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Determining The Stability Of Hiv-1 Rna In Plasma Samples Stored At Various Temperatures In Primary Tubes Of Centrifuged Whole Blood

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Abstract

INTRODUCTION

Lesotho is a developing country faced with challenges of limited resources. Majority of Basotho live in remote areas that are miles and miles from health care facilities.HIV-1 RNA quantification is mainly performed at the Reference Laboratory and a few laboratories where the assay has been decentralized to. Patient's samples often take a long time to reach the laboratory. The study is aimed to evaluate the HIV-1 RNA stability of un-separated plasma stored for one week at different temperatures.

METHODOLODY

Ethylenediaminetetraacetic acid (EDTA) whole blood samples were collected. HIV-1 RNA quantification test was performed within 24 hours of collection on Roche Cobas Ampliprep-Cobas Taqman analyser. This was done to determine samples with detectable values, thereafter three samples with equal volumes were mixed together to make a mini pooling sample. A large volume was obtained and aliquots of four sample batches made and samples at; 25-30^{0}C (a fresh sample analysed within 24 hours), 2-8^{0}C, -20^{0}C and -80^{0}C. Results of a fresh sample were compared to matching HIV-1 RNA concentrations determined from un-separated plasma stored at above mentioned temperatures for a week.

RESULTS AND DISCUSSION

Results in number of copies did not show a significant decrease in HIV-1 RNA copies, for samples that were stored at 2-8^0 C (difference A) a greater percentage of 57% samples, showed the difference in number of copies that was less than 50%. Only 21 % were above 50% difference and the other 21% of the batches stored at this temperature had failed. Ginocchio et.al 2006, Bruistein et.al 1997 and Amellal et.al 2008 in their studies also indicated that samples that were stored at 4^0 C for 14 days, 24 hours and 1 week respectively, did not show a significant decline in number of RNA copies.
Samples stored at -20^0 C and – 80^0 C did not indicate significant decline in RNA copies as 7% and 14% respectively of the samples showed a difference of above 50% number of copies from their initial values.
A log difference of ≥ 0.5 log in HIV-1 RNA viral load testing is said to be clinically significant. The results of this study were below 0.5 log therefore a significant change or decline was not noted.

CONCLUSION

The study showed that there is loss of HIV-1 RNA copies when EDTA samples are stored beyond 24 hours of sample collection, with plasma un-separated from RBCs. However, this loss is significantly shown by the number of HIV-1 RNA copies, logs shows that there is no statistically significant difference.

Keywords: *HIV-1, RNA, Lesotho, temperature, Whole blood*

INTRODUCTION

Lesotho is a small country landlocked by the Republic of South Africa in southern region of Africa; it faces a lot of challenges providing quality health care services. This is because majority of its population live in remote villages that are hours away from the nearest health facility (www.pih.org/country/lesotho).
Determination of Human Immunodeficiency Virus type 1(HIV-1) Ribonucleic Acid (RNA) viral load testing is performed at central and regional laboratories. However, decentralization of HIV-1 viral load testing has slowly started with three laboratories out of sixteen laboratories countrywide. This means high volumes of HIV viral

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load specimen are sent to the National Reference Laboratories (NRL) for testing. This implies that samples are being stored for a long time before testing, posing compromise of sample integrity (WHO, 2018).
There are six laboratories at NRL that provide laboratory services to both remote and nearby health centres. The molecular laboratory is one of these laboratories. It provides HIV-1 monitoring and diagnostic testing services. These tests are HIV-1 viral load, early infant diagnosis of HIV (EID) and confirmation of discordant HIV test results from the hubs or health facilities. However, in this study the focus will be on HIV-1 viral load testing using Roche COBAS Ampliprep/COBAS TaqMan instrument which requires plasma specimen.
WHO 2018, recommends that blood should be collected in sterile Ethylenediaminetetraacetic Acid (EDTA). Whole blood should not be stored at 2-25 ^{0}C beyond 24 hours of sample collection, and plasma should be separated from whole blood within 24 hours of collection. Prior to plasma separate, samples should be centrifuged at 3000 rotation per minute (rpm) for 20 minutes at room temperature. It further elaborates that plasma should then be transferred into sterile polypropylene tube and therefore stored as follows; up to 24 hours at 25 - 30^0 C (room temperature), up to 6 days at 2-8^{o}C and at -20 to -80^{0}C up to 6 weeks. (ICAP, 2016), plasma specimen may be frozen and thawed up to five times, without significant loss of HIV-1 RNA. (WHO, 2018).

In molecular laboratory at NRL specimens are not handled and stored as per WHO, 2018 recommendations. Upon reception samples are centrifuged within the first 24 hours of sample collection. However, the samples are stored without plasma being separated from the cells. These samples are stored at 2-8^{0}C beyond 24 hours of collection (up to 72 hours). Zucker 2016, the study indicates that elevated temperatures (exceeding 25^{0}C) and very cold temperatures can compromise the integrity of RNA specimen especially if still in the primary tube.
Fear that HIV-1 RNA stability may be compromised by poor specimen handling spiked the researcher to conduct this study. The fact that plasma is not separated from blood cells at NRL molecular laboratory gave birth to this study. Whole blood samples are received and centrifuged within 24 hours of collection at NRL molecular. However, these samples are stored at 2-8^{0}C beyond 24 hours. Therefore, the study was intended to determine the stability of HIV-1 RNA viral load in plasma specimens that are not cell free (un-separated) stored at different temperatures for a period of one week.
The test was performed on COBAS AmpliPrep/COBAS Taqman (CAP/CTM) (Taqman 96) using CAP/CTM HIV -1 Test, version 2.0 reagents.HIV-1 viral load test is an assay that determines or measures the number of HIV-1 RNA copies in a millilitre of blood (copies/ml). It can be used to determine the pre therapy (baseline) HIV-1 RNA level or to monitor the effects of the antiretroviral treatment (WHO, n.d).
WHO 2013, HIV treatment guidelines strongly recommend routine viral load (VL) testing six and twelve months after treatment initiation and then at least every year thereafter. Viral load testing enables clinicians to monitor antiretroviral therapy efficacy and to identify treatment failure and/or adherence challenges faced by patients, these two may result in elevated or non-suppressing VL levels. VL results help clinicians to intervene quickly and provide enhanced adherence support that can lead to VL suppression rate increasing (WHO, n.d). It is therefore very important that the laboratory give accurate and reliable results for efficient and effective treatment.
Over four hundred (400) EDTA specimens are received in the molecular lab daily from different health centres within its catchment area. The specimens are collected from sixty local health centres of which send specimens per day. Molecular laboratory serves sixty (60) local health centres and five (5) district laboratories. Each district laboratory serves plus or minus twenty (20) health centres. However, the district laboratories bring specimens in the form of plasma aliquots not EDTA whole blood.
WHO, n.d states: correct sample handling as per Standard Operating Procedures (SOPs) guarantees accurate - reliable results, hence efficient - effective treatment. HIV suppression will be met, and the risk of mismanagement will be lowered. This in turn will reduce the risk of transmission. HIV can be transmitted from mother to child during pregnancy or lactating phase (in mixed feeding, from one partner to the next in sexual activities.HIV-1 viral load samples are not handled as per SOPs and manufacturer specification prior analysis. Accuracy and reliability of results produced maybe affected by poor sample handling. Issuing inaccurate and unreliable results is a health and life hazard that can lead to mismanagement of patients. This can lead to delayed or unnecessary regimen switch of antiretroviral drugs (ARV). Failure to handle samples as per standards may result in reporting misleading patients' results. Statistics may reflect one thing while the patient's clinical status may show another. This may result in laboratory clients losing confidence in their services.

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LITERATURE REVIEW

A few studies have been conducted over the years with the aim of determining the suitable temperature and storage conditions that will best keep the HIV-1 RNA stable. Standard protocols for RNA – based viral load testing require for the RNA to be extracted directly from plasma. Through a few studies EDTA plasma was found to be the better source of cell free viral RNA compared to other anticoagulants. The whole blood EDTA and cell free EDTA plasma can be stored at room temperature for up to 30 hours, at 4^0C up to 14 days and at -70^0C for extended periods without significant decrease in the viral load signal (Ginocchio, 1997, Lew, 1998, Holodniy, 1995 & 2006).

A study was conducted to determine the influence of different temperature storages and anticoagulants on the HIV-1 RNA in EDTA whole blood samples (Bruistein, et al 1997). Nucleic acid sequence-based amplification – quantitative method (NASBA-QT) was the assay used for HIV viral testing in this study. The results indicated that whole blood EDTA sample stored at 25^0C for 72 hours was stable (Bruistein, et al 1997). Blood stored at 4^0C for 24 hours showed a decline. EDTA-plasma HIV-RNA copies did not decline while stored in lysis buffer up to 14 days (336 hours), but a significant decline was noted while stored at 30^oC in 2 days (48 hours). It was then recommended that when quantifying HIV-1 RNA by NASBA-QT, EDTA anticoagulant should be used (Bruistein, et al 1997).

HIV1-RNA quantification was performed using Roche Cobas Ampliprep- Cobas TaqMan (CAP/CTM: Roche Diagnostics). EDTA blood samples were processed within 6 hours of collection. The results were compared with HIV-1 RNA copies determined from plasma that was stored for 1 week at 4, 22, 30 and 37^0C. Samples stored at 4, 22 and 30^0 C did not show any effect on the HIV-1 RNA copies in 1 week. However, samples that were stored at 37^0C for a week showed a significant decrease in HIV-1 RNA copies. It was therefore recommended that plasma can be saved for up to 1 week at 30^0C before shipping to a reference laboratory for HIV-1 RNA quantification (Amellal, et al, 2008).

A study was conducted with the aim of evaluating the use of dried plasma spots to determine HIV-1 RNA viral load. The dried plasma samples on the filter paper (DPS-FS) and in tubes (DPS-T) were compared with corresponding liquid plasma samples. The samples were stored at 4, 22 or 37^0Cfor 7 days, and others were further air- dried up to 54 hours to evaluate effects of temperature and dry on HIV-1 RNA copies. It was concluded that obtaining reliable measurement of HIV-1 RNA viral load plasma must be stored in good conditions. The HIV-1 RNA stability was affected by drying and 1 week storage at 37^0C. The findings indicate that liquid plasma can be kept at 4 or 22^0C with no effect on viral load for a week (Amellal, 2007).

(Bonner, 2014), performed a study where RNA stability was measured, samples from HIV patients were stored at the time and temperature that exceeded manufacturer recommendations. The mean results of these samples were compared to those of the samples that were stored within established thresholds (as per manufacturer recommendations)

Bonner, 2014 further indicates that HIV-1 RNA stability was maintained in whole blood EDTA at all measured time points up to 168 days (1week) when stored at 4^0C. While stored at 25^0C for 72 hours it was also stable with data points before and beyond 72 hours suggesting stability but not reaching statistical significance. EDTA stored at 30^0C maintained stability up to 48 hours. It was concluded that whole blood and plasma samples in EDTA may remain stable under conditions exceeding current manufacture recommendations for HIV viral load testing.

AIM

To determine the effects of different temperature storages on HIV-1 RNA stability in an un-separated plasma sample.

The aim is to establish if HIV-1 RNA stability in un-separated plasma sample stored at different temperature storages will be affected.

OBJECTIVES

- To obtain the value of HIV-1 RNA copies from a freshly drawn (within 24 hours of collection) EDTA whole blood specimen.
- To obtain aliquots of 2-3 ml of blood specimen from a mini pool and store them at different temperatures (2-80C, -200C & -800C) for a week.

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- To determine the value of HIV-1 RNA copies of these aliquots after a week.
- To determine the chances of differences of HIV-1 RNA copies in both copies/ml and logarithms.

METHODOLOGY

Study Location

National Reference Laboratories in the Molecular Laboratory (section) in Lesotho

Study Design

Experimental research incorporating the post-test-control- group design was used in this study to determine whether various temperature storages of the sample's prior analysis from the time of sample collection will affect the number of HIV- 1RNA copies detected in the milliliter of blood as shown on table 1.

Table1 Study Layout

Protocol in question	Received in the lab(hours)	Centrifugation time from collection (hrs)	Aliquot time from collection (hrs)	Storage Temp:^{0}C & Time(hrs)	Sample stored as:	Container (tube)
SOP	24	24	24	25-30(24) 2-8 (144) -20, -80(11088)	Cell free plasma	sterile polypropylene tube
Current	24	24	32 +	2-8 (32-72)	Un-separated Plasma	Primary tube (EDTA)
Study	24	24	Not	2-8 (168) -20 (168) -80 (168)	Un-separated Plasma	Sterile tube

NB: Same colours = procedures agree & different =disagree

STUDY POPULATION

The number of participants

Sixty-eight (68) samples were intended to be used, however only fifty-six (56) were used: batches (21 Samples per rack) of fresh samples will be analysed daily to determine samples that have detectable values of viral load (HIV1 RNA copies). Batches of three (3) samples of detectable copies were made into one sample (mini pooling) to allow huge volumes that made four (4) aliquot (initial value (Int), 2-8 ^{0}C, -20 ^{0}C and -80 ^{0}C). Fourteen of these batches will be made, therefore summing up to 56 samples.

Participant identification

In the study samples were selected through Random sample. The method was used in order to represent the target population without being bias.

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In - and exclusion criteria

Inclusion Criteria

- Fresh specimen analysed as per SOP
- ETDA whole blood specimen to be tested for HIV-1 viral load
- Specimen from all ages and genders
- Specimen for both baseline and treatment monitoring
- Specimen stored beyond manufacturer's threshold
- Specimen not handled as per WHO recommendations

Exclusion Criteria

- Plasma aliquot samples to be tested for HIV-1 viral load

Special investigations

In this study routine viral load samples were used; therefore, no additional methods or technique were required to carry out the study.

Statistical analysis

Purely no statistical analysis was used in the study, because any result that deviated from the fresh sample (one analysed as per SOP) result was of significance.

Ethical aspects and good clinical practice

No names, addresses or patients' identification information were used in this study. Outcome purely served the purpose of the study. Samples were given unique numbers.

Ethical clearance

An ethical approval was granted by the Lesotho Ministry of Health (MOH number 199-2019), before proceeding with this project.

Good Clinical Practice (GCP) / Quality Assurance

All clinical work conducted under this protocol is subjected to the GCP guidelines (The Principles of ICH GCP, 2004). The declaration of Helsinki's basic principle number 3 states that research should be conducted only by scientifically qualified people and under the supervision of adequately qualified people (World Medical Association, 2002).

Safety variables

Laboratory safety rules and procedure were adhered to as per safety SOPs, guidelines and manual.

Project safety

The project was undertaken by laboratory personnel who routinely analyses HIV 1 viral load samples and adheres to protocols and procedures that guide such assays, so is the laboratory supervisor.

Patient's safety

Patients were not affected by this study as samples that used are routine viral load samples.

Premature discontinuation of the study

The study would have been discontinued prematurely if the researcher or any of the supervisors(s) felt that a patient's confidentiality might be breached or if any unethical procedures occurred.

Financial implications for the participant

Patients were not affected financially by this study as samples used were routine viral load samples.

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Withdrawal criteria
Patients were not directly affected by this study as samples used are routine viral load samples.

RESULTS

Table 2: Viral load results expressed in number of copies per ml of blood stored at different temperatures

BATCH No.	25-30^{0}C (Int)	2-8^{0}C	-20	-80^{0}C
B#1	655	325	468	289
B#2	2134	891	1413	1236
B#3	1872	566	879	884
B#4	36744	32307		24428
B#5	10780	7720	10689	9117
B#6	33939	21748	24306	18420
B#7	9119	6123	8688	8857
B#8		927124	926909	836444
B#9		42145	40713	29280
B#10		44709	42981	40052
B#11	22976	16161	16037	12732
B#12	32968	32157	26960	25917
B#13	107	103	84	72
B#14	30176	27314	26720	26710

NB: The red block indicates no results. (The run had failed, and their samples were insufficient to re-run)
Table 2 above shows the results of fourteen batches of samples stored at different temperatures and they are expressed in RNA copies per ml of blood, only ten batches show results as indicated.

Table 3: Viral load results expressed in logs (log_{10}) stored at different temperatures

BATCH No.	25-30	2-8^{0}C	-20	-80^{0}C
B#1	2.8	2.5	2.7	2.5
B#2	3.3	2.9	3.2	3.1
B#3	3.3	2.8	2.9	2.9
B#4	4.6	4.5		4.4
B#5	4.0	3.9	4.0	4.0
B#6	4.5	4.3	4.4	4.3
B#7	4.0	3.8	3.9	3.9
B#8		6.0	6.0	5.9
B#9		4.6	4.6	4.5
B#10		4.7	4.6	4.6
B#11	4.4	4.2	4.2	4.1
B#12	4.5	4.5	4.4	4.4
B#13	2.0	2.0	1.9	1.9
B#14	4.5	4.4	4.4	4.4

NB: The red block indicates no results. (The run had failed, and their samples were insufficient to re-run)
The table 3 above shows the results of fourteen batches of samples stored at different temperatures expressed in logs, only ten batches show results as indicated.

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Table 4: Differences (DX) in number of copies and percentage differences (%X) per batch

BATCH No.	Diff A (2-8^{0}C)	%A	Diff B (-20^0 C)	% B	Diff C (-80^{0}C)	%C
B#1	330	50	187	29	366	56
B#2	1243	58	721	34	898	42
B#3	1306	70	993	53	988	53
B#4	4437	12			12316	34
B#5	3060	9	91	1	1663	15
B#6	12191	36	9633	28	15519	46
B#7	2996	33	431	5	262	3
B#8			215	0	90680	10
B#9			1432	3	1432	3
B#10			1728	4	4657	10
B#11	6815	30	6939	30	10244	45
B#12	811	2	6008	18	7051	21
B#13	4	4	23	21	35	33
B#14	2862	9	2862	9	3466	11

NB: The red block indicates no results. (The run had failed, and their samples were insufficient to re-run)
Table 4 above shows the difference in number of RNA copies and their percentages of samples that were stored at different temperatures, compared to the initial value (Int). Only ten batches show results as indicated.

The following formulae were used:
DX= Int – (new value as per varying temperature storages)

$$\% X = \frac{DX}{Int} \times 100$$

Where x can be replaced by either A, B or C.

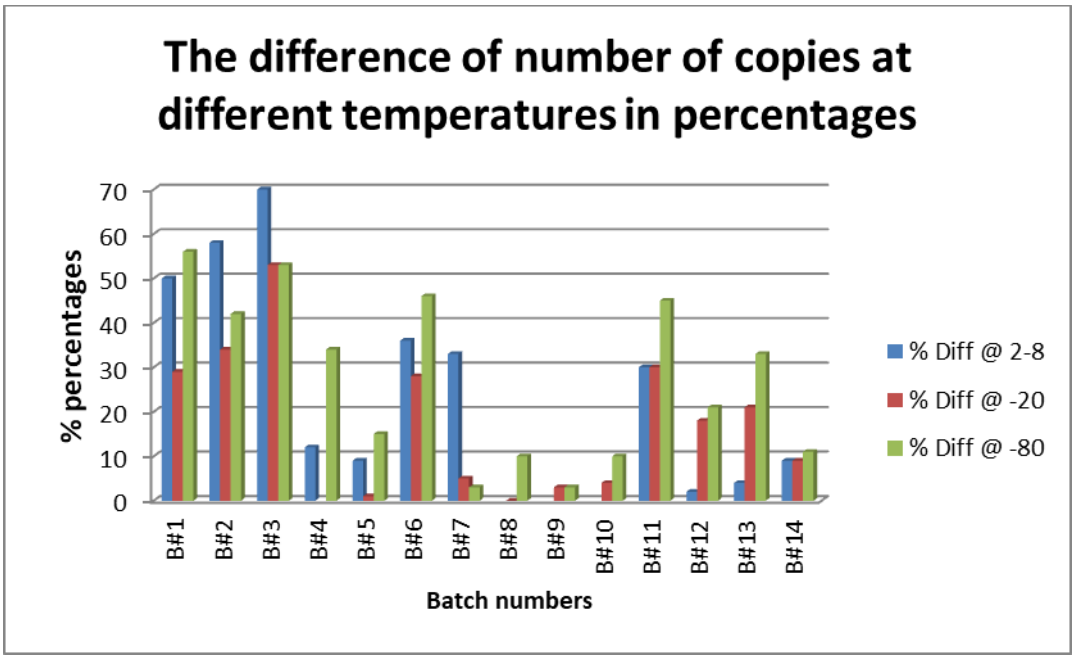

Figure 1: The Bar chart below shows the differences in number of copies at different temperatures per batch expressed in percentages

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The figure 1 above shows the percentage difference in number of RNA copies that were stored at different temperatures, compared to the initial value (Int). The following formula was used:

$$\% X = \frac{DX}{Int} \times 100$$

Where x can be replaced by either A, B or C.

Table 5: Differences in logs expressed in percentage per batch

BATCH No.	% DIFF A	% DIFF B	% DIFF C
B#1	11	5	13
B#2	11	0	10
B#3	16	10	10
B#4	1		4
B#5	4	2	2
B#6	4	3	7
B#7	5	0	1
B#8			
B#9			
B#10			
B#11	4	4	6
B#12	0	2	2
B#13	1	5	8
B#14	1	1	1

NB: The red block indicates no results. (The run had failed, and their samples were insufficient to re-run)
Table 5 above shows the results of fourteen batches of samples stored at different temperatures expressed percentage differences of their logs, only ten batches show results as indicated and four tests failed which resulted in B#8 to 10 not having results due to unavailability of the initial value. Therefore, the formula cannot be applied.

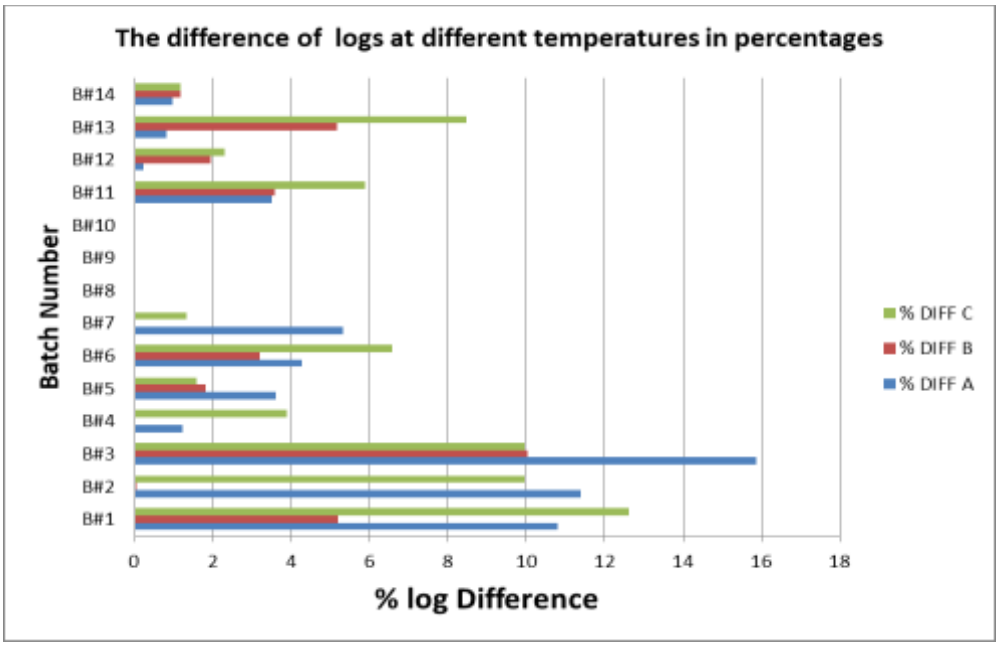

Figure 2: The Bar chart below shows the differences in logs at different temperatures per batch expressed in percentages

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The figure 2 shows the results of fourteen batches of samples stored at different temperatures expressed percentage differences of their logs, only ten batches show results as indicated and four tests failed which resulted in B#8 to 10 not having results due to unavailability of the initial value.

DISCUSSIONS

Results in number of copies did not show a significant decrease in HIV-1 RNA copies, for samples that were stored at 2-8^0 C (difference A) a greater percentage of 57% samples, showed the difference in number of copies that was less than 50%. Only 21 % were above 50% difference and the other 21% of the batches stored at this temperature had failed. Ginocchio et.al 2006, Bruistein et.al 1997 and Amellal et.al 2008 in their studies also indicated that samples that were stored at 4^0 C for 14 days, 24 hours and 1 week respectively, did not show a significant decline in number of RNA copies.

Samples stored at -20^0 C and – 80^0 C did not indicate significant decline in RNA copies as 7% and 14% respectively of the samples showed a difference of above 50% number of copies from their initial values.

A log difference of ≥ 0.5 log in HIV-1 RNA viral load testing is said to be clinically significant. The results of this study were below 0.5 log therefore a significant change or decline was not noted. However, the results were grouped as follows in percentages:

At 2-8^{0}C 72% of the samples showed a 0-11 % variation from the initial value, with a high of 16% deviation consisting 7% of the batch and 21% of them failed. Samples stored at -20^{0}C 64% showed a 0-5 % deviation, 10% deviation was the highest at this temperature with 7% of the samples and 29% had failed. At -80^{0}C 72% of the sampled showed a deviation of 1-10%, 13% was the highest deviation of 7% of the samples. 21% had failed.

Results showed a more significant loss of RNA when expressed in number of copies/ml as opposed to logs. Logs are the tenfold units' measurements and because of these numbers are still within the same range of units the difference will not be seen a significant.

CONCLUSION AND RECOMMENDATIONS

The study showed that there is loss of HIV-1 RNA copies when EDTA samples are stored beyond 24 hours of sample collection, with plasma un-separated from RBCs. However, this loss is significantly shown by the number of HIV-1 RNA copies, logs shows that there is no statistically significant difference. These pose as a challenge as most of our clinicians monitor patients HIV viral load in number of copies. Viral load testing enables clinicians to monitor antiretroviral therapy efficacy and to identify treatment failure and/or adherence challenges faced by patients, if HIV-1 RNA copies are lost over time when samples are stored against manufacturer's recommendations, patient's results may not correlate with their clinical appearance and history.

For the purpose of the study samples were stored for a week; it is most likely that if an EDTA viral load sample was stored over a longer period with plasma un-separated from the cell there would be a great loss of HIV-1 RNA copies in such a sample. Loss of HIV-1 RNA copies in a sample however does not indicate that a patient is HIV-1 negative; rather they will give false monitoring results. Patients will therefore be perceived to have HIV-1 viral load suppression. It is therefore very important to centrifuge ETDA samples and separate plasma from the red blood cells within the first 24 hours of sample collection to avoid loss of HIV-1 RNA copies in such samples.

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