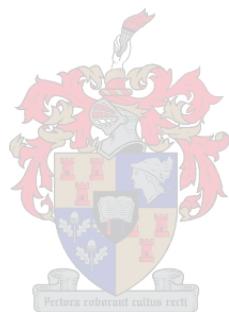


# **HIV-1C Dynamics and Evolutionary Trends in Botswana**

**By**

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**Dissertation presented for the degree of Doctor of Philosophy in  
Medical Sciences (Medical Virology) at Stellenbosch University**

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**December 2016**

## ABSTRACT

**Introduction:** HIV incidence estimates are critical for monitoring HIV transmission dynamics, and for design and evaluation of the impact of interventions. Biomarkers and assays for cross-sectional surveillance of HIV incidence are greatly needed because of the high costs and time needed to maintain prospective cohorts to determine HIV incidence. New cross-sectional assays for estimation of HIV incidence are attractive due to their improved performance and cost-effectiveness. In this dissertation, methods for identification and characterization of recency of HIV infection are described. An in-depth review of HIV recency determination methods, including novel cross-sectional application of molecular methods, is given in “From serological assays to genomics.” Multi-assay approaches were evaluated in order to increase the sensitivity and specificity of the commercial incidence assays in the context of high treatment coverage and stable but high HIV prevalence in Botswana. A novel biomarker based on HIV viral diversity was investigated as a complementary or standalone tool to characterize HIV recency. In this thesis, an innovative use of pairwise diversity and the time to the most recent common ancestor (tMRCA) in a heterosexual HIV-1 subtype C (HIV-1C) epidemic were introduced as novel approaches for HIV incidence estimation. We evaluated the properties of the new potential tools for estimating time since infection, including their specificity and predictive performance in the context of the HIV-1C epidemic in Botswana.

**Methods:** Characterization of HIV recency and novel biomarkers for estimation of HIV infection incidence is based on application of immunologic and molecular methods:

- a) Evaluation of the long-term specificity (false recent classification rates) of serological tests for recent infection, and algorithms for estimating HIV-1C incidence utilizing samples from patients with known long-standing HIV infection.
- b) Application of within-host viral diversity for estimation of HIV-1C recency in Botswana using samples collected from patients with known time since seroconversion in the primary HIV-1C infection cohort.
- c) Investigation of intra-host viral pairwise diversity and the time to the most common recent ancestor (tMRCA), as potential markers for HIV infection recency.

**Results:** We estimated for the first time false recency rates (FRR) of the commercially available BED and Limiting Antigen (LAG) assays in Botswana. We demonstrated that combined algorithms reduce FRR to the recommended < 2%. Including viral load in the assay algorithm resulted in an FRR of 0.4%

for LAg. Analysis of the within-host viral pairwise diversity provided more accurate estimation of HIV recency, as compared with the recommended LAg and BED using the receiver operator characteristic analysis (ROC). We demonstrated that intra-host viral pairwise distances reduce misclassification and increase the accuracy of serologic assays. tMRCA and intra-host viral pairwise distances correlated with time since HIV infection provide additional novel tools for reliable estimation of HIV recency.

**Conclusion:** HIV infection recency can be determined cross-sectionally using a combination of serological and molecular biomarkers. Including viral load and an assessment of prior exposure to ARVs is critical for accurate estimation of HIV incidence. Intra-host pairwise diversity and tMRCA are able to predict time since HIV infection and can be used to improve accuracy in estimation of HIV infection recency.

## OPSOMMING (AFRIKAANS)

**Inleiding:** MIV voorkoms skattings is van kritieke belang vir die monitering van MIV-oordrag dinamika, en vir die ontwerp en evaluering van die impak van intervensies. Biomerkers en toetse vir deursnee-toesig van MIV voorkoms is baie nodig as gevolg van die hoë koste en tyd wat nodig is om voornemende kohorte in stand te hou om MIV voorkoms te bepaal. Nuwe deursnee-toetse vir skatting van MIV voorkoms is aantreklik as gevolg van hul verbeterde prestasie en koste-effektiwiteit. In hierdie verhandeling word metodes vir die identifikasie en karakterisering van resentheid van MIV-infeksie beskryf. 'N In-diepte oorsig van MIV relevante bepaling metodes, insluitend roman deursnee-toepassing van molekulêre metodes, word in "Van serologiese toetse om genomika." Multi-toets benaderings is om die sensitiwiteit en spesifisiteit van die kommersiële voorkoms verhoog geëvalueer toetse in die konteks van 'n hoë dekking behandeling en stabiele maar 'n hoë voorkoms van MIV in Botswana. 'N roman biomarker wat gebaseer is op MIV virale diversiteit ondersoek as 'n aanvullende of selfstandige instrument om MIV relevante kenmerk. In hierdie tesis, 'n innoverende gebruik van paarsgewyse diversiteit en die tyd om die mees onlangse gemeenskaplike voorouer (tMRCA) in 'n heteroseksuele MIV-1 subtip C (HIV-1 C) epidemie is ingestel as nuwe benaderings vir MIV voorkoms skatting. Ons geëvalueer die eienskappe van die nuwe potensiële gereedskap vir die beraming keer sedert infeksie, insluitend hul spesifisiteit en voorspellende prestasie in die konteks van die MIV-1C epidemie in Botswana.

**Metodes:** Karakterisering van MIV relevante en nuwe biomerkers vir skatting van MIV-infeksie voorkoms is gebaseer op die toepassing van immunologiese en molekulêre metodes:

- a) Evaluering van die langtermyn-spesifisiteit (valse onlangse klassifikasie tariewe) van serologiese toetse vir onlangse infeksie, en algoritmes vir die beraming van MIV-1C voorkoms met behulp van die monsters van pasiënte met 'n bekende lang MIV-infeksie.
- b) Toepassing van binne-gasheer virale diversiteit vir skatting van MIV-1C resentheid in Botswana met behulp van monsters van pasiënte met 'n bekende keer sedert sero Omskakeling geconstateerd in die primêre MIV-1C infeksie vorder.
- c) Ondersoek van intra-gasheer virale paarsgewyse diversiteit en die tyd om die mees algemene onlangse voorouer (tMRCA), as 'n potensiële merkers vir MIV-infeksie resentheid.

**Resultate:** Ons beraam vir die eerste keer valse relevante tariewe (FRR) van die kommersieel beskikbare BED en beperking van Antigeen (lag) toetse in Botswana. Ons het getoon dat gekombineerde algoritmes te verminder FRR om die aanbevole <2%. Insluitend virale lading in die

toets algoritme tot gevolg gehad dat 'n FRR van 0,4% vir lag. Ontleding van die binne-gasheer virale paarsgewyse diversiteit verskaf meer akkurate skatting van MIV resentheid, in vergelyking met die aanbevole lag en 'n bed met behulp van die ontvanger operateur eienskap analise (ROC). Ons het getoon dat intra-gasheer virale paarsgewyse afstande te verminder foutieve classifikasie en verhoog die akkuraatheid van serologic toetse. tMRCA en intra-gasheer virale paarsgewyse afstande gekorreleer met die tyd sedert MIV-infeksie bykomende roman gereedskap vir 'n betroubare beraming van MIV resentheid.

**Gevolgtrekking:** MIV-infeksie resentheid kan kruis-sectionally bepaal met behulp van 'n kombinasie van serologiese en molekulêre biomerkers. Insluitend virale lading en 'n evaluering van vorige blootstelling aan ARV is van kritieke belang vir akkurate skatting van MIV voorkoms. Intra-gasheer paarsgewyse diversiteit en tMRCA in staat is om tyd te voorspel, aangesien MIV-infeksie en kan gebruik word om die akkuraatheid in raming van MIV-infeksie relevante verbeter.

## **DECLARATION**

This dissertation includes 5 original papers published in peer reviewed journals and 1 unpublished work under review in peer reviewed journals. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case a declaration is included in the dissertation indication the nature and extent of the contributions of co-authors. I declare that this work has not previously in its entirety or in part submitted it for obtaining any qualification.

Sikhulile Moyo

December 2016

## **DEDICATION**

To the loving memory of my champions (mom & dad), gone too soon!

*Benjamin & Priscilla Bhetswana Mninkuni Moyo*

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Professor Susan Engelbrecht and Professor Tilio de Oliveira their guidance and advice throughout the course of this work. I would like to acknowledge the immense contribution of mentors Drs Eduan Wilkinson, Vladimir Novitsky for all the guidance and contributions. Your contribution to this work is evident in many of these pages. I would like to thank Alain Vandormael, a persistent investigator for his support with analytical and quantitative skills. I would like to express my gratitude to Drs Rosemary Musonda and Simani Gaseitsiwe for unwavering support, motivation, and encouragement. I am greatly indebted to Professor Max Essex for his unwavering support for my training and development, and always keen to see development of our potential. I appreciate the support that I received from the senior management of Botswana-Harvard AIDS Institute, Dr Joseph Makhema and Mrs Ria Madison. To dear friends and colleagues you made this journey look possible with your words of encouragement: Anthony Ogwu, Aida Asmelash, Tumalano Sekoto, Lillian Okui and Gloria Mayondi. I would like to thank Rui Wang, Jia Weng and Lesego Gabaitiri for their assistance with statistical analysis. Many thanks to all the co-authors for all the valuable insights, suggestions and contributions towards manuscripts generated during my studies. I am grateful to Alex Welte and Eduard Grebe from Stellenbosch University & the South African Centre for Epidemiological Modelling and Analysis (SACEMA) for technical assistance and guidance with incidence estimation tools.

Many thanks to research Fellows and Scientists at the Botswana-Harvard AIDS Institute, Botswana-Harvard HIV Reference Laboratory and Essex Lab in Boston who gave me supporting laboratory work, all the troubleshooting and data analysis, and with good suggestions always. I would like to thank Lendsey Melton for editorial assistance.

This work would not have been possible without the generous support from OAK foundation and Fogarty International Grants, as well as funding from the University of Stellenbosch (Division of Medical Virology). I am grateful to the principal investigators of these grants and the Division of Medical Virology for the financial support I received.

I am grateful to my family for believing in me and supporting me at all the times and seasons. To my best friend and beloved wife Natasha, and my three children Moemedi Samuel, Phatsimo Ndumiso and Luyanda Tehillah: your support has been amazing. Thanks for being a pillar of ... at all times! Thank you for being patient while I took time (long hours!! to do this work). I am forever grateful for God's wisdom, protection and guidance!

## ABBREVIATIONS

<b>HIV</b>	Human Immunodeficiency Virus
<b>AIDS</b>	Acquired Immunodeficiency virus
<b>ART</b>	Antiretroviral treatment
<b>ARV</b>	Antiretrovirals
<b>PMTCT</b>	Prevention of Mother to Child Transmission
<b>MTCT</b>	Mother to Child Transmission
<b>PwD</b>	Pairwise Diversity
<b>tMRCA</b>	time to the most common recent ancestor
<b>HAART</b>	Highly Active Antiretroviral Therapy
<b>UNAIDS</b>	The Joint United Nations Programme on HIV/AIDS
<b>NACA</b>	National AIDS Coordinating Agency

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# CHAPTER 1

## INTRODUCTION

### 1.1 The HIV Epidemic in Botswana

Thirty years into the global Human Immunodeficiency Virus (HIV) pandemic, HIV prevalence and incidence remain high in sub-Saharan Africa, with 25.6 million people living with HIV as of the end of 2015 (1). Although sub-Saharan Africa represents only 12% of the world's population, it accounts for approximately 67% of the world's 36.7 million HIV-infected persons, and about two-thirds of the world's 2.1 million new infections occur in this region annually (1).

Botswana, a politically stable country in southern Africa, has experienced an explosive and devastating HIV epidemic since the first case was identified in 1985 (2). HIV prevalence among antenatal clinic attendees increased from 6% in the early 1990s to 36% in 2002 (2). The Sentinel Surveillance surveys conducted over 14 years in Botswana revealed an overall decline in HIV prevalence among pregnant women although this remains above 30% (3-6). General population-based surveys conducted approximately every four years have shown evidence of decline in new HIV infections and stabilisation of HIV prevalence among adults aged 15–49 years and decline in HIV prevalence among the younger age groups (7, 8). Key populations such as female sex workers (FSW) have prevalence rates of over 60% (9).

In response to the epidemic, Botswana has been one of the leaders in adopting and implementing HIV prevention and treatment interventions. In January 2002 Botswana officially initiated a national antiretroviral treatment (ART) program providing highly active antiretroviral therapy (HAART) to its citizens. To date, over 86% of HIV-infected individuals in Botswana are receiving ART (10), and all public health facilities provide ARV for the prevention of mother-to-child transmission (PMTCT) services, resulting in one of the lowest MTCT rates globally (<1.5%) (11, 12).

In 2014, the Joint United Nations Programme on HIV/AIDS (UNAIDS) proposed new targets

directed at ending the AIDS epidemic by 2030 (13). Namely, by 2020, 90% of all people living with HIV should know their HIV status; 90% of all people with diagnosed HIV infection should receive sustained combination ART; and 90% of all people receiving ART should be virologically suppressed. Owing to the substantial progress made in providing HIV treatment and prevention of MTCT services to its citizens, Botswana is one of the few countries worldwide to be close to meeting these targets (10). However, with an estimated 24% of its adult residents (aged 15–49) HIV-infected, Botswana has one of highest adult HIV prevalence and the third highest national HIV incidence rate globally (1, 7, 14, 15). Appropriate tools are therefore required for monitoring HIV incidence in Botswana and for evaluating the impact of the public health response as well as identifying the drivers of the high HIV incidence, both in the general population and in special key populations.

## **1.2 The Challenge of Estimation of HIV Incidence**

The ability to accurately estimate incidence, or the risk of acquiring infection within a given period of time is an essential component of monitoring the transmission dynamics of HIV (16-22) and evaluate prevention efforts, especially in the era of broad scale-up of ART and universal test and treatment (20, 23). Accurate surveillance of recently infected individuals and estimation of HIV incidence can also inform the design and evaluation of intervention strategies (24), and assist in the design of efficacy clinical studies and identify high risk subgroups. Treatment as prevention (TasP) is likely to be more cost effective if individuals in the early infection phase, responsible for disproportionately higher rates of onward transmission, are a primary focus(25-29).

Estimating HIV incidence remains challenging despite the development of several methods (30). A classical approach to estimating HIV incidence is based on longitudinal follow-up of uninfected individuals (31-33). However, this approach is expensive, time consuming and logistically challenging to implement (24). Other approaches include the use of mathematical models such as back-calculation methods that infer incidence from changes in prevalence in serial surveys (34). The development of cross-sectional assays relies on the evolution of the host's immune responses, or use of viral markers (30, 35-37). The main concept behind the cross-sectional approach is based on a specific biological marker (biomarker) associated with changes in the host's immune response to HIV infection, and able to distinguish recent from established (long-term, or chronic) HIV infections (35, 38-40).

In Chapter 2, a detailed description of these methods is provided

### 1.3 Study rationale

Despite the development of cross sectional methods to identify recent infections and estimate HIV incidence, so far no method or biomarker has sufficient properties to provide robust estimations of HIV incidence. New biomarkers and assays for cross-sectional surveillance of HIV incidence are needed as an alternative to the prospective cohorts that are costly and time consuming. Serological tests can also be used cross-sectionally for estimation of recent HIV infections due to improved performance of new assays and their associated algorithms (41). However, these assays can misclassify some long-term infected individuals as recent cases (42). Their specificity is affected by exposure to ART, co-infections, HIV-1 subtype, and long-term non-progressors and viremic controllers (37, 43-45). The false recency rate (specificity) needs to be taken into account in order to improve the utility of these assays (46, 47). To my knowledge there has not been any studies evaluating specificity of the cross sectional incidence assays in Botswana. I also tested the hypothesis that multi-assay algorithms are likely to increase specificity of the serological assays that previously demonstrated non-subtype C HIV-1 infections. Viral diversity-based assays are receiving increasing attention as complementary or new alternatives to immunoassays (38, 48-53) but there is limited data on how accurately these could be applied to characterize HIV recency.

In this dissertation, I assessed the incidence assays and multi-assay approaches to maximise their sensitivity and specificity in the context of high ART coverage and high HIV-1C prevalence in Botswana. I examined whether a novel biomarker based on HIV viral diversity can be used to characterize recency of HIV infection. In this thesis an innovative use of pairwise diversity and the time to the most recent common ancestor (tMRCA) in a heterosexual HIV-1C epidemic are explored as potential approaches for identifying recent infections. I further analyzed the properties of the new tools including their specificity and predictive performance in the context of the HIV-1C epidemic in Botswana. The thesis is organized in the form of manuscripts to address 3 specific aims.

## 1.4 Specific Aims

**Aim 1:** To identify and characterize early founder viruses using cross sectional serological markers.

**Aim 2:** To determine if virus genomic variation (pairwise diversity) can be used to determine recency of HIV infection

**Aim 3:** To determine if coalescence models can be used to estimate the time since HIV infection

## 1.5 Summary of enclosed publications

In Chapter 2, I describe the methods for identifying recent infection using an in-depth review of literature covering serological assays that measure changes in the immune response, application of genomics in the form of diversity based assays and standard application of mathematical models. The paper examines the successes and challenges in application of these methods in surveillance and research, and identifies opportunities for further development. The traditional approach of following HIV negative cohorts is logically complex, very expensive and may be subject to biases related to following up individuals over long periods. The cross-sectional approaches are therefore the most appealing but require evaluation, characterization and optimization in each local context.

The drawback of serological assays includes the variability across subtypes, long-term non-progressors, impact of exposure to ART, impact of HIV-1 viral load and co-infections. In chapters 3 and 4, I examined the strategies for improving the specificity of the cross-sectional assays using multi-assay algorithms.

In the papers enclosed in chapter 5 and 6, I describe the novel use of viral diversity to determine HIV recency of infection using pairwise distances or time to the most common recent ancestor (tMRCA). Viral diversity based assays are potential alternatives or complementary methodologies to immunoassays. Since intra-host diversity increases linearly within hosts from early to chronic phases of infection, I determined if it can be applied in determining HIV recency using a unique set of samples with known estimated time since infection.

## 1.6 Manuscripts enclosed in this thesis

The following papers are enclosed as part of this thesis:

### Chapter 2 – Identifying Recent HIV infections: From Serological Assays to Genomics

*Paper 1: Moyo, S., E. Wilkinson, V. Novitsky, A. Vandormael, S. Gaseitsiwe, M. Essex, S. Engelbrecht and T. de Oliveira (2015). Identifying Recent HIV Infections: From Serological Assays to Genomics. Viruses 7(10): 5508-5524.*

**Number of citations by 01 Aug 2016 = 3 citations**

### Chapter 3 – Improved cross-sectional estimate of HIV-1 subtype C incidence using multi-assay algorithms

*Paper 2: Moyo, S., T. LeCuyer, R. Wang, S. Gaseitsiwe, J. Weng, R. Musonda, H. Bussmann, M. Mine, S. Engelbrecht, J. Makhema, R. Marlink, M. K. Baum, V. Novitsky and M. Essex (2014). "Evaluation of the false recent classification rates of multiassay algorithms in estimating HIV type 1 subtype C incidence." AIDS Res Hum Retroviruses 30(1): 29-36.*

**Number of citations by 01 Aug 2016 = 6 citations**

*Paper 3: Wang, R., J. Weng, S. Moyo, D. Pain, C. D. Barr, D. Maruapula, D. Mongwato, J. Makhema, V. Novitsky and M. Essex (2013). Short communication: effect of short-course antenatal zidovudine and single-dose nevirapine on the BED capture enzyme immunoassay levels in HIV type 1 subtype C infection. AIDS Res Hum Retroviruses 29(6): 901-906.*

*Paper 4: Sikhulile Moyo, Kenanao P Kotokwe, Terence Mohammed,, Corettah Boleo, Lucy Mupfumi,, Samuel Chishala, Lesedi Tsalaile, Hermann Bussmann, Simani Gaseitsiwe, Rosemary Musonda, Joseph Makhema, Marianna Baum, Richard Marlink, Susan Engelbretch, M. Essex & Vladimir Novitsky. Low False Recent Rate of Limiting Antigen-Avidity Assay Combined*

*with HIV-1 RNA Data in Botswana.* [AIDS Res Hum Retroviruses 2016 Sep 7.](#)  
[Epub ahead of print]

## **Chapter 4 – Within-host pairwise diversity and recency of HIV infection**

*Paper 5:* Sikhulile Moyo, Alain Vandormael, Eduan Wilkinson, Susan Engelbrecht, Simani Gaseitsiwe, Kenanao P. Kotokwe, Rosemary Musonda , Frank Tanser , Max Essex , Vladimir Novitsky, Tulio de Oliveira. [PLOS One](#) (2016). *Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana – PLoS One.* 2016 Aug 23;11(8):e0160649.

## **Chapter 5 – Evolutionary dynamics in primary HIV-1C infection**

*Paper 6:* **Sikhulile Moyo**, Eduan Wilkinson, Alain Vandormael, Rui Wang, Jia Weng, Kenanao P. Kotokwe, Simani Gaseitsiwe, Rosemary Musonda, Joseph Makhema, M. Essex, Susan Engelbrecht, Tulio de Oliveira, and Vladimir Novitsky. *Pairwise Diversity and tMRCA as Potential Markers for HIV Infection Recency.* [MEDICINE](#) (Submitted – Under Review)

## **Chapter 6 - Summative Comment**

## **Chapter 7 – Additional Materials**

### **Conference Abstracts related to the thematic areas**

- 1) Conference on Retrovirus and Opportunistic Infections (CROI 2016)  
Moyo, S et al., ***Cross-sectional HIV Incidence in 24 communities in Botswana.***  
(Poster)

- 2) 21<sup>st</sup> International AIDS Society Conference (IAS July, AIDS2016)  
Moyo, S et al., ***Cross-sectional estimates of HIV incidence remain high in rural communities in Botswana in the era of successful scale-up of ART.*** (Oral Poster)
- 3) Viral Evolution and Molecular Epidemiology (VEME, Aug 2016)  
Moyo, S et al., ***Pairwise Diversity and tMRCA as potential markers for HIV Infection Recency.*** (Accepted, Poster)
- 4) HIV Dynamics and Evolution (April 2016)  
Moyo, S et al., ***Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana.*** (Accepted Poster)

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## CHAPTER 2

### **IDENTIFYING RECENT HIV INFECTIONS: FROM SEROLOGICAL ASSAYS TO GENOMICS**

**One paper in this chapter was published**

#### **Paper 1: Identifying Recent HIV Infections: From Serological Assays to Genomics**

**Moyo, S., E. Wilkinson, V. Novitsky, A. Vandormael, S. Gaseitsiwe, M. Essex, S. Engelbrecht and T. de Oliveira (2015). "Identifying Recent HIV Infections: From Serological Assays to Genomics." *Viruses* 7(10): 5508-5524.**

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*Review*

## Identifying Recent HIV Infections: From Serological Assays to Genomics

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Received: 9 July 2015 ; Accepted: 13 October 2015 ; Published: 23 October 2015

Academic Editor: Viktor Müller

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## About this article

### Paper 1: Identifying Recent HIV Infections: From Serological Assays to Genomics

Moyo, S., E. Wilkinson, V. Novitsky, A. Vandormael, S. Gaseitsiwe, M. Essex, S.

Engelbrecht and T. de Oliveira (2015). "Identifying Recent HIV Infections: From Serological Assays to Genomics." *Viruses* 7(10): 5508-5524.

**Abstract:** In this paper, we review serological and molecular based methods to identify HIV infection recency. The accurate identification of recent HIV infection continues to be an important research area and has implications for HIV prevention and treatment interventions. Longitudinal cohorts that follow HIV negative individuals over time are the current gold standard approach, but they are logistically challenging, time consuming and an expensive enterprise. Methods that utilize cross-sectional testing and biomarker information have become an affordable alternative to the longitudinal approach. These methods use well-characterized biological makers to differentiate between recent and established HIV infections. However, recent results have identified a number of limitations in serological based assays that are sensitive to the variability in immune responses modulated by HIV subtypes, viral load and antiretroviral therapy. Molecular methods that explore the dynamics between the timing of infection and viral evolution are now emerging as a promising approach. The combination of serological and molecular methods may provide a good solution to identify recent HIV infection in cross-sectional data. As part of this review, we present the advantages and limitations of serological and molecular based methods and their potential complementary role for the identification of HIV infection recency.

**Keywords:** recent HIV infection; viral diversity; serology-based assays, molecular-based assays

## CHAPTER 3

### IMPROVED CROSS-SECTIONAL ESTIMATE OF HIV-1 SUBTYPE C INCIDENCE USING MULTI-ASSAY ALGORITHMS

There are 3 manuscripts included in this chapter.

The text format is as required by the respective journals where published

**Paper 2: Evaluation of the false recent classification rates of multiassay algorithms in estimating HIV type 1 subtype C incidence**

**Moyo, S., T. LeCuyer, R. Wang, S. Gaseitsiwe, J. Weng, R. Musonda, H. Bussmann, M. Mine, S. Engelbrecht, J. Makhema, R. Marlink, M. K. Baum, V. Novitsky and M. Essex (2014). "Evaluation of the false recent classification rates of multiassay algorithms in estimating HIV type 1 subtype C incidence." *AIDS Res Hum Retroviruses 30(1): 29-36.***

**Paper 3: Effect of short-course antenatal zidovudine and single-dose nevirapine on the BED capture enzyme immunoassay levels in HIV type 1 subtype C infection**

Wang, R., J. Weng, **S. Moyo**, D. Pain, C. D. Barr, D. Maruapula, D. Mongwato, J. Makhema, V. Novitsky and M. Essex (2013). "Short communication: effect of short-course antenatal zidovudine and single-dose nevirapine on the BED capture enzyme immunoassay levels in HIV type 1 subtype C infection." ***AIDS Res Hum Retroviruses 29(6): 901-906.***

**Paper 4: Low False Recent Rate of Limiting Antigen-Avidity Assay Combined with HIV-1 RNA Data in Botswana**

**Sikhulile Moyo**, Kenanao P Kotokwe, Terence Mohammed,, Corettah Boleo, Lucy Mupfumi,, Samuel Chishala, Lesedi Tsalaile, Hermann Bussmann, Simani Gaseitsiwe, Rosemary Musonda, Joseph Makhema, Marianna Baum, Richard Marlink, Susan Engelbretch, M. Essex &Vladimir Novitsky. ***AIDS Res Hum Retroviruses. Sep 7 2016.***

## Paper 2: Evaluation of the false recent classification rates of multiassay algorithms in estimating HIV type 1 subtype C incidence

AIDS RESEARCH AND HUMAN RETROVIRUSES  
Volume 30, Number 1, 2014  
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DOI: 10.1089/aid.2013.0055

### Evaluation of the False Recent Classification Rates of Multiassay Algorithms in Estimating HIV Type 1 Subtype C Incidence

Sikhulile Moyo,<sup>1,2</sup> Tessa LeCuyer,<sup>1,3</sup> Rui Wang,<sup>4,5</sup> Simani Gaseitsiwe,<sup>1,6</sup> Jia Weng,<sup>5</sup> Rosemary Musonda,<sup>1,6</sup> Hermann Bussmann,<sup>1,6</sup> Madisa Mine,<sup>1,7</sup> Susan Engelbrecht,<sup>2,8</sup> Joseph Makheba,<sup>1,6</sup> Richard Marlink,<sup>1,6</sup> Marianna K. Baum,<sup>9</sup> Vladimir Novitsky,<sup>1,6</sup> and M. Essex<sup>1,6</sup>

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DOI: <https://dx.doi.org/10.1089/aid.2013.0055>

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## About this article

**Moyo, S., T. LeCuyer, R. Wang, S. Gaseitsiwe, J. Weng, R. Musonda, H. Bussmann, M. Mine, S. Engelbrecht, J. Makhema, R. Marlink, M. K. Baum, V. Novitsky and M. Essex (2014). "Evaluation of the false recent classification rates of multiassay algorithms in estimating HIV type 1 subtype C incidence." *AIDS Res Hum Retroviruses* 30(1): 29-36.**

### Summary

Laboratory cross-sectional assays are useful for estimation of HIV incidence, but are known to misclassify individuals with long-standing infection as recently infected. The false recent rate (FRR) varies widely across geographic areas; therefore, accurate estimates of HIV incidence require a locally defined FRR. We determined FRR for Botswana, where HIV-1 subtype C infection is predominant, using the BED capture enzyme immunoassay (BED), a BioRad Avidity Index (BAI) assay (a modification of the BioRad HIV1/2+O EIA), and two multi-assay algorithms (MAA) that included clinical data. To estimate FRR, stored blood samples from 512 antiretroviral (ARV)-naïve HIV-1 subtype C-infected individuals from a prospective cohort in Botswana were tested at 18–24 months post-enrolment. The following FRR mean (95% CI) values were obtained: BED 6.05% (4.15–8.48), BAI 5.57% (3.70–8.0), BED-BAI 2.25% (1.13–4.0), and a combination of BED-BAI with CD4 (>200) and viral load (>400) threshold 1.43% (0.58–2.93). The inter-assay agreement between BED and BAI was 92.8% (95%CI, 90.1–94.5) for recent/long-term classification. Misclassification was associated with viral suppression for BED [adjusted OR (aOR) 10.31; p = 0.008 ], BAI [aOR 9.72; p =0.019 ] and MAA1 [aOR 16.6 ; p = 0.006]. Employing MAA can reduce FRR to < 2%. A local FRR can improve cross-sectional HIV incidence estimates.

Key words: HIV-1 Subtype C, False Recent Rate HIV Incidence, Botswana, BED, Avidity

## **Paper 3: Effect of short-course antenatal zidovudine and single-dose nevirapine on the BED capture enzyme immunoassay levels in HIV type 1 subtype C infection**

AIDS RESEARCH AND HUMAN RETROVIRUSES  
Volume 29, Number 6, 2013  
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DOI: 10.1089/aid.2012.0294

### **Short Communication: Effect of Short-Course Antenatal Zidovudine and Single-Dose Nevirapine on the BED Capture Enzyme Immunoassay Levels in HIV Type 1 Subtype C Infection**

Rui Wang,<sup>1,2</sup> Jia Weng,<sup>2</sup> Sikhulile Moyo,<sup>3</sup> Debanjan Pain,<sup>4</sup> Christopher D. Barr,<sup>1</sup> Dorcas Maruapula,<sup>3</sup> Dineo Mongwato,<sup>3</sup> Joseph Makhema,<sup>3,5</sup> Vladimir Novitsky,<sup>3,5</sup> and M. Essex<sup>3,5</sup>

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**DOI:** <https://dx.doi.org/10.1089%2Faid.2012.0294>

Wang, R., J. Weng, **S. Moyo**, D. Pain, C. D. Barr, D. Maruapula, D. Mongwato, J. Makhema, V. Novitsky and M. Essex (2013). "Short communication: effect of short-course antenatal zidovudine and single-dose nevirapine on the BED capture enzyme immunoassay levels in HIV type 1 subtype C infection." **AIDS Res Hum Retroviruses 29(6): 901-906.**

## About this article

### Summary

Cross-sectional prevalence studies based on immunoassays that discriminate between recent and long-term infections, such as the BED assay, have been widely used to estimate HIV incidence. However, individuals receiving highly active antiretroviral therapy tend to have lower BED levels and are associated with a higher risk for being mistakenly classified as recent infections. To assess the effect of short-term antenatal Zidovudine (ZDV) and single-dose Nevirapine (sdNVP) on the BED levels in HIV-1C infection, we measured longitudinal BED optical density (OD-n) levels using stored plasma samples collected pre-and post-natally from 159 pregnant HIV-infected women in Botswana who participated in the randomized clinical Mother-to-Child-Prevention study, the *Mashi study*. All women received ZDV from 34 weeks gestation through delivery and were randomized to receive either sdNVP or placebo during labor. Among 159 subjects, the OD-n levels decreased from baseline to delivery in 93 subjects ( $p=0.039$ ), suggesting that short-course ZDV may decrease OD-n levels. sdNVP at delivery did not affect longitudinal BED OD-n levels post-delivery. However, sdNVP appeared to modify the association between CD4 count at delivery and OD-n levels post-delivery. When estimating HIV incidence with the BED assay, special care may be required regarding women who received short-term ZDV for prevention of mother-to-child transmission.

Key Words: BED, HIV incidence, mother-to-child transmission, Nevirapine, Zidovudine.

## Paper 4: Low False Recent Rate of Limiting Antigen-Avidity Assay Combined with HIV-1 RNA Data in Botswana

AIDS RESEARCH AND HUMAN RETROVIRUSES  
Volume 00, Number 00, 2016  
© Mary Ann Liebert, Inc.  
DOI: 10.1089/aid.2016.0127

SHORT COMMUNICATION

### Low False Recent Rate of Limiting Antigen-Avidity Assay Combined with HIV-1 RNA Data in Botswana

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Simani Gaseitsiwe,<sup>2,4</sup> Rosemary Musonda,<sup>2,4</sup> Joseph Makhema,<sup>2,4</sup> Marianna Baum,<sup>5</sup>  
Richard Marlink,<sup>2,4</sup> Susan Engelbrecht,<sup>1,6</sup> Max Essex,<sup>2,4</sup> and Vladimir Novitsky<sup>2,4</sup>

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PubMed Link: <https://www.ncbi.nlm.nih.gov/pubmed/27481530/>

DOI: <https://dx.doi.org/10.1089/AID.2016.0127>

**Sikhulile Moyo**, Kenanao P Kotokwe, Terence Mohammed, Corettah Boleo, Lucy Mupfumi, Samuel Chishala, Lesedi Tsalaile, Hermann Bussmann, Simani Gaseitsiwe, Rosemary Musonda, Joseph Makhema, Marianna Baum, Richard Marlink, Susan Engelbretch, M. Essex & Vladimir Novitsky. Low False Recent Rate of Limiting Antigen-Avidity Assay Combined with HIV-1 RNA Data in Botswana. *AIDS Res Hum Retroviruses 2016 Sep 7. [Epub ahead of print]*

## About this article

### Summary

Cross-sectional estimation of HIV incidence could misclassify some established or chronic HIV infections as recent. Usually long-term non-progressors, elite and viremic controllers, and individuals on ART contribute to misclassification. Local data on the false recent rate (FRR) could minimize misclassification during estimation of HIV incidence. To improve monitoring of HIV incidence, we estimated local FRR in Botswana. A total of 1036 specimens from individuals infected for least 1.5 to 2 years were sampled between 2004 and 2009, and tested using the Limiting Antigen-Avidity assay (LAG) using a cutoff of 1.5 normalized optical density units (OD-n). The FRR was 0.97% (10/1036; 95% confidence interval (CI) 0.46–1.77). Four samples had HIV-1 RNA >1000 cps/mL, giving an adjusted FRR of 0.39% (4/1036; 95% CI 0.11–0.99). A combination of LAG and HIV-1 RNA load data resulted in FRR below 1% in the Botswana population.

## CHAPTER 4

### WITHIN-HOST PAIRWISE DIVERSITY AND RECENCY OF HIV INFECTION

One manuscripts of this chapter was published

The text format is as required by the respective journals where published

#### **Paper 5: Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana**

Sikhulile Moyo, Alain Vandormael, Eduan Wilkinson, Susan Engelbrecht, Simani Gaseitsiwe, Kenanao P. Kotokwe, Rosemary Musonda , Frank Tanser , Max Essex , Vladimir Novitsky, Tulio de Oliveira. Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana. PLoS One. 2016 Aug 23;11(8):e0160649.

## Paper 5: Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana



RESEARCH ARTICLE

# Analysis of Viral Diversity in Relation to the Recency of HIV-1C Infection in Botswana

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PubMed Link: <https://www.ncbi.nlm.nih.gov/pubmed/27552218/>

DOI: <https://dx.doi.org/10.1371/journal.pone.0160649>

Sikhulile Moyo, Alain Vandormael, Eduan Wilkinson, Susan Engelbrecht, Simani Gaseitsiwe, Kenanao P. Kotokwe, Rosemary Musonda, Frank Tanser, Max Essex, Vladimir Novitsky, Tulio de Oliveira. Analysis of viral diversity in relation to the recency of HIV-1C infection in Botswana. **PLoS One.** 2016 Aug 23;11(8):e0160649.

## About this paper

### Summary

**Background:** Cross-sectional, biomarker methods to determine HIV infection recency present a promising and cost-effective alternative to the repeated testing of uninfected individuals. We evaluate a viral-based assay that uses a measure of pairwise distances (PwD) to identify HIV infection recency, and compare its performance with two serologic incidence assays, BED and LAg. In addition, we assess whether combination BED plus PwD or LAg plus PwD screening can improve predictive accuracy by reducing the likelihood of a false-recent result.

**Methods:** The data comes from 854 time-points and 42 participants enrolled in a primary HIV-1C infection study in Botswana. Time points after treatment initiation or with evidence of multiplicity of infection were excluded from the final analysis. PwD was calculated from quasispecies generated using single genome amplification and sequencing. We evaluated the ability of PwD to correctly classify HIV infection recency within <130, <180 and <360 days post-seroconversion using Receiver Operator Characteristics (ROC) methods. Following a secondary PwD screening, we quantified the reduction in the relative false-recency rate (rFRR) of the BED and LAg assays while maintaining a sensitivity of either 75, 80, 85 or 90%.

**Results:** The final analytic sample consisted of 758 time-points from 40 participants. The PwD assay was more accurate in classifying infection recency for the 130 and 180-day cut-offs when compared with the recommended LAg and BED thresholds. A higher AUC

statistic confirmed the superior predictive performance of the PwD assay for the three cut-offs. When used for combination screening, the PwD assay reduced the rFRR of the LAg assay by 52% and the BED assay by 57.8% while maintaining a 90% sensitivity for the 130 and 180-day cut-offs respectively.

**Conclusion:** PwD can accurately determine HIV infection recency. A secondary PwD screening reduces misclassification and increases the accuracy of serologic-based assays.

**Abbreviations:** PwD, pairwise diversity; BED, Calypte Incidence Assay; LAg, Limiting Antigen Assay; ROC, receiver operator characteristics; FRR, false-recency rate; ART, antiretroviral treatment; TPR, true positive rate; AUC, area under the curve.

**Keywords:** HIV recency rate; False recency rate; Pairwise diversity; HIV1-C; BED; Limiting Antigen Assay; Botswana

## CHAPTER 5

### EVOLUTIONARY DYNAMICS IN PRIMARY HIV-1C INFECTION

**One manuscript (unpublished) was submitted MEDICINE JOURNAL and is under review.**  
The text format is as required by the respective journals where published

#### Paper 6: Pairwise Diversity and tMRCA as Potential Markers for HIV Infection Recency

**Sikhulile Moyo**, Eduan Wilkinson, Alain Vandormael, Rui Wang, Jia Weng, Kenanao P. Kotokwe, Simani Gaseitsiwe, Rosemary Musonda, Joseph Makhema, M. Essex, Susan Engelbrecht, Tulio de Oliveira, and Vladimir Novitsky. MEDICINE (**SUBMITTED - Underview**)

##### About this paper

##### Summary

Intra-host HIV-1 diversity increases linearly over time. We assessed the extent to which mean pairwise distances and the time to the most recent common ancestor (tMRCA) inferred from intra-host HIV-1C env sequences are associated with the estimated time of HIV infection. Data from a cohort of 42 participants with estimated time of seroconversion from the primary HIV-1C infection study in Botswana were used in this analysis. A total of 2,540 HIV-1C env gp120 V1C5 viral sequences were generated by single genome amplification and sequencing, an average of 61 sequences per participant and 11 per time point per participant. Raw pairwise distances were calculated per time point per patient using the ape package in R. The tMRCA was estimated per time per patient using phylogenetic inference implemented in BEAST v1.8.2. Pairwise distances and tMRCA were significantly associated with the estimated time since HIV infection (both  $p < 0.001$ ), and taking into account multiplicity of HIV infection strengthened these associations. HIV-1C env-based pairwise distances and tMRCA can be used as potential markers for HIV recency. However, the estimated tMRCA demonstrated no advantage over pairwise distances.

Keywords: tMRCA, pairwise distances, HIV recency, HIV incidence, HIV-1C.

## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSIONS

The publications included in this dissertation focused on the characterization of recency of HIV infection and the exploration of novel biomarkers for estimation of HIV incidence based on application of immunologic and molecular methods in the context of a HIV-1 subtype C epidemic in southern Africa. These studies could guide and set priorities for further population-based studies addressing cross-sectional HIV incidence, and to design and monitor interventions that aim to reduce and eliminate new HIV infections [1-3]. Although progress has been made in development and validation of cross-sectional tests for recent HIV infection (TRI), new biomarkers and improved algorithms are required.

Research included in the thesis demonstrated that specificity of cross-sectional TRIs can be improved by algorithms that combine multiple assays, clinical data on disease progression (viral load/CD4), and viral diversity. To my knowledge, including viral load in a multi-assay algorithm (MAA) resulted in the one the lowest false recency rate of the Limiting Antigen Avidity assay ever reported (paper 4). This MAA was used for estimating cross-sectional HIV incidence in Botswana [14]. A novel biomarker based on HIV diversity was investigated as a potential tool for characterizing recency of HIV infection and inferring time since infection. This work has major implications for tracking newly transmitted HIV infections and evaluation of interventions, and is particularly important for Southern Africa. With increasing access to antiretroviral therapy in Southern Africa, the rapid and cost effective measurements of HIV incidence are critical for the success of public health interventions.

The research included in the thesis was conducted in the country with one of the highest HIV prevalence in the world, in a predominantly HIV-1 subtype C epidemic [1, 4]. Despite the burden of severe HIV-1C epidemic, Botswana has led one of the strongest responses to the HIV epidemic, being the first African country to offer its qualifying citizens with free antiretroviral drugs, care and laboratory monitoring.

The following topics were investigated:

- a) Evaluation of the long-term specificity (false recent classification rates) of serological tests for recent HIV infection, and algorithms for estimating HIV-1C incidence utilizing samples from patients with known long-standing HIV infection.
- b) Application of within-host viral diversity for estimation of HIV-1C recency in Botswana using samples collected from patients with known time since seroconversion in the primary HIV-1C infection cohort.
- c) Investigation of intra-host viral pairwise diversity and the time to the most common recent ancestor (tMRCA), as potential markers for HIV infection recency.

### ***Characterisation of Serological Tests for Recency of HIV Infection***

In the review paper [5] we detailed the immense progress that has been made in the development of biomarkers for HIV recency estimation. These methods have become a reasonable alternative to the longitudinal approach for rapid assessment of interventions and in surveillance studies. However, serological biomarkers are sensitive to the variability in immune responses modulated by HIV-1 subtypes, viral load and antiretroviral therapy, resulting in low specificity. Implementing algorithms that include viral load, ART status and CD4 can reduce false recency rates [6]. We demonstrated that applying an algorithm including documented ART status resulted in the, to our knowledge, lowest false recent rate of the Limiting Antigen Avidity Assay (LAG) of 0.4% in Botswana. However, this algorithm is limited if ART status is unknown or self-reported. It has become feasible to test for presence of ARV drugs traces to verify use of ART [7]. However, this is costly and not yet widely available. Many countries are going to implement universal test and treat strategies, or general early access to treatment regardless of CD4 status. This means that the serological incidence assays affected by exposure to ARVs may become less useful in identifying recent infections. There is therefore an urgent need for new biomarkers that are less sensitive to ARV use.

### ***Application of within-host viral diversity for estimation of HIV-1C recency***

Viral diversity-based methods are becoming an attractive alternative to serological methods. These methods take advantage of the linear nature of the viral diversification over time [8-10]. Using samples from individuals with known time since HIV infection, we demonstrated that pairwise diversity is significantly correlated with time since infection and can accurately

discriminate between recent and long-standing infection. We also found that presence of multiple founder strains affects the performance of the pairwise diversity assay and developed a method to characterise multiplicity of infection. We also demonstrated that a combination of serological and molecular methods not only improves the performance of these methods but also provides a potentially cost-effective and practical alternative for large population surveys.

Estimating dates of HIV infection is important for HIV incidence estimates and also for understanding the viral dynamics during early infection. These dates are often not known due to the long latency period of HIV and the lack of frequent HIV testing, while establishing primary infection cohorts is challenging and costly. We asked the research question whether we could estimate the time since infection using within-host pairwise viral diversity and time to the most common recent ancestor inferred from a pool of viral quasispecies. To our knowledge this was the first application of tMRCA for HIV recency determination in a heterosexual HIV-1C epidemic compared to one previous application in HIV-1 subtype B-infected men who have sex with men (MSM) and injection drug users. We found that pairwise distances and tMRCA were significantly associated with the estimated time since HIV infection and taking into account multiplicity of HIV infection can further strengthen these associations. This means that HIV-1C env-based pairwise distances and tMRCA can be used as potential markers for HIV recency and can help resolve instances of multiple founder viruses, providing a critical impact on the specificity of the serological assays and other simple measures of diversity.

### ***Application of cross-sectional methods in community based surveys***

Cross-sectional incidence testing is becoming a critical part of designing and calculating needed sample sizes in prevention studies. Some publications included in this thesis provided a good baseline for implementation in ongoing studies [11]. Using a cross-sectional sampling approach, HIV incidence was estimated at the baseline of the Botswana Combination Prevention Project (BCPP) in 30 rural communities from Nov 2013 to Nov 2015. The algorithm for estimation of HIV recency combined Limiting-Antigen Avidity Assay (LAG) data, ART status and HIV-1 RNA load. This study confirmed modelling estimates that suggest that the incidence rate in Botswana is between 1.1 and 1.4% in the past 2 years [1, 12, 13]. Given the high level of ART scale-up [11, 14] in Botswana, studies able to identify HIV transmission sources and reduce HIV incidence are warranted.

### ***Overall limitations of the work***

There are several limitations to this work. The publications on characterisation of serological assays were based on cohorts sampled mostly in the capital city of Botswana and surrounding villages. It would be desirable to have a wider geographical representation of sampling. However, Gaborone is the economic capital of Botswana and has a wide representation of people from different regions. Increasing specificity of the serological assays requires viral load testing and possible ARV drug tracing that may not be available in many resource settings. It is envisaged that with the widespread implementation of test and treat, the UNAIDS 90-90-90 targets, viral load will become more available even in low- to medium-income countries. The application of viral diversity and tMRCA requires single-genome amplification and direct sequencing, which is expensive and labor intensive. The advent of high-throughput sequencing platforms is likely to drive the costs down. As we demonstrated, pairwise diversity could be used as a complementary or confirmatory assay to the serological methods in multi-assay algorithms.

### ***Conclusion***

Cross-sectional estimation of HIV incidence is practical and feasible using a combination set of serological and molecular methods. Specificity of the methods can be improved with use of algorithms that include at least HIV-1 RNA load as described in the thesis publications. Pairwise diversity and tMRCA are promising new tools to refine HIV recency estimation. In addition, the usage of phylogenetic methods to characterise and better understand HIV transmission dynamics is essential for elimination of new HIV infections. Applying these methods in a heterosexually transmitted HIV-1C epidemic in Botswana allowed us to identify sub-epidemics [15] and also estimate population-level HIV incidence estimates [1].

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## CHAPTER 7

### ADDITIONAL MATERIALS

The following materials will be attached as appendices

#### 6.1 List of Conference Abstracts related to the thematic areas

1. Conference on Retrovirus and Opportunistic Infections (CROI 2016)

*Moyo, S et al., Cross-sectional HIV Incidence in 24 communities in Botswana.  
(Poster)*

2. 21<sup>st</sup> International AIDS Society Conference (IAS July, AIDS2016)

*Moyo, S et al., Cross-sectional estimates of HIV incidence remain high in rural  
communities in Botswana in the era of successful scale-up of ART. (Oral Poster)*

3. Viral Evolution and Molecular Epidemiology (VEME, Aug 2016)

*Moyo, S et al., Pairwise Diversity and tMRCA as potential markers for HIV Infection  
Recency. (Accepted, Poster)*

4. HIV Dynamics and Evolution (April 2016)

*Moyo, S et al., Analysis of viral diversity in relation to the recency of HIV-1C  
infection in Botswana. (Accepted Poster)*

## APPENDICES

**APPENDIX 1:** Journal format versions of the open access publications

**APPENDIX 2:** Conference abstracts related to the thematic areas

# Appendix 1

## RESEARCH ARTICLE

# Analysis of Viral Diversity in Relation to the Recency of HIV-1C Infection in Botswana

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**Citation:** Moyo S, Vandormael A, Wilkinson E, Engelbrecht S, Gaseitsiwe S, Kotokwe KP, et al. (2016) Analysis of Viral Diversity in Relation to the Recency of HIV-1C Infection in Botswana. PLoS ONE 11(8): e0160649. doi:10.1371/journal.pone.0160649

**Editor:** Jean-Luc EPH Darlix, "INSERM", FRANCE

**Received:** March 18, 2016

**Accepted:** July 23, 2016

**Published:** August 23, 2016

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**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files. Accession numbers have been provided in the supplementary files

**Funding:** This work was supported was supported from the National Institutes of Health (NIH) Fogarty International Center (Grant # 5D43TW009610) and the OAK Foundation Fellowship (Grant # OUSA-12-025). The primary HIV-1C infection study in Botswana ("Tshedimoso Study") was supported and funded by the NIH R01 AI057027. FT, AV, TD were supported by a South African MRC Flagship grant (MRC-RFA-UFPSP-01–2013/UKZN HIVEPI). FT was partially supported by an Academy of Medical Sciences-

## Abstract

## Background

Cross-sectional, biomarker methods to determine HIV infection recency present a promising and cost-effective alternative to the repeated testing of uninfected individuals. We evaluate a viral-based assay that uses a measure of pairwise distances (PwD) to identify HIV infection recency, and compare its performance with two serologic incidence assays, BED and LAg. In addition, we assess whether combination BED plus PwD or LAg plus PwD screening can improve predictive accuracy by reducing the likelihood of a false-recent result.

## Methods

The data comes from 854 time-points and 42 participants enrolled in a primary HIV-1C infection study in Botswana. Time points after treatment initiation or with evidence of multiplicity of infection were excluded from the final analysis. PwD was calculated from quasispecies generated using single genome amplification and sequencing. We evaluated the ability of PwD to correctly classify HIV infection recency within <130, <180 and <360 days post-seroconversion using Receiver Operator Characteristics (ROC) methods. Following a secondary PwD screening, we quantified the reduction in the relative false-recency rate (rFRR) of the BED and LAg assays while maintaining a sensitivity of either 75, 80, 85 or 90%.

## Results

The final analytic sample consisted of 758 time-points from 40 participants. The PwD assay was more accurate in classifying infection recency for the 130 and 180-day cut-offs when compared with the recommended LAg and BED thresholds. A higher AUC statistic

Newton Advanced Fellowship. TdO is partially supported by an Royal Society-Newton Advanced Fellowship. The funders had no role in the study design, data collection and decision to publish, or in the preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

**Abbreviations:** PwD, pairwise diversity; BED, Calypte Incidence Assay; LAg, Limiting Antigen Assay; ROC, receiver operator characteristics; FRR, false-recency rate; ART, antiretroviral treatment; TPR, true positive rate; AUC, area under the curve.

confirmed the superior predictive performance of the PwD assay for the three cut-offs. When used for combination screening, the PwD assay reduced the rFRR of the LAg assay by 52% and the BED assay by 57.8% while maintaining a 90% sensitivity for the 130 and 180-day cut-offs respectively.

## Conclusion

PwD can accurately determine HIV infection recency. A secondary PwD screening reduces misclassification and increases the accuracy of serologic-based assays.

## 1.0 Background

Identification of HIV infection recency is crucial for the accurate estimation of HIV incidence, the evaluation of the effectiveness of antiretroviral treatment (ART) programs, and the timely linking of HIV-infected individuals (and their partners) to treatment and care services [1–9]. The timing of infection can also be used to identify the immunological and virological characteristics of individuals who have recently acquired HIV and to characterize individuals who are putative transmitters in linked infections [10–14].

The longitudinal cohort design is currently recognized as the standard approach to identify new HIV infections [15–17]. However, frequent HIV testing at the population level is a logistically challenging, time-consuming, and expensive enterprise. For these reasons, large-scale surveillance programs are typically undertaken on a periodic basis of 12 or more months, making it difficult to ascertain the precise date of an HIV infection. Factors associated with illness, work commitments, temporary or cyclical migration, assumed knowledge of current HIV status, and the stigma associated with a positive status, among others, may decrease the frequency at which an eligible individual is captured for HIV testing [18–20]. On the other hand, the identification of new HIV infections is possible for experimental trials where relatively small cohorts (typically <500 individuals) are routinely tested on a weekly or monthly basis [21–23].

There is growing scientific interest in the use of cross-sectional sampling methods to identify individuals recently infected with HIV. Cross-sectional methods can mitigate the impact of infrequent testing and the high lost-to-follow-up rates that are associated with the longitudinal approach [24–28]. Biomarker data collected from cross-sectional sampling has also shown great promise in the ability to differentiate between recent and established HIV infections. Serological assays, for example, the Calypte Incidence Assay (BED) and Limiting Antigen assay (LAG), depend on the markers of evolution of the host immune response to HIV, such as antibody levels, avidity, isotype and proportion [29–35]. Attention is now turning to the improvement of assay-based methods and the use of multi-assay algorithms (MAA) to better predict HIV infection recency [36].

One area that is receiving increasing attention is the use of a viral diversity measure [11, 13, 37–41]. The majority of HIV infections are caused by the transmission of a single founder virus, resulting in a relatively homogeneous population of viral quasispecies during the early stage of HIV infection [42–45]. Due to the error prone nature of the Reverse Transcriptase (RT) enzyme and the host immune response to pressure, the virus is able to diversify rapidly over time. The approximately linear diversification of HIV in early infection [46] provides a rationale for using viral diversity as a marker for HIV infection recency [11, 39, 47, 48]. One example of a time-dependent, viral-based diversity measure is the pairwise nucleotide diversity (PwD). PwD measures the average number of pairwise nucleotide differences per site in DNA

sequences [11, 37, 38, 43, 49, 50]. Assays based on a measure of PwD should be less sensitive to the variability in immune responses modulated by HIV clade, host genetics and routes of transmission. However, viral-based assays are more challenging and costly to implement.

About 20–25% of HIV infections are caused by the transmission of multiple viral variants [43, 51–53]. The rate of HIV-1 super-infection could be comparable with the rate of primary HIV-1 infection [54], although super-infection is less frequent in the HIV-1C epidemic in South Africa [55]. Ignoring multiplicity of HIV infection could mislead analysis and lead to erroneous conclusions due to increased intra-host diversity in cases with multiple transmitted HIV variants, or in super-infection. Using intra-host viral sequences that represent HIV quasispecies provides an opportunity to identify phylogenetically distinct viral lineages and take into account multiplicity of HIV infection.

In this paper, we use data from a frequently tested longitudinal HIV-1C infection cohort (the “Tshedimoso” study from Botswana) for which the exact date of infection is known. We assess the accuracy of the PwD assay to correctly classify HIV infection recency, and compare its performance with the BED and LAg assays. Because of the high cost currently associated with single genome sequencing, we further investigate the use of a MAA (BED plus PwD or LAg plus PwD) to increase accuracy and maintain affordability. We also evaluate the addition of viral load (VL) as a covariate to the MAA algorithm. We discuss the potential of cross-sectional, biomarker information and the use of MAAs as an affordable and accurate alternative to the longitudinal cohort approach.

## 2.0 Methods

### 2.1 Participants and specimens

The data comes from 854 time points and 42 study participants enrolled into a primary HIV-1 subtype C infection longitudinal cohort in Botswana (the “Tshedimoso” study) from April 2004 to April 2008 [56, 57]. Recent HIV-1 infections were identified by a positive HIV-1 RNA test combined with a negative HIV-1 serology in double enzyme immunoassay [58] or by applying a 2-step testing algorithm using the Vironostika HIV-1 Plus O Microelisa System (bioMérieux, Durham, NC) [59]. Acutely infected participants had weekly visits for the first 2 months, biweekly visits for the next 2 months and monthly visits for the first year following the date of seroconversion. Participants were then followed-up on a quarterly basis after the first post-seroconversion year. The study design and participant characteristics are described in greater detail elsewhere [56, 59, 60]. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Boards of Botswana and the Harvard School of Public Health. All patients provided written informed consent for the collection of samples and subsequent analysis.

### 2.2 Serological assays and HIV pairwise diversity for recency determination

Blood specimens from 42 participants were used to generate 594 BED (Calypte Aware BED HIV-1 Incidence Test, Calypte Biomedical Corporation; Portland, USA) and 597 LAg (Limiting Antigen Assay, Sedia BioSciences; Portland, USA) test results according to manufacturers' instructions [34, 35]. All available specimens were included for testing with both serological assays. UNAIDS/WHO guidelines for determining infection recency recommend the removal of specimens with evidence of ART use [33, 61, 62], resulting in the exclusion of 49 time points and one participant from our analysis (see [S1 Fig](#) of the Supplement).

The intra-host viral sequences representing HIV-1C quasispecies were generated by single genome amplification and sequencing, as described elsewhere [47, 63]. The primary goal of sequencing was analysis of viral diversity and evolution during primary HIV-1C infection [47, 63]. The quarterly time points spanning the period from the earliest sample at enrollment to about 500 days post seroconversion were selected from the available sampling points ([S4 Fig](#)). Individuals with acute HIV-1C infection were sampled more frequently than individuals enrolled during Fiebig stages IV-V [64].

The targeted region spanned HIV-1C *env* gp120 V1C5 corresponding to nucleotide positions 6,615 to 7,757 of HXB2. A total of 2,540 single genome amplification sequences were generated from an average of 6 time points per patient and an average of 10 multiple-sequences (quasispecies) per time point. Both viral RNA and proviral DNA were used as templates for amplification and sequencing. Viral sequences were codon-aligned by muscle [65] in MEGA 6.06 [66]. Mean pairwise distances (PWD) were estimated per participant per time point using the Maximum Composite Likelihood model and pairwise deletion of gaps in MEGA 6.06 [66]. The accession numbers of the viral sequences used in this study are KC628761—KC630726.

### 2.3 Multiplicity of HIV infection

Previous research has shown that multiplicity of infection can result in highly variable PWD values [33, 61, 62]. For this reason, we undertook a phylogenetic analysis to identify and exclude time points with multiple founder variants or potential super-infection. Multiplicity was determined by the branching topology of viral quasispecies (~1,200 bp V1C5 region of HIV-1 env gp120) derived from a single time point of sampling. A total of 2,540 viral sequences from 42 subjects were analyzed with 1322 HIV-1 subtype C V1C5 sequences retrieved from the Los Alamos National Laboratory (LANL) HIV Database ([S2 Table](#)). Phylogenetic trees were inferred by the Maximum-Likelihood (ML) using Fasttree v.2.1.8 with a GTR model of nucleotide substitution [67]. Phylogenetic trees were visualized and inspected in FigTree [68]. Monophyletic clustering was interpreted as HIV transmission from a single source including transmission of multiple viral variants from the same source. We excluded 47 time points and one participant with viral quasispecies separated by reference sequence(s), as these were interpreted as HIV transmissions from multiple sources (including potential super-infection). The final sample size was 758 time-points from 40 participants (see [S1 Fig](#) of the Supplement).

### 2.4 Statistical Analysis

We used a receiver operating characteristics (ROC) analysis to compare the accuracy of the BED, LAg and Pwd assays to identify HIV infection recency. Frequent and repeated testing of study participants enabled us to identify the *known* instances of a recent HIV infection. Specifically, known HIV infection recency was defined as any specimen obtained within a <130, <180 or <360-day post-seroconversion period. For each BED, LAg and Pwd assay, we then classified a specimen as a “recent” infection if it was below a threshold value, or classified the specimen as an “established” infection if it was above this threshold. We refer to these as the *classified* instances of a recent HIV infection [69]. For example, we classified specimens with a BED value  $\leq 0.8$  as a recent infection or an established infection otherwise. The recommended threshold values for the BED and LAg assays are 0.8 and 1.5 respectively [34, 70].

The best performing thresholds for the Pwd assay have yet to be definitively established. Previous research has suggested that the rate of increase in the pairwise sequence diversity of the HIV-1 *env* gene region is a constant rate of approximately 0.01 per year during early infection [46]. We therefore used these biological guidelines to select Pwd thresholds of 0.004, 0.005 and 0.01 for the 130, 180 and 360-day cut-offs respectively. For each threshold, we

obtained the sensitivity (recent infections correctly identified) and the specificity (established infections correctly identified) using maximum likelihood estimates from a logistic regression analysis. Because repeated measurements were taken for each participant over time, we calculated the standard errors and 95% confidence intervals (CI) for these estimates using the Huber-White sandwich estimator [71, 72]. Given the evaluation of multiple test thresholds, we used the highest percentage of specimens correctly classified (CC) as a guide to evaluate the performance of each PwD threshold. The CC is computed as the sum of the recent and established specimens correctly classified divided by the total number of specimens classified.

We next evaluated the predictive performance of combination BED plus PwD screening to determine infection recency, and repeated this procedure for combination LAg plus PwD screening. Specifically, our aim was to determine whether the more affordable BED or LAg assay can be combined with the more sensitive PwD assay to reduce the likelihood of a false-recent classification. We first screened for recent infections using a recommended BED threshold of 0.8 for the 180-day cut-off and a recommended LAg threshold of 1.5 for the 130-day cut-off. The threshold and cut-off combination selected for the analysis are based on the work of Kassanjee et al. and Duong et al. [33–35]. We then used the PwD assay with a threshold of 0.005 to reduce the false-recency rate associated with the primary BED and LAg screening assays. Shaw et al. [73] propose to obtain the relative true-recent rate (rTRR) and the relative false-recent rate (rFRR) of the combined BED (or LAg) and PwD assays with:

$$rTRR = \frac{P(BED = +, PWD = + | R)}{P(BED = + | R)} \quad \text{and}$$

$$rFRR = \frac{P(BED = +, PWD = + | \bar{R})}{P(BED = + | \bar{R})}.$$

In the above equations, *BED+* and *PwD+* are the specimens classified as recent infections by the respective assay, *R* denotes the specimens known to be recent infections and  $\bar{R}$  denotes the specimens known to be established infections. When considering the use of a second marker to improve predictive performance, it is expected that a high rTRR (sensitivity) is maintained while the rFRR is reduced, such that the rTRR will be close to 1.0 and the rFRR will be substantially less than 1.0 [73]. We evaluate the percentage reduction in the rFRR by the PwD assay at rTRR (sensitivity) levels of 75%, 80%, 85% and 90%. Further, we show how the addition of viral load (VL) information can improve accuracy. Research has shown that VL measurements <1,000 copies/mL are associated with false-recent infections and can identify individuals with viral suppression [74, 75]. We used the methods of Shaw et al. [73], Janes et al. [76] and Pepe et al. [77] to obtain estimates for the rFRR and its 95% confidence intervals. Statistical analyses were undertaken in Stata 13.1.

The mean duration of recent infection (MDRI), the average time being recent while infected for less than time cut-off time (*T*) was estimated using the Incidence Estimation Tools version 1.0.5.9001 (The *inctools* package in R software version 3.2.4). The *T* value of 2 years and time points with viral load above 1,000 copies/mL were used for the MDRI calculation.

### 3.0 Results

All of the 2,540 sequences from the 42 participants in the cohort were classified as subtype C. To account for multiplicity of HIV infection and avoid inflated estimate of HIV pairwise distances, time points with phylogenetically distinct viral lineages ( $n = 47$ ) were excluded from analysis (see section 2.3 Multiplicity of HIV infection in *Methods*). The final analytic sample consisted of 758 (BED = 554, LAg = 579, and PwD = 238) time-points from 40 participants

(see [S1 Fig](#) of the Supplement for the data flow diagram). Among the study participants, 28 (70%) were female. The median (IQR) age at enrollment was 27 (20–56) years. Participants were followed for a median (IQR) of 45.9 (32.4–53.9) months, with a median (IQR) of 21 (18–27) time points per participant. The mean (SD) and median (IQR) time between tests were 2.0 ( $\pm 2.9$ ) months and 1.1 (0.92–3.0) months respectively. [Table 1](#) shows the summary statistics for the participant characteristics and covariate measures.

We present the maximum likelihood estimates for the PwD assay in [S1 Table](#) of the supplement. Given that there is currently no recommended PwD threshold, we show the sensitivity and specificity estimates for values ranging from 0.0005 to 0.015. For the 130-day cut-off, a PwD threshold of 0.004 gives a sensitivity of 76.2% and a specificity of 79.7%, with 77.8% of the total specimens correctly classified. For the 180-day cut-off, a PwD threshold of 0.005 gives a sensitivity of 74.5% and a specificity of 75.5%, with 74.9% of the total specimens correctly classified. We found that PwD values of 0.0055 and 0.006 performed slightly better than the 0.004 and 0.005 values for both the 130 and 180-day cut-offs, and are biologically plausible given that HIV is known to evolve at a rate of approximately 0.01 per year.

We found the PwD threshold values (reported above) to be more accurate than the recommended LAg = 1.5 and BED = 0.8 threshold values in identifying infection recency. For a 130-day cut-off and a threshold value of 1.5, the LAg assay gives a sensitivity of 71.3% and a specificity of 72.9%, with 72.4% of the total specimens correctly classified. For a 180-day cut-off and a threshold value of 0.8, the BED assay gives a sensitivity of 87.4% and a specificity of 50.2%, with 65.5% of the total specimens correctly classified. For these cut-offs and thresholds, we see that the PwD assay has a higher proportion of specimens correctly classified when compared with the LAg and BED assays.

We also compare the accuracy of the three assays to identify infection recency using the AUC estimate of a ROC graph. An AUC closer to 1.0 indicates a better accuracy, and we show these estimates along with their standard errors and 95% CIs in [Table 2](#). The AUC value for the 130-day cut-off is 0.83 compared with 0.78 for the BED assay and 0.81 for the LAg assay. For the 180-day cut-off, these values are PwD = 0.82, BED = 0.75, and LAg = 0.79 and for the 360-day cut-off these are PwD = 0.78, BED = 0.74, and LAg = 0.72 (see also [Fig 1](#)).

We investigated whether MAA could further distinguish recent from established infections. [Table 3](#) shows the ability of the PwD assay to improve predictive accuracy by reducing the relative false-recent rate (rFRR) of the LAg and BED assays. Here, we are specifically interested in the percentage reduction in the rFRR and so we subtract the rFRR estimate from 100%. As an example, we interpret the result for the LAg plus PwD combination screening for the 130-day

**Table 1. Participant and covariate characteristics.**

Participant Characteristics	n = 40	
Female, N (%)	28	(70)
Age (years), Median (IQR)	27	(20–56)
Time under observation (months), Median (IQR)	45.9	(32.4–53.9)
Difference between time points (months), Median (IQR)	1.1	(0.92–3.0)
Total time points per participant, Median (IQR)	21	(18–27)
Assay time points per participant, Median (IQR)		
BED	14	(10–19)
Lag	14.5	(7–22)
PwD	5	(4–6)
CD4 cells/ $\mu$ l, Median (IQR)	417	(302–569)
Viral load ( $\log_{10}$ copies/mL, Median (IQR)	3.9	(2.65–4.73)

doi:10.1371/journal.pone.0160649.t001

**Table 2.** Area under the curve (AUC) of a receiver-operator characteristics (ROC) graph comparing the accuracy of the PwD, BED, and LAg assays in identifying HIV infection recency.

Assay	AUC	SE	95% CI
<i>130-day cut-off</i>			
PWD	0.83	0.03	0.78–0.88
BED	0.78	0.02	0.74–0.82
LAG	0.81	0.02	0.78–0.85
<i>180-day cut-off</i>			
PWD	0.82	0.03	0.76–0.88
BED	0.75	0.02	0.71–0.79
LAG	0.79	0.02	0.75–0.82
<i>360-day cut-off</i>			
PWD	0.78	0.05	0.68–0.89
BED	0.74	0.03	0.69–0.79
LAG	0.72	0.02	0.68–0.77

The table shows the results for the area under the curve (AUC) of a receiver operating characteristics (ROC) graph. The AUC is an objective measure of the accuracy of a classification schema. The best possible value is 1.0, which represents a 100% sensitivity and 100% specificity of the assay to correctly distinguish recent from established HIV infections. The results show that the PwD assay has the best predictive performance for the three window periods. CI: confidence interval.

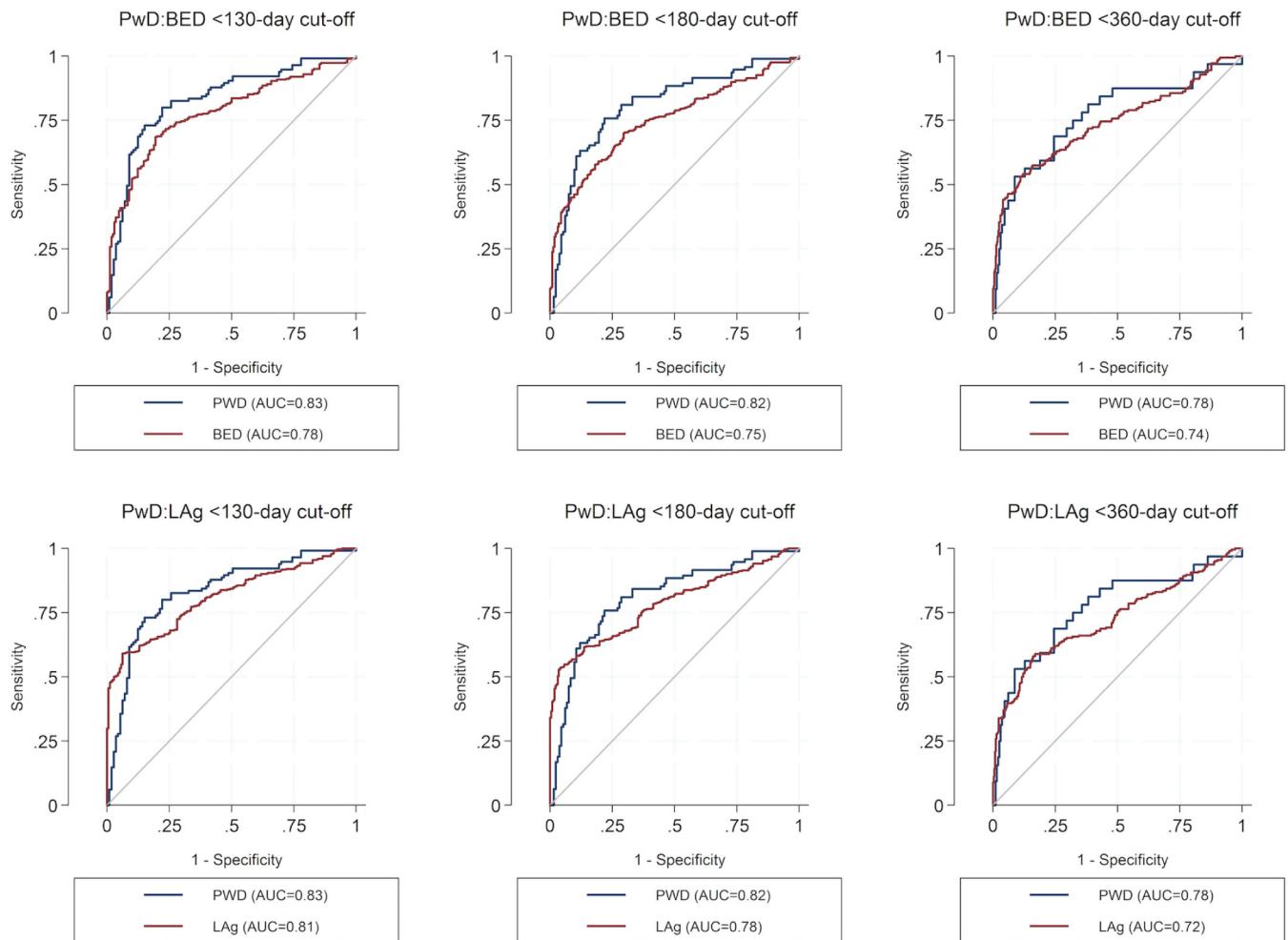
doi:10.1371/journal.pone.0160649.t002

cut-off as follows: The PwD assay reduces the rFRR by (100–48) 52% while maintaining a 90% rTRR (sensitivity) of the LAg assay. We can also interpret this result using the upper bound of the 95% CI: the PwD assay reduces the rFRR by *at least* (100–87.5) 12.5% while maintaining a LAG sensitivity of 90%.

Results show that the PwD assay reduces the rFRR by (100–42.2) 57.8%, or that it reduces the rFRR by *at least* (100–62.8) 37.2%, while maintaining a 90% sensitivity of the BED assay. Panel D of [S2 Fig](#) provides a graphical illustration of the reduction in the rFRR due to the BED plus PwD combination screening. The panel shows the rFRR estimate (red dot) on the ROC graph that corresponds with a 90% sensitivity (y-axis) and a 42.2% false-recent (x-axis) value. The red bar represents the 95% CI of the rFRR. Panels A-C of [S2 Fig](#) show that rFRR estimates at a sensitivity levels of 75%, 80% or 85% respectively, the values of which can be obtained from [Table 3](#).

We further provide a data flow diagram in [S3 Fig](#) to demonstrate the procedure used to produce the results for [Table 3](#). There were 217 time points that had values for *both* the PwD and BED assays, of which 134 were known to be recent. We first used a recommended BED threshold of  $\leq 0.8$  to classify 168 time points as recent infections. We then used a PwD threshold of  $\leq 0.005$  to re-screen these 168 time points in order to improve predictive accuracy. [S3 Fig](#) shows a reduction in the number of false-recent infections from 45 to 16 (64%) due to the PwD screening, while maintaining a BED sensitivity of 91.6%. This result differs slightly from that of [Table 3](#), which is interpreted at an exact sensitivity of 90%.

We then show how additional biomarker information can be used to improve the combination screening procedure. Here we hypothesize that treatment naïve participants with viral loads  $<1000$  copies/mL are less likely to be recently infected with HIV. [Fig 2](#) shows the BED plus PwD screening for the 180-day cut-off. The rFRR estimate is 31.6% (95% CI: 11.0–63.1), which shows that the PwD assay and VL information reduces rFRR by 68.4% (or by *at least* 36.9%) while maintaining a BED sensitivity of 90%. We also show this result for the LAg plus PwD combination screening for the 130-day cut-off in [Fig 3](#).



**Fig 1. ROC graphs comparing the predictive performance of the PwD, LAg and BED assays for determining HIV infection recency for the 130, 180, and 360-day cut-offs.** We used the area under the curve (AUC) of a receiver operator characteristics (ROC) graph to assess the accuracy of the PwD, BED and Lag assays to identify HIV infection recency. The best possible AUC value is 1.0. The ROC graphs are produced by calculating the sensitivity and specificity at different thresholds, which are typically incremented by a fixed value over the minimum and maximum range of the assay. The AUC results show that the PwD assay is the most accurate identifier of infection recency for the three cut-off periods.

doi:10.1371/journal.pone.0160649.g001

Finally, we estimated MDRI's for PwD using a threshold of 0.005, BED and LAg using standard thresholds of 0.8 and 1.5 respectively. PwD had an estimated MDRI of 128 days (95% CI 92–185). BED and LAg had estimated MDRIs of 267 days (95% 212–335) and 129 days (81–190), respectively ([S4 Table](#))

## 4.0 Discussion and Conclusion

There is an urgent need in HIV research to classify infection recency using accurate, practical and cost effective methods [29, 78–81]. In this study, we evaluate the accuracy of a viral-based assay, HIV pairwise diversity (PwD), to identify participants recently infected with HIV. Our study provides information on the best-performing thresholds for the PwD assay, and compares this assay with two serologic-based assays, BED and LAg. We found that PwD threshold values in the range of 0.005 and 0.006 gave a high sensitivity and specificity for the 130 and

**Table 3.** Combination assay screening to identify HIV infection recency for the 130 and 180-day cut-offs periods.

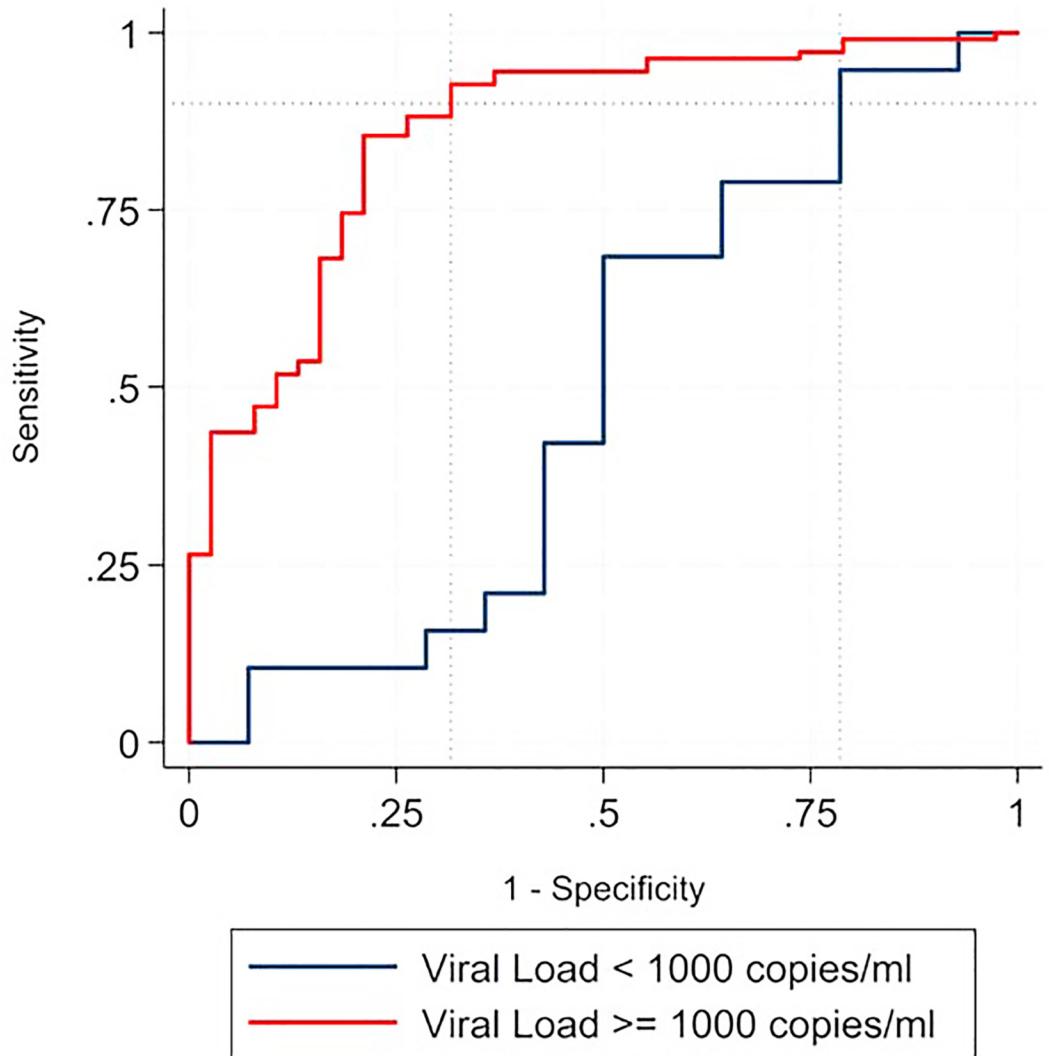
	Sensitivity level	Relative False- Recency Rate	95% Lower bound	95% Upper bound
<i>BED+PwD</i>	75	28.3	13.8	47.9
130-day	80	35.0	17.0	48.3
<i>cut-off</i>	85	36.7	19.4	52.1
	90	40.0	23.2	68.5
<i>BED+PwD</i>	75	28.9	11.8	44.6
180-day	80	31.1	13.8	46.2
<i>cut-off</i>	85	31.1	18.1	53.2
	90	42.2	21.1	62.8
<i>LAG+PwD</i>	75	44.0	25.0	68.2
130-day	80	48.0	28.6	68.4
<i>cut-off</i>	85	48.0	27.5	73.3
	90	48.0	30.9	87.5
<i>LAG+PwD</i>	75	42.1	15.8	71.8
180-day	80	42.1	17.4	73.3
<i>cut-off</i>	85	42.1	18.5	71.8
	90	47.4	22.2	83.3

The table shows the reduction in the relative false-recency rate (rFRR) of the BED and LAg assays due to the PwD assay. A BED = 0.8 or LAg = 1.5 threshold was first used to screen the specimens for HIV infection recency. Specimens classified as recent were then re-screened using the PwD assay in order to reduce the rFRR while maintaining a 75%, 80%, 85% or 90% true-recency rate (sensitivity) of the BED or LAg assay. Since we are interested in the reduction of the rFRR by the PwD assay, we subtract this estimate from 100%. The results can be interpreted as follows: for the 180-day cut-off, the PwD assay reduces the rFRR by (100–42.2 =) 57.8% while maintaining a BED sensitivity of 90%. The table also gives the 95% confidence bounds for the reduction in the rFRR. The same result can be interpreted as follows: for the 180-day cut-off, the PwD assay reduces the rFRR by at least (100–62.8 =) 37.2% while maintaining a BED sensitivity of 90%.

doi:10.1371/journal.pone.0160649.t003

180-day cut-offs. These values are biologically feasible and consistent with previous work. For example, studies have determined that the mean pairwise sequence diversity of the HIV-1 *env* gene region increases at an approximately constant rate of 0.01 per year during early HIV infection [46]. Other studies using a different measure of HIV diversity, namely proportion of ambiguous sites, found that a threshold ranging from 0.0045 to 0.005 gave a high sensitivity for the 180-day cut-off [11]. Xia et. al. [82] show that a 0.006 diversity cut-off distinguished recent infections with both single and multiple infections.

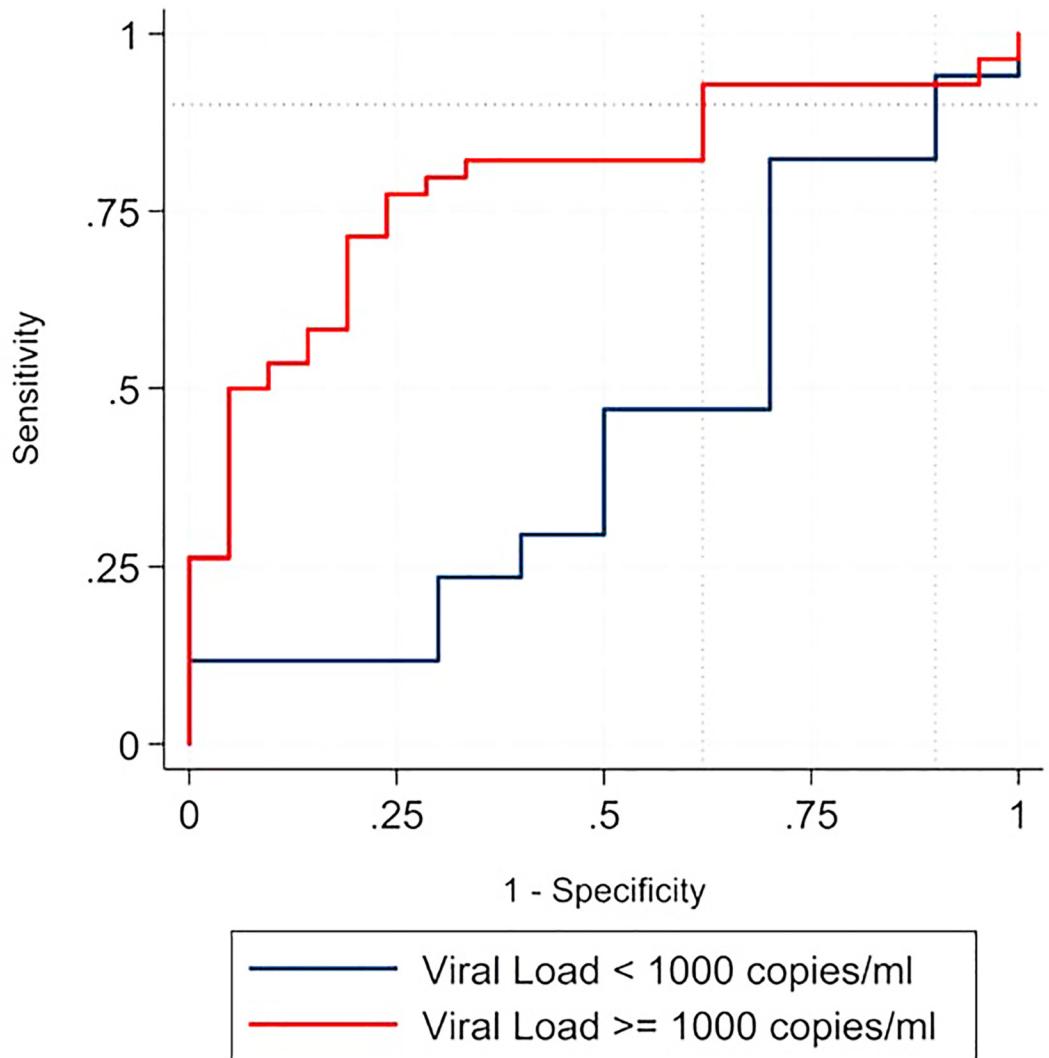
The results of our study show that the PwD assay can accurately identify recent HIV infections. The PwD assay gave the best performance for the 130, 180, 360-day cut-offs according to the AUC estimates. PwD thresholds of 0.004 and 0.005 correctly classified a higher proportion of specimens when compared with BED and LAg thresholds of 0.8 and 1.5 respectively. We also evaluated a multi-assay algorithm (BED plus PwD or LAg plus PwD) to identify HIV infection recency. Our algorithm first uses an affordable, serologic based assay (BED or LAg) to identify a high proportion of true-recent HIV infections, and then the more sensitive PwD assay to reduce the percentage of specimens misclassified as recent infections. Combination screening significantly improved the classification of HIV infection recency. We found that the PwD assay was able to reduce the relative false-recency rate (rFRR) by approximately 52% while maintaining a LAg sensitivity of 90% for the 130-day cut-off. PwD reduced the rFRR by approximately 58% while maintaining a BED sensitivity of 90% for the 180-day cut-off. Results also show an improvement in accuracy when including biomarker information such as participant viral load (VL).



**Fig 2.** Shows a reduction in the relative false-recency rate (rFRR) when viral load information is added to the combination BED plus PwD screening procedure. The figure shows how additional biomarker information can be used to improve the combination screening procedure for the 180-day cut-off. We hypothesize that treatment naïve participants with viral loads  $\leq 1000$  copies/mL are more likely to be recently infected with HIV. Results show an rFRR estimate of 31.6% (95% CI: 11–63.1) at a 90% sensitivity level. Since we are interested in the reduction of the rFRR by the PwD assay, we subtract this estimate from 100%. Thus, the PwD assay reduces the rFRR by 68.4% (or by at least 36.9% given the upper bound of the 95% CI) while maintaining a BED sensitivity of 90% for the subsample of VL  $> 1000$  copies/mL specimens. The figure displays both ROC curves for the viral load covariate and the corresponding rFRR estimates (displayed by the dotted vertical lines).

doi:10.1371/journal.pone.0160649.g002

Prior research has shown that the presence or active use of ART can reduce HIV diversity and result in the misclassification of infection recency [62, 83]. The sensitivity of incidence assays can be maintained if auxiliary patient information on ART usage is collected at the same time as the blood specimen. The collection of additional information, such as VL or CD4 counts, has also been shown to improve the performance of bio-marker based assays to detect infection recency [12, 13, 32]. Our study confirms that the inclusion of VL as a covariate in the analysis significantly reduced the false-recent rate of the BED or LAg assays while maintaining a high sensitivity [12, 84, 85]. Collecting VL or CD4 count information may however increase



**Fig 3.** Shows a reduction in the relative false-recency rate when viral load information is added to the combination LAg plus PwD screening. The figure shows how additional biomarker information can be used to improve the combination screening procedure for the 130-day cut-off. We hypothesize that treatment naïve participants with viral loads  $\leq 1000$  copies/mL are more likely to be recently infected with HIV. Results show an rFRR estimate of 38.1% (95% CI: 15.8–88.6) at a 90% sensitivity level. Since we are interested in the reduction of the rFRR by the PwD assay, we subtract this estimate from 100%. Thus, the PwD assay reduces the rFRR by 61.9% (or by at least 11.4% given an upper bound of the 95% CI) while maintaining a LAg sensitivity of 90% for the subsample of VL  $< 1000$  copies/mL specimens. The figure displays both ROC curves for the viral load covariate and the corresponding rFRR estimates (displayed by the dotted vertical lines).

doi:10.1371/journal.pone.0160649.g003

operational costs. Some of these markers may not be readily available during routine cross-sectional surveys or for previously collected specimens. The PwD MDRI estimates are similar to LAg MDRI recently published [34, 86], although larger sample sets could help to evaluate different thresholds of PwD.

In this paper, we excluded time points with evidence of multiple founder variants or super-infection. Previous research has shown that multiplicity of infection can result in highly variable pairwise distances. PwD values calculated from multi-infection time points are likely to fall outside of the expected range, and do not give an accurate estimate of HIV diversity [61, 87]. Methods to better identify multi-infections in cross sectional sampling are currently being

developed. The PwD assay may be of limited use in men who have sex with men (MSM) [88, 89] due to a high multiplicity of infection. However, more than 80% of all heterosexual HIV infections are seeded by a single founder strain [42–45], which is the main route of transmission in Botswana.

One current limitation associated with the wide-scale use of the PwD assay is the cost of genomic sequencing, which requires expensive laboratory equipment, the training of staff and the technically demanding task of generating single genomes or clonal sequences. The current cost of generating quasispecies from a single time point ranges from \$150–200\$ compared to \$5.29 and \$2.35 per test for LAg and BED, respectively. Nevertheless, we argue that the data generated from genome sequencing can address a range of research questions related to the timing of infections in transmission clusters, the number of strains infecting individuals, tropism of the virus and the selection of optimal drug regimens. In this regard, the costs of genome sequencing would be absorbed into a body of research initiatives and questions, rather than used exclusively for the generation of a viral diversity measure. It is also likely that expensive viral-based assays will become a moot point in the near future as the cost of genomics technology continues to decline.

In conclusion, serologic assays and their algorithms have become increasingly popular in recent years because they are based on antibody laboratory tests that are cheaper, quicker and relatively straightforward to implement at the population level [30, 31, 80, 90]. In this study, we show that a measure of HIV diversity can accurately classify infection recency. Our results show that BED plus PwD or LAg plus PwD combination screening has the potential to correctly identify a high proportion of recent HIV infections in a cost-effective manner. The use of bio-marker based assays and cross-sectional data to identify HIV infection recency presents a promising alternative to the resource-intensive approach of a longitudinal cohort design. With continued development, these assays hold the potential to accurately estimate HIV incidence, monitor the spread of the epidemic, evaluate the impact of treatment interventions and inform the design of vaccine and prevention trials.

## Supporting Information

**S1 Fig. Data flow diagram showing total time points and participants included in the final analysis.**

(PNG)

**S2 Fig. ROC graphs showing a reduction in the relative false-recency rate (rFRR) of the BED assay by the PwD assay for the <180-day cut-off.** The figure gives an example of the reduction in the relative false-recency rate (rFRR) of the BED assay by the PwD assay for the 180-day cut-off. The panels A-D show the ROC curves for the four sensitivity levels. The y-axis is the sensitivity and the x-axis the false-recency rate (1 –specificity); the red point on each graph is the rFRR estimate along with its 95% CI, as shown by the red error bar. The PwD assay reduces the rFRR by 57.8% while maintaining a 90% sensitivity of the BED assay. The ROC graphs show that after performing combination screening, an rFRR estimate can be obtained for any sensitivity value between 0 and 1.0.

(PNG)

**S3 Fig. Flow chart of the combination BED plus PwD screening to identify HIV infection recency for the 180-day cut-off.** Flow chart showing how the PwD assay can be combined with the BED assay to reduce the likelihood of a false-recent result (i.e., established infections misclassified as recent infections). A recommended BED assay threshold value of 0.8 was used to classify infection recency for the N = 217 specimens. This first screening correctly identified

123 of the 134 recent infections for the 180-day cut-off (true positives), giving a sensitivity of 91.8%. However, 45 of the 83 (54.2%) established specimens were falsely classified as recent. A PwD threshold of 0.005 was then used to screen the subset of specimens classified as recent ( $n = 168$ ) by the BED assay. Results show that the secondary PwD screening reduces the false-recent infections by 64% (45 to 16 specimens) at a BED sensitivity of 87.8%. (This result differs slightly from that of [Table 3](#), which is interpreted at an exact sensitivity of 90%).  
(PNG)

**S4 Fig. Distribution of time-points for the BED, LAg and PwD assays.** The figure gives the analysed time points of sampling and sequencing in the study since the known time of seroconversion. Time in days post-seroconversion is shown on the x-axis.  
(PNG)

**S5 Fig. Spaghetti plots for the BED, LAg and PwD time-points.**  
(PNG)

**S1 Table. Performance of PwD threshold values to determine HIV Infection Recency for 130, 180, and 360-day cut-offs.** The table shows the performance of the PwD threshold values to identify HIV infection recency. The range of values were selected according to rate of increase in the pairwise sequence diversity of the HIV-1 *env* gene region, which is approximately a constant rate of 0.01 per year during early infection. For example, a 180-day cut-off corresponds with a PwD value of 0.005. We selected thresholds values in the range of these biological values for each of the cut-off periods. For each threshold we obtained the sensitivity, specificity, their 95% CI, likelihood ratio, and percentage correctly classified. For the 130-day cut-off, a PwD threshold of 0.005 correctly identified 79.37% (95% CI: 62.83–95.9) of the recent infections (sensitivity) and correctly identified 72.57% (95% CI: 61.87–83.26) of the established infections (specificity), giving a percentage correctly classified of 76.15%.  
(DOCX)

**S2 Table. Accession numbers for the reference sequences used.**  
(DOCX)

**S3 Table. Area under the curve (AUC) for the PwD, BED, and LAg assays for shared time-points ( $n = 238$ ).** Table shows the results for the area under the curve (AUC) of a receiver operating characteristics (ROC) graph for the <130, <180- and <360-day cut-offs. Using only shared time-points ( $n = 238$ ) significantly reduces the sample size and therefore the performance of the three assays. The performance of the three assays are therefore indistinguishable given the overlap in the confidence intervals of the AUC estimates.  
(DOCX)

**S4 Table. Mean Duration of Recent Infection for BED, LAg and PwD Assays.** Table shows the estimated Mean Duration of Recent Infection (MDRI), average time ‘recent’ while infected for less than some time cut-off T for the BED, LAg and PwD assays.  
(DOCX)

## Acknowledgments

We are grateful to all participants in the *Tshedimoso* study in Botswana. We acknowledge the support from the staff of the Botswana-Harvard HIV Reference Laboratory and the HIV Research Trust Scholarship program. We are grateful to Alex Welte and Eduard Grebe South African Centre for Epidemiological Modeling and Analysis (SACEMA) for technical assistance and guidance with use incidence assays tools package (inctools) and calculations of the MDRI.

## Author Contributions

**Conceived and designed the experiments:** SM EW SE VN TdO SG RMM.

**Performed the experiments:** SMM KPK.

**Analyzed the data:** SMM AV.

**Contributed reagents/materials/analysis tools:** VN ME SMM AV SE EW TdO.

**Wrote the paper:** SM AV EW SE VN FT ME TdO.

Designed and supervised the primary infection cohort “Tshedimoso”: VN ME, Provided laboratory support for the primary infection cohort: SM.

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## Appendix 2

<b>TITLE</b> <b>Cross-sectional estimates of HIV incidence remain high in rural communities in Botswana in the era of successful scale up of ART</b>
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**Background:** The successful scale up of the national ART program in Botswana has almost reached the UNAIDS "90-90-90" goal (Gaolathe et al., CROI-2016). Cross-sectional estimate of HIV incidence needs to take into account ARV use to avoid mis-classification of HIV recency.

**Methods:** Using cross-sectional sampling HIV recency was estimated at the baseline of the Botswana Combination Prevention Project in 30 rural communities from Nov 2013 to Nov 2015. The algorithm for estimation of HIV recency combined Limiting-Antigen Avidity Assay (LAg) data, ART status and HIV-1 RNA load (as described in Rehle et al., *PLoS One* 2015;10:e0133255). The LAg cut-off normalized optical density was 1.5. ART status was documented. The HIV-1 RNA cut-off was 400 cps/mL. The Mean Duration of Recent Infection was 130 days and the False Recent Rate was zero.

**Results:** During the baseline household survey, a total of 3,596 individuals tested HIV positive among 12,570 individuals with definitive HIV status. Among those testing HIV positive, 3585 (99.7%) had a research blood draw available, of whom 3580 (99.9%) had LAg data generated. Of those, 326 were identified as LAg-recent cases. Among those, 278 individuals were considered chronically infected based on their documented ART status. Among the remaining 48 ART-naïve individuals, 14 had an HIV-1 RNA load ≤400 cps/mL. The Botswana MoH electronic medical records system was queried for these 14, 10 were found in the MoH data and evidence for initiation of ART was found for 5 individuals. ARV-naïve status could not be confirmed in 9 individuals. Thus, 34 LAg-recent, ARV-naïve individuals with HIV-1 RNA above 400 cps/mL were classified as individuals with recent HIV infections. HIV incidence was estimated at 1.06% (95% CI 0.70%-1.42%). Including 9 virologically suppressed individuals with uncertain ART status brings the estimate of HIV incidence to 1.34% (95% CI 0.91%-1.77%).

**Conclusions:** Using an algorithm including LAg-Avidity EIA, documented ART status and HIV-1 RNA load, cross-sectional HIV incidence in 30 rural communities in Botswana was estimated at 1.06%-1.34% in 2013-2015. Given the high level of ART scale-up in Botswana, studies able to identify HIV transmission sources and reduce HIV incidence are warranted.

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**CONFERENCE DATES AND LOCATION:**

February 22–25, 2016 | Boston, Massachusetts

**Abstract Number:**

984

## Cross-Sectional HIV Incidence at Scale-up of ART in 24 Rural Communities in Botswana

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**Abstract Body:**

Direct real-time estimates of HIV incidence using cross-sectional sampling can provide critical information for design and evaluation of HIV prevention interventions. The ongoing scale-up of ART in southern African countries presents a substantial challenge to the accuracy and reliability of cross-sectional methods used for identification of HIV recency.

HIV recency was estimated at the baseline of the Botswana Combination Prevention Project (BCPP). Cross-sectional data were collected during household surveys in 24 rural communities from Nov 2013 to Aug 2015. An HIV incidence testing algorithm combining the Limiting-Antigen Avidity Assay (LAg-Avidity EIA) with ART status and level of HIV-1 RNA load (multi-assay algorithm described in Rehle et al., PLoS One 2015;10:e0133255) was used. The LAg cut-off normalized optical density was 1.5. ART status was documented. The HIV-1 RNA cut-off was 400 cps/mL. The Mean Duration of Recent Infection was 130 days and the False Recent Rate was set to zero.

A total of 2,727 individuals tested HIV-positive among 9,745 individuals with definitive HIV status (28.3% HIV prevalence after adjustment for study design; 95% CI: 25.6%–31.2%) during the baseline household surveys. About 70% of HIV-positive individuals were already on ART. LAg-Avidity EIA data was generated for 2,710 of 2,719 (99.7%) HIV-positive individuals with research blood draw available, and 234 cases were identified as LAg-Avidity EIA recent. Among those, 198 individuals were considered chronically infected based on their documented ART status. Eleven of 36 LAg-Avidity EIA recent, ARV-naïve individuals had an HIV-1 RNA load  $\leq$ 400 cps/mL, and were classified as having long-term HIV infections. Thus, 25 LAg-Avidity EIA recent, ARV-naïve individuals with HIV-1 RNA above 400 cps/mL were classified as individuals with recent HIV infections. HIV incidence across 24 communities was estimated at 1.00% (95% CI 0.60%–1.41%).

The increasing scale-up of ART in southern African communities requires adjustment of cross-sectional methods for identification of HIV recency. An algorithm that combines LAg-Avidity EIA testing with ART status and HIV-1 RNA data was used to estimate baseline HIV recency in 24 rural communities at

adjustment of cross-sectional methods for identification of HIV recency. An algorithm that combines LAg-Avidity EIA testing with ART status and HIV-1 RNA data was used to estimate baseline HIV recency in 24 rural communities at baseline of the BCPP. This algorithm should be validated by longitudinal HIV incidence data in the future. HIV incidence in rural communities in Botswana was estimated at 1.0% in 2013–2015.

**Session Number:**

P-X3

**Session Title:**

HIV Testing and Prevention

**Presenting Author:**

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