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ABBREVIATIONS AND SYMBOLS

Abbreviations:

3TC – lamivudine

Ab – antibody

Ag – antigen

AHI – acute HIV infection

AIDS – acquired immunodeficiency syndrome

ANC – antenatal care

ARV – antiretroviral

ART – antiretroviral therapy / treatment

APOBEC3G – apolipoprotein B mRNA editing enzyme catalytic subunit 3G

ATV/r – ritonavir-boosted atazanavir

AZT – zidovudine

CA – capsid

CAP/CTM – Cobas Ampliprep/Cobas TaqMan

CCR5 – C-C chemokine receptor type 5

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

cDNA – complementary DNA

CI – confidence interval

CIA – chemiluminescent immunoassay

CMV – cytomegalovirus

Cpx – complex

Cpz – chimpanzee

CRF – circulating recombinant form

CXCR4 – C-X-C chemokine receptor type 4

DNA – deoxyribonucleic acid

dNTP – deoxynucleotide triphosphate

EFV – efavirenz

EIA – enzyme immunoassay

ELISA – enzyme linked immunosorbent assay

eMTCT – elimination of mother-to-child transmission of HIV

Env – envelope

FDA – Food and Drug Administration

FIDSSA – Federation of Infectious Diseases Societies of Southern Africa

FTC – emtricitabine

Gag – group-specific antigen

Gor – gorilla

Gp – glycoprotein

HAART – highly active antiretroviral therapy

HCT – HIV counseling and testing

HIV – human immunodeficiency virus

HXB2 – subtype B reference strain of HIV

INSTI – integrase strand transfer inhibitor

IQR – interquartile range

Kb – kilobase

LAg – limiting antigen avidity

LIS – laboratory information system

LPV/r – ritonavir-boosted lopinavir

LTR – long terminal repeat

MA – matrix

MAFFT – Multiple Alignment using Fast Fourier Transform

MDGs – millennium development goals

MgSO4 – magnesium sulphate

Ml – milliliter

mM – millimolar

MTCT – mother-to-child transmission of HIV

MW – molecular weight

mRNA – messenger ribonucleic acid

NAAT – nucleic acid amplification test

NC – nucleocapsid

NCBI – National Center for Biotechnology Information

Nef – negative regulatory factor

NHLS – National Health Laboratory Service

NRTI – nucleoside reverse transcriptase inhibitor

NNRTI – non-nucleoside reverse transcriptase inhibitor

NPV – negative predictive value

NVP – nevirapine

OD-n – normalized optical density

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PEP – post-exposure prophylaxis

PI – protease inhibitor

PMTCT – prevention of mother-to-child transmission of HIV

Pk – pharmacokinetic

POC – point-of-care

Pol – polymerase

PrEP – pre-exposure prophylaxis

R5 viruses – viruses that use CCR-5 co-receptors

R5X4 tropic strains – dual tropic strains of HIV-1 that can use either CCR5 or CXCR4 co-receptors

R – rand

RCF – relative centrifugal force

Rev – regulator of expression of virion proteins

RNA – ribonucleic acid

RT – reverse transcriptase

RT-PCR – reverse transcription polymerase chain reaction

SA – South African

SDGs – sustainable development goals

SDRM – surveillance of drug resistance mutations

SIV – Simian immunodeficiency virus

STI – sexually transmitted infection

TAD – Tshwane Academic Division

Tat – trans-activator of transcription

TDF – tenofovir disoproxil furamate

TMREC – Tshwane Municipality Research Ethics Committee

U – unit

UNAIDS - United Nations Programme on HIV/AIDS

URF – unique recombinant form

US – United States

USD – United States dollar

Vif – viral infectivity factor

VL – viral load

Vpr – viral protein R

Vpu – viral protein U

W. Blot – Western Blot

WHO – World Health Organisation

X4 viruses – viruses that use the CXCR4 co-receptors

Symbols:

~ – approximately

< – less than or below

> – greater than or above

≥ – greater than or equal to

≤ – less than or equal to

µl – microlitre

% – percentage

ºC – degrees celcius

– number

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CHAPTER 1

Introduction and study aims

1.1 HIV prevalence

Human immunodeficiency virus (HIV) is a major global health problem that has disproportionately affected sub-Saharan Africa. This region houses about two thirds (~64%) of the world's burden of HIV infections (Fig. 1.1) [1, 2]. By the end of 2016, there were approximately 36.7 million people living with HIV globally. There has been a gradual decline observed with new HIV infections and acquired immunodeficiency syndrome (AIDS)-related mortality owing to scale up of antiretroviral (ARV) treatment. However, both these indices still occur at high rate as there were 1.8 million new HIV infections and 1 million AIDS-related deaths in 2016 [2].

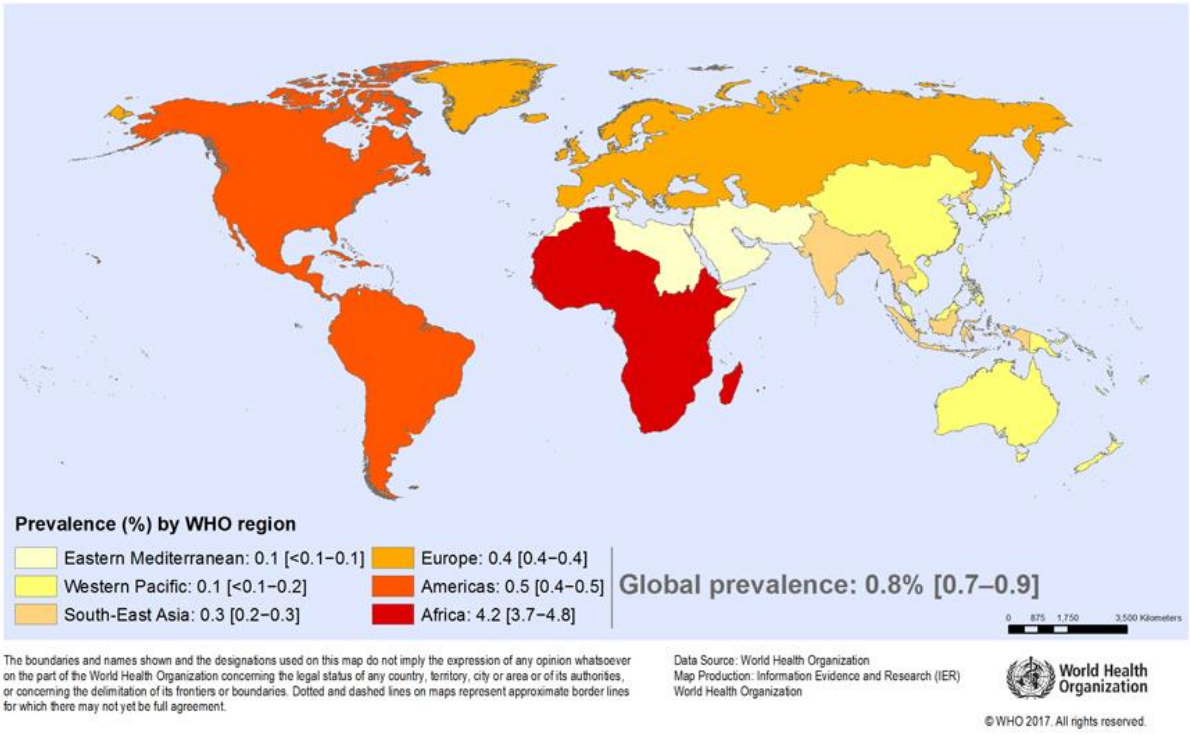

Figure 1.1 Global prevalence of HIV among people aged 15 – 49 years in 2016. Colour coding is used to highlight HIV prevalence in different regions of the world (Open source access) [1].

South Africa has the highest burden of HIV infections in the world [2, 3] with about 7.9 million people living with HIV [4]. Thus, SA contributes approximately 20% of HIV infections to the global HIV pandemic [2] despite having about 1% of the world's population [5]. Halting and reversing the spread of HIV was part of the millennium development goals (MDGs), goal 6A [6]. Although progress has been made in achieving this MDG goal, there were still too many new HIV infections at the end of MDG target period, particularly in high HIV endemic regions [7].

The prevalence of HIV among South Africans aged 15 – 49 years is 20.6% and this varies among SA's provinces, with the highest prevalence (27.0%) found in KwaZulu-Natal province and the lowest prevalence (12.6%) found in Western Cape province (Fig. 1.2) [4]. However, HIV prevalence in SA can be as high as 44.4% in some population groups such as pregnant women (Fig. 1.3) [8].

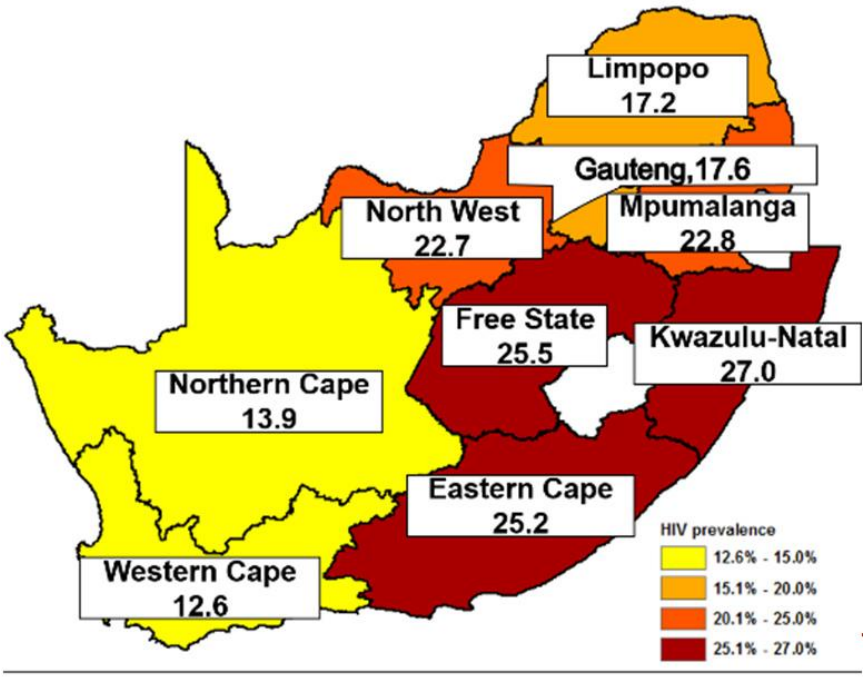

Figure 1.2 HIV prevalence among adults aged 15 – 49 years in South African provinces. Reproduced with permission from ref. # [4].

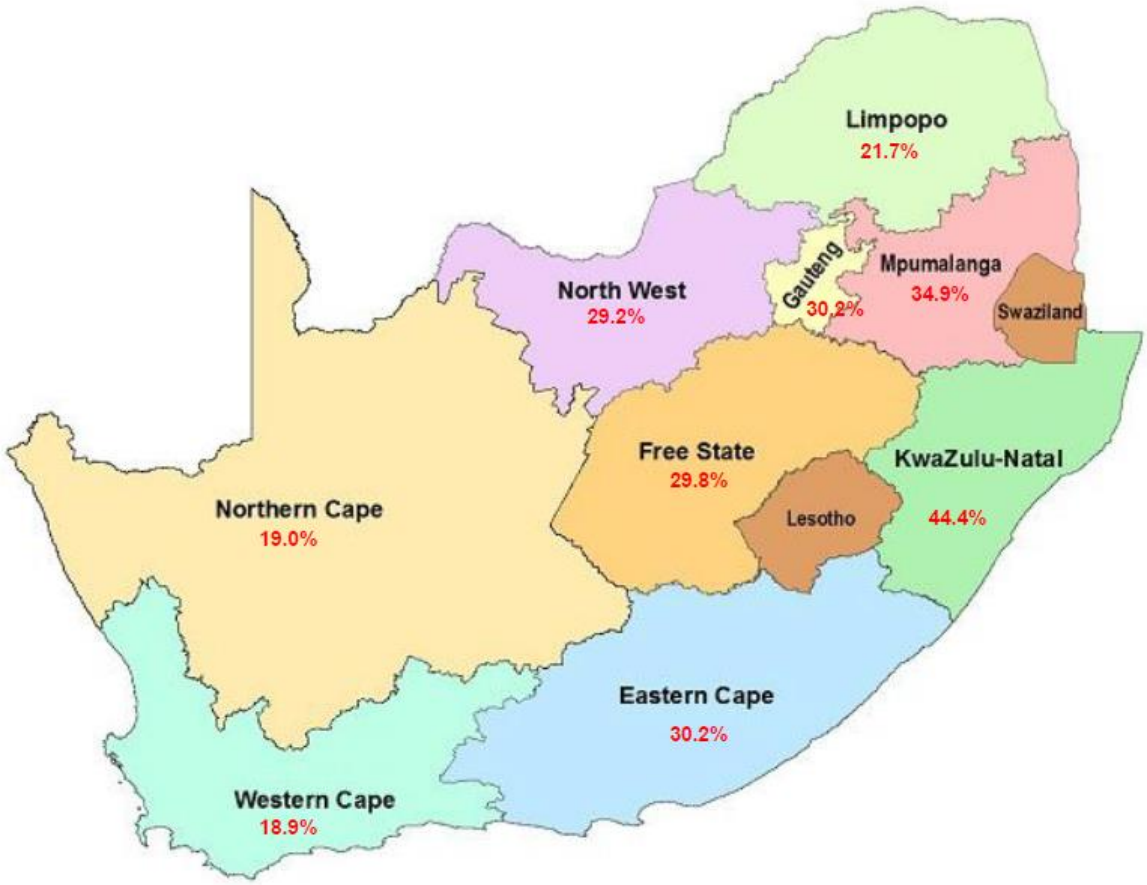

Figure 1.3 HIV prevalence among pregnant women in South African provinces. Lesotho and Swaziland are SA's neighbouring countries. Constructed using data from the last SA national antenatal HIV sentinel survey, which was conducted in 2015 [8].

South African HIV incidence among people aged 15 – 49 years slightly dropped from 2.2% in 2002 –2005 period to 1.9% in both 2005 – 2008 and 2008 – 2012 periods [9]. This maintenance of incidence at 1.9% for an 8 year period highlights an ongoing transmission of HIV, which is driven by highly infectious individuals. The recent survey conducted in 2017 has, however, shown a decline in HIV incidence (0.79%) among people aged 15 – 49 year [4].

1.2 Transmission modes of HIV

HIV is transmitted via the following routes; sexual contact, blood or blood products and vertical transmission (mother to child transmission). Sexual transmission accounts for the majority (~85%) of HIV-1 infections worldwide [10, 11]. The presence of high HIV viral load (VL) of at least 1500 copies per mililitre (ml) in an infected individual is associated with a risk of sexual transmission to uninfected individuals [12]. Unprotected or condomless sex is the major determinant for sexual acquisition or transmission of HIV [13, 14]. Therefore, human behaviour that leads to unprotected sex plays a huge role in HIV transmission.

No use or inconsistent use of condoms, multiple sexual partners, commercial sex work and drug abuse are among the behavioural risk factors that have been identified as contributing to the spread of HIV [15]. The high prevalence of behavioural risk factors coupled with slow rollout or uptake of HIV interventions such as HIV testing and ARV treatment have been implicated as contributory factors to the South African HIV epidemic [9]. The presence of other sexually transmitted infections increases the risk of HIV acquisition as these infections increase the target cells of HIV (CD4+ cells) around the genital mucosa [16]. Male circumcision status also plays a role in HIV acquisition as uncircumcised men are at a much higher risk of HIV infection [14, 17].

Women are at a higher risk of contracting HIV from unprotected sex owing to behavioural, socio-economic and biologic risk factors [18]. HIV infection among women of child-bearing age results in a high risk of transmission to their babies. In the absence of intervention, transmission to the baby is relatively lower in-utero but higher during the intrapartum stage of pregnancy and breastfeeding [19]. However, the prevention of mother to child transmission (PMTCT) programmes through treatment of HIV-infected mothers and post-exposure prophylaxis to their babies, have led to reduction of vertical transmission of HIV [19, 20].

Although blood and its products result in high risk exposure to HIV, they no longer play a significant role in the transmission of HIV nowadays owing to screening of blood with sensitive HIV diagnostic assays in blood centres [21].

1.3 HIV structure and taxonomy

HIV is an enveloped, single stranded ribonucleic acid (RNA) virus, which belongs to the *Lentivirus* genus in the *Retroviridae* family [22]. It has a spherical shape with a diameter of approximately 100 - 120 nanometers (nm), and contains two copies of RNA strands in each virion. The envelope is supported by a matrix layer, and the capsid forms the core of the virus, which encapsidates the RNA genome and replication enzymes (Fig. 1.4) [23].

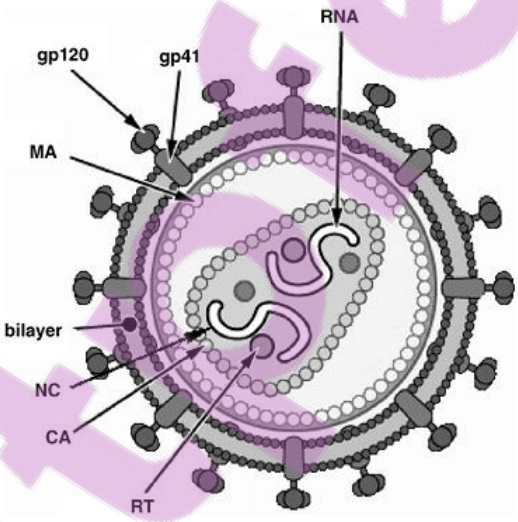

Figure 1.4 The structure of a mature HIV virion. The envelope is a lipid bilayer obtained from the cell during budding. HIV glycoprotein (gp) 41 is embedded in the envelope and gp120 protrudes through the surface of the envelope. Beneath the envelope is the matrix (MA) layer, which supports the envelope and the capsid (CA). The capsid contains HIV genome and enzymes. Nucleocapsid (NC) proteins are tightly associated with the RNA strands. Reproduced with permission from ref. # [23].

1.4 HIV genome and diversity

The genome length of HIV is ~9.8 kilobases (kb) [24]. HIV genome is flanked by long terminal repeat (LTR) regions, which sandwich the major HIV genes; group-specific antigen (gag), polymerase (pol) and envelope (env) genes. The gag and env genes code for structural proteins, while the pol gene codes for non-structural proteins (enzymes). The three HIV enzymes encoded by pol gene are reverse transcriptase, protease and integrase. The HIV-1 genome also encodes for six accessory genes (vif, vpu, vpr, tat, rev and nef), which are not required for replication *in vitro* (Fig. 1.5) [25].

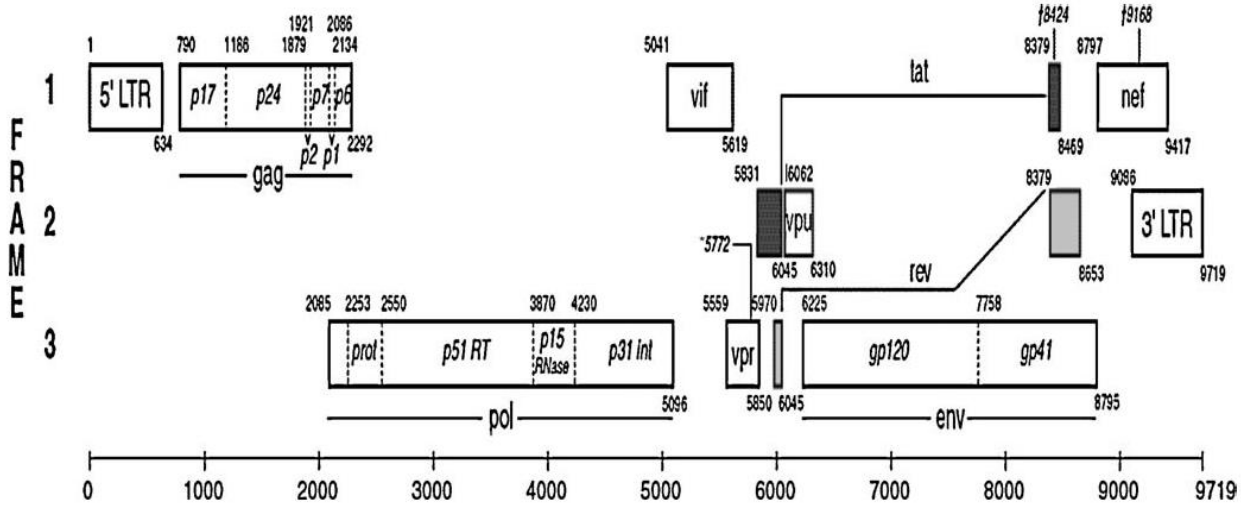

Figure 1.5 The organization of HIV genome according to the HXB-2 reference strain. Long terminal repeats (LTR) are positioned at the 5' and 3' prime ends of the genome, flanking the major genes (gag, pol and env) and the non-essential genes (tat, vif, vpu, vpr, nef and rev) of HIV. The products of each major gene are shown in the blocks that are divided by broken lines. The genome is numbered from 5'-3' direction to show the beginning and the end of each gene and its products. Gag = group-specific antigen, pol = polymerase, env = envelop, PR = protease, RT = reverse transcriptase, gp = glycoprotein, vif = viral infectivity factor, vpr = viral protein R, vpu = viral protein U, tat = trans-activator of transcription, rev = regulator of expression of virion proteins, nef = negative regulatory factor. Reproduced with permission from ref. # [25].

HIV is classified into two types, HIV-1 and HIV-2. The origins of HIV have been tracked to the simian immunodeficiency virus (SIV), which infects non-human primates. HIV-1 originated from SIV that infects chimpanzees (SIVcpz) and gorillas (SIVgor), while HIV-2 originated from SIV that infects sooty mangabey monkeys (SIVsm). HIV-1 accounts for the majority (>80%) of global HIV infections and is further classified into four groups based on genetic diversity: M (main group), O (outliers), N (non-M, non-O) and group P.

Group M is responsible for the majority of HIV infections worldwide, and is further divided into 9 subtypes: A – D, F – H, and J – K. The inter-subtype amino acid variation is 25 - 30% in env gene, 20% in Gag gene, and 10% in POL gene. Group M also contains circulating recombinant forms (CRFs) such as A/E or A/G, and unique recombinant forms (URFs) (Fig. 1.6) [26]. Subtype C is responsible for about 50% of the global HIV infections, and is the predominant subtype in the southern African region [27]. Groups O, N and P HIV infections are commonly found in Cameroon, and some group O infections are also found in the neighbouring central African countries [26]. HIV-2 infections are more confined to the West African region but have been reported in other areas of the world owing to migration [28].

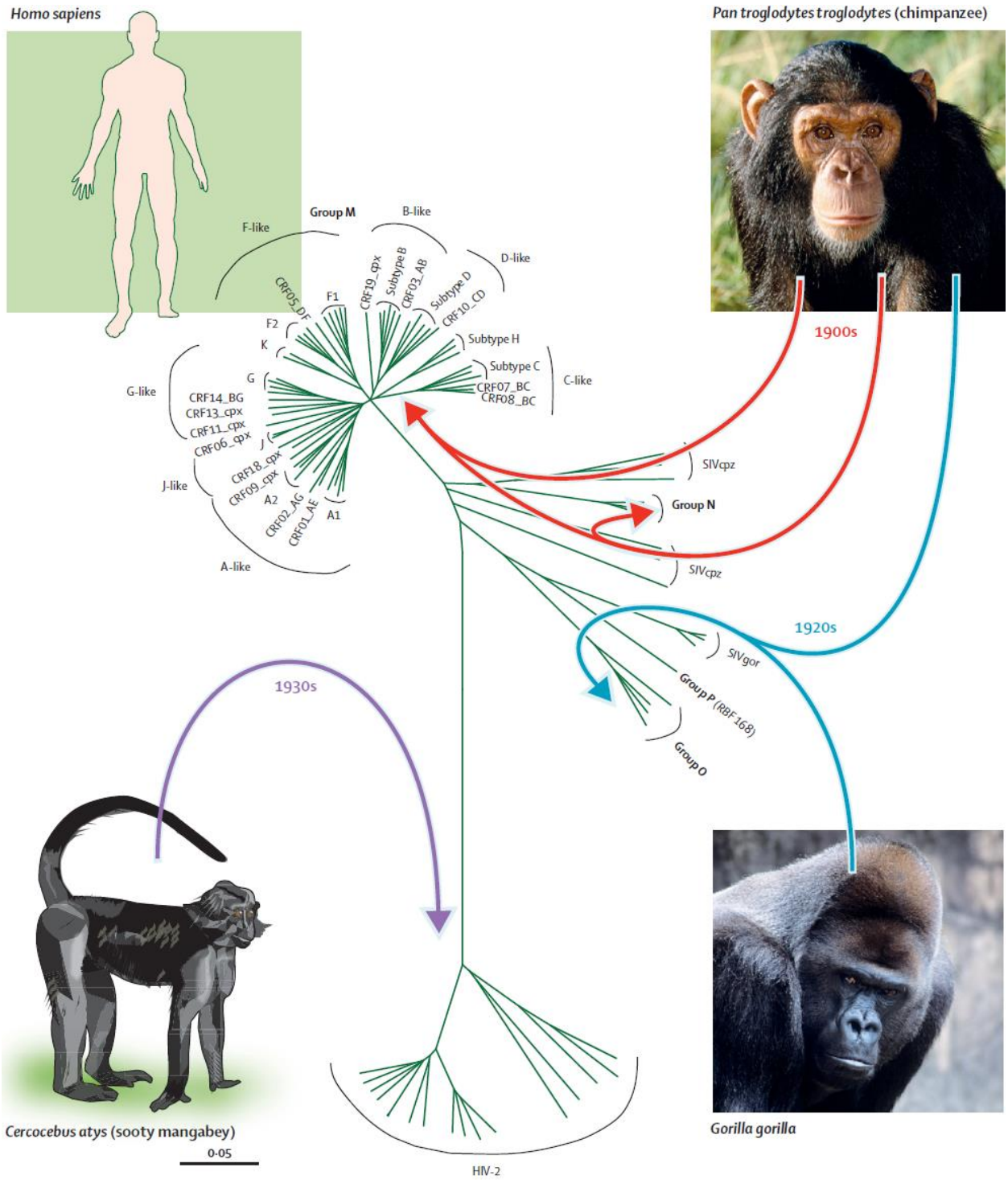

Figure 1.6 Phylogenetic tree showing the origins and classification of HIV, and the estimated times that each type or group emerged in humans. HIV-1 originated from simian immunodeficiency virus (SIV) that infects chimpanzees and gorillas, while HIV-2 originated from the SIV that infects sooty mangabey monkeys. The four groups of HIV-1 (M, N, O and P) are shown as well as group M subtypes and their related circulating recombinant forms (CRFs). Cpz - chimpanzee, gor - gorilla, cpx - complex, SIV - simian immunodeficiency virus. Reproduced with permission from ref. # [26].

1.5 HIV replication

HIV-1 replicates in cells that express cluster of differentiation 4 receptors (CD4+ cells); these are mainly CD4+ T lymphocytes, macrophages and dendritic cells [11]. The virus uses its envelop glycoprotein (gp 120) to initially attach to a CD4 receptor and then to a co-receptor. The second attachment to a co-receptor is important for cell entry. The co-receptors used by HIV are C-C chemokine receptor type 5 (CCR-5) and C-X-C chemokine receptor type 4 (CXCR4) [23, 29]. Following attachment, gp41 undergoes structural conformational changes, which allow the fusion between viral envelop and cellular membrane. This leads to the release of the capsid into the cytoplasm, followed by uncoating and release of the HIV RNA. In the cyctoplasm, reverse transcriptase enzyme facilitates reverse transcription of HIV RNA into complementary DNA (proviral DNA). The proviral DNA is then integrated into the host chromosomes in the nucleus through the action of an integrase enzyme. Transcription of the proviral DNA is performed by cellular DNA polymerases; followed by translation of the different mRNA species into various HIV proteins. Assembly occurs beneath the cellular membrane, followed by budding of immature virions. Cleavage of the gag and gag-pol polyproteins by the protease enzyme leads to maturation of the virions; this makes virions ready to infect new cells [23].

Infection across the genital mucosa is mostly established by the viruses that use CCR-5 co-receptors (R5 viruses), thus these viruses predominate in the early HIV phase. During the course of HIV infection, viral mutants that use the CXCR4 co-receptors (X4 viruses) emerge [11, 23]. Interestingly, the tropism of the R5 and X4 viruses differ; R5 viruses preferentially infect macrophages (M-tropic) while X4 viruses infect T lymphocytes (T-tropic). The X4 viruses are known to be highly virulent as they are often associated with a rapid decrease of CD4 T lymphocytes, leading to a faster progression to AIDS. Dual tropic strains of HIV-1

(R5X4-tropic strains) that can use either CCR5 or CXCR4 co-receptors have been found in some patients [30, 31].

During HIV replication, the reverse transcriptase enzyme makes a lot of nucleotide substitution errors owing to lack of proof reading activity; this results in high diversity of HIV (Fig. 1.6). Other factors that contribute to high HIV diversity include short replication times, relatively shorter genome, and high recombination events [24, 26]. The ability of HIV to establish latency in non-dividing cells very early after infection together with its high diversity have been proven to be the major challenges against curing HIV with the current ARV drugs and developing an effective vaccine [24, 32].

1.6 Natural course of HIV infection

HIV-1 infection starts a process that leads to progressive destruction of CD4 T lymphocytes, which are its main target cells. A typical course of HIV-1 infection in infected individuals is characterised by the following three phases: early (primary) infection, chronic (latent) HIV stage, and the AIDS phase (Fig. 1.7) [33]. On average, it takes about 6 – 10 years for infected individuals to progress to the AIDS stage. However, there is a wide variation of the HIV-1 natural course amongst infected individuals. For instance, some individuals take longer than 10 years to progress to the AIDS stage such as elite controllers and long term non-progressors. There is also a group of infected patients that progresses faster to AIDS stage (within 5 years after infection), known as faster progressors [11, 33, 34].

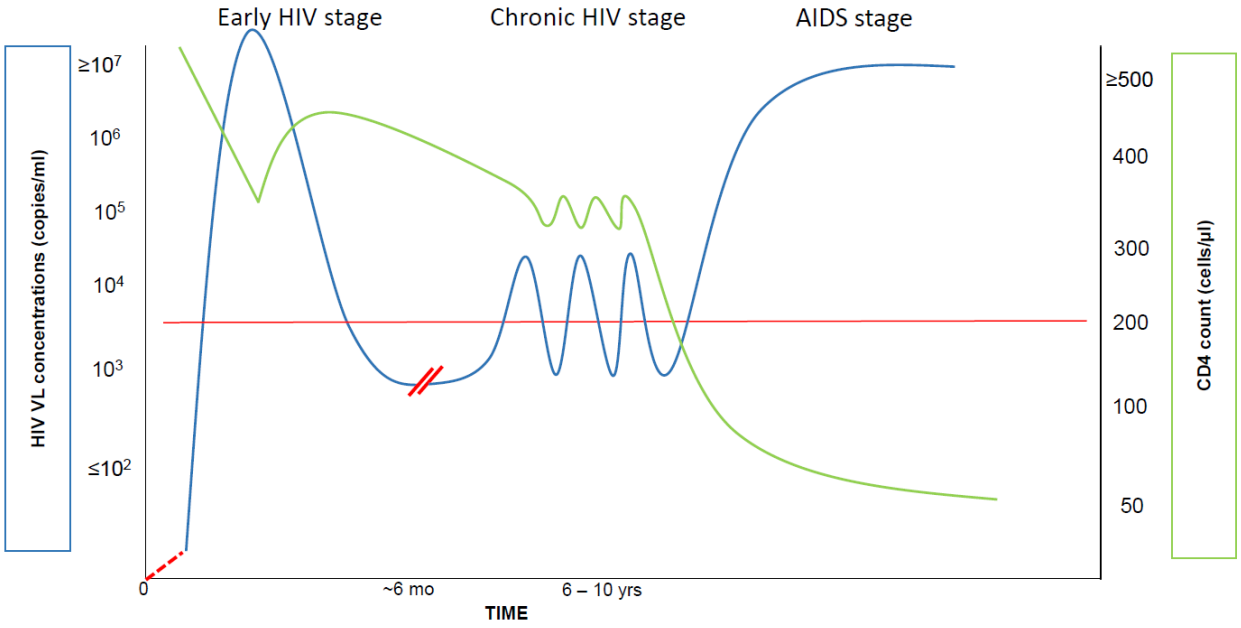

Figure 1.7 Natural history of HIV infection, with all its different stages: early stage, chronic stage and AIDS stage. The dotted red lines mark the period preceding HIV viraemia (blue line). The double red lines represent the lowest point (viral load set-point) to which HIV viral load is controlled during early HIV stage, which marks the end of early HIV stage. Viral load and CD4 count fluctuations occur during the chronic HIV stage owing to continuous HIV replication. The average times for early and chronic HIV stages are reflected. Green line represents CD4 count. Red horizontal line represents CD4 count level of ≤200 cells / µl, which is used for defining the AIDS stage, where a lot of opportunistic diseases occur. Mo – months, yrs – years, ml – millilitres, ul – microliters [33].

Acute HIV infection (AHI) refers to the time of virus acquisition until the appearance of HIV antibodies (seroconversion). The formation of HIV-1 antibodies, usually accomplished at 3 – 12 weeks, marks the completion of seroconversion; however, in some people seroconversion may be longer than this period. The characteristic feature of the AHI is the very high levels of virus concentrations in plasma, often exceeding 10^7 copies/ml. Early (primary) HIV infection, which includes AHI, is regarded as the interval between virus acquisition and the establishment of HIV VL set-point, a lowest point to which VL is suppressed during early HIV infection [35, 36]. The development of HIV-specific cytotoxic T lymphocyte responses remarkably reduces HIV vireamia, and this leads to recovery of CD4 T lymphocytes during the early HIV infection

[34]. It takes about 6 – 12 months for a VL set-point to be established, and this marks the end of early HIV infection phase. The significance of the VL set-point is that it is prognostic for disease progression, as individuals with higher VL set-point are known to progress faster to AIDS compared to those with lower VL set-point [11, 37]. Infected individuals may be asymptomatic or present with non-specific clinical signs and symptoms (often flu-like illness) during the early HIV stage, which makes the clinical suspicion of HIV infection difficult at this stage [37, 38]. Many studies have evaluated a combination of signs and symptoms that can be used to predict early HIV infection, but have yielded inconsistent results [39-43]; this further highlights the complexity of identifying patients in this phase of infection.

The chronic phase of HIV infection begins after establishment of the VL set point, and plasma VL remains relatively lower during this phase. The chronic phase is characterised by a gradual depletion of CD4 T lymphocytes, reflecting the continuous HIV replication in this phase, which finally leads to severe immunosuppression or AIDS stage [33]. AIDS is the terminal stage of HIV infection, which is characterised by an increase in plasma HIV VL and a severe depletion of CD4 T lymphocytes to <200 cells/µl. Most patients present with opportunistic diseases during the AIDS stage owing to severe immunosuppression. Different diseases occur at different levels of immunosuppression. For instance, infection with mycobacterial tuberculosis can occur at any level of immunosuppression, while CMV disease invariably occurs at advanced immunosuppression [34].

1.7 Diagnosis of HIV infection

HIV diagnostic modalities that are routinely used in the diagnostic laboratories mainly include serological tests and nucleic acid amplification tests (NAATs). Serological tests detect the presence of HIV-specific antibodies or antigens, while NAATs detect HIV RNA and/or DNA

in the blood. The commonly used laboratory-based serological assays are enzyme-linked immunosorbent assays (ELISAs) and Western Blot assays. Rapid HIV tests are serological assays that are available for use at the point-of-care (POC) facilities [44-46]. Serological tests are mainly used to diagnose HIV infection in adults, and qualitative NAATs are used to establish HIV infection in children below 18 months of age owing to interference of maternal antibodies with serology tests in this age group [46]. The quantitative NAATs (VL assays) are used for monitoring response to ARV treatment. In some instances, HIV VL assays are employed for diagnostic purposes, particularly during early HIV infection before antibodies are detectable by serological assays [37].

Early HIV infection is classified the into six different stages based on the presence of diagnostic marker(s) in blood [47]. NAATs have markedly reduced the HIV window period as HIV RNA is the first diagnostic marker to appear in blood between 1-2 weeks after HIV infection. The fourth generation HIV enzyme linked immunosorbent assays (ELISAs), which simultaneously detect p24 antigen and HIV antibodies, become positive at approximately 2 weeks after HIV infection; and the third generation HIV ELISAs (antibody-based assay) only become positive from about 3 weeks onwards (Fig. 1.8) [44, 48]. Rapid HIV tests become positive late in the course of early HIV infection as many of them detect antibodies only. Therefore, these tests misdiagnose individuals with early HIV infection.

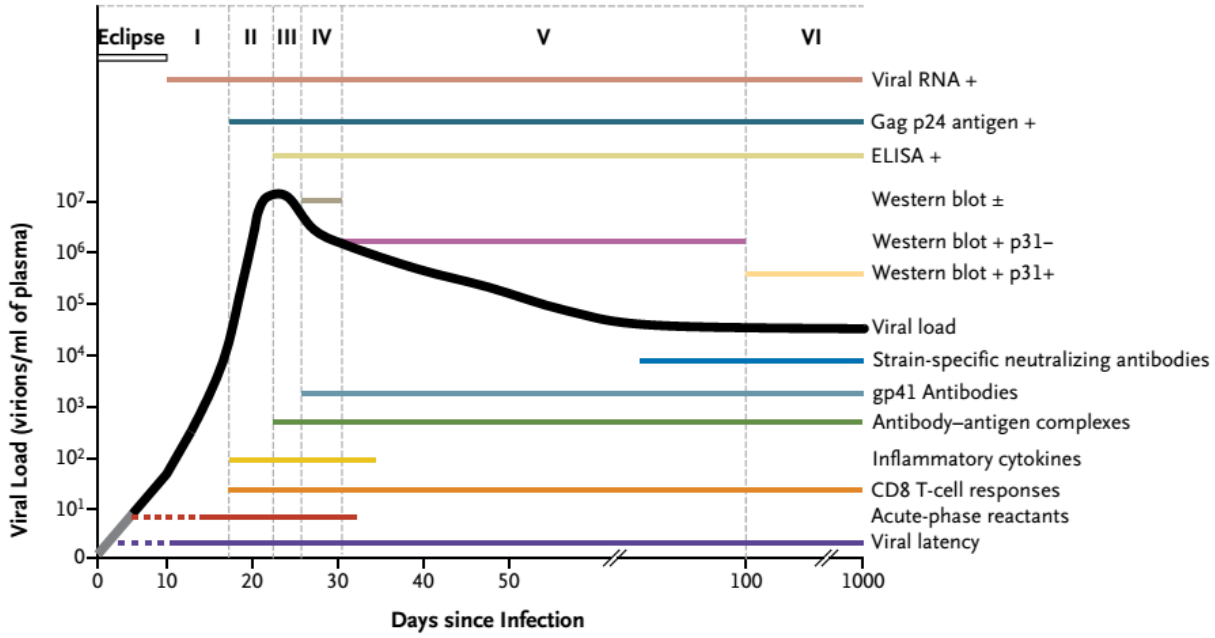

Figure 1.8 Timing of the appearance of HIV diagnostic markers during early HIV infection. Eclipse stage - no HIV marker is detectable at this stage; Stage I – only HIV RNA is detectable; Stage II – HIV RNA and p24 antigen are positive; Stage III – antigen-antibody complexes are detected and HIV ELISAs become positive; Stage IV – inconclusive or negative Western Blot, and free gp41 antibodies are detected during this stage; Stages V and VI – all the HIV markers are positive except that p31 antibodies may still be negative on Western Blot in stage V. Other markers (not used for routine diagnosis of HIV) and immune responses are reflected on this diagram as they appear during the natural course of HIV. Reproduced with permission from ref. # [48].

When Food and Drug Administration (FDA)-approved HIV tests were compared to a positive Western Blot assay, lateral flow rapid tests (the commonly used rapid tests) were found to detect HIV only about 25 days or more after a positive RNA-based test (Fig. 1.9) [45]. The addition of p24 antigen to some of the rapid HIV strips has not increased their sensitivity as the p24 component of these strips has poor sensitivity [49, 50]. The initial HIV antibody responses are directed against the glycoprotein 41 (gp 41), hence HIV serology assays target these antibodies either in isolation or in addition to other HIV antibodies [48, 51, 52].

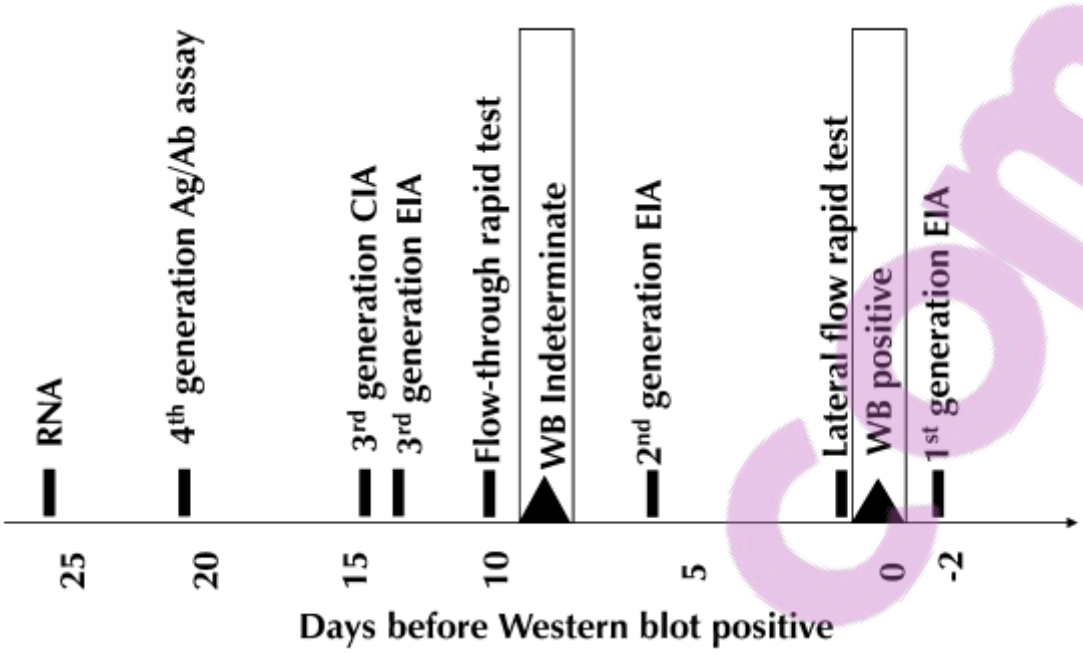

Figure 1.9 Comparison of FDA-approved HIV-1 assays with a positive Western Blot assay. Lateral flow rapid tests that only detect HIV antibodies become positive about 25 days or more after a positive RNA-based test. RNA – ribonucleic acid, Ag/Ab – antigen/antibody, CIA – Chemiluminescent immunoassay, EIA – enzyme immunoassay. Reproduced with permission from ref. # [45].

Understanding the timing of events in the natural history of early HIV infection has played an important role in determining the diagnostic strategies employed for identifying infected individuals during this phase [37]. Direct viral markers such as HIV RNA and p24 antigen play an important role in diagnosing acute HIV infection as HIV antibodies are not yet formed at this stage. Negative or weakly positive enzyme immunoassay (EIA) results, or evolving Western Blot results are often noticed during the course of early HIV infection (Figs. 8 and 9). Automated HIV VL assays have been evaluated and used for diagnosis of early HIV infection owing to their high sensitivity to detect HIV RNA. Some experts use a VL cut-off of >5000 copies/ml for interpretation of test positivity as low plasma level of HIV-1 RNA could be caused by contamination [37]. The other method that is employed for detection of early or recent HIV infections is HIV antibody avidity testing, which is often used in cross-sectional

studies. One of the avidity tests that are commonly used nowadays is limiting antigen (LAg) avidity test. Unfortunately, the currently available avidity tests suffer from a limitation of misclassifying chronic HIV infections as recent [53, 54]. Studies that evaluated the use of avidity tests in multi-assay algorithms (MAAs) that include HIV VL or CD4 count, have found that the MAAs perform better than individual avidity tests for detection of recent HIV infections and minimize misclassification of chronic HIV infections [55, 56].

Currently, SA is using the serial testing strategy for HIV testing in HIV counselling and testing (HCT) clinics, whereby testing is performed on a screening rapid HIV test and results are confirmed on a different rapid test if positive. No confirmatory testing is done if the screening rapid test is negative. If there are discordant results between screening and confirmatory rapid tests, further testing is performed on HIV ELISA. The following rapid HIV tests, Advanced Quality and First Response, have been approved for use in the Gauteng region [57, 58]. Advanced Quality is currently used as a screening test and First Response as a confirmatory test. HIV management guidelines used in most developing countries do not have optimal provisions for the diagnosis of early HIV infection [46, 58-60], mainly due to the high cost of detecting these infections. In the era of test and treat, where individuals who test positive for HIV are initiated immediately on treatment regardless of CD4 count level, detecting individuals with early HIV infection could be of great benefit.

1.8 Treatment of HIV infection

Understanding of the HIV replication cycle has led to identification of treatment sites as the available ARV drug classes target all the three HIV enzymes and the cell entry steps, CCR-5 co-receptors and fusion [61]. There are currently six classes of ARV drugs that are approved for treatment of HIV infection; nucleoside reverse transcriptase inhibitors (NRTI), non-

nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), integrase strand transfer inhibitors (INSTI), CCR-5 co-receptors inhibitors, and fusion inhibitors. There are also pharmacokinetic (PK) boosters that are used to increase the blood levels of ARV drugs [62]. Examples of drugs in each class are tenofovir (TDF) and emtricitabine (FTC) or lamivudine (3TC) in the NRTI class; efavirenz (EFV) and nevirapine (NVP) in the NNRTI class; ritonavir-boosted lopinavir (LPV/r) and atazanavir (ATV/r) in the PI class; raltegravir and dolutegravir in the INSTI class; maraviroc in the CCR-5 inhibitor class; and enfuvirtide in the fusion inhibitor class [46].

Highly active antiretroviral treatment (HAART), which is a combination of three ARV drugs from different classes, is the currently used approach of treating HIV infection. The benefits of HAART at an individual level include suppression of viral replication, immunological recovery, prevention of HIV-associated morbidity and mortality [62, 63]. The benefits of HAART at a population level include reduced risk of secondary transmission risks if most HIV-infected people have fully suppressed viral load [64]. The first-line regimen currently used for adult patients in the SA public sector is a fixed dose combination of TDF, FTC and EFV; while zidovudine (AZT), 3TC and LPV/r are used in the second-line regimen [65].
Emergence of ARV drug-resistance mutation(s) during treatment of HIV-infected individuals limits the efficacy of ARV regimen, and warrants regimen changes. Transmission of the ARV drug-resistant strains has emerged as a serious challenge in management of HIV, as individuals who are primarily infected with these strains are at high risk of treatment failure [66, 67].

1.9 Prevention of HIV

The unavailability of an HIV cure or vaccine has made it very difficult to control the HIV pandemic. A comprehensive plan for controlling the HIV pandemic includes behavioural,

biomedical and structural approaches. Behavioural prevention measures aim at reducing people's HIV risk behaviour, while biomedical approaches include biologic tools of managing HIV such as diagnostic assays and drugs. Structural approaches are government policies that provide guidelines and resources for management and prevention of HIV infections [3, 15, 68, 69]. The behavioural prevention measures such as abstinence, faithfulness to sexual partner, and condom use are usually employed for reduction of new HIV infections. However, the effectiveness of behavioural prevention measures has been inconsistent, highlighting the difficulty to solely rely on these measures for HIV prevention [15].

HIV management requires a continuum of care, which begins from testing where individuals receive HIV counselling, and are later referred for appropriate treatment if tested positive for HIV. Many studies have observed that patients whose HIV VL is fully suppressed by treatment have a markedly reduced risk of transmitting HIV to uninfected individuals via unprotected sex [70-72]. As a result, treatment has become an integral part of newer strategies for controlling the HIV pandemic. For instance the 90-90-90 UNAIDS target includes diagnosing 90% of all HIV-infected individuals, initiating 90% of them on HIV treatment, and having full suppression of HIV VL in 90% of those on treatment [64]. Other global initiatives for controlling the HIV pandemic include the UNAIDS target of ending AIDS by 2030 [73], and having HIV as part of the targets of sustainable development goals (SDGs) [74]. South Africa has also incorporated HIV management and prevention into its government's priorities [75].

Other pharmaceutical modalities of preventing HIV include pre- and post-exposure prophylaxis. Pre-exposure prophylaxis (PrEP) refers to use of ARV drugs by HIV-uninfected individuals at high risk of acquiring HIV in order to prevent HIV infection. The success of PrEP is determined by good adherence to the prophylactic regimen [76]. Post-exposure

prophylaxis (PEP) employs ARV drugs to prevent HIV infection in individuals with recent exposure to HIV. It is commonly used among health care workers in cases of exposure to HIV either through needle-stick injuries or mucosal exposure to infectious fluids. PEP can be used in non-occupational environment as well such as in cases of sexual assault [77]. There is limited use of PrEP and PEP for prevention of HIV in developing countries as ARV treatment is more prioritised for treating HIV-infected people. However, PEP is mainly used as part of PMTCT programme for babies born to HIV-infected mothers [46].

1.10 The importance of diagnosing early HIV infection

Blood HIV VL, which is higher during the early HIV infection period and during advanced disease, is the principal predictor of heterosexual transmission. Quinn et al., found that HIV-1 transmission is rare among persons with VL levels of less than 1500 copies/ml, but highest with levels ≥50000 copies/ml [12]. A study conducted among heterosexual people in Uganda showed a high HIV transmission rate of 43.4% during early HIV infection stage compared to 8.4% and 37.3% in chronic and AIDS stages, respectively [78]. This translates to early HIV stage being 26 times highly infectious compared to the chronic stage of HIV. Other factors that contribute to high infectiousness during early HIV infection include the presence of homogenous CCR-5 viruses in an infected individual, which are efficiently transmitted across the genital mucosa [31]; and that there are no neutralizing antibodies in most part of this phase. The latter often appear at about 3 - 12 months after HIV infection [79]. In the context of pregnancy, women with early HIV infection are at higher risk of transmitting HIV to their babies compared to those with chronic HIV infection [80]. A study that assessed in-utero HIV transmission in Malawi noticed a 3 fold increase of HIV transmission in women with recent HIV infection compared to those with established infection [81].

Advantages of making the diagnosis of early HIV infection include opportunities to enhance immune response if treatment is made available early, and to reduce secondary transmission in the population. Preventing the "unknowing spread" of HIV-1 during the window period (early HIV stage) is an important public health preventive measure because of extremely high VLs during this period. Previously, treatment of early HIV infection was controversial owing to the fact that most patients are not ill at this stage and that ARV drugs had more side effects. So, there was reluctance by most clinicians to treat this phase as they feared that patients would unnecessarily suffer from drug-induced toxicities [37]. However, the damage caused by HIV during early infection such as loss of T-lymphocytes and immune activation was appreciated despite the absence of symptoms [11, 82, 83]. ARV drugs with better side effect profile became available with time [84]. Initial data that showed the benefits of treating early HIV infection was not entirely convincing [85]. As a result, only few HIV management guidelines made provisions for the treatment of early HIV infection [86, 87]. The two landmark studies, START and TEMPRANO trials, unequivocally showed the benefits of treating early HIV infection. These studies found that initiation of ARV treatment in early asymptomatic HIV infection was associated with better outcomes such as good immunological recovery, lower rate of AIDS- and non-AIDS-related events, and lower mortality from any cause [63, 88]. These data led to the adoption of "test and treat" approach, which has now been recommended by many HIV management guidelines, including SA guidelines [46, 59, 60, 89].

A large body of data on detection of early HIV infection comes from developed parts of the world with lower prevalence of HIV [90-95], and in some of these parts, pooled NAAT has been incorporated into the routine screening for HIV in order to identify infections that are missed by the rapid tests [90, 93]. The availability of resources in the developed world enables detection of these infections through pooled NAAT, followed by appropriate HIV preventive

interventions for reducing further spread of HIV. The pooling strategy reduces the costs of NAAT as these tests are very expensive [93]. Detection of early HIV infections allows close monitoring of the HIV epidemiology. For instance, some studies conducted in USA between 2005 and 2009 have observed different prevalence rates of early HIV infection among different population groups. Pilcher et al. reported an early HIV infection prevalence of 0.02% in 2005 in the general population who had HIV testing in North Carolina [90], while Sherpard et al. found a prevalence of 0.08% in New York City in predominantly men who have sex with men (MSM) [91], and Stekler et al. found a prevalence of 0.3% in 2009 in the MSM population from Seattle and King County [92]. This close monitoring of early HIV infection prevalence can be used to assess the impact of the existing HIV diagnostic and management policies, and inform necessary changes to such policies.

There are scanty data on detection of early HIV infection in developing world (including SA) owing to the high costs of NAATs. In 1998, several patients referred to a hospital in SA with suspected tropical diseases such as malaria were found to be having early HIV infection, through detection of p24 antigen in the absence of HIV antibodies [96]. Stevens et al. found a 0.99% prevalence of early HIV infection in 2005 after screening 1200 HIV antibody-negative individuals from Esselen Street Clinic in Johannesburg (SA) with pooled NAAT [97] compared to prevalence rates of 0.13% to 1.1% reported by studies conducted between 2010 and 2011 [98-101]. Other studies conducted in HIV high risk individuals such as patients with genital ulcer disease reported higher prevalence of early HIV infection of up to 11.4% [41, 102]. Only few SA studies followed up patients with early HIV infection for risk reduction counseling and further management in order to prevent forward spread of HIV. Unfortunately, molecular tests such as NAAT that have shortened the HIV window period are expensive and not easily

accessible for routine diagnosis of HIV. This limits the identification of highly infectious individuals that are misdiagnosed by rapid HIV tests in low resource settings.

1.11 Hypothesis

It is feasible to detect and manage early HIV infections in an HIV hyper-endemic setting with limited resources.

1.12 Study aims and objectives

Aims:

1. To assess the feasibility of detecting and managing individuals with early HIV infection in HCT clinics around the Tshwane district of SA
2. To assess transmission of HIV drug-resistant strains as SA has the largest ARV rollout
3. To assess the impact of diagnosing and treating early HIV infections in pregnant women
4. To evaluate evolution of HIV in samples taken at two different time points in participants diagnosed with early HIV infection
5. To develop a questionnaire tool that can be used to screen for individuals at high risk of early HIV infection
6. To assess the cost effective strategy of detecting early HIV infections in South Africa

Objectives:

1. To use NAAT to screen for HIV infection in individuals who tested negative on rapid HIV tests
2. To perform serological characterisation in individuals with NAAT-positive results
3. To sequence detected infections for subtyping, characterisation of viral population

(quasi-species) and assessment of their ARV resistance profile

4. To follow-up babies born to mothers who were diagnosed HIV infection during pregnancy, so as to assess vertical HIV transmission
5. To use a questionnaire to collect and analyse the HIV risk behavioural factors
6. To evaluate a more sensitive rapid HIV test, the INSTI HIV test, at the POC facilities for detection of early HIV infections
7. To document the costs of the assays used for detection of early HIV infection

1.13 Sample size

The study sample size estimate was 10 000 participants (see appendix C).

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CHAPTER 2

Detection of Acute and Early HIV-1 Infections in an HIV Hyper-Endemic Area with Limited Resources

The editorial style of the PLOS ONE journal was followed in this chapter – (minor post-publication amendments have been included).

2.1 Abstract

Background: Two thirds of the world's new HIV infections are in sub-Saharan Africa. Acute HIV infection (AHI) is the time of virus acquisition until the appearance of HIV antibodies. Early HIV infection, which includes AHI, is the interval between virus acquisition and establishment of viral load set-point. This study aimed to detect acute and early HIV infections in a hyper-endemic setting.

Methods: This was a cross-sectional diagnostic study that enrolled individuals who had negative rapid HIV results in five clinics in South Africa. Pooled nucleic acid amplification testing (NAAT) was performed, followed by individual sample testing in positive pools. NAAT-positive participants were recalled to the clinics for confirmatory testing and appropriate management. HIV antibody, p24 antigen, Western Blot and avidity tests were performed for characterization of NAAT-positive samples.

Results: The study enrolled 6910 individuals with negative rapid HIV results. Median age was 27 years (interquartile range {IQR}: 23 – 31). NAAT was positive in 55 samples, resulting in 0.8% newly diagnosed HIV-infected individuals (95% confidence interval {CI}: 0.6-1.0). The

negative predictive value for rapid HIV testing was 99.2% (95% CI: 99.0-99.4). Characterization of NAAT-positive samples revealed that 0.04% (95% CI: 0.0-0.1) had AHI, 0.3% (95% CI: 0.1-0.4) had early HIV infection, and 0.5% (95% CI: 0.4-0.7) had chronic HIV infection. Forty-seven (86%) of NAAT-positive participants returned for follow-up at a median of 4 weeks (IQR: 2 – 8). Follow-up rapid tests were positive in 96% of these participants.

Conclusions: NAAT demonstrated that a substantial number of HIV-infected individuals are misdiagnosed at South African points-of-care. Follow-up rapid tests done within a 4 week interval detected early and chronic HIV infections initially missed by rapid HIV testing. This may be a practical and affordable strategy for earlier detection of these infections in resource-constrained settings. Newer molecular tests that can be used at the points-of-care should be evaluated for routine diagnosis of HIV in hyper-endemic settings.

2.2 Introduction

Halting and reversing the spread of HIV was part of Millennium Development Goal (MDG) 6A [1]. Although good progress has been made in achieving MDG 6A, there were still too many new HIV infections by the end of 2014 and 2015, and two thirds of these infections were found in sub-Saharan Africa [2,3]

Acute HIV infection (AHI) refers to the time of virus acquisition until the appearance of HIV antibodies. Early or primary HIV infection, which includes AHI, is regarded as the interval between virus acquisition and the establishment of HIV viral load (VL) set-point [4]. Chronic HIV stage follows after the set-point is established [5]. People with early HIV infection contribute significantly to the transmission of HIV, as they have very high VLs in blood and genital secretions. It is estimated that early HIV infection stage is 26 times more infectious compared to the chronic stage [6]. This early stage of HIV infection is also known to predominantly produce C-C chemokine receptor type 5 (CCR-5) HIV strains, which are efficiently transmitted across the genital mucosa [7].

Rapid HIV tests play a crucial role in detecting HIV infections, and thereby initiating a cascade of linking infected patients to care. These rapid tests are commonly used for diagnosis of HIV infection in low resource settings such as in sub-Saharan Africa, but have poor sensitivity for detection of early HIV infection [8,9], which results in giving false negative results to highly infectious individuals. The addition of p24 antigen to some rapid HIV tests has led to a slight improvement in sensitivity for detection of early HIV infections, as the p24 antigen component on these tests performs poorly [8,9]. Tests that have shortened the HIV window period such as enzyme-linked immunosorbent assays (ELISAs) and NAATs are costly and not readily available for point-of-care testing [10].

Management of early HIV infection has benefits for the infected individual, and prevents secondary spread of HIV in the population [4,5]. This study aimed to detect acute and early HIV infections in an HIV hyper-endemic setting with limited resources.

2.3 Materials and Methods

2.3.1 Recruitment and enrollment

This was a cross-sectional diagnostic study, conducted between March 2012 to June 2015, which enrolled individuals who had negative rapid HIV results and were 14 years or older. Participants were recruited and enrolled from 5 HIV counseling and testing (HCT) clinics in the Tshwane district of South Africa (SA). Four of these HCT clinics were antenatal clinics and one was a general HCT clinic. Rapid HIV testing was done according to the SA HIV testing guidelines, which recommend a serial HIV testing strategy at the points-of-care [11]. Testing at the HCT clinics was done by HIV counselors, who had received training in HIV testing and counseling. The rapid HIV test that was commonly used for screening during the course of this study was Advanced Quality (Intec Products Inc). Abon (Abon Biopharm) was used for screening in 2014; however, this was replaced with Advanced Quality at the end of 2014. At enrolment, study samples and participants' cell phone numbers were collected. Plasma was separated from whole blood through centrifugation at 1700 relative centrifugal force (RCF) for 20 minutes, and stored at -70ºC within 24 hours after collection until the time of testing.

2.3.2 Ethics statement

Written consent was obtained from all participants before enrollment. The study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number – 295/2015) and by Tshwane Research Ethics Committee (TMREC 2010/26). The legal ages for consenting to HIV testing and medical treatment in South Africa are 12 and 14 years, respectively [12]. During the course of the study we noticed that some people who came for HIV testing were younger than 18 years of age, and came alone without parents or guardians. Hence, we amended our study protocol to include this group, in order to extend the benefit of earlier diagnosis of HIV to them. We applied for inclusion of this group with our Ethics Committee, and were granted approval. So all study participants, including 14-17 years, signed the same written consent forms that were used for participants older than 18 years.

2.3.3 Sample testing

Roche CAP/CTM HIV VL version 2 assay (Roche Diagnostics, Mannheim, Germany) with a lower detection limit of 20 copies/ml was used for NAAT, in a mini-pool of 5 samples, using 200 µl from each sample to constitute a 1 ml sample volume required for testing. An additional volume of about 20 µl from one of the pool samples was used for top-up in order to avoid sample rejection due to insufficient volume. Pools that had undetectable VL were considered negative for HIV. Individual sample testing was done in pools that had detectable VL. A VL threshold of ≥5000 copies/ml in an individual sample was considered as diagnostic for HIV infection. If HIV VL was <5000 copies/ml, a repeat test was done in a follow-up sample in order to exclude a possible contamination in the initial test [5]. All participants were encouraged to voluntarily collect NAAT results; however, those who tested positive on NAAT were contacted on their cell phones to come back to the clinic for further management. During

this follow-up visit, HIV counseling and repeat rapid testing were done, follow-up samples collected, and participants referred for appropriate management.

The following serology tests were done in NAAT-positive samples: 3rd generation Genscreen HIV-1/2 version 2 ELISA (BioRad, Marnes-la-Coquette, France) and HIV Western Blot (Bio-Rad Laboratories, Redmond WA, USA) for antibody detection; p24 antigen (Roche Diagnostics, Mannheim, Germany); and limiting antigen (LAg) HIV avidity assay {Maxim Biomedical Inc., Rockville, USA} for confirmation of early HIV infection in samples with detectable antibodies. LAg avidity assay was repeated on follow-up samples. Acute HIV infection was defined as the presence of HIV RNA with or without p24 antigen in the absence of HIV antibodies. Early HIV infection was defined as the presence of HIV RNA with or without p24 antigen, and presence of HIV antibodies with low avidity as reflected by values <1.5 normalised optical density (OD-n) on LAg avidity assay. Samples found to have HIV RNA with or without p24 antigen, and HIV antibodies with high avidity of >1.5 OD-n were classified as having chronic infection.

Rapid HIV testing was later repeated from stored plasma samples in the laboratory using the same tests that were used at the points-of-care. All the tests were performed and analyzed according to manufacturer's instructions. CD4 count results were later enumerated from the laboratory information system and patient records for NAAT-positive participants.

2.3.4 Statistical analysis

A descriptive analysis was used to present summary statistics (median, proportions and 95% confidence intervals) for the parameters. Proportions of acute, early and chronic HIV infections that were missed at the points-of-care were computed. Comparison of the proportions of newly diagnosed HIV infections between males and females was done using Fisher's exact test. In

addition, median values of HIV VL and CD4 count were compared between the groups of participants with early and chronic HIV infections using the Wilcoxon rank-sum test. All the statistics were performed on the STATA version 14.1 software (StataCorp LP, College Station, TX, USA). A p-value of ≤0.05 was considered statistically significant.

2.4 Results

2.4.1 Demographics and newly diagnosed HIV infections

From March 2012 to June 2015, the study enrolled and tested 6910 participants who had negative rapid HIV test results (Fig 2.1). Their median age was 27 years (IQR: 23 – 31). Females formed a large proportion (87%, n = 6011) of the study group, and 88% (n = 5271) of the female participants were pregnant. NAAT detected HIV RNA in 55 samples, resulting in 0.8% of newly diagnosed HIV-infected individuals (95% CI: 0.6-1.0). This showed a negative predictive value (NPV) of 99.2% (95% CI: 99.0-99.4) for rapid HIV testing at the points-of-care. The newly diagnosed HIV infections were detected in all five study clinics, and were slightly higher in pregnant females (0.9%) compared to non-pregnant females and males, 0.7% (p = 0.83) and 0.6% (p = 0.54), respectively.

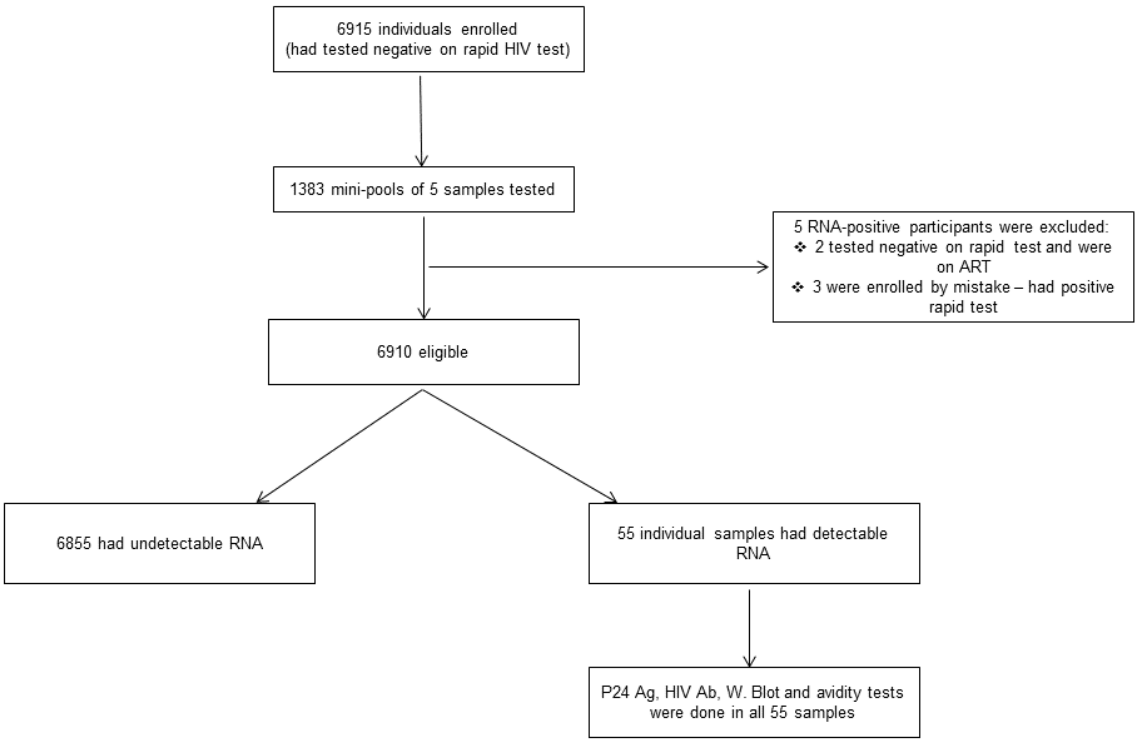

Figure 2.1. Algorithm showing enrolment and testing of participants. Participants' baseline rapid results were reviewed if they had positive HIV RNA results. Those who had negative rapid test and receiving antiretroviral therapy, and those enrolled by mistake (positive baseline rapid test) were excluded. Ag = antigen, Ab = antibody, W. Blot = Western Blot.

2.4.2 Serological characterization of NAAT-positive samples

Of the 55 NAAT-positive participants, 52 (95%) tested positive for HIV antibodies on Genscreen HIV ELISA. Western Blot (W. Blot) was positive in 48 (92%) of these antibody-positive participants. P24 antigen testing was performed on 53 participants, and was positive in 12 (23%) participants. The two samples that were insufficient for p24 antigen test were positive on HIV ELISA. Limiting antigen avidity assay identified 16 antibody-positive participants as having early HIV infection, and classified the remainder (n = 36) as having chronic infection. Limiting antigen avidity was repeated on follow-up samples of participants who came for a follow-up visit, and the results remained the same as in initial testing. The combination of HIV RNA, p24 antigen, HIV antibody, LAg avidity and W. Blot test results enabled staging of HIV infections. Amongst those with early HIV infection, few participants were detected very early before the appearance of HIV antibodies, some were detected at the peak of HIV vireamia, and many others were near the time of HIV VL set-point (Fig 2.2). A significant proportion of HIV-infected individuals were classified as having chronic HIV infection (Fig 2.2).

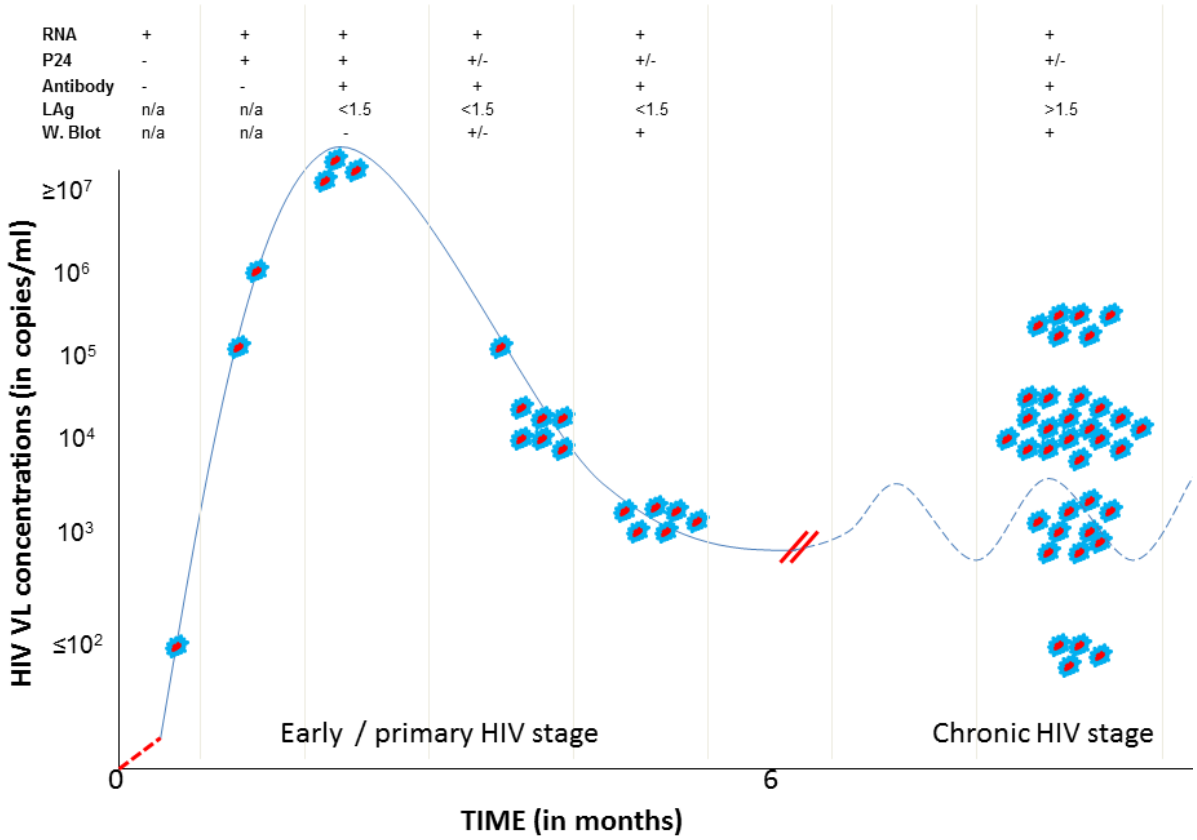

Figure 2.2. Staging of identified HIV infections based on HIV RNA (viral load), p24 antigen, antibodies, limiting antigen (LAg) avidity results and Western Blot (W. Blot). All the red-blue dots represent participants with HIV infections that were misdiagnosed by rapid HIV tests at the points-of-care. Solid blue line represents HIV viral load during early HIV stage, and dotted blue line represents HIV viral load fluctuation during the chronic stage. Red dotted line represents eclipse stage where all HIV tests are negative. Double red lines represent HIV viral load set-point. Participants with LAg avidity results <1.5 OD-n values had early HIV infections and those with LAg results >1.5 OD-n values had chronic HIV infections. N/A = not applicable, + = positive, - = negative, < = less than, > = greater than, +/- = positive or negative results.

Serological characterization of NAAT-positive samples showed that 0.04% (95% CI: 0.0-0.1) had acute HIV infection (n = 3); 0.3% (95% CI: 0.1-0.4) had early HIV infection (n = 19, including those with acute infection) (Table 2.1); and 0.5% (95% CI: 0.4-0.7) had chronic HIV infection (n = 36) (Table 2.2).

Table 2.1. Characteristics of participants diagnosed with acute or early HIV infection

			INITIAL TESTS*						FOLLOW-UP TESTS						
Pt ID	**Sex**	**Marital status**	**Rapid HIV test**	**HIV VL**	**p24 antigen**	**Genscreen 3rd gen ELISA**	**W. Blot**	**LAg avidity**	**F/U interval (weeks)**	**F/U Rapid HIV test**	**F/U HIV VL**	**F/U LAg avidity**	**HIV Staging**	**CD4 count**	**Pregnancy**
9498	F	S	-	509793	+ (10.9)	- (0.05)	n/a	n/a	10	+		n/a	Acute	964	No
9228	M	S	-	94	- (0.3)	- (0.06)	n/a	n/a	10	-	71674	n/a	Acute	384	n/a
8047	M	M	-	1245238	+ (33.8)	- (0.03)	n/a	n/a	2	+		n/a	Acute	---	n/a
9218¥	F	D	-	55099	- (0.4)	+ (5.23)	+	0.143	10	-		0.103	Early	58	No
2066	F	S	-	1010	- (0.5)	+ (4.28)	+	0.058	7	+	38900	0.338	Early	752	No
8575	F	S	-	93079	+ (1.3)	+ (5.06)	+	0.063	8	+		0.171	Early	668	No
7293¥	F	S	-	5078	- (0.3)	+ (4.76)	+	0.487	ND	ND		ND	Early	---	Yes
5041	M	S	-	22921300	+ (434.1)	+ (2.92)	-	0.073	ND	ND		ND	Early	---	n/a
9049¥	F	S	-	16848	- (0.3)	+ (5.06)	+	0.201	3	+		0.397	Early	---	Yes
6638¥	F	S	-	195105	- (0.3)	+ (4.45)	+	0.256	6	+		0.437	Early	457	Yes
261¥	M	S	-	84501600	+ (1383.0)	+ (4.06)	-	0.073	9	+		1.094	Early	386	n/a
6512¥	F	S	-	1763	- (0.3)	+ (4.93)	+	1.312	2	+	1938	1.111	Early	215	Yes
6743	F	S	-	27364	- (0.5)	+ (4.48)	-	0.108	7	+		0.418	Early	638	Yes
6582	F	S	-	6216	INSUF	+ (4.81)	INSUF	0.458	6	+		1.060	Early	818	Yes
6727¥	F	S	-	4874	+ (2.6)	+ (4.48)	+	0.234	2	+	1970	0.258	Early	706	Yes
6737¥	F	S	-	2227	- (0.3)	+ (5.95)	+	0.746	ND	ND	ND	ND	Early	---	Yes
7084	F	S	-	337987200	+ (2884.0)	+ (0.17)	-	0.065	4	+		0.153	Early	411	No
2504¥	F	S	-	37243	- (0.4)	+ (5.95)	+	1.211	2	+		1.391	Early	287	Yes
3469¥	F	S	-	33274	+ (5.6)	+ (5.52)	+	0.832	9	+		0.763	Early	123	Yes

*Initial tests were done from samples obtained at enrolment (i.e. after a negative rapid HIV test result). HIV VL tests (including follow-up) were done first to confirm infection, and all the serology tests were done later. Pt ID = participant identity, F = female, M = male or married, S = Single, D = divorced, gen = generation, ELISA = enzyme-linked immunosorbent assay, W. Blot = Western Blot, LAg = limiting antigen, F/U = follow-up, Insuf = insufficient, ND = not done (participant did not come for follow-up), n/a = not applicable, + = positive, - = negative, --- = not available, ¥ = had positive p31 antibodies on W. Blot. Units: VL = copies/ml; p24 antigen = cut-off index (COI); Genscreen ELISA = sample cut-off (S/CO); LAg avidity = normalized optical density (OD-n); CD4 count = cells/µl.

Table 2.2. Characteristics of participants diagnosed with chronic HIV infection

			INITIAL TESTS*						FOLLOW UP TESTS						
Pt ID	**Sex**	**Marital Status**	**Rapid HIV test**	**HIV VL**	**p24 antigen**	**Genscreen 3rd gen ELISA**	**W. Blot**	**LAg avidity**	**F/U Interval (weeks)**	**F/U Rapid HIV test**	**F/U HIV VL**	**F/U LAg avidity**	**HIV Staging**	**CD4 count**	**Pregnancy**
5054	F	S	-	27820	- (0.29)	+ (4.54)	+	4.246	2	+		3.577	LT	72	Yes
5067	F	S	-	12675	INSUF	+ (4.22)	+	3.317	8	+		2.822	LT	147	Yes
9915	F	M	-	14100	+ (1.36)	+ (5.95)	+	4.175	2	+		3.341	LT	382	Yes
4351	F	S	-	2685	- (0.24)	+ (5.96)	+	3.931	3	+	1248	3.267	LT	---	Yes
639	F	S	-	6579	- (0.25)	+ (4.63)	+	3.853	4	+		3.099	LT	392	Yes
641	F	S	-	70569	- (0.32)	+ (5.23)	+	4.116	4	+		3.366	LT	228	Yes
7959	F	S	-	148776	- (0.16)	+ (4.58)	+	3.835	ND	ND		ND	LT	343	No
8828	F	M	-	41500	- (0.42)	+ (5.95)	+	2.382	14	+		2.849	LT	230	Yes
2678	F	M	-	222853	+ (11.13)	+ (4.69)	+	3.639	4	+		3.135	LT	199	Yes
9895	F	M	-	4880	- (0.29)	+ (4.21)	+	4.305	6	+	7873	3.480	LT	607	Yes
9986	F	S	-	97600	- (0.92)	+ (4.75)	+	3.700	2	+		3.093	LT	160	Yes
843	F	S	-	29712	- (0.29)	+ (4.63)	+	2.252	5	+		2.463	LT	348	Yes
6990	F	S	-	17536	- (0.33)	+ (5.96)	+	3.945	6	+		3.233	LT	269	Yes
2340	F	S	-	14490	- (0.36)	+ (4.63)	+	2.850	ND	ND		ND	LT	---	Yes
6709	F	S	-	53	- (0.34)	+ (4.48)	+	3.162	ND	ND	ND	ND	LT	683	Yes
6748	F	S	-	932	- (0.29)	+ (4.68)	+	4.085	2	+	1707	3.324	LT	---	Yes
6671	F	M	-	14072	- (0.23)	+ (4.48)	+	4.083	3	+			LT	---	Yes
6380	F	S	-	11073	- (0.23)	+ (4.62)	+	4.763	4	+		3.509	LT	385	Yes
6557	F	S	-	614	- (0.33)	+ (4.41)	+	4.049	4	+	265	3.593	LT	353	Yes
6565	F	S	-	5670	- (0.37)	+ (4.32)	+	3.091	3	+		2.969	LT	371	Yes
6509	M	M	-	106713	- (0.34)	+ (4.48)	+	3.640	ND	ND		ND	LT	---	n/a
6596	F	S	-	3873	- (0.36)	+ (5.95)	+	4.385	3	+	1087	3.519	LT	576	Yes
6640	F	M	-	3074	- (0.34)	+ (4.48)	+	2.824	5	+	9887	2.864	LT	407	Yes
6649	F	M	-	21051	- (0.28)	+ (5.95)	+	3.804	2	+		2.867	LT	164	Yes
6738	F	S	-	159539	- (0.37)	+ (5.96)	+	4.384	2	+		ND	LT	682	Yes
1067	F	S	-	1779	- (0.37)	+ (4.75)	+	4.088	2	+	2574	3.470	LT	394	Yes
921	F	M	-	9781	- (0.28)	+ (5.04)	+	4.553	7	+		3.607	LT	469	Yes
3869	F	S	-	217372	- (0.31)	+ (5.52)	+	3.716	4	+		3.264	LT	---	Yes
3912	F	S	-	32008	- (0.29)	+ (5.52)	+	2.314	8	+		2.079	LT	287	Yes
3920	F	S	-	66694	- (0.44)	+ (4.68)	+	4.375	8	+		3.410	LT	306	Yes
3880	F	S	-	7505	- (0.30)	+ (4.62)	+	3.840	3	+		3.206	LT	575	Yes
3935	F	S	-	242663	+ (21.77)	+ (5.95)	+	1.833	8	+		2.157	LT	61	Yes
1117	F	M	-	153	- (0.31)	+ (5.95)	+	3.315	2	+	629	3.249	LT	536	Yes
1121	F	S	-	80287	- (0.34)	+ (5.95)	+	3.634	2	+		3.483	LT	127	Yes
3474	F	S	-	16510	- (0.32)	+ (4.91)	+	2.078	12	+		2.828	LT	675	Yes
1475	F	S	-	44450	+ (1.17)	+ (4.92)	+	4.241	9	+		3.628	LT	255	Yes

*Initial tests were done from samples obtained at enrolment (i.e. after a negative rapid HIV test result). HIV VL tests (including follow-up) were done first to confirm infection, and all the serology tests were done later. Pt ID = participant identity, F = female, M = male or married, S = Single, D = divorced, gen = generation, ELISA = enzyme-linked immunosorbent assay, W. Blot = Western Blot, LAg = limiting antigen, F/U = follow-up, Insuf = insufficient, LT = long term infection, ND = not done (participant did not come for follow-up), n/a = not applicable, + = positive, - = negative, --- = not available. Units: HIV VL = copies/ml; p24 antigen = cut-off index (COI); Genscreen ELISA = sample cut-off (S/CO); LAg avidity = normalized optical density (OD-n); CD4 count = cells/μl.

2.4.3 HIV viral loads and CD4 counts of NAAT-positive participants

Median HIV VL was slightly higher in participants with early HIV infection (4.5 log, IQR: 3.7-5.7) compared to those with chronic infection (4.2 log, IQR: 3.8-4.9) (p = 0.33). Most participants (89%) had HIV VLs >1500 copies/ml. CD4 count results were available for 44 participants. Median CD4 count was significantly higher in participants with early HIV infection (434 cells/μl, IQR: 287-706) compared to those with chronic infection (351 cells/μl, IQR: 228-469) (p = 0.03) (Tables 2.1 and 2.2).

2.4.4 Repeat rapid testing from stored plasma samples

Rapid tests were repeated in 50 frozen antibody-positive plasma samples with sufficient volumes in the laboratory. Advanced Quality rapid tests were clearly positive in 45 samples, low positive in 2 (faint band noticed), and negative in 3 samples. Abon rapid tests were clearly positive in 48 samples, low positive in 1 sample and negative in 1 sample.

2.4.5 Follow-up of NAAT-positive participants

NAAT-positive participants were recalled to the clinics for follow-up, and they came at different intervals based on their availability. Only 47 (86%) participants came back for follow-up at a median of 4 weeks (IQR: 2 – 8), and 45 (96%) of them were positive on follow-up rapid tests. Follow-up rapid testing done at the points-of-care improved NPV from 99.2% to 99.8%. Participants were managed according to the SA HIV management guidelines, which recommended immediate initiation of antiretroviral therapy (ART) in pregnant females, and ART eligibility when a CD4 count was <350 cells/µl from 2012 – 2014 and <500 cells/µl from 2015 [13-16]. Two non-pregnant participants, with confirmed early HIV infection, were still negative on rapid test at a 10 week follow-up interval (Table 2.1). One of them tested positive on the 4th generation ELISA, had a low CD4 count of 58 cells/µl, and was initiated on ART. The other participant tested positive on a second follow-up rapid test done 5 months later, and was still not eligible for treatment as his CD4 count was 384 cells/µl (in 2012). All participants with chronic HIV infection had positive HIV ELISA, positive W. Blot and LAg avidity results of >1.5 OD-n values, and all were positive on follow-up rapid HIV testing done at the points-of-care (Table 2.2). NAAT-positive participants were asymptomatic or had no severe illnesses as they came back to collect results, with the exception of one who was admitted with acute retroviral syndrome.

2.5 Discussion

2.5.1 Frequency of new HIV infections

This study evaluated the detection of early HIV infections in a low resource setting through the use of different HIV assays. The finding of 55 (0.8%) newly diagnosed HIV infections missed by rapid tests shows that a substantial number of HIV-infected individuals are misdiagnosed at the points-of-care in SA. A slightly higher prevalence of these infections in females compared to males was not surprising as HIV prevalence in SA is generally higher in females [17]. The newly diagnosed HIV-infected participants were identified in all the study clinics, highlighting that this could be a bigger problem in SA. Other studies in SA and sub-Saharan African countries have identified individuals with early or chronic HIV infection that were misdiagnosed at the points-of-care [18-23]. This calls for improvement of HIV testing guidelines in HIV hyper-endemic setting in order to facilitate earlier detection of HIV-infected individuals who are misdiagnosed by rapid HIV tests at the points-of-care.

From 2002 – 2012, SA HIV incidence in people aged 15 – 49 years ranged between 1.9 – 2.2% [17]. This maintenance of HIV incidence at around 2% for a 10 year period highlights ongoing transmission of HIV, which is probably driven by highly infectious individuals, particularly those with early HIV infection. Most participants (89%) in this study had HIV viral loads above 1500 copies/ml, which is a known risk factor for sexual transmission of HIV [24]. The 0.3% prevalence of early HIV infections noted in this study is lower compared to a recently published study from SA that showed a prevalence of 1.1% in individuals who were HIV antibody-negative at the point-of-care [21]. This lower prevalence of early HIV infection could be explained by the fact that this study was conducted in an area with a moderate HIV prevalence [17], and at a time where there was higher ART coverage in SA [25].

2.5.2 Earlier detection of HIV-infected individuals misdiagnosed at the points-of-care

The majority of NAAT-positive participants (86%) were successfully recalled to the clinics for further management, showing that it is feasible to detect and manage participants misdiagnosed by rapid HIV testing at the points-of-care. Participants with early HIV infection were detected in all the Fiebig stages of early HIV infection (Fig 2.2) [26]. The finding that most participants with early HIV infection were near the time of HIV VL set-point could mean that most do not present for HIV testing close to the time of exposure to HIV (Fig 2.2). Pooled NAAT has been incorporated into the routine screening for HIV in some parts of developed world in order to identify HIV-infected individuals that are misdiagnosed by the rapid tests, particularly those with early HIV infection [27,28]. The availability of resources in the developed world enables detection of these infections, followed by appropriate HIV management [28]. The pooling strategy reduces the costs of NAAT [28]; however, this strategy is expensive to implement for routine diagnosis in low resource settings.

This study showed that follow-up rapid tests done within a 4 week interval are able to detect individuals with early or chronic HIV infection previously misdiagnosed by rapid HIV tests. It is likely that other participants who came for follow-up later than the 4 week interval would have tested positive on rapid HIV tests if they had presented within this interval (Tables 2.1 and 2.2). The exception to this was the 2 participants with early HIV infection who were still negative at 10 weeks follow-up (Table 2.1). Most HIV testing guidelines used in developing countries recommend a follow-up rapid HIV test to be done 12 weeks later after a negative rapid HIV test [16,29,30]. Implementing a 4 week follow-up interval instead of a 12 week follow-up interval would reduce both HIV diagnostic and transmission window periods. Where possible, the 4 week follow-up interval could be incorporated into the existing 12 week follow-

up interval to have a 0 – 4 – 12 week rapid testing strategy, which could easily be implemented in a setting of frequent clinic visits such as in antenatal clinics. The current WHO HIV testing guidelines recommend follow-up rapid testing at 4 – 6 weeks only for a minority of individuals who identify a specific recent suspected exposure to HIV [31]. However, it is very difficult to predict or clinically diagnose early HIV infections [5,10]. Hence, a routine follow-up rapid testing at 4 weeks would be a good surveillance tool for early detection of HIV-infected individuals previously misdiagnosed by rapid HIV tests at points-of-care.

2.5.3 Alternative strategies for detecting HIV-infected individuals misdiagnosed at the points-of-care

Parallel testing algorithm, where HIV testing at the points-of-care is done on two different rapid strips concurrently, could be considered for improving the sensitivity of rapid HIV testing at points-of-care. This strategy has been reported to have a higher sensitivity [32,33]. However, some data show that serial testing is equivalent to the parallel testing algorithm with an added cost saving benefit [34,35]. Black et al. have identified HIV-infected individuals who were misdiagnosed at the points-of-care despite the use of the parallel testing algorithm [36]. This highlights that parallel testing may not be the best solution for improving HIV testing at the points-of-care. WHO recommends either serial or parallel testing algorithm for HIV testing at points-of-care [37].

The other alternative strategy for detection of HIV-infected individuals misdiagnosed by rapid tests in resource limited countries would be to screen individuals testing negative on rapid HIV test with the 4th generation HIV ELISA. However, HIV ELISA is costly and laboratory-based, and thus may also lead to a high rate of loss to follow-up. Molecular tests are now available for use at the points-of-care. These are not readily available in the developing world owing to high costs [38,39]. If NAAT was done at the points-of-care in this study, those misdiagnosed by the

rapid HIV test would have been detected and immediately linked to HIV care, without any issues of loss to follow-up.

2.5.4 Implications of misdiagnosing HIV infections at the points-of-care

Advantages of making an accurate diagnosis of HIV infection include opportunities to preserve HIV-specific immune responses if treatment is made available early, and prevent secondary transmission in the population [5]. There is now convincing evidence showing the benefits of early treatment of HIV-infected patients regardless of CD4 counts [40]. Misdiagnosing HIV-infected individuals at the points-of-care means that some people do not benefit from early treatment of HIV infection. This could lead to complications that are associated with delayed ART initiation, such as development of AIDS conditions [40].

Preventing the secondary spread of HIV during the window period is an important public health preventive measure because of extremely high viral loads during this period [41]. Individuals with early HIV infection contribute significantly to the secondary transmission of HIV, and thus play a big role in sustaining new HIV infections [4,6]. Other stages of HIV infection also have a significant contribution to secondary transmission of HIV [6].

Undiagnosed or untreated HIV infection in pregnant women results in a high risk of vertical transmission of HIV, and this risk is much higher with early HIV infection [42,43]. Although prevention of mother to child transmission (PMTCT) programme has led to a remarkable reduction of vertical transmission of HIV in SA and other countries [43,44], more needs to be done to eliminate vertical transmission. Accurate diagnosis of HIV infection during antenatal care creates an excellent opportunity for initiation of PMTCT. Thus, strengthening HIV testing at the points-of-care would contribute towards further decrease and elimination of vertical transmission of HIV.

The high frequency of new HIV infections undermines the global efforts of eliminating new HIV infections. The first 90 of 90-90-90 UNAIDS target might not be properly assessed if there are many HIV-infected individuals that are misdiagnosed by rapid tests at points-of-care in resource-constrained settings. A test and treat strategy might not have a desired impact in low resource setting if point-of-care testing for HIV is not strengthened. Misdiagnosis of HIV infection could lead to inadvertent use of pre-exposure prophylaxis in HIV-infected individuals, thus resulting in emergence and transmission of ARV drug-resistant strains.

Detection of new HIV infections allows a close monitoring of the HIV epidemiology. This monitoring can be used to assess the effectiveness of the existing HIV diagnostic, treatment and prevention policies, and inform necessary changes to such policies.

2.5.5 Possible explanations for misdiagnosis of HIV-infected people at the points-of-care

The finding that the majority of participants (95%) who were misdiagnosed at the points-of-care were positive on HIV ELISA is not surprising as the rapid tests have poorer sensitivity for the detection of HIV antibodies, especially during the early HIV phase [45]. In this study most participants with detectable antibodies had positive W. Blot and also tested positive on repeat rapid testing in plasma. The reason for false negative rapid tests at the points-of-care could be due to human errors during testing. These errors could include insufficient volume of blood and/or incorrect volume of diluent used [31].

Other researchers have previously showed inferior performance of some rapid HIV tests on finger-stick whole blood compared to testing done on serum in a setting where testing was done by skilled personnel [8]. Black et al. showed a remarkably lower sensitivity of rapid tests during a field evaluation done by trained nurses on finger-stick whole blood compared to the laboratory evaluation done on serum samples [36]. A recent study that screened for acute HIV

infection in SA also detected a significant number of chronic HIV infections that were missed at the points-of-care [21]. The lower performance of some rapid tests in finger-stick whole blood could be caused by dilution effect from the red blood cells and/or weak antibody binding due to haemolysis [8], and by antigen-antibody complexes predominantly found in early HIV phase before the appearance of free antibodies [41]. These complexes are also found in varying degrees in later stages of HIV infection [46,47]. This shows a need to use rapid HIV tests with higher performance in finger-stick whole blood, as this is a sample type used at the points-of-care. One such test is INSTI, a rapid HIV test approved by the Food and Drug Administration (FDA), which has shown a high sensitivity and specificity in diagnosing HIV infection in whole blood samples [8]. The INSTI rapid test has also been reported to perform well in identifying individuals with early HIV infection [48].

The limitations of this study are that follow-up visits for NAAT-positive participants were done at different intervals and there was no active tracing of their partners. However, all participants identified with HIV infection were encouraged to disclose to their partners, and return with them to the clinics. Repeat rapid testing from plasma samples was not done before NAAT and serology testing. If done earlier, this would have led to faster identification of HIV-infected individuals as most rapid tests were positive in plasma samples. Few participants with chronic HIV infection could have been misclassified as having early HIV infection by the LAg avidity test as this is a limitation of the currently available avidity assays [49,50].

2.6 Conclusions

These data show that it is feasible to detect acute, early and chronic HIV infections initially missed by rapid tests in an HIV hyper-endemic setting with limited resources. The routine implementation of a 4 week follow-up rapid test would reduce both HIV diagnostic and transmission window periods. Rapid HIV tests with higher performance in finger-stick whole blood should be used in resource poor settings with high HIV prevalence. The newer molecular tests that can be used at the points-of-care should be evaluated for routine diagnosis of HIV in HIV hyper-endemic settings.

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CHAPTER 3

High Risk Exposure to HIV among Sexually Active Individuals who Tested Negative on Rapid HIV Tests in the Tshwane District of South Africa – the importance of behavioural prevention measures

The editorial style of the PLOS ONE journal was followed in this chapter - (minor post-publication amendments have been included).

3.1 Abstract

Objective: To assess the prevalence of HIV risk behaviour among sexually active HIV sero-negative individuals in the Tshwane district of South Africa (SA).

Methods: Demographic and HIV risk behaviour data were collected on a questionnaire from participants of a cross-sectional study that screened for early HIV infection using pooled nucleic acid amplification testing (NAAT). The study enrolled individuals who tested negative on rapid HIV tests performed at five HIV counseling and testing (HCT) clinics, which included four antenatal clinics and one general HCT clinic.

Results: The study enrolled 9547 predominantly black participants (96.6%) with a median age of 27 years (interquartile range [IQR]: 23 – 31). There were 1661 non-pregnant and 7886 pregnant participants largely enrolled from the general and antenatal HCT clinics, respectively. NAAT detected HIV infection in 61 participants (0.6%; 95% confidence interval [CI]: 0.5 –

0.8) in the whole study. A high proportion of study participants, 62.8% and 63.0%, were unaware of their partner's HIV status; and also had high prevalence, 88.5% and 99.5%, of recent unprotected sex in the general and pregnant population, respectively. Consistent use of condoms was associated with protection against HIV infection in the general population. Trends of higher odds for HIV infection were observed with most demographic and HIV risk factors at univariate analysis, however, multivariate analysis did not show statistical significance for almost all these factors. A significantly lower risk of HIV infection was observed in circumcised men ($p = 0.04$).

Conclusions: These data show that a large segment of sexually active people in the Tshwane district of SA have high risk exposure to HIV. The detection of newly diagnosed HIV infections in all study clinics reflects a wide distribution of individuals who are capable of sustaining HIV transmission in the setting where HIV risk behaviour is highly prevalent. A questionnaire that captures HIV risk behaviour would be useful during HIV counselling and testing to ensure that there is a systematic way of identifying HIV risk factors and that counselling is optimised for each individual. HIV risk behaviour surveillance could be used to inform relevant HIV prevention interventions that could be implemented at a community or population level.

3.2 Introduction

Human immunodeficiency virus (HIV) is a global health problem that has disproportionately affected sub-Saharan Africa [1]. In this region, the highest burden of HIV infections is found in South Africa (SA) where there are about 6.19 million people living with HIV [2]. Thus, SA contributes approximately 20% of HIV infections to the global HIV pandemic [1] despite having about 1% of the world's population [3].

HIV is transmitted through sexual intercourse, blood or blood products and by vertical transmission. Sexual transmission plays a big role in HIV transmission contributing to about 80% of transmissions or more [4]. Unprotected sex is the major determinant for sexual acquisition and transmission of HIV [5, 6]. The presence of high HIV viral load (VL) of at least 1500 copies in an individual infected with HIV is associated with a risk of sexual transmission to uninfected individuals [7]. Various other factors play a significant role in genital acquisition of HIV during unprotected sex, such as the presence of other sexually transmitted infections (STIs) [8] and male circumcision status [6]. Failure to respond to antiretroviral (ARV) therapy creates opportunities for transmission of ARV drug resistant strains of HIV [9].

Preventing new infections is important for controlling the HIV pandemic. Strategies for preventing HIV infections include behavioural, biomedical and structural approaches. Behavioural prevention measures aim at reducing people's HIV risk behaviour, and form an integral part of any HIV prevention approach as they are also needed for risk management in individuals infected with HIV in order to avoid further spread of HIV. Successes of HIV prevention have been achieved with behavioural measures in some countries, however, these successes were often short-lived and difficult to maintain over a long time [10]. For instance,

in early to mid-1990s a decline of HIV prevalence and incidence was observed in Uganda and Thailand owing to behavioural prevention measures [11-13]. However, this was later followed by increasing trends of HIV prevalence and incidence in the general population and in some key population groups [14-17]. The other challenge of the behavioural prevention measures is that their success could not be reproduced in some parts of the world [10].

The world continues to see a high rate of new HIV infections despite the availability of prevention measures [1]. Of all the HIV prevention measures, the behavioural measures are the most affordable and easier to implement even in low resource settings. Therefore, it is important to continually make efforts to understand and improve behavioural prevention measures against HIV. This study assessed HIV risk behaviours among sexually active HIV sero-negative people attending HIV counselling and testing (HCT) clinics in the Tshwane district of SA.

3.3 Methods

Participants were enrolled as part of a study that screened for early HIV infection from 5 HCT clinics in the Tshwane region of SA. Four of these clinics were antenatal clinics and one was a general HCT clinic. The inclusion criteria for this study were a negative rapid HIV test and recent sexual activity as defined by having had sex within the last 3 months. The pregnant females were enrolled during their first antenatal care visit just after they had done the HIV test. Pooled nucleic acid amplification testing (NAAT) was performed in mini-pools of 5 samples using the Roche CAP/CTM HIV VL version 2 assay (Roche Diagnostics, Mannheim, Germany), followed by individual sample testing in positive pools. NAAT-positive participants

were recalled to the clinics for confirmatory testing and appropriate management. Data on serological characterisation of NAAT-positive individuals have previously been published [18].

At enrolment data on demographics and HIV risk behaviour were collected using a newly designed questionnaire, which captured information on; age, race, marital status, condom use, HIV status of the partner, current number of sexual partners, alcohol and drug use, commercial sex work by females, recent history of STIs (within the last 3 months), male circumcision, sex with other men, recent history of sexual assault (within the last month), history of travel to other countries, and recent flu-like illness (S3.1 Fig). The latter was not included in the analysis as it is not a component of risk behaviour but a manifestation of HIV infection.

Questionnaires were in English and administered by research assistants who had received training in HCT and had been providing HCT at the clinics prior to their involvement in this study. Questionnaire was administered to one participant at a time and this process took about 5 minutes to complete. This was done in a private space, mostly in the counseling room after the prospective participants had just received their negative rapid HIV test results. All the research assistants knew the commonly used languages in SA; hence it was easier that they administer the questionnaires. Participants were given an option of not answering questions that they were not comfortable with. The questionnaire data were then captured on an Excel spreadsheet using codes to simplify capturing. Quality assurance of data capturing included training all the research assistants who captured data into Excel, verification of correct codes in each column of Excel spreadsheet, and spot check verification comparing the original questionnaire documents to what was captured. Validation of the questionnaire tool was done in a subset of participants using a follow up questionnaire at a different time point. A few

questions from the original questionnaire were not included in the validation questionnaire as data for certain parameters would have changed with time (S3.2 Fig).

The study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number–295/2015) and by Tshwane Research Ethics Committee (TMREC 2010/26). The legal ages for consenting to HIV testing and medical treatment in SA are 12 and 14 years, respectively [19]. This study enrolled participants aged 14 years or older. The initial study protocol was approved for participants older than 18 years but it was later amended and approved to include those from 14 years of age to extend the benefits of screening for early HIV infection to the younger age groups, most of whom came to the clinics alone. All study participants agreed to participate and signed written consent forms before enrolment.

Statistical analysis

A descriptive analysis was used to present summary statistics (median, proportions and 95% confidence interval [CI]) for the parameters. Stratification of study participants was done according to general and antenatal HCT clinics, and then further stratification was done in each population group according to demographic and HIV risk factors. Univariate logistic regression was done on stratified data in order to identify factors that are associated with a risk of HIV infection. Distribution of newly identified HIV infections in each parameter was used to assess association with the risk of HIV. All factors associated with the risk of HIV infection in the univariate analysis, with *p*-value of 0.2 or less, were included in the multivariate logistic regression analysis. Association with the risk of HIV infection was further assessed using Fisher's exact test for factors that showed statistical significance on multivariate analysis. Questionnaire parameters that were not applicable to the whole general or pregnant population group were not included in the analysis, but a few of these were analysed separately such as

data for males. Comparison of the proportion of HIV infections for data on circumcision was done using Fisher's exact test. Missing data occurred at a low rate (≤2%) for most parameters and were excluded from the analysis. The only parameter with a high rate of missing data was the "number of current sexual partners" owing to amendment of the initial questionnaire, which assessed "number of lifetime sexual partners." This amendment was done early in the study after enrolling about 300 participants from the general HCT clinic, where the study was initially conducted. Missing data for this parameter were also excluded from the analysis. All the statistics were performed on the STATA version 14.2 software (StataCorp LP, College Station, TX, USA).

3.4 Results

3.4.1 Demographics

From March 2012 to June 2016, the study enrolled 9547 predominantly black participants (96.6%). Their median age was 27 years (interquartile range [IQR]: 23 – 31). There were 1661 non-pregnant participants largely enrolled from the general HCT clinic, while there were 7886 pregnant females largely enrolled from the antenatal clinics. Majority of the non-pregnant (general) population were males (54.7%). Similar trends of demographic and HIV risk factors were observed between the two population groups. For instance, most participants from both population groups were unmarried, had reported inconsistent use or no use of condoms, and had no knowledge of their partner's HIV status (Table 3.1). A significant proportion of participants with consistent condom use reported having had a recent condom burst. Few participants reported having multiple current sexual partners, recent STIs, sexual assault,

alcohol or other drugs consumption, and frequent travel. Those who reported use of other drugs only mentioned use of marijuana. Most men (99%) were heterosexual men and 54.3% said they were circumcised (Table 3.1). None of the study participants reported involvement in commercial sex work. Questionnaire validation was done in a small subset of pregnant participants (n = 133). The questionnaire data that was obtained at a different time point for validation purposes showed similar trends as the data collected at enrolment, indicating that the information obtained on the original questionnaire was reliable (S3.1 Table).

Table 3.1: Demographic and behavioural risk factors of all study participants

FACTORS	SUB-GROUPS	GENERAL POPULATION % (n = 1661)	PREGNANT POPULATION % (n = 7886)
Age	14 - 24 years	21.7 (361)	38.1 (3007)
	25 - 49 years	72.7 (1208)	61.9 (4879)
	50+ years	5.5 (92)	(0)
Gender	Males	54.7 (908)	n/a
	Females	45.3 (753)	100 (7886)
Marital status	Unmarried	72.5 (1205)	69.5 (5479)
	Married	27.4 (455)	30.4 (2400)
	No data	0.1 (1)	0.1 (7)
Condom use	Consistent*	11.4 (190)	0.5 (42)
	Inconsistent	43.1 (716)	51.9 (4092)
	No use	45.4 (754)	47.5 (3742)
	No data	0.1 (1)	0.1 (10)
Partner's HIV status	Positive	12.1 (201)	0.2 (14)
	Negative	25.0 (416)	36.7 (2898)
	Unknown	62.8 (1043)	63.0 (4968)
	No data	0.1 (1)	0.1 (6)
Number of current sexual partners	One	72.5 (1204)	99.6 (7855)
	Multiple	9.7 (162)	0.3 (22)
	No data	17.8 (295)	0.1 (9)
Recent STI (self-reported)	Yes	6.3 (104)	3.0 (240)
	No	93.6 (1555)	96.8 (7637)
	No data	0.1 (2)	0.1 (9)
Recent sexual assault	Yes	0.2 (3)	0.1 (8)
	No	99.7 (1656)	99.7 (7860)
	No data	0.1 (2)	0.2 (18)
Alcohol use	Yes	46.3 (770)	4.7 (373)
	No	53.6 (890)	95.2 (7506)
	No data	0.1 (1)	0.1 (7)
Other drugs	Yes†	1.4 (23)	(0)
	No	98.5 (1637)	99.9 (7876)
	No data	0.1 (1)	0.1 (10)
Frequent travel to other countries	Yes	6.6 (110)	2.0 (161)
	No	93.3 (1549)	97.8 (7716)
	No data	0.1 (2)	0.1 (9)
Male circumcision	Yes	54.3 (487)	n/a
	No	45.7 (410)	n/a
	No data	1.2 (11)	n/a
Sex with other men	Yes	1 (9)	n/a
	No	99 (881)	n/a
	No data	2.0 (18)	n/a

* = condom burst was reported by 52.1% (n = 99) of participants in the general population and by 90.5% (n = 38) in the pregnant population. STI = sexually transmitted infection, < = less than, † = only marijuana was reported, n/a- not applicable.

3.4.2 Newly diagnosed HIV infections

Of all the study participants (i.e. with negative rapid tests at enrolment), 61 tested positive on NAAT, resulting in 0.6% (95% CI: 0.5 – 0.8) of newly diagnosed HIV infections. These infections were identified in all 5 study clinics with variable prevalence, ranging from 0.3% to 1.2%. Serological characterisation was performed and data for the first 55 participants with newly diagnosed HIV infection have been published previously. Participants with newly diagnosed HIV infection consisted of individuals with either early HIV infection or chronic HIV infection, all of whom were missed by rapid tests at the points-of-care [18]. The median VL of participants with newly diagnosed infections was 17536 copies/ml (IQR: 4877 – 85037), and these participants also had other risk factors that enhance secondary transmission of HIV (S3.2 Table).

3.4.3 Risk factors and association with HIV infection

Trends of higher odds for HIV infection were observed with some demographic and HIV risk factors at univariate analysis (Table 3.2). However, multivariate analysis did not show statistical significance for all these factors except for recent sexual assault ($p = 0.001$, CI: 5.22 – 865.53) in the general population, but this was a weak finding as there were very few participants who reported recent sexual assault (Table 3.3).

Table 3.2: Univariate analysis of factors associated with HIV infection

		GENERAL POPULATION			PREGNANT POPULATION		
		HIV INFECTIONS	UNIVARIATE ANALYSIS		HIV INFECTIONS	UNIVARIATE ANALYSIS	
FACTORS	**SUB-GROUPS**	**% (*n* / sub-group)**	**ODDS ratio (95% CI)**	**P-value**	**% (*n* / sub-group)**	**ODDS ratio (95% CI)**	**P-value**
Age	14 - 24 years	0.8 (3/361)	1		0.5 (14/3007)	1	
	25 - 49 years	0.8 (10/1208)	1.0 (0.27 - 3.64)	0.995	0.7 (34/4879)	1.5 (0.80 – 2.80)	0.203
	50+ years	-- (0/92)	--		--		
Gender	Males	0.6 (5/908)	0.52 (0.18 – 1.58)	0.247	--		
	Females	1.1 (8/753)	1		0.6 (48/7886)	--	
Marital status	Unmarried	0.9 (11/1205)	2.09 (0.46 – 9.45)	0.340	0.7 (39/5479)	1.90 (0.92 – 3.94)	0.082
	Married	0.4 (2/455)	1		0.4 (9/2400)	1	
Condom use	Consistent	-- (0/190)	--		2.3 (1/43)*	5.05 (0.66 – 38.69)	0.119
	Inconsistent	0.8 (6/716)	1		0.7 (29/4091)	1.48 (0.82 - 2.66)	0.195
	No use	0.9 (7/754)	1.11 (0.37 – 3.31)	0.853	0.5 (18/3742)	1	
Partner's HIV Status	Positive	1.5 (3/201)	1.96 (0.51 - 7.45)	0.553	-- (0/14)	--	
	Negative	0.5 (2/416)	0.63 (0.13 - 2.96)	0.323	0.4 (13/2898)	0.63 (0.33 – 1.20)	0.163
	Unknown	0.8 (8/1043)	1		0.7 (35/4968)	1	
Number of current sexual partners	One	0.8 (10/1204)	1		0.6 (48/7855)	--	
	Multiple	0.6 (1/162)	0.82 (0.10 - 6.55)	0.855	-- (0/22)	--	
Recent STI (self-reported)	Yes	1.9 (2/104)	2.75 (0.60 – 12.58)	0.192	0.4 (1/240)	0.68 (0.93 – 4.92)	0.699
	No	0.7 (11/1555)	1		0.6 (47/7637)	1	
Recent sexual assault	Yes	33.3 (1/3)	68.5 (5.81 – 807.18)	0.001	-- (0/8)	--	
	No	0.7 (12/1656)	1		0.6 (48/7860)	--	
Alcohol use	Yes	0.6 (5/770)	0.72 (0.23 - 2.21)	0.567	0.5 (2/373)	0.87 (0.21 – 3.61)	0.853
	No	0.9 (8/890)	1		0.6 (46/7506)	1	
Other drugs	Yes[†]	4.3 (1/23)	6.16 (0.77 – 49.41)	0.087	-- (0)	--	
	No	0.7 (12/1637)	1		0.6 (48/7876)	--	
Frequent travel to other countries	Yes	1.8 (2/110)	2.59 (0.57 – 11.83)	0.220	-- (0/161)	--	
	No	0.7 (11/1549)	1		0.6 (48/7716)	--	

CI = confidence interval, **--** = not applicable, STI – sexually transmitted infection, * = the infected participant in this sub-group had reported condom burst, [†] = only marijuana was reported. Data on male circumcision and men who have sex with men were analysed separately.

Table 3.3: Multivariate analysis of factors associated with HIV infection

		GENERAL POPULATION			PREGNANT POPULATION		
		HIV INFECTIONS	MULTIVARIATE ANALYSIS		HIV INFECTIONS	MULTIVARIATE ANALYSIS	
FACTORS	**SUB-GROUPS**	**% (*n* / sub-group)**	**ODDS ratio (95% CI)**	**P-value**	**% (*n* / sub-group)**	**ODDS ratio (95% CI)**	**P-value**
Age	14 - 24 years	0.8 (3/361)	--		0.5 (14/3007)	1	
	25 - 49 years	0.8 (10/1208)	--		0.7 (34/4879)	1.82 (0.96 – 3.45)	0.066
	50+ years	-- (0/92)	--		--		
Gender	Males	0.6 (5/908)	--		--		
	Females	1.1 (8/753)	--		0.6 (48/7886)	--	
Marital status	Unmarried	0.9 (11/1205)	--		0.7 (39/5479)	2.00 (0.89 – 4.49)	0.091
	Married	0.4 (2/455)	--		0.4 (9/2400)	1	
Condom use	Consistent	-- (0/190)	--		2.3 (1/43)*	5.02 (0.63 – 39.66)	0.126
	Inconsistent	0.8 (6/716)	--		0.7 (29/4091)	1.15 (0.60 - 2.22)	0.674
	No use	0.9 (7/754)	--		0.5 (18/3742)	1	
Partner's HIV Status	Positive	1.5 (3/201)	--		-- (0/14)	--	
	Negative	0.5 (2/416)	--		0.4 (13/2898)	0.65 (0.34 – 1.25)	0.198
	Unknown	0.8 (8/1043)	--		0.7 (35/4968)	1	
Number of current sexual partners	One	0.8 (10/1204)	--		0.6 (48/7855)	--	
	Multiple	0.6 (1/162)	--		-- (0/22)	--	
Recent STI (self-reported)	Yes	1.9 (2/104)	2.07 (0.41 – 10.52)	0.382	0.4 (1/240)	--	
	No	0.7 (11/1555)	1		0.6 (47/7637)	--	
Recent sexual assault	Yes	33.3 (1/3)	67.2 (5.22 – 865.53)	0.001¥	-- (0/8)	--	
	No	0.7 (12/1656)	1		0.6 (48/7860)	--	
Alcohol use	Yes	0.6 (5/770)	--		0.5 (2/373)	--	
	No	0.9 (8/890)	--		0.6 (46/7506)	--	
Other drugs	Yes†	4.3 (1/23)	6.15 (0.74 – 51.07)	0.093	-- (0)	--	
	No	0.7 (12/1637)	1		0.6 (48/7876)	--	
Frequent travel to other countries	Yes	1.8 (2/110)	2.69 (0.57 – 12.62)	0.211	-- (0/161)	--	
	No	0.7 (11/1549)	1		0.6 (48/7716)	--	

CI = confidence interval, -- = not applicable, STI – sexually transmitted infection, * = the infected participant in this sub-group had reported condom burst, † = only marijuana was reported. ¥ = statistical significance was also observed on Fisher's exact test (p = 0.023).

Interestingly, no HIV infection was found in participants who reported consistent condom use in the general population despite a high rate of condom burst. The single participant who reported consistent condom use in the pregnant population and found to have HIV infection belonged to a sub-group of participants who reported recent condom burst (Table 3.2). A significantly lower risk of HIV infection was observed in circumcised men as there were no infections found in this group (n = 487) compared to 4 infections in uncircumcised males (n = 410 participants) (p = 0.04). A small proportion (1%) of men reported having sex with other men (Table 3.1), but no HIV infection was found among them.

3.4.4 Other HIV risk behaviours

There was a very high prevalence of recent unprotected sex among study participants as evidenced by the high rate of inconsistent use or no use of condoms and high rate of condom burst in those who reported consistent condom use (Table 3.1). HIV infections were also identified in some married participants and in the group of participants that reported to have HIV-negative partners (Table 3.2). Among the participants with newly diagnosed HIV infection who reported having HIV-positive partners, there was one whose partner was already on ARV therapy. One male participant (#7, Pt ID 5041) with concurrent sexual partners who was diagnosed with new HIV infection had four sexual partners and a very high HIV viral load of 22 million copies/mL (S3.2 Table).

3.5 Discussion

3.5.1 Demographics and HIV infections

This study assessed the prevalence of HIV risk behaviour among sexually active HIV sero-negative individuals who were screened for early HIV infection in the Tshwane district of SA. The finding of 0.6% newly diagnosed HIV infections in the whole study population highlights the problem of ongoing transmission of HIV despite the availability of many HIV prevention measures [1]. A higher prevalence of these infections in females compared to males in the general population (Table 3.2) was not surprising as HIV prevalence is generally higher in females [20]. The newly diagnosed HIV-infected participants were found in all the study clinics, highlighting a wide distribution of individuals who are capable of sustaining the HIV transmission in the setting where there is a high prevalence of unprotected sex (Table 3.1). It is not surprising that HIV infections were only observed in participants aged 14 - 49 years in this study (Table 3.2). This is consistent with other data that have showed a significantly higher incidence of HIV in sexually active people within this age group [20].

The trend of higher risk of HIV infection among unmarried individuals compared to the married ones (Table 3.2) has been observed in a previous South African study [21]. The finding of HIV infections among married participants has also been observed by many other studies in the sub-Saharan Africa [21-24]. Extramarital affairs could be the possible source of HIV infection in married couples [23]. Another possible explanation is if one partner was in the window period if the couple did HIV testing prior to marriage [18]. Individuals who are in sero-discordant relationships are at an increased risk of HIV acquisition especially when the VL of the infected partner is ≥1500 copies/mL [7]. The majority of participants with newly diagnosed HIV infection had VL above this threshold (S3.2 Table) showing a higher likelihood of secondary spread of HIV infection. It was not surprising to find that circumcision was

associated with a significantly reduced risk of HIV infection as it has been reported to offer protection against HIV acquisition [6, 25].

3.5.2 Condom use and HIV infections

The absence of HIV infection among participants who reported consistent condom use in the general population (Table 3.2) highlights the protective effect associated with consistent condom use. The high rate of condom burst reduces the protective effect of consistent condom use as observed in the pregnant population where a single HIV infection was found in the sub-group that reported consistent condom use. This highlights that a proper and consistent use of condoms is important for protective effect of condoms against HIV acquisition. When condoms are used properly, their effectiveness could be as high as 95% in preventing acquisition of HIV [26, 27]. Our study shows that consistency of condom use and recent history of condom burst should be measured by future HIV studies or surveys that look at the protective effect of condoms rather than measuring condom use at last sex. Attempts of reducing condom burst should be made in order to maximise the benefits of consistent condom use.

The risk of HIV infection between the group of participants with inconsistent condom use and the other with no condom use looked similar. This indicates that people with inconsistent condom use are as exposed to HIV infection as those who do not use condoms. Inconsistent use of condoms has been associated with high risk of acquiring HIV infection [26, 28]. Most HIV studies measure condom use at last sex as the proxy for protected sex [20, 29]. This may not be a good practice as people with inconsistent condom use may report having used condoms at last sex, while they were exposed to HIV at the times they did not use condoms. Some researchers have reported impressive rates of last sex condom use [20], but this did not seem

to translate to protection against HIV infection as the rate of new HIV infections remains high globally and in high endemic areas such as SA [1, 20].

3.5.3 Partner's HIV status and HIV infections

The finding that majority of the study participants in both population groups did not know their partner's HIV status (Table 3.1) reflects the observation from other studies that most people are unaware of their HIV status and/or that of their sexual partners [24, 30]. This reflects on low rate of couples' HIV testing, which has been reported for most countries in the sub-Saharan Africa [24]. Couples' HCT is an effective way of counselling as it ensures that both partners know their HIV status, and thus reduces the risk of HIV transmission [22, 24, 31]. Our study data highlight a need to promote couple's HCT in South Africa. Newly diagnosed HIV infections were also identified among participants who reported that their partner's HIV status was negative (Table 3.2). The reason for this could be that their partners were in the window period or had false-negative HIV test results the last time they tested for HIV [18], or that they lied about their HIV status. The diagnosis of HIV infection in a participant whose partner was already on ARV therapy indicates a risk for acquisition of HIV drug resistant virus. South Africa has the biggest ARV programme in the world [32], and has a substantial number of patients who have failed first line regimen [33]. This puts individuals who are exposed to HIV at high risk of acquiring HIV drug-resistant mutants, which compromise response to ARV therapy. Transmission of HIV drug-resistant strains has been reported in SA and other sub-Saharan African countries [9, 34].

3.5.4 Number of current sexual partners and HIV infections

A low rate of concurrent sexual partnerships was observed in this study (Table 3.1). The diagnosis of HIV infection in a male participant who reported having four concurrent sexual partners and found to have a very high HIV viral load of 22 million copies/ml (S3.2 Table) highlights the potential of concurrent sexual partnerships to exponentially increase the number of individuals infected with HIV. Concurrent sexual relationships play a significant role in HIV transmission and epidemiology even though they occur at a lower rate in some settings. A study conducted in Botswana observed a similar trend of a lower rate of concurrent sexual partnerships compared to monogamous partnerships [35]. Some of the participants with newly diagnosed HIV infection reported having changed the sexual partners by the time they came back for follow up. This observation was also made with some HIV-uninfected individuals who were included in the questionnaire validation arm of the study. This could be an indication that although most people are in monogamous relationships, there is a substantially higher rate of sexual partner exchange or serial monogamous relationships, which favours transmission of HIV. Both serial monogamous relationships and concurrent sexual relationships have been implicated in driving the HIV epidemiology in sub-Saharan Africa [36, 37].

3.5.5 Trends on multiple behaviours and HIV infections

Only a small proportion of participants reported sexual assault, and this was associated with a significantly higher risk of HIV infection in the general population. This is probably caused by the fact that abused individuals are not provided with options for safe sex [38]. Even though there was lack of significant association with HIV infection found with other risk factors such as STIs, travel history, and use of alcohol or other drugs use; there are published data that have

shown a higher risk of HIV infection associated with these factors [8, 39, 40]. It is not uncommon to observe non-significant trends of HIV association with risk factors in behavioural studies. The complexity of understanding the specific behaviours that significantly contribute to HIV acquisition has been observed in other studies. These studies, like ours, failed to show statistically significant findings despite observing trends associating HIV infection with certain behavioural risk factors [10, 21, 36, 37]. Coates et al summed up this phenomenon by saying – "failure to show that a specific strategy reduces HIV infections does not render it useless in a comprehensive programme or a multilevel behavioural strategy for HIV prevention [10]." HIV risk behaviour studies probably suffer from the dynamics of a relationship, where a risk is determined by the total couple's behaviour. Thus, enrolling into a study the partner with less behavioural risk(s) who is infected with HIV due to the other partner's behaviour could easily mislead the interpretation of study data. This is reflected in certain parameters in this study, such as identification of HIV infections in married participants and in those who reported that their partners were HIV-negative (Table 3.2).

3.5.6 HIV risk assessment during counselling

Currently, there is no tool used for assessing or capturing HIV risk data during counselling in most South African HCT clinics. Thus, it is difficult to know if all counsellors assess HIV risk factors and address such factors during counselling for each and every individual. A study that assessed the quality of HIV counselling in South Africa noted that HIV risk assessment and reduction were amongst key aspects of HIV that were not adequately discussed with clients during counselling. Instead, counselling mostly focused on details of HIV testing procedure and results interpretation [41]. Another recently published study conducted in South Africa observed that there was no improvement in HIV knowledge after counselling. This was

attributed to quality assurance issues during the counselling process and to possible HCT inconsistencies [29]. Our study suggests that a questionnaire could be used to standardise counselling, whether it is used to collect risk behaviour data for surveillance or just as a reference tool to identify and address HIV risk factors during counselling. Other researchers have also shown that a tool used to collect information on risk behaviour could help in identifying risk of HIV acquisition [42, 43].

The limitations of this study are that it had a cross-sectional design, had fewer participants from the general HCT clinic, only enrolled participants who had negative rapid HIV tests who were screened for early HIV infection, and that it did not assess all the factors that can influence the risk of acquiring HIV such as educational level, socio-economic status and cultural or societal norms. Analysis of some findings may have been limited by the small number of newly identified HIV infections found in this study or small number of individuals who reported to have a certain HIV risk factor. There was also a lack of data on serial monogamous relationships for the majority of participants. Even though the study questionnaire was validated there could still be some inaccuracies in the behavioural data obtained from the participants owing to perceived sensitivity and stigma on certain questions in the context where questionnaires were administered by research assistants.

3.6 Conclusions

Our data show that a large segment of sexually active South Africans in the Tshwane district have high risk exposure to HIV and that combination of risk factors play a role in HIV acquisition. The detection of newly diagnosed HIV infections in all study clinics reflects a wide distribution of individuals who are capable of sustaining HIV transmission in the setting where HIV risk behaviour is highly prevalent. This study also suggests that a questionnaire tool that captures data on HIV risk behaviour would be useful during HIV testing and counselling. This could ensure that there is a systematic way of identifying HIV risk factors during HCT such that counselling is optimised to identify and address the relevant risk factors for each individual. Systematic collection of HIV risk data during counselling would help to identify individuals who are eligible for biomedical interventions such as pre-exposure prophylaxis and circumcision. These data could also be used to inform relevant HIV prevention interventions that could be implemented at a community or population level. For instance, home-based HIV testing could be promoted in areas where most people are unaware of their partner's HIV status as noted in this study. Continuous surveillance of HIV risk behaviour is needed to assess the risk of HIV acquisition in communities and the impact of HIV prevention measures.

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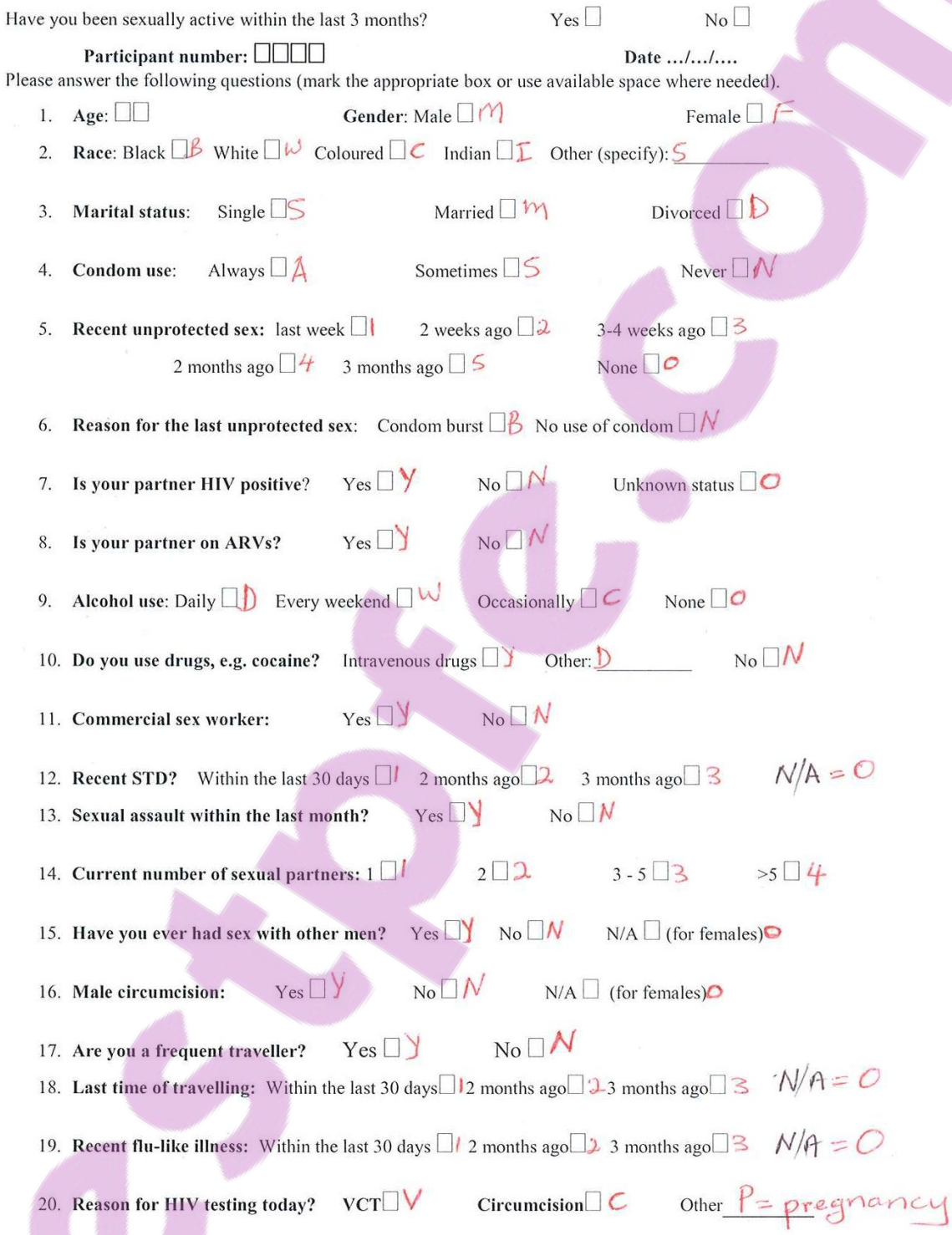
Supporting Information:



Have you been sexually active within the last 3 months? Yes ☐ No ☐

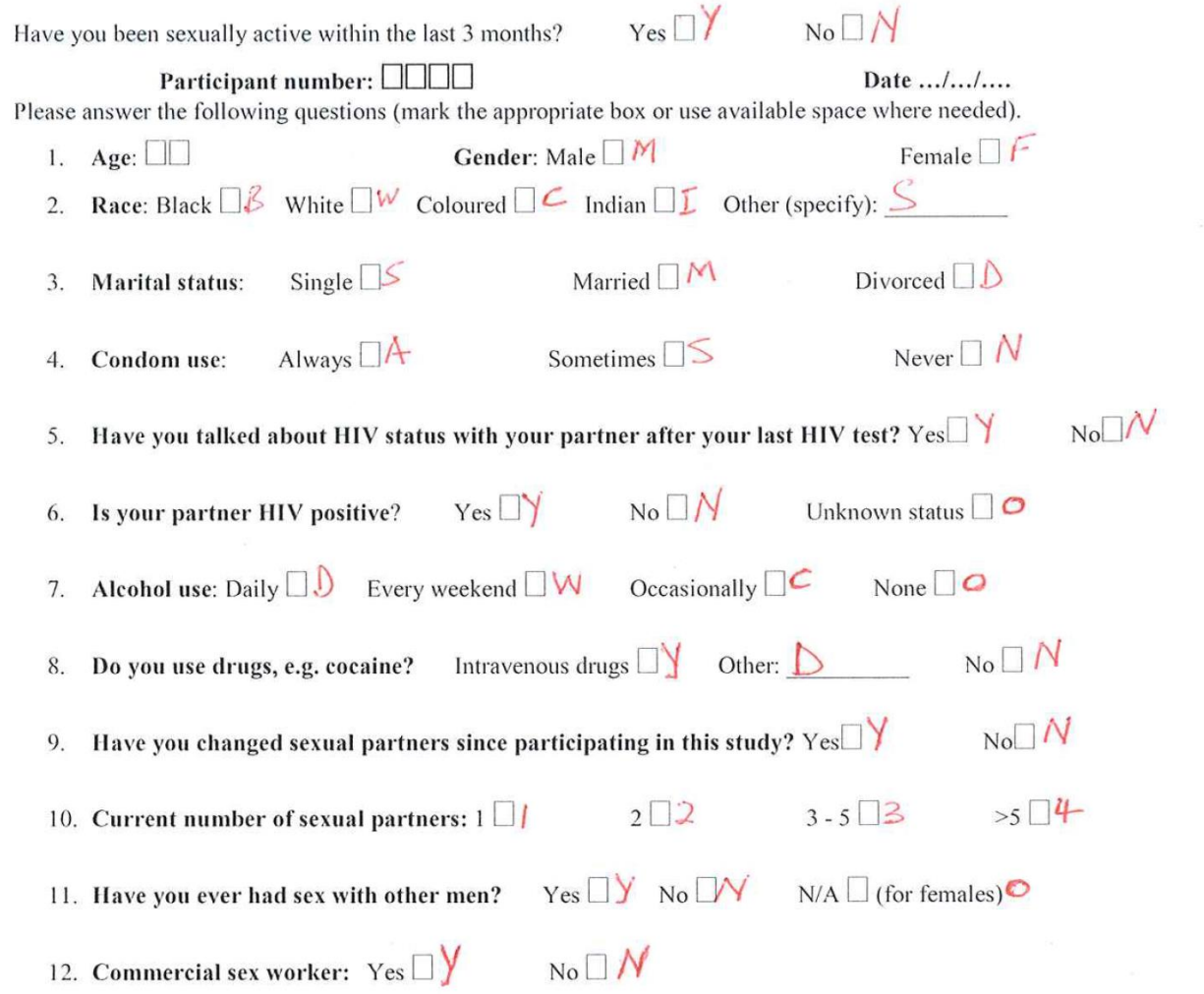
Participant number: ☐☐☐☐ **Date** .../.../....

Please answer the following questions (mark the appropriate box or use available space where needed).

1. **Age:** ☐☐ **Gender:** Male ☐M Female ☐F
2. **Race:** Black ☐B White ☐W Coloured ☐C Indian ☐I Other (specify): S
3. **Marital status:** Single ☐S Married ☐M Divorced ☐D
4. **Condom use:** Always ☐A Sometimes ☐S Never ☐N
5. **Recent unprotected sex:** last week ☐1 2 weeks ago ☐2 3-4 weeks ago ☐3 2 months ago ☐4 3 months ago ☐5 None ☐0
6. **Reason for the last unprotected sex:** Condom burst ☐B No use of condom ☐N
7. **Is your partner HIV positive?** Yes ☐Y No ☐N Unknown status ☐O
8. **Is your partner on ARVs?** Yes ☐Y No ☐N
9. **Alcohol use:** Daily ☐D Every weekend ☐W Occasionally ☐C None ☐O
10. **Do you use drugs, e.g. cocaine?** Intravenous drugs ☐I Other: D No ☐N
11. **Commercial sex worker:** Yes ☐Y No ☐N
12. **Recent STD?** Within the last 30 days ☐1 2 months ago ☐2 3 months ago ☐3 N/A = 0
13. **Sexual assault within the last month?** Yes ☐Y No ☐N
14. **Current number of sexual partners:** 1 ☐1 2 ☐2 3 - 5 ☐3 >5 ☐4
15. **Have you ever had sex with other men?** Yes ☐Y No ☐N N/A ☐ (for females) O
16. **Male circumcision:** Yes ☐Y No ☐N N/A ☐ (for females) O
17. **Are you a frequent traveller?** Yes ☐Y No ☐N
18. **Last time of travelling:** Within the last 30 days ☐1 2 months ago ☐2 3 months ago ☐3 N/A = 0
19. **Recent flu-like illness:** Within the last 30 days ☐1 2 months ago ☐2 3 months ago ☐3 N/A = 0
20. **Reason for HIV testing today?** VCT ☐V **Circumcision** ☐C Other P = pregnancy

S3.1 Fig. A questionnaire tool that was used to collect demographic and HIV risk factors from the study participants. Codes (in red) were used to capture questionnaire data into the Excel spreadsheet. N/A – not applicable was used for participants who had no recent sexually transmitted diseases (number 12) and this also applied for parameters on number 18 and 19. Questionnaire parameters (numbers 5, 6, 8, 15 and 18) that were not

applicable to the whole general or pregnant population group were not included in the group's analysis but some of them were analysed separately.



Have you been sexually active within the last 3 months? Yes ☐Y No ☐N

Participant number: ☐☐☐☐ **Date …/…/….**

Please answer the following questions (mark the appropriate box or use available space where needed).

1. **Age:** ☐☐ **Gender**: Male ☐M Female ☐F
2. **Race**: Black ☐B White ☐W Coloured ☐C Indian ☐I Other (specify): S
3. **Marital status**: Single ☐S Married ☐M Divorced ☐D
4. **Condom use**: Always ☐A Sometimes ☐S Never ☐N
5. **Have you talked about HIV status with your partner after your last HIV test?** Yes ☐Y No ☐N
6. **Is your partner HIV positive?** Yes ☐Y No ☐N Unknown status ☐O
7. **Alcohol use**: Daily ☐D Every weekend ☐W Occasionally ☐C None ☐O
8. **Do you use drugs, e.g. cocaine?** Intravenous drugs ☐Y Other: D No ☐N
9. **Have you changed sexual partners since participating in this study?** Yes ☐Y No ☐N
10. **Current number of sexual partners:** 1 ☐1 2 ☐2 3 - 5 ☐3 >5 ☐4
11. **Have you ever had sex with other men?** Yes ☐Y No ☐N N/A ☐ (for females) O
12. **Commercial sex worker:** Yes ☐Y No ☐N

S3.2 Fig. A questionnaire tool that was used to collect data for validation. This data was collected at a follow up visit.

S3.1 Table. Validation questionnaire data obtained at a different time point

FACTORS	SUB-GROUPS	PARTICIPANTS % (n)
Age	14 - 24 years	34.6 (46)
	25 - 49 years	65.4 (87)
	50+ years	--
Gender	Males	--
	Females	100 (133)
Marital status	Unmarried	72.2 (96)
	Married	27.8 (37)
Condom use	Consistent	1.5 (2)
	Inconsistent	50.8 (67)
	No use	57.1 (63)
Partner's HIV status	Positive	2.3 (3)
	Negative	40.6 (54)
	Unknown	63.0 (76)
Number of current sexual partners	One	100 (132)
	Multiple	--
Alcohol use	Yes	0.7 (1)
	No	99.3 (132)
Other drugs	Yes	--
	No	100 (133)

S3.2 Table. Some behavioural and biologic characteristics of participants with newly diagnosed HIV infection

	Pt ID	Age	Gender	Race	Marital Status	Frequency of condom use	HIV status of the partner	Alcohol use	Marijuana use	Recent STI	Current number of sexual partners	Frequent travel to other countries	HIV viral loads (copies/ml)
1	9498	34	F	B	S	No	Neg	No	No	No	ND	No	5.0×10^5
2	9218	30	F	B	D	Incon	Neg	No	No	Yes	ND	Yes	5.5×10^4
3	9228	28	M	B	S	No	Unk	Occ	Yes	Yes	1	Yes	9.4×10^1
4	2066	22	F	B	S	Incon	Unk	Wknd	No	No	ND	No	1.0×10^3
5	8575	27	F	B	S	Incon	Unk	No	No	No	1	No	9.3×10^4
6	7293	21	F	B	S	Incon	Neg	Occ	No	No	1	No	5.0×10^3
7	5041	23	M	B	S	Incon	Unk	Occ	No	No	4	No	2.2×10^7
8	8047	31	M	B	M	No	Unk	Occ	No	No	1	No	1.2×10^6
9	9049	20	F	B	S	Incon	Unk	No	No	No	1	No	1.6×10^4
10	6638	28	F	B	S	Incon	Unk	No	No	No	1	No	1.9×10^5
11	261	33	M	B	S	No	Pos	Wknd	No	No	1	No	8.4×10^7
12	6512	23	F	B	S	Incon	Unk	No	No	No	1	No	1.7×10^3
13	6743	26	F	B	S	Incon	Unk	No	No	No	1	No	2.7×10^4
14	6582	24	F	B	S	No	Unk	No	No	No	1	No	6.2×10^3
15	6727	28	F	B	S	Incon	Unk	No	No	No	1	No	4.8×10^3
16	6737	24	F	B	S	Incon	Unk	No	No	No	1	No	2.2×10^3
17	7084	28	F	B	S	No	Unk	No	No	No	1	No	3.3×10^8
18	2504	24	F	B	S	Incon	Unk	No	No	No	1	No	3.7×10^4
19	3469	20	F	B	S	No	Neg	No	No	No	1	No	3.3×10^4
20	5054	26	F	B	S	No	Unk	No	No	No	1	No	2.7×10^4
21	5067	20	F	B	S	No	Unk	No	No	No	1	No	1.2×10^4
22	9915	30	F	B	M	No	Unk	No	No	No	1	No	1.4×10^4
23	4351	35	F	B	S	No	Unk	No	No	No	1	No	2.6×10^3
24	639	30	F	B	S	No	Unk	No	No	No	1	No	6.5×10^3
25	641	30	F	B	S	Incon	Neg	No	No	No	1	No	7.0×10^4
26	7959	40	F	B	S	Incon	Pos	No	No	No	1	No	1.4×10^5
27	8828	27	F	B	M	Incon	Unk	No	No	No	1	No	4.1×10^4
28	2678	30	F	B	M	No	Unk	No	No	No	1	No	2.2×10^5
29	9895	31	F	B	M	No	Unk	No	No	No	1	No	4.8×10^3
30	9986	28	F	B	S	No	Unk	No	No	No	1	No	9.7×10^4
31	843	21	F	B	S	Incon	Pos	No	No	No	1	No	2.9×10^4
32	6990	26	F	B	S	Incon	Unk	No	No	No	1	No	1.7×10^4
33	2340	22	F	B	S	Incon	Unk	No	No	No	1	No	1.4×10^4
34	6709	28	F	W	S	Incon	Unk	No	No	No	1	No	5.3×10^1
35	6748	28	F	B	S	Incon	Unk	No	No	No	1	No	9.3×10^2
36	6671	35	F	B	M	No	Unk	No	No	No	1	No	1.4×10^4
37	6380	25	F	B	S	Incon	Unk	No	No	No	1	No	1.1×10^4
38	6557	38	F	B	S	No	Unk	No	No	No	1	No	6.1×10^2
39	6565	28	F	B	S	Incon	Unk	No	No	No	1	No	5.6×10^3
40	6509	36	M	B	M	No	Pos	No	No	No	1	No	1.0×10^5
41	6596	31	F	B	S	Incon	Unk	No	No	No	1	No	3.8×10^3
42	6640	37	F	B	M	No	Unk	No	No	No	1	No	3.0×10^3
43	6649	32	F	B	M	No	Unk	No	No	No	1	No	2.1×10^4
44	6738	28	F	B	S	No	Unk	No	No	No	1	No	1.5×10^5
45	1067	19	F	B	S	Con	Unk	Occ	No	No	1	No	1.7×10^3
46	921	44	F	B	M	Incon	Neg	No	No	No	1	No	9.7×10^3
47	3869	32	F	B	S	Incon	Unk	No	No	Yes	1	No	2.1×10^5
48	3912	27	F	B	S	Incon	Neg	No	No	No	1	No	3.2×10^4
49	3920	20	F	B	S	Incon	Neg	No	No	No	1	No	6.6×10^4
50	3880	30	F	B	S	Incon	Neg	No	No	No	1	No	7.5×10^3
51	3935	33	F	B	S	No	Unk	No	No	No	1	No	2.4×10^5
52	1117	37	F	B	M	Incon	Unk	No	No	No	1	No	1.5×10^2
53	1121	27	F	B	S	Incon	Unk	No	No	No	1	No	8.0×10^4
54	3474	21	F	B	S	Incon	Neg	No	No	No	1	No	1.6×10^4
55	1475	32	F	B	S	No	Neg	No	No	No	1	No	4.4×10^4
56	3387	37	F	B	S	No	Neg	No	No	No	1	No	7.9×10^4
57	3253	28	F	B	S	Incon	Neg	No	No	No	1	No	8.9×10^4
58	1692	18	F	B	S	Incon	Unk	No	No	No	1	No	3.9×10^2

59	3606	24	F	B	S	Incon	Unk	No	No	No	1	No	1.5×10^4
60	2866	35	F	B	S	Incon	Unk	No	No	No	1	No	3.3×10^3
61	1213	36	F	B	S	No	Neg	No	No	No	1	No	3.2×10^4

Pt ID – participant identity, B – black, W – white, S – single, M – male or married, D – divorced, Incon – inconsistent, Con – consistent, Neg – negative, Pos – positive, Unk – unknown, Occ – occasionally, Wknd – every weekend, STI – sexually transmitted infection. ND – no data.

CHAPTER 4

Transmitted Minority and Majority Antiretroviral Drug-Resistance Variants of HIV in the Tshwane District of South Africa

Manuscript under review.

4.1 Abstract

Background: Transmission of antiretroviral (ARV) drug-resistant strains compromises the effectiveness of first-line ARV regimen.

Objective: This study aimed to assess transmission of drug-resistant strains among individuals with early HIV infection.

Study design: Individuals with early HIV infection were identified from five clinics in the Tshwane district of South Africa. Follow-up samples were obtained from them before antiretroviral (ARV) treatment initiation. Complete polymerase (POL) gene was amplified using an in-house nested PCR. Sanger sequencing was performed on baseline samples, while Illumina deep sequencing was performed on both baseline and follow-up samples.

Results: Twenty-two participants were enrolled, with a median age of 26 years (interquartile range: 23 - 29). POL gene was successfully amplified from 17 participants, 10 of whom had sample pairs. Sanger detected transmitted resistance in 11.7% (2/17) of participants. Using ≥1% threshold for transmitted variants, Illumina detected resistance in 20.0% (3/15) of

participants at baseline, and in 33.3% (4/12) of participants at follow-up. There were three sample pairs that only had detectable resistance mutations at follow-up, ranging from 1.3 – 8.6%. Mutations identified at baseline were K103N and T66I, and those identified at follow-up were Y188H, N83D, Y143H and Q148R.

Conclusions: Illumina deep sequencing detected a higher rate of transmitted ARV drug-resistance. Emergence of minority variants in some sample pairs, without ARV drug pressure, highlights their evolution and potential clinical relevance. More studies are needed for further assessment of transmitted ARV drug-resistant strains using deep sequencing.

4.2 Background

The global scale up of antiretroviral (ARV) treatment has led to a decline in AIDS-related deaths and new HIV infections [1]. Unfortunately, this expanded use of ARVs has also led to an increasing trend of ARV drug-resistance prevalence and transmission of drug-resistant strains of HIV [2-4].

Baseline drug-resistance testing is not performed in developing countries with limited resources [5-7], thus individuals who are primarily infected by drug-resistant strains of HIV are likely to have poor response to the first-line regimen [8]. Variants existing below 20% in a virus population are generally missed by Sanger sequencing [9], however, these are detectable by deep sequencing technologies. A 1% threshold is generally considered acceptable for detection of clinically relevant minority variants [9, 10].

South Africa has had a massive scale up of ARV treatment in recent years [11], and has been using a non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing first-line regimen since its first national guidelines in 2004. Evolution of these guidelines included substituting stavudine with tenofovir in 2010, and stopping use of nevirapine (NVP) as a preferred NNRTI drug in the first-line regimen in 2015 [5, 12, 13]. Several studies in SA have detected ARV treatment failure in patients who were on first or second line ARV regimen [14-16], highlighting the risk of transmission of ARV drug-resistant strains. The World Health Organisation (WHO) recommends that first-line ARV regimen changes be considered when there is transmitted or pre-treatment drug-resistance prevalence above 10% [17].

4.3 Objective

The aim of this study was to assess the transmission of ARV drug-resistant strains of HIV among individuals with early HIV infection.

4.4 Study design

4.4.1 Study population

Twenty-two participants with early HIV infection were identified from a study that screened for early HIV infections between 2012 and 2016 in the Tshwane region of SA. Follow-up samples were obtained from them before referral for ARV treatment [18]. All the participants' plasma samples were stored at -70 ºC after enrollment. The study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number–295/2015) and by Tshwane–Metsweding Region Research Ethics Committee (TMREC 2010/26). All participants provided written informed consent.

4.4.2 Extraction of nucleic acids

QIAamp UltraSens Virus kit (Qiagen, Hilden, Germany) was used for extraction of total nucleic acids from plasma samples using a 1 millilitre (ml) input volume for samples with viral load (VL) ≤1000 copies/ml, and 0.5 ml (adjusted to 1 ml using phosphate-buffered saline) for those with VL >1000 copies/ml. Elution was done in a volume of 60 microlitres (µls) and eluates were stored at -70 ºC immediately after extraction.

4.4.3 In-house nested PCR

PCR primers targeting the complete polymerase (POL) gene of HIV-1 were designed from a database that contained a total of 225 HIV-1 full genome reference sequences representing all the HIV-1 groups but predominantly had group M strains (88%), obtained from Los Alamos (www.hiv.lanl.gov) and NCBI (www.ncbi.nlm.nih.gov) websites. Conserved areas were identified for primer design. HXB2 reference strain (K03455) was used for numbering. The following primers were used to amplify the POL gene in most samples; SM-F1 (outer forward) 5'-GCG GCT ACA CTA GAA GAA ATG ATG-3' (1807-30) and SM-R1 (outer reverse) 5'-GTG CCA AGT ATT GTA GAG ATC CTA CCT-3' (5462-88), SM-F3 (inner forward) 5'-AGA ATT GTT AAG TGT TTC AAC TGT GG-3' (1952-76) and SM-R3 (inner reverse) 5'-CTC CTG TAT GCA AAC CCC AAT A-3' (5245-66). The nested primers amplified a 3268 bp fragment. Alternative primers were used in few cases of amplification failure (Supplementary Table 4.1).

SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, California, USA) was used for cDNA synthesis and first round PCR in a 50 µl reaction, which included 5 µls of extracted RNA / DNA template, 10X high fidelity buffer, 50 mM magnesium sulphate (MgSO4), 10 mM deoxynucleotide triphosphates (dNTPs), 10 mM sense primer, 10 mM anti-sense primer, 5 units (U) of enzyme mix, and nuclease free water. Nested PCR was also done in a 50 µl reaction using 0.5 µl of the pre-nested template, 1U of Platinum Taq polymerase, and similar concentrations of reagents mentioned above with the appropriate nuclease free water volume adjustment (www.lifetechnologies.com) (see Supplementary Material for PCR conditions). HIV-negative and -positive controls were included in each experiment.

4.4.4 Sanger Sequencing

All the 2nd round PCR products for baseline samples were sent for Sanger sequencing at Inqaba Biotechnical Industries, which was done in 5 overlapping regions (Supplementary Material). Only two follow-up samples were sequenced by Sanger as there was amplification failure on their corresponding baseline samples owing to low viral load. Sequence analysis and generation of contiguous sequences were done on CLC Main Workbench version 7.8 (www.qiagenbioinformatics.com). MAFFT online version 7.310 program was used for sequence alignment.

4.4.5 Illumina MiSeq sequencing

Deep sequencing was performed on 2nd round PCR products for baseline and follow-up samples at Inqaba Biotechnical Industries. Samples that had a non-specific band were gel-purified using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, California, USA) before they were sent for deep sequencing. Samples were fragmented using Covaris ultrasonication approach (www.covaris.com). Resulting DNA fragments were size selected (300 – 800 bp) using AMPure XP beads (www.beckmancoulter.com). The fragments were then end repaired and Illumina specific adapter sequences were ligated to each fragment. Each sample was individually indexed, and a second size selection step was performed. Samples were then quantified using a fluorometric method, diluted to a standard concentration (4 nanomolar) and then sequenced on Illumina's MiSeq platform using a MiSeq version 3 (600 cycle) kit, following manufacturer's standard protocol (www.illumina.com).

All the samples were processed in one sequencing batch. Illumina MiSeq sequencing data was analysed on three different pipelines; PASeq, HyDRA and DeepChekHIV [19]. During analysis on PASeq, the feature for excluding APOBEC-induced mutations was always used. Quality of generated sequences was assessed on the PASeq pipeline and CLC Genomics

Workbench 11.0 (www.qiagenbioinformatics.com). Transmitted variants were assessed using ≥1% threshold value. Only variants called by all three pipelines were reported. PASeq-generated consensus sequences were used for constructing a phylogenetic tree on MEGA v7.0.21 using Neighbor-Joining statistical method (with a 1,000 bootstrap replicates). Illumina sequencing data were further analysed on Stanford's staging website (http://staging.hivdb.org/hivdb/by-reads/) for assessing the frequency of unusual and APOBEC-induced mutations in samples with drug-resistance minority variants.

4.4.6 Transmitted Drug-resistance Assessment

HIV drug-resistance for Sanger sequences was assessed on HIV Drug-resistance Database version 8.4 on Stanford's website (https://hivdb.stanford.edu/hivdb/by-mutations/). Reporting of mutations identified from Sanger and Illumina MiSeq was done according to the WHO list for surveillance of drug-resistance mutations (SDRMs) [20]. Although INSTI resistance mutations are not yet part of this list, all the major INSTI drug-resistance mutations identified in this study were considered as part of SDRMs.

4.5 Results

4.5.1 Demographics and PCR results

Twenty-two black participants with confirmed early HIV infection were enrolled, with a median age of 26 years (IQR: 23 - 29). Majority (81.8%, n = 18) were female participants, most of whom were pregnant (66.7%, n = 12). The median VL amongst all participants was 30319 copies/ml (IQR: 4874 – 195105). Of the 22 participants with early HIV infection, 17 (77.3%) had successful POL gene amplification by in-house nested PCR. The sensitivity of the PCR

was estimated to be 2628 copies/ml (Supplementary Fig. 4.1), however, there were few samples with VL below this threshold that were successfully amplified.

4.5.2 Sequencing and phylogenetic analysis

Illumina deep sequencing was performed on both baseline and follow-up samples. All sequences were of satisfactory quality, with average quality scores of >20 for both forward and reverse reads. All study sequences clustered with subtype C reference strains except for one, which clustered with AG recombinant reference strain. Sequences from sample pairs significantly clustered together (Fig. 4.1).

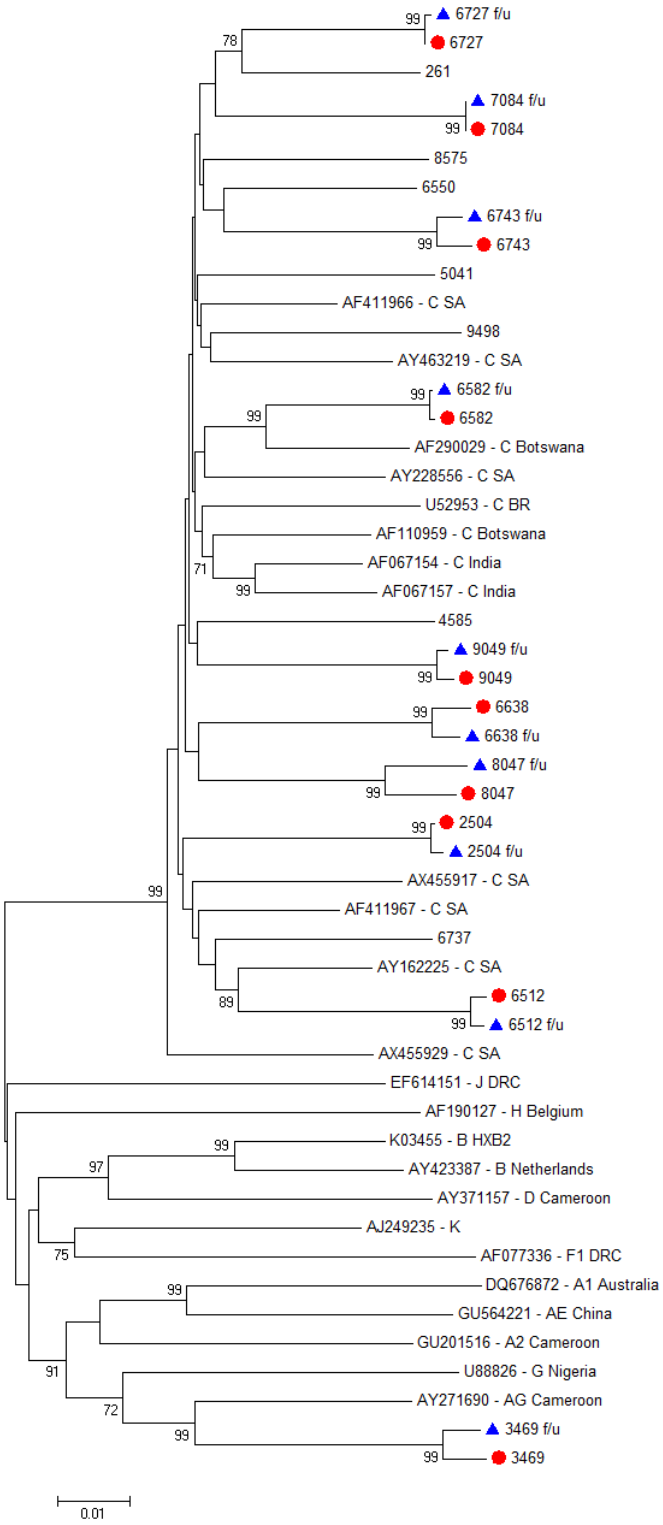


Figure 4.1: Neighbour-Joining phylogenetic tree of the 27 PASeq-generated consensus sequences obtained from 17 participants. Red dots represent baseline sequences, and blue triangles represent follow-up (f/u) sequences. Only bootstrap values >70% are shown as this was the cut-off used for significant clustering. All sequences obtained from sample pairs had 99% bootstrap values. Some participants had no sample pairs.

The three analysis pipelines (PASeq, HyDRA and DeepChekHIV) had good agreement above 20% and 5% thresholds, but gave highly discrepant results around 1% threshold. The strategy of reporting variants that are called by all three pipelines eliminated a lot of variants at 1% threshold that were called by HyDRA and DeepChekHIV (Fig. 4.2).

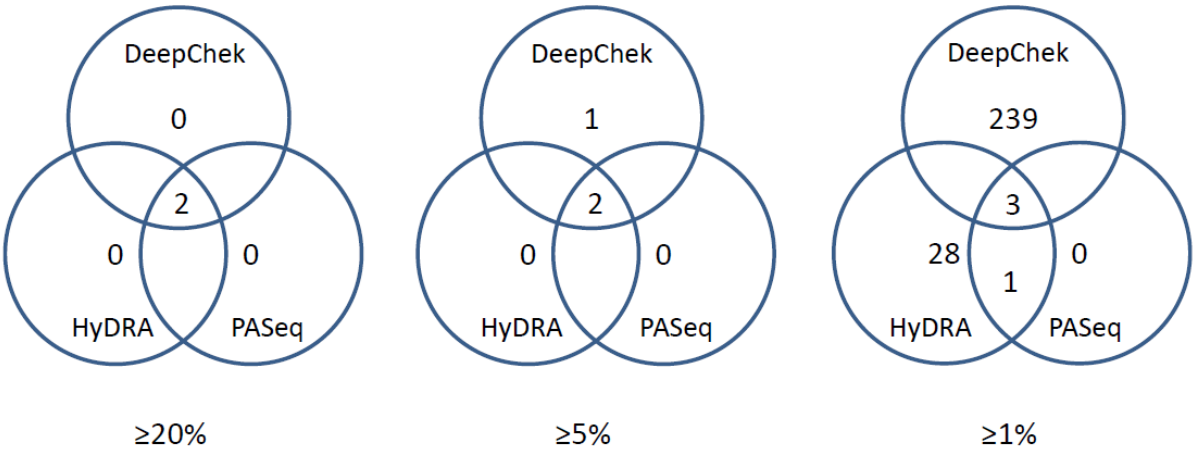


Figure 4.2: Deep sequencing data analysis showing agreement and discrepancies among PASeq, HyDRA and DeepChekHIV pipelines. Deep sequencing was performed in 27 samples belonging to 17 study participants. Only consensus transmitted ARV drug-resistance mutations among these 3 pipelines were reported.

Sanger sequencing only detected K103N mutation in 11.7% (2/17) of participants. Using ≥1% threshold for transmitted variants, Illumina detected resistance in 20.0% (3/15) of participants at baseline, and in 33.3% (4/12) participants at follow-up. There were three sample pairs that only had detectable resistance mutations at follow-up, ranging from 1.3 – 8.6%. Mutations identified at baseline were K103N and T66I, and those identified at follow-up were Y188H, N83D, Y143H and Q148R (Table 4.1). There was a low frequency of unusual mutations observed at 1% analysis threshold in samples that had minority variants, highlighting that these minority variants were unlikely to have been caused by PCR errors. APOBEC-induced drug-resistance mutations were not observed (Supplementary Fig. 4.2).

Table 4.1. Detected minority and majority variants at baseline and follow-up samples

Pt ID	VL results (copies/ml)	SANGER results	ANALYSIS AT ≥1% THRESHOLD (Illumina) PASeq (HyDRA / DeepChek) baseline results	F/U interval (wks)	ANALYSIS AT ≥1% THRESHOLD (Illumina) PASeq (HyDRA / DeepChek) follow-up results
9498	509793	nd	nd	LTF	n/a
9228	94	nd†	failed PCR	10	nd
2066	1010	nd†	failed PCR	7	Y188H - 11.4 (11.2 / 11.1)
8575	93079	nd	nd	8	failed PCR
5041	22921300	K103N	K103N ≥ 95.0 (by all 3 pipelines)	LTF	n/a
8047	1245238	nd	nd	2	nd
9049	16848	nd	T66I - 4.4 (3.6 / 5.8)	3	nd
6638	195105	nd	nd	6	nd
261	84501600	K103N	K103N ≥ 95.0 (by all 3 pipelines)	LTF	n/a
6512	1763	nd	nd	2	nd
6743	27364	nd	*N83D – 0.04 (nd / 0.07)	7	N83D - 1.3 (1.4 / 1.4)
6582	6216	nd	nd	6	nd
6727	4874	nd	nd	2	nd
6737	2227	nd	nd	LTF	n/a
7084	337987200	nd	nd	4	nd
2504	37243	nd	*Y188H - 0.03 (nd / 0.08), *Y143H - 0.07 (nd / 0.58)	2	Y188H - 2.4 (2.2 / 2.5), Y143H - 8.6 (8.5 / 8.5)
3469	33274	nd	*Q148R - 0.4 (nd / 0. 4)	9	Q148R - 1.8 (1.6 / 1.8)

† - Sanger sequencing was done from a follow-up sample. Participants 9228 and 2066 had higher viral loads (71 674 copies/ml and 38 900 copies/ml, respectively) on follow-up samples, hence amplification succeeded on these samples [18]. * - values below 1% are only reported for mutations that existed above 1% analysis threshold in the f/u sample of the pair. Pt – participant, ID – identity, VL – viral load, ml – millilitres, F/U – follow-up, wks – weeks, nd – not detected, n/a – not applicable. Amplification failure on sample 9430 was probably due to markedly reduced VL after 8 weeks of follow-up during early HIV infection (VL was not measured on this sample).

There was a good coverage depth, >4000, for all the identified mutations (Table 4.2). Other drug-resistance and accessory mutations such as E138A and K20R were observed, and a trend of emergence of minority variants only at follow-up was observed in some sample pairs (Supplementary Table 4.2).

Table 4.2. Evolution and coverage depth of all transmitted ARV drug-resistance mutations

Pt ID	Mutation (%) (at baseline)	Coverage depth	Follow-up interval (wks)	Mutation (%) (at follow-up)	Coverage depth
2066	failed PCR		7	Y188H - 11.4	5495
5041	K103N - 99.3	4008	LTF		
261	K103N - 99.3	6988	LTF		
9049	T66I - 4.4	6925	3	T66T - nd†	1747
6743	*N83D – 0.04	10000	7	N83D - 1.3	6227
2504	*Y188H – 0.03	8155	2	Y188H - 2.4	10000
	*Y143H – 0.07	10000	2	Y143H - 8.6	10000
3469	*Q148R – 0.4	10000	9	Q148R - 1.8	4771

Data generated from PASeq pipeline was used for this Table. Coverage depth of all mutations reported in Table 4.1 is shown. The PASeq pipeline reports a maximum coverage value of 10000 for mutations that have a coverage above this threshold, but exact values are given for a coverage below 10000. † - coverage depth for wild type amino acid was used as T66I mutant was not detected in the follow-up sample. * - values below 1% are only reported for mutations that existed above 1% analysis threshold in the f/u sample of the pair. Pt – participant, ID – identity, wks – weeks, f/u – follow-up, LTF – lost to follow-up, n/a – not applicable, nd – not detected.

4.6 Discussion

4.6.1 Study strains

To our knowledge this is the first study that assessed evolution of transmitted ARV drug-resistant strains in sample pairs obtained before treatment initiation using deep sequencing. It is not surprising that the majority of study strains belonged to HIV-1 subtype C as this is the predominant subtype in the Southern African region [21]. The presence of non-subtype C strains in SA has been reported previously [22].

4.6.2 Deep sequencing data analysis

Deep sequencing data analysis pipelines should be used cautiously in order to avoid reporting minority variants that might exist as part of analysis-related errors [23]. The discrepancy observed among the three pipelines around 1% threshold has been observed in a recent study [19]. This highlights the need to confirm deep sequencing results obtained from one pipeline. The analysis strategy employed in this study seems to be more stringent as only consensus variants among the three pipelines were reported.

4.6.3 Total transmitted drug-resistance

The total transmitted ARV drug-resistance of up to 33.3%, observed in this study (Table 4.1) is higher than that observed in previous SA studies [24-26]. This is attributed to the high sensitivity of deep sequencing to detect minority variants. However, this is also consistent with the rising trend of transmitted drug-resistance, which has been observed with the expanded use of ARV drugs [2, 4]. There was a higher rate of transmitted NNRTI drug-resistance compared to other drug-class resistance. This is not surprising as first generation NNRTI drugs have a

low resistance barrier [27], and have always been used in SA first-line ARV regimen since 2004 [5, 12, 13].

The surprise finding was that of INSTI drug-resistance mutations in some participants as this drug-class is not commonly used, but only limited to 3rd line regimen in SA public sector [5]. However, raltegravir, an INSTI drug with low resistance barrier [28] has been available in the SA private sector since 2012, and recommended for use in first-line regimen in cases of contra-indication to NVP and efavirenz [29, 30]. Raltegravir resistance has been reported previously in SA patients on raltegravir-containing regimen, including patients who were enrolled in a clinical trial conducted between 2012 and 2013 [31-33]. The observation of Q148R mutation is worrisome as this confers resistance to all INSTI drugs including dolutegravir. This mutation confers a higher level of resistance when it exists with E138A mutation, which has been observed in some participants (Supplementary Table 4.2) [34]. Raltegravir use should be limited as it has a potential to compromise the efficacy of a dolutegravir-containing first-line regimen [28]. The presence of elvitegravir-selected mutation, T66I (Table 4.1), could be explained by population migration or acquisition of HIV from an individual who participated in a clinical trial as this drug is not used in SA [5, 30].

K103N mutation existed as part of the majority variants in two participants it was detected from. Replication fitness is the likely explanation for this as K103N mutants are known to replicate efficiently [35]. The existence of all other transmitted mutations as minority variants is also attributable to replication capacity, as all these mutations are known to compromise the replication fitness of HIV [27, 28, 35].

4.6.4 Clinical and epidemiological relevance of minority variants

The ARV drug-resistant minority variants have implications on HIV management and epidemiology. The emergence of minority variants over time without drug pressure highlights their potential clinical relevance (Tables 1 and 2). This emergence could have been influenced by the presence of accessory mutations that increase the replication capacity of less fit mutants (Supplementary Table 4.2) [27]. Studies that assessed the clinical impact of minority variants above or below 1% prior to ARV treatment initiation, have observed higher rates of treatment failure among patients who harboured these variants [10, 36-39]. The T66I variant that was observed at baseline but undetectable at follow-up was probably archived [40] or undetectable owing to lower coverage depth on the follow-up sample (Table 4.2).

The detection of transmitted minority variants by deep sequencing in this study highlights that these variants were part of viruses that established HIV infection [41]. Most participants with transmitted drug-resistance had VLs of >1500 copies/ml (Table 4.1), which is a VL threshold associated with transmission of HIV [42]. Thus these participants might have transmitted drug-resistant mutants before they initiated treatment. Some of the participants were pregnant women who posed a risk of transmitting drug-resistant HIV strains to their babies.

4.6.5 Prevention of transmitted drug-resistance

The problem of transmitted drug-resistance could possibly be resolved by implementation of a more robust first-line regimen such as dolutegravir-containing regimen, which is associated with a lower probability of inducing drug-resistance [28]. Dolutegravir has already been incorporated into first-line regimen in some HIV management guidelines, mostly in developed countries [43, 44], but not yet used in most developing countries [5, 6]. WHO HIV treatment guidelines only recommend dolutegravir use as an alternative to the preferred EFV-containing first-line regimen [7].

Scaling up access to HIV VL monitoring in developing countries could result in earlier detection of treatment failure and appropriate treatment changes. The availability of baseline ARV resistance testing for individuals with newly diagnosed HIV infection could also play a role in preventing transmitted resistance. However, this is not affordable in most developing countries owing to limited financial resources [45]. This shows a demand for making drug-resistance testing cheaper or developing less expensive novel techniques for assessing drug-resistance. Strengthening HIV behavioural measures is also needed to improve treatment adherence, and to reduce the risk of HIV acquisition among uninfected individuals [46].

The limitations of this study include a small sample size and amplification failure in some samples. Failed amplification was probably caused by either low viral load (below assay detection limit) or primer mismatch. There could have been PCR bias or sequencing errors that influenced the absence of some mutations. The stringent criteria used for deep sequencing data analysis could have led to under-reporting of mutations. The absence of clinical data on response to ARV treatment is another limitation. However, follow-up of all the study participants is planned to be conducted in the future.

4.7 Conclusions

Illumina deep sequencing detected a higher rate of transmitted ARV drug-resistance. All minority variants were mutations that reduce the replication capacity of HIV; hence they existed at lower proportion. Emergence of minority variants in some sample pairs, without ARV drug pressure, highlights their potential clinical relevance. Strategies to prevent

emergence and transmission of ARV resistance should be strengthened. Deep sequencing could play a crucial role in the surveillance of transmitted ARV drug-resistance. More studies are needed for further assessment of transmitted ARV drug-resistant strains using deep sequencing.

4.8 References

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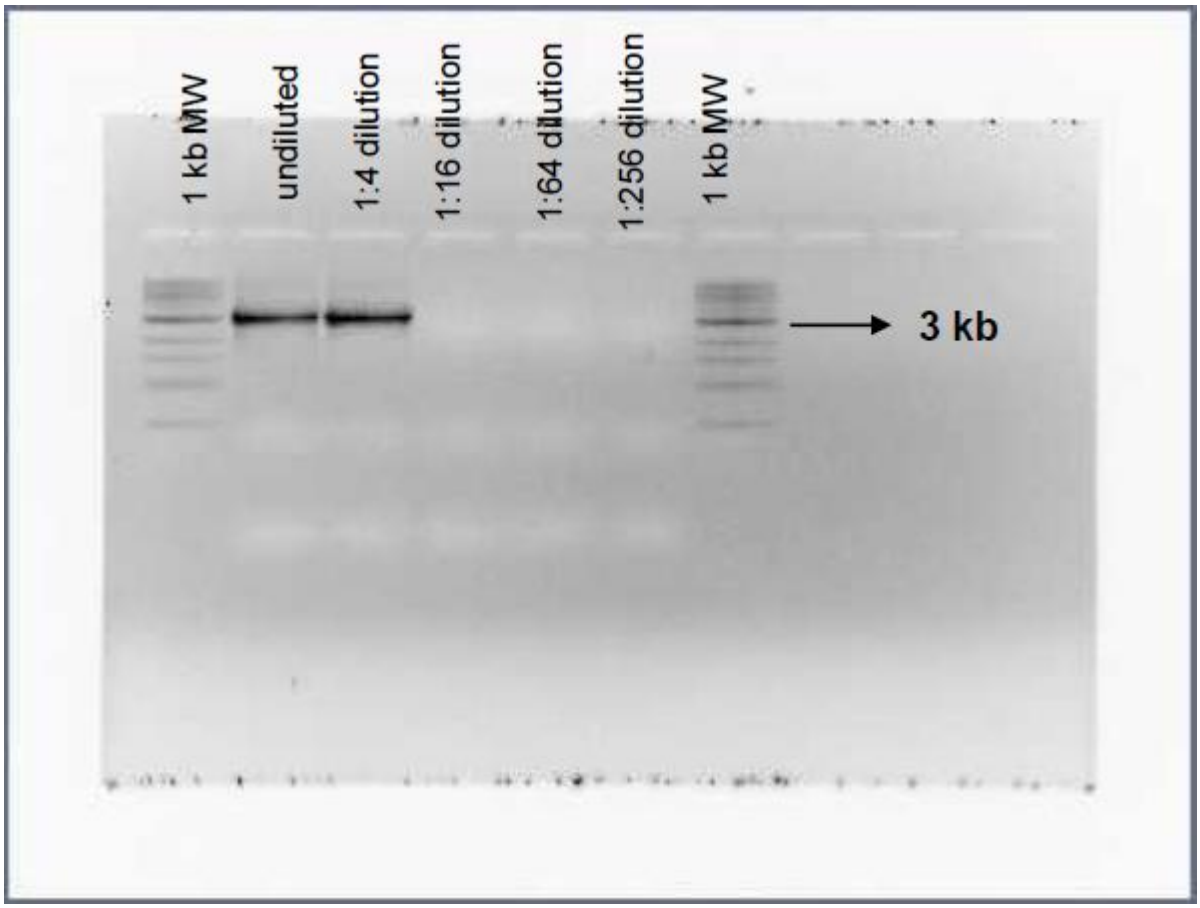
Supplementary Material

PCR conditions

First round PCR conditions were as follows: 1 cycle of cDNA synthesis at 50 ºC for 30 minutes; 1 cycle of pre-denaturation at 94 ºC for 2 minutes; 40 cycles of denaturation at 94 ºC for 15 seconds, annealing at 45 ºC for 30 seconds, extension at 68 ºC for 5 ½ minutes; and final extension at 68 ºC for 10 minutes. Second round PCR conditions were as follows: 1 cycle of denaturation at 94 ºC for 2 minutes; followed by 40 cycles of denaturation at 94 ºC for 15 seconds, annealing at 48 ºC for 30 seconds, extension at 68 ºC for 5 minutes; and final extension at 68 ºC for 10 minutes. PCR products were detected by electrophoresis using 1% agarose gel and 1 kilobase (kb) DNA ladder.

Sanger sequencing

SM-F3 Alt (forward) 5'-GTT AAG TGT TTC AAC TGT G-3' (SM-F3 shorter version) and SM-R3 (mentioned above) were used as outer sequencing primers together with the following inner primers: SM-Int-R1 (reverse) 5'-CTR CAR TCY ACY TGB CCA TG-3' (4380-99), SM-Int-R2 (reverse) 5'-CCA BTC AGG WAT CCA G-3' (3776-91), SM-Int-R3 (reverse) 5'-TGD AGY TCR TAY CCC ATC CA-3' (3234-53). An alternative primer SM-Int-R2 Alt (reverse) 5'-CCA NTC RGG DAY CCA G-3' was used in few samples that could not be sequenced by SM-Int-R2 due to a mismatch. SM-DF3 and SM-DR3 or SM-F2 and SM-R2 primer pair were used as outer sequencing primers for few samples that were successfully amplified with these primers, together with the above mentioned inner sequencing primers. Sequencing was repeated on the same or newly amplified template for samples that initially had failed sequencing.



Supplementary Figure 4.1: A 1% gel electrophoresis picture showing nested PCR products from a sample with serial dilutions. The undiluted sample had an HIV viral load of 21051 copies/millilitre (ml) and was extracted using an input volume of 0.5 ml, which was mixed with 0.5 ml of phosphate-buffered saline (PBS). The final extraction volume was 60 microlitres (µl), which probably contained about 10525 copies as 0.5 ml of the original sample was used for extraction. The 5 µls used as template for the undiluted sample had about 877 copies. A volume of 5 µl was used for serial dilutions, thus there were about 219 copies in 1:4 dilution, 54 copies in 1:16 dilution, 13 copies in 1:64 dilution and 3 copies in 1:256 dilution. The sensitivity was thus estimated to be 2628 copies/ml. 1 Kilobase (kb) molecular weight (MW) marker was used for identifying the correct PCR fragment.

Supplementary Table 4.1. Alternative primers used in few cases of amplification failure

Alternative primers	HXB-2 position
SM-F2 (inner forward) 5'-AGT TTT GGC TGA GGC AAT GAG-3'	1872-92
SM-R2 (inner reverse) 5'-CTT ATG GCA GAG TCT GCA AAA CA-3'	5380-5402
SM-DF1* (outer forward) 5'-GCG GCT ACA CTA GAR GAR ATG ATG-3'	1807-30
SM-DR1* (outer reverse) 5'-GTG CCA AGT ATT GTA RVG AYC CTA CCT-3'	5462-88
SM-DF3* (inner forward) 5'-AGA ATT GTT AAG TGT TTY AAY TGT GG-3'	1952-76
SM-DR3* (inner reverse) 5'-CTC CTG TAT GCA RVC CCC AAT A-3'	5245-66

*degenerate versions of the original primers that were used for amplification in most samples.

Supplementary Table 4.2. Other drug-resistance and accessory mutations

Pt ID	VL results (copies/ml)	Illumina MiSeq (%) PASeq (HyDRA / DeepChekHIV) baseline results	F/U interval (wks)	Illumina MiSeq (%) PASeq (HyDRA / DeepChekHIV) follow-up results
9498	509793	K103R ≥95	LTF	n/a
9228	94	failed PCR	10	F227 - 0.9 (1.2 / 1.9), L10F - 4.6 (4.0 / 4.5), T97A - 1.4 (1.2 / 1.4), I132L - 1.0 (1.0 / 1.7)
2066	1010	failed PCR	7	nd
8575	93079	V82I ≥95	8	failed PCR
5041	22921300	nd	LTF	n/a
8047	1245238	nd	2	nd
9049	16848	L10V ≥95	3	L10V ≥95
6638	195105	E138A ≥95	6	E138A ≥95
261	84501600	K20R ≥95, V82I ≥95	LTF	n/a
6512	1763	K20R ≥95	2	K20R ≥95
6743	27364	K20R ≥95, V179I ≥95, L74I ≥95, E138A ≥95	7	K20R ≥95, V179I ≥95, L74I ≥95, E138A ≥95, *V108I - 3.2 (3.2 / 3.3)
6582	6216	nd	6	nd
6727	4874	K20R ≥95	2	K20R ≥95
6737	2227	L10I ≥95, L74I ≥94	LTF	n/a
7084	337987200	K20R ≥95, T74S ≥95	4	K20R ≥95, T74S ≥95, *F227L - 0.9 (1.2 / 2.2)
2504	37243	L74I ≥95	2	L74I ≥95
3469	33274	K20I ≥95, V82I ≥95	9	K20I ≥95, V82I ≥95, *S230N - 22.5 (21.8 / 21.4)

These are other drug-resistance or accessory mutations that contribute to resistance. Majority variants were detected by all 3 pipelines, hence reported as ≥ a minimum value obtained among these pipelines. If a variant existed at 0.9% on one pipeline but at ≥1% on the other two pipelines, it was considered as part of 1% population. * - mutation was below analysis threshold at baseline. Pt – participant, ID – identity, VL – viral load, ml – millilitres, F/U – follow-up, wks – weeks, nd – not detected, n/a – not applicable. Amplification failure on sample 9430 was probably due to markedly reduced VL after 8 weeks of follow-up during early HIV infection (VL was not measured on this sample) [18].

A

Mutations Statistics

% cutoff	# Usuals	# Unusuals	# DRMs	# APOBECs	# Stops	# APOBEC DRMs
20%	83	0	1	0	0	
10%	84	0	1	0	0	
5%	89	0	2	0	0	
2%	93	5	3	0	0	
1%	102	12	3	0	0	
0.5%	126	24	4	0	1	
0.2%	468	127	18	3	8	0
0.1%	824	302	38	15	29	0

B

Mutations Statistics

% cutoff	# Usuals	# Unusuals	# DRMs	# APOBECs	# Stops	# APOBEC DRMs
20%	93	0	0	0	0	
10%	98	1	0	0	0	
5%	99	1	0	1	0	0
2%	104	3	0	1	0	0
1%	115	8	1	1	1	0
0.5%	185	26	3	1	3	0
0.2%	598	168	22	6	10	1
0.1%	889	318	44	27	35	4

Supplementary Figure 4.2: Mutation statistics which show different analysis cut-offs (in %); and the number of usual, unusual, and drug-resistance mutations (DRMs). The number of APOBEC, stop, and APOBEC-related DRM mutations is also shown. Analysis for two follow-up (f/u) samples is shown; 2504 f/u (**A**) and 3469 f/u (**B**), that had minority drug-resistant variants (see Table 4.1).

CHAPTER 5

Vertical Transmission of HIV among Pregnant Women who were Initially Misdiagnosed by the Rapid HIV Tests in Four South African Antenatal Clinics

Manuscript under review.

5.1 Abstract

Introduction: There is a risk of mother-to-child transmission of HIV (MTCT) during pregnancy and breastfeeding. The aim of this study was to assess vertical transmission of HIV among pregnant women who were initially misdiagnosed by the rapid HIV tests in South African antenatal clinics.

Methods: Pregnant participants were enrolled in a study that used nucleic acid amplification testing (NAAT) to screen for early HIV infection among individuals who tested negative on rapid tests used at the point-of-care (POC) facilities. Participants were enrolled from four antenatal clinics (F.F. Ribeiro, Skinner, Stanza Bopape and Phomolong) in the Tshwane district of SA. All NAAT-positive participants were recalled to the clinics for further management. Vertical transmission was assessed among exposed babies whose HIV polymerase chain reaction (PCR) results were available.

Results: This study enrolled 8208 pregnant participants who tested negative on rapid HIV tests between 2013 and 2016. Their median age was 26 years (interquartile range [IQR]: 23 – 30).

Newly diagnosed HIV infections were detected in 0.6% (n = 51) of all study participants; their distribution was 0.6% (n = 27) at F.F. Ribeiro, 0.5% (n = 7) at Skinner, 1.1% (n = 14) at Stanza Bopape and 0.3% (n = 3) at Phomolong clinics. Forty-seven participants (92.2%) were successfully recalled and referred for antiretroviral treatment initiation as part of prevention of MTCT (PMTCT), except for one who had miscarriage. Most women (61.9%, n = 26) with newly diagnosed HIV infection presented for the first antenatal care visit in the second trimester, 31.0% (n = 13) presented in the third trimester, and 7.1% (n = 3) presented in the first trimester of pregnancy. Only two participants were still in the first trimester at the time of PMTCT initiation. HIV PCR results were available for thirty-two babies, three of whom tested positive (9.4%; 95% confidence interval: 1.98 – 25.02).

Conclusions: This study showed that supplemental HIV testing for pregnant women led to earlier linkage to PMTCT programme, which reduced transmission of HIV to their babies. Misdiagnosis of HIV infection at antenatal clinics is likely to undermine the efforts of eliminating MTCT in certain settings.

5.2 Introduction

HIV infection in women of childbearing age is associated with a risk of transmission to babies during pregnancy and breastfeeding. Without intervention, the risk of vertical transmission is highest during the intrapartum period and breastfeeding, but lower in the antepartum period [1]. Prevention of mother-to-child transmission of HIV (PMTCT) with antiretroviral (ARV) drugs has remarkably reduced the rate of vertical transmission [2], and this has led to elimination of MTCT (eMTCT) in some countries [3]. World Health Organisation (WHO) defines eMTCT in a country as less than 50 paediatric HIV infections per 100 000 live births and a transmission rate below 5% if mothers are breastfeeding or below 2% if they are not breastfeeding for at least 1 year. For achieving eMTCT, WHO requires the following targets to be met; ≥95% antenatal coverage (at least one visit), ≥95% HIV testing coverage, and ≥95% HIV treatment coverage [4].

PMTCT guidelines have evolved over time from use of a single drug during pregnancy [5, 6] to recommending immediate initiation of highly active antiretroviral therapy (HAART), also known as option B+, which is associated with higher efficacy for reduction of MTCT [1, 7]. South Africa (SA) implemented option B+ strategy in March 2013 [8], and has been making a good progress in reducing MTCT [9-11]. Following the first antenatal care (ANC) visit within the first trimester, guidelines for maternity care in SA recommend follow-up ANC visits at gestations 20, 26 – 28, 32 – 34 and 38 weeks, and 41 weeks if still pregnant [12].

HIV screening during pregnancy plays a huge role in identifying HIV-infected individuals who would benefit from PMTCT programme. Unfortunately, some pregnant women with HIV infection are misdiagnosed at SA point-of-care (POC) facilities [13-15]. The aim of this study

was to assess vertical transmission of HIV among pregnant women who were initially misdiagnosed by rapid HIV tests in SA antenatal clinics.

5.3 Methods

Pregnant participants were enrolled in a study that screened for early HIV infection among individuals who tested negative on rapid HIV tests used at the POC facilities from 2013 - 2016. They were enrolled after HIV testing during the first antenatal visit from four clinics (F.F. Ribeiro, Skinner, Stanza Bopape and Phomolong) in the Tshwane district of SA. Pooled nucleic acid amplification testing (NAAT) was performed in mini-pools of 5 samples, followed by individual sample NAAT in positive pools. The average turn-around-time for NAAT was about 2 weeks as samples were batched and tested when a minimum of 10 - 12 pools were available. All NAAT-positive women were recalled to the clinics for HIV confirmatory testing and appropriate management, which included follow-up rapid testing and referral for immediate initiation of HAART according to SA PMTCT guidelines [8].

Data on gestation at first ANC visit, initiation of PMTCT and mode of delivery were collected from the mothers' clinic records. Data on baby's names, date of birth, receipt of HIV prophylaxis, feeding method and HIV PCR results were collected from the babies' clinic records. Where it was difficult to locate clinic records, mothers were phoned for babies' information such as names and date of birth, and this was used to search for baby's HIV polymerase chain reaction (PCR) results from the laboratory information system. Current SA HIV testing guidelines for babies born to HIV-infected mothers, which were implemented in June 2015, recommend first HIV PCR test at birth [16]. Thus, there was no birth HIV PCR

results for babies born before June 2015 as previous guidelines recommended the first HIV PCR test at 6 weeks of age [8]. A descriptive analysis was used to present summary statistics (95% confidence interval [CI]). All the statistics were performed on the STATA version 15.1 software (StataCorp LP, College Station, TX, USA).

The study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number–295/2015) and by Tshwane–Metsweding Region Research Ethics Committee (TMREC 2010/26). The legal ages for consenting to HIV testing and medical treatment in SA are 12 and 14 years, respectively [17]. This study enrolled participants aged 14 years or older. The initial study protocol was approved for participants older than 18 years but it was later amended and approved to include those from 14 years of age to extend the benefits of screening for early HIV infection to the younger age groups, most of whom came to the clinics alone. All study participants agreed to participate and signed written consent forms before enrolment.

5.4 Results

5.4.1 Demographics and newly diagnosed HIV infections

There were 8208 pregnant participants enrolled into the study from March 2013 – November 2016, with a median age of 26 years (interquartile range [IQR]: 23 – 30). The majority of participants (51.8%, n = 4255) were enrolled from F.F. Ribeiro, followed by Skinner (17.5%, n = 1439), Stanza Bopape (16.1%, n = 1324) and Phomolong (14.5%, n = 1190) clinics. All had tested negative on rapid HIV tests used at the POC facilities. Newly diagnosed HIV infections were detected by NAAT in 0.6% (n = 51) of all study participants, of whom 23.1%

(n = 12) had early HIV infection and others had chronic infection [15]. The distribution of newly diagnosed HIV infections among the four clinics was 0.6% (n = 27) at F.F. Ribeiro, 0.5% (n = 7) at Skinner, 1.1% (n = 14) at Stanza Bopape and 0.3% (n = 3) at Phomolong.

All NAAT-positive participants were recalled to their respective clinics for further management, and came at different intervals owing to their availability. Forty-seven participants (92.2%) were successfully recalled to the clinics and referred for HAART initiation after confirmation of HIV infection, except for one participant who had miscarriage (Fig. 5.1). The latter was referred for appropriate management, which at the time (in 2014) recommended initiation of HAART at CD4 count <350 cells/µl [18].

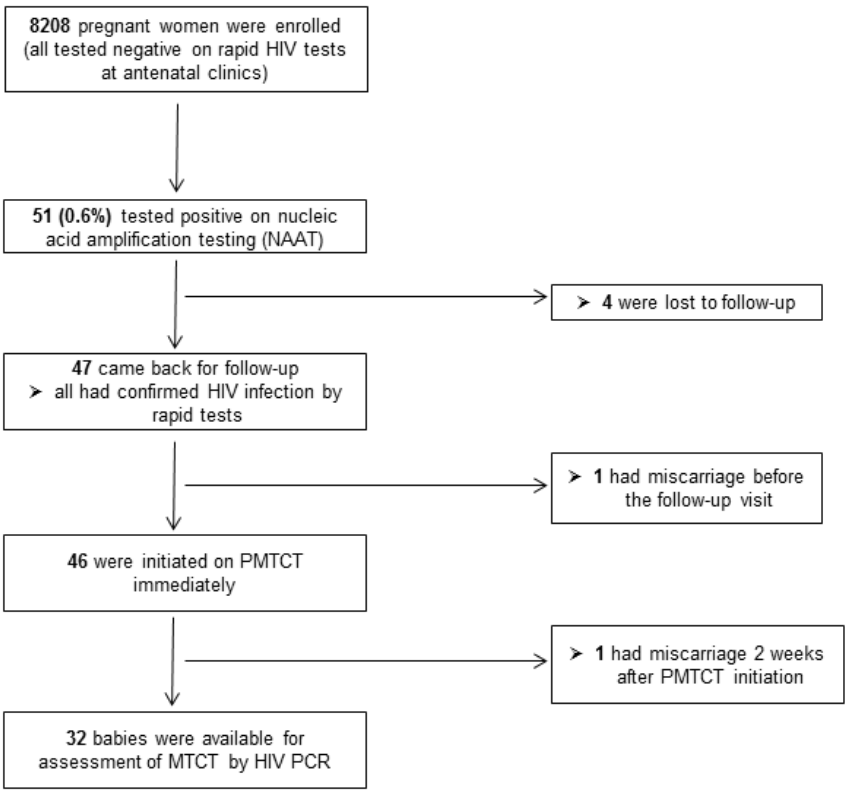


Figure 5.1. Algorithm showing enrollment of study participants. Those who tested positive on nucleic acid amplification testing (NAAT) were recalled for HIV confirmatory test and immediate initiation of highly active antiretroviral therapy (HAART) as part of prevention of mother-to-child transmission of HIV (PMTCT). Lost to follow-up was mainly due to relocation to other clinics within South Africa and neighbouring countries. PCR = polymerase chain reaction.

5.4.2 Pregnancy data and PMTCT initiation

Data on pregnancy was available for 42 women who were initiated on PMTCT programme. Clinic records for four women could not be accessed, and these participants could not be contacted by telephone. Most women (61.9%, n = 26) with newly diagnosed HIV infection presented for the first antenatal care visit in the second trimester, 31.0% (n = 13) presented in the third trimester, and 7.1% (n = 3) presented in the first trimester of pregnancy (Table 5.1). Only two participants were still in the first trimester at the time of PMTCT initiation. One woman was initiated on PMTCT three weeks post-delivery as she presented late for ANC (Table 5.1, #25). Available data on the mode of delivery and infant feeding showed that most women had normal vaginal delivery and exclusively breastfed their babies. Unfortunately, some women were lost to follow-up after PMTCT initiation owing to various reasons such as relocating to other clinics or neighbouring countries. Even though some women presented earlier for ANC and were contacted after positive NAAT results, they only came back very late for a follow-up visit (Table 5.1, #5 and 6).

5.4.3 Assessment of vertical transmission

Thirty-two babies were successfully followed up to assess vertical transmission (Fig. 5.1). Nevirapine prophylaxis was documented for those whose files were accessible. However, other exposed babies whose mothers were already within the PMTCT programme, probably received nevirapine prophylaxis as well. Most babies (60%, n = 18) had more than one HIV PCR results. HIV infection was diagnosed in three babies (9.4%; 95% CI: 1.98 – 25.02), one whose HIV PCR test was positive three days after birth. His mother was initiated on PMTCT at 34 weeks of gestation and defaulted treatment. The mothers of other two babies who contracted HIV were initiated on PMTCT at 20 and 25 weeks of gestation. It was difficult to estimate the time of HIV transmission in them as they had relocated to other clinics after PMTCT initiation, and

their babies had no birth HIV PCR results (Table 5.1). Interestingly, all the mothers who transmitted HIV to their babies had chronic HIV infection, with viral loads of 14072, 66694 and 242663 copies / milliliter before PMTCT initiation [15].

Table 5.1. Data on antenatal care and assessment of mother-to-child transmission of HIV

	MOTHERS' DATA					BABIES' DATA								
#	Pt	Gestation	Rapid	HIV VL	Gestation	Birth time	Delivery	Nevirapine	Feeding	HIV PCR results				Rapid
	ID	at 1st ANC	HIV test	(copies/ml)	at PMTCT initiation	mmm-yy	mode	prophylaxis	method	0 - 5 days	6 - 8 weeks	3 -6 months	7 - 18 months	HIV test
1	5067	19	NEG	1.2×10^4	22	Sep-13	NVD	Yes	BF	--	--	--	--	--
2	9915	30	NEG	1.4×10^4	32	Sep-13	NVD	Yes	BF	--	NEG	NEG	--	--
3	639	16	NEG	6.5×10^3	19	Jun-14	NVD	Yes	BF	--	NEG	--	NEG	NEG
4	641	13	NEG	7.0×10^4	15	Jul-14	--	Yes	BF	--	--	--	--	--
5	6638	24	NEG	1.9×10^5	33	Apr-14	NVD	Yes	BF	--	NEG	--	NEG	NEG
6	8828	25	NEG	4.1×10^4	37	Jul-14	NVD	Yes	BF	--	NEG	NEG	NEG	NEG
7	2678	16	NEG	2.2×10^5	20	Sep-14	C/S	Yes	BF	--	NEG	--	NEG	NEG
8	9895	9	NEG	4.8×10^3	18	Sep-14	--	--	BF	--	--	NEG	NEG	NEG
9	9986	9	NEG	9.7×10^4	11	Dec-14	C/S	--	--	--	NEG	NEG	--	--
10	843	21	NEG	2.9×10^4	24	Oct-14	NVD	Yes	BF	--	NEG	NEG	--	--
11	6512	15	NEG	1.7×10^3	19	Dec-14	--	--	--	--	NEG	--	--	--
12	6990	6	NEG	1.7×10^4	11	--	--	--	--	--	--	--	--	--
13	6671	17	NEG	1.4×10^4	20	Jan-15	NVD	--	BF	--	--	POS x2	--	n/a
14	6380	21	NEG	1.1×10^4	24	Feb-15	NVD	Yes	BF	--	NEG	--	NEG	NEG
15	6557	22	NEG	6.1×10^2	26	Feb-15	NVD	Yes	BF	--	NEG	--	--	--
16	6565	25	NEG	5.6×10^3	27	Jan-15	NVD	Yes	BF	--	NEG	--	NEG	--
17	6582	31	NEG	6.2×10^3	36	Dec-14	NVD	Yes	BF	--	NEG	--	--	--
18	6596	32	NEG	3.8×10^3	35	Dec-15	NVD	Yes	BF	--	NEG	--	--	--
19	6640	22	NEG	3.0×10^3	26	--	--	--	--	--	--	--	--	--
20	6727	20	NEG	4.8×10^3	23	Feb-15	--	Yes	--	--	NEG	--	--	--
21	6649	15	NEG	2.1×10^4	17	--	--	--	--	--	NEG	--	--	--
22	6738	24	NEG	1.5×10^5	26	--	--	--	--	--	--	--	--	--
23	1067*	24	NEG	1.7×10^3	26	--	--	--	--	--	--	--	--	--
24	2504	19	NEG	3.7×10^4	21	--	--	--	--	--	--	--	--	--

25	921	36	NEG	9.7×10^3	post delivery	Apr-15	C/S	--	--	--	NEG	--	NEG	--
26	3869	32	NEG	2.1×10^5	36	May-15	NVD	--	BF	--	--	--	NEG x2	--
27	3912	21	NEG	3.2×10^4	23	Sep-15	NVD	Yes	FF	--	NEG	--	--	--
28	3920	23	NEG	6.6×10^4	25	Aug-15	--	--	--	--	--	--	POS x2	n/a
29	3880	22	NEG	7.5×10^3	26	Aug-15	NVD	Yes	BF		NEG	--	--	--
30	3935	32	NEG	2.4×10^5	34	Jun-15	NVD	Yes	BF	POS	--	POS	--	--
31	1117	24	NEG	1.5×10^2	26	Sep-15	NVD	Yes	BF	NEG	NEG	--	--	--
32	1121	30	NEG	8.0×10^4	32	Jun-15	NVD	Yes	BF	NEG	--	--	--	--
33	3469	23	NEG	3.3×10^4	25	--	--	--	--	--	--	--	--	--
34	3474	28	NEG	1.6×10^4	32	--	--	--	--	--	--	--	--	--
35	1475	27	NEG	4.4×10^4	29	Aug-15	--	--	--	NEG	NEG	--	--	--
36	3387	27	NEG	7.9×10^4	37	Oct-15	NVD	Yes	BF	NEG	--	NEG	--	--
37	3253	13	NEG	8.9×10^4	15	Feb-15	--	--	--	NEG	--	--	--	--
38	1692	16	NEG	3.9×10^2	18	Jan-16	C/S	Yes	FF	--	--	--	NEG	NEG
39	3606	28	NEG	1.5×10^4	32	--	--	--	--	--	--	--	--	--
40	2866	21	NEG	3.3×10^3	24	Jan-16	--	--	--	--	--	NEG	--	--
41	1213	24	NEG	3.2×10^4	26	Aug-16	NVD	Yes	BF	NEG	NEG	NEG	--	--
42	3910	13	NEG	2.9×10^4	16	Jan-17	C/S	Yes	FF	NEG	NEG	--	NEG	--
43	6748†	--	NEG	9.3×10^2	--	--	--	--	--	--	--	--	--	--
44	5054†	--	NEG	2.7×10^4	--	--	--	--	--	--	--	--	--	--
45	9049†	--	NEG	1.6×10^4	--	--	--	--	--	--	--	--	--	--
46	4351†	--	NEG	2.6×10^3	--	--	--	--	--	--	--	--	--	--

Some participants were lost to follow-up at different stages of ANC or post-natal care. HIV viral load (VL) testing was performed from samples obtained at enrolment (i.e. after a negative rapid HIV test). # - participant (pt) numbering, ID – study identity, 1st – first, ANC – antenatal care, ml – millilitre, mmm-yy (month and year), PMTCT – prevention of mother-to-child transmission of HIV, PCR – polymerase chain reaction, NVD – normal vaginal delivery, C/S – Caesarian section, BF – breastfeeding, FF – formula feeding, NEG – negative, POS – positive, -- – no data, x2 – two results available in a testing interval. Participants who transmitted HIV to their babies are highlighted in light brown rows. † - participants were referred for PMTCT initiation during the follow-up visit but their clinic records could not be accessed later, and they could not be contacted by telephone. * - participant had miscarriage two weeks after HAART initiation.

5.5 Discussion

5.5.1 Misdiagnosed HIV infections

This study identified HIV infection in pregnant women who were initially misdiagnosed by rapid HIV tests used at four antenatal clinics in the Tshwane district of SA. The finding of 0.6% newly diagnosed HIV-infected individuals who were initially misdiagnosed at antenatal clinics highlights gaps in the quality of HIV testing at POC facilities. Other researchers have also reported misdiagnosis of HIV infection during pregnancy or seroconversion after an initially negative HIV test [13, 14, 19, 20]. PMTCT initiation was delayed in the study participants as a result of misdiagnosis (Table 5.1). The improved HIV diagnostic strategy employed in this study led to earlier linkage of infected women to PMTCT programme.

5.5.2 Gestation at first antenatal care visit

The majority of women presented for first ANC visit in the second and third trimesters. This shows that most babies were potentially exposed to HIV in-utero for a considerable amount of time before PMTCT was initiated. Late booking for ANC is associated with a higher risk of MTCT [21], and has been reported by previous SA studies [22-24]. In the absence of intervention, the risk of vertical transmission of HIV during antenatal period is between 20 – 25% in the first 28 weeks of pregnancy, but increases to 75 – 80% after 28 weeks of pregnancy [1]. Studies that assessed MTCT among pregnant women who received HAART during antenatal period, from 26 or 28 weeks' gestation through weaning at 6 months post-partum, found that majority of HIV infections were transmitted in-utero [25, 26]. This shows that it is important for pregnant women to present early for ANC, within the first trimester, in order to maximize the benefits of option B+ strategy.

5.5.3 Impact of improving HIV testing in antenatal clinics

Without intervention, the overall risk of MTCT of HIV is up to 40% among infants who are breastfed [1]. The finding of 9.4% transmission rate in this study highlights that improved HIV diagnosis, which led to immediate initiation of PMTCT, contributed to interrupting the vertical transmission of HIV in the majority of women. It is likely that MTCT could have been higher among these women had they not participated in this study. The diagnosis of HIV infection three days after birth in one baby highlights the benefit of HIV PCR test performed around birth for earlier detection of HIV infection that likely occurred in-utero. The rate of vertical transmission of HIV observed in this study is higher compared to findings of other studies that assessed efficacy of option B+ PMTCT strategy [25-28].

Correct diagnosis of HIV during pregnancy initiates a cascade of PMTCT events, where HIV-infected pregnant women are initiated on HAART immediately, and their babies get tested for HIV and receive prophylactic treatment if HIV-negative or HAART if HIV-positive. Thus, misdiagnosis of HIV infection at antenatal clinics is likely to undermine the efforts of eMTCT as the important opportunity for initiating PMTCT is missed for some women. Studies that assessed the effectiveness of PMTCT programme, from HIV testing during ANC period to HIV testing and prophylaxis among HIV-exposed infants, found that there was a higher risk of HIV transmission to the infants if some steps of PMTCT programme were missed [29, 30].

Between 2013 and 2015, SA HIV testing strategy for pregnant women who tested negative at the first ANC visit was as follows; a repeat test was recommended every 3 months throughout pregnancy, at delivery, at 6 weeks post-partum and 3 monthly throughout breastfeeding [8, 16]. This shows that most women in this study should have had one or two HIV tests before delivery if they adhered to the recommended ANC visit schedule [12]. A single false-negative test in a

woman who presented for the first ANC visit at 36 weeks of gestation resulted in her not initiated on PMTCT prior to labour (Table 5.1, #25). She was not tested for HIV during labour at 38 weeks, hence she was only started on PMTCT three weeks post-delivery when she came back for positive NAAT results. This highlights that it could be difficult to offer HIV testing during the labour period. This again emphasizes the importance of early presentation for ANC, as this would create opportunities for follow-up HIV testing and correct diagnosis prior to labour if the initial HIV test was negative.

The current SA HIV testing guidelines, published in 2016, recommend that pregnant women who test negative for HIV at the first ANC visit be retested at every antenatal visit, at delivery and every 3 months during breastfeeding [31]. These guidelines represent an improvement in HIV testing, but their effectiveness would probably be determined by early presentation for ANC. Shortening the HIV retesting interval in individuals with negative HIV test is supported by our previous publication, which found that follow-up rapid tests were positive at a median interval of 4 weeks in individuals who were initially misdiagnosed [15].

The limitations of this study include a small sample size, and high frequency of loss to follow-up that led to missing data for some participants. HIV infection could not be excluded in some babies who only had one HIV PCR result and were still breastfeeding. There is no unique laboratory identity number in SA public sector laboratories that is used to link patient's results at different time points; this could have made it difficult to locate some of the babies' laboratory results. This study is not representative of all HIV-infected women as it assessed vertical transmission only among women who were initially misdiagnosed by the rapid tests at the antenatal clinics.

5.6 Conclusions

This study showed that improved HIV testing for pregnant women led to earlier linkage to PMTCT programme, which reduced the vertical transmission of HIV to their babies. Misdiagnosis of HIV infection at antenatal clinics is likely to undermine the efforts of eMTCT as the important opportunity for initiating PMTCT is missed for some women. Late presentation for the first ANC visit poses a serious challenge to the efficacy of PMTCT. This study shows a need to strengthen HIV diagnosis at the antenatal clinics and to encourage pregnant women to present earlier for ANC.

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CHAPTER 6

Field Performance of the INSTI Rapid HIV-1/2 Antibody Test in Two South African Antenatal Clinics

Manuscript (short communication) under review.

6.1 Abstract

The INSTI HIV test has been shown to have higher sensitivity for detection of antibodies even during early HIV infection. This study assessed the field performance of the INSTI HIV test. The study was conducted in two antenatal clinics in the Tshwane district of South Africa (SA), where Advanced Quality test was used for HIV screening and First Response test only used for confirming positive screening tests. INSTI test was evaluated at the point-of-care (POC) facilities as part of a study that screened for early HIV infection, which used pooled nucleic acid amplification testing (NAAT) in plasma samples of participants who had negative rapid HIV tests.

This study enrolled 386 pregnant women, of whom 334 (86.5%) tested negative on Advanced Quality and INSTI HIV tests. Pooled NAAT was performed for 290 participants, all of whom had negative results. Fifty-two participants (13.5%) tested positive on Advanced Quality, First Response and INSTI HIV tests. Therefore, INSTI HIV test had 100% sensitivity, specificity and negative predictive value for detection of HIV antibodies. This study showed an excellent field performance of the INSTI HIV test, highlighting that this rapid test can be easily implemented for use at SA POC facilities.

6.2 Introduction, Materials and Methods, Results, and Discussion

HIV diagnosis forms a crucial part of the 90-90-90 strategy as this strategy aims to diagnose 90% of all HIV-infected individuals, have 90% of them on antiretroviral therapy (ART), and to have HIV viral load (VL) suppression in 90% of those receiving ART [1]. Rapid HIV tests are commonly used for diagnosis of HIV infection in low resource settings as they are cheaper and easier to use at point-of-care (POC) facilities. Unfortunately, rapid HIV tests have relatively lower sensitivity than enzyme-linked immunosorbent assays (ELISAs) [2-4]. As a result, some HIV-infected individuals are misdiagnosed by rapid tests, especially those with early HIV infection [5].

Antibodies are the last diagnostic marker of HIV to appear in the blood following exposure and infection. Even after their appearance they predominantly exist as antigen–antibody complexes owing to higher antigen load during early HIV infection. This makes antigen-bound antibodies difficult to detect until free antibodies appear later as the HIV VL decreases [6, 7]. Unfortunately, the addition of the p24 antigen to some rapid tests has only led to a slight improvement in sensitivity for HIV diagnosis [8].

INSTI HIV test, a Food and Drug Administration (FDA)–approved test, has been shown to have higher sensitivity for detection of HIV-specific antibodies even during early HIV infection [9]. Making an accurate diagnosis of HIV infection is important for management of an infected individual and interruption of further spread of HIV [1, 10]. The aim of this study was to assess the field performance of the INSTI HIV test.

The study was conducted in 2016 in two antenatal clinics in the Tshwane district of South Africa (SA), one based in the Pretoria City and the other in Mamelodi Township. Participants were recruited during counseling to be tested on the INSTI HIV test in addition to the rapid tests used in the clinics as part of standard HIV testing service. INSTI HIV test evaluation was conducted as part of a study that screened for early HIV infection, where participants who tested negative on rapid tests used in the clinics received further testing on pooled nucleic acid amplification testing (NAAT) [5]. The study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number–295/2015) and by Tshwane–Metsweding Region Research Ethics Committee (TMREC 2010/26). All participants provided written informed consent.

South Africa employs a serial testing strategy for HIV testing at POC facilities, where a screening rapid test is performed first and no further testing is done if the results are negative. Confirmatory testing on a different rapid test is only done if the screening test results are positive. Testing on ELISAs is performed in cases of discrepant results between screening and confirmatory rapid tests [11]. The rapid tests that were used at the clinics were Advanced Quality HIV test for screening, and First Response HIV test for confirmatory testing [12]. INSTI testing was performed while awaiting the results of the screening test. Individuals who tested negative on the Advanced Quality and INSTI HIV tests were also tested on pooled NAAT, which was done in mini-pools of 5 samples using 200 microlitres (µls) from each sample to constitute a 1 millilitre (ml) sample volume required for testing [5]. Those who tested positive on Advanced Quality and INSTI HIV tests received further testing on the First Response test. Testing was done during the first antenatal visit, and results were documented for all the rapid tests.

HIV testing was performed by lay counselors who had received training for counseling and testing. All the counselors also received orientation on the use of INSTI HIV test. The INSTI HIV test employs immunofiltration (flow through) principle as opposed to most rapid tests, which use immunochromatographic (lateral flow) principle [13, 14]. With INSTI testing, finger-stick whole blood is added into a vial containing sample diluent, then the mixture is applied to a testing cartridge, followed by addition of colour developer and clarifying solutions, respectively. The results are read 1 minute after addition of the sample diluent-specimen mixture to the cartridge [13].

There were 386 pregnant women enrolled in this study; 135 (35.0%) from the city-based clinic and 251 (65.0%) from the township-based clinic. All the participants had testing performed on Advanced Quality and INSTI HIV tests. Three hundred and thirty-four (86.5%) participants tested negative on the Advanced Quality test, and all had negative results on the INSTI HIV test as well. Pooled NAAT was performed for 290 participants, all of whom had negative results. Forty-four participants with negative rapid HIV tests were not available for NAAT as they were not seen for antenatal care on the same day of testing, and were lost to follow-up. Fifty-two participants (13.5%) tested positive on the Advanced Quality HIV test, and were also positive on the First Response and INSTI HIV tests (Table 6.1). The HIV prevalence distribution in this study was 10.4% (n = 14) and 15.1% (n = 38) in the city-based and township-based clinics, respectively. There were no discrepancies found among all test results. Therefore, the INSTI HIV test had 100% sensitivity, specificity, and negative predictive value for detection of HIV antibodies compared to all other tests.

Table 6.1. Field performance of the INSTI rapid HIV test

	INSTI HIV test results		
	NEG	POS	Total
NEG (n = 334)[a]	334*	0	334
POS (n = 52)[b]	0	52	52
Total	334	52	386

*Pooled nucleic acid amplification testing (NAAT) was performed for 290 samples in mini-pools of 5 samples. [a]All negative (NEG) samples were obtained from the Advanced Quality rapid test at the clinics. [b]All positive (POS) results were obtained from the Advanced Quality and First Response rapid tests at the clinics.

This study assessed the field performance of the INSTI HIV test in two antenatal clinics. There was an overall HIV prevalence of 13.5%, but the prevalence varied between the two study clinics. This is not surprising as variation in HIV prevalence has been observed among districts within one SA province [15, 16]. The INSTI HIV test demonstrated excellent field performance in this study as it had 100% correlation with rapid tests used at POC facilities and pooled NAAT. INSTI testing provided parallel testing in this study, and highlights that this strategy is costly [17], as all those who tested negative in this study would have been correctly diagnosed by the screening test. The excellent field performance of the INSTI HIV test highlights that this test could be easily used by lay counselors at the SA POC facilities. The ease of use of the INSTI HIV test at a POC facility has been reported previously [18].

The INSTI HIV test provides results within 1 minute compared to most rapid tests whose results are only read in 15 minutes time interval or longer [13, 14, 19]. Therefore, the use of this test could simplify HIV testing at the POC facilities, and create more time that could be dedicated to other aspects of HIV management or prevention. The INSTI HIV kit comes with a calibrated capillary pipette that enables the exact measurement of blood needed for the test, and thus lessens the chances of using inadequate volume for testing [13].

Studies have shown that the INSTI HIV test has higher sensitivity for detection of early HIV infection [9, 19]. One study that compared performance of five rapid tests (including INSTI) on finger-stick whole blood and plasma observed that INSTI had higher sensitivity on finger-stick whole blood than other rapid tests [20]. Many SA studies have reported that some individuals with HIV infection are misdiagnosed by the rapid HIV tests at the POC facilities [5, 21, 22]. This highlights a need to consider evaluation and use of highly sensitive rapid tests at the SA POC facilities. High costs are the only disadvantage of the INSTI HIV test. The cost of using the INSTI HIV test in 2016 was about R83.60 (5.96 USD) compared to R5.20 (0.37 USD) for the Advanced Quality rapid test, and R6.30 (0.45 USD) for the First Response rapid test. This high cost of the INSTI HIV test was closer to that of pooled NAAT, which was about R92.00 (6.56 USD). The limitations of this study include small sample size, and that early HIV infection could not be excluded in few participants who tested negative on rapid tests but were not available for NAAT.

This study showed an excellent field performance of the INSTI HIV test, highlighting that this rapid test can be easily implemented for use in SA. Larger studies are needed for field evaluation of rapid HIV tests, like INSTI test, that have higher sensitivity for diagnosis of HIV infection. There is a need to improve the accuracy of HIV diagnosis at POC facilities, particularly in HIV hyper-endemic areas. When this need is met, it could lead to improved assessment of the first 90 of UNAIDS 90-90-90 strategy.

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CHAPTER 7

Perspectives

This study found 0.6% (n = 63) of newly diagnosed individuals (with acute, early or chronic HIV infection) after screening 10 287 participants who tested negative on the rapid HIV tests used at the point-of-care (POC) facilities in the Tshwane district of South Africa (SA). Newly diagnosed HIV infections were identified in all study sites, ranging from 0.3% - 1.1%, highlighting a broad distribution of missed HIV infections. SA has massively scaled up HIV counselling and testing. In 2015, there were 11 898 308 people who were tested for HIV at the POC facilities, and 380 000 of them were diagnosed with HIV infection [1]. After excluding those with newly diagnosed HIV infection in 2015, a 0.6% misdiagnosis rate translates to about 691 000 missed HIV infections at the POC facilities. As observed in our study and by other researchers, the misdiagnosed individuals also include those with acute or early HIV infection [2, 3], who are highly infectious for HIV [4, 5]. Therefore, misdiagnosed HIV infections probably contribute significantly in sustaining the HIV epidemic in SA. In an antenatal care setting, misdiagnosing HIV infection has serious implications for mother-to-child transmission of HIV [6, 7].

The principle of screening for an infectious disease in low prevalence areas requires confirmatory testing of a positive screening test with two tests due to lower positive predictive value of screening assays in these settings. However, in high prevalence settings, it is the negative predictive value of a screening assay that is compromised [8, 9]. Therefore, more focus should be directed at confirming negative HIV results in high prevalence settings. The false negative rapid HIV tests observed in our study, in all research sites [3], show a need to improve the HIV screening strategy at the SA POC facilities. Rapid tests with higher sensitivity

for HIV diagnosis should be evaluated and made more accessible for use in HIV hyper-endemic regions.

World Health Organisation (WHO) and SA HIV testing guidelines recommend retesting in 4 – 6 weeks after a negative rapid test for individuals at high risk of HIV and special population groups [9, 10]. This is corroborated by our study findings as we observed that follow-up rapid HIV tests were positive at a median of 4 week interval in most participants who previously had a false negative rapid test [3]. However, it is difficult to reliably identify individuals at high risk of HIV infection in whom retesting is recommended within a shorter interval by WHO and SA HIV testing guidelines. This is reflected by our study data, which found HIV infection in some individuals who reported having no behavioural risk factors [11]. HIV epidemiology in most developed countries is mainly sustained by infections in the special population groups such as commercial sex workers and intravenous drug users. However, this picture is different in developing countries as the general population plays a bigger role in sustaining HIV epidemiology, with special population groups still contributing significantly [12]. This highlights a need to encourage HIV retesting after a negative rapid test at 4 – 6 weeks interval for all sexually active adults in hyper-endemic settings.

The other strategy of improving HIV diagnosis is to employ molecular tests that are available for use at the POC facilities, as they are more sensitive for diagnosis of HIV. Laboratory-based molecular testing in our study was associated with loss to follow-up of some participants with newly diagnosed HIV infection. Molecular HIV tests performed at the POC facilities would result in earlier identification of infected individuals and linkage to care, and thus would potentially limit the spread of HIV drug-resistant strains. Most molecular HIV tests are still under evaluation for use at the POC facilities [13-15]. When POC molecular tests are more available for use, antenatal clinics should be prioritised as accurate diagnosis in pregnant

women leads to earlier linkage to prevention of mother-to-child transmission of HIV (PMTCT) programme.

Our data highlighted that counselling could be standardised to screen for HIV risk behavioural factors, and this would allow optimised counselling for each individual. A systematic way of collecting HIV risk factors could inform relevant HIV prevention interventions to be implemented at a community or population level. Strengthening behavioural HIV prevention measures is important in resource-constrained settings as biomedical prevention measures such as pre-exposure prophylaxis are costly to implement at a larger scale.

We showed that deep sequencing is more informative for assessment of transmitted HIV drug-resistant strains, and that some transmitted minority variants take some time to emerge during the course of infection probably due to compromised replication capacity. Where possible, deep sequencing in sample pairs should be considered for detection of mutants that are known to have lower replication capacity. Making deep sequencing more accessible is of paramount significance as it is able to detect minority variants that are missed by Sanger sequencing, however, more data is needed to assess the clinical relevance of drug-resistant minority variants of HIV. Strategies of preventing emergence and transmission of ARV resistance should be strengthened. These could include implementation of a more robust first-line regimen such as a dolutegravir-containing regimen, which is associated with a lower probability of inducing drug-resistance [16].

Continued surveillance for acute and early HIV infections should be encouraged in HIV hyper-endemic regions in both general and special population groups. The availability of POC molecular tests could facilitate the surveillance for acute and early HIV infections. Elimination

of new HIV infections in hyper-endemic regions needs concerted efforts of strengthening HIV diagnosis among other aspects of HIV management. Improved HIV diagnosis at the POC facilities would lead to accurate assessment of the first 90 of 90-90-90 UNAIDS strategy, and earlier linkage of infected individuals to HIV care.

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APPENDIX A (Ethics Committee approval)

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

23/07/2015

Approval Certificate
New Application

Ethics Reference No.: 295/2015

Title: DETECTION AND CHARACTERISATION OF PRIMARY (ACUTE) AND EARLY HIV-1 INFECTIONS IN AN HIV HYPER-ENDEMIC AREA.

Dear Dr Simnikiwe Mayaphi

The **New Application** as supported by documents specified in your cover letter dated 29/06/2015 for your research received on the 30/06/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 22/07/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**295/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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APPENDIX B (Informed Consent form)

PATIENT OR PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: DETECTION AND CHARACTERISATION OF PRIMARY (ACUTE) AND EARLY HIV-1 INFECTIONS IN AN HIV HYPER-ENDEMIC AREA

SPONSOR: NHLS-RT, FIDSSA, MRC-SIR, Discovery Foundation and University of Pretoria research assistant grants

Principal Investigators: Dr S Mayaphi, Prof A. Stoltz and Prof TC Quinn

Institution: University of Pretoria

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):

Daytime numbers: 012 319 2305

Afterhours: 082 786 5483

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

			:	
dd	**mmm**	**yyyy**	**Time**	

Dear Mr. / Mrs. .....….

1) INTRODUCTION

You are invited to volunteer for a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to detect the window period phase of HIV (called primary or early HIV infection stage). By doing so, we wish to learn more about ways of detecting HIV during the window period, and also about the nature of HIV during this phase, such as transmission of viruses that do not respond to antiretroviral (ARV) drugs. HIV infected people who are in the window period often have negative results on rapid HIV tests. These are tests that are commonly used in the South African clinics for detection of HIV. Therefore, this study will include only people who have negative rapid HIV tests.

Window period phase is the early phase of HIV where the virus multiplies at a high rate in the body, but tests that detect antibodies (i.e. body soldiers fighting against HIV) are unable to detect HIV, because the antibodies are not yet formed at this time. It takes about 3 – 12 weeks for the antibodies to be formed after infection with HIV. Most rapid HIV tests that are used in the South African clinics rely on the availability of these antibodies. In this study, we are going to use quantitative PCR (polymerase chain reaction) test, which is able to detect HIV during the window period, as it detects parts of HIV, not antibodies.
From March 2016, we will also use the INSTI HIV rapid test at the clinics, in addition to the rapid tests that are currently used in the clinics. INSTI HIV rapid test is a Food and Drug Administration (FDA) approved test that is known to have a higher performance for detection of early HIV infections. Therefore, the INSTI rapid test might lead to faster identification of early HIV infections.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves answering some questions with regards to HIV exposure and taking blood for study tests. Answering the questionnaire (list of questions) will take about 5 minutes. Blood will be taken from a vein in your arm and the minimum amount of blood needed for the study tests is 15 mls (about one and a half tablespoon). HIV PCR will be done in your blood to see if you were in the window period when you had a negative rapid HIV test. The HIV PCR and ARV resistance results will be made available to you. More blood will be needed from you if HIV PCR test is positive. The maximum amount of blood taken from you will not be more than 60 mls (about 6 tablespoons) if more blood is needed. This study has two visits; the first one will include blood taking and the second one will be for collection of results when they are available. Participants who have tested positive on HIV PCR will be offered HIV counseling in the clinic and referred for further HIV management.
The INSTI rapid test will be done at the same time as the screening rapid test that is currently used in the clinics. If you were tested on INSTI rapid test and the results of this test are different from the results of the rapid test currently used in the clinic, you will have further testing done on HIV ELISA in order to resolve the difference between the rapid test results.

4) RISK AND DISCOMFORT INVOLVED.

The only possible risk and discomfort involved is the taking of blood from a vein. In rare cases, there could be a bruise or ulcer (sore) where blood was taken from.

5) POSSIBLE BENEFITS OF THIS STUDY.

The negative HIV PCR test will confirm the negative results from a rapid HIV test. If HIV PCR or HIV ELISA test is positive, it will enable you to know your HIV status early. You will therefore receive appropriate counseling and early HIV management, which will include immediate initiation of ARVs for pregnant women to reduce chances of HIV transmission to the child. If you are not pregnant, you will be referred for CD4 test, and be started on treatment if you meet treatment criteria.

6) I understand that if I do not want to participate in this study, I will still receive standard treatment for my illness.

7) I may at any time withdraw from this study.

8) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3541677 / 012 3541330 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

9) INFORMATION If I have any questions concerning this study, I should contact:
Dr S Mayaphi……tel : 012 319 2670…..or cell: 082 786 5483.

10) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable.

11) CONSENT TO PARTICIPATE IN THIS STUDY.

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

....
Patient name Date

....
Patient signature Date

....
Investigator's name Date

....
Investigator's signature Date

....
Witness name and signature Date

VERBAL PATIENT INFORMED CONSENT (applicable when patients cannot read or write)

I, the undersigned, Dr ……..…, have read and have explained fully to the patient, named …….. and/or his/her relative, the patient information leaflet,

which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

I hereby certify that the patient has agreed to participate in this study.

Patient's Name _____
(Please print)

Patient's Signature _____ Date _____

Investigator's Name _____
(Please print)

Investigator's Signature _____ Date _____

Witness's Name _____ Witness's Signature _____ Date _____
(Please print)

(Witness - sign that he/she has witnessed the process of informed consent)

APPENDIX C (Sample size calculation)

BIOSTATISTICS UNIT

LETTER OF STATISTICAL SUPPORT

Date: 01/03/2018

This letter is to confirm that the researcher, Dr Sim Mayaphi a Researcher at the Department of Medical Virology, Faculty of Health Sciences, University of Pretoria discussed the Project titled "**DETECTION AND CHARACTERISATION OF PRIMARY AND EARLY HIV-1 INFECTIONS IN AN HIV HYPER-ENDEMIC AREA"** with me.

I hereby confirm that I am aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

DATA ANALYSIS

The aim of this study include identification of individuals with PHI or early HIV infection and follow them up to provide them with appropriate management so as to prevent the spread of HIV in SA, undertake the molecularly characterization of the identified infections in order to assess transmission of resistant strains of HIV and to assess the impact of detecting PHIs or early HIV infections in a pregnant population.

The data analysis will initially be descriptive presenting summary statistics like proportions for the different category of participants (male, pregnant and non-pregnant participants), mean duration of transition from negative status to early HIV infection status. The statistical package will be STATA 14 to achieve the objective.

SAMPLE SIZE

The sample size estimation is based on the prevalence of early HIV infection of about 1% from the previous studies (Stevens et al 2005 - found 0.99% prevalence of early HIV infection; and Bassett et al 2011 - found 1.1% prevalence of early HIV infection). The variance of prevalence between these two studies is roughly 0.1%. Accordingly, the sample size was reviewed in view of the statement above and the fact that it is based on precision based on single proportion.

$$\mathbf{n} = \frac{((\pi)(1-\pi))}{\varepsilon^2} = \mathbf{10{,}000.}$$

Where $\epsilon = 0.001$ is the variation and a prevalence of $\pi = 0.01$. The sample size was power at 99 percent

Reference "Medical Statistics: Betty R. Kirkwood and Jonathan A.C. Stern (Second Edition – 2003)

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APPENDIX D

See attachment for the publication mentioned below:

Mayaphi SH, Martin DJ, Quinn TC, Laeyendecker O, Olorunju SAS, Tintinger GR, Stoltz, AC. Detection of Acute and Early HIV-1 Infections in an HIV Hyper-Endemic Area with Limited Resources. PLoS ONE **2016**; 11: e0164943. doi:10.1371/journal.pone.0164943

APPENDIX E

See attachment for the publication mentioned below:

Mayaphi SH, Martin DJ, Olorunju SAS, Williams BG, Quinn TC, Stoltz AC. High risk exposure to HIV among sexually active individuals who tested negative on rapid HIV Tests in the Tshwane District of South Africa -The importance of behavioural prevention measures. PLoS ONE **2018**; 13: e0192357. https://doi.org/10.1371/journal.pone.0192357