SETTING UP OF HIV-1 ANTIRETROVIRAL RESISTANCE TESTING METHODS AND THEIR CLINICAL APPLICATIONS

By

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Abstract

Introduction: Following the World Health Organization (WHO)'s 3 by 5 initiative, antiretroviral treatment (ART) for HIV disease management has been scaled up rapidly in resource limited settings such as Zimbabwe. First line treatment options include a three antiretroviral (ARV) drug regimens compromising of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside (NNRTI). There are fears that the use of SD NVP for PMTCT and rapid scale-up of ART will be followed by a rapid emergence of primary drug resistance in the population rendering the first line treatment options ineffective. Zimbabwe has instituted 90% coverage of SD NVP: ARVs became widely available in 2003 and currently about 200000 people are on treatment in both the private and public sector. A cross-sectional study was conducted to determine the prevalence of primary drug resistance over a period of two years in a population where ART access is being rapidly scaled up.

Methods: This study was nested in a larger study on the HIV-1 Subtype C drug resistance and pathogenesis. Signed informed consents were obtained from eligible young pregnant (<25 years) women who were attending antenatal clinics in Chitungwiza, Zimbabwe. Whole blood samples were collected in EDTA for CD4 counts, using Partec Cyflow, viral load using Roche HIV monitor Amplicor version 1.5, and estimation of period of infection using BED Calypte Assay as well as presence of HIV-1 drug resistance mutation using population sequencing. Sequence analysis for the prevalence of primary drug resistance was done using the Calibrated Population resistance software of the Stanford HIVDB and the Stanford Drug Resistance Mutations List. Sequences obtained in the analysis for drug resistance were also compared with other sequences from Southern Africa as well as other parts of the world where Subtype C is the dominant HIV-1 strain driving the epidemic.

Results: Three hundred and three women with a mean age of 21 years (95% Confidence Interval, 21 to 22 years) were studied. The median CD4 count was 393 cells/uL, Interquartile range (IQR): 249-509 and among a subset of 107 women, median plasma VL was 3.70 $\log_{10}$ copies/ml, IQR: 3.1-4.2. Thirty two percent (n=236) of the women were considered to be recently infected using the BED assay, (infected within 155 days of sample collection The median CD4 count in recently-infected women was significantly higher than in women with long term infections based on the BED results (p = 0.000). There were only two test specimens (0.85%) out of 236, with evidence of drug resistance. One specimen had the Y181C mutation associated with NNRTI resistance and a second had the I85V mutation associated with protease inhibitor (PI) resistance. There was no evidence of primary drug resistance in the recently infected women. No evidence of geographic clustering of sequences among subtype C Southern African sequences was observed after phylogenetic analysis. Interestingly, subtype C sequences from India, Brazil, China and Ethiopia formed separate clusters distinct from the Southern Africa sequences.

Conclusion: The lack of evidence of HIV-1 primary drug resistance in this population of young pregnant women suggests that the use of genotypic drug resistance data to select the most optimal treatment regimen may not yet be warranted. Currently there is no evidence of evolutionary compartmentalization of the HIV-1 Subtype C epidemic in Southern Africa. This may be indicative of the absence of selective pressure driving differentially adapted sub clusters.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ANC	Ante-Natal Clinic
ART	Antiretroviral Therapy
ARVs	Antiretroviral drugs
BED	HIV-1 Subtypes B, E and D
BHIVA	British HIV Association
CD4	Clusters of Differentiation number 4
CPR	Calibrated Population Resistance
CRF	Circulating Recombinant Forms
DHHS	Department of Health and Human Services
DNA	Deoxyribonucleic Acid
dNTP	deoxy nucleotide triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
EIA	Enzyme Immuno Assay
Env	Envelope
FDA	Food and Drug Administration
Gag	Group Specific Antigens
Gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIVDR	HIV Drug Resistance
HIVDB	HIV Data Base
HIVDR-TS	HIV Drug resistance Threshold Survey
IAS	International AIDS Society

IgG	Immunoglobulin G
ML	Maximum Likelihood
mRNA	messenger ribonucleic acid
NGOs	Non Governmental Organizations
NJ	Neighbor Joining
NNIBP	Non-nucleoside Inhibitor Binding Pocket
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside/Nucleotide Reverse Transcriptase Inhibitor
NVP	Nevirapine
OD	Optical Density
PCR	Polymerase Chain Reaction
PEPFAR	President's Emergency Plan for AIDS Relief
PI	Protease Inhibitor
PMTCT	Prevention of Mother-To Child-Transmission of HIV
QA	Quality Assurance
QC	Quality Control
Rev	Regulator of virion
RLS	Resource limited settings
RRC	Resource rich countries
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT -PCR	Reverse Transcriptase Polymerase Chain Reaction
SCR	Subtype C Resistance
SD	Single Dose

SDRM	Stanford Drug Resistance Mutations
STI	Sexually Transmitted Infections
Tat	Transcriptional Transactivator
TBE	Tris Borate EDTA
TAMs	Thymidine Analogue Mutations
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

The human Immunodeficiency Virus (HIV), the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) was first discovered in 1983[Barre-Sinoussi et al., 1983]. It is a member of the genus *Lentivirus*, part of the family *Retroviridae*. There are currently two types of HIV that infect humans, HIV-1 and HIV-2. The diversity of HIV-1 is greatest in Sub-Saharan Africa, with currently nine subtypes, A to D and F, G, H, J, amd K; 43 Circulating Recombinant Forms (CRFs) and several Unique Recombinat Forms (URFs) [http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html, 2008]. HIV-1 seems to be more virulent, and transmissible than HIV-2 [Gilbert et al., 2003; Kanki et al., 1994; Marlink et al., 1994]. It is the cause of the majority of HIV infections globally. HIV-2 is largely confined to West Africa [Peeters et al., 2003].

According to the Joint United Nations Program on HIV/AIDS (UNAIDS) AIDS Epidemic Update of December 2007 an estimated 33.2 million people are living with HIV globally. The bulk of the epidemic is in Sub Saharan Africa where about 22.5 million adults and children are living with the virus. An estimated 1.7 million people were infected during 2007. The scale and trends of the epidemic in the region vary considerably, with Southern Africa most seriously affected. The sub-region accounted for 35% of all people living with HIV and almost one third (33%) of all new infections and AIDS related deaths globally in 2007 [WHO/UNAIDS, 2008].

Access to therapy, in this region most affected by the pandemic has, however been very limited with less 5% of the patients in sub Saharan Africa in need of treatment being on therapy before 2003. Fortunately, through the World Health Organization (WHO)'s 3 by 5 initiative antiretroviral (ARV) drugs have become increasingly available to treat AIDS in the region and other parts of the developing world. The WHO, the Global Fund for AIDS TB and Malaria, and the US bilateral program for 14 countries in Africa (excluding Zimbabwe) and the Caribbean, the President's Emergency Plan for AIDS Relief (PEPFAR), plans to provide effective treatment of 3 million people in the developing world by the end of 2008.

Pharmaceutical companies are reducing both prices and international trade restrictions on patented drugs, which have been the major impediments to access, to allow more equitable access to essential medicines for AIDS, TB and Malaria. Non Governmental Organizations (NGOs) and donor funded programs are increasing access to antiretroviral therapy (ART) by running ART programs parallel to the government programs in most countries. With increased access, it is hypothesized, that treatment will mitigate the stigma and fatalism associated with HIV infection and AIDS, enhance uptake of voluntary counseling and testing and promote behavioral change in affected communities and ultimately reduce the prevalence of HIV infection in many countries.

However, the gains due to the introduction of highly active antiretroviral therapy (HAART), substantial improvement in the prognosis of HIV infected patients and improvement of their quality of life [Ives et al., 2001] risk being eroded by the emergence of drug resistance. HIV-1 has been shown to have a very high replication rate with 10^{10} viral particles being produced daily in an infected person [Perelson et al., 1996]. In addition, reverse transcriptase (RT) is a very error prone enzyme,

incorporating the wrong nucleotide at least once per replication cycle [Preston et al., 1988; Roberts et al., 1988], thus resulting in large variation in new particles produced. The combination of HIV's high replication capacity and RT's propensity to make errors is of major importance to viral evolution and development of drug resistance in patients on treatment and ultimately leads to treatment failure. These factors also pose great challenges in the development of vaccines against the virus.

In the development of drug resistance, antiretroviral drugs (ARVs) exert selective pressure on naturally mutating virus. In the presence of incomplete viral suppression, quasie-species with a competitive advantage are selected and these drug resistant mutants become the dominant strains thereafter. Drug resistance is one of the most important reasons for treatment failure [Coffin, 1995]. The factors that are important in the selection of drug resistant viruses include inadequate regimen potency, inadequate drug plasma concentrations, the presence of pre-existing drug resistant mutants and poor adherence to ART as shown in Fig 1. Good adherence of the patient to therapy has been shown to be the most critical factor in successful long term ART since resistance can be avoided as drugs will exert their maximal potency. There are many factors which cause poor adherence. They include adverse effects associated with most ARVs and the high tablet load associated with some of the ART regimens.

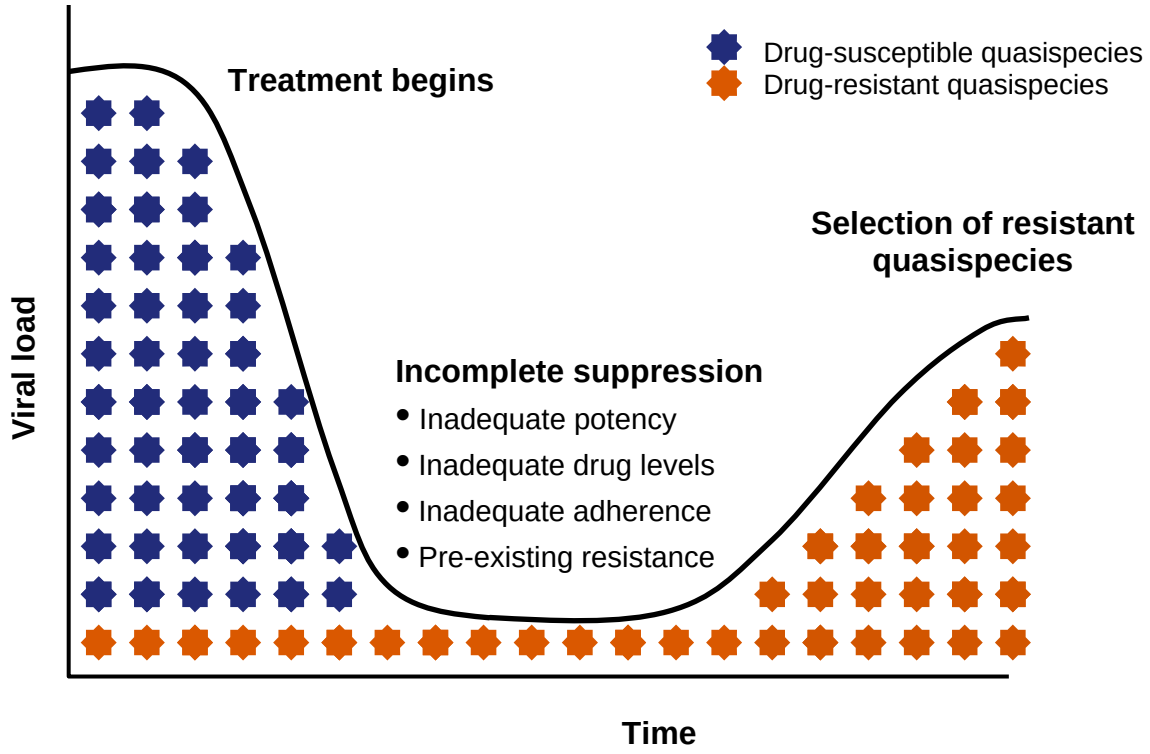


Fig 1. Factors contributing to inadequate viral suppression and the development of drug resistance in patients on treatment (Adapted from **William. A. O'Brien, 2004:** http://www.clinicaloptions.com/HIV/Treatment%20Updates/Resistance%20Testing/Modules/testing_obrien.aspx)

Development of drug resistance has great clinical implications for patients on treatment. It also poses great challenges at a public health level since the drug resistant viruses can be transmitted to newly infected individuals. This reduces their treatment options before they are even exposed to any drugs. It is therefore important to monitor the development of drug resistance in patients on ART as well as to monitor the emergence of transmitted drug resistance at a public health level.

1.1 JUSTIFICATION

The numbers of patients receiving ART in Zimbabwe has been increasing during the past five years. Currently there are about 110 000 patients on ART in both the private and the public sector. Most of them use a standard first line therapy regimen containing two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI). The most commonly used drug combinations are: Stavudine (d4T) or Zidovudine (AZT) plus, Lamuvidine (3TC) plus Nevirapine (NVP) or Efavirenz (EFV). The number of patients on treatment is, however, less that 30% of those who currently need treatment. There is therefore a need to keep expanding the ART programs in Zimbabwe. As has been shown in a number of settings where there has been a substantial increment in ART access, some patients will have treatment failure during the course of their management [Phillips et al., 2005a]. In the Development of Antiretroviral therapy in Africa (DART) study, 70 % of patients who were initiated on therapy, were still on the same combination after a year. Some had changed regimen due to various reasons including unacceptable side effects and treatment failure [DART and Team, 2006].

The Ministry of Health and Child Welfare in Zimbabwe has been using short course AZT or Single dose (SD) NVP for the prevention of mother-to-child transmission of HIV (pMTCT) since around 2000. A number of studies have shown that the use of these drugs in pMTCT may compromise the mother's response to future ART that may include these drugs or other related drugs due to drug resistance [Eshleman et al., 2001; Flys et al., 2005; Kurle et al., 2007]. Since ARVs became publicly available in Zimbabwe, either as SD NVP or short course AZT or HAART, there haven't been any systematic studies on the epidemiology of drug resistance in treatment naïve patients. However, a

small study of 87 patients on AZT/3TC/NVP conducted in Zimbabwe between 2003 and 2005 showed that 29% had developed resistance, mostly to NVP (K103N) and 3TC (M184V), during the first year on treatment [Ledwaba et al., 2007].

1.2 Aim of the Study

To gain understanding of HIV-1 Subtype C drug resistance development and the potential impact of ART roll out programs on the prevalence and the patterns of primary HIV-1 drug resistance in Zimbabwe.

1.3 Objectives

The objectives of the study were to:

1. set up genotyping methods for resistance testing, the oligonucleotide ligation assay (Rapid) and the sequencing of the *RT* and *PR* genes.
2. determine the wild type consensus sequence within subtype C HIV-1 virus for *RT* and *Protease* from RNA samples isolated from patients in the Chitungwiza subtype C study.
3. determine frequency and patterns of resistance post AZT/d4T+3TC+NVP.
4. set up a data base for subtype C HIV-1 antiretroviral resistance
5. determine the incidence of the transmission of RT gene mutations associated with NRTI and NNRTI resistance in subtype C HIV-1 among recently infected women attending antenatal clinic in Chitungwiza, Zimbabwe.

CHAPTER 2: LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 HIV-1 Replication and Antiretroviral Drugs

There are currently more than five classes of ARV drugs which can be used in various combinations for the management of HIV infection and HIV disease (AIDS), Table 1. These drugs target various parts of the replication cycle of HIV as illustrated on Figure 2. HIV enters CD4+ T cells and cells of the monocyte/macrophage lineage by the adsorption of glycoproteins on its surface to receptors on the target cell followed by the fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell.

Entry into the cell begins with the attachment of a viral envelope glycoprotein (gp), 160 to the CD4 receptor. This is then followed by the binding of the gp120 to a chemokine receptor (CCR), either CCR5 or CXCR4, which act as HIV coreceptors on the cell surface [Dragic et al., 1996; Zhang et al., 1998]. The final stage for the entry process is the fusion of the viral and host cell membranes and the entry of the viral capsid into the host cell. These series of events are the target of the first classes of ARVs, the entry inhibitors. *Enfuvirtide*, a synthetic peptide, was the first drug to be developed in this class; it mimics naturally occurring motif found in the viral protein gp41, an HIV transmembrane glycoprotein (amino acid residues 638-673), and prevents the gp41 from forming a secondary structure

necessary for successful binding to the CD4 molecules [Kilby et al., 1998; Krambovitis et al., 2005]. Another drug in this class *Maraviroc,* terminates the entry process by blocking one of the coreceptors, CCR5, necessary for successful membrane fusion and viral entry [Dorr et al., 2005].

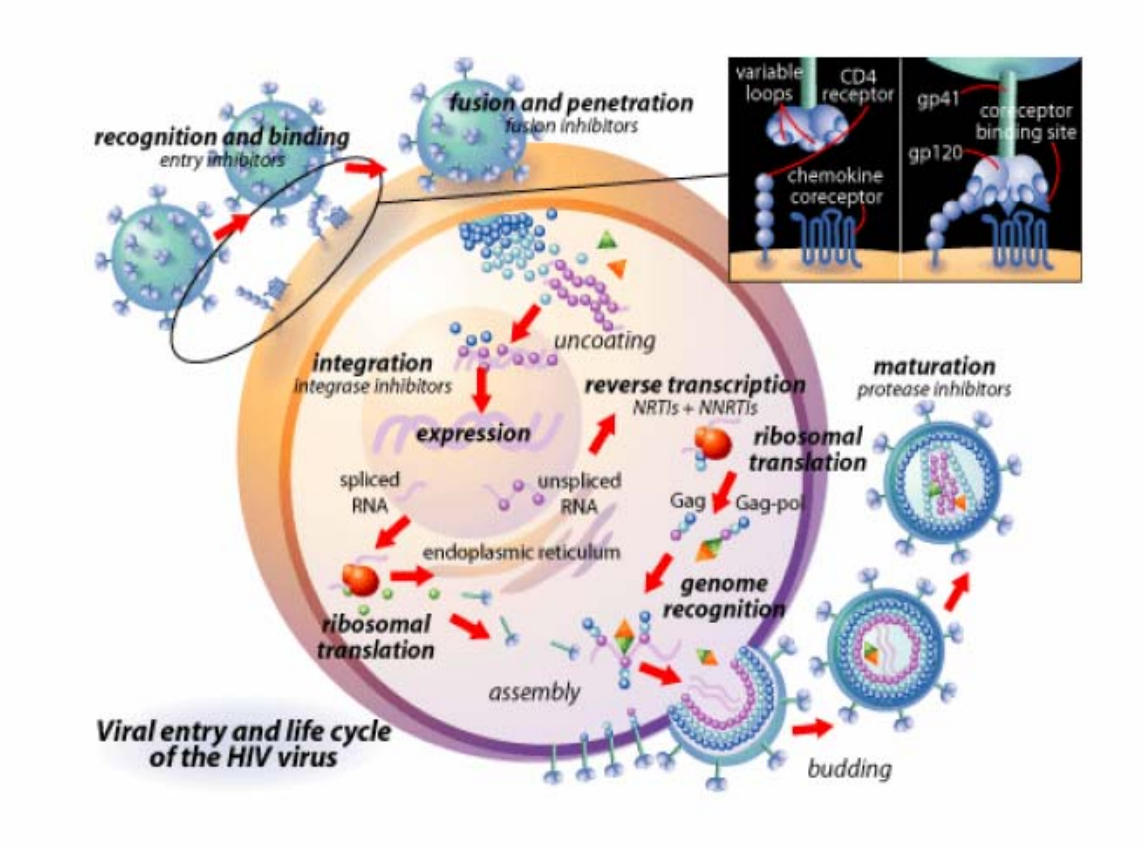


Figure 2. The HIV replication cycle depicting the targets for the current ARVs**. (Adapted from Lythgo, P. A. 2004)**

The viral capsid, composed of 2000 copies of the viral protein, p24, encloses two copies of positive sense single stranded RNA that codes for the virus's nine genes. The RNA is tightly bound to the nucleocapsid proteins, p17 and the enzymes that are needed for the establishment of an infection and

the production of new virions, HIV RT, proteases, ribonucleases and integrase [Benjamin et al., 2005; Ganser et al., 1999]. Once the viral capsid enters the cell, RT liberates the single stranded positive sense RNA from the attached viral proteins and copies it into a complementary DNA strand. Thus RT is an RNA dependent DNA polymerase.

The reverse transcription stage of the HIV infection cycle is the target of two classes of ARVs, the NRTIs and the NNRTIs. Historically, the NRTIs were the first compounds to be used clinically to treat HIV infection with the discovery of the usefulness of AZT in the management of HIV in 1985 and its subsequent registration in 1987 by the Food and Drug Administration (FDA), United States of America (USA) [Mitsuya et al., 1985] [Yarchoan et al., 1986]. AZT had been discovered as an anti cancer agent in the 1970s but had not been registered for that application because it had many side effects. The currently FDA USA registered NRTIs are listed in Table 1.These compounds are nucleoside analogues that lack the 3′-OH group in their ribose ring. When they are phosphorylated, the lack of the 3′-OH differentiates them from the physiological deoxy nucleotide triphosphate (dNTP) substrates. Their mechanism of action involves both competitive action as well as termination of chain elongation once they are incorporated into the growing chain of DNA.

Trade Name	Drug	Abbri-viaton	Analogue Nucleosides (NRTIs only)	Manufacturer	Generic Approvals
Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)					
Epivar™	Lamivudine	3TC	Deoxycytidine	GSK	Yes
Ziagen™	Abacavir	ABC	Deoxyguanosine	GSK	Yes
Retrovir™	Zidovudine	AZT	Deoxythymidine	GCK	Yes
Zerit™	Stavudine	D4T	Deoxythymidine	BMS	Yes
Videx™	Didanosine	DDI	Deoxyadenosine	BMS	Yes
Emtriva™	Emitricitabine	FTC	Deoxycytidine	Gilead	No
Viread™	Tenofovir	TDF	Adenosine Nucleotide	Gilead	Yes
HIVID™	Zalcitabine	DDC	Deoxycytidine	Roche	No
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)					
Rescriptor™	Delavirdine	DLV		Pfizer	No
Sustiva/Stocrin™	Efavirenz	EFV		BMS/MSD	Yes
Intelence	Etravirine	TMC-125		Tibotec	No
Viramune™	Nevirapine	NVP		BI	Yes
Protease Inhibitors (PIs)					
Reyataz™	Atazanavir	ATV		BMS	Yes
Prezista	Duranavir	DRV		Tibotec	No
Telzir/Lexiva™	Fosamprenavir	FPV		GSK	No
Agenerase™	Amprenavir	APV		GSK	No
Crixivan™	Indinavir	IDV		MSD	No
	Lopinavir	LPV		Abbott	No
Norvir	Ritinovir	RTV		Abbott	No
Viracept™	Nelfinavir	NFV		Roche/Pfizer	No
Invirase 500™	Saquinavir	SQV		Roche	No
Aptivus™	Tipranavir	TPV		BI	No
Fusion Inhibitors					
Fuzeon™	Enfuvirtide	T-20		Roche	No
Chemokine Coreceptor Antagonist					
Celsentri/Selzentry™	Maraviroc	MVC		Pfizer	No
Integrase Inhibitors					
Isentress	Raltegravir	RAL		Merck	No

Table 1. Currently FDA USA registered ARVs

For the NRTIs to achieve this they act as triphophates and are phosphorylated intracellularly hence can be considered as prodrugs. Tenofovir exists as a monophosphate and needs two additional phosphorylation steps whereas the rest of the other NRTIs undergo three phosphorylation steps [Balzarini et al., 1989; Dahlberg et al., 1987; Furman et al., 1986; Kim et al., 1987; Vivet-Boudou et al., 2006].

The NNRTIs consist of chemically diverse compounds that bind to a hydrophobic pocket, non-nucleotide inhibitor binding pocket (NNIBP), located approximately 10 angstroms away from the RT polymerase active site. They do not prevent template: primer or dNTP-binding, but cause misalignment of the template: primer in relation to the catalytic site so that the incoming dNTP cannot be incorporated. The currently available NNRTIs, Delavirdine (DVD), NVP, EFV and Etravirine (TMC-125) have adequate to strong potency against wild type HIV strains [Hargrave et al., 1991; Merluzzi et al., 1990; Tantillo et al., 1994; Young et al., 1995].

After reverse transcription, the viral DNA is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by the viral enzyme integrase. The process involves binding of the enzyme onto the cellular DNA and its preparation for integration. The process culminates in the transfer and integration of the viral DNA into the host DNA. This sequence of events is the target of the newest class of ARVs, the integrase inhibitors. *Isentress* is the only drug in this class currently approved.

Once the viral DNA is integrated into the host genome, it may then lie dormant, in a latent stage of the infection where viral replication is minimal. To actively produce new viruses, certain cellular

transcription factors are necessary, the most important of which is Nuclear Factor kappa B (NF kβ), which is up regulated when T-cells become activated. The integrated provirus is copied into mRNA which is then spliced into smaller pieces. These small pieces produce the regulatory proteins, transcriptional transactivator (Tat)-which promotes new virus production and regulator of virion (Rev)-which promotes nuclear export, stabilization and utilization of the unspliced viral mRNAs. As Rev accumulates it starts to inhibit mRNA splicing. At this stage, the structural proteins, the group specific antigens (gag) and the envelope (env) are produced from full length mRNA. The full length RNA is actually the virus genome; it binds to the gag proteins and is packaged into a new virus.

The final step of the viral replication cycle, assembly of the new HIV virions, begins at the plasma membrane of the host cell. The env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by the protease and processed into HIV env glycoproteins gp41 and gp120. These are transported to the host cell surface where gp41 anchors the gp120 to the membrane of the infected cell. The gag (p55) and gag-Pol (p160) polyproteins are also associated with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional proteins and enzymes. The various components are then assembled to produce a mature HIV virion.

The polyprotein cleavage step is a target of another class of ARVs, known as the protease inhibitors (PIs). The HIV protease consists of two identical 99-amino acid subunits, and has an active site that lies at the dimmer interface, with each monomer contributing a single catalytic aspartic (D) acid

residue (D25 and D24'). The substrate-binding cleft that surrounds the active site contains both hydrophobic and hydrophilic elements. Each monomer of the protease also contributes amino acids (position 45-56) to form a flap that extends over the substrate binding cleft. The cleft must be flexible enough to allow entry and exit of the polypeptide substrates. For each substrate, three to four amino acids located on either side of the peptide bond cleavage site are utilized for binding to the substrate cavity of protease. Protease must cleave the immature HIV polyprotein precursors, gag and gag-pol, in at least nine different cleavage sites. All currently approved protease inhibitors are competitive inhibitors of the protease.

2.2 Highly Active Antiretroviral Therapy

The currently available ART regimen combinations cannot completely eradicate HIV from the body hence the main goal of ART is prolongation of life and improvement of the quality of life for the HIV infected patients. Therapies should result in the reduction of the viral burden to levels below the detection limits of the currently available tests (20-50 viral RNA copies/ml) for as long as possible and halt disease progression and prevent or delay progression. This will lead to immune reconstitution that is both quantitative (as measured by CD4 counts) and qualitative (as demonstrated by pathogen specific immune response). Due to the long term nature of ART, it is important that the drugs are sequenced in such a way that they achieve clinical (reversal of symptoms), virologic (reduction of viral load), and immunological (immune reconstitution) goals while maintaining treatment options, limiting drug toxicity and facilitating adherence. Epidemiologically, treatment should be able to reduce HIV transmission and halt the HIV epidemic by suppressing the viral load in potentially infectious patients

thereby reducing the chances of viral transmission if they have unprotected sex with uninfected individuals.

The current general recommendations for commencement of ART is to assess the patients individually and make decisions to treat in each patient based on the appearance of AIDS defining symptoms, the CD4+ T-Cell count, the progressive loss of CD4+T-Cells, HIV plasma viral load, age and social situation including the patient's understanding of the importance of adherence. The absolute CD4 count is the most important consideration in decisions to initiate ART in HIV infected individuals aged 5 years and above (ART commencement in those under age of 5 years is based on CD4% count) according to all major treatment guidelines, WHO, International AIDS Society USA (IAS-US), the USA Department of Health and Human Services (US-DHHS) and the European guidelines. Although a few years ago all these bodies agreed that ART should be commenced at CD4 counts of 200 cells/µl or less, recent guidelines are advocating for commencement of ART at CD4 count levels of 350 cells/µl or less plus WHO clinical stage III and higher. However, there is conflicting data on the benefit of starting treatment late (200 cells/µl or less) [DHHS, 2008; Palella et al., 2003; Phillips et al., 2001; WHO, 2006]. In some resource limited settings (RLS) with no access to CD4 count machines, WHO recommends initiation of ART in patients with WHO clinical disease stage III or greater which include HIV wasting, chronic diarrhea, chronic fever of unknown origin, active pulmonary disease, disseminated military tuberculosis, Kaposi's sarcoma, recurrent invasive bacterial infections or recurrent mucosal candidiasis. Factors that influence ART prognosis include viral load, CD4 count trajectory, age, and the patient's ability to adhere to the prescribed regimen.

All treatment guidelines currently recommend the use of ART in the form of combination therapy for higher efficacy and the minimization of the emergence of drug resistance.HAART**,** which is a combination of three or more drugs from at least two classes, has been shown to be highly efficacious [Brodt et al., 1997; Hammer et al., 1996]. The most common practice for 1st line therapy is to use two NRTIs plus one NNRTI or PI [BHIVA, 2008; DHHS, 2008]. However there is evidence of higher immunological and virological potency for the two NRTI and one NNRTI combination regimen in treatment of ART naïve patients than the use of two NRTI plus one PI combination [Robbins et al., 2003; Staszewski et al., 1999; Torre et al., 2001].

Use of drug resistance data to guide the selection of an ART regimen for patients commencing therapy in settings of high primary drug resistance prevalence or in patients switching therapy has been demonstrated to result in better clinical outcomes than without. The study on genotypic analysis for resistance testing (GART) and the Viradapt study were the first prospective trials to use genotypic resistance testing in patient management [Baxter et al., 2000; Durant et al., 1999].Both studies showed that resistance testing applied to patient management resulted in better short- to medium-term viral suppression than was achieved by expert advice alone. The results of these two studies were substantiated by two other studies, HAVANNA, VIRA 3001 and NARVAL, although the result in the latter study was less convincing [Meynard et al., 2000; Tural et al., 2000]. Remarkably, the quantitative benefit of all these studies was quite similar: about 0.5 $\log_{10}$ copies/ml greater viral suppression than was achieved without use of the test. While the magnitude of viral suppression looked small, such reductions of viral load are similar to the activity of antiviral agents seen in the early days of NRTI monotherapy which were associated with clinically significant reductions in morbidity and mortality, clinically significant improvements in CD4+ cell count, and improved durability of the

suppressive effects of the regimen. Furthermore, patients in both arms of the prospective trials (GART and Viradapt studies) were managed by highly experienced clinicians more likely to empirically select an active regimen for the comparator, non-resistance-tested arms of the studies. Thus, the study results likely minimized the perceived effect of resistance testing. An analysis of the HAVANNA trial results highlighted this effect [Tural et al., 2002]. Clinical experience has often shown much more substantive improvement in viral suppression in individual patients with resistance tests results to aide in the selection of the treatment regimen. Hence the current recommendations to use drug resistance test to guide the selection of the treatment regimen by a number of expert panel guidelines in the US and in Europe including the IAS-USA, the US-DHHS, the British HIV Association (BHIVA), and the EuroGuidelines Group for HIV resistance [BHIVA, 2008; DHHS, 2008; EuroGuidelines Group for HIV Resistance, 2001; Hammer et al., 2006].

Most of the methods available for drug resistance testing are, however, very expensive to be routinely used in most RLS. Thus, WHO recommends a public health approach to ART for RLS instead of personalizing therapy by using drug resistance tests to select the most optimal treatment regimen [Gilks et al., 2006]. The public health approach to ART delivery focuses on optimizing therapy at the population level through standardized sequencing of the available ARVs, delivered to individuals by means of simplified approaches and supported by clinical and basic laboratory monitoring.

As with most other countries that have incorporated ART in routine HIV care, the scale up in ART access in RLS will most likely be accompanied by the emergence of drug resistance. WHO has therefore made recommendations that specific strategies to evaluate and limit HIV Drug Resistance (HIVDR) be included in all national HIV treatment and prevention plans. The goals of these strategies

would be to use a standard methodology for regular population level evaluations of the emergence and transmission of drug resistance; to implement ongoing evaluation of ART program factors potentially associated with the emergence of HIVDR; and to support evidence based recommendations for maintaining the effectiveness of ART regimens and limiting HIVDR transmission.

2.3 Antiretroviral Therapy Monitoring

Success and failure of ART can be evaluated using virological, immunological or clinical criteria. These three are often associated with each other; however they should be reviewed separately. The earliest response to ART is the virologic response as measured by the plasma HIV-1 viral burden. This is followed a little later by the immunological response as measured by the CD4 cell count. In treatment failure, the viral load starts going up, followed by a decline in CD4 cell count and clinical failure only becomes apparent much later. Hence the importance of regular laboratory monitoring through viral load determination and CD4 cell count for all patients on ART.

Virologic treatment success is generally a decrease in viral load to below the level of detection of 50 RNA copies/ml. This is based on the experience that, the more rapid and greater the decrease in viral load, the longer the therapeutic effect [Kempf et al., 1998; Powderly et al., 1999]. For patients on ART, viral load declines in two phases; an initial, very rapid decrease in the first few weeks followed by a slower phase, in which viraemia declines only slowly. It should take 3-4 months to reach levels below detection. This may take longer for patients with very high baseline viral loads. However, a viral load above the level of detection 6 months after initiation of therapy is always considered treatment failure regardless of the baseline viral load [Clumeck et al., 2008; DHHS, 2003; Hammer et al., 2008].

The most important risk factors for virologic ART treatment failure are previous ART, pre-existing resistance (acquired or primary) and poor treatment compliance [Deeks, 2000]. Other factors include psychosocial factors that affect adherence and also pharmacogenetic and pharmacodynamic factors that may affect the therapeutic levels of the drugs as well as the potential to develop severe adverse drug reaction as with the case with abacavir (ABC), EFV and NVP [Bjornsson and Olsson, 2006; Clay, 2002; Haas et al., 2004; Hewitt, 2002; Jackson et al., 2003; Jamisse et al., 2007; Marzolini et al., 2001; Phillips et al., 2005b].

Immunological treatment success is an increase in the CD4+ T cells, however this is not defined as precisely as for the viral load [Mellors et al., 1997]. The most important immunological goal in ART is to achieve a CD4+ T cell count above 200. Treatment failure is usually defined as the absence of an increase or as a decrease in the CD4 count in patients receiving ART [O'Brien et al., 1997]. The CD4 count is the best predictor of an individual's risk to AIDS [O'Gorman and Zijenah, 2008].

It is difficult to individually predict the immunological success of therapy for patients on ART, as it varies significantly from one person to another. As with the decrease in viral load, the increase in CD4 count also occurs in two phases. After a first, usually rapid increase over the first three to four months, further increases are considerably less pronounced. In a prospective study involving some 1,000 patients, the CD4-cell count increased during the first three months by a median of 21.2 CD4 cells/µl per month; in the following months the increase was only 5.5 CD4 cells/µl [Le Moing et al., 2002]. It is still under debate whether the immune system is restored continuously after a long period of viral suppression or whether a plateau is reached after three to four years [Smith et al., 2004; Viard et al.,

2004]. The immunological success is not predictable in individual cases. However, the lower the CD4 cell count at baseline, the less likely they are to normalize completely [Kaufmann et al., 2005; Kaufmann et al., 2003; Valdez et al., 2002]. The immune system often does not recover completely. In the Swiss Cohort, only 39 % of 2,235 patients who had initiated HAART in 1996-97 reached a CD4-cell count above 500/µl [Kaufmann et al., 2003]. The introduction of treatment within the first 3-6 months of infection possibly provides certain clues as to how well the immune system will be restored [Kaufmann et al., 2005].

Immunological treatment success is not necessarily linked to maximal viral suppression; even partial suppression can result in improved CD4-cell count [Kaufmann et al., 1998; Ledergerber et al., 2004; Mezzaroma et al., 1999]. The initial level of viral load is also not significant; what seems to be decisive is that the viral load remains lower than before treatment [Deeks et al., 2002; Ledergerber et al., 2004]. In view of the many factors that occur, which are able to influence the success of therapy as well as the individual regeneration capacity (independent of ART), it does not makes sense to depend on the CD4-cell count as the deciding criterion for the success of ART. Virological success is more appropriate for judging the efficacy of specific regimens. However, for most patients in RLS, the currently available methods for viral load determination are very expensive so they have to rely more on the relatively cheaper CD4 measurement plus clinical monitoring.

2.4 HIV-1 Drug Resistance (HIVDR)

The emergence of drug resistance is likely to develop when the drug selection pressure is suboptimal and permits the virus to continue replicating in the presence of the drug. About 10^{10} HIV virions are

produced in an HIV-1 infected individual daily [Kelly, 1996; Wei et al., 1995]. The replication errors caused by the RT's infidelity in reverse transcription results in the incorporation of wrong nucleotides in the growing DNA chain and may lead to changes in the amino acid sequence of the resultant protein. These changes or mutations are defined as amino acid differences from one of several HIV-1 wild type reference sequences. The most commonly used reference sequences are the laboratory isolates HXB2 and NL43 and a consensus reference sequence comprising the most common amino acid at each position in wild type subtype B viruses (Subtype B consensus) [Ratner et al., 1987]. These sequences are nearly identical, differing in only a few amino acids not involved in drug resistance.

Mathematical modeling have shown that the error rate of RT combined with HIV-1's genome size of 9200 nucleotides plus its replication rate, will allow every possible single-point mutation to occur up to 10000 times per day in an infected person [Coffin, 1995]. So all single mutations, that can contribute to resistance to ARVs may exist before the person has been exposed to the drugs. Incomplete viral suppression due to factors shown earlier in Figure 1 may lead to the selection of drug resistance and ultimately treatment failure.

Apart from its importance in the patient's response to medication, the evolution of drug resistance in populations of HIV infected individuals is a very important tool in efforts at controlling the HIV and AIDS epidemics. In patients who are receiving ART, the presence of drugs exerts some selection pressure on the already existing quasi-species of viruses in circulation. Viral adaptation to the drug pressure is characterized by the initial selection of deleterious mutations that generally reduce drug susceptibility and viral fitness. Such mutations are commonly known as primary or major drug resistance mutations and are relatively specific for each drug. The deleterious effect of the primary

mutations to the virus is efficiently reduced by the selection of additional compensatory mutations known as secondary or minor mutations. Such compensatory mutations result in the restoration of structure and function of the affected protein and generally increase the level of drug resistance. However, despite the accumulations of compensatory mutations, drug resistant viruses generally display reduced fitness compared with wild type viruses. The interruption of ART in treated patients carrying drug resistance viruses usually results in the rapid overgrowth of mutant viruses by wild type viruses from archived populations. Although the clonality of the transmitted virus population is limited, some studies suggest that HIV-1 transmission may involve infection with an oligoclonal viral population [Delwart et al., 2002; Keele et al., 2008]. In this setting and in the absence of treatment, transmitted drug resistant viral populations gradually evolve back to the fit wild type viruses.

As depicted in Figure 3, selection and evolution of drug resistance during treatment is generally associated with a stepwise decrease in drug susceptibility and viral fitness.

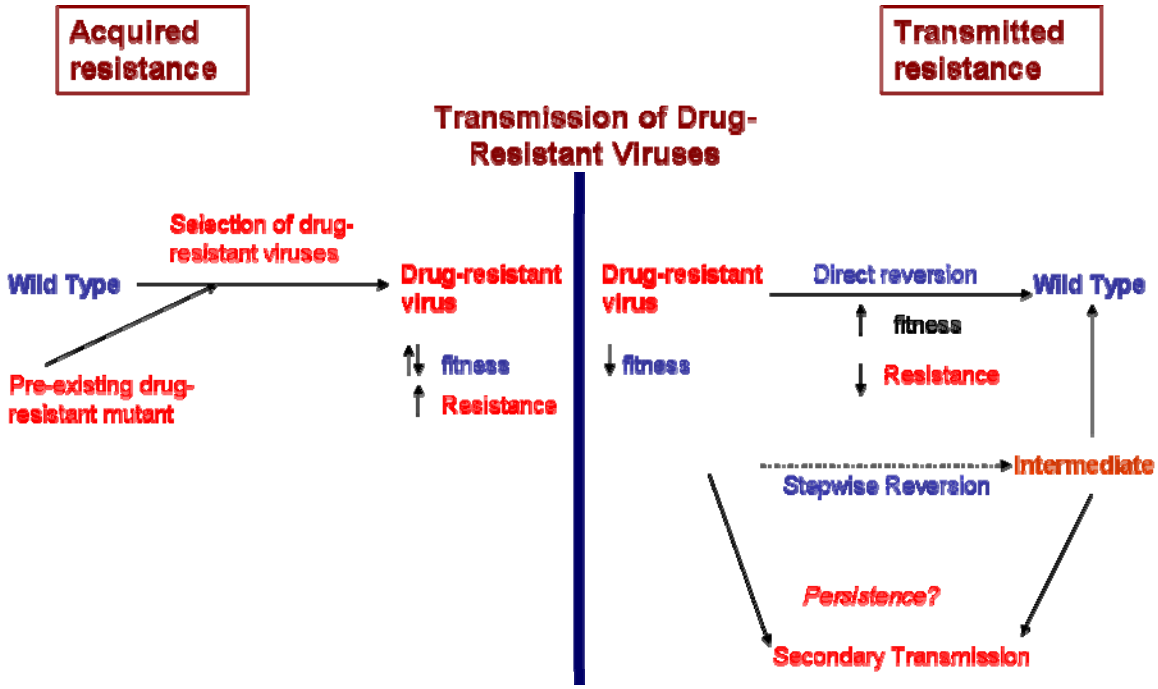


Figure 3. Evolution of acquired and transmitted resistance.

Following transmission, drug resistant viruses may persist for prolonged periods of time or may gradually lose mutations that confer a high fitness cost as they evolve to more fit viruses. Reversion may occur through a direct loss of mutations or through a stepwise process involving intermediates. The rate of reversion and persistence may have important implications to detectability and subsequent secondary transmission.

The determinants of the persistence and pathways of reversions of transmitted resistance are not fully understood. It is generally accepted that mutations that confer a low or moderate fitness cost have the potential to persist for longer periods of time as opposed to those that have a high impact on viral fitness. For instance, fit mutants carrying the lysine (K) to asparagine (N) mutation at codon position 103 (K103N) that is associated with NNRTI resistance are able to persist for as long as 3 years

compared to the relatively rapid reversion observed in less fit mutants carrying the methionine (M) to valine (V) (M184V) mutation associated with NRTI resistance. However, the rate of reversion when low fitness mutants are allowed to replicate may also depend on other factors including the characteristics of the original innocula (oligoclonal or polyclonal), immune selective pressures, and characteristics of the infecting virus such as the number and type of resistance mutations

2.4.1 HIV-1 Drug Resistance (HIVDR) Testing

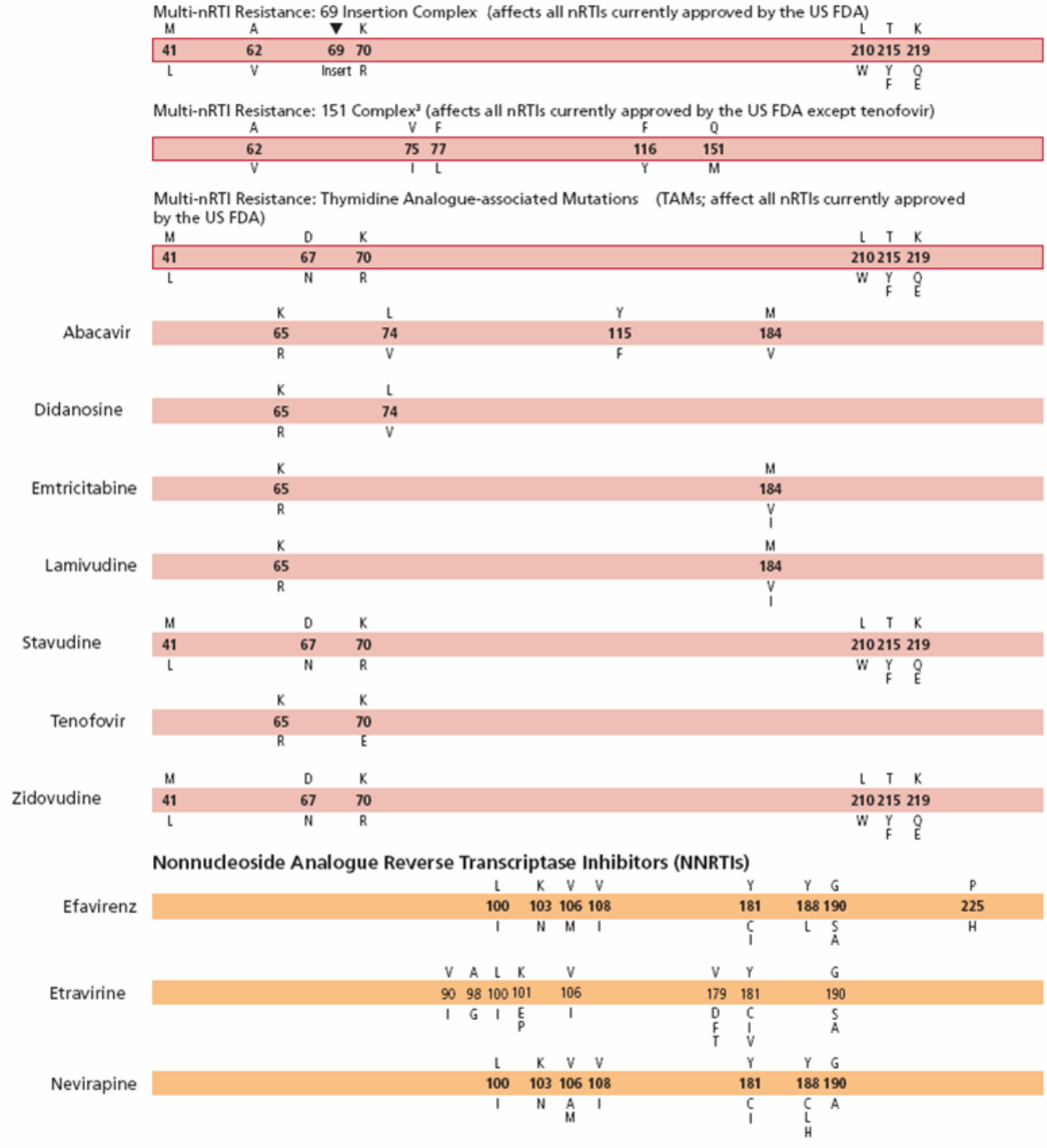
There are two main classes of tests used in HIVDR monitoring, the phenotypic and genotypic tests. Phenotypic tests measure the ability of the viruses to replicate in various concentrations of ARVs in culture. RT and protease gene sequences derived from patient plasma HIV RNA are inserted into the backbone of a laboratory clone of HIV, either by cloning or in vitro recombination. Replication of the recombinant virus at different drug concentrations is monitored by expression of a reporter gene and is compared to a reference strain. The result is commonly expressed as the minimum inhibitory concentration of the drug necessary to inhibit viral replication by a certain percentage, for example 50% inhibitory concentration (IC_{50}) or 90% (IC_{90}). The minimum IC result of the sample is compared to that of the reference viruses and the results are reported as the fold increase in the minimum IC. This provides data for any drug added to the test, including cross resistance. Examples of commercially available phenotypic tests include PhenoSense HIV (Monogram) assays [Qari et al., 2002a; Qari et al., 2002b].

The genotypic tests map the genetic sequences of the virus and compare it to a reference sequence such as the HIV HXB2 sequences. Through this any changes in the amino acid sequence that would have

taken place in the gene of interest are identified. These changes are then compared using various mathematical algorithms to mutations that have been demonstrated to cause drug resistance in clinical specimens or in vitro studies. There are a number of programs that have been developed for use in the analysis of sequence data and interpretation of drug resistance information obtained from those sequences. These include the Stanford HIV Drug Resistance Database (Stanford HIVDB), the Agence Nationale de la Recherche sur le SIDA (ANRS) system, the Retrogram, and the Rega Institute System. There are also other genotypic interpretation systems that are specific to commercially available drug resistance kits and some that are specifically at certain reference laboratories such as Monogram. A list of all known significant resistance associated mutations in the RT, protease and env gene is maintained by the IAS-USA (http://www.iasusa.org/resistancemutations) [Johnson et al., 2008], as shown in Figure 4 below.

MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (nRTIs)



Nonnucleoside Analogue Reverse Transcriptase Inhibitors (NNRTIs)

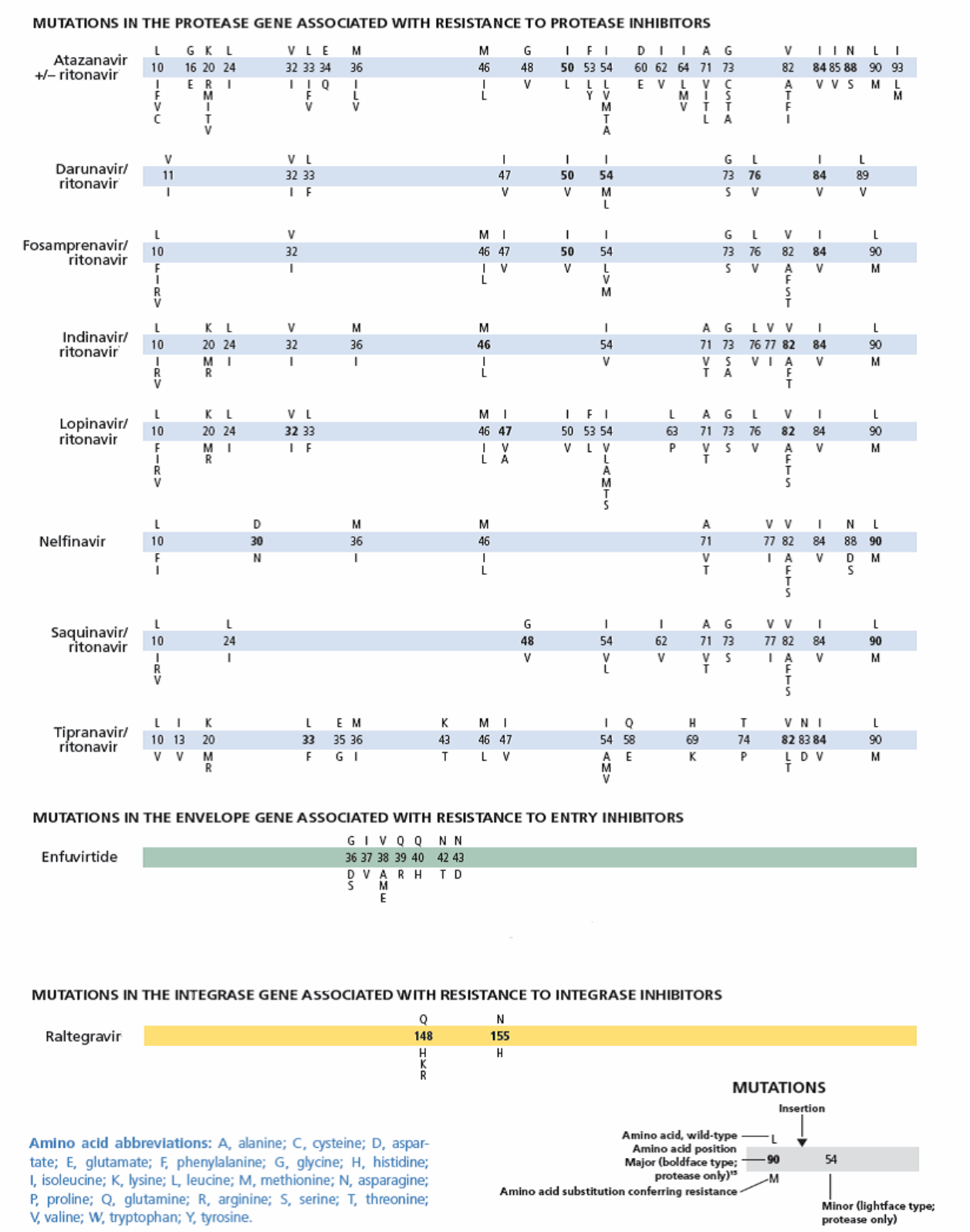


Figure 4. Current list of known HIV-1 drug resistance mutations by the IAS-USA

2.4.2 Epidemiology of HIV-1 Drug Resistance (HIVDR)

HIVDR, whether acquired or transmitted is of major public health concern. Transmitted drug resistance has the potential to rapidly reverse the effectiveness of first line ART at a population level. Countries that have been using ART since the introduction of AZT monotherapy have seen an increase in the prevalence of drug resistance in patients on ART [Richman et al., 1991; Richman et al., 1994; Susman, 2002; Winters et al., 1997]. The use of ART as monotherapy or in combination (HAART) has led to the transmission of drug resistant viruses to primary drug resistance in newly infected patients [Blower et al., 2001]. Transmitted HIVDR has been reported for a number of cohorts in North America and Western Europe. In the United States primary drug resistance varied from 8.3% in a study of 10 US cities to 23% in San Francisco. In Western Europe, these estimates varied from 10% to 16% in the United Kingdom, 11% in a study of 15 European cities and Israel, and 23.7% in Madrid [Fox et al., 2006; Wensing et al., 2005; Wensing et al., 2003]. The high levels of primary drug resistance in these settings may be attributed to the early days of HIV mono- or dual-ART before the advent of the three or more ARV regimens. These mono- and dual treatment regimens were associated with incomplete viral suppression and the selection of drug resistant viral quasie-species.

During the past five years, access to ART has been increasing in most RLS including sub Saharan Africa which has the highest burden of the HIV and AIDS epidemic [UNAIDS, 2007; UNAIDS/WHO., 2005]. Ideally, all the millions of people in need of ART in RLS should commence treatment, however there are fears that aggressive rolling out of ART may pose long term challenges due to the emergence of HIVDR especially when factors such as intermittent drug supply, drug stock-

outs, poor patient monitoring, incorrect prescribing practices and low adherence are not properly addressed.

The impact of pMTCT programs that use SD NVP or short course AZT on the future antiretroviral drug options for the mothers remains controversial [Jackson et al., 2000]. There are currently studies showing that drug resistance mutations acquired after the use of SD NVP may persist for more than six months [Johnson et al., 2005; Kassaye et al., 2007; Lee et al., 2005]. One study has reported virologic ART failure in women with pre-exposure to NVP for pMTCT interventions who commenced a three ART-NVP containing regimen, within six months, for their own HIV disease [Jourdain et al., 2004]. Studies from Southern Africa have reported that treatment failure associated with previous exposure to SD NVP for pMTCT may be related to when ART is commenced following the pMTCT interventions [Ledwaba et al., 2007; Lee et al., 2005; Lockman et al., 2007; Zijenah et al., 2006]. Indeed, drug resistance mutations selected by SD NVP has been shown to become undetectable with time [Coffie et al., 2008; Kassaye et al., 2007]

To monitor the impact of accelerated ART access and pMTCT programs on the emergency of primary and secondary drug resistance, the WHO prepared protocols for monitoring drug resistance in settings were ART is already in use or is rapidly being scaled up. For monitoring transmitted drug resistance the protocol used is the HIVDR threshold survey (HIVDR-TS) which was designed to supplement HIV sentinel surveys, conducted routinely in many RLS with generalized epidemics. HIV DR genotyping is performed on a limited number of eligible HIV positive specimens collected in the HIV sentinel surveys. The target population for most sentinel surveys is generally pregnant women attending antenatal clinics (ANC). Using ANC sentinel surveys, evaluations of recently infected women only is

facilitated by restricting to specimens from women below the age of 25yrs, and on their first pregnancy. The method uses a binomial sequential sampling technique and up to 47 consecutively collected eligible specimens are used in the survey. HIVDR-TS does not precisely estimate the prevalence of drug resistance, instead, it classifies for each drug/drug class as <5%, 5-15%, and >15%. If >5 specimens with HIVDR are found when 14-24 specimens have been genotyped, then the HIVDR prevalence to that drug or drug class is classified as "high prevalence" (>15%). If no specimens with one or more known mutations associated with HIVDR has been found after the 34th specimen is genotyped, the prevalence is classified as "low prevalence" <5%. If the maximum survey sample size (47) is reached without either limit being crossed then sampling stops and the population is classified as moderate prevalence (5-15%). In cases where prevalence is classified as <5%, the survey is repeated two years later. If it is in the higher categories, additional surveys or more resource intense surveillance may be required as well as additional public health actions [Bennett et al., 2008a; Myatt and Bennett, 2008; ResNet, 2005].

The results that have been published to date from a number of African countries have been showing that the prevalence of drug resistance has been below 5%. Almost all these studies were done among young pregnant women attending antenatal clinics [Bessong et al., 2006; Handema et al., 2003; Kassu et al., 2007]. This is because the infrastructure for the ANCs in most African countries has wide coverage and provides easy access to a population that is potentially representative of the whole population. A lot of infrastructure has also been set to support different HIV pMTCT programs that have been going on for a while in most countries. There are other studies that published HIV primary drug resistance data before the implementation of the HIVDR-TS. Most of the results from these studies showed primary drug resistance prevalence below the HIVDR-TS threshold limit of 5%

[Bessong et al., 2006; Handema et al., 2003; Kamoto and Aberle-Grasse, 2008; Kassu et al., 2007; Maphalala et al., 2008; Pillay et al., 2008]. The current studies following the HIVDR-TS protocols are more standardized hence allow easy comparison of results between different studies or countries. These studies provide good baseline data for future monitoring of ART programs. They also provide good baseline data for studies comparing the pathways for the evolution of HIV DR in populations where subtype C is predominant compared to the relatively well characterized patterns in Subtype B.

CHAPTER 3: MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Study Design

The study was nested in a National Institutes of Health USA sponsored larger cross sectional collaborative study between the Stanford University Medical School and the University of Zimbabwe College of Health Sciences, whose primary aims were to determine the prevalence of HIV-1 subtype C drug resistance and co-receptor tropism in a population of ART naïve and HAART experienced women attending ANCs in Chitumgwiza, Zimbabwe as well as to determine the co-receptor usage of the viral samples. The subtype C study enrolled 400 HIV-1 seropositive pregnant women over a period of two years (2006 to 2007). The protocol was approved by the Medical Research Council of Zimbabwe, Research Council of Zimbabwe and the Stanford University Institutional Review Board.

The parent study was conducted in two parts, the first part focussed on primary drug resistance in treatment naïve patients and the second, on secondary drug resistance in patients on HAART. In this study, the focus was on the first part, looking at the prevalence of primary drug resistance in the population studied. This objective was also expanded to look at the extent of geographic clustering of the sequences generated when compared to sequences generated from other countries where HIV-1 Subtype C is the dominant subtype. The second part which was a clinical trial to assess impact of AZT/d4T+3TC+NVP on pMTCT as well as development of drug resistance was not approved by the

Medicines Control Authority of Zimbabwe. Hence the frequency and patterns of resistance post AZT/d4T+3TC+NVP could not be determined.

3.2 Sample Collection, Storage and Use

Young pregnant women attending ANC, who had been counseled, tested and found HIV positive and were not yet on ART, were referred to the anonymous subtype C drug resistance (SCR) study. The SCR study protocol was explained to the volunteers from the ANC and signed informed consent was obtained from the women at enrollment. A comprehensive medical history and demographic data questionnaire was administered, and 2 x 5 ml of whole blood in ethyl diamine tetra acetic acid (EDTA) vacutainer tubes (BD Biosciences, San Jose, CA, USA) were collected from each participant. Two hundred microlitres (200µl) of blood was aliquoted for enumeration of absolute CD4 counts within 6 hours of phlebotomy. Plasma, for quantitation of virus load, BED Calypte assay and pol sequencing for drug resistance was obtained within 6 hours of phlebotomy by centrifuging the remainder of the blood at 200g for 10 minutes using a bench centrifuge (Centronic, J. P. Selecta, sa: Barcelona, Spain) The plasma samples were aliquoted (500µl in each tube) and then stored at -80°C until the assay were performed.

3.2.1 Enumeration of CD4+ T-Lymphocyte Cell counts

Absolute CD4+ T lymphocyte counts were enumerated (within six hours of blood collection) using a Partec Cyflow counter (Cyflow, Partec, Munster, Germany), which is a single platform volumetric flow cytometer with an inbuilt software for automatic calculation of absolute CD4+ T cells. Twenty microlitres (20µl) of whole blood in EDTA tubes was pipetted into a Partec tube, 20µl of CD4 –

phycoerythrin labeled monoclonal antibodies (also supplied by Partec, Munster) was then added to the tube. The 40µl reaction mixture was then incubated for 15 minutes in the dark (cupboard), following which 800 µl of Partec No Lyse Buffer was added to make volume of 840 µl. The contents were then gently mixed after which the tube was plugged into the Partec Cyflow Counter sample port for automatic CD4+ T lymphocyte enumeration. The histogram generated contains two peaks corresponding to two populations of CD4+ cells, the weakly stained and less dense monocytes and the CD4+ lymphocytes on the left and right of the histogram respectively. The CD4+ T cell peak was then used to automatically calculate the absolute CD4+ T cells per microlitre.

3.2.2 HIV-1 Plasma Viral Load Determination

The prototype Amplicor Monitor version 1.5 ultra sensitive assay (Roche Diagnostic Corporation, Indianapolis, IN, USA) with a detection range of 50-100 000 copies per ml was used to quantify virus load in plasma samples.

3.2.2.1 Reagent Preparation

A working master mix (MMX) was prepared by adding 100 µl of HIV-1 Mn^{2+} to a 700 µl solution of HIV-1 master mix containing the dNTPs, biotinynlated primers, Amperase and rTth polymerase (provided with the kit). Fifty microlitres (50µl) of the working MMX was added to 200 µl PCR tubes for each of the samples and control.

3.2.2.2 Specimen and Control Preparation

Frozen plasma samples and normal human plasma (supplied with the kit) were thawed to room temperature, then vortexed for thorough mixing. Five hundred microlitres (500 µl) each for the samples and normal human plasma for the high positive control (HPC), low positive control (LPC) as well as the negative control (NC) were aliquoted into labelled conical microcentrifuge tubes. The tubes were then ultracentrifuged at 23 885 x g (Biofuge primo R, Heraeas and Sorvall® Thermo Electronic Cooperation, Germany) for 1 hour at 4°C. After centrifugation, the supernatants were aspirated leaving viral pellets at the bottom of the microcentrifuge tube from which RNA was extracted.

3.2.2.3 HIV-1 RNA Extraction

A bottle of the guanidine containing lysis buffer was thawed in a water bath at 37°C, ensuring that no guanidine crystal remain in the bottle. A working lysis reagent was prepared by adding 25 µl of an HIV-1 quantitation standard reagent to 9 ml of a lysis buffer. Six hundred microlitres (600 µl) of the working lysis reagent was then added to each sample and the three controls. To each of the three controls 12.5 µl of the NC, LPC, HPC (provided in the kit) was added to the appropriate tubes containing 600 µl of the working buffer. The samples and controls were then incubated at room temperature for 10 minutes after which 600 µl of propanol was added to each tube to precipitate the viral RNA. The tubes were then vortexed followed by centrifugation at 15 000 x g (Biofuge primo R, Heraeas and Sorvall® Thermo Electronic Cooperation, Germany). After centrifugation, the supernatant was aspirated leaving a pellet at the bottom of the tube to which 1 000 µl of 70% alcohol was added to each of the tubes and vortexed at 15 000 x g. (Biofuge primo R, Heraeas and Sorvall® Thermo

Electronic Cooperation, Germany)). The supernatants were then aspirated leaving a pellet containing the viral RNA at the bottom of the tubes to which 100 µl of the HIV-1 Diluent was added.

3.2.2.4 Reverse Transcription and PCR Amplification

Fifty microlitres of the extracted RNA was added to 50 microlitres of the MMX. The tubes were then placed in a thermocycler; reverse transcribed and amplified using the following conditions.

Hold Program:	2 minutes at 50°C
Hold:	30 minutes at 60°C
Cycle Program (8 Cycles):	10 sec at 95°C, 10 sec at 52°C, 10 sec at 72°C
Cycle Program (23 Cycles):	10 sec at 90°C, 10 sec at 55°C, 10 sec at 72°C
Hold Program:	15 min at 72°C

The PCR reaction was stopped during the 15 minutes of the last hold program and the amplicon denatured using 100 µl of a sodium hydroxide containing HIV-1 Denaturation reagent supplied with the kit.

3.2.2.5 Post Amplification, Detection and Quantitation

The detection reagents, which include the microwell plate (MWP); with rows A through F coated with HIV-1 specific oligonucleotide probe and rows G and H coated with the HIV-1 QS-specific oligonucleotide probe, hybridization solution, horse radish peroxidase, substrates A (Hydrogen peroxide) and B (Tetramethylbenzidine-TMB), stop reagent (4.9% Sulfuric acid) were brought to room temperature before use. The wash buffer (WB) was warmed at 37°C (Incubator LC 203-1, Lab Line Instruments, Inc., Melrose Park, U.S.A) to redissolve any precipitate. A working wash buffer was

prepared by mixing one volume of 10X WB to nine volumes of de-ionized water. One hundred microlitres of the Monitor hybridization solution was added into each well on the 96 well MWP using a 12-channel Amplicor electronic pipettor (Matrix Technologies, Lowell, Massachusetts, U.S.A). Twenty five microlitres of each denatured amplicon were added into row A, and mixed up and down ten times with a 12 channel Amplicor electronic pipettor fitted with aerosol barrier tips. A five-fold serial dilution from row B to F was performed by transferring 25 µl from row A to B and mixing as before and the same procedure repeated through to row F. After mixing row F as before, 25 µl was discarded. Twenty-five microlitres of the remaining denatured amplicons were added to row G and mixed ten times as before. A five-fold serial dilution was made by transferring 25 µl from row G to row H. The extra 25 µl after mixing row H as before was discarded. The MWP was covered and incubated at 37°C (Incubator LC 203-1, Lab Line Instruments, Inc., Melrose Park, U.S.A) for one hour. The plate layout after all the samples were loaded and diluted is illustrated in Fig 5 below.

	1	2	3	4	5	6	7	8	9	10	11	12	Dilution factor
A	NC	LPC	HPC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	1
B													5
C													25
D													125
E													625
F													3125
G													1
H													5

Fig 5. HIV-1 Amplicor monitor assay plate layout

Following incubation, the plates were washed five times using an automatic plate washer (ELP-40, Bio-Tek Instruments, Inc., Vermont, U.S.A) by aspiration of the well contents first, filling each well with the working wash buffer, soaking for 30 seconds and then aspiration to dry the plate. This wash

cycle was repeated four more times and then the MWP was tapped dry. After washing, a 100 µl solution containing avidin conjugated horse raddish peroxidase (AV-HRP) was added to each well and then the plate incubated at 37°C (Incubator LC 203-1, Lab Line Instruments, Inc., Melrose Park, U.S.A) for 15 minutes.

After the incubation, the plate was washed as described above to remove any unbound AV-HRP. One hundred microlitres of a working substrate solution made by mixing 12 mls of substrate A and 3 mls of substrate B was added to each of the wells. The plate was then incubated for 10 minutes at room temperature in a dark cupboard and the reaction was stopped using stop solution (supplied by with the kit) containing weak sulfuric acid (H_2SO_4). The optical densities of the plate wells were then measured using an automatic plate reader (ELx 808, Bio-Tek Instruments, Inc., Vermont, USA) at a wavelength of 450nm (OD_{450}).

For each sample, the well containing the highest dilution factor and with an OD_{450} that is ≥0.200 but ≤2.000 OD units was used for the calculation of the results together with the highest dilution factor in the quantitation standard wells with an OD_{450} that is ≥0.300 but ≤2.000 OD units. A background correction was done for the selected wells by subtracting 0.07 OD units. The total HIV-1 OD and the total QS OD were calculated by multiplying respective corrected OD values by their corresponding dilution factors. The following formula was then used to calculate the HIV-1 RNA copies/ml for all the samples and controls:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} \times \text{Input HIV-1 QS copies /PCR} \times 4$$

Where, the Input HIV-1 QS copies/PCR is the number of copies of the QS in each reaction (provided by the kit manufacturer and is lot specific) and 4 is the sample volume factor to convert copies/PCR to copies/ml. For the run to be valid the HIV-1 RNA copies/ml for all the controls had to be within the ranges specified by the kit manufactures.

3.2.3 BED Calypte Assay

The Calypte® HIV-1 BED Incidence EIA is an IgG-capture enzyme immunoassay. In this assay, the wells of a microplate are coated with goat antihuman IgG. When serum or plasma is added to the wells, anti-HIV IgG and non-anti-HIV IgG are captured on the goat-anti-human IgG coated wells. The relative amounts of anti-HIV IgG and non-anti-HIV IgG captured represent IgG antibody populations found in the serum or plasma. Indirectly, the test measures the proportion of HIV-1 specific IgG in a given specimen with respect to total IgG. In this study this assay was used to identify recent seroconverters. Recent seroconverters have a lower proportion of HIV-specific IgG in the serum/plasma than those with long-term infection. Although the same specimens may have high optical density (OD) values on regular diagnostic EIAs, OD values are lower on the Calypte® HIV-1 BED Incidence EIA. Studies have indicated that HIV-specific IgG may continue to increase for more than 2 years after seroconversion when tested by this assay.

3.2.3.1 Sample Preparation and Control

The samples and controls were thawed to room temperature and diluted. The dilutions were made by adding 5 µl of the test samples to 500 µl of sample diluent provided with the kit to make 1:101 dilutions. Two replicate dilutions of a negative control (NC), and 3 replicate dilutions of a calibrator (CAL) which is inactivated human serum containing a low titer of antibodies to HIV-1 antigens, low positive control (LPC), and high positive control (HPC) were also diluted in the same way as the test samples.

3.2.3.2 BED ELISA

The diluted test samples and controls were carefully mixed by pipetting up and down ten times and then 100 µl of each transferred to a microwell plate coated with anti-human Immunoglobulin (IgG) antibodies. The plate was covered to prevent sample evaporations and incubated for 1 hour at 37°C. The test samples and controls were loaded onto the MWP using the plate configuration illustrated in Figure 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	HPC	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 54	Sample 62	Sample 70	Sample 78
B	NC	HPC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47	Sample 55	Sample 63	Sample 71	Sample 79
C	CAL	HPC	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48	Sample 56	Sample 64	Sample 72	Sample 80
D	CAL	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 49	Sample 57	Sample 65	Sample 73	Sample 81
E	CAL	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 50	Sample 58	Sample 66	Sample 74	Sample 82
F	LPC	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 51	Sample 59	Sample 67	Sample 75	Sample 83
G	LPC	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 52	Sample 60	Sample 68	Sample 76	Sample 84
H	LPC	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 53	Sample 61	Sample 69	Sample 77	Sample 85

Figure 6. Plate Layout for the BED screening assay

Within the last 10 minutes of the incubation, the working solution of the HIV-1 BED peptide was prepared by making a 1:1001 dilution of the peptide that comes with the kit. For one plate, 12 µl of the HIV-1 BED peptide was added to 12 ml of the sample diluent and vortexed for thorough mixing. Following incubation, the microwell plate was washed 4 times using an automatic microwell washer (ELx50 Bio-Tek Inc Winoski, VT, USA). Three hundred microlitres of wash buffer was used for each wash cycle. One hundred microlitres of the diluted HIV-1 BED peptide was then added to each well and the microwell plate incubated for 1 hour at 37°C.

Streptavidin-Horse raddish peroxidase conjugate (SA-HRP) provided with the kit was diluted 1 in 1001 in the sample diluent by adding 12 µl of the SA-HRP conjugate to 12 ml of the sample diluent and vortexing approximately 10 seconds for thorough mixing. Following incubation, the plate was washed as described above and 100 µl of the conjugate added to each microwell. The plate was sealed and incubated for 90 minutes at 37°C.

After the 90 minute incubation, the plate was washed and 100 µl of tetramethyl benzidine (TMB) substrate was added to each well. This was followed by a 15 minute incubation at room temperature (25°C). The reaction was then stopped using a stop solution provided with the kit and the plate was read spectrophotometrically at a wavelength of 450nm with a reference wavelength at 630nm (Humareader^{Plus} Human GmbH Wiesbaden, Germany).

3.2.3.3 Run Validation and Calculation of Results

The median optical density (OD) results for the controls and calibrators were determined. The median OD of the calibrator was used to normalize the ODs of the controls and samples to obtain a normalized OD (ODn) using the following formulae:

$$\text{ODn of control or sample} = \frac{\text{median OD of control or sample}}{\text{median OD of CAL}}$$

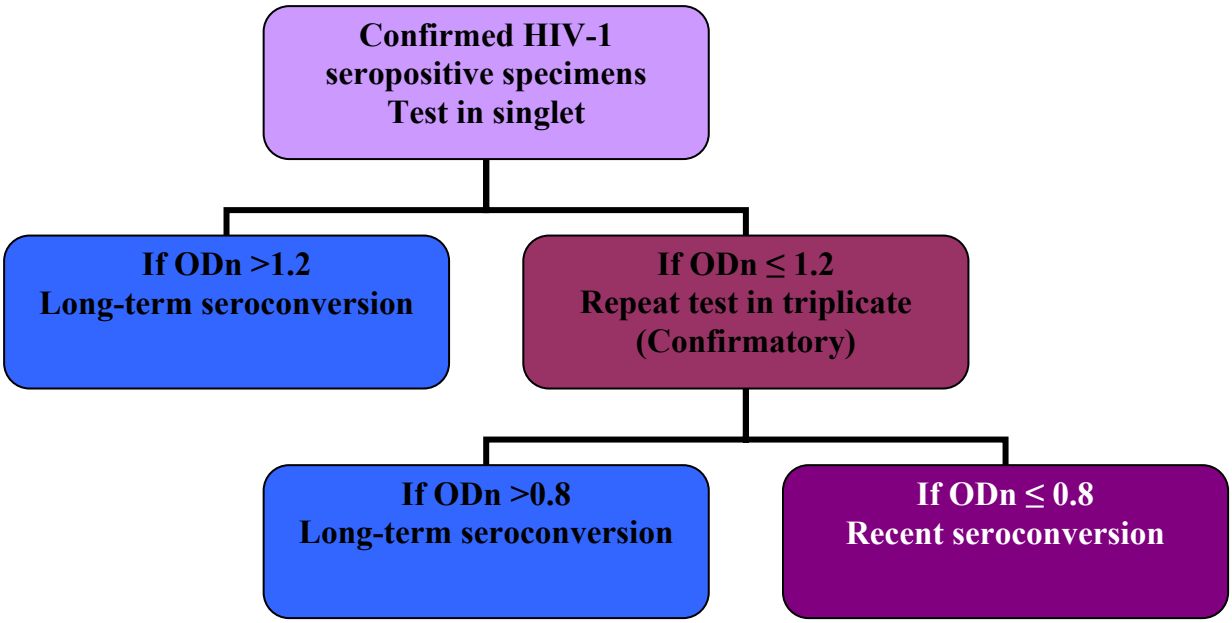
For the assay to be valid, the ODn of the NC, CAL, LPC and HPC had to be in the ranges provided by the kit manufactures and shown in Table 2 below

Table 2. Acceptable ODn ranges of the BED Controls and Calibrator

	NC	CAL	LPC	HPC
Minimum	0.000	1.000	0.400	1.200
Maximum	0.300	1.000	0.750	1.900

3.2.3.4 Interpretation of results

All specimens with ODn greater that 1.2 were considered to be have been cases of long term seroconversion. Samples with ODn $\leq$ 1.2, were tested again in triplicate to confirm their ODn values. In confirmatory testing, specimens with ODn $\leq$ 0.8 were considered to be cases of recent seroconversion, defined as infection within 155 days of sample collection. The algorithm used for testing and interpretation of the results is shown in Figure 7 below.



The ODn of ≤0.8 corresponds to mean seroconversion duration of 155 days

Figure 7. Algorithm for the interpretation of the BED results

3.2.4 HIV-1 RNA Extraction for *pol* sequencing

HIV RNA was extracted from plasma samples using the Roche RNA extraction kit (Roche Molecular Diagnostics, NJ). The plasma samples were thawed and allowed to reach room temperature. Five hundred microlitres of each test sample were pipetted into appropriately labeled 1.5 ml microcentrifuge tubes and centrifuged at 23 000 x g (Biofuge primo R, Heraeas and Sorvall® Thermo Electronic Cooperation, Germany) at 4°C for 45 minutes. After centrifugation, the virus free supernatant was transferred into appropriately labeled clean cryovials. This virus free supernatant was used for the BED assay described above. The pelleted viral particles were lysed using the guanidine containing Roche lysis buffer (Roche Molecular Diagnostics, NJ) by resuspending the pellet in 600µl of the lysis buffer

and incubating for 15 minutes at room temperature. After the incubation, 600 µl of propanol was added to each tube to precipitate the viral RNA. The tubes were then vortexed approximately 5 seconds and then centrifuged at 15 000 x g (Biofuge primo R, Heraeas and Sorvall® Thermo Electronic Cooperation, Germany), for 15 minutes. After centrifugation, the supernatant was aspirated leaving a pellet at the bottom of the tube to which 1 000 µl of 70% alcohol was added to each of the tubes and vortexed for approximately 5 seconds and then centrifuged at 15 000 x g (Biofuge primo R, Heraeas and Sorvall® Thermo Electronic Cooperation, Germany), for 5 minutes. The supernatants were then aspirated leaving a pellet containing the viral RNA at the bottom of the tubes to which 80 µl of the HIV-1 diluent was added.

3.2.5 HIV-1 Reverse Transcription

HIV-1 RNA was reverse transcribed to generate whole genome cDNA using the commercial kit *Superscript III First Strand Synthesis Ki*t. (Invitrogen Corporation, Carlsbad CA) The reverse transcriptase enzyme MMLV and random hexamers which are mixtures of 6 to 8 base pair (bp) primers of varying sequences are used to generate whole genome cDNA.

Reaction mixtures were prepared as shown in tables 3 and 4 below. Four microlitres (4.0 µL) of the dNTP/Random hexamers mix were added to each 200µL PCR reaction tubes followed by addition of 9µL of the RNA templates. The mixture was then incubated at 65°C for 5 minutes. Following incubation, the mixture was then placed on ice and 7µL of the enzyme mix added immediately, followed by incubation for 10 min at 25ºC, 30 min at 45ºC, and 15 min at 70ºC. The generated cDNA was used either immediately in PCR reactions or stored at -70°C.

Table 3. Master Mix used for Reverse Transcription

Component	1 x Reaction	Concentration in the final reaction mixture
50 ng/µl Random Hexamers	2.8µl	7 ng/ µl
25 mM dNTP	1.2µl	1.5mM
TOTAL	**4.0µl**	

Table 4. Enzyme Mix used for Reverse Transcription

Component	1 x Reaction	Concentration in the final reaction mixture
5 x First Strand Buffer	4µl	1 M
0.1M DTT	1µl	0.005M
RNaseOUT (40 U/µl)	1µl	2 U/µl
Superscript III Reverse Transcriptase (200 U/µl)	1µl	10 U/µl
TOTAL	**7.0µl**	**-**

3.2.6 PCR Amplification and Sequencing of the Target Genes

A 1200bp fragment of the *pol* gene covering all the 99 *protease* codons and the first 240 codons of the *RT* gene was amplified using Platinum Taq (Invitrogen Corperation, CA, USA) polymerase by nested PCR. The first 240 codons of the *RT* gene cover most known *RT* mutations associated with resistance to all RT inhibitors. The primers used for the PCR are described in table 6. The cycling conditions for both the 1st and 2nd round PCR were, 94°C for 2 minutes, 30 cycles at 95°C for 30 seconds, 55°C for 20 seconds and 72°C for 2 minutes, followed by 72°C for 10 minutes. Success of the reverse transcription and the nested PCR was checked by running the 2nd round PCR products on 1% TBE agarose gel for 45 minutes at 120V and checking for the 1200bp fragment using a 1Kb plus DNA ladder (Invitrogen Cooperation, CA, USA). Successfully amplified samples were purified using

Qiagen PCR purification kits (QIAGEN Inc. Valencia, CA, USA). After the purification, DNA concentration and quality were determined by spectrophotometry using a nanodrop scanning spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). The purified PCR amplicons were sequenced using the Big Dye Terminator Chemistry (Applied Biosystems Inc, Foster City, CA, USA). The sequencing primers used are described in table 6. The cycling conditions for the sequencing reactions were 95ºC for 30 seconds, 28 cycles at 96ºC for 10seconds, 50ºC for 5 seconds and 60ºC for 4 minutes. After the sequencing reactions, the sequencing products were purified using the Big Dye XTerminator kits (Applied Biosystems Inc, Foster City, CA) to remove unincorporated Big Dye Terminators. Electrophoresis of the purified sequencing products was conducted on an ABI 3130xl Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA).

Table 5. 1^{st} and 2^{nd} Round PCR Master Mix

	1^{st} Round PCR		**2^{nd} Round PCR**	
Reagent	**1 x reaction**	**Final concentration in reaction mixture**	**1 x reaction**	**Final concentration in reaction mixture**
DEPC treated water	76.5	-	78	
10X PCR	10µl	1	10µl	1 x
50 mM $MgCl_2$	5	2.5 mM	3.5	1.75 mM
25 mM dNTP Mix	1	0.25mM	1	0.25mM
Forward Primers 50pmol/µl MAW26(1^{st} Rnd) / RT20(2^{nd} Rnd)	1	0.5pmol /µl	1	0.5pmol /µl
Reverse Primers 50pmol/µl RT21 (1^{st} Rnd) / Pro1 (2^{nd} Rnd)	1	0.5pmol /µl	1	0.5pmol /µl
5 U/ µl Platinum Taq Polymerase	0.5µl	0.025U	0.5µl	0.025U
Total	95	-	95	-

Table 6. PCR and Sequencing primers

Stage	Primer Name	Sequence	Length	Direction	HXB2 Position
1st Rnd	**MAW-26**	TTGGAAATGTGGAAAGGAAGGAC	23	Forward	2028-2050
	RT-21	CTGTATTTCTGCTATTAAGTCTTTTGATGGG	31	Reverse	3539-3509
2nd Rnd	**Pro-1**	CAGAGCCAACAGCCCCACCA	20	Forward	2147-2166
	RT-20	CTGCCAGTTCTAGCTCTGCTTC	22	Reverse	*3462-3441*
Sequencing	**RTC1F**	ACCTACACCTGTCAACATAATTG	23	Forward	2486-2508
	RTC2R	TGTCAATGGCCATTGTTTAACCTTTGG	27	Reverse	*2630-2604*
	RTC3F	CACCAGGGATTAGATATCAATATAATGTGC	30	Forward	2956-2994
	RTC4R	CTAAATCAGATCCTACATACAAGTCATCC	29	Reverse	*3129-3101*
	RT-y	GTGTCTCATTGTTTATACTAGG	22	Reverse	*2967-2946*
	MAW-46	TCCCTCAGATCACTCTTTGGCAACGAC	27	Forward	2251-2277

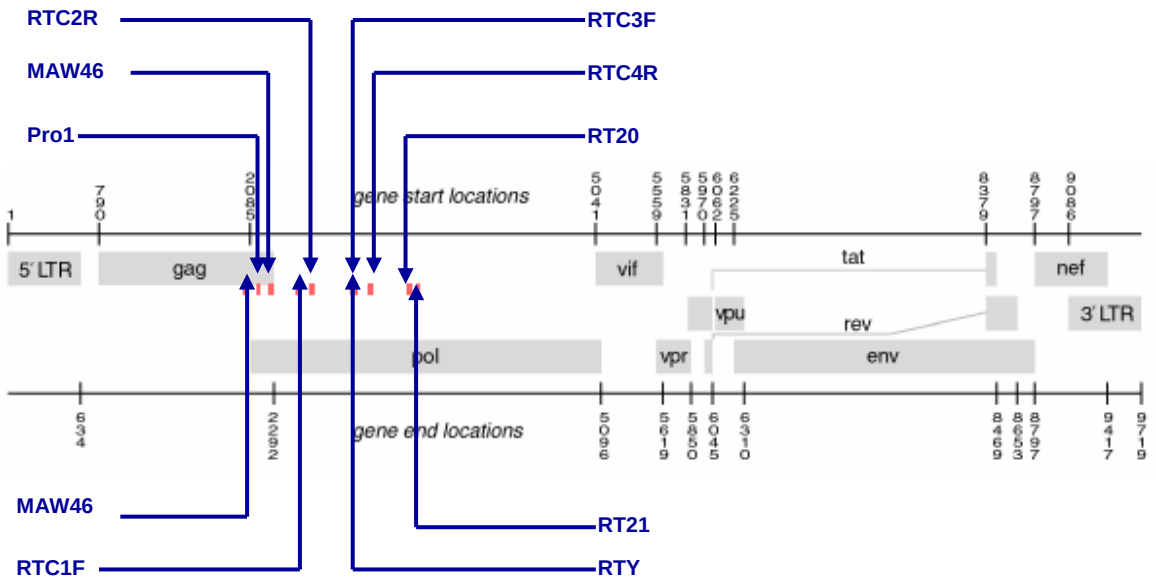


Figure 8. PCR and Sequencing primer map showing the PCR and Sequencing strategies.

3.2.7 Sequence Analysis

The complete sequences for each sample were assembled by building a consensus from the sequences obtained from the six different sequencing primers using Sequencher Version 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Each assembled sequence was analyzed using the Stanford HIVDB for subtype identity, sequence quality and drug resistance. The HIVDB program is an expert system that accepts user-submitted protease and RT sequences and returns inferred levels of resistance to 17 FDA-approved anti-HIV drugs.

Mutations are defined as differences from the consensus B reference sequence (PR and RT). The mutations are divided into those associated with drug resistance ('Resistance Mutations') for mutations associated with RT inhibitors and 'Major and Minor Resistance mutations for mutations associated with PI inhibitors) and those that have no known association with drug resistance ('Other Mutations'). This separation, however, is not always sharp. There are some mutations that appear to be associated with drug therapy but which are not generally considered drug-resistance mutations.

Each drug resistance mutation is assigned a drug penalty score; the total score for a drug is derived by adding the scores of each mutation associated with resistance to that drug. Using the total drug score, the program reports one of the following levels of inferred drug resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance. For subtype analysis each sequence is compared to a list of reference sequences for each of the Main group of HIV-1 sequences representing subtypes A, B, C, D, F, G, H, J, K, CRF01_AE, and CRF02_AG. The subtype of the closest reference sequence is assigned to the submitted sequence. For sequence quality,

the program checks for three types of problems: (i). Positions containing stop codons or frame shifts; (ii) Positions containing highly ambiguous nucleotides: (iii) Positions with atypical mutations, these are mutations that have been observed in <0.1 % in published group M HIV-1 sequences.

The sequences were also analyzed using the Stanford HIVDB's QA/QC program which compares any new sequences to other sequences produced from the same laboratory, for possible contamination. To also aid in quality assurance, neighbor joining phylogenetic trees where also created using Bioedit version 7.0.0 (Ibis Biosciences, Carlsbad CA) for sequence alignments and MEGA version 4.0.2 (The Biodesign Institute, Tempe AZ) for tree generation. The evolutionary relatedness of all the sequences generated from the sequenced specimens was estimated using the maximum likelihood method in the program The phylogenetic trees were generated and viewed using the program Fig Tree. The aligned sequences in Bioedit were used to generate the Zimbabwean HIV-1 Subtype C pol consensus sequence which was then compared with the current Los Alamos HIV-1 Subtypes B and C consensus sequences.

All the sequences were also analyzed using the Calibrated Popullation Resistance (CPR) program of the HIVDB (version 3.0 beta). The program determines the prevalence of resistance to ARVs within the query data set and is summarized using standard lists of mutations suitable for monitoring primary drug resistance. These include the Stanford Surveillance Drug Resistance Mutation (SDRM 2008) list and then confirmed by the IAS-USA major (2007) mutation list. The SDRM provides drug resistance prevalence in terms of WHO guidelines. For the analysis of the 236 sequences, the SDRM 2008 list was used and the results were confirmed by the analysis using the IAS-USA list.

3.3 Statistical Analysis

The sample size (n) was calculated using the following formula:

$$n = \frac{P(100-P)}{(SE)^2}$$

Where P is the expected prevalence of primary drug resistance in the population of women attending antenatal clinics in Chitungwiza studied. Based on a study by Vardavas and Blower on the emergence of primary drug resistance in Botswana which projected the prevalence of primary drug resistance not to exceed 5% before 2009, P was set to be 5% [Vardavas and Blower, 2007]. The standard error (SE) was set at 1.5%. The minimum sample size (n) required to determine the prevalence of primary HIV-1 drug resistance in this study population with an error margin of 1.5% was

$$n = \frac{5(100-5)}{(1.5)^2}$$

$$= 212$$

For the analysis of the results, the statistical program STATA version 10 (StataCorp LP, Texas, USA) was used. P-values were calculated using the Chi squared test for categorical data and the Student T tests for continuous data. For the phylogenetic analysis, 1000 replicates were analyzed for the bootstrapping for each phylogenetic tree generated. The analysis was done using the program PhyML version 2.4.4 [Guindon and Gascuel, 2003]

Chapter 4: RESULTS

4. RESULTS

4.1 Patient Demographic and BED Results

Two hundred and thirty seven (237) samples of the 303 samples analyzed in this study were tested using the BED assay to estimate the infection duration of the study participants. Of the 237 samples tested, only 224 had their results confirmed. Seventy-five (33.5%) of the 224 samples with confirmed results were classified as samples from recently infected women whilst 149 (66.5%) had long term infection. According to the assay design, this means they were infected within 155 days of sample collection. 63 women out of the 75 that were considered to have been recently infected had dates for their estimated date of delivery. Considering the estimated dates for delivery and the window period for the BED assay (155 days) 95% of the 63 recently infected women were infected during pregnancy. Of the 75 women classified as recently infected, three, had received ARVs for pMCTC in their earlier pregnancies. They were all considered to have been misclassified as recently infected by the BED assay, hence were all excluded in all subsequent analyses of recently infected and long term infected women.

After correcting for misclassifications, 32.6% of the women in this study were recently infected, 67.4% had long term infections, and 4% were misclassified as recently infected.

Table 7. summarizes the demographic data of the study participants grouped according to the estimated duration of their infection. The mean age, mean age at first sexual intercourse, and mean age of the husbands of the women did not statistically differ between the women with long term infections and the recently infected women, ($p>0.05$). Interestingly there was a statistically significant difference in the mean age at first menses, $p= 0.04$, between the long term infection (15 yrs) and that of the recently infected (14 yrs).

There was no statistically significant difference between the recently infected women and the long term infected women when the proportions of women who had ever been forced to have sex, who had one or more sexual partners within the past 12 months, had a still birth before, and one or more children dead and who had a history of ART exposure through SD NVP for PMTCT or HAART in the two groups under study ($p>0.05$).

However, there was a statistically significant difference, $p=0.001$, between the long term infected and the recently infected women when the proportions of women who had never been pregnant before were compared. Seventy percent of the recently infected women had never been pregnant before compared to 46% of the long term infected women. There were statistically significant differences in the proportions of women who had one or more children alive and miscarriages in the past in the long term infected compared to the recently infected, with p-values of 0.013 and 0.006 respectively. The proportions were higher in the group of long term infected women. This was also the same for the proportion of women who were treated for STIs, $p=0.044$, with 24% of the long term infected women having been treated for STIs before compared to 13% of the recently infected women.

Table 7. The characteristics of the 221 study participants grouped according to their infection duration based on the BED results.

	Long term Infection N=149	Recently Infected N=72	p-value
Age women, mean (SD)	22(2) N=149	21(2) N=72	0.081
Age of husband, mean (SD)	28(5) N=140	29(5) N=67	0.113
Age at 1st menses, mean (SD)	15(2) N=148	14(1) N=72	0.040
Age at 1st sex, mean (SD)	18(2) N=149	19(2) N=71	0.075
Ever been forced to have Sex	6% N=149	8% N=71	0.507
More than one sexual partner in the past 12 months	18% N=149	16% N=71	0.973
Have never been pregnant before	46% N=149	70% N=71	0.001
One or more children alive	42% N=138	28% N=66	0.013
More that one miscarriage in the past	14% N=137	3% N=66	0.006
Still Birth	1% N=138	0% N=66	0.326
One or more children dead	9% N=138	7% N=66	0.787
Treated for STI	24% N=149	12% N=72	0.044
Have Taken ART	1% N=149	0% N=72	0.486
Have taken ART for PMTCT	1% N=148	0% N=72	0.323
Have taken SD NVP for PMTCT	1% N=131	0% N=66	0.477

N, Number of samples tested

4.2 CD4+ Lymphocyte Cell Count

CD4 counts were enumerated for 250 women. The median CD4 count for the women was 393 cells/µL interquartile range (IQR): 249-509 cells/µL. Figure 9 is a graphical representation of the women's CD4 counts grouped according to their time of infection. Of the196 women included in this analysis, 66 (33.7%) were classified as recently infected using the BED method. The median CD4 count for the women with long term infections was statistically significantly lower 349 cells/µL; IQR: 209-458 cells/µL than that of the recently infected women, 429 cells/µL; IQR: 319-562 cells/µL, p = 0.0003.

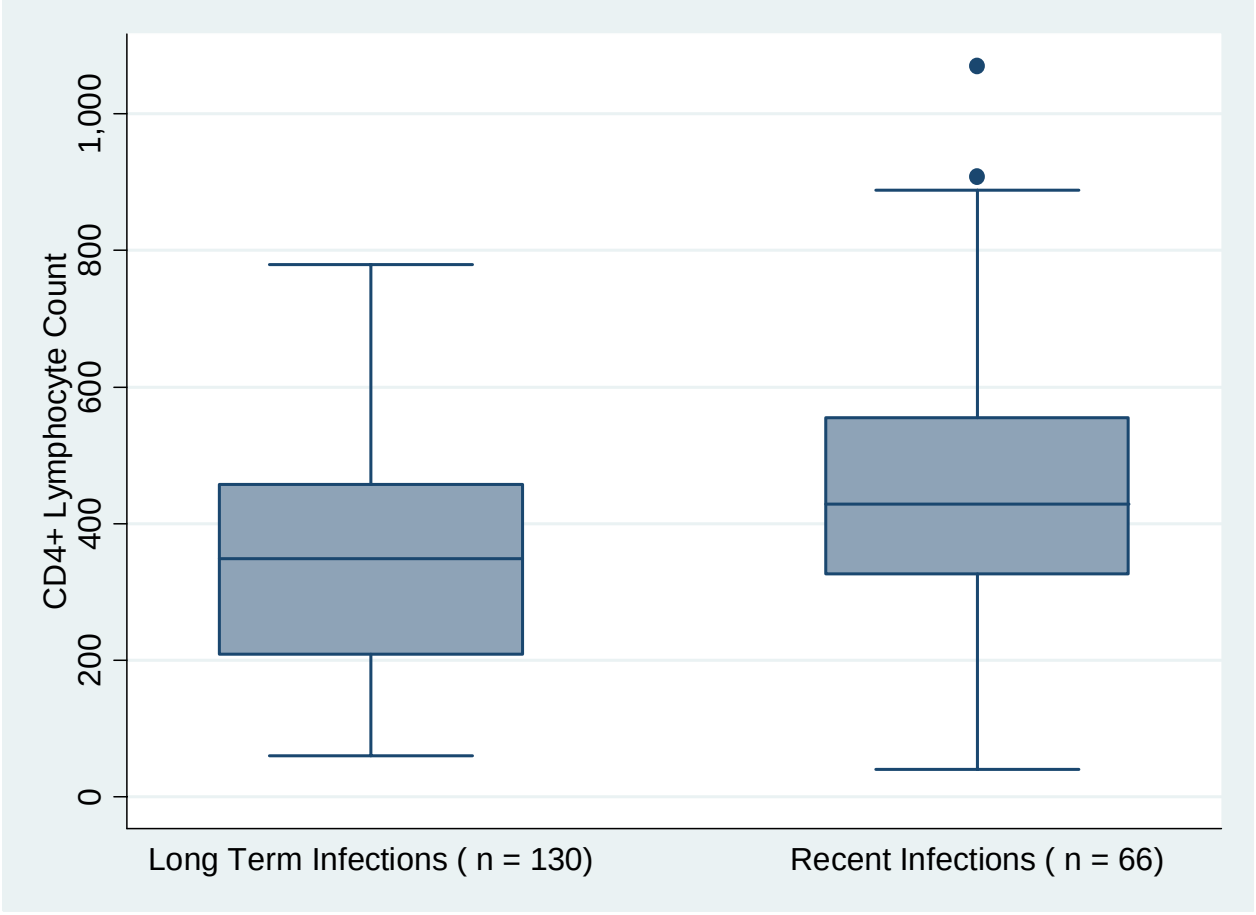


Figure 9. CD4+ Lymphocyte counts for the study participants grouped according to infection durations as defined by the BED Assay.

4.3 HIV-1 RNA Plasma Viral Load

Plasma HIV-1 RNA viral load was quantified in 102 samples. However, only 59 (58%) of these were successfully sequenced. The mean viral load for the successfully sequenced samples was 3.66 $\log_{10}$ HIV-1 RNA Copies/ml, 95% Confidence Interval (CI); 3.44 to 3.88 $\log_{10}$ HIV-1 RNA Copies/ml. Of these, 38 had confirmed BED results but two were excluded from the comparison of long term infections versus recent infection because they were among the three considered to have been misclassified by the BED assay as recently infected.

Fig 10 is a graphical representation of 36 women's viral loads when grouped according to their estimated duration of infection. There were three outliers in the group with long term infections, one of the women had a viral load below the low detection limit of the assay (1.70 $\log_{10}$ HIV-1 RNA Copies/ml). For analysis purposes, she was assigned a viral load of 1.70 $\log_{10}$ HIV-1 RNA Copies/ml (50 HIV-1 RNA copies/ml), the low detection limit of the assay. The viral loads of the other two women were above the upper detection limit of the assay, 5.0 $\log_{10}$ HIV-1 RNA Copies/ml (100 000 HIV-1 RNA copies/ml). For analysis purposes, these two women were assigned viral loads of 5.0 $\log_{10}$ HIV-1 RNA Copies/ml. The mean viral load for the 25 women with long term infections 3.77 $\log_{10}$ HIV-1 RNA Copies/ml (CI: 3.41-4.13 $\log_{10}$ HIV-1 RNA Copies/ml) was not statistically different from that of the 11 women with recent infection 3.62 $\log_{10}$ HIV-1 RNA Copies/ml (CI: 3.18-4.07 $\log_{10}$ HIV-1 RNA Copies/ml), p-value = 0.620.

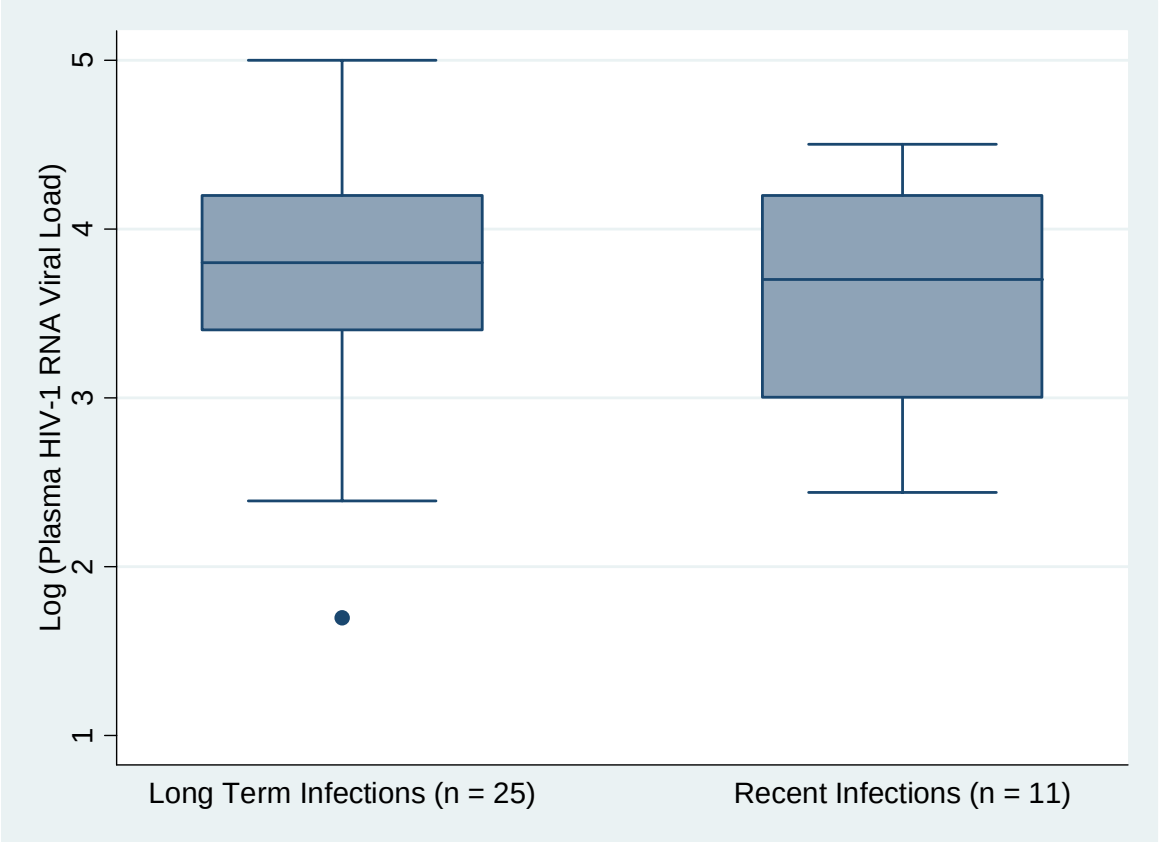


Fig 10. HIV-1 RNA viral loads for the study participants (n = 36) grouped according to their infection duration.

4.4 Sequencing

4.4.1 HIV-1 Drug Resistance

Only one of the two genotypic drug resistance testing, population sequencing of the HIV-1 pol gene, was used in the study. The allele specific oligonucleotide ligation assay (ASOLA), originally developed using subtype B virus, was not explored because during the execution of this study, a paper was published providing evidence that ASOLA had a low sensitivity in the detection of drug resistance in non-subtype B viruses including subtype C which is the predominant subtype in Zimbabwe[Beck et al., 2008].

Out of the 303 samples analyzed, only 236 samples were successfully sequenced. The sequencing success rate was 78%. Different laboratories using in house methods for sequencing have had varying sequencing success rates ranging from 48% to 94%, depending on their experience [Kamoto and Aberle-Grasse, 2008; Maphalala et al., 2008; Nguyen et al., 2008; Pillay et al., 2008; Sirivichayakul et al., 2008; Somi et al., 2008].Sixty seven (67) samples either did not amplify or the sequences obtained did not cover at least the first 230 codons of the RT gene. Of the 236 that amplified, only 175 had sequences covering all the 99 codons of the protease gene and at least the first 230 codons of the RT gene. Sixty one sequences covered the RT gene only without the PR hence PR drug resistance data could not be obtained from these sequences.

Table 8. Summary of the sequencing results

Total Number of samples Analyzed	**303**
Total Sequences Obtained	**236**
Protease (PR) Sequences	**175**
Reverse Transcriptase (RT) Sequences	**236**
PR RT sequences	**175**

When the sequences were analyzed using the Stanford HIVDB's CPR, only two sequences (0.8%) were found to have known primary drug resistance mutations (Table 9). Of the two sequences with drug resistance mutations, one had the I85V mutation. The second sequence had the Y181C mutation.Table10 shows some of the characteristics of the two women that had the drug resistance mutations.

Table 9. Summary of the results obtained from the analyses of the sequences for the presence of drug resistance mutations. .

	Number	**Percentage**
Sequences with any mutation	2	0.8
PR sequence with any PI mutation	1	0.6
RT sequence with any NRTI mutation	0	0
RT sequence with any NNRTI mutation	1	0.4
RT sequence with any NRTI and any NNRTI mutation	0	0
PRRT sequences with any NRTI, any NNRTI and any PI mutation (3 Class Resistance)	0	0

Table 10. Characteristics of the samples with known drug resistant mutations.

	Patient Characteristics				**Drug Resistance Mutation Information**			
Participant I.D	**Age (Years)**	**Infection Duration**	**Exposure to ART**	**CD4 Count (Cells/µl)**	**Mutation**	**Prevalence (dataset)**	**Prevalence in naive (HIVdb)**	**Prevalence in treated (HIVdb)**
SCR423	24	Recent	SD-NVP (2004)	750	I85V	0.43	0.05	2.5
SCR541	22	Long Term	None	388	Y181C	0.42	0.1	8.3

Besides the two primary drug resistance mutations that were detected, there were secondary drug resistance mutations that were also detected. Figure 12 is a graphical representation of the frequencies of the secondary drug resistance mutations identified from the sequences generated. In the same graph, these frequencies are compared to frequencies of the same mutations in sequences from treatment naïve and treatment experienced patients maintained on the HIV DB. The mutations V11I and L33V are from the protease gene and the rest of the mutations are from the RT gene. The mutation V11I is weakly associated with PI therapy and has been shown to result in decreased response to DRV. The mutation L33V is selected by FPV, DRV, LPV, ATV and TPV, and contributes resistance to these drugs. The mutation, K69S is selected by NRTIs but has no known effects on drug resistance. A98G reduces NVP susceptibility by 2 to 3 fold. It occasionally occurs in the absence of ART. V118I, occurs in approximately 2% of untreated persons and with increased frequency in persons receiving multiple NRTIs, it causes low level resistance to 3TC and possibly other NRTIs when present with one or more thymidine analogue mutations (TAMs). V179D/E causes low level reduction in susceptibility to NVP, EFV, and DLV. It occurs in about 1% of untreated persons. K43N and E203K were found in higher frequencies in treated persons than in treatment naïve persons but their effects on drug resistance are not yet known. All the mutation above, were found in higher frequencies in HIVDB treated patients followed by the SCR patients with lowest values in the HIVDB treatment naïve persons, except the V179D mutation where the frequency of the mutation in HIVDB treatment naïve persons was lower than that of the SCR frequency. This is graphically illustrated in figure 12.

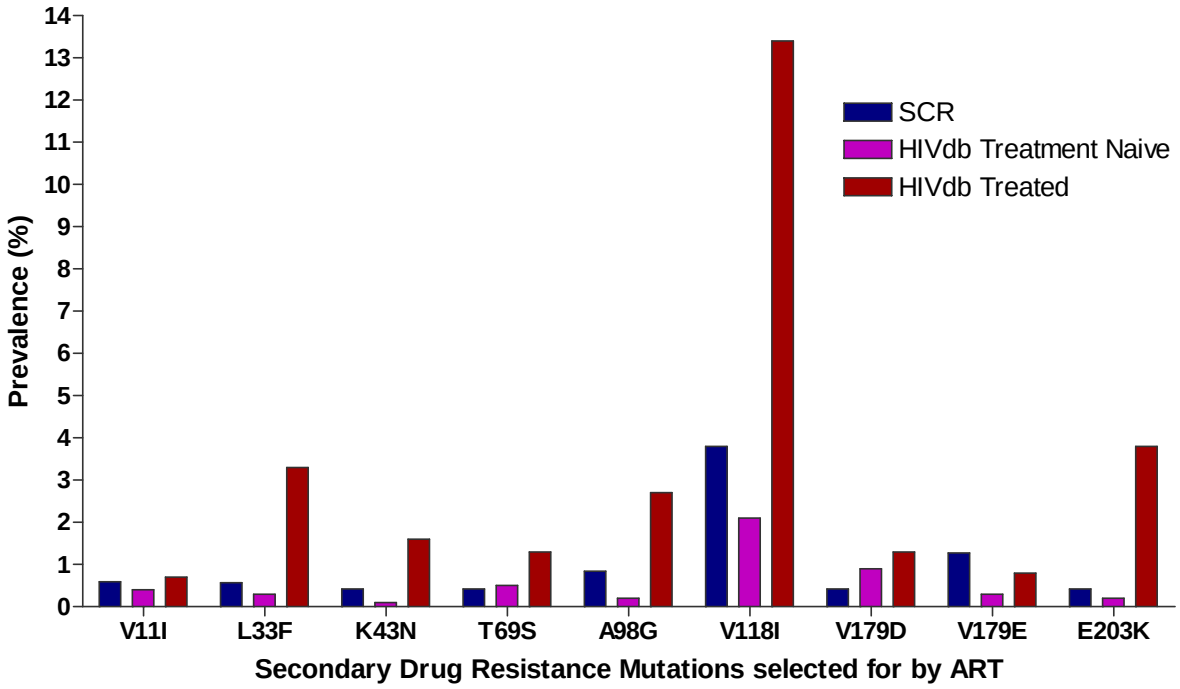


Figure 11. Secondary drug resistance mutation found in the study population compared with data from treated and treatment naïve patients in the HIVdb

Polymorphisms in the RT gene that are not currently associated with drug resistance to any of the RT inhibitors available but were occurring at frequencies greater than 5% in our study population were also evaluated. The frequencies of these polymorphisms in the SCR sequences were compared to HIVDB HIV-1 Subtype C sequences and also Subtype B sequences. The results of this comparison are shown in Figure 13. There were nine mutations that had frequencies greater that 50% for both the SCR samples and the HIVDB Subtype C samples. These mutations, V35T, E36A, T39E, S48T, K173A, D177E, T200A, Q207E and R211K could be HIV-1 Subtype C specific mutations since they were found at very low frequencies or not all in subtype B sequences.

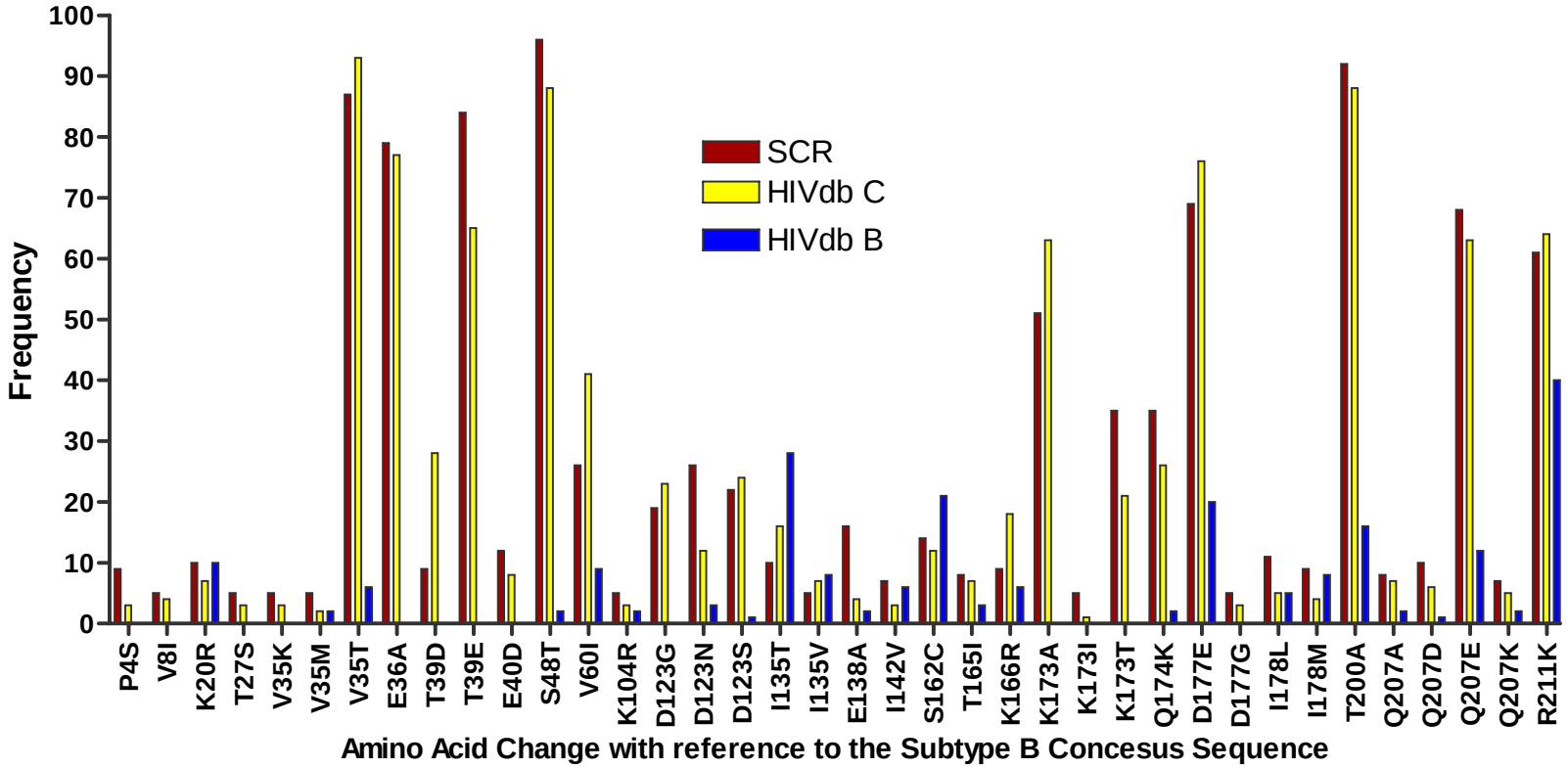


Figure 12. Comparison of polymorphisms found in greater than 5% of the samples with the Stanford sequences

4.4.2 Generation of the HIV-1 Pol Consensus Sequence

The PR and RT majority consensus sequences generated from the 236 sequences were both 100% identical to the HIV-1 Subtype C consensus sequences from the Los Alamos HIV database. When the PR consensus sequence was compared with the subtype B and group M consensus sequences, the similarity was found to be 91% and 95% respectively. The RT sequence was 92% similar to the consensus subtype B sequence and 95% similar to the consensus group M sequence. A small PR portion of the study sequences aligned with the subtype B and C consensus sequences as well as the SCR consensus sequence generated from the 236 sequences used are shown in Figure 14 below.

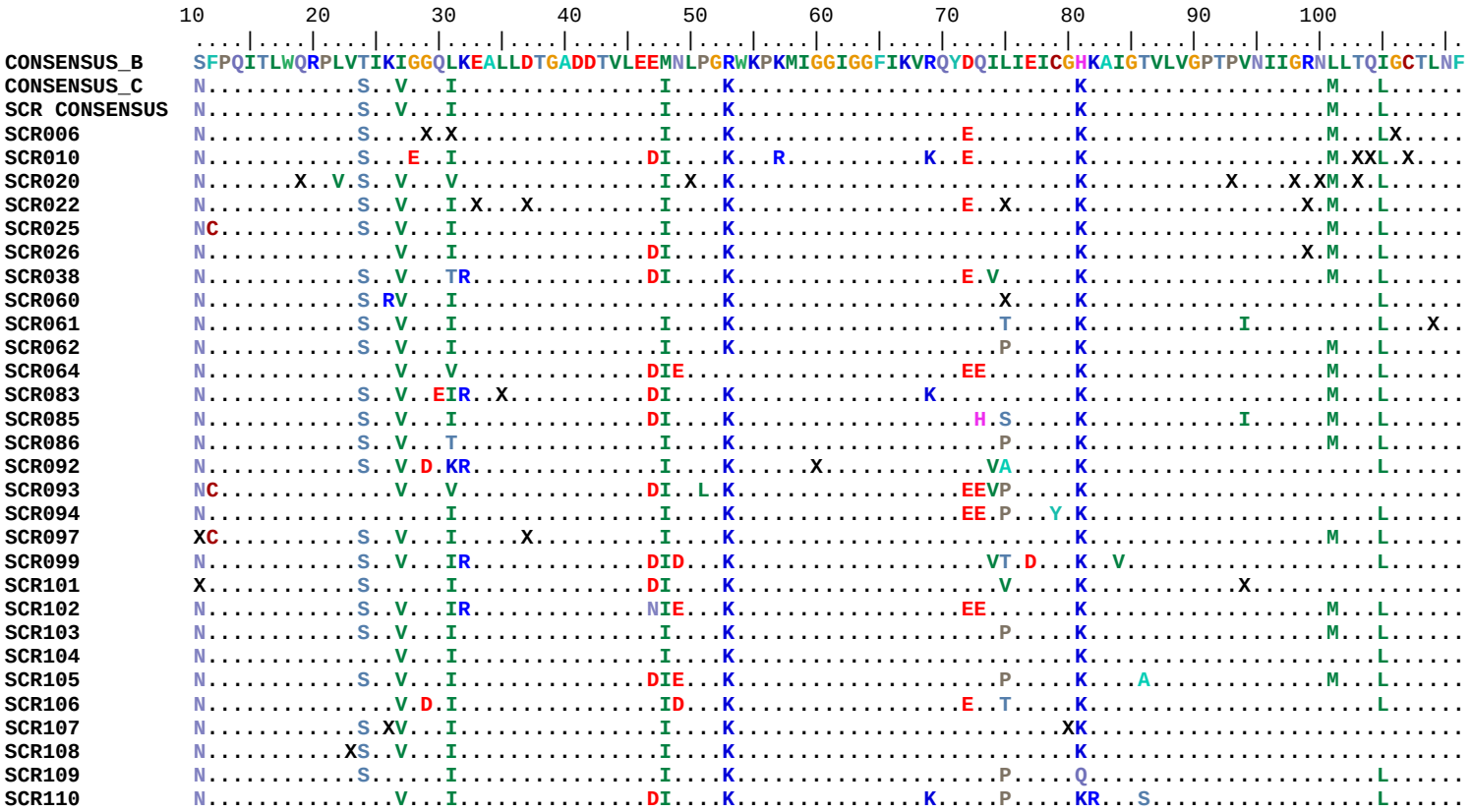


Fig 13. Comparison of the SCR Protease majority consensus amino acid sequence with the Subtypes B and C consensus sequences.

4.4.3 Phylogenetic and Geographic Clustering Analysis

On analysis of the maximum likelihood tree generated from all the sequences that had both the PR and RT genes, no specific clustering of the study sequences were observed. The phylogenetic relationship between all these sequences is represented in the phylogenetic tree shown on Figure 15. The bootstrap values for the majority of the branches were very low so no specific relationships between the different sequences could be inferred from the tree.

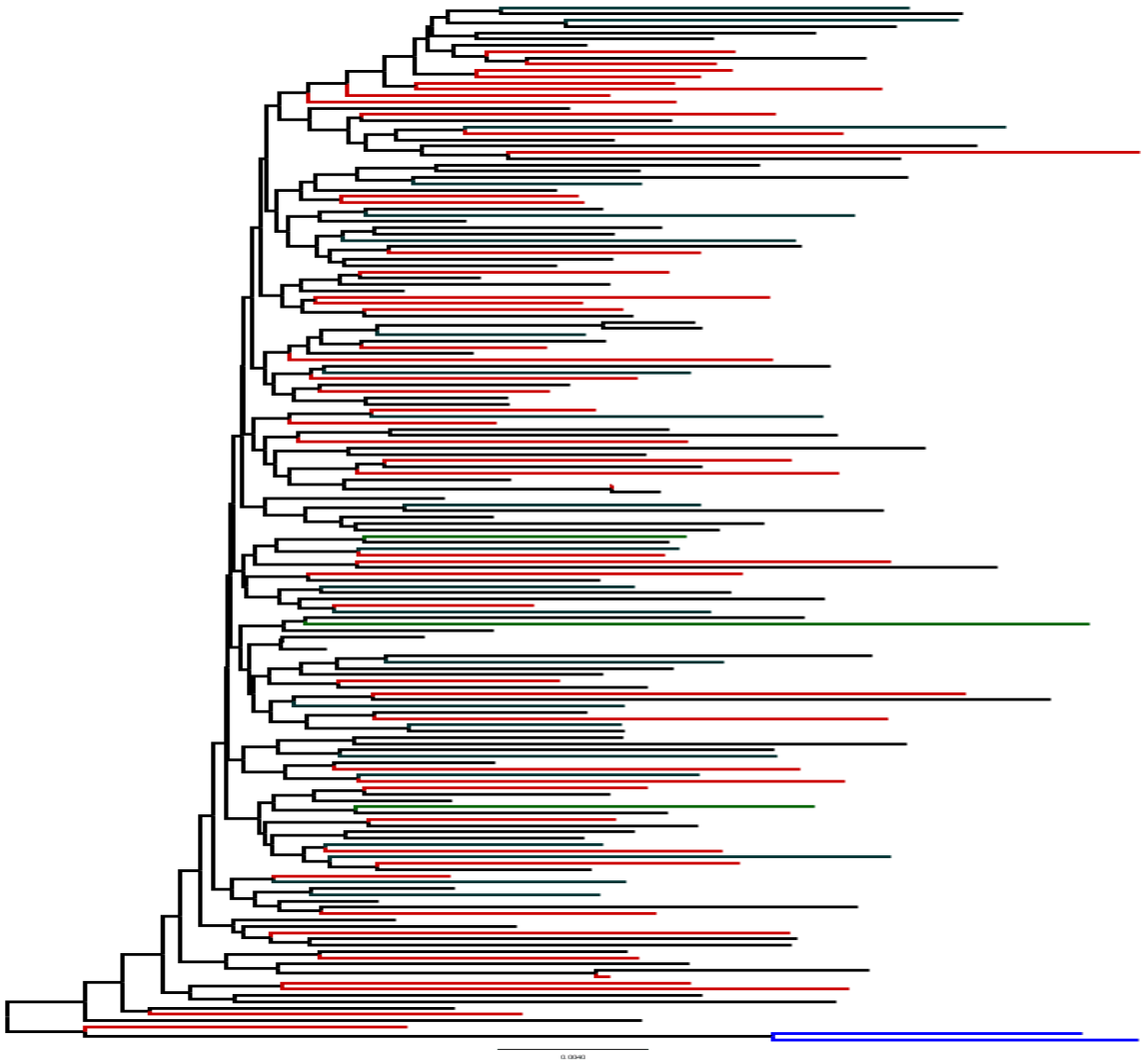


Figure 14. Maximum Likelihood Tree (1000 replicates for Bootstrap) indicating the relationships between all the sequences (175) that had all the protease codons plus the first 240 RT codons. The royal blue colored branches represent subtype B reference sequences, green subtype C reference sequences, red line from patients classified as recently infected, black lines from long term infected patients and dark blue from patients whose infection duration could not be as determined by the BED Enzyme Immunoassay for Incidence testing.

Subtype C sequences from the Los Alamos HIV DB were also retrieved to analyze for any geographic clustering when compared to sequences from Zimbabwe. All the sequences retrieved were from treatment naïve patients. There were no significant clusters for the Southern African samples except for some Zambian sequences that formed a cluster of their own among the other sequences. Other sequences from India, Brazil, China and Ethiopia formed clusters that could be distinguished visually (Figure 15).

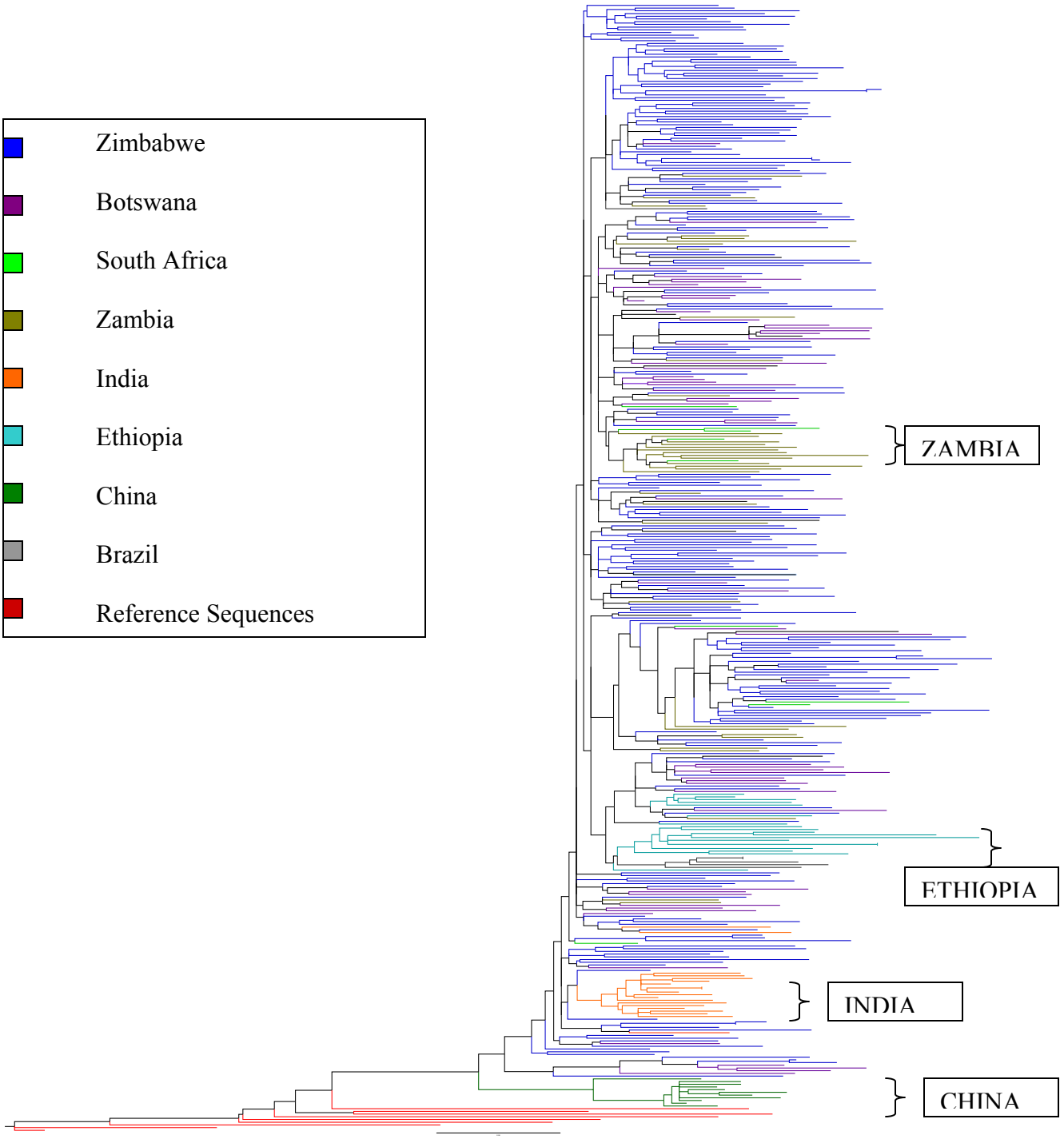


Figure 15. ML tree illustrating the phylogenetic relationship between the sequences from the current study and other regional subtype C sequences and sequences from other settings where Subtype C is a major contributor to the HIV epidemic.

CHAPTER 5:DISCUSSION

From the samples successfully sequenced, the prevalence of drug resistance among the women included in this study was very low, less that 2%. Only two women had mutations associated with drug resistance. Each one of them had a single mutation. SCR541 had the Y181C mutation. This mutation involves a substitution of the amino acid tyrosine at codon 181 of the RT gene by cysteine. It is found at a significantly higher frequency (23.8%) in patients receiving one or more NNRTIs compared to ART naïve patients (0.20%), p = 0.000. It causes high level resistance to NVP and DLV and low level resistance to EFV. It has been found in 28.2% and 26.7% of patients receiving NVP and DLV respectively (http://hivdb.stanford.edu/cgi-bin/Marvel.cgi [accessed 29/01/09). For individuals infected with HIV-1 subtype C, it has been found in 1% of patients receiving NRTIs only and 8.1% of NNRTI treated patients (http://hivdb.stanford.edu/cgi-bin/Marvel.cgi [accessed 29/01/09). However, the Y181C mutation has also been associated with increased AZT and TDF susceptibility (http://hivdb.stanford.edu/cgi-bin/Marvel.cgi [accessed 29/01/09). Surprisingly, SCR541, classified as recently infected by the BED assays, reported no exposure to ART either for pMTCT or for HIV disease. The possibility that she may have acquired the drug resistant virus from the person who infected her cannot be ruled out, although there was no information on whether or not the person who infected her was taking HAART.

SCR423 had the I85V mutation. It involves a substitution of the amino acid isoleucine at the 85th PR codon by valine. It is selected for by PIs and is associated with a decreased virologic response to ATV. It was found to be significantly higher in patients (5.8%) receiving one or more PIs compared to 0.5% of treatment naïve patients from the patient data and sequences curated on the Stanford HIVDB (p = 0.05 after Yates's correction). For Subtype C sequences

only, 5% of the sequences from PI treated patients (n= 282) had the I85V mutation compared to zero percent of the 2145 PI naïve patients (http://hivdb.stanford.edu/cgi-bin/PRPosMutSummary.cgi [accessed 29/01/2009]. SCR423 had previous exposure to SD-NVP taken for pPMTCT in 2004 (approximately two years before she was enrolled into this current study). The SD-NVP could not have selected for the I85V mutation identified. Hence this mutation could have resulted from the transmission of this drug resistant quasie species from her spouse if he was exposed to protease inhibitors. However, the woman had no knowledge of her spouse's exposure to ART. At 750 cell/µl, her CD4 count was still very high making her ineligible for HAART, based on the current recommendations of initiating therapy when CD4 count is equal to or less than 200 cells/µl. Using the BED assay, this woman was considered to have been recently infected (approximately infected within 155 days before sample collection).

There was a small group of women (3%) that had previous exposure to SD NVP through pMTCT interventions. None of these women had evidence of NNRTI drug resistance. Surprisingly, of the three women who had received SD NVP, one had the I85V mutation associated with PI resistance. A number of studies have shown that up to 30% of women who receive SD NVP develop drug resistance mutation which may persist for up to six months as detected by population sequencing but fade away in the absence of therapy [Flys et al., 2005; Loubser et al., 2006]. The sensitivity of population sequencing in detecting NVP resistance mutations selected through pMTCT decreases with time after exposure [Loubser et al., 2006]. Within six months after exposure, the drug resistant quasie species will constitute less than 20% of the total viral population. Hence there was a very remote chance of detecting drug resistance due to their exposure to NVP using population sequencing in the group of women that had previous exposure to SD NVP. However, other more sensitive techniques such as allele specific

real time PCR, cloning or ultra deep pyrosequencing may have been able to detect any NVP selected minority quasie species if they were present.

For the determination of primary drug resistance, only data from patients who were classified as recently infected patients based on the BED results were considered. The low prevalence of primary drug resistance in the studied population is consistent with other studies that have been conducted in settings that are currently scaling up ART in Southern Africa [Kamoto and Aberle-Grasse, 2008; Maphalala et al., 2008; Pillay et al., 2008] which have reported prevalence levels of <5%, the WHO detection threshold limit in antiretroviral naïve populations. Unlike in most developed countries where access to ART started as monotherapy or dual therapy involving same class drugs and was associated with the rapid emergence of primary drug resistance [Fox et al., 2006; Wensing et al., 2005; Wensing et al., 2003], it is anticipated that the use of HAART in current ART scale up projects will result in a slower rate of the emergence of primary drug resistance. This is due to the higher genetic barrier to drug resistance associated with HAART as compared to monotherapy or same class dual therapy that might have fueled it in developed countries. Some mathematical models have shown that if clinicians adhere to good prescribing practices, monitor patients adherence strictly and patients failing their first line regimen are identified early and managed appropriately, it will take at least 10 years for primary drug resistance to go beyond the 5% threshold limit [Blower et al., 2005].

Another set of mutations that were also identified in some of the women in this study which are not considered for the calculation of the primary drug resistance were classified as borderline/suspicious by the CPR, Figure 12. These mutations are those that; (i) have demonstrable associations with antiretroviral exposure, and do not occur as natural

polymorphisms at a level above 0.5% in untreated individuals (based on current data), but have been excluded from the SDRM list because they do not fulfill the criterion of being widely recognized as treatment-associated mutations, or (ii) have some demonstrable association with treatment, but also occur naturally, either as rare polymorphisms close to the cut-off point for consideration as natural polymorphisms (e.g. between 0.5 and 1.0%), or as polymorphisms that are generally uncommon, but that occur at elevated frequencies in certain subtypes. Most studies on HIV-1 drug resistance have been done on HIV-1 Subtype B, however as more patients infected with non-subtype B viruses access therapy some of the mutations that are currently being considered as secondary may emerge to be critical in drug resistance. So, more work needs to be done on the evolution of HIV-1 drug resistance in non B subtypes.

Based on the phylogenetic analysis of the 236 pol sequences there was no evidence of close epidemiologic linkage of the sequences based on phylogenetic clusters supported by high bootstrap values. However, analysis of larger sequences or the env sequences could have shown some linkages if there were any that couldn't be detected by the choice of sequences used in the analysis

The HIV-1 Subtype C consensus sequence for the Pol gene generated from the samples from Chitungwiza was 100% similar to the Los Alamos database HIV-1 Subtype C consensus. When the 236 Zimbabwean sequences were compared with sequences from other countries for geographic clustering, there was no significant clustering amongst the sequences from the Southern African region. However, subtype C sequences from Brazil, India, China and Ethiopia formed separate clusters. This could be evidence of the lack of compartmentalization in the HIV epidemic in Southern Africa due to the free population movement in the region. If this lack of

compartmentalization can also be demonstrated for the rest of the HIV genome, it could be evidence that in vaccine development, a single vaccine for HIV-1 subtype C in Southern Africa would be sufficient to cover the whole region. However, this could not be extended to other regions with HIV-1 Subtype C such as India, Ethiopia and Brazil.

The identification of patients acutely infected for the purposes of assessing the transmission of drug resistance is usually problematic. The ideal population would be patients with documented evidence of recent seroconversion through longitudinal studies where HIV-1 sero-negative participants are followed through seroconversion and their approximate dates of infection are known or with laboratory confirmation of recent infection using a validated assay. Most studies on primary HIV-1 drug resistance in resource limited settings have been targeting young treatment naïve women attending antenatal clinics on their first pregnancies and less than 22 years as the population for the assessment of transmitted drug resistance [Bennett et al., 2008b; Maphalala et al., 2008; Pillay et al., 2008; Somi et al., 2008]. The assumption being that they would be recently infected or at least infected within 2 years of enrollment in the studies. This is also a very convenient population because of the structures and resources already available for ANC surveys that are already in place in most countries.

The BED assay, an antiHIV-1 IgG capture antibody assay that estimates the duration of an infection based on the proportion of HIV-1 specific IgG antibodies out of the total IgG antibodies, was used to try and identify recently infected woman among the women enrolled in our study. Thirty two percent of the enrolled women were classified as recently infected. From the drug resistance analysis of the sequences obtained from these women, there seems to be no evidence of primary drug resistance in the population studied.

The only two samples which had evidence of drug resistance, were excluded from the assessment of primary drug resistance because they were from women not recently infected. SCR541 was classified to have a long term infection. SCR423 was classified as recently infected based on the BED assay; however, she had prior exposure to SD NVP in a previous pregnancy. This could have been a case of misclassification by the BED assay. She had a high CD4 count, 750 cells/µl suggesting that she could have been a slow progressor. The selection criterion for participants in the study for primary drug resistance surveillance was supposed to have excluded those participants who had previous exposure to ART, either through pMTCT programs or HAART programs. Obviously, this exclusion criterion was not adhered to based on the nine (including SCR423) women who received SD NVP enrolled in the study.

Although the BED assay was used to try and identify recently infected women, it also has its own challenges. When used in HIV-1 incidence studies, it was shown that the BED assay produces slightly higher incidence rates [Karita et al., 2007]. The window period for the assay for different HIV-1 Subtypes varies. One prospective study done in pregnant women in Zimbabwe recommended a cut off of 187 days [Hargrove et al., 2008] instead of the 155 days recommended by the kit manufactures after noticing differences in the maximum duration of seropositivity in those testing as recently infected. The assay has been documented to misclassify patients in about 2-3% of cases [McDougal et al., 2006], however this data was obtained from studies on patients in settings where HIV-1 Subtype B predominates. The misclassification is most common in patients with advanced disease, WHO stage III and IV, characterized by severe weight loss (>10%), unexplained chronic diarrhea, pulmonary TB within the last two years, extrapulmonary tuberculosis, KS, among many other opportunistic infection associated with severe immunosuppression (CD4 count < 100 cell/µl). It these patients, the misclassification are

caused by HIV-1 antibody regression caused by the falling of the proportion of the HIV-1 specific antibodies below the levels at which the samples will be classified to have been obtained from recently infected subjects. The misclassifications have also been associated with cases of irregular antibody response due to other pathological conditions such as X-linked agammaglobulinemia, fortunately these are not common in Zimbabwe [National Cancer Registry, Zimbabwe-2004 Data].

For this study, the cut off recommended by the manufacturers, 155 days, was used. A prospective study in post partum women by the ZVITAMBO study group [Hargrove et al., 2008] recommended the use of a cut off of 187 days for the Zimbabwean population instead of the manufacturer recommended cut off. Multiple other studies from different settings including South Africa, Uganda and Zambia have been using the cut off values recommended by the manufacturers[BÃ¤rnighausen et al., 2008; Hall et al., 2008; Karita et al., 2007; McDougal et al., 2006; Mermin et al., 2008; Nesheim et al., 2005]. Using the ZVITAMBO cut off on the current data reduced the percentage of women considered to have been infected during pregnancy from 95% to 86%, which is not a statistically significant difference (P = 0.069). Therefore for the purposes of just distinguishing recently infected from long term infected, any of the two cut offs can be used.

In addition to SCR 423, two other women were classified as recently infected by the BED assay although they had reported prior exposure to ARVs through pMTCT during previous pregnancies. Like SCR 423, these women, SCR059 and SCR269 also had high CD4 counts, 478 and 690 respectively, all were above the median CD4 count of the women classified as recently infected (429 cell/µl). Based on data from settings where HIV-1 Subtype B is predominant, on

average patients seroconvert at CD4 count of 1000 and the count drops by almost a quarter within the first six months of seroconversion [Mellors et al., 1997; Schacker et al., 1996; Stein et al., 1992]. By the end of the first year, the CD4 counts would have dropped by almost 35% and will then decline by approximately 50 cells per year until they reach the AIDS state or are initiated on ART [Hughes et al., 1994; Margolick et al., 1992; Stein et al., 1992]. This could mean that these women were infected within two years prior to enrollment into the study. SCR 059 received ART for PMTCT in 2005, a year before she was enrolled into the study and her spouse was on HAART at the time of her enrollment.

Out of the 72 women who were classified as recently infected using the BED assay, almost 95% of them were infected during pregnancy, based on information from the gestational period. The parent SCR study did not collect data on the HIV status of the spouses of the enrolled women. What would certainly be interesting would be to explore the social and cultural dynamics that might be associated with the behaviors of the male spouses if they too were found to be exposed to and getting HIV infection during pregnancy of their spouses.

Using the WHO recommendation for HIV DR Threshold surveys, thirty two percent (32%), 24 out of 75 of the recently infected women would be excluded from the list of recent infections based on the demographic data for the purposes of determining the prevalence of primary drug resistance because they were pregnant before. Of these, three (SCR059, SCR269 and SCR423) reported use of ARVs for pMTCT in their earlier pregnancies. Both SCR059 and SCR269 took triple HAART regimen combination for pMTCT but did not state the specific ARVs they took. The spouse of SCR059 was on HAART at the time at which she enrolled for the study. SCR423 took SD NVP for pMTCT. An additional five (5) would be excluded because they had CD4

counts that were very low, less than 200cells/μL. For these five, its either they were truly recently infected but were ultra-rapid progressors or they may have been misclassified as recently infected. If they were ultra-rapid progressors, they should not have been excluded in the determination of the prevalence of primary drug resistance [Bennett et al., 2008b]. For the purposes of this study, only the three women, who had received ARVs for pMTCT previously, were excluded from the list of recently infected women. Hence, after the correction, 32% of the women in this study were recently infected, 67% had long term infections, and 1% was misclassified.

There were several limitations encountered in this study. The prevalence of drug resistance in the study population was too low to enable the determination of any significant differences in the prevalence of drug resistance between recently infected and chronically infected women. In addition, we also couldn't determine if there was any relationship between the women's CD4 counts or viral load and the presence of drug resistant mutations. However, it has been shown in other studies that most drug resistant viruses are mutants which are not as fit as the wild type viruses and have lower replicative capacities and would result in lower viral burdens [Croteau et al., 1997]. The numbers of patients that had their viral loads determined were very low. The methods currently validated for HIV-1 viral load measurement are very expensive to run, and thus we were unable to determine viral loads for all the study participants. Hence there is a great need for the evaluation, validation and introduction of low cost methods for viral load measurement. Examples of methods that are currently under evaluation include a method from Partec (Munster, Germany) that combines RT PCR and flow cytometry as well as Real Time PCR methods. However, the later method requires a consistent supply of the appropriate reproducible standards, which are sometimes difficult to find locally. Due to the low numbers in

the samples with viral load results, no definite relationship could be established between the viral load results and the infection duration of the women.

CHAPTER 6:CONCLUSION AND RECOMMENDATIONS

The prevalence of primary drug resistance in Chitungwiza, four years after the initiation of the national ART program, is still below the WHO threshold limit of 5%. To preserve the low prevalence of primary drug resistance, proper prescribing practices of ARVs, as well as adherence ART education have to be maintained. Patients have to be adequately monitored to identify those failing therapy due to drug resistance so as to switch them before they can spread the drug resistant viruses. The high percentage of women who were infected during pregnancy shows the urgent need for more prevention education in pregnant women and behavioral change in their spouses that might be putting their spouses and unborn children at risk of HIV infection.

As the ART coverage in Zimbabwe increases during the next couple of years, more drug resistance monitoring studies in patients failing ART are important to keep track of the evolution of drug resistance as well as surveillance for primary drug resistance. It is also important to monitor the patterns of HIV-1 secondary drug resistance mutation associated with treatment failure. This can help in the development of low cost screening assays for use in patients failing therapy instead of sequencing methods.

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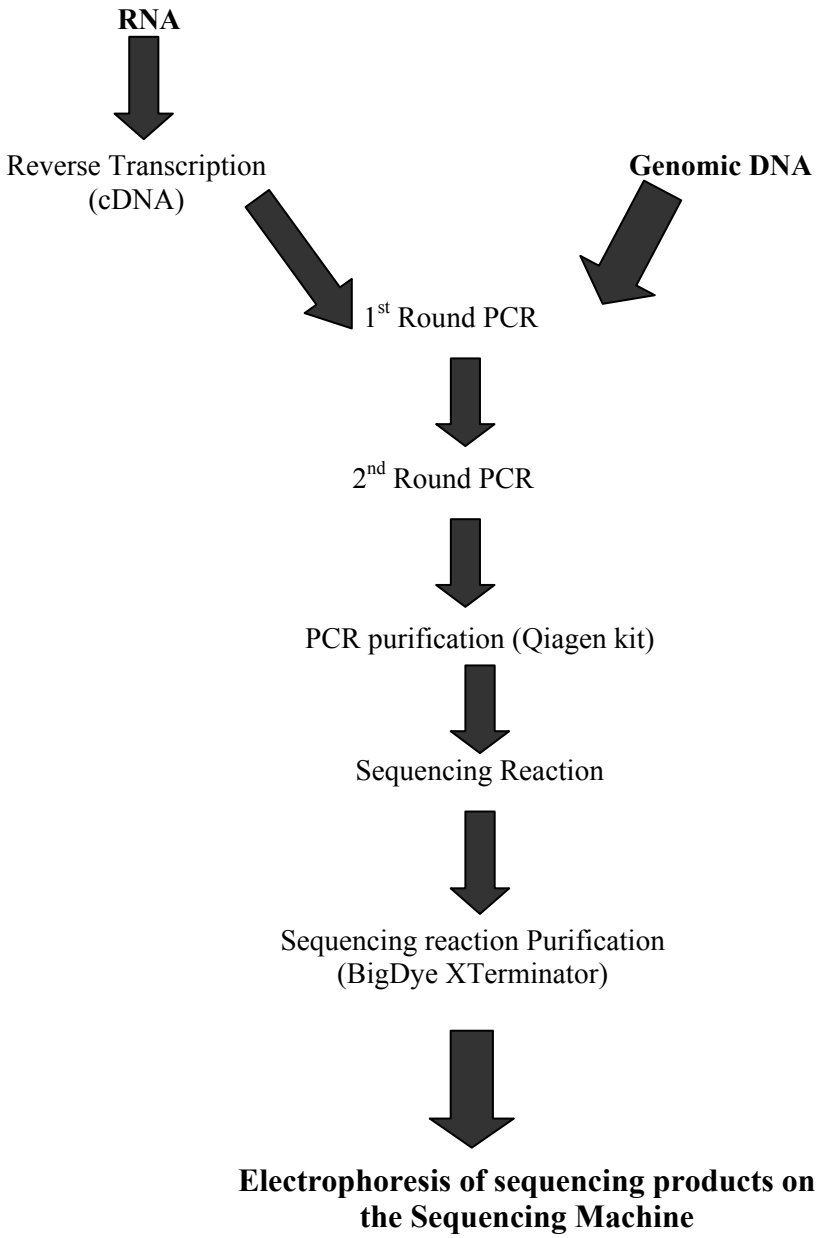
APPENDICES

Appendix I: HIV-1 Pol Sequencing Schema



Appendix II

STANDARD OPERATING PROCEDURE:

Title: **In-house assay for *pol* sequencing to monitor ARV Drug Resistance**

Purpose: **In House Assay for *pol* Sequencing to Monitor ARV Drug Resistance**

Scope

To provide instruction on how to amplify *pol* fragments from RNA using the Superscript III First Strand Synthesis Kit (Invitrogen Corporation, Carlsbad CA, USA) to generate cDNA followed by a nested PCR using the Platinum Taq PCR System (Invitrogen Corporation, Carlsbad CA, USA). This SOP also provides instruction for the sequencing of the amplified pol product using the unidirectional dideoxynucleotide sequencing with the Big Dye Terminator Chemistry (Applied Biosystems Inc, Foster City, CA, USA) and the assembly of the sequences for drug resistance analysis for sequence and drug resistance analysis.

Contents

1.0 Safety

This protocol requires the use of potentially infectious substances (HIV-1,-2, HSV, Hepatitis); therefore, each person to use this protocol must have documented training in laboratory safety. The safety training should cover operator safety and the safe handling and disposal of biological material.

2.0 Principle of the test

- Total nucleic acid is extracted from plasma using the Roche RNA extraction kits.
- The SuperScript III RT-PCR System containing Superscript III RT, a Moloney Murine Leukemia Virus (M-MLV) RT that has been engineered to reduce RNase H activity and provide increased thermal stability, is used to produce high yields of cDNA.
- cDNA is synthesized by using total RNA primed by random primers 45°C.
- RNA targets from 100bp to more than 12kb can be amplified with this system.

3.0 Definitions

Abbreviations

Bp base pairs

nPCR Nested polymerase chain reaction

PCR Polymerase chain reaction

PI Principle Investigator

RT Reverse transcriptase

RPM Revolutions per minute

SOP Standard Operating Procedure

4.0 Materials and equipment

4.1 RNA is extracted from plasma using the guanidine containing Roche RNA extraction kit (Roche Diagnostic Corporation, Indianapolis, USA).

Reagents and solutions supplied as part of the Roche RNA extraction kit

Guanidine containing lysis buffer

Elution Buffer

Materials to be supplied by user
Disposable gloves
Sterile pipette tips with aerosol barrier
2 ml Nuclease microcentrifuge tubes
Serological pipettes
Isopropanol
Ethanol

Equipment
Microcentrifuge
Vortex Mixer
micropipettes
Pipette Aid

4.2 Reverse Transcription using Superscript III RT-PCR kit (Invitrogen Corporation, Carlsbad, CA, USA)

Reagents and solutions supplied as part of Superscript III *RT-PCR* kit
ThermoScript™ RT (15U/µl)
5 X cDNA Synthesis buffer
0,1M DTT
10mM dNTP mix
RNaseOUT™ (40U/µl)

Oligo $(dT)_{20}$ (50µM)
Random hexamers (50ng/µl)
DEPC-treated water
E. coli Rnase H (2U/µl)

Reagents and solutions supplied by user
Molecular Biology grade ultra-pure water

Materials to be supplied by user
0.2ml thin-walled PCR tubes
Sterile pipette tips with aerosol barrier
Disposable gloves
Ice

Equipment
Ice-bucket
Pipette Aid
-20°C Freezer
Thermocycler (ABI 2720)
Microcentrifuge
Timer

4.3 nPCR using Platinum Taq polymerase and Amplicon clean up using Qiagen kits

Reagents and solutions supplied as part of kit
Platinum Taq kit (Invitrogen Corporation, CA, USA)
- Platinum Taq polymerase.
- 10 x PCR Buffer.
- 50 mM $MgCl_2$.

Qiaquick PCR Purification kits (Qiagen Inc. Valencia, CA, USA)
- Spin columns
- Buffer PB
- Buffer PE
- Buffer EB

Reagents and solutions supplied by user
25 mM dNTPs (Invitrogen Corporation, Carlsbad, CA, USA)
Molecular biology grade ultra-pure water
Gene specific primers
1KB plus molecular weight marker (Invitrogen Corporation, CA, USA)

Materials to be supplied by user
0.2ml thin-wall PCR tubes
2ml micro-centrifuge tube
Sterile pipette tips with aerosol barrier
Disposable gloves
Ethanol

Equipment
Ice-bucket
Pipette Aid
-20°C Freezer
Microcentrifuge
Thermocycler (ABI 2720)
NanoDrop scanning spectrophotometer (NanoDrop technologies Inc, Wilmington, DE)

4.4 Big Dye Terminator Sequencing Reaction and clean up

Reagents and solutions supplied as part of kits
Big DyeTerminator v3.1 Cycle sequencing kit (Applied Biosystems Inc, Foster City, CA)
- Big Dye Terminator v3.1 Cycle sequencing kit ready mix
- Big Dye Terminator sequencing buffer 5 X

Big Dye XTerminator kits (Applied Biosystems Inc, Foster City, CA)
- XTerminator solution
- SAM Solution.

Reagents and solutions supplied by user
25 mM dNTPs
Molecular biology grade ultra-pure water
Gene specific primers

Materials to be supplied by user
0.2ml thin-wall PCR tubes
96 well reaction plates
Sterile pipette tips with aerosol barrier
Wide-bore pipette tips
Disposable gloves

Equipment
Ice-bucket
Pipette Aid
-20°C Freezer
Microcentrifuge
Thermocycler (ABI 2720)
Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA)

4.5 Software

Sequencher ver 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA)
Bioedit
MEGA
HIVDB (http://hivdb.stanford.edu/pages/algs/sierra_sequence.html)

5.0 Procedures

5.1 RNA Extraction using the Roche method

RNA extraction room:

Before Starting:

- Incubate lysis buffer in 37^{o}C water bath until all crystals are dissolved.

5.1.1 Add 600 µl lyses buffer to 500 µl plasma in a 2 ml eppendorf tube. Vortex for 15 seconds. (release nucleic acids from viral particles)

5.1.2 Allow to incubate at room temperature for 10 minutes.

5.1.3 Add 600 µl isopropanol. Vortex for 15 seconds. (precipitates out RNA)

5.1.4 Centrifuge for 15 minutes at 15,000 rpm.

5.1.5 Remove supernatant. Add 1 ml 70% ethanol and vortex for 15 seconds. (washes RNA)

5.1.6 Centrifuge for 5 minutes at 15,000 rpm.

5.1.7 Remove supernatant. Add 60 µl elution buffer. Allow to incubate for 1 minute at room temperature then vortex for 15 seconds. Freeze RNA or use in experiment.

5.2 cDNA synthesis (Reverse Transcription)

- Set 2 heating blocks at 65°C and 50°C, respectively, and a water bath at 37°C, or alternatively, a thermocycler can be used for the 3 different temperature incubations.

Note: Always include one positive control (pooled RNA from HIV positive individuals to ensure high viral load that ensures that the PCR reaction works) and one negative control (pooled RNA from HIV negative individuals to control for reagent contamination) with every batch of cDNA samples to be amplified.

Pre-PCR clean room:

5.2.1 Prepare master mix solutions 1 and 2 as indicated in the tables below. Keep all tubes on ice.

5.2.2 Transfer 4µl of master mix 1 to 0.2 ml PCR tubes or 96 tube reaction plates.

5.2.3 Move to the PCR area

Master Mix 1- Annealing Step (primers attach to RNA)

Component	1 x Reaction	Concentration in the final reaction mixture	Comment
50 ng/µl Random Hexamers	2.8µl	7 ng/ µl	Enable whole genome reverse transcription
25 mM dNTP	1.2µl	1.5mM	A, C, T, G nucleotides
TOTAL	**4.0µl**		

Master Mix 2- Reverse Transcription Step

Component	1 x Reaction	Concentration in the final reaction mixture	Comment
5 x First Strand Buffer	4µl	1 M	
0.1M DTT	1µl	0.005M	Disruption of disulfide bond
RNaseOUT (40 U/µl)	1µl	2 U/µl	Inactivates any RNAses if present
Superscript III Reverse Transcriptase (200 U/µl)	1µl	10 U/µl	Reverse transcriptase enzyme
TOTAL	**7.0µl**	**-**	

PCR room:

5.2.4 Add 9µl of RNA template to the 4µl master mix 1 in the reaction tubes and close them.

5.2.5 Heat for 5 minutes at 65^{o}C in thermocycler, then cool to 4^{o}C in thermocycler or take out of thermocycler and rapidly cool on ice.

5.2.6 Without removing the tubes from ice, remove the caps and add 7µl of master mix 2 to each of the tubes.

5.2.7 Recap the tubes with new lids and return them into the thermal cycler. Run one cycle of the following program on the thermocycler:

25 °C 10 min
45 °C 30 min
70 °C 15 min

5.2.8 You now have cDNA! Use right away or freeze at -20 °C or colder for further use.

5.3 nPCR using Platinum Taq polymerase PCR System.

Always include a positive and negative control with every 10 samples to be amplified by PCR.

Prepare PCR master mixes as follows:

5.3.1 Primers for *pol* nPCR

Stage	Primer Name	Sequence	Length	Direction	HXB2 Position
1st Rnd	**MAW-26**	TTGGAAATGTGGAAAGGAAGGAC	23	Forward	2028-2050
	RT-21	CTGTATTTCTGCTATTAAGTCTTTTGATGGG	31	Reverse	3539-3509
2nd Rnd	**Pro-1**	CAGAGCCAACAGCCCCACCA	20	Forward	2147-2166
	RT-20	CTGCCAGTTCTAGCTCTGCTTC	22	Reverse	*3462-3441*

1st Round PCR

Pre-PCR clean room:

5.3.2 Prepare the 1st round PCR master mix as indicated in the table below. Keep all tubes on ice.

1st Round PCR mastermix:

Reagent	1 x reaction	Final concentration in reaction mixture
DEPC treated water	**76.5**	**-**
10X PCR	**10µl**	**1**
50 mM $MgCl_2$	**5**	**2.5 mM**
25 mM dNTP Mix	**1**	**0.25mM**
Forward Primers 50pmol/µl MAW26(1^{st} Rnd) / RT20(2^{nd} Rnd)	**1**	**0.5pmol /µl**
Reverse Primers 50pmol/µl RT21 (1^{st} Rnd) / Pro1 (2^{nd} Rnd)	**1**	**0.5pmol /µl**
5 U/ µl Platinum Taq Polymerase	**0.5µl**	**0.025U**
Total	**95**	**-**

5.3.3 Transfer 95µl of master mix to 0.2 ml PCR tubes or 96 tube reaction plates.

5.3.4 Move to the PCR room.

PCR Room

5.3.5 Add 5 µl cDNA to the tubes with the 95µl of the master mix.

5.3.6 Close the tubes, put the samples in the thermocycler and run the following program:

-Hold: 94°C for 2 min
-30 Cycles:
94 °C 30 sec
55 °C 20 sec
72 °C 2 min
-Hold: 72 °C 10 min
-Hold: 4 °C until ready to use or store

2^{nd} Round PCR:

Pre-PCR clean room:

5.3.7 Prepare the 2^{nd} round PCR master mix as indicated in the table below. Keep all tubes on ice.

Reagent	1 x reaction	Final concentration in reaction mixture
DEPC treated water	78	
10X PCR	10µl	1 x
50 mM $MgCl_2$	3.5	1.75 mM
25 mM dNTP Mix	1	0.25mM
Forward Primers 50pmol/µl MAW26(1^{st} Rnd) / RT20(2^{nd} Rnd)	1	0.5pmol /µl
Reverse Primers 50pmol/µl RT21 (1^{st} Rnd) / Pro1 (2^{nd} Rnd)	1	0.5pmol /µl
5 U/ µl Platinum Taq Polymerase	0.5µl	0.025U
Total	95	-

5.3.8 Transfer 95µl of master mix to 0.2 ml PCR tubes or 96 tube reaction plates.

5.3.9 Move to the PCR room.

PCR Room

5.3.10 Add 5 µl of the 1^{st} round PCR product to the tubes with the 95µl of the master mix.

5.3.11 Close the tubes, put the samples in the thermocycler and run the following program:

-Hold: 94°C for 2 min
-30 Cycles:
94 °C 30 sec
55 °C 20 sec
72 °C 2 min
-Hold: 72 °C 10 min
-Hold: 4 °C until ready to use or store at –20°C.

5.4 Verification of PCR Product: Agarose Gel Electrophoresis

5.4.1 Add 1 g ultraPure agarose gel to 100 ml 1X TBE Buffer to make a 1% agarose gel.

5.4.2 Microwave on high for 2-3 minutes, stopping and swirling occasionally to dissolve agarose into buffer.

5.4.3 Add 100 µl ethidium bromide needed for UV detection of DNA.

5.4.4 Pour gel into a gel casting tray and add combs. Allow 15-20 minutes for gel to set.

5.4.5 To load samples, mix 10 µl of each 2nd round product with a drop of blue loading dye (to visualize what you are loading into the gel) and pipette one sample per well. For ladder, use 1 µl 1Kb Plus Ladder mixed with 9 ul TBE buffer and a drop of blue loading dye.

5.4.6 Allow gel to run for 45 minutes at 120V.

5.4.7 Check for DNA bands by placing gel on UV light and verify amplification of the 1200 bp second round product.

5.5 2nd round PCR product cleaning using the Qiagen PCR product clean up kit.

5.5.1 Add 500 µl of Buffer PB to a 1.5 ml centrifuge tube

5.5.2 Add 100 µl of the 2nd round PCR product to the tube and mix by briefly vortexing

5.5.3 Place a QIAquick spin column in a provided 2 ml collection tube.

5.5.4 To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 sec.

5.5.5 Discard flow-through. Place the QIAquick column back into the same tube.

5.5.6 To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60 sec.

5.5.7 Discard flow-through and place the QIAquick column back in the same tube.

5.5.8 Centrifuge the column for an additional 1 min.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

5.5.9 Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

5.5.10 To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from the 50 µl elution buffer. Store DNA at –20°C or colde

5.6 Determine the DNA concentration for each sample using a NanoDrop ND-1000 Spectrophotometer.

5.7 PCR product sequencing using the Big Dye Terminator V3.1 Cycle Sequencing Ready Reaction Kit.

5.7.1 Primers used for sequencing

Primer Name	Sequence	Length	Direction	HXB2 Position
RTC1F	ACCTACACCTGTCAACATAATTG	23	Forward	2486-2508
RTC2R	TGTCAATGGCCATTGTTTAACCTTTGG	27	Reverse	*2630-2604*
RTC3F	CACCAGGGATTAGATATCAATATAATGTGC	30	Forward	2956-2994
RTC4R	CTAAATCAGATCCTACATACAAGTCATCC	29	Reverse	*3129-3101*
RT-y	GTGTCTCATTGTTTATACTAGG	22	Reverse	*2967-2946*
MAW-46	TCCCTCAGATCACTCTTTGGCAACGAC	27	Forward	2251-2277

Pre-PCR clean room:

5.7.2 Prepare the big dye master mix for each of the above primers as indicated in the table below

Reagent	1 x reaction	Final Concentration in reaction mixture
Water	12µl	-
5 X Sequencing Buffer	3.0µl	0.75 x
BigDye Terminator Sequencing mix	2.0µl	-
2.5 pmol/µl Primer	1.0µl	0.125pmol/µl
Total	**18.0µl**	

5.7.3 Transfer 18µl of master mix to 0.2 ml PCR tubes or 96 tube reaction plates.

5.7.4 Move to the PCR room

5.7.5 Add 2µl of the purified PCR product to the tubes containing the master mix solution. Each sample will be added to four different primers.

5.7.6 Close the tubes, put the samples in the thermocycler and run the following program:

-Hold: 94°C for 30 sec
-28 Cycles:
96 °C 30 sec
50 °C 5 sec
60 °C 4 min
-Hold: 4 °C until ready to use.

5.8 Clean the sequencing products using the Big Dye XTerminator kits.

5.8.1 After the sequencing reaction, briefly centrifiuge the reaction plates *(If you used the 0.2 ml PCR reaction tubes for the sequencing reactions, transfer the sequencing rections to 96 well reaction plates first).*

5.8.2 Pipette 90µl of the SAM solution into each reaction.

5.8.3 Mix the XTerminator solution thoroughly by vortexing and then pipette 20µl into the reaction wells using a wide bored pipette tip.

5.8.4 Seal the plate using a clear adhesive.

5.8.5 Vortex the plate for 30 minutes, then briefly centrifuge it.

5.8.6 Place the reaction plate in the ABI sequence analyzer . Select run module and run the plate.

5.9 Sequence Analysis

5.9.1 Edit and form contiguous sequences for the complete Pol region using the program Sequencher V 4.5 (Gene Codes Corporation, USA).

5.9.2 Align sequences and perform phylogentic tree analysis using ClustalW in BioEdit Sequence Alignment Editor software (Tom Hall, North Carolina State Univerisity) and Mega 2. Include subtype reference alignments provided for sequence analysis from the Los Alamos HIV Database

5.9.3 To detect mutations associated with ARV resistance, submit the nucleic acid sequence to the Stanford HIV database's HIVDB program for analysis (http://hivdb.stanford.edu/pages/algs/sierra_sequence.html).

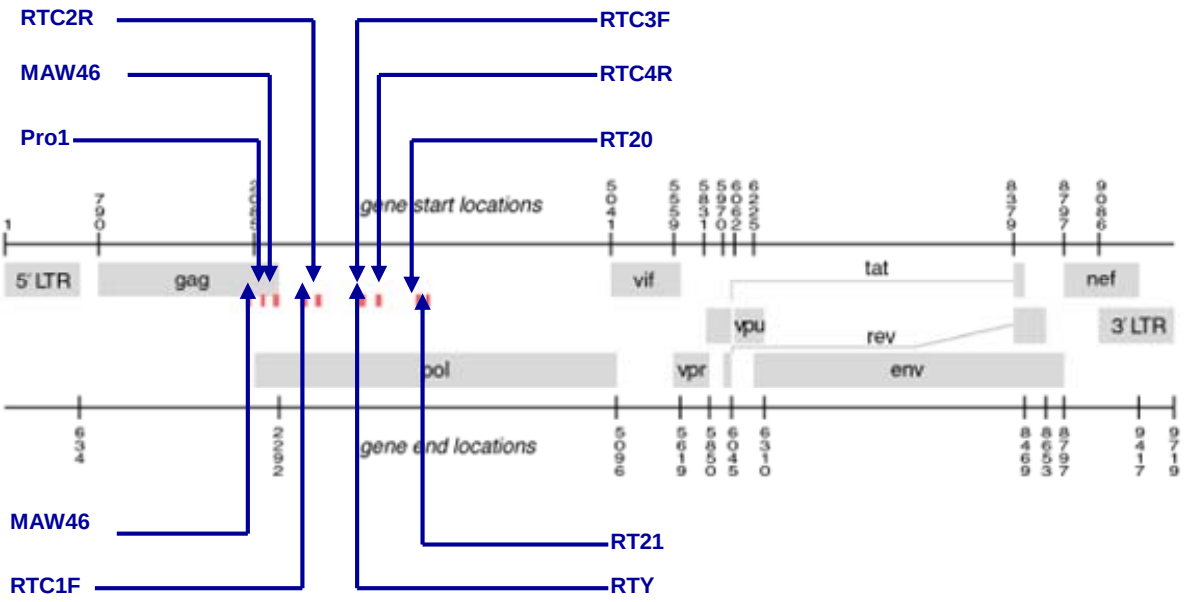
6 Quality Checks and prevention of contamination

6.1 Manipulation of potentially infected blood sample should be performed according to proper biosafety procedures

6.2 Rooms in which DNA and RNA is extracted, as well as reagents, pipetters and other supplies should be separated from rooms where post amplification products are handled according to the PCR guidelines. Always include an HIV negative RNA sample to generate cDNA that is used for subsequent PCR steps to control for false positive results. Always include a HPLC water control in the PCR steps to control for reagent contamination. In addition, when performing sequence analysis, perform checks for sequence contaminants by following the guidelines on the Los Alamos HIV Database (http://www.hiv.lanl.gov/content/hiv-db/CONTAM/contam_main.html)

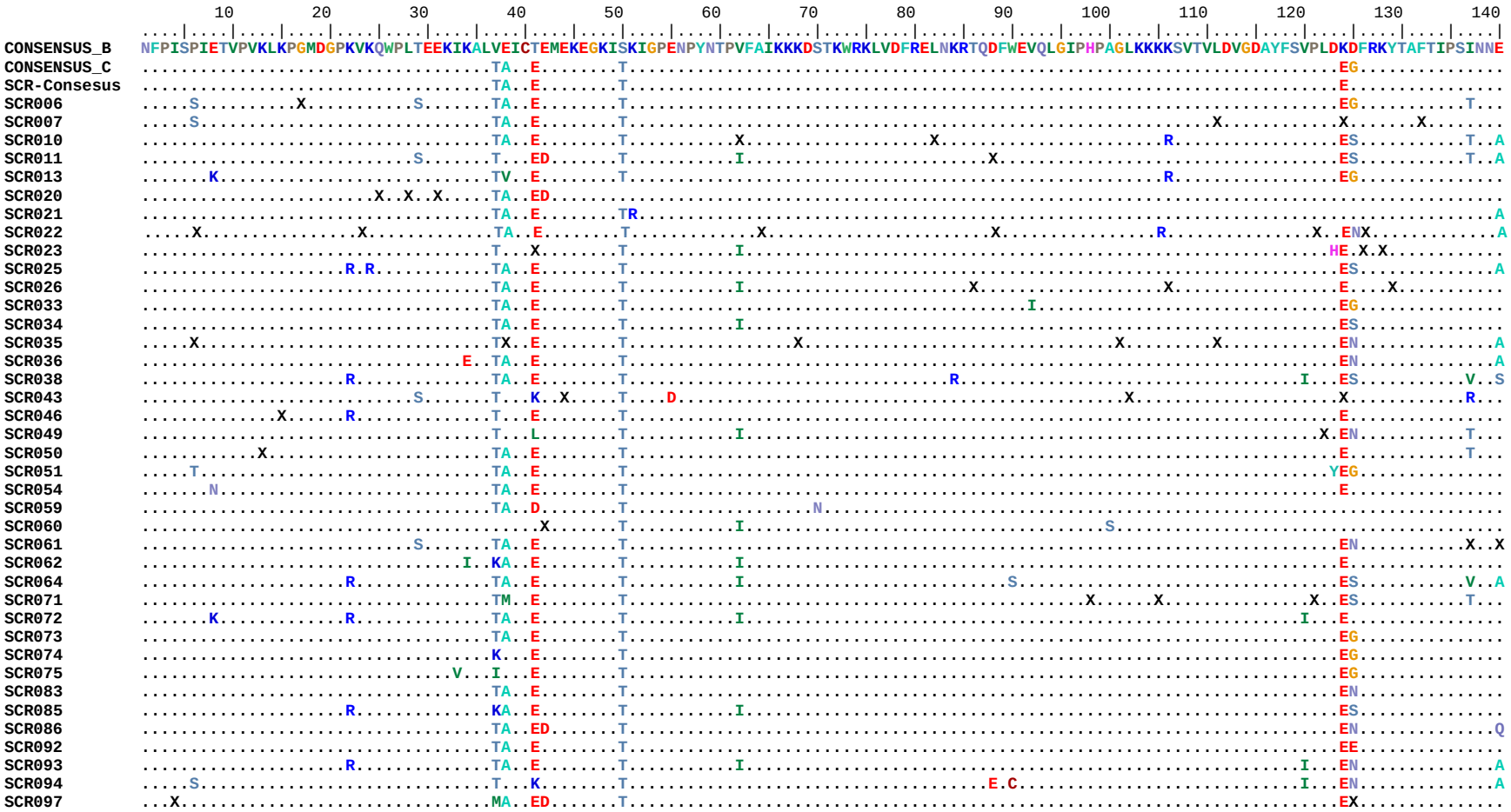
6.3 If PCR is expected to be positive, and it is negative, it could be due to red cell inhibitors. The use of QIAGEN kit instead of the Roche kits for RNA extraction can remove most of the inhibitors.

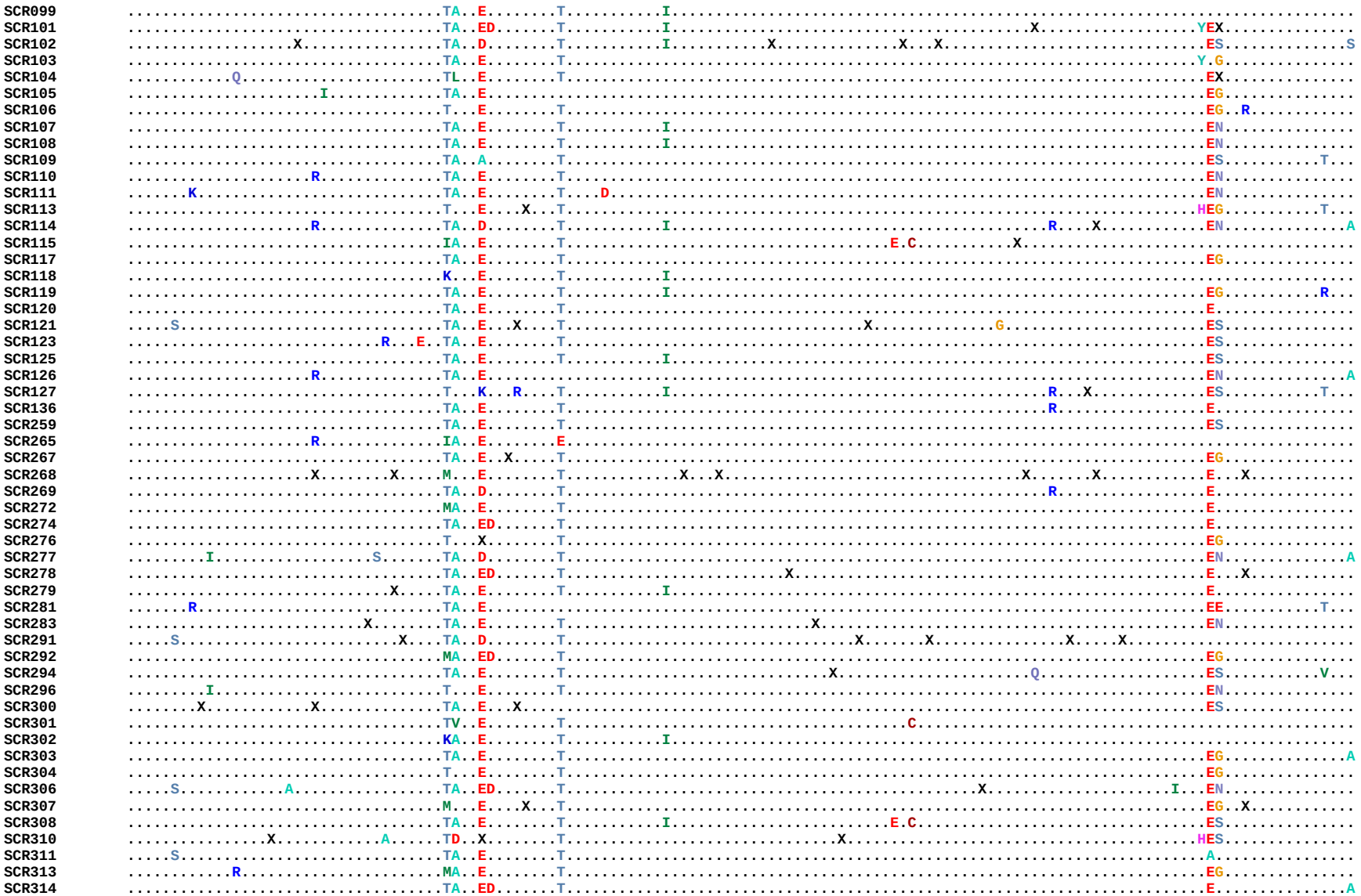
7 Primer Map for the *gag-pol* nPCR and sequencing primers



Appendix III: Sequence alignments

RT Sequence Alignment

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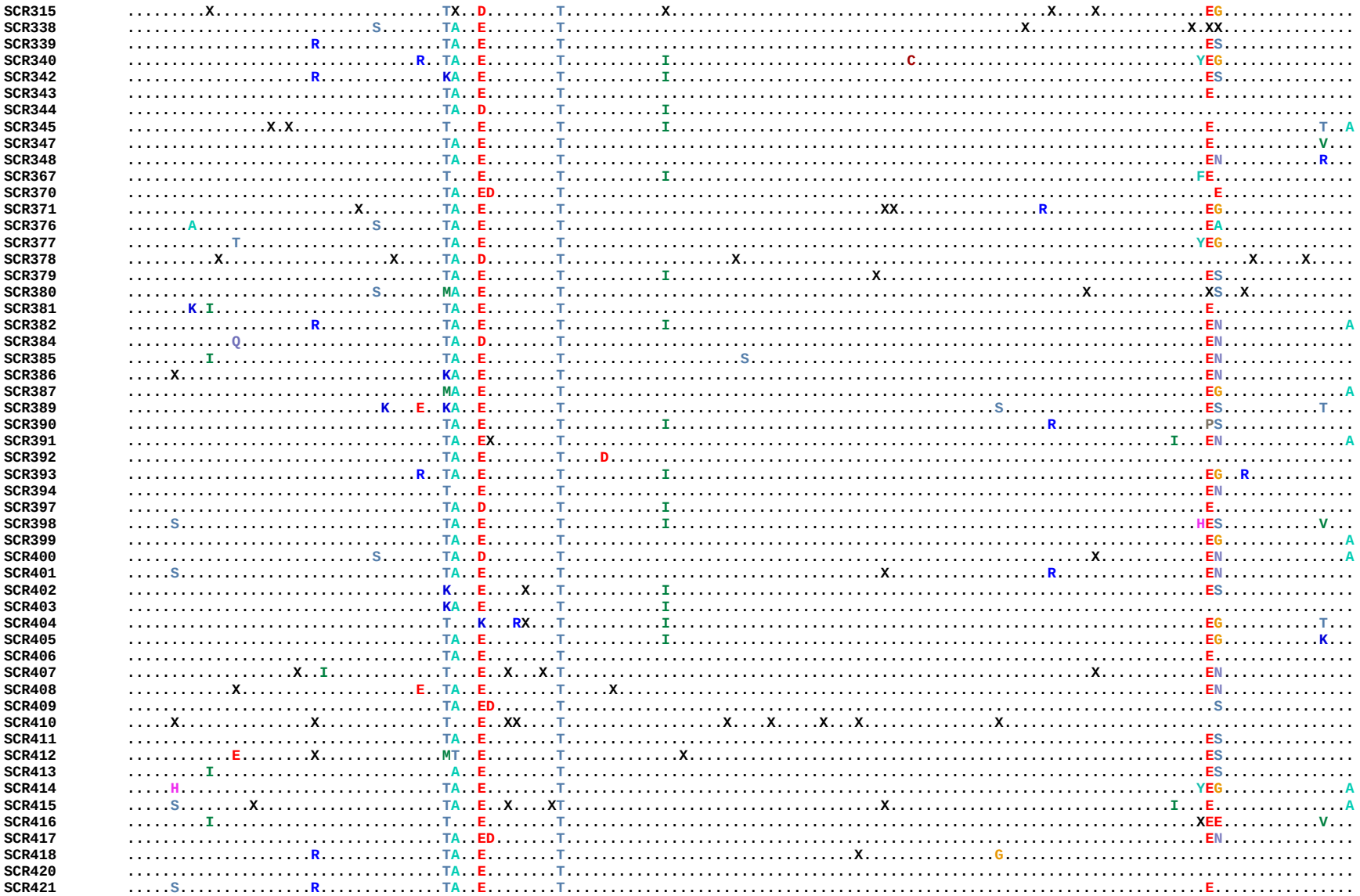
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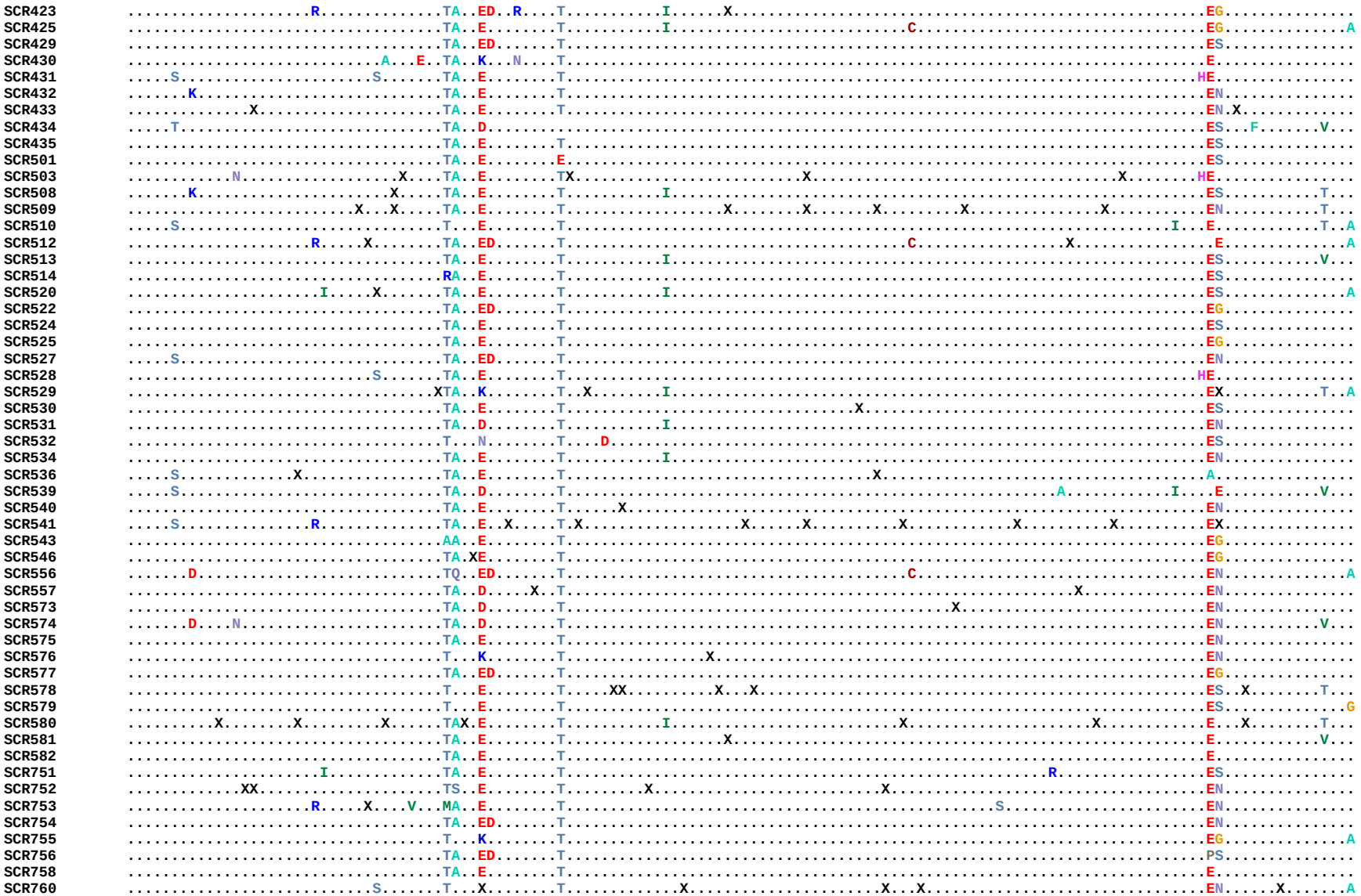
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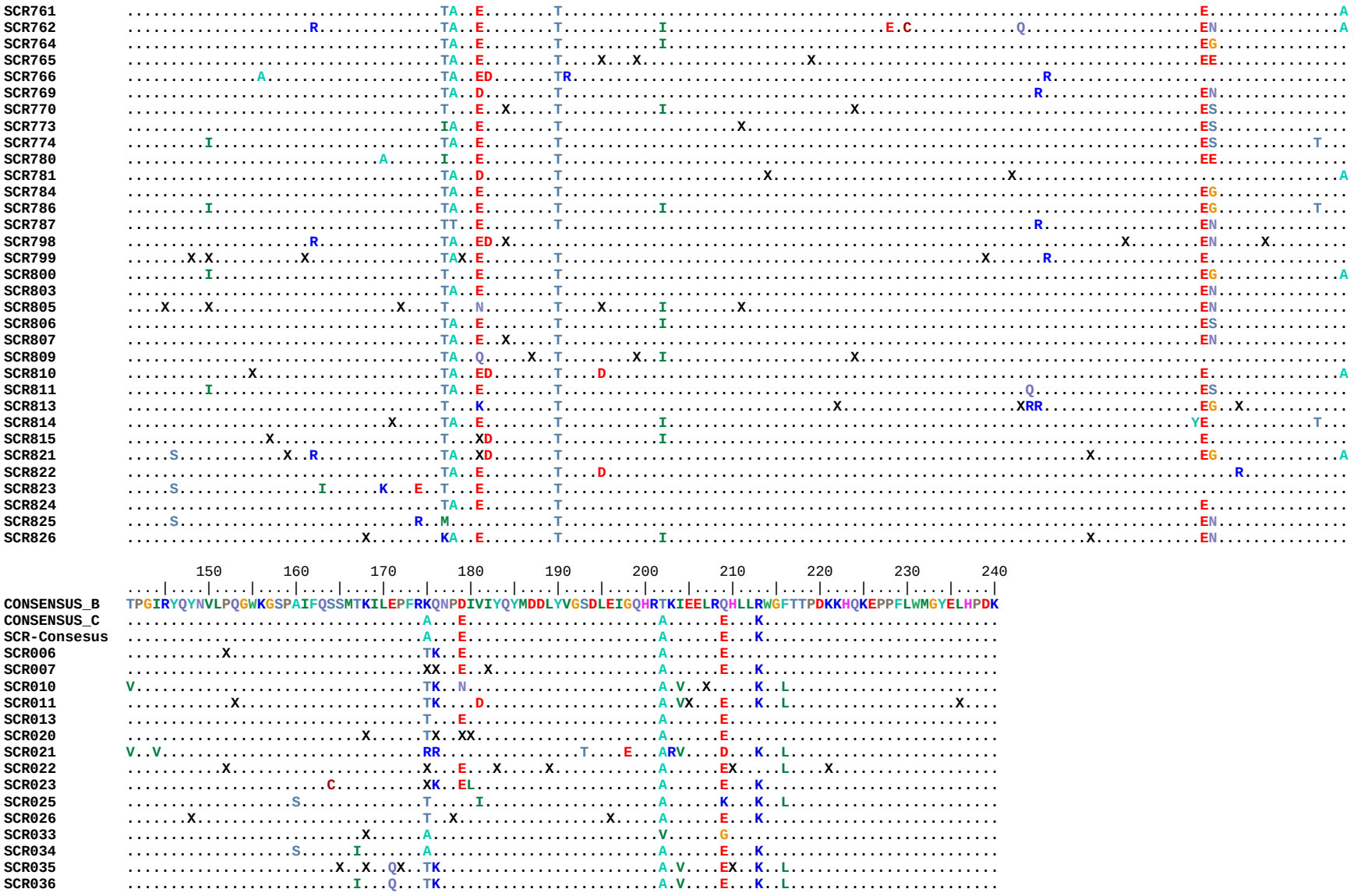
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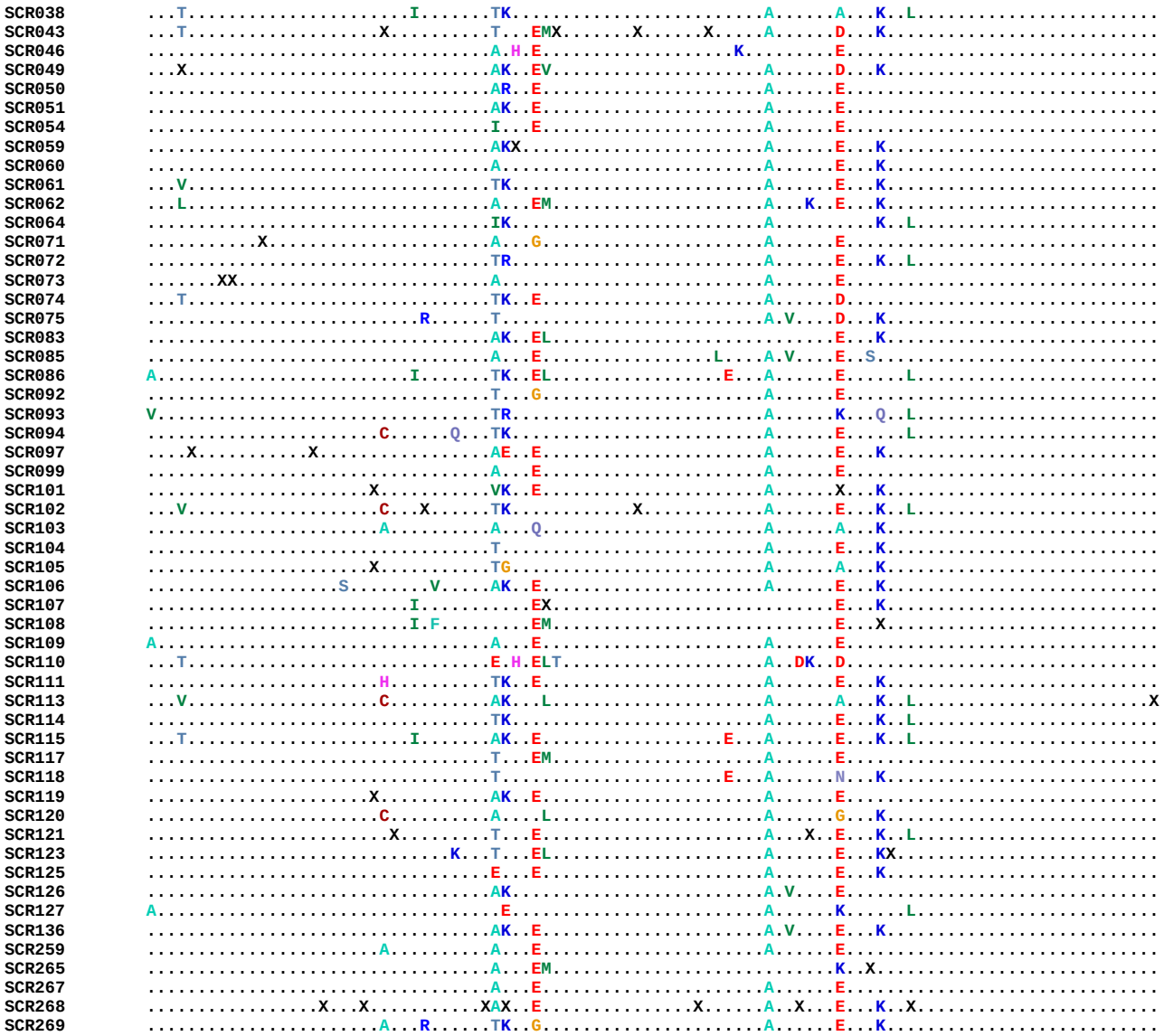
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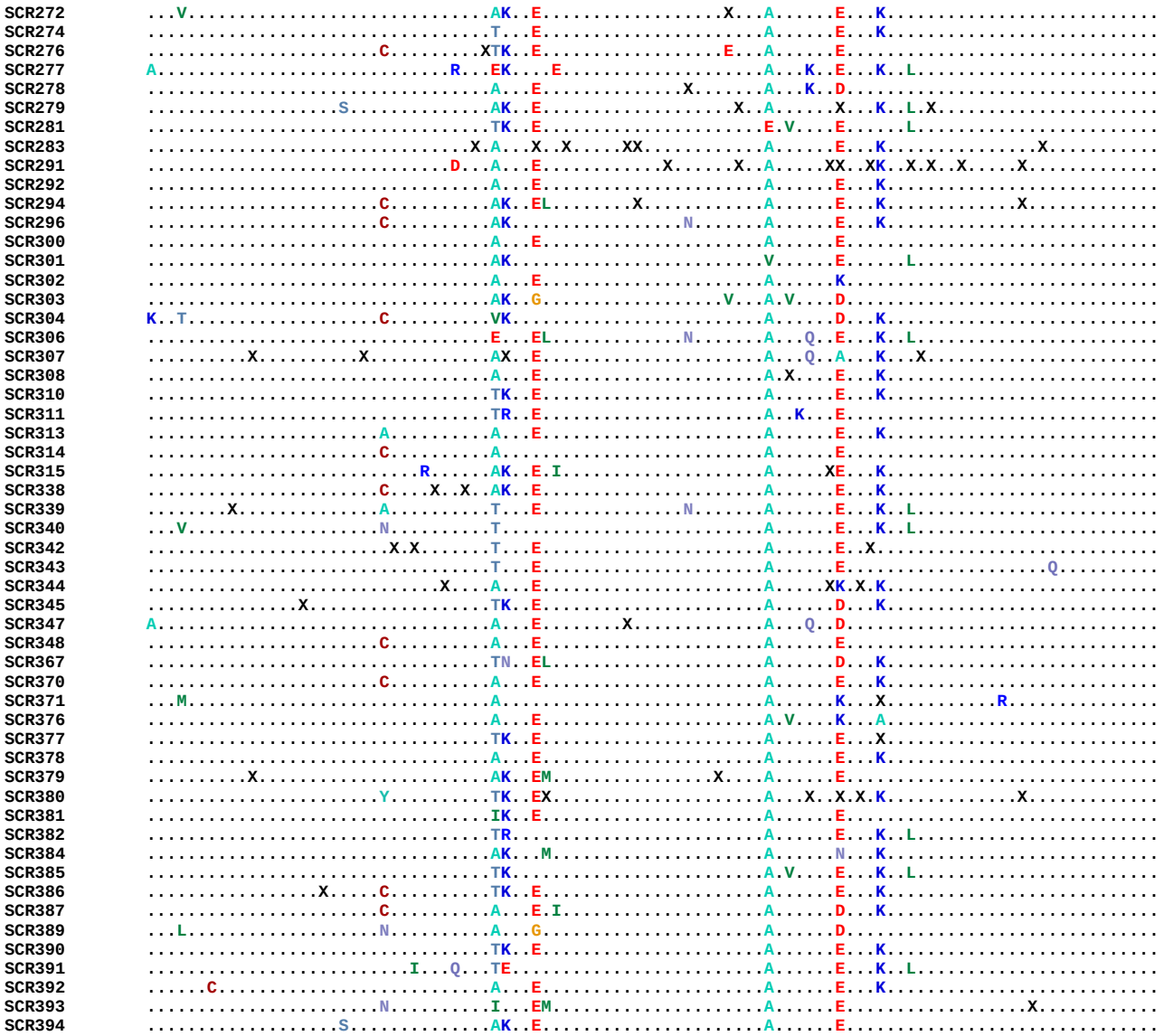
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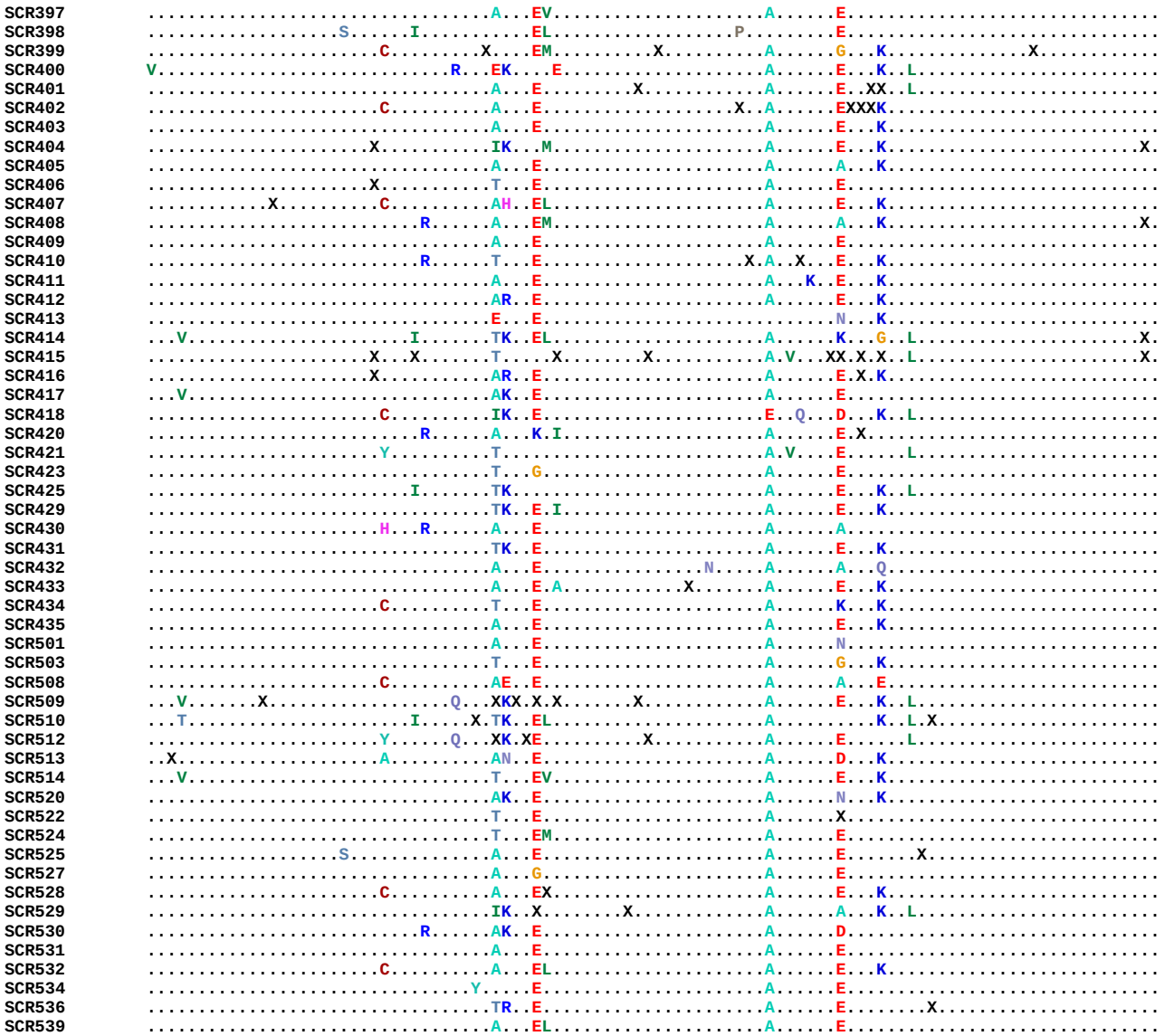
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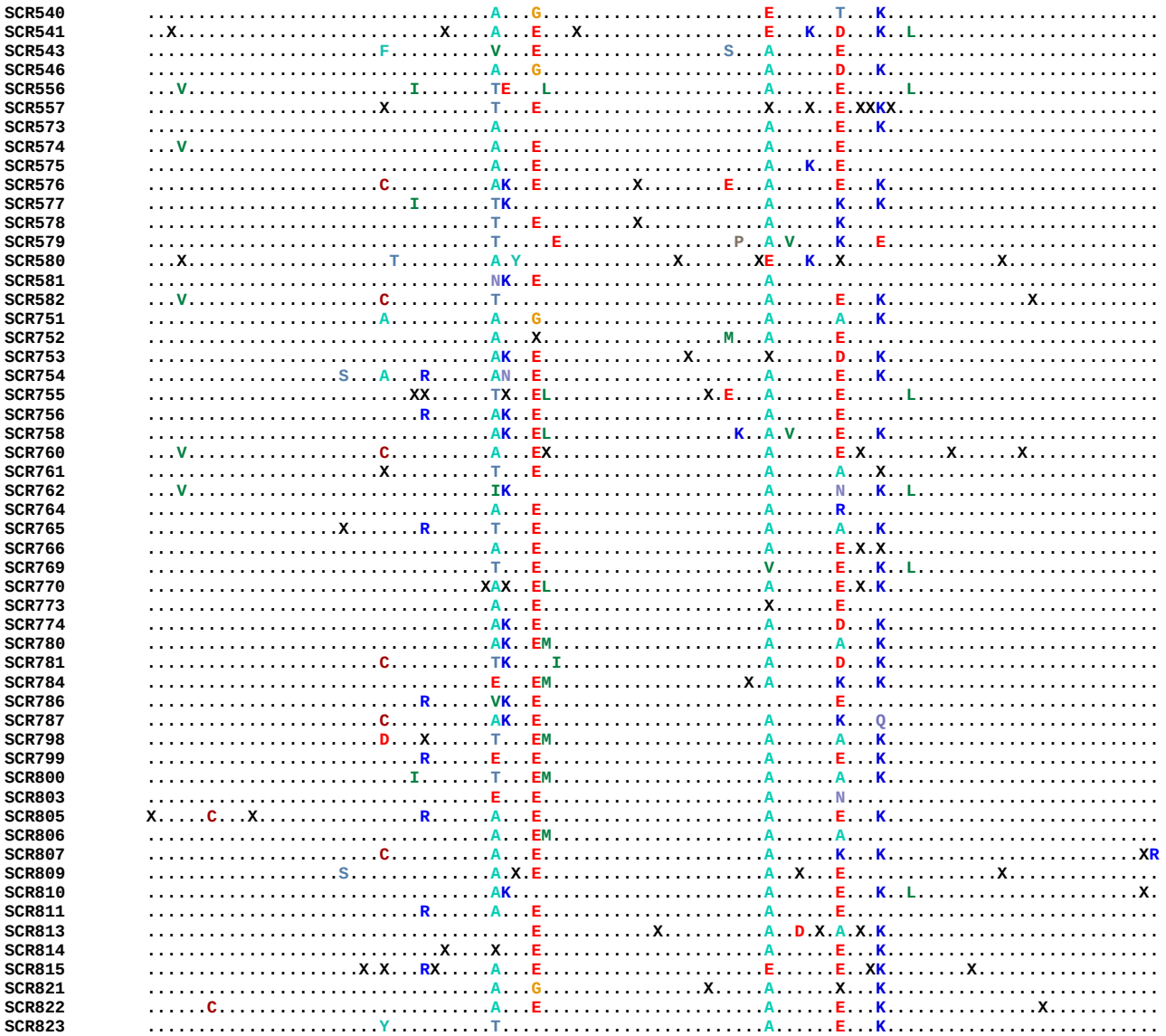
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PR Sequencing alignment

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CONSENSUS_C     N...........S..V...I.................I....K.........................K.................M...L......
SCR CONSENSUS   N...........S..V...I.................I....K.........................K.................M...L......
SCR006          N...........S....X.X.................I....K.................E.......K.................M...LX.....
SCR010          N...........S...E..I................DI....K...R...........K..E.......K.................M.XXL.X....
SCR020          N.......X..V.S..V...V.................I.X..K.........................K...........X....X.XM.X.L......
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SCR038          N...........S..V...TR...............DI....K.....................E.V.....K.................M...L......
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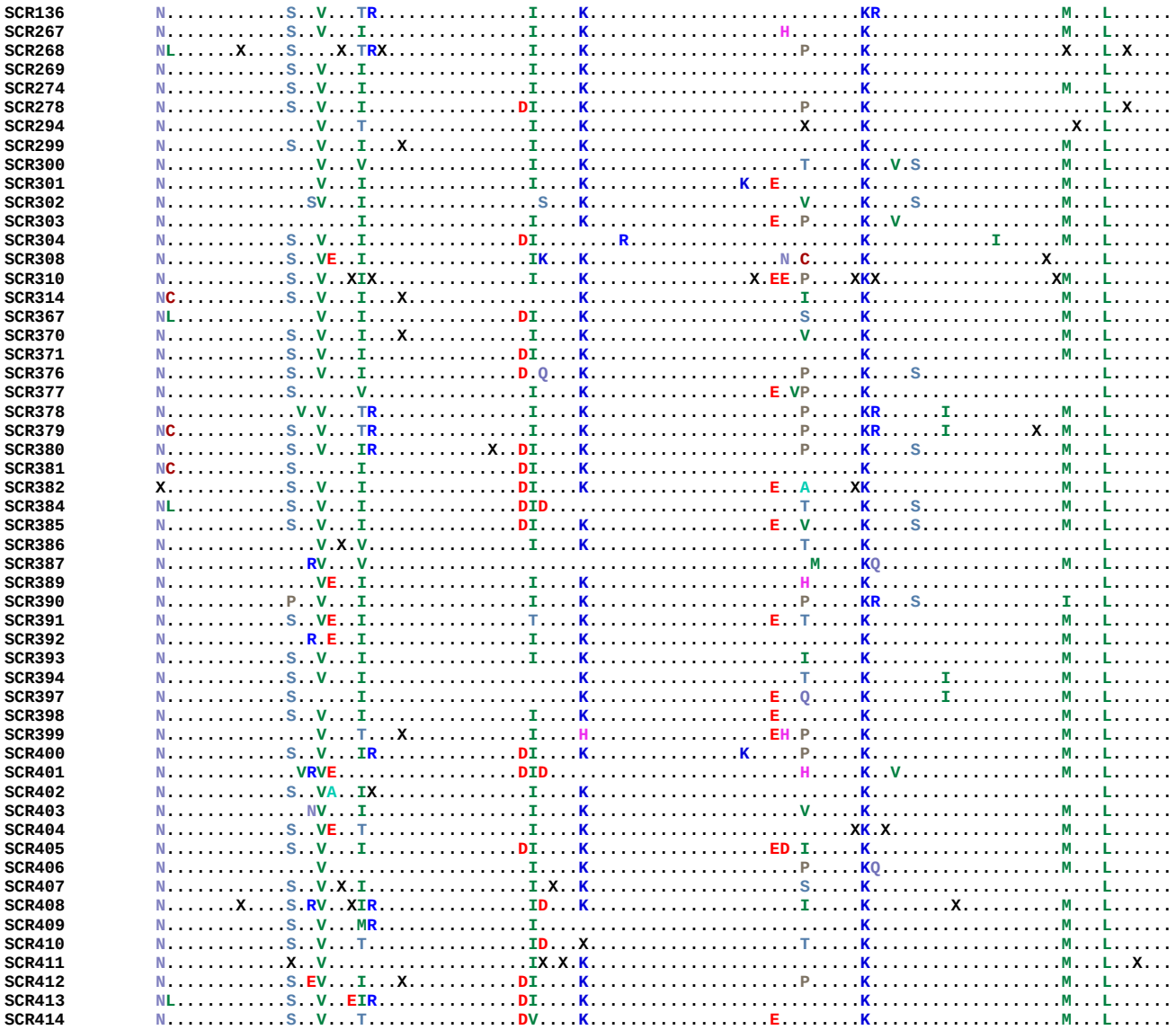
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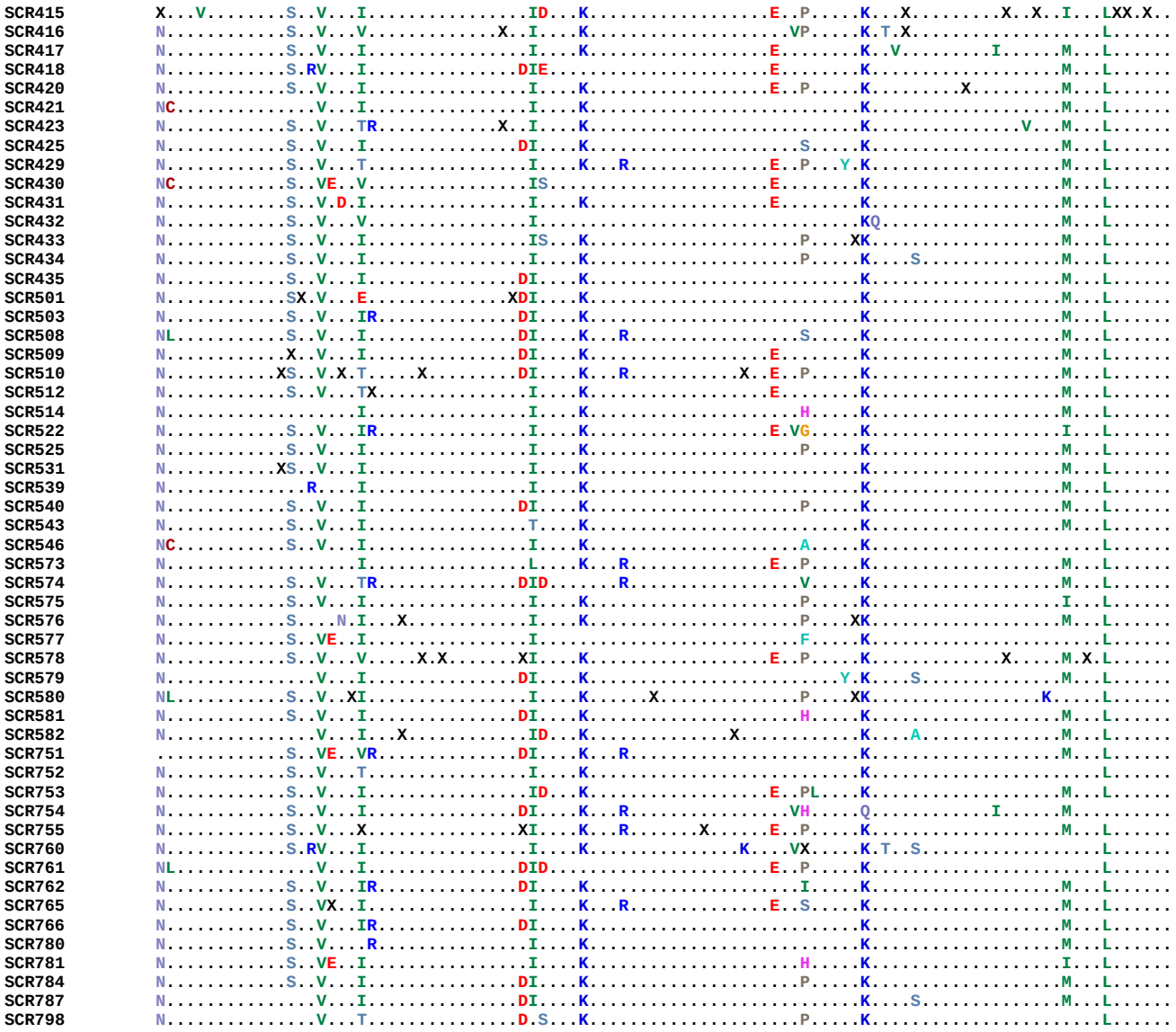
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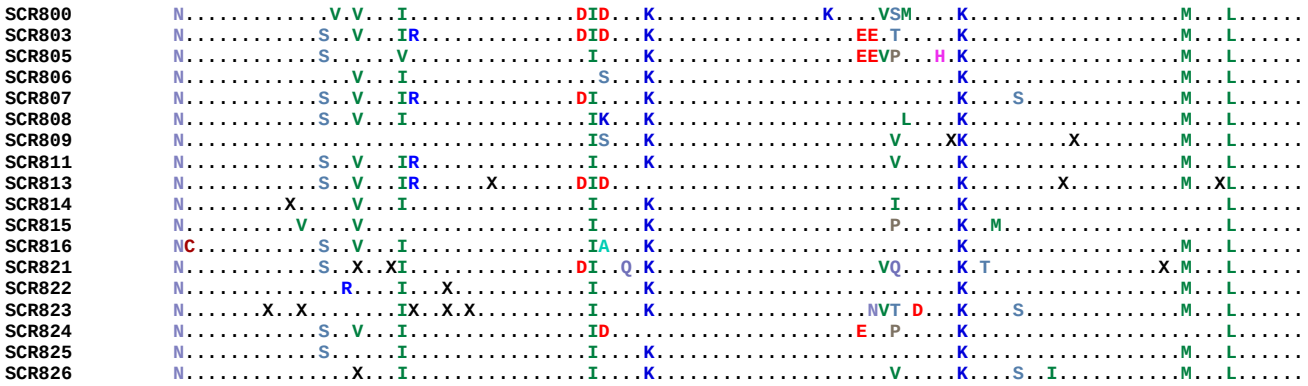
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