Laboratory Indicators of Mastitis Are Not Associated with Elevated HIV-1 DNA Loads or Predictive of HIV-1 RNA Loads in Breast Milk

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Background. Mother-to-child transmission (MTCT) of HIV-1 has been associated with symptomatic and asymptomatic mastitis and with the quantity of HIV-1 RNA and DNA in maternal milk. An improved understanding of the relationship between indicators of inflammation and HIV-1 loads in breast milk could improve MTCT prevention strategies.

Methods. In a cross-sectional study, laboratory indicators of mastitis (breast milk sodium [Na+] concentration, sodium:potassium ratio [Na+:K+], and leukocyte count) were related to breast milk HIV-1 RNA and DNA loads and were evaluated for predicting viral loads in milk.

Results. Mastitis was present in 63 (15%) of 407, 60 (15%) of 407, and 76 (18%) of 412 milk specimens, as defined by Na+ concentration >12 mmol/L, Na+:K+ >1, and total leukocyte counts >10^9 cells/mL, respectively. Each indicator was associated with an increased milk HIV-1 RNA load (P<0.05) but not with HIV-1 DNA load. Neutrophils correlated better with milk HIV-1 RNA load than total leukocytes. However, neither neutrophil count, Na+ concentration, nor Na+:K+ displayed a threshold that was both sensitive and specific for the detection of HIV-1 RNA in milk at thresholds of >50 or >10^4 copies/mL.

Conclusions. HIV-1 DNA loads in breast milk were not increased during mastitis. Neither milk cell counts nor electrolyte concentrations were useful predictors of milk HIV-1 RNA or DNA loads for individual women.

Breast-feeding is a major mode of mother-to-child transmission (MTCT) of HIV-1 in resource-poor communities, accounting for a third or more of all HIV-1 infections in children [1, 2]. Where access to clean water and artificial formula are unreliable, breast milk remains the safest infant food, despite being a potential source of HIV-1 infection [2–6]. HIV-1 RNA and DNA in breast milk have been associated with the risk of MTCT through breast-feeding [7–12]. Therefore, identifying simple and inexpensive indicators of breast-milk HIV-1 loads should be useful in assessing the risk of MTCT and targeting interventions in resource-poor settings.

Mastitis, or inflammation of the breast, has been associated with an increased HIV-1 RNA loads in breast milk [8, 13–15], as well as with increased risk of MTCT [8, 14, 16, 17]. Symptomatic mastitis occurs in >20% of women during lactation [18], and asymptomatic mastitis appears to be even more common [8, 14, 15, 19–21]. Preventing or treating mastitis has been proposed as a way of reducing MTCT through breast-feeding [9, 22, 23]. Although HIV-1 RNA loads increase in milk during mastitis, it is unclear whether breast-milk HIV-1 DNA loads, another strong risk factor for MTCT [7, 11, 12], also increase.

During mastitis, there are increases in inflammatory-
Breast-milk HIV-1 DNA load

cell and cytokine levels, and mammary epithelia become more permeable, resulting in leakage of plasma components into milk [13, 19, 22, 24–27]. Sodium (Na+) concentrations are normally lower in breast milk than in plasma, but they increase during mastitis [13, 19, 20, 28–31]. Thus, in women without symptoms, elevation of Na+ concentrations or the ratio of sodium to potassium concentrations in breast milk has been used to define asymptomatic mastitis [8, 13–15, 28–31]. Similarly, a high total leukocyte count in milk has also been used as an indicator of asymptomatic mastitis [23, 32]. Although the breast-milk HIV-1 RNA load has been associated with mastitis [8, 13–15], the evaluation of different thresholds of electrolytes or leukocytes to predict milk HIV-1 loads or the risk of MTCT has not been reported.

The use of artificial formula to reduce breast-feeding transmission of HIV-1 is costly. Furthermore, withholding breast milk in resource-poor settings may directly or indirectly increase infant morbidity from infections and nutritional deficiencies [6]. Intervention strategies that could effectively target high-risk breast-feeding, conceivably by identifying women with elevated milk HIV-1 loads due to mastitis, could reduce MTCT. In an effort to identify a simple and inexpensive test predictive of HIV-1 shedding in breast milk, we evaluated laboratory indicators of mastitis, including breast-milk electrolyte concentrations and cell counts, for their association with HIV-1 RNA and DNA loads in the breast milk of Zimbabwean women.

PARTICIPANTS AND METHODS

Participants and data collection. Between March and September 2005, lactating women attending clinics for HIV-1–infected mothers and infants in the Zimbabwean towns of Chitungwiza and Epworth who were 6–16 weeks postpartum were asked to participate in a cross-sectional study. All participants had documented HIV-1 infection. Written informed consent and breast-milk and blood samples were obtained, as were responses to a questionnaire regarding current symptoms of breast inflammation (pain, swelling, warmth, or redness) and exposure to antiretroviral medications. The Medical Research Council of Zimbabwe and committees regulating research on human subjects at Children’s Hospital in Seattle and Stanford University approved the study.

Specimen collection and processing. Blood (6 mL) was collected by venipuncture, and breast milk (up to 20 mL) was manually expressed from each breast into separate sterile tubes. Blood and milk specimens were refrigerated and processed within 4 h after collection. Blood plasma was separated by centrifugation and stored at −70°C. DNA was extracted from plasma-poor whole blood using the Gentra Puregene kit (Gentra Systems).

Milk was centrifuged at 1000 g for 15 min to separate the lipid, lactoserum, and cell-pellet fractions. The lipid was discarded, and lactoserum was stored at −70°C. An aliquot of the cell pellet was used for total and differential cell counts. The remainder of the pellet was frozen at −70°C. Cell counts were determined by microscopic examination using a hemocytometer for the total cell count and Wright-Giemsa–stained smears for the proportion of cell types (epithelial cells, neutrophils, lymphocytes, macrophages, and eosinophils).

Electrolyte measurement. Na+ and K+ concentrations in lactoserum were measured using a Roche/Hitachi 902 Autoanalyzer (Roche Diagnostics), after dilution (1:1) in calibration

| Table 1. Laboratory indicators of mastitis associated with HIV-1 RNA but not DNA load in breast milk. |
|-------------------------------|---------------------|---------------------|---------------------|---------------------|
| Indicator of mastitis | Breast-milk HIV-1 RNA load | Breast-milk HIV-1 DNA load |
| | Specimens tested | Virus detected | Copies/mL, median (range) | P |
| | | | | |
| All milk specimens | 409 (100) | 239/409 (58) | 99 (<50–124,254) | .22 |
| Symptoms | 22/409 (5) | 16/22 (73) | 488 (<50–23,636) | .22 |
| Present | 387/409 (95) | 223/387 (58) | 93 (<50–124,254) | .22 |

Na+ concentrations and cell counts, for their association with HIV-

NOTE. Data are no. of specimens tested or no. in which virus was detected/total no. (%), unless otherwise indicated. Data are missing from some categories.

a For differences in the proportion of specimens with detectable virus with and without an indicator of mastitis.

b Milk specimens from breasts with pain, erythema, swelling, and/or increased warmth reported.

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Figure 1. Evaluation of milk sodium (Na⁺) concentration, Na⁺:potassium (K⁺) ratio, and neutrophil count in predicting HIV-1 RNA loads. Laboratory indicators of mastitis, including Na⁺ and K⁺ concentrations in lactoserum and neutrophil count in whole milk, were related to the HIV-1 RNA load in respective lactoserum samples. Na⁺ concentration (A), Na⁺:K⁺ ratio (B), and neutrophil counts (C) were significantly associated with HIV-1 RNA loads in milk collected from the right and left breasts of >200 Zimbabwean women, although with only moderate strength.

solution with 160 mmol/L Na⁺ and 7 mmol/L K⁺, to bring electrolyte concentrations into the linear range of the analyzer. Two aliquots of every tenth specimen were tested, to ensure reproducibility. Asymptomatic mastitis was defined by either Na⁺ concentration >12 mmol/L, Na⁺:K⁺ >1, or total leukocyte count >10⁶ cells/mL of milk [8, 13–15, 19–21, 23, 32].

HIV-1 quantification. HIV-1 RNA in lactoserum and plasma specimens was quantified using the AMPLICOR Monitor kit (version 1.5; Roche Diagnostics). The detection limit for plasma was 400 copies/mL using the standard method, and that for lactoserum was 50 copies/mL using the UltraSensitive method.

DNA was extracted from frozen breast-milk cell pellets derived from 1–14 mL of whole breast milk using the QiaAmp Mini Kit (Qiagen) in 50 μL of elution buffer, in accordance with the manufacturer’s instructions. HIV-1 DNA was quantified by real-time polymerase chain reaction (PCR; iCycler iQ Real-Time Detection System; BioRad Laboratories). A 129-bp region of HIV-1 gag was amplified using primers (forward, 5′-CAAGCAGCCATGCAAATGTT-3′; reverse, 5′-TGCTATGTC-ACTTCCCCCTTGTTCTCT-3′) and a probe (5′-FAM-AAAG-ATACCATCAATGAGGGCTGAGAA-TAMRA-3′). Each specimen was evaluated in duplicate 50-μL reactions that contained 10 μL of extracted DNA, 0.2 μmol/L probe, 0.4 μmol/L of each primer, and BioRad Supermix (BioRad Laboratories). Cycling conditions were 3 min at 95°C, followed by 45 cycles of 30 s at 95°C alternating with 1 min at 64°C. A near-full-length subtype C plasmid, p97ZA012.1 (obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease, National Institutes of Health [Bethesda, MD], from Drs. Cynthia M. Rodenburg, Beatrice H. Hahn, and Feng Gao and the Joint United Nations Programme on HIV/AIDS Network for HIV Isolation and Characterization) [33] was linearized by digestion with EcoR1 and used to generate the standard curve for quantification of HIV-1 DNA. The standard curve was included in each assay and consisted of 10-fold serial dilutions of the plasmid from 10⁵ to 10⁰ copies/reaction in 1 ng/mL of tRNA. Amplification of the standard curve was linear over this range for all valid assays. The detection limit was between 1 and 10 copies of HIV-1 DNA per reaction. β-Globin was also amplified from each sample by real-time PCR, as described elsewhere [34], to test for inhibitors of PCR, as well as to quantify the number of nucleated cells for calculation of the HIV-1 DNA load per cell. Negative controls containing tRNA alone were included in each assay.
Figure 2. Breast-milk total leukocyte and differential cell counts in specimens from breasts with and without mastitis. In milk specimens with mastitis, defined by sodium (Na⁺) concentrations ≥12 mmol/L, neutrophil counts increased more than those of other cell types (eosinophil and basophil counts were negligible; data not shown). All milk specimens with cell counts and Na⁺ concentrations (n = 400; 62 with Na⁺ concentrations ≥12 mmol/L and 338 with Na⁺ concentrations ≤12 mmol/L) were analyzed. Box plots display quartiles and outliers. A greater relative increase in neutrophil counts was also observed when mastitis was defined by a sodium:potassium ratio >1 (data not shown).

Statistical analysis. HIV-1 RNA and DNA measurements were examined both as dichotomous variables (e.g., detectable/undetectable) and as continuous numeric variables (in copies per milliliter). For the analyses focusing on dichotomous outcomes, a generalized estimating equation (GEE) version of logistic regression with robust variance estimation and data clusters defined by individual women was used to generate P values for tests of association, to generate odds ratios (ORs) and accompanying confidence intervals (CIs), and for receiver-operating-characteristic (ROC) analyses. These statistical analyses accounted for the potential within-person correlation between specimens collected from each breast of the same woman. A Gaussian GEE model was used to compare leukocyte, lymphocyte, neutrophil, and macrophage counts in breast milk for specimens with and without mastitis. These variables were log-transformed before analysis, to ameliorate the skewness in their distributions.

For analyses using HIV-1 RNA and DNA loads as continuous variables, specimens with undetectable HIV-1 RNA or DNA loads were assigned a copy number equal to half the lower limit of detection. Spearman’s rank correlation statistic was used for evaluating the strength of the relationship between 2 continuous variables. Overall ρ statistics were computed with the complete data set. To address potential problems with the independence assumption for right- and left-side specimens from the same woman, we used Monte Carlo simulation analysis to evaluate the behavior of the ρ statistic if only 1 independent observation per woman were selected for analysis. Simulations were run for 10,000 replications. The median of the distribution of ρ in the simulation set matched the overall ρ to at least 2 decimal places in all cases. The statistical significance of rank correlation statistics was also confirmed in Monte Carlo analyses. Logistic regression analyses and the Monte Carlo simulations were done using Stata statistical software (version 9.2; StataCorp). CIs for the Spearman rank correlation statistics were generated using StatXact software (version 7; Cytel).

RESULTS

Participants and specimens. A total of 217 breast-feeding women with HIV-1 infection were enrolled; 414 breast-milk specimens were obtained from 216 of the 217 participants. Questionnaire data and blood specimens were obtained from all 217 participants. One hundred eighty-five women (85%) reported receiving single-dose nevirapine for the prevention of MTCT, but no other exposure to antiretroviral drugs was elicited.

Prevalence of mastitis. One or more symptoms of mastitis were reported at the time of enrollment by 17 (8%) of 217 women, in 22 (5%) of 434 breasts. Asymptomatic mastitis, defined by either an Na⁺ concentration >12 mmol/L, Na⁺:K⁺
HIV-1 RNA load

<table>
<thead>
<tr>
<th>Sensitivity of indicator in predicting HIV-1 level in milk specimen</th>
<th>HIV-1 RNA load ≥50 copies/mL</th>
<th>HIV-1 RNA load &gt;10,000 copies/mL</th>
</tr>
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<tbody>
<tr>
<td>Indicator threshold</td>
<td>False-positive rate (1 – specificity), %</td>
<td>Indicator threshold</td>
</tr>
<tr>
<td>≥90%</td>
<td>Neutrophils &gt;221 cells/mL</td>
<td>86</td>
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<tr>
<td>&gt;80%</td>
<td>Neutrophils &gt;1068 cells/mL</td>
<td>69</td>
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<tr>
<td>≥50%</td>
<td>Neutrophils &gt;12,874 cells/mL</td>
<td>29</td>
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<tr>
<td>≥90%</td>
<td>Na+ &gt; 2 mmol/L</td>
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<tr>
<td>&gt;80%</td>
<td>Na+ &gt; 2 mmol/L</td>
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<tr>
<td>≥50%</td>
<td>Na+ &gt; 6 mmol/L</td>
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<td>Na+:K+ &gt; 0.20</td>
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<td>&gt;80%</td>
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<tr>
<td>≥50%</td>
<td>Na+:K+ &gt; 0.57</td>
<td>24</td>
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</tbody>
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NOTE. Na+, sodium concentration; Na+:K+, ratio of sodium to potassium concentrations in breast milk.

HIV-1 RNA loads in breast milk. HIV-1 RNA was detected in 239 (58%) of 409 breast-milk specimens from 142 (66%) of 215 women, respectively. Associations between each indicator of mastitis and HIV-1 RNA and DNA loads in individual milk specimens are shown in table 1. The number of breast-milk specimens with Na+ concentrations >12 mmol/L and Na+:K+ ≥1 was similar; however, a total of 11 (3%) of 407 specimens were discordant, with 7 specimens classified as mastitis by Na+ concentration but not by Na+:K+ and 4 specimens by Na+:K+ but not by Na+ concentration. Seventy-seven (19%) of 400 specimens were discordant with Na+ concentration >12 mmol/L and total leukocyte count >10⁶ cells/mL, with 33 specimens classified as mastitis by Na+ concentration but not by total leukocyte count and 44 specimens by total leukocyte count but not by Na+ concentration.

HIV-1 DNA in breast milk. HIV-1 DNA was quantified in 201 milk specimens. This subset of specimens did not differ significantly from the others with respect to electrolyte concentrations, cell counts, or HIV-1 RNA loads or to HIV-1 RNA loads in linked plasma specimens. HIV-1 DNA was detected in 170 (85%) of 201 breast-milk cell pellets derived from a median of 14 mL (range, 1–14 mL) of milk. The median HIV-1 DNA load was 18 copies/mL (range, 0–97,100 copies/mL) of breast milk and 105 copies/10⁶ cells (range, 0–23,000 copies/10⁶ cells).

Neither the detection nor the load of HIV-1 DNA in milk was associated with indicators of mastitis (table 1). Detection of HIV-1 DNA was concordant with detection of HIV-1 RNA in 120 (60%) of 199 milk specimens, with a positive correlation between the copy number of each (ρ = .43; P < .0001). This correlation was stronger in milk specimens with elevated Na+ concentrations (ρ = .47 vs. .27 [95% CI, 0.35 to 0.60 vs. −0.11 to 0.65]).

Predictive utility of mastitis indicators for HIV-1 RNA shedding. MTCT has been associated with the presence of detectable HIV-1 RNA in breast milk as well as with the quantity of milk HIV-1 RNA [8–10, 12], with an apparent sharp increase in transmission rate observed at levels ≥10⁴ copies/mL [10]. Therefore, breast-milk Na+ concentrations, Na+:K+, and neutrophil counts were evaluated for the ability to predict HIV-1 RNA loads in milk at ≥50 or ≥10⁴ copies/mL by ROC analysis. Neither Na+ concentrations, Na+:K+, nor neutrophil counts displayed a cutoff point with both high sensitivity and specificity for detecting HIV-1 RNA at either ≥50 or ≥10⁴ copies/mL of
lactoserum. For example, the milk neutrophil cutoff associated with 90% sensitivity for the detection of HIV-1 RNA loads $\geq 10^4$ copies/mL had a specificity of only 42%, with a corresponding false-positive rate of 58% (table 2).

**DISCUSSION**

Our hypothesis—that the elevated leukocyte counts [23, 32] or electrolyte concentrations (Na$^+$ and Na$^+$/K$^+$) [8, 13–15, 19–21] associated with mastitis could serve as simple, inexpensive indicators of HIV-1 levels in breast milk—was based on associations in previously studied populations [8, 13–15]. Despite modest correlations, these indicators of breast inflammation did not adequately predict HIV-1 loads in the breast milk of individual Zimbabwean women. When cutoffs were chosen that would identify breast milk at high risk for transmission on the basis of the number of HIV-1 RNA copies [8–10, 12], there was an unacceptable rate of false-positive results for each parameter. The utility of these indicators is further limited in that none correlated with the HIV-1 DNA load in breast milk.

HIV-1–infected cells may play an important role in MTCT, especially early in life, when the infant consumes colostrum with high cell counts [11]. Although HIV-1 DNA in milk has been associated with MTCT [7, 11, 12], it has not previously been related to markers of breast inflammation. The observation that HIV-1 DNA loads were similar in milk regardless of the presence of mastitis suggests that inflammation may allow free virus to pass into milk from plasma through leaky epithelia. However, these findings are also consistent with the possibility of increased viral replication within the breast due to inflammation. If, for example, the rate of infected cell turnover increases as productive infection results in cell lysis, then the number and concentration of HIV-1–infected cells could appear to be similar to those in milk without mastitis.

A greater understanding of the biological determinants of HIV-1 concentrations in breast milk could assist in developing and evaluating interventions to reduce MTCT. Administration of highly active antiretroviral therapy (HAART) to breast-feeding women may curb MTCT by reducing virus in milk or by passing prophylactic levels of antiretrovirals to the infant [35–37]. Importantly, the HIV-1 DNA load in breast milk has not been observed to decrease during HAART [36]. Thus, without prophylactic levels of antiretrovirals, infectious cells could pose a risk to breast-feeding infants.

Identifying mothers at high risk of HIV-1 transmission via breast milk could assist MTCT programs in the cost-effective delivery of interventions. In the women we studied, the simple and inexpensive tests most commonly associated with breast inflammation (i.e., milk Na$^+$ concentration, Na$^+$/K$^+$, and cell counts) did not predict the HIV-1 RNA or DNA load in breast milk adequately enough to be useful for the management of individual women. The poor predictive value of these parameters alludes to the likely interplay of multiple factors in determining virus loads in milk. Additional studies to unravel the determinants of both breast-milk infectivity and infant susceptibility to HIV-1 [16, 38, 39] should lead to more-effective MTCT prevention strategies while promoting infant health through breast-feeding.

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