacity for proofreading and its high rate of genetic recombination, cause the virus to acquire mutations (Johnson, Jones and Clark, 2022). Resistant viruses will outlive strains that lack resistance mutations when selective drug pressure is present. The resistant strains will eventually proliferate and dominate the current virus population (Pennings, 2013; WHO, 2022). The prevalence of a mutation, its selective advantage, and quantities of ARV drugs present, have an impact on how quickly resistance develops and is selected for (Clavel and Hance, 2004). Selective advantage are traits in an organism that enable it to survive longer and reproduce more frequently. This idea is closely related to that of the virus fitness, which is its capacity to propagate and replicate in a given environment. Development of mutations is usually associated with reduced viral fitness; although, compensatory mutations have the capability to restore viral fitness, enhancing viral survival without influencing susceptibility of particular ARV drugs (Johnson, Jones and Clark, 2022). **Figure 1.5 below illustrates the drug resistance selection process.**

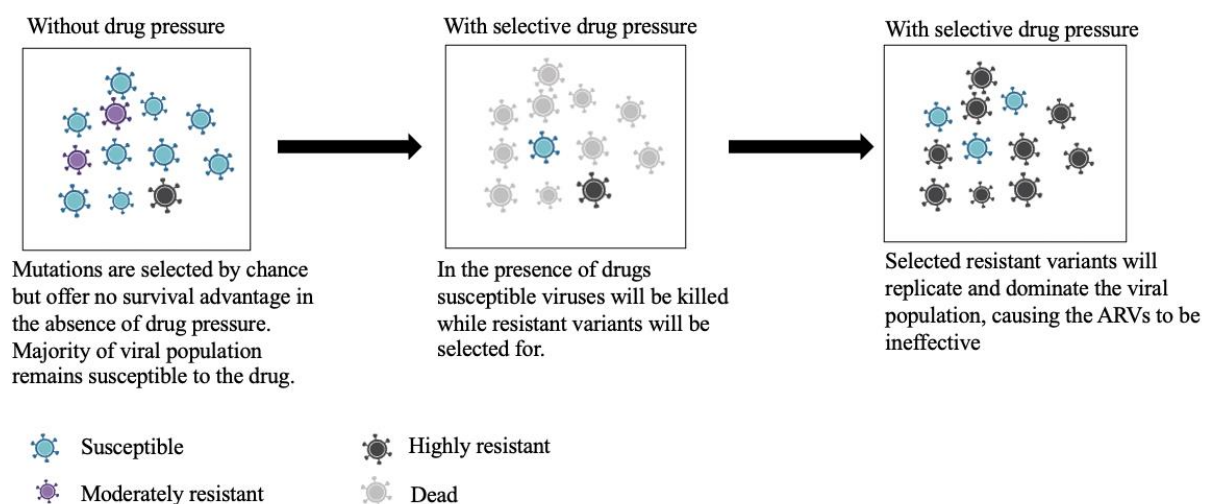


Figure 0.5: Selection of drug resistance

Taken from Sax, P.E. (2006)

Without selective pressure, viral strains with resistance mutations frequently vanish from the predominant circulating virus. Various factors, including number of mutations and the fitness cost of certain mutations necessary for resistance to reverse, affect the rate at which

this occurs (Johnson, Jones and Clark, 2022; Shafer, Kantor and Gonzales, 2000; Gregson *et al.*, 2020). When ARV regimens are re-initiated, variants with specific resistance mutations can quickly emerge from archived genomes in latently infected cells.

1.8.2 Mechanisms of Drug Resistance

Emergence of resistance is dependent on how much the virus continues to replicate during ARV therapy; viral population size and heterogeneity within an individual; acquisition of mutations and how much they change viral fitness and drug susceptibility (Mackie, 2006; Clavel, 2004). The emergence of drug resistance poses a threat to the effectiveness of all ARVs, including newer classes. Failure to prevent this phenomenon can result in a rise in HIV related morbidity and mortality (WHO, 2022). Future drug initiative programs should look into agents that have a high genetic barrier to resistance, to ensure adequate drug efficacy in patients who are failing therapy (Emamzadeh-Fard *et al.*, 2013).

NNRTI resistance occurs when resistance mutations alter the size or physical structure of the hydrophobic pocket found close to the active site of RT. This prevents binding of the ARV drugs to the RT hydrophobic pocket (Mackie, 2006). One mutation has the ability to cause resistance to multiple NNRTIs (Shafer, Kantor and Gonzales, 2000). NNRTI resistance associated mutations, including L100I, Y181C, G190S/A, and M230L occur within the RT hydrophobic pocket. Mutation K103N forms a hydrogen bond in unbound RT, thereby keeping the hydrophobic pocket closed, preventing NNRTIs from entering (Clavel, 2004).

Resistance to NRTIs primarily occurs through two distinct mechanisms, namely discrimination and excision of the inhibitor (Ly *et al.*, 2007). Appearance of specific mutations, namely K65R, K70E/G, L74V, Y115F, Q151M, and M184V/I, allows RT to distinguish between natural nucleotides used in DNA synthesis and NRTIs. This prevents incorporation of the NRTIs onto the growing DNA strand through discrimination (Marcelin, 2006; Clavel, 2004). Mutations M184V/I is the most common among various populations and is linked to significant resistance to lamivudine and emtricitabine (Ly *et al.*, 2007; Gregson *et al.*, 2020). NRTI resistance also occurs when the virus acquires thymidine analogue mutations (TAMs) (M41L, D67N, K70R, L210W, T215Y/F and K219Q/E), which allow the removal of NRTIs from the DNA chain by mediated phosphorylation of adenosine triphosphate (ATP). TAMs are selected for by stavudine and zidovudine, which are both thymidine analogues, and cause resistance to nearly all NNRTIs (Marcelin, 2006; Clavel, 2004). However, the extent to which

the TAMs reduce drug efficacy is different for each NNRTI drug causing resistance to develop much quicker for some drugs than others (Clavel, 2004)

Integrase strand transfer inhibitor drug resistance mutations are associated with altered flexibility of integrase. Mutations N155H when found solo or in combination with Q148H alter the orientation of Mg^{2+} ion on the active site of enzyme integrase, and this completely changes the INSTI binding site. Alteration of the ion coordination affects the terminal nucleotide flexibility close to the active site of integrase, weakening the PI and integrase complex (Anstett *et al.*, 2017; Machado and Guimarães, 2020).

On the other hand, development of resistance to PIs is necessitated by accumulation of major and minor compensatory mutations **that** occur close to the protease active site (Emamzadeh-Fard *et al.*, 2013). However, some protease mutations occur further from the active site and change the structure of the protease enzyme conferring resistance to PIs (Clavel, 2004). Mutations that occur at the substrate binding cleft, just on top of the protease active site prevent binding of the PIs to the protease enzyme causing resistance to PIs (Noortje, 2006). When major PI mutations are absent, minor mutations have little effect on treatment outcome although they can increase the ability of protease to bind to its substrate resulting in more efficient viral progeny production. Major mutations emerge as individuals are continuously exposed to PI containing regimens (Emamzadeh-Fard *et al.*, 2013; Noortje, 2006).

1.8.3 Clinical Implications of Drug Resistance

Since drug resistance affects a significant number of HIV patients receiving therapy and builds up over time, it poses a challenge in treatment (Green *et al.*, 2012). The spread of drug resistant variants exacerbates medication failure and poses a substantial public health risk as new infections occur. Even with the availability of more tolerable regimens, drug resistance still affects up to 15% of first line ART (Cortez and Maldarelli, 2011), and individuals who harbour variants with drug resistance associated mutations have limited treatment options (Gantner *et al.*, 2016). Drug resistance is more likely to be acquired by individuals who poorly adhere to their drug regimens or are receiving suboptimal treatment combinations (Johnson, Jones and Clark, 2022). It is estimated that 60-72% of acquired drug resistance occurs after virologic failure (Hosseinipour *et al.*, 2013). Drug resistance can also be transmitted through infection by drug resistant variants (Pennings, 2013). Transmitted drug resistance is estimated

to be prevalent at 10% in individuals initiating first line NNRTIs, within new infections (WHO, 2022).

1.9 Problem Statement

There currently are reports on HIV-1 variants resistant to all four drug classes used for the management of HIV in Botswana. Previous research has also demonstrated cross-resistance between first generation and second-generation NNRTIs, reduced efficacy to DTG due to pre-treatment NNRTI resistance, and prevailing naturally occurring low resistance to entry inhibitors in entry inhibitor naïve individuals. The presence of DRM substantially reduces therapeutic choices of ARVs, especially in settings with limited treatment options. Data on the prevalence of HIV multi-drug resistance is limited in Botswana, and available data was pre-DTG initiation; however, the trends of multi-drug resistance are increasing. No presumptions can therefore be made on the clinical management of individuals with multi-class drug resistance, who have exhausted available ART options. This leaves a need to monitor the emergence and spread of drug resistance to avoid selection of ineffective regimens.

1.10 Justification

To the best of my knowledge, this study is the first to determine if HIV-1 MDR variants in Botswana are susceptible to newer NNRTIs and entry inhibitors using a large cohort. Currently, the management of PLHIV who have multi-class drug resistant strains is one of the most critical issues in HIV therapy. Therefore, this study was aimed at determining multi-class drug resistance and its predictive factors, and also predict the prevalence of *pol* and *env* mutations associated with resistance to DOR, ETR, RPV, MVC, FTR and T20. Obtained results will give insight into drugs that can be used as alternative treatment for MDR individuals who are not responsive to their current treatment options in Botswana.

1.11 Aim and Objectives

Aim

- To evaluate the potential impact of multi-class drug resistant (MDR) strains on newly approved ART in PLHIV in Botswana

Specific Objectives:

- To determine the prevalence of multi-class drug resistance in Botswana.

- To determine the prevalence of mutations associated with resistance to second generation NNRTI's [Rilpivirine (RPV), Etravirine (ETR) and Doravirine (DOR)] among MDR HIV variants.
- To determine the prevalence of mutations associated with resistance to entry inhibitors [maraviroc (MVC), enfuvirtide (T20) and fostemsavir (FTR)] among MDR HIV variants.
- To identify predictive factors of MDR multi-drug resistance among PLWH in Botswana.

CHAPTER 2: METHODOLOGY

2.1 Study Population

The study population included sequences of HIV isolates from individuals who had enrolled for various studies for PLHIV in Botswana, the Botswana Combination Prevention Program (BCPP) and the Bosele studies, described in **Table 2.1**. Also included were sequences downloaded from the Los Alamos database, representing different studies that were conducted between 1996 and 2001 in Botswana. **BCPP sequences were generated from samples obtained from 30 communities in Botswana (Ranaka, Digawana, Molapowabojang, Otse, Letlhakeng, Lentswelatau, Bokaa, Oodi, Mmathethe, Sefhope, Lerala, Ramokgonami, Maunatlala, Mmadinare, Shoshong, Metsimotlhabe, Tati Siding, Sebina, Nkange, Mandunyane, Rakops, Gweta, Shakawe, Gumare, Tsetsebjwe, Sefhare, Nata and Masunga).** Bosele samples were collected from individuals who were under clinical monitoring at the Infectious Disease Control Centre Princess Marina Hospital (IDCC PMH). All participants had multiple HAART failures. Sequences downloaded from the Los-Alamos database could not be matched with their specific studies, therefore sample collection information could not be obtained.

Table 0.1: Description of the studies whose sequences were used for the analysis

Cohort	Description	Period	Number of sequences
BCPP study	Determining implementation of combination prevention and if it could reduce population-level cumulative HIV incidences in adults.	2013 - 2018	6076
Bosele study	Assessed if HIV-1 mutations other than those on the integrase region were associated with development of virologic failure in HIV-1C infected individuals on treatment with DTG containing regimens.	2015 - 2021	22
Los Alamos Sequences	Database for HIV sequences, including sequences for various HIV studies that were conducted in Botswana.	1996 - 2001	1375
Total			7473

A total of 7473 sequences were analysed in this study, of which 7 were generated from the Bosele study samples and 7466 were readily available from the mentioned studies.

2.1.1 Ethical Considerations

This study was approved by the Ministry of Health & Wellness (HPDME/13/18/1). Previous studies were approved by the Office of Human Research Administration (OHRA) of the Harvard School of Public Health, the MOH Institutional Review Board (IRB) and Health Research and Development Division (HRDC).

2.2 Sample Inclusion Criteria for Bosele Study Samples

Only samples from the Bosele study were available for application of molecular biology techniques in this study. The inclusion criteria outlined in **Figure 2.1** was used for selecting samples that could be used for viral RNA extraction, amplification, sequencing and analysis along with available sequences. From a total of 65 Bosele participants, 43 individuals using DTG as first line therapy were excluded. The remaining 22 participants, using DTG as second line or deep salvage therapy were separated according to availability of RT, PR and IN sequences. A total of 10 plasma samples from individuals with only IN sequences were obtained, and 7 sequences were successfully generated.

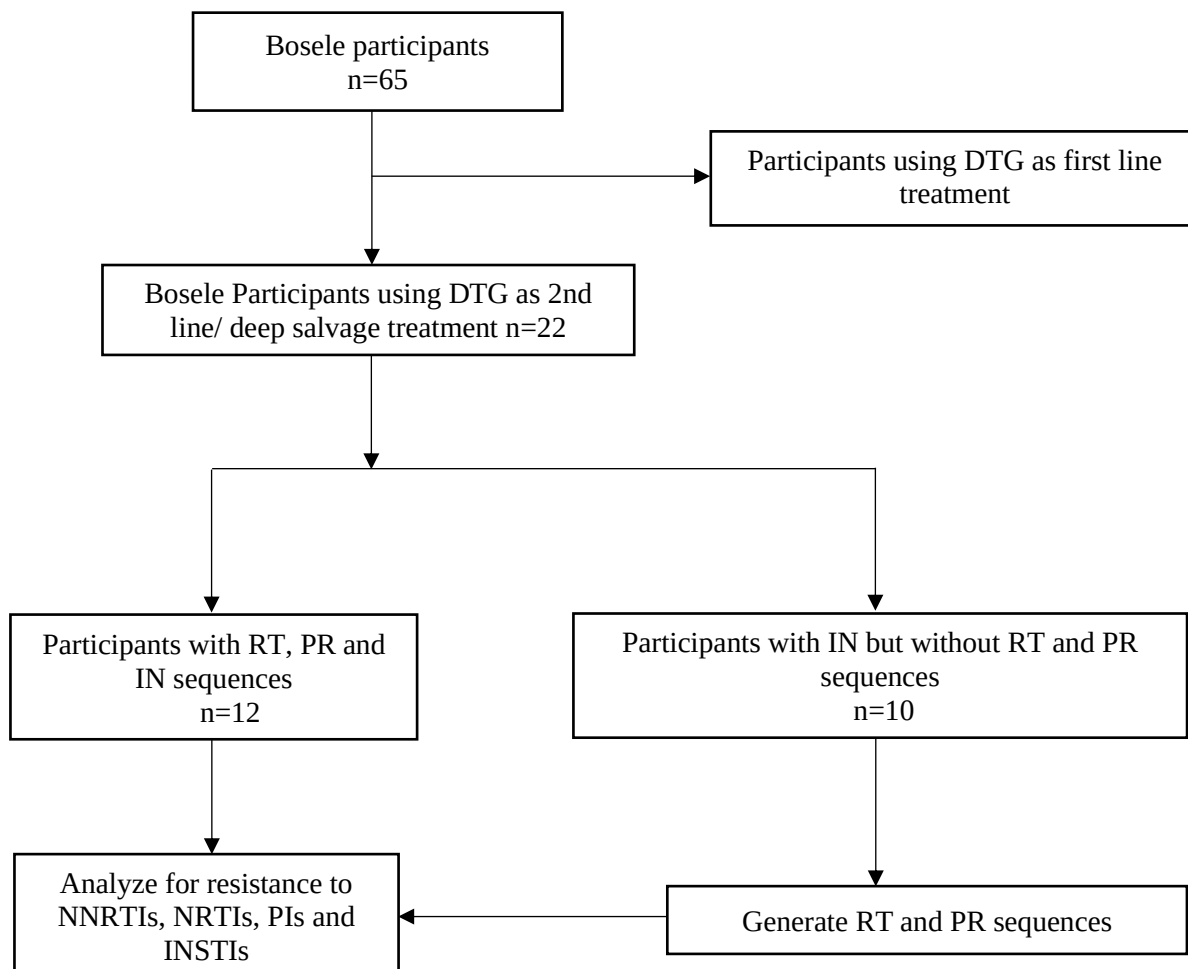


Figure 0.1: Bosele study samples inclusion criteria

2.3 Viral RNA Extraction, Amplification and Sequencing

2.3.1 Viral RNA Isolation from Plasma Samples

The ZYMO QUICK RNA Viral Kit (ZYMO Research, CA, USA) was used to extract viral RNA from 400 µl plasma samples. RNA extraction was performed for 10 samples, at room temperature, with all centrifugation steps done at 16000 xg. Briefly, 800 µl of viral RNA buffer was added to 400 µl of the plasma sample, transferred into a spin column and centrifuged for 2 minutes. The column was then washed twice using 500 µl of wash buffer, centrifuging for 30 seconds each time, and discarding the supernatant. The wash buffer was rinsed off with 95% ethanol, and RNA was eluted using 15 µl of DNase-free water.

2.3.2 Amplification of the HIV-1 *pol* region

A 1.1kb HIV *pol* region containing complete protease and the initial 800 bps of RT was amplified using the Transcriptor one-step RT-PCR kit (Roche Applied Science, Penzberg, Germany), following the manufactures' instructions. The Roche RT-PCR mixture contains four enzymes and a reaction buffer containing dNTPs, a comprehensive solution that gives outstanding sensitivity and performance. Enzymes contained in the mixture include: (1) Transcriptor Reverse Transcriptase, a reverse transcriptase enzyme that guarantees a highly sensitive and robust reverse transcription process with a high yield; (2) Protector RNase Inhibitor, which is thermophilic and ensures maximum template protection during reverse transcription; and a combination of (3) Taq DNA and a (4) proofreading polymerase (Expand System), which reduces the likelihood of mutations while also providing high PCR yield and fidelity. A PCR reaction mixture containing 0.5 µL Transcriptase Enzyme, 12 µL RNase free water, 5 µL of 5X Buffer, mixture of primers CWF1-LNA2 (10 µM) and CWR1-LNA3 (10 µM) at 1.25 µl each (Primer sequences in **Table 2.2**) and 5 µL of template RNA was prepared, making a 25 µL PCR reaction mixture for each sample. The first round PCR parameters for reverse transcribing viral RNA involved the following steps: Reverse Transcription cycle at 50°C for 30 minutes, initial denaturation at 94°C for 7 minutes, 10 cycles of denaturation at 94°C for 10 seconds, annealing at 55.5°C for 30 seconds, and extension at 68°C for 2 minutes increasing each cycle by 10 seconds, and the final elongation at 68°C for 2 minutes and holding at 4°C for a maximum of 18 hours.

Table 0.2: Primers used for the amplification and sequencing of the *pol* region

Primer Name	Primer sequences
CWF1-LNA2	5'-GAA GGA CCA AAT GAA AGA YTG-3'
CWR1-LNA3	5'-GCA TAC TTY CCG TTT TCA G-3'
RT-20C	5'-CTG CCA ATT TCT AAC TGC CTT C-3'
CWCS2	5'-AGA ACT CAA GAC TTT TGG G-3'
CWCS3	5'-TGC TGG GTG CGG TAT TC-3'
CWCS5	5'-TGG TAA ATT TGA TGT CCA T-3'
Seq2.1	5'-GGC CAG GAA TTT TCT TCA GAG C-3'
Seq6	5'-CCA TCC CTG TGG AAG CAC ATT A-3'

The Phusion high fidelity PCR kit (Finnzymes Oy, Thermo Scientific, New England Biolabs) was used for second round PCR with a 25- μ l reaction volume. The prepared reaction mixture contained 9 μ l RNase free water, 12.5 μ l of 2X Buffer, 2.5 μ l of primers CWF1-LNA2 (10 μ M) and RT-20C (10 μ M) (Primer sequences in **Table 2.2**) and 1 μ l of first round PCR product template. Second round PCR conditions involved initial denaturation at 98°C for 30 seconds, 35 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 20 seconds, final elongation at 72°C for 10 minutes and holding at 4°C.

2.3.4 Determining successful amplification of viral DNA

Successful DNA amplification was determined using 1% agarose gel stained with ethidium bromide. A solution of 1 gram agarose powder and 100 ml of 1X TBE buffer was supplemented with 3ml of ethidium-bromide. A 1kb DNA ladder was used as a molecular marker. For each sample, including the negative control, a 3- μ l amplicon and 2- μ l loading dye mixture was loaded into gel wells. The gel was subjected to 100 volts of current for 30-45 minutes and viewed under ultraviolet light at 280 nm wavelength. Availability of a single 1.1 kb band confirmed successful amplification of the *pol* region.

2.3.5 PCR Product Purification

PCR products were purified prior to sequencing using the Shrimp Alkaline phosphatase and Exonuclease 1 enzymes, commercially available as the ExoSAP-IT reagents (Affymetrix

USB, Ohio, USA). A 5- μ l PCR product was mixed with 2 μ l of ExoSAP-IT reagent and incubated for 15 minutes at 37°C to breakdown remaining nucleotides and primers. The ExoSAP-IT reagents were inactivated at 80°C for 15 minutes.

2.3.6 Sequencing Cycle PCR

Sequencing was conducted inhouse using the BigDye terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Primers CWF1-LNA2, CWCS2, CWCS3, CWCS5, Seq2.1, Seq6 and RT-20C (Refer to **Table 2.2** for primer sequences) were used for sequencing. The sequencing cycle reaction mixture of each sample had a 10 μ l volume, containing 4.8 μ l dH₂O, 1 μ l BigDye Terminator, 3 μ l BigDye Sequencing Buffer, 0.2 μ l (10 uM) primer, and 1 μ l of purified DNA template. Sequencing cycle PCR was conducted under the following conditions: 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes, holding the PCR run at 4°C.

2.3.7 Sequence Clean Up and Sequencing

Sequencing cycle amplicons were purified using the Big Dye XTerminator purification kit (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. This involved mixing 10 μ l cycle sequencing product with 45 μ l SAM solution and 10 μ l of the Big Dye XTerminator, followed by vortexing for 45 minutes at 1,800 rpm and centrifuging for 3 minutes at 3000g. Subsequently, the purified sequencing cycle products were analyzed using the ABI Prism 3031xl genetic analyzer (Applied Biosystems, Foster City, USA).

2.4 Mutation Analysis

2.4.1 Sequence base editing

Obtained raw *pol* sequences covering the RT and PR were edited using the Sequencher 5.0 software. Chromatograms representing the bases G, C, T and A were analyzed to determine if they corresponded with the actual sequence bases. In cases where mixed bases were detected through availability of two or more peaks, it was determined whether the peaks were true by ensuring that the second peak was at least 40% of a true peak, representing a mixed base. Contigs were assembled and saved in FASTA format after the base editing process.

2.4.2 Adjusting APOBEC induced hypermutations for proviral DNA sequences

The Los Alamos HIV database Hypermute tool (<http://www.hiv.lanl.gov/>) was used to screen for viral catalytic polypeptide-like (APOBEC) induced hypermutations. These

hypermutations are characterized by Guanine-to-Adenine (G-to-A) transitions that are mediated by apolipoprotein B mRNA editing enzyme (Delviks-Frankenberry *et al.*, 2016). The HIV-1C consensus sequence served as a reference for hypermutation adjustment, which was performed prior to analysis of drug resistance as a quality control measure. Drug resistance mutations identified as hypermutations were excluded from the prevalence of drug resistance in this study.

2.5 Multi-class drug resistance analysis

Sequences were analyzed for known HIV DRM associated with NNRTI, NRTI, PI and INSTI resistance, according to the Stanford University HIV Drug Resistance Database (<https://hivdb.stanford.edu/hivdb/by-sequences/>). Sequences with resistance to at least 2 drug classes were considered as multi-class drug resistant (MDR) and further analysed for resistance to second generation NNRTIs (DOR, ETR and RPV) and entry inhibitors (MVC, T20 and FTR).

2.6 DOR, ETR and RPV drug resistance mutation analysis among MDR individuals

DOR, ETR and RPV resistance associated mutations were identified using the list of major drug resistance and surveillance mutations obtained from the Stanford University HIV Drug Resistance Database algorithm version 9.4 (<https://hivdb.stanford.edu/hivdb/by-sequences/>). The Stanford HIV Drug Resistance database (HIVdb) penalty scores were used to determine the level of resistance to DOR, ETR and RPV within MDR variants. The HIVdb predicts the activity of ARVs against a specific mutant virus based on its activity against a wild type virus. A penalty score is assigned for each DRM, ranging from high ARV activity to loss of ARV activity. Total scores for level of resistance to a drug are calculated by summing scores of mutations known to confer resistance to that drug, and interpreting scores 0-9 as susceptible, 10-14 as potential low level resistance, 15-29 as low level resistance, 30-59 as intermediate resistance and ≥ 60 as high-level resistance (Tang, Liu and Shafer, 2012). From the obtained scores, prevalence of intermediate and high-level resistance to Doravirine, Etravirine and Rilpivirine was evaluated within the MDR variants.

2.7 MVC, FTR and T20 drug resistance mutation analysis among MDR individuals

Sequences were aligned in the BioEdit software (Version 7.0) prior to analysis, using HXB1 as a reference sequence. The gp120 and gp41 regions of MDR variants were extracted from the sequences and analyzed for resistance to entry inhibitors.

2.7.1 MVC resistance analysis

Resistance to MVC was determined by predicting coreceptor usage using the Geno2pheno (g2p) coreceptor and WebPSSM software's. Geno2pheno and WebPSSM are computational tools that predict HIV-1 coreceptor usage based on the genetic information of the V3 region of gp120 (Kalu *et al.*, 2017; Riemenschneider *et al.*, 2016). Using g2p, according to recommendations of the European Consensus Group on Clinical Management, a variant with a 10% False Positive Rate (FPR) was regarded as using CXCR4. Using WebPSSM, sequences scored as 0 were regarded as using CCR5 and those with a score of one were regarded as using CXCR4. In addition, the 11/25 rule was used to determine resistance to MVC by evaluating for the presence of positively charged amino acids at position 11 and 25 of the **gp120 V3** region. Sequences with positively charged amino acids at the two positions were considered as using CXCR4. Sequences scored as either CCR5 or CXCR4 by at least two analysis methods were regarded as CCR5-or-CXCR4-using variants respectively. Usage of the CXCR4 coreceptor denoted MVC resistance.

2.7.2 FTR resistance analysis

FTR resistance was determined according mutations classified in studies by Bouba Y. *et al* (2020), Cahn P. *et al* (2018), Soulie C. *et al* (2013) and Zhou N. *et al* (2014). Sequences were aligned in BioEdit 7.0 software, and gp120 positions identified from the stated studies were analyzed for nucleotide base changes associated with resistance to FTR.

2.7.3 T20 resistance analysis

Resistance to T20 was determined according to the International AIDS Society (IAS USA) HIV Drug Resistance Mutation Update 2022 (<https://www.iasusa.org/resources/hiv-drug-resistance-mutations/>). Mutations of the gp41 HR1 domain, associated with major T20 resistance were used as reference to T20 resistance. Sequences were aligned and analyzed for availability of mutations using the BioEdit 7.0 software.

2.8 Statistical Analysis

The demographics of the study participants were summarized using descriptive statistics. continuous variables were presented as medians along with the first and third quartiles. Categorical variables were presented as percentages. The binomial exact method was used to estimate the prevalence of mutations with 95% confidence. Univariate logistic

regressions were used to determine predictive factors of multi-class resistance and variables included were ART status, VL status, duration on ART, Coreceptor used, gender and age. Variables with $p < 0.2$ in the univariate analysis were selected for inclusion in the multivariate analysis. Statistical analysis was performed using STATA software (StataCorp. 2021. Stata: Release 17), $p < 0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Baseline Demographics and Clinical Parameters

Fundamental characteristics including gender, age, ART status, viral load and duration on ART were evaluated for participants enrolled for the analysed studies that constitute three cohorts in the current investigation. This evaluation aimed to provide a comprehensive overview of the composition of the study population. Participants characteristics were obtained from the BCPP and Bosele study data master lists and are shown in **Table 3.1** below. Demographics for the sequences downloaded from the Los Alamos database could not be obtained. Among the 7473 sequences that were analyzed, 6098 had participants baseline demographics, with 70.7% and 29.3% being female and male, respectively. The median age of the enrolled individuals was 39.9 (Q1, Q3: 32.9, 47.8) years. Participants were either ART naïve or experienced with a suppressed or unsuppressed viral load.

Table 0.1: Baseline demographics and clinical parameters

Variable	Totals (%)
Gender (n=6086)	
Female	4305 (70.7)
Male	1781 (29.3)
Median Age (in years) at enrollment (Q1, Q3)	39.9 (32.6, 47.8)
ART Status (n=6028)	
Naïve	1281 (21.3)
Experienced	4747 (78.7)
VL among ART experienced (n=4747)	
Viral suppression	4525 (95.3)
Virologic failure	222 (4.7)
Median duration on ART in years (Q1, Q3)	6.2 (3.03, 8.95)

3.2: Virus Isolation and Sequencing

Of the 10 samples whose viral RNA was extracted, 7 (70%) were successfully amplified and sequenced. The sequences were subsequently edited and analyzed for drug resistance together with BCPP, Bosele and Los Alamos sequences.

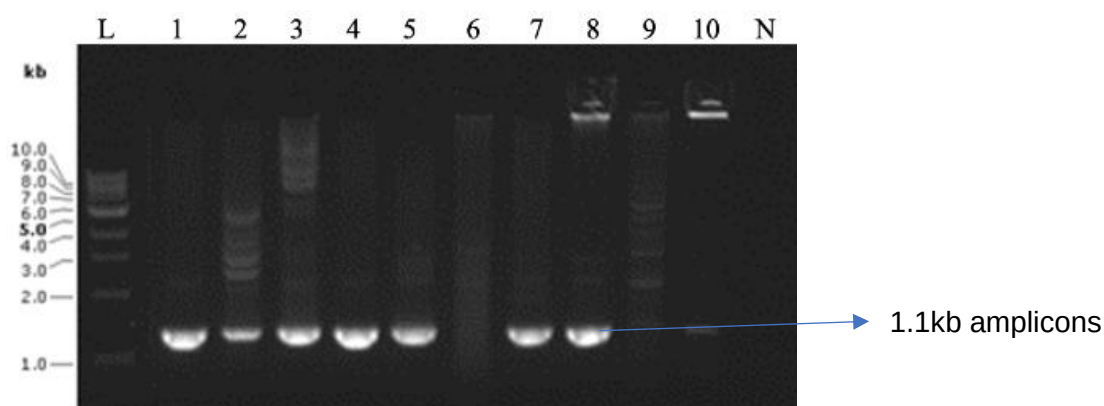


Figure 0.1: Agarose gel image for DNA amplicons

Gel electrophoresis results displaying the amplified *pol* region. The 1-kb molecular weight marker (Ladder), labelled L, was used as reference for amplicon size. PCR products were loaded in lanes 1 to 10, with N being the negative control. Each successfully amplified sample is represented by a 1.1-kb PCR product band.

3.3 Prevalence of Multi-Class Drug Resistance Across Cohorts

In the current study, multi-class drug resistance was defined as resistance to at least two drug classes. So, three cohorts were analysed for multi-class drug resistance, and a total of 682/7473 (9.1%: 95 % CI; 8.3-9.6) sequences showed resistance to ≥ 2 drug classes. Overall resistance to one, two, three and four drug classes was as follows: 1372 (18.34%), 482 (6.45%), 198 (2.67%) and 2 (0.03%), respectively, indicating that resistance to one class was more common. The BCPPP cohort, which represented individuals that were enrolled for a study which sought to assess if combination prevention could reduce HIV incidence at population level in Botswana between 2013 and 2018, exhibited the highest level of multi-class drug resistance at 91.1% (621/682). This was distantly followed by 7.2% (49/682) of the Los Alamos database cohort, for sequences from various HIV studies that were conducted in Botswana between 1996 and 2001. The Bosele cohort had the lowest prevalence of multi-class drug resistance at 1.8% (12/682).

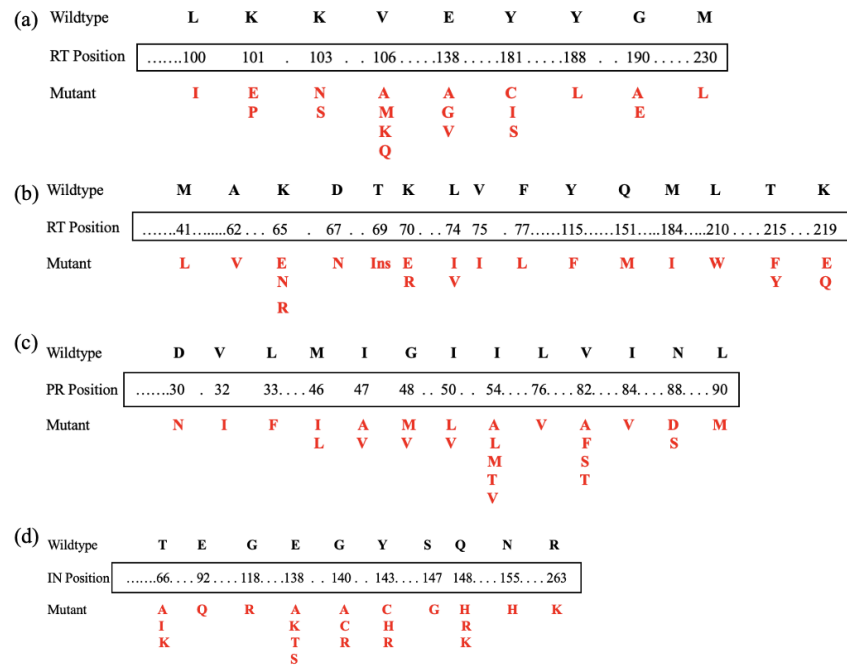
3.3.1 Prevalence of Resistance to NNRTIs, NRTIs, PIs and INSTIs

Major mutations of the reverse transcriptase (RT), protease (PR) and integrase (IN) regions, shown in **Figure 3.2A**, were used to determine resistance to NNRTIs and NRTIs, **PIs** and INSTIs. The trends of resistance across different drug classes varied within the three cohorts that were analysed. All cohorts presented high prevalence of resistance to NNRTIs and NRTIs, with varying trends of resistance for PIs and INSTIs as shown in **Figure 3.2B**.

The Los Alamos sequences had the highest prevalence of resistance to NNRTIs at 98% (48/49), closely followed by the BCPP sequences (83.8%: 519/621) and Bosele sequences (83.3%: 10/12). On the other hand, the Bosele cohort showed the highest prevalence of resistance to NRTIs at 91% (11/12), followed by the Los Alamos cohort at 87.8% and the BCPP cohort at 83.7%. Resistance to PIs varied across the three cohorts, with BCPP exhibiting the highest prevalence of resistance at 58.8% (365/621), and the Bosele and Los Alamos following distantly at 25% (3/12) and 18.4% (4/49), respectively. The Bosele cohort had high prevalence of resistance to INSTI (75%: 9/12), while Los Alamos and BCPP had low prevalence of resistance to INSTI at 12.5% (1/49) and 5% (31/621) respectively.

Overall, MDR variants from the combined cohorts presented high prevalence of resistance to NNRTIs, NRTIs and PIs, with a low prevalence of resistance to INSTIs, as shown in **Figure 3.2B**. Within the 682 MDR variants, resistance to the four drug classes was as follows: NNRTIs 577 (84.6 %: 95 % CI; 81.7- 87.2), NRTIs 572 (83.9%: 95 % CI; 80.9- 86.6), PIs 377 (55.3%: 95 % CI; 51.5- 59.1) and INSTIs 41 (6%: 95 % CI; 4.3- 8.1).

A.



B.

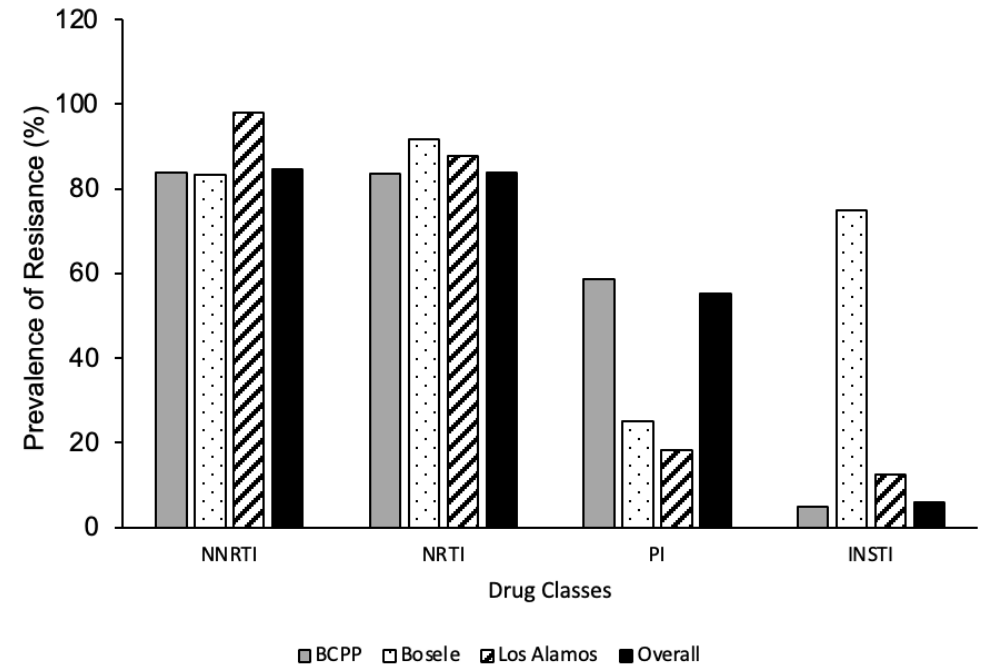


Figure 0.2: Drug resistance prevalence to different classes of drugs in each population cohort

A (a) Mutations of the reverse transcriptase [RT] region associated with major NNRTI resistance. **(b)** Mutations of the RT region associated with major NRTI resistance. **(c)** Mutations of the protease [PR] region associated with major protease resistance. **(d)** Mutations of the integrase [IN] region associated with major integrase resistance. The genome regions are not drawn to scale. **(B)** Prevalence of resistance to NNRTIs, NRTIs, PIs and INSTIs was determined among MDR variants of each cohort. Resistance to each drug class was determined by availability of at least one major mutation according to the Stanford database.

3.4 Prevalence of Resistance to Second-Generation NNRTIs

The study analyzed 682 sequences of MDR variants for resistance to DOR, ETR and RPV to determine their potential use as alternative therapy. The majority of MDR variants, 483 (70.8%), had resistance to one second generation NNRTI, either DOR or ETR or RPV. These were followed by 85 (12.5%) sequences with resistance to all three second-generation NNRTIs (DOR, ETR and RPV). Variants with resistance to two second-generation NNRTIs were 38 (5.6%), of which 25 (65.8%) had resistance to both ETR and RPV and 13 (34.2%) had resistance to both DOR and RPV.

Based on the Stanford HIV drug resistance database, the mutations identified for the NNRTIs were categorised into either intermediate or high-level resistance for each of the three drugs: DOR, ETR and RPV, as presented in **Table 3.2**. Prevalence of resistance to RPV was high within MDR variants. Overall, 541/682 (79.3%; 95% CI; 76.1-82.3) MDR variants exhibited resistance to RPV, 74.9% of these variants had intermediate level resistance, while 25.1% had high-level resistance. Mutations M230I associated with intermediate RPV resistance was observed at a high prevalence of 34.9% within MDR variants showing resistance to RPV. Other mutations associated with intermediate RPV resistance were observed at the following frequencies within MDR variants with RPV resistance: E138K (12.4%), Y181C (7.4%) and K101E (4.8%). Mutation G190E had the highest prevalence within mutations associated with high level RPV resistance at 7.3%, with other high level RPV associated resistance mutations Y118L, M230L, L100I, K101P and Y181L presenting prevalence's below 2%.

The second highest prevalence of resistance was associated with DOR, showing 67.1% resistance at 95 % CI; 63.3-70.6. MDR variants showed a prevalence of 83.6% to intermediate and 16.4% high level DOR resistance. Mutation G190E associated with high-level DOR resistance had the highest prevalence at 8.8% within MDR DOR resistant variants. Mutations Y188L (2%), M230L (0.9%) and V106A (0.4%), associated with high level DOR resistance, were observed at low prevalence's. Mutations V106A, G190S, and P225H associated with intermediate DOR resistance occurred at prevalence's less than 6%. Resistance to ETR was observed in 434/682 (63.6%; 95 % CI; 59.9-67.3) MDR strains; 93.5% intermediate resistance and 6.6% high level resistance. Mutations G190E and Y181C both associated with intermediate ETR resistance had the highest prevalence at 9.2%. Mutations K101P and Y181I associated with high level ETR resistance were both prevalent at 0.5%.

Table 0.2: Prevalence of overall, intermediate and high-level DOR, ETR and RPV resistance

Resistance	DOR n=682	ETR n=682	RPV n=682
Overall	67.1%	63.6%	79.3%
Intermediate	83.6%	93.5%	74.9%
High-level	16.4%	6.5%	25.1%
Specific mutation (%)			
V106A	0.4 ^b	-	-
V106M	5.9 ^a	-	-
P225H	2 ^a	-	-
L100I	-	2 ^a	0.7 ^b
K101E	-	-	4.8 ^a
KI01P	-	0.5 ^b	0.4 ^b
E138K	-	-	12.4 ^a
Y181C	-	9.2 ^a	7.4 ^a
Y181I	-	0.5 ^b	0.4 ^b
Y188L	2 ^b	-	1.7 ^b
G190E	8.8 ^b	9.2 ^a	7.3 ^b
G190S	0.4 ^a	-	-
M230I	-	-	34.9 ^a
M230L	0.9 ^b	0.9 ^a	0.7 ^b

^a Mutation associated with intermediate resistance

^b Mutation associated with high level resistance

- Mutation associated with susceptibility to the drug

Mutations G190E and M230L are both associated with intermediate resistance to ETR, and high-level resistance to DOR and RPV. G190E was observed to have higher prevalence of resistance to DOR, ETR and RPV at prevalence greater than 7%, compared to M230L which prevailed below 1% across all the three drugs. Therefore, mutation G190E is more likely to

cause resistant to DOR, ETR and/or RPV among individuals with multi-class drug resistance than mutation M230L.

3.5 Prevalence of Resistance to Entry Inhibitors

The prevalence of resistance to three entry inhibitors MVC, T20 and FTR, was assessed. Potential use of MVC, a CCR5 antagonist, as alternative therapy for individuals with multi-class drug resistance was determined by evaluating viral coreceptor usage. Geno2pheno, at a 10% False Positive Rate (FPR), and WebPSSM software's were used to identify coreceptor usage through genotypic detection of the gp120 V3 region (Raymond *et al.*, 2008). The 11/25 rule was also used to determine if there were positively charged amino acids at position 11 and 25 of gp120 V3 loop region (Shen *et al.*, 2016). A total of 391/682 MDR sequences had coverage of the gp120 region that could be evaluated for coreceptor usage. Coreceptor analysis showed a low utilization of the CXCR4 coreceptor at 7.2% (28/391) and a high CCR5 usage at 92.8% (363/391). This suggests that the majority of MDR variants use the CCR5 coreceptor to gain entry into host cells.

A total of 623 sequences were analysed for resistance to a viral fusion inhibitor, T20, a peptide with a low genetic barrier to resistance (Ding *et al.*, 2017). Major T20 resistance mutations found on the gp41 HR1 region, listed in **Figure 3.3A**, were used to determine T20 resistance. At least one T20 associated resistance mutation was observed in 313/623 (50.2%) MDR variants. Resistance to T20 was largely attributed by mutation N42T, which was the most prevalent at 70.9% (222/313) within T20 resistant variants, followed by mutation N43K at 44.3% (121/313). Other major T20 resistance mutations observed were G36D, G36S and I37V at prevalence's below 8% as shown in **Figure 3.3B**.

A.

Wildtype	D	I	V	Q	Q	N	N
gp41 HR1 Position	36	37	38	39	40	. 42	43
Mutants	D S	V	A M E	R	H	T	D

B.

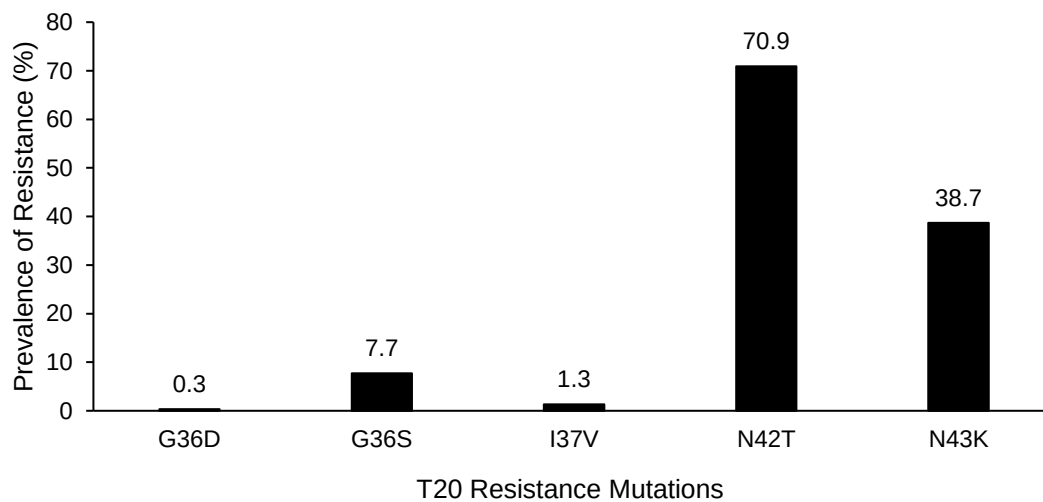


Figure 0.3: Prevalence of T20 resistance associated mutations

(A) Major T20 resistance mutations, of the gp41 HR1 region, used as reference for T20 resistance. **(B)** MDR variants were evaluated for prevalence of mutations associated with T20 resistance. T20 resistance mutations were identified according to the IAS mutations update and calculated using the Stata software.

FTR is a relatively new ARV drug used to inhibit virus entry, as it was approved by the FDA in 2020 (Chahine, 2021; Seval, Frank and Kozal, 2021). Nevertheless, several mutations have been associated with its resistance since its use. A total of 623 MDR sequences were analysed for mutations associated with FTR resistance using mutations shown in **Figure 3.4A** as reference. Of the 632 sequences, 113 (18.1%) MDR variants presented at least one mutation associated with FTR resistance. Further analysis showed mutations M434I (51.3%; 58/113) and M475I (44.3%; 50/113) shown in **Figure 3.4B** as the most predominant. Mutations A204S, S375F/I/T and M434-/T/Y were each observed at a prevalence of 0.9% (1/113).

A.

Wildtype	A	S	M	M	M
gp41 HR1 Position	204.....	375.....	426.....	434.....	475.....
Mutants	- S	- F H I M N T	- I K L R T V	- F I K L T V	- I K L S T V W Y

B.

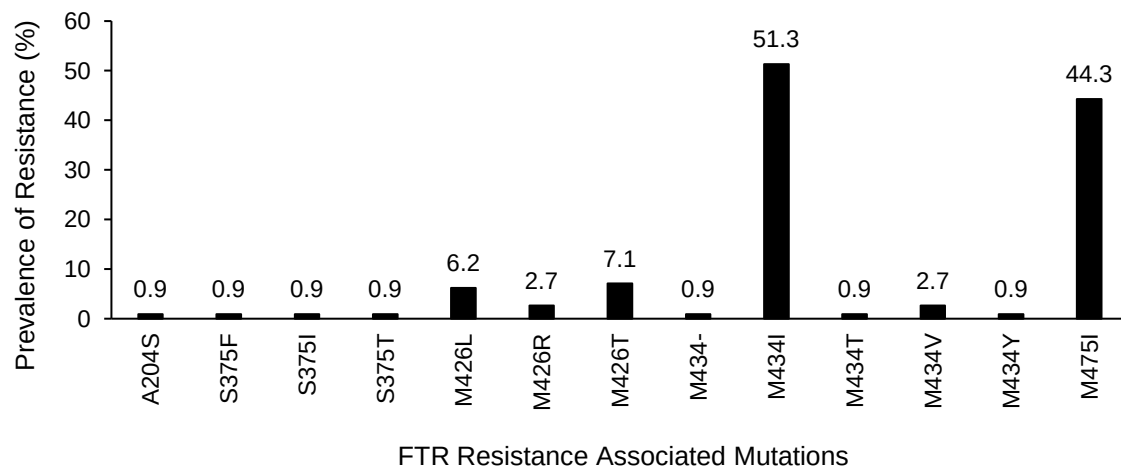


Figure 0.4: Prevalence of FTR resistance

(A) FTR resistance mutations used as reference for FTR resistance through analysing the gp120 region. The minus sign (-) under mutations depicts deletion. Mutations were adapted from previous studies by Bouba Y. *et al* (2020), Cahn P. *et al* (2018), Soulie C. *et al* (2013) and Zhou N. *et al* (2014). **(B)** Prevalence of FTR resistance mutations was evaluated on MDR sequences.

3.6 Factors Associated with Development of Multi-Class Drug Resistance

To shed light on the underlying risk factors of multi-class drug resistance, I evaluated participants demographics and clinical parameters for association with multi-class drug resistance. Characteristics evaluated included ART status, viral load (VL), duration on ART, coreceptor used, gender and age at enrolment of the study. Among 6098 individuals included in this analysis, 633 had multi-class drug resistance. Individuals on ART were at risk of developing multi-class drug resistance compared to ART naïve individuals (adjusted OR: 4.47

95% CI (3.02-6.62) (p value ≤ 0.01)) as shown in **Table 3.3**. Among ART experienced individuals, 4.7% had virologic failure with VL <400 copies/ml, experiencing virologic failure on ART was associated with increased risk of developing multi-class drug resistance when compared to the ART naïve group (unadjusted OR: 1.74, 95% CI (1.02-2.95) (p value = 0.042)). Being a male was significantly associated with developing multi-class drug resistance compared to being a female in univariate analysis. Duration on ART, age at study, enrolment and co-receptor usage were not significantly associated with risk to developing multi-class drug resistance.

Table 0.3: Predictive factors associated with multi-class drug resistance

Variable	Totals n=6098	MDR	non-MDR	Univariate	p-value	Multivariate	p-value
Gender (n=6086)		n=633	n=5453				
Female	4305(70.7)	469 (74.1)	3836 (70.3)	1(ref)	0.008	↓ (0.66-1.05)	0.13
Male	1781(29.3)	164 (25.9)	1617 (29.7)	0.8 (0.68-0.94)			
Age at enrollment	39.9	39	40	0.99 (0.99-1.01)	0.574		
Median (Q1, Q3)	36.6-47.8	32.6-48	32.6-47.8				
ART Status (n=6028)		n=621	n=5407				
Naïve	1281(21.3)	43 (6.9)	1238 (22.9)	1(ref)	<0.01	4.47 (3.02-6.62)	<0.01
Experienced	4747(78.7)	578 (93.1)	4169 (77.1)	3.99 (3.05-5.22)			
VL among ART							
Experienced (n=4747)		n=578	n=4169				
VS	4525(95.3)	536 (92.7)	3989 (95.7)	1(ref)	0.042	*	
VF	222(4.7)	42 (7.3)	180 (4.3)	1.74 (1.02-2.95)			
Duration on ART	6.2	6.61	6.13	1.03 (0.99-1.07)	0.18	*	
Median (Q1, Q3)	3.03-8.95	3.63-9.01	2.99-8.91				
Coreceptor (n=4077)		n=391	n=3686				0.13
CCR5	3602(88.3)	353 (92.1)	3249 (88.1)	1(ref)	0.15	0.75 (.52-1.08)	
CXCR4	475(11.7)	31 (7.9)	437 (11.9)	0.76 (0.52-1.1)			

*Highlighted in grey is additional information to the baseline demographics and clinical parameters in Table 4.1 used in this analysis.

CHAPTER 4: DISCUSSION

Botswana has a mature ART treatment program that started in 2002, and PLHIV have been exposed to first-generation NNRTI (nevirapine-NVP and efavirenz-EFV) and other variety of ARVs. This has greatly contributed to success in managing HIV and improving clinical outcomes for PLHIV. However, extensive use of ART selects for emergence of HIV drug resistant variants that are difficult to manage. Over the years, Botswana has reported a rise in cases of HIV associated drug resistance to all four available ARV drug classes. Multi-class drug resistance is associated with virologic failure, increased disease progression and cross resistance within drugs of the same class, which significantly reduces ARV choices. **This study** therefore aimed to determine if circulating MDR HIV variants would be susceptible to newly approved second-generation NNRTIs (DOR, ETR, and RPV) and entry inhibitors (MVC, T20 and FTR), that have never been used in Botswana.

Overall, MDR strains presented higher prevalence of NNRTI, NRTI and PI resistance, while INSTI- resistance was low. Previous studies have also reported high prevalence of resistance associated with NNRTIs among different groups in Botswana (Bareng *et al.*, 2022a). This is similar to reports from HIV-1C studies which reported higher prevalence of resistance to NNRTIs (98%) and NRTIs (88%) within children failing NNRTIs (Moyo *et al.*, 2020; Saravanan *et al.*, 2017). A study in Kenya observed a 13% prevalence of resistance to PI six to twelve months after initiating PI containing regimens (Bircher *et al.*, 2020). In contrast, I observed 55.3% prevalence of resistance to PI in variants obtained from individuals with multi-drug resistance. High prevalence of NNRTI, NRTI and PI resistance is attributed to accumulation of DRM overtime with prolonged exposure to ARV drugs (Bircher *et al.*, 2020). Possibly, low prevalence of resistance INSTIs is attributed to their later use, being introduced in 2016 in Botswana and used on individuals failing salvage therapy. Also, DTG, an INSTI has a high genetic barrier to resistance (Llibre *et al.*, 2015).

This study observed a high prevalence of resistance to RPV (79.3%), DOR (67.9%) and ETR (63.3%) within MDR HIV variants whose sequences were generated between 1996 and 2021 in Botswana. Findings similar to those observed in this study reported high prevalence of resistance to both RPV (54%) and ETR (65%) in children failing first-generation NNRTIs for at least six months (Saravanan *et al.*, 2017), and 84.8% DOR resistance among patients who were failing NNRTI based regimens in South Africa (Steege *et al.*, 2021). Contrary, a recent study in Botswana reported a 12.3% prevalence of DOR resistance (Bareng *et al.*, 2022b).

However, their findings reported overall resistance regardless of multi-drug resistance status. The differences in the study populations could have therefore attributed to the observed differences. Second-generation NNRTIs are known to have a high genetic barrier to resistance (Boucher *et al.*, 2018), but have also been reported to have cross resistance with first-generation NNRTIs (Diphoko *et al.*, 2018; James *et al.*, 2021). The majority of individuals with RPV and ETR resistance have notably had prior exposure to NVP- and/or EFV-based ART regimens (Diphoko *et al.*, 2018; Ayitewala *et al.*, 2020; Saravanan *et al.*, 2017). Therefore, individuals with NNRTI resistance associated mutations are at a high risk of failing second-generation NNRTIs, with increased chances of cross resistance as the number of mutations increase (Diphoko *et al.*, 2018). This proves to be a challenge in their use as alternative therapy for individuals with multi-class drug resistance.

T20 is a peptide based fusion inhibitor that targets inhibition of the viral envelope gp41 (Araújo and Almeida, 2013), and has previously been associated with development of mutations associated with T20 resistance in T20 naïve individuals. I therefore evaluated for naturally occurring T20 resistance in individuals with multi-class drug resistance and without prior exposure to T20. High prevalence of T20 resistance (50.2%) was observed within MDR HIV variants isolated from individuals who are T20 naïve in this study. Occurrence of T20 resistance in individuals who are T20 naïve suggests that the high replication capacity of the virus possibly selects for development of T20 resistance mutations without T20 selective pressure. T20 has a low genetic barrier to resistance (Ding *et al.*, 2017), therefore, a single mutation can cause T20 resistance. Also, T20 targets inhibition of gp41, a transmembrane glycoprotein of the viral envelope, a region known to have a high genetic variation (Araújo and Almeida, 2013). These combination factors possibly contributed to observed high prevalence of T20 resistance. Also, T20 resistant variants could have been acquired during infection. Previous studies reported T20 resistance prevalence's >16%: 27.5% (Chang *et al.*, 2021), 20.5% (Leung *et al.*, 2010) and 16.7% (Jang *et al.*, 2014), which although high, were lower than what was observed in this study. The different studies constituted of different subtypes which could have attributed to the different prevalence's observed, as naturally occurring T20 resistance has previously been reported to have higher frequencies within non-subtype B variants (Leung *et al.*, 2010; Carmona *et al.*, 2005). I also evaluated for the prevalence of major T20 resistance associated mutations found on the gp41 HR1 domain, which have previously been associated with 5-to-10-fold reduced susceptibility to T20 (Carmona *et al.*, 2005; Leung

et al., 2010; Jang *et al.*, 2014; Wensing *et al.*, 2019). Mutation N42T (70.9%) and N43K (38.7%) were the most prevalent, contrary to observations by Leung *et al* (2010), whose study on various HIV-1 CRFs reported mutation G36D as the most prevalent at 19.9%. Mutation G36D was observed at 0.3% in this study. Different observations could be due to the varying genetic diversity of the gp41 *env* region within different HIV subtypes that were under study.

Maraviroc, a CCR5 antagonist, is highly effective in treating CCR5 coreceptor using HIV-1 variants (Surdo *et al.*, 2015; Sax, 2010). The efficacy of maraviroc is limited when viruses gain the capability to use the CXCR4 coreceptor due to disease progression (Le *et al.*, 2015). When CCR5 antagonists are used to treat R5X4 variants, X4 variants which are more virulent, and likely to increase disease progression, emerge (Abbate *et al.*, 2011). I therefore evaluated susceptibility of MDR variants to MVC by determining viral coreceptor usage. A low prevalence of CXCR4 coreceptor use, 7.9%, within individuals with multi-class drug resistance was observed in this study, similar to 8.2% observed from a meta-analysis of sequences from various countries in Africa (Matume, Tebit and Bessong, 2020). Unlike observations of this study, a previous study in Botswana reported on 14.9% CXCR4 usage in ART naive patients with CD4⁺ T-cell count <200 copies/ml (Smeaton *et al.*, 2011). Also, in disagreement with this **study's observations**, a study on HIV-1C reported a >50% switch from CCR5 to CXCR4 usage in children failing ART. Viral coreceptor switch is associated with treatment failure (Green *et al.*, 2012), ART experience and progressed disease with low CD4⁺ T-cell count (Green *et al.*, 2012; Smeaton *et al.*, 2011). Majority of MDR individuals, 92.7%, analysed in this study were virologically suppressed, whereas the study by Smeaton *et al.*, 2011 evaluated coreceptor usage on individuals with low CD4⁺ T-cell counts at <200 copies/ml, and Green *et al.*, 2012 evaluated children failing ART, which could have conferred the observed differences. Also, a study on a recombinant (CRF01-AE) subtype reported a 47.9% switch from CCR5 to CXCR4 usage (Green *et al.*, 2012; Phuphuakrat *et al.*, 2014), a high prevalence compared to this study observation. Discordance in results could be attributed by different subtypes across different studies as prevalence of X4 variants has been observed to be higher in non-subtype C infections. According to Smeaton *et al* (2011), HIV-1B individuals with significantly advanced disease show $\geq 50\%$ CXCR4 coreceptor usage.

Due to high diversity of gp120, and possible protein structural changes due to amino acid mutations, susceptibility to attachment inhibitors (AI) varies across different HIV subtypes

(Zhou *et al.*, 2014). I evaluated natural resistance to FTR, an AI, within MDR strains. Mutations M434I (53.2%), M475I (45.9%) were the most predominant in this study, while M426L, A204S, S375F/I/T, M434-/T/Y were observed at a <6.5% prevalence. Mutations M434I and M475I have previously been reported as less predominant in FTR naïve HIV-1 subtype B studies (Soulié *et al.*, 2013; Bouba *et al.*, 2020; Zhou *et al.*, 2014). Their prevalence's were relatively lower than observed in this study at 4.2% and 1.6 %, respectively (Bouba *et al.*, 2020). This could suggest that diversity of gp120 in HIV-1 subtype C selects for M434I and M475I, which are possibly responsible for the majority of observed FTR resistance observed, or these mutations may be natural polymorphism of the HIV-1C subtype. Mutations M426L, M475I, S375M and M434I possibly cause resistance through decreasing the binding affinity to gp120 (Zhou *et al.*, 2014). Changes in these positions including L116, A204, M434, and V506 are associated with low susceptibility to fostemsavir (Bouba *et al.*, 2020; Cahn, Fink and Patterson, 2018). Also, because there is limited data on FTR resistance in subtype C variants, there is need to evaluate the impact conferred by these mutations in-vitro, and the role played by FTR polymorphisms in subtype C variants, which could not be determined in this study. However, it is still difficult to know the clinical impact of FTR -associated mutations as there is no software that categorize the mutations into intermediate and high-level resistance, as in the case with mutations associated with other drugs.

To determine factors that can be targeted as tools for alleviating HIV, I identified the possible predictive factors of multi-drug resistance using age, gender, ART status, viral load among ART experienced, duration on ART, and viral coreceptor usage. ART experience, virologic failure (VF) at viral loads > 400 copies/ml and being male were associated with development of multi-drug resistance. Consistent with prior research, experiencing virologic failure was linked to being male and on second-line ART (Hailu *et al.*, 2018; Penot *et al.*, 2014; Samizi *et al.*, 2021). Differences in the way ARVs are processed in the body (Pharmacokinetic and pharmacodynamic) contribute to the increased susceptibility of men to virologic failure. Research has shown that women tend to have higher concentrations of ARV drugs in their bodies, which enhances their virologic efficacy compared to men (Penot *et al.*, 2014). In this study, predictive factors were not further assessed for their potential impact on resistance to RPV, DOR, ETR, MVC, FTR and T20.

The study utilized a large population data set, providing a good representation of PLHIV in Botswana, however it was difficult to assess the trends of multi-drug resistance from

1996 to 2018 due to use of different study designs employed in each study included. Some of PLHIV included did not have clinical data such as ART, gender, age and VL measurement as the sequences were downloaded from the Los Alamos HIV database. The prevalence of multi-drug resistance was not stratified by ART, gender, age and VL measurement but reported as an overall. The available data was from PLHIV (1996-2018) who were exposed to NNRTI-containing regimens, and do not reflect any clinical significance among individuals with multi-class drug resistance who are taking DTG ART. However, the data provides very important information of the impact of the previously used ARV's on the future ART options.

In conclusion, these findings confirm high prevalence of mutations associated with resistance to second-generation NNRTIs and T20 in individuals with multi-drug resistance. High prevalence of resistance mutations associated with DOR, ETR, RPV and T20 limits their potential use as alternative therapy in individuals with multi-drug resistance. Low frequencies of entry inhibitor associated resistance mutations highlight the potential use of MVC and FTR as alternative therapy in individuals with multi-drug resistance in Botswana. However, Botswana would require genotypic testing prior to use and during the course of treatment with these ARVs. In-vitro and clinical studies are warranted to determine the impact of MVC, FTR and T20 associated mutations and polymorphisms and their relevance in drug susceptibility.

4.2 Recommendations

In order to reduce drug resistance through ensuring viral suppression among individuals on ART, and also reduce HIV related illnesses and death, I propose that the Ministry of health in Botswana should consider to increase available ART options. FTR and MVC can potentially be used as alternative therapy for individuals who are MDR and experiencing treatment failure. These ARVs can also be used as 1st and 2nd line therapies. Efficient management of HIV infections requires therapeutically effective ARVs, therefore, genotypic testing should be done prior to, and during the course of treatment with FTR and MVC based regimens in order to determine if patients are eligible for these drugs. This will prevent the emergence of resistance to MVC and FTR, as low prevalence of their resistance was observed in this study. Single genome sequencing can be used to assess resistance to MVC resistance by identifying low levels of R5X4 and X4 variants to prevent inappropriate use of MVC and emergence of X4 variants. The HIV env regions (gp120 and gp41) shows high levels of genetic variation, necessitating additional research to determine the impact of polymorphisms and naturally occurring mutations associated with FTR and T20 resistance.

Future research could focus on analysis of multi-class drug resistance at patient level in order to determine which ARV were associated with development of resistance to second-generation NNRTIs. This will give clinicians direction in decision making on which patients would be eligible for second-generation NNRTI regimens based on previous ART exposure. Baseline sequences were used for analysis in this study, in the future, cumulative drug resistance from sequences generated at different time points, over years, per individuals could be used to evaluate for multi-class drug resistance. This will give better understanding of prevalence patterns of drug resistance and aid in selection of effective ART.

4.3 Study limitations and Future Improvements

This study has limitations. FTR resistance was determined based on HIV-1B FTR resistance profiles due to limited research on FTR resistance in HIV subtype C. Therefore, FTR level of resistance associated with observed mutations could not be determined. There is possibility that different subtypes may exhibit different resistance profiles for ARV susceptibility. To address this limitation, *in vitro* and clinical studies should be done to determine the impact of these mutations in HIV-1C. This study determined multi-class drug resistance at population level, therefore, future research could focus on analysis of multi-class drug resistance at patient level in order to determine which ARV were associated with development of resistance to second-generation NNRTIs. This will give clinicians direction in decision making on which patients would be eligible for second-generation NNRTI regimens based on previous ART exposure. Baseline sequences were used for analysis in this study, in the future, cumulative drug resistance from sequences generated at different time points, over years, per individuals could be used to evaluate for multi-class drug resistance. This will give better understanding of prevalence patterns of drug resistance and aid in selection of effective ART. Also, sequences were not classified according to the years the samples were collected. Earlier sequences, generated prior to INSTI exposure are unlikely to show resistance to four drug classes. Therefore, future studies could stratify sequences according to years in order to give clear drug resistance patterns based on drug exposure.

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