KENYA MEDICAL RESEARCH INSTITUTE

Performance of Blood STASIS DNA/RNA tubes for RNA stabilization in whole blood

Prof Matilu Mwau
Chief Research Officer
Kenya Medical Research Institute
Mbagathi Rd. Nairobi, Kenya
P.O. Box 54840 – 00200
Tel: +254 (0)20 2713349. +254 (0)20 2722541. 0722-205901
mmwau@kemri.org, www.kemri.org
Signature……Date…7/Dec/208

Background

Monitoring HIV positive patients has evolved over the years from using CD4 monitoring every six months and viral load testing only when the capacity exists to using viral load testing as the preferred monitoring approach to diagnose and confirm treatment failure[2, 3]. The current World Health Organization (WHO) guidelines recommend viral load (VL) testing as the preferred monitoring approach for all individuals treated with ART in order to assess treatment response, detect treatment failure and determine the need to switch to second-line regimen in a timely manner[1]. In countries all over the world, viral load testing guidelines and algorithms have been developed in line with these WHO recommendations. The viral load testing algorithm in Kenya recommends that viral load monitoring of patients on ART should be done at 6 months after starting ART, irrespective of baseline viral load results[4].

In Kenya, viral load monitoring is commonly conducted using with the Amplicor HIV1 Monitor v1.5 (Roche Molecular Systems), the COBAS TaqMan (Roche Molecular Systems), the RealTime HIV-1 (Abbott Systems) and the Aptima Quant Dx (Hologic Inc) assays [5, 6]. These are sophisticated reference technologies placed in ten laboratories in the major cities. They differ in performance characteristics, HIV target region, the dynamic range of viral load counts, as well as nucleic acid extraction, amplification and detection methods.

Samples get to these labs through various innovative approaches to meet the challenge of transportation over long, difficult roads.

Up to 35% of the samples delivered to these labs are in the form of dried blood spots, while the remainder is either whole blood or plasma transported in EDTA tubes. Dried blood spots can be kept at room temperature for several weeks without loss of sample integrity for either early infant diagnosis or viral load testing. However, in our hands, dried blood spots present significant challenges during HIV genotyping if stored for more than seven days after spotting.

Two thirds of the samples received at the testing laboratories are either whole blood or plasma, each of which can be stored at room temperature for up to six hours at most. These sample types also often require transportation in ice. Unfortunately, many facilities outside of the big cities are unable to provide the ice packs required to transport plasma or whole blood. In any case, once the samples arrive in the laboratory, they must be tested within a few hours, or stored at minus 80C.

Blood STASIS DNA/RNA are plastic, evacuated tubes used for the collection, anticoagulation, stabilization, transport and storage of venous whole blood as well as the preservation of circulating, cell free DNA.

Justification

DNA Stasis tube are reputed to stabilize whole blood for 21 days at room temperature. Although they have the potential to improve sample handling in field settings in Kenya, their performance characteristics have not been evaluated locally. Additionally, although they have been evaluated for the stabilization of both genomic and cell free DNA, their effect in plasma RNA has not been comprehensively studied.

Kenya's HIV viral load testing policy was revised in the last quarter of 2018. The clinical cutoff was lowered from 1000 copies/ml of plasma to below the detection limit. Detection limits are dependent on the particular technology and the sample type, but can be as low as 20 copies/ml using plasma. Since dried blood spots do not allow detection below hundreds of copies per ml, the net effect of the new policy guidelines was to shift from DBS to plasma or whole blood. Transportation of plasma of whole blood requires a cold chain unless DNA and RNA stabilization can be achieved.

Objectives

The overall objective of this study was to determine the performance of DNA Stasis tube for RNA stabilization in whole blood.

Methodology

This was a methods comparison study conducted amongst consenting HIV positive adults from a cross-section of health facilities in Western Kenya.

Sample size and recruitment of study participants

A total of 523 study participants were recruited for this study: 388 were from Busia County referral hospital, 70 from Nambale and 65 from Alupe Sub-county referral hospitals in Western Kenya. Consecutive sampling was adopted for sample collection. Each participant was presented with an informed consent form to participate to the study. Once consent was provided, participants were recruited to the study and samples were collected from them. From each participant, venous blood samples were collected from a single blood draw to two tubes (EDTA plasma preparation tube (PPT) and Blood STASIS DNA/RNA each of 4ml. Whole blood samples in the EDTA PPT were first spotted on the 5 spots of a Dried Blood Spot (DBS) card with each spot have 50µl of blood. DBS were left at the health facility to dry overnight and transported to the KEMRI Alupe HIV lab on the next day upon drying for routine viral load monitoring. Within 6 hours of blood sample collection, remnant whole blood on the PPT and that in the Blood STASIS tube was transported at 4^0C to the same lab for analysis.

Laboratory testing

In the KEMRI Alupe HIV lab, sample testing was done in three phases: comparison between plasma EDTA on day 0 and Stasis tube on day 5 on Roche CAP/CTM, comparison between plasma EDTA on day 0 and Stasis tube on day 5 on Abbott m2000 and comparison between DBS on day 5 and plasma Stasis tube on day 5 on Abbott m2000.

Comparison on Roche CAP/CTM

Venous whole blood samples collected in both tubes were received for this phase of the study. Immediately, whole blood in the EDTA PPT was centrifuged at 1100g for 10 minutes to obtain plasma. On the day of blood draw, these plasma samples were tested on the Roche Cobas Ampliprep/ Cobas Taqman HIV-1 Test v2.0 assay according to manufactures instructions. Briefly; Plasma samples were transferred into an S-tube. Assay controls were vortexed before use. From the protocol screen, the AMPLILINK equipment software, daily maintenance was selected and performed as per the instructions in the wizard. The machine was disinfected using 70% ethanol.

Sample racks containing S-tubes with Low Positive control, High Positive control, Negative control and samples at different positions were placed in the COBAS® Ampliprep Instrument. HIV-1 v2.0 CS1, HIV-1 v2.0 CS2, HIV-1 v2.0 CS3 and HIV-1 v2.0 CS4 immediately from 2-8°C storage, were placed into reagent racks and put in positions A-E respectively in the COBAS® Ampliprep Instrument. Test consumables sufficient for 24 tests at a time were placed in the COBAS® Ampliprep Instrument at different positions; SPUs loaded in racks were placed at positions J, K/L. K-tips in racks were placed at positions M, N,O/P and K-tubes in racks were placed at positions F,G /H. From the AMPLILINK software protocol screen, HI2CAP96 application was selected for HIV-1 quantitative assay. Sample extraction, amplification and detection was initiated by following the instructions in the wizard. After test completion of COBAS® TaqMan® Analyzer results were archived. Used SPUs, K-tip racks, K-tube racks, reagent cassettes, barcodes from sample racks and k-tubes were put in a biohazard bag and discarded.HIV-1 quantitative assay sample results were interpreted in copies/ml (cp/ml) with the limit of detection being 20cp/ml.

Whole blood collected in Blood STASIS DNA/RNA tubes was maintained at room temperature for 5 days after which the tubes were centrifuged at 1100g for 10 minutes to obtain plasma tested on the Roche assay as previously described.

Results from both tubes were recorded and performance described using results from EDTA PPT as the reference.

Comparison on Abbott m2000

Venous whole blood samples collected in both tubes were also received for this phase of the study. Immediately, whole blood in the EDTA PPT was spotted on a DBS card, with each spot having 50µl of whole blood and left to dry overnight and stored. The remaining whole blood in the EDTA PPT was centrifuged at 1100g for 10 minutes to obtain plasma. This was tested on Abbott m2000 HIV-1 Test v2.0 assay according to manufactures instruction on the day of blood draw.

On the 5th day after sample collection, DBS samples were retrieved and tested on the Abbott m2000 HIV-1 Test v2.0 assay according to manufactures instructions. Briefly; One DBS spots from a Whatman 903 filter paper card was cut into the Abbott master mix tube, mLysis DNA Buffer was added and this mixture was incubated at 560C for 30 minutes. Assay calibrators, controls and

internal controls were thawed at 15-30oC or at 2-8°C. Each calibrator and control was vortexed before use. Amplification reagents were thawed at 15-30oC or at 2-8oC until required for the amplification master mix procedure. The Abbott msample preparation bottles were allowed to equilibrate at room temperature. Low positive control, high positive control, negative control, calibrators and samples in Abbott Master mix tubes were placed onto a sample rack and into the Abbott m2000sp 1ml subsystem carrier. Abbott msample Preparation System Reagents; mlysis 1, mlysis 2, mMicro-particles, mWash1, mWash 2 and mElution Buffer for HIV-1 quantitative assay were placed into reagent racks and onto the Abbottm2000sp. Abbott 96 Deep-Well plate was placed on the Abbott m2000sp worktable.1000ul DiTi Tips were loaded in the DiTi racks on the shelf and rack 1 and 200ul DiTi on rack 2. From the protocol screen, 2.0 ml DBS application was selected for HIV-1 quantitative assay. Sample extraction protocol was initiated by following the instructions in the wizard. Calibrator and control lot specific values in the appropriate fields will be entered. Upon completion of sample extraction process, Abbott 96-well Optical Reaction Plate was placed on the output deck. Amplification reagents and the master mix bottle were loaded on the Abbott m2000sp worktable and the appropriate deep-well plate that matches the corresponding sample preparation extraction was selected. The Abbott m2000sp Master Mix Addition protocol was initiated and the instructions followed as described in the wizard. The Abbott m2000rt instrument was switched on and left to initialize. After completion of the Master Mix Addition Protocol, the Abbott 96-well Optical Reaction Plate was sealed using an optical adhesive cover and transferred to the Abbott m2000rt instrument. From the screen protocol the appropriate application file corresponding to the sample work sheet being tested was selected. The Abbott RealTime HIV-1 protocol for HIV-1 quantitative assay was initiated and the process left to run to completion. The results were archived and interpreted in copies/ml (cp/ml).The limit of detection was 839cp/ml.

Whole blood collected in Blood STASIS DNA/RNA tubes were maintained at room temperature. After 5 days, the tubes were centrifuged at 1100g for 10 minutes to obtain plasma which was tested on the Abbott m2000 HIV 1assay as previously described.

Results from both evaluations were recorded and performance described.

Ethical Considerations

The evaluation was carried out in line with existing ethical guidelines. Permission to conduct this evaluation was sought from the Ethical Review Board of the Kenya Medical Research Institute and the study was reviewed and approved by the Kenya Medical Research Institute Ethical Review Committee. Appropriate ethical considerations were considered to protect the patients included in the study including Informed consent and confidentiality.

Data management and statistical analysis

All data was consolidated into a spread sheet and exported to STATA version 13.1 for Windows. Viral load results were log transformed. Bland Altman analysis was used to determine the limits of agreement and the mean bias. A 2 by 2 table was used to determine misclassification rate at a cut-off of 1000 copies/ml and the coefficient of determination (r^2) was used to describe the linear fit of paired log transformed values of viral loads for both EDTA PPT and DNA Stasis tube on Roche CAP/CTM test platform.

Results

A total of 523 participants provided samples for this study. All were adults aged between 18 and 82 years with a mean age of 41.69. A total of 317 (60.96%) were female while 203 (39.04%) were male. Three participants did not provide data on gender.

Of the 523 participants, 296 provided a PPT and DNA Stasis tube sample for comparison using the Roche CAP/CTM. Two hundred and twenty seven (227) participants provided a PPT, DNA stasis and DBS sample for comparison on Abbott m2000.

Comparison on Roche CAP/CTM

A total of 273 samples collected in EDTA PPT were tested successfully. Of these, 45 (16.48%) samples had undetectable viral loads, 18 (6.59%) samples had counts <20 cp/ml and 210 (76.92%) samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 111 (48.68%) samples had viral load counts <1000cp/ml while 117(51.32%) samples had viral load counts >1000cp/ml.

A total 281 samples collected in DNA stasis tubes were tested successfully. Of these, 20 (7.12%) samples had undetectable viral loads, 6 (2.14%) samples had counts <20 cp/ml and 255 (90.75%)

samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 153 (58.62%) samples had viral load counts <1000cp/ml while 108 (41.38%) samples had viral load counts >1000cp/ml.

The percentage concordance between the two tubes on the Roche CAP/CTM assay at a cut off of 1000 copies/ml was determined as shown in Table 1. Two hundred and seventeen (217) successful paired comparisons were made. Overall concordance was seen in 200 (92.16%) comparisons. Thirteen (13, 11.30%) samples were misclassified downwards and 4 (3.92%) samples were misclassified upwards. Therefore, the sensitivity of the DNA Stasis tube on day 5 was 88.70% and the specificity was 96.08%. These findings are summarized in Table 1 below.

DNA STASIS TUBE	EDTA PPT TUBE		
	<1000cp/ml	>1000cp/ml	Total
< 1000 cp/ml	98 (96.08%)	13 (11.30%)	111(51.15%)
>1000cp/ml	4 (3.92%)	102(88.70%)	106(48.85%)
Total	102 (100.00%)	115(100.00%)	217(100.00%)

Table 1: Sensitivity and specificity of Blood Stasis tube on the CAP/CTM HIV -1 plasma assay

A scatter plot indicated an excellent fit with a coefficient of determination of r^2=0.8308, (95% CI 0.9667-1.0915, $p<0.05$) as shown in Figure 1.

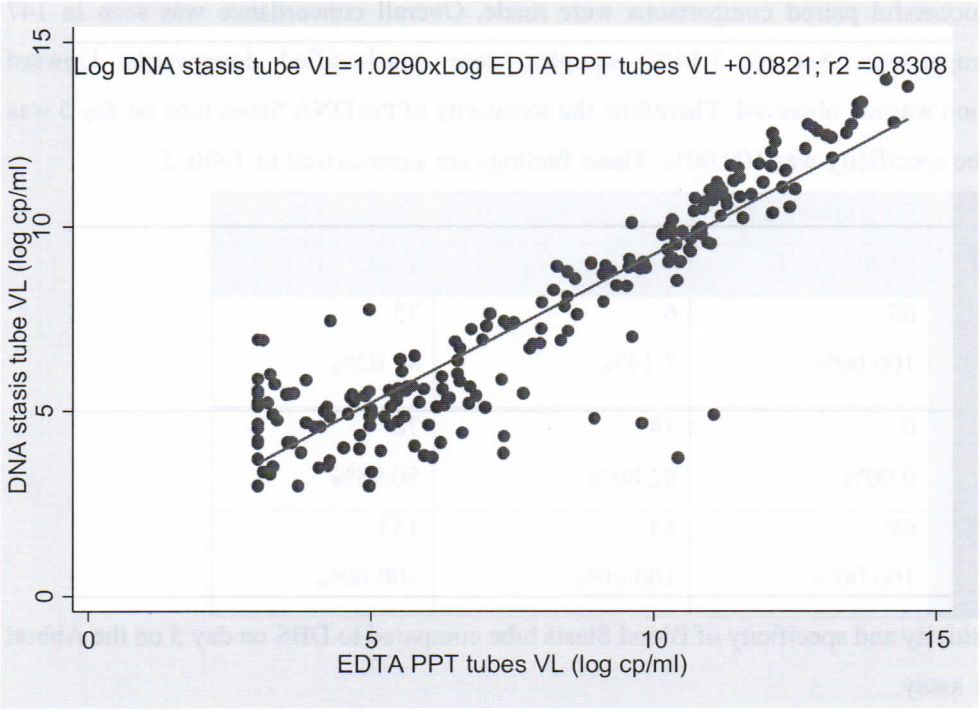

Figure 1: Scatter plot of log transformed viral loads of DNA Stasis tube against EDTA PPT plasma assay on the Roche CAP/CTM test platform.

Comparison between DBS and DNA stasis tube on day 5 Abbott m2000

A total 218 DBS samples were tested successfully. Of these, 65 (29.82%) samples had undetectable viral loads, 67 (30.73%) samples had counts <839 cp/ml and 86 (39.45%) samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 69(45.10%) samples had viral load counts <1000cp/ml while 84(54.90%) samples had viral load counts >1000cp/ml.

A total 119 plasma samples collected in DNA Stasis tubes were tested successfully. Of these, 47 (39.50%) samples had undetectable viral loads, 25(21.01%) samples had counts <40 cp/ml and 47 (39.50%) samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 75(49.02%) samples had viral load counts <1000cp/ml while 78(50.98%) samples had viral load counts >1000cp/ml.

The percentage concordance between DBS and plasma on DNA Stasis tubes on the Abbott m2000 assay at a cut off of 1000 copies/ml was determined as shown in Table 2. One hundred and fifty

three (153) successful paired comparisons were made. Overall concordance was seen in 147 (96.08%) comparisons. Six (6, 7.14%) samples were misclassified downwards. Upward misclassification was not observed. Therefore, the sensitivity of the DNA Stasis tube on day 5 was 92.86% and the specificity was 100.00%. These findings are summarized in Table 2.

BLOOD STASIS TUBE	DBS ON DAY 5		
	<1000cp/ml	>1000cp/ml	Total
< 1000 cp/ml	69 100.00%	6 7.14%	75 49.02%
>1000cp/ml	0 0.00%	78 92.86%	78 50.98%
Total	69 100.00%	84 100.00%	153 100.00%

Table 2: Sensitivity and specificity of Blood Stasis tube compared to DBS on day 5 on the Abbott m2000 HIV-1 assay.

A scatter plot indicated a coefficient of determination of r^2=0.7997, (95% CI 1.1765-1.5405, p<0.05) as shown in Figure 2.

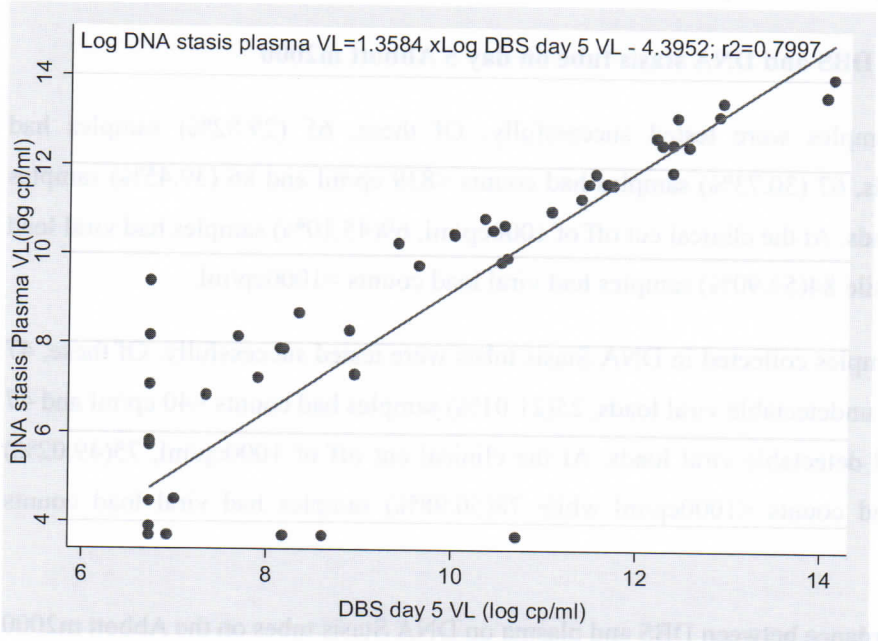

Figure 2: Scatter plot of log transformed viral loads of DNA Stasis tubes plasma against DBS on day 5 on the Abbott m2000 test platform.

Comparison between EDTA PPT and DNA stasis tube on day 5 Abbott m2000

A total 226 samples collected in EDTA PPT were tested successfully. Of these, 84 (37.17%) samples had undetectable viral loads, 49 (21.68%) samples had counts <40 cp/ml and 93 (41.15%) samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 65(45.77%) samples had viral load counts <1000cp/ml while 77(54.23%) samples had viral load counts >1000cp/ml.

A total 226 samples collected in DNA stasis tubes were tested successfully. Of these, 43 (19.03%) samples had undetectable viral loads, 31 (13.72%) samples had counts <40 cp/ml and 152 (67.26%) samples had detectable viral loads. At the clinical cut off of 1000cp/ml, 111 (60.33%) samples had viral load counts <1000cp/ml while 73 (39.67%) samples had viral load counts >1000cp/ml.

The percentage concordance between plasma on EDTA PPT and DNA Stasis tubes on the Abbott m2000 assay at a cut off of 1000 copies/ml was determined as shown in Table 3. One hundred and thirty one (131) successful paired comparisons were made. Overall concordance was seen in 128 (97.71%) comparisons. Three (3, 3.95%) samples were misclassified downwards. Upward misclassification was not observed. Therefore, the sensitivity of the DNA Stasis tube was 96.05% and the specificity was 100.00%. These findings are summarized in Table 3 below.

DNA STASIS TUBE	EDTA PPT TUBE		
	<1000cp/ml	>1000cp/ml	Total
< 1000 cp/ml	55 100.00%	3 3.95%	58 44.27%
>1000cp/ml	0 0.00%	73 96.05%	73 55.73%
Total	55(100.00%)	76 (100.00%)	131(100.00%)

Table 3: Sensitivity and specificity of Blood Stasis tube on the Abbott m2000 HIV -1 plasma assay

A scatter plot indicated an excellent fit, with a coefficient of determination of r^2=0.9743, (95% CI 0.8470-0.8964, p<0.05) as shown in Figure 3.

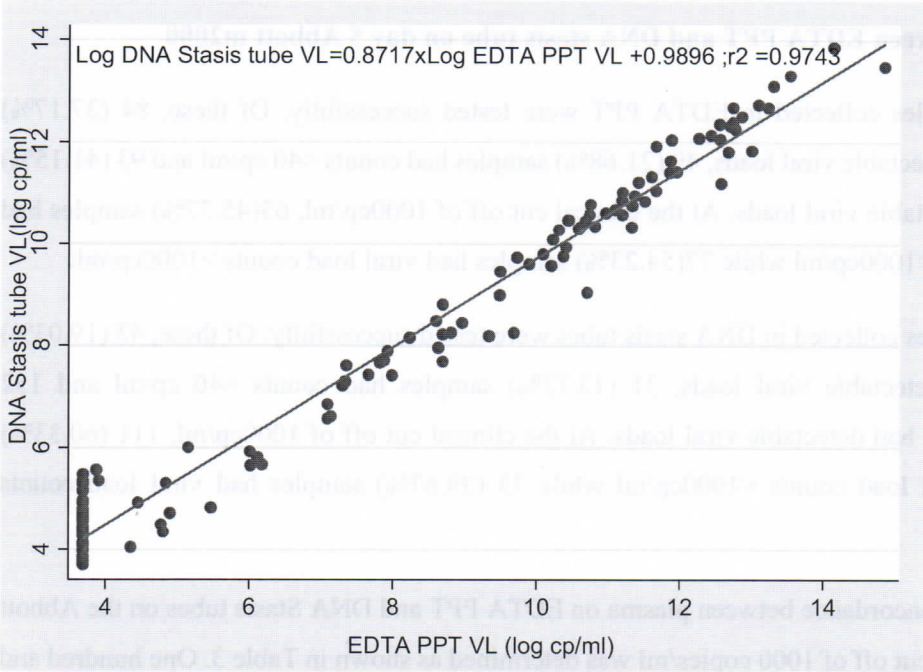

Figure 3: Scatter plot of log transformed viral loads of DNA Stasis tubes against EDTA PPT plasma on the Abbott m2000 test platform.

DISCUSSION

Viral load monitoring guidelines change regularly, with the current guidelines requiring all HIV patients on ART to be virally suppressed i.e. viral copies/ml should be undetectable. Using plasma as the sample type for HIV viral load monitoring, the limit of detection on Abbott is 40cp/ml and 20cp/ml on both Roche CAP/CTM and C8800. Using DBS as a samples type, the limit of detection has been 550 cp/ml on the 2 spot protocol and 839 cp/ml on the current 1 spot protocol. To meet the guideline requirements, plasma testing should be the ideal sample type for HIV clinical patient management.

In Kenya, K2 EDTA plasma preparation tubes (PPT) are used for HIV sample collection routine viral load testing. These tubes have a cloudy gel which separates the plasma and the hematocrit

preventing remixing after centrifugation. Additionally, the gel contains a genetic material stabilizing agent. However, these tubes only stabilize RNA in whole blood for 6 hours and 24 hours after centrifugation. For additional storage, cold chain is required to maintain RNA stability.

The Blood Stasis tube has not been previously evaluated in Kenya. Its strength as a sample collection tube lies on its ability to stabilize RNA in whole blood at room temperature for 5 days.

In the Roche Platform, with EDTA at the clinical cut off of 1000 cp/ml, the concordance was 92.16%, while the sensitivity was 88.70% and the specificity was 96.08%. Pearson Correlation of log transformed viral loads between DNA Stasis tube against EDTA PPT plasma assay on the Roche CAP/CTM test platform showed high concordance with an $r2=0.8044$, $p<0.05$ indicating the significant positive relationship between the two tubes regardless of the difference in testing days.

In the Abbott m2000 Platform, with DBS at the clinical cut off of 1000 cp/ml, the concordance was 96.08%, while the sensitivity was 92.86% and the specificity was 100%. Pearson Correlation of log transformed viral loads between DNA Stasis tube against DBS assay on the Abbott platform showed high concordance with an $r2=0.7997$, indicating the significant positive relationship between the two sample types.

In the Abbott m2000 Platform, with DNA stasis plasma as the sample type at the clinical cut off of 1000 cp/ml, the concordance was 97.71%, while the sensitivity was 96.05 % and the specificity was 100%. Pearson Correlation of log transformed viral loads between DNA Stasis tube against EDTA plasma assay on the Abbott platform showed high concordance with an $r2=0.9743$, indicating the significant positive relationship between the two sample types.

Our performance evaluation therefore indicates that with plasma as the sample type, DNA Stasis tube are comparable and can be used interchangeably with EDTA PPT tubes for HIV-1 viral load testing on either the Roche or the Abbott m2000 platform. They can also be used interchangeably with Dried Blood Spots.

Conclusion

DNA Stasis tube plasma tests was comparable to EDTA PPT tests and DBS tests for viral load quantification. Therefore, the DNA Stasis tube can stabilize RNA in whole blood for 5 days and ultimately be used for accurate HIV viral load monitoring.

In resource limited settings, the ability to stabilize RNA in whole blood at room temperature will obviate the need for cold storage for whole blood samples scheduled for HIV viral load testing.

Recommendation

DNA Stasis tube have met the threshold for use interchangeably with PPT tubes and Dried Blood Spots for viral load testing, and as they do not need cold storage during transportation, we recommend that they be adopted for blood transportation for viral load testing.