Comparative Prediction of Perinatal Human Immunodeficiency Virus Type 1 Transmission, Using Multiple Virus Load Markers

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Maternal plasma human immunodeficiency virus (HIV) type 1 RNA load has a role in perinatal transmission, but significant overlap in the range of plasma virus loads among transmitters and nontransmitters is often observed, which makes it difficult to predict transmission outcome. We measured several virus markers in a drug-naive population of HIV-1–infected mothers in Botswana. Maternal plasma HIV-1 RNA load, peripheral blood mononuclear cell–associated blood HIV-1 DNA load, and cervicovaginal fluid (CVF) HIV-1 DNA load were determined using quantitative real-time polymerase chain reaction. The overall rate of transmission among these mother-infant pairs was 35.7%. Median infant age was 2.5 months. An association between increased plasma HIV-1 RNA load and perinatal transmission was observed (odds ratio [OR], 2.20/1-log virus load; 95% confidence interval [CI], 1.15–4.18). However, the association between increased blood HIV-1 DNA load and perinatal transmission was stronger (OR, 10.30; 95% CI, 2.11–50.38). When blood HIV-1 DNA load was combined with CVF HIV-1 DNA load, the association with transmission increased (OR, 25.0; 95% CI 2.73–228.60).

Although multiple factors contribute to mother-to-child transmission (MTCT) of human immunodeficiency virus (HIV) type 1, maternal plasma HIV-1 RNA from blood and genital fluids of HIV-1–positive mothers has been documented to correlate strongly with MTCT [1–16]. Of importance, several studies indicate that a viral threshold for perinatal transmission may not be clearly definable [1, 3, 7, 10, 15, 17]. Because perinatal transmission among mothers with plasma HIV-1 RNA loads <1000 copies/mL has been documented [18], an improved understanding of viral and host determinants for perinatal transmission may help to predict transmission likelihood and could assist in making treatment decisions.

Botswana remains one of the countries within sub-Saharan Africa and globally most severely affected by HIV. Perinatal transmission is a significant public health concern in this region and in other African countries. Before the implementation of antiretroviral interventions, perinatal transmission rates worldwide were 20%–40%. MTCT has been described to occur in utero, intrapartum, and postpartum through breast-feeding [19–23]. The endemic HIV-1 subtype within Botswana and throughout sub-Saharan Africa is HIV-1C. Recent studies of HIV-1C collectively suggest that this subtype displays a preferential use of the CCR5 coreceptor [24] and altered gene expression [25–27]. Hence, the effects of systemic and genital HIV-1 burden on perinatal transmission need to be better defined for African populations and may differ from those seen in other settings.

Few other studies have evaluated the postpartum persistence of viral or cellular determinants associated with MTCT of HIV. An increase in HIV-1 RNA load has been observed postpartum, whereas HIV-1 DNA load is relatively more stable [2, 28]. The persistence of markers associated with transmission may be informative and useful for future pregnancies and may also
have relevance for sexual transmission. In addition, the wide range of maternal HIV loads at which transmission occurs prompted us to evaluate additional virus markers—namely, HIV-1 DNA levels in the blood and cervicovaginal fluid (CVF). The role of cell-associated virus burden in transmission has not been well documented, although cell-associated virus is potentially more infectious than free virions [29].

Here, we describe the overall rate of perinatal infection in a cross-sectional population of HIV-1–infected, drug-naive, postpartum mothers in Botswana and the association of plasma HIV-1 RNA load and blood and CVF HIV-1 DNA loads with transmission outcome. This study took place in 1999, simultaneously with efforts to establish an MTCT prevention program by the Botswana government, which is now in place.

METHODS

Study design overview. All women presenting at the maternal health clinic for their first postnatal or child welfare visit 6–8 weeks after giving birth were offered voluntary counseling and testing for HIV by a recruiting health officer. After appropriate posttest counseling, those who tested positive for HIV were invited to give their informed consent to participate in the study. Women underwent 1 HIV test and received their results 1 month later, which was at the time of their next scheduled visit. HIV testing was performed using 2 independent ELISAs (Ortho Ab-Capture [Ortho-Clinical Diagnostics] and Murex HIV-1.2.0 [Abbot-Murex, Murex Biotech Limited]). Maternal blood and CVF samples were collected at that time from participating HIV-infected women (median age, 24.5 years), and blood samples were obtained from the babies of these mothers (median infant age, 2.5 months). No exclusion was made on the basis of health status of the mother. Maternal clinical manifestation data were compiled from 9 clinical categories, including severe weight loss, chronic diarrhea, prolonged fever, persistent cough, severe or repeated bacterial infections, generalized pruritic dermatitis, oropharyngeal candidiasis, generalized lymphadenopathy, Kaposi sarcoma, and others. There were no recorded cases of herpes zoster virus infection or cryptococcal meningitis. Study participants were enrolled from 3 different villages: Molepolole, Mochudi, and Lobatse.

Ethical considerations. At the time that this study was conducted, voluntary counseling and testing programs for the prevention of perinatal transmission and antiretroviral treatment were not available in any study location. HIV counseling and testing initiated through this study were in preparation for and occurred in parallel with implementation of an MTCT prophylaxis program. Recruiting health care providers offered women free infant formula if they were found to be HIV infected, to decrease the risk of additional perinatal HIV transmission through breast milk, and educated them in its safe preparation and use. HIV-infected infants were also offered trimethoprim-sulfamethoxazole prophylaxis. This study increased the number of health care providers trained and experienced in voluntary counseling and testing for HIV and facilitated the establishment of a program for the prevention of MTCT of HIV by use of zidovudine. Infant formula was introduced to the study areas shortly after this study ended, with the assistance of these trained staff. The study was approved by the Health Research Unit of Botswana and the institutional review boards of the Harvard School of Public Health and the Boston University School of Medicine. All participating mothers gave written informed consent.

Specimen collection. Blood and CVF samples were collected from mothers, and blood samples were collected from their infants. Approximately 1 mL of blood was placed into a cryovial tube containing 4 mL of RNA/DNA Stabilizing Reagent for Blood/Bone Marrow solution (Roche), and tubes were inverted and stored until processing. To obtain CVF, a dacron swab was inserted and rotated 360 degrees in all 4 quadrants of the vaginal vault. The swab was placed into a sterile tube containing 1 mL of PBS, rinsed, and mixed with 4 mL of Stabilizing Reagent. Total nucleic acid was isolated from all specimens, on the basis of a modified use of the mRNA Isolation Kit for Blood/Bone Marrow (Roche). To obtain RNA from total nucleic acid, specimens were DNase treated (DNA-free; catalog no. 1906, Ambion) and to obtain DNA, specimens were RNase treated (RNase, DNase-free; catalog no. 1119 915, Roche).

Virus load and DNA sequencing. Plasma virus loads were analyzed by use of the Amplicor Monitor assay (version 1.5; Roche Diagnostics), according to the manufacturer’s instructions. To confirm the subtype of specimens in this study, polymerase chain reaction (PCR) products corresponding to the envelope region (primary PCR with primers ED3/ED14, secondary PCR with primers E57/E58; ED3: TTAGGCACTTCC-TATGGCAGGAAGAAGGCG; ED14: TCTTGGCTGGAGCTG-TTTGATGCCCAAGC; E57: CTGTATTAATGGCAGTCTAGC; E58: CACTTCTCAATGGTCCCTCA) were Topo cloned (Invitrogen) and used to generate 6–10 clones/individual, which were sequenced using an automated sequencer. Nucleotide sequences were edited using Sequencher (Gene Codes) or SeqMan (DNAStar). Multiple alignment was performed using ClustalX (available at: http://inn-prot.weizmann.ac.il/software/ClustalX.html) and BioEdit (available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The pairwise evolutionary distances of nucleotide alignment were computed with the Kimura 2-parameter model. To generate phylogenetic trees, alignments were globally gap-stripped. A phylogenetic tree was drawn using PAUP software (Swofford, Smithsonian Institute).

Real-time PCR. Fluorescence-based quantitation was conducted using a LightCycler real-time PCR machine, and duplicate runs were conducted on an ABI 7000 real-time PCR.
machine, with no apparent difference in sensitivity. To quantify HIV-1 gag, we used the primer set SSK145/SSKCC1B 5′-AGTGG-GGGGACATCAAGC-3′ and SSKCC1B 5′-TACTAGTAGGCTGCTAT-3′ and TaqMan SYBR green reagents (Applied Biosystems). Fluorescence-based quantitation was conducted using an ABI 7000 real-time PCR machine to quantify HIV-1 gag with the following primer set: P24-F1, CAAGCAGCCATGCA-AATGTT; p24-R1, CCCTGATGTACTGGATGTAATCT; and p24-FAMprobe, 6FAM-ATCAATGAGGAGGCTGCAGAATG-GGA-TAMRA. Cell-associated viral expression levels were normalized to endogenous 18S (RNA) for RNA measurements or normalized to β-globin level or 18S (DNA) for HIV-1 DNA measurements. gag primers were optimized for dual detection of both subtypes HIV-1B and HIV-1C.

**Statistical considerations and definitions.** Women were considered to be transmitters (TRs) if PCR analysis of their infants yielded positive results in at least 2 viral loci and if specimens tested positive using the Amplicor version 1.5 diagnostic kit. The PCR tests were based on 1 blood specimen from the infants (median age, 2.5 months). Infants were defined as HIV positive if they had at least 2 positive PCR results in separate viral loci and also were positive for β-globin. In 4 cases with limited DNA, the infant was considered to be HIV positive with only 1 positive PCR conducted on duplicate samples tested separately in Botswana and Boston. Infants were considered to be HIV negative if they had at least 2 negative PCR results in separate loci. All specimens were tested for β-globin and, if negative, were excluded from analysis. Seven individuals were indeterminate and excluded from the analysis. Maternal plasma HIV-1 RNA loads, blood HIV-1 DNA loads, and CVF HIV-1 DNA loads were log10-transformed; all calculations were done on the log10-transformed values. Plasma HIV-1 RNA load was measured in copies per milliliter. Blood and CVF HIV-1 DNA loads were measured in fluorescent units and normalized to cell copy number on the basis of β-globin fluorescence. Wilcoxon rank sum test was used for comparing virus load levels between TR and nontransmitter (NTR) groups. Wilcoxon rank test and Spearman’s rank correlations were used instead of t tests and Pearson’s correlations, to make the results robust to possible nonnormality of the log-transformed virus load variables. Logistic regression models were used for computing odds ratios (ORs) of transmission given 1-log differences in the level of virus load. To evaluate whether the virus load factors independently predicted the risk of perinatal transmission, for each virus marker, both unadjusted ORs and ORs adjusted for the other markers were estimated. In the continuous, the virus load variables were dichotomized on the basis of their median values, and Fisher’s exact tests were applied to assess the effect of low virus load in multiple compartments on transmission risk. Spearman’s rank correlation coefficients were used for measuring linear associations between the virus load markers and for assessing potential colinearity of the markers.

**RESULTS**

During May–November 1999, 750 mothers were invited to participate in the study. Of 472 postpartum women who were tested, 133 (28%) were HIV infected. Of those HIV-infected mothers, 91 blood samples were collected from matched babies (median age, 2.5 months; range, 1.0–7.6 months) and were tested. Of the 91 babies tested, 30 were HIV positive, 7 had indeterminate results, and 54 were HIV negative. All but 4 of the TRs had ≥2 positive PCR test results, and all the NTRs had ≥2 negative PCR test results. TRs and NTRs did not differ significantly with regard to age, clinical status or condition, parity, Cesarean section experience, breast-feeding practice, or prevalence of sexually transmitted diseases. We evaluated sample recovery in the infants by quantitative PCR measuring β-globin DNA. On the basis of that analysis, there was no apparent bias in sample recovery between TR and NTR specimens.

The median plasma HIV-1 RNA load was 4.72 log10 copies/mL (range, 3.50–5.89 log10 copies/mL) for TRs and 4.33 log10 copies/mL (range, 2.60–5.87 log10 copies/mL) for NTRs. As shown in table 1, log-transformed plasma HIV-1 RNA load

<table>
<thead>
<tr>
<th>Virus load marker</th>
<th>Wilcoxon P</th>
<th>Mean difference</th>
<th>95% CI for difference</th>
<th>Logistic regression ORa</th>
<th>95% CI for OR of transmission</th>
<th>P for OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LogVL</td>
<td>.018</td>
<td>0.44</td>
<td>0.10–0.79</td>
<td>2.2</td>
<td>1.15–4.18</td>
<td>.016</td>
</tr>
<tr>
<td>LognBLD</td>
<td>.0006</td>
<td>0.94</td>
<td>0.50–1.39</td>
<td>10.3</td>
<td>2.11–50.38</td>
<td>.004</td>
</tr>
<tr>
<td>LognCVF</td>
<td>.0019</td>
<td>0.75</td>
<td>0.16–1.34</td>
<td>2.69</td>
<td>1.12–6.43</td>
<td>.026</td>
</tr>
</tbody>
</table>

*Univariate logistic regression ORs are shown. Models that controlled for exactly 1 of the variables age, parity, Cesarean section, and feeding gave similar results.
Figure 1. Pairwise scatter plots for the 3 virus load measures between maternal nontransmitters (N) and transmitters (T). All Spearman’s rank correlation coefficients ($r_s$) were <0.25: log plasma virus load (LogVL) vs. log virus load in the blood (LognBLD) ($r_s$, 0.23; $P = .13$), LogVL vs. log virus load in cervicovaginal fluid (LognCVF) ($r_s$, 0.21; $P = .18$), and LognBLD vs. LognCVF ($r_s$, 0.23; $P = .14$).
Figure 2. Comparative 1-way box plots for maternal nontransmitter (N) and transmitter (T) specimens. Top. Plasma human immunodeficiency virus (HIV) type 1 RNA virus load using the Amplicor test (Roche Diagnostics). Middle. Blood HIV-1 DNA load (quantitative polymerase chain reaction [PCR], second pair, normalized). Bottom. Cervicovaginal fluid (CVF) HIV-1 DNA load (quantitative PCR, third pair, normalized). All shown are for N and T specimens, respectively. All 3 pairs suggest that T virus load tends to be higher. Scale is log-transformed and shown as arbitrary fluorescence units for HIV-1 DNA in blood and CVF and log-transformed copies/mL for plasma RNA on the X-axis.

Table 2. Evaluation of maternal cell-associated human immunodeficiency virus (HIV) type 1 DNA load in the blood and HIV-1 DNA load in the cervicovaginal fluid (CVF) as independent predictors of transmission in a logistic regression model.

<table>
<thead>
<tr>
<th>Virus load marker</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LognBLD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.02 (2.03–49.42)</td>
<td>.005</td>
</tr>
<tr>
<td>LognCVF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 (1.07–5.85)</td>
<td>.034</td>
</tr>
<tr>
<td>LognBLD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.87 (2.23–177.49)</td>
<td>.0074</td>
</tr>
<tr>
<td>LognCVF&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.64 (1.09–12.15)</td>
<td>.036</td>
</tr>
<tr>
<td>LogVL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.87 (0.48–7.32)</td>
<td>.37</td>
</tr>
<tr>
<td>LogVL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.95 (0.58–6.54)</td>
<td>.28</td>
</tr>
<tr>
<td>LognBLD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.72 (2.04–171.62)</td>
<td>.0096</td>
</tr>
<tr>
<td>LognCVF&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.51 (1.02–12.00)</td>
<td>.046</td>
</tr>
</tbody>
</table>

**NOTE.** Odds ratio (OR) is per 1 unit difference in log virus load. CI, confidence interval; LognBLD, log virus load in blood; LognCVF, log virus load in CVF; LognVL, log virus load in plasma.

<sup>a</sup> Adjusted for plasma virus load.

<sup>b</sup> Adjusted for LognCVF.

<sup>c</sup> Adjusted for LognBLD.

<sup>d</sup> Adjusted for plasma virus load and CVF.

We determined whether maternal blood HIV-1 DNA load (logVL) differed significantly between TRs and NTRs (P = .018). The mean difference in plasma virus load between TR and NTR was 0.44 logs (95% confidence interval [CI], 0.10–0.79; table 1). By use of a logistic regression model, the OR of transmission was 2.20 for each 1.0-log higher plasma virus load (95% CI, 1.15–4.18). Although plasma HIV-1 RNA load was significantly higher for TRs than for NTRs (P = .02), a substantial overlap in the range of virus loads between these 2 groups was apparent, which is consistent with many other studies designed to evaluate virus load and transmission.

We measured whether comparative HIV-1 DNA levels in blood and CVF might similarly be correlated with transmission outcome. Therefore, maternal cell-associated blood and CVF HIV-1 DNA loads were determined using real-time PCR for a subset of specimens: 21 of 24 TR specimens and 19 of 24 NTR maternal specimens present and with sufficient nucleic acid in the laboratory at the time of testing. Other studies have investigated the relationship between HIV levels in plasma and the genital tract [30, 31]. Comparative pairwise scatter plots (figure 1) and 1-way box plots (figure 2) are shown for all 3 virus load measurements between TRs and NTRs. None of the 3 markers was significantly linearly correlated (Spearman’s rank correlation coefficient, <0.25; P > .10). As shown in table 1, the difference between blood HIV-1 DNA load for TRs and NTRs was highly significant (P = .0006) in this study. The mean difference in blood HIV-1 DNA load between TRs and NTRs was 0.94 (95% CI, 0.50–1.39). Using a logistic regression model, the OR of transmission was 10.30 for each 1-log higher blood HIV-1 DNA load (LognBLD: 95% CI, 2.1–50.4). Analysis of CVF HIV-1 DNA load gave similar results (LognCVF: OR, 2.7; 95% CI, 1.1–6.4).

We determined whether maternal blood HIV-1 DNA load was associated with transmission independently of plasma HIV-1 RNA load. When adjusted for plasma HIV-1 RNA load, LognBLD appeared to be a strong independent predictor of transmission risk (table 2). The adjusted OR was 10.0 (95% CI, 2.0–49.4). In this model, logVL was not significant (P = .37). LognCVF was also an independent predictor of transmission risk, when adjusted for logVL. The blood HIV-1 DNA load appeared to be an independent predictor of transmission risk, when we controlled for LognCVF and vice versa. The
Figure 3. Based on a logistic regression model, the lines show the estimated probability of transmission vs. log human immunodeficiency virus (HIV) type 1 DNA load in blood for women with log HIV-1 DNA load in cervicovaginal fluid (CVF) fixed at the 80th, 60th, 40th, and 20th percentiles, respectively (from left to right). Nonparallel lines indicate an interaction. LognBLD, log virus load in blood.

potential problem of colinearity of multiple virus load markers within a model did not occur (figure 1). Nearly identical adjusted ORs were obtained when we adjusted for plasma HIV-1 RNA load, which suggests that the influence of plasma HIV-1 RNA load is comparatively minor ($P = .74$, when LognBLD and LognCVF were taken into account). In this population, plasma HIV-1 RNA load apparently has a minimal contribution to the prediction of transmission outcome, with an estimated OR of 1.32. The overlap in virus load measurements between TRs and NTRs was more apparent in blood and CVF HIV-1 DNA loads. We observed that the distributions of plasma HIV-1 RNA and blood and CVF HIV-1 DNA loads were asymmetric and that the distribution of CVF HIV-1 DNA load had lighter tails than those of a normal distribution. Nevertheless, all 3 virus load measurements exhibited modest departures from normality. We were then interested in evaluating combinations of virus load markers (plasma HIV-1 RNA, blood HIV-1 DNA, and CVF HIV-1 DNA) in relation to transmission outcome. We observed that low virus load in multiple maternal compartments was associated with minimal transmission risk. This result was derived by considering a binary indicator of whether all 3 virus loads were below the median or whether at least 2 of the virus loads were below the median. All 4 subjects with low virus loads in all 3 compartments did not transmit HIV to their infants. In addition, 6 of 7 subjects with low plasma and blood virus loads did not transmit (OR, 10.0; $P = .035$), 5 of 7 with low plasma and CVF virus load did not transmit (OR, 3.65; $P = .22$), and 10 of 11 with low blood and CVF virus loads did not transmit (OR, 25.0; $P < .001$). We also observed a trend toward interaction between maternal blood and CVF HIV-1 DNA loads in their predictive effect on transmission risk (figure 3). Therefore, within this population, the NTRs tended to have lower blood and CVF HIV-1 DNA loads, whereas the TRs tended to have higher blood and CVF HIV-1 DNA loads.

**DISCUSSION**

To improve the predictive value of virus burden for MTCT, we developed a quantitative real-time PCR assay for HIV-1C and compared it with traditional markers for perinatal transmission among HIV-infected, drug-naive, mothers in Botswana. In the present study, blood HIV-1 DNA load appeared to be the strongest independent predictor of transmission and contained much of the predictive information for transmission in this population. Blood HIV-1 DNA load and, to a lesser extent, that in CVF were better correlated with transmission than plasma HIV-1 RNA levels. There was an improved strength when virus load markers were combined as predictors of transmission, especially for blood and CVF HIV-1 DNA loads (OR, 25.0; $P < .001$). A trend toward interaction between blood and CVF HIV-1 DNA loads in their predictive effect on transmission risk was observed at high HIV-1 DNA loads. Collectively, within this population, the NTRs tended to have lower HIV-1 DNA levels in the CVF and blood, whereas the TRs tended to have higher HIV-1 DNA levels. These results suggest that cell-associated virus burden is an important correlate of perinatal transmission.

Because the present study was cross-sectional and retrospective in nature, the exact timing of transmission is uncertain (in utero, intrapartum, or through breast-feeding); thus, we cannot exclude the possibility that differences observed among TRs and NTRs measured post facto were not present before transmission, nor can we confirm whether virus burden subsequently increased proportionately after transmission. There is some evidence that virus load differences between NTRs and TRs are stable before and after delivery [2]. An important limitation of this study is that the virus load measures were based on PCR results from only 1 blood specimen taken from infants (at 2.5 months). In addition, because the HIV serostatus of 7 infants was indeterminate (thus excluding those infants from this analysis) and because, of the 133 HIV-infected mothers,
only 91 blood samples were collected from matched babies, a potential selection bias exists, despite our efforts to avoid bias. Knowledge of the route of transmission, together with knowledge of the relative abundance of the different virus load markers in different body locales and at multiple time points, might help to better understand the cumulative role of virus burden in transmission outcome.

The increased association with HIV-1 DNA load in this population prompted us to consider cell-associated transmission as a potential mechanism. In fact, previous studies have suggested that a role exists for cell-associated HIV-1 during pregnancy. Infant exposure to HIV-1–infected cells may be a critical factor during intrapartum transmission and, an attractive mechanism for in utero transmission has been proposed [32]. In this model, the apical side of syncytiotrophoblast cells can make contact with infected maternal blood that contains both cell-free HIV-1, as well as infected cells. HIV-infected peripheral blood mononuclear cells, in contrast to cell-free HIV-1, might induce efficient HIV-1 translocation across the trophoblastic barrier shortly after cell-to-cell contact. Because the trophoblast barrier appears not to be permeable to cell-free virus, this model adds to the relevance of HIV-1 DNA load and cell-associated perinatal transmission. Although not addressed in this study, the presence of cell-free and cell-associated HIV-1 in breast-milk colostrum (present for the first 3–5 days) needs to be addressed. Whether HIV is transmitted sexually as a predominantly cell-free virus or in a cell-associated form is also not well understood [33]. Future studies that incorporate the measurement of HIV-1 DNA burden may help to better understand the mechanism and likelihood of viral transmission, particularly in cases where viral RNA and DNA burden are discordant (e.g., when plasma RNA load is low [<1000 copies/mL] and HIV-1 DNA burden is high).

References


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