Quantification of Genital Human Immunodeficiency Virus Type 1 (HIV-1) DNA in Specimens from Women with Low Plasma HIV-1 RNA Levels Typical of HIV-1 Nontransmitters[▽]

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Received 18 July 2006/Returned for modification 18 September 2006/Accepted 4 October 2006

Studies of human immunodeficiency virus type 1 (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 are 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 with 100 genomes to 1 genome isolated from a cell line containing one 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, or 5, the average copy number/reaction was 80.2 (standard deviation [SD], 28.3), 9.1 (SD, 5.4), or 3.1 (SD, 2.1), respectively. We used this method to examine genital HIV-1 DNA levels in specimens from women whose low plasma HIV-1 RNA levels are typical of HIV-1 nontransmitters. The median HIV-1 DNA copy number in endocervical secretions from these women (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 versus 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 of cell-associated HIV-1 in transmission.

In human immunodeficiency virus type 1 (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 that 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 levels of HIV-1-infected cells 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 nonpregnant antiretroviral-naïve women (1).

Both cell-free and cell-associated viruses could play a role in transmission of HIV-1 via genital secretions. Macaques inoculated intravenously with either cell-free or cell-associated simian immunodeficiency virus 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. For antiretroviral-naïve women, a recent estimate of cervicovaginal HIV-1 DNA (median, 67 HIV-1 DNA copies/1,000,000 cells) (1) was 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

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[▽] Published ahead of print on 18 October 2006.

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 level of HIV-1 DNA in endocervical and vaginal secretions from 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 old, 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 a Gen-Probe HIV-1 viral load assay) (7) of this cohort have been reported previously (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 nontransmitters in previous studies (21). Thirteen additional women were randomly selected from the remaining 637 women for comparison (four women with 2,201 to 22,000 plasma HIV-1 RNA copies/ml; four women with 22,001 to 220,000 plasma HIV-1 RNA copies/ml; and five women with >220,000 plasma HIV-1 RNA copies/ml).

Clinical specimen collection. Genital secretions were collected using Dacron swabs, which were immersed into 1 ml of freezing medium (70% RPMI 1640, 20% fetal calf serum, and 10% dimethyl sulfoxide). Swabs were stored in cryovials in liquid nitrogen prior to shipment to Seattle, WA, for testing. Endocervical secretions were collected by rotating a swab two full turns after insertion (1 cm) into the endocervical os, and vaginal secretions were collected by rotating a swab three 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 one proviral copy/cell (5). ACH2 genomic DNA was isolated by using a 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 microliters 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 ($\sim 500,000$ [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 μ l of each batch of cells onto a Dacron swab and immersing the swab into 1 ml of freezing medium.

DNA extraction. Genomic DNA was extracted from genital specimens and mock swabs by using a 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 by using 100 to 500 μ l of sample material and elute in volumes of 100 to 200 μ l 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 to 500 μ l was used for DNA extractions. Individual mock swabs were used for one or two genomic DNA extractions, each performed on separate days. Genomic DNA was eluted using either 100 μ l (mock swabs) or 200 μ l (genital specimens) of water. Twenty microliters (mock swabs) or 10 μ l (genital specimens) of eluate was used for *pol*

real-time PCRs, which were performed in duplicate, and 2 μ l of eluate (mock and genital swabs) was used for human β -actin gene real-time PCRs, which were performed in single experiments.

Real-time PCR. Real-time reaction mixtures for both β -actin and *pol* were prepared using TaqMan PCR core reagents (Applied Biosystems, Foster City, CA). For all real-time assays, the reaction volume was 50 μ l. Reaction mixtures for the human β -actin gene were prepared as has been described previously (22). The linear dynamic range of this quantitative β -actin assay is 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 previously (22) and was used with minor modification. Specifically, the concentrations of $MgCl_2$, 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. Reaction mixtures contained 350 μ M $MgCl_2$, 200 μ M deoxynucleoside triphosphates, 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.

Tenfold serial dilutions (ranging from 10,000 copies to 1 copy/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/ μ l stock of herring sperm DNA (Promega, Madison, WI) to ensure that each dilution contained the same amount of total DNA.

Reactions were carried out with 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 cycle threshold for each reaction was set manually and was defined as 10 standard deviations (SD) above the mean ΔR_n level during cycles 3 to 15 for each well for each individual plate.

HIV-1 *pol* real-time reactions using ACH2 dilutions, genital specimens, and mock swabs were 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 including a commercial preparation of human genomic DNA (Promega, Madison, WI) were performed to control for the potential for nonspecific 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 fivefold of one another. For duplicates that did not meet this criterion, up to two 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 an HIV-1 *pol* real-time assay can be used to determine HIV-1 DNA copy number in reactions with extremely low levels of proviral genomes. For this purpose, a panel of low-copy reactions was generated by serially diluting genomic DNA isolated

TABLE 1. Summary data for HIV-1 *pol* and human β -actin DNA real-time PCR assays on serial dilutions of ACH2 genomic DNA

Target copy no.	HIV-1 <i>pol</i> assay			Human β -actin assay		
	Mean no. of copies (SD)/reaction	CV ^a (%)	No. (%) positive	Mean no. of copies (SD)/reaction	CV ^a (%)	No. (%) positive
1	1.0 (1.2)	120.0	21 (50)	1.4 (0.7)	50.0	6 (75)
2	1.6 (1.6)	100.0	28 (67)	1.9 (1.3)	68.4	6 (75)
3	2.8 (1.7)	60.7	38 (90)	3.4 (1.1)	32.4	8 (100)
4	3.6 (2.6)	72.2	38 (90)	4.7 (1.7)	36.2	8 (100)
5	4.4 (2.5)	56.8	40 (95)	6.7 (2.3)	34.3	8 (100)
10	9.8 (4.9)	50.0	42 (100)	13.2 (2.6)	19.7	8 (100)
100	109.4 (24.9)	22.8	42 (100)	120.4 (25.2)	20.9	8 (100)

^a CV, coefficient of variation.

from a chronically infected HIV-1 cell line (ACH2) that contains a single copy of HIV-1 (5). The final target levels of genomes for these reactions were 100, 10, 5, 4, 3, 2, and 1. Human β -actin and HIV-1 *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 *pol* assay (Table 1). HIV-1 proviral DNA was detected in 21/42 (50%) one-copy reactions, and β -actin was detected in 6/8 (75%) one-copy reactions. For both assays, the mean observed copy number at each dilution fell within <1% to 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 *pol* assay was evaluated using the F test for lack of fit (18). This test demonstrated that, for reactions with 10 copies to 1 copy, a linear regression function of the mean observed copy numbers on the target levels

was appropriate for the data ($P = 0.99$) (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 (HeLa) cells. 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%) 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. With the negative mock swabs, we detected HIV-1 *pol* copies in 4 of 109 (3.7%) genomic DNA extractions, of which 3 exhibited a readout of fewer than 1.0 copy/reaction. To examine whether this background could be due to nonspecific amplification, we performed reactions con-

FIG. 1. Means and means $\pm$ 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 to 10 was used to generate the trend line. The inset shows means and means $\pm$ 1 SD of observed HIV-1 *pol* copies at target copy levels 1 to 100 and a trend line representing a regression of mean observed HIV-1 copies on target levels 1 to 100.

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

taining 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 by use of the β -actin assay. The mean cell number in these swabs was 381,881 (SD, 163,505), compared to the predicted cell number of 500,000 (based on cell counts) added to each mock genital sample.

Quantitative analysis of genital HIV-1 DNA in specimens from 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 levels, 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 by using a threshold of $\leq 2,200$ copies/ml, based on the plasma HIV-1 RNA level that has previously been used to define nontransmitters (21). Thirteen women had plasma HIV-1 RNA levels of $\leq 2,200$ 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 numbers of endocervical and vaginal HIV-1 DNA copies/10,000 total cells for these 26 women were quantified by use of our in-house HIV-1 *pol* real-time assay and the real-time assay for β -actin (Table 2). In endocervical secretions, the median number of cells that were sampled for HIV-1 DNA in two PCRs combined was 11,410 (interquartile range [IQR], 5,452 to 16,382), and in vaginal secretions, the median number of cells sampled was 23,348 (IQR, 8,676 to 48,744). The number of endocervical HIV-1 DNA copies was significantly lower in specimens from women with low plasma HIV-1 RNA (median, 1.8 HIV-1 DNA copies/10,000 cells; IQR, 0.7 to 5.1 HIV-1 DNA copies/10,000 cells) (Fig. 3) than in specimens from women with higher plasma HIV-1 RNA (median, 16.6 HIV-1 DNA copies/10,000 cells; IQR, 2.4 to 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 (seven versus eight, respectively). Vaginal HIV-1 DNA was not detected in specimens from any of the 13 women in the low plasma HIV-1 RNA group; by contrast, there was detectable

TABLE 2. Summary of HIV-1 RNA levels in plasma and of HIV-1 DNA copy numbers in endocervical and vaginal secretions from 26 women

Participant no.	Plasma HIV-1 RNA copies/ml	Endocervical HIV-1 DNA copies/ 10^4 cells	Vaginal HIV-1 DNA copies/ 10^4 cells
1	<12	0.5 ^a	0.3 ^a
2	<12	0.2 ^a	0.1 ^a
3	<12	8.1	0.2 ^a
4	214	0.3 ^a	0.1 ^a
5	270	0.7	0.1 ^a
6	312	5.1	0.6 ^a
7	352	2.8 ^a	0.1 ^a
8	838	14.7	0.2 ^a
9	1,294	1.8	0.2 ^a
10	1,320	5.1	2.0 ^a
11	1,640	38.9	1.2 ^a
12	1,940	1.0 ^a	0.1 ^a
13	2,152	1.7 ^a	0.1 ^a
14	2,320	2.5 ^a	0.7 ^a
15	7,120	2.1 ^a	0.2 ^a
16	7,700	2.4 ^a	6.5
17	19,260	0.5 ^a	0.1 ^a
18	47,080	60.3	5.6
19	59,060	0.4 ^a	9.8
20	155,840	20.2	1.7 ^a
21	179,540	9.1	1.0
22	248,880	58.0	0.6 ^a
23	968,540	221.9	10.4
24	1,082,020	65.4	0.1 ^a
25	1,129,580	16.6	34.4
26	2,336,200	38.2	0.6 ^a

^a Values were defined as 0.5 HIV-1 copies divided by the total number of cells sampled, as determined by β -actin levels, and represent genital swab specimens in which no HIV-1 DNA copies were detected.

HIV-1 DNA in specimens from 6 of the 13 (46%) women in the higher plasma HIV-1 RNA group ($P = 0.02$), which had an overall median vaginal HIV-1 DNA level of 1.0/10,000 cells (IQR, 0.6 to 6.5 HIV-1 DNA copies/10,000 cells).

FIG. 3. Box plot of endocervical (black) and vaginal (gray) HIV-1 DNA copies/10,000 cells in specimens from 13 women with plasma virus levels of $\leq 2,200$ copies/ml and in 13 women with plasma HIV-1 RNA levels of $\geq 2,201$ copies/ml. One outlier in endocervical HIV-1 DNA (221.9) values for women with higher plasma HIV-1 RNA levels is not shown.

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 specimens from 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 numbers for HIV-1 provirus in reactions with from 100 copies to as little as a single copy. We obtained similar results for β -actin copies in reactions with commercially available real-time assay reagents and the same dilutions of template, which contained one 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 one copy/unit of volume added to each reaction mixture and that the probability of pipetting at least one copy into each reaction mixture 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 twofold of the target. HIV-1 proviruses were recovered from 100% of mock swab reactions with both predicted 100 and predicted 10 HIV-1 proviruses and from 88% of mock swab reactions with a predicted 5 copies of provirus. The five-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 from antiretroviral-naïve women. We asked whether specimens from 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 than specimens from other women (21). Endocervical secretions from women with low plasma HIV-1 RNA levels had significantly lower median levels of HIV-1 DNA copies/10,000 cells than those from women with higher plasma HIV-1 RNA levels. Vaginal HIV-1 DNA was detected in specimens from 0 of the 13 women with low plasma HIV-1 RNA levels, compared with specimens from 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 versus cell-associated virus in transmission studies. They may also be useful in quantifying changes in levels of HIV-1-infected cells in the genital secretions of women who are treated with highly active antiretroviral therapy.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health through grants AI38518 and AI39996. S.B. was supported in part by the National Institutes of Health predoctoral fellowship GM62819-02 and the Poncin Scholarship Fund.

We are grateful to Christine Rousseau for her valuable work in establishing the HIV-1 *pol* real-time assay. We thank the administration at Coast Provincial General Hospital, Mombasa, Kenya, for allowing this study to take place, and we thank the hospital research staff for their valuable contribution. Finally, we thank the women who contributed their time and effort by participating in this research.

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