HIV Diversity among Pregnant Women and their infants in Harare Peri-urban;

Implications in

Disease Diagnosis, Monitoring and Transmission

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Dedications

This thesis is dedicated to my beloved brother, the late Professor SK Chandiwana in his memory and honour, and to my parents, nine siblings, two lovely sons Munyaradzi and Theophilus K. and dear husband, Godfrey. Last by not least to the special BHAMC cohort.

The late Professor SK Chandiwana

My parents, husband and sons

My siblings

The Unique BHAMC Cohort; from Pregnancy to 10 Year Olds

Golden Words

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Abbreviations and Acronyms

AIDS	**A**cquired **I**mmune **D**eficiency **S**yndrome
ANC	**An**tenatal **C**are
APOBEC3G	**A**poli**po**protein **B** mRNA-editing **E**nzyme-**C**atalytic polypeptide-like 3G
ART	**A**nti**r**etroviral **T**herapy
BHAMC	**B**etter **H**ealth for **A**frican **M**other and **C**hild
CCR5	**C**ysteine-Cysteine **C**hemokine **R**eceptor-5
CXCR4	**C**ysteine-**X**-Cysteine **C**hemokine **R**eceptor-4 (where X is any amino acid)
CD	**C**luster of **D**ifferentiation
CTL	**C**ytotoxic **T** **l**ymphocyte
CRF	**C**irculating **R**ecombinant **F**orm
DC-SIGN	**D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin
DNA	**D**eoxyribo**n**ucleic **A**cid
EDTA	**E**thylene-**D**iamine **T**etra **A**cetate
ELISA	**E**nzyme-**l**inked **I**mmuno-**s**orbent **A**ssay
Env	**Env**elope Gene
EPP	**E**pidemic **P**rojection **P**ackage
Gag	**G**roup-**s**pecific **A**ntigen **G**ene
GALT	**G**ut **A**ssociated **L**ymphoid **T**issue
Gp41	**G**lyco-**P**rotein 41
HAART	**H**ighly **A**ctive **A**nti**r**etroviral **T**herapy
HIV	**H**uman **I**mmunodeficiency **V**irus

HLA	**H**uman **L**eukocyte **A**ntigen
HMA	**H**eteroduplex **M**obility **A**ssay
HTLV	**H**uman **T**-**l**ymphotrophic **V**irus
IDU	**I**ntravenous **D**rug **U**ser
INF	**In**ter**f**eron
KIR	**K**iller cell **I**mmunoglobulin-like **R**eceptor
LAG-3	**L**ymphocyte **A**ctivation **G**ene-3
LAV	**L**ymphadenopathy **A**ssociated **V**irus
LTR	**L**ong **T**erminal **R**epeat
MIP	**M**acrophage **I**nflammatory **P**rotein
MOHCW	**M**inistry **o**f **H**ealth and **C**hild **W**elfare
MRCZ	**M**edical **R**esearch Council of **Z**imbabwe
MTCT	**M**other **T**o **C**hild **T**ransmission
NBSZ	National **B**lood **S**ervice **Z**imbabwe
Nef	**Ne**gative **F**actor gene
NHP	**N**on-**H**uman **P**rimates
NNRTI	**N**on-**N**ucleoside **R**everse **T**ranscriptase **I**nhibitor
PARD3	**Par**titioning **D**efective **3** homolog
PCR	**P**olymerase **C**hain **R**eaction
PITC	**P**rovider **I**nitiated **T**esting and Counseling
PMTCT	**P**revention of **M**other-**T**o-**C**hild **T**ransmission
Pol	**Pol**ymerase gene

RNA	**R**ibo**n**ucleic **A**cid
RANTES	**R**egulated on **A**ctivation **N**ormal **T** cell **E**xpressed and **S**ecreted
RT	**R**everse **T**ranscriptase
RT-PCR	**R**everse **T**ranscriptase-PCR
SdNVP	**S**ingle **D**ose **N**evira**p**ine
SDF-1	**S**tromal **D**erived **F**actor-1
SIV	**S**imian **I**mmuno-deficiency **V**irus
SSA	**S**ub-**S**aharan **A**frica
Tat	**T**rans**a**ctivator of **T**ranscription gene
Tim-3	**T**-cell **I**mmunoglobulin and **M**ucin domain-containing molecule-3
TLR	**T**oll-**L**ike **R**eceptor
TNF	**T**umour **N**ecrosis **F**actor
TRIM5α	**Tri**partite **M**otif-containing protein-5 alpha
URF	**U**nique **R**ecombinant **F**orm
Vif	**V**iral **I**nfectivity **F**actor
Vpr	**V**iral **P**rotein **R**
Vpu	**V**iral **P**rotein **U**
µL	Micro (10^{-3}) litre
WHO	**W**orld **H**ealth **O**rganisation
ZDF	**Z**imbabwe **D**efence **F**orces
ZDHS	**Z**imbabwe **D**emographic and **H**ealth **S**urvey

List of Papers Included in the PhD Thesis

I. **Duri K**, Gumbo FZ, Kristiansen KI, Kurewa NE, Mapingure MP, Rusakaniko S, Chirenje MZ, Muller F and Stray-Pedersen B. **Antenatal HIV-1 RNA load and timing of mother to child transmission; A nested case-control study in a resource poor setting.** *Virol J* 2010;7:176.

II. **Duri K**, Soko W, Gumbo F, Kristiansen K, Mapingure M, Stray-Pedersen B, Muller, F and the BHAMC Group. **Genotypic analysis of Human Immunodeficiency Virus type 1 (HIV-1) env V3 loop sequences: Bioinformatics prediction of co-receptor usage among 28 infected mother-infant pairs in a drug-naive population.** *AIDS Res Hum Retroviruses* 2010; 27(4):411-419.

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women under a PMTCT program at three peri-urban clinics in a resource-poor setting. *J Perinatol* 2010; 30(11):717-723.

IV. Gumbo FZ, Kurewa NE, Kandawasvika GQ, **Duri K,** Mapingure MP, Munjoma MW, Stray-Pedersen B. **Rising mother-to-child HIV transmission in a resource-limited breastfeeding population.** *Trop Doct* 2010; 40(2):70-73.

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VI. Soko W, **Duri K**, Gumbo FZ, Kristiansen KI, Mapingure MP, Muller F and Stray-Pedersen B**. Frequency of host genes CCR2V64i and CCR5-delta-32; Association with HIV-1 infection among pregnant women in Harare, Zimbabwe.** Submitted to *AIDS Res Hum Retroviruses*

VII. Mhandire K, Pharo G, **Duri K**, Kandawasvika GQ, Stray-Pedersen B and Dandara C. **Variation in Human Immunodeficiency Virus restriction genes MBL2 and RANTES and their roles in HIV/AIDS Diseases progression in children born to infected mothers.** Submitted to *AIDS Res Hum Retroviruses*

VIII Duri K. **Coreceptor Usage in HIV infection. In Immunodeficiency** ed. Metodiev K, **Intech Open Science/Open Minds**, Croatia 2012, Chapter **11** pp. 1-34.

Summary of Thesis

Background

Within the African regions, there are striking differences in human immunodeficiency virus (HIV) prevalence yet social and cultural differences are relatively small suggesting that sexual or vertical transmission alone may not explain HIV infections in Sub-Saharan Africa (SSA). Several factors may contribute to the variation in the pandemic distribution in the region, for example, unsafe medical care, differences in host genetics or HIV-1 genetic diversity. The hallmark of HIV-1 is its extensive genetic diversity that emanates mainly from high mutations. Phylogenetically, HIV can be classified into geographically confined groups, types, subtypes and circulating recombinant forms (CRFs) that are subject to change over time. The plasticity of the HIV-1 env gp120 gene may also cause variation in chemokine co-receptors usage, numbers and distribution of potential glycosylation sites (PNGS) including amino acid length polymorphism. HIV genetic diversity may partially explain the observed heterogeneity in HIV prevalence and has also been reported to impact on viral transmissibility and differential rates of disease progression. Zimbabwe is one of the countries in the world with the highest HIV-1 prevalence. Despite the high HIV-1 prevalence in the general populace which translates to high vertical transmission rates, the desire to have future pregnancies among HIV-1 positive mothers has been increasing. Concurrently, the decade long volatile economic climate has forced over 80% of jobless Zimbabweans into self-employment through cross-border trading all over the world. This desperate economic situation may have led some traders to engage in risky sexual behaviour. With the world fast becoming a global village, new HIV strains are emerging in areas where they were originally non-existent. There is paucity of information on the current HIV-1 diversity;

types, subtypes and recombinants circulating in Zimbabwe. Tracking the presence of new HIV strains is important not only for surveillance purposes but also for monitoring disease progression, facilitating personalized targeted therapy as well as using this data for the development of the much anticipated effective vaccines against this scourge.

Aim of the study

The main goal of this study was to characterise HIV genetic diversity among Harare peri-urban pregnant women and ascertain its role in diagnosis, transmission and disease progression.

Materials and Methods

Pregnant women at 36 gestational weeks who were enrolled in a national prevention of mother to child transmission (PMTCT) programme were studied. The design of the study was a case-control study in which the cases and controls were sampled from an antiretroviral therapy (ART) naive PMTCT cohort of pregnant women attending Antenatal Clinics (ANC) around Harare. Single dose nevirapine (SdNVP) was offered to all HIV-1 positive women during labour and their infants within 72 hours post-delivery. Follow-ups were from delivery, six weeks, four and nine months and thereafter three monthly until two years. At each subsequent follow-up visit HIV-1 negative mothers and all exposed infants were re-tested for HIV antibodies and viral DNA, respectively. Similar procedures were followed in subsequent pregnancies. Women who were included in our study were enrolled from an initially sexually transmitted infections (STI) study. Some of these women's spouses also consented to participate in the HIV diversity study. Genotyping of HIV-1 *env* gp120 C2V5 region was done for subtype and viral co-receptor usage determination. Mother-infant viral heterogeneity, potential N-linked glycosylation site(s) (PNGs)

variations and amino acid length polymorphisms were also investigated including immunological and virological markers of disease progression

Results

HIV T**ypes**:

- HIV-1 prevalence was 25.6% and contributed 98.4 % of the HIV infections.
- HIV-2 prevalence was 0%.
- HIV/HIV-2 co-infections contributed 1.6% of the HIV infections.

HIV-1 Subtypes:

- All mother–infant pairs were infected with HIV-1 subtype C virus.
- Sequences clustered closely with other regional HIV-1 subtype C sequences.
- Phylogenetic analysis was suggestive of a localized expansion of the subtype C.
- Unusually high variation in amino acid sequence was observed within the HIV-1 subtype C supposedly constant region 3 (C3) as well as the atypical fairly constant variable region 3 (V3).

Antenatal HIV-1 Co-receptor Usage:

- R5 co-receptor usage was the predominant genotype (82%).
- X4 genotype was significantly associated with higher viral load.
- GPGR amino acid motif within the V3 crown was associated with X4 genotype and lymphadenopathy; p=0.031 and 0.043, respectively.

Mother-Infant(s) HIV-1 env gp120 C2V5 Viral Heterogeneity

- Degree of HIV-1 subtype C viral heterogeneity: mothers> first siblings>second sibling.

Env C2V5 Glycosylation Patterns and Sequence Length Polymorphisms:

- HIV-1 env C2V5 amino acid length and PNGs tended to increase with age and HIV disease progression.
- Directionality of the HIV transmission events with respect to C3 region sequence length polymorphism was suggestive of a 50-50 transmission events in either direction, whether male to female (MTF) or female to male (FTM).

- Increases in PNGs or amino acid lengths within the C3, C4 and V3 sub-regions positively correlated with CD4 counts or percentage (%) but negatively correlated with viral load.

HIV-1 Vertical Transmission:

- The risk of transmission increased by 29% for each unit increase in $\log_{10}$ viral load; p=0.023.
- Transmission rates were 7.5% and 15.3 % for the *in utero* and intra-partum/postpartum periods, respectively.
- 90% of the transmissions occurred below viral load of 16 000 HIV-1 RNA copies /mL.
- Generally more than one maternal variants were responsible for infant's infection.
- Maternal co-receptor genotype was generally preserved in vertical transmission and was predictive of the infant's viral genotype.
- None of the infants had dual R5X4 genotype.
- Vertically infected children were surviving longer than was expected even without ART.

Risk Factors for Vertical Transmission

- High antenatal plasma HIV-1 RNA load, low total lymphocytes count (TLC) and anemia were each significantly associated with vertical transmission.
- Lack of antibody reactivity to HIV *gag* p39 antigen on western blot band profiles was associated with vertical transmission and advanced disease.

HIV Diagnosis

- HIV-1/HIV-2 rapid kits test results concordance was 100%.
- Non- reactivity to *pol* antigens was associated with acute HIV-1 infection; p=0.002.

HIV-1 Disease Monitoring

- 28% of the 64 mothers had undetectable HIV-1 RNA load yet 10% proceeded to transmit to their infants.

Conclusion

Despite the high mobility, there seems to be no new types, subtypes nor CRFs being introduced at least in this population based on the analysis of the HIV-1 env C2V5 region. The sensitivity and specificity of the HIV-1/HIV-2 screening and confirmatory diagnostic tests used were appropriate as concordance was 100%. However, disease monitoring test, viral load determination, may not have been as sensitive as shown by mothers with undetectable viral load who nevertheless transmitted to their infants. Alternatively, this observation is pointing to the complex factors associated with MTCT of HIV. Data are suggestive that subtype C env sequence may be different from that of subtype B and hence extrapolation of subtype B findings to non-B subtypes may not be accurate. Since CCR5 was the most predominant genotype it entails that ART combinations that include R5 entry inhibitors can be used in this population. HIV-1 infected infants inherited their respective mothers' co-receptor genotypes, were more likely to be infected with more than one maternal viral variants and were also surviving longer even without ART. These long term survivors require tailor-made HIV-care especially during the adolescent period. Continuing following up these HIV-1 infected mother-infant pairs inclusive of all subsequently born children is worthwhile for documentation of disease progression and trends in drug resistant mutations under such settings whereby the host factors can be controlled. Future bigger studies comparing HIV-1 transmission rates from a population like ours with exclusive subtype C infection to other cohorts with mixed infections inclusive of subtype C could partly explain the observed heterogeneous distribution of HIV prevalence.

CHAPTER 1

1.0 Background

1.1.0 HIV/AIDS Disease Burden and Geographical Distribution

1.1.1 Historical Background of HIV/AIDS

A syndrome associated with severe immunodeficiency was observed in the United States of America (USA) among previously healthy homosexual men and intravenous drug addicts in 1981 [1]. The aetiological agent was isolated from the lymph nodes of suspected patients two years later [2;3]. By then it was called human T-cell lymphotrophic virus type-3 (HTLV-III) or lymphadenopathy-associated virus (LAV) but was later re-named human immunodeficiency virus (HIV) [4]. Transmission can be through vaginal, anal or oral sex, blood transfusion, hypodermic needles or from a pregnant mother to her unborn child during pregnancy, childbirth or through breastfeeding [5-7]. HIV causes progressive immunodeficiency leading to Acquired Immunodeficiency Syndrome (AIDS). It is currently one of the most devastating infectious diseases in the history of mankind. The earliest anti-HIV-1 sero-positive blood sample was from an individual in Kinshasa, Congo in 1959 [8] yet, globally by 2010, 20 million people had since died from the infection whilst another 33 million are living with HIV/AIDS as shown in **Figure 1.1.**

Sub Saharan Africa (SSA) with just a mere tenth of the world's population, harbors about two thirds of all HIV infections globally and 90% of all pediatric infections [9]. A disturbing phenomenon in Africa is the high HIV/AIDS burden observed amongst women unlike in the USA and Europe, where it is concentrated among hemophiliacs, intravenous drug users (IDUs) and homosexual men [10;11]. In view of the fact that many Africans even in stable

relationships are also infected, there has been growing interests to understand the dynamics and risk factors of HIV-1 transmissions [12].

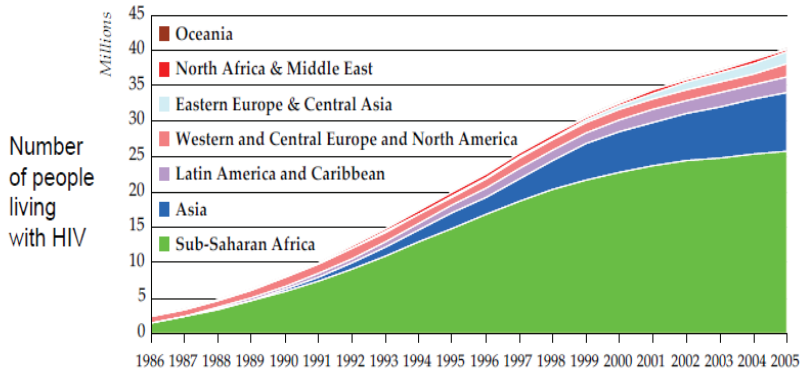


Figure 1.1: Global Heterogeneous HIV Burden [13].

1.1.2. Origin of HIV and Zoonosis

The origin of HIV can be traced back to a Simian Immunodeficiency Virus (SIV) isolated from a Chimpanzee (cpz) sub-species, *Pan troglodytes troglodytes* (SIVcpz) in Southern Cameroon [14]. It is hypothesized that cross species transmission of HIV occurred from its primary host, the SIVcpz to humans. This zoonotic transmission of the virus from the non-human primates (NHPs) to humans is thought to have occurred through practices of hunting and butchering of NHPs or during the process of caring for captive NHPs alongside with poor laboratory handling of their respective virally infected tissues and/or fluids [15;16]. Interestingly, there is another alternative but unsubstantiated propositions of this complex and controversial topic on the origin of HIV [17].

1.1.3. HIV prevalence and Trends in Africa

During the 1980s, researchers in Africa observed a high HIV prevalence among female commercial sex workers and patients attending sexually transmitted infections (STIs) clinics [18-21]. Consequently, a consensus was reached among AIDS experts dealing with Africa that heterosexual and vertical transmissions were the primary modes of HIV acquisition in adults and children, respectively [22-24]. Thus, it is now widely accepted that the HIV-1 epidemic in SSA is mainly driven by heterosexual transmission [25]. Husbands have been shown to acquire HIV-1 infection first from extra marital affairs and then proceed to infect their wives [26;27]. Cultural practices such as inheritance of widows and re-use of sharps by traditional healers have also been implicated in driving the pandemic to alarming levels in some regions. As a result HIV pandemic within the continent reflects many co-existing sub-epidemics in different regions as shown in **Figure 1.2**.

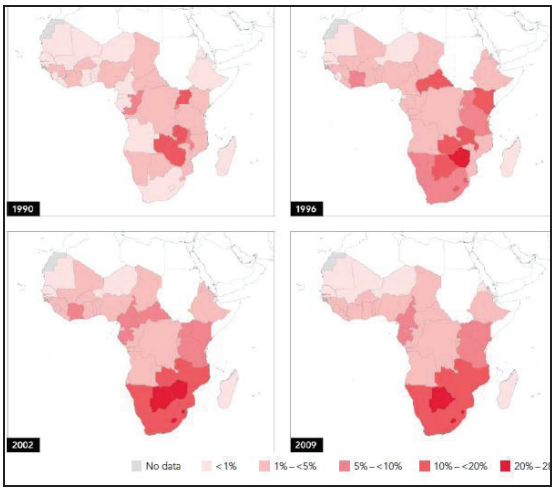


Figure1 2: HIV Prevalence trends among the 15-49 year olds over the past 10 years in SSA [28]

1.1.3.1. Striking Differences in HIV Prevalence in SSA

Within Africa there are striking regional differences in HIV prevalence [29;30]. Among women attending antenatal care (ANC), HIV prevalence increased from 20–26 % between 1997 and 2002 for Southern Africa, but actually declined from 14% to 1.4% and 5% to 4% for Eastern and Western Africa, respectively during the same period [31]. Interestingly, in Kinshasa, the purported region for the origin of HIV, sero-prevalence amongst pregnant women steadily rose from 0.25% in 1970, to 3.0% in 1980 and 5.7% in 1990s [32]. Amongst some neighboring SSA countries huge differences in HIV prevalence have been observed being, 38%, 20%, 14% and 10% for Botswana, Zimbabwe, Mozambique and Tanzania, respectively yet social and cultural differences among these African countries are relatively small [33]. HIV prevalence discrepancies are so distinct suggesting that there could be other possible unique but currently unknown precipitating factors in the high prevalence countries contributing to the pandemic.

1.1.3.2. HIV Transmission Modes in Africa, Controversies

Some researchers in an attempt to explain the observed heterogeneity in HIV prevalence are supportive of the hypothesis that HIV infections in SSA may not be explained by sexual or vertical transmissions alone [34-38]. Studies have demonstrated that STIs facilitate HIV transmission [39-41]. Assuming this synergism, a high burden of STIs should correlate with high HIV prevalence. However, ecological comparative studies from population based surveys from high and relatively low HIV prevalence areas in Zimbabwe and Tanzania respectively, have reported more or less similar burdens of STIs but distinctive HIV prevalence [42]. More interestingly, studies have also shown that Hepatitis B virus (HBV) which has similar modes of transmission to HIV and in addition much more infectious has generally a much lower

prevalence [43]. HBV infection is common in SSA with Mozambique having the highest incidence rate yet this country's HIV-1 prevalence is amongst the lowest in the region [44;45].

HIV infection has been confirmed in a number of pediatric cases where its source has not been adequately explained [46;47]. Studies have observed an unexplained high HIV-1 incidence among pregnant women who were sero-negative at the first antenatal visit but sero-converted later during antenatal and post-partum periods [48;49]. This observation is suggestive that whatever happens during pregnancy and post-partum periods whether iatrogenic, sexual or otherwise accounts for the high HIV incidence rates observed among these generally low risky women. Some researchers argue that the massive increase in use of medical injections for parenteral therapies to treat diseases could have been the possible source of the background effect of high HIV infection in some communities [50-55]. HIV has been shown to stay infectious on a needle for more than two weeks [56]. This hypothesis of unsafe medical injections has been shown to be scientifically implausible as some countries like Egypt where despite the vigorous parenteral anti-schistosomal treatment campaigns has very low HIV prevalence but interestingly the highest hepatitis C virus (HCV) disease burden in the world.

Paradoxically, recent meta-analysis studies have observed relatively large proportions of HIV-1 discordant couples in Africa with women as likely as men to be the index HIV-1 positive partners [57;58]. Even more intriguingly has been the observation that some of these HIV sero-discordant couples continue to bear children implying unprotected sex [57] suggesting that something other than simply heterosexual transmission could be involved. Regional

differences in HIV-1 prevalence of discordant couples vary from 8-31%, and 16-31% for Eastern and Southern Africa respectively, coincidentally, reflecting the same trend with HIV-1 prevalence [59]. Thus, there are research gaps to elucidate the precipitating factors which could have contributed to a relatively much more efficient transmission of HIV-1 in SSA resulting in the virus infecting more than a quarter of the population in some communities [60-63]. Thus, several factors may contribute to the differential spread of the HIV pandemic within the region including behavioral, biological factors, viral characteristics, unsafe medical practices and ethnic variation in host HIV restriction genes. Each of these factors alone or in combination could determine susceptibility to infection and consequently affecting the observed differential rates in progression towards AIDS.

1.2.0. Zimbabwe: Geographical Location, Demographics and Socio-Economics

Figure 1.3: Geographical Location of Zimbabwe and Study Sites

1.2.1. Geographic Profile

Zimbabwe lies north of the Tropic of Capricorn between the Limpopo and Zambezi rivers. Situated in Southern Africa, it is a landlocked country covering an estimated area of 390,784 km². Zimbabwe borders Zambia, Mozambique, Botswana and South African to the north, east, west and south, respectively. A narrow Caprivi Strip is also shared in the north-western border with Namibia. For administrative purposes the country is divided into ten provinces which are further divided into 58 districts. Zimbabwe attained its independence from the British in April 1980 after a protracted armed guerilla struggle. Since then until the late 1990s all sectors of the economy performed well.

Zimbabwe boasts of abundant natural resources that include 9 million hectares of potentially arable land and more than 5 million hectares of forests, national parks, and wildlife estates. The country is adored for its extensive and varied mineral resources such as platinum, gold, asbestos, coal, nickel, iron, copper, lithium, including precious gems like emeralds and diamonds. There are adequate supplies of surface and ground water which are not only enough for domestic and industrial uses but can also be harnessed for generation of hydro-electric power and irrigation of crops. Thus, the economy is diversified but biased towards agriculture, mining and tourism. However, despite the abundance of these natural resources the country has been riddled with profound socio-economic and political challenges in the last decade that nearly drove the economy into oblivion had it not been for the government of national unity (GNU) signed in February 2009 by the three major feuding political parties.

1.2.2. Population Size and Trends

A national census is carried out every ten years since 1931. Currently the 2012 census is ongoing. The population has been doubling almost every 20 years. According to the previous 2002 census, Zimbabwe had 11.6 million people, 1.2 million more than in 1992 and 4.2 million more than in 1982 [64]. There are dissensions to the effect that the 2002 census excluded about three million Zimbabweans who are economic refugees in the Diaspora. About 70% of the population lives in the rural areas. Africans constitutes about 98% of the population. Major ethnic groups are the Shona (82%) and Ndebele (14%) tribes with the rest being other ethnic minorities as shown in **Table 1.1**. Zimbabwe is generally a Christian nation and in some instances mixed with traditional beliefs. About 1% of the population is Muslim. National literacy rate is very high (94%) with almost all the urbanites being literate. The 2002 population pyramid had a wide but tapering base depicting a population experiencing a decline in fertility probably due to previous socio-economic hardships and/or the current HIV/AIDS pandemic. On a lighter note, the current total fertility rate for Zimbabweans is 4.1 children per woman slightly higher than the previous the rate [65].

Table 1.1: Trends of Selected Demographic Indicators in Zimbabwe [64]

Indicator	1992 Census	2002 Census
Total population (thousands)	10,412	11,632
Distribution by ethnic group (percent)		
African	98.8	99.3
European	0.8	0.4
Coloured	0.3	0.2
Asian	0.1	0.1
Distribution by age group (percent)		
0-14	45.1	40.6
15-64	51.3	55.0
65+	3.3	4.0
Not stated	0.3	0.4
Crude birth rate (births per 1,000 population)	34.5	30.3
Crude death rate (deaths per 1,000 population)	9.5	17.2
Number of males per 100 females in the total population	95	94
Life expectancy at birth	61.0	45.0

National surveys which involve HIV testing called Demographic and Health Surveys (DHS) are conducted every five years. The primary objective is to provide current information and statistics on key health indicators such as fertility levels, sexual activity and mortality rates including HIV infection for policymakers, planners and researchers. Though quite expensive, these surveys offer nationally representative statistics as more than 11000 randomly chosen households are enumerated and over 43000 individuals interviewed. According to the latest 2010-11 DHS, females represented 53% of the population whilst the proportion of children under 15 and senior citizens above 65 years of age were 43% and 5%, respectively, [65]. Median ages at first marriage among women and men were 19.7 and 24.8 years, respectively. Eleven percent of married women were married to men already in polygamous unions [65].

1.2.3. Socio-economic conditions

Zimbabwe has been the only country in the Southern Africa Development Community (SADC) region experiencing a negative economic growth rate following political and economic crisis since year 2000. The economy deteriorated from one of Africa's strongest to the world's worst with the official inflation rate estimated at more than 1 000% in 2006 [66]. Excessive demand for foreign currency pushed inflation from 231 million percent in July 2008 to more than 79.6 billion percent per month, thus translating to an annual inflation rate of over 90 sextillion (10^{21}) percent [67]. Zimbabwean currency of billions denomination, as shown in **Figure1.4** below, was literally not worth the paper on which it was printed.

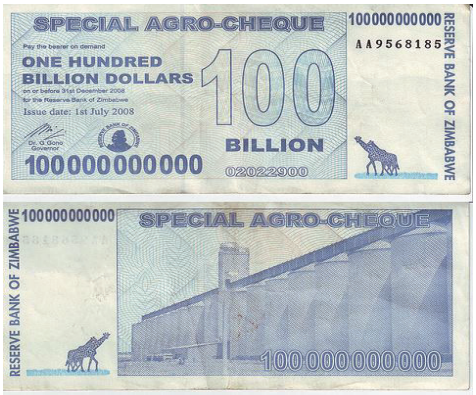


Figure 1.4: Zimbabwean Currency during Hyperinflation Period

The country experienced acute shortages of foreign currency, food stuffs, liquid fuels, electricity, medical equipment and drugs. With over 80% formal unemployment levels, the informal sector has been growing stronger over the years. Compounded by sanctions, hyperinflation has been the major problem for the past decade in Zimbabwe until April 2009 when the new coalition government suspended the use of local currency in favor of multi foreign currencies. Economic challenges encountered in the period 2000-2008 led to acute poverty. Coping strategies to mitigate food and foreign currency shortages were devised. Most jobless Zimbabweans especially women resorted to cross border trading with regional and Asian countries exposing themselves to sexual and other forms of abuse during the execution of their work [68]. As the economy deteriorated further, farmers failed to cope with the economic volatility triggered by land reforms. Consequently, food shortages were inevitable. Low remuneration not commensurate with the then prevailing economic conditions made working in the health sector non-conducive leading to low morale. Consequently, there was massive brain-drain of experienced professionals in all sectors of the economy among the

general population to unprecedented levels. Challenges associated with staff attrition in the health sector negatively impacted on the quality and coverage of HIV/AIDS health programs.

1.2.4. Health Care

Soon after independence, the Zimbabwean government adopted national policies that benefited the black majority such as access to free education and health care. One of the salient policy tenets in post independent Zimbabwe was "Health for all by year 2000". To this end, the government built over 240 new health centres and refurbished and upgraded over 500 pre-existing centres. The Zimbabwean's healthcare system was so good that 85% of the population lived within 10 kilometers of a health care facility. Quality of life of most Zimbabweans improved dramatically as depicted by key health indicators such as life expectancy, maternal and infant mortalities. Sadly, these early socio-economic gains were short lived as maternal mortality rate increased from 283 per 100000 in 1994 to 555 deaths per 100000 live births in 2005 [69]. Infant mortality rate rose from 50 per 1000 live births in 1990 to 60 per 1000 in 2006 [70]. Adult mortality rate sky-rocketed from 286 per 1,000 in 1990 to 751 per 1000 in 2006, aggravated by the fact that over 91% of the population did not have health insurance [70]. This drastic fall in vital health statistics was a consequence in part due to the diminished access to healthcare, closures of public hospitals, scarcity of essential drugs and inadequate or prohibitive medical care services complicated by foreign currency shortages. Most distressing was the fact that average life expectancy at birth fell dramatically from 60 years for both sexes in 1990 to about 40 years in 2006 as shown in **Figure 1.5**.

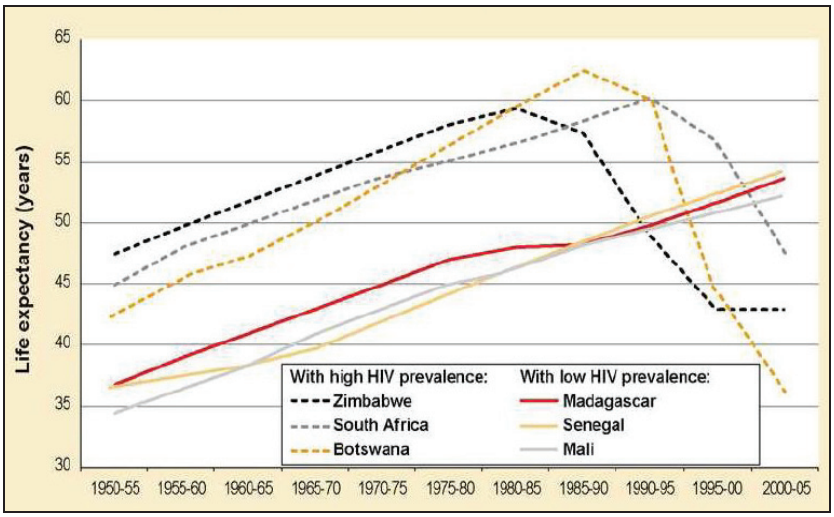


Figure.1.5: Trends in Life Expectancy in Zimbabwe Relative to other African Countries [71]

Malnutrition and high HIV prevalence were major factors that precipitated the decline in life expectancy [72-77]. On a lighter note, the current economic stability and recovery resulting in better health delivery and increased access to HIVAIDS therapy as well as better nutrition have seen marked improvement in welfare of most Zimbabweans [78].

1.3.0. The Zimbabwean HIV/AIDS Situation

1.3.1. HIV/AIDS; the Beginning

The first AIDS case was reported in Zimbabwe in 1985 [79]. Since then more patients began to present with illnesses suggestive of HIV infection. Young adults presented with severe respiratory infections, herpes zosters, persistent generalised lymphadenopathy and diarrhea associated with weight loss [80]. Children were seen who appeared to be suffering from malnutrition but whose socio-economic backgrounds were inconsistent with poverty and such

patients failed to respond to standard nutritional and conventional medical treatments, suggesting an immunodeficiency condition.

1.3.2. HIV in Blood Donors

The foregoing observation was the basis for the introduction of routine HIV-1 testing of donated blood and blood products in August 1985 by the National Blood Service Zimbabwe (NBSZ). Since then, HIV testing has been available to clinicians [81]. HIV-1 sero-prevalence amongst blood donors by then was 2%. Ten years later, the HIV sero-prevalence amongst blood donors had arisen to 8.8% with a sero-incidence of 2.1 per 100 person-years being highest among married first-time blood donors of 21-45 years of age [82;83]. From 1995 onwards, HIV testing included screening for both the two types, HIV-1 and HIV-2 [84]. As of 2010, NBSZ reported an HIV-1 prevalence of 0.74%, a slight decline from 0.77% in 2009 [85]. Within this healthy blood donor population the hepatitis B virus (HBV) and syphilis sero-prevalence were 0.97% and 0.68%, respectively [85]. Over the years there has been a remarkable decline in HIV-1 prevalence among blood donors as shown in **Figure 1.6**.

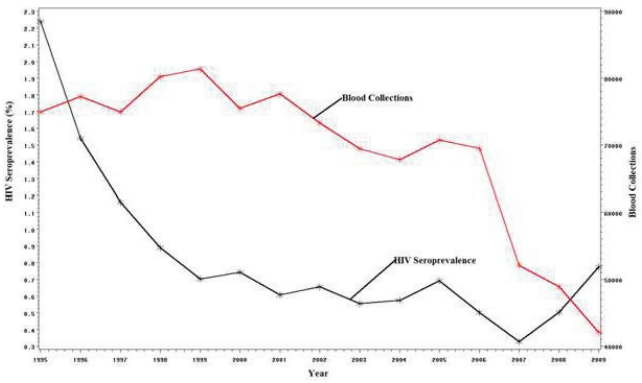


Figure 1.6: HIV Sero-Prevalence Trends among Blood Donors (1995-2009) [85]

1.3.3. HIV-1 in the Military Population

Militaries are generally reluctant to divulge figures on HIV prevalence for security reasons. However, Zimbabwe Defense Forces' (ZDF) figures are believed to be high [95;96]. The risky behaviour of military personnel compounded by the high HIV-1 sero-prevalence within this population has been a cause for concern [97;98]. ZDF personnel have been actively involved in peace keeping mission in Somalia, Rwanda, and Angola. Controversially, they have been deployed in the Democratic Republic of Congo (DRC) and Mozambique to fight civil war in support of the ruling regimes alongside other troops from Angola and Namibia. These external missions, whether offensive in nature or peace keeping have had a bearing on the transmission of HIV/AIDS within the armed forces to and from the civilian population both at home and abroad. There has been a deep concern regarding the possibility of ZDF personnel introducing new infections into the country inclusive of HIV infections. There are suggestions to the effect that the epidemic originated from multiple introduction into the country in the late 1970s during the demobilization phase of war corresponding to rapid influx of native military personnel from neighbouring countries [99] .

1.3.4. HIV-1 Trends and Distribution in the General Population

Following the diagnosis of the first case of HIV-infection in 1986 in the Northern district of Hurungwe, a local hospital based surveillance system was introduced to monitor the spread of the epidemic. This was before the official notification system included the HIV syndrome. AIDS cases increased exponentially from 19 in 1986 to 290 in 1987, 433 in 1988, and 145 during the first quarter of 1989 [86]. As early as 1987 the prevalence had shot up to 3.2% and interestingly all infections were found in the 17-30 years old group [87]. A cross sectional hospital-based study screening for STIs amongst adult volunteers at Murehwa rural district

hospital, 100km north east of Harare demonstrated a 50% HIV-1 sero-positivity in adults with STIs [88]. This fast growing HIV epidemic became a major threat to the health and development of the district, nation, region and the world at large, raising many questions. Where did this infection come from and why so many cases in a very short space of time? Sadly in Zimbabwe, there was so much denial by the government until 1990 when HIV/AIDS issues were debated in the public domain.

The coming together of traditional culture with the colonial legacy of men migrating to cities for employment leaving behind their spouses has influenced family structures and sexual relations. [89]. In Zimbabwe just like the rest of Africa young women continued to bear the brunt of the pandemic. Thirty four percent of women and 21% of men tested for HIV and received their results in the past year in the 2010-11 DHS relative to just 7% for both sexes in the 2005-6 DHS. One percent of the women and 11% of men of the 15-49 age group reported having sex with at least two partners during the past year of which 48% and 33% of the women and men, respectively reported using of a condom during their last sexual intercourse. All in all 15% of adults were HIV-1 positive down from 18% in the previous 2005-6 DHS. When stratified by gender HIV-1 prevalence was 18% and 12% for women and men, respectively and generally HIV was more prevalent in urban settings as depicted in **Figure 1.7**. Interestingly, there was also no clear relationship between wealth and HIV prevalence among both women and men [65]. Similarly no clear relationship between level of education and HIV prevalence has been observed among women. Conversely, HIV prevalence decreased as education level increased amongst men. Circumcised men in the age group 15-49 were slightly more likely to be HIV positive than those who were uncircumcised [65]. Thus, sadly circumcision may be giving a false sense of HIV protection among these men.

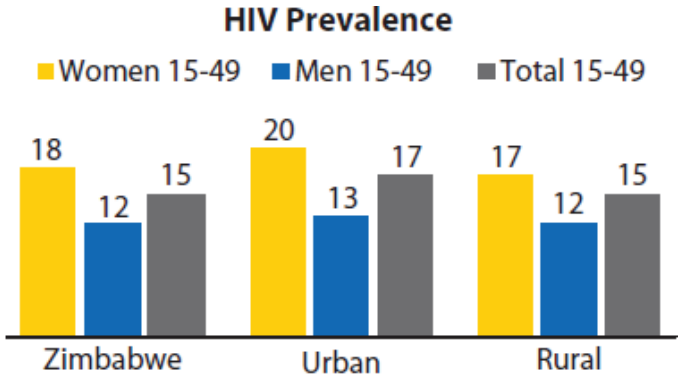


Figure 1.7: HIV-1 Prevalence among the 15-24 years old by Gender and Place of Residence [65].

Recent statistics on 2700 co-habiting couples has shown that in 79% of the cases both partners were HIV negative whilst 10% were both HIV positive [65]. Interestingly, 11% were discordant, that is, one partner was infected with HIV whilst the other was not [65]. Thus, sexual contact with an HIV infected person represents only a necessary, but not sufficient, condition for HIV transmission through sex suggestive that other cofactors may be central in fueling the HIV epidemic in SSA. Studies have observed a synergistic relationship between HIV and co-infections including malnutrition and these have been implicated as possible cofactors for HIV-1 acquisition and transmission [90-93].

The scale of the epidemic at country level reflects its widely disseminated nature with HIV prevalence in small towns, farming estates and mines located in rural areas (22%) exceeding that in the major cities (14.5%). Significant variations in the pandemic prevalence are also observed across the country provinces with Matabeleland South, bordering Botswana showing the highest prevalence. Ironically Harare, the capital city recorded the lowest as shown in **Figure 1.8**.

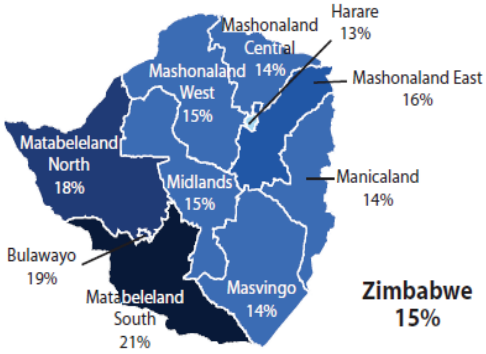


Figure 1.8: HIV Prevalence by Province in Zimbabwe [65].

Using the Epidemic Projection Package (EPP) and Spectrum software, declines have also been observed in both sentinel surveillance of pregnant women and in the National HIV Estimates process that models all available data. Single digit prevalence is being projected from the year 2016 as shown in **Figure 1.9**.

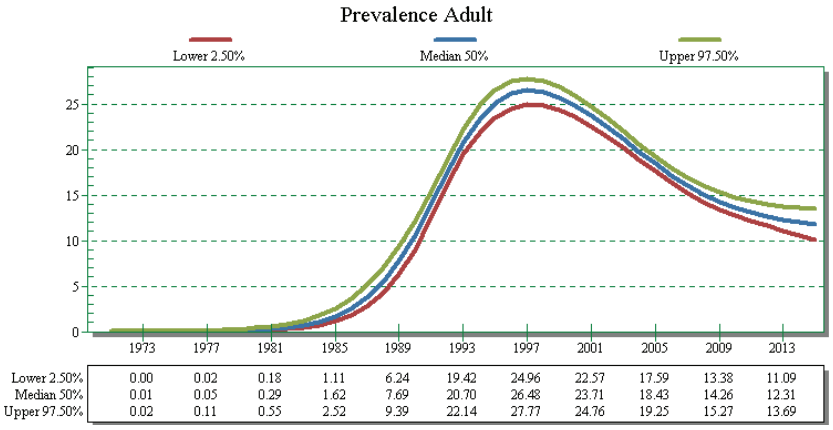


Figure 1.9: Zimbabwean Trends in Adult HIV Prevalence and Projections, 1970-2015 [94].

1.3.5. Impact of HIV/AIDS in Zimbabwe

In 2009 alone 83,000 HIV/AIDS related deaths were recorded. The number of children orphaned that is, a child with one or both parents dead due to HIV/AIDS in Zimbabwe remains unacceptably high (20%). The approximate number of such orphans is estimated to be one million [100]. The Food Agriculture Organisation (FAO) estimated that Zimbabwe lost about 23% of its agricultural workforce due to the HIV/AIDS pandemic [101]. Exacerbated by the apparent shortages of inputs, poor irrigation and low capitalisation levels the agricultural sector experienced a negative average growth rate of minus 8% over the past decade [101] and this had obvious negative repercussions on food security and the general health of the nation at large. One million two hundred thousand people are living with HIV/AIDS of which 200000 are children under 15 years. Access to antiretroviral therapy (ART) is quite limited in Zimbabwe. With over 300,000 people in need of ART, Zimbabwe is among the 20 countries identified by the World Health Organization (WHO) as having the highest unmet needs for ART. AIDS stigma has been an impediment to the uptake of voluntary counseling and testing (VCT) of HIV [102]. On a positive note, access to treatment has mitigated the stigma and fatalism associated with HIV infection and AIDS thereby enhancing uptake of VCT. Maintaining millions of people on treatment throughout their lifetimes is not sustainable and hence the importance of prevention strategies needs not to be over-emphasised.

1.3.6. HIV/AIDS & Legislation

In Zimbabwe discrimination of HIV positive people is prohibited under National HIV and AIDS Policy of 2000 and the Statutory Instrument (SI 202) of 1998 which prohibits HIV screening for purposes of employment. The country has not been able to fund its response to HIV/AIDS through domestic and international sources of finance. It was against the

background that the government of Zimbabwe used the Presidential Powers (Temporary) Regulations to declare HIV/AIDS a national disaster. This consequently legally empowered Zimbabwe to manufacture antiretroviral generic drugs locally. Criminal Law (Codification and Reform) Act 23 of 2004 is an extraordinary piece of legislation which makes it a crime for a person who knows that he or she has HIV to infect another, even between husband and wife. Some authors' summaries it all by saying "that such a law creates a crime not of effect and consequence, but of fear and possibility" [103]. They go on to argue that enacting of HIV-specific laws to criminally punish transmission of, exposure to, or non-disclosure of HIV, is counter-active to good public health conceptions and unacceptable to elementary human rights principles.

1.3.7 Mitigation Strategies

As part of the nation's attempts to raise funds for the control and management of HIV/AIDS the Government of Zimbabwe introduced the National AIDS Trust Fund (also called AIDS Levy) which entails collection of 3% of all taxable individuals and corporates incomes to fund HIV/AIDS programmes. There has been introduction and integration of family planning with HIV/STI and maternal health services voluntary counseling and testing (VCT), prevention of mother-to-child transmission (PMTCT) including primary care to identify the infected individuals with the intention of preventing both horizontal and vertical transmissions. Widowhood has been shown to play an important role in the transmission since it has been associated with 8–17% of all HIV cases [104]. As such family structures of traditional intra-and intergenerational coping mechanisms such as the levirate, whereby a widow is re-married to a close family member of the deceased husband are now discouraged [105]. Since 2009,

Zimbabwe has made available circumcision procedure to adult and adolescent men through a there has been collaborative effort between the government and technical agencies with the aim to reach 1.2 million 15–29 year-olds by 2015 for male circumcision [106;107]. The steady HIV-1 prevalence decline is also attributed to several factors such as behaviour change, condom use or high mortality rate of the infected [108-110]. Hopefully it continues to fall. The severe economic decline in the last decade has played a considerable role in sexual behavior change, particularly partner reduction especially amongst urban men [34]. With less disposable income during the economic meltdown many men were not able to purchase sex or sustain multiple sexual relationships [110;111]. Decline could also be due to the early adoption of a home-based care policy by the Zimbabwean government's which could inadvertently have fast-tracked the process of behavior change. It has been hypothesized that, when AIDS patients die at home, a situation where family members and friends have direct confrontation with AIDS mortality is more likely to instill fear of contracting the infection unlike a situation where such patients are cared for in health institutions [112]. The epidemic in Zimbabwe is also believed to be declining as result of the impact of the prevention programmes such as Prevention of Mother to Child Transmission (PMTCT). Mother to child transmission (MTCT) of HIV is a huge problem in Zimbabwe which has become the major cause of infant and child mortality [118].

1.4.0. Pregnancy, HIV and PMTCT in Zimbabwe

1.4.1. HIV and Pregnancy Disease Burden and Trends

Besides the DHS much of the information on national HIV prevalence in Zimbabwe is derived from surveillance of pregnant women attending ANC. In such generalized epidemics, pregnant constitute an easily accessible population which is generally representative of the

general sexually active population [119]. Routine sentinel surveillance of pregnant women attending ANC commenced in 1990. It has provided the estimated HIV prevalence rates for the adult population. In some border towns sentinel sites the HIV-1 prevalence among pregnant women has been alarming, compared to the national average **Figure 1.10.**

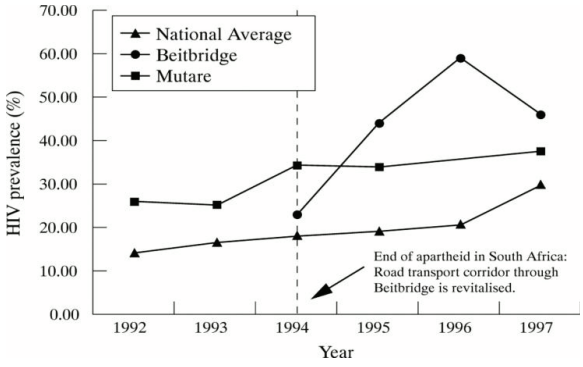


Figure 1.10: HIV prevalence among pregnant women in some border town sentinel sites **[120].**

In Harare, the capital city, the picture was different with incidence and prevalence peaking around year 2000, **Figure 1.11**. A large study apparently spanning the peak of the HIV-1 epidemic among reproductive women in Harare that recruited over 14 000 pregnant women reported HIV-1 prevalence from 0% among the 14-year-olds to over 45% among women aged 29–31 years, falling to 20% among the >40 years age group, with an alarming overall prevalence of 32% [122;123]. On a positive note, HIV prevalence among women attending ANC declined from around 32% in 2000 to about 13% in 2011 [118;122;124-130].

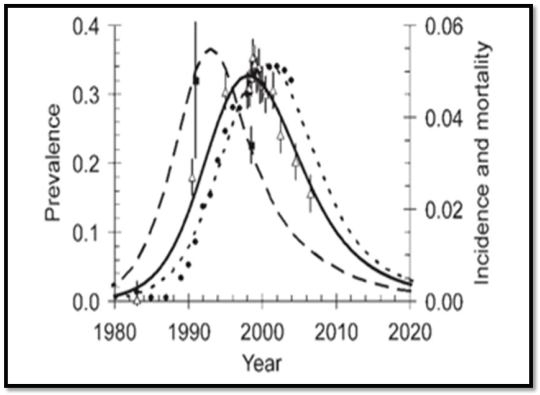


Figure 1.11: Estimated and fitted curves, HIV incidence(----), prevalence ( ) and deaths (....) among women attending antenatal and maternal clinic in Harare [121]

1.4.2. Mother-to-Child Transmission (MTCT) of HIV

Despite the high HIV-1 prevalence in the general populace which translates to high vertical transmission rates, the desire to have future pregnancies among HIV-1 positive mothers has increased from 3% to more than 55% over the years, more so with the advent of HIV-1 PMTCT initiatives [114;131;132]. Annual births stand at about 379000 with more recent neonatal and infant mortality rates of 36/1000 and 56/1000, respectively [133-135]. Out of these 47,494 pregnant women are HIV infected resulting in about 17,370 new pediatric HIV infections annually [135]. MTCT of HIV is the most significant source of HIV infection in children below the age of 15 years [118]. In the absence of ART, MTCT of HIV-1 can occur during pregnancy, intra-partum or postpartum through breastfeeding with risks of 10%, 25% or 40%, respectively [136]. Comprehensive PMTCT services based on single dose Nevirapine (SdNVP) to reduce mother-to-child transmission (MTCT) was initiated in 1999. It was only after December 2008 that the country started rolling out multiple dose PMTCT regimens [137]. Between 1980 and 2005, among 10 million children born in Zimbabwe, a cumulative 504,000 were vertically infected with HIV [138]. As of 2010 it is estimated that about 120000 children

between the ages of 0-15 are living with HIV/AIDS of which 3.4% of children aged 10 years are long-term survivors of MTCT [139]. ART has proved effective in reducing rates of MTCT of HIV-1 to very low levels not only in resource-rich countries but also in some resource-limited settings [140;141] as shown in **Figure 1.12**.

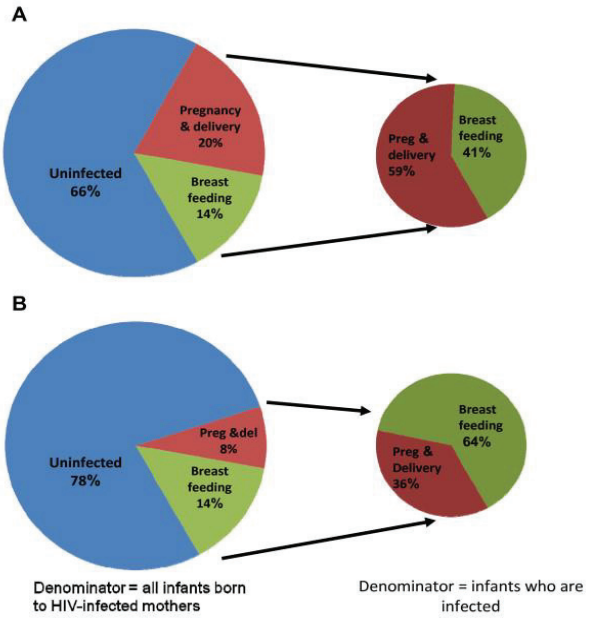


Figure 1.12: Transmission rates and proportions of infections. Panel A without interventions and Panel B with short course antiretroviral interventions provided [142]

1.4.3. PMTCT Practices in Zimbabwe

The goal of PMTCT in Zimbabwe is to reduce PMTCT of HIV infection, thereby leading to reduction of infant morbidity and mortality. PMTCT practices are carried out during the antenatal period, labour and delivery and post-natal period including after hospital/clinic discharge as summarised in **Figure 1.13** below.

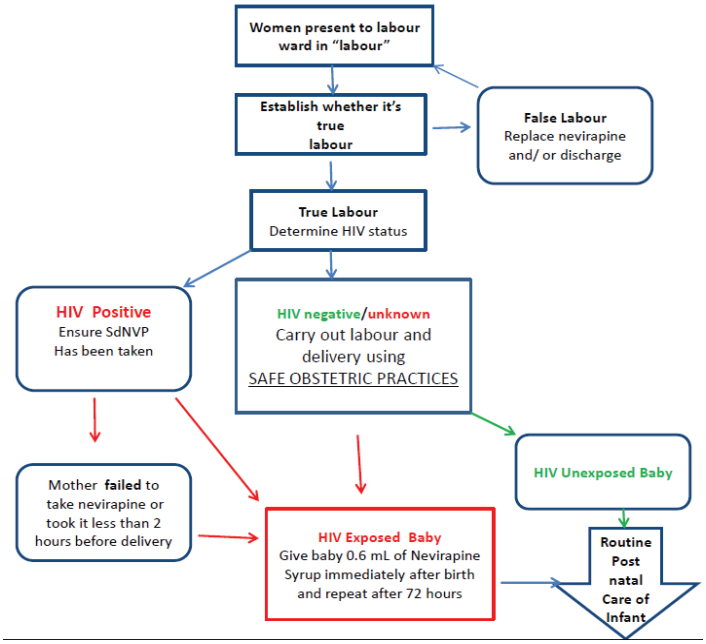


Figure 1.13: Summary of PMTCT practices during labour and delivery including postnatal during the time of the study.

Sadly, most women go for pregnant registration when their pregnancies are at advanced stages, a situation which put their unborn babies at risk. Consequently, the MTCT transmission rate remains high [143;144]. Studies have shown that if effective antiretroviral drugs are not provided, abstinence from breastfeeding or early weaning may result in no benefit for HIV-free survival in resource poor settings [145;146]**.** Exclusive breast feeding and provision of extended prophylactic HAART to the infant have been the practical option effective in prevention of transmission in such settings [147], **Figure 1.14.** This option is still to be implemented in Zimbabwe.

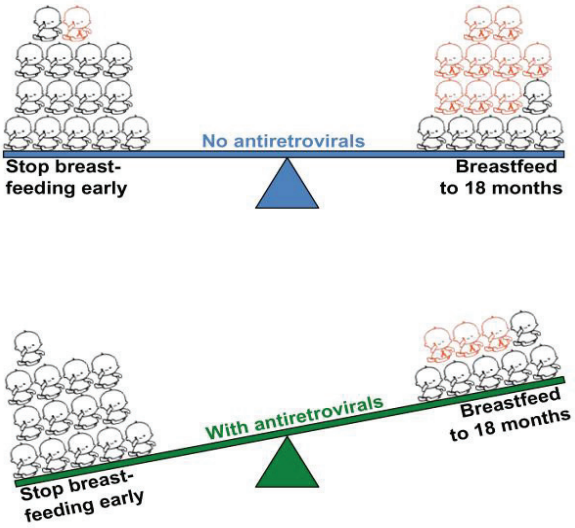


Figure 1.14: Balancing adverse outcomes in breastfed and non-breastfed infants. In red colour are the infected infants [145].

Promotion of exclusive breastfeeding within the first 6 months of life has become the cornerstone of child survival programs in Zimbabwe regardless of the infant's or mother's HIV status. Despite encouraging exclusive breast feeding for infants under 6 months of age only about 6% of the mothers strictly follow this instruction [78]. Early HIV infant diagnosis using the HIV DNA PCR testing was introduced at National Medical Reference Laboratory in 2008 [148].

1.4.4. PMTCT Coverage in Zimbabwe

Coverage (50%) and acceptance (42%) have been relatively low resulting in a relatively slow decline in MTCT rates [149;150]. Lately most HIV/AIDS services have been decentralised to clinics thus improving coverage and access to services in both urban and rural settings. As a

result comprehensive PMTCT sites increased from 710 in December 2007 to 920 in December 2008 and up to 960 in 2009. Provider Initiated Testing and Counseling (PITC) initiative has resulted in a further dramatic increase in HIV testing. This has been achieved through training large numbers of healthcare workers accompanied by more health care facilities providing testing and counseling services. The country has seen a general increase in uptake of PMTCT by pregnant women, although, worrisomely has been the vast gap of uptake of such services between mothers and infants as depicted in **Figure 1.15**. With continued efforts to reach women with PMTCT services and renewed commitment to address gaps in ANC access national targets for PMTCT can be met. By year 2015, at least 85% of all HIV positive pregnant women are to receive antiretrovirals for PMTCT whilst all HIV-exposed infants are to have virologic testing within 6 weeks of life.

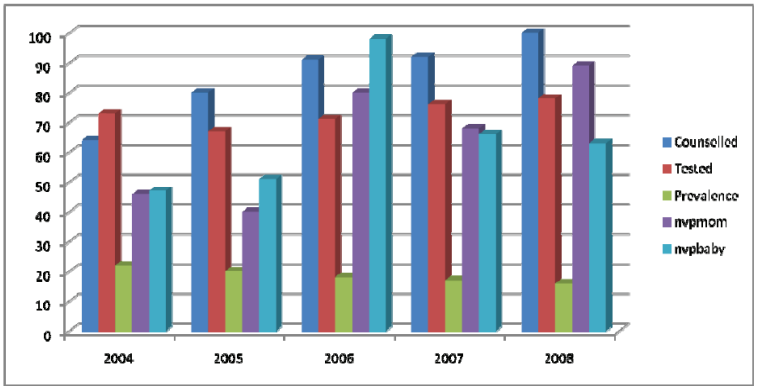


Figure 1.15: PMTCT program performance over 5 years; 2004-2008 [148].

1.4.5. PMTCT Impact and Challenges

There are claims that between 2002 and 2005, SdNVP PMTCT program have averted 4600 infections [138]. However, more infections would have been averted if more efficacious regimens could have been used. More efficacious regimens for PMTCT will start at 14 weeks

gestation including extended use of nevirapine for infants. It is sad to note that most of the sites are still offering SdNVP with very few sites offering more efficacious regiments. Brain drain causing high staff attrition rates has been counter-productive as there is constant need to train and re-train. Moreover, there is limited access to lab services especially disease monitoring tests such as CD4 testing and PCR for viral load testing as they are beyond the reach of many. Most HIV/AIDS patients on ART in Zimbabwe also generally use traditional herbal remedies to supplement treatment [115-117]. There is a gap in knowledge on the safety of concurrent use of the traditional herbal remedies and antiretroviral drugs.

1.4.6 Risk Factors for Vertical Transmission

Cognizance that about 70% of the HIV-1 exposed infants remain unaffected even in the absence of antiretroviral prophylaxis means that other factors also come into play in PMTCT. Genetic variations in HIV-1 co-receptors and determinants of immunity have been shown to influence the outcome of MTCT of HIV-1 [151;152]. HIV-1 variants that result in either increased CCR5 expression or a non-functional receptor (32 base-pair deletion variant) have been shown to influence the risk of vertical transmission [153;154]. Genetic determinants of innate immunity such as the toll-like receptor-9 and mannose-binding protein have also been shown to affect the risk of MTCT [155;156]. Discordance at the human leukocyte antigen (HLA) class I loci between mother and child have been shown to protect against MTCT [157]. Dendritic cell–specific ICAM-3 grabbing-non-integrin (DC-SIGN, encoded by CD209) is a C-type lectin that binds to many pathogens including HIV-1 [158] resulting in viral capture. Studies have reported significant associations between DC-SIGN genetic variants that modulate DC-SIGN expression in placental macrophages and increased risk of MTCT [159]. High maternal viral loads in serum or breast milk and low CD4 cell counts as well as other obstetric factors such

as prolonged membrane rupture, preterm and vaginal deliveries have been correlated with increased risk of MTCT of HIV-1 [152;160;161]. Previous reports have shown that treating women with low plasma CD4 count reduces postnatal HIV transmission, **Figure 1.16**

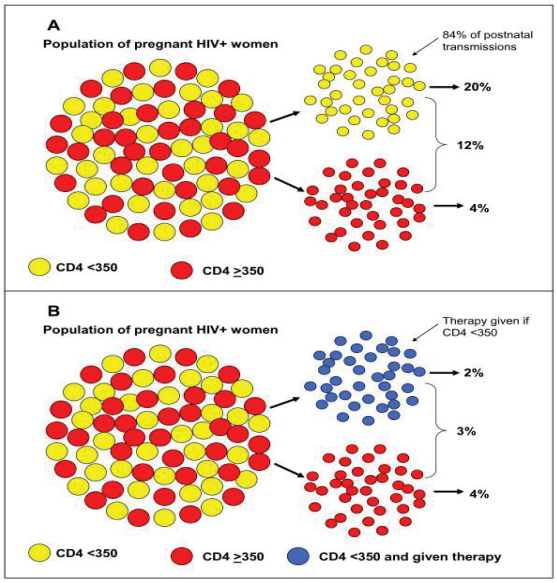


Figure 1.16: Postnatal transmission rates and maternal immunity with and without intervention. Panel A: Without intervention postnatal HIV transmission rates in the population are an average of low rates among women with high CD4 counts and high rates among women with low CD4 counts. Panel B: With ARTs given to women with low CD4 counts, the postnatal HIV transmission rate in this group, and in the overall population, declines to low levels [162].

HIV diversity has been shown to play a pivotal role in transmission as some variants have been shown to be more transmissible than others. Consequently, the efficacy of regimens of PMTCT administered only at labour may not be as protective in different geographical regions with different subtypes. The control of MTCT requires not only a deep understanding of MTCT of HIV but also the interplay between host-viral factors.

CHAPTER 2

2.0 Introduction

2.1 HIV Structure and Gene Organisation

HIV-1 is a member of the genus *Lentivirus* within the family of *Retroviridae* [163]. The term *Lentivirus* is derived from the Latin word *lentus*, meaning slow thus relating to the slow nature of the course of disease caused by these viruses. The HIV virion has a diameter of about 100 nm [164]. Host derived lipid bi-layer acquired during budding envelopes the virus. [165]. The genome is approximately 9 kb consisting of 9 genes encoding 15 different proteins [166]. Two copies of single stranded RNA genome are packaged in the virus particle alongside with enzymes as shown in **Figure 2.1**.

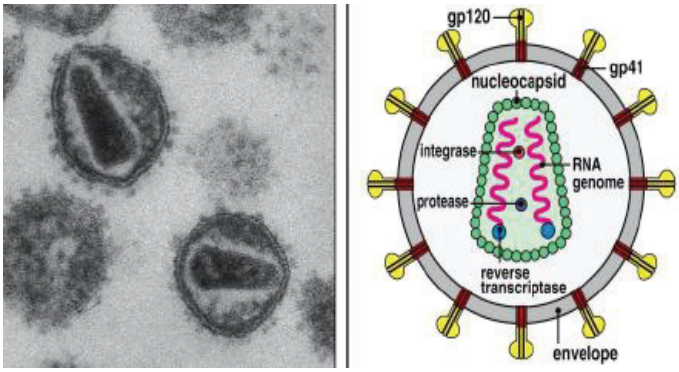
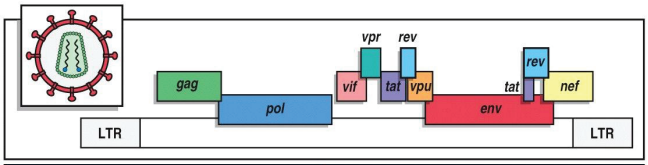


Figure 2.1: HIV Structure Adopted from Reference [167]

HIV genes can be either structural or regulatory. Structural genes are *pol gag* and *env* as shown in **Figure 2.2** below. *Pol* gene products include enzymes reverse transcriptase (RT) (p66), integrase (p32) and protease (p10) [168]. RT has strand–switching activity but lacks proof reading mechanisms causing mutations [169]. Integrase facilitates incorporation of viral DNA

into host chromosomal DNA, whilst the protease cleaves the group specific antigen (gag) and pol protein precursors into their respective individual components [170;171] .



Gene		Gene product/function
gag	Group-specific antigen	Core proteins and matrix proteins
pol	Polymerase	Reverse transcriptase, protease, and integrase enzymes
env	Envelope	Transmembrane glycoproteins. gp120 binds CD4 and CCR5; gp41 is required for virus fusion and internalization
tat	Transactivator	Positive regulator of transcription
rev	Regulator of viral expression	Allows export of unspliced and partly spliced transcripts from nucleus
vif	Viral infectivity	Affects particle infectivity
vpr	Viral protein R	Transport of DNA to nucleus. Augments virion production. Cell cycle arrest
vpu	Viral protein U	Promotes intracellular degradation of CD4 and enhances release of virus from cell membrane
nef	Negative-regulation factor	Augments viral replication *in vivo* and *in vitro*. Decreases CD4, MHC class I and II expression

Figure 2.2: HIV-1 Gene Organisation, Adopted from [167]

Gag poly-protein is cleaved into four structural components namely; the matrix (p17), capsid (p24), nucleocapsid (p7) and p6 that are each involved in nuclear importation of HIV pre-integration, binding cyclophilin A, binding RNA and interaction with viral protein R (Vpr), respectively [172;173]. Accessory or regulatory genes modulate virus replication. These include (Vpr; p15), viral infectivity factor (Vif; p23), viral protein U (Vpu; p18), negative regulation factor (Nef; p24), transcriptional activator; (Tat; p14) and regulator of viral gene expression (Rev; p19) [174]. Roles of the accessory genes are summarised in **Figure 2.2**. Long terminal repeats (LTR) constitute the control regions that bind to host transcription factors whilst nuclear factor kappa beta (NFκβ) and nuclear factor of activated T lymphocytes (NFAT) are critical in the initiation of transcription [175;176].

2.1 1 HIV Envelope (env) Glycoprotein (gp)

HIV *env* gene encodes the viral gp 120 and gp41 which both recognize and bind to host cell surface receptors [177]. The gp120 is composed of relatively conserved constant (C) C1 to C5 and variable (V) V1 to V5 sub-regions [178]. HIV env is ranked one of the most heavily glycosylated proteins known in nature with potential N-linked glycans (PNGs) constituting over 55% of its molecular weight [179]. This extensive glycosylation is known to play a critical role in viral evasion of the host immune response by masking key neutralization epitopes such that the glycosylated env (glycan shield) is presented to the immune system as "self" [180]. The *env* gene displays considerable plasticity which enables it to change its three-dimensional configuration consequently allowing escape from antibody-mediated neutralization [181]. Changes in the number of *env* gp 120 PNGs and variable regions amino acid length polymorphisms have been associated with striking a balance between transmission competence and resistance to immune challenges [182]. Under natural host immune response or anti-retroviral therapy (ART) selection pressures, it is postulated that HIV-1 evolves towards a denser glycan shield [181;183]. Thus, shorter variants with fewer glycans are expected during earlier phases of infection whilst longer V1-V5 variants with more glycans evolve at later stages of HIV-1 infection [184-187].

2.1.2 Envelope (Env) Protein and HIV Cellular Entry

HIV-1 gp120 on the surface of the virion binds to the CD4 receptor on helper T cells, macrophages and dendritic cells, and either the α-chemokine receptor CXCR4 (T cell-tropic) or the β-chemokine receptor CCR5 co-receptor (macrophage-tropic) [188]. Based on chemokine co-receptor usage, HIV-1 can be classified as CCR5 (R5), CXCR4 (X4), or dual tropic

(R5X4) [189]. Most new infections are due to CCR5 HIV-1 variants [190]. CXCR4-tropic viruses generally appear during the late stages of infection and are associated with increased pathogenicity [191]. Gp120 V3 loop amino acid sequence is the critical genetic determinant of cellular co-receptors usage [192]. Mutations within this region have been linked to changes in viral co-receptor usage [193]. Interaction of env gp120 and CD4 surface molecules expressed on target cells results in conformational changes that exposes the co-receptor binding sites [194], as summarised in **Figure 2.3**.

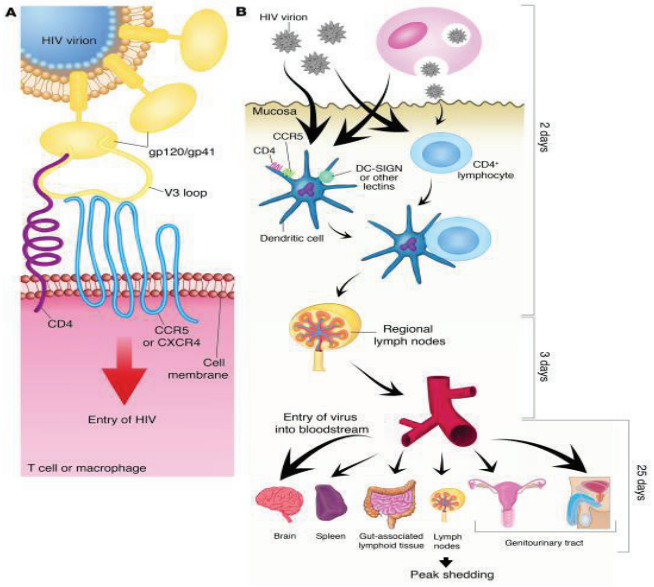


Figure 2.3: HIV Infection and Spreading [195].

HIV virions are bound by DCs through the C-type lectin receptor, dendritic cell-specific ICAM-grabbing non-integrin-related (DC-SIGNR) also known as CD209L thereby augmenting viral spread by carrying virus to activated T-lymphocytes [196;197]. Particles also bind B-lymphocytes through the complement receptor CD21 [198]. Virus or virus-infected cells ultimately reach the draining lymph nodes where by encounter activated CD4+CCR5+ T-

lymphocytes that propagate further infection [199]. The HIV promoter embedded in the 5′ long terminal repeats is able to engage the host's transcription machinery for viral gene expression [200;201]. **Figure 2.4** summarises the life cycle of HIV.

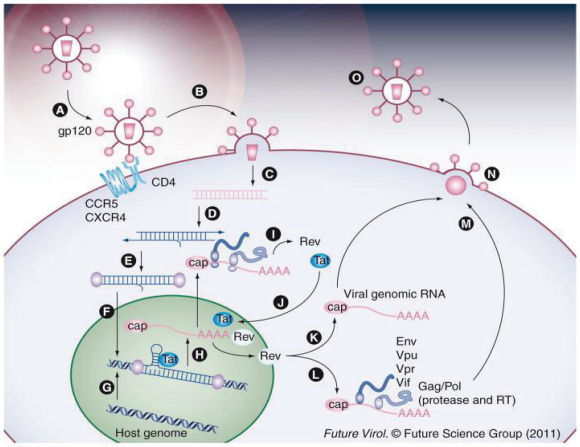


Figure 2.4: HIV life cycle Adopted from [202]. DNA is shown in blue while RNA is shown in pink. (A) Adsorption. (B) Fusion. (C) Uncoating. (D) Reverse transcription of the viral RNA genome into cDNA. (E) Pre-integration complex formation. (F) Nuclear import of pre-integration complex. (G) Integration of viral cDNA into the host. (H) Transcription of the proviral DNA. (I) Translation of Tat and Rev. (J) Import of Tat and Rev into the nucleus. (K) Rev facilitates the export of HIV-1 RNA genome for packaging. (L) Rev exports HIV-1 transcripts to the cytoplasm. (M) Assembly. (N) Budding. (O) Maturation.

Eventually HIV disseminate to secondary lymphoid tissue throughout the body with a particular predilection for gut associated lymphoid tissue (GALT) where activated CD4+CCR5+ effector memory T-lymphocytes are present in high numbers [203].

2.2.3 Susceptibility to HIV Infection

Why some individuals remain uninfected despite repeated sexual exposure to HIV-1 remains a mystery [204]. However, this observation offers a unique opportunity for studying host genetic factors conferring susceptibility to HIV infection and differential progression to AIDS. Among host genetic factors identified for their roles in HIV-1 acquisition and/or transmission include polymorphisms in the genes encoding chemokine receptors CCR5, CCR2, stromal derived factor 1 (SDF-1) and human leukocytes antigens (HLA) ligands [205;206]. Some alleles are protective [207;208] whilst others increase susceptibility to HIV acquisition [209-213]

2.2 Acute HIV Infection

Acute or primary HIV infection is defined as the first period of infection from the detection of plasma HIV RNA up until the formation of HIV-specific antibodies 3-4 weeks post infection [214]. Consequently plasma viral load increases exponentially reaching a peak 21-28 days post infection. See **Figure 2.5**. Acute HIV infection leads to depletion of CD4+ T-lymphocytes in GALT subsequently causing irreversible damage to the host immune system [215].

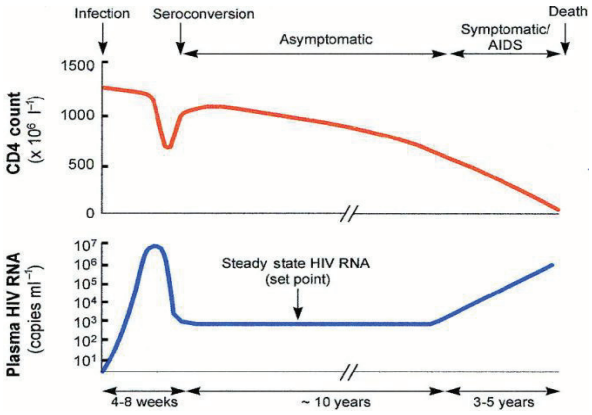


Figure 2.5: Natural History of HIV disease [216].

Viral reservoirs established during the primary infection facilitate HIV latency and persistence. More so, they have slower rate of decay relative to T-lymphocytes such that overall the virus cannot be eliminated even by highly active antiretroviral treatment (HAART) within the life time of the patient [217].

2.2.1 Signs, symptoms and Diagnosis

Acute infection is characterised by plasma HIV RNA levels of greater than 10,000 copies per milliliter (mL) [218;219]. Patient(s) may develop symptoms of the acute retroviral syndrome which include influenza-like illness with fever, sore throat, lymphadenopathy and exanthema [220]. Frequently observed hematological abnormalities include thrombocytopenia, anemia, leucopenia, lymphopenia and monocytosis [221]. CD4 counts are usually decreased and there is a reversal of the CD4:CD8 cell ratio [222]. Tools used to detect primary HIV infection in the absence of sero-conversion include p24 antigen assays and nucleic acid testing [223;224]. Assays for p24 antigen are widely available and relatively cheap. However, the test of choice is the polymerase chain reaction (PCR) for HIV-1 RNA which is rather expensive.

2.2.2 Viral Load and Set point

Eventually the viral load decreases over 12-20 weeks to reach a viral set point [225]. HIV set point is the viral load that stabilises after acute HIV infection which is maintained at a plateau during the asymptomatic phase [226]. Measurement of plasma HIV-1 RNA load is an important predictor of disease progression [227;228]. The higher the viral load of the set point the faster the patient will progress to AIDS whilst the lower the value the longer the patient will remain in

clinical latency. See **Figure 2.6** for the host and viral dynamics for typical, rapid and slow progressors including long term non-progressors.

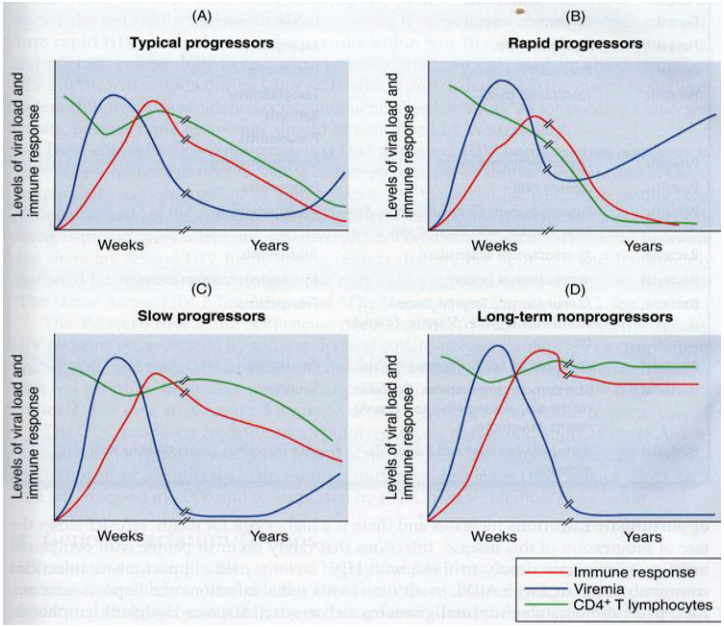


Figure 2.6: Viral and host dynamics and progression to AIDS adopted from [229]

Without antiretroviral therapy, the median time from initial infection to immune failure or AIDS amongst typical progressors is 7-10 years (75-80% of all patients) whilst the remaining patients experience either a more rapid progression to AIDS within 2-3 years (rapid progressors, (10-15%) or more extended periods without clinical disease progression >10 years, long term non-progressors, (5-10%) [230]. Interestingly, there is also a small unique subset of HIV sero-positive antiretroviral naive patients who remain aviremic with viral load below the detection limit of less than 50 HIV RNA copies/ml) for extended periods (elite suppressors (<1%) [230].

2.3 Control of Viremia

Primary HIV infection presents with a high HIV titre that is initially controlled by a CD8+ cytotoxic T-lymphocyte (CTL) response alongside with anti-HIV antibodies [231;232]. An effective cell mediated immune response is also characterised by increased numbers of natural killer cells and high cytokine levels such as INF-γ, TNFα and IL-1β [233]. Humoral response is initially effective but declines as the disease progresses with neutralizing antibodies tending to be weak and lacking broad cross-reactivity [234]. Despite the impediment of masking of env epitopes by the glycan shield, high HIV-1 genetic diversity is also a major challenge to antibody–mediated neutralization. Mutant viruses notoriously resistant to antibody neutralization are generated and archived in memory cells of viral reservoirs. HIV-1 diversity is one among several challenges that needs to be combated in attempts to design effective anti-HIV vaccines as generating broadly neutralizing antibodies that can efficiently inactivate or neutralize HIV variants remains elusive.

CTLs are a heterogeneous population of cells that vary in their antiviral efficacy [235]. They bear the CD8 molecule and are the major immunological mechanism in the control of viremia [236]. Non progression of HIV may associated with certain populations of HIV-specific CD8+ T-lymphocytes that display poly-functional characteristics and/or proliferative capacity that ensure maintenance of low plasma viral load. Up to 20% of circulating CD8+ T-lymphocytes can be HIV-specific in untreated chronically infected patients [237]. Activation of CTLs also results in the release of soluble antiviral factors which inhibit progeny viruses from entering target cells [238]. Such factors include the beta chemokines RANTES (regulated on activation normal T cell expressed and secreted), macrophage inflammatory protein 1 alpha and beta (MIP-1α and MIP-1β) which are all active against CCR5 viruses [239].

Mammalian cells harbor intrinsic cell-autonomous activities which can suppress viral replication, collectively called host restriction factors. Major classes of host restriction factors comprise, HLA alleles, killer-cell immunoglobin-like receptors (KIR), [240] apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) [241] proteins, tripartite motif-containing protein 5 alpha (TRIM5α) and tetherins [242-245] as shown in **Figure 2.7.**

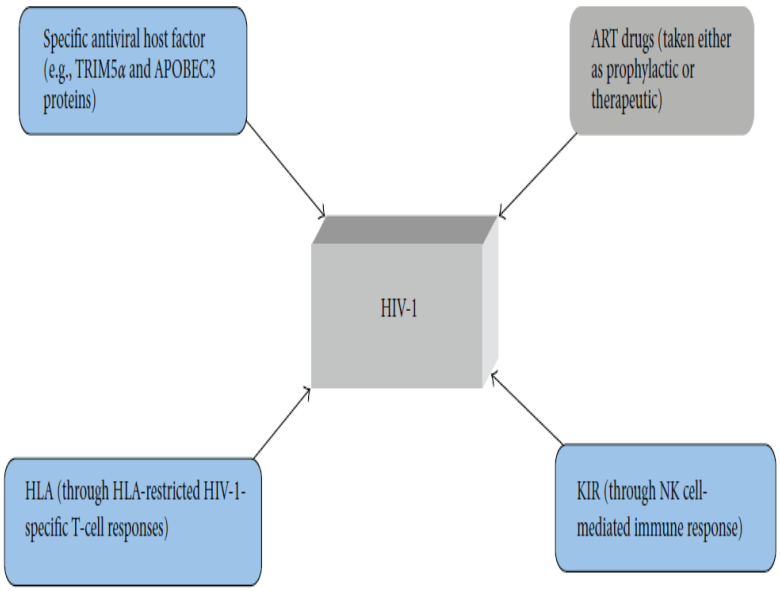


Figure 2.7: Host Restriction factors to HIVinfection. ART drugs block is shown in grey colour as it is not natural as drugs exert viral suppression only in patients undergoing therapy [246].

ART drugs are also used to control viremia and details will be discussed in **Section 2.4.5.** In the absence of therapy HIV-specific CD8+ T-lymphocytes cannot completely clear the infection and consequently a chronic infection develops.

2.4. Chronic HIV Infection

Chronic HIV-1 infection has been shown to be associated with persistent high level viral replication with half-life of plasma HIV RNA ranging from 0.5-6 hours such that half the plasma virus pool is replaced in thirty minutes [247]. Major part of the chronic phase of HIV-1 infection is a clinically asymptomatic period characterised by remarkably stable but subdued HIV-1 RNA load [248]. While acute HIV infection is characterized by widespread explosive infection and massive depletion of memory CD4+ T-lymphocytes, chronic HIV infection is associated with gradual loss of the remaining CD4+ T-lymphocytes alongside with insufficient replenishment of the lost cells and persistent immune activation [249;250].

2.4.1. Immune Activation

Chronic activation of the immune system is a hallmark of progressive HIV infection that better predicts disease outcome than plasma viral load [251;252;253]. Intriguingly, viral constituents such as gp 120, nef, including viral nucleic acids produced during viral replication including pro-inflammatory type 1 cytokines have been shown to be central players in the immune activation process [254;255]. A vicious cycle is established during which HIV-1 replication promotes immune activation and immune activation promotes HIV-1 replication [256]. Nonetheless, the extent of activation during the course of HIV-1 infection is such that the stimulation with viral antigens may not solely account for the complete phenomenon of immune activation observed [257]. The massive depletion of CD4+ T cells in the mucosal lymphoid tissues can result in the disruption of the different immune components that constitute the mucosal barrier in the gut thereby compromising its integrity resulting in

microbial translocation from the gut to the systemic immune system [258;259;260]. Translocation of bacterial products causes profound activation of the innate immune response involving lipopolysaccharide (LPS), flagellin and CpG DNA, which are all toll-like receptor (TLR) ligands known to directly stimulate peripheral macrophages and DCs to produce a wide range of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β [257]. Antigenic stimulation during HIV-1 infection may also be induced by some viruses, such as CMV and EBV as shown in **Figure 2.8**.

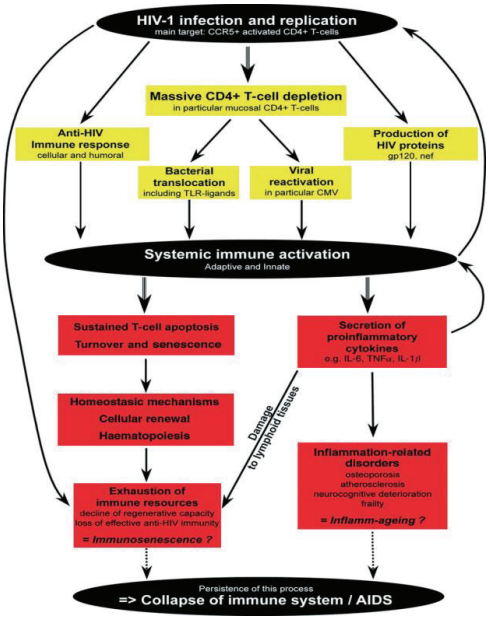


Figure 2.8: Causes and consequences of immune activation are in yellow or red, respectively. Consequences of immune activation that make a parallel with human ageing are in italic [257]

Activation leads to robust proliferation and acquisition of effector functions, accompanied by cell surface phenotype expression changes that reflect the activation such as CD38, HLA-DR and Ki67 on both the CD8+ and CD4+ T-lymphocytes [261]. Tenacious antigenic stimulation

and viral replication during chronic HIV infection may lead to immune exhaustion a phenomenon that has also been correlated with HIV disease progression.

2.4.2 Immune Exhaustion

Due to persistent viral replication and stimulation, HIV-specific CD8+ T-lymphocytes may be gradually driven towards an irreversible exhaustion of their replicative capacities and become worn-out cells [262]. The term "immune exhaustion" is defined by loss of proliferative capacity and diminished effector functions of memory T-lymphocytes as the disease progresses. Markers of immune exhaustion include programmed-death 1 (PD-1), T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) and lymphocyte activation gene-3 (LAG-3) [258]. Despite the high expression of PD-1 in exhausted T-lymphocytes during HIV infection, not all exhausted cells display PD-1, suggesting the role of other inhibitory molecules [263].

2.4.3 Acquired Immunodeficiency Syndrome (AIDS)

Overall, the immune system of HIV-1-infected individuals faces major challenges of coping with massive T cellular destruction at the same time trying to contain viral replication such that with time deterioration of the immune system is inevitable. The dropping of CD4 T-lymphocyte count to 200 cells/mL signals the onset of AIDS. AIDS is defined by the occurrence of infections associated with immune system deficiency called opportunistic infections. AIDS-Defining Conditions such as HIV-1 co-infections with cytomegalovirus (CMV), Herpesviruses, *Pneumocystis carinii*, *Mycobacterium avium*, *Toxoplasma gondii* and *Candida* among others have been shown to be associated with increased risk of death [264-267],

see **Figure 2.9.** At this stage the host's immune system is immuno-compromised to such an extent that without the initiation of HAART the infection progress to AIDS at rapid pace.

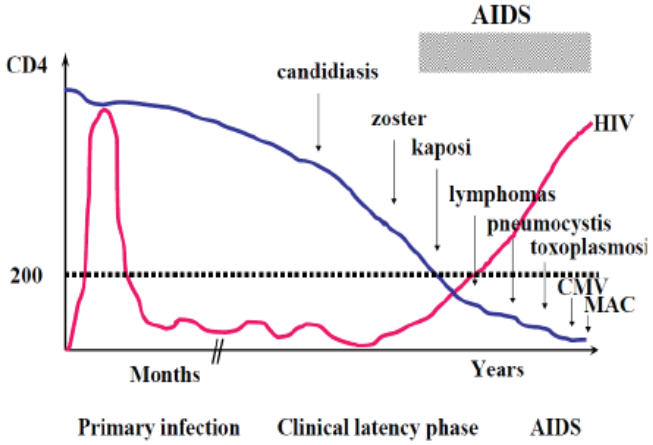


Figure 2.9: CD4 T-lymphocyte depletion and progression to AIDS, adopted from [268]

2.4.4. Highly active anti-retroviral therapy (HAART)

HAART refers to a combination of anti-HIV drugs directed at different stages of HIV life cycle and or host surface cell co-receptors. Antiretroviral drugs include entry, (non)-nucleoside/nucleotide reverse transcriptase, protease, integration and assembly inhibitors as shown in **Figure 2.10**. Therapy aims to suppress HIV replication, restore or preserve immune function and subsequently reducing HIV related morbidity or mortality with minimal toxicity. Thus, HAART essentially improves the quality of life of the infected individuals. Currently it is recommended to initiate therapy when the CD4 count is ≤350 cells/µL and also during pregnancy to prevent vertical transmission [269]. HIV RNA infected T-lymphocytes persist in the lymphoid tissues despite years of effective HAART even with undetectable viral load [270;271].

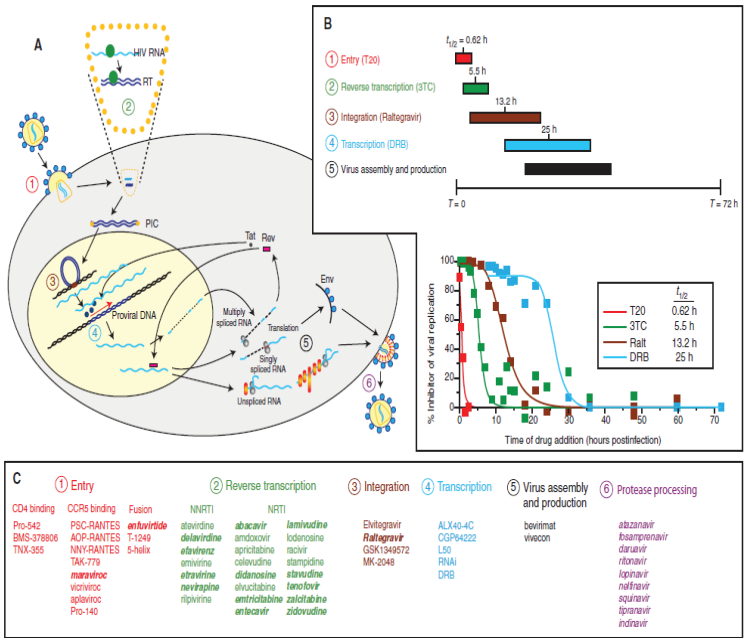


Figure 2.10: Potential and current targets for antiretroviral drugs in HIV-1 life cycle. FDA-approved (bold italic text), preclinical/abandoned (normal text) inhibitors [272].

Unfortunately HAART cannot eradicate the virus and unfortunately viremia can be re-activated following cessation of therapy [273]. Despite restoring immune function, prolonged use of HAART represents additional risk factors for the development of metabolic complications [274].

2.4.5. Immune Recovery Following HAART

Following successful HAART, there is a prompt and dramatic rise in circulating memory CD4+ T-lymphocytes attributed to reduction in apoptosis, redistribution of immune cells from lymphoid tissues, peripheral expansion of memory T-lymphocytes with limited T cell receptor

(TCR) diversity followed by thymic synthesis of naïve T-lymphocytes with broader TCR diversity alongside with restored thymic function [275;276]. Within 6 months following the initiation of HAART, T-lymphocyte reactivity to recall antigens is restored in most patients [277]. Function of CD4+ cells also improves post-HAART evidenced by restored proliferation and cytokine response to mitogens [278]. Whilst the amount of circulating T-lymphocytes subsequently returns close to normal, CD4+ T-lymphocyte numbers in the GALT remain severely reduced even after HAART [279]. Restoration of cells may not occur if the patient is depleted of circulating naïve T-lymphocytes prior to therapy. It remains debatable whether starting HAART in acute HIV infection preserves or restores GALT CD4+ T-lymphocyte number and function. There has been significant decline in the incidence of Kaposi sarcoma, non-Hodgkin lymphomas and cervical cancers since the advent of HAART suggesting that it may be necessary to treat earlier in order to prevent some of these AIDS related tumors [280-282].

2.4.6. Immune Reconstitution Inflammatory Syndrome (IRIS) of HIV

IRIS refers to the adverse clinical manifestation that occurs in HIV-infected individuals successfully treated with HAART. It is characterised by a paradoxical deterioration of clinical status despite the remarkable improvement in $CD4^+$ T-lymphocyte counts [283;284]. IRIS occurs in the first few months of HAART and is also characterized by a markedly pro-inflammatory response to a number of pathogens that commonly cause AIDS-associated opportunistic infections [285]. This inflammatory response is known as either “unmasking” or “paradoxical,” depending on whether the provoking opportunistic infection is previously undiagnosed or whether an already known infection worsens following treatment, respectively [286]. Factors

affecting response to HAART include host's ethnicity, nutritional status, presence of other co-infections, pre-HAART CD4 count, genetics as well as HIV genetic diversity [246;275;287;288].

2.4.7. HAART Induced HIV Mutations

With increasing numbers of people on ART, there is also an increased probability of development of HIV drug resistance mutations due to drugs selection pressures [289;290]. Such mutations may also be present in drug-naïve patients but at lower frequencies [291]. Shortages of drugs in some resource limited settings may exacerbate development of such mutations [292]. The importance of setting up an efficient and effective HIV drug resistance surveillance infrastructure to track and combat the emergence of HIV variants resistant to multiple antiretrovirals may not be over-emphasised. Despite these drug-induced mutations other intrinsic viral factors also contribute to HIV diversity.

2.5. HIV-1 genetic diversity

The hallmark of HIV-1 is its extensive genetic diversity [293-296]. Diversification is due to errors encountered during viral replication including host immune response selection pressures. Diversity is manifested as sequence variability particularly within the env V regions [297] Variability not only makes it difficult for the immune system to identify the virus but it also facilitates the rapid viral immune escape. High level of genetic diversity has important implications in screening, diagnostic testing, disease monitoring and treatment outcome [224;298-305]. Questions have been raised on whether diversity may also affect viral transmissibility and pathogenicity [186;306-310]. Sadly, genetic diversity has been the major impediment in the

effective vaccine design and development since the human immune response is HIV strain-specific [311]. Four factors *vis-a-viz*, the infidelity of RT, recombination, superinfection and high replication rate of the virus contribute to the development of the extensive HIV genetic variation [312;313].

2.5.1. Properties of Reverse Transcriptase (RT) Enzyme and Recombination

The infidelity of HIV RT enzyme confers mutations at an approximate rate of one error per genome per replication cycle [314]. RT also accounts for genomic heterogeneity in progeny viruses through its role in recombination. Genetic recombination is an evolutionary strategy for survival in a changing environment for viral variants with superior fitness at an average of $1.38 \times 10{-}4$ recombination events/adjacent sites/generation *in vivo* [315]. It occurs when an individual is co-infected with at least two different HIV strains that are multiplying in the same cell [316;317]. It is caused by high selection pressure from either the natural host immune response or ART drugs [318]. Recombinants between highly similar HIV-1 strains are formed at highest frequencies while recombination between distant HIV-1 strains occur at very low frequencies [319]. Infections with dual or even triple HIV-1 variants have been reported [320;321]. HIV superinfections allow a mechanism for genetic recombinants between distant variants [322-327]. Superinfection and co-infection which both involve re-infection by at least two genetically distinct viral variants differ based on whether the second infection is contracted prior to or after the primary host immune response has been mounted [328]. These are associated with high viral loads and accelerated rates of disease progression [329;330]. HIV-1 superinfection presents an additional concern to the already challenging problem of HIV-1 vaccine design in the face of the virus's rapid evolution [331].

2.5.2. High Turnover Rates of HIV-1 *in vivo*

HIV-1 virions are produced and cleared at an extremely rapid pace. Since the HIV-1 genome is about 10^3 base pairs in length, then the baseline rate of viral production is approximately 10^{10} virions per day [247]. This rapid turnover has been considered the major factor underlying the pathogenesis of HIV/AIDS alongside with the destruction of CD4+ T-helper lymphocytes [247]. Besides the viral RT, host RNA polymerase II makes minimal contributions to retroviral frame shift mutations [332]. Diversity may also be enhanced by different genetic factors, including HLA in patients from different regions of the world. Viral genetic factors include proteins such as Tat, Vif and Rev that interact with human genetic factors such as APOBEC, langerin, tetherin and CCR5 and HLA B27, B57, DRB1*1303, KIR and PARD3B [333]. The inability of Vif to counteract host APOBEC3 proteins lead to the deamination of cytidine to uridine consequently, causing viral guanosine to adenosine hypermutations [334]. Some error causing mechanisms contributing to HIV-1 variations are shown in **Figure 2.11**.

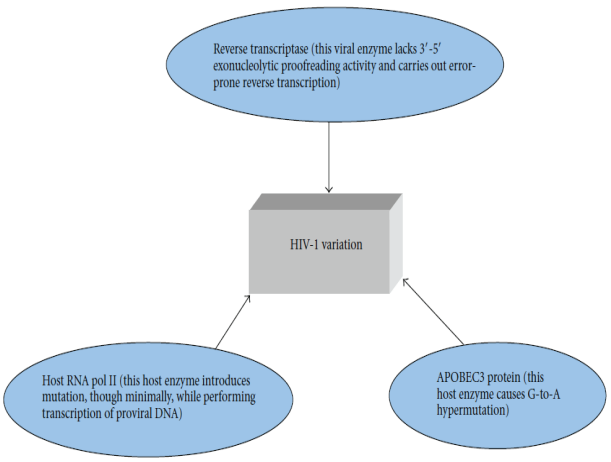


Figure 2.11: A schematic sketch of error-causing machinery causing HIV-1 genetic diversity [246].

Genetic variation helps the virus evade the immune system and consequently this viral heterogeneity allows for a quick adaptation to the human immune system, antiretroviral drugs, or both leading to viral fitness/positive selection in the face of pharmacologic or immunologic selection pressures [335]. Everyday millions of genetic variants accumulate in latently infected cells only to be re-activated at some time in the future [336]. Thus, the extensive diversity of HIV resulting in a myriad of HIV variants has necessitated the need for its classification. This taxonomy facilitates better utilization of the ever growing viral sequence database through comparison with previously published works.

2.6. Classification of HIV

HIV-1 strains are not randomly distributed across the globe but they display a distinctive geographical distribution [337]. Prior to 1992, HIV-1 strains were classified into two main classes on the basis of their respective geographical origin being then, the North American and African variants [338]. Thus, HIV variation is highest among viruses from different geographical locations, higher among isolates from different individuals within the same location. However, variants present as relatively similar quasi-species within the same individual [339;340]. A quasi-species is a cloud or swarm of genetically diverse variants that are linked through mutations that interact cooperatively on a functional level and collectively contributing to the characteristics of the viral population [341].

With the advent of phylogenetic analysis the *env* gene has revealed the existence of multiple phylogenetic clusters that were used in the compilation of the 1992 HIV classification

compendium based on viral sequence similarities [342;343]. As the env sequence database increased over the years the *gag* and *pol* gene sequences were also incorporated in the classification process consequently identifying HIV types, groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs) [296;344-356], as summarised in **Figure 2.12**.

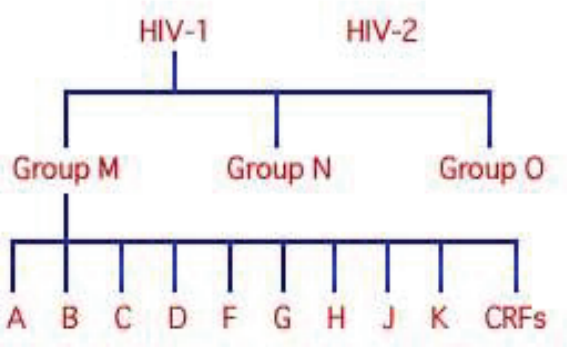


Figure 2.12: Summary of HIV Classification [357]

2.6.1. HIV types

Phylogenetically HIV can be classified into two types; type 1 (HIV-1) and type 2 (HIV-2) [353]. Both viral types cause AIDS. HIV-1 is the first in the class of human retroviruses and accounts for most of the world's HIV infections. Its origin can be traced back to a Simian Immunodeficiency Virus (SIV) isolated from a Chimpanzee (cpz) sub-species, *Pan troglodytes troglodytes* (SIVcpz) cross species transmission to humans [15;358;359]. HIV-2 is the second in the same class of human retroviruses but is largely confined to West Africa. The primate reservoir of HIV-2 is sooty mangabey, *Cercocebus atys* (green monkey) [360;361]. Thus, (SIVCPZ) is closely related to HIV-1, while SIV from sooty mangabey (SIVSM) is closest to

HIV-2 [362]. HIV-1 and HIV-2 are closely related viruses with nucleotide sequence homology of 58%, 59% and 39% in the *gag, pol* and *env* genes, respectively [363]. Despite similar modes of transmission, HIV-2 is not as efficient in transmission horizontally and vertically [353;364;365]. Relative to HIV-1, HIV-2 has a reduced rate of disease development and has shown natural resistance to readily available NNRTIs [366]. Genetic recombination between HIV types-1 and-2 has been reported [367]. Distinction of HIV types is not only essential for accurate surveillance or diagnosis purposes but is also critical for correct administration of appropriate antiretroviral therapies.

2.6.2. HIV Groups

Phylogenetic analysis of HIV-1 suggested that zoonosis occurred on at least three independent cross species transmission events from chimpanzee, *Pan troglodytes* (*pts*), *Pan troglodytes troglodytes* (*ptts*) or gorilla (*gor*) resulting in three main distinct HIV groups called the major (**M**), outlier (**O**) or non–M/non–O (**N**) as shown in **Figure 2.13**. Studies have estimated the timing for the zoonosis of each lineage of groups M, O, and N at around 1931, 1920 and 1963, respectively [368]. Group M is responsible for more than 90% of the world HIV infections [369]. Interestingly, the genetic analysis of sequences from clinical materials obtained from members of a Norwegian family infected much earlier than 1971 showed that they carried viruses of the group O, mainly restricted to West Africa [8]. HIV groups have genetic sequence differences of >40% in some coding regions [370;371]. More recently, a new putative group, designated P, was reported in France from a Cameroonian female immigrant [372]. Group P viral sequences have been shown to form a distinct HIV-1 lineage with SIV sequences from western gorillas (SIVgor; *Gorilla gorilla gorilla*), suggesting that group P originated from

gorillas [373]. Reports have indicated that HIV-1 group P infections are rare, accounting for only 0.06% of HIV infections in Cameroon [374]. Unlike groups O, N and P, group M has been classified into subtypes.

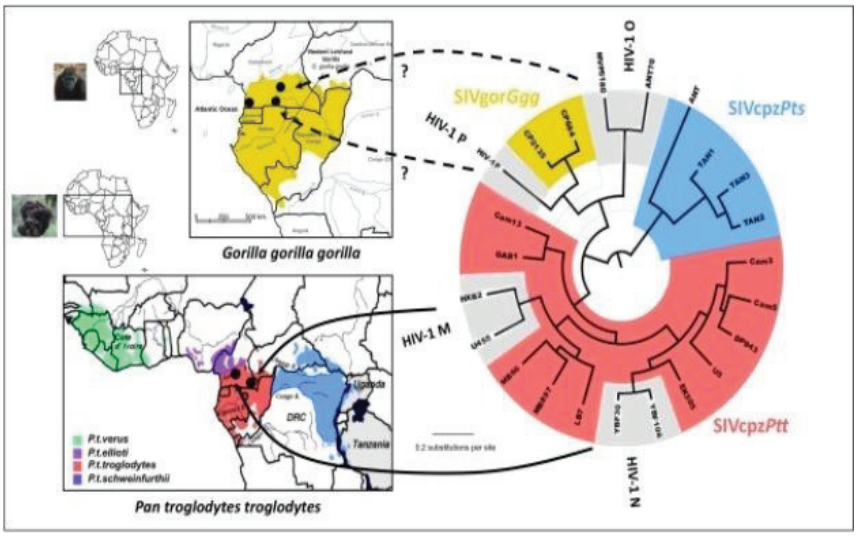


Figure 2.13: Evolutionary relationships of HIV groups**.** SIVcpzPts (blue), SIVcpzPtt (red), SIVgor (yellow), and HIV-1 group M, N, O, and P (gray) strains based on partial env (gp41) sequences. Arrows indicate the ape reservoirs of the different HIV groups. Dotted arrows indicate that the direct reservoirs for HIV-1 groups O and P remain elusive [375].

2.6.3. HIV-1 subtypes

Subtypes are phylogenetically linked strains of HIV-1 that are approximately the same genetic distance from one another. Group M has been classified into nine distinct subtypes, also called clades or genotypes, denoted with letters, A, B, C, D, F, G, H, J and K, thus making the development of effective blanket diagnostic and monitoring tests or vaccine a challenge [370;376]. Inter-subtype variation is about 30% with respect to the *env* gene sequence and 15% for both the *gag* and *pol* genes sequences [377]. Different risk groups for HIV infection are

associated with certain subtypes with IDUs including the gay communities and heterosexual population generally acquiring subtype B and non-B subtypes, respectively [378-380].

2.6.4. HIV-1 Sub-Subtypes

Within each subtype numerous HIV-1 variants exist that exhibit minor intra subtype genetic diversity of within 10% called sub-subtypes [381]. These are distinctive HIV-1 lineages that are closely related to a particular subtype lineage, but are not genetically distant enough to justify calling them new subtypes. Sub-subtypes are denoted by numerals for instance in the case for subtype A these have been named A1, A2 or A3 [382]. Recent studies have demonstrated the need for HIV classification using full-length genomic sequences if new distinctive subtypes are to be accurately identified rather than relying on sequencing of different viral gene fragments as has been the standard.

2.6.5. HIV recombinants

Full genome sequencing of HIV has resulted in the discovery of circulating and unique recombinant forms (CRFs) and URFs, respectively. Recombinants are unique in the sense that they may be described in isolated individuals without any evidence of epidemic spread. To be classified as a CRF, a virus strain must be detected in at least three epidemiologically unlinked individuals and must be capable of establishing an epidemic on its own. Thus, these mosaic HIV-1 strains reflecting a mixture of subtypes circulating in different populations may have altered pathogenic and/or transmissibility properties [383]. CRFs are referred to by their number that is assigned according to the order of their discovery, underscore and the

respective subtypes involved for example CRF02_AG or by their number(s) followed by the letters `cpx' (for complex), when more than two subtypes are involved for example CRF04_cpx or CRF06_cpx [384]. One of the most prevalent group M CRF common in Southeast Asia was earlier on incorrectly designated subtype E but was later correctly re-named CRF01_AE following full HIV genome sequencing [385]. To date more than 21 CRFs and several URFs have been described [386]. All CRFs together account for 18% of the world's HIV-1 infections [387]. HIV-1 subtypes and recombinants may differ with respect to plasma viral load levels [388] transcriptional activation levels, disease progression and response to chemotherapy including drug induced/natural resistance patterns [389-392].

2.7. Distribution of HIV-1 Subtypes and Recombinants

Over 50 different subtypes and CRFs have been described [291;393]. Subtype B is geographically confined to North America, Western Europe and Australia. See **Figure 2.14**. Paradoxically subtype B is quite rare in Africa, the purported origin of HIV. Global proportions of HIV-1 subtypes and recombinants have shown that subtype C accounts for more than 50% of world's infections followed by 12%, 10%, 6% and 3% for subtypes A, B,G and D respectively, whilst subtypes F, H, J and K together accounted for about 0.94% of all the infections [377]. CRF01_AE and CRF02_AG are each responsible for 5% of the global infections while CRF03_AB is responsible for 0.1% with the other recombinants responsible for the remaining 8% of the infections [377].

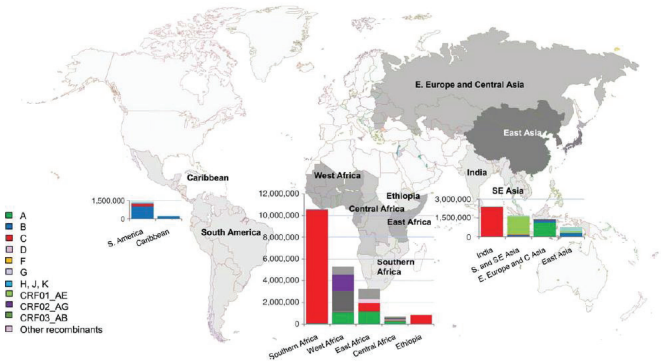


Figure 2.14: Global distribution of HIV-1 subtypes and recombinants [394]

In most Southern African nations subtype C predominate, contributing 93-100% of the HIV-1 infections amongst individual countries [377;395]. Interestingly, the greatest diversity of subtypes and recombinants is present in Central Africa, Central African Republic, Gabon, Angola and Chad harboring only about 5% of the world's infected individuals [395]. Thus, a general observation is that higher diversity of subtypes is associated with relatively slower epidemics whilst explosive epidemics generally have only one predominant subtype.

2.7.1. Subtypes Trends and Distribution in Zimbabwe

Previous Zimbabwean studies in the 1990s and early 2000 have observed a predominant HIV-1 subtype C infection [396-398]. The origins and evolutionary history of HIV-1 subtype C in Zimbabwe with respect to the *pol* sequence data sets generated from four sequential cohorts of antenatal women in Harare, from 1991–2006 has demonstrated increasing sequence divergence over the 15-year period. This data also dated the most recent common HIV ancestor to be around 1973 with three epidemic growth phases: an initial slow phase (1970s)

followed by exponential growth (1980s), and a linearly expanding epidemic to the present day [99]. However, current HIV subtype(s) distribution in Zimbabwe remain elusive in view of the influence of the population movements in the past decade as result of the economic meltdown which could have facilitated subtype inter mixing. Generally, subtype specific variations may exist that influence differential transmissibility in different regions [399-401].

2.7.2. HIV Diversity, Transmission and Disease Progression

Following sexual transmission of HIV the virus initially replicates locally in the vaginal or rectal mucosa [402]. Genetic diversity of HIV is lost during horizontal transmission as the virus gradually evolves towards a common ancestral sequence once in the new host [306;403]. Newly infected individuals acquire a subset of the viruses that would be circulating in the transmitting partner [404]. Studies have correlated high HIV replication capacity with increased transmission rates [405]. Understanding the quantitative relationship between plasma HIV-1 RNA and HIV-1 transmission risk has been the cornerstone for ART preventive interventions that strive to reduce plasma HIV-1 levels that in turn reduce the risk of HIV-1 transmission [406]. Interestingly, Langerhans cells have shown minimal susceptibility to infection with subtype B virus but has demonstrated substantially greater sensitivity to infections by subtype C viruses [407] **.** In the Rakai, Ugandan study, subtype A viruses have been shown to have a significantly higher rate of heterosexual transmission relative to subtype D viruses. [408]. Differential subtype transmission efficiency may be important for HIV vaccine evaluation especially for the subtype-specific HIV epidemic in SSA. HIV-1-discordant couples are increasingly viewed as a valuable source of participants for HIV vaccine and prevention trials [57]. Interestingly, HIV-1 subtype C has been found to be the predominant subtype in sero-

discordant couples followed by subtypes B and A, respectively [409]. Increasing HIV-1 replication efficiency has also been related to a concomitant increase in HIV-1 diversity, which in turn has been the determining factor in disease progression [410;411]. Non-A subtype infections have been shown to progress to AIDS faster than those infected with subtype A [412]. More so subtype D has been associated with the most rapid disease progression relative to subtypes A, C and CRFs [413;414;415].

Pregnancy has been shown to increase the risk of female-to-male HIV-1 transmission by two folds [416]. Pregnant women infected with subtype C shed significantly more HIV-1-infected vaginal cells than those infected with subtypes A or D [417]. Increased HIV-1 shedding has been correlated with a more complex population of HIV-1 quasi-species in the genital tracts of parturient women, which may increase the probability of transmission of fetotropic viral strains [418].

2.7.3. HIV Diversity and vertical transmission

Maternal neutralizing antibody response with broad specificity and low viral load may protect the child from HIV-1 infection [419;420]. Factors associated with an increased risk of perinatal HIV transmission include advanced maternal disease, prolonged duration of ruptured membranes and increased quantity of HIV in maternal blood at delivery [421]. Maternal DC-SIGNR expressed at the maternal-fetal interface play a crucial role in MTCT of HIV-1 as impaired placental DC-SIGNR expression has been shown to increase the risk of transmission [422]. Presence or absence of some PNGs at specific sites on the gp120 env has also been associated with increased HIV-1 MTCT [423]. Transmission can be in uterine, intra-partum or

postpartum mainly through breast milk [424;425]. Different factors may influence HIV transmission during each of these time periods, and hence interventions strategies to reduce transmission need to be period specific. The probability of HIV-1 infection per liter of breast milk ingested by an infant has been shown to be similar in magnitude to the probability of heterosexual transmission of HIV-1 per unprotected sex act in adults [426]**.** Infants may be infected with the most prevalent maternal strain, a minor maternal variant or multiple maternal quasi-species [427;428]. *In utero* transmitters have been shown to be more likely to transmit single or multiple maternal viral variants whilst intrapartum transmitters are more likely to transmit minor HIV-1 variants [429]. After transmission HIV-1 infected infants harbour either homogenous or heterogeneous virus populations [430;431]. HIV-1 inter-subtype recombinants may also be effectively transmitted vertically to infants [432]**.**

SdNVP given to mothers before delivery and to newborns after delivery can reduce the risk of transmission by 10-15% [433] although this can select for nevirapine resistant variants which decrease with time, but remain above pre-dose levels [434]. The rate of nevirapine resistance mutations after SdNVP has been shown to be significantly higher in women with HIV-1 subtype C than in women with subtype A or D [435]. This observation has a bearing on the efficacy of subsequent antiretroviral therapy containing nevirapine or other NNRTIs [436]. Efficiency of MTCT of HIV may be among those properties that vary with HIV diversity. A few and controversial results have been described so far with respect to subtype and vertical transmission. Subtype C has been shown to be more transmissible than other subtypes [417;437]. However, other studies have shown no apparent differences in the rate of MTCT of HIV-1 among women with different subtypes [438-440]. As a result the efficacy of regimens of PMTCT administered only at labour may not be as protective in different geographical regions with

different subtypes. In vertical transmission, a closely related maternal and infant HIV-1 diversity is suggestive of late pregnancy or perinatal transmission. On the other hand, wide genetic differences are suggestive of an earlier transmission during pregnancy [441]. Subtype specific identification of such patterns of MTCT points may be useful in the PMTCT programmes. V3 region of the *env* gene is a key determinant of MTCT [442;443]. There is need for further research in HIV-1 diversity and transmission patterns to achieve remarkable reduction of MTCT rates especially for developing countries. Subtype and CRFs determination is generally done using the *gag/env* heteroduplex mobility assay (HMA) originally developed by Delwart [444] which was later modified by Heyndrick [445]*et al*, in the year 2000. Sequencing remains the gold standard although partial sequencing also gives good results at reasonable cost.

2.8. Rationale of the study

The geographic distribution of subtypes is subject to constant change. Recombinant forms of the virus will continue to appear as long as the different subtypes of HIV-1 continue to circulate between continents and recombination continues to occur. With the world fast becoming a global village new HIV strains are emerging in areas where they were originally non-existent. Thus importation and exportation of new types, subtypes and even CRFs of HIV is possible. Due to political and socio-economic challenges most Zimbabweans have resorted to cross border trading within the region and abroad. Furthermore the ZDF personnel have been actively involved in peace keeping mission all over the world. The risky behaviour of military personnel plus high HIV-1 sero-prevalence within this group may have facilitated the introduction of new HIV types, subtypes or recombinants within the armed forces themselves and to the general population both at home and abroad. There is a paucity of data on the

current HIV diversity in Zimbabwe. Tracking the presence of new HIV strains is important for surveillance purposes, effective chemotherapy, diagnosis and disease monitoring including vaccine design and development. Identifying the specific genetics characteristics of successfully transmitted variants is also paramount in the development of an effective vaccine.

2.9. Hypothesis

There are no new types, subtypes and CRFs in the population and no biological and genetic differences exist between HIV-1 subtypes

2.10. Aim of the study

The main goal of this study to determine and characterise genetic diversity of HIV among pregnant women and their infants in Harare peri-urban and ascertain its role in diagnosis, disease monitoring and transmission

2.11. Objectives

To investigate:

1. Antenatal plasma viral load and its role in vertical transmission
2. HIV types and or co-infections among Zimbabwean pregnant women
3. The distribution of HIV-1 subtypes and CRFs among women and their infants
4. HIV viral co-receptor usage genotype of the mothers and their infants
5. HIV subtype C env gp 120 glycosylation patterns and diversity following horizontal and vertical transmission
6. The role of HIV-1 gp120 env PNGs variations and sequence length polymorphism following transmission events

CHAPTER 3

3.0. Materials and Methods

3.1. Study Population and Design

This was a nested case-control study within a PMTCT cohort of ART naive pregnant women and their infants. A case was a sero-positive mother who transmitted HIV-1 to her infant (transmitter). All transmitting mothers were included. The transmitter was matched to one HIV-1 positive but non-transmitting mother (control). Noteworthy is that antiretroviral drugs were not readily available in Zimbabwe at the time of recruitment of study participants. HIV positive women who consented for themselves and their infants were eligible to participate. Mothers who were too ill were excluded.

3.2. Study Sites

Harare peri-urban antenatal clinics approximately 20 kilometres from the city centre namely Epworth, Seke North and Saint Mary's Chitungwiza were the study sites. See site map, **Figure 1.3**. Residents living in these communities are generally of low socio-economic status, without piped water supplies or if the piped water infrastructure is available, supplies are erratic. Most of the adult population is formally unemployed making them informally employed.

3.3. Sampling and Procedures

Women were sampled from a bigger cohort of 691 pregnant women. Matching of cases and controls was done with respect to maternal age, educational level, marital and socio-economic status, parity, current STIs, the date of last menstruation, and uptake of SdNVP prophylaxis. SdNVP prophylaxis was offered to all HIV-1 positive mothers during labour and their infants within 72 hours post-delivery. All mothers answered a structured questionnaire at enrolment from which information regarding their socio-demographics, sexual behavior, obstetric and reproductive health issues was obtained. A gynecologist performed physical and gynecological examinations.

Study participants were classified into of two groups as shown in **Figure 3.1**. The main group comprised of pregnant women who were HIV-1 positive at enrolment, referred to as having chronic HIV-1 infections and a minor group of women who were HIV-1 negative during pregnancy but sero-converted after delivery during the follow-up period, regarded as having acute HIV-1 infections. Follow-up of women was from delivery, six weeks, four and nine months and thereafter three monthly until two years generally coinciding with infant immunization visits. At each subsequent follow-up visit, previously HIV-1 negative mothers and infants were re-tested for HIV-1 antibodies and HIV-1 DNA, respectively. A pediatrician examined infants. Date of birth, birth weight, gender, SdNVP therapy and breastfeeding patterns for each infant were recorded. Infant deaths were also documented during the follow-up period. Mothers were encouraged to exclusively breastfeed during the first six months following delivery. HIV-1 positive mothers were followed up again in cases of subsequent pregnancies and similar procedures were followed. Since this was initially an STI cohort, four of mothers' spouses also consented to participate in the HIV diversity study. Plain and EDTA

blood samples were collected from the mothers at each follow up visit. Samples were processed, aliquoted and appropriately stored until testing. Concurrently, infants' venous EDTA whole blood samples were collected and stored at -86°C until testing.

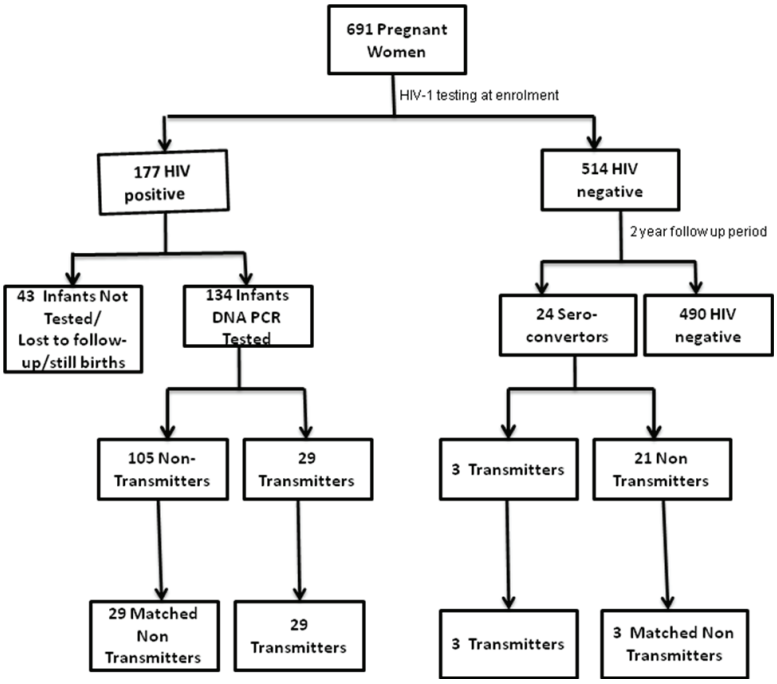


Figure 3.1: Summary of enrolment procedures of the 32 cases and 32 controls

3.4.0. HIV Testing

3.4.1. Mothers' HIV-1/HIV-2 Screening

Better sensitivity and specificity of almost 100% are achieved using an algorithm combining two tests for HIV antibodies, a practice currently considered the best option for low-resource settings[446]. Serial HIV-1/2 algorithm antibody tests were done using Determine (Abbott Diagnostics, Illinois USA) and OraQuick (Abbott Diagnostics, Illinois, USA) rapid kits on serum samples.

3.4.1. Determine T.M. Test

Abbott Determine TM HIV-1/2 kit is a qualitative immuno-assay that is used to visually detect antibodies to HIV-1 and or HIV-2 in human plasma, serum or whole blood. The test is based on the immuno-chromatography principle. Briefly, the sample is added on to the sample pad, flows up to mix with the selenium colloid antigen conjugate in the conjugate pad and finally forms a red line on the patient window site on the solid phase of immobilized HIV-1/2 recombinant antigens and synthetic peptides as shown on **Figure 3.2**.

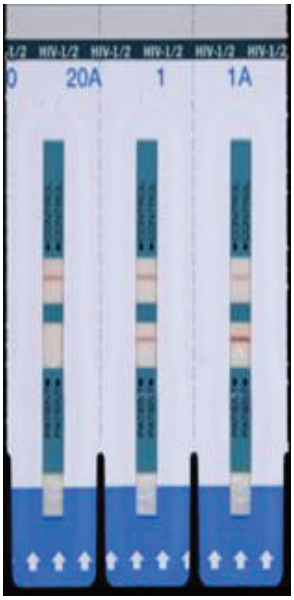


Figure 3.2: The Determine HIV-1/2 Test Strip**.** The white arrows point to the direction of the sample flow from sample pad. First strip from the left shows non-reactive sample without a red line on the patient's window. The second and third strips are both reactive regardless of differences in red lines intensities.

Thus, the second HIV test used in our study was Oraquick.

3.4.2. OraQuick HIV-1/2 Antibody Test

OraQuick® ADVANCE Rapid HIV-1/2 Antibody Test is also a qualitative immunoassay that detect viral antibodies in oral fluid, fingerprick whole blood, venipuncture whole blood and plasma specimens. Comprised of a single-use test device and a vial containing buffered developer solution, the test is based on a lateral flow immuno-assay technique. The test strip contains synthetic peptides from the HIV *env* region and a goat anti-human IgG procedural control immobilized onto a nitrocellulose membrane in theTest (T) zone and the Control (C) zone, respectively.

Figure 3.3: OraQuick Test Kit. The first and second strips showing a reactive and non-reactive samples, respectively.

The developer solution facilitates the flow of the specimen into the device, rehydrates the protein-A gold colorimetric reagent contained in the device, continues migrating up until it reaches the T zone where the presence of HIV-1/-2 antibodies to the immobilized antigens on the nitrocellulose membrane causes the appearance of a reddish-purple line. With this new technology of rapid testing, the turn around time has been reduced to about twenty to thirty minutes, rather than waiting for weeks for the results as has been the case with traditional testing methods. However, for both the rapid tests the intensity of the test line does not

necessarily correlate with HIV antibody titre in the sample. Due to the antibody testing limitations regarding the window period all HIV-negative patients were scheduled for re-testing after three months.

3.4.2. Mothers' HIV-1/HIV-2 Western Blot Testing

Western blot (WB) is another HIV testing technique that provides additional information not readily gathered from rapid test such as the immuno-dominant proteins [447-449]. WB test continues to be of value in confirming results from antibody tests. HIV proteins used in western blotting can be produced by recombinant DNA through a technique called recombinant immunoblot *assay* where viral proteins are separated and transferred on a nitrocellulose strip. Diluted serum is applied to the membrane and if antibodies are present in the serum they bind to some of the HIV antigens. Antibodies that do not bind to viral antigen(s) are washed away. Enzyme-linked antibodies with the capability to attach to the patient's antibodies determine to which HIV antigens the person has antibodies to.

Figure 3.4 Serodia WB Testing kits used.

Confirmation of HIV-1/2 rapid test results was done at the Norwegian Institute of Public Health using the WB test (HIV blot 2.2, MP Diagnostics, Singapore) according to the

manufacturer's instructions, kit shown in **Figure 3.4**. Interpretation of the WB test results was done in line with the World Health Organization (WHO) guidelines [450]. Though very specific WB test is rather expensive and there is no universal criterion for interpreting the test results.

3.5 Determination of Total Lymphocyte Counts (TLC)

EDTA-anti-coagulated venous blood samples were processed within six hours for full blood counts using Abbott Diagnostic Cell Dyne 3500R SL Hematology Analyser. TLC was enumerated as the total white blood cell count multiplied by the lymphocyte percentage. In this resource poor setting, TLC was used as a surrogate marker for CD4 cell count since by then, the capacity to determine the latter was not readily available at the University of Zimbabwe due to prohibitive costs [451]. TLC of 1200 cells/mm^3 was the threshold value used equivalent to a CD4 count of 200 cells/mm^3 [452;453].

3.6. CD4 cell counts enumeration

A CD4 count has been a useful marker used to monitor immune system function and disease progression in HIV-positive individuals. $CD4^+$ T lymphocytes were enumerated using a Partec Cyflow counter (Cyflow, Partec, Munster, Germany) within 6 hours of blood collection as previously described [454]. This test was done only on a few family samples.

3.7. HIV-1 RNA Load Determination

Serum and plasma samples were shipped on dry ice to the Institute of Microbiology at the University of Oslo in Norway for further laboratory analysis. Maternal baseline serum samples were quantified for HIV-1 RNA load using an automated TaqMan Roche Amplicor 1.5 Monitor Test (Cobas AmpliPrep/Cobas TaqMan, Roche Diagnostics, Branchburg NJ), according to the manufacturer's instructions. HIV-1 viral load testing is considered essential when initiating antiretroviral therapy ART, monitoring ART response, and when considering switching ART regimens.

3.8. Infants' Qualitative HIV-1 DNA PCR Test

Qualitative HIV-1 proviral DNA PCR tests have three main diagnostic applications. These include direct detection of viral sequences in the pre-seroconversion window period which may be positive up to 8 days prior to the development of HIV specific antibodies; resolution of indeterminate HIV serological tests and in the diagnosis of neonates born to seropositive mothers where maternal antibodies may be detectable for up to 15 months postpartum. Early detection of HIV-1 infection in infants is complicated by the persistence of maternal antibodies and by diverse HIV-1 subtypes [455]. Detection of infants' HIV-1 infection was determined using a qualitative 1.5 Roche Amplicor HIV-1 DNA PCR kit (Roche Diagnostics Incorporation, Branchburg, New Jersey). The test amplifies and detects several target sequences located in specific HIV genes, such as gag or pol [456] .Testing was done in the Obstetrics and Gynecology Department, Medical School, University of Zimbabwe. Infants that tested HIV-1 DNA PCR positive on whole blood collected within 10 days of birth were considered to be infected in utero. Infants having negative HIV-1 DNA PCR results within the first 10 days of life and positive HIV-1 results at six weeks postpartum and/or thereafter were

considered to be infected intra-partum/postpartum [144;457]. HIV antigen testing using such a nucleic-acid-based technology has shortened the window period between infection and detectability of disease. However, the issue of testing infant samples with respect to the volume of blood required remains a challenge as the volumes of sample required are often difficult to obtain from little infants [458] .

3.9. Nucleic acid extraction

Total RNA was extracted from plasma using the NucliSENS isolation kit**,** based on the Boom *et al.,* method [459]. Briefly samples were ruptured in a lysis buffer containing a chaotropic agent, guanidine thiocyanate. Cells, bacteria and viruses in the samples were lysed whilst proteins such as nucleases were denatured and inactivated. DNA and RNA bound to silica particles whilst everything else was washed following several washing steps with the wash buffer. Finally the nucleic acids were eluted from the silica particles using the elution buffer.

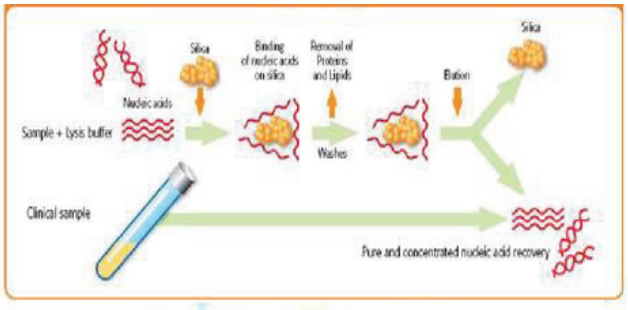


Figure 3.5: Boom Technology Principle**,** Adopted from [460]

The eluate was highly purified and concentrated enough for PCR amplification. It is a smart method where no columns are needed and different specimens can be used.

3.10. DNA amplification

3.10.1. *Taq* Polymerase

Taq polymerase is a thermostable DNA polymerase named after the hot spring *Thermus aquaticus* bacterium[461]. It is often abbreviated to "*Taq* Pol" or simply "Taq". Frequently used in PCR reaction for amplifying short segments of DNA, *Taq* is a heat stable enzyme able to withstand the protein-denaturing temperatures required during PCR [462]. It can replicate a 1000 base pair strand of DNA in less than 10 seconds at 72°C [463]. However, one of *Taq*'s drawbacks is its relatively low replication fidelity as it lacks the 3' to 5' exonuclease proofreading activity and consequently translating to an error rate of at about 1 in 9,000 nucleotides [464]

3.10.2. First Round: RT PCT

The viral RNA is extracted from the patient's plasma and was first converted to cDNA using RT. The PCR process is then applied, using two primers unique to the virus's env region and *Taq*. The primary PCR amplified an approximately 800-base pair (bp) fragment spanning the V3 and V4 region of the envelope (positions 6948–7537) on the HIV-HXB2 genome using HIV primers ENV 2 and NY3.

Table 3.1: RT PCR Reagent Preparations

Reagent	Volume/Sample (µL)
Buffer (5x)	10
dNTPs	2

NY3 Primer 1 (10 pmol/µL)	3
ENV primer 2 (10 pmol/µL)	3
Enzyme mix (RT and *Taq*)	2
RNAase (40U/µL)	0.5
Template (from boom extraction)	10
RNA free water	19.5
Total Volume	**50**

Table 3.2: Reverse transcription (RT) Thermal cycler cycles

Temperature (ºC)	**Time (minutes)**
50	30
95	15

Table 3.3: Amplification with *Taq* polymerase

Temperature (ºC)	**Time (seconds)**
94	15
55	30
72	60
	29 cycles in total
72	120
04	∞

3.10.3. DNA amplification Nested PCR

Secondary or nested PCR amplified a 535-bp env gene fragment.

Table 3.4: Nested PCR Reagent Preparations

Reagent	**Volume/Sample (µL)**
Ammonium Buffer (10x)	5

20mM dNTPs	2
JA168 primer 1 (10 pmol/µL)	2
ES 8 primer 2 (10 pmol/µL)	2
50mM $MgCl_2$	2
Taq Pol	0.5
Template (from RT PCR)	1
RNA free water	35.5
Total Volume	**50**

Table 3.5: Nested PCR cycles Programmed on the Thermal Cycler

Temperature (ºC)	**Time (seconds)**
95	60
94	20
55	30
72	60
	24 cycles in total
72	120
04	∞

3.11. Detections of Nested PCT Amplicons

Detection and quantification of secondary PCR amplicons were done using a 1% agarose gel electrophoresed together with a standard mass ladder and then stained with SYBR safe stain, **Figure 3.6a and b**.

Figure 3.6: Loading PCR Amplicons on a gel & Gene Doc Gel Reader **(Bio-Rad)**

3.12. Purification of Nested PCR amplicons

In preparation for sequencing excess primers and salt were removed using Microspin columns (Amersham Bioscience), **Figure 3.7.**

Figure 3.7: Microspin columns for purification of the extracted DNA.

3.13. Dye-Terminator Cycle-Sequencing

PCR products that produced the expected band sizes were sequenced together with negative controls for procedural quality assessment. Amplicons were diluted to a final concentration of 5–20ng of template DNA prior to sequencing. For each sample two reaction mixtures were

prepared with either the upstream or downstream primers in separate reaction tubes to facilitate both forward and reverse sequencing. Big dye-terminator cycle-sequencing technology was used where the purified template DNA was first subjected to PCR with Big dye which contains *Taq* DNA polymerase, dNTPs (deoxynucleotides) in excess concentration, ddNTPs (dideoxy nucleotides) with fluorescent dyes in low concentration. Dideoxy nucleotides are nucleotides that lack a 3'-OH group and these causes the termination of the extension process each time they are encountered during the PCR reaction.

Table 3.6: Big Dye PCR Reaction Mixture

Reagent	**Volume (µL)/Microplate well**
Template	1
Primer (ES8/ JA168)	1
RNAase free water	13
*Master Mix	5
Total Volume	**20**

***Components of Master Mix**

Reagent	**Volume (µL)**
Buffer	3.5
RNAase water	0.5
Big Dye	1.0
Total	**5.0**

Thus, the Big Dye contained dNTPs and ddNTPs in a mixture of approximately 100:1 such that each time the polymerase added a nucleotide, there was a small chance that it would have added a ddNTP. Consequently, repeating the PCR-cycle many times resulted in a population of fragments ranging from the length of the primer plus one nucleotide to the length of the

primer plus all nucleotides of the entire PCR product. Since these extension products were labeled with different fluorescent dyes depending on the base composition, the composition of the 3' nucleotide bases were identified by separating the fragments on a gel and scanning the fluorescence pattern with a UV laser that energized the dyes attached to each ddNTP. Each dideoxy nucleotide, ddCTP, ddATP, ddTTP, or ddGTP, had a different fluorescent dye attached to it with one unique color for each ddNTP. When exposed to a UV laser, the four different ddNTPs fluoresced at four different wavelengths which were detected and recorded by a sensor on the DNA sequencer, **Figure 3.8.** The outputs were chromatograms depicting the nucleotide sequence for both the forward and reverse primers. Contigs were constructed which were later aligned and analysed.

Figure 3.8: ABI 3730 DNA analyzer (Applied Biosystems/HITACHI, Tokyo, Japan).

3.14. TOPO Cloning

In the event that direct sequencing failed to yield clean chromatographs then cloning was done using an Invitrogen TOPO TA cloning kit version J, 2006. *Taq* pol has a non-template-dependent terminal transferase activity that such it makes adenine (A) overhangs at the 3' ends

of its products. The linearized vector supplied in the kit had overhanging 3′deoxythymidine (T) residues which allowed PCR inserts to ligate efficiently in the vector without ligase, post-PCR procedures nor requirement of PCR primers. Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand [465] and thus facilitating the ligation of purified nested PCR product into the pCR™2.1-TOPO vector as shown in **Figure 3.9.**

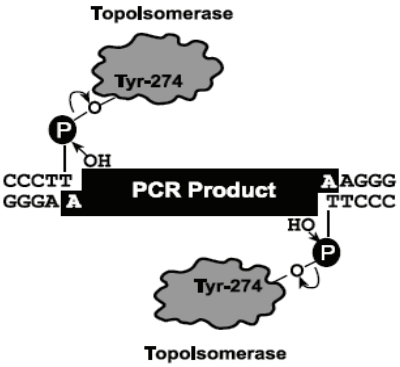


Figure 3.9: Ligaton of the PCR product into the TOPO vector using Topoisomerase Adopted from [466]**.**

The transformed the recombinant vector was taken up and expressed in chemically competent *E. coli* (C4040-03) as per the manufacturer's instructions [466]. Recovery and plating was done on Luria-Bertani (LB) plates with kanamycin and 5 bromo-4-chloro-3 indolyl-beta-D-galactopyranoside (x-gal) as the selective agents for the chemical transformation.

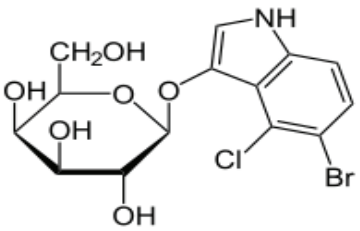


Figure 3.10: X-gal Structure

X-gal is an analog of lactose that was hydrolyzed by the β-galactosidase enzyme, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole which was further oxidised to 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble. See **Figure 3.11**. Thus, in the presence of X-gal and the artificial inducer of the Lac operon, isopropyl thiogalactoside (IPTG) in the agar medium, bacterial colonies with the functional Lac Z gene were blue. However, E coli transformed by the PCR inserts in the Lac Z open reading frame were not able to make β galactosidase enzyme and hence appeared as white colonies.

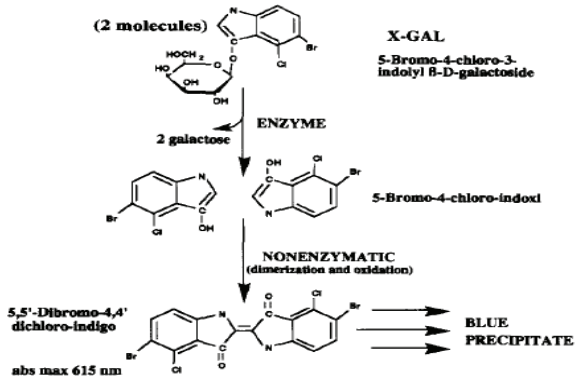


Figure 3.11: Formation of the insoluble blue product from X-gal**,** adopted from [467]**.**

Thus, in gene cloning X-gal is used to indicate whether or not there is expression of the β galactosidase enzyme which is encoded by the LacZ gene, a technique called "blue/white screening". For each sample four white colonies streaked separately on LB plates containing kanamycin and incubated over night at 37º C. Finally plasmid DNA isolation was done using the mini-preparation method and checked on a 1% agarose gel before sequencing.

3.15. Data analysis

The data were collected and analyzed using SPSS (version17.0, Chicago, IL). Viral load values were $\log_{10}$ transformed. The Student's t-test was used to compare means. Regression analysis was used to investigate the associations. Tests of statistical significance included the 95% confidence interval of unadjusted relative risks, two-sided p values based on Chi-square and Fisher's exact tests. Sequences contigs were assembled using the Vector NTI Advance 10 program. Alignment was attained using Gene Doc, BioEdit, and Clustal X2 sequence alignment programs including manual editing to ensure that deletions or insertions did not alter the reading frame. Prediction of co-receptor usage genotype was automatically generated on a sub-C Position-Specific Scoring Matrices (PSSM on website: http://mullinslab.microbiol.washington.edu/computing/pssm/6). HIV-1 subtype was determined using BioAfrica-Bioinformatics tool (version 2.0), website http://www.bioafrica.net/. MEGA version 5.0 was used in sequence phylogenetic analysis and tree drawings.

3.16. Ethical Issues

The study was approved by the Medical Research Council of Zimbabwe (MRCZ), reference number MRCZ/A/1407 and the Ethical Review Committee of Norway. Written consent to participate in the research study was obtained from the mothers and they were free to discontinue at any given time without any prejudice. Mothers also consented to have their blood samples and that of their infants used for future HIV related research. Participants were assured of confidentiality See participants consent form sample in both English and vernacular languages (Shona) in the Appendix section. All mother-infant pairs were offered free treatment for other ailments other than HIV and were re-imbursed their bus fare to and from the clinic.

CHAPTER 4

4.0. Some Experimental Results

4.1. First and Second Round PCR Amplicons ran on a 1% Agarose gel

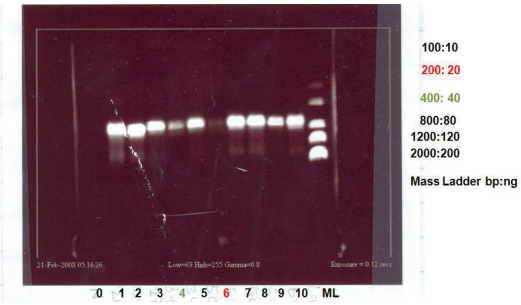


Figure 4.1: Gel Picture, Samples were in wells 1-10; ML denote molecular ladder in well number 11. On the right are the molecular weights for each ladder and the corresponding approximate DNA concentration of in nonagrams (ng). Gel was stained with sybr green.

4.2. A Typical Clean Chromatogram

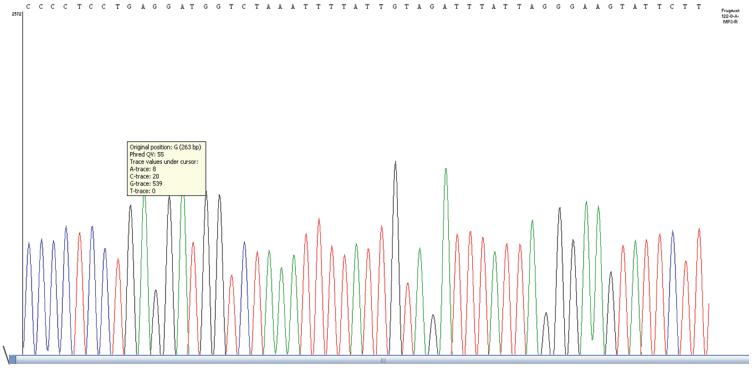


Figure 4.2: A Portion of a clean chromatogram from the Vector NTI program of the reverse primer viral sequence of Mother ID 122. Peaks in blue, red, black and green colours represent nucleotide cytosine (C), thymidine (T), guanosine and adenine, respectively. Size of the peak is directly proportional to concentration of the nucleotide(s).

4.3. Typical Raw Data from the DNA Analyser

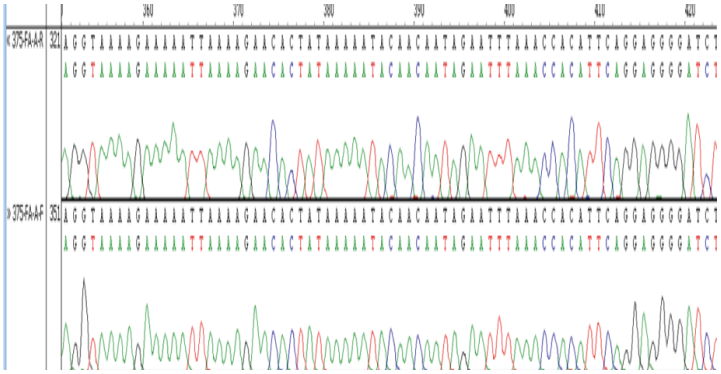


Figure 4.3: Typical Sequence Raw data for Father ID 375 showing sequences for both the forward (F) and reverse ® primer sequences

4.4. Mother-infant Nucleotide Sequence Alignment

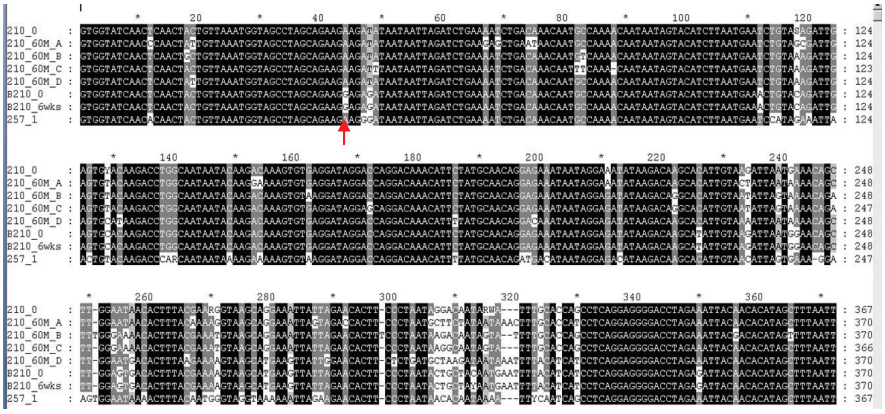


Figure 4.4: GeneDoc nucleotide alignments for mother-infant pair ID 210 and mother ID 257. The darker the shed the more the similar the nucleotide sequences. Region 306-333 looks like a hot spot for mutations. Red arrow shows all mother sequences had an adenine on position 44 whilst the baby sequences had a guanine on that same position**.**

4.5. Family 205 part Amino Acid Sequence Alignment

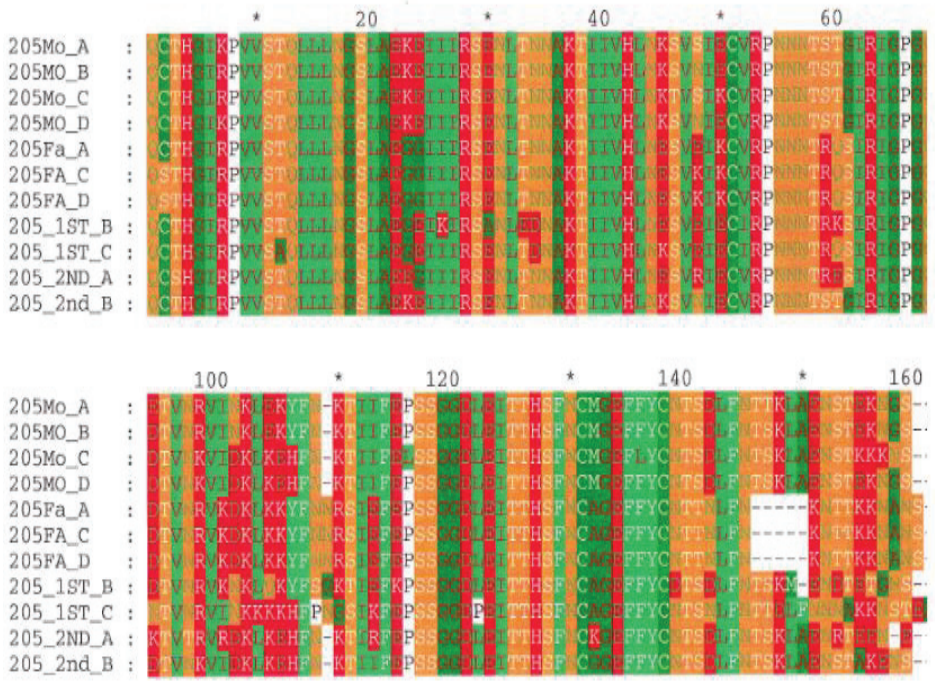


Figure 4:5: Amino acid Clustal X program alignment for the viral variants infecting family 205. FA, MO, 1ST and 2ND represent father, mother first sibling and second sibling viral sequences, respectively followed by a letter representing a clone. Coloured letters represent the amino acid. Amino acids with similar or related chemical properties are shown in the same shade of colour. Generally the viral variant are closely related. However, there are some mutations for example deletions in positions 111 for all mothers' and second siblings' viral DNA. All father's sequences have 4 amino acids deletions on positions from 146-150.

4.6. Phylogenetic Analysis 4 Family Sequences

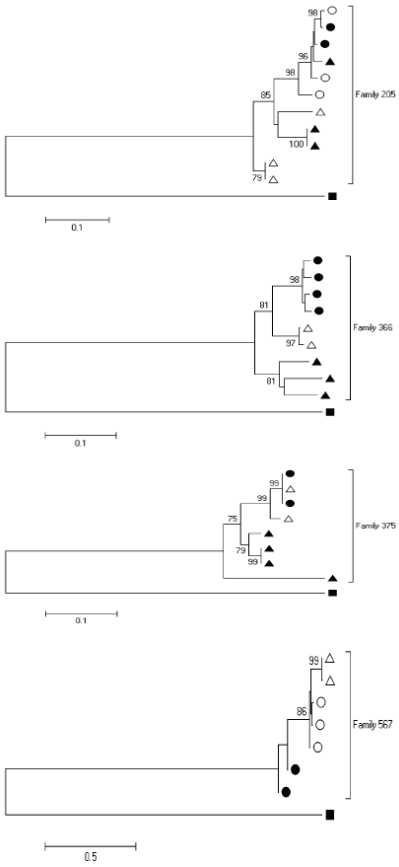


Figure 4.6: Rooted Neighbour joining tree for HIV-1 env (C2V5) sequences for the 4 families. Bootstrap values were expressed as percentages per 1000 replicates and only proportions of > 70% are shown as shaded triangles and circles represent fathers' and mothers' sequences, respectively. Open triangles and circles represent sequences of first and second siblings, respectively whilst filled in squares represent Outlier, HIV group O sequence.

4.7. Phylogenetic Analysis of Family sequences in relation to other subtype C sequences from different regions

Figure 4.7: Phylogenetic relationships between families' sequences and other subtype C from different geographical regions; AR: Argentina, BW: Botswana, ZA: South Africa, TZ: Tanzania, IN: India, ZW: Zimbabwe & CN: China. Bootstrap values expressed as percentages per 1000 replicates and only proportions of > 70% are shown. Generally the family subtype C viruses are closely related to other regionally subtype C as shown by the high boot strap values.

4.8. Unique HIV subtype C env V3 and C3 sequences

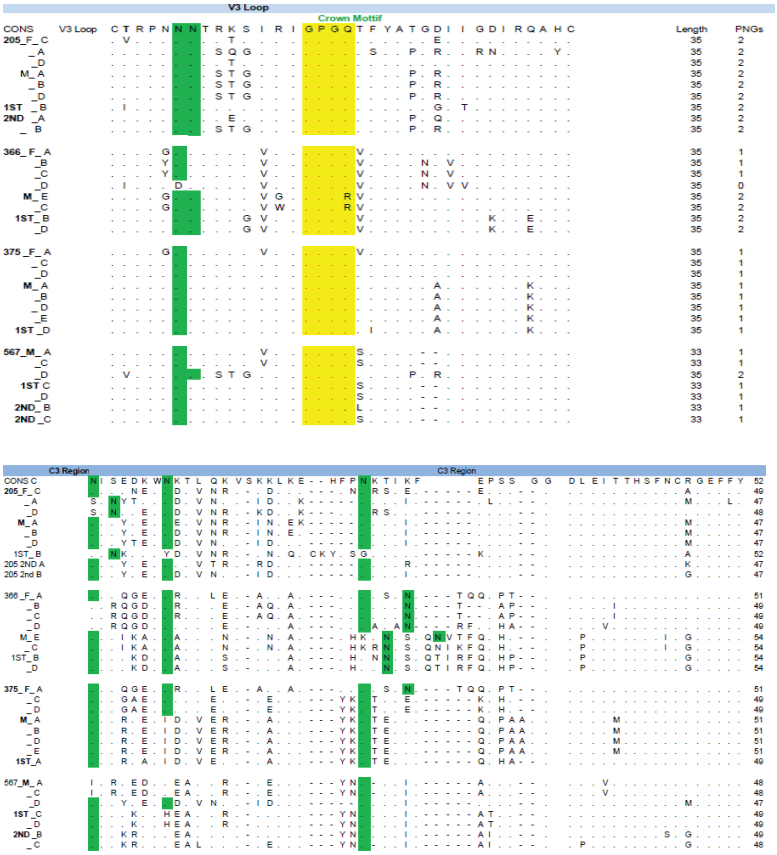


Figure 4:8: Unique HIV subtype C env V3 and C3 sequences**.** According to subtype B sequence V3 region and C3 regions are hypervariable region and constant regions, respectively. However, our subtype C V3 region is relatively constant (top part) whilst the supposedly constant C3 region is rather hypervariable (lower part) especially the first part. Dots represent similarity and each letter denotes an amino acid.

CHAPTER 5

5.0. Published Papers

5.1. Paper I

Antenatal HIV-1 RNA load and timing of mother to child transmission; a nested case-control study in a resource poor setting;

Duri K, Gumbo FZ, Kristiansen KI, Kurewa NE, Mapingure MP, Rusakaniko S, Chirenje MZ, Muller F and Stray-Pedersen B

Virology Journal 2010, 7: 176

Objective

To determine HIV-1 RNA load during the third trimester of pregnancy and evaluate its effect on in utero and intra-partum/postpartum transmissions in a breastfeeding population

Design

A nested case-control study within a PMTCT cohort of antiretroviral therapy naive pregnant women and their infants

Methods

A case was a mother who transmitted HIV-1 to her infant (transmitter) who was matched to one HIV-1 positive but non-transmitting mother (control).

Results

From a cohort of 691 pregnant women, 177 (25.6%) were HIV-1 positive at enrolment and from these 29 (23%) transmitted HIV-1 to their infants, 10 and 19 during in utero and intra-

partum/postpartum respectively. Twenty-four mothers sero-converted after delivery and three transmitted HIV-1 to their infants. Each unit increase in $\log_{10}$ viral load was associated with a 178 cells/mm^3 and 0.2 g/dL decrease in TLC and hemoglobin levels, p=0.048 and 0.021 respectively, and a 29% increase in the risk of transmission, p=0.023. Intra-partum/postpartum transmitters had significantly higher mean viral load relative to their matched controls, p=0.034.

Conclusion

Antenatal serum HIV-1 RNA load, TLC and hemoglobin levels were significantly associated with vertical transmission but this association was independent of transmission time. This finding supports the rationale for preventive strategies designed to reduce vertical transmission by lowering maternal viral load.

5.2. Paper II

Genotypic Analysis of Human Immunodeficiency Virus Type 1 (HIV-1) env V3 Loop Sequences: Bioinformatics Prediction of Co-receptor Usage among 28 Infected Mother–Infant Pairs in a Drug-Naive Population.

Duri K, Soko W, Gumbo F, Kristiansen K, Mapingure M, Stray-Pedersen B, Muller, F and the BHAMC Group.

AIDS Research and Human Retroviruses 2010, 26; 11

We sought to predict virus co-receptor utilization using a simple bioinformatics method based on genotypic analysis of human immunodeficiency virus types 1 (HIV-1) env V3 loop

sequences of 28 infected but drug-naïve women during pregnancy and their infected infants and to better understand co-receptor usage in vertical transmission dynamics. The HIV-1 env V3 loop was sequenced from plasma samples and analyzed for viral co-receptor usage and subtype in a cohort of HIV-1-infected pregnant women.

Results

Predicted maternal frequencies of the X4, R5X4, and R5 genotypes were 7%, 11%, and 82%, respectively. Antenatal plasma viral load was higher, with a mean $\log_{10}$ (SD) of 4.8 (1.6) and 3.6 (1.2) for women with the X4 and R5 genotypes, respectively, p=0.078. Amino acid substitution from the conserved V3 loop crown motif GPGQ to GPGR and lymphadenopathy were associated with the X4 genotype, p = 0.031 and 0.043, respectively. The maternal viral co-receptor genotype was generally preserved in vertical transmission and was predictive of the newborn's viral genotype. Infants born to mothers with X4 genotypes were more likely to have lower birth weights relative to those born to mothers with the R5 genotype, with a mean weight (SD) of 2870 (332) and 3069 (300) grams, respectively.

Conclusion

These data show that at least in HIV-1 subtype C, R5 co-receptor usage is the most predominant genotype, which is generally preserved following vertical transmission and is associated with the V3 GPGQ crown motif. Therefore, antiretroviral-naive pregnant women and their infants can benefit from ARV combination therapies that include R5 entry inhibitors following prediction of their co-receptor genotype using simple bioinformatics methods.

5.3 Paper III

Human Immunodeficiency Virus (HIV) types Western blot (WB) band profiles as potential surrogate markers of HIV disease progression and predictors of vertical transmission in a cohort of infected but antiretroviral therapy naïve pregnant women in Harare, Zimbabwe

Duri K, Muller F, Gumbo FZ, Kurewa NE, Rusakaniko S, Chirenje MZ, Muller F and Stray-Pedersen B.

BMC Infectious Diseases 2011, 11:7

Background

Expensive CD4 count and viral load tests have failed the intended objective of enabling access to HIV therapy in poor resource settings. It is imperative to develop simple, affordable and non-subjective disease monitoring tools to complement clinical staging efforts of inexperienced health personnel currently manning most healthcare centres because of brain drain. Besides accurately predicting HIV infection, sequential appearance of specific bands of WB test offers a window of opportunity to develop a less subjective tool for monitoring disease progression.

Methods

HIV type characterization was done in a cohort of infected pregnant women at 36 gestational weeks using WB test. Student-t test was used to determine maternal differences in mean full blood counts and viral load of mothers with and those without HIV *gag* antigen bands. Pearson Chi-square test was used to assess differences in lack of bands appearance with vertical transmission and lymphadenopathy.

Results

Among the 64 HIV infected pregnant women, 98.4 % had pure HIV-1 infection and one woman (1.7%) had dual HIV-1/HIV-2 infections. Absence of HIV pol antigen bands was associated with acute infection, p=0.002. All women with chronic HIV-1 infection had antibody reactivity to both the HIV-1 envelope and polymerase antigens. However, antibody reactivity to gag antigens varied among the women, being 100%, 90%, 70% and 63% for p24, p17, p39 and p55, respectively. Lack of antibody reactivity to gag p39 antigen was associated with disease progression as confirmed by the presence of lymphadenopathy, anemia, higher viral load, p=0.010, 0.025 and 0.016, respectively. Although not statistically significant, women with p39 band missing were 1.4 times more likely to transmit HIV-1 to their infants.

Conclusion

Absence of antibody reactivity to pol and gag p39 antigens was associated with acute infection and disease progression, respectively. Apart from its use in HIV disease diagnosis, WB test could also be used in conjunction with simpler tests like full blood counts and patient clinical assessment as a relatively cheaper disease monitoring tool required prior to accessing antiretroviral therapy for poor resource settings. However, there is also need to factor in the role of host-parasite genetics and interactions in disease progression.

5.4 Paper IV

Phylogenetic Analysis of Human Immunodeficiency Virus type 1 (HIV-1) Subtype C Env gp 120 sequences among four drug naïve families following subsequent heterosexual and vertical transmissions

Duri K, Gumbo FZ,Kristiansen KI, Mapingure MP, Munjoma M, Rusakaniko S, Chirenje MZ, Stray-Pedersen B and Muller F

AIDS Res and Hum Retroviruses Journal 2012, 28(8): 885-893

We sought to characterise phylogenetic relatedness of plasma HIV-1 RNA subtype C *env* gp120 viral variants capable of establishing an infection following heterosexual and subsequent vertical transmission events by sequencing a 520 base pair fragment of the C2V5 sub-region from four HIV-1 infected families. Analysis was done using MEGA software.

Phylogenetic analysis performed on families' sequences suggested a localized expansion of the subtype C pandemic and that several mechanisms may be involved in both vertical and heterosexual transmission. Second siblings' sequences were homogeneous and clustered in a single branch whilst first siblings' sequences were more heterogeneous, clustering in separate branches, suggestive of more than one donor variants responsible for the infection or evolution from founder variant(s) could have occurred. Though the directionality for heterosexual transmission could not be determined, homogeneous viral variants was a unique characteristic of maternal variants *vis-a–viz* the more heterogeneous paternal variants. Sequences clustered quite closely with other regional HIV-1 subtype C sequences supported by a bootstrap value of 86%. Bigger studies are warranted to address the caveats of this study and build on the strengths. Our study could be the beginning of family-based HIV-1 intervention research in Zimbabwe.

5.5 Paper V

HIV-1 Env gp120 C2V5 Potential N-Linked Glycosylation site(s) (PNGs) and amino acid length polymorphisms following among infected family members

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Objective

To ascertain the role of HIV-1 gp120 env PNGs variations and sequence length polymorphism following transmission events as possible supporting forensic evidence to determine directionality of HIV transmission

Method

An observational study of HIV-1 infected family members, where median and range values of the amino acid lengths and PNGs for the genotyped C2V5 region were calculated. Wilcoxon rank-sum test was used to determine differences in these parameters between different family members.

Results

For heterosexual transmission, two mothers had longer C3 sequences relative to that of their spouses; p=0.006 and=0.025 whilst the opposite was observed for one mother, p=0.028. No clear trends were observed for PNGs. In three families, index children had longer C2V5 amino acid sequences compared to their mothers; p=0.013, 0.040 and 0.043. Second siblings' V4 and V5 sequences were generally shorter relative to the maternal ones; p=0.039 and 0.028, respectively. Generally adults had longer V3 amino acid sequences compared to the children; p=0.018. Similar trends were also observed regarding PNGs within the entire C2V5 region, C3 and V4 sub-regions; p=0.0025, 0.005 and 0.008, respectively. First siblings' C2V5 and C3 sequence lengths were significantly longer relative to those of the second siblings; p=0.005 and 0.007, respectively.

Conclusion

Our results are suggestive that HIV-1 env C2V5 amino acid length and PNGs tend to increase with age and HIV disease progression. Though sensitive and should be cautiously handled, it is tempting to propose the directionality of the HIV transmission events with respect to C3 region length polymorphism. Correlating HIV-1 env C2V5 sequence lengths and age of infection may be the first step towards a possible valuable piece of forensic evidence which may be useful in criminalisation of willful HIV infections. However, bigger studies are warranted to substantiate the authenticity of this potentially useful application.

CHAPTER 6

6.0 Discussion

6.1 Study Design

In such poor resource settings of our study the nested case control design reduced costs and efforts of data collection considerably with relatively minor loss in statistical power [468-471]. Despite the relatively small sample size, this retrospective case-control study is novel in HIV diversity and transmission mainly among mothers and their infants and to a lesser extent the respective fathers in that it enunciates new observations pertinent to the scientific fraternity especially for resource limited communities. Additionally, numerous new avenues for further studies have been opened.

6.2 HIV Spread and Diagnosis

Worryingly, mothers did not know when and how they got infected by HIV-1. On a positive note our study observed a decline of HIV-1 prevalence as compared to previous studies [123]. Certain viral types, groups or subtypes are found in particular regions of the world or in certain populations. Travellers contribute to the spread of HIV-1 genetic diversity worldwide, a situation exacerbated by migration of rural populations and civil wars in developing countries [472]. Different categories of travelers ranging cross border traders, immigrants, military personnel, seamen, tourists, expatriates, diplomats to high profile business persons are at risk "exporting" and "importing" HIV infections from different locations across the globe. In our study **s**ero-conversion of HIV-1 was more likely observed amongst mothers who reported having a travelling spouse or sexual partner, **Paper I** [473], supporting the hypothesis of the association of travel and acquisition of HIV infection. Host genetic effects, transmission bottlenecks, social/behavioral and environmental limitations, founder effect and other viral

factors could have contributed to variable spread through the human population [309]. This differential spreading of HIV-1 variants has implications for diagnostic, disease monitoring, treatment, and vaccine development. Continued developments in HIV testing technology and practices are the cornerstone for all HIV prevention strategies if the curbing of this pandemic is to be realised [474;475].

6.2.1. Serological HIV Diagnosis

6.2.1.1 Rapid HIV Tests

Serial testing method was used although the relatively more expensive parallel algorithm could have been more appropriate to reduce false results. Ideally HIV antibody tests should be sensitive enough to detect all known HIV variants. At least in our study concordance of HIV rapid testing was 100% for the two kits used as illustrated in **Paper III** [476]. However, the bigger mother cohort showed a rapid test results discordance of 5% [477]. The development of blood screening reagents is nearly always based on conserved viral antigens or viral sequences derived from 'prototype' strains or antibodies raised against these prototype strains. Therefore in situations where an individual is infected by a viral strain that is genetically and antigenically distantly related to the prototype strain used in the development of the test, screening failure may occur [478]. Cases of antibody-negative HIV infections have been reported [479 480]. Depending on tests or algorithms used, up to 6% of HIV-1 group M and 80% of HIV-1 O infected patients may be misdiagnosed [481]**.** Co-infections especially with tuberculosis which is high among HIV positive people in SSA has been associated with increase in the diversity of HIV quasi-species [482] may be also be the reasons behind some inaccurate HIV diagnosis. Apart from obvious human errors and HIV genetic diversity, discrepancy in the results could be due to antibody cross reaction of patient specimen with

some kit components or non-specific immune reactivity [446]. Generally the interpretation of indeterminate or discrepant results between different rapid tests on one sample poses a challenge and under such circumstances WB test is generally used as the tie breaker.

6.2.1.2. WB Test

No challenges of indeterminate results were among the mothers. Regardless of that observation all our mothers' rapid test results were confirmed on WB. Although WB test remains relatively more expensive compared to rapid tests it could be worthwhile using it as it provides more information on patients' serology **Paper III** [476]. Studies have shown African sera to exhibit a significantly higher number of indeterminate WB patterns [483]. None of our women showed indeterminate WB test results. Unfortunately most kits are developed and validated in developed countries using their predominant subtype B antigen preparations which contribute less than 10% of all global HIV infection. However, the same kits when used in regions with non-B subtypes may not be as effective and accurate.

6.2.2. Qualitative DNA PCR

Like the Rapid tests most molecular diagnostic assays for the detection of HIV infection have been approved and licensed specifically for subtype B. Despite the high viral variability observed, some essential portions of the HIV genome are highly conserved and such regions are used in universal primer design which theoretically should pick up all the viral variants. Accordingly, some studies have successfully detected non-B subtype infections using USA Food and Drug Administration licensed subtype B specific diagnostic assays [484]. In our study infant HIV-1 status was determined using qualitative Roche DNA PCR which previous studies have demonstrated 100% sensitivity and 100% specificity at least amongst

Zimbabwean infants and adults with predominant HIV-1 subtype C [485]. For our study there was no other test employed to validate the Roche DNA PCR qualitative test results and as such some few misdiagnoses could not be ruled out completely. Constant HIV-1 genomic variability within and across strains plays a major role in relation to the sensitivity of such tests, sometimes leading to misdiagnosis [300;486-489]. Thus some studies have shown that Amplicor DNA PCR HIV-1 test does not detect all subtypes with equivalent sensitivity [490]. Clinicians should be aware of this particular limitation of this commonly used assay. Therefore constant monitoring of the performance of such molecular screening tests is very critical. It is imperative that alternative cheaper in-house test(s) be urgently developed taking cognizance that the Roche DNA qualitative PCR test is rather expensive and beyond the reach of many in resource limited settings where ironically HIV prevalence is unacceptably high.

6.3 HIV Monitoring

Monitoring antiretroviral therapy requires that HIV-1 viremia assays be applicable to all distinct HIV variants. Unfortunately, Like the diagnostic test excellent viral load quantitation results have been observed for subtype B compared to other subtypes or CRFs [491-499;499]. Accurate HIV-1 RNA quantitation have been shown to be compromised by primer and probe sequence polymorphisms as a result of the extensive tremendous genetic diversity as a result of ongoing HIV-1 evolution. Inaccurate quantification could be the underlying reason for the observation of pregnant mothers in our study who despite having undetectable plasma viral loads, went on to transmit to their infants, **Paper I** [473]. Thus, non-detection of HIV RNA among these women could be explained by subtype B specific primers mismatches in subtype C amplifications. This has been confirmed by previous studies that have shown that approximately 30% of non-B subtypes specimens have been shown to be under-quantified by

at least -0.51 $\log_{10}$ by the Amplicor version 1.5 PCR [500;501]. Such observations emphasise the importance of efficient designing of primers and probes for optimal quantitation of plasma or serum HIV-RNA in non-B subtypes. Studies have shown similar sensitivity and performance with serum or plasma [502]. Mothers' host factors such HLA and CYP polymorphisms and psychosocial factors also remain important predictors of disease progression they may impact on viral load [229].

Other more affordable and easily measurable markers of disease progression such as TLC, hemoglobin, body mass index and delayed type hypersensitivity may be used in resource poor settings in desperation particularly at this time of scaling-up of antiretroviral therapies [229]**.** Taking cognizance of the high cost per test, there is an urgent need for low-cost, simple, and accurate HIV-1 RNA load monitoring technologies tailor made for resource-limited settings In our study indeed TLC and hemoglobin levels correlated with viral load **Paper I** [473]. Since unintentional viral load underestimation may lead to further infections or inappropriate treatment decisions other sequence-independent tests remain valuable requirements for confirming a low viral load.

6.4 HIV Diversity and Transmission

6.4.1 Types

HIV-2 was not found in our mothers yet its prevalence of this viral type is relatively high in neighbouring Mozambique and Angola. These countries are amongst those frequented by most Zimbabwean cross border traders and ZDF personnel in the past three decades.

However, transmission of HIV-2 in Zimbabwe has remained very low if any. This observation could be attributed to the low transmissibility properties of HIV-2 relative to HIV-1 [365].

6.4.2. Subtypes /CRFs

Despite the high mobility there seems to be no new HIV types being introduced into this population, supporting the null hypothesis. Based on the sequencing of the C2-V5 HIV-1 *env* gene exclusive subtype C was observed among the mothers and their infants. Our study results are consistent with previous Zimbabwean studies that have demonstrated subtype C as the predominant subtype [396-398] except for one study with unconvincing methodology that reported several double and triple recombinants [503]. HIV-1 env subtype C clusters were clearly distinguished with high bootstrap values suggestive of infections of monophyletic origin or a localized expansion of the subtype C epidemic. Previous studies also done in Harare in the same population have also observed the same picture but with the *pol* gene [99]. E*nv* C2-V5 sequences clustered with other HIV -1 subtype C from South Africa, Malawi, Botswana, Tanzania, India, China and Argentina as well as from other previous studies Zimbabwean subtype C sequences as evidenced by a high bootstrap values, **Paper IV** [504]**,** thus confirming the challenges associated with classifying subtype C sequences on a geographical location basis.

Generally the regional distributions of individual subtypes are mostly stable, although CRFs may play an increasing role in the HIV pandemic [505]. The geographic distribution of subtypes and CRFs is subject to constant change. Historically the North American and Western European are dominated by HIV-1 subtype B virus. However, this paradigm is changing rapidly as migrants and refugees from developing countries with non-B subtype infections

often now present for care in the developed world, and travelers to developing countries acquire non-B subtype infection abroad and present for care at home [506]. Thus continued diversification and global redistribution of HIV groups, subtypes and CRFs makes it imperative that these monitoring molecular assays be constantly or periodically designed and evaluated to ensure reliable performance to detect all HIV variants. Interestingly some studies have indicated that the dominance of HIV-1 subtype C in the current epidemic might be related to the lower virulence of this subtype compared with other subtypes hypothesizing HIV-1 as has either reached peak virulence or has already started the slow path to attenuation [507]. The most severe HIV-1 pandemic is occurring in Southern Africa. It is caused by HIV-1 subtype C [377 508]**.** Consequently, understanding the molecular phylogeny and genetic diversity of HIV-1 subtype C viruses may not be over-emphasised especially for the design and evaluation of an effective HIV vaccine.

6.4.3 Subtype C Uniqueness

Most of what is known about HIV is based on information from subtype B studies. However, questions are being raised around the possibility of genetic and phenotypic differences in HIV-1 regarding transmissibility efficiency, infectivity and pathogenicity, in addition to responses to therapy and vaccines. Findings in this study are suggestive of fact that subtype C may different from subtype B probably due to its unique genetic and biological properties, **Papers II, IV, V** [504;509;510]. Compared to other subtypes, subtype C has been shown to replicate and to be transmitted more efficiently [388;511-513]. It is possible the transmissibility of HIV-1 subtype C is so high such that even with very low or undetectable viral load in some mothers vertical transmission was inevitable, **Paper I** [473]. Subtype C may also be biologically different as demonstrated by the predominant R5 co-receptor usage, **Paper II** [509], a phenotype

that has been shown to persist throughout the course of infection whereas in the case of subtype B switching from R5 to X4 is observed as the disease progresses in 50% of the patients [514]. Traditionally, V3 region has been considered a variable domain based on analysis of subtype B sequences. Surprising, our study population's V3 region sequences were relatively constant, **Paper IV** [504]. Interestingly, similar results of a relatively constant subtype C V3 region have been obtained elsewhere [293;515-518]. Another consistent unique feature of our sequence analysis showed the highest levels of variation within the *env* gp120 C3 regions, challenging whether the so-called 'constant region' with respect to subtype B is really a constant one when it comes to subtype C. Other distinctive findings included the extensive deletion of the V4 region, amino acid length polymorphism of the V3 sequence and a characteristic GPGR motif on the crown of the V3 loop **Papers II, IV and V** [504;509;510]. Thus HIV-1 subtype C envelope seems to show significant differences and unique characteristics compared to its subtype B counterpart, thus refuting the null hypothesis. In view of these observations extrapolation of findings from one subtype to the other should be discouraged.

6.5. Vertical Transmission

However, figures for both vertical and heterosexual transmission of HIV-1 were still unacceptably high in this population, **Papers I; III** [473;476] . Of note was the highly significant relationship between antenatal HIV-1 RNA load, at 36 weeks gestational period with vertical transmission, **Paper I** [473]. Similar to other studies [420], no threshold for transmission was observed in this cohort that could predict transmission or non-transmission. Risk factors for vertical transmission in our study included high antenatal viral load, low TLC and anemia with the risk of transmission increasing by about 30% for each unit increase in $\log_{10}$ viral load supporting preventive interventions that lower maternal viral load **Paper I** [473]**.** It was also

interesting to note that maternal co-receptor genotype was preserved upon vertical transmission **Paper II** [509]. Findings are suggestive of the fact that the child acquired more or less the same number of PNGs from the mother which tends to decrease with disease progression **Paper IV** [504]. Phylogenetic analysis performed for each family sequences set suggested that several mechanisms may be involved in both vertical and heterosexual transmission as also previously demonstrated [519;520]. There is need for further research on the possible reasons behind the observed highly efficient vertical transmission phenomenon observed in some mothers who had undetectable viral load. There is also need to address subtype C specific research questions rather than extrapolating and applying subtype B findings if curbing of the pandemic is to be realized.

6.6. Horizontal Transmission

Homogeneous viral variants were a unique characteristic of maternal variants *vis-a–viz* the more heterogeneous paternal variants. Similar results have also been previously reported [521]. This study also showed that C2V5 amino acid length and PNGs tended to increase with age and disease progression. This relationship can applied and used as potential supporting forensic evidence to determine the directionality of HIV transmission in court cases of willful HIV transmission where it is currently difficult to prove who infected who, **Paper V** [510] Anecdotal of verbal autopsy suggests that generally Zimbabweans think most men infect their spouses whether willingly or unwillingly. However, this pilot study suggests otherwise pointing to a possible 50-50 transmission in either direction whether male to female (MTF) or female to male (FTM) transmission **Paper V** [510]. Bigger studies are warranted to address the caveats of this study and build on its strengths.

6.7. Methodological Issues

The gold standard in subtype determination entails that the whole HIV genome be sequenced but this approach has its own technical challenges and prohibitive costs. The second best approach of determining subtype is based on sequencing all the three main HIV genes, env, pol and gag or any two. HIV-1 subtypes determination using at least were determined using two or three genes, *gag, pol,* and *env* using several methods [522]. Thus at least one more gene, for example the pol gene could also have been sequenced for subtype determination which would in addition give an idea of HIV-1 pol gene mutation profiles in the absence of selection of antiretroviral therapy. When at least one gene is genotyped, subtype determination for each gene can be concordant or discordant which could be indicative of recombination [523]. However, there is also a chance of missing out on CRFs in other regions of the HIV genome. More so due to the same bottlenecks, the number of clones cloned per sample of about 4 on average fell short of the expected. Normally an average of 12 clones per sample could have been the ideal practice for more conclusive observations.

Originally it was planned that direct sequencing be done for all the samples. However, it was very challenging to directly sequence 50% of the samples probably due to mutations hence the need to first TOPO clone such samples. Consequently, we ended up having some samples assayed by either method. Comparing such data has been a challenge. We had also anticipated comparing viral heterogeneity between transmitting and non-transmitting mothers which can only be done after controlling for such methodological challenges. Complete genomes are often not available and there is need for a method which accurately determines the subtype of strains for which only a segment of the genome has been sequenced. Relatively new methods

[524;525] have been developed to improve the accuracy of HIV-1 subtype classification [73526;526;527].

6.8. Strength of the study

This is a unique, advanced and technologically high-powered study which applied state-of the art techniques and equipment in biomedical research and analysis. As such, it opens countless windows of opportunities for North to South technology transfer, institutional and national capacity building and mutual co-operation in biomedical research. This study could be the beginning of research including family-based HIV-1 intervention research in Zimbabwe where males can be persuaded to participate in the PMTCT studies. The study's unique cohort could be a sound and solid basis for lengthy follow-up periods which must be continued as most of the study participants are still available and willing to participate in research.

6.9 Limitation of the study

6.9.1 Sample Size and Follow up

The sample size used is generally small. All the transmitters were included in this study as planned but to increase the statistical power of this study it was originally envisaged that each transmitter would be matched to four non-transmitting mothers but failed due to other technical and financial constraints. Due to the foregoing constraints, a one to one matching was settled for in anticipation that this would be more of a pilot study which could be scaled up depending on the results obtained. However, all transmitting and non-transmitting mothers selected in the study may not be a full representation of all the cases and controls in the original cohort due to failure to follow up all the mothers and infants. Nonetheless, the follow-up rate was generally good. The socio-economic situation deteriorated fast during the follow

up period and some of our study participants relocated to the rural areas where cost of living was relatively cheaper there-by up-setting follow up rates. Some infants were seen at delivery and only reappeared after 15 months of age, **Paper I** [473]. Since they did not turn up for their six week visits, it was impossible to know whether they were infected intra-partum or postpartum. Consequently, this resulted in the unusual categorization of time of infection of infants as intra-partum/postpartum, **Paper I** [473].

6.9.2 Methodological Challenges

In poor resource settings, subtype and CRFs determination is generally done using the *gag/env* HMA originally developed in 1993 and later modified by others seven years later [444;445]. However, full genome sequencing remains the gold standard method though expensive. Partial sequencing gives good results at reasonable cost. In our study subtype determination was done by genotyping a relatively long fragment of the most variable region of HIV which was very appropriate. Generally, it appears that no new subtypes or recombinants were being introduced in our study population.

CHAPTER 7

7.0 Conclusion and Recommendations

1. Based on the HIV-1 env gp 120 C2V5 there seems to be no new types or subtypes despite high mobility of the Zimbabweans.

2. The strongest predictor for sero-conversion was having a travelling partner.

3. High viral load was a risk factor for vertical transmission. The diagnostic (qualitative DNA-PCR) and disease monitoring (viral load test) kits used may not have been sensitive enough for this population.

4. R5 co-receptor genotype was the most predominant in both the mothers and their infants and co-receptor usage genotype and PNGs were preserved upon vertical transmission.

5. Mother viral heterogeneity was more or less the same with that of the second sibling whilst that of the first siblings showed diverse heterogeneity.

6. Our subtype C clustered closely with other subtype C from other geographical regions and had unique V3 and C3 sequences.

The following recommendations are being made:

Besides accurately predicting HIV infection, sequential appearance of specific bands on WB, test results offer a window of opportunity to develop a less subjective tool to monitor disease progression in poor resource settings.

In resource poor settings with predominant R5 HIV-1 genotype, patients may benefit from ARV combination therapies that include anti-R5 entry inhibitors such as Maraviroc which can be taken with or without food and does not require refrigeration. If the poor communities are to benefit, the cost of the tropism test as well as the drug itself must be curtailed.

Extrapolation of one subtype findings to another should be discouraged. Genetic differences observed may translate to variations in biological properties which in turn may translate to differences in transmission efficiency, disease progression, diagnostic and monitoring test sensitivity as well as treatment outcome.

It is imperative to set up health care facilities to cater for long term survivors of vertical infection as HIV-1 infected children reaching their fifth birthday without ART and possibly maturing into adolescents. Currently existing institutions and facilities do not seem to cater for this generation of children. Their health conditions may demand more frequent and specialised medical attention.

Lastly but more importantly technology transfer from North to South initiated by this collaboration should be taken to a higher level where a state of art molecular biology laboratory should be established in the Zimbabwe for advancement of research for the benefit of the nation and SSA region at large. This will be a step in the right direction towards addressing problems unique to this region that include co-infections and malnutrition and how these modify HIV acquisition and disease progression.

CHAPTER 8

8.0 Further Studies

There is need to assay the same population but this time sequencing at least two HIV gene fragments or even sequencing the whole HIV subtype C genome to conclusively rule out presence or absence of CRFs.

Necessary are bigger multi-centred studies in regions with different HIV-1 disease burdens but having overlapping subtypes to address transmissibility efficiencies of HIV. These bigger studies will also have to substantiate this pilot study's findings:

a) The underlying reason(s) behind undetectable viral load from both the viral and host aspects including validating the assays in our own settings

b) To substantiate the antenatal reactivity of p39 antigen on WB test as a possible predictor of vertical transmission

c) To further determine the role of amino acid length polymorphism and PNGs variation in transmission and or disease progression

d) To further assess and evaluate the potential of env length polymorphism and PNGs to predict the directionality of heterosexual transmission.

e) Further follow up the infected children into adolescence studying the immunological and virological markers and their association with disease progression.

Further research to identify the selective factors governing which variants are transmitted, how the compartmentalization of HIV in different cells and tissues contributes to transmission, and the influence of host immunity, viral diversity, and recombination on MTCT may provide insight into new prevention strategies and the development of an effective HIV vaccine.

Additional prospective research study is warranted to determine the concordance of HIV-1 genetic diversity in different compartments in addition to the blood such as the genital tract (GT) and breast milk and their roles in transmission.

CHAPTER 9

9.0 Reference List

(1) Centres for Disease Control (CDC). Kaposi's sarcoma and Pneumocystis pneumonia among heterosexual men. **30**, 305-308. 1981. New York city and California, *Morb Mortal Wkly Rep*.

(2) Chermann JC: HIV: the etiologic agent of AIDS and associated diseases. Biomed Pharmacother 1988; 42(1):3-4.

(3) Chang SY, Bowman BH, Weiss JB, Garcia RE, White TJ: The origin of HIV-1 isolate HTLV-IIIB. Nature 1993; 363(6428):466-469.

(4) Coffin CM: Current issues in transfusion therapy. 1. Risks of infection. Postgrad Med 1986; 80(8):219-224.

(5) Johnson AM, Laga M: Heterosexual transmission of HIV. AIDS 1988; 2 Suppl 1:S49-S56.

(6) van der Graaf M, Diepersloot R: Sexual transmission of HIV: routes, efficiency, cofactors and prevention. A survey of the literature. Infection 1989; 17(4):210-215.

(7) Pape JW, Johnson W, Jr.: Perinatal transmission of the human immunodeficiency virus. Bull Pan Am Health Organ 1989; 23(1-2):50-61.

(8) Zhu T, Korber BT, Nahmias AJ et al.: An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature 1998; 391(6667):594-597.

(9) UNAIDS JUNPoHA. Global report: UNAIDS report on the global AIDS epidemic 2010. 2011. Geneva.

(10) Lansky A, Brooks JT, DiNenno E et al.: Epidemiology of HIV in the United States. J Acquir Immune Defic Syndr 2010; 55 Suppl 2:S64-S68.

(11) Kilmarx PH: Global epidemiology of HIV. Curr Opin HIV AIDS 2009; 4(4):240-246.

(12) Malamba SS, Mermin JH, Bunnell R et al.: Couples at risk: HIV-1 concordance and discordance among sexual partners receiving voluntary counseling and testing in Uganda. J Acquir Immune Defic Syndr 2005; 39(5):576-580.

(13) UNAIDS/WHO. AIDS epidemic 2004 by UNAIDS/WHO working group on HIV/AIDS/STI. 2004. Geneva, WHO.

(14) Hahn BH, Shaw GM, De Cock KM, Sharp PM: AIDS as a zoonosis: scientific and public health implications. Science 2000; 287(5453):607-614.

(15) Gao F, Bailes E, Robertson DL et al.: Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 1999; 397(6718):436-441.

(16) Switzer WM, Qari SH, Wolfe ND et al.: Ancient origin and molecular features of the novel human T-lymphotropic virus type 3 revealed by complete genome analysis. J Virol 2006; 80(15):7427-7438.

(17) Hooper E: Experimental oral polio vaccines and acquired immune deficiency syndrome. Philos Trans R Soc Lond B Biol Sci 2001; 356(1410):803-814.

(18) Le BF, Mason PR, Gwanzura L, Robertson VJ, Latif AS: HIV and other sexually transmitted diseases at a rural hospital in Zimbabwe. Genitourin Med 1993; 69(5):352-356.

(19) Latif AS: Sexually transmitted diseases in Africa. Genitourin Med 1990; 66(4):235-237.

(20) Muwanga F: HIV and STDs: how are they linked? [letter]. Afr Health 1995; 17(3):40.

(21) Mehta SD, Erbelding EJ, Zenilman JM, Rompalo AM: Gonorrhoea reinfection in heterosexual STD clinic attendees: longitudinal analysis of risks for first reinfection. Sex Transm Infect 2003; 79(2):124-128.

(22) Scarlatti G, Hodara V, Rossi P et al.: Transmission of human immunodeficiency virus type 1 (HIV-1) from mother to child correlates with viral phenotype. Virology 1993; 197(2):624-629.

(23) Wolfs TF, Zwart G, Bakker M, Goudsmit J: HIV-1 genomic RNA diversification following sexual and parenteral virus transmission. Virology 1992; 189(1):103-110.

(24) Christenson B, Lundbergh P: [HIV spreads fast in Africa. Women and children especially, are at high risk]. Lakartidningen 1994; 91(22):2255-2256.

(25) Piot P, Bartos M, Ghys PD, Walker N, Schwartlander B: The global impact of HIV/AIDS. Nature 2001; 410(6831):968-973.

(26) Carael M, Van de Perre PH, Lepage PH et al.: Human immunodeficiency virus transmission among heterosexual couples in Central Africa. AIDS 1988; 2(3):201-205.

(27) Smith DJ: Modern marriage, men's extramarital sex, and HIV risk in southeastern Nigeria. Am J Public Health 2007; 97(6):997-1005.

(28) Joint United Nations Programme on HIV/AIDS (UNAIDS). Global report: UNAIDS report on the global AIDS epidemic 2010. 2010.

(29) Hu DJ, Dondero TJ, Rayfield MA et al.: The emerging genetic diversity of HIV. The importance of global surveillance for diagnostics, research, and prevention. JAMA 1996; 275(3):210-216.

(30) Buve A: HIV epidemics in Africa: what explains the variations in HIV prevalence? IUBMB Life 2002; 53(4-5):193-195.

(31) Asamoah-Odei E, Garcia Calleja JM, Boerma JT: HIV prevalence and trends in sub-Saharan Africa: no decline and large subregional differences. Lancet 2004; 364(9428):35-40.

(32) Fleming AF: AIDS in Africa-an update. AIDS Forsch 1988; 3(3):116-138.

(33) UNAIDS 2. 2008 report on the global AIDS epidemic. 2011. Geneva.

(34) Gisselquist D, Rothenberg R, Potterat J, Drucker E: HIV infections in sub-Saharan Africa not explained by sexual or vertical transmission. Int J STD AIDS 2002; 13(10):657-666.

(35) Mapingure MP, Msuya S, Kurewa NE et al.: Sexual behaviour does not reflect HIV-1 prevalence differences: a comparison study of Zimbabwe and Tanzania. J Int AIDS Soc 2010; 13:45.

(36) Lingappa JR, Lambdin B, Bukusi EA et al.: Regional differences in prevalence of HIV-1 discordance in Africa and enrollment of HIV-1 discordant couples into an HIV-1 prevention trial. PLoS One 2008; 3(1):e1411.

(37) Bulterys M, Chao A, Dushimimana A, Parekh BS: Unsafe injections and transmission of HIV-1 in sub-Saharan Africa. Lancet 2004; 363(9421):1650.

(38) Gisselquist D, Potterat JJ, Brody S: Running on empty: sexual co-factors are insufficient to fuel Africa's turbocharged HIV epidemic. Int J STD AIDS 2004; 15(7):442-452.

(39) Grosskurth H, Plummer F, Mhalu F, Mabey D: STD research in Africa. Lancet 1993; 342(8884):1415-1416.

(40) Grosskurth H, Mosha F, Todd J et al.: Impact of improved treatment of sexually transmitted diseases on HIV infection in rural Tanzania: randomised controlled trial. Lancet 1995; 346(8974):530-536.

(41) Mavedzenge SN, Pol BV, Cheng H et al.: Epidemiological synergy of Trichomonas vaginalis and HIV in Zimbabwean and South African women. Sex Transm Dis 2010; 37(7):460-466.

(42) Boerma JT, Gregson S, Nyamukapa C, Urassa M: Understanding the uneven spread of HIV within Africa: comparative study of biologic, behavioral, and contextual factors in rural populations in Tanzania and Zimbabwe. Sex Transm Dis 2003; 30(10):779-787.

(43) Giesecke J, Scalia-Tomba G, Furucrona A: HIV infectivity--the hepatitis B lesson. Scand J Infect Dis 1988; 20(4):385-387.

(44) Kiire CF: The epidemiology and control of hepatitis B in sub-Saharan Africa. Prog Med Virol 1993; 40:141-156.

(45) Kiire CF: Hepatitis B infection in sub-Saharan Africa. The African Regional Study Group. Vaccine 1990; 8 Suppl:S107-S112.

(46) Hiemstra R, Rabie H, Schaaf HS et al.: Unexplained HIV-1 infection in children--documenting cases and assessing for possible risk factors. S Afr Med J 2004; 94(3):188-193.

(47) Gisselquist D, Potterat JJ, Brody S, Minkin SF: Does selected ecological evidence give a true picture of HIV transmission in Africa? Int J STD AIDS 2004; 15(7):434-439.

(48) Mbizvo MT, Kasule J, Mahomed K, Nathoo K: HIV-1 seroconversion incidence following pregnancy and delivery among women seronegative at recruitment in Harare, Zimbabwe. Cent Afr J Med 2001; 47(5):115-118.

(49) Munjoma MW, Mhlanga FG, Mapingure MP et al.: The incidence of HIV among women recruited during late pregnancy and followed up for six years after childbirth in Zimbabwe. BMC Public Health 2010; 10:668.

(50) Schmid GP, Buve A, Mugyenyi P et al.: Transmission of HIV-1 infection in sub-Saharan Africa and effect of elimination of unsafe injections. Lancet 2004; 363(9407):482-488.

(51) Frank C, Mohamed MK, Strickland GT et al.: The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 2000; 355(9207):887-891.

(52) Gisselquist D: HIV transmission through health care in sub-Saharan Africa. Lancet 2004; 364(9446):1665-1666.

(53) Gisselquist D, Minkin SF, Okwuosah A, Salerno L, Minja-Trupin C: Unsafe injections and transmission of HIV-1 in sub-Saharan Africa. Lancet 2004; 363(9421):1648-1649.

(54) Hoelscher M, Riedner G, Hemed Y et al.: Estimating the number of HIV transmissions through reused syringes and needles in the Mbeya Region, Tanzania. AIDS 1994; 8(11):1609-1615.

(55) Okwen MP, Ngem BY, Alomba FA et al.: Uncovering high rates of unsafe injection equipment reuse in rural Cameroon: validation of a survey instrument that probes for specific misconceptions. Harm Reduct J 2011; 8(1):4.

(56) Abdala N, Stephens PC, Griffith BP, Heimer R: Survival of HIV-1 in syringes. J Acquir Immune Defic Syndr Hum Retrovirol 1999; 20(1):73-80.

(57) Guthrie BL, de BG, Farquhar C: HIV-1-discordant couples in sub-Saharan Africa: explanations and implications for high rates of discordancy. Curr HIV Res 2007; 5(4):416-429.

(58) Dunkle KL, Stephenson R, Karita E et al.: New heterosexually transmitted HIV infections in married or cohabiting couples in urban Zambia and Rwanda: an analysis of survey and clinical data. Lancet 2008; 371(9631):2183-2191.

(59) Eyawo O, de WD, Ford N et al.: HIV status in discordant couples in sub-Saharan Africa: a systematic review and meta-analysis. Lancet Infect Dis 2010; 10(11):770-777.

(60) Mishra V, Assche SB, Greener R et al.: HIV infection does not disproportionately affect the poorer in sub-Saharan Africa. AIDS 2007; 21 Suppl 7:S17-S28.

(61) Gouws E, Stanecki KA, Lyerla R, Ghys PD: The epidemiology of HIV infection among young people aged 15-24 years in southern Africa. AIDS 2008; 22 Suppl 4:S5-16.

(62) Wester CW, Bussmann H, Moyo S et al.: Serological evidence of HIV-associated infection among HIV-1-infected adults in Botswana. Clin Infect Dis 2006; 43(12):1612-1615.

(63) Bernasconi D, Tavoschi L, Regine V et al.: Identification of recent HIV infections and of factors associated with virus acquisition among pregnant women in 2004 and 2006 in Swaziland. J Clin Virol 2010; 48(3):180-183.

(64) Central Statistical Office H. Census 1992; Zimbabwe National report. 2002.

(65) Zimbabwe National Statistics Agency (ZIMSTAT) and ICF International. *Zimbabwe Demographic and Health Survey 2010-11*. 2012. Calverton, Maryland, ZIMSTAT and ICF International Inc.

(66) Economist Intelligence Unit. Zimbabwe Country Report. 2008.

(67) Cato Institute. New Hyperinflation Index (HHIZ) Puts Zimbabwe Inflation at 89.7 Sextillion Percent. 2008.

(68) Index Mundi. Zimbabwe unemployment rate. 2009.

(69) DFID. Reducing maternal deaths in Africa. 2008.

(70) WHO. WHO Statistical Information System (WHOSIS). 2008. Geneva.

(71) United Nations (UN) Department of Economic Social Affairs. World Population Prospects, the 2000 Revision. 2001. Geneva.

(72) Mathers CD, Sadana R, Salomon JA, Murray CJ, Lopez AD: Healthy life expectancy in 191 countries, 1999. Lancet 2001; 357(9269):1685-1691.

(73) Sibanda A: A nation in pain: why the HIV/AIDS epidemic is out of control in Zimbabwe. Int J Health Serv 2000; 30(4):717-738.

(74) Ikeogu MO, Wolf B, Mathe S: Pulmonary manifestations in HIV seropositivity and malnutrition in Zimbabwe. Arch Dis Child 1997; 76(2):124-128.

(75) Chimhuya S, Kambarami RA, Mujuru H: The levels of malnutrition and risk factors for mortality at Harare Central Hospital-Zimbabwe: an observation study. Cent Afr J Med 2007; 53(5-8):30-34.

(76) Ticklay IM, Nathoo KJ, Siziya S, Brady JP: HIV infection in malnourished children in Harare, Zimbabwe. East Afr Med J 1997; 74(4):217-220.

(77) Serdula M: Diet, malnutrition in sub-Saharan Africa. Ann IFORD 1988; 12(2):35-63.

(78) Zimbabwe National Nutrition Survey, 2010- Preliminary findings. 2010.

(79) Mapenzauswa S. Zimbabwe Holds First National Meeting on AIDS Crisis", June 15, 2004. 2004. *Reuters NewsMedia*.

(80) Dehne KL, Dhlakama DG, Richter C et al.: Herpes zoster as an indicator of HIV infection in Africa. Trop Doct 1992; 22(2):68-70.

(81) Topley JM: HIV infection in Zimbabwe. Arch Dis Child 1988; 63(7):842-844.

(82) McFarland W, Mvere D, Shamu R, Katzenstein D: Risk factors for HIV seropositivity among first-time blood donors in Zimbabwe. Transfusion 1998; 38(3):279-284.

(83) McFarland W, Mvere D, Shandera W, Reingold A: Epidemiology and prevention of transfusion-associated human immunodeficiency virus transmission in sub-Saharan Africa. Vox Sang 1997; 72(2):85-92.

(84) Mertens T: [Modern serologic diagnosis of HIV 1 and HIV 2 infection]. Verh Dtsch Ges Inn Med 1991; 97:375-381.

(85) National blood services Zimbabwe. National blood services Zimbabwe Annual report 2010. 2010. Harare , Zimbabwe.

(86) Denhe K, Dhlakama D, Richter C et al.: Pattern of HIV-infection in Hurungwe district, Mashonaland West, Zimbabwe. Cent Afr J Med 1992; 38(4):139-143.

(87) Mertens T, Tondorf G, Siebolds M et al.: Epidemiology of HIV and hepatitis B virus (HBV) in selected African and Asian populations. Infection 1989; 17(1):4-7.

(88) Tswana SA, Nystrom L, Moyo SR et al.: A sero-epidemiology cross-sectional nationwide study of the prevalence of human immunodeficiency virus in Zimbabwe 1989-1991. Afr J Health Sci 1996; 3(3):96-100.

(89) Bassett MT, Mhloyi M: Women and AIDS in Zimbabwe: the making of an epidemic. Int J Health Serv 1991; 21(1):143-156.

(90) Simonsen JN, Fowke KR, MacDonald KS, Plummer FA: HIV pathogenesis: mechanisms of susceptibility and disease progression. Curr Opin Microbiol 1998; 1(4):423-429.

(91) Paulo M, Borges AB, Duarte G et al.: The environmental cofactors in carcinogenesis in high risk HPV/HIV-positive women. Braz J Infect Dis 2007; 11(2):189-195.

(92) Tobian AA, Quinn TC: Herpes simplex virus type 2 and syphilis infections with HIV: an evolving synergy in transmission and prevention. Curr Opin HIV AIDS 2009; 4(4):294-299.

(93) Koethe JR, Chi BH, Megazzini KM, Heimburger DC, Stringer JS: Macronutrient supplementation for malnourished HIV-infected adults: a review of the evidence in resource-adequate and resource-constrained settings. Clin Infect Dis 2009; 49(5):787-798.

(94) Ministry of Health and child Welfare Z. National HIV Estimates 2009. 2009.

(95) Zimbabwe Institute of Development Studies and the UNDP. Zimbabwe Human Development Report. 2003.

(96) Matchaba-Hove R. HIV/AIDS in the Zimbabwe defence forces; A civil Society Perspective. In: *The Enemy Within.* 2005.

(97) Whiteside A, Winsbury R: Vancouver AIDS conference: special report. The role of the military: to protect society and themselves. AIDS Anal Afr 1996; 6(4):4.

(98) Yeager R: Armies of east and southern Africa fighting a guerrilla war with AIDS. Special report: AIDS and the military. AIDS Anal Afr 1995; 5(6):10-12.

(99) Dalai SC, De OT, Harkins GW et al.: Evolution and molecular epidemiology of subtype C HIV-1 in Zimbabwe. AIDS 2009; 23(18):2523-2532.

(100) Mangoma J, Chimbari M, Dhlomo E: An enumeration of orphans and analysis of the problems and wishes of orphans: the case of Kariba, Zimbabwe. SAHARA J 2008; 5(3):120-128.

(101) Kwaramba P. The socio-economic impact of HIV/AIDS on communal agricultural production systems in Zimbabwe. 1997. Harare Zimbabwe, Zimbabwe farmers Union and Friederich Ebert.

(102) Sambisa W, Curtis S, Mishra V: AIDS stigma as an obstacle to uptake of HIV testing: evidence from a Zimbabwean national population-based survey. AIDS Care 2010; 22(2):170-186.

(103) Cameron E, Burris S, Clayton M: HIV is a virus, not a crime: ten reasons against criminal statutes and criminal prosecutions. J Int AIDS Soc 2008; 11:7.

(104) Lopman BA, Nyamukapa C, Hallett TB et al.: Role of widows in the heterosexual transmission of HIV in Manicaland, Zimbabwe, 1998-2003. Sex Transm Infect 2009; 85 Suppl 1:i41-i48.

(105) Adetunji JA: HIV/AIDS and young age widowhood in sub-Saharan Africa. J Health Hum Serv Adm 2001; 24(3):259-278.

(106) Strategy for safe medical male circumcision scale up to support comprehensive HIV prevention in Zimbabwe. Ministry of Health. 2010.

(107) Halperin DT, Fritz K, McFarland W, Woelk G: Acceptability of adult male circumcision for sexually transmitted disease and HIV prevention in Zimbabwe. Sex Transm Dis 2005; 32(4):238-239.

(108) Bateman C: HIV prevalence in Zimbabwe dropping like a stone. S Afr Med J 2011; 101(1):10-11.

(109) Gregson S, Gonese E, Hallett TB et al.: HIV decline in Zimbabwe due to reductions in risky sex? Evidence from a comprehensive epidemiological review. Int J Epidemiol 2010; 39(5):1311-1323.

(110) Halperin DT, Mugurungi O, Hallett TB et al.: A surprising prevention success: why did the HIV epidemic decline in Zimbabwe? PLoS Med 2011; 8(2):e1000414.

(111) Muchini B, Benedikt C, Gregson S et al.: Local perceptions of the forms, timing and causes of behavior change in response to the AIDS epidemic in Zimbabwe. AIDS Behav 2011; 15(2):487-498.

(112) Hansen K, Woelk G, Jackson H et al.: The cost of home-based care for HIV/AIDS patients in Zimbabwe. AIDS Care 1998; 10(6):751-759.

(113) McClellan MK, Patel R, Kadzirange G, Chipatod T, Katzenstein D: Fertility desires and condom use among HIV-positive women at an antiretroviral roll-out program in Zimbabwe. Afr J Reprod Health 2010; 14(2):27-35.

(114) Moyo W, Mbizvo MT: Desire for a future pregnancy among women in Zimbabwe in relation to their self-perceived risk of HIV infection, child mortality, and spontaneous abortion. AIDS Behav 2004; 8(1):9-15.

(115) Mudzviti T, Maponga CC, Khoza S, Ma Q, Morse GD: The impact of herbal drug use on adverse drug reaction profiles of patients on antiretroviral therapy in zimbabwe. AIDS Res Treat 2012; 2012:434171.

(116) Bepe N, Madanhi N, Mudzviti T et al.: The impact of herbal remedies on adverse effects and quality of life in HIV-infected individuals on antiretroviral therapy. J Infect Dev Ctries 2011; 5(1):48-53.

(117) Monera TG, Wolfe AR, Maponga CC, Benet LZ, Guglielmo J: Moringa oleifera leaf extracts inhibit 6beta-hydroxylation of testosterone by CYP3A4. J Infect Dev Ctries 2008; 2(5):379-383.

(118) Mahomva A. Zimbabwe PMTCT program, Annual Report. 2007.

(119) UNAIDS/WHO Working Group on Global HIV/AIDS and STI Surveillance. Guidelines for conducting HIV sentinel sero-surveys among pregnant women and other groups. 2003. Geneva, UNAIDS.

(120) Decosas J, Padian N: The profile and context of the epidemics of sexually transmitted infections including HIV in Zimbabwe. Sex Transm Infect 2002; 78 Suppl 1:i40-i46.

(121) Hargrove JW, Humphrey JH, Mutasa K et al.: Improved HIV-1 incidence estimates using the BED capture enzyme immunoassay. AIDS 2008; 22(4):511-518.

(122) Humphrey JH, Hargrove JW, Malaba LC et al.: HIV incidence among post-partum women in Zimbabwe: risk factors and the effect of vitamin A supplementation. AIDS 2006; 20(10):1437-1446.

(123) Humphrey JH, Nathoo KJ, Hargrove JW et al.: HIV-1 and HIV-2 prevalence and associated risk factors among postnatal women in Harare, Zimbabwe. Epidemiol Infect 2007; 135(6):933-942.

(124) Zimbabwe 2005-06: results from the Demographic and Health Survey: Stud Fam Plann 2008; 39(3):227-232.

(125) Mbizvo MT, Mashu A, Chipato T et al.: Trends in HIV-1 and HIV-2 prevalence and risk factors in pregnant women in Harare, Zimbabwe. Cent Afr J Med 1996; 42(1):14-21.

(126) Forland F, Eriksen K: [HIV-prevalence in Mutoko, Zimbabwe. A study among pregnant women and patients with sexually transmitted diseases]. Tidsskr Nor Laegeforen 1994; 114(9):1050-1052.

(127) Obi CL, McAdoo HP, Murray M, Tswana SA, Moyo SR: HIV infection and HIV-1 clades among pregnant women in Harare, Zimbabwe. Cent Afr J Med 1997; 43(7):188-192.

(128) Mbizvo EM, Msuya SE, Stray-Pedersen B et al.: HIV seroprevalence and its associations with the other reproductive tract infections in asymptomatic women in Harare, Zimbabwe. Int J STD AIDS 2001; 12(8):524-531.

(129) Gonese E, Dzangare J, Gregson S et al.: Comparison of HIV prevalence estimates for Zimbabwe from antenatal clinic surveillance (2006) and the 2005-06 Zimbabwe Demographic and Health Survey. PLoS One 2010; 5(11):e13819.

(130) Mahomva A, Greby S, Dube S et al.: HIV prevalence and trends from data in Zimbabwe, 1997-2004. Sex Transm Infect 2006; 82 Suppl 1:i42-i47.

(131) Gregson S, Zhuwau T, Anderson RM, Chandiwana SK: Is there evidence for behaviour change in response to AIDS in rural Zimbabwe? Soc Sci Med 1998; 46(3):321-330.

(132) Smee N, Shetty AK, Stranix-Chibanda L et al.: Factors Associated With Repeat Pregnancy Among Women in an Area of High HIV Prevalence in Zimbabwe. Womens Health Issues 2011; 21(3):222-229.

(133) State of the World's Children Special Edition, Statistical Tables, UNICEF 2009.

(134) UNICEF UNDPD. Levels & Trends in Child Mortality, Report 2010, Estimates Developed by the UN Inter-agency Group for Child Mortality Estimation, 2010.

(135) UNICEF 2011. State of the World's Children, Statistical Tables. 2011.

(136) Marinda ET, Moulton LH, Humphrey JH et al.: In utero and intra-partum HIV-1 transmission and acute HIV-1 infection during pregnancy: using the BED capture enzyme-immunoassay as a surrogate marker for acute infection. Int J Epidemiol 2011.

(137) Ministry of Health and Child welfare. More Efficacious ARV Prophylaxis of Mother to Child Transmission (PMTCT), Module 9. 2008.

(138) Dube S, Boily MC, Mugurungi O et al.: Estimating vertically acquired HIV infections and the impact of the prevention of mother-to-child transmission program in Zimbabwe: insights from decision analysis models. J Acquir Immune Defic Syndr 2008; 48(1):72-81.

(139) Ferrand RA, Corbett EL, Wood R et al.: AIDS among older children and adolescents in Southern Africa: projecting the time course and magnitude of the epidemic. AIDS 2009; 23(15):2039-2046.

(140) Marazzi MC, Liotta G, Nielsen-Saines K et al.: Extended antenatal antiretroviral use correlates with improved infant outcomes throughout the first year of life. AIDS 2010; 24(18):2819-2826.

(141) Dryden-Peterson S, Jayeoba O, Hughes MD et al.: Highly active antiretroviral therapy versus zidovudine for prevention of mother-to-child transmission in a programmatic setting, Botswana. J Acquir Immune Defic Syndr 2011; 58(3):353-357.

(142) Kuhn L, Sinkala M, Thea DM, Kankasa C, Aldrovandi GM: HIV prevention is not enough: child survival in the context of prevention of mother to child HIV transmission. J Int AIDS Soc 2009; 12(1):36.

(143) Nathoo K, Rusakaniko S, Zijenah LS et al.: Survival pattern among infants born to human immunodeficiency virus type-1 infected mothers and uninfected mothers in Harare, Zimbabwe. Cent Afr J Med 2004; 50(1-2):1-6.

(144) Zijenah LS, Moulton LH, Iliff P et al.: Timing of mother-to-child transmission of HIV-1 and infant mortality in the first 6 months of life in Harare, Zimbabwe. AIDS 2004; 18(2):273-280.

(145) Kuhn L, Reitz C, Abrams EJ: Breastfeeding and AIDS in the developing world. Curr Opin Pediatr 2009; 21(1):83-93.

(146) Kuhn L, Aldrovandi GM: Clean water helps but is not enough: challenges for safe replacement feeding of infants exposed to HIV. J Infect Dis 2009; 200(8):1183-1185.

(147) Horvath T, Madi BC, Iuppa IM et al.: Interventions for preventing late postnatal mother-to-child transmission of HIV. Cochrane Database Syst Rev 2009;(1):CD006734.

(148) Ministry of Health and Child welfare. PMTCT Programme 2008 Annual Report, AIDS & TB Unit, MOHCW. 2008.

(149) Gumbo FZ, Kurewa NE, Kandawasvika GQ et al.: Rising mother-to-child HIV transmission in a resource-limited breastfeeding population. Trop Doct 2010; 40(2):70-73.

(150) Gumbo FZ, Kandawasvika GQ, Duri K et al.: Reduced HIV transmission at subsequent pregnancy in a resource-poor setting. Trop Doct 2011.

(151) Singh KK, Spector SA: Host genetic determinants of human immunodeficiency virus infection and disease progression in children. Pediatr Res 2009; 65(5 Pt 2):55R-63R.

(152) Kourtis AP, Bulterys M: Mother-to-child transmission of HIV: pathogenesis, mechanisms and pathways. Clin Perinatol 2010; 37(4):721-37, vii.

(153) Singh KK, Hughes MD, Chen J et al.: Associations of chemokine receptor polymorphisms With HIV-1 mother-to-child transmission in sub-Saharan Africa: possible modulation of genetic effects by antiretrovirals. J Acquir Immune Defic Syndr 2008; 49(3):259-265.

(154) Pedersen BR, Kamwendo D, Blood M et al.: CCR5 haplotypes and mother-to-child HIV transmission in Malawi. PLos One 2007; 2(9):e838.

(155) Ricci E, Malacrida S, Zanchetta M et al.: Toll-like receptor 9 polymorphisms influence mother-to-child transmission of human immunodeficiency virus type 1. J Transl Med 2010; 8:49.

(156) Mangano A, Rocco C, Marino SM et al.: Detrimental effects of mannose-binding lectin (MBL2) promoter genotype XA/XA on HIV-1 vertical transmission and AIDS progression. J Infect Dis 2008; 198(5):694-700.

(157) Mackelprang RD, John-Stewart G, Carrington M et al.: Maternal HLA homozygosity and mother-child HLA concordance increase the risk of vertical transmission of HIV-1. J Infect Dis 2008; 197(8):1156-1161.

(158) Tsegaye TS, Pohlmann S: The multiple facets of HIV attachment to dendritic cell lectins. Cell Microbiol 2010; 12(11):1553-1561.

(159) Boily-Larouche G, Milev MP, Zijenah LS et al.: Naturally-Occurring Genetic Variants in Human DC-SIGN Increase HIV-1 Capture, Cell-Transfer and Risk of Mother-To-Child Transmission. PLos One 2012; 7(7):e40706.

(160) Humphrey JH, Marinda E, Mutasa K et al.: Mother to child transmission of HIV among Zimbabwean women who seroconverted postnatally: prospective cohort study. BMJ 2010; 341:c6580.

(161) Mark S, Murphy KE, Read S, Bitnun A, Yudin MH: HIV Mother-to-Child Transmission, Mode of Delivery, and Duration of Rupture of Membranes: Experience in the Current Era. Infect Dis Obstet Gynecol 2012; 2012:267969.

(162) Kuhn L, Semrau K, Ramachandran S et al.: Mortality and virologic outcomes after access to antiretroviral therapy among a cohort of HIV-infected women who received single-dose nevirapine in Lusaka, Zambia. J Acquir Immune Defic Syndr 2009; 52(1):132-136.

(163) Clewley JP: Enigmas and paradoxes: the genetic diversity and prevalence of the primate lentiviruses. Curr HIV Res 2004; 2(2):113-125.

(164) Palmer E, Goldsmith CS: Ultrastructure of human retroviruses. J Electron Microsc Tech 1988; 8(1):3-15.

(165) Checkley MA, Luttge BG, Freed EO: HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. J Mol Biol 2011; 410(4):582-608.

(166) Frankel AD, Young JA: HIV-1: fifteen proteins and an RNA. Annu Rev Biochem 1998; 67:1-25.

(167) Janeway CA, Travers P, Walport M, Shlomchik MJ. Failures of Host Defense Mechanisms. In: ***Immunobiology: The Immune System in Health & Disease***. Lawrence E, ed. Sixth Edition ed. **Garland Science** , New York, 2005, pp. 491-508.

(168) Hill M, Tachedjian G, Mak J: The packaging and maturation of the HIV-1 Pol proteins. Curr HIV Res 2005; 3(1):73-85.

(169) Seyoum E, Wolday D, Girma M et al.: Reverse transcriptase activity for quantitation of HIV-1 subtype C in plasma: relation to RNA copy number and CD4 T-cell count. J Med Virol 2006; 78(2):161-168.

(170) Chen N, Morag A, Almog N et al.: Extended nucleocapsid protein is cleaved from the Gag-Pol precursor of human immunodeficiency virus type 1. J Gen Virol 2001; 82(Pt 3):581-590.

(171) Wu X, Liu H, Xiao H et al.: Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. EMBO J 1997; 16(16):5113-5122.

(172) Sengupta S, Jana S, Roy P et al.: Phylogenetic analysis of the p24-p7 region of the human immunodeficiency virus type 1 gag gene to determine subtype distribution among female sex workers in Calcutta, India. J Clin Microbiol 2005; 43(11):5787-5791.

(173) Waheed AA, Ono A, Freed EO: Methods for the study of HIV-1 assembly. Methods Mol Biol 2009; 485:163-184.

(174) Scriba TJ, de VT, Treurnicht FK et al.: Characterization of the South African HIV type 1 subtype C complete 5' long terminal repeat, nef, and regulatory genes. AIDS Res Hum Retroviruses 2002; 18(2):149-159.

(175) Stroud JC, Oltman A, Han A, Bates DL, Chen L: Structural basis of HIV-1 activation by NF-kappaB-a higher-order complex of p50:RelA bound to the HIV-1 LTR. J Mol Biol 2009; 393(1):98-112.

(176) Le Grice SF: "In the beginning": initiation of minus strand DNA synthesis in retroviruses and LTR-containing retrotransposons. Biochemistry 2003; 42(49):14349-14355.

(177) Berger EA, Murphy PM, Farber JM: Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 1999; 17:657-700.

(178) Starcich BR, Hahn BH, Shaw GM et al.: Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell 1986; 45(5):637-648.

(179) Zhang M, Gaschen B, Blay W et al.: Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology 2004; 14(12):1229-1246.

(180) Kwong PD, Wyatt R, Sattentau QJ, Sodroski J, Hendrickson WA: Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. J Virol 2000; 74(4):1961-1972.

(181) Wei X, Decker JM, Wang S et al.: Antibody neutralization and escape by HIV-1. Nature 2003; 422(6929):307-312.

(182) Dieltjens T, Loots N, Vereecken K et al.: HIV type 1 subtype A envelope genetic evolution in a slow progressing individual with consistent broadly neutralizing antibodies. AIDS Res Hum Retroviruses 2009; 25(11):1165-1169.

(183) Bunnik EM, Pisas L, van Nuenen AC, Schuitemaker H: Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. J Virol 2008; 82(16):7932-7941.

(184) Derdeyn CA, Decker JM, Bibollet-Ruche F et al.: Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science 2004; 303(5666):2019-2022.

(185) Derdeyn CA, Hunter E: Viral characteristics of transmitted HIV. Curr Opin HIV AIDS 2008; 3(1):16-21.

(186) Zhang H, Tully DC, Hoffmann FG et al.: Restricted genetic diversity of HIV-1 subtype C envelope glycoprotein from perinatally infected Zambian infants. PLoS One 2010; 5(2):e9294.

(187) Chohan B, Lang D, Sagar M et al.: Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J Virol 2005; 79(10):6528-6531.

(188) Wu L: The role of monocyte-lineage cells in human immunodeficiency virus persistence: mechanisms and progress. 2011; 6(3):129-132.

(189) Choe H, Farzan M, Sun Y et al.: The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996; 85(7):1135-1148.

(190) Chen Z, Zhao X, Huang Y et al.: CD4+ lymphocytopenia in acute infection of Asian macaques by a vaginally transmissible subtype-C, CCR5-tropic Simian/Human Immunodeficiency Virus (SHIV). J Acquir Immune Defic Syndr 2002; 30(2):133-145.

(191) Suresh P, Wanchu A: Chemokines and chemokine receptors in HIV infection: role in pathogenesis and therapeutics. J Postgrad Med 2006; 52(3):210-217.

(192) Zhang C, Xu S, Wei J, Guo H: Predicted co-receptor tropism and sequence characteristics of China HIV-1 V3 loops: implications for the future usage of CCR5 antagonists and AIDS vaccine development. Int J Infect Dis 2009; 13(5):e212-e216.

(193) Hoffman TL, Stephens EB, Narayan O, Doms RW: HIV type I envelope determinants for use of the CCR2b, CCR3, STRL33, and APJ coreceptors. Proc Natl Acad Sci U S A 1998; 95(19):11360-11365.

(194) Gorry PR, Ancuta P: Coreceptors and HIV-1 pathogenesis. Curr HIV /AIDS Rep 2011; 8(1):45-53.

(195) Pilcher CD, Eron JJ, Jr., Galvin S, Gay C, Cohen MS: Acute HIV revisited: new opportunities for treatment and prevention. J Clin Invest 2004; 113(7):937-945.

(196) Geijtenbeek TB, Torensma R, van Vliet SJ et al.: Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000; 100(5):575-585.

(197) Wu L, KewalRamani VN: Dendritic-cell interactions with HIV: infection and viral dissemination. Nat Rev Immunol 2006; 6(11):859-868.

(198) Moir S, Malaspina A, Li Y et al.: B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells. J Exp Med 2000; 192(5):637-646.

(199) Salazar-Gonzalez JF, Salazar MG, Keele BF et al.: Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med 2009; 206(6):1273-1289.

(200) Nilson KA, Price DH: The Role of RNA Polymerase II Elongation Control in HIV-1 Gene Expression, Replication, and Latency. Genet Res Int 2011; 2011:726901.

(201) Fukuhara T, Hosoya T, Shimizu S et al.: Utilization of host SR protein kinases and RNA-splicing machinery during viral replication. Proc Natl Acad Sci U S A 2006; 103(30):11329-11333.

(202) Chung J, Rossi JJ, Jung U: Current progress and challenges in HIV gene therapy. Future Virol 2011; 6(11):1319-1328.

(203) Brenchley JM, Schacker TW, Ruff LE et al.: CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J Exp Med 2004; 200(6):749-759.

(204) Ball TB, Ji H, Kimani J et al.: Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers. AIDS 2007; 21(9):1091-1101.

(205) Philpott S, Burger H, Charbonneau T et al.: CCR5 genotype and resistance to vertical transmission of HIV-1. J Acquir Immune Defic Syndr 1999; 21(3):189-193.

(206) Merino A, Malhotra R, Morton M et al.: Impact of a functional KIR2DS4 allele on heterosexual HIV-1 transmission among discordant Zambian couples. J Infect Dis 2011; 203(4):487-495.

(207) Carrington M, O'Brien SJ: The influence of HLA genotype on AIDS. Annu Rev Med 2003; 54:535-551.

(208) Rousseau CM, Daniels MG, Carlson JM et al.: HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load. J Virol 2008; 82(13):6434-6446.

(209) Choi RY, Farquhar C, Juno J et al.: Infant CD4 C868T polymorphism is associated with increased human immunodeficiency virus (HIV-1) acquisition. Clin Exp Immunol 2010; 160(3):461-465.

(210) Poonia B, Kijak GH, Pauza CD: High affinity allele for the gene of FCGR3A is risk factor for HIV infection and progression. PLoS One 2010; 5(12):e15562.

(211) Shankarkumar U, Pawar A, Ghosh K, Bajpai S, Pazare A: Human leucocyte antigen class II DRB1 and DQB1 associations in human immunodeficiency virus-infected patients of Mumbai, India. Int J Immunogenet 2010; 37(3):199-204.

(212) Naicker DD, Werner L, Kormuth E et al.: Interleukin-10 promoter polymorphisms influence HIV-1 susceptibility and primary HIV-1 pathogenesis. J Infect Dis 2009; 200(3):448-452.

(213) Chatterjee A, Rathore A, Yamamoto N, Dhole TN: Mannose-binding lectin (+54) exon-1 gene polymorphism influence human immunodeficiency virus-1 susceptibility in North Indians. Tissue Antigens 2011; 77(1):18-22.

(214) McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF: The immune response during acute HIV-1 infection: clues for vaccine development. Nat Rev Immunol 2010; 10(1):11-23.

(215) Malhotra U, Huntsberry C, Holte S et al.: CD4+ T cell receptor repertoire perturbations in HIV-1 infection: association with plasma viremia and disease progression. Clin Immunol 2006; 119(1):95-102.

(216) Barry M, Mulcahy F, Back DJ: Antiretroviral therapy for patients with HIV disease. Br J Clin Pharmacol 1998; 45(3):221-228.

(217) Dinoso JB, Kim SY, Wiegand AM et al.: Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy. Proc Natl Acad Sci U S A 2009; 106(23):9403-9408.

(218) Morrison CS, Demers K, Kwok C et al.: Plasma and cervical viral loads among Ugandan and Zimbabwean women during acute and early HIV-1 infection. AIDS 2010; 24(4):573-582.

(219) Hayes RJ, White RG: Role of acute infection in HIV transmission. Lancet 2011; 378(9807):1913-1914.

(220) Kahn JO, Walker BD: Acute human immunodeficiency virus type 1 infection. N Engl J Med 1998; 339(1):33-39.

(221) De Santis GC, Brunetta DM, Vilar FC et al.: Hematological abnormalities in HIV-infected patients. Int J Infect Dis 2011; 15(12):e808-e811.

(222) Gomo E, Ndhlovu P, Vennervald BJ, Nyazema N, Friis H: Enumeration of CD4 and CD8 T-cells in HIV infection in Zimbabwe using a manual immunocytochemical method. Cent Afr J Med 2001; 47(3):64-70.

(223) Ly TD, Ebel A, Faucher V, Fihman V, Laperche S: Could the new HIV combined p24 antigen and antibody assays replace p24 antigen specific assays? J Virol Methods 2007; 143(1):86-94.

(224) Cohen MS, Gay CL, Busch MP, Hecht FM: The detection of acute HIV infection. J Infect Dis 2010; 202 Suppl 2:S270-S277.

(225) Schacker TW, Hughes JP, Shea T, Coombs RW, Corey L: Biological and virologic characteristics of primary HIV infection. Ann Intern Med 1998; 128(8):613-620.

(226) Pantaleo G, Fauci AS: Immunopathogenesis of HIV infection. Annu Rev Microbiol 1996; 50:825-854.

(227) Mellors JW, Rinaldo CR, Gupta P, White MR, Todd JA and Kingsley LA. Prognosis of HIV-1 infection predicted by the quantity of virus in plasma. Science 272, 1167-1170. 1996.

(228) Pantaleo G, Cohen OJ, Schacker T et al.: Evolutionary pattern of human immunodefiency virus (HIV) replication and distribution in lymph nodes following primary infection: implication for antiviral therapy. Nat Med 1998; 4:341-345.

(229) Langford SE, Ananworanich J, Cooper DA: Predictors of disease progression in HIV infection: a review. AIDS Res Ther 2007; 4:11.

(230) Sharma G, Kaur G, Mehra N: Genetic correlates influencing immunopathogenesis of HIV infection. Indian J Med Res 2011; 134(6):749-768.

(231) Daar ES, Moudgil T, Meyer RD, Ho DD: Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. N Engl J Med 1991; 324(14):961-964.

(232) Clark SJ, Saag MS, Decker WD et al.: High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. N Engl J Med 1991; 324(14):954-960.

(233) Sinicco A, Biglino A, Sciandra M et al.: Cytokine network and acute primary HIV-1 infection. AIDS 1993; 7(9):1167-1172.

(234) Koup RA, Safrit JT, Cao Y et al.: Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994; 68(7):4650-4655.

(235) Keefe D, Shi L, Feske S et al.: Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity 2005; 23(3):249-262.

(236) Goulder PJ, Rowland-Jones SL, McMichael AJ, Walker BD: Anti-HIV cellular immunity: recent advances towards vaccine design. AIDS 1999; 13 Suppl A:S121-S136.

(237) Betts MR, Ambrozak DR, Douek DC et al.: Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. J Virol 2001; 75(24):11983-11991.

(238) Cocchi F, Devico AL, Garzino-Demo A et al.: Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995; 270(5243):1811-1815.

(239) Gonzalez E, Dhanda R, Bamshad M et al.: Global survey of genetic variation in CCR5, RANTES, and MIP-1alpha: impact on the epidemiology of the HIV-1 pandemic. Proc Natl Acad Sci U S A 2001; 98(9):5199-5204.

(240) Liu R, Paxton WA, Choe S et al.: Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 1996; 86(3):367-377.

(241) Snoeck J, Fellay J, Bartha I, Douek DC, Telenti A: Mapping of positive selection sites in the HIV-1 genome in the context of RNA and protein structural constraints. Retrovirology 2011; 8:87.

(242) Malim MH: APOBEC proteins and intrinsic resistance to HIV-1 infection. Philos Trans R Soc Lond B Biol Sci 2009; 364(1517):675-687.

(243) Sheehy AM, Gaddis NC, Choi JD, Malim MH: Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 2002; 418(6898):646-650.

(244) Lin TY, Emerman M: Determinants of cyclophilin A-dependent TRIM5 alpha restriction against HIV-1. Virology 2008; 379(2):335-341.

(245) Neil SJ, Zang T, Bieniasz PD: Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature 2008; 451(7177):425-430.

(246) Sampathkumar R, Shadabi E, Luo M: Interplay between HIV-1 and Host Genetic Variation: A Snapshot into Its Impact on AIDS and Therapy Response. Adv Virol 2012; 2012:508967.

(247) Ho DD, Neumann AU, Perelson AS et al.: Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 1995; 373(6510):123-126.

(248) Salazar-Gonzalez JF, Martinez-Maza O, Nishanian P et al.: Increased immune activation precedes the inflection point of CD4 T cells and the increased serum virus load in human immunodeficiency virus infection. J Infect Dis 1998; 178(2):423-430.

(249) Aukrust P, Liabakk NB, Muller F, Espevik T, Froland SS: Activation of tumor necrosis factor--alpha system in HIV-1 infection: association with markers of immune activation. Infection 1995; 23(1):9-15.

(250) Bass HZ, Nishanian P, Hardy WD et al.: Immune changes in HIV-1 infection: significant correlations and differences in serum markers and lymphoid phenotypic antigens. Clin Immunol Immunopathol 1992; 64(1):63-70.

(251) Villinger F, Rowe T, Parekh BS et al.: Chronic immune stimulation accelerates SIV-induced disease progression. J Med Primatol 2001; 30(5):254-259.

(252) Fahey JL, Taylor JM, Manna B et al.: Prognostic significance of plasma markers of immune activation, HIV viral load and CD4 T-cell measurements. AIDS 1998; 12(13):1581-1590.

(253) Fahey JL: Cytokines, plasma immune activation markers, and clinically relevant surrogate markers in human immunodeficiency virus infection. Clin Diagn Lab Immunol 1998; 5(5):597-603.

(254) Lee C, Liu QH, Tomkowicz B et al.: Macrophage activation through. J Leukoc Biol 2003; 74(5):676-682.

(255) Wang JK, Kiyokawa E, Verdin E, Trono D: The Nef protein of HIV-1 associates with rafts and primes T cells for activation. Proc Natl Acad Sci U S A 2000; 97(1):394-399.

(256) Kawakami K, Scheidereit C, Roeder RG: Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-kappa B) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc Natl Acad Sci U S A 1988; 85(13):4700-4704.

(257) Appay V, Sauce D: Immune activation and inflammation in HIV-1 infection: causes and consequences. J Pathol 2008; 214(2):231-241.

(258) Mogensen TH, Melchjorsen J, Larsen CS, Paludan SR: Innate immune recognition and activation during HIV infection. Retrovirology 2010; 7:54.

(259) Brenchley JM, Price DA, Schacker TW et al.: Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006; 12(12):1365-1371.

(260) Brenchley JM, Douek DC: Microbial translocation across the GI tract. Annu Rev Immunol 2012; 30:149-173.

(261) Groux H, Torpier G, Monte D et al.: Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med 1992; 175(2):331-340.

(262) Almeida JR, Price DA, Papagno L et al.: Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med 2007; 204(10):2473-2485.

(263) Khaitan A, Unutmaz D: Revisiting immune exhaustion during HIV infection. Curr HIV /AIDS Rep 2011; 8(1):4-11.

(264) Leroy V, Salmi LR, Dupon M et al.: Progression of human immunodeficiency virus infection in patients with tuberculosis disease. A cohort study in Bordeaux, France, 1988-1994. The Groupe d'Epidemiologie Clinique du Sida en Aquitaine (GECSA). Am J Epidemiol 1997; 145(4):293-300.

(265) Manabe YC, Clark DP, Moore RD et al.: Cryptosporidiosis in patients with AIDS: correlates of disease and survival. Clin Infect Dis 1998; 27(3):536-542.

(266) Spector SA, Wong R, Hsia K, Pilcher M, Stempien MJ: Plasma cytomegalovirus (CMV) DNA load predicts CMV disease and survival in AIDS patients. J Clin Invest 1998; 101(2):497-502.

(267) Chaisson RE, Gallant JE, Keruly JC, Moore RD: Impact of opportunistic disease on survival in patients with HIV infection. AIDS 1998; 12(1):29-33.

(268) Mocroft A, Youle M, Phillips AN et al.: The incidence of AIDS-defining illnesses in 4883 patients with human immunodeficiency virus infection. Royal Free/Chelsea and Westminster Hospitals Collaborative Group. Arch Intern Med 1998; 158(5):491-497.

(269) Hammer SM, Eron JJ, Jr., Reiss P et al.: Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. JAMA 2008; 300(5):555-570.

(270) Andersson J, Fehniger TE, Patterson BK et al.: Early reduction of immune activation in lymphoid tissue following highly active HIV therapy. AIDS 1998; 12(11):F123-F129.

(271) Guadalupe M, Sankaran S, George MD et al.: Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. J Virol 2006; 80(16):8236-8247.

(272) Arts EJ, Hazuda DJ: HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb Perspect Med 2012; 2(4):a007161.

(273) Hamlyn E, Ewings FM, Porter K et al.: Plasma HIV Viral Rebound following Protocol-Indicated Cessation of ART Commenced in Primary and Chronic HIV Infection. PLoS One 2012; 7(8):e43754.

(274) Hester EK: HIV medications: an update and review of metabolic complications. Nutr Clin Pract 2012; 27(1):51-64.

(275) Battegay M, Nuesch R, Hirschel B, Kaufmann GR: Immunological recovery and antiretroviral therapy in HIV-1 infection. Lancet Infect Dis 2006; 6(5):280-287.

(276) Ledergerber B, Lundgren JD, Walker AS et al.: Predictors of trend in CD4-positive T-cell count and mortality among HIV-1-infected individuals with virological failure to all three antiretroviral-drug classes. Lancet 2004; 364(9428):51-62.

(277) Guihot A, Tubiana R, Breton G et al.: Immune and virological benefits of 10 years of permanent viral control with antiretroviral therapy. AIDS 2010; 24(4):614-617.

(278) Pinzone MR, Di RM, Cacopardo B, Nunnari G: HIV RNA suppression and immune restoration: can we do better? Clin Dev Immunol 2012; 2012:515962.

(279) Guadalupe M, Reay E, Sankaran S et al.: Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. J Virol 2003; 77(21):11708-11717.

(280) Biggar RJ, Chaturvedi AK, Goedert JJ, Engels EA: AIDS-related cancer and severity of immunosuppression in persons with AIDS. J Natl Cancer Inst 2007; 99(12):962-972.

(281) Malfitano A, Barbaro G, Perretti A, Barbarini G: Human immunodeficiency virus-associated malignancies: a therapeutic update. Curr HIV Res 2012; 10(2):123-132.

(282) Tserenpuntsag B, Kolacinska A, Jablonowska E: [AIDS associated cancers in the era of highly active antiretroviral therapy (HAART)]. Przegl Epidemiol 2007; 61(3):529-534.

(283) Shelburne SA, Montes M, Hamill RJ: Immune reconstitution inflammatory syndrome: more answers, more questions. J Antimicrob Chemother 2006; 57(2):167-170.

(284) Tappuni AR: Immune reconstitution inflammatory syndrome. Adv Dent Res 2011; 23(1):90-96.

(285) Appay V, Hansasuta P, Sutton J et al.: Persistent HIV-1-specific cellular responses despite prolonged therapeutic viral suppression. AIDS 2002; 16(2):161-170.

(286) Huis i', V, Sun HY, Hung CC, Colebunders R: The immune reconstitution inflammatory syndrome related to HIV co-infections: a review. Eur J Clin Microbiol Infect Dis 2012; 31(6):919-927.

(287) Kaufmann GR, Perrin L, Pantaleo G et al.: CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study. Arch Intern Med 2003; 163(18):2187-2195.

(288) Gras L, Kesselring AM, Griffin JT et al.: CD4 cell counts of 800 cells/mm3 or greater after 7 years of highly active antiretroviral therapy are feasible in most patients starting with 350 cells/mm3 or greater. J Acquir Immune Defic Syndr 2007; 45(2):183-192.

(289) Mansky LM, Bernard LC: 3'-Azido-3'-deoxythymidine (AZT) and AZT-resistant reverse transcriptase can increase the in vivo mutation rate of human immunodeficiency virus type 1. J Virol 2000; 74(20):9532-9539.

(290) Kolber MA, Buendia P, DeGruttola V, Moore RD: HIV-1 diversity after a class switch failure. AIDS Res Hum Retroviruses 2010; 26(11):1175-1180.

(291) Coffin JM: HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 1995; 267(5197):483-489.

(292) Chipunza P. **Acute ARV shortage hits Zimbabwe**. The Herald . 11-9-2012.

(293) Lynch RM, Shen T, Gnanakaran S, Derdeyn CA: Appreciating HIV type 1 diversity: subtype differences in Env. AIDS Res Hum Retroviruses 2009; 25(3):237-248.

(294) McCutchan FE, Viputtigul K, de Souza MS et al.: Diversity of envelope glycoprotein from human immunodeficiency virus type 1 of recent seroconverters in Thailand. AIDS Res Hum Retroviruses 2000; 16(8):801-805.

(295) McCutchan FE, Artenstein AW, Sanders-Buell E et al.: Diversity of the envelope glycoprotein among human immunodeficiency virus type 1 isolates of clade E from Asia and Africa. J Virol 1996; 70(6):3331-3338.

(296) McCutchan FE, Salminen MO, Carr JK, Burke DS: HIV-1 genetic diversity. AIDS 1996; 10 Suppl 3:S13-S20.

(297) Almond D, Kimura T, Kong X et al.: Structural conservation predominates over sequence variability in the crown of HIV type 1's V3 loop. AIDS Res Hum Retroviruses 2010; 26(6):717-723.

(298) Mercado JM, Di DR, Pradal MG: Genetic diversity of HIV-1 subtype F from Brazil: failure of HIV-1 viral load testing based on molecular biology amplification methods. AIDS 1999; 13(15):2183-2185.

(299) Brenner BG, Oliveira M, Doualla-Bell F et al.: HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture. AIDS 2006; 20(9):F9-13.

(300) Clewley JP: Genetic diversity and HIV detection by PCR. Lancet 1995; 346(8988):1489.

(301) Diversity of HIV strains impacts diagnostic test accuracy: AIDS Patient Care STDS 2009; 23(3):220.

(302) Apetrei C, Loussert-Ajaka I, Descamps D et al.: Lack of screening test sensitivity during HIV-1 non-subtype B seroconversions. AIDS 1996; 10(14):F57-F60.

(303) Bolivar H, Geffin R, Manzi G et al.: The challenge of HIV-1 genetic diversity: discordant CD4+ T-Cell count and viral load in an untreated patient infected with a subtype F strain. J Acquir Immune Defic Syndr 2009; 52(5):659-661.

(304) Julg B, Goebel FD: HIV genetic diversity: any implications for drug resistance? Infection 2005; 33(4):299-301.

(305) Spira S, Wainberg MA, Loemba H, Turner D, Brenner BG: Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance. J Antimicrob Chemother 2003; 51(2):229-240.

(306) Edwards CT, Holmes EC, Wilson DJ et al.: Population genetic estimation of the loss of genetic diversity during horizontal transmission of HIV-1. BMC Evol Biol 2006; 6:28.

(307) Lange A, Ferguson NM: Antigenic diversity, transmission mechanisms, and the evolution of pathogens. PLoS Comput Biol 2009; 5(10):e1000536.

(308) Papathanasopoulos MA, Hunt GM, Tiemessen CT: Evolution and diversity of HIV-1 in Africa-a review. Virus Genes 2003; 26(2):151-163.

(309) Tebit DM, Nankya I, Arts EJ, Gao Y: HIV diversity, recombination and disease progression: how does fitness "fit" into the puzzle? AIDS Rev 2007; 9(2):75-87.

(310) Saag MS, Hammer SM, Lange JM: Pathogenicity and diversity of HIV and implications for clinical management: a review. J Acquir Immune Defic Syndr 1994; 7 Suppl 2:S2-10.

(311) Lal RB, Chakrabarti S, Yang C: Impact of genetic diversity of HIV-1 on diagnosis, antiretroviral therapy & vaccine development. Indian J Med Res 2005; 121(4):287-314.

(312) Bonhoeffer S, Holmes EC, Nowak MA: Causes of HIV diversity. Nature 1995; 376(6536):125.

(313) Takebe Y: [HIV-1 genetic diversity: mechanism and its biological implication]. Uirusu 2001; 51(2):123-134.

(314) Oelrichs RB, Shrestha IL, Anderson DA, Deacon NJ: The explosive human immunodeficiency virus type 1 epidemic among injecting drug users of Kathmandu, Nepal, is caused by a subtype C virus of restricted genetic diversity. J Virol 2000; 74(3):1149-1157.

(315) Shriner D, Rodrigo AG, Nickle DC, Mullins JI: Pervasive genomic recombination of HIV-1 in vivo. Genetics 2004; 167(4):1573-1583.

(316) Fang G, Weiser B, Kuiken C et al.: Recombination following superinfection by HIV-1. AIDS 2004; 18(2):153-159.

(317) Machuca A, Tang S, Hu J et al.: Increased genetic diversity and intersubtype recombinants of HIV-1 in blood donors from urban Cameroon. J Acquir Immune Defic Syndr 2007; 45(3):361-363.

(318) Vijay NN, Vasantika, Ajmani R, Perelson AS, Dixit NM: Recombination increases human immunodeficiency virus fitness, but not necessarily diversity. J Gen Virol 2008; 89(Pt 6):1467-1477.

(319) Delviks-Frankenberry K, Galli A, Nikolaitchik O et al.: Mechanisms and factors that influence high frequency retroviral recombination. Viruses 2011; 3(9):1650-1680.

(320) van der Kuyl AC, Cornelissen M: Identifying HIV-1 dual infections. Retrovirology 2007; 4:67.

(321) Templeton AR, Kramer MG, Jarvis J et al.: Multiple-infection and recombination in HIV-1 within a longitudinal cohort of women. Retrovirology 2009; 6:54.

(322) Chohan B, Lavreys L, Rainwater SM, Overbaugh J: Evidence for frequent reinfection with human immunodeficiency virus type 1 of a different subtype. J Virol 2005; 79(16):10701-10708.

(323) Hu DJ, Subbarao S, Vanichseni S et al.: Frequency of HIV-1 dual subtype infections, including intersubtype superinfections, among injection drug users in Bangkok, Thailand. AIDS 2005; 19(3):303-308.

(324) McCutchan FE, Hoelscher M, Tovanabutra S et al.: In-depth analysis of a heterosexually acquired human immunodeficiency virus type 1 superinfection: evolution, temporal fluctuation, and intercompartment dynamics from the seronegative window period through 30 months postinfection. J Virol 2005; 79(18):11693-11704.

(325) Gross KL, Porco TC, Grant RM: HIV-1 superinfection and viral diversity. AIDS 2004; 18(11):1513-1520.

(326) Plantier JC, Lemee V, Dorval I et al.: HIV-1 group M superinfection in an HIV-1 group O-infected patient. AIDS 2004; 18(18):2444-2446.

(327) Gottlieb GS, Nickle DC, Jensen MA et al.: Dual HIV-1 infection associated with rapid disease progression. Lancet 2004; 363(9409):619-622.

(328) Allen TM, Altfeld M: HIV-1 superinfection. J Allergy Clin Immunol 2003; 112(5):829-835.

(329) Grobler J, Gray CM, Rademeyer C et al.: Incidence of HIV-1 dual infection and its association with increased viral load set point in a cohort of HIV-1 subtype C-infected female sex workers. J Infect Dis 2004; 190(7):1355-1359.

(330) Saathoff E, Pritsch M, Geldmacher C et al.: Viral and host factors associated with the HIV-1 viral load setpoint in adults from Mbeya Region, Tanzania. J Acquir Immune Defic Syndr 2010; 54(3):324-330.

(331) Chohan BH, Piantadosi A, Overbaugh J: HIV-1 superinfection and its implications for vaccine design. Curr HIV Res 2010; 8(8):596-601.

(332) Zhang J: "Host RNA polymerase II makes minimal contributions to retroviral frame shift mutations". Journal of General Virology 2004; 85(8):2389-2395.

(333) Eberle J, Gurtler L: HIV types, groups, subtypes and recombinant forms: errors in replication, selection pressure and quasispecies. Intervirology 2012; 55(2):79-83.

(334) Jern P, Russell RA, Pathak VK, and Coffin JM: "Likely role of APOBEC3G-mediated G-to A mutations in HIV-1 evolution and drug resistance". PloS Pathogens 2009; 5(4).

(335) Brander C, Frahm N, Walker BD: The challenges of host and viral diversity in HIV vaccine design. Curr Opin Immunol 2006; 18(4):430-437.

(336) Keele BF, Tazi L, Gartner S et al.: Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1. J Virol 2008; 82(11):5548-5561.

(337) Kuiken C, Thakallapalli R, Esklid A, de RA: Genetic analysis reveals epidemiologic patterns in the spread of human immunodeficiency virus. Am J Epidemiol 2000; 152(9):814-822.

(338) Wain-Hobson S, Myers G: Human immunodeficiency viruses. Too close for comfort. Nature 1990; 347(6288):18.

(339) Sankale JL, De La Tour RS, Marlink RG et al.: Distinct quasi-species in the blood and the brain of an HIV-2-infected individual. Virology 1996; 226(2):418-423.

(340) Goodenow M, Huet T, Saurin W et al.: HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions. J Acquir Immune Defic Syndr 1989; 2(4):344-352.

(341) Lauring AS, Andino R: Quasispecies theory and the behavior of RNA viruses. PLoS Pathog 2010; 6(7):e1001005.

(342) Jackson MM, Stanley SR, Nelson P, Richardson DR, White GB: Compendium of HIV/AIDS positions, policies and documents. April 1992. ANA Publ 1992;(PR-8 .6M):i, 1-i,M1.

(343) Louwagie J, Janssens W, Mascola J et al.: Genetic diversity of the envelope glycoprotein from human immunodeficiency virus type 1 isolates of African origin. J Virol 1995; 69(1):263-271.

(344) Abebe A, Pollakis G, Fontanet AL et al.: Identification of a genetic subcluster of HIV type 1 subtype C (C') widespread in Ethiopia. AIDS Res Hum Retroviruses 2000; 16(17):1909-1914.

(345) Archer J, Robertson DL: Understanding the diversification of HIV-1 groups M and O. AIDS 2007; 21(13):1693-1700.

(346) Aulicino PC, Kopka J, Mangano AM et al.: Circulation of novel HIV type 1 A, B/C, and F subtypes in Argentina. AIDS Res Hum Retroviruses 2005; 21(2):158-164.

(347) Bartolo I, Rocha C, Bartolomeu J et al.: Highly divergent subtypes and new recombinant forms prevail in the HIV/AIDS epidemic in Angola: new insights into the origins of the AIDS pandemic. Infect Genet Evol 2009; 9(4):672-682.

(348) Bredell H, Hunt G, Casteling A et al.: HIV-1 Subtype A, D, G, AG and unclassified sequences identified in South Africa. AIDS Res Hum Retroviruses 2002; 18(9):681-683.

(349) Gao F, Yue L, Robertson DL et al.: Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J Virol 1994; 68(11):7433-7447.

(350) Geretti AM: HIV-1 subtypes: epidemiology and significance for HIV management. Curr Opin Infect Dis 2006; 19(1):1-7.

(351) Diaz RS, Zhang L, Busch MP, Mosley JW, Mayer A: Divergence of HIV-1 quasispecies in an epidemiologic cluster. AIDS 1997; 11(4):415-422.

(352) Gould K: Infection with HIV-1 group O. AIDS Patient Care STDS 1997; 11(6):399-405.

(353) Kanki PJ, Peeters M, Gueye-Ndiaye A: Virology of HIV-1 and HIV-2: implications for Africa. AIDS 1997; 11 Suppl B:S33-S42.

(354) Pando MA, Eyzaguirre LM, Segura M et al.: First report of an HIV-1 triple recombinant of subtypes B, C and F in Buenos Aires, Argentina. Retrovirology 2006; 3:59.

(355) Ramirez BC, Simon-Loriere E, Galetto R, Negroni M: Implications of recombination for HIV diversity. Virus Res 2008; 134(1-2):64-73.

(356) Stebbing J, Moyle G: The clades of HIV: their origins and clinical significance. AIDS Rev 2003; 5(4):205-213.

(357) McCutchan FE, Viputtigul K, de Souza MS et al.: Diversity of envelope glycoprotein from human immunodeficiency virus type 1 of recent seroconverters in Thailand. AIDS Res Hum Retroviruses 2000; 16(8):801-805.

(358) Hahn BH, Shaw GM, De Cock KM, Sharp PM: AIDS as a zoonosis: scientific and public health implications. Science 2000; 287(5453):607-614.

(359) Keele BF, Van HF, Li Y et al.: Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science 2006; 313(5786):523-526.

(360) Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR: An African primate lentivirus (SIVsm) closely related to HIV-2. Nature 1989; 339(6223):389-392.

(361) Johnson PR, Gravell M, Allan J et al.: Genetic diversity among simian immunodeficiency virus isolates from African green monkeys. J Med Primatol 1989; 18(3-4):271-277.

(362) Sharp PM, Hahn BH: Origins of HIV and the AIDS Pandemic. Cold Spring Harb Perspect Med 2011; 1(1):a006841.

(363) Clavel F, Guyader M, Guetard D et al.: Molecular cloning and polymorphism of the human immune deficiency virus type 2. Nature 1986; 324(6098):691-695.

(364) Kanki PJ, Travers KU, Mboup S et al.: Slower heterosexual spread of HIV-2 than HIV-1. Lancet 1994; 343(8903):943-946.

(365) Marlink R, Kanki P, Thior I et al.: Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science 1994; 265(5178):1587-1590.

(366) Adje-Toure CA, Cheingsong R, Garcia-Lerma JG et al.: Antiretroviral therapy in HIV-2-infected patients: changes in plasma viral load, CD4+ cell counts, and drug resistance profiles of patients treated in Abidjan, Cote d'Ivoire. AIDS 2003; 17 Suppl 3:S49-S54.

(367) Motomura K, Chen J, Hu WS: Genetic recombination between human immunodeficiency virus type 1 (HIV-1) and HIV-2, two distinct human lentiviruses. J Virol 2008; 82(4):1923-1933.

(368) Korber B, Muldoon M, Theiler J et al.: Timing the ancestor of the HIV-1 pandemic strains. Science 2000; 288(5472):1789-1796.

(369) Liao HX, Sutherland LL, Xia SM et al.: A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. Virology 2006; 353(2):268-282.

(370) Robertson DL, Anderson JP, Bradac JA et al.: HIV-1 nomenclature proposal. Science 2000; 288(5463):55-56.

(371) Korber B, Muldoon M, Theiler J et al.: Timing the ancestor of the HIV-1 pandemic strains. Science 2000; 288(5472):1789-1796.

(372) Plantier JC, Leoz M, Dickerson JE et al.: A new human immunodeficiency virus derived from gorillas. Nat Med 2009; 15(8):871-872.

(373) Takehisa J, Kraus MH, Ayouba A et al.: Origin and biology of simian immunodeficiency virus in wild-living western gorillas. J Virol 2009; 83(4):1635-1648.

(374) Vallari A, Holzmayer V, Harris B et al.: Confirmation of putative HIV-1 group P in Cameroon. J Virol 2011; 85(3):1403-1407.

(375) Locatelli S, Peeters M: Cross-species transmission of simian retroviruses: how and why they could lead to the emergence of new diseases in the human population. AIDS 2012; 26(6):659-673.

(376) Salminen MO, Koch C, Sanders-Buell E et al.: Recovery of virtually full-length HIV-1 provirus of diverse subtypes from primary virus cultures using the polymerase chain reaction. Virology 1995; 213(1):80-86.

(377) Hemelaar J, Gouws E, Ghys PD, Osmanov S: Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 2006; 20(16):W13-W23.

(378) Mastro TD, Kunanusont C, Dondero TJ, Wasi C: Why do HIV-1 subtypes segregate among persons with different risk behaviors in South Africa and Thailand? AIDS 1997; 11(1):113-116.

(379) Van HJ, Wood R, Lambrick M et al.: An association between HIV-1 subtypes and mode of transmission in Cape Town, South Africa. AIDS 1997; 11(1):81-87.

(380) Louwagie J, McCutchan F, van der Groen G et al.: Genetic comparison of HIV-1 isolates from Africa, Europe, and North America. AIDS Res Hum Retroviruses 1992; 8(8):1467-1469.

(381) Triques K, Bourgeois A, Vidal N et al.: Near-full-length genome sequencing of divergent African HIV type 1 subtype F viruses leads to the identification of a new HIV type 1 subtype designated K. AIDS Res Hum Retroviruses 2000; 16(2):139-151.

(382) Meloni ST, Kim B, Sankale JL et al.: Distinct human immunodeficiency virus type 1 subtype A virus circulating in West Africa: sub-subtype A3. J Virol 2004; 78(22):12438-12445.

(383) Carr JK, Salminen MO, Albert J et al.: Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. Virology 1998; 247(1):22-31.

(384) Thomson MM, Najera R: Molecular epidemiology of HIV-1 variants in the global AIDS pandemic: an update. AIDS Rev 2005; 7(4):210-224.

(385) Gao F, Robertson DL, Morrison SG et al.: The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J Virol 1996; 70(10):7013-7029.

(386) Casado G, Thomson MM, Sierra M, Najera R: Identification of a novel HIV-1 circulating ADG intersubtype recombinant form (CRF19_cpx) in Cuba. J Acquir Immune Defic Syndr 2005; 40(5):532-537.

(387) Hemelaar J, Gouws E, Ghys PD, Osmanov S: Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 2006; 20(16):W13-W23.

(388) Neilson JR, John GC, Carr JK et al.: Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J Virol 1999; 73(5):4393-4403.

(389) Montano MA, Nixon CP, Ndung'u T et al.: Elevated tumor necrosis factor-alpha activation of human immunodeficiency virus type 1 subtype C in Southern Africa is associated with an NF-kappaB enhancer gain-of-function. J Infect Dis 2000; 181(1):76-81.

(390) Blackard JT, Renjifo B, Fawzi W et al.: HIV-1 LTR subtype and perinatal transmission. Virology 2001; 287(2):261-265.

(391) Renjifo B, Fawzi W, Mwakagile D et al.: Differences in perinatal transmission among human immunodeficiency virus type 1 genotypes. J Hum Virol 2001; 4(1):16-25.

(392) Odaibo GN, Donbraye E, Adewumi MO et al.: Reliability of testing and potential impact on HIV prevention in Nigeria. Afr J Med Med Sci 2006; 35 Suppl:131-135.

(393) Nabel G, Makgoba W, Esparza J: HIV-1 diversity and vaccine development. Science 2002; 296(5577):2335.

(394) Shao Y, Williamson C: The HIV-1 Epidemic: Low- to Middle-Income Countries. Cold Spring Harb Perspect Med 2012; 2(3):a007187.

(395) Hemelaar J, Gouws E, Ghys PD, Osmanov S: Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS 2011; 25(5):679-689.

(396) Batra M, Tien PC, Shafer RW, Contag CH, Katzenstein DA: HIV type 1 envelope subtype C sequences from recent seroconverters in Zimbabwe. AIDS Res Hum Retroviruses 2000; 16(10):973-979.

(397) Tien PC, Chiu T, Latif A et al.: Primary subtype C HIV-1 infection in Harare, Zimbabwe. J Acquir Immune Defic Syndr Hum Retrovirol 1999; 20(2):147-153.

(398) Guevara H, Johnston E, Zijenah L et al.: Prenatal transmission of subtype C HIV-1 in Zimbabwe: HIV-1 RNA and DNA in maternal and cord blood. J Acquir Immune Defic Syndr 2000; 25(5):390-397.

(399) Walter BL, Armitage AE, Graham SC et al.: Functional characteristics of HIV-1 subtype C compatible with increased heterosexual transmissibility. AIDS 2009; 23(9):1047-1057.

(400) Ndung'u T, Lu Y, Renjifo B et al.: Infectious simian/human immunodeficiency virus with human immunodeficiency virus type 1 subtype C from an African isolate: rhesus macaque model. J Virol 2001; 75(23):11417-11425.

(401) Osmanov S, Pattou C, Walker N, Schwardlander B, Esparza J: Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. J Acquir Immune Defic Syndr 2002; 29(2):184-190.

(402) Gupta P, Collins KB, Ratner D et al.: Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. J Virol 2002; 76(19):9868-9876.

(403) Herbeck JT, Nickle DC, Learn GH et al.: Human immunodeficiency virus type 1 env evolves toward ancestral states upon transmission to a new host. J Virol 2006; 80(4):1637-1644.

(404) Sagar M, Laeyendecker O, Lee S et al.: Selection of HIV variants with signature genotypic characteristics during heterosexual transmission. J Infect Dis 2009; 199(4):580-589.

(405) Eshleman SH, Lie Y, Hoover DR et al.: Association between the replication capacity and mother-to-child transmission of HIV-1, in antiretroviral drug-naive Malawian women. J Infect Dis 2006; 193(11):1512-1515.

(406) Lingappa JR, Hughes JP, Wang RS et al.: Estimating the impact of plasma HIV-1 RNA reductions on heterosexual HIV-1 transmission risk. PLoS One 2010; 5(9):e12598.

(407) Essex M, Soto-Ramirez LE, Renjifo E, Wang WK, Lee TH: Genetic variation within human immunodeficiency viruses generates rapid changes in tropism, virulence, and transmission. Leukemia 1997; 11 Suppl 3:93-94.

(408) Kiwanuka N, Laeyendecker O, Quinn TC et al.: HIV-1 subtypes and differences in heterosexual HIV transmission among HIV-discordant couples in Rakai, Uganda. AIDS 2009; 23(18):2479-2484.

(409) Mehta PR, Nema S, Paranjpe S et al.: Study of HIV-1 subtypes in serodiscordant couples attending an integrated counselling and testing centre in Mumbai using heteroduplex mobility analysis and DNA sequencing. Indian J Med Microbiol 2010; 28(4):290-294.

(410) Troyer RM, Collins KR, Abraha A et al.: Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. J Virol 2005; 79(14):9006-9018.

(411) Archary D, Gordon ML, Green TN et al.: HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. Retrovirology 2010; 7:92.

(412) Kanki PJ, Hamel DJ, Sankale JL et al.: Human immunodeficiency virus type 1 subtypes differ in disease progression. J Infect Dis 1999; 179(1):68-73.

(413) Vasan A, Renjifo B, Hertzmark E et al.: Different rates of disease progression of HIV type 1 infection in Tanzania based on infecting subtype. Clin Infect Dis 2006; 42(6):843-852.

(414) Kaleebu P, French N, Mahe C et al.: Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. J Infect Dis 2002; 185(9):1244-1250.

(415) Kiwanuka N, Laeyendecker O, Robb M et al.: Effect of human immunodeficiency virus Type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. J Infect Dis 2008; 197(5):707-713.

(416) Mugo NR, Med M, Heffron R et al.: Increased risk of HIV-1 transmission in pregnancy: a prospective study among African HIV-1 serodiscordant couples. AIDS 2011.

(417) John-Stewart GC, Nduati RW, Rousseau CM et al.: Subtype C Is associated with increased vaginal shedding of HIV-1. J Infect Dis 2005; 192(3):492-496.

(418) Panther LA, Tucker L, Xu C et al.: Genital tract human immunodeficiency virus type 1 (HIV-1) shedding and inflammation and HIV-1 env diversity in perinatal HIV-1 transmission. J Infect Dis 2000; 181(2):555-563.

(419) Scarlatti G, Leitner T, Hodara V et al.: Neutralizing antibodies and viral characteristics in mother-to-child transmission of HIV-1. AIDS 1993; 7 Suppl 2:S45-S48.

(420) Arvold ND, Ngo-Giang-Huong N, McIntosh K et al.: Maternal HIV-1 DNA load and mother-to-child transmission. AIDS Patient Care STDS 2007; 21(9):638-643.

(421) Gardella B, Preti E, Zanchi S, Roccio M, Spinillo A: [Perinatal transmission of HIV]. Minerva Ginecol 2007; 59(2):139-149.

(422) Boily-Larouche G, Iscache AL, Zijenah LS et al.: Functional genetic variants in DC-SIGNR are associated with mother-to-child transmission of HIV-1. PLoS One 2009; 4(10):e7211.

(423) Baan E, de RA, Luchters S et al.: HIV Type 1 Mother-to-Child Transmission Facilitated by Distinctive Glycosylation Sites in the gp120 Envelope Glycoprotein. AIDS Res Hum Retroviruses 2011.

(424) Kumar SB, Handelman SK, Voronkin I et al.: Different regions of HIV-1 subtype C env are associated with placental localization and in utero mother-to-child transmission. J Virol 2011.

(425) Volmink J, Marais B: HIV: mother-to-child transmission. Clin Evid (Online) 2008; 2008.

(426) Richardson BA, John-Stewart GC, Hughes JP et al.: Breast-milk infectivity in human immunodeficiency virus type 1-infected mothers. J Infect Dis 2003; 187(5):736-740.

(427) Matala E, Hahn T, Yedavalli VR, Ahmad N: Biological characterization of HIV type 1 envelope V3 regions from mothers and infants associated with perinatal transmission. AIDS Res Hum Retroviruses 2001; 17(18):1725-1735.

(428) Zhang H, Orti G, Du Q et al.: Phylogenetic and phenotypic analysis of HIV type 1 env gp120 in cases of subtype C mother-to-child transmission. AIDS Res Hum Retroviruses 2002; 18(18):1415-1423.

(429) Dickover RE, Garratty EM, Plaeger S, Bryson YJ: Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants in utero and intrapartum. J Virol 2001; 75(5):2194-2203.

(430) Verhofstede C, Demecheleer E, De CN et al.: Diversity of the human immunodeficiency virus type 1 (HIV-1) env sequence after vertical transmission in mother-child pairs infected with HIV-1 subtype A. J Virol 2003; 77(5):3050-3057.

(431) Scarlatti G, Leitner T, Halapi E et al.: Comparison of variable region 3 sequences of human immunodeficiency virus type 1 from infected children with the RNA and DNA sequences of the virus populations of their mothers. Proc Natl Acad Sci U S A 1993; 90(5):1721-1725.

(432) Steain MC, Wang B, Saksena NK: Analysis of HIV-1 sequences vertically transmitted to infants in Kisumu, Kenya. J Clin Virol 2006; 36(4):298-302.

(433) Bartelsman M, Veeken H: [The HIV pandemic in the year 2007, an overview]. Ned Tijdschr Geneeskd 2007; 151(48):2655-2660.

(434) Palmer S, Boltz V, Martinson N et al.: Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. Proc Natl Acad Sci U S A 2006; 103(18):7094-7099.

(435) Eshleman SH, Hoover DR, Chen S et al.: Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. J Infect Dis 2005; 192(1):30-36.

(436) Solis V, I, Munoz GE, Ramos Amador JT et al.: [Maternal characteristics of a cohort of pregnant women with HIV-1 infection]. Med Clin (Barc) 2006; 127(4):121-125.

(437) Renjifo B, Gilbert P, Chaplin B et al.: Preferential in-utero transmission of HIV-1 subtype C as compared to HIV-1 subtype A or D. AIDS 2004; 18(12):1629-1636.

(438) Tapia N, Franco S, Puig-Basagoiti F et al.: Influence of human immunodeficiency virus type 1 subtype on mother-to-child transmission. J Gen Virol 2003; 84(Pt 3):607-613.

(439) Martinez AM, Hora VP, Santos AL et al.: Determinants of HIV-1 mother-to-child transmission in Southern Brazil. An Acad Bras Cienc 2006; 78(1):113-121.

(440) Li GH, Chen ZW, Chen Z et al.: [Study on the distribution of human immunodeficiency virus-1 subtypes in different regions of China and mother-to-child transmission]. Zhonghua Liu Xing Bing Xue Za Zhi 2004; 25(12):1013-1018.

(441) Fischetti L, Danso K, Dompreh A et al.: Vertical transmission of HIV in Ghanaian women diagnosed in cord blood and post-natal samples. J Med Virol 2005; 77(3):351-359.

(442) Halapi E, Gigliotti D, Hodara V et al.: Detection of CD8 T-cell expansions with restricted T-cell receptor V gene usage in infants vertically infected by HIV-1. AIDS 1996; 10(14):1621-1626.

(443) Becker-Pergola G, Mellquist JL, Guay L et al.: Identification of diverse HIV type 1 subtypes and dual HIV type 1 infection in pregnant Ugandan women. AIDS Res Hum Retroviruses 2000; 16(12):1099-1104.

(444) Delwart EL, Shpaer EG, Louwagie J et al.: Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science 1993; 262(5137):1257-1261.

(445) Heyndrickx L, Janssens W, Zekeng L et al.: Simplified strategy for detection of recombinant human immunodeficiency virus type 1 group M isolates by gag/env heteroduplex mobility assay. Study Group on Heterogeneity of HIV Epidemics in African Cities. J Virol 2000; 74(1):363-370.

(446) Klarkowski DB, Wazome JM, Lokuge KM et al.: The evaluation of a rapid in situ HIV confirmation test in a programme with a high failure rate of the WHO HIV two-test diagnostic algorithm. PLoS One 2009; 4(2):e4351.

(447) Garland FC, Garland CF, Gorham ED, Brodine SK: Western blot banding patterns of HIV rapid progressors in the U.S. Navy Seropositive Cohort: implications for vaccine development. Navy Retroviral Working Group. Ann Epidemiol 1996; 6(4):341-347.

(448) Ragni MV, O'Brien TA, Reed D, Spero JA, Lewis JH: Prognostic importance of antibodies to human immunodeficiency virus by recombinant immunoassay and Western blot techniques in HIV antibody-positive hemophiliacs. AIDS Res Hum Retroviruses 1988; 4(3):223-231.

(449) Sudha T, Lakshmi V, Teja VD: Western blot profile in HIV infection. Indian J Dermatol Venereol Leprol 2006; 72(5):357-360.

(450) World Health Organisation: Proposed WHO criteria for interpretating results from Western blot assays for HIV-1, HIV-2 and HTLV-I/HTLV-II. Wkly Epidemiol Rec 1990; 37:281-283.

(451) Obirikorang C, Quaye L, Acheampong I: Total lymphocyte count as a surrogate marker for CD4 count in resource-limited settings. BMC Infect Dis 2012; 12:128.

(452) World Health Organisation. Scaling up antiretroviral therapy in resource limited settings: Guidelines for a public health approach. 2002.

(453) Daka D, Loha E: Relationship between total lymphocyte count (TLC) and CD4 count among peoples living with HIV, Southern Ethiopia: a retrospective evaluation. AIDS Res Ther 2008; 5:26.

(454) Zijenah LS, Kadzirange G, Madzime S et al.: Affordable flow cytometry for enumeration of absolute CD4+ T-lymphocytes to identify subtype C HIV-1 infected adults requiring antiretroviral therapy (ART) and monitoring response to ART in a resource-limited setting. J Transl Med 2006; 4:33.

(455) Creek TL, Sherman GG, Nkengasong J et al.: Infant human immunodeficiency virus diagnosis in resource-limited settings: issues, technologies, and country experiences. Am J Obstet Gynecol 2007; 197(3 Suppl):S64-S71.

(456) Bogh M, Machuca R, Gerstoft J et al.: Subtype-specific problems with qualitative Amplicor HIV-1 DNA PCR test. J Clin Virol 2001; 20(3):149-153.

(457) Bryson YJ, Luzuriaga K, Sullivan JL, Wara DW: Proposed definitions for in utero versus intrapartum transmission of HIV-1. N Engl J Med 1992; 327(17):1246-1247.

(458) Piwowar-Manning E, Lugalia L, Kafufu B, Jackson JB: Comparison of results obtained with Amplicor HIV-1 DNA PCR test version 1.5 using 100 versus 500 microliters of whole blood. J Clin Microbiol 2008; 46(3):1104-1105.

(459) Boom R, Beld M, Weel J, Goudsmith J and Wertheim-van Dillen P: Improved silica-guanidinium thiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. J Clin Microbiol 1999; 37(3):615-619.

(460) Perrotte M. Boom Extraction Principle. 2007.

(461) Chien A, Edgar DB, Trela JM: Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. J Bacteriol 1976; 127(3):1550-1557.

(462) Saiki RK, Gelfand DH, Stoffel S et al.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239(4839):487-491.

(463) Lawyer FC, Stoffel S, Saiki RK et al.: High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. PCR Methods Appl 1993; 2(4):275-287.

(464) Tindall KR, Kunkel TA: Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 1988; 27(16):6008-6013.

(465) Shuman S: Recombination mediated by vaccinia virus DNA topoisomerase I in Escherichia coli is sequence specific. Proc Natl Acad Sci U S A 1991; 88(22):10104-10108.

(466) Invitrogen-TOPO. TOPO TA Cloning kit. 2010.

(467) Fire A: Histochemical techniques for locating Escherichia coli beta-galactosidase activity in transgenic organisms. Genet Anal Tech Appl 1992; 9(5-6):151-158.

(468) Colditz GA: Overview of the epidemiology methods and applications: strengths and limitations of observational study designs. Crit Rev Food Sci Nutr 2010; 50 Suppl 1:10-12.

(469) Ernster VL: Nested case-control studies. Prev Med 1994; 23(5):587-590.

(470) Booysen FR, Arntz T: The methodology of HIV/AIDS impact studies: a review of current practices. Soc Sci Med 2003; 56(12):2391-2405.

(471) Barnett ML, Hyman JJ: Challenges in interpreting study results: the conflict between appearance and reality. J Am Dent Assoc 2006; 137 Suppl:32S-36S.

(472) Perrin L, Kaiser L, Yerly S: Travel and the spread of HIV-1 genetic variants. Lancet Infect Dis 2003; 3(1):22-27.

(473) Duri K, Gumbo FZ, Kristiansen KI et al.: Antenatal HIV-1 RNA load and timing of mother to child transmission; a nested case-control study in a resource poor setting. Virol J 2010; 7:176.

(474) Owen SM: Testing for acute HIV infection: implications for treatment as prevention. Curr Opin HIV AIDS 2012; 7(2):125-130.

(475) Daskalakis D: HIV diagnostic testing: evolving technology and testing strategies. Top Antivir Med 2011; 19(1):18-22.

(476) Duri K, Muller F, Gumbo FZ et al.: Human Immunodeficiency Virus (HIV) types Western blot (WB) band profiles as potential surrogate markers of HIV disease progression and predictors of vertical transmission in a cohort of infected but antiretroviral therapy naive pregnant women in Harare, Zimbabwe. BMC Infect Dis 2011; 11:7.

(477) Kurewa NE, Munjoma MM, Chirenje ZM et al.: Compliance and loss to follow up of HIV negative and positive mothers recruited from a PMTCT programme in Zimbabwe. Cent Afr J Med 2007; 53(5-8):25-30.

(478) Barin F, Laperche S, Courouce AM: [Genetic diversity of viruses. Consequences for screening and prevention]. Transfus Clin Biol 2000; 7(5):472-478.

(479) Preiser W, Brink NS, Hayman A et al.: False-negative HIV antibody test results. J Med Virol 2000; 60(1):43-47.

(480) Novitsky V, Gaolathe T, Woldegabriel E, Makhema J, Essex M: A seronegative case of HIV-1 subtype C infection in Botswana. Clin Infect Dis 2007; 45(5):e68-e71.

(481) Aghokeng AF, Mpoudi-Ngole E, Dimodi H et al.: Inaccurate diagnosis of HIV-1 group M and O is a key challenge for ongoing universal access to antiretroviral treatment and HIV prevention in Cameroon. PLoS One 2009; 4(11):e7702.

(482) Collins KR, Mayanja-Kizza H, Sullivan BA et al.: Greater diversity of HIV-1 quasispecies in HIV-infected individuals with active tuberculosis. J Acquir Immune Defic Syndr 2000; 24(5):408-417.

(483) Schindzielorz AH, Belshe RB, Mufson MA: Occurrence, characteristics, and patterns of HIV-1 and HIV-2 western blot indeterminate sera in low risk populations in West Virginia and pre-AIDS Africa. Am J Trop Med Hyg 1990; 42(5):460-464.

(484) Lyamuya E, Olausson-Hansson E, Albert J, Mhalu F, Biberfeld G: Evaluation of a prototype Amplicor PCR assay for detection of human immunodeficiency virus type 1 DNA in blood samples from Tanzanian adults infected with HIV-1 subtypes A, C and D. J Clin Virol 2000; 17(1):57-63.

(485) Zijenah LS, Humphrey J, Nathoo K et al.: Evaluation of the prototype Roche DNA amplification kit incorporating the new SSK145 and SKCC1B primers in detection of human immunodeficiency virus type 1 DNA in Zimbabwe. J Clin Microbiol 1999; 37(11):3569-3571.

(486) Aulicino PC, Gomez CM, Kopka J et al.: HIV-1 genetic diversity in Argentina and early diagnosis of perinatal infection. Medicina (B Aires) 2006; 66(4):319-326.

(487) Cunningham P, Marriott D, Harris C et al.: False negative HIV-1 proviral DNA polymerase chain reaction in a patient with primary infection acquired in Thailand. J Clin Virol 2003; 26(2):163-169.

(488) Kline NE, Schwarzwald H, Kline MW: False negative DNA polymerase chain reaction in an infant with subtype C human immunodeficiency virus 1 infection. Pediatr Infect Dis J 2002; 21(9):885-886.

(489) Lee S, Hu J, Tang S et al.: Evaluation of FDA licensed HIV assays using plasma from Cameroonian blood donors. J Med Virol 2006; 78 Suppl 1:S22-S23.

(490) Toro C, Amor A, Soriano V: [Diagnosis of HIV-1 non-B subtypes and HIV-2]. Enferm Infecc Microbiol Clin 2008; 26 Suppl 13:66-70.

(491) Holguin A, de MM, Yebra G, Lopez M, Soriano V: Increase of non-B subtypes and recombinants among newly diagnosed HIV-1 native Spaniards and immigrants in Spain. Curr HIV Res 2008; 6(4):327-334.

(492) Wang Y, Song A, Xu S et al.: Impact of HIV-1 genetic diversity in China on the measurement of viral load. J Med Virol 2008; 80(1):1-8.

(493) Swanson P, de MC, Joshi Y et al.: Impact of human immunodeficiency virus type 1 (HIV-1) genetic diversity on performance of four commercial viral load assays: LCx HIV RNA Quantitative, AMPLICOR HIV-1 MONITOR v1.5, VERSANT HIV-1 RNA 3.0, and NucliSens HIV-1 QT. J Clin Microbiol 2005; 43(8):3860-3868.

(494) Swanson P, Harris BJ, Holzmayer V et al.: Quantification of HIV-1 group M (subtypes A-G) and group O by the LCx HIV RNA quantitative assay. J Virol Methods 2000; 89(1-2):97-108.

(495) Gobbers E, Fransen K, Oosterlaken T et al.: Reactivity and amplification efficiency of the NASBA HIV-1 RNA amplification system with regard to different HIV-1 subtypes. J Virol Methods 1997; 66(2):293-301.

(496) Xu S, Song A, Nie J et al.: Comparison between the automated Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 assay and its version 1 and Nuclisens HIV-1 EasyQ version 2.0 assays when measuring diverse HIV-1 genotypes in China. J Clin Virol 2012; 53(1):33-37.

(497) Szabo S, Moffett LE, Cantwell-McNelis K, James CW, Joseph A: Low-level viremia associated with the use of TaqMan assay. J Int Assoc Physicians AIDS Care (Chic) 2010; 9(4):203-205.

(498) Tang N, Huang S, Salituro J et al.: A RealTime HIV-1 viral load assay for automated quantitation of HIV-1 RNA in genetically diverse group M subtypes A-H, group O and group N samples. J Virol Methods 2007; 146(1-2):236-245.

(499) Plantier JC, Gueudin M, Damond F et al.: Plasma RNA quantification and HIV-1 divergent strains. J Acquir Immune Defic Syndr 2003; 33(1):1-7.

(500) Rouet F, Chaix ML, Nerrienet E et al.: Impact of HIV-1 genetic diversity on plasma HIV-1 RNA Quantification: usefulness of the Agence Nationale de Recherches sur le SIDA second-generation long terminal repeat-based real-time reverse transcriptase polymerase chain reaction test. J Acquir Immune Defic Syndr 2007; 45(4):380-388.

(501) Sizmann D, Glaubitz J, Simon CO et al.: Improved HIV-1 RNA quantitation by COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 using a novel dual-target approach. J Clin Virol 2010; 49(1):41-46.

(502) Ethridge SF, Wesolowski LG, Nasrullah M et al.: Comparative evaluation of Aptima HIV-1 Qualitative RNA assay performance using plasma and serum specimens from persons with established HIV-1 infection. J Clin Virol 2011; 52 Suppl 1:S63-S66.

(503) Obi CL, McAdoo HP, Onigbinde AO et al.: Subtypes of HIV-1 and the impact of dual infections of HIV-1 and measles virus on micronutrient levels of pregnant women in Harare, Zimbabwe. Cent Afr J Med 1997; 43(6):165-172.

(504) Duri K, Gumbo F, Kristiansen K et al.: Phylogenetic analysis of human immunodeficiency virus type 1 subtype C env gp120 sequences among four drug-naive families following subsequent heterosexual and vertical transmissions. AIDS Res Hum Retroviruses 2012; 28(8):885-893.

(505) Lihana RW, Ssemwanga D, Abimiku A, Ndembi N: Update on HIV-1 diversity in Africa: a decade in review. AIDS Rev 2012; 14(2):83-100.

(506) Thomson MM, Najera R: Travel and the introduction of human immunodeficiency virus type 1 non-B subtype genetic forms into Western countries. Clin Infect Dis 2001; 32(12):1732-1737.

(507) Arien KK, Vanham G, Arts EJ: Is HIV-1 evolving to a less virulent form in humans? Nat Rev Microbiol 2007; 5(2):141-151.

(508) Bredell H, Martin DP, Van HJ et al.: HIV type 1 subtype C gag and nef diversity in Southern Africa. AIDS Res Hum Retroviruses 2007; 23(3):477-481.

(509) Duri K, Soko W, Gumbo F et al.: Genotypic analysis of human immunodeficiency virus type 1 env V3 loop sequences: bioinformatics prediction of coreceptor usage among 28 infected mother-infant pairs in a drug-naive population. AIDS Res Hum Retroviruses 2011; 27(4):411-419.

(510) Duri K, Kristiansen KI, Mapingure MP et al.: HIV-1 Env gp120 C2V5 Potential N-Linked Glycosylation Site(s) (PNGs) and amino acid length polymorphisms among infected family members. Advances in Infectious Diseases 2011; 1:1-13.

(511) Sundaravaradan V, Das SR, Ramakrishnan R et al.: Role of HIV-1 subtype C envelope V3 to V5 regions in viral entry, coreceptor utilization and replication efficiency in primary T-lymphocytes and monocyte-derived macrophages. Virol J 2007; 4:126.

(512) Soares EA, Martinez AM, Souza TM et al.: HIV-1 subtype C dissemination in southern Brazil. AIDS 2005; 19 Suppl 4:S81-S86.

(513) McCormack GP, Glynn JR, Crampin AC et al.: Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi. J Virol 2002; 76(24):12890-12899.

(514) Casper C, Fenyo EM: Mother-to-child transmission of HIV-1: the role of HIV-1 variability and the placental barrier. Acta Microbiol Immunol Hung 2001; 48(3-4):545-573.

(515) Korber BT, MacInnes K, Smith RF, Myers G: Mutational trends in V3 loop protein sequences observed in different genetic lineages of human immunodeficiency virus type 1. J Virol 1994; 68(10):6730-6744.

(516) Patel MB, Hoffman NG, Swanstrom R: Subtype-specific conformational differences within the V3 region of subtype B and subtype C human immunodeficiency virus type 1 Env proteins. J Virol 2008; 82(2):903-916.

(517) Stanfield RL, Gorny MK, Zolla-Pazner S, Wilson IA: Crystal structures of human immunodeficiency virus type 1 (HIV-1) neutralizing antibody 2219 in complex with three different V3 peptides reveal a new binding mode for HIV-1 cross-reactivity. J Virol 2006; 80(12):6093-6105.

(518) Gilbert PB, Novitsky V, Essex M: Covariability of selected amino acid positions for HIV type 1 subtypes C and B. AIDS Res Hum Retroviruses 2005; 21(12):1016-1030.

(519) Abrahams MR, Anderson JA, Giorgi EE et al.: Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. J Virol 2009; 83(8):3556-3567.

(520) Nowak P, Karlsson AC, Naver L et al.: The selection and evolution of viral quasispecies in HIV-1 infected children. HIV Med 2002; 3(1):1-11.

(521) Long EM, Martin HL, Jr., Kreiss JK et al.: Gender differences in HIV-1 diversity at time of infection. Nat Med 2000; 6(1):71-75.

(522) Neogi U, Bontell I, Shet A et al.: Molecular epidemiology of HIV-1 subtypes in India: origin and evolutionary history of the predominant subtype C. PLoS One 2012; 7(6):e39819.

(523) Nyombi BM, Kristiansen KI, Bjune G, Muller F, Holm-Hansen C: Diversity of human immunodeficiency virus type 1 subtypes in Kagera and Kilimanjaro regions, Tanzania. AIDS Res Hum Retroviruses 2008; 24(6):761-769.

(524) Pandit A, Sinha S: Using genomic signatures for HIV-1 sub-typing. BMC Bioinformatics 2010; 11 Suppl 1:S26.

(525) Hraber P, Kuiken C, Waugh M et al.: Classification of hepatitis C virus and human immunodeficiency virus-1 sequences with the branching index. J Gen Virol 2008; 89(Pt 9):2098-2107.

(526) Gale CV, Myers R, Tedder RS, Williams IG, Kellam P: Development of a novel human immunodeficiency virus type 1 subtyping tool, Subtype Analyzer (STAR): analysis of subtype distribution in London. AIDS Res Hum Retroviruses 2004; 20(5):457-464.

(527) Dwivedi SK and Sengupta S: Classification of HIV-1 Sequences Using Profile Hidden Markov Models. PLos One 2012; 7(5):e36566.

Chapter 10

Appendices

Published Papers

Consent Forms

Paper I

Duri et al. Virology Journal 2010, 7:176
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VIROLOGY JOURNAL

RESEARCH Open Access

Antenatal HIV-1 RNA load and timing of mother to child transmission; a nested case-control study in a resource poor setting

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Abstract

Objective: To determine HIV-1 RNA load during the third trimester of pregnancy and evaluate its effect on in utero and intra-partum/postpartum transmissions in a breastfeeding population.

Design: A nested case-control study within a PMTCT cohort of antiretroviral therapy naive pregnant women and their infants.
Methods: A case was a mother who transmitted HIV-1 to her infant (transmitter) who was matched to one HIV-1 positive but non-transmitting mother (control).

Results: From a cohort of 691 pregnant women, 177 (25.6%) were HIV-1 positive at enrolment and from these 29 (23%) transmitted HIV-1 to their infants, 10 and 19 during in utero and intra-partum/postpartum respectively. Twenty-four mothers sero-converted after delivery and three transmitted HIV-1 to their infants. Each unit increase in $\log_{10}$ viral load was associated with a 178 cells/mm_3 and 0.2 g/dL decrease in TLC and hemoglobin levels, p = 0.048 and 0.021 respectively, and a 29% increase in the risk of transmission, p = 0.023. Intra-partum/postpartum transmitters had significantly higher mean viral load relative to their matched controls, p = 0.034.

Conclusion: Antenatal serum HIV-1 RNA load, TLC and hemoglobin levels were significantly associated with vertical transmission but this association was independent of transmission time. This finding supports the rationale for preventive strategies designed to reduce vertical transmission by lowering maternal viral load.

Introduction

Sub-Saharan Africa continues to be the epicentre of the HIV-1 epidemic contributing more than 90% of the 370 000 infants who acquire the infection from their mothers annually worldwide [1]. More than half of the HIV-1 infected children die before their second birthday [2]. The HIV-1 epidemic among pregnant women poses a challenge to child health and survival of future generations.

Zimbabwe is among the Sub-Saharan countries with the highest HIV-1 prevalence in the world. Among 600 000 women who get pregnant annually, HIV-1 prevalence peaked to 30% in 1997 [3] but has steadily declined over the years to 15.6% in 2006 [1,4]. Without any intervention, 30-49% of the children born to HIV-1 positive mothers are infected by the virus [5]. In Zimbabwe the estimate of mother to child transmission rate of HIV-1 has been shown to be 30% [6]. The reason why some mothers transmit to their infants whilst the majority does not is not well documented.

Maternal HIV-1 RNA load has been shown to be the strongest predictor of vertical transmission [7,8]. In Zimbabwe, among exclusively breastfed infants, in utero and intra-partum transmission has been shown to be 9.4% and 16%, respectively [6] with a postpartum transmission rate of 12% [9]. However, both studies have made no reference to maternal viral load. More so, other previous studies have pooled the three transmission periods; in utero, intra-partum and postpartum cases and this may underestimate time specific risk factors of vertical transmission [8].

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Despite the high HIV-1 prevalence in the general populace which translates to high vertical transmission rates, the desire to have future pregnancies among HIV-1 positive mothers has increased from 3% to more than 55% more so with the advent of HIV-1 Prevention of Mother To Child Transmission (PMTCT) initiatives [10,11]. Therefore there is a need for the development of a simple, effective and time specific vertical transmission preventive strategy to curb this epidemic. This study aims to determine HIV-1 RNA load during the third trimester of pregnancy and evaluate its association with in utero and intra-partum/postpartum vertical transmissions.

Methodology

Study Design and Setting

This was a nested case-control study in which the cases and controls were sampled from an antiretroviral therapy naive PMTCT cohort of pregnant women attending Antenatal Clinics at Epworth, Seke North and Saint Mary's Chitungwiza, all around Harare. Antiretroviral drugs were not readily available in Zimbabwe at the time of recruitment of study participants.

Study Population and Procedures

The study population consisted of two groups of HIV-1 positive pregnant women. The main group comprised of pregnant women who were HIV-1 positive at enrolment, referred to as having chronic HIV-1 infections and a minor group of women who were HIV-1 negative during pregnancy but later on sero-converted after delivery during the follow-up period, regarded as having acute HIV-1 infections. Each HIV-1 positive mother who transmitted the virus to her infant (case) was matched to one HIV-1 positive but non-transmitting mother (control). Matching of cases and controls was done with respect to maternal age, educational level, marital and socio-economic status, parity, alcohol intake, sexually transmitted infections, the date of last menstruation, and uptake of single dose nevirapine therapy.

Pregnant women were enrolled at 36 gestational weeks in a national PMTCT program between April and September 2002. Pre-and post-HIV test counseling was provided. Single dose nevirapine therapy was offered to HIV-1 positive mothers during labour and to their infants within 72 hours post delivery. Mothers were encouraged to exclusively breastfeed during the first six months. Follow-up was from delivery, six weeks, four and nine months and thereafter three monthly until two years. Follow up visits generally coincided with infant immunization visits. At each subsequent follow-up visit, HIV-1 negative mother and infants were re-tested for HIV-1 antibodies and HIV-1 DNA, respectively. Serum samples from the HIV-1 negative mothers and their infants were aliquoted and appropriately stored for further tests in the event that they sero-converted.

Mothers and Infants Demographic characteristics and Examination

All mothers answered a structured questionnaire at enrolment and information regarding their socio-demographics, sexual behavior, obstetric and reproductive health issues was obtained. A gynecologist performed physical and gynecological examinations.

A pediatrician examined infants. Date of birth, birth weight, gender, single dose nevirapine therapy and breastfeeding patterns were recorded. Infant deaths were also recorded during the follow up period.

Mothers' Tests

Serial HIV-1/2 algorithm antibody tests were done using Determine (Abbott Diagnostics, Illinois USA) and Ora-Quick (Abbott Diagnostics, Illinois, USA) rapid kits on mothers' serum samples. EDTA-anti-coagulated venous blood samples were processed within six hours for full blood counts using Abbott Diagnostic Cell Dyne 3500R SL Hematology Analyser. Total Lymphocyte Count (TLC) was enumerated as the total white blood cell count multiplied by the lymphocyte percentage. In this resource poor setting, TLC was used as a surrogate marker for CD4 cell count since by then, the capacity to determine the latter was not readily available to the general public due to prohibitive costs. TLC of 1200 cells/mm^3 was the threshold value used equivalent to a CD4 count of 200 cells/mm^3 [12,13].

Blood samples were shipped on dry ice to the Institute of Microbiology at the University of Oslo in Norway for further laboratory analysis. Maternal baseline serum samples were quantified for HIV-1 RNA load using an automated TaqMan Roche Amplicor 1.5 Monitor Test (Cobas AmpliPrep/Cobas TaqMan, Roche Diagnostics, Branchburg NJ) according to the manufacturer's instructions. As for sero-converters, the first HIV-1 positive sample was quantified. The linear range of the test was between 40 ($1.6\log_{10}$) and 10^7 ($7\log_{10}$) copies/mL and the detection limit of the assay is 40 copies/mL based on a sample volume of 1 mL thus the detection limit for this study was 400 copies/mL based on a serum sample volume of 100 L that was topped up to 1 mL with HIV-1 negative serum.

Infants' Tests

Infants' venous EDTA whole blood samples were collected at each follow up visit. Samples were stored at -86°C until testing. Detection of infants' HIV-1 infection was determined using a qualitative 1.5 Roche Amplicor HIV-1 DNA PCR kit (Roche Diagnostics Incorporation, Branchburg, New Jersey). Testing was done in the

Obstetrics and Gynecology Department, Medical School, University of Zimbabwe. Infants that tested HIV-1 DNA PCR positive on whole blood collected within 10 days of birth were considered to be infected in utero. Infants who had negative HIV-1 DNA PCR results within the first 10 days of life and positive results at six weeks postpartum and/or thereafter were considered to be infected intra-partum/postpartum [14].

Statistical Analysis

Data were collected and analyzed using STATA version 10 from Texas and SPSS version 16.0 from Illinois, USA. Viral load values were log $_{10}$ transformed. Viral load values of below the detection limit were assigned half the value of the detection limit. The Student t-test was used to compare mean log $_{10}$ viral load between transmitting and non transmitting mothers, chronic and acute HIV-1 infections, in utero and intra-partum/post-partum transmitters. Mean log $_{10}$ viral load of each of these groups was also compared with their respective matched controls. Regression analysis was used to investigate the association between log$_{10}$ viral load, TLC or hemoglobin levels, and vertical transmission. Tests of statistical significance included the 95% confidence interval of relative risks, two sided p values based on Chi-squared and Fisher's exact tests.

Ethical Consideration

The study was approved by the Medical Research Council of Zimbabwe and the Ethical Review Committee in Norway. Written consent to participate in the research study was obtained from the mothers and they were free to discontinue at any given time without any prejudice. Mothers also consented to have their blood samples and that of their index infants' used for future HIV related research.

Results

Demographic and reproductive health characteristics of 32 transmitters and matched 32 non-transmitters

There was no statistical significant difference with respect to socio-demographic characteristics, sexual behavior, reproductive genital tract infections and medical history between the 64 HIV-1 positive mothers constituting this study population and the rest (113) of the HIV-1 positive mothers in the cohort. However, the 32 transmitters and matched 32 non-transmitters were more likely to have more children relative to the other 113 HIV-1 positive but non transmitting mothers who were not part of the study population, p = 0.016.

Mothers' mean age (SD) was 26.0 (5.6) years with that of transmitters and non-transmitters being 26.3 (5.6) and 25.6 (5.6) years respectively p = 0.610. All the mothers had spontaneous vaginal deliveries. There were no statistically significant differences between transmitting and non-transmitting mothers with respect to age, level of education, parity, type of marriage, socio-economic status and number of life sexual partners. The transmitters and non-transmitters also had comparable burdens of reproductive tract infections and obstetric history, see table 1.

When the transmitters were stratified by time of infecting their infants, there were no statistically significant differences with respect to demographics, sexual behaviour, reproductive health characteristics and medical history between those who transmitted during in utero and those who transmitted intra-partum/postpartum.

Mothers with acute HIV-1 infection, the sero-converters, were generally younger relative to HIV-1 negative mothers in the cohort, with mean age of 21.8 (4.6) and 23.7(5) years respectively although not statistically significant, p = 0.06. There were also no statistical significant differences with respect to parity, level of education, age of sexual debut and reproductive tract infections between these two groups. Sero-converters were more likely to be single, have more than one sexual partner(s), syphilis, clinical warts, and a history of blood transfusion with p values of 0.000, 0.019, 0.041, 0.002 and 0.033 respectively. Transmitting sero-converters were more likely to report having a travelling partner, p = 0.022 and were significantly younger than transmitters with chronic HIV-1 infections, with mean age of 20(1.7) and 27(5.5) years respectively, p = 0.04.

HIV-1 Prevalence and Transmission

At enrolment, 691 pregnant women attending national PMTCT program were sampled between April and September 2002. Of these, 177 (25.6%) and 514 (74.4%) were HIV-1 positive and negative respectively. There were two stillbirths each among the HIV-1 positive and negative mothers and these were excluded from analysis. From the 176 mothers with chronic HIV-1 infections that delivered live births 134 (76%) mother-baby pairs were successfully followed up and tested, see figure 1. There were no statistically significant differences with respect to socio-demography and reproductive health characteristics between the 42 women lost to follow up and the 134 with complete data sets. Twenty-nine (22%) mothers transmitted the virus to their infants, 10 (34%) and 19 (66%) during in utero and intra-partum/postpartum transmissions with rates of 7.5% and 15.3% respectively.

Out of the 514 HIV-1 negative mothers at baseline, 24 sero-converted during the 2 year follow-up period, giving an HIV-1 cumulative incidence rate of 2.3 per hundred women years. Among the 24 sero-converters with

Table 1 Socio-demographics, sexual behavior, medical history and baby characteristics of the 32 transmitters and 32 non-transmitters

Variable	Transmitter N = 32 (%)	Non Transmitters N = 32 (%)	RR (95% CI)
Age in years			
Mean (sd)	26.3 (5.6)	25.6 (5.6)	1.01 (0.95-1.08)
Years in school			
<8	4/32 (13)	3/32 (9)	1.16 (0.58-2.33)
Parity			
At least 1 child	28/32 (88)	25/32 (78)	1.40 (0.60-3.30)
Polygamous marriage			
Yes	4/31 (13)	4/30 (13)	0.98 (0.47-2.06)
Subsidised income			
Yes	4/32 (13)	8/32 (25)	0.62 (0.27-1.43)
Age at sexual debut			
≤ 16 years	6/32 (19)	3/32 (9)	1.41 (0.82-2.42)
Life time partners			
>1	17/32 (53)	16/32 (50)	1.06 (0.65-1.74)
Vaginal discharge			
Abnormal	15/32 (47)	13/31 (42)	1.10 (0.68-1.79)
Genital ulcer			
Present	5/32 (16)	3/30 (10)	1.25 (0.69-2.28)
Dysuria			
Yes	7/32 (22)	4/31 (13)	1.32 (0.78-2.25)
Lymphadenopathy			
Yes	3/28 (11)	1/31 (3)	1.65 (0.87-3.12)
Abortion history			
Yes	7/32 (22)	3/32 (9)	1.51 (0.92-2.49)
Infant death history			
Yes	4/32 (13)	7/32 (22)	0.69 (0.30-1.57)
Schistosomiasis infection history			
Yes	7/32 (22)	4/31 (13)	1.32 (0.78-2.24)
Mothers'ARV Prophylaxis			
No	17/32 (53)	16/32 (50)	1.00 (0.60-1.66)
Infant gender			
Male	14/30 (47)	12/29 (41)	1.11 (0.67-1.83)
Birth weight			
<2500	2/31 (6)	1/30 (3)	1.36 (0.58-3.15)
Deceased infant			
Yes	9/31 (29)	2/30 (7)	4.35 (1.02-18.52)*
Breastfed			
Yes	28/31 (90)	19/25 (76)	1.79 (0.69-4.64)
Baby ARV prophylaxis			
Yes	16/27 (59)	13/25 (52)	0.87 (0.51-1.49)

acute HIV-1 infections, three (13%) transmitted the virus to their infants through breastfeeding around 9 months postpartum. All the three infants were exposed, through breast milk for about three months before acquiring HIV-1 infection at about 12 months postpartum.

Thus there were a total of 32 transmitting mothers in this cohort, giving an overall transmission rate of 21.3%.

Maternal Viral Load and Transmission

Of the 32 transmitters and 32 matched non-transmitters, 26 (81%) and 20 (63%), respectively had detectable serum HIV-1 RNA load ranging from 400 to 3 000 000 copies/mL. Vertical transmission occurred throughout the entire range with 90% of the transmissions occurring below 16 000 HIV-1 RNA copies/mL. The mean (95% Confidence Interval) log $_{10}$ viral load was 3.55(3.15-3.96) and 2.92

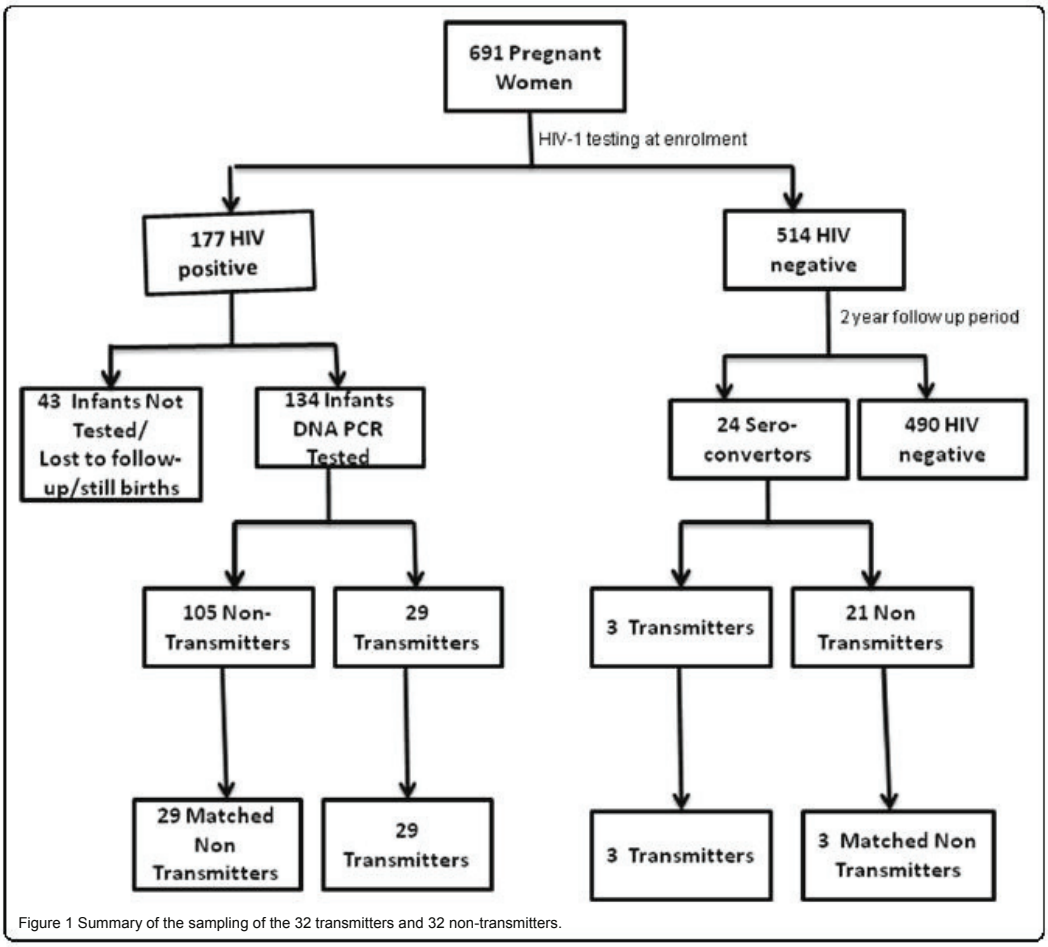

Figure 1 Summary of the sampling of the 32 transmitters and 32 non-transmitters.

(2.59-3.26) for transmitters and non-transmitters respectively, p = 0.018, see table 2. For each unit increase in log $_{10}$ viral load, the risk of transmission increased by 29%, p = 0.023. Mean log $_{10}$ (SD) viral load of mothers with acute and chronic HIV-1 infection was 4.22 (1.01) and 3.55 (1.09) respectively, p = 0.317. Mean log$_{10}$ (SD) viral load of transmitting sero-converters and non-transmitting sero-converters were 3.99 (1.34) and 2.77 (0.81) respectively, p = 0.248. There was no statistical significant difference in mean log$_{10}$ viral load between in utero

Table 2 Baseline HIV-1 RNA load, TLC and hemoglobin levels of 32 transmitters and non-transmitters

Variable	Transmitters N = 32	Non-transmitters N = 32	RR (95% CI)
Hemoglobin g/dl			
<10	7/32 (22)	2/32 (6)	1.71 (1.08-2.69)*
TLC			
Mean cells/mm$_3$(sd)	2147 (111)	2505 (132)	0.99 (0.99-0.99)*
Viral load			
Mean log10 copies/ml	3.55 (1.12)	2.92 (0.92)	1.29 (1.07-1.55)*

and intra-partum/postpartum transmitters. In-utero transmitters generally had higher mean log_{10} viral load compared to their matched controls though not statistically significant and similarly intra-partum/postpartum transmitters had significantly higher mean viral load relative to their respective matched controls, p = 0.034.

Among the 32 transmitting and 32 non-transmitting mothers 6 (19%) and 12 (37.5%), respectively had undetectable viral load respectively and none of them were from the acute infection subgroup. Mothers with undetectable viral load were less likely to transmit when compared to mothers with detectable viral load. There were no statistically significant differences regarding socio-demographic and reproductive health characteristics between mothers with detectable and undetectable viral loads.

HIV-1 RNA load, TLC, Hemoglobin levels and Transmission

Mean TLC for transmitting mothers and non-transmitting mothers were 2147 cells/mm_3 and 2505 cells/mm_3 respectively, p = 0.04. HIV-1 RNA load negatively correlated with TLC, correlation coefficient of -0.254. Each unit increase in log_{10} viral load was associated with a 178 cells/mm_3 decrease in TLC, (p = 0.048). There were no statistical significant differences in mean TLC of mothers with acute and chronic HIV-1 infections and also between in utero transmitters and their respective controls. However, there was a statistically significant difference in mean TLC between intra-partum/postpartum transmitters and their matched controls, p = 0.030, see table 3.

Each unit increase in log_{10} viral load was associated with a 0.2 g/dL decrease in hemoglobin levels, p = 0.021. There were no statistically significant differences in hemoglobin levels between mothers with acute and chronic HIV-1 infection and also among in utero and intra-partum/postpartum transmitters, p = 0.870 and 0.980 respectively. Mean hemoglobin levels were significantly different between intra-partum/postpartum transmitters relative to their matched controls p = 0.038, see table 3. Anaemic mothers with hemoglobin levels of less than 10 g/dL were 1.7 times more likely to transmit compared to those with hemoglobin levels of more than 10 g/dL in univariate analysis. After controlling for the effect of viral load and TLC this relationship ceased to be significant.

Infant Factors, Mortality and Transmission

Infant sex, birth weight, single dose nevirapine therapy and breastfeeding patterns were not significantly different neither between transmitters and non-transmitters nor among in utero and intra-partum/postpartum transmitters. HIV-1 infected infants were 4 times more likely to die compared to those uninfected (p = 0.003), see table 1. The odds of dying were 14 (p = 0.04) for infants infected in utero compared to their respective uninfected controls.

Discussion

This is a first study in Zimbabwe where viral load was determined in pregnant women and was related to time point vertical transmission. This nested case-control study of Harare peri-urban pregnant women provided data on risk factors of vertical transmission by assessing maternal HIV-1 RNA load, TLC and hemoglobin levels of transmitting and non-transmitting mothers, who were otherwise similar with respect to demographic and reproductive health characteristics.

Of note was the highly significant relationship between antenatal HIV-1 RNA load, at 36 weeks gestational period, with vertical transmission. Similar to other studies [7,8,15,16], transmitting mothers had a significantly higher viral load compared to non-transmitting mothers.

No threshold for transmission was observed in this cohort that could predict transmission or non-transmission, as transmission occurred throughout the whole range of viral load values, contrary to previous

Table 3 Comparison of in utero and intra-partum/postpartum transmitters and their respective matched non-transmitting controls with respect to viral load, TLC and hemoglobin levels

Variable	In utero transmitters N = 10	In utero matched controls N = 10	RR (95%CI)	Intra/Postpartum Transmitters N = 22	Intra-/postpartum Matched Controls N = 22	RR (95% CI)
Hemoglobin						
Mean g/dl (sd)	10.7 (0.9)	10.5 (1.3)	1.10 (0.74-1.65)	10.5 (1.5)	11.4 (1.0)	0.82 (0.71-0.94)*
TLC						
Mean cells/ml (SD)	2133 (724)	2207 (443)	0.99 (0.98-1.00)	2153 (576)	2632 (790)	0.98 (0.97 = 0.99)*
Viral load Mean log10 copies/ml	3.5 (1.2)	3.0 (0.9)	1.60 (0.70-3.79)	3.6 (1.1)	2.9 (1.0)	2.0 (1.02-3.81)*

studies [17]. More so, no threshold of HIV-1 RNA load was associated with in utero and intra-partum/postpartum transmissions contrary to some studies [18-20]. Our findings are analogous to those by Garcia et al., where serum HIV-1 RNA levels predicted the risk but not the timing of vertical transmission [21]. While viral load was an important determinant of vertical transmission, it was not the only one, as six percent of non-transmitting mothers had high viral loads of >100 000 copies/mL yet they did not transmit. Besides high levels of viremia, other risk factors of vertical transmission such as maternal host genetic factors, neutralizing antibodies, HIV-1 phenotype and/or genetic diversity could have also played a role in transmission.

Eighteen (28%) of the 64 mothers had undetectable viral load yet some (n = 6) still transmitted the virus to their infants. A Spanish study has also observed some pregnant women with undetectable plasma viral load who were at risk of vertically transmitting the HIV-1 RNA during vaginal delivery [22]. Quantification of HIV-1 RNA in cervico-vaginal secretions has been shown to be more useful when investigating vertical transmission risk associated with vaginal delivery [23]. African mothers who are immigrants in Europe have been shown to have lower HIV-1 RNA loads but were more likely to vertically transmit relative to their non-African counterparts [23-26] probably due to differences in HIV-1 subtypes and host genetic factors. This group of mothers with undetectable viral load could be elite controllers [27-29]. Elite controllers have been shown to maintain high levels of $CD4^+$ $CD25^+$ regulatory T cells in their peripheral blood [30]. These are of high research interest as they may provide novel insights regarding host mechanism of virus control. The percentage of the mothers with undetectable viral load in this study was relatively higher compared to previous Zimbabwean studies done in the late 1990 s which was around 10% [8,31]. This could be attributed to differences in quantitation methods used. The fully automated COBAS AmpliPrep/COBAS TaqMan Viral RNA load test used has been shown to excellently satisfy the requirements for reliable quantification of HIV-1 RNA in clinical specimens of all HIV-1 subtypes [32,33] and the automation itself reduced inter and intra assay variation.

Infant HIV-1 status was successfully determined using qualitative Roche DNA PCR. This test has shown 100% sensitivity and 100% specificity at least in Zimbabwean infants and adults with predominant HIV-1 subtype C [6,34]. The observed in utero and intra-partum/postpartum transmission rates of 7.5%, and 15.3% were quite comparable but lower relative to a previous Zimbabwean study that has shown in utero, intra-partum/early postpartum and late postpartum transmission rates of 9.4%, 16% and 5.3%, respectively [6]. The rates were also quite comparable to those obtained in a Tanzanian cohort with an in utero and intra-partum transmission rates of 8.4% and 16.1% respectively [19]. The overall vertical transmission rate of 21.3% observed was much lower compared to that obtained from previous studies prior to antiretroviral prophylaxis era of 30.7% and 27% [6,8]. This coincides with the general decrease in HIV-1 prevalence in the general population and could be attributed to better access to antiretroviral prophylaxis. However, in this cohort receiving single dose nevirapine was not protective against HIV-1 vertical transmission [35]. This could possibly be due to a relatively small sample size. Intra-partum/postpartum transmissions constituted the majority, 69% of the infections. Other African studies have also shown such high transmission rates through breastfeeding [36]. In resource poor settings, where a large proportion of infants are infected through breastfeeding, concerted efforts should be made towards interventions aimed at reducing such transmissions by advocating for more effective HAART during pregnancy and or breastfeeding, encouraging exclusive breastfeeding for six months, with ongoing breastfeeding thereafter, during the introductions of complementary feeds [37].

Generally male infants were more at risk of HIV-1 vertical transmission though this was not statistically significant, unlike previous studies where in utero transmission was significantly higher among girl than boy infants [38]. Consistent with other studies was the fact that, in utero infected infants were 2.5 times more likely to die relative to intra-partum infected infants probably because they would have been infected for longer periods [39].

As early as 1964, it was recognized that a decrease in the TLC was associated with immune suppression [40]. The equipment and skills to perform total white blood cell count and differentials are readily available in most hospitals and clinics in resource-poor settings, and performing a TLC costs much cheaper compared to CD4 cell count measurements. We applied WHO guidelines that acknowledge that TLC may be used as surrogate marker for CD4 counts in situations where CD4 cell count measurements may not be affordable. Observed was a negative correlation between HIV-1 RNA load and TLC. Pregnant women with high TLC were less likely to transmit to their infants compared to those with low counts and such findings have been observed by others [16,18]. Anaemic mothers were more likely to transmit to their babies. A mean decline of 0.46 g/dL hemoglobin level per unit increase in log $_{10}$ viral load has been observed in a South African study [41]. This value is relatively higher compared to 0.2 g/dL decrease in hemoglobin levels observed in our study. This is

probably due to the fact that the former study sampled only patients with acute HIV-1 infection which was not the case with our study.

In this cohort being single, having multiple partners and having a history of blood transfusion constituted significant risk factors for HIV-1 sero-conversion following delivery. Transmitting sero converters were more likely to be young and have a travelling partner. Prevention strategies should address these risk factors associated with sero-conversion to reduce HIV-1 incidence rates in the general population. In such poor resource settings a nested case control design reduced costs and efforts of data collection considerably with relatively minor loss in statistical efficiency [42]. However, all transmitting and non transmitting mothers selected in the study may not be a full representation of all the cases and controls in the original cohort due to failure to follow up all the mothers and infants, though generally the follow-up rate was good.

Conclusion

We concluded that antenatal serum HIV-1 RNA viral load, TLC and hemoglobin levels in the third trimester were significantly associated with vertical transmission and this association was independent of transmission time. These data support the rationale for preventive strategies designed to reduce vertical transmission through lowering maternal viral load by introducing more effective HAART during pregnancy, delivery and breastfeeding. Unclear are the factors that contribute to the low viral load levels which were observed in some transmitting mothers. Further research is warranted to determine host genetic factors among these mothers who had undetectable viral load but still transmitted to their infants.

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Authors' contributions
DK collected data, carried out the laboratory analysis and drafted the manuscript, GFZ collected data, KKI participated in laboratory analysis, KNE collected data, MMP carried out data analysis and interpretation of results, RS supervised data analysis and interpretation of results, CMZ participated in designing of the study, MF supervised laboratory analysis, SB participated in designing of the study. All authors read and corrected the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. UNAIDS: Epidemiology Fact Sheet on HIV and AIDS, Zimbabwe, UNAIDS Geneva. 2008.
2. Brahmbhatt H, Kigozi G, Wabwire-Mangen F, Serwadda D, Lutalo T, Nalugoda F, Sewankambo N, Kiduggavu M, Wawer M, Gray R: Mortality of HIV-1 infected and uninfected children of HIV-1 infected and uninfected mothers in rural Uganda. J Acquir Immun Defic Syndrome 2006, 41(Suppl 4):504-508.
3. Mbizvo EM, Msuya SE, Stray-Pedersen B, Sundby J, Chirenje MZ, Hussain A: HIV sero-prevalence and its association with the other reproductive tract infections in asymptomatic women in Harare, Zimbabwe. International Journal of STD and AIDS 2001, 12:524-531.
4. Gregson S, Gonese E, Hallet TB, Taruberekera N, Hargrove JW, Lopman B, Corbett EL, Dorrington R, Dube S, Dehne K, Mugurungi O: HIV decline due to reduction in risky sex? Evidence from a comprehensive epidemiological review. Int J Epidemiol 2010.
5. Kourtis AP, Bulterys M, Nesheim SR Lee FK: Understanding the timing of HIV transmission from mother to infant. JAMA 2001, 285:709-712.
6. Zijenah LS, Moulton LH, Iliff P Nathoo K, Munjoma MW, Mutasa K, Malaba L, Zvandasara P, Ward BJ, Humphrey J, ZVITAMBO Study Group: Timing of mother to child transmission of HIV-1 and infant mortality in the first 6 months of Life in Harare, Zimbabwe. Acquir Immun Defic Syndrome 2004, 18:273-280.
7. Arvold ND, Ngo-gian-Huong N, McIntosh K, Suraseranivong V, Warachit B, Piyaworawong S, Changchit T, Lallemant M, Jourdain G, Perinatal HIV Prevention Trial (PHPT-1), Thailand: Maternal HIV-1 DNA load and mother to child transmission. AIDS patient Care and STDs 2007, 21(Suppl 9):638-643.
8. Katzenstein DA, Mbizvo M, Zijenah L, Gittens T, Munjoma M, Hill D, Madzime S, Maldonado Y: Serum level of maternal Human Immunodeficiency Virus (HIV) RNA, infant mortality and vertical transmission of HIV in Zimbabwe. JID 1999, 179:1382-1387.
9. Ilif PJ, Piwoz EG, Tavengwa NV, Zunguza CD, Marinda ET, Nathoo KJ, Moulton LH, Ward BJ, Humphrey JH, ZVITAMBO study group: Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. AIDS 2005, 19(Supp l7):699-708.
10. Gregson S, Zhuwau T, Anderson RM, Chandiwana SK: Is there evidence for behaviour change in response to AIDS in rural Zimbabwe? Social Science & Medicine 1998, 46:321-330.
11. Moyo W, Mbizvo MT: Desire for a future pregnancy among women in Zimbabwe in relation to their self-perceived risk of HIV infection, child mortality, and spontaneous abortion. AIDS and Behavior 2004, 8(Suppl 1):9-16.
12. Lee S, Wong K: The use of total lymphocyte count (TLC) as an independent criterion for initiating HAART in resource-poor countries. J of Infection 2005, 50(1):66-67.
13. WHO: Scaling up antiretroviral therapy in resource-limited settings: Guidelines for a public health approach. 2002 [http://www.who.int].
14. Bryson YJ, Luzuriaga K, Sullivan JL, Wara DW: Proposed definitions' for in utero versus intrapartum transmission of HIV-1. N Eng J Med 1992, 327:1246-1247.
15. Cao Y, Krogstad P, Korber BT, Koup RA, Muldoon M, Macken C, Song JL, Jin Z, Zhao JQ, Clapp S, Chen IS, Ho DD, Ammann AJ: Maternal HIV-1 viral load and vertical transmission of infection. Nature Medicine 1997, 3(Suppl 5):549-552.
16. O' Shea S, Newell ML, Dunn DT, Garcia-Roudriguez MC, Bates I, Mullen J, Rostron T, Corbett K, Aiyer S, Butler K, Smith R, Banatvala JE: Maternal viral load, CD4 cell count and vertical transmission. Journal of medical virology 1998, 54:113-117.
17. Fang G, Burger H, Grimson R, Tropper P, Nachman S, Mayers D, Weislow O, Moore R, Reyelt C, Hutcheon N, Baker D, Weiser B: Maternal plasma human immunodeficiency virus type 1 RNA level: A determinant and projected threshold for mother to child transmission. Proc Natl Acad of Sci of the USA 1995, 92:12100-12104.

18. Jourdain G, Mary JY, Coeur SL, Ngo-Giang-Huong N, Yuthavisuthi P, Limtrakul A, Traisathit P, McIntosh K, Lallemant M, Perinatal HIV Prevention Trial Group, Thailand: Risk factors for in utero or intra-partum mother to child transmission of Human Immunodeficiency Virus type in Thailand. J Infect Dis 2007, 196:1629-1636.
19. Fawzi W, Msamanga G, Renjifo B, Spiegelman D, Urassa E, Hashemi L, Antelman G, Essex M, Hunter D: Predictors of intrauterine and intra-partum transmission of HIV-1 among Tanzanian women. AIDS 2001, 15:1157-1165.
20. Magder LS, Mofenson L, Paul ME, Zorrilla CD, Blattner WA, Tuomala RE, LaRussa P, Landesman S, Rich KC: Risk factors for in utero and intra-partum transmission of HIV. J Acquir Immun Defic Syndrome 2005, 38(Suppl 1):87-95.
21. Garcia PM, Kalish LA, Pitt J, Minkoff H, Quinn TC, Burchett SK, Kornegay J, Jackson B, Moye J, Hanson C, Zorrilla C, Lew JF: Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Babies transmission study group. N Engl J Med 1999, 341(Suppl 6):394-402.
22. Garcia-Bujalance S, Ruiz G, De Guevara CL, Pena JM, Bates I, Vazquez JJ, Gutierrez A: Quantitation of Human Immunodeficiency Virus type 1 in cervico-vaginal secretions in pregnant women and relationship between viral loads in the genital tract and blood. Eur J Clin Microbiol Infect Dis 2004, 23(Suppl 2):111-115.
23. Mayaux MJ, Blanche S, Rouzioux C, Le Chenadec J, Chambrin V, Firtion G, Allemon MC: Maternal factors associated with perinatal HIV-1 transmission, The French cohort Study, 7 years of follow-up observation. J Acquir Immun Defic Syndrome 1995, 8:188-194.
24. Mayaux MJ, Dussaix E, Isopet J, Rekacewicz C, Mandelbrot L, Ciraru-Vigneron N, Allemon VC, Katlama C, Delfraissy JF, Puel J: Maternal Virus load during pregnancy and mother to child transmission of Human Immunodeficiency Virus type 1: The French Perinatal Cohort Studies. J Infect Dis 1997, 175:172-175.
25. Gottesman BS, Grosman Z, Lorber M, Levi I, Shitrit P, Mileguir F, Gottesman G, Chower MY: Measurement of HIV RNA in patients infected by subtype C by assays optimized for subtype B results in an underestimation of the viral load. J Med Virol 2004, 73:167-171.
26. Jasseron C, Mandelbrot L, Tubiana R, Teglas JP, Faye A, Dollfus C, Le Chenadec J, Rouzioux C, Blanche S, Warszawski J, ANRS French Perinatal Cohort: Prevention of mother to child HIV transmission: similar access for Sub Saharan African immigrants and for French women. AIDS 2008, 22(Suppl 12):1503-1511.
27. Goulder PJ, Watkins DI: HIV and SIV CTL escape implication for vaccine design. Nat Rev Immunol 2004, 4:630-640.
28. Bailey JR, Williams TM, Siliciano RF, Blankson JN: Maintenance of viral suppression in HIV-1 infected HLA- B*57+ elite suppressors despite CTL escape mutation. J Exp Med 2006, 203(Suppl 5):1357-1369.
29. Goepfert PA, Lumm W, Farmer P, Matthews P, Prendergast A, Carlson JM, Deedeyn CA Tang J, Kaslow RA, Bansal A, Yusim K, Heckerman D, Mulenga J, Allen S, Goulder PJR, Hunter E: Transmission of HIV-1 Gag immune escape mutation is associated with reduced viral load in linked recipients. J Exp Med 2008, 205(Suppl 5):1009-1017.
30. Chase AJ, Yang H, Zhang H, Blankson JN, Siliciano RF: Preservation of FoxP3+ regulatory T cells in the peripheral blood of Human Immunodeficiency Virus Type-1 infected Elite suppressors correlates with low CD4+ T cell activation. J Virol 2008, 82(Suppl 17):8307-8315.
31. Guevara H, Johnston E, Zijenah L, Tobaiwa O, Mason P, Contag C, Mahomed K, Hendry M, Katzenstein D: Prenatal Transmission of Subtype C HIV-1 in Zimbabwe: HIV-1 RNA and DNA in Maternal and Cord Blood. JAIDS 2000, 25(Suppl 5):390-397.
32. Schumacher W, Frick E, Kauselmann M, Maier-Hoyle V, van der Vliet R, Babiel R: Fully automated quantification of Human Immunodeficiency Virus (HIV) type 1 RNA in human plasma by the COBAS AmpliPrep/ COBAS TaqMan system. J Clin Virol 2007, 38(Suppl 4):304-312.
33. Michael NL, Herman SA, Kwok S, Dreyer K, Wang J, Christopherson C, Spadoro JP, Young KK, Polonis V, McCutchan FE, Carr J, Mascola JR, Jagodzinski LL, Robb ML: Development of calibrated viral load standards for group M subtypes of human immunodeficiency virus type 1 and performance of an improved Amplicor HIV-1 MONITOR test with isolates of diverse subtypes. J Clin Micro 1999, 37:2557-2563.
34. Zijenah LS, Humphrey J, Nathoo K, Malaba L, Zvandasara P, Mahomva A, Iliff P, Mbizvo MT: Evaluation of the Prototype Roche DNA Amplification Kit Incorporating the new SK145 and SKCC1B primers in detection of Human Immunodeficiency Virus type 1 DNA in Zimbabwe. J Clin Microbiol 1999, 37(Suppl 11):3569-3571.
35. Gumbo FZ, Kurewa NE, Kandawasvika GQ, Duri K, Mapingure MP, Munjoma MW, Pazvakavambwa IE, Rusakaniko S, Chirenje MZ, Stray-Pedersen B: Rising mother-to-child transmission in a resource-limited breastfeeding population. Trop Doct 2010, 40:70-73.
36. Nduati R, John G, Mbori-Ngacha D, Richardson B, Overbaugh J, Mwatha A, Ndinya-Achola J, Bwayo J, Onyango FE, Hughes J, Kreiss J: Effect of breastfeeding and formula feeding on transmission of HIV-1: A randomized clinical trial. JAMA 2000, 283:1167-1174.
37. WHO Report, 2010. Exclusive breastfeeding. [http://www.who.int/nutrition/topics/exclusive_breastfeeding/en/print.html].
38. Piwoz EG, Humprey JH, Mutasa K, Moulton LH, Iliff PJ: Effects of baby sex on mother to child transmission of HIV-1 according to timing of infection in Zimbabwe. AIDS 2006, 20(Suppl 15):1981-1983.
39. Kuhn L, Steketee RW, Abrams EJ, Lambert G, Bamji M, Schoenbaum E, Farley J, Nesheim SR, Palumbo P, Simonds RJ, Thea DM: Distinct risk factors for intrauterine and intrapartum human immunodeficiency virus transmission and consequences for disease progression in infected children. Perinatal AIDS Collaborative Transmission Study. J Infect Dis 1999, 179:52-58.
40. Isom JB, Gordy PD, Selner JC, Brown LJ, Willis M: Immuno-suppression and Infection. N Eng J Med 1964, 12(Suppl 271):1068-1069.
41. Mlisan K, Auld SC, Grobler A, van Loggerenberg F, Williamson C, Iriogbe I, Sobieszczyk ME, Abdool Karim SS, CAPRISA Acute Infection Study Team: Anaemia in acute HIV-1 subtype C infection. PLos One 2008, 3(Suppl 2): e1626, 1-5.
42. Ernster VL: Nested case-control studies. Prev Med 1994, 23(5):587-590.

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Paper II

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Genotypic Analysis of Human Immunodeficiency Virus Type 1 (HIV-1) env V3 Loop Sequences: Bioinformatics Prediction of Coreceptor Usage among 28 Infected Mother–Infant Pairs in a Drug-Naive Population

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Abstract

We sought to predict virus coreceptor utilization using a simple bioinformatics method based on genotypic analysis of human immunodeficiency virus types 1 (HIV-1) env V3 loop sequences of 28 infected but drug-naive women during pregnancy and their infected infants and to better understand coreceptor usage in vertical transmission dynamics. The HIV-1 env V3 loop was sequenced from plasma samples and analyzed for viral coreceptor usage and subtype in a cohort of HIV-1-infected pregnant women. Predicted maternal frequencies of the X4, R5X4, and R5 genotypes were 7%, 11%, and 82%, respectively. Antenatal plasma viral load was higher, with a mean $\log_{10}$ (SD) of 4.8 (1.6) and 3.6 (1.2) for women with the X4 and R5 genotypes, respectively, p ¼ 0.078. Amino acid substitution from the conserved V3 loop crown motif GPGQ to GPGR and lymphadenopathy were associated with the X4 genotype, p ¼ 0.031 and 0.043, respectively. The maternal viral coreceptor genotype was generally preserved in vertical transmission and was predictive of the newborn's viral genotype. Infants born to mothers with X4 genotypes were more likely to have lower birth weights relative to those born to mothers with the R5 genotype, with a mean weight (SD) of 2870 (Æ332) and 3069 (Æ300) g, respectively. These data show that at least in HIV-1 subtype C, R5 coreceptor usage is the most predominant genotype, which is generally preserved following vertical transmission and is associated with the V3 GPGQ crown motif. Therefore, antiretroviral-naive pregnant women and their infants can benefit from ARV combination therapies that include R5 entry inhibitors following prediction of their coreceptor genotype using simple bioinformatics methods.

Introduction

Human immunodeficiency virus type 1 (HIV-1) enters target cells through interaction of the viral envelope (env) glycoprotein (gp) 120 with a host cellular receptor CD4 molecule and a chemokine coreceptor. Based on chemokine coreceptor usage, HIV-1 can be classified as CCR5 (R5), CXCR4 (X4), or dual tropic (R5X4).[1] The genetic determinants of HIV-1 coreceptor usage are localized in the V3 loop of gp120, which has a highly conserved crown motif and glycosylation sites.[2] More so, this V3 region is also crucial in viral replication, transmission, infectivity, and neutralization.[3] Genetic variation within this region has been linked to changes in coreceptor usage.[2]

Frequencies of R5 HIV-1 variants vary among different populations, being 80% and 50% in drug-naive individuals and patients receiving antiretroviral therapy, respectively.[4,5] During the course of HIV-1 infection, the virus changes its coreceptor usage from R5 to X4 with or without concurrent use of R5 in 50% of HIV-1 subtype B-infected individuals.[6] This switch of coreceptor usage is associated with an accelerated decrease in CD4 cells and hence it could be an important

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determinant of HIV pathogenesis and disease progression.[7] However, some subtype C and D studies have observed X4 variants in newly infected individuals.[8,9]

Irrespective of the transmission route or HIV-1 subtype, R5 viruses are preferentially transmitted in both adults and children except for subtype D.[6,10] At least in subtype B, maternal viral phenotype can be predictive of the newborn's viral phenotype while the R5X4 phenotype is predominantly lost during vertical transmission.[11] Contrary to this finding, vertical transmission of dual-tropic HIV-1 has been demonstrated.[9] V3 loop genotypic characteristics with special emphasis on the predominant HIV-1 subtype C leading to preferential vertical transmission of a particular coreceptor genotype from mother to infant remains unclear, yet this information is critical for the development of effective transmission-preventive strategies.

With the recent introduction of HIV-1 chemokine receptor antagonists on the market as components of antiretroviral therapy, it is increasingly important to screen HIV patients' coreceptor usage prior to therapy.[12,13] Hence simple and efficient methods for routinely characterizing and monitoring HIV-1 coreceptor usage are needed to replace slow and resource-intensive phenotypic assays. Excellent correlations between the HIV-1 V3 genotype and phenotype have been observed.[14,15] Bioinformatics methods have been developed to improve the genotypic prediction of HIV-1 coreceptor usage from V3 sequences.[16] There is little information regarding the bioinformatics' prediction of HIV-1 coreceptor usage in Zimbabwe, yet this information is important for drug or vaccine design and development. This study aimed to predict virus coreceptor usage using a simple bioinformatics method based on HIV-1 V3 sequences from infected but drug-naive mother–infant pairs to better understand coreceptor genotypes in the dynamics of vertical transmission.

Materials and Methods

Study design and setting

This was an antiretroviral therapy-naive Prevention of Mother-to-Child Transmission (PMTCT) cohort study of pregnant women attending three antenatal clinics all around the city of Harare.[17]

Study population and procedures

Two groups of pregnant HIV-1-positive women who later transmitted their virus to their infants were studied. The main group consisted of pregnant women who were HIV-1 positive at baseline, regarded as having chronic HIV-1 infections, and a minor group of women who were HIV-1 negative during pregnancy but later on seroconverted after delivery during the follow-up period, regarded as having acute HIV-1 infections (Fig. 1).

Consent was obtained from the women followed by enrollment at 36 gestational weeks in a national PMTCT program between April and September 2002. Pre- and post-HIV test counseling was offered. The mode of HIV-1 acquisition was most likely heterosexual and generally all the women were asymptomatic at enrollment. HIV-1-positive mother and infant pairs were offered 200 mg of single dose nevirapine during labor and 2 mg/kg body weight within 72 h postdelivery, respectively.[18] Mothers were encouraged to exclusively breastfeed during the first 6 months. Infants' venous EDTA whole blood and plasma samples were collected at delivery, at 6 weeks, and at 4 and 9 months postpartum, and thereafter every 3 months until 2 years, thus generally coinciding with infant immunization visits. Samples were stored at Å868C until testing. At each subsequent follow-up visit, HIV-1-negative mothers and previously DNA PCR test result-negative but exposed infants were retested for HIV-1 antibodies and antigen, respectively. In addition to HIV testing, plasma samples of seronegative mothers and their respective infants were aliquoted and stored for further analysis in the event that they seroconverted.

FIG. 1. Summary of the sampling of the subset of 28 transmitters from the original cohort of 691 pregnant women.

Mothers' and infants' demographic characteristics and examination

All mothers answered a structured questionnaire at enrollment addressing information regarding their sociodemographics, sexual behavior, and obstetric and reproductive health issues. A gynecologist performed physical and gynecological examinations.

A pediatrician examined the infants and recorded their date of birth, birth weight, gender, and single-dose nevirapine therapy administration.

Mothers' tests

Serial HIV-1/2 algorithm antibody tests were done using Determine (Abbott Diagnostics, Illinois) and Ora-Quick (Abbott Diagnostics, Illinois) rapid kits on mothers' plasma samples. EDTA-anticoagulated venous blood samples were processed within 6 h for full blood counts using the Abbott Diagnostic Cell Dyne 3500R SL Hematology Analyser. Mothers were screened for sexually transmitted infections as previously described by Gumbo et al.[18] Serum samples were shipped on dry ice to the Department of Microbiology,

Rikshospitalet in Oslo for further laboratory analysis. Maternal baseline plasma samples were quantified for HIV-1 RNA load using the automated TaqMan Roche Amplicor 1.5 Monitor Test (Cobas AmpliPrep/Cobas TaqMan, Roche Diagnostics, Branchburg, NJ) according to the manufacturer's instructions. As for seroconverters, the first positive sample available was quantified. Baseline total RNA was extracted for V3 loop sequencing.

Infants' tests

Detection of infants HIV-1 infection was performed using qualitative the 1.5 Roche Amplicor HIV-1 DNA PCR kit (Roche Diagnostics). Infants that tested HIV-1 DNA PCR positive on whole blood collected within 10 days of birth were considered to be infected in utero. Infants who had negative HIV-1 DNA PCR results within the first 10 days of life but had positive results at 6 weeks were considered to be infected intrapartum and those positive thereafter were considered infected after birth.

The first DNA PCR-positive sample available was HIV-1 env V3 loop sequenced. Seven infants had longitudinal samples within the 2-month follow-up period.

Nucleic acid extraction PCR amplification and cloning

Total RNA was extracted from plasma using the Boom et al.[19] method. The primary PCR amplified an approximately 800-base pair (bp) fragment spanning the V3 and V4 region of the envelope (positions 6948–7537) in the HIV-HXB2 genome using outer sense and antisense primers, 5′-GTCAGCACA GTACAATGTACACAT-3′ and 5′-GCGCCCATAGTGCTTC CTGCTGC-3′, respectively. Secondary PCR amplified an approximately 535-bp env gene fragment using inner sense and antisense primers 5′-ACAATGYACACATGGAATTARG CCA-3′ and 5′-GGAGGGGCATACATTGCT-3′, respectively. Both positive and negative controls were included in all the PCR reactions to rule out any possible contamination and for assay sensitivity assessment. Detection and quantification of secondary PCR amplicons were done using a 1% agarose gel electrophoresed together with a standard mass ladder and then stained with SYBR safe stain. Gel reading was done using Gel Doc 2000 analyzer (Bio-Rad). The standard mass ladder was used to estimate molecular weights of the amplicons and the respective band intensity was used to estimate the quantities. Removal of salts and excess primers in amplicons was done using Microspin columns (Amersham Bioscience). Amplicons were diluted to a final concentration of 5–20 ng of template DNA for direct sequencing.

If direct sequencing was not possible cloning was done using an Invitrogen TOPO TA cloning kit version J, 2006. Secondary PCR products were cloned in an Invitrogen plasmid vector, PCR®2.1-TOPO®, using the chemical transformation method followed by expression in competent Escherichia coli cells (C4040-03). In the presence of galactosidase substrate, X–gal (5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside) and an inducer, isopropyl thiogalactoside (IPTG) on an agar medium on a culture plate, transformed E. coli with PCR inserts in their LacZ open reading frame were unable to make b-galactosidase enzyme and presented as white colonies. Four randomly selected white colonies were streaked on a quarterly subdivided Luria-Bertani plate medium containing 50 mg/ml kanamycin and incubated overnight at 378C. Plasmid DNA isolation was done using the mini-preparation method.

DNA sequencing and analysis

Sequencing of both sense and antisense strands of the diluted secondary amplicons was done using BigDye terminator sequencing standard kit version 3.1 (Applied Biosystems, Foster City, CA) using the inner amplification primers. Sequencing reaction products were analyzed on an ABI 3730 DNA analyzer (Applied Biosystem/HITACHI, Tokyo, Japan). Forward and reverse sequences were assembled using the Vector NTI Advance 10 program. Alignment was done using Gene Doc, BioEdit, and Clustal X2 sequence alignment programs with manual editing to ensure that deletions or insertions did not alter the reading frame. Samples V3 loop sequences were aligned against a Los Alamos subtype C

Table 1. Maternal Baseline Social Demographic, Reproductive Health, and Markers of Disease Progression Including Infant Characteristics among 25 Mothers with Chronic HIV-1 Infection

Variable	X4/R5X4 genotypes	R5 genotype	p Value
Age in years			
Mean (SD)	30.8 (3.8)	27.0 (5.0)	0.176
Parity			
No child, n (%)	0 (0)	1 (100)	
! One child, n (%)	3 (14)	18 (86)	0.684
HSV infection			
Not infected, n (%)	1 (50)	1 (50)	
Infected, n (%)	2 (11)	16 (89)	0.144
Syphilis			
Not infected, n (%)	5 (25)	15 (75)	
Infected, n (%)	0 (0)	2 (100)	0.421
Trichomonas			
Not infected, n (%)	3 (18)	14 (82)	
Infected, n (%)	0 (0)	3 (100)	0.430
Genital ulcer disease			
Not infected, n (%)	5 (25)	15 (75)	
Infected, n (%)	0 (0)	1 (100)	0.567
Lympadenopathy			
Absent, n (%)	3 (15)	17 (85)	
Present, n (%)	2 (67)	1 (33)	0.043
Baseline Hb			
Mean (SD)	11.0 (1.8)	10.9 (1.0)	0.925
Log_{10} viral load			
Mean (SD) (copies/ml)	4.8 (1.6)	3.6 (1.2)	0.078
GPGQ crown motif			
Absent, n (%)	2 (67)	1 (33)	
Present, n (%)	3 (14)	19 (86)	0.031
Maternal death			
No, n (%)	4 (17)	19 (83)	
Yes, n (%)	1 (50)	1 (50)	0.269
Baby delivery weight			
Mean (SD) (g)	2870 (332)	3069 (300)	0.205
Baby sex			
Female, n (%)	2 (22)	7 (78)	
Male, n (%)	1 (9)	10 (91)	0.413

n is for number tested; SD, standard deviation.
X4 and R5X4 genotypes were grouped together.

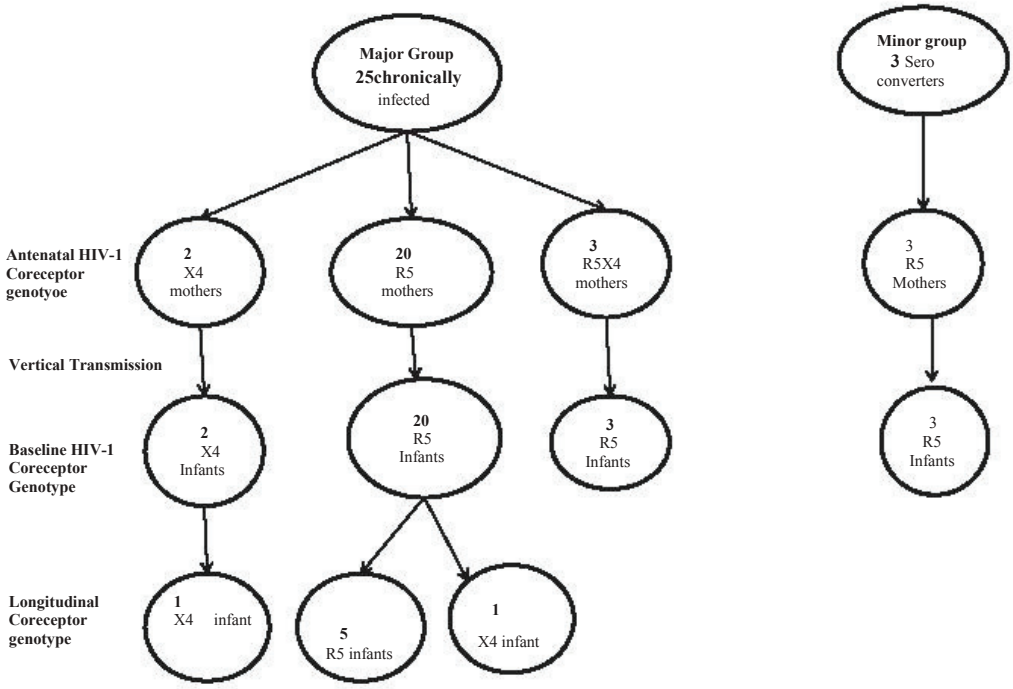

FIG. 2. Baseline mother–infant pair coreceptor genotypes and infants' longitudinal genotypes.

consensus. A year 2000 local Zimbabwean HIV-1 subtype C V3 loop consensus was also included in the alignment.[8]

Coreceptor usage genotype prediction

Samples V3 loop sequences were aligned to the program training sequence. Gaps were introduced for optimization of homologous amino acid residues. Following alignment of the approximately V3 loop 35 amino acid residues, prediction of coreceptor usage genotype was automatically generated on a subC Position-Specific Scoring Matrices (PSSM) algorithm freely available online at the following website: http://

[16]mullinslab.microbiol.washington.edu/computing/pssm/. Sequence scores of lower than the 5th percentile and higher than the 95th percentile were assigned to the R5 and R4 genotypes, respectively. The intermediate score were assigned a genotype prediction based on amino acid residues charge at either position 11 or 25.[16] A mixture of both R5 and R4 genotype prediction in one sample was regarded as having a dual R5X4 genotype.

Subtype determination

HIV-1 subtype was determined using secondary PCR amplicons of the env gene fragment (535 bp). Sequences in their respective Fasta format were entered into the REGA HIV-1 BioAfrica-Bioinformatics tool (version 2.0), website http://www.bioafrica.net/, and the sequences were automatically subtyped.

Data analysis

The data were collected and analyzed using SPSS (version 17.0, Chicago, IL). The frequency and transmission pattern(s) of HIV-1 coreceptor usage genotype, the crown GPGQ motif,

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FIG. 3. Mother–infant pairs of HIV-1 subtype C V3 loop sequences aligned with a Los Alamos consensus reference and their respective predicted coreceptor usage genotype. *Mother–infant pairs are identified by identification number (ID) with infant ID with a B (baby) prefix before the ID number. Infants' ID and sequences are in italics. ID numbers with an asterisk indicate seroconverters (mothers with relatively acute HIV-1 infection). The number after the underscore indicates the time of blood collection for sequencing. 0 denotes at enrollment and delivery for the mothers and infants, respectively. WKS denotes weeks postpartum. M denotes months postpartum and seroconversion from delivery for infants and postdelivery for mothers. In parentheses at the end of each sequence are the number of V3 loop sequences analyzed for each sample. Generally all samples were directly sequenced except for IDs 39, 118, 205, and 366, which were also cloned. Within the alignment dots indicate identity with the consensus sequence and dashes indicate deletion for optimization of alignment. Paragraphs were used to separate different mother–infant pairs.

ID No.	10 20 30	CCR5/CXCR4 Usage
	....|....|....|....|....|....|....|....|	
Los Alamos	CTRPNNNT-RKSIRI-GPGQAFYA-TGEI-IGDIRQAHC	
Zim Cons	.....-.....-.....-.....DD.-.....	R5
39_0	.....-...V..-....T...-....-..-.....(1)	R4
39_0_A	.A..SK..-.....-...RV...-ND-.-.....E.Y.(1)	R4
39_0_B	.....-...V..-...RT...-....-..-.....(3)	R5
B39_4M	.....-...*V*..-.....-.*N.V*-.....*Y.(3)*	*R5*
46_6M*	.....-.....-....V...-..N.-.....(2)	R5
B46_12M	.....-.....-....*V*...-..*N.*-.....*(2)*	*R5*
67_0	.....-.....-....T...-.NG.-.....Y.(2)	R5
B67_9M	.....-...*V*..-.....-.*ND*.-.....*(2)*	*R5*
118_0	....S...-.....-....V...-ND-.-.....E...(2)	R5
B118_0	.*A..S*...-.....-....*V..S*-.*ND*.-..*N..E...(2)*	*R5*
B118_9M	.*A..SK*..-.....-....*V*...-.*ND*.-..*N..E...(2)*	*R5*
139_0	.V..S...-.....-....T...-....-.....(2)	R5
B139_6wks	.*V..S*...-.....-....*T*...-....-.....*(2)*	*R5*
B139_9M	.*V..S*...-.....-....*T*...-....-.....*(2)*	*R5*
143_0	.....-.....-....T...-..D.-.....(2)	R5
B143_9M	.....-.....-....*T*...-..*D.*-.....*(2)*	*R5*
157_0	.....-...V..-....V..T-ND-.-.....E...(2)	R5
B157_0	.....-...*V*..-....*V..T-ND*-.-.....*E...(2)*	*R5*
165_0	.....-...V..-....T...-....-..N.....(2)	R5
B165_9M	.....-...*M*..-....*T*...-..*D.*-.....*(2)*	*R5*
205Mo_0	.....-...V..-....V...-..N..-.....K...(2)	R5
205Mo_0_A	.V.....-STG...-....T...-P.R.-.....(4)	R4
B205_12M_B	.*I*.....-.....-....*T*...-..*G.-T.N*.....*(3)*	*R5*
B205_18M_C	.*I*.....-.*Q*....-....*T*...-*P*.....*(2)*	*R5*
210_0	....G...-.Q.V..-....T...-....-..N.....(2)	R5
210_24M	.....-...V..-....T...-....-..N.....(2)	R5
B210_0	....*G*...-.*Q.V*..-....*T*...-....-.....*Y.(2)*	*R5*
B210_6wks	....*G*...-.*Q.V*..-....*T*...-....-.....*Y.(2)*	*R5*
228_0	.....-.....-....T..T-....-.....Y.(2)	R5
B228_4M	.....-.....-....*T..T*-....-.....*Y.(2)*	*R5*
279_0	.I.....-.....-.....F.-.TD.-..N....Y.(2)	R5
B279_0	.*I*.....-.....-.....*F.-.TD*.-..*N*....*Y.(2)*	*R5*
344_0_A	.....-.R....-.....-..D.-.....K...(2)	R5
B344_18M	.*A*.....-.....-....*T..T-N-D*.-..*N*.....*(2)*	*R5*
345_0	.....-.R....-.....-..D.-..N..E...(2)	R5
B345_9M	.*A*.....-.....-....*T*...-..*D.*-.....*(2)*	*R5*
366_MO_C	.....-...VW.-...RV...-..D.-.....(4)	R4
B366_12M	.....-..*GV*..-....*V*...-..*D.*-..*K..E...(4)*	*R5*
375_MO_A	.....-.....-....T.F.-..A.-..N..K...(4)	R5
B375_12M	.....-.....-....*TIF*.-..*A.*-..*N..K...(4)*	*R5*
453_6WKS	.....-.....-....TYF.-..D.-..N.....(2)	R5
B453_6WKS	.....-.*Q.V*..-.....*F.-..D.*-.....*K.Y.(2)*	*R5*
504_0_A	.....-...V..-....T...-....-V.N.....(2)	R5
B504_0	.....-...V..-....T...-..D.-..N.....(2)	R5
B504_6WKS_B	.....-...*V*..-....*T*...-..*D.*-..*N*.....*(2)*	*R5*
506_0_B	...H....-...V..-.....-.N..-.....K...(2)	R5
506_0_C	.M.....-...V..-....T...-.ND.-.....(1)	R5
B506_0	.*M*.....-...*V*..-....*T*...-.*ND*.-.....*(2)*	*R5*
541_0_A	.....-.....-....T...-..D.-..N.....(2)	R5
B541_9M_A	.....-.....-....*T*...-..*D.*-..*N*.....*(2)*	*R5*
567MO_0_A	.....-...V..-....S...-..D.-.....(4)	R5
567Mo_0_D	.V.....-ST...-....T...-P.R.-.....(1)	R4
B567_12M_C	.....-.....-....*S*...-*D*-.-.....*(3)*	*R5*
683_0	...C....-QQ....-....T...-.D-.-..N.....(2)	R5
B683_6wks	.....-...*V*..-....*T*...-..-.-..*N*.....*(2)*	*R5*
B683_15M	.....-.....-...*R*.....-*N*-.-.....*(2)*	*R4*
714_9M_A*	....S...-.....-.....-.D-.-.....(4)	R5
B714_9M-A	....*S*...-.....-.....-.*D*-.-.....*(2)*	*R5*
743_6WKS	.V.....-.Q....-...R..F.-.KG.-..N....Y.(2)	R4
B743_6WKS	.*V*.....-.*Q*....-.....*F.-.KG*.-..*N*....*Y.(2)*	*R4*
B743_4M	.*V*.....-.*Q*....-.....*F.-.KG*.-..*N*....*Y.(2)*	*R4*
3221_9M*	.I..G...-.....-....T...-...V-..N.....(2)	R5
B3221_18M	.*I*.....-.*Q..R.*-....*T*...-...*V*-..*N*.....*(2)*	*R5*
3351_0_A	.....-.....-....T...-....-..N....Y.(4)	R5
B3351_21M-A	.....-.....-....*T*...-....-.....*Y.(2)*	*R5*
B3351_21M-B	.....-.....*V*-....*T*...-....-..N.....*(1)*	*R5*
3551_6WKS	.....-.R....-....S...-..D.-.....(2)	R5
B3551_17M	.....-.....-....*T*...-....-.....*Y.(2)*	*R5*
4031_0	.I.....-.Q....-....T...-....-.....(2)	R5
B4031_0_A	.*I*.....-.*Q*....-....*T*...-....-.....*(2)*	*R5*

and the potential glycosylation site, six amino acids upstream of the first cysteine, were determined in both the mothers and infants. Viral load values were $\log_{10}$ transformed. The Student's t-test was used to compare mean maternal age, $\log_{10}$ viral load, hemoglobin (Hb) levels, and baby delivery weights of mothers with antenatal X4 and R5 genotypes. For analysis purposes women with X4 and R5X4 genotypes were grouped together as X4 and R5/X4. Regression analysis was used to investigate the association between mothers, infant genotypes, and vertical transmission. Tests of statistical significance included the 95% confidence interval of unadjusted relative risks, two-sided p values based on Chi-square, and Fisher's exact tests.

Results

From a cohort of 177 pregnant women with chronic HIV-1 infections, 29 mothers transmitted the virus to their infants and of these 25 had their samples successfully sequenced. Out of the 514 HIV-1-negative pregnant mothers at baseline, 24 seroconverted during the follow-up period and of these three infected their infants (acute HIV infections). Hence for both groups, a total of 28 transmitting mothers had their V3 loop successfully sequenced (Fig. 1).

When coreceptor usage was compared with other parameters, some trends were observed that were, however, nonsignificant statistically. Mothers with X4 variants were more likely to be older, mean age (SD) 30.8 (3.8) versus 27.0 (5.0) years, and also to have more children, p ¼ 0.176 and 0.684, respectively. Interestingly, relatively younger women with the R5 genotype were generally more likely to have reproductive tract infections relative to their older counterparts with the X4 genotype, although this was also not statistically significant. Lymphadenopathy was significantly more common among mothers with the X4 genotype (67%) versus those with R5 variants (33%), p ¼ 0.043. Higher viral load was associated with the X4 genotype, mean $\log_{10}$ (SD) viral load 4.8 (1.6) and 3.6 (1.2), respectively, p ¼ 0.078 (Table 1).

HIV-1 subtypes

All the 28 mothers and their respective infants had purely HIV-1 subtype C virus.

Predicted coreceptor genotypes for the 28 pregnant women

Overall, for the two groups of women, predicted maternal HIV-1 coreceptor usage frequencies for the X4, R5X4, and R5 genotypes were 7%, 11%, and 82%, respectively, although the frequencies were somewhat different after stratification by maternal time of HIV-1 infection, whether chronic or acute.

Predicted maternal coreceptor usage for 25 mothers and infants with chronic HIV-1 infection

Antenatal frequencies of X4, dual R5X4, and R5 genotypes among the 25 mothers with chronic infections were 8%, 12%, and 80%, respectively (Fig. 2). Maternal viral genotype was generally preserved in vertical transmission and was predictive of the newborn's viral genotype. Infants born to mothers with X4 variants were more likely to have lower birth weights relative to those born to mothers with the R5 genotype, mean (SD) 2870 (332) g and 3069 (300) g, respectively, p ¼ 0.205.

The infants' baseline (first HIV-1-positive sample) coreceptor use genotypes were 8% and 92% for the X4 and R5, respectively. No infant had a dual-tropic genotype. There was no association between antenatal HIV-1 coreceptor usage and the time of infection of the infants, be it in utero, intrapartum, or postpartum, p ¼ 0.365. Seven infants had two longitudinal samples for coreceptor use determination within the 24-month follow-up period. Interestingly, six of these infants had the R5 genotype at baseline and of these, one switched coreceptor usage to the X4 genotype 15 months later. This infant's mother had an R5 genotype at baseline. One of the seven infants acquired an X4 genotype from the mother at 6 weeks after delivery and had maintained it by 4 months of age (Fig. 3).

Coreceptor usage of three mothers and infants with acute HIV-1 infection

Three mothers seroconverted during the follow-up period, one at 6 months and two at 9 months postdelivery, also infecting their respective infants. All the mothers had baseline R5 genotypes. Like their counterparts in the main group with chronic HIV-1 infection, maternal baseline coreceptor genotype was predictive of the newborn's genotype. Dual tropism was also absent in these infants.

V3 loop crown motif

Substitution from GPGQ to GPGR was associated with the X4 genotype (p ¼ 0.031). The conserved GPGQ crown motif at the tip of the V3 loop was present in 88% of the mothers with chronic HIV-1 infection whereas all the seroconverters had this motif, indicating a possible functional significance for this site.

Ninety-six percent of the infants had the conserved GPGQ site with only one infant having the GPGR motif. This infant was infected at 6 weeks postpartum and had a baseline GPGQ motif. However, at 15 months of age the motif had switched to GPGR, associated with the X4 genotype. The mother had a baseline sample only, with the GPGQ motif.

All infants born to seroconverting mothers had GPGQ motifs.

Glycosylation site

Potential glycosylation sites located six amino acids upstream from the first cysteine were conserved in 96% of the mothers with chronic HIV-1 infection with only one infant losing this site. All the recent seroconverters and their infants had this site conserved. Generally for both the chronic and acute infections, there was no systemic loss or acquisition of glycosylation sites during vertical transmission.

Discussion

This study is the first study in Zimbabwe where frequencies of coreceptor usage genotypes have been determined in a population of pregnant women and related to vertical transmission, more so applying a relatively new bioinformatics tool to predict HIV coreceptor usage based on genotypic data from HIV-1 V3 loop sequences of the env gp120. This tool has been confirmed to have a high accuracy and has been specifically optimized for HIV-1 subtype C.[16,20] The recent introduction of entry inhibitors in the clinics as components of

antiretroviral therapy has diversified research in coreceptor usage in HIV-1 infection. Interestingly, the anti-R5 inhibitor, maraviroc, can be taken with or without food and does not require refrigeration and hence can be very suitable for resource-poor settings where coincidentally subtype C predominates with the R5 genotype being present throughout, regardless of disease stage.[12]

Prediction of R5 coreceptor usage of at least 82% among the mother and their infants was observed, which is in agreement with previous studies[21] not from Zimbabwe.[4] A previous report from Zimbabwe was different, studying mostly patients on antiretroviral therapy who had an R5 phenotype frequency of 50%. A generally high R5 genotype is quite common with HIV-1 subtype C, which is the predominant subtype in resource-poor sub-Saharan Africa. Such high levels of R5 variants could possibly be attributed to the relatively high levels of immune activation caused by parasitic infections.[22]

Studies have shown that X4 viruses are more common among pretreated patients with high viral loads.[23] Similarly in our study, surrogate markers of disease progression such as viral load and lymphadenopathy, showed a consistent trend with the X4 genotype being associated with disease progression; however, this was not statistically significant, possibly due to the relatively smaller sample size. This was also confirmed by the fact that mothers with X4 variants were significantly more likely to have infants with lower birth weights, possibly due to more advanced disease.

In our study the R5 genotype was preferentially transmitted from mother to infant, similar to HIV-1 subtype B where maternal viral phenotype was generally preserved in vertical transmission and was predictive of the newborn's viral phenotype. Likewise, X4 viruses have also been shown to be rarely transmitted to newborns.[11] Similar to the findings of Casper and others in 2002, the X4 viral genotype in HIV-1-infected children was related to the presence of X4 in their mothers. The complete agreement between maternal and infant genotypes was also observed by Casper et al. from 11 mother–infant pairs in the first year of life with discordant mother–infant coreceptor genotypes occurring much later at more than 12 months.[6] An early infant genotype switch has been shown to be related to the transmission from the mother, whereas a late switch may be related to development of mutations in HIV-1. However, Clevestig et al. have shown that the X4 phenotype in children actually evolves from their own previous R5 population implying that at least in subtype D, the X4 genotype in infants is not caused by transmission.[24] We could not determine whether the presence of X4 in the infant who was infected after delivery was due to transmission or was from evolution from R5, since these infants were not genotyped soon after infection.

The dual-tropic R5/X4 genotype was present in 11% of the mothers at baseline, whereas no dual tropism was detected in infants. This finding is in agreement with Cavarelli et al., who postulate that the R5X4 phenotype is predominantly lost during transmission.[11] However, contrary to this finding, a Ugandan study has demonstrated that the X4 and R5X4 can be transmitted from mother to infant before, during, or shortly after delivery.[25]

An 8% frequency of X4 variants among infants in our study is quite comparable to the 9% observed in a Ugandan study with subtypes A and D-infected infants. Similarly, survival of the infants with the R5 variant was not significantly different from survival of the infants with X4 variants as also observed by Church et al. [26] Generally studies have shown that children can progress to AIDS without evidence of X4 virus, and some with X4 variants can remain asymptomatic for more than 1 year.[27]

Of the three more recently infected mothers with acute HIV-1 infection, one had R5 genotypes as expected since the X4 genotype is normally associated with advanced disease or is more common in patients receiving antiretroviral therapy.[4] Nevertheless, X4 variants are not very rare in early infection. In Zimbabwe among male seroconverters, X4 phenotype frequency has been shown to be 12%.[8] Huang et al. observed a 4% frequency of X4 viruses in a cohort of 150 recently HIV-1 subtype D-infected individuals.[9] The presence of X4 variants in recent infection may have implications for antiretroviral therapy and vaccine development.

X4 variants were also associated with a substitution of the conserved GPGQ crown motif to GPGR. Similar observations were also observed by Coetzer et al. in a South African cohort, also with a predominant HIV-1 subtype C.[28]

The potential N-linked glycosylation site within the V3 loop region is present in almost all the mothers' and infants' viral isolates, suggesting its importance in CCR5 interaction.[29] Similar findings have been observed in a South African study where X4 viruses were associated with loss of the glycosylation site.[28] The loss of this glycan has been shown to assist in more efficient use of CXCR4 and thus might be an important factor in the switching of the R5 to R4 viruses.[30] Studies on subtype B virus have shown that the number of potential N-linked glycosylation sites increased significantly over time in individuals who do not switch from R5 to X4, whereas no change was observed for those who switched.[31] Since glycans are an important part of the viral defense against antibodies, it is possible that the difference in evolution of potential N-linked glycosylation sites may reflect differences in antibody responses directed toward switching and nonswitching populations.[32]

These data show that HIV-1 R5 coreceptor usage is the most predominant genotype among pregnant women and is associated with a highly conserved GPGQ crown motif and glycosylation site. Antenatal HIV-1 subtype C coreceptor usage is generally preserved in vertical transmission and can be predictive of the newborn's viral genotype. Hence, the majority of antiretroviral-naive pregnant women and later their infants can benefit from antiretroviral combination therapy that includes R5 entry inhibitors following screening of virus genotype using bioinformatics methods.

Author Disclosure Statement

No competing financial interests exist.

References

1. Berger EA, Doms RW, Fenyo EM, Korber BT, and Littman DR: A new classification for HIV-1. Nature 1998;391:240.
2. Hoffman TL and Doms RW: HIV-1 envelope determinants for cell tropism and chemokine receptor use. Mol Membr Biol 1999;16:57–65.
3. Sundaravaradan V, Das SR, Ramakrishnan R, Sehgal S, Gopalan S, Ahmad N, and Jameel S: The role of HIV-1 subtype C V3 to C5 region in viral entry, co-receptor utilization and replication efficiency in primary T-lymphocytes and monocyte derived macrophages. Virol J 2007;4:126.

4. Johnston ER, Zijenah LS, Mutetwa S, Kantor R, Kittinunvorakoon C, and Katzenstein DA: High frequency of syncytium-inducing and CXCR4-tropic viruses among human immunodeficiency virus type 1 subtype C infected patients receiving antiretroviral treatment. J Virol 2003; 77(13):7682–7688.
5. Moyle GJ, Wildfire A, and Mandalia S: Epidemiology and predictive factors for chemokine receptor use in HIV-1 infection. J Infect Dis 2005;191:866–872.
6. Casper CHE, Clevestig P, Carlenor E, Leitner T, Anzen B, Lidman K, Belfrage E, Albert J, Bohlin AB, Naver L, Lindgren S, Fenyo EM, and Ehrnst AC: Link between the X4 phenotype in human immunodeficiency virus type 1 infected mothers and their children, despite the early presence of R5 in the child. J Infect Dis 2002;186:914–921.
7. Regoes RR and Bonhoeffer S: The HIV co-receptor switch: A population dynamics perspective. Trends Microbiol 2005; 13:269–277.
8. Tien PC, Shafer RW, Contag CH, Katzenstein DA, and Batra M: HIV type 1 envelope subtype C sequences from recent sero-converters in Zimbabwe. AIDS Res Hum Retroviruses 2000;16(10):973–979.
9. Huang W, Toma J, Stawiski E, Frasen S, Wrin T, Parkin N, Whitcomb JM, Coakley E, Hecht FM, Deeks SG, Gandhi RT, Eshleman SH, and Petropoulos CJ: Characterisation of human immunodeficiency virus type 1 population containing CXCR4-using variants from recently infected individuals. AIDS Res Hum Retroviruses 2009;25(8):795–802.
10. Huang W, Eshleman SH, Toma J, Stawiski E, and Whitcomb JM: Co-receptor tropism in human immunodeficiency virus type 1 subtype D: High prevalence of CXCR4 tropism and heterogeneous composition of viral population. J Virol 2007; 81:7885–7893.
11. Cavarelli M, Karlsson I, Zanchetta M, Antonsson L, Plebani A, Giaquinto C, Fenyo EM, De Rossi A, and Scarlatti G: HIV-1 with multiple CCR5/CXCR4 chimeric receptor use is predictive of immunological failure in infected children. PLoS ONE 2008;3:e3292.
12. Sayana S and Khanlou H: Maraviroc: A new CCR5 antagonistic. Expert Rev Anti Infect Ther 2008;7(1):9–19.
13. Hunt JS and Romanelli F: Maraviroc, a CCR5 coreceptor antagonist that blocks entry of human immunodeficiency virus type 1. Pharmacotherapy 2009;29(3):295–304.
14. Delobel P, Nugeyre MT, Cazabat M, Pasquier C, Marchou B, and Massip P: Population based sequencing of the V3 region of env for predicting the coreceptor usage of human immunodeficiency virus type I quasispecies. J Clin Microbiol 2007;45:1572–1580.
15. Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, Sandres-Saune K, Cuzin L, Marchou B, Massip P, and Izopet J: Correlation between genotype predictions based V3 sequences and phenotypic determination of HIV-1 tropism. AIDS 2008;22:F11–F16.
16. Jensen MA, Coetzer M, van't Wout AB, Morris L, and Mullins JI: A reliable phenotype predictor for human immunodeficiency virus type 1 subtype C based on envelope v3 sequences. J Virol 2006;80:4698–4704.
17. Kurewa NE, Munjoma MM, Chirenje ZM, Rusakaniko S, Hussain A, and Stray-Pedersen B: Compliance and loss to follow up of HIV negative and positive mothers recruited from a PMTCT Programme in Zimbabwe. Cent Afr J Med J 2007;53(5/8):25–30.
18. Gumbo FZ, Duri K, Kandawasvika GQ, Kurewa NE, Mapingure MP, Munjoma MW, Rusakaniko S, Chirenje MZ, and Stray-Pedersen B: Risk factors of HIV vertical transmission in a resource-poor setting. J Perinatol 2010; advance online publication: doi: 10.1038/jp.2010.31.
19. Boom R. Sol C, Beld M, Weel J, Goudsmit J, and Wertheim-van Dillen P: Improved silica-guanidinium thiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. J Clin Microbiol 1999; 37(3):615–619.
20. Garrido C, Chueca N, Aguilera A, Skrabal K, Poveda E, Zahonero N, Carlos S, Garcia F, Faudon JL, de Medoza C, and Soriano V: Prevalence of X4 viruses in patients infected with HIV-1 non B subtypes. 14th Conference on Retroviruses and Opportunistic Infection, Los Angeles, CA, 2007. Abstract D-177.
21. Moore JP, Kitchen SG, Pugach P, and Zack JA: The CCR5 and CXCR4 co-receptors: Central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 2004; 20:111–126.
22. Clerici M, Butto S, and Lukwiya M: Immune activation in Africa is environmentally-driven and is associated with upregulation of CCR5. Italian-Ugandan AIDS Project. AIDS 2000;14:2083–2092.
23. Poveda E, Briz V, de Mendoza C, Benito JM, Corral A, Zahonero N, Gonzalez-Lahoz LS, and Soriano V: Prevalence of X4 tropic HIV-1 variants in patients with differences in disease stage and exposure to antiretroviral therapy. J Med Virol 2007;79:1040–1049.
24. Clevestig P, Maljkovic I, Casper. C, Carlenor E, Lindgren S, Naver L, Bohlin AB, Fenyo EM, Leitner T, and Ehrnst A: The X4 phenotype of HIV type 1 evolves from R5 in two children of mothers carrying X4 and is not linked to transmission. AIDS Res Hum Retroviruses 2005;21(5):371–378.
25. Huang W, Eshleman SH, Toma J, Stawiski E, Whitcomb JM, Jackson JB, Guay L, Musoke P, Parkin N, and Petropoulos CJ: Vertical transmission of X4 tropic and dual tropic HIV-1 in five Ugandan mother-infant pairs. AIDS 2009;23(14): 1903–1908.
26. Church JD, Huang W, Mwatha A, Toma J, Stawiski E, Donnell D, Guay LA, Mmiro F, Musoke P, Jackson JB, Parkin N, and Eshelman SH: HIV-1 tropism and survival in vertically infected Ugandans infants. J Infect Dis 2008;197:1382–1388.
27. Fitzgibbon JE, Gaur S, Gavai M, Gregory P, Frenkel LD, and John JF Jr: Effect of the HIV-1 syncytium-inducing phenotype on disease stage in vertically-infected children. J Med Virol 1998;55:56–63.
28. Coetzer M, Cilliers T, Ping L, Swanstrom R, and Morris L: Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates. Virology 2006; 356:95–105.
29. Polzer S, Dittmar MT, Schimitz H, Meyer B, Muller H, and Krausslich HG: The N- linked glycan g15 within the V3 loop of the HIV-1 external glycoprotein gp120 affects co-receptor usage, cellular tropism and neutralization. Virology 2002;304(1):70–80.
Nabatov AA, Pollakis G, Linnemann T, Kliphius A, Chalaby MI, and Paxton WA: Intra-patient alteration in the human immunodeficiency virus type 1 gp120 V1V2 and V3 regions differentially modulate coreceptor usage, virus inhibition by CC7CXC chemokines, soluble CD4 and b12 and 2G12 monoclonal antibodies. J Virol 2004;78(1):524–530.
Mild M, Kvist A, Esbjornsson J, Karlsson I, Fenyo EM, and Medstrand P: Differences in molecular evolution between

switch (R5 to R5X4/X4-tropic) and non switch (R5 tropic only) HIV-1 populations during infection. Infect Genet Evol 2009;584:1–9.
32. Sagar M, Wu X, Lee S, and Overbaugh J: Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. J Virol 2006;80(19):9586–9598.

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Paper III

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Human Immunodeficiency Virus (HIV) types Western blot (WB) band profiles as potential surrogate markers of HIV disease progression and predictors of vertical transmission in a cohort of infected but antiretroviral therapy naïve pregnant women in Harare, Zimbabwe

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Abstract

Background: Expensive CD4 count and viral load tests have failed the intended objective of enabling access to HIV therapy in poor resource settings. It is imperative to develop simple, affordable and non-subjective disease monitoring tools to complement clinical staging efforts of inexperienced health personnel currently manning most healthcare centres because of brain drain. Besides accurately predicting HIV infection, sequential appearance of specific bands of WB test offers a window of opportunity to develop a less subjective tool for monitoring disease progression.

Methods: HIV type characterization was done in a cohort of infected pregnant women at 36 gestational weeks using WB test. Student-t test was used to determine maternal differences in mean full blood counts and viral load of mothers with and those without HIV gag antigen bands. Pearson Chi-square test was used to assess differences in lack of bands appearance with vertical transmission and lymphadenopathy.
Results: Among the 64 HIV infected pregnant women, 98.4% had pure HIV-1 infection and one woman (1.7%) had dual HIV-1/HIV-2 infections. Absence of HIV pol antigen bands was associated with acute infection, p = 0.002. All women with chronic HIV-1 infection had antibody reactivity to both the HIV-1 envelope and polymerase antigens. However, antibody reactivity to gag antigens varied among the women, being 100%, 90%, 70% and 63% for p24, p17, p39 and p55, respectively. Lack of antibody reactivity to gag p39 antigen was associated with disease progression as confirmed by the presence of lymphadenopathy, anemia, higher viral load, p = 0.010, 0.025 and 0.016, respectively. Although not statistically significant, women with p39 band missing were 1.4 times more likely to transmit HIV-1 to their infants.

Conclusion: Absence of antibody reactivity to pol and gag p39 antigens was associated with acute infection and disease progression, respectively. Apart from its use in HIV disease diagnosis, WB test could also be used in conjunction with simpler tests like full blood counts and patient clinical assessment as a relatively cheaper disease monitoring tool required prior to accessing antiretroviral therapy for poor resource settings. However, there is also need to factor in the role of host-parasite genetics and interactions in disease progression.

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Background

Acquired Immunodeficiency Syndrome (AIDS) is currently one of the most devastating diseases caused by HIV. Globally, in 2007 alone, 33 million people were living with HIV/AIDS and 20 million had died [1]. Studies have shown a cross-species transmission of HIV from a primate lentivirus to humans and the virus can be phylogenetically classified into two types; 1 and 2 [2]. This distinction is essential for accurate surveillance and diagnosis as well as administration of appropriate antiretroviral therapies within a population.

HIV type 1 (HIV-1) is the first in the class of human retroviruses and accounts for more than 95% of the world's HIV infections. Its origin can be traced back to a Simian Immunodeficiency Virus (SIV) isolated from a Chimpanzee (cpz) sub-species, Pan troglodytes troglodytes (SIVcpz)[3]. Both HIV-1 and SIVcpz have a unique Vpu gene in their respective genomic structures [2]. HIV-2 is the second in the same class and is largely confined to West Africa. Its closest relative is a monkey, sooty mangabey (sm), Cercocebus atys, SIVsm. A unique Vpx gene characterises both viruses' gene structures [4,5]. However, HIV-2 and HIV-1/HIV2 co-infections have also been documented outside West Africa [6]. HIV-1 and HIV-2 are closely related viruses with nucleotide sequence homology of 58%, 59% and 39% in the group specific antigen (Gag), Pol and Env genes encoding the viral nucleocapsid, polymerase enzymes and envelope glycoproteins, respectively [7]. Relative to HIV-1, HIV-2 has a reduced rate of transmissibility, disease development and has shown natural resistance to readily available non-nucleoside reverse-transcriptase inhibitors [8,9].

Classical algorithm of laboratory diagnosis of HIV infection has been the detection of anti-HIV antibodies using rapid tests with WB immunoassay as the gold standard method for validating screening test results [10,11]. However, in Zimbabwe, a large proportion of HIV diagnoses are currently being done without WB confirmation yet its banding profiles can yield valuable patient information. In this setting, WB test is only used as a tie-breaker in cases of discrepancy in the results. Unlike screening tests that detect antibodies to one or all HIV antigen(s) without specifying which antigen reacts to which antibody, the WB test with separated viral proteins immobilized on a membrane, generates specific information on the reactivity of patient antibodies to specific HIV antigens. Positive reactions appear as bands of numerous patterns [12]. Variations in WB band intensities, numbers, or their sequential order of appearance during different stages of HIV infection have been observed [13]. Following sero-conversion, anti-gag antibodies to p17, p24 and its precursor p55 appear first and tend to decrease with the onset of clinical symptoms [14]. A reduced prevalence of core antibodies has also been shown to be associated with the development of immunodeficiency [15]. In contrast, antibodies to env antigens have been detected in virtually all HIV infected persons regardless of clinical stage [16]. This sequential appearance of specific WB bands offers a window of opportunity to develop a simple and non-subjective disease assessment tool and also to predict the likelihood of vertical transmission.

High cost of CD4 count and viral load tests has hampered the intended objective of accessing HIV therapy in poor resource settings. Hence, there is a need for alternative initiative towards development of simple, accurate, affordable and non subjective disease monitoring tools. In view of the current brain drain challenge, this development would complement clinical staging efforts of inexperienced health personnel currently manning most healthcare centres.

WB test has been in use in Zimbabwe for some time now, mainly for HIV diagnosis. However, critical analysis of the band profiles regarding their additional potential applications has been overlooked. This study aimed to characterize HIV types among pregnant women using the WB test and to determine whether the presence or absence of particular band(s) correlated with HIV-1 disease progression or predicted vertical transmission.

Methods

Study Design and Setting

This was a nested case-control study in which the cases and controls were sampled from a cohort of pregnant women attending 3 antenatal clinics around the city of Harare, Zimbabwe. All participants were part of a national Prevention of Mother-To-Child Transmission (PMTCT) program and were naïve to antiretroviral therapy. The primary end point was an HIV-1 positive mother who transmitted the virus to her infant, transmitting mother (case). Each case was matched to one HIV-1 positive but non-transmitting mother (control). Matching of cases and controls was done with respect to important risk factors of HIV disease progression and vertical transmission notably maternal age, baseline sexually transmitted infections (STIs), clinical signs, the date of last menstruation and single dose nevirapine therapy, see figure 1.

Study Population and Procedures

Pregnant women were enrolled at 36 gestational weeks between April and September 2002. Pre-and post-HIV test counseling services were readily available. HIV-1 positive mother and infant pairs were offered 200 mg single dose nevirapine during labour and 2 mg/kg body weight within 72 hours post delivery, respectively. Mothers were encouraged to exclusively breastfeed during the first six months post-delivery.

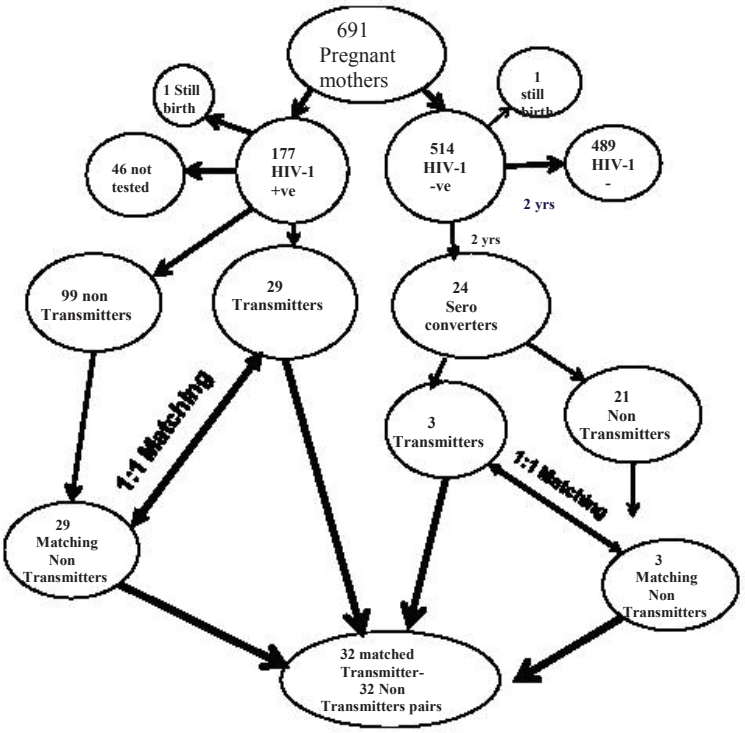

Figure 1 Summary of how the 32 Transmitters and 32 non-Transmitters were sampled from a cohort of pregnant mothers attending antenatal clinics around Harare.

The study population consisted of two groups of pregnant HIV-1 positive women. The main group consisted of pregnant women who were HIV-1 positive at enrolment, considered to be having chronic HIV-1 infection, and a subgroup of pregnant women who were HIV-1 negative during pregnancy but sero-converted after delivery, thus regarded as having acute HIV-1 infection. Follow-up of HIV-1 negative mothers together with HIV-1 exposed infants was from delivery, 6 weeks, 4 and 9 months and thereafter 3 monthly until 2 years, thus generally coinciding with infant immunization visits. At each subsequent follow-up visit, HIV-1 negative mothers and exposed infants were re-tested for HIV-1 antibodies and antigens, respectively. Besides HIV testing, serum samples of sero-negative mothers and their respective infants were aliquoted and stored for further analysis.

Mothers' and Infants' Demographic characteristics, Examination and sample collection

At enrolment all mothers answered a structured questionnaire and information regarding their socio-demographics, sexual behavioural, obstetric and reproductive health issues was obtained. A gynecologist performed physical and gynecological examinations.

A pediatrician examined infants. Date of birth, birth weight, gender and single dose nevirapine therapy were recorded. Five milliliters of maternal venous blood samples were collected in EDTA tubes at baseline and each follow-up visit in the cases of HIV-1 negative mothers. Two milliliters of venous EDTA whole blood samples were collected at each follow-up visit for HIV-1 negative but HIV-1 exposed infants. Samples were stored at -86°C until tested.

Mothers' Tests

Serial HIV-1/-2 algorithm antibody tests were performed on plasma samples using Determine (Abbott Diagnostics, Illinois USA) and Ora-Quick (Abbott Diagnostics, Illinois, USA) rapid kits. Confirmation of screening HIV-1/2 rapid test results was done at the Norwegian Institute of Public Health using the WB test (HIV blot 2.2, MP Diagnostics, Singapore) according to

the manufacturer's instructions. Interpretation of the WB test results was done in line with the World Health Organization guidelines [17]. A WB test was considered positive if at least two of the three envelope antigen bands for HIV-1 or glycoprotein (gp) 36 for HIV-2 and any of the four gag antigens or at least any one of the three pol antigens were present. A WB test result was considered to show dual reactivity when sera reacted with at least two env glyco-proteins and one core protein of each virus. Specimens with reactive gp36 antigen were re-run on a WB test specific to HIV-2.

Full blood counts were done using Abbott Diagnostic Cell Dyne 3500R SL Hematology Analyser. Plasma samples were shipped on dry ice to the Institute of Microbiology in Oslo to be quantified for HIV-1 RNA load using an automated TaqMan Roche Amplicor 1.5 Monitor Test (Cobas AmpliPrep/Cobas TaqMan, Roche Diagnostics, Branchburg NJ) according to the manufacturer's instructions as previously described [18]. The first available HIV-1 positive sample was quantified in the cases of sero-converters.

Infants' Test

Detection of infants' HIV-1 infections was performed using qualitative 1.5 Roche Amplicor HIV-1 DNA PCR kit (Roche Diagnostics). Since this was a breastfeeding population, the criteria used to determine time of infection was similar to that used by Bertolli et al. [19]. Infants that tested HIV-1 DNA PCR positive on whole blood collected within 10 days of birth were considered to be infected in utero. Infants who had negative HIV-1 DNA PCR results within the first 10 days of life but had positive results at six weeks were regarded as infected during intra-partum and those testing positive thereafter were considered infected after birth.

Statistical Analysis

Data were entered and analyzed using STATA version 10. The frequency of WB bands were determined among the pregnant women in general and also after stratifying by the time of HIV infection (acute or chronic) and vertical transmission, as transmitting or non-transmitting mothers. A graph was plotted to show the frequency of different WB gag antigen bands between the two groups of mothers. Student-t test was used to determine differences in mean viral load and maternal hemoglobin between mothers with and those without gag antigen bands. Pearson Chi-square test was used to assess differences in the absence HIV gag antigen bands with vertical transmission and lymphadenopathy. Comparisons of the appearance of the HIV env, pol and gag antigens band profiles of mothers with chronic and those with acute HIV-1 infections were also done. Tests of statistical significance included the 95% confidence intervals of unadjusted relative risks and p values of less than 0.05 were considered statistically significant.

Ethical Consideration

The study was approved by the Medical Research Council of Zimbabwe and the Ethical Review Committee of Norway. Written consent to participate in the research study was obtained from the mothers and they were free to discontinue at any given time without any prejudice.

Results

Demography and Reproductive Health Characteristics of the 58 mothers: 29 transmitting and their 29 matched non-transmitting mothers with chronic HIV infection

Mean age (standard deviation) of the women was 26.6 (5.2) years, being 26.3 (5.6) and 25.6 (5.6) years for transmitters and non-transmitters respectively, p = 0.610.

All the women had at least 7 years in school and were not formally employed. Ninety-three percent were married and 90% had at least one child. All the mothers had spontaneous vaginal deliveries and were generally asymptomatic for HIV infection at enrolment.

There were two stillbirths, one among the HIV-1 positive and the other within the HIV negative group. These two were excluded from analysis. From the 176 HIV-1 positive mothers that delivered live births 126 (72%) mother-baby pairs were successfully followed up and tested. There were no differences with respect to socio-demographic characteristics, sexual behavior, reproductive genital tract infections and medical history between the 58 mothers with chronic HIV-1 infection constituting the main group in this study population and the rest of the mothers were HIV-1 positive at enrolment but were excluded or lost to follow-up. However, these 58 mothers were more likely to have more children, p = 0.016.

HIV Prevalence, Types and Vertical Transmission of the 29 Transmitting and their Matched 29 Non-Transmitting Mothers with Chronic Infection

At baseline 691 pregnant women were enrolled of whom 177 (25.6%) and 514 (74.4%) were HIV-1 sero-positive and sero-negative, respectively. Performance concordance of the two serial HIV-1 rapid test results was 100%. Confirmatory WB tests of the 58 women with chronic HIV infection showed a 98.3% pure HIV-1 infection. None was found with solely HIV-2 infection. Only one woman (1.7%) had dual HIV-1/HIV-2 infections.

Twenty nine (23%) mothers transmitted the virus to their infants 10 (34%) and 19 (66%) during in utero and intra-partum/postpartum transmissions respectively.

HIV Incidence, Type(s) and vertical transmission among 6 sero-converters: 3 transmitting and 3 non-transmitting mothers

Out of the 512 HIV-1 negative mothers that delivered live births, 24 sero-converted during the two year

follow-up period, giving an HIV-1 incidence rate of 2.3 per hundred women years. Eighty-five percent of the mothers sero-converted after weaning their infants from breast-milk. Mothers with acute HIV-1 infections were generally younger relative to HIV-1 negative mothers in the cohort, with mean ages of 21.8 (4.6) and 23.7(5) years respectively, p = 0.06. More so, sero-converters were generally younger compared with mothers having chronic HIV-1 infection, mean (SD) ages, 21.8(4.6) and 26(5.5) years respectively, p = 0.04. There were no differences with respect to level of education, age of sexual debut, reproductive tract infections and STIs between the mothers with acute and those with chronic HIV-1 infections.

Among the 24 sero-converters with acute HIV-1 infection, three (13%) transmitted the virus to their infants through breastfeeding around 9 months postpartum. All the three infants were exposed, through breast milk for about three months before acquiring HIV-1 infection. All the sero-converting mothers had solely HIV-1 infection. Neither HIV-2 nor HIV-1/HIV-2 co-infections were detected in this subgroup.

antibody reactivity to HIV-1 gag demonstrated no relationship with maternal age, marital status, age of sexual debut, the number of sexual partners the women had had, current nor history of STIs.

Gag p39 and p55 antigens were the most commonly missing bands among transmitting mothers. Generally band appearance was not significantly different when compared with band profiles of the non-transmitting mothers, see figure 2. Mothers who had gag p39 antigen bands missing were 1.4 times more likely to transmit the virus to their infants compared to those who had this band present though their number was too small to reach statistical significance, p = 0.104.

Lack of antibody reactivity to gag p39 antigen was significantly associated with disease progression as demonstrated by the presence of maternal lymphadenopathy, anaemia and higher viral load, p = 0.010, 0.025 and 0.016 respectively, see table 1. Women with p39 gag antigen band missing were about 6 times more likely to have a viral load of more than 10 000 copies/mL relative to their counterparts who had that band present, 5.58 [1.74-17.86].

Presence of gag p17 antigen band was associated with established or advanced HIV-1 infection in mothers with chronic HIV-1 infection p = 0.002, see table 2.

Frequency of HIV-1 WB Bands among 58 mothers with chronic HIV infection

Reactivity to all the 10 WB HIV-1 proteins was observed in 78% of the HIV-1 positive women. All specimens showed a strong positive reaction to both the HIV-1 envelope glycoproteins (gp160, gp120 and gp41) and the polymerase antigens (p31, p51 and p66). However, antibody reactivity to the gag core antigens varied among the women, being 100%, 90%, 70% and 63% with the p24, p17, p39 and p55 respectively. Absence of maternal

Frequency of HIV WB Bands among mothers with acute HIV-1 infection

All the mothers with acute HIV-1 infection had antibody reactivity to all the 3 env antigens, gp41, 120 and 160. However, reactivity to HIV-1 gag antigens varied, but because of the small number of women in this subgroup, it was difficult to make any conclusive remarks. Lack of antibody reactivity to pol antigens, p31, p51 or

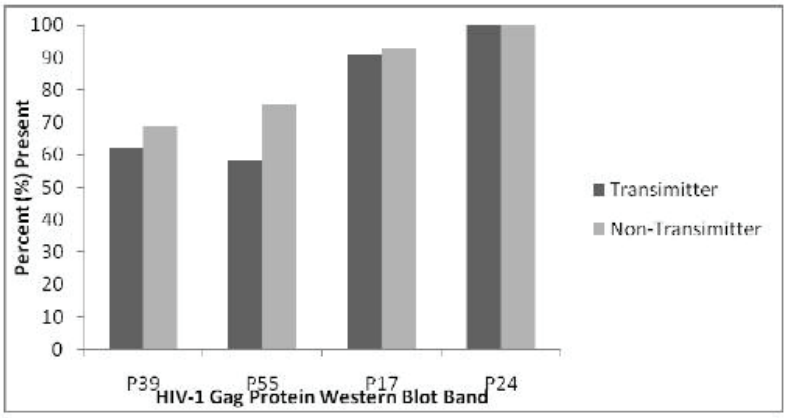

Figure 2 Frequencies of different gag protein among transmitters and non-transmitters

Table 1 Associations of antenatal surrogate markers of disease progression of 58 pregnant women (chronic HIV-1 infections) with presence or absence of gag proteins reactivities

Antenatal variable	HIV-1 WB gag antigen band reactivity			
	Band	P17	P39	P55
Lymphadenopathy Number/Total (%)	Present	4/51 (8%)	0/32 (0%)	2/38 (5%)
	Absent	0/2 (0%)	4/21 (19%)	3/15 (13%)
	p-value	0.680	0.010	0.316
Hemoglobin Mean (SD)	Present	10.8 (1.3) N = 54	11.0 (1.2); N = 33	10.9 (1.4); N = 39
	Absent	11.7 (1.0) N = 2	10.4 (1.5); N = 23	10.6 (1.0); N = 17
	p-Value	0.312	0.025	0.395
Viral load log_{10} Mean (SD)	Present	3.3(1.0); N = 56	3.0 (0.8); N = 35	3.1 (0.9); N = 41
	Absent	2.3 (0); N = 2	3.7 (1.2); N = 23	3.6 (1.2); N = 17
	P-value	0.167	0.016	0.072
Vertical Transmission Number/Total (%)	Present	29/56 (52%)	15/36 (42%)	20/41 (49%)
	Absent	0/2 (0%)	14/22 (64%)	9/17 (53%)
	P-value	0.150	0.104	0.773

p66 was significantly associated with early infection, hence absence of these bands on a WB test result could predict acute HIV-1 infection, see table 2.

WB band Profiles of the mother with HIV-1/-2 co-infection

In addition to the weak HIV-2 specific p36 band, reactivity to all the env, pol and gag antigens was observed. The mother was generally well with a relatively low baseline viral load of 690 viral copies per mL and did not transmit the viruses to her baby.

Discussion

This study is a first attempt to correlate simple WB band profiles with disease progression and to some extent vertical transmission in Zimbabwe. The WB test is simple and easily interpreted by skilled users.

Table 2 Reactivity of different env, pol and gag proteins of mothers with chronic HIV-1 infection and sero-converters

Positive HIV-1 antigen Band	Chronic infections	Sero-converters	p-value
	N = 58 (%)	N = 6 (%)	
gag			
p17	57 (98)	3 (50)	0.002
p24	57 (98)	5 (83)	0.183
p39	35 (60)	2 (33)	0.186
p55	41 (71)	2 (33)	0.251
pol			
p31	58 (100)	3 (50)	0.001
p51	58 (100)	4 (67)	0.002
p66	58 (100)	4 (67)	0.002
Env			
Gp41	58 (100)	6(100)	-
Gp120	58(100)	6(100)	-
Gp160	58(100)	6(100)	-

Key: P: protein, gp: glycoprotein

Nevertheless, rapid tests are even simpler and can be conducted in rural settings without electricity. Although WB test remains relatively more expensive compared to rapid tests, it could be worthwhile using provided it yields a wealth of information on patients' serology. Besides accurately predicting HIV infection, sequential appearance of specific bands on WB test result offers a window of opportunity to develop a less subjective tool to monitor disease progression.

In our study of pregnant women recruited around Harare, HIV 1 was the predominant type with only one HIV-1/HIV-2 co-infected mother. This observation is in agreement with a previous bigger study, coincidentally also done in Harare in the same population that showed an HIV-2 and HIV-1/HIV-2 prevalence of 1.3% and 0.5%, respectively [20].

A recent study has shown that serological cross-reactivity for HIV-2 in HIV-1 infected individuals is rare when using synthetic peptide based assays which was the case with the MP Diagnostic HIV BLOT 2.2 kit we used in our study [21]. Similar to the findings of the same study, a weak reactivity to gp36 band was observed in the co-infected mother. However, a more specific HIV-2 PCR should have been done to confirm the HIV-2 immuno-blot test result. With the world fast becoming a global village, the possibility of importing and/or exporting new HIV types is inevitable, more so for most unemployed Zimbabweans who have resorted to cross-border trading with regional countries such as Mozambique and Angola with HIV-2.

Antibodies to the env and pol antigens were well detected in all the HIV infected pregnant women. Similar findings of band reactivities were reported earlier [13,16,22,23]. These results emphasise the importance of considering both the env and pol antigens in the interpretation criteria of WB HIV positive test results at least

in this population. Antibodies to gag antigens, p17, p39 and p55 were not expressed efficiently in these women as was the case with other previous related studies [22,23]. Unlike the Indian study, where p55 antigen band was not detected at all in the patients with WHO clinical stage 1, our study observed a 63% expression of this antigen [22]. Interestingly in our study population, this antigen was the least expressed of all the WB test HIV antigens. This difference could be due to the fact that most of our women had surpassed the WHO clinical stage 1.

Fiebig et al., have classified primary infection of HIV into seven stages incorporating WB test results. A characteristic band appearance indicative of an indeterminate test result has been shown to occur at stage IV with a true positive WB test result at stage V but without the p31 antigen band which only appears in the final acute infection stage VI [24]. Also observed in our study amongst sero-converters, was the absence of reactivity to pol antigens hence these could be predictors of sero-conversion. Analogous results have also been demonstrated by Sudha et al., who have shown p31 antigen to be a predictor for early HIV infection [13]. Presence of p17 antigen band was associated with chronic infections and hence could be a predictor of established HIV infection contrary to the results observed by some studies [14,15]. Lack of antibody reactivity to p39 and p55 antigens was associated with disease progression as confirmed by the presence of lymphadenopathy, anemia, high viral load of above 10 000 viral RNA copies per mL and a higher likelihood of vertical transmission. Similar findings were also obtained elsewhere [25].

Without any intervention, majority HIV-1 positive pregnant women do not transmit the virus to their infants. Highly active antiretroviral therapy (HAART) is not yet readily available in PMTCT programs in resource limited settings. The long term side effect of single dose nevirapine therapy offered to most HIV positive pregnant mothers poses a threat to the health of the naturally non-transmitting mothers should they require nevirapine later as part of their antiretroviral combination therapy. Hence, it is critical to precisely predict mothers who are likely to vertically transmit and offer them nevirapine monotherapy. Accurately predicting pregnant mothers likely to transmit the virus to their infants has long term benefits of saving on drugs and minimizing drug resistance problems. Lack of antibody reactivity to gag p39 antigen during the last trimester of pregnancy could be a predictor of vertical transmission although bigger studies are necessary to verify this observation. The current small sample size could not permit conditional logistic regression analysis to control for variables that could have had an effect on disease progression other than WB band profiles. Hence results should be interpreted cautiously because disease progression and transmission also depend on other factors including host-parasite genetics and interaction.

Control of viral replication following infection has been attributed partly to cytotoxic T lymphocyte (CTL) CD8+ activity. Studies have shown that CTLs directed against gag antigens correlate with improved clinical markers of disease progression [26]. Hence absence of antibody responses to p39 antigen could interfere with normal host neutralization of virus and may contribute to disease progression. Missing bands among the HIV chronically infected women were likely to have been due to diminished antibody responses with progressive disease whilst in recent sero-convertors the missing bands may have been due to immature antibody responses or possibly due to mutations in the pol gene [27].

Conclusion

These data support the rationale of using WB band profiles plus simple laboratory tests like differential counts together with clinical symptoms such as lymphadenopathy in establishing and evaluating disease progression before accessing antiretroviral therapy. This could have important practical applications especially in resource poor settings, where over 95% of the 40 million HIV infected people live, who unfortunately cannot afford costly viral load and CD4 cell tests. However, bigger studies are necessary to shed more light on the use of simple WB band profiles to determine the likelihood of vertical transmission as an initiative to reduce HIV-1 vertical transmission in developing countries.

Conflict of interests
The authors declare that they have no competing interests.

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Authors' contributions
KD collected data and drafted the manuscript, FM supervised laboratory analysis, FZG collected data, NEK collected data, SR participated in designing of the study, MZC participated in designing of the study, MPM performed the statistical analysis and interpretation of results, BS participated in designing and coordination of the study. All authors read and corrected the final version of the manuscript.

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References
1. UNAIDS: 2008 Report on the global AIDS epidemic; Executive Summary. Geneva, Switzerland; 2008.
2. Hahn BH, Shaw GM, De Cock KM, Sharp PM: AIDS as a zoonosis: Scientific and public implications. Science 2000, 287(5453):607-614.
3. Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, Bibollet-Ruche F, Chen Y, Wain LV, Liegeois F, Loul S, Ngole EM, Bienvenue Y, Delaporte E, Brookfield JF, Sharp PM, Shaw GM, Peeters M, Hahn BH: Chimpanzee Reservoirs of Pandemic and Non-pandemic HIV-1 Chimpanzee Reservoirs of Pandemic and Non-pandemic HIV-1. Science 2006, 313:523-526.
4. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg : Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 1999, 397:436-441.
5. Hirsch VM, Omsted RA, Murphey-Corb M, Purcell RH, Johnson PR: An African primate lentivirus (SIVsm) closely related HIV-2. Nature 1989, 339:389-392.
6. Grez M, Dietrich U, Balfe P, Briesen von H, Maniar JK: Genetic analysis of human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-) mixed infections in India reveals a recent spread of HIV-1 and HIV-2 from a single ancestor for each of these viruses. J Virol 1994, 68:2161-68.
7. Clavel F, Guétard D, Brun-Vézinet F, Chamaret S, Rey MA, Santos-Ferreira MO, Laurent AG, Dauguet C, Katlama C, Rouzioux C: Isolation of a new human retrovirus from West Africa patients with AIDS. Science 1986, 233(4761):343-346.
8. Marlink R, Kanki P, Thior I, Travers K, Eisen G, Siby T: Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science 1994, 265(5178):1587-1590.
9. Adje-Toure CA, Cheingsong R, Gerardo Garcia-Lerma , Eholie S, Borget MY, Bouchez JM: Antiretroviral therapy in HIV-2 infected patients. Changes in plasma viral load, CD4 cell counts, and drug resistance of patients trated in Abidjan, Cote d'Ivoire. AIDS 2003, 17(Suppl3):S49-S54.
10. Gurtler L: Difficulties and strategies of HIV diagnosis. Lancet 1996, 348:176-179.
11. Dax EM, Arnott A: Advances in laboratory testing for HIV. Pathology 2004, 36:551-560.
12. Saah AJH, Farzadegan R, Fox P, Nishanian CR, Rinaldo JP Jnr, Phair JL: Detection of early antibodies in human immunodeficiency virus infection by enzyme-linked immunosorbent assays, Western blot, and radioimmunoprecipitation. J Clin Microbiol 1987, 25:1605-1610.
13. Sudha T, Lakshmi V, Teja VD: Western blot profile in HIV infection. Ind J Derma, Ven and Virol 2006, 72(5):357-360.
14. Lange JMA, Wolf FD, Krone WJA, Danner SA, Coutinho RA, Goudsmit J: Decline of antibody reactivity to outer core protein p17 is an earlier serological marker of disease progression in human immunodeficiency virus infection than anti-p24 decline. AIDS 1987, 1:155-159.
15. Franchini G, Robert-Guroff M, Aldovini A, Kan NC, Wong-Staal F: Spectrum of natural antibodies against five HTLV-III antigens in infected individuals: correlation of antibody prevalence with clinical status. Blood 1987, 69(2):437-41.
16. Lange JDA, Paul DA, Huisman HG: Persistent HIV antigenemia and decline of HIV core antibodies associated with transition to AIDS. Brit Med J 1986, 293:1459-62.
17. World Health Organisation: Proposed WHO criteria for interpretin results from Western blot assays for HIV-1, HIV-2 and HTLV-I/HTLV-II. Wkly Epidemiol Rec 1990, 37:281-283.
18. Duri K, Gumbo FZ, Kristiansen KI, Kurewa NE, Mapingure MP, Rusakaniko S, Chirenje MZ, Muller F, Stray-Pedersen B: Antenatal HIV-1 RNA load and timing of mother to child transmission; a nested case-control study in a resource poor setting. Virol J 2010, 7:176.
19. Bertolli J, St Louis ME, Simonds RJ, Nieburg P, Kamenga M, Brown C, Brown C, Tarande M, Quinn T, Ou CY: Estimating the timing of mother-to-child transmission of human immunodeficiency virus in a breast-feeding population in Kinshasa, Zaire. J Infect Dis 1996, 174(4):722-726.
20. Humphrey JH, Nathoo KJ, Hargrove JW, Iliff PJ, Mutasa KE, the Zvitambo Study Group: HIV-1 and HIV-2 prevalence and associated risk factors among postnatal women in Harare, Zimbabwe. Epidemiol Infect 2007, 135:933-942.
21. Amor A, Ainhoa S, Salgado M, Rodes B, Soriano V, Toro C: Lack of significant cross-reactivity for HIV-2 immunoblot in HIV-1 infected patients. J AIDS 2009, 50:339-340.
22. Sivakumar MR, Kumar S, Viswanath R, Thatchinamoorthy G, Jacob M, Samuel NM: Western blot pattern in HIV positive individuals in Namakkal, South Indian. The Internet J Infect Dis 2008, 6(2):1-9.
23. Srlkanth P, Babu PG, Sridharan G, John TJ, Mathai D: Immunoblot reactivity in relation to Human Immunodeficiency Virus disease progression. Ind J Med Microbiol 1998, 16(3):118-120.
24. Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L, Heldebrant C, Smith R, Conrad A, Kleinman SH, Busch MP: Dynamics of HIV viremia and antibody seroconversion in plasma donor: implicatioms for diagnosis and staging of primary HIV infection. AIDS 2003, 17:1871-1879.
25. Garland FC, Garland CF, Gorham ED, Brodine SK: Western blot banding patterns of HIV rapid progressors in the U.S. Navy Seropositive Cohort: implications for vaccine development. Navy Retroviral Working Group. Ann Epidemiol 1996, 6(4):341-347.
26. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, Goepfert PA: Magnitude of functional CD8+ T cell response to gag protein of human immunodeficiency virus type I correlates inversely with viral load in plasma. J virol 2002, 76:2298-2305.
27. Dykes C, Demeter LM: Clinical significance of human immunodeficiency virus type-1 replication fitness. Clin Micribiol Rev 2007, 20:550-578.

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Paper IV

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Phylogenetic Analysis of Human Immunodeficiency Virus Type 1 Subtype C Env gp120 Sequences Among Four Drug-Naive Families Following Subsequent Heterosexual and Vertical Transmissions

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Abstract

To characterize phylogenetic relatedness of plasma HIV-1 RNA subtype C env gp120 viral variants capable of establishing an infection following heterosexual and subsequent vertical transmission events a 650-base pair fragment within the C2-V5 subregion was sequenced from four HIV-1-infected families each consisting of biological parent(s), index children (first), and subsequent (second) siblings. None of the family members had received antiretroviral therapy at the time of sample collection. Sequence alignment and analysis were done using Gene Doc, Clustal X, and MEGA software programs. Second siblings' sequences were homogeneous and clustered in a single branch while first siblings' sequences were more heterogeneous, clustering in separate branches, suggestive of more than one donor variants responsible for the infection or evolution from founder variant(s) could have occurred. While the directionality for heterosexual transmission could not be determined, homogeneous viral variants were a unique characteristic of maternal variants as opposed to the more heterogeneous paternal variants. Analysis of families' sequences demonstrated a localized expansion of the subtype C infection. We demonstrated that families' sequences clustered quite closely with other regional HIV-1 subtype C sequences supported by a bootstrap value of 86%, confirming the difficulty of classifying subtype C sequences on a geographic basis. Data are indicative of several mechanisms that may be involved in both vertical and heterosexual transmission. Larger studies are warranted to address the caveats of this study and build on the strengths. Our study could be the beginning of family-based HIV-1 intervention research in Zimbabwe.

Introduction

Sub-Saharan Africa (SSA) is the region hardest hit by the HIV/AIDS pandemic, harboring 63% of the world's 40 million HIV-infected individuals.[1] Heterosexual and vertical transmissions are the primary modes of HIV acquisition in adults and children, respectively.[2,3] Each transmission route represents a distinct microenvironment and consequently a unique set of factors influencing transmission of selected viral variants.[4] The nature of the genital mucosal surfaces, gender, age of the transmitter or recipient, host immunology, and viral characteristics play important roles during HIV transmission.[5] The observation that 70% of HIV-1-exposed infants remain uninfected even in the absence of any antiretroviral therapy underlines the importance of viral determinants in vertical transmission.[6–8] Almost 30 years into the HIV scourge, mechanisms of transmission are still poorly understood, especially for the predominant subtype C. Identifying viral characteristics capable of establishing an infection also remains elusive, a gray research area worth exploring in the current desperate attempt to develop effective HIV transmission preventive strategies.

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Single or multivirus transmission event(s) can initiate HIV-1 infections.[9–11] Despite the fact that the transmitter harbors heterogeneous variants, newly transmitted HIV-1 gp120 env sequences in the recipient show relative uniformity until the immune responses drive the founder virus to diversify into a quasispecies, closely related viral swarms.[3,12–15] HIV-1 subtype B studies have shown a linear increase in env diversity during the first few years of infection, which tends to stabilize or even decrease at some point but often becomes homogeneous once more as the immune system wanes.[16–18] The founder variants have been shown to reappear as the dominant quasispecies in plasma later during infection.[19] Diversity of recent heterosexually transmitted variants has been shown to be greater in women than men.[20] Studies have shown that the use of hormonal contraceptives and sexually transmitted infections increase the likelihood of acquiring heterogeneous variants from a single donor.[21–23] Acquisition of homogeneous viral quasispecies is a unique and consistent feature of perinatal HIV-1 transmission, suggesting the presence of selective host pressures.[24–26] However, some studies have reported infants with exclusive heterogeneous viral populations,[27,28] while other studies suggest multiple mechanisms with different proportions of both homogeneous and heterogeneous viral populations.[29–32] At least for HIV-1 subtype A, homogeneous viral populations have been observed in 50% of vertically infected infants.[33]

The current knowledge on HIV-1 transmission is biased toward homosexually or parenterally acquired subtype B and is confined to Europe and America at the expense of the most widespread subtype C. Phylogenetic assessments of the HIV-1 subtype C env gp120 region during both acute and chronic infections are limited in Zimbabwe, particularly, following concurrent heterosexual and vertical transmission events. Four HIV-1-infected families provided an opportunity to characterize phylogenetic relatedness of the virus variants capable of establishing an infection following heterosexual and subsequent vertical transmission events by genotyping a 650-base pair fragment of the HIV-1 subtype C env gp120 C2-V5 subregion.

Materials and Methods

Study population and procedures

Four HIV-1-infected families labeled 205, 366, 375, and 567 consisting of biological parent(s)-infected siblings, an index child (older), and an index child's sibling (younger) constituted the study population. The unit of analysis was a family and these four were willing to participate in the phylogenetic study. The index child in this study was defined as the first child to be recruited into our study. Two families comprised both parents and a respective biological index child. The other two families had parent(s) and two subsequent biological children, the first and second siblings. In family 567 the father figure was missing as he was working in another regional country. Each member of the four families was HIV-1 infected and none had received antiretroviral therapy at the time of sample collection. We hereby describe a unique HIV-1 transmission clusters of four families for which the time and directionality of vertical transmission were known but were unknown for heterosexual transmission.

Consent was obtained from the four pregnant mothers of each family participating in the national PMTCT program in the periurban Harare mother and child clinic who were known to be HIV-1 positive at 36 weeks gestations. Spouses also consented to participate in the family phylogenetic study. Similar recruitment and procedures were followed as previously described for the mothers and infants.[34] Despite being encouraged to exclusively breastfeed during the first 6 months of life, all the infants were exposed to breast milk for at least 9 months. First siblings' plasma samples were collected at 60 – 10 months of age as there were insufficient sample volumes from their respective first HIV-positive samples. The first available HIV-1-positive sample was genotyped for the second sibling at about 15 – 3 months; for details see Table 1. Sexually transmitted infection (STI)

Table 1. Family Members' Demography and HIV-1 Clone Characteristics Based on Nucleotide Sequences

Parent/sibling	Age (years)	Unique clones	Intrapatient min-max diversity (%)	Intrapatient genetic distance (%)	Family members mean diversity	Interfamilies mean diversity	Entire population diversity
205							
F	39	3/5	7.7 (0.0–11.6)	4.70			
M	30	3/6	1.5 (0.8–2.0)	1.53	3.41		
1st_	5.3	3/4	0.0 (0.0–0.0)	0.00	2.04		
2nd\	1.2	2/4	5.7 (0.02–8.1)	3.80	6.55		
366							
F	39	3/6	10.6 (0.3–14.9)	4.86			
M	38	4/6	3.1 (0.0–6.2)	1.50	6.56		
1st\	5.8	2/5	0.6 (0.0–1.3)	0.25	5.12	6.41	
375							
F	36	3/5	14.5 (0.03–21.7)	6.95			
M	36	4/6	0.0 (0.0–0.0)	0.00	7.36		
1st\	4.9	1/4	0.0 (0.0–0.0)	0.00	4.77	5.75	
567							
M	35	2/5	14.4 (0.01–21.6)	8.90			
1st_	5	2/4	0.3 (0.0–0.7)	0.50	9.43		
2nd_	1.3	3/4	2.23 (0.0–4.5)	1.07	7.94	3.89	16.42

F, father; M, mother; 1st, first siblings; 2nd, second siblings; gender symbols denoting male _ and female \.

screening, nucleic acid extraction, polymerase chain reaction (PCR) amplification, cloning, and DNA sequencing methods for the HIV-1 env gp120 C2-V5 region were done as previously described and so was HIV-1 subtype determination.[35] Briefly, the primary PCR amplified an approximately 800-base pair (bp) fragment spanning the C2 and V5 region of the envelope, positions 6948–7537 in the HIV-HXB2 genome while the secondary PCR amplified an approximately 650-bp env gene fragment.

DNA sequence analysis

Phylogenetic and molecular evolutionary analyses are pivotal in the clarification of transmission patterns of HIV. To visualize the extent of the genetic relatedness of the HIV-1 env gp120 C2-V5 region among members of the four families following heterosexual and vertical transmission env amino acid sequences were analyzed including the construction of a phylogenetic tree using MEGA 5.0 software.[36] Using the Clustal X program positions with gaps were excluded before tree building, consequently DNA sequences had an equal length before alignment. The most popular test for tree reliability, bootstrap, was used. The bootstrap value is a percentage of how often each branch is present in exactly the same topology in all the resampled trees. A bootstrap cut-off value of > 70% signified at least a 95% probability that the topology of a branch is real.[37] An HIV-1 group O sequence obtained from the Los Alamos national database of HIV Sequence Compendium 2009 was used as the outgroup for rooting each of the families' trees.[38] Families' sequences were compared with other HIV-1 group M subtype reference sequences retrieved from the same database including other subtype C sequences from different countries within the SSA and other geographic regions such as Argentina, China, and India.

Genetic distance between two HIV-1 sequences is the count of the number of differences arising due to mutations and genetic drift resulting in genetic diversity. Differences were computed using Tamura and Tamura-Nei distribution-based distances also in MEGA.[36] Diversity, which is a measure of genetic variation at a given time, was calculated by measuring nucleotide diversity (p) implemented in MEGA 5. Homogeneity or heterogeneity of the C2-V5 regions in the viral clones of fathers, mothers, and first and second siblings was evaluated by comparing the number of unique DNA sequences among multiple clones per family member. Comparison of the genetic diversity and maximum genetic distance between families and different groups of family members, fathers, mothers, index children, or index children siblings was done. An arbitrary cut-off value of less than 1% was used to define viral homogeneity.[20]

Results

Characteristics of the four families

Three of the four mothers were in a monogamous marriage except for mother 375 who was in a polygamous relationship. All parents had at least 7 years in school and were of low economic status. Mothers were generally 5 years younger than their spouses. Table 1 summarizes the demography of family members.

HIV infections

Parents of the four families were HIV-1 positive but did not know when and how they got infected. Parents' mode of HIV-1 acquisition was most likely heterosexually as none mentioned any history of blood transfusion, drug abuse, or homosexuality except for one mother, 366, who had a history of blood transfusion. The index children of families 366, 375, and 567 and second siblings of families 205 and 567 were HIV-1 DNA PCR negative at delivery and 6 weeks postpartum but later became infected through breastfeeding. Index child 205 was HIV-1 DNA PCR negative at delivery but was not at the 6 week visit, hence the exact time of infection could not be established.

Mothers' reproductive health and single-dose nevirapine (SdNVP) prophylaxis

All mothers had spontaneous vaginal deliveries. One lifetime sexual partner was generally reported except for mother 375 who reported two. Due to religious beliefs mother 205 never used any method of contraception and moreover she refused to take SdNVP for herself and for both her infants. Otherwise the other mothers were on oral contraceptives and received SdNVP for themselves and their respective infants. All mothers had negative results for the RPR syphilis test but two of the four mothers were HSV-2 positive at enrollment.[39] Mothers 375 and 567 reported itchy genitals but no discharges were observed upon examination.

Subtypes and phylogenetic analysis

A total of 64 sequences from four family members were cloned of which a total of 35 unique clones were analyzed. On average four (three–five) clonal nucleotide sequences were determined for the env C2-V5 gp120 env region (650 bp) from each infected family member. Phylogenetic analysis of env amino acid sequences showed that HIV-1 subtype C had infected each one of the four families' members. The neighbor-joining phylogenetic tree showed that sequences were genetically linked and formed interfamilial clusters of HIV as shown in Fig. 1. Clusters were clearly distinguished with high bootstrap values suggestive of infections of monophyletic origin or a localized expansion of the subtype C epidemic at least among these four families. Our families env C2-V5 sequences clustered with other HIV-1 subtype C from South Africa, Malawi, Botswana, Tanzania, India, China, Argentina, and also from other previous Zimbabwean subtype C studies as evidenced by a bootstrap value of 86% (see Fig. 1). Sequences from families 205, 375, and 567 clustered more closely with sequences from South Africa, Malawi, and Botswana.

Genetic distances

Although all the families sequences turned out to be subtype C with respect to the env gene, the interfamilies mean genetic distances varied being furthest apart between families 205 and 366 (20%) while families 205 and 567 showed the least genetic distances of 16.5% (see Table 2). The mean pairwise genetic distance was higher among the fathers, mean 18.13% (0.00–28.38), being highest between fathers of families 205 and 366. Mothers' percentage mean genetic distances were significantly lower, 17.21% (0.00–24.28), and the longest distance was between mothers of families 366 and 375. The mean

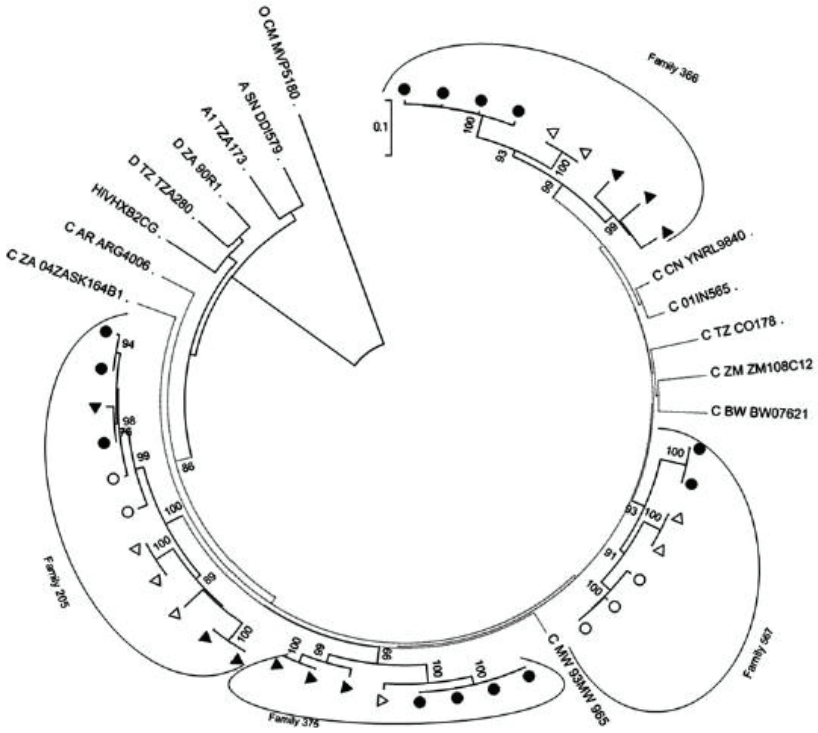

FIG. 1. Phylogenetic relationships between families' nucleotide sequences and other subtype C from different geographic regions. The first letter represents the subtype followed by the name of the country: AR, Argentina; BW, Botswana; ZA, South Africa; TZ, Tanzania; IN, India; ZM, Zimbabwe; CN, China. Shaded triangles and circles represent fathers' and mothers' sequences, respectively, while open triangles and circles represent first and second siblings sequences, respectively. Bootstrap values are expressed as percentages per 1000 replicates and only proportions of ‡ 70% are shown.

genetic distance was statistically higher among the adult population, 16.5% relative to that of children, 11.5%. The intergroup mean genetic distance between fathers and first siblings was 17.7% (see Table 3).

Heterosexual transmission

Transmission events were epidemiologically linked, supported by high bootstrap values, suggestive of predominantly monogamous relationships. Paternal sequences showed the highest average number of unique isolates by nucleotide sequence. Father 205 had the most heterogeneity followed by 366 and then 375. Comparison of all viral populations revealed that fathers' sequences exhibited the most heterogeneous viral quasispecies, which were observed to cluster in several separate branches suggestive of multiple variants. However, mothers showed a consistent pattern of limited viral diversity. Consequently both single and multiple transmission events may have occurred in these close contacts. Since the direction of transmission was not known we could not show how diversity changed with heterosexual transmission.

Vertical transmission

Mothers and second siblings had limited heterogeneity, indicative of a relatively recent infection or suggestive of

Table 2. Percent Nucleotide Genetic Distances Between Families

Family	205	366	375
205			
366	20.4		
375	17.5	19.0	
567	16.5	19.9	18.4

Table 3. Nucleotide Genetic Distances Between Families' Groups

	F	M	1st
F			
M	16.9		
1st	17.7	16.8	
2nd	17.2	15.3	15.9

F, fathers; M, mothers; 1st, first siblings; 2nd, second siblings.

vertical transmission of a single or very few closely related maternal variants. However, a more heterogeneous virus population was generally observed for first siblings of families 205, 375, and 366, demonstrated by the intermingling of their sequences with the parental ones. Viral sequences were distributed into several branches suggesting multiple distinct lineages probably as a result of evolution away from maternal viral sequences through immune selection pressures.

Phylogenetic analysis of family 205 sequences

Figure 2a represents the neighbor-joining phylogenetic tree for family 205 family member's sequences intermingled with each other. Despite the limited number of clones per family member's sample, genetic heterogeneity was detected in the father's sequences, which intermingled with sequences from the mother and both the two children. The less prevalent paternal strain clustered in a single branch of the tree supported with a 98% bootstrap value with both the maternal and second sibling variants. The most prevalent strain of the first sibling appeared on its own branch supported with the lowest bootstrap value of 79%. The topology of the tree reflects the vertical transmission of at least two maternal variants that diverge over time as seen in the first sibling's sequences. It is worthwhile to note that in this family there was no SdNVP prophylaxis selection pressure.

Phylogenetic analysis of family 366 sequences

Figure 2b represents the neighbor-joining phylogenetic tree for family 366. The high bootstrap values of > 95% indicated the sequences are closely related and monophyletic. In contrast to family 205 sequences, homogeneous infections were presumed based on the level of genetic diversity. The child sequences were in between the parent sequences. All sequences of mothers and child clustered tightly on one branch with paternal sequences on the other, but all sequences were supported with a bootstrap value of 81%. In this case the child could have been infected with a single maternal variant, but because of the time factor in between, the variant could also have evolved under host immunological selective pressure.

Phylogenetic analysis of family 375 sequences

Like family 366, paternal sequences clustered tightly on one branch while the mother–child sequence also clustered tightly on another branch with a bootstrap value of 99%, indicative of a relatively more recent infection, as shown in Fig. 2c. The overall family tree is supported by a high bootstrap value. As with family 205, the child sequences were also intermingled with those of the mother and consequently this case supports the transmission of multiple maternal variants. Since it is the father with the most divergent variants it can be assumed that he was infected much earlier and probably infected his spouse with a minor variant, which has also undergone selective pressure over the years. In family 375, the appearance of child HIV-1 variants closely related to antenatal maternal sequences at 55 months of age possibly supports the hypothesis of the reappearance of the founder virus later on during infection.

Phylogenetic analysis of family 567 sequences

The mother's sequences were closer to those of the second sibling' while the first sibling's sequences were further apart.

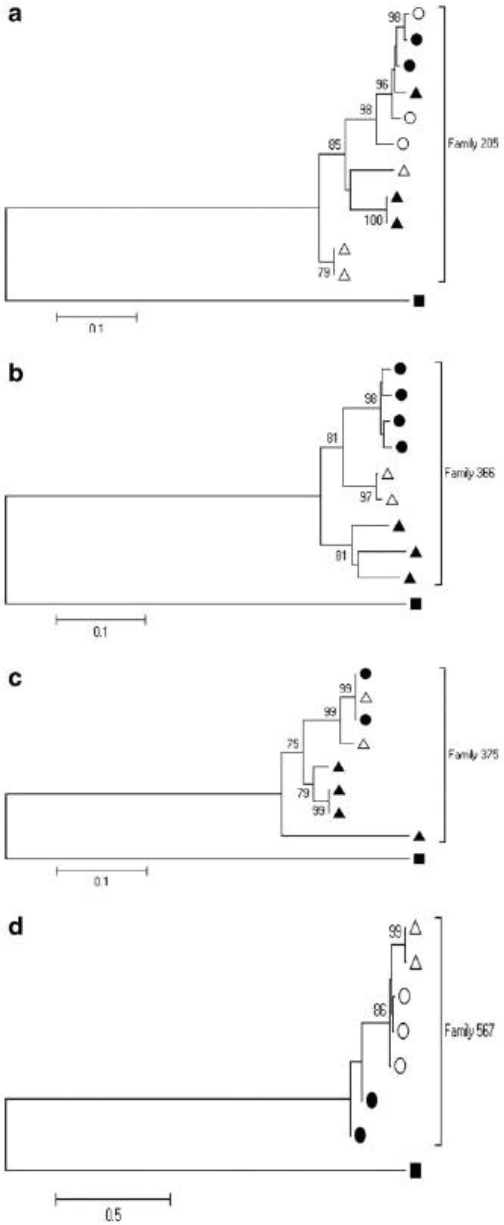

FIG. 2. Rooted neighbor-joining trees of HIV-1 env (C2-V5) amino acid sequences for four family members (a–d). Bootstrap values are expressed as percentages per 1000 replicates. Only bootstrap values greater than 70% are shown. Shaded triangles and circles represent fathers' and mothers' sequences, respectively, while open triangles and circles represent first and second siblings' sequences, respectively. Filled in squares stand for the divergent HIV group O sequences for rooting the trees.

Family sequences were supported by a bootstrap value of 86% (see Fig. 2d).

Discussion

In the absence of antiretroviral therapy, studies have shown that HIV-1 replicative fitness, which in turn impacts virus transmission, is largely determined by the functions of the envelope gene that was genotyped in our study.[40–42] This is the first report attempting to assess HIV-1 subtype C envelope gp120 C2-V5 phylogenetic relatedness among close contacts in Zimbabwe following concurrent heterosexual and subsequent vertical transmission. In SSA where HIV prevalence is high, it is culturally acceptable for men to have more than one wife and/or extramarital sexual relationships. Ironically, the underrepresentation of these male sexual partners' involvement in PMTCT programs in this setting where HIV-1 transmission is predominantly heterosexual has compromised holistic HIV control strategies.

In 80% of heterosexual transmission cases, single viruses have been shown to establish infection[9,43–45] with women harboring homogeneous env sequences being less likely to transmit per sexual act.[46] Limited heterogeneity observed in our mothers suggests that a single variant could have been acquired from the local site of infection. This could be a gender difference during pregnancy of HIV-1 subtype C possibly influenced by hormonal balances. In our study the directionality of heterosexual transmission, whether it was female to male (FTM) or male to female (MTF), could not be ascertained. Some subtype C studies have demonstrated similar FTM and MTF transmission rates.[47] Semen-derived viral populations have been shown to exhibit lower genetic diversity relative to the blood variants and this could probably be the reason for the limited heterogeneity observed in the mothers.[48] It could be worthwhile to explore similar but larger studies with many clones from the plasma and other compartments.

Contrary to our findings, some studies have demonstrated that women are often infected with multiple variants.[20] Diverse virus population in such studies could be attributed to reinfection by multiple partners since this other study's population was a cohort of female sex workers in Nairobi, Kenya with predominant A and D and to a lesser extent C HIV-1 subtypes. In the Kenyan study none of the five men and only two of 32 women had HIV-1 subtype C and interestingly both women had homogeneous virus populations. Homogeneous subtype C viruses have also been described elsewhere.[49,50] This is suggestive of the fact that subtype could influence the pattern of viral transmission.

Differences between the two studies could be due to variations in the study populations, the exact env region being analyzed, possibly variations in the sampling times at different stages of HIV-1 infection, and also different subtypes. Studies have shown that heterosexual and vertical transmission of HIV-1 subtype C viruses spread more rapidly due to increased mucosal and vaginal shedding.[51–53] Compared to other subtypes, subtype C has also been shown to replicate and be transmitted more efficiently.[54–56] Hence, there is a need to address subtype C-specific research questions rather than extrapolating and applying subtype B findings if curbing of the pandemic is to be realized.

Similar to previous observations a low degree of maternal HIV-1 genetic heterogeneity has been shown to correlate with vertical transmission contrary to observations by others.[31,57,58] If diversity remained low, then it could be likely that fewer variants could be harbored and hence there could be a narrow breadth of maternal neutralizing antibodies associated with increased risk of vertical transmission. Other important factors could be associated with infant exposure such as diverse viral inoculum, including other maternal factors such as nutritional status, human leukocyte antigen (HLA) genotype, coreceptor expression, or the presence of STIs among others. Successful transmission of maternal escape mutants has been reported in children sharing HLA alleles with their mothers.[59] Coinfection with human simplex virus type 2 (HSV-2) at delivery has been associated with increased intrapartum transmission of HIV-1.[60]

Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Our observation of distant first siblings' sequences is consistent with previous studies where viral divergence has been found to increase over time in children.[11] The first siblings were much older, about 60 months old, hence their HIV-1 could have evolved further from the maternal HIV, which was more or less closer to that of the second siblings who were about 15 months old.

The route of transmission may influence the genetic diversity, with some authors postulating that HIV homogeneity and heterogeneity patterns are likely to be different between infants and adults due to different exposures, different viral dynamics and set points following infection, and different immunity maturities in the recipients.[61] Studies have shown that most HIV-infected infants have a deficiency in cytotoxic T lymphocyte (CTL) responses to HIV[62,63] and inadequate CD4 T cell help.[64,65] Infection of infants with maternal CTL escape variants may further compromise the infant's ability to contain the virus.[66] On a positive note diversity may also disrupt the function of the env gene resulting in attenuation of HIV-1 virulence probably resulting in long-term nonprogressors in the absence of antiretroviral therapy observed in these pediatric patients.[67,68] Differences in mother–infant pair viral heterogeneity may also be due to virus compartmentalization between the plasma and breast milk variants. The immunological milieu of breast milk has been found to be distinct from that in blood as it contains a high concentration of HIV-1-specific T cells, antibodies, chemokines, and innate factors that modulate HIV-1 transmission risk.[69–71] However, a subtype C study of breastfeeding mothers found no differences between breast milk and blood variants.[72]

Families' subtype C intermingled with subtype C sequences from other regions, confirming the difficulty of classifying subtype C sequences on a geographic basis.[73] Due to the limited number of clones sequenced per family member it was not possible to determine the minor or major variants. More so, the directionality of heterosexual transmission could not be ascertained. It could be worthwhile to explore the determination of directionality of transmission using glycosylation patterns and amino acid lengths of HIV-1 env variable regions.

Few subtype C studies have looked at variation of the V1-V2 on transmission.[74,75] It would have been more interesting if the whole env region was sequenced including the V1-V2 region for comparison. However, sequencing of longer fragments has its own challenges. Phylogenetic analysis performed for each family sequence set suggested that several

mechanisms may be involved in both vertical and heterosexual transmission. The star-shaped families tree suggested a localized expansion of the subtype C epidemic at least among these our families. Generally families' sequences clustered quite closely with sequences from South Africa, Malawi, and Botswana. Paternal sequences exhibited the most heterogeneous viral quasispecies while maternal sequences were relatively homogeneous. First siblings viral sequences were distributed into several branches suggesting multiple distinct lineages probably as a result of the evolution away from maternal viral sequences through immune selection pressures during their 5–6 years of life while second siblings' HIV-1 sampled between 12 and 15 months of age had relatively homogeneous viral populations closely related to maternal variants. Larger studies are warranted to address the caveats of this study and build upon its strengths. Our study could be the beginning of a family-based intervention in HIV research in Zimbabwe.

Sequence Data

Sequences were submitted to GenBank and the accession numbers assigned were JQ070719–JQ070752.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Bartelsman M and Veeken H: [The HIV pandemic in the year 2007, an overview]. Ned Tijdschr Geneeskd 2007;151(48): 2655–2660.
2. Scarlatti G, Albert J, Rossi P, et al.: Mother-to-child transmission of human immunodeficiency virus type 1: Correlation with neutralizing antibodies against primary isolates. J Infect Dis 1993;168(1):207–210.
3. Wolfs TF, Zwart G, Bakker M, and Goudsmit J: HIV-1 genomic RNA diversification following sexual and parenteral virus transmission. Virology 1992;189(1):103–110.
4. Biesinger T and Kimata JT: HIV-1 transmission, replication fitness and disease progression. Virology (Auckl) 2008;2008(1): 49–63.
5. Corey L, Wald A, Celum CL, and Quinn TC: The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: A review of two overlapping epidemics. J Acquir Immune Defic Syndr 2004;35(5):435–445.
6. Ahmad N: Maternal-fetal transmission of human immunodeficiency virus. J Biomed Sci 1996;3(4):238–250.
7. Scott GB, Fischl MA, Klimas N, et al.: Mothers of infants with the acquired immunodeficiency syndrome. Evidence for both symptomatic and asymptomatic carriers. JAMA 1985; 253(3):363–366.
8. Mok JQ, Giaquinto C, de RA, et al.: Infants born to mothers seropositive for human immunodeficiency virus. Preliminary findings from a multicentre European study. Lancet 1987;1(8543):1164–1168.
9. Keele BF, Giorgi EE, Salazar-Gonzalez JF, et al.: Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci USA 2008;105(21):7552–7557.
10. Sagar M, Laeyendecker O, Lee S, et al.: Selection of HIV variants with signature genotypic characteristics during heterosexual transmission. J Infect Dis 2009;199(4):580–589.
11. Nowak P, Karlsson AC, Naver L, et al.: The selection and evolution of viral quasispecies in HIV-1 infected children. HIV Med 2002;3(1):1–11.
12. Frost SD, Liu Y, Pond SL, et al.: Characterization of human immunodeficiency virus type 1 (HIV-1) envelope variation and neutralizing antibody responses during transmission of HIV-1 subtype B. J Virol 2005;79(10):6523–6527.
13. Simmonds P, Balfe P, Ludlam CA, Bishop JO, and Brown AJ: Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. J Virol 1990;64(12):5840–5850.
14. Zhang LQ, MacKenzie P, Cleland A, et al.: Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. J Virol 1993;67(6):3345–3356.
15. Karlsson AC, Gaines H, Sallberg M, Lindback S, and Sonnerborg A: Reappearance of founder virus sequence in human immunodeficiency virus type 1-infected patients. J Virol 1999;73(7):6191–6196.
16. Mullins JI and Jensen MA: Evolutionary dynamics of HIV-1 and the control of AIDS. Curr Top Microbiol Immunol 2006;299:171–192.
17. Shankarappa R, Margolick JB, Gange SJ, et al.: Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol 1999;73(12):10489–10502.
18. Delwart E, Magierowska M, Royz M, et al.: Homogeneous quasispecies in 16 out of 17 individuals during very early HIV-1 primary infection. AIDS 2002;16(2):189–195.
19. Herbeck JT, Nickle DC, Learn GH, et al.: Human immunodeficiency virus type 1 env evolves toward ancestral states upon transmission to a new host. J Virol 2006;80(4):1637–1644.
20. Long EM, Martin HL Jr, Kreiss JK, et al.: Gender differences in HIV-1 diversity at time of infection. Nat Med 2000;6(1): 71–75.
21. Sagar M, Lavreys L, Baeten JM, et al.: Identification of modifiable factors that affect the genetic diversity of the transmitted HIV-1 population. AIDS 2004;18(4):615–619.
22. Keele BF and Derdeyn CA: Genetic and antigenic features of the transmitted virus. Curr Opin HIV AIDS 2009;4(5):352–357.
23. Haaland RE, Hawkins PA, Salazar-Gonzalez J, et al.: Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. PLoS Pathog 2009;5(1):e1000274.
24. Contag CH, Ehrnst A, Duda J, et al.: Mother-to-infant transmission of human immunodeficiency virus type 1 involving five envelope sequence subtypes. J Virol 1997;71(2): 1292–1300.
25. Ahmad N, Baroudy BM, Baker RC, and Chappey C: Genetic analysis of human immunodeficiency virus type 1 envelope V3 region isolates from mothers and infants after perinatal transmission. J Virol 1995;69(2):1001–1012.

26. Wolinsky SM, Wike CM, Korber BT, et al.: Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. Science 1992;255(5048):1134–1137.
27. Wike CM, Korber BT, Daniels MR, et al.: HIV-1 sequence variation between isolates from mother-infant transmission pairs. AIDS Res Hum Retroviruses 1992;8(7):1297–1300.
28. van't Wout AB, Kootstra NA, Mulder-Kampinga GA, et al.: Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. J Clin Invest 1994;94(5):2060–2067.
29. Pasquier C, Cayrou C, Blancher A, et al.: Molecular evidence for mother-to-child transmission of multiple variants by analysis of RNA and DNA sequences of human immunodeficiency virus type 1. J Virol 1998;72(11):8493–8501.
30. Lamers SL, Sleasman JW, She JX, et al.: Persistence of multiple maternal genotypes of human immunodeficiency virus type I in infants infected by vertical transmission. J Clin Invest 1994;93(1):380–390.
31. Briant L, Wade CM, Puel J, Brown AJ, and Guyader M: Analysis of envelope sequence variants suggests multiple mechanisms of mother-to-child transmission of human immunodeficiency virus type 1. J Virol 1995;69(6):3778–3788.
32. Dickover RE, Garratty EM, Plaeger S, and Bryson YJ: Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants in utero and intrapartum. J Virol 2001;75(5):2194–2203.
33. Verhofstede C, Demecheleer E, De CN, et al.: Diversity of the human immunodeficiency virus type 1 (HIV-1) env sequence after vertical transmission in mother-child pairs infected with HIV-1 subtype A. J Virol 2003;77(5):3050–3057.
34. Gumbo FZ, Kandawasvika GQ, Duri K, et al.: Reduced HIV transmission at subsequent pregnancy in a resource-poor setting. Trop Doct 2011;41(3):132–135.
35. Duri K, Soko W, Gumbo F, et al.: Genotypic analysis of human immunodeficiency virus type 1 env V3 loop sequences: Bioinformatics prediction of coreceptor usage among 28 infected mother-infant pairs in a drug-naive population. AIDS Res Hum Retroviruses 2011;27(4):411–419.
36. Tamura K, Peterson D, Peterson N, et al.: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28(10):2731–2739.
37. Hillis DM, Bull JJ, White ME, Badgett MR, and Molineux IJ: Experimental phylogenetics: generation of a known phylogeny. Science 1992;255(5044):589–592.
38. Yamaguchi J, Bodelle P, Kaptue L, et al.: Near full-length genomes of 15 HIV type 1 group O isolates. AIDS Res Hum Retroviruses 2003;19(11):979–988.
39. Munjoma MW, Kurewa EN, Mapingure MP, et al.: The prevalence, incidence and risk factors of herpes simplex virus type 2 infection among pregnant Zimbabwean women followed up nine months after childbirth. BMC Womens Health 2010;10:2.
40. Ball SC, Abraha A, Collins KR, et al.: Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C. J Virol 2003;77(2):1021–1038.
41. Marozsan AJ, Fraundorf E, Abraha A, et al.: Relationships between infectious titer, capsid protein levels, and reverse transcriptase activities of diverse human immunodeficiency virus type 1 isolates. J Virol 2004;78(20):11130–11141.
42. Kong X, West JT, Zhang H, et al.: The human immunodeficiency virus type 1 envelope confers higher rates of replicative fitness to perinatally transmitted viruses than to nontransmitted viruses. J Virol 2008;82(23):11609–11618.
43. Abrahams MR, Anderson JA, Giorgi EE, et al.: Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. J Virol 2009;83(8):3556–3567.
44. Liu Y, Nonnemacher MR, Stauff DL, et al.: Structural and functional studies of CCAAT/enhancer binding sites within the human immunodeficiency virus type 1 subtype C LTR. Biomed Pharmacother 2010;64(10):672–680.
45. Bar KJ, Li H, Chamberland A, et al.: Wide variation in the multiplicity of HIV-1 infection among injection drug users. J Virol 2010;84(12):6241–6247.
46. Baeten JM and Overbaugh J: Measuring the infectiousness of persons with HIV-1: Opportunities for preventing sexual HIV-1 transmission. Curr HIV Res 2003;1(1):69–86.
47. Fideli US, Allen SA, Musonda R, et al.: Virologic and immunologic determinants of heterosexual transmission of human immunodeficiency virus type 1 in Africa. AIDS Res Hum Retroviruses 2001;17(10):901–910.
48. Pillai SK, Good B, Pond SK, et al.: Semen-specific genetic characteristics of human immunodeficiency virus type 1 env. J Virol 2005;79(3):1734–1742.
49. Karlsson AC, Lindback S, Gaines H, and Sonnerborg A: Characterization of the viral population during primary HIV-1 infection. AIDS 1998;12(8):839–847.
50. Rademeyer C, Van Harmelen JH, Ramjee G, Karim SS, and Williamson C: Heterosexual transmission of multiple highly conserved viral variants in HIV-1 subtype C-infected seronegative women. AIDS 2004;18(15):2096–2098.
51. John-Stewart GC, Nduati RW, Rousseau CM, et al.: Subtype C is associated with increased vaginal shedding of HIV-1. J Infect Dis 2005;192(3):492–496.
52. Overbaugh J, Kreiss J, Poss M, et al.: Studies of human immunodeficiency virus type 1 mucosal viral shedding and transmission in Kenya. J Infect Dis 1999;179(Suppl 3):S401–S404.
53. Renjifo B, Gilbert P, Chaplin B, et al.: Preferential in-utero transmission of HIV-1 subtype C as compared to HIV-1 subtype A or D. AIDS 2004;18(12):1629–1636.
54. Sundaravaradan V, Das SR, Ramakrishnan R, et al.: Role of HIV-1 subtype C envelope V3 to V5 regions in viral entry, coreceptor utilization and replication efficiency in primary T-lymphocytes and monocyte-derived macrophages. Virol J 2007;4:126.
55. Soares EA, Martinez AM, Souza TM, et al.: HIV-1 subtype C dissemination in southern Brazil. AIDS 2005;19(Suppl 4): S81–S86.
56. McCormack GP, Glynn JR, Crampin AC, et al.: Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi. J Virol 2002;76(24): 12890–12899.
57. Matala E, Crandall KA, Baker RC, and Ahmad N: Limited heterogeneity of HIV type 1 in infected mothers correlates with lack of vertical transmission. AIDS Res Hum Retroviruses 2000;16(15):1481–1489.
58. Panther LA, Tucker L, Xu C, et al.: Genital tract human immunodeficiency virus type 1 (HIV-1) shedding and inflammation and HIV-1 env diversity in perinatal HIV-1 transmission. J Infect Dis 2000;181(2):555–563.
59. Leslie A, Kavanagh D, Honeyborne I, et al.: Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. J Exp Med 2005;201(6):891–902.
60. Cowan FM, Humphrey JH, Ntozini R, et al.: Maternal herpes simplex virus type 2 infection, syphilis and risk of intra-

partum transmission of HIV-1: Results of a case control study. AIDS 2008;22(2):193–201.
61. James MM, Wang L, Musoke P, et al.: Association of HIV diversity and survival in HIV-infected Ugandan infants. PLoS One 2011;6(4):e18642.
62. Luzuriaga K, Koup RA, Pikora CA, Brettler DB, and Sullivan JL: Deficient human immunodeficiency virus type 1-specific cytotoxic T cell responses in vertically infected children. J Pediatr 1991;119(2):230–236.
63. Buseyne F and Riviere Y: HIV-specific CD8 + T-cell immune responses and viral replication. AIDS 1993;7(Suppl 2):S81–S85.
64. Huang S, Dunkley-Thompson J, Tang Y, et al.: Deficiency of HIV-Gag-specific T cells in early childhood correlates with poor viral containment. J Immunol 2008;181(11):8103–8111.
65. Thobakgale CF, Ramduth D, Reddy S, et al.: Human immunodeficiency virus-specific CD8 + T-cell activity is detectable from birth in the majority of in utero-infected infants. J Virol 2007;81(23):12775–12784.
66. Shalekoff S, Meddows-Taylor S, Gray GE, et al.: Identification of human immunodeficiency virus-1 specific CD8 + and CD4 + T cell responses in perinatally-infected infants and their mothers. AIDS 2009;23(7):789–798.
67. Arien KK, Troyer RM, Gali Y, et al.: Replicative fitness of historical and recent HIV-1 isolates suggests HIV-1 attenuation over time. AIDS 2005;19(15):1555–1564.
68. Keet IP, Veugelers PJ, Koot M, et al.: Temporal trends of the natural history of HIV-1 infection following seroconversion between 1984 and 1993. AIDS 1996;10(13):1601–1602.
69. Kourtis AP, Butera S, Ibegbu C, Beled L, and Duerr A: Breast milk and HIV-1: Vector of transmission or vehicle of protection? Lancet Infect Dis 2003;3(12):786–793.
70. Habte HH, De BC, Lotz ZE, et al.: Inhibition of human immunodeficiency virus type 1 activity by purified human breast milk mucin (MUC1) in an inhibition assay. Neonatology 2008;93(3):162–170.
71. Garofalo RP and Goldman AS: Cytokines, chemokines, and colony-stimulating factors in human milk: The 1997 update. Biol Neonate 1998;74(2):134–142.
72. Heath L, Conway S, Jones L, et al.: Restriction of HIV-1 genotypes in breast milk does not account for the population transmission genetic bottleneck that occurs following transmission. PLoS One 2010;5(4):e10213.
73. Gaschen B, Taylor J, Yusim K, et al.: Diversity considerations in HIV-1 vaccine selection. Science 2002;296(5577):2354–2360.
74. Kwiek JJ, Russell ES, Dang KK, et al.: The molecular epidemiology of HIV-1 envelope diversity during HIV-1 subtype C vertical transmission in Malawian mother-infant pairs. AIDS 2008;22(7):863–871.
75. Sagar M, Wu X, Lee S, and Overbaugh J: Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. J Virol 2006;80(19):9586–9598.

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Paper V

Consent Form in English

CONSENT FORM

STUDY TITLE

HIV Diversity among Pregnant Women and their infants in Harare Peri-urban;

Implications in Disease Diagnosis, Monitoring and Transmission

Name of Clinic: ……

Study Number: ……

Study Explanation

There are different types of HIV variants called subtypes. Currently we do not know which HIV subtype you harbour and hence we are going to determine maternal and infant subtype that is if your baby if also HIV positive. This is important for more effective chemotherapy and also forms the basis for vaccine development, which so far has been a challenge. There is also need to find out whether subtype plays a role in vertical transmission. For HIV and subtype determination, 3ml and 1ml of EDTA blood will be drawn from the mothers and babies respectively.

There is no risk in participating in this study except for the pain you may experience during bleeding. You are free to participate in this sub-study if you wish and should you decide to join the study, you are free to withdraw from this study at any time. If for some reasons you are not willing to participate in the sub-study you are still entitled to all your full benefits from the main study. You will be reimbursed your bus-fare for coming to the clinic and will also have the privilege to be examined by a gynaecologist and your baby by a paediatrician. Do you have any questions pertaining to this study? Should you have any questions in future feel free to contact Mrs. K Duri on telephone number 791631. If you have understood and are willing to participate in the study you can show by signing this form on the space provided below.

Date _____ Participant's Signature _____

Date _____ Interviewer's Signature _____

Consent Form in Vernacular Language (Shona)

GWARO RETSANANGUDZ YECHIRONGWA NECHIBVUMIRANO

HIV Diversity among Pregnant Women and their infants in Harare Peri-urban;

Implications in Disease Diagnosis, Monitoring and Transmission

(Mhando dzeutachiwana hweHIV-1 (subtypes): Kukosha kwemhando idzi pakutapurirwa kwehutachiona kubva kuna amai kumwana wake)

Name of Clinic: ……
Study Number: ……

Tsanangudzo yechirongwa

Sezvo paine mhando dzakasiyana-siyana dzehutachiwana hweHIV-1 pari zvino hatisati tavakuziva kuti mune mhando ipi. Chirongwa chino chiri kuenderera mberi chichiongorora kuti mune mhando ipi yeutachiwana. uye kuti utachiwana hwenyu hwashanduka zvakadii mumakore apfuura. Izvi zvakakosha mumatanho ari kuitwa pasi rese ekutsvaga mushonga wokudzivirira nekurapa chirwere cheHIV usati wavanikwa pari zvino. Tinoda kuongorora zvakare kuti kusiyana kwemhando dzeutachiwana kune chekuita here pakutapurirwa kungaita utachiwana kubva kuna amai kumwana wake. Zvichirewa kuti tinoda kuona kuti utachiwana huri muna amai hwakasiyana sei nehuri mune mwana wake.

Kuti izvi zviitike ropa rinoita mamiririta matatu (3ml) richange richizotorwa kwamuri imi pamwe chete nemwana (1) ml kana achinge ainewo utachiwana. Hapana zita renyu richashandiswa paongororo iyi asi tinoshandisa namba chete. Zita renyu, kero yenyu pamwe chete nezvimwe zvakabvunzwa pamusoro penyu zvichange zvakachengetedzwa. Vashandi wemuchirongwa vanoongorora ropa renyu havafi vakaziva zvinhu izvi. Kana musinganzwe zvavakanaka, pana chiremba wemadzimai nevevana anokwanisa kukuona imi kana mwana wenyu. Vana chiremba ivavo vanogona kukutumidzirai kuzvipatara zvikuru kana zvichikodzera. Chirongwa chichahadhara mari dzinenge dzichizodiwa kana mapiwa mubhedha, mishonga pamwe neongororo dzamungangoitwa. Muchadzorerwa zvakare mari yenyu yebhazi yamunenge mashandisa kuuya kuchirongwa. Hapana njodzi inowanikwa mukupinda muchirongwa kunze kwemarwadzo angangowanikwa pakutorwa kweropa. Munhu haamanikidzwe kupinda muchirongwa chino. Kana muchinge mapinda muchirongwa chekutanga mune kodzero yekuramba kuenderera mberi nechirongwa chero zvayo nguva. Kuramba kwenyu hakushandure mabatirwo amunoitwa muchirongwa chekutanga. Mune mibvunzo here pamusoro petsanangudzo yechirongwa ichi? Mukazoita zveimwe mibvunzo ridzirai Mai Duri runhare panhamba dzinoti 791631. Kana manzwisisa chirongwa ichi sainai pazasi zvichireva kuti mabvuma kupinda muchirongwa.

Date _____ Participant's Signature _____

Date _____ Interviewer's Signature _____