Effect of Sample Management on Quantitative HIV-1 Viral Load Measurement at Saint Paul Hospital Millennium Medical College, Addis Ababa

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List of Study Teams and Study Investigators

Name	Qualification	Institution
Gadissa Gutema	MSC	EPHI
Belete W/semayat	BSc	EPHI
Hiwot Amare	(BSc, MSc)	EPHI
AmeleworkYilma	(BSc, MSc)	EPHI
Saro Abdela	(BSc, MPH)	EPHI
Mulu Girma	(BSc, MSc)	EPHI
Eleni Kidane	(BSc, MSc)	EPHI
Sisay Adane	BSc	EPHI
Sisay Tadele	BSc	EPHI
Ajanaw Yizengaw	BSc	EPHI
Gutema Bulti	Bsc	EPHI
Mengistu Yimer	Bsc	EPHI
Atsbeha G/ Egziabher	(BSc, MSc)	EPHI
Tigist Nigussie		EPHI
Abebech		EPHI
Dereje Leta	(BSc, MSc)	EPHI
Tolewak Sura	(BSc)	EPHI
Gebeyehu Yihenew	(Bsc,MSc)	SPHMMC
Habteyes Tola	(Bsc, Msc and PhDCandidate)	EPHI (advisor)
Dr. Aster Tsegaye	(MSc, PhD)	AAU(advisor)

List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretro viral Therapy
CD	Cluster of Differentiation
CDC	Center for Disease Control
cDNA	Complementary Deoxyribose Nucleic Acid
EDTA	Ethylene Di amine Tetra Acetic acid
EPHI	Ethiopian Public Health Institute
FMOH	Federal Ministry of Health
HIV	Human Immunodeficiency Virus
IC	Internal Control
IRB	Institutional Review Board
NHIVRL	National HIV Reference laboratory
OWA	One World Accuracy
PBS	Phosphate Buffered Saline
PLWHIV	People Living with Human Immunodeficiency Virus
PPT	Plasma Preparation Tube
RANOVA	Repeated Analysis of Variance
RNA	Ribonucleic Acid
RT PCR	Real Time polymerase Chain Reaction
RVI	Retroviral Infection
SD	Standard Deviation
SOP	Standard Operating Procedure
SPHMMC	Saint Paul Hospital Millennium Medical College
SPSS	Statistical Package for the Social Sciences
Tth	Thermos Thermopiles
UNAIDS	United Nations Program HIA/AIDS
WHO	World Health Organization

Abstract

Background: Proper specimen handling prior to quantification of plasma HIV-1 RNA viral load is important, since some reports suggest that variations in specimen handling may affect detection and quantification of plasma RNA. However, there is limited evidence on the effect of time of plasma separation, storage, freeze-thawing and dilution on the HIV-1 RNA viral load.

Objective: To determine the effect of sample management on HIV-1 RNA viral load measurement in St. Paul Hospital Millennium Medical College from April to July 2019.

Methods: Experimental study design was conducted in St. Paul Hospital Millennium Medical College from April to July 2019 GC in people living with HIV. Whole blood sample was collected into two EDTA test tubes from 88 eligible participants. The viral load test was done by Abbott m2000sp/rt analyzer. Data was entered into Microsoft excel and analyzed by SPSS version 20. Repeated measure analysis of variance was used to compare HIV RNA viral load mean difference between different time of plasma separation, storage, freeze-thawing and dilution. Post-hock analysis was employed to locate the place of significant difference. Level of significance was set at 5%.

Results: There was significant HIV-1 RNA viral load log mean difference between plasma separation time at 6 hours (hrs) and 24hrs ($p<0.001$). Similarly, there was significant HIV-1 RNA viral load log mean difference between plasma tested within 6hrs, stored at 2-8^{0}c for 6 and 15 days ($p = 0.006$ *and* <0.001). HIV-1 RNA viral load log mean difference was not observed when plasma was stored at 2-8^{0}c for 6 days ($p= 0.999$) and at - 20^{0}c for either 30 ($p = 0.899$) or 60 days ($p = 0.999$). There was significant log mean difference between plasma that exposed to 4th cycle of freeze-thawing in -20^{0}c when compared with plasma tested within 6hrs ($p = 0.013$). For the three dilution proportions (1:2, 1:3 and 1:5) there was no significant difference on mean RNA copies when compared to each other and tested within 6hrs ($p = 0.999$).

Conclusion and recommendation: Only plasma separated at 24hrs, stored at 2-8^{o}c for 15days and freeze-thaw 4th cycle had statistically significant effect on HIV RNA viral load variation. Though the differences were not clinically significant at a cut-off viral load level of 0.5 $\log_{10}$, therefore focusing on these factors could improve the result quality and ultimately improve patient care.

Key words: *Human immunodeficiency virus, HIV-1 RNA viral load, quantitative RT PCR, Abbott m2000sp/rt analyzer, Ethiopia.*

1. Background and Justification

The human Immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) disease progression and antiretroviral therapy (ART) success as well as failure are monitored by immunological markers Cluster of Differentiation (CD4 T-cell quantification), clinical criteria, and viral load monitoring [1]. However, plasma viral load is the gold standard predictor for detecting of HIV-1 disease progression and treatment failure [2]. It also enables clinicians to assess the success of treatment and detect treatment failure prior to onset of clinical symptoms [3].

The reproducibility and accuracy of HIV-1 Ribonucleic Acid (RNA) viral load quantification are still a major concern, because HIV naked RNA is extremely unstable in whole blood or plasma [4]. Careful specimen handling prior to testing of plasma for HIV-1 RNA levels is important since way of handling may profoundly affect the detection and quantification of plasma HIV-1 RNA [5, 6]. Moreover, controversies exist as to whether values of HIV-1 RNA obtained under different conditions may not vary significantly [7].

According to World Health Organization (WHO) technical recommendations, plasma sample is recommended for HIV viral load [2] and it must be separated within 6 hours at room temperature after whole blood collection [2, 8]. Evidences indicate, plasma sample can stay at room temperature without viral degradation for up to 24 hours, 5 days at 2–8 ° C and for longer periods at -80^{O}C [2]. If not separated, there is a large amount of viral RNA degradation during the first 3 to 6 hours [6, 9]. Studies, however, have shown that viral RNA level found in plasma or whole blood is relatively remained stable after 6 hours of sample collection at 4°C for 24 to 48 hours [6, 9].

Studies also demonstrated decline of different degree of HIV-1 RNA concentration when analyzing samples after storage as whole blood for 24hrs, although, it was not statistically significant [10, 11]. In addition, certain studies showed that HIV-1RNA were stable up to 72hrs at room temperature as a whole blood, [12, 13, and 14]. A study which determined stability of HIV-1 RNA in whole blood samples stored in EDTA tubes showed a decrease in viral load over time and a statistically significant decrease were observed at day 7 [15].

Evidence about stability of HIV RNA in plasma specimens stored at 4^{0}c indicates that keeping plasma at 4 ^{o}c for 1 week did not affect HIV RNA measurement when compared with HIV-1 RNA concentrations determined from fresh plasma [16, 17]. Besides, it was also found that HIV-1RNA copy numbers were maintained within 0.5 log10 in plasma samples held at room

temperature for up to 3 days [12] and up to 28 days [18]. In contrast, HIV-1 RNA levels decreased significantly when plasma was stored at 37 ^{o}c [16].

RNA is relatively stable in Ethylene Di amine Tetra Acetic acid (EDTA) tube, [19] thus, plasma prepared in EDTA tube is recommended to maintain RNA stability.

Another factor that can affect the stability of HIV quantification is frequency of freeze-thaw cycle [12]. It is indicated that there is direct and close association with HIV viral load quantification [12]. Evidence also showed no statistically significant difference in HIV viral load measurement because of freezing of plasma [20]. Moreover, plasma was stable despite frequent freeze-thaw for three [6] and four [21] cycles.

Plasma could be diluted by diluents when the volume is insufficient and the concentration of HIV-1 RNA is above the reading limit of a given instrument. However, plasma dilution by phosphate buffered saline (PBS) has been shown to have an effect on the HIV-1 RNA concentration [22].

Another factor that could affect HIV-1 RNA quantification is the level of the concentration of the virus in the specimen. Measurement result of plasma viral load <250 copies/ml and those close to the lower level of detection of each assay are interestingly over-represented than values 500 copies/ml [23]. In general any inconvenience happened from sample collection to amplification could affected the final result [24].

Poor specimen handling could result in RNA degradation which may leads to underestimation of concentration and leads to unsuppressed HIV viral load result to be wrongly reported as suppressed [9]. It also leads to drug regimen shift delay and hence resulting in the dissemination of resistant strain. In so doing, it misleads policy makers and other stakeholders by assuming viral suppression is good in the population [25].

Taken together, the studies reviewed above indicated that HIV-1 RNA level quantification could be affected by specimen type, storage temperature and duration of storage though some of the studies found no effect. Moreover, laboratory infrastructure and samples transportation are the main complications that affect HIV-1 RNA viral load quantification in developing countries [26]. Thus, there is a need to see the effect of specimen handling in the local context. Ethiopia is among high HIV prevalence countries with a recent national prevalence of 0.9% [27] and according to the latest Spectrum modeling, an estimated 610,335 people were living with HIV in 2018[28]. Ethiopia has introduced routine HIV viral load testing for ART monitoring which is the preferred monitoring tool for diagnosing and confirming ART failure

[29], as per the WHO recommendation [30]. The country has also adopted the UNAIDS 90–90-90 by 2020 target [31]. To achieve this goal, particularly the third target which aims at achieving 90% virologic success, access to viral suppression monitoring by viral load testing is needed. To scale up the routine viral load monitoring service, the country follows a sample referral linkage system; thus, proper specimen handling is highly mandatory to ensure the quality of the test result. Studies regarding the effect of sample management on the quantification of HIV viral load are lacking and hence this study tried to address this gap.

2. OBJECTIVE

2.1. General objective

To determine the effect of sample management on HIV -1 RNA viral load measurement in Saint Paul Hospital Millennium Medical College from April to July, 2019.

2.2. Specific objectives

- To compare the effect of HIV VL plasma separation time at six and 24 hours.
- To determine the effect of plasma sample storage time duration (24hrs and 48hrs at room temperature; 6 days and 15 days at 2-8^{0}C; 30 days and 60days at -20^{0}C).
- To identify the effect of sample freezing and thawing cycle on viral load measurements
- To evaluate the effect of sample dilution on HIV viral load count
- To determine clinical significance for every specific objectives

3. Materials and Methods

3.1. Study design

Experimental study design was conducted to determine the effect of plasma separation time, storage temperature and duration of storage, freeze-thaw cycles and dilution proportion on HIV 1 RNA concentration in Saint Paul Hospital Millennium Medical College (SPHMMC) from April to July, 2019 GC.

3.2. Study area and period

The study was conducted at Saint Paul Hospital Millennium Medical College from April to July, 2019 GC. St Paul Hospital Millennium Medical College is selected for this study based on its closeness for Ethiopian Public Health Institutes (EPHI) to minimize the factor that can affect during transportation. It is found in Gulelle Sub-city, Arbegnoch Street, Addis Ababa, Ethiopia. It is a referral hospital that administrated under Ethiopian Federal Ministry of Health (FMOH). It is the second largest public hospital in the country established in 1961.The hospital has more than 2800 clinical, academic, administrative and support staffs. The hospital provides medical specialty services to patients who are referred from all over the country [32]. The hospital has 309 beds for inpatients and provides referral services for over 1.5 million populations. It also provides care and treatment for PLWHIV. Recently, there were 4,352 total active PLWHIV in the ART clinic of St Paul's hospital according to records of the ART clinic.

3.3. Study population

The source population was all known HIV positive patients who were receiving ART services at the ART clinic of St. Paul Hospital Millennium Medical College, during the study period.

3.4. Study sample

Study population was those who were on ART at ART clinic of St Paul's Hospital Millennium Medical College for at least six months; provide their sample for viral load testing during the study period and fulfill the eligibility criteria.

3.5 Eligibility criteria

3.5.1 Inclusion criteria

- Being on ART at least for six months
- Age greater than or equal to 18 years old
- Willingness to participate in the study

3.5.2 Exclusion criteria

- Severely ill and patients with co morbidity

3.6. Sampling method and sample size determination

3.6.1. Sample size determination

Sample size was determined by using comparison of two means with equal sample size and variance using Epi Info sample size calculation tool. X_1 and X_2 were assumed as two means in different conditions, 80% power and 95% confidence level. Table 1 depicts values of the two means used to calculate sample size for each main outcomes of the study (separation time, storage temperature, freezing-thaw and dilution). In addition, 0.2log [12] difference was used as variance in this sample size calculation.

Table 1: Sample size calculation

Condition	**Separation time mean**		**Storage temperature**		**Free - Thaw**		**Dilution**	
	X_1	X_2	X_1	X_2	X_1	X_2	X_1	X_2
6hrs Vs 24hrs	4.2	3.61						
Plasma 24hrs RT Vs 48hrs			4.1	3.9				
Plasma Within 6hrs Vs freeze – thaw					4.45	4.46		
Undiluted Vs diluted							0.4	0.49
Sample size	11		88		34,915		432	

RT-Room temperature, Vs-Verses

The values of each mean obtained from previous studies [5, 20]. Sample sizes were calculated for each study main outcomes based on the specific mean difference and the largest determined sample size was 34,915 which determined by using freeze-thaw cycle. However, a total of 88 participants which was determined by using plasma 24 hours at room temperature and 48hours at room temperature were included to this study. This sample size was used based on the cost of test because viral load test is

very expensive to run for several participants. We believe this sample size is enough because our experiment is within individual comparison.

3.6.2. Sampling Technique

St Paul Hospital Millennium Medical College was selected purposively for this study based on its closeness for EPHI to minimize the factor that can affect HIV viral load during transportation. Special sampling technique was not employed to enroll individual participants because all clients attending the hospital for HIV viral load monitoring during the study period were included consecutively.

3.7. Study variables

3.7.1 Dependent variable

HIV viral load count

3.7.2 Independent variable

Plasma separation time, storage temperature, storage period, freeze-thaw cycle and dilution were independent variables

3.8. Data Collection

3.8.1. Data and specimen collection

Fig 1 depicts the overall procedure of specimen collection procedure. Samples were collected by the hospital phlebotomists after training obtained on method of consent request, objective of the study, accident management method and data collection tools. The samples were transported from SPHMMC to EPHI by triple packaging and in temperature monitored condition at least within three hours of collection. The transportation was performed by EPHI NHIVRL staffs. Ten ml of whole blood samples were drawn from each participant into two different EDTA test tubes (each 5ml). Structured data extraction format was used to follow the sample quality, storage temperature, time of sample collection, sample dilution and freeze-thaw cycle. Valid individual patient code was used to trace the viral load result of each participants to return their viral load results. Standard operational procedures (SOPs) and Job aids were prepared for each procedures of testing and employed during operation process.

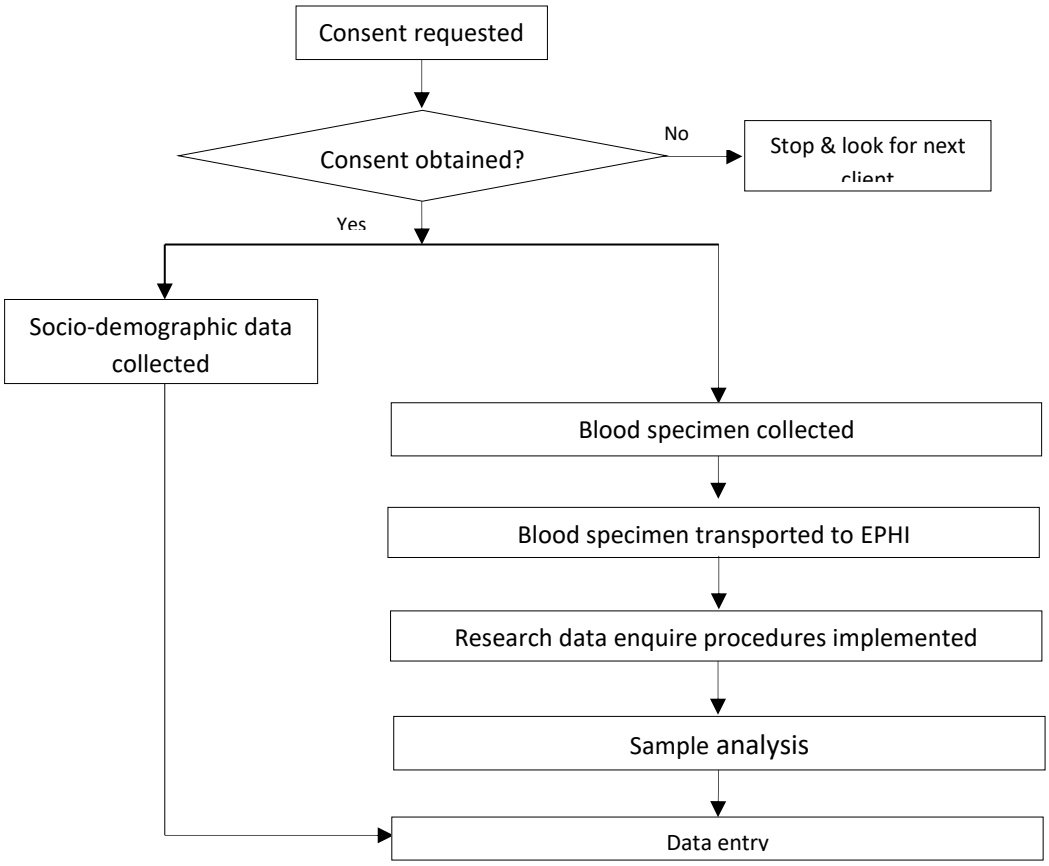

Figure 1: Specimen and data collection flow chart

Experiment

Ten ml whole blood was collected from all participants, transported within three hour of collection to EPHI NHIVRL. From the 10ml whole blood which was collected in to two 5ml EDTA tubes 2ml was subtracted in other EDTA tube and left at room temperature. The 8ml blood was centrifuged at 5000rpm for 5minutes and the plasma is aliquoted into six nunc tubes with the volume of 0.35ml. The plasma aliquots were stored as follows: two aliquots were stored at room temperature, two at 2-8^0 and two at -20^{0}c. One remaining aliquot with 0.2ml volume was diluted in the concentration of 1:2, one with 0.1ml diluted 1:3, one with 0.1ml diluted with 1:5 based on the actual dilution practice in different laboratories in Ethiopia. The remaining one with 1.5ml was used for freeze-thawing experiment. The dilutions and 1.5 ml plasma aliquots were tested within six hour of collection (taken as time zero) and the leftover of the 1.5ml plasma were used for freeze-thaw and analyzed for four cycles. The remained 2ml was stored as whole blood for 24hrs processed the next day. Mean viral load log and RNA copies used for comparison.

The samples were collected and aliquoted/distributed as follows:

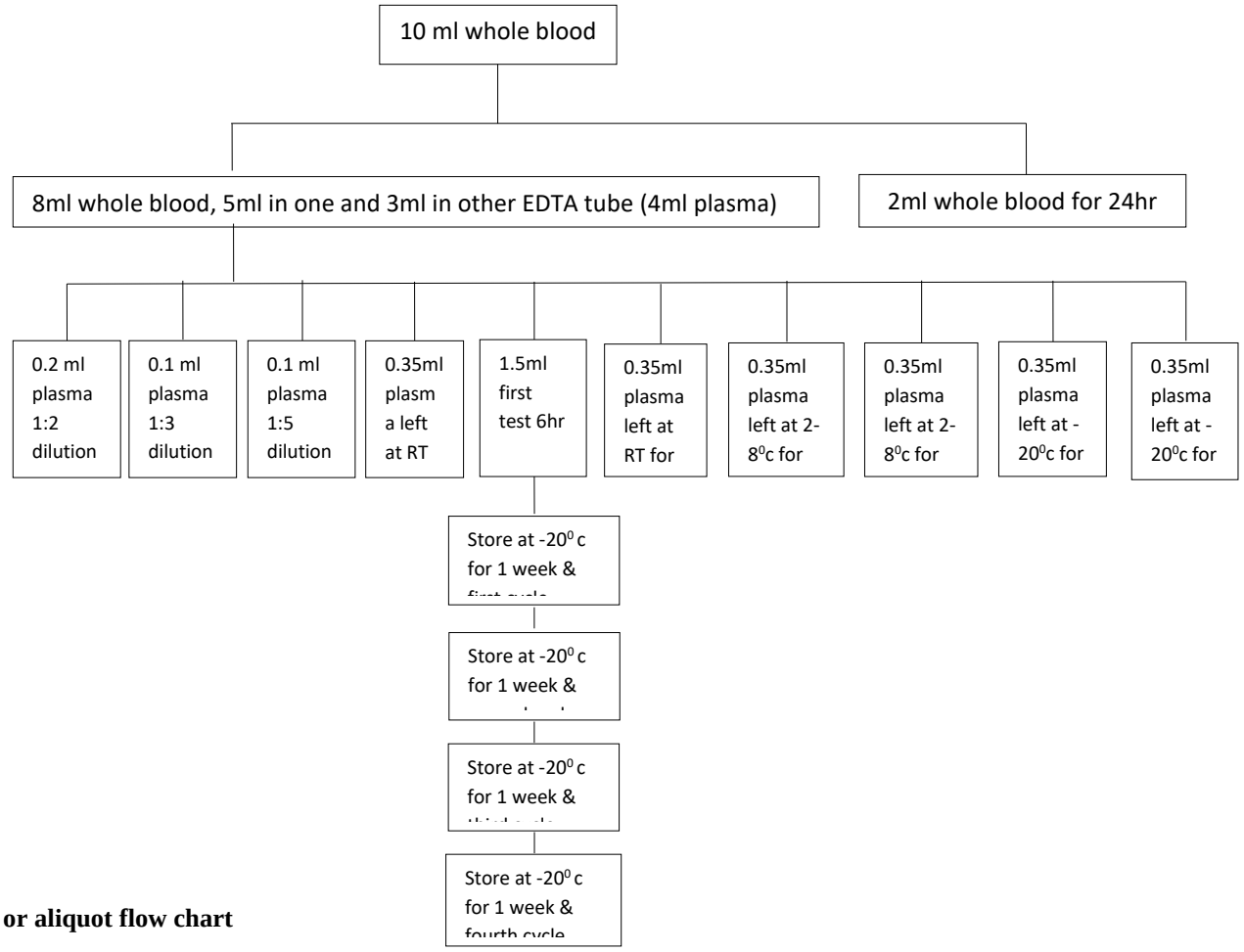

Figure 2: sample distribution or aliquot flow chart

3.8.2 Laboratory analysis

3.8.2.1 Sample collection

Depicts [Fig 1] show how whole blood specimens were collected by hospital phlebotomists and transported to EPHI NHIVRL. The collected and transported samples were separated in to plasma and required research procedures conducted [Fig 2].

3.8.2.2 Principle of detection

The working principle of the Abbott instrument used to analyze the sample in this research was customized from the user manual of the instrument [33]. The Abbott Real Time HIV-1 assay uses RT-PCR to generate amplified product from the RNA genome of HIV-1 in clinical specimens. RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeding correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labelled oligonucleotide probes on the Abbott *m*2000*rt* instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott *m*2000*rt* is proportional to the *log* of the HIV-1 RNA concentration present in the original sample. In the presence of template RNA, a target-specific reverse primer and manganese ions, the Tth DNA polymerase, transcribes the RNA into a DNA strand. The product of reverse transcription is a hybrid, consisting of one strand RNA and the first molecule cDNA. Now, the PCR start, in the first step, the RNA/DNA hybrid is separated by heat denaturation. When the temperature is lowered, the forward and the reverse primers anneal to their target sequences. In the next step the Tth polymerase elongates the primers, thus synthesizing new, complementary DNA strands. These DNA copies again serve as templates in subsequently performed PCR cycles.

3.8.3. Sample rejection criteria

Test result with insufficient information, at least one invalid test result, clot, result below the lower limit of the instrument and insufficient sample volume were rejected.

3.9. Quality assurance

During data collection important variables such as plasma separation time, storage temperature, storage period, freeze-thaw cycle, and dilution proportions were controlled by recording on data and result extraction formats and temperature recording formats, to avoid any miss match and traceability issue. Standardized SOP were developed and used for every procedure. Every procedure was performed by competent laboratory personnel and internal quality control, and assay calibration was tested. Moreover, EPHI NHIVRL participates in external quality assurances with One World

Accuracy (OWA) three times a year and two times with Center for Disease Control (CDC) Atlanta. The results are always 100%.

3.10. Data analysis

The data was entered into Microsoft office Excel and analyzed using SPSS version 20. Before main analysis data was checked for normality, linearity, univariate and multivariate outliers, and homogeneity of variance-covariance matrices with no serious violations noted [Fig 3]. Descriptive statistics was employed to explain socio-demographic characteristics distribution and HIV viral load copies. Repeated Measure Analysis of Variance (RANOVA) was done to determine the mean difference between different scenarios. RANOVA was used because the viral load of each experimental scenario did not seriously violate the normality and other assumptions. Post-hock analysis was employed to locate the place of significance between more than two categories. Level of significance was set at 5%. $P < 0.05$ was taken as statistically significant. The mean differences >0.5log or >±2SD were used as clinical significance [12, 13].

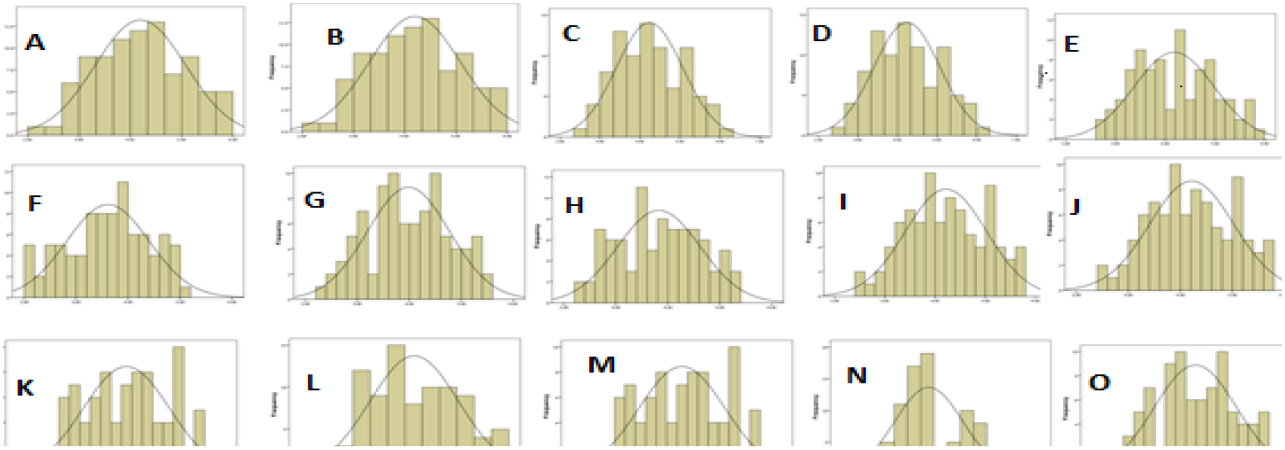

Figure 3: Normality curve to assess normality of the outcome variables
A- HIV viral load within 6hr, B- HIV viral load for one time Freeze at -20^{0}c for one week, C- HIV viral load refreeze for one week at -20^{0}c, D- HIV viral load refreeze for one week at -20^{0}c, E- HIV viral load refreeze for one week at -20^{0}c, F- 1:2 Dilution, G-1:3 Dilution, H-1:5 Dilution, I- HIV Plasma viral load stored at room temperature for 24hr, J- HIV plasma viral load stored at room temperature for 48hr, K- Plasma HIV viral load separated from whole blood after 24hr, L- HIV viral load for plasma stored at 2-8^{0}c for 6 days, M- HIV viral load for plasma stored at 2-8^{0}c for 15 days, N- HIV viral load for plasma stored at - 20^{0}c for 30 days, and O- HIV viral load for plasma stored at 2-8^{0}c for 60 days.

3.11. Ethical clearance

Ethical clearances were obtained from Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, Ethiopian Public Health Institute and St. Paul Millennium Medical College Scientific Ethical Review Committees. Data access was limited to data manager and the principal investigator through keys locked and password used for electronic files. All information that exposes the identity of each patient was removed from the result of this study and the codes and patient medical record number were used to return the viral load results. All participants were provided both

oral and written informed consent after they thoroughly understood the objective and procedure of this study.

3.12 Dissemination of results

The study findings will be presented at the Department of Medical laboratory sciences of Addis Ababa University and copies shared to the university, EPHI and St. Paul Millennium Medical College. The findings will be presented in national and international conferences. Manuscript will also be published in peer reviewed journals.

3.13. Operational definitions

Freeze-thawing: Freeze-thaw cycle is the process where frozen plasma homogenized by letting it on the bench at room temperature for the maximum of 6hrs and freezing it again in deep freezer.

Clinically significant viral load level: is the practical importance of a treatment effect whether it has a real genuine, noticeable effect on patient management. Viral load less than $0.5\log_{10}$ or difference of ±2SD is considered as normal assay variation of plasma RNA levels, while changes greater than $0.5\log_{10}$ or ±2SD indicates significant clinical difference.

Dilution proportion: is the proportion of the concentration of plasma to the diluents (PBS)

4. Results

4.1 Ccharacteristics of study participants

A total of 88 PLWHIV were included from the ART clinic at St Paul's Hospital Millennium Medical College. The majority 60(68.2%) of study participants were female. The mean (± SD) age of the participants was 37.3 (±8.44) years with the age range of 18 to 53 years.

4.2 The mean viral load value of the participants

The mean viral load copies of the WHO recommendations (within 6hour/Gold standard/, 2-8^{0}c and -20 ^{0}c) were 4.24, 4.23, and 4.21 respectively [Table 2].

Table 2: Mean and median distribution of variables of participants at SPHMMC 2019

Variable	Mean (±SD)	Median (IQR)
HIV viral load at 6hr	4.24(0.82)	4.25(3.53 – 4.94)
HIV viral load 1 week freeze thaw first cycle	4.24(0.83)	4.25(3.56 – 4.93)
HIV viral load 1 week refreeze thaw second cycle	4.2(0.89)	4.13(3.5 – 4.94)
HIV viral load 1 week refreeze thaw for third cycle	4.22(0.86)	4.20(3.54 – 4.92)
HIV viral load 1 week refreeze thaw for fourth cycle	4.18(0.80)	4.2(3.51 – 4.84)
HIV viral load result of RT storage of plasma for 24hrs	4.22(0.81)	4.19(3.6 – 4.9)
HIV viral load result of RT storage of plasma 48hr	4.20(0.81)	4.15(3.6 – 4.91)
HIV viral load result of HB separated after 24hr	4.1(0.83)	4.06(3.42 – 4.8)
HIV viral load result of storage of plasma at 2-8 for 6days	4.23(0.84)	4.25(3.6 – 4.91)
HIV viral load result of storage of plasma at 2-8 for 15 days	4.18(0.85)	4.1(3.59 – 4.9)
HIV viral load result of storage of plasma at -20 for 30 days	4.21(0.83)	4.03(3.67 – 4.9)
HIV viral load result of storage of plasma at -20 for 60 days	4.20(0.86)	4.16(3.59 – 4.84)

HIV viral load result of 1 to 2 dilution(RNA copies)	3.98(0.79)	3.94(3.42 – 4.56)
HIV viral load result of 1 to 3 dilution (RNA copies)	3.83(0.8)	3.85(3.16 - 4.41)
HIV viral load result of 1 to 5 dilution (RNA copies)	3.61(0.79)	3.64(3.02 – 4.2)

RT-Room temperature, WB-Whole blood, HIV, SD-Standard deviation, IQR-Inter quartile range, HIV-Human Immunodeficiency syndrome and RNA-Ribonucleic Acid

4.3 Viral load level with different separation time

There was statistically significant difference between mean viral load tested within 6hrs and separated after 24hrs: *F (1, 87) =33.11, p < 0.001*. The mean (± SD) viral load was decreased when it stored as whole blood for 24hrs as compared to viral loads tested within 6hrs 4.1(0.83) versus 4.24(0.82) [Table 3]. With mean difference of 0.14 log/ml which was not clinically significant at <0.5log/ml.

4.4 Viral load level of plasma storage time at different temperature

There was no statistically significant difference between mean viral load at 6hrs and stored at 24hrs and 48hrs at room temperature: *F (2, 86) =1.11, p=0.336.* There was slightly higher mean (± SD) viral load levels within 6hrs, plasma stored at room temperature for 24hrs and 48hrs at room temperature 4.24(0.82), 4.22(0.81), and 4.20(0.81), respectively [Table 3].

There was statistically significant difference between mean viral load at 6hrs and plasma stored in 2-8^{0}C for 6 and 15 days: *F (2, 86) =10.16, p < 0.001*. With Post-hock analysis still there were statistically significant difference between mean viral load at 6hrs and plasma stored in 2-8^{0}C for 15 days: *F (1, 87) = 10.17, p= 0,006.* Similarly, there was statistically significant difference on mean viral load of plasma stored in 2-8^{0}C for 6 days and for 15days: *F (1, 87) = 10.17, p< 0,001* [Table 3]. However, these differences were not clinically significant based on the mean difference 0.06log/ml for 6 days and 0.05log/ml for 15 days based on the < 0.5log clinical significance cut-off point.

There was no statistically significant difference between mean viral load at 6hrs and plasma stored in -20^{0}c for 30 and 60 days: *F (2, 86) =0.55, p = 0.576* [Table 3].

Table 3: Repeated measurement analysis of variance in log/ml for plasma separation time, storage at room temperature, 2-8^{0}c and -20^{0}c.

Variables	Measurement Indices			
	Mean(±SD) log/ml copies	Mean Difference	F- Statistics	P- value
HIV VL within 6hrs - HIV VL Separated after 24hrs (log copies/ml)	4.24(0.82) - 4.1(0 .83)	0.145	33.11	**<0.001**
Within group comparison			1.106	0.336
HIV VL within 6hrs - HIV VL 24hrs RT	4.24(0.82) -4.22(0.81)	0.025	1.106	0.999
HIV VL within 6hrs - HIV VL 48hrs RT	4.24(0.82) -4.20(0.81)	0.038	1.106	0.430
HIV VL 24hrs RT - HIV VL 48hrs RT	4.22(0.81)-4.20(0.81)	0.013	1.106	0.999
Within group comparison of (log copies/ml)			10.164	**<0.001**
HIV VL within 6hrs- HIV VL 2-8^{0}c for 6 days	4.24(0.82)- 4.23(0.84)	0.007	10.164	0.999
HIV VL within 6hrs- HIV VL 2-8^{0c} for 15 days	4.24(0.82)-4.18(0.85)	0.060	10.164	**0.006**
HIV VL 2-8^{0}c for 6 days- HIV VL 2-8^{0c} for 15 days	4.23(0.84) -4.18(0.85)	0.052	10.164	**<0.001**
Within group comparison of (log copies/ml)			0.554	0.576
HIV VL within 6hrs- HIV VL -20^{0}c for 30 days	4.24(0.82)-4.21(0.83)	0.029	0.554	0.899
HIV VL within 6hrs- HIV VL -20^{0c} for 60 days	4.24(0.82)- 4.20(0.86)	0.036	0.554	0.999
HIV VL -20^{0}c for 30 days- HIV VL -20^{0c} for 60 days	4.21(0.83)-4.20(0.86)	0.007	0.554	0.999

VL-Viral load, RT- Room temperature, WB-Whole blood, SD-Standard deviation, HIV-Human Immunodeficiency syndrome and RNA-Ribonucleic Acid

4.5 Viral load level of freeze-thaw cycle of plasma

The mean viral load within 6hrs and plasma freeze-thawed for four consecutive cycles in -20^{0}c for seven days duration between each cycle revealed a statistically significant difference: $F\ (4, 84) = 3.6$, $p = 0.009$ [Fig 4]. Post-hock analysis indicated a statistically significant difference between mean viral load within 6hr and the fourth cycle of freeze-thawing $F\ (1, 87) = 3.6$, $p= 0.013$ [Fig 4]. However, the mean difference was log 0.063 which was not clinically significant as it was < 0.5 log, which is the cut-off for clinical significance.

4.6 Viral load level of plasma dilution

There was no statistically significant difference between mean viral load for undiluted and diluted plasma: $F\ (3, 85) =0.47$, $p = 0.707$ [Fig 4].

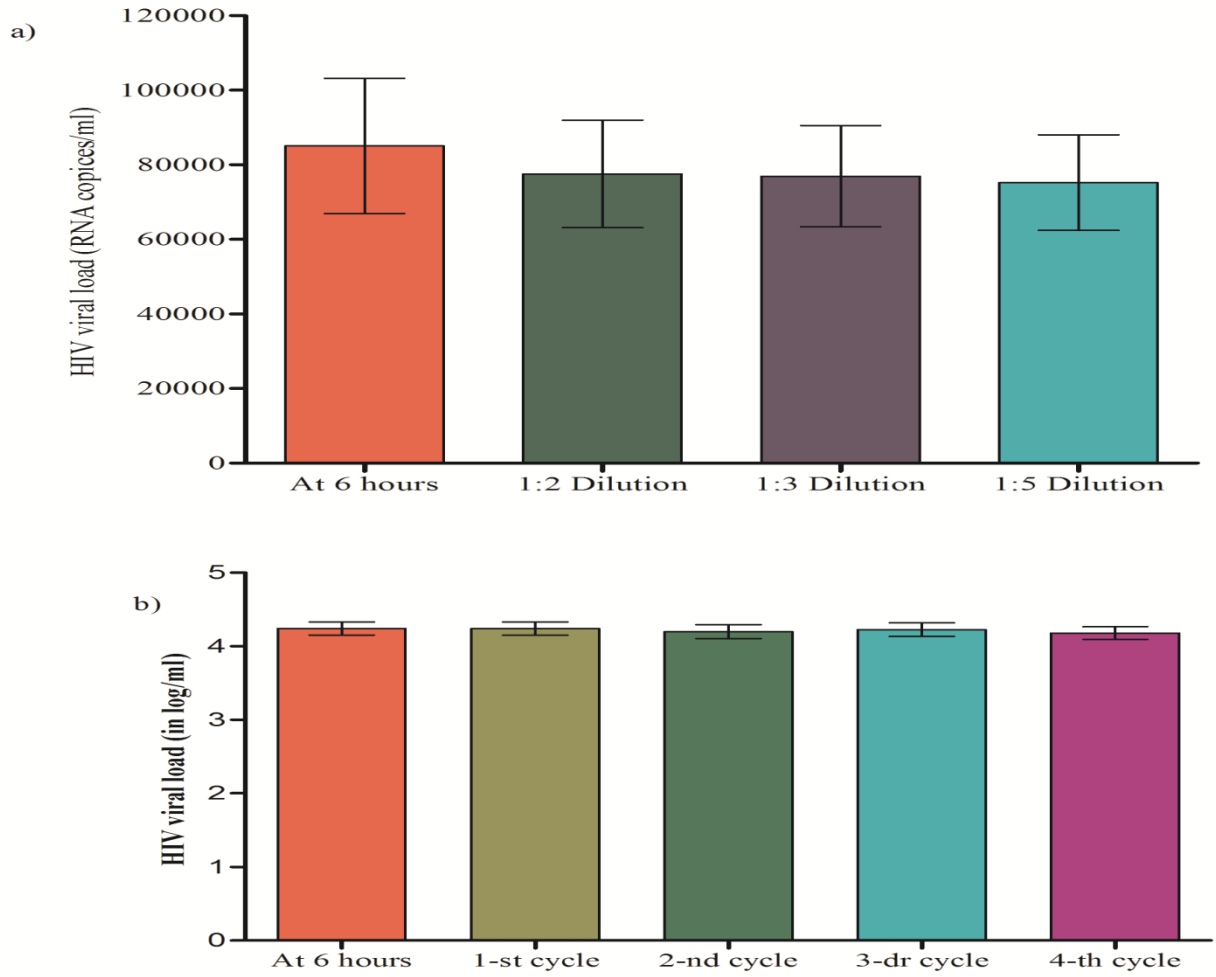

Figure 4: Graphic summary of freeze thaw cycle and Dilution of plasma with PBS

The lines of the box's indicates the mean value while the cross on the vertical lines shows the standard deviation, a) HIV viral load measurement after dilution of plasma With PBS b) HIV viral load measurement of four cycles of freeze thaw

5. **Discussion**

Several studies have focused on identifying problems related to blood collection and processing parameters that will affect accuracy and reproducibility of quantitative HIV-1 RNA viral load testing. However, there are few studies on the effects of plasma separation time, storage time and temperature, freeze-thawing cycle and dilution proportion for HIV-1 RNA viral load. Thus, this study aimed to investigate effects of sample management on HIV viral load measurement in a total of 88 participants from ART clinic at St Paul's Hospital Millennium Medical College.

This study revealed that mean difference in HIV-1 RNA viral load with plasma separation time at 24hrs post collection, plasma stored in 2-8^{0}c for 15 days and the fourth cycle of freeze-thawing were statistically significant compared to mean viral load tested at six hrs. However, these variables though they were statistically significant the changes were not clinically significant at the recommended cut-off point 0.5log$_{10}$ (two times SD). With regards to storage time, there was no significant mean difference in viral load of all scenarios as compared with the viral load tested within 6hrs of collection.

Evidence indicated that HIV viral load significantly decreases when stored at room temperature for 72hrs [12]. Moreover, mean HIV viral load decreases significantly when stored at room temperature for 30hrs when compared to viral load tested at 2hrs [11]. These studies findings were similar with the current finding in which the mean HIV viral load was significantly different between viral load tested within 6hrs of collection and after 24hrs.

In contrast, a study demonstrated that the mean viral load was not significantly different between HIV viral load tested within 4hrs of collection and after 24hrs [7]. In addition, HIV RNA was found to be stable up to 48hrs in whole blood at room temperature [6, 9]. Even longer stability of HIV RNA until 3 days [13] and 7 days [4] at room temperature were reported. This difference may be due to HIV strains variation, the level of damaged HIV RNA in the plasma and the testing condition variation.

In this study, there was statistical significant difference between the HIV viral load test at 6hr and 24hrs. However, this significance was not clinically significant based on the cut-off point proposed by previous studies such as >±2SD or a variation of < 0.5log10 difference [12, 13, 22]. A previous study revealed a clinically significant difference in the mean HIV viral load when viral load was tested at 6hrs and 24hrs [12]. This finding was opposite to the current study finding in which the mean viral load was not clinically significantly different between viral load tested at 6hrs and 24hrs. This difference most probably due to statistical test used, time duration of test and sample size difference. In the previous study they used paired t test which can increase type one error when used for more than two categories [34]. However, in the current study repeated measure analysis of variance was used which is free from the problem indicated above [34]. Furthermore, the study reported by Sebire *et al* [12] conducted the experiment on 20 participants. However, in the current study case, 88 participants were included which could increase the precision of comparison.

The difference in the mean HIV viral load quantified from plasma stored at 2-8^{0}c for 15 days was statistically significant when compared with viral load tested within 6hrs and that tested after 6 days in current study. This finding was in line with a previous study in which viral load was significantly different when tested within 2hrs and 72hrs for the plasma stored in 4^{0}c [12]. In contrast viral load remained stable at 4°c for up to 24 to 48hrs [6], 30hrs [5], 14 and 28 days [18]. This difference could be due to temperature fluctuation and poor temperature regulation in the different set ups where viral load testing took place. Although the difference in mean HIV viral load of plasma stored at 2-8^{0}c for 15 days was statistically significant in the current study, it was not clinically significant at the cut-off point of 0.5log$_{10}$. This difference might be due to strain variations and increased amount of damaged HIV RNA in the specimens.

This study also tried to see the effect of freeze-thawing. The mean HIV viral load had statistical significance different in fourth cycle of freeze-thawing after storage in -20^{0}C as compared with the mean HIV viral load within 6hrs of testing. However, the difference was not statistically significant up to three cycles of freeze-thaw by comparison with mean HIV viral load tested within 6hrs. In contrast, to this finding the plasma stored in -70^{0}C refrigerator and freeze-thaw for the 4th cycle was not statistically significantly different [6]. This difference is most probably because of temperature difference and might suggest lower storage temperature gives better result when freeze thawing is unavoidable. Of note, although the mean difference was statistically significant it was not clinically significant as the mean difference was <0.5 log$_{10}$, which is the cut-off for clinical significance. Thus, care has to be taken when interpreting statistical significance as it is demonstrated in the current study where the observed statistically significant mean differences were more likely to be clinically irrelevant.

6. Strength and Limitation of the Study

6.1 Strength

The strength of current study was inclusion of relatively large sample size compared to earlier studies which increases our estimation precision. In addition, this study was carried out at EPHI molecular laboratory which routinely practices internal quality control and participates in external quality assurance scheme delivered by One World Accuracy and CDC, USA. The temperature and storage equipment are also well controlled and regularly serviced.

6.2 Limitation

Even though standard operating procedure (SOP) was followed strictly, personal variation in test procedure and lower limit of detection of Abbott analyzer could affect the results of this study. Transportation condition in real situation is not studied. Electric power fluctuation could also impact the freeze-thaw test result of this study. However, since the laboratory has a backup generator power the impact of temperature fluctuation is minimal. In the current study -80^0C freezer was not used for freeze-thawing process, which could limit our study conclusion of freeze-thawing cycle at this storage temperature. Nevertheless, the study used storage temperature being used in the viral load testing sites which do not have -80^0C freezer, and hence studying the effect with the same storage condition as the testing sites is logical.

7. Conclusion and Recommendation

7.1. Conclusion

This study results was shown that the HIV RNA in plasma maintained stability and not degraded greater than 0.5 $\log_{10}$ for all scenarios of the experiment. The different storage, separation, freeze-thaw and dilution conditions tested in this study do not appear to have a large effect on viral load stability. However, there was statistically significant difference on the mean viral load between viral load tested at 24hrs, stored in 2-8^{0}C and 4th cycle of freeze-thaw when compared to viral load tested at 6hrs. All the statistically significant variables were not clinically significant.

7.2. Recommendation

Though it did not reach clinical significance level, statistical differences have been noted. Thus, to be on the safe side, plasma should be separated before 24hrs for the whole blood stayed at room temperature. In addition, plasma should not stay in 2-8^{0}C beyond 15 days and should not be freeze-thawed for more than three cycles. Moreover, future strong research which will address limitations of this study such as transportation is required.

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