Title: Understanding the BED Capture Enzyme Immunoassay (CEIA): Measuring HIV-1 incidence in cross-sectional studies

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Declaration

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

Edmore Marinda 28 Day of May 2012

Abstract

Measuring HIV incidence has proved challenging over the years. A number of serological HIV assays have been proposed, and among these, the BED Capture Enzyme Immunoassay (CEIA) is one of the more widely used. Although the assay performs well among known seroconverting panels, it has been shown to classify some long term infected patients as being recently infected. Information on the performance of the BED assay among low CD4 cell count patients and those on anti-retroviral therapy is limited. The risk of onwards transmission of HIV has been reported to be elevated around the seroconversion period compared to the chronic stage of infection. RNA viral load has been reported as the strongest predictor of HIV transmission compared to other HIV markers. Understanding how these markers influence the relationship between the likelihood of being recently infected and the BED assay might help in understanding some of the shortcomings of the BED assay. The main aim of this study was to understand the properties of the BED assay. The performance of the BED assay among advanced HIV disease patients and the influence of ART on BED levels once patients started treatment was investigated. The BED assay and CD4 cell count were used to quantify the risk of *in utero* and intra-partum transmission to their infants among women believed to have seroconverted during pregnancy. The influence of viral load, haemoglobin and mid-upper arm circumference was investigated on the relationship between the probability of being recently infected and BED ODn levels.

Methods

Cryopreserved plasma samples from HIV patients on the national antiretroviral treatment (ART) rollout programme at Tygerberg Hospital HIV clinic, South Africa,

were used to investigate the effect of ART on BED ODn levels once patients commenced treatment. Mixed effect logistic regression models accounting for multiple readings per patient were used.

To investigate the risk associated with seroconversion during pregnancy HIV seropositive women who had just given birth were classified into mutually exclusive groups according to their likelihood of having recently seroconverted using BED and CD4 cell count levels. Multinomial logistic regression models adjusting for other factors were used to assess the risk of MTCT *in utero* and intra-partum infection comparing these groups.

To investigate the relationship between BED ODn levels and the probability of being recently infected, BED data from known HIV infected women and women who seroconverted over a 2 year period was used. Fractional polynomial regression models that allow for non-linear functions to be fitted were used, and the influence of viral load, haemoglobin and mid-upper arm circumference was assessed through multi-variable models. Data from the Zimbabwe Vitamin A for Mothers and Babies (ZVITAMBO) project, a double blinded treatment-placebo trial was used for these last two objectives.

Results

Patients with very low CD4 cell counts were more likely to test false recently infected according to the BED assay than other patients. ART changed BED ODn kinetics among HIV patients on treatment. Over half of advanced disease stage patients were likely to be classified as being recently infected according to the BED assay 2 years into ART treatment.

Women who seemed to have seroconverted during pregnancy had elevated risk of transmitting HIV *in-utero* compared to chronic HIV patients. BED and CD4 cell count were not predictive of risk of intra-partum infections attributed to seroconversion during pregnancy.

The relationship between the probability of being recently infected with HIV and BED ODn levels was described better using Fractional Polynomial regression models than using a linear model in BED ODn or a model in which the BED ODn was categorised. Viral load and haemoglobin were important independent predictors of incident infections.

Conclusions

If the BED assay is to be used for HIV incidence estimations patients on ART should be accounted for. The BED assay together with other HIV serological markers can be used as prognostic tools to assess the risk of HIV transmission.

The risk of *in-utero* transmission of HIV is higher among women who seroconvert during pregnancy. Repeat HIV testing among pregnant women may help in identifying women who seroconvert during pregnancy, and these women will benefit from Prevention of Mother-to-Child transmission (PMTCT) programmes.

It was found that additional markers such as viral load and haemoglobin did not alter the relationship between the probability of having been recently infected and BED ODn.

Keywords: BED, IgG, HIV-1, HIV incidence, *in utero*, intra partum, seroconversion, fractional polynomials

Publications

1. **Marinda ET**, Hargrove J, Preiser W, Slabbert H, van Zyl G, Levin J, Moulton LH, Welte A, Humphrey J. *Significantly diminished long-term specificity of the BED capture enzyme immunoassay among patients with HIV-1 with very low CD4 counts and those on antiretroviral therapy*. J Acquir Immune Defic Syndr. 2010 Apr 1;53(4):496-9. PMID: 20306555

2. **Edmore T Marinda**, Lawrence H Moulton, Jean H Humphrey, John W Hargrove, Robert Ntozini, Kuda Mutasa, Jonathan Levin. In utero *and intra-partum HIV-1 transmission and acute HIV-1 infection during pregnancy: using the BED capture enzyme-immunoassay as a surrogate marker for acute infection*. Int. J. Epidemiol. (2011) 40 (4): 945-954. DOI: 10.1093/ije/dyr055

3. **Edmore Marinda,** Jonathan Levin, Lawrence Moulton, Jean Humphrey, John Hargrove, Robert Ntozini, Kuda Mutasa. *Modelling BED Capture Enzyme Immunoassay (CIEA) in new HIV infections*. Journal: submitted to Statistics in Medicine (01 November 2011)

Dedications:

In loving memory of my late brother Kingston Marinda (04 January 1967 to 02 August 2003) and his late wife, Raika Imedi (15 June 1979 to 18 April 2006).

If only we were not too scared to ask,

If only we were not too scared to know,

If only we were not too quick to judge,

If only yesterday was today,

If only.....

....and to my beings Michael Tinotenda, Kimberly Tafadzwa and Nissy Tawananyasha, and to the one I chose Dorothy for all the love, support, encouragement and patience, and to my parents, Michael and Angeline for believing in me.

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Abbreviations

Ab – Antibodies

AIC – Akaike Information Criterion

AIDS Acquired Immunodeficiency Syndrome

aOR – Adjusted Odds Ratio

ART – Antiretroviral therapy

AZT - Zidovudine

CDC – Centre for Disease Control

CEIA – Capture Enzyme Immunoassay

CHR – Committee on Human Research

CI – Confidence Interval

df – Degrees of freedom

DNA – deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

EIA – Enzyme Immunoassay

ELISA - Enzyme-linked immunosorbent assay

FTC - Emtracitabine

HIV-1 – Human Immunodeficiency virus -1

IDU – Intravenous Drug Use

IgG – Immunoglobulin –G

IgG-3 Immunoglobulin G3

IgM – Immunoglobulin-M

IQR – Inter-quartile range

LL – Log likelihood

$\log_{10}$ – logarithmic base 10

LS – Less sensitive

MGHEC – Montreal General Hospital Ethics Committee

mg/dL – milligrams per decilitre

mm^3 – Cubic millimetres

MRC – Medical Research Council

MTCT – Mother-to-Child-Transmission

MUAC – Mid upper arm circumference

NVP - Nevirapine

ODn – Normalized Optical density

OD – Optical Density

OR – Odds Ratio

PCR – Polymerase Chain Reaction

PHI – Primary HIV infection

PMTCT – Prevention of Mother-to-Child-Transmission

RNA – Ribo Nucleic Acid

sdNVP – Single Dose Nevirapine

SE – Standard error

TDF - Tenofovir

TB – tuberculosis

UNAIDS - United Nations program for HIV/AIDS

VL – Viral load

ZVITAMBO – Zimbabwe Vitamin A for Mothers and Babies

% - Percentage

μL – Micro-litres

χ^2 – Chi-squared test

CHAPTER ONE

INTRODUCTION

1. Introduction

The Human Immunodeficiency syndrome (HIV) epidemic has increased to an estimated 25 million infections worldwide, with 80% of these infections in Sub-Saharan Africa. The United Nations program for HIV/AIDS (UNAIDS) estimated that 2.6 million new HIV-1 infections (range 2.3 – 2.8 million) occurred in 2009 worldwide, translating to 7 119 new infections a day. One million eight hundred thousand (range 1.6 million – 2 million) of these new infections were in Sub-Sahara Africa [1]. Paediatric HIV/AIDs has reversed substantial gains in child survival achieved earlier as a result of successful immunization and other programs. Among breastfeeding populations without anti-retroviral prophylaxis, it was estimated that *in utero* transmission contributed 23%, intra-partum 65% and breastfeeding between 12 – 14% of new HIV infection in infants [2-3]. Vertical paediatric transmission can be reduced to below 4% if current guidelines on anti-retroviral prophylaxis are followed [4]. The 2010 South African guidelines on Prevention of Mother-to-Child transmission of HIV (PMTCT) state that all HIV infected women who are not already on lifelong antiretroviral (ART) treatment should be initiated on Zidovudine (AZT) starting at 14 weeks gestation until the onset of labour. At the onset of labour, these mothers should get Single Dose Nevirapine (sdNVP) and AZT 3 hourly until delivery. The same women get Tenofovir (TDF) and Emtracitabine (FTC), while their infants are initiated on Nevirapine (NVP) for 6 weeks or for the duration of breastfeeding. Mothers who are not on lifelong treatment are assessed at 6 weeks post delivery to determine whether they need lifelong ART. Pregnant women who are already on lifelong treatment continue on their ART regimen during and after delivery, and their infants are put on NVP for 6 weeks after birth [5]. There are still problems however in rolling this out to all eligible pregnant women partly because some women do not

know their HIV status and/or because some women have no access to antenatal services.

Cumulative disease incidence is defined as the proportion of people in a well defined population that acquire the condition of interest in a defined period of time. It is relatively straightforward to test and measure HIV-1 prevalence, which is the cumulative number of cases (both new and old), but measuring incidence is challenging, resulting in scarcity of HIV-1 incidence data. Methods for measuring HIV-1 incidence have largely been unsuccessful or too expensive for the most affected areas. As much as 30% of primary HIV infection remains undiagnosed, thus the extent of new infections may be poorly defined [6-7]. Although changes in prevalence may give indications of trends in HIV-1 incidence, this does not guarantee accurate or reliable results, because HIV prevalence in cohort studies besides reflecting changes in incidence also show changes in other population factors such as migration, deaths and aging as well as survey coverage [8]. A considerable number of Sub-Saharan countries (29 countries), have reported no incidence data at all, while in other countries that including Benin, the Democratic Republic of Congo and Rwanda the data are outdated [9].

Simple and valid ways of estimating HIV incidence such as the use of cross-sectional assays would greatly improve this situation. Sub-Saharan Africa, one of the poorer regions of the world, has the highest burden of HIV-1, making it extremely important that a simple and practical method of identifying recently infected persons is available. This will help in monitoring and controlling the spread of the disease.

1.1 Literature Review

1.1.1 Importance of reliable estimates of HIV Incidence

It is important to get reliable and timely estimates of HIV-1 incidence because information on recent infections is used for estimating HIV-1 trends. The impact of prevention and intervention programs can be evaluated by using information on new infections that occur over the intervention period [10]. Incidence data are required for identifying and selecting appropriate cohorts in preparation for vaccine trials and for microbicide trials [11-14]. A valid methodology for estimating HIV-1 incidence from cross-sectional surveys would be valuable for planning randomised controlled trials; specifically, for doing sample size calculations, as well as obviating the necessity for extended follow-up prior to the trial. Reliable data on the number of new HIV-1 infections is of help in identifying high risk groups, so that targeted educational interventions can be implemented to maximize benefits [10, 15]. There is thus a need to strive to improve HIV intervention programs by combining proven effective methods, as well as the need to identify high incidence populations in which to efficiently evaluate these methods.

Incidence screening methods would provide an entry point for identifying individuals for investigating characteristics of viral subtypes being transmitted. Patho-physiology and/or pharmacotherapy studies require the enrolment of newly infected individuals [10].

Although current HIV-1 laboratory assays are neither sensitive nor specific enough to clinically identify new infection, available assays may be used for screening for high risk individuals, thus minimizing onward transmission. For example HIV incidence

assays that can be applied cross sectionally would have a large impact in public heath surveillance programmes. These would provide the opportunity to get accurate and timely measures of recent transmissions, thus preventive programs can be targeted and evaluated properly. The future extent of the HIV disease can be predicted with better certainty and finite resources can be allocated more appropriately [16]. Correctly diagnosing newly infected individuals mean effective antiretroviral treatment that reduces viral replication following primary infection can be initiated [17-18]. Early treatment is likely to reduce the establishment of tissue virus reservoirs and leads to better prognosis [19-21]. It is however unlikely that in resource constrained settings preference for treatment would be given to newly infected individuals rather than chronic cases that qualify for treatment under existing guidelines.

1.1.2 Methods for measuring/estimating HIV-1 incidence

The 'gold standard' for measuring HIV-1 incidence is through conducting longitudinal follow-up studies where an initially HIV-1 negative cohort is recruited and followed up with regular HIV-1 testing [8, 22-26]. Repeated cross-sectional sero-prevalence studies have been used to estimate HIV-1 incidence, using the rate of change of the sero-prevalence curve over time [27]. Some researchers have used the number of reported AIDS cases in the youngest age groups (13-25 years) as a measure of HIV-1 incidence, because it is thought that these individuals have relatively short incubation time from infection to AIDS [28]. Mathematical models such as the 'backward calculation' model that combines number of AIDS cases with the distribution of the incubation period to infer how many HIV infections occurred during specified time periods have also been used [29-30]. Biological methodologies

such as the ‘capture-recapture’ method have also been proposed, in which individuals are ‘tagged’, repeatedly sampled and tested resulting in identifying initially HIV-1 negative individuals who become positive providing a numerator for incidence estimation [31-32].

The majority of the methods mentioned above require measurements to be taken over a long period of time, which has major implications for both the cost and exact timing of the measurements [33]. Repeated cross-sectional sero-prevalence studies, for example, are logistically difficult to carry out especially in large populations. It would therefore be beneficial to have methods of estimating HIV-1 incidence from cross-sectional surveys. There are a number of laboratory assays that were developed to distinguish new from old HIV infections. These assays define and differentiate the duration (window period) of a transient state related to changing virologic or immunologic response observed in early infection such as the detection of the virus before antibody seroconversion or they detect characteristics in the initial antibody response that differ from those of established infection [30, 34-36].

1.1.3 HIV-1 Incidence Assays

The natural history of the Human Immunodeficiency Virus type-1 (HIV-1) has features that evolve over time which can be used to differentiate recent from long term infections, Figure 1 [35]. In the early stages of primary infection, HIV-1 antigen or nucleic acid is detectable. After seroconversion antibody response evolves and matures, thus antibodies are detectable [30, 35]. The majority of HIV-1 laboratory assays are designed to detect antibodies, to identify antigen, to detect/monitor viral nucleic acids or estimate T-lymphocyte counts. Antigen tests and nucleic acid

detection methods are useful as an early detection tool in the period immediately following contact with an infected person. For HIV-1 diagnosis to be accurate, detection tests should be effective in identifying HIV-1 antibodies, and not antibodies directed to other infectious agents that may be antigenically similar. Antigens used in HIV-1 diagnostic tests should be appropriately specific, and these are normally purified antigens from viral lysates, or antigens produced through recombinant or synthetic peptides technology [10].

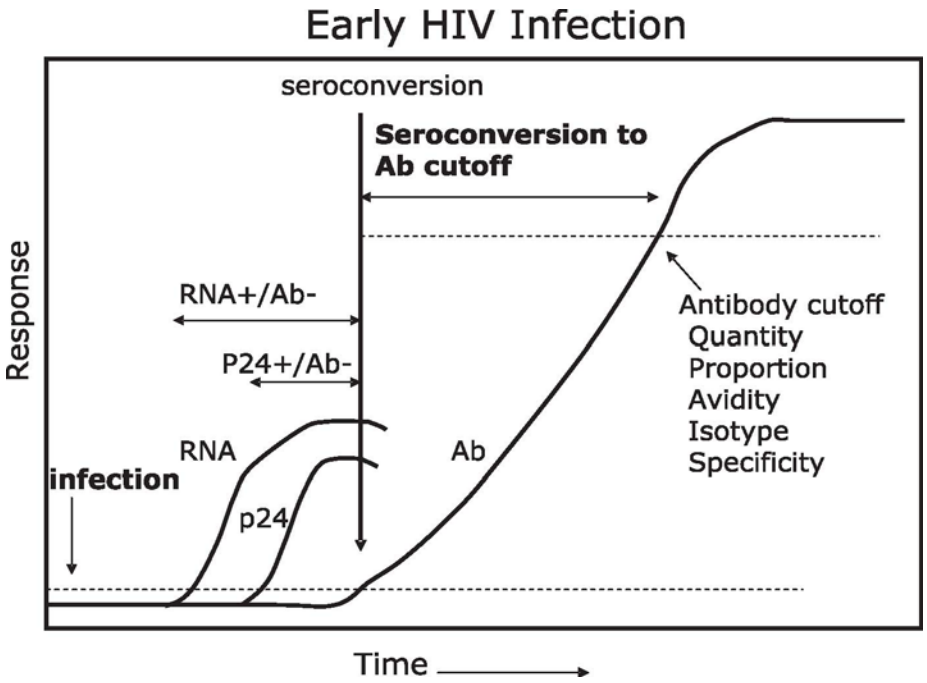

Fig-1:A schematic diagram showing various parameters that define early HIV-1 infection. HIV-1 RNA and p24 detection are useful in pre-seroconversion period while several antibody-based parameters are useful in post-seroconversion period [15].

Table 1 below lists some laboratory assays that have been developed, or are currently under investigation, for measuring HIV-1 recent infection. The assays can be classified into pre-seroconversions and post-seroconversion assays.

1.1.3.1 Pre-seroconversion tests

The detection of HIV-1 RNA or p24 antigen in the absence of specific antibodies (Ab) indicates primary HIV infection (PHI), and this has been used to indicate new infections [19, 21, 37-40]. This acute or primary HIV-1 infection period can be diagnosed by p24 antigen or viral RNA via Polymerase Chain Reaction (PCR - RNA), and during this period symptoms of the acute retroviral syndrome occur [30, 38, 40-42]. The duration during which RNA/p24 appears without antibodies is very short (~ 1-2 weeks), thus limiting the practical use of RNA or p24 in incidence estimation [10, 43]. For this assay to give accurate estimates of HIV incidence, a large number of HIV negative people would have to be tested and/or the incidence rate should be relatively high, more than 5% per year [28]. Testing for HIV-1 primary infection using these techniques is technically complex, expensive, and requires testing of the negative population. This method is thus not suitable for wide scale implementation for determination of HIV-1 incidence [15, 44].

Table 1 Assay for recent HIV infection [15]

Assay	Test for:	Window Interval		
		From	To	duration
HIV - RNA	Plasma RNA	RNA+	seroconversion	~ 19 days
HIV p24	Plasma p24	P24+	seroconversion	~ 14 days
LS-EIAs	HIV Ab titer	seroconversion	Titer cutoff	130 – 170 days
BED - CEIA	HIV Ab/total IgG	seroconversion	Ab proportion cutoff	153 – 187 days
Avidity	HIV Ab avidity	seroconversion	Avidity cutoff	Variable
Affinity	HIV Ab affinity	seroconversion	Affinity cutoff	Variable
IgG3 isotype	IgG3 anti-HIV Ab	seroconversion	Undetectable IgG3 Ab	~ 80 days

LS – EIAs, less sensitive – enzyme immunoassay;
BED – CEIA, BED – capture enzyme immunoassay

1.1.3.2 Post seroconversion test

A number of tests that measure changing levels of antibodies (Ab) have been developed. The tests require relatively few individuals since only HIV-1 infected individuals are tested. They rely on the concept of a 'window period' which is the

time period sero-positive people stay below a pre-defined threshold on the test following seroconversion.

The Less-Sensitive Enzyme Immunoassay (3All -LS EIAs) or detuned assay identifies persons in the early infection period when antibody titre is increasing but before peaking with persistently high antibody response [30, 45]. Among individuals testing positive to a standard ELISA test, tested blood specimens from newly infected individuals are reactive on the sensitive part of 3A11-LS but not on the less sensitive part of the assay, and thus are classified as recently infected. Individuals reactive on both the sensitive 3A11-LS and 3A11-LS less sensitive part are classified as long term infected. The 3A11-LS assay was assessed in subtypes B and E and great variation was found in the window periods for these subtypes. Parekh *et al*. reported that 4% of participants (subtype E) remained below the threshold for recent infection on the LS EIA two years after seroconverting [46]. In the same study 8.7% of AIDS patients were also misclassified as recently infected by the assay. The wide use of the 3A11 LS has been limited because it is not easily available, it needs specialised laboratory expertise and it needs dedicated equipment, and it does not perform as well in non-B subtypes of HIV [46].

Other tests that measure antibody activity have been studied. Measurement of antibody affinity, which is the strength of the reaction between an antigenic determinant and a single combining site on the antibody, is complex and not suitable for high throughput, while the avidity assay which measures the binding strength of antibodies to multiple antigenic determinants on natural antigens have largely remained in-house tools. Immunoglobulin M (IgM) is an antibody isotype that is

detectable early, but it has a short duration and is of variable intensity [47-51]. One study showed detection of IgM in patients with long term infection, which was probably a result of periodic viremia and antigenic stimulation [50], thus making detection of specific IgM an unreliable marker of recent HIV infection.

1.1.3.3 BED Capture Enzyme Immunoassay (BED CEIA)

The BED capture-enzyme immunoassay (BED CEIA) quantitatively reflects the proportion of Immunoglobulin G (IgG) HIV-1 antibody, and this proportion gradually rises and levels off in the first year of infection. The BED assay is performed in a single well, EIA format and, unlike other assays, is suitable for high throughput testing, with up to 96 samples being tested at one time. The IgG BED CEIA is suitable for different HIV-1 subtypes because it was designed to include a peptide with divergent sequences from the immunodominant region of the trans-membrane protein, gp41 [35].

Individuals who are seropositive and test below a given threshold of the BED normalized optical density (ODn) are classified as recently infected and those who test above the threshold are classified as long term infected [35]. The window period of the BED assay, the time following seroconversion after which infections are no longer considered to be recently infected varies with subtype, and is estimated to be between 153 days (for subtype B and E) and 187 days (for subtype C) [35, 52]. An assay such as BED has a reasonably long window period thus providing a useful dynamic range for distinguishing recent from long term infections [35].

Although the BED assay is commercially available and has been widely used, [34, 52-56] it has been shown to overestimate HIV incidence as a proportion of subjects who in fact have long term infections, falsely test positive as being recently infected [34, 36, 52, 57].

Non-progressors on the BED assay are individuals whose proportion of HIV-1-specific IgG never rises above the recency threshold within the window period, while regressors are individuals who despite being HIV-1infected for a long time have BED ODn values that are below the cutoff threshold after having previously progressed above it. Viral suppression due to immune reconstitution once antiretroviral treatment (ART) starts, concurrent infections, which are non-ignorable in the most affected countries, and late-stage HIV disease are some of the suggested biological reasons that may cause BED ODn levels of long term infected patients to fall below the threshold cutoff levels [57].

1.2 Problem statement and Rationale

It has been reported that BED produced HIV incidence estimates were 2-3 times higher than those derived from follow-up of negative cohorts or derived from prevalence surveys [34, 36, 52]. Specimens of 456 individuals who had AIDS from the USA and Thailand were tested using the BED CEIA assay, and 4.38% were classified as being new infections. A further 1.7% out of 178 non AIDS patient who had been infected for more than a year registered as recently infected in the same study. It was observed in this study that RNA viral loads differed by sub-type, with individuals presenting with sub-type E having relatively higher viral load three months after infection than those with sub-type B [35]. In another study in Rwanda

and Zambia individuals misclassified as recently infected by the BED CEIA assay after a year of infection had lower plasma viral load compared to those correctly classified as long term infected (median copies/ml 14 773 versus 93 560; p = 0.02). In the same study clinical presentation and HIV-1 sub-type was not associated with BED misclassification in seroconverter samples [34]. In an intravenous drug usage (IDU) study of 594 individuals in Bangkok, 17.3% [95% CI 12.8 to 24.2] of participants were estimated by BED to have been recently infected in the previous year, almost twice the observed incidence of 9.0 % [95% CI 6.7 to 11.9%] per year [35]. A study in Zimbabwe among women known to have been infected in the first two years post-partum gave BED derived HIV-1 incidence estimates of 4.1 [95% CI 3.3 to 4.9] infections per 100 susceptible persons per year. When the BED assay was used on all sero-positive post-partum women regardless of known timing of infection, HIV incidence was estimated to be 7.6 infections per 100 susceptible persons per year. This estimate was 2.2 times higher than the longitudinal follow-up estimates of 3.5 infections [95% CI 2.8 to 4.2] per 100 person years of follow-up [52].

Serial serological panels of known HIV seroconvertors with known date of seroconversion are used in validating assays such as the BED assay. Optimal cutoffs for defining timing of infection as well as estimating assay window periods are determined from these panels. Only recently infected patients with panel data are used in validating these assays. In the ZVITAMBO study, initially HIV negative post-natal women were followed up and regularly tested for HIV, every 3 months for up to 2 years [52]. The timing of HIV seroconverstion was estimated using the last negative and first positive ELISA tests.

With increased availability of antiretroviral treatment for HIV infected individuals, more and more people are living longer, thus the pool of individuals who could be wrongly classified as recently infected by the BED assay is likely to increase. Anti-retroviral drugs result in immune reconstitution, thus various immunological markers including BED levels may change with ARV treatment. It is important to investigate how ART affects BED ODn levels among patients starting treatment and on ART.

The risk of mother-to-child transmission through breast feeding has been shown to be higher if a woman seroconverts during breast feeding compared to a woman who has been infected for a long time [58]. Seroconversion is associated with high levels of the HIV virus and higher viral load has been reported as a risk factor for all modes of HIV transmission [59-63]. It is not clear if the risk of transmission of HIV from the mother to her unborn child (*in utero* infection) increases if the mother seroconverts during pregnancy. Reported risk factors for *in utero* infection include high viral load and low CD4 cell count among others, but little data is available on the risk of MTCT associated with maternal seroconversion during pregnancy. It is not easy to identify recently infected pregnant women because of infrequent testing during this period.

It is important to understand the mathematical form of the relationship between the probability of being recently infected and the BED ODn, and how this relationship may be affected by other serological markers such as viral load, CD4 cell count and haemoglobin. Slow HIV disease progressors, sometimes referred to as HIV suppressors, have been known to present with low viral loads, but there are few results on the relationship between BED ODn and other markers such as haemoglobin. Understanding the relationship between the probability of recent

infection and BED ODn, and how this relationship may be altered by viral load, haemoglobin, and CD4 cell count may help in understanding and refining new testing algorithms. Understanding the properties of the BED CEIA assay and how the assay is associated with other serological markers of HIV-1 infection may shed light on some of the shortcomings of the assay.

This PhD project aims to investigate the properties of the BED assay. The project investigated the performance of the BED assay among patients on ARV treatment, and how this performance change with length of time on ARVs. The project showed how the BED assay in conjunction with CD4 cell count could be used as a surrogate marker of recent infection in order to investigate whether women infected during pregnancy were at increased risk of transmitting HIV to their infants *in-utero* and intra-partum. The relationship between the BED assay and the likelihood of being recently infected was investigated among HIV infected patients. The effect of other markers viral load, haemoglobin and mid upper arm circumference (MUAC) was explored.

1.3 Aims and Objectives

1.3.1 General Aims

The aim of the study was to understand the association between BED Optical Density (ODn) values and other HIV-1 markers such as viral load, and haemoglobin, as well as how anti-retroviral treatment affects BED ODn values. The project investigated the relationship between BED and other proxies of disease such as mother-to-child HIV-1 transmission. The next three chapters presented as standalone publications address the following three main objectives of the research thesis.

1.3.2 Specific Objectives

1. To investigate the performance of the BED assay in patients with advanced disease and how the assay performs among patient on antiretroviral treatment.
2. To estimate the risk for *in utero* and intra-partum HIV-1 infection by combining the BED CEIA and CD4 cell count.
3. To investigate and describe the functional form of the relationship between the probability of being recently infected and BED values and how this relation is affected by other markers such as viral load, haemoglobin and maternal mid-upper arm circumference.

1.3.3 Methods

1.3.3.1 Study samples and data:

The data used in this research came from the Zimbabwe Vitamin-A for Mothers and Babies (ZVITAMBO) project, Harare, Zimbabwe and from Tygerberg Hospital in Cape Town, South Africa.

1.3.3.2 ZVITAMBO data and samples

The ZVITAMBO Project trial, which is described in detail elsewhere [64], was a large randomized double blinded placebo controlled clinical trial whose main objective was to measure the impact of a single large post-partum dose of vitamin A given to mothers and/or neonates on a number of clinical outcomes; infant mortality and morbidity, Mother-to-Child-Transmission (MTCT), incident HIV-1 infection among women and maternal morbidity among other outcomes. Fourteen thousand, one hundred and ten (14 110) postpartum mothers and their newborn babies were

enrolled within 4 days of delivering a single baby. The study started recruitment at 14 maternity clinics in Harare, Zimbabwe in November 1997 and ended in January 2000. Various methods, including structured questionnaires were used to collect data. Mother-infant pairs were followed up at 6 weeks, 3 months, and then every 3 months for up to 12 or 24 months.

1.3.3.2.1 Maternal Serological samples

Mothers' blood was collected into EDTA and plain tubes at recruitment. Mothers EDTA samples were then stored at ~ 20^{o} C and all other samples at ~ 10^{o} C before being processed within 2 hours of phlebotomy. Maternal plasma and an aliquot of serum were stored at - 70^{o} C. The remaining serum was immediately used to test for HIV-1 using a rapid test [Immuno Chemical Laboratories (ICL) Path Seattle, Washington] and then batched for subsequent ELISA testing. The ICL did not have good enough sensitivity and specificity for diagnostic purposes, but was used to identify probable infected samples so that CD4 cells could be enumerated within 48 hours of phlebotomy (Faccount, Becton Dicknson International, Erembodegem, Belgium). Maternal sera were tested for HIV by two ELISA assays (HIV 1.0.2 ICE: Murex Diagnostic, Edenvale, South Africa, and GeneScreen HIV 1 / 2: Sanofi Diagnostics Pasteur, Johannesburg, South Africa) run in parallel. Discordant ELISA results were resolved by a Western Blot assay (HIV Blot 2.2: Genelabs Diagnostics SA, Geneva, Switzerland). Among mothers who tested HIV positive at recruitment, serum RNA concentration (viral load) was measured (Roche Amplicor HIV-1 Monitor test version 1.5 Roche Diagnostics, Alameda, CA, USA). Haemoglobin was measured at baseline for all women recruited from 1 October 1998 to the end of the study (60% of total samples) on a drop of blood from the EDTA tube using HamoCue

hemoglobinometer (HemoCue, Mission Viejo CA) on the same day of collection. Stored sera for all HIV-1 ELISA positive women were run on the BED IgG (cat. Bo 98003; Calypte Biomedical Corporation, Lake Oswego, OR USA). Results of samples with BED ODn values greater than 1.2 or less than 0.8 were returned as final readings. BED results between 0.8 and 1.2 were rerun. The last result was taken as the final result if the resultant rerun was above 1.2; otherwise the sample was run in triplicate. The median value of the three readings for this third level run was taken as the final reading.

There were 9562 HIV-1 ELISA negative women at recruitment of whom 352 later tested ELISA positive. All sera from these 352 seroconverting women were run on the BED CEIA assay. There were 4595 baseline HIV-1 ELISA positive women in the ZVITAMBO study. BED CEIA was run at baseline, six months and at 12 months for sera from these women who tested positive at baseline.

1.3.3.2.2 Baby Serological samples

Cell pellets (Roche Diagnostics Systems, Alameda, CA) and plasma were prepared from whole blood collected at baseline and follow-up visits and stored at –70°C for babies born to HIV-1 positive women. At the end of all patient follow-up, the last available sample from each infant was tested (pellet by Roche Amplicor version 1.5 qualitative DNA PCR assay [Roche Diagnostic Systems] for samples collected prior to 18 months; serum by GeneScreen ELISA for samples collected after 18 months). If this sample was negative, the child was classified as HIV-negative; if it was positive, then earlier samples were tested to determine timing of infection.

1.3.3.3 Tygerberg data and samples

Plasma samples from 501 HIV-1 infected patients enrolled for the South African

National treatment programme at the Tygerberg Hospital HIV clinic, Cape Town, South Africa, were used to investigate the association between BED and being on antiretroviral therapy (ART). All available samples beginning from just before the initiation of ART and continuing up to 31 months after starting treatment were tested using the BED assay. The testing protocol was similar to that used in the ZVITAMBO study and followed the instructions of the manufacturer of the test kit.

The thesis comprises three papers which are briefly described in the following sections and then presented in detail in chapters 2-4. Chapter 5 summarizes the results of the research and suggests further research on the topic.

1.3.4.1 Paper 1 Significantly diminished long –term specificity of the BED Capture Enzyme Immunoassay among patients with very low CD4 count and those on antiretroviral therapy.

In chapter 2, we investigate the specificity of the BED assay among long term infected patients about to start ART treatment. The performance of the assay is also assessed once these patients commence treatment, using samples collected at 6 months, 12 months, 18 months and at 2 years after initiation of ART treatment. Anti-retroviral treatment has been shown to change specific HIV-1 antibody levels and it is likely that it also affects IgG levels [65-66]. In this study archived samples from patients referred to the Tygerberg hospital in Cape Town, South Africa for ART as part of the South African ART Roll Out program were used. Those presenting with BED ODn values below 0.8 were classified as false recently infected, while those with BED values 0.8 or higher were classified as long term infected. Patients' BED levels were measured over a 2 year period once ART had commenced. Logistic

regression models were fitted using as outcome low vs high BED levels; multiple visits during the two-year follow-up were accounted for by the use of population average mixed effect models, allowing for both random slopes and random intercepts. Viral load and CD4 cell count were used as explanatory variables in addition to time.

1.3.4.2 Paper 2 *In utero* and intra-partum HIV-1 transmission and acute HIV-1 infection during pregnancy: Using the BED Capture Enzyme Immunoassay as a surrogate marker for acute infection

Primary HIV-1 infection may increase the risk of HIV-1 transmission to the unborn child both during pregnancy (*in utero*) and during delivery (intra-partum). Baseline data for women who tested HIV-1 positive from the ZVITAMBO Project was used for this paper. To compare the risk of *in utero* and intra-partum infection between recent infection and chronic infection, we fitted both unadjusted and adjusted multinomial logistic regression models, with an outcome variable with three levels namely *in utero*, intra-partum transmission or PCR negative at 6 weeks. Women were classified into 6 distinct exposure groups using two BED ODn cut offs (< 0.8 and ≥ 0.8) and three CD4 cell counts cut offs (< 200, $200 – 350$ and ≥ 350). This paper investigated how the BED assay can be applied to stored samples in order to provide further evidence of the increased risk of MTCT when a woman is infected during the course of her pregnancy.

1.3.4.3 Paper 3 Modelling the BED Capture Enzyme Immunoassay (CEIA) in new HIV infections

In chapter 4, we investigate the functional form of the relationship between the probability of being classified as recently infected and the BED ODn readings. Fractional polynomial (FP) models are found to be useful in modelling this relationship [67-68]. The impact of including RNA viral load, haemoglobin and mid-upper arm circumference on this relationship is also investigated. The data used to answer this question came from the ZVITAMBO project, (described above and in the paper). Briefly, women who tested HIV-1 positive at the time of recruitment and women who seroconverted during the post-partum follow-up period were considered for the study. Two populations of women were used in the analysis, with the population being defined by the degree of certainty in classifying the women as either recent seroconvertors or long term-infected respectively. To be included in either population, women had to have complete data on BED, viral load, haemoglobin and MUAC; complete data was required to enable the comparison of different statistical models. We used the logit link, with baseline positive women classified as long term infected (coded 0), and known seroconverting women as recently infected (coded 1). Three logistic regression models where used; the logit of recent infection fitted on:

1. Linear regressors BED, viral load, haemoglobin and MUAC. In this model we fitted:

$$logit(p_i) = \beta_0 + \beta_1 * ODn_i + \beta_2 * VL_i + \beta_3 * HB_i + \beta_4 * MUAC_i$$

   where $i = 1$ to n indexes the women used in the analysis and ODn denotes BED ODn, VL denotes viral load, HB denotes the haemoglobin and MUAC denotes the mid-upper arm circumference, all fitted as continuous variables.

2. Regressors BED, viral load, haemoglobin and MUAC were fitted in the logistic regression model as categorized variables, i.e.

$$logit(p_i) = \beta_0 + \sum_{j=1}^{r-1} \beta_j * ODn_{ji} + \sum_{k=1}^{s-1} \beta_k * VL_{ki} + \sum_{l=1}^{t-1} \beta_l * HB_{li} + \sum_{m=1}^{u-1} \beta_m * MUAC_{mi}$$

for i = *1* to *n*, and *j = 1* to *(r -1)* for *(r-1)* BED ODn dummy variables, *k = 1* to *(s – 1)* for *(s – 1)* dummy variables for viral load, *l = 1* to *(t – 1)* for *(t – 1)* haemoglobin dummy variables, and *m = 1* to *(u – 1)* for *(u – 1)* dummy variables for MUAC.

3. All regressors, BED ODn, viral load, haemoglobin and MUAC were allowed to take any form including non-linear forms using fractional polynomials. In the model the linear part of the *logit(p_i)* is allowed to assume a nonlinear function;

$$\eta_i(X; \boldsymbol{\beta}; \boldsymbol{p}) = \beta_o + \beta_1 X^{p_1} + \beta_2 X^{P_2} + .. + \beta_r X^{P_r}$$

where β_i are regression parameters, $X^{p_i} = X^{p_i} log_e(X)$ if $p_j = p_{j-1}$ or X^{pj} *if* $p_j \neq p_{j-1}$, and X^p denote the Box-Tidwell transformation defined as X^p if $p_j \neq p_{j-1}$ and $X^0 \equiv log_e(X)$ [69]. A restricted list of integers and non-integers: (-2, -1,-0.5,0,1,0.5,1,2,3) such that $p_1 < ... < p_r$, have been proposed for values of p_i , and these have been shown to be sufficient to cover a wide range of linear (when p = 1) and non-linear functions commonly encountered in medical epidemiology [67]. A wide range of link(***η***) functions that include the identity, logit, probit, poisson, negative binomial among others can be fitted. The logit link was used in this study. The fitted probability of being recently infected was obtained from the expression:

$$\hat{p}i = \frac{e^{\boldsymbol{X_i'}\hat{\boldsymbol{\beta}}}}{1 + e^{\boldsymbol{X_i'}\hat{\boldsymbol{\beta}}}}$$

where $\boldsymbol{X}'$ is a row vector of regressors for the i^{th} observation and $\hat{\beta}$ is a vector of fitted coefficients. Univariable models for outcome recent and regressor BED and multivariable models adjusted for viral load, haemoglobin and MUAC were fitted.

All models were fitted using maximum likelihood methods. The likelihood ratio test and the Akaike Information Criterion (AIC) were used to choose between linear, categorical and fractional polynomial models [70]. Model fit was assessed using the Hosmer-Lemeshow Goodness-of-Fit test [71].

1.3.5 Statistical methods and Data Management

ZVITAMBO data were stored in Visual Dbase, a relational database that links laboratory information with follow-up data. Additional data from Tygerberg were stored in MicroSoft Excel. Data cleaning was carried out in both Visual Dbase and during analysis. Data analysis was carried out using STATA release 10 (STATACORP version 10; College Station, TX). In general estimates were reported with 95% confidence intervals, and where statistical tests were done, the 5% significance level was used.

1.3.6 Ethics

The ZVITAMBO Project obtained ethical clearance from the Medical Research Council of Zimbabwe, Johns Hopkins Bloomberg School of Public Health Committee on Human Research, and Research Ethics Committee of the McGill University Health Centres (CHR#H.22.96.06.17.B , REC#96-064, MRCZ/A). Ethical approval for the Tygerberg study was granted by the Committee for Human Research of the Faculty of Health Sciences, Stellenbosch University (project number N07/06/137). The current study obtained ethical clearance from the University of the Witwatersrand's Postgraduate Ethics Committee on Human research (clearance number M080224). In both studies patients provided written signed informed consent, and confidentiality was maintained as per standard laboratory protocols.

CHAPTER TWO

SIGNIFICANTLY DIMINISHED LONG-TERM SPECIFICITY OF THE BED CAPTURE ENZYME IMMUNOASSAY AMONG PATIENTS WITH HIV-1 WITH VERY LOW CD4 COUNT AND THOSE ON ANTIRETROVIRAL THERAPY

Significantly Diminished Long-Term Specificity of the BED Capture Enzyme Immunoassay Among Patients With HIV-1 With Very Low CD4 Counts and Those on Antiretroviral Therapy

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Objective: To estimate the proportion who test as *recent* infections by the BED capture enzyme immunoassay (BED) among patients about to commence, and those receiving, antiretroviral therapy.

Design: Cryopreserved plasma samples from HIV patients on the national antiretroviral treatment (ART) rollout program at Tygerberg Hospital HIV clinic, South Africa, were tested using the BED assay.

Participants: Five hundred five patients qualifying for ART were included in this study.

Method: All plasma samples from each patient were tested by BED. Basic demographic data, HIV-1 viral load, and CD4 count results were obtained from the laboratory database.

Main Outcome: The proportion presenting as false recently infected is reported.

Results: Among patients, with presumed long-term HIV-1 infections, about to commence ART, 11.2% [95% confidence interval (CI): 8.3 to 14.5%] tested recent by BED. The proportion was higher among patients with CD4 counts <50 cells per microliter [odds ratio 2.63, 95% CI: 1.39 to 5.00] and log10 HIV-1 viral load less than 4 [odds ratio 3.03, 95% CI: 1.05 to 9.09]. Proportions testing false recent increased from 11.2% before ART to 17%, 25%, 38%, and 56% at 0.5, 1, 1.5, and 2 years, respectively, after ART initiation.

Conclusions: If the BED method is to be used for the accurate estimation of HIV incidence from cross-sectional surveys, it will be essential, before other statistical adjustment methods, to identify, at least, all cases who are on ART and all those with CD4 counts < 50 cells per microliter. The more general remaining problem is the unequivocal identification of all persons with long-term HIV infections.

Key Words: BED, HIV-1, HIV incidence, IgG, specificity

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INTRODUCTION

Numerous attempts have been made to measure HIV-1 incidence in a timely and inexpensive manner.[1–8] Although some success has been achieved, obstacles still remain to widespread use of these methods, especially in the developing world, where such methods and technologies are most urgently required.[1,9] The most reliable way of measuring HIV incidence is following up HIV-uninfected but susceptible cohorts, measuring new infections over specified time periods. This, however, is subject to a number of biases, is expensive, and involves following up large numbers of people over long periods of time.[9–11]

Accurate and timely measures of HIV incidence would provide ways of evaluating impacts of interventions, estimating epidemiological trends in HIV studies, and providing baseline measures at the start of clinical trials.[4,10,12] Several laboratory assays have been proposed for estimating HIV incidence, with the BED capture enzyme immunoassay (henceforth BED; Calypte Biomedical Corporation, Rockville, MD) being one of the most promising. The assay measures the changes in anti-HIV-1 immunoglobulin G (IgG) activity relative to total IgG and is typically used to classify individuals as HIV-1 *recently* infected or *long term* infected depending on whether a normalized absorbance reading lies below or above a preset optical density cutoff point (*C*).[4,13] The

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time taken post-seroconversion for the BED's optical density to exceed *C* is termed the window period. The assay is easy to perform, does not need specialized equipment, and has inbuilt quality control.[4,13] Several analytical methods have been proposed to infer incidence from the distribution in a cross-sectional survey of HIV-negative, recently infected, and long-term infected individuals. These methods systematize, under different assumptions, the intuitive idea that a cross-sectional survey finding a large fraction of *recently infected* individuals is indicative of a high incidence. Studies in the United States, Asia, and Africa have however shown that estimates produced using this assay are 2 to 3 times higher than those obtained using prospective follow-up data.[4,10,11,14] This is essentially a consequence of the proportion of individuals who test as recently infected, despite being HIV-1 positive for more than twice the window period. A proportion of these are patients who remain below the target threshold of the assay for all times. Others may *revert* to being misclassified as recently infected when they reach end-stage AIDS or on antiretroviral treatment (ART).[15] We studied the latter problems by estimating the proportion testing as recent infections by BED among patients immediately before, and while they were undergoing, ART.

MATERIALS AND METHODS

Study Population

We used cryopreserved plasma samples from HIV-infected (predominantly clade C) patients referred for ART to the South African national rollout program at the Tygerberg Hospital HIV clinic, Western Cape province, South Africa.

Most of the Tygerberg patients did not have evidence of previous positive HIV test results. To qualify for ART, HIV-seropositive patients had to have a CD4 count of less than 200 cells per microliter or had to suffer from an AIDS-defining condition, both of which almost invariably imply long-standing HIV infection. We therefore consider it safe to assume that patients qualifying for inclusion in the ART rollout program, that is, fulfilling the above-cited criteria, have been infected for several years, that is, more than twice the BED assay's window period.[11,14]

All patients were assumed to have been ART naive before enrollment at Tygerberg. Routine laboratory diagnosis at the time included measurement of HIV-1 viral load (i.e., quantitative HIV-1 RNA in plasma using the NucliSens EasyQ HIV-1 V1.1 system; bioMérieux bv, Boxtel, the Netherlands) and CD4 count at baseline, that is, when enrolling for ART. Once enrolled on ART, patients are normally monitored for HIV-1 viral load and CD4 count at 6-month intervals. Residual EDTA plasma specimens left over after routine HIV-1 viral load testing has been performed are routinely stored in the −20°C specimen cryobank at the Division of Medical Virology, National Health Laboratory Service Tygerberg.

Inclusion criteria for our study were being on ART and at least 1 residual specimen being available in the cryobank. All specimens were collected between January 14, 2004, and August 3, 2006. Wherever available, serial samples were selected; the first one taken at enrollment for ART and later ones from the same patient at subsequent clinic visits. For follow-up samples, the minimum duration of HIV infection is determined by the time since the baseline sample. Ideally, we tried to select samples at 12- and 24-month follow-up intervals. However, due to suboptimal adherence to follow-up schedules, many follow-up samples were obtained at intermittent intervals, that is, 6 and 18 months.

All available cryopreserved plasma samples from each patient were tested by the BED assay. Basic demographic data and HIV-1 viral load and CD4 count results were retrieved from the laboratory database. Viral load results were log10 transformed.

BED Testing

Cryopreserved plasma samples were thawed and then immediately tested by the Calypte HIV-1 BED Incidence EIA (IgG-capture HIV-EIA; BED-CEIA) strictly following the manufacturer's instructions (cat. no. 98003; Calypte Biomedical Corporation, Lake Oswego, OR).

BED measures levels of HIV-1–specific IgG antibodies as a proportion of total serum IgG antibodies; it detects recent seroconversion through low levels of HIV-1–specific IgG. Specimens with normalized optical density ($ODn = OD_{specimen}/OD_{calibrator}$) of less than 0.8 are classified as recent infections and those with values of 0.8 or more as long-term infections. According to the test kit insert, all samples with ODn values of 1.2 or less must be retested in triplicate (confirmatory testing); the median ODn value of the 3 repeats is then used to classify the sample as recent (if ODn <0.8) or long term (if ODn ≥ 0.8).

We retested the first 81 samples with initial ODn values ≤ 1.2. Of these, 47 had ODn values of ≤ 0.8 and 34 had ODn values >0.8 and ≤ 1.2. Retesting did not change classification in 70 of the 81 samples (86.4%). Four samples were classified by retesting as long term despite an initial ODn ≤ 0.8 and 7 as recent despite an initial ODn >0.8. The observed high level of concordance between the initial and the confirmatory test results is in line with the results of a previous evaluation by Dobbs et al.[13] We therefore decided to abandon retesting for the remainder of the study as it resulted in negligible difference on a population basis.

Statistical Methods

Data were entered into Microsoft Excel and exported to STATA (STATACORP version 10; College Station, TX) for analysis. Ninety-five percent confidence intervals (CIs) are reported for all estimates. We used 5% significance levels for all comparisons. The rate of false recent was estimated as a percentage with 95% binomial CI. We used 2-tailed *t* tests or nonparametric equivalents to compare BED recent versus BED long term infected for continuous variables and χ^2 or Fisher exact tests for categorical variables.

To investigate factors associated with false-recent BED status before commencement of ART, multiple logistic regression models were used. To investigate trends in BED ODn values over the treatment period, we fitted random effect multiple logistic regression models to account for repeated readings for individuals over time.

Ethics and Confidentiality

Ethics approval was granted by the Committee for Human Research of the Faculty of Health Sciences, Stellenbosch University (project number N07/06/137) and the University of the Witwatersrand's Postgraduate Ethics Committee on Human research (clearance number M080224). Confidentiality was maintained as per standard laboratory protocol.

RESULTS

In total, 1061 samples collected from 505 patients were analyzed with the BED assay. Not all patients had BED readings at the first visit. There were 430 baseline BED ODn readings and 433, 127, 31, and 9 readings at 0.5, 1, 1.5, and 2 years of follow-up, respectively. Of the 505 patients in the study (median age 33 years), 132 (31%) were male (median age 37 years, range 22–79 years) and 298 (69%) were female (median age 32 years, range 18–58 years).

Proportion Testing as Recent HIV-1 Infections Just Before Commencing ART

Univariate analysis provided an overall BED false-recent rate of 11.2% (95% CI: 8.3 to 14.5%), independent of patient gender and age (P = 0.67 and 0.16, respectively). Among patients with CD4 counts below 50 cells per microliter, 18 of 89 (20.2%, 95% CI: 12.4 to 30.1) tested recent by BED compared with 30 of 341 (8.8%, 95% CI: 6.0 to 12.3) of those with higher CD4 counts (P = 0.002 for the difference).

In multivariate analysis with independent variables log10 viral load (stratified above and below 4) and CD4 count (stratified above and below 50 counts/μL), patients with low CD4 and low viral loads were 2.9 (95% CI: 1.5 to 5.6) (P = 0.01) and 3.8 (95% CI: 1.39 to 11.11) (P = 0.001) times more likely to test BED false recent than those with high CD4 and high viral loads, respectively.

Performance of the BED Assay When ART Commences

In univariate analysis, the percentage of patients testing recent by BED increased from 11.2% pre-ART to 17%, 25%, 38%, and 56% at 0.5, 1, 1.5, and 2 years, respectively, after treatment commenced (Fig. 1).

After adjusting for CD4 count, stratified as above, the odds of testing recent by BED, compared with the baseline visit, were 2.1 (95% CI: 1.6 to 2.9), 3.5 (95% CI: 2.2 to 5.7), 6.8 (95% CI: 3.0 to 15.7), and 12.2 (95% CI: 3.2 to 46.1) at visits 0.5, 1, 1.5, and 2 years, respectively, after ART initiation. The wide CIs for estimated odds ratios are a consequence of sparse data for later time-points. Low CD4 count (below 50), but not viral load, remained predictive of testing recent in a random effect multiple logistic regression model.

DISCUSSION

Previously published analysis of data from the AIDSVAX B/B vaccine trial and Zimbabwe Vitamin-a for Mother and Babies Project (ZVITAMBO) studies showed that, if the BED were to be at all useful for estimating HIV incidence, it would be necessary to adjust for a proportion of patients infected with HIV-1 for long periods of time (much in excess of 1 year) who nonetheless continued to test as recent infections by BED.[11,14] In these 2 studies, there were, however, few cases with CD4 counts below 50 cells per microliter and nobody was on ART.

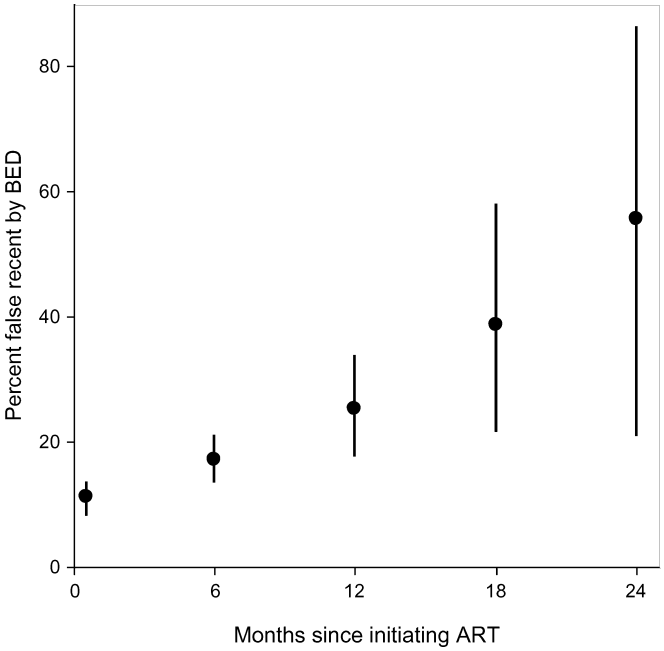

FIGURE 1. Percent false recent by BED as a function of time since initiating ART. Vertical bars indicate 95% CI.

The present study shows that the proportion testing recent increased significantly among pre-ART clients with CD4 counts below 50 cells per microliter, and even more sharply with time spent on ART, its value being significantly raised only after 6 months on ART. This poses serious problems because BED incidence estimates are very sensitive to changes in the proportion of long-term false-recent cases.[14] Moreover, as previously observed, patients with a log10 viral load of 4 or less than 4 (i.e., <10,000 viral copies/μL) were more likely to test false recent than those with higher viral loads.[16] If the BED method is to be used for the accurate determination of HIV incidence from cross-sectional surveys, we must be able to identify, and classify as long-term infections, cases who are on ART and/or have extremely low CD4 counts. Failure to do so will lead to unknown, but probably very large, errors in incidence estimates.

It is unclear why differences exist, between studies in the same region and among people infected with the same clade of HIV-1, in the proportion of long-term HIV-1 infections who continue to test recent by BED. Suggested factors influencing BED recency threshold include viral suppression, immune reconstitution on ART, concurrent infections, and late-stage HIV disease. Participants in the ZVITAMBO study were generally free of life-threatening conditions at recruitment (only 15/4495 HIV-1–positive individuals presented with CD4 count of less than 50 cells/μL), and a substantial number of patients with AIDS-defining conditions were thus excluded.[14]

In the Tygerberg study, conversely, patients either had to have a CD4 counts below 200 cells per microliter and/or an AIDS-defining condition to qualify for ART. Depending on the stage of the HIV epidemic in the population, patients at the AIDS stage are expected to be relatively fewer than those at other stages. The false-recent proportion of 11.2% for all patients, or 8.8% for patients with CD4 counts of 50 cells per microliter or above, in the Tygerberg study probably overestimates the true false-recent ratio among long-term HIV-1 infections in the population. (This problem would have been exacerbated if our unverifiable assumption that all patients recruited into this treatment center were ART naive was violated.) Conversely, because ZVITAMBO excluded AIDS patients, it is possible that the 5.2% figure from that study underestimated the true proportion in the female population.

By contrast, the Africa Center study reports a false-recent proportion of 1.7% among clients known to be infected with HIV-1 for more than 1 year, much lower than other similar clade C estimates.[17] The study is one of the few that covers the whole spectrum of HIV-infected individuals: both sexes, different age-groups, and individuals at different HIV disease stage. Further studies are clearly indicated to discover the basis of the variability in the proportion of false-recent infections between populations. Such studies must be based on appropriate sample sizes and carried out in a variety of situations to assess variations with geography, HIV subtype, ART treatment, and other demographic factors. All factors that potentially influence BED ODn threshold levels, such as CD4 count, viral load, ART, and coinfections, should be investigated and appropriately used to aid in the estimation of robust HIV incidence estimates.

Algorithms that combine results of more than 1 test, either sequentially or in parallel, thus improving predictive values of the assay, could be used to improve incidence estimates.[1] But such protocols and the use of clinical information to improve assay performance are unlikely to be feasible in resource-constrained setting. The essential problem in using the BED method to estimate HIV incidence thus continues to lie in the lack of unequivocal identification of persons with long-term HIV infections. If some alternative test can be developed to achieve this end, it could be used with or without other assays to render the statistical corrections unnecessary, and this should be a primary research aim.[11]

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CHAPTER THREE

IN UTERO AND INTRA-PARTUM HIV-1 TRANSMISSION AND ACUTE HIV-1 INFECTION DURING PREGNANCY: USING THE BED CAPTURE ENZYME-IMMUNIOASSAY AS A SURROGATE MARKER FOR ACUTE INFECTION.

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In utero and intra-partum HIV-1 transmission and acute HIV-1 infection during pregnancy: using the BED capture enzyme-immunoassay as a surrogate marker for acute infection

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Objective The BED assay was developed to estimate the proportion of recent HIV infections in a population. We used the BED assay as a proxy for acute infection to quantify the associated risk of mother-to-child-transmission (MTCT) during pregnancy and delivery.

Design A total of 3773 HIV-1 sero-positive women were tested within 96 h of delivery using the BED assay, and CD4 cell count measurements were taken. Mothers were classified according to their likelihood of having recently seroconverted.

Methods The risk of MTCT *in utero* and intra-partum was assessed comparing different groups defined by BED and CD4 cell count, adjusting for background factors using multinomial logistic models.

Results Compared with women with BED $\geqslant$ 0.8/CD4 $\geqslant$ 350 (typical of HIV-1 chronic patients) there was insufficient evidence to conclude that women presenting with BED < 0.8/CD4 $\geqslant$ 350 (typical of recent infections) were more likely to transmit *in utero* [adjusted odds ratio (aOR) = 1.37, 96% confidence interval (CI) 0.90–2.08, $P = 0.14$], whereas women with BED < 0.8/CD4 200–349 (possibly recently infected patients) had a 2.57 (95% CI 1.39–4.77, P-value < 0.01) odds of transmitting *in utero*. Women who had BED < 0.8/CD4 < 200 were most likely to transmit *in utero* (aOR 3.73, 95% CI 1.27–10.96, $P = 0.02$). BED and CD4 cell count were not predictive of intra-partum infections.

Conclusions These data provide evidence that *in utero* transmission of HIV might be higher among women who seroconvert during pregnancy.

Keywords BED, CD4, *in utero*, intra-partum, seroconversion, HIV

Introduction

Primary infection[1–8] is characterized by elevated viral load[9–18] which is a strong risk factor for sexual transmission[15] and all modes of mother-to-child transmission (MTCT) of HIV.[19] We recently reported data from the ZVITAMBO vitamin A trial demonstrating that breastfeeding-associated HIV transmission was 2.9–4.6 times higher among mothers who seroconverted post-natally compared with mothers who were already infected at delivery. Among 17 mothers who were seroconverted during delivery (i.e. their blood tested negative by HIV ELISA but positive by HIV RNA PCR), 75% of their infants died or became infected by 9 months post-partum.[20] Thus within ZVITAMBO, maternal seroconversion during the intra-partum and post-natal periods was associated with high risk of MTCT.

Reports have been inconsistent, however, regarding whether maternal seroconversion during pregnancy increases *in utero* MTCT.[21–24] It is important to understand whether primary HIV infection during pregnancy increases transmission risk: firstly, because the risk of HIV acquisition may be higher during pregnancy than non-pregnancy;[25–27] secondly, to strengthen HIV prevention messages targeting reproductive-aged couples; and lastly, to determine whether repeat HIV testing and screening of antenatal women is warranted.

Within the ZVITAMBO trial we previously noted that women testing HIV-negative were younger and were more educated than those testing HIV-positive. Moreover, we noted that among HIV-positive women, those who transmitted *in utero* were younger, were more educated and also had a higher CD4 cell count than those who transmitted intra-partum or post-natally.[28] We hypothesized that the group that transmitted *in utero* may have included a large number of women who seroconverted during pregnancy.

We used the BED capture enzyme-immunoassay (henceforth BED), a technically simple and relatively cheap assay, to measure plasma concentration of HIV-specific antibodies (IgG) that increase with time following primary infection. Individuals presenting with 'low' (<0.8) normalized optical density (ODn) values on BED are likely to have been infected within the past half year.[29] Although the BED assay was designed to estimate the proportion of recently acquired HIV at the population level, in this analysis we used BED in combination with CD4 cell count as a proxy for acute infection to distinguish HIV-positive women who were likely to have acquired HIV during pregnancy from chronically infected women. We then compared *in utero* and intra-partum transmission rates between these groups to estimate the risk of *in utero* and intra-partum MTCT associated with primary HIV infection during pregnancy.

Methods

The ZVITAMBO project protocol and primary outcomes have been reported elsewhere.[28–31] In brief, 14 110 mother–infant dyads were enrolled within 96 h of delivery between November 1997 and January 2000. Mother–infant pairs were eligible if both were free of acutely life-threatening conditions, the baby was a singleton with birth weight $\geqslant 1500$ g and the mother planned to stay in Harare after delivery. Written informed consent was obtained.

Baseline data were collected by interview, medical record transcription or direct measurement. Gestational age was estimated using the Capurro method.[32] Infant birthweight and maternal mid-upper arm circumference (MUAC) were measured using published methods.[33] Mother–infant pairs were followed up at 6 weeks, 3 months and then every 3 months to 12 or 24 months. The trial preceded availability of HIV testing and anti-retroviral prophylaxis for antenatal women in Harare public sector facilities.

Laboratory procedures

At baseline, all mothers were tested for HIV using an algorithm that included two parallel enzyme-linked immunosorbent assays [HIV 1.0.2 ICE (Murex Diagnostics); GeneScreen HIV 1/2 (Sanofi Diagnostics Pasteur)] and Western blot [HIV Blot 2.2; (Genelabs Diagnostics)] where results were discordant. Haemoglobin (Hb) was measured for women enrolled from 1 October 1998 onwards (~60% of the total sample) (HemoCue, Mission Viejo, CA, USA). For HIV-positive women, CD4 cells were enumerated (FACsCount; Becton Dickinson) and plasma assayed by the Calypte HIV-1 BED Incidence EIA (BED-CEIA), (cat. No. 98003; Calypte Biomedical Corporation, Lake Oswego, OR, USA).

From infants born to HIV-positive mothers, cell pellets (Roche Diagnostics Systems, Alameda, CA, USA) and plasma were prepared from whole blood collected at baseline and follow-up visits and stored at –70°C. Following all patient contact, the last available sample from each infant was tested [pellet by Roche Amplicor version 1.5 qualitative DNA PCR assay (Roche Diagnostic Systems) for samples collected prior to 18 months; serum by GeneScreen ELISA for samples collected after 18 months]. If this sample was negative, the child was classified as HIV-negative; if it was positive, then earlier samples were tested to determine timing of infection.

The ZVITAMBO trial was approved by the Medical Research Council of Zimbabwe (MRC-Z), the Medicines Control Authority of Zimbabwe, the Johns Hopkins Bloomberg School of Public Health Committee on Human Research (CHR) and the Montreal General Hospital Ethics Committee (MGHEC). BED analysis of archived specimens was approved by MRC-Z, CHR, MGHEC and CDC Program Ethics Review Board, whereas the

University of the Witwatersrand Human Research Ethics Committee (Medical) approved the current research.

Statistical analysis

Infant HIV infection groups

HIV-exposed children were classified into one of three HIV status groups: (i) *in utero* infected (infant-tested PCR-positive at baseline), (ii) intra-partum infected (infant-tested PCR-negative at baseline and PCR-positive at 6 weeks) and (iii) not infected at 6 weeks (infant-tested PCR-negative at 6 weeks or later).[34]

Maternal likelihood of recent seroconversion

We used the BED assay together with CD4 cell count to categorize HIV-1-positive women according to their likelihood of having acquired HIV during pregnancy. Recently-infected individuals are likely to present with low BED values (ODn < 0.8)[29] and relatively high CD4 cell counts, Figure 1.[35] People with advanced stage HIV-1 may also present with low BED readings,[36] but they usually have low CD4 cell counts. On fitting multinomial logistic regression models using three outcome levels, it was found that there was a significant interaction term (P-value $= 0.03$) between BED and CD4 cell count as continuous variables. We categorized women according to their BED and CD4 cell count values informed by this significant interaction term and the natural history of HIV infection as illustrated in Figure 1.[35]

Women were classified into one of six discrete groups according to their BED ODn and CD4 cell count interpreted as the following HIV duration and severity:

(i) BED $\geqslant 0.8$/CD4 $\geqslant 350$ cells/μl (characteristic of chronic asymptomatic infection).
(ii) BED $\geqslant 0.8$/CD4 200–349 (characteristic of chronic intermediate stage infection).
(iii) BED $\geqslant 0.80$/CD4 < 200 (characteristic of chronic end-stage infection).
(iv) BED < 0.8/CD4 $\geqslant 350$ (characteristic of recent infection).
(v) BED < 0.8/CD4 200–349 (characteristic of the short interval of primary infection when CD4 cell count falls precipitously as illustrated in Figure 1).
(vi) BED < 0.8/CD4 < 200 (characteristic of very severe end-stage infection where BED declines due to depressed antibody production), Table 3.

The primary comparison was between group 1 and each of groups 4 and 5. We performed sensitivity analysis by combining groups (1 and 2) and (1, 2 and 3), and comparing these new groups with combined group (4 and 5). We assessed the effect of missing CD4 cell count data by comparing models with

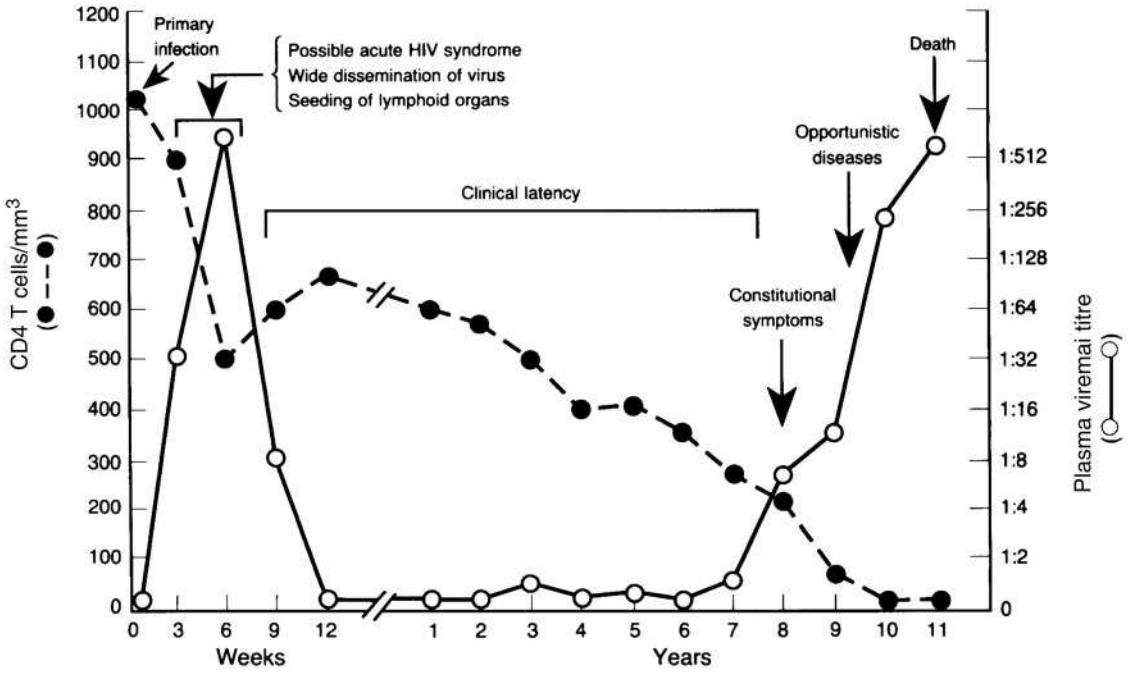

Figure 1 Relationship among peripheral blood $CD4^+$ T-cell count, plasma viremia and clinical disease progression. During the early period after primary infection, there is widespread dissemination of virus and a sharp decrease in the number of CD4 T cells in peripheral blood. An immune response to HIV ensues, with a decrease in detectable viremia followed by a prolonged period of clinical latency. The CD4 T-cell count continues to decrease during the following years, until it reaches a critical level below which there is a substantial risk of opportunistic diseases. (From Pantaleo G, Graziosi C, Fauci AS. The immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med* 1993;**328**:327–35, with permission.)

complete data with models where CD4 was imputed.[37] Maternal age, MUAC, Hb and plasma viral load were used to estimate for missing CD4 cell counts.

The data were analysed in STATA release 10 (STATACORP version 10; College Station, TX). Baseline characteristics were compared across infant infection status categories using ANOVA for continuous variables, except in the case of extreme non-normal variables where a Kruskal–Wallis test was used. Chi-squared tests were used for categorical variables. To compare the risk of *in utero* and intra-partum infection between recent infection and chronic infection, we fitted both unadjusted and adjusted multinomial logistic regression models, using three outcome levels namely *in utero*, intra-partum or PCR-negative at 6 weeks. We adjusted for maternal factors: Hb level, MUAC, age, education, mode of delivery and duration from rupture of membranes to delivery. We also adjusted for the infant's gender, gestational age and birthweight. We did not adjust for maternal viral load since the main question of interest was whether *in utero* or intra-partum transmission was associated with incident infection during pregnancy. Estimates were reported with 95% CIs.

Results

A total of 4495 women enrolled in the ZVITAMBO trial tested HIV positive at baseline; 382 and 508 of their neonates were infected during the *in utero* and intra-partum periods, respectively, 2883 tested PCR-negative at 6 weeks and the remainder (722) had an undefined or missing HIV status. As previously reported, mothers who transmitted HIV during the *in utero* period were younger and had better education than mothers who transmitted intra-partum or who did not transmit by 6 weeks, and mothers transmitting *in utero* had higher CD4 cell counts than mothers who transmitted intra-partum. A new finding not previously published, is that the *in utero* group also had higher plasma viral load (despite having higher CD4) compared with the intra-partum group, Table 1. Infants not included in the analysis due to missing timing of HIV infection had a lower mean birthweight (2.83 vs 2.93 kg), were born slightly earlier (gestational age 38.9 vs 39.2 weeks), their mothers had lower median CD4 cell count (382 vs 403 cells/μl) and their mothers' plasma viral load was higher ($\log_{10}$ 4.20 vs 3.99 copies/μl) compared with infants included in the analysis.

Maternal baseline factors and BED assay results

Of the 4495 HIV-positive mothers, 4466 had a baseline BED reading and 509 (11%) of these had a low BED (<0.8 ODn) reading. Compared with mothers with BED values ⩾0.8, mothers with a low BED were younger, had better education and higher CD4 cell counts (Table 2). Viral load values were more widely distributed among women with low BED readings than in those with high BED readings (IQR = 3.26–4.71 compared with 3.39–4.57). Similarly, the proportion of women with both undetectable viral load (<2.6 $\log_{10}$) and very high viral load (>4.6 $\log_{10}$) was greater among women with low BED readings.

Risk of *in utero* and intra-partum transmission according to BED and CD4 cell count

Among chronically infected women (BED ⩾ 0.8/CD4 ⩾ 350), 156 (9.1%) of their neonates tested PCR positive at birth compared with 34 (13.4 %) among women described as recently infected (BED < 0.8/CD4 ⩾ 350), Table 3. Among women with BED ⩾ 0.8/CD4 ⩾ 200 (a more liberal definition of chronic infection), 225 (9.1%) neonates tested PCR positive at birth, and this percentage hardly changed (275 or 9.4%) when BED ⩾ 0.8 readings irrespective of CD4 count were considered. Among women with BED < 0.8/CD4 ⩾ 200, a broader definition of recent infections, 48 (14.9%) neonates tested PCR positive at birth. In the intra-partum groups, 180 (10.5%) and 19 (7.5%) were classified in the chronic and recently infected groups, respectively, and these percentages hardly changed when different definitions of chronic and recent infections were used.

In unadjusted models, a low BED (<0.8) reading was a risk factor for *in utero* transmission (OR = 1.68, 95% CI 1.26–2.25) and a protective factor for intra-partum transmission (OR = 0.63, 95% CI 0.44–0.90). The odds of *in utero* infection ranged from 1.37 (95% CI 0.90–2.08) to 2.64 (95% CI 1.37–5.06) times higher when comparing women with BED < 0.8/CD4 ⩾ 350 and BED < 0.8/CD4 200–349 to women with BED ⩾ 0.8/CD4 ⩾ 350 in adjusted multinomial models, Table 4. In sensitivity analysis the odds of *in utero* transmission were 1.54 (95% CI 1.08–2.20; $P = 0.02$) and 1.47 (95% CI 1.03–2.09; $P = 0.03$) times higher when comparing a group with BED < 0.8/CD4 ⩾ 200 (4 and 5) with BED ⩾ 0.8/CD4 ⩾ 200 (1 and 2) and BED ⩾ 0.8/all CD4 groups (1, 2 and 3) in adjusted models respectively.

Based on multiple adjusted models, the odds of intra-partum infection ranged from 12% (95% CI −127; 66%) to 23% (–28; 54%) lower in recently infected women when compared with chronically infected, although the variances of the estimates were large. When the recently infected group (4 and 5) was compared with the two definitions of chronically infected groups (1 and 2; and 1, 2 and 3) in sensitivity analysis, the odds were 0.69 (95% CI 0.43–1.07; $P = 0.10$), and 0.60 (95% CI 0.38–0.93; $P = 0.03$), respectively.

In sensitivity analysis models with imputed CD4 cell count data, the aOR estimates for *in utero* infection were 2.85 (95% CI 1.55–5.26, $P < 0.01$) and 1.38

Table 1 Baseline characteristics of infant–mother dyads in which HIV transmission occurred intra-uterine and intra-partum and post-natal/did not get infected[a]

Characteristics	Intra-uterine ($n=382$) n (%)	Intra-partum ($n=508$) n (%)	PCR negative at 6 weeks ($n=2883$) n (%)	Overall P-value[b]
Infant				
Male sex	153 (40.2)	250 (49.2)	1473 (51.1)	<0.001
Birth weight, g [$\bar{x}$(SD)]	2.79 (0.47)	2.88 (0.49)	2.96 (0.45)	<0.001
Gestational age, weeks [$\bar{x}$(SD)]	39.0 (1.6)	38.9 (1.6)	39.2 (1.4)	<0.001
Maternal				
Age, years [$\bar{x}$(SD)]	24.8 (4.6)	26.3 (5.4)	25.7 (5.0)	<0.001
Schooling, years				
<8	12 (3.2)	38 (7.5)	142 (4.9)	
8 to <12	170 (44.6)	245 (48.2)	1334 (46.3)	
⩾12	199 (52.2)	225 (44.3)	1402 (48.7)	0.016
Mode of delivery				
Vaginal	351 (93.9)	465 (92.4)	2597 (90.6)	
Caesarean section	23 (6.1)	38 (7.6)	270 (9.4)	0.062
Rupture of membranes to delivery (hours)				
>4	147 (39.8)	231 (47.4)	1052 (40.0)	<0.001
MUAC, cm [$\bar{x}$(SD)]	25.6 (2.9)	25.4 (2.6)	25.8 (3.0)	0.001
Hb, g/dl [$\bar{x}$(SD)]	10.9 (2.2)	10.7 (2.0)	11.2 (1.9)	<0.001
CD4 cell count, cells/100 µl				
Median (IQR)[c]	400 (262–542)	322 (185–492)	418 (276–588)	<0.001
<200	56/328 (17)	123/436 (28)	317/2521 (13)	
200–349	83 (25)	115 (26)	628 (25)	
⩾350	189 (58)	198 (45)	1576 (63)	<0.001
Plasma viral load, $\log_{10}$ copies/µl				
Median (IQR)[c]	4.58 (4.00–5.09)	4.37 (3.90–4.90)	3.95 (3.42–4.46)	<0.001
<3.38[d]	35/376 (9.3)	57/493 (11.6)	840/2830 (29.7)	
3.38–4.6	164 (43.6)	238 (48.3)	1463 (51.7)	
>4.6	177 (47.1)	198 (40.2)	527 (18.6)	<0.001
Undetectable viral load	6/376 (1.6)	13/493 (2.6)	245/2830 (8.7)	<0.001

[a]All value are n (%) except where noted otherwise.
[b]Chi-squared/ANOVA/Kruskal–Wallis test as appropriate.
[c]Kruskal–Wallis test used.
[d]<3.38 is below lower quartile (Q1), and >4.6 is above upper quartile (Q3).

(95% CI 0.95–2.00, $P=0.09$) for BED < 0.8/CD4 200–349 and BED ⩾ 0.8/CD4 ⩾ 350, respectively, compared with BED ⩾ 0.8/CD4 ⩾ 350. The estimates were 0.98 (0.41–2.32, $P=0.96$) and 0.65 (0.41–1.04, $P=0.07$) for intra-partum infection comparing BED < 0.8/CD4 200–349 and BED ⩾ 0.8/CD4 ⩾ 350 relative to BED ⩾ 0.8/CD4 ⩾ 350.

Other factors associated with *in utero* and intra-partum transmission

Other factors that were independently associated with *in utero* infection in multiple multinomial logistic models were: gender adjusted odds ratio (aOR) 1.56 times for females compared with males, birthweight aOR 0.50, 50% lower odds for every kilogram increase in birthweight, maternal age aOR 0.96, 4% lower odds for a year increase in mother's age, mode of delivery aOR 0.54, 46% less odds for caesarean delivery compared with vaginal deliveries and maternal Hb aOR 0.36, 64% lower odds for HBs >11 mg/dl compared with HBs <7 mg/dl, Table 4. Gestational age aOR 0.92, 8% lower likelihood of intra-partum infection for a week increase in gestational age, mother's education aOR 0.62, 38% less odds of infection comparing

Table 2 Baseline characteristics of HIV-positive post-partum women according to BED assay results[a]

Characteristic	BED < 0.8 'low' ($n = 422$)[b] n (%)	BED ⩾ 0.8 'high' ($n = 3350$)[b] n (%)	P-value[c]
Maternal			
Age, years [$\bar{x}$(SD)]	23.9 (4.9)	25.9 (5.0)	0.001
Schooling, years			
<8	13 (3.1)	179 (5.4)	
8 to <12	175 (41.6)	1574 (47.0)	
⩾12	233 (55.3)	1593 (47.6)	0.005
MUAC[d], cm [$\bar{x}$(SD)]	25.6 (2.6)	25.8 (2.9)	0.175
Hb, g/dl [$\bar{x}$(SD)]	11.3 (2.1)	11.1 (1.93)	0.070
CD4 cell count, cells/100 µl			
<200	25/346 (7.2)	469/2937 (16.0)	
200–349	67 (19.4)	759 (25.8)	
⩾350	254 (73)	1709 (58.2)	<0.001
Plasma viral load, $\log^{10}$ copies/µl			
<3.38	116/414 (28)	816/3285 (24.8)	
3.38–4.6	178 (43.0)	1687 (51.3)	
>4.6	120 (29.0)	782 (23.8)	0.005
Percentage undetectable RNA	57/414 (13.8)	207/3285 (6.3)	<0.001

[a]All values are n (%) except where noted otherwise.
[b]Missing values if numbers do not add-up to this total.
[c]Chi-squared tests for categorical comparisons and t-test for continuous variables.
[d]Mid-upper arm circumference.

Table 3 *In utero* and intra-partum transmission according to baseline maternal BED ODn and CD4 cell count

	Maternal baseline BED ODn and CD4 cell count groups		Transmission n (%)	
Group	BED ODn and CD4 count (cells/ml) group	HIV duration and severity interpretation	*In utero* $n = 382$	Intra-partum $n = 508$
1	BED ⩾ 0.8 and CD4 ⩾ 350	Chronic early stage	9.1 (156)	10.5 (180)
2	BED ⩾ 0.8 and CD4 200–349	Chronic intermediate stage	9.1 (69)	14.5 (110)
3	BED ⩾ 0.8 and CD4 < 200	Chronic end-stage	10.7 (50)	24.6 (115)
4	BED < 0.8 and CD4 ⩾ 350	Recent early stage	13.4 (34)	7.5 (19)
5	BED < 0.8 and CD4 200–349	Recent intermediate stage	20.9 (14)	7.5 (5)
6	BED < 0.8 and CD4 < 200	Severe end-stage[a]	20.0 (5)	28.0 (7)

[a]During severe end-stage HIV, antibody production declines; this category of low BED and low CD4 probably reflects this disease stage.

women with ⩾12 years of education with women with 0–7 years spent at school, mode of delivery 0.57, 43% lower odds for intra-partum infection for babies delivered by caesarean section compared with vaginal deliveries, prolonged duration of rupture of membranes aOR 1.49 times higher odds of infection intra-partum for duration of rupture to delivery of ⩾4 h compared with periods of <4 h and maternal HBs aOR 0.39, 61% lower odds for HBs >11 mg/dl compared with <7 g/dl.

Discussion

Maternal acute HIV infection during the post-partum period has been associated with increased risk of MTCT in breastfeeding populations.[11,20,38,39] Although 50–90% of individuals with primary HIV infections do present with symptomatic seroconversion illnesses, their symptoms are usually non-specific and are similar to many common febrile illnesses, with pregnancy further clouding the issue.[40–42] Most

Table 4 Risk of *in utero* and intra-partum infection for women with low BED and high CD4 count at delivery

	Intrauterine transmission			Intrapartum transmission		
	Univariable	Multivariable		Univariable	Multivariable	
Risk Factors (levels)	OR (95% CI)	OR (95% CI)	*P*-value	OR (95% CI)	OR (95% CI)	*P*-value
Infant factors						
Sex						
Males	1	1		1		
Females	1.56 (1.25–1.94)	1.56 (1.21–2.00)	0.01	1.08 (0.89–1.30)		
Gestational age, weeks	0.91 (0.85–0.98)			0.88 (0.83–0.94)	0.92 (0.84–0.99)	0.03
Birth weight, g	0.44 (0.35–0.56)	0.50 (0.33–0.61)	<0.01	0.66 (0.54–0.82)		
Maternal factors						
Age, years	0.96 (0.94–0.98)	0.96 (0.93–0.99)	<0.01	1.02 (1.01–1.04)		
Education, years						
0–7	1			1	1	
8–11	1.51 (0.82–2.78)			0.69 (0.47–1.01)	0.66 (0.42–1.04)	0.80
⩾12	1.68 (0.91–3.08)			0.60 (0.41–0.88)	0.62 (0.39–0.99)	0.04
MUAC, cm	0.98 (0.94–1.02)			0.95 (0.92–0.99)		
Hb, g/dl						
<7	1	1		1	1	
7–10	0.58 (0.29–1.18)	0.53 (0.23–1.26)	0.15	0.60 (0.32–1.15)	0.59 (0.26–1.32)	0.20
⩾11	0.40 (0.20–0.80)	0.36 (0.15–0.84)	0.02	0.40 (0.21–0.76)	0.39 (0.17–0.87)	0.02
Mode of delivery						
Vaginal	1	1		1	1	
Caesarean section	0.63 (0.41–0.98)	0.54 (0.30–0.97)	0.04	0.79 (0.55–1.12)	0.57 (0.36–0.92)	0.02
Prolonged rupture of membrane (h)						
<4	1			1	1	
⩾4	1.08 (0.87–1.35)			1.47 (1.21–1.79)	1.49 (1.20–1.86)	<0.01
BED/CD4 interaction terms						
BED × CD4 (CD4 continuous)	0.87 (0.75–1.01)			0.93 (0.77–1.11)		
BED × CD4 both continuous	1.05 (1.01–1.10)			0.99 (0.95–1.03)		
BED						
⩾0.8 (long-term)	1			1		
<0.8 (recent)	1.68 (1.26–2.25)			0.63 (0.44–0.90)		
CD4						
<200 (end-stage)	1			1		
200–349 (intermediate stage)	0.76 (0.52–1.09)			0.47 (0.35–0.63)		
⩾350 (early stage)	0.69 (0.50–0.95)			0.33 (0.25–0.42)		
Group BED/CD4						
1. BED ⩾ 0.8 and CD4 ⩾ 350	1	1		1	1	
2. BED ⩾ 0.8 and CD4 200–349	1.45 (0.78–1.41)	1.10 (0.80–1.51)	0.56	1.45 (1.12–1.87)	1.45 (1.11–1.90)	0.01
3. BED ⩾ 0.8 and CD4 < 200	1.46 (1.04–2.06)	1.61 (1.12–2.32)	0.01	2.91 (2.23–3.79)	2.07 (2.03–3.60)	<0.01
4. BED < 0.8 and CD4 ⩾ 350	1.49 (1.00–2.22)	1.37 (0.90–2.08)	0.14	0.72 (0.44–1.19)	0.77 (0.46–1.28)	0.31
5. BED < 0.8 and CD4 200–349	2.57 (1.39–4.77)	2.64 (1.37–5.06)	<0.01	0.80 (0.31–2.03)	0.88 (0.34–2.27)	0.80
6. BED < 0.8 and CD4 < 200	3.39 (1.19–9.64)	3.73 (1.27–10.96)	0.02	4.12 (1.62–10.45)	2.71 (0.93–7.86)	0.07

prevention of mother to child transmission (PMTCT) programmes in developing countries provide a single test sometime during the antenatal period. Mothers who test positive are then likely to be enrolled into PMTCT programmes. Major reductions have been reported in intra-partum and breastfeeding transmission (the two MTCT modes contributing the biggest portion of all MTCT) due to HIV prophylaxis and feeding options.[34,43] It is thus likely that *in utero* transmission will contribute proportionately more to total MTCT compared with other transmission times.[44,45] Women with low BED reading and a moderate CD4 cell count were more likely to transmit to their unborn children than women with high BED and high CD4 cell count (aOR = 2.64, $P < 0.01$), although the effect for women with a low BED reading and high CD4 cell count was smaller (aOR = 1.37, $P = 0.14$). Reproductive-aged women should be educated about this higher risk of transmitting to their unborn child associated with seroconversion during pregnancy.[17]

We have previously reported a 2.90–4.60 times increased risk of post-natal MTCT among breastfed children if the mother seroconverted during this period.[20] This risk is much higher than the 1.37–2.64 times risk reported here, suggesting that the transmission risk associated with maternal primary infection may be lower for placental than breastfeeding-associated transmissions. This is further supported by the fact that intra-partum and post-natal transmissions individually contribute more to total MTCT where anti-retroviral treatment is not available.[31,44]

We acknowledge that our interpretation of HIV duration and severity for each BED/CD4 group is imperfect and may have resulted in misclassification, especially among women with BED < 0.8 and CD4 200–350, whose risk of *in utero* transmission was higher than that of chronically infected women. We judged that this combination of BED and CD4 cell count values reflected the nadir in CD4 count that occurs during the acute phase of HIV infection and therefore classified these women as having been recently infected. However, this BED/CD4 combination may also reflect a period of advanced disease when antibody production is depressed, but CD4 count has not yet dropped below 200. If any women in this BED < 0.8/CD4 200 < 350 group were truly chronically infected, our estimate of excess *in utero* transmission risk due to maternal primary infection during pregnancy would have been biased towards the null if the misclassified women had a lower risk compared with the truly acutely infected women.

In our previous publication, the infant death or infection rate among the 17 women whose blood sample near delivery tested HIV ELISA negative but HIV RNA PCR positive was 75%; clearly mothers who deliver during HIV seroconversion (when HIV antibodies remain undetectable and viral load is extremely high) are at very high risk of peri-partum transmission.[20] In this report, women likely to have acquired HIV infection during pregnancy, based on their BED/CD4 group, were not at increased risk of intra-partum transmission compared with chronically infected women. Indeed, our risk estimates of intra-partum infection were consistently lower for women classified as recently infected compared with chronically infected women although all OR CIs overlapped 1 except in one comparison. This is probably because all the women included in the current report tested positive for HIV-antibodies at delivery, indicating they had completed the brief 'window period' of the ELISA assay (when HIV antibodies are undetectable but viral load is very high) and were now in a much lower risk period of early asymptomatic disease characterized by high antibody, high CD4, low viral concentrations.

In sensitivity analysis models with imputed CD4 cell count, adjusted estimates for *in utero* and intra-partum infections were consistent with those obtained with observed data, although they had smaller CIs (i.e. smaller standard errors).

Similar to other studies, the risk for *in utero* infection was inversely related with birthweight, with smaller babies at increased risk of infection.[19,34,39,44,46] It is not clear whether an infected fetus fails to grow properly or fetal growth impairment predisposes the unborn child to increased risk of HIV infection.[44,47–48] The proportion of infants with low birthweight was 16% overall in this study, 24% of those infected in *in utero*, 22% in intra-partum and 14% among infants who tested negative at 6 weeks. Prematurity has been cited as a risk factor for infection because of immature immune systems, permeability of neonatal mucosal barriers and low levels of maternal antibodies which are normally transferred in the latter half of pregnancy.[34,49,50]

There was a 43–46% lower risk of infection among babies born by caesarean section than those delivered vaginally in both the *in utero* and intra-partum groups.[1] Prolonged rupture of membranes before delivery resulted in a 50% excess risk in intra-partum infection as reported elsewhere.[34,39,44,48] Among vaginal deliveries in our study, 37% had rupture-of-membranes-to-delivery durations of >4 h, whereas among emergency caesarean sections, 72% had prolonged rupture of membranes and only 17% among elective caesarean sections. Babies are exposed to cervico-vaginal secretions during prolonged rupture of membranes thus increasing the likelihood of infection.[51] Long duration of ruptured membranes before delivery and low CD4 cell count high viral load have been reported to increase risk of HIV transmission.[39,52] It is unlikely that elective caesarean sections will ever become the choice of delivery in the most affected HIV regions because of the huge challenges in cost, personnel and equipment. Other efforts to reduce the risk of infection such as reducing viral load have to thus be scaled up in these areas.

Research on optimal and easy-to-apply anti-retroviral treatment protocols should continue.

In conclusion, our findings indicate that the risk of *in utero* HIV transmission is likely to be greater among women who have their primary HIV infection during pregnancy compared with women who were infected prior to conception. Our estimates of this excess risk ranged from 1.37 (95% CI 0.90–2.08) to 2.64 (95% CI 1.37–5.06), which are lower than the excess risk of transmission during breastfeeding among women who seroconvert during the breastfeeding period. This suggests that the placenta may be a more effective barrier to the high viral load of primary infection than the mammary gland.

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CHAPTER FOUR

MODELLING BED CAPTURE ENZYME IMMUNOASSAY (CIEA) IN NEW HIV INFECTIONS

Modelling the BED Capture Enzyme Immunoassay (CIEA) in new HIV infections

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Abstract

The aim of this paper is to describe the functional form of the relationship between the probability of being recently infected with HIV (as opposed to having been infected for a long time) and BED optical density levels, and to examine how this relationship is modified by other markers such as viral load, haemoglobin and mid-upper arm circumference.

Participants

Initially HIV negative women with known seroconversion dates and women who tested ELISA HIV-1 positive at the time of recruitment had BED normalized Optical Density (ODn), ribo-nucleic acid viral load, haemoglobin, and mid-upper arm circumference measured.

Methods

Fractional Polynomial (FP), linear and categorical independent factors were used to assess the functional form of the BED ODn on the probability of being recently infected. The influence of viral load, haemoglobin and maternal mid-upper arm circumference were investigated in multivariable logistic regression models.

Results

Fractional Polynomial regression models described the functional form of the relationship between the probability of being recently HIV-infected and BED ODn better than either linear or categorical specifications of BED ODn. Use of a linear BED ODn term over-estimated the likelihood of recent infection for lower values of BED ODn, while categorical models had unrealistic jumps in the probability of recent infection.

Conclusions

Viral load, haemoglobin and mid-upper arm circumference did not change the relationship between probability of being recently infected and BED ODn levels.

Key Words: Fractional polynomial, BED assay, HIV recent infection

Word count: Abstract 227

Introduction

A number of laboratory assays have been proposed to distinguish between recent HIV-1 infections and long term HIV-1 infections [15, 30, 43, 72]. The assays are based on qualitative and/or quantitative changes in antibody levels before or after HIV-1 sero-conversion and involve the duration of a transient state related to changing virologic or immunologic response in early HIV-1 infection. This can be achieved through the detection of the virus before antibody sero-conversion (HIV-1 RNA or HIV-1 p24 assays) or through characterizing the initial antibody response that differs from that of established infection (anti-body titer, proportion specificity, isotpye or avidity) [15, 73]. HIV infected individuals are said to be recently infected if HIV virus is detectable in their blood and no detectable antibodies to the virus or if they are below certain cut-off levels on given antibody markers.

The BED Capture Enzyme Immunoassay (CEIA), hereafter referred to as BED, is an HIV-1 incidence assay that quantitatively measures the proportion of IgG antibodies associated with HIV-1; this proportion rises over the first year of infection [72]. IgG is a protein whose concentration in the blood reflects the cumulative exposure of the immune system to antigens such as bacterial or viral infections. The level of IgG used to classify individuals as recently infected or not was determined by modeling the kinetics of individual normalized optical density values (ODn) over time. The within subject profiles were found to be non-linear over time, with ODn levels rising before leveling off after 153 to 187 days hence the modeling of sero-conversion time was carried out using a linear mixed effects model of the square root transformed ODn level on log-time, with random effects to account for the correlation of responses within subjects. Survival type models were also investigated [52, 72, 74] . Estimates

of HIV incidence based on BED are sensitive to individual biological factors such as HIV-1 sub-type and population level factors such as HIV-1 prevalence [56, 75].

A number of measures are used to assess the health status of HIV-1 infected individuals. These include serological markers such as RNA viral load, CD4 cell count and haemoglobin, and measures of general nutritional status, such as mid-upper arm circumference (MUAC). These measures are likely to differ between recently infected individuals and those with long established infections [76-79]. Recently infected people are likely to be relatively healthy and thus should have relatively high CD4 cell counts, high haemoglobin levels, and normal MUAC, but high HIV-viral loads until after optimal concentrations of HIV-specific antibodies are produced. Such recently infected people would be expected to have low BED ODn. In contrast, long term infected individuals in the chronic pre-AIDS stage of HIV-1 infection have lower CD4 cell count and haemoglobin concentration, may show signs of wasting (thus low MUAC), but may have lower viral load and should have high BED ODn values.

Unadjusted estimates of HIV-1 incidence based on BED have been reported to be higher than estimates found prospectively from cohort studies [52, 72, 80]. People testing "false-recent" by the BED assay may include individuals in whom the ODn assay values rise slowly in the first year of infection and never reach the cut-off that discriminate between new infections and long-term infections, as well as individuals with AIDS in whom antibody production has declined, yielding low ODn values.

The BED assay is used cross sectionally, thus it is important to model the probability of being recently infected as a function of the assay and to investigate how other markers affect this relationship. In this paper the probability of being newly infected is modeled among women enrolled in the Zimbabwe Vitamin A for Mother and Babies Project (ZVITAMBO).. Women and their new born babies were recruited into the study within 96 hours of parturition and followed up to 12 months postpartum [52, 64, 81]. Women were tested for HIV at baseline and those testing negative were retested at 12 months to identify women who seroconverted during the postpartum year. The objective of the paper is to examine the form of the relationship between the probability of having acquired HIV during the 12 month postpartum period and BED ODn levels and the effect of baseline viral load, haemoglobin and MUAC on this relationship. Understanding the properties of the BED assay in identifying new HIV infections, and how this is related to other markers would provide insights into how the assay works. This can be used in defining new algorithms that combine these serological markers that better discriminate new from old HIV infections. The discriminating ability of the validated ODn cut off of 0.8 was assessed using fractional polynomial regression models in two groups of subjects.

Methods

The data used in this paper came from the ZVITAMBO project in which 14110 women were recruited between November 1997 and January 2000 in Harare Zimbabwe [52, 64]. Written informed consent was obtained.

At baseline, all mothers were tested for HIV-1 using an algorithm that included two parallel enzyme-linked immune-sorbent assays (HIV 1.0.2 ICE [Murex Diagnostics];

GeneScreen HIV 1/2 [Sanofi Diagnostics Pasteur]) and Western blot (HIV Blot 2.2; [Genelabs Diagnostics]) when results were discordant. Haemoglobin (HB) was measured for women enrolled from Oct 1, 1998 onwards (HemoCue, Mission Viejo, CA). For HIV-positive women, CD4 cells were enumerated (FACsCount; Becton Dickinson) and plasma assayed by the Calypte HIV-1 BED Incidence EIA (BED-CEIA), (cat. No. 98003; Calypte Biomedical Corporation, Lake Oswego. OR). For baseline HIV-1 positive women and women who seroconverted between baseline and 12 months, stored sera were analyzed by the BED assay. Normalized Optical Density (ODn) of less than 0.8 or greater than 1.2 were returned as the final readings.If ODn values were between 0.8 and 1.2 the samples were retested once and if the value was above 1.2 then that reading was taken as the final value, otherwise the sample was run in triplicate. The median value for these three readings was then considered as the final reading. Serum RNA concentration (viral load) was quantified using the Roche Amplicor HIV-1 monitor test (version 1.5, Roche Diagnostics, Alameda, CA, USA). Viral load values for undetectable samples were set to the assay detection limit (400 copies/μL) in analyses where viral load was treated as a continuous variable. Viral load values were $\log_{10}$ transformed. CD4 cell testing was done on the first positive sample for seroconverting women.

For participants to be included in this analysis they had to have complete data on viral load, haemoglobin and MUAC in addition to BED ODn readings. The first analysis set of women used in the analysis consisted of women at 12 months of follow-up who were known to be HIV infected at baseline and had complete data (n=1120) and women whose sero-conversion time was known to be within three months of the last negative and the first positive test (n=52). This analysis set defined as the restricted

analysis set, although small, has the advantage that subjects are clearly either long-term infected (n=1120) or recently infected (n=52). A second, larger analysis set of women was also considered, namely women who tested HIV-1 positive at baseline (n=1565) as well as women who seroconverted postnatally regardless of the exact date of seroconversion (n=98). The advantage of this larger group defined as the full analysis set, in addition to the larger sample size, is that it is more representative of the actual population that would be encountered in a cross-sectional survey. The disadvantage is that there is a risk that some women would be misclassified, since some of the women who tested positive at baseline may have in fact been infected within the previous 6 months, which is the approximate window period of the BED assay.

Models: Functional form of BED ODn for recent infection

The logit of the binary outcome recent/long term infection based on observed follow-up data was used as the outcome variable. In order to provide a comparison for fractional polynomial models, conventional simple linear logistic regression models were fitted first:

$$logit(p_i) = \beta_0 + \beta_1 Odn$$

Categorized BED ODn values with r-1 dummy variables on the logit of infection were then considered next

$$logit(p_{i)} = \beta_0 + \beta_1 ODn_1 + \beta_2 ODn_2 + \ \ldots + \beta_{r-1} ODn_{r-1}$$

The categorization included the manufacturer's recommended 0.8 cut-off, as well as a grouping with cutoffs (min to 0.2), (>0.2 to 0.4), (>0.4 to 0.6), (> 0.6 to 0.8), (>0.8 to 1.0), (>1.0 to 2.0), (>2.0 to 3.0) and (> 3.0 to max), (Tables 3 and 4). Thirdly, to investigate non-linearity in the relationship between the logit of infection and BED

ODn, fractional polynomials were used [67, 82]. The general form of the FP model is given by

$$\eta_i(X;\boldsymbol{\beta};\boldsymbol{p}) = \sum_{j=0}^{d} \beta_j g_j(X)$$

where for $j = 0$ to d where d is the number of the independent variables fitted in the model, $g_j(X)$ is $X^{p}{}_j$ *for* $p_j \neq p_{j-1}$ and $g_{j-1}(X)$ is $X^{p}ln(X)$ for $p_j = p_{j-1}$ and X^{p} denote the Box Tidwell transformation defined as X^{p} if $p_j \neq p_{j-1}$ and $ln(X)$ if $p_j = 0$. The coefficients and powers of the model are contained in the vectors $\boldsymbol{\beta} = (\beta_1, \ldots, \beta_d)$ and $\boldsymbol{p} = (p_1, \ldots, p_d)$ such that $p_1 < \ldots < p_d$ respectively. The power list which is restricted to a pre-determined set of integers and non-integers: (-2, -1,-0.5,0,1,0.5,1,2,3) includes the reciprocal, logarithm, square root, square and repeated-powers transformations [67]. $\boldsymbol{\eta}$ is an appropriate link function, and in this paper the logit link is used. The first-degree FP model with the smallest deviance (defined as minus twice the log likelihood) was selected as a basis for comparison with higher degree FP models if it was better than a null model. Among all second-degree FP models, the best second-degree FP was selected based on the smallest deviance, and only when there was a statistically significant difference in deviance between the best first degree and the best second-degree FP would the second-degree model be selected as a better model. Third-degree FP models were considered only when there was an improvement in the first or second degree FPs. We used the likelihood ratio test and the Akaike Information Criterion (AIC) to determine the best model among linear, categorical and fractional polynomial models [70] . We plotted the probability of being recently infected versus BED ODn values for linear, categorical and fractional polynomials models for a graphical impression of fitted models. All models were fitted using STATA (STATA Version 10; College Station TX).

To assess the effect of other markers, univariable fractional polynomials were fitted first for all variables (viral load, Haemoglobin and MUAC) following the same procedures as described for BED ODn models i.e. fit first-degree, second-degree and third-degree models. Factors with p-values of 0.20 or less in unvariable models were considered for entry into the multivariable FP model. The final multivariable FP model had factors which were significant at the 5% significance level but including BED. The influence of other disease markers for linear and categorical models were assessed in a similar way, starting by univariable models and building multivariable models, assessing significance of factors using likelihood ratio tests.

The full analysis set was used to illustrate FP model building (Table 1), in which univariable and multivariable linear, categorical and FP models were compared. The main comparisons and results are however shown for both analysis sets.

The Medical Research Council of Zimbabwe (MRC-Z), the Medicines Control Authority of Zimbabwe, the Johns Hopkins Bloomberg School of Public Health Committee on Human Research (CHR), and the Montreal General Hospital Ethics Committee (MGHEC) approved the ZVITAMBO trial. BED analysis of archived specimens was approved by MRC-Z, CHR, MGHEC and CDC Program Ethics Review Board, and the University of the Witwatersrand Human Research Ethics Committee (Medical) approved the current research.

Results:

One thousand six hundred and sixty three (1663) women; 1565 women who tested positive at time of recruitment and 98 women who sero-converted had complete data on BED ODn, viral load, haemoglobin and MUAC. One hundred and twenty four (124, 10%) of those who tested positive at baseline had undetectable viral load compared to 14 (14%) of sero-convertors (p-value = 0.03). When only women with well defined timing of HIV infection were used i.e. the restricted analysis set, 1120 baseline positive and 52 sero-convertors had complete data. When CD4 cell was considered only 16 sero-convertors had complete data, thus CD4 count was excluded from all models.

Table 1 shows the best first-degree, second-degree and third-degree fractional polynomial models for BED, viral load, haemoglobin and mid-upper arm circumference respectively. The best FP model for BED ODn was a first-degree FP (0.5), although a second-degree FP was marginally significant, (likelihood ratio test p = 0.06). The second-degree FP model for BED ODn had powers (-2 ; 0.5) and it was statistically significant in adjusted multivariable FPs. A second-degree FP model for viral load with powers (1 ; 1) was chosen as the best model among first, second and third-degree FP models. Haemoglobin had first-degree FP model with power (3) as the best model. All FP models fitted for mid-upper arm circumference were no better than the null model.

The best second-degree FP models (-2 ; 0.5) and (1 ; 1)on BED ODn fitted the data better compared to the linear and categorical models using the restricted and the full

analysis set respectively, (Figures 1a and 1b). The linear model had a flatter gradient than both FP models for lower values of BED ODn, thus it failed to capture the rapid decline in the probability of being recently infected. The linear model overestimated infection probabilities for ODn values below 2. The categorical model did not capture the continuous rapid decline in risk for lower values of BED ODn. The step function had probability values (0.41, 0.14, 0.05, 0.04, 0.2 and 0.1) and (0.85, 0.39, 0.15, 0.09, 0.03 and 0.004) for the full and restricted analysis set respectively for BED ODn groups (min to 0.2), (>0.2 to 0.4), (>0.4 to 0.6), (>0.6 to 0.8) (0.8 to 1.0) and (>1 to max). Using the manufacturer's 0.8 cut off gave two probability levels 0.29 and 0.02 for BED ODn levels below and above 0.8 respectively using the full analysis set.

In multivariable logit models based on the full analysis set, BED ODn, viral load, haemoglobin and mid-upper arm circumference fitted as linear independent factors were significant co-factors for recent infection, (Table 2). In this model the univariable coefficient for BED ODn was -1.85 and it changed to -1.82 in an adjusted multivariable model. In multivariable models with all factors categorised, BED ODn and viral load were the only significant factors where the coefficient for BED ODn changed from 3.21 in a univariable model to 3.14 in a multivariable model. BED ODn with powers (-2 ; 0.5), viral load power (1), and haemoglobin power (1) in multivariable FP model were predictive of the probability of being recently infected. The coefficients for the FP model in univariable model for BED ODn were (0.0005 ; -3.2007) and became (0.0007 ; -3.1158) in adjusted models. Viral load and haemoglobin were fitted as linear factors in multivariable FP models. Whereas mid-upper arm circumference was statistically significant in linear models (p-value = 0.04), it was not significant in multivariable FP models (p-value = 0.44). The

categorical model had the lowest AIC (406), although the FP model (AIC = 475) described the functional form of BED ODn and the probability of being recently infected better, (Figures 1a and 1b).

The likelihood of being recently infected was high for low ODn values when the full analysis set was considered (Table 3 and Figure 1a). Adjusting for viral load and haemoglobin did not change the odds of being classified as recently infected that much when BED ODn was assessed. Using ODn cut off of 0.8 in all FP models the odds was above 1 for BED values below 0.8 and was below 1 for values of BED above 0.8. When strict definitions for timing of infections were used, the separation between being recently infected and being a chronic case was very apparent in adjusted models, (Table 4 and Figure 1b). Adjusting for viral load and haemoglobin resulted in 'noisy' estimates of probability of being recently infected for lower levels of BED ODn values, (Figure 2).

Discussion:

To fully understand the utility of the BED assay for HIV-1 recent infection, it is necessary to evaluate how the assay relates to other markers such as viral load and hemoglobin, and MUAC. It is thus important to correctly describe the functional forms of these assays in HIV-1 infected individuals at different stages of infection. FP models were used to investigate the functional form of the BED ODn on the probability of being recently infected among HIV infected women. FP allows for the investigation of non-linear relationships, allows for correct adjustment of confounding and the modeling of complex interaction relationships [82-86]. The square root (on

ODn) and natural log (time) transformations were used to linearize the functional forms of the BED ODn over time using a panel of known HIV sero-convertors when estimating the window period of the BED assay [52, 72]. In this current study, FP fitted the probability of being recently infected against BED ODn levels best. Fitting BED ODn as an independent linear function resulted in over-estimation of the probability of being recently infected for low values of BED ODn, while the step functions resulted in unrealistic groupings for probability estimates, a tendency observed in other functional modeling studies [82, 84].

The functional forms for ODn for the restricted analysis set, powers (1 ; 1); $\text{logit}(p_i) = \beta_0 + \beta_1 ODn + \beta_2 ODn * \log_e(ODn)$ was different from the full analysis set , powers (-2 ; 0.5),$\text{logit}(p_i) = \beta_0 + \beta_1 ODn^{-2} + \beta_2 ODn^{0.5}$, (Figure 2). There was better discrimination by the BED assay between recently infected and long term infected individuals when analysis was restricted to participants with well defined timing of HIV infections. In the restricted analysis set, all women classified as long term infected had been infected for over one year, whereas in the full analysis data set some women could have been misclassified as having been infected for a long time when in fact they had been infected within the last six months. Adjusting for viral load and haemoglobin seemed to have little impact on this separation ability. This has been shown as one of the major short-coming of the BED assay. When validated within known sero-converting panels, the BED assay performed very well, but when it was applied to truly cross-sectional samples regardless of timing of infection, the assay incorrectly classified a considerable number of long term infected participants [34, 52, 87-89]. This problem is likely to be more profound in generalized HIV-1 epidemics characterized by large proportions of long term infected individuals. This

misclassified long term infected group inflates the numerator used in the calculation of HIV-1 incidence considerably, thus over estimating HIV incidence. In practice the problem of misclassifying the long term infected group might be lessened by removing AIDS patients [90]. It should however be noted that haemoblogin was measured at baseline only so its influence on the probability of being recently infected and BED may not have been properly captured.

Viral load and hemoglobin were the only independent co-factors in the relationship between the likelihood of being recently infected and BED ODn. Viral load is expected to be elevated during the acute phase of infection before the host's immune system is able to fully respond to the virus, as well as during the AIDS disease stage when the immune system cannot cope with high viral replication [91] . The role and relationship between these markers and iron levels is not clearly understood thus further research is needed. Adjusting for viral load and hemoglobin resulted in a lot of noise in the likelihood of being classified as recently infected for individuals with low BED ODn values. This could partly be a function of the relatively small number of recently infected participants. The odds of being recently infected remained relatively stable in models adjusted for viral load and hemoglobin, suggesting that these two factors are independent co-factors for being recently infected. Because of the lower detection limits for viral load, we used the lower limit of detection (400 copies/μl) for all readings with undetectable levels. This might result in biased results because these readings are left censored.

The effect of CD4 cell count on the relationship between the probability of having been recently infected and BED ODn could not be explored in this study because only

16 CD4 count readings were available for recently infected participants. CD4 count levels are expected to drop during the acute phase of infection, but recover at lower levels of original values once antibodies to HIV-1 have been established. The nadir at which CD4 cell count levels rebound after sero-conversion is not well documented, although data from Uganda (subtype A and D) suggest that this nadir may be relatively high (above 400) with wide variations in the actual counts (personal communication Dr. Jonathan Levin, MRC/UVI Uganda Research Unit on AIDS). Current World Health Organization (WHO) antiretroviral treatment guidelines stipulate that treatment should commence at 350 CD4 cell counts or lower [92]. While low levels of CD4 count may be an indication of advanced disease, more research is need to understand CD4 level profiles around acute infection.

Other alternatives to FP models to investigate non-linear relationships exist. These include Spline Regression which however easily become cumbersome as the number of factors to be fitted and/or the number of parameters to be estimated increase [93-94]. FP models can however be affected by outliers and high influence values, thus resulting in wrong functional relationships between outcome and independent variables.

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Table 1: Fractional polynomial model building for logit (recent infection)

Model	1st degree (M1)			2nd – degree (M2)				3rd degree (M3)			
	power	LL df = 2$	AIC	Powers	LL df = 4$	AIC	Chi-2 Gain, p-value M2-M1	Powers	LL df = 6$	AIC	Chi-2 Gain, p-value M3-M2%
Odn	**0.5**	**-246.9082**	**497.82**	-2 0.5	-244.0339	494.07	5.75 (0.06)	-2 -2 0	-242.1001	492.20	3.87 (1.00)
Viral load	3	-357.3576	718.72	**1 1**	**-352.9346**	**711.87**	**8.85 (< 0.01)**	-2 -2 -2	-351.2212	710.44	3.43 (0.18)
Hemoglobin	**3**	**-368.6369**	**741.25**	0 0	-366.5401	739.08	4.17 (0.12)	3 3 3	-363.42	734.84	6.23 (0.18)
Muac	***-2***	***-371.3739***	***746.75***	*3 3*	*-371.2262*	*748.45*	*0.30 (0.86)*	*3 3 3*	*-371.2119*	*750.42*	*0.32 (0.99)*

This table uses full analysis set: All available data n = 1663

Bold – Best model across each variable comparing powers 1, 2 and 3.

Italics – These models are not any better than the NULL model

% Except for MUAC where models are compared to M1 because M2 is not an improvement on M1

$1-df for each estimated coefficient and 1-df for each power term

Table 2: Final multivariable models for logit recent HIV infection for women who seroconverted postnatally

Variable	*LL (df)*	*β*	SE	p-value[@]
a). Assuming a linear function for continuous factors	-247.61 (4)			
Odn		-1.82	0.22	< 0.01
Viral load		0.53	0.13	< 0.01
Haemoglobin		0.015	0.001	0.02
Mid-upper arm circumference		0.08	0.04	0.04
b). Categorical approach	-226.45 (3)			
ODn				
>= 0.8		ref		
< 0.8		3.14	0.26	< 0.01
Viral load				
< Q1		ref		
Q1-Q3		-0.05	0.29	0.85
> Q3		0.97	0.30	< 0.01
Haemoglobin				
< 90		ref		
90 – 110		Out		
> 110		Out		
Mid-upper arm circumference				
< 23				
23 – 25		Out		
> 25		Out		
c) Fractional polynomial model	-232.98 (8)			
Odn^{-2}		0.0007	0.0004	0.04
$Odn^{0.5}$		-3.1158	0.3084	< 0.01
Viral load[1]		0.5589	0.1391	< 0.01
Haemoglobin[1]		0.0171	0.0067	0.01
Mid-upper arm circumference[1]		Out		

This table uses the full analysis set: All available data, n = 1663

[@]p-values are from likelihood ratio tests

Table 3: 2nd degree FP model for HIV incident infection on ODn, preserving the usual information from an analysis based on categories for all infected women regardless of timing of infection, (full analysis set).

ODn*		Number			OR (Model based)			
					Un- adjusted		Adjusted*	
Range	Comparison point	At risk	Infected	Naïve proportion recently infected (95% CI)	Estimate	95% CI	Estimate	95% CI
0 – 0.2	0.15	85	46	0.541 (0.430 – 0.650)	5.18	3.96 – 6.84	5.00	3.81 – 6.58
> 0.2 – 0.4	0.25	58	12	0.207 (0.112 – 0.334)	3.56	2.85 – 4.46	3.45	2.75 – 4.33
> 0.4 – 0.6	0.45	55	9	0.164 (0.078 – 0.288)	2.05	1.80 – 2.33	2.01	1.76 – 2.30
> 0.6 – 0.8	0.65	60	7	0.117 (0.048 – 0.226)	1.33	1.26 – 1.40	1.39	1.25 – 1.39
> 0.8 – 1.0	0.90	39	2	0.051 (0.006 – 0.173)	0.84	0.81 – 0.87	0.84	0.82 – 0.87
> 1.0 – 2.0	1.50	412	12	0.029 (0.015 – 0.050)	0.35	0.29 – 0.42	0.36	0.29 – 0.44
> 2.0 – 3.0	2.50	510	8	0.016 (0.007 – 0.031)	0.11	0.07 – 0.17	0.12	0.08 – 0.18
> 3.0	4.00	444	2	0.004 (0.001 – 0.016)	0.03	0.02 – 0.06	0.03	0.02 – 0.06

*$\text{logit}(p_i) = \beta_0 + \beta_1 ODn^{-2} + \beta_2 ODn^{0.5} + \beta_3 VL + \beta_4 HB$, where coefficients are as in table-2. Model is adjusted for viral load, haemoglobin#. The reference point for all these comparisons is ODn = 0.8

Note that viral load and haemoglobin are fitted as linear functions.

Table 4: 2nd degree FP model for HIV incident infection on ODn, preserving the usual information from an analysis based on categories for women with well defined timing of HIV infection (restricted analysis set).

ODn		Number			OR (Model based)			
					Unadjusted		Adjusted*	
Range	Comparison point	At risk of being new infection	Recent infections	Naïve recent Incidence probability (95% CI)	Estimate	95% CI	Estimate	95% CI
0 – 0.2	0.15	40	34	0.85 (0.70 – 0.94)	57.52	(20.12 – 164.42)	62.21	20.85 – 185.61
> 0.2 – 0.4	0.25	23	9	0.39 (0.20 – 0.61)	23.75	(9.09 – 62.05)	25.37	9.35 – 68.84
> 0.4 – 0.6	0.45	23	2	0.09 (0.01 – 0.28)	5.87	(2.98 – 11.57)	6.12	3.02 – 12.37
> 0.6 – 0.8	0.65	20	3	0.15 (0.03 – 0.37)	1.97	(1.44 – 2.69)	2.01	1.45 – 2.77
> 0.8 – 1.0	0.90	33	1	0.030 (0.001 – 0.158)	0.67	(0.53 – 0.84)	0.66	0.53 – 0.84
> 1.0 – 2.0	1.50	252	1	0.004 (0.0001 – 0.0219)	0.12	(0.02 – 0.69)	0.11	0.02 – 0.67
> 2.0 – 3.0	2.50	352	0	0 (0 – 0.01)	0.04	(0.0003 – 4.28)	0.03	0.0002 – 4.15
> 3.0	4.00	429	2	0.005 (0.0006 – 0.017)	0.07	(3.6×10^{-6} – 1482.43)	0.05	1.83×10^{-6} – 1443.73

* $\text{logit}(p_i) = \beta_0 + \beta_1 ODn + \beta_2 ODn * \log_e(ODn) + \beta_3 VL$, where $\beta_1 = -6.90$, $\beta_2 = 3.34$, $\beta_3 = -6.76$. Model is adjusted for viral load#. The reference point for all these comparisons is ODn = 0.8, the manufacturer's cut off for BED.

Note that viral load is fitted as a linear functions.

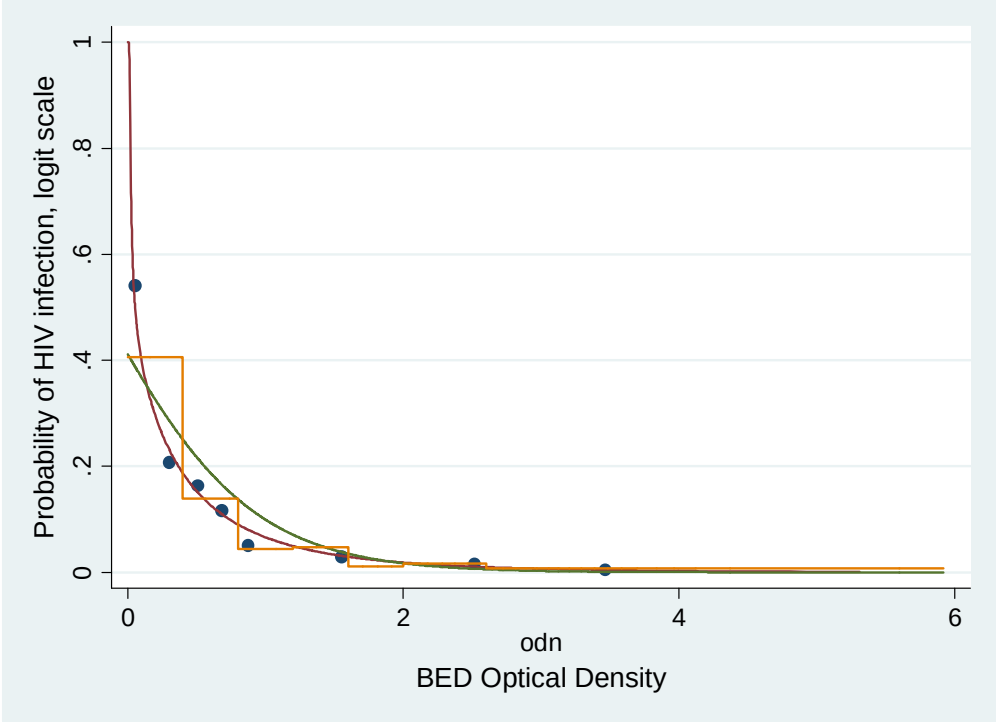

Figure 1a: Fitted estimated probability of recent incident HIV infection by BED ODn using full analysis set : blue dots –proportion recent infections in BED group (see Table 3), green - linear model, yellow - categorical step model, red - FP model (-2 ; 0.5).

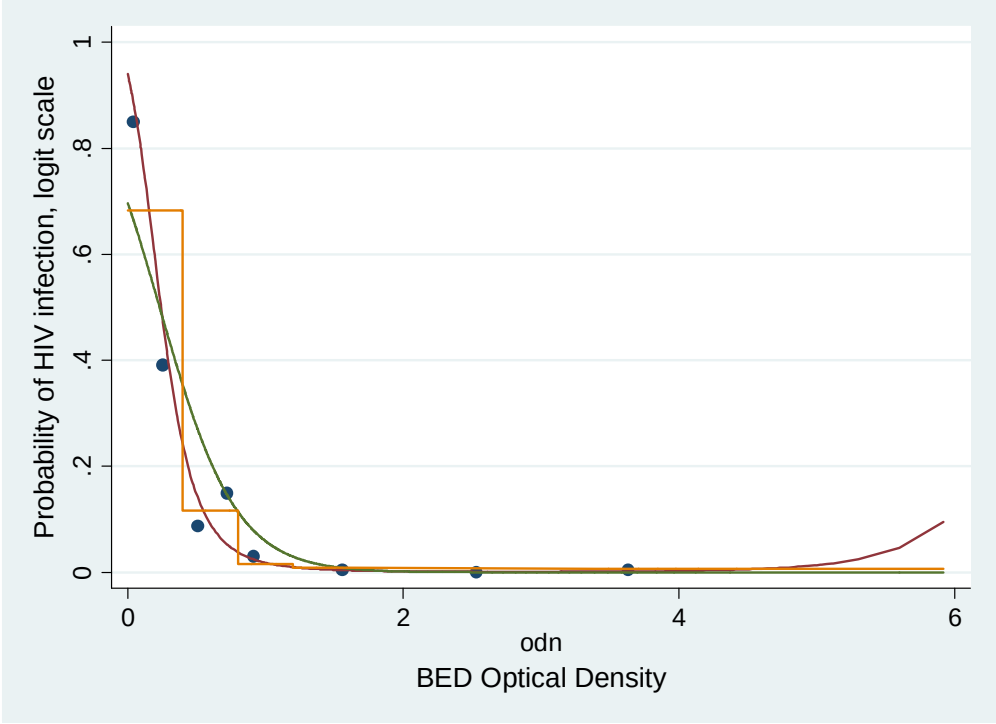

Figure 1b: Fitted estimated probability of recent incident HIV infection by BED ODn using restricted analysis set : blue dots –proportion recent infections in BED group (see Table 4), green - linear model, yellow - categorical step model, red - FP model (1 ; 1).

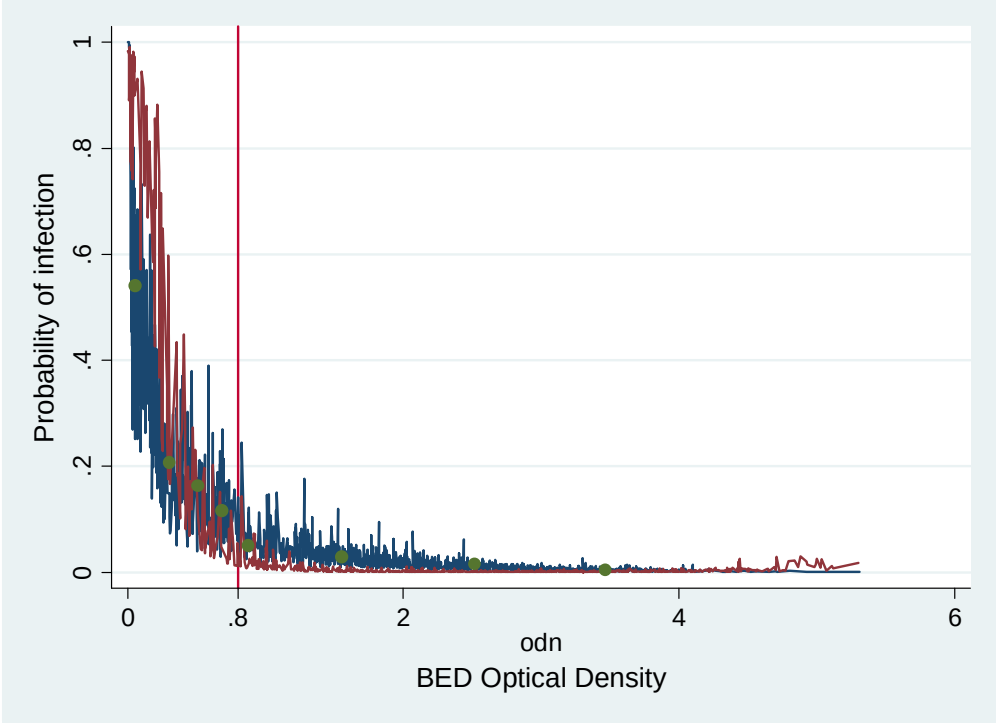

Figure 2: Fitted estimated probability of recent incident HIV infection by BED ODn using: red line - restricted analysis set – blue line - full analysis set and green line - proportion recent.

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CHAPTER FIVE

SUMMARIZING DISCUSSION AND CONCLUSIONS

Chapter 5 Summarizing Discussion and Conclusions

Simple laboratory assays that can be applied cross-sectionally hold great promise in getting timely and inexpensive estimates of HIV incidence urgently needed in the most affected areas of the world. Jassen *et al.*'s study in 1998 probably represents the turning point in the interest and possible resolution of availing timely usable assays that measure HIV incidence [30, 33, 46, 95-97]. A number of such assays have been proposed and among these assays the BED and 3A11 – LS are thought to be much more reliable for estimating HIV-1 incidence [16].

Current cross-sectional assays used in incidence testing are not specific enough to give satisfactory results in incidence estimation; neither can they be used for clinical purposes to identify recently infected individuals. It is thus important to understand the properties of the BED assay and how other HIV disease markers such as viral load, CD4 cell count, and initiation of ART relate to the performance of this assay. Although the BED assay was validated using serial samples of seroconvertors with known timing of infection, this validation process misses important information on long term infected patients. Ignoring these long term infected patients affects the specificity of the BED assay, giving imprecise estimates of HIV incidence when applied in cross-sectional samples.

The 3A11-LS had short-comings similar to those of the BED assay; 4% of chronically HIV infected patients remained below the threshold years after seroconversion , and 8.7% of AIDS patients were classified as recently infected [30]. Both the 3A11-LS and BED assays falsely classified 2% to 5% of AIDS patients as recent infections [15]. The rate of false recent infection was higher among patients

about to start ART, and this rate was even higher when patients with very low CD4 cell counts where considered [98].

AIDS patients were more likely to be misclassified (4.38%), than chronic, (1.69%) patients known to have been infected for more than a year [35]. Immune function declines during the AIDS stage of HIV disease, and high viral antigens can combine with antibodies effectively reducing viral antigens available for titer, proportion or affinity, thus resulting in AIDS patients being falsely classified as new infections [33]. In our study on patients about to start ART, 11.2 % of patients who were about to start ARV treatment had BED ODn values below 0.8, and this percentage was even higher (20.2%) among patients with very low CD4 counts. Although there was no data to differentiate patients who started treatment based on CD4 count or AIDS defining conditions, it is possible that those with very low CD4 counts were also likely to have other co-morbidities. In the ZVITAMBO study, among post-partum women, disease state seemed not to be related to being falsely classified as recent; for women with CD4 counts < 200, 200-399, 400 – 599, 600 – 799 or 800 or more, the false positive percentage was 4.5, 3.0, 6.1, 6.5, and 5.4 % respectively. In the same study, women known to have been infected for at least 12 months who subsequently died between 12 and 24 months after recruitment had a false positive percent of 5.4%, which was not very different from 5.2 % among women last seen alive between 12 and 24 after recruitment [52]. In another study AIDS patients had a similar rate of false positivity to that observed among long term infected people without AIDS [35].

In the ZVITAMBO study mother and/or child were excluded from the study if either had a life threatening condition or if the baby was of very low birth weight (< 1500

g). HIV infection is possibly associated with increased risk of abortions, still births, early neonatal deaths and low birth weight [99]. This means exclusion from the study was likely to be associated with advanced maternal disease, but some excluded participants could have been due to poor child outcomes associated with maternal seroconversion during pregnancy. The prognosis of babies early on in life for these infected *in utero* has been reported to be worse than those infected intra-partum and those infected post-partum through breastfeeding [100]. The exclusion criteria could have resulted in biased estimates of *in-utero* infection associated with maternal seroconversion during pregnancy, i.e. *in utero* transmission rates could in fact have been under-estimated. The BED assay could be used on stored samples to investigate whether seroconversion during pregnancy is associated with adverse pregnancy outcomes.

Although the transmission rate from acutely infected individuals is much higher than that from chronically infected individuals, in an advanced epidemic there are far more chronically infected persons than acutely infected persons, and thus the chronically infected persons account for a larger percentage of incident infections [101-102]. Pinkerton *et al*. estimated that about 8.6% of around 32 000 sexually transmitted HIV infections acquired each year in the US were due to acute-phase transmission [102]. Xiridou *et al.* estimated that 11% of incident HIV infections among gay men in Amsterdam could be attributed to acute-phase infection [103].

5.1 Properties of the BED assay

The shape of the relationship between the probability of being recently infected and the BED assay was investigated in a sample of new and long term infected post-

partum women. The influence of viral load, haemoglobin and MUAC on this relationship was investigated.

Viral load has been reported as the strongest predictor of the risk of all forms of HIV transmission [62-63]. The natural history of HIV serological markers show that these markers are likely to be correlated. In fractional polynomial regression models although BED, viral load and haemoglobin were all significant predictors of the probability of being recently infected, viral load and haemoglobin seemed to capture other aspects of HIV disease from that captured by the BED assay. The proportions of correctly classified individuals were similar in a univariable model containing BED and a multivariable model adjusted for viral load and haemoglobin. In this study, using the restricted analysis population in which the recent and long-term infected were well defined, resulted in a good separation between those recently infected and those known to have been infected for at least a year, which is similar to results found from validation studies using serial validation panels with known timing of infection. Addition of viral load and haemoglobin, however, did not improve the classification of BED, yielding at most a 1% absolute change in agreement. In another study all individuals with mean uncertainty of 10 days between last negative test and first-positive test were correctly classified, but this declined to 73% when the mean uncertainty increased to 140 days [35]. In our study the restricted analysis population had a higher likelihood of being correctly classified than the full analysis population where the timing of infection was less certain. In McNicholl *et al.*'s study, BED derived cross sectional HIV incidence estimates were similar to longitudinal incidence estimates when the assay was applied to people with known timing of being recently infected. In the same study, when the analysis included long-term infections, the

incidence estimates by BED were higher [104]. This has been cited as the main weakness of the BED assay.

5.2 Variability in HIV serological markers

The proportions of long term HIV infected individuals who present with low BED readings seems to vary widely, depending on subtype, population prevalence, and time [89]. The BED assay, similar to other HIV serological markers such as the LS assay, CD4 cell count and viral load is variable within and between infected patients.

If populations differ in concentrations of IgG, then the BED assay would not be expected to perform the same in these different populations. For example, if normal IgG concentration ranges are lower in Caucasian than African populations, seroconversion durations may be shorter in Caucasian populations, assuming similar HIV antibodies synthesis kinetics [33]. Total IgG levels are known to be significantly elevated among Africans, and malnutrition, which is a common occurrence in Africa, has been linked to immunologic dysfunction [105-106]. Conditions or co-infections that elevate total IgG (hypergammaglobulinemia) may result in long term infected individuals falsely classifying as new infections, a situation that may be true in Sub-Saharan Africa. The onset of opportunistic infections due to advanced HIV disease and immune reconstitution after the commencement of antiretroviral therapy seem to result in a decrease of HIV specific IgG [107-108]. The effect of other co-infections such as TB, pneumonia and a host of other opportunistic infections commonly associated with HIV were not evaluated in this study. Further research is needed on the relationship between BED and other specific opportunistic infections.

There have been observed differences in immune response to HIV between African and European populations especially regarding CD4 cell counts [109]. Some studies have reported that individuals with low CD4 count/high viral loads are much more likely to be misclassified on the BED assay than infected individuals with high CD4 count/low viral loads [98]. CD4 cell count levels vary greatly both within and among populations for reasons that are not yet fully understood. It has been noted that individuals' CD4 counts may vary by as much as 200/µl in readings taken a few weeks apart [110]. There were no observed differences in CD4 cell levels at time of seroconversion between individuals whose progression to AIDs was normal, rapid or slow within a 2 to 6 year follow-up multi-centre study [111-112].

BED cut-off levels used in classifying HIV infected patients were first determined empirically and later refined and confirmed using statistical models [35]. For a BED cut-off of 1.0 corresponding to a mean window period of 158 days, the BED assay had sensitivity and specificity of 82% and 89%, respectively, and an accuracy rate in incidence calculation of 93% in combined subtypes B and E [35]. Subtype B in the same study had a slightly longer window (186 days) period than E (152 days). Karita *et al.* reported unusually high proportions of persistently high false recent rates (26.7 % to 57.1%) on the BED assay among HIV patients infected for at least a year. According to the same study, the proportions seemed to vary with subtype; 30.4 % in subtype-C and 57.1 % in subtype A1.The authors reported that plasma viral load was significantly lower over a year of follow-up among volunteers who tested false positive as being recently infected compared with those whose BED values correctly indicated them as being long-term infected. Sensitivity (81.2%) in this study was similar to the Parekh *et al.* study, but specificity was much lower (67.8%). The

authors reported that the optimal cut-off on the ROC curve that would give acceptable sensitivity and specificity was 0.5, corresponding to a window period of 80 days [34].

Although Karita *et al.*'s estimates are based on relatively small sample sizes; this highlights the issues of wide variability in BED performance across subtypes and regions. Hallett *et al.* did a systematic review of the BED assay and reported that the BED assay performs differently across regions, time and subtype [89]. The ZVITAMBO study had an estimated mean window of 187 days using a cut-off of 0.8 among post-natal women carry subtype C [52]. The window period from the VAX004 study was 133 days compared to 153 days derived from independent seroconversion panels [36].

It is still not well understood why the BED assay performs differently in different HIV subtypes. A slower HIV IgG synthesis in subtype B infection than E infection or a poor recognition of the subtype B peptide by homologous antibodies may explain some of these differences [35]. The B peptide used in the assay may perform differently because there is heterogeneity even within the same subtype [113]. Disease level such as viral load kinetics may explain some of the differences in seroconversion duration. For example there was significantly higher HIV viral RNA in subtype E compared to subtype B during the first three months of infection [21]. The lower viral load in subtype B infection could have resulted in slower kinetics of the HIV specific IgG synthesis [35]. Viral kinetics over time as indicated by various disease stages such as clinical AIDS where IgG are presumably lower can affect performance of the assay thus leading to misclassifications [114-115]. Primary HIV infection results in a burst of viral replication that is thought to be controlled by viral

specific immune response of the host. Broad HIV specific immune responses may be more effective in controlling viral replication than restricted ones [116-118]. Some HIV infected individuals are elite viral suppressors, and these individuals' BED ODn profiles progress much more slowly than HIV rapid progressors. The observed ODn values of these elite suppressors remain below the cut-off threshold for a long time after initial infection [107, 119]. Adjusting for viral load and haemoglobin did not improve the performance of the BED assay in our study.

Although the results of antibody assays are reported dichotomously (recent or long standing), these categories are defined by quantitative, assay-specific cut-offs. Algorithms that make use of the more quantitative nature of these assays might provide better predictions of incidence than currently observed. Consideration should be made to see if instead of using a single cut-off point, a number of cut-offs with varying assigned probabilities of belonging to a class can be more useful. Assigning probability values of being recently infected modelled on BED assay values and other correlates of recent infection might be practically more useful than using single cut offs. The probability models can investigate a wider range of lymphocyte counts besides CD4 cell count. Similar models have been successfully used in vaccine efficacy research [120-121].

BED ODn levels are affected by ART; in our study over 50% of the patients who had been on ART for over two years tested below the 0.8 cut off of the ODn assay [98]. Antiretroviral treatment slows the rate of HIV virus replication, thus altering HIV specific response quantitatively (antibody level) or qualitatively (avidity) resulting in lower levels of the BED assay [122-123]. It has been reported that complete

seroconversion can be achieved much earlier by initiating HAART during acute/early HIV infection [124-125]. The BED assay has been successfully used in the USA because of readily available medical records for HIV infected patients. This kind of data is unlikely to be available in the most affected countries, thus other ways of identifying patients on treatment as well as those who have been infected for long periods have to be devised. ART provision has increased substantially in some of the most affected areas of the world [126], thus data on the number of HIV-infected individuals on ART may progressively become more available. HIV infected individuals may be asked to indicate if they are on ART therapy. Since ART affects BED ODn levels, the number of HIV-1 infected individuals on ART should be taken into account when estimating HIV incidence.

5.3 Risk of MTCT for women who get infected during pregnancy

It has been shown that the acute phase of infection is associated with an increased risk for onward transmission compared to any other times [58, 127]. It is important to compare and contrast similarities and differences between different timing of HIV transmission. The different mechanisms for MTCT of HIV for example, cannot be assumed to be the same, and may be expected to be very different from horizontal ways of HIV transmission. It has been shown that without ART, the risk of HIV infection is highest during delivery, and lowest during pregnancy [2-3, 128]. But within each of these modes of transmission is the risk similar between those with acute infection and those with chronic infection?

The probability of transmission from a single coital act during acute infection was found to be 43 times than the probability of transmission from a single act during

chronic infection [129]. In this study in Rakai, Uganda, as much as 89.1% of all infection was estimated to be possibly due to acute infection. Model estimates suggest that the rate of heterosexual HIV-1 transmission per coital act follows a U-shaped curve, being highest during the post-seroconversion period, lower during latency, and increasing with advanced disease [130-132]. This is supported by findings that serum HIV viral load, which is high during the post-seroconversion period and during advanced disease, is the principal predictor of heterosexual transmission [61, 133-134]. It has been shown that high serum viral load is associated with an increased probability of heterosexual transmission, thus transiently high viremia during acute infection might translate to increased likelihood of onward transmission [58, 135-136] . Men with acute HIV infection are biologically more infectious because of increased genital shedding of HIV. During acute infection, HIV viral load increases and thereafter decreases in semen, with these changes approximately paralleling those that occur in blood. Viral dynamics in semen suggest that on average individuals are hyper-infectious at the onset of the acute retroviral syndrome period and continuing for ~ 6 weeks thereafter [137]. Men in sub-Saharan Africa with clade C have been shown to have 3-4 fold higher viral load compared to clade B and E predominant in Europe, even without STD co-infections [109, 137].

It is important to understand the serological kinetics associated with different modes of HIV infection. It cannot just be assumed that transmission dynamics for mother-to-child are similar to heterosexual transmission. For example, although serum viral loads are usually used as a measure of infectiousness, the key driver of sexual transmission is genital fluids (and not blood), thus making it difficult to model HIV transmission during acute infection on the basis of blood alone. This is partly

explained by the fact that acute HIV infection represents a period of initial establishment of anatomic HIV-1 reservoirs; therefore the viral dynamics in blood, which have been well described for acute HIV infection [138-141] cannot be assumed to apply to the genital tract. If viral load were to increase more rapidly in genital fluids than in the systemic compartment, for instance, the probability of transmission during acute HIV-1 infection would be greater than that predicted on the basis of concurrent blood HIV-1 load. If on the other hand semen viral load were to increase slowly, relative to blood viral load, it is possible that no peak in probability of transmission would occur at all, despite elevated blood VL during acute infection. These same arguments can be used when assessing HIV risk associated with MTCT of HIV. In a study of post-partum women who were breastfeeding, women who seroconverted had median plasma viral load of $\log_{10}$ 5 (IQR 3.5 – 5.8) at time of infection, and $\log_{10}$ 4.1 (IQR 3.7 – 4.7) one year after the last negative test. Breast milk supernatant viral load for the same group of women, was lower ; $\log_{10}$ 2.2 (IQR 1.9 – 3.1) and $\log_{10}$ 1.4 (IQR 1.4 – 2.1) for the same time points respectively [58].

We used BED and CD4 cell count to estimate the risk associated with low BED reading typical of recent infections. The risk of *in utero* infection was highest among women with low BED values (ODn < 0.8) and low (< 200) to moderate (200 - 349) CD4 cell counts, 3.4 times and 2.6 times higher compared to women with high BED (≥ 0.8) and high CD4 cell counts (≥ 350) [142]. Yet the risk patterns in intra-partum infections were somewhat different to *in utero* infections; women with low BED and low CD4 counts had twice the risk of transmitting HIV to their babies intra partum compared to those with high BED (≥ 0.8) and high CD4 cell counts (≥ 350). The risk group with the highest HIV transmitting probability probably consists mainly of

women who seroconverted during pregnancy. Although this kind of classification has its own limitations, it was clear that various groupings of BED and CD4 cell counts quantified different risk levels posed by pregnancy and delivery. This suggests that HIV negative pregnant women must be tested more than once during antenatal visits.

In a breast feeding study, women who seroconverted in the post partum period were more likely to transmit HIV to their breastfed babies compared to women who were already HIV infected at the time of delivery. Although in high prevalence areas like Zimbabwe where post-natal incidence rates were estimated to be 3.4 per 100 person years[52], post-natal seroconversion has been estimated to contribute as much as 20% of all breastfeeding transmission [58].

5.4 Use of the BED and other serological assays

Serological assays such as the BED or the 3A11-Ls assay can be used at the population level for estimating HIV-1 incidence, and for identifying subjects with early infection for therapeutic trials and pathogenesis studies, and at public health level for focusing and evaluating HIV prevention efforts [30]. It should however been noted that these assays have poor specificity.

Antibodies to HIV develop during primary HIV infection, thus may not be detected by HIV antibody tests. If effective identification and thus appropriate intervention programs targeted specifically to new infection are to be made, assays that identify recently infected patients with high levels of certainty are needed.

Montaner *et al.* proposed what they termed "test and treat", where universal testing of the whole population takes place and everyone found to be HIV infected immediately commences on ART [143]. The argument used is similar to HIV prophylaxis used for PMTCT programs. Treated mothers are less infectious, thus MTCT rates has been reduced to very low levels. Mathematical models have suggested that the test and treat strategy could result in new infections coming down substantially [144-146]. Strong arguments for this radical approach are that current HIV preventive methods have shown little effectiveness, effective preventive vaccines seem a long way off and that current treatment methods cannot eradicate HIV infection [147-151]. Although the test and treat algorithm is an attractive proposal, treating every HIV infected person is unlikely to be feasible and in a resource constrained setting it would be ethically challenging to treat those acutely infected in preference to treating patients with advanced HIV disease who qualify for treatment under current guidelines. Algorithms that incorporate assays such as the BED assay may be used to develop modified test and treat protocols that are more practical and feasible. These assays can be used to identify high risk groups thus targeted interventions implemented. These should include identification of possible new infections for partner notifications as well as identifying sexual networks, thus reducing onward transmission [30].

Viral load is known to be elevated during the acute phase of infection and towards end stage disease (AIDS stage). This means people at extreme ends of the disease can present with similar viral loads, thus there is need to use other disease markers such as BED, CD4 and haemoglobin to define different risk algorithms that differentiate risk profiles in order to reduce the risk of onward transmission.

It has been proposed that despite the BED assay's imperfections, differences in estimated incidence in the same population over time or between subgroups of the same population are probably valid because errors, biases, and variances in the assay are expected to be stable over time [95, 152-158]. This means these anti-body incidence assays may be used to examine trends of HIV-1 incidence over time in the same population and to assess the effectiveness of pre and post interventions and to compare incidence between two or more groups from the same population or to assess trends, a common objective in public health. Well known established epidemiological facts should guide the use and interpretation of results obtained in incidence data using these assays.

5.5 Limitations of cross-sectional serological assays

Questions have been raised as to how comparable cross-sectional HIV incidence assay based estimates are to longitudinal estimates. BED measures historical exposure that may not span the same time period as cohort studies. The rate of misclassification on the BED assay may depend on time if individuals with fully developed immune responses live longer than other HIV patients [107, 119]. This means that the assumption of constant false positive rate may only be true in relatively short periods. McDougal *et al.* demonstrated increased incidence estimates from 2.94% to 5.45% when prevalence changed from 3% to 20% with a fixed false recent percentage of 5% [36].This means that changes in or differences in prevalence even within the same group might affect incidence estimates substantially. In generalised epidemics the proportion of long term infected may be quite high. Sakarovitch *et al.* demonstrated that different assays would overestimate HIV incidence by different amounts when true incidence is very low relative to prevalence. For example,

assuming an incidence of 1% and prevalence of 30%, the IDE-V3 assay gave HIV incidence estimates that were 3 times higher, Vironostika 17 times higher, BED 20 times higher and the avidity test gave HIV incidence estimates that were 36 times higher than the true incidence value. In this study, the observed bias in estimated incidence increased when the simulated incidence rate decreased, which raises the question as to whether these tests can be used to reliably monitor incidence trends over time [159]. In a study using data from Abidjan where prevalence was 10% [160] and incidence was 1% [161], four HIV incidence assays (Vironostike, Avidity, IDE-V3 and BED) would have failed to identify a decrease of 50% in incidence; the BED would have given an incidence decrease from 6.2% to 5.1% (a 5% relative decrease), Vironostika 5.5% to 5.1% (7% relative decrease), IDE-V3 1.2 % to 1.0% (17 % relative decrease) and avidity 11.2 to 11% (2% decrease) [159].

Incidence estimates derived from cross sectional samples measure the number of recent infections per period, e.g. 186 days for subtype C or the appropriate window depending on subtype. Extrapolation of this value to a longer period for example when calculating an annualized estimate assumes constant rate of infection during this one year period. A non constant rate over the estimation period might result in biased results [104]. The magnitude of bias associated with false-recent positives using the BED assay can be substantial, increasing with prevalence as noted in a number of studies [35-36, 52, 162-164].

Individuals who are elite or viremic controllers with low or undetectable viral loads may remain in the window period of the BED assay until AIDS or death, thus creating what Brookmeyer *et al*. termed an assay shadow [165]. According to the author long

shadows (2 years or longer) can create discrepancies between cohort and biomarker incidence estimates especially if incidence changes sharply over time. This means for instance longitudinally obtained estimates may be biased when used to validate assay based estimates because these two methods are measuring HIV incidence at different time points.

A number of ways to improve the performance of the BED assay or similar assays have been proposed. A single specimen can be tested for levels of antibodies to two or more different proteins or peptides representing early and late infection [33]. Two or more different methods such as proportion or titer of HIV-IgG and antibody avidity can be combined into a single assay [33]. Because of wide variation in immune response, single stand alone serological approaches for measuring HIV infections are unlikely to better the 85% observed sensitivity and specificity of the BED assay [35]. Issues such as individuals with late stage disease or opportunistic infections, or those receiving therapy need to be addressed before applying these tests to reduce the bias in the estimates of incidence. To account for these, certain specimens could be excluded on clinical grounds, secondary confirmatory assays may be used or mathematical analytical adjustments may be used [36]. Participants who self report or otherwise are known to be long term HIV-1 positive [166-167] patients with AIDS [30, 168], or patients receiving antiretroviral therapy [65] are unlikely to be recently infected and likely to register as recent by the assay, thus should be accounted for when estimating HIV incidence. More stringent testing algorithms can be used, for example requiring a confirmatory test with a second test for recent infection or asking patients if they are on ART if they present with low BED ODn readings. Mathematical adjustments have been proposed, and they rely on getting an accurate

estimate of false recent rates [35, 52, 164]. A number of studies have reported the percentage of HIV patients who present with low BED ODn levels despite being infected for periods longer than the BED window period [34, 52, 169-170] and this varies from 1.7% in SA [169] to as high as 27% in Rwanda and Zambia [34]. This brings into question the generalizability of applying mathematical methods in incidence calculations to account for the rate of false positivity. The use of false positive proportions that are not locally derived have resulted in unrealistically high estimates of incidence in Cote d'Ivoire[170], South Africa [55, 169] and Uganda [171]. The recency period (window) may differ by subtype [172] requiring selection of appropriate window periods for populations in the same area such as Thailand, where more than one subtype predominates [104]. This limits the wide use of the BED, because genotyping may be required, thus making the use of these assays even more complex. One promising strategy is based on algorithms involving multiple assays such as CD4, BED, avidity assays, p24 antigen, HIV RNA screening and presence of antiretroviral drugs [107]. Some of the proposed strategies are however likely to make these assay expensive and technically complex to implement, a huge drawback for the most affected regions of the world. Janssen *et al*. propose using CD4 cell count to supplement the assay classification for clinical care of patients [30].

A number of population level epidemiological adjustment methods have been proposed, and these correct the naïve incidence estimates by correcting assay imperfections: window period, short and long term false recent rate and sensitivity of the test [36, 52, 173] .

5.6 Key Summary Findings

i. It was shown that ART reduces HIV specific IgG levels; over half of patients presented with BED levels typical of recently infected patients. Most patients put on ART are likely to be advanced stage patients, thus they should be accounted for when estimating HIV incidence using the BED assay.

ii. The proportion of long term infected patients testing as recently infected can be substantial, as much as 11% of HIV advanced stage patients had low BED ODn levels. This rate seems to be higher than other rates obtained in a general HIV infected population. Patients with very low CD4 count were more likely to present with low BED values.

iii. The BED assay, in combination with other serological markers can be used as a prognostic tool to assess historical risk of in-utero HIV infection in stored sera. Different combinations of BED and CD4 cell count presented with different risk measures of *in utero* and intra-partum infection

iv. HIV negative pregnant women who normally have a single HIV test earlier on during the antenatal period may benefit from retesting. The women carry a disproportionate risk of transmitting to the unborn child if they get infected, thus may not benefit from PMTCT programs if they are not tested again.

v. The BED assay and other serological markers such as viral load and haemoglobin seem to measure different aspects of the HIV disease. Addition of viral load and haemoglobin seem not to have changed the predictive ability of the BED assay for recent infections in a cross-sectional sample.

5.7 Future work

Although the BED assay is not specific enough to correctly identify new HIV infections, it has great potential in other HIV programmes. Measuring viral load is relatively expensive and it requires specialised equipment and expertise. Algorithms combining CD4 count, which is routinely collected and BED ODn which is technically simpler to perform might be good proxies for viral load in assessing HIV risk.

Novel proposals on 'test and treat' have been made. These proposals though theoretically very attractive are unlikely to be implementable in the near future because of costs and commitment from funders of HIV programs. Algorithms profiling different risks can be developed and these might be useful in the management of the HIV disease.

The BED assay is based on changing levels of HIV specific IgG as a function of total IgG. The effect of other co-infections on BED readings is not clear, thus future work should be carried out to investigate how co-infections such as TB, malaria and pneumonia affect BED levels. Anti-retroviral treatment is known to alter and reduce BED ODn levels. The effect of other drugs such as anti-TB drugs should also be investigated to see if they have a similar effect on BED readings.

Questions have been raised as to whether the BED assay can be used to evaluate HIV prevention interventions, where the aim is not to get precise estimates of incidence but to capture changes in incidence over time. Future work should be done to assess if the BED assay can be used to measure the impact of interventions. These studies should

use independent confirmatory methods in order to assess possible biases associated with using the BED assay. The studies have to cover wide HIV incidence ranges in order to measure the sensitivity of the assay.

The role of other health markers such as haemoglobin on the likelihood of testing recent on the BED assay is not yet fully understood. In our study, haemoglobin was only measured at baseline, and there was limited CD4 cell count data on seroconvertors, thus limiting our inference on the full effects of these two markers. Further research on the relationship between these various markers might shed more light on why some HIV infected individuals present with low BED readings a long time after being infected.

Current proposed HIV incidence formulae adjust for the non specificity of the BED assay using the window period of the assay [36, 52, 173-174]. The assay window period is derived from BED ODn panel data. Further research using different weighting systems based on the window period and/or BED ODn levels might give more precise estimates of incidence.

5.8 Conclusion

Major gains have been made in reducing the number of babies born with HIV by using HIV prophylaxis during pregnancy and after delivery. These reductions are mostly observed on the number of intra-partum and breastfeeding associated transmission where rates have gone down to single digit figures in most areas. HIV transmission to the unborn child is thought to occur late during pregnancy. Pregnancy associated transmissions to the unborn child will increasingly become the focus of

PMTCT research. Current HIV prophylactic guidelines for pregnant women say treatment should commence as soon as a women test positive or at least at 14 weeks gestation [175]. Women who acquire HIV during pregnancy may pose a higher risk of infecting their unborn children than long term infected women. Identifying this high risk group may be missed because of a single HIV test. Pregnant women should thus be encouraged to seek antenatal care early in order to achieve optimal benefits of PMTCT programmes. Repeat HIV tests for pregnant women in high HIV prevalent areas as well as in high risk groups may identify recently infected women. Although incidence rates may be relatively small, the absolute number of infected babies may be substantial especially in settings with high HIV prevalence, thus prevention strategies to avoid new infections should continue.

There are benefits in identifying recently infected individuals because these individuals carry a disproportionately high risk of onward transmission of HIV. More conservative criteria such as lowering the cut-off of the BED assay may be used to increase the likelihood of picking up truly recently infected individuals as well as such other high risk individuals [35]. Besides antenatal and maternal patients, all such patients with elevated risk of onward HIV transmission would benefit from ARV treatment. An easy and fast way of identifying newly HIV-1 infected individuals can result in prescribing ART early, thus reducing the chance of establishment of reservoirs of tissue of the HIV virus [19-20, 176]. Early treatment coupled with appropriate counselling and partner notification might help in reducing secondary transmission from newly infected individuals to their sexual partners [38, 156, 177]. The potential clinical benefits of antiretroviral treatment for individual patients who are diagnosed during antibody-negative acute infection further emphasize the need for

improved and early identification of cases of HIV-1 infection [178]. Treatment consideration for recent sex contacts should include prospective screening for acute and chronic HIV infection and appropriate screening for other sexually transmitted pathogens. It is conceivable that antiretroviral treatment in early infection may reduce the risk of onward transmission, and treated patients may benefit from better immune control and lower set points of viremia [136, 179]. With less than 100% ART coverage for people in need of treatment, there are serious ethical issues associated with who should be considered for treatment, recently infected person to reduce the risk of onward transmission or sick people who may die if treatment is withheld. It is however important to actively seek out recently infected persons and to propose counselling to reduce high risk behaviour during this critical period [136, 153].

Cross-sectional assays such as the BED are advantageous because they can measure historical risks such as risk of MTCT during pregnancy. The risks associated with different pregnancy periods (trimesters) can thus be compared.

Symptomatic seroconversion illnesses are nonspecific such that they are usually misdiagnosed and as such a large number of HIV infected patients do not seek medical care [180-182]. This means clinical assessment of primary HIV infection is limited, emphasizing the urgent need for cross sectional assays such as the BED assay that can be used to assess risk of recent infection, enabling targeted versions of test and treat to be implemented.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Marinda

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080224**
PROJECT	Understanding the BED Capture Enzyme Immunoassay: Measuring HIV-1 incidence in cross-sectional studies
INVESTIGATORS	Mr E Marinda
DEPARTMENT	School of Public Health
DATE CONSIDERED	08.02.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.04.02 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr/Prof J/L Levin/Moulton

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES