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ASSESSMENT OF HIV-1 DNA COPIES PER CELL BY REAL-TIME POLYMERASE CHAIN REACTION

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1. ABSTRACT

Measurements of HIV-1 DNA and plasma RNA levels represent unique entities, thus clinically and molecularly, data obtained from each can be used independently in assessing therapy or experiments. Plasma HIV-1 RNA levels are used to make clinical decisions regarding treatment strategies, but viral DNA can still be detectable when plasma RNA levels are undetectable. At the molecular level, accurate assessment of HIV-1 DNA copies/cell could increase the ability to target specific tissues for further analysis such as identification of site-specific integration of HIV in cellular DNA. Using real-time polymerase chain reaction (PCR), HIV-1 copies/cell were determined in peripheral blood mononuclear cells (PBMC), bone marrow (BM), and tissue. Duplicate specimens were analyzed for plasma HIV-1 RNA levels and for viral DNA copies/cell from 24 HIV-1 infected individuals. DNA from an additional 58 PBMC and 34 other tissue specimens were also assayed with the results reported as a log of HIV-1 DNA copies/cell. The log viral DNA copies/cell of the 24 matched specimens ranged from –2.699 to 0.278 with no correlation to the plasma HIV-1 RNA levels (range 52 to 2 X 10^5 copies/mL). Similar range in log HIV-1 DNA copies/cell was found in the other specimens. Real-time PCR assay for viral DNA copies/cell provides a rapid assessment of HIV-1 copies/cell in specimens independent of plasma HIV-1 RNA levels. From selected cases with relatively high HIV-1 DNA copies/cell, inverse PCR successfully identified viral integration. This type of assay could facilitate further studies when relatively high viral copies/cell are needed for screening.

2. INTRODUCTION

Measuring human immunodeficiency virus type 1 (HIV-1) RNA in plasma is important in assessing treatment efficacy and to study the pathogenesis of infection (1, 2). The clinical significance of plasma HIV-1 RNA load, though, should be taken in context with other viral characteristics given the pathogenesis of HIV-1 infection. A response or lack of response in plasma HIV-1 RNA load by antiretroviral therapy may be independent of any response in the viral DNA copies/cell (3-5). The two values are independent measures of different aspects of viral infection because plasma viral load primarily reflects HIV replication within lymphoid tissues compared to viral DNA present in circulating PBMC, which do not typically express HIV. Persistence of viral DNA in patients on therapy with undetectable viral load heralds ongoing interest and debate related to persistent disease or reactivation upon cessation of therapy (3, 4). Comparing plasma HIV-1 RNA load and HIV-1 DNA values in the same clinical specimens illustrates the dichotomous nature of virus measurements reflecting different pathogenic pathways of HIV (3-10). This persistence of viral DNA, integrated or unintegrated, provides further challenges in eradicating the virus and further understanding disease progression.

At the molecular biology level, HIV integration in the human genome has recently been identified favoring active genes and preferential target sites, thus revealing unexpected specificity in integration targeting by the virus (11). Other obstacles in elucidating the pathophysiology of the infection include the presence of virus in different tissue compartments, which may play different roles in the development of HIV-associated complications, such as virus in the brain, bone marrow, etc. (12-15). Identification of HIV-1 DNA copies in tissue compartments can be useful in understanding the pathogenesis of HIV-related complications. In addition, with new therapeutic strategies such as HIV integrase inhibitors being tested, modalities to assess efficacy of integration inhibition could be useful (16, 17). Previous reports using real-time PCR to study viral integration studied the kinetics and preintegration complexes of HIV-1 focusing on the mechanisms of integration as a therapeutic

platform for integration inhibitors (18, 19). Recently, other PCR-based methods have compared quantitation of integrated HIV-1 DNA, which relied on PCR and Southern hybridization (20, 21). Others have used different real-time PCR strategies in determining HIV-1 DNA copies, but our method with Syber® green may offer an advantage with its uniformity and efficiency (22-24). The general aim of the current study was to develop a more efficient and quantitative assay for identifying viral DNA copies per cell by real-time PCR and to obtain reproducible and consistent results for the purpose of screening specimens for specific assays requiring relatively high viral copy number. In this regard, our approach was to use real-time PCR to enable accurate quantification over a wide range with low intra- and inter-assay variation and consistent results followed by using IPCR to identify site-specific integration (25, 26).

3. MATERIALS AND METHODS

3.1 Specimens

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) as per guidelines by the University of Hawaii (UH) Institutional Review Board from specimens collected after informed consent through the University of Hawaii NeuroAIDS Specialized Neuroscience Research Program and Hawaii AIDS Clinical Research Program. Other specimens were obtained and tested through collaborative efforts as per guidelines at Johns Hopkins University (JHU) Institutional Review Board. The quantity and quality of the DNA was determined using UV spectrophotometry (assessing DNA concentration and OD 260/280 absorbance ratio) and PCR analysis using HLADQ-alpha primers as housekeeping oligomers. DNA for control and standards was extracted from OM-10.1 cell line (NIH AIDS Research and Reference Reagent Program, Rockville, MD), which contains one integrated virus/cell. Additional control DNA was prepared from non-HIV-1 infected PBMC. For quantitation purposes, two plasmid DNAs were used: 1) p89.6 (NIH AIDS Research and Reference Reagent Program), which contains a full length (9.7kb) HIV genome derived from the 89.6 viral isolate; and 2) pMito, which contains a fragment of nuclear DNA from CD95L Fas ligand (27). For the specimens from UH, chromosomal DNA, extraction and purification was carried out in the presence of SDS and NaCl, allowing the supernatant containing extrachromosomal DNA to be extracted separately (19). The DNA obtained from the JHU specimens was isolated using standard phenol extraction (28).

For specimens in which HIV-RNA levels were obtained at the University of Hawaii, plasma was sent to a certified local clinical laboratory to perform plasma HIV-1 RNA load (Amplicor HIV-1 Monitor UltraSensitive Test, Roche Diagnostics, Switzerland).

3.2 Quantitative Real-Time PCR Assay

Two independent methodologies were designed to compare the consistency of the results. Both methods utilized the real-time PCR technology in order to calculate HIV-1 copy/cell. To verify uniformity in the reactions and data, and to determine the dynamic range of the standards, 6 logs of the starting plasmid concentration, from 10^3 to 10^9 plasmids per reaction, were used. The mean threshold cycle for the reactions was calculated through the software program accompanying the real-time iCycler system (BioRad, Hercules, CA) to determine uniformity and inter-assay consistency. Extracted DNA was diluted in H2O at a concentration corresponding to 40,000 cell equivalents/10 microL. The number of cell equivalents was calculated assuming that 6.6 ng of DNA was equivalent to 103 cells (29).

The first method used, as a standard for HIV-1 quantitative PCR analysis, DNA from OM-10.1 cell line and HIV gag/pol primers (sense: GGG TAC CAG CAC ACA AAG GA; antisense: TCA CTA GCC ATT GCT CTC CAT CA) and c-jun primers (for nuclear DNA; sense: CTG CAT GGA CCT AAC ATT CG; antisense: CCC GCT TTG TGT TCT TAA GG). DNA extracted from 5 X 106 OM-10.1 cells.

The second method utilized p89.6 and pMito to assay virus copy and nuclear DNA, respectively. The same gag primers listed above were used and the genomic primers, GenDIR (GGC TCT GTG AGG GAT ATA AAG ACA) and GenREV (CAA ACC ACC CGA GCA ACT AAT CT) were specific for the region of the genome encoding the Fas ligand. Quantitative PCR conditions were optimized for amplification efficiency and melt curve analysis on the iCycler system (BioRad) using Syber® Green I with DNA from OM-10.1, p89.6, and pMito with the appropriate primers.

Standardization of real-time PCR was performed using Quantitect Sybr® Green PCR Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. A dilution series of each of the control plasmids containing the targeted viral DNA and genomic DNA was prepared to set up the standard curves. Each sample and standard was set up in triplicate containing: Sybr Green Master Mix, 5 microL Glycerol, and 15 picomoles of each primer, with 100 ng sample DNA. PCR cycling conditions were: 95ºC for 15 minutes, 40 cycles of 95ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 30 seconds, followed by a 7 minute final extension step at 72ºC. At the conclusion of the PCR, a melt curve analysis was programmed with the following conditions: beginning at 48ºC, the temperature increased half a degree every 10 seconds for 90 cycles. In addition to the melt curve analysis, PCR products were size fractionated on a 1%/1.5% agarose/low-melting point agarose to verify that a single amplified product was present in each reaction. To evaluate the reproducibility of the intra-assays (replicates within the same real-time PCR run) and inter-assays (replicates between four different runs), the calculated HIV-1 DNA copies/cell were compared.

In the analyses of the clinical specimens, reactions were set up in triplicate with triplicate standards and triplicate positive and negative controls. At the conclusion of every run, a melt curve analysis was performed to verify the efficiency of the reactions. The unknown specimens were analyzed against the standards curve generated by the iCycler Real-Time Detection System Program (BioRad, Hercules, CA) and calculated as the log viral DNA copies/cell.

Table 1. Clinical Specimens Analyzed

Specimen	HIV Status	n	DNA Quality*
Matched PBMC/Plasma	Positive	24	24
PBMC	Positive	58	58
PBMC	Negative	4	4
Bone Marrow	Positive	4	4
Bone Marrow	Negative	12	12
Other Tissue	Positive	33	33
Total		135	135

*Assessed by PCR analysis using HLADQ-alpha primers as housekeeping oligomers

Table 2. Real-Time PCR Intra-Assay Reproducibility

Standard No.	Log HIV-1 DNA Copy/Cell	Mean	Coefficient of Variation
1	-3.754 -3.735 -3.745 -3.742	-3.744	0.002
2	-2.683 -2.800 -2.796 -2.775	-2.764	0.020
		Mean	0.011 (1.1%)

Table 3. Real-Time PCR Inter-Assay Reproducibility

Standard No.	Log HIV-1 DNA Copy/Cell	Mean	Coefficient of Variation
1	-4.921 -4.854 -4.796 -4.800	-4.843	0.012
2	-3.777 -3.745 -3.759 -3.755	-3.759	0.004
		Mean	0.014 (1.4%)

3.3. Analysis

The analysis and standardization of the assay was performed using internal standards and compared against known standards. Quadruplicate runs were set up to assess the intra-assay and inter-assay consistency and to calculate mean coefficients of variations.

3.4 Inverse PCR

To test the utility of screening specimens for HIV-1 DNA copies/cell in identifying samples which might yield viral integration, we performed inverse PCR (IPCR) on selected specimens as previously described (30, 31). The specimens that were chosen were identified as those with relatively high viral DNA copies/cell. An aliquot of DNA (1 microgram) was digested with *Pst I* (Promega, Madison, WI) and religated with T4 DNA ligase (Promega). Genomic/HIV hybrid sequences were amplified using nested PCR with Expand High Fidelity Taq Polymerase system (Roche Biomedical) using primers: outer primers, LTR 1-115 5'-TTG TTG GCT TCT TCT AAC TTC TCT GG-3' and *gag* 1-H 5'-CTT TAA ATG CAT GGG TAA AAG TAG TRG-3'; inner primers, LTR 2:-116 5'-TGG TAC TAR CTT GWA GCA CCA TCC A-3' and *gag* 2- J 5'-TGA TAC CCA TGT TTT CAG CAT TAT CAG-3'; and cycling conditions: 94° 1 minute, 10 cycles of 94° 15 seconds, 62° 30 seconds, 68° 3 minutes, 35 cycles of 94° 15 seconds, 57° 30 seconds, 68° 3 minutes with a final elongation of 68° 8 minutes for the outer primers; and 94° 1 minutes, 35 cycles of 94° 15 seconds, 56° 30 seconds, 68° 3 minutes with a final elongation of 68° 8 minutes for the inner primers. Amplification was performed using GeneAmp System 9700 thermalcycler (Perkin Elmer, Emeryville, CA). PCR products were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA), screened with inner IPCR primers 116/J, and sequenced.

4. RESULTS

The specimens obtained and analyzed in this study had adequate and good quality DNA available (Table 1). Paired/matched PBMC and plasma were available from HIV-1 seropositive individuals, from which plasma HIV-1 RNA levels were obtained at the same time that the specimens for DNA extraction for real-time PCR were available. The other 58 PBMC specimens were from HIV seropositive individuals in which plasma viral load was not performed at the same time point as the real-time PCR assay because the viral loads were performed at a different time. PBMC from controls (HIV seronegative) were also available. DNA from bone marrow (BM) from both HIV seropositive and seronegative individuals were available for analysis. The other specimens were obtained from autopsy material and included different anatomical sites (brain, lung, spleen, lymph node, bone marrow).

Standardization of the quantitative real-time PCR assay was set up using internal standards and compared against known standards. The threshold cycle values of the HIV and genomic standards, and the standard curves generated from the plasmids showed intra-assay and inter-assay consistency, as indicated by the mean coefficients of variations. An example of the calculated viral DNA and genomic copies shows the standard curve generated along with unknown specimens (Figure 1). Unknown specimens and controls were run and plotted against the standards in triplicate corresponding to the log starting quantity copy number. The analysis of HIV copy of the replicates of each PCR run (intra-assay) and in different runs (inter-assay) for the standards showed a mean coefficient of variation of 1.1% and 1.4%, respectively, (Tables 2 and 3). With the efficient reproducibility of the standards established, analysis of DNA from clinical specimens was performed.

DNA from 135 specimens were analyzed for HIV-1 DNA copies/cell and reported as the log HIV-1 DNA copies/cell. In addition to the standard curves generated using serial dilution of standards, two separate known concentrations were set up in triplicate independently and analyzed with each run. The known concentrations were: 1 copy of HIV-1/cell and 1 HIV-1 copy/10^5 cells. In the assessment and calculations of the standards, 1 microgram of genomic DNA was calculated to correspond to 3.4 x 10^5 copies of a single-copy gene. Analysis of matched PBMC and plasma specimens

Table 4. Comparison of Log Viral DNA Copies/Cell versus Plasma Viral RNA Levels

	Log HIV-1 DNA (Copies/Cell)	Plasma HIV-1 RNA Level (Copies/mL)
1	-2.699	625
2	-2.155	1081
3	-2.000	246
4	-1.824	2645
5	-1.569	5037
6	-1.509	10353
7	-1.387	208847
8	-1.357	80092
9	-1.292	3326
10	-1.284	135522
11	-1.237	52
12	-1.201	907
13	-1.137	125872
14	-1.051	2337
15	-1.032	2230
16	-1.013	1059
17	-0.943	56030
18	-0.772	31317
19	-0.572	76
20	-0.333	137
21	-0.308	480
22	-0.197	414
23	0.120	24125
24	0.278	25003

comparing log viral DNA copies/cell with plasma HIV-1 RNA load, (Table 4), demonstrates that there is little correlation between the two measurements on specimens obtained at the same time point. The other clinical specimens, consisting of PBMC and BM from HIV-1 seropositive and seronegative individuals, and necropsy brain, lung, spleen, lymph node, and BM, were successfully analyzed for viral copies/cell. The results ranged from log HIV-1 DNA copy/cell of –7.000 (limit of detection) for the specimens from HIV-1 seronegative individuals to 0.278. Similar coefficients of variation were demonstrated for the unknown specimens, in which the log viral DNA copies/cell ranged from –2.699 to 0.278. Of note, two PBMC specimens were found to have relatively high viral DNA copies/cell (log), specimens #23 and 24 (Table 4). The clinical information currently available from these two patients (plasma viral load, antiretroviral therapy, CD4 counts, age, gender) do not appear to be an different from the other patients, but further analysis of these two particular specimens are planned to determine if viral integration can be detected in the specimens.

5. DISCUSSION

We have developed a real-time PCR assay to assess HIV-1 DNA copies/cell with good sensitivity and precision. The assay displayed low inter-assay and intra-assay variability, with relatively low coefficients of variation. This type of assay would be useful in understanding HIV-1 integration as a non-random event or in the context of assessing therapeutic interventions using HIV integrase inhibitors (11, 17, 21, 31). This primary focus of this particular report was to highlight the efficiency of the method in analyzing a number of specimens as part of a screening tool. A more comprehensive description of the results of the assay with respect to clinical parameters is being planned as a follow up. As previously reported, the discordance between viral DNA copies/cell and plasma HIV-1 RNA levels in specimens from the same patient obtained at the same time reflects viral entities being measured and therefore each assay should be used independently in assessing disease pathogenesis (3-10, 22-24). The significance of the dichotomy between the two values, HIV-1 DNA and RNA levels, stresses the importance of the role of highly active antiretroviral therapy but also highlights the fact that viral DNA may be more of a factor in the chronicity of the disease. Thus accurate assessment of HIV-1 DNA could become an important tool in studying the clinical and basic nature of HIV infection. In quantifying viral copies, rapid and efficient methods would be useful clinically and in further advancing the understanding of the pathogenesis of HIV-1 infection.

In this assay, HIV-1 DNA copies/cell was calculated in specimens to be analyzed for viral integration. The results showed that varying levels of viral copies/cell were calculated independent of the plasma HIV-1 RNA level of the specimens. In the analyses performed to date, the log of viral DNA copies/cell ranged from –7.000 to 0.278. A limitation of the DNA specimens analyzed in the current study is that the DNA was extracted using two different techniques from the two institutions. While the quantity and quality of the DNA was good, a confounding factor is that in the course of DNA extraction, it is possible that unintegrated forms of virus may be present. The supernatant from the PBMC from many of the specimens will be tested for viral DNA in the future. Because each group of DNA specimens was isolated in a uniform fashion, from the standpoint of using this assay as a screening tool, the efficient and rapidity of this procedure offers a great advantage over previous methods (6, 22, 24). The current method reliably uses Syber® green as an intercalating compound, which is shown for our standardized protocol to be free of background artifact. In this context, the technique is fast and efficient. The current Syber® green method offers the advantage of high efficiency, rapid results, and accurate relative assessment of HIV-1 DNA copies/cell, which could be useful clinically as well as a screening tool in research models studying viral integration. Assaying for viral integration is a labor-intensive task, but being able to focus the analysis on cells or specimens which harbor the virus would be a great benefit. We have previously reported preliminary data on HIV-1 DNA integration in 3 specimens, which were retrospectively screened for viral DNA copies/cell, blindly (32, 33). These 3 specimens were blindly included in the pool of other unknowns being analyzed for this study. These three specimens had log viral DNA copies/cell

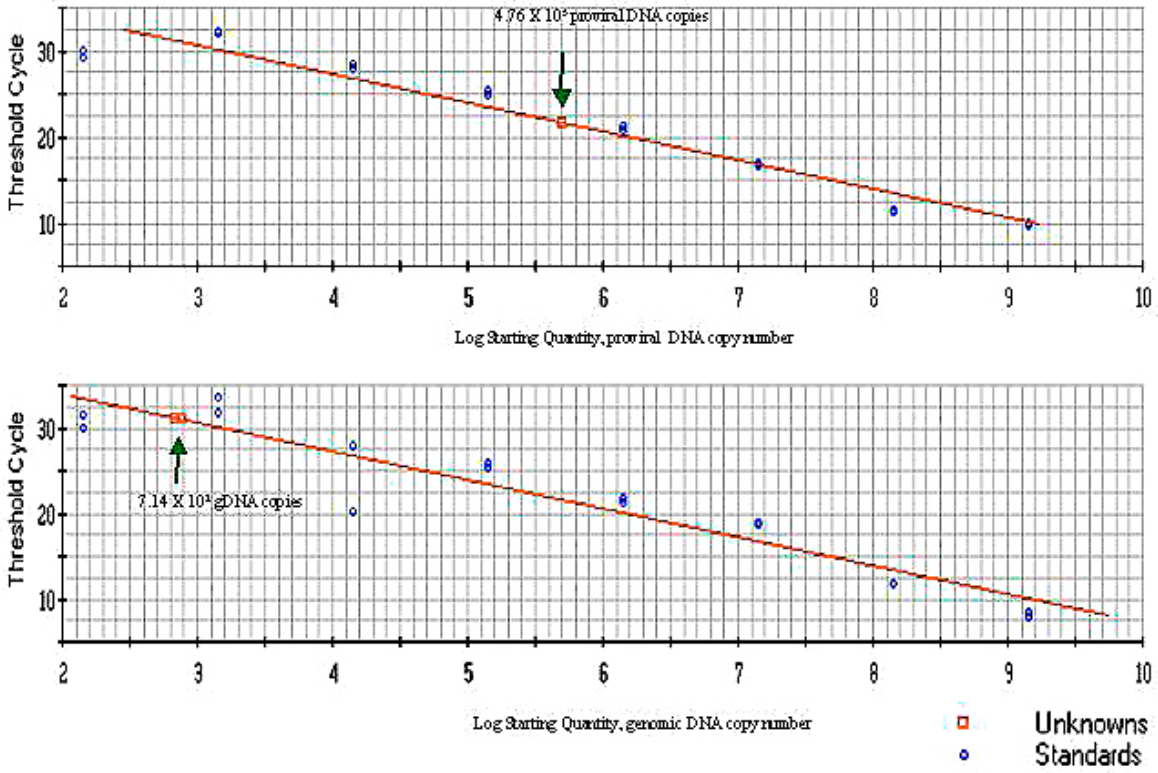

Figure 1. Standard curves for HIV-1 gag region (A) and nuclear gene (B). Unknown specimens (red squares) are plotted on the standard curves (blue circles) corresponding to the log starting quantity copy number. Specimens were run in triplicate, with the standard curve. Each run was completed with a final melt curve analysis to verify efficiency. HIV-1 copy/cell was calculated by: (viral DNA copy/genomic DNA copy) X 2.

values ranging from –0.097 to 0.041. When compared to the 135 specimens assayed in the current study, these 3 had relatively high viral copies/cell. One of the specimens previously analyzed, a bone marrow sample, had a log viral DNA value of -0.046 (33). Further analysis by IPCR demonstrated viral sequence within the integrin beta 4 subunit gene (ITGβ4), within intron 26 (Genbank #Y11107). While the significance of integration near ITGβ4 is unclear, identifying site-specific integration in a specimen with relatively high viral copies/cell suggest that the real-time quantitative PCR assay may be useful in screening other specimens for analysis. The utility of the current real-time PCR method could be useful for studying subsets of cellular compartments or fine needle aspirates of tissue, which could be screened relatively quickly for viral DNA copy. Knowing the relative HIV-1 copy/cell in a specimen can complement other diagnostic or research tools in order to more appropriately target specific assays with appropriate HIV-1 DNA copy or in assessing treatment response to integrase inhibitors.

The quantitative viral DNA assay described here provides a valuable addition to the analytical tools already available to monitor viral replication. As shown by other investigators, analysis of sequential samples from patients on therapy showed that HIV-1 DNA levels in PBMCs remained detectable and quantifiable despite plasma HIV-1 RNA levels that were undetectable for up to 1 year (3, 20, 34). The presence of detectable DNA is consistent with the successful isolation of virus from PBMCs of patients with undetectable plasma HIV-1 RNA (35, 36). Further work is needed with a larger sample size and longitudinal analysis to determine if our assay could be clinically efficient and effective in further screening for viral integration. The availability of this new standardized assay will be important in at least the following ways: 1) facilitate the comparison of the efficacy of different HAART regimens on elimination of viral reservoirs; 2) permit evaluation of differences in HIV-1 suppression in patients with nonquantifiable plasma viral loads and viral DNA copies/cell; and 3) be a useful screening tool in evaluating viral integration and other assays looking at the role of HIV in disease progression. In conclusion, this assay has been developed to quantitate viral DNA copies/cell and has a number of potential applications clinically and in basic science in further studying the pathogenesis of HIV-1 infection.

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