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Alec Goldberg April 3, 2017

Heterosexual Transmission of HIV-1 Despite Reported Use of ART in Couples from Zambia and Rwanda

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Abstract

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Background:

The use of antiretroviral therapy (ART) has become a common and effective form of HIV-1 treatment. The effectiveness of ART in preventing transmission was established by the HPTN 052 trial, which reported a 96% reduction in transmission between discordant couples with the early initiation of ART in the HIV-1 positive partners (1). In this study we sought to understand why HIV-1 transmission occurred in Zambian and Rwandan serodiscordant couples despite the reported use of highly effective antiretroviral treatment.

Methods:

We selected 9 Zambian and 8 Rwandan early seroconverting/RNA positive couples where the chronic partner reported ART use for at least one month prior to initial testing. As a follow up study, we identified 50 Zambian non-transmitting serodiscordant couples where the positive partner self reported ART use to determine if a significant fraction of Zambian partners who claim to be on ART are non-adherent or resistant to ART. Plasma samples from the transmitting/positive partners were subjected to mass spectroscopy to determine if antiretroviral (ARV) drug(s) were present and quantitative RT-PCR to measure the HIV-1 viral load. Viral genotyping was performed for the transmitting partner and seroconverter to identify drug resistance mutations (DRMs).

Results:

Seven out of the nine Zambian transmitting partners had no detectable ARVs in the plasma. Of the two index partners with detectable ARVs, only one had effective concentrations. We detected DRMs in the transmitting partner and most were passed on to the seroconverting partner. In the follow up study of 50 Zambian newly identified serodiscordant couples, only 8% of the positive partners had no detectable ARVs in the plasma. Out of the 8 Rwandan serodiscordant transmitting couples, five transmitting partners had multiple detectable ARVs, two had one detectable ARV, and one had no detectable ARVs.

Conclusions:

Our analysis of Zambian and Rwandan serodiscordant couples exemplifies that ART alone is not sufficient to prevent HIV-1 transmission. The development of a low cost viral load test is needed in low-income countries to detect lack of adherence to ART and antiretroviral treatment failure. Additionally, our data reveals the need for measures to educate the population about the benefits and the importance of adhering to ART.

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Table of Contents

INTRODUCTION **1**

METHODS **7**

Study Population 7
Mass Spectroscopy I 7
Mass Spectroscopy II 8
Viral Load Testing 9
RT-PCR Screening Procedure For Sample Viral RNA 9
Viral Genotyping 10

RESULTS **12**

Analysis of 9 Zambian Transmission Pairs **12**
VL Quantitation 12
HPLC Analysis of ARVs 12
Sequence Analysis for DRMs 13
Analysis of 50 Zambian Discordant Couples for Adherence and Resistance **13**
Qualitative RT-PCR Screening 13
VL Quantitation 14
HPLC Analysis of ARVs 14
Sequence Analysis for DRMs 15
Analysis of 8 Rwandan Transmission Pairs **15**
VL Quantitation 16
HPLC Analysis of ARVs 16

DISCUSSION **17**

FIGURES AND TABLES **22**

Table 1 22
Figure 1 22
Table 2 23
Figure 2 23
Table 3 24
Figure 3 24
Table 4 25
Table 5 25
Figure 5 26
Table 6 27
Table 7 27
Table 8 28
Table 9 28
Table 10 29
Table 11 29
Table 12 30

REFERENCES **31**

Introduction

Acquired Immunodeficiency Syndrome (AIDS), the late-stage clinical manifestation(s) of HIV-1 infection, was initially described in 1981, but the first drug to treat HIV/AIDS was not available until 1987 (2, 3). The development of antiretroviral (ARV) drugs to treat HIV/AIDS began in 1985 with the first clinical trials for Retrovir® (zidovudine, AZT), a dideoxynucleoside reverse transcriptase inhibitor (NRTI) (4), yet it was not until 1987 that it was approved for patients with advanced HIV-1 (3). With the development of new NRTIs, several investigators began to test the efficacy of ARVs in combination, known as combination antiretroviral therapy (cART) (5). The next big advance came with the development of non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PI). Saquinavir, the first PI, and nevirapine, the first NNRTI, were approved in 1995 and 1996 respectively (6, 7). In 1996, highly active antiretroviral therapy (HAART) became the standard form of treatment, combining ARVs from the different drug classes (7). New drugs and drug regimens continue to be created to successfully treat HIV-1, but a major challenge has been getting individuals affordable treatment throughout the world.

The use of antiretroviral therapy (ART) has become a common and effective form of HIV-1 treatment. The 2015 World Health Organization (WHO) "Guideline on When to Start Antiretroviral Treatment" suggests that individuals should receive ART at any CD4 cell count regardless of their age (8). The use of ART suppresses HIV-1 at multiple stages of infection thereby improving the health of the treated person. Several studies have evaluated the effectiveness of ART in preventing transmission between HIV-1

discordant couples (one HIV-1 positive partner and one HIV-1 negative partner). In 2011, the HIV Prevention Trials Network (HPTN) 052 Study evaluating the efficacy of antiretroviral therapy for preventing transmission in discordant couples was published. They sought to understand whether treating the positive partner of HIV-1 discordant couples with ART could prevent transmission to the negative partner. This monumental study looked at 1,763 discordant couples from 9 different countries comparing early versus delayed ART. They observed a 96% reduction in transmission from the early initiation of antiretroviral therapy group relative to those who initiated therapy later (1). This was a breakthrough in the field, and established that antiretroviral treatment, when started early, is an effective measure to significantly inhibit HIV-1 transmission. However, this study is limited because it only analyzed transmission in couples where the positive partner had ART-induced virologic suppression. Outside of a clinical trial not all patients will become virologically suppressed due to factors such as lack of adherence to the drug regimen and the presence of viruses with drug resistance mutations. Even though ART was effective in a clinical setting, this treatment method must be evaluated in the real world.

Following the breakthrough of HPTN 052, the Partners PrEP Study was published which evaluated the effectiveness of pre-exposure prophylaxis (PrEP) in preventing HIV-1 transmission in discordant couples. PrEP is an antiretroviral treatment intended for seronegative individuals that are at a high risk for HIV-1 acquisition. The Partners PrEP study evaluated the efficacy of PrEP in 4,756 discordant couples from Kenya and Uganda. The seronegative partners were randomly assigned to a once daily regimen of tenofivir (TDF), tenofovir disoproxil and emtricitabine (FTC/TDF) or a placebo.

Adherence to PrEP by the seronegative partners was measured through the return of drug bottles and the estimated level of adherence was 92.1% (9). They found that the use of oral tenofivir (TDF) and tenofovir disoproxil and emtricitabine (FTC/TDF) significantly reduced HIV-1 transmission to the uninfected partner by 67% and 75% respectively (10). However, one of the major concerns of PrEP usage is infection by HIV-1 with drug resistance mutations due to selective pressure from the presence of ARVs in PrEP. In the study, two out of the eight (25%) seroconversions that occurred in the experimental groups resulted in HIV-1 infection with virus encoding drug resistance mutations to the study medications (9).

Despite the potential effectiveness of ART, HIV-1 can still be transmitted in the presence of this treatment. The availability of ARVs and the degree of adherence to the treatment regimen both impact the level of protection afforded to patients by ART. In addition, one of the possible risks of widespread usage of ARVs is an increase in drug resistance mutations (DRMs) in the treated population. Drug resistance can be a consequence of selective pressure from using ART positively selecting for naturally occurring or transmitted polymorphisms from the donor quasispecies that reduce the efficacy of the drugs (11). This pressure can ultimately lead to the transmission of HIV-1 variants with DRMs (or transmitted drug resistance mutations; TDRM), rendering ART treatment ineffective. As the medical community continues to expand and improve ART, it is imperative to monitor the effectiveness of the medication by consistently measuring a patient's viral load (VL) (3). VL should be very low or undetectable if the medication is working, but a high VL is an indication that a new treatment program must be

implemented (12). Unfortunately, VL monitoring is rare in many developing countries due to high cost and limitations on the availability of testing facilities (13).

Though ART is an effective preventative measure, especially in developed countries, it may not be the most realistic strategy to overcome the spread of HIV-1 worldwide. Other strategies, such as Couples Voluntary Counseling and Testing (CVCT) have been shown to successfully prevent transmission in resource-limited areas. In 1988, Dr. Susan Allen discovered that 14% out of 1500 research subjects she was studying had a different HIV-1 status than their partner, illuminating a common misconception in the medical community that couples always share the same HIV-1 status (14). This revealed the need for couples to be tested and counseled together. In an initial study, Dr. Allen discovered a 50-70% lower incidence of HIV-1 in counseled couples compared to uncounseled couples (14). This led to the establishment of the Rwanda Zambia HIV Research Group (RZHRG), which works with couples to provide testing and counseling. Couples are counseled together on how to make healthy decisions based on serologic test results revealing concordance or discordance. RZHRG has observed that CVCT reduces transmission by about two-thirds in discordant couples and can prevent more than half of new HIV-1 infections in adults (15, 16).

In 2011, it was estimated that 12.5% of adults in Zambia had HIV-1 (17). As a result, the Zambian Ministry of Health created a program for HIV-1 treatment and in 2011 about 400,000 Zambians that met the requirements for ART treatment were reported to be receiving care (17). According to UNAIDS, Rwanda and Zambia reported that 80% or more eligible adults were receiving ART under the 2010 WHO guidelines (18). ART distribution continues to increase with the new WHO guidelines broadening

the eligibility requirements for ART treatment. However, it is estimated that 75% of adults in sub-Saharan Africa are not virologically suppressed despite this increase in treatment (18). With the increase of ART in Zambia and Rwanda there is also a risk for an increase in drug resistance mutations. A recent study conducted in East and Southern Africa supports this assertion. They found an increase in the diversity and presence of TDRMs over time in their cohorts using ART (11). This study demonstrates that there is an increase in HIV-1 resistance to treatment over time and raises the question, does drug resistant HIV-1 contribute to transmissions in HIV-1 discordant couples where the HIV-1 positive partner reports ART use? In Zambia, where couples were attending government CVCT clinics, the rate of transmission where the transmitting partner reported being on ART prior to CVCT was only reduced by 40%. Similarly, in Rwanda transmission was also observed in some couples where the positive partner reported adherence to ART (16).

In this study we are looking to understand why Zambian and Rwandan serodiscordant couples transmitted HIV-1 despite reporting the use of ART. We hypothesized that the transmitting (index) partner was either not adhering to the ART treatment regimen or had DRM rendering the ART treatment ineffective. To test these hypotheses, we first looked at nine Zambian serodiscordant couples where the donor reported use of ART at the time of HIV-1 transmission. As a follow up study, we identified 50 Zambian serodiscordant couples where the positive partner (25M, 25F) self reported ART use to determine if a significant fraction of Zambian partners who claim to be on ART are non-adherent or resistant to ART. Lastly, we looked at 8 Rwandan couples where the donor reported ART use at the time of HIV-1 transmission. Plasma

samples from the transmitting/positive partners were subjected to mass spectroscopy to determine if antiretroviral (ARV) drug(s) were present and quantitative RNA PCR to measure the HIV-1 VL. In addition, to identify drug resistance we amplified the 5' half of the HIV-1 genome and sequenced the gene encoding reverse transcriptase, protease and integrase (*pol)* for each partner in the couples. We were able to determine if any DRM(s) were present by uploading the sequences to the Stanford University HIV Drug Resistance Database. Understanding how HIV-1 transmission occurs despite reported use of ART will allow better interventions to be implemented and get us closer to our goal of eliminating HIV-1 transmission.

Methods

Study Population

The Rwanda Zambia HIV Research Group (RZHRG) initiated a Couples Voluntary Counseling and Testing (CVCT) program in 1986 to prevent transmission of HIV-1 among long-term partners. At Government clinics in Zambia and Rwanda, couples participate in a serologic test during the initial visit. The results are shared with the couples and they are referred for counseling and treatment based on whether they are concordant positive (both partners are HIV-1 positive) or discordant (one partner is HIV-1 positive while the other partner is HIV-1 negative) (19). Serodiscordant couples were asked to return at month 1 and month 3 after initial serologic testing. Seroincidence was determined for individuals who seroconverted at month 1 or who were found to be viral RNA positive at initial testing. Transmission within the couples was confirmed by viral sequencing. In this study, we selected early seroconverting/RNA positive couples where the chronic partner reported use of ART for at least one month prior to initial testing in order to determine if the transmitted virus was resistant to ART. We chose plasma and PBMC DNA from the seroconversion time point and matched that with the identical time point in the transmitting partner. The HIV-1 sequences from the subjects are HIV-1 Subtype A from Rwanda and Subtype C from Zambia.

Mass Spectroscopy I (9 Zambian Couples)

In order to confirm that partners reporting ART use were in fact taking their drugs, we used High Performance Liquid Chromatography (HPLC) analysis of plasma. This analysis was completed in collaboration with the Schinazi Lab (Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory

University School of Medicine, Atlanta, GA 30322, USA; & Veterans Affairs Medical Center, Decatur, GA 30033, USA). In the patient plasma samples, 10 ARVs were monitored (Lamivudine (3TC), Zidovudine (AZT), Emtricitabine (FTC), Tenofovir (TFV), Stavudine (d4T), Efavirenz (EFV), Abacavir (ABC), Lopinavir (LPV), Ritonavir (RTV) and Nevirapine (NVP)) (**Table 1**) and quantified by liquid chromatography-mass spectrometry (LC-MS). The instrument used was API5000, Column: phenomenex Kinetex XB-C18 (50 x 2.1mm, 2.6 μm). To prepare the samples, 100 μL human plasma was extracted with 500 μL of methanol containing Amdoxovir (DAPD) (MW: 252.2) and Indinavir (MW: 613.7) as internal standards. The supernatant was divided into two equal fractions, air-dried, then reconstituted in two buffers respectively: 1) 2 mM ammonium acetate with 0.1% formic acid, 2) 2 mM ammonium acetate with 0.1% formic acid and methanol (v:v = 40:60). Then the sample was analyzed using LC-MS. The LC buffers used were 2mM ammonium acetate with 0.1% formic acid (buffer A), and acetonitrile (buffer B). The LC gradients for 3TC, AZT, FTC, TFV (also TDF), d4T and ABC, increased from 0% to 70% of buffer B in 7 minutes. The LC gradients for EFV, LPV, RTV and NVP, increased from 20% to 90% of buffer B in 7 minutes.

Mass Spectroscopy II (50 Zambian Couples and 9 Rwandan Couples)

The same approach was taken for the analysis of the 9 Zambian Couples with the following modifications: the instrument used was TSQ Quantiva, Column: phenomenex EVO C18 (100 x 2.1mm, 5 μm). The supernatant was divided into two equal fractions, air-dried, then reconstituted in two buffers respectively: 1) 0.1% formic acid, 2) 0.1% formic acid with methanol (v:v = 40:60). The LC buffers used were 0.1% formic acid (buffer A), and acetonitrile (buffer B). The LC gradients for 3TC, AZT, FTC, TFV, d4T

and ABC, increased from 0% to 70% of buffer B in 5 minutes. The LC gradients for EFV, LPV, RTV and NVP, increased from 10% to 90% of buffer B in 6 minutes (**Figure 1**).

VL Testing

VL testing was performed on plasma using the Abbott m2000 assay, in collaboration with the Center for AIDS Research (CFAR) Virology Core. The Abbott Realtime HIV-1 Assay is an in vitro reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantitation of HIV-1 on the automated m2000 System in human plasma from HIV-1 infected individuals over the range of 40 to 10,000,000 copies/mL (**Figure 1)**.

RT-PCR Screening Procedure For Sample Viral RNA

Plasma samples were initially screened in the field, by Clive Michelo, using an in-house RT-PCR assay that targeted 3 regions of the genome *Gag*, *Pol* and *Env* (gp41). Plasma samples from HIV-1 sero-positive partners were selected based on oral affirmation by the positive partner for adherence to ART (n=50). The multiplex RT-PCR for the *gp41*, *gag* and *pol* regions of HIV-1 was used on 150ul of donor undiluted plasma and 150ul of the same sample diluted 1:10 (v/v) with water prior to RNA extraction using the E.Z.N.A Viral RNA extraction kit (Cat No. R6874-02) and the extracted RNA used in the RT-PCR procedure (SuperScript III One-Step RT-PCR System; ThermoFisher Scientific). A second round of nested PCR was done for each region and the PCR products were run on a 1% agarose gel.

Viral Genotyping

Samples of plasma or Qiagen mini-column prepared PBMC DNA were used to amplify the 5' half of the HIV-1 genome in order to sequence the gene encoding reverse transcriptase, protease and integrase (*pol)* of HIV-1 in order to perform population sequencing. A volume of 140µl of plasma was applied to a QIAamp Viral RNA MiniKit (Qiagen, Limburg, Netherlands) to extract viral RNA, and perform cDNA synthesis. First half genome amplification was performed with either cDNA or Qiagen DNA minikit purified PBMC DNA through two rounds of PCR amplification using Q5 Hot Start High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA). Different first round PCR primers were used for each subtype. The Zambia primers included GOF and ViFOR for the first round and GIF and ViFIR for the second round. The Rwanda primers were GOF_SubtypeA1 and ViFOR_SubtypeA2 for the first round and GIF_SubtypeA1 and ViFIR_Subtype A1 for the second round (**Table 2**). After completion of the second round PCR, 5µl of the second round PCR product and 2µl of loading buffer were run in a 1% agarose-Tris-Acetate-EDTA gel to identify positive amplicons with a length of 4.5 kb. All of the positive amplicons from the same subject were pooled in order to have the best representation of the viral population present. The combined products were purified using SV Gel & PCR Clean Up System (Promega), if there were no additional bands to the 4.5 kb band, and sent off to GenScript for population sequencing using six *pol* primers (**Figure 2**). If there were additional identifiable bands, the pooled products were re-run through a 1% agarose-TAE gel. Then, the 4.5 kb product was extracted from the gel, gel purified using SV Gel & PCR Clean Up System (Promega), and sent off to GenScript for population sequencing using six *pol* primers (**Table 2**). After analyzing the

sequences in the software program, Geneious, they were uploaded to the Stanford University HIV Drug Resistance Database to identify drug resistance mutations that may affect the virus.

Results

1. Analysis of Nine Zambian Transmitting Pairs

Nine Zambian serodiscordant couples where the donor reported ART use for at least one month prior to transmission of HIV-1 to their partner were studied. Plasma samples from the nine transmitting partners were first subjected to VL testing and mass spectroscopy as described in Methods.

VL Quantitation:

VL testing was performed to determine if there was measurable HIV-1 in the plasma. All of the transmitting partners had measurable VLs ranging from 7,852 to 444,440 copies/mL, and a median VL of 47,600 copies/mL (**Table 3**). The VL data suggests that the majority of patients were not adhering to the ART treatment regimen because these VLs are similar to those seen in chronically infected individuals not on treatment in the Zambia-Emory HIV Research Project (ZEHRP) cohort (**Figure 3**).

HPLC Analysis of ARVs:

Our analysis revealed that only two out of the nine transmitting partners had detectable levels of ARVs in the plasma. MSH596F had detectable levels of a single drug, Zidovudine (AZT), at a concentration above the effective concentration 90 (EC90). MON3076M had detectable levels of Emtricitabine (FTC) and Efavirenz (EFV) above the EC90, Tenofovir (TFV) above the effective concentration 50 (EC50), and low levels of Nevirapine (NVP) (**Table 4**). The other seven index partners had no detectable ARVs in the plasma.

Sequence Analysis for DRMs:

In parallel, we amplified the 5' half of the HIV-1 genome from viral RNA in the plasma of each transmitting and seroconverting partner. Then, we sequenced the gene encoding reverse transcriptase, protease and integrase (*pol)* from each of the 9 couples to determine whether drug resistance could explain transmission. As expected, in the seven couples where ARV concentrations were undetectable, we did not find any drug resistance mutations (DRM). No drug resistance mutations were identified in the MSH596 pair despite the presence of AZT in MSH596F. In contrast, MON3076M had multiple NRTI and NNRTI drug resistance mutations, and most mutations were transmitted to the seroconverting partner (**Table 5**). The estimated level of resistance to each antiretroviral drug for both partners in the MON3076 pair was calculated through the Stanford University HIV Drug Resistance Database. Both partners are still susceptible to Zidovudine (AZT), but they have high-level resistance to all of the other ARVs available in Zambia (**Figure 4)**.

2. Analysis of 50 Zambian Discordant Couples for Adherence and Resistance:

To determine if the adherence problem observed in the 9 Zambian couples applies to the Zambian epidemic as a whole, we selected a larger cohort of fifty newly identified HIV-1 serodiscordant couples (25 male, 25 female), where the positive partner self-reported ART use.

Qualitative RT-PCR Screening:

Plasma samples were first screened, on site in Lusaka, for the presence of HIV-1 viral RNA using an in-house qualitative RT-PCR assay that targeted *gag*, *pol* and *env*

(gp41). From the 50 positive partners, 40 (80%) had undetectable levels of HIV-1 viral RNA compared to 10 (20%) with detectable HIV-1 viral RNA (**Table 6**). Out of the 10 partners with detectable HIV-1 viral RNA, seven were women and three were men.

VL Quantitation:

VL testing was then performed on the 10 individuals that had detectable HIV-1 viral RNA and for 9 randomly selected individuals that had undetectable HIV-1 viral RNA as a control. VL was measured for the 9 control samples in order to confirm that these individuals had undetectable HIV-1 viral RNA as observed in the initial qualitative RT-PCR screening and for the 10 HIV-1 viral RNA positive individuals to obtain quantitative values for VL. All of the control samples had VLs <160 copies/mL confirming that there was no detectable HIV-1 viral RNA (**Table 7**). The 10 individuals with detectable HIV-1 viral RNA had VLs ranging from 260 to 194,929 copies/mL (**Table 8)**.

HPLC Analysis of ARVs:

Mass spectroscopy analysis was conducted to determine the ARVs present in the 9 control individuals and the level of adherence to ART in the 10 individuals with measurable VLs. Each of the 9 individuals with undetectable VLs had high concentrations of ARVs in the plasma (**Table 9**). The most common treatment regimen included Lamivudine (3TC) or Emtricitabine (FTC), Tenofovir (TFV), and Efavirenz (EFV).

The 10 individuals with measurable VLs had varied levels of adherence to ART.

Three (6%) of the individuals, KAM3297F, KAM3895F, and KAM3921F, had high levels of ARVs in the plasma but were not fully virologically suppressed (VL range– 254-2,840). Four (8%) of the individuals, GEO7093F, GEO6685F, GEO6809M and MAT6133F, had no detectable ARVs in the plasma (VL range– 2,948-194,928). The remaining three (6%) individuals, CHI3121M, MAT6371F, and GEO6959M, were poorly adherent to ART with only one or two detectable ARVs (VL range– 1,232-168,992) (**Table 10**).

Sequence Analysis for DRMs:

We were able to amplify the 5' half of the HIV-1 genome and sequence the gene encoding reverse transcriptase, protease and integrase (*pol*) for five of the ten individuals with measurable HIV-1 viral RNA. GEO6809M, GEO7093F and MAT6371F had no evidence of drug resistance mutations. CHI3121M had no drug resistance mutations despite the use of Lamivudine (3TC), which was at a concentration above the EC50. GEO6959M had one NNRTI drug resistance mutation E138A causing low-level resistance to Rilpivirine (RPV) and potential low-level resistance to Etravirine (ETR). However, GEO6959M is still susceptible to Efavirenz (EFV) and Nevirapine (NVP).

3. Analysis of Eight Rwandan Transmission Pairs

Lastly, we looked at HIV-1 subtype A Rwandan couples to see if our previous observations about the Zambian couples are subtype specific. Eight Rwandan serodiscordant couples where the donor reported ART use for at least one month prior to HIV-1 transmission were first subjected to VL and mass spectroscopy testing.

VL Quantitation:

The transmitting partners had VLs ranging from <160 to 914,100 copies/mL. KIM168F and RUG001F had high VLs (VL > 80,000 copies/mL), but the rest of the transmitting partners had relatively low VLs (VL < 10,000 copies/mL) (**Table 11**).

HPLC analysis of ARVs:

Mass spectroscopy was performed to determine if a quantifiable level of ARVs were present in the plasma of the transmitting partners. All of the transmitting partners had detectable ARVs except for KIM169F. KRU001M and RUG2M had high levels of multiple ARVs present in the plasma and suppressed viral replication (VL < 160 copies/mL). KIN001M, COR150M and COR140M had multiple ARVs detectable in the plasma at high concentrations but still had measurable VLs (VL > 160 copies/mL). BGO003M and RUG001F only had one detectable antiretroviral drug with EFV above the EC90 and NVP below the EC50, respectively. The most common treatment regimen consisted of Lamivudine (3TC), Tenofovir (TFV), and Efavirenz (EFV) (**Table 12**).

Discussion

With the current WHO recommendation leading to greater ART distribution, HIV-1 transmission should theoretically decline with increased ART use (7). The effectiveness of ART in preventing transmission was established by the HPTN 052 trial, which reported a 96% reduction in transmission between discordant couples with the early initiation of ART in the HIV-1 positive partners (1). However, there are various factors that can affect ART efficacy leading to transmission during ART use. In this study, we explored HIV-1 transmission in Zambian and Rwandan serodiscordant couples despite reported use of ART by the HIV-1 positive partner. Through our analysis, we were able to determine that lack of adherence and drug resistance pose serious challenges for preventing HIV-1 transmission.

Our analysis of the 9 Zambian serodiscordant couples exemplifies the importance of adhering to the ART treatment regimen in order to avoid transmitting HIV-1. We found that seven out of the nine (78%) Zambian index partners had no detectable ARVs in their plasma demonstrating that the majority of HIV-1 transmissions to the negative partners were due to lack of adherence to ART (**Table 4**). Our analysis was limited due to the small sample size of the study population and our selection criteria for transmitting pairs. Nevertheless, if we extrapolate our data to the 1.2 million HIV-1 positive individuals in Zambia (20), this would present a serious public health concern as an enormous portion of the HIV-1 infected Zambian population is at a high risk of transmission due to unsuppressed viral replication from non-adherence to ART. However, lack of adherence is not a generalized situation in Zambia which we determined by comparing our data from the 9 Zambian serodiscordant couples to a larger,

more representative Zambian study population. When we looked at 50 Zambian HIV-1 positive partners who self-reported ART use we only saw 8% of the positive partners non-adherent to ART. This sample was a better representation of the Zambian population than the smaller study group because the couples were newly identified as serodiscordant and randomly selected. Even though we found that the majority of positive partners were adherent to ART, any evidence of an adherence issue should not be overlooked as it could present a significant obstacle to our goal of eliminating HIV-1 transmission.

Our data relates to a recent study conducted in Zambia looking at adherence to ART in a large cohort of 131,767 patients from 56 public sector clinics. Adherence was measured by looking at pharmacy records to determine the medication possession ratio (MPR). MPR was calculated through the amount of time patients had ART in their possession and when they should return to the pharmacy to pick up more medication. They found the median MPR to be 85.8% with variations among individuals and clinics (21). These findings support our data by illustrating that while adherence was generally high, there were a significant number of individuals that were not adherent to ART. Lack of adherence can be due to many factors such as a negative perception of ART, lack of family support, economic constraints, stigma, discrimination, travel distance to obtain ART, and side affects (22). Taken together, these data reveal the need for measures to educate the population about the benefits and the importance of adhering to ART.

Through our sequencing analysis we were able to identify individuals with drug resistant HIV-1 in the Zambian study populations. From the 9 Zambian HIV-1 serodiscordant couples, we determined that transmission occurred in one pair (11%) due to drug resistant HIV-1. Additionally, DRMs were identified in one out of the five

individuals we were able to sequence from the 50 Zambian HIV-1 positive partners. We also suspect that KAM3297F, KAM3895F, and KAM3921F may have DRMs due to multiple detectable ARVs and measurable VLs (**Table 12**). Therefore, we estimate that 8% of the 50 Zambian HIV-1 positive partners have HIV-1 with varying levels of drug resistance. This is consistent with a study published in 2010 which found that that 6% of their Zambian study population had HIV-1 with DRMs to first line ARVs (23). Taken together, these results have significant implications for the HIV-1 epidemic in Zambia. If we extrapolate our data to the 400,000 HIV-1 positive individuals in Zambia who have initiated ART, then a sizable number of the population has drug resistant virus (17). Furthermore, a significant number of transmissions occurring in Zambia would result in the circulation of drug resistant HIV-1 decreasing the efficacy of first line treatments and increasing the need for less effective, more expensive second line therapies (13, 24).

The Zambian data demonstrates the need for VL testing to detect lack of adherence to ART and antiretroviral treatment failure. However, at the present time VL testing is uncommon in low-income countries due to high costs, limited resources, and lack of technical expertise needed to perform the assay (13). Therefore, lack of adherence and drug resistance are not being detected making it difficult to prevent HIV-1 transmission. This reveals the need for the development of a low cost VL test, otherwise the impact of ART treatment may not be fully realized.

Conversely to the Zambian transmitting couples, we saw that the majority of Rwandan HIV-1 positive transmitting partners were adherent to ART. Five out of the eight (62.5%) index partners had multiple detectable ARVs and only one (12.5%) transmitting partner had no detectable drugs. Interestingly, KRU001M and RUG2M had

suppressed VLs, but still transmitted HIV-1 to the negative partner. Transmission linkage was confirmed for the pairs with the RUG2 Pairwise Distance (PWD) of 2.3% and the KRU001 PWD of 1.0%. Since our samples were taken close to the seroconversion time point, we suspect that transmission likely occurred when drugs were stopped for a brief period of time resulting in a VL rebound in the transmitting partner.

The data from the Rwandan couples has similar implications as our results from the Zambia studies. We suspect that several transmissions occurred due to the presence of DRMs while the rest of the transmissions were due to adherence issues. Despite our small sample size, the message conveyed through our data is noteworthy. Issues of adherence and drug resistance are leading to the transmission of HIV-1 in both Zambia and Rwanda. While we saw less lack of adherence in Rwanda, it was still present suggesting that adherence may be an issue in the larger population. With the goal of eliminating HIV-1 transmission entirely, continuous effort should be made to educate the population about the importance of following the ART treatment regimen. Additionally, with more patients adherent to ART in the Rwanda cohort, there may be a larger issue of drug resistance in Rwanda. This further supports the need for a cost effective method to monitor patient VL to ensure that ART is working in order to prevent selection for drug resistant HIV-1. Once we finish sequencing the Rwandan couples we will be able determine the prevalence of drug resistance in our study population.

Through our analysis of Zambian and Rwandan serodiscordant couples, we have determined that ART alone is not sufficient to prevent HIV-1 transmission. CVCT may be the ultimate solution with the ability to cost effectively test and counsel couples together (16). Preliminary data from a study conducted in Khayelitsha, South Africa

found that 70% of patients remained on original treatment four years after initiation with combined VL testing and counseling (13). Another study conducted by Dr. Susan Allen suggests that CVCT could prevent up to 60.3% of heterosexual HIV-1 transmission in the Zambian population. Additionally, Dr. Allen's team estimates that 20% of serodiscordant couples will transmit HIV-1 without CVCT (15). CVCT has proven to be a successful, economical method for preventing the spread of HIV-1 and should be highly considered in low-income countries with limited resources as the preferred prevention strategy. In 2012, the WHO released recommendations, for a public health approach, outlining the benefits and endorsing the implementation of CVCT worldwide (25). With the increase in CVCT and the development of a low cost VL test, ART efficacy will increase taking us closer to our goal of eliminating HIV-1 transmission.

Figures and Tables

Drug Name	Abbreviation	Drug Class	EC50 (nM); EC90 (nM)
Lamivudine	3TC	NRTI	3.1; 75
Zidovudine	AZT	NRTI	1.3; 13
Stavudine	d4T	NRTI	55; 350
Tenofovir	TFV	NRTI	43; 180
Abacavir	ABC	NRTI	2400; 13800
Nevirapine	NVP	NNRTI	57; 350
Efavirenz	EFV	PI	1.1; 5.2
Ritonavir	RTV	PI	14; 48
Lopinavir	LPV	PI	5.5; 13
Emtricitabine	FTC	PI	2.3; 58

Table 1: ARVs screened for during LCMS. The Effective Concentration (EC) 50 and EC90 values for each drug were used to calculate the detectable drug concentration in the plasma for each patient measured by LCMS.

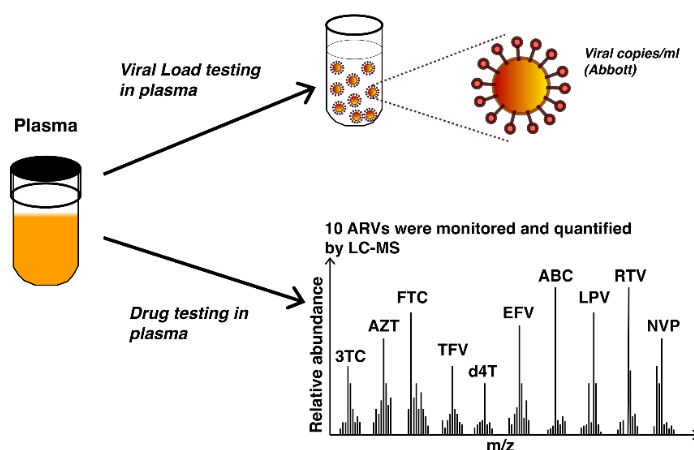

Figure 1: Viral load and ART drug testing

Plasma samples were analyzed for VLs (Abbott) and ART drug presence (LC-MS, Instrument: TSQ Quantiva, Column: phenomenex EVO C18 (100 x 2.1mm, 5 μm). 10 ARVs (3TC, AZT, FTC, TFV, d4T, EFV, ABC, LPV, RTV and NVP) were monitored and quantified by LC-MS.

Primer Name	1st/2nd Round/Sequencing	Subtype	Forward/Reverse	Sequence
GOF	1st round	C	Forward	5'-ATTTGACTAGCGGAGGCTAGAA-3'
VIFOR	1st round	C	Reverse	5'-TTCTACGGAGACTCCATGACCC-3'
GIF	2nd round	C	Forward	5'-TTTGACTAGCGGAGGCTAGAAGGA-3'
VIFIR	2nd round	C	Reverse	5'-TCCTCTAATGGGATGTGTACTTCTGAAC-3'
GOF_SubtypeA1	1st round	A	Forward	5'-TTTTGACTAGCGGAGGCTAGAA-3'
ViFOR_SubtypeA2	1st round	A	Reverse	5'-TTCTATGGAGACCCCATGACC-3'
GIF_SubtypeA1	2nd round	A	Forward	5'-TTTGACTAGCGGAGGCTAGAAGGAG-3'
ViFIR_Subtype A1	2nd round	A	Reverse	5'-TCCCCTAATGGGATGTGTACTTCTGAAC-3'
POLS1	Sequencing	Both	Forward	5'-CCTCAAATCACTCTTTGGC-3'
POLS2	Sequencing	Both	Forward	5'-AGAACTCAAGACTTTTGGG-3'
POLS3	Sequencing	Both	Reverse	5'-TGCTGGGTGTGGTATTC-3'
POLS4	Sequencing	Both	Reverse	5'-CCATGTACTGGTTCTTTTAG-3'
POLS5	Sequencing	Both	Forward	5'-CAATGGACATATCAAATTTACCA-3'
POLS6	Sequencing	Both	Reverse	5'-CCCTATTAGCTGCCCCATCTACATA-3'

Table 2: Zambian and Rwandan primers used for first half genome amplification and sequencing.

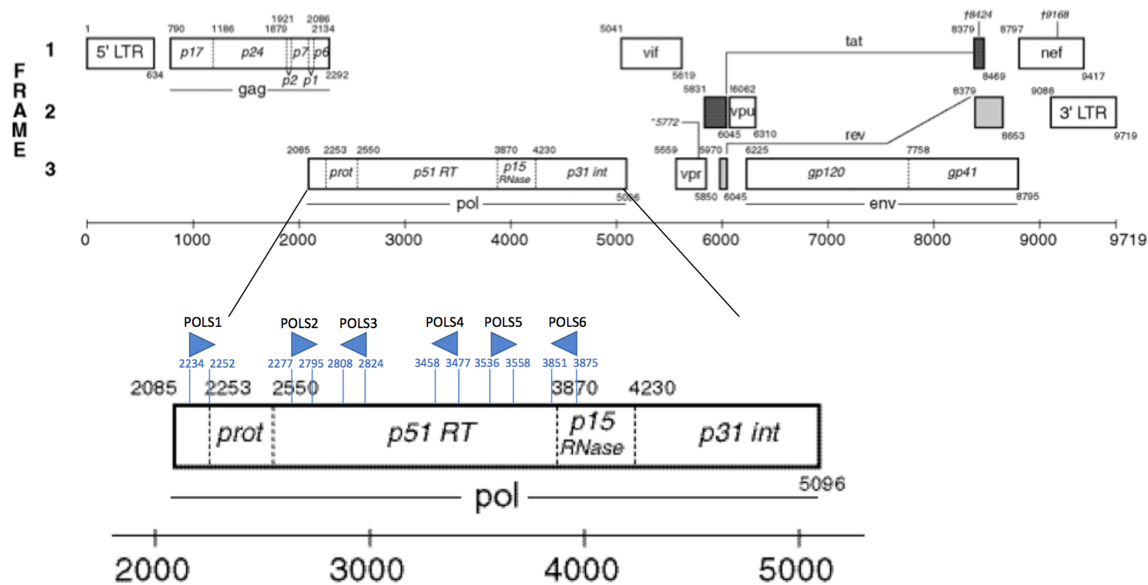

Figure 2: Sequencing primer binding sites on the HIV-1 *pol* gene (26).

ID (Test Site + CVTID)	Sample Date	Viral Load (copies/mL)
CHI 2766M	2-Feb-13	54,220
IPU 2188M	18-May-14	47,600
ITP 911F	29-Sep-12	7,852
KWA 1802M	14-Jul-13	7,912
MAN 5764M	14-Dec-13	444,440
MAT 2702M	1-Sep-13	47,600
MON 3076M	9-Mar-13	21,796
MSH 596F	18-Nov-12	10,612
MTE 2143M	10-Aug-13	186,148

Table 3: Donor Viral Loads at Linked Recipient's Time of Seroconversion

VLs from 9 seropositive partners reporting ARV usage. The Abbott RealTime HIV-1 assay, an *in vitro* RT-PCR based assay, was used for the quantitation of HIV-1 RNA copies in plasma on the m2000 system over the range of 40 to 10,000,000 copies/mL.

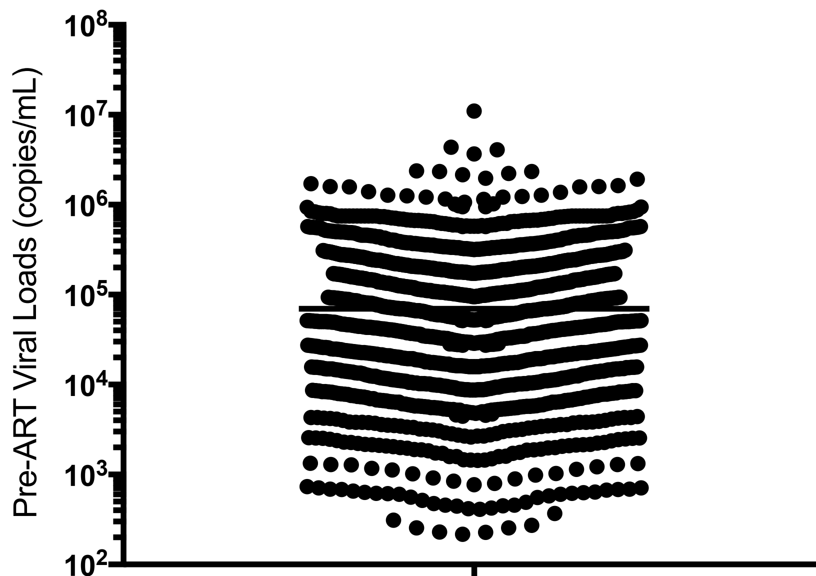

Figure 3: Dot plot of viral loads from 1652 chronically infected individuals not on ART from the Zambia-Emory HIV Research Project (ZHERP) cohort. The median viral load (denoted by thick horizontal line) is 66,000 copies/mL.

Test Site + CVCT ID	3TC	AZT	FTC	TFV	d4T	EFV	ABC	LPV	RTV	NVP	Viral Load
CHI 2766M	-	-	-	-	-	-	-	-	-	-	54,220
IPU 2188M	-	-	-	-	-	-	-	-	-	-	47,600
ITP 911F	-	-	-	-	-	-	-	-	-	-	7,852
KWA 1802M	-	-	-	-	-	-	-	-	-	-	7,912
MAN 5764M	-	-	-	-	-	-	-	-	-	-	444,440
MAT 2702M	-	-	-	-	-	-	-	-	-	-	47,600
MON 3076M	-	-	194++	33.0+	-	19572++	-	-	-	0.69	21,796
MSH 596F	-	36.1++	-	-	-	-	-	-	-	-	10,612
MTE 2143M	-	-	-	-	-	-	-	-	-	-	186,148

Table 4: Antiretroviral (ARV) Concentrations in Donor Partners

10 ARVs were monitored and quantified (in ng/mL) in 9 HIV+ patient (donors) plasma samples by liquid chromatography mass spectrometry (LC-MS) analysis. Drugs that were below the level of quantification in the plasma are denoted by (-). In individuals with detectable ARV levels, the concentrations are reported and concentrations above the effective concentration 90 (EC90) are indicated by (++) and concentrations above the effective concentration 50 (EC50) are indicated by (+). VL is measured in copies/mL.

HTID	MON3076M (index)	MON3076F (seroconverter)
NRTI Resistance Mutations	K65R, M184V	K65R, M184V
NNRTI Resistance Mutations	V106M, Y181C, G190A, N348I	Y181C, G190A, N348I

Table 5: Drug resistance mutations identified in reverse transcriptase

DRMs were detected in one couple. Mutations in red are shared by the transmitting (index) partner and the seroconverter.

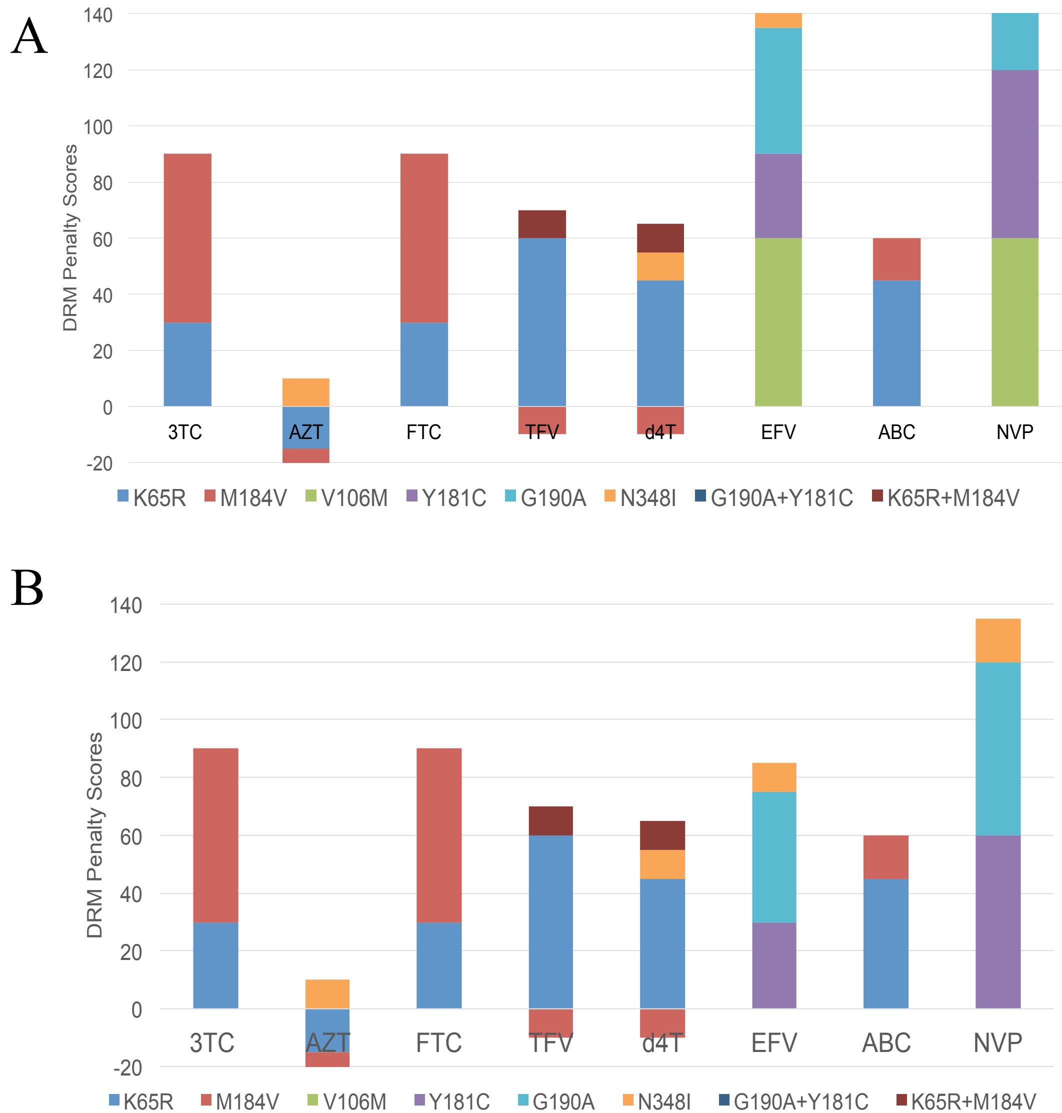

Figure 5: Transmitted Drug Resistance in One Couple (MON3076)

Major DRMs in index (A) and seroconverting (B) partner at the time of transmission. Each DRM contributes to ARV resistance positively (values greater than 0) or negatively (values less than 0). The contribution of each DRM is estimated by penalty scores (generated by the Stanford University HIV Drug Resistance Database). The cumulative penalty score determines the level of drug resistance (between 0-9: susceptible; 10-14: potential low level resistance; 15-29, low level resistance; 30-59, intermediate resistance; >/=60, high level resistance).

Viral Load Status	Number (n=50)	Percentage (%)
Undetectable Viral RNA	40	80
Detectable Viral RNA	10	20

Table 6: Qualitative RT-PCR Screening Results

VL measurement for monitoring ART adherence can be costly and may not be readily accessible or economical in most low and/or middle income countries. The Zambia Emory HIV Research Project developed an inexpensive (~$8), qualitative in-house PCR screen for the presence of viral RNA (~400 copies/mL cut-off) by amplification of regions in gag, pol and gp41.

ID (Test Site + CVTID)	*Sample Date*	*Viral Load (copies/mL)*
GEO7098F	25-Oct-15	<160
GEO6682F	24-Jun-15	<160
KAM3936F	11-Oct-15	<160
GEO6305F	8-Feb-15	<160
GEO6347F	26-Apr-15	undetectable
GEO7269M	8-Dec-15	<160
MAT6479M	20-Sep-15	<160
KAM3065M	28-Jun-14	<160
CHI2933M	20-Sep-14	<160

Table 7: Confirmation of Viral Loads From 9 Control Samples

VL testing was performed using the Abbott RealTime HIV-1 assay for 9 HIV-1 positive partners that had undetectable HIV-1 viral RNA from the initial RT-PCR screening to confirm the results.

ID (Test Site + CVTID)	Sample Date	Viral Load (copies/mL)
KAM3279F	10-Jan-15	768
KAM3895F	8-Aug-15	264
KAM3921F	20-Sep-15	2,840
GEO7093F	23-Oct-15	23,800
GEO6685F	24-Jun-15	2,948
GEO6809M	6-Aug-15	22,160
MAT6133F	31-Jan-15	194,928
CHI3121M	4-Jun-15	168,992
MAT6371F	8-Jul-15	1,232
GEO6959M	20-Sep-15	7,984

Table 8: Viral Load Testing For Individuals With Detectable Viral RNA

VL testing was performed using the Abbott RealTime HIV-1 assay for the 10 HIV-1 positive partners with detectable HIV-1 viral RNA from the initial RT-PCR screening to obtain quantitative VL values.

CVCTID	3TC	FTC	TFV	ABC	NVP	EFV	RTV	LPV	AZT	d4T	Viral Load
GEO7098F	298++	-	33.3+	-	-	2580++	-	-	-	-	<160
GEO6682F	259++	-	40.3+	-	-	3334++	-	-	-	-	<160
KAM3936F	290++	-	47.3+	-	21352++	-	-	-	-	-	<160
GEO6305F	1213++	-	-	564	39223++	-	-	-	-	-	<160
GEO7269M	244++		33.8+	-	-	25675++	-	-	-	-	<160
GEO6347M	-	305	28.4+	-	-	4119++	-	-	-	-	undetectable
MAT6479M	822++	-	12.1	-	-	3049++	-	-	-	-	<160
KAM3065M	-	419	24.4+	-	-	2064++	9.9	-	-	-	<160
CHI2933M	-	217	22.8+	-	-	2172++	-	-	-	-	<160

Table 9: Mass Spectroscopy Analysis For 9 Control Samples

10 ARVs were monitored and quantified (in ng/mL) in 9 HIV+ patients with undetectable viral RNA by liquid chromatography mass spectrometry (LC-MS) analysis. Concentrations above the effective concentration 90 (EC90) are indicated by (++) and concentrations above the effective concentration 50 (EC50) are indicated by (+). ARVs below the level of quantification in the plasma denoted are by (-). VL is measured in copies/mL.

CVCTID	3TC	FTC	TFV	ABC	NVP	EFV	RTV	LPV	AZT	d4T	Viral Load
KAM3297F	78.9++	-	9.44	-	-	1252++	-	-	-	-	768
KAM3895F	214++	-	16.5+	-	-	14210++	2.83	-	-	-	264
KAM3921F	-	4181++	82.1++	-	45284++	-	-	-	-	-	2,840
GEO7093F	-	-	-	-	-	-	-	-	-	-	23,800
GEO6685F	-	-	-	-	-	-	-	-	-	-	2,948
GEO6809M	-	-	-	-	-	-	-	-	-	-	22,160
MAT6133F	-	-	-	-	-	-	-	-	-	-	194,928
CHI3121M	3.31+	-	-	-	-	-	-	-	-	-	168,922
MAT6371F	-	-	-	-	-	-	2.25	-	-	-	1,232
GEO6959M	259++	-	23.9+	-	-	-	-	-	-	-	7,984

Table 10: LCMS for the 10 Individuals with Measurable Viral Loads

10 ARVs were monitored and quantified (in ng/mL) in 10 HIV+ patients by liquid chromatography mass spectrometry (LC-MS) analysis. Concentrations above the effective concentration 90 (EC90) are indicated by (++) and concentrations above the effective concentration 50 (EC50) are indicated by (+). ARVs below the level of quantification in the plasma denoted are by (-). VL is measured in copies/mL.

ID (Test Site + CVTID)	*Sample Date*	*Viral Load (copies/mL)*
BGO003M	22-Mar-13	6,148
COR140M	19-Sep-12	1,168
COR150M	20-Sep-13	6,556
KIM168F	23-Jan-13	914,100
KIN001M	17-Sep-13	268
KRU001M	8-Nov-13	<160
RUG001F	28-Jan-13	84,622
RUG2M	9-Apr-13	<160

Table 11: Donor Viral Loads at Linked Recipient's Time of Seroconversion

VLs from 8 seropositive partners reporting antiretroviral (ARV) usage. The Abbott RealTime HIV-1 assay, an *in vitro* RT-PCR based assay, was used for the quantitation of HIV-1 RNA copies in plasma on the m2000 system over the range of 40 to 10,000,000 copies/mL.

CVCTID	3TC	FTC	TFV	ABC	NVP	EFV	RTV	LPV	AZT	d4T	Viral Load
BGO003M	-	-	-	-	-	6.06++	-	-	-	-	6,148
COR140M	425++	-	39.1+	-	-	5,680++	-	-	-	-	1,168
COR150M	1,526++	-	-	-	67,855++	-	-	-	35.6++	-	6,556
KIM168F	-	-	-	-	-	-	-	-	-	-	914,100
KIN001M	622++	-	47.4+	-	-	5,969++	-	-	-	-	268
KRU 001M	543++	-	33.7+	-	-	7,764++	-	-	-	-	<160
RUG001F	-	-	-	-	0.34	-	-	-	-	-	84,622
RUG2M	1,591++	-	47.2+	185	135,275++	-	-	-	-	-	<160

Table 12: Antiretroviral (ARV) Concentrations in Transmitting Partners

10 ARVs were monitored and quantified (in ng/mL) in 8 HIV+ patient (donors) plasma samples by liquid chromatography mass spectrometry (LC-MS) analysis. Concentrations above the effective concentration 90 (EC90) are indicated by (++) and concentrations above the effective concentration 50 (EC50) are indicated by (+). Drugs below the level of quantification in the plasma are denoted by (-). VL is measured in copies/mL.

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