In vitro HIV-1 drug resistance phenotyping, genotyping and novel virological failure detection tools for clinical patient management

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree

of

Doctor of Philosophy

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DECLARATION

I, Michelle Bronze declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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DEDICATION

The AIDS

Virus you are virus

Because you live in blood only

You are a syndrome

Collection of diseases

You kill young adult young child

You kill heros and zeros

By MN

Primary school KaNyamazane, South Africa

Dedicated to all the people whose lives have been affected by HIV

And

Dedicated to my father, whose absence maintains a gaping wound in my soul,

the late Alvaro Bronze (10.06.1949 - 17.03.2013)

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publications

Bronze M, Steegen K, Wallis CL, De Wolf H, Papathanasopoulos MA, Van Houtte M, Stevens WS, de Wit TR, Stuyver LJ; ART-A Consortium. HIV-1 phenotypic reverse transcriptase inhibitor drug resistance test interpretation is not dependent on the subtype of the virus backbone. PLoS One. 2012;7(4):e34708. **(Chapter 2)**

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Conference Presentations

Wallis CL, Struck D, Perez Bercoff D, **Bronze M**, Denisov G, Schmit JC, Rinke de Wit T, Stevens W, on behalf of the ART-A consortium. Evaluation of an automated sequence analysis software programme in high-throughput laboratories. 8th European HIV Drug Resistance Workshop 17-19 March 2010, Sorrento, Italy.

Bronze M, Steegen K, Dumont S, Wallis C, Stevens W, Dekker J, Rinke de Wit T, Stuyver L. Genotypic and phenotypic characterization of subtype C HIV-1 isolates from patients failing antiretroviral therapy. 7th European HIV Drug Resistance workshop 2009, Stockholm, Sweden.

Steegen K, **Bronze M**, Van Craenenbroeck E, Winters B, Van der Borght K, Wallis CL, Stevens W, Rinke de Wit T, Stuyver L; ART-a consortium. An affordable genotypic HIV drug resistance protocol for truncated Reverse Transcriptase compatible to the vircoTYPE prediction tool, 3rd INTEREST workshop, Lusaka, Zambia, 26-29 May 2009.

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Bronze M, Aitken SC, Wallis CL, Steegen K, Stuyver LJ, Stevens W, Rinke de Wit. Evaluation of an HIV-1 virological failure assay and short reverse transcriptase genotyping protocol in a South African reference laboratory. 10th European Meeting on HIV Hepatitis Treatment strategies & Antiviral Drug Resistance; Barcelona, Spain (2012).

Bronze M, Steegen K, Papathanasopoulos MA, Wallis CL, Stevens WS, Rinke de Wit TF, Stuyver LJ. HIV-1 genotypic antiretroviral drug resistance testing of HIV-1 subtype C samples reflects phenotypic resistance. 1st International Conference of the African Society for laboratory Medicine, Cape Town, South Africa (2012).

ABSTRACT

Of the 22.5 million individuals infected with the human immunodeficiency virus (HIV) in sub-Saharan Africa, 62% of patients requiring treatment had access to highly active antiretroviral therapy (HAART) in 2011. The delivery of HAART and the appropriate laboratory monitoring of HIV positive individuals in sub-Saharan African countries has become a public health priority, an intervention which has and will continue to dramatically reduce HIV-related morbidity and mortality. Routine laboratory monitoring of HIV infected individuals should ideally include $CD4^+$ T cell testing to assess when to start ART, viral load monitoring to assess virological failure on ART and when indicated, HIVDR genotyping. However, this is often not implemented in resource limited settings due to challenges such as inadequate infrastructure and laboratory capacity, amongst others. Thus the Affordable Resistance Testing for Africa (ART-A) initiative was established to develop an affordable HIV drug resistance testing (HIVDR) algorithm applicable to Africa. The objective of this study was to evaluate the role of *in vitro* HIVDR phenotyping in the context of HIV-1 subtype C (the most prevalent circulating subtype in sub-Saharan Africa), genotyping and genotypic interpretation tools using existing algorithms, as well as novel virological failure detection tools for clinical patient management.

Current gold standard HIVDR phenotyping technologies use an HIV-1 subtype B backbone to create recombinant viruses with patient-derived polymerase (protease and partial reverse transcriptase). This backbone could impact on the *in vitro* phenotyping results of non-B subtypes, and therefore it was deemed necessary to establish the applicability of HIVDR phenotypic testing of subtype C polymerase when a commercially available subtype B backbone is used. One hundred and fourteen HIV-1 subtype C samples were HIVDR phenotyped against 17 antiretroviral drugs using both subtype B and C backbones and

showed a high level of concordance between the two backbone phenotypic resistance profiles (95.8%; 1590 of 1660 fold change comparisons). Natural assay variability was largely responsible for discordant results. Results confirmed that HIV-1 phenotypic reverse transcriptase inhibitor drug resistance test interpretation is independent of the virus backbone subtype. No conclusions could be made for protease inhibitor resistance since limited samples from 2nd line failure were available. Subsequently, the HIVDR genotypic and phenotypic results of the 114 patient samples were compared to determine whether genotyping is a viable alternative to phenotyping. Results showed a 92.3% concordance between genotyping and phenotyping of individual drug comparisons for a number of HIVDR profiles. Discrepancies were attributed to phenotypic assay variability in addition to the role of mutation mixtures, which impacted genotypic interpretations. Overall, HIVDR genotyping is a reliable tool to detect and interpret antiretroviral drug resistance in HIV-1 subtype C infected patients, and can thus be used for clinical patient management.

Once the accuracy of HIVDR genotyping was established, the development, validation and evaluation of a potential virological failure assay (ARTA-VFA) and a simplified HIVDR (ARTA-HIVDR^{ultralight}) assay was undertaken. A simplified and conceptually novel approach using a qualitative viral load assay with a pre-determined cut-off that gives a threshold above which virological failure (VF) could be confirmed and below which treatment success was likely, was tested. A real-time PCR (ARTA-VFA) assay was developed which involved the amplification of a short sequence of the HIV-1 LTR region from RNA extracted either from plasma and/or dried blood spots (DBS). The ARTA-VFA was tested on 409 patient samples, and successfully amplified samples from all major HIV-1 group M subtypes with equal specificity. The VF was qualitatively classified as a viral load >1000 RNA copies/ml in plasma samples, and >5000 RNA copies/ml in DBS samples. Comparative testing yielded

accurate VF determination for therapy-switching in approximately 93% of clinical cases tested, compared to current gold standard quantitative viral load assays.

A simplified HIVDR genotyping assay (ARTA-HIVDR^{ultralight}) targeting the region of RT harboring all major RT inhibitor resistance mutation positions, thus providing all relevant susceptibility data for first-line regimen failures was developed and assessed. The ARTA-HIVDR^{ultralight} assay was designed to be practical, faster, and more affordable, show flexibility with respect to equipment (open platform), use DBS or plasma as starting material and amplify and sequence a smaller amplicon (RT). The assay performed well when compared to the in-house assay used in the laboratory at the time for both 212 plasma and 25 DBS samples, yielding identical mutations and subsequent resistant profiles. Furthermore, a theoretical *in silico* exercise to investigate the consequences of using 125,329 shortened RT genotype (ARTA-HIVDR^{ultralight}) as compared to full-length RT sequences showed >95% and >90% concordance when using the Stanford HIVdb algorithm and the virco®TYPE tool, respectively. Differences noted were minor and unlikely to have any impact on clinical decision-making. Overall, this study illustrated that the short RT sequences can be reliably used to generate HIVDR genotypes using the Stanford HIVdb and virco®TYPE algorithms and reduce sequencing costs substantially. A field evaluation using the ARTA-VFA and ARTA-HIVDR^{ultralight} on 288 clinical samples was conducted, showing that the accuracy and precision of both assays (using 248 plasma or 40 DBS sampling methods) compared well to the reference methodology, thereby extending access of testing to more remote settings. These assays were designed to either be used as a testing strategy of initially assessing VF, and once confirmed performing an HIVDR assay, or alternatively to be used separately as stand-alone, or within different laboratory tiers in resource limited settings. It is envisaged that the ARTA-VFA could be used in the middle laboratory tier, and if confirmatory, patient

samples can be referred to a reference laboratory with the available infrastructure for HIVDR testing using the ARTA-HIVDR^{ultralight}. Lastly, an automated sequence analysis and editing software for use in correct base calling of nucleotide/mutation mixtures in HIVDR genotyping was validated on 1624 sequences. Compared to reference software, where interpretation is often operator dependent, this software performed extremely well, with minor discrepancies noted. The automated software can be used to reduce subjectivity, time taken for analysis which is often the rate-limiting step and thus improving the turn-around time and clinical relevance of HIVDR genotyping.

Overall, the results obtained describe the validation of using HIVDR genotyping as an alternative tool to phenotyping, and the subsequent development and validation of simple, affordable, "open-platform" alternatives to currently used methods for virological failure monitoring, and accommodate a centralized approach to HIVDR with DBS testing in resource limited settings.

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ABBREVIATIONS AND SYMBOLS

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
AMC	Academic Medical Centre
APV	Amprenavir
APV	Fosamprenavir
ART	Antiretroviral Therapy/ Treatment
ARTA or ART-A	Affordable Resistance Test for Africa
ARTA-HIVDR^{ultralight}	Affordable Resistance Test for Africa human immunodeficiency virus drug resistance ultralight assay
ARTA-VFA	Affordable Resistance Test for Africa Virological Failure Assay
ARV	Antiretroviral
ATV	Atazanavir
AZT	Zidovudine
CA	Capsid protein
CDC	Centres for Disease Control
CRFs	Circulating recombinant forms
CTL	Cytotoxic $CD8^+$ T lymphocytes
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic Acid
DRV	Darunavir
EFV	Efavirenz

ELISA	Enzyme-linked immunosorbent assay
ENF	Enfurvirtide
ETR	Etravirine
FDA	United States Food and Drug Administration
FTC	Emtricitabine
HAART	Highly active antiretroviral therapies
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 1
HIVDR	Human Immunodeficiency Virus Type 1 Drug resistance
HTLV-III	Human T lymphotropic virus type III
IC_{50}	The concentration of antiretroviral required to inhibit viral replication of a test virus by 50% relative to the control virus
IC_{90}	The concentration of antiretroviral required to inhibit viral replication of a test virus by 90% relative to the control virus
IDV	Indinavir
IN	Integrase
LAV	Lymphadenopathy-associated virus
LPV	Lopinavir
LTR	Long terminal repeat
MA	Matrix proteins
MTCT	Mother-to-child transmission
MVC	Maraviroc
NC	Nucleocapsid
NC	Nucleocapsid protein
NFV	Nelfinavir

NNRTIs	Non-nucleotide reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NtRTIs	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
OLA	Oligonucleotide ligation assay
ORFs	Open reading frames
PCR	Polymerase chain reaction
PI	Protease Inhibitors
PMTCT	Prevention of mother-to-child transmission treatment
PR	Protease
RAL	Raltegravir
RNA	Ribonucleic Acid
RPV	Rilpivirine
RT	Reverse transcriptase
RTV	Ritonavir
SQV	Saquinavir
SU	Surface units
TAM	Thymidine Analog mutation
TB	Tuberculosis
TDF	Tenofovir
TDR	Transmitted drug resistance
TM	Transmembrane units
TPV	Tipranavir
UMCU	University Medical Centre Utrecht
UN	United Nations
UNAIDS	United Nations Programme on HIV-1/AIDS
URF	Unique recombinant form

VCT	Voluntary counselling and testing
VL	Viral load
WHO	World Health Organization

Chapter 1

Introduction & Research Objectives

1 An introduction to the Human Immunodeficiency Virus (HIV)

1.1 The history of HIV infection and Acquired Immune Deficiency Syndrome (AIDS)

The earliest blood sample ever identified, to date, as being Human Immunodeficiency Virus (HIV) positive was from a stored plasma sample taken from an adult male from the Democratic Republic of Congo in 1959[1]. It is suggested that the ancestor of this strain dates back to the 1940's or 1950's, and may have been introduced into humans a decade earlier[1]. Two major classifications of HIV exist, HIV-1 and HIV-2. HIV-1, which causes the vast majority of global HIV infections, is related to a virus found in the chimpanzee subspecies *Pan troglodytes troglodytes*, and HIV-2, which is mostly confined to West Africa, is related more closely to a virus found in the sooty mangabey *subspecies Cercocebus atys atys*[2,3].

In 1981, increasing numbers of cases of the previously uncommon *Pneumocystis jiroveci* pneumonia and Kaposi's sarcoma were being reported among previously healthy young homosexual and bisexual men in the United States of America[4]. Other life threatening infections and neoplastic diseases were observed in these patients, which were found to be associated with an unexplained defect in cell mediated immunity[5]. In 1982, the Centers for Disease Control (CDC) named this group of disease entities as AIDS[6]. The causative viral agent responsible for AIDS was first discovered and isolated in 1983[7], later known as lymphadenopathy-associated virus (LAV), and again by another group in 1984[8], who renamed the virus human T lymphotropic virus type III (HTLV-III). In 1985, reports showed that these two viruses were indeed the same[9], and the virus was renamed HIV[10].

Since AIDS was first recognized in 1981, it has resulted in approximately 30 million deaths worldwide[11]. The United Nations Programme on HIV-1/AIDS (UNAIDS) estimated that approximately 34 million people were living with HIV-1 worldwide in 2011[12]. The highest

prevalence of HIV-1/AIDS is in the developing world, with sub-Saharan Africa having an estimated 23.5 million people infected, representing 69% of the global HIV burden[13]. A snapshot overview of the global epidemic in 2011 is illustrated in Table 1.

Table 1: Number of people living with HIV, number of people newly infected with HIV and number of AIDS deaths worldwide, 2011. Copied from: http://www.who.int/hiv/data/2012_epi_core_en.png.[14]

Number of people living with HIV	Total	34.0 million [31.4-35.9 million]
	Adults	30.7 million [28.2-32.3 million]
	Women	16.7 million [15.4-17.6 million]
	Children (<15 years)	3.3 million [3.1-3.8 million]
People newly infected with HIV in 2011	Total	2.5 million [2.2-2.8 million]
	Adults	2.2 million [1.9-2.4 million]
	Children (<15 years)	330000 [280000-390000]
AIDS deaths in 2011	Total	1.7 million [1.5-1.9 million]
	Adults	1.5 million [1.3-1.7 million]
	Children (<15 years)	230 000 [200 000-270 000]

1.2 HIV-1 structure and genome

HIV belongs to the genus *Lentivirus*, which is part of the Retroviridae family of viruses. These are enveloped and positive-stranded RNA viruses. Following infection with this group of virus, the single-stranded RNA genome is reverse-transcribed into double-stranded DNA. The resulting double-stranded DNA is subsequently irreversibly integrated into the host genome.

HIV virions have a spherical morphology of 100-120nm in diameter (Figure 1). The virus consists of a lipid bilayer membrane that surrounds a dense, truncated cone-shaped nucleocapsid (NC) or core. The capsid protein p24 forms the NC which contains two single stranded RNA copies, the viral protease (PR), reverse transcriptase (RT), integrase (IN), some of the accessory and regulatory proteins (p6, Nef, Vif and Vpr) and some cellular factors[15,16]. The envelope is largely derived from the host cell plasma membrane and is dotted with approximately 72 spikes derived from the viral envelope glycoprotein (gp160). Each spike is composed of surface units (gp120, SU) that are non-covalently linked with the transmembrane units (gp41, TM), to form functional trimers. The inner surface of the lipid bilayer is lined with matrix proteins (MA).

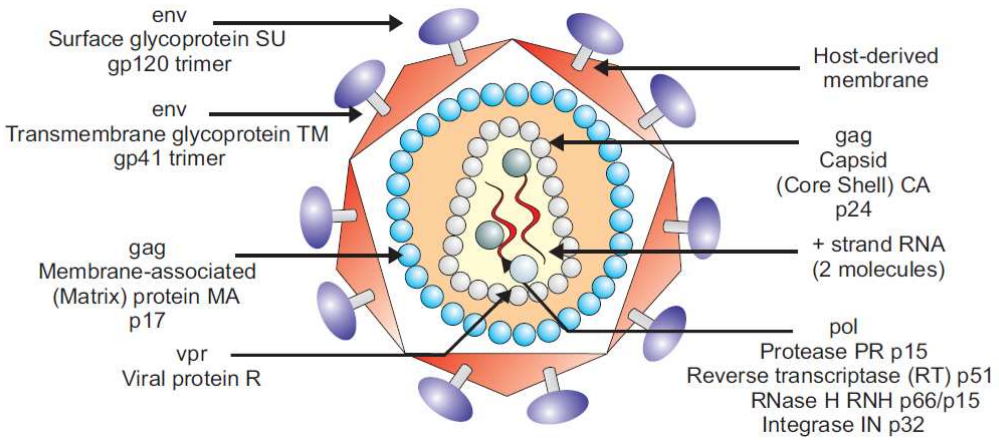

Figure 1: HIV-1 virion structure. ***Copied from: Molecular Medicine, 2009***[17]

The HIV-1 genome consists of two identical, approximately 9.2kb, single-stranded RNA molecules within the virion, whereas the persistent form of the HIV-1 genome is proviral double-stranded DNA within infected cells[18].The HIV-1 genome contains nine open reading frames (ORFs; Figure 2), three of which encode for the *gag*, *pol* and *env* precursor polyproteins which are subsequently proteolysed into individual proteins. The *gag* gene provides the basic infrastructure of the virus and encodes for the precursor of the matrix

proteins (MA, p17), the capsid protein (CA, p24), the nucleocapsid protein (NC, p7) and p6. The *pol* gene provides the basic mechanism by which retroviruses reproduce, encoding for the viral enzymes: protease (PR, p10), reverse transcriptase and RNaseH (RT, p66/51) and integrase (IN, p32). The *env* gene encodes for the precursor (gp160) of the envelope glycoproteins gp120 (SU) and gp41 (TM). The HIV-1 genome also encodes for two regulatory proteins (Tat and Rev) and four accessory proteins: Vif, Vpr, Vpu and Nef. Once HIV-1 has integrated into the host genome, the ORFs are flanked by two identical long terminal repeat (LTR) regions, which are essential for integration and contain promoters for the transcription of the viral genes[19,20].

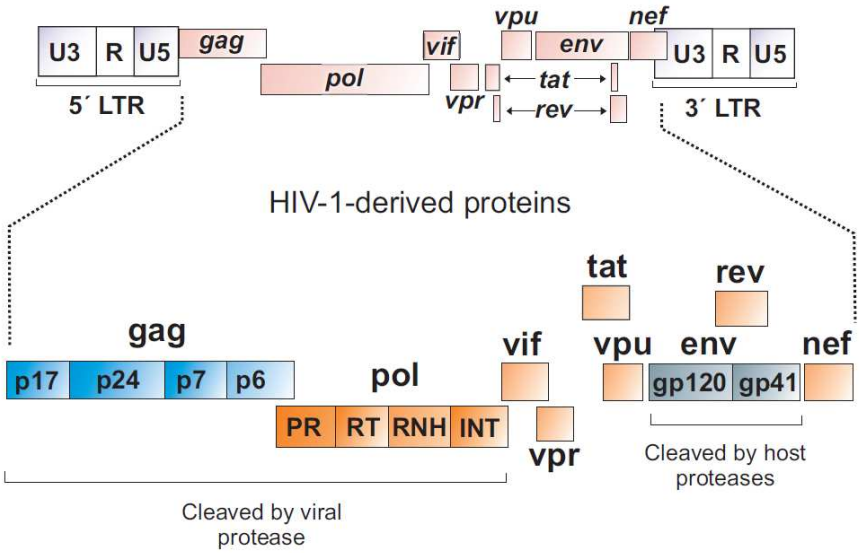

Figure 2: Genomic structure of the human immunodeficiency virus type 1. Copied from *Molecular Medicine, 2009*[17]

1.3 HIV-1 Replication cycle

The main steps in the HIV-1 replication cycle can be divided into the 8 steps depicted in Figure 3. The initiation of infection of a target $CD4^+$ T cell by HIV-1 is elicited by an interaction between the extra-cellular domain of the viral envelope glycoprotein (gp120) and both the CD4 receptor and appropriate co-receptor (CCR5 or CXCR4) located on the host's

cellular membranes (Step 1). These receptors are found in all cell types, including macrophages and T cells. This results in a conformational change in the transmembrane units, leading to the fusion of the viral envelope and cellular membrane, and the release of the viral nucleocapsid into the cytoplasm (Step 2). The nucleocapsid is then uncoated to expose the viral nucleoprotein complex consisting of MA, RT, IN, Vpr and viral RNA (Step 2a). The viral RNA is then reverse transcribed into cDNA by RT (Step 3)[17].

The resulting double-stranded proviral DNA then migrates as part of a pre-integration complex into the cell nucleus and is irreversibly integrated into the cellular DNA through a step catalyzed by the IN enzyme (Step 4). Viral transcripts are then expressed from the promoter in the 5' LTR while Tat enhances the rate of transcription. The processing of the RNA transcripts is regulated by the viral Rev protein. Most of the resulting transcripts are double spliced to generate the Tat, Rev and Nef proteins. When sufficient Rev protein is produced, it protects the viral transcripts from being spliced and mediates their transport to the cell plasma. This allows single spliced and unspliced transcripts to be produced (Step 5). The single spliced transcripts are then translated into Gag, Gag-Pol polyprotein and Env precursors by cellular polysomes (Step 6). Splicing of the Env precursor, gp160, by cellular proteases results in gp120 and gp41 products, which are then transported from the endoplasmic reticulum to the cellular membrane. The Gag and Gag-Pol polyproteins, the unspliced RNA transcripts and the Vif, Vpr and Nef proteins are assembled into a new virion. This new virion starts to bud from the cell membrane, taking a part of the host membrane embedded with the Env glycoprotein trimers with it, and is subsequently released from the cell as an immature virion (Step 7). During the maturation step, the Gag and Gag-Pol polyproteins are fully cleaved by viral protease (Step 6), forming the mature virion that is now ready to infect a new cell[17]. The replication cycle takes approximately 2.5 days and

accounts for the high levels of virion production, reaching up to approximately 10^{10} to 10^{12} virions/day[18,21].

Each step of the viral life cycle represents a potential antiretroviral drug target. Currently validated drugs target the RT, PR, IN enzymes, CCR5 co-receptor binding and the fusion process.

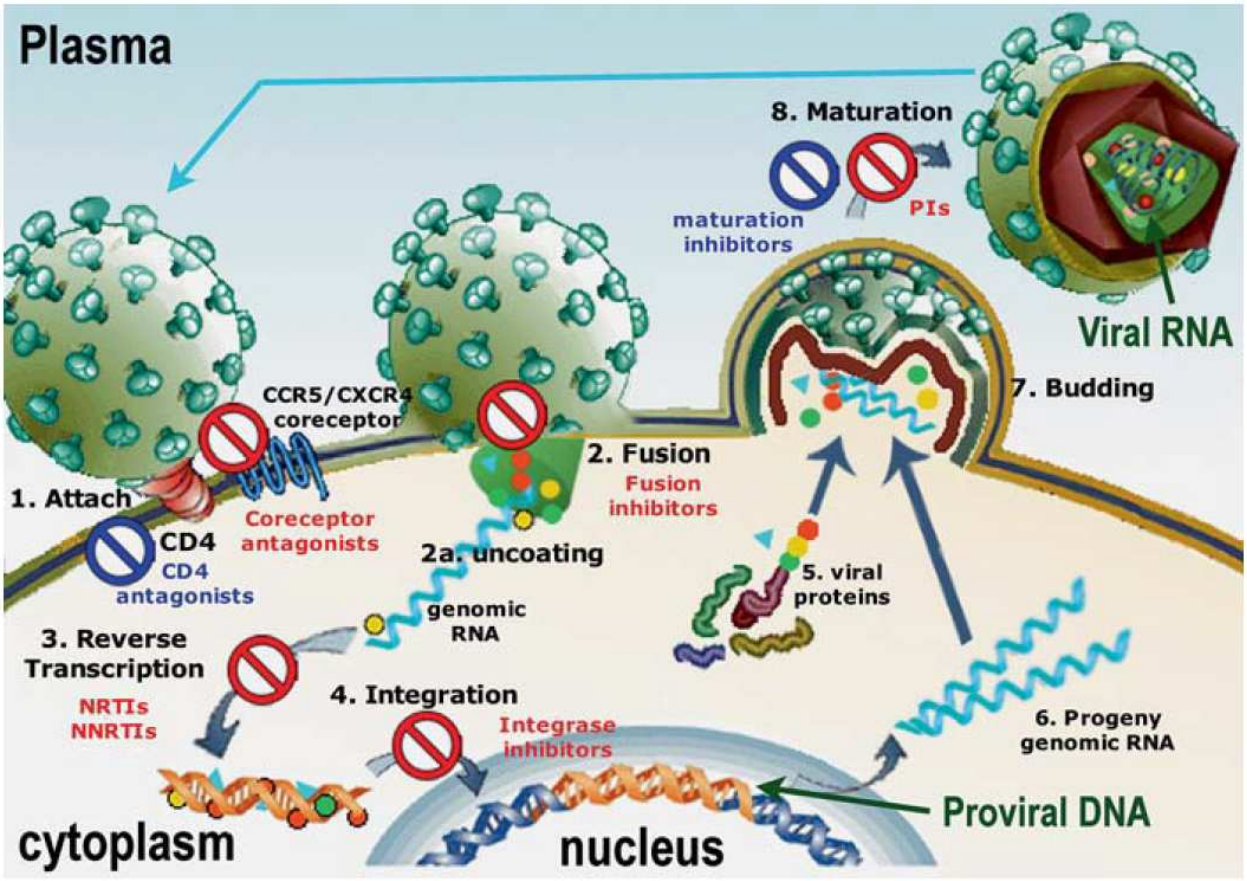

Figure 3: Replicative Cycle of HIV-1. Steps 1 to 8 correspond to the descriptions in the text in section 1.3. ***Copied from Intervirology 2012; 55:89-97***[22]

1.4 HIV Transmission

The main mode of HIV transmission is through sexual contact (homosexual or heterosexual), vertically through mother-to-child transmission (MTCT) or through the transfer of blood or blood-containing products. The transmission of HIV from an HIV-positive mother to her

child may occur during pregnancy, labour, delivery or breastfeeding. In the absence of any interventions, MTCT rates range from 15-45%, but this rate can be reduced to levels below 5% with effective interventions[23]. Blood transmission is most commonly a result of the sharing of infected needles by intravenous drug users, to a lesser extent through health care worker accidental blood exposures, or as in the early 1980's and 1990's through inadequately screened blood transfusions[24].

1.5 Disease Progression to AIDS

Initially, during the acute/primary phase of HIV-1 infection, some patients remain asymptomatic while others develop flu-like symptoms. When HIV-1 replicates uncontrollably, it can reach viral titers as high as 10^8 HIV-1 RNA copies/ml and the number of $CD4^+$ T lymphocytes decrease significantly[25] (Figure 4). During this initial phase, the emergence of HIV-1 specific cytotoxic $CD8^+$ T lymphocytes (CTL) ultimately bring viral replication under control, and the viral load starts to decline to a patient-specific plateau or set point. The production of binding antibodies against different HIV-1 viral proteins signals seroconversion.

The asymptomatic phase or clinical latency is initially associated with a transient increase of $CD4^+$ T lymphocytes. The viral set point determines the rate of disease progression to AIDS. A typical progressor will develop AIDS symptoms within eight to ten years post infection, while a subset of patients (10 - 15%) progress to AIDS within two to five years (rapid progressors), and long-term non-progressors or elite controllers (<5%) remain asymptomatic for at least 10 years[26]. Although vigorous anti-HIV cellular and humoral immune responses are developed, they ultimately cannot control viral replication, nor eradicate the virus. Although the binding of neutralizing antibodies to viral particles mediate the destruction of virus by phagocytes, this action does not reduce the viral infectivity significantly[27].

As the disease progresses, a persistent decline in the immune system is noted, with a decline in $CD4^+$ T lymphocytes and an increase in viral load. As the $CD4^+$ T lymphocyte count declines, the immune system fails to control the virus and other pathogens, which results in the patient becoming vulnerable to opportunistic infections. A patient with a $CD4^+$ T cell count of < 350 cells/mm^3 will be diagnosed with AIDS[28,29]. In the absence of antiretroviral drug treatment a patient will likely die within 2 years of an AIDS diagnosis.

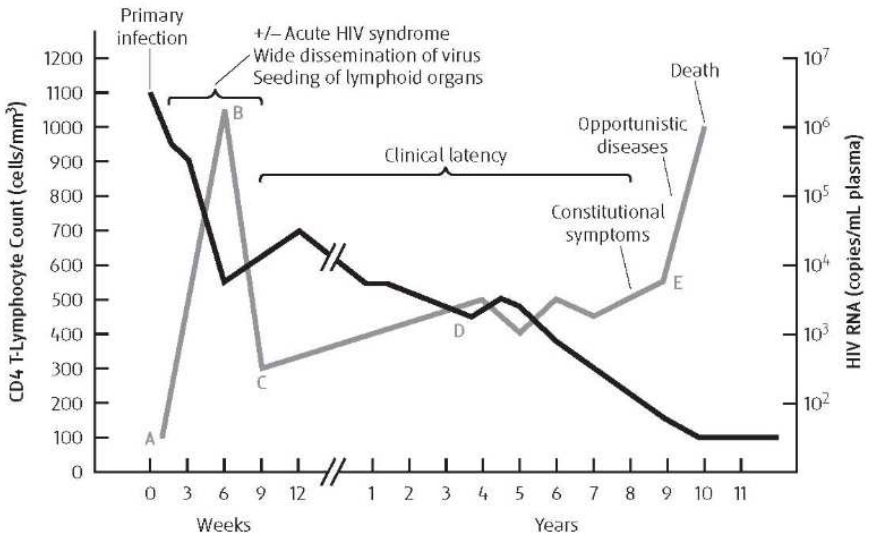

Figure 4: The natural evolution of HIV-1 infection and disease progression to AIDS.

Copied from http://ftguonline.org[30]

1.6 HIV-1 Latency

In addition to HIV-1 enabling itself as a mature virion, the virus is also able to form viral reservoirs throughout its host. A viral reservoir is a cell type or anatomical site in association with which a replication-competent form of virus accumulates and persists with more stable kinetic properties than the main pool of actively replicating virus[31]. A lymphatic tissue reservoir is established within the first few weeks post-infection[31]. HIV-1 infection can therefore persist despite long-term administration of the currently available highly active antiretroviral therapies (HAART). The mechanism of this persistence appears to result

primarily from viral infection of $CD4^+$ T-lymphocytes that have the ability to duplicate and revert into a quiescent state[32]. The low level ongoing viral replication in patients on suppressive HAART may also contribute to the stability of this reservoir. HIV-1 infection of resting memory or naïve $CD4^+$ T cells, macrophages or mononuclear cells leads to a non-productive latent infection[31]. Although the number of latently infected cells harboring replication competent virus is very low (approximately one million cells), there is minimal decay and it appears to be sufficient to guarantee lifelong persistence of HIV in the majority of patients on current antiretroviral regimens. Studies have shown that eradication of HIV-1 from the latent reservoirs would require approximately 73.4 years of suppressive therapy. To date, HIV-1 latency represents a major barrier to complete virus eradication[31,32].

1.7 HIV diversity and geographical distribution

Genetic diversity is a hallmark of HIV infection with regard to the expansion of distinct viral groups and subtypes in different geographical regions. The turnover of the virus is short and about 10^{10} virions are produced daily[33]. The RT enzyme lacks proofreading activity which results in a high rate of incorrect nucleotide substitutions (10^{-4}/nucleotide or 1 mutation per replication cycle). Moreover, recombination processes make the exchange of larger genomic fragments possible[34].

HIV-1 and HIV-2 are the two types of HIV recognized, based on differing antibody binding assay reactions and phylogenetic analyses of genetic sequences show over 50% differences at the nucleotide level. HIV-1 is divided into 3 distinct genetic groups: M, N, O and P[35]. Groups N and O have been found to be responsible for a minority of infections in central Africa. Group M is responsible for more than 90 % of the HIV-1 global infections, and to date, it has been recognized to consist of nine subtypes (A-D, F-H, J and K), 58 circulating recombinant forms (CRFs)[36] and multiple unique recombinant forms (URFs)[36]. Subtype B is the

predominant subtype in Europe and Northern America, whereas non-B subtypes are mostly represented within the African continent, with CRF01_AE being common in Asia[35]. Subtype C is responsible for 50% of global infections[37] and 56% of infections in Africa and is mainly distributed along the southern and eastern part of the continent[38]. The global prevalence of HIV-1 subtypes is illustrated in Figure 5a as a percentage of the total number of HIV-1 isolates worldwide, and the proportions of each subtype within distinct geographic regions are shown in Figure 5b.

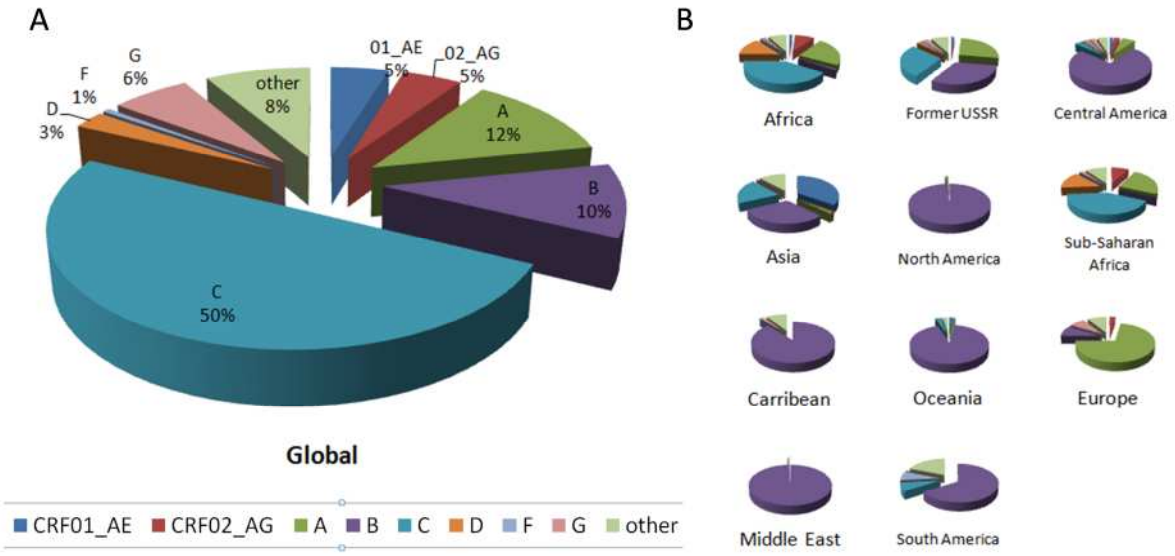

Figure 5: The global prevalence of HIV-1 subtypes (A) as a percentage of the total number of HIV-1 isolates worldwide, and the proportions of each subtype within distinct geographic regions as per the Los Alamos Database (B). ***Adapted from J Infect Dis. 171: 1411–1419 (1995)[37] and the Los Alamos database[35]***

2 Antiretroviral Therapy (ART)

2.1 History of Antiretroviral Therapy (ART)

The first strategies implored in designing ART targeting HIV, aimed to inhibit RT and PR, which are viral enzymes exclusively expressed by the virus. RT inhibitor development began with the discovery of the antiretroviral activity of the nucleoside RT inhibitor (NRTI)

zidovudine (AZT) in 1987[22]. Shortly after that, other NRTI's like didanosine (ddI), stavudine (d4T) and lamivudine (3TC) were discovered. Over the next few years other RT inhibitors became available and were used as monotherapy[22]. However, treatment with monotherapy of these nucleosides was soon challenged, as rapid antiretroviral drug resistance development by the virus was noted, specifically in the case of 3TC, where approximately 80% of patients developed resistance after only 2 weeks of 3TC monotherapy[39]. ART was improved upon by the development of the first non-nucleoside RT inhibitors (NNRTI's) in the mid-1990s[40]. With the licensing of NNRTI's like nevirapine and efavirenz, the definition of HAART included dual NRT and NNRTI combinations. A different antiretroviral strategy was tested, which entailed blocking the viral PR. This led to the approval of the first protease inhibitor (PI), saquinavir (SQV), in 1995 which lead to a substantial enrichment of ART[22]. It was only in 1996 that a breakthrough trial was announced, which showed the potent antiretroviral activity of the triple combination of two RT inhibitors and one PI[41]. This triple therapy, also known as HAART was introduced into the clinical setting and had a dramatic impact on the mortality and morbidity among HIV-infected patients[42,43]. During the evolution of PI therapy, it was found that including a pharmaceutical booster like ritonavir with the existing regimen lead to increased and more stable drug levels, and to a drastic reduction of viral resistance development, from approximately 50% of all observed therapy failures to 0-15% against all drugs[44,45].

The first United States Food and Drug Administration (FDA) approved fusion inhibitor, enfuvirtide (T-20) was placed on the market in 2003[46]. As this drug is cost prohibitive and is subcutaneously administered twice daily, its clinical use to salvage regimens is limited, even though it is quite effective and still successfully in use[22]. The first CCR5 receptor antagonist, maraviroc (MVC) was approved in 2007, but can only be used in patients who are infected exclusively with R5-tropic viruses[47]. In 2009, the first IN strand transfer inhibitor raltegravir

(RAL), which took substantially longer to formulate than the RT inhibitors, was approved[48]. To date, 26 antiretroviral drugs have been approved by the FDA, excluding fixed-dose drug combinations[49] (Table 2). Overall, treatment should ideally incorporate antiretroviral drugs from at least 2 different drug classes to ensure a high genetic barrier to the development of antiretroviral drug resistance[22]. Successful ART is measured as an undetectable viral load (<50 RNA copies/ml) within 12 weeks of treatment initiation[22].

Table 2: The five classes and multiclass combination FDA Approved antiretroviral drugs. ***Adapted from http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm.***[49]

NRTIs	NNRTIs	PI s	Fusion and Entry Inhibitors	Integrase Inhibitors	Multiclass Combination Drugs
Abacavir (ABC)	Rilpivirine (RPV)	Amprenavir (APV)	Enfuvirtide (ENF)	Raltegravir (RAL)	Atripla (EFV + FTC +TDF)
Didanosine (ddI)	Etravirine (ETR)	Atazanavir (ATV)	Maraviroc (MVC)		Complera (FTC + RPV +TDF)
Emtricitabine (FTC)	Delavirdine (DLV)	Darunavir (DRV)			Stribild (ELV + FTC +TDF + cobistat)
Lamivudine (3TC)	Efavirenz (EFV)	Fosamprenavir (APV)			Combivir (3TC + AZT)
Stavudine (d4T)	Nevirapine (NVP)	Indinavir (IDV)			Trizivir (ABC + AZT + 3TC)
Tenofovir (TDF)		Lopinavir (LPV)			Truvada (TDF + FTC)
Zalcitabine (ddC)		Nelfinavir (NFV)			
Zidovudine (AZT)		Ritonavir (RTV)			
		Saquinavir (SQV)			
		Tipranavir (TPV)			

2.2 Mode of action and resistance associated mutations of antiretroviral drugs

The NRTI's and Nucleotide RTIs (NtRTIs) are analogues of the natural substrates used to synthesize viral DNA, which compete with natural nucleotides for incorporation into the growing viral DNA chain. The RT inhibitors lack a 3'-hydroxyl group on the deoxyribose moiety, so any subsequent incorporation of nucleotides into the nascent DNA is blocked[50].

Resistance to this class of drugs are a result of amino acid changes that increase the discrimination between natural nucleotides and NRTIs (eg. A62V, K65R, L74V, V75T/I, F77L, Y115F, F116Y, V118I, Q151M and M184V) or by promoting the removal of NRTIs,

caused by the thymidine analog mutations (TAMs) (eg. M41L, D67N, K70R, L210W, T215Y and K219Q/E)[50].

The non-nucleotide RTIs (NNRTIs) display a high affinity for a hydrophobic pocket of RT that is located close to the active site of the enzyme. The NNRTIs function by impairing the mobility of particular domains of the RT enzyme, and block the DNA polymerization reaction. Due to naturally occurring amino acid polymorphisms, these drugs are generally inactive against HIV-2 RT enzymes[51].

Several mutations in the HIV-1 RT gene, selected by antiretroviral drug pressure, cause resistance by altering the tertiary structure or charge of the RT[51]. Since the lipophilic binding pocket is built mainly by three sites of the amino acid primary sequence, all but one of the NNRTI resistance mutations observed can be found in these regions (amino acids 98-108, 178-190, 225-238), with the exception being amino acid 138, which is associated with rilpivirine and possibly etravirine resistance[51].

The PI's are derived from the natural peptide substrates of the HIV protease enzyme. In the absence of an active viral PR, the shell of viral particles remains rigid and fails to condense into the characteristic conical virus core, which contains the entire replicative machinery of the virus. The immature particles are unable to initiate infection of new target cells and therefore inhibitors of protease have been proven to be among the most active agents in HIV treatment[52].

Resistance to PIs is achieved through mutations located in the substrate-binding pocket (D30N, V32I, L33F, M46I/L, I74A/V, G48V, I50L/V, V82A/F/L/S/T and I84V) leading to conformational changes in the PR that reduce PI incorporation or binding. The conformational changes decrease the overall fitness of the virus, so compensatory mutations are needed to hold on to viral fitness, thereby resulting in a higher level of resistance[53].

Moreover, some mutations in the *gag* gene can adapt the *gag/gag-pol* cleavage site or improve the incorporation of PR in the virion in order to optimize the cleavage activity of a mutated PR enzyme[52].

Entry inhibitors act by preventing the virus from infecting the cell by either inhibiting fusion between the viral and the host cell membrane or by blocking the co-receptors. The fusion inhibitor, T-20 inhibits fusion by binding to the gp41 HR1 region, thereby preventing gp41 undergoing a fusogenic conformation[54]. Mutations in the gp41 amino acids 36-45 have been related to ENF resistance and therapy failure[54].

The co-receptor antagonist MVC binds to the CCR5 receptor, thereby preventing gp120 binding and significantly hindering HIV replication[55]. As CCR5 antagonists only prevent viral entry of R5 viruses, a tropism test is mandatory before these drugs can be administered[55]. Interestingly, the virus develops resistance to MVC by adapting to binding to CCR5 in the presence of the drug[56].

The IN inhibitor raltegravir prevents the strand transfer process of viral cDNA into the host chromosome[22]. Resistance to RAL has been associated with amino acid substitutions at three key positions in the IN protein: Y143R/C, Q148H/R/K, or N155H, alone, or accompanied by other mutations[57].

Next generation inhibitors within the existing antiretroviral drug classes, as well as other novel targets are being investigated, including inhibitors of CD4 binding, CXCR4 antagonists and maturation inhibitors[22].

2.3 Antiretroviral treatment regimens

The WHO recommends that all adolescents and adults, including pregnant women, infected with HIV-1 and a CD4 count ≤350 cells/mm^3 should start HAART, regardless of the

presence or absence of clinical symptoms. Patients with severe or advanced clinical disease should start ART irrespective of their CD4 cell count. In terms of first-line therapy, the WHO suggests it consists of one NNRTI and two NRTIs, one of which should be AZT or TDF. Second line therapy should consist of a RTV-boosted ATV or LPV as the preferred PIs[58].

WHO treatment guidelines for infants and children <5 years of age, recommend initiation of therapy irrespective of WHO clinical stage. Children >5 years of age should initiate treatment once CD4 ≤ 350 cells/mm^3 (as in adults). Initiation of therapy should proceed in all children with clinical stages 3 and 4, irrespective of CD4 count. HAART regimens for infants and children generally include NVP or EFV, if not exposed to NVP during prevention of mother-to-child transmission treatment (PMTCT) plus 2 NRTI's. In case of pre-exposure to NVP or other NNRTI's, the regimen should be RTV-boosted LPV (LPV/r) with 2 NRTIs. For infants and children, the NRTI backbone should be one of the following in preferred order: 3TC + AZT or 3TC + ABC or 3TC + d4T[59].

The 2010 South African ARV treatment guidelines[60] define first and second line HAART regimens as shown in Tables 3 and 4. Patients failing second line regimens are asked to "consult an expert".

Table 3: Current ARV drug guidelines for adults and adolescents in South Africa.

Copied from *The South African Antiretroviral treatment Guidelines*. (2010)[60]

First line		
All new patients needing treatment, including pregnant women	TDF + 3TC/FTC + EFV/NVP	For TB co-infection EFV is preferred. For women of child bearing age, not on reliable contraception, NVP is preferred
Currently on d4T based regimen with no side-effects	d4T + 3TC + EFV/NVP	Remain on d4T if well tolerated. Early switch with any toxicity. Substitute TDF if at high risk of toxicity (high BMI, low Hb, older female)
Contraindication to TDF: renal disease	AZT + 3TC + EFV/NVP	
Second Line		
Failing on a d4T or AZT-based first line regimen	TDF + 3TC/FTC + LPV/r	
Failing on a TDF-based first line regimen	AZT + 3TC + LPV/r	
Salvage		
Failing any second line regimen	Specialist referral	

Table 4: Current ARV guidelines for children in South Africa. Copied from *The South African Antiretroviral treatment Guidelines*. (2010)[60]

First Line		
All infants and children under 3 years	ABC + 3TC + LPV/r	
Children 3 years or over	ABC + 3TC + EFV	
Currently on d4T-based regimen with no side-effects	Can continue	Substitute - once lipodystrophy suspected
Second Line		
Children above 3 years Failed ABC + 3TC + EFV	AZT + ddI + LPV/r	
Failed on AZT or ddI-based regimen	ABC + 3TC + LPV/r	
Failed on LPV-based regimen	Refer	Specialist advice necessary and/or hospital referral
Infants under 3 years failing 1st line	Refer	Specialist advice necessary and/or hospital referral
Salvage		
Failing any 2nd line	Specialist referral	

Recently, the South African Minister of Health announced the availability of new first line ARV for state patients from April 2013, which combines tenofovir, emtrivitabine and efavirenz (a generic form of Atripla)[61]. The introduction of ARV drug therapy has been accompanied by an increase in the diagnostic testing required to monitor patients, including $CD4^+$ T cell counts, viral loads and HIVDR testing, amongst others (see section 4 below).

2.4 Access to ART in Sub-Saharan Africa

As mentioned previously, the latest WHO/UNAIDS report estimates that 34 million individuals are infected with HIV at the end of 2011[12]. Although the number of newly infected individuals each year remained relatively stable since 2007, an increase in the total number of infected individuals can be attributed to the roll-out of ARV programs worldwide that have increased the lifespan of infected individuals, thereby decreasing the number of AIDS deaths. Access to antiretroviral therapy (ART) has increased in the past decade, in particular in Sub-Saharan Africa, from 300 000 in 2002 , to an estimated 6.65 million of

infected individuals by 2011[62]. This translates to approximately 62% ART coverage of HIV-infected patients requiring ART in sub-Saharan Africa, and 57% coverage worldwide (Figure 6). The WHO has suggested that if the considered "Test and Treat" approach is adopted, involving regular screening of entire populations for HIV-1 infection, and initiating immediate treatment for those found to be HIV-positive, this would result in a total of 32 million people being eligible for ART[63]. However, this would pose numerous logistical and financial challenges, particularly in resource limited settings. Although constant efforts to reduce drug prices for ART, and efforts to increase the availability of generic products, as well as drugs for second- and third-line treatments are being made, these are still scarce in resource-limited settings as compared to the Western world[64].

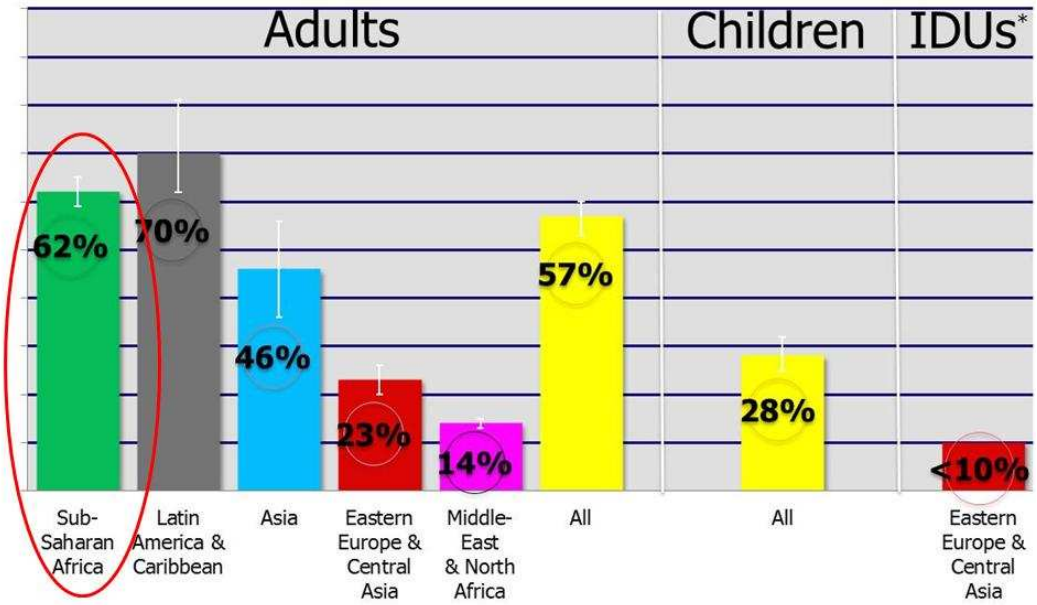

Figure 6: ART coverage per region, 2012.*Copied from http://pag.aids2012.org/flash.aspx?pid=1547* [65]. IDU: Intravenous drug users

Treatment failure attributed to numerous factors, including non-compliance, and the emergence of antiretroviral drug resistance, is the biggest threat to successful patient clinical outcomes and national treatment programs[66,67]. The emergence of HIV antiretroviral drug

resistance (HIVDR) is exacerbated by inadequate resources and insufficient health care infrastructures. In Africa, different factors play a role in HIVDR, which include intermittent drug supplies[68,69], patient adherence challenges exacerbated by weak patient support systems[70], the use of sub-optimal regimens, such as NVP for PMTCT, d4T with its higher toxicity[71], tuberculosis treatment drug interactions (NVP, EFV and rifampicin)[72], the late reporting of patients to clinics and the lack of patient virological monitoring[73,74].

The net result is an ever increasing accumulative number of patients who harbour resistance mutations (acquired), and have the potential to also transmit ARV drug resistant viruses. Therefore, acquired or transmitted HIVDR is likely to be a determining factor for the long term success of treatment programs.

3 An overview of HIVDR

Therapeutic drug resistance is a major confounding factor in the effective treatment of human pathogens, particularly those which account for the majority of the global infectious disease burden eg. malaria, tuberculosis and HIV-1[75]. The evolution/emergence of drug-resistant mutants is an intrinsic aspect of HIV-1 replication in light of life-long treatment, high replication and mutation rates. HIV-1 is known to develop resistance to all known antiretroviral drugs. Under conditions that allow ongoing viral replication in the presence of ART pressure, drug-resistant mutants acquire a selective advantage over wild-type virus[76]. Furthermore, any alteration of viral genes which play a role in the replication cycle, as well as in the evasion of HIV-1 from the immune response or from ART pressure can affect the viral fitness. Fitness refers to the ability of an organism to adapt and reproduce in a defined environment[76]. The impairment of viral replication appears to vary widely among viral strains that are resistant to ARV's.

There are many factors which determine the relative rate of resistance selection with different drugs and drug combinations, and this is reflected in the 'genetic barrier' to resistance. Certain mutations may be antagonistic, whereas others may confer resistance to some drugs while conferring hypersusceptibility to others[76]. Cross-resistance occurs when mutations appear and cause resistance to more than one ARV drug within a drug class[76]. The selection of ARV drug resistant viruses becomes possible when either of two conditions are fulfilled: 1) the resistant virus already exists within the complex population of HIV quasispecies found in an infected patient before treatment (transmitted or pre-treatment drug resistance) or 2) the treatment is insufficiently suppressive, allowing continuous repetition of cycles of random introduction of mutations and selection of viruses with increasing levels of resistance (acquired drug resistance).

The 2012 WHO Resistance report states that 10-17% of ARV-naive HIV positive individuals treated in Australia, Japan, the United States of America and Europe are infected with HIV-1 quasispecies resistant to at least one antiretroviral drug (transmitted or pretreatment). Pretreatment ARV drug resistance was reported early after HAART was introduced in many high-income countries in the late 1990's but have since reached a plateau. The proportion however, of people achieving virological suppression (treatment success) has increased over time, thus reducing the emergence of acquired drug resistance and its subsequent transmission[77]. The current state of HIV-1 drug resistance in sub-Saharan Africa are presented below.

3.1 Transmitted Drug Resistance (TDR)

Transmitted drug resistance (TDR) occurs when previously uninfected individuals are infected with a drug resistant virus[77]. Transmitted antiretroviral drug resistant viruses may either persist over time or alternatively, the transmitted resistant variant may be less fit than the wild type virus. The resistant variant may then, in the absence of selective drug pressure,

be overgrown by the wild type virus, decreasing the proportion of the resistant variant within the total quasispecies, which only resurges when selected for by drug pressure[78].

TDR is a potential threat to the treatment success for individuals who are eligible for their first-line ARV treatment[79]. In 2008, WHO published guidelines regarding the surveillance of TDR in settings scaling-up antiretroviral treatment[80]. Although many countries, including South Africa still report TDR prevalence below 5%[79], other countries like Uganda[81–83] and Cameroon[84] show that the longer HAART is available within a population in a particular setting, the higher the prevalence of TDR.

Hamers *et al*. (2011)[79] showed that the prevalence of TDR within a single country may vary greatly. In South Africa, for example, the TDR prevalence in Tshwane (formerly Pretoria) only reached 1.1% whereas the TDR prevalence in the Johannesburg and White-River population nearly reached the 5% threshold in 2008. A recent study conducted in Limpopo even revealed a TDR prevalence of 9.3% in an adult population attending a voluntary counseling and testing (VCT) facility[85]. A study of TDR from 9 different surveys in two provinces of South Africa, suggested that while TDR remains low (<5%) in Gauteng Province, it may be increasing in KwaZulu-Natal, with most recent surveys showing moderate (5-15%) levels of resistance to NNRTIs[86]. Containment of TDR levels in South Africa is likely to have resulted in part from the tight regulation of first and second line drug regimens. A second and plausible reason for the containment of resistance has been the effective use of adherence counsellors in the public sector. According to a mathematical model, the time from HAART roll-out, and reaching adequate treatment coverage are important predictors of TDR prevalence[87]. As more drugs are added to the marketplace and as guidelines offer a broader range of options, a greater variety and complexity of mutations is expected to be encountered.

3.2 Pre-treatment (baseline) Drug Resistance

The distinction between transmitted and pre-treatment HIVDR is difficult to discern, because the time of infection is often not known. In African settings, this resistance is of particular importance, as patients report to clinics quite late for HAART initiation, and from a clinical perspective it would be helpful to assess if some HIVDR is detectable before the commencement of HAART.

By contrast, pre-treatment drug resistance emerges as a natural consequence of the viral replication cycle, and the error prone RT giving rise to quasispecies that contain HIVDR mutations, in treatment-naïve patients. The WHO therefore recommends HIVDR testing within the first 3 years of infection, due to the risk of reversion to 'wild-type', in order to reliably detect transmitted resistance[80].

3.3 Acquired Drug Resistance

Acquired drug resistance occurs when resistance mutations evolve/emerge because of drug-selective pressure in patients receiving HAART[77].

In an assessment of 16,511 samples from 11,492 treatment experienced individuals in seven European countries between 1999 and 2008, 80.1% had at least one drug resistance mutation, with 17.2% showing resistance to three classes[88]. After adjusting for confounding factors, individuals failing therapy in more recent years showed a decline in overall resistance to NRTIs and PIs, but not to NNRTIs[77]. This finding is probably associated with the use of improved first- and second-line regimens of HAART with greater potential to fully suppress viral replication.

A large meta-analysis on sub-Saharan African HIVDR data[66] from 89 different studies, revealed that the current first line ARV regimens are very effective in suppressing the virus, especially in the short term. The study indicated that 78% of individuals showed viral

suppression after 6 months of treatment, 76% after 12 months and 67% after 24 months. Of those patients who failed ARV treatment (VL>1000 RNA copies/ml), approximately 60% have evidence of HIVDR. Hamers *et al.*[89] reported large variations in the rate of acquired HIVDR in 12 African countries. The HIVDR prevalence in individuals failing first line ARV treatment varied from 3.7% to 49% after 24 to 163 weeks of treatment.

The accumulation of resistance mutations such as Thymidine Analog mutations (TAM's), which infer cross resistance to all NRTIs, has been associated with patients left on a continued failing HAART regimen[90–92].

Several studies have been conducted in South Africa to assess the prevalence of HIVDR mutations in patients failing their first line regimen[91–97]. Despite good virological suppression in the majority of the patients, 66 to 88% of patients failing first line regimens developed at least one drug resistance mutation (DRM). As expected, the most common mutations are K103N (~45%) and M184I/V (~65%) conferring resistance to some NNRTIs and NRTIs, respectively. In addition up to 32% of the patients develop TAMs which reduces the available treatment options for second-line regimens. Although the prevalence of K65R and Q151M is relatively low (<5%), these mutations also jeopardize future treatment options and are therefore a reason of concern. Most adult patients included in these studies received d4T+3TC+NVP/EFV as a first line regimen. In the meantime, the South African guidelines have changed the preferred first line regimen to TDF+3TC+NVP/EFV[98]. A TDF containing regimen has been shown to produce a higher rate of K65R than d4T regimens, causing cross-resistance to other NRTI's, thus potentially limiting NRTI use in second-line therapy[99,100]. It is expected the complete integration of the TDF containing regimen will take a significant period of time. Cross-sectional observations of the HIVDR patterns acquired under pressure of both these regimens are of uttermost importance, and are ongoing.

Children under 3-years of age start with a boosted protease inhibitor based regimen which consists of d4T, 3TC and LPV/r. This regimen is associated with a low prevalence of PI resistance, except in children who had a period of exposure to RTV as a single PI[101]. As TDF cannot be used in children under 25 kg, ABC replaced d4T in the most recent (2010) recommendations[60]. Limited data is available on the prevalence of resistance in HIV-1 infected children with subtype C patients using these new regimens and surveillance is critically needed.

Only a few studies have been conducted in South Africa addressing second line regimen HIVDR patterns. A recent study found that 39% of the patients failing a LPV/r based second-line regimen did not have any HIVDR mutations, indicating poor adherence as the reason for virological failure[102]. Only 7% of the failing patients developed major LPV/r mutations. A study from Gauteng province found only 6% with PI resistance[93] and one from the Western Cape detected 6% of HIV-1 sequenced having major PI resistance mutations[103]. These results suggest that HIVDR is not the main barrier to future viral suppression but that inadequate drug levels and/or compliance are primary contributors[104].

3.4 Impact of HIV subtypes on drug resistance

Most published literature on the impact of HIVDR mutations on the effectiveness of HAART has been derived from studies focused on HIV-1 subtype B, which represents less than 15% of HIV-1 infections worldwide[105]. Subtype B is prevalent in the Western world, with non-B predominating in the rest of the world. However, the proportion of non-B subtypes in North and South America and Western Europe is increasing[106–108]. Significant sequence differences have been observed amongst both the structural and regulatory genes of the different subtypes, which can influence the biological properties of the virus, and in turn possibly affect ARV drug resistance pathways[109]. Several amino acid substitutions have been found to

occur at high frequencies as naturally occurring polymorphisms in certain non-B viruses at positions known to be associated with HIVDR in subtype B viruses[110] (Table 5).

Table 5: Examples of polymorphisms and mutations in protease, reverse transcriptase, and integrase of different subtypes that may impact on emergent resistance to nucleoside reverse transcriptase inhibitors (NRTIs) and non NRTIs, protease inhibitors (PIs), and integrase strand transfer inhibitors (INSTIs). Copied from Mol Biol Int 2012, 256982 (2012)[110].

Drug class	Type/ group/ subtype	Polymorphism or mutation associated with drug resistance	Drug(s) affected	Mutation(s) or their consequences
Reverse transcriptase				
NRTI	C	64-65-66 KKK motif	ddI, d4T, TDF	K65R
NRTI	HIV-2	T69N, V75I, V118I, L210N, T21S, K219N	NRTIs	TAMs/K65R
NNRTI	C	V106V	EFV, NVP	V106M
NNRTI	G	A98S	NNRTIs	
NNRTI	HIV-2	Y181I, Y188L, G190A, K101A, V106I, V179I	All NNRTIs	Cross-NNRTI resistance
NNRTI	O	Y181C, A98S, K103R, V179E	All NNRTIs	Cross-NNRTI resistance
Protease				
PI	Non-B	M36I	PIs	
PI	G, AE	K20I	PIs	
PI	G	V82I	PIs	I82M/T/S
PI	A, C, F, G, AE, AG	L89M	PIs	L89I
PI	HIV-2	L10I/V, K20V, V32I, M36I, M46I, I47V, L63E/K, A71V, G73A, V77T, V82I/L	PIs	APV and other PIs
Integrase				
INSTIs	B	R263	MK-2048, DTG	R263K
	C	G118	MK-2048, DTG	G118R

ddI: didanosine; d4T: stavudine; TDF: tenofovir; EFV: efavirenz; NVP: nevirapine; TAMs: thymidine analog mutations; MK-2048: second generation integrase inhibitor; DTG: dolutegravir; APV: Amprenavir

Furthermore, the genetic barrier to the emergence of some resistance mutations such as K65R, V106M and D30N are lower in certain subtypes. The K65R mutation in RT confers cross-resistance to NRTI's, and its prevalence is noted to be higher amongst subtype C infected patients, with studies supporting regional differences among subtype C sub-epidemics from Ethiopia, Brazil, and sub-Saharan Africa, that impact on NRTI resistance rates as a result of different NRTI-based regimens[111,112]. The higher rates of K65R in subtype

C suggest that these viruses may have a particular predisposition toward acquiring this mutation. Subtype C viruses apparently have an intrinsic difficulty in synthesizing stretches of adenine homopolymeric runs that lead to template pausing at codon 65, facilitating the acquisition of K65R under selective drug pressure, whereas subtype B templates favour pausing at codon 67, which may facilitate D67N and TAMS rather than K65R. A template-dependent dislocation mechanism has been shown to potentiate K65R mutation in subtype C[113–115].

Similarly, the V106M mutation has been shown to commonly develop in subtype C viruses following drug pressure with NVP or EFV, whereas a V106A mutation is more commonly selected in subtype B. This difference is due to a nucleotide polymorphism at codon 106 in RT[116,117], and the clinical importance of V106M in non-B subtypes has been confirmed in multiple studies showing that V106M is frequently seen in subtype C and CRF01_AE after therapy with NVP or EFV[118–120].

By contrast D30N, which confers resistance to NFV, is seemingly more frequent in subtype B viruses, and is caused by increasing the PR flap region and destabilizing the PR inhibitor complex[121]. In subtype C, D30N requires the accessory N83T mutation to confer resistance and rescue fitness, possibly elucidating its lower prevalence in comparison to subtype B[122].

An example of disparity in overall resistance among subtypes is that of single dose NVP for PMTCT, with frequencies of 69, 36, 19 and 21% against NVP in women with subtypes C, D, A and CRF02_AG infections, respectively. Often this was linked to the presence of pretreatment resistance[123–126]. In other studies, minority species detection revealed a higher

incidence of NVP resistance mutations (K103N, Y181C) in 70-87% of individuals infected with subtype C compared with 42% of individuals infected with subtype A[127–129].

Overall, a solid body of evidence exists indicating that drug resistance pathways vary between different subtypes[130–134]. Each subtype may have a distinct resistance profile and differences in resistance pathways may also impact on cross-resistance and the choice of regimens to be used in second-line therapy. Although responsiveness to first-line therapies should theoretically not be affected by considerations of viral subtype and drug resistance, well-designed clinical studies involving patients infected by viruses of different subtypes should be carried out[110].

4 Clinical and laboratory assessments in HIV infected patients

HIV-1 infection is initially confirmed by an HIV antibody test. Thereafter, clinical staging is part of the baseline assessment on entry into care and treatment programs. Additionally, $CD4^+$ T cell counts (cells/mm^3) in the blood and HIV RNA viral load (RNA copies/ml) in the plasma are used as laboratory markers to determine disease progression and the need for treatment. Once on treatment, HIVDR testing can be used to assess resistance to HAART in patients classified as having virological failure.

Given that most HAART programs in resource-limited settings rely on infrequent clinical or immunological criteria for switching therapy, accumulation of multiple ARV drug resistance mutations is expected. Currently, South Africa is an exception, where virological failure is monitored and used for HAART treatment switches. A recent study in South Africa showed that an early clinical intervention to treatment failure (loss of viral suppression) is essential to

re-establish viral control in 40-45% of patients[135]. This opportunity is potentially lost when VLs are infrequently (less than biannually) checked, if at all.

4.1 HIV screening and confirmatory testing

An HIV positive status should be based upon the outcome of two or more different tests. When two test results are discrepant (one is reactive, the other non-reactive), a third test should be performed as a tie-breaker. Initial screening for HIV infection is done using either a rapid antibody-based assay (in resource limited settings) or an enzyme-linked immunosorbent assay (ELISA). Ideally, if the test shows the patients to be HIV-positive, a confirmatory ELISA or Western blot is requested, where patient blood is sent to the laboratory[136].

4.2 Clinical Staging

Clinical staging is used in predicting disease progression and treatment failure when combined with immunological staging or even viral load monitoring, when available. The WHO clinical staging system remains an essential and cheap tool to assess the disease status at baseline, to guide the decision to start HAART and follow-up patients on treatment. As discussed previously, in clinical stage 1 (primary infection), patients may remain asymptomatic, but may present with a persistent generalized lymphadenopathy. Patients with mild clinical symptoms like herpes zoster infection, recurrent oral ulcerations, chronic upper respiratory tract infections among others, are classified as being in clinical stage 2. Clinical stage 3 is characterized by more advanced symptoms, with chronic diarrhea, persistent fever, severe weight loss, bacterial infections, tuberculosis (TB), oral candidiasis, oral hairy leukoplakia, and acute necrotizing ulcerative diseases. Finally, in clinical stage 4, the occurrence of Kaposi's Sarcoma, Pneumocystis pneumonia, severe wasting, encephalopathy and extrapulmonary TB point to the manifestation of AIDS[29].

4.3 $CD4^+$ T cell testing

The $CD4^+$ T cell count serves as an indicator of the immunocompetence of the HIV infected individual. The number of $CD4^+$ T cells is the most important consideration in the decision of HAART initiation. The WHO and South African 2010 national guidelines recommend to start ARV drug treatment before $CD4^+$ T cells drop below 350 cells/mm^3 in HIV/TB con-infected individuals and pregnant women, and ARV drug treatment for individuals with $CD4^+$ T cells ≤ 200 cells/mm^3 [60,137]. South African guidelines recommend a baseline $CD4^+$ T cell count at the first visit after HIV infection has been confirmed by an antibody test[60]. Furthermore, an increase in $CD4^+$ T cells of 100 cells/mm^3 per year is an indication of adequate viral suppression on treatment[138].

4.4 Viral load testing

Since the goal of HAART is to suppress viral replication to a level that viral RNA is no longer detectable in the plasma, the HIV-1 RNA viral load measurement in plasma is a critical parameter and an early marker for evaluating HAART response[139]. Effective regimens and good adherence should result in a viral load decrease of at least 1 log RNA copies/ml per month and the achievement of an undetectable viral RNA level (<50 RNA copies/ml) within 12 to 24 weeks[140]. South African guidelines recommend that VL testing be done 6 months after HAART initiation, upon successful HAART, the VL should be undetectable[60]. If a VL >1000 RNA copies/ml is detected, adherence will be addressed with the patient and a repeat sample will be tested in 3 months. In case the VL is still >1000 RNA copies/ml, adherence counselling is suggested, and if again 3 months later the VL is not suppressed, empiric treatment switch is considered[60] or an HIVDR test is recommended, as per the Southern African HIV Clinicians Society 2012 recommendations for drug resistance testing[141]. An annual VL is otherwise indicated should the VL be <1000 RNA copies/ml. A confirmed rebound in plasma viral load may indicate poor adherence and/or the development of

HIVDR, hence virological failure[142]. The inability to detect virological failure, due to unavailability of viral load assays as is often the case in resource-limited settings, may lead to an accumulation of resistant mutations, including TAMs, and the selection of viruses with broad cross-resistance to an entire class of ARVs[74,143]. This might jeopardize future treatment options for the individual patients and for recently infected patients who could be infected with a circulating resistant virus.

4.5 Resistance Testing

When virological failure in a patient on HAART is determined using clinical, immunological and/or virological parameters, an antiretroviral drug resistance test can provide information about the presence of resistance mutations and ARV drug susceptibilities.

The use of HIVDR testing has become an integral part of HIV clinical care in Western countries. The first clinical description of HIVDR was published in 1989, in patients receiving AZT monotherapy[144]. Subsequently, HIV variants resistant to every available ARV have been identified in viral culture and in HIV-infected patients. As was previously discussed, pre-treatment, transmitted and acquired resistance is prevalent in different proportions around the world, and with increasing access to HAART, resistance will inevitably become an issue that will need to be further addressed on a global level, with greater intensity.

HIVDR information can aid the clinician on the selection of optimal therapy for their patients, and it can be a useful tool to prevent unnecessary treatment switches in patients with poor adherence. HIVDR testing provides an indication of the susceptibility or resistance of a particular virus to one or more ARV's. Resistance testing can be performed by analyzing the

nucleic acid sequence of the virus (genotyping), or by direct analysis of the ability of the virus to infect cells and/or replicate within them *in vitro* in the presence of ARVs (phenotyping). Although phenotyping is still restricted to specialized laboratories, phenotyping assays that were previously cumbersome and time consuming can now be performed within a matter of weeks, making them more accessible. Phenotyping does however require an expensive infrastructure, highly trained personnel, and a high level of funding to maintain this type of laboratory, so in essence this type of testing is really only feasible in high-income settings. Genotyping assays are now largely automated and both commercial and home-brew assays are widely available. The selection of one assay over another depends on specific factors such as access, cost, turnaround time and the availability of expert interpretation. Genotyping has been shown to be cost-effective at first-line antiretroviral failure in the South African setting, provided timely response to the results is observed[145,146].

4.5.1 Phenotypic resistance testing

Although there are several different methods for HIV phenotyping, they all follow the same basic principles. Sufficient virus must be produced in the laboratory, either by direct isolation from infected plasma or peripheral blood mononuclear cells (PBMC's) followed by *in vitro* culture, or by using recombinant technology to produce recombinant virus carrying sequences derived from clinical samples. Plaque reduction assays were initially used for testing laboratory isolates, but as they required co-cultivation of virus with HeLa-CD4+ cells (lacking the CCR5 receptor), these assays could only test syncytium inducing (CXCR4 tropic) viruses. PBMC-based assays were however suitable for testing most clinical isolates, but were laborious and time-consuming. Morevoer, PBMC-based assays were expensive

because virus growth in PBMC's cannot be monitored visually, but require the specific measurement of p24 antigen.

Commercially available standardized assays amenable to high-throughput performance are available eg. Janssen Diagnostics (Antivirogram, Belgium) and Monogram (PhenoSense, California). These assays use PCR to amplify *protease*, *reverse transcriptase* and some of *gag* from HIV-1 RNA extracted from patient plasma. The amplified material is incorporated into a *pol*-deleted recombinant virus construct to create a recombinant HIV-1 isolate. The PhenoSense assay uses direct ligation to create a recombinant virus, and tests the recombinant construct during a single cycle of replication using a luciferase reporter gene assay designed to be highly sensitive to HIV-1 replication. The Antivirogram assay uses homologous recombination in cell culture to combine the patient's *pol* gene with the *pol*-deleted HIV-1 vector, and cultures the virus for several replication cycles using cell killing as a measure of virus replication. The recombinant virus is used to infect host cells and the ability of the recombinant viruses to replicate in cell culture (*in vitro*) in the presence of different ARV concentrations, compared with a drug-susceptible reference recombinant virus (wild-type) is measured[147–149].

Recombinant virus susceptibility assays have several advantages over non-recombinant assays in that plasma can be used as opposed to PBMC isolations, the assays can be performed under highly uniform conditions because the backbone of the virus construct is constant, and the use of PCR to amplify the protease and RT genes decreases the need for virus culture. A drawback of these assays, other than expense, is the lengthy timescale (~ 4 weeks) and that they are population-based phenotyping assays. This has implications on the possibility of outgrowth of a major highly resistant strain (with diminished replciative capacity) by a minor non resistant strain during initial culture. The use of clonal phenotyping could reduce this effect, but would be even more time consuming and expensive to perform.

The raw data output of a phenotyping assay is the concentration of ARV required to inhibit viral replication by 50% or 90% (IC_{50} or IC_{90}, respectively) relative to the control. Results are usually expressed as the IC_{50} of the ARV being tested for the patient-derived virus divided by the IC_{50} for the reference virus. The value of this ratio is commonly referred to as a fold change in susceptibility (Figure 7).

From a clinical perspective, the most pertinent method of interpretation of phenotypic data is based on the use of 'clinical cut-offs'. These are derived using clinical response data from treatment-experienced patients by determining the relationship between fold changes measured at baseline and the reduction in viral load after a defined period of treatment. To reflect the gradual, rather than discrete, loss of ARV activity observed with increasing resistance to the drugs, two phenotypic cut-off points are often derived for each ARV. The first cut-off defines the fold change at which there is a reduction in antiviral activity (lower cut-off), whereas the second cut-off defines the fold change above which all ARV activity is lost (upper cut-off)[150].

Even though the interpretation of phenotypic tests might seem uncomplicated, because the susceptibility of the virus is directly measured, an expert opinion is still needed as 'clinical cut-offs' are not available for all drugs, and there needs to be an understanding of the natural variability of this assay which needs to be considered. Clinicians may also depend on biological cut-offs that are derived from *in vitro* susceptibility experiments with clinical isolates from drug-naive patients[151].

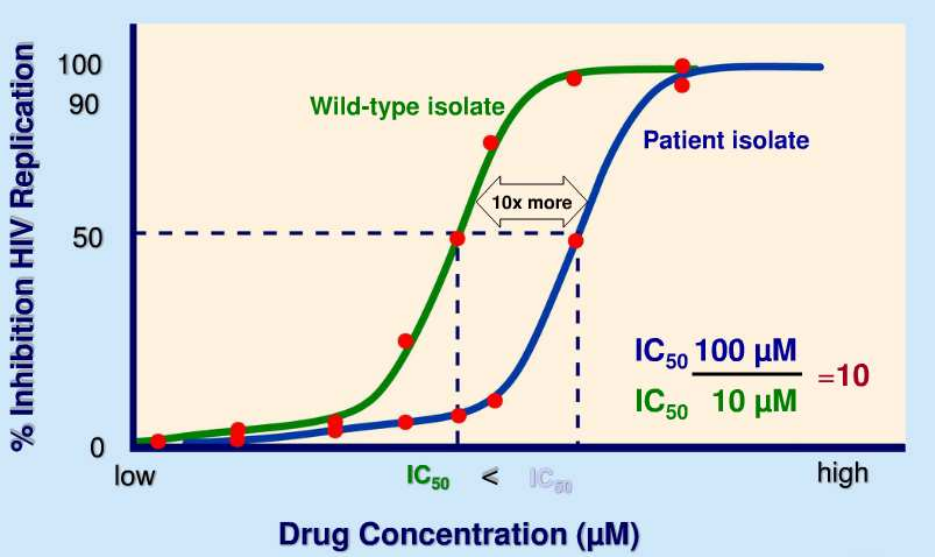

Figure 7: Phenotypic drug susceptibility curves. The green curve represents a wild-type drug-susceptible virus. The shift to the right of the blue curve, representing the patient strain tested, indicates a reduction in drug susceptibility to a higher IC_{50} value[152].

4.5.2 Genotypic Resistance Testing

Genotyping infers resistance or susceptibility to a particular antiretroviral drug by sequencing the HIV-1 gene of interest and feeding this data into a predictive algorithm. There are various algorithms available, the majority of which predict antiretroviral drug resistance based on data derived from HIV-1 subtype B viruses. The Stanford HIVdb (http://hivdb.stanfordd.edu/index.html), Agence Nacional de Recherche sur le SIDA (ANRS) (http://www.hivfrenchresistance.org/) and Rega Institute (Rega) (http://regaweb.med.kuleuven.be/software/rega_algorithm/) algorithms are rule-based algorithms, providing scores for specific combinations of mutations. The scores are then translated into different levels of susceptibility based on literature and expert opinion[153]. The virco®TYPE and PhenoSense® GT are hybrid algorithms that combine phenotypic data with genotypic testing. The methodology employs statistical analyses of genotypic and phenotypic

data generated from a large number of previously tested samples to find and calculate the impact of significant mutations and mutation pairs[154].

Currently two major molecular based techniques are used to detect population-based drug resistance related mutations: dideoxynucleotide sequencing assays and single point mutation assays. The dideoxynucleotide sequencing is a viral population-based sequencing method that determines the genetic code of the polymerase chain reaction (PCR)-amplified target gene, based on the incorporation of fluorescently labeled dideoxynucleotides. This method is most commonly used as there is no limit on the number of mutations that can be detected. This method is less sensitive for minor variants, as this sequencing reads only up to 20-25% of the target viral quasispecies population sequence[155]. Several sequencing methods are commercially available, though a number of laboratories have their own home-brew/in house methods available, so as to reduce costs.

Although genotyping is less costly than phenotyping, it still remains unaffordable to the majority of ART programs in Africa, with a few exceptions[145,146]. Genotyping has the disadvantage of only producing a list of mutations, which uses an interpretation algorithm to depict ARV resistance.

The single point mutation assays are more sensitive to detecting minor variants, although the variability of the HIV genetic code can pose problems for using this approach. Typically, the single point mutation assays make use of a selective PCR, by performing two separate PCRs; one to amplify the wild-type sequence, and the other to amplify the mutant sequence. The primers are specifically designed to hybridize with either the mutant or wild-type sequence. Amplification will only take place when the primer perfectly matches the target sequence[156]. Selective PCRs have previously been used to detect HIVDR mutations in minor variants [157,158].

The oligonucleotide ligation assay (OLA) consists of two phases, a multiplex PCR amplification and a multiplex OLA, in a single-tube format. In the initial reaction a PCR primer is hybridized to the target sequence. The second reaction is a ligation reaction. A common primer contains a fluorescent dye marker (FAM, TET, HEX) at the 3' end and meets the first primer right over the nucleotide position that will be altered in a mutant allele[159]. If the 3' end of the first primer matches perfectly with the target DNA, it will be brought into close enough proximity to the second oligonucleotide in order that both primers can be ligated together. No ligation occurs when there is a mismatch between the 3' end of the first primer and the target DNA. Several groups showed that this assay is more sensitive compared to population sequencing assays[160,161], including B and non-B subtypes[162].

The study of resistance mutations of minor variants has been facilitated through the use of ultra-deep sequencing (UDS). UDS refers to next generation high-throughput DNA sequencing platforms that produce very high coverage by sequencing many short reads in parallel. The technology can perform direct sequencing of every single amplicon (i.e. quasispecies) present within the PCR mixture, and can therefore infer co-occurrences of drug resistance mutations and accurately estimate viral genetic diversity, all of which are known to play a role in HIV drug resistance evolution, but cannot be assessed by population based sequencing. Further applications of the technology include the pooling of patient samples, each with unique identifier tags, to develop more sensitive and low-cost alternatives capable of addressing the anticipated increase in throughput in resource-limited settings[163].

5 Research Objectives

Overall, it is clear that as national ARV programs expand to achieve the UN General Assembly health-related Millennium Development Goals by 2015[164], which include universal

access to HAART[164], there is an urgent need to expand the technology, laboratory capacity, accessibility and affordability to perform HIVDR testing in resource limited settings.

South Africa currently has the highest number of people living with HIV-1 worldwide, with approximately 5.7 million people infected by the end of 2011[13]. The national ARV roll-out program was initiated in April, 2004 and has expanded dramatically, with the latest statistics showing that >1.79 million South Africans are accessing the national roll-out programme[13]. Our laboratory recognized the need to develop an affordable resistance testing platform, and established the HIV Genotyping Laboratory in 2005 in anticipation of future needs.

The Affordable Resistance Test for Africa (ART-A; www.arta-africa.org) project was founded to address the need to develop an affordable resistance test. In this project, private, academic and civil society stakeholders joined forces to develop the first affordable, comprehensive HIV drug resistance protocol which could be universally applied to individual patients by a network of clinics, hospitals and laboratories in Africa. The public-private consortium launched the ART-A project on the 1st of January 2008. The project was co-ordinated by the PharmAccess Foundation (www.pharmaccess.org), a non-profit organisation, affiliated with the Amsterdam Institute for Global Health and Development (http://aighd.org/) and the Academic Medical Centre (AMC) in Amsterdam. Partners in the public-private consortium are the University Medical Centre Utrecht (UMCU), the Netherlands, the Centre de Recherche Public de la Santé in Luxembourg, the University of the Witwatersrand in South Africa, Contract Laboratory Services in South Africa and Virco BVBA (now Janssen Diagnostics) in Belgium. Two PhD students, including the present candidate, were selected to complete their PhD’s with the support of this consortium.

Thus, the overall aim of this study was to develop and evaluate the role of *in vitro* HIV- drug resistance phenotyping, genotyping and novel virological failure detection tools for clinical patient management within the ART-A network.

This was achieved by the following objectives:

1. To assess whether an HIV-1 subtype B-backbone recombinant virus can be used to accurately phenotype HIV-1 subtype C ARV drug resistant samples.
2. To compare HIVDR genotypic testing in combination with the Stanford HIVdb interpretation algorithm, with HIVDR phenotyping.
3. To develop and evaluate an affordable real-time qualitative assay for determining HIV-1 virological failure (ARTA-VFA), in plasma and dried blood spots (DBS) and a novel RT-only (ARTA-HIVDR^{ultralight}) HIV-1 genotyping assay.
4. To perform a comparative bioinformatics analysis of HIVDR interpretation based on the ARTA-HIVDR^{ultralight} sequences versus full RT sequences.
5. To conduct a field evaluation of the ARTA-VFA and ARTA-HIVDR^{ultralight} assays developed in objective 3.
6. To develop and validate an automated sequence analysis and editing software for HIVDR testing.

The above mentioned comprehensive approach focusing on the issues relating to improving access to affordable virological failure identification and HIVDR resistance testing in resource-limited settings is addressed by the subsequent publications.

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Chapter 2

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HIV-1 Phenotypic Reverse Transcriptase Inhibitor Drug Resistance Test Interpretation Is Not Dependent on the Subtype of the Virus Backbone

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Abstract

To date, the majority of HIV-1 phenotypic resistance testing has been performed with subtype B virus backbones (e.g. HXB2). However, the relevance of using this backbone to determine resistance in non-subtype B HIV-1 viruses still needs to be assessed. From 114 HIV-1 subtype C clinical samples (36 ARV-naïve, 78 ARV-exposed), *pol* amplicons were produced and analyzed for phenotypic resistance using both a subtype B- and C-backbone in which the *pol* fragment was deleted. Phenotypic resistance was assessed in resulting recombinant virus stocks (RVS) for a series of antiretroviral drugs (ARV's) and expressed as fold change (FC), yielding 1660 FC comparisons. These Antivirogram® derived FC values were categorized as having resistant or sensitive susceptibility based on biological cut-off values (BCOs). The concordance between resistance calls obtained for the same clinical sample but derived from two different backbones (i.e. B and C) accounted for 86.1% (1429/1660) of the FC comparisons. However, when taking the assay variability into account, 95.8% (1590/1660) of the phenotypic data could be considered as being concordant with respect to their resistance call. No difference in the capacity to detect resistance associated with M184V, K103N and V106M mutations was noted between the two backbones. The following was concluded: (i) A high level of concordance was shown between the two backbone phenotypic resistance profiles; (ii) Assay variability is largely responsible for discordant results (i.e. for FC values close to BCO); (iii) Confidence intervals should be given around the BCO's, when assessing resistance in HIV-1 subtype C; (iv) No systematic resistance under- or overcalling of subtype C amplicons in the B-backbone was observed; (v) Virus backbone subtype sequence variability outside the *pol* region does not contribute to phenotypic FC values. In conclusion the HXB2 virus backbone remains an acceptable vector for phenotyping HIV-1 subtype C *pol* amplicons.

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Introduction

Within the past decade, access to antiretroviral therapy (ART) for HIV-1 infection has increased exponentially in low- and middle-income countries. More than six million people were receiving highly active antiretroviral therapy (HAART) in these countries at the end of 2010, as compared to just 400 000 at the end of 2003 [1]. However, a major hurdle to sustainable, successful ART is the inevitable emergence of HIV-1 drug resistance. In addition, inadequate resources and health care infrastructure in these regions, as well as the introduction of ART, can create conditions for the accelerated development of HIV-1 resistance to antiretrovirals (ARVs) [2], further compromising the patients' future treatment options. Hunt *et al.* (2011) showed that an average of 34% of South African children under the age of 24 months had developed non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance, in particular the Y181C mutation, when they were previously exposed to single dose nevirapine (sdNVP) [3].

HIV-1 transmitted drug resistance mutations (TDRMs) were evaluated in recently infected individuals from some East and Southern African countries, and showed a 5.0 and 5.6% prevalence respectively [4], [5]. Hamers *et al.* (2010) [6] found that HIV-1 drug resistance mutations were present in 6% of

patients initiating ART in Lusaka, Zambia. Levels of transmitted resistance have been shown to be 8.6% in Kampala, Uganda [7]. In light of these findings, focus should be placed on optimal frequency of both viral load testing and appropriate antiretroviral drug resistance testing.

HIV-1 ARV drug resistance is usually measured by genotypic testing. It still remains an expensive test and is not yet an option for individual patient management in resource poor settings, but is a vital tool for resistance surveillance of large-scale HIV treatment programs. During genotypic resistance testing, the nucleotide sequence of specific HIV-1 genes, which are responsible for ARV drug resistance, are determined and fed into a predictive algorithm, describing the susceptibility to a range of ARVs. The *pol* region is sequenced when the drug therapies of the patient contain nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Most of the algorithms for predicting drug resistance are based on data derived from *in vivo* (clinical outcome data) *or in vitro* phenotypic testing of subtype B virus (virco®TYPE and PhenoSense® GT).

However, HIV-1 subtype C accounts for over 48% of all global infections, and is the predominant circulating subtype amongst the heterosexual population in sub-Saharan Africa [8]. The prevalence of HIV-1 subtype C resistance amongst patients failing first-line HAART has been shown to be 82% in the South African public sector [9] and in a study by Murphy *et al.* 2010 [10], it was noted that 87% of patients on HAART for 12 months had developed at least one resistance mutation.

In contrast to HIV-1 genotype resistance testing, phenotyping is an *in vitro* assay, which measures the ability of a virus to replicate in the presence of a drug. Currently, most available phenotyping assays are based on recombining patient-derived sequences into a subtype B backbone deleted for the corresponding patient sequences. HIV-1 phenotyping is considered to be the gold standard in resistance testing, although it is has only been performed, using subtype B backbones. Phenotyping is not a tool that could be adapted to resource limited settings due to its high cost, infrastructural requirements, and technical skill needed. Until recently, it remained, however, unclear whether a recombinant virus assay using a subtype B backbone would correctly measure drug resistance when the patient-derived sequences are of subtype C. The Antivirogram® assay [11] recombines patient-derived PR and RT sequences into an HIV-1 subtype B (HXB2) backbone deleted for these sequences [12]. Nauwelaers *et al.* (2011) [13] constructed an HIV-1 subtype C-backbone within the Antivirogram® assay setting, and tested eight subtype C samples on a clonal level within both an HIV-1 subtype B- and C- backbone. Resistance profiles generated were similar in both backbones. The present study is an extension of the work by Nauwelaers *et al. (2011)*, comparing population-based phenotypic HIV-1 drug resistance profiles of subtype C gag-protease-reverse transcriptase (GPRT) sequences generated using a subtype B- and C-backbone. The aim was to assess whether an HIV-1 subtype B- backbone could be used with a high level of confidence to phenotype subtype C samples.

Materials and Methods

1. Ethics Statement

Ethical clearance for this study was obtained and approved for by the Human Research Ethics Committee (HREC) at the University of the Witwatersrand (Clearance Number M090688), and for the PASER-M cohort from the Academic Medical Center Institutional Review Board and the University of Zambia Research Ethics Committee. Informed consent was obtained for samples from the PASER-M cohort, but not for those from the University of the Witwatersrand, as it was not required for the ethical approval obtained from the HREC. The data derived from this work was for research and development purposes and for method validation only. According to HREC policies, for this type of study, these specimens did not require patient enrollment or informed consent, and a waiver was hence granted.

2. Patient samples used in this study

Plasma samples received for routine population-based HIV-1 drug resistance genotyping were analyzed with the genotyping assay described by Wallis *et al.* (2010) [14]. A total of 265 samples were used for further phenotypic testing. Two hundred and fifteen (215) samples were obtained from treatment-experienced patients attending clinics in the public sector in Johannesburg, South Africa, and selected specifically for the presence of HIV-1 ARV drug resistance. Fifty (50) treatment-naïve samples were selected from the PASER-M cohort [15], based on available genotypic information.

3. Viral RNA Extraction

Viral RNA was isolated from all patient plasma samples using the MagNA pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) with a sample input of 200 µl, and a 50 µl elution volume, as per manufacturer's instructions. Viral RNA from recombinant virus stocks (RVS) was extracted using the QIAamp Virus BioRobot MDx kit (Qiagen, Belgium) or the NucliSENS easyMAG (bioMérieux Inc, Belgium), starting with an input volume of 600 µl and eluting in 25 µl, as per manufacturer's instructions.

4. Gag-Protease-Reverse Transcriptase (GPRT) amplification and sequencing

Samples were analysed in a two-step approach. First, an RT-PCR amplification protocol [14] was used to amplify a 1.5 kb *pol* fragment. This is later referred to as protocol 1. The resulting HIV-1 genotype was used to select for resistant samples for this study. Secondly, a 1.9 kb GPRT fragment was amplified (One-Step SuperscriptIII High Fidelity, Invitrogen, CA, USA) using the "3′-RT" and "5′-OUT" primers [13], with a 10 µl RNA input in a total volume of 35 µl. Nested PCR was performed using the Expand High Fidelity PCR System (Roche Diagnostics GmbH, Manheim, Germany), with 8 µl of first round amplicon and primers 3′IN, and 5′IN in a final volume of 100 µl [13] resulting in a final amplicon encompassing nucleotides 2012 to 3879 in *pol* (according to HXB2 numbering – genbank: AF033829). This second protocol is further referred to as protocol 2. Amplification products were analyzed by 1% agarose gel electrophoresis, and amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, CA, USA) as described previously [13]. Cycle sequencing purification was performed using the DyeEX (Qiagen, Hilden, Germany) purification kit, according to manufacturer guidelines. The ABI3730 XL (Applied Biosystems, CA, USA) performed the sequence detection and analysis was performed using the Sequencher v.4.5 software (Gene Codes Corporation, MI, USA).

5. Genotypic Analysis

Sequence data generated from first-step analysis was submitted to the Stanford University HIV drug resistance database [16] to

generate an ARV drug resistance profile and subtype. Based on the ARV drug resistance profiles, resistant samples were selected for the GPRT amplification, and subsequent phenotypic drug resistance analysis. The second-step (GPRT amplicon) sequence data was submitted to the Stanford University HIV drug resistance database.

6. In vitro phenotypic ARV drug resistance analysis

Recombinant virus stocks (RVS) were generated through homologous recombination of each of the GPRT amplicons into subtype B- and subtype C- backbones for use in the Antivirogram® assay, as described by Hertogs *et al.* (1998) [12]. Generation of the subtype C recombinant viruses was performed in MT4/eGFP cells [13], whereas subtype B recombinant viruses were generated in MT4 cell lines, respectively using eGFP expression and Cytopathic Effect (CPE) scoring, respectively to monitor adequate viral growth. One hundred (100) µl of harvested subtype B and C recombinant viruses were then titrated in MT4/eGFP cells. A panel of 18 ARV drugs (see below) was used in the antiviral experiment to establish the resistance profile of the RVS.

Each GPRT amplicon was initially used to generate a full subtype C recombinant virus (RVS_C) which was phenotyped in the Antivirogram® assay. After phenotyping, the GPRT region of the recombinant subtype C- backbone virus was PCR amplified as described above, and the resulting amplicon was genotyped and used to generate a recombinant subtype B- backbone virus. The recombinant subtype B- backbone virus (RVS_B) was subsequently phenotyped, followed by PCR amplification and genotyping of the GPRT region. The genotyping of the GPRT region was performed at all three time points (plasma, RVS-C and RVS-B) to ensure that the genetic background of the recombinant viruses was identical throughout the phenotyping experiments (Figure 1). This strategy was undertaken to first phenotype in a C-backbone because C-backbone phenotyping took 10 to 23 days to harvest virus, whilst only 5 to 10 days in a B-backbone. During this extended time to harvest with the C-backbone, it was a concern that there may have been some significant viral evolution during that time, therefore the RVS_C that was harvested was used as input into the B-backbone phenotyping.

Wild type subtype B (HXB2) and C [13] cell line adapted viruses were used as reference viruses for the subtype B- and C-backbone experiments, respectively. All 50% inhibitory concentration (IC_{50}) values were calculated from 8 readings for each recombinant virus against the different ARV drugs. Fold changes (FC) were calculated by dividing these IC_{50} values by the IC_{50} of the appropriate reference virus. Viruses were characterized as being susceptible or resistant based on pre-established biological cut-off values (BCOs) [17].

ARV drugs and drug concentrations (µM) used in the antiviral experiment included lamivudine (3TC; 0.12 to 31.25), zidovudine (AZT; 4.88 10^{-3} to 1.25), stavudine (D4T; 0.24 to 62.50), didanosine (DDI; 0.24 to 62.5), abacavir (ABC; 0.49 to 125), emtricitabine (FTC; 7.63 10^{-4} to 5.00), tenofovir (TDF; 0.12 to 31.25), nevirapine (NVP; 4.88 10^{-3} to 1.25), efavirenz (EFV; 1.53 10^{-4} to 10.00), etravirine (ETR; 3.82 10^{-5} to 2.50), indinavir (IDV; 4.88 10^{-3} to 1.25), fosamprenavir (APV; 4.88 10^{-3} to 1.25), atazanavir (ATV; 0.48 10^{-5} to 0.31), saquinavir (SQV; 0.48 10^{-5} to 0.31), darunavir (DRV; 3.82 10^{-5} to 2.50), lopinavir (LPV; 0.24 10^{-5} to 0.16), and tipranavir (TPV; 0.02 to 5.00).

7. Statistical Analysis

Possible inter-batch IC_{50} differences between the wild-type viruses, HXB2 and wildtype C were investigated for each ARV by means of an analysis of variance (ANOVA). This was done by comparing the IC_{50}'s of all drugs repeatedly tested throughout 10 batches of experiments.

The comparison of resistance and susceptibility call rates between the B- and C-backbone phenotyping were assessed by calculating the sensitivity and specificity of the C-backbone phenotype as compared to the B-backbone phenotype (regarded as the gold standard). Sensitivity was calculated as (true sensitive/(true sensitive + false resistant)×100) and specificity was calculated as (true resistant/(true resistant + false sensitive)×100). Hence, sensitivity measures the proportion of sensitive calls, which are correctly identified, while specificity measures the proportion of resistant calls, which are correctly identified.

Fold Change comparisons for which a discordant resistance call was observed between B-backbone and C-backbone were further assessed within the context of the intra-assay variability. Initially, all FC values were "normalized" against BCO's for the various drugs, by subtracting the relevant ARV's BCO from that FC value. Hence, all FC values, regardless of the ARV that was tested, could be compared collectively. FC values that gave the same resistance call, regardless of their backbone of descent were subtracted and used to set the acceptable FC variability limits between both backbones. The mean +/−2 standard deviations (2SD) was calculated for the concordant FC comparisons (susceptible and resistant viruses separately). The difference of log FC values of the discordant data points were calculated, and plotted on a Bland-Altman plot using the mean +/−2SD derived from the concordant samples as cut-offs. Any values outside these cut-offs were considered to not fall within these acceptable limits of assay variation, and hence truly discordant data points.

In addition, the capacity of the two backbones to detect resistance caused by predominant subtype C ARV drug resistance associated mutations was also assessed using Receiver of Operator Characteristics (ROC) [18].

Results

1. Generating recombinant virus stocks from subtype C- and B- viruses

A total of 265 clinical isolates were available for testing, 215 from ARV experienced patients, and 50 from therapy naïve patients. In the protocol 2 amplification procedure [13], 237 GPRT amplicon were obtained, 190 and 47 from treatment-experienced and naïve patients respectively. The 237 GPRT amplicons were used to generate 132 subtype-C recombinant virus stocks (RVS-C), which were subsequently GPRT amplified, and recombined with a subtype B- backbone. One-hundred and fourteen (114) subtype B RVS were generated. These RVS were GPRT sequenced and compared. Only RSV-C and RSV-B strains with identical genotypic analysis were included in further analysis. Finally, 114 paired B- and C- backbone recombinant viruses (78 from treatment-exposed +36 from treatment-naive) were retained for further analysis. The process flow phenotypic testing is given in Figure 1.

2. Genotypic analysis of the 114 sequences

The HIV-1 drug resistance mutation profiles [19] for the treatment-exposed group (n = 78) were analyzed. The most prevalent mutations in this dataset included: K103N (n = 30; 38.5%), M184V (n = 26; 33.3%), T215Y (n = 13; 16.7%), T215F (n = 8; 10.3%), M41L (n = 9; 11.5%), V106M (n = 8; 10.3%), D67N (n = 9; 11.5%), V108I (n = 8; 10.3%). The treatment naïve group (n = 36) had no ARV drug resistance mutations.

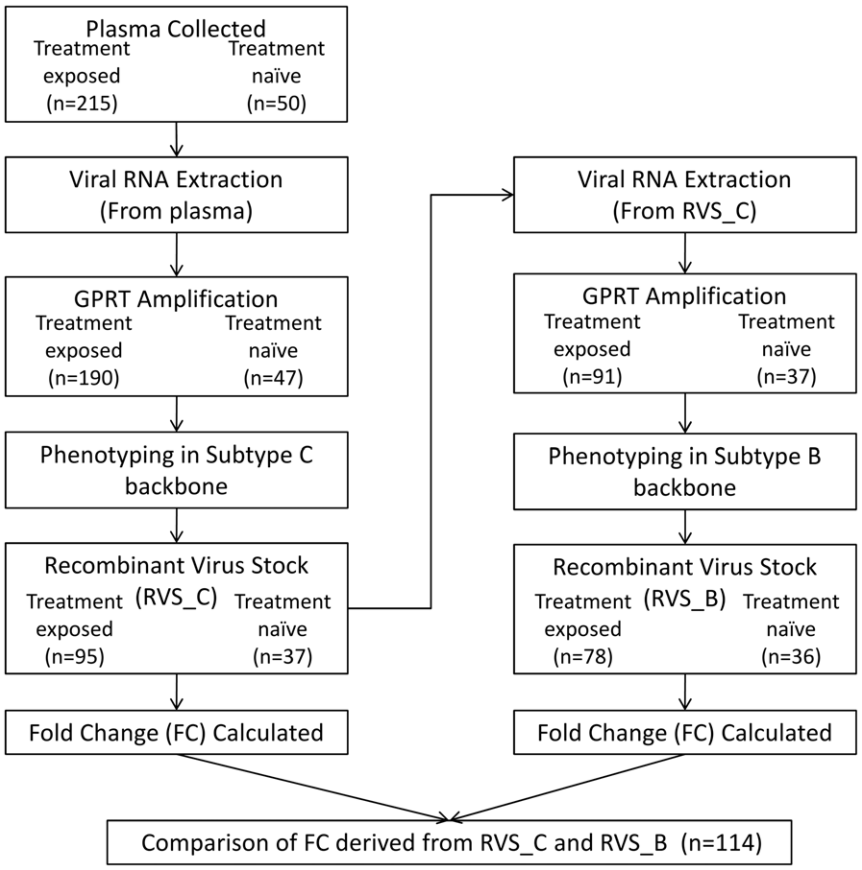

Figure 1. Process flow diagram for phenotyping procedure within subtype B- and C- backbones.
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3. Evaluation of the subtype C virus assay variability in phenotypic testing

Prior to the IC_{50} –values determination of the 114 RVS-C and RVS-B preparations, assay variability for the subtype C Antivirogram® assay was determined. The wild-type virus IC_{50} values were compared over the course of all experiment batches (n = 10) performed and for each drug tested, to determine assay variability. No difference in the variance of the IC_{50} –values of the wild-type viruses was noted amongst all drugs tested for the 10 experiments performed (p = 0.41).

4. Analysis of the phenotypic resistance determinations on 114 RVS-B and RVS-C strains

FC calculations, using respective wild-type viruses, were performed to determine whether an RVS was sensitive or resistant to a specific ARV. A summary of the subtype B- and C-backbone phenotyping resistance profiles is shown in Table 1. Not all 114 paired comparisons were obtained for every ARV. For example, for AZT a total of 82 of the expected 114 comparisons were obtained, resulting in an AVE success rate of 71.9% for AZT. Using the Antivirogram® BCO's [17], the FC values for the RVS-B (gold standard) were designated as being either sensitive (n = 1272) or resistant (n = 378) to a particular ARV. Using the same BCO's as for the B- backbone, the C-backbone phenotype resistance calls were determined as sensitive (n = 1192) and resistant (n = 458). A total of 1650 paired subtype B- and C- backbone derived FC comparisons were obtained, with an overall AVE success rate for all drugs of 85.1%.

Resistance and susceptibility call rates were compared to obtain sensitivities and specificities of the C- backbone phenotyping (Table 1). Sensitivity ranged from 69.9% to 89.9% (overall mean 87.5%), and specificity from 20.0% to 100.0% (overall mean 72.4%). A low sensitivity depicts that the C- backbone is over-calling resistance, and a low specificity means that the C-backbone is under-calling resistance. The observed resistance call discordances were further assessed for their relationship to genotypic predictions (Table 2), and biological variation, which may affect resistance calling in those samples with FC values close to the BCO (Figure 2).

5. Concordance and discordance analysis

The concordant and discordant data points are summarized in Table 2. Firstly, 1118 data points were called sensitive and 304 called resistant in both backbones. This resulted in 86.2% (1422/1650) concordant data points. Secondly, comparisons which were sensitive in the B- backbone and resistant in the C- backbone (B'sens/C'res) (n = 154) were 58.4% (90/154) concordant with a sensitive genotypic call in the genotypic interpretations from the Stanford HIV Drug Resistance database [16]. The comparisons which were resistant in the B- backbone and sensitive in the C-backbone (B'res/C'sens) were 16.2% (12/74) concordant with a resistant genotypic interpretation.

6. Analysis of the observed variability of FC values between backbones

To ascertain the acceptable variability of FC values generated in both backbones, the mean FC difference +/−2SD of the

Table 1. Comparison between HIV-1 subtype B and C backbone phenotyping resistance profiles.

ARV	BCO (FC)	AVE success rate (%)	B Backbone Phenotype		C Backbone Phenotype		Total paired Comparisons (n)	SENSITIVITY (%)	SPECIFICITY (%)
			SENSITIVE (n)	RESISTANT (n)	SENSITIVE (n)	RESISTANT (n)			
AZT	2.5	71.9	74	8	72	10	82	97.3	100.0
3TC	2.1	76.3	45	42	51	36	87	91.1	76.2
DDI	2.3	95.6	73	36	61	48	109	69.9	72.2
D4T	2.2	79.8	89	2	82	9	91	92.1	100.0
ABC	2.3	96.5	80	30	66	44	110	78.8	90.0
FTC	3.1	85.1	52	45	46	51	97	84.6	95.6
TFV	2.2	96.5	101	9	89	21	110	83.2	44.4
NRTI		**86.0**	**514**	**172**	**467**	**219**	**686**	**85.3**	**82.6**
NVP	6.0	93.0	52	54	46	60	106	86.5	98.1
EFV	3.3	85.1	42	55	36	61	97	81.0	96.4
ETR	3.2	86.8	79	20	67	32	99	77.2	70.0
NNRTI		**88.3**	**173**	**129**	**149**	**153**	**302**	**81.6**	**88.2**
IDV	2.3	74.6	76	9	77	8	85	98.7	77.8
SQV	1.8	74.6	82	3	77	8	85	92.7	66.7
APV	2.2	89.5	90	12	93	9	102	98.9	66.7
LPV	1.6	81.6	73	20	68	25	93	78.1	45.0
ATV	2.1	86.0	86	12	82	16	98	91.9	75.0
TPV	1.7	96.5	94	16	96	14	110	91.5	37.5
DRV	2.0	78.1	84	5	83	6	89	94.0	20.0
PI		**83.0**	**585**	**77**	**576**	**86**	**662**	**92.3**	**55.5**
All_Drugs		**85.1**	**1272**	**378**	**1192**	**458**	**1650**	**87.5**	**72.4**

ARV abbreviation: lamivudine(3TC), zidovudine (AZT), stavudine (D4T), didanosine (DDI), abacavir (ABC), emtricitabine (FTC), tenofovir (TFV), nevirapine (NVP), efavirenz (EFV), etravirine (ETR), indinavir (IDV), fosamprenavir (APV), atazanavir (ATV), saquinavir (SQV), darunavir (DRV), lopinavir (LPV), tipronavir (TPV). Sensitivity was calculated as (true sensitive/(true sensitive + false resistant)×100) and specificity was calculated as (true resistant/(true resistant + false sensitive)×100). BCO: Biological Cut-off. AVE: Antiviral Experiment. FC: Fold Change.

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concordant samples were calculated. This was plotted on a Bland Altman plot, to visualize truly discordant B- and C- backbone comparisons, with the log FC of B- backbone derived data on the x-axis, and the difference between the B- and C- backbone derived log FC's on the y-axis (Figure 2). Under these settings, 95.8% (i.e. 1590 from 1660 comparisons) of the phenotypic data derived from subtype B- backbone viruses had a concordant resistance call with subtype C- backbone derived data. Sixty-three (63) and 7 data points lie outside of the mean ± 2SD for the B'sens/C'res (Figure 2A) and B'res/C'sens (Figure 2B) data, respectively. Collectively, these 70 data points were considered discordant data points. The 70 discordant comparisons were predominantly from samples with a resistant genotype, who were treatment experienced (60/70), with 10 out of the 70 being from sensitive and treatment naïve samples.

7. ROC curves

Finally, the capacity of the different backbone phenotyping to predict ARV drug resistance associated with the M184V, K103N and V106M mutations was assessed. Only these 3 mutations were assessed, as other mutations in this sample set had too few observations for meaningful data interpretation. Table 3 summarizes ROC curve analyses, which demonstrates the tradeoff between sensitivity and specificity for the subtype B- and C-backbone based phenotyping assays in detecting decreased sensitivity caused by M184V, K103N and V106M mutations. The tests of differences between the areas under the empiric ROC curves show that regardless of the range of BCOs, the two backbones report the same detection sensitivities for ARV drug resistance to these 3 mutations.

Discussion

The emergence of ARV drug resistance in HIV-1 infected patients requires that clinicians make informed decisions when selecting the next ARV regimen based on genotypic and/or phenotypic drug resistance testing. However, based on its cost and logistic requirements, genotyping may not yet be an HIV-1 drug resistance monitoring tool in resource limited settings. Nonetheless, this methodology can certainly be used in monitoring HIV resistance on a population-based level at selected sites in Africa. The current study is primarily meant to ensure that the genotyping data generated through such monitoring programs for Africa are supported by phenotyping as a gold standard. This study evaluated the feasibility of using a phenotypic assay (Antivirogram®) based on a subtype B- backbone for resistance testing of subtype C infected patient samples.

Choe *et al.* (2007) [20] reported that the interpretation of ARV drug susceptibility using the Phenosense® phenotypic assay was not dependent on the subtype of the backbone vector (B vs. C). Furthermore, comparative analyses of commercially available

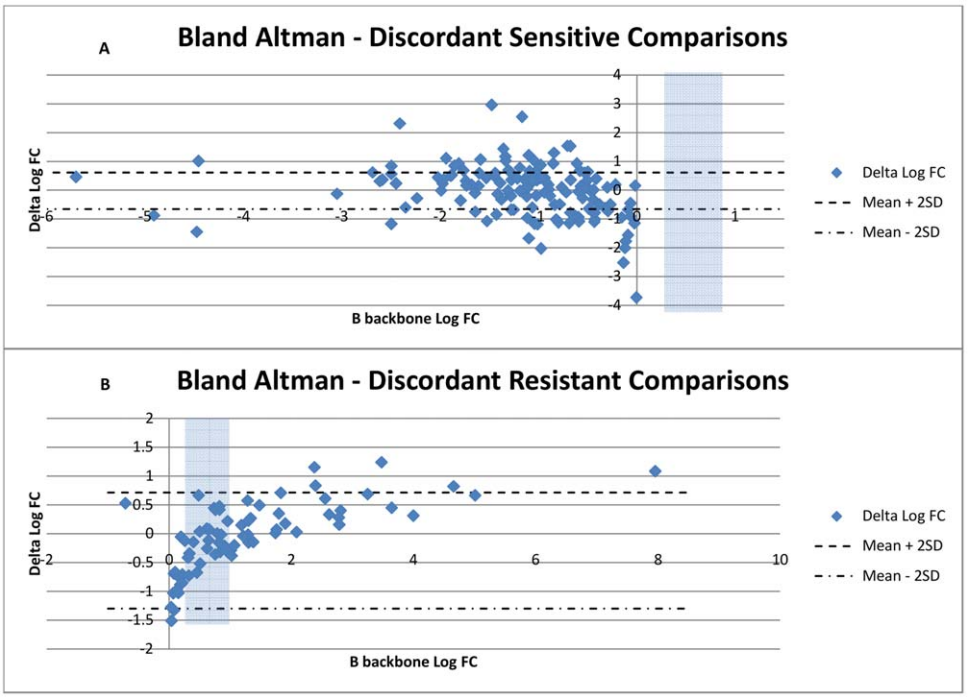

Figure 2. Plots showing discordant phenotypic resistance comparisons, and variation around the BCO. The FC of the B backbone derived viruses (assumed to be the gold standard) are shown on the x-axis, and the difference in log FC values (B backbone FC – C backbone FC) on the y-axis. The shaded region on the plots is the region wherein the log FC biological cut-offs lie for the 18 ARVs tested. The mean +2SD and mean −2SD are drawn in to illustrate the natural variation around the BCO. Any point s found above the mean +2SD or below the mean −2SD are considered to be truly discordant. Figure 2A is a plot of comparisons within the B′sens/C′res group. Figure 2B is a plot of comparisons within the B′res/C′sens group.
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phenotypic assays, Antivirogram® with PhenoSense® [21], and the virco®TYPE HIV-1 (*virtual***Phenotype**) with PhenoSense® [22] found that results correlate well, despite the use of different testing strategies. These two assays amplify the 3′ part of *gag*, including the p7/p1 and p1/p6 cleavage sites, the entire *protease*, and most of *RT* (Antivirogram®: RT amino acids 1–400 and PhenoSense®: RT amino acids 1–311) [12]; [23]. With the minor difference in the length of RT sequence (aa 311–400) used in these two assays, it is thus expected that the subtype of the backbone used in the Antivirogram® assay should also not impact on the interpretation of ARV drug susceptibility of non-B subtypes. Although the IAS guidelines [19] suggest that no known resistance mutations appear between amino acids 311 and 400, the virco®TYPE HIV-1 algorithm [24] and the Stanford University drug resistance database [16] have listings of resistance mutations within this region. No difference in these assays are expected, however, since Steegen *et al.* [24] showed that using a shortened RT sequence (aa41–238) for genotyping, still gave comparable genotypic resistance results as sequencing a full RT.

Table 2. Stanford University HIV Drug Resistance Database Resistance Profiles of Phenotypic Comparisons.

Phenotype category	Stanford HIVdb ARV Drug Resistance Profiles				
	Total (n)	Sensitive + Potential Low Resistance (n)	Low Level Resistance (n)	Intermediate Level Resistance (n)	High Level Resistance (n)
B′sens/C′sens†	**1118**	NP	NP	NP	NP
B′res/C′res‡	**304**	NP	NP	NP	NP
B′sens/C′res*	154	90	25	25	14
B′res/C′sens**	74	62	6	6	0

NP: Comparison not performed
Stanford HIVdb genotypic data not shown for concordant comparison, as this was only done to try ascertain if one backbone's phenotype was consistently miscalling (with reference to genotype).
†B′sens/C′sens are those comparisons which were sensitive in both the B and C backbone.
‡B′res/C′res‡are those comparisons which were resistant in both the B and C backbone.
*B′sens/C′res are those comparisons which were sensitive in the B backbone and resistant in the C backbone.
**B′res/C′sens are those comparisons which were resistant in the B backbone and sensitive in the C backbone.
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Table 3. Receiver of Operator Characteristic (ROC) statistics for 3 common HIV-1 subtype C resistance mutations.

Resistance Mutation	Antiretroviral Drug	Number of Observations		ROC Curve Statistics		
		With resistance mutation	Without any resistance mutations	Difference (B–C)	CI	P-value
M184V	3TC	27	33	0.01	−0.21, 0.18	0.896
	FTC	39	27	0.02	−0.05, 0.01	0.218
K103N	NVP	37	31	0.01	−0.04, 0.01	0.278
	EFV	34	29	0.0009	−0.003, 0.001	0.480
V106M	NVP	14	31	0.14	−0.39, 0.11	0.272
	EFV	12	29	0.17	−0.43, 0.08	0.187

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Amplification of the region encompassing *gag*-PR-RT was necessary for recombination with the Antivirogram® subtype B- and C- backbone [13]. Unlike Nauwelaers *et al.* (2011) [13], who used a clonal phenotyping approach, this work used population-based phenotyping, which depicts what would happen in a clinical setting. This population-based approach is preferred due to ease of use in comparison to the clonal approach.

Of the 133 amplicons initially phenotyped in a C- backbone, 114 paired subtype B and C phenotypes were obtained. During the phenotyping process, some of the transfections failed to yield recombinant viruses especially in the C- backbone. Often it was noted that even though adequate virus was scored with eGFP scoring for the C- backbone, when titrated, the desired yield of virus was not obtained. The standard CPE scoring is not an option for C-backbone viruses, as subtype C viruses do not produce CPE in these cell types [25], [13], and therefore other alternatives were required (eg. eGFP scoring using MT4/EGFP cells). A suggestion is that a more direct measurement of virus concentration should be used for the scoring of these viruses (eg. p24).

With 114 paired B- and C-phenotype comparisons, and 17 drugs tested, the expected number of FC comparisons would be 1938. Only 1650 comparisons were obtained however, with an overall AVE success rate of 85.1%. A lowered AVE success rate was generally noted to be a result of failure to meet the quality control criteria set for Antivirogram® analyses of either a B- or C-backbone derived IC_{50} reading. Fluorescent pixel intensity readings for subtype C- backbone derived viruses were often too high for accurate IC_{50} calculations to be made. This could be attributed to the different scoring method used for C-backbone viruses or altered replication capacities of the different virus subtypes, but was not investigated further in this study.

Resistance and susceptibility calls were compared between the phenotypes derived from the two backbones, as one of the primary objectives of the study was to analyze the concordance of results by phenotypic category because of the direct implications for patient management. Sensitivity and specificity was calculated for each ARV drug tested, using the subtype B- backbone as the gold standard, and hence subtype B derived BCO's as the ubiquitous cut-offs. An 87.9% (average sensitivity) of susceptible calls (FC<BCO) were correctly identified in the C- backbone. For some drugs, like ddI, ABC, ETR and APV, with sensitivities of less than 80%, it appears that the C- backbone is over-reading resistance as compared to B-backbone results. In terms of specificity, a 72.4% average is noted overall, with markedly low specificities observed within the PI group. The interpretation of which, is that there is some over-calling of resistance occurring in the B- backbone, particularly within the PI group. This may be a result of there being an insufficient amount of data illustrating HIV-1 subtype C drug resistance to PIs in this study, as PIs are mainly part of the second-line regimen in South Africa, which has resulted in few patients developing PI resistance.

A high number of concordant resistance calls were reported, with 1118 comparisons within the B'sens/C'sens category, and 304 within the B'res/C'res category, thus accounting for 86.2% (1422/1650) of comparisons tested. The discordant resistance calls resulting were compared with genotypic profiles as per the Stanford University drug resistance database [16] to investigate which backbone is theoretically giving the correct call (Table 2). Ultimately, subtype B backbone phenotyping was being assessed for use with subtype C specimens. In the following discordant B- and C-backbone resistant calls, a comparison was made of how the B-backbone resistance calls fare with a genotypic algorithm. Data shows that for discordant comparisons, the sensitive B- backbone phenotype agreed with genotypic sensitive calls for 58.4% (5.5% (90/1650) of resistance over-calling) of these cases, and only 16.2% of resistant B- backbone phenotype corresponded to the resistant genotype. The implications of the 16.2% correct calling of resistance in the B backbone was that, in terms of the genotypic prediction algorithm the B- backbone had a 0.7% rate of under-calling resistance in these particular cases. Notably, these discordant samples only account for 13.8% (228/1650) of total comparisons made. These discordant comparisons were quite diverse, with no trends being noted in terms of resistance mutations and/or ARV resistance profiles affected, hence results could not be further elaborated upon. Nonetheless, the clinical implications of the under-calling of resistance would be that patients would remain on a failing drug regimen whilst accumulating more resistance. The over-calling of resistance would mean that patients would be switched too early onto second-line regimens.

Restrictions encountered with these analyses are that only subtype B derived BCO's are available, and these discrepancies may be a result of using inappropriate BCO's for subtype C-backbone viruses. The above analyses are also reliant on a single BCO value, not taking into account any variation around that cut-off nor of the assay variation. If a subtype-specific C-backbone is to be used, it would need to be investigated whether subtype-specific BCO's would need to be derived. Phenotypic output throughout various assays is shown to have expected inter- and intra- assay variability due to the nature of this *in vitro* assay [20,21,26,27]. By indirectly taking these confounding factors into account (i.e. Figure 2), the truly discordant comparisons were targeted (outside the mean +/−2SD range). Collectively, it was calculated that 95.8% (1590/1660) of all phenotypic data derived from the

subtype B- backbone virus had a concordant resistance profile with that of the subtype C- backbone derived viruses. Of the 70 discordant values found, only 10 comparisons were from treatment naïve (sensitive virus as per Stanford drug resistance database predictions), re-iterating that the B- and C- backbone assay variability is similar. Similar results were noted in a study by Choe *et al.* (2007) [20], who performed an analogous experiment within the PhenoSense® assay, showing a concordance of 95.8% of pair wise FC value comparisons across all drugs for all subtype C viruses tested in B- and C- backbone.

Another minor restriction of this analysis is that the majority of recombinant viruses carried the M184V, the K103N and the V106M mutations, which provide high-level resistance to 3TC and FTC with M184V or EFV and NVP with K103N and V106M. The virus backbone in such cases may theoretically have a decreased, if any, impact on the phenotypic result. In the context of the sample set used in this analysis, the prevalence of other mutations considered to not be classed as high-level resistance were not present in high amounts, and as such this could not be further studied.

An assessment of the sensitivities of these two backbones to measuring resistance in the presence of the M184V, K103N, or V106M mutation was performed. These particular mutations were selected for this analysis, in the context that subtype C viruses are under investigation in these experiments, and these mutations have previously been shown to be some of the most prevalent drug resistance mutations in subtype C treatment failures [10]. It would have been of interest to look at the K65R and thymidine analogue mutations (TAMs), but there were insufficient data points available for appropriate ROC analysis to be performed. ROC curves were plotted (data shown in Table 3), and no statistical differences noted with any of these 3 mutations. The detection sensitivity of both backbones was equal in measuring resistance to these prominent drug resistance mutations.

No systematic resistance under- or overcalling of the subtype C amplicons in the B- backbone phenotyping was noted. It appears that the virus backbone susceptibility outside of the GPRT region does not contribute to any changes in phenotypic FC values. The practical question being considered in this work is whether or not it is reliable to use a subtype B- backbone (as is currently the case with all available phenotypic assays) when assessing HIV-1 subtype C. In clinical practice, what this data suggests is that in the instance of subtype C, it is reliable for 95.8% of cases to use a B-backbone for phenotyping, once assay variability is to taken into account. Assay variability is especially important when FC values are close to the BCO's. Clinical decisions should not only be made merely according to the resistance call, but rather actual FC values should be considered. Caution should also be taken even when assessing resistance of non-B subtypes in a subtype B- backbone. This study indirectly suggests that the vircotype tool, which is built upon the Antivirogram database, is an equally reliable algorithm for genotyping subtype C samples. The Antivirogram® assay therefore remains an acceptable tool for phenotyping non-B GPRT amplicons.

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Author Contributions

Conceived and designed the experiments: MB KS CLW WSS TRDW LJS. Performed the experiments: MB KS. Analyzed the data: MB KS HDW MVH LJS. Contributed reagents/materials/analysis tools: LJS WSS CLW. Wrote the paper: MB KS MAP LJS. Provided significant comments and editing on the paper: CLW HDW MVH WSS TRDW.

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Chapter 3

Genotypic HIV-1 antiretroviral drug resistance testing of HIV-1 subtype C is a good substitution for phenotyping.

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Genotypic HIV-1 antiretroviral drug resistance testing of HIV-1 subtype C is a good substitution for phenotyping

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Abstract

Phenotyping is the HIV-1 drug resistance assay gold standard. However, genotyping is cheaper and requires reduced technical complexity. A South African reference laboratory performs genotyping predominantly for HIV-1 subtype C infected patients using an in-house assay. This study validates this protocol compared to phenotyping.

Treatment-experienced (n=78) and treatment-naive (n=36) patient samples were phenotyped (Antivirogram®) against a panel of 17 antiretroviral drugs (ARVs), and results compared to genotypic results obtained, interpreted using the Stanford HIVdb algorithm.

Matched reisstance profiles accounted for 92.3% (1603/1737) of individual drug comparisons. Sixty-one (53.5%) patients showed concordance for the full drug complement tested (n=17). Fifty-three (53) patients had 1 (n=4), 2 (n=17) or 3 (n=32) ARV genotypes misclassified compared with phenotyping. Discordant patients had at least one ARV with a sensitive phenotype and matched low resistance (n=36; 31.5%), or intermediate genotype (n=8; 7.0%). Nine (9; 7.9%) patients were classified as phenotypically resistant and genotypically potential-low-resistant in at least one ARV. Discordant categories were not clearly sensitive or resistant with both assays, and thus regarded as borderline profiles.

Good concordance between resistance profiles was reported. Genotyping is a reliable tool to detect resistance in HIV-1 subtype C infected patients and can be used in a clinical setting.

1. Introduction

In 2011 there were an estimated 1.79 million HIV-1 positive patients receiving antiretroviral drugs as part of the South African government's antiretroviral treatment roll-out program (Johnson 2012), and the program aims to increase this number dramatically in the near future (Conradie et al. 2012). The success of the program can be hampered by the inevitable emergence of antiretroviral drug resistance mutations in patients, which diminishes the virological response to antiretroviral therapy (Derdelinckx et al. 2004; Little et al. 2002; Wensing et al. 2005). To date, the South African government does not recommend HIV-1 antiretroviral drug resistance testing as part of the routine clinical monitoring of patients on treatment. In the latest Southern African HIV Clinicians Society recommendations on resistance testing however (Conradie et al. 2012), baseline resistance testing is recommended for children who have been exposed to ART for prevention of mother-to-child-transmission therapy and subsequently become HIV-infected. In addition, recommendations include resistance testing for South African adults after virological failure of standard first- and second-line ART regimens, although these are not yet established guidelines by the South African national department of health.

HIV-1 antiretroviral drug resistance can be evaluated by phenotypic and/or genotypic laboratory testing. *In vitro* phenotypic testing is the most direct method of assessing antiretroviral drug resistance as it measures the ability of a primary virus isolate or recombinant virus to replicate in the presence of varying concentrations of antiretroviral drugs. Phenotyping is regarded as the gold standard in resistance testing, however it is very costly, has specific infrastructure requirements, must be performed by highly skilled technical staff and is therefore not used for routine patient management. Genotyping is less costly, although it remains unaffordable by the majority of antiretroviral treatment programs in Africa with South Africa as a potential exception (Rosen et al. 2011; Levison et al. 2012). Genotyping infers resistance or susceptibility to a particular antiretroviral drug by sequencing the HIV-1 gene of interest and feeding this data into a predictive algorithm. There are various algorithms available, the majority of which predict antiretroviral drug resistance based on data derived from HIV-1 subtype B viruses. The Stanford HIVdb, ANRS and Rega algorithms are rule based algorithms, providing scores for specific combinations of mutations. The scores are then translated into different levels of susceptibility based on literature and expert's opinion (Frentz et al. 2010). The virco®TYPE and PhenoSense® GT are hybrid algorithms that combine phenotypic data with genotypic testing. The methodology employs statistical analyses of genotypic and phenotypic data generated from a large number of previously tested samples to find and calculate the impact of significant mutations and mutation pairs (Vermeiren et al. 2007).

In Western countries, HIV-1 drug resistance testing and viral load testing are standardly used to guide clinical treatment options. However, in resource-limited settings this is not affordable. Instead, according to the WHO-recommended public health approach (WHO 2010), standardized first-line antiretroviral drug regimen combinations are prescribed empirically. Switching to 2nd line treatment is based mostly on clinical and immunological information and sometimes (like in South Africa) on virological evaluations. As the number of South African patients classified as virological failures increase, antiretroviral drug resistance testing will need to be implemented to guide in the successful clinical management of patients (Conradie et al. 2012). This should be combined with refining programmatic issues such as feeding back viral load and HIVDR results, to ensure that this system becomes fully functional and applicable. The obvious assay choice to routinely implement HIV drug resistance testing is genotyping. A small subset of national South African laboratories has

started to offer HIV-1 genotypic testing as a tool for individual patient monitoring(Wallis et al. 2010; van Zyl et al. 2011). In case of national implementation, the number of labs offering these services will have to increase significantly. HIV-1 Subtype C is responsible for 50% of global infections(Buonaguro, Tornesello, and Buonaguro 2007) and 56 % of infections in Africa and is mainly distributed along the southern and eastern part of the continent(Hemelaar et al. 2006). Previous data has highlighted inconsistencies in current genotyping interpretation algorithms inadequately applied to non-B HIV-1 subtypes (Santos et al. 2009). Thus, this work aims to establish the reliability of using genotypic testing, in combination with the Stanford HIVdb interpretation algorithm, for antiretroviral drug resistance testing in a South African setting, as compared to phenotyping gold standards.

2. Methodology

2.1 Patient samples used in this study

A total of 114 plasma samples were selected based on their antiretroviral drug resistance mutation profiles (drug resistant and susceptible samples), and used for the purposes of this study. These included 78 samples from treatment-experienced patients attending clinics in the public sector in Johannesburg, South Africa that were sent for routine population-based HIV-1 drug resistance genotyping, and 36 treatment-naïve samples from the PASER-M cohort (Hamers et al. 2011). Samples were collected between October 2008 and October 2009, and were genotyped using an in-house assay assay (Wallis, 2010). Only samples with a viral load above 1,000 RNA copies/ml were assessed. The median viral load range for the tested samples was 87,900 RNA copies/ml (3,460 – 320,000 RNA copies/ml). All samples were phenotyped using the Antivirogram® assay (Janssen Diagnostics, Belgium). Ethical clearance for this study was obtained by the Human Research Ethics Committee (HREC) at the University of the Witwatersrand (Clearance Number M090688).

2.2 Viral RNA Extraction

Viral RNA was isolated from all patient plasma samples using the MagNA pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) with a sample input of 200µl, and a 50µl elution volume, as per manufacturer's instructions. Viral RNA from recombinant virus stocks (RVS) used in phenotyping was extracted using the QIAamp Virus BioRobot MDx kit (Qiagen, Belgium) or the NucliSENS easyMAG (bioMérieux Inc, Belgium), starting with an input volume of 600µl and eluting in 25µl, as per manufacturer's instructions.

2.3 HIV-1 antiretroviral drug resistance genotyping

The validated Gag-protease-reverse transcriptase (GPRT) genotyping protocol (Nauwelaers et al. 2011) was used to generate amplicons as input into the Antivirogram phenotyping assay, as well as to genotype the resulting recombinant virus stocks for quality control purposes. Amplification products were analyzed by 1% agarose gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, CA, USA), and detected on the ABI3730 XL automated sequencer (Applied Biosystems, CA, USA). Contigs were generated in the Sequencher v.4.5 software (Gene Codes Corporation, MI, USA), and fasta files were exported and submitted to the Stanford University HIV drug resistance database (Stanford HIVdb, version 6.0.3) to confirm antiretroviral drug resistance profiles and subtypes.

2.4 HIV-1 Antivirogram® phenotyping

Recombinant virus stocks (RVS) from each of the 114 patient samples were generated through homologous recombination of amplicons produced by the GPRT amplification protocol into a subtype B backbone for use in the Antivirogram® assay (Hertogs et al. 1998). The resistance profiles of the generated RVS were determined against a panel of 17 antiretroviral drugs (listed in Table 2) (Bronze et al. 2012). The GPRT region was genotyped before and after each patient sample was phenotyped, to ensure that the genetic background of each of the recombinant viruses was identical throughout the phenotyping experiments.

Wild type subtype B cell line adapted viruses (HXB2) were used as reference viruses for the antiviral experiments. All 50% inhibitory concentration (IC_{50}) values were calculated from 8 readings for each recombinant virus against the different antiretroviral drugs. Fold changes (FC) were calculated by dividing these IC_{50} values by the IC_{50} of the HXB2 reference virus. Recombinant viruses were characterized as being susceptible or resistant based on pre-established biological cut-off values (BCOs) (Winters et al. 2009).

2.5 Data Analysis

The comparison of drug resistance classifications between the genotypic and phenotypic results was performed by calculating the sensitivity and specificity of the genotype as compared to the phenotype. Sensitivity was calculated as (true sensitive/ (true sensitive + false resistant) x 100) and specificity was calculated as (true resistant/ (true resistant + false sensitive) x 100). Sensitivity, therefore, measures the proportion of sensitive calls, which are correctly identified, while specificity measures the proportion of resistant calls, which are correctly identified. Phenotypic calculations were resulted as either resistant or sensitive, whereas the Stanford HIVdb algorithm depicts viruses ARV susceptibilities in five categories. In order to compare the data from both assays, the sensitive (S) and potential low resistant (PLR) classifications from genotypic results were equated to as being sensitive, and the low (LR), intermediate (IR) and high resistance (HR) equated to as being resistant.

Fold change comparisons for which a discordant resistance call was observed between genotyping and phenotyping were further assessed within the context of phenotypic intra-assay variability. Initially, all FC values were “normalized” against BCO’s for the various drugs, by subtracting the relevant ARV’s BCO from the observed FC value. Hence, all FC values, regardless of the ARV that was tested, could be compared collectively. A 2-fold difference in FC was regarded as acceptable intra-assay variability (Choe, Stawiski, and Parkin 2007). Any values outside these cut-offs were considered to not fall within these acceptable limits of assay variation, and hence truly discordant data points.

3. Results

3.1 ARV exposure and drug resistance profiles of patients

All samples analysed were classified as HIV-1 subtype C, using the Rega subtyping algorithm(Stanford University HIVdb). No ARV drug resistance mutations were detected in the treatment naïve group (n=36). The HIV-1 drug resistance mutation profiles for the treatment-exposed group (n=78) were

analyzed (see Table 1). The most prevalent mutations in this dataset included: K103N (n=30; 38.5%), M184V (n=26; 33.3%), T215Y (n =13; 16.7%), M41L (n =9; 11.5%), D67N (n = 9; 11.5%), V106M (n= 8; 10.3%), T215F (n=8; 10.3%), and V108I (n= 8; 10.3%).

Table 1. Antiretroviral drug exposure profiles of treatment-exposed patients (n=78)

First Line therapy	Number of patients	Treatment switches	
		Drug switches	Patient number with treatment switches (n)
3TC+D4T+EFV	30	3TC+**AZT**+**LPV**	1
		3TC+**DDI**+**LPV**	3
3TC+D4T+NVP	5	**AZT**+**DDI**+**LPV**	2
3TC+D4T+LPV	4	**AZT**+**DDI**	1
3TC+DDI+EFV	9	3TC+**AZT**+**LPV**	3
3TC+DDI+NVP	2		
3TC+DDI+LPV	1		
3TC+TDF+EFV	17	3TC+**AZT**+**LPV**	1
		3TC+TDF+**LPV**	1
3TC+TDF+LPV	1		
AZT+DDI+EFV	5	AZT+DDI+**LPV**	1
AZT+DDI+LPV	2		
AZT+ABC+LPV	1		
AZT+D4T+EFV	1		
Total Drug exposed	78		13

ARV's highlighted in bold are those which have been introduced as a treatment switch

NRTI: Nucleoside reverse transcriptase inhibitor; NNRTI: Non-nucleoside reverse transcriptase inhibitor; PI: Protease Inhibitor; 3TC: Lamivudine; D4T: Stavudine; DDI:Didanosine; TDF: Tenofovir; AZT: Zidovudine; ABC: Abacavir; LPV; Lopinavir/ ritonavir boosted

3.2 Comparison of genotypic and phenotypic results

A total of 17 drugs were tested within the phenotyping assay, for 114 samples. Out of the expected 1,938 comparisons, 1,737 comparisons were obtained (89.6%), as not all comparisons had results for all ARVs tested, due to an average antiviral experiment (AVE) success rate of 85.1%. Table 2 shows the comparison statistics for all 17 ARVs tested, as well as combined results for NRTI, NNRTI, PI and all drugs. The concordance between genotypes and phenotypes for the 1,737 comparisons performed was 92.3% (1,603/1,737). The drug class concordance is noted at 92.7% (673/726) for NRTIs, 91.1% (288/316) for NNRTIs, and 92.4% (642/695) for PIs. The number of discordant comparisons was 134 (7.7%). The overall sensitivity of the genotypic results compared to gold standard phenotyping was 93.9%, and the specificity 87.4%.

Table 2. Comparison between HIV-1 genotypic and phenotypic resistance profiles.

ARV	Phenotypic vs. genotypic comparisons (#)	Resistant (phenotype)-resistant (genotype) (#)	Resistant (phenotype)-sensitive (genotype) (#)	Sensitive (phenotype)-sensitive (genotype) (#)	Sensitive (phenotype)-resistant (genotype) (#)	Sensitivity (%)	Specificity (%)	Number of matched resistance profiles	Number of mismatched resistance profiles
AZT	91	7	0	79	5	94.0	100.0	86	5
3TC	97	47	1	45	4	91.8	97.9	92	5
DDI	110	27	6	73	4	94.8	81.8	100	10
D4T	96	7	0	79	10	88.8	100.0	86	10
ABC	110	31	4	71	4	94.7	88.6	102	8
FTC	110	51	5	53	1	98.1	91.1	104	6
TFV	112	13	5	90	4	95.7	72.2	103	9
NRTI	**726**	**183**	**21**	**490**	**32**	**93.9**	**89.7**	**673**	**53**
NVP	111	59	0	49	3	94.2	100.0	108	3
EFV	104	55	4	43	2	95.6	93.2	98	6
ETR	101	21	2	61	17	78.2	91.3	82	19
NNRTI	**316**	**135**	**6**	**153**	**22**	**87.4**	**95.7**	**288**	**28**
IDV	101	11	3	86	1	98.9	78.6	97	4
SQV	89	2	1	80	6	93.0	66.7	82	7
APV	103	9	2	91	1	98.9	81.8	100	3
LPV	96	8	7	79	2	97.5	53.3	87	9
ATV	103	6	6	89	2	97.8	50.0	95	8
TPV	111	11	1	87	12	87.9	91.7	98	13
DRV	92	1	6	82	3	96.5	14.3	83	9
PI	**695**	**48**	**26**	**594**	**27**	**95.7**	**64.9**	**642**	**53**
All drugs	**1737**	**366**	**53**	**1237**	**81**	**93.9**	**87.4**	**1603**	**134**

Antiretroviral (ARV) abbreviations: lamivudine(3TC), zidovudine (AZT), stavudine (D4T), didanosine (DDI), abacavir (ABC), emtricitabine (FTC), tenofovir (TFV), nevirapine (NVP), efavirenz (EFV), etravirine (ETR), indinavir (IDV), fosamprenavir (APV), atazanavir (ATV), saquinavir (SQV), darunavir (DRV), lopinavir (LPV), tipranavir (TPV). Sensitivity (diagnostic) was calculated as (true sensitive/(true sensitive + false resistant) x 100) and specificity (diagnostic) was calculated as (true resistant/ (true resistant + false sensitive) x 100).

The clinical implication of concordant and in particular discordant comparisons was investigated on a patient level. Sixty-one (53.5%) patients showed concordant results for the full drug complement tested (n=17). The 53 discordant patient samples had 1 (n=4), 2 (n=17) or 3 (n=32) ARV drug genotypes misclassified, 9 of which were drug naïve and 44 which were drug exposed. Ninety-eight (98) of the mismatched resistance profiles were classified as susceptible by phenotyping, but had low- (n=78) or intermediate resistance (n=20) by the Stanford HIVdb algorithm, attributed to resistance mutation mixtures. The remaining 36 mismatched resistance profiles involved borderline resistance calls such as PLR (n=23) or LR (n=13) on Stanford HIVdb, and did not contain any mutation mixtures. In these cases, when a LR was genotypically scored, this was shown as being susceptible in phenotyping, and when a PLR call was genotypically scored, phenotyping calculated this virus as being resistant (Table 3). The corresponding phenotypic results of these 36 samples were equally regarded as borderline, where the FC values were within a 2-fold range (Figure 1.) All in all, there was no systematic under- or over-calling of resistance from genotyping compared to phenotyping.

Table 3. Characteristics of mismatches drug resistance profiles

Genotypic mutation mixtures	Number	Paired ARV Phenotypic and Genotypic profiles		Number of patients profiles affected (1 ARV)	Number of patients profiles affected (2 ARVs)	Number of patients profiles affected (3 ARVs)	Total numbers of patient profiles affected
		Phenotype	Genotype				
Mixtures present	78	S	LR	2	11	18	31
	20	S	IR	0	4	4	8
Mixtures not present	13	S	LR	0	2	3	5
	23	R	PLR	2	0	7	9
Total	**134**			**4**	**17**	**32**	**53**

Sensitive (S), Low Resistance (LR), Intermediate Resistance (IR) and Resistance (R). Genotypic mixture mutations are described, where the presence of a mixture of a known HIV-1 drug resistance mutation confers the relevant resistance profile, as per genotyping.

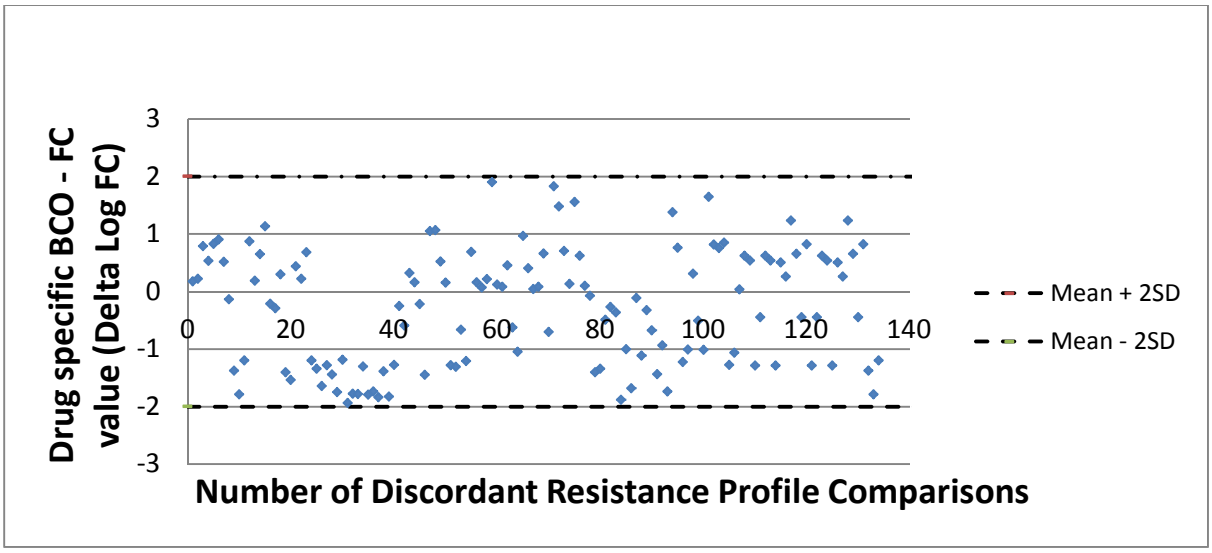

Figure 1. Plots showing discrepant phenotypic resistance comparisons as compared to genotyping, and variation around the BCO.

4. Discussion

The purpose of this study was to compare an in-house HIV-1 genotyping protocol in conjunction with the Stanford HIVdb algorithm on HIV-1 subtype C samples from South Africa with gold standard HIV-1 phenotyping. This was considered important in light of the recent developments with respect to the national antiretroviral treatment program in South Africa, where HIV drug resistance testing might become a routine application for large groups of patients (Conradie et al. 2012). Since the majority of these patients are infected with HIV-1 subtype C, it was deemed important to establish the reliability of the most commonly used HIV-1 genotypic interpretation algorithm against the phenotyping gold standard as it will be scaled up to more laboratories in South Africa in the near future.

The implementation of viral load monitoring should be a priority in routine patient monitoring, and accordingly resulting of viral loads should be easily accessed by the appropriate health care provider, to enable adequate clinical management of the patient. Since viral loads are widely rolled out in the South African healthcare system, the logical follow-up testing required, should the patient be in

virological failure, is that of HIVDR, as to further guide clinicians on relevant treatment options and adherence interventions. This diagnostic algorithm will be most effectively used in conjunction with the scale-up of 1st line treatment, as well as an increase in 2nd line treatment options. Genotypic resistance testing has been shown to be cost-effective at first-line antiretroviral failure in the South African setting, provided timely response to the results is observed (Levison et al. 2012; Rosen et al. 2011).

The phenotyping used in this study was performed as commercially available, which entails using a subtype B backbone, and recombining the patient-derived subtype C amplicons, thereby creating a subtype B-C chimeric virus. Previous work, testing the same subtype C amplicons recombined into both a subtype B and C backbone, showed that there was no significant difference in resulting drug resistance profiles, and as the B-backbone still remains the gold standard for phenotyping, it was used in this study (Bronze et al. 2012). The use of a subtype B backbone should therefore not have been a source for any of the discordant comparisons noted.

The comparison of the genotypic predictions and phenotypic results indicated a concordance of 92.3% (1,603/1,737). There are few published studies using this type of comparison and analysis. Previous work showed that phenotyping detected reduced antiretroviral drug susceptibility and hypersusceptibility in HIV from some antiretroviral-naive Ugandan adults that was not predicted by genotyping (Eshleman et al. 2009). In our work we did not find this to be the case, and there does not appear to be any systematic under- or over resistance calling by genotyping. In an earlier study, a concordance of 81% for NRTIs, 91% for NNRTIs and 90% for PIs was determined on subtype B samples (Dunne et al. 2001). The current study using subtype C samples resulted in similar concordance per drug class of 91.1% (288/316) for NNRTIs, and 92.4% (642/695) for PIs (Table 2), with a higher concordance for NRTIs: 92.7% (673/726). The work by Dunne et al. (2001) did not use the same phenotyping assay (PhenoSense^{TM} HIV (ViroLogic)) and therefore comparisons with the current study should be considered carefully.

Complete concordance between phenotype and genotype for all 17 drugs evaluated was observed in 61/114 (53.5%) of patients, whereas (Dunne et al. 2001) found only 17% of patients with full drug complement concordance. This difference between studies can be explained by the fact that over time the HIV-1 genotyping algorithms have been refined, and a better overall level of concordance with phenotyping is being obtained.

The categories of discordance are described (Table 3), which show the majority (91/134 (68.0%)) of these differences entailing a sensitive phenotype paired with a LR genotype. In the clinical context these cases would likely be treated with increased caution, as a LR profile does not rule out the use of a particular ARV, instead of changing treatment, this rather alerts a clinician to the decreased susceptibilty of this drug, and not a fully resistant profile. Twenty-three (23) comparisons had a resistant phenotype, with a PLR genotype, which may result in a patient staying on a failing therapy, instead of being switched, as a PLR genotype is usually interpreted as a sensitive profile. This would mean that 9/114 (7.9%) of patients would possibly be maintained on a failing ARV if genotyping were used. Twenty (20) ARV profiles, translating to 8 patient samples were shown to have a sensitive phenotype, with an IR genotype, suggesting that in 7.0% of this patient cohort an ARV drug may have been erroneously deleted from a regimen. The discordant categories may be regarded as

borderline resistance profiles, indicating that they were not clearly genotypically sensitive or resistant i.e PLR, LR and IR. Equally borderline, were the paired phenotyping results for all discordant cases which showed an acceptable variability around the BCO (Figure 1). This data implies that although different phenotypic/ genotypic resistance profiles were noted in these cases, they are not extreme cases, but may not be treated equally in a clinical setting, should the clinician have been supplied either a phenotypic or genotypic report. Any resistance demonstrated by either assay puts the patients in a higher risk category. It should be noted that HIV treatment switches are not merely reliant on HIVDR reporting, and need to take into account patient-specific information such as drug toxicity, adherence knowledge, drug interactions and other clinical details.

This work proposes that genotyping is a reliable tool to detect and interpret antiretroviral drug resistance in HIV-1 subtype C samples. However clinicians should still remain cautious about altering HIV therapies in the individual cases when a genotype leads to a borderline prediction (PLR, LR or IR) or when mutation mixtures are present.

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Chapter 4

Development and evaluation of an affordable real-time qualitative assay for determining HIV-1 virological failure in plasma and dried blood spots.

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Development and Evaluation of an Affordable Real-Time Qualitative Assay for Determining HIV-1 Virological Failure in Plasma and Dried Blood Spots

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Virological failure (VF) has been identified as the earliest, most predictive determinant of HIV-1 antiretroviral treatment (ART) failure. Due to the high cost and complexity of virological monitoring, VF assays are rarely performed in resource-limited settings (RLS). Rather, ART failure is determined by clinical monitoring and to a large extent immunological monitoring. This paper describes the development and evaluation of a low-cost, dried blood spot (DBS)-compatible qualitative assay to determine VF, in accordance with current WHO guideline recommendations for therapy switching in RLS. The assay described here is an internally controlled qualitative real-time PCR targeting the conserved long terminal repeat domain of HIV-1. This assay was applied to HIV-1 subtypes A to H and further evaluated on HIV-1 clinical plasma samples from South Africa ($n = 191$) and Tanzania ($n = 42$). Field evaluation was performed in Uganda using local clinical plasma samples ($n = 176$). Furthermore, assay performance was evaluated for DBS. This assay is able to identify VF for all major HIV-1 group M subtypes with equal specificity and has a lower detection limit of 1.00E+03 copies/ml for plasma samples and 5.00E+03 copies/ml for DBS. Comparative testing yielded accurate VF determination for therapy switching in 89% to 96% of samples compared to gold standards. The assay is robust and flexible, allowing for "open platform" applications and producing results comparable to those of commercial assays. Assay design enables application in laboratories that can accommodate real-time PCR equipment, allowing decentralization of testing to some extent. Compatibility with DBS extends access of sampling and thus access to this test to remote settings.

In 2010, the HIV-1 epidemic was estimated to include 34.0 million (range, 31.6 to 35.2 million) infected adults and children across the globe. An alarming 67.4% ($n = 22.9$ million) of the total global infections are people residing in sub-Saharan Africa. As a result of antiretroviral therapy (ART) scale-up initiatives, 6.65 million infected individuals requiring treatment were receiving it in sub-Saharan Africa by the end of 2010 (1). However, particularly in resource-limited settings (RLS), effective treatment faces challenges, which include failing supply chains resulting in drug shortages, drug toxicity of older first- and second-line drugs, failure of patient adherence, drug interactions, lack of qualified health care staff, or failing adherence support, etc. As a result, HIV-1 can develop drug resistance to ART, leading to virological failure (VF) and subsequently ART failure. A recent report has shown that the prevalence of pre-ART HIV-1 drug resistance in 13 sites in various countries in sub-Saharan Africa is 5.6%, ranging from 1.1% in South Africa to 12.3% in Uganda (2). Recent scientific findings have led to the consideration of "treatment as prevention," which according to the most intensive "test and treat" scenario could ultimately increase the number of HIV patients qualifying for ART to 32 million (3). With rapidly increasing numbers of HIV patients on ART in RLS with weak health systems, the risk of further increase of HIV-1 drug resistance is imminent.

The success of increased access to ART in RLS has largely been due to massive donor funding and important reduction of costs of selected first-line drugs. However, reduced susceptibility to these first-line drugs and the consequent switching to second-line drugs would at least partly undo early ART successes and result in higher expenditures and increasing numbers of patients on failing regimens with no options for effective second-line or salvage therapies (4, 5).

According to the definition of the WHO, VF is a repeated viral load of ≥5.00E+03 RNA copies/ml in an individual taking ART for at least 4 to 6 months (6). Timely detection of VF by viral load (VL) testing, which is routine in industrialized countries (7), is necessary to prevent accumulation of HIV drug resistance (8) or to identify poor adherence to the treatment. However, in RLS, high costs and technical complexity limit VL monitoring, and treatment failure is determined primarily by clinical monitoring for stage three and four AIDS-defining illnesses and, if available, immunological monitoring using CD4 counts (6). The inadequacies of CD4 counts for determining true treatment failure have been described on many occasions (9–12). The clinical-immunological monitoring approach results in individuals being left on suboptimal regimens for an extended period of time with the risk

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TABLE 1 HIV-1 isolates used from the BBI HIV-1 subtype reference panel

HIV-1 subtype	Strain	Country of origin	NCBI accession no.
A	UG275	Uganda	AB485632
B	BK132	Thailand	AY173951
C	ZB18	Zambia	AB485641
D	SE365	Senegal	AB485648
CRF01_AE	CM240	Thailand	AF067154
F	BZ126	Brazil	AY173957
G	BCFDIOUM	Zaire	AB485661
H	BCPKITA	Zaire	AB485665

of accumulating drug resistance mutations or unnecessary switching to second-line therapy based on non-VL-supported decisions (4). Both scenarios limit future treatment options and may cause increased costs associated with second-line therapy (5).

The current paper addresses the challenge of determination of VF in RLS by taking several premises into consideration that reflect the actual public health situation in these settings. First of all, the standpoint was taken that determination of an exact VL is not required to determine ART failure, and therefore a less complex, and thus less expensive, assay that classifies a sample as either above or below a treatment success threshold would suffice. Second, in order to implement the WHO recommendations of task shifting and decentralization of ART to remote settings, the consequence would be that complex procedures, including drawing blood, isolation and storage of plasma samples, and cold chain shipments to qualified labs for VL testing, should be avoided. Rather, VF should be detectable on dried blood spots (DBS), a sampling alternative that is inexpensive and easy to collect and transport and has proven application for VL testing (13, 14). Third, given the fact that for accurate detection of VF, a nucleic acid amplification step remains necessary and taking into consideration the realities of contamination risks in remote labs, it was decided to concentrate on a real-time PCR approach. This allows for VF determination in a closed system and with equipment that is continuously evolving, regularly reducing in price, and being adapted to local circumstances through battery and solar energy applications. Finally, it was considered essential that the protocol for VF testing should be generic, "open platform," applicable on a wide array of real-time PCR instruments in various African settings, and freely available in the public domain.

With the above assumptions in mind, the Affordable Resistance Test for Africa (ARTA) consortium was established, consisting of a unique combination of academia, industry, and nongovernment organizations both in Africa and Europe (the members of the consortium are listed in the Acknowledgments). Here we report on the results of ARTA research to develop a real-time PCR assay that can be used as a screening tool to determine VF in RLS. This virological failure screening assay (VFA) can be readily applied in basic laboratories, using either plasma samples or DBS as the sample input. The VFA is applicable for all major HIV-1 group M subtypes and is specifically designed to identify VF as defined by the WHO as a VL of ≥5.00E+03 copies/ml (6).

MATERIALS AND METHODS

Samples. (i) HIV-1 subtype reference panel. A panel of virus isolates consisting of HIV-1 subtypes A through H (Table 1) was obtained from BBI Biotech Research Laboratories Inc. (Gaithersburg, MD, USA) for assay optimization and evaluation at the University Medical Centre Utrecht (UMCU), Utrecht, the Netherlands. Serial dilutions were prepared from these stocks using HIV-1-negative human plasma samples. These dilutions were also used to spike HIV-1-negative whole-blood samples for dried blood spot (DBS) preparation.

(ii) Clinical samples. Clinical plasma samples from HIV-1-infected individuals from several African sites were included for further evaluation at the UMCU, Utrecht, the Netherlands. Samples were selected to include several subtypes with a variety of viral loads (VL) in accepted ranges for subsequent analysis. Samples from South Africa ($n = 191$) were plasma samples sent for routine VL testing, performed on the Cobas AmpliPrep/Cobas TaqMan system v2 (Roche, Penzberg, Germany), and represented HIV-1 subtype C with a VL range of 1.30E+03 to 3.00E+06 (median, 5.50E+04) copies/ml. Samples from Tanzania ($n = 42$) were part of an ongoing prevention-of-mother-to-child-transmission (PMTCT) study (15), where VL was determined using the COBAS Amplicor HIV-1 Monitor test v1.5 (Roche). Samples included subtypes A ($n = 23$), C ($n = 10$), and D ($n = 6$), and three samples with undetermined subtype with a VL range of 6.65E+02 to 3.07E+05 (median, 2.67E+04) copies/ml.

In addition, as part of a technology transfer program, the assay described here was applied in three Joint Clinical Research Centre (JCRC) laboratory sites in Uganda, where retrospective plasma samples collected from HIV-1-positive individuals as part of the PharmAccess African Studies to Evaluate Resistance (PASER) program were included (16). These samples represented baseline and follow-up clinical samples at yearly intervals after therapy initiation. For these samples, routine VL testing had been performed in Uganda using the Cobas AmpliPrep/Cobas TaqMan system v2 (Roche). A total of 176 plasma samples were tested, including subtypes A ($n = 89$) and D ($n = 64$) and 23 with an unknown subtype, with a VL range of 1.00E+02 to 1.00E+06 (median, 1.00E+04) copies/ml. Twenty-five confirmed HIV-1-negative plasma samples were included for assay specificity control.

To investigate the application of the assay with DBS samples, DBS were prepared from EDTA-collected whole-blood samples for participants of the PASER program (16). The same blood sample was then centrifuged, and the plasma was removed for analysis. These samples are subsequently referred to as paired plasma and DBS samples. A total of 82 paired samples were tested in Uganda, with a VL range of 4.40E+01 to 7.18E+06 (median, 2.61E+03) copies/ml. DBS samples were stored at −70°C for 270 to 515 (median, 485) days ($n = 31$), −20°C for 45 to 112 (median, 82) days ($n = 21$), or room temperature for 2 to 192 (median, 126) days ($n = 30$).

(iii) Internal control. An internal control (IC) was added to each clinical sample at a fixed amount of 10% of the elution volume at the start of nucleic acid isolation. The IC consisted of the nonhuman RNA virus encephalomyocarditis virus (EMCV) and was prepared at the UMCU, Utrecht, the Netherlands, in batches of single-use aliquots and stored at −80°C until use.

Virological failure screening assay (VFA). (i) Nucleic acid isolation from plasma samples. At the UMCU, Utrecht, the Netherlands, viral nucleic acid (NA) isolation was performed using NucliSENS magnetic extraction reagents in combination with the Minimag (bioMérieux, Boxtel, the Netherlands). For each sample, an input of 100 μl plasma was used, or two DBS of 50 μl each, and 2.5 μl IC. Positive and negative controls were included in each run. Upon completion of the isolation procedure, purified nucleic acids were eluted in 25 μl elution buffer.

In Uganda, NA isolation was performed using the QIAamp RNA kit (Qiagen GmbH, Germany) per the manufacturer's instructions. Input was 100 μl of plasma, or two 50-μl DBS, and 5 μl IC. Isolated NA was eluted in 50 μl elution buffer.

Upon completion of both isolation procedures, the eluates were used immediately for reverse transcription (RT), and the remaining nucleic acids were stored at −20°C.

TABLE 2 Primer and probe sequences for the HIV-1 virological failure screening assay

Primer or probe[a]	Sequence	Function	Target[b]
EMC-forward	5′-TGACCACGCCACCGC-3′	Forward primer	EMCV
EMC-reverse	5′-TAAAGATTTCCCTTGCCCCG-3′	Reverse primer	EMCV
EMC-VIC	5′-TGTGAGCCAGTCGTGATTGTGCTCC-3′	TAMRA probe	EMCV
HIV-LTR S4	5′-AAGCCTCAATAAAGCTTGCCTTGA-3′	Forward primer	HXB2 nt 520–543
3′UNI-KS-6	5′-GAGGGATCTCTAGTTACCAGAGTCACA-3′	Reverse primer	HXB2 nt 574–600
3′UNI-KS-6-AG	5′-GAGGGATCTCTAGTTACCAGAGTCCTA-3′		
HIV-LTR-FAM	5′-TAGTGTGTGCCCGTCTG-3′	MGB probe	HXB2 nt 554–570

[a] In the primer and probe designations, EMC stands for encephalomyocarditis virus and LTR stands for the long terminal repeat region of HIV-1.
[b] EMCV, encephalomyocarditis virus (internal control). HXB2 is a HIV-1 reference sequence. nt, nucleotides.

(ii) Nucleic acid isolation from DBS. At both sites, a preincubation step for DBS was performed. DBS samples were excised by hand using scissors, which were decontaminated between samples with 70% ethanol. For the Nuclisens method, DBS were placed in the provided 2-ml lysis buffer (bioMérieux) in a 9-ml tube. For the QIAamp RNA method (Qiagen GmbH), DBS were placed in 700 μl of the provided buffer, AVL lysis buffer that was aliquoted in 2-ml Eppendorf tubes for use. For both methods, samples were incubated at room temperature with gentle rotation for 30 min, after which filters were removed and NA was isolated as described above for plasma samples.

(iii) Reverse transcription. Purified NA, containing both HIV-1 RNA and IC RNA, was reverse transcribed using the TaqMan real-time PCR system random hexamer RT kit (Life Technologies, Foster City, CA) according to the manufacturer's instructions. An input of 10 μl NA isolate was used in a final reaction volume of 25 μl. Reactions were carried out according to the following conditions: 25C° for 10 min, 48°C for 30 min, and 95°C for 5 min. The cDNA generated was used immediately for real-time PCR or stored at 4°C.

(iv) Real-time PCR. HIV-1 and IC cDNA fragments were amplified in the multiplex format. A 25-μl real-time PCR mixture contained 12.5 μl universal TaqMan master mix (Life Technologies), 10 μl cDNA, 300 nM primer EMC-forward (EMC stands for encephalomyocarditis virus), 900 nM primer EMC-reverse, 100 nM tetramethylrhodamine (TAMRA) probe EMC-VIC, 300 nM forward primer LTR S4 (LTR stands for the long terminal repeat region of HIV-1), a mixture of 600 nM HIV-LTR reverse primers 3′UNI-KS-6 and 3′UNI-KS-6-AG, and 100 nM minor groove binder (MGB) probe HIV-LTR-FAM (Table 2). The assay was performed at the UMCU, Utrecht, the Netherlands, using an Applied Biosystems 7500 real-time PCR system (ABI7500) (Life Technologies), and in Uganda using a MiniOpticon real-time PCR detection system (Bio-Rad, Hercules, CA). Both systems included a temperature profile allowing for dUTP/uracil-*N*-glycosylase (UNG) decontamination, namely, 50°C for 2 min; 95°C for 10 min; 45 cycles, with 1 cycle consisting of 95°C for 15 s and 60°C for 1 min. In order to enable run-to-run comparison, a fixed threshold was established for both systems (data not shown).

(v) Assay controls. Positive and negative controls were included in each run. Appropriate performance of the run was judged based on the results of these controls. The positive control consisted of a plasma sample spiked with a fixed concentration of 2.50E+04 copies/ml HIV-1, the threshold cycle (C_T) value acceptance range of which was determined for each real-time system. For this evaluation, the positive-control C_T range was set at 29 to 32. The IC was used to monitor for inhibition of each individual sample. As with the positive control, the C_T value acceptance range of the IC was determined for each real-time system. For this evaluation, the positive-control C_T range was set at 30 to 33. Three negative controls were included, an isolation negative control that consisted of HIV-1-negative human plasma and IC and negative RT and PCR controls that consisted of nuclease-free water and no IC. The result obtained for a sample was considered valid when the positive and IC controls were within their predetermined ranges, and the negative controls were below detection.

Data analysis. A 5-fold serial dilution series of viral RNA for plasma and DBS for all panel subtypes (Table 1) was used to assess dynamic range, level of detection (LOD), and inter- and intra-assay reproducibility. For the ABI7500 (Life Technologies), the serial dilution series ranged from 5.00E+06 to 3.20E+02 copies/ml, and for the MiniOpticon (Bio-Rad), the serial dilution series ranged from 1.00E+06 to 1.6E+03 copies/ml. Linearity was determined and reported as a coefficient of determination (R^2) value and slope gradient. Positive-control and IC C_T values were averaged to determine assay precision and reproducibility. Multiple measurements of 5.00E+03 copies/ml dilutions were performed and averaged to establish the VF C_T cutoff range. Theoretically, a one-C_T difference reflects a 2-fold change in target NA in the amplification reaction. The slope of gradient determined using the dilution series gave the number of C_Ts difference to result in a 1-log-unit change in VL, which were used to interpret an equivalent $\log_{10}$ copies/ml value to assess precision and reproducibility.

For clinical samples, a chi-squared test was performed in order to determine the proportion of virological failures detected using the 5.00E+03 copies/ml cutoff range according to the determined C_T value. A positive result was regarded as a C_T value equal to or lower than the C_T value range designated for 5.00E+03 copies/ml, and a negative result was regarded as a C_T value greater than that C_T value range. C_T values that were within the 5.00E+03 copies/ml C_T range were considered positive, with a suggestion to repeat in a follow-up test. Samples that were positive or negative in the VFA, but not in the corresponding commercial VL assay were classified as false positive or false negative, respectively. Sensitivity was calculated as the number of true-positive results/(number of true-positive and false-negative results. Specificity was calculated as the number of true-negative results/(number of true-negative and false-positive results).

RESULTS

Assay design. An alignment of the long terminal repeat regions of HIV-1 (LTR) sequences obtained from the Los Alamos database was created representing all HIV-1 reference subtypes ($n = 37$) and circulating recombinant forms (CRFs) ($n = 18$). A 145-nucleotide fragment of the HIV-1 5′ long terminal repeat (LTR) R/U5 region was subsequently used for real-time PCR assay design using ABI Primer Express 2.0 software (Life Technologies, CA, USA). Mixed nucleotides were introduced at positions of intersubtype heterogeneity. The most efficient combinations of designed virological screening failure assay (VFA) primers-probes and a previously published 5′LTR-based VL primer (17) (Table 2) were optimized and extensively tested in several independent runs with all isolated samples of the subtype panel.

Analytical sensitivity. All HIV-1 subtypes in the panel could be detected with equal efficiency. The assay demonstrated good overall linearity across subtypes, determined by plotting mean C_T values for all subtypes tested. The ABI7500 (Life Technologies)

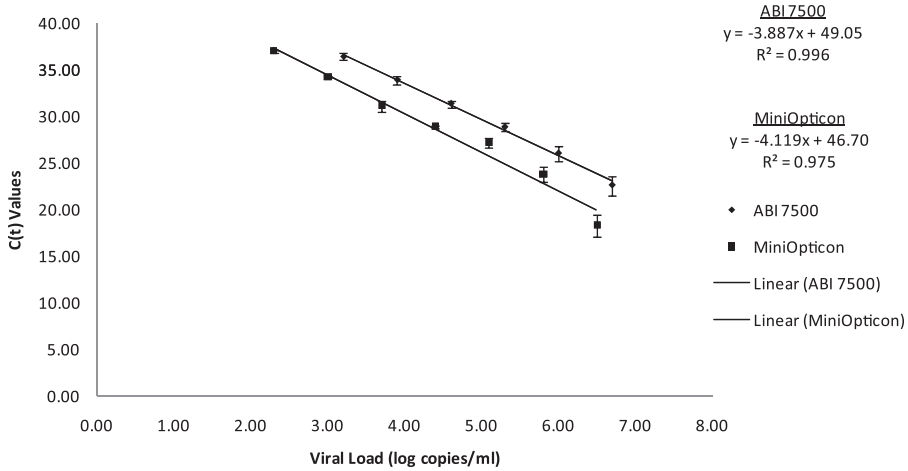

FIG 1 Linearity of VFA results using two different instruments, ABI7500 and MiniOpticon. The threshold cycle values are shown on the *y* axis.

and the MiniOpticon (Bio-Rad) had coefficients of determination (R^2) of 0.996 and 0.975, respectively (Fig. 1). The level of detection (LOD) for both plasma and dried blood spot (DBS) samples was defined by the lowest concentration where no negative VFA result for any subtype was observed in replicates. Results from the serial dilutions determined the LOD for plasma to be 1.00E+/+03 copies/ml with a mean C_T of 36.65 and SD of 1.12 (data not shown).

Assay precision and reproducibility. To determine precision, the assay was performed by two different operators using positive controls ($n = 12$) with a viral load (VL) of 2.50E+04 copies/ml (4.40 $\log_{10}$ copies/ml) in 12 individual runs. Results were highly reproducible for both the HIV-1-positive controls, with a mean VL of 4.17 $\log_{10}$ copies/ml and a SD of 0.10 $\log_{10}$ copies/ml, and the corresponding IC values, with a mean VL of 4.14 $\log_{10}$ copies/ml and SD of 0.17 $\log_{10}$ copies/ml. Intra-assay precision was further assessed in quadruplicate, from isolation to result, for each isolate of the subtype panel, with a VL of 5.00E+03 copies/ml (3.70 $\log_{10}$ copies/ml). The mean, SD, and percent coefficient of variation (%CV) of the VL were 3.98 $\log_{10}$ copies/ml, 0.24 $\log_{10}$ copies/ml, and 8.0%, respectively, for plasma samples, and 3.50 $\log_{10}$ copies/ml, 0.33 $\log_{10}$ copies/ml, and 13.2%, respectively, for DBS.

Interassay reproducibility was determined at two of the JCRC laboratory sites in Uganda. The assay was performed on 10 high-VL plasma samples with VL of 1.25E+05 to 2.0E+06 copies/ml by 4 different users on different days. The results were highly reproducible with a mean SD of 0.13 $\log_{10}$ copies/ml (range, 0.04 to 0.19 $\log_{10}$ copies/ml). For all high-VL sample runs ($n = 40$ reactions), the IC results were highly comparable, with a mean SD of 0.11 $\log_{10}$ copies/ml (range, 0.03 to 0.23 $\log_{10}$ copies/ml).

Accuracy. Three sample sets are shown in Table 3 depicting the qualitative comparison of the VFA and commercial assays using plasma samples. A total of 91.6% (175/191) of the South African samples were accurately classified compared to the commercial assay. Two samples were invalid in the assay, 10 (5.2%) were overestimated (false positive) and six samples (3.1%) were underestimated (false negative) by the assay (Table 2). The sensitivity and specificity were 96.2% and 79.2%, respectively. A total of 92.9% (39/42) of the samples from Tanzania were accurately classified compared to the commercial assay. An additional three samples (7.1%) were overestimated, and no underestimation was observed, with a sensitivity and specificity of 100.0% and 76.9%, respectively.

TABLE 3 Qualitative comparison and method agreement summary of clinical plasma and DBS samples for the VFA[a]

Standard assay	Sample standard	Sample VFA	n[c]	No. of samples (%) with the following result[d]: True Pos.	True Neg.	Correctly classified	False Pos.	False Neg.	Incorrectly classified	Sensitivity (%)	Specificity (%)
TaqMan[e]	Plasma	Plasma	191	153	38	175 (91.6)	10	6	16 (8.4)	96.2	79.2
Amplicor[e]	Plasma	Plasma	42	32	10	39 (92.9)	3	0	3 (7.1)	100.0	76.9
TaqMan[f]	Plasma	Plasma	176	93	83	169 (96.0)	7	0	7 (4.0)	100.0	92.2
TaqMan[f]	Plasma	DBS[b]	82	62	20	73 (89.0)	4	5	9 (11.0)	92.5	83.3
VFA[f]	Plasma	DBS	82	68	14	75 (91.5)	0	7	7 (8.5)	90.7	100.0

[a] The results for the virological failure screening assay (VFA) were compared to the results for two commercial assays, Cobas TaqMan system v2 (Roche) (TaqMan) and Cobas Amplicor HIV-1 monitor test v1.5 (Roche) (Amplicor), and a VFA performed in the field.
[b] DBS, dried blood spot.
[c] *n*, number of samples tested.
[d] True positive (True Pos.), identified by the standard assay as having ≥5.00E+03 copies/ml; true negative (True Neg.), identified by the standard assay as having <5.00E+03 copies/ml; false positive (False Pos.), identified by VFA as having ≥5.00E+03 copies/ml and as negative by the standard assay; false negative (False Neg.), identified by VFA as having <5.00E+03 copies/ml and identified as positive by the standard assay.
[e] The test was performed at UMCU, Utrecht, The Netherlands.
[f] The test was performed at JCRC laboratory sites in Uganda.

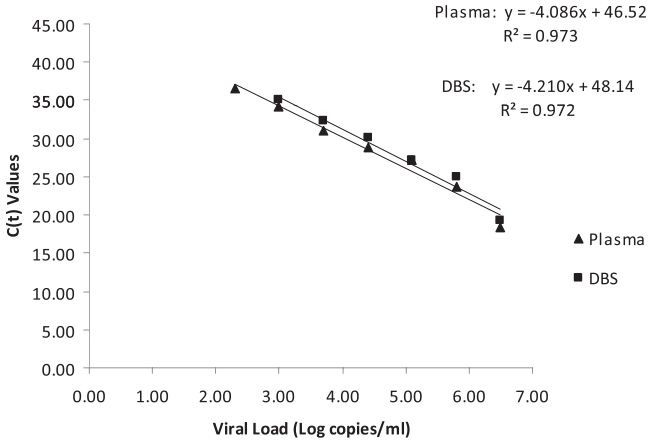

FIG 2 Standard curve of HIV-1 subtype A plasma and DBS samples determined using the ABI7500.

Results from all the JCRC laboratory sites in Uganda showed a 96.0% (169/176) comparable classification of the VFA performed compared to the commercial assay (Table 3). Seven samples (4.0%) were overestimated, and no underestimation was observed, with a specificity of 100.0% and a sensitivity of 92.2%. In addition, 100.0% of the HIV-1-negative plasma samples ($n = 25$) were not detected in the VFA.

Plasma samples versus DBS. Assay linearity and within-run precision were determined for both plasma and DBS samples for subtype A of the subtype panel by plotting mean C_T values for the serial dilutions using the ABI7500 at the UMCU, Utrecht, the Netherlands. The slope and R^2 of standard curves derived from plasma samples and DBS were highly comparable with values of −4.067 and −4.224 and values of 0.951 and 0.971, respectively. On average, the results for plasma samples were 0.32 $\log_{10}$ copies/ml lower than the results for DBS of the same dilution, while the mean SD for plasma was 0.18 versus 0.19 $\log_{10}$ copies/ml for DBS (excluding 2.0E+02 dilution) (Fig. 2).

The performance of the VFA on DBS samples was determined at the JCRC laboratory sites in Uganda. Results were compared with plasma VL results of the same sample that were previously measured with the commercial assay and the VFA. Figure 3 shows a comparison of VFA results for paired plasma and DBS ($n = 31$) samples given in log copies/ml, determined using a standard curve, compared to plasma VL results generated using TaqMan system v2 (Roche). Above 3.00 $\log_{10}$ copies/ml, it is clear to see comparable qualitative classifications between sample types and assays. In Table 3, there is a summary of qualitative results from all paired plasma and DBS samples. A total of 89.0% (73/82) of the samples compared with the TaqMan were accurately classified. Four samples (4.9%) were overestimated and five samples (6.10%) were underestimated, with a sensitivity and specificity of 92.5% and 83.3%, respectively. The DBS VFA results compared to the plasma VFA results showed a 91.5% (75/82) comparability in classification, with seven samples (8.5%) incorrectly classified, and a sensitivity and specificity of 90.7% and 100.0%. Five of the seven false-negative samples were the same samples that were false negative compared to the TaqMan plasma results, four of which had been stored at room temperature for 40, 70, 184, and 192 days. The remaining three had been stored at −70°C for 330, 455, and 486 days.

DISCUSSION

We have developed and evaluated a qualitative assay to screen for virological failure (VF) during antiretroviral treatment (ART) with particular emphasis on application in resource-limited settings (RLS). The VFA described here can assess ART adherence and inform therapy switching, earlier and with predicted better specificity than clinical and immunological monitoring. Informed therapy switching can prevent unnecessary treatment switching (4) to more expensive and less accessible second-line therapies. Moreover, early detection of VF using the VFA can prevent extended exposure to a failing regimen and possible accumulation of drug resistance mutations that may confer cross-resistance to other drugs or drug classes (8). Using the VFA for early detection of treatment failure could also prevent HIV-1 transmission (18). Viral load (VL) monitoring to determine treatment failure is recommended, including in RLS such as sub-Saharan Africa (19), and should preferably be performed according to the WHO guidelines (6), which suggest targeted use to confirm suspected clinical or immunological failure to prevent unnecessary therapy switching, or earlier use, within 4 to 6 months after ART initiation, to assess adherence and introduce adherence counseling if necessary. The assay presented here meets these WHO requirements and is suitable for use in decentralized settings with less trained medical personnel, compatible with task shifting of ART implementation.

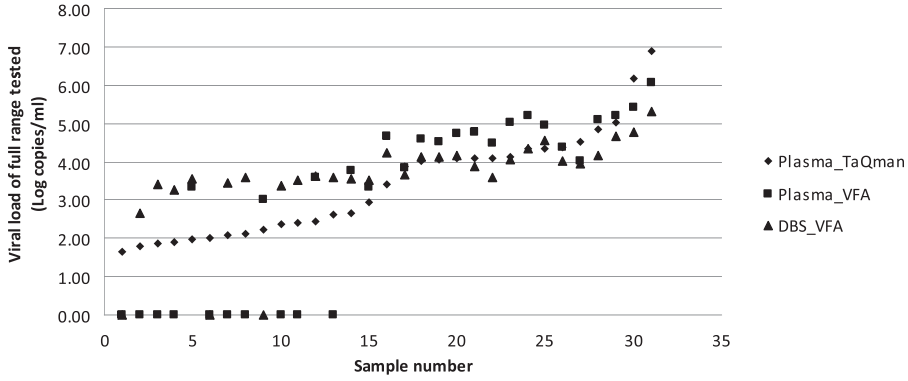

FIG 3 Comparison of viral loads obtained for plasma samples on the TaqMan system v2 (Roche) and for plasma and DBS samples for the same samples using the described VFA method.

The assay was designed as "open platform": all primer sequences and protocols are openly accessible, and the assay can use various equipment and reagents that can be ordered from multiple manufacturers. This open access and open platform approach increase the affordability and scalability of molecular diagnostics in Africa. Reagent costs for the assay are country dependent, with a per sample cost, based on a run of 10 samples, including controls, of 22.00 U.S. dollars (USD) calculated for UMCU, Utrecht, the Netherlands, and 27.00 USD for JCRC, Uganda. The Southern African Treatment Resistance Network (SATuRN) and ARTA are key supporters of this open movement. SATuRN has negotiated discounted reagents and technical support with Life Technologies in order to decrease the cost and increase access of HIV genotypic drug resistance testing in Africa (http://www.bioafrica.net/saturn/). The VF screening assay described here has the potential to use the same approach. In addition, SATuRN and ARTA have been providing extensive training on the usage of molecular diagnostics for treatment monitoring with more than 1,500 physicians, nurses, and health care workers trained in Africa. These organization training platforms can be used to expand and support the usage of this VF screening assay in Africa.

It is possible to use the VFA in a central laboratory with higher-throughput systems such as the Applied Biosystems 7500 real-time PCR system (Life Technologies), but the VFA has also shown remarkable ease of use in smaller, district laboratories with the MiniOpticon real-time PCR detection system (Bio-Rad), as we have shown in Uganda. This is important, as decentralizing VL testing enables faster turnaround times in result reporting to clinicians and their patients, and consequently more efficient treatment monitoring. In addition, a compact real-time thermocycler requires minimal maintenance due to the use of light-emitting diodes (LEDs) instead of xenon lamps and lasers, has no filter wheels, and is easily transportable. However, it has to be emphasized that the assay still requires a laboratory equipped for some molecular diagnostic techniques and staff with medium- to high-level training.

The VFA is designed to control for all steps in the laboratory procedure, ensuring quality and reliability of results. The performance of the assay has demonstrated good correlation to other available VL screening assays in this evaluation and between the two instruments and three Ugandan field sites tested. Evaluation of the VFA for plasma and DBS samples determined the lower limit of detection to be 1.00E+03 and 5.00E+03 copies/ml, respectively. Although the assay was not designed for quantitative application, assay linearity was shown to be adequate, with comparable equations for plasma samples and DBS. The assay demonstrated good intra- and interassay precision, with highly reproducible results at the 5.00E+03 copies/ml cutoff for plasma samples and DBS. The accuracy of this assay to determine VF showed good correlation with VL results previously determined using commercial VL assays.

The next step to improving access to VL monitoring and reducing associated costs in RLS is routine application with DBS sampling. The use of DBS with commercial and in-house VL assays has already been shown to have some success (20, 21). Preliminary data using spiked whole-blood samples have shown that the current VFA performs well with DBS but with reduced sensitivity compared to plasma samples, which has been previously described (20, 22). Possible reasons for this decreased sensitivity could be due to RNA degradation during storage or loss of sample due to incomplete elution from the filter paper as part of the nucleic acid isolation process. Accuracy of VF determination by commercial assays can also be affected by the DBS method of collection, specifically when blood is either collected directly from finger or heel prick or spotted with a dropper instead of a pipette from EDTA-blood. Unless blood is spotted in exact volumes, it is not possible to determine precise VL using commercial assays. A prospective clinical validation into these collection methods would be needed to investigate their effect on VF determination using the assay described here.

In summary, we have developed a robust and affordable test for VF determination that is open platform and compatible with finger or heel prick DBS collection and pediatric applications and is particularly suitable for application in RLS, such as sub-Saharan Africa. The unique aspect of the assay described here is its multiplex design enabling detection of an internal control in each sample, ensuring accurate and reliable results from isolation to amplification. The VFA could contribute to improved quality of ART and prevention of the development of HIV drug resistance. Further explorations are needed to assess the performance of this test in clinical patient management in African settings. A study along these lines has been performed in Uganda (23).

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Chapter 5

A Pragmatic Approach to HIV-1 Drug Resistance Determination in Resource-Limited Settings using a Novel RT-only Genotyping Assay.

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A Pragmatic Approach to HIV-1 Drug Resistance Determination in Resource-Limited Settings by Use of a Novel Genotyping Assay Targeting the Reverse Transcriptase-Encoding Region Only

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In resource-limited settings (RLS), reverse transcriptase (RT) inhibitors form the backbone of first-line treatment regimens. We have developed a simplified HIV-1 drug resistance genotyping assay targeting the region of RT harboring all major RT inhibitor resistance mutation positions, thus providing all relevant susceptibility data for first-line failures, coupled with minimal cost and labor. The assay comprises a one-step RT-PCR amplification reaction, followed by sequencing using one forward and one reverse primer, generating double-stranded coverage of RT amino acids (aa) 41 to 238. The assay was optimized for all major HIV-1 group M subtypes in plasma and dried blood spot (DBS) samples using a panel of reference viruses for HIV-1 subtypes A to D, F to H, and circulating recombinant form 01_AE (CRF01_AE) and applied to 212 clinical plasma samples and 25 DBS samples from HIV-1-infected individuals from Africa and Europe. The assay was subsequently transferred to Uganda and applied locally on clinical plasma samples. All major HIV-1 subtypes could be detected with an analytical sensitivity of 5.00E+3 RNA copies/ml for plasma and DBS. Application of the assay on 212 clinical samples from African subjects comprising subtypes A to D, F to H (rare), CRF01_AE, and CRF02_AG at a viral load (VL) range of 6.71E+2 to 1.00E+7 (median, 1.48E+5) RNA copies/ml was 94.8% ($n = 201$) successful. Application on clinical samples in Uganda demonstrated a comparable success rate. Genotyping of clinical DBS samples, all subtype C with a VL range of 1.02E+3 to 4.49E+5 (median, 1.42E+4) RNA copies/ml, was 84.0% successful. The described assay greatly reduces hands-on time and the costs required for genotyping and is ideal for use in RLS, as demonstrated in a reference laboratory in Uganda and its successful application on DBS samples.

There are currently more than 3.9 million HIV-1-infected individuals receiving highly active antiretroviral treatment (HAART) in sub-Saharan Africa (1), with recent studies showing an estimated prevalence of 5.6% antiretroviral (ARV) drug resistance prior to the initiation of treatment, ranging from 1.1% in South Africa to 12.3% in Uganda (2). The use of HIV-1 drug resistance (HIVDR) genotyping in resource-limited settings (RLS) is limited to clinical research studies and for preauthorized private medical care. Limited routine resistance testing is performed due to the high cost, infrastructure requirements, and the complexity of available commercial assays. The use of HIV-1 drug resistance genotyping is vital for advising policy makers on the status of HIV-1 drug resistance profiles to ensure that optimal HAART options are maintained, but it should also be considered for individualized treatment management.

Treatment monitoring of individuals receiving therapy primarily includes clinical evaluation with or without CD4 testing. When available and affordable, it is recommended to perform viral load (VL) monitoring. Current guidelines do not recommend HIV-1 drug resistance genotyping be included as part of treatment management in RLS (3), primarily due to the cost and complexity of the assays and limited ARV drug options.

Current ARV regimens for the treatment of HIV-1 in adults and adolescents in RLS, as recommended by WHO guidelines, comprise a first-line regimen consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and one nonnucleoside reverse transcriptase inhibitor (NNRTI) (3). There is limited use of protease inhibitor (PI)-containing regimens, restricted to second-line therapy, and the rate of PI drug resistance is still low (4, 5). According to a systematic review by Barth et al. on the success of ARV treatment programs in sub-Saharan Africa, 94% of African adult patients on ART received a combination of NRTI and NNRTI first-line therapy (6). When therapy failure occurs, more than 83% of subjects failing first-line regimens harbor mutations that confer resistance to both NRTIs and NNRTIs. The most common mutation observed is the M184V, followed by several NNRTI mutations, such as K103N, Y181C, and V106M (7). Complex resistance to NRTIs such as K65R, thymidine analogue mutations (TAMs), and Q151M is also observed, with frequencies increasing when viral load monitoring is not observed (7, 8).

All major mutations that affect the efficacy of RTI therapy, as defined by the International AIDS Society (IAS) drug resistance mutation list, are located between reverse transcriptase (RT) amino acids (aa) 41 and 238 (9). Furthermore, *in silico* analysis of genotyping profiles of this specific RT region demonstrated that

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TABLE 1 Summary of HIV-1 isolates in the subtype reference panel

Subtype	Strain	Country of origin	GenBank accession no.
A	UG275	Uganda	AB485632
B	BK132	Thailand	AY173951
C	ZB18	Zambia	AB485641
D	SE365	Senegal	AB485648
CRF01_AE	CM240	Thailand	AF067154
F	BZ126	Brazil	AY173957
G	BCF-DIOUM	Zaire	AB485661
H	BCP-KITA	Zaire	AB485665

the predicted drug susceptibilities were as informative as sequences that more broadly cover the RT gene (aa 1 to 400) (10). The studies therefore indicate that for HIV-1 drug resistance genotyping for individuals experiencing therapy failure while receiving the current ARV regimens in RLS, an assay that targets the RT region only would be effective for monitoring.

As part of the Affordable Resistance Test for Africa (ART-A) initiative to develop affordable resistance testing for use in Africa (www.arta-africa.org), we have used this knowledge base to design an RT-specific assay for a simplified genotyping screen. For application in RLS, the potential use of the assay on dried blood spots (DBS) was considered an important addition. This choice, in combination with a smaller fragment for PCR amplification, has enabled the development of a test that fulfils the criteria with an analytical sensitivity of 5.00E+3 RNA copies/ml at a more affordable cost. We evaluated the assay on both plasma and DBS in a WHO reference laboratory in The Netherlands and subsequently evaluated its application in a reference laboratory in Uganda.

MATERIALS AND METHODS

Samples. A selection of reference viruses for subtypes A, B, C, D, F, G, H, and circulating recombinant form 01_AE (CRF01_AE) (Table 1) from the BBI panel (BBI Biotech Research Laboratories Inc., Gaithersburg, MD) was used to evaluate the sensitivity and specificity of the assay. Virus stocks for each subtype were used to prepare dilution series of plasma and spiked whole blood for DBS preparation in concentrations of 1.00E+4, 5.00E+3, 1.00E+3, and 5.00E+2 RNA copies/ml, which were used to optimize the assay.

The assay was subsequently evaluated on a selection of clinical samples from Africa and The Netherlands that were available at the University Medical Centre (UMC), Utrecht. A total of 212 samples with a VL range of 6.71E+2 to 1.00E+7 (median, 1.35E+4) RNA copies/ml, comprising subtypes A ($n = 47$; 22.1%), B ($n = 20$; 9.4%), C ($n = 72$; 33.9%), D ($n = 18$; 8.4%), CRF01_AE ($n = 35$; 16.5%), and CRF02_AG ($n = 10$; 4.7%) and rare subtypes H, G, and F ($n = 10$; 4.7%), were used. For the clinical samples, plasma was separated from EDTA tube blood and stored at −80°C prior to analysis.

The clinical samples available at the UMC Utrecht were included to assess the assay application for various African subtypes. For these samples, the VL was determined using the COBAS AmpliPrep/COBAS TaqMan System v2 (Roche, Penzberg, Germany). These samples were obtained from two separate sources, RNA isolates previously genotyped using the ViroSeq HIV-1 genotyping system (Celera Diagnostics, Alameda, CA, USA) or with a laboratory-developed genotyping assay ($n = 88$) (11) and plasma samples from a study to monitor for the development of drug resistance mutations in children failing therapy in Rwanda ($n = 67$), which were only genotyped with the described method. In addition, clinical samples from South Africa ($n = 57$) sent for routine VL testing were included. VLs were determined in South Africa using NucliSENS EasyQ HIV-1 version 1.2 (bioMérieux, Boxtel, The Netherlands). Plasma samples were stored at −80°C and shipped on dry ice to The Netherlands.

Application of the assay with clinical DBS samples was evaluated with a selection of 25 clinical DBS samples from South Africa sent for genotyping with a laboratory-developed genotyping assay at the UMC Utrecht. Selected samples had a VL range of 1.02E+3 to 4.49E+5 (median, 1.42E+4) RNA copies/ml, and all samples were HIV-1 subtype C.

Following development and evaluation in The Netherlands, the assay was transferred to, and applied in, a reference laboratory in Kampala, Uganda. A total of 132 pretreatment baseline plasma samples from the PASER-M cohort (12) were used for further evaluation. These samples were chosen to represent a distribution of subtypes A ($n = 60$; 45.4%) and D ($n = 43$; 32.6%) and unassigned subtypes ($n = 29$; 22.0%), with a VL range of 1.05E+3 to 1.00E+7 (median, 6.92E+4) RNA copies/ml. All of these samples were isolated and amplified at the local laboratory. A selection of 50 amplicons was subsequently sequenced in order to confirm compatibility with the local sequencing method.

Nucleic acid purification. (i) UMC Utrecht, The Netherlands. Viral RNA was isolated using the manual NucliSENS miniMAG (bioMérieux). For the subtype reference panel plasma dilutions and the clinical samples, an input volume of 100 μl was used. In the case of DBS samples, two 50-μl spots were used as input material. Prior to processing, the excised spots were incubated in 2 ml NucliSENS lysis buffer (bioMérieux) at room temperature for 30 min with gentle shaking, after which the filter papers were removed and the isolation proceeded according to the manufacturer's instructions. Isolated RNA was eluted in 50 μl elution buffer and was either used immediately for RT-PCR ($n = 67$) or stored at −20°C for 50 to 120 weeks ($n = 145$) prior to amplification. For each isolation, a positive and a negative control were used, consisting of subtype C virus from the subtype panel (2.00E+4 RNA copies/ml) and negative plasma, respectively.

(ii) Joint Clinical Research Centre, Kampala, Uganda. Viral RNA was isolated using the QIAamp viral RNA minikit (Qiagen GmbH, Germany). For the clinical samples, 100 μl of plasma was used as the input volume. Isolated RNA was eluted in 50 μl elution buffer and was either used immediately for RT-PCR amplification or stored at −20°C for up to 4 weeks. For each isolation, a positive and a negative control were used, consisting of HIV-1 subtype C virus from the subtype panel and nuclease-free water, respectively.

Amplification (The Netherlands and Uganda). Isolated viral RNA was reverse transcribed and amplified using a single-round RT-PCR encompassing the RT gene from aa 41 to 238. The RT-PCR was performed using the SuperScript III-one-step RT-PCR system with Platinum Taq high-fidelity polymerase (Life Technologies, Foster City, CA) as follows. Ten microliters of isolated RNA was amplified in a reaction mixture consisting of 0.4 μM forward primer, 0.4 μM reverse primer (Table 2), 2×

TABLE 2 Amplification and sequencing primers

Primer	Position[a]	Length (nt[b])	Sequence	Application
ART-A_2611_M13-40	2611–2635	42	5′-GTTTTCCCAGTCACGACTTAAACAATGGCCATTGACAGAAGA-3′	Forward amplification
ART-A_3349_SR	3370–3348	23	5′-ATCCCTGSRTAAATCTGACTTGC-3′	Reverse amplification/sequencing
M13-40		17	5′-GTTTTCCCAGTCACGAC-3′	Forward sequencing

[a] According to HIV-1 HXB2.
[b] nt, nucleotides.

TABLE 3 Assay sensitivity determined using a dilution series performed in duplicate using isolates from the subtype panel

Subtype	Sensitivity[a] at a dilution (RNA copies/ml) of:			
	10,000	5,000	1,000	500
A	++	++	++	+−
B	++	++	++	++
C	++	++	++	−+
D	++	++	++	−+
F	++	++	++	++
G	++	++	++	++
H	++	++	++	++
CRF01_AE	++	++	++	−+

[a] +, positive amplification; −, negative amplification.

reaction buffer, 0.5 μl SuperScript III RT/Platinum Taq high-fidelity enzyme mix, and nuclease-free water in a final volume of 25 μl. The combined cDNA and amplification reactions were carried out in a GeneAmp 2720 thermal cycler (Life Technologies) under the following conditions: 1 hold at 50°C for 30 min, 1 hold at 94°C for 2 min, 2 cycles at 94°C for 15 s, 61°C for 30 s, and 68°C for 1 min, 14 cycles at 94°C for 15 s, 60°C (with a decrease of 0.5°C with every cycle) for 30 s, and 68°C for 1 min, 34 cycles at 94°C for 15 s, 53°C for 30 s, and 68°C for 1 min, and a final extension step at 68°C for 7 min.

Amplification products were visualized on an agarose gel, and positive reactions were purified using QIAquick PCR purification columns (Qiagen GmbH, Germany) according to the manufacturer's instructions.

Sequence analysis. (i) Applied Biosystems-based sequencing, UMC Utrecht, The Netherlands. The cycle sequencing master mix for each primer consisted of 0.2 μM primer (Table 2), 1× BigDye sequencing buffer, 10 to 40 ng of PCR product, 2 μl BigDye v3.1, and nuclease-free water in a final volume of 20 μl. The cycle sequencing reactions were performed using a GeneAmp 2720 thermal cycler and consisted of 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The reaction products were purified using an EDTA-ethanol precipitation, subsequently run on an ABI 3730 automated sequencer (Life Technologies), and analyzed using SeqScape data analysis software v2.6 (Life Technologies).

(ii) Beckman Coulter-based sequencing, Joint Clinical Research Centre, Kampala, Uganda. The cycle sequencing master mix for each primer consisted of 0.2 μM primer (Table 2), GenomeLab DTCS quick start kit mix (Beckman Coulter, Inc., Brea, CA), 10 to 40 ng of PCR product, and nuclease-free water in a final volume of 20 μl. The cycle sequencing reactions were performed using a 9800 Fast thermocycler (Life Technologies) and consisted of 30 cycles at 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min. Reaction products were purified using an EDTA-ethanol precipitation, subsequently read on a CEQ800 genetic analysis system (Beckman Coulter), and analyzed using BioEdit v7.0 (13).

(iii) Phylogenetic analysis. Consensus sequences were aligned using MEGA 4.1 software (http://www.megasoftware.net/) to control for contamination. To determine the subtype and the HIV-1 drug resistance (HIVDR) profile, the NCBI subtyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and GRADE analysis programs (http://www.hiv-grade.de/grade/deployed/grade.pl?program=hivalg) were used, respectively. Consensus sequences, obtained as described above, were compared at the nucleotide level to sequences for the same samples generated using both the ViroSeq and an in-house genotyping assay (11) for quality control purposes.

RESULTS

Assay design. Based on the optimal RT amplicon to contain all the RT resistance mutations that are relevant after an RT-based first-line failure, a single-round RT-PCR assay targeting RT aa 41 to 238 was developed. Primers were designed using a database of 267,841 sequences from nine subtypes and seven CRFs in order to have optimal sensitivity and specificity for all major HIV-1 subtypes and CRFs.

Nucleic acid amplification (RT-PCR sensitivity). The assay was optimized for the amplification of plasma samples containing ≥5.00E+3 RNA copies/ml. The initial amplification of a dilution series of the subtype panel indicated that amplification from plasma virus was achievable from 5.00E+2 RNA copies/ml and was reproducibly achievable for samples with ≥5.00E+3 RNA copies/ml (Table 3). The results of the dilution series of DBS samples for the subtype panel indicated reliable amplification of samples with ≥5.00E+3 RNA copies/ml. All subtypes were amplified and sequenced with equal sensitivities and success rates.

Clinical samples from UMC Utrecht, The Netherlands. A total of 205 of 212 clinical samples (96.7%) were successfully amplified using the single-round RT-PCR. Of the 205 successfully amplified samples, full bidirectional sequencing of RT aa 41 to 238 (Table 4) was obtained 98% of the time (201 samples), giving an overall genotyping success rate of 94.8% (201/212). Of the seven samples that did not amplify, five had a VL of <5.00E+3 RNA copies/ml, all subtype C, and the remaining two were subtypes C and D, with VLs of 6.30E+3 and 8.78E+3 RNA copies/ml, respectively. Four samples resulted in incomplete sequences for subtypes A ($n = 1$), C ($n = 2$), and D ($n = 1$).

The sequences generated with the described method were compared to the sequences previously generated ($n = 30$), using either ViroSeq or the in-house assay, and demonstrated average homologies of 98.9% and 99.3% at the nucleotide level, respectively. Sequences from a total of 73 samples were compared to

TABLE 4 Amplification and sequencing results of clinical samples tested at the UMC Utrecht, The Netherlands

VL[a] range (RNA copies/ml)	No. of samples	No. of samples of the following subtypes[b]:							Success rate (no. of successes/total no. [%]) of:	
		A	B	C	D	AE	AG	Rare	Amplification	Bidirectional sequencing
>125,000	35	10	6	1	2	12	2	2	35/35 (100)	35/35 (100)
25,000–125,000	45	12	2	8	7	9	3	4	45/45 (100)	44/45 (97.8)
5,000–25,000	89	14	8	47	3	10	5	2	87/89 (97.8)	84/87 (96.6)
1,000–5,000	43	11	4	16	6	4	0	4	38/43 (88.4)	38/38 (100)
Total	212	47	20	72	18	35	10	12	205/212 (96.7)	201/205 (98.1)

[a] VL, viral load.
[b] Indicated subtypes as assigned by the NCBI and GRADE.

TABLE 5 Amplification results of clinical samples tested at the Joint Clinical Research Centre, Kampala, Uganda

VL[a] range (RNA copies/ml)	No. of samples	No. of samples			Amplification success rate (no. of successes/ total no. [%])
		A[b]	D[b]	X[c]	
>125,000	48	23	14	11	48/48 (100)
25,000–125,000	36	14	13	9	36/36 (100)
5,000–25,000	31	19	11	1	31/31 (100)
1,000–5,000	17	4	5	8	15/17 (88.2)
Total	132	60	43	29	130/132 (98.5)

[a] VL, viral load.
[b] Indicated subtype previously assigned.
[c] Unknown subtype, not previously sequenced.

those from the in-house assay alone and demonstrated a 99.2% homology. The differences observed were almost entirely due to mixture calling (347/43,362 positions), with only six positions at which the nucleotides were different, all at nonresistance positions.

For the selection of 25 clinical DBS samples genotyped with the described method, the amplification success rate was 95.0% for samples with >5.00E+3 RNA copies/ml (19/20) and 88.0% for samples with >1.00E+3 RNA copies/ml (22/25). The sequencing was 95.5% successful for the amplified samples (21/22). The overall genotyping success rate was 84.0% (21/25). The generated sequences showed 97.0% nucleotide homology to the previously generated sequences from plasma.

Field implementation in Kampala, Uganda. A total of 130 of 132 local plasma samples (98.5%) were amplified using the described method (Table 5) with the alternative viral RNA isolation method. The samples that did not provide a positive amplification result had <5.00E+3 RNA copies/ml; however, amplification of lower-VL samples ranging from 1.05E+3 to 4.92E+3 (median, 2.12E+3) RNA copies/ml was also successful (15/17 samples; 88.2%). Using the Beckman Coulter sequencing method was successful, with full bidirectional sequences obtained for the 50 amplicons processed.

Cost. The cost of this assay is lower than that of other laboratory-developed assays and commercially available genotyping assays due to the limitation of the amplification and sequencing regions to the minimal region required for sequencing of first-line RTI therapy in RLS. The use of a single-round RT-PCR and reduced amplification and elution volumes and the need for only two sequencing reactions equate to a reduction in reagents required. From a reagent perspective, using the described assay would result in a >75% savings compared to using a commercial assay such as the ViroSeq genotyping system version 2.0 (Celera Diagnostics, USA) and an approximate 40% savings compared to using our current in-house assay (11). Furthermore, the quicker laboratory protocol and shorter sequence to be analyzed result in a decrease in the labor required compared to currently available methods (11, 14, 15).

DISCUSSION

We have developed a simplified, specific, lower-cost assay for the determination of HIV-1 drug resistance associated with first-line therapy that can be performed at reference laboratories in RLS and is suitable for use with DBS.

The unique feature of this assay which sets it apart from currently available commercial and laboratory-developed assays is the one-step RT-PCR specifically focusing on the analysis of the most relevant part of RT. The size of the amplicon is roughly half the length of those generated using commercial and in-house HIV-1 drug resistance genotyping assays (11, 15, 16). By amplifying as little as possible of the RT using highly fine-tuned primer combinations, focusing on the region encompassing all of the relevant HIV-1 drug resistance mutations, it was possible to achieve a genotyping success rate of 94.8% for clinical plasma samples with ≥1.00E+3 RNA copies/ml in a single-round RT-PCR. Subsequent sequencing requiring only a single forward and a single reverse primer, compared to the four to six primers needed for commercial and in-house assays (11, 14–16), increases the throughput for processing and decreases the analysis time per sample. In turn, decreasing the number of reactions required decreases the overall cost of the assay and minimizes the hands-on time, contamination risk, and turnaround time.

The described assay has been specifically designed with several key features for use in RLS. The primers were carefully designed and selected to cover all major HIV-1 group M subtypes and CRFs, as shown in Tables 3, 4, and 5. The nonnested approach to amplification strongly reduces the risk for sample contamination. The reduced number of reactions required for amplification and sequencing ensures the efficient use of reagents and allows for greater sample throughput. To demonstrate these principles, the assay was transferred to, and applied in, a reference laboratory in Kampala, Uganda. The results showed a very high success rate for amplification, with 99% (n = 130/132) amplification success for samples with a VL of >1.00E+3 RNA copies/ml.

Genotyping from DBS has become a popular sampling method used to overcome the impediments associated with the cost and logistics of transport and storage of plasma in RLS, and it has been shown to give comparable results from plasma (16–18). Recently, the WHO identified DBS as the primary sample type for genotyping in RLS (19). The described RT-specific genotyping assay using a DBS dilution series and application with clinical DBS samples show that this method displays adequate sensitivity for use with DBS samples, with 95% and 84% genotyping success rates for samples with VLs of ≥5.00E+03 and ≥1.00E+3 RNA copies/ml, respectively. Research is under way in Uganda and South Africa as part of the ART-A project to demonstrate the scalability of HIV-1 drug resistance genotyping using DBS sampling. In this approach, DBS samples are collected from HIV-1-infected individuals and sent to a reference laboratory to screen for virological failure. Samples that test positive for treatment failure, classified as having a VL of >5.00E+3 RNA copies/ml (3), are selected for HIV-1 drug resistance genotyping from the same DBS sample using the described assay.

HIV-1 drug resistance testing for individual patient management is currently not recommended in RLS, mostly due to cost limitations. However, targeted monitoring and surveillance of HIV-1 drug resistance on sentinel populations are increasingly mentioned as a necessity for guiding national ART programs (2). The potential use of this test in DBS-supported applications would markedly increase its application in remote settings. This, in combination with the ability to transport specimens at an ambient temperature to a centralized reference laboratory where actual HIV-1 drug resistance genotyping is performed, will contribute to more affordable population-level HIV-1 drug resistance

data collection, which is necessary for keeping national ART programs effective in the long term.

In conclusion, we have designed and tested a simplified method for HIV-1 RT drug resistance genotyping. The sensitivity, broad subtype inclusivity, and compact nature of this assay make it ideal for HIV-1 drug resistance testing in RLS. The described assay generates the most vital information necessary at a lower cost and in a shorter time compared to currently available HIV-1 drug resistance genotyping assays.

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Chapter 6

A comparative analysis of HIV drug resistance interpretation based on short reverse transcriptase sequences versus full sequences.

Steegen K, Bronze M, Van Craenenbroeck E, Winters B, Van der Borght K, Wallis CL, Stevens W, Rinke de Wit TF, Stuyver LJ, the ART-A consortium.

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AIDS RESEARCH AND THERAPY

RESEARCH Open Access

A comparative analysis of HIV drug resistance interpretation based on short reverse transcriptase sequences versus full sequences

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Abstract

Background: As second-line antiretroviral treatment (ART) becomes more accessible in resource-limited settings (RLS), the need for more affordable monitoring tools such as point-of-care viral load assays and simplified genotypic HIV drug resistance (HIVDR) tests increases substantially. The prohibitive expenses of genotypic HIVDR assays could partly be addressed by focusing on a smaller region of the HIV reverse transcriptase gene (RT) that encompasses the majority of HIVDR mutations for people on ART in RLS. In this study, an *in silico* analysis of 125,329 RT sequences was performed to investigate the effect of submitting short RT sequences (codon 41 to 238) to the commonly used virco®TYPE and Stanford genotype interpretation tools.

Results: Pair-wise comparisons between full-length and short RT sequences were performed. Additionally, a non-inferiority approach with a concordance limit of 95% and two-sided 95% confidence intervals was used to demonstrate concordance between HIVDR calls based on full-length and short RT sequences.
The results of this analysis showed that HIVDR interpretations based on full-length versus short RT sequences, using the Stanford algorithms, had concordance significantly above 95%. When using the virco®TYPE algorithm, similar concordance was demonstrated (>95%), but some differences were observed for d4T, AZT and TDF, where predictions were affected in more than 5% of the sequences. Most differences in interpretation, however, were due to shifts from fully susceptible to reduced susceptibility (d4T) or from reduced response to minimal response (AZT, TDF) or vice versa, as compared to the predicted full RT sequence. The virco®TYPE prediction uses many more mutations outside the RT 41-238 amino acid domain, which significantly contribute to the HIVDR prediction for these 3 antiretroviral agents.

Conclusions: This study illustrates the acceptability of using a shortened RT sequences (codon 41-238) to obtain reliable genotype interpretations by virco®TYPE and Stanford algorithms. Implementation of this simplified protocol could significantly reduce the cost of both resistance testing and ARV treatment monitoring in RLS.

Introduction

In most developed countries, HIV treatment monitoring guidelines recommend regular viral load (VL) testing and HIV drug resistance (HIVDR) testing in the case of virologic failure and prior to treatment initiation [1,2]. In contrast, current clinical practice in resource-limited settings (RLS) is predominantly based on clinical staging and/or CD4 measurements [3]. However, the latest WHO recommendations promote strategic introduction of VL monitoring as well as greater access to CD4 testing for treatment initiation [4]. In 2003 WHO and UNAIDS initiated a public health approach to HIV management by recommending standardized antiretroviral (ARV) treatment regimens in order to improve the access to HIV treatment in RLS [5]. This approach has been successful and the number of patients on treatment in low- and middle-income countries has since increased 10-fold to more than 4 million at the end of 2008 [6]. Despite these joint efforts, laboratory tools to monitor patients on treatment are still lacking in many

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parts of the world, due to the lack of infrastructure and financial resources.

Several studies have shown that CD4 measurements are inaccurate in predicting treatment failure [7-11], which has resulted in the aforementioned WHO recommendations. Therefore it is of utmost importance to develop simple and affordable alternatives to the currently available VL and HIVDR tests that could be better implemented in RLS. In the context of these challenges a public-private consortium, aiming to bring an affordable HIV monitoring algorithm to Africa (ART-A: affordable resistance testing for Africa) was established in 2008 with partners in South-Africa, Luxembourg, the Netherlands and Belgium [12]. The overall aim of the ART-A project is to develop a more affordable HIV treatment monitoring system which can be universally applied for both individual patient management and public health purposes. In order to achieve this, the project will look at the use of dried blood spots and combine this with a cost-effective qualitative VL testing and subtype-independent confirmatory HIVDR genotyping with automated base-calling software to reduce operator errors in identifying pure mutations and mixture mutations. One strategy to reduce the costs of HIVDR testing is to focus on a partial region of the HIV-1 reverse transcriptase (RT) from codon 41 to 238. This region covers all HIVDR mutations recognized by the IAS [13]. This approach can be justified because 98% of the patients on treatment in RLS receive a first-line drug regimen based on RT-inhibitors only [6]. Moreover, the mutations, commonly present in patients failing a first-line drug regimen in RLS (M41L, D67N, K65R, K70R, K103N, V106A/M, Y181C, M184V, G190A, L210W, T215Y/F and K219Q/E) are all present in the shorter RT sequence [8,14-17].

In this study, the potential effect on the prediction of HIVDR by submitting a short RT sequence from amino acid 41 to 238 to the virco®TYPE and Stanford resistance interpretation algorithms was investigated through an *in silico* analysis. It was not our intention to compare the performance of virco®TYPE versus Stanford.

Materials and methods

Amplification of a short RT sequence useful in HIVDR testing

As of today, HIV resistance testing is based on amplifying and sequencing of the viral protease and reverse transcription genes. This requires multiple rounds of amplification and at least 6-8 sequencing reactions. For RLS, we assumed that a cost-reduction could be implemented by sequencing a short RT region. Amplification of this short RT region (codon 41-238) is feasible using a one-step single round amplification followed by a simplified sequencing protocol. Proof of principle for this cost-reduction approach is available [18].

Virco database analysis

A total of 125,323 full length RT sequences (codon 1-400) were retrieved from the Virco database. For all these sequences, virco®TYPE interpretations were generated for the paired full-length RT (codon 1-400) and short RT sequences (codon 41-238) on 8 FDA-approved RT inhibitors commonly used in RLS [6] (lamivudine = 3TC, abacavir = ABC, zidovudine = AZT, stavudine = d4T, didanosine = ddI, tenofovir = TDF, efavirenz = EFV and nevirapine = NVP). A similar approach on non-B subtypes (n = 17,131) was used for the Stanford HIVDR interpretation algorithm.

virco®TYPE HIVDR interpretation tool

virco®TYPE calculates the phenotypic drug susceptibility from a genotype, based on a linear regression model [19]. The phenotypic drug susceptibility is expressed as a fold change (FC) i.e. the ratio of inhibitory concentration 50% (IC_{50}) of a patient-derived sample to the IC_{50} value of a reference strain (IIIB). *virco®TYPE* provides a data-driven identification of mutations affecting FC and the magnitude of their effect [19]. The calculated FC per drug is interpreted using cut-off values. The virco®-TYPE report uses clinical cut-offs (CCOs), where available [20]. Clinical cut-offs are used to facilitate the interpretation of fold change and drug resistance. They represent thresholds on the fold change continuum to indicate loss in clinical drug activity due to resistance. These cut-offs are determined based on observational studies in treated patients. When the calculated FC falls below the lower CCO, a maximal response (MA) to treatment with that drug is predicted, whereas a minimal response (MI) is expected if the FC falls above the higher CCO value. A calculated FC that falls between the lower and higher CCO predicts reduced response (RE). When CCOs are not available for a particular drug (EFV and NVP), biological cut-offs (BCOs) are used. A biological cut-off is based on laboratory observations of viruses derived from treatment naïve patients, and gives an indication of the normal range of in vitro susceptibility of wild-type viruses. The virus is predicted to be susceptible (S) or resistant (R) to a specific drug when the calculated FC is below or above the BCO, respectively [21].

In this analysis virco®TYPE VPT4.3.00 was used, with the clinical and biological cut-offs currently in use on the virco®TYPE report [20]. The optimal sequence length for virco®TYPE analysis is from codon 1 to 99 of the protease region and from codon 1 to 400 of the RT region. The minimal accepted sequence lengths are

from codon 10 to 95 and from codon 41 to 238 for protease and RT respectively. Any missing sequence length should be filled with "***" or a reference strain sequence. The virco®TYPE linear regression model then calculated the resistance profile.

In this study the output from full RT sequences (codon 1-400) were compared to the resistance prediction of short RT sequences (codon 41-238), whereby the protease gene and RT codon 1-40 were replaced by the HXB2 reference strain sequence.

Stanford HIVDR interpretation tool

The Stanford HIV database interpretation algorithm is a qualitative HIVDR interpretation tool that assigns a mutation penalty score to each HIV mutation that is, according to published studies, associated with drug resistance [22]. The total score for a drug is derived by adding up the scores of each mutation associated with HIVDR to that drug. The interpretation tool subsequently reports one of the following levels of inferred drug resistance: susceptible (S), potential low-level resistant (pLR), low-level resistant (LR), intermediate resistant (I) and high-level resistant (R) [22]. To simplify the analysis, pLR was regarded as susceptible and LR was interpreted as intermediate. Stanford algorithm version 5.0.0 was used in this analysis.

In contract to virco®TYPE, Stanford has no restrictions on the sequence length input. For the Stanford analysis the output from full RT sequences (codon 1-400) were compared to the resistance prediction of short RT sequences (codon 41-238).

Pair wise comparisons between HIVDR calls generated from full RT and short RT

A pair wise comparison of the predicted HIVDR profile (or resistance call) for each full-length and short RT sequence pair was performed for both the virco®-TYPE and Stanford HIVDR interpretation algorithms. Changes in resistance calls between the full-length RT and the short RT sequence were categorized in major and minor call changes. Major HIVDR call changes are defined as a switch from S to R and MI to MA, or vice versa. Minor call changes include a switch from RE to MA, RE to MI, I to R and I to S, or vice versa (Figure 1). A non-inferiority approach with a concordance limit of 95% and two-sided 95% confidence intervals was used to show if at least 95% of the HIVDR calls based on the short RT sequence (codon 41-238) were concordant with HIVDR calls based on the standard RT sequence (codon 1-400).

Results

The dataset used for this analysis contained 125,329 RT sequences. Only HIV subtypes with at least 500 sequences in the database were included for analysis. The majority of sequences were derived from subtype B viruses (n = 108,198), but other non-B subtypes were also represented (n = 17,131). An assortment of 'sensitive' (S or MA) and 'resistant' (RE, MI and R) profiles towards different drugs was observed. The majority of the subtype B sequences were susceptible to RT inhibitors ranging from 52.6% (ABC) to 72.3% (d4T). Due to the delayed introduction of ART in RLS, the proportion of 'sensitive' profiles among the non-B subtypes is higher with the exception of the rare subtypes F1 and CRF12_BF. For the latter two subtypes, specific collaborations had been set up to obtain resistant viruses to enrich the Virco database. A descriptive dataset distribution is depicted in Figure 2.

The HIVDR call changes between full RT and short RT were analyzed per drug in two groups: group 1: sequences that were attributed a 'susceptible' profile (MA or S), based on virco®TYPE analysis of the full RT sequence; and group 2: sequences that were attributed a 'resistant' profile (RE, MI or R), based on virco®TYPE analysis of the full RT sequence.

Sequences interpreted by virco®TYPE

The *virco®TYPE* interpretation based on a full length RT sequence (codon 1-400) was compared to the prediction based on the shortened RT sequence (codon 41-238).

Figure 3a shows that in the 'susceptible' group (group 1) the minor call changes remained below 2%, when all subtypes were pooled together. Subtype-specific analysis demonstrated that at least 95% of HIVDR call-concordance was observed for the majority of the drugs with the exception of d4T. However, across the different drugs, subtype F1 showed a higher proportion of minor call changes, ranging from 3.2% for AZT to 8.0% for d4T. Across subtypes, most minor changes were observed for d4T, ranging from 1.3% for subtype B to 9.1% for subtype A1.

Less than 1.3% major call changes were detected when all sequences from the 'susceptible' group were analyzed. However, 2.6% of the subtype G sequences showed major changes for EFV (Figure 3b).

The analyses for subtype F1 (3TC, ABC and TDF) and subtype G (d4T) were inconclusive. This can be explained by the smaller sample size for subtype F1 (N = 745) and G (N = 560) as compared to the other subtypes (N >1000).

In the other analyses, comparisons between the HIVDR calls based on short and full length RT sequences were concordant in at least 95% of the cases, except for d4T in subtype A1, C, CRF01_AE and F1, with concordance values of 90.87% (95% CI 90.25-91.49%), 94.58% (95% CI 94.30-94.86%), 94.25% (95% CI 93.78-94.72%), 92.02% (95% CI 90.80-93.25%)

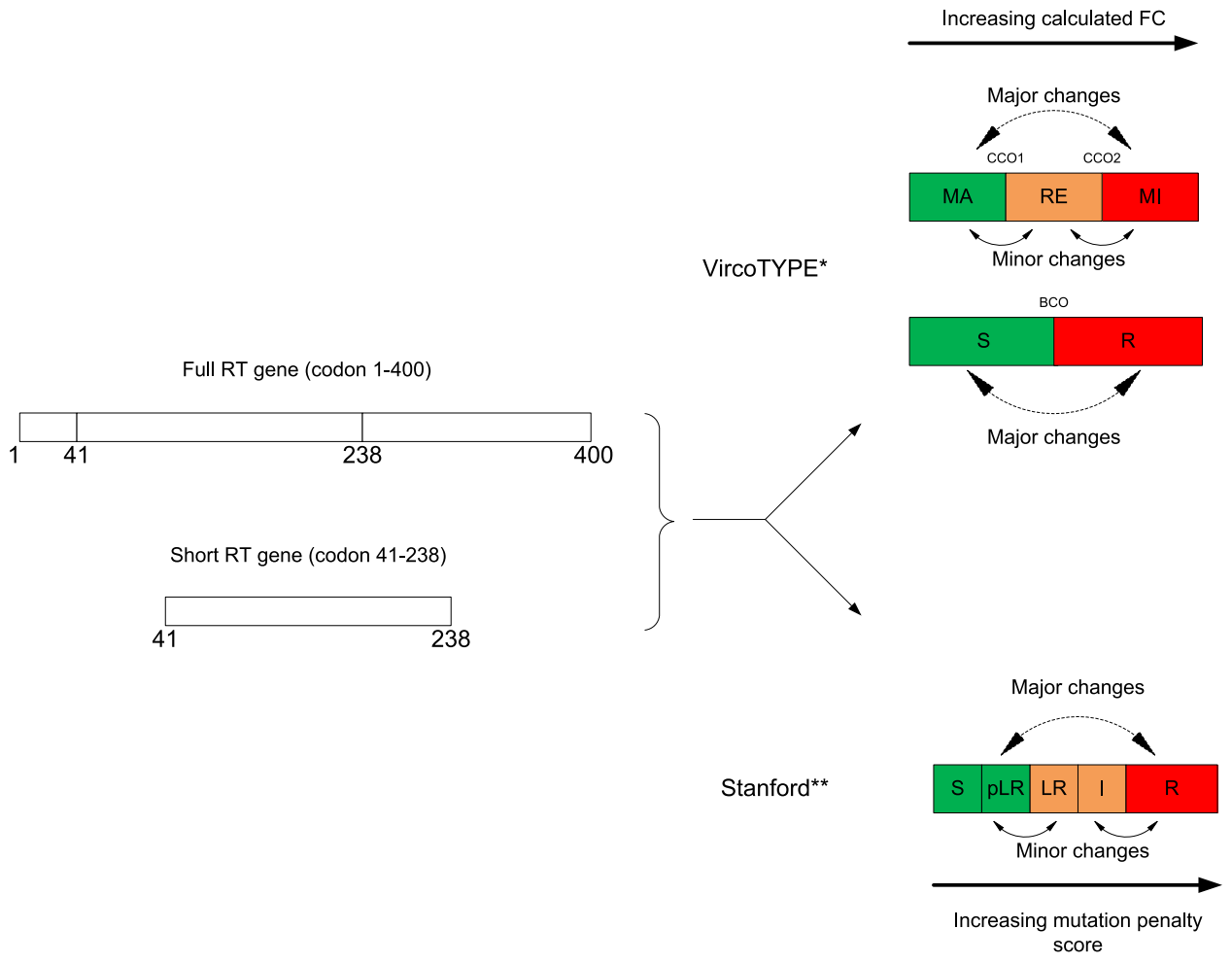

Figure 1 Representation of the definition of minor and major changes in predicted HIVDR calls between full RT and short RT sequences. *MA: maximal response; RE: reduced response; MI: minimal response; CCO1: lower clinical cut-off; CCO2: upper clinical cut-off; S: susceptible; R: resistant; BCO: biological cut-off . **S: susceptible; pLR: potential low-level resistant; LR: low-level resistant; I: intermediate resistant; R: high-level resistant.

respectively. Of note, all discordances were caused by minor call changes.

As expected, the proportion of call changes increased in the group of 'resistant' samples (group 2), see Figure 4. Overall, there were fewer than 12.6% minor call changes but subtype-specific call changes of up to 19.6% were detected for AZT on subtype G sequences (Figure 4a).

The highest number of major call changes in group 2 were seen among the subtype D samples for NVP (2.7%) and EFV (6.3%), see Figure 4b for more details.

Due to small sample sizes the analyses were inconclusive for the following groups: d4T (subtype A1, D and F1), subtype G (ABC) and CRF01_AE (ABC). Non-inferiority analysis in the remaining groups revealed an inferior HIVDR prediction when using short RT sequences for ABC in subtype A1 sequences, d4T in CRF01_AE, EFV in subtype D, AZT and TDF for all subtypes. As previously observed, all discordances were caused by minor call changes, with the exception of TDF on subtype A1 and B sequences, whereby just a small subset of call changes was of the major type (0.22% and 0.02%, respectively).

Sequences interpreted by Stanford

The Stanford HIVDR interpretation algorithm was applied only to the non-B sequences (n = 17,131). Neither minor nor major call changes were observed for 3TC, ABC, d4T ddI and TDF. The HIVDR calls for the remaining drugs (AZT, EFV and NVP) changed only in a few cases, with all changes being minor. For AZT, 7 sequences (0.04%) gave a different result when the short RT sequence was submitted to Stanford. The HIVDR level only changed in two sequences (0.01%) for EFV and in 13 sequences (0.08%) for NVP.

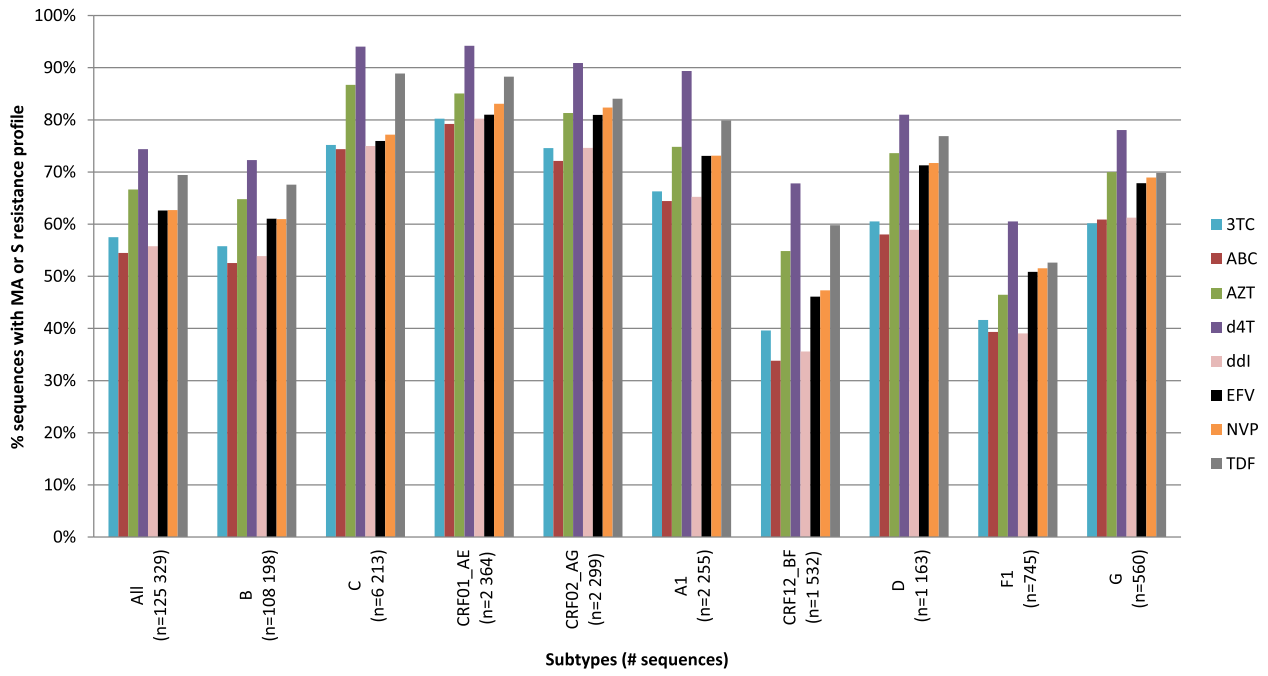

Figure 2 Dataset, based on subtype and drug-specific full-length RT HIVDR profile by virco®TYPE. The subtypes are arranged by decreasing prevalence of in the Virco database. MA: maximal response, S: susceptible.

Discussion

There is an increased need for affordable and robust HIV monitoring tools in RLS, including point-of-care VL assays and simplified HIVDR testing protocols. Attempts are being made to simplify currently available technologies in order to make them more accessible for RLS. This study evaluated the use of reducing the sequence length used to interpret HIVDR patterns.

The use of the shorter RT sequences in the virco®-TYPE HIVDR interpretation tool was not inferior to the full RT sequence for most drugs. An inferior HIVDR interpretation in more than 5% of the cases was detected only for d4T (subtype A1, C, F1 and CRF01_AE) in the group of 'sensitive' sequences. These HIVDR interpretation changes were caused by minor changes: from fully susceptible as predicted by the full RT sequence to reduced susceptibility as predicted by the short RT sequence. Moreover, recent WHO treatment guidelines recommend to phase out the use of d4T as preferred component of first-line treatment [4]. Therefore the clinical impact of HIVDR interpretation for d4T will be limited. In the 'resistant' group, the HIVDR prediction for AZT and TDF is of concern, as more than 5% of the sequences yielded a different HIVDR call for all subtypes when the short RT sequence was submitted to virco®TYPE. However, all call changes were minor (from 'reduced response' to 'minimal response', or vice versa), except for TDF for subtype A1 and B samples (0.22% and 0.02% major call changes, respectively). It is therefore unlikely that these HIVDR interpretation changes will have a major clinical impact. Because there is no clinical cut-off available for the NNRTIs NVP and EFV, only major call changes could be observed. The resistance call changes for those two drugs were in most cases limited to less than 3%, which is under our 5% cut-off. Moreover, the clinical relevance for the resistance prediction of these drugs is limited because they are not recommended in second line regimens.

When the sequences were submitted to Stanford, call changes were observed in less than 0.08% of the cases for AZT, EFV and NVP, showing no inferiority of using the short sequence in any of the non-B subtypes (B sequences were not analyzed). Observed HIVDR interpretation differences between full RT and shortened RT sequences in virco®TYPE can be explained by the fact that the Virco algorithm includes resistance weight factors for a substantial number of codon positions outside the RT codon 41-238 region which are depicted in Table 1. The Stanford algorithm is based on mutations that all lie within the region of RT codon 41 to 238, except for 333D, 333E and 318F, (see Table 1). The latter three mutations influence HIVDR only towards AZT, EFV and NVP and were only present in 2% of the non-B subtypes (405/17,131). One could argue that part of the observed differences

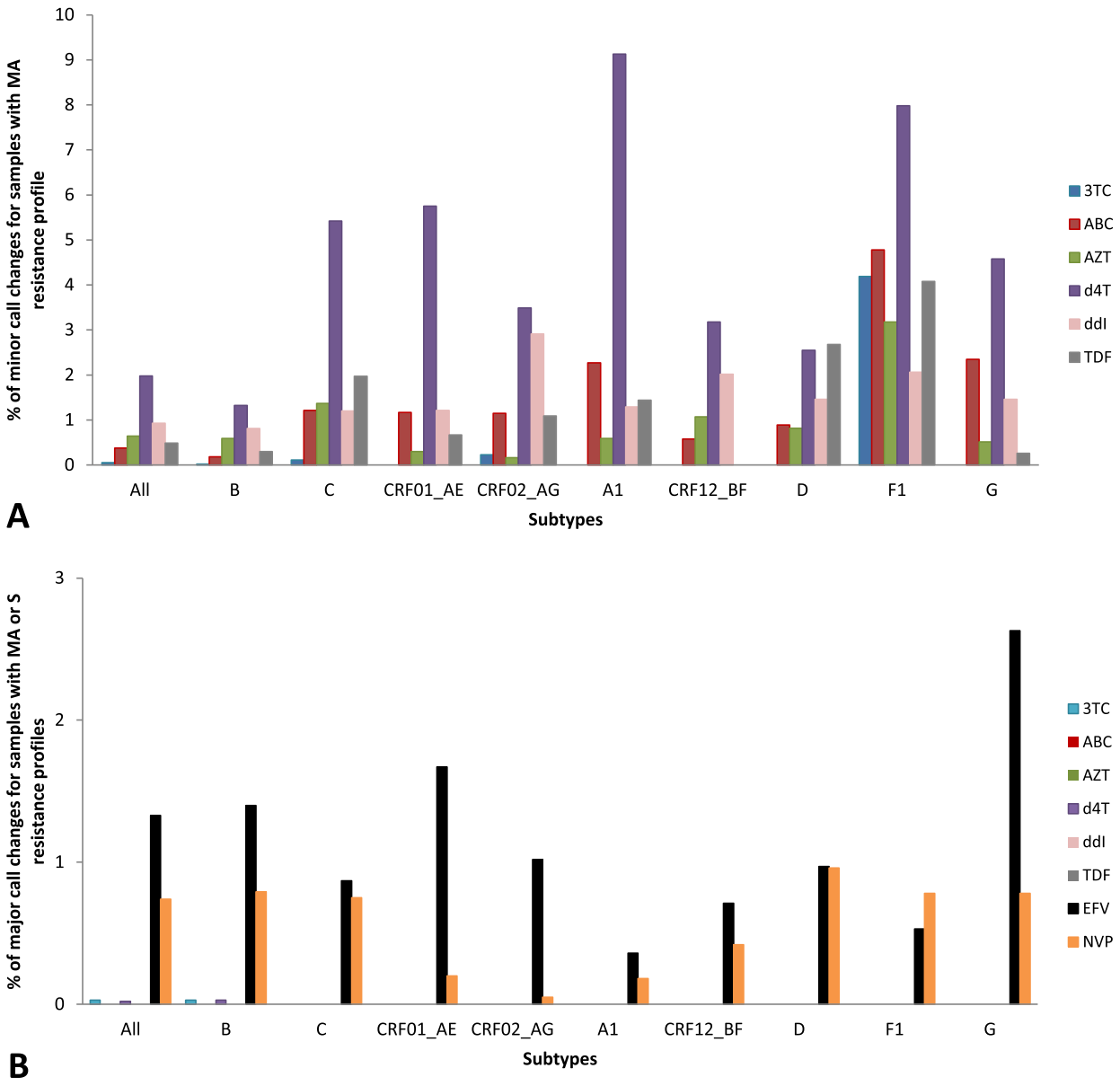

Figure 3 virco®TYPE call changes between full length and short RT HIVDR interpretations for sequences with a 'susceptible' profile, based on full RT interpretation (group 1). A. Minor call changes. Minor changes are not possible for drugs with a BCO only as a shift can only occur from susceptible to resistant (or vice versa), which is a major call change; therefore EFV and NVP are not depicted in this graph. B. Major call changes MA: maximal response, S: susceptible.

between Stanford and virco®TYPE could be explained by the fact that the reference strains, used in Stanford and virco®TYPE, are different (consensus B versus HXB2 respectively). However, both reference strains only differ from each other at four positions (codon 122, 214, 376 and 400). Moreover, in most cases, resistance mutations at those four positions would be picked up by both algorithms as they are different from the reference amino acids found in either HXB2 or the consensus B sequence.

Overall this study shows that the use of a shorter RT sequence genotype results in >95% concordance with results obtained from full length RT sequences obtained from two routinely used interpretation systems, virco®-TYPE and Stanford. The results provide initial validation that the simpler shorter genotype can be considered for use in a new ARV-treatment monitoring system for use in RLS.

Nevertheless, this study also has some limitations. Firstly, despite a good representation of non-B subtypes

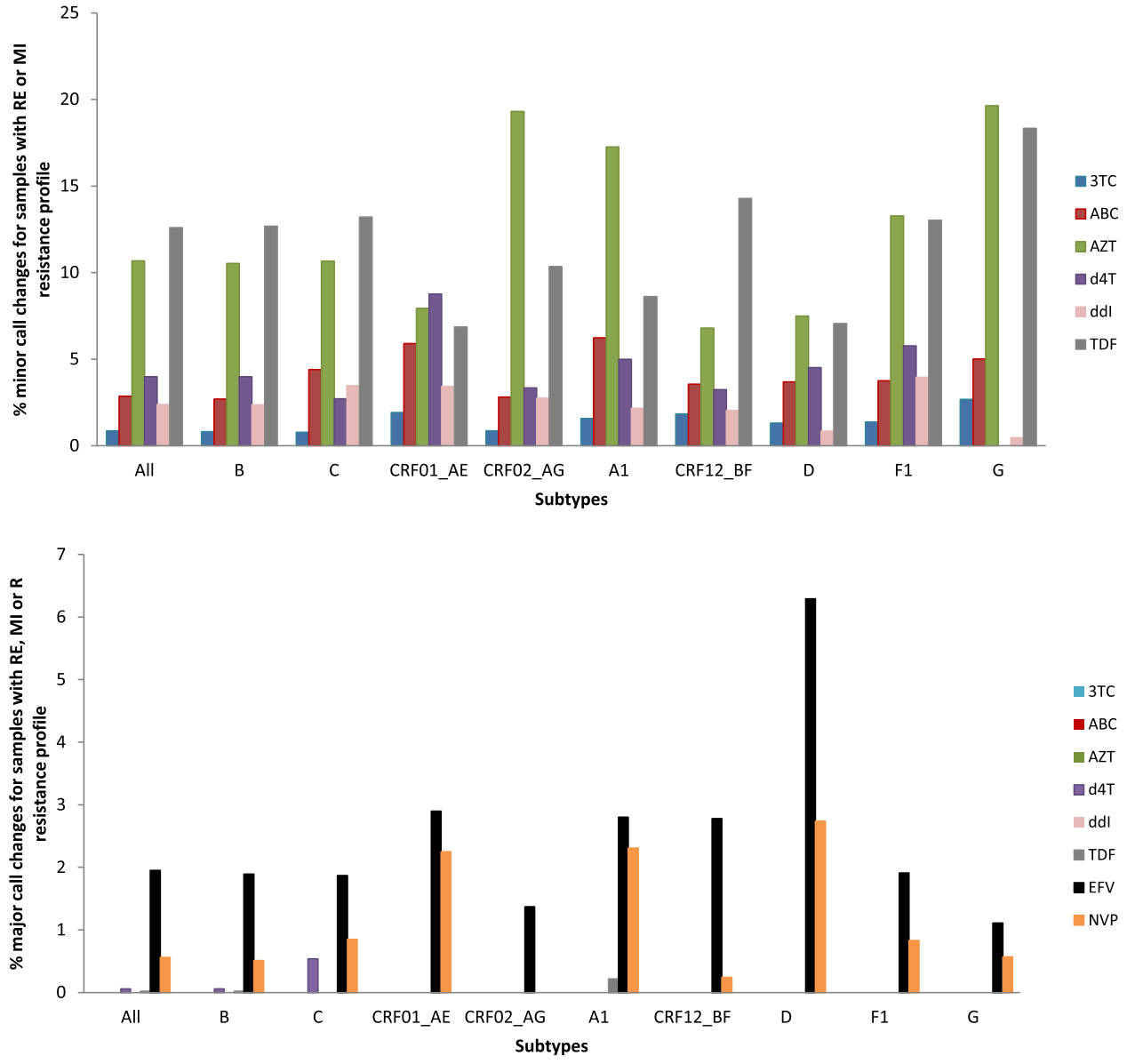

Figure 4 virco®TYPE call changes between full length and short RT HIVDR interpretations for sequences with a 'resistant' profile based on full RT interpretation (group 2). A. Minor call changes. Minor changes are not possible for drugs with a BCO only as a shift can only occur from susceptible to resistant (or vice versa), which is a major call change; therefore EFV and NVP are not depicted in this graph. B. Major call changes RE: reduced response, MI: minimal response, R: resistant.

(n= 17,131) in the dataset used, the majority of sequences in this database are subtype B, which is less relevant for RLS. To accommodate this limitation, further analysis on the effect of sequencing a short RT fragment for HIVDR testing of RLS samples accessing first-line regimens will be done in collaboration between ART-A and the PASER (PharmAccess African Studies to Evaluate Resistance) network [23]. Secondly, the treatment data of the patients from which these sequences were derived is missing and therefore we could not make a clear differentiation between the resistance interpretations in a treatment naïve group versus a treatment exposed group. This issue will also be addressed in the future study (mentioned above) as treatment naïve and treatment failing patients will be included. Thirdly, this simplified resistance assay only focuses on assessing resistance in the RT gene, which is relevant for RLS at the moment as most of the patients receive a combination ARV regimen of RT inhibitors only. However, when protease inhibitors will become

Table 1 Amino acid positions outside RT codon 41-238 contributing to the HIVDR interpretation algorithms. ROI: region of interest

ROI	ARV drugs	Stanford	virco®TYPE
RT codon 1-40	3TC	none	7, 8, 13, 35, 36, 40 (n = 6)
	ABC	none	3, 13, 21, 33, 35, 39, 40 (n = 7)
	AZT	none	none
	d4T	none	3, 13, 33, 35, 36, 40 (n = 6)
	ddI	none	3, 4, 33, 35, 36, 39, 40 (n = 7)
	TDF	none	4, 7, 13, 21, 33, 40 (n = 6)
	EFV	none	16, 20, 22, 27, 28, 31, 33, 34 (n = 8)
	NVP	none	21, 31, 35 (n = 3)
RT codon 239-400	3TC	none	240, 248, 277, 313 (n = 4)
	ABC	none	334, 348 (n = 2)
	AZT	333 (n = 1)	240, 242, 244, 245, 282, 296, 297, 313, 334, 335, 350, 357, 359, 360, 375, 377, 386, 395 (n = 18)
	d4T	none	334, 348, 357, 359 (n = 4)
	ddI	none	348, 359, 360, 395 (n = 4)
	TDF	none	242, 245, 249, 277, 297, 329, 334, 335, 353, 357, 359, 395 (n = 12)
	EFV	318 (n = 1)	240, 241, 243, 244, 245, 250, 251, 257, 271, 272, 274, 282, 283, 286, 292, 297, 313, 317, 318, 329, 333, 334, 335, 338, 339, 348, 353, 356, 357, 358, 365, 366, 369, 370, 371, 375, 376, 377, 379, 381, 382, 385, 386, 390, 393, 394, 395, 400 (n = 48)
	NVP	318 (n = 1)	244, 245, 248, 250, 272, 283, 286, 293, 297, 313, 317, 318, 329, 333, 334, 335, 338, 339, 348, 353, 356, 357, 358, 365, 366, 369, 370, 371, 374, 375, 376, 377, 379, 382, 385, 386, 390, 393, 394, 395, 399, 400 (n = 42)

more readily available in RLS there will be a need to include the protease gene as well.

Although this simplified HIVDR interpretation algorithm still requires a lab infrastructure, skilled personnel and investment in major equipment, it also has several advantages. Firstly, amplification of the short RT region is feasible using a one-step single round amplification protocol [18], which reduces the risk for contamination, minimizes hands-on work and cuts down the reagent cost as only one amplification primer set is needed. Secondly, the sequencing is also simplified by reducing the number of primers from 8 (in Virco's in-house assay) to only 2. Thirdly the analysis time of the obtained short RT sequence is also reduced compared to the analysis of a full RT sequence. The obtained short RT sequence can subsequently be submitted to either Stanford or virco®TYPE. However, the biological starting material for this simplified HIVDR algorithm is plasma, which might pose a problem in RLS, as cold-chain transport and deep frozen storage is still a challenge in many places. Therefore, the ART-A team is currently investigating the feasibility of using dried blood spots as a source material to overcome this issue.

In conclusion, this comparative analysis has shown that HIVDR interpretation, based on shorter RT sequence, is not inferior compared to the use of full RT sequences for most of the commonly used HIV RT inhibitors in RLS.

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Authors' contributions

KS designed the study, performed the analysis and prepared the manuscript. MB assisted in drafting the manuscript. EvC and KvdB performed the analysis. BW took care of the statistical analysis. WS, CW and TRdW and LS assisted in designing the study and provided substantial intellectual content to the manuscript. All authors critically reviewed and approved the final manuscript.

Competing interests

KS, EVK, BW, KvdB, and LJS are employees of Tibotec-Virco Virology BVBA. The company commercializes HIV drug resistance testing technology on the codon 1-400 RT domain. While the present study does not represent a commercial activity, products using the complete RT codon are commercialized by the company in the western world. However, no commercial activities are planned for RLS specifically. Other authors declare that they have no competing interests.

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Chapter 7

Evaluation of an affordable HIV-1 virological failure assay and antiretroviral drug resistance genotyping protocol.

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Evaluation of an affordable HIV-1 virological failure assay and antiretroviral drug resistance genotyping protocol☆

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HIV-1 RNA viral load is the preferred tool to monitor virological failure during antiretroviral therapy (ART) exposure. Timely detection of virological failure can reduce the prevalence and complexity of HIV-1 drug resistance. This field evaluation further characterizes a two-step approach to identify virological failure, as a measure of ART adherence, and detect HIVDR mutations in the reverse transcriptase (RT) gene of HIV-1. Two hundred and forty-eight (248) samples were tested; 225 from South African HIV-1 participants enrolled in the PharmAccess African Studies to Evaluate Resistance (PASER) cohort, forty of which had paired dried blood spot (DBS) samples and 23 HIV-1 negative samples. A newly developed virological failure assay (ARTA-VFA) was used on all samples, and those with a viral load >5000 RNA copies/ml were genotyped with a shortened RT protocol to detect HIVDR (ARTA-HIVDR^{ultralight}). The ARTA-VFA showed good precision and linearity as compared to a commercial reference assay (NucliSENS® EasyQ v1.2, Roche) with an R^2 of 0.99. Accuracy studies illustrated standard deviations of <1 log RNA copies/ml for plasma and DBS ARTA-VFA results compared to the reference method. The ARTA-VFA's intended use was to deliver qualitative results either < or >5000 RNA copies/ml. No significant differences in the proportion of results < or > either the 5000 RNA copies/ml or 1000 RNA copies/ml cut-off were noted for plasma indicating either cut-off to be useful. Significant differences were noted in these proportions when DBS were used (P=0.0002), where a 5000 RNA copies/ml cut-off was deemed more appropriate. The sensitivity and specificity of the ARTA-VFA with plasma were 95% and 93% and 91% and 95% for DBS using a 5000 RNA copies/ml cut-off. The ARTA HIVDR^{ultralight} assay was reliable for plasma and DBS samples with a viral load >5000 RNA copies/ml, with amplification and sequencing success rates of 91% and 92% respectively for plasma, and 95% and 80% respectively for DBS. HIVDR profiles for plasma and DBS were 100% concordant with the reference assay. This study evaluated a previously described combination of two assays potentially useful in assessing HIV-1 virological failure and resistance, showing good concordance with reference assays. These assays are simple to perform and are affordable, viable options to detect virological failures in certain resource limited settings. The assays' compatibility with DBS sampling extends the access of HIV-1 virological monitoring to more remote settings.

1. Introduction

Access to antiretroviral therapy (ART) has increased in the past decade, in particular in Sub-Saharan Africa where an estimated 6.65 million (47%) of infected individuals requiring ART are receiving it (WHO, 2011). The World Health Organization (WHO), has suggested that if the considered "Test and Treat" approach is adopted, involving regular screening of entire populations for HIV, and initiating immediate treatment for those found to be HIV-positive, this would result in a total of 32 million people being eligible for ART (WHO, 2012). Antiretroviral therapy is potentially threatened by

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the emergence of HIVDR which is compounded when infrastructure is inadequate to detect virological failure and identify subsequently the presence of resistance.

Viral load monitoring is the preferred tool to monitor HIV-1 positive patients. However, in resource-limited settings, routine viral load testing is often not feasible, due to a variety of reasons, including the use of limited numbers of centralized reference laboratories entails logistical challenges of failure to report back the results to remote sites and individual patients.

When a patient's viral load is measured to be above the selected cut-off it is necessary to assess the reason(s) for this failure. Initially, once virological failure is detected, patient adherence is investigated. If virological failure is confirmed despite intensified adherence counselling, the presence of HIVDR should be assessed. HIVDR testing can guide clinicians on appropriate therapy switches or alternatively behavioural intervention. HIV-1 genotypic drug resistance assays which sequence the protease (PR) and partial reverse transcriptase (RT) genes of HIV-1 are routinely available, but are technically complex and expensive. Major expense resides in the cost of equipment and skilled labour.

A review of ART treatment programmes in sub-Saharan Africa showed that 94% of patients on ART receive a combination of NRTI/NNRT first-line therapy, and hence when resistance occurs, it affects one or both of these drug classes (Barth et al., 2010). Moreover, those patients who are on 2nd line therapy have been shown to have only 7% resistance to PI's (Wallis et al., 2011), as second line failure is mostly attributed to inadequate adherence resulting from poor tolerance to 2nd line therapy (El-Khatib et al., 2010; Van Zyl et al., 2011). This implies that for the for majority of African patients, it is sufficient to sequence the RT gene of HIV-1 today and for several years to come, as long as the current 1st and 2nd line treatment combinations are used as recommended by the WHO public health approach and/or in-country guidelines. More specifically, all the key HIVDR mutations measured within the Stanford HIV drug resistance (HIVdb) algorithm are found between amino acid 41 and 238 of the RT gene (Brehm et al., 2012; Rhee et al., 2003). It is in light of this information that a short RT genotypic test covering this region was designed and evaluated in the laboratory. The current study describes a field evaluation of the combined ARTA-VFA and HIVDR test in the South African setting (Aitken et al., 2013a, 2013b).

The assays under field evaluation in this study have been previously developed by the ARTA consortium (www.arta-africa.org). These tests initially screen for patients with virological failure and, if failing, subsequently for HIV-1 drug resistance, using either plasma or DBS as starting material. The goal for the development of these assays was that these would not be fully or in-part limited to reference laboratories, but would enable medium-sized throughput laboratories to perform part of these assays. In light of the expected increase in numbers of South African patients qualifying for HIVDR testing, as recommended by the Southern African HIV Clinicians Society (Conradie et al., 2012), the ARTA tests could represent an interesting alternative to more costly approaches, which would require sequencing large portions of RT and protease regions of the genome.

2. Materials and methods

2.1. Samples

HIV-1 subtype C samples were collected as part of the PASER-M study from sites within South Africa (Hamers et al., 2011). Ethical clearance was obtained by the Research on Human Subjects (Medical) committee at the University of the Witwatersrand (Clearance Number: M090688). All samples, except the HIV negatives, had previously been processed for viral load using a commercial assay (NucliSENS EasyQ® HIV-1 version 1.2, the assay in use in South African laboratories at the time of the clinical study) and for HIV-1 drug resistance using an in-house assay (Wallis et al., 2010). The plasma samples were divided into two groups. The first set (set 1) comprised 208 plasma samples with viral loads ranging from undetectable (<40 RNA copies/ml) to >125,000 RNA copies/ml, and included 23 HIV-1 negative samples, which had been previously tested for HIV-1 in a routine diagnostic setting. A second set (set 2) included 40 matched plasma and DBS, with a viral load range of 270–15,519,576 RNA copies/ml (median: 14,322 RNA copies/ml). The DBS were spotted at the time of patient sample collection, and stored with desiccants at −20 °C for a mean time of 16 months (13–21 months). All samples with available sequences were subtyped with the REGA subtyping tool – Version 2.0 (Rhee et al., 2003).

2.2. Nucleic acid extractions for ARTA-ARTA-VFA and ARTA-HIV^{ultralight}

Nucleic acids from plasma were extracted using the NucliSENSEasyMAG® system (bioMérieux) as per manufacturer's instructions, with an on-board lysis incubation. A plasma sample input volume of 100 μl was assigned to a sample vessel, and spiked with 5 μl of the internal control (Aitken et al., 2013a), resulting in a 50 μl nucleic acid eluate. DBS nucleic acid extractions were performed using an initial off-board lysis step, using 2 DBS (estimated 50 μl whole blood/plasma per spot) and incubating them in the NucliSENS® Lysis Buffer (2 ml) for an hour. The DBS paper was removed from the lysis buffer, and 5 μl of the internal control then spiked into the sample. The resulting 2 ml of fluid was aliquoted into a NucliSENSEasyMAG® sample vessel and eluted in 25 μl. The downstream extraction process was conducted in the same manner as for the plasma samples. The resulting eluate from this extraction was used as the nucleic acid input for both the ARTA-VFA and the ARTA HIVDR^{ultralight}.

2.3. Viral load testing

The NucliSENSEasyQ® HIV-1 version 1.2 was used to measure the HIV-1 viral load in all plasma samples tested in this study. This assay was considered the reference method for the time of the study.

The ARTA-VFA to be evaluated in this study was designed to be used as a qualitative viral load test, resulting in a positive (virological failure) or negative (non-virological failure) result. The assay is based on real-time PCR targeting the long terminal repeat domain (LTR) of HIV-1 (Aitken et al., 2013b), and is designed to identify virological failure where viral load's are greater than 5000 RNA copies/ml, as was the cut-off defined by the at time of study design and completion (WHO, 2010). The South African guidelines identify virological failure as a viral load greater than 1000 RNA copies/ml measured on two consecutive occasions (National Department of Health, 2010). Since this study was conducted in a South African laboratory, both of these cut-offs were used in this evaluation. The ARTA-VFA was performed as previously described (Aitken et al., 2013b). The assay was performed on the ABI 7900HT Real-Time PCR system (Life Technologies, CA, USA).

2.4. HIV-1 drug resistance testing

The reference HIVDR assay for this evaluation was as previously described (Wallis et al., 2010). The resulting amplicon analyzed is 1544 base pairs in length, encompassing nucleotides 2066–3610 (as per HXB2), which includes both protease and RT genes. The ARTA HIVDR^{ultralight} evaluated in this study is based on a single-round PCR using the One-Step Superscript-III High Fidelity system

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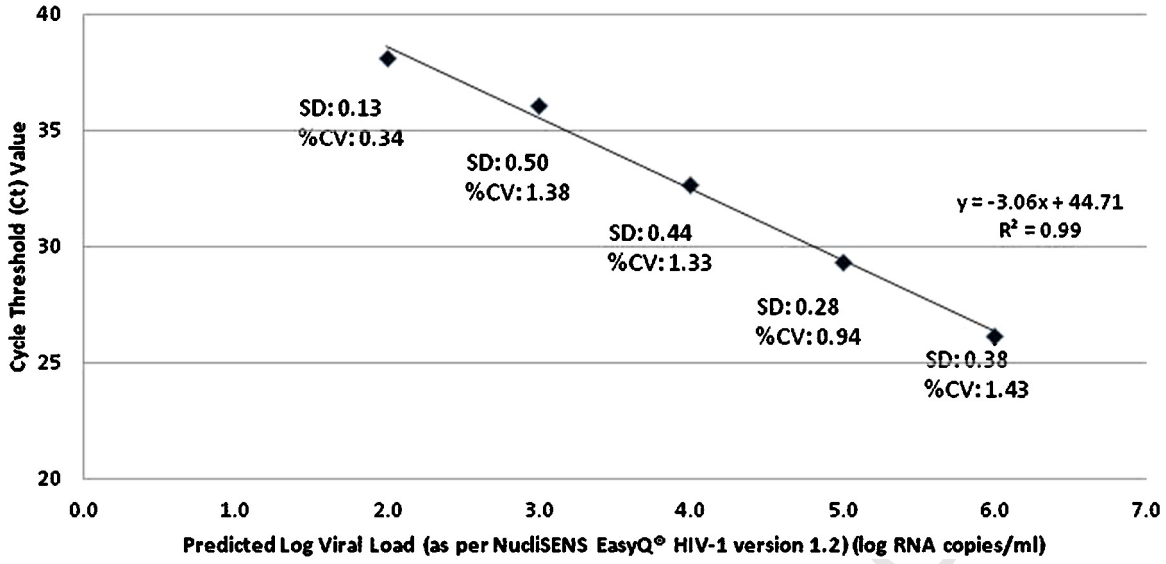

Fig. 1. Precision and linearity of ARTA-VFA over the range log 2 to log 6.

(Invitrogen, CA, USA) as previously described (Aitken et al., 2013a). The post-PCR steps were identical to the in-house method (Wallis et al., 2010), with the exception that only two sequencing primers were used as opposed to five. This short HIVDR protocol produces a 591 base pair amplification product encompassing nucleotides 2673–3264 (as per HXB2), which translates to amino acid 41–238 of the RT gene.

2.5. Assay evaluation

2.5.1. Virological failure assay precision

Within-assay precision was established by four repeated measurements of serial dilutions of plasma with known viral load (as per NucliSENSEasy Q® HIV-1 v1.2) which were further used to create the standard curve. A 1:10 dilution series was performed from stock plasma sample with a viral load measuring 7.0 log RNA copies/ml. Between-assay precision was measured by assessing viral loads obtained by the ARTA-VFA from a positive control sample (25,000 RNA copies/ml) tested throughout the various assay runs performed.

2.5.2. Virological failure assay accuracy

The accuracy of the assay using plasma samples was determined by performing the Bland–Altman statistics (Bland and Altman, 1986). The Bland–Altman graph for the plasma samples was generated by plotting the NucliSENSEasy Q® HIV-1 v1.2 viral load (log RNA copies/ml) against the difference between the NucliSENSEasy Q® HIV-1 v1.2 and the VFA (log RNA copies/ml). Assay accuracy using DBS was also assessed by comparison of viral load results obtained using paired DBS and plasma within the VFA.

2.5.3. Evaluation of ARTA HIVDR^{ultralight} protocol

The amplification and sequencing success rates of the ARTA HIVDR^{ultralight} as compared to the reference protocol (Wallis et al., 2010) was calculated over four viral load ranges for both plasma and DBS specimens. Mutation profiles from the two methods were compared.

2.5.4. Assay costing

The cost of the assay reagents under evaluation were calculated and compared with the cost of the reference assays. A time saving analysis was performed and incorporated into the costing analysis.

2.5.5. Statistics

HIV-1 viral load values (RNA copies/ml) were $\log_{10}$-transformed before statistical analyses. For precision testing, the mean, standard deviation (SD) and percentage coefficient of variance (%CV) were calculated for each log range assessed in the construction of the standard curve of known viral load (as per NucliSENS EasyQ v 1.2) versus cycle threshold (Ct) values measured. This was regarded as the within-run precision. The linear regression was calculated and presented as the R^2 value, and the equation of the linear regression shown on the respective plots. Between-run precision was assessed by measuring results of a positive control (viral load: 25,000 copies/ml) which was added to every run, and calculating the same statistics as per within-run precision.

The Bland–Altman plot for accuracy testing used the viral load for the matched plasma samples as the reference data set. Secondly, percentage similarity statistics (Scott et al., 2003) included calculating the mean, standard deviation, and percent of coefficient of variance. Thirdly, proportionality of calls around a 1000 RNA copies/ml and 5000 RNA copies/ml cut-off was measured using a Chi-squared test (for plasma comparisons) and Fisher's exact test (for DBS comparisons due to small sample sizes). Finally, the sensitivity and specificity of the ARTA-VFA was also tested against the reference assay. The proportion of percentage results obtained (e.g. sensitivity, specificity) was calculated using a Z-test to test significance of sample proportions.

3. Results

A total of 248 plasma and 40 DBS samples were tested with the VFA. All samples which had a sequence available, as per the reference HIVDR assay, were sub-typed as HIV-1 subtype C.

3.1. Virological failure assay evaluation

Within-assay precision of the ARTA-VFA was calculated for viral load from 2 log RNA copies/ml up to 6 log RNA copies/ml (Fig. 1). The standard deviations and % coefficient of variance are both shown for each log range measured, with a measured average of 0.35 log RNA copies/ml and 1.08% respectively. Between-assay precision resulting statistics showed a mean Ct: 30.00, median Ct: 29.87, maximum Ct: 30.91, minimum Ct: 28.94, the standard deviation (SD) of Ct: 0.49 and the mean ± 2SD of Ct was 30.97; 29.02 (Data not shown).

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Table 1
Samples tested for ARTA-VFA evaluation as compared with NucliSENS EasyQ® HIV-1 v1.2.

Sample set	Viral load range (RNA copies/ml)	Total tested (*n*)	Number of VFA results obtained (*n*)	Undetermined VFA results (*n*)	Undetermined IC results (*n*)	Number of VFA results with amplified IC (*n*)	5000 copies/ml cut-off		1000 copies/ml cut-off	
							Correctly classified call (*n* (%))	Misclassified call (*n* (%))	Correctly classified call (*n* (%))	Misclassified call (*n* (%))
1 (Plasma)	>125,000	35	34	0	1	34	34 (100)	0(0)	34 (100)	0 (0%)
	25,000–125,000	25	25	0	0	25	23 (92)	2* (8)	23 (92)	2 (8)
	5000–25,000	25	24	0	1	24	22 (92)	2* (8)	22 (92)	2 (8)
	1000–5000	25	21	4	0	25	18 (72)	7# (28)	18 (72)	7# (28)
	200–1000	25	14	10	1	24	24 (100)	0 (0)	22 (92)	2# (8)
	40–200	25	1	15	9	16	16 (100)	0 (0)	16 (100)	0 (0)
	<40	25	0	25	0	25	25(100)	0 (0)	25 (100)	0 (0)
	HIV negative	23	0	23	0	23	23(100)	0 (0)	23 (100)	0 (0)
	Total (set 1)	**208**	**119**	**77**	**12**	**196**	**185 (94)**	**11 (6)**	**183 (93)**	**13 (7)**
	>5000	21	21	0	0	21	19 (91)	2 (9)	21 (100)	0 (0)
2(DBS)	<5000	19	2	17	0	19	18 (95)	1 (5)	11 (58)	8 (42)
	Total (set 2)	**40**	**23**	**17**	**0**	**40**	**37 (93)**	**3 (7)**	**32 (80)**	**8 (20)**

VFA, virological failure assay; IC, internal control.
* These samples had Ct values translating to viral load's <5000 RNA copies/ml, and therefore misclassified due to under-calling, i.e. called non-virological failures (5000 RNA copy/ml cut-off) when in reality there was virological failure.
# These samples had cycle threshold (Ct) values translating to viral load's >5000 RNA copies/ml, and therefore misclassified due to over-calling, i.e. called virological failure (5000 RNA copies/ml) when in reality it was a non-virological failure.
#* Denotes samples that had Ct values translating to viral load's >1000 copies/ml, misclassified due to over-calling, i.e. called virological failure (1000 RNA copies/ml cut-off) when in reality is a non-virological failure.

Table 2
Bland–Altman, percentage similarity statistics and diagnostic sensitivities and specificities for VFA compared to the reference method.

Bland–Altman parameters							% Similarity			Sensitivity, specificity, PPV and NPV							
Comparison	Bias (log RNA copies/ml)	SD of difference (log RNA copies/ml)	Limits of agreement	Distance over limits (log RNA copies/ml)	Fraction (%) of outliers					5000 Copies/ml cut-off				1000 Copies/ml cut-off			
					>0.5 log RNA copies/ml	>1 log RNA copies/ml	Mean	SD	%CV	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV%)	NPV (%)
Plasma VFA vs. NucliSENS EasyQ® HIV-1 vl 2	−0.28	0.67	1.04; −1.60	2.64	58/196 (30)	19/196 (10)	103.2	8.56	8.30	95	93	92	96	90	98	98	86
DBS VFA vs. NucliSENS EasyQ® HIV-1 vl.2	−0.05	0.86	1.64; −1.74	3.37	6/21 (29)	3/21(14)	101	7.98	7.90	91	95	96	91	100	70	72	100
DBS VFA vs. Plasma VFA	0.20	0.37	0.93; −0.52	1.45	5/21 (24)	0/21 (0)	98.11	3.93	4.01	91	91	95	91	100	72	76	100

SD: standard deviation; CV, coefficient of variance; PPV, positive predictive values; NPV, negative predictive value.
Sensitivity was calculated as True virological failure/(True virological failure + False non-virological failure).
Specificity was calculated as True non-virological failure/(True non-virological failure + False virological failure).
Positive predictive value was calculated as True virological failure/(True virological failure + False virological failure).
Negative predictive value was calculated as True non-virological failure/(True non-virological failure + False non-virological failure).

Ct values obtained were extrapolated into log RNA copies/ml, using the standard curve equation. The calculated Ct for 5000 RNA copies/ml was 33.92. The SD of the Ct was calculated as 0.51, and hence the mean ± 2SD was 34.94; 32.90, with a minimum of 32.57 and a maximum of 34.77. A Ct value of >34.94 was classified as a value <5000 RNA copies/ml, and a value <32.90 as >5000 RNA copies/ml. Samples measuring Ct values within this range were repeated, and if still within the range, regarded as >5000 RNA copies/ml (i.e. virological failures).

From a total of 248 samples tested, 142 samples produced a Ct value and 94 did not have a measured Ct value with the ARTA-VFA. The latter 94 samples did however have an internal control amplified, and hence regarded as having a non-detectable viral load (non-virological failure's). Twelve (12) samples had a failed amplification of internal control, and were therefore not measured against reference assay results. Table 1 represents a summary of the samples used to determine assay accuracy, including the details of correct and misclassified calls around the 5000 and 1000 RNA copies/ml cut-offs. For set 1 samples 94 and 93% were correctly classified using the 5000 and 1000 RNA copies/ml cut-off, respectively. All samples <1000 RNA copies/ml were correctly classified as non-virological failures, using the 5000 RNA copies/ml cut-off, whilst 2 samples (8%) were classified as virological failure's with the 1000 RNA copies/ml cut-off. All the HIV negative samples were classified as negative. Set 2 (DBS) samples with a viral load >5000 RNA copies/ml had 91% (19/21) samples correctly classified as virological failure's using the 5000 RNA copies/ml cut-off, and 100% (21/21) using the 1000 RNA copies/ml cut-off. Those samples with a viral load <5000 RNA copies/ml as per reference assay had 95% and 58% correctly classified as non-virological failure's as per the 5000 RNA copies/ml and 1000 RNA copies/ml cut-off's (Table 1).

Chi-squared statistics comparing proportion of classified and misclassified calls generated by the two cut-offs of 1000 and 5000 RNA copies/ml showed that there was no statistical difference for plasma comparisons, set 1 ($P = 0.83$) and set 2 comparisons ($P = 0.19$) (Table 1). The resulting sensitivities and specificities of the ARTA-VFA (plasma) compared to the reference method were noted to be >90% for both cut-off's used (Table 2). The viral load's from the resulted samples were used to determine the accuracy of the ARTA-VFA by comparing these results with those obtained with the reference assay (Table 2).

A collection of 40 paired DBS and plasma samples were obtained for this analysis and comparison was done using the 5000 RNA copies/ml cut-off. The plasma ARTA-VFA samples for this group, with a viral load <5000 RNA copies/ml, failed to yield a Ct value with the ARTA-VFA, in the presence of an amplified internal control (IC) (Fig. 2A). This denotes that these samples did not have a detectable viral load with the ARTA-VFA, i.e. accurately classifying these samples as <5000 RNA copies/ml. Fig. 2B illustrates the comparison of viral load's of matches samples with a reference viral load >5000 RNA copies/ml, with two matched plasma and DBS samples being under-called for virological failure. Bland–Altman and percentage similarity statistics for the DBS and plasma comparisons were calculated (Table 2). The sensitivity and specificity of the DBS calls compared to those resulted from plasma testing was 91% using the 5000 RNA copies/ml cut-off, but the specificity decreased to 72% once a 1000 RNA copies/ml cut-off was used ($P = 0.0002$). The implications on positive and negative predictive values are shown in Table 2.

3.2. ARTA-HIVDR^{ultralight} assay evaluation

Table 3 summarizes the amplification and sequencing success rates of the ARTA-HIVDR^{ultralight} assay using both plasma and DBS starting material. Above 5000 RNA copies/ml, the amplification rates were 91% and sequencing rates were 92% from plasma. Using the 1000 RNA copies/ml cut-off, the amplification and sequencing rates from plasma changed to 76% and 93% respectively. DBS samples had a 95% amplification and 76% sequencing success rate above 5000 RNA copies/ml.

The comparison between sequences obtained from plasma ($n = 21$) using the reference protocol (Wallis et al., 2010) and the currently evaluated ARTA-HIVDR^{ultralight} protocol for set 2 samples showed identical resistance profiles. All but one of these sequenced samples had known HIVDR mutations. The mutation frequency in these samples was as follows: K103N/R ($n = 7$), M184V ($n = 4$), E138A/Q ($n = 6$), and a frequency of one ($n = 1$) of the M41L, A62V, T69S, V90I, A98G, K101E, V106M, V10I, T215D and Y318F mutations was noted. The ARTA-HIVDR^{ultralight} protocol sequenced 16 paired plasma and DBS samples (set 2), 7 of the DBS samples required repeat sequencing, as initial sequences were of bad quality. A 100% concordance in resistance profiles was obtained between these 16 matched plasma and DBS samples.

3.3. Assay cost

The ARTA-HIV^{ultralight} protocol showed a cost saving of approximately 51% against the reference HIV-1 drug resistance assay, whilst the ARTA-VFA assay did not show a cost saving, but rather a reduced hands-on time component. The time saving analysis showed the ARTA-VFA had a 31% reduced hand's on time, and 51% decreased instrument time per sample compared to the reference method, mostly attributed to the sample number per run being higher in the ARTA-VFA ($n = 96$) than in the reference method ($n = 24$). The ARTA-HIV^{ultralight} protocol showed a 32% time reduction in hands-on time, but no change in instrument time compared to the in-house protocol. Time savings were incorporated into the final cost calculations (Table 4).

4. Discussion

The proposed ARTA stepwise approach allows for different healthcare facilities/lab infrastructures to contribute to viral load and HIVDR testing as per their competencies and mandates: the most basic facilities can collect DBS and send these to the medium category facilities, that can perform the qualitative viral load test and return results; while those samples that have detectable viral load can be sent on to more central reference labs for HIVDR testing. The choice of a limited fragment of the HIV genome to amplify for HIVDR determination, has allowed for increased sensitivity of the HIVDR component of the assay and therefore made it suitable for DBS-applications, which are generally typically less sensitive than plasma-based methods.

Both the ARTA-VFA and the ARTA^{ultralight} were initially tested on HIV-1 subtype panels during their validations (Aitken et al., 2013a, 2013b). However, further analyses on a greater sample size of clinical isolates and in an actual field setting was deemed necessary to evaluate the assays more adequately. The current evaluation involved South African HIV-1 subtype C samples. A similar validation was done in Uganda where subtype A and D are prevalent (Balinda et al., 2012).

The ARTA-VFA was designed using principles of a quantitative viral load assay, with the intent of reporting results as a qualitative test with either a 5000 or a 1000 RNA copies/ml cut-off. The linearity plot of the ARTA-VFA showed the R^2 to be 0.99, which is comparable to how other viral load assays perform in linearity studies (Crump et al., 2009; Scott et al., 2009a, 2009b). Within-assay precision demonstrated (Fig. 1) that the upper range of viral load show the most variability, which is has been noted in other viral load assays (Brambilla et al., 2000). The viral load's calculated from the Ct values (Fig. 1, legend) show a variability

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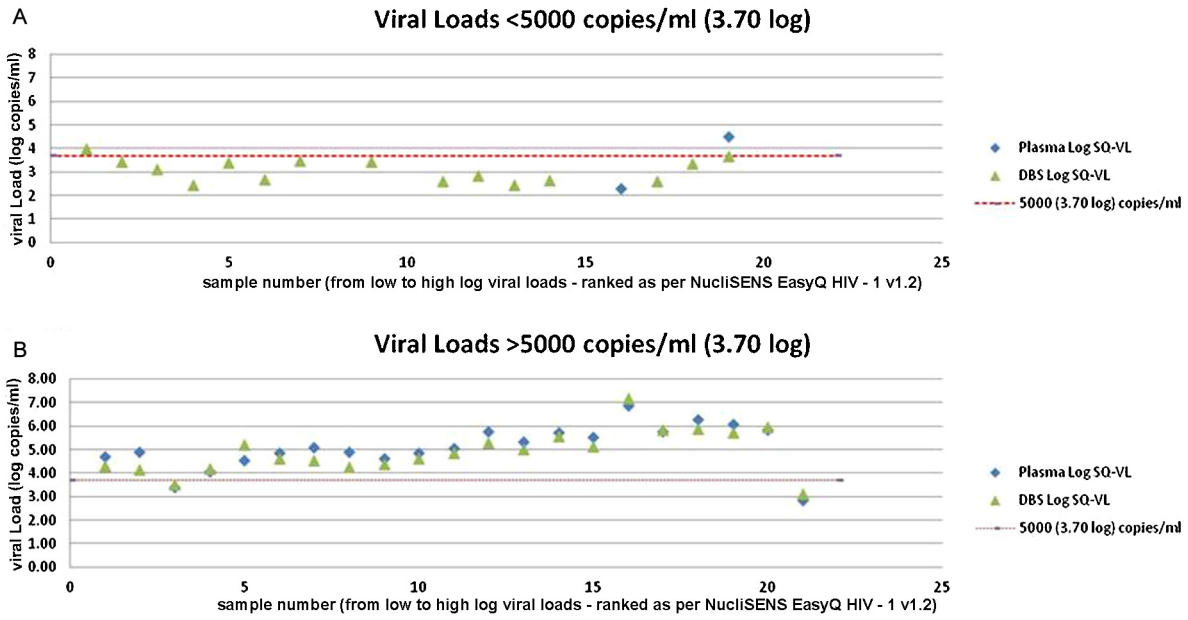

Fig. 2. Comparison of viral loads obtained with ARTA-VFA in matched plasma and DBS samples.

Table 3
ARTA-HIVDR[ultralight] amplification and sequencing success rates.

Sample set	Sample size (*n*)	Viral load (RNA copies/ml)	Amplification success rate (%)		Sequencing success rate (%)	
			Plasma	DBS	Plasma	DBS
1	35	>125,000	100 (35/35)	na	97 (34/35)	na
	25	25,000–125,000	92 (23/25)	na	91 (21/23)	na
	25	5000–25,000	76(19/25)	na	84(16/19)	na
2	25	1000–5000	28 (7/25)	na	100 (7/7)	na
	21	>5000	100 (21/21)	95 (20/21)	95 (20/21)	80(16/20)
	20	<5000	15 (3/20)	40(8/20)	100 (3/3)	15 (3/8)

which concurs with the findings of other studies (Martin et al., 2006; Scott et al., 2009a, 2009b). Method comparison between results obtained when analyzing plasma with the ARTA-VFA and the NucliSENS EasyQ® HIV-1 v1.2 showed a negative mean bias of −0.28 log RNA copies/ml, which is within acceptable ranges of 0.04 and 0.48 log RNA copies/ml. The variance of the bias (precision) was 0.67 log RNA copies/ml, which is higher than the expected 0.5 log RNA copies/ml (Scott et al., 2009a, 2009b), but as the ARTA-VFA is intended to provide only qualitative results, this may only be an issue of the resulted Ct value translating to a viral load around the 5000 RNA copies/ml cut-off, in such a borderline case a repeat test should be considered. The use of DBS instead of plasma did not influence the performance of the ARTA-VFA. Table 2 shows that the SD of DBS ARTA-VFA results compared to the reference method was 0.86 log RNA copies/ml, which is lower than the accepted limit of 1 log RNA copies/ml noted in other studies. The percentage similarity %CV was 7.90, indicating an acceptable assay variance (Scott et al., 2009a, 2009b).

Proportion testing of > or < the 1000 or 5000 RNA copies/ml cut-off of the ART-VFA as compared with the gold standard showed that the proportion of virological failure's within the plasma ARTA-VFA results did not differ significantly whether either cut-off was used. This indicates that either cut-off may be used confidently when plasma is the input material. The DBS ARTA-VFA results however, showed a decrease in specificity from the 5000 to the 1000 RNA copies/ml cut-off ($P = 0.0002$). This may be explained by the

Table 4
Cost of compared HIV-1 viral load and HIV-1 drug resistance genotyping assays.

	Viral load/virological failure assay		HIVDR assay	
	NucliSENS EasyQ® HIV-1 v1.2 (run size = 22 samples)	ARTA-VFA (run size = 92 samples)	In-house HIVDR (run size = 19 samples)	ARTA HIVDR[ultralight] (run size = 48 samples)
Time analysis (per run)				
Hands-on time (min)	35	50	320	260
Instrument time (min)	100	210	665	725
Time analysis (per samples)				
Hands-on time (min)	1.6	0.5	16.8	5.4
Instrument time (min)	4.5	2.3	35.0	38.2
Labour cost analysis ($)	0.6	0.2	6.5	2.1
Fixed instrument expenses cost analysis ($)	1.1	0.3	1.1	0.4
Reagent costs ($)	33	28	90	45
Total costs ($)	34.7	28.5	97.6	47.5

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possibility that the ARTA-VFA could be detecting proviral DNA as opposed to just HIV-1 RNA from the DBS, as has previously been reported (Masciotra et al., 2007; Monleau et al., 2010). This implies that using a 1000 instead of 5000 RNA copies/ml cut-off, increases the number of false virological failure's, which in practice would possibly cause clinicians to unnecessarily change the ART regimen for those patients. It is therefore suggested that a 5000 RNA copies/ml cut-off be used when testing DBS within the ARTA-VFA, as was suggested by the validation article by Aitken et al. (2013b).

The performance of the ARTA-HIVDR^{ultralight} protocol was assessed, by genotyping samples that had previously been genotyped by a reference method. Overall, both the amplification and sequencing success rates indicate that a cut-off of 5000 RNA copies/ml can be used for this assay for both plasma and DBS samples (Table 3). Below 5000 RNA copies the success rates drop quite sharply, suggesting a limitation of this assay in terms of detection limits, as patients failing therapy with a viral load <5000 RNA copies/ml may not be genotyped successfully. Resistance calls were identical between the reference assay and the ARTA-HIV^{ultralight}, and also between plasma and DBS, indicating that the ARTA-HIV^{ultralight} can reliably be applied to both specimen types.

The cost of the ARTA-HIV^{ultralight} protocol showed a cost saving of 51%, which would reduce the total cost of this two-step protocol significantly. The instrument and hands-on time analysis of the assays showed a reduction in time spent when using the ARTA protocols, which in turn would reduce fixed costs as well as labour costs. As instruments in South Africa are generally on a lease agreement it was not possible to factor in maintenance and purchasing costs of those instruments used. Depending on which countries implement such assays, these costs will vary considerably. In using the ARTA-HIV^{ultralight} in conjunction with an automated sequencing analysis software (Struck et al., 2012), this would further reduce the time spent on the analysis, and reduce labour costs.

With good correlations with reference assays, it is suggested that the ARTA protocols are a viable option for appropriate patient monitoring of virological failure, including ART adherence, and HIVDR surveillance in settings where NRTI and NNRTI's are used in first line ART. ARTA-VFA could preferably be used as an "adherence assay" during the crucial first months of ART of patients on 1st line ART. A positive VFA would imply: immediate adherence counselling and support, followed by a second VFA approximately 4 weeks later. If this second VFA is again positive, the clinician should request a HIVDR test, which could be done on the same sample through the downstream ARTA-HIVDR^{ultralight}. These assays performed well in plasma and more importantly in DBS samples, elucidating their use and compatibility with the more stable starting material.

Uncited references

Q3

Hamming (1987), Johnson et al. (2011), and Steegen et al. (2010).

Author contributions

MB, SCA, KS, CLW, WS, TRdW: conceived and designed the experiments; MB: performed the experiments; MB, CLW: analyzed the data; University of the Witwatersrand: contributed reagents/materials/analysis tools; MB: wrote the paper; SCA, KS, TRDW, WS, CLW: provided significant comments and editing on the paper.

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Appendix.

Consortium partners:

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Chapter 8

Automated sequence analysis and editing software for HIV drug resistance testing.

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Automated sequence analysis and editing software for HIV drug resistance testing

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ABSTRACT

Background: Access to antiretroviral treatment in resource-limited-settings is inevitably paralleled by the emergence of HIV drug resistance. Monitoring treatment efficacy and HIV drugs resistance testing are therefore of increasing importance in resource-limited settings. Yet low-cost technologies and procedures suited to the particular context and constraints of such settings are still lacking. The ART-A (Affordable Resistance Testing for Africa) consortium brought together public and private partners to address this issue.
Objectives: To develop an automated sequence analysis and editing software to support high throughput automated sequencing.
Study design: The ART-A Software was designed to automatically process and edit ABI chromatograms or FASTA files from HIV-1 isolates.
Results: The ART-A Software performs the basecalling, assigns quality values, aligns query sequences against a set reference, infers a consensus sequence, identifies the HIV type and subtype, translates the nucleotide sequence to amino acids and reports insertions/deletions, premature stop codons, ambiguities and mixed calls. The results can be automatically exported to Excel to identify mutations. Automated analysis was compared to manual analysis using a panel of 1624 PR–RT sequences generated in 3 different laboratories. Discrepancies between manual and automated sequence analysis were 0.69% at the nucleotide level and 0.57% at the amino acid level (668,047 AA analyzed), and discordances at major resistance mutations were recorded in 62 cases (4.83% of differences, 0.04% of all AA) for PR and 171 (6.18% of differences, 0.03% of all AA) cases for RT.
Conclusions: The ART-A Software is a time-sparing tool for pre-analyzing HIV and viral quasispecies sequences in high throughput laboratories and highlighting positions requiring attention.

1. Introduction

The vast majority of HIV infections occur in resource-limited-settings (RLS). Access to antiretroviral treatment (ART) programs in these settings has been widely improved following WHO recommendations and international funding efforts. As a consequence, high throughput diagnostic techniques such as viral load monitoring and automated sequencing are gradually gaining importance in RLS. First-line treatment regimens in Sub-Saharan Africa are mainly based on combinations of 3 reverse transcriptase (RT) inhibitors,[1] and second-line ART is becoming more accessible. As a consequence, the emergence of HIV drug resistance is becoming an increasing concern. Regular viral load monitoring and resistance testing in the case of virological failure are recommended in Western countries[2,3] and are being implemented in clinical practice to complete and replace inaccurate patient follow-up based on CD4 counts and clinical staging.[4–6] In Africa, direct patient testing for HIV drug resistance is (financially and technically) an option generally restricted to few reference laboratories; however population-based HIV drug resistance monitoring is feasible and targeted HIV drug resistance testing on patients failing 1st

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or 2nd line treatment is becoming more widespread as therapeutic options progressively become available.

In this setting, the ART-A consortium was established in 2008 and brought together public and private partners in South-Africa, Luxembourg, the Netherlands and Belgium to develop and deploy an affordable HIV resistance monitoring protocol for Africa (ART-A). The protocol includes patient sample collection using dried-blood-spots, semi-quantitative subtype-independent cost-effective viral load testing for assessing treatment efficacy, viral protease (PR) and RT genotyping in cases where virological failure is observed and sequence interpretation. In the majority of resource limited areas, viral load measurements, genotyping and sequence interpretation are centralized at the reference laboratory level and these laboratories are faced with the burden of processing and resulting large numbers of specimens.

2. Objectives

One aspect of the ART-A protocol was to develop and evaluate the use of an automated, easy-to-use sequence basecalling, analysis and editing software. In this study we describe the ART-A Software and its features, as well as the validation for HIV-1 PR–RT genotyping sequence analysis comparison between this automated method and 'manual-analysis'.

3. Methods

3.1. ART-A Software set-up

Most parts of the analysis pipelines are multi-threaded to fully exploit the parallel processing capacities of modern computers. The ART-A Software is protected by a GNU/GPL license and is freely available for non-commercial users. The software is implemented in the Java language to be deployed on Windows or Linux operating systems. The code (size of 16 M) holds on a memory stick or can be downloaded via the internet (http://arta.retrovirology.lu).

3.2. ART-A Software description and settings

The ART-A Software was developed to automatically process (i.e. basecall, align, analyze and edit) chromatograms generated in either the ABI format or FASTA files, and therefore includes calibration tables for the Applied Biosystems (ABI, Foster City, CA, USA) 3100, 3130, 3730, 3700pop6, 3700pop5 automated sequencers.

TraceTuner was chosen among the three potentially freely available basecalling modules for its capacity to call mixed-bases, an absolute requirement for population sequencing of viral quasispecies. TraceTuner[7] binaries were translated into Java for basecalling, assigning quality values (QV) and inferring a consensus sequence.

3.3. ART-A Software functions

The software allows to select the appropriate (here HIV) reference sequence as well as the name format of samples to undergo 'automated-analysis'. Basecalls with poor quality values (threshold: QV < 20 within a window of 10 bases) at the extremities are automatically trimmed. Chromatograms from one specimen are automatically assembled, a consensus sequence is inferred (TraceTuner module translated into Java). Local pairwise alignment against the selected reference sequence is performed by the JAligner implementation of the Smith–Waterman algorithm with Gotoh's improvement.[8] The consensus nucleotide sequence is then automatically analyzed and translated into an amino-acid (AA) sequence. Differences relative to the reference sequence are identified and reported in a tabular overview which summarizes sample identification, HIV type and subtype, gene (PR, RT, IN, Env), total number of traces, average QV, number of mixed-bases detected, as well as problems requiring attention (frameshifts, STOP codons, insertions/deletions, contradictory basecalls). For detailed inspection, the user can visualize chromatograms through a Biojava library[9] and directly "jump" to the highlighted regions by clicking on the appropriate button in the sequence window, and make the required amendments and/or manually trim the sequence.

Sequence analysis results can be stored as a project in an internal database (H2 database[10]).

Alternatively, FASTA files rather than chromatograms can be imported directly, read, identified, analyzed (STOP codons, frameshifts, ambiguities, insertions/deletions) and stored.

From the overview page, the FASTA file or final consensus sequence can either be exported in a comma-separated values (CSV) or Excel format listing mutations/polymorphisms for further analysis, exploiting the Apache POI library,[11] or be uploaded to sequence interpretation algorithms to retrieve a resistance report, as the ART-A software is not intended to provide a genotypic interpretation of resistance to ART or to predict treatment outcome, for which other algorithms have been developed by others[12–16]).

Table 1
Subtype representation and distribution of PR–RT sequences used for comparison of manual versus automated sequence analysis using the ART-A Software. Subtype was assessed using COMET v0.2.

Subtype	CRP-Santé	Wits	UMCU	Total
Pure subtypes				
A1	15	16	17	47
B	383	1	437	820
C	49	271	34	357
D	7	5	3	15
F1	11	–	3	23
F2	1	–	–	1
G	44	–	3	47
Recombinant forms				
CRF01_AE	32	–	11	43
CRF02_AG	61	–	36	97
CRF03_AB	–	–	1	1
CRF07_BC	–	–	1	1
CRF42_BF	41	–	–	41
CRF46_BF	11			
06cpx	6	–	4	6
09cpx	2	–	–	2
11cpx	1	–	–	1
18cpx	–	–	2	2
URF	90	6	19	121

3.4. HIV-1 PR–RT sequence panel

Chromatograms from 1624 HIV-1 PR–RT sequences generated in three independent laboratories (754 from CRP-Santé, 299 from Wits and 571 from UMCU) using the ViroSeq kit (CRP-Santé and IMCU) or an in-house method (Wits)[17] and a 3100 or 3130 ABI sequencer were included. Major subtypes, determined using COMET v0.2 (http://comet.retrovirology.lu) were included (Table 1). Of note, full-length RT (AA 1-332) sequences were compared for the CRP and UMCU datasets, while Wits sequences comprise RT positions 1–250 because of poor trace quality after AA250. Sequences included forward and reverse traces.

Chromatograms were submitted to the ART-A Software and the resulting FASTA files (referred to as 'automated-analysis') were compared to 'manual-analysis' performed in each laboratory using SeqScape.[18] In 'manual-analysis', chromatograms from each sample were aligned against the HXB2 reference and correct pairwise alignment, sequence quality (chromatogram neatness),

consideration of mixtures and manual trimming when necessary were performed by the technician visualizing each sequence. 'Automated-analysis' was compared to 'manual-analysis' because it is the standard and most widespread procedure in most laboratories performing sequencing and genotyping, despite lack of standardization and user-linked variability. To ensure maximum objectivity, chromatograms were submitted to the ART-A Software by two researchers who had not seen the 'manually analyzed' FASTA file results until after completion of the 'automated-analysis'. Furthermore, FASTA files edited by the ART-A Software were not processed further, even when problematic issues were reported.

3.5. Statistical analyses

Differences between results obtained in different laboratories were compared using paired *t*-tests or one-way ANOVA tests (GraphPad Prim, version 5). Differences were considered statistically significant if $p < 0.01$. The specificity of the ART-A Software was calculated by setting the manual basecall as "True", using the following formula: Correct basecall/(Correct-basecall + False-basecall).

4. Results

4.1. Comparison of 'manual' versus 'automated' sequence analysis

FASTA files generated by the ART-A Software without further manual amendments were compared at the nucleotide and at the AA levels, examining partial and complete discrepancies (hamming distance). 'Automated-' and 'manual-analysis' differed by 0.69% at the nucleotide level (2,008,508 nucleotides analyzed) and by 0.57% at the AA level (668,047 AA analyzed) (Table 2). The specificity of the ART-A Software was 0.9939 for pure bases and 0.8903 for mixed calls (all disagreements with the manually called base was considered false). Differences between laboratories were not statistically significant ($p > 0.01$). Differences between subtypes could not be assessed because of the reduced number of non-B, non-C subtype samples.

Discrepancies were distributed evenly throughout the PR–RT region analyzed at both the nucleotide and at the AA level, without hotspots of discordance (data not shown). The number of fully discordant basecalls was low (Table 2) and the vast majority of discrepancies involved calling mixtures versus pure bases: 'manual-analysis' detected mixtures while 'automated-analysis' detected a pure base in 11,675 cases; conversely, in 1154 cases, 'automated-analysis' detected mixed-bases where 'manual-analysis' called a pure base, suggesting that the majority of differences between 'manual-' and 'automated-analysis' are due to the threshold set for the detection of mixtures, and that the ART-A Software might underestimate the presence of mixtures.

4.2. Characterization of discrepancies at resistance mutations

Given their potential clinical impact in patient management, differences at AA positions involved in resistance to protease inhibitors (PIs) and NRTIs/NNRTIs were further investigated.[19] Of the 4008 AA differing between 'manual-' and 'automated-analysis', 3291 involved polymorphisms that do not affect resistance and 484 involved AA known as minor mutations within PR and RT[19] (Table 3). Differences at major resistance positions[19] were found in 62 cases (4.83% of differences, 0.04% of all AA) for PR and 171 (6.18% of differences, 0.03% of all AA) cases for RT. In line with results at the nucleotide level, one difference within PR and 6 within RT were due to a different basecall, while all others involved detecting a pure versus a mixture of AA (Table 3) and all but 2, one in RT, one in PR, were inclusive (data not shown). If we assume 'manual-analysis' to be the standard, resistance at major positions was missed by 'automated-analysis' (false negative) in the vast majority of cases

Table 2
Comparison of manual versus automated PR–RT sequence analysis using the ART-A Software. Sequences analyzed manually or using the ART-A sequence analysis software were compared at the nucleotide level and at the amino acid (AA) level, and percent agreement and hamming distances were calculated. The total number of AA (668,047 AA) analyzed is lower than 1/3 of the total number of nucleotides analyzed (2,008,508 nucleotides) because AA analysis by the software was limited to the PR–RT genes (therefore nucleotides upstream of pol were automatically excluded. Furthermore, since AA are coded by 3 bases, single or duplet nucleotides read at the end of the RT read were also excluded.

PR–RT sequences			CRP-S	Wits	UMCU	Total	Mean
Number samples analyzed			754	299	571	1,624	–
Time for automated analysis (sec)			144.44	74.48	253.33	472.25	–
Nucleotides	Total		979,492	312,377	716,639	2,008,508	–
	Differences		3,829	1,559	8,399	13,787	–
	Percent differences		0.39%	0.50%	1.17%	–	0.69%
	Percent agreement		99.61%	99.50%	98.83%	–	99.31%
Detailed comparison	Manual analysis	ART-A Software					
Agreement	Pure[a]	Pure (same)	969,958	308,418	704,233	1,982,609	98.71%
	Mix	Mix (same)	5,678	2,397	3,997	12,072	0.60%
Partial agreement	Pure	Mix (inclusive)	646	340	168	1,154	0.06%
	Mix	Pure (inclusive)	2,824	990	7,861	11,675	0.58%
	Mix	Mix (overlap)	18	1	48	67	~0%
Disagreement	Pure	Pure (different)	121	12	138	271	0.01%
	Pure	Mix (exclusive)	27	6	16	49	~0%
	Mix	Pure (exclusive)	0	1	7	8	~0%
	Mix	Mix (exclusive)	33	32	16	81	~0%
	Deletion	No deletion	4	177	16	197	0.01%
	No deletion	deletion	156	0	129	285	0.01%
Amino Acids	Total		325,809	103,921	238,317	668,047	–
	Total differences (including deletions)		1,347	355	2,306	4,008	–
	Percent agreement		99.59%	99.66%	99.03%	–	99.36%
	Percent differences		0.41%	0.34%	0.97%	–	0.57%

[a] Pure: only one base was read; mix: two or more bases were read; overlap: both manual and automated analysis detect a mix and one of the bases in the mix is the same but the other differs; inclusive: the pure base called is included in the mixture; exclusive: the pure base detected using one method differs from those detected in the mixture called by the other method.

Table 3
Comparison of AA positions involved in resistance (IAS major and minor mutations) in PR and RT between 'manual' and 'automated' analysis.

Gene			CRP-S	Wits	UMCU	Total	Mean
PR	Agreement		73,060	29,072	55,124	157,256	
	Disagreement:total		374	109	759	1,242	
	Disagreement: detailed comparison						
	Manual-analysis Polymorphism Resistance positions (IAS)	ART-A Software Polymorphism	219	78	455	752	60.55%
	Major	Major different	16	2	18	36	2.90%
	Pure	Pure different	0	0	0	0	0%
	Pure	Mix	0	0	0	0	0%
	Mix	Pure	12	2	17	31	2.50%
	Mix	Mix	4	0	1	5	0.40%
	Major	No mutation	11	1	11	23	1.85%
	Pure	Pure different	0	0	0	0	0%
	Pure	Mix	0	0	0	0	0%
	Mix	Pure	9	1	11	21	1.70%
	Mix	Mix	2	0	0	2	0.16%
	No mutation	Major	1	2	0	3	0.24%
	Pure	Pure different	1	0	0	1	0.08%
	Pure	Mix	0	0	0	0	0%
	Mix	Pure	0	2	0	2	0.16%
	Mix	Mix	0	0	0	0	0%
	Minor	Minor different	72	16	123	211	16.99%
	Minor	No mutation	42	5	147	194	15.62%
	No mutation	Minor	13	5	5	23	1.85%
RT	Agreement		251,359	74,438	180,875	506,672	–
	Disagreement: total		972	248	1,546	2,766	–
	Disagreement: detailed comparison						
	Manual-analysis Polymorphism Resistance positions (IAS)	ART-A Software Polymorphism	879	220	1,440	2,539	91.79%
	Major	Major different	37	11	36	84	3.04%
	Pure	Pure different	0	0	0	0	0%
	Pure	Mix	2	1	0	3	0.11%
	Mix	Pure	29	9	33	71	2.57%
	Mix	Mix	6	1	3	10	0.36%
	Major	No mutation	36	8	39	83	3.00%
	Pure	Pure different	4	1	0	5	0.18%
	Pure	Mix	0	0	0	0	0%
	Mix	Pure	30	6	38	74	2.67%
	Mix	Mix	2	1	1	4	0.14%
	No mutation	Major	3	1	0	4	0.14%
	Pure	Pure different	1	0	0	1	~0%
	Pure	Mix	0	1	0	1	~0%
	Mix	Pure	0	0	0	0	0%
	Mix	Mix	2	0	0	2	0.07%
	Minor[a]	Minor different	8	3	3	14	0.50%
	Minor	No mutation	7	4	28	39	1.41%
	No mutation	Minor	2	1	0	3	0.11%

[a] Minor mutations in RT are those that affect susceptibility to ETV (1).

(23 cases in PR and 83 cases in RT, against 1 and 4 falsely detected resistance mutations in PR and RT respectively) (Table 3).

Manual inspection of the chromatograms showed that full discordances were generally due to a frameshift in the sequence, while partial discordances leading to underestimation of resistance were not due to failed detection of a mixed base by TraceTuner (the module performing basecalling, assigning QV and inferring the consensus in both SeqScape, used for 'manual-analysis' and the ART-A Software) but rather to the weight it was given among all traces when inferring the consensus. For example, when one or two traces called a mixture and one trace called a pure, the consensus sequence inferred a pure base. However, in 'manual-analysis', such a case would be solved by the user while scanning through the sequence whereas the ART-A Software will record it as a 'contradictory basecall' in the report table, and the user will be addressed to the issue by clicking on the 'contradictory basecall' button. Please note that for this comparison, ART-A-edited FASTA files were not amended prior to comparison with 'manually-edited' FASTA files to avoid introduction of further human-related bias.

Taken together, these data show that 'automated-analysis' can be a useful support to alleviate genotyping, although it does not fully exclude manual verification.

5. Discussion

Here we describe a new automated sequence-analysis and editing software suited for quasispecies sequencing of HIV or other viruses. Automated sequencing techniques generate vast amounts of DNA sequence data at a faster rate than can be processed. Many softwares, including TraceTuner, SeqScape (ABI), Phred/Phrap, Sequencher,[7,18,20–22] have been developed to automate basecalling, alignment and inferring a consensus sequence

from the electropherograms. The ART-A software performs theses functions based on an adapted version of TraceTuner, but also edits and pre-analyzes the sequences, i.e. automatically detects and highlight dubious positions requesting attention and visual interpretation (insertions/deletions, mixed-bases, frameshifts, STOP codons). These tasks are generally performed manually by scanning through the aligned chromatograms from each sample. The ART-A software cannot "decide" how to handle a problematic region, but orients the user directly to it on the aligned chromatograms to make amendments. This software is intended for rapid and automated sequence alignment and pre-editing for laboratories dealing with large batches of samples to test for routine drug resistance monitoring, or tropism, where such a tool allows significant time sparing. 'Automated-analysis' of the 1624 PR–RT sequences was achieved in less than 8 min (Table 2), while a trained technician generally analyzes and edits 10–15 sequences in 2 h.

The vast majority of discrepancies between 'manual-' and 'automated-analysis' consisted of partial discrepancies, i.e. mixed basecall versus pure basecall, and more specifically mixed calls involving one common base, rather than exclusive disagreements (Table 2). Overall, the ART-A software underestimated mixed-calls in comparison to 'manual-analysis'. This observation held true at resistance positions. Tuning the TraceTuner thresholds for detecting mixed bases (min_ratio) and calling a mixed-base over noise (noise_fraction) to increase the ability of the software in calling mixed-bases, i.e. increasing aggressiveness of the software in calling a mixed-base, modified the balance between pure versus mixed-calls but also increased the number of false positive mixtures at the nucleotide level, decreasing specificity and increasing type II errors in comparison to 'manual-analysis' (data not shown). In most cases, mixtures had been detected in at least one trace by the ART-A Software, but inferring the consensus sequence from discordant traces generally favored the pure over the mixed base in the consensus. Such situations are frequent in population sequencing of viral quasispecies, and are generally dealt with and solved by manual inspection. Importantly, when mixed bases were detected in each of the overlapping traces, the mixture was inferred correctly in the consensus. The ART-A Software however highlights those positions where different traces differ in the base called as a "contradictory basecall" (e.g. A in one trace versus A+G in another trace at a given position), and the user would have seen the mixed call and amended the consensus sequence as needed.

The detection of mixtures and minority variants remains a crucial issue in HIV genotyping, as it directly impacts resistance monitoring and is constrained by the ability of bulk Sanger sequencing to detect minorities on one hand, and on sequence interpretation on the other hand.[23–25] Although resistance to most PIs and NRTIs involves the cumulative effect of more than one mutation, which is expected to dim the weight of such discrepancies on resistance reports inferred by genotypic interpretation algorithms,[12–15] this issue becomes crucial for inhibitors with a low genetic barrier, as lamivudine and efavirenz/nevirapine, which are included in all first-line regimens in RLS.[1,3] Accordingly, genotypic interpretation of the paired 'manually-edited' and 'automatically-edited' FASTA files, i.e. resistance reports generated using the Stanford, the Rega and the ANRS algorithms[12–15] did not always disagree where 'manual-' and 'automated-analysis' disagreed at one of the major or minor mutations involved in resistance. Furthermore, in most cases, discrepant sequences disagreed according to one algorithm, but not according to the other(s). For clarity, and because it is the 'worst-case' scenario, discordances at IAS minor and major resistance positions were considered in this analysis.

Because the ART-A Software was developed as a component of the ART-A program and therefore initially intended to ease genotyping of viral (HIV) populations in RLS, it was tested and validated for HIV PR–RT sequence editing using three independent datasets, but it can readily be further widespread and adapted for other HIV genes and for other viral quasispecies (HCV, HBV, FluA) by selecting the appropriate reference sequence for alignment, as well as for pyrosequencing data.

In this study, 'automated-analysis' was compared to 'manual-analysis', despite the lack of standardization of such a reference, as it is the most commonly used procedure. Therefore, to estimate the relevance of automating sequence editing, 'manual-analysis' of a subset of chromatograms (Wits dataset) by different technicians in different laboratories was compared (data not shown): FASTA files disagreed partially in 1233/328,396 (0.38% against 0.64% in 'manual-' versus 'automated-analysis') and fully disagreed in 43/328,396 (0.01%, as for 'manual-' versus 'automated-analysis') cases (hamming distance = 1461), highlighting that differences between 'automated-analysis' and 'manual-analysis' were comparable to interpersonal differences ($p > 0.05$, data not shown). Large-scale comparisons would be needed to gain further insight and a better estimate of the extent of variability in population-sequencing interpretation.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

Not required.

Authors' contributions

DS developed the ART-A automated sequence analysis and editing software and performed the validation. CW, SA and MB tested the software and contributed to software validation; TRdW designed and coordinated the ART-A algorithm; WS, RS and JCS provided assistance and guidance in preparing the ART-A program; GD substantially councelled and assisted in adapting TraceTuner to the ART-A Software requirement; DPB coordinated the software development, contributed to its validation and to data analysis. DS and DPB drafted the manuscript. All authors critically reviewed and approved the final manuscript.

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Appendix A.

Collaborating centers: Contract Laboratory Services, Johannesburg, South Africa; Center for Poverty-related Communicable Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; Centre de Recherche Public de la Santé, Luxemburg; PharmAccess Foundation, Amsterdam, The Netherlands; University Medical Center Utrecht, Department

of Virology; Utrecht, The Netherlands; Virco BVBA, Mechelen, Belgium; Wits Health Consortium, University of the Witwatersrand, Johannesburg, South Africa.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2012.01.018.

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Chapter 9

Conclusion

Concluding chapter

The WHO estimates that 34 million individuals were infected globally with HIV by the end of 2011, with 22.5 million of those infections occurring in sub-Saharan Africa[1]. Of these, 12.6 million (56%) HIV positive individuals with CD4 count largely less than 200 CD4 cells/mm^3 are on HAART[1]. The management of HAART in the developed world is based on individualized specialist care, and generally includes access to all FDA approved antiretroviral drugs, frequent monitoring of plasma viral load to detect treatment failure, and drug resistance testing to guide regimen choice. However, more than 17 years after the initial introduction of HAART for HIV infected individuals, only 62% of patients requiring treatment in sub-Saharan Africa have access to therapy[1]. Thus the WHO has recommended a public health approach to HAART scale-up in resource limited settings, based on the inclusion of standardized HAART regimens, limited laboratory monitoring and decentralized service delivery[2]. Despite this, as in developed countries many patients are lost to follow-up along the value chain in patient management from diagnosis, to initiation and maintenance, thus in addition to the work conducted in this project, the newly anticipated WHO guidelines 2013 will hopefully strengthen the linkage to care and retention in care (to be launched in April).

In light of the current status of the HIV pandemic, the delivery of HAART in sub Saharan African countries has become a public health priority, an intervention which has and will continue to dramatically reduce HIV-related morbidity and mortality. However, each country in sub Saharan Africa is faced with unique challenges, such as inadequate infrastructure, laboratory capacity, social structures, varying levels of political commitment, etc, which affect their response to their epidemics. Although clinical or immunological definitions of

treatment failure are commonly used in the absence of monitoring of plasma viral load, routine laboratory monitoring of HIV infected individuals should ideally include CD4 testing to assess when to start ART, viral load monitoring to assess virological failure on ART and when indicated, HIVDR testing. It is well established that a CD4 count is a suboptimal marker for monitoring of treatment failure on ART and, and the use of it as such has led to significant misclassifications of therapeutic responses or decisions relating to treatment switch to second line[3–7].Thus, a much wider introduction of viral load measurements, coupled with HIVDR testing where available, for clinical monitoring of patients on HAART is needed. A working group has been established to accelerate this process and produce a position paper highlighting current thinking in this arena.

If a "Test and Treat"[8] approach is adopted in sub-Saharan Africa, which would involve regular voluntary screening of entire populations for HIV infection, and initiating immediate treatment for those found to be HIV-positive, this would ultimately result in a drastic increase of people being eligible for ART (up to 32 million today). Since these settings have the highest burden of HIV, this would pose enormous logistical and financial challenges, and appropriately validated and cost-effective monitoring tools would have to rapidly become available. For example, in South Africa in the next financial year (2013/2014), if the added target of initiating 700 000 new patients on HAART using CD4 T cell counts and continuing the monitoring of patients on therapy, the public sector will have to double their services to deal with approximately 7 million addition tests. Currently, government policy does not include routine HIVDR testing when required for the clinical management of patients. However, the 2012 Southern African ARV drug resistance testing guidelines recommend incorporation of HIVDR testing at specific time points, with the ultimate aim of incorporating these into the national guidelines [9]. Patient adherence and/or the emergence of HIVDR are

likely to be one of the most relevant factors determining the long term success of treatment programs.

The ART-A consortium was set up to try to overcome some of the challenges associated with implementation and scaling up of HAART laboratory monitoring. Specifically, the ART-A initiative objective was the establishment of an affordable HIVDR testing algorithm applicable to Africa, which could be adapted, either in part, or in full at different tiers of laboratories, using either plasma or DBS as the starting material. The Genotyping laboratory at the University of the Witwatersrand Medical School was one of the ART-A partner sites, and hence development, validation and evaluation of the ART-A protocols was performed locally. Two PhD students, including the present candidate, were selected to complete their PhD's with the support of this consortium.

South Africa is well equipped to manage the local epidemic having significantly more laboratory capacity for toxicity tests, CD4 and viral load assays than many other resource-limited countries. This country has a three-tier laboratory system, consisting of regional tertiary reference laboratories (21% of laboratories), secondary or district facilities (10%) and primary health care laboratories (69%)[10]. Tertiary care laboratories are capable of implementing sophisticated virological assays, CD4 and HIVDR assays, handle large volumes of work, and for the most part possess appropriate technical resources, including electricity, freezer capacity, dedicated laboratory space, skilled personnel, training programs, reliable internet connections and appropriate quality control systems. The second tier includes the medium volume laboratories that also perform CD4 and viral load assays but with a reduced repertoire of tests, and do not have specialized facilities. These laboratories generally run less complex techniques providing medium or low throughput processing. The primary

care laboratories or clinics conduct technically non-demanding assays, which typically include rapid and point-of-care tests [10]. To date, this has largely focused on HIV and syphilis rapid tests. However, the high burden of disease and ever-increasing testing volumes is placing an enormous workload on the current public sector laboratory networks and innovative diagnostic solutions are needed for the future as HAART coverage expands.

The overall objective of this study, in line with the ART-A objectives, was to establish an affordable HIVDR testing algorithm applicable to Africa. This entailed evaluating the role of *in vitro* HIVDR phenotyping in the context of HIV-1 subtype C, genotyping and genotypic interpretation tools using existing algorithms, as well as novel virological failure detection tools for clinical patient management.

Chapter 2 evaluated whether HIV-1 Phenotypic Reverse Transcriptase Inhibitor Drug Resistance Test Interpretation is dependent on the subtype of the virus backbone. Since HIVDR phenotyping is the gold standard for predicting HIVDR in patients, it was first established whether the most common Southern African HIV-1 variants (subtype C) would be accurately phenotyped using the existing technologies. The current phenotyping technologies use a subtype B backbone to create recombinant viruses with patient-derived protease and partial reverse transcriptase. There is controversy in the literature with respect to the influence of the subtype backbone in the phenotypic drug susceptibility assay[11]. This backbone could impact on the *in vitro* results and therefore it was deemed necessary by the ART-A consortium to establish the applicability of HIVDR phenotypic testing of HIV-1 subtype C viruses when a commercially available subtype B backbone is used.

Current genotyping HIVDR prediction algorithms are updated based on both phenotypic and genotypic correlation studies. The reliability of these assays in the setting of a subtype C epidemic is thus critical information. The dilemma was resolved by obtaining clinical HIV-1 subtype C samples, from South Africa and phenotyping them with both a subtype B backbone and a newly created subtype C backbone. Results indicated there was a high level of concordance between the two backbone phenotypic resistance profiles (95.8%), where natural assay variability was largely responsible for discordant results. The outcome included the proposal that confidence intervals should be reported around the biological cut-off's in phenotyping where such variation exists. There was no systematic under- or overcalling of resistance observed for phenotypic testing using either backbone. The conclusion of this work was that the commercially available phenotypic assay used in this work is as reliable as gold standard assays in the setting of assessing subtype C HIVDR.

Chapter 3 addressed whether genotypic HIV-1 antiretroviral drug resistance testing of HIV-1 subtype C is a good substitution for phenotyping. Since HIVDR phenotyping is too expensive to implement in a routine diagnostic laboratory, genotyping algorithms based on phenotypic results are often used to predict HIVDR. The phenotypic results obtained in Chapter 2 were compared to their matched genotypes using the Stanford HIVdb algorithm (http://hivdb.stanford.edu). This work showed a 92.3% concordance between genotyping and phenotyping of individual drug comparisons for a number of HIVDR profiles. Where there was a discrepancy, the phenotypic assay variability may have played a role in addition to the contribution of mutation mixtures. The overall conclusion reached was that HIVDR genotyping is a reliable tool to detect and interpret resistance in HIV-1 subtype C infected patients, and can thus be used reliably for clinical patient management.

Once the feasibility of HIVDR genotyping was established (Chapter 3), the development, validation and evaluation of a potential virological failure assay (ARTA-VFA; Chapter 4) and a simplified HIVDR (ARTA-HIVDR^{ultralight}; Chapter 5) assay was undertaken.

Chapter 4 describes the development and evaluation of an affordable real-time qualitative assay for determining HIV-1 virological failure in plasma and dried blood spots (DBS). DBS have been used for other testing purposes in HIV diagnostics some with more success than others and has includes detection of HIV antibodies [12–16], HIV antigen detection [17,18], early diagnosis of perinatally infected infants (EID) [19–23], quantification of CD4 T cells [24], quantification of HIV-1 viral loads [25–31], and HIVDR testing[32–35]. In cases where samples need to be transported between the clinic and laboratory or between laboratories for appropriate testing, this adds further complexity and cost to the management of sample transportation and storage, particularly for viral load and HIVDR testing, both requiring cold chain management and significant logistical support. If the need for a cold chain can be avoided, samples can be collected in the field and sent to a centralized reference laboratory where the required equipment and infrastructure are available at a significantly lower cost. The DBS approach is a well- recognized form of collection in many countries, and an ideal medium for blood collection in the field. Following on-site air drying using a variety of stands, the DBS can be handled as non-infectious and transported to a central laboratory by regular air mail or other courier services[36]. Furthermore, DBS collection avoids the need for centrifugation, and obviates the need for phlebotomy skills which are often not available in remote sites.

The ART-A team proposed that a simplified, affordable, conceptually novel approach could be taken by using a qualitative viral load assay with a pre-determined cut-off that gives a

threshold above which virological failure could be confirmed and below which treatment success was likely. This involved the development of a real-time PCR assay for use in Tier 1 and 2 laboratories where the relevant equipment was already available. This was the principle that supported the need for the virological failure assay (ARTA-VFA), which relies on optimizing an RNA extraction step from patient plasma or DBS samples, a cDNA synthesis component and a real-time PCR amplification of a short sequence of the HIV-1 LTR region. Its applicability using either plasma or DBS was confirmed, with virological failure being qualitatively classified as a viral load >1000 RNA copies/ml in plasma samples, and >5000 RNA copies/ml in DBS samples. The ARTA-VFA identified all major HIV-1 group M subtypes with equal specificity. Comparative testing yielded accurate virological failure determination for therapy-switching in approximately 93% of clinical cases tested, compared to current gold standard quantitative viral load assays.

Chapter 5 assessed a newly developed simplified HIVDR genotyping assay (ARTA-HIVDR^{ultralight}) targeting a shorter region of RT harboring all major RT inhibitor resistance mutation positions, thus providing all relevant susceptibility data for first-line regimen failures. Protease sequencing was excluded in this assay. Patients with virological failure, despite adherence intensification, should ideally be tested for HIVDR. Thus the ARTA-HIVDR^{ultralight} assay was developed to meet these needs, and also take the following specifications into account: to be practical and affordable; flexible with respect to equipment choices (open platform); include the option of DBS or plasma; amplify and sequence a smaller amplicon (RT) and develop generic software (Chapter 8).

Since many sub Saharan countries only have access to 1st line HAART regimens, typically including one NNRTI and two NRTIs, any HIVDR resistance mutations would only exist in

the RT gene. This justifies only sequencing of amino acids 41-238 of the RT gene. This region contains all of the key RT resistance mutations according to the IAS guidelines[37], but exclude the Y318F, N348I and the G333D/E mutations according to the virco®TYPE HIV-1 algorithm [38] and the Stanford University drug resistance database [39]. Since no key resistance mutations were missed, the assay approach was determined to be clinically useful. [38]. The novelty of this assay is that it requires only a one-step RT-PCR as opposed to traditional HIVDR assays where a 2 or 3 step PCR approach is used. Furthermore, the assay uses only 2 sequencing primers, due to its smaller amplicon size, as opposed to the 5 or 6 sequencing primers used in other HIVDR assays, thus substantially reducing assay cost. The assay performed well when compared to the in-house assay used in the laboratory at the time for both plasma and DBS yielding identical mutations and subsequent resistant profiles. The cost and time analysis of the ARTA-HIVDR^{ultralight} demonstrated significant savings over the current approaches**.**

Chapter 6 is a substudy supporting Chapter 5, and encompasses a theoretical *in silico* exercise to investigate the consequences of using the shortened RT genotype (ARTA-HIVDR^{ultralight}). Pair-wise comparisons between full-length and short RT sequences were performed. Results showed that when using the Stanford HIVdb algorithm, more than 95% concordance was obtained. When using the virco®TYPE tool, however, some differences were observed for d4T, AZT and TDF, where predictions were affected in more than 5% of the sequences. Differences were regarded as minor and unlikely to have any impact on clinical decision-making. Overall, this study illustrated that the short RT sequences can be reliably used to generate HIVDR genotypes using the Stanford HIVdb and virco®TYPE algorithms and reduce sequencing costs substantially.

Subsequently, when the relevant assays were developed and validated (as described in Chapters 4 and 5) a field evaluation using the ARTA-VFA and ARTA-HIVDR^{ultralight} on clinical samples was conducted **(Chapter 7).** The accuracy and precision of both assays, the ARTA-VFA and the ARTA-HIVDR^{ultralight} assays compared well to the reference methodology. The compatibility of the assays for use with both plasma and DBS sampling extends the access of testing to more remote settings. These assays were designed to either be used as a testing strategy of initially assessing virological failure, and once confirmed performing an HIVDR assay, or alternatively to be used separately as stand-alone, or within different laboratory tiers. Since large scale commercial assays are commonly used in reference laboratory service areas with a large burden of HIV-1, and primary health care centres ideally use rapid or point-of-care assays, the ARTA-VFA could potentially be used in the middle laboratory tier. This would involve screening patients for virological failure with a qualitative outcome (a >1000 RNA copies/ml cut-off for plasma samples, and a >5000 RNA copies/ml cut-off for DBS), followed by referral to a Tier 1 laboratory with the available infrastructure for HIVDR testing using the ARTA-HIVDR^{ultralight} if indicated.

Moreover, to ensure results could be reproduced in different centres, field evaluations were conducted in Uganda where subtype A and D are prevalent[40,41] and South Africa (high subtype C prevalence) by the ARTA team (Chapter 4 &7). Future work should focus on evaluating the ARTA-VFA and ARTA-HIVDR^{ultralight} in regions where other HIV-1 group M subtypes are circulating.

Chapter 8 describes the development and validation of an automated sequence analysis and editing software for HIVDR testing. The analysis of mutation mixtures in sequencing analysis currently remains labour intensive and fairly subjective depending on the experience

of the user. The automated software can be used to reduce subjectivity, time taken for analysis which is often the rate-limiting step and thus improving the turn-around time and clinical relevance of the assay. This software is capable of performing base-calling, assigns quality values, aligns query sequences against a set reference, infers a consensus sequence, identifies the HIV type, and subtype, translates the nucleotide sequence to amino acids and reports insertions/deletions, premature stop codons, ambiguities and mixed calls. Compared to reference software, which is very operator dependent such as Sequencher (Gene Codes Corporation, USA) and SeqScape (Applied Biosystems, USA), this software performed extremely well, with minor discrepancies noted**.** Other freeware software able to perform automated basecalling and editing has subsequently been made available online, capable of performing the same/similar functions [42].

Overall, the results of the work summarized above meet the ARTA objectives in that they offer simple, affordable, "open-platform" alternatives to currently used methods for virological failure monitoring, and accommodate a centralized approach to HIVDR with DBS testing in resource limited settings.

Future Perspectives

The success of the HAART programmes in many sub-Saharan African countries confirms that the logistical challenges of public health infrastructure, training of staff and the supply of ARV drugs can be overcome. There remain, however, many challenges to refine many of these systems. Funding remains a critical factor in the availability of ARV drugs and on the design and implementation of appropriate diagnostic tools and the frequency at which they are used. It is essential that increased and continued funding programmes (both local and

international) are available in resource-limited settings to reach the goal of universal access and to improve the quality of HIV treatment in the public sector.

Emerging evidence suggests that HIVDR prevalence is increasing as a result of the ART scale-up programmes in sub-Saharan Africa, particularly in East Africa[43–45]. There is thus an ongoing urgent need for even more affordable viral load assays, as well as cheaper and simpler HIVDR assays that provide the appropriate quality of care to HIV-infected individuals, which can be expanded for implementation in all laboratory tiers. The work described here provides the basis for affordable HIVDR algorithms, and can be expanded to include sub Saharan countries affected with various HIV-1 subtypes. In addition, the feasibility of implementing these assays in other low income countries such as India, where HIV-1 subtype C is the predominant circulating subtype could be investigated.

An attractive alternative is the implementation of novel sequencing technologies, such as next generation sequencing which can be adapted to pooling patient samples (each with unique identifier tags) to develop more sensitive and low-cost alternatives to current HIVDR genotyping methods. Options for simultaneous viral load detection and possible resistance mutations should be further explored. Appropriate training of staff in these diagnostic assays is imperative to ensure the success of such implementations. Furthermore, the extended capacity of quality-assured HIVDR testing will accommodate future testing of increased volumes of patient samples to be tested, as treatment programmes continue to grow. Current Sanger sequencing based platforms will never be feasible in wide-scale use in South Africa where program needs may conservatively require 50 0000-100 000 HIV drug resistance sequences once guidelines are accepted.

Aside from increased diagnostic and laboratory capacity, the ARV drug availability should be guaranteed to avoid ARV drug stock-outs at clinics, and based on HIVDR surveillance data, appropriate drug changes in ARV regimens should be made to circumvent certain resistance patterns. Repeated population-based HIVDR surveillance should continue to be conducted annually to inform HIV treatment programs on the prevalence of transmitted and pre-treatment drug resistance, as well as the suitability of recommended regimens.

Overall, appropriate virological monitoring of patients on HAART is possible in resource limited settings, and technological advancements are expected to make these assays more accessible in the future. This, coupled with a concerted effort from funding agencies, politicians and infrastructural advancement programmes will ensure the best quality of care for HIV-infected patients.

References - Chapter 9

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