



Research Article

Validation of Small Plasma Volume to Monitor HIV-1 Viral Load by Reverse Transcriptase Enzyme Activity Assay

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Abstract

Background: Reverse transcriptase enzyme activity (RT) assay is recommended to monitor viral load (VL) for HIV-infected patients in resource-limited settings by WHO, which has many advantages such as low-cost, less technical expertise requirement, no contamination issues and excellent-concordance with the gold standard nucleic acid amplification test (NAAT). Monitoring VL in pediatric patients is still a big challenge due the large plasma volume requirement of the test. Our study aims to validate whether small plasma volume is feasible to monitor HIV-1 VL in pediatric patients by RT assay.

Materials and Methods: In RT assay, a gel-separation step isolates virions from plasma components. The virions are then lysed and the lysates undergo a modified ELISA to measure the activity of reverse transcriptase enzyme. Two small plasma volume 0.2 mL [dilution factor 5 - DF5] and 0.5 mL [DF2] (top up to 1mL with HIV-1 sero-negative plasma or saline 0.9%) were compared to undiluted plasma on the linearity percentage to determine plasma volume and topping-up buffer in experiments with the known viral load plasma. The modified RT assay was used to measure VL for 420 pediatric patients in HIVCHI collaboration project between Sweden and Vietnam. All positive VL results were compared to RT-PCR by Bland-Altman test.

Results: Results from DF5 had 95.39% linearity better than 88.68% (DF2) but DF5 was not chosen for further testing due to its instability with higher deviation (10.91 in DF5 vs. 8.02 in DF2). Results from DF2 had 88.86% linearity (top-up with HIV-1 sero-negative plasma) and 96.01% (top-up with saline 0.9%), however, there was no significant difference ($p=0.23$) i.e. 0.5 mL saline 0.9% could be used as top-up buffer for 0.5 mL plasma. There were 25 positive VL over 420 pediatric patients detected by RT assay with 0.5mL plasma volume. The Bland-Altman comparison of results to RT-PCR (NAAT) showed that 95% limits of agreement were between $-1.09 \text{ Log}_{10} \text{ VL}$ and $0.33 \text{ Log}_{10} \text{ VL}$ with mean difference -0.377 (%95CI: -0.524 to -0.229). And the Spearman's paired correlation r^2 was 0.89.

Conclusion: The new RT assay using only 0.5 mL volume plasma (DF2) topped up with saline 0.9% to 1 mL produces VL results with high linearity and comparable to RT-PCR. RT assay with small plasma volume is feasible to monitor HIV-1 VL for pediatric patients.

Keywords

HIV-1 viral load; Reverse transcriptase enzyme activity assay; Pediatric patients

Background

Reverse transcriptase enzyme activity assay (RT assay) is a non-NAAT based on the detection and quantification on HIV viral enzymes and proteins, which can be used as a correlate measure of viral RNA. The RT assay has many advantages such as low-cost, less technical expertise requirement, no contamination issues and excellent-concordance with the gold standard NAAT.

The RT assay commercial kit named ExaVirLoad (Cavidi) had excellent concordance with gold standard HIV-1 NAAT, $r^2=0.96$ was observed with good correlation in paired samples taken at week 0 ($r^2=0.84$) and at week 4 ($r^2=0.77$) of antiretroviral treatment [1]. In Vietnamese adult patients the RT assay had specificity ~ 99% and sensitivity 1fg/ml equivalent 200 copies/mL compared to NAAT showing a correlation of $r^2=0.97$ and good agreement with a mean difference in $\text{Log}_{10} \text{ VL}$ of 0.34 [2]. Other previous studies also showed good correlation compared to NAAT [3-5].

HIVCHI project - collaboration study between Sweden and Vietnam assesses VL in HIV-1 pediatric cohort. Sample requirement of 1 mL plasma limits the implementation of RT assay for small volume sample from pediatric patients. This issue is also challenging NAAT, for instance COBAS TaqMan HIV-1 test also required 1mL of plasma and it was validated with smaller volume plasma diluted with phosphate buffered saline [6]. For RT assay, this issue has not been solved yet. Our study aims to validate whether small plasma volume in RT assay is feasible to monitor HIV pediatric VL.

Material and Methods

ExavirLoad kit version 3 from Cavidi was used to measure VL in experiments with median low limit: 1fg RT/mL equivalent to 200 copies/ml; median high limit: 2,258 fg RT/ml equivalent to 451,600 copies/ml (as in manufacturer's instruction). Pooling HIV-1 positive VL (HIV-pos) plasmas then quantifying with ExavirLoad to make the large amount of known VL plasma was used for experiments. The known VL plasma has the amount RT enzyme (159 fg/mL) equivalent to 31,800 copies/mL (1fg/mL ~ 200 copies/ml as manufacturer's instruction), which were quantified by RT assay. HIV-1 sero-negative (HIV-neg) plasma prepared from blood donor bank was used as top-up buffer. Commercial sterilized saline 0.9% for parenteral (intravenous) application and tissue irrigation was used as top-up buffer. Plasma samples from 420 pediatric patients in HIVCHI were tested with the new modified RT assay.

Methods

In RT assay, a gel-separation step isolates virions from plasma components. The virions are then lysed and the lysates undergo a modified ELISA to measure the activity of reverse transcriptase enzyme. All detail steps were followed as manufacturer's instruction.

Experiment 1: Dilution factor (DF) / Volume of top-up buffer test

Small plasma volume needs topping up with buffer to 1 mL and ExavirLoad limit of detection (200 copies/mL) will increase. To reserve the acceptable limit of detection (<1000 copies/mL), DF2 and DF5 were chosen to test. The limit of detection would be 400 copies/mL in DF2 (0.5 mL plasma) and 1000 copies/mL in DF5 (0.2 mL plasma). HIV-

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neg plasma was used since it could minimize the changes of plasma characteristics. In the experiment 1, HIV-pos plasma (0.2 mL and 0.5 mL) was topped up with HIV-neg plasma to 1 mL and then measured VL. Each DF was done duplex in 1 run and in 3 different runs.

Experiment 2: Top-up buffer test

HIV-neg plasma as top-up buffer would be insufficient for routine test. Meanwhile, commercial sterilized saline 0.9% is often used as top-up buffer in many different ELISA assays and it is ready in a large amount at any laboratory settings. So saline 0.9% was tested as an alternative top-up buffer. HIV-pos plasma was topped up to 1 mL with HIV-neg plasma or saline 0.9% at DF2 and DF5. Each top-up buffer w

Experiment 3 New modified protocol evaluation

The new modified protocol was used to monitor HIV viral load for 420 children under ART at two laboratories (National Pediatric Hospital laboratory and Children's Hospital Number 1 laboratory). All positive VL samples were sent to HIV laboratory of Pasteur Institute Hochiminh city (independent laboratory) for testing with RT-PCR (NAAT).

Data analysis

The linearity percentage is calculated as in the following formula:

$$\% \text{ Linearity} = (\text{observed concentration} * \text{dilution factor} * 100) / \text{undiluted concentration}$$

Mean linearity percentage should be 80 – 120%, preferably 90 – 110%. Bland-Altman plot (Bland & Altman, 1986 and 1999), or difference plot, is a graphical method to compare two measurement techniques. In this graphical method the differences (or alternatively the ratios) between the two techniques are plotted against the averages of the two techniques. Alternatively (Krouwer, 2008) the differences can be plotted against one of the two methods, if this method is a reference or “gold standard” method. Horizontal lines are drawn at the mean difference, and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences. If the differences within mean \pm 1.96 SD are not clinically important, the two methods may be used interchangeably.

Spearman's paired correlation was used to test for result association between paired samples (RT and NAAT). Spearman's correlation coefficient $[-1 \leq r \leq 1]$ measures the strength of association between two ranked variables. A perfect positive correlation has a coefficient of 1.0; a perfect negative correlation has a coefficient of -1.0. When there is no association between two variables, the correlation coefficient has a value of 0. A good positive correlation should be from / above 0.70.

Results

DF2 (0.5 mL HIV-pos plasma + 0.5 mL HIV-neg plasma) produced 88.68 % linearity of RT enzyme amount (fg/mL) to 1 mL plasma. DF5 could produce better linearity (95.39% vs. 88.68%) but its deviation was higher than DF2 (10.91 vs. 8.02) i.e. the stability of DF2 was higher than DF5. So DF2 data were used for analyzing in the experiment 2.

HIV VL monitoring in 420 HIV-infected children, 25 samples were positive with Log_{10} VL mean is 4.47 ± 0.63 [3.06 – 6.12] (RT assay) and 4.85 ± 0.77 [3.60 – 6.93] (RT-PCR). The Bland-Altman comparison of results from two assays (RT assay vs. RT-PCR) showed that the mean difference was $-0.377 \text{ Log}_{10} \text{ VL}$ (%95CI: -0.524 to -0.229) and the rank of agreement was from $-1.09 \text{ Log}_{10} \text{ VL}$ to $0.33 \text{ Log}_{10} \text{ VL}$. The mean difference -0.377 is

nearly equal 0, it means that the difference is not clinically important, the two methods may be used interchangeably (Figure 1). Furthermore, the Spearman's paired correlation with 0.89 of two assay results showed that the new modified RT assay worked well with small plasma volume from pediatric samples (Figure 2).

Discussion

HIV-1 pediatric VL is challenging for both RT and NAAT assay due to the small plasma volume of samples. For RT assay, Vicki Greengrass et al. had validated small volume plasma (0.5 mL and 0.25 mL) top-up with HIV-1 sero-negative plasma produced the results not significantly different to those obtained from 1mL of plasma ($p=0.17$) [7]. In our study, smaller volume plasma (0.2 mL - DF5) top-up with HIV-1 sero-negative plasma could produce comparable VL result to undiluted plasma with linearity 95.39 % even higher than 0.5 mL (88.68%). However, to avoid the dilemma in result interpretation due to the same value 1000 copies/ml (limit of detection of assay and WHO cut-off of virological treatment), 0.5 mL - DF2 was chosen as the small volume plasma in the new modified ExaVirLoad protocol for HIV-1 pediatric VL test and Vicki Greengrass studied on small plasma volume in RT assay used HIV-1 sero-negative plasma as top-up buffer [7]. HIV-1 sero-negative plasma can minimize the changes in plasma sample (viscosity, pH, anti-coagulation) hence it is considered as a good top-up buffer, especially for ExaVirLoad kit since it has some reactions with the ionic binding virions to gels. However, it is impossible to apply HIV-1 sero-negative plasma in routine. Blood donor plasma is prioritized for medical treatment, not for laboratory works (Tables 1 and 2). Meanwhile, saline 0.9% is cheap, readily available and often used as dilution buffer in many laboratory assays

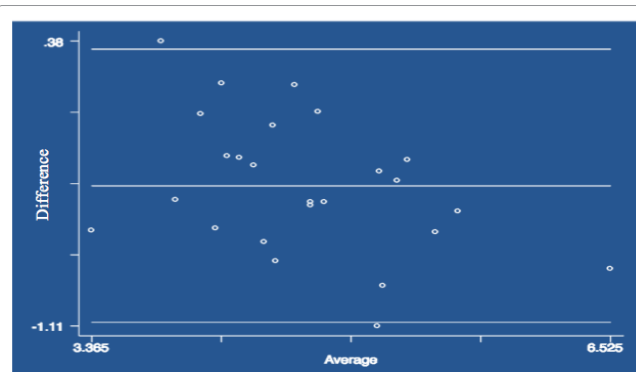


Figure 1: Bland-Altman plot Log_{10} VL results between RT assay and RT-PCR.

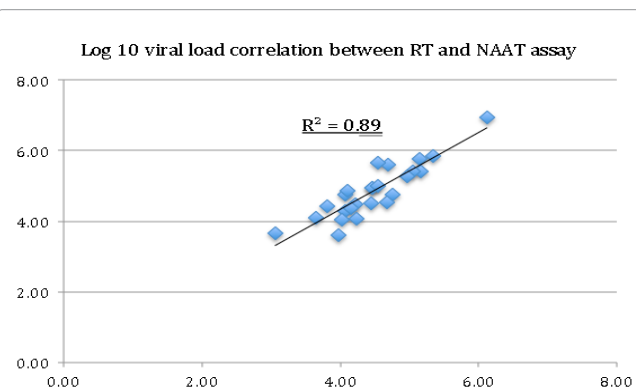


Figure 2: Spearman's paired correlation Log_{10} VL between RT assay and RT-PCR.

Table 1: RT enzyme (fg/mL) and % linearity of DF2, DF5 to DF0.

RUN	Results in DFs	RT enzyme (fg/mL) [% linearity]		
		DF0 HIV-pos (1mL)	DF2 HIV-pos (0.5 mL) HIV-neg (0.5 mL)	DF5 HIV-pos (0.2 mL) HIV-neg (0.8 mL)
1 st	159 [100%]		149 [93.71%]	167.5 [105.35%]
2 nd			151 [94.97%]	160 [100.63%]
3 rd			123 [77.36%]	127.5 [80.19]
RT enzyme amount (mean)			141 ± 12.75	151.67 ± 17.36
% Linearity (mean)			88.68 ± 8.02	95.39 ± 10.91

Table 2: RT enzyme (fg/mL) and % linearity of top-up buffers.

RUN	Results in Buffers	RT enzyme (fg/mL) [% linearity]		
		HIV-pos (1mL)	HIV-neg (0.5 mL) HIV-pos (0.5 mL)	Saline 0.9% (0.5 mL) HIV-pos (0.5 mL)
1 st	159 [100%]		149 [93.71%]	166 [104.4%]
2 nd			151 [94.97%]	143 [89.9%]
3 rd			123 [77.36%]	149 [93.71%]
RT enzyme amount (mean)			141 ± 12.75	152.67 ± 9.74
% Linearity (mean)			88.68 ± 8.02	96.01 ± 6.12
p-value			0.23 ^c	

^(c)No significant difference in mean % linearity (88.86% vs. 96.01%), t-test paired data

since it does not make dramatically changes in plasma properties like pH, ionic concentration [8]. Our study was the first study using saline 0.9% as top-up buffer in RT assay. The phosphate buffered saline (1× PBS) was used as top-up buffer in NAT, but it was not considered in RT assay. PBS has a strong ionic strength so it may interfere the binding reaction in RT assay. Interestingly, the linearity percentage with saline 0.9% as top-up buffer was even better than HIV-1 sero-negative plasma (96.01% vs. 88.68%). Perhaps, the reaction with 50% (v/v) plasma and 50% (v/v) saline 0.9% has less reaction inhibitors than the reaction with 100% (v/v) plasma. With less inhibitor, the reaction yield might be higher. This has been shown for other reactions especially NAT (nucleic acid amplification technology) with pre-treatment procedures such as dilution of the samples, storages in refrigerator/freezer, re-suspending dried endocervical swabs in saline, delayed testing, etc., can decrease the number of PCR-inhibited samples [3,5].

In previous studies, ExaVirLoad (1 mL of plasma) for adults was compared to TaqMan PCR (Roche) with the correlation $r^2=0.97$ and a good agreement with a mean difference in \log_{10} VL of 0.34 [2,5,7]. In our study, Spearman's paired correlation ($r^2=0.89$) was lower than previous studies. The correlation may be affected from small plasma sample [9-11]. The Bland-Altman comparison with mean difference -0.377 showed that RT assay produced VL results lower than NAAT assay. As only 25 samples with detectable viral load were tested on standard NAAT, the RT assay may have been under-quantifying the VL, which would not have been picked up by the new modified RT assay. This is considered as a major limitation of this study.

Conclusion

The small volume plasma for RT assay is 0.5 mL, reducing 50% compared to sample requirement (1 mL plasma). The saline 0.9% can be used as top-up buffer in RT assay. The new modified RT assay produced VL results comparable to NAAT assay. RT assay with small plasma volume (0.5 mL) is feasible to monitor HIV viral load for pediatric patients.

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