Use of an HIV-1 reverse-transcriptase enzyme-activity assay to measure HIV-1 viral load as a potential alternative to nucleic acid-based assay for monitoring antiretroviral therapy in resource-limited settings

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An inexpensive and technically less-demanding methodology to quantify HIV-1 viral load would be of great value for resource-limited settings, where the nucleic-acid amplification technique (NAAT) is impractical and/or resource-prohibitive. In this study, an HIV-1 reverse-transcriptase enzyme-activity assay (ExaVir Load assay, version 1) was compared with the gold standard RT-PCR assay, Roche HIV-1 Amplicor Monitor, version 1.5. A total of 121 plasma specimens were used for the evaluation. ExaVir Load had a sensitivity of 97 % and a specificity of 71 % in identifying specimens with <400 copies ml^{-1} in the Roche RT-PCR assay as being less than the detection limit of the assay (5500 copies ml^{-1}). The mean difference (95 % limits of agreement) between Roche RT-PCR and ExaVir Load was -0.23 (-1.59 to 1.13) $\log_{10}(\text{copies } \text{ml}^{-1})$ by Bland–Altman analysis. Significant negative correlations were seen between CD4^{+} T-cell counts and the ExaVir Load assay ($r = -0.32$, $P < 0.05$), and between CD4^{+} T-cell counts and the Roche RT-PCR ($r = -0.38$, $P < 0.01$). The present study with HIV-1 showed a strong correlation between the ExaVir Load assay and the RT-PCR assay. Hence, the ExaVir Load assay could be considered for use in resource-limited settings as an alternative viral-load assay to the standard NAAT-based assay after further evaluation with prospective specimens.

Received 18 June 2007

Accepted 27 August 2007

INTRODUCTION

The management of HIV-infected patients is based mainly on CD4^{+} T-lymphocyte cell counts and HIV plasma viral load (PVL), with increasing emphasis on PVL monitoring of responses to antiretroviral therapy (ART) (Braun *et al.*, 2003; Sivapalasingam *et al.*, 2005). The routinely used PVL assays are based on amplification of virion RNA (nucleic-acid amplification technique; NAAT), and as a result require infrastructure facilities for PCR, complex equipment and skilled technicians, and are generally expensive. An inexpensive and technically less-demanding methodology to quantify HIV-1 would be of great value for settings where nucleic acid-based assays are impractical and/or resource-prohibitive (Sivapalasingam *et al.*, 2005).

Abbreviations: ART, antiretroviral therapy; IQR, interquartile range; NAAT, nucleic-acid amplification technique; PVL, plasma viral load, RT, reverse transcriptase.

Recent studies have shown a positive correlation between the standard RNA-based assays and the ExaVir Load assay. A boosted p24 antigen assay that uses heat dissociation to allow quantification of HIV-1 p24 antigen has been investigated (Jennings *et al.*, 2005; Stevens *et al.*, 2005) but is currently recommended by the company for paediatric diagnosis rather than monitoring PVL. An assay that quantifies virion-associated reverse-transcriptase (RT) enzyme activity (Malmsten *et al.*, 2003; Jennings *et al.*, 2005; Braun *et al.*, 2003; Stevens *et al.*, 2005; Sivapalasingam *et al.*, 2005; Shao *et al.*, 2003) (Cavidi ExaVir Load) has shown sensitivity and reproducibility comparable to those of RNA PCR-based PVL assays in clinical monitoring of patients undergoing therapy. However, most of these studies have been carried out with HIV-1 subtype B, although worldwide patients are predominantly infected with subtype C (Osmanov *et al.*, 2002). The objective of the present study was to compare

the performance of the ExaVir Load (version 1) RT assay for HIV PVL determination with a standard RT-PCR assay, Roche Amplicor HIV-1 Monitor (version 1.5).

METHODS

Setting. The YRG Centre for AIDS Research and Education (YRG CARE), Chennai, is the largest community-based tertiary HIV care institution in India. Since 1996, it has provided a continuum of care for more than 10 000 HIV-infected individuals. This study was done using plasma samples from patients who were requested for HIV-1 viral load by clinicians during the period between February 2002 and September 2004. Whole blood was collected in a closed system of collection using K3 EDTA tubes (Becton Dickinson).

Viral-load assays. The plasma HIV-1 RNA levels of the patients were determined by RT-PCR using the Amplicor HIV-1 Monitor version 1.5 assay with a lower limit of detection of 400 copies ml⁻¹ (Roche Diagnostics), and the remaining plasma was stored at -70 °C. Our laboratory participates in external quality-assessment programs for HIV-1 RNA PCR (Roche COBAS Amplicor HIV-1 Monitor Assay, version 1.5) and has been certified by the Virology Quality Assessment (VQA) program, Rush University, Chicago, IL, USA, from 2002 to the present. The remaining stored samples were processed for HIV-1 RT activity using the ExaVir Load kit, version 1.0 (Cavidi Tech), as per the instructions of the manufacturer. In brief, the assay uses HIV RT enzyme purified from plasma specimens to catalyse the conversion of RNA to cDNA. Using a virion-binding gel, the virus particles are purified from the plasma and bound virions are washed to remove inhibitors, including plasma antibodies and inhibitor drugs used for ART. Virions are then lysed and lysates transferred to a 96-well plate for assay of RT activity. In an overnight incubation, RT enzyme in the lysate incorporates 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdUTP) into a DNA strand complementary to the poly-A template (bound to the wells). Subsequently, an anti-BrdU antibody conjugated to alkaline phosphatase is added and the amount of incorporated BrdU detected using a substrate. The colour intensity with respect to RT activity in each well is read using a standard ELISA plate reader (Multiskan Ascent, Labsystems) at 405 nm with a reference at 620 nm. Results are compared to a standard curve, and HIV RT activity is determined and expressed as fg (ml plasma)⁻¹ and converted to HIV RNA copies ml⁻¹ equivalent using the conversion factor of 210 provided by the manufacturer.

Flow cytometry for CD4 cell enumeration. CD4⁺ T-lymphocyte enumeration was done for each specimen with a two-colour, single-platform flow cytometer (FACSCount, Becton Dickinson), as per the manufacturer's instructions. Our laboratory has established ongoing participation in a quality-control program with the UK National External Quality Assessment Service (UK-NEQAS) for leukocyte immunophenotyping, and has been certified for flow cytometry since 2001.

Statistical analysis. A normality check was performed, and the PVL data were seen to follow a non-normal distribution and were hence transformed on a logarithmic scale (log₁₀) for statistical analysis. The descriptive statistics of mean, median and interquartile range (IQR) were used to describe the study samples. Bland-Altman analysis (Bland & Altman 1986) was performed to examine the level of agreement between the ExaVir Load and Roche RT-PCR assays. Bland-Altman analysis included only the samples with results within the limits of the assay detection ($n=44$). Those samples beyond the detection limits [<400 and $>750\,000$ copies ml⁻¹ in Roche RT-PCR, and <5500 equivalent copies ml⁻¹ in the ExaVir Load assay ($n=77$)] were excluded in the Bland-Altman analysis. Sensitivity and

specificity were determined to study the agreement between the assays at the RT-PCR limit of detection (<400 copies ml⁻¹). The correlation between CD4⁺ T-cell absolute counts and ExaVir Load count was analysed using Pearson's correlation coefficient. All statistical analysis was done using SPSS software, version 13.0. $P<0.05$ was considered statistically significant.

RESULTS

Of the 121 samples, 83 (69%) were from males, and the mean age of the study population was 36 years. Thirty-two (27%) were drug-naïve and 89 (74%) were on ART. The median (IQR) CD4 count of the samples was 238 (155–455) cells mm³. Using the Roche RT-PCR assay, a total of 41 (34%) patients had a PVL of <400 copies ml⁻¹. The lower detection limit of the ExaVir Load assay was found to be <5500 copies ml⁻¹. In the ExaVir Load assay, 63 (52%) individuals had a PVL of <5500 copies ml⁻¹. The ExaVir Load assay had a sensitivity of 97% and a specificity of 71% in identifying specimens with <400 copies ml⁻¹ in the Roche RT-PCR assay as being less than the detection limit of the assay (5500 copies ml⁻¹; Table 1). Twenty-three specimens (28%) were detected as having <5500 copies ml⁻¹ in the ExaVir Load assay, and they had an HIV viral load of >400 copies ml⁻¹ in the standard PCR assay (median 2500; range 620–4710). The mean difference (95% limits of agreement) between Roche RT-PCR and ExaVir Load was -0.23 (-1.59 to 1.13) log₁₀(copies ml⁻¹) by Bland-Altman analysis (Fig. 1). Significant negative correlations were seen between CD4⁺ T-cell counts and the ExaVir Load assay ($r=-0.32$, $P<0.05$), and between CD4⁺ T-cell counts and Roche RT-PCR ($r=-0.38$, $P<0.01$).

DISCUSSION

The present study of patients living in south India, where it is evident that HIV-1 subtype C is prevalent (Osmanov *et al.*, 2002), has shown a strong correlation between the ExaVir Load RT assay and the standard Roche RT-PCR assay to measure PVL. The study includes samples from patients for whom PVL was measured before and after the initiation of ART. It is estimated globally that $>50\%$ of HIV infection is due to subtype C (Osmanov *et al.*, 2002); evaluations of PVL assays using subtype C-infected individuals are helpful for resource-poor settings, including India and African countries. This is believed to be the first evaluation report of the ExaVir Load assay from India, where HIV-1 subtype C is prevalent. Earlier studies, mainly with subtype B, have also shown a good correlation between the ExaVir Load assay and standard NAAT-based assays (Jennings *et al.*, 2005; Braun *et al.*, 2003).

The Bland-Altman analysis of our data has shown that the two methods have a high level of agreement and can be used interchangeably. The bias of -0.23 log₁₀(copies ml⁻¹) may reflect the fact that the ExaVir Load assay overestimates PVL slightly more than the RT-PCR assay. This

Fig. 1. Bland-Altman plot of ExaVir Load assay results and those of the standard PCR, using a transformed logarithmic scale ($n=44$). Assay results are in $\log_{10}(\text{copies ml}^{-1})$. The solid line indicates the bias and the dotted lines show 95 % limits of agreement.

may be due to the fact that ExaVir Load quantitates the RT enzyme rather than the viral genome. However, for a clinical decision, these minor differences between the two assays may not be significant. In the ExaVir Load assay, the lower detection limit differs in each run, depending on the standard curve of each run. We have selected <5500 copies ml^{-1} for our analysis, as it was the highest lower detection limit seen in five different runs. However, the ExaVir Load assay has greater sensitivity (97 %) than that of the RT-PCR assay (<400 copies ml^{-1}) to correctly identify PVL values. The ExaVir Load assay has reduced specificity in detecting specimens with >400 copies ml^{-1} compared with the standard PCR assay. Since the ExaVir Load assay has different detection limits in different runs, some specimens may have been assigned different results. These specimens may be of clinical importance, since virological failure is defined as any detectable amount of viral load (>400 copies ml^{-1}) in two consecutive specimens within the interval of 1 month. However, these considerations may be superseded by the newly introduced ExaVir Load assay version 2.0, which has a lower detection limit of <400 copies ml^{-1} .

In other reports evaluating the ExaVir Load assay, a good correlation has been reported with a standard RT-PCR assay in patients infected with HIV-1 subtype B strains (Jennings *et al.*, 2005), with CRF02_AG strains (Sivapalasingam *et al.*, 2005) and other non-subtype B viruses, including subtype C (Braun *et al.*, 2003; Stevens *et al.*, 2005). A recent study observed 100 % specificity with the ExaVir Load RT assay using HIV-seronegative samples (Jennings *et al.*, 2005).

The advantages of the ExaVir Load assay over the standard RT-PCR assay are: (1) it is simple to perform; (2) it is

inexpensive ($< \$20$ US); and (3) it does not require sophisticated infrastructure facilities and expensive equipment. There is another important feature of the ExaVir Load assay, in that it is an ELISA-based assay and can utilize the same equipment and technical staff who perform routine antibody tests for HIV diagnosis. The disadvantages of the ExaVir Load assay are: (1) lengthy incubation procedures that take the assay time to more than 2 days (although the actual amount of 'hands-on' labour is similar on a per-test basis to that of the Roche RT-PCR); (2) a requirement for 1 ml of plasma, which is difficult for paediatric patients; and (3) a lack of standard positive and negative controls.

To set up the ExaVir Load assay, a start-up kit is available ($\sim \$2500$ US), which includes a sample box and lid, four 3 l wash buckets, a vacuum pump, plastic bottles for buffer, a waste collector, a sample collector, a column holder and a rack for holding tubes. An ELISA reader, a mechanical rocker, an incubator, pipettes and a -20°C refrigerator are also needed to perform the assay, and these are not provided in the kit. The ExaVir Load assay is also provided with a preprogrammed Excel spreadsheet, which can give copies ml^{-1} values upon entering the optical density obtained.

In the present study, a negative correlation was seen between plasma RNA levels and the CD4^+ T-cell count of the study population; however, this is not universal, as some individuals with a high CD4^+ T-cell count may have a high PVL and vice versa (Kannangai *et al.*, 2001; García *et al.*, 1997; Ledergerber *et al.*, 2000). Although the absolute CD4^+ T-cell count is widely used as an HIV disease progression marker for treatment initiation and therapy monitoring, the PVL has been shown to be the best surrogate marker for monitoring ART (Braun *et al.*, 2003; Sivapalasingam *et al.*, 2005). In this study, the ExaVir Load assay was found to reflect decreases in PVL that occurred in patients who commenced ART.

Table 1. Performance characteristics of the ExaVir Load assay to identify viral copies below and above the detection limit (400 copies ml^{-1}) of the standard PCR assay

ExaVir Load result (copies ml^{-1})	Standard PCR result (copies ml^{-1})	
	<400	>400
<5500	40*	23
>5500	1	57
ExaVir Load performance characteristic		
	Sensitivity (%)	Specificity (%)
	97	71

*Values show the number of samples in each category.

Considering the fact that globally the most affected individuals with HIV infection are from poor families (Solomon *et al.*, 2006a), ART monitoring with the standard RT-PCR is unaffordable for management of their disease (Cecelia *et al.*, 2006; Solomon *et al.*, 2006b). Hence, there is an urgent need to assess and introduce lower-cost assays for monitoring PVL. This is particularly so since ART has become increasingly available to larger numbers of patients worldwide through the reduced prices of generic drugs and access to global programmes such as the UNAIDS initiative. The newly introduced, ultrasensitive ExaVir Load RT assay version 2.0 is about seven times more sensitive than version 1.0. Hence, this RT assay could be considered for ART monitoring in resource-limited settings after further evaluation using longitudinal samples from patients who are on different common drug regimens. To conclude, the ExaVir Load assay will be highly useful for the low-cost monitoring of blood viraemia levels in resource-limited settings.

ACKNOWLEDGEMENTS

Dr E. M. Shankar's help with this manuscript is gratefully acknowledged. We also thank the laboratory staff of YRG CARE for helping in the completion of this work.

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