

# Distribution and Analysis of HIV-1 Variants in the Uterine Cervix and as Related to HIV-1 in the Peripheral Blood

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## Abstract/Introduction

**Background:** The uterine cervix has high concentrations of T-lymphocytes that may serve as a compartment for evolution of unique HIV-1 variants. Understanding the biology of HIV-1 in the female genital tract, because of its role in heterosexual and peripartum transmission, has important implications for treatment and vaccine development. In previous studies, HIV-1 DNA sequences from cervical tissues had low genetic diversity, suggesting that a single punch biopsy may sample only related viruses that spread between adjacent cells. To examine HIV-1 diversity within the cervix and the highly related viral clusters observed in cervical tissue, we compared HIV-1 sequences from multiple locations within the cervix to each other and to sequences from the peripheral blood mononuclear cells (PBMC).

**Methods:** Multiple single genomes of HIV-1 DNA from PBMC and three cervical biopsies per woman were analyzed in a cross-sectional study. HIV-1 DNA was PCR amplified from a single specimen from each subject on separate days to minimize the chance of cross-contamination between specimens. Up to 40 single genome sequences of the C2-V5 gp120 region of *env* were derived per specimen and subjected to phylogenetic analysis for compartmentalization. The distribution of sequence diversity between PBMC and cervical tissues from each woman was compared using a permutation analysis.

**Results:** Viruses from six of eight women (median of 65 sequences/woman) clustered into unique clades with significant compartmentalization between the blood and cervical sequences ( $p < 0.0043$  to  $p < 0.0001$ ). Closely related and monomorphic HIV-1 sequences were found within and among all three biopsies from each woman. The frequency of identical sequences with a biopsy affected whether compartmentalization was observed. Of note, in 2/8 women a single biopsy had significantly ( $p < 0.007$ ) less diversity compared to examining all three biopsies. Each woman's phylogenetic tree also had clades with both PBMC and cervical sequences.

**Conclusions:** Compartmentalization between cervical and peripheral blood HIV-1, and between individual biopsies, appears to result from bursts of viral replication or clonal expansion of infected cells within each woman. A single biopsy may result in sampling viruses with low genetic diversity that skews the analysis towards compartmentalization. Sampling a larger number of sequences from the blood and cervix consistently demonstrated trafficking of HIV-1 between these tissues, suggesting that the female genital tract does not constitute an isolated compartment of viruses.

## Hypotheses

Single biopsies of the cervix, which we previously observed have genetically identical and closely related variants, under represent HIV-1 genital tract diversity. We hypothesize that viruses obtained from multiple biopsies of the cervix will reveal greater diversity compared to a single biopsy, and will demonstrate an overall greater diversity in the genital tract compared with the PBMC.

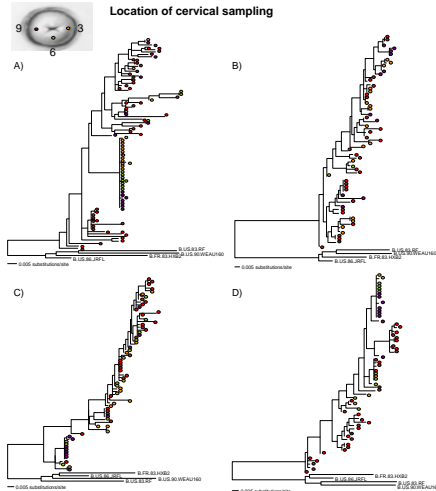
## Methods

- DNA from peripheral blood mononuclear cells (PBMC) and cervical biopsies was isolated using commercial extraction kits (GenTrix Kit, QIAGEN, USA)
- HIV-1 RNA was quantified in plasma and uterine cervical secretions using a real-time PCR assay (RW Coombs, in preparation)
- DNA was subjected to nested multiplex 1st round PCR followed by single region (*pol*/*RT*, *pol*/*RT* and *env* C2-V5) second round PCR<sup>1</sup>, and then cloned into the TOPO-TA plasmid
- Single clones from the C2-V5 gp120 region of *env* were sequenced from ~30 individual PCR per specimen<sup>1</sup>
- Evolutionary models were selected using PAUP<sup>2</sup> 4.0b10 with Modeltest 3.7 under the Akaike Information Criterion<sup>3,4</sup> and used for the construction of maximum likelihood trees using PAUP
- Sequences were evaluated for compartmentalization using phylogenetic (Slatkin and Maddison) analysis<sup>5,6</sup> and genetic diversity was calculated using PAUP<sup>2</sup>
- A Bonferroni correction was performed to account for multiple comparisons, a **p-value of <0.0074** was considered significant.

## Results

Table 1. Clinical Parameters of Eight Women Studied

	Years Post Diagnosis	ART status	CD4	Plasma HIV-1 RNA x1000 copies/mL	Genital HIV-1 RNA x1000 copies/mL
1004	6	ART	507	Undetectable	Undetectable
1013	22	ART	267	Undetectable	Undetectable
1021	7	ART	500	Undetectable	Undetectable
1025	14	ART	556	Undetectable	Undetectable
1002	8	"Failing" ART	97	>1,000	1.3
1009	11	"Failing" ART	381	133	0.133
1010	4	NO ART	667	36	Undetectable
1014	3	NO ART	388	23	Undetectable



**Figure 1.** Phylogenetic analysis of single-genome HIV-1 *env* sequences from peripheral blood and cervical tissue reveals clades of monomorphic and closely related sequences shared between the two tissues. Participants 1013 (A) and 1021 (B) were receiving ART; participant 1002 (C) was "failing" ART; and participant 1014 (D) was naive to ART. Sequences were rooted to Subtype B sequences from GenBank. Red circles represent sequences derived from PBMC; yellow, green, and purple circles represent viruses from different cervical biopsies, as shown in the photo, above.

Table 2. Compartmentalization of HIV-1 *env* genotypes using Slatkin-Maddison (p-values)<sup>5</sup>

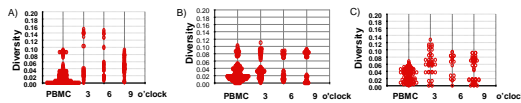
Subject ID	PBMC vs. all Cervix	PBMC vs. 3 o'clock Cervix	PBMC vs. 6 o'clock Cervix	PBMC vs. 9 o'clock Cervix
1004	< 0.0001	1	0.0538	0.0007
1013	< 0.0001	0.0003	0.5594	0.3996
1021	0.0335	0.0617	NP*	0.0257
1025	0.0312	0.0337	0.1245	1
1002	0.0043	0.0075	1	0.1112
1009	< 0.0001	0.0001	0.0032	< 0.0001
1010	< 0.0001	< 0.0001	NP*	0.0001
1014	< 0.0001	1	0.0009	0.0001

The table lists the p value for genetic flow between tissues. Significant values are indicated in red. In a separate analysis monomorphic (identical) sequences were "collapsed" into a single sequence, which reduced significant compartmentalization between the genital tract and PBMC from 6/8 to 3/8 women. \*Permutation analysis was performed using the Slatkin and Maddison (1989)<sup>5</sup> method as applied by Poss et al., (1998)<sup>6</sup>. \*NP= Not performed due to low sequence numbers.

Table 3. Genetic diversity in PBMC and cervical biopsies separately and combined

Subject ID	Median HIV-1 Genetic Diversity (25 <sup>th</sup> , 75 <sup>th</sup> percentile)				
	PBMC	3 o'clock Cervix	6 o'clock Cervix	9 o'clock Cervix	Cervix Combined
1004	0.017 <sup>ns</sup> (0.008, 0.027)	0.025 (0.017, 0.038)	0.035 (0.016, 0.056)	0.045 <sup>†</sup> (0.035, 0.056)	0.040 <sup>†</sup> (0.021, 0.055)
1013	0.034 (0.027, 0.044)	0.004 (0.00, 0.038)	0.002 (0.00, 0.036)	0.038 (0.002, 0.049)	0.033 (0.00, 0.042)
1021	0.053 (0.038, 0.064)	0.046 (0.029, 0.059)	NP*	0.057 (0.031, 0.072)	0.049 (0.031, 0.061)
1025	0.030 (0.020, 0.041)	0.030 (0.022, 0.044)	0.030 (0.027, 0.050)	0.039 (0.028, 0.048)	0.034 (0.025, 0.047)
1002	0.016 <sup>ns</sup> (0.016, 0.026)	0.028 <sup>†</sup> (0.017, 0.042)	0.022 (0.016, 0.077)	0.021 (0.003, 0.082)	0.024 <sup>†</sup> (0.011, 0.076)
1009	0.035 <sup>ns</sup> (0.018, 0.045)	0.060 <sup>†</sup> (0.038, 0.084)	0.071 (0.023, 0.082)	0.057 (0.012, 0.074)	0.061 <sup>†</sup> (0.017, 0.077)
1010	0.035 <sup>ns</sup> (0.027, 0.045)	0.004 (0.00, 0.038)	0.002 <sup>†</sup> (0.00, 0.025)	0.037 <sup>†</sup> (0.002, 0.048)	0.032 (0.00, 0.041)
1014	0.035 (0.023, 0.045)	0.024 (0.020, 0.032)	0.020 (0.002, 0.029)	0.012 (0.00, 0.020)	0.018 (0.002, 0.029)

HIV-1 genetic diversity was calculated using PAUP version 4.0b10. Orange highlighting indicates biopsy tissues with very low diversity, these women had been diagnosed with HIV-1 for 22 (1013) and four (1010) years. Of note, between 27-80% of the sequences from these two women were identical. In the 2/3 women with significantly greater diversity in the genital tract there were perturbations in their vaginal flora including elevated *Gardnerella vaginalis*, enterococcus, and low levels of *H2O2* producing lactobacilli (data not yet available in 1/3 women). In 4/8 women the diversity was similar between the genital tract and PBMC and one woman had greater diversity in PBMC. These 5 women had *H2O2* producing lactobacilli and no *G. vaginalis*. Comparisons of viruses from one biopsy to others from the women showed no significant differences in diversity. \*ND= No data. DNA was not recovered from this biopsy. <sup>†</sup> $p < 0.0074$  for comparisons between PBMC vs. combined cervix, <sup>†</sup>PBMC vs. 3 o'clock, <sup>†</sup>PBMC vs. 6 o'clock and <sup>†</sup>PBMC vs. 9 o'clock.



**Figure 2.** The 3 women with greater genetic diversity in the genital tract compared to the PBMC had two sequence populations in the cervix, one with high and the other with low diversity. Bacterial vaginosis was present in two of these women while the data is not yet available for the third. The participants are: (A) 1004 on ART (B) 1002 "failing" ART, and (C) 1009 "failing" ART.

## Discussion

Several contradictory findings are suggested by these studies:

- First, HIV-1 sequences from each cervical biopsy mix within most clades of the phylogram, as did sequences from PBMC, suggesting that viral variants were not tissue or location specific but flowed between tissues
- Second, HIV-1 are compartmentalized between the cervix and PBMC in 75% (6/8) of women regardless of their ART status

In reconciling these two findings it is notable that:

- When monomorphic sequences are collapsed to a single variant the frequency of compartmentalization was reduced (from 6/8 to 3/8 women)
- Two phenomena could make cervical sequences appear compartmentalized from PBMC:
  - ~ Clonal expansion of infected cells
  - ~ Replicative bursts with localized spread of virus

Cervical sequences that mix with the blood clades may be an artifact of HIV-1 infected PBMC circulating in the cervix at the time of the biopsy and supports genetic flow between tissues

## Conclusions

Multiple biopsies (compared to a single biopsy) more thoroughly measure the HIV-1 population in the genital tract

Greater HIV-1 diversity in the genital tract compared to blood suggests that the female genital tract may serve as a viral reservoir

Genital viruses are compartmentalized from blood in statistical analysis, however:

- Phylogenetic analysis showed mixing of HIV-1 variants from cervix and PBMC
- Compartmentalization may be an artifact of cross sectional studies, due to over sampling of replicating viruses or variants expressed for a short time

Implications of findings for HIV-1 pathogenesis:

- Monomorphic viruses may reflect:
  - ~ Homing of HIV-1 infected cells to the cervix or local proliferation of HIV-1 infected cells due to inflammatory signals
  - ~ Bursts of viral replication
- Cervix as a viral reservoir:
  - ~ Includes viruses from the initial or multiple exposures
  - ~ Archive of viral variants that evolve during infection

## References

- Tobin N, et al. *J. Virol.*, (2005) 79: 9625-9634
- Swofford DL PAUP<sup>4.0</sup>. Phylogenetic Analysis Using Parsimony (\*and Other Methods), edn 4.0b2a. Sunderland, MA: Sinauer Associates, Inc.: 1999.
- Akaike H. *IEEE Trans. Automat. Contr.*, (1974) AC 19: 716-723
- Possada D and Crandall K. *Bioinformatics* (1998) 14: 817-816
- Slatkin M, and Maddison W.P. *Genetics*, (1989) 123: 603-613
- Poss, et al., *J. Virol.*, (1998) 72: 8240-8251

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