Immunity to hepatitis B in South African students following childhood vaccination

by

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DECLARATION OF INDEPENDENT WORK

I, Dineo Monaheng, student number _____ hereby declare that this dissertation submitted to the Central University of Technology, Free State for the degree Master of Health Sciences in Biomedical Technology, is my own independent work that has not been submitted before to any institution, by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

Signature: **Date: 19 Aug 2025**

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TABLE OF CONTENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In this empirical research, I discuss the long-term humoral and cellular immune response to childhood hepatitis B virus (HBV) vaccination among South African medical students. Healthcare workers (HCWs) are considered at a high risk of HBV exposure and have a four times greater probability of contracting the virus than the general population, simply because HCWs are exposed to blood and body fluids as well as needle stick and sharps injuries.

The study was conducted in Bloemfontein where samples were collected at three different timepoints from 122 Central University of Technology (CUT) Biomedical and University of the Free state (UFS) medical students. Participants were South African students born during and after 1995 who had not been vaccinated at any point against HBV after their childhood vaccination. It took approximately 7 months to observe the response to the complete booster vaccination schedule. The first samples were obtained from February to March 2018. The second samples were obtained a month after the initial sampling, and the third samples 7 months after the initial blood draw. Students were also immunized at the first and second time point, with the last vaccination administered a month before the last blood draw (6 months after the initial vaccination) to adhere to a standard 0-, 1- and 6-month vaccination schedule. Information collected at the first time point included date of birth, age, gender, race, vaccination and clinical history. However, if this information was not available, it did not exclude the participant from the study. The baseline sample draw and first dose of immunizations were done at both institutions. The samples were drawn before vaccination so that the baseline HBsAb titre results reflected those obtained from childhood vaccination. Two blood samples were drawn, one without an anticoagulant (clotted tube) for serological tests and one lithium-heparinized tube to perform the interferon-gamma (IFN-y) and interleukin-2 (IL2) release assays. The second samples were collected one month after baseline sampling. One tube without anticoagulant (clotted tube) was taken at this time point for serological testing. The second shot of HBV vaccine was administered at this time point. Clotted blood samples were obtained again at the 7-month collection. Samples were collected in the same manner as previous collections; however, no

vaccination was done at the 7-month interval. Vaccination was done at 6 months after baseline vaccination (a month before the 7-month blood draw), as it is recommended that immunity following HBV vaccination is tested approximately one month following the third dose. Samples were analysed for hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), interleukin 2 (IL-2) and interferon gamma (IFN-y). HBsAb and HBcAb tests were conducted on the Liaison® XL Murex instrument. The IFN-y and IL-2 were manual ELISA tests which were read on the Biotek-ELx800.

At baseline, 52% tested negative for HBsAb, while 43% tested positive and 5% were equivocal. Following a single booster dose of HBV vaccine, 92% of participants showed adequate HBsAb responses. At the end of vaccinations, all participants tested positive for HBsAb. IFN-y results grouped and associated with baseline HBsAb results showed to be statistically not significant, while at second sampling these proved to be statistically significant, with a p value of 0.03. For IL-2, the association with both baseline and second sampling HBsAb proved to be statistically significant with a p-value of 0.02 at baseline and <0.01 at second sampling.

Only one participant in this study provided childhood immunization records. The remainder were unaware of their vaccination status and did not have access to their childhood immunization records. This indicates the need for exploring alternative methods of documenting immunization in order to improve record keeping and provide better continuity of care.

This vaccination programme is expected to provide long-term protection against HBV infection. However, more than half of the participants (52%) tested negative for HBsAb at baseline. This is suggestive of waning humoral immunity. Possible factors that could induce waning are weight, age and the type of vaccination administered. At second sampling, 92% of the participants tested positive to HBsAb. This indicates a strong anamnestic response and this is an indication that most participants have likely been vaccinated previously during childhood and have developed long-term memory immune responses.

At baseline, the association between IL-2 results and HBsAb gave a p-value of 0.02 indicative of a statistically significant association. The sensitivity and specificity of IL-2 results to predict HBsAb status was 93% and 26%, respectively. IL-2 seemed to be a more reliable assay as it produced much higher results at baseline and during second sampling. The participants who tested positive had the highest median of 92pg/mL. After booster vaccinations, the sensitivity and specificity were 85% and 71%, respectively, with a p-value of >0.01. Participants who tested positive also had the highest median of 65 pg/mL

When comparing the two ELISA tests, we can confirm based on the sensitivity, specificity and p-value that IL-2 is a much more reliable marker to use in order to evaluate cellular immunity that has been induced by vaccination. This can be seen prior to and after booster vaccination. However, we cannot conclude the above, because there a high number of participants were involved during IL-2 testing; therefore, giving a much more accurate platform to make a conclusion.

Both the IFN-y and IL-2 assays are involved in the cellular immune response. However, in this study, they were used as proxies for existing immunological memory. The immune system of the participants has been exposed previously to HBV antigens and this can be seen by the cytokines response when stimulated by the presence of antigens, which can either be through HBV infection or vaccination.

It is recommended that HB booster vaccination be included in policies as part of a routine programme for all individuals, more especially, individuals in healthcare settings and other high-risk areas because of the waning immunity in more than half of the participants.

IL-2 assay has proven to be a more reliable assay due to its high sensitivity and specificity; therefore, it can be implemented as one of the routine checks to use in order to assess cellular immunity that is induced due to vaccination.

Only one participant produced his/her vaccination records for the use of the study, while the rest of the participants did not have their records available. Therefore, it is recommended that in future, more digital records should be used that will be accessible at all healthcare settings.

LIST OF ABBREVIATIONS

ALT	- Alanine Transaminase
Anti-HBc Igm	- Hepatitis B core IGM antibody
Anti-HBe/HBeAb	- Hepatitis B e antibody
cccDNA	- Covalently closed circular DNA
CLEIA	- Chemiluminescent enzyme immune assay
CLIA	- Chemiluminescent immunoassay
CUT	- Central University of Technology
DM	- Diabetes mellitus
DNA	- Deoxyribonucleic acid
EPI	- Extended programme on immunizations
ER	- Endoplasmic reticulum
HBcAb/anti-HBc	- Hepatitis B core antibody
HBcAg	- Hepatitis B core antigen
HBeAg	- Hepatitis B e antigen
HBIG	- Hepatitis B immune globulin
HBsAb/anti-HBs	- Hepatitis B surface antibody
HBsAg	- Hepatitis B surface antigen
HBV	- Hepatitis B Virus
HCC	- Hepatocellular carcinoma
HCV	- Hepatitis C virus
HCW	- Healthcare worker

HIV	- Human Immunodeficiency Virus
IFN	- Interferon
IFN-y	- Interferon gamma
IGRA	- Interferon gamma release assay
IL-2	- Interleukin 2
IQR	- Interquartile ranges
mRNA	- Messenger Ribonucleic Acid
NHLS	- National Health Laboratory Services
NK	- Natural killer
PCR	- Polymerase Chain Reaction
pgRNA	- Pregenomic RNA
RLU	- Relative light units
RT	- Reverse transcription
SHBs	- Small hepatitis B surface antigens
SANDOH	- South African national department of health
SVP	- Subviral spherical or filamentous envelope particles
UFS	- University of the Free State
WHO	- World Health Organization

CHAPTER 1: LITERATURE REVIEW

1.1 Background

The hepatitis B virus (HBV) is a vaccine-preventable viral infection that results in liver disease (Kane, 1998). HBV chronic infection is estimated to have a prevalence of 4.1% globally. This explains the 316 million people who are hepatitis B surface-antigen (HBsAg) seropositive carriers. These carriers are more prevalent in developing and low-income countries (Sheena et al., 2022; Yuen et al., 2018). There are at least 65 million chronic HBsAg carriers in Africa and of these, approximately 2.5 million reside in South Africa (Kramvis and Kew, 2007; Tsebe et al., 2001). Healthcare workers (HCW) are at high risk of contracting the virus, as HBV is a well-known occupational hazard in healthcare settings, together with the hepatitis C virus (HCV) and human immunodeficiency virus (HIV). Up to 37.6% of HBV infections in HCW result from occupational exposure via needle stick injuries (World Health Organisation, 2002).

The expanded programme on immunization (EPI) is a World Health Organization programme that was established in 1974 in order to expand immunization programmes and improve access to vaccines globally (Keja et al., 1988). According to the recommendations for routine immunization made by the World Health Organization, a three-dose HBV vaccine should be given to all children worldwide. All infants, including those of low birth weight and those who are premature, should receive the first dose of HBV vaccine as soon as possible, preferably within 24 hours of birth. This is because perinatal and postnatal transmission is one of the most important sources of chronic HBV infection globally. Administration of a birth dose with two or three additional doses, which must be four weeks apart, should be followed as part of the primary series of HBV vaccinations. The latter applies more especially for premature children or children with a low birth weight. Unvaccinated children should be vaccinated with a 0-, 1- and 6-month schedule (World Health Organisation,17a). In South Africa, HBV vaccination was introduced into the EPI programme in 1995. This was an excellent initiative, since horizontal infection of children is the principal route of transmission in Africa. However, no birth dose is currently administered (Kiire, 1996). As many as 98% of inhabitants of Africa

are at some point in their lives infected with the virus, with millions chronically infected with HBV (Kew, 1996). Individuals who are exposed to blood such as HCWs; those with chronic diseases e.g. diabetes; and individuals who engage in behavioural activities such as having multiple partners are all groups at the highest risk of acquiring HBV. These individuals require vaccination if they have not received a complete primary course of vaccine and should be considered for post-vaccination testing to ensure that adequate antibody levels are obtained (World Health Organization, 2017a).

The efficacy of HBV vaccine introduction is evident in many areas of the world. Vaccination of all newborns began in 1984 in Taiwan, resulting in 85% of infants obtaining adequate levels of protective antibodies at 18 months of age. The vaccination programme controlled the rate of infection in many ways. Firstly, the HBV carrier rate decreased from 11% to 1% from 1994 to 2004 (Chen et al., 1996; Hsu et al., 1986; Ni et al., 2001; Ni et al., 2007). Secondly, the infant mortality rate due to fulminant HBV decreased from 5.36/100 000 to 1.7/100 000 and almost disappeared in children older than one year of age (Chen et al., 2004; Kao et al., 2001). Thirdly, the average annual incidence of hepatocellular carcinoma (HCC) in children 6–14 years of age declined from 0.57/100 000 between 1986 and 1990 to 0.36/100 000 between 1990 and 1994. The decline in HCC incidence extended to teenagers and young adults two decades post vaccination (Chang et al., 1997).

In China, routine infant HBV vaccination was recommended from 1992, but parents were required to pay for vaccination. A free birth dose was implemented in 2002 and free vaccinations were provided for all infants from 2005. These interventions decreased the carrier rate from 9.8% in 1992 to 7.2% in 2006 (Liang et al., 2013). By 2014, the coverage rate was 99%. A national survey from the Chinese CDC found that the prevalence of HBV in age groups 1–4, 5–14 and 15–29 years was 0.36%, 0.94% and 4.38%, respectively. This suggests that the HBV vaccine can successfully decrease the prevalence of HBV, but emphasizes the importance of catch-up vaccination in older children who may not have been vaccinated, as well as vaccination of high-risk adults in order to prevent infection in these age groups. The increased prevalence in the age groups 15–29 years

could suggest waning or non-vaccination, as the individuals were born prior to 2005 (Wang et al., 2019)

Although childhood vaccination is expected to provide long-term protection against HBV infection, reports of waning memory immunity have been described following neonatal vaccination. Causes of waning immunity remain unknown; however, immune memory is a key characteristic in determining long-term protection (Lu et al., 2008). Booster vaccinations are not required for immunocompetent subjects for at least 15 years after vaccination, but behavioural risks and medical institutions where HBV is at a high prevalence pose a risk to acquiring HBV infection (Kane, 1998; Lu et al., 2008; Wang et al., 2019). Behavioural risks include sexual activities, the use of needles during drug use, tattoos and scarification of the skin during ritual initiation ceremonies or when given treatment, and lastly at medical institutions where HBV exposure is at its peak. HBV exposure can be due to HCW being exposed to potentially infectious blood and body fluids, which at times they do not recognize because of the ability of HBV to remain infectious for prolonged periods on environmental surfaces and as it is transmissible even in the absence of visible blood (Beltrami et al., 2001).

The risk of contracting HBV infection due to needlestick injury is 100 times higher than that of contracting HIV (Steyn, 2002). HIV-positive individuals have a higher likelihood of being infected with HBV than HIV-negative individuals (Herrero, 2001; Rogers et al., 2000). According to UNAIDS, more than 35 million people were infected with HIV globally by 2013, with the majority residing in sub-Saharan Africa. Sub-Saharan Africa contains 12% of the global population, but accounts for 71% of global HIV infections. South Africa accounts for 25% of the people living with HIV in sub-Saharan Africa (Kharsany and Karim, 2016). From the above-mentioned, it is quite evident that HBV is a significant public health issue in South Africa.

1.2 HBV classification, structure and replication

1.2.1 Classification

HBV belongs to the *Hepadnaviridae* family that has 18 species, divided among five genera. The five genera are *Orthohepadnavirus*, *Avihepadnavirus*, *Herpetohepadnavirus*, *Metahepadnavirus* and *Parahepadnavirus.* HBV is classified under the genus *Orthohepadnavirus,* which contains 12 species. They are HBV, capuchin-monkey HBV, Chinese shrew HBV, ground-squirrel hepatitis virus, long-fingered bat HBV, Pomona-bat HBV, roundleaf-bat HBV, Tai-Forest hepadnavirus, tent-making bat HBV, woodchuck hepatitis virus, domestic-cat HBV and woolly-monkey HBV (Lytras et al., 2021). HBV is divided into four major serotypes (adr, adw, ayr, ayw) based on the antigenic epitope present on its envelope protein, HBsAg. These serotypes are based on a common determinant (a) and 2 mutual determinant pairs (d/y and w/r).

To date, at least 10 HBV genotypes (A–J) have been described according to the overall nucleotide sequence variation of the genome, of which five are further divided into at least 24 subtypes (Hundie, 2017; Lin and Kao, 2017; Liu et al*.,* 2021). Genotype A has seven subtypes (A1 to A7). Genotype B has six subtypes (B1 to B6). Genotype C has eleven subtypes (C1 to C11). Genotype D is divided into seven subtypes (D1 to D7) and genotype E has no subtypes. Genotype F is subdivided into four subtypes (F1 to F 4). Genotype G, H, I and J have no subtypes (Lin and Kao, 2017).

1.2.2 Structure

The HBV virion is a spherical, double-shelled structure 42 nm in diameter. The nucleocapsid consists of hepatitis B core antigen (HBcAg), viral polymerase and the viral DNA genome (Hundie et al., 2017). The hepatitis B e antigen (HBeAg), which is associated with the nucleocapsid, is surrounded by the lipid envelope. It also circulates as soluble proteins in serum.

The genome of HBV is made of circular deoxyribonucleic acid (DNA), which is not fully double-stranded. One end of the full-length strand (long or L strand) is linked to the viral

DNA polymerase. The genome is 3 020–3 320 nucleotides long for the full-length strand, and 1 700–2 800 nucleotides long for the shorter strand, known as the short or S strand (Kay and Zoulim, 2007). The short strand extends from a variable distance from a fixed s' terminus, which is approximately at 250 nucleotides 5' to the nick in the L strand (Delius, 1983; Kay and Zoulim, 2007). There are four open reading frames (ORF), namely pre- S/S, polymerase, pre-Core/core and X. The pre-S/S ORF encodes the envelope proteins which is divided into three different surface molecules (large, middle and small) that form the surface antigen, HBsAg. The polymerase encodes for the reverse transcriptase (RT) domain of the HBV polymerase (POL gene). The pre-Core/core encodes for the C gene, which consists of two proteins (HBcAg and HBeAg). HBcAg forms the nucleocapsid and the HBeAg is a secretion protein that is a marker for HBV replication and infectivity. The X regulatory protein is encoded by the X gene. The X protein function is not fully understood; however, the establishment of the infection and viral replication have been proposed as possible functions (Caligiuri et al., 2016)

The HBV virion is also known as a Dane particle, which carries viral nucleic acid in core particles enveloped by three proteins, namely large S (LS), middle S (MS) and major S (SS) proteins (Tiollais et al., 1985). These three proteins are embedded in a lipid bilayer originating from the host cell. The SS is also known as HBsAg and it consists of 226 amino acids. It is in the p24 and gp27 form (Gavilanes et al., 1982). The MS has an additional 55 amino acids at the N terminus of the SS and these additional amino acids are referred to as pre-S2. MS has three forms: p30, gp33 and gp36 (Gerlich et al., 1980; Gerlich and Thomssen, 1975). LS has an additional 108–119 amino acids often called the pre-S1 at the N terminus of the MS and its forms are p39 and gp42 (Klinkert et al., 1986; Theilmann et al., 1986). Subviral spherical or filamentous envelope particles (SVPs) are empty particles that bud intracellularly due to the production of HBV surface proteins. The SVPs are 20 nm in diameter and are secreted in larger quantities than HBV virions. The SVPs, which lack the nucleocapsid, are formed at the rough endoplasmic reticulum (ER) and, due to them being non-infectious, they were used as the basis of most vaccines against HBV (Dubois et al., 1980; Ganem, 1991; Heermann et al., 1984).

1.2.3 Replication

HBV is one of the few viruses that undergo reverse transcription as part of the replication process by generating DNA by reverse transcription of an RNA intermediate (Summers and Mason, 1982). Replication occurs in the following steps (Firnhaber et al., 2008):

Attachment: the virus enters the host cell by binding to the receptor on the surface of the host cell.

Penetration: the virus membrane fuses with the host cell membrane, therefore releasing core proteins and DNA into the cytoplasm of the host cell.

Uncoating: the viral DNA genome is transferred to the host cell nucleus where the core proteins dissociate from the partially double stranded viral DNA. The viral DNA then becomes fully double stranded and is transformed into a covalently closed circular DNA (cccDNA) that assists in the transcription of four viral messenger ribonucleic acid strands (mRNA).

Replication: the largest mRNA is then used to make new DNA genomes, capsid, core proteins, and viral DNA polymerase.

Assembly: the four mRNAs return to the nucleus where they are recycled. The new DNA genome, capsid, core proteins and viral polymerase then assemble and undergo additional processing to form a new virus.

Release: this new virus then moves to the cytoplasm, then out of the host cell where it goes on to infect other host cells.

1.3 Epidemiology

In 2019, 296 million people were living with chronic HBV globally with a prevalence of approximately 3.8% in the general population. Of these, 6 million were children who were younger than five years of age. In 2015, four million new cases of hepatitis were reported per year. This number had fortunately dropped to 1.5 million in 2019 (Devarbhavi et al.,

2023; Noubiap and Ndoula, 2022; Zuccaro et al., 2015). HBV distribution varies globally in prevalence depending on the geographical area. The WHO reports that the prevalence of HBV is the highest in Africa at 7.5% in the general population. In this region, the prevalence among children younger than five years of age is 2.5%; therefore 4.3 million out of the 82.3 million people living with HBV infection are children younger than five years. The lowest prevalence is in the Americas at 0.5% and a prevalence 0.1% in children younger than five years, with children younger than five years therefore accounting for 51 000 out of the 54 million people living with HBV infection. Similarly, the HBV mortality rate is the highest in African regions (80 000 in 2019) and lowest in American regions (15 000 in 2019) (Devarbhavi et al., 2023). The global deaths associated with HBV complications such as liver cirrhosis and hepatocellular carcinoma has decreased from 0.6 - 1 million by 2008 to 820 000 by 2019 (Devarbhavi et al., 2023; Noubiap and Ndoula, 2022; Zanettiet al., 2008). The western pacific region accounts for 57% of global deaths due to HBV (Devarbhavi et al., 2023; World Health Organisation , 2017). China, India and Nigeria are the top three countries which carry the burden of HBV at 74 million, 17 million and 15 million, respectively, with China in the lead globally at 29% and the other two countries at 6.6% and 5.8% of the global burden of hepatitis B respectively (Devarbhavi et al., 2023).

The ten genotypes are geographically distributed in certain parts of the world.

- Genotype A is prevalent in Europe, Africa, India and America and accounts for 75% of HBV in southern Africa.
- Subtype A1 is located in sub-Saharan Africa and India; A2 is located in Northern Europe and India; A3 in western Africa and A4 to A7 in Gambia and Nigeria.
- Genotype B and C are prevalent in the Asian-pacific region.
- Subtype B1 is located in Japan; B2 to B5 in east-Asia, Taiwan, China, Indonesia, Vietnam and the Philippines; B6 in Alaska, northern Canada and Greenland.

- Genotypes C1 to C3 are prevalent in Taiwan, China, Korea, Japan and southeast Asia; C4 in Australia; C5 in the Philippines and Vietnam and C6 to C11 in Indonesia.
- Genotype D with seven subtypes, D1 to D7, is more prevalent in Africa, Europe, the Mediterranean area and India. Genotype D has also been reported in Indonesia and Australia. It also accounts for 20% of HBV in South Africa.
- Genotype E is restricted to west and central Africa as well as Saudi Arabia.
- Genotype F is found in central and southern America. Genotype G has been found in France, Germany and in the United States.
- Genotype H is prevalent in central America and Mexico.
- Genotype I is found in Vietnam and Laos and lastly, genotype I is found in Japan (Lin and Kao, 2017).
- By 2021, more subtypes were established such as subtype A8, B7 to B10, C12 to C17, D8 to D12, F5 to F6, H2, and I2 (Kew, 2008; World Health Organisation, 2021).

1.4 The prevalence of HBV in South Africa

In 1996, there were approximately 2.5 million carriers of HBV residing in South Africa, with more than 70% of the South African population being exposed to HBV. By 2008, 3–4 million black South Africans were chronically infected with HBV, with the carriage being more common in males than in females. Before HBV vaccination in South Africa, the prevalence of HBsAg of chronic carriage in black South Africans was 9.6%, with 76% previously exposed, as indicated by one or more serological markers to HBV. More than a decade later, 15.8% of rural black males and 2.0% urban black females presented with HBsAg positivity. After various studies conducted within different geographical sites in South Africa, it has been established that HBV prevalence is 5–16% among rural black males, 8–9% among urban black males, 4–12% among rural black females and 2–4%

among urban black females. Some contributing factors to the high rural carriage rate are horizontal transmission at school-going age and at the time of sexual activity (Kew, 2008; Kiire,1996). Caucasians and Indians have a carrier rate of 0.2% and an exposure rate of 5%, while those of mixed descent (European-African) have a carrier rate of 0.4–3% and an exposure rate of 18–25%, while South Africans of Chinese descent had a carrier rate of 5.2% and an exposure rate of 50% (Kew, 1996).

In a more recent South African study, the prevalence of HBV was measured for a five-year period (2015-2019) and was conducted at different age groups, namely 0–4 years, 5–9 years, 10–14 years, 15–19 years, 20–24 years, 25–49 years, and lastly 50 years and older. It was established that under the age of 15, the age group 0–4 years had the highest HBV prevalence, followed by the 10–14 year-olds and the 5–9 year-olds. The age group 15–19 years had the lowest HBV prevalence when compared to the age group 20–24 years. The age group 25–49 years had the highest prevalence among all the age groups within the study, while 50-year-olds and older showed a decrease in HBV. The study has also established that the annual national HBV prevalence rate per 100 000 population had increased from 56.14–74.17% in 2015 to 2017, and then decreased to 62.81% in 2018. However, it increased in 2019 to 67.76%. The prevalence rate was consistently higher in males than females. The study also looked into establishing the HBV prevalence among provinces and the Gauteng province had the highest HBV rate, followed by the Eastern Cape and KwaZulu-Natal, with the lowest rate in the Northern Cape (Moonsamy et al., 2022).

Approximately 2,7 million people are coinfected with HIV and HBV within the WHO regions, of which 8% are from sub-Saharan Africa (Coffie et al., 2017; World Health Organisation , 2021). In a study conducted in Johannesburg, the prevalence of HBV co-infection in a South African urban HIV government clinic was established. It showed that 4.8% of the participants where HBsAg positive, with 47% of the participants showing evidence of HBV exposure. The study also concluded that HBV infection is 5 times more common in HIV-infected individuals in urban South Africa than individuals who are not infected with HIV (Firnhaber et al., 2008). Another study established that there was a high prevalence of HBV in pregnant women; therefore, increasing the risk of perinatal

HBV transmission and decreasing the transfer of hepatitis B surface antibody (HBsAb) due to immunosuppression. Fortunately, after 1995 when the use of HBV vaccine in the EPI was introduced in SA, there was a reduction in the prevalence of HBV amongst children, ranging from 0.0% in children with unknown HIV status to 2.7% in children who are HIV positive (Burnett et al., 2012).

1.5 Transmission

HBV can survive outside the body for at least seven days; therefore, during this time, it is still infectious when it enters the body of an individual who is not immune (Emerenini and Inyama, 2017). The virus can be detected within 30 to 60 days after infection and can persist and develop into chronic hepatitis B (World Health Organisation, 2017b). HBV can be found in blood, saliva, menstrual, vaginal and seminal fluids, tears and breast milk (Schillie et al., 2013; World Health Organisation, 2017). Although studies have established that HBV is found at lower levels in saliva, tears or breast milk than in other body fluids, it is present at high enough levels to transmit the virus (Schillie et al., 2013). Transmission occurs when the body fluid of an infected person enters the body of another person via a number of routes including parenteral, permucosal and percutaneous (through breaks in the skin) (Nelson et al., 2016). The age at which infection occurs has a significant impact on the risk for developing chronic infection. The rates of chronicity are between 80–90% for infected infants, but drop to approximately 30% in children infected before the age of 6 years and
<5% when infection occurs in adulthood (Hyams, 1995).

There are two types of routes of transmission described for HBV, namely vertical (perinatal) and horizontal transmission. Examples of horizontal transmission are sexual contact, sharing of needles, work tasks where blood is being handled by personnel (especially HCWs and laboratory personnel), transmission between children, body piercings, tattoos, the use of infected persons' toiletry if there is blood and in rare cases through blood transfusion products, donated livers and other organs (Nelson et al., 2016). HBV exposure during childhood can lead to a large proportion of adolescents being infected by the time they reach the age of sexual maturity (Burnett et al., 2005). This then causes sexual transmission to become the dominant route of transmission, with 3–5% of

those infected at this age progressing to chronicity (Andre, 2000; Ayoola, 1988; Burnet et al., 2005; Kew, 1996; Kiire, 1996; Lin and Kirchner, 2004; Mphahlele, 2002; World Health Organisation, 1940). In South Africa, the most common route of transmission is the horizontal route of transmission, which is transmission from person to person and it increases or decreases, depending on the endemic level of HBV in the region (Nelson et al., 2016).

Horizontal transmission of HBV in young children can lead to chronic HBV. Three studies conducted have proved the above-mentioned statement. The first study was conducted in Alaska. It established that 29% of the participants younger than the age of five developed chronic HBV; 16% of participants between the ages of five and 10 years developed chronic HBV; and 16% of participants who were more than 30 years of age developed chronic HBV. A second study conducted in Taiwan proved that 23% of its participants who acquired HBV before the age of five developed chronic HBV. In the third study conducted in Senegal, 68% of participants had chronic HBV at the age of one; 82% of participants younger than six months of age; and 54% of participants between six months to one year (Nelson et al., 2016). In South Africa, about 20–30% of individuals infected horizontally before the age of five years progress to HBV chronicity (Andre, 2000; Ayoola, 1988; Lin and Kirchner, 2004).

With vertical transmission, also known as perinatal transmission, an infected woman transmits the virus to her newborn *in utero* or, more commonly, during the delivery process. Perinatal transmission accounts for the majority of HBV transmission worldwide (Nelson et al., 2016; World Health Organisation, 2017b). Transmission can also occur through breast milk while breast feeding during the post-partum period. This can lead to the development of a chronic infection (World Health Organisation, 2017b).

HBeAg is a serological marker that represents high viral replication and therefore high levels of HBV DNA. The risk of acquiring HBV can reach 100% in unvaccinated infants who were born to HBeAg-positive mothers. A study conducted before vaccination was made publicly available in Taiwan proved that 85% of infants born to HBeAg-positive mothers became chronically infected with HBV, compared to the 32% of those born to HBeAg-negative mothers (Nelson et al., 2016).

HCWs are considered at a high risk of HBV exposure and have a four times greater probability of contracting the virus than the general population, simply because HCWs are exposed to blood and body fluids as well as needle stick and sharps injuries (Di Giuseppe, 2007; Lamberti et al., 2015). HBV among HCWs remain high in South Africa due to policies that often require of students to pay for their own vaccination and post-vaccination testing. With many students requiring financial support, it becomes a challenge for these students to be vaccinated as required (Burnett et al., 2021).

In a healthcare setting, blood from persons infected with HBV contains the highest HBV titres of all body fluids. The following body fluids are considered potentially infectious: cerebrospinal fluid, synovial fluid, pleural fluid, peritoneal fluid, pericardial fluid and amniotic fluid. It has been documented in some studies that tears and saliva contain HBV. However, these fluids do not pose an occupational risk for HBV infection unless they contain blood. Semen and vaginal secretions sexually transmit HBV; therefore, they are also not seen as an occupational risk for HCW. HBsAg can also be detected in milk, bile, faeces, nasopharyngeal washings and sweat, but with low quantities of infectious HBV. Sputum, urine and vomit are not considered potentially infectious unless they contain blood (Beltrami et al., 2001). In studies of HCWs who sustained injuries from needles contaminated with blood containing HBV, the risk for developing clinical hepatitis if the blood was HBsAg positive and HBeAg positive was 22–31% and the risk for developing serological evidence of HBV infection was 37–62%. The study also proved that in HBsAg-positive participants, the risk of being HBeAg positive versus HBeAg negative is 10:1. Laboratory assays used in the study also established that 55% of participants who were HBsAg negative were positive for HBeAg (Beltrami et al., 2001; Werner and Grady, 1982). HBV, HCV and HIV are the top blood-borne infections that are contracted through occupational exposure, with HBV at 37% and the other viruses at 39% and 4.4%, respectively (Prüss-Ustun et al., 2005). The percentage of incidents of HBV among HCWs depend on the number of HCWs per global setting. However, incident underreporting is a major contribution to the quality and validity of estimates obtained. Studies conducted in South Africa have established that 24% of needlestick injuries (NSI) occur in primary-care nurses and 69% among junior doctors per annum (Karstaedt and

Pantanowitz, 2001; Kruger et al., 2012). In HCW, the risk of transmission following a percutaneous NSI is 6–30% for HBV and approximately 0.3% for HIV (Baggaley, 2006; Beltrami et al., 2000).

1.6 Immunity

There are 2 types of immunity: innate immunity and adaptive immunity.

1.6.1 Innate immunity

Immediately after HBV infection, the innate immunity responds to limit viral replication (Wieland et al., 2004). This type of immunity is the first line of defence against infection and it is characterized by type 1 interferon production and natural killer (NK) cell activation (Abbas et al., 2014). The innate immunity serves three important functions. Firstly, it is the initial response to microbes, where it controls, prevents and eliminates infection of the host by many microbes. Secondly, it recognizes the products of damaged and dead host cells and serves to eliminate these cells and to initiate the process of tissue repair. Lastly, it stimulates adaptive immune response to make them optimally effective against different type of microbes (Abbas et al., 2014).

1.6.1.1 Interferon

As one of the first lines of defence against invading pathogens, interferons (IFNs) restrict HBV replication by affecting multiple steps in the viral life cycle. These steps include HBV RNA synthesis, pregenomic RNA (pgRNA) encapsulation that occurs during the 4th and 5th stages of the replication process, the turnover rate of viral proteins, and modulation of cccDNA formation by inducing numerous IFN- stimulated genes. IFNs are classified into three types, type I, II and III, based on the structure of their receptors on the cell-wall surface. The early phase of infection is characterized mainly by the production of type I IFN (Wieland et al., 2004).

1.6.1.2 Natural Killer cells

NK cells account for approximately 30% of the intrahepatic lymphocytes compared to 5–15% of the peripheral blood lymphocytes (Mackay, 2002). NK cells possess receptors

allowing them to sense and respond to viral and bacterial patterns. NK cells produce interferon gamma (IFN-y) once infection has occurred (Lu et al., 2008). Regulation of NK cell activation is made possible by cytokines such as type 1 IFN, interleukin 2(IL-2), IL-12, IL-15 and IL-18 (Cooper et al., 2001; Kimura et al., 2002).

1.6.2 Adaptive immunity

The adaptive immunity can recognize a much broader range of substances and, unlike innate immunity, it displays memory of antigen encounters and specialization of effector mechanisms (Abbas et al., 2014). The adaptive immunity involves coordination between antigen-presenting cells, T-cells and B-cells lymphocytes. These cells help activate pathogen-specific immune responses, create immunologic memory and maintain the body's immune balance. Upon the first encounter with an antigen or pathogen, long-lived memory T- and B-cells are established. Once there is an encounter with the same pathogen again, these memory cells are quickly activated to produce a faster and stronger immune response (Bonilla and Oettgen, 2010).

There are two types of adaptive immune responses, namely humoral immunity and cell mediated immunity (Abbas et al., 2014). Humoral immunity is mediated by antibodies that are produced by the B lymphocytes, also known as the B-cells. The antibodies recognize microbial antigens, neutralize the infectivity of the microbes and target microbes for elimination by the use of various effector mechanisms. Humoral immunity is the main defence mechanism against extracellular microbes and their toxins because the secreted antibodies bind to the microbes and toxins and assist in their elimination. Antibodies have different effector mechanisms; for example, different types of antibodies promote the ingestion of microbes by host cells (phagocytosis), bind to and trigger the release of inflammatory mediators from cells, and are actively transported into the lumens of mucosal organs and through the placenta to provide defence against the ingested and inhaled microbes and against infections of newborns, respectively.

Cell-mediated immunity, also called cellular immunity, is mediated by T lymphocytes, also known as T-cells. Viruses and bacteria that reside inside phagocytes and other host cells

are destroyed together with the infected cells by cell-mediated immunity so that all reservoirs of infection are eliminated. B cells are the only cells that produce antibodies by differentiating into antibody-secreting plasma cells once they recognize antigens. T lymphocytes consist of various cells, namely T-helper cells and cytotoxic cells. T-helper cells secrete proteins called cytokines, which function as the 'messenger' molecules of the immune system. The cytokines stimulate proliferation, differentiation and activation of B cells, macrophages and other leukocytes. Other T-cells called regulatory T-cells, inhibit immune response and natural killer T-cells (NKT) have a role that is not well understood (Abbas et al., 2014).

Once the immune system is exposed to a foreign antigen, it has the ability to respond again to that antigen, should it come into contact with the very same antigen in future. This is called the secondary immune response or anamnestic response, which is more rapid, larger and powerful than with the first exposure. The first exposure is known as the primary immune response. During the primary immune response, naïve cells, which are lymphocytes that have not encountered a particular antigen, meaning they are immunologically inexperienced, are exposed to an antigen. This generates long-lived memory cells specific for the antigen. These memory cells are more numerous than the naïve T-cells and they have special characteristics that make them more efficient at responding to and eliminating the antigen than the naïve lymphocytes that have not previously been exposed to the antigen. For example, the memory B lymphocytes produce antibodies that bind to antigens with higher affinities than do antibodies produced in the primary immune response. Memory T-cells react more rapidly and vigorously to antigen challenge than do naïve T-cells (Abbas et al., 2014).

The major histocompatibility complex (MHC) are proteins of the host that recognize peptides that are derived from foreign proteins. MHC is divided into 2 different classes, namely MHC class I and MHC class II. Class I MHC molecules display peptides to CD8 T-cells and class II MHC molecules display peptides to CD4 T cells. Class I CD8 cells kill the infected cells, while class II CD4 cells recognize antigens that are captured and presented by dendritic cells in lymphoid organs, but their main function is to activate

macrophages and B lymphocytes (Abbas et al., 2014). IFN-y is the principal cytokine involved in the stimulation of expression of class II molecules. IFN-y may be produced by the NKT cells during an immune response (Abbas et al., 2014).

1.7 Pathogenesis

When a person can clear acute HBV infection, the HBsAg disappears within 6 months, but if the HBsAg persists for more than 6 months then it is considered a marker for chronic HBV infection (Gerlich, 2013). One of the key factors that determine whether one will be chronically infected with HBV infection is age (World Health Organisation, 2017b). Children younger than 6 years of age are at a higher risk of developing chronic HBV infection. While 80–90% of infants will develop chronic HBV if they become infected during their first year of life, only 30–50% of children who were infected with HBV before the age of 6 years will develop chronic HBV (World Health Organisation, 2017b). In adults, less than 5% of healthy persons infected with HBV will develop chronic infection, while 20–30% of adults infected chronically with HBV will develop cirrhosis and/or liver cancer (World Health Organisation, 2017b). Chronic viral hepatitis is a liver disease of variable severity that can lead to cirrhosis and HCC. Many studies suggest that up to 100% of hepatocytes may be infected with HBV without any histological or biochemical evidence of liver disease. This means that HBV may not be cytopathic in hepatocytes, but that the liver injury may be immune mediated (Guidotti et al., 1999; Thimme et al., 2003).

Persistent HBV is characterized by a weak adaptive immune response, which is responsible for the viral clearance and disease pathogenesis (Chisari et al., 2010). As mentioned previously, the humoral antibody response contributes to the clearance of circulating virus particles, while the cellular immune response eliminates the infected cells. A few of the factors that contribute to HBV persistence are immunological tolerance, mutational epitope inactivation, T-cell receptor antagonism, incomplete down regulation of viral replication, and infection of immunologically privileged tissue (Gerlich, 2013; Chisari et al., 2010). In adults, HBV infection may persist due to mutational escape, which leads to the inactivation of B and T cells. However, in infants, persistence following mother-to-child transmission (MTCT) may be due to immune

tolerance, which is induced by HBeAg, as it is able to cross the placenta and cause neonatal tolerance to HBV and also due to the fact that an effective immune response does not begin for years or decades (Chisari et al., 2010; Gerlich, 2013).

1.8 Clinical picture

HBV infection clinically presents in various ways, depending on whether it is acute or chronic. During the acute phase, the incubation period is 1–6 months, in which the majority of patients are asymptomatic. Clinically, one would find that a patient affected by acute HBV will experience symptoms of tiredness and vague abdominal discomfort. The patient may be anorexic and present with nausea and vomiting. The patient may also complain of pain in the upper right quadrant. Other acute clinical manifestations may include hepatic encephalopathy, mental confusion, gastro-intestinal bleeding, dark urine and coagulopathy. Patients with chronic HBV infection may present with hepatomegaly and splenomegaly, while patients with cirrhosis may present with jaundice, gynecomastia and testicular atrophy (World Health Organisation, 2002). Of the patients with acute HBV, 90% are able to clear the infection, meaning they are no longer infected and therefore cannot infect other individuals, 1% of the patients develop fulminant HBV (rapid liver failure, which can be life threatening) and 9% of patients have persistence for more than 6 months, which is classified as chronic HBV. Of the patients that develop chronic HBV, 50% may clear up the infection and the other 50% may fall under three chronic categories.

The first category is the asymptomatic carrier state where the patient shows no symptoms of the disease but are able to infect other individuals. The second and third categories are chronic persistent and chronic active hepatitis, which later results in extra-hepatic disease such as polyarteritis nodosum and glomerulonephritis. Chronic active hepatitis can later develop into cirrhosis, which may present with jaundice, gynecomastia and testicular atrophy, as well as hepatocellular carcinoma, which will eventually lead to death (Taylor et al., 2009; World Health Organisation, 2002). Hepatocellular carcinoma is one of the causes of death in 54–75% of patients with liver cirrhosis of various aetiologies. In viral related cirrhosis, HBV/HCV and HBV/HDV coinfections increase the HCC risk (Fattovich et al., 2004).

1.9 Laboratory diagnosis

Several laboratory tests can be performed in order to diagnose HBV infection and to distinguish whether a patient has acute or chronic HBV infection (World Health Organisation, 2017b). The laboratory tests that serve as serological markers for HBV are HBsAg, hepatitis B surface antibodies (anti-HBs or HBsAb), total hepatitis B core antibodies (anti-HBc or HBcAb), hepatitis B core IgM antibodies (anti-HBc IgM), hepatitis B e antigen (HBeAg), and hepatitis B e antibodies (anti-HBe or HBeAb). HBsAg is a marker that is present in acute or chronic infection and the presence of HBsAg therefore indicates that the person is currently HBV infected and is able to infect other individuals (Porstmann and Kiessig, 1992). HBsAg may not be present early in the infection and it may be undetectable later on in the infection due to host clearing the infection (Aba and Aminu, 2016).

In most cases, the presence of HBsAg indicates that the individual is a carrier of HBV; therefore, HBsAg testing is used as a screening assay for HBV infection. It has been recommended that this test be considered as one of the prenatal screening tests, as babies born to mothers who are HBsAg-positive need to be given HBV vaccine, together with hepatitis B-immune globulin (HBIG) immediately at birth (Porstmann and Kiessig, 1992). Without immediate intervention, a mother who is positive for HBsAg has a 20% chance of infecting her baby with HBV (Porstmann and Kiessig, 1992). After clearing of the infection, HBsAg is followed by IgG antibodies called anti-HBs. HBsAg-negative, but anti-HBs-positive results is an indication of immunity to HBV. Either the infection has been cleared, or there has been previous vaccination. Anti-HBs is the test done to assess whether HBV vaccine is effective and has elicited protective immunity. An effective vaccine response is indicated by an HBsAb titre of ≥ 10 IU/ml. HBcAg is an inner ‘core particle’ which encloses the viral genome of an infectious virion. HBcAg may be cleared by IgM antibodies to HBcAg known as anti-HBc IgM. Anti-HBc is a marker that indicates current or previous infection with HBV. It does not develop after immunization with HBV vaccine; however, it does develop after exposure to HBV and it may persist for many years. Anti-HBc IgM indicates likely acute infection as this marker usually becomes negative within six months after infection. Anti-HBc IgM, together with HBsAg, should be

tested if an acute infection is suspected (Porstmann and Kiessig, 1992). HBeAg is a marker that indicates high infectivity and high levels of replication. HBeAg will only be present in a person who is HBsAg positive. A mother who is HBeAg and HBsAg positive has a 70–90% chance of infecting her baby with HBV and if the infant receives postnatal immunoprophylaxis, the chance of transmission decreases to 5–15%. If the mother is HBeAg negative, the transmission chances are even lower at 10% (Beasley et al., 1983; Mast et al., 2005; Stevens et al., 1987; Xu et al., 1985). Anti-HBe is a marker for decreased replication and possible resolution. This means that an individual who is HBeAg positive has developed immunity against the antigen. If, however, the individual remains HBsAg positive and HBeAb positive, then this simply means that the individual is still infectious to others but to a much lesser degree than when they were HBeAg positive (Gu et al., 2020). Table 1 below illustrates HBV serologic tests and their interpretation.

Table 1: serological tests and their interpretation

TESTS	**RESULTS**	**INTERPRETATION**
HBsAg Anti-HBc Anti-HBs	Negative Negative Negative	Susceptible
HBsAg Anti-HBc Anti-HBs	Negative Positive Positive	Immune due to natural infection
HBsAg Anti-HBc Anti-HBs	Negative Negative Positive	Immune due to hepatitis B vaccination
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Positive Negative	Acutely infected
HBsAg Anti-HBc IgM anti-HBc	Positive Positive Negative	Chronically infected

Anti-HBs	Negative	
HBsAg Anti-HBc Anti-HBs	Negative Positive Negative	Interpretation unclear; four possibilities: Resolved infection where HBsAb has waned (most common) False positive anti-HBc, thus susceptible “low level” chronic infection (occult HBV infection) Resolving acute infection (where HBsAb is increasing but not yet detectable)

All these tests are done using immunoassays such as an enzyme-linked immunosorbent assay (ELISA) technique, which combines the specificity of antibodies with the sensitivity of simple enzyme assays by using an antibody or antigen coupled to an enzyme (Porstmann and Kiessig, 1992). ELISAs are used to measure the antibody or antigen concentration. More recent techniques used are the chemiluminescent immunoassay (CLIA) and the chemiluminescent enzyme-immune assay (CLEIA). The CLEIA technique is an indirect luminescence measurement that uses an enzyme-labelled antigen or antibody and enzyme-catalysing chemiluminescent reagents to generate light. CLIA is a much simpler method that measures luminescence by using reagents to directly label the antigen or antibody, without the involvement of an enzyme (Zhang and Qi, 2011).

HBV DNA is a marker that indicates active HBV replication. HBV DNA is at its peak during the acute phase of hepatitis and it gradually decreases and disappears when the infection resolves (Porstmann and Kiessig, 1992). HBV DNA is normally tested quantitatively to check whether patients are eligible for HBV treatment or not. The HBV DNA level is used in conjunction with other markers such as alanine aminotransaminase (ALT) to determine the need for treatment and also to monitor treatment response for patients on therapy (SA NDOH, 2019). The detection of HBV DNA can be done by using different molecular methods. The most widely used method is polymerase chain reaction (PCR) (Kaneko et al., 1989). Other methods used are the hepatitis B bDNA signal amplification assay (Urdea et al.,1991), the HBV DNA hybridization assay (Kuhns, 1988), and the HBV DNA direct-membrane hybridization method (Fujiyama et al., 1983). In order to monitor

whether successful treatment was achieved, HBV DNA quantification, together with repeated ALT determinations and HBeAg/HBeAb antibody assessment in HBeAg-positive patients should be conducted (Dienstag et al., 1995; Dienstag, 1999; Lai et al., 1987; Lok et al., 1988; Perrillo et al., 1990; Lai et al., 1987)

1.0 Prevention

1.10.1 HBV vaccination

Safe and effective plasma-derived vaccines against HBV have been available since 1982; however, recombinant vaccines became available in 1986 (World Health Organisation, 2017a). This vaccine is made synthetically, which means that the vaccine does not contain any blood products. The most common type of recombinant hepatitis vaccine used contains small hepatitis B surface antigens (SHBs). It is indicated for prevention of infection caused by all known subtypes of HBV and is approved for use in individuals of all ages (Lacson et al., 2005; World Health Organisation, 2017a)

HBV vaccine is available as a single antigen formulation and also in combination with other vaccines. The most commonly used vaccines are Recombivax HB, Engerix-B (which are monovalent HBV vaccines) and Twinrix (which is a combination vaccine with hepatitis A) (Nelson et al., 2016; Shefer et al., 2011). A study conducted in Korea confirmed HBV vaccine to be highly immunogenic, resulting in a seroprotection rate of 98% (Kane, 1998).

1.10.1.1 Childhood vaccination

Childhood vaccination programmes can significantly decrease the prevalence of HBV infection. The National Children Immunization Program of 2002 in China has reduced HBV prevalence among 1 to 4-year-old children from 9.7% in 1992 to 1% in 2006. However, the population of 1–59 years still has a high prevalence at 7.2% (Liang et al., 2013). All infants should receive their first dose of hepatitis B vaccine immediately after birth or within 24 hours, followed by two or three additional doses (World Health Organisation, 2010).

1.10.1.2 Adult immunization

Primary HBV vaccination of adults usually consists of three doses of 10 ug or 20 ug of recombinant HBsAg protein, administered intramuscularly into the deltoid muscle at 0, 1 and 6 months (Mast et al., 2006; Shefer et al., 2011). Acceleration schedules are administered in special situations such as travelling on short notice to places where the individual may be exposed to HBV, or for emergency responders to disaster areas. For such cases, the vaccine is administered at 0, 7 and 21–30 days, with a booster at 12 months and a combination vaccine such as Twinrix can be administered to provide concurrent immunity to hepatitis A virus (Centers for Disease Control and Prevention, 2013).

1.10.1.3 Vaccination side effects

The most common side effects reported with HBV vaccination are pain at the injection site and a rise in temperature (Mast et al., 2006). Persons with moderate to severe acute illness with or without fever should not be vaccinated at the time of illness until it resolves. The vaccine is not harmful in pregnant or lactating woman, because the vaccine contains non-infectious HBsAg. Therefore, it poses no risk to the foetus or infant and is also safe to administer to immunocompromised individuals (Alter et al., 2001; Ayoola and Johnson, 1987; Levy and Koren, 1991; Mast et al., 2006,).

1.10.1.4 Vaccination among HCWs

Despite the South African National Department of Health (SA NDoH) recommending all HCWs to be vaccinated against HBV before being exposed to patients, there are only 30.6 –52.4% of SA HCWs seroprotected, i.e. with HBsAb ≥10 IU/ml (Mosendane et al., 2012; Vardas et al., 2002). It is therefore recommended that HCWs undergo quantitative HBsAb testing 1–2 months after the last dose has been administered to confirm adequate immunity. HCWs with HBsAb titres of less than 10 mIU/ml should get an additional 3 doses; therefore 6 doses in total, whereafter they need to be tested 1–2 months after their last dose has been administered to confirm adequate immunity (Schillie et al., 2013).

1.10.1.5 Non-responders to vaccination

International guidelines have defined a non-responder as a person who does not develop protective surface antibodies after completing two full series of HBV vaccine and for whom an acute or chronic HBV infection has been ruled out (Centers for Disease Control and Prevention, 2011). Several factors are associated with the lack of development of a protective response, namely the male gender, vaccination at >40 years of age, a high Body Mass Index (BMI), tobacco smoke, drug use, and immunosuppression (Alimonos et al., 1998; Clemens et al., 1997; Filippelli et al., 2014; Kamath et al., 2014; Yang et al., 2016; Zuckerman, 2006;). Immunosuppression can be associated with chronic diseases such as chronic renal failure, alcoholism, type 1 diabetes mellitus (DM), HIV, or cancer (Chen, 2009).

Recommendations for the management of non-responders, especially those who are HCWs who handle infectious samples such as blood and other body fluids, are to have 3 additional doses at 0, 1 and 6 months, to double the dosage, to use a vaccine with a high antigen content, to have intradermal administration of the vaccine, or to have the vaccine administered orally and/or nasally, if available (Filippelli et al., 2014; Kamath et al., 2014; Walayat et al., 2015; Zuckerman, 2006). New vaccines have been explored such as a triple-action antigen vaccine, adjuvants using granulocyte-macrophage colony-stimulating factors and antigen-pulsed blood dendritic cells (Akbar et al., 2007; Cruciani et al., 2007; Jha et al., 2001; Zuckerman et al., 2001). In a meta-analysis published in 2015, there is a comparison of various additional dosages, which were 20 ug intramuscular (IM-20), 40 ug intramuscular (IM-40), 5 ug intradermal (ID-5), and 20 ug intradermal (ID-20). It was found that approximately 50% seroconversion was achieved with IM-20, 90% for ID-20, 53% for IM-40, and 85% for ID-5 (David et al., 2015). We can therefore conclude from the different doses that ID-20 has the highest percentage of seroconversion and could be recommended. A 2008 study asserts that three doses of low dose intradermal vaccine, followed by intramuscular boosters to non-responders, is immunogenic and cost effective (Sangfelt et al., 2008).

1.10.1.6 HBIG administration

HBIG is prepared from human plasma and it is known to contain a high titre of HBsAb. HBIG provides temporary protection for 3–6 months (Mast et al., 2006). The chief indications for administering HBIG are when there is a single acute percutaneous or mucocutaneous exposure to HBV; unprotected sexual exposure; mother-to-infant transmission; prevention of reinfection after liver transplantation; non-responders to HBV vaccine; and immunosuppressed patients (Zuckerman, 2007). Contraindications to HBIG occur in patients who suffer from anaphylactic or severe system reaction and in patients who have an IgA deficiency.

1.10.1.7 Post-vaccination waning of immunity

The HBsAb antibodies decline with time, but usually persist for at least 10–15 years after vaccination (Fujisawa et al., 1996; Kao and Chen, 2005). An anamnestic response (a rapidly high level of antibody production) is noted when booster vaccines are administered and this simply means that the individuals remain protected by memory cells since they recognize the HBsAg from previous immunizations (World Health Organisation, 2017a).

Many studies have been conducted globally that have tried to establish how much time it will take for immunity to wane in individuals who have been vaccinated. Some studies conclude 10 years, others 15 years, and yet others 20 years before waning occurs (Lu et al., 2008). Recommendations were made that there should be a birth dose of HBV vaccine given, since anti-HBs geometric mean titres have shown to be much higher in individuals who received a HBV vaccine birth dose (Dhillon, 2010). Some authors recommend that a targeted HBV vaccination approach should be administered at 12 years of age if the individual's road to health cards show that they were not fully vaccinated as infants (Burnett et al., 2012). Another study that aimed to evaluate the long-term cell-mediated immune memory response to booster vaccination in vaccine recipients 20 years after neonatal HBV immunization showed that 42.6% of the participants were still sero-protected (HBsAb titre≥10 IU/L), and of the remaining participants, 83% and 81% showed an increase in the mean concentration level of IL2 and IFN, respectively. This

indicates that cell-mediated immune memory was present in these patients (Saffar et al., 2014).

1.11 Problem statement

With such a high percentage of study participants showing a lack of protection prior to boosting, it raises concerns regarding the medical students that have joined, or will still be joining the medical professionals at the hospitals, clinics and other medical institutions in South Africa. This brings us to the aim of this study, which is to describe long-term humoral and cellular immunity in South African students who received childhood HBV vaccination, more especially the medical and laboratory students who are to undergo medical training at the medical institutions. The objectives of this study is to investigate humoral immunity in young adults following childhood vaccination and to investigate memory cellular immunity in young adults following childhood vaccination.

CHAPTER 2: METHODOLOGY

2.1 Study location

This study was conducted in Bloemfontein at the Division of Virology, National Health Laboratory Service (NHLS)/University of the Free State (UFS) as well as the Faculty of Health and Environmental Sciences at the Central University of Technology, Free State (CUT).

2.2 Study design

This study was a prospective longitudinal study, as samples were collected at three timepoints. Samples and data were acquired from the main study (HSREC 79/2017; UFS-HSD2017/0788) and stored for later testing for this study (UFS-HSD2018/0783/3107).

2.3 Study population:

Subjects were recruited from approximately 160 first-year medical students from the Faculty of Health Sciences, UFS and approximately 160 first- and second-year Biomedical Technology students from the Faculty of Health and Environmental Sciences, CUT.

2.4 Inclusion and exclusion criteria:

Certain criteria were put in place to ensure appropriate participant recruitment.

2.4.1 Inclusion criteria

As childhood HBV vaccination was included in the South African EPI programme from 1995 and the aim of the study was to describe and investigate long-term humoral and cellular immunity in young adults following childhood vaccination, students aged between 18 and 23 years of age were included in the study (i.e. adults born during or after 1995).

Only students that were MBChB first-year students from the UFS as well as first- and second-year Biomedical Technology students from the CUT took part in the study, as these students had not yet received booster doses of HBV vaccine.

2.4.2 Exclusion criteria

Students that were vaccinated for HBV at any time following their childhood vaccination before their first year at the UFS and the CUT were excluded from the study because their inclusion would not show a true reflection of the responses obtained during childhood vaccinations.

Students that were not South African citizens were also excluded because the administration of HBV vaccination as part of EPI programmes was initiated at different time intervals globally.

2.5 Study layout

The UFS students were notified about the study in the form of a written letter in 2017 during the time of selection for the MBChB programme. The letter explained the purpose and procedure of the study, for which recruitment took place during 2018. It is a requirement for UFS students to get vaccinated against HBV; however, for the purpose of this study, the letter clearly stated that students should not undergo vaccination before starting their first year if they agreed to take part in the study, because the results obtained from the study would be affected. Since CUT students are routinely provided with HBV immunization during their first year, it was not necessary to inform them before registration. At the specific time during which the study was performed, the second-year Biomedical technology students were not immunized during their first year due to a global shortage of HBV vaccine and it was therefore possible to include them in study recruitment.

Orientation sessions were held at both institutions in 2018, where information regarding the study was presented to the students. Students were divided into groups where further explanation about the study and signing of consent forms was conducted. It took

approximately 7 months to observe the response to the complete booster vaccination schedule. The first samples were obtained from February to March 2018. The second samples were obtained a month after the initial sampling and the third samples, 7 months after the initial blood draw. Students were also immunized at the first and second time point, with the last vaccination administered a month before the last blood draw (6 months after the initial vaccination) to adhere to a standard 0-, 1- and 6-month vaccination schedule. Information collected at the first time point included date of birth, age, gender, race, vaccination and clinical history. However, if this information was not available, it did not exclude the participant from the study. Where possible, the information included the provision of records by the participant, showing that he/she had received childhood immunizations against HBV, as well as the number of doses received.

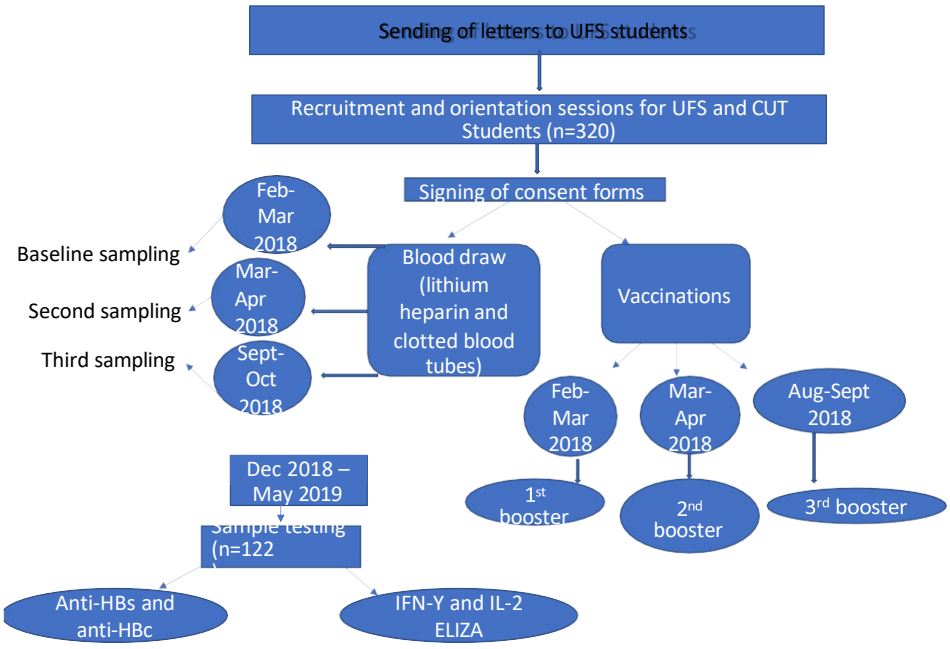


Figure 1: Illustration of study layout

2.6 Sample collection

2.6.1 Baseline

The baseline sample draw and first dose of immunizations were done at both institutions. The samples were drawn before vaccination so that the baseline HBsAb titre results

reflect those obtained from childhood vaccination. Two blood samples were drawn, one without an anticoagulant (clotted tube) for serological tests and one lithium-heparinized tube to perform the interferon gamma (IFN-y) and interleukin-2 (IL2) release assays.

2.6.2 Collection after one month

The second samples were collected a month after baseline sampling. One tube without anticoagulant (clotted tube) was taken at this time point for serological testing. The second shot of HBV vaccine was administered at this time point.

2.6.3 Collection after seven months

Clotted blood samples were obtained again at the seven-month collection. Samples were collected in the same manner as previous collections; however, no vaccination was done at the 7-month interval. Vaccination was done at 6 months after baseline vaccination (a month before the 7-month blood draw), as it is recommended that immunity following HBV vaccination is tested approximately 1 month following the third dose.

2.7 Sample processing and storage

The clotted samples from each time point were centrifuged at 3 000 rpm for 10 minutes whereafter the serum was aliquoted and stored at -80 °C for later testing. From the lithium samples drawn , 1 ml of whole blood was added to each of three tubes. One tube served as a positive control containing Staphylococcal enterotoxin B (SEB) at a final concentration of 1 µg/ml (Sigma-Aldrich, USA). SEB is a bacterial superantigen, derived from the *Staphylococcus aureus*, which can bind MHC class II molecules on antigen-presenting cells and T-cell receptors in order to stimulate cytokine production. The second tube served as a negative control containing phosphate buffered saline and the third tube contained the HBV peptides (PEPMix HBV), which served as HBV antigens. The PEPMix HBV (JPT Peptide Technologies, Berlin, Germany) includes 98 peptides that are 15 mers, with an 11 mer overlap covering the large envelope protein (s antigen) of HBV. The PEPMix HBV includes approximately 15 nmol/peptide used at a final concentration of 5 µg/ml. The three tubes were incubated at 37 °C for 24 hours followed by centrifugation

at 14 000 rpm for 1 minute. The supernatant was stored at -80 °C until further testing was conducted.

2.8 Laboratory analysis:

All laboratory work was conducted following the final specimen collection. HBsAb and HBcAb tests were conducted on the Liaison® XL Murex instrument. The IFN-y and IL-2 were manual ELISA tests that were read on the Biotek-ELx800.

2.8.1 Anti-HBs

Anti-HBs (HBsAb) is a test that is done to check whether participants have protective antibody levels prior to and after boosting with the HBV vaccine. The specimens for this test were collected at three different time intervals; therefore, three sets of testing were done on each participant.

2.8.1.1 Anti-HBs principle

Anti-HBs were analysed on the Liaison® Anti-HBs II assay (DiaSorin, Saluggia, Italy) for quantitative determination of anti-HBS based on the direct sandwich chemiluminescence immunoassay (CLIA). During the solid phase, magnetic particles are coated using recombinant HBsAg. The HBsAg also forms an isoluminol-HBsAg conjugate. Anti-HBs found in calibrators, samples or controls form a sandwich by binding to the solid phase as well as the isoluminol-HBsAg conjugate during incubation. After incubation, a wash cycle process occurs where unbound particles are removed. After all reagents in the reagent integral have been added, a chemiluminescence reaction is induced. The anti-HBs concentration is measured in the form of relative light units (RLU) using a photomultiplier. The photomultiplier does this by measuring the light signal and the amount of isoluminol HBsAg conjugate.

2.8.1.2 Reagent and materials

2.8.1.2.1 Reagent integrals

Consumables used on the Liason®XL Murex instrument were cuvettes, disposable tips, starter kit (reagent integrals), wash/system liquid and waste bags. The reagents required were in the form of reagent integrals. Each integral includes 2.5 ml of magnetic particles that are coated with HBsAg obtained in mammalian cells, BSA, PBS buffer and <0.1% sodium azide; 2.3 ml of calibrator 1 that consists of human serum/plasma that has low levels of anti-HBs, foetal calf serum, EDTA, 0.2% Proclin®300 and preservatives; and 2 ml of calibrator 2, which contains the same contents as calibrator 1. However, calibrator 2 has an inert blue dye, 20 ml of conjugate that consists of heat-treated human HBsAg conjugated to an isoluminol derivative, BSA, PBS buffer, EDTA, 0.2% Proclin®300, preservatives and an inert red dye, and a specimen diluent that consists of human serum/plasma, EDTA, 0.2% Proclin®300, preservatives and an inert blue dye. Each reagent integral is sufficient for 200 tests.

2.8.1.2.2 Reagent loading

Before loading reagents on the Liason®XL, the following was taken into account: the magnetic particles must be fully resuspended and this can be done by rotating the small wheel at the magnetic vial. Fortunately, some Liaison®XL instruments are able to resuspend magnetic particles; no formation of bubbles should exist in vial two and three following resuspension of magnetic particles. If bubbles were present then the integrals were left unused on the instrument until the bubbles disappeared. Once the integral was ready for use, it was inserted into the instrument with the label facing left. Reagents were left to stand for 15 minutes before use.

2.8.1.2.3 Stability

The anti-HBs reagent integral has to be kept away from light and is stable at 2–8 °C for 12 weeks once opened and until the expiry date if it is sealed. The integral should be stored in an upright position in order to facilitate the proper resuspension of magnetic particles.

2.8.1.3 Test procedures

Anti-HBs were tested on the samples that were collected over the seven-month period using the instrument, Liason® XL. The aliquoted samples that were frozen at -80 °C after collection and centrifugation were thawed. Once samples reached room temperature, they were thoroughly mixed. The aliquoted serum was transferred to a secondary tube (cuvette) and placed in the instrument racks. Clots and air bubbles were checked and removed if present before sample testing. 300 uL of serum were pipetted into the cuvette of which 150 uL were analysed by the instrument and 150 uL were the dead volume.

2.8.1.4 Controls and calibrations

Calibrations are done after every 8 weeks or when there was a new lot for the starter kit/integral to be used. Calibrators were also run when the instrument needed to be serviced or when the control values lay outside the expected range. Negative and positive controls were used to check the reliability of the assay. The negative control contained human serum/plasma without antibodies, TRIS buffer, and 0.2% Proclin®300 preservatives, while the positive control contained human serum/plasma with antibodies, TRIS buffer, and 0.2 % Proclin®300 preservatives. After controls had been run, they were stored at 2–8 °C for up to 12 weeks as per the manufacturer's instructions.

2.8.2 Anti-HBc

This specific test was done to see whether the participants have acquired a past or present HBV infection. As the HBV vaccine only contains HBsAg, the presence of anti-HBc is used to differentiate natural infection from vaccination, as this marker will not be present following vaccination alone.

2.8.2.1 Anti-HBc principle

Anti-HBc (HBcAb) determination was done qualitatively using the Liaison® Anti-HBc assay (DiaSorin, Saluggia, Italy). Anti-HBc determination took place by means of a two-step competitive chemiluminescence immunoassay (CLIA). For the solid phase,

recombinant HBcAg coats the magnetic particles and the antibodies to HBcAg (mouse monoclonal) are linked to isoluminol to form an isoluminol-antibody conjugate. There are two incubation periods for anti-HBc determination. During the first incubation, binding of the anti-HBc present in calibrators, samples or controls to a fixed amount of recombinant HBcAg that is bound to the solid phase takes place. In the second incubation period, the antibody conjugate links to the free recombinant HBcAg epitopes. The wash cycle then removes the unbound particles after incubation has occurred. Reagents from the integrals are then added to induce a chemiluminescence reaction. The photomultiplier then measures the amount of isoluminol-antibody conjugate as relative light units (RLU) that are inversely proportional to the anti-HBc concentration found in calibrators, samples and controls.

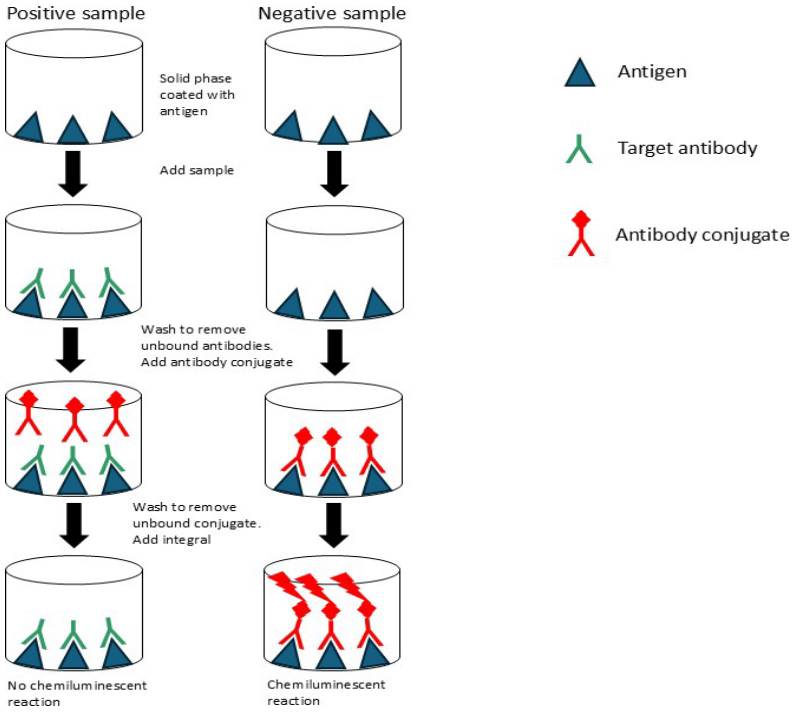


Figure 2: Basic principle of competitive ELISA technique for antibody detection

2.8.2.2 Reagents and materials

2.8.2.2.1 Reagent integrals

A reagent integral for anti-HBc contains 2.3 ml of magnetic particles that are coated with HBcAg obtained in E.coli by recombinant DNA technology, BSA, phosphate buffer, and

0.1% sodium azide; 1.4 ml of calibrator consisting of calf serum, which has high anti-HBc antibody levels, 0.2% Proclin®300, preservatives and an inert blue dye; 11 ml of buffer F, which is acetate buffer; and lastly, 23 ml of conjugate to an isoluminol derivative, human serum/plasma, newborn calf serum, phosphate buffer, EDTA, 0.2% Proclin®300, preservatives and an inert blue dye. Each integral was able to conduct 100 tests.

2.8.2.2.2 Reagent loading

Before loading reagents on the Liason®XL, the following was taken into account: the magnetic particles must be fully resuspended and this can be done by rotating the small wheel at the magnetic vial. Fortunately, some Liaison®XL instruments are able to resuspend magnetic particles; no formation of bubbles should exist in vial two and three following resuspension of magnetic particles. If bubbles were present then the integrals were left unused on the instrument until the bubbles disappeared. Once the integral was ready for use, it was inserted in the instrument with the label facing left. Reagents were left to stand for 15 minutes before use.

2.8.2.2.3 Stability

The anti-HBc reagent integral has to be kept away from light and is stable at 2–8 °C for 12 weeks once opened and until the expiry date if it is sealed. The integral should be stored in an upright position in order to facilitate later, the proper resuspension of magnetic particles.

2.8.2.2.4 Test procedure

Anti-HBc were tested on samples that were collected over the seven-month period using the instrument, Liason®XL. The aliquoted samples that were frozen at -80 °C after collection and centrifugation were thawed. Once samples reached room temperature, they were thoroughly mixed before tested. The aliquoted serum was transferred to a secondary tube (cuvettes) and placed in the instrument racks. Clots and air bubbles were checked and removed if present before sample testing. A total of 260 uL of serum sample was required for testing, of which 110 uL was used for analysis and 150 uL was the dead volume.

2.8.2.2.5 Controls and calibrators

Calibrations were done after every 8 weeks, or when there was a new lot for the starter kit/integral to be used. Calibrations were also done when the instrument needed to be serviced or when the control values lay outside the expected range. Negative and positive controls were used to check the reliability of the assay. The positive control contained human plasma/serum containing anti-HBc antibodies (human), 0.2% Proclin®300 and preservatives. The negative control contained human plasma/serum without anti-HBc antibodies (human), 0.2% Proclin®300 and preservatives.

2.8.3 Maintenance of Liason®XL analyser

Maintenance was only done when the instrument notified that maintenance was due. The instrument also guided the user on how the procedure was to be conducted. The instrument should be switched off and disconnected from the main supply before performing cleaning procedures. Once all maintenance procedures were complete, the instrument was switched on. Once the instrument prompted the user for maintenance, the following was done: a light check, which is required when running a system test weekly, and monthly probe cleaning by preparing 1 litre of 1% liquid-nax® solution beforehand. The left and right pipette probes were cleaned with the above-mentioned solution, then with distilled water, and lastly with dry tissue to remove excess fluid. Monthly analyser interior cleaning involves the wiping of each sample rack, sample module surfaces, ancillary plate and starter reagent area, disposal of liquids from buffer tanks, primary and intermediate tanks. The buffer tanks should be filled with wash solutions, while the primary and intermediate tanks should be with DI water; followed by monthly cleaning of instrument covers, flaps, monitors and extensible board with tissue saturated with an alcoholic/disinfectant solution; and lastly, automatic overnight back-up and clean-up of archive results by the instrument itself.

2.8.4 Safety precautions

All laboratory safety rules were applied when running samples. 0.5% sodium hypochlorite solution was used in the event of spillages. All waste was disposed of in compliance with the laboratory guidelines and the statutory provisions.

2.8.5 IFN-y and IL-2 release assays

For this study, two manual ELISA tests were used, the interferon gamma release assay (IGRA) and the interleukin 2 (IL-2) release assay. Both these assays are specialized tests that are performed in the laboratory to detect memory T-cell responses by stimulating whole blood with HBV antigens in order to release IFN-Y and/or IL-2, which is detected by means of an ELISA. A positive result indicates the presence of memory T-cells which can recognize the HBV antigen, meaning the individual has been exposed to HBV vaccination or previous infection. IFN-y plays a role in controlling HBV infection by firstly, recruiting and activating T-cells; secondly, it can induce T-cells towards developing antiviral effector functions for effective control of HBV infection; thirdly, it can regulate MHC expression on infected liver cells and then promote antigen processing and presentation; and lastly, it can perform direct antiviral functions. The IL-2, which is also an antiviral cytokine, regulates more the cellular immunity during HBV infection.

The lithium heparin blood samples were aliquoted into three tubes (1 ml) each to test for IFN-y and IL-2 as described above, in order to identify a memory T-cell response. The IFN-y and IL-2 release assays are fast tests that determine whether patients have memory T-cells that respond to HBV antigens. If positive, it means the patient has been exposed to HBV before and that the memory T-cells are able to recognize the HBV antigens. The heparinized blood was stimulated by the HBV antigens in the form of peptides. Once the T-cells in the whole blood recognize the HBV antigens, a release of IFN-y and/or IL-2 will result and this can be detected by means of an ELISA. A positive result indicates the presence of memory T-cells that were stimulated during primary vaccination or infection.

2.8.5.1 Principle

ELISA is a method of target antigen (or antibody) capture in samples using a specific antibody (or antigen) followed by target molecule detection and quantitation using an enzyme reaction with its substrate. In ELISA, various antigen-antibody combinations are used, including an enzyme-labelled antigen or antibody with enzyme activity, which is measured colorimetrically. The enzyme activity is measured using an enzyme substrate that changes colour when modified by the enzyme. Light absorption of the product formed after substrate addition is measured and converted to a numeric value.

2.8.5.2 Reagents and materials

2.8.5.2.1 IFN-Y

The Human IFN-γ ELISA MAX™ Deluxe kit (BioLegend, San Diego, USA) was used to quantify IFN-γ at each time point. Reagents used were human IFN-y ELISA MAX™ capture antibody (200X), human IFN-y ELISA MAX™ detection antibody (200X), human IFN-y, human IFN-y standard, Avidin-HRP (1000X), Substrate solution A, Substrate solution B, coating buffer (5X), Assay diluent (5X) and Nunc™ MaxiSorp™ ELISA plates that are uncoated. Additional materials used were phosphate-buffered saline (PBS), wash buffer, stop solution and plate sealers. No sensitivity and specificity information was provided by manufacturer

2.8.5.2.1 IL-2

The Human IL-2 ELISA MAX™ Deluxe kit (BioLegend, San Diego, USA) was used to quantify IL-2 at each time point. Reagents used were human IL-2 ELISA MAX™ capture antibody (200X), human IL-2 ELISA MAX™ detection antibody (200X), human IL-2, human IL-2 standard, Avidin-HRP (1000X), Substrate solution A, Substrate solution B, coating buffer (5X), Assay diluent (5X), and Nunc™ MaxiSorp™ ELISA plates that are uncoated, phosphate-buffered saline (PBS), wash buffer, stop solution and plate sealers. No sensitivity and specificity information was provided by manufacturer.

2.8.5.3 Reagent preparation

The following reagents were prepared per one plate: 2.4 ml of coating buffer A (5X) were

diluted in 9.6 ml of distilled (DI) water, 60 ul of capture antibody (200X) in 12 ml of 1X coating buffer A, 12 ml of assay diluent A (5X) in 48 ml of PBS, 60 ul of detection antibody (200X) in 12 ml of 1X assay diluent A and 12 ul of Avidin-HRP in 12 ml of 1X assay diluent A.

2.8.5.3.1 IFN-y

For the standard reconstitution, the lyophilized human IFN-y standard was reconstituted by adding 0.2 ml of 1X assay diluent A to make 45 ng/ml standard stock solution. The reconstituted standard was allowed to sit for 10–15 minutes at room temperature thereafter briefly vortexed to mix completely. 1 000 ul of top standard at 500 pg/ml were prepared by adding 11.1 ul of reconstituted stock standard solution to 988.9 ul of 1X assay diluent A.

2.8.5.3.2 IL-2

For the standard reconstitution, the lyophilized human IL-2 standard was reconstituted by adding 0.2 ml of 1X assay diluent A to make 150 ng/ml standard stock solution. The reconstituted standard was allowed to sit for 10–15 minutes at room temperature and thereafter briefly vortexed to mix completely. 1 000 ul of top standard at 500 pg/ml were prepared by adding 3.3 ul of reconstituted stock standard solution to 996.7 ul of 1X assay diluent A.

For each analyte, six two-fold serial dilutions of 500 pg/ml top standard with 1X assay diluent A were performed in eight separate tubes. The first tube contained the 500 pg/ml of the above-mentioned mixture. The second tube contained 250 pg/ml, the third, 125 pg/ml, the fourth, 62.5 pg/ml, the fifth, 31.25 pg/ml, the sixth, 15.625 pg/ml, the seventh, 7.8125 pg/ml, and the eighth was 1x assay diluent A, which served as the zero standard blank (0 pg/ml).

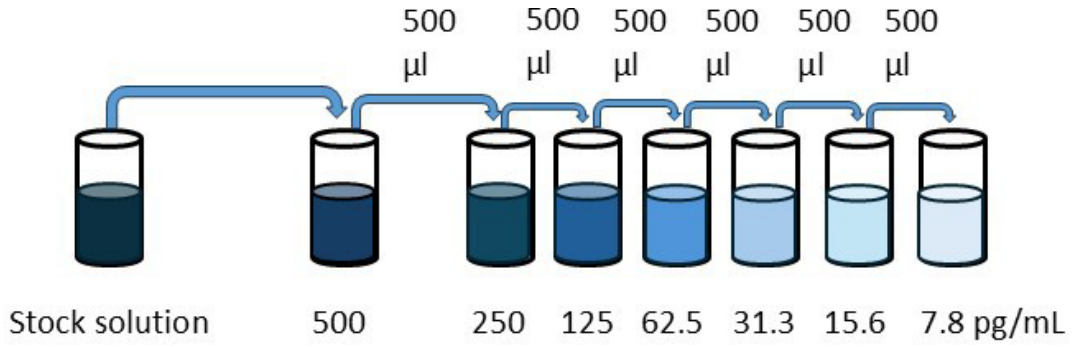


Figure 3: IL-2 and IFN-y standard preparation using serial dilutions.

For TMB substrate solution, a mixture of equal volumes of substrate solution A (5.5 ml) and substrate B (5.5 ml) was done in a clean container.

2.8.5.4 ELISA procedure for IFN-y and IL-2

The ELISA procedure takes two days to complete each run. On day one, 100 ul of diluted capture antibody solution are added to each well of the 96-well plate in order to coat the plate. The plate is then sealed and incubated overnight at 2–8 °C. A wash buffer solution of PBS with 0.05% Tween-20 was also prepared to be used during day two.

On day two, heparinized samples which were stored at -80 °C were then thawed out, the plate was washed four times using an automated washer that was set to dispense 300 µl per well for each wash cycle and residual wash buffer was removed by tapping the plate firmly onto paper towel. All subsequent wash steps were performed in the same manner. Thereafter, the plate was blocked by adding 200 ul of 1X assay diluent to each well. The plate was then sealed and incubated at room temperature for one hour on a shaker at approximately 500 rpm and a circular orbit of 0.3 cm. After one hour the plate was then washed four times, as previously, then 100 ul of standard dilutions and samples were added to the appropriate wells. The plate was then incubated for two hours with shaking. Below is an illustration of a 96 well plate used for IL-2 and IFN-y testing.

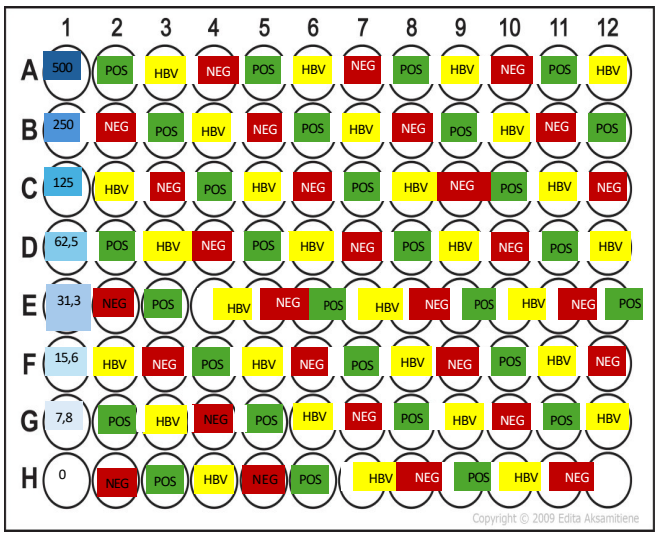


Figure 4: 96 well plate for IFN-y and IL-2 testing.

Well A1-G1 represents the standard dilutions used with H1 being the blank, with Well A2-G12 having positive control, negative control and the target analyte (IFN-y or IL-2), respectively. Well H12 is an unused well as samples a run in triplquets of three therefor leaving the last well unused.

After two hours of incubation, the plate was again washed four times and 100 ul of diluted detection antibody solution were added to each well. The plate was then sealed and incubated at room temperature for one hour with shaking. After an hour, the plate was then washed four times and 100 ul of diluted Avidin-HRP solution were added to each well. The plate was then sealed and incubated at room temperature for 30 minutes with shaking. After incubation, the plate was washed five times with soaking for 30 seconds to one minute per wash. After the final wash, 100 ul of freshly mixed TMB substrate solution were added to each well and then incubated in the dark for 20 minutes for IFN-y testing and 30 minutes for IL-2 testing. Once the respective incubations had passed, 100 ul of stop solution (2N H_sSO_4) were added to each well. The absorbance was then read at 450 nm and 570 nm on the Biotek ELx800 plate reader, within 15 minutes of adding the stop solution. The results obtained from the absorbance at 570 nm were subtracted from the results at 450 nm.

2.8.5.5 Standards

The ELISA plate used for each investigation included standard dilutions prepared as described above and added to the first row of the ELISA plate . At the conclusion of each test, the optical density of the standard solutions was read along with the samples. As previously noted, dilutions were carried out in eight tubes and these standards were used to construct standard curves and to interpolate corresponding concentrations for each sample absorbance reading using GraphPad Prism version 7.04 with a 4-parameter logistics curve-fitting algorithm (GraphPad Software, San Diego, United States). Each plate included its own standard dilutions, which led to a unique graph. The outcome of the standard curve was determined by the absorbance values on the *y* axis and the concentration values on the *x* axis. Once the standard curve has been plotted, it is then easier to determine the concentration of the sample, the negative and positive controls. This can be done by drawing a horizontal line from the point of absorbance to the stand-curve point of intersection. At this point, a vertical line can be drawn to determine the concentration.

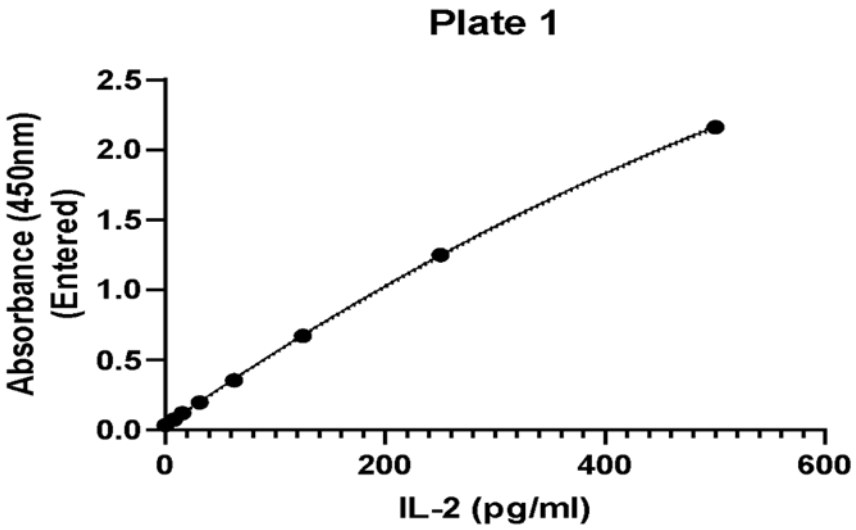


Figure 5: IL-2 standard curve: an example of the standard curve calculated for the IL-2 assay using GraphPad Prism

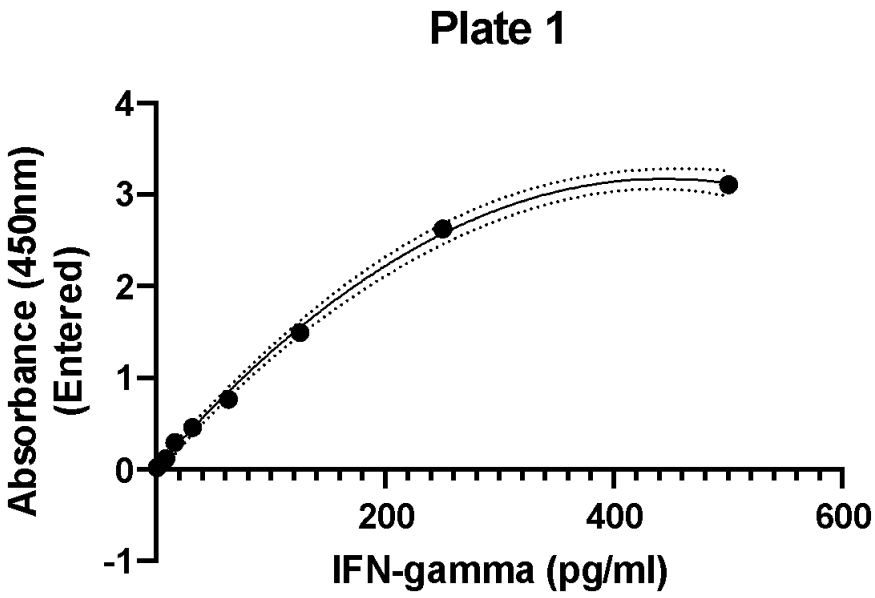


Figure 6: IFN-Y standard curve: an example of the standard curve calculated for the IFN-γ assay using GraphPad Prism

2.8.5.6 Maintenance

The Biotek-ELx800 is a microplate reader for absorbance used in clinical and life science research. For maintenance procedures, dust particles are removed regularly from the surface of the instrument to avoid accumulation of dust.

The BioTek ELx50 is an automated strip washer with an eight-channel, dual-action manifold. The Dual-Action Manifold has two sets of aspiration and dispensing tubes per microplate well. The instrument works well with solid-bottom microplate plates traditionally used for cell-based and ELISA assays. Maintenance on the ELx50 is done weekly as well as before use by means of priming to prevent blockages within the pipes.

2.9 Special investigations

For cases whereby a participant was found to be a non-responder, i.e. the protective levels were found to be below 10 ml/UL after the final booster of HBV vaccine, further tests were conducted, including HBsAg, to rule out the possibility of a current infection. If HBsAg was found to be positive, then further testing of HBeAg, anti-HBe and HBV DNA

testing would be done. Counselling and advice regarding the further management of HBsAg participants were provided by Dr Dewald Steyn (Infectious Disease Physician), where needed.

2.10 Statistical analysis

All statistical analysis was performed by Prof. G. Joubert from the Department of Biostatistics at the UFS. Deidentified data were captured electronically using Microsoft Excel. Results were summarized by frequencies and percentages (categorical variables) and medians, interquartile ranges (IQR) and ranges (numerical variables, due to skew distributions). Associations between categorical variables were assessed using Fisher's exact tests due to sparse cells. Subgroup differences regarding numerical variables were assessed using Kruskal-Wallis tests. Positive and negative predictive values as well as sensitivities and specificities were calculated.

2.11 Ethical aspects and good clinical practice

2.11.1 Ethical clearance

Ethical approval for the main study was obtained from the Health Science Research Ethics Committee (HSREC) at UFS (HSREC 79/2017; UFS-HSD2017/0788). The current study was submitted as a sub-study for HSREC approval (UFS-HSD2018/0783/3107), following evaluation by the Faculty Research and Innovation Committee (FRIC), CUT. Institutional approval to conduct the study was obtained from the Head of Department and Assistant Dean: Research, Innovation and Engagement for CUT and the Head of School of Pathology, Dean of the Faculty of Health Sciences and Vice-Rector: Research for the UFS.

2.11.2 Safety variables

2.11.2.1 Patient safety

Administration of vaccines and drawing of blood were done by qualified doctors who are well trained and experienced in phlebotomy, in order to ensure patient safety. Students

were monitored for a minimum of 30 minutes following administration of the vaccine to detect any immediate hypersensitivity reactions or anaphylaxis.

2.11.3 Premature discontinuation of the study

The study would have been discontinued prematurely if the researcher or any of the study leaders felt that participants' confidentiality might be breached or if any unethical procedures occurred.

2.11.4 Good clinical practice (GCP)/quality assurance

All clinical work conducted under this project was subjected to the GCP guidelines (the principles of ICH, GCP,2004). The declaration of Helsinki's basic principle number three states that research should be conducted only by scientifically qualified people under the supervision of adequately qualified people (World Medical Association, 2002).

2.11.5 Financial implications to the patient

Participants were not paid to be in this study; however, participants from the UFS had no need to pay for their immunizations, as it was provided for them as part of the study and all participants were provided with the final anti-HBs results to submit as proof of immunity to the relevant institution. No financial input was required from the participants.

2.11.6 Withdrawal criteria

Participation was completely voluntary. Participants had the right to withdraw from this particular study at any time, irrespective of the reason(s), without detriment to their medical care presently or in the future. The elimination of a patient from this particular study did not involve any penalties.

2.11.7 Subject information and informed consent

All participants were informed about the purpose and necessity of the research project, the financial implications and the risk/benefit, as well as the possible adverse effects and their right to withdraw without any effects on them. Participants signed an informed

consent form and received an information sheet that they could keep. The HSREC had approved all study documentation. Both the consent form and the information sheet were made available in English, as this is the language of tuition at both institutions.

2.11.8 Confidentiality

No personal details of the participants were used for this study, as samples were collected by a bigger study and data were deidentified prior to use in the current study and were identified only by means of a unique study identification number. A separate list of participant names linked to the relevant study number was maintained by the study leader (main study principal investigator, Prof. D. Goedhals) to which other members of the research team did not have access, in order to allow collation of longitudinal data. Following the collection of data for the final time point and provision of final anti-HBs results to participants as proof of immunity, the list of identifiers was destroyed.

2.12 Funding

Joint UFS/CUT funding of R100 000 was obtained for the parent study, which was utilised for purchasing of vaccines, sample collection requirements and HBV peptides. An application for DHET funding was submitted for the remaining costs, including laboratory consumables and laboratory reagents. Additional funding was also made available from the supervisor's UFS incentive funding.

2.13 Limitations to the study:

Possible limitation to the study is the uncertainty of whether participants have had immunizations after the EPI programme. Although particiapnts verbally confirmed that they did not receive immunization after early childhood vaccination it does not rule out the possibility that participant could have been Immunized before the study but after their childhood vaccinations. Although this does not occur in South Africa, we do acknowledge the possibility of immunization after early childhood vaccination.

CHAPTER 3: RESULTS

3.1 DEMOGRAPHICS

For this study, a total of 320 students from two institutions, namely the UFS and the CUT, were approached during orientation sessions regarding study participation. Out of 320 students, only 122 were recruited into the study. Of these, 67 participants were students from the CUT and 55 participants were students from the UFS. The majority of those who did not take part in the study were excluded as they elected not to participate. However, some of the students that did not take part in the study were students that did not meet the inclusion or the exclusion criteria. Factors such as having received HBV vaccination prior to the start of the study, age and citizenship played a role in the exclusion in these cases. Recruitment and analysis took place for a period of 16 months and results were obtained after the experimental phase of the study.

The EPI was initiated in 1995 in South Africa; therefore, only students who were born after 1995 could be part of the study, as students born prior to this would be unlikely to have received the vaccine during childhood. Students that were younger than 18 years old (i.e. minors) were also excluded; therefore, only students between the ages of 18 and 22 years of age were included in the study. As indicated in Table 2 below, 42 students (34%) were 18 years old, 34 students (28%) were 19 years old, 21 students (17%) were 20 years old, 22 (18%) were 21 years, and 3 (3%) were 22 years old. Both male and females participated in the study, and out of the 122 participants, 74 students (61%) were females, and 48 students (39%) were males. Even though citizenship played a role in the decrease in the number of participants, many different races of South African origin took part in the study, as self-reported by the participants. Ninety-two (92) students (75%) were Africans, 9 (7%) were Asians, 1 (1%) was of coloured origin, and 20 (17%) were whites.

Table 2: Participant demographic characteristics.

Participant demographics	**n (%)**
TOTAL	**122**
INSTITUTION	
CUT	67 (55)
UFS	55 (45)
AGE GROUPS (YEARS)	
18	42(34)
19	34 (28)
20	21 (17)
21	22 (18)
22	3 (3)
GENDER	
Male	48 (39)
Female	74 (61)
RACE	
African	92 (75)
Asian	9 (7)
Coloured	1 (1)
White	20 (17)

3.2 CLINICAL DATA

3.2.1 Introduction

At the baseline visit, information was collected from participants regarding their childhood HBV vaccination history and clinical history including a history of previous jaundice, previous confirmed HBV infection, and history of other liver disease.

3.2.1 HBV vaccination

During the orientation session, students were asked whether they had received childhood

HBV vaccinations, and if so, they were asked to confirm the number of doses, preferably by providing their *EPI Road to Health Booklet* or similar document containing immunization records. Only one out of the 122 participants confirmed that they had been vaccinated during childhood and provided immunization records that stated that the participant was administered with three doses of HBV vaccine during early childhood. The remaining participants were unaware of their childhood HBV vaccination status.

3.2.2 HBV infection

Participants were further questioned regarding whether they were previously diagnosed with HBV and/or whether they had had any other liver diseases besides HBV. A total of 113 students (93%) confirmed that they had no history of previous HBV infection, while 9 (7%) were not certain whether they had previously been infected with HBV or not.

3.2.3 Liver disease

The students were also asked whether they had other past or present liver diseases such as jaundice. Regarding a history of jaundice, 2 (2%) reported that they had neonatal jaundice, 116 (95%) had no previous history of jaundice, 1 (1%) was not certain and 3 (2%) were previously diagnosed with jaundice without a specified cause. For other liver conditions apart from jaundice, 117 (96%) confirmed that they had no other liver conditions, while 5 (4%) were uncertain (Table 2).

Table 3: History of past/present liver disease as self-reported by study participants (N=122)

Liver disease	**n (%)**
Jaundice	
No history of jaundice	116 (95)
Uncertain	1 (1)
Neonatal jaundice	2 (2)
Previously diagnosed with jaundice	3 (2)
Other liver conditions	
No other liver conditions	117 (96)
Uncertain	5 (4)

3.3 LABORATORY ANALYSIS

3.3.1 Introduction

During the experimental phase of the study, various laboratory tests were conducted to fulfil the aim of determining long-term humoral and cellular immunity in South African medical students following childhood HBV vaccination.

3.3.2 HBsAb testing

HBsAb results were classified as negative (<9.0 mIU/mL), equivocal (90-11.0 mIU/mL), or positive (≥11 mIU/mL) according to the Liaison® XL Anti-HBs package insert (DiaSorin, Saluggia, Italy), based on the initial testing result.

3.3.2.1 Baseline

After participants were tested for HBsAb at baseline sampling, it was found that 64 (52%) tested negative, 52 (43%) tested positive, and 6 (5%) participants showed equivocal results (Table 4).

Table 4: Baseline hepatitis B surface antibody (HBsAb) results of the participants

HBsAb result at baseline	**n (%)**
Negative (<9.0 mIU/mL)	64 (52)
Positive (≥11 mIU/mL)	52 (43)
Equivocal (9.0-11.0 mIU/mL)	6 (5)
Total	122 (100)

As previously mentioned, HBsAb baseline samples were collected before vaccination was administered. Therefore, these percentages reflect responses to childhood vaccination or subsequent infection. It is therefore evident that 52% of participants showed signs of absent or waning immunity, while 43% of participants still had protective levels of HBsAb. The six equivocal results are results that are uncertain, meaning they are lower than the positive results, but they are not negative; therefore, suggesting that the participants could be non-responders or have waning immunity.

3.3.2.2 Second sampling

Participants returned for collection of a second sample after one month and it was found that of the 122 participants, 2 participants did not return for sample collection. Therefore, from the 120 samples received, 9 participants (8%) tested negative, while 111 (92%) tested positive (Table 5).

Table 5: Hepatitis B surface antibody (HBsAb) results of the participants tested one month after a booster dose of hepatitis B vaccine

HBsAB result at second sampling point	**n (%)**
Negative (<9.0 mIU/mL)	9 (8)
Positive (≥11 mIU/mL)	111 (92)
Equivocal (9.0-11.0 mIU/mL)	0 (0)
Total	120 (100)

From the above table, when compared to Table 4, it is quite evident that an anamnestic response has taken place in majority of the participants.

3.3.2.3 Third sampling

Participants returned for a final blood sample 6 months after the second sampling, having received 3 booster doses of HBV vaccine and it was found that 3 more participants did not return for sample collection. Therefore, there were a total of 119 participants, all of whom tested positive for HBsAB at the final time point.

Figure 7 represents the HBsAb results of participants over a seven-month period, illustrating the participants' responses to HBV vaccination.

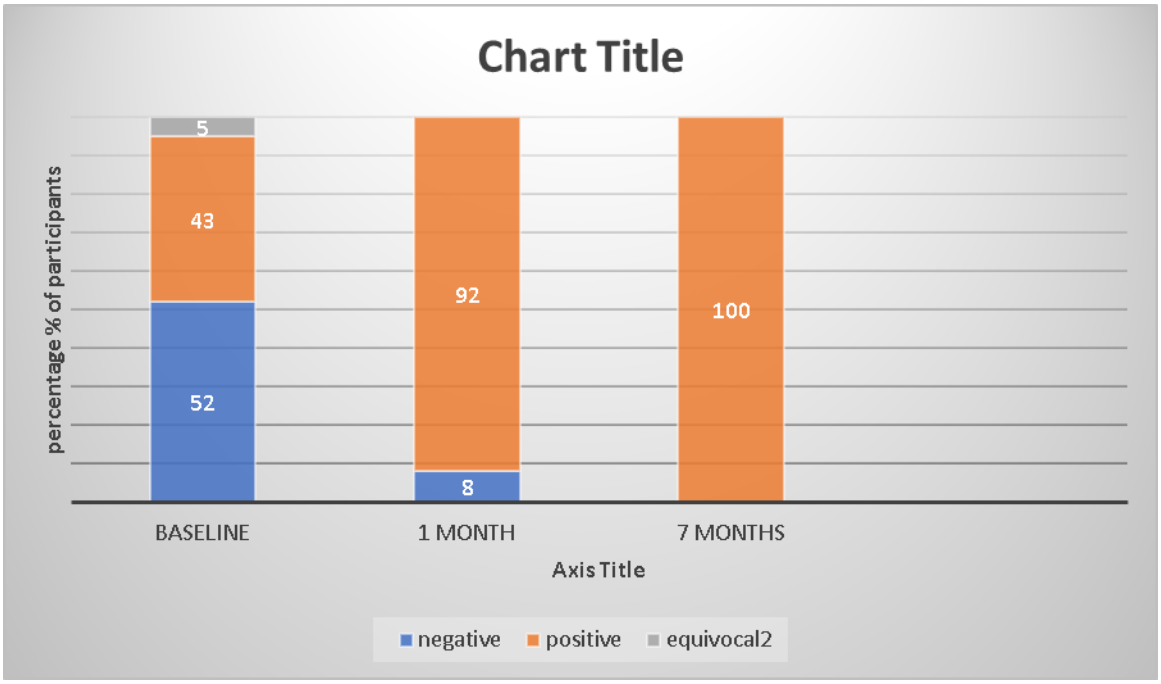


Figure 7: Hepatitis B surface antibody (HBsAb) results at different time points over a seven-month period following hepatitis B vaccine booster doses at baseline, month one and month six

The quantitative HBsAb results at the third time point were as follows, as illustrated in Table 6. 115 students (96%) had an HBsAb level of above 1000 mIU/mL, 1 (1%) was 969 mIU/mL, 1 (1%) was 476 mIU/mL, 1 (1%) was 436 mIU/mL, and 1 (1%) was 11.2 mIU/mL. The sample that gave a result of 11.2 ml/Ul was tested for HBsAg due to the value being close to that of a non-responder and the results were negative, indicating that the participant did not have a current HBV infection.

Table 6: Positive HBsAB result levels amongst participants

HBsAb level (IU/L)	**n (%)**
Above 1000	115 (96)
969	1 (1)
476	1 (1)
436	1 (1)
11	1 (1)
Total	119 (100)

3.3.3 HBcAb testing

HBcAb testing was performed on all samples at baseline to identify current or previous HBV infection. HBcAb results were classified as negative (≥1.1), equivocal (0.9-1.1) or positive (<0.9) according to the index values as recommended in the Liaison® XL Anti-HBc package insert (DiaSorin, Saluggia, Italy).

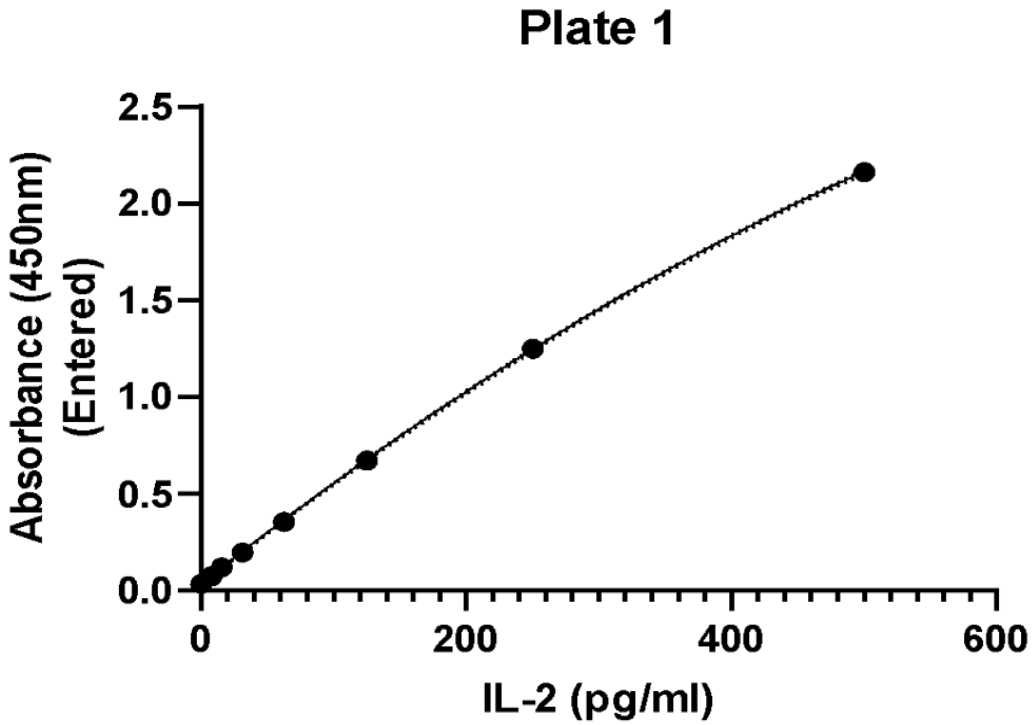
Only one participant tested HBcAb positive (1%), while the remaining 121 (99%) tested negative (Figure 8). The single participant who tested HBcAb positive also tested HBsAb positive, with a titre of 418 mIU/mL, which increased to >1000 mIU/mL following the first booster dose of HBV vaccine. The presence of both HBcAb and HBsAb indicates previously resolved HBV infection.

3.3.4 IFN-Y and IL-2 testing

IFN- Y and IL-2 testing was done by means of ELISA tests on all 122 samples at baseline, using 96-well plates in which the first column represents the standards used, except for plate 1 for IFN-Y testing, where the standard values were situated in column six. Five plates for IFN-Y and five plates for IL-2 testing were used. The absorbance was read at 450 nm with the reference wavelength being 620 nm. Wells that did not have any sample added are indicated as ‘NS’ in the plate illustration, which stands for No Sample. Below are the plate layouts representing the actual results obtained after analysis. As mentioned in the previous chapter, each plate ran for IL-2 and IFN-y had their own standard curve graph because each plate had their own standards. From all the above-mentioned plates there is a graph below each plate to show its respective standard curve, which was generated in order to quantify the amount of IFN- Y or IL-2 in each sample.

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.1630	1.4510	0.0290	0.0490	1.4820	0.4540	OVER	0.7890	0.0500	0.0240	1.4410	0.6660
B	1.2500	0.0280	0.8060	0.9600	0.1330	1.7060	0.2670	0.5710	0.0510	0.0330	0.1110	1.4360
C	0.6730	0.0290	0.0350	1.6790	2.0200	0.0440	0.1410	0.0770	0.0270	1.3540	0.4530	0.0310
D	0.3580	1.5550	0.0350	0.0320	0.1300	0.0760	2.4310	0.6640	0.0420	0.0500	1.8380	0.0320
E	0.1990	0.0600	0.8660	1.1570	0.0450	1.9360	0.4450	1.2430	0.9630	0.7260	0.0290	1.3800
F	0.1210	0.5400	0.1160	1.9330	0.0460	0.9590	0.0910	0.2130	0.0240	1.5020	0.4690	0.0220
G	0.0760	1.0510	0.8350	0.0870	1.5470	1.2150	1.6550	0.6180	0.2030	0.0210	1.5160	0.6010
H	0.0370	0.0270	0.9900	0.5600	0.9880	1.3430	0.0710	0.0270	1.9160	0.2330	0.0600	NS

A

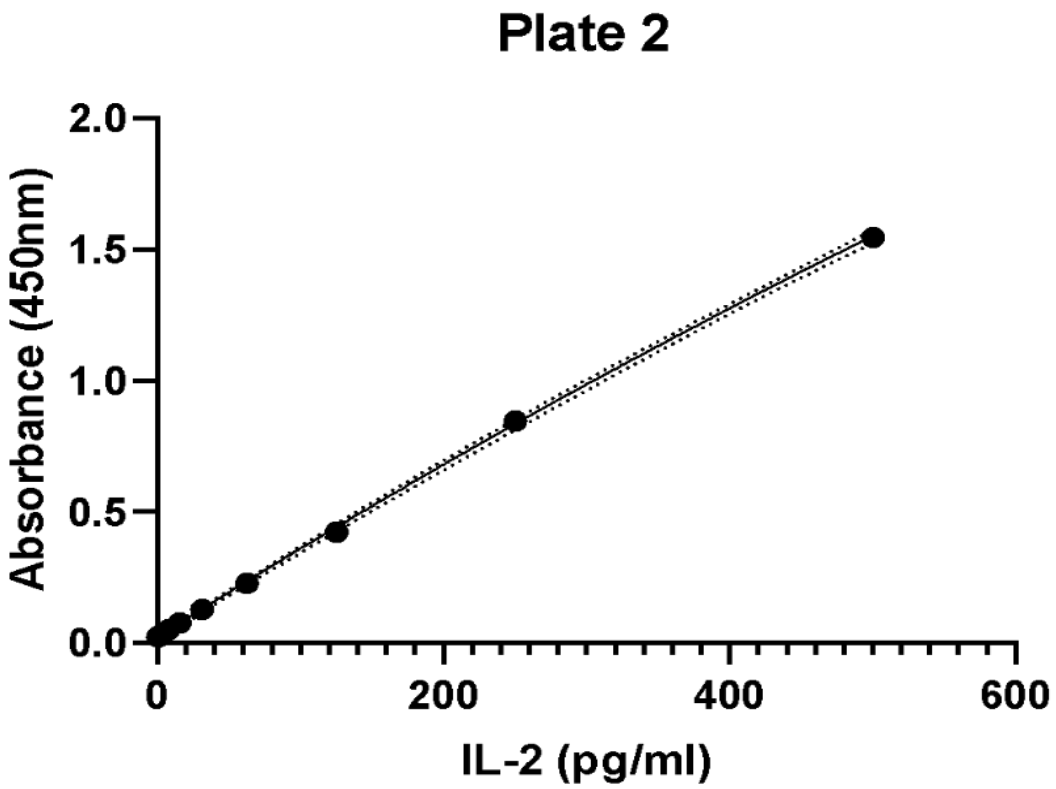


B

Figure 9: Interleukin-2 results for ELISA plate 1 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5480	0.5160	0.2210	0.0530	0.4550	0.0430	2.8940	0.3470	0.0690	0.0440	0.3270	0.1380
B	0.8470	0.0920	0.9600	0.4910	0.0340	0.2810	0.2130	0.0340	0.2090	0.1390	0.0240	0.7460
C	0.4240	0.6370	0.0350	0.7430	0.0290	0.0370	0.6880	0.1280	0.0620	0.2380	0.3010	0.1700
D	0.2290	1.0840	0.0350	0.0990	0.1160	0.0570	0.0490	0.5080	0.1610	0.0290	0.3160	0.3440
E	0.1290	0.0570	1.0110	0.6180	0.0410	0.5590	0.0530	0.0200	0.1700	0.0320	0.1240	0.4040
F	0.0780	0.2100	0.0750	1.3060	0.0840	0.0290	0.2110	0.1120	0.0650	0.6370	0.2920	0.0330
G	0.0530	0.6270	0.2540	0.0270	0.6130	0.1770	0.0440	0.6450	0.1940	0.0780	0.4060	0.1160
H	0.0240	0.0240	0.7120	0.0280	0.0400	0.2390	0.0850	0.0470	0.5270	0.2210	0.0490	NS

A

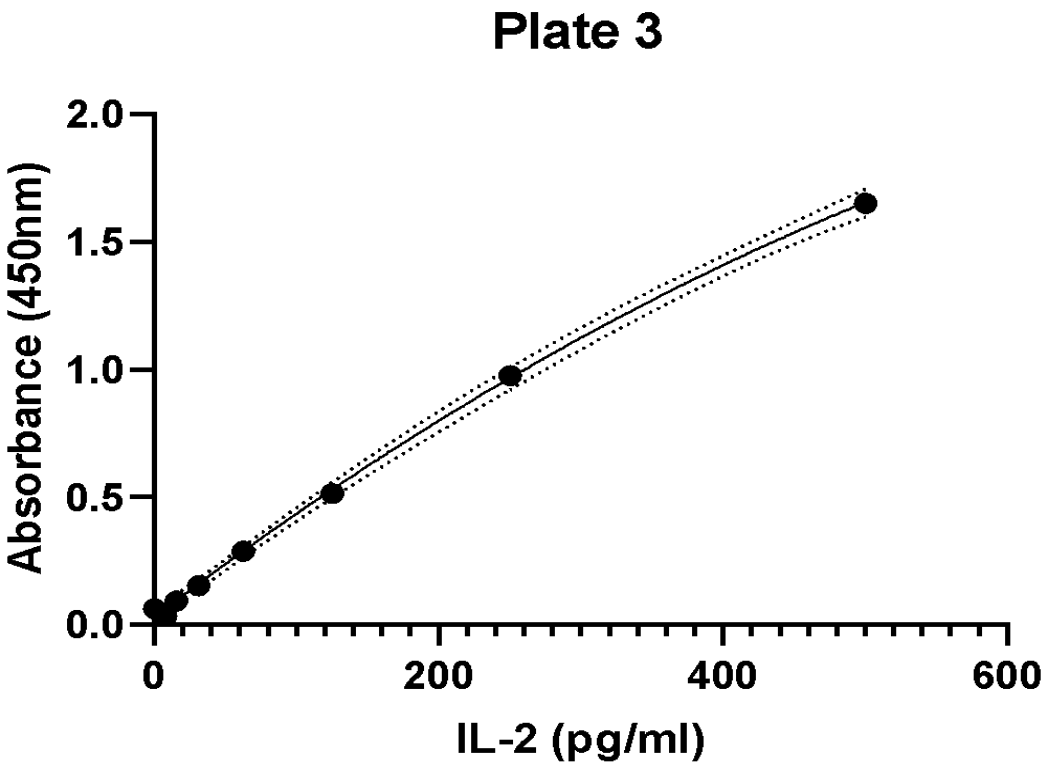


B

Figure 10: Interleukin-2 results for ELISA plate 2 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.6520	0.4330	1.7230	0.0760	0.8740	0.2530	0.0550	1.0340	0.3370	0.0470	0.4390	0.4790
B	0.9770	0.0910	0.0420	0.0770	0.1020	0.9080	0.0480	0.0380	0.6160	0.0440	0.1480	0.5970
C	0.5150	0.7810	0.0360	0.9260	0.4340	0.1690	1.6340	0.5830	0.0210	0.8810	0.4420	0.0480
D	0.2890	0.4850	0.1010	0.0250	1.0030	0.1150	0.0370	0.8590	0.0620	0.0870	0.4290	0.0470
E	0.1540	0.0340	0.7530	0.5470	0.0810	0.8650	0.6980	0.0890	0.5820	0.4910	0.0600	0.7620
F	0.0940	0.7550	0.0690	1.0450	1.7060	0.0520	0.9420	0.5860	0.0350	0.8580	0.1290	0.0290
G	0.0360	0.9800	0.2260	0.0610	0.4140	0.3140	0.0430	0.9940	0.4300	0.0430	0.0640	0.2400
H	0.0640	0.0490	0.9560	0.6450	0.0330	0.5060	0.3870	0.0590	0.9960	0.5110	0.0690	NS

A

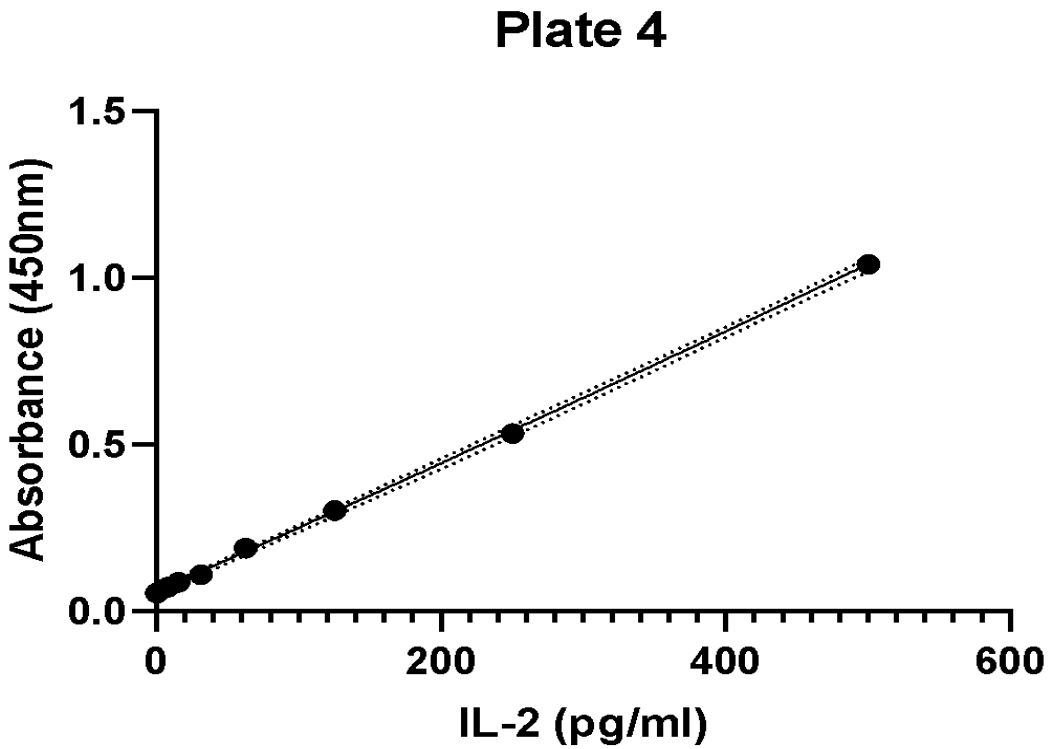


B

Figure 11: Interleukin-2 results for ELISA plate 3 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.0420	0.0860	0.2520	0.0330	0.3060	0.6540	0.0370	0.5720	0.1610	0.0660	0.4450	0.0310
B	0.5340	0.0270	0.5870	0.4610	0.0310	0.6810	0.4280	0.0910	0.4650	0.2970	0.1000	0.5570
C	0.3030	0.0250	0.0420	0.5350	0.0280	0.0830	OVER	0.3450	0.0650	0.2770	0.3040	0.0710
D	0.1910	0.6180	0.2620	0.0850	0.6570	0.4380	0.0280	0.4080	0.3150	0.0520	0.5330	0.2590
E	0.1100	0.0820	0.3410	0.3080	0.0320	0.6440	0.1070	0.0360	0.5980	0.0530	0.0260	0.3970
F	0.0880	0.0970	0.0420	0.3140	0.0320	0.0800	0.7820	0.2290	0.0630	0.4260	0.0210	0.0700
G	0.0730	0.4330	0.1660	0.0320	0.9660	0.0800	0.0480	0.2160	0.0510	0.0290	0.0150	0.4290
H	0.0560	0.0340	0.5200	0.3260	0.0270	0.4590	0.4580	0.0590	0.7410	0.1700	0.0320	NS

A

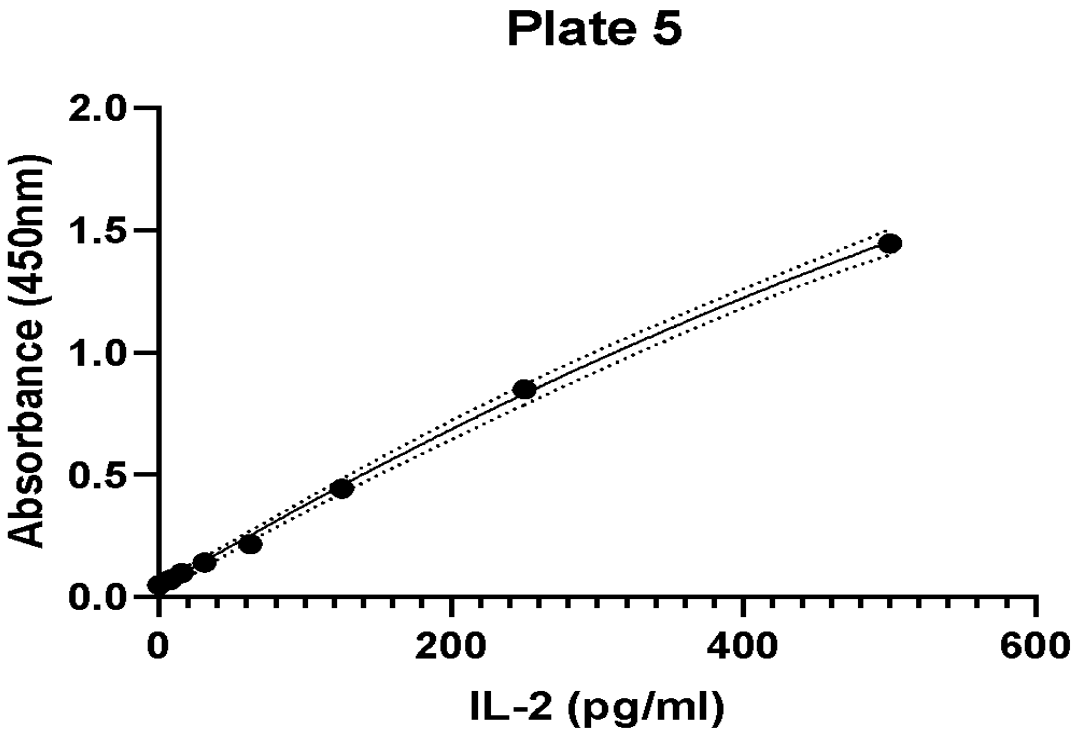


B

Figure 12: Interleukin-2 results for ELISA plate 4 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.4480	0.4220	0.0460	0.0900	0.2600	NS	NS	NS	NS	NS	NS	NS
B	0.8510	0.0480	1.0940	0.0950	0.2000	NS	NS	NS	NS	NS	NS	NS
C	0.4440	0.2630	0.0470	0.6140	0.0520	NS	NS	NS	NS	NS	NS	NS
D	0.2170	0.5340	0.8060	0.0540	NS	NS	NS	NS	NS	NS	NS	NS
E	0.1420	0.0630	0.8910	0.3140	NS	NS	NS	NS	NS	NS	NS	NS
F	0.0990	0.4560	0.0270	0.6820	NS	NS	NS	NS	NS	NS	NS	NS
G	0.0730	0.6550	0.7220	0.0690	NS	NS	NS	NS	NS	NS	NS	NS
H	0.0510	0.0420	0.7950	0.0770	NS	NS	NS	NS	NS	NS	NS	NS

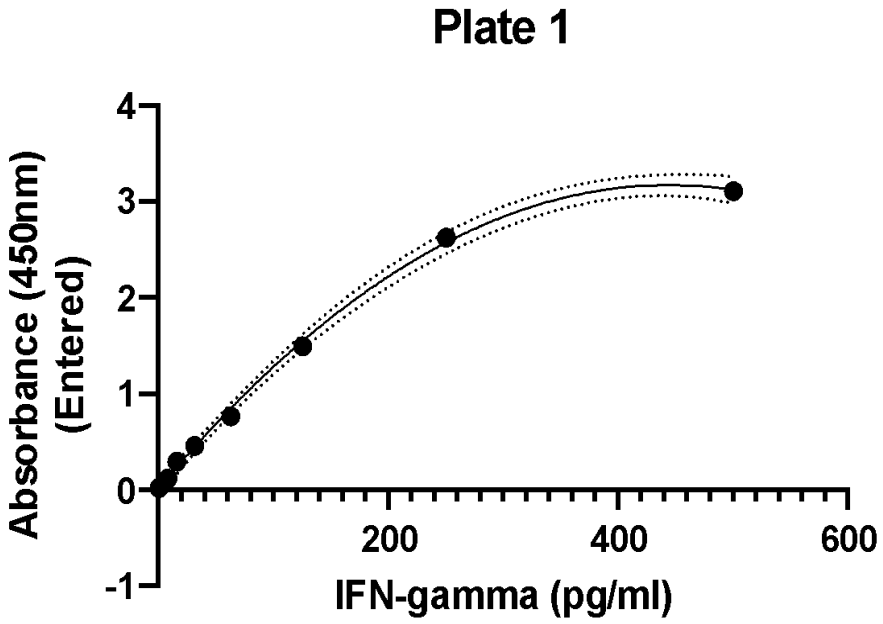
A



B Figure 13: Interleukin-2 results for ELISA plate 5 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	NS	NS	NS	NS	NS	3.1160	0.0880	0.1020	0.0500	0.1010	0.0820	0.0640
B	NS	NS	NS	NS	NS	2.6320	0.0580	0.2680	0.1000	0.0760	0.7320	0.0990
C	NS	NS	NS	NS	NS	1.5000	3.6760	0.0490	1.5070	0.0650	0.0380	0.1360
D	NS	NS	NS	NS	NS	0.7740	0.3930	0.0650	0.1450	1.6570	0.0590	0.1290
E	NS	NS	NS	NS	NS	0.4630	0.0490	0.3010	0.0970	0.2510	0.1220	0.1340
F	NS	NS	NS	NS	NS	0.3010	0.0660	0.1050	0.5700	0.2330	0.0740	0.3550
G	NS	NS	NS	NS	NS	0.1280	0.8210	0.1440	0.0900	0.4700	0.2850	0.1460
H	NS	NS	NS	NS	NS	0.0260	0.1170	0.4470	0.1310	0.0870	1.7330	0.1320

A

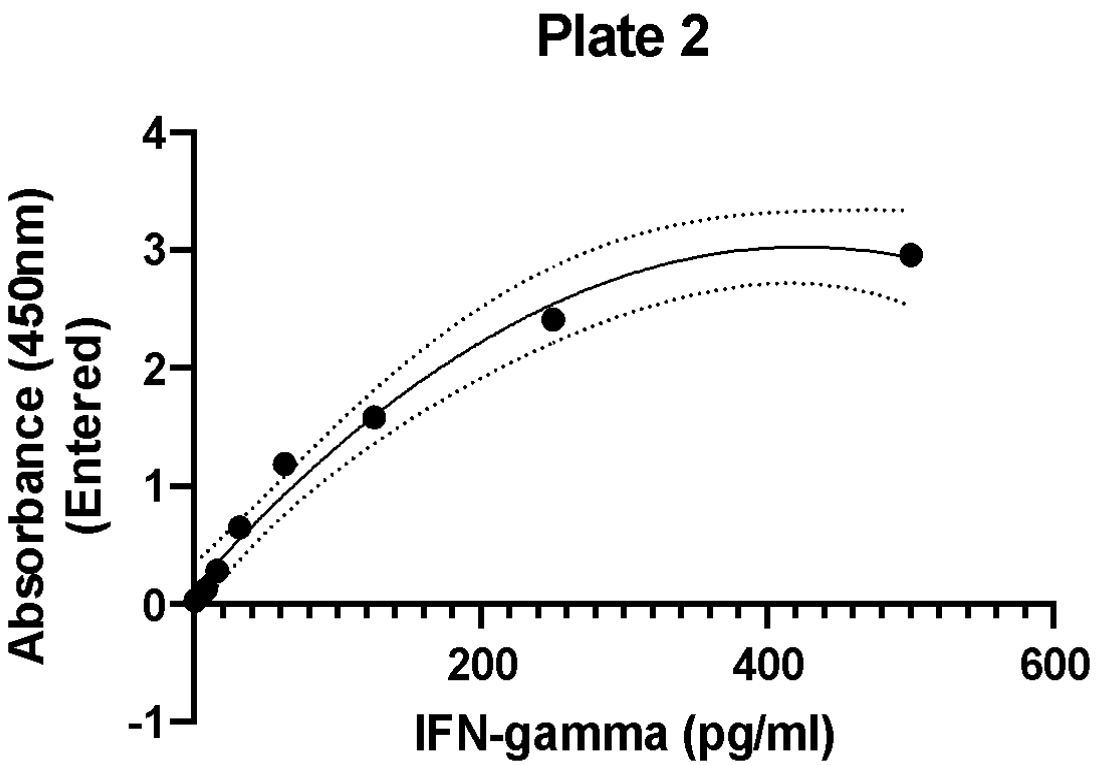


B

Figure 14: Interferon gamma results for ELISA plate 1 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.9660	1.9420	0.3420	0.2590	0.6100	0.1140	0.1140	0.0970	0.0340	0.9760	0.1040	0.1010
B	2.4180	0.1880	0.4940	0.2790	0.1120	0.1850	0.0520	0.1690	0.1850	0.1050	0.1290	0.1370
C	1.5880	0.2350	0.2610	0.3590	0.0900	0.0530	3.6070	0.0590	0.5160	0.0740	0.0940	2.3640
D	1.1940	1.2140	0.3000	0.2660	0.9300	0.0420	1.0120	0.0670	0.0760	0.1120	0.0570	0.0910
E	0.6570	0.0590	2.3900	0.3070	0.0450	0.1020	0.0610	1.4660	0.0980	0.0590	1.2050	0.1510
F	0.2890	0.0570	0.1640	0.2980	0.0360	0.0320	0.0890	0.2790	0.3110	0.0760	0.0580	0.1910
G	0.1320	1.1750	0.2040	0.2350	0.2060	0.0800	0.1400	0.4260	0.0330	0.9440	0.0440	0.0560
H	0.0370	0.3130	0.6200	0.2470	0.2120	NS	0.0870	1.6890	0.0500	0.0530	0.1930	0.0730

A

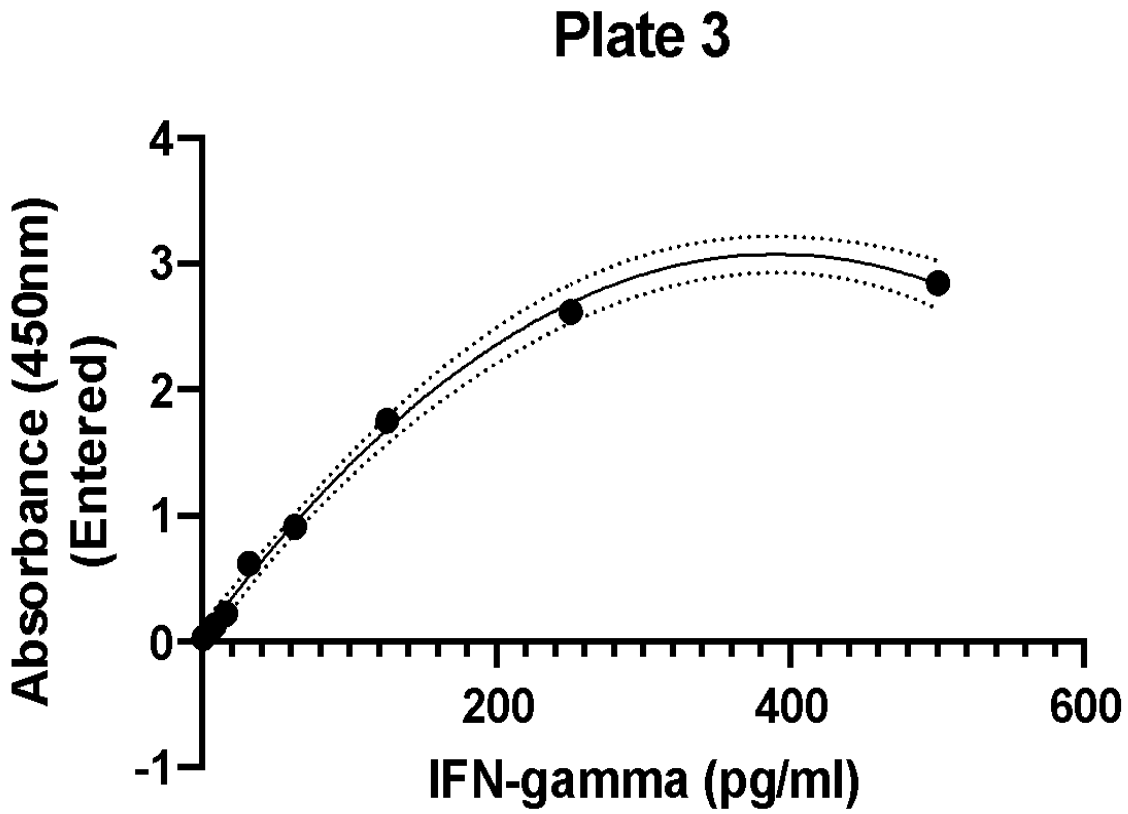


B

Figure 15: Interferon gamma results for ELISA plate 2 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.8510	2.1090	0.7360	0.0850	1.9900	1.1740	2.2250	1.2680	0.1080	1.8640	0.7730	0.0850
B	2.6240	0.1060	2.1140	0.8140	0.1710	1.4900 D	0.0600	0.1760	0.4520	0.2970	2.3570	0.8170
C	1.7590	0.7750	0.2980	1.4050	0.8850	0.1390	3.6230	0.1610	1.6380	0.6750	0.1480	2.1470
D	0.9200	2.5370	0.4500	0.0440	1.9570	1.2920	1.6600 D	0.2050	0.0920	2.1430	0.8070	0.1590
E	0.6260	0.0760	1.7000	0.0410	0.0990	2.1740	0.1780	2.0300 D	0.8430	0.1930	1.6050	1.2980
F	0.2290	0.7230	0.2470	1.8880	1.1520	0.1220	1.1580	0.1030	0.9570 D	1.0470	0.0760	1.2540
G	0.1320	2.5740	1.2390	0.0840	1.7500	0.1490	0.7960	1.0440	0.1460 D	1.1470	0.5730	0.1410
H	0.0350	0.2090	2.3400	0.5110	0.8550	2.9200	0.2170	2.6250	0.7460	NS	1.7700	1.0270

A

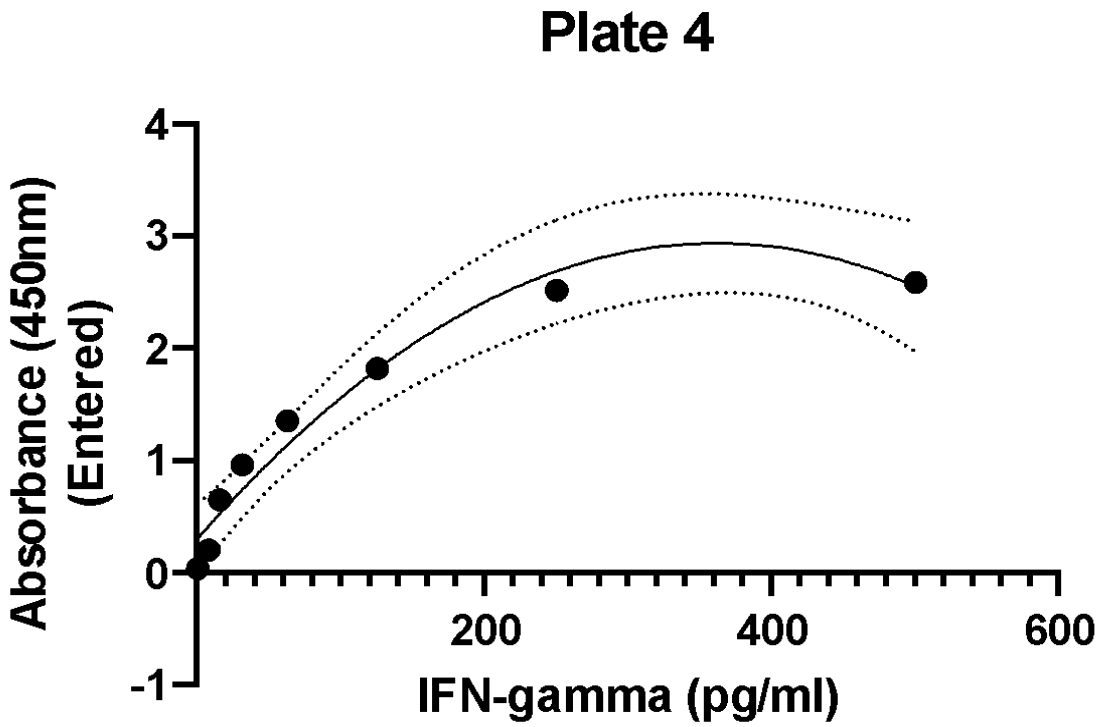


B

Figure 16: Interferon gamma results for ELISA plate 3 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.5920	2.2250	0.7080	0.1540	2.4410	1.7150	1.8430	0.8420	0.0650	1.9050	1.9820	0.0810
B	2.5230	0.1270	2.5650	0.1930	0.0640	2.0540	0.0740	2.1090	0.7160	0.1780	2.4890	1.1070
C	1.8250	1.5130	0.0620	1.9320	1.0790	0.1970	3.5680	0.2560	2.0430	0.4030	0.1170	2.6220
D	1.3610	2.0250	0.1030	0.1200	1.9090	1.4550	2.7050	0.8520	0.0970	1.8540	1.8320	0.1670
E	0.9670	0.1050	2.2420	1.2710	0.0840	2.0270	0.1430	2.4550	0.7530	0.0700	2.5510	0.9910
F	0.6570	0.9180	1.5110	2.0470	1.3840	0.3290	1.1100	0.2160	1.7430	0.1200	0.1670	2.4080
G	0.2120	2.2290	2.4530	0.2160	2.2210	1.2610	2.7300	1.5590	0.0830	2.6480	1.3810	0.3080
H	0.0430	0.1430	1.2890	1.3100	0.6660	NS	0.1390	2.4170	0.9710	0.8040	2.1250	1.3120

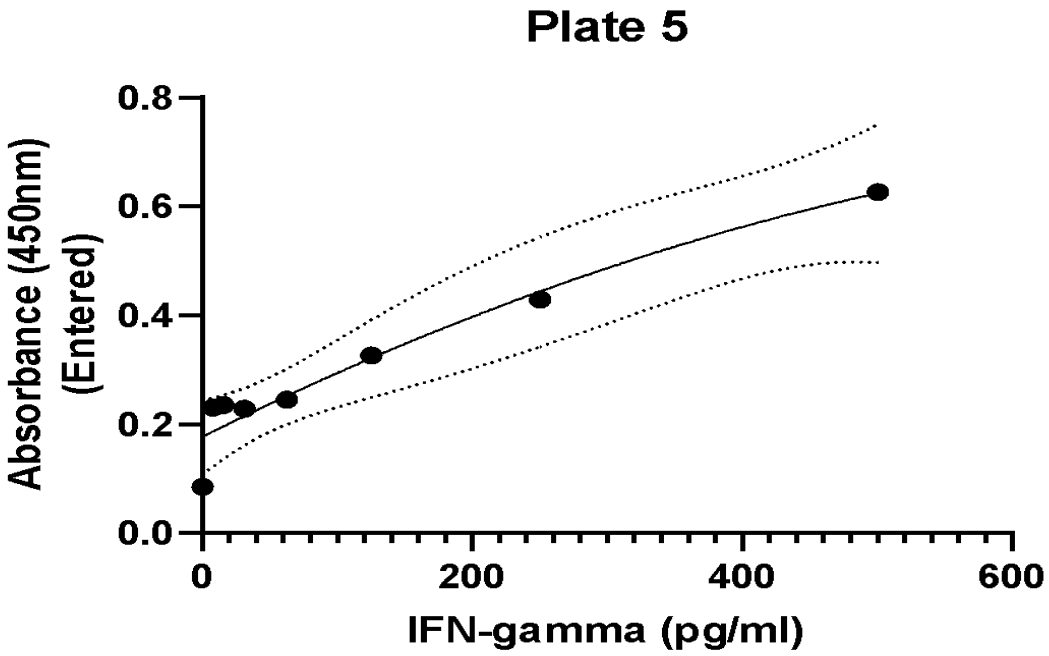
A



B: Figure 17: Interferon gamma results for ELISA plate 4 indicating the optical density readings (A) and resulting standard curve (B)

<>	1	2	3	4	5	6	7	8	9	10		12
A	0.6270	0.4530	0.6210	0.3260	0.2430	0.2500	1.2750	0.8980	0.9100	0.4930	NS	NS
B	0.4300	0.5210	1.2290	0.2720	0.1450	0.3280	1.1060	0.4230	0.8610	0.5110	NS	NS
C	0.3270	0.5450	1.0420	0.5180	0.1590	0.1480	0.7340	0.3550	0.3160	0.4880	NS	NS
D	0.2460	0.3350	1.1860	1.0580	1.8770	0.1470	0.4310	0.3050	0.2930	0.1010	NS	NS
E	0.2290	0.3160	2.6170	1.2650	1.9810	0.4080	3.5040	0.3320	0.2050	0.0800	NS	NS
F	0.2360	0.1950	2.5850	0.5410	1.8010	0.2550	0.2900	0.2840	0.6750	NS	NS	NS
G	0.2310	0.5830	2.7150	0.6480	0.2550	0.4280	1.0440	0.2660	0.6370	NS	NS	NS
H	0.0860	0.6740	0.3210	0.6580	0.2070	0.0830	1.1900	1.0200	0.5180	NS	NS	NS

A



B: Figure 18: Interferon gamma results for ELISA plate 5 indicating the optical density readings (A) and resulting standard curve (B)

3.3.4.1 INF-y release assay

For IFN-γ, quantitative results were generated for 71 of the 122 baseline samples. Failed standards in plate 5 meant that quantitative results could not be calculated for the 22 samples tested on this plate. For a further 29 samples, failure of either the positive or negative control wells resulted in invalid results. Due to limited reagents for the IFN-γ ELISA assay, repeat testing of the invalid samples was not possible.

The IFN-γ results were grouped according to HBsAb result at baseline (Table 7). For the negative HBsAb samples, the IFN-γ results ranged from 0-96 pg/mL, with a median of 3 pg/mL (IQR 0 to 60). For the positive HBsAb samples, the IFN-γ results ranged from 0-120 pg/mL, with a median of 29 pg/mL (IQR 0 to 58). Lastly, for the equivocal HBsAb results, the IFN-γ results ranged from 0-90 pg/mL, with a median of 2 pg/mL (IQR 0 to 11). These values did not differ statistically significantly (p=0.49).

Table 7: Interferon gamma (IFN-γ) results grouped according to baseline hepatitis B surface antibody (HBsAb) result

HBsAb	**Number**	**IFN-γ range (pg/mL)**	**IFN-γ median (pg/mL)**	**IFN-γ interquartile range (pg/mL)**
Negative	35	0 – 96	3	0 – 60
Positive	31	0 - 120	29	0 – 58
Equivocal	5	0 - 90	2	0 - 11

Table 8 shows the association between IFN-y results and HBsAb at baseline. The p-value was 0.68, which indicates that there was no statistically significant association. The positive and negative predictive values of IFN-y results to predict HBsAb status were 47% and 55%, respectively, with sensitivity of 74% and specificity of 34%.

Table 8: Association between interferon gamma (IFN-γ) and baseline hepatitis B surface antibody (HBsAb)

	HBsAb Equivocal	**HBsAb Negative**	**HBsAb Positive**	**Total**
IFN-γ Not detected	2 (9%)	12 (55%)	8 (36%)	22
IFN-γ Not done	1 (2%)	29 (57%)	21 (41%)	51
IFN-γ Detected	3 (6%)	23 (47%)	23 (47%)	49
Total	6	64	52	122

Similarly, the IFN-γ results were grouped according to the HBsAb result at time of the second sampling (Table 9). For the negative HBsAb samples, there were no IFN-γ results above 0 pg/mL. For the positive HBsAb samples, the IFN-γ results ranged from 0-120 pg/mL, with a median of 12 pg/mL (IQR 0 – 60). The difference in these values was statistically significant (p=0.03).

Table 9: Interferon gamma (IFN-γ) results grouped according to hepatitis B surface antibody (HBsAb) result at second sampling (one month after HBV vaccine booster dose)

HBsAb	**Number**	**IFN-γ range (pg/mL)**	**IFN-γ median (pg/mL)**	**IFN-γ interquartile range (pg/mL)**
Negative	9	0	0	0
Positive	111	0 - 120	12	17 - 131

Table 10 shows the association between IFN-γ results and HBsAb at second sampling. The p-value was 0.03, which indicates that there was a statistically significant association. The positive and negative predictive values at this time point were 100% and 14%, respectively, with sensitivity 72% and specificity 100%.

Table 10: Association between baseline interferon gamma (IFN-γ) and hepatitis B surface antibody (HBsAb) result at second sampling (one month after HBV vaccine booster dose)

	HBsAb Negative	**HBsAb Positive**	**Total**
IFN-γ Not detected	3 (14%)	19 (86%)	22
IFN-γ Not done	6 (12%)	44 (88%)	50
IFN-γ Detected	0 (0%)	48 (100%)	48
Total	9	111	120

3.3.4.2 IL-2 release assay

For IL-2, quantitative results were generated for 107 samples. Failure of either the positive or negative control wells for the remaining 15 samples resulted in invalid results for these samples, which therefore could not be included in the analysis. Similar to the INF-γ assay, limited reagents prevented repeat testing for invalid IL-2 results.

The IL-2 results were grouped according to HBsAb result at baseline (Table 11). For the negative HBsAb samples, the IL-2 results ranged from 0-239 pg/mL, with a median of 26 pg/mL (IQR 0-101). For the positive HBsAb samples, the IL-2 results ranged from 0-526 pg/mL, with a median of 92 pg/mL (IQR 53-167). Lastly, for the equivocal HBsAb results, the IL-2 results ranged from 0-89 pg/mL, with a median of 29 pg/mL (IQR 0-45). These values were shown to be statistically significantly different ($p<0.01$).

Table 11: Interleukin-2 (IL-2) results grouped according to baseline hepatitis B surface antibody (HBsAb) result

HBsAb	**Number**	**IL-2 range (pg/mL)**	**IL-2 median (pg/mL)**	**IL-2 interquartile range (pg/mL)**
Negative	57	0 – 239	26	0 – 101
Positive	45	0 - 526	92	53 – 167
Equivocal	5	0 - 89	29	0 - 45

Table 12 shows the association between IL-2 results and HBsAb at baseline. The p-value was 0.02, which indicates that there was a statistically significant association.

The positive and negative predictive values of IL-2 results to predict HBsAb status were 48% and 75%, respectively, with sensitivity of 93% and specificity of 26%.

Table 12: Association between interleukin-2 (IL-2) and baseline hepatitis B surface antibody (HBsAb)

	HBsAb Equivocal	**HBsAb Negative**	**HBsAb Positive**	**Total**
IL-2 Not detected	2 (10%)	15 (75%)	3 (15%)	20
IL-2 Not done	1 (7%)	7 (47%)	7 (47%)	15
IL-2 Detected	3 (3%)	42 (48%)	42 (48%)	87
Total	6	64	52	122

The IL-2 results were grouped according to the HBsAb result at the time of the second sampling (Table 13). For the negative HBsAb samples, the IL-2 results ranged from 0–8 pg/mL, with a median of 0 pg/mL (IQR 0–2). For the positive HBsAb samples, the IL-2 results ranged from 0–526 pg/mL, with a median of 65 pg/mL (IQR 17–131). The difference in these values was statistically significant ($p<0.01$).

Table 13: Interleukin-2 (IL-2) results grouped according to hepatitis B surface antibody (HBsAb) result at second sampling (one month after HBV vaccine booster dose)

HBsAb	**Number**	**IL-2 range (pg/mL)**	**IL-2 median (pg/mL)**	**IL-2 interquartile range (pg/mL)**
Negative	9	0 – 8	0	0 – 2
Positive	111	0 – 526	65	17 – 131

Table 14 shows the association between IL-2 results and HBsAb at second sampling. The p-value was <0.01, which indicates that there was a statistically significant association. The positive and negative predictive values at this time point were 98% and 25%, respectively, with sensitivity of 85% and specificity of 71%.

Table 14: Association between baseline interleukin-2 (IL-2) and hepatitis B surface antibody (HBsAb) result at second sampling (one month after HBV vaccine booster dose)

	HBsAb Negative	**HBsAb Positive**	**Total**
IL-2 Not detected	5 (25%)	15 (75%)	20
IL-2 Not done	2 (12%)	13 (97%)	15
IL-2 Detected	2 (2%)	83 (98%)	85
Total	9	111	120

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Demographics

Approximately 316 million people are carriers of HBV and of this number, 65 million reside in Africa, with 2.5 million being in South Africa (Kramvis and Kew, 2007; Sheena et al., 2022, Tsebe et al., 2001; Yuen et al., 2018). Among the WHO regions, Africa and the Western Pacific regions have the highest HBV prevalence at 6.1% and 6.2%, respectively by 2017 and currently at 17.2% and 82.8%, respectively (Devarbhavi et al., 2023; World Health Organisation, 2017b). By 2015, 4 million new cases of hepatitis were reported per year; however, this number has fortunately dropped to 1.5 million in 2021 (Noubiap and Ndoul, 2022; Zuccaro et al., 2015). The HBV mortality rate is the highest in the African regions (80 000 deaths in 2019) (Devarbhavi et al., 2023). HBV has 18 species, 4 major serotypes, 10 genotypes and at least 24 subtypes (Hundie et al., 2017; Lin and Kao, 2017; Lytras et al., 2021). Of the 10 genotypes, Genotype A accounts for 75% of HBV in Southern Africa and Genotype D for 20% of HBV in South Africa (Kew, 2008; World Health Organisation, 2021). South Africa has a lower carriage rate in urban areas than in rural areas. A few contributing factors to the latter are the horizontal transmission at school-going age and at the time of sexual activity (Kew, 2008; Kiire,1996). Studies have established that there is a high prevalence of HBV among pregnant woman in South Africa; therefore, increasing perinatal HBV transmission and decreasing the transfer of HBsAb due to immunosuppression. Children exposed to HBV during childhood go on to infect other adolescents once they reach sexual maturity, making sexual transmission of HBV the dominant horizontal route of transmission during adolescent stages (Andre, 2000; Ayoola, 1988; Burnett et al., 2005; Kew, 1996; Kiire, 1996; Lin and Kirchner, 2008; Mphahlele, 2002). The prevention of HBV is therefore of great relevance in the South African setting, particularly amongst high-risk groups.

The WHO has formulated several global public plans addressing HBV vaccination, rapid viral testing and advanced antiviral therapies to prevent HBV infection and HBV-related disease progression (World Health Organisation, 2017b). One of the public plans established by the WHO is the EPI programme. This programme allows infants from as early as birth to be vaccinated against HBV, including infants of low birth weight and older children who did not receive vaccination as infants (World Health

Organisation, 2017a). The EPI programme has been extremely effective where implemented and this was made evident in China, where chronic HBV infections decreased by 33.9% (World Health Organisation, 2017a). Vaccination for HBV was included in the South African EPI from 1995. In the current study, 122 South African participants were recruited from two tertiary education institutions in the Free State, namely the Central University of Technology (CUT) and the University of the Free State (UFS). The inclusion and exclusion criteria included factors such as age, where participants had to be between the ages of 18 and 22. Participants also had to be South African citizens. These criteria allowed the study to have a high number of participants vaccinated with HBV vaccine during childhood due to the initiation of the EPI programme in South Africa as from 1995. The study therefore aimed to see whether they still had detectable antibody responses and how well they responded to booster doses of HBV vaccine.

HCWs are considered at a high risk of HBV exposure and have a four times greater probability of contracting the virus than the general population, simply because HCWs are exposed to blood and body fluids as well as needle stick and sharps injury (Di Giuseppe et al., 2007; Lamberti et al., 2015). HBV infection rates among HCWs remain high in South Africa due to policies that require of students to pay for their own vaccinations and post-vaccination testing. Many students require financial support; therefore, it becomes a challenge for most students to be vaccinated as required (Burnette et al., 2021). Fortunately, the EPI programme has accommodated HCWs to be vaccinated in childhood and this has been beneficial for participants of this study, as they are medical and Biomedical students who will be undergoing training and later work in an environment where HBV is quite prevalent and in which they will be exposed to patients who may be infected with HBV.

In South Africa, the highest racial population group are Africans, and when comparing gender, South Africa has a higher number of females than males (Statistics South Africa, 2022). This explains the predominantly African population participation in this study (75%), with a higher female participation (61%) than males (39%).

4.2 Clinical data

Persistent HBV is characterized by a weak adaptive immune response, which could be caused by factors such as immunological tolerance, mutational epitope inactivation,

T-cell receptor antagonism, incomplete down regulation of viral replication and infection of immunologically privileged tissue (Chisari et al., 2010; Gerlich, 2013). 80–90% of infants will develop chronic HBV if they become infected during their first year of life, while 30–50% of children who were infected with HBV before the age of 6 years will develop chronic HBV (World Health Organisation, 2017). In adults, less than 5% of healthy persons infected with HBV will develop chronic infections, while 20–30% of adults infected chronically with HBV will develop cirrhosis and/or liver damage (World Health Organisation, 2017b). One of the participants tested positive for HBcAb, meaning that the participant has either a current or previous HBV infection. As HBsAb was also present in this participant, it confirms that the individual has resolved the past infection. This infection could have occurred as far back as during early childhood.

The majority of the participants were unaware of whether they had any history of jaundice or liver disease. However, 4% were aware of one of the liver diseases, which simply suggests that these participants could have undiagnosed hepatitis or other liver-related diseases. The fact that the majority of participants are not aware of their history of liver-related diseases is a concern, as this could mean that the majority of the general population are not aware of whether they have chronic HBV or not. Therefore, the need for more information and education on HBV should be prioritized amongst the general population.

Only one participant in this study provided childhood immunization records. The remainder were unaware of their vaccination status and did not have access to their childhood immunization records. This indicates the need for exploring alternative methods of documenting immunization in order to improve record keeping and provide better continuity of care.

4.3 Laboratory data

4.3.1 Introduction

This study describes long-term humoral and cellular immunity among South African students who received childhood HBV vaccination, with the first objective to investigate humoral immunity in young adults following childhood vaccination. HBsAb and HBcAb were the two tests used to fulfil this objective by detecting whether participants still have protective antibody levels following their childhood vaccinations,

as well as to check whether participants might have had a past or current infection. Rapid HBsAb production following exposure to a booster dose of HBV vaccine is an indication that there is a memory B-cell response induced by the initial vaccine course or subsequent HBV exposure. Only one participant tested positive for HBcAb and HBsAb, indicating a previous resolved infection with HBV. This amounts to 0.8%, which is lower than the prevalence of HBV in South Africa. Confirmation to the latter is in the literature mentioned in the study, which varies, because the prevalence of HBV varies according to the race, gender and location (rural vs urban areas). The literature states a study that has established the prevalence of HBV to be the highest amongst rural black males at 15.8% (Kew, 2008; Kiire,1996). In another South African study that measured the HBV prevalence for a five-year period (2015–2019), it was established that the annual national HBV prevalence rate per 100 000 population had increased from 56.14-74.17 in 2015 to 2017. It then decreased to 62.81 in 2018 and then increased again to 67.76 in 2019 (Moonsamy et al., 2022). All participants in this study should have been vaccinated against HBV during early childhood as part of the EPT programme established in 1995 in South Africa, because all participants were born after 1995 and are South African citizens. The low rate of HBV infection in this study brings us to the hypothesis that childhood vaccinations have provided protection against HBV infection, therefore reducing the rate of infection

Currently, the first- and second-year students recruited for this study should have been immunized against HBV during childhood as part of the EPI programme. This vaccination programme is expected to provide long-term protection against HBV infection. However, more than half of the participants (52%) tested negative for HBsAb at baseline. This is suggestive of waning humoral immunity, which supports a study that has proven that HBsAb levels decline with time, but usually persists for at least 10–15 years after vaccination (Fujisawa et al., 1996; Kao and Chen, 2005). Causes of waning immunity remain unknown; however, immune memory is a key characteristic in determining long-term protection (Lu et al., 2008). Possible factors that could induce waning are weight, age and the type of vaccination administered.

4.3.2 HBsAb

During the study, vaccination was provided to participants on three occasions, namely at 0, 1 and 6 months as per current recommendations (World Health Organisation,

2017a). At baseline, 43% of participants tested positive to HBsAb. At second sampling, 92% of the participants tested positive to HBsAb. This indicates a strong anamnestic response and this is an indication that most participants have likely been vaccinated previously during childhood. Even though the baseline results (43%) are suggestive of antibodies that have waned among the participants, the rapid anamnestic response has proven that the participants have developed immunological memory. This highlights the importance of routine testing for HBsAb levels following vaccination in high-risk groups, in order to confirm adequate protection in the event of HBV exposure. During the third sampling, three participant samples were not received; therefore, 119 samples were analysed and one of these samples had a titre value of 11.2 mIU/mL, while the rest of the samples gave a titre level of >1000 mIU/mL. All participants therefore tested positive for HBsAb at the end of the three vaccinations, indicating that there were no non-responders in this group.

4.3.2 IFN-y and IL-2 assays

Another objective of this study was to investigate memory cellular immunity among young adults following childhood vaccination. IFN-y and IL-2 analysis was used to achieve this by stimulating whole blood with HBV antigens which, if positive, will release IFN-y or IL-2 that functions to restrict HBV replication by affecting multiple steps in the viral life cycle (Wieland et al., 2004) and this will be evidence to prove the presence of memory T-cells produced during childhood vaccination.

4.3.2.1 IFN-y

At baseline, the sensitivity and specificity were 74% and 34%, respectively, with a p-value of 0.68. Due to the p-value exceeding 0.05, it was concluded to be statistically insignificant. This could be caused by a low number of participants due to calibration failure in one of the plates as well as invalid results. A negative predictive value of 55% and a positive predictive value of 47% are suggestive of variability. This therefore decreases the ability of distinguishing between immune and non-immune participants based on the baseline IFN-y values.

After booster vaccinations, the sensitivity and specificity was 72% and 100%, respectively with a p-value of 0.03; therefore, making this statistically significant.

4.3.2.2 Il-2

At baseline, the sensitivity and specificity was 93% and 26%, respectively, with a p-value of 0.02, making it statistically highly significant. IL-2 seemed to be a more reliable assay, as it produced much more higher results at baseline and during second sampling. The participants who tested positive had the highest median of 92 pg/mL After booster vaccinations, the sensitivity and specificity were 85% and 71%, respectively with a p-value of >0.01. Participants who tested positive also had the highest median of 65 pg/mL

When comparing the two ELISA tests, we can confirm, based on the sensitivity, specificity and P-value, that IL-2 is a much more reliable marker to use to evaluate cellular immunity that has been induced by vaccination. This can be seen prior to and after booster vaccination. However, we cannot conclude the above, because a high number of participants were involved during IL-2 testing; therefore, giving a much more accurate platform to make a conclusion.

Both the IFN-y and IL-2 assays are involved in the cellular immune response. However, in this study, they were used as proxies for existing immunological memory. The immune system of the participants has been exposed previously to HBV antigens and this can be seen by the cytokines response when stimulated by the presence of antigens, which can either be through HBV infection or vaccination.

4.4 Conclusion

It is recommended that HB booster vaccination be included in policies as part of a routine programme for all individuals, especially amongst individuals in healthcare settings and other high-risk areas because of the waning immunity in more than half of the participants.

IL-2 assay has proven to be a more reliable assay due to its high sensitivity and specificity; therefore, it can be implemented as one of the routine checks to use in order to assess cellular immunity that is induced due to vaccination.

Only one participant produced his/her vaccination records for the use of the study, while the rest of the participants did not have their records available. Therefore, it is

recommended that in future, more digital records should be used that will be accessible at all healthcare settings.

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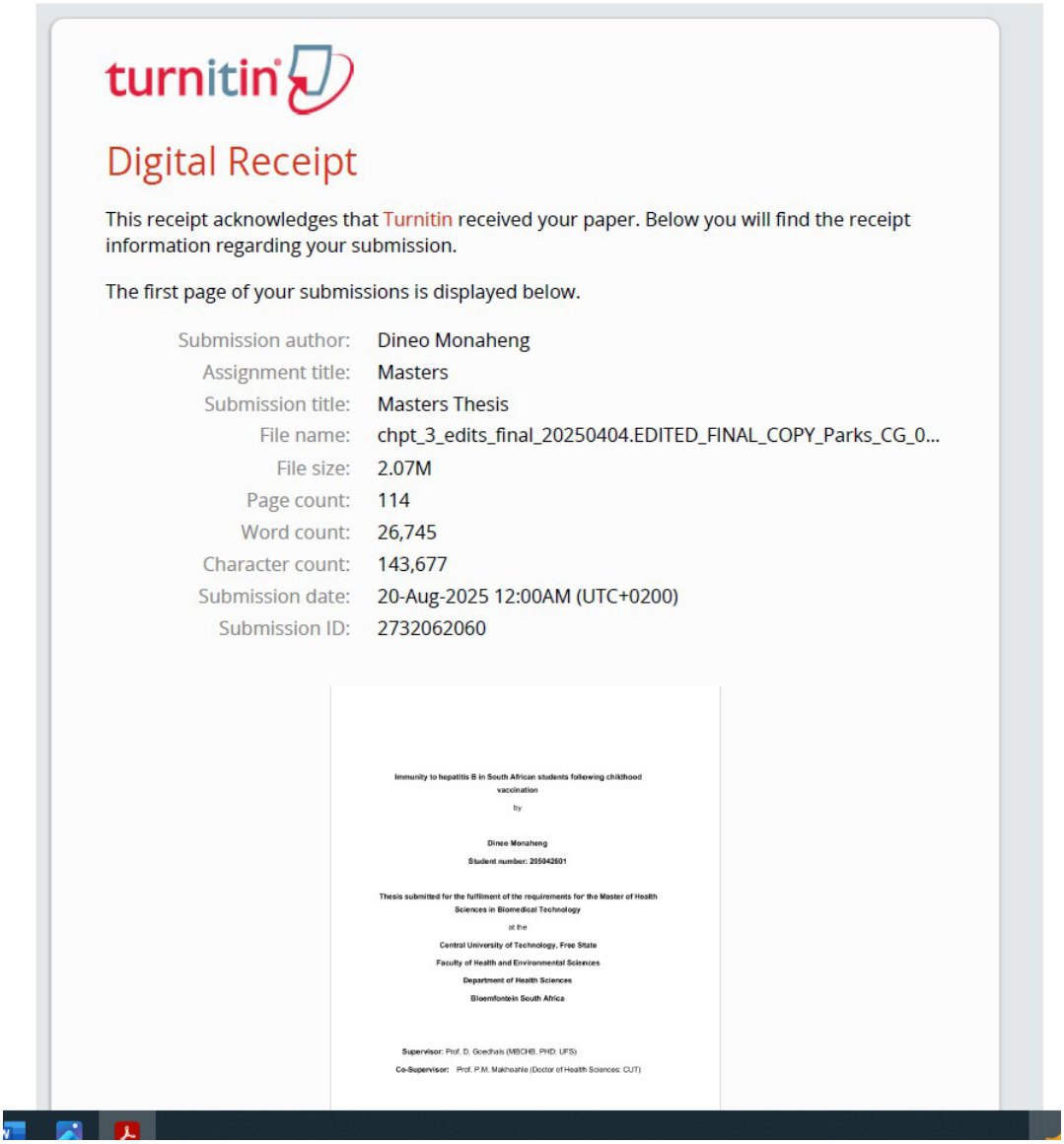

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Immunity to hepatitis B in South African students following childhood vaccination

by

Dineo Monaheng

Student number: 205042601

Thesis submitted for the fulfilment of the requirements for the Master of Health Sciences in Biomedical Technology

at the

Central University of Technology, Free State

Faculty of Health and Environmental Sciences

Department of Health Sciences

Bloemfontein South Africa

Supervisor: Prof. D. Goedhals (MBChB, PHD: UFS)

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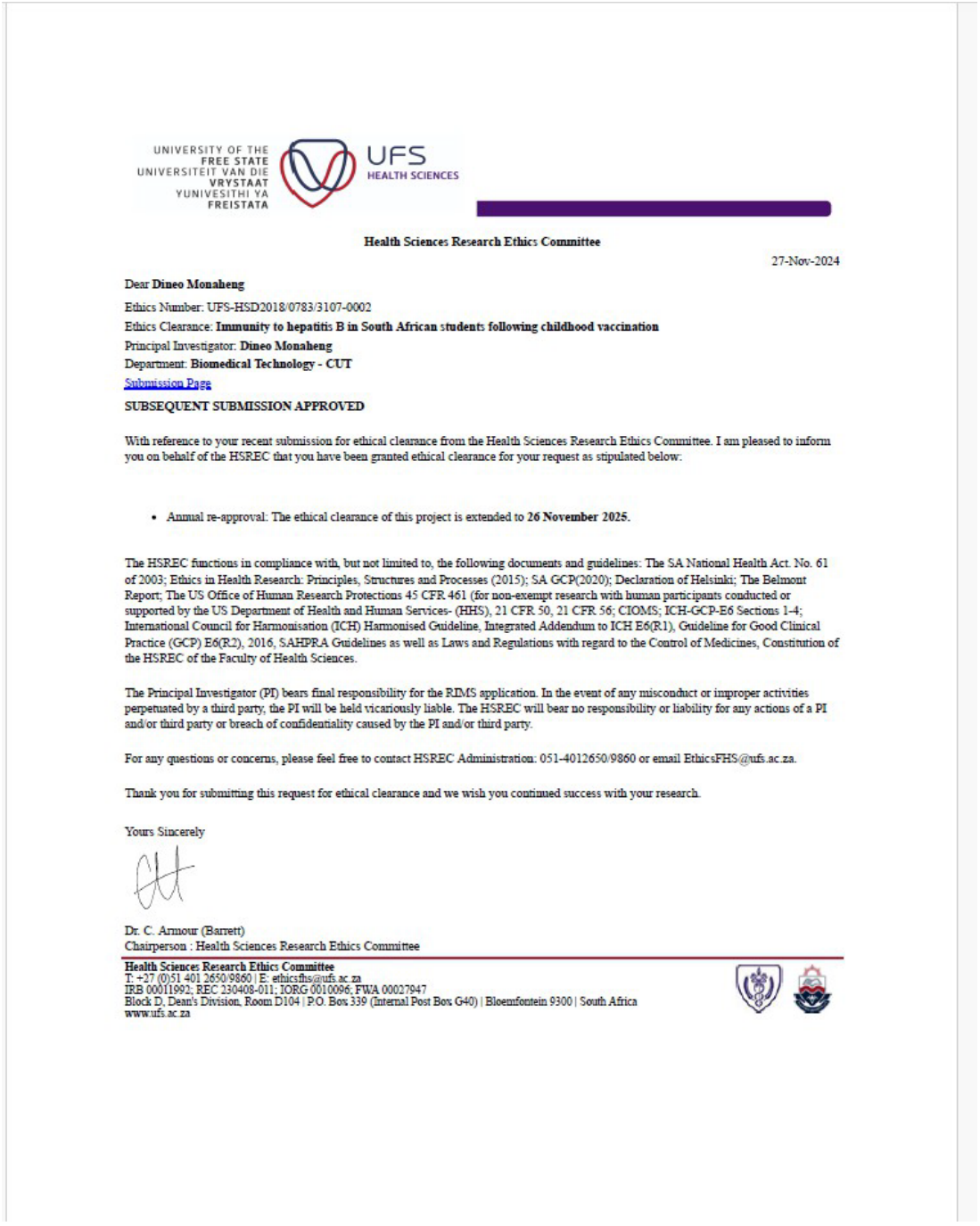

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Health Sciences Research Ethics Committee

27-Nov-2024

Dear **Dineo Monaheng**

Ethics Number: UFS-HSD2018/0783/3107-0002

Ethics Clearance: **Immunity to hepatitis B in South African students following childhood vaccination**

Principal Investigator: **Dineo Monaheng**

Department: **Biomedical Technology - CUT**

Submission Page

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

- Annual re-approval: The ethical clearance of this project is extended to **26 November 2025.**

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2020); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetuated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of confidentiality caused by the PI and/or third party.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4012650/9860 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Dr. C. Armour (Barrett)
Chairperson : Health Sciences Research Ethics Committee

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Herewith I, **Cornelia Geldenhuys (ID 521114 0083 088)** declare that I am a qualified, accredited language practitioner and that I have edited the following master's dissertation:

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CUT Permission Letter request letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

10 March 2018

Dr A Szubarga
Central University of Technology
Bloemfontein

Dear Dr Szubarga,

Re: Permission to perform research project "Immunologic memory to hepatitis B vaccine in South African students after neonatal vaccination."

I hereby request permission for participation of students from the Faculty of Health and Environmental Sciences, Central University of Technology in the above mentioned study. This is a joint project between Mr P Makoahle at the Faculty of Health and Environmental Sciences, CUT and the Division of Virology, UFS. The study will also contribute towards the MTech qualification of Mrs D Monaheng who is employed in the Faculty of Health and Environmental Sciences, CUT. Funding has been obtained from the CUT & UFS Joint Research Programme and the Department of Higher Education and Training.

The study will recruit 1st and 2nd year students in Biomedical Technology from CUT and 1st year Medical students from UFS at the time of their hepatitis B vaccination. Currently three doses of hepatitis B vaccine are recommended for all students followed by laboratory testing for proof of immunity. As the current student cohort was vaccinated during childhood as part of the South African Department of Health Expanded Programme on Immunization, we would like to determine whether the current vaccination policy requires adjustment. The students who participate in the study will be provided with the result of laboratory testing which can be used as proof of immunity, and those without responses will be counseled by a medical doctor regarding further management.

Approval for the study has been obtained from the Health Sciences Research Ethics Committee of the UFS, the Acting Head of the Department of Health and Environmental Sciences (Dr E Vermaak) and Assistant Dean: Research, Innovation and Engagement (Prof C van der Westhuizen) at CUT, and the Vice-Rector: Research (Prof C Witthuhn) at UFS.

We aim to begin recruitment of CUT students in March 2018 in order to complete the three dose vaccination series before the end of the 2018 academic year. I apologise for the urgency of this submission, but was not aware that further permission was required from your office. Please feel free to contact me should you require any additional information.

Yours sincerely,

DGoedhals

Dominique Goedhals

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UNIVERSITY OF THE FREE STATE
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Permission Letter

Central University of Technology, Free State

ACADEMIC PLANNING

DR D GOEDHALS
STAFF NR: 0096727
HEAD: DIVISION OF VIROLOGY
MEDICAL MICROBIOLOGY AND VIROLOGY
HEALTH SCIENCES
UNIVERSITY OF THE FREE STATE

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PERMISSION FOR DR GOEDHALS AND MR MAKHOAHLE TO CONDUCT RESEARCH PROJECT "IMMUNOLOGIC MEMEORY TO HEPATITIS B VACCINE IN SHOURTH AFRICAN STUDENTS AFTER NEONATAL VACCINATION" WITH PARTICIPATION OF STUDENTS IN BIOMEDICAL TECHNOLOGY FROM CUT

Dear Dr Goedhals/ Mr Makhoale

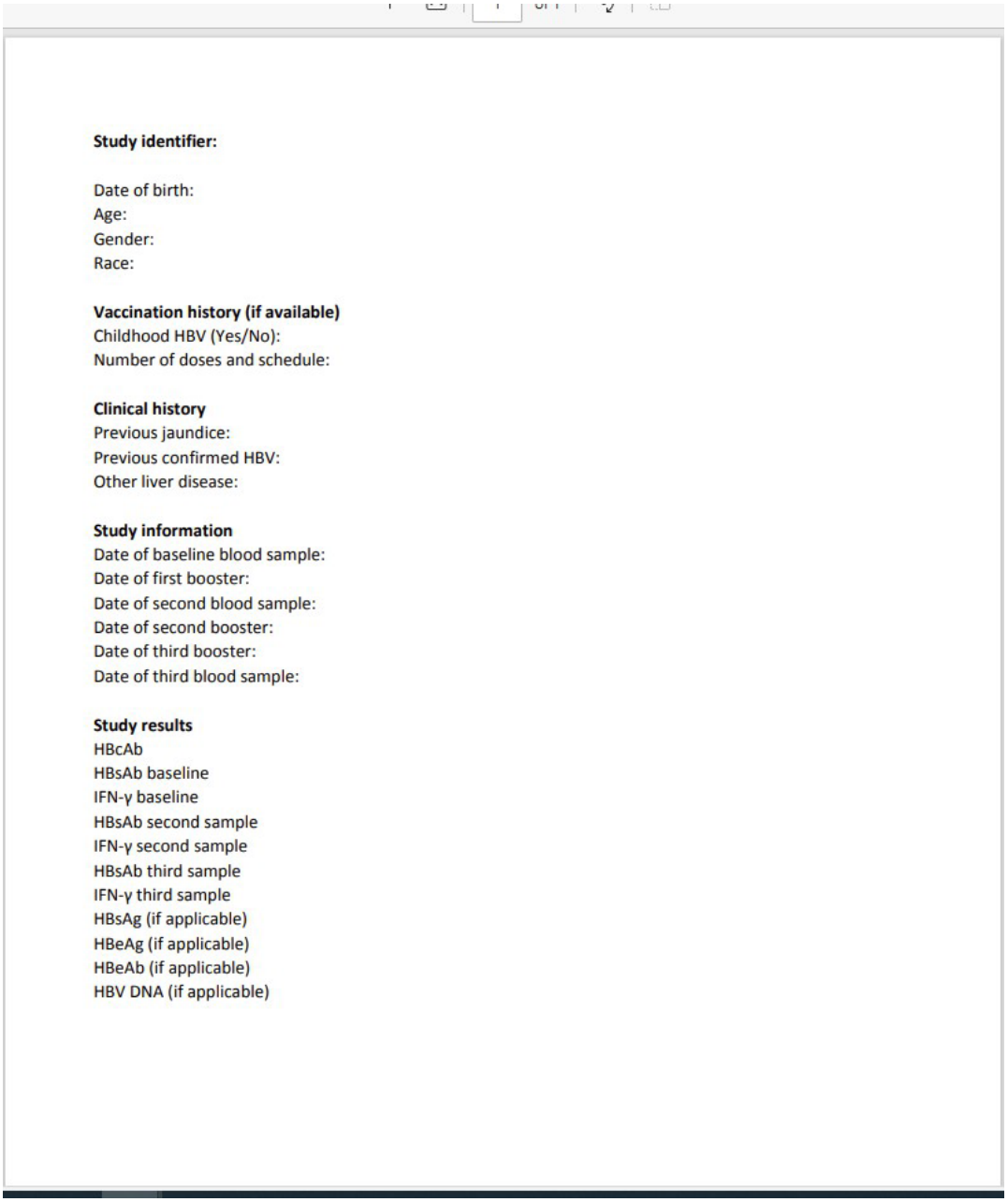
This is to confirm that you have been granted permission to conduct research project "Immunologic memory to hepatitis B vaccine in South African students after neonatal vaccination" with participation of students in Biomedical Technology from CUT.

The conditions of the conditional permission are:

- The research will not interrupt any of the official activities at the CUT;
- You will supply us with the copy of your report;
- The cost of all related activities will be covered by yourself;
- Recruitment of participants is the sole responsibility of yourself;
- Voluntary nature of the potential participant's decision to consent to participate should be strictly observed;
- You should not disclose a potential participant's decision to participate or otherwise to any other party;
- Permission does not compel, in any sense, participation of staff members or students in your survey.

ACTING DIRECTOR: INSTITUTIONAL PLANNING AND QUALITY ENHANCEMENT
DR A SZUBARGA
12 MARCH 2018

Data Collection form:



Study identifier:

Date of birth:
Age:
Gender:
Race:

Vaccination history (if available)
Childhood HBV (Yes/No):
Number of doses and schedule:

Clinical history
Previous jaundice:
Previous confirmed HBV:
Other liver disease:

Study information
Date of baseline blood sample:
Date of first booster:
Date of second blood sample:
Date of second booster:
Date of third booster:
Date of third blood sample:

Study results
HBcAb
HBsAb baseline
IFN-γ baseline
HBsAb second sample
IFN-γ second sample
HBsAb third sample
IFN-γ third sample
HBsAg (if applicable)
HBeAg (if applicable)
HBeAb (if applicable)
HBV DNA (if applicable)

Example of Consent form

CONSENT DOCUMENT (version 1.1)

PROJECT TITLE: Immunologic memory to hepatitis B vaccine in South African students after neonatal vaccination. (ECUFS number:__)

You have been asked to participate in a research study. You have been informed about the study by medical staff from the Department of Medical Microbiology and Virology.

You may contact Dr Dominique Goedhals at 051 4053162 at any time if you have questions about the research or if you are injured as a result of the research. You may contact the Secretariat of the Health Sciences Research Ethics Committee, UFS at telephone number 051 4017794/5 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

1. I will donate blood on 3 separate occasions as described in the information document
2. My blood may be stored for future research
3. Research data obtained from the study may be published anonymously in a scientific journal

Name of participant:

Telephone number:

Email:

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date
(Where applicable)

_____ _____
Signature of Translator Date
(Where applicable)