Detection of Hepatitis B virus using dried blood spots and plasma separation cards

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DECLARATION

I, Tsholofelo Moabi, declare that the dissertation entitled 'Detection of Hepatitis B Virus using dry blood spots and plasma separation cards', submitted for the degree Master of Science in Biology at the North-West University, Mafikeng Campus, has not previously been submitted by me to this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.



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DEDICATION

I dedicate this work to myself, Tsholofelo Moabi for not giving up on my studies even in difficult times. I held my head up high with confidence and no fear and continue with the commitment I made for myself.

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I would like to express my sincere appreciation to the following persons and institutions for their contributions and support towards the completion of this study:

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ABSTRACT

Hepatitis B virus (HBV) infection is one of the major public health problems in the world. Testing and diagnosis are the first steps that provide access to prevention and treatment services; however, many undiagnosed infections remain in resource-limited settings. This is because the current tests require the use of plasma samples which can only be obtained with venepuncture and some people have poor veins that are difficult to draw blood from and some are afraid of venous pricks and therefore not willing to give venous blood for testing. Thus, the collection of blood by a small finger prick and spotting on dried blood spots (DBS) is an alternative method that can overcome the problems seen with venepuncture blood collection. Moreover, there is a newly developed blood collection method called plasma separation card (PSC) which has similar features as DBS but rather separates plasma from whole blood, and offers excellent sample stability and a higher volume capacity. Thus, the aim of this study was to evaluate the feasibility of DBS and PSC for the detection of HBV infection in resource-limited areas.

A total of 60 EDTA donated blood samples, 30 HBV positive and 30 HBV negative were used in the study. The Determine^{TM} HBsAg 2 Rapid test was used to test for HBV in the samples. DBS and PSC samples were prepared from the 60 blood samples and the remainder of the samples were used to obtain plasma samples. Ten randomly selected samples were stored at different temperatures, that is, 4 °C and room temperature (RT), for 3 days to assess the stability of the DBS and PSCs. The detection of HBV from DBS, PSC and plasma samples was then evaluated using Architect i1000sr and GeneXpert assays. Viral DNA was also extracted from all the samples and the complete HBV surface gene was amplified by nested PCR. The PCR amplicons were then purified and sequenced using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) on the AB 3500 xL Genetic Analyser DNA sequencer (Applied Biosystems, California, USA). DBS and PSC showed no significant change in HBsAg and HBV VL after 3 days at 4ºC and RT compared to samples stored at -20 ºC.

HBsAg was detected in 47 positive plasma samples, 35 positive DBS samples and 32 positive PSC samples using the Architect HBsAg assay. The sensitivity and specificity

of the DBS and PSC methods were compared with the gold standard plasma method and were determined using the area under the ROC curve (AUROC) analysis method. The DBS method was 76% sensitive and 56% specific while the PSC method was 80% sensitive and 56% specific.

HBV viral load (VL) was detected in 26/47 plasma, 19/34 DBS and 22/31 PSC samples using GeneXpert. The mean difference of HBV DNA VL detected with DBS vs plasma samples was 0.5 log10 IU/ml while that of PSC vs plasma samples was 0.8 log10 IU/ml with a strong correlation of $R^2 = 0.6019$; p-value < 0.00 and $R^2 = 0.6726$; p-value < 0.01 respectively. The Bland-Altman analysis plot showed great agreement when detecting HBV VL between plasma samples and card samples (DBS and PSC) for all storage conditions. DBS and PSC methods were able to detect HBsAg and HBV DNA VL even for samples stored at 4 °C and room temperature for 3 days.

Some of the DBS and PSC samples were PCR amplified and compared with plasma samples. The samples were used further for phylogenetic analysis using Mega 7 software. Three genotypes (genotypes A, D and E) were detected. The study recommends the use of DBS and PSC blood collection methods as the best alternative to the venepuncture blood collection method.

Keywords: Hepatitis B Virus, Dried Blood Spots, Plasma Separation Cards, Venepuncture blood collection, HBV Diagnosis, HBV Genotyping

LIST OF ABBREVIATIONS

ART:	Antiviral Treatment
CHB:	Chronic HBV
CVI:	Centre of Vaccine and Immunology
DBS:	Dry Blood Spots
EDTA:	Ethylenediaminetetraacetic acid
FP:	False positive
FN:	False negative
HBV:	Hepatitis B Virus
HBsAg:	Hepatitis B surface antigen
HCC:	Hepatocellular Carcinoma
HBcAg:	Hepatitis core antigen
HBeAg:	Hepatitis e antigen
NICD:	National Institution of Communicable Disease
NWU:	North-West University
NHLS:	National Health Laboratory Services
ORF:	Open Reading Frame
PCR:	Polymerase Chain Reaction
PSC:	Plasma Separation Card
RT:	Room Temperature
ROC:	receiver operating characteristic curve
SA:	South Africa

SSA:	Sub-Saharan Africa
S/CO:	Signal per Cut Off
SPEX:	Specimen Pre Extraction
TP:	True positive
TN:	True negative
VL:	Viral load
WHO:	World Health Organization
Xpert:	GeneXpert®

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CHAPTER 1

1.1 INTRODUCTION

Hepatitis B virus (HBV) is one of the most prevalent blood-borne viruses affecting the liver and causes acute and chronic hepatitis and cirrhosis and hepatocellular carcinoma (HCC) (Ortonne *et al.*, 2022; Shenge & Osiowy, 2021). Although there are available effective vaccines and anti-viral treatments, HBV infection remains a major public health problem worldwide (Woldemedihn *et al.*, 2021; Bogler *et al.*, 2018). It is estimated that 350 million people have been infected with HBV and only a few have been diagnosed (Ortonne *et al.*, 2022). There are about 1 million death cases reported each year due to HBV liver complications (Chevaliez & Pawlotsky, 2018).

The majority of infected individuals are unaware of their HBV status of infection (Martínez-Campreciós *et al.*, 2021; Amini *et al.*, 2017). This is because viral hepatitis is generally asymptomatic until advanced liver disease develops, which also increases the chance of HBV transmission (Chevaliez & Pawlotsky, 2018; Spearman *et al.*, 2017). HBV infection is mostly transmitted through the perinatal route (during birth), sexually and by drug injection. However, the likelihood of being chronically infected is higher at a young age as it has been reported in about 90% of infants, 30-50% of children between the ages of one to five years and 5% of adults.

The World Health Organization (WHO) has announced a goal to eliminate chronic viral hepatitis, including HBV as a public health threat, by the year 2030 by reaching full coverage of prevention, diagnosis and treatment monitoring (Jackson *et al.*, 2021; Bogler *et al.*, 2018). The most significant way to increase awareness about HBV infection and to prevent delay in diagnosis is by improving access to HBV testing (Amini *et al.*, 2021; Shenge & Osiowy, 2021). However, it is challenging to access HBV testing facilities in low and middle income countries such as sub-Saharan Africa (SSA) where the prevalence of chronic HBV is high (Woldemedihn *et al.*, 2021; Vinikoor *et al.*, 2015). This is because in rural areas there is limited access to healthcare services and a lack of sophisticated laboratories with appropriate collection, storage, and transportation of specimens (Hans *et al.*, 2021).

Generally, susceptible people are tested through laboratory means using serum or plasma to detect HBV antigens, antibodies, or DNA and to assess the stage of liver disease for treatment and/or monitoring of the infection (Cruz *et al.*, 2020; Xiao *et al.*, 2020; Song, 2016). Serological tests are the first tests to be performed for the diagnosis of chronic hepatitis B (CHB) by detecting Hepatitis B surface antigen (HBsAg) followed by quantification of the HBV viral load (VL) as a confirmatory test (Martínez-Campreciós *et al.*, 2021; Amini *et al.*, 2017). HBsAg is one of the serological markers that indicate acute infection (Guvenir & Arikan, 2020). It has been reported that the persistence of positive HBsAg for more than 6 months suggests chronic infection (Woldemedihn *et al.*, 2021; Guvenir & Arikan, 2020). Therefore, the detection of HBsAg is essential in identifying patients who have been exposed to HBV infection and determining their clinical phase of infection (Coffin *et al.*, 2019; Song, 2016; Mendy *et al.*, 2006). Quantification of HBV DNA in serum is also important in predicting the clinical outcome of the infection and is usually performed to monitor antiviral therapy in order to identify patients with treatment failure (Bezerra *et al.*, 2021; Roger *et al.*, 2020).

It is noteworthy that the sampling of HBV diagnostic tests requires the use of large volumes of blood, professional phlebotomists, reliable plasma transportation and continuous cold-chain facilities for storage of samples and reagents which are not available in resource-limited settings (Carmona *et al.*, 2019; Jackson *et al.*, 2019). The other challenge is the cost of the diagnostic tests which is more costly than treatment in many low-income countries (Xiao *et al.*, 2020). As a result, patients in rural areas are less likely to know their HBV status or have their VL tested thus management of their infection is limited compared to patients in urban areas (Nichols *et al.*, 2020; Xiao *et al.*, 2020; Girdwood *et al.*, 2019). Therefore, there is a need for a more feasible and cost-effective approach to HBV sampling and diagnosis (Zeh *et al.*, 2017).

Research has recently moved to alternative methods other than plasma samples to overcome these challenges and improve access to and uptake of VL testing. One of the alternative methods is the use of dried blood spots (DBS) (Nichols *et al.*, 2020; Carmona *et al.*, 2019). DBS is a blood sample collection technique which is easily drawn from a finger prick and spotted on a filter paper card, and requires a small volume of blood compared to venipuncture (Hans *et al.*, 2021). There are also newly

developed blood-collecting devices known as plasma separation cards (PSC) that are similar to DBS except that they separate whole blood from plasma and collect the plasma, which offers additional sample stability (Marins *et al.*, 2020; Vubil *et al.*, 2020).

DBS and PSC samples are easy to transport and can easily be stored at room temperature for longer periods without degradation of the viral nucleic acid (Su *et al.*, 2018; Zeh *et al.*, 2017; Stene-Johansen *et al.*, 2016). It has also been shown that DBS and PSC can help with people being more receptive to testing for hepatitis B, particularly in resource-limited areas as samples can be collected any day without the need for special transportation and cold-chain storage (Nichols *et al.*, 2020; Yamamoto *et al.*, 2020). Although they have been widely used in settings where nucleic acid testing is unavailable and for people with poor venous access (Ortonne *et al.*, 2021; Poiteau *et al.*, 2020), there is a limited number of publications, particularly in sub-Saharan Africa, that have evaluated the use of PSC on HBV testing.

1.2 Problem statement

Chronic Hepatitis B virus (HBV) remains a major public health problem worldwide, despite availability of effective treatment and prevention (Schweitzer *et al.*, 2015). It is a major health issue with a reported global estimated incidence of 296 million infected people in 2023 and almost 750,000 people die from hepatitis-related infections every year (World Health Organization, 2023). Traditionally, the diagnosis of HBV infection is usually performed with the detection of hepatitis B surface antigen (HBsAg), hepatitis B surface IgM antibodies and the presence of HBV DNA (Guvenir & Arikan, 2020; Song, 2016). These detection methods require the collection of large volumes of blood by venipuncture, thus resulting in an urgent need for other forms of sampling that may greatly reduce the amount of pain experienced by infected people and the volume of blood, but which will produce the same result as the gold standard i.e., using plasma samples. Improved sampling methods will be helpful to identify most patients who are infected with HBV on time and the WHO goal of eliminating HBV by 2030 can be achieved.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

This study aimed to detect HBV surface antigen (HBsAg), viral load and genotyping of infected blood samples using dry blood spots (DBS) and plasma separation cards (PSC), respectively, in comparison to plasma samples.

1.3.2 Objectives

1. To determine the performance of HBsAg tests of DBS and PSC samples in comparison with plasma samples.
2. To determine the performance of HBV viral load of DBS and PSC samples in comparison with plasma samples.
3. To isolate HBV DNA from DBS and PSC eluates and plasma samples.
4. To assess the performance of HBsAg, viral load and genotyping on DBS and PSC samples stored at varying temperatures.
5. To amplify the complete surface (S) gene of HBV from DBS and PSC eluates and plasma samples.
6. To sequence the PCR amplicons and analyse the sequences from DBS and PSC samples in comparison with the plasma samples.

CHAPTER 2

2.1 LITERATURE REVIEW

2.1.1 Structure of Hepatitis B Virus

Hepatitis B virus (HBV) is a small (32 kb) circular, partially double-stranded DNA virus that belongs to the family hepadnaviridae (Ambachew *et al.*, 2018; Kondo *et al.*, 2013) The nucleocapsid of HBV contains four overlapping open reading frames (ORF) namely, the surface protein (pre-S/S), pre-core/core (pre-C/C), DNA polymerase (P) and transcriptional co-activator (X) (Song, 2016). The pre-S/S open reading frame is responsible for the large, middle, and small envelope proteins. The pre-C/C codes for two antigens, the hepatitis core antigen (HBcAg) which is responsible for forming the protein shell of the nucleocapsid, and the hepatitis e antigen (HBeAg) which is a marker for active viral replication (Guvenir & Arikan, 2020; Zhang *et al.*, 2016; Villar *et al.*, 2015). The DNA polymerase is essential for reverse transcription, and the X gene regulates infection processes such as transcription, signal transduction, apoptosis and protein degradation pathways (Song, 2016; Pollicino *et al.*, 2014).

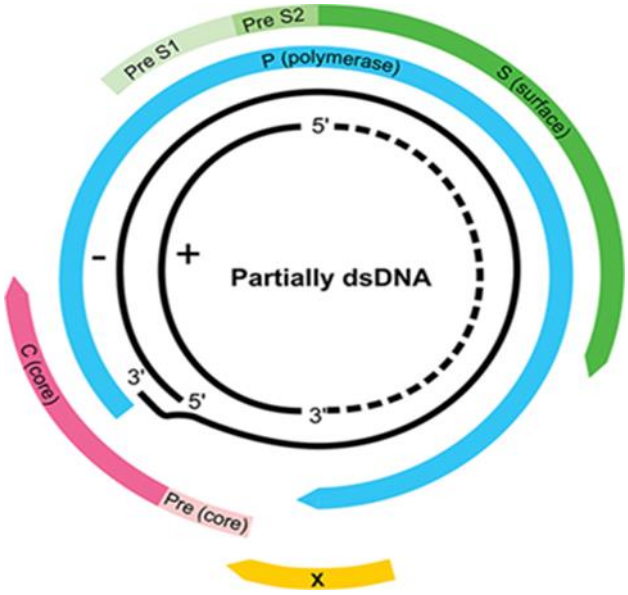


Figure 2. 1 Hepatitis B virus genome partially double-stranded DNA with the four open reading frames (ORFs) is shown with the pre-core/core (preC/C) encoding for HBcAg and HBeAg highlighted in pink, polymerase (P) encoding for reverse transcriptase in blue, PreS1/PreS2/S encoding for the surface proteins in green and X the transcriptional trans-activator protein in yellow. Adapted from Bell and Kramvis (2016).

HBV viral particles exist in the serum of an infected person in two forms, a mature virion of 42 nm in diameter with viral DNA and a subviral particle of 22 nm without viral DNA (Chang, 2007; Lu & Block, 2004). A mature virion can replicate up to 10^8 virions/ml or more in the infected person (Weinberger *et al.*, 2000). However, viral infection is associated with a higher production of subviral particles that are 100 to 100,000-fold more than mature virions (Coffin *et al.*, 2019; Wei *et al.*, 2010).

2.1.2 Transmission of HBV infection

HBV DNA is transmitted through contact with infected blood and other body fluids such as saliva, tears, sweat, semen and vaginal secretions of infected individuals (Paoli *et al.*, 2018; MacLachlan & Cowie, 2015; Franco *et al.*, 2012). Most HBV infections worldwide are acquired through vertical transmission (perinatal), horizontal transmission (between young children through sharing of contaminated objects) and sexual transmission (Guvenir & Arikan, 2020; Bogler *et al.*, 2018).

HBV causes acute infection and chronic liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) that put infected individuals at high risk of death (Cayarga *et al.*, 2019). During the acute phase of infection, most individuals do not show symptoms, while few might have a progressed infection with symptoms that later develop into acute liver failure leading to death (Zhang *et al.*, 2016; Papastergiou *et al.*, 2015). The immune system of other individuals can control the infection by eradicating the virus from the bloodstream without being diagnosed with liver diseases, while others fail to clear the virus and develop chronic infection (Ott *et al.*, 2012; Bertoletti & Gehring, 2006). A chronic infection develops when a person has been infected with HBV for more than six months while clearance occurs when a person has recovered from a previous HBV infection. It is advised that everyone who has been in contact with an HBV-infected person should be tested and vaccinated against HBV to prevent transmission (Bogler *et al.*, 2018). The Hepatitis B vaccine is the most effective measure used to decrease HBV transmission and prevent infection and it is available in clinical settings (Xiao *et al.*, 2020; Franco *et al.*, 2012). South Africa (SA) introduced the 3-dose HBV vaccine in the national vaccination schedule for infants 6, 10 and 14 weeks in 1995 (Burnett *et al.*, 2012).

Several studies including those from SA reported a significant reduction in HBV infection through vaccination programmes (Prabdial-Sing *et al.*, 2019; Chevaliez & Pawlotsky, 2018; Ghosh *et al.*, 2016). However, its implementation remains inadequate in low-income countries and does not benefit people who are already infected (Xiao *et al.*, 2020; Ghosh *et al.*, 2016).

2.1.3 Epidemiology of HBV

The World Health Organization (WHO) reported 296 million people, who are chronically infected worldwide with about approximately 2.5 million from SA (World Health Organization, 2023). Every year, there is a record of about 1 million death cases that are caused by acute and chronic HBV infection (Yamamoto *et al.*, 2020; Pourkarim *et al.*, 2014). Although HBV infection is found all over the world, its prevalence differs amongst the different populations worldwide (Papastergiou *et al.*, 2015). The difference is due to HBsAg levels, the higher the level of HBsAg the higher the transmission (Guvenir & Arikan, 2020; Nguyen *et al.*, 2020) and this led to countries being divided into three HBsAg level categories, that is, high (≥8%), intermediate (2–7%) and low (<2%) (Bogler *et al.*, 2018; Caligiuri *et al.*, 2016; Papastergiou *et al.*, 2015). In high-prevalence countries such as Southeast Asia and Africa, transmission is generally perinatal and horizontally between children (Papastergiou *et al.*, 2015; Schweitzer *et al.*, 2015). Perinatal transmission occurs mostly with HBV infected mothers that are positive for HBeAg with high viral loads (VL) (Béguelin *et al.*, 2018), while in low prevalence countries the infection rate is higher in young adults and usually transmitted through injection drug use and high-risk sexual behaviours (Béguelin *et al.*, 2018; Schweitzer *et al.*, 2015; Mohamed *et al.*, 2013).

Other vulnerable groups that acquire HBV infection are healthcare workers, sex workers, prisoners, intravenous drug users and immigrants to highly endemic countries (Nguyen *et al.*, 2020; Mohamed *et al.*, 2013; Franco *et al.*, 2012). The high risk of HBV infection in healthcare workers is due to work-related exposure to infected body fluids without proper or no personal protection equipment and contaminated medical equipment (Bogler *et al.*, 2018; Trépo *et al.*, 2014)). A major concern is in high-prevalence countries, where a majority of infected individuals are unaware of their HBV status of infection (Martínez-Campreciós *et al.*, 2021; Franco *et al.*, 2012). This is

because viral hepatitis is generally asymptomatic until advanced liver disease develops, which also increases the chance of HBV transmission (Chevaliez & Pawlotsky, 2018). Poor access to health care services, lack of sophisticated laboratories and the high cost of tests are factors that increase the burden of infection in high-prevalence countries (Nguyen *et al.*, 2020; Amponsah-Dacosta *et al.*, 2014)).

Although an estimated number of HBV infections has been reported globally, the overall infection number may often be miscalculated because vulnerable populations are not captured in national prevalence estimates (Nguyen *et al.*, 2020; Papastergiou *et al.*, 2015). Furthermore, the miscalculation of HBV infection does not only occur in underdiagnosed populations but also in occult HBV infection (OBI) carriers (Subic & Zoulim, 2018). OBI is an HBV infection with detectable HBV DNA in the absence of HBsAg in the serum (Kuhns *et al.*, 2019; Subic & Zoulim, 2018). The burden of HBV will continue to rise if governments, healthcare workers and societies do not raise awareness and improve the accessibility of care (Béguelin *et al.*, 2018; Trépo *et al.*, 2014). Consequently, WHO has announced a goal to eliminate chronic viral hepatitis as a public health threat by the year 2030, aimed at reaching full coverage of prevention and monitoring treatment and diagnostic processes even in lower-income countries (Dionne-Odom *et al.*, 2018; Spearman *et al.*, 2017; Schweitzer *et al.*, 2015).

2.1.3.1 The burden of HBV infection in South Africa

In South Africa, the main infected population group is Blacks, especially in rural areas as compared to urban areas (Prabdial-Sing *et al.*, 2019; Burnett *et al.*, 2012). A previous study showed a prevalence rate of 15.5% in the Eastern Cape and as low as 7.4% in Durban and 1.3% in Soweto, Johannesburg (Burnett *et al.*, 2012). The high rate of human immunodeficiency virus (HIV) infection in SA is also a concern, and studies have reported that the development of chronic liver disease to fibrosis and cirrhosis is high in HIV/HBV coinfected patients (Prabdial-Sing *et al.*, 2019; Dionne-Odom *et al.*, 2018; Chotun *et al.*, 2015). Patients with HIV are at a higher risk of developing HBV-related liver disease (Béguelin *et al.*, 2018; Dionne-Odom *et al.*, 2018).

2.1.4 Replication of HBV

Replication of HBV takes place only in the liver cells where the virus enters the liver through the bloodstream (Wei *et al.*, 2010; Shepard *et al.*, 2006). Once HBV has entered a cell, it uncoats and moves the relaxed partially double-stranded genome to the nucleus, where it will be converted into a covalently closed circular HBV DNA (cccDNA) by the reverse transcription enzyme (Lin & Kao, 2017; Moucari & Marcellin, 2011) as shown in figure 2.2. The cccDNA will remain stable and resistant in an infected hepatocyte nucleus, and then formulate its own mRNAs known as pregenomic RNA (pgRNA) for the production of the core (capsid) particle (Seto *et al.*, 2018). This pgRNA is then converted into a DNA genome by the DNA polymerase within the capsid in the cytoplasm, which can either re-enter the nucleus for another cccDNA formation or be enveloped and released from the cell (Lin & Kao, 2017; Trépo *et al.*, 2014).

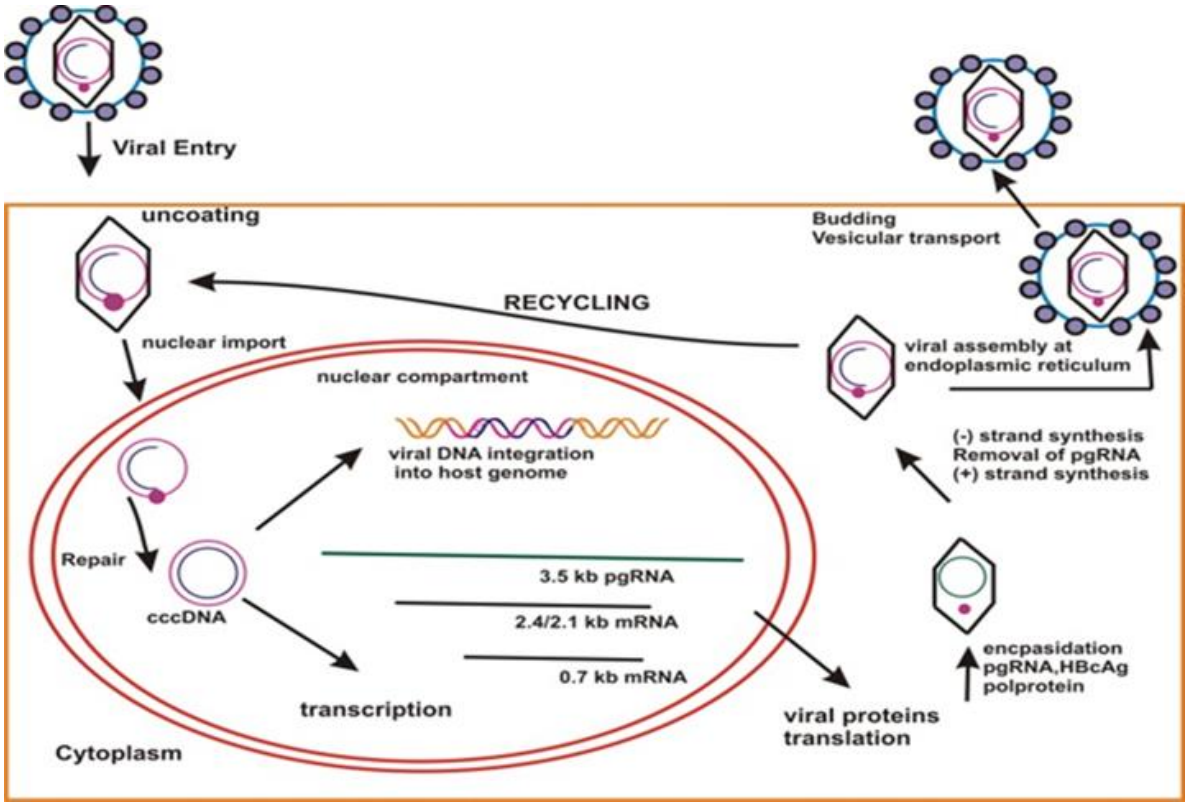


Figure 2. 2 Hepatitis B virus replication. Adapted from Jayalakshmi *et al.* (2013).

Although HBV is a DNA virus, it replicates through reverse transcription of pregenomic RNA (3.5 kb) initiated by viral polymerase from the covalently closed circular DNA (cccDNA) in the cell nucleus (Kramvis, 2018; Lin & Kao, 2017; Kramvis, 2014). Studies

report that the efficiency of the virus to infect depends on the stability of the ccc DNA (Trépo *et al.*, 2014). The cccDNA is a minichromosome found in infected hepatocytes which functions as a template that produces viral mRNAs (Song, 2016; Tong & Revill, 2016; Trépo *et al.*, 2014). Therefore to cure chronic HBV, cccDNA has to be eliminated (Bogler *et al.*, 2018; Nassal, 2015). However, there is little information in the literature on the molecular mechanisms of cccDNA formation and degradation (Nassal, 2015).

The reverse transcription enzyme has a deficient proofreading activity that results in a high mutation rate within the viral genome compared to other DNA viruses (Liu *et al.*, 2017; Pourkarim *et al.*, 2014). Thus, the HBV genome changes rapidly over time with a nucleotide substitution rate of 1.4 - 3.2 × 10-5 sites/year (Liu *et al.*, 2021; Zhang *et al.*, 2016). This unique replication strategy causes HBV variants to be classified into genotypes, subgenotypes, mutations, recombinants and even quasispecies (Liu *et al.*, 2021; Araujo *et al.*, 2020; Lin & Kao, 2017). Some of these variants are capable of escaping diagnosis, prevention and treatment (Zhang *et al.*, 2016; Pourkarim *et al.*, 2014). Furthermore, several studies have also reported that a high level of overlapping open reading frames of the HBV genome causes more mutations (Seto *et al.*, 2018). Kania *et al.* (2014) stated that the mutation rate of the different regions of the HBV genome also differs due to the open reading frame restrictions. During mutation, the restrictions are caused by extreme overlapping of the open reading frames which limits the possibility of fixing the mutation rate.

2.1.5 HBV Genotypes

There are ten HBV genotypes (A to J) that have been identified based on nucleotide divergence of more than 8% over the entire genome sequence (Paoli *et al.*, 2018; Caligiuri *et al.*, 2016; Sunbul, 2014). These genotypes develop as a result of mis-banding nucleotides during the replication process (Attaullah *et al.*, 2011). Some of the genotypes are further divided into several sub-genotypes with nucleotide divergence of between 4% and 7.5% (Ambachew *et al.*, 2018; Pourkarim *et al.*, 2014). HBV genotypes and sub-genotypes are geographically distributed worldwide as shown in Table 2.1 and can be associated with the mode of transmission (Araujo *et al.*, 2020; Caligiuri *et al.*, 2016; Sunbul, 2014).

Table 2. 1 Geographic distribution of Hepatitis B Virus genotypes. Adopted from Nguyen *et al.* (2020).

HBV genotype	Geographic lotion
A	Sub-Saharan Africa, India, northern Europe, Western Africa, Gambia, Nigeria
B	Japan, East Asia, Taiwan, China, Indonesia, Vietnam, Philippines, Alaska, northern Canada, Greenland
C	Taiwan, China, South Korea, Japan, Southeast Asia, Australia, Philippines, Vietnam, Indonesia
D	Africa, Europe, Mediterranean countries, India, Indonesia, Australia
E	Western and Central Africa, Saudi Arabia
F	Central and South America
G	France, Germany, United States
H	Central America
I	Vietnam and Laos
J	Japan

Genotypes B and C are found in high-endemic regions where transmission is through perinatal or vertical, while the other remaining genotypes prevail in regions with horizontal transmission (Velkov *et al.*, 2018; Sunbul, 2014; Lin & Kao, 2011).

HBV epidemiological studies and genotype studies are essential for monitoring the spread of the disease and to guide the efficiency of antiviral treatment (Velkov *et al.*, 2018; Song, 2016; Kramvis, 2014). Severe chronic liver disease is associated with genotypes A and C (Coffin *et al.*, 2019) while genotype D is associated with a higher risk of HBV reappearance and death after a liver transplant (Papastergiou *et al.*, 2015; Ma *et al.*, 2011). Thus, genotypes are an important element in the study of acquisition and geographic distribution of HBV strains (Al Baqlani *et al.*, 2014; Lin & Kao, 2011).

There are variations amongst HBV genotypes and subgenotypes regarding symptoms, pathogenicity and response to antiviral treatment (Liu *et al.*, 2021; Araujo *et al.*, 2020; Al Baqlani *et al.*, 2014). However, the mechanisms of the different HBV genotypes to cause disease are not yet known (Araujo *et al.*, 2020; Sunbul, 2014). A better response

to interferon therapy has been reported in patients infected with genotypes A and B than in patients infected with genotypes C and D (Velkov *et al.*, 2018). On the contrary, Al Baqlani *et al.* (2014) stated that the treatment response of nucleoside/nucleotide analogues does not depend on HBV genotypes, however, they affect the efficacy of the HBV vaccine (Al Baqlani *et al.*, 2014). Although HBV genotyping is not necessary for diagnosis, it is important to determine the genotype and mutations for epidemiological data and to guide treatment options with regard to genotypes with drug resistance patterns (Liu *et al.*, 2021; Guvenir & Arikan, 2020).

Africa is one of the highly endemic regions for HBV, with five predominating HBV genotypes (A-E) (Ambachew *et al.*, 2018; Kramvis, 2018; Al Baqlani *et al.*, 2014). However, there is limited knowledge about the distribution of HBV genotypes in Africa (Ambachew *et al.*, 2018). Several studies have listed immigration as a significant factor in global HBV distribution which has greatly changed the geographic pattern of HBV genotypes (Wolf *et al.*, 2021; Zhang *et al.*, 2016). Genotype A which is the first genotype to be identified is generally found in southeast Africa, including South Africa, and southern Asia (Kramvis, 2018; Croagh *et al.*, 2015; Kramvis, 2014). Genotype D has been reported throughout Africa but prevails mostly in northern Africa while Genotype E is found in western Africa (Ambachew *et al.*, 2018; Kramvis, 2018).

Genotype A is classified into seven subgenotypes (A1-A7) and it has been found in specific regions in Africa such as Cameroon, Mali, Gabon, Nigeria and the Congo (Wolf *et al.*, 2021; Zehender *et al.*, 2015). However, subgenotypes A1 and A2 have been detected in many parts of the world (Wolf *et al.*, 2021). The origin of subgenotype A1 is in Africa while that of subgenotype A2 is in Europe (Wolf *et al.*, 2021; Kramvis, 2018; Zehender *et al.*, 2015). Other studies have reported that subgenotype A2 also belongs to southern Africa and it was introduced in Europe by migration of the slave trade back in the 19th century (Wolf *et al.*, 2021; Zehender *et al.*, 2015; Kramvis & Kew, 2007).

2.1.6 Diagnosis

The most important way to reduce the burden of HBV infection apart from active anti-HBV vaccination is through the diagnosis of acute and chronic HBV (CHB) infection (Bezerra *et al.*, 2021; Song, 2016). HBV infection is traditionally diagnosed by detecting

the following serum markers: HBeAg, HBsAg, anti-HBc (IgM and IgG), and anti-HBs using serological tests (Guvenir & Arikan, 2020; Cayarga *et al.*, 2019; Song, 2016)), while the nucleic-acid amplification tests and sequencing are used to detect HBV DNA (qualitative and quantitative), mutations and genotypes (Shenge & Osiowy, 2021; Liu *et al.*, 2017). The detection of these serum markers is essential to identify patients who have been exposed to HBV infection, to determine the clinical phase of infection and to monitor antiviral therapy (Coffin *et al.*, 2019). However, it is important to know about the latest or current diagnostic tests that are available so that patients can be accurately screened, diagnosed and treated (Shenge & Osiowy, 2021; Nguyen *et al.*, 2020).

2.1.7 Serology

2.1.7.1 Hepatitis B surface antigen

The HBsAg is the first serologic marker that indicates active infection (Xiao *et al.*, 2020; Ott *et al.*, 2012; Jaroszewicz *et al.*, 2010). HBsAg is an envelope protein found on the surface of infectious virions called Dane particles (Guvenir & Arikan, 2020). It is highly secreted by the infected cells as a subviral particle and has been reported as a causative agent of HBV that evades immune response (Coffin *et al.*, 2019; Karra *et al.*, 2016; Jaroszewicz *et al.*, 2010). The secretion of HBsAg in the blood occurs before the appearance of symptoms, within the first two weeks of exposure to HBV (Guvenir & Arikan, 2020; Song, 2016). The presence of HBsAg in blood for more than six months indicates chronic infection (Guvenir & Arikan, 2020).

The development of CHB is through four different phases, namely the immune-tolerant phase (IT), immune clearance phase (IC), low-replicative phase (LR) and Hepatitis B e antigen-negative hepatitis (Coffin *et al.*, 2019; Karra *et al.*, 2016; Pollicino *et al.*, 2014). It is generally believed that HBsAg levels vary amongst the four phases of CHB infection (Karra *et al.*, 2016; Wursthorn *et al.*, 2011). A systematic analysis evaluating serum HBsAg in different phases of CHB has reported a higher median HBsAg titre in IT (4.62 log10 IU/ml) compared to the LR phase (2.76 log10 IU/ml) (Karra *et al.*, 2016). This shows that there is no immune response against HBsAg during the first phase of HBV infection (IT), hence the high antigen level in the blood (Hong *et al.*, 2019; Karra *et al.*, 2016).

Seroclearance of HBsAg is considered an effective immunological control of active HBV and the main functional cure for CHB (Cornberg *et al.*, 2017; Karra *et al.*, 2016; Liaw, 2011). Thus, for the development of a new HBV treatment, serum HBsAg kinetics are necessary for routine tests (Cornberg *et al.*, 2017). Moreover, recovery or immunity acquired through vaccination is indicated by serum hepatitis B surface antibodies (anti-HBs) (Guvenir & Arikan, 2020; Song, 2016). However, it has been reported that in the first phase of the infection, some hosts may suppress the level of HBsAg without developing HB antibodies and therefore remain infected (Guvenir & Arikan, 2020; Hong *et al.*, 2019; Karra *et al.*, 2016).

There has been an increased interest in serum HBsAg quantification since studies discovered that the level of serum HBsAg in the blood correlate with the transcription activity of cccDNA in the liver (Cornberg *et al.*, 2017; Song, 2016; Liu & Yao, 2015)). The discovery of quantitative HBsAg detection assisted greatly in monitoring the disease and treatment response (Karra *et al.*, 2016; Liu & Yao, 2015). The first methods for detecting HBsAg which were introduced 20 years ago using electron microscopy, radioimmunoassay and enzyme Immunoassay have been reported to be difficult to perform, they involve a great deal of manual work and are confined to research settings with the need for highly trained personnel (Amini *et al.*, 2017; Liu & Yao, 2015).

Recently, there have been new HBsAg quantitative assays that are automated, standardized, more reproducible, and available for clinical use (Karra *et al.*, 2016; Liu & Yao, 2015; Jaroszewicz *et al.*, 2010). One of the automated systems is the Architect HBsAg QT (Abbott Laboratories, Abbott Park, IL, United States). It is an automated chemiluminescent microparticle immunoassay method that is widely used in clinical practice. It is a two-step immunoassay with flexible assay protocols referred to as Chemiflex, for quantitative measurement of HBsAg concentrations in serum and plasma. The Architect system can detect as low as 0.2 ng/mL HBsAg with a dynamic range of 0.05 - 250 IU/mL (Liu & Yao, 2015).

The diagnosis of HBsAg can also be detected with rapid diagnostic tests in lateral flow devices which are one-step methods, easy to use and disposable (Shenge & Osiowy, 2021; Xiao *et al.*, 2020; Amini *et al.*, 2017). RDTs can easily be performed as a point-of-

care diagnosis method, at home or outside medical facilities without compromising the accuracy of the test (Shenge & Osiowy, 2021; Guvenir & Arikan, 2020; Amini *et al.*, 2017). Three HBV RDTs that have been prequalified by the WHO include Determine HBsAg (Alere Medical Co. Ltd, Chiba-ken, Japan), VIKIA HBsAg (bioMerieux SA, Marcy-l'E'toile, France) and SD Bioline HBsAg (Abbott Diagnostics Korea Inc. Giheung-gu, Republic of Korea) (Shenge & Osiowy, 2021; Xiao *et al.*, 2020). Rapid testing with adequate diagnostic accuracy is expected to help improve the rate of HBV diagnosis in the future (Béguelin *et al.*, 2018).

2.1.7.2 HBV viral load

The diagnosis of acute and chronic HBV infection is generally performed with the use of serologic methods. However, the level of HBV DNA in serum is also essential and is a more reliable marker compared to HBeAg that confirms viral replication (Cayarga *et al.*, 2019; Liu *et al.*, 2017). HBV DNA is detectable at the onset of infection and increases to high levels of about 10^8 copies/ml within 3 months which then slowly develops into chronic infection (Song, 2016; Villar *et al.*, 2015). Pawlotsky *et al.* (2008) confirmed that the risk of developing liver disease is associated with an increased HBV VL of $\geq 10^4$ copies/ml.

The levels of HBV DNA in chronically infected patients are not the same in the different stages of the CHB infection (Terrault *et al.*, 2018; Liu *et al.*, 2017; Morris *et al.*, 2013). However, treatment has been recommended for all patients with cirrhosis irrespective of the HBV DNA level and stage of CHB infection (Coffin *et al.*, 2019; Terrault *et al.*, 2018; Lange *et al.*, 2017). For patients without cirrhosis, treatment is recommended when HBV DNA levels are above 2,000 IU/mL (Bezerra *et al.*, 2021; Coffin *et al.*, 2019; Lange *et al.*, 2017).

Hans *et al.* (2021) cautioned that with a completely effective treatment, even at a low HBV DNA concentration, the remaining DNA can lead to the reappearance of HBV infection. Other studies have shown that a delay in changing treatment in patients who have failed the first-line antiviral treatment increases morbidity and mortality and may also cause resistance mutations that reject second-line antiviral treatment (Rutstein *et*

al., 2015; Pawlotsky *et al.*, 2008). However, with VL monitoring, patients who fail treatment are identified in time and receive suitable treatment which helps to alleviate resistance mutations (Carmona *et al.*, 2019; Rutstein *et al.*, 2015). Nonetheless, there is a lack of access to HBV-DNA testing in resource-constrained settings (Coffin *et al.*, 2019; Lange *et al.*, 2017).

2.1.8 Quantification of HBV viral load

Quantitative serum HBV DNA is a standard measure that determines the risk of disease progression (Guvenir & Arikan, 2020; Béguelin *et al.*, 2018). It is also believed that the measurement of HBV DNA is useful in ruling out OBI and equally quantifies all HBV genotypes (Ortonne *et al.*, 2021; Villar *et al.*, 2015). Successful clinical management of HBV can be achieved by using a sensitive and accurate nucleic acid amplification assay (Ortonne *et al.*, 2021; Aretzweiler *et al.*, 2019; Mendy *et al.*, 2006). However, the available HBV DNA quantification assays can only be used with plasma and serum samples (Poiteau *et al.*, 2020).

A more sensitive assay with a lower limit of detection of 10 IU/ml is recommended by WHO in the clinical management of early HBV detection (Ortonne *et al.*, 2021; Maasoumy *et al.*, 2020). Recently, several automated real-time PCR systems that are rapid, more sensitive, with a broad dynamic range (upper range of quantification of 7-8 Log10 IU/mL and low limit of detection of 10 lU/mL) have been introduced (Marcuccilli *et al.*, 2021; Han *et al.*, 2017; Villar *et al.*, 2015). The Cobas® AmpliPrep/Cobas® TaqMan HBV assay, version 2.0 (Roche Molecular Systems, California, USA) and the Alinity™ m HBV assay (Abbott Molecular, Illinois, USA) are the most widely used real-time systems in clinical practice (Ortonne *et al.*, 2021; Poiteau *et al.*, 2020). They are fully automated and have less risk of contamination as amplification and detection are performed simultaneously in a closed-vessel system (Song, 2016; Villar *et al.*, 2015).

These advantages are making diagnostic laboratories switch their labour-intensive quantification methods to real-time technologies (Niesters, 2001). However, the systems are highly expensive and require skilled laboratory personnel to operate which is a challenge in resource-constrained settings (Jackson *et al.*, 2021; Gupta *et al.*, 2017; Kania *et al.*, 2014). They are designed to test multiple specimens concurrently;

however, they need at least one full day to acquire data (Marcuccilli *et al.*, 2021; Poiteau *et al.*, 2020; Gupta *et al.*, 2017). In resource-constrained settings this delays the decentralization of data and causes a backlog of VL testing and patients have to wait a few weeks to receive their results (Gupta *et al.*, 2017).

2.1.9 Xpert® HBV Viral Load Test

The GeneXpert system (Cepheid, Sunnyvale, California, USA) is a diagnostic device that can amplify nucleic acids and quantify the amplicons simultaneously in one cartridge (Shenge & Osiowy, 2021). The system is approximately the size of a coffee machine, and it consists of an instrument, a computer and a disposable cartridge that completes both nucleic acid extraction and real-time PCR within 40 minutes (Xiao *et al.*, 2020; Lawn & Nicol, 2011). The VL tests are run in a closed disposable cartridge which is easy to use without any risk of cross-contamination (Jackson *et al.*, 2021). However, the GeneXpert systems are costly particularly for resource-limited countries due to the need for uninterrupted power supply (Shenge & Osiowy, 2021; Xiao *et al.*, 2020). Nonetheless, clinical practices in endemic regions have been granted a price discount (Jackson *et al.*, 2021; Shenge & Osiowy, 2021).

Although in some countries the GeneXpert regulatory approvals have not yet been granted (Shenge & Osiowy, 2021; Woldemedihn *et al.*, 2021) and the limited data on the systems' analysis performance (Xiao *et al.*, 2020), the system has an opportunity for hepatitis B VL testing to be implemented in resource-constrained areas where access to HBV-DNA testing is limited (Woldemedihn *et al.*, 2021; Wursthorn *et al.*, 2011). Recent studies that evaluated the performance of GeneXpert HBV VL tests using serum samples showed adequate quantification of VL compared to the "gold standard" Roche Cobas® TaqMan® and Abbott systems (Jackson *et al.*, 2021; Woldemedihn *et al.*, 2021; Abravanel *et al.*, 2020). However, the GeneXpert HBV VL kit has not been evaluated for use with whole blood obtained from dried blood spots or plasma separation cards (Poiteau *et al.*, 2020), thus, there is a need to evaluate such samples for increased HBV screening in the quest to alleviate HBV infections worldwide.

2.1.10 Dry blood spots

The collection of blood on dry blood spot (DBS) (Figure 2.3) filter papers was first introduced by Guthrie and Susi in 1963 to detect phenylketonuria in new-borns (McAllister *et al.*, 2015; Ross *et al.*, 2013; Lakshmy, 2008). The filter paper cards have since been successfully used in the detection, quantification and identification of other biological analytes such as viruses, nucleic acids, antibodies, and antigens, in a single sample (Ortonne *et al.*, 2022; Carmona *et al.*, 2019; McAllister *et al.*, 2015). The WHO has recommended the use of DBS to test HBV DNA where nucleic acid testing is unavailable at the place of the collection but can be sent to a laboratory further away and/or in people with poor venous access. However, there is no approved commercial assay that can test whole blood obtained from DBS (Poiteau *et al.*, 2020; Cruz *et al.*, 2018).



Figure 2. 3 Correct spotting on dried Dry blood spot (DBS), with five 1.3 cm spotting circles of the Whatman 903 Protein Saver Cards**.** Adopted from Buchanan *et al.* (2016).

The blood samples on DBS cards are easily obtained from a finger or heel prick with a sterile non-reusable lancet and spotted on a perforated filter paper (Chevaliez & Pawlotsky, 2018; Mohamed *et al.*, 2013). Once spotted, the filter cards are left to dry at room temperature for 2-3 hours and stored in the presence of silica gel desiccant sachets for subsequent analysis (Nichols *et al.*, 2020; Yamamoto *et al.*, 2020; Chevaliez & Pawlotsky, 2018). Several studies confirmed the use of DBS cards and have reported

that any analyte that can be detected in whole blood, serum and plasma can also be detected using DBS (Martínez-Campreciós *et al.*, 2021; Vinikoor *et al.*, 2015). Laboratory personnel with less experience can also prepare DBS cards and send them via mail to prescribed laboratories for testing (Martínez-Campreciós *et al.*, 2021).

DBS cards are easy to transport since they cannot leak like liquid samples or be broken during transportation or delivery, therefore posing minimal threat to humans and the environment (Carmona *et al.*, 2019; Mahajan *et al.*, 2018). The processing of DBS cards is quicker and easier as it cuts out the process of centrifugation and sera separation steps that are followed when using the 'gold standard' method with plasma or sera (Marins *et al.*, 2020; Mahajan *et al.*, 2018; Mohamed *et al.*, 2013). There is also no need for cold-chain storage as most of the analytes remain stable for long periods at room temperature without degradation (Stene-Johansen *et al.*, 2016; Ross *et al.*, 2013). Villar *et al.* (2015) have detected all HBV markers in DBS samples stored in a sealed plastic bag for 63 days at −20 °C, 4 °C and 22–25 °C. The stability of DBS samples to detect all HBV markers, even after long storage periods, makes DBS cards the best blood sample collection technique to use in poor resource settings where storage of samples is challenging (Stene-Johansen *et al.*, 2016; Villar *et al.*, 2011).

Although the use of DBS offers many advantages, several studies have reported a few limitations such as the elution of red blood cells along with the analyte of interest from a filter paper (Hans *et al.*, 2021; Ross *et al.*, 2013; Lakshmy, 2008). It is thought that the cellular components rupture when whole blood is dried on the filter, which is then subsequently released into the sample solution when reconstituted (Lakshmy, 2008). Thus, the amount of cellular nucleic acid in the elution might lead to over-estimation, low specificity or even false positive VL results in DBS compared to plasma (Hans *et al.*, 2021; Carmona *et al.*, 2019). Other issues concerning the use of DBS are the variability associated with the paper used, efficiency of elution of the analyte of interest, staff not filling the spot with enough blood, using forceps to cut out the discs as well as difficulty in automating card-cutting (Martínez-Campreciós *et al.*, 2021; Chevaliez & Pawlotsky, 2018).

2.1.11 Plasma separation cards

A plasma separation card (PSC) is a blood collection device which is similar to DBS except that it collects plasma rather than whole blood and offers additional sample stability (Marins *et al.*, 2020; Vubil *et al.*, 2020). A PSC has a porous membrane which separates plasma from whole blood and collects it into an underlying polyester fleece that is impregnated with a DNA stabilizing reagent (Figure 2.4) (Martínez-Campreciós *et al.*, 2021; Marins *et al.*, 2020; Carmona *et al.*, 2019). It was introduced as an improved version of the DBS cards as it provides high volume capacity and an excellent stabilisation membrane which allows samples to be stored and transported at high temperatures and humid conditions (Martínez-Campreciós *et al.*, 2021; Velásquez-Orozco *et al.*, 2021).

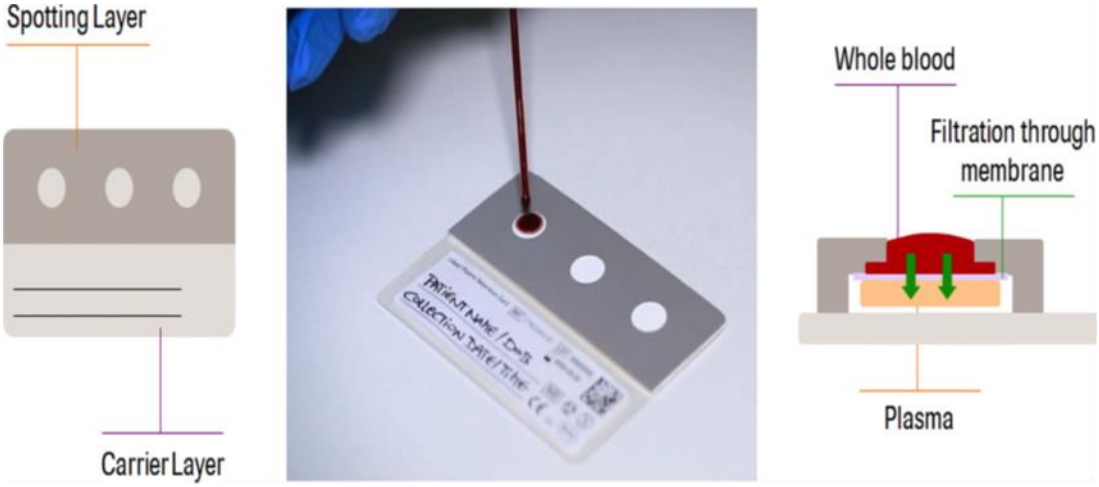


Figure 2. 4 Plasma separation card (PSC) showing a porous membrane which separates plasma from whole blood by allowing it to pass through the membrane to collect into an underlying polyester fleece. Adapted from Carmona *et al.* (2019).

The first study to evaluate the use of PSC was in SA for a diagnosis of HIV; the study revealed the PSC to be a successful method particularly in the area of equivalency, limit of detection and sample stability (Carmona *et al.*, 2019). Similar observations were later reported, for example, a study assessed the performance of PSCs for the detection of

HCV (Marins *et al.*, 2020) and another study showed that PSC testing is useful for serologic and virologic diagnosis of both HBV and HCV infections (Colucci *et al.*, 2022).

CHAPTER 3

3.1 MATERIALS AND METHODS

3.1.1 Ethics approval

Ethics clearance for the study was granted by the North-West University (NWU) Health Research Ethics Committee with ethics number NWU-00465-20-A1 (Appendix A). The ethics committee approved the recruitment of study participants who were willing to donate blood samples as outlined in the participants' informed consent form (Appendix B). The participants were recruited from Mafikeng Provincial Hospital, Gelukspan Hospital, Makouspan Clinic and Setlagole Clinic, all in North West province and samples were sent to the Centre for Vaccines and Immunology (CVI) at the National Institute for Communicable Diseases (NICD) in Johannesburg, for testing. Permission to access the hospitals for participant recruitment was granted by the National Health Laboratory Services (NHLS) Academic Affairs and Research and Management System (Appendix C) and the Department of Health, North West Province (Appendix D). The inclusion criteria of the study participants was Hepatitis B virus infected patients (30) and non-HBV infected patients (30) as controls. The exclusion criteria were individuals that presented with any other hepatitis virus infection other than HBV.

3.1.2 Study samples

The recruitment started in August 2022 to April 2023 in North West and proceeded to NICD. The study enrolled 32 clinical participants (HBV positive), that is, one from Makouspan Clinic, one from Setlagole Clinic and 30 participants' samples were obtained from CVI at NICD. The consent from the two participants younger than 18 years was granted by their mothers who accompanied them to the hospital. Thus, a total number of 60 whole blood samples (including HBV negative samples) were collected in EDTA (ethylenediaminetetraacetic acid) tubes. That is, two EDTA tubes of 5 mL of blood were drawn from each participant. Each tube was clearly labelled with an anonymised number. Demographics and clinical data were also recorded as shown in Table 3.1. Majority were African (78%), and only 6 participants have been vaccinated for HBV while 12 were HIV positive.

Table 3. 1 Descriptive sample demographics.

Demographics	Number	%
Gender		
Male	27	45.0 %
Female	33	55.0 %
Race		
African	49	81.7 %
White	8	13.3 %
Indian	3	5 %
Age		
17 and younger	2	3.3 %
18 - 35	22	36.7 %
36 - 53	30	50.0 %
Older	6	10.0 %
HBV vaccinated		
Vaccinated	6	10 %
Not Vaccinated	9	15 %
Unknown	45	75 %
HIV status		
HIV positive	12	20.0 %
HIV negative	11	18.0 %
Unknown	37	62.0 %
HIV Treatment		
ARVs	10	17.0 %
Tenofovir	6	10.0 %
Unknown	44	73.0 %

3.1.3 HBV Rapid Test

The Hepatitis B surface antigen (HBsAg) Rapid test, Determine^{TM} HBsAg 2 package insert (Abbott Diagnostics Medical Co., Ltd, Chiba, Japan), was used according to the manufacturer's instructions to determine whether the donated blood samples were HBV

positive or negative. This was performed at the hospital sites during recruitment. It is a rapid test that is visually read, a qualitative immunoassay for detection of HBsAg in human serum, plasma or whole blood. The analytical sensitivity of Determine^{TM} HBsAg 2 is 0.1 IU/mL. The specimen is added to the sample pad as shown in Figure 3.1 (a). Briefly, two drops of whole blood were spotted at the bottom white patch of the sample pad, and the sample was allowed to absorb for one minute. Then a drop of Chase buffer was added to the sample pad and allowed to absorb for 15 minutes before interpreting the results. The sample pad has two reading bars, that is, the control bar and test bar. If HBsAg was present (positive sample) a red line would appear in both the control and test bars. The red line appeared only on the control bar then the sample is negative, without HBsAg as shown in Figure 3.1(b).

From Mafikeng provincial hospital two participants tested positive for HBV and 28 were HBV negative and they were all enrolled in the study. The other 30 HBV positive samples were available from CVI at NICD, making a total of 60 samples that were used in the study, 32 HBV positive and 28 HBV negative samples.

The blood samples in EDTA tubes were then transported from the hospitals to the laboratories. The Mafikeng samples were transported to the Virology laboratory at the NWU for preparation.

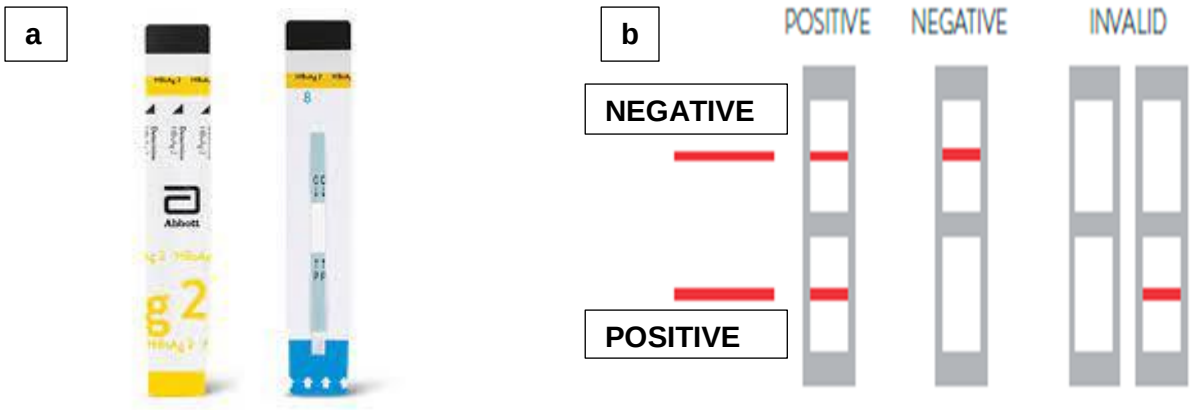


Figure 3. 1 (a) Determine^{TM} HBsAg 2, Abbott point of care strip test. (b) Interpretation of the results samples from HBsAg positive participants yielded two line bars on the strip test. Samples from HBsAg negative participants yielded only one line on the negative bar. When there is no line yielded or when a line reflects only in the positive bar the test is considered invalid.

3.1.4 Sample preparation

Once the samples arrived at the laboratories, DBS and PSC cards were prepared. DBS samples were prepared according to Kouam´e *et al*. (2023) protocol where 50 µL of blood was pipetted on each of the five 1.3 cm spotting circles of the Whatman 903 Protein Saver Cards (GE Healthcare, Piscataway, NJ) as shown in Figure 2.3. The PSC samples were prepared according to Marins *et al*. (2020) protocol. Briefly 140 µL of blood was pipetted on each of the three 1.0 cm spotting circles of the Cobas® Plasma Separation Cards (Roche Diagnostics GmbH, Mannheim, Germany) shown in Figure 2.4. After spotting, the DBS and PSC cards were left to dry for 4 hours at room temperature (RT). They were then placed in individual zipped plastic bags with silica gel desiccant sachets (1 g for DBS and 4 g for PSC) and stored at -20ºC until use (Marins *et al*., 2020). Ten (10/30) positive samples were randomly selected, and their DBS and PSC samples were stored at different temperatures, that is, 4ºC and RT (average 20ºC to 24ºC) for three days before use, to assess the stability of the cards. The remaining blood was centrifuged for 10 minutes at 3,000 rpm to separate plasma from whole blood. The plasma was then aliquoted into microcentrifuge tubes and stored at -20ºC until use. However, five samples were insufficient to separate plasma, that is, samples: 465-008, 465-010, 465-013, 465-014 and 465-015.

To avoid contamination every sample preparation step was performed on working bench or fume hood sterilized with 70% ethanol. The removal of the disc from the cards was done using forceps which were sterilized with 70% ethanol for 30 second for every use. Preparation of every sample was done separately, that is, for HBsAg testing, DBS samples were first prepared followed by PSC preparation and all the prepared samples were then tested. PPE were wear. A new pipetting tip was used for each pipetting step.

3.1.5 HBsAg qualitative test

An HBsAg qualitative test was performed to detect the HBV surface antigen in the samples and to confirm the HBsAg Rapid test results. The HBsAg test was done on the Architect i1000sr system (Abbott Laboratories, Illinois, USA) as shown in Figure 3.4. The Architect i1000sr is a 2-step sandwich chemiluminescent microparticle

immunoassay technology. Sample and acridinium-labeled conjugate are mixed with HBsAg coated with paramagnetic microparticles. The resulting chemiluminescent reaction is measured as relative light units (RLUs). There is a direct relationship between the amount of analytes in the sample and the RLUs detected by the system optics. HBsAg is determined qualitatively and the system calculate results by using the ratio of the sample RLU (S) to the cut off RLU (CO) represented as S/CO for each specimen and control (Won et al., 2020). The cut off value of the Architect i1000sr system is 1 (S/CO) with a dynamic range of 0.05 – 250.0 IU/mL. The results are interpreted as reactive if the sample value is >1.00 or more, and nonreactive when the value is <1.00.

To test the plasma samples, each tube of plasma was vortexed thoroughly and 190 µL of plasma was added to the sample cups placed on sample carriers as shown in Figures 3.2 and 3.3 below. The sample carriers were then loaded on the robotic sample handler (RSH) to run the tests as per manufacturer's Instruction. To test the DBS and PSC sample a method by Martínez-Campreciós *et al.* (2021) was followed, one disc (per sample) was removed from the DBS cards and suspended in a 1.5 mL Eppendorf tube with 400 µL of PBS buffer. Then the eluted samples were shaken in the MB100-2A Thermo shaker incubator (Apex scientific, Durban, South Africa) at 8,000 rpm for 2 hours at RT. For PSC samples, one disc (per sample) was removed from the cards and suspended in 600 µL of PBS buffer and shaken overnight at 8,000 rpm in RT in the MB100-2A Thermo shaker. The DBS and PSC eluates were added into the Architect i1000s sample cups as described for the plasma tests above.

To dissolve/ elute DBS and PSC different methods were used because the volume of samples (blood) spotted in the cards is not the same…for every amount of sample spotted on the card it is assume that 50% of it will be recovered by the elution buffer.



Figure 3. 2 Architect i1000sr immunoassay instrument.

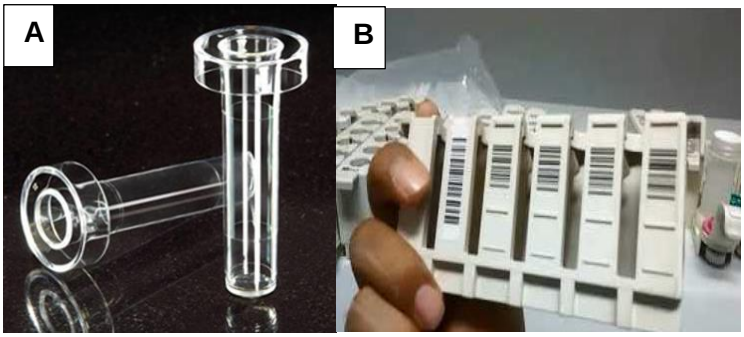


Figure 3. 3 (a) Architect i1000sr immunoassay sample cups. (b) Architect i1000sr immunoassay sample carriers.

3.1.5.1 Confidence interval of the HBsAg assay results

The characteristics of a desirable diagnostic test depend on the accuracy, sensitivity, specificity, and the positive and negative predictive values of the test with a confidence interval of at least 95%-99%. To calculate a true positive (TP) test, a true negative (TN) test, and a false positive (FP) and false negative (FN) tests, the method in Table 3.2 was used. The calculations were applied with a cut-off value of 1.00 S/CO for the Architect HBsAg assay.

Table 3. 2 Method used to evaluate the accuracy of the Architect HBsAg detection with DBS and PSC samples compared to plasma samples.

Test	**Plasma sample**	
DBS / PSC sample	TP	FP
	FN	TN

TP: True positive, FP: False positive, FN: False negative, TN: True negative.

To calculate the predictive values for sensitivity, specificity, accuracy, positive and negative tests the following equations were used:

Sensitivity = $\frac{TP}{TP+FN}$

Specificity = $\frac{TN}{TN+FP}$

Positive predictive value = $\frac{TP}{TP+FP}$

Negative predictive value = $\frac{TN}{TN+FN}$

Accuracy = $\frac{TP+TN}{TP+TN+FP+FN}$

3.1.5.2 ROC analysis of DBS and PSC methods

A ROC curve (receiver operating characteristic curve) was used to evaluate the overall diagnostic accuracy of DBS and PSC methods. ROC curve is a graph that shows the performance of a test at different threshold values. It plots the sensitivity against specificity of the test in order to select an optimal cut off value for the detection of infection. The ROC area under curve (AUC) score is the area under the ROC curve that shows the relative score produced by the assay instead of differentiating between positive and negative results across the different values for performance classification of the test. The ROC AUC score ranges from 0 to 1 where values from 0 – 0.5 indicate

random guessing (bad test), 0.6 – 0.7 are good scores, 0.7 – 0.9 are best scores and 1 indicate perfect performance.

3.1.6 Viral load testing

To test the VL from DBS samples, two discs (per sample) were removed from the DBS cards and suspended in a 1.5 mL Eppendorf tube with 1.0 mL of specimen pre-extraction (SPEX) buffer (Roche Molecular Systems, California, USA). The eluted samples were then incubated in an Eppendorf thermomixer F0.5 (Eppendorf, Hamburg, Germany) at 56ºC for 30 minutes at 1,000 rpm. For PCS samples, one dried plasma disc (per sample) was removed from the cards and incubated at 56ºC with 950 mL of SPEX buffer for 30 minutes at 1,000 rpm in a thermomixer (Marins *et al*., 2020). Thereafter, 650 µL of plasma, DBS and PSC eluates were added to the HBV viral load GeneXpert cartridges (Cepheid, California, USA) for VL detection on an eight-module GeneXprt as per manufacturer's instructions. A correction factor was used to adjust the viral load from DBS and PSC.

3.1.6.1 Linear regression analysis of viral load results

Linear regression graphs were plotted using the Xpert HBV viral load results to compare the plasma samples with the DBS and PSC samples. The comparison was performed by using the best fit line of a linear equation Y =mx + b, where X is the independent variable, Y is the dependent variable, the slope of the line is m, and b is the intercept. Bland-Altman plot was used to analyse the HBV VL detection agreements between plasma samples and the cards samples (DBS & PSC) measured with GeneXpert (Garg *et al*., 2022).

3.1.6.2 Bland-Altman analysis viral load results

A Bland-Altman plot was used to analyse the HBV VL detection agreements between plasma samples and the cards samples (DBS & PSC) measured with GeneXpert (Cepheid). The Bland-Altman graph is plotted such that the y-axis is the difference between the two measurements and the X-axis is the mean difference between the measurements. If the data points are close to the mean of the difference (Bias) or fall within the 95% limits of agreement it is interpreted as a good agreement between the

methods. If the data points are far from the mean of the difference or fall outside the 95% limit of agreement it is interpreted as a weak agreement between the two methods.

3.1.7 Sample processing for DNA extraction

A DNA elution protocol for DBS and PSC samples was developed and optimised in-house at the NICD. Two DBS and two PSC discs (per sample) were suspended in 400 µL of ATL Buffer (QIAGEN, Hilden, Germany) separately by heating in a water bath (United Scientific, Johannesburg, South Africa) at 13,000 rpm for 2 hours at 37ºC. Viral DNA was extracted and purified from 200 µL of the eluted DBS and PSC samples as well as the plasma samples (200 µL), with the use of the QIAamp Viral DNA extraction kit (QIAGEN) according to the manufacturer's instructions.

3.1.8 Amplification of HBV Surface antigen (S) gene

PCR optimization cycles were as follows: 95°C for 15 min initial denaturing followed by 40 cycles of 94°C for 1 min, 63°C - 65°C for 1 min and 72°C for 3 min. A final extension was done at 72°C for 10 min. The final PCR cycling conditions were as follows: 95°C for 15 min initial denaturing followed by 40 cycles of 94°C for 1 min, 66°C for 3 min and 72°C for 3 min. A final extension was done at 72°C for 10 min. Three times replicates of the PCR experiments were done.

The extracted DNAs from DBS, PSC and plasma samples were used to amplify the HBV Surface (S) gene using the GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Massachusetts, USA). A final volume of the first round of PCR consisted of 25 µL PCR master mix made up of 2.5 µL DNA, 12.5 µL HotStarTaq DNA Polymerase (Qiagen), 8.5 µL of nuclease free water and 0.75 µL of forward S1F primer and reverse S1R primer (Table 3.3) at 20 µM each (Yousif *et al.*, 2014). The first-round thermal cycling conditions are shown in Table 3.4.

Table 3. 3 List of PCR primers

Sense	Primers
S1F	TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC
S1R	TCC AGA CCG GCT GCG AGC AAA ACA
S2F	AAT GTT AGT ATT CCT TGG ACTCAT AAG GTG GG
S2R	AGT TCC GCA GTA TGG ATC GGC AGA GGA

Table 3. 4 First round PCR thermal cycling conditions

Thermal cycling step	Temperature (ºC)	Duration (minutes)	Cycles
Initial denaturation of DNA template	95	15	x1
Denaturation	94	1	x40
Annealing	66	1	
Extension	72	3	
Final extension	72	10	x1
Temperature hold	4	∞	

The second round PCR reaction mixture consisted of 25 µL HotStarTaq master mix (Qiagen), 1.5 µL of forward S2F primer and reverse S2R primer (Table 3.3) at 20 µM each and 17 µL of nuclease free water. To this reaction mixture, 5 µL of the first round PCR product was added for a total volume of 50 µL. The second-round thermal cycling conditions were performed as shown in Table 3.5.

Table 3. 5 Second round PCR thermal cycling conditions.

Thermal cycling step	Temperature (ºC)	Duration (minutes)	Cycles
Initial denaturation of DNA template	95	15	x1
Denaturation	94	1	x40
Annealing	65	1	
Extension	72	3	
Final extension	72	10	x1
Temperature hold	4	∞	

3.1.9 Gel electrophoresis

The HBV Surface DNA amplification was confirmed with agarose gel electrophoresis. Briefly, the amplicons were run on a 1% (w/v) agarose gel made of 1 x TAE buffer [20 mM Acetate, 40 Mm Tris (Merck, Johannesburg, South Africa) and 1 mM EDTA (Merck)

at pH 8.0 with 3 µl of GelRed Nucleic Acid Stain (Thomas scientific, New Jersey, USA). The GelRed stain assists with visualisation of the DNA under ultraviolet light. A DNA molecular weight marker of 100 bp – 4 Kbp (Roche) was used to confirm the fragment sizes. The amplicons (5 µl) were mixed with 3 µl of gel loading dye (Qiagen, Hilden, Germany) and loaded into the gel. The gel was run at 120 V for 60 minutes. The DNA bands were visualized using the G:Box F3- gel instrument (Syngene, Vacutec, Rooosevelt Park, South Africa). Successful PCR amplification of the complete S gene yielded a 1,202 bp fragment.

3.1.10 PCR product purification

PCR was performed on all samples (plasma, DBS and PSC) with detectable HBV-DNA of >100 IU/mL to evaluate the performance of DBS and PSC for HBV genotyping. HBV-DNA of 100 IU/mL is enough to undergo PCR purification since the purpose of the purification is to remove unwanted substances and to ensure that a clean and reliable DNA sample is obtained for further analysis. The PCR products were purified using the QIAquick PCR-purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A volume of 250 µL of Binding buffer was added to 45 µL of amplicon. The mixture was then transferred into a spin column, placed in the collection tube and centrifuged at RT for 60 seconds at 8 000 rpm. The flow-through was discarded and 0.75 mL of Buffer PE was added to the spin column and centrifuged as above. The flow-through was discarded and an additional centrifugation step was carried out for 1 minute to remove excess wash buffer. The spin column was placed in a 1.5 mL microcentrifuge tube and 30 µL of Buffer EB was added and incubated for 60 seconds at RT. The tubes were then centrifuged at 13,000 rpm for 1 minute to elute the purified DNA.

To ensure good quality DNA for sequencing, the PCR amplicons were run on a 1% agarose gel using ethidium bromide staining to visualise the DNA bands. A Nanodrop spectrophotometer (Thermo fisher scientific, Waltham, United States) was used to measure the amplified samples.

3.1.11 Sequencing PCR of the HBV Surface DNA

The purified HBV Surface DNA was sequenced using BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA). The DNA was sequenced using three overlapping forward primers (Table 3.6) in a 96 well plate. The primers were designed to allow sufficient overlap between the DNA fragments to ensure the correct dominant population of amplicons are assembled as the complete S gene region as shown in Figure 3.1.

Table 3. 6 List of sequencing primers.

Forward sense	Primers
2497F	TTC CTT GGA CTC ATA AGG TG
3188F	AG TCA GGA AGG CAG CCT AC
591F	ATT GCA CCT GTA TTC CCA TCC

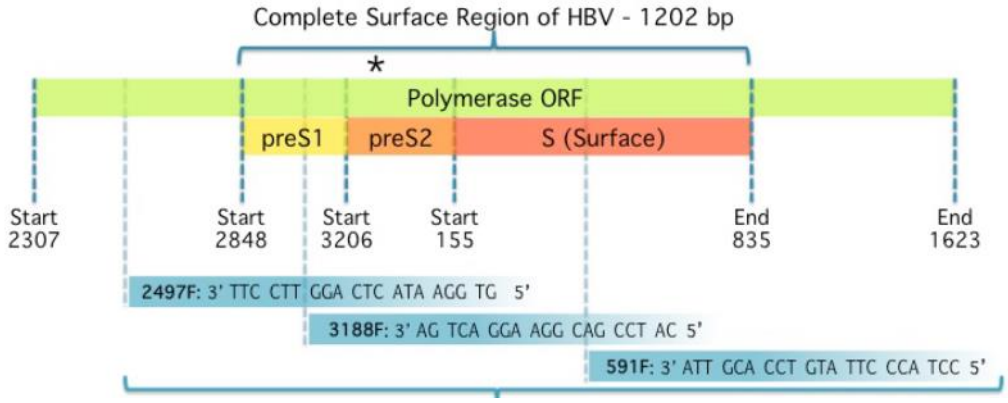


Figure 3. 4 An illustration of the merged primer fragments on Fragment Merger Tool. Adopted from (Yousif *et al.*, 2014).

The sequencing PCR reaction mixture consisted of a total of 10 µL made up of 1 µL of 3.2 pmol primer (for each primer), 2 µL of 5× sequencing buffer, 2 µL of big dye terminator, 4 µL of nuclease free water and 1 µL of DNA. The sequencing PCR was carried out using the GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with the cycling conditions listed in Table 3.7.

Table 3. 7 Sequencing PCR thermal cycling conditions.

Thermal cycling step	Temperature (ºC)	Duration	Cycles
Initial denaturation of DNA template	96	1 min:30 sec	x1
Denaturation	96	0:30 seconds	x25
Annealing	50	0:15 seconds	
Extension	60	1 min:30 sec	
Temperature hold	4	∞	

3.1.12 Sequence amplicon purification

The 96-well plates with the sequence mixture were centrifuged for 1 minute. To each well with 10 µL of sequence amplicons and 45 µL of SAM solution (Applied Biosystems, California, USA) was added. The plate was placed in a shaking incubator for 30 minutes at 1,800 rpm with shaking and then centrifuged for 2 minutes at 1,000 rpm. The purified products were sequenced at NICD using the AB 3500 xL Genetic Analyser DNA sequencer (Applied Biosystems, California, USA). The sequencing results were merged using a Fragment merger tool (Bell & Kramvis, 2013) and a phylogenetic tree was drawn using Mega 7 software (Kumar *et al*., 2016).

The sequence results were merged using the Fragment Merger Tool, an online software used to trim poor quality ends of chromatograms and merge overlapping long sequence fragments (Bell & Kramvis, 2013). The fragments of the chromatograms that were trimmed to a length limit of 50% or more were merged and selected for phylogenetic tree analysis (http://hvdr.bioinf.wits.ac.za/tools). The National Center for Biotechnology Information Blast search online tool was used to identify similar published sequences to use as references for genotyping (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The analysis was performed using molecular evolutionary genetics analysis (Mega) 7 version 0.26 and the sequences were aligned together with the published sequences using Muscle on Mega 7. A phylogenetic tree was constructed using a neighbor-joining statistical method of analysis with a bootstrap of 1,000 replicates and a Kimura 2-parameter substitution method.

CHAPTER 4

4.1 RESULTS

4.1.1 Serology

4.1.1.1 HBsAg rapid test

An HBsAg rapid test (Determine^{TM} HBsAg 2) was used to screen for the HBsAg in the study samples to determine whether they were HBV positive or negative. In total, the rapid test detected 29 HBV positive and 31 HBV negative samples (Table 4.1). Two other participants were declared HBV positive by the hospital tests/personnel however, they all tested negative with the HBsAg rapid test while one tested positive with the Architect HBsAg qualitative test. Nonetheless, they were tested further with the rest of the samples (with sample IDs 465-022, 465-025 and 465-054).

Table 4. 1 HBsAg rapid test results

HBsAg Rapid Test	**Number**	**%**
HBV surface antigen	29	48%
positive	31	52%
HBV negative		

Using the Architect as the gold standard method of HBV detection, the sensitivity and specificity results of the rapid test are shown in table 4.2.

Table 4. 2 Sensitivity and specificity results of the HBsAg Rapid test compared to Architect HBsAg qualitative test.

Architect Tests	**HBsAg Rapid test**	
	Sensitivity (%)	**Specificity (%)**
Plasma samples	61.7%	100%
DBS samples	80%	100%

PSC samples	90.6%	100%

4.1.1.2 Architect HBsAg assay

The DBS and PSC HBsAg results were compared with the HBsAg results of the gold standard assay (plasma samples). The architect HBsAg assay detected 47 positive and 13 negative plasma samples, 35 positive and 25 negative DBS samples (Table 4.3) as well as 32 positive and 28 negative PSC samples (Table 4.4). The raw data of all the Architect HBsAg qualitative test results obtained from plasma, DBS and PSC samples stored at different temperatures is shown in Table A of Appendix E.

Table 4. 3 DBS Architect HBsAg test results compared with plasma results.

HBsAg results	Plasma HBsAg positive (n=47)	Plasma HBsAg negative (n=13)	Total (%)
DBS HBsAg positive (n=35)	33	2	35 (58.3)
DBS HBsAg negative (n=25)	14	11	25 (41.7)
Total	47	13	60 (100)

Table 4. 4 PSC Architect HBsAg test results compared with plasma results.

HBsAg results	Plasma HBsAg positive (n=47)	Plasma HBsAg negative (n=13)	Total (%)
PSC HBsAg positive (n=32)	31	1	32 (53.3)
PSC HBsAg negative (n28)	16	12	28 (46.7)
Total	47	13	60 (100)

The HBsAg qualitative test results of the ten randomly selected positive DBS and PSC samples that were stored at different temperatures. The raw data is shown in Table B of Appendix E and all are positive for HBsAg.

4.1.1.3 Sensitivity and specificity of DBS and PSC methods

ROC curve was drawn to evaluate the cut off value of HBsAg in DBS and PSC. When using an S/CO value > 1.00, area under the curve (AUC) for DBS is shown to be 0.60 (60%), with sensitivity of 76% and specificity of 56% that is indicated by a red circle on figure 4.1. The area under the curve for PSC is shown to be 0.64 (64%), with sensitivity of 80% and 56% specificity that is indicated by a red circle in figure 4.2. The AUC of PSC ROC curve is greater than the AUC of DBS ROC curve, which means POC is a good diagnostic method.

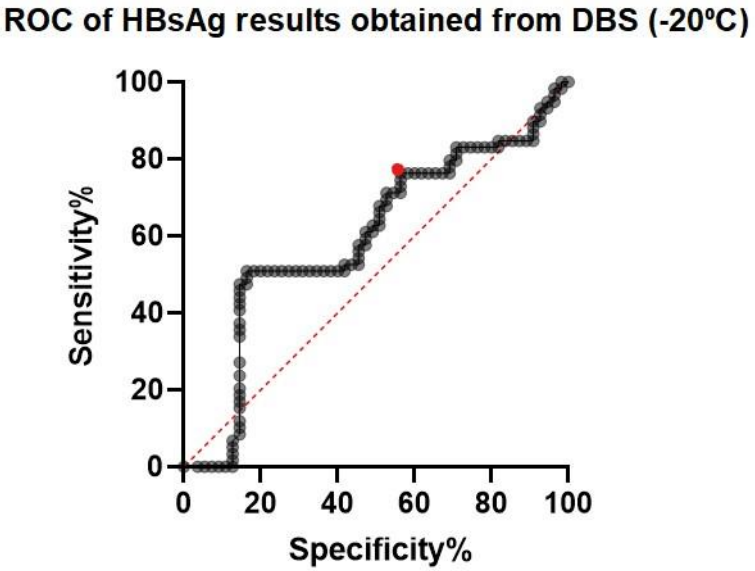


Figure 4. 1 The sensitivity and specificity of DBS HBsAg detection with the Architect i1000sr system reflected in a ROC curve.

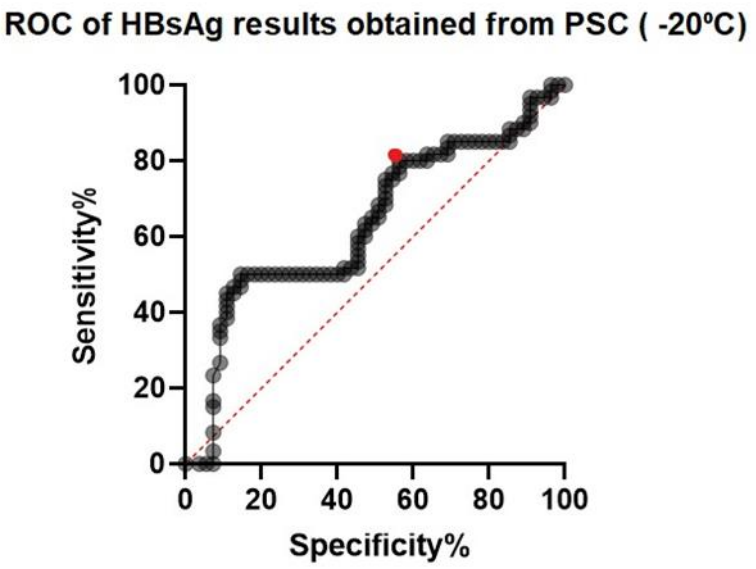


Figure 4. 2 The sensitivity and specificity of PSC HBsAg detection using the Architect i1000sr system reflected in a ROC curve.

4.1.2 HBV viral load tests

The samples that tested positive for HBsAg at a detection level of ≥1 S/CO on the Architect HBsAg system were further tested for HBV viral load (VL) using the Xpert viral load kit (Cepheid) with a cut off value of 10 IU/mL. The raw data is shown in Table C of the Appendix E. Overall, 112 samples were tested with the Xpert VL kit, that is, 47 plasma, 34 DBS and 31 PSC samples and HBV VL was detected in 26/47 plasma, 19/34 DBS and 22/31 PSC samples. Of note, 3/26 plasma samples stored at -20ºC had VL levels of less than 10 IU/mL. Moreover, VL could not be detected from the other 21 plasma samples that were also stored at -20ºC, while 1/21 plasma samples had an error reading (Table C of the Appendix E).

The correction factor of DBS and PSC samples was calculated as outlined in section 4.1.2.1 below.

4.1.2.1 Correction factor of DBS and PSC samples

The purpose of calculating a correction factor was to correct the DBS and PSC values in order to take into account the reduced volume of plasma in these samples. That is, for DBS samples, 50 µL of whole blood was spotted onto the DBS card per spot and

two spots of DBS were used for VL detection with the assumption that there was 50% volume of plasma in the DBS eluates (in SPEX buffer). Thus, 2 × 50 µL of whole blood × 50% = 50 µL of plasma. Therefore, the DBS dilution factor was calculated based on the following: 2 spots of 50 µL whole blood and 1,000 µl of SPEX buffer provided a total volume of 1,100 µL. A total volume of plasma in DBS would then be at a dilution factor of 22 (1,100/50 µL) (Ed Marins, Roche diagnostics, Department of Virology, personal communication). Thus, the DBS uncorrected results in Table C of the Appendix E and Table D of the Appendix E were multiplied with the normalization coefficient of 22 to correct for volume difference and sample type. For example, the VL for DBS sample 465-016 (Table C of Appendix E) was calculated as follows: 1810 IU/mL of uncorrected VL × 22 = 39820 IU/mL corrected VL which is equal to 4.6 IU/ml corrected log VL.

For PSC samples, 140 µL of whole blood were spotted onto the PSC cards per spot. In Table C of Appendix E one spot of PSC was used for VL detection with the assumption that there was 50% volume of plasma in the PSC eluates (with SPEX buffer). Thus, 1 × 140 µL of whole blood × 50% = 70 µL of plasma. Thus, the PSC dilution factor was calculated based on the following: 1 spot of 140 µL whole blood and 950 µl of SPEX buffer provided a total volume of 1,090 µL. Therefore, a total volume of plasma in PSC would give a dilution factor of 8 (1,090/140 µL (Ed Marins, Roche diagnostics, Department of Virology, personal communication).

HBV VL was also detected in 9/10 (90%) DBS samples that were stored at 4ºC however, 3 of those samples had VLs less than 10 IU/mL (Table D of the Appendix E). For the DBS samples that were stored at RT HBV VL were detected in 9/10 (90%) DBS samples and 4 of those samples had VLs less than 10 IU/mL (Table D of Appendix E).

HBV VL was again detected in 7/10 (70%) PSC samples stored at 4ºC and two of those samples had VLs less than 10 IU/Ml (Table E of the Appendix E). For PSC that was stored at RT HBV VL were detected in 6/10 (60%) PSC samples and 2 of those had VLs less than 10 IU/mL (Table E of Appendix E).

4.1.2.2 Linear regression of DBS and PSC viral loads vs. plasma viral loads

Linear regression graphs were plotted using the Xpert HBV viral load results to compare the plasma samples with the DBS and PSC samples as explained in chapter 3, section 6.1.2.1. DBS and PSC samples were plotted on the y-axis (dependent variable) and plasma samples were plotted on the x-axis (independent variable) as shown in Figures 4.3 - 4.8. The correlation of the two methods plotted per graph is represented by the R^2 value which measures the strength and the direction of a linear relationship between two variables. The viral load was transformed to log scale prior to regression analysis (Garg *et al*., 2022).

A positive correlation (R^2 = 0.60; p value < 0.00) was observed between corrected DBS VL and plasma VL, and also between corrected PSC VL and plasma VL (R^2 = 0.67; p value < 0.01) as shown in Figures 4.3 and 4.4 respectively. Thus, the regression model correlated the plasma method with the DBS and PSC methods by 60% and 67% respectively. The mean difference of HBV DNA VL detected in corrected DBS vs plasma samples was 0.5 log10 IU/ml and the mean difference detected in corrected PSC vs plasma samples was 0.8 log10 IU/ml.

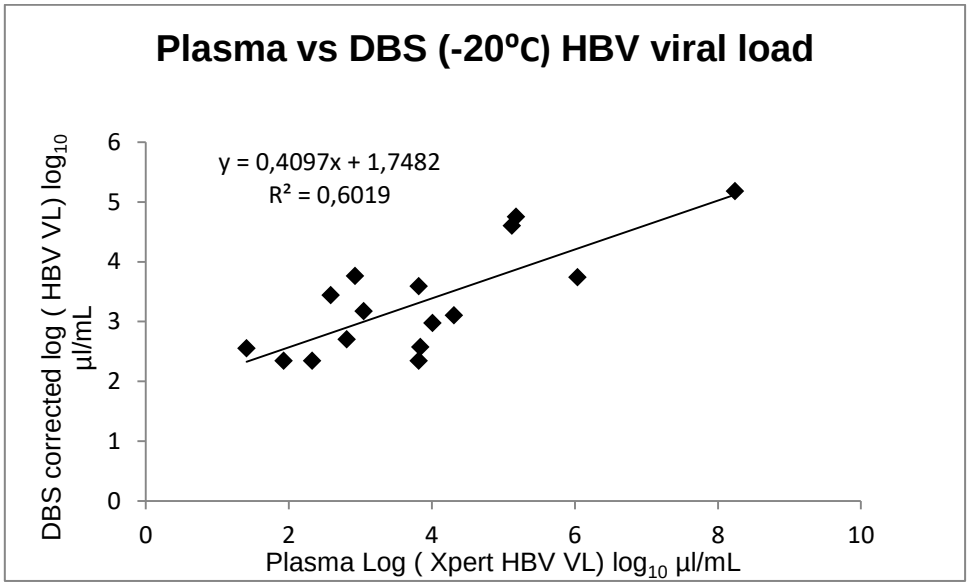


Figure 4. 3 HBV viral load correlation between plasma samples and corrected DBS samples detected by GeneXpert.

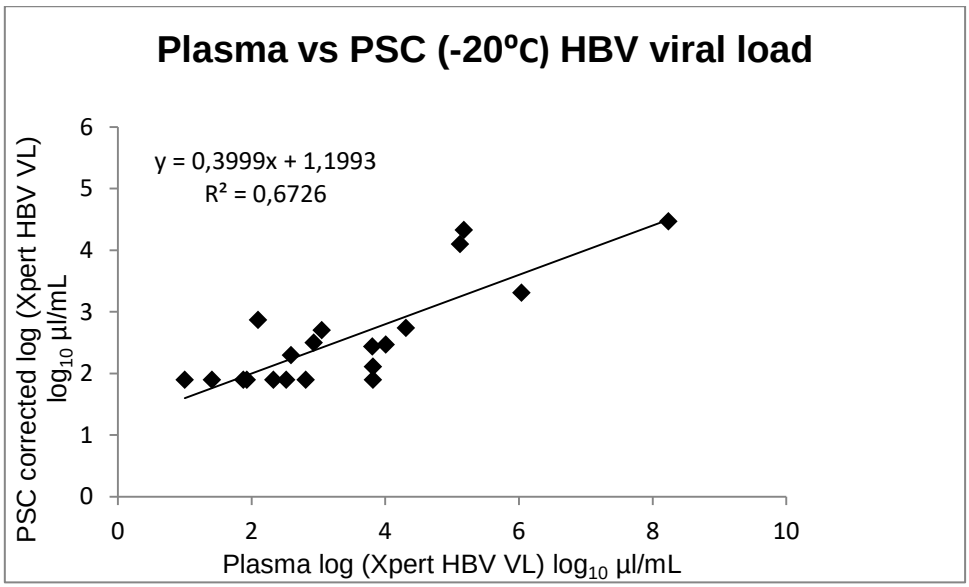


Figure 4. 4 HBV viral load correlation between plasma samples and corrected PSC samples detected by GeneXpert.

A strong positive correlation was observed with the DBS samples that were stored at 4ºC and RT as shown in Figures 4.5 and 4.6. The plasma method was shown to correlate with the 4ºC DBS and the RT DBS methods by 92% (R^2 = 0.93; p value < 0.03 and 0.05) and 90% (R^2 = 0.89; p value < 0.00) respectively.

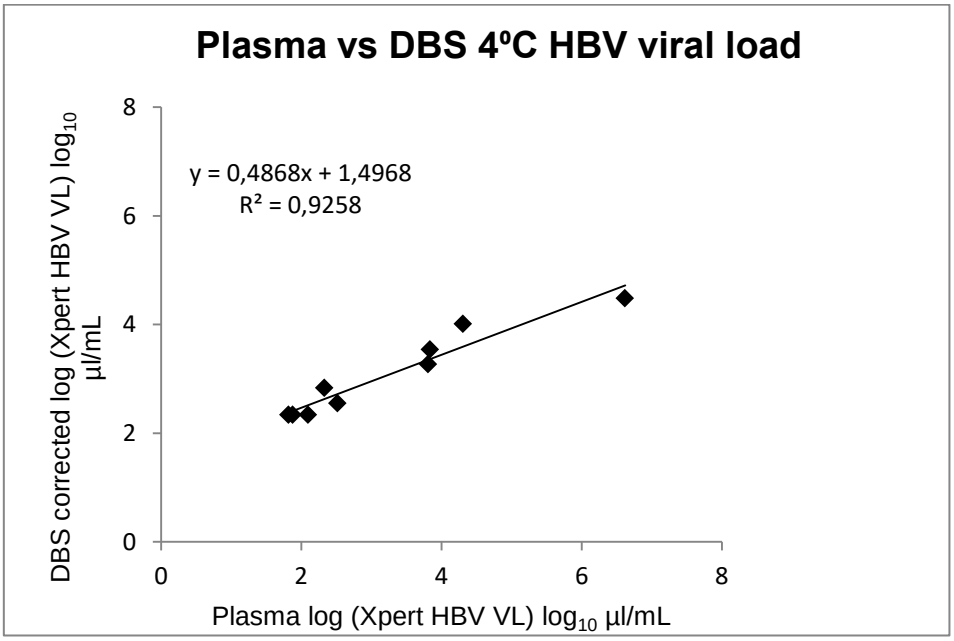


Figure 4. 5 HBV viral load correlation between plasma samples and corrected DBS samples stored at 4ºC and detected by GeneXpert.

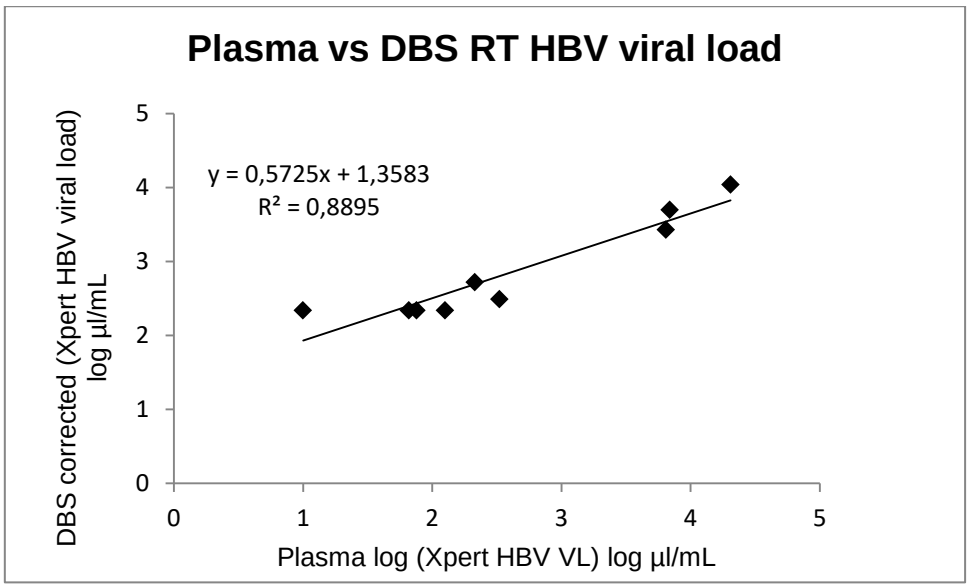


Figure 4. 6 HBV viral load correlation between plasma samples and corrected DBS samples stored at RT and detected by GeneXpert.

Another strong positive correlation was observed with the PSC samples that were stored at 4ºC and RT as shown in Figures 4.7 and 4.7. The plasma method was shown to correlate with the 4ºC PSC and the RT PSC methods by 99% (R^2 = 0.99) and 87% (R^2 = 0.87) respectively.

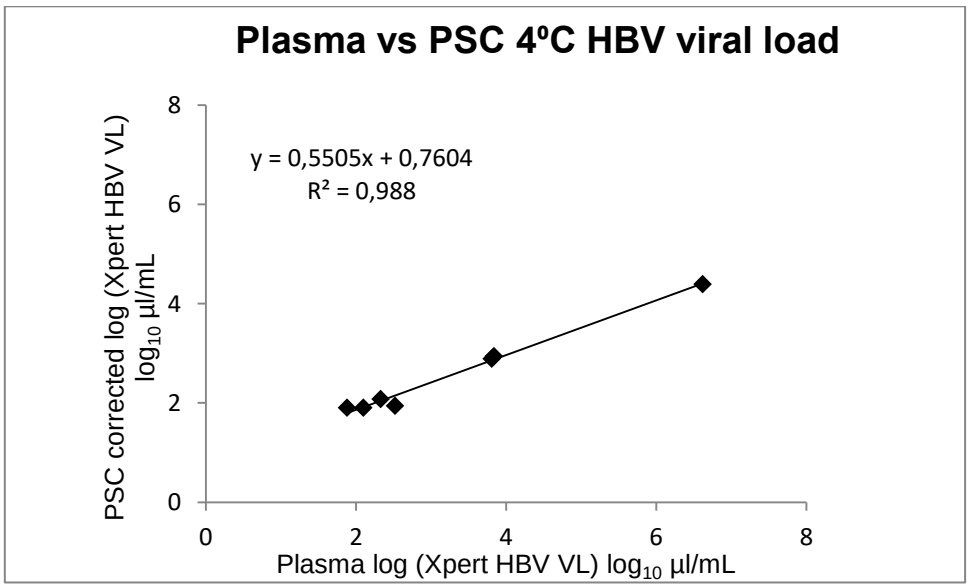


Figure 4. 7 HBV viral load correlation between plasma samples and corrected PSC samples stored at 4ºC and detected by GeneXpert.

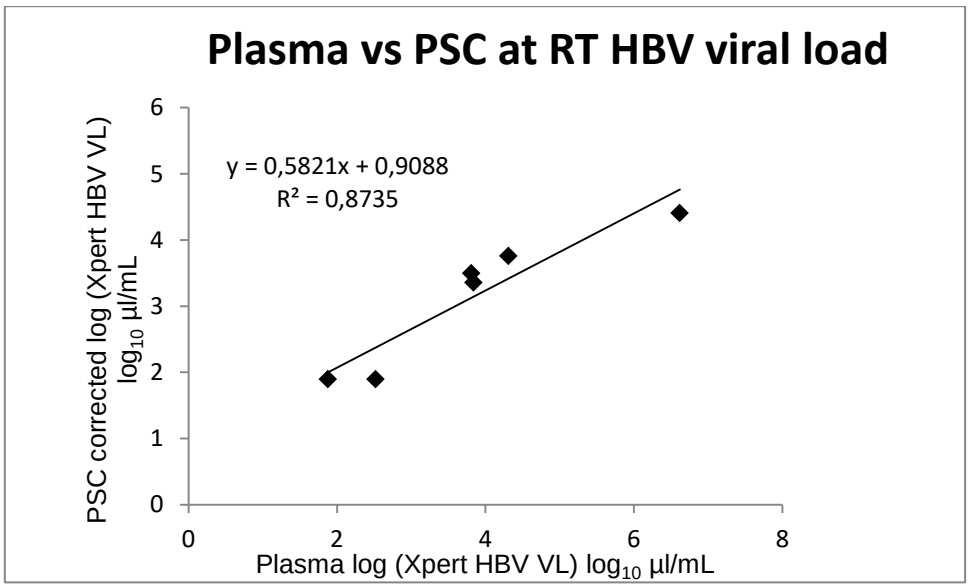


Figure 4. 8 HBV viral load correlation between plasma samples and corrected PSC samples stored at RT and detected by GeneXpert.

4.1.2.3 Bland-Altman analysis between DBS and PSC vs. plasma HBV viral loads from GeneXpert

A Bland-Altman plot was used to analyse the HBV VL detection agreements between plasma samples and the cards samples (DBS & PSC) measured with GeneXpert as outlined in chapter 3, section 3.1.6.3 and shown in Figures 4.8 - 4.13. The black middle lines indicate the mean difference, and the dotted green lines indicate the limit of confidence for the measurements. The results were obtained by calculating bias, the standard deviation, lower limit of agreement (lower loa) and upper limit of agreement (upper loa) which helps with measuring the random fluctuations around the mean difference. Calculations are as follows:

Mean: (Plasma-Card (DBS or PSC))/2

Difference: Plasma – card (DBS or PSC)

For upper limit of agreement

Upper loa = mean + 1.96 × standard deviation of the difference

Lower limit of agreement

Lower loa = mean -1.96 × standard deviation of the difference

Bias is the mean of the difference

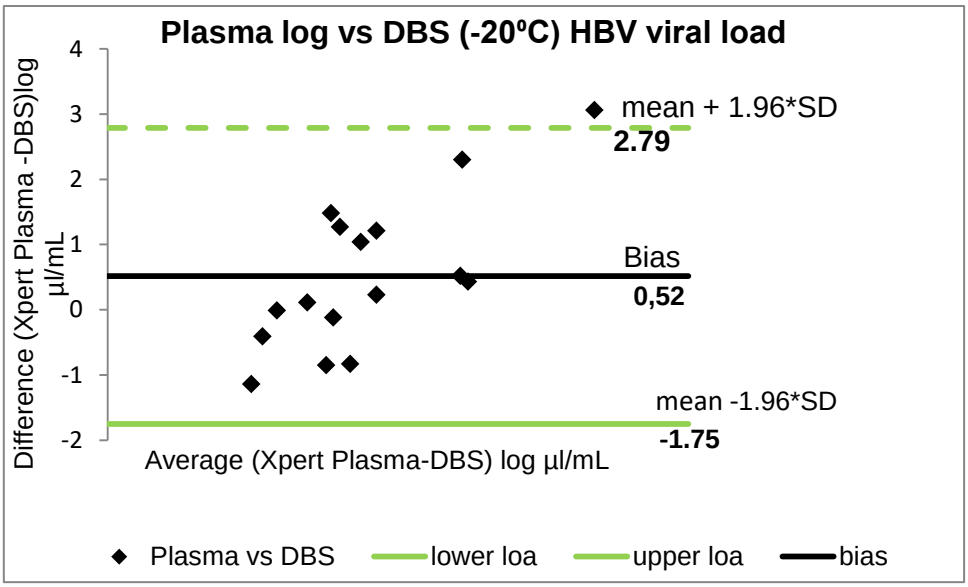


Figure 4. 9 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected DBS (-20ºC) samples.

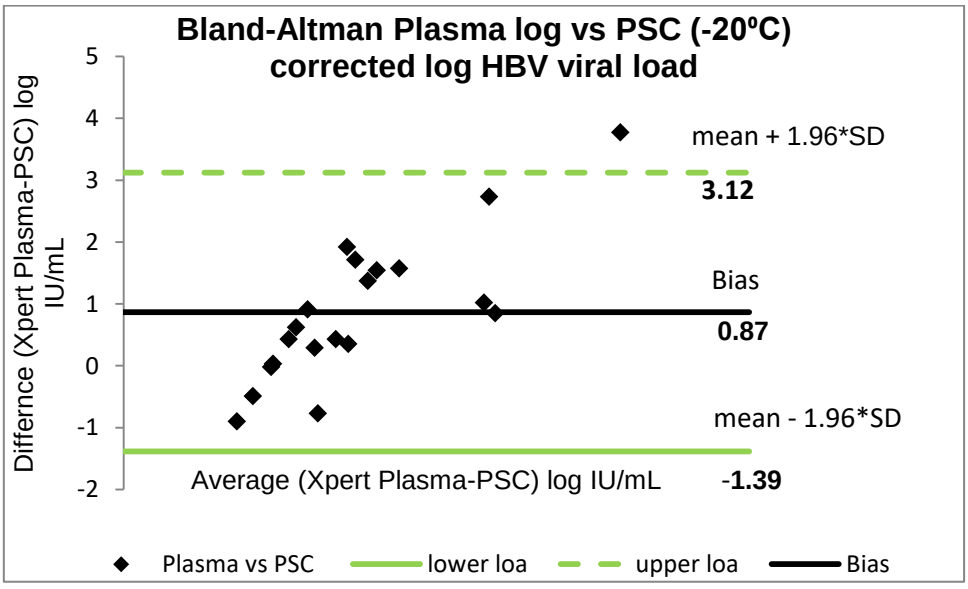


Figure 4. 10 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected PSC (-20ºC) samples.

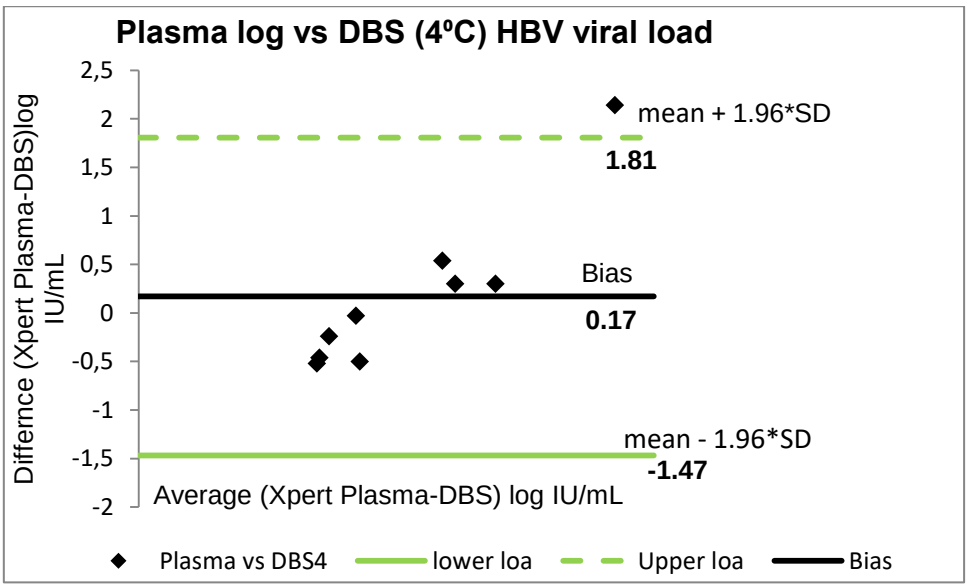


Figure 4. 11 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected DBS 4ºC samples.

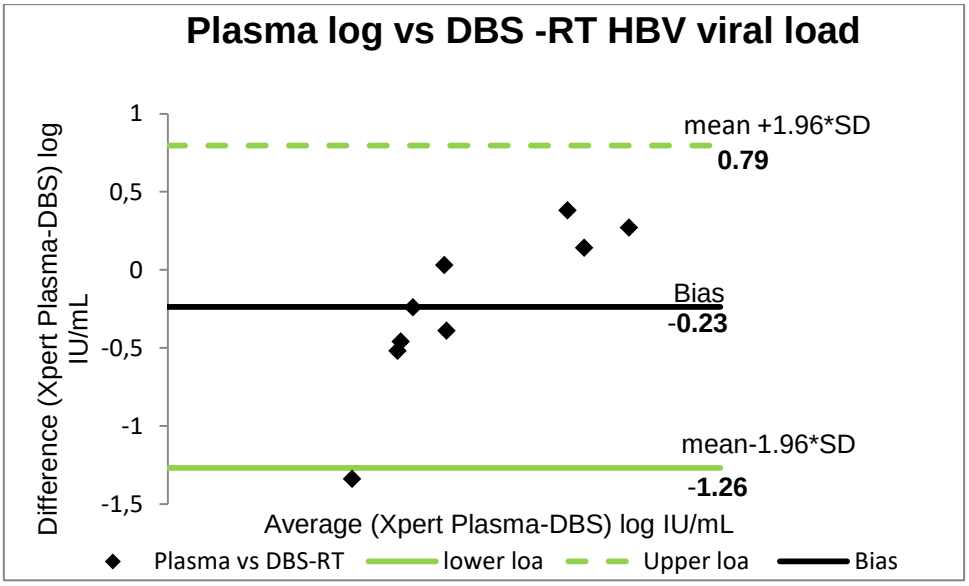


Figure 4. 12 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected DBS RT samples.

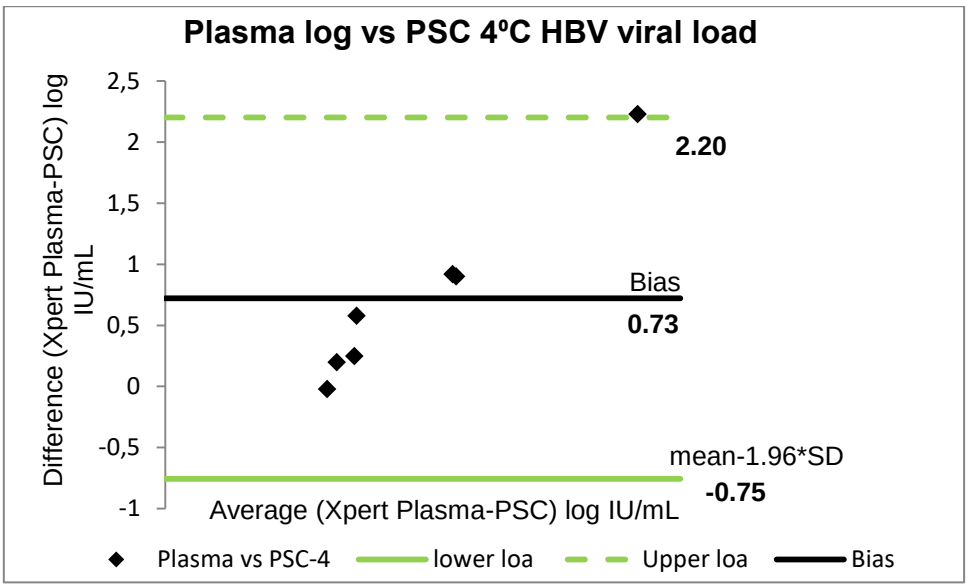


Figure 4. 13 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected PSC 4ºC samples.

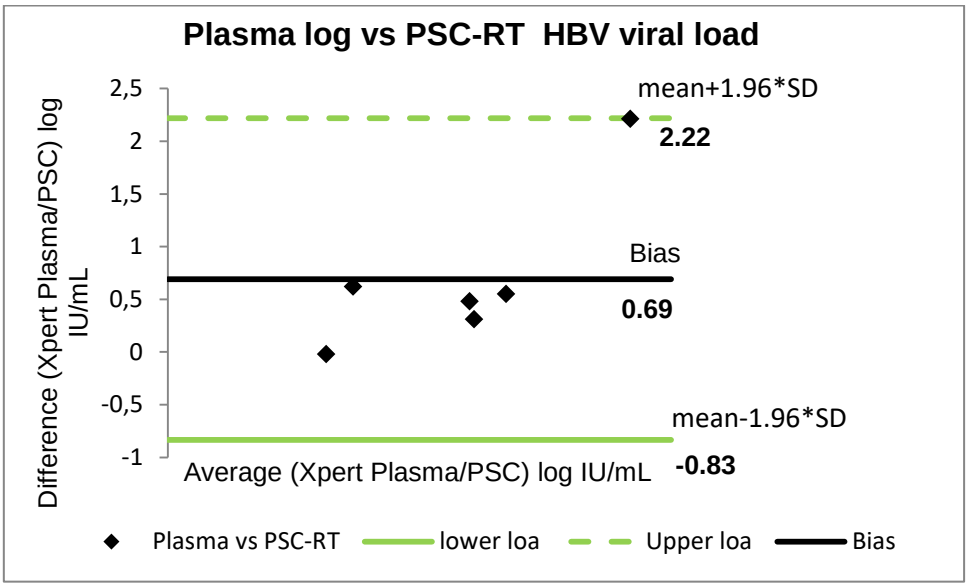


Figure 4. 14 A Bland-Altman plot showing a limit of agreement between HBV viral load of the plasma samples and the corrected PSC RT samples.

4.1.3 HBV DNA amplification of DBS and plasma samples

DNA extraction from plasma, DBS and PSC samples was performed with the samples that detected HBV viral load levels of ≥100 IU/mL using the Xpert VL kit. A complete S

region of the HBV genome was amplified as described in the previous chapter 3, section 3.1.10. The amplified fragment was then resolved on 1% (w/v) agarose gel and the expected HBV DNA band of 1202 bp was observed as shown in Figures 4.14 and 4.15. The concentration of the amplicons was also determined (Figure A of Appendix F). The test was preceded to the next step which is sequence step, and the quality of the sequences proved that there was no contamination, and the study samples were amplified as HBV samples.

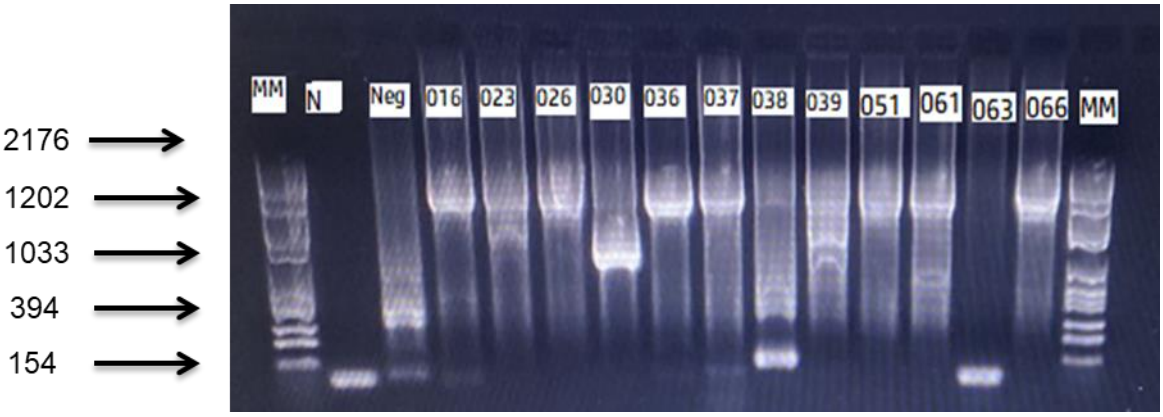


Figure 4. 15 A 1% agarose gel showing HBV PCR amplicons of plasma samples. A molecular weight marker (MM) of 154 bp - 2.1 kb was used. N indicates negative water control; NEG indicate a known negative sample and 016 - 066 represent the plasma amplicons.

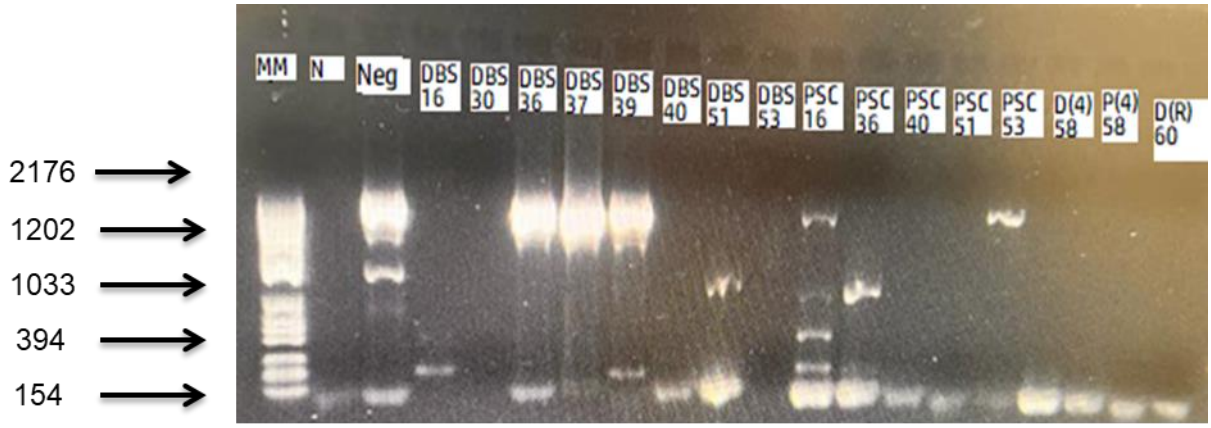


Figure 4. 16 A 1% agarose gel showing HBV PCR amplicons of some DBS and PSC samples. MM of a 154 bp - 2.1 kb ladder was used. N indicates negative water control, and the numbers 16-60 represent the DBS and PSC amplicons.

The data in Figure A, Appendix F shows the purification of the samples prior sequencing to ensure accurate results. The negative controls are samples used to indicate that there was no contamination during sample preparation and testing.

4.1.4 Sequencing results

The sequence results were trimmed and analysed as outlined in chapter 3, section 3.1.12. The sequences that were used to draw the phylogenetic trees were those that were able to be trimmed to a length limit of ≥ 50% and merged with the Fragment Merger Tool software (http://hvdr.bioinf.wits.ac.za/tools) (Figures 4.17 - 4. 18). The blank spaces in tables 4.10 and 4.11 represent the sequences that were not merged because the chromatograms were trimmed to below 50% or to a length of zero (according to the tool protocol), as well as the samples that did not show good bands on the agarose gel (chapter 3, section 3.19). The results of some samples with good quality of sequence data (chromatograms) that was obtained are shown in Figure B-D of the Appendix F.

Table 4. 5 Sample sequences that were stored at -20ºC and selected by the Fragment Merger Tool software for phylogenetic analysis.

Sample ID	Plasma (-20°C)	DBS (-20°C)	PSC (-20°)
465-016	Genotyped	Genotyped	
465-023	Genotyped		
465-036	Genotyped	Genotyped	Genotyped
465-037	Genotyped		
465-038	Genotyped		
465-039	Genotyped	Genotyped	
465-040		Genotyped	Genotyped
465-051	Genotyped		
465-053		Genotyped	Genotyped
465-056	Genotyped		

Table 4. 6 DBS and PSC sample sequences that were stored at different temperatures and selected by the Fragment Merger Tool software for phylogenetic analysis for comparison with the plasma sequences stored at -20ºC.

Sample ID	Plasma(-20ºC)	DBS (4ºC)	DBS (RT)	PSC (4ºC)	PSC (RT)
465-057	Genotyped	Genotyped			Genotyped
465-059	Genotyped				
465-060	Genotyped	Genotyped			
465-061	Genotyped				
465-062	Genotyped				

The phylogenetic tree of the complete surface region of HBV (1 202 bp) in Figure 4.18 revealed that the study samples that were stored at -20ºC clustered into three different HBV genotypes, that is, genotype A, D and E. In the tree (Figure 4. 18), plasma and DBS sample ID 465-016 are closely clustered with genotype A1 accession number MH464828 with root of 75%. The DBS and PSC sample ID 465-053 are closely clustered with genotype A1 accession number KF922428 with root 99%. Plasma sample ID 465-056 is closely clustered with MT426098 genotype A2 with root of 100%. Plasma sample ID 465-023 is clustered together with genotype E accession number of Mt426116 and Plasma sample ID 465-037 is closely clustered with HBV M2097842 genotype D with root of 77%.

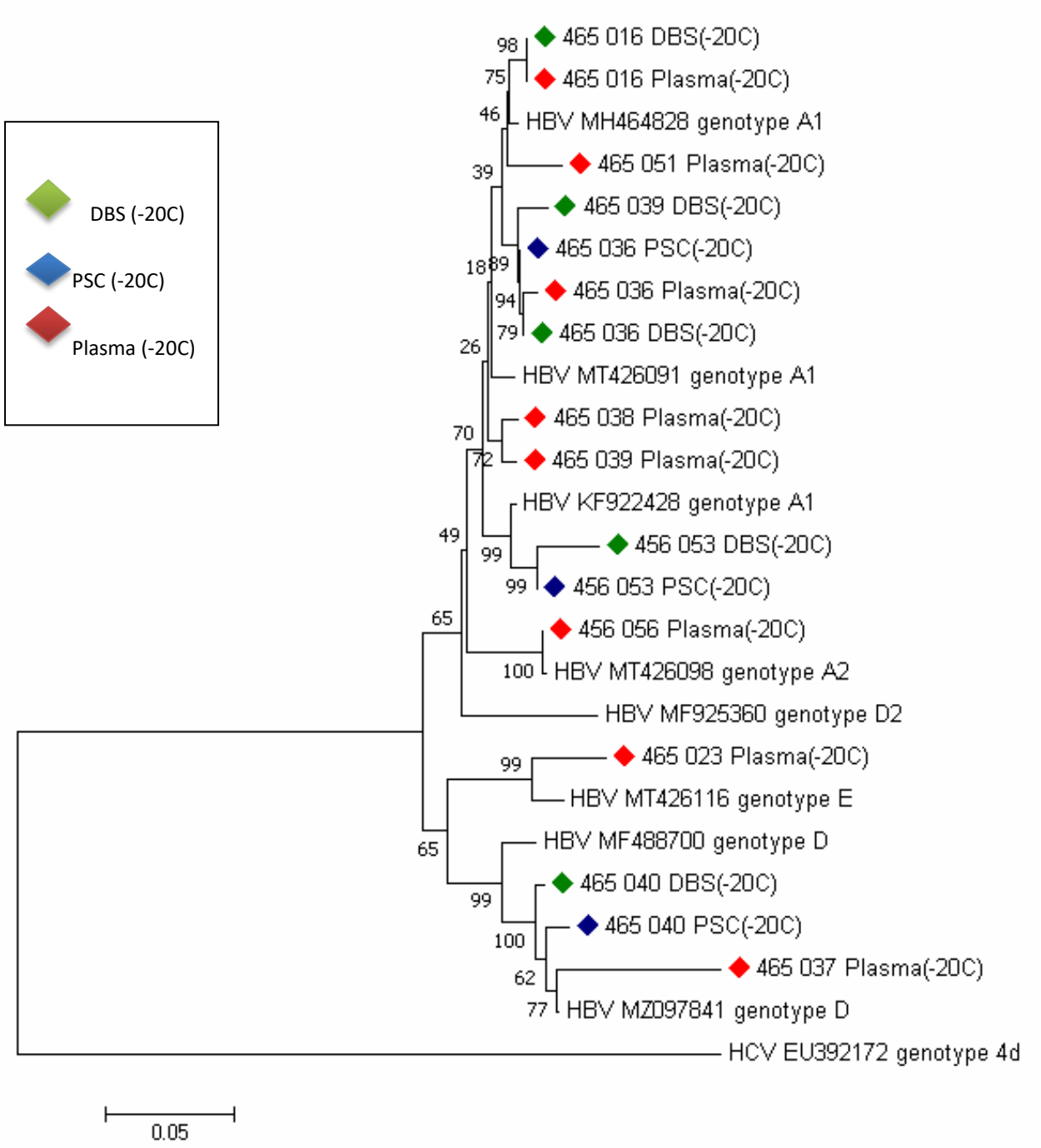


Figure 4. 17 A neighbor-joining phylogenetic tree of the complete surface region of the HBV genome established using bootstrap statistical analysis of 1000 bootstraps and a Kimura 2-parameter. The tree shows the DBS, PSC and plasma samples that were stored at -20ºC.

A phylogenetic tree of the complete surface region of HBV (1202 bp) for the samples that were stored at different temperatures for 3 days is shown in Figure 4.19. The sample sequences clustered together with genotype A1 published sequences.

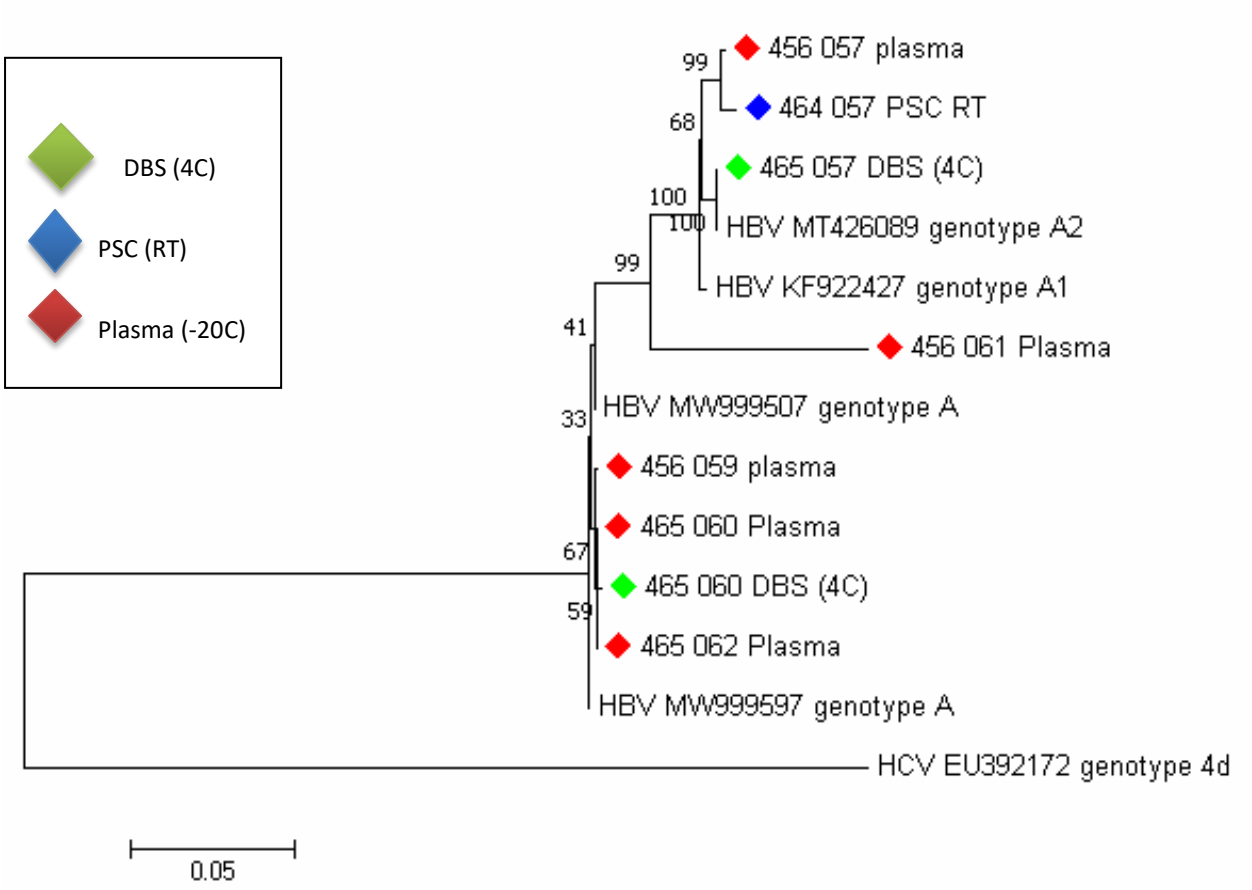


Figure 4. 18 A neighbor-joining phylogenetic tree of the complete surface region of the HBV genome established using bootstrap statistical analysis of 1000 bootstraps and a Kimura 2-parameter. The tree shows DBS, PSC stored at 4ºC and RT, and plasma (-20ºC) sample sequences that clustered with genotypes A.

CHAPTER 5

Discussion and Conclusion

In 2017 the WHO announced a target date to eliminate viral hepatitis B and C by 2030 and to reduce HBV mortality rate by 90% and new chronic HBV infections by 65% (World Health Organazition, 2017). However, there still remains a challenge of access to health care services in limited resource settings, and this results in people not knowing their HBV status and thus, increases the spread of infection. The ability to collect capillary whole blood with a finger prick or heel prick in infants has been shown to be an effective alternative method of drawing whole blood to improve accessibility and uptake of diagnosis compared to the venepuncture method (Martínez-Campreci´os *et al*., 2021). The capillary method that is generally used at point of care facilities also makes sample storage and transportation much easier to manage. For HBV testing, diagnostic tests such as the Determine^{TM} HBsAg 2 Rapid test also simplifies the clinical screening of HBV infection. The study explored the use of venepuncture blood HBV testing with the use of dry blood spot and plasma separation card methods in comparison to the gold standard method of using plasma extracted directly from whole blood. The study confirmed the possibility of using DBS and PSC methods for HBV diagnosis. This was first shown by the correlation between the Architect i1000sr HBsAg qualitative test and DBS method with 76% sensitivity as well as PSC method with 80% sensitivity compared with plasma samples. The specificity was 56% with all the samples. The mean difference between DBS and plasma samples was 0.5 log 10 while PSC and plasma samples mean was 8 log 10 IU/mL with a correlation of (R^2=0.60). PSC shows to be more stable at 4ºC and RT for up to 3 days compare to DBS. Majority of tests in the literature on detection of HBsAg are performed with average sensitivities and specificity of 100% and 99%, respectively**.** Thus, the study needs to be repeated to confirm the results; and to improve the accuracy of DBS and PSC data the number of the disc eluted samples should be increased. A phylogenetic analysis further confirmed that the samples are indeed HBV samples as they clustered with published HBV strains.

The Determine^{TM} HBsAg 2 Rapid test using whole blood collected by venepuncture showed a good correlation with plasma samples as well as DBS and PSC samples that

were tested using the Architect i1000rs qualitative HBsAg assay (Abbott). There were no false positive results obtained with the Determine^{TM} HBsAg 2 Rapid test, and all samples were tested positive with the Architect i1000sr HBsAg qualitative test using a cut off 1 S/CO. Overall, Determine^{TM} HBsAg 2 Rapid test had 61% sensitivity compared with plasma, 80% with DBS and 90.6% with PSC. Specificity was 100% with all the samples. In the study of Avellon *et al.* (2020) Determine^{TM} HBsAg had a sensitivity of 97.2% and specificity of 99.0% with venous whole blood. The Determine^{TM} HBsAg demonstrated an average performance (61% sensitivity) as a diagnostic method for detection of HBsAg at point of care. The current study had limited number of HBV positive samples (n≤ 30) compared to other studies that have been mentioned. That is, according to literature low concentration of HBsAg, viral load and specific genotypes and HBsAg mutation can affect the sensitivity of Determine^{TM} HBsAg 2 Rapid test (Avellon *et al*., 2020; Xiao *et al*., 2020), and since most of the participants in the study were on treatment it is possible that the level of HBsAg and viral load titre were low.

High sensitivity and rapid assay are needed for early detection of the disease. The WHO has recommended the use of DBS as a sample collection method but there is no commercially approved assay using whole blood obtained from DBS yet (Poiteau *et al.*, 2020). The study used Architect i1000sr HBsAg quantitative assay and Xpert HBV VL to evaluate DBS and PSC samples. In the present study, the cut off value of DBS and PSC for the detection of HBsAg was recalculated using ROC curve method. However, low accuracies of 64% and 60% were observed in both DBS and PSC methods when a new cut off value was determined and was different from the one reported in the previous study (Villar *et al.*, 2011). These differences are due to a small number of samples in the current study. A low sensitivity and specificity was observed with DBS using ROC curve. The HBsAg sensitivity obtained by DBS (76%) in the a current study is similar to 78.6% reported in a study that assessed the use of DBS in routine serological diagnosis of HBV infection from Nigeria (Forbi *et al.*, 2010) and higher than 37% reported by Cruz *et al.* (2020). However, the specificity of 56% seen in the current study was lower than the one reported in the similar studies (Cruz *et al.*, 2020; Forbi *et al.*, 2010).

PSC method detected HBsAg with 80% sensitivity and 56% specificity using a ROC curve. However, high sensitivity and specificity of 98.4 % and 96% respectively, was reported in a similar study from Spain (Martínez-Campreciós *et al.*, 2021) without recalculation of the cut off value. The sensitivity of PSC samples was higher than the DBS samples in the current study, which prove that PSC is a better collection method than DBS even though the sensitivity is bit lower due to small volume of the eluate. However, the detection of HBsAg in the current study was different from previous studies due to the small number of samples tested and preparation of the cards with capillary whole blood compared to EDTA venous blood used in the present study (Cruz *et al.*, 2020; Martínez-Campreciós *et al.*, 2021).

The type of assay used might be another explanation to the observations due to the cut off values presented by each assay. For accurate diagnosis, it is also important to carefully consider the type of elution buffer used to filter the cards before the analysis can begin (Kouamé *et al.*, 2023; Villar *et al.*, 2011). According to Kouamé *et al.* (2023) PBS buffer was not suitable for screening HBsAg (100% sensitivity and 61% specificity). However, other studies have reported good performance when using PBS buffer to detect HBsAg on ELISA (Mohamed *et al.*, 2013).

The Bland-Altman analysis plot shows a great agreement with the detection of HBV VL between plasma and cards (DBS and PSC samples) of all storage conditions. A great agreement of Bland Altman analysis is shown by the diminished scattering points which lie closely to the line representing mean (bias). All data points fall within the 95% limits of agreement which indicates that the difference between the methods is acceptable. However, DBS and PSC samples which were stored at 4ºC and RT showed a strong correlation of R^2=0.99 and 0.93; p values <0.03 and 0.05, respectively in 4ºC and R^2 = 0.89 and 0.87; p value < 0.0001 and 0.006, respectively at RT compared to DBS and PSC stored at -20ºC (R^2=0.60 and 0.67; p value <0.00 and 0.01). The correlation that was observed with DBS stored at 4ºC is similar to the one observed by Poiteau *et al.* (2020) when DBS was stored at -20ºC using Xpert HBV VL. The DBS method could detect HBV VL in 61% (16/26) of plasma samples while the PSC method could detect HBV VL in 65% (17/26) only when HBV VL was higher than 10 IU/mL. The using of GeneXpert assay does not only provide results on time but could also detected HBV

DNA level as low as 10 -10^9 IU/ml even with DBS and PSC samples. Although, HBV treatment is recommended for patient with HBV DNA level of >2000 ul/ml (3.3 log) unless the symptoms of liver disease present (Mohamed *et al.*, 2013). The current study proves that PSC and DBS could correctly Identify patient eligible for HBV treatment.

The mean difference observed between DBS (-20ºC) and plasma samples of 0.5 log 10 IU/mL was lower than the 2 log 10 IU/mL reported by Poiteau *et al.* (2020) using Xpert HBV VL but similar to previous studies (0.5 log 10 IU/mL and 0,59 log 10 IU/mL) using different assays (Garg *et al.*, 2022; Stene-Johansen *et al.*, 2016). For PSC samples, the 8 log 10 IU/mL observed in the current study is higher than the 1 log IU/mL observed by Martínez-Campreciós *et al.* (2021) when compared with plasma samples.

The current study however, has proven that DBS and PSC samples can remain stable for up to 3 days after sample collection and storage at 4ºC and RT without degradation of serum and nucleic acids. A sensitivity of 90% and specificity of 10% was observed when HBV VL was detected by DBS samples both at 4ºC and RT in the present study. The sensitivity of HBV VL obtained by DBS sample was higher than 80% when reported by Garg *et al.* (2022), and was stored at RT (37°C) for up to 14 days. While Stene-Johansen *et al.* (2016) showed a good stability at RT (average 20–25°C) for up to 12 weeks. A sensitivity of 70% and sensitivity 30% was observed when HBV VL was detected by PSC samples at 4ºC, and 60% sensitivity and 40% specificity when stored at RT in the present study. The ability for DBS and PSC samples to remain stable for a long period, could be relevant in limited resource settings where blood samples are often delayed to be transported to reference laboratories for testing without cold storage.

DBS and PSC samples were also amplified with the conventional PCR method in this study. A few of the DBS and PSC samples were able to be amplified by PCR compared to plasma samples. This might be due to a very low concentration of the virus in the samples (cards).

Phylogenetic analysis of the complete HBV surface genome showed clustering of the sequences of all the frozen samples (-20ºC) with genotypes A/A1, D and E. However, the sequences of DBS and PSC samples that were stored at 4ºC and RT clustered only with genotype A1. The phylogenetic analysis also confirmed that the samples used in the study were indeed HBV positive samples as they all clustered away from the HCV sequence that was used to root the tree (EU392172). A high rate of chronicity have been reported in genotype A and D and they mostly circulate in the African continent including South Africa compared to other genotypes (Wolf *et al.*, 2021; Kramvis, 2018). The majority of the study samples that clustered with genotype A had high HBV VLs of >1000 IU/mL while those that clustered with genotype D has low HBV VLs <1000 IU/mL. These suggested that patient infected with genotype A are at high risk of developing cirrhosis and HCC and they urgently need to be put on treatment. A published HBV genotype A with accession number MT426089 that clustered together with the DBS sample (465-057) that was stored at 4ºC with 100% similarity can also be observed clustering with plasma sample (465-056) stored at -20ºC.

It was interesting to note the plasma sample (ID 465-023) that clustered together with a published genotype E sequence. Although, there is limited of information about the clinical characteristics of genotype E, patients infected with genotype E have been considered to be at higher risk of developing liver disease (Ingasia *et al.*, 2021). Thus, further investigation on genotype E Is needed. The phylogenetic analysis confirm that the filter cards can successfully be used to test for HBV and even used to genotype the virus if prepared and stored correctly no matter the temperature.

The strength of this study is that some of the samples that were used are from limited resource settings (Mafikeng, North West), which makes our findings more relevant than studies that are carried out in urban areas with quick access to laboratory tests. The limitation of the study is that DBS and PSC samples were prepared using whole blood collected with venipuncture rather than capillary whole blood from fingerpick.

In conclusion, data from the study revealed that dried blood spots (DBS) and plasma separation cards (PSC) can be used for HBV testing in limited resource settings. However, due to the small number of positive samples which has affected the sensitivity

and specificity of the assays used, further investigation is required before DBS and PSC testing can be implemented.

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APPENDICES

APPENDIX A

Ethics approval

NWU®
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

Private Bag X6001, Potchefstroom
South Africa 2520
Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Faculty of Health Sciences Ethics Office for Research, Training and Support

Tel: 018 299 2092
Email: wayne.towers@nwu.ac.za

30 November 2022

Dr HT Mufhandu
Microbiology

Dear Dr Mufhandu

FEEDBACK ON NWU-HREC ANNUAL MONITORING REPORT: NWU-00465-20-A1

We would like to thank you for submitting the annual monitoring report for your project entitled, ***"Detection of Hepatitis B virus using dry blood spots and plasma separation cards"***, to the North-West University Health Research Ethics Committee (NWU-HREC) in a timely manner. Please find below the decision of the NWU-HREC regarding the continuation of your project.

Classification	Mark with X	Comment	
Clarification			
Completion (Final report)			
Suspended			
Continuation	X	**Date of next monitoring report:**	30 November 2023
Termination			

Please note: Due to the current status of the study i.e. (collection of blood samples from patients and control individuals in a public health facility by members of staff in the facility, followed by collection and molecular analysis of these samples by the researchers outside the facility), this study will be able to proceed during the current alert level, following submission of an amendment request to include a COVID-19 risk mitigation strategy to protect researchers and the public from possible COVID-19 infection (as setup according to the document entitled "COVID-19 research risk assessment and management approach" (Prof Minrie Greeff, 29 June 2020)"). When wanting to amend an approved research study impacted by the COVID-19 pandemic, the existing SOP (2.2.4_SOP_Ethics_1.4) for this process still remains in place. The process to be followed is clearly discussed under Section 8.4 of this SOP entitled, "Application for an amendment to an approved study". The process as described in this SOP is handled via the expedited process. This SOP is freely available on our website at the following URL i.e. http://healthsciences.nwu.ac.za/healthethics/sops. The process that should be followed, is as indicated below:

a. Ensure all your amendments are indicated in yellow highlight in the amended documents.
b. Submit your request to Ethics-HRECApply@nwu.ac.za, with a cover letter with a specific subject title indicating, "Amendment request (COVID-19): NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted.
c. Name the emails, to which you attach the documents that you send, with a specific subject line indicating that it is an amendment request e.g. "Amendment request (COVID-19): NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment.

APPENDIX B

Participants' informed consent form

Private Bag X1290, Potchefstroom
South Africa 2520
Tel: +2718 299-1111/2222
Fax: +2718 299-4910
Web: http://www.nwu.ac.za

NWU Health Research Ethics Committee
(NWU - HREC)
Faculty of Health Sciences
2022 -11- 02
NWU®
NWU-HREC Stamp

INFORMED CONSENT DOCUMENTATION FOR PATIENTS PRESENTING WITH HEPATITIS B VIRUS (HBV) SYMPTOMS BLOOD SAMPLES (5 ml X 2 EDTA tubes per participant) WILL BE DRAWN ONCE.

TITLE OF THE RESEARCH STUDY: Detection of Hepatitis B Virus using dry blood spots and plasma separation cards

ETHICS REFERENCE NUMBERS: NWU-00465-20-S1

PRINCIPAL INVESTIGATOR: Dr Hazel T Mufhandu

POST GRADUATE STUDENT: Miss Tsholofelo Moabi

ADDRESS: Building A12, Office G38, Mahikeng Campus

CONTACT NUMBER: 018 389 2416

You are being invited to take part in a **research study** that forms part of a Masters study. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research is about and how you might be involved. Also, your participation is **entirely voluntary** and you are free to say no to participate. If you say no, this will not affect you negatively in

APPENDIX C

National Health Laboratory services (NHLS) Academic Affairs and Research Management System

Permission letter for access to samples

NATIONAL HEALTH LABORATORY SERVICE

Academic Affairs and Research
Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 386 6142
Fax: +27 (0)11 386 6296
Email: babatyi.kgokong@nhls.ac.za
Web: www.nhls.ac.za

08 December 2020

Applicant: Hazel Tumelo Mufhandu
Institution: North West University
Department: Bophelong Hospital
E-mail Address: hazel.mufhandu@nwu.ac.za
Tel: 018 389 2416
Cell: 072 301 6629

CC: Nishi Prabdial Sing – Centre for Vaccines and Immunology

Re: Provisional approval to access National Health Laboratory Service (NHLS) Samples

Your application to undertake a research project **"Detection of Hepatitis B Virus using dry blood spots and plasma separation cards", ref no: PR20172** using samples from the NHLS has been reviewed. This letter serves to advise that the application has been provisionally approved ***without patient names*** to conduct the proposed study as outlined in the submitted application.

Please note that the approval is granted on your compliance with the NHLS conditions of service and that the study can only be undertaken provided that the following conditions have been met.

- Full Ethics clearance have been obtained from an approved local Ethics Committee
- Processes are discussed with the relevant Laboratory Manager and Business Manager and are agreed upon
- Confidentiality is maintained at participant and institutional level and there is no disclosure of personal information or confidential information as described by the NHLS policy.
- A final report of the research study and any published paper resulting from this study are submitted and addresses to the NHLS Academic Affairs and Research office and the NHLS has been acknowledged appropriately.

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research. Once all requirements have been met, Laboratory Manager and Business Manager may be contacted for advise and support.

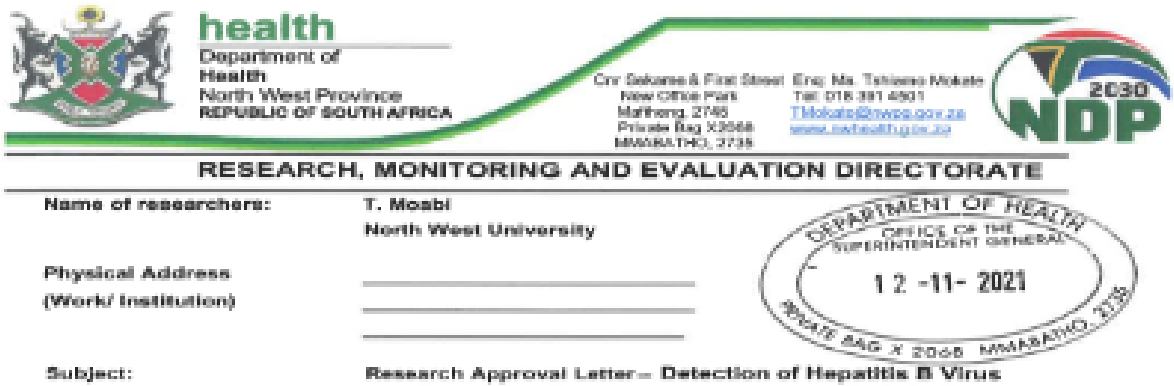
Yours sincerely,

Dr Babatyi Malope-Kgokong

APPENDIX D

Department of Health, North West Province

Permission letter for access to samples



health
Department of
Health
North West Province
REPUBLIC OF SOUTH AFRICA

Cnr Sekame & First Street
New Office Park
Mafikeng, 2745
Private Bag X2068
MMABATHO, 2735

Enq: Ms. Tshiamo Mokate
Tel: 018 381 4901
TMokate@nwpg.gov.za
www.nwhealth.gov.za

2030 NDP

RESEARCH, MONITORING AND EVALUATION DIRECTORATE

Name of researchers: **T. Moabi**
North West University

Physical Address
(Work/ Institution)

DEPARTMENT OF HEALTH
OFFICE OF THE SUPERINTENDENT GENERAL
12 -11- 2021
PRIVATE BAG X 2068 MMABATHO 2735

Subject: **Research Approval Letter – Detection of Hepatitis B Virus using dry blood spots and plasma separation cards.**

This letter serves to inform the Researcher that permission to undertake the above mentioned study has been granted by the North West Department of Health. The Researcher must arrange in advance a meeting with the District Chief Director and District Director to introduce their research team/members on the proposed research to be undertaken. Further to the above the researcher must produce this letter to the District and chosen facilities as proof that the research was approved by the NWDoH.

This letter of permission should be signed and a copy returned to the Department. By signing, the Researcher agrees, binds him/herself and undertakes to furnish the Department with an electronic copy of the final research report. Alternatively, the Researcher can also provide the Department with electronic summary highlighting recommendations that will assist the Department in its planning to improve some of its services where possible. Through this the Researcher will not only contribute to the academic body of knowledge but also contributes towards the bettering of health care services and thus the overall health of citizens in the North West Province.

Below are the contact details of Office of the Chief Director and District Director of Ngaka Modiri Molema District.

1

Healthy Living for All

APPENDIX E

Table A. Architect HBsAg qualitative test results obtained from plasma, DBS and PSC samples stored at -20ºC.

The colour coding in Table A of Appendix E indicate false positive and negative samples: yellow colour is for false positive samples and grey colour is for false negative samples.

Sample ID	Rapid Test Result	Plasma -20ºC	DBS -20ºC	PSC -20ºC
		S/CO		
465-001	Negative	0.31	0.87	0.40
465-002	Negative	7.61	0.75	0.48
465-003	Negative	3.99	0.70	0.51
465-004	Negative	0.33	0.90	0.38
465-005	Negative	12.71	0.75	0.36
465-006	Negative	5.27	0.58	0.35
465-007	Negative	29.95	0.66	0.38
465-008	Negative		0.86	0.36
465-009	Negative	17.53	0.96	0.58
465-010	Negative		0.87	0.40
465-011	Negative	111.66	0.72	0.39
465-012	Negative	9.54	0.74	0.34
465-013	Negative		0.83	0.35
465-014	Negative		0.78	0.35
465-015	Negative		0.82	0.44
465-016	Positive	730.68	6454.05	6249.94
465-017	Negative	9.19	0.59	0.40
465-018	Negative	17.13	0.87	0.43
465-019	Negative	0.70	0.86	0.36
465-020	Negative	21.51	1.04	0.38
465-021	Negative	50.96	0.87	0.36
465-022	Negative	83.16	0.57	0.37
465-023	Positive	3050.06	5093.26	6285.78
465-024	Negative	14.71	0.84	0.38
465-025	Negative	0.28	0.88	0.34
465-026	Positive	4093.57	1300.49	1193.88

465-027	Positive	4267.41	2686.72	2094.88
465-028	Positive	6250.54	7175.38	6374.47
465-029	Negative	3.05	1.92	1.13
465-030	Positive	3749.76	4479.50	4127.04
465-031	Positive	4202.38	7077.03	7400.98
465-032	Negative	0.28	0.96	2.16
465-033	Negative	0.43	0.84	0.42
465-034	Negative	0.54	3.07	0.40
465-035	Negative	9.11	3.97	0.39
465-036	Positive	4233.39	8324.12	8049.26
465-037	Positive	8316.65	4624.80	3290.12
465-038	Positive	7113.16	5701.64	3099.86
465-039	Positive	8480.96	8257.14	6782.38
465-040	Positive	5095.86	7075.47	6656.80
465-041	Positive	6111.20	5026.04	5904.99
465-042	Positive	5645.67	6687.89	4530.23
465-043	Negative	0.39	1.17	0.41
465-049	Negative	4.40	1.33	0.53
465-050	Positive	5329.56	3404.35	1922.74
465-051	Positive	6017.72	7960.90	5954.42
465-053	Positive	3648.56	6423.00	4985,37
465-054	Negative	2884.46	267.14	227,45
465-055	Positive	812.44	54.46	66,09
465-056	Positive	4998.06	752.93	483,31
465-057	Positive	7343.40	3187.53	1760,59
465-058	Positive	7027.96	3337.90	2929,53
465-059	Positive	5752.44	2483.06	2347,29
465-060	Positive	1617.78	113.08	174,64
465-061	Positive	4010.39	491.99	234,78
465-062	Positive	459.93	304.50	169,35
465-063	Positive	5851.92	1365.36	814,1
465-064	Positive	5398.31	1298.18	594,25
465-065	Positive	5271.84	595.64	260,76
465-066	Positive	5555.59		790,13

Table B. Architect HBsAg qualitative test results obtained from ten selected DBS and PSC samples stored at different temperatures

Sample ID	DBS 4ºC	DBS RT	PSC 4ºC	PSC RT
	(S/CO)			
465-057	3098.56	2945.38	2065.72	1615.96
465-058	3285.83	3108.67	2817.73	2854.97
465-059	2512.68	2118.79	2418.42	2338.16
465-060	109.25	95.40	188.87	190.95
465-061	490.01	428.69	166.1	242.63
465-062	143.84	126.73	156.36	66.34
465-063	1383.21	640.43	732.26	674.23
465-064	1272.03	1218.39	518.77	454.28
465-065	1162.50	997.10	529.94	541.78
465-066	1429.06	1294.93	630.85	624.73

Table C. Xpert HBV viral load results of the plasma, DBS and PSC samples stored at -20ºC.

The highlighted entries in Table C of the Appendix E represent the samples that could not be tested due to Architect HBsAg levels of less than 1 S/CO.

Sample ID	Plasma viral load log	DBS uncorrected viral	DBS corrected log viral load	PSC uncorrected viral load	PSC corrected log viral load
		(IU/mL)			
465-002	HBV NOT DETECTED				
465-003	HBV NOT DETECTED				
465-005	HBV NOT DETECTED				
465-006	HBV NOT DETECTED				
465-007	HBV NOT DETECTED				
465-009	HBV NOT DETECTED				
465-011	HBV NOT DETECTED				
465-012	HBV NOT DETECTED				
465-016	5.12	1810	4.6	1560	4.1
465-017	HBV NOT DETECTED				
465-018	HBV NOT DETECTED				
465-020	HBV NOT DETECTED				
465-021	HBV NOT DETECTED				
465-022	HBV NOT DETECTED				
465-023	3.82	<10	2.34	16	2.11
465-024	HBV NOT DETECTED				
465-026	2.81	23	2.7	<10	1.9
465-027	1.11	HBV NOT DETECTED		HBV NOT	

				DETECTED	
465-028	1.93	<10	2.34	<10	1.9
465-029	HBV NOT DETECTED	HBV NOT DETECTED		HBV NOT DETECTED	
465-030	3.82	178	3.59	<10	1.9
465-031	HBV NOT DETECTED	<10	2.34	HBV NOT DETECED	
465-035	HBV NOT DETECTED	HBV NOT DETECTED			
465-036	8.24	6850	5.18	3730	4.47
465-037	2.59	125	3.44	25	2.3
465-038	3.05	67	3.17	63	2.7
465-039	2.93	262	3.76	39	2.5
465-040	5.18	2530	4.75	2670	4.33
465-041	1.41	16	2.55	<10	1.9
465-042	HBV NOT DETECTED	<10	2.34	<10	1.91
465-043		HBV NOT DETECTED			
465-049	HBV NOT DETECTED	HBV NOT DETECTED			
465-050	1	HBV NOT DETECTED		<10	1.9
465-051	6.04	2480	3.74	256	3.31
465-053	Error	1260	4.44	4040	4.51
465-054	1	HBV NOT DETECTED		HBV NOT DETECTED	
465-055	HBV NOT DETECTED	HBV NOT DETECTED		HBV NOT DETECTED	
465-056	4.01	42	2.97	37	2.47
465-057	2.52	HBV NOT DETECTED		<10	1.9
465-058	6.62	INVALID		ERROR	
465-059	1	HBV NOT DETECTED		HBV NOT DETECTED	
465-060	3.81	ERROR		35	2.44
465-061	3.84	17	2.57	ERROR	
465-062	2.33	<10	2.34	<10	1.9
465-063	4.31	56	3.1	68	2.74
465-064	1.88	HBV NOT DETECTED		<10	1.9
465-065	1.82	HBV NOT DETECTED		HBV NOT DETECTED	
465-066	2.1	HBV NOT DETECTED		93	2.87

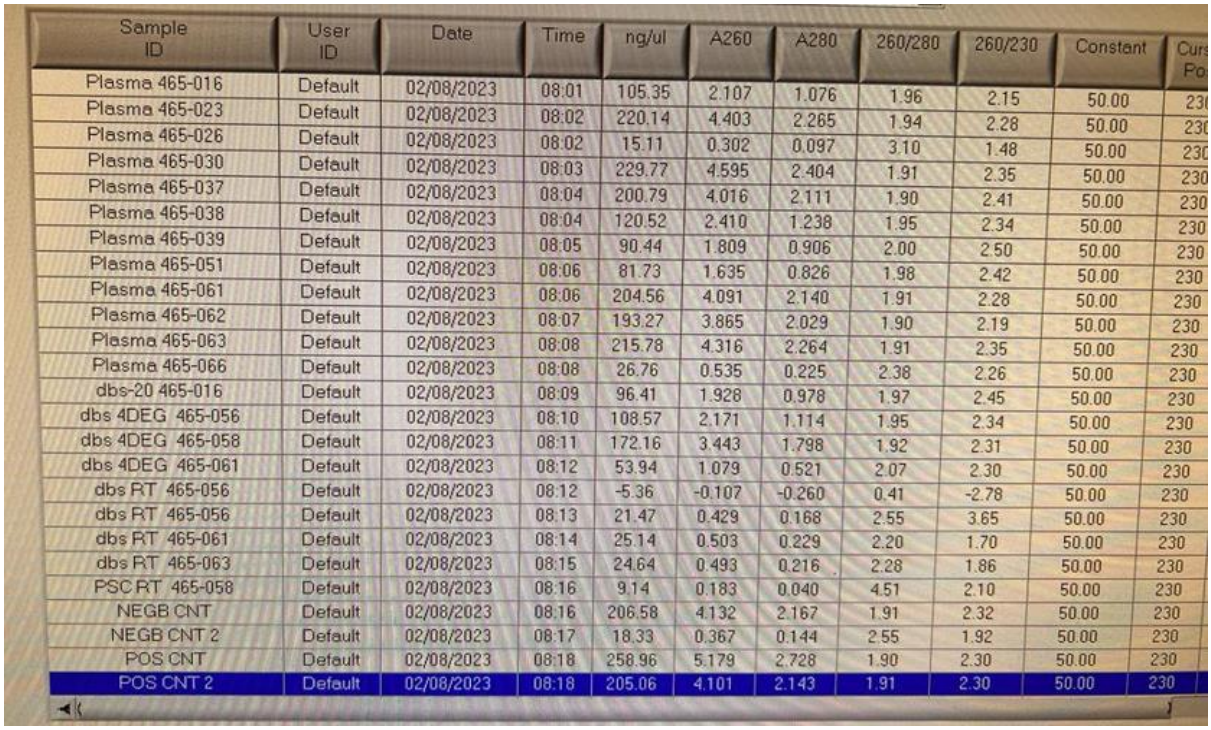
Table D. Xpert HBV viral load results of DBS samples stored at 4ºC and room temperature.

Sample ID	DBS uncorrected (4ºC) viral load	DBS corrected (4ºC) log viral load	DBS uncorrected (RT) viral load	DBS corrected (RT) log viral load
	(IU/mL)			
465-057	16	2.55	14	2.49
465-058	1370	4.48	INVALID	
465-059	HBV NOT DETECTED		<10	2.34
465-060	85	3.27	121	3.43
465-061	156	3.54	223	3.7
465-062	31	2.83	24	2.72
465-063	467	4.01	499	4.04
465-064	<10	2.34	<10	2.34
465-065	<10	2.34	<10	2.34
465-066	<10	2.34	<10	2.34

Table E. Xpert HBV viral load results of the PSC samples stored at 4ºC and room temperature.

Sample ID	PSC uncorrected (4ºC) viral load	PSC corrected (4ºC) log viral load	PSC uncorrected (RT) viral load	PSC corrected (RT) log viral load
	(IU/mL)			
465-057	11	1.94	<10	1.9
465-058	3010	4.39	3210	4.41
465-059	HBV NOT DETECTED		HBV NOT DETECTED	
465-060	97	2.89	391	3.5
465-061	109	2.94	285	3.36
465-062	15	2.08	ERROR	
465-063	ERROR		715	3.76
465- 064	< 10	1.9	<10	1.9
465-065	HBV NOT DETECTED		ERROR	
465-066	<10	1.9	HBV NOT DETECTED	

APPENDIX F



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Curs Po
Plasma 465-016	Default	02/08/2023	08:01	105.35	2.107	1.076	1.96	2.15	50.00	23(
Plasma 465-023	Default	02/08/2023	08:02	220.14	4.403	2.265	1.94	2.28	50.00	23(
Plasma 465-026	Default	02/08/2023	08:02	15.11	0.302	0.097	3.10	1.48	50.00	23(
Plasma 465-030	Default	02/08/2023	08:03	229.77	4.595	2.404	1.91	2.35	50.00	230
Plasma 465-037	Default	02/08/2023	08:04	200.79	4.016	2.111	1.90	2.41	50.00	230
Plasma 465-038	Default	02/08/2023	08:04	120.52	2.410	1.238	1.95	2.34	50.00	230
Plasma 465-039	Default	02/08/2023	08:05	90.44	1.809	0.906	2.00	2.50	50.00	230
Plasma 465-051	Default	02/08/2023	08:06	81.73	1.635	0.826	1.98	2.42	50.00	230
Plasma 465-061	Default	02/08/2023	08:06	204.56	4.091	2.140	1.91	2.28	50.00	230
Plasma 465-062	Default	02/08/2023	08:07	193.27	3.865	2.029	1.90	2.19	50.00	230
Plasma 465-063	Default	02/08/2023	08:08	215.78	4.316	2.264	1.91	2.35	50.00	230
Plasma 465-066	Default	02/08/2023	08:08	26.76	0.535	0.225	2.38	2.26	50.00	230
dbs-20 465-016	Default	02/08/2023	08:09	96.41	1.928	0.978	1.97	2.45	50.00	230
dbs 4DEG 465-056	Default	02/08/2023	08:10	108.57	2.171	1.114	1.95	2.34	50.00	230
dbs 4DEG 465-058	Default	02/08/2023	08:11	172.16	3.443	1.798	1.92	2.31	50.00	230
dbs 4DEG 465-061	Default	02/08/2023	08:12	53.94	1.079	0.521	2.07	2.30	50.00	230
dbs RT 465-056	Default	02/08/2023	08:12	-5.36	-0.107	-0.260	0.41	-2.78	50.00	230
dbs RT 465-056	Default	02/08/2023	08:13	21.47	0.429	0.168	2.55	3.65	50.00	230
dbs RT 465-061	Default	02/08/2023	08:14	25.14	0.503	0.229	2.20	1.70	50.00	230
dbs RT 465-063	Default	02/08/2023	08:15	24.64	0.493	0.216	2.28	1.86	50.00	230
PSC RT 465-058	Default	02/08/2023	08:16	9.14	0.183	0.040	4.51	2.10	50.00	230
NEGB CNT	Default	02/08/2023	08:16	206.58	4.132	2.167	1.91	2.32	50.00	230
NEGB CNT 2	Default	02/08/2023	08:17	18.33	0.367	0.144	2.55	1.92	50.00	230
POS CNT	Default	02/08/2023	08:18	258.96	5.179	2.728	1.90	2.30	50.00	230
POS CNT 2	Default	02/08/2023	08:18	205.06	4.101	2.143	1.91	2.30	50.00	230

Figure A. Measurement of some of the amplified samples using the Nanodrop spectrophotometer.

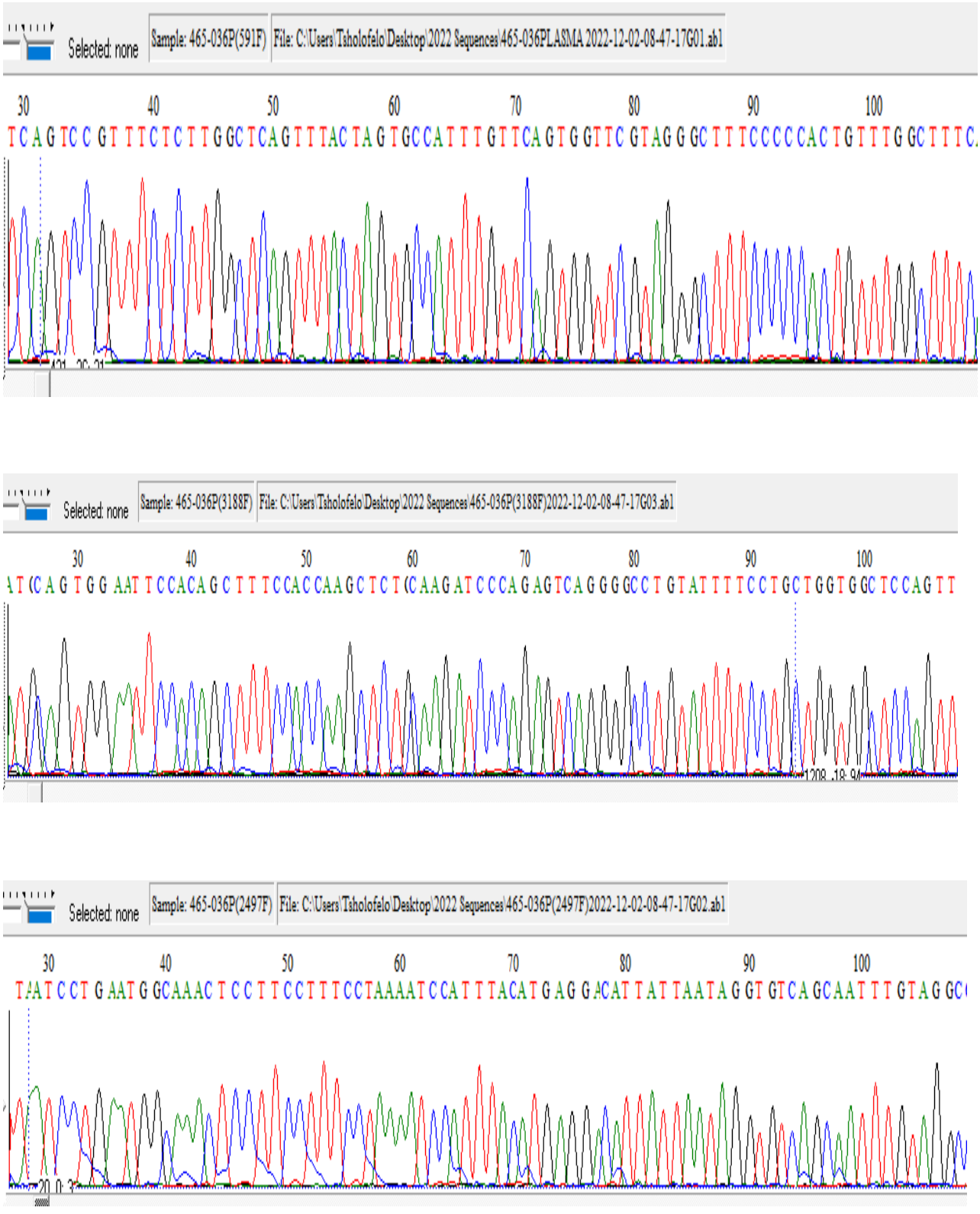


Figure B. Results of 465-036 plasma sample with good quality of sequence data (chromatogram) that was obtained from all forward primer (591F, 3188F and 2497F).

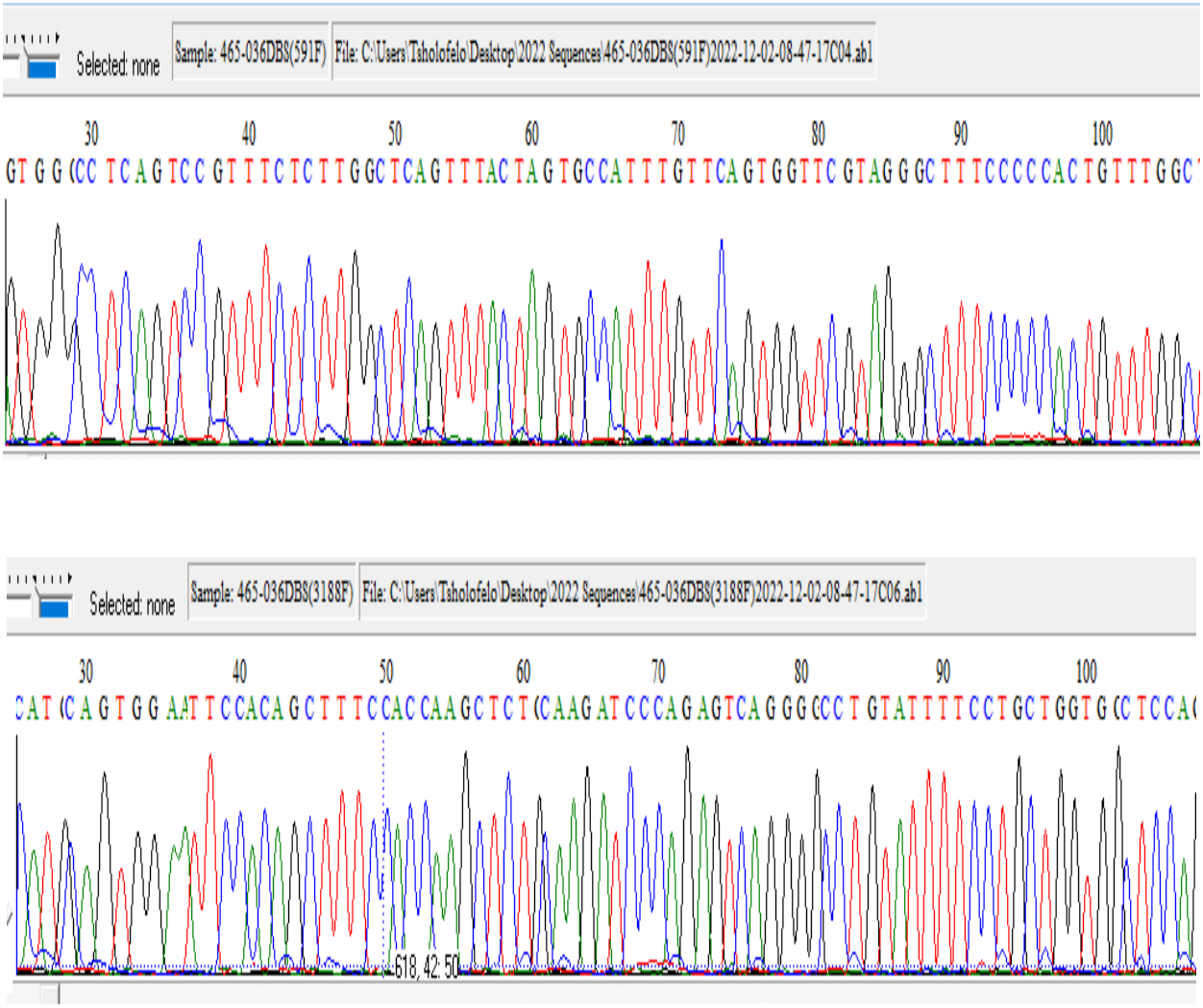


Figure C. Result of 465-036 DBS sample with good quality of sequence data (chromatogram) that was obtained from all forward primer (519F, 3188F and 2491F).

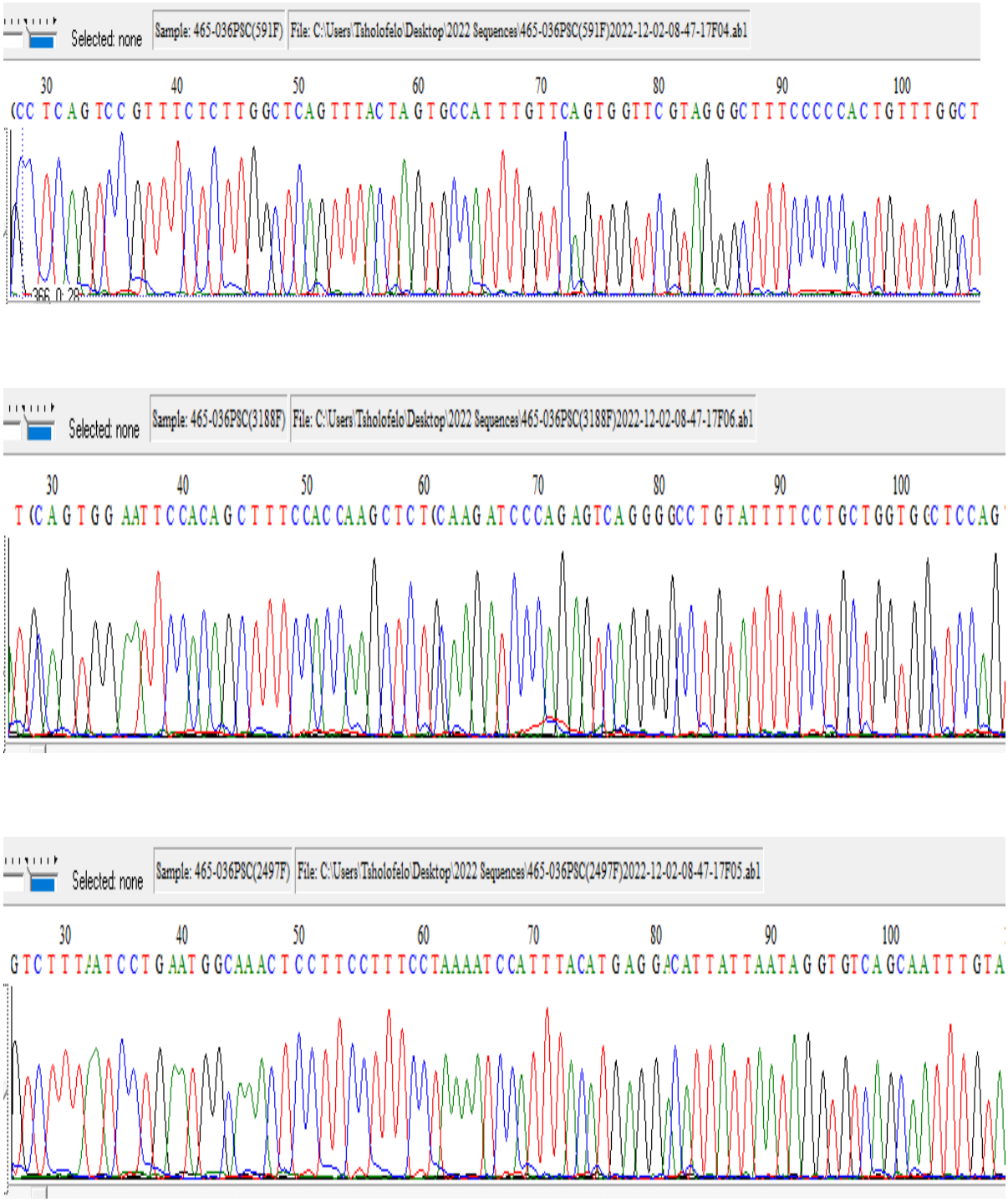


Figure D. Results of 465-036 PSC sample with good quality of sequence data (chromatogram) that was obtained from all forward primer (591F, 3188F and 2491F).