Published in final edited form as:
J Clin Virol. 2010 January ; 47(1): 18. doi:10.1016/j.jcv.2009.10.001.

HIV-1 Viral Load and Phenotypic Antiretroviral Drug Resistance Assays Based on Reverse Transcriptase Activity in Comparison to Amplification Based HIV-1 RNA and Genotypic Assays

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Abstract

Background—Amplification based HIV-1 viral load and genotypic resistance assays are expensive, technologically complex and may be difficult to implement in resource limited settings. Inexpensive, simpler assays are urgently needed.

Objectives—To determine the suitability of the ExaVir™ Load and ExaVir™ Drug assays for use in patient monitoring.

Study Design—Specimens from 108 adults were used to compare ExaVir™ Load HIV-1 RT to Amplicor HIV-1 Monitor® HIV-1 RNA, and ExaVir™ Drug phenotype to HIV GenoSure™ genotype.

Results—HIV-1 RT and HIV-1 RNA levels were comparable (Pearson correlation coefficient 0.83). Most (94%) had detectable results in both assays. The mean difference (HIV-1 RT minus HIV-1 RNA) was -0.21 $\log_{10}$ cps/mL equivalents. Relationship between HIV-1 RT and HIV-1 RNA was not affected by RT mutations, CD4 cell count, or efavirenz (EFV) or nevirapine (NVP) use. Phenotypes were generally consistent with genotype findings for EFV, but not for NVP. Most patients (93.9%) with phenotypic EFV resistance had at least one EFV mutation, while 78.0% of patients with phenotypic NVP resistance had at least one NVP mutation. Eleven of 49 samples tested for EFV susceptibility were found resistant (n=2) or with reduced susceptibility (n=9) despite the absence of genotypic resistance. Eleven of 45 samples tested for NVP susceptibility were found resistant (n=9) or with reduced susceptibility (n=2) with no evidence of genotypic mutations.

Conclusions—The ExaVir™ Load assay performed well and may be an alternative to amplification based techniques for HIV-1 RNA quantification. The ExaVir™ Drug assay for phenotypic resistance testing requires further evaluation, especially for NVP.

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Presented in part at the 13th Conference on Retroviruses and Opportunistic Infections, Denver, CO, February 5-8, 2006, and the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 3-6, 2008.

Keywords

Cavidi; HIV-1; phenotype assay; genotype assay; viral load

Background

Access to antiretroviral drugs is rapidly expanding around the globe; however, issues of cost and complexity have limited access to monitoring tools such as HIV-1 viral loads, CD4 cell counts, and genotypic and phenotypic resistance assays.[1] Prompt detection of virologic failure is essential for preventing further evolution of antiretroviral drug resistant HIV isolates and preserving future treatment options.[2, 3] High rates of antiretroviral drug resistance emerging on therapy have already been documented in countries with antiretroviral availability but without available laboratory monitoring.[4-7] Inexpensive, technologically simpler assays are therefore urgently needed.

Objectives

One lower cost, technologically simpler assay is the ExaVir™ Load assay which measures the reverse transcriptase (RT) activity in plasma.[8, 9] Once the RT enzyme has been isolated and quantitated, its ability to function in the presence of non-nucleoside RT inhibitors and thymidine analog nucleoside RT inhibitors can also be determined, thereby measuring susceptibility or resistance of the virus to certain antiretroviral drugs. We evaluated the performance characteristics of the ExaVir™ Load and ExaVir™ Drug assays and compared these assay results to those of HIV-1 RNA RT-PCR quantitation and HIV-1 sequencing analysis, respectively to determine the suitability of these assays for use in patient monitoring in resource limited settings.

Study Design

HIV-1 Viral Load Assays

The ExaVir™ Load assay, version 2.0 (Cavidi Tech AB, Uppsala, Sweden) was performed, following manufacturers' package inserts, on EDTA plasma stored at -80°C and frozen/thawed once, from a random sample of adults with clade B HIV-1, participating in the UNC CFAR HIV Clinical Cohort Study (n=108).[10] Patient samples were eligible to be randomly selected if a sample was available on a day where: (1) an HIV-1 RNA was performed and was greater than 1000 cps/mL; and (2) an HIV-1 genotype was also performed. Results were reported as fentograms RT per milliliter (fg/mL) and converted to HIV-1 RNA copies per milliliter equivalents (cps/mL eqs) using the ExaVir™ Load Analyzer version 1.62 These results were compared to HIV RNA levels (Amplicor HIV-1 Monitor® Test, version 1.5, Roche Diagnostics, Branchburg, NJ, USA).

HIV Drug Resistance Assays

The ExaVir™ Drug assay (ExaVir™ Drug assay, version 1.0, Cavidi Tech AB, Uppsala, Sweden) [11] was used to test for phenotypic resistance to efavirenz (EFV) (n=48), nevirapine (NVP) (n=37), and both EFV and NVP (n=23). The percentage of inhibition was calculated for each drug concentration as the ratio of HIV-1 RT activity with drug present to HIV-1 RT activity with drug absent (×100). Samples were considered resistant if the sample half maximal inhibitory concentration (IC_{50}) was ≥ the IC_{50} of the mutant referent, susceptible if the sample IC_{50} was < 2 times the IC_{50} of the wild type referent, and with reduced susceptibility otherwise, based on the assay provided software. The manufacturer recommends a minimum 10 fg/ml RT level for phenotypic drug susceptibility testing which is approximately 4000 cps/ml eqs (3.6

log cps/ml eqs). These phenotypic results were compared to HIV-1 genotyping (HIV GenoSure™, LabCorp, Research Triangle Park, NC, USA).

Statistical Analysis

HIV-1 RNA and HIV-1 RT levels were $\log_{10}$ transformed, and results less than the assay limit of detection were imputed at half the lower limit. Log_{10} HIV-1 RT level (cps/mL eqs) assay values were compared to the $\log_{10}$ HIV-1 RNA level (cps/mL) assay values via descriptive statistical methods, Bland-Altman analysis of agreement, Pearson's correlation coefficient (r), and linear regression methods. Multivariable linear regression was used to investigate whether the differences between HIV-1 RT and HIV-1 RNA were affected by: (1) current or prior use of EFV and NVP; (2) cumulative number of RT mutations; (3) specific RT mutation; and (4) contemporaneous CD4 cell count. The following RT mutations were considered based on the International AIDS Society – USA Panel Guidelines[12]: M41L, A62V, K65R, D67N, T69Insert, K70R, L74V, V75I, F77L, Y115F, F116Y, Q151M, M184I/V, L210W, T215F/Y, and K219E/Q; including the following EFV and/or NVP related mutations: L100I, K103N, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A/S, and P225H. All statistical computations were performed using SAS software (version 9.1, SAS Institute, Inc., Cary, NC).

Results

Plasma samples from 108 individuals with HIV-1 RNA levels >1000 cps/mL were used to compare HIV-1 RT and HIV-1 RNA assay results. Most (N=102) had detectable results in both assays, and 94 had quantifiable HIV-1 RT and HIV-1 RNA (Figure 1A). Eight samples gave measurable HIV-1 RT but HIV-1 RNA was above the assay linear range (i.e., >5.88 $\log_{10}$ cps/mL; median HIV-1 RT=5.71 $\log_{10}$ cps/mL eqs (Interquartile range [IQR]; 5.06, 5.73). In six samples HIV-1 RT was below assay limit of detection but HIV-1 RNA result was measurable (median HIV-1 RNA=3.60 $\log_{10}$ cps/mL (IQR; 3.45, 4.06).

The difference between the two assays was within 0.3, 0.5 and 1.0 $\log_{10}$ cps/mL eqs in 53.2%, 75.5% and 92.6% of samples with results on both assays (N=94), respectively. When the HIV-1 RNA result was >1000, >5000 or >10,000 cps/mL, 102 of 108 (94.4%), 82 of 84 (97.6%), and 64 of 66 (97.0%) had quantifiable HIV-1 RT results, respectively.

The means of the HIV-1 RT and HIV-1 RNA assays were 4.26 and 4.47 $\log_{10}$ cps/mL (SD=1.04, 0.86); and the medians were 4.15 and 4.30 $\log_{10}$ cps/mL eqs (IQR; 3.52, 5.21; 3.78, 5.13), respectively. The mean difference (HIV-1 RT minus HIV-1 RNA) was -0.21 $\log_{10}$ cps/mL eqs (SD=0.59) [lower and upper 95% limits of agreement=-1.36 (95% Confidence Interval [CI]; -1.55, -1.17), and 0.94 (95% CI; 0.75, 1.13)], and did not appear to be influenced by the amount of virus present (Figure 1B). The Pearson's correlation coefficient was r=0.83 [0.76, 0.88].

The difference between HIV-1 RT and HIV-1 RNA was not appreciably affected by current NNRTI (N=27), EFV (N=16), or NVP use (N=11), or prior/never NNRTI use (N=53), with mean differences of: -0.37 $\log_{10}$ cps/mL eqs (SD=0.49), -0.43 (SD=0.55), -0.27 (SD=0.40), and -0.16 (SD=0.61), respectively. All contrasts of the mean differences by NNRTI use had likelihood ratio test p-values > 0.10.).

Of the 108 patients, 83 had virus with ≥1 RT, 65 with ≥1 EFV or NVP, and 69 with ≥1 NRTI mutation. The median number of RT mutations was 3 (IQR; 1, 5). The most common mutations were M184V (N=49), K103N (N=39), T215Y (N=33), M41L (N=32), and Y181C (N=27). In multivariable analyses neither the number of RT, EFV or NVP mutations, nor any specific mutation, affected the relationship between HIV-1 RT and HIV-1 RNA. Current CD4 cell

count also did not appear to affect the difference between HIV-1 RT and HIV-1 RNA (p-value=0.35).

Phenotypic drug susceptibility was undertaken for EFV (N=71) and NVP (N=60), but was unmeasurable due to low HIV-1 RT levels (< 10 fg RT/ml) in 22 (31%) EFV and 15 (25%) NVP attempts. The median HIV-1 RT and HIV-1 RNA levels in these 37 unmeasurable samples were 3.13 and 3.76 $\log_{10}$ cps/mL eqs (IQR; 2.79, 3.65; 3.45, 4.08), respectively.

Of the 49 available EFV phenotypes, 33 were resistant, 12 had reduced susceptibility, and 4 were susceptible. Of the EFV resistant patients (N=33), all 16 with NVP phenotype data were also NVP resistant. Among the 12 patients with EFV reduced susceptibility, 7 had available NVP data and 4 were NVP resistant, 1 had NVP reduced susceptibility and 2 were NVP susceptible. None of the EFV susceptible patients had available NVP phenotypes.

In general EFV phenotype resistance findings were consistent with RT genotype results (Figure 2). Of 33 patients with phenotypes indicating EFV resistance, 31 had ≥1 EFV associated mutation. No EFV mutations were observed in patients with phenotypes indicating EFV susceptibility (N=4). However, of the 12 patients with phenotypic results indicating reduced EFV susceptibility, 9 had no evidence of EFV mutations, and 5 had never received EFV or NVP.

Of the 45 NVP phenotypes, 41 were resistant, 2 had reduced susceptibility, and 2 were susceptible (Figure 2). Nine of 41 patients with phenotypes indicating NVP resistance, and all with NVP reduced susceptibility, had no evidence of NVP mutations, or any RT mutations, including an expanded list of NNRTI mutations including etravirine associated mutations. Of these eleven patients with NVP resistance or reduced susceptibility but without corresponding NVP mutations only one had any history of EFV or NVP use.

Discussion

The required technology and equipment used in the ExaVir™ Load assay are relatively simple and inexpensive, and the output of HIV-1 RNA cps/mL equivalents offers a surrogate for HIV-1 RNA values readily interpretable to clinicians. The RT extract may also be used to obtain a susceptibility phenotype to NNRTI and thymidine analogue NRTI antiretroviral drugs, a potentially low cost alternative to genotyping.[11]

Our findings contrasting the ExaVir™ Load assay to HIV-1 RNA RT-PCR quantitation (Amplicor HIV-1 Monitor® Test) confirm prior work indicating that HIV-1 RT and HIV-1 RNA results, in general, are comparable. Our observed mean difference (HIV-1 RT minus HIV-1 RNA) of -0.21 $\log_{10}$ cps/mL (eqs), was similar to results from a cohort of patients predominantly infected with non-clade B HIV-1 virus (mean difference -0.23 $\log_{10}$ cps/mL [eqs].[13]

The Pearson correlation coefficient in this study (r=0.83, CI=[0.76, 0.88]), was only slightly lower than those reported in prior studies (ranging from r=0.85 to r=0.90).[8, 9, 14-21] Although the majority of studies have not observed differences in assay performance comparing clade B and non-clade B populations,[14] a few studies have reported weaker correlation coefficients (r=0.65 to r=0.81) in samples from patients with non-clade B HIV-1 virus.[17, 22, 23]

Among samples with HIV-1 RNA greater than 1000 cps/mL, 94% had quantifiable HIV-1 RT results. When a higher HIV-1 RNA cut-off was used a slightly greater proportion of samples also had quantifiable HIV-1 RT results (e.g., 97% of samples with HIV-1 RNA greater than 10,000 cps/mL). This observation may be clinically relevant as more complex resistance

patterns are seen in patients in resource limited settings failing initial therapy with viral loads greater than 10,000 cps/mL.[7, 24]

The binding mode of inhibition of EFV to RT is known to be much tighter than that of NRTIs or the other NNRTIs,25 raising the possibility of HIV-1 RT suppression by the presence of EFV in the sample. A prior study did not find a difference by EFV exposure, with correlation coefficients for HIV-1 RT and HIV-1 RNA among EFV users and non-users of 0.86 and 0.89, respectively.[15] In this study the correlation coefficients for current and never/prior EFV users were 0.83 and 0.87, respectively. In additional analyses contrasting the mean differences (HIV-1 RT minus HIV-1 RNA) by EFV use, differences were not statistically significant. Given the available data it appears that current EFV use has a small and possibly negligible effect on the performance of the HIV-1 RT.

Greengrass and colleagues, observed an effect of NNRTI resistance mutations on the relationship between HIV-1 RT and HIV-1 RNA suggesting a possible decrease in RT fitness. [15] Using the same analytic approach we did not find evidence that the presence of at least one EFV or NVP mutation affected the association between HIV-1 RT and HIV-1 RNA.

Only 70-75% of the samples had sufficient RT levels to perform the phenotypic assays. The EFV phenotype assay worked fairly well. Only 6% of samples determined to be phenotypically resistant to EFV did not harbor EFV associated mutations, and all of the samples determined to be phenotypically susceptible had wild type RT. However, only 25% of samples determined to have phenotypic reduced susceptibility had detectable EFV mutations. These results are in contrast to those of Basson, et al.,[26] who found that only 6.5% of subtype C specimens demonstrated phenotypic resistance in the absence of genotypic mutations. All of the specimens we tested were subtype B. Since the phenotypic reduced susceptibility category appears to have substantial misclassification problems, the overall assay performance could be improved by changing the cutoff for determining susceptibility or by only including two categories (resistant and susceptible).

The phenotypic NVP resistance findings based on the ExaVir™ Drug assay were discordant in comparison to genotyping results in a large number of patient samples. One-fifth of samples indicating NVP resistance based on phenotypic drug susceptibility testing had no NVP associated mutations. These results were not explained by NVP or EFV use, since the majority of these patients were NNRTI-naïve. Moreover, the majority of these patients did not have mutations conferring resistance to any NRTI, or to a broader list of NNRTI mutations. While a small number of discrepant results between a resistant phenotype and an apparently sensitive genotype could occur due to undetected or unrecognized mutations we believe this is an unlikely explanation for our observations given the extensive study and characterization of EFV and NVP resistance over the last 10-15 years.

Single dose NVP is the most common antiretroviral regimen used for the prevention of mother to child transmission in resource limited settings and resistance frequently develops in mothers and infected infants.[27, 28] Therefore, given the findings of this study, the ExaVir™ Drug assay needs further evaluation, especially for NVP resistance. In addition, the phenotypic assay can only be performed on NNRTIs and T-analogue NRTIs which limits its utility. On the other hand, the ExaVir™ Load assay HIV-1 RT appears comparable to HIV-1 RNA based on RT-PCR quantitation, and is not appreciably affected by NNRTI use or RT mutations.

Acknowledgments

Acknowledgements and Conflicts of Interest

Funding: This work was supported by National Institute of Allergy and Infectious Diseases (NIAID) grants AI068636 and AI069423 (AIDS Clinical Trials Group Central Grant) and AI068632 [International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT) Central Grant]. IMPAACT is also funded by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and the National Institute of Mental Health (NIMH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID, NICHD, NIMH, or NIH. This research was also supported by The University of North Carolina at Chapel Hill, Center for AIDS Research, National Institutes of Health funded program P30 AI50410. Competing interests: None declared. Ethical approval: This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

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Abbreviations

CI	Confidence intervals
Cp/mL eqs	Copies per milliliter equivalents
EFV	Efavirenz
IC_{50}	Half maximal inhibitory concentration
IQR	Interquartile range
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor

NVP	Nevirapine
RT	Reverse transcriptase
SD	Standard deviation

FIGURE 1A.

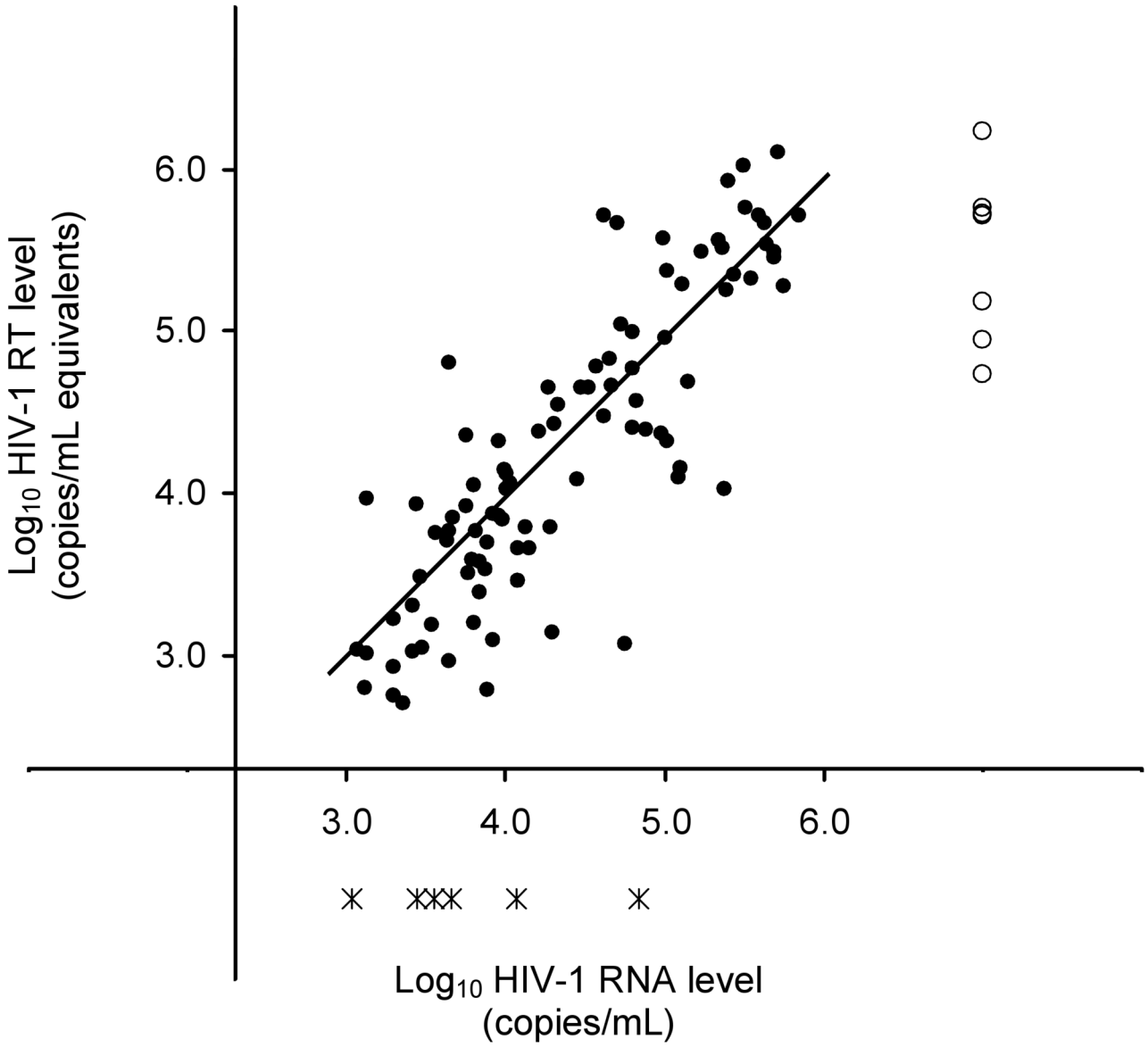

FIGURE 1B.

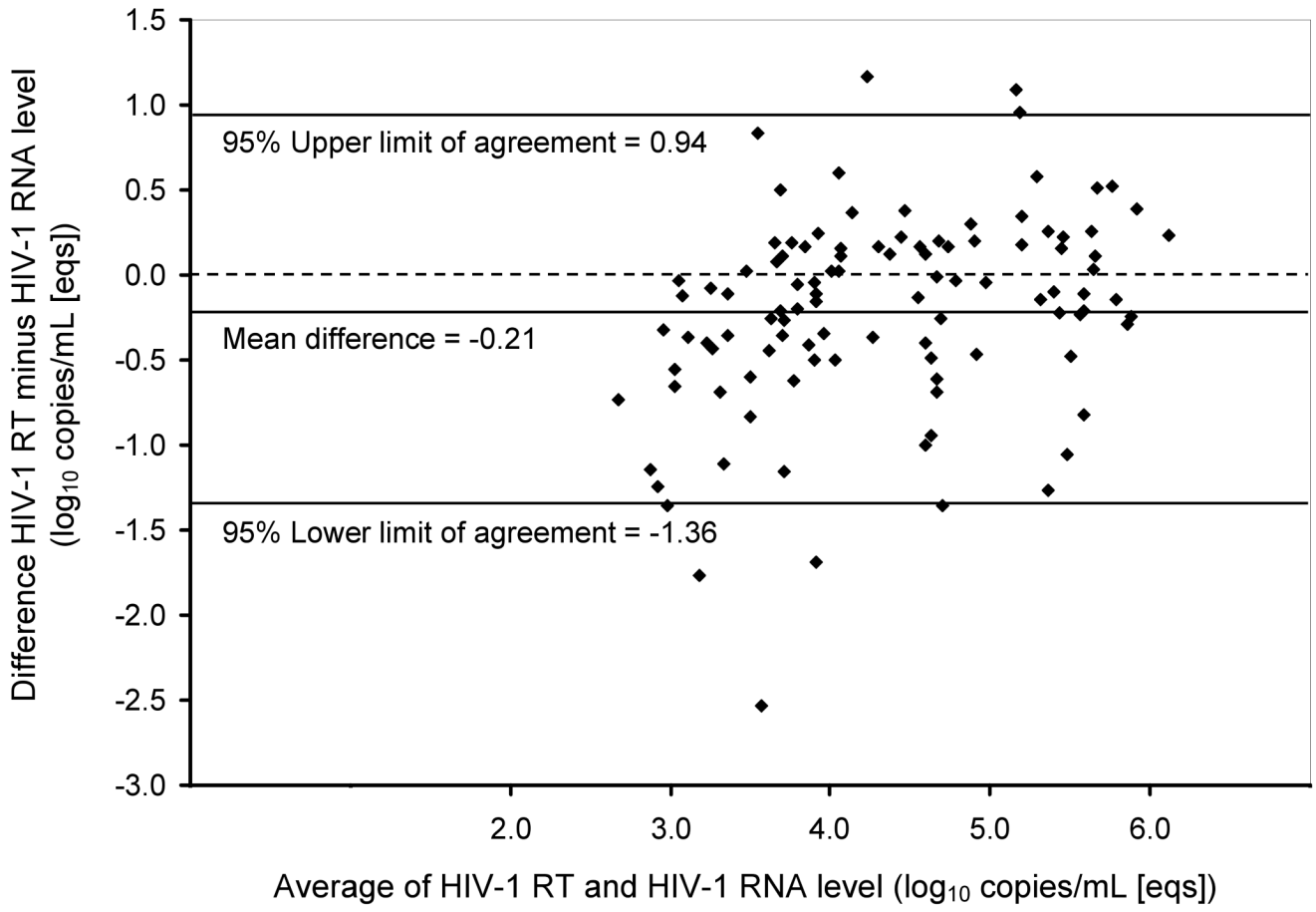

FIGURE 1.

ExaVir™ Load assay $\log_{10}$ HIV-1 RT level and Amplicor HIV-1 Monitor® Test $\log_{10}$ HIV-1 RNA level with the line of equality (A). HIV-1 RT and HIV-1 RNA measurable N=94, denoted by (●); HIV-1 RT measurable and HIV-1 RNA above the linear range of the assay N=8, denoted by (○); and HIV-1 RT below the linear range of the assay and HIV-1 RNA measurable N=6, denoted by (*). Bland-Altman difference plot of difference in HIV-1 RT minus HIV-1 RNA versus average values of HIV-1 RT and HIV-1 RNA, with mean difference and 95% limits of agreement (B).

NNRTI use	EFV Phenotype (N)	NVP Phenotype (N)	Genotype (mutations)					
			100	103	108	181	188	190
Yes	Resistant (9)	Resistant (4)		■				
Yes	Resistant (3)	Resistant (3)				■		
Yes	Resistant (3)	Resistant (2)					■	
Yes	Resistant (2)							■
Yes	Resistant (2)	Resistant (1)		■	■			
Yes	Resistant (3)	Resistant (8)		■		■		
Yes	Resistant (1)	Resistant (3)		■				■
Yes	Resistant (1)	Resistant (3)	■	■				
Yes	Resistant (1)	Resistant (2)			■	■		
Yes	Resistant (4)	Resistant (4)				■		■
Yes	Resistant (1)	Resistant (1)			■	■		■
Yes		Resistant (1)		■	■	■		
No	Resistant (1)		■	■				
Yes		Resistant (1)						
No	Resistant (2)	Resistant (8)						
Yes	Red. Suscept. (1)			■				
Yes	Red. Suscept. (1)					■		
Yes	Red. Suscept. (1)			■		■		
Yes	Red. Suscept. (4)							
No	Red. Suscept. (5)	Red. Suscept. (2)						
Yes	Susceptible (2)	Susceptible (1)						
No	Susceptible (2)	Susceptible (1)						

FIGURE 2.

Comparison of the ExaVir™ Drug (phenotype) assay and HIV GenoSure™ (genotype) mutation results for assessing efavirenz resistance (N=49) and nevirapine resistance (N=45). The following efavirenz mutations were included: L100I, K103N, V106M, V108I, Y181C/I, Y188L, and G190A/S; and no patient had V106M or P225H. The following nevirapine mutations were included: L100I, K103N, V106A/M, V108I, Y181C/I, Y188C/L/H, and G190A; and no patient had V106M.

NOTE: Non-nucleoside reverse transcriptase inhibitor (NNRTI); efavirenz (EFV); nevirapine (NVP); Reduced Susceptibility (Red. Suscept.).