Quantification of Genital HIV-1 DNA in Women with Low Plasma HIV-1 RNA Levels

Typical of HIV-1 Non-Transmitters

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Running head: Genital HIV-1 DNA in Women with Low Plasma HIV-1 RNA

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ABSTRACT

Studies of HIV-1 transmission suggest that genital HIV-1 RNA and DNA may both be determinants of HIV-1 infectivity. Despite its potential role in HIV-1 transmission, there is limited quantitative data on genital HIV-1 DNA. Here we validated an in-house real-time PCR method for quantification of HIV-1 DNA in genital specimens. In reactions including 100 to 1 genomes isolated from a cell line containing 1 HIV-1 provirus/cell, this real-time PCR assay is linear and agrees closely with a commercially available real-time PCR assay specific for a cellular housekeeping gene. In mock genital samples spiked with low numbers of HIV-1 infected cells such that the expected HIV-1 DNA copy number/reaction was 100, 10, and 5, the average copy number/reaction was 80.2 (standard deviation (SD), 28.3), 9.1 (SD, 5.4), and 3.1 (SD, 2.1), respectively. We used this method to examine genital HIV-1 DNA levels in women whose low plasma HIV-1 RNA levels are typical of HIV-1 non-transmitters. In these women, the median HIV-1 DNA copy number in endocervical secretions (1.8 HIV-1 DNA copies/10,000 cells) was lower than that for women with higher plasma HIV-1 RNA levels (16.6 HIV-1 DNA copies/10,000 cells) ($P = 0.04$), as was the median HIV-1 DNA copy number in vaginal secretions (undetectable vs 1.0 HIV-1 DNA copies/10,000 cells). These data suggest that women with low plasma HIV-1 RNA, and thus a predicted low risk of HIV-1 transmission, have low levels of genital HIV-1 cell-associated virus. The assay described here can be utilized in future efforts to examine the role for cell-associated HIV-1 in transmission.
INTRODUCTION

In HIV-1 infected women, genital secretions are a potential source of transmissible virus both to male sexual partners and to neonates during vaginal delivery. The level of virus in the genital compartment has been presumed to be a primary determinant of HIV-1 infectivity (3, 6, 27); however, most studies of the viral determinants of HIV-1 transmission have focused on systemic virus. In these analyses, systemic HIV-1 RNA level was a significant predictor of HIV-1 transmission in antiretroviral-naïve serodiscordant couples, and individuals with low plasma HIV-1 RNA levels exhibited low or no risk of transmission (8, 10, 21, 28). The presumption that low systemic HIV-1 burden is a surrogate marker for low genital HIV-1 burden in these studies is supported by the observations that systemic HIV-1 RNA levels correlate with genital virus levels in cross-sectional studies (9, 11, 13, 15, 24), and HIV-1 RNA levels in maternal genital secretions at the time of delivery are associated with vertical HIV-1 transmission (4, 14, 19). Fewer studies have examined HIV-1 infected cell levels in genital secretions (1, 13, 24), which in part reflects the limited availability of sensitive methods for quantifying HIV-1 DNA in genital specimens. Only one study to date has reported quantitative data on HIV-1 DNA levels in non-pregnant antiretroviral-naïve women (1).

Both cell-free and cell-associated virus could play a role in transmission of HIV-1 via genital secretions. Macaques inoculated intravenously with either cell-free or cell-associated SIV can become infected (23). One recent study showed that breast milk HIV-1 DNA level, adjusted for breast milk HIV-1 RNA level, predicted HIV-1 vertical
transmission in antiretroviral-naïve women (22). Similarly, HIV-1 DNA level, but not
RNA level, in cervicovaginal secretions was an independent predictor of vertical
transmission in treated women (25). These data suggest that cell-associated virus may
play an important role in HIV-1 transmission.

Sensitive methods are needed to quantify the number of HIV-1 DNA copies,
which provide a measure of the number of HIV-1 infected cells, in the genital
compartment. In antiretroviral-naïve women, a recent estimate of cervicovaginal HIV-1
dNA (median, 67 HIV-1 DNA copies/1,000,000 cells) (1) is more than 10-fold lower than
that for blood (average, 1,199 HIV-1 DNA copies/1,000,000 cells) (26). This estimate of
genital HIV-1 DNA copies was obtained using a modification of the Amplicor HIV
Monitor Test 1.5 for DNA, which has a lower limit of quantification of 10 copies/reaction.
Here we examined whether a recently developed in-house real-time HIV-1 PCR assay
(22), can be used to quantify low numbers of HIV-1 DNA in genital specimens. This
assay, like the Amplicor assay, is sensitive for multiple HIV-1 subtypes (22).

We used this method to examine the relationship between plasma HIV-1 RNA
burden and the level of HIV-1 DNA in endocervical and vaginal secretions in women
with low plasma virus levels predictive of a low risk of HIV-1 transmission.

MATERIALS AND METHODS

Study Population. A subgroup of women was selected, on the basis of HIV-1
plasma virus level, from a larger cohort of 650 HIV-1 seropositive antiretroviral-naïve
Kenyan women (2, 16, 17). Women had been excluded from this cohort if they were <
18 or > 45 years, pregnant, had a sexually transmitted disease, or used vitamin
supplements or oral contraceptive pills. Data on the clinical characteristics, and the
HIV-1 plasma virus levels (assessed by the Gen-Probe HIV-1 Viral Load Assay) (7), of
this cohort have been reported (17). Ethical review committees at the University of
Washington, the Fred Hutchinson Cancer Research Center, and at the University of
Nairobi approved the study protocol.

Women with low plasma HIV-1 RNA levels \((N = 13)\) were defined using a
threshold of \(\leq 2,200\) copies/mL by the Gen-Probe Assay, because this level
 corresponds to the plasma HIV-1 RNA level (1,500 copies/mL by Roche Amplicor) (7)
 used to define HIV-1 non-transmitters in previous studies (21). Thirteen additional
women were randomly selected from the remaining 637 women (4 with 2,201-22,000; 4
with 22,001-220,000; and 5 with > 220,000 plasma HIV-1 RNA copies/mL) for
comparison.

**Clinical Specimen Collection.** Genital secretions were collected using Dacron
swabs, which were immersed into 1 mL of freezing media (70% RPMI 1640, 20% fetal
calf serum, and 10% DMSO). Swabs were stored in cryovials in liquid nitrogen prior to
shipment to Seattle, WA for testing. Endocervical secretions were collected by rotating
a swab 2 full turns after insertion (1 cm) into the endocervical os, and vaginal secretions
were collected by rotating a swab 3 full turns against the lateral vaginal wall.

**Generation and Testing of ACH2 Cell Dilutions.** Serial dilutions of HIV-1
proviruses were generated using genomic DNA isolated from ACH2 cells, which are
reported to contain 1 proviral copy/cell (5). ACH2 genomic DNA was isolated using the
QIAamp DNA Blood Maxi Kit (Qiagen Inc. Valencia, CA) according to the
manufacturer’s instructions. The concentration of genomic DNA was determined by UV spectroscopy, and converted to genomes per unit volume (assuming a cellular genome weighs 6 pg). Dilutions of ACH2 genomic DNA were made in water. Four sets of serial dilutions were generated on different days. Ten µL of each DNA dilution was used for quantification of the human β-actin gene and the HIV-1 pol gene, as described below.

**Generation of Mock Genital Samples.** Mock genital samples were generated using batch mixtures of HeLa (HIV-1 negative) and ACH2 (HIV-1 positive) cells at total cell concentrations that were expected to approximate the number of cells on a typical genital swab specimen (~ 500,000; S. Benki, unpublished data). All mock samples were generated and processed in a hood dedicated for HIV-1. Uninfected mock swab samples (prepared to control for the presence of contamination during DNA extraction) consisted of 500,000 HeLa cells/swab alone. The panel of spiked mock genital samples consisted of 500,000 HeLa cells/swab together with low numbers of ACH2 cells. This panel was generated such that the target levels of proviral copies added to each pol PCR would be 100, 10, and 5, after accounting for the fraction of sample added to the PCR. For the purpose of generating batch cell mixtures, cell counts were performed using a hemacytometer. Individual swabs were generated by pipetting 100 µL of each batch of cells onto a Dacron swab, and immersing the swab into 1 mL of freezing media.

**DNA Extraction.** Genomic DNA was extracted from genital specimens and mock swabs using the QIAamp 96 DNA Blood Kit (Qiagen Inc. Valencia, CA) according to the manufacturer’s instructions. In pilot studies we found that we could isolate
proviruses from mock swabs and genital specimens using 100-500 µL of sample
material, and elute in volumes of 100-200 µL without impacting recovery (data not
shown). Genital specimens and mock swab samples were thawed at room temperature
and vortexed prior to removal of sample material. For genital specimens and for mock
swabs, 200-500 µL was used for DNA extractions. Individual mock swabs were used
for 1 or 2 genomic DNA extractions, each performed on separate days. Genomic DNA
was eluted using either 100 µL (mock swabs) or 200 µL (genital specimens) of water.
Twenty µL (mock swabs) and 10 µL (genital specimens) of eluate were used for pol
real-time PCRs, which were performed in duplicate, and 2 µL of eluate (mock and
genital swabs) were used for human β-actin gene real-time PCRs, which were
performed in singlet.

Real-time PCR. Real time reactions for both β-actin and pol were prepared
using Taqman PCR Core Reagents (Applied Biosystems, Foster City, CA). For all real-
time assays, reaction volumes were 50 µL. Reactions for the human β-actin gene were
prepared as has been described (22). The linear dynamic range of this quantitative β-
actin assay ranges from 1.7 to 17,000 copies (22) (data not shown).

The real-time PCR method for detection of HIV-1 pol sequences, including the
primer and probe sequences, has been described (22), and was used with minor
modification. Specifically, the concentrations of MgCl₂, primers, and probe were further
optimized for the detection of product, as determined by an increase in fluorescent
signal above the threshold level, in single copy reactions for which the specific PCR
product could be visualized by gel electrophoresis. Reactions contained 350 µM MgCl₂,
200 µM dNTPs, 900 nM each forward and reverse primer, 150 nM probe, 1.5 U of
AmpliTaq Gold DNA polymerase, and the volume of Buffer A recommended by the
manufacturer.

Ten-fold serial dilutions (ranging from 10,000-1 copies/reaction, and including a 5
copy level for mock swab reactions) of a stock of a full-length clone of a subtype A HIV-
1 (Q23-17; (20, 22)) were used to generate a standard curve for quantification of HIV-1
DNA. The concentration of the plasmid stock was ascertained by UV spectroscopy.
Dilutions of plasmid were made in a 10 ng/µL stock of herring sperm DNA (Promega,
Madison WI) to ensure that each dilution contained the same amount of total DNA.

Reactions were carried out in a 7900HT sequence detector (Applied Biosystems,
Foster City, CA). HIV-1 pol reactions were analyzed over 42 cycles. Analyses of real-
time PCR results were performed using Sequence Detection Software, version 2.1
(Applied Biosystems, Foster City, CA). The threshold for determining the C\textsubscript{T}
for each
reaction was set manually, and was defined as 10 standard deviations above the mean
\Delta Rn level during cycles 3-15 for each well for each individual plate.

For HIV-1 pol real-time reactions using ACH2 dilutions, genital specimens, and
mock swabs, an HIV-1 pol real-time reaction was defined as positive if the readout from
the assay was 1.0 copy/reaction or greater. Reactions for which the assay readout was
between 1.0 and 0.5 were defined as positive if the presence of the pol PCR product
could be confirmed on a gel. All other reactions were considered negative. Reactions
containing a commercial preparation of human genomic DNA (Promega, Madison, WI)
were performed to control for the potential for non-specific amplification.
For genital specimens and for mock swabs, HIV-1 pol real-time reactions were performed in duplicate. For genital specimens, HIV-1 pol duplicate reactions were considered acceptable only if the readout values were within five-fold one another. For duplicates that did not meet this criterion, up to 2 additional sets of duplicate reactions were performed until acceptable results were obtained, and only these results were used for analysis. Results from negative reactions were arbitrarily assigned a value of 0.5 for the purpose of applying this criterion.

For genital specimens, the ratio of HIV-1 infected cells to uninfected cells was calculated. The number of HIV-1 pol DNA copies/reaction was rounded to the nearest whole integer and summed for all acceptable reactions/specimen. This sum was divided by the total number of cellular genomes (ascertained by the human β-actin assay) that were cumulatively sampled in all acceptable reactions/specimen. If no HIV-1 proviral copies were detected, the sum was assigned a level of 0.5. These ratios were expressed as the number of HIV-1 DNA copies/10,000 cells, which reflects the median number of cells sampled in genital specimens (see results).

**Statistical Analysis.** Statistical analyses were performed using Stata 7.0 (Stata Corp. College Station, TX). The F Test For Lack of Fit (18) was used to test whether a linear regression function was a good fit for observed HIV-1 pol values. Univariate comparisons were evaluated using the Mann-Whitney U test for continuous data and Fisher's exact test for binary data.
RESULTS

Validation of a Real-Time PCR Method to Quantify Low Numbers of HIV-1

Proviruses. We validated whether a HIV-1 \textit{pol} real-time assay can be used to
determine HIV-1 DNA copy number in reactions that have extremely low levels of
proviral genomes. For this purpose, a panel of low copy reactions was generated by
serially diluting genomic DNA isolated from a chronically infected HIV-1 cell line (ACH2)
that contains a single copy of HIV-1 (5). The final target level of genomes in these
reactions was 100, 10, 5, 4, 3, 2, and 1. Human β-actin and HIV-1 \textit{pol} copy numbers
were compared for each sample.

At each level of copy number in the panel of serial dilutions, there was good
agreement between the mean level observed using the β-actin assay and the mean
level observed using the HIV-1 \textit{pol} assay (Table 1). In 1-copy reactions, HIV-1 proviral
DNA was detected in 21/42 (50%) reactions, and β-actin was detected in 6/8 (75%)
reactions. For both assays, the mean observed copy number at each dilution fell within
< 1% - 40% of the target copy number, as determined by UV spectroscopy of purified
DNA. The linearity of the relationship between the target level of proviral copies and the
mean observed level of proviral copies as quantified by the HIV-1 \textit{pol} assay was
evaluated using the F Test for Lack of Fit (18). This test demonstrated that, for
reactions containing 100 to 1 copies, a linear regression function of the mean observed
copy numbers on the target levels was appropriate for the data ($P = .99$) (Fig. 1). We
obtained similar results for this test whether we included the 100 copy reactions or not
(data not shown).
Validation of a Real-Time PCR Method to Quantify HIV-1 Provirus in Genital Swab Samples. To validate whether low numbers of HIV-1 proviral copies can be recovered from genital specimens, a panel of mock genital specimens was generated by mixing various ratios of infected (ACH2) and uninfected cells (HeLa). Genomic DNA was isolated from these samples and tested for HIV-1 pol levels. We detected HIV-1 proviruses in 33 of 33 (100%), 33 of 33 (100%), and 29 of 33 (87.9%) of genomic DNA extractions in the 100, 10, and 5 copy reaction panels, respectively (Fig. 2). Mean levels of HIV-1 proviruses recovered from the spiked samples were 80.2 (SD, 28.3), 9.1 (SD, 5.4), and 3.1 (SD, 2.1) for the 100, 10, and 5 copy reactions, respectively. In the negative mock swabs, we detected HIV-1 pol copies in 4 of 109 (3.7%) genomic DNA extractions, of which 3 exhibited a read-out of fewer than 1.0/reaction. To examine whether this background could be due to non-specific amplification, we performed reactions containing human genomic DNA alone and no HIV-1 DNA. In these reactions, readout values were always less than 0.1 (data not shown). Total cell number was quantified in a subset of mock swabs using the ß-actin assay. The mean cell number in these swabs was 381,881 (SD, 163,505), compared to the predicted cell number (500,000), based on cell counts, added to each mock genital sample.

Quantitative analysis of genital HIV-1 DNA in women with low plasma HIV-1 RNA levels. To assess HIV-1 DNA levels in endocervical and vaginal secretions in relation to plasma HIV-1 RNA level, we identified 26 women with low and higher plasma HIV-1 RNA levels from a larger cohort of 650 women. We defined a low plasma HIV-1 RNA group using a threshold of ≤ 2,200 copies/mL, based on the plasma HIV-1 RNA
level that has previously been used to define non-transmitters (21). Thirteen women
had plasma HIV-1 RNA levels \( \leq 2,200 \) copies/mL and were compared with 13 additional
women who were randomly selected from the remaining 637 women with higher plasma
HIV-1 RNA levels. The number of endocervical and vaginal HIV-1 DNA copies/10,000
total cells was quantified in these 26 women using our in-house HIV-1 pol real-time
assay and the real-time assay for \( \beta \)-actin (Table 2). In endocervical secretions, the
median number of cells that were sampled for HIV-1 DNA in 2 PCRs combined was
11,410 (IQR, 5,452-16,382), and in vaginal secretions, the median number of cells
sampled was 23,348 (IQR, 8,676-48,744). The number of endocervical HIV-1 DNA
copies (median, 1.8 HIV-1 DNA copies/10,000 cells, IQR, 0.7-5.1 HIV-1 DNA
copies/10,000 cells) (Fig. 3) was significantly lower in women with low plasma HIV-1
RNA as compared with women with higher plasma HIV-1 RNA (median, 16.6 HIV-1
DNA copies/10,000 cells, IQR, 2.4-58.0 HIV-1 DNA copies/10,000 cells) \((P = 0.04)\),
although similar numbers of women had detectable HIV-1 DNA in both groups (7 vs 8,
respectively). Vaginal HIV-1 DNA was not detected in any of the 13 women in the low
plasma HIV-1 RNA group; by contrast, there was detectable HIV-1 DNA in 6 of the 13
(46\%) women in the higher plasma HIV-1 RNA group \((P = .02)\), who had an overall
median vaginal HIV-1 DNA level of 1.0/10,000 cells (IQR, 0.6-6.5 HIV-1 DNA
copies/10,000 cells.

**DISCUSSION**

In this study, we have demonstrated that a real-time PCR assay (22) can be
used for quantification of low levels of HIV-1 DNA copies in genital swab specimens,
and we have demonstrated the utility of this assay for examination of genital HIV-1 DNA
levels in women with low plasma HIV-1 levels.

Using our in-house HIV-1 real-time assay, we observed good agreement
between the target and the observed copy number for HIV-1 provirus in reactions
containing from 100 to as few as a single copy. We obtained similar results for β-actin
copies in reactions prepared using commercially available real-time assay reagents and
the same dilutions of template, which contained 1 HIV-1 provirus/genome (5). A
regression of observed mean HIV-1 copies on target levels demonstrated that this
assay is linear at copy levels below 10 and including 1. In our reactions for which the
target copy level was 1, 50% of reactions were positive by the HIV-1 pol assay and 75%
of reactions were positive by the β-actin assay. These percentages are within range of
the expected 63% positive reactions, given the assumptions that the starting
concentration of template is truly 1 copy/unit of volume added to each reaction, and that
the probability of pipetting at least 1 copy into each reaction follows a Poisson
distribution (12).

In mock genital specimens, the mean recovery of HIV-1 proviruses from mock
genital swabs containing low numbers of HIV-1 infected cells was within 2-fold of the
target. HIV-1 proviruses were recovered from 100% of mock swab reactions that
included both a predicted 100 and 10 HIV-1 proviruses, and from 88% of mock swab
reactions that included a predicted 5 copies of provirus. The 5 copy target translates to
25 HIV-1 infected cells/DNA extraction, given the amount tested. In less than 4% of
HIV-1 negative mock swab samples, we detected HIV-1 specific product at levels that
were at or near the limit of detection. This background contamination may reflect, in part, the challenges of processing samples in a hood dedicated for HIV-1 and using a highly sensitive PCR that detects a single copy.

Using the highly sensitive real-time PCR methods described here, we examined HIV-1 DNA levels in endocervical and vaginal secretions in antiretroviral-naïve women. We asked whether genital HIV-1 DNA levels in 13 women predicted to have a very low risk of transmitting HIV-1 to sexual partners, given their low plasma HIV-1 RNA levels, would exhibit lower levels of genital HIV-1 DNA as compared with other women (21). Women with low plasma HIV-1 RNA levels had significantly lower median level HIV-1 DNA copies/10,000 cells in endocervical secretions as compared with women with higher plasma HIV-1 RNA levels. Vaginal HIV-1 DNA was detected in 0 of the 13 women with women with low plasma HIV-1 RNA levels, compared with 6 women with higher virus loads, a difference that was statistically significant. The quantitative assays described here will be useful for helping to further define the role of cell-free vs cell-associated virus in transmission studies. They may also be useful in quantifying changes in HIV-1 infected cell levels in the genital secretions of women who are treated with highly active antiretroviral therapy.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG. 1. Mean and mean +/- 1 SD of observed HIV-1 pol copies in serial dilutions of ACH2 genomic DNA. A regression of mean observed HIV-1 pol copy levels on target levels 1-10 was used to generate the trendline. Inset shows mean and mean +/- 1 SD of observed HIV-1 pol copies at target copy levels 1-100, and a trendline representing a regression of mean observed HIV-1 copies on target levels 1-100.

FIG. 2. Observed HIV-1 copies in genomic DNA extractions of mock genital samples. Data represents duplicate HIV-1 pol tests. Black bars indicate the mean HIV-1 pol copies for each target level. The expected number of copies/reaction was 5, 10, and 100.

FIG. 3. Boxplot of endocervical (black) and vaginal (gray) HIV-1 DNA copies/10,000 cells in 13 women with plasma virus levels ≤ 2,200 copies/mL and in 13 women with plasma HIV-1 RNA levels ≥ 2,201 copies/mL. One outlier in endocervical HIV-1 DNA values for women with higher plasma HIV-1 RNA levels (221.9) is not shown.
TABLE 1. Summary data for HIV-1 \textit{pol} and human \(\beta\)-actin DNA real-time PCR assays on serial dilutions of ACH2 genomic DNA.

<table>
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<th>Target</th>
<th>\textit{HIV-1} \textit{pol} assay</th>
<th>Human (\beta)-actin assay</th>
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<td>No.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>100</td>
<td>109.4 (24.9)</td>
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TABLE 2. Summary of HIV-1 RNA levels in plasma and of HIV-1 DNA copy numbers in endocervical and vaginal secretions for 26 women.

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<tr>
<th>Participant</th>
<th>Plasma HIV-1 RNA copies/mL</th>
<th>Endocervical HIV-1 DNA copies/10^4 cells</th>
<th>Vaginal HIV-1 DNA copies/10^4 cells</th>
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<sup>a</sup> Genital swab specimens in which no HIV-1 DNA copies were detected; values were defined as 0.5 HIV-1 copies divided by the total number of cells sampled, as determined by β-actin levels.
FIG. 1.

Observed HIV-1 pol copies/reaction, means and means +/- SDs

Target HIV-1 pol copy number/reaction

Y = 1.1x + (-0.7)

Y = 1.0x + (-0.3)
FIG. 2.

Target HIV-1 copy level/reaction

Observed HIV-1 pol copies/reaction
FIG. 3.

![Graph showing HIV-1 DNA copies/10,000 cells for women with plasma HIV-1 RNA ≤ 2,200 copies/mL and ≥ 2,201 copies/mL.](image-url)