Assessing the recency of HIV infections and estimating HIV incidence in two rural districts of Lesotho

by

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DECLARATION

I TSEPANG MOHLOANYANE hereby declare that this research dissertation submitted to the Central University of Technology for the degree Master of Health Sciences in Biomedical Technology is my own independent work and has not been submitted to any other institution by myself or any other person(s) for the requirements for the attainment of any qualification

Tsepang Mohloanyane:  **Date: 31.03.22 / Revision Date: 05.03.23**

DEDICATION

This study is dedicated to my beloved parents who have instilled in me the power of dreams and not giving up from a tender age. To my brother, sister, and friends who shared their words of encouragement and advice until the end. Lastly, to my loving partner who was always there for me from the very beginning of this project.

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LIST OF ABBREVIATIONS

Ag/Ab	Antigen/antibody
AIDS	Acquired Immunodeficiency Syndrome

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ART	Antiretroviral therapy
bDNA	Branched-chain DNA
BED-cEIA	BED capture-EIA
CAGE	Craving, Annoyed, Guilt, Eye opener
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
CTL	Cytotoxic T Lymphocytes
CrAg	Cryptococcal antigen
CRFs	Circulating Recombinants Forms
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme-Linked Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
Env	Envelope
FcR	Fragment Crystallisable Receptor
FRR	False Recency Rates
Gag	Group Specific Antigen
GCP	Good Clinical Practice
GP	Glycoprotein
HIV	Human Immunodeficiency Virus
HTS	HIV Testing Services
IDE	Immunodominant Epitope
IDR	immunodominant region
IFA	Indirect Immunofluorescence Assay
Ig	Immunoglobulin
Lag	Limiting Antigen Avidity assay
M	Major
MCH	Major Histocompatibility Complex
MDRI	Mean Duration for Recent Infections
mRNA	Messenger RNA
N	New
NASBA	Nucleic Acid Sequence Based Amplification
NAT	Nucleic Acid Testing
O	Outlier

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PCR	Polymerase Chain Reaction
POC	Point of Care
Pol	Polymerase
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RTRI	Rapid Test for Recent Infection
RITA	Recent Infection Testing Algorithm
SIV	Simian Immunodeficiency Viruses
STI	Sexually Transmitted Infections
TRACE	Tracking with Recency Assays to Control the Epidemic
UNAIDS	Joint United Nations Programme on HIV/AIDS
VIBRA	Village-Based Refill of Antiretroviral Therapy
VL	Viral Load
WB	Western Blot
WHO	World Health Organisation

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ABSTRACT

Background

HIV recency assays are used to distinguish recently acquired infection from long-term infection among individuals newly diagnosed with HIV and thus identify hotspots of transmission. Additionally, since 2015, UNAIDS and the World Health Organisation (WHO) advise the use of HIV recency assay together with HIV viral load (VL) quantification as an incidence measure.

Objectives

Based on randomly selected samples of new HIV infections in two districts of Lesotho, we determined recent HIV infections, described and assessed risk factors for these recent infections, and estimated HIV incidence.

Methods

A total of 243 samples had been collected from newly diagnosed HIV- positive patients during a door-to-door HIV testing campaign from August 2018 to May 2019 in randomly selected rural villages of two districts in Lesotho (Butha-Buthe and Mokhotlong). The MSc candidate tested these samples for HIV recency using the Asanté HIV-1 Rapid Recency Assay and VL using the Roche Cobas System, according to the recent infection testing algorithm recommended by WHO. Moreover, she extracted the clinical and socio-demographic information from the trial database and conducted the descriptive (characteristics and incidence calculation) as well as inferential statistical analyses (risk factor analysis).

Results

Participants were recruited from August 2018 to May 2019. Among the 11,291 participants tested for HIV, 243 (2.2%) samples of new HIV diagnoses were available for recency and VL testing. Among the 243 samples, we found 18 (7.4%; 95% confidence interval 4.4%-11.5%) recent infections, while 225 (92.6%) were classified as long-term HIV infections. The majority of the participants were female (142 [58%]) with a median age of 37 years (interquartile range [IQR] 31-50 years). Although some characteristics showed a certain trend, only female gender was predictive for a recent HIV infection (adjusted odds ratio 3.74 (95% confidence interval 1.05-13.36); p-value 0.041). The estimated annual incidence was I_a = 0.29%.

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Conclusion

During door-to-door testing among a general population sample in two rural districts of Lesotho, 7.4% of newly diagnosed HIV infections were recent (< 6 months) as well as a significant incidence rate. Women were more likely to have acquired HIV recently. Our results imply significant ongoing transmission in these rural communities.

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

There is a lot of evidence that proves incredible progress over the past years in the reduction of human immunodeficiency virus (HIV) transmission and scale-up of HIV treatment. The Joint United Nations Programme on HIV/AIDS (UNAIDS) shows that the number of HIV infections has declined globally since 2000, however, there are still regions where new infection rates, i.e. HIV incidence, are rising. In 2018, about 1.7 million people became newly infected with HIV. In the same year, Lesotho had approximately 340,000 individuals that were living with HIV and an estimated 13,000 new infections (UNAIDS, 2020).

HIV incidence is defined as the rate at which a population acquires HIV infection. Determining the best method to measure incidence is still a challenge. Traditional strategies of estimating HIV incidence are expensive and time-consuming. To save costs and to have an easier implementation when estimating incidence, simpler methods are required (WHO, 2015). The combination of serological and molecular methods may provide a good solution to identify recent HIV infections in cross-sectional data (Moyo et al., 2015). Thus, since 2015, UNAIDS and World Health Organisation (WHO) advised the use of HIV recency assay together with viral load quantification to estimate incidence (WHO, 2015).

HIV recency assays are used to distinguish recently acquired infection from “long-term” infection among individuals newly diagnosed with HIV. Besides its use for incidence measurement, it is pivotal to identify populations and geographic areas where current transmission is occurring (Kim et al., 2019). Rapid identification of newly infected persons has a profound impact on breaking the transmission chain and ending epidemics. With new developments in laboratory technology, rapid detection of recent HIV infection is now possible using a validated rapid test for recent infection (RTRI) that diagnoses HIV and detects recent infection within minutes (Kim et al., 2019). The Asanté HIV-1 Rapid Recency Assay is a recently developed lateral flow assay to determine the time point of infection based on the avidity of HIV antibodies. Combined with HIV viral load testing, this test offers the possibility of an accurate incidence measurement (Sedia HIV-1 Lag-Avidity EIA Product Insert, 2015).

HIV continues to cause a substantial disease burden in Lesotho, which has one of the highest prevalences of HIV in the world (Frank et.al, 2019). In Lesotho, the monitoring

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and evaluation of the HIV programme are done by a routine collection of patient data across several service delivery points. Tools used in this system are mainly paper-based registers intended for the clinical management of patients. From these registers, one can identify whether patients are newly diagnosed, new in care or new on treatment (Ministry of Health Lesotho, 2016). HIV case reports reflect HIV incidence imperfectly because there is a time gap between the time when a person gets infected with HIV and the time when this person gets diagnosed with HIV (Hofmann et al., 2017). A recent population-based HIV prevalence survey in Lesotho, conducted by Low, Thin and Davia et al. (2019) among adolescent girls and young women, estimated HIV incidence with the above-mentioned RTRI and showed that annualised HIV incidence among this sub-population was 1.8%. However, recency data from Lesotho are scarce.

The Village-Based Refill of Antiretroviral Therapy (VIBRA) trial was a cluster-randomized trial in rural villages of two districts in Lesotho from August 2018 to May 2019 and was used as a platform for this study (Amstutz et al., 2020). The trial assessed the effect of village-based antiretroviral therapy (ART) refill through village health workers following same-day ART initiation during a home-based HIV testing campaign. Eligible trial participants were people living with HIV who were either newly diagnosed during the testing campaign or known to be HIV-positive but not on ART. From eligible and consenting participants, baseline point-of-care laboratory tests were performed (e.g. CD4) and venous blood was taken and stored. These blood samples from over 240 people newly found to be living with HIV and not on ART are the basis for this recency testing study.

1.2 AIM

This research aims to provide valuable HIV epidemic data for Lesotho concerning two aspects:

1) To assess the proportion of recently acquired HIV infections during a door-to-door campaign in randomly selected rural villages of two districts (VIBRA trial) using the Asanté HIV-1 Rapid Recency Assay and describe these infections in terms of demographics, risk factors, and geographical distribution.

2) To estimate the annual HIV incidence of the study area using an incidence formula based on the Asanté HIV-1 Rapid Recency Assay.

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1.3 OBJECTIVES

- To determine the recency of HIV infection among HIV-positive individuals not on ART identified during the VIBRA trial, using the Asanté HIV-1 Rapid Recency Assay
- To describe the demographic and sociodemographic characteristics (sex, age, district, employment status, schooling/education status, regular sexual partner status, alcohol consumption, local cannabis use, HIV/ART history, CD4 count, viral load) as well as some geographical information of the study participants with recent HIV infection
- To assess risk factors associated with recent infection versus long-term infection
- To estimate annual HIV incidence in the period of the VIBRA trial for Butha-Buthe and Mokhotlong districts.

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CHAPTER 2: LITERATURE REVIEW

2.1 HIV – STRUCTURE AND GENOME

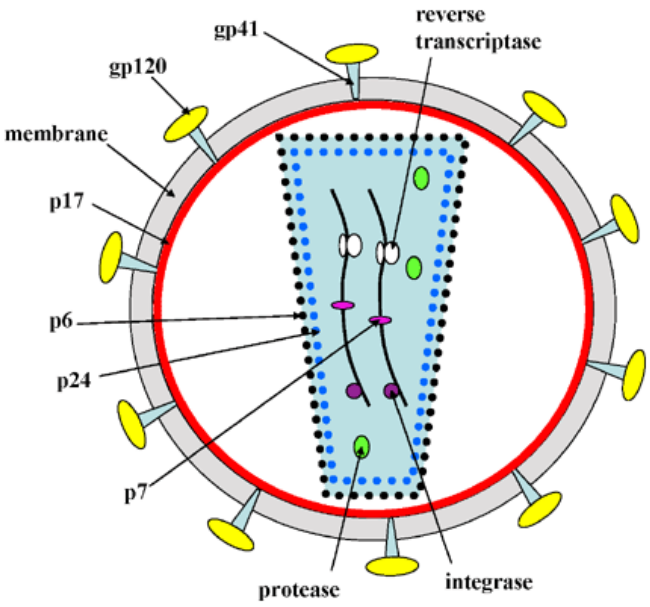
HIV is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). HIV-1 was first isolated in 1983, and HIV-2 in 1986. Phylogenetics discovered that HIV-1 and HIV-2 originated from the Simian Immunodeficiency Viruses (SIVs) of primates. SIV of chimpanzees (SIVcpz) gave rise to HIV-1 and the SIV of the sooty mangabey monkey (SIVsm) to HIV-2. Thus, HIV-1 and HIV-2 each had a zoonotic origin but are now spreading directly from human to human. There is no clear explanation on how exactly this transmission occurred, but these primates are found in areas in Central and Western Africa. After the initial transmission event, individuals infected with these primate SIVs then transmitted the human form of the viruses (HIV-1, HIV-2) to people in their communities and beyond (Tang and Chan, 2020).

2.1.1 Structure

HIV is a member of the retroviruses in the genus of lentivirus. HIV is spherical and around 100nm in diameter (Figure 1). It is composed of two copies of single-stranded ribonucleic acid (RNA) enclosed by a cylindrical capsid made of 2000 copies of viral protein 24 (p24). The single-stranded RNA is bound to nucleocapsid proteins p7 and other enzymes required for the replication of HIV such as the reverse transcriptase, protease, ribonuclease, and integrase. To ensure the integrity of the virion particle, a matrix made of viral protein p17 surrounds the capsid. The matrix is surrounded by an outer lipid envelope, where the trimeric transmembrane glycoprotein (gp) 41 and the attached surface glycoprotein gp120 are embedded (Tang and Chan, 2020).

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Figure 1. A simplified schematic version of HIV



Footnote: Figure from Tang and Chan, 2020

2.1.2 Genome

The HIV RNA has nine genes that contain information to make new viruses. It has three major genes, the group-specific antigen (gag), polymerase (pol) and envelope glycoprotein (env), that encode structural proteins and six non-structural genes that encode various proteins that are involved in viral replication. The gag gene encodes the core and matrix proteins. The pol gene encodes the enzymes (protease, reverse transcriptase,and integrase). The env gene encodes the outer (gp120) and transmembrane (gp41) envelope proteins. The non-structural genes are further divided into regulatory genes that encode the regulatory proteins and accessory genes that encode the accessory proteins (Li and De Clercq, 2016).

2.2 HIV REPLICATION CYCLE

The HIV replication cycle defines the steps and changes the virus undergoes from its first contact with a target cell to the production of new infectious viral particles that can initiate the next round of replication (Kirchhoff, 2013).

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2.2.1 Binding

To infect cells, the HIV gp120 attaches to the CD4 receptors on the surface of some T lymphocytes, macrophages, monocytes, and dendritic cells. This binding initiates a change in shape in the envelope, which in turn promotes binding of the virion to a specific subset of chemokine co-receptors. Twelve different chemokine receptors function as HIV co-receptors, but only two (CCR5 and CXCR4) are normally used in vivo (Doms, 2000).

2.2.2 Fusion

Due to binding, the transmembrane envelope glycoprotein gp41 gets exposed, leading to the HIV envelope and the CD4 membrane joining together. After this, the HIV contents are injected into the cytoplasm of the cell (Kirchhoff, 2013).

2.2.3 Reverse Transcription

To enable the HIV RNA to be incorporated into the genome of the CD4 cell, it must first be converted to deoxyribonucleic acid (DNA). This process is called reverse transcription. Reverse transcription is one of the features that distinguishes retrovirus from other viruses. It is called “reverse” transcription because it reverses the order of events that take place during the regular transcription process. In reverse transcription, the enzyme reverse transcriptase transcribes the single RNA molecules into a double-stranded DNA molecule (Kirchhoff, 2013).

2.2.4 Integration

Once the double-stranded DNA has been formed, it can now enter the genome of the host cell for gene expression and productive infection. The enzyme integrase inserts the double-stranded DNA into the host DNA. The new HIV DNA is also called “proviral DNA” (Kirchhoff, 2013).

2.2.5 Transcription

The proviral DNA can either be in a latent state or an active state. The active proviral DNA is then transcribed back to viral RNA as well as messenger RNA (mRNA) by the enzyme RNA polymerase II. Once synthesized, HIV mRNA goes through the nuclear pores into the rough endoplasmic reticulum to the host cell's ribosomes where it is translated into HIV structural proteins, enzymes, glycoproteins, and regulatory proteins (Libretexts, 2020).

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2.2.6 Assembly and Budding

The final steps include assembly, where newly formed viral nucleic acid and the structural proteins are brought together to produce a HIV virion. The new virion pushes through the cell wall of the host cell by budding, now containing all the components needed to infect other cells. However, the virion has to mature before it can infect other cells. The mature virion that is released infects other immune cells, thus continuing the process of replication (Libretexts, 2020).

2.3 GLOBAL GENETIC DIVERSITY AND EPIDEMIOLOGY

The various lineages of HIV-1 are divided into three groups, the major (M), new (N) and outlier (O), which are linked to three separate zoonotic transfers. The M group accounts for 99% of 40 million HIV infections and is globally distributed. Group M has nine different subtypes, being A, B, C, D, F, G, H, J and k. There are also recombinant forms, whereby the separate strains are combined into a new viral genome within a dually infected host. The existence of clear recombinants with genomes from distinct lineages has led to the introduction of a system for recognizing them. They are formally called circulating recombinant forms (CRFs). The subtypes E and I have been re-classified as CRFs after a thorough analysis of their genome (Volberding et al., 2012).

A study conducted by Bbosa et al. (2019), whose purpose was to provide a summary of the current data on the global HIV subtype diversity and distribution by region, showed the following: Subtype C is mostly found in Southern Africa, while subtype A is predominant in Eastern Africa. Subtype B is mostly spread in Europe, North and South America, and Australia. Various subtypes circulate in Asia, which has numerous recombinant viruses with several CRFs. Subtype A predominates in Russia and other former Soviet Union countries although CRF02_AG is common in Kyrgyzstan. CRF01_AE and CRF07_BC predominate in China; new CRFs have been found in China more than in any other country. In Southeast Asia, CRF01-AE is the most prevalent, while subtype C predominates in India. The Middle East and North African countries are grouped because subtype B is the predominant subtype in the region, together these countries show a relatively low prevalence and limited sampling of infections (Bbosa et al., 2019).

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2.4 HIV TRANSMISSION

While HIV-1 is predominantly transmitted through sexual contact across mucosal surfaces, it can also be transmitted by blood and blood products, mother to child in utero or during birth or breastfeeding through breast milk (Shaw and Hunter, 2012).

Approximately 80% of HIV infections worldwide are transmitted through sexual contact. A higher risk of HIV transmission is seen in homosexual men, people with multiple sexual partners, and when there are concomitant sexually transmitted infections (STI) (Maartens, Celum, and Lewin, 2014).

Countries in Southern Africa have the highest rates of HIV in the world with the largest number of people living with HIV in South Africa. eSwatini and Lesotho have extremely high prevalence (27.4%, 23.6%) while the prevalence in Angola (2%) is low (UNAIDS, 2019).

HIV prevalence is higher among young women than young men in most countries in the sub-Sahara African region. In Lesotho, the annual incidence of HIV among adults ages 15 to 59 years is 1.47%: 1.74% among females and 1.22% among males (Thin et al., 2017). The HIV epidemic is high in female sex workers with a percentage of 71.9%, followed by factory workers with 42.7 %, and thirdly men who have sex with men accounting for 32.9% of infections (avert, 2018). In Lesotho, there are several other groups, in addition to key populations, that are particularly vulnerable to HIV infection. These include migrant workers, people with disabilities, and herd boys (Ministry of Health Lesotho, 2016).

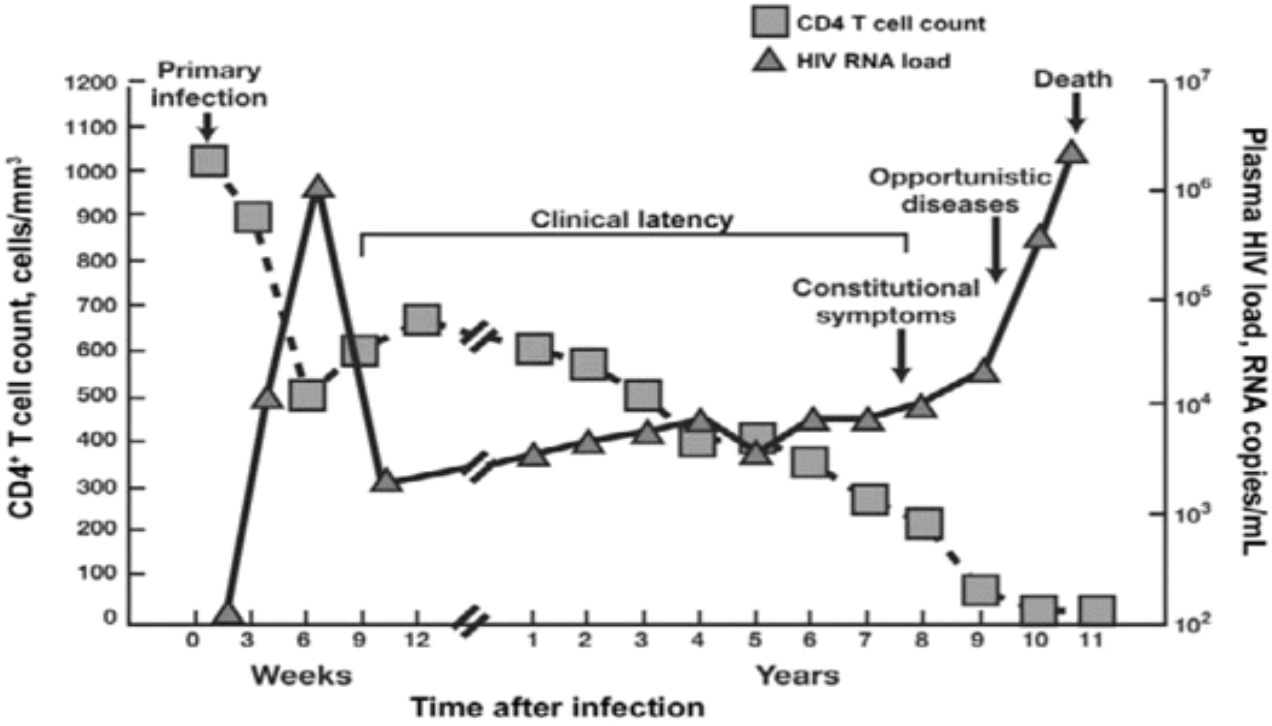
2.5 PATHOGENESIS

HIV/AIDS disease results from depletion of helper T cells leading to severe immunodeficiency. Once the virus has gained entry into the body and multiplies in the target immune cells, it destroys the CD4+ cells. The virus escapes immune-mediated clearance and the virion is released from the damaged cells found within the regional lymph nodes, causing viremia. This usually occurs within 2-6 weeks after infection when a threshold of replication is reached. During the primary viremia phase, there is high plasma viral load and a temporary fall in the CD4 count. As the virus further replicates, T cells activate the immune system which results in the release of various proinflammatory cytokines. The viral effects and the presence of the cytokines cause

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depletion, poor production, and dysfunction of CD4 helper cells. The period and the duration of clinical latency after the primary infection depend upon the individual's immune system. The infected host is prone to opportunistic infections when CD4 is below 200 cells/μL and will progress to an advanced stage of HIV known as AIDS (Kasper et al., 2015). The relationship between CD4 T lymphocyte count and viral load in an untreated HIV- infected individual is shown in figure 2.

Figure 2. CD4 count and viral load evolution in an untreated HIV infected individual



Footnote: Figure from Clin Infect Dis, Volume 45, Issue Supplement_4, December 2007, Pages S206–S212, https://doi.org/10.1086/522540

2.5.1 Immune Response

Progression to a disease state may take up to ten years, due to an immune response that normally reduces the impact of viremia during primary infection. Humoral and cell-mediated immunity are activated due to antigenic determinants of the virion and also against viral proteins which are usually expressed on the surface of infected cells (Kasper et al., 2015).

2.5.2 Humoral Immunity

Antibodies specific to HIV usually appear within 3-6 weeks of the primary infection. The stage where HIV-specific antibodies can be detected is referred to as seroconversion,

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which usually takes place up to three months after infection. This excludes individuals who are unable to produce HIV-specific antibodies. The majority of screening tests are designed to detect these antibodies, for example, Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot (WB). Only after the initial decrease in plasma viral load, do neutralizing antibodies appear and are closely associated with the arrival of HIV-specific CD8+ T lymphocytes. The first antibodies which appear are against the immunodominant region of the envelope protein gp41. This is followed by antibodies to gag protein p24 and the gag precursor p55. The antibodies to gp120 (envelope), p17 (gag), and p31 and p66 (pol protein) appear after antibodies to p24. Envelope proteins gp120 and gp41 are the only proteins that can elicit neutralizing antibodies. These neutralizing antibodies are considered as both protective and may also contribute to the pathogenesis of HIV. The virus can escape the neutralizing antibodies because of the high rate of mutation. One important mechanism of immune escape is the formation of a glycan shield by linking the glycosylation sites, which interferes with its envelope recognition (Kasper et al., 2015).

The main HIV envelope sites that evoke the production of neutralizing antibodies are:

i. CD4 binding site of gp120

ii. Glycan dependent epitopes in V1/V2 region of gp120

iii. Near the base of the V3 region of gp120.

Some antibodies engage in antibody-dependent cellular cytotoxicity, whereby an antibody-bearing Fragment Crystallisable Receptor (FcR) binds to a target cell, typically a natural killer cell with an Fc receptor. This cross-linking of the FcRs leads to the lysis of the target cell bearing HIV antigens on its surface. This usually occurs in the early stage of infection (Kasper et al., 2015).

2.5.3 Cellular Response

HIV has a huge impact on the immune system cells. Once HIV enters the body, it is usually recognised by the macrophages. Macrophages are phagocytic cells that present antigens to other immune cells. These antigens are then presented to CD4 helper cells, ultimately activating T-helper cells and CD8+ killer T cells. These cells help HIV-specific B cells and CD8+ T cytotoxic cells in mounting an immune response. The B cells are responsible for antibody production (Constantine, 2005). Signaling

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occurs through the major histocompatibility complex (MCH) class 1 to proteins between cytotoxic cells and the virus-infected cell and, once activated by the T helper cells, the CD8 cells produce cytotoxic proteins, which then lyse the infected cell. Hence CD8 production is associated with fast disease progression (Constantine, 2005). Cytotoxic T lymphocytes (CTL) have been detected within weeks of HIV infection and before the appearance of the virus in the blood. Two types of CTL activity are seen in HHIV-infected individuals. The first type is known as spontaneous CTL activity where the CTL directly lyses appropriate target cells. The second type of CTL activity is the ability of CD8+ T lymphocytes to inhibit the replication of HIV without killing infected targets, also known as non-cytolytic CD8+ T cell-mediated suppression of HIV replication (Kasper et al., 2015).

2.6 STAGES OF HIV INFECTION

HIV infection progression can be indicated by CD4+T cells count and plasma viral load. Patients not taking anti-retroviral treatment, typically HIV goes through the following four stages of HIV (Calles et al., 2010).

2.6.1 Primary Infection, or Acute Retroviral Syndrome

Primary infection refers to the time when HIV first infects the body. During this period, the viral load gets elevated, and the CD4+ count in the blood decreases remarkably. 2-4 weeks after infection, newly infected persons often experience signs of the acute retroviral syndrome. These are flu-like symptoms such as fever, nausea, vomiting, diarrhea, night sweats, weight loss, and rash. HIV antibody testing using ELISA also referred to as enzyme immunoassay (EIA) may yield positive or negative results depending on the time of seroconversion. DNA Polymerase Chain Reaction (PCR) and RNA PCR will be positive, but confirmation with WB analysis may yield an indeterminate result because seroconversion can take up to 2–8 weeks to occur. The average time to seroconversion is 25 days (Calles et al., 2010).

2.6.2 Clinical Latency/Asymptomatic Disease (Clinical Stage 1)

During this phase, the immune system starts to produce antibodies in an attempt to fight against the virus. HIV continues to replicate, either at a high rate or a slow rate, this is what determines the disease progression in individuals. CD4+ count returns to

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near normal and the viral load drops dramatically. During latency, HIV-infected patients may or may not have signs and symptoms of HIV infection. In HIV-infected adults, this phase may last 8–10 years. The HIV enzyme-linked immunosorbent assay and WB or immunofluorescence assay will be positive (Calles et al., 2010).

2.6.3 Mild Signs and Symptoms of HIV (Clinical Stage 2)

HIV-infected people may appear to be healthy for years before minor signs and symptoms of HIV infection begin to appear. They may develop candidiasis, lymphadenopathy, molluscum contagiosum, persistent hepatosplenomegaly, papular pruritic eruptions, herpes zoster, and/or peripheral neuropathy. The viral load increases and the CD4+ count falls. Once patients are in this stage they remain in this stage. They may be reassigned to stage 3 or 4 if a condition from one of those will occur, however they cannot be reassigned to stage 1 or 2 if they become asymptomatic again (Calles et al., 2010).

2.6.4 Advanced Signs and Symptoms of HIV (Clinical Stage 3)

In this stage the viral load keeps on increasing while the CD4+ count continuously decreases. Due to this, the immune system gets weakened and makes patients susceptible to life-threatening infections or other opportunistic infections. Opportunistic infections may include recurrent bacterial pneumonia, pulmonary and lymph tuberculosis, fever that last for longer than a month and many others. These patients may also lose a significant amount of weight (Calles et al., 2010).

2.6.5 Clinical Stage 4

Patients with advanced HIV disease, now called AIDS, acquire opportunistic infections such as *Pneumocystis jirovecii* pneumonia, cytomegalovirus infection, Toxoplasmosis infection, *Mycobacterium avium* complex infection, Cryptococcal meningitis, progressive multifocal leukoencephalopathy, may develop Kaposi sarcoma and other severe conditions. The viral load is usually greater than 10 000 copies/mL the CD4+ count is less than 200 cells/μL. At this point in the course of the disease, death is imminent (Calles et al., 2010).

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2.6.6 Long-term Non-progression

These are individuals who show no symptoms for long periods without ART while the CD4 count remains high. With time this status may be lost if left untreated (Sabin, 2013).

2.6.7 Elite Controllers or Viral controllers

These are individuals who have a suppressed viral replication to a point where their viral load remains undetectable without ART (Sabin, 2013).

2.7 HIV TESTING

Testing for HIV is the cornerstone of preventing HIV infections globally. Since the discovery of AIDS in the 1980s, HIV was initially tested using culture-derived viral antigen preparations. There have been five generations of enzyme immunoassays (EIAs) since the mid-1980s (Parekh et al., 2019). Laboratory diagnostics made use of different biological markers for different purposes. For example determining a person's serostatus using antibodies, determining a recent infection from a long-term infection using antibodies, VL or p24 antigen, and identification and monitoring of treatment using VL (Parekh et al., 2019).

There are different HIV tests and the reason for testing usually decides the type of test to be used. The variety of tests available include the following (Hurt, 2017):

- Antibody tests which may use serum, plasma, whole blood, oral fluids, and urine
- Viral antigen tests
- Nucleic tests
- Viral sequencing tests to identify viral resistance
- Viral culture
- Immunophenotyping tests.

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2.7.1 Antibody Tests

These assays do not detect HIV itself, but rather the antibodies that are produced due to an immune response to the virus. Since the production of antibodies may take weeks, these tests may give false-negative results if the person being tested is still within the window period. Tests that detect antibodies can be classified as screening assays or confirmatory tests. Screening tests are designed to detect infected individuals. They possess a high degree of sensitivity (low false-negative rate), while confirmatory assays are designed to differentiate people who test false-positive to those who are truly infected, i.e. they have a high specificity (low false-positive rate) (Constantine, 2005). The detection of antibodies at an early period depends on which antigens are incorporated in the test (Constantine, 2005).

The initial tests developed in 1995 are known as ELISA/EIA. These tests are easy to use and can test a large number of samples within a few hours and are easily adapted to automation. Not long after ELISAs were developed rapid and simple assays became available. Rapid testing is usually completed within a few minutes (Constantine, 2005).

2.7.2 Combined Antibody/Antigen Tests

Antigen/antibody (Ag/Ab) combination tests (formerly fourth-generation test) pair an IgM/IgG sensitive antibody test with simultaneous, separate p24 antigen detection. Antigen from the patient specimen is first captured by immobilized, anti-p24 antibody on the solid phase of the assay, and then a separate, labelled anti-p24 antibody is applied, forming an “antibody sandwich.” Some of these p24/IgM/IgG sensitive tests report a reactive result if any element is detected, while others yield separate results for p24, anti-HIV-1 antibodies, and anti-HIV-2 antibodies. Detecting p24 shortens the median window period down to just 18 days following infection (Hurt, 2017).

2.7.3 Nucleic Acid Testing

HIV infection can also be diagnosed by detecting the presence of viral nucleic acid (i.e. viral RNA or viral DNA) often called nucleic acid testing (NAT). NAT works by extracting, amplifying, and detecting HIV nucleic acids. Most of these assays rely on the amplification of the genetic material of HIV by polymerase chain reaction (PCR) and can be classified as qualitative or quantitative (Hurt, 2017).

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A range of methods for NAT also allows quantification of HIV RNA (=HIV viral load) and are widely used for monitoring the progression of HIV disease and response to ART in children and adults (WHO, 2010). As the level of viral replication is very high during acute HIV infection, NAT can be used for the diagnosis of early HIV infection (Rich et al., 1999). There are three most commonly used NAT methods for detecting and quantifying HIV RNA. They include reverse transcription-polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA), and branched-chain DNA (bDNA). These methods all share three steps beginning with sample preparation and/or viral nucleic acid extraction, progressing onto target nucleic acid sequence amplification or the amplification of the signal generated from the detection of target viral RNA, and finishing with the detection and/or quantification of amplified products (WHO, 2010).

2.7.3.1 Reverse Transcriptase-Polymerase Chain Reaction Technology

PCR artificially replicates nucleic acid sequences of the target which are then made available for detection. The amplified target sequences (amplicons) are captured and visualized through a colourimetric reaction (enzyme: substrate reaction). Detection of the amplicons can either be during the amplification process as the product accumulates in 'real time' or at the end of the amplification reaction, 'endpoint.' RT-PCR is a method of PCR using a Reverse Transcriptase (RT) enzyme to convert viral RNA into complementary DNA (cDNA). The cDNA undergoes replication and detection. RT-PCR is used to quantify HIV RNA and to determine viral load (WHO, 2010).

2.7.3.2 Nucleic acid Sequence-based Amplification Technology

Nucleic acid sequence-based amplification(NASBA) is an isothermal method of amplification that removes the need for heat-stable enzymes and thermocycling instruments that are required for performing RT-PCR. In this method, the target RNA is exponentially amplified by a three-enzyme system and quantified by a competitive co-amplification of laboratory-generated controls. Endpoint electrochemiluminescence detection completes the process (WHO, 2010).

2.7.3.3 Branched DNA Technology

Branched DNA (bDNA) technology is a method of HIV RNA quantification whereby a signal, not the target is amplified (WHO, 2010).

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2.7.3.4 Quantitative Real-time PCR

Quantitative real-time polymerase chain reaction (q-PCR) also called quantitative polymerase chain reaction and real-time polymerase chain reaction is a technique based on the conventional polymerase chain reaction (PCR) which is used to amplify and simultaneously quantify a target DNA (Singh and Roy-Chowdhuri, 2016). As opposed to the conventional PCR in which quantification, if performed, is based on "end-point" analysis of the amount of the amplicon, q-PCR allows for the product quantification as the process takes place in real-time (Singh and Roy-Chowdhuri, 2016). Conventional PCR with end-point analysis usually requires additional steps such as gel electrophoresis to separate the PCR product, and although quantification can be performed, it is not a simple task. In contrast, qPCR allows PCR quantification and analysis during amplification and the amplicon is measured in a "closed-tube" system by using various detection systems that employ melting curve analysis and/or fluorescent detection systems. The qPCR can detect, quantify, as well as perform genotyping, which sets it apart from other techniques. An optical thermocycler is used to monitor the fluorescent molecules through excitation and quantification of the fluorescent emission. Although many different designs for fluorophores are possible, including being linked to an oligonucleotide to form a labelled primer or probe, or being directly bound to double-stranded DNA, the common feature is that there must be a change in fluorescence during PCR that would allow detection and quantification of the product in real-time (Singh and Roy-Chowdhuri, 2016).

2.7.3.5 Storage and Collection of HIV Samples for Viral Load and Antibody Testing

HIV viral load testing is used to determine the number of copies of HIV RNA present per millilitre (ml) of plasma or serum. It is expressed as copies/ml or log scale. Numerous studies have examined the protocol of blood collection and the storage for viral load testing. Most of these studies showed that whole blood collected in EDTA anticoagulant tubes is most stable and has insignificant changes in the viral load when stored at room temperature for up to 30 hours and as plasma between 2°C and8°C for up to 5 days (Sebire et al., 1998, Griffith et al., 1997 and Ginocchio et al., 1997). Studies have also demonstrated that HIV-1 RNA levels are stable in EDTA-anticoagulated plasma stored at less than –70°C for at least 5 years. In addition, multiple freeze-thaw cycles do not appear to affect viral load levels (Ginocchio, 2001). In a study conducted by Jennings et al. 2018 assessing the long-term stability of HIV-1 RNA in EDTA-plasma samples stored at -80°C, which was done by comparing the

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data generated after a median of 6.7 years of storage with the HIV-1 RNA data generated in real-time as the clinical trials were being conducted. The results showed that there was no significant loss of RNA seen in samples containing either a low concentration (50 copies/ml) or a high concentration (50 copies/ml) of HIV-1 RNA (P0.10 and P0.90, respectively) regardless of the time in storage. These data confirm that samples can be stored for a long period at -80ºC without compromising the quality.

Antibodies have an important role in serological testing. Due to their binding affinity for antigens, the integrity of the structure of antibodies is vital for this interaction. Biological specimens have been stored for many years, for later confirmation of diagnosis or for future research. Also, the availability of viable stored material enables making inquiries about health problems in the past with the use of current and advanced technological resources. Some of the benefits resulting from the use of stored biological samples in research proved to be of great social relevance. A study was done to investigate the reproducibility of HIV antibodies reactivity, to assess the stability of the sera samples stored at -20ºC for two to eighteen years. In this study, serum sample stability was assessed by ELISA/EIA, indirect immunofluorescence assay (IFA), and WB for detecting anti-HIV antibodies. The previously found results (1988-2004) and those obtained in 2006 were compared statistically. The results showed that regarding the HIV serologic test, the serum samples were stable for 18 years in ELISA/EIA and for 4 years in IFA technique, however, for the WB methodology it was not possible to determine the time of stability of the anti-HIV antibodies (Castejon et al., 2014).

Another important test in HIV monitoring is ART resistance testing. This is due to the growing prevalence of HIV strains resistant to ART and the transmission of drug-resistant strains increases. Genotyping is the cheapest and most commonly used test for drug resistance as opposed to phenotyping (Volberding, 2012).

2.8 HIV TESTING IN LESOTHO

The Ministry of Health and Social Welfare are responsible for developing policies and executing programmes for the prevention and control of HIV in Lesotho. The goal of the HIV Testing Services (HTS) is to identify people living with HIV in time through the provision of quality testing services for all and effectively link them to appropriate prevention, care and treatment and support services. Lesotho has rolled out HTS to all

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its public health facilities and the community level. There is still a gap in increasing access to HIV testing and identification of those who are at high risk of getting infected and remain undiagnosed. There has been a gradual increase in the number of people testing for HIV, however, new infections have remained a challenge since 2011 (Ministry of Health Lesotho, 2016).

The US President's Emergency Plan for AIDS Relief supported Lesotho together with other 15 countries in 2019 as they approached the 90–90–90 goals for implementing the TRACE initiative (Tracking with Recency Assays to Control the Epidemic) to establish HIV recent infection surveillance systems to detect, characterize, monitor, and respond to recent infection among newly diagnosed people living with HIV. TRACE uses an RTRI as a supplementary test in HTS to detect recent infection among newly diagnosed people living with HIV. Following regulatory approval, such an RTRI may be included in routine national HTS algorithms (Kim et al., 2019).

2.9 HIV INCIDENCE ESTIMATION AND RECENCY TESTING

2.9.1 Incidence Estimation

Regardless of the significance of incidence as a public health indicator, the majority of prevention programmes and surveillance systems measure HIV prevalence instead of incidence. Prevalence does not give a clear picture of the rate of current HIV transmission due to factors like migration, birth rate and mortality rate. Furthermore, as the use of ART has improved in most countries and the survival rate of people living with HIV has increased, the calculation of prevalence has become less important than before (WHO, 2011).

Incidence is a better indicator for two different reasons. It can be used to determine trends of infection over time in a population, which will help in policy-making decisions when allocating resources. Also, it can be used to assess the impact of preventative interventions and treatment in a population (WHO, 2011).

Several methods for estimation of incidence have been exploited, but they all have their limitations in terms of accuracy, practicability or costs. Some of these methods are described below.

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2.9.2 Direct Measurement of Incidence

In this method, follow-up of HIV-negative individuals is done longitudinally with re-testing to determine the proportion that has been infected with HIV. This method is considered the best way to estimate incidence, however, it is difficult to apply on a large scale because of the resources needed for longitudinal follow-up (WHO, 2011).

2.9.3 Indirect Measurement of Incidence

HIV incidence can also be estimated using HIV prevalence. In this model, it is assumed that the difference that occurs in HIV prevalence in a series of cross-sectional surveys is the net effect of incidence when subtracting the mortality rate and migration of people living with HIV. Some of the limitations to this method are that there is a lack of reliable information regarding the death and migration of people living with HIV. It also requires a lot of time to pass before incidence can be measured (WHO, 2011).

2.9.4 Using Laboratory Tests for Incidence Measurement

Another method for assessing HIV incidence is the use of lab-based techniques that can discern recent HIV infection from long-term HIV infection in blood examples from HIV-positive people. The advantage of the RTRI methodology over the above-mentioned methods of incidence estimation is that RTRIs do not require following members longitudinally nor gathering sequential blood tests. Also, when using RTRIs for incidence estimation, information about migration and mortality is not needed (Mastro et al., 2010).

The WHO formed a Working Group on HIV Incidence in 2008 to discuss the concerns and challenges associated with assay-based HIV incidence estimation methods (UNAIDS/WHO, 2011). Several significant milestones have been accomplished since the group's founding, including the publication of UNAIDS/WHO guidelines on when and how to use assays for recent infection to measure HIV incidence at the population level (UNAIDS/WHO, 2011) and UNAIDS/WHO guidance on tracking the effects of the HIV epidemic using population-based surveys (UNAIDS/WHO, 2015). The WHO and the US Center for Disease Control and Prevention (CDC) have recommended a recent infection testing algorithm (RITA) that incorporates recency assays as well as HIV-1 viral load for incidence calculations (UNAIDS/WHO, 2015). RITA is defined by the WHO (2011) as a laboratory test or series of tests, or a set of tests plus supplementary laboratory and clinical data used to assess whether an HIV infection is recent or not.

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The majority of the tests for recent HIV infection are based on the principle of maturation and evolution of HIV-1 specific antibodies during seroconversion (Shepherd et al., 2015). During the window period, HIV infection can only be discovered through detection of circulating p24 antigen or presence of viral RNA or proviral DNA. Once the antibodies appear, titres increase progressively for about 5 months, after which levels peak and then remain constant throughout the infection. During this period of early infection, antibodies usually have low avidity (binding strength), and their avidity increases as the infection progresses. This helps to determine recent infection from long-term infection (Constantine, 2005).

The assays' outputs are influenced by a variety of factors, including the predominant HIV subtype in the survey setting, low anti-HIV antibody titers observed in late-stage HIV infection, ART, and long-term non-progressors/"elite controllers" (Mastro et al., 2010).

Antiretrovirals (ARV) minimize viral load, which decreases antibody levels. Thus, patients on ART must be detected and removed from the analysis, either by collecting declarative information (from the clinician or the patient) or by detecting drugs in serum specimens, using for example mass spectrometry (Le Vu et al., 2009). The virus subtype has also an effect on test outcomes. All tests developed predominantly on specimens from patients infected with subtype B viruses have been shown to provide inconsistent results when used for infections with non-B subtypes. Data on sex, mode of infection, testing patterns, and likely virus subtypes must be gathered to better interpret incidence estimates (Le Vu et al., 2009).

Several different assays have been developed as tests for HIV recency testing (WHO, 2020). There are eight types of assay tests for recent HIV infection. Among these assays, some have been developed specifically for identifying recent infections, while others are modifications of commercial HIV diagnostic tests (Shepherd et al., 2015).

2.9.5 Desensitized or Less Sensitive Enzyme Immunoassay Method

This method is based on the gradual increase of HIV-1 antibodies with time after seroconversion. Recent infection is determined by using a confirmed HIV-positive sample and then retesting it using the less sensitive EIA. Individuals with recent HIV infections have lower levels of HIV antibodies and therefore they test negative in the less-sensitive EIA (WHO, 2016). This immunoassay was developed based on HIV-1

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subtype B antigens, this method is thus less accurate in populations with primarily non-subtype B infections (Constantine et al., 2003).

2.9.6 Proportional Assay

In an attempt to overcome desensitized assays' limitations, the BED capture-EIA (BED-cEIA) was developed. This assay measures the proportion of all the immunoglobulin G (IgG) in an individual's serum which is specifically against HIV. The principle is that the proportion of IgG is lower in recent infections than in long-term infections (WHO, 2016). The proportional assay uses a synthetic antigen called BED containing sequences from multiple subtypes and a simple capture format to allow the measurement of the proportion of HIV-1 antibodies which increase over time after seroconversion (Parekh et al., 2002). It has been used in many studies but high false recency rates (FRR), the frequency of false recent results from long-term infections, which in return overestimates HIV-1 incidence, have been reported with this method (Guy et al., 2009).

2.9.7 Avidity Assay

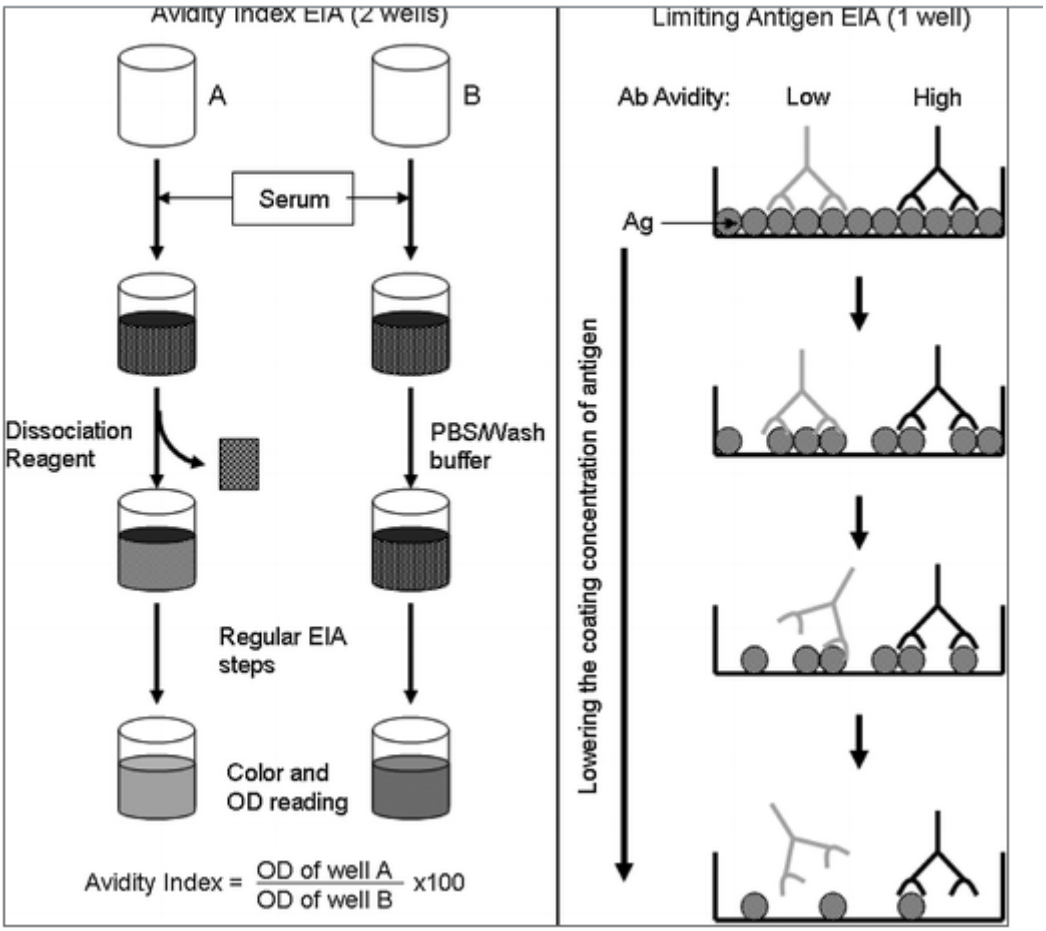
Antibodies and antigens have more than one binding site, which means they are multivalent. Avidity is the strength of the bond between an antibody and an antigen at every binding site. Avidity assays are based on the principle that antibodies increase their binding capacity (avidity) over the progress of the infection, i.e. low avidity is suggestive of a recent infection. The relative avidity of antibodies can be estimated serologically based on the resistance of the antigen-antibody complex resistance to agents which separate the antigen-antibody complex. The results can be measured in a reader in terms of a ratio referred to as the avidity index. The avidity index is calculated as optical density (OD) of the well treated with denaturation agent divided by OD of well washed with phosphate buffered saline. Avidity index of ≤ 0.80 is considered as a recent infection and an avidity index > 0.80 is considered as a long-term infection (Suligoi et al., 2002).

Subtype bias may be seen in some avidity assays based on commercial assays or based on a single antigenic subtype, thus new avidity assays now incorporate a recombinant protein, rIDR-M, that contains divergent sequences from the immunodominant region (IDR) of gp41 from all major subtypes and recombinants of HIV-1 group M. The protein is used together with a pH 3.0 buffer to dissociate low-

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avidity antibodies present during early infection, either using a two-well avidity index assay (AI-EIA) or a new one-well limiting antigen avidity assay (LAg-avidity EIA) including a one-well avidity assay using limiting amounts of antigen (Wei et al., 2010, Figure 3).

Figure 3. Schematic representation of principles underlying two-well avidity index EIA (left panel) and one-well limiting antigen EIA (right panel)



Footnote: Figure from Wei et al., 2010

The one-well limiting antigen avidity assay is based on the following principle (Fig.3 right panel). At high concentrations of antigen, both high- and low-avidity antibodies can bind and the binding can occur with both binding sites because of the high density of antigen. Lowering the coating concentration of antigen should permit binding of only high-avidity antibodies. Moreover, because the antigen is not available to bind both binding sites simultaneously low-avidity antibodies will not be able to bind strongly to

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wells with low antigen density. Therefore, this approach may be able to differentiate recent and long-term infections based on antibody avidity, with or without dissociation treatment (Wei et al., 2010).

A study done by Hassan et al. (2016) evaluated three avidity immunoassays: Sedia HIV Limiting Antigen Avidity assay (LAg), Sedia BED HIV-1 Incidence immunoassay and a modified Bio-Rad Genetic Systems HIV-1/HIV-2 plus O enzyme immunoassay. The study showed that the positive predictive value for the Lag, BED and Bio-Rad assays were 98%, 90% and 93.3% respectively, sensitivity was 100% for all assays and specificity was 100%, 100% and 97.2% respectively. Overall, the LAg has shown the best accuracy among the three immunoassays (Hassan et al., 2016).

2.9.8 IDE-V3 Assay (Immunodominant Assay)

The IDE-V3 assay is based on two conserved immunogenetic sequences found in the envelope glycoprotein of HIV-1. One is the immunodominant epitope (hence IDE) of gp41, which comprises two oligopeptides of 30 amino acids; one from group M and the other from subtype D. The second is from the V3 loop of gp120, which contains five oligopeptides from subtypes A, B, C, D and E. This assay uses a mathematical formula that combines the quantitative responses to antigens from each region to distinguish recent from established infections (WHO, 2020). A study conducted in Cote d'Ivoire found that the IDE-V3 assay, while having good specificity (96.3%), had poor sensitivity (42.3%), and this suggests strongly that its seroconversion window is likely to be considerably shorter than six months (Sakarovitch et al., 2007).

2.9.9 p24 Antigen

The p24 antigen (p24Ag) is usually detectable within a few days after onset of HIV viremia and before detectable HIV antibodies are present. The level of p24Ag usually falls as the host immune system initiates a response. Detection of p24Ag in the absence of anti-HIV antibody may be used as a marker of recent infection. However, its presence is unreliable and brief (1–2 weeks) and therefore the test has limited utility in detecting recent infections (WHO, 2020).

2.9.10 HIV RNA

Detection of RNA in the absence of anti-HIV antibodies can also be used to identify recent HIV infections. As HIV RNA can be detected earlier than p24Ag, a more

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accurate period for classification of recency can be used. Additionally, testing of pooled HIV RNA leads to an increase in the accuracy of RNA amplification assays and significantly lowers testing costs. However, the use of this method to determine HIV incidence requires very large sample population sizes and the level of viremia does not necessarily correlate well with the onset of infection (WHO, 2020).

2.9.11 IgG3 Anti-p24

IgG isotypes formed in response to an infection may vary during the infection. Isotype IgG3 is usually present transiently during the first few months of HIV-1 infection and the antigen against which the IgG3 response is most reliable is p24. A simple EIA-based procedure has been developed where IgG3 to p24Ag is typically detectable for only the first one to four months of infection. The findings from initial studies of this assay, however, have not yet been generalized to different populations with different subtypes of HIV infection (WHO, 2020).

2.9.12 Line Immunoassay

A line immunoassay is similar to a WB but uses a limited range of synthetic oligopeptides and recombinant antigens of both HIV-1 and HIV-2. Routinely, this type of assay is used as a confirmatory test to validate the presence of antibodies against HIV. The Inno-LIA™ HIV I/II Score, a line immunoassay, can be used to interpret results as either recent or non-recent infection. This assay is very costly but may be of value in settings where it is routinely used as the confirmatory diagnostic test (WHO, 2020).

2.10 THE ASANTE HIV-1 RAPID RECENCY ASSAY

The Asanté HIV-1 Rapid Recency Assay is a rapid in-vitro immunoassay that is designed to differentiate HIV infections in terms of their recency; whether they are long-term infections or recent infections (within the previous 6 months) (Parekh et al., 2017).

This assay expands upon the technology of the Sedia HIV-1 Lag-Avidity EIA, based on the principle of antibody avidity including the use of the same rIDR-M, but in a rapid lateral flow format instead of a laboratory-based EIA (Asante HIV-1 Rapid Recency Assay package insert, 2019).

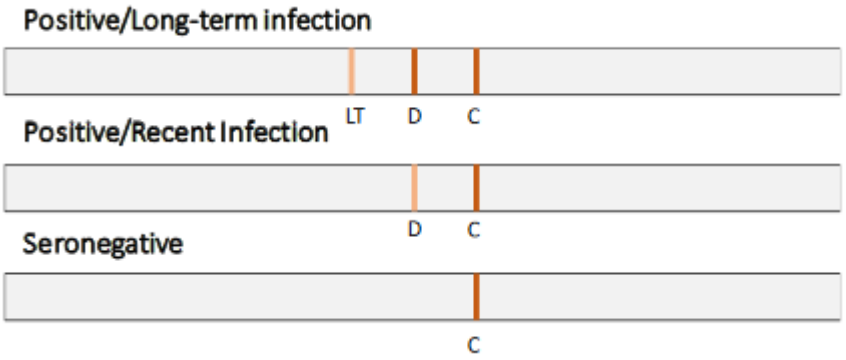
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The assay consists of a specimen loop, a capped tube containing 0.5ml sample buffer and a test strip. The specimen loop is used to collect the sample of interest, which can be either in the form of blood, serum or plasma containing HIV antibodies. The loop will then be placed into the sample buffer and mixed with the buffer. The test is started by placing the test strip into the buffer where the sample and buffer mixture gets absorbed by the absorbent pad. The absorbent pad contains reagents that prepare the mixture to react properly as it moves along the test strip. The mixture continues to move along the test strip until it gets in contact with a reagent made of Protein A conjugated to a colloidal gold reagent. When this rehydration occurs the conjugate releases a reddish-purple colour to the mixture which is used to visualise the results (Asante HIV-1 Rapid Recency Assay package insert, 2019).

The coloured liquid containing the sample, protein A and sample buffer continues to move along the strip to a nitrocellulose membrane that has three lines. The first line the sample buffer mixture encounters is the Long-term/Recent line, this line contains HIV-1 rIDR-M recombinant antigen bound to the membrane. If HIV-1 antibodies of high avidity are present in the specimen they will bind to the rIDR-M antigen causing a reddish-purple line,, this reaction indicates a long-term infection and the absence of this line indicates a recent infection. The second line is the verification line which contains p24, gp41 and gp36 recombinant HIV viral antigens bound to the membrane, which bind HIV-1 and HIV-2 antibodies present in the specimen. The appearance of this line proves that the specimen contains HIV. The last line is the control line that has goat antibodies reactive to human antibodies and will bind human antibodies irrespective of the presence or absence of HIV. The absence of the control line indicates that the device is not functioning properly (Asante HIV-1 Rapid Recency Assay package insert, 2019). Figure 4 outlines the summary of the result interpretation.

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Figure 4. Representation of Asante HIV-1 Recency Assay interpretation based on presence or absence of lines



Footnote: Picture taken from Parekh et al., 2017

When using the Asanté HIV-1 Rapid Recency Assay test in cross-sectional surveys for incidence estimation, the FRR (False Recency Rate) mainly caused by individuals already on ART needs to be defined and incorporated. However, estimation of local FRR values can be challenging or even impractical in some areas. Because of this, it has been recommended by UNAIDS/WHO and the US CDC that the users incorporate viral load testing into the recency test algorithm when using the Asanté HIV-1 Rapid Recency Assay (Parekh et al.,2017).

A study that evaluated the performance of this assay in the laboratory using a well-characterized panel of specimens was done and showed that sensitivity was 99.14% and specificity 98.7% and showed a high correlation with LAg-Avidity EIA results. The assay uses blood (fingerprick or venous), serum or plasma and can be used as a point of care (POC) test or in the laboratory (Parekh et al., 2017).

As a POC test, this rapid recency assay has the potential to play a key role in HIV prevention and treatment programs. The rapid incidence assay is currently being recommended for wider use globally to detect hot spots of new infections (Parekh et al., 2017).

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CHAPTER 3: METHODOLOGY

3.1 STUDY LOCATION

This nested study is embedded in the VIBRA trial, a cluster-randomized trial that was conducted in two districts of Lesotho (Butha-Buthe and Mokhotlong) from August 2018 to May 2019 (Amstutz et al., 2020). The two districts are located in the north of Lesotho. These districts are predominantly rural and have a population of approximately 220,000 inhabitants. Mokhotlong and Butha-Buthe have an estimated HIV prevalence of 26.1% and 17.8%, respectively (Thin et al., 2019). The samples included in this nested study are from consenting and eligible VIBRA trial participants from more than 100 villages randomly scattered over the two districts. Viral load testing and recency testing – the key laboratory assessments of this research – were done at Butha-Buthe Government Hospital Laboratory.

3.2 STUDY DESIGN

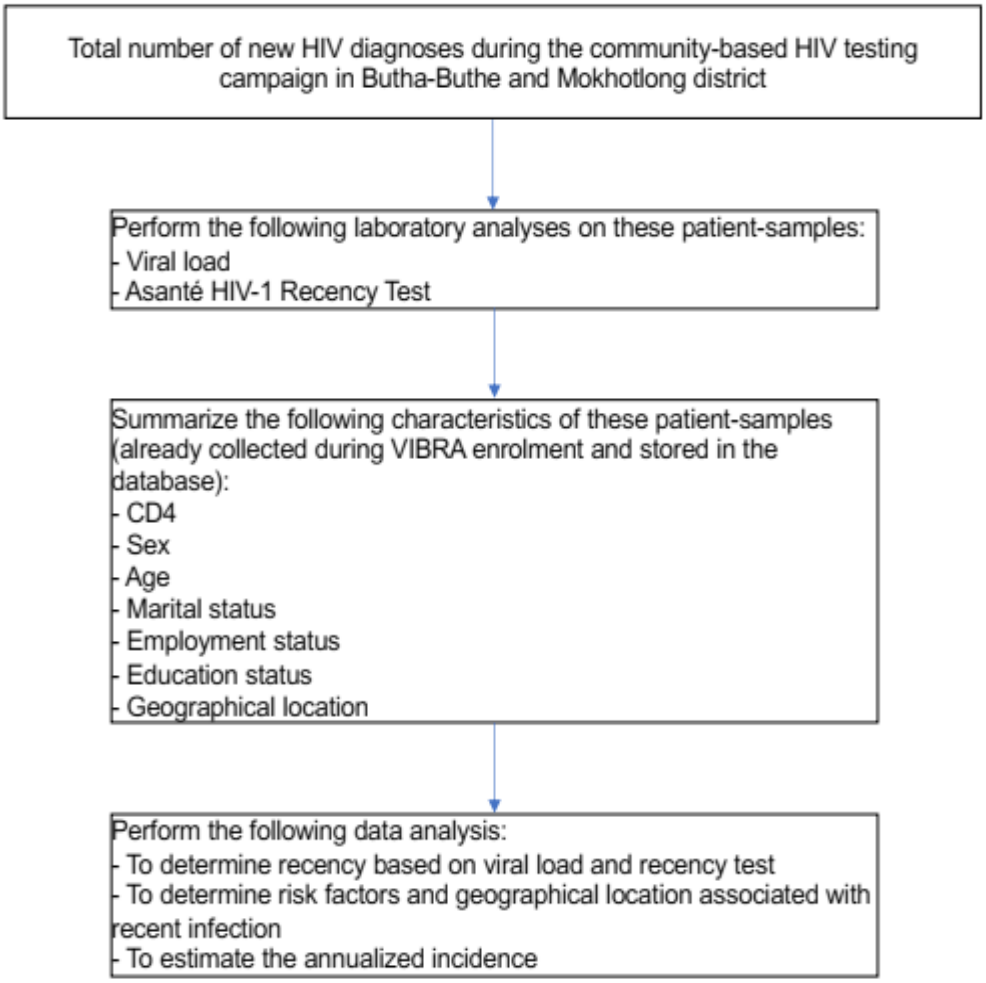
This study is a laboratory-based cross-sectional study embedded in a cluster-randomized clinical trial, called the VIBRA trial. The VIBRA trial assessed the effect of village-based ART refill following same-day ART initiation versus clinic-based ART refill among individuals found living with HIV not on ART during a home-based door-to-door HIV testing campaign. Blood samples were drawn from consenting eligible individuals at enrollment. Serum tubes of the individual participants were labelled with a unique study identifier and stored in a -80ºC biobank fridge at Butha-Buthe Government Hospital Laboratory. HIV viral load and recency of HIV infection were determined for all the specimens collected using the Cobas 4800 and the Asanté HIV-1 Rapid Recency test, respectively.

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3.4 STUDY LAYOUT

Figure 5 below shows the steps taken in the implementation of the study.

Figure 5. Study flow diagram



3.5. STUDY POPULATION

3.5.1 The Number of Participants

The sample size of the study was determined by the number of participants and the samples that were taken during the VIBRA trial, which had a sample size of 257 participants. However, not all samples from the participants were available in the biobank (see Results section: Figure 10, study sample flow).

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3.5.2 Participant Identification

Identification of participants was done during a door-to-door HIV testing campaign in 117 villages in the rural Butha-Buthe and Mokhotlong districts. The villages were randomly selected from the overall Ministry of Health village list of these two districts. However, due to feasibility and a stronger HIV programme in Butha-Buthe, proportionally more villages from Mokhotlong were randomly selected than from Butha-Buthe. The two campaign teams consisted of 6-10 counsellors, one campaign coordinator and one study nurse. In every consenting household, the study team proposed blood-based HIV testing and counselling as well as clinical screening for tuberculosis, assessment for harmful alcohol use and HIV prevention services to all present household members. POC blood-based HIV testing was offered to all present household members with unknown HIV status. Individuals who tested HIV-positive or presented proof of a known HIV-positive status but were not taking ART were offered participation in the VIBRA trial. The blood samples from eligible and consenting VIBRA participants are the basis for this study.

3.5.3 In- and Exclusion criteria

The eligibility criteria for samples of this study are based on the VIBRA trial:

Inclusion criteria:

- The individual is a member of the visited household i.e. the individual is acknowledged by the household head or their representative as part of the household and sleeps in the household regularly (at least once a month)
- The individual is confirmed HIV-positive, as determined by two reactive blood-based HIV antibody tests according to the Lesotho national guidelines
- The individual has never taken ART (“ART naïve”) or has stopped ART more than 30 days prior (“ART defaulter” according to the Lesotho national guidelines)
- The individual is ≥10 years and body weight ≥35kg

Exclusion criteria:

- The individual is on ART or has stopped less than 30 days ago
- The individual is physically, mentally or emotionally not able to participate in the study, according to the nurse

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- The individual is already in care for hypertension or diabetes
- The individual wishes to get care outside the two study districts

Additional exclusion criteria for this study:

- No participant sample is in the biobank or insufficient sample quality to run viral load quantification or/and Asanté HIV-1 Rapid Recency testing

3.6 METHODS AND MATERIAL

3.6.1 Preparation

Already developed Standard Operating Procedures were used for viral load testing of the study samples. For recency, testing manufacturer instructions from the package insert of the test were used. This ensured the reliability and validity of the results.

3.6.2 Data and Sample Collection

During the VIBRA trial door-to-door HIV testing campaign, household members with unknown HIV status were tested for HIV by the counsellors according to national HIV testing guidelines (Amstutz et al., 2019). Those with already known HIV-positive status but not taking ART and those who tested HIV-positive during the campaign (including confirmatory testing by the study nurse), were assessed for eligibility into the VIBRA trial according to the above-mentioned eligibility criteria. Once the participant was deemed eligible for the VIBRA trial, several data and laboratory assessments were conducted. After obtaining written informed consent, venous blood draw as well as a finger prick was done. The finger prick was done to perform four point-of-care tests (CD4, haemoglobin, serum creatinine and cryptococcal antigen (CrAg) test if CD4 < 200 cells/µL) to assess necessary parameters for treatment initiation on the day of diagnosis. The venous blood was drawn by the study nurse for storage in the -80°C biobank and to be used in this nested study. The venous blood was taken in three 5ml Ethylenediaminetetraacetic acid (EDTA) tubes, labelled using a unique study participant code.

Additionally, a questionnaire was administered to assess clinical, socio-demographic data, which included age, sex, gender, district, HIV/ART history, employment, schooling and education, regular sexual partner, as well as alcohol and cannabis use among others. Alcohol abuse was defined using the CAGE questionnaire which

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includes four questions that have been proven to assess alcohol dependency: The C stands for Craving (“Have you ever felt like you should cut down on your drinking?”), A for Annoyed (“Have people annoyed you by criticising your drinking?”), G for Guilt (“Have you ever felt guilty about your drinking?”) and E for Eye opener (“Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover?”) (Ewing, 1984). The data was stored in the password-protected VIBRA cloud database and could only be accessed through regulated user profiles.

3.6.3 Sample Storage and Transportation

Whole blood samples from the field were transported in cooler boxes containing ice packs to the nearest hospital laboratory within 30hours (Mokhotlong hospital laboratory, Butha-Buthe hospital laboratory or Seboche Mission hospital laboratory). On arrival at the laboratories, the EDTA whole blood samples were centrifuged for 10 minutes at 1300g to separate plasma and were divided into aliquots. At Mokhotlong hospital and Seboche hospital, the plasma was then stored in a -20ºC Celsius freezer and shipped once a week to the biobank in the main study laboratory at Butha-Buthe Government Hospital Laboratory. In Butha-Buthe Government Hospital Laboratory the samples were stored in a -80ºC biobank freezer. The samples will be stored for up to 5 years and then incinerated by the waste management team of the hospital.

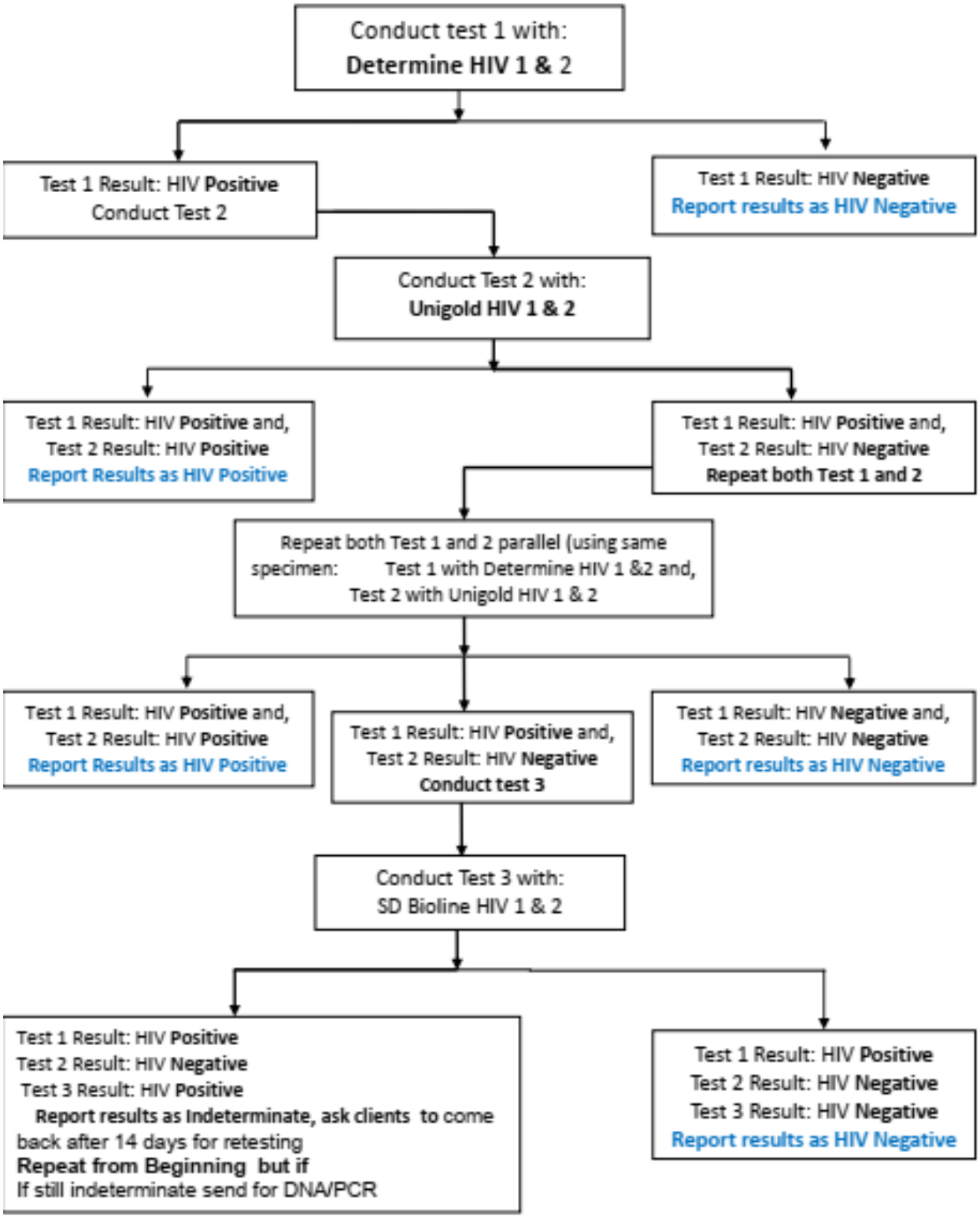
3.6.4 Laboratory Procedures during VIBRA Trial and in this Nested study

3.6.4.1 HIV Testing Among VIBRA Participants

HIV testing during the VIBRA trial was done based on the Lesotho HTS guidelines (2016) as shown in figure 6 below. Every positive HIV test was confirmed by a second study staff (study nurse).

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Figure 6. Lesotho HIV testing algorithm



Footnote: Picture taken from Lesotho HTS guidelines (2016)

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Additional retesting to confirm the HIV-1 positive status of the collected samples was done in Butha-Buthe Government Hospital Laboratory using DNA/PCR on the Cobas 4800 system (Roche Molecular Diagnostics).

3.6.4.2 HIV viral load testing

HIV viral load testing in Butha-Buthe Government Hospital Laboratory was performed using the Cobas 4800 system (Roche Molecular Diagnostics), which is an automated multi-channel pipetting instrument for in-vitro nucleic acid amplification for the detection and quantitation of HIV-1 in EDTA-Plasma using real-time PCR (Roche Diagnostics cobas® 4800 System. Instrument Manual Version 1.0).

Reagents that were used for HIV-1 PCR were:

- COBAS 4800 system wash buffer
- COBAS 4800 system lysis kit
- COBAS HIV-1 quantitative nucleic acid test kit
- COBAS HBV/HCV/HIV-1 control kit
- COBAS 4800 system sample preparation kit2 (Elution buffer and MGP2)

Materials that were required included:

- Biosafety Cabinet
- 1000 microL Pipetting tips
- 50mL Reagent Reservoir
- 200mL Reagent Reservoir
- Cobas 4800 Extraction plate
- Cobas 4800 Deepwell plate
- Cobas 4800 microwell plate
- Sealing foil applicator
- 24 position carrier
- Wastebin
- Vortex Mixer

The stored frozen plasma samples were retrieved from the biobank and then left at 2-8ºC until completely thawed. All samples were thawed once in 3 batches. The samples were then checked for clots and haemolysis. Samples with such were not processed (See Results section: Figure 10, study sample flow). All reagents used were from Roche Molecular Systems. Each sample had an internal control (β-globin) to monitor

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cell adequacy, and each run included a set of controls: high, low, and negative control. Three runs were required to complete the testing.

The study samples were vortexed manually, uncapped, and placed on the sample carrier. The reagents were loaded in respective reagent reservoir carriers and the carries were then placed into the Cobas. After sample and reagent loading, sample preparation, isolation and extraction of DNA were done automatically with the Cobas x480 instrument. The final DNA products were collected into a microwell plate. The microwell plate with the PCR-ready samples was unloaded, sealed manually with COBAS sealing film, and transferred to the analyser (COBAS z480) for amplification and detection using real-time PCR technology. Results were then be taken from the laboratory information system. Negative controls would have yielded a “Target Not detected” result, while the positive controls yielded a “Target Detected” result. For the samples, various result outcomes were possible:

1. Target not detected: Ct value for HIV-1 is below the limit of the assay or no Ct value for HIV-1 is obtained. Results are reported as “Lower than detectable limit”
2. <20 copies/ml: Calculated cp/ml are below the limit of detection of the assay. Results are reported as “HIV-1 RNA detected, <20 RNA cp/ml”.
3. ≥20 cp/ml and ≤ 1.00E+07 cp/ml: Calculated cp/ml are within the linear range of the assay. Results are reported with the exact number of cp/ml given by the instrument.

   >1.00E+07 cp/ml: Calculated cp/ml are above the range of the assay. The original sample was diluted 1:100 with HIV-1 negative human EDTA-plasma and the test was repeated. The result is then multiplied by the dilution factor and gives a quantitative result, reported as “quantitative result 1.00E+07 cp/ml”.

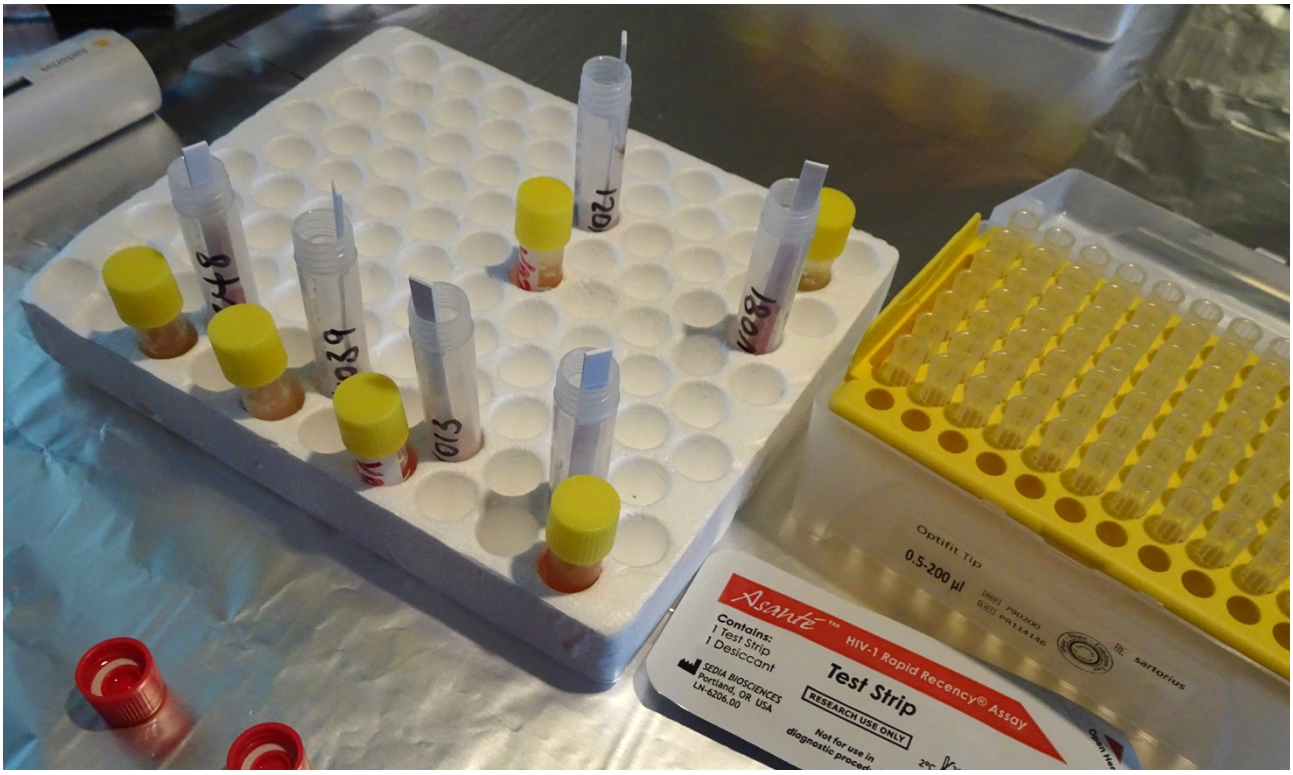
3.6.4.3 Recency Testing

After viral load testing, recency testing was done on the thawed samples. Using a mechanical pipette, 5 microlitres of plasma was pipetted into a tube containing 0.5ml sample buffer. The assay test strip was then inserted into the tube containing the sample-buffer mixture. The mixture was absorbed into the absorbent pad of the test strip. Results were then read visually immediately after 20 minutes**.** The appearance of all three lines is considered as a long-term infection while a recent infection is determined when only two lines are visible; the control line and the verification line

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(Asante HIV-1 Rapid Recency Assay package insert, 2019). This recency assay has a sensitivity of 99.14% and a specificity of 98.7% (Parekh et al., 2017). Figures 7 and 8 below were taken during the study and show the procedure of this recency test.

Figure 7. Asanté HIV-1 Rapid Recency Assay test material



Footnotes: The test strips are placed in the tube containing the sample-buffer, next to the participant-sample cryotube. On the bottom right, a closed test strip is visible.

Figure 8. Asanté HIV-1 Rapid Recency Assay test strips and outcome



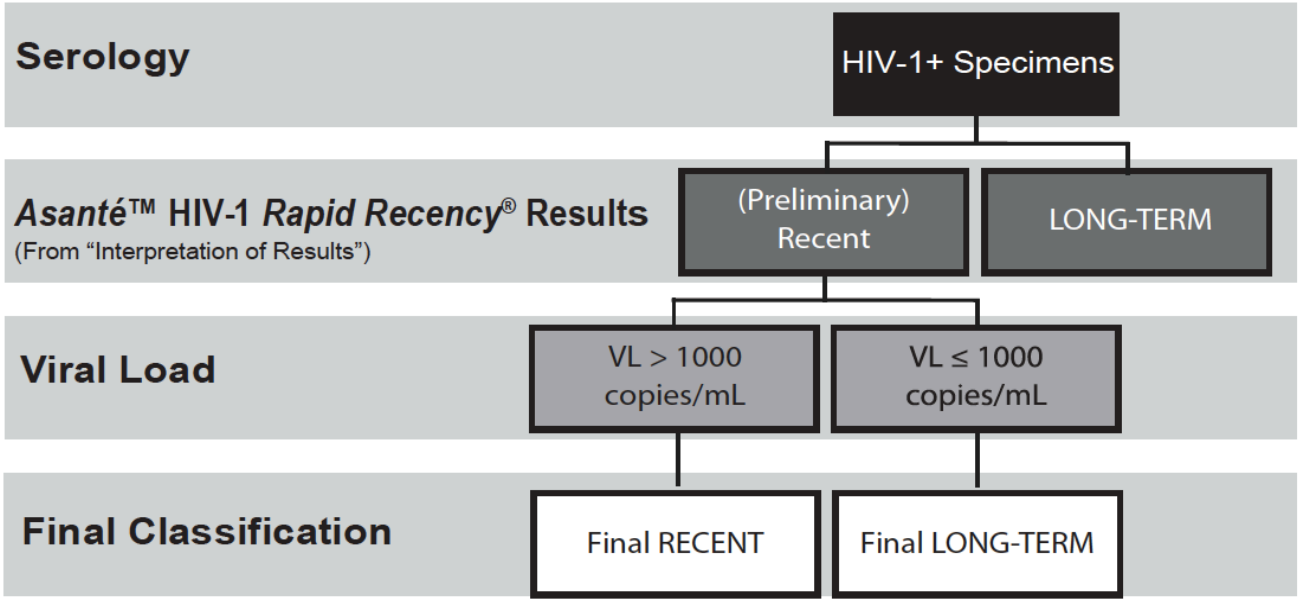
Footnotes: A test strip showing 3 lines, indicating a long-term test outcome

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3.6.4.4 Using RITA

To determine the recency outcome we applied the RITA below (Figure 9). This RITA includes Asanté HIV-1 Recency testing and VL testing as an additional parameter to ascertain the outcome. The final classification of recency was determined by the following criteria. Samples that tested recent with the Asanté HIV-1 recency assay but had a VL greater than 1000 copies/ml were classified as a true recent infection. Those that were initially tested as recent but had a viral load that was equal to or less than 1000 copies/ml were classified as long-term infections (Asanté HIV-1 Rapid Recency Assay package insert, 2019).

Figure 9. RITA using Asanté HIV-1 Rapid Recency Assay and HIV viral load



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3.6.5 Estimation of Incidence

To estimate the population level annual incidence based on a recency assay, the formula (Formula 1) recommended by the US CDC, the office of the global AIDS coordinator and the UNAIDS/WHO working group on Global HIV/AIDS and STI Surveillance was used (WHO, 2011).

Formula 1. Recommended incidence formula based on the Asanté HIV-1 Rapid Recency Assay

$$I_F = \frac{R - (FRR * P)}{(1-FRR)\,\omega N} X\ 100$$

I_F = annual HIV incidence rate
N = number of HIV-negative samples in the survey
P = number of HIV-positive samples in the survey
R = number of HIV-positive samples testing as recent on the test
ω = mean duration of recent infections for the test specified in years
FRR = false recency rate for the test

Findings from independent validations of LAg-only recency testing suggest that the FRR is 1.5% (Kassanjee et al., 2016). However, once combined with VL testing to minimize misclassification of people on ART, the FRR is assumed to be 0 (Asanté HIV-1 Rapid Recency Assay package insert, 2019). The mean duration for recent infections (MDRI) for the Asanté HIV-1 Rapid Recency Assay was estimated at 180 days (0.5 years) as suggested by the manufacturer and the US CDC for the Southern African setting (Parekh et al., 2017). If samples are missing at random due to various reasons such as contamination of the sample or insufficient volume, it is advised to exclude those samples and to scale down the number of HIV-negative samples appropriately according to Formula 2 (WHO, 2011):

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Formula 2. Adaptation of the incidence formula when there is missing data

$$\overline{N} = \frac{N(P-M)}{P}$$

$$\overline{P} = P - M$$

M is the number of HIV-positive samples with a missing RITA test

N is the number of HIV-negative samples in the survey

P is the number of HIV-positive samples in the survey

The annual incidence rate may be converted into the annual risk of infection using the following Formula 3 (WHO, 2011):

Formula 3. Conversion of annual incidence rate into the annual risk of infection

$$I_a = 1 - \exp(-I_F)$$

I_a is the annual risk of infection

I_F is the annual incidence rate

3.7 DATA MANAGEMENT AND STATISTICAL ANALYSIS

Data integrity checks were written into the database that was used for data collection during the home-based HIV testing campaign to limit missing fields or the entry of incorrect data during the data collection. Access to the database was regulated by user identification code and password. A study data manager monitored data quality and completeness every week. Queries about the data were regularly sent to the local principal investigators for follow-up and correction as needed.

Appropriate descriptive statistics were used in this project, i.e. absolute and relative frequencies for categorical data and medians and interquartile ranges for continuous variables. The CD4 cell count results were grouped according to the CDC stage classification (CDC, 1997): <200 cells/µL vs 200-499 cells/µL vs >500 cells/µL.

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Inferential statistical testing was performed to assess risk factors between the characteristics of the patient samples and recent HIV diagnoses. We used a logistic regression model to determine the factors associated with recent HIV infections. We will fit the multivariable logistic regression model using only clinically relevant factors based on literature, rather than statistically significant variables from the univariable logistic regression model. And we will only fit a maximum of one predictive variable for every 5-10 events as suggested as a the one-in-ten or one-in-five rule.
Results are presented as odds ratios with 95% confidence intervals. All analyses were performed using Stata (version 14, Stata Corporation, Austin/Texas, USA). For all tests, we used complete case analysis and two-sided p-values with an alpha 0.05 level of significance.

3.8 ETHICAL ASPECTS AND GOOD CLINICAL PRACTICE

3.8.1 Ethical Clearance

The VIBRA trial has been approved by the National Health Research and Ethics Committee of the Ministry of Health of Lesotho (ID06–2018) and the ethics committee in Switzerland (Ethikkomission Nordwest- und Zentralschweiz; ID2018–00283). Submission of the first study protocol to ethics committees happened in Lesotho in February 2018 and Switzerland in April 2018. The approval followed on 25 April 2018 (Lesotho) and 8 May 2018 (Switzerland). Afterwards, four minor amendments to the study protocol were submitted and approved by the National Health Research and Ethics Committee of the Ministry of Health of Lesotho, where the last amendment was submitted specifically for approval of this nested study (Version 9).

3.8.2 Safety Variables

3.8.2.1 Project safety

During the sample analysis, appropriate personal protective equipment was used at all times to ensure safety.

3.8.2.2 Patient's Safety and Risk or Benefit to the Patient

The study posed minimal risks to the participants. During the survey the participants might have found it distressing to be found HIV-positive, however, all the participants got pre and post counselling from trained counsellors. Also, they might have

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experienced slight discomfort during phlebotomy. Regardless of all of these factors, participants benefited greatly from the study since participants could get to know their HIV status and were linked to care as soon as possible, with baseline tests already in hand. Their progress was also monitored after enrolment in care. The assessment and outcomes of this nested recency study add important epidemiological data.

3.8.2.3 Premature discontinuation of the study

There was no necessity to discontinue the study since patient confidentiality protocols were overserved and no unethical procedures were performed

3.8.2.4 Good Clinical Practice (GCP) and Quality Assurance

All clinical work conducted under this protocol is subjected to the GCP guidelines (The Principles of ICH GCP, 2004). The declaration of Helsinki's basic principle number 3 states that research should be conducted only by scientifically qualified people and under the supervision of adequately qualified people (World Medical Association, 2002).

3.8.3 Financial implications to the participant

There were no financial implications to the patient.

3.8.4 Withdrawal criteria

Participation was completely voluntary. Patients had the right to withdraw from the VIBRA trial at any time, irrespective of the reason, without detriment to their medical care presently or in the future.

3.8.5 Subject information and informed consent

The study nurse obtained separate written informed consent from all eligible persons during the home-based testing campaign of the VIBRA trial. All the participants were informed about the purpose and necessity of the research project, the financial implications and the consequences as well as the adverse effects and their right to withdraw without any effects on them or their doctor nurse-patient relationship. The informed consent included the collection of data and blood draw for storage and additional analyses such as this nested study. Illiterate participants provided a thumb-print and a witness (independent of the trial and > 21 years old), chosen by the participant, who co-signed the informed consent form. For participants aged < 18

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years, a literate caregiver > 21 years old provided written consent. The informed consent form was in the local language, Sesotho, and each participant was given a copy.

3.8.6 Confidentiality

All samples that were analysed were assigned unique identification numbers to anonymize the data and protect confidentiality. At no point during the study the source of the samples, i.e. participants' names, were revealed. This data will be stored for 10 years according to the GCP guidelines.

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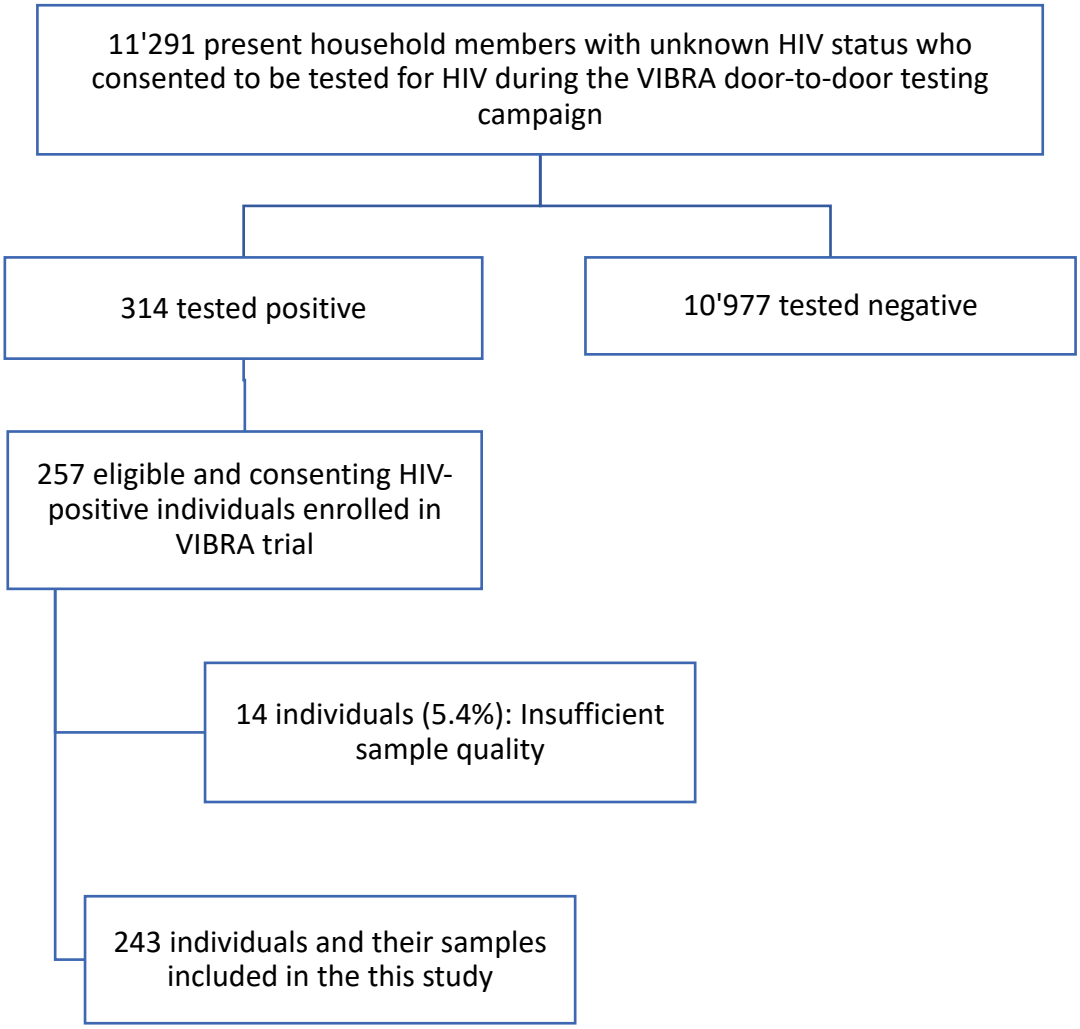
CHAPTER 4: RESULTS

4.1 OVERALL STUDY SAMPLE FLOW

During the VIBRA door-to-door HIV testing campaign between August 16th, 2018, and May 28th, 2019, 11291 present household members with unknown HIV status consented to be tested for HIV (Figure 10). 314 among them were HIV-positive on the day of enrolment as outlined in chapter 3 and 257 of them were enrolled into the VIBRA trial.

From all enrolled individuals, blood samples were collected and stored in the -80°C biobank at Butha-Buthe Government Hospital Laboratory. However, out of the 257 samples, 14 samples (5.4%) were excluded from the study due to insufficient sample quality, leaving a total of 243 participant samples included in this nested study.

Figure 10. Study sample flow

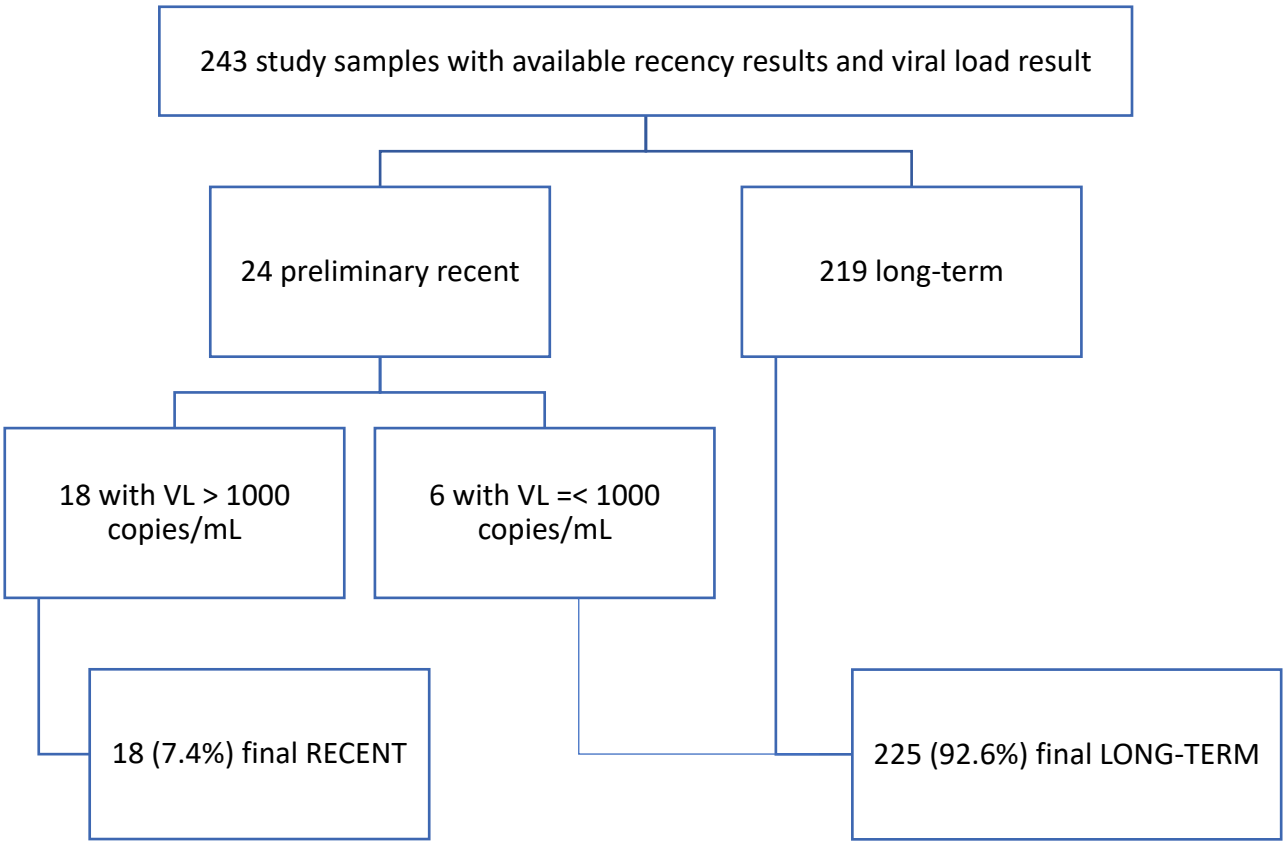


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4.2 RECENCY OUTCOMES

Figure 11 outlines the outcomes along with the recency algorithm as described in chapter 3 (figure 9), i.e. the RITA based on the Asanté recency test and HIV viral load. The samples were classified as recent or long-term accordingly. 24 samples were defined as preliminary recent, 6 of these samples were then reclassified as final long-term because their viral load was below 1000 copies/ml. Overall, among the 243 samples included in this study, we found 18 (7.4%; 95% confidence interval 4.4%-11.5%) recent infections, i.e. acquired within the previous 6 months, while 225 (92.6%) were classified as long-term HIV infections.

Figure 11. Recency outcomes



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4.3 STUDY PARTICIPANT CHARACTERISTICS OF INCLUDED SAMPLES

The demographic and socio-demographic characteristics of the 243 participants included in this study are shown in table 1 below. The majority of the participants were female (142 [58%]) with a median age of 37 years (interquartile range [IQR] 31-50 years). The majority of the participants were from Mokhotlong district (216/243 [89%]). Most participants were unaware of their HIV-positive status (184 [76%]). Only 59 (25%) participants already knew their status which included 28 (12%) participants who had once been on ART and 31 (13%) who had never taken ART before.

From the catchment area of five health facilities, more than 10% of new diagnoses were recent HIV infections: Malefiloane health center in Mokhotlong (22%), Mapholaneng health center in Mokhotlong (11%), Moeketsane health center in Mokhotlong (11%), Molikaliko health center in Mokhotlong (17%) and Ngoajane health center Butha-Buthe (11%).

Lack of employment with no regular income was relatively high (202 [83%]) while only 41 (17%) participants were employed or self-employed with regular income. In addition, 192 (79%) participants completed primary school only or had no schooling at all, which reflects also in the fact that 148 (61%) participants had less than 7 years of schooling. Only 50 (21%) participants had secondary or higher education.

163 (67%) participants reported no regular use of alcohol, 21 (9%) participants showed signs of alcohol dependency (assessed by the CAGE questionnaire, see Chapter 3.6.2), whereas the remaining 59 (24%) used alcohol but showed no signs of alcohol dependency. The use of cannabis seemed low; only 33 (14%) participants had a history of local regular cannabis use, the rest of the participants (86%) reported they have not used cannabis before.

A few participants reported having several sexual partners (20 [8%]). 110 (45%) participants reported that they have one regular sexual partner, 106 (44%) said they had no sexual partner and 7 (3%) participants refused to answer.

The CD4 cell count was missing for 43 (18%) study participants. The median CD4 cell count among all participants with available CD4 cell count was 390 cells/µL (IQR 256-534 cells/µL). According to CDC stage classification, 32 (13%) participants were stage 3, 107 (44%) stage 2 and 61 (25%) stage 1. All participants had a viral load available, median viral load was 36’200 copies/mL (IQR 7510-145’000 copies/mL) and 132 (54%) participants had a viral load above 30’000 copies/mL.

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Table 1. (Socio-)Demographics of study participants by recency outcome

	Total (N=243)	Long-term (n=225)	Recent (n=18)
Sex			
Female	142 (58%)	127 (56%)	15 (83%)
Male	101 (42%)	98 (44%)	3 (17%)
Age in years, median (IQR)	37 (31-50)	37 (31-49)	40 (28-53)
Age groups			
16-29	51 (21%)	45 (20%)	6 (33%)
30-39	84 (35%)	81 (36%)	3 (17%)
40-49	46 (19%)	43 (19%)	3 (17%)
50-81	62 (26%)	56 (25%)	6 (33%)
District			
Butha-Buthe	27 (11%)	24 (11%)	3 (17%)
Mokhotlong	216 (89%)	201 (89%)	15 (83%)
Health facility			
Libibing HC MKG	14 (6%)	14 (6%)	0 (0%)
Linakaneng HC MKG	19 (8%)	18 (8%)	1 (6%)
Linakeng HC BB	2 (1%)	2 (1%)	0 (0%)
Makhunoane HC BB	7 (3%)	6 (3%)	1 (6%)
Malefiloane HC MKG	30 (12%)	26 (12%)	4 (22%)
Mapholaneng HC MKG	27 (11%)	25 (11%)	2 (11%)
Moeketsane HC MKG	10 (4%)	8 (4%)	2 (11%)
Mokhotlong Hospital MKG	29 (12%)	28 (12%)	1 (6%)
Molikaliko HC MKG	16 (7%)	13 (6%)	3 (17%)
Motete HC BB	1 (0%)	1 (0%)	0 (0%)
Ngoajane HC BB	5 (2%)	3 (1%)	2 (11%)
Rampai HC BB	1 (0%)	1 (0%)	0 (0%)
Seboche Hospital BB	2 (1%)	2 (1%)	0 (0%)
St. James HC MKG	18 (7%)	18 (8%)	0 (0%)
St. Martins HC MKG	36 (15%)	35 (16%)	1 (6%)
St. Paul HC BB	1 (0%)	1 (0%)	0 (0%)
St. Peters HC BB	7 (3%)	7 (3%)	0 (0%)
Tlhanyaku HC MKG	17 (7%)	16 (7%)	1 (6%)
Tsime HC BB	1 (0%)	1 (0%)	0 (0%)
HIV/ART history			
Newly diagnosed	184 (76%)	171 (76%)	13 (72%)
Known+/ART naive	31 (13%)	27 (12%)	4 (22%)
Previous ART	28 (12%)	27 (12%)	1 (6%)
Schooling in years, median (IQR)	5 (2-7)	5 (2-7)	7 (3-9)
7 years or more of schooling			
No	148 (61%)	140 (62%)	8 (44%)
Yes	94 (39%)	84 (37%)	10 (56%)
Missing	1 (0%)	1 (0%)	0 (0%)
Education			
Secondary or higher education	50 (21%)	44 (20%)	6 (33%)
No schooling or only primary	192 (79%)	180 (80%)	12 (67%)
Missing	1 (0%)	1 (0%)	0 (0%)
Employment			
No employment and no regular income	202 (83%)	184 (82%)	18 (100%)

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(Self-)Employment with regular income	41 (17%)	41 (18%)	0 (0%)
Alcohol abuse (using CAGE)			
No	59 (24%)	57 (25%)	2 (11%)
Yes	21 (9%)	21 (9%)	0 (0%)
Not applicable because no regular use	163 (67%)	147 (65%)	16 (89%)
History of local regular cannabis use			
No cannabis use	210 (86%)	193 (86%)	17 (94%)
Cannabis use	33 (14%)	32 (14%)	1 (6%)
Regular sexual partner			
Yes, one	110 (45%)	102 (45%)	8 (44%)
Yes, several	20 (8%)	20 (9%)	0 (0%)
No	106 (44%)	98 (44%)	8 (44%)
Refused to answer or not applicable	7 (3%)	5 (2%)	2 (11%)
CD4 count, median (IQR)	390 (256-534)	396 (256-534)	366 (258-504)
CD4 count groups			
<200 cells/µL	32 (13%)	29 (13%)	3 (17%)
200-499 cells/µL	107 (44%)	98 (44%)	9 (50%)
>500 cells/µL	61 (25%)	57 (25%)	4 (22%)
Missing	43 (18%)	41 (18%)	2 (11%)
Viral load, median (IQR)	36200 (7510-145000)	36000 (7210-140000)	40350 (16700-145000)
Viral load above 30'000c/mL			
No	111 (46%)	104 (46%)	7 (39%)
Yes	132 (54%)	121 (54%)	11 (61%)

4.4 PREDICTIVE RISK FACTORS FOR RECENCY

The association of demographics and a recent HIV infection were explored using a logistic regression model (see Chapter 3.7) and the results are displayed in table 2. Although some characteristics showed a certain trend, only female gender was predictive for a recent HIV infection (odds ratio 3.86 (95% confidence interval 1.09-13.70); p-value 0.037). The multivariate logistic regression model was fitted with the most important clinical factors (age and gender and sexual partners) and did not change the association between female gender and recency (adjusted odds ratio 3.74 (95%CI 1.05-13.36); p-value 0.041). Because none of the participants with recent infection was (self-) employed with regular income nor showed alcohol abuse (see table 1), the logistic regression led to a nonsensical result.

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Table 2. Univariate and multivariate logistic regression on recent vs. long-term outcome

	Univariate logistic regression			Multivariate logistic regression		
Characteristic	**Odds Ratio (95% CI)**	**beta coeff.**	***P* - value**	**Adjusted Odds Ratio (95% CI)**	**beta coeff.**	***P* - value**
Female (vs Male)	3.86 (1.09-13.70)	1.35	0.037	3.74 (1.05-13.36)	1.32	0.041
Age (per year)	1.00 (0.96-1.04)	0.00	0.949	1.00 (0.96-1.03)	-0.00	0.928
Butha-Buthe (vs Mokhotlong)	1.68 (0.45-6.21)	0.52	0.440			
HIV/ART History						
Newly Diagnosed	1					
Known+/ART naive	1.95 (0.59-6.42)	0.67	0.273			
Previous ART	0.49 (0.06-3.88)	-0.72	0.497			
Schooling (per year)	1.07 (0.95-1.20)	0.07	0.289			
No schooling/only primary (vs secondary/higher education)	0.49 (0.17-1.37)	-0.72	0.175			
(Self-)Employed with regular income (vs No employment/no regular income)	n/a[1]					
Alcohol abuse (vs No alcohol abuse)	n/a[1]					
Cannabis use (vs No cannabis use)	0.35 (0.05-2.76)	-1.04	0.322			
Regular Sexual Partners						
Yes, any	1					
No or refused to answer	0.68 (0.26-1.77)	-0.39	0.426	0.76 (0.28-2.03)	-0.28	0.580
CD4 count groups						
<200 cells/µL	1					
200-499 cells/µL	0.89 (0.23-3.50)	-0.12	0.865			
>500 cells/µL	0.68 (0.14-3.24)	-0.39	0.626			
Viral load >30'000 copies/mL (vs. viral load <30'000 copies/mL)	1.35 (0.51-3.61)	0.30	0.549			

Footnotes: [1]nonsensical due to low numbers.

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4.5 INCIDENCE ESTIMATION

Based on the recommended incidence formula (Chapter 3.6.5, formula 1) we calculated the annual HIV incidence rate, using our results:

R= 18
FRR= 0
P= 314
N= 10977 (negative tested individuals, see Figure 10)
w= 0.5

Taking into account the adaptation of missing data (formula 2), in total 14 in our sample, it resulted in an annual incidence rate of I_F **= 0.34%**. Using formula 3, we estimate the annual risk of infection at I_a **= 0.29%.**

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CHAPTER 5: DISCUSSION

This cross-sectional study assessed the proportion of recently acquired HIV infections – using the Asanté HIV-1 Rapid Recency Assay – among new diagnoses found during a door-to-door HIV testing campaign in 2018/2019 in two rural districts of Lesotho. We found 7.4% recent infections (within the 6 months before the campaign) among the 243 study samples. Most of them were from women and being female was a predictor for a recent (versus a long-term) infection. Based on our data, we estimated an annual incidence rate of 0.34% for that area. Among the 225 study participants that had a long-term infection, 76% were unaware of their HIV-positive status during enrollment. The CD4 cell counts were not very different between long-term vs recently infected individuals, similar proportions had CD4 cell count >500 cells/μL, and <200 cells/μL. We may have expected a different pattern, e.g., lower CD4 for long-term and higher CD4 for recent infections. However, the progress of HIV without ART is highly variable and usually takes much more time than 6 months until CD4 cell count decreases.

A similar recency study, based on stored samples from 2007, was conducted in Kenya. In this study, 1025 HIV-antibody-positive specimens were tested finding 64 (6.2%) recent infections and establishing some risk factors associated with recent infection such as being married/widowed, having several sexual partners, a sexually transmitted co-infections, no condom use at last intercourse, and being below 30 years old (Kim et al., 2016). While our data showed a similar proportion of recent infections, the risk factor analysis revealed fewer risk factors. This may be due to the small sample size of our study which may have impacted our risk factor analysis. Moreover, some of the characteristics (such as co-infection) were not available in our study population.

A study based on HIV routine programmes for female sex workers (FSW), women attending antenatal care (ANC) and general HTC facilities in Kenya and Zimbabwe in 2018 assessed the recency of new diagnoses using a similar RITA (Rice et al., 2020). The study found recency rates of 2.3% among ANC clients in Kenya, 10.5% among FSW in Zimbabwe and 8.6% among general HTC clients in Nairobi, Kenya. It did not find any risk factors associated with recent infections. Comparing these findings to our study, the recency proportion for ANC clients and FSWs is quite different, which makes sense since this is a different population than in our study. The general population recency proportion of 8.6% is comparable to our findings.

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Another similar cross-sectional recency study was done in 2017 in Nigeria (Negudu et al., 2021). Out of a total of 370 plasma samples that were collected during a household survey, a total of 11 samples (2.9%) were identified as recent infections using the same recency test and RITA. No risk factor analysis was performed. These recency results were lower than our results. However, nearly 20% of the 370 participants in the study of Negudu et al., were virally suppressed, indicating a high rate of people on ART in their sample. And these are probably people with a longer-term infection. Our study did not entail any participants taking ART. In the study from Negudu et al., nearly 50% had a CD4 count greater than 500 cells/mm3. In our study there were only 25% of participants with such a high CD4 count, the others had a lower count indicating a severely compromised immune system and high risk for co-infections and death.

Between October 2015 and August 2016, a survey was conducted in Zimbabwe to determine progress towards epidemic control (Gonese et al., 2020). The study enrolled 20,577 adults aged 15-64 years old. They conducted interviews using a questionnaire, performed a rapid HIV test, and, if positive, testing for recency using the same RITA but including ARV level detection. In the end, 2901 HIV-seropositive individual samples were tested for HIV recency. Overall, 26 individuals (0.9%) had a recent infection (men: 8; women: 18). Risk factors were not assessed.

Between 2015 and 2017 population-based surveys were conducted in Zimbabwe, Malawi, Zambia, eSwatini, and Lesotho, to estimate HIV recency and incidence among adolescents in these countries (Low et al., 2021). Adolescents were tested for HIV using national rapid testing algorithms. Recent infection was measured using the same recency assay as in our study, but excluding adolescents with viral suppression or with detectable ARVs in the blood. The study found that among the 707 adolescents living with HIV, 22 (3.1%) were classified as recent, which was lower than our finding. This is an interesting finding since this study population was exclusively adolescents who are generally a key group more likely to be infected.

Another similar study among young people was conducted in Malawi in 2019 (Ministry of Health Malawi, 2019). Its main objective was to detect recent HIV infection among newly diagnosed adolescent girls and young women (AGYW), aged 15-24 years old, seeking antenatal care, by using a similar RITA as in our study. Among the 589 study participants, 11.5% had a recent infection. This may be explained by the inclusion of young women (19-24 years old) in addition to adolescent women (15-18 years old).

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This result is higher than in our study, probably because our study sample consisted of a general population sample. Also, this study assessed risk factors and found that the first trimester of pregnancy was significantly associated with a recent infection, which could not be explained. No other variables were significantly associated with a recent HIV infection among pregnant women.

A similar study conducted in Lesotho estimated HIV incidence among 2358 adolescent girls who were tested for HIV between November 2016 and May 2017 (Low et al., 2019). In this study, the same HIV-1 limiting antigen avidity enzyme immunoassay combined with viral load was used. The estimated incidence of HIV in girls and young women was 1.8% (0.8-2.8). This estimated incidence was higher than our incidence estimate. Again, however, this may be caused by the difference in the population in this study, which was young women, and our study, which included both sexes and a general population. It is well-known that adolescent girls and women aged 15-24 are a vulnerable key group highly affected by HIV in sub-Saharan Africa. Despite representing only 10% of the population, this group accounts for 25% of all new HIV infections globally (UNAIDS, 2019). The general annual incidence of HIV among adults (ages 15 years and older) in Lesotho was 0.45% in 2019, 0.64% among women and 0.28% among men (LePHIA, 2020). This is a bit higher than our estimates, but the two districts are not the two most affected districts in the country.

5.1 LIMITATIONS AND FUTURE RESEARCH

This study has revealed a few limitations. Since this study was conducted only in Butha-Buthe and Mokhotlong, the findings may not reflect the characteristics of other districts in Lesotho. Future research including other districts in Lesotho and a larger sample size may allow for an in-depth risk factor analysis and give a true reflection of the conditions in Lesotho. Furthermore, a reliable incidence estimation requires a large sample, preferably from a large-scale cross-section survey, not necessarily from a trial. Hence, our incidence estimate has to be viewed with caution.

This project did not measure the presence of ARV to exclude participants on ART from the recency sample. However, all samples were tested for VL and the VIBRA trial team verified every new diagnosis they found in the field with the surrounding health facilities, thus minimizing the risk of having someone already on ART in our study.

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Moreover, it has been shown that ARV use has generally little impact on incidence estimates (Huerga et al., 2017).

Moreover, it is important to note that we included study participants that once took ART but were off treatment for at least 1 month. These were only 28 people and 1 person still tested as recently infected with HIV.

5.2 CONCLUSIONS

This cross-sectional study assessed the proportion of recently acquired HIV infections – using the Asanté HIV-1 Rapid Recency Assay – among new diagnoses found during a door-to-door HIV testing campaign in 2018/2019 in two rural districts of Lesotho. We were able to distinguish long-term HIV-1 infections from recent HIV-1 infections among HIV-positive individuals using the Asanté HIV-1 Rapid Recency Assay together with viral load testing. We found 7.4% recent infections (within the 6 months before the campaign) among the 243 study samples. Most of them were from women, and being female was a predictor for a recent (versus a long-term) infection. Based on our data, we estimated an annual incidence rate of 0.34% for that area. Among the 225 study participants that had a long-term infection, 76% were unaware of their HIV-positive status during enrollment. The most recent infections were found in the catchment area of only 5 health facilities, most in Mokhotlong district.

The use of a recency assay together with HIV viral load can help determine hot spots for ongoing HIV transmission. In a low-resource country like Lesotho this provides a useful strategy in the allocation of prevention resources and may be routinely considered.

These findings have various important implications. In general, more HIV testing and prevention efforts are needed in these areas of Lesotho among the general population – and in particular for women and in Mokhotlong district – to prevent new infections. It is crucial to understand better why women are more at risk for recent HIV infection in order to target the root cause of this. Moreover, we need to continually implement HIV prevention strategies such as condom distribution, safe sex practice, pre-exposure prophylaxis, voluntary medical male circumcision, as well as long-term ART for everyone who is living with the virus. Also, we need to continue to educate not only the community but health care workers about these various prevention methods and offer

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testing, including recency testing, at every level of society. Only then, we may be able to curb the ongoing transmission of HIV in Lesotho.

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CHAPTER 6: REFERENCES

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APPENDICES

A. Approval from VIBRA study Principal Investigator

Swiss TPH

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January 19, 2020

To whom it may concern

APPROVAL TO USE VIBRA RESEARCH SAMPLES FOR MASTERS PROJECT

As principal investigator of the study entitled *"Village-based refill of ART after same-day ART start vs clinic-based ART refill for HIV-positive individuals not on ART during home-based HIV testing – a cluster randomized controlled trial in rural Lesotho"*[1] (VIBRA study), I confirm that I agree providing Mrs Ts`epang Mohloanyane the baseline samples from the VIBRA study to perform HIV viral load quantification and Asanté™ recency test®.

The VIBRA trial enrolled individuals found HIV positive during home-based HIV testing in Butha-Buthe and Mokhotlong district in Lesotho. In November 2019 we submitted an amendment to the study protocol to allow for Asanté™ recency tests® in the baseline samples, aiming to provide information on recency of newly diagnosed HIV infections in the two study districts. Attached to this letter you may find the submitted amendment as well as the approval of the National Ethics and Research Committee of Lesotho.

I am convinced that this nested study provides an interesting opportunity to a Master student to conduct his/her thesis by a) conducting the tests, b) reviewing the literature on the validity of the recency tests and c) interpreting the findings in the broader context of the dynamics of the HIV epidemic in Lesotho. I was thus looking for a Master student in Lesotho to conduct, analyse and interprete these tests. I am convinced that with Mrs Mohlanyane I found the ideal candidate to conduct these tests and that she will use the results for a successful thesis.

[1] Amstutz, A., Lejone, T.I., Khesa, L., Muhairwe, J., Nsakala, B.L., Tlali, K., Bresser, M., Tediosi, F., Kopo, M., Kao, M., Klimkait, T., Battegay, M., Glass, T.R., Labhardt, N.D., 2019. VIBRA trial – Effect of village-based refill of ART following home-based same-day ART initiation vs clinic-based ART refill on viral suppression among individuals living with HIV: protocol of a cluster-randomized clinical trial in rural Lesotho. Trials 20, 522. https://doi.org/10.1186/s13063-019-3510-5

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Attached to this letter you may find

- VIBRA study protocol v9_10.11.19
- Approval letter from NH-REC Lesotho, dated Dec 09, 2019

Please do not hesitate to contact me in case of any questions, kind regards

Prof. Dr. Niklaus Labhardt
Principal Investigator VIBRA study

copy to:
- Dr. Josephine Muhairwe, Coordinator of VIBRA study implementation, SolidarMed Lesotho

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B. Cover letter to Ethics to perform the nested study with recency testing

Swiss TPH
Swiss Tropical and Public Health Institute
Schweizerisches Tropen- und Public Health-Institut
Institut Tropical et de Santé Publique Suisse

Assoziiertes Institut der Universität Basel

SOLIDAR MED

Department of Medicine
Clinical Research Unit

Prof. Dr. Niklaus Labhardt, MD, MIH, DTM&H
Research Group Leade

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T +41 61 284 8255
M +41 79 870 1859

To
Chairperson of
National Health Research and Ethics Committee
Ministry of Health of Lesotho
Maseru

Basel / Butha-Buthe / Maseru, 10.11.2019

SUBMISSION of AMENDMENT of GET ON research project (ID06-2018) with the two inter-linked substudies:

***Part A) HOSENG study**: Home-based plus self-testing to improve HIV testing coverage in rural Lesotho – a cluster randomized controlled trial in rural Lesotho*

***Part B) VIBRA study**: Village-based refill of ART after same-day ART start vs clinic-based ART refill for HIV-positive individuals not on ART during home-based HIV testing – a cluster randomized controlled trial in rural Lesotho*

Dear Chairperson of the NH-REC

Thank you again for approving our GET ON research project with the two interlinked substudies (HOSENG study and VIBRA study) on April 25th 2018 and the recent extension of the project on June 3rd 2019 (see approval letters attached).

We would like to submit an amendment to the current approved protocol version (v8_01.05.19) of the VIBRA substudy.
As planned, submitted and approved we will perform resistance testing among all baseline samples (Chapter 7 "Biomolecular Research", page 27). We would like to perform the resistance testing in Durban, at the KRISP (Kwazulu-Natal Research Innovation and Sequencing Platform, www.krisp.org.za). Moreover, we would like to use the information obtained from the resistance testing not only to inform clinical care but to build an HIV transmission model using phylogenetics methods and recency testing, for example for the entire Mokhotlong district. This would allow the District Health Management Team in Mokhotlong a more targeted approach for HIV prevention and services. Therefore, we would like to amend the protocol as follows:

Chapter 7 (page 27):
"The resistance testing will be performed regionally, in Durban, at the KRISP (Kwazulu-Natal Research Innovation and Sequencing Platform, www.krisp.org.za). Moreover, the information obtained from the resistance testing of all baseline samples will be used for an HIV transmission clustering model using phylogenetic methods. In order to add incidence information to the model we will perform Asanté HIV-1 recency test and viral load test among all baseline samples."

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Swiss TPH

Thank you for reviewing the modified VIBRA study protocol (modified submission):

- **VIBRA study_study protocol_v9_10.11.19**

Kind Regards,

Dr Josephine Muhairwe MD MPH
Coordinator of study implementation
Country Director
SolidarMed

Prof. Dr Niklaus Labhardt MD, MIH, DTM&H
Principal Investigator VIBRA study
Swiss TPH

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C. Ethics approval Ministry of Health Lesotho

LESOTHO

Ministry of Health
P.O. Box 514
Maseru 100

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

REF: ID06-2018-Modify 03

Date: December 09, 2019

To
Josephine Muhairwe, MD, MPH
Study Coordinator
SolidarMed, Lesotho

Dear Dr. Josephine,

RE: GET ON Research Project with two Interlinked Studies: HOSENG study and VIBRA study

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

This approval includes review of the following attachments:
[x] Study Protocol_v9_10.11.19: **VIBRA study _v9_10.11.19**
[ ] English & Sesotho consent forms
[ ] Case Report Form
[ ] Data collection forms/question guide in English
[ ] Participant materials *[insert types, versions, dates]*
[x] Other materials: Letter of request for amendment dated 10.11.2019

This approval is **VALID** until June 03, 2020.

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

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

We look forward to receiving your progress reports and a final report at the end of the study. If you have any questions, please contact the Research and Ethics Committee at rcumoh@gmail.com (or) 22226317.

Sincerely,

Dr. Nyane Letsie
Director General Health Services

Dr. M. M. Moteetee
Chairperson NH-REC

D. Informed Consent and Information Sheet

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Foromo ea Tumello e nang le Tsebo

Foromo ena e fanang ka tumello e nang le tsebo ke ea bakuli ba fumanoeng ba ena le kokoana-hloko ea HIV ba sa noeng litlhare nakong eo ho etsaong liteko tsa HIV metseng, ba menngoeng ho nka karolo lipatlisisong tsa morero o bitsoang *"GET ON – GETting tOwards Ninety"*.

Lipatlisiso tsena li etsoa ka t'sebelisano 'moho le litsi tse hlano:
- Swiss Tropical and Public Health Institute: Principal Investigators: Dr Niklaus Labhardt / Dr Tracy Glass
- SolidarMed: Study Physicians; Dr Bienvenu Nsakala, Dr Alain Amstutz
- District Health Management Team: Butha-Buthe (Dr L. Sao), Mokhotlong (Dr K. Matjeane)
- Research Coordination Unit, Ministry of Health of Lesotho: Dr Kyaw Thin
- Department of Biomedicine, University of Basel: Prof. Thomas Klimkait

Foromo ena e fanang ka tumello e likarolo li peli:
- Karolo ea Pele: Leqephe la litaba (le arolelana litaba tsa morero ona oa lipatlisiso le uena)
- Karolo ea Bobeli: Lengolo la Tumello (moo u tekenang haeba u lumela ho nka karolo)

U tla fumana kopiea foromo ena ea Tumello.
Likhutsufatso: ART = anti-retroviral therapy (=HIV medication)

KAROLO EA PELE: Leqephe la Litaba

1. Selelekela
Ke_____, ea sebeletsang SolidarMed, mokhatlo o ikemetseng o t'sehetsang tlhokomelo ea bophelo Lesotho nako ea lilemo tse fetang tse mashome a mahlano. Ke tlo u fa litaba ke u meme hore u be karolo ea lipatlisiso tsena. Ho ka ba le mantsoe ao u sa a utloisiseng. Ke kopa u mpolelle ke emise 'me ke tla hlalosa. Haeba u na le lipotso hamorao, u ka mpotsa kapa oa botsa emong oa batho ba ngotsoeng ka tlase mona.

2. Sepheo sa morero ona oa lipatlisiso
Ho uena motho ea nang le kokoana-hloko ea HIV, ho bohlokoa ho qeta bophelo bohle ba hau u noa litlhare tsa HIV tse tsebahalang ka hore ke "anti-retroviral therapy" (ART). Ha u nka ART kamehla, kokoana-hloko e fokotseha haholo 'meleng, le 'mele oa hau u tseba ho sebetsana le kokoana-hloko 'me u ka labella ho phela bophelo bo t'soanang le ba motho ea hlokang t'soaetso. Leha ho le joalo, hona le lit'sitiso tse ngata ho fumaneng ART kapa ho tsitsa ho eona. Morero ona oa lipatlisiso o reretsoe ho lekola lit'sitiso tsena le mekhoa e fapaneng ea ho hlokomela HIV. T'sitiso e kholo ke ho eketsa li ART ka nako e tloaelehileng setsing sa bophelo. Boithuto bona bo reretsoe ho lekola mokhoa o mocha oa tlhokomelo ea HIV, o bitsoang VIBRA. Mohlala ona oa VIBRA o kenyeletsa khetho ea ho fumana SMS ea khopotso, t'sehetso ea batho ba utloisisang boemo ba ka fanang ka tlhabollo ea t'sehetso le, e bohlokoa haholo, mokhoa oa ho lata ART ho Basebeletsi ba tsa Bophelo ba Metseng (Village Health Workers – VHW) ka kotloloho. Re nahana hore, ka liteboho ho mohlala ona oa tlhokomelo ea HIV, ho ka ba bonolo hore batho ba tsitse ho ART, empa ha re tsebe. Ke ka lebaka leo re etsang lipatlisiso tsena.

3. Khetho ea batho ba nkang karolo
Re mema batho ba fumanoeng ba na le kokoana-hloko ea HIV lihlahlobong tse entsoeng malapeng ba sa nkeng ART 'me ba le lilemong tse leshome ho ea holimo bao sekaleng ba leng kaholimo ho 35kg.

4. Tsamaiso
Re eletsa hore u qale ART 'me u etele Mosebeletsi oa tsa Bophelo Metseng (VHW) ho etsa t'salo-morao, ho lata lipilisi tse ling tsa ART 'me u etele setsi sa bophelo kamora khoeli tse t'seletseng kamehla. Lipalo tsa hau tsa bokuli li tla nkuoa. Kamora likhoeli tse t'seletseng ho isa ho tse leshome le metso e 'meli, mooki oa setsing sa bophelo sa haeno o tla kha mali ho nt'setsa pele liteko tse hlokomelang kokoana-hloko. U khothaletsoa hore u batle thuso setsing sa bophelo ha u ikutloa u sa phela hantle.

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5. Ho kha mali

Motho ea noang ART- ho sa tsotellehe hore na o morerong ona oa lipatlisiso kapa chee- o hloka ho etsa liteko tsa mali ha a ntse a noa ART. Ho ea ka melao ea tataiso ea naha ea Lesotho, mali a tlameha ho khuoa pele ART e qaloa, ebe kamora likhoeli tse t'seletseng ho isa ho tse leshome le metso e 'meli. Re tla tsepama tlhophiso ena.. Mali a khuoeng a tla hoamisoa a bololokoe setsing sa polokelo sa sepetlele sa 'muso Butha-Buthe. Maling a nkuoeng pakeng tsa likhoeli tse t'seletseng le tse leshome le metso e 'meli re tla etsa liteko tse khothaletsoang motho ea noang ART ho ea ka melao ea tataiso ea naha. Ha hona mali a tla nkuoa ka thoko ho a hlokahalang bakeng sa tlhokomelo e tloaelehileng ho latela tsamaiso ea lipatlisiso; empa haeba a khuoa ho latela lipehelo tsa booki, retla fumana sephetho sa hau ele karolo ea morero oa lipatlisiso tsena. Re ikemiselitse hape ho sheba mali a hoamisitsoeng ho hlahloba na u na le kokoana-hloko e hanang litlhare 'me e li sitisa ho sebetsa. Re tla u tsebisa ka sephetho sena.

6. Nako

Morero ona o lebeletsoe ho nka khoeli tse leshome le metso e 'meli 'me mali a khuoang ka nako eno a tla fana ka sephetho sa mantlha. Kamora lemo tse peli re rerile ho hlahlobisa lipalo tse entsoeng tseleng ea t'sebetso. Haeba re rera ho batla litaba tse ling hape kamora lemo tse peli, re tla u fa foromo 'ngoe hape e fanang ka tumello.

7. Kotsi

Re hatella hore haeba u nka karolo lipatlisisong tsena u tla fumana feela ART e lumelletsoeng, e se ntse sebelisoa Lesotho, e bileng e fetisitsoeng litekong tsa ho bolokeha tse nang le motheo o hlakileng. ART tsohle li ka ba le litla-morao. U tla fumana tlhalosetso. Re batla ho hatella hore bo-VHW bohle ba u fang ART ba tla lula tlasa tataiso le tlhokomelo ea manese a koetlisitsoeng a nang le tsebo ea ART (ART nurse). Re u khothaletsa hore u ikopanye le e mong oa moifo oa lipatlisiso haeba u na le ho t'soenyeha hona joale kapa hamorao.

8. Melemo le lipuseletso

Ho nka karolo hoa hao ho tla re thusa ho fumana likarabo tsa potso ea bohlokoa ea lipatlisiso hore na re ka ntlafatsa joang tlhokomelo ea HIV Lesotho. Ha u na fumana chelete kapa limpho hore u nke karolo lipatlisisong tsena.

9. Boithaopo le tokelo ea ho ikhula

Ha u nka karolo, u lumela ho botsoa lipotso hona joale ha u ngolisoa, le ho batloa ha u lahlehile tlhokomelong le hore re ka hlahlobisa lipalo tsa hau tsa bophelo (sephetho sa liteko tsa mali) ka tsela e patileng boitsebiso ba hau (sheba tlase leqepheng). Ho nka karolo morerong ona oa lipatlisiso ho etsoa ka boithaopo bo feletseng. Ke khetho ea hau hore na u nka karolo kapa chee. Ha u nka karolo kapa u sa e nke, u ntse u tla fumana lit'sebeletso tsohle tse tloaelehileng tse fuoang batho ba nang le kokoana-hloko ea HIV Lesotho. U ka emisa ho nka karolo lipatlisisong nako eohle ha u batla, 'me ha u na lahleheloa ke litokelo tsa hau u le mokuli.

10. Lekunutu

Litaba tseo re li bokelletseng lipatlisisong li tla bolokoa ele lekunutu. Litaba tsohle tseo re li bokeletseng ka uena li tla bolokoa ka thata 'me haho motho ea tla li fumana ntle le batho ba etsang lipatlisiso. Mali a polokelong le litaba tsohle tse amanang le uena li tla fuoa nomoro e sa tsebahaleng sebakeng sa lebitso la hau. Batho ba etsang lipatlisiso ke bona feela ba tla tseba nomoro ea hau 'me ba tla sireletsa litaba tseo kaofela. Basebeletsi ba bophelo ba amehang ba tla tseba litaba tsohle tsa bohlokoa tsa bophelo ba hau ele ho beha leihlo ka thata ho okoeng ha hau. Sephetho se tla hlaha polokelong ea lipalo tsa kokoana-hloko e maling, moo lekala la bophelo le tla ba le tumello ea ho sheba, li tla hlahisoa ka boitsebiso ba linomoro tse sa tsebahaleng. Re batla ho arolelana tsebo ea lipatlisiso tsena le sechaba, ele hore batho ba nang le thahasello batle ba ithute ho tsona. Le boemong boo, litaba tsa lekunutu (joaloka lebitso la hau) ha li na fuoa sechaba.

11. Batho bao u ka ikopanyang le bona

Haeba u na le lipotso, u ka botsa hona joale kapa hamorao, le kamorao hore morero ona oa lipatlisiso o qale. Haeba u lakatsa ho botsa lipotso hamorao, u ka ikopanya le batho ba latelang ba moifo oa lipatlisiso ka nako eohle:

- **Thabo Lejone, SolidarMed Butha-Buthe, 62000584 (Research Manager & Community ART Nurse)**
- **Lefu Khesa, SolidarMed Mokhotlong, 56425205/63166525 (Community ART Nurse)**

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KAROLO EA BOBELI: Lengolo la Tumello

1. Ea batho ba tsebang ho ngola ba lilemo li 18 ho ea holimo

Botona/Botsehali ba motho ea nkang karolo: ☐ mosali ☐ monna

Ngola lebitso la motho ea nkang karolo:_____

Tekeno ea motho ea nkang karolo:_____

Letsatsi: _____ (letsatsi / khoeli / selemo)

Ke balile litaba tse ngotsoeng mona, kapa ke li balletsoe. Ke bile le monyetla oa ho botsa lipotso 'me lipotso tsaka li arabuoe ka tsela e nkhotsofatsang. Ke lumela ka boithaopo ho nka karolo lipatlisisong tsena.

2. Ea batho ba sa tsebeng ho bala le ho ngola kapa bana ba lilemo li ka tlase ho 18

- *Bakeng sa batho ba sa tsebeng ho bala le ho ngola*: Paki e lilemo li 21 ho ea holimo e tsebang ho bala le ho ngola (ha ho khoneha motho enoa a khethoe ke motho ea nkang karolo 'me a hloke kamano le moifo oa lipatlisiso). Motho ea nkang karolo ea sa tsebeng ho bala le ho ngola a fane ka tumello ka ho bua le ho nomora hoa monoana oa hae o motona.
- *Bana ba lilemo li ka tlase ho ho 18*: Mohlokomeli ea tsebang ho bala le ho ngola (motho ea hlokomelang ngoana ena a le lilemo tse 21 kapa ho feta) o tlameha ho fana ka tumello e ngotsoeng. Ngoana ea nkang karolo u tlameha ho fana ka tumello ka ho bua, le tumello e ngotsoeng kapa ho nomora hoa monoana o motona.

motho ea nkang karolo:

Sex: ☐ mosali ☐ monna

Ngola lebitso la motho ea nkang karolo:_____

Monoana o motona: ☐

Ke balile litaba tse boletsoeng mona kapa ke li baletsoe. Ke fumane monyetla oa ho botsa lipotso 'me lipotso tsaka li arabetsoe ka tsela e nkhotsofatsang. Ke lumela ke sa qobelloa ho nka karolo lipatlisisong tsena.

paki/mohlokomeli:

Ngola lebitso la paki/mohlokomeli: _____

Tekeno ea paki/mohlokomeli:_____

Letsatsi: _____ (letsatsi / khoeli / selemo)

Ke bone hore motho ea nkang karolo o baletsoe foromo e fanang ka tumello,o fumane monyetla oa ho botsa lipotso. O fane ka tumello a sa qobelloa.

Mofuputsi ea amohelang tumello

Ngola lebitso la mofuputsi/motho ea amohelang tumello:_____

Tekeno ea mofuputsi/motho ea amohelang tumello: _____

Letsatsi: _____ (letsatsi/khoeli/selemo)

Ke balile ka hloko leqephe la litaba ho motho ea ka nkang karolo, 'me ke lekile ka hohle ho netefatsa hore oa utloisisa boithuto bona le tsohle tse amanang le bona. Ke tiisa hore motho ea nkang karolo o fumane monyetla oa ho botsa lipotso ka boithuto bona, 'me lipotso tsohle tseo a li botsitseng li arabetsoe ka nepo ka hohle ka moo nka khonang. Ke tiisa hore motho enoa ha a qobelloa ho fanoa ka tumello, le hore tumello e fanoe ka tokoloho le boithaopo. Ke tiisa hore moo motho ea nkang karolo a leng lilemo tse ka tlase ho 18, o fane ka tumello ka ho bua le hore o fumane kopi ea foromo e fanang ka tumello e nang le tsebo.

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E. Proof of submission of the article to PLOS One

Gmail **Tsepang Mohloanyane <tsepimohloa@gmail.com>**

Submission Confirmation for PONE-D-22-02237 - [EMID:16c2b9430e8d2793]

1 message

PLOS ONE <em@editorialmanager.com> Sun, Jan 23, 2022 at 11:18 PM
Reply-To: PLOS ONE <plosone@plos.org>
To: Tsepang Mohloanyane <tsepimohloa@gmail.com>

PONE-D-22-02237
The recency of newly diagnosed HIV infections among the rural general population in Lesotho: Secondary data from the VIBRA cluster-randomized trial
PLOS ONE

Dear Dr. Mohloanyane,

Thank you for submitting your manuscript entitled 'The recency of newly diagnosed HIV infections among the rural general population in Lesotho: Secondary data from the VIBRA cluster-randomized trial' to PLOS ONE. Your assigned manuscript number is PONE-D-22-02237.

We will now begin processing your manuscript and may contact you if we require any further information. You will receive an update once your manuscript passes our in-house technical check; you can also check the status of your manuscript by logging into your account at https://www.editorialmanager.com/pone/.

If during submission you selected the option for your manuscript to be posted on the bioRxiv preprint server (http://biorxiv.org), we will be assessing the manuscript for suitability shortly. If suitable, your preprint will be made publicly available on bioRxiv and you will receive an email confirmation from them when it has posted. Please check your response to this question and email us as soon as possible at plosone@plos.org if it has been answered incorrectly. Further information about our partnership with bioRxiv to facilitate the rapid availability of life sciences research is available at http://journals.plos.org/plosone/s/preprints.

If you have any inquiries or other comments regarding this manuscript please contact plosone@plos.org.

Thank you for your support of PLOS ONE.

Kind regards,
PLOS ONE

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