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

When flood comes, fish eat ants & when flood recedes, ants eat fish. Only time matters. Just hold on, God gives opportunity to everyone!
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Abbreviations and Acronyms

AIDS  Acquired Immune Deficiency Syndrome
ANC  Antenatal Care
APOBEC3G  Apolipoprotein B mRNA-editing Enzyme-Catalytic polypeptide-like 3G
ART  Antiretroviral Therapy
BHAMC  Better Health for African Mother and Child
CCR5  Cysteine-Cysteine Chemokine Receptor-5
CXCR4  Cysteine-X-Cysteine Chemokine Receptor-4 (where X is any amino acid)
CD  Cluster of Differentiation
CTL  Cytotoxic T lymphocyte
CRF  Circulating Recombinant Form
DC-SIGN  Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA  Deoxyribonucleic Acid
EDTA  Ethylene-Diamine Tetra Acetate
ELISA  Enzyme-linked Immuno-sorbent Assay
Env  Envelope Gene
EPP  Epidemic Projection Package
Gag  Group-specific Antigen Gene
GALT  Gut Associated Lymphoid Tissue
Gp41  Glyco-Protein 41
HAART  Highly Active Antiretroviral Therapy
HIV  Human Immunodeficiency Virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HMA</td>
<td>Heteroduplex Mobility Assay</td>
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<tr>
<td>HTLV</td>
<td>Human T-lymphotrophic Virus</td>
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<tr>
<td>IDU</td>
<td>Intravenous Drug User</td>
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<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell Immunoglobulin-like Receptor</td>
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<tr>
<td>LAG-3</td>
<td>Lymphocyte Activation Gene-3</td>
</tr>
<tr>
<td>LAV</td>
<td>Lymphadenopathy Associated Virus</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MOHCW</td>
<td>Ministry of Health and Child Welfare</td>
</tr>
<tr>
<td>MRCZ</td>
<td>Medical Research Council of Zimbabwe</td>
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<tr>
<td>MTCT</td>
<td>Mother To Child Transmission</td>
</tr>
<tr>
<td>NBSZ</td>
<td>National Blood Service Zimbabwe</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative Factor gene</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-Human Primates</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>PARD3</td>
<td>Partitioning Defective 3 homolog</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PITC</td>
<td>Provider Initiated Testing and Counseling</td>
</tr>
<tr>
<td>PMTCT</td>
<td>Prevention of Mother-To-Child Transmission</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase gene</td>
</tr>
</tbody>
</table>
RNA  Ribonucleic Acid
RANTES  Regulated on Activation Normal T cell Expressed and Secreted
RT  Reverse Transcriptase
RT-PCR  Reverse Transcriptase-PCR
SdNVP  Single Dose Nevirapine
SDF-1  Stromal Derived Factor-1
SIV  Simian Immuno-deficiency Virus
SSA  Sub-Saharan Africa
Tat  Transactivator of Transcription gene
Tim-3  T-cell Immunoglobulin and Mucin domain-containing molecule-3
TLR  Toll-Like Receptor
TNF  Tumour Necrosis Factor
TRIM5α  Tripartite Motif-containing protein-5 alpha
URF  Unique Recombinant Form
Vif  Viral Infectivity Factor
Vpr  Viral Protein R
Vpu  Viral Protein U
μL  Micro (10^{-3}) litre
WHO  World Health Organisation
ZDF  Zimbabwe Defence Forces
ZDHS  Zimbabwe Demographic and Health Survey
List of Papers Included in the PhD Thesis


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

V. **Duri K.** Gumbo FZ, Kristiansen KI, Mapingure MP, Chirenje MZ, Rusakaniko S, Muller F and Stray-Pedersen B. **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 doi:10.4236/aid.2011.11001

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Other Papers Co-authored Related to my PhD Work but not Included in this Thesis

I. **Duri K.** Gumbo FZ, Kristiansen KI, Mapingure MP, Rusakaniko S, Muller F and Stray-Pedersen B. **HIV-1 subtype C Pediatric envelope (Env) region amino acid length polymorphism and glycosylation variation; Association with markers of disease progression.** Submitted to *BMC Virology Journal*

II. **Duri K.** Muller F, Kristiansen KI, Mapingure MP, Chirenje MZ, Rusakaniko S and Stray-Pedersen B. **HIV-1 subtype C envelope (env) region amino acid length polymorphism and glycosylation variation; Association with markers of disease progression in adults.** Submitted to *BMC Virology Journal*

III. Gumbo FZ, **Duri K.** Kandawasvika GQ, Kurewa NE, Mapingure MP, Munjoma MW, Stray-Pedersen B. **Risk factors of HIV vertical transmission in a cohort of**


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*

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 Types:
- 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. The aetiological agent was isolated from the lymph nodes of suspected patients two years later. 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). 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. 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 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. 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. 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\textsuperscript{12}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hiv_burden.png}
\caption{Global Heterogeneous HIV Burden\textsuperscript{13}.}
\end{figure}

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, \textit{Pan troglodytes troglodytes} (SIVcpz) in Southern Cameroon\textsuperscript{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\textsuperscript{15;16}. Interestingly, there is another alternative but unsubstantiated propositions of this complex and controversial topic on the origin of HIV\textsuperscript{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. 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. Thus, it is now widely accepted that the HIV-1 epidemic in SSA is mainly driven by heterosexual transmission. Husbands have been shown to acquire HIV-1 infection first from extra marital affairs and then proceed to infect their wives. 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.

Figure 1.2: HIV Prevalence trends among the 15-49 year olds over the past 10 years in SSA
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. 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.

HIV infection has been confirmed in a number of pediatric cases where its source has not been adequately explained. 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. 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. HIV has been shown to stay infectious on a needle for more than two weeks. 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. Even more intriguingly has been the observation that some of these HIV sero-discordant couples continue to bear children implying unprotected sex suggesting that something other than simply heterosexual transmission could be involved.
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

**Southern Africa**

**Zimbabwe**

![Geographical Location of Zimbabwe and Study Sites](image)

*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. 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.

Table 1.1: Trends of Selected Demographic Indicators in Zimbabwe

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

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. 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. 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. 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. Infant mortality rate rose from 50 per 1000 live births in 1990 to 60 per 1000 in 2006. 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. 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

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

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. 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. 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. 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. From 1995 onwards, HIV testing included screening for both the two types, HIV-1 and HIV-2. As of 2010, NBSZ reported an HIV-1 prevalence of 0.74%, a slight decline from 0.77% in 2009. Within this healthy blood donor population the hepatitis B virus (HBV) and syphilis sero-prevalence were 0.97% and 0.68%, respectively. 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)
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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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. 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. 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. 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. 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 200,000 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 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. 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. 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. With less disposable income during the economic meltdown many men were not able to purchase sex or sustain multiple sexual relationships. 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. 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.

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
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.
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. Annual births stand at about 379000 with more recent neonatal and infant mortality rates of 36/1000 and 56/1000, respectively. Out of these 47,494 pregnant women are HIV infected resulting in about 17,370 new pediatric HIV infections annually. MTCT of HIV is the most significant source of HIV infection in children below the age of 15 years. 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. 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. Between 1980 and 2005, among 10 million children born in Zimbabwe, a cumulative 504,000 were vertically infected with HIV. 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{147}, Figure 1.14. This option is still to be implemented in Zimbabwe.
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 breastfeeding for infants under 6 months of age only about 6% of the mothers strictly follow this instruction. Early HIV infant diagnosis using the HIV DNA PCR testing was introduced at National Medical Reference Laboratory in 2008.

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. 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](image)

1.4.5. PMTCT Impact and Challenges
There are claims that between 2002 and 2005, SdNVP PMTCT program have averted 4600 infections. 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. 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.

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.

![HIV Structure](image.png)

*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\textsuperscript{170,171}.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product/function</th>
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<tbody>
<tr>
<td>gag</td>
<td>Group-specific antigen</td>
</tr>
<tr>
<td>pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>env</td>
<td>Envelope</td>
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<tr>
<td>tat</td>
<td>Transactivator</td>
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<tr>
<td>rev</td>
<td>Regulator of viral expression</td>
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<tr>
<td>vif</td>
<td>Viral infectivity</td>
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<tr>
<td>vpr</td>
<td>Viral protein R</td>
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<tr>
<td>vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>nef</td>
<td>Negative-regulation factor</td>
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</table>

**Figure 2.2:** HIV-1 Gene Organisation, Adopted from\textsuperscript{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 \textit{viral protein R} (Vpr), respectively\textsuperscript{172,173}. Accessory or regulatory genes modulate virus replication. These include (Vpr; p15), \textit{viral infectivity factor} (Vif; p23), \textit{viral protein U} (Vpu; p18), \textit{negative regulation factor} (Nef; p24), \textit{transcriptional activator}; (Tat; p14) and \textit{regulator of viral gene expression} (Rev; p19)\textsuperscript{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 \textit{nuclear factor kappa beta} (NF\textsubscript{κ}B) and \textit{nuclear factor of activated T lymphocytes} (NFAT) are critical in the initiation of transcription\textsuperscript{175,176}. 30
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 \(\alpha\)-chemokine receptor CXCR4 (T cell-tropic) or the \(\beta\)-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
Most new infections are due to CCR5 HIV-1 variants. CXCR4-tropic viruses generally appear during the late stages of infection and are associated with increased pathogenicity. Gp120 V3 loop amino acid sequence is the critical genetic determinant of cellular co-receptors usage. Mutations within this region have been linked to changes in viral co-receptor usage. Interaction of env gp120 and CD4 surface molecules expressed on target cells results in conformational changes that exposes the co-receptor binding sites, as summarised in Figure 2.3.

Figure 2.3: HIV Infection and Spreading.

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. Particles also bind B-lymphocytes through the complement receptor CD21. Virus or virus-infected cells ultimately reach the draining lymph nodes where by encounter activated CD4+CCR5+ T-
lymphocytes that propagate further infection. The HIV promoter embedded in the 5′ long terminal repeats is able to engage the host’s transcription machinery for viral gene expression. Figure 2.4 summarises the life cycle of HIV.

**Figure 2.4:** HIV life cycle Adopted from 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.
2.2.3 Susceptibility to HIV Infection
Why some individuals remain uninfected despite repeated sexual exposure to HIV-1 remains a mystery. 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. Some alleles are protective whilst others increase susceptibility to HIV acquisition.

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. 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.

![Figure 2.5: Natural History of HIV disease](image)

Figure 2.5: Natural History of HIV disease.
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 lifetime of the patient.

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). Patient(s) may develop symptoms of the acute retroviral syndrome which include influenza-like illness with fever, sore throat, lymphadenopathy and exanthema. Frequently observed hematological abnormalities include thrombocytopenia, anemia, leucopenia, lymphopenia and monocytosis. CD4 counts are usually decreased and there is a reversal of the CD4:CD8 cell ratio. Tools used to detect primary HIV infection in the absence of sero-conversion include p24 antigen assays and nucleic acid testing. 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. HIV set point is the viral load that stabilises after acute HIV infection which is maintained at a plateau during the asymptomatic phase. Measurement of plasma HIV-1 RNA load is an important predictor of disease progression. 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. 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. Humoral response is initially effective but declines as the disease progresses with neutralizing antibodies tending to be weak and lacking broad cross-reactivity. 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. They bear the CD8 molecule and are the major immunological mechanism in the control of viremia. 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. Activation of CTLs also results in the release of soluble antiviral factors which inhibit progeny viruses from entering target cells. 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.
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), apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) proteins, tripartite motif-containing protein 5 alpha (TRIM5α) and tetherins as shown in Figure 2.7.

**Figure 2.7:** Host Restriction factors to HIV infection. ART drugs block is shown in grey colour as it is not natural as drugs exert viral suppression only in patients undergoing therapy.

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\textsuperscript{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 TNF\(\alpha\), IL-6 and IL-1\(\beta\)\textsuperscript{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\textsuperscript{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\textsuperscript{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. 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). 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.

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,
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.

![AIDS Diagram](image)

**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].
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-vis*, 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.

![Diagram](image.png)

**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. Everyday millions of genetic variants accumulate in latently infected cells only to be re-activated at some time in the future. 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. 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. 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. 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.

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. 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), as summarised in Figure 2.12.

Figure 2.12: Summary of HIV Classification

2.6.1. HIV types

Phylogenetically HIV can be classified into two types; type 1 (HIV-1) and type 2 (HIV-2). 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. 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). Thus, (SIVCPZ) is closely related to HIV-1, while SIV from sooty mangabey (SIVSM) is closest to
HIV-2. 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. Despite similar modes of transmission, HIV-2 is not as efficient in transmission horizontally and vertically. Relative to HIV-1, HIV-2 has a reduced rate of disease development and has shown natural resistance to readily available NNRTIs. Genetic recombination between HIV types-1 and-2 has been reported. 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. Group M is responsible for more than 90% of the world HIV infections. 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. HIV groups have genetic sequence differences of >40% in some coding regions. More recently, a new putative group, designated P, was reported in France from a Cameroonian female immigrant. 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\textsuperscript{373}. Reports have indicated that HIV-1 group P infections are rare, accounting for only 0.06\% of HIV infections in Cameroon\textsuperscript{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\textsuperscript{375}.](image)

**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\textsuperscript{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\textsuperscript{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. One of the most prevalent group M CRF common in Southeast Asia was earlier on incorrectly designated subtype E but was later correctly renamed CRF01_AE following full HIV genome sequencing. To date more than 21 CRFs and several URFs have been described. All CRFs together account for 18% of the world’s HIV-1 infections. HIV-1 subtypes and recombinants may differ with respect to plasma viral load levels, transcriptional activation levels, disease progression and response to chemotherapy including drug induced/natural resistance patterns.

2.7. Distribution of HIV-1 Subtypes and Recombinants

Over 50 different subtypes and CRFs have been described. 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. 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.
In most Southern African nations subtype C predominate, contributing 93-100% of the HIV-1 infections amongst individual countries. 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. 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. 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. 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. Non-A subtype infections have been shown to progress to AIDS faster than those infected with subtype A. More so subtype D has been associated with the most rapid disease progression relative to subtypes A, C and CRFs.

Pregnancy has been shown to increase the risk of female-to-male HIV-1 transmission by two folds. Pregnant women infected with subtype C shed significantly more HIV-1-infected vaginal cells than those infected with subtypes A or D. 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.

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. 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. 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. Presence or absence of some PNGs at specific sites on the gp120 env has also been associated with increased HIV-1 MTCT. Transmission can be in uterine, intra-partum or...
postpartum mainly through breast milk \textsuperscript{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 \textsuperscript{426}. Infants may be infected with the most prevalent maternal strain, a minor maternal variant or multiple maternal quasi-species \textsuperscript{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 \textsuperscript{429}. After transmission HIV-1 infected infants harbour either homogenous or heterogeneous virus populations \textsuperscript{430,431}. HIV-1 inter-subtype recombinants may also be effectively transmitted vertically to infants \textsuperscript{432}.

SdNVP given to mothers before delivery and to newborns after delivery can reduce the risk of transmission by 10-15\% \textsuperscript{433} although this can select for nevirapine resistant variants which decrease with time, but remain above pre-dose levels \textsuperscript{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 \textsuperscript{435}. This observation has a bearing on the efficacy of subsequent antiretroviral therapy containing nevirapine or other NNRTIs \textsuperscript{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 \textsuperscript{417,437}. However, other studies have shown no apparent differences in the rate of MTCT of HIV-1 among women with different subtypes \textsuperscript{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. 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. 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 which was later modified by Heyndrick 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 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. 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™ 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.](image)

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 the Test (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.](image)

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. 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\textsuperscript{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\textsuperscript{451}. TLC of 1200 cells/mm\textsuperscript{3} was the threshold value used equivalent to a CD4 count of 200 cells/mm\textsuperscript{3}\textsuperscript{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\textsuperscript{+} T lymphocytes were enumerated using a Partec Cyflow counter (Cyflow, Partec, Munster, Germany) within 6 hours of blood collection as previously described\textsuperscript{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. 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. 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 \textsuperscript{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 \textsuperscript{458}.

3.9. **Nucleic acid extraction**

Total RNA was extracted from plasma using the NucliSENS isolation kit, based on the Boom \textit{et al.}, method \textsuperscript{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 \textsuperscript{460}](image)

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. 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. It can replicate a 1000 base pair strand of DNA in less than 10 seconds at 72°C. 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.

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/Sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (5x)</td>
<td>10</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>95</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 3.3: Amplification with Taq polymerase

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>15</td>
</tr>
<tr>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>72</td>
<td>60</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/Sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Buffer (10x)</td>
<td>5</td>
</tr>
</tbody>
</table>
20mM dNTPs          2
JA168 primer 1 (10 pmol/μL)       2
ES 8 primer 2 (10 pmol/μL)        2
50mM MgCl₂          2
Taq Pol          0.5
Template (from RT PCR)        1
RNA free water       35.5

**Total Volume** 50

<table>
<thead>
<tr>
<th>Table 3.5: Nested PCR cycles Programmed on the Thermal Cycler</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
</tr>
<tr>
<td>95</td>
</tr>
<tr>
<td>94</td>
</tr>
<tr>
<td>55</td>
</tr>
<tr>
<td>72</td>
</tr>
<tr>
<td><strong>24 cycles in total</strong></td>
</tr>
<tr>
<td>72</td>
</tr>
<tr>
<td>04</td>
</tr>
</tbody>
</table>

**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.**
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.


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 5–20 ng 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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)/Microplate well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>1</td>
</tr>
<tr>
<td>Primer (ES8/ JA168)</td>
<td>1</td>
</tr>
<tr>
<td>RNAase free water</td>
<td>13</td>
</tr>
<tr>
<td>*Master Mix</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

*Components of Master Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>3.5</td>
</tr>
<tr>
<td>RNAase water</td>
<td>0.5</td>
</tr>
<tr>
<td>Big Dye</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.0</strong></td>
</tr>
</tbody>
</table>

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).](image)

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 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: Ligatong of the PCR product into the TOPO vector using Topoisomerase Adopted from 466.](image)

The transformed the recombinant vector was taken up and expressed in chemically competent *E. coli* (C4040-03) as per the manufacturer’s instructions. 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.
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 (version 17.0, 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-imburused 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 nanograms (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 bootstrap 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 log10 (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
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-vis 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*

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


**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. 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. 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. 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 sero-conversion of HIV-1 was more likely observed amongst mothers who reported having a travelling spouse or sexual partner, Paper 1, 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. 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.

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. However, the bigger mother cohort showed a rapid test results discordance of 5% . 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 . Cases of antibody-negative HIV infections have been reported. 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. 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 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. 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. Studies have shown African sera to exhibit a significantly higher number of indeterminate WB patterns. 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. 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. 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. Thus some studies have shown that Amplicor DNA PCR HIV-1 test does not detect all subtypes with equivalent sensitivity. 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. 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. 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.

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 except for one study with unconvincing methodology that reported several double and triple recombinants. 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. Env 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, 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. 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. 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. The most severe HIV-1 pandemic is occurring in Southern Africa. It is caused by HIV-1 subtype C. 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. Compared to other subtypes, subtype C has been shown to replicate and to be transmitted more efficiently. 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. Subtype C may also be biologically different as demonstrated by the predominant R5 co-receptor usage, Paper II, 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. 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, \textbf{Paper IV}. Interestingly, similar results of a relatively constant subtype C V3 region have been obtained elsewhere. 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 \textbf{Papers II, IV and V}. 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, \textbf{Papers I; III}. Of note was the highly significant relationship between antenatal HIV-1 RNA load, at 36 weeks gestational period with vertical transmission, \textbf{Paper I}. Similar to other studies, 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 \textbf{Paper I}. It was also
interesting to note that maternal co-receptor genotype was preserved upon vertical transmission Paper II. 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. 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. 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-vis the more heterogeneous paternal variants. Similar results have also been previously reported. 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. 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. 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. 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. 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
have been developed to improve the accuracy of HIV-1 subtype classification.

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.
up period and some of our study participants relocated to the rural areas where cost of living was relatively cheaper thereby upsetting follow up rates. Some infants were seen at delivery and only reappeared after 15 months of age, Paper I. 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.

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. 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.
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


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Chapter 10

Appendices

Published Papers

Consent Forms
Paper I
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 OraQuick (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 Dyn 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/ mm3 was the threshold value used equivalent to a CD4 count of 200 cells/mm3[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 (1.6log10) and 10 (7log10) 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 (5.8) 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 lifetime 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 = 0.08. 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 enrollment, 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Transmitter N = 32 (%)</th>
<th>Non Transmitters N = 32 (%)</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>26.3 (5.6)</td>
<td>25.6 (5.6)</td>
<td>1.01 (0.95-1.08)</td>
</tr>
<tr>
<td>Years in school</td>
<td>4/32 (13)</td>
<td>3/32 (9)</td>
<td>1.16 (0.58-2.33)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At least 1 child</td>
<td>28/32 (88)</td>
<td>25/32 (78)</td>
<td>1.40 (0.60-3.30)</td>
</tr>
<tr>
<td>Polygamous marriage</td>
<td>4/31 (13)</td>
<td>4/30 (13)</td>
<td>0.98 (0.47-2.06)</td>
</tr>
<tr>
<td>Subsidised income</td>
<td>4/32 (13)</td>
<td>8/32 (25)</td>
<td>0.62 (0.27-1.43)</td>
</tr>
<tr>
<td>Age at sexual debut ≤ 16 years</td>
<td>6/32 (19)</td>
<td>3/32 (9)</td>
<td>1.41 (0.82-2.42)</td>
</tr>
<tr>
<td>Life time partners</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>17/32 (53)</td>
<td>16/32 (50)</td>
<td>1.06 (0.65-1.74)</td>
</tr>
<tr>
<td>Vaginal discharge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>15/32 (47)</td>
<td>13/31 (42)</td>
<td>1.10 (0.68-1.79)</td>
</tr>
<tr>
<td>Genital ulcer</td>
<td>5/32 (16)</td>
<td>3/30 (10)</td>
<td>1.25 (0.69-2.28)</td>
</tr>
<tr>
<td>Dysuria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7/32 (22)</td>
<td>4/31 (13)</td>
<td>1.32 (0.78-2.25)</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>3/28 (11)</td>
<td>1/31 (3)</td>
<td>1.65 (0.87-3.12)</td>
</tr>
<tr>
<td>Abortion history</td>
<td>7/32 (22)</td>
<td>3/32 (9)</td>
<td>1.51 (0.92-2.49)</td>
</tr>
<tr>
<td>Infant death history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4/32 (13)</td>
<td>7/32 (22)</td>
<td>0.69 (0.30-1.47)</td>
</tr>
<tr>
<td>Schistosomiasis infection history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7/32 (22)</td>
<td>4/31 (13)</td>
<td>1.32 (0.78-2.24)</td>
</tr>
<tr>
<td>Mothers’ ARV Prophylaxis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17/32 (53)</td>
<td>16/32 (50)</td>
<td>1.00 (0.60-1.66)</td>
</tr>
<tr>
<td>Infant gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14/30 (47)</td>
<td>12/29 (41)</td>
<td>1.11 (0.67-1.83)</td>
</tr>
<tr>
<td>Birth weight &lt;2500</td>
<td>2/31 (6)</td>
<td>1/30 (3)</td>
<td>1.36 (0.58-3.15)</td>
</tr>
<tr>
<td>Deceased infant</td>
<td>9/31 (29)</td>
<td>2/30 (7)</td>
<td>4.35 (1.02-18.52)*</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breastfed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28/31 (90)</td>
<td>19/25 (76)</td>
<td>1.79 (0.69-4.64)</td>
</tr>
<tr>
<td>Baby ARV prophylaxis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16/27 (59)</td>
<td>13/25 (52)</td>
<td>0.87 (0.51-1.49)</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Variable</th>
<th>Transmitters (N = 32)</th>
<th>Non-transmitters (N = 32)</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin g/dl &lt;10</td>
<td>7/32 (22)</td>
<td>2/32 (6)</td>
<td>1.71 (1.08-2.69)*</td>
</tr>
<tr>
<td>TLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cells/mm3 (sd)</td>
<td>2147 (111)</td>
<td>2505 (132)</td>
<td>0.99 (0.99-0.99)*</td>
</tr>
<tr>
<td>Viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean log10 copies/ml</td>
<td>3.55 (1.12)</td>
<td>2.92 (0.92)</td>
<td>1.29 (1.07-1.55)*</td>
</tr>
</tbody>
</table>
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 of 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>
<thead>
<tr>
<th>Variable</th>
<th>In utero transmitters N = 10</th>
<th>In utero matched controls N = 10</th>
<th>RR (95% CI)</th>
<th>Intra/Postpartum Transmitters N = 22</th>
<th>Intra-/postpartum Matched Controls N = 22</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean g/dL (sd)</td>
<td>10.7 (0.9)</td>
<td>10.5 (1.3)</td>
<td>1.10 (0.74-1.65)</td>
<td>10.5 (1.5)</td>
<td>11.4 (1.0)</td>
<td>0.62 (0.71-0.94)*</td>
</tr>
<tr>
<td>TLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cells/ml (SD)</td>
<td>2133 (724)</td>
<td>2207 (443)</td>
<td>0.99 (0.98-1.00)</td>
<td>2153 (576)</td>
<td>2632 (790)</td>
<td>0.98 (0.97 = 0.99)*</td>
</tr>
<tr>
<td>Viral load</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean log_{10} copies/ml</td>
<td>3.5 (1.2)</td>
<td>3.0 (0.9)</td>
<td>1.60 (0.70-3.79)</td>
<td>3.6 (1.1)</td>
<td>2.9 (1.0)</td>
<td>2.0 (1.02-3.81)*</td>
</tr>
</tbody>
</table>
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 resolution to HIV-1 RNA loads, and such findings have been observed in African counterparts [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 log10 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, TLR 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. SB 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


Paper II
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

Kerina Duri, White Soko, Felicity Gumbo, Knut Kristiansen, Munyaradzi Mapingure, Babill Stray-Pedersen, Fredrik Muller, and the BHAMC Group

Abstract

We sought to predict virus coreceptor utilization using a simple bioinformatics method based on genotypic analysis of human immunodeficiency virus type 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 = 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 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 and subtype, HIV-1 can be classified as CCR5 (R5), CXCR4 (X4), or dual tropic (R5X4). 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. More so, this V3 region is also crucial in viral replication, transmission, infectivity, and neutralization. Genetic variation within this region has been linked to changes in coreceptor usage. 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. 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, where 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.\(^7\) Contrary to this finding, vertical transmission of dual-tropic HIV-1 has been demonstrated,\(^5\)\(^6\) 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 mothers and 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 –80°C 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.

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 3500SL Hematology Analyser. Mothers were screened for sexually transmitted infections as previously described by Gumbo et al.\(^11\) 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 secovers, 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 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'-GTACGGACA GTACAAGTGACATACAT-3' and 5'-GGCCCCCATAGTGCTTGCTCTGTCACACAT-3', respectively. Secondary PCR amplified an approximately 535-bp env gene fragment using inner sense and antisense primers 5'-ACAAATGACATGAAATGTTTCCA-3' and 5'-GGAGGGCCATACATTGCTTCTGCT-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 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 performed with the standard mass ladder. 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, PCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA). 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, PCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA). 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.

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 GeneDoc, 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>X4/R5X4 genotypes</th>
<th>R5 genotype</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (Mean (SD))</td>
<td>30.8 (3.8)</td>
<td>27.0 (5.0)</td>
<td>0.176</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No child, n (%)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>One child, n (%)</td>
<td>3 (14)</td>
<td>18 (88)</td>
<td>0.684</td>
</tr>
<tr>
<td>HSV infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not infected, n (%)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td></td>
</tr>
<tr>
<td>Infected, n (%)</td>
<td>2 (11)</td>
<td>16 (89)</td>
<td>0.144</td>
</tr>
<tr>
<td>Syphilis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not infected, n (%)</td>
<td>5 (25)</td>
<td>15 (75)</td>
<td>0.421</td>
</tr>
<tr>
<td>Infected, n (%)</td>
<td>0 (0)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td>Trichomonas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not infected, n (%)</td>
<td>3 (18)</td>
<td>14 (82)</td>
<td></td>
</tr>
<tr>
<td>Infected, n (%)</td>
<td>0 (0)</td>
<td>3 (100)</td>
<td>0.430</td>
</tr>
<tr>
<td>Genital ulcer disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not infected, n (%)</td>
<td>5 (25)</td>
<td>15 (75)</td>
<td>0.567</td>
</tr>
<tr>
<td>Infected, n (%)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>Lymphadenoapathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent, n (%)</td>
<td>3 (15)</td>
<td>17 (85)</td>
<td>0.043</td>
</tr>
<tr>
<td>Present, n (%)</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td></td>
</tr>
<tr>
<td>Baseline Hb (Mean (SD))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log viral load (Mean (SD)) (copies/ml)</td>
<td>11.0 (1.8)</td>
<td>10.9 (1.0)</td>
<td>0.925</td>
</tr>
<tr>
<td>GPGQ crown motif</td>
<td></td>
<td></td>
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<tr>
<td>Absent, n (%)</td>
<td>4.8 (1.6)</td>
<td>3.6 (1.2)</td>
<td>0.078</td>
</tr>
<tr>
<td>Present, n (%)</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td></td>
</tr>
<tr>
<td>Maternal death</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No, n (%)</td>
<td>3 (14)</td>
<td>19 (86)</td>
<td>0.031</td>
</tr>
<tr>
<td>Yes, n (%)</td>
<td>4 (17)</td>
<td>19 (83)</td>
<td></td>
</tr>
<tr>
<td>Baby delivery weight Mean (SD) (g)</td>
<td>1.50 (50)</td>
<td>1 (50)</td>
<td>0.269</td>
</tr>
<tr>
<td>Baby sex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Female, n (%)</td>
<td>2870 (332)</td>
<td>3069 (300)</td>
<td>0.205</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>2 (22)</td>
<td>7 (78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (9)</td>
<td>10 (91)</td>
<td>0.413</td>
</tr>
</tbody>
</table>

n is for number tested; SD, standard deviation. X4 and R5X4 genotypes were grouped together.
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://16mullinslab.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. 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,
<table>
<thead>
<tr>
<th>ID No.</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>CCR5/CXCR4 Usage</th>
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<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
<td></td>
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</tr>
<tr>
<td>Los Alamos</td>
<td>CTRPNNNT-RKSIRI-GPGQAFYA-TGEI-IGDIRQAHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zim Cons</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>39_0</td>
<td>V</td>
<td>T</td>
<td></td>
<td></td>
<td>R4</td>
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<tr>
<td>39_0_A</td>
<td>A...2R...VR...ND...K...V...T...D...V...Y...N...V...T...</td>
<td>R5</td>
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<tr>
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ID No. represents different samples or conditions, while CCR5/CXCR4 Usage indicates the usage pattern of CCR5 and CXCR4 in these samples.
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 log₁₀ transformed. The Student’s t-test was used to compare mean maternal age, log₁₀ 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, nonsignificantly. 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 log₁₀ 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 age 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.

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 < 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. 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, not from Zimbabwe. 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.24 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 of discordant mother–infant coreceptor genotypes occurring much later at more than 12 months.25 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.26 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.11 Nevertheless, X4 variants are not very rare in early infection. In Zimbabwe among male seroconverters, X4 phenotype frequency has been shown to be 12%.28 Huang et al. observed a 4% frequency of X4 viruses in a cohort of 150 recently HIV-1 subtype D-infected individuals. 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 Coetzee 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.29 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 phenotype. 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.

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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

Kerina Duri1*, Fredrik Müller2, Felicity Z Gumbo3, Nyaradzai E Kurewa4, Simba Rusakaniko5, Mike Z Chirenje6, Munyaradzi P Mapingure5, Babill Stray-Pedersen4

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.
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 Chimp panzee (cpz) sub-species, Pan troglodytes troglodytes (SIVcpz)[3]. Both HIV-1 and SIVcpz have a unique Vpx 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 immunocassay 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.
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’ 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

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 -80°C until tested.
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 Dyn 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 Incidence, Type(s) and vertical transmission among 6 seer-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-converters had solely HIV-1 infection. Neither HIV-2 nor HIV-1/HIV-2 co-infections were detected in this subgroup.

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 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 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
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

<table>
<thead>
<tr>
<th>Antenatal variable</th>
<th>Band</th>
<th>P17</th>
<th>P39</th>
<th>P55</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Present</td>
<td>Absent</td>
<td>p-value</td>
</tr>
<tr>
<td>Lymphadenopathy Number/Total (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>4/51 (8%)</td>
<td>0/32 (0%)</td>
<td>2/38 (5%)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0/2 (0%)</td>
<td>4/21 (19%)</td>
<td>3/15 (13%)</td>
<td>0.361</td>
</tr>
<tr>
<td>Hemoglobin Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>10.8 (1.3); N = 54</td>
<td>11.0 (1.2); N = 33</td>
<td>10.9 (1.4); N = 39</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>11.7 (1.0); N = 2</td>
<td>10.4 (1.5); N = 23</td>
<td>10.6 (1.0); N = 17</td>
<td></td>
</tr>
<tr>
<td>p-Value</td>
<td>0.312</td>
<td>0.025</td>
<td>0.395</td>
<td></td>
</tr>
<tr>
<td>Viral load log10 Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3.3 (1.0); N = 56</td>
<td>3.0 (0.8); N = 35</td>
<td>3.1 (0.9); N = 41</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>2.3 (0); N = 2</td>
<td>3.7 (1.2); N = 23</td>
<td>3.6 (1.2); N = 17</td>
<td></td>
</tr>
<tr>
<td>p-Value</td>
<td>0.167</td>
<td>0.016</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>Vertical Transmission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>29/56 (52%)</td>
<td>15/36 (42%)</td>
<td>20/41 (49%)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0/2 (0%)</td>
<td>14/22 (64%)</td>
<td>9/17 (53%)</td>
<td></td>
</tr>
<tr>
<td>p-Value</td>
<td>0.150</td>
<td>0.104</td>
<td>0.773</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Positive HIV-1 antigen Band</th>
<th>Chronic infections</th>
<th>Sero-converters</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 58 (%)</td>
<td>N = 6 (%)</td>
<td></td>
</tr>
<tr>
<td>gag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p17</td>
<td>57 (98)</td>
<td>3 (50)</td>
<td>0.002</td>
</tr>
<tr>
<td>p24</td>
<td>57 (98)</td>
<td>5 (83)</td>
<td>0.183</td>
</tr>
<tr>
<td>p39</td>
<td>35 (60)</td>
<td>2 (33)</td>
<td>0.186</td>
</tr>
<tr>
<td>p55</td>
<td>41 (71)</td>
<td>2 (33)</td>
<td>0.251</td>
</tr>
<tr>
<td>pol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p31</td>
<td>58 (100)</td>
<td>3 (50)</td>
<td>0.001</td>
</tr>
<tr>
<td>p51</td>
<td>58 (100)</td>
<td>4 (67)</td>
<td>0.002</td>
</tr>
<tr>
<td>p66</td>
<td>58 (100)</td>
<td>4 (67)</td>
<td>0.002</td>
</tr>
<tr>
<td>Env</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp41</td>
<td>58 (100)</td>
<td>6 (100)</td>
<td>-</td>
</tr>
<tr>
<td>Gp120</td>
<td>58 (100)</td>
<td>6 (100)</td>
<td>-</td>
</tr>
<tr>
<td>Gp160</td>
<td>58 (100)</td>
<td>6 (100)</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

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-converters 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.

**Acknowledgements**

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**Authors’ contributions**

KD collected data and drafted the manuscript, FM supervised laboratory analysis, FZG collected data, NEXK 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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Paper IV
Phylogenetic Analysis of Human Immunodeficiency Virus Type 1 Subtype C Env gp120 Sequences Among Four Drug-Naive Families Following Subsequent Heterosexual and Vertical Transmissions

Kerina Duri, Felicity Gumbo, Knut Kristiansen, Munyaradzi Mapingure, Marshall Munjoma, Mike Chirenje, Simbarashe Rusakaniko, Babill Stray-Pedersen, and Fredrik Muller

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. Heterosexual and vertical transmissions are the primary modes of HIV acquisition in adults and children, respectively. Each transmission route represents a distinct microenvironment and consequently a unique set of factors influencing transmission of selected viral variants. 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. 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. 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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8Department of Microbiology, University of Oslo and Oslo University Hospital, Rikshospitalet, Oslo, Norway.
Single or multivirus transmission event(s) can initiate HIV-1 infections.\textsuperscript{3, 4} 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.\textsuperscript{5, 6, 7} 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.\textsuperscript{8, 9} The founder variants have been shown to reappear as the dominant quasispecies in plasma later during infection.\textsuperscript{10} Diversity of recent heterosexually transmitted variants has been shown to be greater in women than men.\textsuperscript{11} Studies have shown that the use of hormonal contraceptives and sexually transmitted infections increase the likelihood of acquiring heterogeneous variants from a single donor.\textsuperscript{12} Consistent feature of perinatal HIV-1 transmission, which tends to stabilize or even decrease at some point but of minor diversity until the immune responses drive the founder virus to diversify into a quasispecies, closely related viral swarms.\textsuperscript{13, 14} HIV-1 subtype C sequences generally harbor heterogeneous variants, newly transmitted HIV-1 having been shown to reappear as the dominant quasispecies in plasma later during infection.\textsuperscript{15}

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 perinatal 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.\textsuperscript{16} 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) patients.

Table 1. Family Members’ Demography and HIV-1 Clone Characteristics Based on Nucleotide Sequences

<table>
<thead>
<tr>
<th>Parent/sibling</th>
<th>Age (years)</th>
<th>Unique clones</th>
<th>Intrapatient min-max diversity (%)</th>
<th>Intrapatient genetic distance (%)</th>
<th>Family members mean diversity</th>
<th>Interfamilies mean diversity</th>
<th>Entire population diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>205</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>39</td>
<td>3/5</td>
<td>7.7 (0.0–11.6)</td>
<td>4.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>30</td>
<td>3/6</td>
<td>1.5 (0.8-2.0)</td>
<td>1.53</td>
<td>3.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>5.3</td>
<td>3/4</td>
<td>0.0 (0.0-0.0)</td>
<td>0.00</td>
<td>2.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>1.2</td>
<td>2/4</td>
<td>5.7 (0.02–8.1)</td>
<td>3.80</td>
<td>6.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>366</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>39</td>
<td>3/6</td>
<td>10.6 (0.3–14.9)</td>
<td>4.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>38</td>
<td>4/6</td>
<td>3.1 (0.0–6.2)</td>
<td>1.50</td>
<td>6.56</td>
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<td></td>
</tr>
<tr>
<td>1st</td>
<td>5.8</td>
<td>2/5</td>
<td>0.6 (0.0–1.3)</td>
<td>0.25</td>
<td>5.12</td>
<td>6.41</td>
<td></td>
</tr>
<tr>
<td>375</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>F</td>
<td>36</td>
<td>3/5</td>
<td>14.5 (0.03–21.7)</td>
<td>6.95</td>
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<td></td>
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<tr>
<td>M</td>
<td>36</td>
<td>3/6</td>
<td>0.0 (0.0-0.0)</td>
<td>0.00</td>
<td>7.36</td>
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</tr>
<tr>
<td>1st</td>
<td>4.9</td>
<td>1/4</td>
<td>0.0 (0.0-0.0)</td>
<td>0.00</td>
<td>4.77</td>
<td>5.75</td>
<td></td>
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<tr>
<td>567</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>35</td>
<td>2/5</td>
<td>14.4 (0.01–21.6)</td>
<td>8.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>5</td>
<td>2/4</td>
<td>0.3 (0.0–0.7)</td>
<td>0.50</td>
<td>9.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>1.3</td>
<td>3/4</td>
<td>2.23 (0.0–4.5)</td>
<td>1.07</td>
<td>7.94</td>
<td>3.89</td>
<td>16.42</td>
</tr>
</tbody>
</table>

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. 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. 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. 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. 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. Diversity, which is a measure of genetic variation at a given time, was calculated by measuring nucleotide diversity (π) 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.

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 heterosexual 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. 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.

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.
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               |     |     |
| 1st             | 16.9| 16.8|
| 2nd             | 17.2| 15.3|
| 2nd             | 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.
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.45-49 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 infection41,43-45 with women harboring homogeneous env sequences being less likely to transmit per sexual act.45 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.46 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.47 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.48 Di- verse virus population in such studies could be attributed to re-infection 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 C 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.51,57-58 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.60 Coinfection with human simplex virus type 2 (HSV-2) at delivery has been associated with increased intrapartum transmission of HIV-1.61

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.62 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.63 Studies have shown that most HIV-infected infants have a deficiency in cytotoxic T lymphocyte (CTL) responses to HIV,64 and inadequate CD4 T cell help.65,66 Infection of infants with maternal CTL escape variants may further compromise the infant’s ability to contain the virus.67 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 classify- ing subtype C sequences on a geographic basis.73-75 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.76-78 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.

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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)
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: ..............................................

Tsangudzo 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 zvakadai mumakore apfuira. Izvi zvakakosha mumatanho ari kuitwa pasi
rese ekutsvaga mushonga wokudzivirira nekurapa chirwere cheHIV usati wawanikwa pari
zvino. Tinoda kuongorora zvakare kuti kusiyanwa kwemhando dzakasiyana dhwe-
chekuita here pakutapurirwa kungaita utachiwana kubva kuna amai kumwana wake.
Zvichirewa kuti tinoda kuona kuti utachiwana huri muna amai hwakasiyana sei nehuri
mune mwanu wake.

Kuti izvi zviitike ropa rinoita mamiririta matatu (3ml) richange richizotorwa kwamwe
chete nemwana (1) ml kana achinge ainewo utachiwana. Hapana zita renyu
richashandiswa paongororo iyi asi tinoshandisa namba chete. Zita renyu, kero yenyu
pamwe chete nezvimeve zvakabvunzwa pamusoro penyu zvichangwe zvakachengeredzwa.
Vashandi wemuchirongwa vanoongorora ropa renyu havafii vakaziva zvinhu izvi. Kana
musinganzwe zvavakakanaka, pana chiremba wemadzimai nevevana anokwanisa kukuona
imi kana muna wenyu. Vana chiremba ivavo vanogona kukuongorora kuyepi kuti
zvichirewa kuti tinoda kuona kuna amai hwakasiyana sei nehuri
mune mwanu wake. Vana chiremba ivavo vanogona kukuongorora kuyepi kuti
zvichirewa kuti tinoda kuona kuna amai hwakasiyana sei nehuri
mune mwanu wake.

Date  _______  Participant’s Signature _______________________
Date  _______  Interviewer’s Signature _______________________