

Evaluation of the Cavidix Exavir load reverse transcriptase assay (version 3.0) for HIV viral load against the Roche Amplicor Monitor (version 1.5) for use in monitoring viral load in patients infected with the HIV -1 virus.

By

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DECLARATION

I, Norah Gwete hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a degree to any other University or Institution.

Signed.....

ABSTRACT

Title: Evaluation of the CaviDi ExaVir load Reverse Transcriptase Assay (version 3.0) for HIV Viral Load against the Roche Amplicor Monitor (version 1.5) for use in monitoring viral load in patients infected with the HIV virus.

Introduction. There are two types of HIV, HIV-1 and HIV-2 and both types are known to cause AIDS, although infection with HIV-1 is more common worldwide. The concentration of HIV RNA in plasma is a critical marker for predicting disease progression and for monitoring the efficacy of antiretroviral drug therapy. However, most viral load assays are not readily available in Zimbabwe due to the complexity and the need for nucleic acid amplification hence the need to explore other available alternatives.

The objective of this study was to evaluate the CaviDi ExaVir load Reverse transcription Assay Version 3.0 for quantifying HIV-1 viral load using the Roche Amplicor monitor version 1.5 as the gold standard.

Method

This was a cross sectional comparative study of two kits where 21 whole blood samples being submitted for viral load testing at Flow cytometry centre were centrifuged and separated within 2 hours of collection and then stored for batched testing with 2 aliquots per sample. The samples were then thawed for testing using the CaviDi Exavir load v 3.0 at flow cytometry centre. The second aliquot was tested using the Roche Amplicor Monitor v1.5 at UZ-UCSF laboratory. The results were then correlated using STATA biostatistics software.

Results: There was a very strong linear correlation in viral load measurement between the CaviDi Exavir Load v 3.0 and the Roche Amplicor Monitor v 1.5 ($r=0.94$ and $p < 0.05$).

85.7% of the samples showed an agreement of $\leq 0.68 \log_{10}$ between the two methods (fivefold) which is equal to the total variation that can be expected in viral load measurements.

Conclusion: The CaviDi Exavir load v 3.0 correlates strongly with the Roche Amplicor monitor v 1.5 in measuring plasma viral load concentrations and can be used to monitor viral load in clinical specimens.

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ABBREVIATIONS

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
RNA	Ribonucleic Acid
RT	Reverse transcriptase Assay
PCR	Polymerase Chain Reaction
DNA	Deoxy Ribonucleic acid
MDG	United Nations Millennium Development Goals
ARV	Anti Retroviral Drugs
FDA	Food and Drug Administration
ART	Anti retroviral therapy
VCT	Voluntary Counseling and testing
ZAPP	Zimbabwe AIDS Prevention Project
ANC	Anti Natal Clinic
Ft	Femtograms
ML	Milliliter
IRB	Institutional Review Board
MRCZ	Medical research Council of Zimbabwe
μ	Microlitre
CDNA	Complementary DNA
AF	Amplification Failure

CHAPTER 1: INTRODUCTION

1.1 Background

The Human Immunodeficiency Virus (HIV) was first identified in 1983[1]. The elucidation of the complete nucleotide sequence of the viral genome followed soon afterwards and the viral proteins were characterized. There are two types of HIV, HIV-1 and HIV-2. Both types are known to cause AIDS, although infection with HIV-1 is more common worldwide. HIV-1 is a retrovirus of the lentivirus subfamily. Retroviruses are RNA viruses that replicate via DNA intermediates using viral enzyme reverse transcriptase (RT). The mature virion of HIV- 1 is an icosahedral sphere with a diameter of approximately 100nm. The outer envelope, which is formed from the host cell membrane, is a lipid bilayer that contains host cell proteins and spikes of the viral protein envelope glycoproteins (gp120 and gp 41). Inside the lipid bilayer are the internal structural capsid and core proteins p17, p24, p7 and p6[2]. These proteins enclose two copies of the single- stranded RNA genome and multiple reverse transcriptase molecules in the virus particle. The enzyme reverse transcriptase catalyzes the synthesis of proviral DNA using the viral RNA as a template. [2]

Zimbabwe is a southern African country with a population of 11.6 million in 2002. Approximately 58% of the population resides in rural areas, 32% in urban areas and 10% in areas that are not classified as strictly urban or rural. The first AIDS case was identified in 1987 and sentinel surveillance of pregnant women receiving antenatal care services at public clinics has been ongoing since 1989. The government of Zimbabwe remains committed to meeting targets set by world health Assembly declaration of 1991 and the United Nations Millennium Development Goals (MDG) of halting and reversing the HIV epidemic by 2015 as well as meeting targets of the 3

by 5 initiative for scaling up antiretroviral therapy. To this end there are several programmes that are providing antiretroviral therapy to the general public in Zimbabwe at this stage in time[3].

Tremendous strides have been made in treating HIV -1 infection in industrialized countries. Combination therapy with antiretroviral (ARV) drugs suppresses virus replication, delays disease progression and reduces mortality. The concentration of HIV RNA in plasma is a critical marker for predicting disease progression and for monitoring the efficacy of antiretroviral drug therapy[4, 5]. In industrialized countries plasma viral load assays are used in combination with CD4 cell counts to determine when to initiate therapy and when a regimen is failing. The best and clearest way to show that ARVs are working to control HIV is through monitoring viral load and CD4/CD8 cells before and during treatment.[5]

Human immunodeficiency virus type 1 (HIV-1) viral load has become the mainstay for monitoring antiretroviral (ARV) therapy for HIV infection. To this end there are three viral load assays that have been approved by the Food and Drug Administration (FDA) namely the Amplicor HIV-1 Monitor test from Roche Diagnostics, Indianapolis, the Quantiplex HIV-1 assay (bDNA by Bayer Corporation New Jersey) and the Nuclisens HIV-1 QT assay by bioMerieux Inc[5, 6]. However, these routinely used viral load assays are based on amplification of nucleic acid and as a result require skilled technicians, dedicated laboratory space, and complex equipment and are generally expensive. As a result, these tests are not readily available in areas where resources are limited[5]. Currently in Zimbabwe RNA monitoring of HIV viral loads is not accessible to the general public. It is only available to research participants and the people in the more affluent levels of society. An inexpensive and technically less demanding approach to quantify HIV-1 would be of great value for

places where nucleic acid testing is impractical or prohibited because of resource limitations. Two potential methods include an assay that detects virion associated reverse transcriptase activity (Cavidi Tech) and a “boosted” p24 antigen assay (Perkin Elmer life sciences) that uses heat dissociation to allow detection of HIV-1 p24 antigen with sensitivity and reproducibility reported to be comparable to those of RNA viral load testing. Most studies to date have been carried out with HIV-1 subtype B-infected patients although a few studies suggest that the Cavidi RT assays may also work with non-B subtypes. In this study only one of them; The Cavidi Tech Reverse Transcriptase Assay will be evaluated against the Roche Amplicor Monitor V1.5 which is assay most commonly used in the country at the moment. This is important in Zimbabwe because it does not require sophisticated equipment and also most of the hospitals at the provincial and district levels already have the basic equipment needed to carry out this assay.

1.2 Literature Review

The ExaVir load kit is intended for determination of the activity of the enzyme Reverse Transcriptase as a marker of retroviral replication.

The ExaVir Load kit procedure is divided into two main parts: the separation part and the RT-Assay. In the separation part the plasma is first treated to inactivate cellular enzymes and then virus particles are then separated from the plasma by use of a gel that binds the virion. At this stage disturbing factors such as antibodies and antiretroviral drugs are washed away. The virion is then lysed to obtain the reverse transcriptase and this is collected for analysis using the RT-assay. In the RT –assay the lysates are added to a 96 microplate well which has wells coated with an RNA template at the bottom together with a reaction mixture containing primer and RT substrate. If the lysates contain any RT the enzyme will synthesize a DNA strand

which is detected colorimetrically using an alkaline phosphatase conjugated alpha-BrdU antibody. The product can then be quantified by addition of a colorimetric AP substrate. The difference between version 3.0 and the earlier versions is mainly in sensitivity. Version 3.0 of the assay has a lower limit of detection of 200 RNA copies per ml as compared to 400 copies/ml for version 2.0 of the assay.

Performance of the CaviDi ExaVir load has been evaluated against a number of different HIV viral load assays and the various results indicate that there is potential for the test to provide good estimates of plasma viral load at a lower cost than the conventionally used HIV-1 RNA assays. Some of the evaluation results from literature are discussed below. The CaviDi Tech ExaVir load has been shown to have a good correlation with the Roche Amplicor Monitor version 1.5 in HIV-1 subtype B populations. Stevens and colleagues compared the Roche RNA assay with both P24 and the ExaVir version 1.0 and found excellent correlation between RNA and RT results. Crowe and colleagues have tested the more sensitive version 2 of the ExaVir load in patients in Australia and reported a 95% sensitivity in samples with HIV RNA levels > 1 000 copies/ml and also that RT activity closely followed the trend for HIV-1 RNA levels in samples in longitudinal studies [5].

Seyoum and colleagues monitored the change in Human immunodeficiency virus viral load using version 1.0 of the Reverse transcriptase assay and an RNA based assay in samples from a prospective study of HIV-1 subtype C infected, untreated Ethiopians followed twice yearly over a period of up to five years. They established that there was significant correlation between the data obtained by RT assay and by the Nuclisens HIV QT test. They also established that during follow up the median RT and RNA levels increased more or less in parallel up to approximately four times

the values at admittance while CD4 cell counts which had already been determined previously were decreasing thereby demonstrating an inverse correlation between CD4 T-cell counts and RT activity.[7] In another study Greengrass and colleagues demonstrated a positive correlation between the $\log_{10}$ HIV RNA copies/ml and $\log_{10}$ HIV RT fg/ml using Spearman's rank correlation ($\rho = 0.92$, $p < 0.0001$) in a population presumed to be subtype B.[8] Jennings and colleagues demonstrated a sensitivity of 54 to 100% using samples spiked with subtypes A,C,D and F at viral load concentrations $>10\,000$ copies/ml and a sensitivity of 68% when the RT assay was applied on clinical samples.[9] Jennings and colleagues evaluated the CaviD ExaVir load Version 2.0 assay with the Roche Amplicor HIV-1 Monitor test v 1.5 using panels of clinical samples (subtype B) from HIV positive subjects and HIV spiked samples (subtype A, C, D, CRF_01AE, CRF_02AG, and F). The CaviD assay detected 54% to 100% of spiked samples with virus loads $>10\,000$ copies /ml and 68% of clinical samples with a correlation coefficient $r = 0.84$ to 0.99 and they concluded that the CaviD Reverse transcription assay offers a feasible alternative to frequent HIV RNA testing in resource-limited settings but will need to be augmented with less frequent confirmation testing [9].

In Zimbabwean samples the version 2.0 of the CaviD ExaVir assay was evaluated using the Bayer Versant kit and the Nucleisens assay and was demonstrated to have very good correlations but there is no Zimbabwean data on how the test compares to the Roche Amplicor monitor which is the kit currently used in the country [10]. A critical review of the existing literature shows that few data for version 1.0 and 2.0 is available on the assay and even less for version 3.0 of the assay. There is currently no Zimbabwean data on how version 3.0 of the CaviD ExaVir load compares against the Roche Amplicor Monitor version 1.5 which is the more readily available test amongst

the three FDA approved assays for viral load. The differences in the versions of the CaviDi Reverse transcriptase assay mainly in sensitivity. Version 3.0 of the assay now has a lower limit of detection of 200 RNA copies/ml as compared to 400 copies/ml for version 2.0 of the assay.

A critical review of literature reveals that the CaviDi ExaVir load version 2.0 is a good candidate for a more affordable alternative viral load assay for a resource limited country and is comparable in terms of sensitivity, specificity, precision, reproducibility, dynamic range and linearity. However most of the assays were done on non B subtypes with only a few C subtypes. There is therefore need for Zimbabwe to determine if the assay will quantify subtypes common in the region (subtype C) and if the technology is appropriate for the technical staff, available laboratory equipment and infrastructure (water, reliable electricity, air conditioning, refrigeration and other equipment).

1.3 Justification of the study

The human immunodeficiency virus (HIV) pandemic has affected countries worldwide, but the impact on resource-limited countries has been especially devastating. Tremendous strides have been made in treating HIV-1 infection in industrialized countries. Combination therapy with antiretroviral (ARV) drugs suppresses virus replication, delays disease progression and reduces mortality. Pressure to lower the cost of antiretroviral therapies (ART) has been critical in fighting this battle so that ART can be accessible to people living with HIV/AIDS in resource limited countries. To this end ART is now fairly accessible to eligible patients (according to the WHO guidelines) in Zimbabwe through the national programmes and other Non governmental Organizations and churches. Optimal

management of HIV -1 disease requires accurate quantitation of viral RNA concentrations in plasma. In industrialized countries plasma viral load assays are used in combination with CD4 counts cell counts to determine when to initiate therapy and when a regimen is failing [5].

In Zimbabwe ARVs are now generally available through the national ARV roll out program and CD4 counts are reasonably available through the public health system. However the routinely used viral load assays which are based on nucleic acid amplification are generally expensive and are not readily available in resource limited settings. Currently, in Zimbabwe RNA monitoring of Viral load is mainly available to research participants and people in the more affluent levels of society. An inexpensive and technically less demanding approach to quantify HIV-1 would be of great value for places where nucleic acid testing is impractical or prohibited because of resource limitations. The current challenge is to identify simplified assays for monitoring patients on ART that are less expensive and less technically demanding with respect to facilities and instrumentation without a compromise in assay quality in terms of sensitivity, specificity, precision, reproducibility, dynamic range and linearity[9].

In Zimbabwe version 2.0 of the CaviDi ExaVir load assay was evaluated using 96 plasma samples collected from Howard hospital using the FDA approved Nuclisens assay as the gold standard. The Nuclisens assay was done in Canada because the assay is not routinely done in Zimbabwe and the CaviDi ExaVir load was done at Howard hospital. The study demonstrated a very good correlation between the ExaVir load and the Nuclisens assay with a correlation coefficient $r^2 = 0.90$ and a lower detection limit or sensitivity of 300 copies /ml. However there is no data in Zimbabwe as to how the CaviDi ExaVir load assay compares to the Roche Amplicor monitor 1.5

which is the gold standard in the country at the moment. Furthermore the CaviDi Tech of Uppsala Sweden has since moved to version 3.0 and there is no data yet in Zimbabwe as to how this version compares to the more commonly used Roche Amplicor Monitor version 1.5. The main aim of this study was to evaluate the CaviDi ExaVir load version 3.0 against the more commonly used Roche Amplicor monitor assay to determine if the assays performance as compared to the Roche Amplicor was acceptable.

1.4 Research question

Can the CaviDi Reverse Transcriptase Assay (ExaVir load version 3.0) be used to monitor HIV viral load in our Zimbabwean population with non B HIV subtypes.

Null hypothesis

There is no correlation in HIV viral load between the CaviDi ExaVir load v 3.0 and the Roche Amplicor monitor v 1.5 ($H_0: r=0$)

Alternative Hypothesis

There is a correlation in viral load between CaviDi ExaVir load v 3.0 and the Roche Amplicor monitor v 1.5 ($H_a: r \neq 0$)

1.5 Aims and objectives

Main Aim: To evaluate the correlation of viral load results obtained using CaviDi ExaVir load Reverse transcription Assay with those obtained using the Roche Amplicor Monitor v1.5. The CaviDi ExaVir Load Reverse Transcriptase assay uses HIV RT enzyme purified from plasma samples to catalyze the conversion of RNA to cDNA.

Specific objectives:

- 1) To determine viral load in plasma samples using the CaviDi Exavir reverse transcriptase assay (version 3.0).
- 2) To determine viral load using in the same plasma samples using the Roche Amplicor Monitor v 1.5.
- 3) To correlate the results obtained using the CaviDi assay to those obtained using the Roche assay.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

The reagents required were 1 kit of the Cavidix Exavir load assay and 1 kit of the Roche Amplicor assay. Please see appendix C for a complete list the materials required for the Cavidix assay. Please see appendix E for a package insert with the complete list of materials required for the Roche Assay.

2.2 Study design

This was cross sectional laboratory comparative study.

2.3 Setting

The study took place at the Flow Cytometry centre which is private lab in Harare Zimbabwe for the Cavidix assay and the UZ-UCSF laboratory in the Department of Obstetrics and Gynaecology, University of Zimbabwe for the Roche Amplicor monitor testing

2.4 Reference population

The reference population was HIV positive patients seeking medical care in Harare.

2.5 Source population

The source population was patients who were submitting samples for viral load testing at the Flow cytometry centre.

2.6 Study Factor

The study factor is performance of the Cavid Reverse Transcriptase Assay in quantifying HIV-1 viral load in plasma as compared to the Roche Amplicor Monitor v1.5 assay.

2.7 Outcome factor

The outcome factors were viral load measurements for the Roche and Cavid Assays.

2.8 Sample Size

The calculated sample size was 109 plasma samples for 80% power at the 5% level of significance for a two tailed test (See Appendix A for sample size calculations).

2.9 Laboratory methods

The 22 whole blood samples (EDTA) were centrifuged and then the plasma was separated from the red blood cells within 2 hours of collection and stored frozen at -20 degrees Celsius until testing (3-6 days for the Cavid Assay) and at -70 degrees for 6 months for the Roche Amplicor Assay. For the study only plasma samples which had sufficient volume (2 mls or more) to be run using both methods were selected. Before testing the samples on the Cavid Exavir load v3.0 they were thawed at room temperature and vortexed (see Appendix C for full method). The Cavid ExaVir Load Reverse Transcriptase assay uses HIV RT enzyme purified from plasma samples to catalyze the conversion of RNA to cDNA. The virus particles were purified from plasma using a virion binding gel and bound virions were washed to remove inhibitors including antiretroviral RT inhibitor drugs or antibodies. The virions were then lysed and the lysates were transferred to a 96 well plate assay of RT activity. In an overnight incubation, RT enzyme in the lysate incorporated BrdUTP into a DNA strand complementary to the polyA template which is bound to the 96 -micro well plate. Subsequently an anti-BrdU antibody conjugated to alkaline phosphatase was added and the amount of incorporated BrdU is detected using a substrate.

The color intensity of each well was read using a standard plate reader at a wavelength of 405 nm with a reference filter of 620nm. Results were then compared to a standard curve and HIV RT activity was determined and expressed as femtograms (fg) HIV RT activity/ml plasma (fg/ml). RT activity (fg/ml) was then converted to HIV RNA copies/ml equivalents using the conversion factor supplied by the manufacturer[12]. Cavid RT assay was done at Flow cytometry centre and the results were noted down. A negative and positive control was included in the assay and Good Clinical and Laboratory Practices were followed. On visual inspection none of the samples were haemolysed.

An aliquot of the same plasma sample was then taken to UZ-UCSF laboratory for testing using the Roche Amplicor Monitor v 1.5 assays. The samples were transported on ice and were checked for thawing at arrival at the UZ-UCSF lab and all of them were noted to be still frozen after the transfer. For the Roche Amplicor HIV-1 Monitor test .v1.5 the five major processes namely specimen preparation, reverse transcription of target RNA to generate cDNA, PCR amplification of target cDNA using HIV-1 specific complementary primers, hybridization of amplified products to oligonucleotide probes specific to targets and detection of the probe-bound amplified products by calorimetric determination were done at UZ-UCSF laboratory. The Amplicor HIV-1 Monitor test allows the simultaneous reverse transcription and PCR amplification of HIV-1 and HIV-1 quantitation standard RNA. The quantitation of HIV viral RNA was performed using the HIV-1 Quantitation Standard which was incorporated in the master mix (see Appendix D for full method).

After testing the optical densities for each sample were recorded on the worksheets. Results for the Cavid RT assay were then calculated using the Cavid RT analyzer program and those for the Roche Amplicor assay were calculated using the Roche

RNA excel calculation program. Controls were included in the run and all the equipment that was used was serviced as per the laboratory's Quality Management System. Good clinical and laboratory practices were followed during performance of the Roche Amplicor Monitor test.

2.10 Ethical Considerations

Permission was sought from the Flow cytometry centre to carry out the study in that laboratory and it was granted. Permission was granted on the understanding that the samples would be unlinked to the participants so names or other demographic data like age sex were collected. Permission was also sought from the Laboratory Director at UZ-UCSF to use the laboratory for Roche Amplicor Monitor v 1.5 testing and was granted. The results of the study will not be disseminated to participants but will be shared with Flow cytometry centre laboratory for use in verifying the accuracy of the method as this was a validation procedure.

CHAPTER 3: RESULTS

3.1 Data presentation and description

The viral load measurements that were obtained from the two assays are shown in table as well as the differences in viral load measurements between the 2 methods are shown in below.

Table 1. Viral load Results

Sample Identity	Cavidi Result (RT femtograms/ml)	ExaVir (RT Equivalence/ml)	Roche Amplicor result (RNA copies /ml)	Difference copies/ml
1	<1.0	<200	<400	0
2	6	1 101	2744	1 643
3	13	2 529	5817	3 288
4	8	1 584	9283	7 699
5	130	26 049	165926	139 877
6	644	128 725	119000	9 725
7	25	4 985	6915	1 930
8	221	44 219	292665	248 446
9	12	2 414	1670	744
10	79	15 705	52047	36 342
11	129	25 708	110211	84 503
12	494	98 697	129043	30 346
13	95	18 982	38077	19 095
14	266	53 236	107822	54 586
15	1124	224 706	117387	107 319
16	1035	206 990	297634	90 644
17	131	26 103	22331	3 772
18	14	2 867	AF	n/a
19	<1.0	<200	<400	0
20	<1.0	<200	<400	0
21	<1.0	<200	<400	0
22	25	4983	6544	1 561

The lower limit of detection for the Cavidi assay was the equivalents of <200 RNA copies/ml and that of the Standard Roche Amplicor was <400 copies/ml. 4 samples had undetectable viral loads on both the Cavidi RT assay and the Roche Amplicor assay. Out of the 22 samples that were analyzed 1 sample had a detectable viral load using the Cavidi RT assay but had inhibition on the Roche Amplicor Monitor so viral

load for the Roche Assay could not be determined. This sample was excluded from the analysis.

3.2 Statistical Analysis

The data was cleaned to make sure that there are no typographical errors in the calculation and interpretation of the viral load measurements for all the samples. The results for the CaviDi ExaVir load version 3.0 were obtained as RT femtograms/ml and then converted to copies /ml equivalent using the software supplied by the manufacturer.

Viral load measurements for both the Roche Amplicor Monitor 1.5 and the CaviDi RT assay viral load measurements did not follow a normal distribution.

Both variables CaviDi HIV RNA result and Roche Amplicor HIV RNA copies/ml were log₁₀ transformed prior to statistical analysis. To determine the best method to analyze the data viral load measurements from both variables were assessed to find out if viral load measurements followed a normal distribution. For purposes of result analysis CaviDi viral load concentrations of <200 copies/ml were handled as <400 copies/ml.

Log 10 RNA copies between the two methods were calculated and are presented in table 2 below.

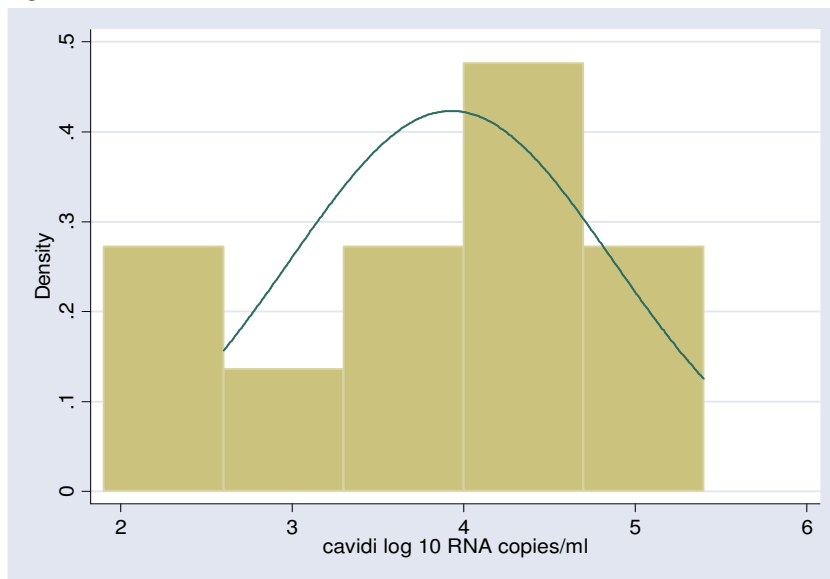
Table 2 Log 10 RNA copies/ml

Specimen Number	Sample ID	Cavidi ExaVir Result log 10 RNA copies/ml Equivalent	Roche Amplicor result log 10 RNA copies /ml	Difference in log 10 viral load copies/ml
1		<2.3	<2.6	0
2		3.0	3.4	0.4
3		3.4	3.8	0.4
4		3.1	4	0.9
5		4.4	5.2	0.8
6		5.1	5.1	0.0
7		3.7	3.8	0.1
8		4.6	5.5	0.9
9		3.4	3.2	-0.2
10		4.2	4.7	0.5
11		4.4	5.0	0.6
12		5.0	5.1	0.1
13		4.3	4.6	0.3
14		4.7	5.0	0.3
15		5.4	5.1	-0.3
16		5.3	5.5	0.2
17		4.4	4.3	-0.1
18		3.5	QS failure	Qs failure
19		<2.3	<2.6	0.0
20		<2.3	<2.6	0.0
21		<2.3	<2.6	0.0
22		3.7	3.8	0.1

The distribution of the differences in log 10 RNA copies/ml between the two methods was assessed for normality and the results are shown below in Figure 1 below.

After log₁₀ transformation the distribution of viral load measurements using Roche Amplicor showed an approximately normal distribution

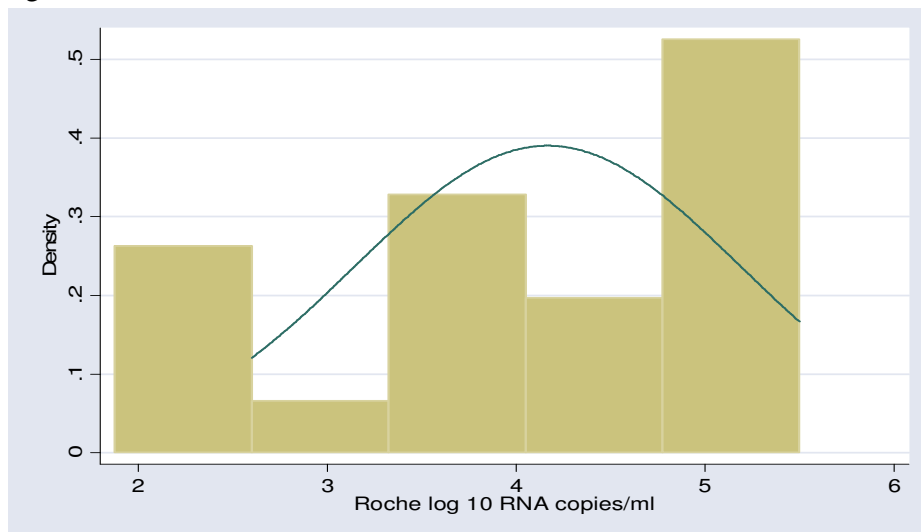
Figure 1



Normal density curve for CaviD log 10 RNA copies /ml measurements.

After log10 transformation the distribution of RNA viral load measurements using the CaviD RT assay showed an approximately normal distribution.

Figure 2

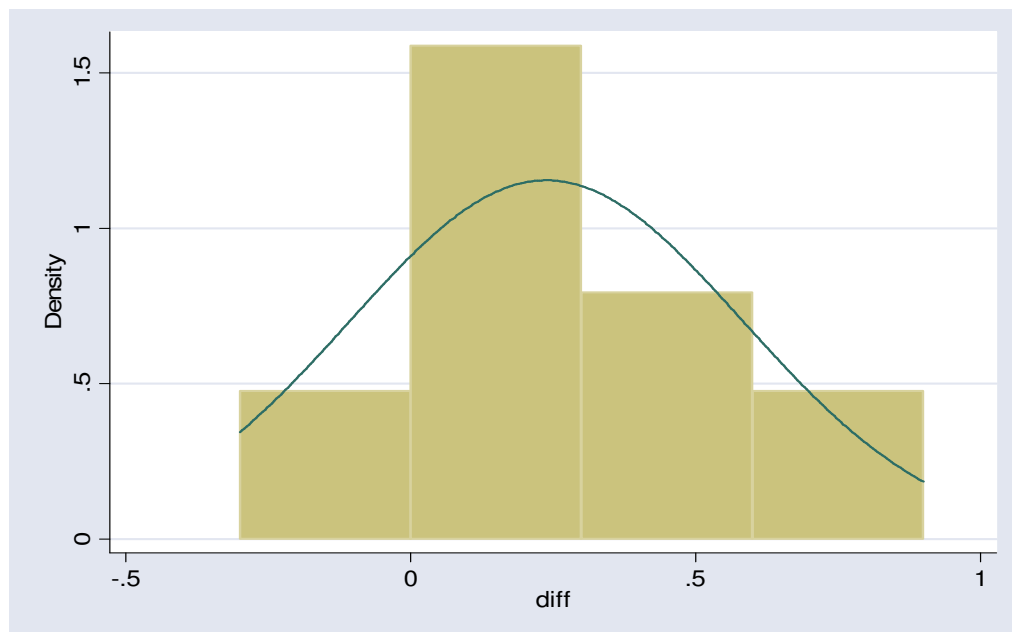


Normal Density Curve for Roche Viral Load Measurements

After log10 transformation the distribution of viral load measurements using Roche Amplicor showed an approximately normal distribution

The distribution of the differences in log 10 RNA copies/ml between the two methods was assessed for normality and the results are shown below in Graph 3 below.

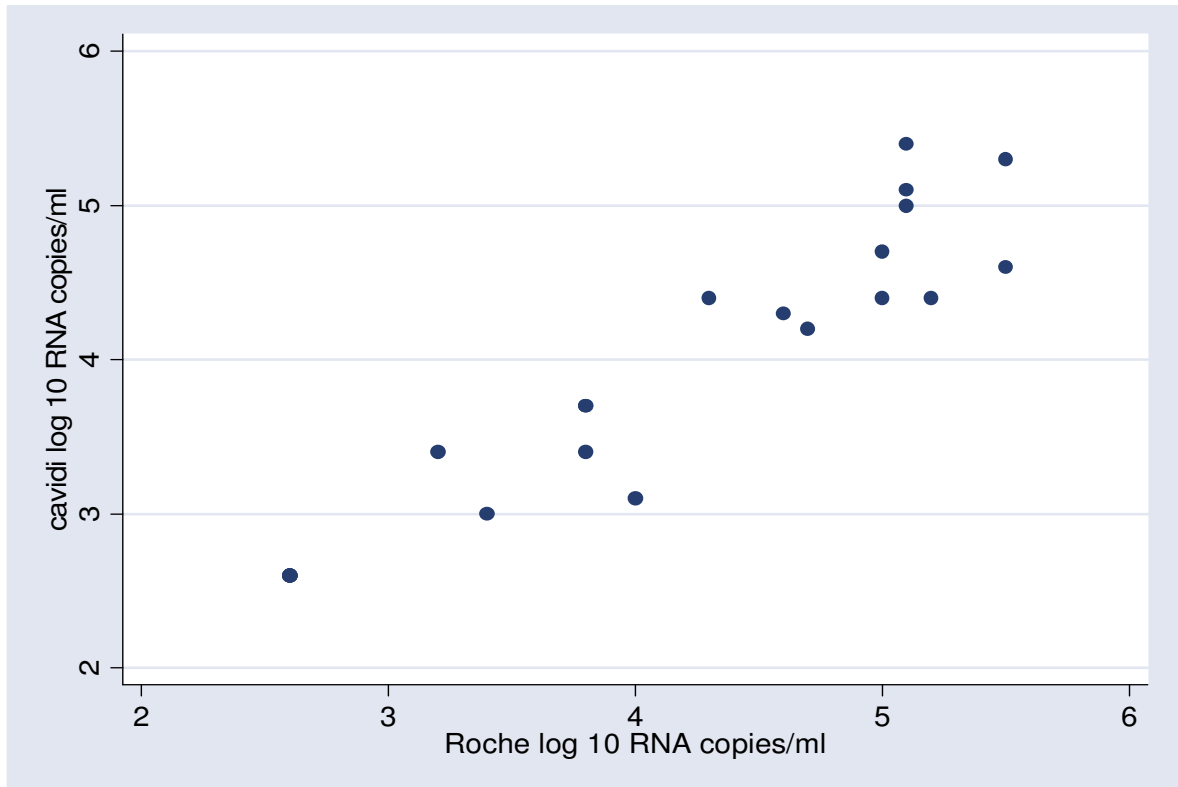
Figure 3



Normal Density curve for difference in viral load between Roche Amplicor and Cavid RT assays.

The Roche log 10 RNA copies/ml were then correlated to the CaviDi log 10 RNA copies /ml on a scatter plot using STATA.

Figure 4



Scatter plot of Roche LOG 10 RNA concentrations against CaviDi Log 10 RNA concentration. $r=0.94$, $p<0.05$

RNA concentration show a roughly elliptical shape indicating that there is a linear relationship between Roche Log 10 RNA measurements and Log 10 CaviDi RNA measurements. 21 data points were included in the analysis but only 17 are appearing because some of the points were identical and so they are lying on top of each other.

To test the strength and direction of the linear relationship a correlation test between Roche Log 10 RNA and CaviDi Log 10 RNA was done in Stata and the output is shown table below.

Table 3. Correlation between Roche Amplicor Monitor and CaviDi RT Assay.

```
. correlate Roche caviDi
(obs=21)
```

```
      |   Roche   CaviDi
-----+-----
Roche |  1.0000
caviDi | 0.9413  1.0000
```

The correlation shows that the correlation coefficient of viral load measurements between the Roche Amplicor Monitor version 1.5 and the CaviDi Reverse Transcriptase Assay is 0.94. The one percent point for 19 degrees of freedom is 0.55 which means that at the 1 % level of significance with 19 degrees of freedom you will need to have a correlation coefficient of greater than 0.55 to be significant. The correlation coefficient for this analysis is 0.94 which is above 0.55 so this implies that it is significant.

Simple linear regression was also used to assess the relationship between Roche Viral Load Measurements and CaviDi Viral load measurements and the results are shown table 4 below.

Table 4 Simple linear Regression model for Roche measurements versus CaviDi Measurements.

```
. regress Roche caviDi
```

```
      Source |      SS      df      MS              Number of obs =    21
-----+-----
      Model | 18.5047311    1 18.5047311          F( 1, 19) = 147.61
      Residual | 2.38193543   19  .125365023          Prob > F    = 0.0000
-----+-----
      Total | 20.8866666   20  1.04433333          R-squared   = 0.8860
                                      Adj R-squared = 0.8800
                                      Root MSE    = .35407
```

```
-----+-----
      Roche |      Coef.   Std. Err.      t    P>|t|   [95% Conf. Interval]
-----+-----
      caviDi |  1.020669   .0840102    12.15  0.000   .8448338   1.196504
      _cons |  .1568952   .3389632     0.46  0.649  - .552563   .8663534
```

The results of the regression indicates that for a t-distribution with 19 degrees of freedom, $p = 0.000$ which is less than 0.05 so we reject the null hypothesis ($H_0 : r=0$) that there is no correlation between viral load measurements between the Roche Amplicor version 1.5 and the CaviDi Reverse transcriptase version 3.0 assay. The coefficient for the CaviDi assay is 1.0, which means that if there is an increase of 1 log 10 RNA copies/ml on the Roche assay there will also be a increase of 1 log 10 RNA copies per/ml when tested using the CaviDi RT assay. The 95% confidence interval is (0.84; 1.2). This does not include a 0, thus showing a significant linear relationship between Roche RNA measurements and CaviDi RNA measurements. To test if the differences obtained between the 2 methods are statistically significant a paired t-test was done (because $n < 100$ and the differences are normally distributed and the means of the two groups are almost equal 3.9 log 10 copies/ml compare to 4.1 log 10 copies/ml). The results are shown below.

Table 5 Paired t –test for difference between Roche Amplicor and CaviDi Viral load Measurements.

. ttest Roche=cavidi

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
Roche	21	4.166667	.2230026	1.021926	3.701491	4.631842
cavidi	21	3.928571	.2056515	.9424134	3.49959	4.357553
diff	21	.2380952	.0754277	.3456533	.0807557	.3954347

$H_0: \text{mean(Roche - cavidi)} = \text{mean(diff)} = 0$

Ha: $\text{mean(diff)} < 0$	Ha: $\text{mean(diff)} \neq 0$	Ha: $\text{mean(diff)} > 0$
t = 3.1566	t = 3.1566	t = 3.1566
P < t = 0.9975	P > t = 0.0050	P > t = 0.0025

The results indicate that the probability that the difference in measurement between the two methods is $=0$ is 0.005 which is less than 0.05 so it is statistically significant

3.3 Discussion

This study assessed the correlation in viral load measurements between the Caviiti Exavir Load assay v 3.0 and the Roche Amplicor Monitor version 1.5 assays to establish if the Caviiti can be used reliably to Monitor HIV viral load in clinical specimens. The results indicate that there is a statistically strong positive linear relationship (correlation) between viral load measurements performed using the Roche Amplicor Monitor version 1.5 and the Caviiti RT Assay version 3.0 in clinical plasma samples submitted at the clinical laboratory.

The correlation coefficient is 0.94 with a p value of <0.005 indicates means the null hypothesis ($H_0: r=0$) is rejected and the alternative hypothesis ($H_a: r \neq 0$) is accepted. A correlation coefficient of 0.94 indicates a very strong positive linear relationship in viral load measurements between the two assays. A positive linear relationship shows that Viral load measurement increase and decrease at the same time in both methods.

Eighty five % of the samples showed an agreement of $\leq 0.68 \log_{10}$ (fivefold) between the two methods which is equal to the total variation that can be expected in viral load measurements in clinical practice. This data supports findings of previous studies (Greengrass and colleagues) that have shown a strong linear correlation between the two methods. However because the sample size was small as (22 compared to 109 which were originally planned) further evaluations will still need to be done to verify the results.

Technically the CaviDi ExaVir load assay was found to be relatively simple to perform especially for laboratory scientist with experience in ELISA technique. It also does not require any specialized equipment and it can easily fit into existing space within the lab as opposed to Molecular work which requires 3 separate rooms. The challenge experienced with the CaviDi ExaVir load assay was the lengthy incubation period (3 days) although the hands on time is almost the same as that for the Roche amplicor Monitor (approximately 6 hours). In Zimbabwe the kits were also not readily available at the time of this study so that was also another challenge. The assay also lacks standard positive and negative controls so previously tested samples were used as controls. In Zimbabwe using the assay may become a challenge in laboratories without a backup power supply as it needs to be incubated at 37 degrees Celsius for 3 days. Water purity is also another factor that needs to be considered before setting up this assay in Zimbabwe as contaminated water leads to a higher lower limit of detection (>200 copies/ml) according to the kit manufactures.

A cost analysis was not done for this study but according Jennings et al the cost per test for viral load using the CaviDi Exavir load is approximately US \$30 whereas that for the Roche Amplicor monitor test is approximately US \$90. There was a long time in between testing the samples on the CaviDi Assay due to unavailability of kits but this should not affect the results as the samples were stabilized by freezing. Samples for viral load testing are routinely frozen at -70 degrees for periods up to 1 year before they are analyzed. The required sample size of 109 was not reached due to lack of funding to buy the required kits so the analysis is based on 21 samples. The smaller sample size might affect the significance of the produced results so follow up studies to verify the results are required. The study samples were tested real time using the CaviDi Exavir load assay and in retrospect on the Roche assay. However both

methods have been demonstrated to work well in previously frozen samples so this should not have caused any bias.

CHAPTER 4: CONCLUSION

There is a very strong positive linear correlation in viral load measurement between the Cavidir Exavir Load v 3.0 and the Roche Amplicor Monitor v 1.5. ($r=0.94$ and $p < 0.05$).

85.7% of the samples showed an agreement of $\leq 0.68 \log_{10}$ copies/ml between the two methods (fivefold difference) which is equal to the total variation that can be expected in viral load measurements. This data supports findings of previous studies that have shown a strong linear correlation between the two methods. In conclusion, the Cavidir Reverse Transcription assay v 3.0 correlated very strongly to the Roche Amplicor Monitor v1.5 in quantifying HIV-1 viral load in clinical samples submitted for viral load testing in the laboratory and it appears that it can be used to monitor HIV-1 viral load in HIV positive patients in Zimbabwe. However the sample size was not large enough to sufficiently power the study so follow up studies are required to confirm the findings. The Cavidir Reverse Transcriptase assay was found to be relatively simple to perform in laboratory personnel who have experience in ELISA technique. However water quality will need to be controlled and a backup power supply is required to avoid loss of power during the 3 day incubation period.

Due to the small sample size larger studies are recommended to eliminate the effect of lack of sufficient power in this study. The follow up studies should also include a sample size that is representative of the whole Zimbabwean population in order for the results to be generalized to the whole Zimbabwean population and environment. Assay performance will also need to be evaluated stratified by ART usage, stage of HIV disease as well as stratified and compared at low, medium and high viral loads before the assay can be fully evaluated. Based on this study the assay does seem to be

very ideal for monitoring of HIV viral load in laboratories which have personnel experienced in running ELISA assays.

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APPENDICES

APPENDIX A

Sample Size Calculation

Viral load measurements using the Roche Amplicor monitor have been shown to have a total variation (standard deviation) of $0.26 \log_{10}$ copies/ml and the true difference in viral load measurements that is biologically significant is $0.68 \log_{10}$ copies/ml (five fold difference) [6].

In order for the study to have sufficient statistical power to detect whether there is a difference in quantitation of HIV viral load between the Roche Amplicor version 1.5 and the Cavid tech Exavir load version 3, the required sample size will be calculated using the formula $n = 2s^2/\Delta^2 \times f(\alpha, \beta)$ where:

Δ =the true difference between the population means whose magnitude would be biologically important. In this case a difference in viral load of at least $0.68 \log_{10}$ copies/ml (five fold difference) or greater is considered to be significant and is usually taken as an indication of failing treatment or resistance.

S = the standard deviation of the observation and in this case it is $0.26 \log_{10}$ which is the standard deviation of the Roche Amplicor Monitor version 1.5 tests as established by Bramblia et al [11]

And α is 0.05 and power $1 - \beta$, is 0.8 and $f(\alpha, \beta)$ is equal to 7.85 for a two tailed test.

$$N = \frac{2s^2}{\Delta^2} \times f(\alpha, \beta)$$

$$\Delta^2$$

$$N = \frac{2(0.68 \log_{10})^2}{(0.26 \log_{10})^2} \times 7.85$$

$$N = \frac{2(4.7863)^2}{(1.8197)^2} \times 7.85$$

$$N = 45.8174 / 3.3113 \times 7.85$$

$$N = 13.8366 \times 7.85$$

$$N = 108.6178$$

$$N = 109$$

Therefore 109 participants in each comparison group are required in order to have 80 % power at the 5% level of significance for a two-tailed test to be able to detect the biologically significant difference of 0.68log₁₀ difference in HIV viral load as measured by the Roche Amplicor Monitor version 1.5 and the Cavid Exavir load version 3.0. To maintain this power even at the analysis stage an additional 10% of samples will be added to the 109 participants to make them 120 participants to cater Quantitation standard failures and invalid results that might occur.

APPENDIX B: MATERIALS NEEDED FOR THE ROCHE AMPLICOR MONITOR

1. HIV MONITOR KITS
2. Thermal cycler
3. Micro Amp reaction tubes
4. Plastic resealable bag
5. Pipettors capacity 200µl and 1000µl
6. 2.0ml polypropylene screw-cap tubes, sterile, non siliconized, conical
7. 1.5ml polypropylene screw-cap tubes, sterile, non-siliconized.conocal
8. 95% ethanol ,reagent grade
9. Isopropyl alcohol, reagent grade
10. Sterile fine tip transfer pipettes
11. Sterile disposable serological pipets (5ml, 10ml and 25ml.
12. Microcentrifuge
13. Vortex mixer
14. Disposable gloves , powderless
15. Multichannel pipettor (25 µl and 100 µl)
16. Aerosol barrier pipettor tips (25 µl and 100 µl)
17. Microwell plate washer
18. Microwell plate reader
19. Incubator 37 °C $\pm$ 2° C
20. Disposable Reagent troughs
21. Graduated vessels
22. Distilled or Deionized water

APPENDIX C: Materials and Methods required for the CaviDi Exavir load v3.0 Assay

1. ExaVir load Assay

The following materials are required for the ExaVir load version 3

3 ExaVir load kits version 3

1 ExaVir Load Analyzer

Sample box and lid

Column Holder

Waste Collector

Sample Collector

Tube rack

Vacuum pump

Waste Container

Buffer dispenser

Vacuum Tubing

5 liter Container

250ml Bottle

1-litre bottle

2-litre Bottle

Rack containing 96 storage Tubes

Elisa plate reader

Incubator set at 33°C

Vortex

Single channel pipettes 100-1000µl, 10-200µl

Reservoirs for multichannel pipettes

Pipette filter tips (1000µl)

Pipette tips (200µl)

25ml Bottle/Tube

Absorbing paper.

Computer with Microsoft Excel version 98 or later and Adobe reader.

Method for Carrying out the ExaVir load assay

Frozen plasma should be thawed to room temperature and vortexed before testing.

1. Add 100 µl of Plasma Treatment Additive to Plasma Processing Tube.
2. Add 1ml plasma to the tube then vortex and incubate
3. Add 1.5 ml of Separation Gel to the tube then incubate and vortex
4. Pour contents of tube into column and let the waste come out. Filter in the bottom of the column will stop the gel, with the virions bound to it from passing through.
5. Wash column 4 times with 8ml of Gel wash buffer.
6. Was 2 times with 8mls of gel reconditioning buffer
7. Add 500µl of Lysis buffer and collect the lysate in a tube for the RT-assay.
8. Add lysate to a 96 well plate with RNA-template bound to wells and incubate
9. Wash plate
10. Add Monoclonal antibodies conjugated with alkaline phosphatase
11. Wash plate
12. Add calorimetric substrate which will give a yellow color proportional to the amount of RT in the sample.
13. Read optical densities using a microwell plate reader connected to a computer which has the ExaVir load analyzer program installed.

APPENDIX D: ROCHE AMPLICOR MONITOR V 1.5 RNA WORKSHEET

APPENDIX E: ROCHE AMPLICOR RNA PACKAGE INSERT

