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Diagnosis and monitoring of HIV in infants: Investigating the first fourth generation rapid test and two viral load technologies for use in the South African setting

Kapila Bhowan



A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree

of

Master of Science in Medicine
Johannesburg, 2013

DECLARATION

I, Kapila Bhowan declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



31st day of, March, 2014.

DEDICATION

To my wonderful husband,

Sachin,

And our precious angels,

Leisha and Tavish

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publications:

1. Bhowan, K., Kalk, E., Khan, K., Sherman, G., *Identifying HIV infection in South African women: how does a fourth generation HIV rapid test perform?* African Journal of Laboratory Medicine, 2011. **1**(1) (Appendix B and C).

2. Bhowan, K. and G.G. Sherman, Performance of the first fourth-generation rapid human immunodeficiency virus test in children. Pediatr Infect Dis J. 32(5): p. 486-8 (Appendix D and E).

Presentations:

1. K. Bhowan, Lesley Scott', Sergio Carmona, Gayle Sherman. *Precision of HIV viral load (VL) testing on dried blood spots (DBS) in adult and paediatric specimens* : 6th IAS Conference on HIV Pathogenesis and Treatment: Abstract no. CDB083.

ABSTRACT

Human immune deficiency virus (HIV) infection contributes to child mortality rates in South Africa. Investigations of newer technologies for improving early infant diagnosis of HIV in the South African setting could reduce child mortality as life saving treatment can be accessed early in life. This study investigated three technologies: a fourth generation rapid HIV test and two viral load (VL) platforms.

Determine Combo (DC) is a qualitative fourth generation rapid test that is able to detect HIV antibodies and p24 antigen simultaneously. The performance of DC was evaluated in the field on samples from pregnant and postpartum women; in the laboratory, on stored samples from children and with the addition of heat denaturation.

In the maternal DC study 90 (8.8%) of 1019 women tested HIV positive of whom 59 (17.1% prevalence) were pregnant and 31% (4.6% prevalence) were postpartum. The sensitivity and specificity of the antibody component of DC on plasma was 100%(Confidence Interval (CI): 95.9-100%) and 99.8%(CI: 99.2-99.9%) respectively. Three postpartum patients tested false positive for HIV antibodies (n=2) and p24 antigen (n=1). No true positive p24 antigen was detected

DC was performed on stored samples from 182 (90%) HIV-exposed and 20 (10%) HIV-unexposed children aged from birth to six years. The DC HIV antibody component returned false negative results in 2 HIV-infected children; one clinically symptomatic and one asymptomatic aged 7 and 23 months respectively. The sensitivity of DC HIV antibody was 100% (CI:94.3-100%) in infants aged 6 months and younger with a specificity of 100% (CI:81.6-100%) for all ages. Of the 61 HIV-infected infants tested, the DC p24 antigen was reactive in only one clinically symptomatic infant resulting in a sensitivity for detection of HIV infection of 1.7% (CI 0.3-8.9%).

A heat denaturation technique designed to improve p24 antigen detection was applied to HIV-infected samples but failed to enhance p24 antigen detection on DC.

HIV viral load (VL) molecular assays are used to confirm an HIV-infected diagnosis and for VL monitoring. In South Africa, plasma is the gold standard sample for VL monitoring in infants even though dried blood spots (DBS) are the preferred specimen type in resource-constrained settings and for early infant diagnosis. The use of DBS specimens for HIV VL monitoring would convenience resource limited settings. The DBS matrix therefore requires validation to determine accuracy (for establishing diagnosis) and precision (for VL monitoring) compared to plasma VL.

This study investigated the accuracy and precision limits of DBS VL on the Roche Cobas AmpliPrep-Cobas TaqMan HIV-1 v2.0 assay (CAP/CTM) and the Abbott RealTime HIV-1 assay (m2000) platforms on samples from HIV-infected adults and children. The CAP/CTM was investigated on DBS containing 75µl blood and the m2000 was investigated using one (50µl) and two (2x50µl) DBS.

Compared to plasma VL, DBS VL from adults and children were higher in the lower range (<3log,<1000copies/ml) and lower values in the higher range (>5log, >185,000copies/ml) on the CAP/CTM in the study of DBS VL accuracy. Additionally, DBS VL values were >log1.0 higher in 42/100 (42%) of adult and 16/49 (33%) of measurements from children, which will have clinical significance. On the m2000 platform, the differences between plasma and DBS VL were lower in the range >5 log and higher in the range 2 log copies/ml (100 copies/ml) to 4 log copies/ml (10000 copies/ml). Compared to plasma VL, DBS VL values were >log1.0 higher in 20/82 (24%) adult and 7/43 (16%) of measurements from children.

Both platforms demonstrated 100% specificity in testing stored DBS from HIV-uninfected infants who were diagnosed negative on HIV DNA PCR.

Acceptable limits for plasma VL precision is a coefficient of variation (CV) <35% and standard deviation (SD) ≤0.19 log. Where plasma VL ≤5log, DBS VL demonstrated poor precision with CV>40% in 8/10 patients and total SD>0.30 log in 4/10 patients on the CAP/CTM. The m2000 total SD was >2log between adult plasma and DBS VLs under the 4 log copies/ml cut-off,

irrespective of the number of DBS used. DBS VLs were unreliable when using precision limits used on plasma VLs on both platforms.

In conclusion the DC test does not offer any advantage over currently available rapid tests in diagnosing new infection in women and children. The two VL platforms can be used to establish an HIV status in treatment naive patients in view of the 100% specificity. HIV-infected patients on treatment with undetectable plasma VL will always have detectable DBS VL on CAP/CTM, but equally undetectable DBS VL on the m2000. With DBS, the CAP/CTM assay generates higher VL values in the lower VL range than on plasma likely due to amplification of proviral DNA. Both platforms display poor intra- and inter- assay precision, using plasma VL based criteria and the variances would potentially affect clinical decision making. The acceptable limits for plasma VL precision cannot be applied to DBS VL on either platform.

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NOMENCLATURE

Ab	HIV antibodies
Ag	p24 antigen
ART	Antiretroviral Therapy / Treatment
CAP/CTM	COBAS AmpliPrep COBAS TaqMan
CDC	Centers For Disease Control and Prevention
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CV	Coefficient of variance
DBS	Dried blood spots
DC	Determine Combo rapid test
DNA	Deoxyribonucleic acid
DoH	Department of Health
EasyQ	NucliSens EasyMag and EasyQ
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
LR	Likelihood ratio
m2000	RealTime HIV-1 Assay

MTCT	Mother to Child Transmission
NASBA	Nucleic Acid Sequence Base Amplification
PCR	Polymerase chain reaction
PMTCT	Prevention of Mother to Child Transmission
RMMCH	Rahima Moosa Mother and Child Hospital
RNA	Ribonucleic acid
SD	Standard deviation
VL	Viral load
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION TO DIAGNOSIS AND MONITORING OF HIV IN INFANTS

Sub Saharan Africa carries the largest burden of the global HIV pandemic. Of the 34 million people that are HIV-infected globally, 23.5 million (69.1%) people live in African countries within resource limited settings [1]. Although much progress has been made in the battle against HIV, challenges such as early identification of HIV-infected pregnant and postpartum mothers in need of antiretroviral therapy (ART) and paediatric HIV diagnosis at point of care remain challenging issues for resource limited settings [2]. Mother to child transmission is the predominant source of paediatric HIV infection. A groundbreaking study showed that early diagnosis and treatment of HIV-infected pregnant women and their HIV-exposed infants reduces paediatric mortality [3], but access to therapy is only possible once a definitive diagnosis of infection is made. The World Health Organization (WHO) recognises the need for newer, inexpensive, straightforward tests to aid the identification, diagnosis and management of HIV-infected persons and has recommended that these tests be assessed as a research priority [4].

The increasing access to ART in countries that are most affected by HIV necessitates an expansion of appropriate testing technologies that aid early diagnosis of persons that have been exposed to HIV and monitoring of those who have been initiated on treatment. Studies of newer testing assays are necessary to collect enough evidence to influence practice. As such, a major drawback for infant testing guidelines is that commercially available rapid HIV tests are often validated

on adult populations [5-7] and disseminated in the field without evaluation in paediatric populations. Findings in adult studies may not always be applicable to children, which necessitates the validation of HIV rapid tests and HIV monitoring technologies in paediatric populations before recommendations for their widespread use can be made. Data on the performance of newer assays and the performance of new applications on existing assays could refine the current diagnostic algorithm in resource limited settings [4].

Presently, in view of ART expansion, the largest viral load (VL) laboratory in the world, based at the National Health Laboratory Services, Johannesburg, is able to provide 45 000 plasma VL results monthly. Plasma VL tests have been used to confirm an HIV-infected status and to monitor patient response to ART in infants in South Africa. VL testing on Dried blood spots (DBS) would potentially increase the number of monthly VL tests and consequentially raise the number of adults and children that gain access to life saving ART, but is not as yet approved by some manufacturers of VL technologies as a sample type for routine care. DBS VL as an alternative strategy to plasma VL testing, would benefit resource limited settings. Collection of plasma requires venipuncture skills whereas DBS sample collection is uncomplicated and addresses the lack of phlebotomy skills in healthcare workers. DBS transport logistics are simpler than those for plasma as DBS samples can be stored and transported at room temperature [8]. Plasma samples require cool storage if testing does not take place within 30 hours of sample collection [9] [10].

This study aimed to address outstanding questions that could lead to improved HIV care by investigating the utility of a fourth generation rapid test in identification of

HIV-exposed and-infected infants and pregnant women. DBS VL accuracy (for the diagnosis of HIV) and precision (for HIV monitoring) on two technologies was investigated to provide evidence for improved healthcare delivery.

To address the objectives, the study was divided into two components:

(1) To assess the Determine Combo HIV-1/2 Ag/Ab Combo Test (Inverness Medical, Japan Co., Ltd) (DC), a fourth generation rapid test in the identification of HIV-infected mothers and their infants.

(2) To assess the accuracy and precision limits of DBS VL testing performed on two VL platforms, namely, Abbott RealTime HIV-1 m2000sp/m2000rt (Abbott Molecular Inc, Des Plaines, USA) and COBAS AmpliPrep/COBAS TaqMan version 2.0 (Roche Molecular Systems, Branchburg, NJ).

CHAPTER 2

DETERMINE COMBO RAPID TEST STUDY

2.1 INTRODUCTION

Early diagnosis of HIV in infants is imperative for the infant to access life-saving ART [3] and for monitoring the efficacy of Prevention of Mother to Child Transmission (PMTCT) programs. This is a dynamic field in which questions of how best to deliver quality, affordable diagnostics are constantly raised by WHO [11] to assist countries such as South Africa in reducing maternal and paediatric HIV-related mortality.

South African child and maternal mortality attributable to HIV was 35% and 43% respectively and an estimated 1.3 million children were maternal orphans in 2011[12]. *The Global Plan towards elimination of new HIV infections among children by 2015 and keeping their mothers alive* campaign lists South Africa as one of the 22 countries for the reduction of new childhood infection by 90% and the number of maternal deaths by 50% [2]. It is therefore important to identify HIV-infected women and children for treatment at their first contact with health care services [4] , by testing for acute and established diagnosis with enhanced, affordable and sensitive rapid tests.

2.1.1 BIOLOGICAL CHARACTERISTICS OF HIV INFECTION

Persons newly infected with HIV experience an acute phase of infection which is characterised by high titres of virus in peripheral blood and associated with increased transmission rates [13]. Diagnosis of HIV in the acute phase of infection can be made by the detection of HIV viral antigens. p24 antigen (Ag), a structural protein from the core of the HIV virus, is the earliest detectable marker of HIV infection and is detectable in blood 16 days after exposure (Figure 2.1). Viral p24 Ag levels are at the highest at the beginning of infection and in late stage disease [13]. In both instances HIV antibodies (Ab) are absent or present in low titres respectively allowing the p24 Ag to exist unbound from the HIV Ab.

Production of HIV Ab marks the end of the window period, the interval between HIV-exposure and the production of HIV Ab. HIV Ab are proteins produced by the host immune system in response to the HIV virus 21 days after HIV-exposure in a process called seroconversion (Figure 2.1) [14]. The formation of the HIV Ab and p24 Ag immune complex renders p24 Ag undetectable [15].

HIV Ab detection is adequate to establish an HIV-infected diagnosis in adults and children over 18 months of age. However, testing for HIV infection in children younger than 18 months of age differs from that for older children. Infants born to HIV-infected mothers (also referred to as HIV-exposed infants) acquire maternal HIV Ab that may remain detectable in the infants bloodstream until 18 months of age.

Therefore, HIV Ab detection in infants younger than 18 months of age is indicative of the HIV-exposure status and not the HIV-infection status of the infant [16].

Laboratory viral detection tests remain the gold standard for diagnosis in infants younger than 18 months of age [11].

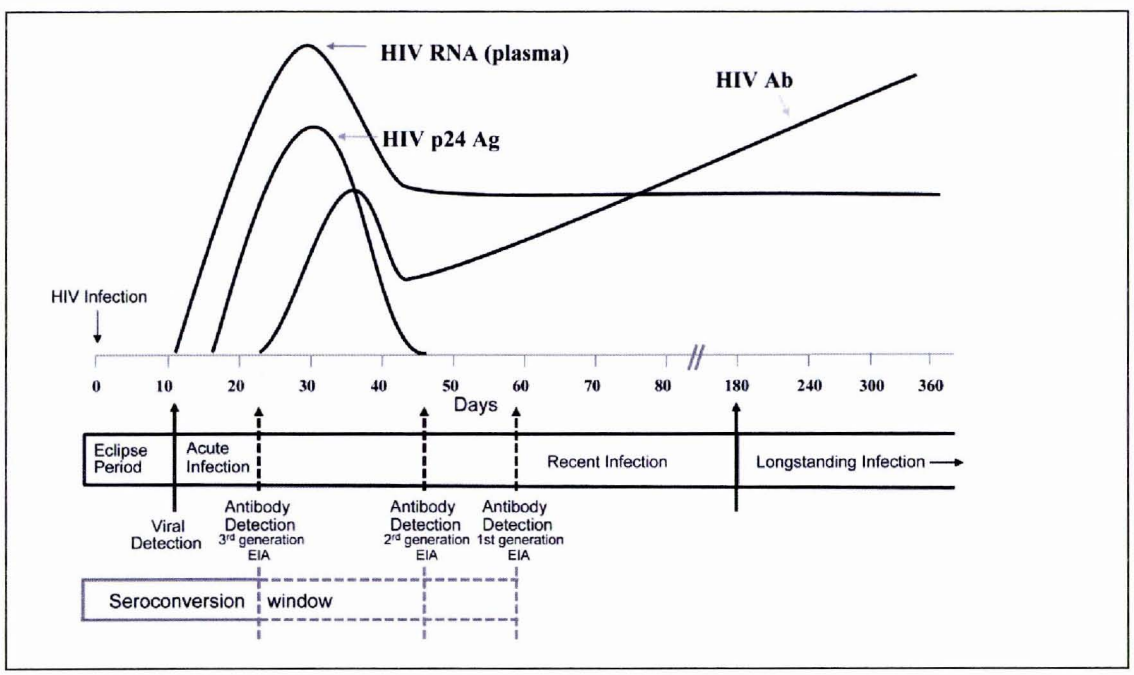


Figure 2.1: Kinetics of HIV markers during early infection [14]

2.1.2 SCREENING AND CONFIRMATORY HIV TESTING

2.1.2.1 HIV Ab DETECTION ASSAYS

Assays that detect HIV Ab (e.g. Enzyme-linked immunosorbent assay (ELISA) and rapid tests) are simple, accurate tools for HIV diagnosis. Rapid tests have expanded the opportunity to screen and test for HIV infection in adults and infants older than 18

months of age in resource limited settings at point of care as they do not require any special instrumentation or phlebotomy skills and provide an advantage over laboratory tests in that the patient receives the test result on the same day at a single clinic visit. The WHO recommends that rapid tests used for screening have sensitivity and specificity of >99% and >98% respectively [11] for accurate screening in the field (Table 2.1).

HIV Ab detection on rapid tests is indicative of HIV-exposure in infants younger than 18 months but prompts further virological testing to establish the infection status in the infant [17]. Presently available third generation rapid tests cannot detect components of the HIV virus and are superseded by laboratory fourth generation ELISA which are HIV Ab and HIV virus detecting assays.

Limitations of currently available third generation rapid tests in ill, symptomatic persons who have been documented to have low or undetectable HIV Ab levels, is the risk of a false negative diagnosis. An additional limitation of third generation HIV rapid tests is that individuals with acute infection will be missed for diagnosis as the test is limited to HIV Ab detection [18].

Efforts have been made to develop rapid tests for viral detection [19] to aid the diagnosis of HIV in children.

2.1.2.2 VIRAL DETECTION ASSAYS

Viral detection assays such as DNA and RNA Polymerase chain reaction (PCR) and p24 Ag assays that detect components of the HIV virus are the benchmark for

diagnosing HIV infection in children younger than 18 months of age [11, 16]. However, the cost and infrastructure requirements for viral detection assays render them less suitable for resource limited settings [11].

Although p24 Ag assays have a specificity of 100% , they are used less commonly than DNA and RNA assays for diagnosing HIV because their sensitivity is very low at around 50% [20] (Table 2.1). The reason for this is that p24 Ag binds to HIV Ab in an immune complex that prevents detection of p24 antigen. Heat dissociation or acid denaturation has been used to disrupt the immune complex thereby increasing the sensitivity of the p24 Ag detection [15, 21]. Ultrasensitive p24Ag assays have a much improved sensitivity as compared to the earlier p24 Ag assays but are not readily commercially available [22, 23].

Table 2.1: Summary of laboratory tests that are used to diagnose HIV in children and their general performance characteristics [11]

HIV viral detection assays	***HIV Ab detection assays**
<18 months of age	*>18 months of age*
RNA and DNA PCR assays Sensitivity >98% Specificity >98%	ELISA (third and fourth generation) and rapid tests Sensitivity >99% Specificity >98%
p24 Ag assays Sensitivity ± 50% in children Specificity ± 100%	
Ultrasensitive p24 Ag assays Sensitivity ±96% in children <6 weeks of age Specificity ±100%	

*Detect HIV-exposure, not HIV infection in children younger than 18 months of age [16].

2.1.3 IDENTIFICATION AND TESTING OF PREGNANT WOMEN AND CHILDREN IN SOUTH AFRICA

South Africa's PMTCT (Prevention of Mother to Child Transmission) programme utilizes the provider-initiated counselling and testing strategy for screening patients [24]. The current South African PMTCT guidelines recommend that pregnant women should be tested at their first antenatal clinic visit and between 32- 34 weeks of pregnancy if their initial HIV test was negative [25] thereby expanding the search for identifying mother and baby pairs in need of prophylactic care [26].

Patients are tested on a rapid test and considered to be HIV-uninfected if the test is negative. If the rapid test is positive, a second, different rapid test is used to confirm the HIV-infected status. Laboratory HIV ELISA tests are used to resolve discordant rapid test results [24]. The Western Blot assay can be used to confirm a reactive ELISA result as the test is designed to detect specific HIV proteins. The test can also be used to rule out false positive ELISA results. Western blot tests are not requested routinely as they are difficult to perform and require technically skilled staff.

In line with the National Department of Health's (DoH) policy to eradicate paediatric HIV infection, women at the Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg, South Africa are offered a further opportunity to establish their HIV status by means of a third generation HIV rapid test immediately postpartum if their HIV status is unknown and if more than six weeks have passed since their last negative HIV test result [26]. The relevance of repeating the test after 6 weeks is to accommodate the window period. Currently available third generation HIV rapid tests

are limited to HV Ab detection. Women with acute HIV infection in the window period will test negative on the third generation HIV rapid tests as they have no HIV Ab at the window stage of infection. HIV viral antigens peak during the window period and the risk of MTCT through birth or breastfeeding is increased [13, 27]. Efforts are underway to develop sophisticated point of care tests for diagnosis of acute infection (by detection of HIV Ag during the window period) [14, 28, 29]. Theoretically, an HIV Ag detecting point of care test would enable women to learn their HIV status during the period in which they are hyper infectious, enabling appropriate, early treatment intervention.

The significance of re-testing pregnant women has been demonstrated by studies in which 1.3% and 3.4% of women who tested HIV negative at their first antenatal visit, subsequently seroconverted during pregnancy or within a year after delivery[30, 31]. A Kenyan study showed that 2.6% (53/2035) women contracted the HIV infection between pregnancy and the 6-week post partum period demonstrating the potential risk of infant HIV infection between delivery and the first postnatal visit [32].

HIV-exposed babies are largely identified for prophylaxis though PMTCT programmes that have identified the mother to be HIV-infected [26]. These HIV-exposed babies that are younger than 18 months of age cannot be tested on currently available HIV Ab detection assays to establish their HIV-infection status as the assays cannot differentiate between maternal and infant HIV Ab [16]. During early HIV infection in infected infants, maternal HIV Abs are present to complex with p24 Ag hindering p24 Ag detection. As maternal HIV Ab wane (process of

seroreversion), increasing infant HIV Ab titres participate in immune complex formation. In advanced disease, HIV-infected infants with hypogammaglobulinaemia (including low HIV Ab titres), high viral loads and waning maternal HIV Ab are the likeliest to have uncomplexed, detectable p24 Ag [20]. Studies have shown the seroreversion process to begin as early as 3 months [33]. Whilst maternal HIV Ab wane, HIV infected infants will produce their own HIV Ab and have high levels of p24 Ag as a result of viral replication. The balance of p24 Ag relative to HIV Ab may change in favour of more unbound and therefore detectable p24 Ag. As a result, the sensitivity of p24 detection assays may increase during the period that maternal HIV Ab wane [20, 34].

The WHO guidelines on infant HIV testing recommends that all HIV-exposed infants are tested at 4 to 6 weeks of age by a DNA PCR test [11]. The PCR test results are communicated to the caregiver at the infants 10 week immunisation visit. The lag between the initial laboratory PCR test and initiation of Highly Active Antiretroviral Therapy (HAART) results in the initiation of therapy around eleven weeks of age for HIV-infected infants [35] even though it has been established that ART initiated at a median of seven weeks of age reduces infant mortality by 75% [3]. Early diagnosis and initiation of treatment would be enhanced if a rapid test, at point of care, could potentially identify HIV-infected children presenting with illness and clinical features of HIV. At present there are no rapid tests that provide a definitive HIV infected diagnosis for use in children younger than 18 months. Infants in resource limited settings where laboratory viral detection assays are unavailable are further disadvantaged and would benefit from such a rapid test. More expensive and

complex point of care units that aid the early diagnosis of infants are being developed and evaluated [29], but are not yet available.

2.1.4 THE FIRST FOURTH GENERATION POINT OF CARE TEST FOR HIV DIAGNOSIS

The Determine Combo HIV-1/2 Ag/Ab Combo Test (DC) (Inverness Medical, Japan Co.,Ltd) is the first fourth generation rapid test. [36]. It surpasses third generation tests by providing simultaneous differentiated HIV Ab and p24 Ag detection in a single test [19]. The DC rapid test is reported to have the potential to diagnose HIV infection five days (range 2-20 days) earlier than third generation rapid tests [36]. The DC test expands the diagnostic spectrum by identifying persons during the acute phase of infection; the phase in which p24 Ag unbound to HIV Ab is detectable [7]. The DC test does not require refrigeration making it easier to use in low resource environments.

Potential advantages of the DC test over current third generation rapid tests in pregnant women are that newly infected women in the window period will test p24 Ag positive and their infants, at high risk of acquiring HIV during an acute maternal infection, will be identified for intervention. A disadvantage of using DC over third generation rapid tests is its higher cost.

The theoretical advantages of the DC test being able to detect p24 Ag in children include an earlier definitive diagnosis of HIV infection and more reliable identification of children with late stage HIV infection who may have very low levels of HIV Ab known to result in false negative third generation rapid tests [37]. The DC test therefore has the potential to act as the first point of care test for a definitive diagnosis of HIV infection in children aged less than 18 months in resource limited settings. If the sensitivity of DC for detecting p24 antigen is the same as earlier laboratory p24 antigen assays, then the DC rapid test may correctly diagnose a positive HIV infection status in 50% of HIV-exposed, infected infants [20]. Assuming the sensitivity of HIV Ab detection in DC is >99% (like third generation rapid tests), then it may be a useful tool for the identification of HIV-exposure in infants and HIV-infection in children older than 18 months of age.

The reported sensitivity and specificity of the DC Ab component is 100% (95% CI 98-100) and 100% (95% CI 98.2-100) respectively and the sensitivity of the p24 Ag component is 86.6% (95% CI 76-93.7) on DC by using samples that were collected in the window period [38]. The specificity of DC p24 Ag was 96.6% as reported by the manufacturer [36]. No sensitivity for p24 Ag detection in established HIV infection is reported.

The performance of the DC in 40 HIV-infected infants aged 3-months or less demonstrated a sensitivity for HIV Ab detection of 100% (95% CI 95.9-100%) and for p24 Ag detection of 2.5% (95% CI 0.1-14.7%) [39]. No clinical details on the single, 3-month old infant identified as infected by the DC p24 Ag were recorded. No data

on the performance of the DC test in older infants and children are available particularly to assess whether it may be useful in specific clinical scenarios such as advanced disease. The DC test has not been widely assessed in children.

2.1.5 AIMS AND OBJECTIVES

This study evaluated the performance of the first fourth generation rapid HIV test in a

1. prospective maternal evaluation

- in pregnant women for detecting early infection (by p24 Ag detection) and established infection (by HIV Ab detection) in order to identify HIV-infected women.

2. retrospective paediatric evaluation

- in children for detecting HIV-infection and HIV-exposure on stored samples from children of varying ages and clinical scenarios

A small subset of maternal and paediatric samples were evaluated to assess whether heat denaturation increased the sensitivity of p24 Ag detection by the DC test.

2.2. MATERIALS AND METHODS

The study has been described in three sections The prospective study assessed the performance of the DC rapid test on pregnant women at a clinical site. The retrospective component describes the testing of stored paediatric samples on the DC test. Section 3 is a heat denaturation study of stored samples from Sections 1 and 2 (figure 2.2).

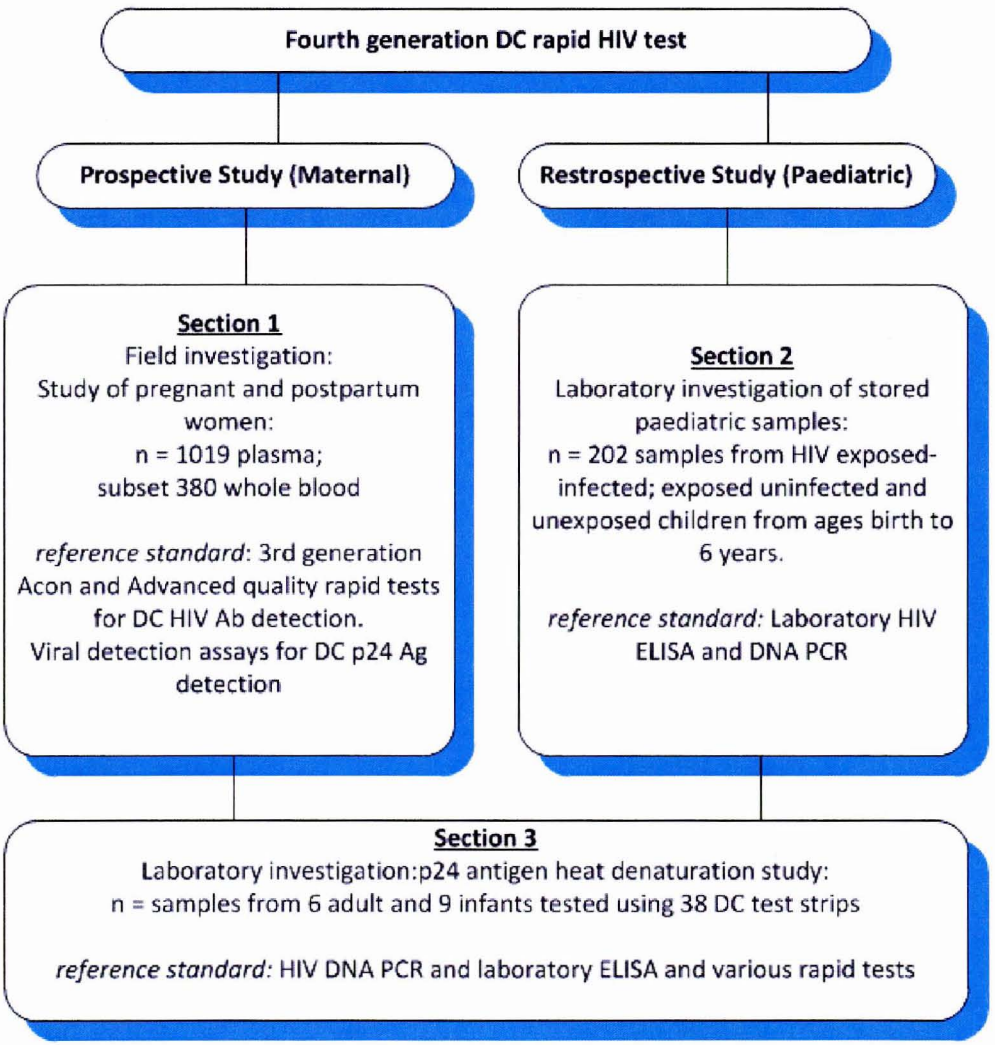


Figure 2.2: Flow diagram presentation of the components of the rapid test study section.

Ethics approval (M091133) to conduct this study was granted by the Human Research Ethics Committee at the University of the Witwatersrand, Johannesburg (Appendix A).

2.2.1 SECTION 1: MATERNAL DC STUDY

The study was conducted at RMMCH, situated in the west of Johannesburg in the Gauteng province. This tertiary hospital provides health care services, including PMTCT, for women from the surrounding geographical area. The number of deliveries at RMMCH annually [26] were considered adequate to enrol the sample size required for this study within a reasonable time period. The convenience of having the antenatal clinic and delivery ward in close proximity of each other at RMMCH facilitated the enrolment of pregnant and postpartum patients for the study. Women attending the antenatal clinic and delivery ward were invited to participate in the study.

The medical staff at the study site consisted of two counsellors, two phlebotomists, a principal medical officer and two medical technologists. The latter were responsible for performing and documenting the routine HIV tests and the fourth generation rapid tests. The counsellors interviewed the participants, provided pre- and post-test counselling and documented whether women verbally reported or had documented evidence of their HIV status and when they last had an HIV test. Women with an unknown or reported negative HIV status as well as those with a documented HIV

negative result performed more than six weeks previously were invited to participate in the study. Women who reported an HIV positive status but had no supporting documentation were also invited to retest. Women that had documented evidence of their HIV-infected status were excluded. Written informed consent was obtained from all participants who agreed to test for HIV.

2.2.1.1 SAMPLE SIZE

The Centers for Disease Control and Prevention (CDC) guidelines for prospective evaluation of HIV testing technologies recommend that 200 HIV-infected and 200 HIV-uninfected plasma samples be included in the test evaluation to provide 95% confidence intervals for the calculation of sensitivity and specificity [40]. The total number of samples to be included in the study depended on the HIV prevalence within the test settings [40]. The prevalence of HIV amongst pregnant women testing antenatally and postpartum at RMMCH was 15% and 4% respectively in 2008 [26]. It was estimated that a total of 1450 patients would need to be enrolled in the study to meet the CDC guidelines and it was anticipated to take six months to achieve based on RMMCH records that showed that approximately 200 women presented for testing monthly (unpublished).

In order to assess the performance of the DC test on whole blood as compared to plasma, a subset of the 1450 samples was tested on both plasma and whole blood, until 20 HIV-infected and 80 HIV-uninfected DC tests results were obtained on whole blood.

In addition to the calculation of sample size for overall infection rate the number of incident (number of newly diagnosed cases of HIV over a period of time) infections that could be expected at RMMCH site was calculated. The number of newly diagnosed cases of HIV over a period of time (incidence) at RMMCH for the first antenatal HIV test is not known. Repeat testing of HIV-negative women at the time of delivery [26], demonstrated that 4.5% seroconverted confirming that incident infections do occur. South African women aged 20-39 years had an HIV incidence of ±3 - 5.6% per annum [41] with higher rates expected in pregnancy. It was expected that over a 6-month period, approximately 1.5 - 2.8 cases of newly infected women would be detected per 100 HIV-uninfected women tested. In a sample size of 1250 HIV-uninfected women (assuming that 200 women out of 1450 would be HIV-infected), 18-35 incident or early HIV infections would be diagnosed by DC as p24 Ag positive and HIV Ab negative. If incident infection rate over 6 months was 1% then in 1250 postpartum women,13 newly infected women should be detected by the fourth generation DC test.

The number of new infections detected by the DC test in this study would provide information on how many new cases of HIV infection in pregnant women could be detected every six months at the RMMCH service.

2.2.1.2 HIV TESTING PROCEDURE

Five millilitres of whole blood was collected into an Ethylenediaminetetraacetic acid (EDTA) tube for testing at a laboratory located at RMMCH study site. The single

EDTA tube was used for the routine HIV rapid tests as well as for the study to avoid drawing extra blood from the patient. Whole blood was separated into two equal aliquots and labelled with the patient name and laboratory reference number. One aliquot was centrifuged at 3000rpm for five minutes to obtain plasma. Third generation rapid tests Advance Quality HIV rapid test (In Tec Products, Inc. Xianen, China) and Acon HIV 1/2/0 Tri-line Rapid Test Device (Acon Laboratories, Inc., San Diego, USA) were the tests stipulated by the DoH for use in the National testing program and were performed according to Figure 2.3 with the DC test performed in parallel on plasma. A subset sample of DC tests were conducted on whole blood in addition to plasma. Whole blood testing required the use of a chase buffer.

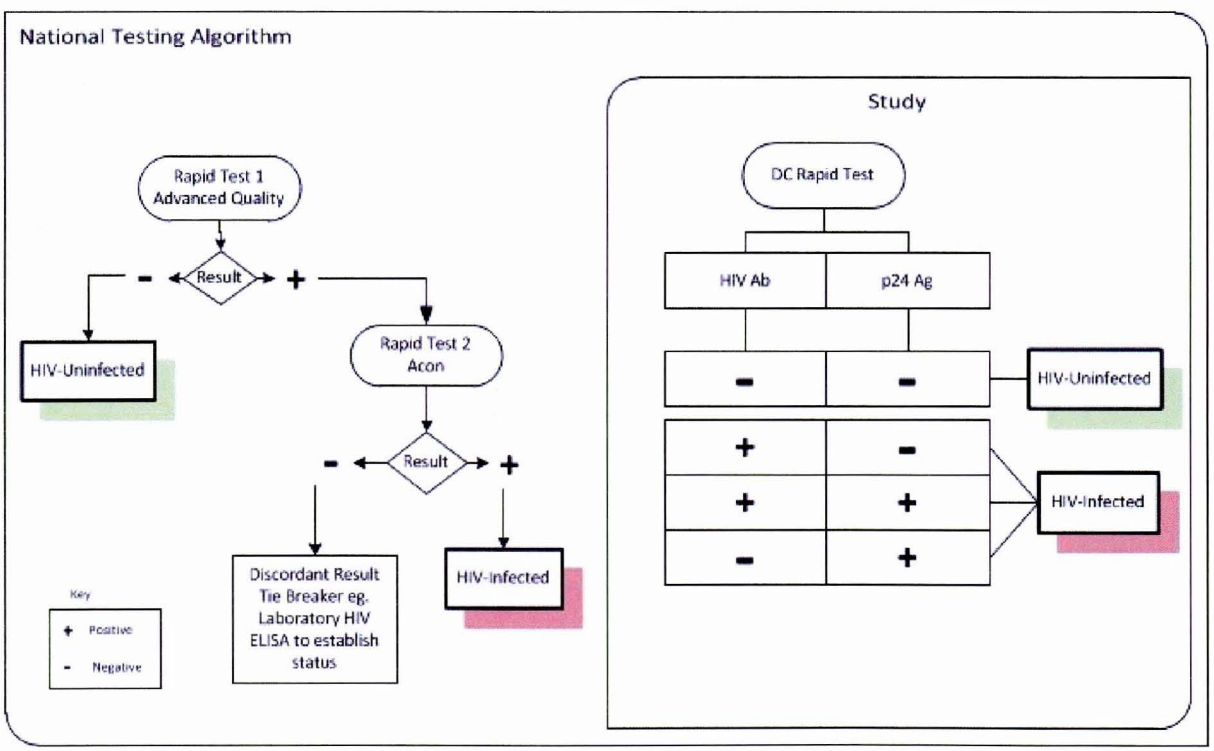


Figure 2.3: The Determine Combo rapid test was performed in parallel with the routine algorithm [42]

2.2.1.3 INTERPRETATION OF TEST RESULTS

The DC is a qualitative immunochromatographic test presented in a lateral flow device for use on plasma, whole blood or serum that takes 20 minutes to perform and is read visually. Each test strip incorporates a procedural positive control and the test is considered valid only if the positive control is detected [36]. The test strip is divided into an HIV Ab window and an HIV p24 Ag window (Figure 2.4). The presence of a pink line in the p24 Ag or HIV Ab windows was indicative of HIV infection (Figure 2.4). The DC HIV Ab test principle is based on the binding of HIV Ab (if present) to an antigen selenium colloid and recombinant antigen to form the pink line in the HIV Ab window. The p24 Ag detection is based on the binding of unbound p24 Ag to a biotinylated and selenium colloid anti-p24Ab and immobilised avidin to form a pink line in the HIV p24 Ag window [36](Figure 2.4).

The four hour turnaround time between pre-test counselling, sample collection, rapid testing and post test counselling ensured that women received their test results within the day.

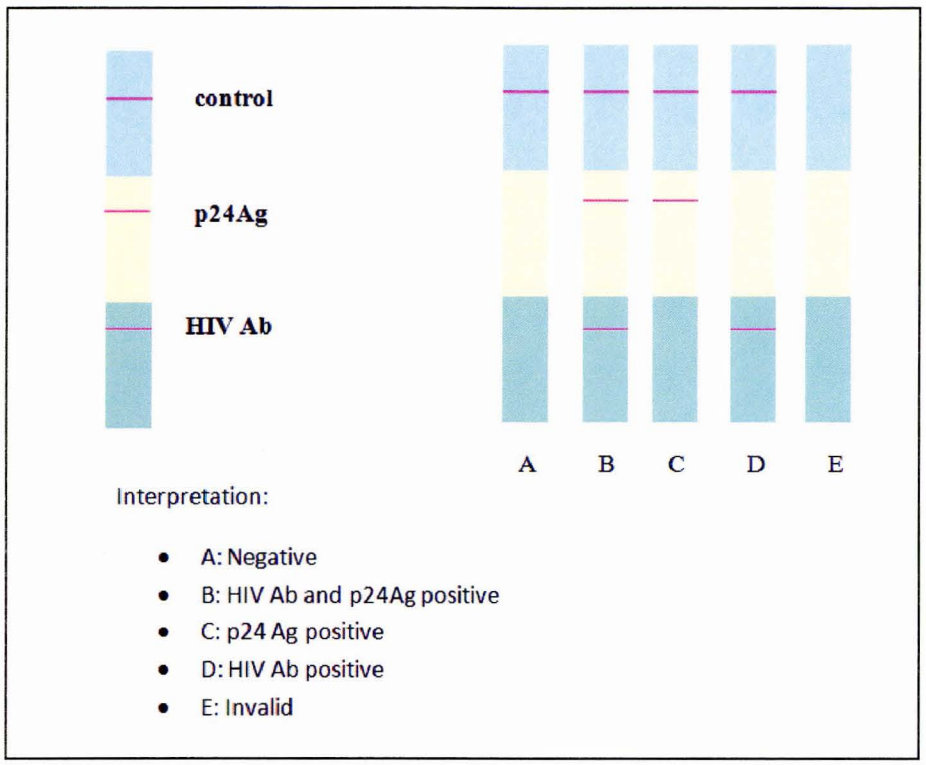


Figure 2.4: Interpretation of DC test results. The DC test strip comprises the control, p24 Ag and HIV Ab windows. Pink lines represent a valid test and an HIV positive result. Interpretation of results of the HIV Ab and p24 Ag components of the DC rapid are described from test strips A-E.

Patients that tested HIV positive on the routinely used third generation rapid tests were referred to ARV clinics. Where the third generation rapid test results were discordant, samples were referred for a confirmatory laboratory fourth generation ELISA (ARCHITECT® HIV Ag/Ab Combo assay; Abbott Diagnostics; Wiesbaden, Germany) and disclosure of the patient's HIV status was delayed (Figure 2.3). The results from DC did not influence the diagnosis of the patient even if p24 Ag was detected and the third generation Advanced quality screening test was negative because the DC test was under validation. The DC p24 Ag test results were kept confidential and not disclosed to the patient unless an HIV-infected status was confirmed by additional viral detection assays, in which case the patient was

contacted for retesting. Samples that tested p24 Ag positive on DC were verified with a p24 Ag detection assay (Vironostika HIV-1 Antigen; bioMerieux; Bosiend, The Netherlands) and a viral load test (NucliSens EasyQ-EasyMag HIV-1, version 1.2 assay; bioMerieux; Boxtel, The Netherlands). Plasma remaining from routine testing was stored at -20°C at the RMMCH facility for 48 hours and subsequently shipped to an accredited storage facility for long term storage at -80 °C to enable further testing in cases where the laboratory gold standard HIV ELISA or viral detection assays yielded discordant results or in cases of misplaced samples and laboratory accidents.

2.2.1.4 STATISTICAL ANALYSIS

Descriptive statistical analyses including sensitivity (*true positives / (true positives + false negatives)*, specificity *(true negatives / (true negatives+ false positive)*, positive and negative likelihood ratios (LR) were used to evaluate the performance of DC in detecting established and new HIV infections in women. Sensitivity described the probability of a positive rapid test when HIV is truly present. Specificity refers to the probability of a negative test result when HIV is truly absent. The LR were calculated instead of predictive values because predictive values depended on prevalence and a difference in HIV prevalence was anticipated between women tested in the antenatal and postpartum period. A positive LR *(sensitivity/(1-specificity))* >10 strongly predicts HIV-infection, whereas a negative LR *((1-sensitivity)/specificity)* <0.1 virtually excludes the condition.

2.2.2 SECTION 2: PAEDIATRIC DC STUDY

This component of the study used stored paediatric samples from prospective longitudinal [43] and cross sectional study cohorts enrolled in 2002 and 2005 [44] respectively. Clinical data records were reviewed for sample selection. Criteria for inclusion were dependent on the child's age, HIV-exposure and -infection status, CDC clinical disease stage and a minimum volume of 50µl sample being stored.

The CDC system of classification categorises patients on the basis of conditions associated with the HIV infection and assists clinicians to stage the infection. For the purposes of this study, children classified as CDC clinical stage A and N were considered clinically asymptomatic whilst those classified as CDC clinical stage B and C were considered clinically symptomatic [45]. This study attempted to model real-life circumstances of children presenting for testing in the field by using four clinical scenarios, namely: 1) HIV-exposed, -infected and clinically asymptomatic (CDC A and N) children; 2) HIV-exposed, -infected and clinically symptomatic (CDC B and C) children; 3) HIV-exposed uninfected children and 4) HIV-unexposed children. Expected outcomes for the DC HIV Ab and p24 Ag components in the various ages and clinical scenarios are detailed in (Table 2.2). The ages of the children chosen for this study ranged from birth to six years (Table 2.3).

Table 2.2: Anticipated DC test results based on age, HIV status and clinical stage.

Clinical scenario	Determine Combo HIV Ab result	Determine Combo p24Ag result
HIV-exposed, HIV-infected, asymptomatic	Positive at all ages	Positive in ±50% *
HIV-exposed, HIV-infected, symptomatic	Positive at all ages #	Positive in ±50% *
HIV-exposed, HIV-uninfected	Positive in <18 months of age ^ Negative in ≥ 18 months of age	Negative in <18 months of age Negative in ≥18 months of age
HIV-unexposed, HIV-uninfected	Negative at all ages	Negative at all ages

* more than 50% of infants may be detected if the p24 Ag/ Ab balance is in favour of unbound p24 Ag during the time that maternal HIV Abs wane, infant HIV Ab production increases and viral replication is unchecked

in severely symptomatic infants with hypogammaglobulinaemia, HIV Abs may be undetectable however, the DC p24 Ag should be positive and avoid the false negative results of the current third generation rapid tests

^ older infants and children may seroревert earlier and test HIV Ab negative before 18 months of age. Together with a negative p24 Ag test, HIV infection can be excluded (in the absence of breastfeeding)

All children had a prospectively determined HIV status with documented HIV Ab and viral detection assay results. The reference standards against which the DC results were measured were the Roche Amplicor HIV-1 DNA version 1.5 (Roche Diagnostics Systems, Inc., Branchburg, NJ) viral detection assay for the ability of the DC p24 Ag component to diagnose infection; and the HIV-1/HIV-2III Plus, IMx System (Abbott Diagnostics Division, Wiesbaden, Germany) third and fourth generation ELISA Ab detection assays and rapid tests (Table 2.3) for the ability of the DC Ab component to diagnose HIV-exposure in infants <18 months of age and HIV- infection in older children.

2.2.2.1 SAMPLE SIZE

The paediatric samples available for testing were collected six to nine years prior to this study. The scarcity of such samples from children hampers the ability to test larger sample sizes unless large multicentre studies are conducted over long periods of time. A convenience sample of 202 infants that had a minimum stored sample volume of either serum or plasma of 50µl, required to perform the DC test, were retrieved from -80°C storage in an accredited laboratory. Preference was given to samples from each of the studies that fulfilled the criteria for the different clinical scenarios and had the most age-appropriate HIV test results available for comparison (Table 2.3).

2.2.2.2 HIV TESTING PROCEDURE

Samples were retrieved from storage, thawed once in batches at room temperature, centrifuged at 3000 rpm for 5 minutes and tested over two days in an accredited laboratory and by a single operator in accordance with manufacturer instructions.

Table 2.3: Paediatric samples tested on DC. Samples were selected from two prospectively conducted studies in which children's HIV infection status was determined by age-appropriate HIV tests.

n	study	Reference assay for DC HIV Ab and p24 Ag result comparison		Age
		HIV Ab detection	*Viral detection*	
77	Longitudinal cohort	ELISA* and rapid tests∞	DNA PCR	6-weeks, 3, 7-12 months
125	Cross-sectional cohort	ELISA*† and rapid tests∞▪	DNA PCR	Birth to 6 years

*Third generation *ELISA HIV-1/HIV-2III Plus, IMx System* (Abbott Diagnostics Division, Wiesbaden, Germany

∞*Pareekshak HIV-1/2 Triline Card* (Bhat Biotech, Bangalore, India); *Insti HIV-1 RT* (BioLytical Laboratories, British Columbia, Canada); *Smart Check* (Globalmed, Vancouver, USA); *First response HIV card 1-2.0* (Premier Medical Corporation, Daman, India); *Determine™ HIV- 1/2* (Abbott Laboratories, Illinois, USA)

†Fourth generation *ELISA HIV-1/HIV-2 III Plus, IMx System* (Abbott Diagnostics Division, Wiesbaden, Germany

▪*UniGold (*Trinity Biotech, Co Wicklow, Ireland)

2.2.2.3 STATISTICAL ANALYSIS

The statistical analyses were essentially the same as those conducted in section 2.2.1.4. The sensitivity, specificity, positive and negative LR of the DC Ab and p24

Ag component were calculated to determine the ages and clinical scenarios in which the DC test may add value to clinical practice.

2.2.3 SECTION 3: p24 Ag HEAT DENATURATION STUDY

Maternal and paediatric samples were used to investigate whether heat denaturation would improve the sensitivity of p24 Ag detection by the DC test. Using a technique described by Schupbach [15], samples were boiled to dissociate the HIV Ab and p24 Ag immune complex and enable detection of free p24 Ag [15]. Although the requirement for heat denaturation is not ideal for a point of care test, boiling of samples could conceivably be achieved in resource-constrained environments if p24 Ag detection was increased by this method and thereby aid the diagnosis of acute infection in the field.

2.2.3.1 SAMPLE SIZE

A convenience sample of six adult and nine infant samples from HIV-infected individuals were retrieved from storage for this exploratory study.

2.2.3.2 HIV TESTING PROCEDURE

Samples from HIV-infected women and children, known to be HIV Ab positive, were retrieved from -80°C storage and thawed at room temperature. The samples had

tested positive on the Acon and Advanced Quality rapid tests (Figure 2.3) and on laboratory HIV ELISA tests (Table 2.3) respectively.

The reference standard used for detection of p24 Ag was the high positive quality assurance control from the HIV p24 Ag kit (Preci Control PC HIV-3 Roche Diagnostics GmbH, Mannheim, Germany) which was used to measure the accuracy of HIV p24 Ag detection in an automated ELISA test. The control was tested neat on the DC test prior to boiling and subsequently boiled undiluted and in 1:3 and 1:6 dilutions for testing on DC to assess the sensitivity of the DC p24 Ag component.

Heat denaturation in maternal samples was investigated in dilution. Distilled water was used to make 1:3 and 1:6 dilutions of maternal plasma in 1.5ml Eppendorf tubes. The serum and plasma samples from children were not diluted as there was insufficient volume for multiple tests. Samples from mothers and children were boiled in a water bath at 100°C for 5-7 minutes to disrupt the HIV Ab and p24 Ag immune complexes and cooled on the bench for 10 minutes [15]. Thereafter 50µl of the heat denatured samples were pipetted onto the sample pad of the DC rapid test strip and the result was read within 20 minutes.

2.3 RESULTS

2.3.1 SECTION 1: MATERNAL DC STUDY

Of the 1099 pregnant and postpartum women that registered for care at RMMCH over the six month study period March to August 2010, 1019 (92.7%) participated in the study. Enrolment of the original sample size of 1450 women including 200 HIV-infected women was therefore not achieved. This resulted from a policy change at RMMCH during the study period to down refer antenatal cases to clinics. Therefore, most women were tested postpartum and had a lower HIV prevalence rate than women tested antenatally.

Of the 1019 participants, 345 (33.9%) were pregnant and 674 (66.1%) were postpartum. Ninety of the 1019 patients (8.8%) tested HIV Ab positive on the routine third generation rapid tests. Among these 90 HIV positives, 59 (65.5%) were pregnant and 31 (34.4%) were postpartum. The antenatal and postpartum HIV prevalence was 17.1% and 4.6% respectively.

The DC rapid test was performed on plasma samples of all 1019 participants and on whole blood samples on a subset of 380 women. A total of 1399 valid test results were obtained as the positive control was visible on all the test strips.

The overall sensitivity of DC HIV Ab component was 100% in the antenatal and postpartum populations on plasma and whole blood (Table 2.4). This parameter may have been affected by the reduced sample size of 90 instead of 200 patients [40] and it is possible that enrolling more patients may have reduced the sensitivity and/or narrowed the 95% confidence intervals.

The specificity of DC HIV Ab was 100% (CI: 95.9-100%) on whole blood and plasma in the antenatal population but lower at 98.9% (CI: 96.4-99.7%) on whole blood and 99.6% (CI: 98.8-99.9%) on plasma in the postpartum population (Table 2.4).

There were a total of three women with false positive results on the DC assay. All were in the postpartum group and all false positive results occurred in both plasma and whole blood samples. The Acon and Advanced quality tests for these three women were negative whereas DC was positive for HIV Ab (n=2) and p24 antigen (n=1). Results from the laboratory fourth generation ELISA, laboratory p24 Ag assay and viral load test results confirmed that the patients were HIV uninfected. Not a single new or early HIV-infection was detected considering that the DC would provide a major advantage over other rapid tests by p24Ag detection. The DC p24 Ag band was not detected in any of the 90 HIV-infected women tested nor in any previously uninfected women tested.

Table 2.4: Performance of the fourth generation DC on whole blood and plasma from pregnant and postpartum women [42].

	sample type	N	Prevalence	DCAb+/ HIV+	Sensitivity% (95% CI)	95% LR+	DCAb-/ HIV-	Specificity% (95% CI)	95% LR-
Antenatal									
	plasma	345	17.1%	59/59	100(93.8-100)	569.2	286/286	100(98.6-100)	0
	whole blood	151	-	59/59	100 (93.8-100)	184.5	92/92	100(95.9-100)	0
Postnatal									
	plasma	674	4.6%	33/31*	100(88.9-100)	321.5	641/643	99.6(98.9-99.9)	0
	whole blood	229	-	32/30*	100(88.6-100)	99.5	197/199	98.9(96.4-99.7)	0

DC= Determine Combo rapid HIV test; Ab = HIV Antibodies; LR+ and LR- = Positive and Negative Likelihood ratios

*False positives were obtained in two women on both sample types

The third generation and fourth generation rapid HIV tests performed equally in diagnosing 31 (4.6%) HIV positive cases in these 674 women (Table 2.5). There were 505 postpartum women who had tested HIV negative 6 -12 weeks prior to testing in this study (Table 2.5). Of the 505, 12(2.4%) were HIV positive upon retesting. This reflects an incidence rate of 2.4% over the 12 week period. Despite these new infections occurring in the postpartum population, the DC test did not detect any new HIV infections i.e. no DC test returned a positive p24 Ag and negative Ab DC test result. Of the 674 postpartum women that participated in the study, 4 women reported being HIV infected but had no documented evidence of HIV testing. Half of the women reporting a positive HIV status (without documentation)

were HIV uninfected. A much higher seroprevalence in the 'unknown HIV status' category was documented as 24% of women were HIV-infected [42].

Table 2.5: HIV status of postpartum women prior to enrolment and after testing with the National testing algorithm and the DC test [42].

Reported or documented HIV status	*n*	*HIV-infected*	*HIV-uninfected*	*% Positive*
Tested HIV negative 6-12 weeks before the study	505	12	492	2.4
Tested HIV negative >12 weeks before the study	107	3	104	2.8
Unknown HIV status	58	14	43	24.1
Positive: reported but not documented	4	2	2	50.0
Total	*674*	*31*	*641*	*4.6*

2.3.2 SECTION 2: PAEDIATRIC DC STUDY

Plasma samples from 202 infants, 182 (90%) HIV-exposed and 20 (10%) HIV-unexposed, were tested using DC. Of the 182 HIV-exposed infants, 121 were HIV-uninfected and 61 (33.5%) were HIV-infected with documented HIV ELISA and DNA PCR results. The HIV prevalence in this study of 202 infants was 30.2% (Table 2.6).

The HIV Ab component of DC demonstrated 100% specificity as no false positive results were obtained in the samples from the HIV-unexposed children born to HIV-uninfected mothers (Table 2.6).

The sensitivity of the DC Ab component varied across the age categories. DC achieved 100% sensitivity in detecting HIV Ab in infants younger than six months old (Table 2.6).

In contrast, a lower DC Ab sensitivity was obtained in infants older than 6 months because the DC Ab test results were inconsistent with the third and fourth generation laboratory HIV ELISA results in four cases. The DC Ab test failed to detect two HIV-infected children viz. a 6.9-month old symptomatic infant and a 22.7-month old asymptomatic child. Additionally, the DC Ab test failed to detect two 7-month old HIV-exposed, uninfected infants possibly because they were in the process of seroreverting [46].

Table 2.6: The performance of the HIV Ab component of DC rapid test in HIV-exposed and HIV-unexposed infants. DC diagnosed HIV exposure in 64 infants younger than six months of age, of which seven were clinically symptomatic resulting in 100 %sensitivity [46].

		STUDY POPULATION				DC HIV ANTIBODY RAPID TEST RESULTS					
		HIV-exposed, uninfected		HIV-exposed, infected	HIV-unexposed, uninfected						
Age (mo)	n	ELISA+ PCR-	ELISA- PCR- *	ELISA+ PCR+[a] (%)	ELISA- PCR-	DC Ab+/ ELISA+	Sensitivity % [95% CI]	LR+	DC Ab- / ELISA-	Specificity % [95% CI]	LR-
≤ 6	79	43	0	21 [7] (26.6)	15	64/64	100 [94.3-100]	30.7	15/15	100 [79.6-100]	0.00
>6 - ≤18	76	33	31	10 [6] (13.2)	2	40/43	93.0 [81.4-97.6]	62.3	33/33	100 [89.6-100]	0.07
>18	47	0	14	30 [22] (63.8)	3	29/30	96.7 [83.3-99.4]	33.8	17/17	100 [81.6-100]	0.03
Total	**202**	**76**	**45**	**61[35] (30.2)**	**20**	**133/137**	**97.1 [83.3-99.4]**		**65/65**	**100 [81.6-100]**	

Ab = Antibodies, DC = Determine Combo, LR = Likelihood Ratio, + = Positive, - = Negative

Superscript denotes number of symptomatic children (CDC clinic stage B and C), [a](%) denotes prevalence;

*Children in the HIV-exposed, uninfected category with negative ELISA results had undergone seroreversion.

The DC detected p24 Ag in just one of the 61 HIV-infected infants tested. This three month old infant was clinically symptomatic. The sensitivity of DC p24 Ag was 1.7% (95% CI 0.3-8.9%). Twelve other HIV-infected infants younger than 18 months were clinically symptomatic but none tested p24 Ag positive on DC. This finding suggests the hypothesis that DC would be more likely to detect p24 Ag in advanced stages in infant HIV infection is not true.

No false positive p24 Ag results were obtained resulting in 100% (95% CI 97.3-100%) specificity.

The LRs were calculated separately for the three age categories (Table 2.6). The formula provided in the methods section would result in an undefined result due to the 100% specificity which leads to a denominator of zero for all the age groups. The LR values were recalculated by replacing zero as the number of false positive and false negative tests by 0.5 to create an understanding of a pretest probability that could assist in the assessment and interpretation of the DC test which could be useful to clinicians that need to interpret the test. The high positive LR and low negative LR demonstrated that the HIV Ab component of DC was reliable for exclusion of HIV Ab detection in HIV-exposed or infected infants.

Twenty seven samples (13%) from the 202 infants tested yielded invalid DC results because there was no control band on the DC test strip. These samples were retested and valid test results obtained which were used in the analysis.

2.3.3 SECTION 3: p24 AG HEAT DENATURATION STUDY

The DC test detected HIV Ab in all 15 undiluted samples from HIV-infected mothers (n=6) and children (n=9) prior to boiling. The 1:3 diluted heat denatured maternal samples yielded 4 invalid tests and two HIV Ab false negative results. Of the five heat denatured samples tested in 1:6 sample dilution, two tests were invalid, two tests were HIV Ab false negative and only one was HIV Ab positive. Of the nine undiluted heat denatured samples from children, six DC tests were invalid and three were DC HIV Ab false negative.

No patient samples tested DC p24 Ag positive, prior to or after heat denaturation.

The high positive control from the p24 Ag Preci Control kit tested positive on the DC p24 Ag strip in the neat and the heat denatured 1:3 and 1:6 diluted samples proving the test kit's capability to detect p24 Ag.

Twenty DC test strips were used with denatured samples of which 12 (60%) were invalid.

Heat denaturation rendered all tests except one either invalid or false negative for HIV Ab and did not improve detection of HIV p24 Ag.

2.4 DISCUSSION

2.4.1 SECTION 1: MATERNAL DC STUDY

The sample of women enrolled in the study is likely to be representative of the population seeking care at RMMCH as 92.7% of women attending antenatal and postpartum care over the six month period agreed to participate. The HIV prevalence of 17.1% and 4.6% in women testing antenatally and postpartum respectively is similar to a previous description at RMMCH in 2008 [26]. The high HIV prevalence rates amongst pregnant women continues to put children at risk of contracting HIV infection.

The fourth generation DC rapid test achieved equal sensitivity in detecting HIV Ab in pregnant and postpartum women in comparison to the reference Advanced Quality and Acon third generation rapid tests. The specificity of the DC assay in excluding the presence of HIV Ab was slightly reduced in whole blood and plasma in postpartum women in comparison to women tested antenatally, although still within the WHO recommended limits of a specificity of >98% [47]. Two false positive results were obtained in the postpartum population. False positive HIV Ab test results during pregnancy or shortly thereafter are potentially caused by the cross reactivity between HIV Ag and non-specific Ab that are produced by a stimulated immune system [48]. In order to maintain diagnostic accuracy and reduce the possibility of false positives, it has been recommended that pregnant and postpartum women are tested with two rapid tests at the same time (parallel testing) in the field

[49]. South Africa presently implements a rapid testing system in which a rapid test has to be positive before a second rapid test can be done (serial testing).The DC Ab test is sensitive enough for use as an initial screening test but less so as a confirmatory second test in the National testing algorithm, because it is less specific than currently used third generation tests.

In this study, DC demonstrated equal sensitivity on whole blood & plasma and all the DC test results were valid. A previous study reported that the sensitivity of DC was better on serum than on blood and that 16% of DC tests were invalid [5]. The difference in outcomes may be accounted for by the presence of EDTA anticoagulant in samples from this study which was not present in fingerstick samples. In resource limited settings the fingerstick sample collection method is more likely to be used for HIV testing in the field and findings in this study may over rate DC performance. DC was not assessed on finger stick blood as the study was initiated when there was limited literature available on the performance of DC. The study took place at a clinical site which routinely uses EDTA blood and plasma for rapid HIV testing and was the site that would give DC the best chance for assessing performance. It was logistically impractical and unethical to prick the patient twice for blood.

It was found that 2.4% of 505 postpartum women who reported an HIV negative status an average of nine weeks prior to repeat testing, had seroconverted within this period. This seroconversion rate is less than the 4.5% rate that was described previously for this site over a similar time period of five and a half months [26].

Other studies support the finding of incident infections in pregnant and postpartum women and draw attention to the problem of newly acquired infections in mothers that fuel MTCT in infants [31, 50-52]. A study of pregnant women in Botswana demonstrated that incident cases of maternal HIV infection accounted for 43% of infant HIV infection because these women were not identified to receive PMTCT [50]. Unless acute infections in women can be detected as part of a PMTCT strategy, it may be difficult to attain the global plan that aims for a 90% reduction of new childhood infections and a 50% reduction in maternal deaths by the year 2015[2]. An improvement in the sensitivity of the DC p24 Ag could aid the detection of early maternal HIV infection and thereby potentially curb the spread of HIV from mother to child. Laboratory plasma p24Ag testing or DNA PCR or HIV VL testing are used to verify recent infection in an ideal world but the costs of these of these tests are astronomical for low resource settings.

The seroconversion rate of 2.4% was an indication that new infections were occurring during pregnancy, yet no true positive p24 Ag positive cases were detected by the DC test. Aside from the poor sensitivity of the DC p24 Ag component, it may be that no women had an acute infection at the time of enrolment into this study which may be the reason why not a single true positive p24 Ag positive and HIV Ab negative case was found.

The false positive p24 Ag result found in this study is similar to the findings of a Malawian study of DC in which 14 false positive p24 Ag results were obtained among 838 HIV-uninfected persons [7], which infers that the finding of p24 Ag on DC

in the field would require confirmatory laboratory testing to ascertain and disclose to the patient their true HIV status.

Furthermore, in a French study of DC in which 200 HIV-infected patients were tested, two patients were in the seroconversion phase as confirmed by Western blot and laboratory fourth generation ELISA, but were p24 Ag negative on DC [5]. Another study reinforces that DC p24 Ag lacks sensitivity as no patients with p24 Ag <50pg/ml were detected by DC, and DC detected only 4 out of 9 patients with p24 Ag between 50-400pg/ml [53]. It was anticipated that DC p24 Ag would have reduced sensitivity as compared to laboratory viral detection assays, some of which are able to detect p24 Ag levels of 4-5pg/ml [53] considering that rapid tests are potentially less sensitive than laboratory based viral detection assays [6, 38]. Evidence from these reports reaffirm that the DC p24 Ag component lacks sensitivity for diagnosis of acute infection.

A limitation of this study was that the projected sample size of 200 HIV-infected women to be tested was not reached which may have affected the sensitivity and 95% CI reported here for DC Ab results. However, not reaching the CDC recommended sample size of infected women does not affect the evaluation of DC p24 Ag sensitivity in diagnosing acute infections, since the population of women required to test p24Ag sensitivity is HIV-uninfected women.

The fact that no new cases were found in the RMMCH population over half a year suggests that DC is not useful in our context for identifying acute infections. Furthermore, the DC test delivered one false positive p24 Ag and 2 false positive HIV Ab results thereby falsely diagnosing new and established infections, which was

less accurate than the performance of the third generation rapid tests presently used for screening at point of care.

2.4.2 SECTION 2: PAEDIATRIC DC STUDY

Studies have been conducted to determine the most appropriate age at which rapid tests can be used to effectively diagnose HIV-exposure and HIV infection in infants [44, 54, 55]. Currently available third generation rapid tests are practical for diagnosing HIV-exposure in infants younger than 18 months of age and for diagnosing HIV-infection in older children. The DC test is the first rapid test that broadens the diagnostic spectrum with the potential to diagnose HIV infection in children of all ages.

This is the first study to evaluate a fourth generation rapid test to diagnose HIV infection in infants and children. After development DC was evaluated at 9 clinical laboratory sites by the manufacturer using commercial seroconversion panels and samples from adults. This study was important for assessing the performance of the DC test in children, a research priority recommended by the 2008 WHO infant diagnosis guidelines [4].

Study samples were selected to emulate the clinical scenarios in which infants present to healthcare workers at point of care. Irrespective of the clinical status, DC would not aid clinicians in the field in diagnosing acute infection in infants and children as it failed to detect p24 Ag in 98% of the HIV-infected infants in this study. A previous study undertaken in the laboratory on infants aged <3 months and of unknown clinical status had similar findings of 1 out of 40 HIV-infected infants testing

p24 Ag positive [39]. The poor sensitivity of DC p24 Ag in samples from children is comparable to the p24 Ag sensitivity of DC on adult samples [7, 56-58].

The DC HIV Ab component is comparable to third generation HIV rapid tests for use at point of care to screen for HIV-exposure as the test is 100% sensitive in detecting HIV Ab in infants younger than 6 months old. However, the reduced sensitivity of DC HIV Ab in infants >6-≤18 months of age as well at >18 months of age as described in our study falls short of the WHO recommended sensitivity of 99% required of rapid tests to be used as a screening tool [47].

Two HIV-exposed, HIV-uninfected patients who were likely in the process of seroreversion contributed to the reduced sensitivity of DC in detecting HIV Ab. The two 7 month old infants had detectable maternal HIV Ab on laboratory ELISA tests but none on DC. The gold standard HIV PCR result confirmed that the infants were HIV-uninfected.

The DC Ab component failed to identify two HIV-infected infants. The implication of a misdiagnosis in the field by a rapid test is that the infants would remain at risk of HIV-related morbidity and mortality despite having made contact with health care services.

The development of a dipstick p24 Ag assay for earlier detection of infection in infants is reportedly 96% (95% CI 88-99) sensitive and 99% specific (95% CI 98-100) in laboratory evaluations [28]. Prospective improvements to the sensitivity of the lateral flow assays and prototype units that aid the heat shocking process at point of care [28] could augment early infant diagnosis.

Limitations of this study include the small number of children tested in each category and the use of samples that were stored for a prolonged period of time. However, samples were suitable for this study despite the period of storage as viral p24 Ag is reportedly stable in samples stored for 10 years [59]. The study samples appear to have undergone no or minimal sample degradation considering that HIV Ab was detected in 133 of 137 cases (97%) [46] .

In this study of stored serum and plasma samples, 13% of DC tests were invalid. Other studies reported that invalid DC tests were obtained on finger-stick blood without anticoagulant [5, 57], but fewer invalid tests occurred when testing on serum [5]. Pavie suggests that the invalid tests are caused by high HIV Ab titres in the patient sample which consume the conjugate at the test line in the HIV Ab window [5]. The incidence of invalid DC tests in various studies suggests that the DC control band needs improvement and that serum should be used to test on DC to minimise the number of invalid tests.

The fourth generation DC test cannot be recommended for a definitive diagnosis of HIV- infection in children younger than 18 months. There are more sensitive rapid tests to use for detection of HIV-exposure in young infants and for diagnosis of HIV-infection in older children.

2.4.3 SECTION 3: p24 Ag HEAT DENATURATION STUDY

Boiling of diluted serum and plasma samples releases HIV p24 Ag from the immune complex that is formed with HIV Ab and permits the detection of free p24 Ag [15]. The DC test was evaluated using heat denatured samples in an exploratory study since boiling samples may be possible in some resource limited settings and such a method and test could be implemented and useful for the diagnosis of acute infection.

Plasma samples in this study coagulated to an egg white sticky consistency upon boiling as was the finding in Schupbach's study [15], making the sample difficult to test on the DC strip. Serum samples did not coagulate but had increased viscosity post boiling and therefore were not easily absorbed into the DC sample pad.

The DC HIV Ab component performed inconsistently in the heat denatured samples since HIV Ab that was detectable prior to boiling was destroyed in the heating process [15], hence the false negative DC HIV Ab results. Even so, in the absence of HIV Ab, the p24 Ag remained undetectable on DC. The fact that the DC detected p24 Ag in the control p24 Ag sample demonstrates that the DC rapid tests can detect p24 Ag but only in high concentrations. The DC p24Ag component of the test requires improved sensitivity to detect p24 Ag in the 50 microliters of blood used as sample input on the DC strip.

A limitation to the study was the small number of samples tested. The study was not extended to a larger sample set as 60% of DC tests were invalid, signifying that the boiling method was not feasible. The heat denaturation method used in our study did not increase p24 Ag detection in a rapid test and would not work in resource limited settings. A de-tuned ELISA or serological testing algorithm for recent HIV

seroconversion (STARHS) has been investigated in Australia for detection of recent seroconversion. The test has the potential to detect new infections if it can be implemented at point of care.

2.5 CONCLUSION

It is important that rapid HIV tests are evaluated in the laboratory and in the field before the tests are recommended for use. In this study, the DC HIV Ab component did not perform as well as the two South African Department of Health approved third generation rapid HIV tests recommended for use in women and children. The DC p24 Ag lacked sensitivity in the field and the laboratory for diagnosing acute HIV infection in mothers and definitively determining a positive HIV status in infected children less than 18 months of age respectively . The sensitivity of the DC p24 Ag could not be improved by a heat dissociation method.

The fourth generation DC test does not offer an advantage over the currently available third generation rapid HIV tests for maternal and child health.

CHAPTER 3

VIRAL LOAD STUDY ON DBS

3.1 INTRODUCTION

Substantial progress has been made in managing HIV. Eight million people globally and 2.3 million more people in sub-Saharan Africa have gained access to life saving ART between 2010 and 2012. South Africa's scaled up treatment program has initiated a further 75% of HIV infected people onto ART over this two year period to reach 1.7 million people. [60].

The expansion of access to ART requires adequate laboratory infrastructure to facilitate HIV VL testing. Quantification of VL is standard of care in the management of adult and paediatric populations [61]. VL tests are the WHO preferred test for confirmation of an HIV- infected diagnosis in infants that have been identified as being HIV-exposed [11], as well as for the monitoring of VL therapy and virological failure for those already on treatment [62]. Resource constrained settings face many challenges in providing extensive VL testing in spite of bearing the major portion of the HIV diseased populations. Some of the challenges of expansion of testing for resource limited settings are related to the cost of developing a laboratory testing site and the purchase of equipment; the training and availability of qualified laboratory staff and the difficulties in accessing technical support for troubleshooting[63]. Further challenges in some areas are the lack of physical

resources such as electricity, clean water and refrigeration facilities that are necessary for storage of samples and reagents.

Presently, VL testing is conducted predominantly on plasma in South Africa. A VL test quantifies the number of HIV RNA copies in 1 ml of plasma [64]. Whole blood collected by the phlebotomist needs to be centrifuged so that the separated plasma can be tested within 6 hours of sample collection or be refrigerated during transportation to the testing site. Rural areas are often less equipped with tools required for plasma sample preparation, storage or transportation. Plasma Preparation tubes (PPT) are an alternative to EDTA blood collection as they do not require plasma separation prior to transportation to the testing site. A disadvantage is that the PPT tubes do require cool transportation which is a limitation in resource poor settings [47]. DBS have been proposed as a less costly, practical alternative sample type to plasma for quantification of VL [8, 65-69]. Whole blood is spotted on to filter paper from a finger or heel prick eliminating the need for skilled venipuncture. The filter paper is air dried, stored and transported for testing in a sealed package containing a desiccant sachet. Studies have investigated the stability of HIV RNA at varying temperatures. One study reported a loss of RNA at 0.026 log copies/ml when DBS were stored at room temperature for 7 days [66]. A study of infant samples reported a decrease of more than 0.5 log copies/ml in DBS samples stored at 37°C for more than 3 months [70]. In contrast, HIV RNA in DBS samples used for infant diagnosis and HIV VL monitoring reported that RNA is stable for 10 weeks at room temperature [71]. A multicentre study of DBS described that HIV RNA was stable for one year in samples stored at room temperature [8], making DBS the preferred

specimen type for rural settings in which challenges such as refrigerated storage and cold transportation remain.

Dried blood spots have been used successfully in resource limited settings for early infant diagnosis of HIV [43, 70, 72] . The 2010 South African National PMTCT guidelines [73] recommended that all infants that test positive on the gold standard HIV DNA PCR test at 6-weeks weeks of age have a confirmatory VL assay on liquid blood at 10-weeks of age even though DBS are much more practical to collect, store and transport. Facilities that are able to submit a DBS for the 6 week infant HIV PCR screening test often lack phlebotomy skills required to submit liquid blood for the confirmatory VL assay [43].

Platforms for quantifying VL for monitoring were universally designed to test plasma but have been investigated for DBS VL testing [63]. DBS VL testing has been studied more extensively in samples from adults [8, 74-76] and in fewer studies on samples from children [70-72]. Studies of adult HIV-RNA document high correlation between VLs in plasma and DBS samples, [8, 74, 75, 77-80]. Investigations of the relationship between samples from children and adults and DBS VL are required to elucidate whether DBS VL performs differently in adults as compared to children.

3.1.1 VL TECHNOLOGIES

Viral load technologies from various manufacturers differ in the methods of nucleic acid extraction, amplification, detection of the target region using PCR. The PCR technique discovered by Kary Mullis in 1983 is a molecular biology method which

amplifies DNA into thousands or millions copies of the DNA sequence. [81]. The PCR quantitative application of PCR is used to determine VL. The principle of PCR is based on a chain reaction in which a DNA molecule is used to produce multiple copies of specific proteins known as polymerases (enzyme which binds individual DNA into a molecular strand). The PCR process comprises the denaturation, annealing and extension stages. In the denaturation step, DNA is denatured at temperatures from 90-97 degrees Celsius. Primers anneal to the DNA template strand in the annealing phase in which an enzyme "Taq polymerase" synthesizes two new strands of DNA at temperatures of 50-60 degrees celcius. In the extension phase which takes place at 72 degrees celcius, annealed primers create a complementary strand which is an identical copy of the DNA template resulting in doubling of DNA quantity through the PCR cycle. A major development of PCR is the real time reverse transcription of PCR which enables detection and quantification of the products of the PCR process by the use of an oligonucliotide probe which was designed to hybridize within the target sequence. The the real time reverse transcription of PCR used to determine VL [82] Three such VL technologies are the NucliSens EasyQ-EasyMag HIV-1 assay (bioMerieux,Boxtel, Netherlands) further referred to as the EasyQ, Abbott RealTime HIV-1 m2000sp/m2000rt (Abbott Molecular Inc, Des Plaines, USA) further referred to as m2000 and COBAS AmpliPrep/COBAS TaqMan version 2.0 docked system (Roche Molecular Systems, Branchburg, NJ) further referred to as CAP/CTM.

The EasyQ assay HIV RNA quantification is based on nucleic acid sequence based amplification (NASBA) technology. Total nucleic acids are extracted using a method

described by Boom *et al.* with amplification that is specific for the *gag* region of the HIV-1 target sequence. The NASBA technique allows for continuous amplification of nucleic acids at 41 degrees celcius [83].The m2000 and CAP/CTM platforms utilise reverse transcription PCR with real time amplification of the *pol* and *gag* regions respectively [47]. The m2000 comprises a m2000sp component for RNA extraction and m2000rt for amplification and detection. The CAP/CTM is composed of the AmpliPrep which performs automated sample extraction and the TaqMan which performs the amplification and RNA detection simultaneously ie a real-time assay.

In September 2010, the EasyQ platform in the South African public healthcare sector was replaced by the m2000 and CAP/CTM platforms for routine plasma VL testing. The VL measurements on any platform must be precise as decisions regarding ART are made according to longitudinal VL measurements and impacts patient management. Precision is the degree to which repeated measurements show the same result [84], irrespective of the operator, kit lot number, analyser or laboratory [85]. Repeated measurements that have the same value are deemed precise but may not be accurate [86]. Accuracy is defined as 'the closeness of agreement between a test measurement and a true value" [84]. The acceptable precision limits for VL in plasma is a coefficient of variation (CV) of <35% on the untransformed copies/ml value and a standard deviation (SD) of ≤0.19 copies/ml on the log transformed VL value as reported by the international Viral Quality Assurance (VQA) programme (Rush Presbyterian- St Luke's Medical Center, Chicago, IL) and two other studies [87, 88]. The SD and CV precision limits for DBS samples are not well documented.

Although VL testing on DBS is not yet approved for diagnostic use by the manufacturers of m2000 and CAP/CTM, studies to provide data on DBS VL testing are necessary in order to improve HIV testing programs in resource limited settings [77]. Presently, plasma VL results are utilised by clinicians to ascertain the baseline HIV RNA copies/ml prior to initiation of ART and determine the need to change therapy [89]. Baseline VL are expected to be high. As treatment progresses VL results are expected to reduce and therefore are markers of successful therapy [14]. An increase in 1log copies/ml VL is clinically relevant to alert the need for follow up and decision to switch therapy[62] The aim of ART is to maintain a VL below the lower limit of detection of the testing platform [90]. The lower limit of detection also known as a threshold is the point at which the minimal number of false positive results occur [91]. The DBS VL lower limit of detection varies according to the platform used and various VL lower limits of detection for DBS have been reported for the same platform [91].

A study conducted in Cameroon reports a 3 log copies/ml threshold using the m2000 platform [79] in comparison to the 3.72 log copies/ml threshold reported by Garrido *et al.* [76] on the m2000 and EasyQ platforms on DBS. The difference in the DBS VL threshold between 3.0 and 3.7 log copies/ml represents changes in VL copies of 1000 to 5000 copies/ml [91]. The 2013 WHO guidelines and South African treatment guidelines on the use of ART describe an increase in plasma VL of >1000 copies/ml (change of 3log copies/ml) over two consecutive measurements two months apart as virological failure [62, 89] in patients that had previous undetectable VL. The guidelines recommend a higher threshold at 3000-5000 copies/ml (3.5 - 3.7 log copies/ml) when testing on DBS [62]. For clinical decision making, one needs to be able to translate a DBS VL into a plasma VL along the continuum of care. Therefore

the relationship between DBS and plasma VL measurements need to be further elucidated in the South African setting if DBS are to be used for HIV-monitoring purposes in the future. The DBS VL limits of detection and VL precision are issues that remain poorly described [91].

Data on the specificity of DBS VL on m2000 and CAP/CTM would allow for an evidence-based decision on whether DBS could be substituted for plasma samples in VL testing for confirmation of a positive HIV infection status without using a threshold. The implication of a high specificity is that the platforms are accurate and could therefore potentially be utilised to diagnose HIV-infection.

A study of the EasyQ assay as a modality for early infant diagnosis in a South African subtype C population yielded 6/157 (3.8%) false positives and 96.2% specificity in DBS samples from infants younger than three months of age [72]. The study demonstrated that using a threshold of >4 log (10000 copies/ml) to diagnose HIV infection minimized the number of false positive results. The danger of a low false positive confirmatory VL result is that an HIV-uninfected infant could be erroneously commenced on life-long ART [72]. However, if the specificity of the m2000 and CAP/CTM VL assays on DBS exceed 98%, [11] then the platforms could be used with confidence by clinicians for diagnosis. A high specificity reaffirms that the assays are HIV RNA specific and therefore would not detect VL in HIV-uninfected persons [85].

A potential complication for using a DBS sample for the m2000 assay is that the work done to date has required the use of two spots (±100ul) per DBS card [75, 77-79]. In routine practice, only three spots per DBS card are generally collected from infants leaving too little sample should a second VL test be required e.g. in the event

of failure of test controls where the test sample would need to be retested. In this study, the m2000 platform was also assessed on one spot (± 50ul) with a view to enabling easier implementation of DBS testing.

The aim of this study was to evaluate the performance characteristics of DBS VL testing on the CAP/CTM and m2000 technologies to assess whether DBS can be used to confirm a diagnosis of HIV-infection and for VL monitoring. Due to the scarcity of infant blood samples with sufficient volumes for routine and research testing, adult samples were included in the study. The findings would establish whether adult DBS VL results could be generalised to children using the platforms currently in use in South Africa.

The objectives were to assess DBS VL by:

1) *Establishing DBS VL accuracy*
    - By comparing the VL results of HIV-infected children and adults obtained on DBS and plasma (gold standard) on the CAP/CTM and m2000 platforms
    - By ascertaining the specificity of DBS VL on the CAP/CTM and m2000 platforms using DBS from HIV-exposed, uninfected infants

2) Establishing DBS *precision limits*
    - intra-assay precision (repeatability) by replicate testing of DBS VL on the same sample by a single operator, on the same day, using the same reagents and equipment.

- inter-assay precision (reproducibility) of DBS VL which measures whether the test result of the same sample, could be replicated by another operator at different time points.

3) Establishing the accuracy and precision of the m2000 using 1 DBS spot versus 2 DBS spots.

3.2 MATERIALS AND METHODS

3.2.1 STUDY LAYOUT AND SAMPLES

A prospective study of DBS VL accuracy and precision limit on the CAP/CTM and m2000 platforms were conducted on 10 adult and 10 children per platform (Figure 3.1). DBS were made from EDTA blood collected from HIV-infected adults attending a wellness clinic, whereas DBS from children were made from EDTA samples submitted to Charlotte Maxeke Johannesburg Academic Hospital's (CMJAH) PCR lab for VL testing. Plasma derived from the EDTA blood was tested for VL on both platforms. The plasma VL result was the gold standard used to assign patient DBS samples into five stratified VL categories namely, undetectable, low (≤3 log), medium (>3log and ≤4 log), high (>4log and ≤5 log) and >5 log. Two patients were tested in each VL category (Figure 3.1). Furthermore, plasma VL in the undetectable category included samples in which the target was not detected and reflected as a zero plasma VL. Plasma samples and DBS with RNA below the detectable limit of 20 copies/ml on CAP/CTM and below 40 copies/ml on m2000 were reflected as having 19 copies/ml (1.3 log) for both platforms.

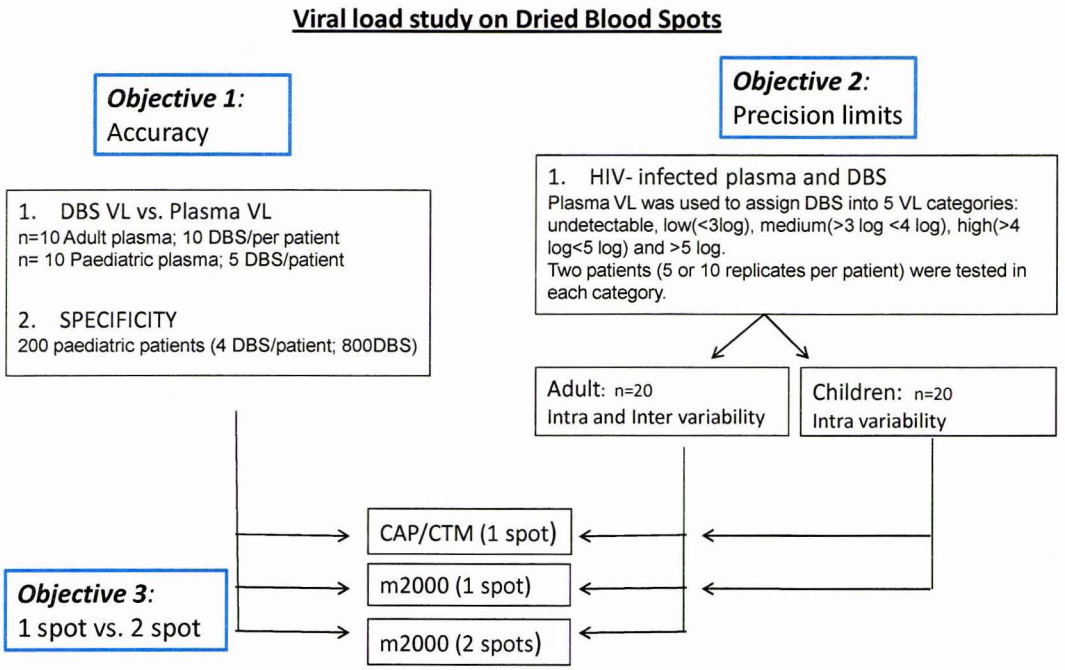


Figure 3.1: A flow diagram that describes the objectives and number of samples tested in the accuracy and precision study of DBS VL on the CAP/CTM and m2000 platforms.

The accuracy study assessed in five viral load categories, the difference between a single gold standard plasma VL result and multiple DBS VL results. Ten DBS VL results were obtained per adult and five DBS V results per child.

The study of DBS VL accuracy was extended to a study of specificity in which 800 stored DBS samples from 200 HIV-uninfected infants were used to ascertain the false positive rate on CAP/CTM and m2000 platforms. Two hundred VL tests were utilised to test 200 single DBS samples on CAP/CTM. Six hundred DBS were tested on the m2000 such that 200 DBS were tested in the one spot assay (using 200 tests) and 400 DBS were tested in the 2 spot assay (using 200 tests). In total, six hundred

assay tests were utilised to test 800 DBS between the two platforms to assess specificity (Figure 3.1).

The qualitative result (e.g. detectable or undetectable) from the DBS VL assays were compared to the gold standard negative HIV DNA PCR result .

The DBS VL precision study was conducted to establish the precision limits (SD and CV) of DBS on the CAP/CTM and m2000 platforms. Inter-assay and intra-assay precision studies were conducted on adult DBS, however only intra-assay precision was evaluated on DBS collected from children due to the limited amount of blood available from children (Figure 3.1).

No direct comparison of the VL data was made as the patients tested on the CAP/CTM platform were not the same patients that were tested on the m2000 platform.

Ethics approval (M091133) to conduct VL studies on adult and paediatric populations was granted by the Human Research Ethics Committee at the University of the Witwatersrand, Johannesburg (Appendix A).

3.2.1.1 DBS SAMPLE PREPARATION - ADULTS

Patients at the Helen Joseph hospital's Themba Lethu clinic were invited to participate in the study if they were HIV-infected, irrespective of their ART history, if

they were older than 18 years of age. Informed consent was obtained, one 5ml tube of EDTA blood was drawn, labelled with a study number without patient identifying details, packaged and transported to Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) within 6 hours of collection for DBS VL sample preparation and gold standard plasma VL testing. At CMJAH, Whatman 903 filter paper cards (GE Healthcare Bio-Sciences Corp. Westborough, MA, USA), each containing five pre-printed circles were labelled with a study number. The EDTA samples were mixed by gentle inversion before the DBS cards were spotted under a laminar flow unit. For the CAP/CTM platform, ten spots (2 cards) were prepared consisting of 75µl whole blood per spot (as per manufacturer suggestions). DBS for the m2000 were prepared by ten spots (2 cards) containing 50µl were made for the 1 spot evaluation. An additional twenty spots (4 cards) of 50µl blood were made for the m2000 2 spot assessment (as per manufacturer suggestions).

The DBS cards were labelled and left to dry at ambient temperature (20°C) in drying racks for a minimum of three hours before packaging individually into plastic zip-lock bags with desiccant sachets and stored at room temperature (20-25°C) .

The remaining EDTA blood was centrifuged and 1ml plasma aliquots were labelled and stored at -70°C until sufficient samples had been collected to commence batch testing. Plasma aliquots were subsequently defrosted at room temperature and tested on CAP/CTM using the COBAS Ampliprep/COBAS CAP/CTM HIV-1 test, version 2.0 kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Plasma

reference standard VL on the m2000 was obtained using the Abbott RealTime HIV-1 kit (Abbott Molecular Inc., Des Plaines, IL,USA).

3.2.1.2 DBS SAMPLE PREPARATION – CHILDREN

Whole blood EDTA-anticoagulated samples submitted for routine HIV VL testing at the PCR laboratory at CMJAH were intercepted at the sample receiving office and screened using the laboratory information system. Patients whose ages ranged from birth to 15 years and were identified to be HIV-infected using their previous VL records were selected for the DBS VL study. Blood from identified HIV-infected patients were anticipated to contain undetectable or low VL if the patients had accessed ART, but no clinical data was available on any of the patients to verify their ART status. Samples from paediatric patients that had no previous record of VL testing were also included as they were anticipated to have high VLs assuming that these were baseline VL samples submitted prior to the initiation of ART. DBS and whole blood for the study were prepared only if a minimum of 3ml EDTA blood had been submitted by the clinician so as not to compromise the sample volume required for routine testing.

For the study, one DBS card (consisting of 5 x 50µl spots) per patient was made, and an aliquot of 1200µl of whole blood was separated from the EDTA sample before the routine testing was performed. The DBS were packaged and stored with a desiccant sachet at room temperature after drying overnight.. The 1200µl EDTA blood was centrifuged to obtain plasma which was stored at -70°C. Plasma samples

were thawed in batches of 24 samples and tested either on the m2000 or CAP/CTM. The plasma VL results from each platform were used to assign DBS samples to that platform in the five VL categories. DBS were used to test intra-precision exclusively as the number of DBS that could be made were limited by sample volume and adequate samples could not be made from samples from children to incorporate inter-precision testing. The CAP/CTM and m2000 intra-precision was assessed on one 50ul spot in replicates of 5 per patient.

3.2.2 ACCURACY TESTING: STUDY DESIGN

The accuracy of DBS VL was measured in two ways:

Firstly, by comparing gold standard plasma VL to 10 DBS VL replicates for each of the two patients tested in the 5 VL categories. In paediatric patients, each plasma VL was paired to 5 replicate DBS VL.

Secondly, specificity was also measured. Stored DBS samples were sourced from a 2010 study in which DBS samples were prospectively collected from a well characterized cohort of 6-week old infants of known HIV-exposed, uninfected status, as determined by an HIV DNA PCR test [92]. DBS cards that had four filled spots per patient were selected to ensure that the same patient could be tested on both the CAP/CTM (using 1 spot) and m2000 platforms (1 spot and 2 spot)

3.2.2.1 CAP/CTM DBS VL ACCURACY TESTING

One DBS was excised under a safety hood using scissors that were swabbed with 2% bleach and 70 % ethanol and dried between samples. The spot was placed into the conical part of the sample processing unit using sterile forceps, and eluted for 10 minutes at 56°C during continuous shaking in 1ml of pre-extraction lysis reagent per the manufacturer's instructions. The sample pre-extraction procedure allowed for nucleic acid release from the white blood cells and stabilised the nucleic acids against enzyme degradation. The samples were subsequently loaded onto the AmpliPrep analyser in batches of 24 samples (21 samples and 3 controls). The COBAS AmpliPrep/COBAS TaqMan HIV-1 test, version 2.0 kit (Roche Molecular systems, Inc., Branchburg, NJ 08876 USA) and the COBAS Ampliprep/COBAS TaqMan HIV-1 test Dried Fluid spot protocol (HI2DFS96) was used for the HIV RNA extraction and detection for plasma and DBS respectively.

The CAP/CTM instrument used in this study was a docked system, with no need to manually transfer the test reaction from the AmpliPrep to the TaqMan as the system was fully automated after samples were loaded.

HIV-infected high positive, low positive and HIV-uninfected plasma quality controls as supplied in the test kits were included in every run on both platforms and the DBS VL results were only considered valid if all three control results were within the range specified by the manufacturer.

The DBS samples from children were processed and tested in the same way as the adult DBS samples on the CAP/CTM.

The CAP/CTM has a reportable range of 20 to 1.0E+7 HIV RNA copies/ml [93]

3.2.2.2 m2000 DBS VL ACCURACY TESTING

One 50ul spot for the adult intra- and inter-precision study and for the child intra-precision study was excised using a 12mm hand held punch. The punch was cleaned between patient samples by punching five spots from blank Whatman 903 DBS card using a method described by Driver [94]. Similarly, two DBS were cut for the comparison study of 1 spot VL versus 2 spot VL.

The DBS were placed in 50ml sterile conical shaped tubes to which 1.7ml sample preparation buffer was added and eluted after 20 minutes. The lysis buffer aided in the release of nucleic acid from the white blood cells. The 1.7ml lysate, excluding the DBS, was pipetted to 5ml reaction vessels and loaded onto the m2000sp system for RNA extraction. An internal control which reinforced non-inhibition of the real time PCR reaction was included in every sample tested. Additionally, three levels of plasma controls as supplied by the manufacturer, were included in every batch of testing. Study results were considered to be valid only if the controls were within range as described by the manufacturer.

The DBS VL were tested on the Open mode 1ml DBS HIV-1 RNA protocol application for 90 minutes. Post the 90 minute RNA extraction, a reaction plate containing HIV RNA eluate was manually transferred to the m2000rt for RNA amplification and detection. The m2000 assays detects VL in the range of 40 to 1.0E+7 HIV RNA copies/ml [95].

3.2.3 PRECISION TESTING: STUDY DESIGN:

3.2.3.1 CAP/CTM DBS VL PRECISION TESTING

The precision testing on samples from adults and children on the CAP/CTM platform was performed by a single operator on a sole analyser designated to this study in a high throughput laboratory. Intra-assay precision (repeatability) was measured by replicate testing of five DBS samples from each patient on the same day, within a single run using the same reagents and equipment. The inter-assay precision (reproducibility) was tested to evaluate if the DBS VL results could be replicated in separate test batches over five days. DBS samples were processed and were the same samples as described in the accuracy study in section 3.2.2.1

3.2.3.2 m2000 DBS VL PRECISION TESTING

The intra and inter precision limits of DBS from adults and children on the m2000 platform took place using the processes described for the CAP/CTM in section 3.2.3.1. The study of DBS VL accuracy and precision limits were conducted on the same sample set and were processed as described in section 3.2.2.2.

3.2.4 STATISTICAL ANALYSIS

Accuracy study: Bland Altman statistical plots [96] were used to assess the agreement between gold standard plasma VL results and the DBS VL method on log

transformed VL results. The difference between plasma and DBS VL was plotted on the Y-axis. The log values of gold standard plasma viral load (not the average between plasma and DBS as shown in conventional Bland Altman plots) were presented on the X-axis on the CAP/CTM and m2000 plots.

The bias was calculated as the mean paired difference between plasma and DBS VL. SD of the bias was a measure of the spread of values and was calculated by the square root of the variance. Variance was the average of the squared difference from the mean (average), and was calculated by ascertaining the mean of the data set. The mean was subtracted from each data point and the difference was squared. The CV was calculated as a percentage from the ratio of the SD to the mean and was a measure of the variability and stability of data. Adult and child populations for each platform were plotted separately.

The upper limit and lower limits of agreement were calculated as SD±1.96 multiplied by the mean to show the overall difference for 95% of the data. The distance over the limits of agreement was calculated as the difference between the upper and lower limits of agreement. The percentage of outliers represented the DBS VL measurements that were outside the limits of agreement [97]. The number and percentage of RNA copies/ml outliers for >0.5 log copies/ml, >1.0 log copies/ml, >2log copies/ml and >3log copies/ml were reported to ascertain the extent of the difference between DBS and plasma VL in each of the 5 VL categories.

The percentage similarity plots [97] were created to measure the agreement between plasma and DBS VL. To calculate percentage similarity the average between the gold standard plasma VL and DBS VL (both log copies/ml) was divided by gold standard plasma VL results and represented as a percentage of the gold standard. The comparison was visualised though a 100% similarity reference that is common to the m2000 and CAP/CTM plots [97]. The formula [({a+b}/2)/a] x 100 was used, where "a" was the reference plasma VL and "b" was the test method of DBS VL [97]. The mean percentage similarity values between plasma and DBS VL were calculated to be 100% if the VL in the two methods were the same. When DBS VL results were higher than plasma VL results, the mean percentage similarity was calculated as being greater than 100%. The converse of DBS VL being lower than plasma VL resulted in mean percentage similarity value calculation of less than 100%.

The specificity *(true negatives / (true negatives+ false positive)*, of the CAP/CTM and m2000 (1 and 2 spot) assays was calculated using HIV-DNA PCR results as the reference standard.

Study of precision limits: The DBS intra- and inter- assay precision limits were assessed by calculating the SD on log transformed VL values and the CV on the absolute VL values for the five VL categories. The SD and CV values were calculated as described in the accuracy study. Plasma VL results were the reference standard for intra- and inter- assay precision measurements. An SD value of ≤0.19 log copies/ml and a CV of ≤35% was considered acceptable [88]. The total assay SD

on intra- and inter-assay precision was calculated on adult samples using the formula: Total assay SD = $\sqrt{(intra\ SD)^2 + (inter\ SD)^2}$.

3.3 RESULTS

3.3.1 SAMPLE YIELD

DBS were stored at room temperature (20-25 degrees Celsius) for a six month period from sample collection to testing. Of the 400 prospectively made DBS from adults and children, 150 DBS spots were tested on CAP/CTM and 250 DBS on the m2000 platform. The CAP/CTM and m2000 assays yielded 149 (99.3%) and 195 (78%) valid results respectively. Of the 100 tests performed on 1 and 2 spot DBS from adults, 75(75%) and 77 (77%) valid results were obtained respectively. Several automation and technical errors were encountered, using the m2000 assay and are described in Table 3.1. The majority of errors on the m2000 assay occurred in adult samples as compared to paediatric samples.

Table 3.1: Number of valid tests and errors obtained on the DBS precision tests performed on the m2000 and CAP/CTM platforms.

		m2000 assay			CAP/CTM assay	
n	**error code**	**adult**		**child**	**adult**	**child**
		1 spot	**2 spot**	**1 spot**	**1 spot**	**1 spot**
n tests		100	100	50	100	50
valid results		84	83	43	100	49
errors	*invalid	0	0	0	0	1
	3118	6	3	0	0	0
	4442	10	14	3	0	0
	3131	0	0	2	0	0
	4457	0	0	2	0	0

*invalid: sample failed ; 3118: Clot limit passed error, 4442: internal control cycle number too high; 3131: more liquid than expected error; 4457: internal control failure

3.3.2 ACCURACY STUDY:

The Bland-Altman plots (Figures 3.2; 3.3; 3.5-3.7) represent the difference in VL between plasma and DBS in samples from children and adults.

3.3.2.1 CAP/CTM DBS VL

The CAP/CTM generates 3 types of VL results, namely 'target not detected'; 'target detected but not quantifiable' and 'target detected and quantifiable'. In this study of the CAP/CTM, 'target not detected' VL results obtained on plasma and DBS samples are reflected on the Bland-Altman plot as zero VL (Figure 3.3A) and in Figure 3.4 which compares DBS VL to plasma VL.

The lower limit of RNA detection on the CAP/CTM is ≤20 copies/ml and is resulted as ' target detected but not quantifiable'. Such DBS and plasma VL results were reflected on the Bland-Altman plot as 1.3 log copies/ml (19 copies/ml) (Figure 3.2A).

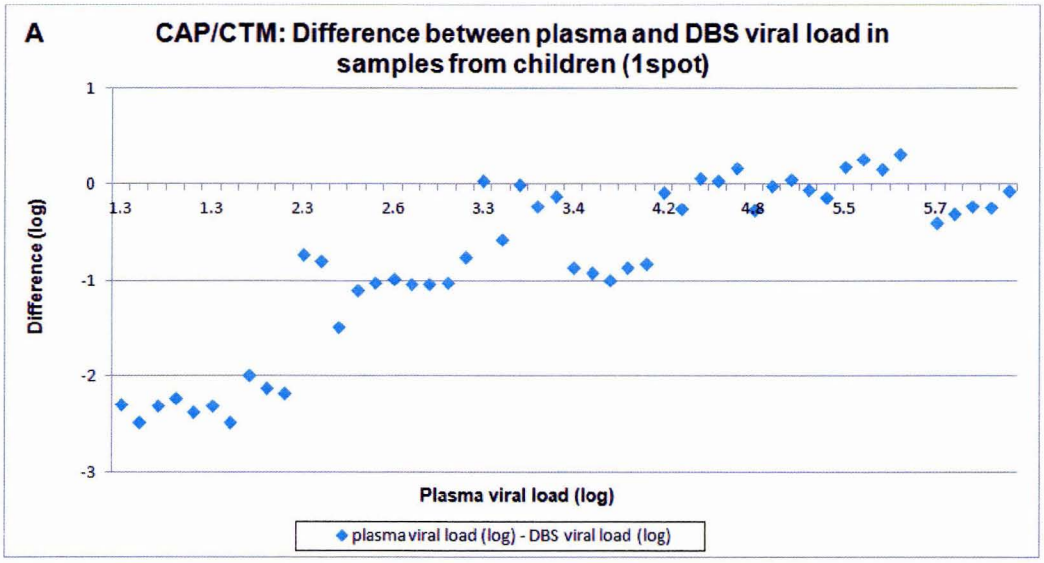
In both adult and paediatric samples, DBS VL was detectable and quantifiable where plasma VL was undetectable. DBS VL values were higher than plasma VL by 2 log copies/ml in 90% (9/10) of samples from children and by 3 log copies/ml in 70% (14/20) of adult samples. Additionally, DBS VL values were higher than plasma VL by >2 log copies/ml in the lower range (<3log, 1000 copies/ml) and lower than plasma VL values by <0.5 log copies/ml in the higher range (>5log, ≥185 000c/ml) (Figure 3.2 A and 3.3 A). DBS and plasma VL showed the least differences in percentage similarity closest to 100% at VL>4log copies/ml (Figure 3.2 B and 3.3 B).

Differences and outliers of >1000 copies/ml-between plasma and DBS VL occurred between plasma VL undetectable samples to plasma VL's of 4log (10 000 copies/ml) (Table 3.2). The difference of >1000 copies/ml is clinically significant in patients that previously had undetectable VL. Compared to plasma VL, DBS VL values were >0.5log higher in 53% (26/49) of specimens. Of these outliers, 16 DBS VL measurements had a difference of >1.0log in children (Table 3.2). Similarly in adults, DBS VL results were 0.5 log higher than plasma VL in 59% of (59/100) observations. The outliers included 42 DBS VL in which a difference of >1log was reported (Table 3.2). Differences of >2 log between plasma and DBS VL were obtained in 18% and 31% of child and adult DBS VL measurements respectively (Table 3.2) in the plasma VL <3log category (Figure 3.2 and 3.3). The scale on x-

axis of figure 3.2B and the subsequent plots represents the plasma VL from the ten patients tested.

Bias values close to zero were interpreted as favourable and a zero difference implied equality between the two methods [97]. In children, the bias of plasma and DBS VL measurements was closer to zero at >4logs and at >5logs in adults. The bias and disparity between plasma and DBS VL methods was largest at <3logs (Table 3.2).

The variability in the differences between plasma and DBS VL measurements was assessed by calculating the SD of the differences. The lower the SD of the differences, the lower the variance. High CV values were associated with the greater variability .



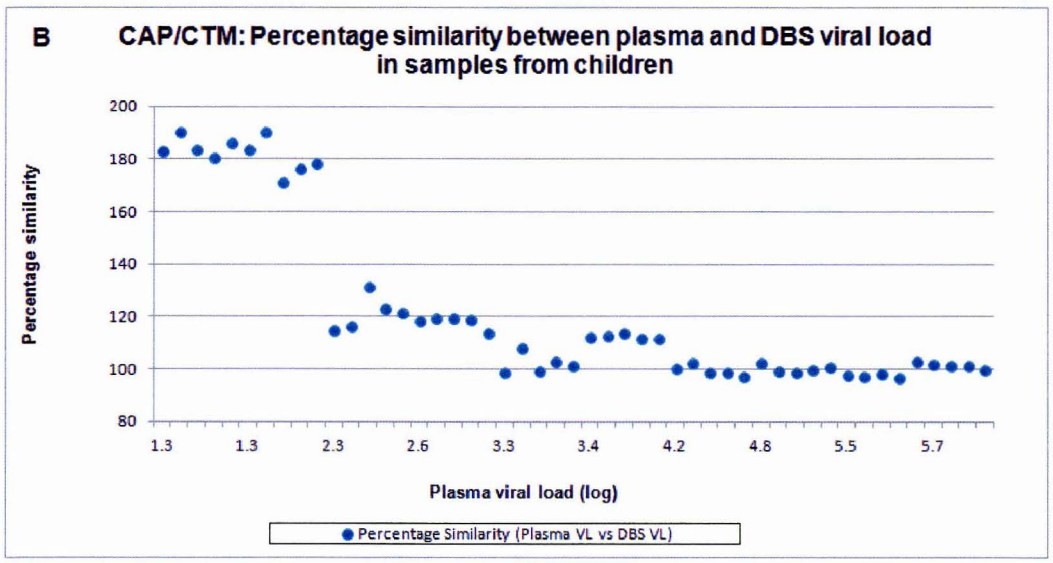
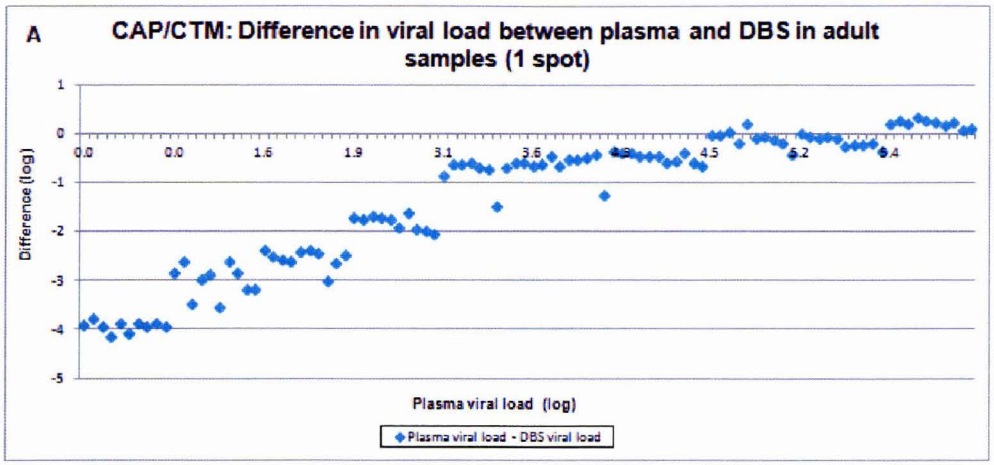


Figure 3.2: CAP/CTM on samples from children. *A:* Bland- Altman plot showing that the differences between plasma and DBS VL were larger at lower range of plasma viral loads platform. VL was detectable in DBS even where plasma VL was undetectable. *B*: DBS VL were similar to plasma VL at plasma VL measurements >4log.



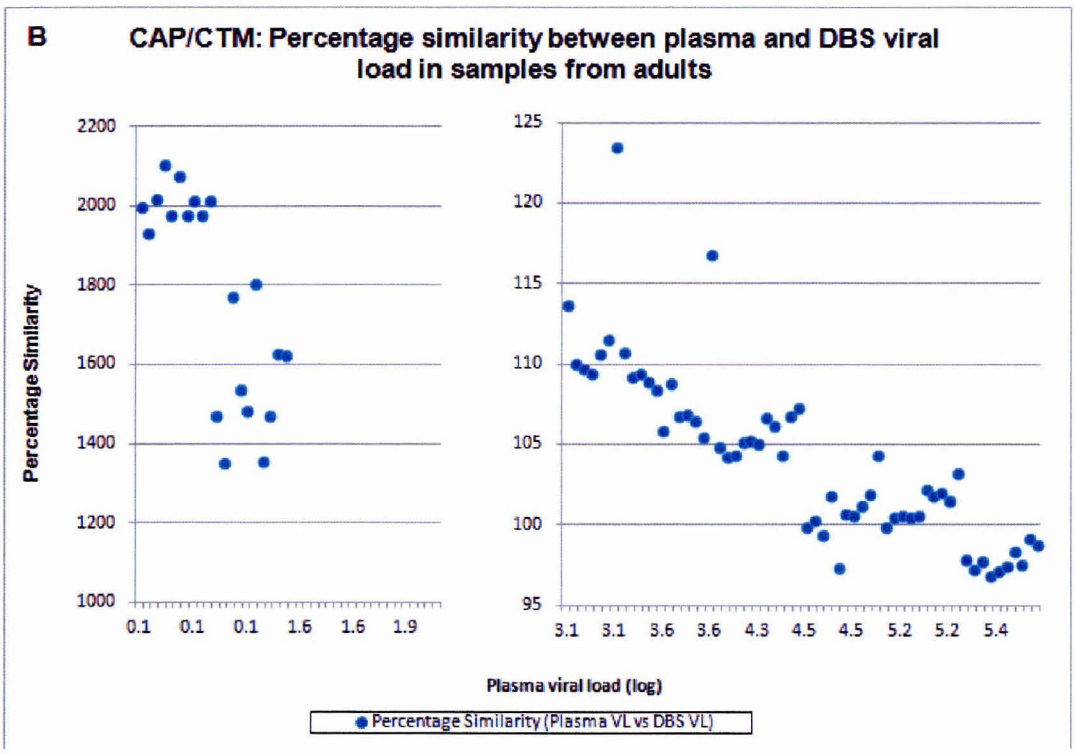


Figure 3.3: CAP/CTM: Difference between plasma and DBS VL in adults. ***A:*** The differences between plasma and DBS viral load were lowest at the higher range of plasma viral loads. VL was detectable in DBS even where plasma VL was undetectable. ***B:*** The percentage similarity between plasma and DBS VL was closer to 100% in the higher plasma VL.

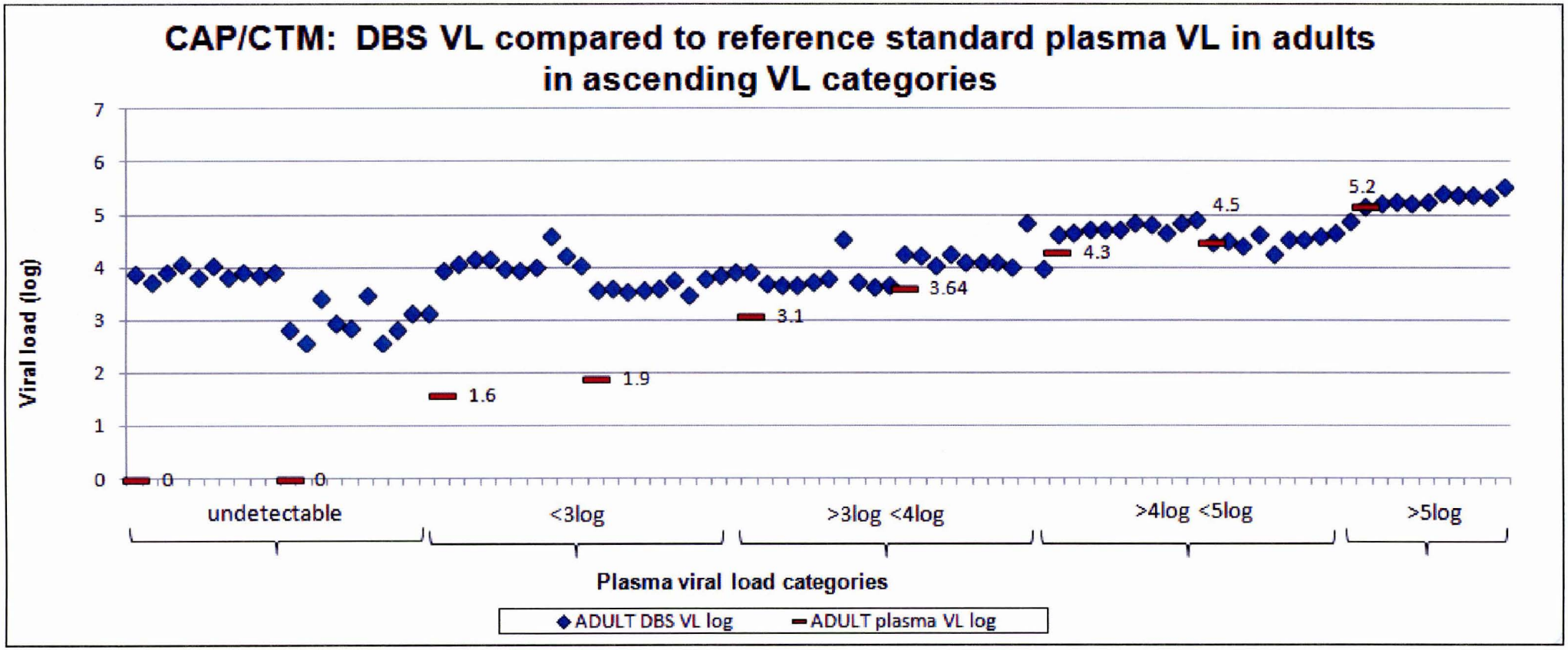


Figure 3.4: Dried blood spot VL as compared to plasma VL on CAP/CTM

Table 3.2: Bland-Atman and percent similarity statistical parameters for measuring DBS VL accuracy in adults and children on the CAP/CTM platform.

CAP/CTM: Bland-Altman Parameters										% Similarity		
						No. (%) of outliers						
Comparison	VL categories	Bias (log RNA copies/ml)	SD of difference (log RNA copies/ml)	Limits of agreement	Distance over limits (log RNA copies/ml)	>log 0.5 log RNA copies/ml	>log 1 log RNA copies/ml	>log 2 log RNA copies/ml	>log 3 log RNA copies/ml	Mean	SD	%CV
Plasma vs DBS (children)	undetectable	-2.25	0.15	-4.26; 4.57	-8.83	10/10 **(100)**	10/10 **(100)**	9/10 **(90)**	0/10 **(0)**	193.90	6.40	3.30
	≤3 log	-0.98	0.22	-1.70; 2.13	-3.82	10/10 **(100)**	6/10 **(60)**	0/10 **(0)**	0/10 **(0)**	120.09	4.95	4.12
	>3 - ≤4 log	-0.52	0.41	-0.60; 1.43	-2.03	6/10 **(60)**	0/10 **(0)**	0/10 **(0)**	0/10 **(0)**	107.63	6.03	5.60
	>4 - ≤5 log	-0.03	0.14	0.08; 0.19	-0.11	0/10 **(0)**	0/10 **(0)**	0/10 **(0)**	0/10 **(0)**	100.28	1.60	1.59
	>5 log	-0.02	0.27	11.25; -10.72	21.97	0/9 **(0)**	0/9 **(0)**	0/9 **(0)**	0/9 **(0)**	100.12	2.41	2.40
	summary	-0.77	0.87	0.94; -2.49	3.42	26/49(53)	16/49 (33)	9/49 (18)	0/49**(0)**	124.90	36.34	29.10
Plasma vs DBS (adult)	undetectable	-3.46	0.52	-6.26; 7.31	-13.57	20/20 **(100)**	20/20 **(100)**	20/20 **(100)**	14/20 **(70)**	*15640.30	5319.31	34.01
	≤3 log	-2.17	0.41	-3.83; 4.66	-8.50	20/20 **(100)**	20/20 **(100)**	11/20 **(55)**	1/20 **(5)**	163.30	17.15	10.50
	>3 - ≤4 log	-0.66	0.26	-1.02; 1.55	-2.58	15/20 **(75)**	2/20 **(10)**	0/20 **(0)**	0/20 **(0)**	109.91	4.28	3.89
	>4 - ≤5 log	-0.28	0.25	-0.30; 0.79	-1.09	4/20 **(20)**	0/20 **(0)**	0/20 **(0)**	0/20 **(0)**	103.21	2.85	2.76
	>5 log	0.04	0.21	0.30; 0.12	0.17	0/20 **(0)**	0/20 **(0)**	0/20 **(0)**	0/20 **(0)**	99.62	1.98	1.99
	summary	-1.30	1.37	1.38; -3.99	5.37	59/100 **(59)**	42/100 **(42)**	31/100 **(31)**	15/100**(15)**	119.01	27.46	23.07

*Mean percentage similarity is raised due to the presence of proviral DNA

3.3.2.2 m2000 DBS VL

Plasma VL measurements in which RNA was undetectable were matched by undetectable DBS VL demonstrating 100% mean percentage similarity between DBS and plasma in samples from children (Figure 3.5B) and adults (Figure 3.6B and 3.7B). VL was undetected in 39 DBS samples (n=9 paediatric and n=30 adult) of which VL was detectable in the corresponding plasma samples (Figure 3.5- 3.7) and 9). The false negative measurements appear as outliers on the points plot that depict differences of up to 4 log copies/ml between plasma and DBS VL (Figure 3.5A, 3.6A and 3.7A). DBS VL were not detected in samples below 4.5 log copies/ml resulting in the lowest mean percentage similarity for m2000 of 87.35% in children and 77.38% in adults (Table 3.3).

Compared to plasma viral loads DBS VL results were:

1) >0.5log higher in 49% (21/43) and >1.0log higher in 16% (7/43) of DBS replicates from children (Table 3.3).
2) >0.5log higher in 61% (51/83) and >1.0log higher in 22% (18/83) adult 1 spot DBS VL measurements (Table 3.3).
3) >0.5log higher in 39% (32/82) and >1.0log higher in 24% (20/82) adult 2 spot replicates (Table 3.3)

Overall, the DBS VL bias was greatest between 3-4log copies/ml in samples from adults and children.

DBS VL at >3log demonstrated higher VL from 2 spots as compared to one DBS spot (Figure 3.8).

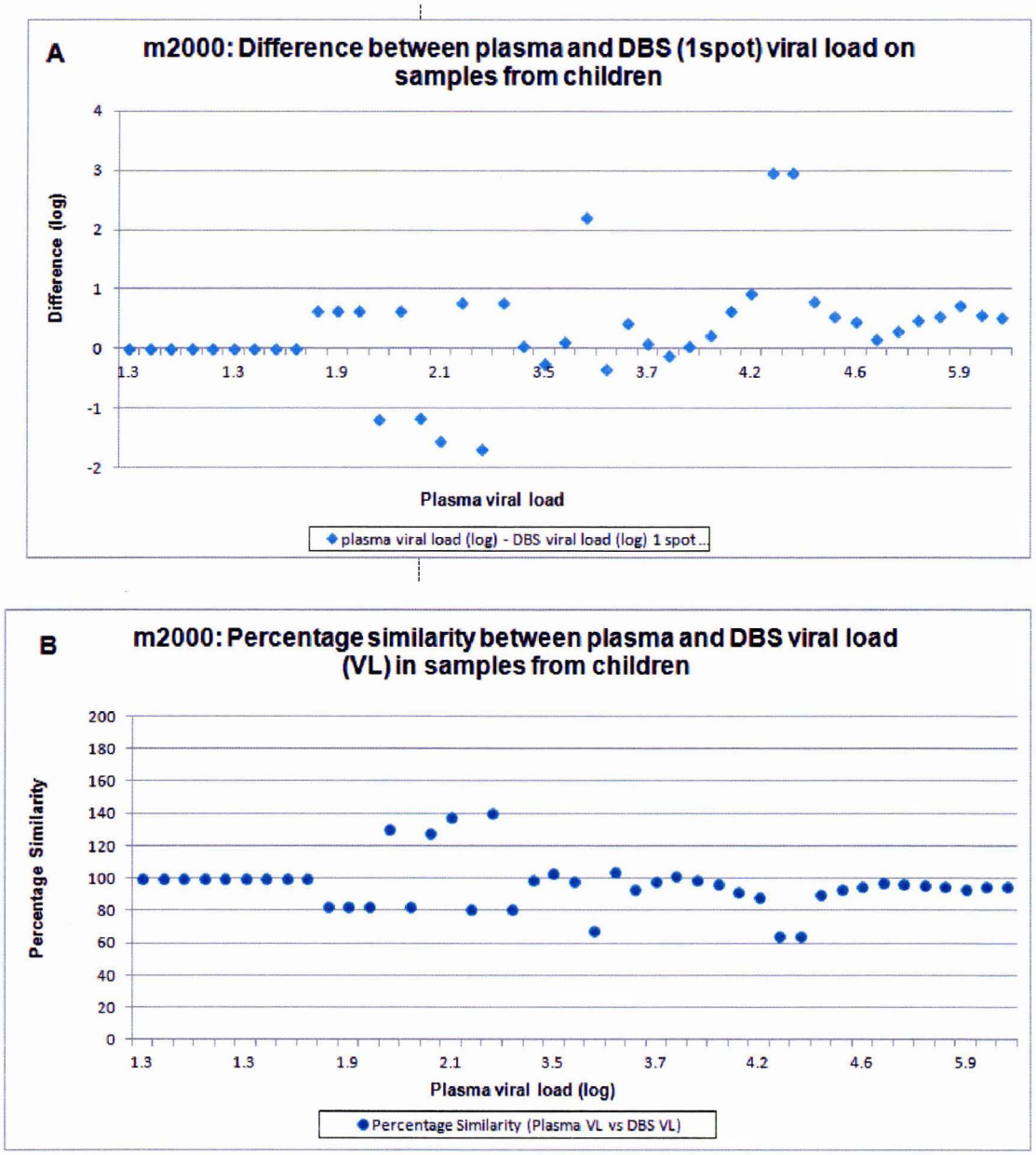
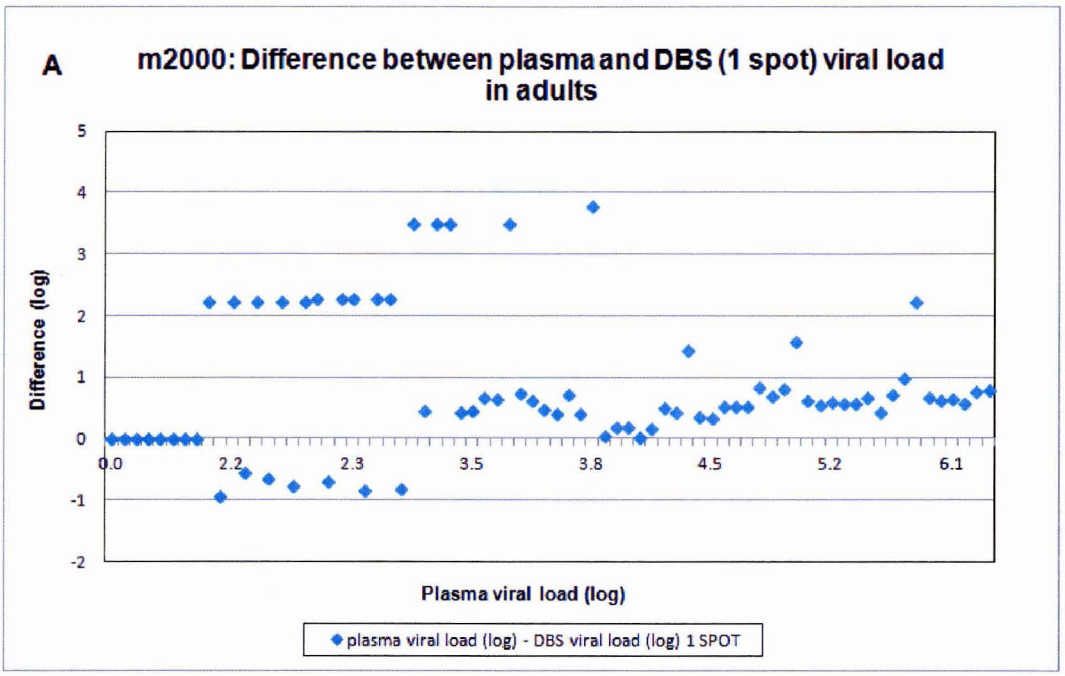


Figure 3.5: m2000: DBS and plasma VL in samples from children. ***A:***Bland-Altman plots depicting plasma and DBS VL differences are greatest between 1.5 and 4.5 logs. ***B:***Percentage similarity plots depict that similarity is the greatest when plasma VL are undetectable.



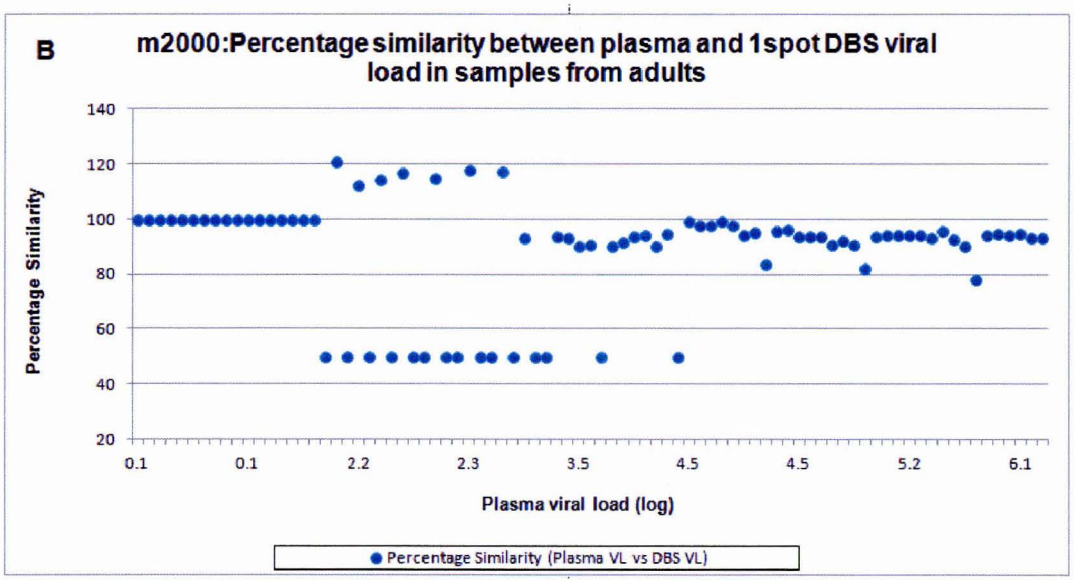
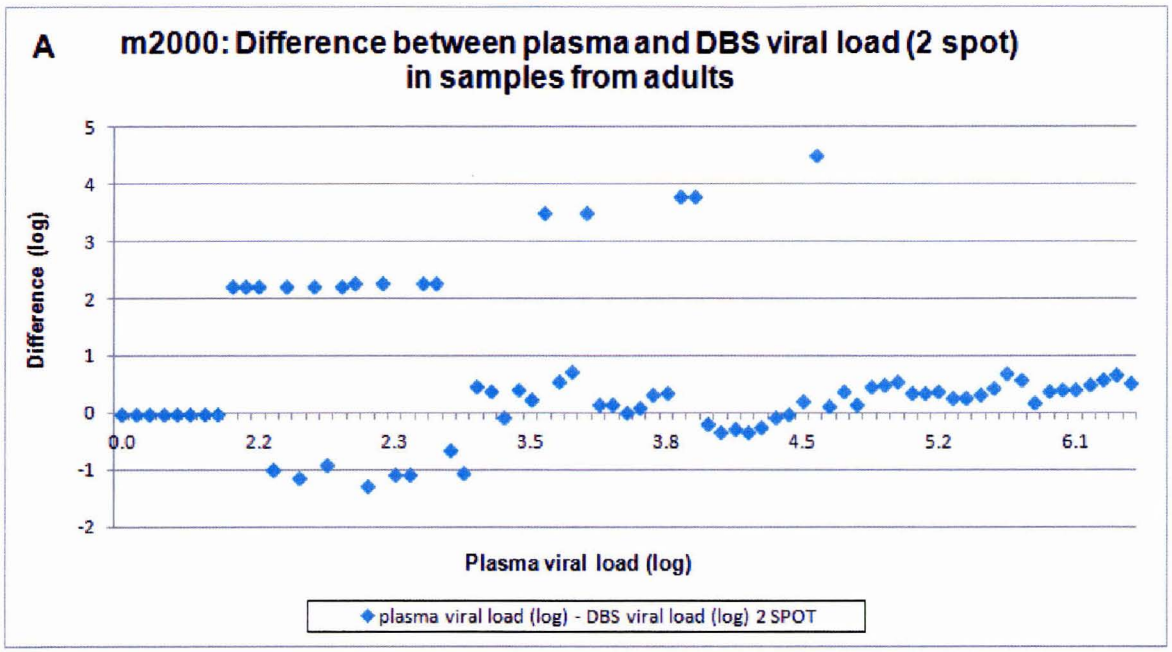


Figure 3.6: m2000 : Plasma and single DBS VL in samples from adults. ***A:***Bland-Altman plots depicting the difference between plasma and 1 spot DBS VL. ***B:*** Percentage similarity plots showing that the percentage similarity is 100% when plasma VL are undetectable



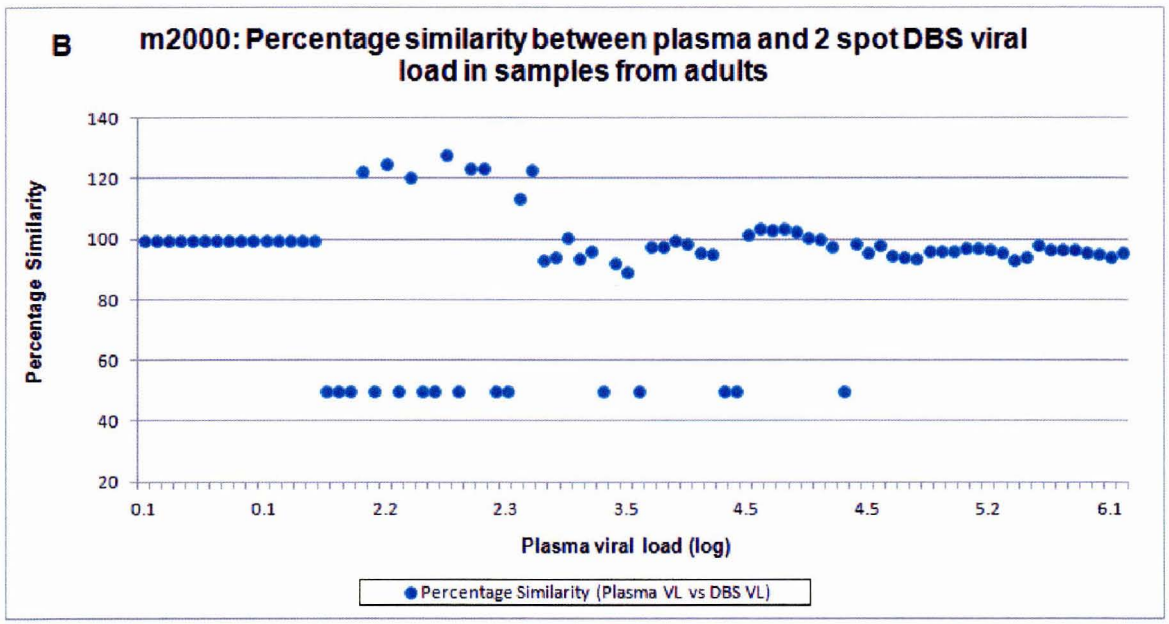


Figure 3.7: m2000: Plasma and DBS VL (2 spots) in samples from adults on m2000. ***A:***Bland-Altman plots depicting the difference between plasma and 2 spot DBS VL. ***B:*** Percentage similarity plots showing that the percentage similarity is 100% when plasma VL is undetectable and >3.5 log copies/ml with some outliers

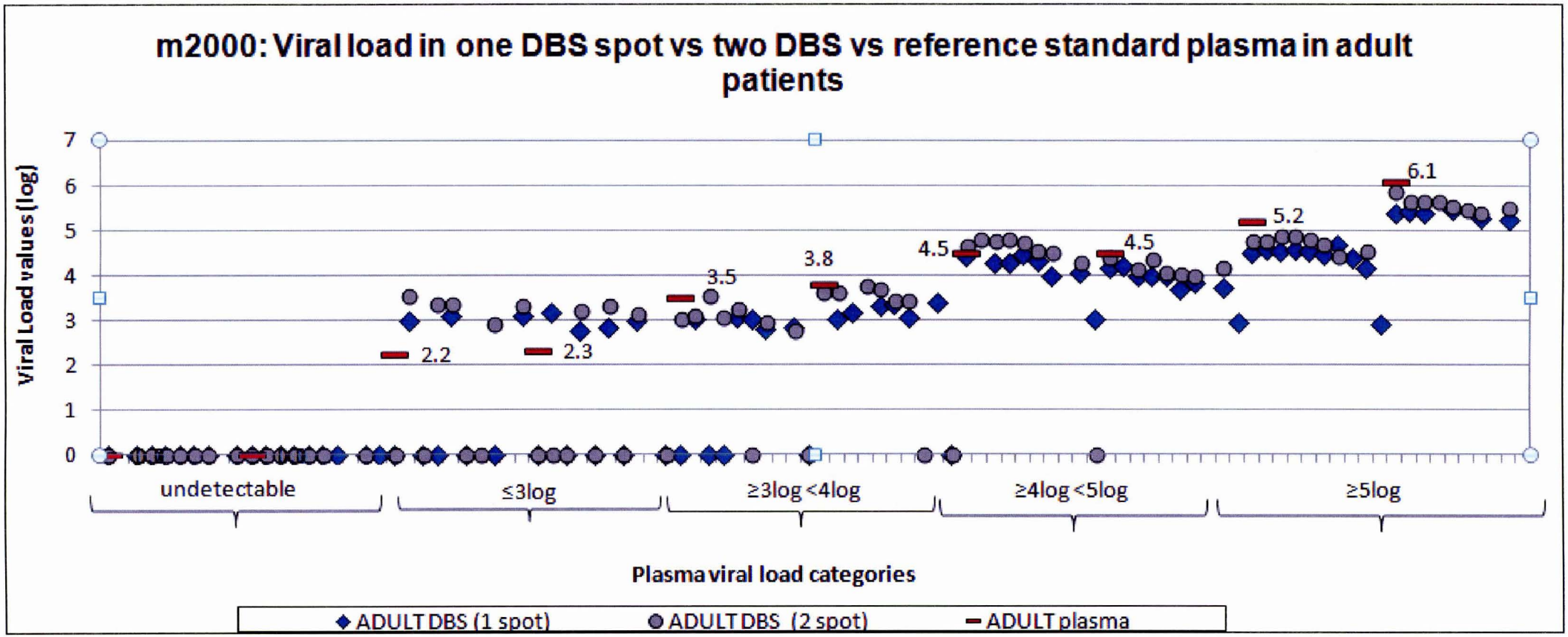


Figure 3.8:Dried blood spot VL in one and 2 spots as compared to gold standard plasma VL. Viral load in two DBS performed better than 1 spot as data points are closer to the plasma gold standard VL as compared to the data generated from the one spot data.

Table 3.3 : Bland-Atman and percent similarity statistical parameters for measuring DBS VL accuracy in adults and children on the m2000 platform.

m2000: Bland-Altman Parameters										% Similarity		
						No.(%) of outliers						
Comparison	VL categories	Bias (log RNA copies/ml)	SD of difference (log RNA copies/ml)	Limits of agreement	Distance over limits (log RNA copies/ml)	>log 0.5 log RNA copies/ml	>log 1 log RNA copies/ml	>log 2 log RNA copies/ml	>log 3 log RNA copies/ml	Mean	SD	%CV
Plasma vs DBS (children)	undetectable	0.00	0.00	0.0; 0.0	0.00	0/9 **(0)**	0/9 **(0)**	0/9 **(0)**	0/9 **(0)**	100.00	0.00	0.00
	≤3 log	-0.14	1.09	0.81; 1.37	-0.55	10/10 **(100)**	4/10 **(40)**	0/10 **(0)**	0/10 **(0)**	103.19	27.02	26.18
	>3 - ≤4 log	0.25	0.73	1.22; 0.24	0.98	1/10 **(10)**	1/10 **(10)**	1/10 **(10)**	0/10 **(0)**	96.49	10.40	10.77
	>4 - ≤5 log	1.08	1.08	3.21; -1.04	4.25	6/9 **(66)**	2/9 **(22)**	2/9 **(22)**	0/9 **(0)**	87.35	12.91	14.79
	>5 log	0.57	0.09	1.22; -1.03	2.24	4/5 **(80)**	0/5 **(0)**	0/5 **(0)**	0/5 **(0)**	95.11	0.80	0.84
	summary	0.32	0.89	1.52; 0.27	1.25	21/43 **(49)**	7/43 **(16)**	3/43 **(7)**	0/43 **(0)**	96.43	15.58	16.11
Plasma vs DBS (adult) 1 spot	undetectable	0.00	0.00	0.00; -0.00	0.00	0/17 **(0)**	0/17 **(0)**	0/17 **(0)**	0/17 **(0)**	100.00	0.00	0.00
	≤3 log	1.02	1.53	3.53; -0.48	4.01	17/17 **(100)**	10/17 **(59)**	10/17 **(59)**	0/17 **(0)**	77.38	33.78	43.65
	>3 - ≤4 log	1.49	1.45	4.37; -1.47	5.84	10/16 **(63)**	5/17 **(29)**	5/17 **(29)**	5/17 **(29)**	79.23	20.41	25.75
	>4 - ≤5 log	0.54	0.44	1.50; -0.62	2.13	9/17 **(53)**	2/17 **(12)**	0/17 **(0)**	0/17 **(0)**	94.01	4.84	5.15
	>5 log	0.76	0.41	1.90; -1.07	2.97	15/16 **(94)**	1/16 **(6)**	1/16 **(6)**	0/16 **(0)**	93.02	4.09	4.39
	summary	0.75	1.07	2.55; -0.40	2.96	51/83 **(61)**	18/83 **(22)**	16/83 **(19)**	5/83**(6)**	85.90	21.12	24.59
Plasma vs DBS (adult) 2 spots	undetectable	0.00	0.00	0.00; 0.00	0.00	0/15 **(0)**	0/15 **(0)**	0/15 **(0)**	0/15 **(0)**	100.00	0.00	0.00
	≤3 log	0.80	1.68	3.24; 0.11	3.13	18/18 **(100)**	15/18 **(83)**	10/18 **(56)**	0/18 **(0)**	82.21	37.16	45.19
	>3 - ≤4 log	1.08	1.48	3.61; -0.64	4.25	6/17 **(35)**	4/17 **(24)**	4/17 **(24)**	4/17 **(24)**	85.09	20.25	23.80
	>4 - ≤5 log	0.36	1.14	1.86; 0.43	1.43	2/16 **(13)**	1/16 **(6)**	1/16 **(6)**	1/16 **(6)**	95.96	12.72	13.25
	>5 log	0.44	0.15	1.01; -0.72	1.73	6/16 **(38)**	0/16 **(0)**	0/16 **(0)**	0/16 **(0)**	96.06	1.31	1.36
	summary	0.56	1.19	2.28; 0.09	2.19	32/82 **(39)**	20/82 **(24)**	15/82 **(18)**	5/82 **(6)**	89.53	23.07	25.77

Specificity

Of the 600 DBS from HIV-uninfected 6 week old infants tested on the m2000 platform, 315 (78.7%) samples were valid. Errors occurred in the one and two spots runs and are described in Table 3.4. The specificity of the m2000 assay was 100% (95% CI 98.8-100).

The CAP/CTM yielded 100% results from the 200 DBS tested from HIV-uninfected 6 week old infants as no errors and no false positive results were incurred. The specificity of the CAP/CTM assay was also 100% (95% CI 98.1-100)

Table 3.4 : Specificity of the CAP/CTM and m2000 assays. The m2000 produced fewer valid results.

	Tests				Results		
Platform	**1 spot**	**2 spot**	**total tests analysed**	**n DBS tested**	**n valid results**	**n false positives**	**specificity**
m2000	200	200	400	600	*315 (78.7%)	0	100%(98.8-100%)
CAP/CTM	200	-	200	200	200 (100%)	0	100%(98.1-100%)

***errors occurred in one and two spot DBS sample**

3.3.3 PRECISION STUDY

3.3.3.1 CAP/CTM DBS VL PRECISION

For CAP/CTM, the intra-variability was less variable than the inter-variability in adults. Using plasma precision limits (SD= <0.19log copies/ml) as the acceptable limit, (the total SD was acceptable in 5/10 (50%) of adult patients across the 5 VL categories. The total SD could not be calculated for the samples from children as the calculation required inter-precision as well as intra-precision measurements. DBS from children were only tested for intra precision and the SD obtained was acceptable in 8/10 (80%) of children across the 5 VL categories (Table 3.5).

The acceptable %CV limit for plasma precision is <35%. High %CV values were observed in samples from adults and children in all VL categories with the exception of the ≥5log copies/ml VL category (Table 3.5 and 3.6). The inter-precision %CV values that were >35% ranged from 36.80% to 117.72% in adults (Table 3.5). In children, only intra-precision was measured and the %CV values >35% ranged from 39.77% to 77.84% (Table 3.6).Of the 10 adult and 10 children tested for intra-precision, 90% of adult samples and 60% of samples from children met the acceptable plasma VL limits of CV <35%.

Table 3.5: CAP/CTM: Adult DBS VL performance (SD and CV) in 5 VL categories.

		Plasma VL		DBS VL				
				Intra precision		Inter precision		
VL category	Panel member	log	copies/ml	SD (log)	% CV (copies/ml)	SD (log)	% CV (copies/ml)	Total SD (log)
undetectable	1	0.00	undetectable	0.13	31.32	0.08	19.60	0.15
	2	0.00	undetectable	0.31	86.02	0.34	76.79	0.46
≤3 log	3	1.63	43	0.11	23.94	0.25	71.92	0.28
	4	1.91	81	0.03	7.25	0.16	30.99	0.17
>3 - ≤4 log	5	3.08	1208	0.11	27.89	0.37	117.72	0.39
	6	3.64	4347	0.10	21.75	0.36	113.47	0.37
>4 - ≤5 log	7	4.46	28521	0.14	31.29	0.14	36.80	0.20
	8	4.34	22122	0.04	9.34	0.10	20.14	0.11
>5 log	9	5.20	158301	0.03	6.77	0.07	17.03	0.08
	10	5.39	245999	0.05	10.64	0.08	18.65	0.09

Red highlight: SD>log 0.19; CV >35%

Table 3.6: CAP/CTM: DBS VL intra precision investigated in children in 5 VL categories using plasma VL as the reference standard.

		Plasma viral load		DBS viral load			
Viral load category	**Panel member**	**log**	**copies/ml**	**SD (log)**	**% CV (copies/ml)**	**n replicates tested**	**n valid results obtained**
undetectable	1	1.30	19	0.10	22.95	5	5
	2	1.30	19	0.19	44.96	5	5
≤3 log	3	2.58	382	0.12	23.30	5	5
	4	2.30	199	0.30	77.84	5	5
>3 - ≤4 log	5	3.31	2046	0.24	66.11	5	5
	6	3.41	2574	0.07	15.83	5	5
>4 - ≤5 log	7	4.81	64919	0.12	29.75	5	5
	8	4.16	14484	0.16	39.77	5	5
>5 log	9	5.65	392215	0.12	26.40	5	5
	10	5.53	336489	0.07	17.03	5	4

Red highlight: SD>log 0.19; CV >35

3.3.3.2 m2000 DBS VL PRECISION

On the m2000, the SD and CV values overall are higher than the plasma limits of acceptance <0.19log copies/ml and <35% across the VL categories due to several undetected VL in DBS which had quantifiable plasma values. The DBS VL SD is acceptable in 2/10 (20%) of patients and the % CV is only acceptable in the plasma undetectable category and in one patient with plasma VL>5 logs in children (Table 3.7).

In comparing the performance of 1 DBS and 2 DBS, the intra-variability is less variable than inter-variability in both instances in samples from adults demonstrating that overall, 2 DBS may have performed better than 1 DBS across all the VL categories (Table 3.8 and 3.9). The total SD is acceptable in the lowest and the highest VL categories irrespective of the number of spots used (Table 3.8 and 3.9).

Table 3.7: m2000: Child DBS VL intra-precision in 5 viral load categories using plasma VL as the reference standard. The DBS viral load SD and CV are only acceptable in the plasma undetectable category and in one patient with plasma VL>5 logs.

		Plasma viral load		DBS viral load			
				Intra precision			
Viral load category	**Panel member**	**log**	**copies/ml**	**SD (log)**	**% CV (copies/ml)**	**n replicates tested**	**n valid results obtained**
undetectable	1	1.30	19	n/a#	n/a#	5	5
	2	1.30	19	n/a#	n/a#	5	4
<3 log	3	2.06	116	0.27	108.42	5	5
	4	1.93	86	0.92	208.19	5	5
>3 log <4 log	5	3.70	4996	0.21	45.60	5	5
	6	3.50	3181	0.22	75.84	5	5
>4 log <5 log	7	4.23	16989	0.14	97.82	5	5
	8	4.61	40735	0.17	38.32	5	4
>5 log	9	5.86	725937	0.09	19.65	5	5
	10	6.06	392212	*		5	0

*SD and CV were not calculated as there were <3 valid results for the replicates in this category

SD and CV could not be calculated as there was no difference between plasma and DBS VL values in the undetectable category

Red highlight: SD>0.19; CV>35%

Table 3.8: m2000: Adult DBS (1 spot) precision in 5 VL categories using plasma VL as the reference standard.

		Plasma Viral load		DBS Viral load						
				Intra precision		Inter precision				
Viral load category	**Panel member**	**log**	**copies/ml**	**SD (log)**	**% CV (copies/ml)**	**SD (log)**	**% CV (copies/ml)**	**Total SD (log)**	**n replicates tested**	**n valid results obtained**
undetectable	1	0.0	0.0	0.00	n/a#	0.00	n/a#	0.00	10	9
	2	0.0	0.0	0.00	n/a#	0.00	n/a#	0.00	10	9
<3 log	3	2.3	196	1.67	139.55	1.79	173.21	2.45	10	9
	4	2.2	169	1.64	155.68	1.69	117.58	2.35	10	8
>3 log <4 log	5	3.8	6135	0.14	31.68	1.66	111.78	1.67	10	7
	6	3.5	3055	1.67	200.24	1.46	73.95	2.22	10	9
>4 log <5 log	7	4.5	31198	0.08	18.79	0.56	81.73	0.57	10	8
	8	4.5	35299	0.10	80.74	0.41	57.07	0.42	10	9
>5 log	9	5.2	142550	0.03	6.18	0.71	76.57	0.71	10	6
	10	6.1	1167215	0.04	8.49	0.02	114.07	0.04	10	10

%CV could not be calculated as a ratio of the SD and mean since the SD and mean values were 0. These values were zero because they were not-quantifiable, and listed as undetectable.

Red highlight: SD>0.19; CV>35%

Table 3.9: m2000: Adult DBS (2 spot) precision in 5 VL categories using plasma VL as the reference standard.

		Plasma viral load		DBS viral load						
				Intra precision		Inter precision				
Viral load category	Panel member	log	Copies/ml	SD (log copies/ml)	CV copies/ ml	SD (log)	CV (copies/ ml)	Total SD (log)	n replicates tested	n valid results obtained
	1	0.0	0.0	0.00	n/a#	0.00	n/a#	0.00	10	7
undetectable	2	0.0	0.0	0.00	n/a#	0.00	n/a#	0.00	10	7
	3	196	2.3	1.88	97.61	1.82	136.33	2.62	10	9
<3 log	4	169	2.2	1.44	223.61	1.88	121.64	2.37	10	9
	5	6135	3.8	0.07	17.88	2.00	94.56	2.00	10	9
>3 log <4 log	6	3055	3.5	0.21	58.74	1.65	120.36	1.67	10	8
	7	31198	4.5	0.06	13.56	2.23	74.24	2.23	10	9
>4 log <5 log	8	35299	4.5	0.18	38.40	0.10	23.88	0.20	10	8
	9	142550	5.2	0.06	13.38	0.12	25.77	0.13	10	9
>5 log	10	1167215	6.1	0.12	29.11	0.07	15.16	0.13	10	8

%CV could not be calculated as a ratio of the SD and mean since the SD and mean values were 0. These values were zero because they were not-quantifiable, and listed as undetectable.

Red highlight: SD>0.19; CV>35%

3.4 DISCUSSION

Plasma VL molecular tests are used in clinical practice to confirm an HIV infected status in newly diagnosed children and to monitor disease progression in HIV-infected patients on ART [89]. Some limitations of plasma VL testing include the requirement for cool transportation and storage conditions to maintain HIV RNA integrity and the relatively large sample volumes (0.2ml – 1.0ml plasma) required from the paediatric population. DBS have been investigated as an affordable and more practical alternative sample type for VL testing with potential to increase access to testing. The relevance of a study of DBS VL accuracy would be an expansion in the diagnosis of infants in resource limited settings. The study of DBS VL precision would provide evidence on the feasibility of replacing plasma VL with DBS VL for HIV monitoring in the paediatric (and adult) population in South Africa.

Evidence from a multicentre study of DBS RNA stability demonstrated that HIV RNA in DBS is stable at ambient temperature (15-30° C) for one year [8]. Based on this evidence, rural areas would be able to submit DBS samples for VL testing without refrigeration, as RNA would be stable between sample collection and testing at locations in which laboratory services are highly centralised. Overall, DBS RNA is stable during storage, provided that high humidity is avoided (by inclusion of desiccant sachets in the packaging) and that storage temperatures do not exceed 37°C [98]. DBS samples require storage at -70°C to preserve HIV RNA if samples were to be stored for more than a year [8]. The DBS RNA in this laboratory study

was considered to be stable for the duration of the study as DBS samples were stored at room temperature and tested within a year of collection as described in the multicentre study of DBS.

In comparing the accuracy of DBS VL to plasma VL on the CAP/CTM platform the finding that DBS VL were detectable and quantified where plasma VL were undetectable was consistent with the findings in other studies of DBS VL [71, 74, 99]. Amplification of proviral DNA from peripheral blood mononuclear cells (PBMC cells) and the total nucleic acid extraction methodology contributed to the total DBS VL [77, 100, 101]. It is suggested that DBS samples treated with DNase during sample preparation would better reflect the true VL since DNA in the sample would be destroyed thereby allowing for RNA to be quantified exclusively [100]. However, methods that require multiple test kits for sample preparation add to the cost of the test and may not be feasible for resource constrained settings nor for high throughput laboratories in which minimal manual sample preparation is preferred.

The CAP/CTM would be highly sensitive for confirming the presence of HIV and therefore ideal to use as confirmation of a positive HIV infection status in children less than 18 months old with an initial positive HIV DNA PCR. In contrast to the CAP/CTM, the m2000 DBS HIV VL assay was impartial to proviral DNA due to the predominantly "RNA only" extraction of this assay and therefore amplification of predominantly HIV RNA nucleic acids [95]. DBS samples matched plasma samples that were RNA undetectable in 100% of cases from adults and children. The clinical application of this finding for routine HIV monitoring is that DBS VL on the m2000 could be used to reaffirm treatment success. Due to the effect of proviral DNA,

patients on ART monitored on the CAP/CTM platform using DBS are unlikely to attain an undetectable VL result, despite the success of their treatment regimen as based on the evidence in this study.

The DBS and plasma VL method comparison on both the CAP/CTM and the m2000 platforms was illustrated in the Bland Altman plots. The CAP/CTM did not show good agreement overall between the two sample types tested, as 29.10% and 23.07% percentage similarity CV in children and adults respectively was obtained. The greatest bias (least accuracy) was obtained at <3log and undetectable plasma VL categories. Overall , a negative bias was obtained demonstrating that the DBS VL measurements were higher than the gold standard plasma VL measurements in most of the VL categories. The least bias (greatest accuracy) was obtained in the higher VL category at VL >5log.

The m2000 comparison of plasma and DBS VL accuracy also did not demonstrate good agreement between the two sample types tested. The percentage similarity CV was lower in children (16.11%) as compared to the percentage CV in adults, but similar percentage CV values were obtained in the 1 spot (24.59%) and two spot (25.77%) comparison on DBS samples from adults. The greatest bias (least accuracy) was obtained at plasma VL <4log copies/ml. Overall, a positive bias was obtained demonstrating that the DBS VL measurements were lower than the gold standard plasma VL measurements in most of the VL categories. The least bias (greatest accuracy) was obtained where plasma VL were undetectable. The clinical implication of using the m2000 and CAP/CTM platforms for monitoring HIV VL on

DBS is that the platforms may not be used interchangeably. Patients tested on either platform would have to be retested on the same platform since DBS VL measurements on CAP/CTM produces higher VL values than on the m2000.

The m2000 in this study did not detect VL in 39 combined adult and child DBS samples in which RNA was detectable at levels of <4log copies/ml in the corresponding plasma sample and is consistent with the findings in two other studies in which 25 DBS samples were HIV RNA undetectable although the matched plasma samples had low levels of detectable RNA in samples from infants [71] and adults [76]. A lower limit of detection or threshold of 4 log copies/ml in this study would minimise the number of false negative DBS VL measurements on both the CAP/CTM and m2000 platforms. The threshold in this study is much higher than the 3.0log [74, 79] and 3.7 log [76, 78] suggested in other studies, but consistent with the threshold described in a study of DBS VL for early infant diagnosis [72]. The WHO guidelines for managing virological failure describes a threshold of 1000 copies/ml (3log) when testing on plasma. The sensitivity of DBS VL is reduced, therefore a higher threshold at 3000-5000 copies/ml (3.5-3.7log copies/ml) is recommended when testing on DBS [62], which fits in with the threshold described by Neogi [78] and Garrido [76]. It can be concluded that DBS VL thresholds vary depending on the nucleic acid extraction techniques and the platforms used for RNA amplification [63, 91].

Meaningful comparisons of studies of DBS VL are difficult mostly due to a lack of standardisation of methodology, the use of modified commercial kits, and varying

platforms and methods for RNA extraction and detection [91, 98]. Studies have reported better correlation between plasma and DBS when the EasyQ boom technology was used for HIV RNA extraction [74, 76, 100]. A study by Monleau confirmed that the boom extraction method used on NucliSens EasyQ-EasyMag yielded the most accurate and sensitive RNA extraction as compared to four other RNA extraction methods on DBS [100]. Resource limited setting are often not equipped with multiple VL platforms and therefore are restricted to using a single supplier for extraction and amplification for VL monitoring. This study investigated two platforms in their extraction and detection equipment pairs. During the m2000 manual extraction method the disintegration of DBS in the lysis buffer during the incubation period may have contributed to the large number of errors obtained on this platform. The CAP/CTM manual extraction method yielded minimal errors making the CAP/CTM the better choice for sample extraction in this study.

Although only 78.7% of valid DBS VL results were obtained on the m2000 as compared to the 100% on CAP/CTM, both platforms demonstrated 100% specificity in diagnosing HIV in 6 week old HIV-exposed, HIV-uninfected infants. The high specificity obtained in this study is consistent with the 100% specificity obtained in two other studies [70, 71] in which DBS were evaluated for early infant diagnosis and VL monitoring. The clinical application of zero false positives DBS VL obtained is that clinicians could utilise these two platforms for diagnosis of HIV in adults and children without the need for a threshold.

The precision of DBS VL was below standard for VL monitoring. DBS VL may not be useful for VL monitoring unless possibly performed in a semi-quantitative way. The

number of errors on the m2000 platform need to be resolved before the platform can be used for clinical DBS samples. In this study of DBS VL precision on the CAP/CTM and m2000 platforms, DBS intra-precision showed less variability than inter-precision. The SD and CV values overall were lower for intra-precision measurements in adult samples. Testing of HIV monitoring emulates inter-precision (except for biological variation). Just as samples are tested in different batches over a period of time, so too, patient VL are tested at the beginning of ART and thereafter periodically over a few months to monitor changes in VL levels which indicate patient response to therapy. Changes in serial measurement of RNA are significant to the caregiver as the laboratory findings should correlate with patient clinical presentation [85]. DBS VL values on CAP/CTM were higher than DBS VL values on m2000 and is consistent with an evaluation of plasma VL precision on these two platforms [102]. Based on these findings, patients tested longitudinally during VL monitoring cannot be tested interchangeably on the two platforms [102], no matter the sample type.

The investigation of the accuracy of m2000 2 spot versus one spot demonstrated that the use of 2 spots yielded mean percentage similarity values closer to 100% (exact match) to the gold standard than 1 spot for VL testing on m2000. The interpretation of this finding is that the m2000 may be difficult to use for paediatric DBS VL monitoring if inadequate sample such as less than 2 full spots of DBS sample is received. The CAP/CTM tests adult and paediatric VL in a single DBS and no technical errors were obtained.

This study of DBS VL had several limitations. One such limitation was that DBS were made from EDTA blood (ideally in the real world DBS cards would be spotted directly from patient by finger- or heel-pricks) and possible EDTA interference was not accounted for in this study. A further limitation was the small sample size comprising a total of 20 adults and 20 children that were tested across five VL categories and only two patients were tested in each VL category. This is a super low sample size, and claims that ~4 log copies/ml should be used as a threshold need further investigation since only four patient samples were tested above and below that cut-off. Future work with a larger sample number is needed to reaffirm and establish whether this threshold can be used confidently. No direct comparison of the two platforms was made (using the same samples) as the samples tested on the CAP/CTM platform were not the same patients that were tested on the m2000 platform.

A multicentre study on DBS VL to standardise nucleic acid extraction and to establish a universal threshold is needed until manufacturers of VL technologies are able to develop apparatus specifically for DBS VL testing [90]. Alternative strategies to improve access to VL testing such as point of care plasma or whole blood VL would be more appropriate than DBS VL testing since plasma VL testing is already the gold standard. Perhaps point of care plasma VL testing [29] would reduce the problems that are incurred with sample transportation and storage to testing sites. An ideal VL test for a resource limited setting would be performed on whole blood, with RNA extraction and or DNAse treatment for monitoring, but whole blood and total nucleic acid detection for diagnosis.

3.5 CONCLUSION

The DBS VL testing method can be used to make a diagnosis of HIV infection in infants due to the high specificity obtained on both platforms. In particular, the CAP/CTM assay would be the preferred platform for confirmation of HIV infection in infants because the sensitivity of the test is increased by proviral DNA. In the era of more and longer ARV regimens for PMTCT, which consequently lowers RNA as described from daily dose Nevirapine, detection of proviral DNA is relevant. However, HIV-infected infants on ART cannot be monitored accurately on DBS without a VL threshold being considered. The DBS VL threshold for both platforms in this study, using the extraction and detection instruments in their pairs as supplied by the manufacturer, was ~4log copies/ml. Furthermore, DBS VL do not perform within the plasma precision limits and inter precision performed worse than intra precision on both platforms, therefore longitudinal clinical monitoring of patient response to ARVs may not be feasible. The m2000 platform performs more accurately and precisely on two DBS spots as compared to one DBS spot.

CHAPTER 4

CONCLUSION TO DIAGNOSIS AND MONITORING OF HIV IN INFANTS

Improvement in technologies that aid the detection and monitoring of infection is essential in the battle against HIV, which has already claimed numerous lives of women and children. Many of the evolving technologies would essentially need to be functional in resource-limited settings. Although newer rapid HIV tests such as DC address the cold storage issue in resource limited settings by changes in design that allow storage of the test at room temperature, the greater challenges of early identification of infants at risk of acquiring HIV and detection of acute HIV infection at point of care remain. A fourth generation rapid test intended for detection of early infection requires further development to enhance the sensitivity of the test and offers no advantage over currently available tests.

Technological advances in presently available HIV VL monitoring platforms are needed before DBS VL can replace the gold standard plasma VL method based on the findings in this study. Although DBS VL were shown to compare with plasma VL (with the proviso that a threshold is used) and this has been demonstrated in many studies, correlation data is not sufficient to draw conclusions that DBS VL can be implemented with confidence for HIV monitoring for patients (adults and children) on treatment. The findings in this study of poor DBS VL precision, despite the limitations of the study, does not support the implementation of the DBS VL method for HIV monitoring. The DBS VL method, however, can be used with confidence to ascertain

an HIV status on treatment naïve patients, based on the high specificity obtained in this study.

Since technology continues to play a major role in achieving goals such as reduced infant mortality and improved HIV health care for infants in South Africa, other alternatives that address the gaps in diagnosis and patient management require ongoing investigation.

Appendix A: Ethics Approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Kapila Bhowan

CLEARANCE CERTIFICATE	**M091133**
PROJECT	Diagnosis and Monitoring of HIV in Infants the Investigating the First Fourth Generation Rapid Test and Two Viral Load Technologies for Use in the South African Setting (revised title)
INVESTIGATORS	Ms Kapila Bhowan.
DEPARTMENT	Molecular Medicine & Haematology
DATE CONSIDERED	2009/11/27
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 31/05/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof I Scott

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

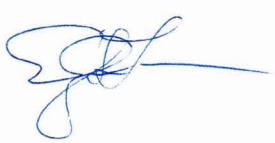
Appendix B: Permission from African Journal of Laboratory Medicine

Dear Ms. Bhowan,

It is my pleasure to grant you permission to include your article, "Identifying HIV infection in women: How does a fourth generation HIV test perform?" (Afr J Lab Med. 2011;1(1)), in your MSc dissertation.

Thank you for your valuable contribution to the African Journal of Laboratory Medicine, and the public health world in general. We hope you will consider submitting to AJLM again in the future.

Best of luck on your dissertation,



Elizabeth Luman, PhD

Managing Editor

African Journal of Laboratory Medicine

Identifying HIV infection in women: How does a fourth generation HIV rapid test perform?

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Background: HIV rapid tests (RT) play an important role in tackling the HIV pandemic in South Africa. Third generation RT that detect HIV antibodies are currently used to diagnose HIV infection at the point of care. Determine Combo (DC) is the first fourth generation RT that detects both p24 antigen (p24Ag) and HIV antibodies (Ab), theoretically reducing the window period and increasing detection rates. Early detection of maternal HIV infection is important to mitigate the high risk of vertical transmission associated with acute maternal infection.

Objectives: We assessed the performance of the DC RT against third generation RT in antenatal and post-partum women.

Methods: Third generation RT Advance Quality and Acon were used in a serial algorithm to diagnose HIV infection in antenatal and post-partum women over six months at a tertiary hospital in Johannesburg, South Africa. This data provided the reference against which the DC RT was compared on plasma and whole blood samples.

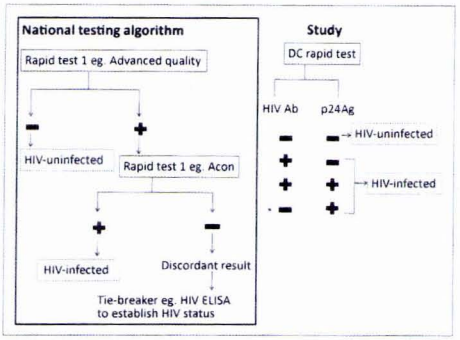
Results: The 1019 participants comprised 345 (34%) antenatal and 674 (66%) post-partum women. Ninety women (8.8%) tested HIV-positive of whom 59 (66%) were tested antenatally, and 31 (34%) post-partum yielding prevalence rates of 17.1% and 4.6% respectively. The sensitivity and specificity of the Ab component of DC on plasma antenatally was 100% (93.8% – 100%) and 100% (98.6% – 100%) respectively and post-partum was 100% (88.9% – 100%) and 99.6% (98.8% – 99.9%) respectively. One false positive and not a single true positive p24Ag was detected. Of 505 post-partum women who tested HIV-negative 6–12 months prior to enrolment, 12 (2.4%) seroconverted.

Conclusion: The fourth generation DC offered no advantage over current third generation RT in the diagnosis of HIV infection.

Introduction

HIV rapid tests (RT) play an important role in addressing the HIV and AIDS pandemic in South Africa. They can be conducted at the point of care because they are easy to perform and require no special instrumentation. The advantage of point of care RT is that the patient can receive their HIV test result at the same clinic visit, which reduces loss to follow-up and fast tracking patients into care.[1] RT are less costly than laboratory-based assays for antibody (Ab) detection namely HIV Enzyme-linked Immunosorbent Assays (ELISA) and viral detection namely HIV DNA or RNA or p24 Antigen (p24Ag).

In South Africa, pregnant women are offered counselling and testing for HIV at their first antenatal clinic visit and at 34 weeks of pregnancy if their initial HIV test was negative.[2] Women at the Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg, South Africa are also offered an HIV test immediately post-partum if their HIV status is unknown or more than six weeks have elapsed since their last negative HIV test. The importance of HIV retesting has been demonstrated by a South African study in which 3.4% of women who tested HIV-negative at their first antenatal visit, subsequently seroconverted during pregnancy or within a year after delivery.[3] The Advance Quality HIV Rapid Test (In Tec Products, Inc. Xianen, China) and Acon HIV 1/2/0 Tri-line Rapid Test (Acon Laboratories, Inc., San Diego, USA) are currently used to diagnose HIV infection in women in Prevention of Mother-to-Child transmission (PMTCT) programmes in Gauteng province, South Africa. These third generation RT detect HIV Ab that are produced in response to the virus by a serial testing algorithm as recommended by the South African PMTCT guidelines[2] (Figure 1). Advance Quality is used to screen for HIV Ab and if positive, the Acon test is performed to confirm HIV status. Laboratory-based HIV ELISA and, less commonly, viral detection assays can be used as a tiebreaker to confirm an HIV status if serial RT results are discordant.[2]



+, positive; –, negative.
*, The combination of Determine Combo rapid test results consistent with early maternal HIV-infection were followed up with fourth generation enzyme-linked immunosorbent assays (ELISA) and nucleic acid testing.

FIGURE 1: Study methodology: The Determine Combo rapid test (DC RT) was performed at the same time as the routine algorithm.

The Determine Combo HIV-1/2 Ag/Ab Combo Test (DC) (Inverness Medical, Japan Co.,Ltd) is the first fourth generation RT and can be performed on either plasma or whole blood samples.[4] It is an enhancement of the third generation tests as it can detect both HIV Ab and p24Ag in a single test.[5] The p24Ag is a marker of early HIV infection and is detectable in blood during the window period before HIV Ab become detectable.[6] The DC RT is reported to have the potential to identify HIV infection five days (range 2–20 days) earlier than third generation RT. The reported sensitivity and specificity of the DC Ab component is 100% (95% confidence interval 98% – 100%) and 100% (95% confidence interval 98.2% – 100%) respectively and the sensitivity of the p24Ag component is 86.6% (95% confidence interval 76% – 93.7%).[7]

Detection of early HIV infection during the window period of third generation RT would allow more maternal HIV infections to be detected. Furthermore, during early maternal HIV infection the levels of the virus in the blood are at their peak and the risk of transmission to the infant during birth and breast-feeding is very high.[8] Detection of early infection would allow more women and their infants at a high risk of vertical transmission to access PMTCT.

The performance of the fourth generation HIV DC RT in diagnosing HIV infection status antenatally and in the early post-partum period in comparison to the third generation HIV RT in routine use at public healthcare facilities in Gauteng, South Africa was assessed. The advantage offered by the DC RT over third generation RT of detecting HIV infection earlier to increase identification of women at a high risk of vertical transmission for PMTCT was investigated.

Methods

Study participants

Women attending the antenatal clinic and delivery unit at RMMCH in Johannesburg were invited to test for HIV infection. Counsellors interviewed the participants to establish their HIV status. Women with an unknown HIV status, those who had tested HIV-negative more than 6 weeks previously and those who reported an HIV-positive status but had no documented evidence thereof on their maternal card were invited to participate in the study. Women with a documented HIV-positive status were excluded. Written informed consent was obtained from all participants who agreed to test for HIV infection. Ethics approval (M091119) for the study was granted by the Human Research Ethics Committee at the University of the Witwatersrand, Johannesburg.

Sample size

The prevalence of HIV infection amongst antenatal women in Gauteng province, South Africa in 2008 was 29.9% (95% confidence interval 28.4% – 31.2%)[9]; however, the prevalence of HIV infection amongst women testing antenatally and shortly after delivery at RMMCH in 2008 was lower at 15% and 4.2% respectively since women with a known, documented HIV-positive status are excluded and proportionately more women fall into this category after delivery than antenatally.[10] From RMMCH HIV testing records we expected approximately 200 women to present for testing per month with a prevalence that depended on the proportion of women testing before or after delivery. A convenience sample of all women eligible for HIV testing at RMMCH who agreed to participate in the study was chosen to assess the number of additional women the DC RT could identify as being infected over a 6-month period on plasma samples. For assessment of whole blood samples, the Centers for Disease Control and Prevention recommendations to include samples from at least 20 HIV-infected and 80 HIV-uninfected women were followed.[11]

HIV testing

Five millilitres of whole blood was drawn into an ethylenediaminetetraacetic (EDTA) tube for testing at the study site. Samples were centrifuged to obtain plasma on which the RT were performed. DC RT were performed on whole blood prior to centrifugation. All RT were performed within one hour of blood sampling by a single laboratory technician according to the manufacturer's instructions. Third generation RT Advance Quality and Acon were used serially on plasma according to the national testing algorithm[2] to diagnose HIV infection (Figure 1) and were the reference standards against which the DC results were compared.

Interpretation of rapid test

The DC is a qualitative immunochromatographic test which is read visually. The test strip is divided into an HIV Ab window and an HIV p24Ag window. The presence of a pink line in either or both of the windows is indicative of HIV infection. Each test strip incorporates a procedural positive control and the test is considered valid only if the positive control is detected.

The women received their RT results and post-test counselling within four hours of blood sampling. Patients that were HIV-positive on both third generation RT were referred to antiretroviral treatment clinics. The results of the DC RT were not disclosed to the patient. However, where the third generation and DC HIV Ab RT results were discordant, samples were referred for confirmatory fourth generation ELISA (ARCHITECHT® HIV Ag/Ab Combo assay; Abbott Diagnostics; Wiesbaden, Germany). Patients that had detectable p24 Ag on DC were followed up with three confirmatory tests that is viral detection assay (Vironostika HIV-1 Antigen; bioMerieux; Bosiend, The Netherlands), viral load testing (NucliSENS EasyQ-EasyMag HIV-1, version 1.2 assay; bioMerieux; Boxtel, The Netherlands) as well as fourth generation ELISA. Disclosure of the patient's HIV status was delayed for 48 hours.

Analysis

Likelihood ratios were calculated instead of predictive values because predictive values depend on prevalence and a difference in HIV prevalence was anticipated between women tested in the antenatal and post-partum period. A positive likelihood ratio (sensitivity/(1 – specificity)) > 10 strongly predicts HIV-infection, whereas a negative likelihood ratio ((1 – sensitivity)/specificity) < 0.1 virtually excludes the condition.

Results

Between March and August 2010, 1019 (92.7%) of the 1099 women eligible for HIV testing at RMMCH were enrolled in the study. Of the 1019 participants, 345 (33.9%) were tested antenatally and 674 (66.1%) post-partum. According to the routine third generation RT diagnostic algorithm, 90 (8.8%) of the 1019 patients tested positive for HIV infection without the need to use a tiebreaker. Of the 90 HIV-infected women, 59 (65.6%) were antenatal and 31 (34.4%) were post-partum. The HIV prevalence amongst the women tested antenatally was 17.1% and those tested post-partum was 4.6%.

Knowledge of the women's HIV status prior to undergoing HIV testing on the study was documented for those tested in the early post-partum period only (Table 1). Of the 505 women that had reported or tested HIV-negative between 6 and 12 weeks prior to study enrolment, 12 (2.4%) tested positive demonstrating that new HIV infections were occurring in this population. Not all of these women had their negative HIV status documented on their maternal record and the possibility that some reported their status incorrectly cannot be excluded.

The DC RT was performed on plasma samples of the 1019 women and on whole blood samples on a subset of 380 women. All 1399 tests performed demonstrated positive control strips therefore no DC RT was invalid. Sensitivity, specificity and likelihood ratios for the DC RT were calculated separately for women who were tested antenatally and post-partum (Table 2). The sensitivity of the DC RT Ab component was 100% in all groups of women tested irrespective of the sample type. The specificity of the DC RT Ab component in women tested post-partum was less than those tested antenatally.

The DC RT results concurred with the third generation RT results in all patients except for three post-partum women on whom both plasma and whole blood samples were tested. In two patients the Ab component of the DC RT was positive on both their plasma and whole blood samples but negative on both third generation RT. Laboratory based fourth generation ELISA tests on both cases were negative confirming two false positive DC HIV Ab results.

TABLE 1: HIV status of post-partum women prior to enrolment and after testing with the National testing algorithm.

Reported or documented HIV status	Time since last test (weeks)	*n*	HIV-infected	HIV-uninfected	Percentage positive
Negative	6–12	505	12	492	2.4
	> 12	107	3	104	2.8
Unknown HIV status	-	58	14	43	24.1
Positive: reported but not documented	-	4	2	2	50.0
Total	-	**674**	**31**	**641**	**4.6**

n, sample size.
Women of unknown HIV status had a high prevalence of HIV infection. Half of the women who reported a positive HIV status but had no documentation to substantiate a positive HIV test, tested HIV-negative.

TABLE 2: Performance of Determine Combo rapid test HIV antibody (DC RT Ab) component in plasma and whole blood samples in women tested antenatally and post-partum.

Time test was conducted	Sample type	*n*	Prevalence	Determine Combo HIV Ab+/ HIV+	Sensitivity	95% confidence interval	LR+	Determine Combo HIV Ab-/HIV-	Specificity	95% confidence interval	LR-
Antenatal	Plasma	345	17.1	59/59	100	93.8–100.0	569.2	286/286	100.0	98.6–100.0	0
	Whole blood	151	-	59/59	100	93.8–100.0	184.5	92/92	100.0	95.9–100.0	0
Post-partum	Plasma	674	4.6	33/31†	100	88.9–100.0	321.5	641/643	99.7	98.9–99.9	0
	Whole blood	229	-	32/30†	100	88.6–100.0	99.5	197/199	99.0	96.4–99.7	0

n, sample size.
†, False positive results obtained in 2 women on both sample types. One additional women in this group tested HIV p24Ag false positive on DC.
HIV+ and HIV- are HIV-infected and HIV-uninfected women respectively as defined by the third generation rapid test (RT)-algorithm.
LR+ and LR- are the positive and negative likelihood ratios.
The high positive likelihood ratios (LR+) and low negative likelihood ratios (LR-) demonstrate that the Determine Combo rapid test was a strong predictor of HIV-infection in HIV positive women and excluded HIV infection in HIV-uninfected women respectively, regardless of sample type and timing of testing.

The DC p24Ag was reactive on plasma and the whole blood of only one patient of all 1019 women tested. This patient was suspected of having an early infection since both third generation RT and the DC Ab component tested negative. However the laboratory based fourth generation HIV ELISA was negative and the viral load was undetectable indicating a false positive DC p24Ag result. No p24Ag bands on DC were obtained on any of the 90 HIV-infected women nor was a single case of early HIV infection detected.

Discussion

Considering the high enrolment rate, the sample of women tested in this study is likely to be representative of women who are tested for HIV-infection at RMMCH over half a year. Furthermore, the HIV prevalence of 17.1% and 4.6% in women testing antenatally and immediately post-partum respectively is similar to a previous description at RMMCH.[10] An HIV prevalence of 24.1% in women of unknown HIV status is also comparable to the 28% prevalence previously described in 2008. However, the seroconversion rate in post-partum women of 2.4% is less than the previously described rate of 4.5%.[10] The implication that new maternal infections are occurring in this population remains. In practice, a positive p24Ag DC test would require confirmation of early seroconversion by fourth generation ELISA or nucleic acid testing which may delay initiation of PMTCT. In contrast to a study that reported 16% invalid DC RT tests due to failure to detect the control,[12] all DC RT in this study were valid possibly because we used fresh, not stored samples.

Sensitivity and specificity of the DC RT was comparable to plasma and whole blood in contrast to a previous report that demonstrated lower sensitivity of the DC RT in whole blood compared to serum samples.[12] The sensitivity of the fourth generation DC RT in detecting HIV Ab was comparable to that of the reference third generation RT in antenatal and post-partum women in plasma and whole blood samples. The specificity of the DC RT in detecting HIV Ab was slightly reduced in whole blood and plasma in post-partum women owing to the false positive results in two women, but was still within the World Health Organization recommended range of more than 98%.[13]

The fourth generation DC RT did not detect a single true positive p24Ag, even in the 90 HIV-infected women. The sensitivity in detecting p24Ag in HIV-infected women was 0% as compared with the claim of 86.6% obtained on HIV-infected samples.[7] The reason for this may be that p24Ag forms immune complexes with HIV Ab and thus no free p24Ag is present for detection by the DC.[6] The DC RT therefore did not identify any new cases of maternal HIV infection over the 6 month study period. Possible reasons for this include that the sensitivity of the DC p24 Ag component is poor or that no women with acute HIV infection were enrolled. The former concurs with previous reports that the DC RT p24 Ag component lacks sensitivity particularly where levels of p24 Ag are below 400 pg/mL.[12,14] Additionally, the p24Ag component of the DC RT has reduced sensitivity in comparison to other fourth generation viral detection assays,[7] some of which are able to detect p24Ag at levels of 4 pg/mL – 5 pg/mL.[15] The initial laboratory based p24Ag assays also demonstrated poor sensitivity that was subsequently improved by denaturation of the immune complex and signal amplification to enhance p24Ag detection.[16]

A limitation of this study is that the incidence of HIV infection in women undergoing HIV testing at RMMCH is unknown therefore, although new maternal infections were demonstrated, it is possible that no women with acute HIV infection were enrolled during the short window period before HIV Ab and subsequent immune complex formation. Nevertheless, the DC RT did not identify any new infections over those identified by the 3rd generation RT assays over six months in the RMMCH PMTCT programme.

Conclusion

The DC RT failed to demonstrate any advantage over third generation RT currently in use in our setting in either determining HIV infection status or in identifying recently infected women. Improved sensitivity of p24 Ag detection is required before fourth generation RT will offer an advantage over their third generation counterparts in the field.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

All of the authors have made substantial contribution to the manuscript. G.S. (Wits Health Consortium) conceptualised and planned the study as well as performed critical revision of the manuscript. K.B. (Wits Health Consortium) analysed and interpreted the study data and prepared the manuscript. S.K. (Wits Health Consortium) performed the testing of samples, maintained quality assurance in the laboratory and performed critical revision of the manuscript. E.K. (Wits Health Consortium) assisted with patient recruitment and management in the field as well as contributed to the writing and editing of the manuscript.

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Performance of the First Fourth-generation Rapid Human Immunodeficiency Virus Test in Children

Kapila Bhowan, ND and Gayle G. Sherman, MD, PhD*†‡*

Abstract: Diagnosing HIV infection in infants by p24 antigen detection at point of care is likely to reduce infant morbidity and mortality. A fourth-generation rapid test evaluated on 202 stored samples from children of known age and clinical presentation demonstrated a sensitivity of <2% for detecting p24 antigen in 61 HIV-infected infants and demonstrated 100% sensitivity and specificity for detecting HIV antibodies in infants aged 6 months and younger.

Key Words: children, human immunodeficiency virus, fourth generation, p24 antigen, rapid test

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Point of care (POC) tests for diagnosing HIV infection in infants in resource-constrained settings may reduce HIV-related infant mortality by improving detection of infected infants and facilitating earlier initiation of antiretroviral therapy.[1]

Rapid HIV tests currently in use are commonly third-generation tests that detect HIV antibodies (Abs). These rapid tests are used at POC to diagnose HIV infection in children older than 18 months of age and adults. In children younger than 18 months of age, reactive third-generation rapid tests indicate HIV exposure (ie, babies born to HIV-infected women) rather than HIV infection because passively acquired maternal HIV Ab can persist to 18 months of age. In these younger children, laboratory-based viral detection assays such as HIV DNA or RNA polymerase chain reaction or p24 antigen (Ag) tests are required to determine an HIV infection status, causing delays in access to treatment and increased morbidity and mortality.

Determine Combo HIV-1/2 Ag/Ab Combo Test (DC) (Inverness Medical Japan Co., Ltd., Tokyo, Japan) is the first fourth-generation rapid test for detection of both HIV Ab and p24Ag. DC is an immunochromatographic test for the qualitative detection of HIV Abs and p24Ag in the seroconversion phases of HIV infection. This test is intended for use as a first screening tool in areas with limited laboratory infrastructure because it does not require any heat or acid manipulation for immune complex dissociation. By virtue of its ability to detect p24Ag, the DC has the potential to diagnose HIV infection in children younger than 18 months of age at POC, providing an earlier definitive diagnosis of HIV infection at a single clinic visit.

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The p24Ag is diagnostic of HIV infection, but in the presence of HIV Abs and the resultant immune complex formation, the sensitivity for detecting p24Ag is low unless dissociation by heat or acid denaturation is performed.[2] In adults, the sensitivity of p24Ag detection is highest in the early and late stages of HIV infection, when HIV Abs are absent or present in low titers, respectively. In early HIV infection in vertically infected infants, maternal HIV Abs are present to complex with p24Ag. As maternal HIV Abs wane, increasing infant HIV Ab titers would participate in immune complex formation. In advanced disease, HIV-infected infants with hypogammaglobulinemia (including low HIV Ab titers), high viral loads, and waning maternal HIV Abs are the likeliest to have uncomplexed, detectable p24Ag. Theoretically, these infants with advanced disease also are the most likely to test false-negative on third-generation HIV rapid tests because of low HIV Ab titers.

In adults, the reported sensitivity and specificity of the DC HIV Ab component is 100%,[3] with 95% confidence intervals (CI) of 98% to 100% and 98.2% to 100%, respectively. The sensitivity of the p24Ag component is 86.6% (95% CI, 76%–93.7%).[3] The performance of the DC in 40 HIV-infected infants aged 3 months or younger demonstrated a sensitivity for HIV Ab detection of 100% (95% CI, 95.9%–100%), and for p24Ag detection was 2.5% (95% CI, 0.1%–14.7%).[4] No clinical details of the single 3-month-old infant identified by the DC p24Ag were recorded. No data of the performance of the DC test in older infants and children are available, particularly those to assess whether it may be useful in specific clinical scenarios such as advanced disease.

This study evaluated the ability of the DC to detect p24Ag and to provide a diagnosis of HIV infection at POC in stored samples from children aged younger than 18 months with known clinical presentations. The performance of the DC in detecting HIV Ab in children younger than 18 months of age and establishing HIV infection status in older children also was investigated.

MATERIALS AND METHODS

The Ethics Committee of the University of Witwatersrand approved the study. Data from 2 previous study cohorts enrolled in 2002 and 2005, respectively, were reviewed to identify cases that met criteria for 4 clinical scenarios representative of children in the field presenting for HIV testing.[5,6] A history of HIV exposure and clinical staging of disease progression, according to Centers for Disease Control guidelines, were used to assign cases to the following scenarios: (1) HIV-exposed, uninfected; (2) HIV-exposed, HIV-infected, and clinically asymptomatic; (3) HIV-exposed, HIV-infected, and symptomatic; and (4) HIV-unexposed. Children classified as Centers for Disease Control and Prevention clinical stages N and A were considered clinically asymptomatic whereas those classified as Centers for Disease Control and Prevention clinical stages B and C were considered symptomatic.[7] A convenience sample of 202 children with a minimum stored plasma or serum sample volume of 50 μL was selected. Samples were retrieved from the accredited −80°C storage facility at which they were stored for a period of between 6 and 9 years and tested by a single operator in accordance with manufacturer instructions.

TABLE 1. Performance of the HIV Ab Component of the DC Rapid Test on HIV-exposed and HIV-unexposed Infants

		Study Population					DC HIV Ab Rapid Test Results				
		HIV-exposed, Uninfected		HIV-exposed, Infected	HIV-unexposed, Uninfected	DC Ab*/ ELISA*	Sensitivity‡ (95% CI)	LR*	DC Ab†/ ELISA†	Specificity§ (95% CI)	LR†
Age (mo)	n	ELISA* PCR†	ELISA† PCR†‖	ELISA* PCR* (%)	ELISA† PCR†						
≤6	79	43	0	21^{7} (26.6)	15	64/64	100 (94.3–100)	30.7	15/15	100 (79.6–100)	0.00
>6 to ≤18	76	33	31	10^{6} (13.2)	2	40/43	93.0 (81.4–97.6)	62.3	33/33	100 (89.6–100)	0.07
>18	47	0	14	30^{22} (63.8)	3	29/30	96.7 (83.3–99.4)	33.8	17/17	100 (81.6–100)	0.00
Total	202	76	45	61^{35} (30.2)	20	133/137	97.1 (83.3–99.4)		65/65	100 (81.6–100)	

Ab indicates antibodies, DC, determine combo, ELISA, enzyme-linked immunosorbant assay; LR, likelihood ratio; PCR, polymerase chain reaction assay.
Superscript digits denote numbers of symptomatic children (Centers for Disease Control and Prevention clinic stages B and C).
*Positive test results.
†Negative test results.
‖Children in the HIV-exposed, uninfected category with negative ELISA results had undergone seroreversion.
‡Prevalence.

The reference standard against which the DC p24Ag results were compared was the Roche Amplicor HIV-1 DNA version 1.5 (Roche Diagnostics Systems, Inc., Branchburg, NJ) viral detection assay results. The reference standard against which the DC Ab results were compared to diagnose HIV exposure in children aged younger than 18 months and HIV infection in older children was the HIV-1/HIV-2III Plus IMx System (Abbott Diagnostics Division, Wiesbaden, Germany) enzyme-linked immunosorbant assay (ELISA). Both reference standard tests were performed at the time the original study was performed. Sensitivity, specificity, and likelihood ratios were calculated. Likelihood ratios were used instead of predictive values because the HIV prevalence in the study differed from that in the field.

Positive likelihood ratios of >10 indicate that positive DC Ab results strongly predict for the presence of HIV Abs in all age groups. Negative likelihood ratios of <0.1 indicate that negative DC Ab results are strongly correlated with the absence of HIV Abs.

RESULTS

The DC was performed on stored samples from 20 (10%) HIV-unexposed and 182 (90%) HIV-exposed children, including 61 HIV-infected children with an overall HIV prevalence of 30.2%. A summary of patient age, exposure/infection status, test results, sensitivity, and specificity are presented in Table 1. The DC Ab rapid test results were consistent with the HIV ELISA results for all but 4 children when the DC Abs returned false-negative results for 2 HIV-exposed, uninfected 7-month-old children and in 2 HIV-infected children, 1 who was 7 months old and symptomatic and 1 who was 23 months old and asymptomatic. The DC Ab component was 100% sensitive in infants aged 6 months and younger. The DC Ab component of the test demonstrated no false-positive results, hence the overall specificity of 100% with 95% CI of 81.6% to 100% (Table 1).

Of the 61 HIV-infected infants tested, the DC p24Ag was reactive in only a 3-month-old clinically symptomatic infant, resulting in sensitivity for detection of HIV infection of 1.7% (95% CI, 0.3%–8.9%). The other 12 clinically symptomatic infected children aged younger than 18 months tested negative for the DC p24Ag.

No false-positive DC p24Ag results were obtained, resulting in a specificity of 100% (95% CI, 97.3%–100%).

DISCUSSION

Child health programs require rapid HIV tests to detect HIV exposure, to exclude HIV infection in infants, and to diagnose HIV infection status in older children. In theory, a fourth-generation rapid HIV test should add the option of a POC test for the diagnosis of HIV infection in infants to this repertoire. This is the first study to explore the performance of the DC test in the context of clinical scenarios expected to present in child health programs in low-resource settings.

The DC rapid test failed to detect p24Ag in 98% of HIV-infected infants. These findings are similar to the results obtained in the younger cohort of infants tested, including the observation that the only infant testing p24Ag-positive was also 3 months of age.[4] However, even at 3 months of age, the sensitivity of the p24Ag component in detecting HIV infection is <10% (data not shown).

Among the 12 clinically symptomatic children was a 6.9-month-old infected infant who tested DC Ab-negative, contradicting our hypothesis that the DC would be able to detect an increase in unbound p24Ag in the setting of waning maternal HIV Abs and late-stage infant HIV infection.

The DC HIV Ab component is comparable with other third-generation HIV rapid tests to screen for HIV exposure in infants younger than 6 months of age. For older children, the DC test sensitivity is <99%, which is recommended by the World Health Organization (Table 1).[8] The inability of the DC Ab component to detect HIV Abs in the 2 HIV-exposed 7-month-old uninfected infants may be because these infants were in the process of seroreverting.

Limitations of this study include a small sample size in each clinical and age group category and prolonged storage of samples. However, HIV Abs were detected in 133 (97%) of 137 cases, suggesting limited, if any, Ab degradation. Viral p24Ag is reportedly stable in samples stored for 10 years.[9]

Recently, the performance of POC p24Ag detection on a dipstick to diagnose HIV infection in infants has been dramatically improved by devices that separate plasma from whole blood and use heat to disrupt p24Ag immune complexes before testing.[10,11] A sensitivity of 96% (95% CI, 88%–99%) and specificity of 99% (95% CI, 98%–100%) have been demonstrated in the laboratory and field testing results are awaited.

The fourth-generation DC rapid test cannot be recommended for POC diagnosis of HIV infection in infants, even at 3 months of age or during advanced-stage disease. The sensitivity of the DC for detection of HIV exposure and diagnosis of HIV infection in children is no better than that of third-generation HIV rapid tests.

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