HIV-1 Clade B pol Evolution following Primary Infection

George K. Hightower1, Susanne J. May2, Josué Pérez-Santiago3, Mary E. Pacold3, Gabriel A. Wagner1, Susan J. Little1, Douglas D. Richman1,4, Sanjay R. Mehta1, Davey M. Smith1,4*, Sergei L. Kosakovsky Pond1,4

1 Department of Medicine, University of California San Diego, La Jolla, California United States of America, 2 Department of Biostatistics, University of Washington, Seattle, Washington United States of America, 3 Life Technologies, San Francisco, California United States of America, 4 Veterans Administration San Diego Healthcare System, San Diego, California, United States of America

Abstract

Objective: Characterize intra-individual HIV-1 subtype B pol evolution in antiretroviral naive individuals.

Design: Longitudinal cohort study of individuals enrolled during primary infection.

Methods: Eligible individuals were antiretroviral naive participants enrolled in the cohort from December 1997-December 2005 and having at least two blood samples available with the first one collected within a year of their estimated date of infection. Population-based pol sequences were generated from collected blood samples and analyzed for genetic divergence over time in respect to dual infection status, HLA, CD4 count and viral load.

Results: 93 participants were observed for a median of 1.8 years (Mean = 2.2 years, SD = 1.9 years). All participants classified as mono-infected had less than 0.7% divergence between any two of their pol sequences using the Tamura-Nei model (TN93), while individuals with dual infection had up to 7.0% divergence. The global substitution rates (substitutions/nucleotide/year) for mono and dually infected individuals were significantly different (p<0.001); however, substitution rates were not associated with HLA haplotype, CD4 or viral load.

Conclusions: Even after a maximum of almost 9 years of follow-up, all mono-infected participants had less than 1% divergence between baseline and longitudinal sequences, while participants with dual infection had 10 times greater divergence. These data support the use of HIV-1 pol sequence data to evaluate transmission events, networks and HIV-1 dual infection.

Citation: Hightower GK, May SJ, Pérez-Santiago J, Pacold ME, Wagner GA, et al. (2013) HIV-1 Clade B pol Evolution following Primary Infection. PLoS ONE 8(6): e68188. doi:10.1371/journal.pone.0068188

Editor: Sunil K. Ahuja, South Texas Veterans Health Care System and University Health Science Center San Antonio, United States of America

Received December 18, 2012; Accepted May 27, 2013; Published June 28, 2013

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: This work was supported by the Veterans Affairs and grants from the National Institutes of Health: DA034978, AI69432, A007384, A0080193, A0096113, A0100970, A0143638, M612512, MO083352, A0177304, A136214, A0077455, A174621, A0080353, A1100665, GM093939; the International AIDS Vaccine Initiative; and the James B. Pendleton Charitable Trust. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: One author, Mary Pacold, does work for Life Technologies. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: davey@ucsd.edu

† These authors contributed equally to this work.

Introduction

HIV-1 is one of the fastest evolving organisms [1,2,3] and within a newly infected individual, quickly evolves into a highly diverse viral population [4,5,6]. This rapid diversification is driven by a low fidelity reverse transcriptase (2.5–3.4 × 10−3 mutations per site per generation), high replication rate (1010 virions produced daily), high recombination rate, genetic plasticity of viral proteins [7], and strong host immune selective pressures [3]. The proportions of sites that undergo substitution differs both within and between HIV-1 coding regions and so studying how specific coding regions evolve provides important insight into HIV-1 pathogenesis and disease because [6,8,9].

Although, HIV-1 genotyping is part of the standard care in the US, its clinical use is largely limited to pol because of the time and cost involved in sequencing the entire HIV-1 genome. With its ubiquitous use in the surveillance and characterization of antiretroviral resistance, HIV-1 pol is likely the most sequenced gene from any organism (as of July 2012 there were over 167,000 sequences in the Stanford HIV Drug Resistance Database) [10]. Subsequently, analyses of large collections of pol sequences have provided important insights into HIV-1 epidemiology and geographic spread [11,12,13] however, such approaches have been limited, by the use of cross-sectional methodology and relatively limited clinical data. For example, HIV-1 pol evolution in acutely monoinfected vs. dual infected individuals has not been extensively examined. Our study used longitudinally collected blood samples and clinical data from newly infected ART naive individuals to better determine intra-individual HIV-1 subtype B genetic variability, provide more accurate estimates of genetic divergence, as well as characterize its relation to disease progression in mono- and dually-infected hosts.
Methods

Participants
Study participants were enrolled in the San Diego Primary Infection Cohort from December 11, 1997 through December 21, 2005, and some participants were followed up until as recently as January 26, 2010. Inclusion was limited to participants, who (1) were infected with HIV-1 clade B, (2) had a blood sample collected within a year of estimated date of infection (EDI), and (3) remained ART naïve through the sequencing of HIV-1 RNA extracted from at least two longitudinally collected blood samples. Available participant data included demographics, EDI, reported HIV risk behaviors, HLA haplotype, CD4 counts (flow cytometry), and blood plasma viral load (Amplicor, Roche) [14,15]. Dual infection was determined by ultradeep sequencing (454 FLX Roche) of three HIV-1 coding regions (env, gag and reverse transcriptase), using a published bioinformatics protocol [16].

HIV-1 pol Sequencing and Analysis
The ViroSeq™ HIV genotyping system was used for population-based pol sequencing per manufacturer instructions (Applied Biosystems, Foster City, CA). Sequencing was performed on an ABI 31000 Genetic Analyzer, and sequences were manually reviewed and edited (Viroseq version 2.4.2). Drug resistance was interpreted by the algorithm available with the Viroseq™ package [20]. Branches between any two sequences were determined as outlined above. Maximum HIV-1 viral load and minimum CD4 counts within the first year following the estimated date of infection were used as covariates. Chi-square and correlation analyses were performed to test whether HLA haplotype, HIV-1 viral load, and CD4 counts were associated with the final inferred HIV-1 substitution rates. We took particular care to characterize genetic variability at sites with ambiguous nucleotides, as these are common in bulk HIV-1 pol sequences and potentially informative of population level viral polymorphism. However, standard phylogenetic approaches treat ambiguous nucleotides as partially missing data, which is conceptually equivalent to resolving ambiguities in a way to maximize sequence similarity [23]. When computing pairwise TN93 distances, we considered two alternatives. First, we resolved ambiguities to minimize pairwise differences between two sequences (TN93-resolved). Second, we averaged all possible complete resolutions of sequences assuming that they are equally likely (TN93-averaged). For instance, when comparing two sequences with ambiguous bases from the electropherogram-ACY and ARC, there are ½ (C:A) and ½ (C:G) differences between them with the resolved approach, and ½ (C:A), ½ (C:G) and ½ (C:T) differences using the averaged approach. TN93-averaged is necessarily greater or equal to TN93-resolved for any pair of sequences.

To determine the global rate of intra-individual HIV-1 pol divergence, we used a maximum likelihood approach and inferred a global rate of nucleotide substitutions under molecular clock with the TN93 model from all sequence data jointly (see approach (1) above). We also considered a model where the global rate differed between mono- and dually-infected individuals, and tested for equality between these rates using LRT [21].

Intra-individual HIV-1 pol Evolution and Disease
HLA haplotype, HIV-1 viral load, and CD4 counts were determined as outlined above. Maximum HIV-1 viral load and minimum CD4 counts within the first year following the estimated date of infection were used as covariates. Chi-square and correlation analyses were performed to test whether HLA haplotype, maximum viral load, and minimum CD4 counts were associated with the final inferred HIV-1 substitution rates.

The Effect of CTL-mediated Immunity on pol Evolution
We used the current annotation of CTL-restricted epitopes available from the Los Alamos Immunology Database (http://www.hiv.lanl.gov/content/immunology/index.html), and participated specific HLA haplotypes (two-digit resolution) to partition pol sequences for each individual into putative CTL-targeted epitopes (CTL*) and non-targeted (CTL) regions. Phylogenetic rate estimation was performed, as previously described above, on CTL* and CTL regions of the alignment separately, for each individual and then jointly to estimate the global substitution rate. Lastly, we measured whether the mean selective pressure, estimated by the dN/dS ratio under the MG94 model, were significantly higher in CTL* regions, both within individuals and globally [24].

Subtype B Divergence and Diversity in the U.S
All unique HIV-1 pol sequences annotated as subtype B from the United States in the Los Alamos National Laboratory
The SCUreal method was used as an additional filter to remove potential mislabeled or intra-subtype recombinant sequences [18]. Sequences were also removed if phylogenetic analysis using LANL Treemaker program demonstrated they clustered with isolates from outside the United States. [http://www.hiv.lanl.gov/components/sequence/HIV/treemaker/treemaker.html]. Finally, we computed pairwise genetic distances (TN93) between all remaining sequences, and used the resulting distribution to determine the 10%, 5% and 1% percentiles of inter-host pairwise genetic distances for subtype B HIV-1.

**Results**

**Participant Characteristics**

Between December 1997 and December 2005, 108 ART naive individuals were enrolled in the San Diego primary infection cohort. 15 of the 108 cohort participants were excluded from this study: nine started ART before at least one follow-up sample was taken, three were infected with non-clade B HIV-1, and for three individuals the time between EDI and first available sequence data exceeded one year (GenBank accession numbers KC814216–KC814576). The 93 eligible individuals were all male, with a median viral load of 38,350 HIV RNA copies/ml (min = 50, max = 5.8 × 10^6) and median CD4 556 cells/ml (min = 121, max = 1193). 16 (17%) had HIV-1 dual infection documented by ultra-deep sequencing [16], 81% were white, and 97% reported sex with other men as their major HIV risk factor (Table 1). At baseline, the median EDI was 81 days (79 for mono-infected and 94 for dually infected), and participants were observed for a median of 1.8 years (mean 2.2, SD = 1.9, min = 41 days, max = 8.6 years) following enrollment. At least one major antiretroviral resistance mutation was found in 24% of participants at time points (p = 0.0025, LRT). It is important to note that for dually infected participants this rate estimate is confounded by the introduction of the second divergent strain, and does not suggest that the intrinsic substitution rate is an order of magnitude faster in those patients. In none of the participants studied, nucleotide substitutions did not occur at a constant rate–in individuals with ≥3 timepoints available, the hypothesis of a strict molecular clock could be rejected in 18 of the 39 mono-infected (at p ≤ 0.05) and 5 of 9 dually infected cases. As expected, on average, pol evolved under purifying selection and we could not reject the hypothesis of purifying selection or neutral evolution for any of the within-host samples (p ≤ 0.05, LRT).

**Intra-individual HIV-1 pol Genetic Evolution**

HIV-1 pol population-level evolution occurred at a low rate, with 37/95 individuals showing no nucleotide substitutions (under the phylogenetic TN93 model) over the entire period of observation. The median substitution rate (including mono and dually-infected individuals) was less than 0.01 substitutions/site/year for all four genetic distance estimates (Table 1). The estimated global substitution rate for mono-infected individuals was $2.2 \times 10^{-4}$ (5%–95% = 6.1 × 10^{-4}–8.4 × 10^{-4}) substitutions/site/year, which is slower than the estimates of the substitution rate based on comparing pol isolates from different individuals under relaxed molecular clock models (1-2 × 10^{-3}, e.g. [11,26,27]). The overall rate for dually infected individuals was $9.7 \times 10^{-3}$ (8.7 × 10^{-3}–1.1 × 10^{-2}) (Figure 1). Global substitution rates for mono and dually infected individuals were significantly different (p < 0.001, LRT). The median EDI in days to last follow-up Median (min, max) 79 (11, 177) 81 (11, 189) 22 (24%).

**Effect of Evolutionary Model**

Since a poorly selected model of evolution can lead to statistical issues during inference [11–12], we assessed the sensitivity of within-host rate inference in the phylogenetic context to the choice of evolutionary model. We compared TN93 to both simpler (JC69 and F81) and more complex (GTR, GTR+G) models, and found that the inference was essentially unaffected. The estimates of within-host substitution rates under all 4 models were nearly perfectly linearly correlated (adjusted R^2 of 0.98 or greater), and the only substantial differences were observed for 2 dually infected cases. As expected, on average, pol evolved under purifying selection and we could not reject the hypothesis of purifying selection or neutral evolution for any of the within-host samples (p ≤ 0.05, LRT).

| Table 1. Participant demographics and major outcomes. |
|-----------------------------------------------|
| **Singly infected** | **Dually infected** | **All** |
| Male gender, n (%) | 77 (100%) | 16 (100%) | 93 (100%) |
| Age, mean (SD, min, max) | 33 (8.7, 19, 58) | 34 (8.2, 22, 54) | 33 (8.6, 19, 58) |
| Risk, Sex with Men, n (%) | 74 (96%) | 16 (100%) | 90 (97%) |
| Race, white, n (%) | 61 (79%) | 14 (88%) | 75 (81%) |
| BL CD4, median (min, max) | 552 (121, 1180) | 571 (263, 1193) | 556 (121, 1193) |
| BL RNA, median (min, max) | 41,600 (50, 58 × 10^6) | 34,900 (779, 21 × 10^5) | 38,350 (50, 58 × 10^5) |
| Any resistance, n (%) | 19 (25%) | 3 (19%) | 22 (24%) |
| EDI in days to first visit Median (min, max) | 79 (11, 189) | 94 (11, 177) | 81 (11, 189) |
| EDI in days to last follow-up Median (min, max) | 741 (116, 3274) | 617 (236, 2351) | 696 (116, 3274) |
| TN93 phylogenetic distance. Substitutions/site/year. Mean (Median; 5%–95%) | 0.0007 (0.0004; 0.0000–0.0031) | 0.0192 (0.0014; 0.0000–0.0905) | 0.0039 (0.0004; 0.0000–0.0052) |
| MG94 phylogenetic distance. Synonymous substitutions/site/year. Mean (Median; 5%–95%) | 0.0003 (0.0000; 0.0000–0.0018) | 0.0131 (0.0003; 0.0000–0.0603) | 0.0025 (0.0000; 0.0000–0.0036) |
| TN93-resolved (first, last). Substitutions/site/year. Mean (Median; 5%–95%) | 0.0006 (0.0003; -0.0000–0.0031) | 0.0103 (0.0005; -0.0000–0.0726) | 0.0023 (0.0003; -0.0000–0.0033) |
| TN93-averaged (first, last). Substitutions/site/year. Mean (Median; 5%–95%) | 0.0025 (0.0014; 0.0003–0.0062) | 0.0154 (0.0026; 0.0008–0.0774) | 0.0047 (0.0015; 0.0003–0.0120) |

doi:10.1371/journal.pone.0068188.t001
patients with $\geq 3\%$ annual rate (between GTR+G and all other models, see Figure S1). This is not surprising, since similar model performance is expected in the setting of very low sequence divergence. Thus, we conclude that the choice of the TN93 model does not unduly influence our results.

**Effect of Distance Metric**

Evolutionary rate estimates from the phylogenetic TN93 and TN93-resolved distances were strongly correlated. This was especially true when restricting the analysis to the 0–0.02 range, which included the vast majority (89/93) of individuals ($p<0.001$, adjusted $R^2 = 0.89$, linear model). Similarly, when restricted to the 0–0.02 range, estimates from phylogenetic TN93 correlated with phylogenetic MG94 (synonymous rates): $p<0.001$, adjusted $R^2 = 0.73$). Under the TN93-averaged metric, non-zero distances were assigned to all but 2 individuals and so linear relationships to the other metrics were poor; however, rank-based testing provided clear evidence of correlation with the other metrics ($p<10^{-9}$ in all pairwise comparisons, Wilcoxon signed-rank test).

**The Impact of CTL-mediated Immune Selection on HIV-1 pol Evolution**

We found that the rates of evolution within pol regions containing epitopes that have been reported as targeted by the individual-specific HLA alleles (2 digit resolution) were consistently higher than outside those regions. Globally, the rate of nucleotide evolution for monoinfected individuals was $9.5 \times 10^{-4} \pm 7.5 \times 10^{-5}$ substitutions/site/year in HLA regions, and $6.0 \times 10^{-4} \pm 4.8 \times 10^{-5}$ for dually infected individuals, the estimates were $1.1 \times 10^{-3} \pm 9.5 \times 10^{-4} \pm 1.3 \times 10^{-5}$, and $9.0 \times 10^{-3} \pm 7.8 \times 10^{-4} \pm 1.0 \times 10^{-3}$, respectively. In both cases, the rates were significantly higher in CTL$^+$ regions based on the phylogenetic likelihood ratio test, $p=0.005$ (monoinfected), $p=0.04$ (dually infected). A similar comparison within individuals revealed that CTL$^+$ region rates were faster in 14 cases and slower in 3 cases ($p\leq0.05$, LRT).

Consistent with multiple literature reports (i.e. [25,28,29,30,31]), we found that selective pressure was elevated in CTL$^+$ regions. Joint estimation for monoinfected individuals, which allowed synonymous substitution rates to vary among individuals, yielded $dN/dS = 0.98 \pm 0.28$ for CTL$^+$ and $dN/dS = 0.49 \pm 0.37$ for CTL$^-$ (the hypothesis that these values were the same can be rejected at $p<0.001$ using LRT). For dually infected individuals, these values were $0.15 \pm 0.12$ and $0.31 \pm 0.24$ (also significantly different ($p<0.001$), $dN/dS$ was also markedly ($p\leq0.05$, LRT) elevated in CTL$^+$ regions for 11 individuals.

**Intra-individual HIV-1 pol Evolution and Disease Progression**

Genetic divergence and nucleotide substitution rates (TN93-resolved, TN93-averaged, MG94) tended to be smaller for individuals with HLA haplotype B27 and B51 ($p$-values between 0.05 and 0.10) when all individuals were considered. It is important to note, this finding was likely driven by a few dually infected individuals with high substitution rate. When only mono-infected individuals were considered (all $p$-values $>0.20$ in similar analysis the results were not statistically significant. In mono-infected individuals TN93-averaged substitution rates were inversely and significantly associated with the observed nadir CD4 count within one year of

---

**Figure 1. Divergence from baseline, based on the phylogenetic estimates under the TN93 model without assuming a molecular clock for each of the 93 study participants.** The solid line represents the mean evolutionary trajectory inferred from mono-infected samples. The dashed horizontal line depicts the 1% genetic distance cutoff, used previously to infer potential epidemiological linkage [11–13].

doi:10.1371/journal.pone.0068188.g001
mediated selection pressure, as others have reported [30–32], it is substitution rates and nadir CD4, likely reflects waning CTL viral load or the presence of transmitted resistance associated with nadir CD4 count, but not with HLA haplotype, respectively. In other words, the genetic distance between 99% of any two random HIV-1 pol sequences from U.S. individuals with subtype B infection is expected to be 3.3% or greater, which exceeds the intra-host viral genetic divergence observed in all of the mono-infected patients in this study.

Conclusions
The utility and widespread use of HIV-1 pol sequencing has facilitated the study of HIV-1 molecular epidemiology and evolution [11,12,13]; however many of these studies have relied on non-longitudinal and limited clinical data. To further characterize intra-host HIV-1 clade B pol evolution in recently infected individuals, who were previously characterized as mono- or dually-infected, we analyzed pol sequences generated from longitudinally collected blood samples in conjunction with HLA haplotype and markers of disease progression.

Overall, HIV-1 pol evolution was slow, with the gene accumulating fewer than 0.01 substitutions/nucleotide/year. Estimated global substitution rate for mono-infected individuals was \( 7.2 \times 10^{-4} \) (5%-95% = \( 6.1 \times 10^{-4} \) to \( 3.4 \times 10^{-4} \)) substitutions/site/year, which is slower than previous estimates that relied on comparing pol isolates from different individuals under relaxed molecular clock models (1-2\( \times 10^{-3} \)) [11,26,27]). For the majority (52%) of mono-infected participants, population-level pol sequences were unchanged after resolving ambiguous nucleotides. Even after an average of 19 months of observation and nearly 9 years of follow-up, divergence remained below 2% for all mono-infected individuals. These findings indicate that the 1% genetic distance cutoff invoked in previous epidemiological linkage studies is likely a conservative estimate to infer individuals belonging to the same transmission network [32,33,34].

In line with previous studies, we observed insignificantly faster nucleotide substitution and higher dN/dS ratios within putative CTL targeted regions of pol, indicating that immune selective pressure is an important factor driving divergence [25,29,30,31]. However, for mono-infected individuals pol substitution rates were associated with nadir CD4 count, but not with HLA haplotype, viral load or the presence of transmitted resistance associated mutations. While, the observed relationship between higher substitution rates and nadir CD4, likely reflects waning CTL mediated selection pressure, as others have reported [30–32], it is difficult to interpret the relationship between pol evolution and other markers of disease progression given there was little or no change in observed viral genotype for most individuals studied. The low observed viral change is an important limitation of this observation and should be pursued in future investigations.

HIV dual infection is defined by the presence of two (or more) concurrently circulating and genetically distinct viral populations in an individual [13], thus it is not surprising that this study inferred a higher rate of viral genetic divergence for individuals with dual infection. The pronounced differences in genetic divergence among dual infected individuals, likely reflect instances in which the original HIV-1 pol sequence has been replaced with the superinfecting strain [16]. Of note, the four dually infected individuals with highest genetic divergence did not differ significantly from the other 12 cases with respect to disease progression (data not shown), which is different from previous reports [16], but this is likely secondary to sample size. Significantly higher dN/dS substitutions within CTL epitopes compared to non-CTL regions, provided clear evidence of host specific selection contributing to divergence, which mirrored our findings in mono-infected individuals.

In summary, this study provides additional evidence that dual infection provides increased amounts of viral genetic material to the infecting viral population and allows for faster rates of observed molecular evolution. For mono-infected individuals, however, this study provides important empirical evidence that the rates of intra-host pol evolution are slow, and lends credence to using pol sequence similarity for transmission network or linkage inferences, even after long periods between sampling and putative transmission events.

Supporting Information
Figure S1 Comparison between nucleotide models demonstrate that inference is insensitive to the choice of substitution model. This is mostly because for very low divergence within individuals, even the simplest models (e.g. F81) provide an adequate approximation to the most general (GTR+G) model.

(TIF)

Acknowledgments
Written informed consent was obtained from all participants and the human experimentation guidelines of the U.S. Department of Health and Human Services and the individual institutions were followed in conducting this research. The U.S. Department of Health and Human Services has issued a Confidentiality Certificate to all of the UCSD studies involving acute and early HIV infection.

Author Contributions
Conceived and designed the experiments: GH SRM DS SKP. Performed the experiments: GH MEP GW SRM DS SKP. Analyzed the data: GH SM JPS MEP GW SRM DS SKP. Wrote the paper: GH SM JPS MEP GW SJL DR SRM DS. Acknowledgments

References
1. Korber B, Muldoon M, Theiler J, Gao F, Gupta R, et al. (2000) Timing the ancestor of the HIV-1 pandemic strains. Science 288: 1789–1796.
2. Leitner T, Kumar S, Albert J (1997) Tempo and mode of nucleotide substitutions in gag and env gene fragments in human immunodeficiency virus type 1 populations with a known transmission history. J Virol 71: 4761–4770.
3. Frost SD, Wrin T, Smith DM, Kosakovsky Pond SL, Liu Y, et al. (2005) Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. Proc Natl Acad Sci U S A 102: 18314-18319.
4. McNearney T, Hornickova Z, Markham R, Birdwell A, Arens M, et al. (1992) Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease. Proc Natl Acad Sci U S A 89: 10247–10251.
5. Wolfs TF, Zwart G, Bakker M, Goudsmidt J (1992) HIV-1 genomic RNA diversification following sexual and parenteral virus transmission. Virology 199: 103–110.
6. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, et al. (2008) Identification and characterization of transmitted and early founder virus...
envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A 105: 7552–7557.

7. Lemey P, Rambaut A, Pybus OG (2006) HIV evolutionary dynamics within and among hosts. AIDS Rev 8: 125–140.

8. Price DA, Goudey PJ, Klemerman P, Sewell AK, Easterbrook PJ, et al. (1997) Positive selection of HIV-1 cytoplasmic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci U S A 94: 1890–1895.

9. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, et al. (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77: 2081–2092.

10. Rhee SV, Taylor J, Wadhera G, Ben-Hur A, Brutlag DL, et al. (2006) Genotypic predictors of human immunodeficiency virus type 1 drug resistance. Proc Natl Acad Sci U S A 103: 17355–17360.

11. Abecasis AB, Vandamme AM, Lemey P (2009) Quantifying differences in the tempo of human immunodeficiency virus type 1 subtype evolution. J Virol 83: 12917–12924.

12. Gifford RJ, de Oliveira T, Rambaut A, Pybus OG, Dunn D, et al. (2007) Phylogenetic surveillance of viral genetic diversity and the evolving molecular epidemiology of human immunodeficiency virus type 1. J Virol 81: 13050–13056.

13. Smith DM, May SJ, Tweeten S, Drumright L, Pacold ME, et al. (2009) A public health model for the molecular surveillance of HIV transmission in San Diego, California. AIDS 23: 223–232.

14. Little SJ, Holte S, Routy JP, Daar ES, Markowitz M, et al. (2002) Antiretroviral drug resistance among patients recently infected with HIV. N Engl J Med 347: 451–456.

15. Hecht FM, Wang L, Collier A, Little S, Markowitz M, et al. (2006) A multicenter observational study of the potential benefits of initiating combination antiretroviral therapy during acute HIV infection. J Infect Dis 194: 725–733.

16. Pacold ME, Pond SL, Wagner GA, Delpotro W, Bourque DL, et al. (2012) Clinical, virologic, and immunologic correlates of HIV-1 intraclade B dual infection among men who have sex with men. AIDS 26: 157–165.

17. Pacold M, Smith D, Little S, Cheng PM, Jordan P, et al. (2010) Comparison of methods to detect HIV dual infection. AIDS Res Hum Retroviruses 26: 1291–1298.

18. Kosakovsky Pond SL, Posada D, Stavinski E, Chappell C, Poon AF, et al. (2009) An evolutionary model-based algorithm for accurate phylogenetic breakpoint mapping and subtype prediction in HIV-1. PLoS Comput Biol 5: e1000581.

19. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526.

20. Pond SL, Frost SD, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. Bioinformatics 21: 676–679.

21. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 386–376.

22. Muse SV, Gaut BS (1994) A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. Mol Biol Evol 11: 715–724.

23. Aldous JL, Pond SK, Poon A, Jain S, Qin H, et al. (2012) Characterizing HIV Transmission Networks across the United States. Clin Infect Dis.

24. Pond SL, Frost SD, Grossman Z, Gravenor MB, Richman DD, et al. (2006) Adaptation to different human populations by HIV-1 revealed by codon-based analyses. PLoS Comput Biol 2: e62.

25. Liu Y, McNevin J, Cao J, Zhao H, Genowati I, et al. (2006) Selection on the human immunodeficiency virus type 1 proteome following primary infection. J Virol 80: 9519–9529.

26. Wertheim JO, Fournet M, Kosakovsky Pond SL (2012) Inconsistencies in estimating the age of HIV-1 subtypes due to heterotachy. Mol Biol Evol 29: 451–456.

27. Huy S, Pillay D, Clewley JP, Pybus OG (2005) Genetic analysis reveals the complex structure of HIV-1 transmission within defined risk groups. Proc Natl Acad Sci U S A 102: 4425–4429.

28. Brumme ZL, Brumme CJ, Carlson J, Streeck H, John M, et al. (2008) Marked epistatic- and allele-specific differences in rates of mutation in human immunodeficiency virus type 1 (HIV-1) Gag, Pol, and Nef cytoplasmic T-lymphocyte epitopes in acute/early HIV-1 infection. J Virol 82: 9216–9227.

29. Henn MR, Boutwell CL, Charlebois P, Lennon NJ, Power KA, et al. (2012) Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. PLoS Pathog 8: e1002529.

30. Brumme ZL, Tao I, Szeto S, Brumme CJ, Carlson JM, et al. (2008) Human leukocyte antigen-specific polymorphisms in HIV-1 Gag and their association with viral load in chronic untreated infection. AIDS 22: 1277–1286.

31. Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, et al. (2007) Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. PLoS Pathog 3: e94.

32. Buskin SE, Ellis GM, Pepper GG, Frenkel LM, Pergam SA, et al. (2008) Transmission cluster of multiclass highly drug-resistant HIV-1 among 9 men who have sex with men in Seattle/King County, WA, 2005–2007. J Acquir Immune Defic Syndr 49: 205–211.

33. Hao D, Fisher M, Hue S, Dean G, Murphy G, et al. (2005) Transmission of HIV-1 during primary infection: relationship to sexual risk and sexually transmitted infections. AIDS 19: 85–90.

34. Hue S, Clewley JP, Gane PA, Pillay D (2004) HIV-1 pol gene variation is associated with viral load in chronic untreated infection. Aids 18: 719–728.