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Inferring Viral Population Structures Using Heteroduplex Mobility and DNA Sequence Analyses

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Abstract

Heteroduplex mobility (HMA) and tracking assays (HTA) are used to assess genetic relationships between DNA molecules. While distinguishing relationships between clonal or nearly clonal molecules is relatively straightforward, inferring population structures is more complex. To address this issue, HIV-1 quasispecies with varying levels of diversity were studied using both HTA and DNA sequencing. Viral diversity estimates and the temporal features of virus evolution were found to be generally concordant between HTA and DNA sequencing. In addition, the distribution of pairwise differences and the rates of virus divergence were similar between the two methods. These findings support the use of HTA to characterize variant populations of DNA and strengthen previous inferences concerning the evolution of HIV-1 over the course of infection.

Keywords

HIV-1; HETERODUPLEX MOBILITY ASSAY; HETERODUPLEX TRACKING ASSAY; DNA SEQUENCING; VIRAL EVOLUTION; VIRAL DIVERSITY; VIRAL DIVERGENCE

1. Introduction

The heteroduplex mobility assay (HMA) was developed as a laboratory method to determine genetic distances between related DNA sequences, producing data that can be used to group sequences, including by construction of phylogenetic trees (Delwart et al., 1993). HMA involves mixing two related DNA sequences or sequence populations (quasispecies), denaturing and then annealing the DNA mixture to reform homoduplexes as well as form heteroduplexes between related but non-identical sequences. The reannealed DNA mixtures are then subjected to acrylamide gel electrophoresis at neutral pH, in which heteroduplexes migrate more slowly than homoduplexes.

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A strong relationship between DNA heteroduplex mobility in acrylamide gels and differences in DNA sequence was found and used to develop the heteroduplex mobility (HMA) and tracking (HTA) assays, initially to characterize the genetic subtype and phylogenetic relatedness between HIV-1 genomes (Bachmann et al., 1994; Delwart et al., 1995; Delwart et al., 1994b; Delwart et al., 1993).

HMA kits have been developed, initially to determine the viral genetic subtype of HIV-1 *env* sequences, and have been made freely available by the National Institutes of Health (NIH) AIDS Reagent Program (www.aidsreagent.org) and the National Institute for Biological Standards and Control (NIBSC) (www.nibsc.ac.uk), and used extensively worldwide (Apetrei et al., 1995; Avila et al., 2002; Bobkov et al., 1997; Bobkov et al., 1998; Bobkova et al., 2001; Buonaguro et al., 1995; Buonaguro et al., 2004; Cardoso et al., 2010; Carrion et al., 2009; Castro et al., 2003; Esteves et al., 2003; Esteves et al., 2002; Gadkari et al., 1998; Gaywee et al., 1996; Guimaraes et al., 2012; Hussein et al., 2000; Lakhashe et al., 2008; Lasky et al., 1997; Li et al., 1999; Loussert-Ajaka et al., 1998; Mandal et al., 2002; Mandal et al., 2000; Menu et al., 1999; Monteiro et al., 2009; Osmanov et al., 2002; Pando et al., 2007; Parreira et al., 2006; Ramalingam et al., 2005; Russell et al., 2000; Saad et al., 2006; Sabino et al., 1996; Sahni, Kapila, and Gupta, 2008; Sahni, Prasad, and Seth, 2002; Santiago et al., 1998; Sarkar et al., 2011; Sarraimi-Forooshani et al., 2006; Sawadogo et al., 2003; Siddappa et al., 2004; Teixeira et al., 2004; Tripathy et al., 2005; Tscherning-Casper et al., 2000; Velarde-Dunois et al., 2000; Wasi et al., 1995b; Zhu et al., 1995). An HIV-1 *gag* subtyping HMA kit (Heyndrickx et al., 2000; Sengupta et al., 2005; Tatt, Barlow, and Clewley, 2000) is also available from the NIH and NIBSC, and assays have been developed for HIV-1 *env*-gp41 (Agwale et al., 2001) and *tat* (Diaz et al., 1999). The ability to subtype different regions of the viral genome made it possible for HMA to identify inter-subtype recombinants (Cham et al., 2000; Heyndrickx et al., 2000; Sarkar et al., 2011; Sawadogo et al., 2003). HMA-based subtyping has also been developed for other viral pathogens, including Hepatitis C (Calvo et al., 1998; Li et al., 2008; White et al., 2000), feline immunodeficiency virus (Bachmann et al., 1997), small-ruminant lentiviruses (Germain, Croise, and Valas, 2008), Influenza (Zou, 1997) and enteric adenoviruses (Soares et al., 2004). The accuracy of HMA subtyping in many of the above studies has been evaluated using DNA sequencing and compared by some to peptide-based serologic methods (Bobkova et al., 2001; Gaywee et al., 1996; Hussein et al., 2000; Wasi et al., 1995a). No erroneous subtyping due to HMA has been reported.

As there is pathogenetic significance of HIV-1 variants that use CXCR4 as a coreceptor (Koot et al., 1993; Tersmette et al., 1989), HMA-based methods have been developed to identify and characterize HIV-1 strains with CCR5 versus CXCR4 coreceptor specificities (Coetzer et al., 2006; Low, Swenson, and Harrigan, 2008; Nelson et al., 2000b; Nelson, Fiscus, and Swanstrom, 1997). Recently, an HMA was developed (Shi et al., 2012) to identify coreceptor usage variants using the enhanced differential migration of heteroduplexes under denaturing conditions (Doukhan and Delwart, 2001; Upchurch, Shankarappa, and Mullins, 2000) and primer modifications.

HMA-based assays targeting different regions of viral genomes have helped track the evolution of the virus over the course of infection (Delwart et al., 1994b; Ince et al., 2009a) and mutations that confer resistance to antiviral drugs (Kapoor et al., 2004a; Resch et al., 2001; Resch et al., 2005; Schnell, Ince, and Swanstrom, 2008b). HMA has been used to document epidemiologically linked infections (Manigart et al., 2012) and to identify distinct variants in different cell types (Jadhav et al., 2011). HMA has also been used to assess compartmentalization of viruses in different fluids, such as the genital tract (Ping et al., 2000), cerebrospinal fluid (Schnell et al., 2010) and saliva (Freel et al., 2001). Similarly, HMA has been used to detect HIV-1 dual or superinfection (Chen et al., 2012; Diaz et al.,

2005; Kraft et al., 2012; Manigart et al., 2004; Powell et al., 2008; Powell, Urbanski, and Nyambi, 2008; Rachinger et al., 2010a) and has been shown to be more sensitive than bulk sequencing to detect superinfection (Rachinger et al., 2010d). Multiple studies have also used HMA to assess changes in the HIV-1 genome during the natural history of infection and during antiviral therapy (Delwart et al., 1994a; Doukhan and Delwart, 2001; Dykes et al., 2000; Ince et al., 2009a; Kitrinos et al., 2003; Li et al., 1999; Rachinger et al., 2012; Troyer et al., 2005).

HTA, a variant of HMA, involves labeling a single sequence or one of the two populations used to form heteroduplexes. Labels have included radioactivity nucleic acid precursors, biotin, and fluorescent dyes as in the current study. Detection of the label after acrylamide gel electrophoresis results in visualization of only those heteroduplexes formed with the probe, hence, specific heteroduplexes can be “tracked” within a population. HTA is also used to quantitate variants within virus competition assays (Abraha et al., 2005).

In addition, a number of studies targeting the minor subpopulations, such as the study of drug-resistance mutations, compartmentalization, superinfection and coreceptor-usage variants, have shown that HMA/HTA are cost-effective, efficient, and often superior to conventional Sanger-based methods (Barlow, Green, and Clewley, 2000; Ince et al., 2009b; Kapoor et al., 2004b; Manigart et al., 2004; Nelson et al., 2000a; Powell et al., 2008a; Powell, Urbanski, and Nyambi, 2008b; Rachinger et al., 2010b; Rachinger et al., 2010c; Schnell, Ince, and Swanstrom, 2008a; Zhu et al., 1996). The level of sensitivity in the assessment of minor populations can also be further enhanced by PCR amplifying the portion of the gel containing heteroduplexes (Delwart and Gordon, 1997) or separating specific heteroduplexes using biotin-labeled probes (Schnell et al., 2008b). Preparation of single-stranded DNA HTA probes also allows detection of minor species within mixed sequence populations, such as viral quasiespecies, at levels as low as ~0.5% (Zhu et al., 1996).

This study characterized a collection of HIV-1 *env* C2V5 heteroduplexes, each of which was generated using an estimated number of virus templates sampled over the course of infection of multiple individuals. Viral evolution over time was examined from heteroduplex mobility patterns using a tracking probe (HTA) and was compared to data derived from sequencing a sample of virus templates from each pool. An earlier study (Shankarappa et al., 1999) had reported highly consistent features in the evolution of HIV-1 using about 10–15 sequences per sample from nine individuals, sampled over 6–12 years of infection starting from primary infection. The current study used HTA on the same samples, but containing 50–100 viral templates, to test for the consistency in estimates of viral variation between HTA and DNA sequence data.

2. Materials and Methods

2.1 Patient samples and specimen preparation

The mobility of heteroduplexes from seven subjects from a previous study (Shankarappa et al., 1999) (Pt 1, 2, 5, 6, 7, 8, and 9, a total of 86 samples) were evaluated in the current study. Samples were collected prospectively for 6 to 11 years at semiannual intervals, and were available starting from the first post-seroconversion sample. PBMCs were processed as described previously (Shankarappa et al., 1999). Serial end-point dilutions of DNA were amplified using primers encompassing HIV-1 *env* gp160 in the first round and the C2-V5 region in the second round, as outlined previously (Shankarappa et al., 1999). Positive versus negative results from these PCRs were used in an endpoint calculator (Rodrigo et al., 1997) web tool (<http://indra.mullins.microbiol.washington.edu/quality/>) to determine the number of amplifiable viral templates present in the sample. Second round PCR products

were pooled to contain 50–100 virus genome templates, and the pools were used for HTA as well as for deriving cloned sequences from each sample. For Pt 1, viral RNA from plasma samples was used for the last five time points since no PBMC samples were available.

2.2 Design and Construction of Fluorescent-Labeled Probes

Four types of HTA probes were used to study virus population structure. 1) HIV-1 founder populations were used as probes to assess virus divergence from those sequences over the course of infection. 2) Relationships between late-stage viruses and those sampled over the course of infection were made using probes derived from the last sample obtained from each individual. 3) Probes were derived from all samples collected over the course of infection in a subset of individuals to more critically assess the temporal features of viral evolution. Each of these probes were used to generate heteroduplexes separately with each of the longitudinally collected samples from that individual. 4) Probes derived from cloned viral DNA was used to explore population shifts at different stages of infection within the individuals from which the probes were derived. The diversity for the first three types of probes in seven subjects is illustrated in Table 1. Diversity of founder sequence probes ranged from 0.31–1.83% (mean: 0.88%), when estimated using DNA sequence data, and 1.01–2.00% (mean: 1.25%), when estimated using HTA. Estimates of the diversity of final sample probes ranged from 2.01–5.06% (mean: 3.76%) using DNA sequences and 1.59–5.48% (mean: 3.09%) using HTA. The average diversity of the group 3) probes estimated using DNA sequence and HTA data were 4.02 and 3.78%, respectively. The downstream PCR primer used to generate the above probes was fluorescently labeled at its 5' end using the FAM-dye (Applied Biosystems, Foster City, CA) in a PCR reaction using the same conditions described in (Shankarappa et al., 1999).

2.3 Heteroduplex formation and analyses

Labeled molecules from one time point (Probe) were hybridized with a 100-fold excess of unlabeled viral variants from another time point (Driver). Specifically, 0.5 µl labeled probe from one sample was mixed with 2 µl of unlabeled driver, the latter corresponding to a 25-fold concentrated PCR-amplified product from the same or a second sample, and 0.5 µl of 5X HMA annealing buffer, for a total volume of 3 µl (for a final concentration of 100 mM NaCl, 10 mM Tris HCl, pH 7.8, 2 mM EDTA). To generate heteroduplexes, each specimen or probe-driver mix was incubated at 100°C for 3 minutes and quickly transferred to ice. Heteroduplexes were mixed with gel loading buffer containing Dextran blue and molecular size markers labeled with the fluorescent dye ROX (Applied Biosystems, Foster City, CA) prior to loading the gel. Electrophoresis was performed on an ABI 373 gel fragment analyzer using 24 cm plates containing 0.8 mm thick 5% polyacrylamide (29% acrylamide, 1% bis) gels in TBE buffer (89 mM Tris-HCl, 89 mM Sodium borate, 2 mM EDTA). To minimize thermal artifacts, electrophoresis was done at a low, constant 5 Watts for 3200 VoltHours (~12 hours). Gel images were acquired using GeneScan software (Applied BioSystems, Foster City, CA) and processed using Adobe Photoshop (Adobe Systems, San Jose, CA). Using gel images without molecular weight markers, fluorescence intensity and migration distance of the heteroduplex bands in each lane were measured using NIH Image software (<http://rsb.info.nih.gov/nih-image>). Since nearly all heteroduplexes formed by the ~600 bp HIV-1 fragments migrated farther than the 2026 bp size marker, the data capture between subjects and runs was normalized by limiting the study of heteroduplexes to those migrating between the 554 and 2026 bp Rox-labeled markers.

2.4 Data Analyses

The relative mobility of each heteroduplex was considered to be a proxy for the pairwise difference between two divergent sequences, and the mobility of heteroduplexes was assumed to be inversely proportional to pairwise differences. Therefore, if two

heteroduplexes migrated similar distances, their genetic differences were assumed to be similar. Thus, for a lane with multiple heteroduplex bands of varying intensities, each successive heteroduplex band was expected to mimic a pairwise difference and the band's intensity was considered to be proportional to its frequency. To quantify genetic distances, a composite value expressed by $pq/10$ was derived for each comparison, in which “ p ” was the fluorescence intensity at each pixel and “ q ” the reciprocal migration distance.

DNA distances were derived using methods described previously (Shankarappa et al., 1999). Briefly, DNA sequences were aligned using Pileup (Genetics Computer Group, Madison, WI) or ClustalW (Thompson, Higgins, and Gibson, 1994) and manually edited to align codons. Uncorrected pairwise DNA distances were derived using MEGA (Kumar et al., 2001). StatPlus:mac (AnalystSoft Inc) in Microsoft Excel (Microsoft, Redmond, WA) were used to derive R^2 , the squared value of the sample correlation coefficient, and its standard error (SE), to assess the concordance between DNA- and HTA-distances, and to determine the rates of viral divergence from the founder sequences.

3. Results

3.1 Characterization of Virus Populations by HTA

Figure 1 illustrates the heteroduplex mobilities formed from PCR fragments derived from 50–100 viral templates, and the comparison of diversity estimated using heteroduplex mobilities and DNA sequences for viruses sampled in seven individuals. The upper half of the figure (panels A, B, and C) is analogous to viral divergence from the founder, as reported in an earlier study (Shankarappa et al., 1999). Panel A illustrates the mobility of heteroduplexes formed between the relatively homogenous founder virus sequences present early in infection as the probe, and the increasingly diverse populations present at subsequent time points as the drivers in the formation of heteroduplexes. The composite HTA distance values are plotted in panel B and the average DNA distances estimated using DNA sequence data from 10–15 viral genomes for each of the corresponding comparisons are illustrated in panel C.

Figure 1 Panels D, E and, F illustrate the same relationships, this time using probe sequences derived from the final time point studied, when the probe population had high diversity. The heteroduplex profiles generated using the early founder and the late sequence probes were generally consistent with the distribution of pairwise DNA distances (compare heteroduplexes in panels A and D with the distribution of individual pairwise distances in panels C and F, respectively). The presence of outliers appeared to be concordant in many instances. For example, a set of outlier pairwise DNA distances in the fourth visit of Pt 8 in panel C correlated with the slow moving heteroduplexes for the corresponding time point in panel A. In addition, the trajectories of viral population changes observed using heteroduplex mobilities were similar to those observed using the average DNA distances (compare panel B with C, and panel E with F).

3.2 Estimating Genetic Diversity for Pools of Variants Using HTA

Figure 2 and Table 2 illustrate comparisons of the genetic distances estimated using heteroduplex profiles and DNA sequences. The paired HTA- and DNA-distance estimates derived from the 86 samples represent a comparison between these estimates at two levels. In Figure 2, panel A, distance estimates were plotted separately for each subject as a function of time following seroconversion to reveal any systematic artifacts associated with viruses within an individual or viruses sampled at certain times during infection. Similarly, distance estimates were plotted compared to founder or final sequence probes in panel B.

In general, the distance estimates were more consistent between HTA and DNA data when the low-diversity founder sequences were used as probes (Figure 2, Table 2). R^2 values for HTA distances derived using the founder and final sequence probes for seven individuals averaged 0.43 and 0.34, respectively. R^2 for the founder sequence probes were higher than that for the final sequence probes in six of seven individuals. Individually, the strongest associations were seen for Pts 2 and 9 founder sequence probes with R^2 values of 0.98 and 0.96, respectively. The weakest associations were observed for probes derived from the final sequences in Pts 1, 7, and 8 with R^2 values of less than 0.1.

3.3 Assessment of Temporal shifts in Viral Quasispecies

Earlier studies documented two features consistent with a continual turnover of the virus population over the course of infection: (i) viruses within a given sample were more closely related to each other as compared to variants sampled at other time points, and (ii) as the length of time between samples increased, the viral sequences became progressively more divergent (Shankarappa et al., 1999). To assess these features using HTA, viruses derived from each sample were used as probes to form heteroduplexes with viruses from each of the other samples as the driver. Figure 3, panel A illustrates the results for samples from Pt 8 and is representative of analyses from other individuals (data not shown). These results revealed that heteroduplexes migrating farthest down the gel always corresponded to the intra-time point comparison. In addition, heteroduplex profiles in each lane appeared unique yet more similar to those of the adjoining time points, becoming progressively less similar with increasing time separating the probe populations. These inferences were generally consistent between the charts depicting HTA and DNA sequence analyses in the assessment of temporal features of viral evolution.

Heteroduplex mobilities decreased roughly linearly with time using the first and last time point sequences as probes (Figure 1, panels A vs. D). For Pt 8, the intersection of these two linear trajectories centered on V15 at 3.4 years post-seroconversion (Figure 3A). Figure 3, panel B, illustrates the assessment of virus population dynamics using two cloned HTA probes, 8V14xA and 8V15x03, derived from samples collected at 2.9 and 3.4 years post-seroconversion, respectively. The V14 clone was more closely related to the population of variants present earlier in infection whereas the V15 clone was more closely related to the population of variants present at subsequent time points. Even though X4 viruses appeared for the first time in this subject during the above period (Shankarappa et al., 1999), both probes corresponded to R5 viral genomes.

Divergence from the founder virus results from a combination of genetic drift and selective forces acting on the virus, including those of the host's immune response and an expanding viral tropism. Table 3 illustrates the rates of virus divergence estimated using HTA and DNA sequences. In three individuals (Pts 1, 7, and 8), the rates of divergence estimated by these two methods differed by less than 10%, and in one (Pt 2) by 27%. In the other three individuals (Pts 5, 6, and 9), HTA markedly underestimated the rates of divergence. However, in two of these individuals (Pts 6, and 9) this underestimation was mostly due to aberrant low estimates for a few samples, especially towards later stages of the disease (Figure 2A). On the other hand, HTA data for Pt 5 was strongly affected by the clustering of heteroduplexes around 3 or 4 regions for all samples collected after 1.6 years of infection (Figure 1A) even though their DNA sequences diverged. Therefore, data from Pt 5 appears to be an exception among the generally concordant estimates of viral divergence using HTA and DNA sequences.

4. Discussion

Comparing sequence data (10–15 sequences per specimen) against HTA data (from 50–100 molecules), consistent features in the evolution of HIV-1 sampled over the course of infection (Shankarappa et al., 1999) were observed. The concordance between the two techniques was inexact, as expected by the fact that the two methods are differentially impacted by indels (insertions and deletions) within the viral genomes studied. Indels reduce heteroduplex mobility much more than mispairings (Delwart et al., 1994a; Upchurch et al., 2000), whereas indels and adjacent regions of substantial heterogeneity are usually stripped out of DNA sequence alignments prior to analyses. The effect of indels can be limited by focusing the study to regions of the viral genome that are known to contain few if any indels, whereas the region used in this study had several indels in order to accentuate differences between variants.

When HTA probes were derived from the early founder sequences with low diversity (mean DNA diversity, 0.88%) the profile of heteroduplexes tended to be sharper and the correlation between the estimated values stronger. On the other hand, using the more diverse final sequence probes (mean DNA diversity, 3.76%), heteroduplexes tended to smear and the correlation between the estimated values discordant due to an underestimation (Pt 9) or overestimation (Pt 2) of DNA distances by HTA (Figure 2). Using a single probe sequence to represent a pool of variants may reduce these artifacts. Such probes can be derived using the ancestral and center of tree approach (Gao et al., 2004; Mullins et al., 2004; Nickle et al., 2003) for use at the individual-level (Kesturu et al., 2006). Another factor contributing to the sometimes weak correlations between HTA- and DNA-distances was the clustering of Pt 5 heteroduplexes around 3 or 4 major bands, in spite of clear incremental differences in the DNA sequences (Figure 1). Such aberrant, potentially structure-induced HMA artifacts can be overcome by using partially denaturing electrophoretic conditions (Upchurch et al., 2000). Lastly, the 5–10 fold difference between the number of templates examined by DNA sequencing versus HTA could have led to differences attributable to DNA sequence sampling artifacts.

While DNA sequencing methods are unquestionably more informative than HMA and HTA, they remain costly and technologically beyond the scope of some laboratories, particularly in developing countries. Whereas this study used an expensive technology, other labeling and simpler electrophoresis technologies can be substituted, as reviewed in the Introduction.

The methods used in this study largely overcame sampling artifacts by the simultaneous evaluation of large numbers of viral templates, on par with next-generation technologies, without the full costs of Sanger sequencing used in methods such as single genome sequencing (SGS) (Edmonson and Mullins, 1992; Frenkel et al., 2003; Liu et al., 1996; Palmer et al., 2005; Simmonds et al., 1990). Thus, HMA offers the opportunity to interrogate viral population structure with lower infrastructure requirements and supply costs than DNA sequencing.

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Highlights

- Viral subtyping and phylogenies generated with heteroduplex mobility (HMA) and tracking (HTA) assays are reliable
- The utility of HTA for describing complex viral quasispecies was addressed in the current study
- Concordance was usually found between heteroduplex mobilities to DNA sequence distances
- Occasional discordance was likely to the effect of indels
- HTA is a useful technology for describing the breadth of complex viral populations

Figure 1. Assessment of genetic changes in HIV-1 over the course of infection using HTA and DNA sequence data

The gel images of the heteroduplexes formed between variants present at each time point and heteroduplex tracking probes derived from early, founder sequences (panel A), or late (final time point) sequences (panel D) are shown for each individual. Rox dye labeled molecular size markers were incorporated into each HTA and their sizes in basepairs are indicated on the left. Genetic distances estimated using heteroduplex mobilities (HTA-distance) are plotted in panels B and E for the probes derived from the founder and final sequences, respectively. Panels C and F illustrate the profile of pairwise DNA distances between sequences sampled at each time point and the founder (panel C) or final (panel F) sequence populations. For DNA distances, each pairwise distance value is plotted as an open diamond with the average values connected by a line. X-axes show the years following seroconversion (not plotted to scale).

Figure 2. Comparison of genetic distances estimated using HTA and DNA sequences according to the individual, time following infection, and the type of probe used in HTA experiments
 Panel A illustrates the genetic distances plotted with respect to time following seroconversion, separately for each subject. Panel B shows the scatter plots of HTA- versus DNA-distance data, derived using the founder sequence (upper graph) and the final sequence probes (lower graph).

Figure 3. Temporal features in the evolution of HIV-1 assessed using HTA and DNA sequence data

Panel A illustrates intra-time point diversity and the HTA using probes as indicated at the top and driver sequences from each sample. The upper and middle sections of panel A illustrate the HTA distances and the source heteroduplexes, respectively, and the bottom section illustrates the DNA distances. Each pairwise DNA distance is depicted as an open diamond with a line connecting the average values. The purple colored open bar spanning all three sections of Panel A identifies the sample from which the probe sequences were derived. Panel B illustrates heteroduplexes formed between the labeled cloned probe DNA sequence from V14 (2.9 years post-seroconversion) and V15 (3.4 years post-seroconversion) and the driver virus population in samples over the course of infection. Highlighted lanes identify the sample from which cloned probes were derived.

Table 1

HTA Probes Used in the Study

<u>Probe</u>	<u>Patient/Visit ID</u>	<u>Diversity (%)</u>	
		<u>DNA</u>	<u>HTA</u>
Founder Sequences	Pt 1	0.31	1.01
	Pt 2	0.80	1.05
	Pt 5	1.83	1.08
	Pt 6	0.59	0.86
	Pt 7	0.79	1.33
	Pt 8	0.27	2.00
	Pt 9	1.56	1.45
	<u>Mean</u>	<u>0.88</u>	<u>1.25</u>
Final Sequences	Pt 1	2.74	4.36
	Pt 2	4.37	2.02
	Pt 5	3.81	1.59
	Pt 6	2.01	2.01
	Pt 7	4.58	5.48
	Pt 8	5.06	3.32
	Pt 9	3.77	2.84
	<u>Mean</u>	<u>3.76</u>	<u>3.09</u>
Pt 8	V09	0.27	2.00
	V10	1.22	3.21
	V11	1.61	2.83
	V13	3.83	4.26
	V14	3.41	3.65
	V15	3.12	3.93
	V16	3.65	4.14
	V17	4.02	2.48
	V18	5.06	3.54
	V19	5.06	4.47
	<u>Mean</u>	<u>4.02</u>	<u>3.78</u>

Table 2

Concordance between HMA- and DNA-distances

Patient ID	Probe	Time Points (N)	R²	SE
All Pts	All **	262	0.36	0.01
	Founder&FinalSeq	172	0.38	0.01
	FounderSeq	86	0.49	0.01
	FinalSeq	86	0.16	0.01
	Founder&FinalSeq	30	0.30	0.02
Pt 1	FounderSeq	15	0.61	0.01
	FinalSeq	15	0.02	0.02
	Founder&FinalSeq	28	0.86	0.01
Pt 2	FounderSeq	14	0.98	0.00
	FinalSeq	14	0.89	0.00
	Founder&FinalSeq	32	0.05	0.02
Pt 5	FounderSeq	16	0.05	0.01
	FinalSeq	16	0.65	0.00
	Founder&FinalSeq	20	0.26	0.01
Pt 6	FounderSeq	10	0.25	0.01
	FinalSeq	10	0.16	0.01
	Founder&FinalSeq	18	0.22	0.01
Pt 7	FounderSeq	9	0.09	0.02
	FinalSeq	9	0.02	0.01
	All **	110	0.38	0.01
Pt 8	Founder&FinalSeq	20	0.37	0.01
	FounderSeq	10	0.09	0.01
	FinalSeq	10	0.06	0.00
	Others **	90	0.10	0.01
Pt 9	Founder&FinalSeq	24	0.76	0.01
	FounderSeq	12	0.96	0.01
	FinalSeq	12	0.55	0.01

**

Includes Pt 8 data from Figure 3

Table 3

Rates of divergence from the early founder viruses estimated using HMA and DNA sequences

Pt ID	DNA sequencing		HMA	
	Percent/Year	SE	Percent/Year	SE
Pt 1	0.92	0.09	0.91	0.08
Pt 2	0.98	0.03	0.72	0.04
Pt 5	0.96	0.05	0.28	0.15
Pt 6	1.20	0.10	0.26	0.29
Pt 7	1.01	0.04	0.92	0.25
Pt 8	0.86	0.06	0.92	0.11
Pt 9	0.78	0.06	0.34	0.04

The rates of virus divergence were calculated using data points from the first post-seroconversion sample to the time of divergence stabilization, as identified previously (Shankarappa et al., 1999).