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Can HIV reverse transcriptase activity assay be a low cost alternative for viral load monitoring in resource-limited settings?

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Abstract

Objective: To evaluate the performance and cost of an HIV reverse transcriptase-enzyme activity (HIV-RT) assay in comparison to a HIV-1 RNA assay for routine viral load monitoring in resource limited settings.

Design: Cohort based longitudinal study.

Setting: Two antiretroviral therapy (ART) centers in Karnataka state, South India, providing treatment under the Indian AIDS control program

Participants: Cohort of 327 HIV-1 infected Indian adult patients initiating first-line ART.

Outcome measures: The performance and cost of an HIV-RT assay (ExaVir Load v3) in comparison to a gold standard HIV-1 RNA assay (Abbott m2000rt) in a cohort of 327 Indian patients before (WK00) and 4 weeks (WK04) after initiation of first-line therapy.

Results: Plasma viral load was determined by HIV-1 RNA assay and HIV-RT assay in 629 samples (302 paired samples and 25 single time-point samples at WK00) obtained from 327 patients. Overall, a strong correlation of r=0.96 was observed, with good correlation at WK00 (r = 0.84) and at WK04 (r = 0.77). Bland-Altman analysis of all samples showed a good level of agreement with a mean difference (bias) of 0.22 $\log_{10}$copies/mL. The performance of ExaVir Load v3 was not negatively affected by a nevirapine/efavirenz based anti-retroviral regimen. The per test cost of measuring plasma viral load by Abbott m2000rt and ExaVir Load v3 assay in our setting was $36.4 and $17.3 respectively.

Conclusions: The strong correlation between HIV-RT and HIV-1 RNA assay suggests that the HIV-RT assay can be an affordable alternative option for monitoring patients on anti-retroviral therapy in resource-limited settings.

Strengths and Limitations of this study:

- To our knowledge the current study is the most thoroughly evaluated study of ExaVir Load v3 (HIV-RT assay) from India to date.
- This study was performed in a large number of subjects in a longitudinal manner looking into the effects of the NNRTI based therapy and drug resistance mutations on the performance of the HIV RT-enzyme activity assay.
- Though the assay has been validated before in other non-C subtype settings, in the present study the HIV-RT assay was validated on a larger scale in a subtype C predominant setting and subtype C is the most predominant subtype globally, more so affecting the resource limited settings.
- This study was limited to patients hailing from Southern India and a performance evaluation and cost-effectiveness of the HIV-RT assay needs to be accessed on a national level.

Introduction

The principle aim of anti-retroviral therapy (ART) is durable suppression of replicating plasma virus to undetectable levels, thereby delaying disease progression and prolonging survival.[1 2] Expanding access to ART in resource-limited settings along with close monitoring is needed for successful treatment outcomes. In high income settings this is achieved by performing quantitative viral load monitoring every 3-6 months,[3] as viral load monitoring detects early treatment failure. However in resource-limited settings therapeutic outcome is evaluated based on either CD4 T cell count or on clinical findings,[4] neither of which accurately predicts viral suppression.[5] Early detection of viral-failure by monitoring viral load also provides the opportunity to intensify adherence counseling to improve adherence to ART potentially leading to re-suppression of viral load before the evolution of drug resistant virus can take place.[6]

The currently used viral load assays are based on the amplification of HIV-1 virion RNA, which is considered impractical for wide-scale use in resource-limited settings, as it requires infrastructure, facilities for molecular diagnostics, expensive equipment and skilled technicians which are often unavailable.[7] Simpler, less expensive viral load assays would be very useful in the resource-limited environments that are most impacted by this epidemic.

An alternative to measure the HIV-1 RNA is to measure the activity of the viral reverse transcriptase (RT) enzyme. ExaVir Load assay (Cavidi, AB, Sweden), a low-cost and technically less-demanding assay using an enzyme linked immune sorbent assay (ELISA)-based method to measure RT enzyme activity has shown promising results.[8-19] Although previous versions of the test have been evaluated against several polymerase chain reaction (PCR)-based HIV tests that measure viral RNA, there are few comparative studies between ExaVir Load version 3 (v3) and such molecular real time assays.[14 16 18] The most recent version 3 of the ExaVir Load assay has an enhanced sensitivity (lower detection limit 200 copies/mL) and reduced turnaround time compared to version 2.[14 18]

HIV-1 subtype C (HIV-1C) is the dominant strain in most low middle-income countries like India, South Africa and Ethiopia [20] and the need for a simple low cost viral load monitoring tool is a priority in these settings. Data available from countries

dominated by the HIV-1C epidemic are limited. Where available, evaluations have been mainly performed with earlier version assays (ExaVir Load v1 and v2), which had lower detection limits of 400 copies/mL.[7 11 21 22] Studies evaluating Exavir Load v3 have been mainly performed in non-C dominated countries.[14 16 18 19]

Thus, we aimed to evaluate the performance of the HIV RT-enzyme activity (HIV RT) assay (ExaVir Load v3) in comparison to a "gold standard" HIV RNA load (Abbott m2000rt real-time PCR) assay in a cohort of patients before and after initiation of first-line ART in Indian settings. We also compared viral load measurements from both assays in a subset of patients with drug resistant mutations at baseline. Further, we studied the difference in costs of the two viral load assays in the context of our laboratory setting.

Materials and Methods

Study participants and samples

Between April 2010 and September 2011, EDTA plasma samples were collected from HIV-1 infected adult patients attending Infectious Disease Clinic, St. John's Medical College and Hospital, Bangalore (main site) and ART center, Krishna Rajendra Hospital, Mysore (peripheral site) enrolled in the HIVIND randomized controlled trial (Trial registration: ISRCTN79261738).[23] All the patients included in the study initiated ART with reverse transcriptase inhibitor (RTI) drugs, i.e. two nucleoside reverse transcriptase inhibitors (NRTI), zidovudine (AZT) or stavudine (d4T) with lamivudine (3TC) + one non-nucleoside reverse transcriptase inhibitors (NNRTI), either Nevirapine (NVP) or Efavirenz (EFV) as per the standard national AIDS program guidelines.[24]

Plasma samples were collected at two time points; (i) prior to ART initiation (not longer than 3 months prior) and (ii) 4 weeks after ART initiation. The plasma samples were separated within 6 hours of EDTA whole blood collection, aliquoted and stored at -80°C in the main site. Plasma samples aliquoted in the peripheral site were stored at -20°C and were transported to the main site on dry ice (every 2 weeks) and then stored at -80°C prior to testing.

Ethical statement

Ethical approvals for the conduct of the trial were obtained from Institutional Ethical Review Board of St John's Medical College Hospital, Bangalore (IERB 1/369/08–92/2008) and Krishna Rajendra Hospital, Mysore (NO/PS/173/2010). All patients participating in the HIVIND study have given their written consent.

Plasma HIV-1 RNA assay

Plasma HIV-1 RNA load was measured in the patient cohort using Abbott Real Time PCR, m2000rt system with manual RNA extraction procedure on m2000sp sample preparation system as per the manufacturer's instructions. The assay was performed using an initial volume of 0.2 mL plasma, which provides limits of quantification between 150 copies/mL (lower limit of detection) to 10,000,000 copies/mL (upper limit of detection). In every run a negative control, a low positive control and a high positive control supplied in the Abbott Realtime HIV-1 control kit were included. This measure using Real Time PCR was considered as the gold standard. This protocol was validated by an external quality control program by the Quality Control for Molecular Diagnostics, Glasgow, Scotland (QCMD, http://www.qcmd.gov) on 2010 panel (Consisting of four HIV-1B samples, two HIV-1C samples, one HIV-1A/G sample and one HIV-1 negative sample) and obtained highly satisfactory score.

Plasma HIV RT enzyme activity assay

The viral RT enzyme activity was quantified using Cavidi ExaVir Load v3 as per the instructions of the manufacturer [14]. In an ELISA based format, the RT activity of the RT enzyme recovered from 1mL of patient plasma was determined and compared to a standard curve based on known amounts of a HIV 1 recombinant RT constructed from the BH10 isolate. The RT activity in the sample was expressed as femtogram HIV-1 RT activity/mL (fg/mL) plasma that was translated into RNA copies/mL equivalent by the ExaVir Load analyzer software. The lower detection limit of the assay was ≥1fg/mL i.e. ≥200copies/mL. In every run a single positive control and a negative control were used to monitor the performance of the RT extraction process and to

access between run variations. The positive control was prepared by pooling the EDTA plasma from a high HIV-1 viral load sample and HIV-1 negative plasma; 1.2mL was aliquoted and stored in -80^{o}C freezer. The laboratory personnel running the ExaVir Load v3 assay were blind to the plasma HIV-1 RNA values.

HIV-1 subtyping and baseline drug resistance

Genotypic resistance testing (GRT) was performed on the baseline plasma samples. Briefly, the reverse transcriptase (RT) region of the HIV-1 *pol* gene was amplified and sequenced using the primers described by us previously.[25] HIV-1 subtyping was determined based on the *pol* gene as well as the *env* gene (wherever sequence data was available).[26-28] using the maximum likelihood (ML) phylogenetic tree based on reference sequences downloaded from Los Alamos Database (www.hiv.lanl.gov). Primary drug resistance analysis was evaluated using World Health Organization list of mutations from 2009 (WHO_SDRM 2009).[29]

Comparative cost analysis

Components used for cost comparison included costs for instruments and start-up kits, human resource costs, annual maintenance, operator-supplied basic instruments, reagents and other consumables for a period of 5 years time. The costs were compared based on patient numbers in the ART Center of our hospital. There are at present, 1500 ART-experienced patients in our ART center. Assuming that viral load monitoring of these patients will require to be performed every six months, there will be 3000 samples a year.

For HIV-1 RNA load by Abbott m2000rt, assays were done in a batch of 24 reactions, each batch comprising 21 samples and 3 controls. Calibration of the HIV-1 amplification kits was performed once for every 5 kits of the same lot. Reagent costs were calculated based on this degree of usage. In the HIV RT assay, 30 samples with two controls (prepared by end user) were analyzed using a single ExaVir Load assay kit. Basic instruments were included as capital costs, as these are necessary for a new laboratory set-up. In the cost comparison analysis we did not consider costs associated

with the sample collection, storage and transportation as these are common to both tests.

Statistical Analysis

All statistical analyses were performed after the HIV RT and HIV-1 RNA level values were $\log_{10}$ transformed. For analysis, the lower limit of detection of HIV RT assay (≤200 copies/mL) was considered; samples showing <200 copies/mL by any of the assays were assigned a value of 199 copies/mL. With Exavir Load v3 we achieved a varying upper detection limit ranging from >360,000 to >770,000 in different runs. Thus samples with a viral load of >360,000 (the lowest range of upper detection limit obtained for ExaVir Load v3) by any of the assays were assigned a viral load of 360,000 copies/mL. The diagnostic agreement between HIV RT assay and HIV-1 RNA assay at different viral load cut-offs was determined from the kappa statistic. Pearson's correlation coefficient (r) was calculated to study the correlation between $\log_{10}$ HIV RT activity (copies/ml equivalents) and $\log_{10}$ HIV RNA (copies/ml). However as this coefficient does not take in account the possibility that one measure may differ consistently from the other, we further assessed the level of agreement using pair-wise Bland-Altman plots. This plot compares the measures between the two tests by plotting the difference in the two VL measures against the average of the two measures.

Results

Sample characteristics

Plasma viral load was obtained from 629 samples collected from 327 HIV-1 infected adult patients, of which 302 were paired (before ART and 4 Weeks after ART initiation). HIV-1C was the predominant subtype observed in 98.1% (313/319) of the patients. Six out of 319 genotyped patients (1.9%) showed presence of non-C subtype strains namely BC recombinant (1), BD recombinant (1), A1C recombinant (2) and HIV-1A1 (2). Eleven patients (3.4%) showed presence of single primary drug resistant

mutations, with six samples harboring NRTI associated drug resistant mutations (DRMs) and five samples with NNRTI-associated mutations. (Table 1).

TABLE 1. Comparison of HIV-1 plasma VL levels measured by the HIV RT and HIV-1 RNA assays by antiretroviral treatment (ART) status, HIV-1 subtypes and RT-drug resistant mutations

Sample Type	Number of Samples	Mean viral load ± SD in log_{10}copies/mL		Mean log_{10} viral load difference ± SD in log_{10}copies/mL
		ExaVir Load v3	Abbott m2000rt	
All samples	629	3.98 ± 1.3	4.19± 1.3	0.22±0.3
ART Status				
Naïve (baseline at WK00)	327	5.07±0.6	5.33±0.5	0.25±0.3
Experienced (WK04)	302	2.79±0.5	2.97±0.6	0.19±0.4
Subtype at WK00 (n=319)*				
C	313	5.08±0.6	5.33±0.4	0.25±0.3
Non-C	6	5.05±0.5	5.3±0.4	0.25±0.3
DRMs at WK00 (n=319)*				
Wild type (No DRM)	308	5.07±0.6	5.32±0.5	0.25±0.3
NRTI mutations	6	5.41±0.3	5.55±0.01	0.15±0.3
NNRTI mutations	5	5.26±0.2	5.62±0.2	0.36+0.2

*Genotyping performed only in baseline samples

Comparison between HIV RT activity and HIV-1 RNA load assay

There were 54 samples (8.5%) that were quantifiable by HIV-1 RNA assay but were below the detection limit of the HIV RT assay (Table 2). At a lower limit of quantification of 200 copies/mL, 90.7% of the samples showed quantifiable virus by HIV RT assay. The percentage of the samples with quantifiable viral load by HIV RT assay increased with higher viral load cut-offs by the HIV-1 RNA assay as shown in Table 2. Over all there was acceptable agreement observed between HIV RT and HIV-1 RNA assay, with excellent agreement observed at higher values of plasma viral load ≥ 3.0 log_{10}copies/mL (Kappa = 0.76). Of all the samples, 81.7% (514/629) had viral

load values by HIV RT assay which, differed by< 0.5 log_{10} units from the HIV-1 RNA values; while 99.2% (624/629) of the samples differed by < 1.0 log_{10} units.

TABLE 2. Agreement between HIV RT assay and HIV-1 RNA assay at different plasma viral load levels.

PVL by HIV-1 RNA In copies/mL (log_{10} copies/mL)	Agreement	Kappa Value	No. of samples detected by Abbott m2000rt	Percentage of Samples detected by ExaVir Load v3
≥ 200 (2.3)	89.1	0.46	580	90.7
≥ 400 (2.6)	88.1	0.57	550	93.5
≥ 1000 (3.0)	89.7	0.76	458	97.6
≥ 5000 (3.7)	94.4	0.89	344	99.7
≥ 10,000 (4.0)	96.8	0.94	324	100

A strong positive correlation was observed between the plasma viral load values by HIV RT and HIV-1 RNA assay (r = 0.96) in all the samples. A good correlation was noted in ART-naïve samples (r = 0.84) as well as in samples at Week 04 of ART (r = 0.77) (Fig. 1).

Bland-Altman plots for all the samples showed good levels of agreement with a mean difference (bias) of 0.22 log_{10}copies/mL, with acceptable limits of agreement (-0.45 and +0.89 log_{10}copies/mL). A good level of agreement was also observed separately at baseline [mean difference bias of 0.25; range of acceptable limit of agreement: -0.39 and +0.89 log_{10}copies/mL] and at WK04 [mean difference bias of 0.19; range of acceptable limit of agreement: -0.52 and +0.89 log_{10}copies/mL]. (Fig. 2)

Influence of current anti-retroviral therapy and drug resistant mutations on RT-enzyme activity

Table 1 shows that the mean log_{10} difference between HIV RT and HIV-1 RNA assay both before and after initiation of ART were not significantly different (<0.25 log_{10} Copies/mL) and well within the clinically accepted limit of 0.5 log_{10}copies/mL. Thus the performance of HIV RT assay is not affected by the presence of NNRTI

(nevirapine/efavirenz) based ART regimen. Also presence of either NRTI associated DRM's (n=6; M41L: 1, D67N: 1, T69D: 1, M184I: 1 and T215S: 2) and NNRTI associated DRM's (n=5; Y181C: 1, K101E: 1 and K103N: 2) showed an acceptable change (<0.4 $\log_{10}$ Copies/mL) in mean $\log_{10}$ difference from the corresponding value among wild types. Although the samples with mutations are small, it indicates that the presence of NRTI and NNRTI DRM's did not negatively impact the test performance.

Cost comparison of the assays

The laboratory cost of viral load monitoring of HIV-infected patients analyzed in our cohort by both Abbott m2000rt (HIV-1 RNA) and ExaVir Load v3 (HIV RT) is shown in Table 2. The per test cost of the plasma viral load measure by Abbott m2000rt and ExaVir Load v3 was $36.4 and $17.3 respectively. Thus, by using the ExaVir Load v3, $19.1 per test can be saved. In a laboratory with a pre-existing basic set up for ELISA based assays, the ExaVir Load v3 can be performed at $16.8, saving $19.6 per test. Most of the expense saved by using ExaVir Load v3 were due to (a) lower capital costs (instruments $37750 against $2000) (b) the lower cost of the assay reagents ($15/test against $31/test) (Table 3).

TABLE 3. Cost comparison between HIV RT assay and HIV-1 RNA assay for a laboratory doing 6000 tests/ year

Parameters	Abbott m2000rt Cost ($)	ExaVir Load v3 Cost ($)
Capital Instruments	37750	2000
Annual Maintenance Cost for 5 years	4458	923/0*
Operator supplied Instruments	840	6830/0*
Reagents for 5 years	465000	225000
Consumables for 5 years	23076.9	9230.8
Salary costs @ $250/month for 5 years	15000	15000
Total expenditure for 5 years	5,46,124.9	2,58,983.8/2,51,230.8*
Cost/test ($)	36.41	17.27/16.75*

*Cost for laboratories already furnished with a set up for ELISA based assays.

Discussion

A good correlation between the HIV RT and the HIV-1 RNA assays was observed in the current HIV-1C predominant setting in India. The agreement between the tests was not significantly affected by the NRTI/NNRTI based anti-retroviral regimen used. Earlier studies performed on panels of different subtypes and recombinants have suggested that the HIV RT assay detects all HIV- 1 and HIV- 2 subtypes with similar efficiency.[14 15 30] This assay, ExaVir Load version 3, can therefore be an attractive option for viral load monitoring in Indian settings.

The current study compared the ExaVir Load assay v3 with Abbott m2000rt HIV-1 RNA assay and observed an excellent correlation (r=0.96). An earlier study from London comparing the same tests observed a similar correlation (r=0.94).[14] Strong correlations between ExaVir Load v3 and Roche HIV-1 RNA-based assays have also been observed by two other studies, by Greengrass *et al.* from Australia (Roche Cobas Amplicor; r=0.85) and Huang *et al.* from China (Roche Cobas TaqMan 48; r=0.95).[16 18] Neither of these studies was performed in HIV-1C dominated settings. The HIV RT assay showed a good agreement with the HIV-1 RNA assay at the clinically important viral load threshold of 1000 copies/mL, which is used by the World Health Organization (WHO) to define viral-failure to first line therapy and is also most often used as the cut-off for drug resistance genotyping.[31] The performance of Exavir Load below 1000 copies/mL is moderate.

In general, we observed an under estimation of viral load of 0.22 $\log_{10}$ RNA copies by the HIV RT assay, which is similar to what have been observed in other studies.[14, 16, [32] These two surrogate assays use very different methods for quantifying the plasma viral load. The HIV-1 RNA assays quantify the amount of viral RNA irrespective of RNA functionality, while the HIV RT assay quantifies the amount of active RT enzyme. The calibration constant used to translate RT activity into RNA copies was estimated from a study of an Australian cohort [18] and is not completely accurate for all combinations of HIV RNA assays and cohorts with varying subtype compositions. The variation observed is, however, well within the acceptable limit of <0.5 log10 copies.

NNRTI drugs bind to the RT enzyme, inhibit its activity and prevent viral replication. Several articles have discussed the possibility that enzymatically inactive RT-drug

complexes could result in under quantification of RT in relation to RNA.[29, 30, [33] These studies were however, cross-sectional and never found any evidence for reduced RT activity during NNRTI therapy. In contrast to previous cohorts, the longitudinal sampling in our study provides optimal material for evaluation of the effects of NNRTI based drug regimens. When comparing HIV viral load data from the same patient cohort before and after onset of ART, we found a mean $\log_{10}$ difference between ExaVir Load v3 and Abbott m2000rt of 0.25 for naïve patients and 0.19 for experienced patients (Table 1). The difference between the tests did thus not increase after onset of therapy. This supports evidence that the current NNRTI containing therapy does not adversely influence the recovery of RT enzyme activity.

On a small number of samples, we assessed if the presence of drug resistant mutations decreased the RT-fitness so as to influence the performance of HIV RT assay. We had 6 samples with single NRTI mutations and 5 samples with single NNRTI mutations and observed no evidence that their presence caused any significant difference in the association between RT-enzyme activity and HIV-1 RNA load. Despite the small sample number, our results support evidence from previous studies by Napravnik *et al.* and Van Rooijen *et al.* indicating that the presence of NRTI or NNRTI mutations do not affect the relationship between RT-enzyme activity and HIV-1 RNA load. [34 35]

To our knowledge the current study is the most thoroughly evaluated study of ExaVir Load v3 from India to date. Thus far, there have been three comparative studies from India that have been reported from the states of Andhra Pradesh (Anantpur),[36] Tamil Nadu (Chennai),[7] and New Delhi.[32] Iqbal *et al*. from Chennai cross-sectionally evaluated ExaVir Load assay version 1 and Roche Amplicor Monitor assay. They found a good agreement between the two tests and a significant inverse correlation between ExaVir Load and CD4+ T-cell count.[7] Alvarez-Uria *et al.* from Anantpur compared the accuracy of ExaVir version 3 with Roche Cobas TaqMan HIV-1 test and Roche Amplicor HIV-1 DNA assay for early infant diagnosis. ExaVir performed well showing 100 % sensitivity and 99 % specificity, but no quantitative correlations were evaluated.[36] A more recent study by Kokkayil *et al.* compared ExaVir Load v3 with Roche Cobas TaqMan among 75 ART naïve patients and reported no statistically significant difference between the two assays.[32]

There are a few drawbacks of the HIV RT assay. The long turnaround time of 48 hours makes it appear labor intensive though the actual hands-on time is approximately 5 hours. The prolonged incubation time is critical to achieve assay sensitivity. For standard performance, the assay requires 1 mL of plasma, which is high in comparison to the requirement for HIV-1 RNA assays, thus limiting its possible usefulness in pediatric populations. However a recent study by Greengrass *et al*. observed that sample volumes down to 0.25 ml with VL>800 copies/mL can be utilized for pediatric monitoring.[19] The ExaVir Load assay does not provide a standard positive and negative control, thus requiring the lab to prepare its own controls, which may compromise the quality assurance of the assay. Additionally, we noted that the quality of the water used for washing is important as impurities and bacterial contaminants present in water may contain polymerases which can create background noise and increase the level of the lower detection limit.

In spite of these caveats, the HIV RT assay has advantages over the HIV-1 RNA assays in resource-limited settings because it is an ELISA based assay and can be performed in any routine lab at a lower cost. The ExaVir Load assay requires a cheaper and maintenance free startup kit as compared to real time assays. We observed that performing the HIV RT assay routinely in our center would save us $19.6 per sample as compared to a HIV-1 RNA assay. A more basic laboratory, which requires installing basic ELISA equipment, would save $19.1 per sample (Table 3).

The use of CD4 cell count as a prognostic marker has been debated, it is argued that this count may not reflect the actual viral load status of the patient.[37] The cost associated with viral load monitoring using HIV-1 RNA assays, despite being lower than PCR assays, is a major limiting factor for its implementation. Currently in India, viral load testing has been phased in to support patients failing first line ART. In the year 2012, about 4157 viral load tests were performed under National AIDS Control Organisation (NACO).[38] Considering $19.6 could have been saved per sample by performing HIV RT assay, the cost saving for these 4157 viral load tests could have amounted to $ 81,477 if a HIV RT assay had been used.

NACO is now considering taking up the monitoring approach recommended by WHO to diagnose and confirm ART failure. Considering that there currently are 604,987 HIV-1 infected individuals receiving first line of ART at 380 centers spread across the

country the cost reduction of utilizing HIV RT compared to HIV RNA plasma load can be substantial.[39]

Scaling up ART requires the critical support of HIV-1 viral load monitoring. Evidence from the comparative performance of the HIV RT assay with HIV-1 RNA assays from ours and other studies from India, indicate that the ExaVir Load assay could serve as an affordable alternative to monitor patients on ART.

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Authors Contribution

Conceived the Study: UN ADC CK PA AS. Designed the experiments: SG UN HS ADC CK AS. Performed the experiments: SG RPC. External lab monitoring and interim quality assessment: PA. Analyzed the data: SG UN ADC CK AS. Contributed reagents/materials/analysis tools: CK AS. Contributed to the writing of the manuscript: SG RP UN HS PA ADC CK AS.

Competing interests

Clas Källander is an employee of Cavidi AB, Uppsala, Sweden. All the other authors have declared that no competing interests exist.

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Data sharing statement

All relevant data are included within the paper. No additional data available.

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Figure Legends:

Figure 1: Correlation between Abbott m2000rt and ExaVir Load v3 assay for A) all 629 samples showing *r = 0.96* B) 327 baseline (WK00) samples samples showing *r = 0.84* and C) 302 4-weeks post-ART (WK04) samples showing *r = 0.77.*

Figure 2: Bland-Altman plot with 95% CI of limits of agreement between HIV-1 viral loads measured with Abbott Real-Time m2000rt assay and ExaVir Load v3 assay for A) all 629 samples showing a mean bias of 0.22 with 95% limits of agreement ranging from -0.45 to 0.89 B) 327 baseline (WK00) samples showing a mean bias of 0.25 with 95% limits of agreement ranging from -0.39 to 0.89 C) 302 4-weeks post-ART (WK04) samples showing a mean bias of 0.19 with 95% limits of agreement ranging from -0.52 to 0.89.

Figure 1A

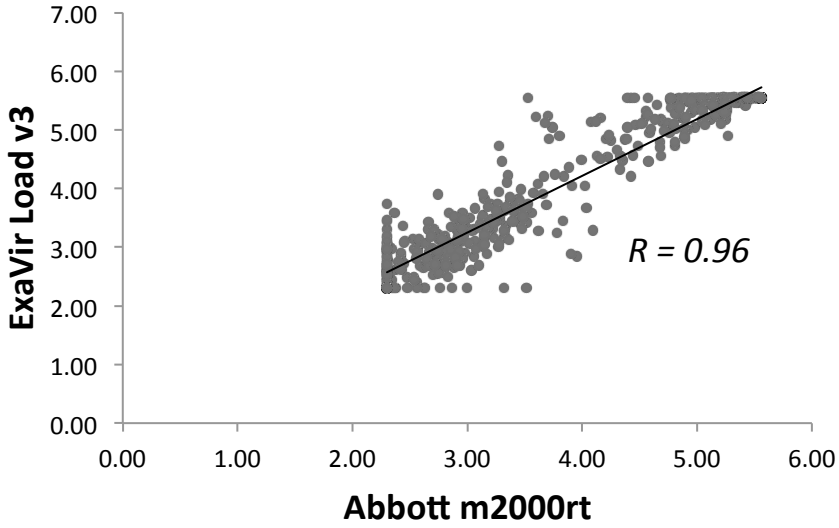


Figure 1B

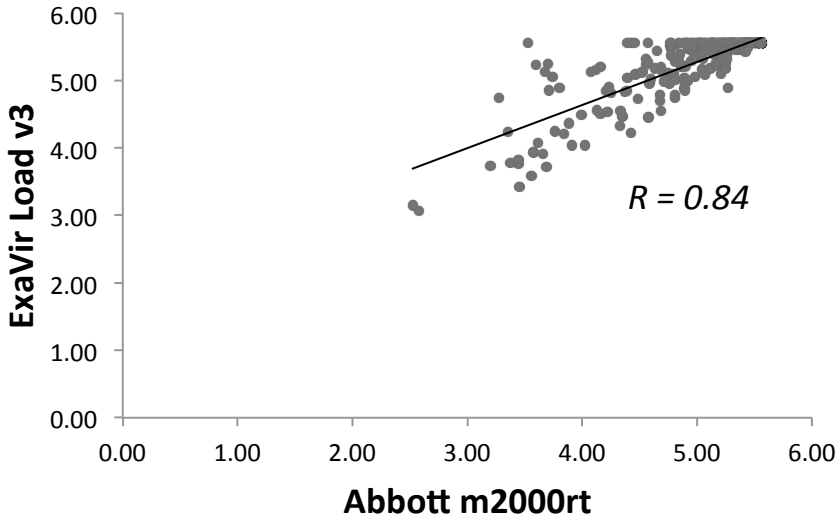


Figure 1C

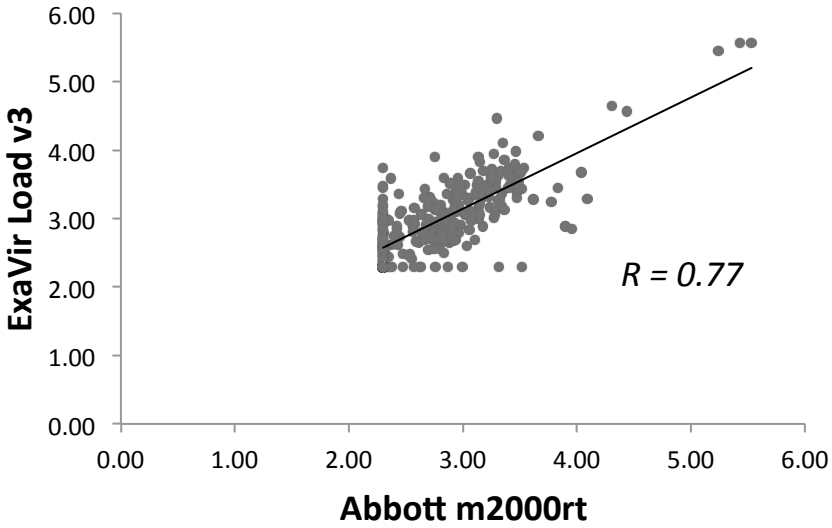


Figure 2A

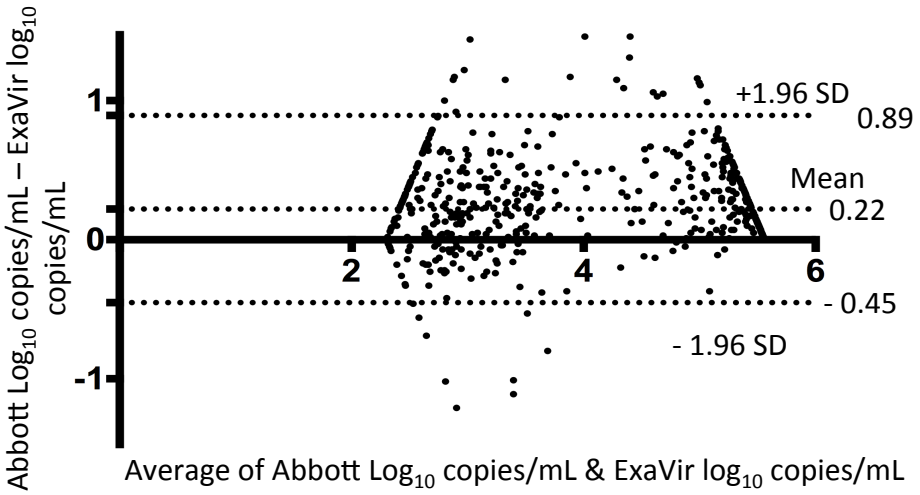


Figure 2B

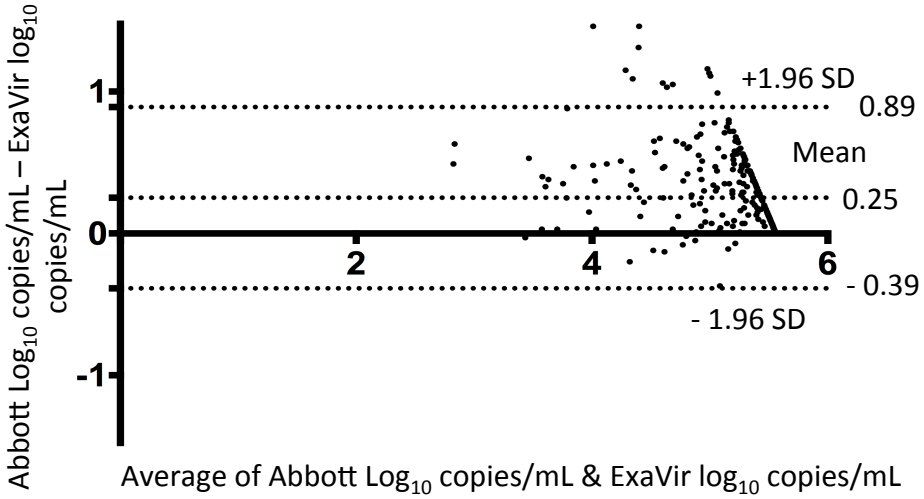
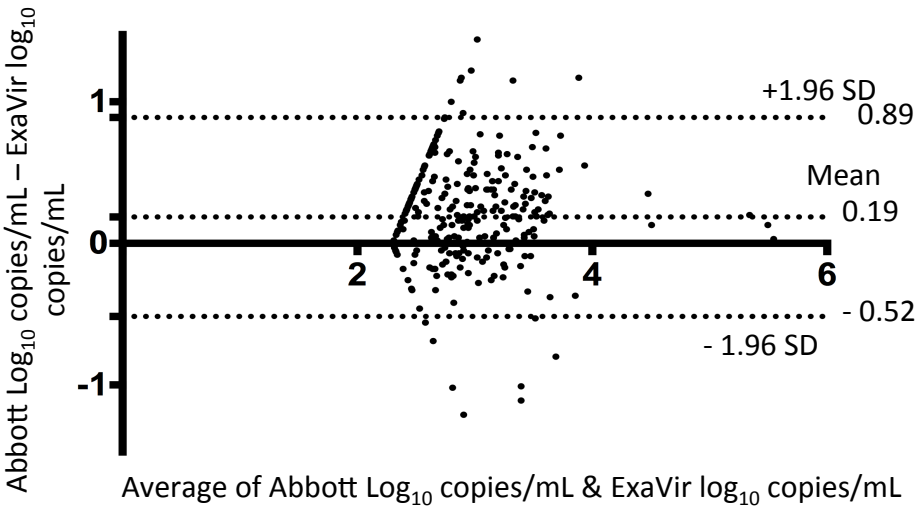


Figure 2C



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Can HIV reverse transcriptase activity assay be a low cost alternative for viral load monitoring in resource-limited settings?

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Abstract

Objective: To evaluate the performance and cost of an HIV reverse transcriptase-enzyme activity (HIV-RT) assay in comparison to a HIV-1 RNA assay for routine viral load monitoring in resource limited settings.

Design: Cohort based longitudinal study.

Setting: Two antiretroviral therapy (ART) centers in Karnataka state, South India, providing treatment under the Indian AIDS control program

Participants: Cohort of 327 HIV-1 infected Indian adult patients initiating first-line ART.

Outcome measures: The performance and cost of an HIV-RT assay (ExaVir Load v3) in comparison to a gold standard HIV-1 RNA assay (Abbott m2000rt) in a cohort of 327 Indian patients before (WK00) and 4 weeks (WK04) after initiation of first-line therapy.

Results: Plasma viral load was determined by HIV-1 RNA assay and HIV-RT assay in 629 samples (302 paired samples and 25 single time-point samples at WK00) obtained from 327 patients. Overall, a strong correlation of r=0.96 was observed, with good correlation at WK00 (r = 0.84) and at WK04 (r = 0.77). Bland-Altman analysis of all samples showed a good level of agreement with a mean difference (bias) of 0.22 $\log_{10}$copies/mL. The performance of ExaVir Load v3 was not negatively affected by a nevirapine/efavirenz based anti-retroviral regimen. The per test cost of measuring plasma viral load by Abbott m2000rt and ExaVir Load v3 assay in a basic lab setting was $36.4 and $16.8 respectively.

Conclusions: The strong correlation between HIV-RT and HIV-1 RNA assay suggests that the HIV-RT assay can be an affordable alternative option for monitoring patients on anti-retroviral therapy in resource-limited settings.

Strengths and Limitations of this study:

- To our knowledge the current study is the most thoroughly evaluated study of ExaVir Load v3 (HIV-RT assay) from India to date.
- This study was performed in a large number of subjects in a longitudinal manner looking into the effects of the NNRTI based therapy and drug resistance mutations on the performance of the HIV RT-enzyme activity assay.
- Though the assay has been validated before in other non-C subtype settings, in the present study the HIV-RT assay was validated on a larger scale in a subtype C predominant setting and subtype C is the most predominant subtype globally, more so affecting the resource limited settings.
- This study was limited to patients hailing from Southern India and a performance evaluation and cost-effectiveness of the HIV-RT assay needs to be accessed on a national level.

Introduction

The principle aim of anti-retroviral therapy (ART) is durable suppression of replicating plasma virus to undetectable levels, thereby delaying disease progression and prolonging survival.[1 2] Expanding access to ART in resource-limited settings along with close monitoring is needed for successful treatment outcomes. In high income settings this is achieved by performing quantitative viral load monitoring every 3-6 months,[3] as viral load monitoring detects early treatment failure. However in resource-limited settings therapeutic outcome is evaluated based on either CD4 T cell count or on clinical findings,[4] neither of which accurately predicts viral suppression.[5] Early detection of viral-failure by monitoring viral load also provides the opportunity to intensify adherence counseling to improve adherence to ART potentially leading to re-suppression of viral load before the evolution of drug resistant virus can take place.[6]

The currently used viral load assays are based on the amplification of HIV-1 virion RNA, which is considered impractical for wide-scale use in resource-limited settings, as it requires infrastructure, facilities for molecular diagnostics, expensive equipment and skilled technicians which are often unavailable.[7] Simpler, less expensive viral load assays would be very useful in the resource-limited environments that are most impacted by this epidemic.

An alternative to measure the HIV-1 RNA is to measure the activity of the viral reverse transcriptase (RT) enzyme. ExaVir Load assay (Cavidi, AB, Sweden), a low-cost and technically less-demanding assay using an enzyme linked immune sorbent assay (ELISA)-based method to measure RT enzyme activity has shown promising results.[8-19] Although previous versions of the test have been evaluated against several polymerase chain reaction (PCR)-based HIV tests that measure viral RNA, there are few comparative studies between ExaVir Load version 3 (v3) and such molecular real time assays.[14 16 18] The most recent version 3 of the ExaVir Load assay has an enhanced sensitivity (lower detection limit 200 copies/mL) and reduced turnaround time compared to version 2.[14 18]

HIV-1 subtype C (HIV-1C) is the dominant strain in most low middle-income countries like India, South Africa and Ethiopia [20] and the need for a simple low cost viral load monitoring tool is a priority in these settings. Data available from countries

dominated by the HIV-1C epidemic are limited. Where available, evaluations have been mainly performed with earlier version assays (ExaVir Load v1 and v2), which had lower detection limits of 400 copies/mL.[7 11 21 22] Studies evaluating Exavir Load v3 have been mainly performed in non-C dominated countries.[14 16 18 19]

Thus, we aimed to evaluate the performance of the HIV RT-enzyme activity (HIV RT) assay (ExaVir Load v3) in comparison to a "gold standard" HIV RNA load (Abbott m2000rt real-time PCR) assay in a cohort of patients before and after initiation of first-line ART in Indian settings. We also compared viral load measurements from both assays in a subset of patients with drug resistant mutations at baseline. Further, we studied the difference in costs of the two viral load assays in the context of our laboratory setting from a provider perspective.

Materials and Methods

Study participants and samples

Between April 2010 and September 2011, EDTA plasma samples were collected from HIV-1 infected adult patients attending Infectious Disease Clinic, St. John's Medical College and Hospital, Bangalore (main site) and ART center, Krishna Rajendra Hospital, Mysore (peripheral site) enrolled in the HIVIND randomized controlled trial (Trial registration: ISRCTN79261738).[23] All the patients included in the study initiated ART with reverse transcriptase inhibitor (RTI) drugs, i.e. two nucleoside reverse transcriptase inhibitors (NRTI), zidovudine (AZT) or stavudine (d4T) with lamivudine (3TC) + one non-nucleoside reverse transcriptase inhibitors (NNRTI), either Nevirapine (NVP) or Efavirenz (EFV) as per the standard national AIDS program guidelines.[24]

Plasma samples were collected at two time points; (i) prior to ART initiation (not longer than 3 months prior) and (ii) 4 weeks after ART initiation. The plasma samples were separated within 6 hours of EDTA whole blood collection, aliquoted and stored at -80°C in the main site. Plasma samples aliquoted in the peripheral site were stored at -20°C and were transported to the main site on dry ice (every 2 weeks) and then stored at -80°C prior to testing.

Ethical statement

Ethical approvals for the conduct of the trial were obtained from Institutional Ethical Review Board of St John's Medical College Hospital, Bangalore (IERB 1/369/08–92/2008) and Krishna Rajendra Hospital, Mysore (NO/PS/173/2010). All patients participating in the HIVIND study have given their written consent.

Plasma HIV-1 RNA assay

Plasma HIV-1 RNA load was measured in the patient cohort using Abbott Real Time PCR, m2000rt system with manual RNA extraction procedure on m2000sp sample preparation system as per the manufacturer's instructions. The assay was performed using an initial volume of 0.2 mL plasma, which provides limits of quantification between 150 copies/mL (lower limit of detection) to 10,000,000 copies/mL (upper limit of detection). In every run a negative control, a low positive control and a high positive control supplied in the Abbott Realtime HIV-1 control kit were included. This measure using Real Time PCR was considered as the gold standard. This protocol was validated by an external quality control program by the Quality Control for Molecular Diagnostics, Glasgow, Scotland (QCMD, http://www.qcmd.gov) on 2010 panel (Consisting of four HIV-1B samples, two HIV-1C samples, one HIV-1A/G sample and one HIV-1 negative sample) and obtained highly satisfactory score.

Plasma HIV RT enzyme activity assay

The viral RT enzyme activity was quantified using Cavidi ExaVir Load v3 as per the instructions of the manufacturer [14]. In an ELISA based format, the RT activity of the RT enzyme recovered from 1mL of patient plasma was determined and compared to a standard curve based on known amounts of a HIV 1 recombinant RT constructed from the BH10 isolate. The RT activity in the sample was expressed as femtogram HIV-1 RT activity/mL (fg/mL) plasma that was translated into RNA copies/mL equivalent by the ExaVir Load analyzer software. The lower detection limit of the assay was ≥1fg/mL i.e. ≥200copies/mL. In every run a single positive control and a negative control were used to monitor the performance of the RT extraction process and to

access between run variations. The positive control was prepared by pooling the EDTA plasma from a high HIV-1 viral load sample and HIV-1 negative plasma; 1.2mL was aliquoted and stored in -80^{o}C freezer. The laboratory personnel running the ExaVir Load v3 assay were blind to the plasma HIV-1 RNA values.

HIV-1 subtyping and baseline drug resistance

Genotypic resistance testing (GRT) was performed on the baseline plasma samples. Briefly, the reverse transcriptase (RT) region of the HIV-1 *pol* gene was amplified and sequenced using the primers described by us previously.[25] HIV-1 subtyping was determined based on the *pol* gene as well as the *env* gene (wherever sequence data was available).[26-28] using the maximum likelihood (ML) phylogenetic tree based on reference sequences downloaded from Los Alamos Database (www.hiv.lanl.gov). Primary drug resistance analysis was evaluated using World Health Organization list of mutations from 2009 (WHO_SDRM 2009).[29]

Comparative cost analysis

We did an analysis comparing the costs between the Abbott m2000rt and the ExaVir Load v3 from a provider (laboratory service provider) perspective. We used costs from our lab for this purpose. Costs considered included annuitized capital costs for the two different instruments including operator-supplied instruments. These are instruments, which are necessary in case of a new laboratory. In case of Abbott the operator-supplied instruments included single-channel micropipettes, two dry baths and a vortex. For the ExaVir Load v3, micropipettes (both single-channel and multi-channel), an ELISA plate reader, an incubator, a rocker and a vortex.

Cost for start-up kits, human resource costs (including time for training), annual maintenance, reagents and other consumables were also considered. . We assumed the working life for the Abbott m2000rt and the ExaVir Load v3 to be five years, and a discount rate of 5% was applied.

Assumptions:

Number of patients: There are at present, 1500 ART-experienced patients in our ART center. Assuming that viral load monitoring of these patients will require to be performed every six months, there will be 3000 samples a year.

Maintenance costs: In the case of the Abbott m2000rt the costs for servicing, maintenance of instrument and calibration of laser head. In the case of ExaVir Load, there are no formal annual maintenance cost, however costs for maintenance of the operator supplied equipment has been assumed to be 10% of the cost of purchase of these equipment.

Human resource skills and training requirements: From our experience, 1 month was required to train a technician on the Abbott m2000rt. One week was required for training on the ExaVir load v3. We also considered that the technician handling the Abbott m2000rt would require to be more senior and experienced (salary 300 USD per month) compared to the technician working with the ExaVir Load v3 (salary 200 USD per month).

Time for each method of testing: For the Abbot m2000rt, a batch of 24 reactions which comprise 21 samples and 3 controls will involve a total time of 8 hours (from the beginning of RNA extraction up until obtaining results), of which 5 hours involve the technician's time. For Exavir Load v3, assay is done in batches of 30 samples. Though the turnaround time is 48 hours, it involves 5 hours of actual hands-on time per batch. Costs for time of the technicians was calculated against the salaries mentioned above

In the cost comparison analysis we did not consider costs associated with the sample collection, storage and transportation as these are common to both tests.

Statistical Analysis

All statistical analyses were performed after the HIV RT and HIV-1 RNA level values were $\log_{10}$ transformed. For analysis, the lower limit of detection of HIV RT assay ($\leq$200 copies/mL) was considered; samples showing <200 copies/mL by any of the assays were assigned a value of 199 copies/mL. With Exavir Load v3 we achieved a varying upper detection limit ranging from >360,000 to >770,000 in different runs.

Thus samples with a viral load of >360,000 (the lowest range of upper detection limit obtained for ExaVir Load v3) by any of the assays were assigned a viral load of 360,000 copies/mL. The diagnostic agreement between HIV RT assay and HIV-1 RNA assay at different viral load cut-offs was determined from the kappa statistic. Pearson's correlation coefficient (r) was calculated to study the correlation between $\log_{10}$ HIV RT activity (copies/ml equivalents) and $\log_{10}$ HIV RNA (copies/ml). However as this coefficient does not take in account the possibility that one measure may differ consistently from the other, we further assessed the level of agreement using pair-wise Bland-Altman plots. This plot compares the measures between the two tests by plotting the difference in the two VL measures against the average of the two measures.

Results

Sample characteristics

Plasma viral load was obtained from 629 samples collected from 327 HIV-1 infected adult patients, of which 302 were paired (before ART and 4 Weeks after ART initiation). HIV-1C was the predominant subtype observed in 98.1% (313/319) of the patients. Six out of 319 genotyped patients (1.9%) showed presence of non-C subtype strains namely BC recombinant (1), BD recombinant (1), A1C recombinant (2) and HIV-1A1 (2). Eleven patients (3.4%) showed presence of single primary drug resistant mutations, with six samples harboring NRTI associated drug resistant mutations (DRMs) and five samples with NNRTI-associated mutations. (Table 1).

TABLE 1. Comparison of HIV-1 plasma VL levels measured by the HIV RT and HIV-1 RNA assays by antiretroviral treatment (ART) status, HIV-1 subtypes and RT-drug resistant mutations

Sample Type	Number of Samples	Mean viral load ± SD in log_{10}copies/mL		Mean log_{10} viral load difference ± SD in log_{10}copies/mL
		ExaVir Load v3	Abbott m2000rt	
All samples	629	3.98 ± 1.3	4.19± 1.3	0.22±0.3
ART Status				
Naïve (baseline at WK00)	327	5.07±0.6	5.33±0.5	0.25±0.3
Experienced (WK04)	302	2.79±0.5	2.97±0.6	0.19±0.4
Subtype at WK00 (n=319)*				
C	313	5.08±0.6	5.33±0.4	0.25±0.3
Non-C	6	5.05±0.5	5.3±0.4	0.25±0.3
DRMs at WK00 (n=319)*				
Wild type (No DRM)	308	5.07±0.6	5.32±0.5	0.25±0.3
NRTI mutations	6	5.41±0.3	5.55±0.01	0.15±0.3
NNRTI mutations	5	5.26±0.2	5.62±0.2	0.36+0.2

*Genotyping performed only in baseline samples

Comparison between HIV RT activity and HIV-1 RNA load assay

There were 54 samples (8.5%) that were quantifiable by HIV-1 RNA assay but were below the detection limit of the HIV RT assay (Table 2). At a lower limit of quantification of 200 copies/mL, 90.7% of the samples showed quantifiable virus by HIV RT assay. The percentage of the samples with quantifiable viral load by HIV RT assay increased with higher viral load cut-offs by the HIV-1 RNA assay as shown in Table 2. Over all there was acceptable agreement observed between HIV RT and HIV-1 RNA assay, with excellent agreement observed at higher values of plasma viral load ≥ 3.0 log_{10}copies/mL (Kappa = 0.76). Of all the samples, 81.7% (514/629) had viral load values by HIV RT assay which, differed by< 0.5 log_{10} units from the HIV-1 RNA values; while 99.2% (624/629) of the samples differed by < 1.0 log_{10} units.

TABLE 2. Agreement between HIV RT assay and HIV-1 RNA assay at different plasma viral load levels.

PVL by HIV-1 RNA In copies/mL (log_{10} copies/mL)	Agreement	Kappa Value	No. of samples detected by Abbott m2000rt	Percentage of Samples detected by ExaVir Load v3
≥ 200 (2.3)	89.1	0.46	580	90.7
≥ 400 (2.6)	88.1	0.57	550	93.5
≥ 1000 (3.0)	89.7	0.76	458	97.6
≥ 5000 (3.7)	94.4	0.89	344	99.7
≥ 10,000 (4.0)	96.8	0.94	324	100

A strong positive correlation was observed between the plasma viral load values by HIV RT and HIV-1 RNA assay (r = 0.96) in all the samples. A good correlation was noted in ART-naïve samples (r = 0.84) as well as in samples at Week 04 of ART (r = 0.77) (Fig. 1).

Bland-Altman plots for all the samples showed good levels of agreement with a mean difference (bias) of 0.22 log_{10}copies/mL, with acceptable limits of agreement (-0.45 and +0.89 log_{10}copies/mL). A good level of agreement was also observed separately at baseline [mean difference bias of 0.25; range of acceptable limit of agreement: -0.39 and +0.89 log_{10}copies/mL] and at WK04 [mean difference bias of 0.19; range of acceptable limit of agreement: -0.52 and +0.89 log_{10}copies/mL]. (Fig. 2)

Influence of current anti-retroviral therapy and drug resistant mutations on RT-enzyme activity

Table 1 shows that the mean log_{10} difference between HIV RT and HIV-1 RNA assay both before and after initiation of ART were not significantly different (<0.25 log_{10} Copies/mL) and well within the clinically accepted limit of 0.5 log_{10}copies/mL. Thus the performance of HIV RT assay is not affected by the presence of NNRTI (nevirapine/efavirenz) based ART regimen. Also presence of either NRTI associated DRM's (n=6; M41L: 1, D67N: 1, T69D: 1, M184I: 1 and T215S: 2) and NNRTI associated DRM's (n=5; Y181C: 1, K101E: 1 and K103N: 2) showed an acceptable change (<0.4 log_{10} Copies/mL) in mean log_{10} difference from the corresponding value

among wild types. Although the samples with mutations are small, it indicates that the presence of NRTI and NNRTI DRM's did not negatively impact the test performance.

Cost comparison of the assays

The laboratory cost of viral load monitoring of HIV-infected patients analyzed in our cohort by both Abbott m2000rt (HIV-1 RNA) and ExaVir Load v3 (HIV RT) is shown in Table 2. The per test cost of the plasma viral load measure by Abbott m2000rt and ExaVir Load v3 was $36.4 and $16.8 respectively. Thus, by using the ExaVir Load v3, $19.6 per test can be saved. In a laboratory with a pre-existing basic set up for ELISA based assays, the ExaVir Load v3 can be performed at $16.1, saving $20.2 per test. Most of the expense saved by using ExaVir Load v3 were due to (a) lower capital costs (instruments $37750 against $2000) (b) the lower cost of the assay reagents ($15/test against $31/test) (Table 3).

TABLE 3. Cost comparison between HIV RT assay and HIV-1 RNA assay for a laboratory doing 3000 tests/ year

Cost items ($)	Abbott m2000rt Cost ($)	ExaVir Load v3 Cost ($)
Annuitized costs of Capital Instruments	8719	462
Annuitized cost of Operator supplied Instruments#	293	1540
Annual Maintenance Cost	1126	666
Costs of kits per year	93000	45000
Consumables per year	4616	1846
Training time for lab staff to run the test	300	50
Salary costs (3000 tests per year)	1200	1000
Total ($)	109634	50534
Cost/test ($)	**36.4**	**16.8**

1 USD= 60 INR.

Discussion

A good correlation between the HIV RT and the HIV-1 RNA assays was observed in the current HIV-1C predominant setting in India. The agreement between the tests was not significantly affected by the NRTI/NNRTI based anti-retroviral regimen used. Earlier studies performed on panels of different subtypes and recombinants have suggested that the HIV RT assay detects all HIV- 1 and HIV- 2 subtypes with similar efficiency.[14 15 30] This assay, ExaVir Load version 3, can therefore be an attractive option for viral load monitoring in Indian settings.

The current study compared the ExaVir Load assay v3 with Abbott m2000rt HIV-1 RNA assay and observed an excellent correlation (r=0.96). An earlier study from London comparing the same tests observed a similar correlation (r=0.94).[14] Strong correlations between ExaVir Load v3 and Roche HIV-1 RNA-based assays have also been observed by two other studies, by Greengrass *et al.* from Australia (Roche Cobas Amplicor; r=0.85) and Huang *et al.* from China (Roche Cobas TaqMan 48; r=0.95).[16 18] Neither of these studies was performed in HIV-1C dominated settings. The HIV RT assay showed a good agreement with the HIV-1 RNA assay at the clinically important viral load threshold of 1000 copies/mL, which is used by the World Health Organization (WHO) to define viral-failure to first line therapy and is also most often used as the cut-off for drug resistance genotyping.[31] The performance of Exavir Load below 1000 copies/mL is moderate.

In general, we observed an under estimation of viral load of 0.22 $\log_{10}$ RNA copies by the HIV RT assay, which is similar to what have been observed in other studies.[14, 16, [32] These two surrogate assays use very different methods for quantifying the plasma viral load. The HIV-1 RNA assays quantify the amount of viral RNA irrespective of RNA functionality, while the HIV RT assay quantifies the amount of active RT enzyme. The calibration constant used to translate RT activity into RNA copies was estimated from a study of an Australian cohort [18] and is not completely accurate for all combinations of HIV RNA assays and cohorts with varying subtype compositions. The variation observed is, however, well within the acceptable limit of <0.5 log10 copies.

NNRTI drugs bind to the RT enzyme, inhibit its activity and prevent viral replication. Several articles have discussed the possibility that enzymatically inactive RT-drug

complexes could result in under quantification of RT in relation to RNA.[29, 30, [33] These studies were however, cross-sectional and never found any evidence for reduced RT activity during NNRTI therapy. In contrast to previous cohorts, the longitudinal sampling in our study provides optimal material for evaluation of the effects of NNRTI based drug regimens. When comparing HIV viral load data from the same patient cohort before and after onset of ART, we found a mean $\log_{10}$ difference between ExaVir Load v3 and Abbott m2000rt of 0.25 for naïve patients and 0.19 for experienced patients (Table 1). The difference between the tests did thus not increase after onset of therapy. This supports evidence that the current NNRTI containing therapy does not adversely influence the recovery of RT enzyme activity.

On a small number of samples, we assessed if the presence of drug resistant mutations decreased the RT-fitness so as to influence the performance of HIV RT assay. We had 6 samples with single NRTI mutations and 5 samples with single NNRTI mutations and observed no evidence that their presence caused any significant difference in the association between RT-enzyme activity and HIV-1 RNA load. These results were not unexpected and support evidence from previous studies by Napravnik *et al.* and Van Rooijen *et al.* indicating that the presence of NRTI or NNRTI mutations do not affect the relationship between RT-enzyme activity and HIV-1 RNA load. [34 35] Resistance to NRTIs is mediated by a primitive DNA editing function that is introduced into the HIV RT by certain mutations. An energy dependent base excision reaction removes the last base in the growing DNA chain. This requires an energy donator, usually ATP or GTP, and might decrease RT reaction velocity. This happens readily *in vivo*, but the reaction conditions in the current RT assay do not support this reaction. [36]

To our knowledge the current study is the most thoroughly evaluated study of ExaVir Load v3 from India to date. Thus far, there have been three comparative studies from India that have been reported from the states of Andhra Pradesh (Anantpur),[37] Tamil Nadu (Chennai),[7] and New Delhi.[32] Iqbal *et al.* from Chennai cross-sectionally evaluated ExaVir Load assay version 1 and Roche Amplicor Monitor assay. They found a good agreement between the two tests and a significant inverse correlation between ExaVir Load and CD4+ T-cell count.[7] Alvarez-Uria *et al.* from Anantpur compared the accuracy of ExaVir version 3 with Roche Cobas TaqMan HIV-1 test and

Roche Amplicor HIV-1 DNA assay for early infant diagnosis. ExaVir performed well showing 100 % sensitivity and 99 % specificity, but no quantitative correlations were evaluated.[37] A more recent study by Kokkayil *et al.* compared ExaVir Load v3 with Roche Cobas TaqMan among 75 ART naïve patients and reported no statistically significant difference between the two assays.[32]

There are a few drawbacks of the HIV RT assay. The long turnaround time of 48 hours makes it appear labor intensive though the actual hands-on time is approximately 5 hours. The prolonged incubation time is critical to achieve assay sensitivity. For standard performance, the assay requires 1 mL of plasma, which is high in comparison to the requirement for HIV-1 RNA assays, thus limiting its possible usefulness in pediatric populations. However a recent study by Greengrass *et al*. observed that sample volumes down to 0.25 ml with VL>800 copies/mL can be utilized for pediatric monitoring.[19] The ExaVir Load assay does not provide a standard positive and negative control, thus requiring the lab to prepare its own controls, which may compromise the quality assurance of the assay. Additionally, we noted that the quality of the water used for washing is important as impurities and bacterial contaminants present in water may contain polymerases which can create background noise and increase the level of the lower detection limit.

In spite of these caveats, the HIV RT assay has advantages over the HIV-1 RNA assays in resource-limited settings because it is an ELISA based assay and can be performed in any routine lab at a lower cost. The ExaVir Load assay requires a cheaper and maintenance free startup kit as compared to real time assays. We observed that performing the HIV RT assay routinely in our center would save us $20.2 per sample as compared to a HIV-1 RNA assay. A more basic laboratory, which requires installing basic ELISA equipment, would save $19.6 per sample (Table 3).

The use of CD4 cell count as a prognostic marker has been debated, it is argued that this count may not reflect the actual viral load status of the patient.[38] The cost associated with viral load monitoring using HIV-1 RNA assays, despite being lower than PCR assays, is a major limiting factor for its implementation. Currently in India, viral load testing has been phased in to support patients failing first line ART. In the year 2012, about 4157 viral load tests were performed under National AIDS Control Organisation (NACO).[39] Considering $19.6 could have been saved per sample by

performing HIV RT assay, the cost saving for these 4157 viral load tests could have amounted to $ 81,477 if a HIV RT assay had been used.

In our cost-comparison analysis we have used a provider perspective (lab service). We acknowledge that this is a narrower perspective than a societal one, which would include patient costs, opportunity costs among other costs. However the purpose of our analysis was to provide information to laboratories in resource constrained settings, often faced with decisions in the face of tight budgets and thus a societal perspective was not considered necessary. A laboratory manager faced with a limited budget would concentrate entirely on costs that have an immediate impact on her/his own budget; this is the perspective adopted in this study.

NACO is now considering taking up the monitoring approach recommended by WHO to diagnose and confirm ART failure. Considering that there currently are 604,987 HIV-1 infected individuals receiving first line of ART at 380 centers spread across the country the cost reduction of utilizing HIV RT compared to HIV RNA plasma load can be substantial.[40]

Scaling up ART requires the critical support of HIV-1 viral load monitoring. Evidence from the comparative performance of the HIV RT assay with HIV-1 RNA assays from ours and other studies from India, indicate that the ExaVir Load assay could serve as an affordable alternative to monitor patients on ART.

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Authors Contribution

Conceived the Study: UN ADC CK PA AS. Designed the experiments: SG UN HS ADC CK AS. Performed the experiments: SG RPC. External lab monitoring and interim quality assessment: PA. Analyzed the data: SG UN ADC CK AS. Contributed reagents/materials/analysis tools: CK AS. Contributed to the writing of the manuscript: SG RP UN HS PA ADC CK AS.

Competing interests

Clas Källander is an employee of Cavidi AB, Uppsala, Sweden. All the other authors have declared that no competing interests exist.

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Data sharing statement

No additional data available.

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Figure Legends:

Figure 1: Correlation between Abbott m2000rt and ExaVir Load v3 assay for A) all 629 samples showing *r = 0.96* B) 327 baseline (WK00) samples samples showing *r = 0.84* and C) 302 4-weeks post-ART (WK04) samples showing *r = 0.77.*

Figure 2: Bland-Altman plot with 95% CI of limits of agreement between HIV-1 viral loads measured with Abbott Real-Time m2000rt assay and ExaVir Load v3 assay for A) all 629 samples showing a mean bias of 0.22 with 95% limits of agreement ranging from -0.45 to 0.89 B) 327 baseline (WK00) samples showing a mean bias of 0.25 with 95% limits of agreement ranging from -0.39 to 0.89 C) 302 4-weeks post-ART (WK04) samples showing a mean bias of 0.19 with 95% limits of agreement ranging from -0.52 to 0.89.

Figure 1A

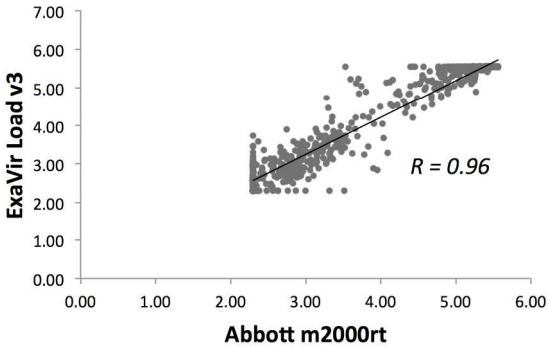


Figure 1B

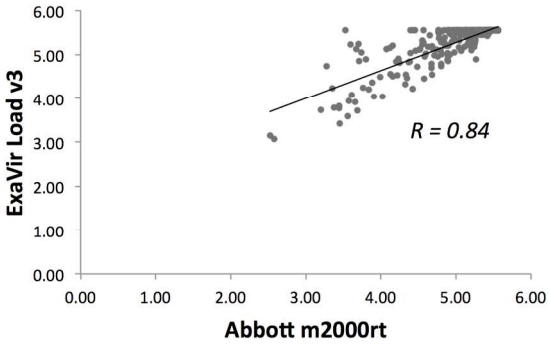
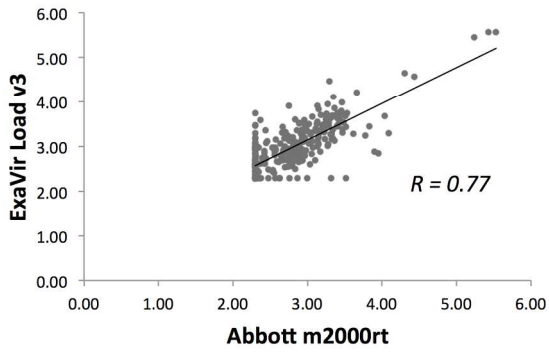


Figure 1C



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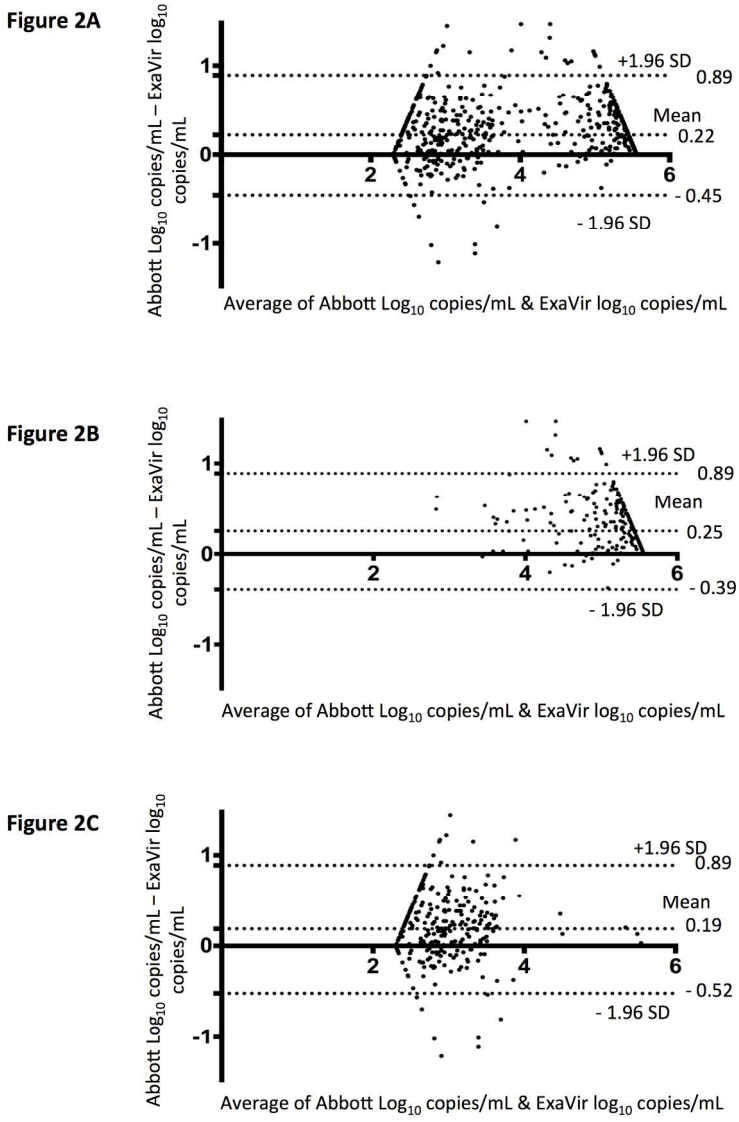

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159x246mm (300 x 300 DPI)