Variance of Plasma Human Immunodeficiency Virus Type 1 RNA Levels Measured by Branched DNA within and between Days

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Previous studies have shown that CD4-positive T cells vary in a predictable manner over 24 h. This diurnal variance has significant clinical implications. Recently, viral RNA measurements have been increasingly used as a standard marker in the management of human immunodeficiency virus (HIV)—infected patients. Little detailed analysis of the variability of this marker has been conducted. To define the variance of plasma HIV-1 RNA levels within days, 11 clinically stable patients with established HIV infection and a baseline viral RNA level >40,000 copies/mL were studied. Following the patients' admission to an inpatient research unit, plasma samples were obtained frequently over 48 h and analyzed for HIV-1 RNA levels by use of a quantitative branched chain DNA assay (bDNA). No diurnal pattern was detected. In these clinically stable patients, viral RNA levels exhibited a variance of ~0.4 log.

The clinical utility of an assay depends, in large part, on its variability. In a clinically stable patient, plasma human immunodeficiency virus type 1 (HIV-1) RNA levels reflect a steady state, in which rates of virion clearance approximate rates of virion production. In patients with a baseline viral RNA level >500 copies/mL, plasma RNA levels have a week-to-week variability of ~0.5 log [1]. Factors influencing variation include inherent biologic changes and those introduced by the performance characteristics of the particular assay.

CD4 cell counts demonstrate significant within-patient variability, limiting their usefulness for monitoring a patient’s response to therapy [1, 2]. The cause of this variance is multifactorial but includes predictable diurnal fluctuations. Absolute levels of CD4 cells are lowest in the morning (8 A.M.) and highest in the late evening (10 P.M.). The impact of diurnal variation can be considerable, with counts increasing by as much as 100% during the day. Similar variation has been seen in healthy volunteers and HIV-infected adults [3–5]. Although the cause for this variation is not completely understood, diurnal fluctuations in plasma cortisol and an associated redistribution of CD4 T cells from lymphoid organs to the peripheral circulation or the demargination of CD4 T cells within the vasculature may be contributing factors [6, 7]. However, it remains unclear whether HIV-1 RNA levels exhibit a diurnal pattern. In one 8-patient study using an early polymerase chain reaction assay, levels did not vary significantly when assayed at 8 A.M. and 5 P.M. [8].

To better understand the performance characteristics of the branched DNA (bDNA) assay, and to explore whether HIV-1 RNA levels vary in a predictable manner over a 24-h period and from day to day, we performed repeated HIV-1 RNA testing on 11 HIV-infected adults. Given the rapid rate of clearance of HIV-
1 virions from circulation, we predicted that if levels of HIV-1 production vary significantly within a day, they would be expected to result in measurable changes in plasma HIV-1 RNA levels.

Methods

This single-site observational study involved 11 HIV-1–infected subjects recruited from a university-based public health AIDS clinic. Primary inclusion criteria included CD4 T cell count <400 cells/mm³, HIV RNA-1 copy number >40,000 copies/mm³, stable or no antiretroviral therapy for 3 months prior to enrollment, and a hemoglobin level >10 g/dL. All subjects were clinically stable during the study period and free of intercurrent illness in the previous 4 weeks or of an AIDS-defining complication within the prior 3 months. Subjects were admitted to the General Clinical Research Center at San Francisco General Hospital for 48 h.

HIV-1 RNA specimen handling. An indwelling intravenous catheter was placed, and blood samples were drawn according to a protocol. Before each sample was obtained, 3–5 mL of blood was obtained and then discarded. On day 1, samples were obtained every 4 h (8 A.M., 12 noon, 4 P.M., 8 P.M., 12 midnight, and 4 A.M.). On day 2, samples were obtained every 8 h (8 A.M., 4 P.M., and 12 midnight). Each viral RNA blood specimen tube was gently inverted five times after being drawn (to mix the blood with the anticoagulant). Whole blood was obtained, and then plasma was promptly separated by centrifugation for 10–15 min at 1000 g, divided into two 2.5-mL aliquots, and stored in sterile plastic screw-capped tubes at −70 to −80°C. Viral RNA plasma specimens were shipped on dry ice to Chiron (Emeryville, CA) for analysis. Viral HIV RNA levels were measured by use of the commercially available Chiron Quantiplex bDNA signal amplification assay as described elsewhere [9].

Statistical methods. To determine if plasma HIV-1 RNA levels vary in a diurnal pattern, repeated-measures analysis of variance was used to test whether there was an overall time effect. Analyses were undertaken to examine the within-day pattern; samples taken every 4 h on the first day were analyzed in which time was the only factor in the model. The analysis was done using SAS (version 6.09, procedure GLM; SAS Institute, Cary, NC). The univariate test was used unless the test for lack of sphericity was significant, when the Huynh-Feldt adjustment to the univariate test was used.

The components of variance were estimated using SAS (version 6.09, procedure PROC-MIXED). The default method for estimating the variance components (restricted maximum likelihood method) was used. By use of these estimates, the 95% tolerance limit was determined. The 95% tolerance limit is the upper 95% confidence limit on the estimate of the 95th percentile; that is, there is a 95% probability that 95% of all future differences between any two measurements will fall within that interval.

Results

Subject characteristics. Eleven HIV-1–positive persons were recruited for this study. The mean age was 42 years (range, 25–54). The subjects were predominantly male (10/11) and white (8/11). Mean CD4 T cell count at baseline was 142 cells/mL (range, 9–326). The median HIV-1 RNA level drawn at the screening visit was $178 \times 10^3$ copies/mL (range, $54–1679 \times 10^3$ copies/mL). Three patients had an AIDS diagnosis (1987 Centers for Disease Control criteria). Among the 11 patients, 5 were antiretroviral-naive, 2 had used antiretroviral agents in the remote past, and 4 had been receiving a stable antiretroviral regimen for a minimum of 5 months. All 11 patients completed the study as planned. A total of 3 samples (1 each from subjects 3, 4, and 9) were not assayed because of gross hemolysis.

Diurnal patterns of plasma viral RNA. Figure 1 shows viral HIV-1 RNA levels at each time point over the 48-h study. To examine the pattern of change of HIV-1 RNA within a day, the mean of the RNA levels from specimens collected every 4 h over the first 24 h was determined, as shown in figure 2. Only small changes in HIV-1 RNA were observed during this 24-h period. The results of a repeated-measures analysis indicated that these changes in RNA levels were not significant (P = .13) and that the observed changes could be explained by random variation. No clear diurnal pattern was evident.

To determine if there was day-to-day effect on RNA levels, the mean of RNA levels of all specimens collected at 8 A.M. on days 1, 2, and 3 was determined. Again, only small changes over the 3 days was observed. The results of a repeated-measures analysis indicated that these day-to-day changes in RNA level were not significant (P = .25).

HIV RNA variance. Assuming that changes over a day are random, an estimate of the within-day variance can be made. By use of this variance estimate, a 95% tolerance limit can be determined such that 95% of the future differences between any two measurements will fall within that interval [10]. If a change in HIV-1 RNA levels within this interval is noted, then that change can be explained by assay variability. In this cohort of patients, this variation includes variation due to biologic phenomena plus within-plate assay variation.

By use of the restricted maximum likelihood method [11] to estimate the within-day variance components, the 95% tolerance limit was calculated. Expressed in linear terms, this tolerance limit is 2.4-fold in either direction (or log₁₀ difference of 0.38 in either direction). This result suggests that a change within a day of >2.4-fold is unlikely to have resulted from random chance alone. This 2.4-fold change includes the within-day biologic HIV variation plus the within-run assay variation.

Effect of number of samples on variance. Since HIV-1 RNA levels, when expressed on a linear scale, exhibit large variability, clinicians have been encouraged to obtain and average 2 samples as a baseline [12]. However, the actual effect of multiple sampling on variance is unknown. To explore this issue, we used the data in this cohort to develop the following model. The 10 time points for each individual patient reflect the combined effects of intrinsic biologic variation and within-plate events. Extrapolated over time, the variability inherent in these values reflects that seen in stable patients. With the values obtained in this study, the effect of 1 versus the average of 2 samples on predicted variability expressed in linear terms (fold differences) was determined. In this cohort, comparing 1 sample with a sample from a later time point resulted in an observed variance of 2.4-fold (in either direction). If the
As determined by repeated-measures analysis, there was no evidence that the time of day a sample was obtained had an effect on the result. Observed variance appeared to be random. These results are in contrast to several studies that showed significant diurnal variation in absolute CD4 cell levels [3–5].

Considering the close relationship between CD4 T cell activation and the level of HIV replication [13, 14], plasma HIV-1 RNA levels could vary with absolute CD4 cell levels. However, this was not observed. One possible explanation is that the diurnal patterns seen with CD4 cells reflects trafficking patterns from lymphoid tissue into the peripheral blood or the demargination of CD4 cells from the endothelial cells lining the vasculature; total CD4 cell levels in the body are unlikely to vary significantly over a 24-h period. If this was true, HIV-1 RNA levels would not vary in a diurnal pattern.

In contrast to CD4 cell levels and other common clinical assays, HIV-1 RNA levels have a large dynamic range. In the natural course of HIV infection, HIV-1 RNA levels vary over several logs (from $<100$ copies/mL to $>1$ million copies/mL). When considered against this wide range, variation in HIV-1 RNA over 24 h was low (0.38 log, or 2.4-fold in either direction). This degree of variance is consistent with recently published observations using another assay (reverse transcriptase–polymerase chain reaction–based assay; Amplicor; Roche Molecular Systems, Mississauga, Canada), in which week-to-week variability of HIV-1 RNA levels was $\approx 0.5$ log [1]. Changes beyond this limit cannot be explained by inherent biologic or assay variability and likely reflect a biologically and clinically relevant change in the level of plasma HIV-1 RNA.

To reduce the variability of these assays, published reports recommend that 2 baseline samples be obtained [12]. The results of this study do not support this recommendation if samples are analyzed in a batched fashion, as they were in this study (see methods). Since recent data suggest that up to 80% of the observed variance in HIV-1 RNA levels over time is

![Figure 1. Diurnal patterns of plasma HIV-1 RNA. Patients are grouped according to baseline RNA levels (A: $<80,000$ copies/mL; B: $80,000–500,000$ copies/mL; C: $>500,000$ copies/mL).](image)

mean of 2 consecutive or random samples is used as a baseline, the observed variability is reduced to 2.2-fold. If 2 samples are used at follow-up, and compared with 2 baseline samples, then the predicted fold change is 1.9.

**Discussion**

In this cohort of 11 subjects with moderate to advanced disease, HIV-1 RNA levels did not exhibit a diurnal pattern.

![Figure 2. Mean plasma HIV-1 RNA levels in specimens collected over the first 24 h from 11 subjects.](image)
due to technical factors rather than biologic factors, batching of samples and running them on the same plate will likely reduce variability significantly [1]. If samples are assayed in "real time" (and assayed on different plates at different times), predicted variability would be greater than that reported here. Since real-time analysis is typically required in the clinical setting, 2 baseline samples might still be beneficial.

The results of this study have direct implications for the routine use of HIV-1 RNA assays. In contrast to CD4 cell levels, the time of day the sample is obtained has no significant effect on the result, at least in patients with moderate-to-high HIV-1 RNA levels. However, clinicians should expect significant variation in HIV-1 RNA levels, even in the absence of any intervention or clinical events. Relative to the wide dynamic range of the assay, this variance is low. When samples are batched and run together on the same assay plates, the predicted variance in a clinically stable patient should be ~2.4 in either direction (or 0.38 log in either direction). The predicted variance may be greater if samples are run in real time on different assay plates. Obtaining and averaging 2 samples may potentially reduce the observed variance, supporting recommendations that 2 baseline samples be obtained before initiating or changing antiretroviral therapy.

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References