Short Communication
Decreased Incidence of Dual Infections in South African Subtype C-Infected Women Compared to a Cohort Ten Years Earlier

Zenda Woodman,1 Koleka Mlisana,2 Florette Treurnicht,3 Melissa-Rose Abrahams,3 Ruwayida Thebus,3 Salim Abdool Karim,4 and Carolyn Williamson,3 for the CAPRISA Acute Infection Study Team

Abstract
Previously, we determined the incidence of dual infections in a South African cohort and its association with higher viral setpoint. Ten years later, we compare the incidence and impact of dual infections at transmission on viral setpoint in a geographically similar cohort (n = 46) making use of both the heteroduplex mobility assay (HMA) and the more recent single genome amplification (SGA) approach. HIV incidence was lower in this cohort (7% compared to 18%), and we find a similar reduction in the number of dual infections (9% compared to 19%). Unlike the previous study, there was no association between either dual infection (n = 4) or multivariant transmission (n = 7) and disease progression. This study emphasized the importance of monitoring changes in the HIV epidemic as it may have important ramifications on our understanding of the natural history of disease.

Introduction
As the HIV epidemic evolves, the contribution of factors that increase viral load and disease progression, such as HIV-1 dual infection,1,2 may change. The incidence of dual infections, defined as infection with two epidemiologically unlinked HIV variants, could influence the disease profile in populations and therefore public health planning. In a previous study we determined that the incidence of subtype C dual infections in a cohort of female sex workers recruited from 1996 to 1999 in KwaZulu-Natal, South Africa was 19%.1 Ten years after the first analysis, this study aims to determine the incidence of dual infections in the more recent CAPRISA 002 cohort in KwaZulu-Natal, and the role of dual infections in rapid disease progression in this cohort.3 Similar to the initial study, we used the heteroduplex mobility assay (HMA) together with sequencing to identify dual infections. HMA can detect variants present as low as 1%; however, it is limited in that it cannot differentiate very closely related viruses.1 We thus verified this approach on a subset of samples using single genome amplification (SGA) followed by sequencing. The SGA approach has become the standard method for defining the transmission bottleneck as it can reliably detect transmission of closely related viruses, although it will only be 95% confident of detecting variants present at a frequency greater than 15% when sampling at least 20 sequences.4

It is well established that there is an extreme virus population bottleneck following transmission,5–7 with an estimated 80% of infections being a result of transmission of a single virus or virus-infected cell. The remaining 20% are infected with multiple variants (quasispecies) where more than one virus, transmitted from the donor, results in productive clinical infection. Like dual infection, multiple variant transmission has been associated with rapid disease progression.5 Factors that have been shown to affect susceptibility to HIV infection8 also appear to increase the chance of multiple variant transmission: studies in Kenya, Rwanda, and Zambia have shown that women with sexually transmitted infections (STIs) or on hormonal contraceptives were more likely to harbor diverse viral populations following transmission.9,10 Furthermore, a study in Tanzania showed that frequency of infection with a diverse viral population was higher in high-risk women compared to women with lower-risk behavior.11

In this study we characterized the frequency of dual infection and multiple variant transmission in 46 individuals of...
whom 12 (26%) were identified as rapid disease progressors based on CD4+ cell counts less than 350 μl/ml within the first year of infection.\(^1\) We also investigated the role/impact that STIs might have in multiple variant transmissions and disease progression.

**Materials and Methods**

Plasma samples were obtained within 3 months of infection from the CAPRISA 002 acute infection cohort, Durban, South Africa.\(^3\) Genital samples were collected every 6 months and tested for sexually transmitted infections \([Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Trichomonas vaginalis, Bacterial vaginosis, syphilis, and herpes simplex virus type 2 (HSV-2)]\) as previously described.\(^1,3\)

Viral RNA was reverse transcribed using the Superscript III Reverse Transcriptase (Invitrogen) with either Env-N (nt 9171–9145) or ED33 (nt 7380–7361) primers (primer numbering according to HXB2). Population polyclonerase chain reaction (PCR) was carried out with a median input of 105 copies of viral RNA as determined using the plasma viral load measurement, with two participants where only two RNA copies were supplied. The Env C2–C3 510-bp region was amplified using nested PCR.\(^1\) Minor viral populations infecting CAP37 were identified using two antisense strain-specific primers: 37-R2 (nt 9053–9033) and 37-R1 (nt 8870–8847). Env sequences obtained using SGA were acquired from GenBank (FJ443128 to FJ444632).\(^7\)

HMA was performed as described.\(^1,4\) PCR products (510 bp) were cloned using the pGEM-T-Easy TA cloning kit (Promega) and sequenced using ABI PRISM dye terminator Prism (version 4; GraphPad Software).

**Results**

This study compares the incidence and impact of dual infection on disease progression in two cohorts of high-risk women from KwaZulu-Natal, South Africa. The previous study (Du Cohort) recruited 31 women between 1996 and 1999,\(^1\) whereas the current study (CAPRISA 002 cohort) characterizes recent infection in 46 women recruited between 2004 and 2009.\(^3\) The HIV incidence in the Du cohort was 18% compared to 7% in the CAPRISA 002 cohort (Table 1).\(^1,3,5\) with participants in the Du cohort having a higher number of sexual partners (median = 20 clients/week, range of 5–40)\(^1,6\) compared to those in the CAPRISA cohort (median = 2 clients/week; range of 0–30).\(^3\)

Most early studies have used the HMA to screen for diversity following transmission,\(^1,2,6\) however, more recently the SGA and sequencing approach was shown to be more accurate as it controls for both resampling and recombination during amplification, both of which can confound the interpretation of the results.\(^7,8\) To assess the reliability of the C2–C3 region HMA to detect multiple variants, we compared the DNA distance between complete env SGA sequences to those of the C2-C3 regions alone. We found that the maximum DNA distance of env of the multiple variants infecting 14 individuals identified by Abrahams et al.\(^7\) ranged from 0.73 to 8.49% and for the C2-C3 region ranged from 1.04 to 10.57%. Only sequences from a fifteenth participant, classified by env SGA sequencing as infected with multiple but very closely related variants, had no changes in C2–C3. As it was previously shown that a single band was visible on an HMA gel when DNA distance was <0.86%,\(^1\) we hypothesized that HMA should be able to identify the majority of multivariant infections. We then directly compared the SGA and HMA approaches on samples from a further 19 individuals. We found concordant results for 17/19 participants of which 13 were classified as homogeneous and the remaining 6 as heterogeneous infections. Of the two discordant samples (CAP84 and CAP221) (Fig. 1A and B), SGA classified these as homogeneous whereas they were classified as heterogeneous by HMA. CAP84 was studied using SGA at the 2 wpi visit whereas HMA demonstrated heteroduplex banding patterns 2 weeks later (4 wpi); thus it is possible that CAP84 either became infected with a second variant between sampling or fluctuations in the viral population occurred so that the second variant became more dominant over time. For CAP221, 58 clones were tested by HMA whereas the SGA approach sequenced only 21 amplicons so it is possible that the minor variants were missed due to limited sampling (Fig. 1C). It has previously been shown that by sequencing only 20 env sequences, there is only a 95% confidence interval of detecting variants that occur at 15% or greater frequency within the virus population.\(^8\)

As HMA reliably identified viral variants with high diversity, we screened 39 individuals for genetic complexity following transmission using HMA. Nine of 39 individuals (23%) were classified as multivariant transmissions based on unique banding patterns and high diversity (maximum C2–C3 DNA distance 0.8–16.9%). Of these nine, five participants were infected with variants forming a single phylogenetic cluster, while three participants harbored viruses with sequences that separated into distinct phylogenetic lineages, confirming that these individuals were dually infected (Fig. 1D). The ninth individual, CAP37, was classified as a potential dual infection using the SGA approach\(^7\) and was confirmed in this study using strain-specific PCR (Fig. 1D). By combining the 39 participants screened by HMA and 7 participants obtained from Abrahams et al.,\(^7\) 11/46 individuals were shown to be infected with multiple variants with 4/11 individuals demonstrated to be infected with phylogenetically distinct strains (Fig. 1D).

To define factors that could predispose individuals to infection with diverse virus populations we determined whether multiple variant infection was associated with concurrent STIs (Table 1). *Bacterial vaginosis* was excluded from the analysis due to its high incidence (34/46; 74%) within the cohort. Although nearly 50% (22/46) of the cohort was infected with one or more STIs, we found more women infected with multivariants having concomitant STIs (8/11; 73%) than women with single variant transmissions (14/35; 40%) (\(p = 0.0848\)) (Table 1).

When we compared CD4 cell counts at 12 months postinfection between those infected with single (\(n = 34\)) vs. multiple variants (\(n = 10\)) (Table 1), the median values (422 cells/μl; range = 241–1030 and 374 cells/μl; range = 188–695, respectively) did not differ significantly between the two groups.
Similarly, the median viral load of individuals infected with single variants (23,900 copies/ml; range 400–1,680,000) was not significantly lower \((p = 0.7902)\) than those infected with multiple variants (47,650 copies/ml; range 400–1,230,000). In this cohort 12 of the 46 individuals were classified as rapid progressors based on CD4\(^+\) counts below 350 cells/\(\mu\)l within the first year of infection and 6 were identified as controllers with viral loads below 2000 RNA copies/ml.\(^6\) Of the rapid progressors only two individuals were dual infected and a further three were infected with multiple variants at transmission. Although, the median CD4\(^+\) cell count (345 cells/\(\mu\)l; range 274–479 cells/\(\mu\)l) of the four dual-infected individuals was lower than those individuals infected with single variants (422 cells/\(\mu\)l; range 241–1030 cells/\(\mu\)l), this association was not significant \((p = 0.1908)\). Thus, despite several independent studies demonstrating an association between dual infection and disease progression, including the study of the Du cohort, this factor does not appear to be a major factor accounting for the high number of rapid disease progressors in this cohort.\(^1,2,5\)

**Discussion**

Characterization of transmitted viruses and factors that affect the transmission bottleneck are important for both vaccine studies and to understand HIV pathogenesis. This
FIG. 1. The heteroduplex mobility assay (HMA) identified multiple variant infection. (A) Representative HMA gels of CAP84 (i) and CAP221 (ii) showing the 510-bp C2–C3 clonal amplicons (clone number prefixed with c) mixed with a reference clone. In some lanes, two clones were mixed with the reference clone. Only representative clones of unique banding patterns were sequenced and these are labeled, whereas lanes showing amplicons that were not sequenced are marked with an asterisk. The 1-kb molecular weight marker was used in lane M (Promega). (B) Neighbor-joining trees of CAP84 (i) and CAP221 (ii) showing the phylogenetic relationship between the respective clonal C2–C3 sequences. (C) Comparison between the C2–C3 clonal sequences and the SGA derived sequences (indicated by a black dot) of CAP84 (i) and CAP221 (ii) aligned to the consensus of the SGA sequences using Highlighter (www.hiv.lanl.gov). (D) Neighbor-joining tree of the C2–C3 region of all 46 participants and reference subtype C (www.hiv.lanl.gov) with replicates of 500 and bootstrap values >70 indicated. The sequences of CAPRISA participants infected with homogeneous variants are shown by either black squares (HMA identified clones) or black circles [C2–C3 region identified by single genome amplification (SGA)] and the participants infected with diverse strains are indicated by participant ID. To clearly illustrate infection with diverse strains, the two sequences with the highest maximum DNA distance from each participant with multivariant infection are shown. For two participants (CAP137 and CAP37) sequences from two time points are illustrated (wpi, weeks postinfection). For CAP37, strain-specific primers were designed to the gp41 region of a recombinant identified by SGA at 2 wpi and used to selectively amplify the second strain at 7 wpi. Color images available online at www.liebertonline.com/aid
study utilized HMA together with sequencing, as well as previously published SGA-derived sequences to further elucidate the relationship between Env viral diversity at transmission and disease progression. We also compared this data to a study completed 10 years earlier to identify possible changes in trends over time that may be driving the high number of rapid disease progressors in this cohort. Changes in the HIV epidemic have important ramifications on our understanding of the natural history of disease and our design of preventive measures such as vaccines.

This study shows that HMA based on the Env C2–C3 region, followed by cloning and sequencing, can reliably detect most multivariant transmission events when compared to the more stringent SGA sequencing method. Although the SGA approach provides the numeration of transmitted variants and in-depth sequence information, there are practical limitations on the number of sequences analyzed and therefore it may underestimate multiple variant transmission due to limited sampling. Although HMA may not detect closely related variants, the advantage of screening numerous clones enables the detection of variants present at low frequency. The HMA approach also requires sufficient input template to sample all quasispecies present and may thus underestimate diversity in individuals with low viral loads. Taken together, both SGA and HMA approaches are likely to provide only a minimum estimation of frequency of dual infection and multivariant transmission.

The earlier study identified 19% (6/31) of participants with dual infection following transmission by HMA, cloning, and sequencing. Using both HMA and SGA, the current study detected only 9% of individuals dually infected with phylogenetically distinct viruses following infection. A further 15% of the women were infected with multiple variants (quasispecies). Thus a total of 24% of participants were infected with variants of high diversity, in concordance with other studies. This study also provides further evidence that diagnosis of STIs at enrollment may be associated with disruption of the genetic bottleneck, suggesting that the mucosal barrier plays a role in restricting transmission. Although this association was not statistically significant there was a trend toward association as 73% of individuals infected with multiple variants were diagnosed as carrying one or more STIs compared to only 40% with single variant infection. This result is most likely due to the high incidence of different STIs in the cohort with only some associated with increased susceptibility to HIV infection.

Our study identified 9% dual infections compared to 19% in the Du cohort study 10 years earlier from the same geographic region. The frequency of dual infection has been associated with risk behavior and this reduction in frequency is in line with the difference in HIV incidence between the two cohorts (the incidence in the CAPRISA cohort was 77%) compared to 18% in the Du cohort). As well as differences in risk behavior. Recently, dual infections were not identified in a chronic infection cohort from the same region, and this is most likely due to the differences between the cohorts such as stage of disease and exposure to antiretroviral (ARV) therapy. This highlights the complexity of intercohort comparisons whereby changes in cohort characteristics can impact disease outcomes.

In conclusion, previously, an association between dual infection and disease progression was found in the Du cohort using the same experimental approach. However, here we find in the CAPRISA 002 cohort that rapid disease progression was neither significantly associated with dual infection nor infection with multiple variants. It is not clear why individuals with dual infections are not rapid progressors, however, virological characteristics are only one of the factors affecting disease progression that is influenced by complex interactions between host genetics, immunological responses, and coinfections. The two cohorts were recruited from similar populations with similar HLA profiles and it is therefore unlikely that host genetics would account for the different results in these studies. Previous studies on the CAPRISA 002 cohort have shown that neither HIV-1-specific interferon gamma (IFN-γ) responses in acute infection nor development of autologous neutralizing antibody responses appear to impact disease progression. Thus far, studies on the same cohort have shown that viral control is associated with HLA-B*5801 CTL escape mutations and the development of a subset of CD8+ memory cells. However, higher viral loads and maintenance of CD4+ cells in the CAPRISA 002 cohort are associated with elevated inflammation in the genital tract and increased levels of specific cytokines in the plasma during acute infection. We have yet to determine the major factor driving the high rates of disease progression observed in this cohort.

Accession Numbers

Env sequences were acquired from GenBank: FJ443128 to FJ444632.

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References


Address correspondence to:
Zenda Woodman
Department of Molecular and Cell Biology
Faculty of Science
University of Cape Town
Cape Town 7701
South Africa

E-mail: zl.woodman@uct.ac.za