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Off-Label Evaluation of Alternative Specimen Types: Cobas® Plasma Separation Card for HIV Viral Load and Dried Blood Spots for COVID-19 Serology Testing



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Haematology and Molecular Medicine.

DECLARATION

I, Thabiso Mmammitsi Mampa declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Haematology and Molecular Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

_____11___day of____November 2022_____in_Johannesburg_____

In memory of my beautiful mother

Eunice Machipu Mampa

1974 - 2018

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

- Wits Faculty of Health Sciences research day & postgraduate expo (Poster Presentation) – 15 October 2020 – Virtual conference
    1. **Mampa T**, Noble L, Scott L, Stevens W. Evaluation of the Plasma Separator Devices for HIV viral load testing on the Abbott M2000 platform.

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    2. Noble L, **Mampa T**, Scott L, Stevens W. The performance of the cobas® plasma separation card for HIV viral load testing using the Abbott m2000 platform.

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- Mampa T, Noble L, Stevens W, Jani I, Scott L
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- Noble L, Mampa T, Scott L, Stevens W
  A landscape review of Plasma separator devices
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ABSTRACT

Plasma is the preferred specimen for HIV viral load (VL) monitoring and COVID-19 serology testing but poses a challenge in resource-limited settings due to the need for venous blood, skilled phlebotomy, and cold storage for specimen integrity. In this study dried blood spots and novel plasma separation devices (PSC, HSSE, and VLPlasma) versus plasma were investigated as alternative specimen types.

The plasma separation devices (PSD) were compared to DBS to determine if eliminating cell-associated nucleic acids could improve HIV VL performance. Paired PSD (n=72), DBS (n=72) and plasma (n=72) were prepared from HIV positive residual whole blood. Similarly, paired PSC, DBS (n=91) and plasma (n=91) were prepared from HIV positive prospective whole blood to assess PSC as an alternative specimen for use on the Abbott m2000. The eluates were processed on the GeneXpert (residual blood), Abbott m2000 (residual and prospective blood) and Roche cobas® 68/8800 (prospective blood). Using plasma as reference, residual blood: DBS outperformed PSC, HSSE and VLPlasma in terms of accuracy 91.8%, compared to 87.8%, 79.1% and 75%. Prospective blood: PSC had improved performance over DBS in terms of sensitivity (92.2% and 87.1%), specificity (65% and 61.9%), and accuracy (86.9% and 80.7%).

Additionally, the performance of DBS was evaluated for COVID-19 serology testing in 45 PCR-confirmed, COVID-19 positive individuals by preparing laboratory paired DBS-plasma samples. DBS were eluted using two diluents followed by manual ELISA and results compared to reference plasma testing. DBS-PBS and DBS-manufacturer's diluent showed the same accuracy (93.6%). Kappa values (0.817 and 0.845) and sensitivity (100% and 91.4%) were similar, but DBS-PBS showed low specificity (75%) compared to DBS-diluent (100%).

Off-Label use of the cobas® PSC for HIV VL and DBS for COVID-19 serology testing provides expanded options for testing in resource-limited settings. Further evaluation on capillary blood and automated laboratory workflow optimisation would still be required prior to scaled implementation.

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ABBREVIATIONS

ACE2: Angiotensin converting enzyme 2

AIDS: Acquired Immunodeficiency Syndrome

ART: Antiretroviral Therapy

CAP/CTM: Cobas AmpliPrep/Cobas TaqMan

CD4: Cluster of Differentiation 4

CD8: Cluster of Differentiation 8

cDNA: complementary deoxyribonucleic acid

CI: Confidence Interval

CMJAH: Charlotte Maxeke Johannesburg Academic Hospital

COVID-19: Coronavirus Disease 2019

cp/ml: Copies per milliliter

DBS: Dried Blood Spot

DNA: deoxyribonucleic acid

DOH: Department of Health

DPS: Dried Plasma Spot

EDTA: Ethylenediamine tetraacetic acid

EID: Early Infant Diagnosis

ELISA: Enzyme-Linked Immunosorbent Assay

FDP: Filtered Dried Plasma

HIV: Human Immunodeficiency Virus

HRP: Horseradish peroxidase

HSSE: HemaSpot SE

IgG: Immunoglobulin G

LOA: Limit of Agreement

LOD: Limit of Detection

µL: Microlitre

NAAT: Nucleic Acid Amplification Test

NP: Nasopharyngeal

NPV: Negative Predictive Value

PBS: Phosphate Buffered Saline

PSC: Plasma Separation Card

PSD: Plasma Separation Devices

POC: Point of Care

PPV: Positive Predictive Value

RLS: Resource Limited Setting

RNA: ribonucleic acid

RT-PCR: Real time polymerase chain reaction

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

SAVQA: South African Viral Quality Assessment

SOC: Standard of Care

VL: Viral Load

WHO: World Health Organization

UNAIDS: Joint United Nations Programme on HIV/AIDS

U = U: Undetectable = Untransmittable

PROJECT OVERVIEW

The study consists of two sections, the first section is the HIV viral load (VL) testing and the second section is the COVID-19 IgG antibody testing. The purpose of the study is to evaluate the Cobas® Plasma Separation Card (PSC) and the Dried Blood Spot (DBS) for use alternatively or interchangeably in HIV viral load monitoring and COVID-19 serology testing.

SECTION 1 – HIV VIRAL LOAD

CHAPTER 1.1 – INTRODUCTION

1.1.1 HIV Burden

The human immunodeficiency virus (HIV) is a virus that leads to the development of Acquired Immunodeficiency Syndrome (AIDS) if an infected individual is not treated, (1-3). The most recent data from the Joint United Nations Programme on HIV/AIDS (UNAIDS) (4) reported that 38 million people worldwide were living with HIV: 36.2 million adults and 1.8 million infected children below 15 years. Western and Southern Africa reported 20.6 million cases of people living with HIV and 280 000 AIDS related deaths (4). In South Africa, 7.8 million people were living with HIV in the year 2020, with 7.5million adults above the age of 15 years and 310 000 children; 230 000 individuals were newly infected and there were 83 000 deaths (5).

1.1.2 HIV Diagnosis

HIV is commonly transmitted from person to person by bodily fluids during unprotected sex or needle-sharing in drug-related cases (6). During HIV infection (**Figure 1**), HIV attacks CD4 cells in large numbers and replicates uncontrollably within these cells (7). The amount of virus increases rapidly and viral ribonucleic acid (RNA) is detected in the bloodstream, activating antibody production by B cells and CD8 killer T cells to control the infection (7). This leads to a reduction in viral concentrations resulting in partial recovery of CD4 cells.

During the asymptomatic period of the infection (clinical latency), HIV continues to replicate at low levels (7).

If left untreated, CD4 cell count can fall below 200 cells/µl and the infected individual progresses to AIDS; which is due to the CD4 cells being destroyed at a faster rate than they can be replaced, causing the human immune system to become exhausted and prone to opportunistic infections (7).

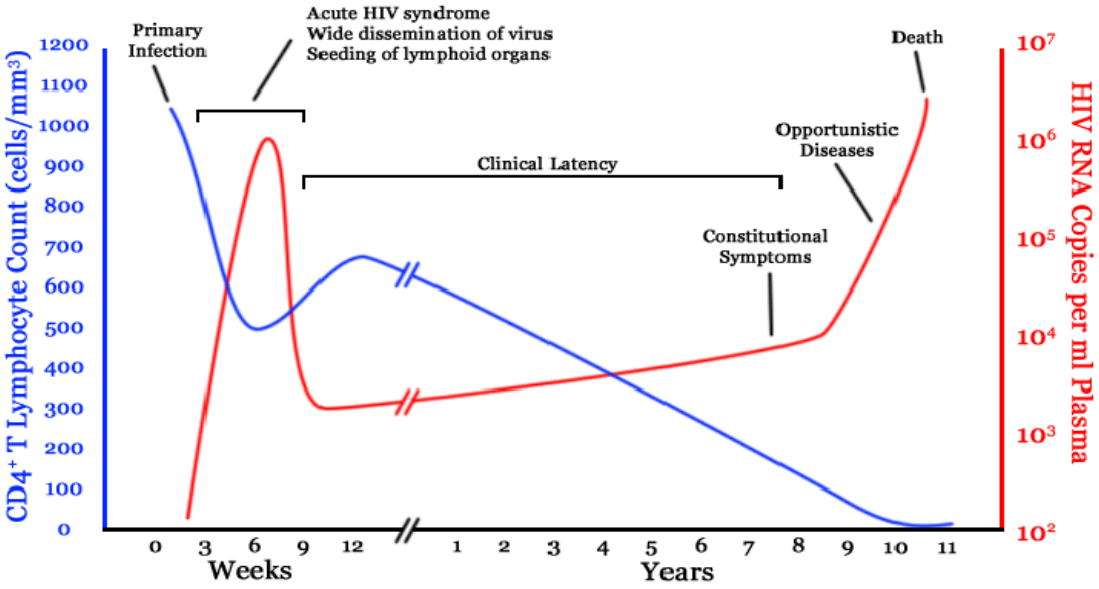


Figure 1: Comparison of the CD4 cell concentration to HIV RNA copies over time after the initial HIV infection. Figure from Omari M, and Ouifki R, 2020 (8).

The World Health Organization (WHO) and UNAIDS recommend "universal access to knowledge of HIV status" (9). HIV diagnosis is essential for treatment initiation and management in infected patients (10). Knowing one's status leads to the immediate start of ART, which can help suppress the viral load and thus prevent transmission of the virus (9). Thus, UNAIDS in 2014 (11) introduced the 90:90:90 targets, which aim to have 90% of HIV-positive patients diagnosed, 90% of HIV-positive patients on Antiretroviral Therapy (ART) and 90% of HIV-positive patients on ART have a suppressed viral load by 2020. A fast track target of 95:95:95 by 2030 was also implemented to prevent the rebound of the AIDS epidemic, which, if successful, will result in 28 million HIV infections and 21 million AIDS-

related deaths averted (11). In 2020, globally 84% of people knew their HIV status, 87% of them were on ART, and 90% of those on ART had achieved a suppressed viral load (VL) (12). By December 2021, South Africa's progress towards the 90:90:90 target was that 92% of HIV positive patients knew their status, 72% were on ART, and 66% of those on treatment were virally suppressed (12).

1.1.3 Antiretroviral therapy (ART)

The purpose of ART is to disrupt viral replication, reducing the concentration of the virus and thus the process of progressing to AIDS (7). ART is accessible to 26 million people worldwide (4). HIV patients on ART have an increased life expectancy, but an inability to control HIV infection can lead to treatment failure (13, 14). Antiretroviral drugs function by inhibiting the HIV replication cycle (15) as illustrated in **Table 1** .

Table 1: An overview of ART drugs and their respective functions (16)

Antiretroviral drugs	**Function**
Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)	Inhibits reverse transcription process by targeting reverse transcriptase
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Blocks the process of reverse transcription by binding to the reverse transcriptase
Integrase inhibitors	Inhibits the insertion of the virus into the human cell DNA by targeting integrase
Entry inhibitors	Blocks HIV from entering the cells
Protease inhibitors (PIs)	Inhibits protease enzyme activity
Post-attachment inhibitors	Inhibits attachment to CD4 receptors
Booster drugs	Enhances protease inhibitors

The Prevention Access Campaign launched an initiative titled: Undetectable = Untransmittable (U=U) in 2016, which promotes the message that HIV positive individuals on ART who maintained an undetectable VL cannot transmit the virus sexually (17). The principles of achieving and maintaining an undetectable VL depend on adhering to the prescribed ART treatment, time taken to reach viral suppression, VL testing as per recommendations, and the risks associated with halting ART (17). The U=U campaign significantly reduces the stigma associated with HIV, encourages individuals to test and be on treatment if infected, and improves adherence (18).

Undetectable VL is an indication of the efficacy of ART, and an increase in the VL levels in an individual is due to ineffective ART (7). Factors such as poor pharmacokinetics and non-adherence, both leading to low drug concentrations, can cause treatment failure and contribute to the virus becoming resistant to the treatment (HIV drug resistance) (7).

1.1.4 HIV viral load (VL) monitoring

The WHO recommends VL monitoring as part of routine care for HIV-positive patients on ART. This is important in order to assess VL suppression and prevent treatment failure due to mutations and non-adherence (19). The VL assays measure the concentration of the virus in an ethylenediaminetetraacetic acid (EDTA) plasma specimen (15). The significance of VL monitoring is to assess treatment, identify challenges related to adherence and determine the optimal time to switch therapy regimes in an instance of treatment failure (20). CD4 cell count and clinical criteria assess treatment failure in cases where VL monitoring is unavailable (21). VL is preferable to CD4 because CD4 count and clinical criteria have poor sensitivity and positive predictive values for the early detection of virological failure (22, 23), thus switching treatment regimens unnecessarily or delaying the switch in patients with undetectable virological failure (24). Seventeen ART programmes in poorer regions reported that patients having their VL monitored could switch to a second line of therapy with a higher CD4 count and earlier than those without their VL monitored (25); thus, viral load is superior in predicting the progression of the disease than CD4 (26).

WHO also recommends routine VL testing six months after ART initiation, repeated at 12 months and performed annually (27). Plasma is currently the gold standard specimen type for HIV VL monitoring (27). A detectable VL of greater than 1000 copies per millilitre (cp/ml) is

regarded as treatment failure (28). Studies have shown that peripheral blood plasma with a higher VL is correlated to an increased risk of clinical progression of HIV disease, while undetectable plasma VL is correlated to a decreased risk of clinical progression (29-31). To reach the UNAIDS 90:90:90 goals, effective and sustainable HIV diagnosis, VL and ART monitoring should be prioritised (32). Bachmann and colleagues reported that frequent monitoring of VL (every three months or twice in a year) results in low cases of multiple drug resistance due to the ability to detect virological failure and periods of non-adherence early (33).

In line with WHO guidelines, the standard of care in South Africa for assessing patient response to ART and prognosis of HIV is by the measurement of HIV-1 RNA blood plasma concentration (i.e. VL) using nucleic acid-based monitoring assays (30, 34-38). South Africa's National Health Laboratory Service (NHLS) provides 80% of the population with diagnostic pathology and public health services. In the 2020/2021 financial year, the NHLS laboratories conducted 5.7 million VL tests (39). However, challenges related to the integrity of specimens and delivery of results are experienced, which ultimately affects the clinical decision making and the provision of adherence counselling (40).

Point of care (POC) VL testing technologies provide alternatives to overcome challenges experienced with regards to VL monitoring in resource-limited settings (RLS). The POC technologies increase access to VL through being placed in remote areas or areas unable to access VL testing and help reduce the turnaround time (TAT) to results, thus making it easy for patients to start treatment early and monitor ART (41). Girdwood *et al.* (41) evaluated the cost of implementing POC VL instruments in South Africa and reported that the POC technologies cost 40 dollars more per person virally suppressed than the central laboratories network. Therefore, POC testing is better applied in a targeted manner in facilities that have high viral failure rates. (41).

1.1.5 Molecular testing platforms

Viral load testing uses nucleic acid-based tests, which amplify the viral nucleic acid to measure the concentration of HIV per millilitre of plasma (42). Molecular testing platforms (**Table 2**) such as the Abbott m2000 Real-time system, Alinity m, Cobas® 6800/8800 systems, Cobas® AmpliPrep/cobas® TaqMan® and Cepheid GeneXpert, in use by the NHLS, are flexible platforms, which use RNA and real-time polymerase chain reaction (RT-PCR) technology. The extraction to detection processes is automated, increasing efficiency and improving service (43).

Table 2: Molecular testing platforms used by the NHLS for HIV VL testing.

Platform	Description	Images (reference)
Abbott m2000 RealTime system (Abbott Molecular, Des Plaines, IL, U.S.A)	A multipurpose and fully automated system that detects HIV nucleic acids using real-time Polymerase Chain Reaction (PCR) fluorescence detection (44). **Volume required**: 0.2 mL, 0.5 mL, 0.6 mL, (Plasma) and 1.0 mL (DBS). **Sample type**: EDTA plasma or DBS **TAT**: 3 hours and 30 minutes for both specimens **Specificity:** Plasma: 100% and DBS: 76.0% **Sensitivity:** Plasma 97.3% and DBS: 89.7% **LOD:** 39 copies/mL for both	 (44)
Alinity m instrument (Abbott Molecular, Des Plaines, IL, U.S.A)	An automated system that detects HIV-1 RNA using RT-PCR and applies the ReadiFlex technology (45). **Volume required:** 600 µL of plasma/serum or 70 µL DBS card **Sample type:** EDTA Plasma or DBS **TAT:** One hour and 55 minutes for both **Specificity:** 100% for both **Sensitivity:** Plasma: 95% and DBS: 78.1% **LOD:** 20 cp/ml for plasma/serum and 400 cp/ml for DBS	 (46)
GeneXpert Cepheid, (Sunnyvale, CA, U.S.A)	An automated, cartridge-based platform that detects HIV-1Viral Load using RT-PCR properties (47) **Volume required**: 1000 µl of plasma **Sample type**: Plasma **TAT**: 90 minutes **Specificity**: Plasma: 98.50% **Sensitivity**: Plasma: 94.14% **LOD**: 18.3 cp/mL	 (48)

Cobas® AmpliPrep/cobas® TaqMan® (Roche Molecular, Pleasanton, CA, U.S.A)	A fully automated platform that processes specimens using the cobas® Ampliprep and amplifies and detects specimens using cobas® TaqMan® (49) **Volume required**: 1000 µL plasma and 70 µL of DBS **Sample type**: Plasma or DBS **TAT**: 3 hours for both **Specificity:** 100% for both **Sensitivity:** 99.9% for both **LOD:** 40 copies/mL for plasma and 300 copies/mL for DBS	 (50)
cobas® 6800/8800 systems (Roche Molecular, Pleasanton, CA, U.S.A)	A fully automated and integrated platform that amplifies and detects HIV-1 nucleic acids (51). **Volume required**: 650 µL and 1300 µL **Sample type:** Plasma and PSC **TAT:** 3 hours 30 minutes **Specificity:** 94.1% for both **Sensitivity:** 94.6% for both **LOD:** 13.2 cp/mL for plasma (500µL) and for PSC 790.2 cp/mL	 (51)

TAT: Turnaround time; LOD: limit of detection; PSC: Plasma separation card

1.1.6 Challenges with specimen collection and stability

The significant challenges faced when using plasma are: (i) the procedure of drawing blood is invasive as opposed to heel stick or finger stick, and it requires trained phlebotomists, (ii) whole blood requires centrifugation to separate plasma, and (iii) the specimens need cold storage for stability purposes (52). Each manufacturer has specific requirements for whole blood stability, i.e. the maximum time it takes from whole blood collection to plasma separation. The time requirement for Abbott RealTime HIV-1 is 24 hours at room temperature (15–30°C) or 48 hours refrigerated (2–8°C), for Cepheid's Xpert HIV-VL it is 8 hours at room temperature (15–30°C) or 72 hours refrigerated (2–8°C), and for cobas 68/8800 systems it is 24 hours at room temperature (2–25°C) or 24 hours refrigerated (2–25°C) (20). Whole blood samples stored beyond their recommended limit are reported to be stable, but are at increased risk of contamination with intracellular material released during cells lysis, leading to samples requiring re-centrifugation before testing (53).

The temperature at which samples are stored influences the VL detection; however, the overall results are still the same with minor false positives. A study showed that 20% of results initially reported as undetectable VL, were reported as low VL concentrations (14.7% viral load of <100 copies/mL, 5.7% it was 101–500 copies/mL and in 0.4% > 500 copies/mL) on a second test after being stored at 4°C, 20°C, and 30°C for 168 hours (20). This study further showed that 51% of specimens with initial undetectable results had detectable VL (17.5% were < 100 copies/ml, 21% were 101–500 copies/ml, 1.8% was 501–1000 copies/ ml and 10.5% were >1000 copies/ml) on repeat testing due to the plasma not being centrifuged again after being stored at room temperature for 48 hours (20). Common challenges and solutions are listed in Table 3. These challenges make it difficult for specimens to reach the central laboratories within a suitable time (54, 55) for HIV VL testing. The overall outcome will significantly affect the UNAIDS 2030 95:95:95 target of patients diagnosed with HIV and on ART with a suppressed VL (11).

Table 3: Challenges encountered, and solutions implemented in resource-limited settings.

	Challenges	Solutions
Human resources required	Phlebotomists	Caregivers/ community health workers to perform finger stick
Specimen collection	Venipuncture	Finger stick and heel stick
Specimen container	4 ml EDTA tube	DBS card or PSD
Specimen transportation	Cold chain to maintain integrity	With a desiccant at ambient temperatures
Specimen preparation	Centrifuged to separate plasma from whole blood.	Elute nucleic acids from DBS
Specimen storage	2°C to 25°C up to 24 hours	Ambient temperature for weeks

Ref: (54) and (55). EDTA: Ethylenediamine tetraacetic acid; DBS: Dried Blood Spot; PSD: Plasma Separation Devices.

1.1.7 Dried Blood Spots (DBS)

DBS (**Figure 2**) facilitate collecting blood specimens in areas where phlebotomists and resources such as centrifuges are limited on-site (56-59). DBS uses blood drawn with minimally invasive procedures such as a heel stick for Early Infant Diagnosis (EID) of infants exposed to HIV (60) and a finger stick for HIV VL monitoring (61). DBS make it possible to easily collect, store and transport specimens for analysis (62) due to the stability of the dried specimen (63). In the laboratory, DBS are incubated with lysis buffer in a heating block or thermomixer to release the target materials into the solution, prior to analysis (64).

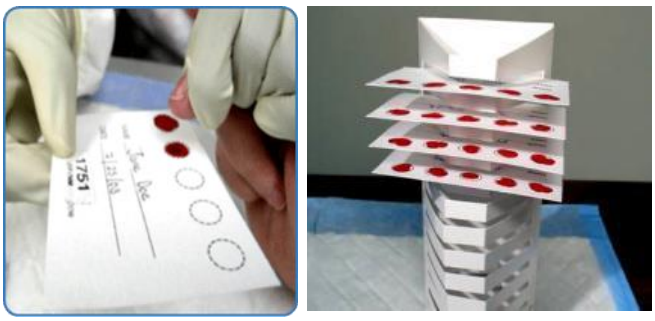


Figure 2: DBS card and drying rack. Figure adapted from World Health Organisation, 2005 (65).

WHO recommends DBS specimens for HIV VL testing in settings experiencing storage, infrastructural and logistical challenges (20, 27). DBS have been implemented in several countries, including South Africa, to assist in the access and scale-up of VL testing. Independent evaluations of DBS using Abbott RealTime HIV-1 one-spot and two-spot protocols reported sensitivity and specificity of 88.26% (49.64–98.28), 99.07% (68.38–99.98) and 93.13% (83.72–97.27), 91.11% (82.35–95.75), respectively, while the Roche COBAS TaqMan HIV-1 Specimen Pre-Extraction Reagent (SPEX) had a low specificity of 48.49% (22.63–75.18) but good sensitivity of 98.23% (95.85–99.26) (66). In contrast, the phosphate buffered saline (PBS) eluted DBS had an improved specificity (100%) (67).

The use of DBS specimens, however, has several limitations that may result in virological misclassification of patients on ART (68). One of the limitations of DBS is that contamination can occur during the collection and processing of DBS specimens due to the extensive handling of the DBS cards (61). Further, DBS preparation uses whole blood, which may result in overestimating viral load results from the extracted and detected intracellular RNA and proviral DNA in addition to the free target RNA circulating in the plasma (20) which may lead to reduced specificity (69). It is reported that when compared to plasma, DBS have reduced sensitivity due to the low input copy number caused by the limited volume of blood (50 to 75 µl) compared to 500 µl to 1 mL used for plasma testing (43). A 1000 cp/ml threshold is clinically relevant for diagnosing virological failure (28). Inaccurate classification of the VL as below or above this threshold is termed downward (virological success) or upward (virological failure) misclassification, respectively (70).

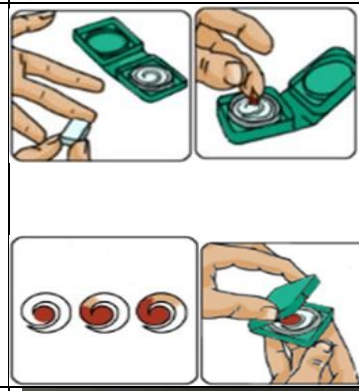
1.1.8 Plasma Separator Devices

Plasma separator devices (PSD) are a possible alternative to DBS and plasma. They do not need centrifuges, freezers or refrigerators, require small amounts of blood through finger-stick or heel stick and can be stored and transported at ambient temperatures (20). Plasma separators separate plasma from whole blood at POC, using membrane-based separation methods. Drops of whole blood are spotted onto the device, and the device membrane prevents the flow of whole blood cells while permitting the free flow of serum soluble particles. Like DBS, they improve HIV and viral load testing coverage by enabling simple

specimen collection, storage, and stability during transportation over long distances and in harsh environmental conditions (71). Unlike DBS, there is less cell-associated RNA/DNA, which qualifies PSD as better for HIV testing and VL monitoring, and the separated plasma is enclosed within the PSD, preventing contamination (61).

A study from Cameroon (72) compared DBS prepared using 50 µL of whole blood with Dried Plasma Spots (DPS) prepared using 50 µL plasma from centrifuged whole blood, with the reference plasma sample using the Abbott m2000 instrument. The sensitivities of DBS and DPS were 88.2% and 91.1%, respectively and specificity was 100% for both specimen types. A Kenyan study (43) evaluated the DBS and DPS using Abbott m2000; the sensitivity and specificity of DBS on Abbott m2000 were 93.9% and 88.8% respectively, while for the DPS the sensitivity was 97.3% and the specificity was 98.1%. The plasma separation devices are explained in detail **Table 4**.

Table 4: Plasma Separation Devices

PSD	Principle	Method	Image
Plasma Separation Card (PSC). Roche Molecular Diagnostics, Pleasanton, CA, USA	Simple, easy to use specimen collection card proposed for HIV plasma viral load testing in Resource Limited Settings (RLS) (73). Thepermeable membrane separates plasma from whole blood obtained from venous blood or finger stick (74). A stabiliser membrane stabilises the specimen for transportation at extreme heat and humidity for 21 days maximum (74).	The primary packaging is removed, a finger stick is prepared, three drops of blood are collected and applied to the three spots on the card, and the card is left to dry. It is compatible with the COBAS ampliprep/COBAS TaqMan system and cobas 6800/8800 systems (74).	 (75)
Hemaspot-SE (HSSE). Spot On Sciences, Inc., Austin, TX, USA	A device that separates plasma/serum from whole blood. It uses a single finger stick to obtain whole blood, which is spotted at the centre of the device where the form prevents the flow of large particles (red blood cells, leukocytes, and platelets) while permitting the passage of plasma/serum soluble particles. Therefore, the large molecules get concentrated at the centre of the spiral-shaped form while the filtrate (plasma soluble particles) collects at the spiral arm of the form (76).	A finger is pricked with a lancet, then three drops of whole blood are added to the centre of the device. After two minutes of blood soaking through and separating plasma, the lid is closed to prevent contamination, and the device is shipped at ambient temperatures (76).	 (76)
VLPlasma® separation device. Nanjing BioPoint Diagnostic Technology Co. Ltd., Nanjing, China	It is a lateral flow point of care test that does not require specimen preparation (77), and samples are transported at ambient temperature. It was developed for the purpose of viral load testing for HIV; the cell-free plasma is separated from whole blood using lateral flow chromatography (77). The device has a nitrocellulose membrane and glass fibre for trapping leukocytes and erythrocytes while permitting plasma flow, collected by the filter paper (78).	Whole blood (100 µl) is pipetted in the sample cavity of the device and incubated for 3 minutes. A supplied plastic pipette is used to aliquot 90 µl of phosphate-buffered saline (PBS) to the sample cavity. After that, the VLPlasma device is stored at room temperature (77).	 (77)

As a novel alternative to plasma for HIV VL monitoring, PSD could widen access to testing and help detect mutations in patients on ART caused by a sudden treatment regimen change.

CHAPTER 1.2 – AIMS AND OBJECTIVES

The purpose of the study is to assess PSC as an alternative to EDTA plasma for use in HIV viral load monitoring on various molecular testing platforms to potentially widen access to patient care in resource-limited settings, which could ultimately lead to national policy development and implementation of a roadmap.

1.2.1 AIMS:

i. To evaluate PSC on two molecular testing platforms for HIV VL monitoring.

ii. To develop a framework for statistical method comparison of HIV VL testing.

1.2.2 STUDY OBJECTIVES

- Verify PSC processing on the Abbott m2000 and GeneXpert using a modified 21-member plasma quality panel: The South African Verification Quality Assessment (SAVQA) panel (79) for HIV VL.
- Determine the sensitivity, specificity and accuracy of PSC, HSSE, VLPlasma® separation device, and DBS prepared from residual whole blood by processing on the GeneXpert and Abbott m2000 instruments.
- Determine the sensitivity, specificity and accuracy of PSC and DBS prepared from prospective whole blood by processing on the cobas 8800 and Abbott m2000 instruments
- Develop and apply a framework of statistical method comparison for HIV VL testing

CHAPTER 1.3 – METHODS AND MATERIALS

The plasma separator devices (PSC, HSSE and VLPlasma) were evaluated using the residual EDTA whole blood (**1.3.2**) and processed on the GeneXpert and Abbott m2000 instruments. PSC and DBS were further evaluated using prospective EDTA whole blood (**1.3.3**) and processed on the cobas 8800 and Abbott m2000.

1.3.1 Study site and ethics clearance

The study was performed in the Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, South Africa. The department has blanket ethics approval obtained from the University of the Witwatersrand Human Research Ethics Committee (**Appendix 1.1** #M150160) for using HIV positive residual whole blood samples with an amendment to include HIV VL testing. Ethics approval (**Appendix 1.2** #161110) for the collection of prospective HIV positive whole blood samples has been obtained as part of the Wits Reproductive Health and HIV Institute (WRHI, Johannesburg, Gauteng, South Africa) HSTAR003A protocol.

1.3.2 Residual EDTA whole blood

1.3.2.1 Selection criteria

Specimens processed on the cobas 8800 in the Flow Cytometry Laboratory of the National Health Laboratory Service (NHLS) (Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, Gauteng, South Africa) were used in this study. The Standard of Care (SOC) data (VL and CD4 count) of these samples was obtained from the laboratory information system, TrakCare, to select samples that met our inclusion criteria of residual whole blood with a CD4 count of <900 cells/µl, HIV VL of >800 cp/ml, no clots or haemolysis, and stored for less than 24 hours at ambient temperature prior to collection from the laboratory for the study. The exclusion criterion was specimens with a CD4 count of <100 cells/µl, as they were required for routine tests.

1.3.2.2 Sample collection

HIV positive residual EDTA whole blood specimens (n=128) were obtained from Flow Cytometry Laboratory of the NHLS (Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, Gauteng, South Africa). Fifty-six samples were rejected: 28 samples had target not detected, 25 had VL < 600 cp/ml, and three had clotted or haemolysed blood. Plasma, DBS, PSC VLPlasma, and HSSE were prepared from 72 samples (**Figure 3**).

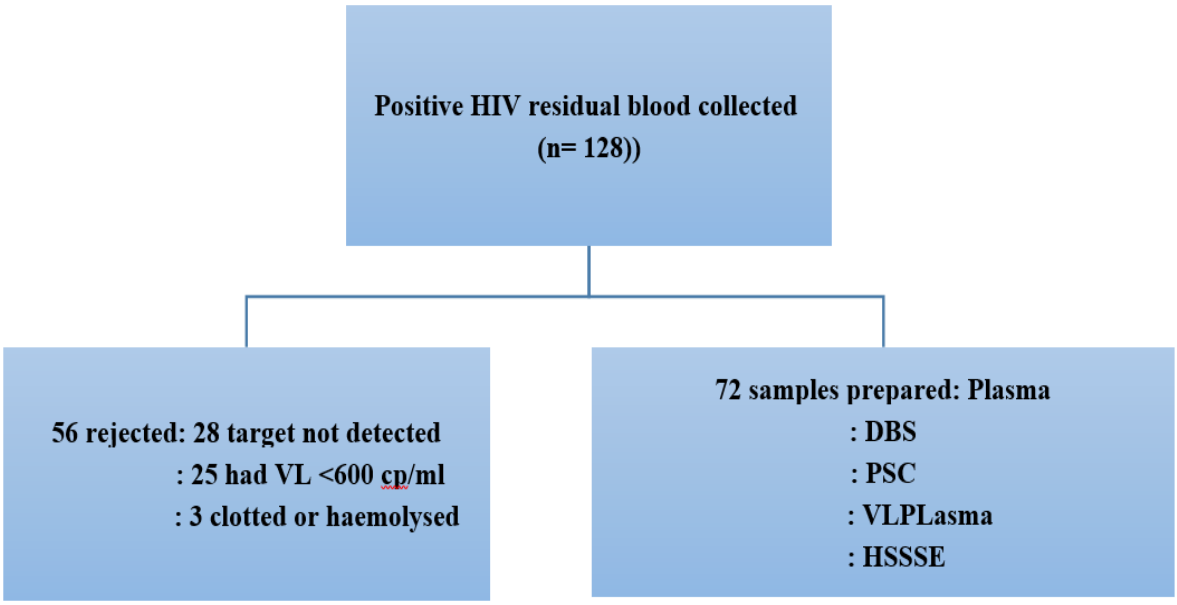


Figure 3: A breakdown of the total number of samples collected, selected according to the criteria and prepared. PSC: Plasma Separation Card; DBS: Dried Blood Spot; HSSE: HemaSpot SE

1.3.2.3 Sample preparation

For standardisation purposes all PSD (PSC, VLPlasma, and HSSE) samples, Plasma and DBS were prepared from a single 5 ml EDTA tube of residual blood, which was inverted five times prior to dispensing appropriate volumes using a precision pipette (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as outlined below (**Table 5**).

Table 5: Plasma, DBS, PSD preparation procedure.

Plasma	DBS	PSC	VLPlasma	HSSE
Whole blood samples were centrifuged at 2000g for 10 minutes, and 1ml plasma aliquoted into 2 ml microtubes (Merck KGaA, Darmstadt, Germany). The samples were stored at -80°C.	Three spots on the Whatman 903 five-spot protein saver DBS cards were prepared using 70 µl of whole blood per spot. The prepared samples were dried at room temperature overnight and stored at -80°C with a 2g desiccant pack in an impermeable, resealable plastic bag.	Three PSC spots were prepared as follows: whole blood (140 µl) was pipetted onto each of the three spots on the spotting layer of the PSC. The prepared samples were dried and stored as for DBS.	The VLPlasma samples were prepared as follows: 100 µl whole blood was pipetted into the sample cavity of the device and incubated for 3 minutes. Two drops from the supplied PBS ampoules were added into the sample cavity to facilitate blood separation. The prepared samples were dried and stored as for DBS.	HSSE was prepared as follows: 150 µl of whole blood was aliquoted onto the centre of the HSSE membrane and after two minutes the cassette was closed to prevent contamination. The prepared samples were dried and stored as for DBS.

1.3.3 Prospective EDTA whole blood

1.3.3.1 Selection criteria

HIV positive individuals visiting the Wits Reproductive Health and HIV Institute (WRHI, Johannesburg, Gauteng, South Africa) were selected for the study after obtaining informed consent.

1.3.3.2 Samples collection

Prospective specimens (n=275) with an unknown CD4 count and VL were collected from WRHI and shipped within 4 hours at ambient temperature to be processed at Clinical Laboratory Services (Braamfontein, Gauteng, South Africa) upon arrival.

1.3.3.3 Sample preparation

One 6ml BD Vacutainer® EDTA blood collection tube (BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) of prospective whole blood from each patient was used to prepare EDTA plasma, PSC, and DBS as described in **Table 5**. Plasma (n=275), PSC (n=275), and 258 DBS (shortage of DBS cards for 17 samples) were prepared as outlined in **Figure 4**.

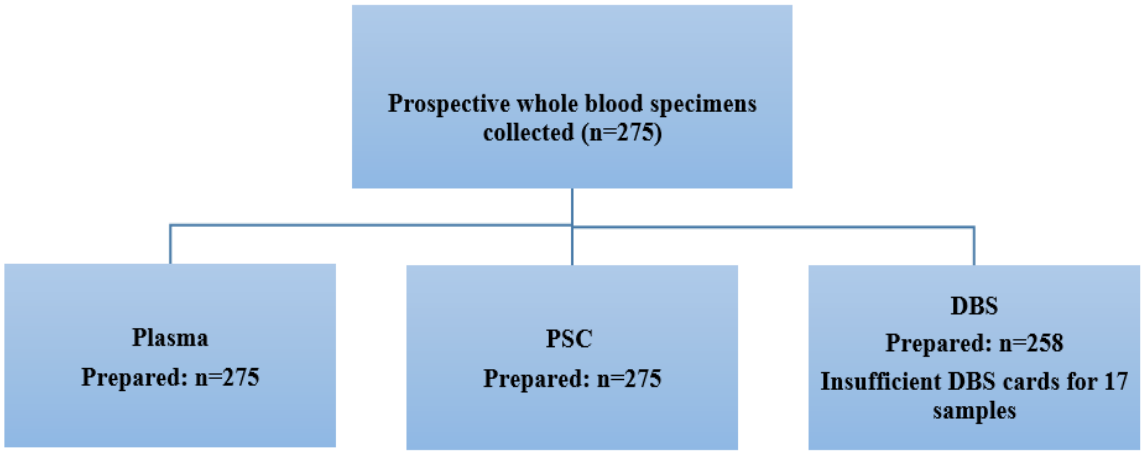


Figure 4: A breakdown of the total number of samples collected and prepared using prospective HIV positive whole blood samples. PSC: Plasma Separation Card; DBS: Dried Blood Spot

1.3.4 Instrument verification

Scott *et. al* (79) developed a 42-member plasma verification panel termed the South African Verification Quality Assessment (SAVQA) panel. The panel is made of bulk plasma lots: 17 negatives and five repeats positives with different VL ranges (500 cp/ml, 2.7 log cp/ml; 1000 cp/ml, 3.0 log cp/ml; 5000 cp/ml, 3.7 log cp/ml; 50000 cp/ml, 4.7 log cp/ml; and 100000 cp/ml, 5.0 log cp/ml). The purpose of the panel is to verify HIV testing platforms that are newly installed or moved and to evaluate assays for precision, accuracy, carryover (contamination of the platform) and limit of blank (the correct identification of HIV-negative specimens) (79).

To assess the ability of the instruments (GeneXpert and Abbott m2000) to process PSC eluate for VL monitoring, 3 SAVQA panel plasma samples with different known VL concentrations of log 3, log 3.7 and log 4.7 were selected. The PSC was prepared as described in **1.3.2.3**. The GeneXpert and Abbott m2000 instruments does not have a PSC elution and processing protocol for HIV VL testing. However, the Abbott m2000 has the DBS elution protocol (1.0 ml HIV-1 RNA DBS protocol). The 1.0 ml HIV-1 RNA DBS protocol was used for eluting PSC and processed using the RealTime 0.6 ml HIV-1 RNA plasma protocol for Abbott m2000 HIV VL testing. Regarding the GeneXpert, the elution protocol by Noble *et. al*., (80) was followed when eluting the PSC and it uses the Cobas Pre-Extraction Reagent buffer (SPER) (Roche Molecular Diagnostics, Pleasanton, CA, USA).

1.3.5 Molecular testing instruments

The Xpert HIV-1 VL, Cobas HIV-1 quantitative nucleic acid test and Realtime HIV-1 VL assays were followed as per the manufacturers' instructions when processing the specimens for testing on each respective molecular instrument (GeneXpert, Abbott m2000 and Cobas 8800).

1.3.5.1 GeneXpert HIV-1 VL testing

DBS and PSC processing on the GeneXpert instrument

The DBS and PSC spot were tested separately; the DBS protocol is illustrated in **Figure 5**. One DBS and PSC spot were placed separately into 2 ml Eppendorf tubes, and 1.5 ml of Cobas Pre-Extraction Reagent buffer (SPER) added, according to an in-house protocol adapted from the Cepheid DBS protocol by Noble *et. al* (80). The specimens were incubated in elution buffer for 15 minutes on a thermomixer (Eppendorf AG, Hamburg, Germany) set at 500 rpm and 56℃. Pasteur pipettes supplied with the kit were used to aliquot 1.2 ml of the lysate of each PSC into the Xpert HIV-1 VL cartridges. The cartridges with DBS or PSC lysates were loaded into the GeneXpert Dx instrument as per the protocol developed by the manufacturer (Cepheid, Sunnyvale, CA, USA), processed, and results recorded.



Figure 5: The process of quantitating the VL of the DBS on the GeneXpert instrument. A similar procedure was followed when testing the PSC. Figure from Jacon P, 2016 (81).

1.3.5.2 Abbott m2000 HIV-1 VL testing

The DBS, PSD and plasma were processed in separate batches on the instrument. DBS were processed following the 1.0 ml HIV-1 RNA DBS protocol, while plasma and PSD were processed following the 0.6 ml HIV-1 RNA plasma protocol.

Elution procedure

DBS and PSD elution: The m2000 DBS elution protocol was used to elute nucleic acids from DBS and PSD (PSC spots, VLPlasma, and HSSE). Briefly, DBS spots, PSC spots, VLPlasma filter pads, and HSSE tails were placed separately into Abbott master mix tubes. Abbott DBS buffer (1.3 ml) was aliquoted into each tube and incubated at 55℃ for 30 minutes on a heating block (**Figure 6**). The PSC spots, VLPlasma filter pads, HSSE tails and DBS were removed from the master mix tubes, the tubes were centrifuged at 2000 g and 24℃ for 10 minutes.

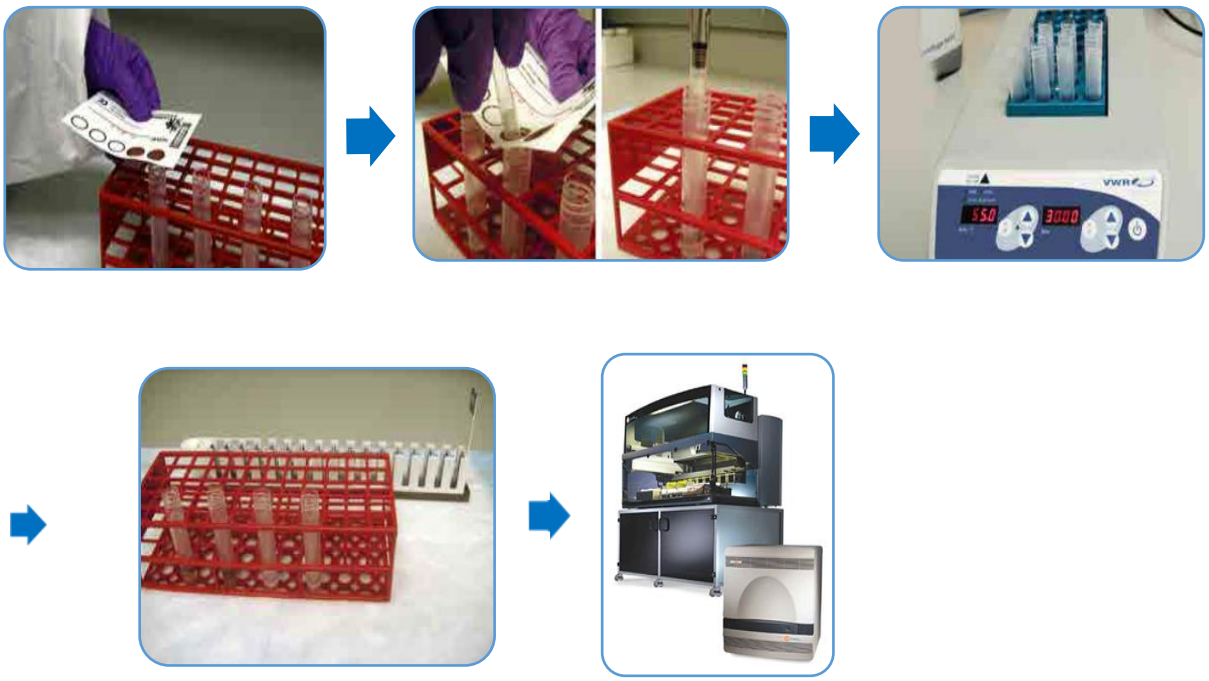


Figure 6*:* The process of testing DBS on the Abbott m2000 (82). The same procedure was followed when quantitating VL from PSC, HSSE and VLPlasma.

Extraction and master mix procedures

Briefly, plasma, DBS and PSD eluates were placed on the instrument in separate runs. Assay controls (negative control, low and high positive controls), calibrators (A and B) and sample master mix tubes were placed on a 13 mm diameter rack and then placed on the instrument. The internal control (armored RNA, 500 µl) was pipetted into one bottle of mLysis buffer and this, along with the other sample preparation consumables (microparticles, mLysis,

mWash 1, mWash 2 and mElution buffer), were inverted ten times and loaded on the Abbott m2000sp instrument with the reagent vessels. Thereafter, the extraction protocol was initiated. After sample extraction, the amplification reagents were loaded onto the instrument with the PCR plate and the master mix protocol was initiated.

Amplification procedure

To amplify the extracted HIV RNA, the PCR plate was sealed and transferred to the Abbott m2000rt, the amplification protocol initiated, and results recorded.

1.3.5.3 Cobas 8800 HIV-1 VL testing

Elution procedure

RNA was eluted by placing PSCs into Cryo.s 5 ml tubes (Roche Diagnostics) (**Figure 7**) separately thereafter, 1.3 ml of Cobas Pre-Extraction Reagent (SPER; Roche Diagnostics) added to each tube. The tubes were capped then incubated at 56℃ for 10 minutes using the Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany) set at 1000 rpm.

Extraction and master mix procedures

The PSC is validated for use on the Cobas 8800 instrument (**Figure 7)**. The reagents and consumables were loaded as per the manufacturer's instructions. Wash reagents, lysis reagents and diluents, processing plates, and amplification plates were loaded onto the instrument. Furthermore, the magnetic glass particles, control cassettes (negative control, low and high positive controls), and tip racks were also loaded onto the instrument as well as the rack for waste and blocked tips. The tubes with 1150 µl PSC lysates of which 850 µl is used for processing were transferred onto racks, de-capped and then loaded onto a Cobas 8800 system, and the run started.

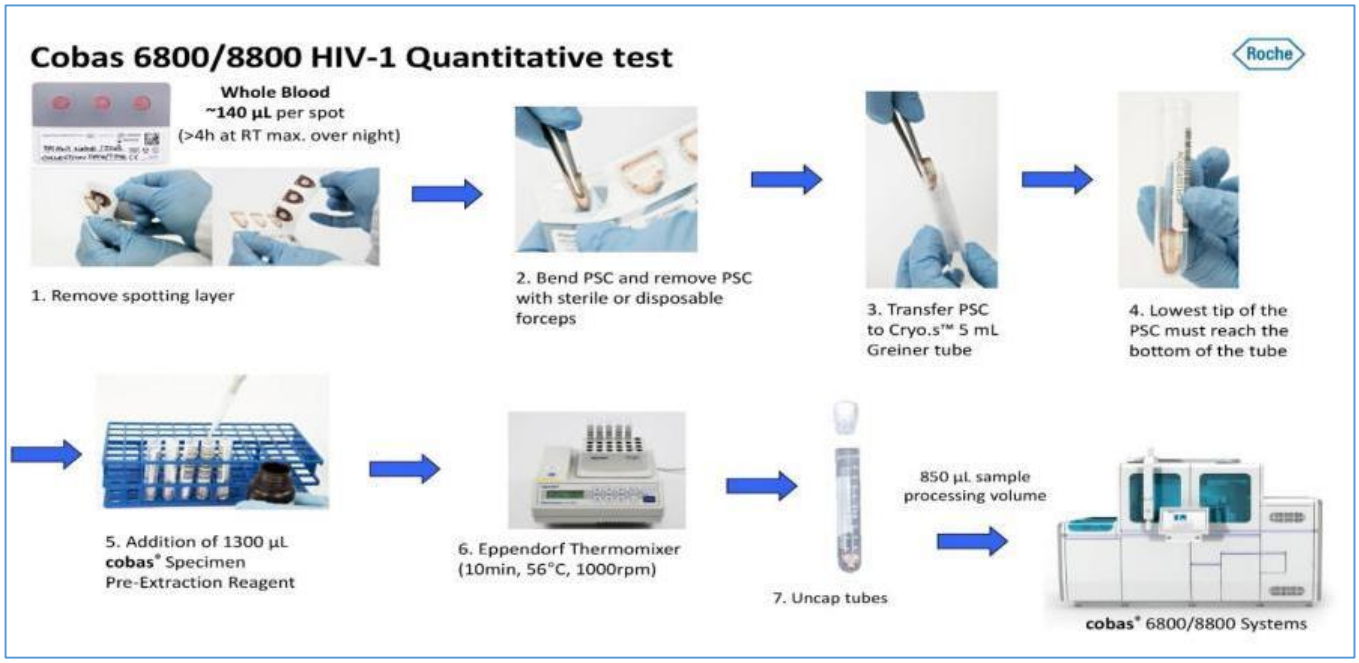


Figure 7*:* The procedure of processing the PSC on the Cobas 68/8800. Figure adapted from Roche, 2020 (83).

CHAPTER 1.4 - DATA ANALYSIS

MedCalc software Version 19.0.5 (Ostend, Belgium) and Microsoft Excel (Microsoft, Redmond, Washington, United States) were used for all data and statistical analysis. Plasma is the gold standard specimen type (reference). PSC, DBS, Hemaspot and VLPlasma VL results were compared to those of the reference.

1.4.1 VL Testing

The sensitivity, specificity, and positive and negative predictive values were calculated to assess treatment failure at the clinically relevant range of log 3 cp/ml (1000 cp/ml). The misclassifications were calculated, representing the VL values incorrectly identified as above or below 1000 cp/ml compared to the plasma specimen (66). The strength of agreement between PSC and plasma, DBS measurements and plasma, was assessed using concordance correlation, which evaluates the alignment of the data pairs on the 45° line from the origin (84), and a Bland-Altman plot, which plots the difference between the two methods against their mean (85).The percentage similarity was used to assess the similarities between the DBS, PSC and plasma results (86). The agreement between plasma and DBS/PSC was also visualised using Passing-Bablok plots, which determine the degree of alignment of the data pairs on the best fit line (precision) and the alignment of the best fit line on the 45° line from the origin (accuracy) (87).

The correction factor used for PSC was [Absolute VL * (1300/70) *(1300/600)] and HSSE was [Absolute VL * (1300/75) *(1300/600)] while for VLplasma it was [Absolute VL * (1300/50)].

Currently, the statistical methods for evaluating new methods for HIV VL testing are not standardised. We reviewed the different method comparison statistics to understand and form a standardised framework of new method comparison. This is seen in the manuscript (**Appendix 1.4**), which is intended for submission to the African Journal of Laboratory Medicine at the end of September 2022.

CHAPTER 1.5 - RESULTS

1.5.1 Instrument verification

To verify the GeneXpert and Abbott m2000 instruments, a SAVQA reference panel of plasma with known VLs was used to make PSCs, which were processed on the GeneXpert and the Abbott m2000 instruments. The PSC specimens were compared to the original SAVQA panel plasma results, GeneXpert reported an average of 1 log cp/ml lower than the reference VL concentrations; in contrast, Abbott m2000 results were 0.3 log cp/ml lower **(Figure 8)**.

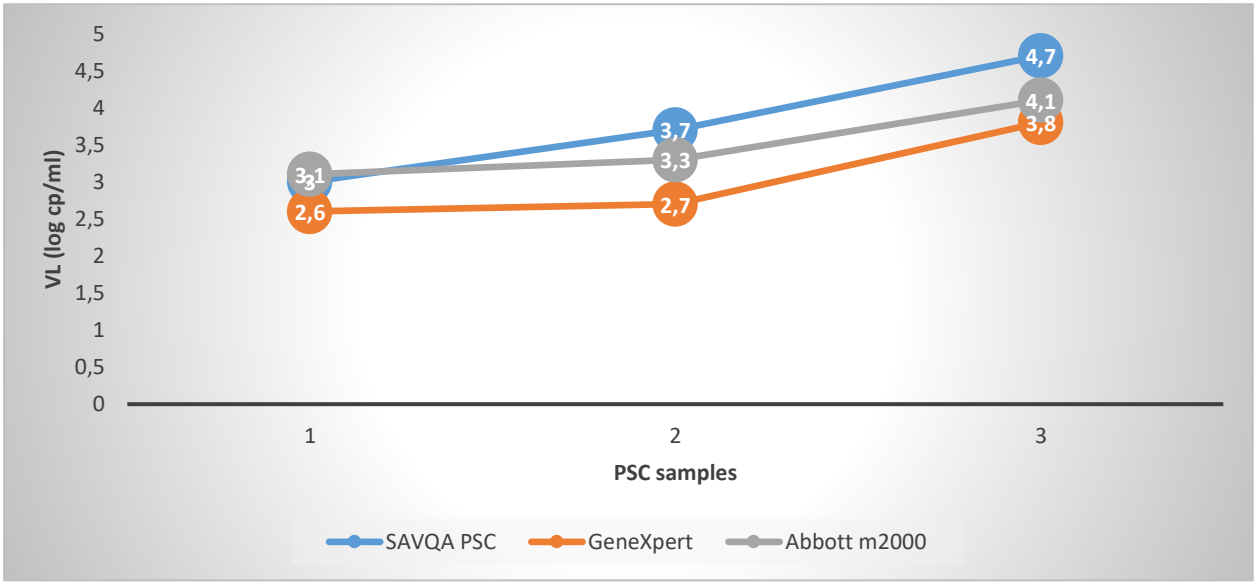


Figure 8: SAVQA PSC processed on GeneXpert and the Abbott m2000 compared to the original SAVQA plasma panel results. Y-axis is the log VL concentrations measured in cp/ml, and X-axis shows the three samples. The results are sorted in ascending order using the SAVQA PSC results.

1.5.2 Residual Whole Blood Results Overview

PSDs (PSC, VLPlasma and HSSE) and DBS were processed, and their VL results were compared to plasma. Overall, 72 plasma samples were processed on the Abbott m2000, and 51 had detectable VL, five had an undetectable VL and 16 showed instrument related errors. PSD (n=51) and DBS (n=50, one sample missing) paired to the 51 detectable plasma samples were also processed on the Abbott m2000 instrument (**Figure 9**). DBS had 49 detected VL results and zero errors compared to the rest of the PSD. Plasma, VLPlasma and HSSE samples were insufficient for processing on the GeneXpert instrument and only 12 PSC samples and 10 DBS were processed on the GeneXpert. Plasma SOC results from the Cobas 8800 were used as a reference when comparing the GeneXpert results.

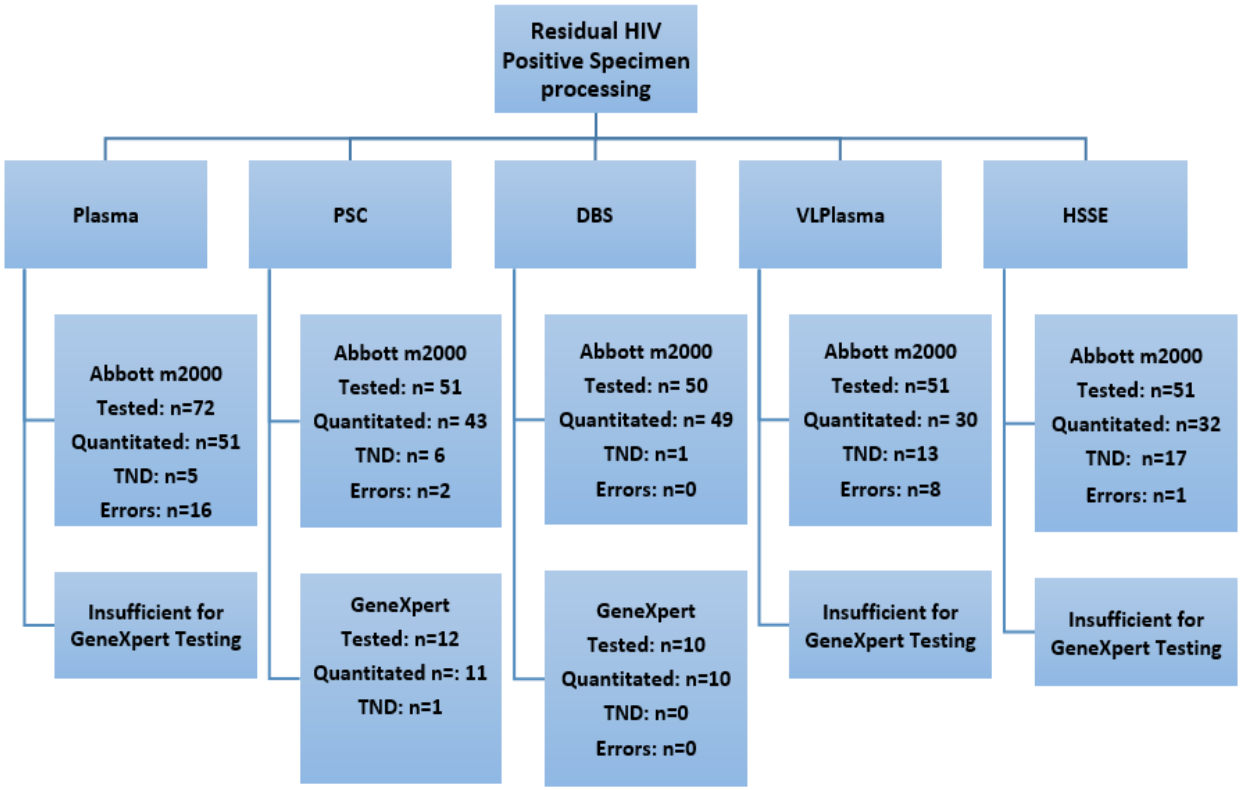


Figure 9: Plasma, PSD and DBS from residual HIV VL samples were processed on the Abbott m2000 and GeneXpert instruments. PSC: Plasma Separation Card; DBS: Dried Blood Spot; HSSE: HemaSpot SE; TND: Target Not Detected

1.5.3 Residual HIV positive whole blood processed on the GeneXpert

1.5.3.1 DBS and PSC VL compared to SOC plasma on a Scatter plot

The VL results of DBS and PSC processed on the GeneXpert were compared to the cobas SOC plasma results on a scatter plot (**Figure 10**). At plasma VL concentrations of ≥ 3 log cp/ml, PSC had one undetectable VL and downward misclassifications of n=4 combined with DBS results. Both showed a similar pattern of VL results lower than that of the SOC plasma results. At <3 log cp/ml, both PSC (n=1) and plasma SOC (n=1) results were similar **(Figure 10).**

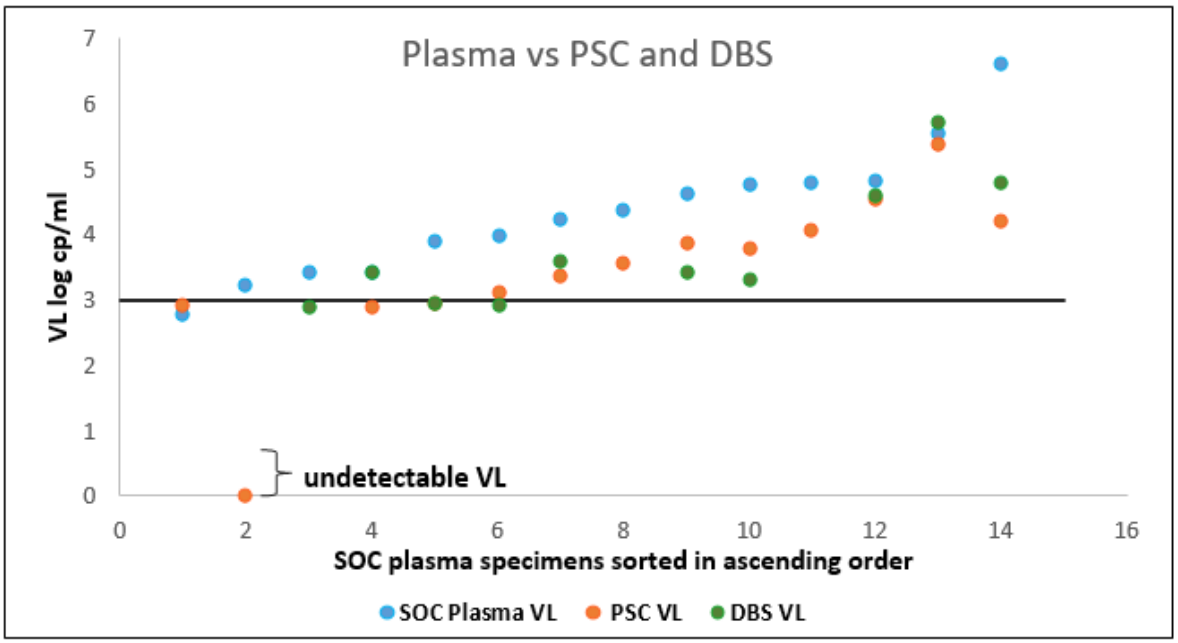


Figure 10: PSC and DBS GeneXpert VL results compared to the reference (SOC plasma) VL results. The vertical axis represents the log VL, and the horizontal axis represents the specimens sorted in ascending order using SOC plasma results (cp/mL). The black horizontal line represents the clinically relevant 3 log cp/ml threshold. Undetected VL values are at 0.

1.5.3.2 Qualitative and quantitative comparison of PSC and DBS with SOC plasma

The DBS sample size was insufficient with only ten samples available for calculating the sensitivity and specificity. The PSC sample size (n=12) was small but sufficient to calculate sensitivity, specificity, accuracy, and misclassification. At the clinically relevant range of log 3 cp/ml (**Table 6**), which determines treatment success or failure, the sensitivity of PSC was 75% and specificity of 100%, with positive predictive value (PPV) and negative predictive value (NPV) of 100% and 25% respectively. Furthermore, PCS reported accuracy of 76.92%. DBS reported a higher percentage similarity and concordance correlation of 93.5% and 0.58.

Table 6: PSC and DBS compared to plasma at log 3 cp/ml.

Specimen type	PSC (N=12)	DBS (N=10)
Sensitivity	75.00% (42.81% to 94.51%)	-
Specificity	100.00% (2.50% to 100.00%)	-
Positive predictive value	100%	-
Negative predictive value	25.00% (11.12% to 47.04%)	-
Accuracy	76.92% (46.19% to 94.96%)	-
Misclassifications	3/13 (23.08%)	-
Percentage similarity	89.6%	93.5%
Concordance coefficient	0.52 (0.16 to 0.76)	0.58 (0.14 to 0.82)

1.5.3.3 Bland Altman plot comparison of PSC and DBS with SOC plasma

The strength of agreement between log PSC and log DBS compared to SOC plasma results is illustrated using Bland Altman plots (**Figure 11.1** and **11.2**). Both PSC and DBS results had lower VL concentrations compared to SOC plasma, with a bias of (-0.78 log cp/ml) and limit of agreement (LOA) of -2.00 log cp/ml to 0.44 log cp/ml while DBS had a bias of -0.77 log cp/ml and LOA of -2.03 log cp/ml to 0.48 log cp/ml. Furthermore, DBS (93.5%) had a better percentage similarity as opposed to the PSC (89.6%) when compared to SOC plasma (**Table 5**). However, both had poor strength of agreement with concordance correlation of DBS 0.58 (0.14 to 0.82) and PSC 0.52 (0.16 to 0.76).

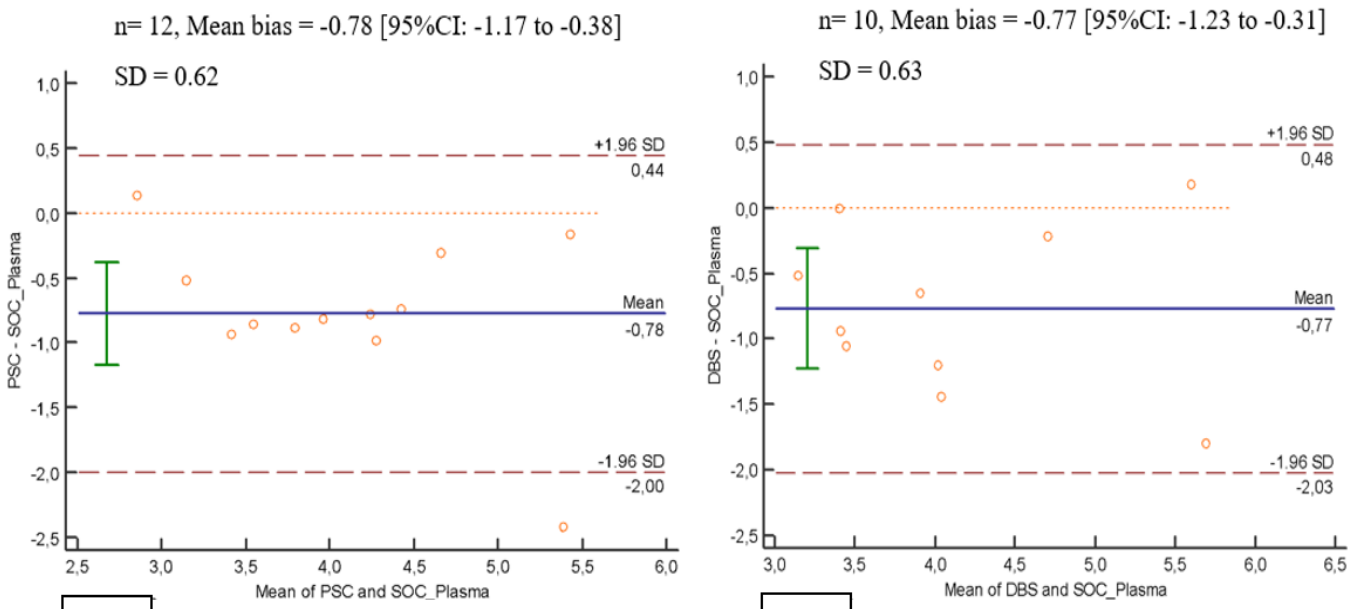


Figure 11.1 - 2: A Bland Altman plot of PSC and DBS GeneXpert VL compared to the SOC plasma reference values processed on cobas 8800. The mean bias (solid line) and limit of agreement (dotted lines) for both PSC and DBS are recorded in log cp/ml**.**

1.5.4 Residual HIV positive whole blood processed on Abbott m2000

The statistical analysis was prepared following the "**Best practices for statistical method comparison to evaluate new plasma separation devices as alternatives to plasma-based HIV viral load monitoring**" manuscript mentioned in **Appendix 1.4**, which delineated and formed a framework of the best statistical methods to use for method comparison. This framework is for standardising statistical analysis to compare new methods to gold-standard references in VL monitoring.

1.5.4.1 The detection rate of PSD and DBS from residual blood on the Abbott m2000

The detection rate is the ability of the instrument to detect the HIV VL. Compared to the detected plasma VL concentrations (n=51), DBS had a higher detection rate followed by PSC. In contrast, HSSE and VLPlasma had the lowest detection rates. VLPlasma had the highest error rate due to instrument clogging while DBS had no errors (**Table 7**).

Table 7: Detection rates of PSD and DBS VL compared to the reference (plasma) VL.

Specimen type	Tested Paired VL	Detectable VL	Percentage of detectable VL	Error Rate
DBS	50	49	98%	-
PSC	51	43	84%	3.9% (2/51)
HSSE	51	32	63%	2 % (1/51)
VLPlasma	51	30	59%	15.7% (8/51)

1.5.4.2 PSD and DBS VL compared to the reference (plasma) VL on a scatter plot

The PSD and DBS VL concentrations were individually compared to the absolute plasma VL concentrations to assess the trend specifically at the clinically relevant range of 3 log cp/ml. PSC concentrations at VL of ≥ 3 log cp/ml showed a pattern of lower VL than the plasma VL concentrations and had few similar VL concentrations **(Figure 12.1**). The limit of detection of PSC on the Abbott m2000 has not been established yet, but **Figure 12.1** illustrates that PSC is not sensitive enough to detect VL <3 log cp/ml as all (n=6) of the PSC values <3 log cp/ml were undetectable. **Figure 12.2** illustrates a pattern of similar VL concentrations of VLPlasma compared to plasma at VL of ≥ 3 log cp/ml while at <3 log cp/ml, 13 VLPlasma samples had an undetectable VL. The VL concentration of one sample in both VLPlasma and HSSE scatter plots was incorrectly overestimated. At ≥3 log cp/ml, HSSE underestimated the plasma values (**Figure 12.3).** DBS VL concentrations were higher compared to plasma VL results at the ≥ 3 log cp/ml. Furthermore, only one sample had an undetectable VL at <3 log cp/ml (**Figure 12.4**).

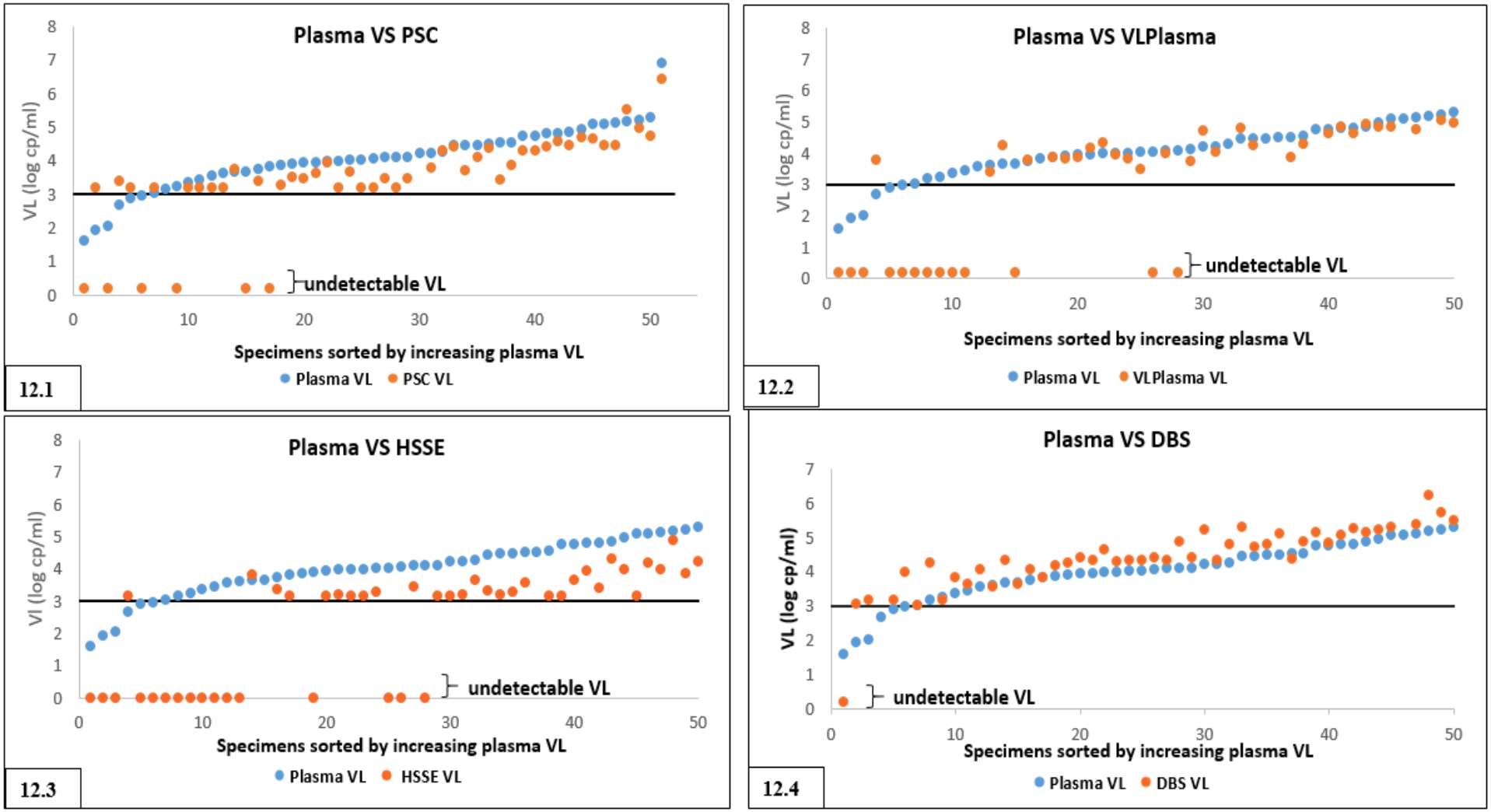


Figure 12.1- 4: PSD and DBS VL compared to the reference plasma specimen. The dark horizontal line represents the clinically relevant range of virological success or failure. The VL values are logged and sorted in ascending order according to plasma VL.

1.5.4.3 Qualitative and quantitative comparison of PSD and DBS with plasma

The sensitivity and specificity of PSC, VLPlasma, HSSE and DBS were reported at the clinically relevant range of 3 log cp/ml to determine virological failure or success (**Table 8**). DBS had highest sensitivity (100%) but with low specificity (20%), followed by PSC (93%) while VLplasma and HSSE were the most specific with similar specificities of 83% (**Table 8**). The PPV and NPV of PSC, VLPlasma, HSSE and DBS were 93.02% and 50.00%; 96.67% and 38.46%; 96.88% and 31.25%; 89.8% and 100.00. PSC had the closest similarity to plasma (98%), with the rest of the devices reporting much bigger differences compared to plasma.

Table 8: Assessment of the qualitative agreement between PSD and DBS VL to the reference plasma.

Plasma	PSC (N = 49)	VLPlasma (N= 43)	HSSE (N= 50)	DBS (N= 50)
Sensitivity	93.02% (80.94% to 98.54%)	78.38% (61.79% to 90.17%)	73.81% (57.96% to 86.14%	100.00% (91.96% to 100.00%)
Specificity	50.00% (11.81% to 88.19%)	83.33% (35.88% to 99.58%)	83.33% (35.88% to 99.58%)	16.7% (0.4% to 64.1%)
PPV	93.02% (85.64% to 96.75%)	96.67% (82.78% to 99.43%)	96.88% (83.70% to 99.47%l	89.8% (77.8% to 96.6%)
NPV	50.00% (20.53% to 79.47%)	38.46% (23.50% to 55.98%)	31.25% (19.63% to 45.83%)	100.00%
Accuracy	87.76% (75.23% to 95.37%)	79.07% (63.96% to 89.96%)	75.00% (60.40% to 86.36%)	91.84% (80.40% to 97.73%)
Concordance Coefficient (Pc)	0.78 (0.64 to 0.86)	0.67 (0.45 to 0.81)	0.42 (0.24 to 0.57)	0.82 (0.73 to 0.88)
Misclassification	6/49 (12%)	9/43 (21%)	12/48 (25%)	4/44 (9%)
Percentage similarity	98.22%	103.68%	94.58%	105.76%

1.5.4.4 Bland Altman plot comparison of PSD and DBS with plasma

PSD and DBS VL concentrations were compared to plasma VL concentrations using the Bland Altman plots to determine their strength of agreement. DBS (**Figure 13.1**) had a smaller variation around the mean bias (SD = ±0.33). HSSE (**Figure 13.2**) had a highest mean bias (-0.88) thus reports lower VL concentrations than plasma. The PSC (**Figure 13.3**) had the second lowest mean bias of log -0.32. VLPlasma (**Figure 13.4**) had a lowest mean bias of log -0.2 illustrating a similar VL concentration to plasma VL.

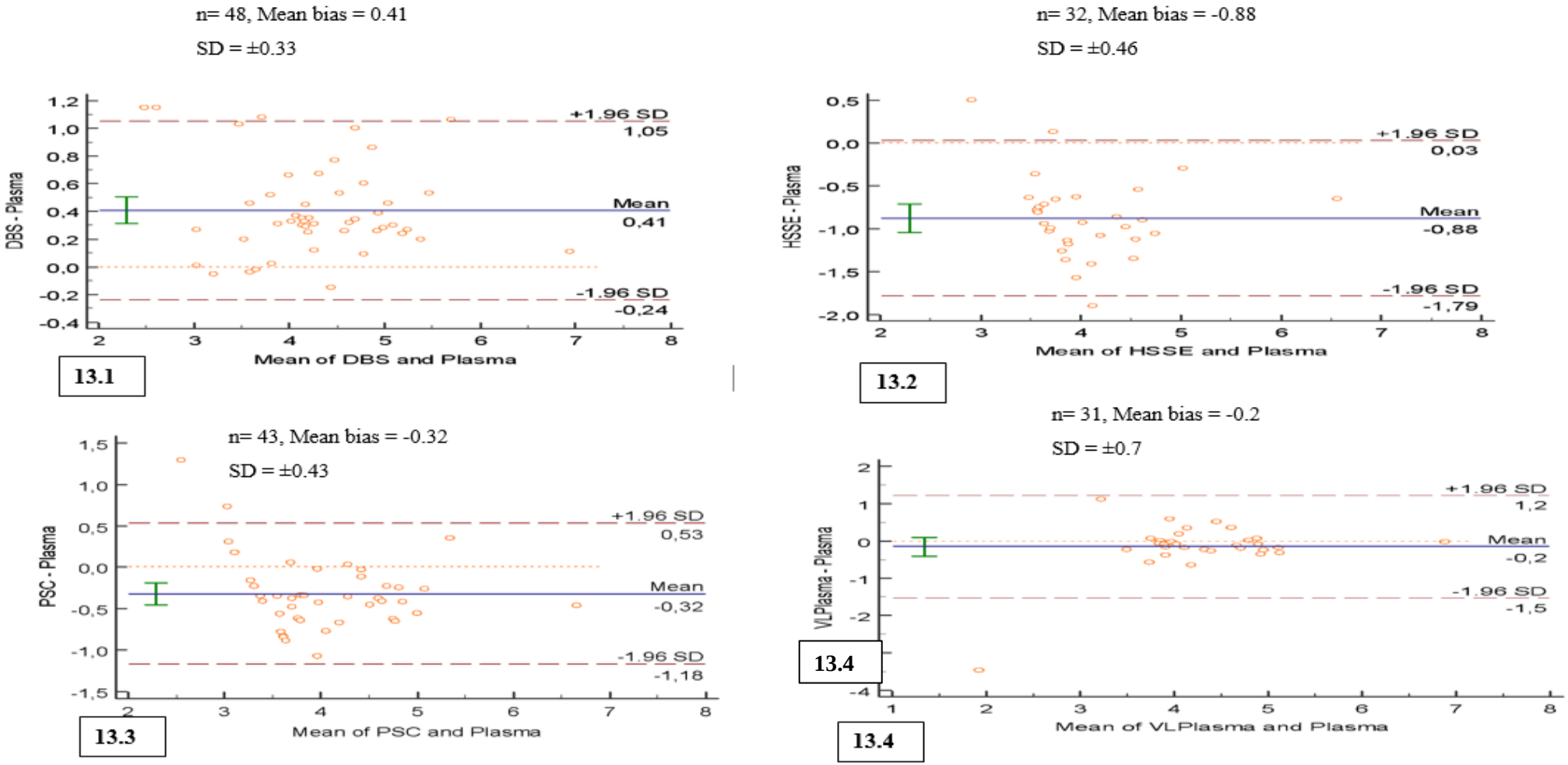


Figure 13.1- 4: The Bland Altman plot of the PSD and DBS VL compared to plasma to evaluate the strength of agreement between the new alternative specimen types (PSD) and existing methods (DBS and plasma). The mean bias is the solid horizontal line and Limit of Agreement (LOA) is the dotted horizontal lines shown on the graphs.

1.5.4.5 Passing Bablok plot comparison of PSD and DBS with plasma

The Passing Bablok plot measures accuracy and precision and provides the overall strength of agreement between measured variables. **Figure 14.1** depicts the log DBS compared to log plasma VL concentrations; DBS is precise but not accurate as it overestimates the plasma values as shown by the clustering of the values above the best-fit line. **Figure 14.2** illustrates that VLPlasma is accurate and precise with most values clustered around the best-fit line. HSSE of the values are clustered below the line of best fit (**Figure 14.3**). PSC is accurate and precise, with most values scattered around the line of best fit similar to VLPlasma (**Figure 14.4**).

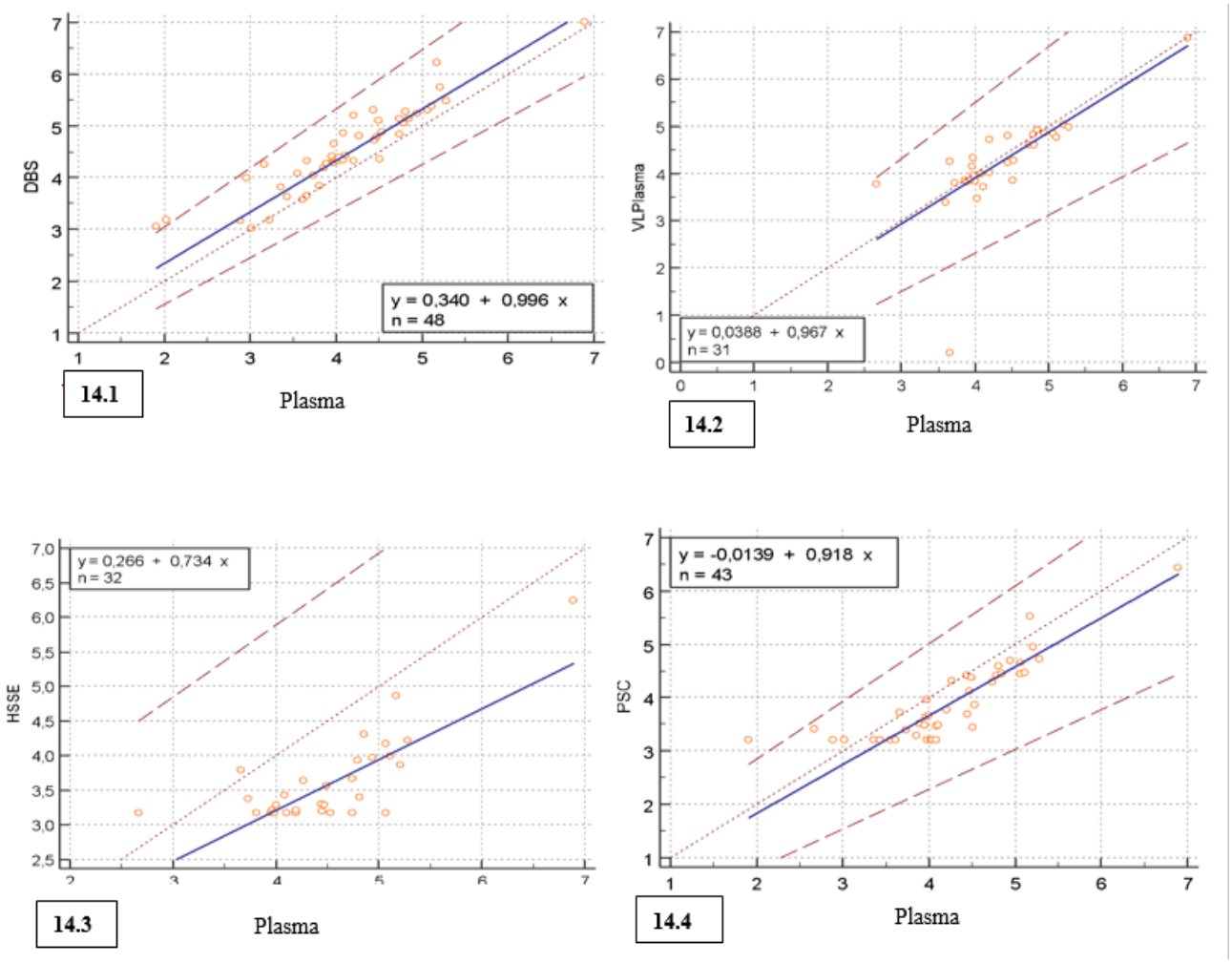


Figure 14.1- 4: Passing Bablok plots for the visualization of PSD and DBS VL to Plasma VL. The blue line represents the best fit line (precision), and the dotted line represents the 45° line from the origin (accuracy)

1.5.4 Prospective Whole Blood Results Overview

The residual HIV positive whole blood specimen evaluation of the PSDs was further investigated in South Africa for the PSC and DBS products using prospectively collected specimens. Through collaboration with the iLEAD Mozambique team (innovation in Laboratory Engineered Accelerated Diagnostics supported by the Bill and Melinda Foundation), the PSC was further investigated and compared to DBS as described in **Figure 15**. The DBS and PSC samples were processed using the Abbott m2000 and Cobas 8800 instruments. Out of 258 PSC samples processed on the Cobas 8800 instrument, only 95 PSC samples had a detectable VL. Plasma (n=91, four samples insufficient), DBS (n=91, four samples insufficient) and PSC (n=95) paired to the detectable PSC samples were further processed on the Abbott m2000 instrument. Plasma had 85 samples with a detectable VL, DBS had 77 samples with detectable VL and PSC had 72 samples with detectable VL. There were no errors from the plasma specimens, while PSC and DBS samples produced 2 and 3 errors, respectively.

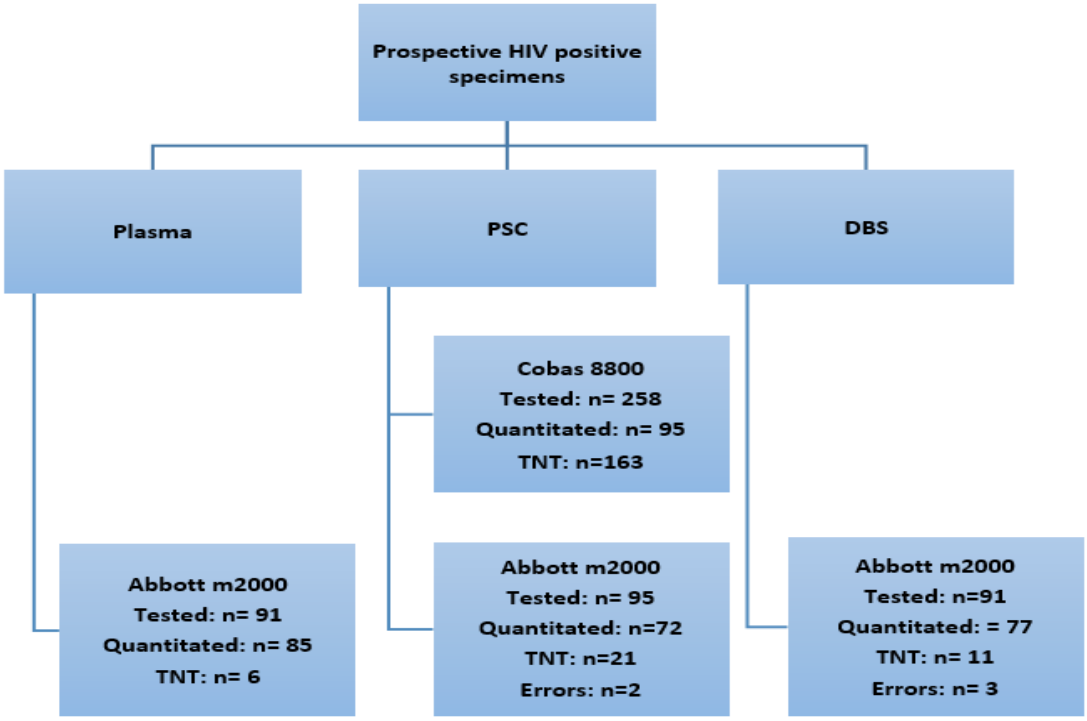


Figure 15: Overview of the processed plasma, DBS and PSC from prospective HIV positive blood samples. PSC was processed on the cobas 8800 (reference instrument) and Abbott m2000 (novel instrument), plasma was processed on the Abbott m2000 (reference instrument), and DBS was processed on the Abbott m2000 (reference instrument). PSC: Plasma Separation Card; DBS: Dried Blood Spot; TNT: Target Not Detected

1.5.5 Prospective HIV positive whole blood processed on the Abbott m2000

1.5.5.1 Abbott PSC and Cobas PSC compared to the reference (Abbott plasma) VL on a scatter plot

The Abbott m2000 log PSC results were compared to the Cobas log PSC results on a scatter plot using Abbott m2000 log plasma results as the reference (**Figure 16**). At >3 log cp/ml, the Abbott log PSC reported lower VL concentrations to plasma with some downward misclassifications (undetectable VL), while the Cobas log PSC VL concentrations were higher than plasma. The PSC has been validated for use on the Cobas 8800, the limit of detection of the Cobas PSC on the Cobas 8800 is 738 cp/ml as reported by the manufacturer; this is illustrated below by two samples with detected VL concentrations at < 3 log cp/ml. In contrast, similar to the residual blood sample results in **1.5.4.2**, the Abbott m2000 log PSC was undetected at < 3 log cp/ml as the PSC has not yet been evaluated and validated for use on the Abbott m2000 instrument, so the PSC concentrations have not yet been adjusted for the instrument.

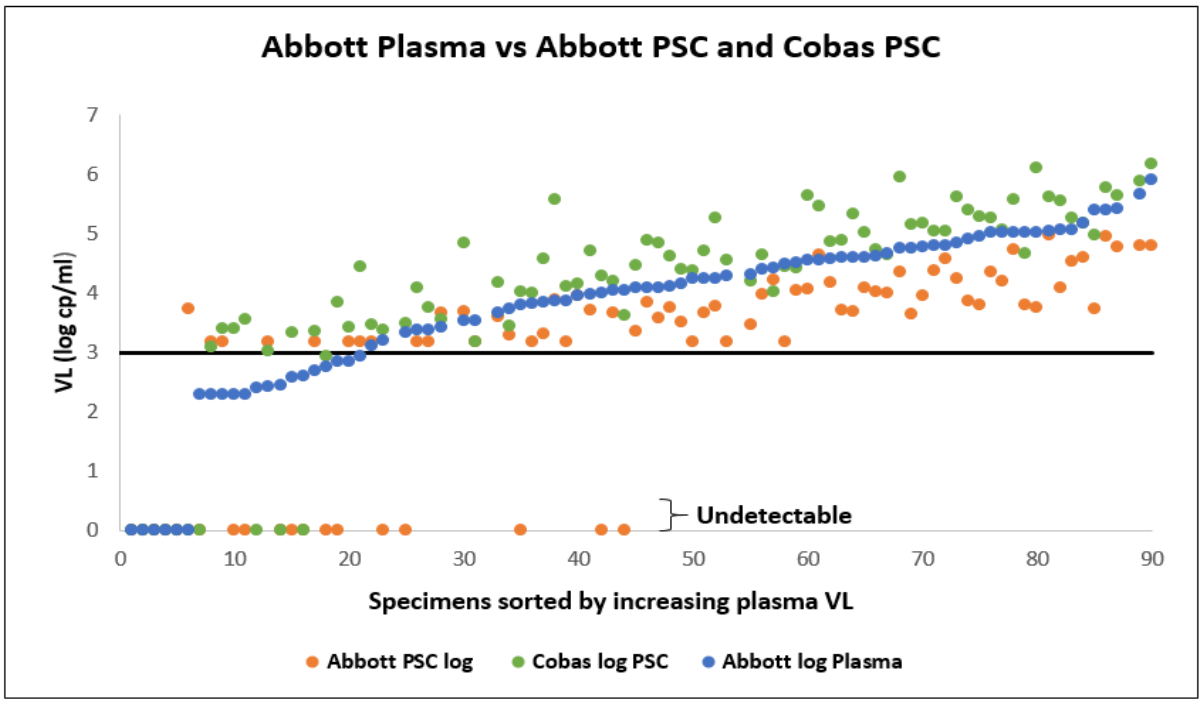
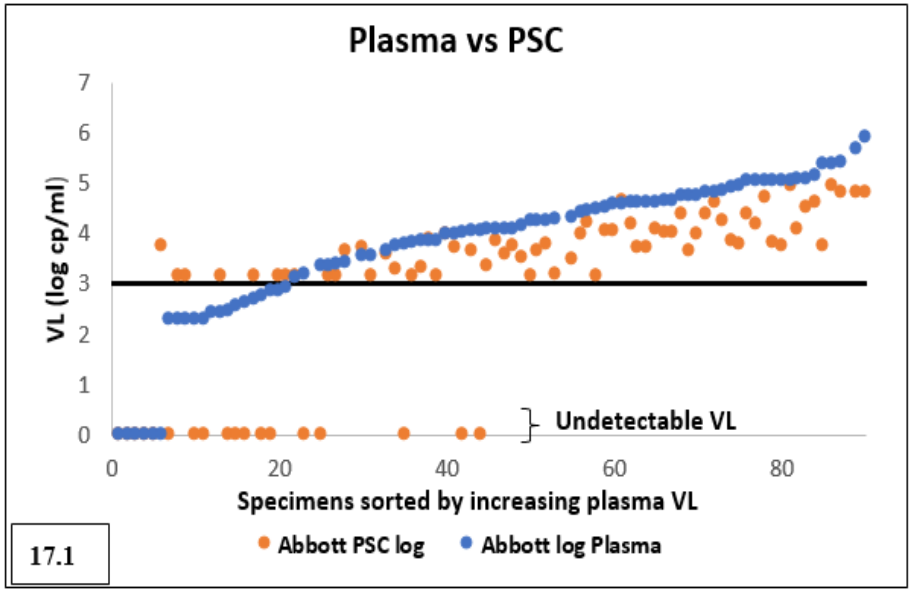


Figure 16: Cobas PSC VL results and Abbott m2000 PSC VL compared to Abbott m2000 plasma results sorted by increasing plasma results. The dark black horizontal line represents the clinically relevant threshold of 3 log cp/ml. The vertical axis is the logged VL values. The undetectable results are illustrated at the bottom of the chart.

1.5.5.2 Abbott PSC and DBS VL compared to the reference (Abbott plasma) VL on a scatter plot

Abbott PSC was compared to plasma using a scatter plot (**Figure 17.1).** PSC VL concentrations below 3 log cp/ml were undetected thus PSC misclassified the detectable plasma VL concentrations at < 3 log cp/ml. At ≥ 3 log cp/ml, PSC results were lower than those of plasma with some misclassifications, while some results were similar to those of plasma. Overall, PSC followed the increasing pattern of plasma VL results.

DBS and Plasma were compared using a scatter plot (**Figure 17.2**). At plasma VL concentrations of < 3 log cp/ml, some DBS (four) VL concentrations were detected as DBS as supported by the limit of detection of 839 cp/ml. However, upward misclassifications were noted at plasma VL concentrations of < 3 log cp/ml. At plasma VL > 3 log cp/ml, the DBS results follow the increasing plasma VL concentration pattern with an overall spread pattern around them (plasma) with few misclassifications.



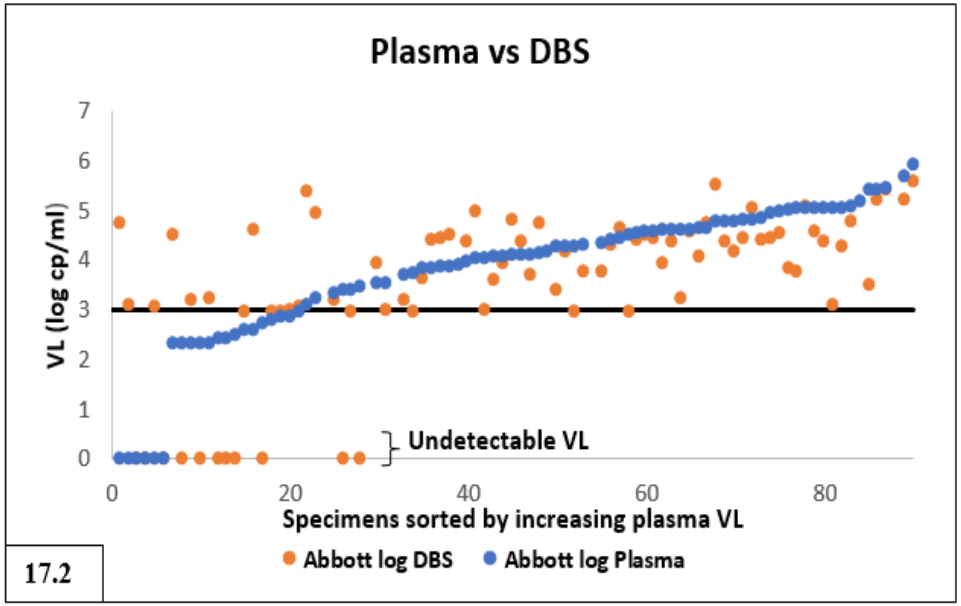


Figure 17.1-2: Abbott PSC and DBS VL compared to the reference plasma sample. The VL values are logged and sorted in ascending order using the plasma results, and the undetectable VL are indicated at the bottom of the plots. The solid dark horizontal line represents the clinically relevant range of 3 log cp/ml.

1.5.5.3 Qualitative and quantitative comparison of PSC and DBS with plasma

To assess the identification of treatment failure or success at the clinically relevant threshold of log 3 cp/ml (1000 cp/ml), the specificity, sensitivity, PPV, NPV, accuracy, misclassification and error rate were reported (**Table 9)**. PSC reported higher sensitivity (92.2%) and specificity (65.00%) than DBS (87.1% and 61.9%). Furthermore, PSC is more sensitive (82.4%) at lower VL and more specific (100%) at higher VL while DBS has an average sensitivity and specificity. Both PSC and DBS have an accuracy of ≥ 80% but a poor (0.59 and 0.49) strength of agreement. Overall, DBS was more similar (93.9%) to plasma than PSC (89%).

Table 9: PSC and DBS VL compared to plasma at the clinical threshold of log 3 cp/ml.

	PSC (N=72)	**DBS (N=77)**
Sensitivity (Overall)	92.2% (82.7% to 97.4%)	87.1% (76.1%to 94.3%)
At log 3	82.4% (56.6% to 96.2%)	68.8% (41.3% to 89.0%)
At log 4	46.8% (32.1% to 61.9%)	67.4% (52.0% to 80.5%)
Specificity (Overall)	65.00% (40.8% to 84.6%)	61.9% (38.4% to 81.9 %)
At log 3	65.0% (40.8% to 84.6%)	61.9% (38.4% to 81.9%)
At log 4	100.0% (90.5% to 100.0%)	73.0% (55.9% to 86.2%)
PPV (Overall)	89.4% (79.4% to 95.6%)	87.1% (76.1% to 94.3 %)
At log 3	66.7% (43.0% to 85.4%)	57.9% (33.5% to 79.7%)
At log 4	100.0% (84.6% to 100.0%)	75.6% (59.7% to 87.6%)
NPV (Overall)	72.2% (46.5% to 90.3%)	61.9% (38.4% to 81.9% 72.2% (46.5% to 79.7%)
At log 3	81.3% (54.4% to 96.0%)	64.3% (48.8% to 78.4%)
At log 4	59.7% (46.4% to 71.9%)	
Accuracy	86.90% (77.78% to 93.28%)	80.72% (70.59% to 88.56%)
Pc	0.59 (0.45 to 0.73)	0.49 (0.33 to 0.66)
Misclassification	11/84 (13%)	16/83 (19%)
Error Rate	2/86 (2.3%)	3/86 (3.5%)
Percentage similarity	89.0%	93.9%

Pc: Concordance correlation

1.5.5.4 Bland Altman and Passing Bablok plots comparison of PSC and DBS with plasma

The strength of agreement between reference plasma and PSC and DBS was assessed visually using the Bland Altman and Passing Bablok plots (**Figures 181-4**). PSC results had a difference of 0.46 compared to plasma results with a narrow SD (± 0.52) and three outliers. DBS results had a smaller mean bias (0.12) compared to plasma results but had high variability around the mean SD (±0.8). The Passing Bablok illustrates that PSC is precise due to the values falling on the line of best fit, but it is not accurate as most VL concentrations aggregate below the 45° line. DBS is reported as neither precise nor accurate as most of the values are not spread on the line of best fit nor on the 45° line.

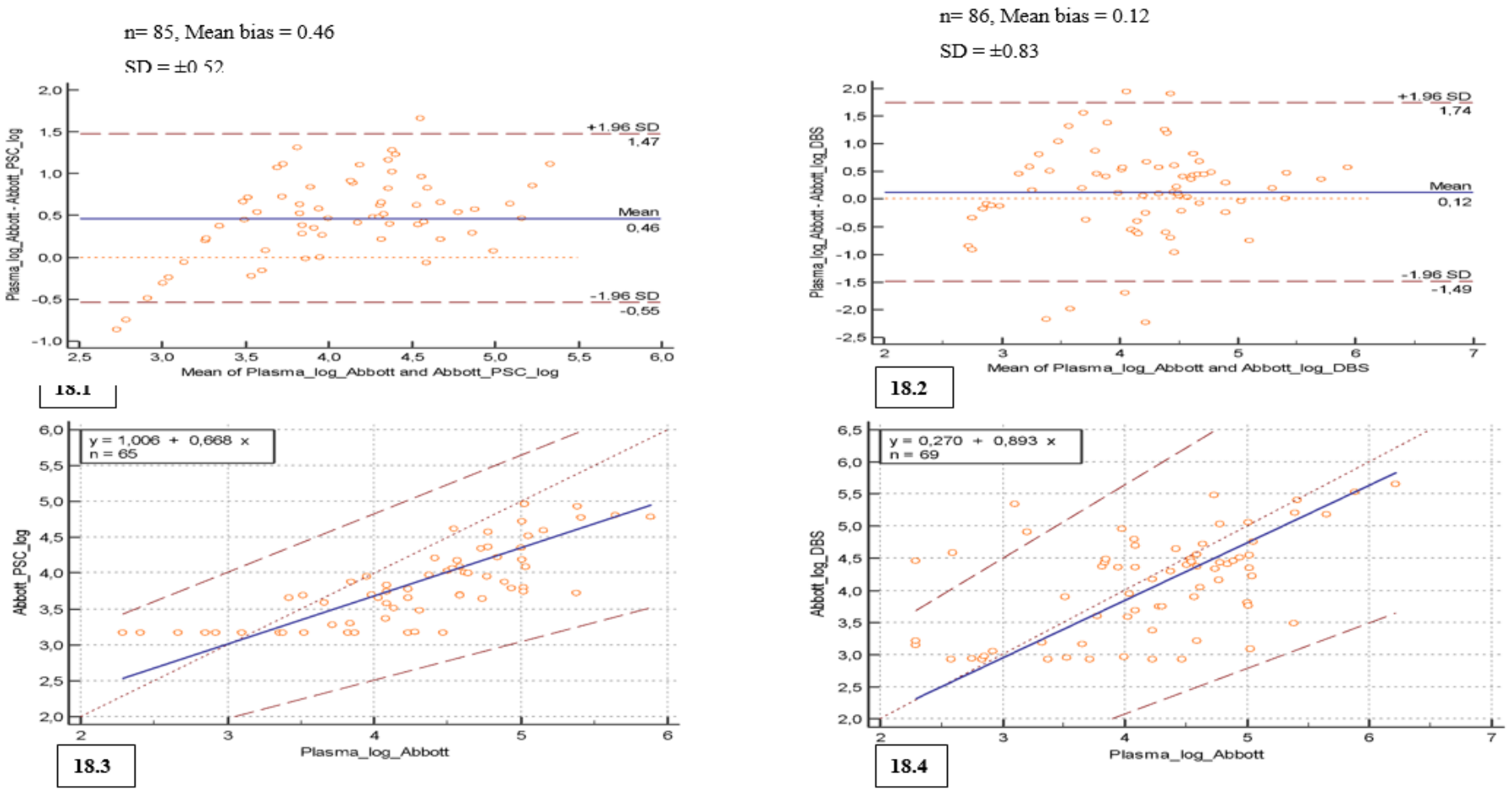


Figure 18.1-4: Visual comparison of PSC and DBS VL with plasma on the Bland Altman (**18.1-2**) and Passing Bablok (**18.3-4**) plots. The solid horizontal line on the Bland Altman plots reports the mean bias, and the dotted lines illustrate the confidence intervals. The solid blue line on the Passing Bablok plot represents the line of best fit, while the dotted line from the origin represents the 45° line. The two opposite lines represent the 95% CI. The horizontal axis represents the reference specimens, and the vertical axis is the newly evaluated method.

CHAPTER 1.6 – DISCUSSION

Resource-limited settings have restricted access to HIV VL monitoring, and the reference specimen type (plasma) requires a phlebotomist for collection, cold storage for transportation to the central laboratories and a centrifuge for preparing and separating plasma (88). Furthermore, the blood requires testing within a few hours post venesection. Thus, PSC and DBS were evaluated for use interchangeably with plasma on the Abbott m2000 instrument. DBS has been validated for use on the Abbott m2000 instrument (89), while PSC, a Roche dried plasma card, has not yet been validated. The cards were prepared following manufacturers' guidelines. The Abbott m2000 instrument was verified for processing PSC by quantitating known PSC HIV VL SAVQA panels. PSD prepared using residual HIV positive whole blood reported a reduced performance due to the low sample numbers. The PSC and DBS were given further attention utilizing prospective HIV positive whole blood samples. These samples were processed on the Abbott m2000 instrument. Our findings suggest that the PSC could be processed on the Abbott m2000 instrument for HIV VL monitoring but may require further optimisation with buffers during pre-processing and result conversion as the latter is currently only automated on the m2000rt platform for DBS testing (**Table 9**).

1.6.1 Evaluation of PSD and DBS prepared using residual blood samples

PSD (PSC, HSSE and VLPlasma) (n=51) and the DBS (n=50) prepared from residual HIV-positive blood samples were processed on the Abbott m2000 instruments. The PSD produced detectable VL results above the clinical threshold of log3 cp/ml but the target was not detected below the threshold; possibly, the detection limit is above log3 cp/ml (**Figure 12.1-4**). Because the PSD was an off-label evaluation that has not been validated on the Abbott m2000 instrument, the correction factor (**section 1.4.1**) was applied when calculating the results. DBS, on the other hand, has been certified for use on the Abbot m2000 instrument and has an 839 cp/ml detection limit (89). VLPlasma and HSSE specificity at log 3 cp/ml were similar. **Table 8** shows that while the PSC and DBS outcomes had higher sensitivity, they had lower specificity.

PSC and DBS can reliably quantify HIV virological failure, but because of their low specificity, they may misclassify virological suppression; this could be due to the inclusion criteria requiring residual HIV positive whole blood samples that were 24 hours old (**1.3.2.1**). Although VLPlasma and HSSE had strong sensitivity and specificity, their strength of agreement was poor when compared to plasma, with the majority of misclassifications. Residual whole blood specimens stored for more than 24 hours may lead to misclassifications. If samples are stored at room and low temperatures of 4ºC, they are prone to HIV RNA decay (90). In contrast at high room temperature of 30ºC the cells gradually lyse thus realising cell-associated viral nucleic acids. In this study, the exact temperature that the samples were stored at NHLS CMJAH prior to collection is unknown (90). PSC and DBS had good strength of agreement and least misclassifications (**Table 8)**. PSC has a higher percentage similarity compared to DBS as DBS reported values that were 5% higher than those of the plasma, leading to an overestimation, which was supported by the presence of proviral nucleic acids in the whole blood used to make DBS.

A recent study (91) found that prospective whole blood samples were more effective than residual specimens in evaluating PSD and DBS on molecular testing platforms. PSC, a novel specimen collection method validated for use on the Cobas 68/8800 instruments (92), and DBS, an existing specimen collection method validated for use on the Abbott m2000 (89) instrument, were selected for further testing on the Abbott m2000 using prospective samples similar to those used in previous studies. With the Abbott m2000 instrument assigned to most central laboratories in South Africa for HIV VL monitoring (40), the aim is to evaluate alternative specimen collection methods for use on the instrument.

1.6.2 Evaluation of PSC and DBS prepared using prospective whole blood samples.

Previous investigations have shown that DBS preparation and processing on the Abbott m2000, are not standardized. DBS was prepared by spotting the card with 50µl of whole blood for each spot (93, 94), while other groups used ≥70µl (43, 95-97). Different elution protocols (95, 97) used 1.3 to 1.4 ml elution buffer whereas the rest of the studies used ≥ 1.7 ml of elution buffer. The sample elution procedure differed amongst the different studies, with some taking as little as 15 to 30 minutes and others taking ≥ 60 minutes (94, 95, 98-103). All the studies mentioned above had two things in common: they used two DBS spots

from each patient and the tubes with the spots and elution solution were mixed during the incubation (43, 94, 95, 97, 99-103). Therefore, the volume required to prepare the DBS cards and the processing of the cards to elute the VL are the key variations between the study procedures. As a result, the mean bias and variability differ.

Arredondo and colleagues (103) measured the mean difference between DBS and plasma at various VL levels and found that the mean difference was higher at low VL and smaller at high VL; however, the overall mean difference measured using the Bland Altman plot was lower with narrow detection limits. Similar to Arredondo, Vidya *et. al* (104) divided the VL into three categories: 1000 cp/ml, 1000 – 3000 cp/ml, and > 3000 cp/ml, with 62 percent, 88 percent, and 100 percent sensitivities, respectively. DBS is less sensitive at lower VLs, and at 3000 cp/ml, it overestimates plasma VL values. When comparing DBS and plasma on the Abbott m2000 (91), other investigations indicated a sensitivity and specificity of about 90%, with a high false-positive rate at lower VL (97).

The WHO accepted Abbott RealTime HIV-1 (m2000sp) on its list of prequalified tests in 2017 (71). There was no literature to compare because PSC had not yet been validated for use on Abbott m2000 equipment. We used the DBS manufacturer-approved protocol to elute RNA from PSC because there was no PSC elution protocol available. However, as PSC is a plasma separator card, we followed the plasma protocol to process it on the Abbott m2000.

An off-label protocol was used to prepare and process ninety-one paired PSC-plasma and DBS-plasma samples in this study. Thus DBS was prepared using 70 µl of whole blood and eluted in 1.4 ml of elution buffer for 30 minutes at 54 ℃. We analysed the Abbott-quantitated PSC and DBS, comparing them to plasma quantitated on the same instrument. At <3 log cp/ml (clinical threshold), DBS had quantifiable results supporting the manufacturers limit of detection of 839 cp/ml (**Figure 17.1-2),** while PSC results were target not detected**.**

The sensitivity and specificity of the PSC and DBS were evaluated at 3 log cp/ml and 4 log cp/ml VL concentrations (**Table 9**). The PSC is more sensitive at 3 log cp/ml VL concentrations (82.4%), whereas the DBS is more sensitive at 4 log cp/ml VL concentrations (67.4%). Their specificities increase with an increasing VL concentration (73% to 100%). Thus the higher the VL concentration, the easier it is to detect, as also evidenced by prior research which compared DBS and plasma by analysing the VL in a stratified manner (94,

102). PSC and DBS had overall sensitivity of 92% and 87%, respectively, and specificity of 65% and 62%. Because mixing was not specified in the procedure for DBS and PSC processing, the samples were not mixed during incubation. One DBS and PSC spot was eluted as opposed to the two spots as observed in previous research (43, 95, 97, 99-103). Therefore, further optimisation may be required. Because two DBS/PSC cards are more concentrated than one, the analytes in the eluates can be detected and VL success and failure can be distinguished easier. Despite the fact that the DBS processing methods used here differed from earlier literature (94), the reported sensitivity was within the range of previously reported findings. PSC and DBS have a high sensitivity, positive predictive value, and accuracy, indicating that they can detect VL concentrations in HIV positive persons and hence aid in the identification of patients who are experiencing treatment failure where plasma testing is a limitation. Previous literature on CAPCTM and Roche 8800 platforms showed a better performance of the PSC to identify patients with VL below 1000 copies/ml (105, 106). Our study is an off-label evaluation, which may have influenced the relatively low performance of the PSC in this study. Overall, the PSC tends to perform better than DBS.

1.6.3 Study limitations

Limitations of this study were its limited sample size and off-label assessment of PSC on the Abbott m2000. COVID-19 caused a three-month delay in sample processing, causing longer than expected storage time that may also have influenced the DBS and PSC outcomes overall.

CHAPTER 1.7 – CONCLUSION

1.7.1 HIV Viral load

In resource-constrained areas, an alternative specimen type to plasma could improve HIV VL monitoring access and help achieve the UNAIDS objective of 95% of HIV infected people knowing their status, on treatment, and with a suppressed VL by 2030. PSC demonstrated the ability to distinguish between virological success and virological failure in this study, as evidenced by HIV VL results that had fewer misclassifications and higher sensitivity and accuracy than DBS, which is approved for use on the Abbott m2000 for HIV VL monitoring (89). PSC processed on the Abbott m2000 detected HIV VL with a good sensitivity but average specificity, therefore, PSC might be evaluated further with a bigger sample size to examine the specificity. Following that, based on the results, PSC maybe recommended for use on the Abbott m2000 device for HIV VL monitoring alternately with DBS and plasma.

SECTION 2 – COVID-19

CHAPTER 2.1 – INTRODUCTION

2.1.1 Epidemiology

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan province, China, in late 2019 and causes the novel coronavirus disease 2019 (COVID-19) (107). On 11th March 2020, WHO announced COVID-19 as a global pandemic (108). As of April 2022, the global cases reported were over 500 million with approximately 6 million deaths and 400 million recoveries. South African cases surpassed the 3.7 million mark with more than 100 000 deaths and 3.6 million recoveries by April 2022 (109).

Several factors affect the transmission of the disease, such as the Ro (basic reproduction number), which has a range of 2-4; thus, an infected individual can infect 2.76 people (110) with a median serial interval of 4.6 days (111). Dense population increases the risk of transmission, such as at large gatherings, health care facilities, indoor events etc (112).

The virus is transmitted through close contact with an infected person or surface, and respiratory droplets of an infected person when they cough or sneeze, and social distancing is not observed (113). The virus can stay in the air for a long time in closed environments, leading to droplets being inhaled when masks are not worn (114).

2.1.2 Viral genome structure

Coronaviruses are large, single-stranded positive-sense RNA viruses with a diameter of 120 to 80 nm (115). The genome encodes the envelope (E), nucleocapsid (N), Spike (S) and membrane (M) glycoprotein (116) as outlined in **Figure 19**. The M protein mainly functions in making the envelope and introducing the virus to the host; the E protein undergoes proliferation and maturation to form the virus's envelope. The N protein forms a capsule

around the viral RNA (117). The S protein binds to the Angiotensin-converting enzyme 2 (ACE2) receptor of the host and fuses with the cellular membranes using the two functional subunits, S1 (responsible for attaching to the host receptor) and S2 (fuses the viral and cellular membranes) (117). The ACE2 is highly expressed in the heart, kidney, lung, bladder and ileum (118) making these organs vulnerable. The S protein elicits an immune response similar to the N protein and serological tests detect the antibodies expressed against these antigens (119).

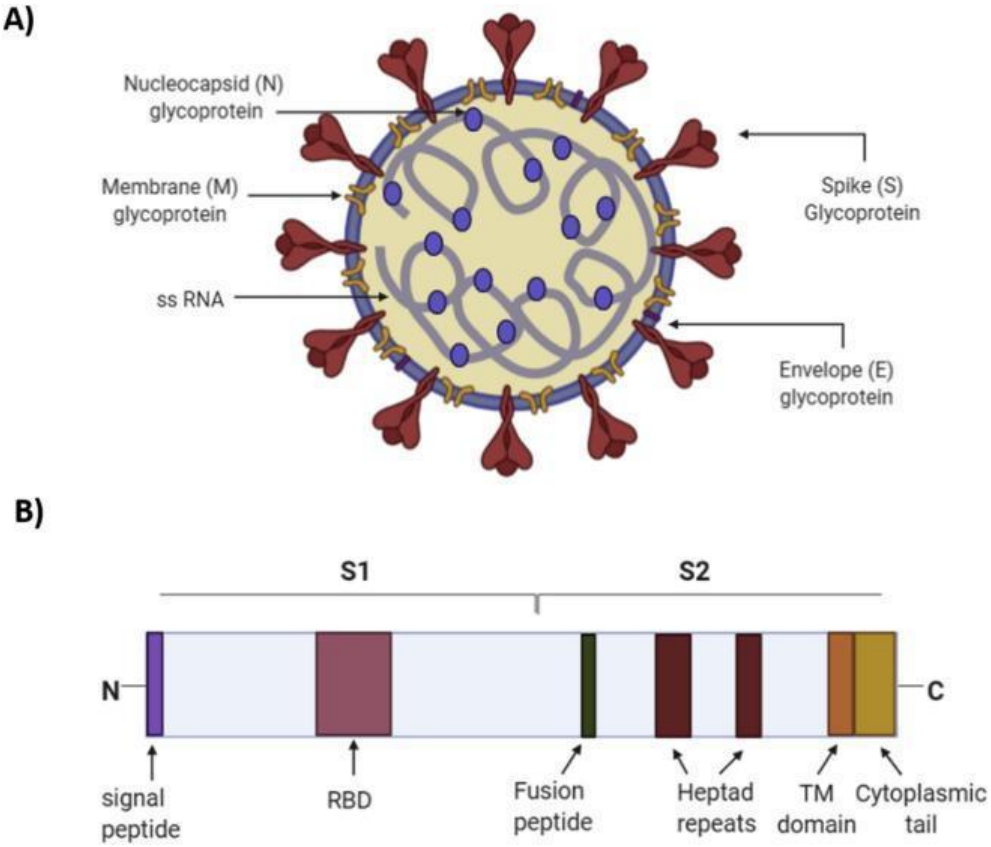


Figure 19: The SARS-CoV-2 viral structure (A) including the two subunits of the S protein (B). Figure from Mahmoud IS, 2020 (120).

2.1.3 SARS-CoV-2 antibody response

After SARS-CoV-2 infection, B cells produce antibodies specific to the virus, which function by stopping the spread of the disease through neutralisation (121). The process of the host-antibody response to the infection (**Figure 20**) is as follows: after viral entry into the host cell, replication and assembly of new SARS-CoV-2 virions occur, leading to daughter viruses

being released from the host cell (121, 122). The antigen-presenting cells take up and break down the daughter virions; after that, antigenic epitopes are presented to T cells which form an interaction with B cells, thus activating them (121). Activated B cells have specific SARS-CoV-2 antigen receptors such as Immunoglobulin A (IgA), Immunoglobulin M (IgM) and Immunoglobulin (IgG) antibodies, and these lead to the process of neutralisation upon antibody-antigen interaction (121).

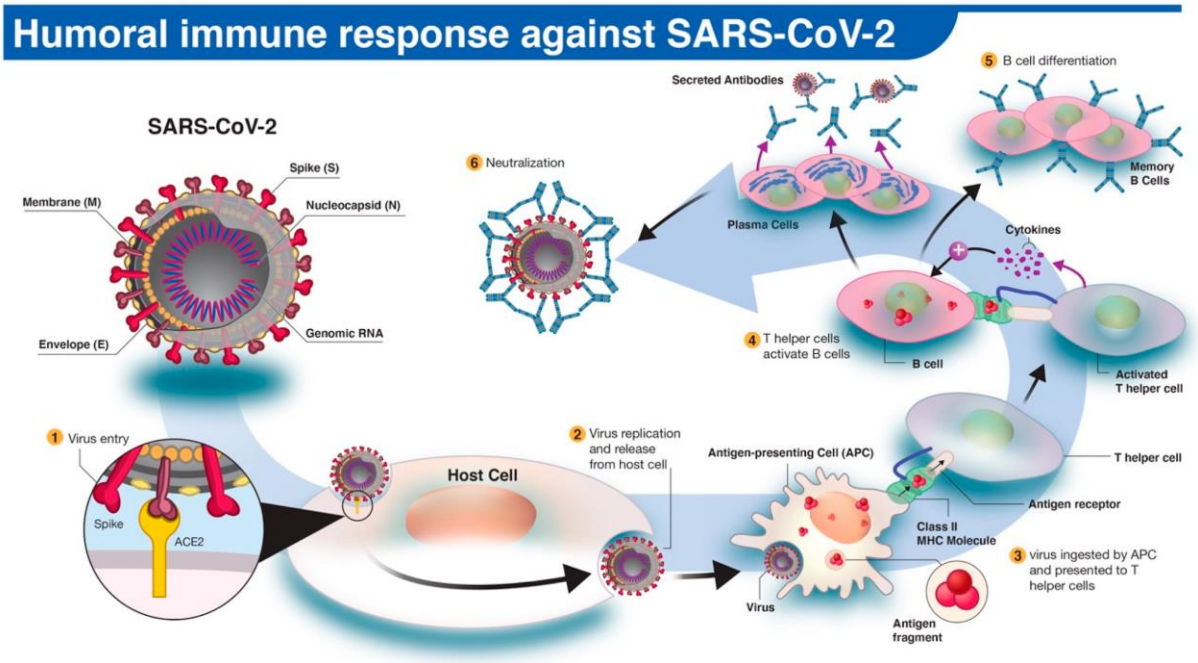


Figure 20: The development of immunity to SARS-CoV-2 after infection. Figure from Ghaffari A et al., 2020 (121).

The different antibodies (IgG, IgM, and IgA) respond differently to SARS-CoV-2 in terms of the duration and titre (123). IgM might be associated with severe disease as it is low to undetected as shown in most children infected with COVID-19, while IgG showed high titres in asymptomatic and mild cases (124-128). Compared to males, females produce IgG antibodies earlier, which correlates to experiencing less severe symptoms and lower death rates (129-131).

2.1.4 Pathogenesis

COVID-19 infected patients present with various symptoms from asymptomatic to mild and severe symptoms (132). During the first two days of the SARS-CoV-2 virus entering the nasal cavity, the virus is actively replicating; this is the asymptomatic stage (133) and the viral load concentration peaks during this period (134). Two to fourteen days after infection, symptoms, such as headaches, dry cough, fever, fatigue, shortness of breath and diarrhoea, appear (132, 133). As the disease progresses to a severe stage, the patients experience shortness of breath, hypoxia, and lung destruction (135). Key risk factors associated with severe outcomes of COVID-19 are hypertension, diabetes, lung disease and coronary artery disease (136). Carriers that are asymptomatic and pre-symptomatic may still transmit the virus (137, 138).

2.1.5 Diagnosis

The gold standard specimen type for testing is provider-collected, nasopharyngeal (NP) swabs used for nucleic acid-based tests (139). The testing algorithm is as follows: A patient who meets the clinical criteria will be tested using nucleic acid amplification test (NAAT). A positive NAAT confirms the case, and a negative NAAT result from a suspected case requires resampling for NAAT retesting. Negative results may be further evaluated using an antibody testing algorithm. (**Figure 21**). Day 2 to 7 post-exposure is the optimal testing period; the virus sheds and is easily transmitted (116).

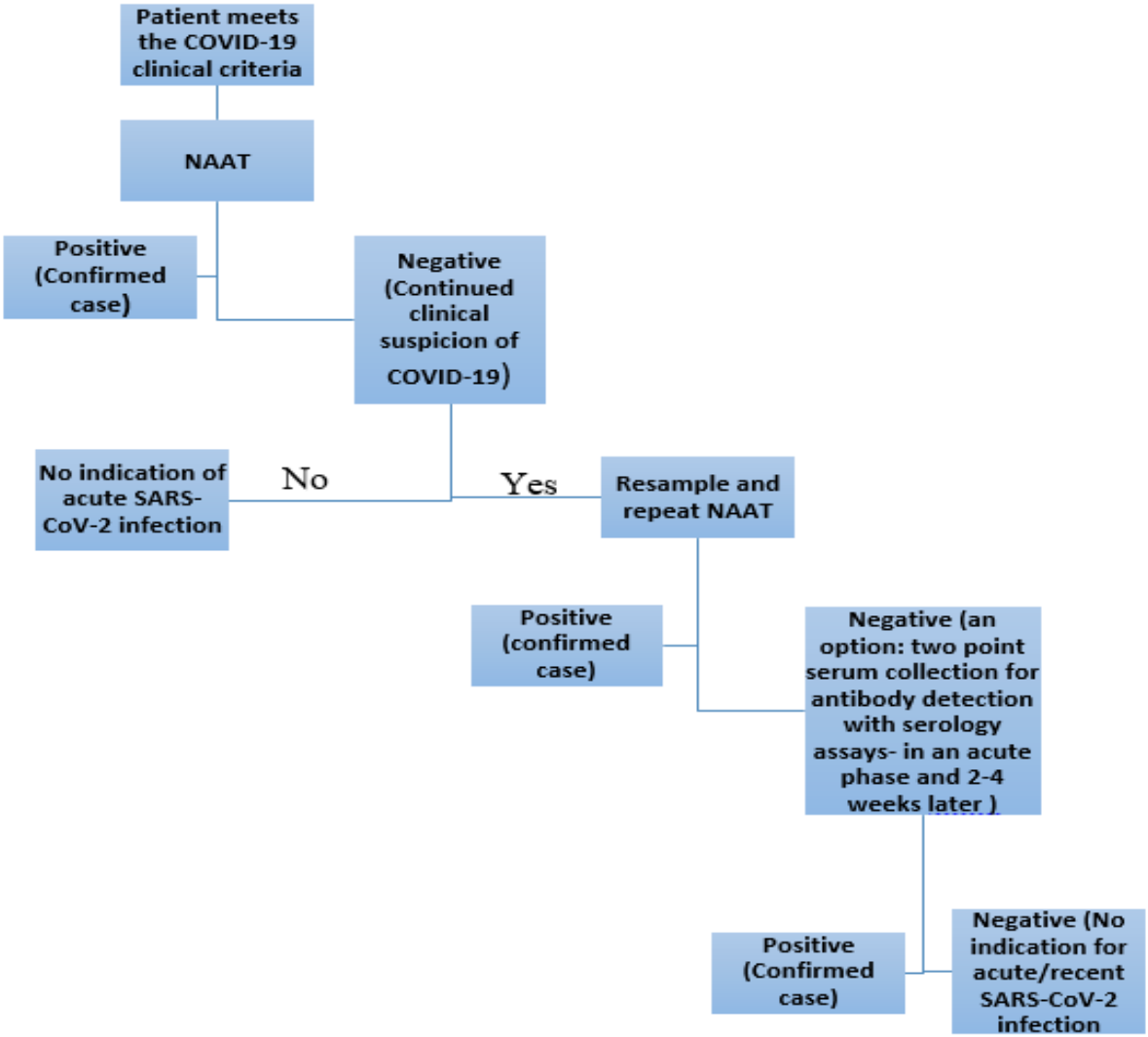


Figure 21: Acute SARS-CoV-2 diagnostic algorithm of individuals with a clinical suspicion of COVID-19. Figure adapted from World Health Organisation, 2020 (140). NAAT: Nucleic Acid Amplification Test

SARS-CoV-2 rapid antigen detection assays function in detecting the SARS-CoV-2 proteins; they are affordable, rapid and can be used as point of care testing (141) and may provide an alternative solution to challenges related to RT PCR.

Viral detection varies; in some patients, it lasts several days, while in others the virus is still detected after several weeks (140). Some NAAT have a moderate sensitivity, leading to false-negative results, particularly at the first week after onset (136), while the rapid antigen testing assays are more accurate in symptomatic patients (116). Challenges to testing include

shortage of personal protective equipment, testing reagents, and NP swabs, which lead to the limited efficiency in tracing the spread of the virus within communities (140).

2.1.6 Serology Antibody Testing

Serological testing plays a role in detecting antibodies produced after antigen invasion (121). The predominant serological assays are the enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), rapid diagnostic test (RDT) and neutralisation tests. ELISA is a laboratory-based test that uses surface antigens to detect their antibodies, producing results within two to five hours (146). CLIA is a high throughput laboratory test that produces light emissions when probes interact with labelled specific antibodies, and results are obtained within one to two hours (121). RDT are self-test strips based on lateral flow technology and can be used at point of care; antibodies are detected from a blood drop administered on the test producing results within 10 to 30 minutes. Neutralisation tests assess the ability of the patient antibodies to recognise and destroy antigens *in vitro*. These require laboratories with proper biosafety certificates to use live viral and cell cultures (121).

With the recent vaccine developments, serological testing will be utilised for screening individuals to monitor antibody response to the vaccine (93), Serological assays could be combined with nucleic acid detection tests to determine the prevalence of the disease (93), to prevent missed cases of COVID-19 and to detect antibodies of previously infected patients to use their plasma for therapeutic purposes (119). These assays can identify the absence or presence of antibodies prior to testing infected individuals using neutralisation assays, and assist in seroprevalence studies to map out the transmission patterns over time and assess herd immunity if/when it is reached (93). Antibody testing is recommended two weeks after symptom onset (142). McDade *et al.* 2020 (139) reported that specific antibodies to the SARS-CoV-2 proteins appear 3-10 days post-infection, with IgM appearing earlier than IgG, which is detected 14 days post-infection (**Figure 22**). Therefore, the results should be interpreted according to the clinical history (142).

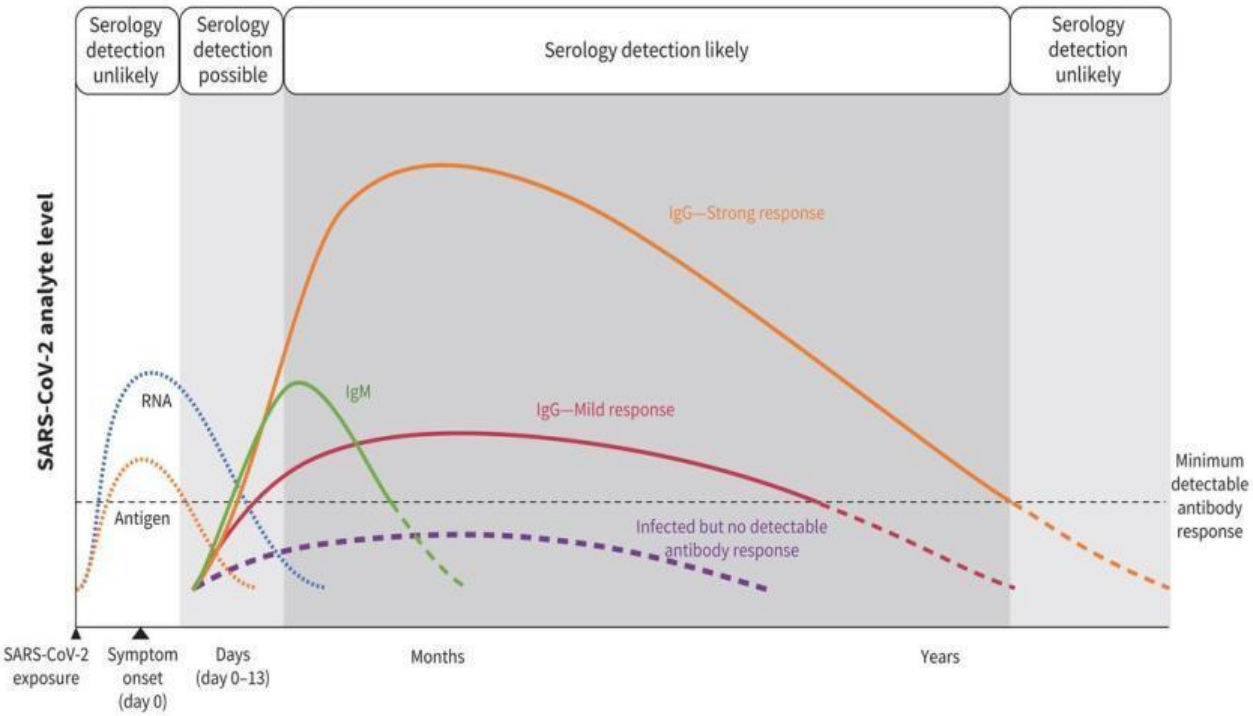


Figure 22: The antibody response to SARS-CoV-2 infection. Figure from Van Caeseele P, 2020 (116).

2.1.7 Specimen type for COVID-19 serology test

The gold standard specimen type for antibody testing is serum/plasma, which requires phlebotomy, cold storage and centrifugation compared to DBS (143). Furthermore, serum/plasma requires the patient to come to the testing centres for in-person collection, thus limiting access for the poor and those with disabilities (144). DBS is an alternative specimen type prepared from heel sticks and finger sticks, which are easy to perform and use a small volume of blood. DBS can be easily transported to the central laboratories (143) and provide an alternative for self-sample collection at home to prevent spreading the virus at the testing facility or contracting it at the facilities (145). DBS can increase access to serological testing in resource-poor areas by transporting them to the central laboratories using postal services. DBS has been used successfully in serology testing for hepatitis and has lower costs than lateral flow rapid POC test (146).

Similar to DBS, the PSC is easy to use, convenient and may be transported at ambient temperature (75). Advantages of the PSC compared to DBS is the higher volume capacity of 140 µl compared to 70 µl of DBS, separation of plasma from whole blood cells, which minimises interference from the whole blood used in DBS (147). PSC has stabilising factors

on the membrane, which assists with ease of storage and transportation in extreme environmental weather conditions (75).

CHAPTER 2.2 – AIMS AND OBJECTIVES

The purpose of the study is to assess PSC and DBS as an alternative specimen type to EDTA plasma for COVID-19 serology testing to widen access in antibody testing.

2.2.1 AIMS

i. To evaluate PSC for COVID-19 serology using an off-label automated EUROIMMUN analyser I-2P instrument.
ii. To evaluate different elution protocols for DBS using the manual EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay.

2.2.2 OBJECTIVES

- Determine the sensitivity, specificity and accuracy of PSC processed on an automated EUROIMMUN analyser I-2P instrument.
- Determine the sensitivity, specificity and accuracy of DBS eluted by manufacture's diluent and processed using manual EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay.
- Determine the sensitivity, specificity and accuracy of DBS eluted by PBS and processed using manual EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay.

CHAPTER 2.3 – METHODS AND MATERIALS

2.3.1 Study site and ethics clearance

The study took place at the Clinical Laboratory Services (CLS) (Braamfontein, Gauteng, South Africa). The study's ethics approval (**Appendix 1.3** #M200468) was obtained from the University of the Witwatersrand Human Research Ethics Committee. The specimens used in this study are from a parent COVID-19 serology study named "Fourway validation of serological and rapid point-of-care testing for Severe Acute Respiratory Viral-2 Coronavirus (SARS-CoV2 or COVID-19) in South Africa". The study included participants who were previously diagnosed with COVID-19. The participants consented to the nurse visiting them at their homes to collect the specimens. CLS biorepository assisted with preparing the plasma, serum, DBS and PSC using the collected whole blood EDTA specimens. The PSC and DBS specimens used the EUROIMMUN protocol and then quantitated the VL using the EUROIMMUN analyser I-2P instrument. Their absolute values were compared to plasma/serum on a scatter plot, and their strength of agreement was measured using the kappa coefficient. The dilution factor was considered when doing the statistics.

2.3.2 Selection Criteria

Whole blood specimens (n=77) of individuals confirmed positive for SARS-CoV-2 using RT-PCR regardless of the cycle threshold (Ct) values were selected for use in the study. Specimens without a positive RT-PCR confirmation were excluded from the study. According to the manufacturer (148), the EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay has a sensitivity of 94.4% and specificity of 99.6% 10-day post symptoms/positive direct detection (recommended time frame). The specimens selected were from day one post PCR detection to the 120th day to evaluate the feasibility of detecting IgG antibodies. The whole blood specimens were stored at 2˚C and 8˚C post venesection and were used to prepare DBS (n=75) and PSC (n= 76) within 24 hours post collection. The plasma (n=77) sample results were obtained from the parent study and were used as the reference.

2.3.3 Sample preparation

A 6 ml single whole blood EDTA tube was used to prepare plasma, PSC and DBS samples as follows: whole blood (70 µl) was pipetted onto each of the three DBS spots, and 140 µl pipetted onto each of the three spots on the PSC spotting layer. The remaining blood in the tube was centrifuged at a temperature of 8℃ at 2000 g for 10 minutes using the Rotina 380 centrifuge (Hettich Laboratory, Tuttlingen, Germany). The plasma (2ml) was removed and aliquoted into microtubes (Merck KG, Darmstadt, Germany).

2.3.4 Automated COVID-19 ELISA Antibody Testing

2.3.4.1 DBS elution process

Different elution buffers could be used to elute DBS and PSC specimens such as sample diluent buffer (Euroimmun, Germany) (149) , Phosphate Buffered Saline (PBS) and Triton X-100 buffer solution (150) . The latter two require overnight incubation, while the sample diluent only requires incubation for an hour. (149)

The manufacturer has validated DBS for use with the EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay (Lübeck, Germany) (149) not the EUROIMMUN analyser I-2P instrument. The DBS extraction protocol (149) was modified due to being unable to obtain the 3.2 mm automated puncher. Therefore, the DBS spot was cut in half (**Figure 23**) to accommodate the small elution buffer and one half was placed into an empty 8 ml sample tube. The PSC is not validated on the EUROIMMUN instrument and the DBS protocol was adapted for the PSC. The PSC spotting layer was removed, and the whole PSC spot was picked up with forceps and placed in an 8 ml sample tube. As recommended by the manufacturer, 250 µl of sample diluent buffer (149) was pipetted into each 8 ml tube. The sample tubes were closed and incubated in a heating block for 1 hour at 37℃.

2.3.4.2 EUROIMMUN analyser I-2P instrument ELISA procedure

The EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay is a semi-quantitative assay for detecting human IgG antibodies against SARS-CoV-2. PSC and DBS eluates were processed following the same protocol as plasma specimens and were run in a single batch

The PSC and DBS eluates were quantified on the EUROIMMUN analyser I-2P instrument (**Figure 23**). The instrument is set to automatically dilute the specimens (plasma and eluates) using a 1:100 dilution factor. The manufacturer's protocol is as follows: **(1)** Controls, reagents and plasma (100 µl) / eluates (250 µl) were placed on racks and loaded onto the instrument. The calibrator, positive and negative controls, including the diluted samples, were transferred to each well pre-coated with recombinant S1 domain of the spike protein of SARS-CoV-2. **(2)** The well plate was then incubated for 1 hour at 37°C. **(3)** To remove the unbound antibodies, the wells were washed three times with 300 µl wash buffer and dried. **(4)** Horseradish peroxidase (HRP)-conjugated anti-human IgG enzyme conjugate (100 µl), which binds to the antibodies, was added to the microplate wells followed by a 30-minute incubation at 37°C. **(5)** The microplate was washed a further three times, as described above, to remove unbound HRP-conjugated anti-human IgG. **(6)** To elicit a colour reaction, 100 µl of chromogen/substrate solution was pipetted into each well and incubated at room temperature for 30 minutes. **(7)** To stop the reaction, 100 µl of sulphuric acid (0.5 M) was added to each of the wells. **(8)** Thirty minutes after adding the stop solution, the absorbance at a wavelength of 450 nm and 620 nm was measured.

The manufacturer recommends that the results be read: ratio <0,8: negative; ratio ≥ 0.8 to <1.1: borderline; ratio ≥ 1.1: positive. However, 0.8 was considered the cut-off ratio for sensitivity and specificity, with ≥ 0.8 listed as positive and <0.8 listed as negative. The recommended (by the manufacturer) dilution factor is a 1:100 which is pre-installed on the instrument. Taking the double dilution into account the OD results were multiplied by four (second dilution of 1000µl divided by initial dilution of 250µl) to obtain the true values of DBS and PSC. **Figure 23** demonstrates the steps followed when processing the PSC and DBS on the automated EURIMMUN analyser.

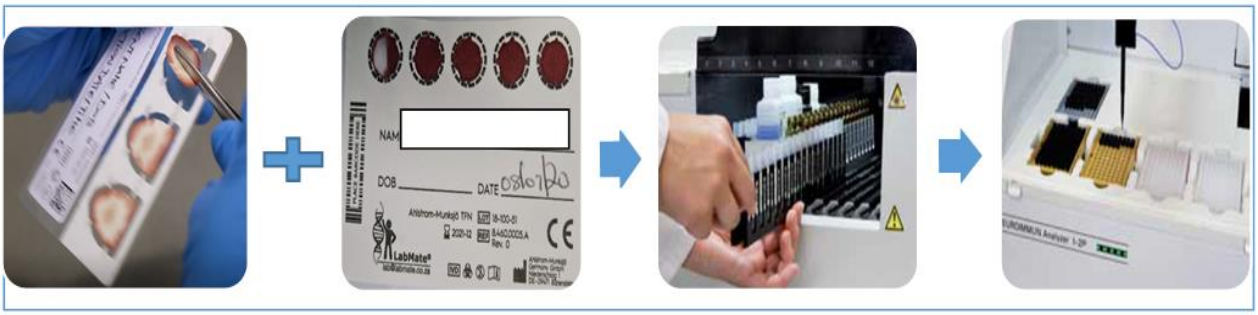


Figure 23: The processing of PSC and DBS using the automated EUROIMMUN analyser I-2P instrument.

2.3.5 Manual COVID-19 ELISA antibody testing.

The automated instrument produced bias results due to over diluting the diluted PSC and DBS eluates and using bigger PSC and DBS spots than manufacturer recommended. Following the findings of bias in the results, the PSC specimens were not further investigated, however the duplicate DBS were processed using the automated instrument, and further evaluated comparing two elution protocols using the EUROIMMUN manual protocol for the detection of the COVID-19 antibody detection.

2.3.5.1 DBS elution procedure

To compare the DBS-diluent and DBS PBS elution processing, DBS were eluted according to the manufacturer's instructions for use (149) or using a protocol by Morley G. *et al*. (151) respectively. The manufacturer's sample diluent and PBS were warmed at 37°C in a mini incubator. A circular piece was punched out from the centre of the DBS card using a 4.5 mm puncher. The DBS spot was transferred to a 1.5 ml Eppendorf tube and 250µl sample diluent was added. Similarly, a new DBS spot from the same patient was added to a separate Eppendorf tube and 250µl PBS (154) added to the tube. The Eppendorf tubes were incubated at 37°C ±1°C for 60 minutes in a heating block (**Figure 24**).

A similar method to the automated instrument above was followed for manual ELISA.

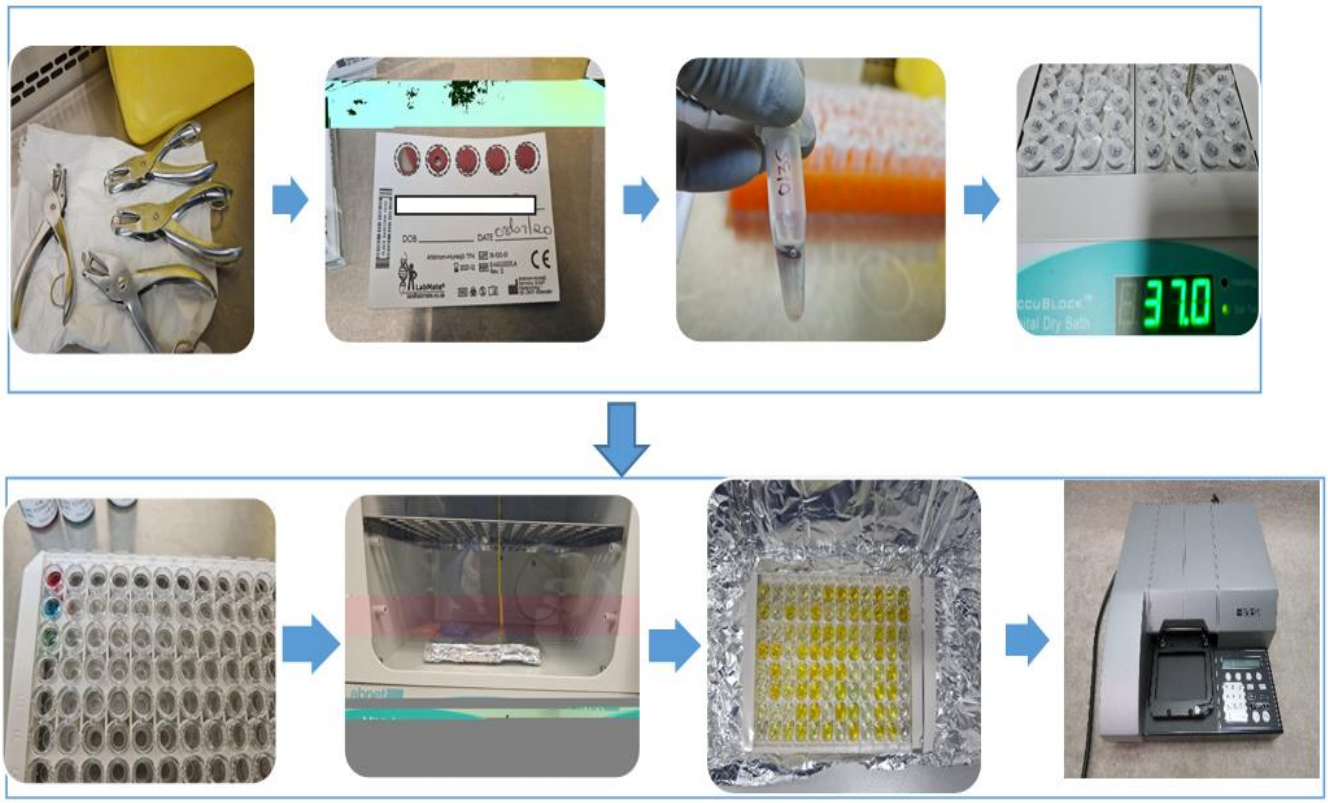


Figure 24: Manual ELISA for COVID antibody detection from DBS, OD measured using the BioTek ELX800 Microplate reader.

CHAPTER 2.4 – DATA ANALYSIS

MedCalc software Version 19.0.5 (Ostend, Belgium) and Microsoft Excel (Microsoft, Redmond, Washington, United States) were used for all data and statistical analysis. The optical density (OD) cut-off values (as specified by the manufacturers) of the DBS and PSC for serology testing were compared to the reference (serum/plasma) results to measure accuracy, specificity, and sensitivity with a 95% confidence interval. The agreement between the DBS values and plasma, and PSC values and plasma were assessed using Cohen's kappa(κ), which measures the similarities between the two methods and compares them to the overall ratings (152).

CHAPTER 2.5 – RESULTS

2.5.1 Characteristics of the COVID-19 positive patients

The patients were enrolled as part of an umbrella study and the patients fitting our study criteria were selected, which are those confirmed positive for SARS-CoV-2 by RT-PCR. Information on gender, symptoms and underlying diseases was unavailable for 44.1%, 46.8% and 44.1% of patients, respectively. From the information available (**Table 10**), the mean age of the patients was 49 years, 42% male, with 43% of the patients experiencing symptoms and 41% without underlying diseases.

Table 10: Patient demographics.

		n	%
Sex	Female	11	14.1
	Male	32	41.6
	Unavailable	34	44.1
Age	Mean ±SD	49.1 ± 12.0	
Symptoms	Yes	33	42.9
	No	8	10.4
	Unavailable	36	46.8
Underlying Disease	Yes	11	14.3
	No	32	41.6
	Unavailable	34	44.1

2.5.2 Overview of sample processing and results

This study evaluated the feasibility of detecting SARS-CoV-2 IgG antibodies from DBS and PSC. An overview of the number of samples processed using the analyser I-2P instrument and the reported positivity rate is provided in **Figure 25**.

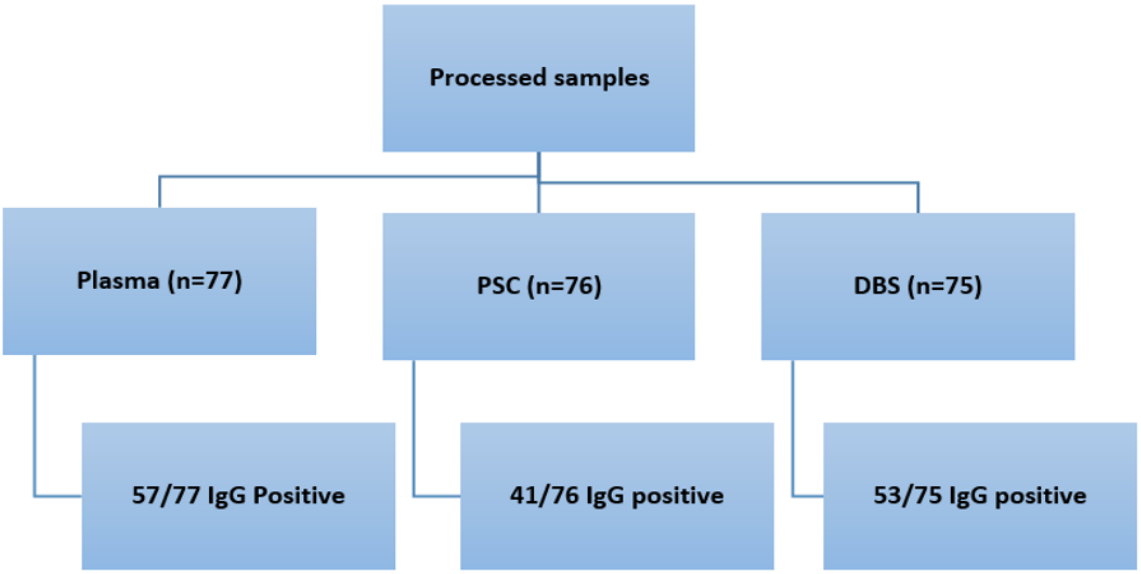


Figure 25: A summary of the specimens processed and their SARS-CoV-2 antibody positivity rate. PSC: Plasma Separation Card; DBS: Dried Blood Spot; IgG: Immunoglobulin G

2.5.3 The automated PSC and DBS ELISA results compared to plasma on a scatter plot.

The automated PSC and DBS ELISA results were compared to plasma samples (reference) using a scatter plot (**Figure 26**). The clinical cut off is at 0.8; ≥0.8 indicates positive results and <0.8 indicates negative results, the dilution factor recommended by the manufacturer is a 1:101 ratio, which is pre-installed on the instrument. Taking that into account and the volume used to elute the DBS and PSC cards, the OD was multiplied by 4 to obtain the true values of DBS and PSC. The DBS results at <0.8 followed the pattern of the reference results and had few upward misclassifications (n= 2 false positives), while at ≥0.8, there was an overall pattern of lower results to the reference and thus many false negatives (n=18). The PSC indicated a similar overall pattern of results to that of DBS at ≥0.8 but with fewer false negatives (n=12), and false positives (n=1) at <0.8, compared to DBS.

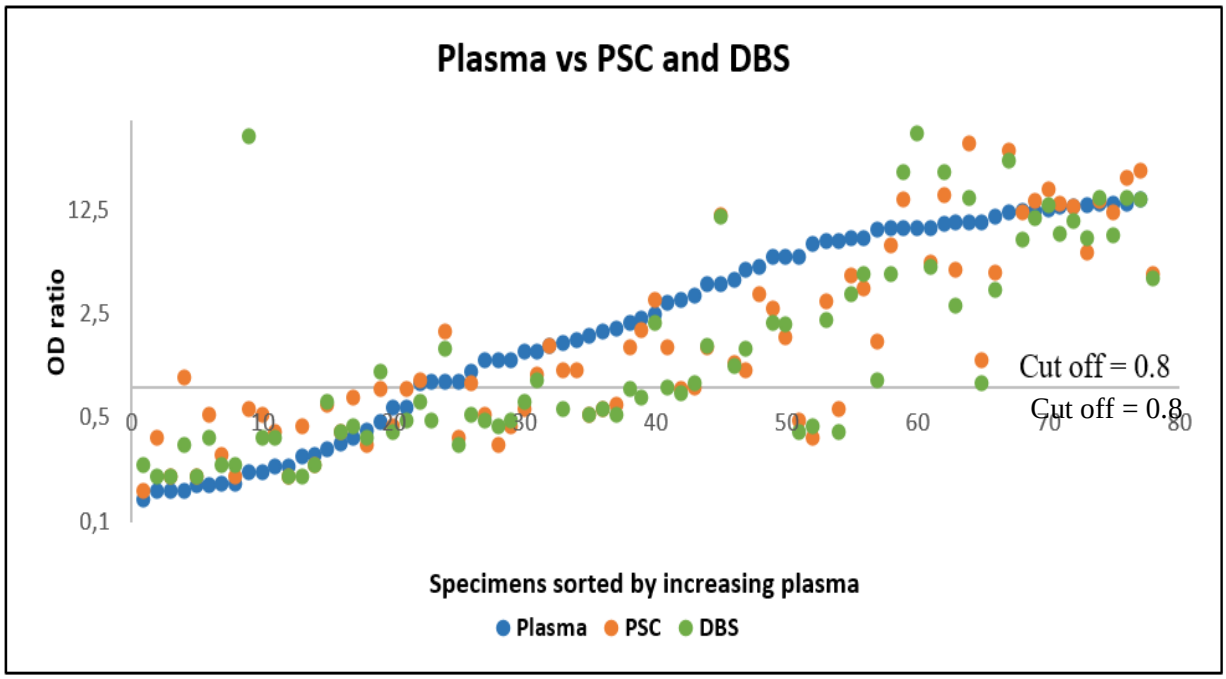


Figure 26: Visual representation of the PSC, DBS compared to the reference specimen (plasma). The horizontal line represents specimen OD ratio sorted in ascending order using the reference. The y-axis represents the optical density (OD) ratio. ● = Plasma results; ● = Plasma Separation Card (PSC) results; ● = Dried Blood Spot (DBS) results.

2.5.4 Qualitative and quantitative comparison of PSC, DBS and plasma.

Pathogens induce antibody response thus; with SARS-CoV-2 the antibodies are detected from 3-10 days post-infection. The results were stratified into time intervals from day 0 to <50 to evaluate the antibody detection rate at different days' post PCR positive confirmation (**Table 11**). Day 11 to 20, DBS had the lowest sensitivity, specificity and accuracy of 31.25%, 66.67% and 40.91% this is due to the high misclassification rate within that interval.

DBS reported a higher misclassification rate (27%) and poor strength agreement with a kappa coefficient of 0.46 compared to plasma. At day 0 to 20, PSC sensitivity was poor at approximately 62.50%.

Table 11: Automated PSC, DBS OD results compared to plasma sectioned into different intervals of the days post-positive PCR results.

Days post PCR	DBS (N= 75)	PSC (N=75)
Overall:		
Sensitivity	66.0% (51.7% to 78.5%)	77.8% (64.4% to 88.0%)
Specificity	90.5% (69.6% to 98.8%)	95.2% (76.2% to 99.9%)
Accuracy	72.97% (61.39% to 82.65 %)	82.67% (72.19% to 90.43%)
Sensitivity % (95%CI)		
Day 0 to 10 (n= 13)	62.50% (24.49% to 91.48%)	62.50% (24.49% to 91.48%)
Day 11 to 20 (n= 22)	31.25% (11.02% to 58.66%)	62.50% (35.43% to 84.80%)
Day 21 to 30 (n= 13)	83.33% (35.88% to 99.58%)	85.71% (42.13% to 99.64%)
Day 31 to 50 (n= 12)	90.91% (58.72% to 99.77%)	90.91% (58.72% to 99.77%)
Day > 50 (n= 13)	90.00% (55.50% to 99.75%)	90.00% (55.50% to 99.75%)
Specificity % (95%CI)		
Day 0 to 10 (n= 13)	100.00% (47.82% to 100.00%)	100.00% (47.82% to 100.00%)
Day 11 to 20 (n= 22)	66.67% (22.28% to 95.67%)	100.00% (54.07% to 100.00%)
Day 21 to 30 (n= 13)	100.00% (54.07% to 100.00%)	100.00% (54.07% to 100.00%)
Day 31 to 50 (n= 12)	100.00% (2.50% to 100.00%)	100.00% (2.50% to 100.00%)
Day > 50 (n= 13)	100.00% (15.81% to 100.00%)	50.00% (1.26% to 98.74%)
Accuracy % (95%CI)		
Day 0 to 10 (n= 13)	76.92% (46.19% to 94.96%)	76.92% (46.19% to 94.96%)
Day 11 to 20 (n=22)	40.91% (20.71% to 63.65%)	72.73% (49.78% to 89.27%)
Day 21 to 30 (n= 13)	91.67% (61.52% to 99.79%)	92.31% (63.97% to 99.81%)
Day 31 to 50 (n= 12)	91.67% (61.52% to 99.79%)	91.67% (61.52% to 99.79%)
Day > 50 (n= 13)	91.67% (61.52% to 99.79%)	83.33% (51.59% to 97.91%)
Misclassifications, N (%)	20/74 (27%)	13/75 (17%)
Kappa	0,46 (0,28 to 0,64)	0,62 (0,46 to 0,80)

During the COVID-19 antibody detection analysis from DBS and PSC processed using the automated EUROIMMUN Analyser I - 2P instrument, it was concluded that there was a bias due to over dilution of the eluted DBS and PSC spots. The over dilution resulted in lower sensitivities of DBS and PSC at the early days of antibody detection at approximately 0 to 20 days. This results in the inability of the assay to detect the majority of the positive patients at day 0 to 20. The over dilution was caused by 1st the elution procedure and the 2nd dilution by

the automated instrument. The procedure was as follows: the dried half DBS spot and full PSC spot were eluted in 250 µl of diluent provided by the manufacturer. The instrument further dilutes the samples at a 1:100 dilution protocol pre-set on the instrument according to the manufacturers assay (148). Overall, this resulted in poor strength of agreement and bias, as mentioned above. Therefore, since DBS is validated for EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) assay, it was selected for further evaluation following the manual EUROIMMUN protocol.

2.5.5 Manual ELISA for IgG COVID-19 antibody detection on DBS

DBS was eluted with manufacturer-provided diluent (DBS-diluent) and in-house PBS (DBS-PBS) and were processed following a manual ELISA protocol. A study (151) previously reported the use of PBS to elute DBS, and we followed a similar protocol for our in house DBS-PBS elution. The plasma was used as the reference compared to DBS-diluent and DBS-PBS.

The reference was visually compared to the DBS diluent, and DBS PBS on a scatter plot (**Figure 27).** As mentioned above, the cut off value is 0.8, the values below are considered negative, and those above are considered positive. Overall, DBS diluent and DBS PBS followed a similar pattern of reporting higher values than the reference at <0.8 cut off value and reporting lower values than the reference at ≥0.8 cut off. However, they misclassified the reference results close to the cut off value. DBS diluent reported three false negatives. In contrast, DBS PBS reported three false positives.

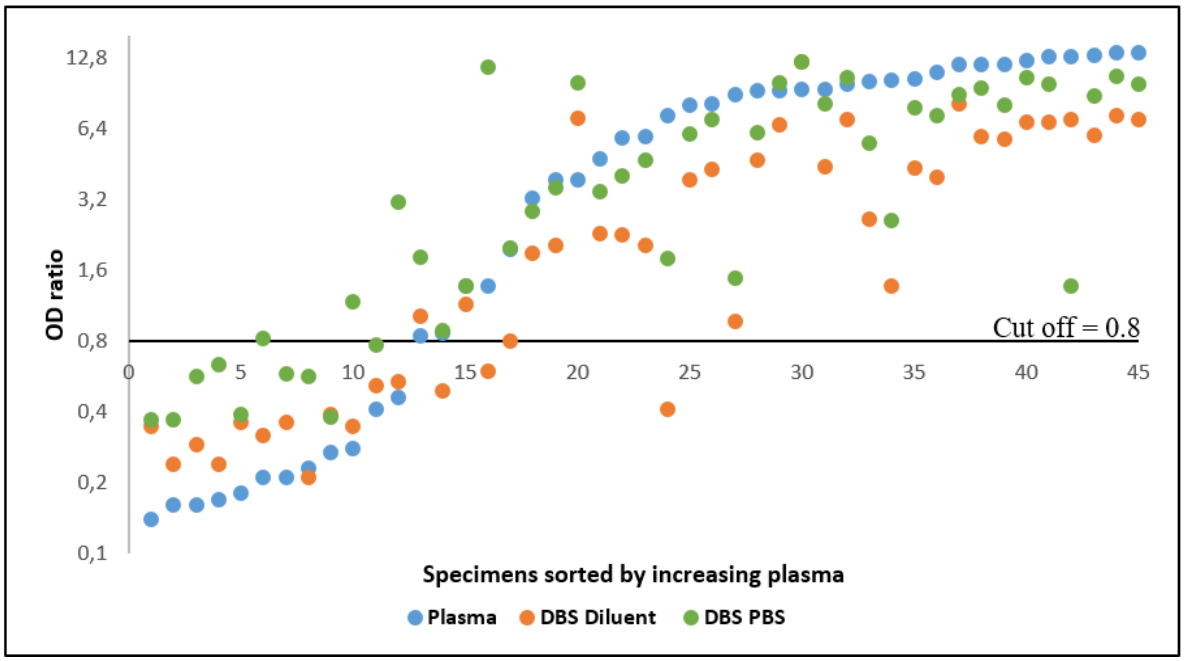


Figure 27: DBS diluent and DBS PBS visually compared to the reference. The horizontal line represents specimen OD ratio sorted in ascending order using the reference and the y-axis represents the optical density (OD) ratio. ● = Plasma results; ● = DBD-Diluent results; ●= DBS-PBS.

The DBS diluent and DBS PBS were compared to the reference plasma sample using the sensitivity, specificity, accuracy, misclassification and kappa coefficient (**Table 12).** Overall, the DBS diluent reported specificity of 91.4% compared to the DBS PBS of 75%. However, both reported the same accuracy (93.62%). They reported similar misclassification rates of 8.5% and a kappa coefficient of >0.8, representing a good strength of agreement compared to the reference**.**

The DBS diluent and DBS PBS were evaluated at different time intervals of days post-PCR positive results. The DBS PBS reported a sensitivity of 100% across the different days post-PCR, while the DBS diluent reported a low sensitivity between days 0 to 10 and after day 50. However, DBS diluent reported 100% specificity while DBS diluent reported low specificity at days 0 to 20 post PCR positive results.

Table 12: Qualitative comparison of PSC, DBS to plasma sectioned into different intervals of the days post-positive PCR results.

Days post PCR	DBS Diluent (N= 45)	DBS PBS (N=45)
Overall:		
Sensitivity	91.43% (76.94% to 98.20%)	100.00% (90.00% to 100.00%)
Specificity	100.00% (73.54% to 100.00%)	75.00% (42.81% to 94.51%)
Accuracy	93.62% (82.46% to 98.66%)	93.62% (82.46% to 98.66%)
Sensitivity % (95%CI)		
Day 0 to 10 (n= 13)	75.00% (34.91% to 96.81%)	100.00% (66.37% to 100.00%)
Day 11 to 20 (n= 5)	100.00% (29.24% to 100.00%)	100.00% (29.24% to 100.00%)
Day 21 to 30 (n= 6)	100.00% (29.24% to 100.00%)	100.00% (29.24% to 100.00%)
Day 31 to 50 (n= 9)	100.00% (66.37% to 100.00%)	100.00% (66.37% to 100.00%)
Day > 50 (n= 12)	90.91% (58.72% to 99.77%)	100.00% (71.51% to 100.00%)
Specificity % (95%CI)		
Day 0 to 10 (n= 13)	100.00% (47.82% to 100.00%)	80.00% (28.36% to 99.49%)
Day 11 to 20 (n= 5)	100.00% (15.81% to 100.00%)	0.00% (0.00% to 84.19%)
Day 21 to 30 (n= 6)	100.00% (29.24% to 100.00%)	100.00% (29.24% to 100.00%)
Day 31 to 50 (n= 9)		
Day > 50 (n= 12)	100.00% (2.50% to 100.00%)	100.00% (2.50% to 100.00%)
Accuracy % (95%CI)		
Day 0 to 10 (n= 13)	84.62% (54.55% to 98.08%)	92.86% (66.13% to 99.82%)
Day 11 to 20 (n=5)	100.00% (47.82% to 100.00%)	60.00% (14.66% to 94.73%)
Day 21 to 30 (n= 6)	100.00% (54.07% to 100.00%)	100.00% (54.07% to 100.00%)
Day 31 to 50 (n= 9)		
Day > 50 (n= 12)	91.67% (61.52% to 99.79%)	100.00% (73.54% to 100.00%)
Misclassifications, N (%)	3/47 (8.5%)	3/47 (8.5%)
Kappa	0.845 (93.62%)	0.817 (93.62%)

CHAPTER 2.6- DISCUSSION

2.6.1 COVID-19

Before implementing a test technique, it must be evaluated and verified. A highly sensitive test should identify all positive individuals (153). A highly specific test should exclude all the negative individuals, but the reality is that no test consistently possesses those characteristics (153) A Cochrane review (154) of 57 publications analysing the accuracy of SARS-CoV-2 antibody testing found that overall IgM and IgG sensitivity of 96% at 22-35 days post-symptom onset, and specificities were greater than 98% for all antibody tests.

2.6.2 SARS-Cov-2 IgG antibody detection using automated ELISA

The study investigated the detection of anti-SARS-Cov-2 IgG antibodies from DBS and PSC. DBS and PSC were evaluated and compared to plasma samples. COVID-19 positive PSC (n=76) and DBS (n=75) samples paired to plasma samples were collected and quantitated using the EUROIMMUN instrument. The DBS (149) was approved by the manufacturer for use in SARS-CoV-2 antibody testing. The results illustrated a bias caused by the samples being diluted twice. The first dilution was achieved by eluting the DBS and PSC spots in a volume of 250 µl, this volume did not cover the entire PSC and DBS spots. The 2nd dilution was that the DBS and PSC eluates from the first dilution, was further diluted in a 1:100 dilution pre-set on the automated instrument. The sensitivity and specificity of DBS declined between day 11 and 20, which may have been caused by the concentration of antibodies on the DBS, since IgG antibodies are beginning to progressively build up in the body during this time (**Figure 22)**.

PSC was evaluated for use in HCV antibodies (147) and reported discordant results at low and medium antibody titers. DBS and PSC had the most misclassifications (**Table 11**) which is supported by the visual presentation (**Figure 26)** of PSC and DBS illustrating false-negative results at plasma OD values of ≥0.8.

When compared to plasma, an at-home collected DBS study (144) demonstrated 100% sensitivity and specificity Similarly, Morley et al (151) compared DBS to serum to assess the possibility of detecting antibodies (IgG, IgM, and IgA) against S glycoprotein, reporting a

Cohen's kappa of 0.975, sensitivity and specificity of 98.1% and 100%, respectively. The manufacturer (148) reported that at ≥10 days post infection, plasma had a sensitivity of greater than 94%, while <10 days post-symptoms and PCR positive detection, the sensitivity was 44% as a result of IgG emerging roughly 14 days after symptoms (155).

2.6.3 SARS-Cov-2 IgG antibody detection using a manual ELISA

Due to the bias observed when processing the DBS and PSC using the automated instrument, DBS was further evaluated, and the results between DBS diluent and DBS PBS compared at the cut off of 0.8 visually using a scatter plot (**Figure 27**). The DBS was eluted using the manufacturer's diluent and in-house PBS. Overall, the DBS PBS findings were similar to the DBS diluent results in terms of accuracy (93.62%), and kappa coefficient (= 0.817) (**Table 12).** However, similar to above, at an interval of 11 to 20 days, the DBS PBS had low specificity (0%) and accuracy (60%). This might be due to the elution quality when antibody concentrations are low, as IgG antibodies are gradually starting to increase at that interval (**Figure 22).** The manufacturer's DBS diluent had a high sensitivity (91.43 %) and specificity (100 %), as well as a high accuracy (93.62 %) and strength of agreement (= 0.845) (**Table 12**). The DBS PBS eluted overnight at room temperature using PBS, overestimated the plasma results, as shown by the poor specificity.

According to the FDA (156) the EUROIMMUN has an overall sensitivity of 90% and a specificity of 100% when evaluated with a manual ELISA, which is identical to the results reported in our investigation with the DBS diluent. The EUROIMMUN anti-SARS-CoV-2 IgG kit was evaluated (150) using DBS and reported a high concordance correlation of $\kappa > 89$, which is an almost perfect strength of agreement, and this falls within a similar range as our reported κ value.

2.6.4 Limitations

The PSC has not yet been validated on the EUROIMMUN instrument. During elution, the remnants/particles of the PSC spots may clog the instrument leading to errors. The EUROIMMUN elution technique uses a 3.2 mm DBS spot and a 250 µl elution buffer. The volume was insufficient to cover the full PSC and DBS spot, which could explain why the PSC and DBS results differed from the plasma results. The sample size was small overall.

CHAPTER 2.7 – CONCLUSION

2.7.1 COVID-19

PSC and DBS specimen types could be used to broaden SARS-CoV-2 antibody detection for mass epidemiological surveillance in resource-constrained situations and locations remote from central laboratories. Plasma and serum are the reference specimens and come with challenges that limit access to SARS-CoV-2 serology testing. DBS and PSC are comparable in that they require less blood volume and are stable for a long length of time, which could be advantageous for transport to central laboratories. PSC, on the other hand, outperformed DBS (approved by EUROIMMUN for COVID-19 testing), but both had bias. Therefore, we further analysed DBS comparing the different elution methods of using a manufacturer's diluent and an in-house PBS protocol. Overall, eluted DBS using a manual ELISA yielded superior results than DBS eluted using an automated instrument, owing to the fact that DBS was eluted using a technique that was similar to the manufacturer's. Irrespective of the small sample size compared to the original size used on the automated instrument, IgG antibodies against SARS-CoV-2 were detected from the DBS sample using the SARS-CoV-2 IgG antibody assay. Therefore, DBS could help track the transmission route and contact tracing in resource-constrained settings, allowing more people to participate in seroprevalence investigations.

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APPENDIX 1.1

R14/49 Prof Lesley Scott

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150160

NAME: (Principal Investigator)	Prof Lesley Scott
DEPARTMENT:	Molecular Medicine & Haematology National Health Laboratory Service
PROJECT TITLE:	Research and Development Blanket Ethics Approval (Previously M000107, M041007 and M090688
DATE CONSIDERED:	30/01/2015
DECISION:	Renewal approved
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	04/03/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

APPENDIX 1.2

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa Tel: +27-11-274 9200 Fax: +27-11-274 9291

Mr M Majam,

EMAILED & COURIERED

Wits Reproductive Health and HIV Institute (
Hillbrow Health Precinct
Hugh Solomon Building
Esselen Street, Hillbrow
2001

07 March 2019

Fax: 086 634 7576

Dear Mr Majam,

PROTOCOL: HSTAR-003 - A CONTROLLED, OBSERVED TRIAL OF HIV SELF-TESTING IN THE HANDS OF UNTRAINED USERS

ETHICS REFERENCE NO: 161119

RE : APPROVAL FOR AMENDMENTS TO PROTOCOL HSTAR003

We acknowledge receipt of your letter dated 06 March 2019 with the following documentation pertaining to the above-captioned trial.

Amendment Date:	06-Mar-2019	Amendment Version.	Version 8
Amendment Number:		Received Date:	06-Mar-2019

The following has been approved by the Wits Human Research Ethics Committee: (Medical)

* Protocol HSTAR-003, WRHI069, Version 8 dated 06 March 2019 (tracked)
* Information Leaflet and Informed Consent Form, Version 5.0 dated 06 March 2019 (tracked)

Noted:
The HSTAR003 Protocol has been amended to include an additional 4ml blood draw from participants for validation of viral load testing. Currently the protocol accommodates for 9ml blood draw from participants. A total of 13ml will be required per participant.

The protocol has also been amended to include an additional oral-based HIV self-test device Avacheck in addition to Chembio (Sure Check), Atomo HIV self-test, Bioaure (blood based device). Biolytical Insti (blood-based device) and Orasure (oral-based device) stipulated in version 1, version 2, version 3, version 4, version 5, version 6 and version 7.

Ethics Approval Date: 07 March 2019

The University of the Witwatersrand, Human Research Ethics Committee Approval Granted for the above mentioned study is valid for five years. Where required by Sponsor to have approval on a more frequent basis it remains the responsibility of the Sponsor and Investigator to apply for continuing review and approval, or for the duration of the Trial.

1. THIS APPROVAL IS SUBJECT TO THE FOLLOWING PROVISOS.

* A copy of the SAHPRA Approval and/or SAHPRA Notification letter must be submitted to the Ethics Regulatory Office Secretariat before the study commences / or where an Amendment may be implemented (IF SAHPRA APPROVAL / NOTIFICATION IS APPLICABLE). It remains the responsibility of the Principal Investigator and/or Sponsor to ensure that the relevant approvals are in place.

* The study is conducted according to the protocol submitted to the University of the Witwatersrand, Human Research Ethics Committee. Any amendments to the protocol must first be submitted to the Human Research Ethics Committee for approval.

APPENDIX 1.3

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2020/10/26

Professors E Mayne, L Scott, W Stevens, et al
School of Pathology
Department of Immunology
National Health Laboratory Service

Sent by e-mail to: Lesley.Scott@wits.ac.za

Dear Professor Scott

Re: **Protocol Ref No: M200468**
Protocol Title: *4-way validation of serological and rapid point-of-care testing for Severe Acute Respiratory Viral-2 Corona Virus (SARS CoV2 or COVID-19) in South Africa*
Principal Investigators: Professors E Mayne, L Scott, W Stevens, et al

I refer to your letter of 2020/08/19 and our subsequent e-mail exchange.

I confirm that we have noted and approve of your proposal to add the validation of one or more nucleic acid amplification, near-patient, rapid diagnostic tests. The first of these will use the *Mobidiag Novodiag* platform. We further note the addition of Dr E Shoul to the investigative team.

Thank you for keeping us informed.

Yours Sincerely

J. Burns

.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

CB Penny

.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

APPENDIX 1.4

Title

Systemic review of statistical methods for evaluating new HIV viral load monitoring technologies.

Abstract

Background: The implementation of new diagnostic devices requires validation and verification procedures. Different studies have been using different statistical methods to evaluate the alternative specimen types and testing instruments for HIV viral load (VL). The lack of consistency and standardisation leads to questionable results.

Aim: To review and put forward a statistical framework for new method comparison in HIV VL monitoring.

Methods: A total of 119 publications were reviewed. Search words included were: VL, HIV/AIDS, DBS, plasma, DPS, PSD, PSC, Cobas® 6800/8800 systems, Abbott m2000 RealTime system, GeneXpert Cepheid, Cobas® AmpliPrep/cobas® TaqMan®, and method comparison. The data was then grouped by author, year, sample size, and molecular platform.

Results: The predominant platform used to monitor HIV VL was the Roche CAP/CTM (n=69). The different manuscripts used n=20 different statistical methods interchangeably. The predominant methods used were Bland-Altman analysis (n=94), sensitivity (n=59), specificity (n=54), and Pearson correlation (n=51).

Conclusion: A standardised framework comprising of data description, qualitative agreement and quantitative agreement was developed to assess new HIV VL testing methods.

Keywords: Plasma separator devices, HIV Viral Load, statistics, plasma, Dried Blood Spot, Plasma Separation Device, review, standardised framework

Introduction

In South Africa 7.8 million people are living with Human Immunodeficiency Virus (HIV) by 2020 (5). The available option for people living with HIV is anti-retroviral therapy (ART) to achieve viral load suppression and lessen the risk of transmission (88). Patients on ART have an increased life expectancy and non-adherence to the treatment may lead to the risk of developing mutations and resistance to the drugs (157). Viral load monitoring has been put in place by the World Health Organisation to monitor the effectiveness of ART and drug resistance (158). The UNAIDS goals of 90% (2020) and 95% (2035) of people infected with HIV knowing their status, being on treatment and having a suppressed viral load (4) require coordinated and multifaceted measures to reach them.

Resource-limited settings are facing challenges related to implementing universal VL monitoring (88). Plasma is the gold standard specimen for HIV viral load testing, there are logistic and economic challenges affecting the process of specimen collection, transportation and storage in virological and pharmacological settings (159). Dried blood spots (DBS) have been recommended by the World Health Organization (2013) for use as an alternative specimen collection method for viral load testing (160). Dried plasma spots (DPS) are considered as alternative method to plasma as well, some studies have successfully managed to quantify the HIV RNA and assess genotypic drug resistance using both DBS and DPS (161). The ease of blood collection, storage, transportation and reduced costs are advantages of the DBS and DPS (159). In Early infant diagnosis (EID), DBS have proven to be advantageous due to requiring a small volume of whole blood which is obtained from a heel prick (162). Similar results were observed when DBS was compared to DPS (56, 163), but cell associated nucleic acids and the low limit of detection associated with DBS leads to challenges in their use (164). The DBS leads to misclassifications that cause discontent results (70).

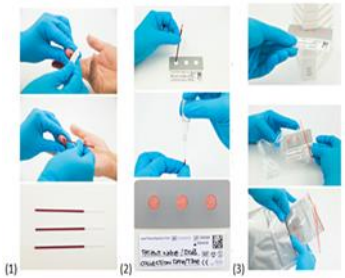
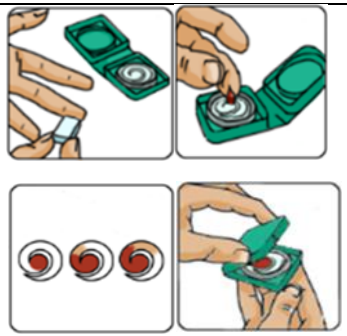
Plasma separator devices may have the ability to circumvent the plasma and DBS related challenges. They are point of care blood collection devices which separate plasma from whole blood using membrane based separation methods. A finger is prepared and pricked with a lancet, whole blood droplets are spotted onto the devices (76). The membrane allows soluble particles to pass through while preventing the flow of the large particles such as blood cells (165). Similarly to the DBS and DPS, the plasma separation devices enable easy blood collection, storage and transportation over long distances in extreme weather conditions (71) and in contrast to DBS, are free of cell associated RNA/DNA (61). The separated plasma is enclosed within the separation device and thus prevent sample contamination (61) (**Table 1)**.

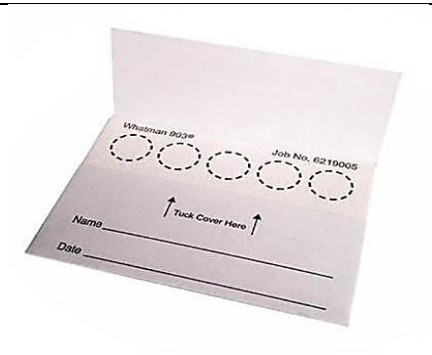
Point of Care (POC) is the near patient testing and provide an advantage of reducing the turn-around time thus improve HIV management (88). POC devices have a potential to expand viral load coverage, measure treatment progress and help reach the USAID goals (88). Ideal POC device should be able to illustrate virologic failure or success at the clinical relevant range of 1000 cp/ml, withstand extreme environmental conditions, require no power supply or need minimum, have automated features (for minimal training) and easy sample collection (166, 167). Preceding the implementation of the POC devices and other viral load testing devices (plasma separator devices) proper validation are required before use. Currently, the available POC devices are the m-PIMA by Abbott, GeneXpert by Cepheid, SAMBA by Diagnostics for the real World and Molbio Diagnostics (**Table 1**.)

Molecular Testing platforms such as Abbott m2000, Cobas 6800/8800, and Roche CAP/CTMv2 are flexible, multipurpose platforms used to quantify nucleic acids in real time. These testing platforms use RNA specific assays based on real-time amplification and detection. The instruments have a high thorough put and the entire process is automated from extraction to detection which help increase efficiency and improve services. The molecular testing platforms are further detailed in **Table 1**. One of the plasma separator devices - PSC was designed by Roche to be run on the Cobas 6800/8800 and Roche CAP/CTMv2 instruments, WHO prequalification for use of the PSC on the Roche CAP/CTMv2 and Cobas 6800/8800 instrument has been approved (71, 92).

Prior to implementing new POC, PSD, and molecular testing instruments, verification and validation is required, which will compare to the reference (gold standard) methods to prevent providing patients with inaccurate results. The aim of this meta-analysis review is to provide the best statistical practices for use in HIV VL method comparison studies by reviewing technologies used for HIV VL testing, review current statistical approaches applied in VL technology evaluations, and develop a framework for standardised evaluations of new technologies.

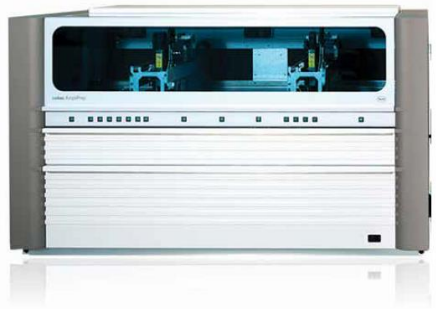
Table 1: POC devices, Plasma separator devices and Molecular testing platforms.

Device	Description	Sample type & volume	Image	Reference
Plasma Separation Card (PSC) (Roche Molecular Diagnostics, Pleasanton, CA, USA)	The finger is prepared and pricked with a lancet and blood drops (~ 140 µl) collected by a graduated capillary. The capillary is used to transfer the blood to the three spots of the spotting layer of the card. The is left to dry overnight. It is compatible with the COBAS ampliprep/COBAS TaqMan system and cobas 6800/8800 systems.	140 µl		(165)
Hemaspot-SE (HSSE) (Spot On Sciences, Inc., Austin, TX, USA)	A lancet is used to prick a finger; three whole blood droplets are added to the device centre. The blood soaks through the membrane for two minutes thereafter, the lid is closed and the device is ready to be shipped.	150 µl		(76)

VLPlasma® separation device (Spot On Sciences, Inc., Austin, TX, USA)	A hundred microliters of whole blood are aliquoted to the sample cavity of the device using a calibrated pipette and incubated for 3 minutes. Phosphate buffered saline (90 µl) is transferred to the device cavity using a supplied plastic pipette, thereafter the device is stored at room temperature.	100 µl		(77)
Whatman 903 Proteinsaver (Whatman plc, Buckinghamshire, UK)	A finger is prepared and then pricked with a lancet, whole blood droplets are transferred to the circles of the card. The Whatman 903 proteinsaver card is left to dry overnight and thereafter, stored in room temperature.	75-80 µl		(168)
GeneXpert Cepheid, (Sunnyvale, CA, U.S.A)	Automated and uses cartridges to quantify RNA from HIV positive plasma sample using the Xpert HIV-1 Viral Load assay (47). The cartridges minimize cross contamination between the specimens and at the detection limit of 1000 copies/ml, the sensitivity of the assay is 94.14% and specificity is 98.50% (47)	Plasma, 1000µl		(47)

m-PIMA (Abbott Park, IL, USA)	Automated, battery powered instrument. It uses reverse transcriptase PCR to amplify and quantitate HIV RNA. Has lower limit of detection 800 cp/ml to 1000 cp/ml and takes 70 minutes (89). The assay sensitivity and specificity is 96.8% and 47.8% (89)	Plasma, 50 µl		(88)
Molbio Diagnostics (Verna Industrial Estate, Verna, Goa 403722, India)	Battery powered, fully automated instrument. The sample is prepared in a different instrument to the amplification and detection instruments (169, 170). The results are reported within 55 minutes (88).	Plasma, 500 µl Blood , 250 µl		(88, 169, 170)

SAMBA I (Diagnostics For The Real World Ltd, Sunnyvale, CA, USA)	A semi-automated instrument which has separate units for sample preparation, amplification, and detection. Has the capability of simultaneously processing four units. Reported a concordance of 98.1% against the Roche COBAS AmpliPrep (171).	Plasm, 200 µl		(88, 171-173)
SAMBA II (Diagnostics For The Real World Ltd, Sunnyvale, CA, USA)		Blood, 120 µl		

Abbott m2000 RealTime system Abbott Molecular, Des Plaines, IL, U.S.A	A multipurpose system that runs the RealTime HIV-1 Viral Load Assay which has been configured for quantifying HIV nucleic acids in EDTA plasma and DBS specimens using real time Polymerase Chain Reaction (PCR) fluorescence detection (60). The entire process of extraction, amplification and quantitation is automated (50). At a threshold of 1000 cp/ml, the sensitivity and specificity are 76.0% and 89.7% (72).	0.2 mL, 0.5 mL, 0.6 mL, and 1.0 mL.		(174)
Cobas® AmpliPrep/cobas® TaqMan® Roche Molecular, Pleasanton, CA, U.S.A	The CAP/CTM instrument is a WHO prequalified diagnostic platform which amplifies nucleic acids in vitro including HIV RNA (49). The cobas® Ampliprep is used to amplify the nucleic acids and cobas® TaqMan® detects the nucleic acids in the specimens (49). Its sensitivity is 98.3% and specificity is 99.4% (173).	1000 µL		(50)

cobas® 6800/8800 systems Roche Molecular, Pleasanton, CA, U.S.A	An automated instrument which runs the cobas® HIV-1 quantitative nucleic acid assay with systems that are integrated (73). The instrument reduces the turnaround time, permits mix batching, and it is configured for medium (6800 system) output and high (8800 system) out thus increasing efficiency (175).The specificity is 99.95% and the sensitivity is 100% (176).	650 µL and 1300 µL		(51)

Methods

Eligibility criteria

The research studies included in this review were those related to HIV VL testing using plasma, DBS, DPS, and/or the PSD, which were quantified using the different molecular testing platforms, such as Cobas® 6800/8800 systems, Abbott m2000 RealTime system, and GeneXpert Cepheid. The articles were published from 2005 to 2020.

Search strategy

The searched databases include PubMed, Google Scholar, Google and Scopus databases through online searches. The following search words were included: *VL, HIV/AIDS, DBS, plasma, DPS, PSD, PSC, Cobas® 6800/8800 systems, Abbott m2000 RealTime system, GeneXpert Cepheid, Cobas® AmpliPrep/cobas® TaqMan®, and method comparison.*

Data collection

The extracted data was grouped by author, year, sample size, molecular platforms, specimen collection type and statistical methods and was analysed on Microsoft Excel spreadsheet (Redmond, Washington, United States).

The different steps followed in a HIV VL method comparison studies

Method comparison is the process of comparing two or more methods to evaluate a new one against the reference method as the new method might be faster, more efficient and inexpensive and may lead to the replacement of the old method (177).

The description of data is the first step in analysing data for method comparison study. Parametric testing is for normal distributed variables which is described as mean and standard deviation (± SD) and non-parametric variables are described using median and interquartile range (159).

The different methods being compared must measure a similar variable and it is significant to examine the data before starting with the analysis (178). The World Health Organisation recommended 1000 copies/ml as the standard for evaluating virologic

failure and success. In a state where the measured variables give dichotomous or binary results, such as falling into one or two categories, the validity will be evaluated using sensitivity, specificity, positive predictive value and negative predictive value (177). When comparing two methods which give numerical variables that have greater than two ordered categories, the Bland Altman plot and Lin's concordance correlation coefficient can be used (177). The bias measures the overestimation and underestimation of the new method compared to the reference method (178). The standard deviation is used to measure the repeatability and provides the range of values of agreement between the methods with upper and lower SDs are called the limits of agreement (178). Furthermore, similar methods should have small difference (average close to zero) when compared against each other thus producing a narrow limit of agreement (179).

Table 2*:* Various statistical methods used when evaluating specimen types and instruments (assays) in HIV VL testing.

Statistical Method	Description	Reference
Sensitivity	The ability of a test to identify the people infected with the disease	(177)
Specificity	The ability of the test to identify the people without the disease	
Positive predictive value	A proportion of patients which tested positive for the disease and have the disease	
Negative predictive values	A proportion of individuals who tested negative for the disease and do not have the it	
Deming regression	It assess accuracy and precision by taking into account the errors in both the reference and test method.	(33)
Accuracy	Measures the true value of the variable obtained when the new instrument is compared to the reference instrument.	
Precision	Measures the ability of a method to produce similar results when run repeatedly.	(178)
Bland Altman plot	Measures bias by plots the difference between the methods against their mean and	(32)

Lin's concordance correlation (LCC)	Assess the closeness of a range of values on the line of best fit (precision) and the closeness of the line of best fit to the 45° line from the origin (accuracy)	(180)
Passing-Bablok plot	A visualisation method which determines the degree of alignment of the data pairs on the best fit line (precision) and the alignment of the best fit line on the 45° line from the origin (accuracy)	(181)
Percentage similarity	Measures the similarities between the two methods	(86)
Cohen's kappa	Statistical measure of the degree of agreement between two methods that produce categorical data	(177)
Spearman rank	Non-parametric measure of the direction of the association between the ranked variables and their strength.	(182)
McNemars test	A non-parametric test which measures the difference between paired dependant groups	(183)
Pearson correlation	A measure of direction of association and strength between two continuous variables	(182)
Wilcoxon Signed Rank sum	A non-parametric comparison of two related groups using their means.	(184)
Mann Whitney	Compares two independent methods which have an ordinal or continuous dependent variable	(185)
T Test	Measures whether there is a significant difference between two groups using their mean.	(186)
Chi square test	Measures what is expected as compared to observed data.	(187)

Results

Summary of the collected data.

The data recovered from the manuscripts (n=119) was sorted by year, sample size, molecular platform, specimen type and statistical methods applied. The mean sample size

of the evaluated specimens was 326 (range: 6, 3114). From the year 2005 to 2020, an upsurge of manuscripts cited was observed in 2009. The manuscripts evaluated different specimen types using molecular testing platforms to quantify the HIV viral load.

Specimen types

The different specimen types were compared to assess the frequent used amongst the different manuscripts for HIV VL testing. The reference specimen for HIV VL is plasma, and it was the frequently used (n=113). WHO, recommended using DBS as an alternative specimen to plasma and it is used in EID. It was the second most used specimen (n=50). Dried Plasma Spot (n=9) and PSD (n=3) were the least, with multiple manuscripts using more than one reference specimen. The specimens were quantified using different assays and platforms.

Molecular testing platforms

The Roche CAP/CTM (69) and Abbott m2000 (61) were the predominant testing platforms used with many manuscripts using both technologies. The manuscripts used different assays/ platforms to quantify VL which lead to a variability in the statistics used to evaluate the instruments.

Statistical methods

The different manuscripts n=20 different statistical methods interchangeably. The statistical methods predominantly used were Bland-Altman analysis (n=94), sensitivity (n=59), specificity (n=54), and Pearson correlation (n=51) (**Figure 1**). This illustrates the non-standardised approach to evaluation studies. This variation requires a standardized framework to evaluate new plasma separator devices and POC devices used in HIV VL testing.

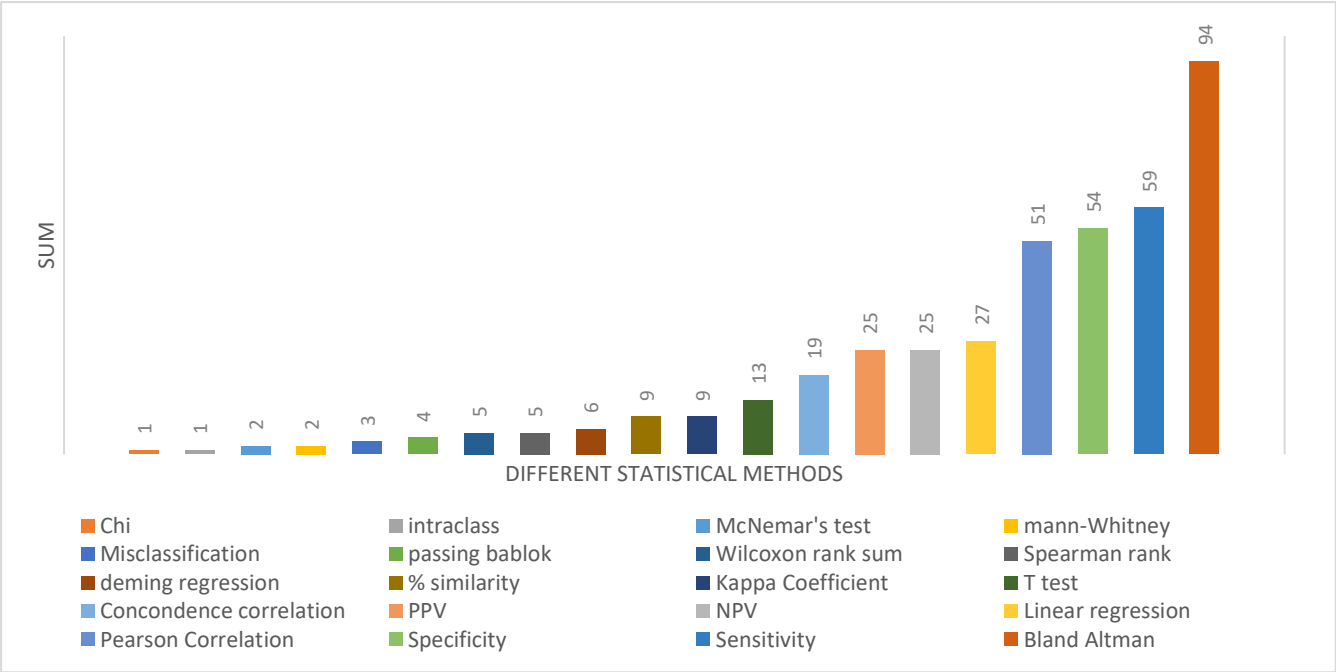


Figure 1: Comparison of the different statistical methods used in the different manuscripts, sorted in ascending order.

Standardised Framework

A framework was developed to ensure standardised evaluations of PSD that accommodate potential increased variability due to specimen collection method, type, volume and storage. Data description should include scatter plots sorted by reference VL and testing sequence. Qualitative agreement must include sensitivity, specificity and clinical misclassification, while quantitative agreement should be evaluated using Lin's concordance correlation coefficient, Bland-Altman analysis, Passing-Bablock analysis

Data description (with visualisation)	Qualitative agreement (with visualisation)	Quantitative agreement (with visualisation)
• Scatter plots sorted by reference VL and testing sequence	• Sensitivity • Specificity • Clinical misclassification	• Concordance correlation coefficient • Bland-Altman analysis • Passing-Bablock analysis • Percentage similarity

and percentage similarity. Ideally, all evaluations will include visualisation of the statistics.

Conclusions

Viral load monitoring helps HIV positive patients find out if they have virologic failure or virologic success. A variety of devices for quantifying VL are verified and validated before being used in routine central labs. This is ensuring that the instruments are at a required standard for quality diagnosis. A new method needs to be assessed by comparing it to the reference method. To make a sound decision on whether to implement the new method or not depends on what their data illustrates.

The Pearson's correlation, McNemar's, T- test, Wilcoxon rank sum, Spearman rank, and Chi square test measure associations and does not take into account the errors of the reference method, significant difference, measures expected and observed data and related variables, therefore might produce variable results when evaluating a new device for HIV viral load. The two variables measured are not directly proportional to each other and both of their errors have to be taken into account to properly assess the new technology put in place.

These data from the manuscripts highlight the variability between HIV-VL evaluations and the need for standardised statistical method comparison. The developed framework incorporates the necessary statistical methods required to evaluate new methods for HIV VL. It visualizes the data for clear head to head comparison, evaluates the strength of agreement and bias, and assesses the similarities between devices. The recommended framework needs to be applied to relevant datasets evaluating PSD for HIV VL to validate this approach.