Classification of HIV Virological Failure Using Whole Blood versus Plasma Viral Load

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Declaration

I, ……*Aabida Khan…*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Abstract

Introduction

HIV viral load testing is the preferred monitoring approach for HIV infected patients on combination antiretroviral therapy (cART) as it is more sensitive than CD4 count and clinical monitoring. In resource limited settings, timely plasma separation and transportation to testing laboratories is a major barrier to the access of HIV viral load testing. The 2015 World Health Organisation guidelines recommend that cART should be initiated in all adults and children living with HIV regardless of disease stage or CD4 count, thereby escalating the demand for HIV viral load testing. Potential solutions to expand implementation and scale up of viral load testing in low and middle income countries are whole blood testing through point of care (POC) viral load assays or dried blood spots (DBS) collected at the health facility. Utilization of whole blood instead of plasma would simplify sample collection, storage and transportation requirements and be cost effective. However, the paucity of studies comparing whole blood HIV viral load across different test platforms, especially in the correct classification of virological failure, has resulted in the lack of a standardised programmatic approach to whole blood viral load testing.

Methods

We evaluated four HIV whole blood viral load test methods namely Alere q HIV-1/2 POC, Abbott RealTime HIV-1 DBS original and updated protocols, and Roche CAP/CTM DBS free virus elution (FVE) protocol, against the standard of care, plasma viral load, on 299 samples across the viral load spectrum from South African patients on cART. Virological failure was defined at >1000 copies/ml. Proportions of correct classification of virological failure and overall correlation with plasma were used for evaluating each method's performance.

Results

Alere q, Abbott original and updated, and Roche FVE correctly classified virological failure in 61%, 89%, 87% and 76% of all samples tested respectively. The performance varied across plasma viral load categories. Alere q showed good correlation above plasma viral load of 1000 copies/ml, with correct classification of virological failure in 100% of samples. However, below the plasma threshold of 1000 copies/ml, Alere q demonstrated significant over-quantification, resulting in reduced specificity and upward misclassification of virological failure in 39% of all samples tested. Abbott original and updated also had good sensitivity of 98% and 91% respectively and the best overall correlation with plasma (r^2 = 0.76 and 0.72 respectively), but there was upward misclassification in 10% and 8% of samples tested respectively. Roche FVE had the best specificity of 99% but with significantly reduced sensitivity of 53%, especially between 1000–10,000 copies/ml of plasma, resulting in downward misclassification in 24% of all samples tested. Greatest variability between the different testing methods was seen when plasma viral load was 40-1000 copies/ml. Correlation was best for all whole blood viral load assays at >10,000 copies/ml.

Conclusion

The key finding highlighted by this study is the great variability between the different whole blood test methods. Various factors influence the ability to quantify whole blood HIV viral load such as input volume used in each assay vary, sample treatment/processing (DBS versus fresh blood samples versus FVE), extraction (RNA selective, total nucleic acid extraction), amplification target and detection methods are different for each of the platforms tested. Based on our study, Alere q and Abbott DBS need to raise their whole blood threshold for virological failure in order to reduce upward misclassification and Roche FVE needs to achieve better sensitivity around its limit of detection. Receiver operating characteristic curve analysis can be used to determine the optimum threshold of virological failure for each assay.

Chapter 1: Literature Review

HIV/AIDS remains one of the world's most significant public health challenges, particularly in low and middle income countries[1]. In 2015, there were 36.7 million (34–39.8 million) people living with HIV[2]. Sub-Saharan Africa, especially southern Africa, has the highest global burden of HIV (70%)[3]. As of December 2015, 17 million people living with HIV were accessing combination antiretroviral therapy (cART), an increase from 15.8 million in June 2015[2]. In sub-Saharan Africa itself, 10.3 million people were accessing cART, with treatment coverage increasing from 24% in 2010 to 54% in 2015[4]. HIV prevalence is therefore increasing worldwide because people on cART have increased life expectancies[3].

Monitoring individuals receiving cART is important to ensure successful treatment (virological suppression), identify adherence problems and treatment failure[5]. The 2016 World Health Organisation (WHO) consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection recommend HIV viral load testing as the preferred monitoring tool for diagnosing and confirming the failure of cART as it is more sensitive and an earlier indicator of treatment failure than CD4 count or clinical monitoring, thereby reducing the accumulation of drug-resistance mutations and improving clinical outcomes[6]. Virological failure as defined by WHO is plasma HIV RNA > 1000 copies/ml based on two consecutive viral load measurements three months apart with adherence support after at least six months of starting a new cART regimen[7].

Identifying treatment failure early enables patient adherence counselling and may enable patients to stay on first line cART longer, thereby avoiding unnecessary switches to more expensive second line regimens[8]. Viral load testing also enables clinicians to switch failing patients earlier to second and third line regimens, before the accumulation of drug resistance mutations, thereby reducing the spread of highly resistant virus[8].

The South African National guidelines released in 2014 also advocate use of HIV viral load for monitoring treatment success and early identification of treatment failure and advise to consider switching patients on the first line regimen if the patient has experienced virological failure on at least two occasions two months apart despite good adherence[9]. After initiating cART, monitoring with viral load is recommended at month six, one year on cART and then annually[9].

In South Africa the threshold for initiation of cART rose to CD4 count ≤500/µl and the prevention of mother to child transmission (PMTCT) programme adopted the B+ approach, which entitles every pregnant and breastfeeding woman to lifelong cART regardless of CD4 count or clinical staging, which was implemented in January 2015 [9]. The department of health has a 2020 target of having 90% of people tested for HIV and 90% of those eligible for treatment on treatment, with at least 90% of those on treatment virally suppressed which is in line with the UNAIDS goal to help end the AIDS pandemic[9, 10].

In the WHO guideline on when to start cART and on pre-exposure prophylaxis for HIV, released in September 2015, it is recommended that cART should be initiated in all adults and children living with HIV at any CD4 cell count, with evidence for both public health and individual benefit from the START and TEMPRANO trials published in 2015[11-13]. The South African Minister of Health, Dr. Aaron Motsoaledi announced in the budget speech in 2016 that from September 2016 everyone with HIV will be eligible for cART irrespective of CD4 count[14]. These encouraging changes mean greater access to cART with more patients receiving care and monitoring, leading to reduced morbidity and mortality. With an expanding number of HIV infected patients on cART, there is a greatly increased demand and workload on laboratories in producing HIV viral load results accurately and timeously.

Plasma HIV viral load is the current universal standard of care using ethylenediaminetetraacetic acid (EDTA) tubes or plasma preparation tubes (PPT)[15]. Plasma viral load determination requires venous blood collection, processing (centrifuging) of that blood to obtain plasma within a certain timeframe, cold chain and storage of specimens by trained personnel (Table 1)[16, 17]. Generating plasma samples requires trained phlebotomists and electricity in health care facilities to power the centrifuges[15]. Most cART programmes in resource limited settings still do not have access to viral load testing and continue to rely on clinical and immunological monitoring[5]. This restricted access to viral load testing has been identified as a key reason for the lower than expected rates for switching cART regimens in resource limited settings[5]. Its availability has been severely restricted because of prohibitively high costs, complex specimen collection, efficient transportation and storage requirements and the need for well-established laboratory infrastructure and well-trained personnel[17].

Factors which could affect HIV RNA stability include the effects of cellular fractions, pre-processing storage temperature, and time delays to processing[18]. In a study on HIV RNA stability, HIV RNA levels were stable for up to 30 hours after collection when stored at either room temperature (mean standard deviation (SD) = 0.101 $\log_{10}$ units) or at 4°C (mean SD = 0.102 $\log_{10}$ units) as plasma or as EDTA-anticoagulated whole blood (mean SD = 0.109 $\log_{10}$ units)[18]. Commercial viral load assays recommendations for specimen storage are as follows: freshly drawn specimens (whole blood) may be held at 15-30°C for up to 6 hours or at 2-8°C for up to 24 hours, prior to centrifugation[19]. After centrifugation, plasma may be stored at 15-30°C for up to 24 hours or at 2-8°C for up to 5 days[19]. In a systematic review by Bonner et al in 2014, they found extended HIV RNA stability compared to the recommended times and temperatures, however this is based on nine studies done in Europe and U.S. with most participants being cART naive, therefore they recommend further studies in low resource settings and warmer climates as well as assessing HIV RNA stability in patients with low viral load close to the virological failure threshold of 1000 copies/ml[15].

Table 1. HIV RNA stability times at different temperatures[17]

Temperature	37°C	15-30°C	4°C	-20°C	-70°C
Whole blood (EDTA)	6 hours	6 hours	24 hours	N/A	N/A
Plasma	24 hours	24 hours	5 days	1 year	5 years
DBS	1-2 weeks	1-2 weeks	2-52 weeks	3-36 months	1 year

Table 1 is an excerpt from the WHO technical and operational considerations for implementing HIV viral load testing and includes a summary of published studies and manufacturer recommendations for time of transport and storage at various conditions for plasma, whole blood and DBS specimens for HIV viral load testing[17]. N/A= not applicable.

In rural areas which are far from the centralised laboratories, sample transport networks do not function well resulting in long delays reaching the laboratory with potential degradation of sample, poor accuracy of results, long turnaround times and loss to follow up of patients. Such restrictive transportation and storage requirements greatly limits access to viral load testing to only those in close proximity to national or regional laboratories[15].

Several solutions have been sought to improve access to viral load testing globally. The price per viral load test can range widely (U.S. 10-60 dollars) depending on the manufacturer and country[17]. Several manufacturers have now committed to regional agreements in which reduced pricing for

viral load reagents may be obtained for as low as U.S. 10.50 dollars per test[17]. The 2016 WHO consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection advocate dried blood spots (DBS) using venous or capillary whole blood as a tool to increase access to viral load testing in settings where logistical, infrastructural or operational barriers prevent routine viral load monitoring using plasma specimens[6]. New platforms for high volume testing are also becoming available, allowing cost effective consolidation of testing in high volume centres (e.g. super-laboratories)[16]. A much anticipated alternative solution to expand access would be to decentralise testing by using point of care (POC) technologies that will enable testing in remote health care facilities[8]. Differential care models proposed such as viral load informed differentiated care is a cost effective means of tailoring care so that those with suppressed viral load visit the clinic less frequently and attention is focussed on those with unsuppressed viral load to promote adherence and timely switching to a second line regimen[20].

Whole blood testing in the form of DBS has been evaluated in several studies as a method to greatly expand HIV early infant diagnosis and viral load access in rural settings[21]. Utilization of DBS samples for viral load will greatly simplify the transport of samples, providing enhanced stability and ease of use for health care workers and is also cost effective[16]. DBS remains stable over extended time and temperature periods, often making this the only practical transport option in remote areas[15]. In South Africa DBS is routinely used for infant HIV diagnostic PCR testing[22]. DBS is simple to collect and prepare and stable at room temperature therefore easy to transport with no need for cold chain or worry about transportation delays. A drop of blood can be obtained in the field from a finger or heel prick, applied to a piece of filter paper, air-dried, and stored until measurement of HIV viral load[23]. The reduction in required materials, less biological waste production and no need of highly skilled personnel at clinic level, decreases costs considerably in comparison to standard blood collection methodologies by venipuncture[24]. Transport associated risks are minimal as DBS cannot break or leak and can be sent at ambient temperatures, without the need for cold packs or expensive dry ice[24]. Using existing networks for early infant HIV diagnosis, they can be transported to a regional or national laboratory with results subsequently returned to the clinic by, for example, mobile phone text messaging[20].

An appealing solution to improve access is to bring the testing closer to the site of patient care, thereby minimising the time to obtain a result and expedite clinical decision making [25, 26]. With the goal of improving the accessibility and affordability of cART, there is a growing demand for simple, affordable, reliable and quality assured POC diagnostics for use in resource limited settings[16]. POC viral load testing would reduce the need for expensive laboratory infrastructure and highly skilled laboratory workers, and also could lower the cost of testing[16]. Rapid, reliable and affordable POC viral load, with minimal equipment and training requirements, would have a major impact on treatment outcomes by facilitating timely detection of virological failure and swift clinical decision making as well as reduce loss to follow up[27, 28]. There are several POC technologies in the pipeline for HIV viral load which are being evaluated in both laboratory and field conditions[8].

POC testing will not be a magic bullet and will most likely complement and not replace conventional testing platforms[29]. The appropriate mix of high-volume laboratories and POC testing will be country specific, and will depend on such factors as the urban/rural split of the country, the expected volume of testing and the ability to effectively transport samples between collection sites and laboratories and ensure the efficient return of laboratory results back to collection sites[16]. Realistically, it also will depend on the comparative all-in cost of centralised versus decentralised testing[16]. Improved access likely will be achieved through a mix of diagnostic services in most countries that combines high volume laboratories in high density areas, and lower volume POC testing in less densely populated regions[16]. POC implementation will still require training of staff,

implementation of quality control mechanisms, and operational research to assess POC performance and the benefits of POC testing over conventional testing[29].

Although DBS is routinely used for early infant diagnosis, its use for viral load testing has not become routine practice. This is due to the interpretation of whole blood viral load in comparison to plasma and the lack of standardization and previous guidelines. The utilisation of DBS for HIV viral load testing has been evaluated by several studies including systematic reviews[21]. However, the number of studies and sample sizes are small, different methodologies, protocols and viral load thresholds were used, and mostly tested in a controlled laboratory environment and not in the field. This has resulted in a lack of standardization and therefore implementation into programmes.

The studies do characterise the intrinsic properties of whole blood HIV viral load testing which should guide further studies in optimization and selection of a threshold for virological failure. Technical issues related to the adaptation of viral load assays to DBS, especially with respect to sensitivity (limit of detection), specificity (cell free RNA in plasma versus cell associated DNA or RNA in whole blood), and assay method, affect the interpretation of a viral load result from DBS[30]. There are two sides of a coin to whole blood HIV viral load testing affecting both sensitivity and specificity. Sensitivity can be reduced by the smaller volumes used in comparison to routine plasma viral load testing especially at lower viral loads. Specificity can also be reduced as whole blood measures both extracellular HIV RNA and intracellular HIV RNA and proviral DNA while plasma measures only extracellular HIV RNA. This has an impact on determining the optimum threshold for classification of virological failure.

Reduced sensitivity of DBS compared to plasma is caused by reduced input copy number from DBS related to limitations on the volume of blood per DBS (50-75ul) and number of DBS (usually not more than two) that can be subjected to nucleic acid extraction, reduced efficiency of nucleic acid extraction, and the presence of interfering substances in DBS (such as haemoglobin) that can inhibit amplification or detection[30]. Reduced specificity of DBS compared to plasma results from cell associated HIV nucleic acids[30]. Unlike a plasma HIV viral load which measures only cell free HIV RNA, a DBS viral load also measures proviral DNA as well as cell associated RNA, potentially leading to false positive results when using DBS[21]. Cell associated HIV nucleic acids may be in the form of proviral or unintegrated DNA, transcribed spliced/unspliced RNA or viral RNA in particles bound to cells such as platelets[30]. The relative contribution of each type of nucleic acid is likely to vary between patients depending on clinical parameters and treatment status[30]. The degree to which DNA can impact the viral load result will depend on how much DNA is present in the extracted nucleic acid, and by the detection technology used for quantitation (real time PCR is non-specific, while nucleic acid sequence based amplification (NASBA) is specific for RNA)[30]. Depending on the total amount of nucleic acid present in these compartments relative to viral RNA in plasma, and on the extraction and amplification procedure of the assay, the viral load from DBS can be higher than the corresponding value obtained from plasma[30]. This effect is most prominent at low or undetectable levels of plasma RNA, since decay of nucleic acid in latently infected cells is slower than that of plasma virus[30]. When plasma viral RNA is suppressed to below the detection limit of the assay, this can result in a “false positive” result from DBS since proviral DNA and spliced RNA may continue to be detectable in latently infected T cells[30]. This can contribute to low specificity and lead to misclassification of patients with suppressed plasma virus as experiencing treatment failure[30].

The extent to which DNA and RNA will contribute to the signal in a viral load assay is dependent on the selectivity of the extraction method and the specificity of the detection method used[30]. Extraction methods that are selective for RNA (closer correlation with plasma) include RNA-specific

nucleic acid extraction, DNAse pretreatment to remove DNA, and virus particle elution[8]. BioMérieux uses total nucleic acid extraction with NASBA while Abbott uses RNA-selective extraction with real time PCR (both methods close to plasma RNA)[8]. Roche uses total nucleic acid extraction followed by real time PCR (RNA and DNA copy number) and has also validated a "free virus elution" (FVE) method with results closer to plasma RNA[8, 31]. Viral load tests using fresh whole blood will have the same issues with cell associated RNA and proviral DNA as DBS, unless a plasma separation step is included[8]. For non selective assays, the contributions of cell associated RNA and proviral DNA can add a "baseline" to the plasma viral load detected below 3000–5000 copies/ml (plasma RNA dominates the assay above 5000 copies/ml)[8]. These relative contributions can vary for treated versus untreated patients and healthy versus immunocompromised patients[8].

There is also a difference between venous and capillary blood. Capillary blood is easier to collect by finger or heel prick than venous blood which requires phlebotomy however there is more variability in volume spotted and presence of interfering or diluting fluids in capillary blood[30]. Capillary blood has been shown to be feasible and performs comparably to venous blood in several studies [32-34].

In a systematic review of dried fluid spots for HIV viral load and resistance genotyping by Hamers et al in 2009, the data indicated that HIV viral load determination by DBS is feasible, yielding comparable test performances, even after storage[35]. Limitations include reduced analytical sensitivity resulting from small volumes (approximately 3.5 $\log_{10}$ copies/ml at 50ul sample volume), nucleic acid degradation under extreme environmental conditions, impaired efficiency of nucleic acid extraction, potential interference of archived proviral DNA and the excision of spots from the filter paper cards in high volume testing[35]. They indicate that consistently improved analytical sensitivity is needed for routine application of DBS for the monitoring of drug resistance in individuals receiving cART, particularly at the onset of treatment failure[35]. They recommended that further studies should be directed towards further optimization and standardization of assay protocols, sensitivity and precision, nucleic acid stability under extreme storage conditions, and that comparative studies of test performance of various commercial viral load assays are warranted[35].

In a more recent systematic review of the use of DBS for monitoring HIV viral load published by Smit et al in March 2014, they included thirteen peer reviewed publications and found that depending on the technology and the viral load distribution in the study population, the percentage of DBS samples that are within 0.5 $\log_{10}$ of viral load in plasma ranged from 52–100%[21]. Due to the smaller input sample volume, there is a risk of false negatives with DBS[21]. Sensitivity of DBS viral load was found to be 78–100% compared to plasma at viral load below 1000 copies/ml, but this increased to 100% at a threshold of 5000 copies/ml[21]. The systematic review showed that specificity was close to 100% at DBS viral load above 5000 copies/ml, and this threshold would be the most reliable for predicting true virological failure using DBS[21].

In an update on the technical and operational considerations for implementing HIV viral load testing published by WHO in 2014 it is advised that all manufacturers of viral load assays should provide a protocol for DBS specimens and pursue regulatory approval for in vitro diagnostics using DBS[17]. It also indicates that the performance of DBS for HIV viral load testing varies by platform compared with plasma[17].

The optimal threshold for defining virological failure and for switching cART regimens has not been established[5]. Most standard viral load assays have good diagnostic accuracy at 1000 copies/ml (Table 2)[5, 30]. However, the sensitivity of DBS for viral load determination at this threshold may be reduced[5]. The 2013 WHO consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection recommend that programmes relying on DBS for viral load assessment

should consider a higher threshold (3000–5000 copies/ml) until sensitivity at lower thresholds is established[5]. In the updated 2016 WHO consolidated guidelines, it is now recommended to use a threshold of 1000 copies/ml to determine virological failure using DBS, based on a systematic review of 43 studies that compared DBS to plasma showing acceptable sensitivity and specificity (Table 3)[6]. However this is stated as a conditional recommendation with low quality evidence[6]. Modelling suggests that if DBS viral load testing can be performed with reasonable sensitivity and specificity (>85%) then costs and outcomes are similar to plasma based testing[6]. An important limitation is that the majority of studies included in the review used venous whole blood specimens prepared in the laboratory using precision pipettes to dispense the blood onto the filter paper rather than based on specimens obtained in clinical settings[6].

Table 2. Commercially available HIV viral load assays[30]

Assay	Nucleic acid extraction specificity	Detection method specificity	LOQ plasma (copies/ml)	LOQ DBS (copies/ml)
Abbott RealTime HIV-1	Iron oxide microparticle capture - RNA selective	Real time PCR – non-specific	40	500-800
Artus HI Virus-1 RG	Total nucleic acid	Real time PCR – non-specific	60	Unknown
Biocentric Generic HIV Charge Virale	Qiagen extraction – total nucleic acid	Real time PCR – non-specific	188-300	740-5000
BioMérieux NucliSENS EasyQ HIV-1 v2.0	Boom method – total nucleic acid	NASBA – RNA specific	25	800
Roche COBAS Taqman HIV-1 v2.0	Magnetic glass particle purification – total nucleic acid	Real time PCR, dual probe – non-specific	20	400
Versant HIV-1 RNA 1.0	Total nucleic acid	Kinetic PCR	37	866

Commercially available assays for HIV viral load are listed in the following table with their nucleic acid extraction specificity, detection method specificity and limit of quantification in plasma and DBS. LOQ = limit of quantification.

Table 3. Performance characteristics of DBS HIV viral load for detection of virological failure[6, 17]

Assay	Sensitivity (mean and 95% CI)	Specificity (mean and 95% CI)	N
Abbott RealTime HIV-1	95% (82-99%)	92% (79–97%)	1529
Biocentric Generic HIV Charge Virale	95% (71-99%)	55% (35-74%)	531
BioMérieux NucliSENS EasyQ HIV-1 v2.0	84% (79-89%)	95% (86-98%)	1062
Roche COBAS TaqMan HIV-1 v2.0 (free virus elution protocol)	85% (77-91%)	94% (85-98%)	229
Roche COBAS TaqMan HIV-1 v2.0 (SPEX protocol)	99% (97-100%)	44% (18-74%)	2314
Versant HIV-1 RNA 1.0	91% (69-98%)	88% (75-94%)	144

Data on performance characteristics for commercially available molecular HIV viral load assays using DBS compared with plasma at 1000 copies/ml cut-off. Pooled estimates of sensitivity and specificity based on published data up to June 2015[6]. 95% CI = 95% confidence interval. N = number of samples.

In conclusion, various strategies have been proposed to expand access to HIV viral load testing due to the restrictions from plasma based testing. The most promising is whole blood testing in the form of DBS or POC tests. DBS is currently recommended by WHO in remote areas, and has been evaluated in several studies and found to be cost effective and performs reasonably. Novel POC technologies are also anticipated as a means to decentralise testing and facilitate prompt clinical decision making to detect virological failure. However whole blood viral load presents its own inherent challenges in terms of reduced sensitivity and specificity and therefore interpretation. The reduced sensitivity of DBS compared to plasma due to lower input volume does not negatively impact on the detection of virological failure as the limit of detection of most assays evaluated is below 1000 copies/ml. The major issue with whole blood viral load testing is the reduced specificity leading to upward misclassification. A higher threshold should be used to avoid misclassification. The precise threshold to be used is likely to be different for each assay and patient, depending on assay methodology and patient factors such as CD4 count, HIV DNA and intracellular RNA copy number[30]. Methods may be required that increase the specificity of the test such as DNAse treatment, RNA specific nucleic acid extraction or detection, and removal of cellular components before extraction[30]. The optimum threshold for the correct classification of virological failure in whole blood requires further studies.

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Chapter 2: Manuscript

1. Background

The global HIV/AIDS pandemic is undoubtedly one of the most significant public health challenges faced in our time with approximately 36.7 million people currently infected[1, 2]. The brunt of the disease burden lies in sub-Saharan Africa where disadvantaged socioeconomic circumstances have led to reduced access to health care and quality of life. However tremendous gains have been achieved in the last decade especially in increasing treatment coverage to advance the UNAIDS 90-90-90 goal, whereby 90% of people living with HIV know their HIV status, 90% of people who know their HIV-positive status are accessing treatment and 90% of people on treatment have suppressed HIV viral loads[3].

HIV viral load is an essential part of routine monitoring of HIV infected patients on combination antiretroviral therapy (cART) as it is a measure of virological suppression, adherence and will identify virological failure[4]. Plasma HIV viral load is the current universal standard of care. Virological failure as defined by the World Health Organisation (WHO) is plasma HIV RNA > 1000 copies/ml on two consecutive viral load measurements within a three month interval with adherence support after at least six months of cART use[5]. Viral load should be tested at 6 months after cART initiation and then annually[4].

Although HIV viral load is recommended as the gold standard for HIV treatment monitoring, many low and middle income countries have limited access[6]. With an expanding number of HIV infected patients on cART, there is an increased demand and workload on laboratories in producing HIV viral load results accurately and timeously. Currently HIV viral load testing by real time PCR is complex, expensive and restricted to centralised laboratories with trained personnel and infrastructure[7]. In rural or remote areas, timely plasma separation and transportation to testing laboratories is a major barrier to the access of HIV viral load testing. Sampling requires collection of a venous whole blood sample that is stable at room temperature for only up to six hours and therefore requires cold chain storage and has to be centrifuged prior to testing[8]. Sample transport networks are often not available or not well functioning resulting in significant delays in processing of samples[9].

There exists a burgeoning need for cost effective methods to improve access and implement scale up of viral load testing. Potential solutions explored are the utilization of whole blood HIV viral load testing in the form of dried blood spot (DBS) testing or direct use of whole blood on point of care (POC) testing platforms which would simplify the whole process of sample collection, storage and transportation[10].

In South Africa DBS is routinely used for infant HIV diagnostic PCR testing[11]. DBS is simple to collect and prepare and stable at room temperature therefore easy to transport with no need for cold chain or worry about transportation delays. Use of DBS would reduce the costs incurred from materials and waste disposal of standard blood collection and the need for a skilled phlebotomist[12].

The routine use of POC devices for HIV diagnostics such as the lateral flow rapid HIV test has facilitated the HIV care and testing programme so that patients are counselled, tested and provided HIV test results all at the same visit thus fundamentally improving access to HIV testing and care. POC HIV viral load assays will facilitate earlier identification of treatment failure[13]. POC viral load testing could form part of a tiered implementation model that includes both POC testing and different tiers of laboratory testing to ensure access for the entire national population[13].

However there is a paucity of studies that have compared whole blood HIV viral load results across platforms. There are various commercial HIV viral load assays and real time PCR platforms available with differences in nucleic acid extraction, PCR target, amplification and detection methodologies hence the difficulty arises in directly comparing these different platforms head to head. Interpretation of whole blood HIV viral load is also complex especially at low viral loads[14]. Previous studies have shown reduced specificity due to cell associated HIV nucleic acids as well as reduced sensitivity due to smaller sample volumes used in comparison to plasma[15].

Due to the limited data available with a demand identified for alternative simpler methods of HIV viral load testing strategies and a need for comparative studies of test performance of various commercial viral load assays using whole blood, we embarked on this study to evaluate whole blood HIV viral load testing using DBS on all the commercially available platforms in our setting as well as on a POC device. Correlation with plasma HIV viral load as gold standard and correct classification of virological failure were used to evaluate performance. This evaluation will help determine the clinical utility of using whole blood HIV viral load in the monitoring of patients on cART in resource limited settings.

2. Methods

2.1 Study design and population:

This was a laboratory-based cross sectional study comparing different whole blood HIV viral load methodologies through testing of routine clinical HIV plasma viral load sent to Groote Schuur Hospital, Virology Laboratory, National Health Laboratory Service. Whole blood HIV viral load methodologies were evaluated on four assays namely Alere q HIV-1/2 point of care device, Abbott RealTime HIV-1 DBS original open-mode protocol and updated new prototype protocol, and Roche CAP/CTM HIV-1 v2.0 DBS free virus elution (FVE) protocol (Table 4). The study population from which the samples were selected comprised of patients from the South African public health care sector in the Western Cape, the majority of whom are on cART. Clinical and laboratory factors that might have played a role in the discrepancy between various HIV viral load methodologies were retrieved from the laboratory information system. These parameters included age, gender, CD4 count current and nadir, treatment history, duration on cART and viral load history.

2.2 Sampling and Procedures

Plasma HIV viral load was used as the gold standard and was performed using Abbott RealTime HIV-1 (Abbott Laboratories, Chicago, USA) according to the manufacturer's instructions[16]. To ensure coverage across the viral load spectrum, we selected plasma viral load results to include at least 50 specimens from each of four categories: lower than detectable limit, detectable – 1000 copies/ml, 1000 – 10,000 copies/ml and > 10,000 copies/ml. Parallel whole blood HIV viral load testing was performed using whole blood samples submitted for CD4 testing taken at the same time as the plasma HIV viral load sample. 25ul of whole blood from the CD4 count sample was used for HIV viral load testing on Alere q HIV-1/2 point of care device, at the same time the DBS was prepared by applying 75ul of whole blood to each individual spot on a Whatman 903 filter-paper card. This was done within 72 hours of sample receipt to ensure no delays that could have resulted in poor quality degraded EDTA sample. The DBS cards were kept on racks to dry overnight away from sunlight, and once dry packed with silica gel dessicant in sealed plastic bags to protect from humidity. DBS preparation and all testing were performed in the laboratory. Testing was performed in collaboration with another viral load reference centre, HIV Molecular Laboratory at Charlotte Maxeke Johannesburg Academic Hospital. Abbott updated protocol and Roche FVE protocol were tested by this laboratory.

2.3 Ethics

Ethics approval for the study was received from the Human Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town (HREC REF: 819/2014).

2.4 Analysis

Data collected was entered in tabulated form in Microsoft Excel 2007. Linear correlation of the plasma and whole blood viral load comparison and Bland-Altman plots were plotted and analysed using Microsoft Excel 2007, while receiver operating characteristic (ROC) curve analysis, sensitivity, specificity, positive predictive value, negative predictive value and concordance were calculated using Stata version 11. Proportions of correct classification of virological failure using the plasma threshold of 1000 copies/ml and overall correlation with gold standard were used for evaluating each method's performance. Viral load results are reported in copies/ml or $\log_{10}$ transformed copies/ml.

Table 4. HIV viral load test methods

Method	Assay (Abbreviation)	Sample Type	Sample Volume	Reportable Range copies/ml
1	Standard of care: Abbott RealTime HIV-1[16] (Plasma VL)	Plasma Gold standard	600ul or 200ul	40 – 10 million (600ul) 150 - 10 million (200ul)
2	Point-of-care: Alere q HIV-1/2 [17, 18] (Alere q)	EDTA whole blood	25ul	2491 – 10 million
3	Abbott RealTime HIV-1 original 'open mode' protocol[19] (Abbott original)	DBS	2 x 75ul spots	550 - 10 million
4	Abbott RealTime HIV-1 updated new prototype protocol [20] (Abbott updated)	DBS	1 x 75ul spot	1000 - 10 million
5	Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0 - free virus elution protocol[21] (Roche FVE)	DBS	1 x 75ul spot	400 – 10 million

Table 4 provides a list of the assays evaluated in the study: their sample type, volume used and reportable range in copies/ml. For details of each assay methodology, refer to cited references.

3. Results

3.1 Proportion of plasma viral load categories:

299 samples that were tested with the standard of care plasma VL were selected across a wide quantitative range, of which 94 (32%) were below detectable limit, 52 (17%) between 40-1000 copies/ml, 52 (17%) between 1000-10,000 copies/ml, 53 (18%) between 10,000-100,000 copies/ml and 48 (16%) >100,000 copies/ml.

3.2 Patient demographics data (Table 5):

199 samples were from females and 100 were from males. Median age of patients was 35 years (IQR 28-41). 21/297 (7%) of samples were from children (<= 12 years old) and 276/297 (93%) from adults. The median CD4 count was 417 x 10^6/l (IQR 220-568). The median plasma viral load was 3.03 $\log_{10}$ copies/ml (IQR LDL - 4.57). 65% of patients were on 1st line cART regimens and 12% were on 2nd line cART regimens. 22/299 (7%) of samples were pre-initiation cART viral loads and 48/299 (16%) had no treatment history available on the laboratory information system. Out of the remaining 229 patients, 83 (36%) had a past history of virological failure with one or more plasma VL >1000 copies/ml and 15 (7%) had no previous plasma VL on the laboratory information system.

Table 5. Patient demographics

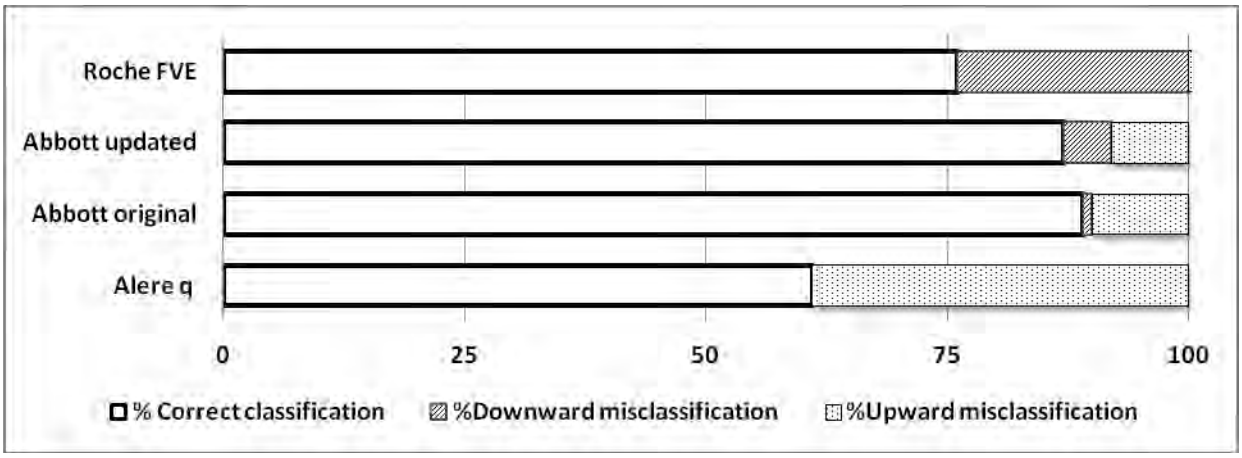
Patient demographics data	**N = 299**
Male	N = 100 (33%)
Female	N = 199 (67%)
Median age (range)	35 (39 days – 60 years old)
Median CD4 count (range)	417 (4 – 2231 x 10^6/l)
cART:	
1st line	N = 194 (65%)
2nd line	N = 35 (12%)
Past history of virological failure	N = 83/229 (36%)
No previous plasma VL	N = 15/229 (7%)

Median age of the study population was 35 years old, with majority female (67%), and on 1st line cART (65%), and median CD4 count was 417 x 10^6/l. 1st line cART regimen consisted mainly of FDC (fixed drug combination) of TDF (tenofovir), 3TC/FTC (lamivudine/emtricitabine) and EFV (efavirenz) while 2nd line cART regimens comprised AZT (zidovudine) or TDF + 3TC + lopinavir/ritonavir. Of those with a previous viral load, 36% had a past history of virological failure with a plasma VL > 1000 copies/ml. N = number.

3.3 Overall correct classification of virological failure (Fig. 1):

Alere q, Abbott original, Abbott updated, and Roche FVE correctly classified virological failure in 61%, 89%, 87% and 76% of all samples tested respectively. Alere q, Abbott original and Abbott updated demonstrated over-quantification compared to plasma resulting in upward misclassification in 39%, 10% and 8% of all samples tested respectively. Downward misclassification was seen in 1%, 5% and 24% of all samples tested by Abbott original, Abbott updated and Roche FVE respectively. There was no downward misclassification by Alere q.

Fig.1 Percentage correct classification, downward misclassification and upward misclassification of virological failure by each of the whole blood assays compared to plasma



Correct classification of virological failure was best by Abbott original and updated at 89% and 87% respectively, followed by Roche FVE at 76% and Alere q at 61%. Upward misclassification which is misclassifying as virological failure when plasma VL is <1000 copies/ml, was evident by Alere q, Abbott original and updated at 39%, 10% and 8% respectively. Downward misclassification which is not detecting virological failure when plasma VL is >1000 copies/ml, was mostly demonstrated by Roche FVE at 24%, with none by Alere q, and 1% and 5% of all samples by Abbott original and updated.

3.4 Correct classification according to plasma VL categories:

The performance varied across plasma VL categories (Table 6, Fig.2 and 3).

3.4.1 Plasma VL lower than detectable limit (LDL):

When plasma VL was LDL, correct classification of virological failure was 22%, 87%, 89% and 100% by Alere q, Abbott original, Abbott updated, and Roche FVE respectively. Over-quantification of viral load resulting in upward misclassification was significant by Alere q at 78% (73/94) when plasma VL was LDL, and moderate by Abbott original and updated at 13% (12/94) and 11% (10/94) respectively (Fig.2). There was no upward misclassification by Roche FVE. Mean $\log_{10}$ copies/ml difference between plasma VL and Alere q, Abbott original, Abbott updated and Roche FVE was -2.85 (95% CI -3.15 – -2.55), -0.60 (95% CI -0.86 - -0.35), -0.82 (95% CI -1.10 - -0.54), and -0.46 (95% CI -0.67- -0.25) respectively (Fig.3).

3.4.2 Plasma VL detectable-1000 copies/ml:

Correct classification at this range was lowest for most test methods at 14%, 63%, 75% and 98% for Alere q, Abbott original, Abbott updated and Roche FVE respectively. Upward misclassification was highest in this category for Alere q, Abbott original and updated. Only one sample was upwardly misclassified by Roche FVE. The mean $\log_{10}$ copies/ml difference between plasma VL and Alere q, Abbott original, Abbott updated and Roche FVE was -0.94 (95% CI -1.31 - -0.57), 0.81 (95% CI 0.37 - 1.25), 0.14 (95% CI -0.23 – 0.51), and 1.10 (95% CI 0.75 - 1.44) respectively (Fig.3). Due to the greatest variability in this category, we performed further analysis using plasma VL of 400 copies/ml as a cut-off, to assess if there was a difference above 400 copies/ml indicative of impending virological failure, but there was no difference.

3.4.3 Plasma VL 1000-10,000 copies/ml:

Above plasma VL of 1000 copies/ml, Alere q showed 100% correct classification of virological failure demonstrating 100% sensitivity (Fig.2a). Abbott original and updated correctly classified 94% and 75% of samples respectively with downward misclassification in 6% and 25% respectively. Roche FVE showed 0% sensitivity at this range by significant under-quantification of viral load resulting in failure of detecting virological failure in any of the plasma VL samples (0/50) tested at this range (Fig.2d). Mean $\log_{10}$ copies/ml difference between plasma VL and Alere q, Abbott original, Abbott

updated and Roche FVE was -0.54 (95% CI -0.65 - -0.43), -0.15 (95% CI -0.34 – 0.05), 0.19 (95% CI 0.02-0.37), and 1.30 (95% CI 0.99-1.60) respectively (Fig.3).

3.4.4 Plasma VL >10,000 copies/ml:

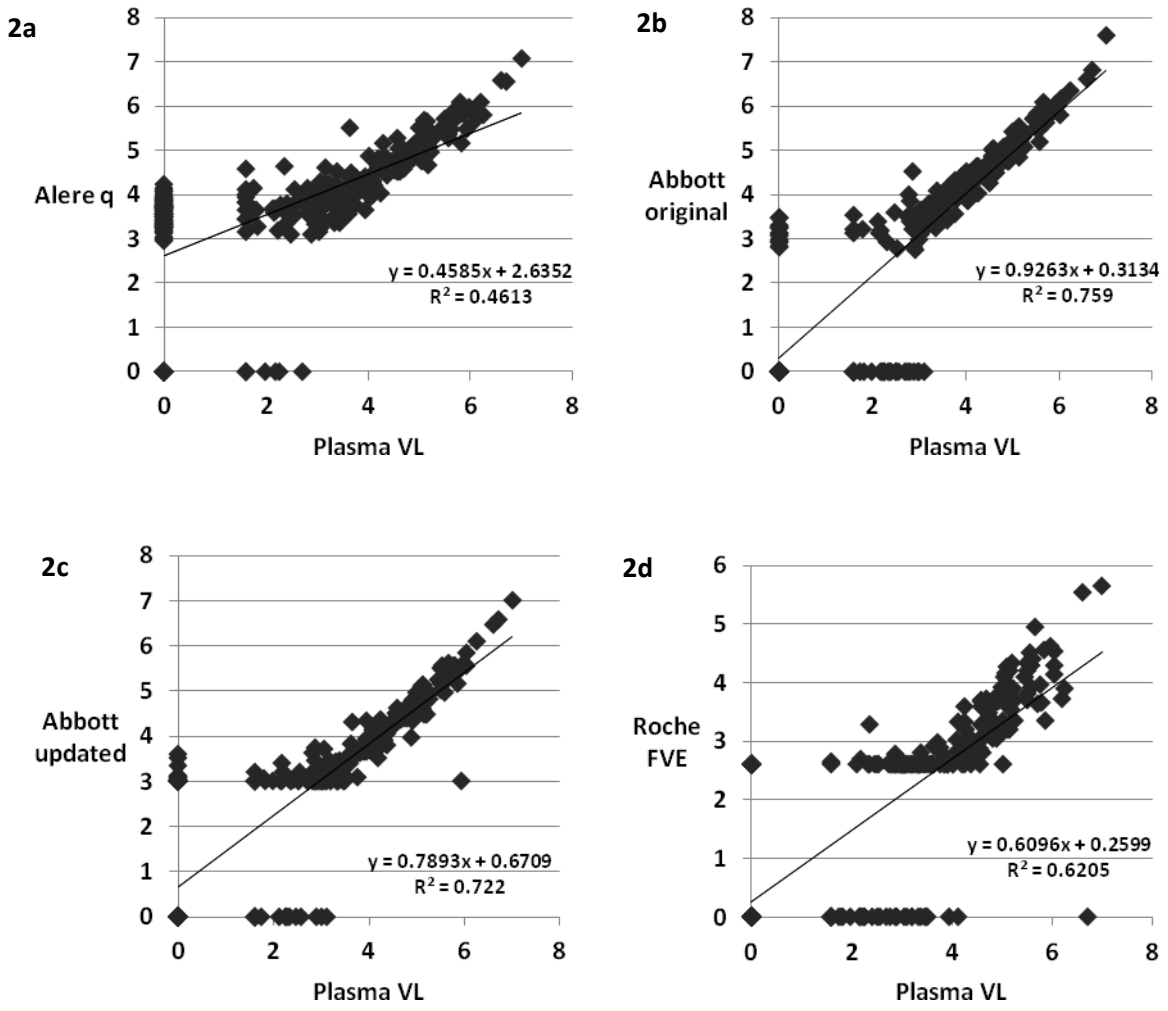
Excellent correlation above 10,000 copies/ml was observed for Alere q, Abbott original and updated with correct classification of virological failure at 100%, 100% and 99% respectively (Fig.2). Roche FVE demonstrated better sensitivity at this range with correct classification of virological failure in 80% (76/95) of samples tested and downward misclassification in 20% (19/95) of samples tested. Under-quantification by Roche FVE at this plasma VL stratum ranged from LDL – 2.96 log_{10} copies/ml (median = 2.67). The mean log_{10} copies/ml difference between plasma VL and Alere q, Abbott original, Abbott updated and Roche FVE was -0.09 (95% CI -0.14- -0.03), -0.12 (95% CI -0.16 - -0.86), 0.27 (95% CI 0.21-0.34), and 1.52 (95% CI 1.38-1.67) respectively (Fig.3).

Table 6: Percentage of correct classification of virological failure by whole blood testing methods according to plasma VL categories

Plasma VL	LDL	Detectable - 1000 copies/ml	1000-10,000 copies/ml	>10,000 copies/ml
N=	94	52	52	101
Alere q	22%	14%	100%	100%
Abbott original	87%	63%	94%	100%
Abbott updated	89%	75%	75%	99%
Roche FVE	100%	98%	0%	80%

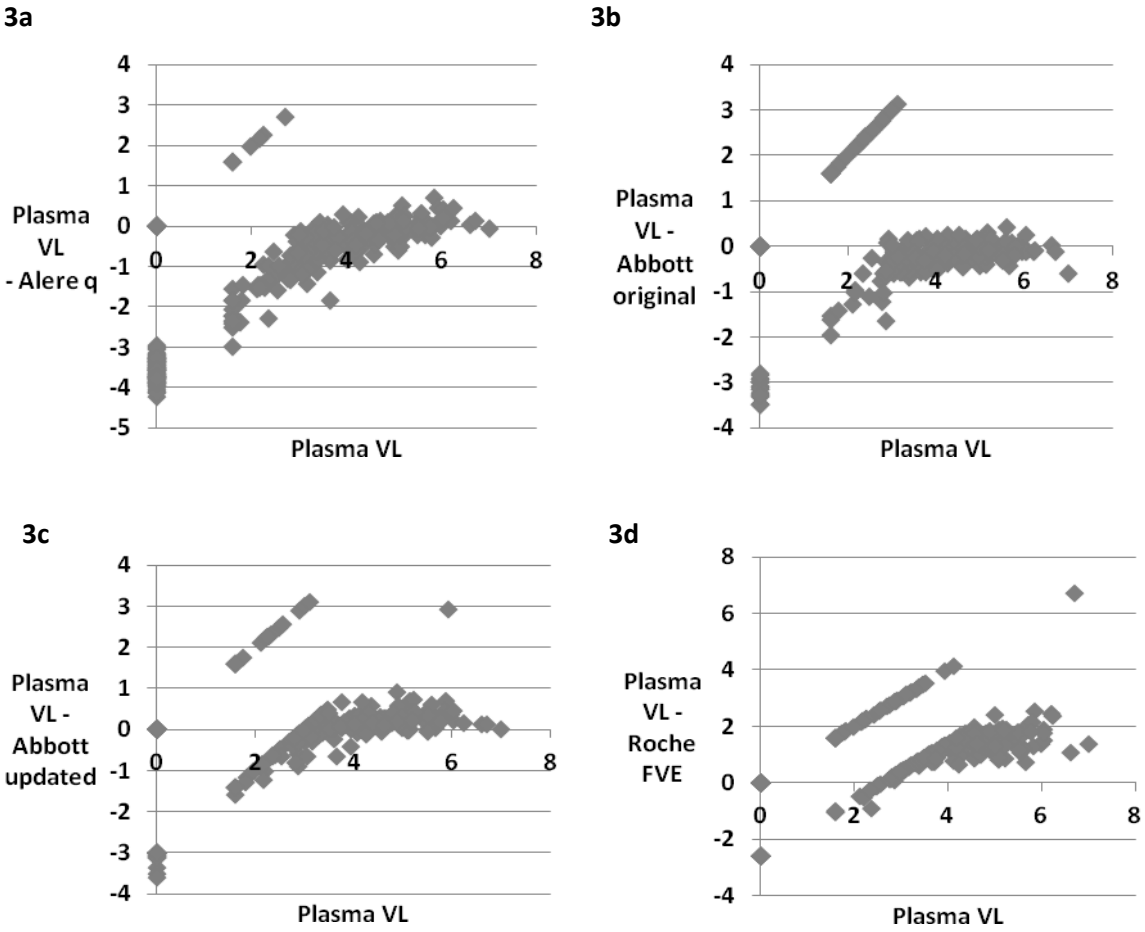
The performance of each whole blood assay differs according to the plasma VL category. Alere q showed 100% sensitivity above plasma VL of 1000 copies/ml, however below this level there was over-quantification resulting in upward misclassification in 78% of the plasma VL that were LDL and 86% in the detectable-1000 copies/ml category. For Abbott original and updated, sensitivity was best above 10,000 copies/ml, there was 6% and 25% downward misclassification in the 1000-10,000 copies/ml category, and there was 37% and 25% upward misclassification in the detectable-1000 copies/ml category as well as 13% and 11% upward misclassification in the LDL category. Roche FVE demonstrated the opposite performance with reduced sensitivity of 0% in the 1000-10,000 copies/ml category. Roche FVE had the best specificity of 100% when plasma VL was LDL and 98% in the detectable-1000 copies/ml category. Correct classification was best for all methods at >10,000 copies/ml.

Fig.2 Correlation plots between plasma VL and each of the whole blood assays in log_{10} copies/ml



Correlation plots between plasma VL and 2a) Alere q - R^2= 0.46. Correlation was good above plasma VL of 3 log_{10} copies/ml, resulting in 100% sensitivity in detecting virological failure. However below plasma VL of 3 log_{10} copies/ml, there was significant overestimation with upward misclassification in 117/145 (81%) of samples by Alere q. *2b) Abbott original* – had the best overall correlation with plasma VL (R^2 = 0.76). There was linear correlation above plasma VL of 3 log_{10} copies/ml. Below plasma VL of 3 log_{10} copies/ml there was overestimation resulting in upward misclassification in 31/146 (21%) samples. *2c) Abbott updated* – 2.9 log_{10} has been used on plot for samples <1000 copies/ml (lower limit of quantification). Correlation was similar to Abbott original (R^2 = 0.72). Correlation was excellent above 4 log_{10} copies/ml. When plasma VL was 3-4 log_{10} copies/ml, Abbott updated under-quantified 13/52 (25%) of samples resulting in downward misclassification. Below plasma VL of 3 log_{10} copies/ml there was upward misclassification in 23/145 (16%). *2d) Roche FVE* - R^2= 0.62. 2.6 log_{10} has been used on plot for Roche FVE samples <400 copies/ml (lower limit of quantification). Below the plasma VL threshold of 3 log_{10} copies/ml, there was good correlation with Roche FVE correctly classifying 139/140 samples (99% specificity). For plasma VL >3 log_{10} copies/ml, there was significant underestimation by Roche FVE especially between plasma 3-4 log_{10} copies/ml, with downward misclassification in 69/145 (48%). Results below the detectable limit are plotted as 0 for all the plots.

Fig.3 Bland Altman plots between plasma VL and each of the whole blood assays in log_{10} copies/ml



Overall mean log_{10} copies/ml difference between plasma VL and 3a) Alere q = -1.18 (95% CI -1.36 – -1.01). *3b) Abbott original* = -0.12 (95% CI -0.24 – 0.01). *3c) Abbott updated* = -0.11 (95% CI -0.24 – 0.02). *3d) Roche FVE* = 0.78 (95% CI 0.63 – 0.93). Note that viral load that was LDL was plotted as 0 on graph, <1000 copies/ml for Abbott updated was plotted as 2.9 log_{10}, and <400 copies/ml for Roche FVE was plotted as 2.6 log_{10}.

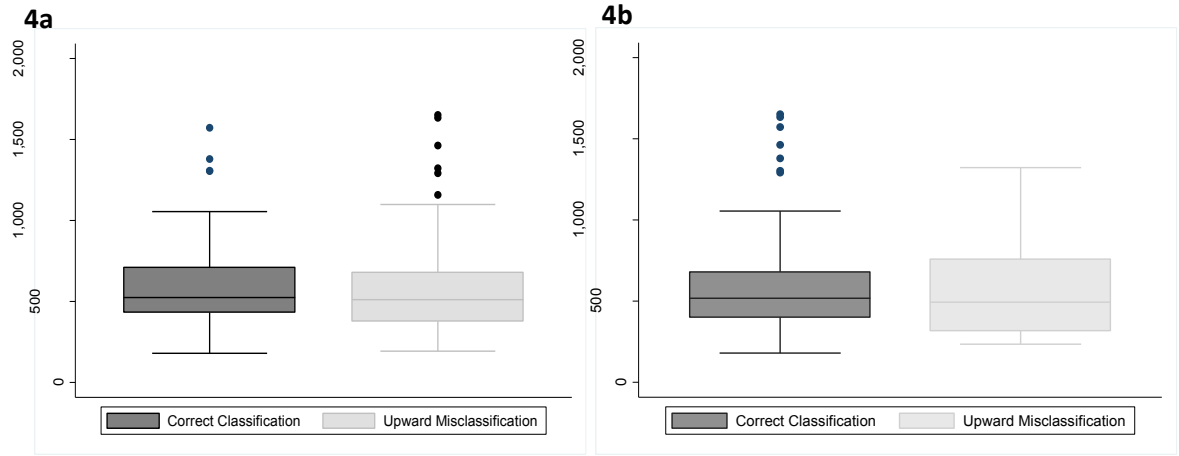
3.5 Evaluation of clinical and laboratory factors associated with discrepancy between plasma and whole blood viral load:

Among patients who were virologically suppressed, we analysed for clinical and laboratory factors that could potentially contribute to the over-quantification by whole blood viral load. Data was retrieved from the laboratory information system which in 22/128 (17%) of current plasma VL<400 copies/ml were missing or incomplete. We assessed for history of virological failure especially recent (in the last 3-6 months) in those who had a current plasma VL <400 copies/ml. In those patients with current plasma VL <400 copies/ml, there was no difference in median CD4 count in those who had a history of virological failure (median CD4 count = 431) and those with no history of virological failure (median CD4 count = 524). There was also no difference in whole blood viral load in patients who had a history of virological failure and those who didn't among the plasma virologically suppressed. Median whole blood VL when plasma VL was < 400 copies/ml was 3.51 log_{10} copies/ml in patients with no history of virological failure and 3.40 log_{10} copies/ml in patients with history of virological failure for Alere q. Median whole blood VL when plasma VL was < 400 copies/ml was LDL in both patients with history of virological failure and with no history of virological failure for Abbott original and updated and Roche FVE.

Among patients with current plasma VL<400copies/ml, 14/105 (13%) had a past history of virological failure. There was 71% upward misclassification rate by Alere q among those with past history of virological failure, while there was 84% upward misclassification among those with no history of virological failure (chi = 1.20). Therefore past history of virological failure did not affect the upward misclassification by Alere q.

We assessed for an association between CD4 count and upward misclassification in whole blood when plasma VL was <1000 copies/ml. For Alere q there was no difference in median CD4 count of correctly classified samples and upwardly misclassified samples of 524 and 512 respectively (Fig. 4a). The same was also noted for Abbott original of median CD4 count of 517 for correctly classified samples and 494 for upwardly misclassified samples (Fig.4b).

Fig.4 CD4 count according to correct classification and upward misclassification of virological failure when plasma VL <1000 copies/ml

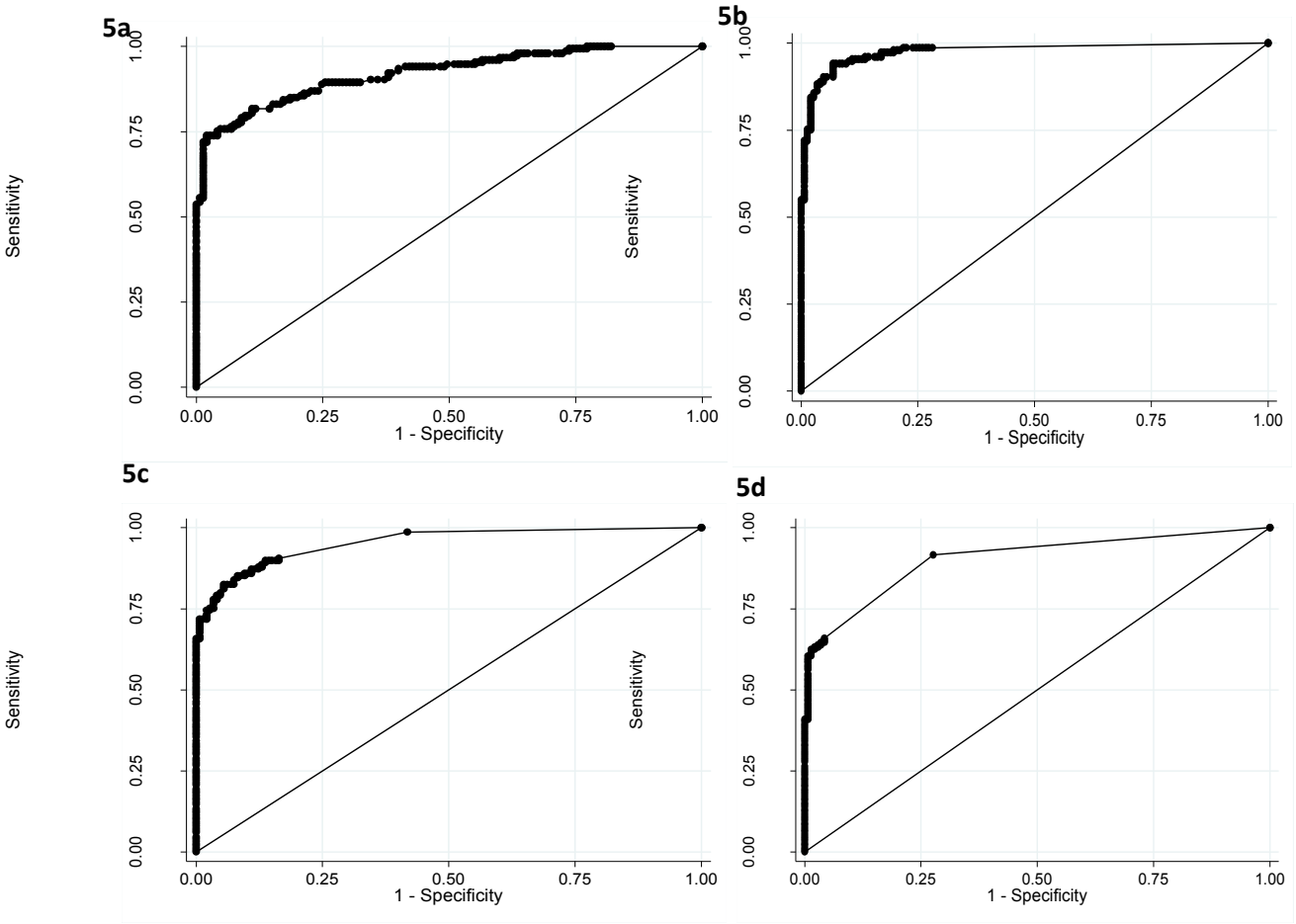


No difference in the median CD4 count was noted between correctly classified and upwardly misclassified samples for both 4a) Alere q and 4b) Abbott original.

3.5 Receiver operating characteristic (ROC) curve analysis (Fig.5):

ROC curve analysis provided the optimum threshold for classification of virological failure for each whole blood method. For the samples tested, 9249 copies/ml (3.97 $\log_{10}$) provided the best overall classification of 85% for Alere q with a sensitivity of 82% and a specificity of 89%. 2697 copies/ml (3.43 $\log_{10}$) provided the best overall classification of 94% for Abbott original with a sensitivity of 94% and a specificity of 93%. For Abbott updated, 1134 copies/ml (3.07 $\log_{10}$) provided the best overall classification of 88% with a sensitivity of 90% and a specificity of 86%. For Roche FVE, 400 copies/ml (2.60 $\log_{10}$) provided the best overall classification of 82% with a sensitivity of 92% and a specificity of 73%.

Fig.5 ROC curve analysis of the whole blood test methods using the threshold of 1000 copies/ml for virological failure



ROC curve analysis for 5a) Alere q shows area under ROC curve = 0.92 (95% CI 0.89-0.95), 1419 copies/ml (3.15 $\log_{10}$) and 44062 copies/ml (4.64 $\log_{10}$) provided 100% sensitivity and 100% specificity respectively. *5b) Abbott original* area under ROC curve = 0.98 (95% CI 0.96-0.99), 974 copies/ml (2.99 $\log_{10}$) and 34025 copies/ml (4.53 $\log_{10}$) provided 99% sensitivity and 100% specificity. *5c) Abbott updated* area under ROC curve = 0.95 (95% CI 0.93-0.97), 999 copies/ml (2.99 $\log_{10}$) and 5995 copies/ml (3.78 $\log_{10}$) provided 99% sensitivity and 100% specificity. *5d) Roche FVE* area under ROC curve = 0.90 (95% CI 0.87-0.94), 400 copies/ml (2.60 $\log_{10}$) and 2134 copies/ml (3.33 $\log_{10}$) provided 92% sensitivity and 100% specificity.

4. Discussion

The utilization of whole blood viral load testing offers an attractive solution to the scale up and widespread implementation of HIV viral load monitoring in resource limited settings. As access increases, patients will be routinely monitored and virological failure will be detected and treatment switched earlier, with lower accumulation of drug resistance mutations, improved quality of life and lower rates of acquired and transmitted drug resistance. Viral load informed differentiated cART care using DBS was found to be cost effective in sub-Saharan Africa in a modelling study for sustainable HIV treatment[(10)].

However whole blood viral load testing presents its own challenges in terms of interpretation of whole blood viral load which measures intracellular HIV RNA and/or proviral DNA in addition to extracellular HIV RNA that is measured from plasma. This is a potential cause of overestimation of whole blood viral load in comparison to plasma VL. Another limitation is the use of smaller sample

volumes for DBS and POC testing platforms in comparison to plasma which can lead to reduced sensitivity. Previous studies have mainly evaluated DBS using a single methodology in comparison to plasma using the threshold of 1000 copies/ml.

In our study we evaluated whole blood HIV viral load using different platforms and methodologies for testing as a comparison to plasma VL categories and to gauge the performance using the recommended WHO threshold of 1000 copies/ml for virological failure. The key finding highlighted in our study is the great variability between each of the methods evaluated. The differences are most likely attributed to various factors such as input volume used in each assay, sample treatment/processing (DBS versus fresh blood samples versus free virus elution), extraction (RNA selective, total nucleic acid extraction), amplification target and detection methods which are different for each of the platforms tested, and software cutoff algorithm is not standardized.

Above plasma VL of 1000 copies/ml, classification of virological failure was good by three of the whole blood viral load methods: Alere q, Abbott original and updated. There was excellent classification of virological failure by these methods above plasma VL of 10,000 copies/ml. However, the opposite was demonstrated by Roche FVE of reduced sensitivity resulting in significant downward misclassification of virological failure when plasma VL was 1000 – 10,000 copies/ml. Reduced sensitivity of DBS compared to plasma is caused by reduced input copy number from DBS related to limitations on the volume of blood per DBS (50-75ul) and number of DBS (usually not more than two) that can be subjected to nucleic acid extraction, reduced efficiency of nucleic acid extraction, and the presence of interfering substances in DBS (eg. haemoglobin) that can inhibit amplification or detection[14]. The reduced sensitivity of Roche FVE is presumably due to the free virus elution protocol used so that only cell free virus is measured in conjunction with the lower input volume of DBS. Increasing the software correction factor or using two spots may help to improve the sensitivity.

Greatest variability between the different testing methods was seen when plasma VL was 40-1000 copies/ml resulting in upward misclassification of virological failure. Reduced specificity of whole blood viral load compared to plasma results from cell associated HIV nucleic acids which may be in the form of proviral or unintegrated DNA, transcribed spliced/unspliced RNA or viral RNA in particles bound to cells such as platelets [14]. The relative contribution of each type of nucleic acid is likely to vary between patients depending on clinical parameters and treatment status[14]. There was significant over-quantification of whole blood viral load by Alere q, Abbott original and updated resulting in upward misclassification of virological failure. When plasma VL was LDL, there was significant upward misclassification by Alere q. There was also some over-quantification but to a much smaller degree by Abbott original and updated when plasma VL was LDL. Over-quantification is likely due to measurement of intracellular HIV RNA in whole blood in combination with extracellular HIV RNA, different extraction methods and amplification targets. The contribution of cell associated RNA and proviral DNA can add a “baseline” to the plasma VL detected below 3000–5000 copies/ml (plasma RNA dominates the assay above 5000 copies/ml)[22].

The Abbott HIV viral load method for extraction is selective for RNA therefore the over-quantification is likely due to intracellular HIV RNA. The extraction method for Alere q is also selective for RNA however the over-quantification is significantly greater, probably due to other differences such as assay target, detection methodology, in conjunction with cell associated HIV RNA. The Roche FVE measures only extracellular HIV RNA similar to plasma therefore it

demonstrated the best specificity. Extraction methods that are selective for RNA have a closer correlation with plasma and include RNA-specific nucleic acid extraction, DNAse pretreatment to remove DNA, and virus particle elution[22].

Therefore the threshold for whole blood virological failure needs to be raised in comparison to the plasma threshold of 1000 copies/ml to overcome reduced specificity. However due to the variability between the different testing platforms, it would be difficult to utilize a uniform threshold across all platforms for whole blood virological failure. Performing receiver operating characteristic curve analysis for each testing method helps to determine the optimum threshold for each method. However, the thresholds from our ROC curve analysis may be artificial due to the small sample size and does not take into account individual patient clinical differences.

Our study is one of the preliminary studies evaluating POC Alere q viral load. The advantages of POC Alere q are ease of use, minimal training required, small portable instrument with minimal reagents, closed system, very small sample volume required, built in controls, results in less than an hour, molecular detection and quantification of HIV-1 and HIV-2, low error rate, and excellent sensitivity for detection of virological failure as demonstrated in our study. Disadvantages include cost per test, low throughput, and reduced specificity. Another study recently published on POC Alere q viral load using finger-prick capillary whole blood samples in a primary health care centre in Mozambique also had similar results to ours albeit with better specificity – the sensitivity was 96.83% and the specificity was 47.8% using the threshold of 1000 copies/ml for virological failure[23]. They also suggested that a raised cut-off of 10,000 copies/ml is a better predictor of virological failure for Alere q[23].

In a study evaluating DBS for virological failure by Sawadogo et al., they state that the WHO recommendations for DBS viral load monitoring should not treat different assay versions, types and RNA extraction methods used with DBS as homogeneous and that explicit recommendations for viral load testing with DBS for each of the prequalified viral load assay versions and types should be included, or recommendations should be further qualified until evidence for or against their use becomes conclusive[24].

Our study clearly illustrates the differences with each of the different test methods for whole blood viral load and the difficulty assigning a uniform threshold for virological failure. Solutions are required so that whole blood viral load monitoring can be implemented as routine testing in rural settings where the utilization of whole blood is the best practical alternative to plasma. Possible solutions include: the threshold for whole blood virological failure can be raised (>5000 copies/ml) to ensure optimum specificity (reduce upward misclassification) across different test methods, alternatively each whole blood test method would define its own threshold by ROC curve analysis (however this would be complicated on a programmatic level for HIV guidelines and implementation). Further work needs to be done to harmonize or standardize the whole blood viral load methodologies such that a universal threshold with optimal sensitivity and specificity for virological failure can be used.

The reduced sensitivity of DBS compared to plasma due to lower input volume does not negatively impact on the detection of virological failure as the limit of detection of most whole blood assays evaluated is below 1000 copies/ml. The major issue with whole blood viral load testing is the reduced specificity leading to upward misclassification. A higher threshold should be used to avoid misclassification. The precise threshold to be used is likely to be different for each assay and patient, depending on assay methodology and patient factors such as CD4 count, HIV DNA and intracellular

RNA copy number[14]. Methods may be required that increase the specificity of the test such as DNAse treatment, RNA specific nucleic acid extraction or detection, and removal of cellular components before extraction[14]. POC technologies are being developed that include an internal or external plasma separation step[23].

Patient factors such as CD4 count and treatment status impact on HIV DNA and intracellular HIV RNA copy number. In untreated patients or patients failing cART and as CD4 count declines, the HIV DNA and intracellular HIV copy number increases. In our study no associations could be made with the discrepancy in whole blood testing methods with clinical and laboratory factors collected from the laboratory information system such as CD4 count and previous history of virological failure. However it was difficult to gather clinical information from the laboratory information system with many request forms being incompletely filled or no histories available.

The prevalence of virological failure might impact on the utility of these assays. For example, with a 5% prevalence of virological failure Alere q has a positive predictive value (PPV) of only 6% and a negative predictive value (NPV) of 100%, if the prevalence is higher at 30% then the PPV is still low at 35% and the NPV remains 100%. Alere q has an excellent NPV to rule out virological failure but a positive result has a poor PPV and would need supplemental testing with a more specific test. The other alternative that could be helpful especially in low prevalence settings would be to raise the threshold for virological failure for Alere q to 10,000 copies/ml, which according to our ROC curve analysis would improve specificity to 90% although reducing sensitivity to 80%. Roche FVE has a PPV of 74% and NPV of 98% with a prevalence of 5%, and with a higher prevalence of 30% the PPV is 96% and the NPV is 83%. In contrast to the other whole blood assays, Roche FVE has a better PPV and the NPV drops with increasing prevalence. Roche FVE needs to increase sensitivity especially for use with high prevalence of virological failure. Although the whole blood assays perform differently in comparison to each other, their unified strength is an excellent PPV above 10,000 copies/ml. A result below this threshold would need additional testing, preferably repeating after a specified time period to monitor the viral load trajectory. Assessing the utility of this approach would require collection of longitudinal data to monitor kinetics of whole blood viral load in patients on cART.

Currently DBS seems to be the easiest option for increasing access and is also recommended by WHO. Health care providers at primary health care facilities are already trained in collecting DBS as it is part of the HIV early infant diagnosis programme. Centralised laboratories with high throughput will need to transition from plasma based testing to DBS, especially taking care to avoid contamination during DBS processing. Pre-punched DBS cards are preferable which obviates the need of cutting spots. As POC technologies become more accessible and affordable, it would be helpful to use in certain settings especially in remote areas where results are required swiftly to guide clinical decision making. POC testing can serve as a complementary tool to expand access to viral load.

Limitations of the study include plasma and whole blood viral load not tested simultaneously, DBS prepared in the laboratory using venipuncture blood and not in the health care facility, DBS and POC whole blood viral load performed in a controlled laboratory environment, difficulty collecting clinical information from the laboratory information system for analysis of clinical factors, whole blood HIV viral load testing methods are currently under research use and not being used for routine

diagnostics with individualized non-standardized protocols. True performance in a real world setting may be more variable compared to our results.

5. Conclusion

Variability was noted between the different whole blood viral load assays. Alere q and Abbott original and updated had excellent sensitivity but had over-quantification below plasma VL of 1000 copies/ml resulting in significant upward misclassification of virological failure. Roche FVE had excellent specificity however a significant proportion of plasma samples between 1000 – 10,000 copies/ml were misclassified as virologically suppressed. Factors such as sample input volume, sample treatment/processing, sample extraction methods and amplification targets influences the ability to quantify whole blood viral load. Based on our study, Alere q and Abbott original and updated need to raise their whole blood threshold for virological failure in order to reduce upward misclassification and Roche FVE needs to achieve better sensitivity around its limit of detection. Receiver operating characteristic curve analysis will help to determine the optimum threshold of virological failure for each assay.

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Abbreviations

(alphabetical order)

AIDS – Acquired immunodeficiency syndrome

Alere q – Alere q HIV-1/2 point of care

Abbott original – Abbott RealTime HIV-1 original open-mode protocol

Abbott updated – Abbott RealTime HIV-1 updated new prototype protocol

AZT - Zidovudine

cART – Combination antiretroviral therapy

CD4 count – CD4 T lymphocyte count

DBS – Dried blood spot

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

EFV - Efavirenz

FTC - Emtricitabine

HIV – Human immunodeficiency virus

IQR – Interquartile range

LDL – Lower than detectable limit

LOQ – Limit of quantification

N - Number

N/A – Not applicable

NASBA – Nucleic acid sequence based amplification

NPV – Negative predictive value

PCR – Polymerase chain reaction

Plasma VL – Plasma viral load

PMTCT – Prevention of mother to child transmission

POC – Point of care

PPT – Plasma preparation tube

PPV – Positive predictive value

RNA – Ribonucleic acid

ROC curve – Receiver operating characteristic curve

Roche CAP/CTM FVE – Roche COBAS Ampliprep/Taqman HIV-1 v2.0 free virus elution protocol

SD – Standard deviation

START – Strategic timing of antiretroviral therapy trial

TDF - Tenofovir

TEMPRANO - A trial of early antiretrovirals and isoniazid preventive therapy in Africa

UNAIDS – The Joint United Nations Programme on HIV/AIDS

US – United States

WHO – World Health Organisation

3TC – Lamivudine

95% CI – 95% confidence interval

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1 December 2014

HREC REF: 819/2014

Dr M Hsiao
Medical Virology
C18
NGSH

Dear Dr Hsiao

PROJECT TITLE: CLASSIFICATION OF HIV VIROLOGICAL FAILURE USING WHOLE BLOOD VERSUS PLASMA VIRAL LOAD (MMed-candidate Dr A Khan)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th December 2015.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

We acknowledge that the student, Dr Aabida Khan will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

HREC 819/2014

The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

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