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TaqMan RT-PCR and VERSANT® HIV-1 RNA 3.0 (bDNA) assay Quantification of HIV-1 RNA viral load in breast milk

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Abstract

Background: Transmission of HIV via breast milk is a primary cause of pediatric HIV infection in developing countries. Reliable methods to detect breast milk viral load are important.

Objective: To correlate the ability of the VERSANT HIV 3.0 (bDNA) assay to real-time (RT) TaqMan PCR in quantifying breast milk HIV-1 RNA.

Study design: Forty-six breast milk samples that had been spiked with cell-free HIV-1 and eight samples spiked with cell-associated HIV-1 were assayed for HIV-1 RNA by both VERSANT HIV 3.0 and TaqMan RNA assays.

Results: Only assays on the cell-free samples were statistically compared. Both a Deming regression slope and a Bland–Altman slope indicated a linear relationship between the two assays. TaqMan quantitations were on average 2.6 times higher than those of HIV 3.0. A linear relationship was observed between serial dilutions of spiked cell-free HIV-1 and both the VERSANT HIV 3.0 and the TaqMan RNA assays.

Conclusion: The two methods correlated well although the VERSANT HIV 3.0 research protocol quantified HIV-1 RNA slightly lower than TaqMan.

Keywords: Breast milk viral load; Mother-to-child transmission; bDNA; TaqMan; HIV

1. Introduction

A major route of mother-to-child transmission of HIV is postnatal infection due to breastfeeding (Dunn et al., 1992). In the absence of antiretroviral therapy, it is estimated that one-third to one-half of HIV positive infants in sub-Saharan Africa acquired HIV via breastfeeding (De Cock et al., 2000). Studies have shown that elevated levels of HIV-1 RNA in breast milk are closely linked to an increased risk of transmitting HIV-1 infection (Richardson et al., 2003; Semba et al., 1999); data suggest that for each 10-fold reduction in breast milk viral load, a 2-fold reduction in transmission is observed (Rousseau et al., 2003). Thus, accurate quantification

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of HIV-1 RNA viral load may be critical for guiding appropriate prevention efforts. The VERSANT® HIV-1 RNA 3.0 (bDNA) assay, an in vitro diagnostic test to aid in the management of individuals infected with HIV-1, has shown greater reliability than the Amplicor™ 1.5 for quantifying HIV-1 in human plasma but was shown to be less sensitive than real-time (RT) TaqMan™ PCR for quantitation of SIV RNA (Elbeik et al., 2002; Leutenegger et al., 2001). Although previous data show that Roche Amplicor 1.0 has excellent correlation between input nominal HIV-1 breast milk RNA copy number and number of copies detected (Ghosh et al., 2003), comparative data on more recent HIV RNA assays of breast milk samples are limited. The aim of the present study was to correlate the ability of the VERSANT HIV 3.0 assay to RT TaqMan PCR in detecting breast milk HIV-1 RNA.

2. Materials and methods

Human breast milk samples were spiked with 1×10^1 to 1×10^8 clade B cell-free HIV-1, or 1×10^1 to 1×10^5 HIV-1-infected cells/mL. We used a modified VERSANT HIV 3.0 plasma assay (Bayer HealthCare, Diagnostics Division) protocol and compared these detection results to those obtained using RT TaqMan PCR. Unspiked breast milk samples were included as controls. As heat treatment is currently being evaluated as a means to neutralize HIV-1 in breast milk samples, both spiked and unspiked heat-treated samples were included in the analysis.

Breast milk samples spiked with either cell-free HIV-1 or cell-associated HIV-1 and unspiked breast milk samples were assayed for HIV-1 RNA by both VERSANT HIV 3.0 and TaqMan RNA assays. The VERSANT HIV 3.0 assay for viral load quantification was performed according to the manufacturer's instructions and has been described elsewhere (Gleaves et al., 2002), with the exception that centrifugation of 1 mL of sample to obtain the pellet was not used. In order to remove the non-specific materials in breast milk samples, in this protocol the target capture reagent from the VERSANT HCV RNA Qualitative Assay (TMA) (Transcription-Mediated Amplification) (Bayer HealthCare, Diagnostics Division) was used to isolate HIV RNA. Virus was lysed, viral RNA stabilized and nucleases inactivated. The reagent contained magnetic beads bound with HIV- and HCV-specific oligonucleotides complementary to regions on the HIV and HCV RNA genomes (the beads are a component of the Procleix HIV-1/HCV assay, distributed by Chiron Corporation). These beads hybridized and captured the HIV RNA target sequence in the sample. For the cell-free samples, 200–1000 μL of sample was added directly to the Test Tube Unit (TTU used in the TMA assay). If these results came above the upper limit of detection then 5 and 10 μL were tested and this data was used to calculate the correct final result; for the cell-associated samples (received as "pellets" suspended in PBS), 400 μL of the target capture reagent was added to the sample tube, mixed, and added to the TTU. The samples in the TTUs were processed through the lysis step of the TMA procedure. Captured hybrids were separated with a magnetic field. Cell debris, plasma, proteins and extraneous nucleic acids were removed by washing. Next, the captured hybrids were tested for HIV-1 RNA using the VERSANT HIV 3.0 assay. The dynamic range of the VERSANT HIV 3.0 (bDNA) assay as marketed outside the U.S. is 50–500,000 copies/mL. In the U.S., the range of the FDA-approved version of this assay is 75–500,000 copies/mL.

RT TaqMan PCR oligonucleotides were based on HIV subtype B gag gene sequences (gag sequences, all 5′–3′, were: HIVB-579f, ACATCAAGCAGCCATGCAAAT; HIVB-682r, TCTGGCCTGGTGCAATAGG; HIVB-612p, CTATCCCATTCTGCAGCTTCCTCATTGATG).

RNA was extracted from 140 μL of aqueous supernatant using the Viral RNA Kit (Qiagen, Valencia, CA). Genomic DNA was extracted from cell pellets using the Tissue DNA Kit (Qiagen) according to the manufacturer's recommendation. The method has been described elsewhere (Leutenegger et al., 2001). The dynamic range of the RT TaqMan PCR is 50–5,000,000,000 copies/mL. All PCR reactions were carried out in a 7700 ABI Prism Sequence Detector (Applied Biosystems).

Eight of the unspiked samples, and 20 each of the cell-free and cell-associated HIV-1 spiked samples had undergone simple heat treatment as a means to inactivate HIV-1 in breast milk. The heating methods have been described elsewhere (Israel-Ballard et al., 2004).

Linearity between HIV 3.0 and TaqMan was evaluated using the Deming regression procedure (Strike, 1991). Deming regression is similar to least-squares regression except that it accounts for the imprecision inherent in both variables. A 95% confidence interval (CI) for slope that did not contain 1.0 indicated a non-linear relationship between assays.

Assays were also compared using the Bland–Altman procedure (Bland and Altman, 1986). For each sample, the log difference between respective quantitations was plotted against the log mean of the two assays. The 95% confidence intervals for slope and mean log difference were calculated. Statistical significance was demonstrated if the confidence interval did not contain zero. All statistical analyses were performed using SAS, Version 8.02, SAS Institute, Cary, NC.

Linearity of quantitation for each method compared to the serial dilution was assessed by least-squares regression of observed log-quantitation versus log of the dilution factor. A 95% confidence interval for slope that did not contain 1.0 indicated that the assay was non-linear with respect to dilution.

Use of human subjects was approved by the required institutional review boards and informed consent was obtained from all volunteer study participants.

3. Results

Results were obtained for 134 breast milk samples, but statistical analyses were performed on only those samples with

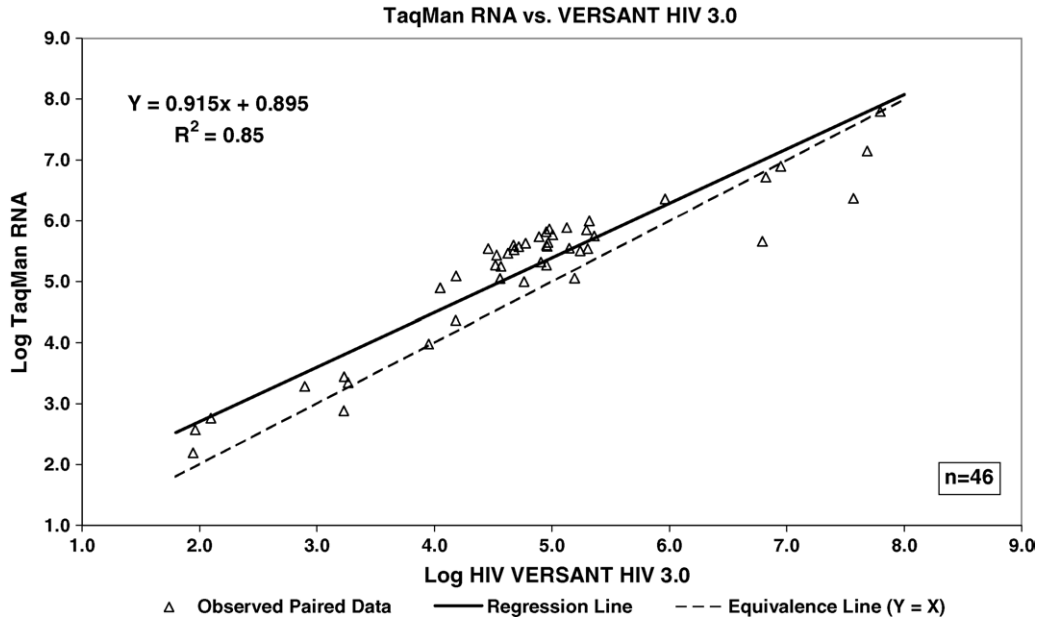

Fig. 1. Deming regression slope, which supports a linear relationship between VERSANT HIV 3.0 and TaqMan.

quantitations from both VERSANT HIV 3.0 and TaqMan RNA assays and whose values were within the dynamic range of the assays. A total of 46 cell-free spiked samples (of which 20 had been heated) were included in the analysis. Based on the limited cell-associated HIV-1 sample size, we evaluated the relationship between VERSANT HIV 3.0 and TaqMan only for cell-free HIV-1 RNA in breast milk. The Deming regression slope was 0.915 (95% CI: 0.782, 1.008), which was not significantly different from 1.0 ($p = 0.07$). This supports a linear relationship between VERSANT HIV 3.0 and TaqMan, although a slope of 1.0 would be expected for perfect agreement (Fig. 1). The Bland–Altman slope was −0.106 (95% CI: −0.227, 0.014), which was not significantly different from zero ($p = 0.08$). Likewise, a linear relationship between the assays is supported. The mean log difference of 0.409 (95% CI: 0.259, 0.559) was significantly different from zero ($p < 0.0001$) indicating that TaqMan quantitations are on average 2.6 times higher (95% CI: 1.8, 3.6) than those of HIV 3.0 within the range of values tested (Fig. 2).

The sample size of 46-paired observations provides 90% power to detect a mean difference of ±0.25 logs (1.8-fold),

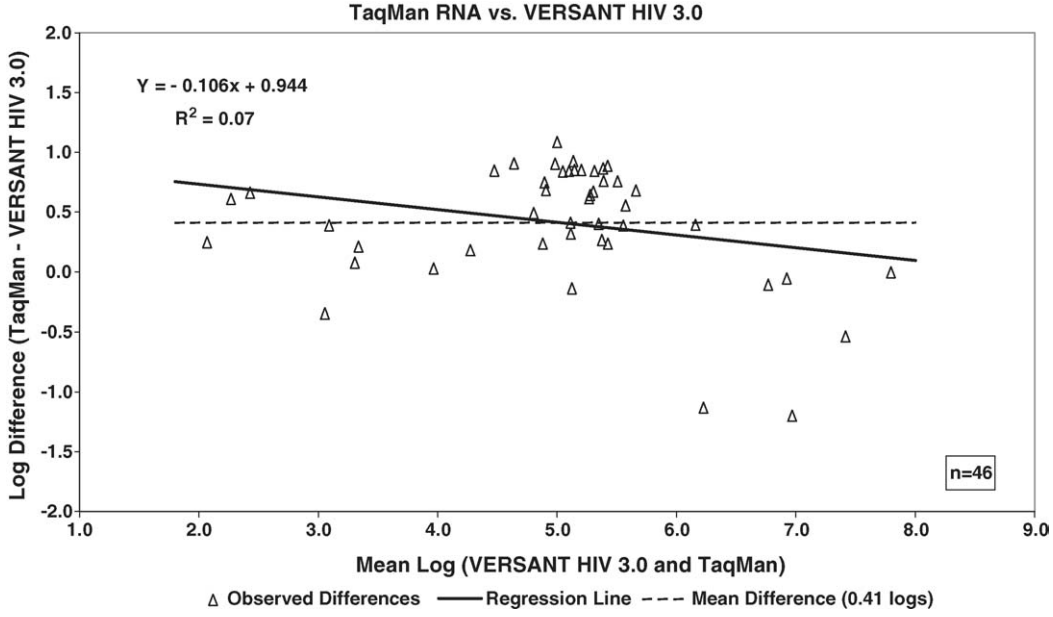

Fig. 2. Bland–Altman slope, which indicates a linear relationship and that TaqMan™ quantitations were higher.

assuming the observed standard deviation of the difference (0.51 logs) and a two-tailed alpha level of 0.05. This same sample size provides 80% power to detect a slope difference of ±0.16 relative to 1.0, assuming the observed standard error of the slope (0.056) and a two-tailed alpha of 0.05.

Analysis of linearity with respect to dilution was restricted to unheated, cell-free samples that fell within the range of the respective assay and for which the dilution factor was known. A total of 26 HIV 3.0 and 28 TaqMan samples were available for this analysis. Both HIV 3.0 and TaqMan quantitations were linear with respect to dilution, as neither slope was significantly different from 1.0. The slope of log quantitation versus log dilution factor was 1.063 for VERSANT HIV 3.0 ($p = 0.15$; 95% CI: 0.976, 1.150) and 0.937 for RT TaqMan $p = 0.20$; 95% CI: 0.836, 1.037).

In addition, assays were performed on eight samples spiked with cell-associated HIV-1, which had quantitations from both VERSANT HIV 3.0 and TaqMan RNA assays and whose values were within the dynamic range of the assays. Mean log values of detected cell-associated RNA copies/mL were 4.59 (S.D. 0.518) and 5.30 (S.D. 0.524) for VERSANT HIV 3.0 and RT TaqMan, respectively.

Samples from an HIV-1 negative panel were assayed as well, with 14/14 (100%) whole breast milk samples testing negative by both methods and 7/8 (88%) heat-treated samples showed negative by VERSANT HIV 3.0 (<75 copies/mL) while 8/8 (100%) heat-treated samples tested negative by RT TaqMan PCR (<50 copies/mL). As VERSANT HIV 3.0 does not detect non-denatured dsDNA, no effort was made to detect proviral DNA for comparison to TaqMan HIV-1 DNA results.

4. Discussion

This study showed that both the VERSANT HIV 3.0 and RT TaqMan PCR assay methods for detecting HIV-1 RNA in breast milk correlated well, although TaqMan quantitations were approximately 0.41 logs (2.6-fold) higher than VERSANT HIV 3.0. While 1/8 (12%) of the heat-treated negative samples in this study tested positive by VERSANT 3.0, we are unable to draw a conclusion regarding false-positives due to the small sample size and acknowledge the manufacturer's data indicating a specificity of 97.6% (#127418 VERSANT® HIV-1 RNA 3.0 Package Insert). HIV-1 RNA was isolated from breast milk using the target capture reagents and the protocol from the HCV TMA Qualitative assay prior to quantification using the VERSANT HIV 3.0 assay. This extraction alternative linked to VERSANT HIV 3.0 reduced background and offered a simple, sensitive, and specific method to quantify HIV-1 RNA in human breast milk. These findings indicate that, in addition to RT TaqMan PCR, this research protocol using VERSANT HIV 3.0 may prove useful to reliably assay for HIV-1 in breast milk.

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