Hepatitis B virus mother-to-child transmission in Namibia: transmission dynamics and possibilities for elimination

By

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Declaration

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Abstract

Introduction: Hepatitis B virus (HBV) remains endemic in sub-Saharan Africa (SSA). While the roll-out of pediatric HBV immunization from six weeks of age has had an impact on horizontal transmission of the virus, mother-to-child transmission (MTCT) has been identified as the driver of the current HBV epidemic in the region. Given the high likelihood of developing chronic HBV infection (CHB) if the infection is acquired during infancy, preventing HBV MTCT in SSA is vital. Where MTCT occurs, it is essential to identify the HBV-infected children for appropriate management, especially in the context of HIV. Current antiretroviral therapy (ART) for the management of HIV infection includes tenofovir for children ≥ 10 years old. Children below the age of 10 years are treated with lamivudine. However, many of the HIV/HBV co-infected children are left on lamivudine treatment for more than ten years and are at risk of developing HBV drug resistance and uncontrolled HBV infection. Uncontrolled HBV infection is a known factor for increased risk of severe liver damage. **Aim:** This research project aimed to (1) assess the molecular character of HBV and the liver health of HIV/HBV co-infected children who have been on long-term lamivudine treatment, (2) to determine the feasibility of a screen-treat-vaccinate intervention to prevent HBV MTCT, and (3) to measure the costs and health outcomes of combined prophylactic measures against HBV MTCT, in Namibia. **Methodology:** Three sub-studies were conducted as part of this research project, to answer each of its aims. The first sub-study involved HIV/HBV co-infected children and adolescents below the age of 18 years old, and who have been exposed to lamivudine. Venous blood samples were collected from these children for HBV serological testing (HBsAg, HBeAg, anti-HBe and anti-HBc total) using Murex ELISA assays. Dried blood spots (DBS) samples were used for HBV DNA levels measurement and genotyping. HBV DNA measurements were completed using the automated AmpliPrep/COBAS TaqMan HBV test V2.0. Genotyping and mutation analyses were performed using online tools. Liver health was assessed through AST platelet ratio index (APRI). An APRI score > 0.5 was considered a sign of liver fibrosis. Mothers attending with these children and adolescents were also enrolled in the study, to determine the role of HBV MTCT in these pediatric HBV infections. DBS were collected from these mothers for HBV molecular characterization as well. The second sub-study focused on pregnant women attending antenatal clinics (ANCs) in Windhoek. These women were recruited following informed consent and screened for HBV using the Alere Determine™ HBsAg rapid test.

HBsAg positive pregnant women were tested for further HBV serological markers (HBeAg, anti-HBe, anti-HBc IgM), and HBV viral loads were measured to determine the risk of MTCT. Positive mothers at high risk of MTCT were reviewed for antiviral prophylaxis and offered treatment where necessary. HBV-exposed babies were immunized as per Namibian guidelines, and followed-up to determine the rate of MTCT. The feasibility of offering routine antenatal HBV rapid testing was assessed quantitatively and qualitatively. The former involved determining the diagnostic accuracy of the rapid test used for HBsAg screening, and the latter focused on the perceptions of this antenatal care service by healthcare workers (HCWs). In the third sub-study, the costs and health outcomes of four interventions against HBV MTCT were assessed through a cost-effectiveness analysis. The interventions included: (1) universal birth dose (BD) vaccination, (2) BD vaccination and HBIG, (3) BD vaccination, HBIG, and maternal antiviral prophylaxis informed by sequential HBV viral load testing, and (4) BD vaccination, HBIG, and maternal antiviral prophylaxis informed by sequential HBeAg testing. All resources including consumables, HCW's time, building space and facilities (and their quantity) were measured and valued to determine the unit costs of HBsAg screening at the ANCs, providing antenatal treatment and administering pediatric immunoprophylaxis. Health outcomes were measured in terms of the number of pediatric HBV infections averted. The incremental cost-effectiveness ratios (ICERs) of these interventions were calculated and were used to compare each intervention to the previous less expensive one. **Results:** Fifteen HIV/HBV co-infected children/adolescents and six mothers attending with the children were enrolled in the first sub-study. Ten serum samples obtained from Windhoek were further tested for HBV serological markers; seven were HBeAg positive/anti-HBe negative (7/10; 70%), three were HBeAg negative (3/10; 30%), and all were reactive for anti-HBc (total) (10/10; 100%). Among HBeAg negatives, one was anti-HBe negative and two were anti-HBe positive. Eight of the fifteen children (8/15; 53.3%) were HBV DNA positive. The viral strains were grouped with genotype E (6/8; 75%) and genotype D3 (2/8; 25%) and harbored lamivudine drug-associated resistance variants and immune escape mutants. Liver health was assessed in nine children: five with detectable levels of HBV DNA and four with undetectable levels of HBV DNA. An abnormal APRI score of 0.713, was detected in one HBV DNA positive child (1/9; 11.1%). In the second sub-study an HBsAg seroprevalence of 5.4% was observed among 515 (28/515) pregnant women enrolled at ANCs in Windhoek. Three pregnant women (3/28; 10.7%) were positive for HBeAg; of whom one was HIV/HBV co-infected and the other two were HBV mono-infected. The two

(2/28; 7.14%) HBV mono-infected/HBeAg-positive patients presented with viral load > 10^5 IU/ml, the study cut-off for antenatal treatment to prevent HBV MTCT; one received antiviral prophylaxis with tenofovir, the other was offered prophylaxis but did not receive it. Postpartum, 25 of the 28 HBV-exposed babies (25/28; 89.3%) were traced and followed-up to determine their HBV status and the rate of HBV MTCT. Fourteen (14/25; 56%) were males, and eleven were females (11/25; 44%). All babies had been vaccinated against HBV at birth, and 15 (15/25; 60%) had received hepatitis B immunoglobulin (HBIG). The 25 babies were tested for HBsAg at a median age of seven weeks (Range: 5.57 weeks – 20.29 weeks). All were non-reactive for HBsAg, including both babies born to the highly viremic women. With regards to the feasibility of HBV rapid testing as part of antenatal care services, the Determine™ HBsAg rapid test had a 100% diagnostic sensitivity and specificity. HCWs found the test simple to use and showed a preference for rapid testing over laboratory testing for routine antenatal screening of HBV. They believed that this method would improve early diagnosis and treatment of HBV of pregnant women. In the cost-effectiveness analysis conducted in the third sub-study, a preventive strategy with universal BD vaccination alone was the cheapest option but was less effective. Adding HBIG to BD vaccination, and providing maternal antiviral prophylaxis was the most effective and the most costly strategy. The strategy that includes antiviral prophylaxis with sequential HBeAg testing added to BD vaccination and HBIG had an ICER of US$6 262.42 per infection averted, in comparison to the strategy including BD vaccination and HBIG only. The BD vaccination and HBIG strategy had an ICER of US$4 550.34/ pediatric HBV infection averted, in comparison to BD vaccination alone. These ICERs were highly sensitive to the prevalence of highly infectious pregnant women, the cost of the HBeAg test, and the effectiveness of each strategy for preventing MTCT in highly infectious pregnant women. **Conclusion:** Results from this research project reemphasized the issue of pediatric CHB, especially in HIV/HBV co-infected children. The data described in this study also showed that elimination of MTCT of HBV in Namibia is achievable, through routine antenatal HBsAg screening, treating pregnant women at high risk of MTCT, and providing HBV vaccination from birth. Screening using rapid testing was found cheap, and a feasible alternative for detecting HBV infection in pregnant women. The costs and health benefits of implementing antenatal antiviral prophylaxis and HBIG presented in the study provide background data for further assessment of the value for money of these interventions in SSA, and to explore alternatives excluding HBIG for HBV PMTCT.

Abstrakt

Inleiding: Die Hepatitis B virus (HBV) bly endemies in Afrika suid van die Sahara (SSA). Terwyl die uitrol van pediatriese HBV-immunisasie vanaf ses weke 'n impak gehad het op die horisontale oordrag van die virus, is moeder-tot-kind-oordrag (MTCT) geïdentifiseer as die belangrikste dryfveer van die huidige HBV-epidemie in die streek. Gegewe die hoë waarskynlikheid om chroniese HBV-infeksie (CHB) te ontwikkel indien die infeksie gedurende die kinderjare verwerf word, is die voorkoming van HBV MTCT in SSA noodsaaklik. Waar MTCT plaasgevind het, is dit noodsaaklik om die HBV-geïnfekteerde kinders te identifiseer en voortaan te monitor vir gepaste hantering, veral in die konteks van MIV infeksies. Huidige antiretrovirale terapie (ART) vir die behandeling van MIV-infeksie sluit tenofovir vir kinders ≥ 10 jaar in. Kinders onder die ouderdom van 10 jaar word behandel met lamivudien. Baie van die kinders met MIV/HBV ko-infeksie is egter al meer as tien jaar aan lamivudienbehandeling blootgestel en loop die risiko om HBV-middelweerstand en ongekontroleerde HBV-infeksie te ontwikkel. Ongekontroleerde HBV infeksie is 'n bekende faktor vir verhoogde risiko van ernstige lewerskade. **Doelwitte:** Hierdie navorsingsprojek het ten doel om (1) die molekulêre eienskappe van HBV en die lewergesondheid van MIV/HBV mede-besmette kinders te beoordeel wat op langtermyn lamivudienbehandeling was, (2) om die lewensvatbaarheid van 'n sifting-behandeling-inenting ingreep om HBV MTCT te voorkom, te bepaal en (3) om die koste en gesondheidsuitkomste van gekombineerde profilaktiese maatreëls teen HBV MTCT in Namibië te meet. **Metodiek:** Drie substudies is uitgevoer om elkeen van die studiedoelwitte aan te spreek. Die eerste substudie het kinders en adolessente met MIV/HBV ko-infeksie onder die ouderdom van 18 jaar, en wat aan lamivudien blootgestel is, ingesluit. Veneuse bloedmonsters is van hierdie kinders versamel vir HBV serologiese toetsing (HBsAg, HBeAg, anti-HBe en totale anti-HBc) deur die Murex ELISA toetse. Gedroogde bloedvlek (DBS) monsters is gebruik om HBV DNA vlakke te meet en die virus te genotpieer. HBV DNA-metings is voltooi met behulp van die geoutomatiseerde AmpliPrep / COBAS TaqMan HBV toets V2.0. Genotipering en mutasie analises is uitgevoer met behulp van aanlyn-metodes. Lewer gesondheid is geassesseer deur die AST bloedplaatjie verhouding indeks (APRI) te gebruik. 'n APRI-telling> 0.5 is beskou as 'n teken van lewerfibrose. Betrokke moeders is ook in die studie ingeskryf om die rol van HBV MTCT in die pediatriese HBV-infeksies te bepaal. DBS is ingesamel uit hierdie moeders vir HBV molekulêre karakterisering. Die tweede substudie het

gefokus op swanger vroue wat voorgeboorteklinieke (ANC's) in Windhoek bygewoon het. Hierdie vroue is gewerf na ingeligte toestemming en gesif vir HBV met behulp van die Alere Determine ™ HBsAg vinnige toets. HBsAg positiewe swanger vroue is verder getoets vir HBV serologiese merkers (HBeAg, anti-HBe, anti-HBc IgM) en HBV virale ladings is gemeet om die risiko van MTCT te bepaal. Positiewe moeders met 'n hoë risiko van MTCT is oorweeg vir antivirale profilakse en behandeling is aangebied waar nodig. HBV-blootgestelde babas is geïmmuniseer volgens Namibiese riglyne, en gevolg om die tempo van MTCT te bepaal. Die uitvoerbaarheid van die aanbied van roetine voorgeboortelike HBV vinnige toetsing is kwantitatief en kwalitatief geassesseer. Eersgenoemde behels die bepaling van die diagnostiese akkuraatheid van die vinnige toets wat gebruik word vir HBsAg-screening, en laasgenoemde het gefokus op die persepsies van hierdie voorgeboortesorgdiens deur gesondheidswerkers (HCWs). In die derde substudie is die koste en gesondheidsuitkomste van vier intervensies teen HBV MTCT geassesseer deur 'n koste-effektiwiteitsanalise. Die intervensies sluit in: (1) BD-inenting, (2) BD-inenting en HBIG, (3) BD-inenting, HBIG en antivirale profylaksis van die moeder wat ingelig word deur sekwensiële HBV virale ladingstoetsing en (4) BD-inenting, HBIG, en antivirale profylaxe van die moeder geïnformeer deur sekwensiële HBeAg toetsing. Alle hulpbronne, insluitend verbruiksgoedere, HCW se tyd, gebouruimte en fasiliteite (en hul hoeveelheid) is gemeet en gewaardeer om die eenheidskoste van HBsAg-screening by die ANC te bepaal, wat voorgeboortelike behandeling en die administrasie van pediatriese immunoprofielaksis verskaf. Gesondheidsuitkomste is gemeet in terme van die aantal pediatriese HBV-infeksies afgewend. Die inkrementele koste-effektiwiteitsverhoudings (ICER's) van hierdie intervensies is bereken en is gebruik om elke intervensie met die vorige goedkoper een te vergelyk. **Resultate:** Vyftien MIV/HBV mede-besmette kinders/adolessente en ses moeders was in die eerste substudie ingeskryf. Tien serummonsters wat van Windhoek verkry is, is verder getoets vir HBV serologiese merkers; sewe was HBeAg positief/anti-HBe negatief (7/10, 70%), drie was HBeAg negatief (3/10; 30%) en almal was reaktief vir anti-HBc (totaal) (10/10; 100%). Onder HBeAg negatiewe, was een anti-HBe negatief en twee was anti-HBe positief. Ag van die vyftien kinders (8/15; 53,3%) was HBV DNA positief. Die virusstamme is gegroepeer met genotipe E (6/8; 75%) en genotipe D3 (2/8; 25%) en bevat lamivudien-middel-verwante weerstandsvariante en immuun-ontwyk-mutante. Lewer gesondheid is in nege kinders geassesseer: vyf met waarneembare vlakke van HBV DNA en vier met onopspoorbare vlakke van HBV DNA. 'n Abnormale APRI-telling van 0.713 is

opgespoor in een HBV DNA positiewe kind (1/9, 11.1%). In die tweede substudie is ' n HBsAg seroprevalensie van 5,4% waargeneem onder 515 (28/515) swanger vroue wat by ANC's in Windhoek ingeskryf is. Drie swanger vroue (3/28; 10.7%) was positief vir HBeAg; van wie een MIV/HBV ko-infeksieen die ander twee slegs HBVinfeksie gehad het.. Die twee (2/28, 7.14%) HBV mono-geïnfekteerde/HBeAg-positiewe pasiënte het 'n viruslading> 10^5 IE / ml, die studie afgesny vir voorgeboortelike behandeling om HBV MTCT te voorkom; een het antivirale profilakse met tenofovir ontvang, die ander is profilakse aangebied, maar het dit nie ontvang nie. Postpartum, 25 van die 28 HBV-blootgestelde babas (25/28, 89.3%) is opgevolg om hul HBV status en die tempo van HBV MTCT te bepaal. Veertien (14/25, 56%) was mans, en elf was vroue (11/25; 44%). Alle babas is teen geboorte teen HBV ingeënt en 15 (15/25, 60%) het hepatitis B immunoglobulien (HBIG) ontvang. Die 25 babas is getoets vir HBsAg op mediane ouderdom van sewe weke (Bereik: 5.57 weke – 20.29 weke). Almal was nie-reaktief vir HBsAg, insluitende albei babas wat gebore is aan die hoogs viremiese vroue. Met betrekking tot die haalbaarheid van HBV vinnige toetsing as deel van voorgeboortesorgdienste, het die Determine ™ HBsAg vinnige toets 'n 100% diagnostiese sensitiwiteit en spesifisiteit gehad. HCWs het die toets maklik gevind om te gebruik en het 'n voorkeur getoon vir vinnige toetse oor laboratoriumtoetse vir roetine-voorgeboortelike screening van HBV. Hulle het geglo dat hierdie metode vroeë diagnose en behandeling van HBV van swanger vroue sal verbeter. In die koste-effektiwiteitsanalise wat in die derde substudie gedoen is, was 'n voorkomende strategie met universele BD-inenting alleen die goedkoopste opsie, maar was minder effektief. Die toevoeging van HBIG tot BD-inenting, en die voorsiening van moeder se antivirale profylaksis was die mees effektiewe en die duurste strategie. Die strategie wat antivirale profilakse sluit met sekwensiële HbeAg toets bygevoeg BD inenting en HBIG het 'n ICER van $6 per infeksie afwyk, in vergelyking met die strategie, insluitende BD-inenting en slegs HBIG. Die BD-inenting en HBIG-strategie het 'n ICER van US $ 4 550.34/pediatriese HBV-infeksie afgeneem, in vergelyking met BD-inenting alleen. Hierdie ICER's was hoogs sensitief vir die voorkoms van hoogs aansteeklike swanger vroue, die koste van die HBeAg-toets en die effektiwiteit van elke strategie om MTCT te voorkom in hoogs aansteeklike swanger vroue. **Slotsom:** Resultate van hierdie navorsingsprojek het die kwessie en ernstigheid van pediatriese CHB infeksie herbeklemtoon, veral in MIV/HBV mede-besmette kinders. Die data wat in hierdie studie beskryf is, het ook getoon dat die eliminasie van MTCT van HBV in Namibië bereik kan word deur roetine-voorgeboortelike HBsAg-sifting, behandeling van

swanger vroue met hoë risiko van MTCT en die verskaffing van HBV-inenting vanaf geboorte. Sifting-toetsingmet behulp van vinnige toetsing is goedkoop en 'n haalbare alternatief vir die opsporing van HBV infeksie in swanger vroue. Die koste en gesondheidsvoordele van die implementering van voorgeboortelike antivirale profilakse en HBIG wat in die studie beskryf word, verskaf data vir verdere assessering van die waarde vir geld van hierdie intervensies in SSA en om alternatiewe te ondersoek wat HBIG vir HBV PMTCT uitgesluit het.

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Scientific outputs

Conference publications

1. Tamandjou, C., Kaindjee-Tjituka, F., Brandt, L., Cotton, M., Nel, E., Preiser, W. and Andersson, M., 2017. A cross-sectional study of hepatitis B virus infection in HIV-infected children in Windhoek, Namibia. *BMJ Global Health, 2* (Suppl 2), pp.A47-A48.

Poster presentations

1. Tamandjou Cynthia, Cleary Susan, Wiysonge Charles, Mufenda Josef, Diergaardt Dorothea, Preiser Wolfgang, Andersson Monique. Routine point of care antenatal screening of hepatitis B virus in Windhoek, Namibia: feasibility of implementation. International Liver Congress 2019, Vienna, Austria. 10 – 14 April 2019.
2. Tamandjou Cynthia, Mufenda Josef, Jacobs Graeme, Landuleni Wilson, Nel Etienne, Diergaardt Dorothea, Wiysonge Charles, Preiser Wolfgang, Andersson Monique. Prevention of Mother-To-Child Transmission of Hepatitis B Virus in Windhoek, Namibia. Conference on liver disease, Nairobi, Kenya. 13 – 15 September 2018.
3. Tamandjou Cynthia, Kaindjee-Tjituka Francina, Brandt Laura, Cotton Mark, Nel Etienne, Preiser Wolfgang, Andersson Monique. A cross-sectional study of hepatitis B virus infection in HIV-infected children in Windhoek, Namibia. Eighth EDCTP Forum, Lusaka, Zambia. 6 – 9 November 2016.

Oral presentations

1. Tamandjou Cynthia, Wiysonge Charles, Preiser Wolfgang, Andersson Monique, Cleary Susan. A cost-effectiveness analysis of antenatal screening with maternal antiviral prophylaxis and active-passive immunoprophylaxis in Namibia. School of public health and family medicine annual research day, University of Cape Town. 20 September 2018.
2. Tamandjou Cynthia, Mufenda Josef, Jacobs Graeme, Landuleni Wilson, Nel Etienne, Diergaardt Dorothea, Wiysonge Charles, Preiser Wolfgang, Andersson Monique. Prevention of Mother-To-Child Transmission of Hepatitis B Virus in Windhoek, Namibia. Conference on liver disease, Nairobi, Kenya. 13 – 15 September 2018.

3. Tamandjou Cynthia, Mufenda Josef, Landuleni Wilson, Nel Etienne, Diergaardt Dorothea, Wiysonge Charles, Preiser Wolfgang, Andersson Monique. Mother-to-child transmission of hepatitis B virus in Windhoek, Namibia: transmission dynamics and prevention. Annual Academic day, Stellenbosch University. 29 August 2018.
4. Tamandjou Cynthia, Kaindjee-Tjituka Francina, Brandt Laura, Cotton Mark, Nel Etienne, Preiser Wolfgang, Andersson Monique. A study of hepatitis B virus infection in HIV-infected children in Windhoek, Namibia. Namibia National AIDS Conference. 28 – 30 November 2016.

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List of Abbreviations

aa – amino acid

AASLD – American Association for the Study of Liver Diseases

ALT – Alanine aminotransferase

ANC – Antenatal clinic

Anti-HBc (total) – total antibodies to hepatitis B core antigen

Anti-HBc IgM − Antibody to hepatitis B core antigen immunoglobulin M

Anti-HBe − Antibody to Hepatitis B e antigen

APASL – Asian Pacific Association for the Study of the Liver

APRI – AST Platelet ratio index

AST – Aspartate aminotransferase

ART – Antiretroviral therapy

BCP – Basal core promoter

BD – Birth Dose

bp – base pairs

BREC – Biomedical Research Ethics Committee

CBA – Cost-benefit analysis

CEA – Cost-effectiveness analysis

CET – Cost-effectiveness threshold

CHB – Chronic hepatitis B

CI – Confidence interval

CLIA – Chemiluminescence immunoassay

COR – Crude odds ratio

CP – Core promoter

CPI – Consumer price index

CUA – Cost-utility analysis

CURS - Core upstream regulatory sequence

DALYs - disability-adjusted life years

DBS – Dried blood spots

DNA – Deoxyribonucleic acid

dNTPs – Deoxynucleotide triphosphate

ddNTPs – Dideoxynucleotide triphosphates

ECLIA – Electrochemiluminescence immunoassay

EIA – Enzyme immunoassay

EASL – European Association for the Study of the Liver

EPI – Expanded programme on immunization

E/C/F/TAF – Tenofovir alafenamide co-formulated with elvitegravir, cobicistat, and emtricitabine

FDA – Food and drug administration

g – G-force

HBcAg – Hepatitis B core antigen

HBeAg − Hepatitis B e antigen

HBIG – Hepatitis B immunoglobulin

HBsAg − Hepatitis B surface antigen

HBV − Hepatitis B virus

HBx – Hepatitis B virus X region

HCC – Hepatocellular carcinoma

HCV – Hepatitis C virus

HCW – Healthcare worker

HIV – Human immunodeficiency virus

HREC – Health Research Ethics Committee

ICER – Incremental cost-effectiveness ratio

IU − International unit

kb – kilobase

Lamivudine – 3TC

LHBs − Large hepatitis B surface protein

LDL – Lower than the detection limit

LoQ – Limit of quantification

mCMV – murine cytomegalovirus

MHBs – Middle hepatitis B surface protein

ml – millilitres

MoHSS – Ministry of Health and Social Services

mRNA – messenger ribonucleic acid

MTCT – Mother-to-child transmission

NAT – Nucleic acid testing

NC – negative control

NHP − Normal human plasma

NIBSC – National Institute for Biological Standards and Control

NRCT – Non-randomized controlled trial

NRTI – Nucleoside reverse transcriptase inhibitor

nt – Nucleotide

NTC − No template control

OD – Optical density

ORF – Open reading frame

PC – Positive control

PCR – Polymerase chain reaction

pgRNA – pregenomic ribonucleic acid

PMTCT – Prevention of mother-to-child transmission

POC – point-of-care

POCT – Point-of-care testing

Pol – Polymerase

PT –Proficiency testing

Pre-C – Pre-core

Pre-S – Pre-surface

PT – proficiency testing

QA – Quality assurance

QALYs – Quality-adjusted life years

QC – Quality control

QS – Quantitation standard

RCT – Randomized controlled trial

RDT – Rapid diagnostic test

RMC – Research Management Committee

RNA – Ribonucleic acid

RPR – Rapid plasma reagin

RT – Reverse transcriptase

S/CO – Signal-to-cut-off

SHBs – Small hepatitis B surface protein

SID – Study identification number

SOPs – Standard operating procedures

SSA – Sub-Saharan Africa

Surf – Surface

SVPs – sub-viral particles

TAE – Tris Acetate EDTA

TAF – Tenofovir alafenamide fumarate

Taq – Thermus aquaticus

TBD – Timely birth dose vaccine

TDF – Tenofovir disoproxil fumarate

TPHA – Treponema pallidum haemagglutination

UN – United Nations

URR – Upper regulatory region

US$ – United States dollars

µL – microlitres

VL – viral load

WHO – World Health Organization

YMDD – tyrosine-methionine-aspartate-aspartate

CHAPTER 1: Introduction

Hepatitis B virus (HBV) infection causes inflammation of the liver which may be acute and resolve within six weeks or may become chronic. While the former usually manifests as a self-limiting illness, the latter may result in chronic infection and over the years lead to a number of liver related complications. About 15% to 40% of chronic cases lead to liver cirrhosis; of which 5% to 30% progress to primary liver cancer, specifically hepatocellular carcinoma (HCC), later in life **(Tang et al., 2018)**. HCC may also develop from chronic hepatitis, in the absence of cirrhosis **(Chayanupatkul et al., 2017)**. The likelihood of becoming a chronic carrier of HBV is inversely related to the age at which the infection is acquired. HBV-infected infants and young children are more likely than adults to develop chronic hepatitis B. Those with infections from early childhood present with the highest risk of HCC as a result of viral persistence **(Shimakawa et al., 2013)**. Thus, preventing pediatric (both early infancy and childhood) HBV infection constitutes a key aspect of disease control. Access and scale-up of the HBV vaccine have significantly reduced the prevalence of early childhood infections, from 4.7% to 1.3% in children under the age of five years old in 2015, globally **(WHO, 2017a)**. However, infections in newborns are still occurring **(Wiseman et al., 2009; Chasela et al., 2014; Chotun et al., 2015; Keane et al., 2016, Babatunde et al., 2018)**. Since chronic infection is most likely to occur following infection in neonates, prevention of mother-to-child transmission (MTCT) should be a priority.

Rationale of the study

It has been widely thought that horizontal transmission is the major mode of the spread of HBV in sub-Saharan Africa (SSA); based on highest prevalence of the virus found in children older than five years of age **(Prozesky et al., 1983; Botha et al., 1984)**, and the lower rate of hepatitis B e antigen (HBeAg) seropositivity in pregnant women, when compared to Asian populations. However, an increasing amount of data has emerged, reporting on high rates of HBeAg among both human immunodeficiency virus (HIV)-uninfected **(Andersson et al., 2013; Fomulu et al., 2013; Bayo et al., 2014; De Paschale et al., 2014)** and HIV-infected pregnant women **(Andersson et al., 2013; Thumbiran et al., 2014)** across the region. The presence of HBeAg is usually indicative of high viral replication activity; thus, HBeAg positive pregnant women often present with high levels of HBV deoxyribonucleic acid (DNA) and are at elevated risk of transmitting HBV to their infants **(Okada et al., 1976; Beasley et al., 1977)**. Based on a

large body of data from Asia, the likelihood of an HBsAg/HBeAg positive pregnant woman to transmit HBV to her infant has been estimated to about 87.5%, as opposed to 13.2% from HBsAg positive/HBeAg negative women, both in the absence of pediatric prophylaxis after birth **(Edmunds et al., 1996)**. With timely HBV birth dose (BD) vaccination of infants, this risk drops to about 24% and below 0.5% from HBeAg positive women and HBeAg negative women, respectively **(Lee et al., 2007; Machaira et al., 2015)**. Recent data from SSA suggest a 39% risk of transmission from HBeAg positive women and a 4% risk from HBeAg negative women without pediatric immunoprophylaxis, based on a small subset of data **(Keane et al., 2016)**. Even though HBV MTCT may occur at low prevalence in SSA, as compared to Asia, it plays an essential role in the sustained endemicity of the disease across the region. An estimated 367 250 newborns are infected with HBV at birth, annually, in the region **(Keane et al., 2016)**. These statistics highlight the urgent need for controlling HBV MTCT in SSA. In the absence of effective preventive measures in place such as BD vaccination, continued transmission of HBV to infants would further increase the pool of persons with chronic HBV at risk of developing HBV-associated liver diseases and perpetuate transmission of the virus in communities.

Antiretroviral therapy (ART) effective against HBV include the nucleos(t)ide analogs tenofovir (TDF) and entecavir as the drugs of choice owing to their low probability of inducing viral resistance. In the context of HIV, first-line adult ART includes TDF and lamivudine. In children below 10 years of age, lamivudine is the only available drug against HBV. Although potent, long-term therapy with lamivudine alone results in the emergence of a group of HBV mutations known as the tyrosine-methionine-aspartate-aspartate or YMDD motif mutations in the polymerase (pol) gene of the HBV genome. This group of mutations leads to therapy failure, characterized by increased viral replication and seroreversion to HBeAg. The incidence of these mutations is higher in HIV-infected individuals and is dependent on the duration of treatment: 20% at one year of treatment, 50% at two years, 73% at two to four years, and 94% after four years of lamivudine monotherapy **(Benhamou et al., 1999; Matthews et al., 2010)**. Owing to the overlap of the surface (surf) gene and pol gene reading frames, lamivudine-induced mutations arising in the pol gene will cause alterations on the surf (HBsAg) gene. This may result in the selection of potentially drug-resistant or vaccine escape mutant viruses **(Torresi et al., 2002)**, thus exposing HIV-HBV co-infected children to a higher risk of liver damage. This is another reason to support the need to prevent pediatric HBV infection.

HBV vaccination commencing immediately after birth is the cornerstone of preventing infection in newborns. In much of SSA, HBV BD is not part of the national vaccination schedule, and most infants only start HBV vaccination as from six weeks of age. The risk of transmission can be further reduced by adding passive immunization with hepatitis B immunoglobulin (HBIG) to BD vaccination, especially for those who are most at risk **(Lee et al., 2006, 2007)**. This practice requires identifying HBV-infected HBeAg positive pregnant women. Still, breakthrough HBV infection in infants born to highly viremic HBV-infected mothers may still occur despite both active and passive immunoprophylaxis **(Ngui et al., 1998; Wiseman et al., 2009; Foaud et al., 2015; Sellier et al., 2015)**. For this reason, reducing maternal viral load during pregnancy using antiviral drugs would be of added value **(Xu et al., 2009; Pan et al., 2012; Tan et al., 2016)**. BD vaccination and access to antiviral medication could be a feasible way to prevent MTCT in SSA. In areas where it is available, HBIG may be of added value in those infants at highest risk. Implementation of national policies for the prevention of HBV MTCT not only relies on robust epidemiological data. It would also require an assessment of the costs and health benefits associated with these interventions, alone or combined, to increase social benefits and for practical use of the resources available.

Context of the study – why Namibia?

While HBV statistics are available from most parts of SSA, data on the epidemiology of HBV in Namibia is profoundly lacking. Available HBsAg seroprevalence figures date from studies conducted more than two decades ago in Northern Namibia. These include a 1983 study among the general population living in the Kavango province, where the authors detected a 13.6% HBsAg carrier state **(Joubert et al., 1985)**. Ovambo children and their mothers showed 15% and 11% HBsAg carrier rates, respectively, while a higher seroprevalence (17%) was described among Ovambo men **(Botha et al., 1984)**. The higher prevalence of HBV in men as opposed to women was noted in another study in Eastern Caprivi **(Joubert et al., 1991)**. Joubert et al. (1991) reported a 19.0% (90/475) HBs antigenemia rate in males as compared to 10.7% (45/419) in females **(Joubert et al., 1991)**.

Our study is built on an earlier retrospective study conducted in HIV-infected children and adolescents in two regions of Namibia, namely Ohangwena and Omusati, both in Northern Namibia. HBV prevalences as high as 6.5% and 13.3% were observed among children aged less than one and older than ten years, respectively **(Brandt et al., 2012)**. These data suggested

a high prevalence of HBV pediatric infections, potentially resulting from both vertical and horizontal transmission. As a result of being HIV co-infected, the majority of these children were exposed to lamivudine monotherapy against HBV for long periods of time and may have developed resistant strains. These children may have formed part of a reservoir of HBV infections that may be continuously transmitted in communities. Further research is needed to identify whether this reservoir may form part of an ongoing reservoir of infection.

In 2014, Namibia introduced HBV BD vaccination for all infants. Although this is World Health Organization policy recommendation **(WHO, 2017b)**, Namibia is one of only nine African countries to have implemented this policy **(WHO, 2018)**. It is also one of the few countries in SSA with a documented policy of routine antenatal HBV screening; pregnant women are tested for HBV infection as part of the routine tests performed at first antenatal care visits **(MoHSS, 2011)**. Part of infant care includes the provision of HBIG, in addition to HBV BD vaccination, to babies born to HBsAg positive mothers **(MoHSS, 2014a)**. Given the availability of these national policies, evaluating the feasibility of adding maternal antiviral prophylaxis for pregnant women with high viral loads to avert HBV MTCT would serve as a proof of concept on the possibility of controlling HBV MTCT in SSA. The costs and health benefits associated with a screen-vaccinate (BD +HBIG) health intervention, and with a screen-treat-vaccinate intervention, through economic evaluations, would also be valuable for policy-making nationally and across the SSA region.

Chapter 2 is a literature review, presenting the epidemiology of HBV, both in the pediatric and the antenatal populations of SSA as well as HBV MTCT and preventive measures against HBV MTCT. It also discusses the published literature on the economic analyses of these preventive interventions, alone or combined.

Chapter 3 describes the investigation of the molecular epidemiology of HBV in HIV/HBV co-infected children and adolescents.

Chapter 4 focuses on the feasibility of implementing a screen, treat, and vaccinate health intervention for the prevention of HBV MTCT. The feasibility is assessed in terms of the rate of HBV MTCT, defined as the number of perinatal HBV infections detected in mother-child pairs in the studied cohort. In an attempt to identify those at risk of HBV, the risk factors associated with HBsAg seropositivity in the studied cohort are also reported.

The costs, as well as the health consequences of this intervention, are reported through a cost-effectiveness analysis in Chapter 5. This chapter also reports on the costs and health benefits

of the current strategy in place in Namibia for the prevention of HBV MTCT and universal BD vaccination alone.

Chapter 6 summarizes the significance of findings presented in chapters 3, 4 and 5 and discusses their implications for addressing HBV MTCT in both Namibia and SSA. Concluding remarks are also presented.

CHAPTER 2: Literature Review

This chapter describes hepatitis B virus (HBV) and its epidemiology, specifically within the obstetrics and pediatric populations in sub-Saharan Africa (SSA). This is followed by a breakdown of the natural history of the infection and available HBV diagnostic technologies. The dynamics of transmission of the virus within the African population are also elaborated on, with a focus on mother-to-child transmission (MTCT). The strategies to prevent transmission are reviewed, and finally, economic evaluations of the different prophylactic options are discussed.

2.1 Virology of hepatitis B virus

2.1.1 Viral taxonomy

HBV is a prototype of the *Hepadnaviridae* family of small hepatotropic enveloped DNA viruses. Viruses within this family are grouped in two genera: while *Avihepadnaviruses* infect birds, *Orthohepadnaviruses* mainly infects mammals. Members of the *Orthohepadnaviruses* genus include HBV, the Woodchuck Hepatitis Virus (WHV) and Ground Squirrel Hepatitis Virus (GSHV) affecting humans and apes, woodchucks, and ground squirrels, respectively **(Howard, 1994)**.

2.1.2 Viral morphology

Electron microscopy of the virus reveals the presence of three morphologically distinct forms of the virus. Spherical and filamentous particles of 20-22 nm in diameter are distinguished from a double-shelled particle of 42 nm in diameter; that is the complete virion known as the Dane particle **(Dane, Cameron & Briggs, 1970)**. The virion is surrounded by the Envelope – a lipid bilayer in which are embedded viral surface proteins (HBsAg). This bilayer protects an icosahedral nucleocapsid core enclosing the DNA genome, the core antigen (HBcAg) and the e antigen (HBeAg). The 20 nm particles, referred to as the sub-viral particles (SVPs), do not contain the viral genome **(Farag & Mansour, 2016)** and are not infectious. They represent an excess of the HBsAg produced during viral replication, usually exceeding the concentration of Dane particles by a factor of 10^3 to 10^5 **(Howard, 1986; Ganem & Prince, 2004)**. SVPs are thought to play a role in immune evasion **(Ganem, 1991; Ortega-Prieto & Dorner, 2017)**, and appear to increase HBV infectivity **(Bruns et al., 1998)**.

2.1.3 Organization of the HBV genome

The HBV genome is a partially double-stranded deoxyribonucleic acid (DNA) molecule consisting of a 3200 base pairs (bp) complete (+) strand and an incomplete (-) strand, shorter by about 700 nucleotides (nt) (~2500 bp). The DNA molecule is held in a circular conformation by a cohesive overlap between the 5'ends of the two DNA strands. The viral proteins are encoded by four open reading frames (ORFs) namely polymerase, surface, core, and X, overlapping each other (See Figure 2.1) **(Howard, 1986; Ganem & Prince, 2004)**. The polymerase (P) ORF is made of three functional domains namely: the terminal protein (TP) – priming domain involved in the priming of the minus strand synthesis (initiation of reverse transcription), the reverse transcriptase (RT) domain coding for the viral Polymerase enzyme used during viral replication, and the RNase H domain. The RNase H domain encodes the RNase H enzyme that cleaves the template messenger ribonucleic acid (mRNA) used for the synthesis of the minus DNA strand during reverse transcription. The surface (S) ORF encodes for the large (LHBs), middle (MHBs) and small (SHBs) surface glycoproteins. The translation of these proteins is initiated by three distinct start codons situated in the S, pre-S2 and pre-S1 domains, coding for LHBs, MHBs and SHBs, respectively. All proteins share a common C-terminus. Pre-core (pre-C) and core are encoded from the core ORF through two in-frame initiation start sites (AUG), yielding two small proteins. Synthesis of the Core protein (HBcAg) is prompted by the translation of the internal AUG **(Glebe & Bremer, 2013)**. HBcAg is a structural protein that is essential for viral assembly and encapsidation of the viral genome and polymerase **(Birnbaum & Nassal, 1990)**. The translation of the upstream AUG results in the synthesis of a precursor polypeptide. Further processing of this polypeptide in the cells' endoplasmic reticulum leads to the secretion of a non-structural protein named hepatitis B e antigen (HBeAg). HBeAg plays no role in the viral life cycle but is a serological marker of high viral replication. It is used as a proxy to assess infectivity **(Siegert et al., 1979)**. The protein behaves like an immune tolerogen, and as such has been associated with the establishment of viral chronicity **(Milich et al., 1990; Chen et al., 2004; Walsh & Locarnini, 2012)**. The smallest ORF codes for a 154 amino acid (aa) long regulatory protein called the X protein. HBx is a multi-functional protein that modulates a number of host and cellular processes, such as signal transduction and gene expression **(Ganem & Prince, 2004; Bouchard & Schneider, 2004)**. The protein is essential in initiating and maintaining viral replication post infection **(Lucifora et al., 2011)**, and has been associated with HBV-induced hepatocarcinogenesis **(Poussin et al., 1999; Zhang et al., 2005; Park et al., 2007)**.

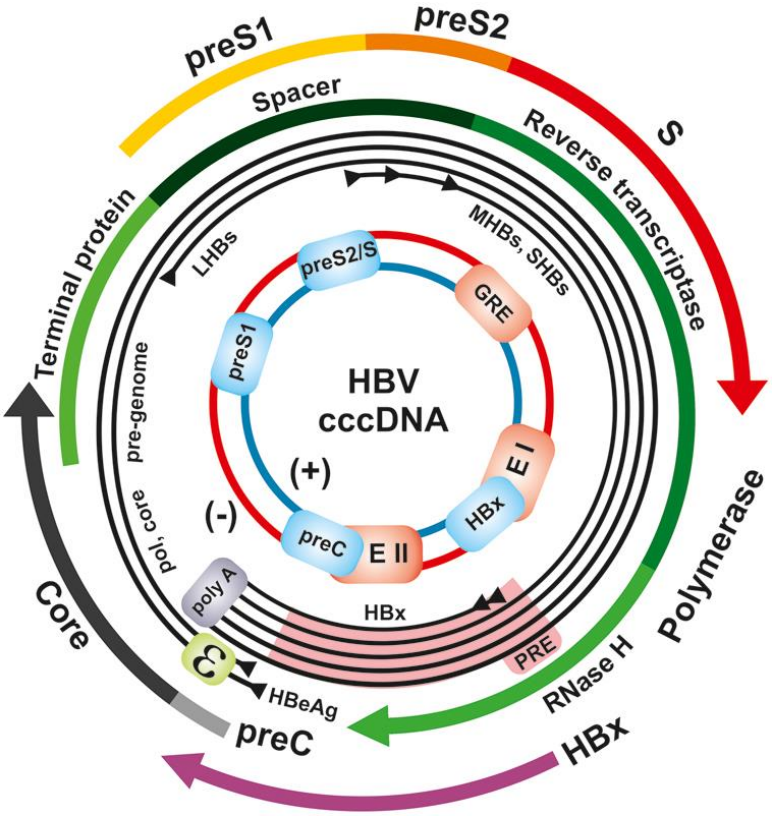


Figure 2.1: The genome of the hepatitis B virus (HBV). The 3.2kb DNA genome is double-stranded with a complete minus (-) strand and the incomplete plus (+) strand. The genome consists of four overlapping frames (ORFs): P, S, C and X. Four polyadenylated 3'co-terminal RNAs of different length ((0.7kb, 2.1kb, 2.4kb and 3.5 kb; represented as black circles), initiated by four viral promoters (pre-C, preS1, preS2/S and HBx; in blue) and two enhancers (Enh1 and Enh2; in red) are used for viral transcription of these four ORFs. During the viral replication cycle, the pre-genome transcript of 3.5kb (pgRNA) is translated to the core protein (the ORF in black), and the viral polymerase (the ORF in green). The other lengthy transcript serves for the expression of the core protein as well as the HBeAg protein. The 2.4 kb mRNA transcript is translated to the large HBV surface protein (LHB; the yellow, orange and red segments of the S ORF) whilst the middle (MHBs; the red and orange segments of the S ORF), and small (SHBs; the red segment of the S ORF) proteins are encoded from the 2.1kb transcript. The smallest mRNA (0.7kb) encodes the X protein (the ORF in purple). E (epsilon) is the signal located in the pgRNA. PRE, posttranscriptional regulatory element, allows the nuclear export of viral mRNAs without splicing **(Nassal & Schaller, 1993; Harrison, Dusheiko & Zuckerman, 2009; Glebe & Bremer, 2013).** Reproduced from Glebe & Bremer, 2013 with permission.

2.1.4 Viral molecular diversity

To date, HBV has evolved to ten phylogenetically distinct genotypes, defined as divergence greater than 8% at the nucleotide level across the complete genomic sequence. These genotypes are named A to J (Table 2.1). Genotypes A, B, C, D, and F are further classified in sub-genotypes, with a 4% intergroup difference in the surface gene **(Lin & Kao, 2017)**. Little is known about genotypes I and J that seem to be products of recombination events between other HBV genotypes **(Tran, Trinh & Abe, 2008; Tatematsu et al., 2009)**. Figure 2.2 depicts the geographical distribution of HBV genotypes.

The molecular diversity of the virus has been implicated as one, among other factors, in the pathogenesis and pattern of transmission of HBV. Genotypes B and C prevail in Southeast Asia, where perinatal transmission of the virus is commonly observed. The predominance of this mode of transmission has been attributed to the high HBeAg seropositivity rate (indicative of high viral replication) of HBV infection observed in women of child-bearing age in the region **(Okada et al., 1976; Tran et al., 2015)**. HBV genotype C infection has been associated with an increased risk of HCC compared to HBV genotype B-related infections **(Yang et al., 2008)**. In Africa, genotypes A (sub-genotypes A1, A3, and A4), D (sub-genotypes D1 and D3) and E are found. Genotype A is predominantly found in SSA, followed by genotype D. Whilst genotype E is more common in West and Central Africa, forming the "genotype E crescent". Reports of this strain outside of the region follow migration patterns from Africa and is often seen among West African immigrants elsewhere **(Palumbo et al., 2007; Rivas et al., 2013; Thurnheer et al., 2017; Malagnino et al., 2018)**. Compared to patients infected with genotype B and C strains, early seroconversion from HBeAg positive to HBeAg negative is seen in individuals infected with genotype A or D viruses **(Livingston et al., 2007)**. Consequently, a lower rate of HBeAg positivity is usually found among pregnant women in Africa; thus, horizontal transmission prevails over perinatal transmission in the region **(Derso et al., 1978; Sellier et al., 2015)**. With regards to pathogenesis in African populations, a greater hepatocarcinogenic potential is seen among patients chronically infected with HBV genotype A compared to carriers of genotype D **(Kew et al., 2005)**. Earlier HBeAg seroconversion and decay of viral replication, and a lower frequency of HCC were observed among patients infected with HBV genotype E compared to genotype A infections **(Shimakawa et al., 2016a)**. A higher prevalence of genomic mutations in the core gene has also been reported among HBV genotype E-infected patients in comparison to genotype A- and D-infected patients **(Thurnheer et al., 2017)**. Genotype F, with four sub-genotypes (F1 – F4), has been isolated in Central and South America **(Kramvis, 2014)**. Reports of genotype G viruses come from France, Germany and the United States **(Stuyver et al., 2000; Lin & Kao, 2017)**. Interestingly, HBV genotype G-related infections can only be established in the presence of other HBV genotypes, mainly genotype A. Phylogenetically this genotype seems to be closer to genotype E, owing to a shared unique 30-basepair segment in the pre-S region **(Lindh, 2005; Kramvis, 2014)**. Genotype H is also found in Central America and Mexico **(Lin & Kao, 2017)**. The new I and J genotypes were isolated in Vietnam and Laos (**Tran, Trinh & Abe, 2008; Thuy et al.,**

2010) and Japan **(Tatematsu et al., 2009)**. Very little is known with regards to the pathogenesis of these viruses.

Table 2.1: Virological characteristics of HBV genotypes (Kramvis, 2014). Reproduced with permission, S. Karger AG Publishers.

Genotype	Length (kb)	Differentiating features	Sub-genotypes
A	3221	6-nucleotide insert at carboxyl end of core gene	A1, A2, quasi-sub-genotype A3 (A3, A4, A5)*, A4 (A6)
B	3215		B1, B2, quasi-sub-genotype B3 (B3, B5, B7–B9, B6 China)* B4, B5 (B6)*
C	3215		C1, quasi-sub-genotype C2 (C2, C14, undefined sequences)*, C3, C4, C5, C6 – C12, C13 – C15, C16
D	3182	33-nucleotide deletion at the amino terminus of the preS1 region	D1, D2, D3, D4, D5, D6
E	3212	3-nucleotide deletion at the amino terminus of the preS1 region	
F	3215		F1, F2, F3, F4
G	3248	36-nucleotide insert, 3′ of position 1905; 3-nucleotide deletion at the amino terminus of the preS1 region; stop codons at positions 2 and 28 of the pre-C region	
H	3215		
I	3215		I1, I2
J	3182	33-nucleotide deletion at the amino terminus of the preS1 region	

*Earlier sub-genotype designation

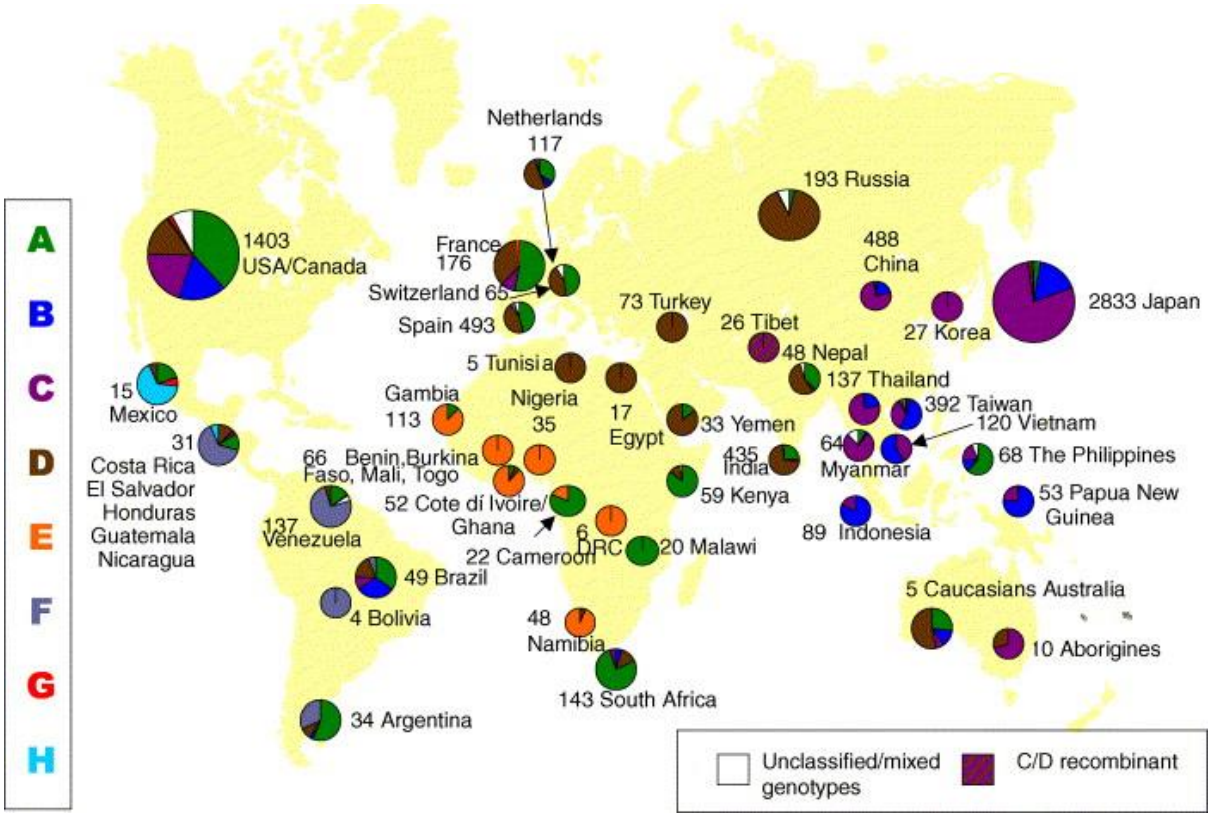


Figure 2.2: Geographic distribution of HBV genotypes. This map presents the global distribution of HBV genotypes. Genotypes A, E, and D are predominantly seen in Africa; with genotype E constrained in West Africa, genotype D dominating Northern Africa and genotype A found in Southern and Eastern Africa. A mixture of these strains is found in Northern and Southern Africa. Central America and Southern America are dominated by genotype F. Genotypes B and C are found in Asia. The numbers seen next to the pie charts represent the number of isolates genotyped. **(Kramvis, Kew & François, 2005)**. Reproduced from Kramvis, Kew & François, 2005 with permission.

2.1.5 Viral genomic mutations

A unique feature of HBV, as a DNA virus, is its replication strategy. Viral replication includes a reverse transcription step, catalyzed through the HBV-encoded RT enzyme. However, the lack of proofreading activity of the RT enzyme leads to transcription errors, resulting in nucleotide substitutions. The frequency of substitution is currently accepted at about 10^{-4} to 10^{-5} nucleotide substitutions per site per year (s/s/t) **(Zhou & Holmes, 2007; Harrison et al., 2011)**. However, recent data suggest that substitution may be occurring at a slower rate of 10^{-6} to 10^{-7} s/s/t **(Littlejohn, Locarnini & Yuen, 2016)**. The overlap of the viral ORFs means that a substitution may affect two frames and possibly lead to a change in amino acids of either or both proteins. These substitutions may have consequences for therapy or prevention, which may be beneficial or harmful to the host.

Mutations in the polymerase gene

The most common group of mutations described in the polymerase gene are found in the tyrosine-methionine-aspartate-aspartate (YMDD) motif, the catalytic site of the DNA polymerase enzyme involved in viral replication **(Cai et al., 2016; Mokaya et al., 2018)**. The selection of mutations in this genomic region is induced by prolonged therapy with anti-HBV nucleos(t)ide analogs targeting the YMDD motif. Seven oral anti-HBV nucleos(t)ide analogs have been approved by the United States of America (USA) Food and Drug Administration (FDA). These are adefovir (Gilead Sciences, California, USA), emtricitabine (Gilead Sciences), entecavir (Bristol-Myers Squibb, New York City, USA), lamivudine (GlaxoSmithKline, Middlesex, United Kingdom (UK)), telbivudine (Novartis, Basel, Switzerland), and tenofovir (tenofovir disoproxil fumarate (TDF), and recently available as the prodrug tenofovir alafenamide fumarate (TAF) (Gilead Sciences). The pressure induced by these drugs may lead to the selection of adaptive mutations that reduce the sensitivity of the virus to the hindering effects of the drugs, thereby causing antiviral resistance. Polymerase mutations are frequently observed in patients following lamivudine-based antiviral therapy. Antiviral resistance to this drug requires the selection of a single mutation: rtM204V/I **(Allen et al., 1998; Ono-Nita et al., 1999)**. The selection of this mutant also causes resistance to emtricitabine and telbivudine. The presence of the rtM204V/I has been detected in 70% of HBV mono-infected **(Lok et al., 2003; Lai et al, 2003)** and 90% of HIV/HBV co-infected patients **(Benhamou et al., 1999)**, after four years of monotherapy with lamivudine (Figure 2.3). On the other hand, resistance to emtricitabine is noted in 18% of HBV mono-infected patients following two years of therapy **(Gish et al., 2005)**. In the same timeframe, 25% of HBeAg positive and 11% of HBeAg negative patients would mount resistance against telbivudine **(Liaw et al., 2009)**. The selection of rtN236T or rtA181V/T mutants confers viral resistance to adefovir and happens following five years of therapy at a rate of 30% **(Hadziyannis et al., 2005, 2006)**. Entecavir has a lower resistance rate of 1.2% among treatment-naïve patients following five years of monotherapy. Viral resistance to the drug is due to the selection of three missense mutations: rtL180M, rtM204V and either rtT184G/S or rtS202I/G or rtM250V **(Tenney et al., 2009)**. Unlike the other nucleos(t)ide analogs, the primary mutation associated with TDF resistance has not been clearly defined. Conflicting results have been reported concerning the rtA194T change as a TDF-resistant substitution **(Sheldon et al., 2005; Delaney et al., 2006; Amini-Bavil-Olyaee et al., 2009)**, but require further investigations. Still, reduced susceptibility to the drug has been observed in the presence

of the aa substitutions rtP177G and rtF249A **(Qin et al., 2013)**, and rtN236T+ rtA181T **(Murakami et al., 2016)** through *in vivo* and *in vitro* models. Virological breakthrough has also been noted among TDF-treated patients harboring multi-site RT mutations including rtL80M, rtL180M, rtM204V/I, rtA200V, rtF221Y, rtS223A, rtT184A/L, rtR153Q, and rtV191I **(Lee et al., 2014)**, and more recently the rtS78T aa substitution **(Shirvani-Dastgerdi et al., 2017)**. To date, no mutation associated with TAF has been reported. A summary of drug resistance-associated mutations is outlined in Table 2.2 below.

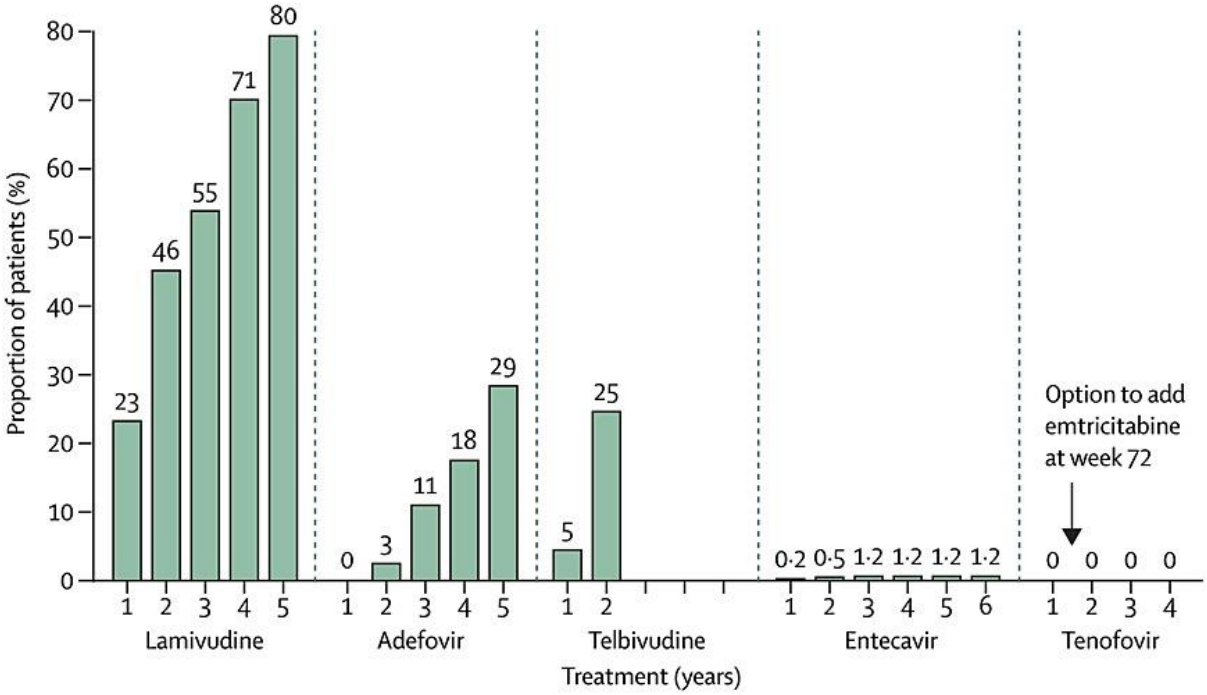


Figure 2.3: The cumulative incidence rate of resistance following anti-HBV therapy in HBV mono-infected patients. The incidence rate portrayed for adefovir concerns HBeAg negative patients **(De Clercq, 2015)**. Reproduced with permission.

Table 2.2: Amino acid substitutions in the RT region of the polymerase gene associated with nucleoside analog resistance. (Menéndez-Arias, Alvarez & Pacheco, 2014). Reproduced with permission.

Antiviral drug	**Major mutations**
Adefovir	rtL80I/V, **rtA181S**, rtA181T, **rtA181V**, rtE218G, **rtN236T**
Emtricitabine	rtA181S, **rtM204I/V**
Entecavir	rtI169T, rtT184A/F/G/L/M/S, **rtS202C/G/I**, **rtM204I/V**, **rtM250I/V/L**
Lamivudine	rtL80I/V, rtV173L, **rtL180M**, rtA181S, rtA181T, rtT184S, **rtM204I/V**, rtM204Q, rtV207I
Telbivudine	**rtM204I/V**
Tenofovir	rtS78T, rtP177G, rtA181S, rtA181T, rtN236T, rtF249A

Underlined bold amino acid substitutions are primary mutations and by themselves confer high-level resistance to the drug. Important resistance mutations are indicated in **bold**.

Mutations in the surface gene

Amino acid changes in the surface gene, precisely the S domain of the gene, may arise as a result of nucleos(t)ide analog-resistance mutations in the overlapping RT domain of the polymerase gene. These changes are commonly referred to as antiviral drug-associated S gene mutations. From a clinical and public health perspective, the selection of these mutants may have diagnostic repercussions in the form of false-negative HBsAg serology results, or lead to vaccine escape where the RT changes cause alterations in the "a" determinant of the HBsAg protein **(Pollicino et al., 2014; Zhang et al., 2016; Valaydon & Locarnini, 2017)**. The pre-S domain is also prone to alterations ranging from deletions, insertions, to missense mutations. As illustrated in Figure 2.4, several epitopes for T and B cells are found in the pre-S1 and pre-S2 domains; hence play essential roles in the interaction with the host immune system **(Chen, 2018)**. In-frame deletions may, therefore, serve as a mechanism of viral immune escape. Pre-S mutations have been extensively described among genotypes B and C patients **(Liu et al., 2009; Wang et al., 2015a; Chen, 2016)**. Still, pre-S2 deletions have also been observed in HBV genotype D and genotype A patients co-infected with HIV in South Africa **(Makondo et al., 2012)**. Then again, Biswas et al. (2012) observed pre-S1 deletions and pre-S2 start codon mutations among genotype D and A infected patients, respectively, in Eastern India **(Biswas et al., 2012)**. The oncogenic potential of mutations in these two genomic domains, mostly the pre-S2, has been well studied in Asia. Case-control Asian studies have reported a higher prevalence of in-frame deletions in HCC patients as compared to non-HCC patients **(Qu et al., 2014a; Zhang et al., 2015)**, thereby suggesting their potential role in HCC risk prediction. Longitudinal studies have demonstrated a cumulative selection of pre-S mutations as the chronic disease progresses, leading to the development of HCC **(Qu et al., 2014a; Zhang et al., 2015, 2017)**.

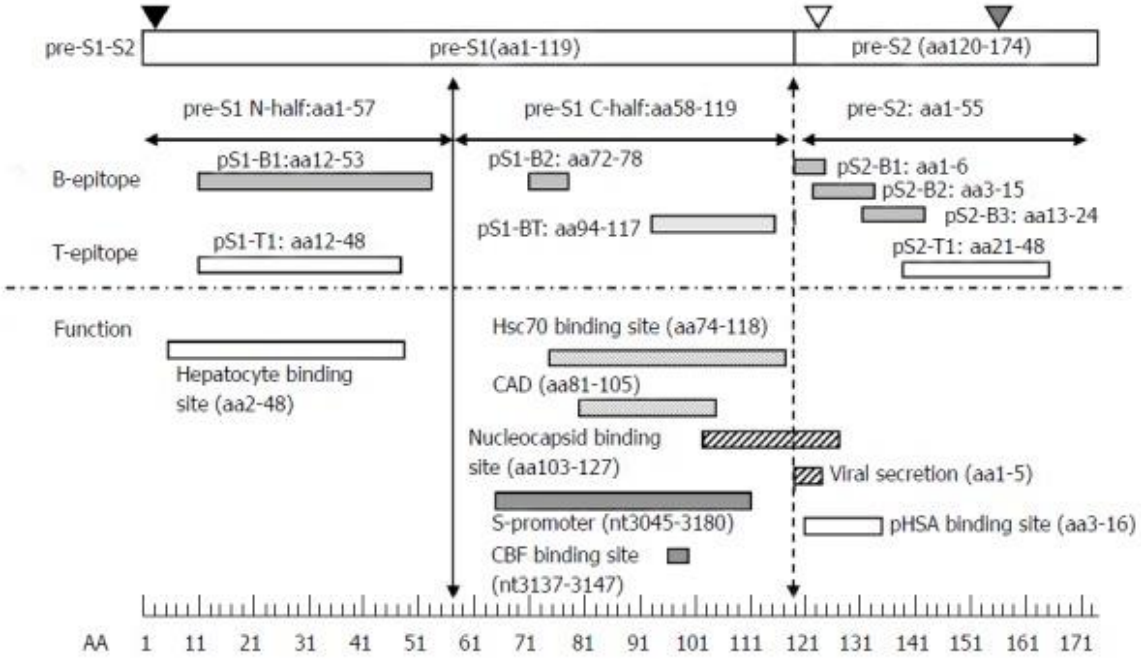


Figure 2.4: Immune epitopes and functional domains in the pre-S1 and pre-S2 domains. The pre-S1 domain consists of an N-terminus (amino acids 1-57) and a C-terminus (amino acids 58-119); both form a total of 119 amino acids. The pre-S2 domain is made up of 55 amino acids (amino acids 120-174). Myristylation at the second amino acid is represented by the black triangle. The N-link glycosylation at N-4 of the MHB protein is denoted by the white triangle indicates, and the O-link glycosylation at T-37 of the MHB protein is shown by the gray triangle. The B-epitopes are pS1-B1, pS1-B2, pS2-B1, pS2-B2, and pS2-B3. T-epitopes are pS1-T1 and pS2-T1. B- and T-epitope: pS1-BT **(Chen, 2018)**. Reproduced with permission, Baishideng Publishing Group.

Mutations in the core gene

Pre-core (pre-C) and basal core promoter (BCP) mutations are the most common and the largest group of mutants described in the core gene. Pre-C mutants have been associated with reduced or abolished expression of the HBeAg protein. The most common pre-C mutation is G1896A. The mutation creates a stop codon TAG at nucleotide (nt) 1896 (or codon 28: TGG). G1896A occurs in the encapsidation signal (ε) structure of the pre-C region and stops the production of HBeAg at a translational level without affecting viral replication **(Jammeh et al., 2008)**. The mutant enables the stabilization of the ε structure by maintaining its stem-loop structure, through base pairing between nt 1858 and nt 1896 **(Lok et al., 1994)**. The incidence of the mutant would depend on the base at position 1858. T-A pairing is more stable than T-G pairing **(Lok et al., 1994)**; thus the mutant is frequently found in genotypes with a T1858 such as genotypes B, C, D, and E. C1858 is found in genotypes A and F, hence the rare occurrence of the G1896A mutant in these two genotypes **(Caligiuri et al., 2016)**. The G1896 stop mutation may or may not occur in conjunction with G1899A. Selection of both mutations was shown to

increase the stability of the ε stem-loop structure **(Lok et al., 1994)**. BCP mutants, on the other hand, may influence both viral replication and the expression of HBeAg. For instance, the C1766T/T1768A double BCP variant has demonstrated enhanced production of both viral particles, and of HBeAg as compared to wild-type viruses in transfection studies **(Jammeh et al., 2008)**. Other BCP nucleotide substitutions may take place at positions 1653, 1753-1757, 1762 and 1764. These changes may or may not occur together, and have been associated with upregulation of viral DNA replication while having an effect on the production of HBeAg **(Quarleri, 2014; Caligiuri et al., 2016; Zhang et al., 2016)**. Interestingly, the combined presence of both BCP and pre-C variants have been associated with advanced liver disease, as demonstrated by Asian studies, and may be used in algorithms for liver disease prediction **(Jang et al., 2012; Lyu et al., 2013; Park et al., 2014; Qu et al., 2014b; Lapalus et al., 2015; Tseng et al., 2015)**. Viral changes can also take place in the upstream part of the core promoter, known as the upper regulatory region (URR) (Figure 2.5). The URR comprises both positive and negative regulatory elements that modulate the transcription of both the pre-C messenger RNA (mRNA) and pregenomic RNA (pgRNA) **(Kramvis & Kew, 1999)**. Limited data are available on the type and effects of variants in this genomic domain.

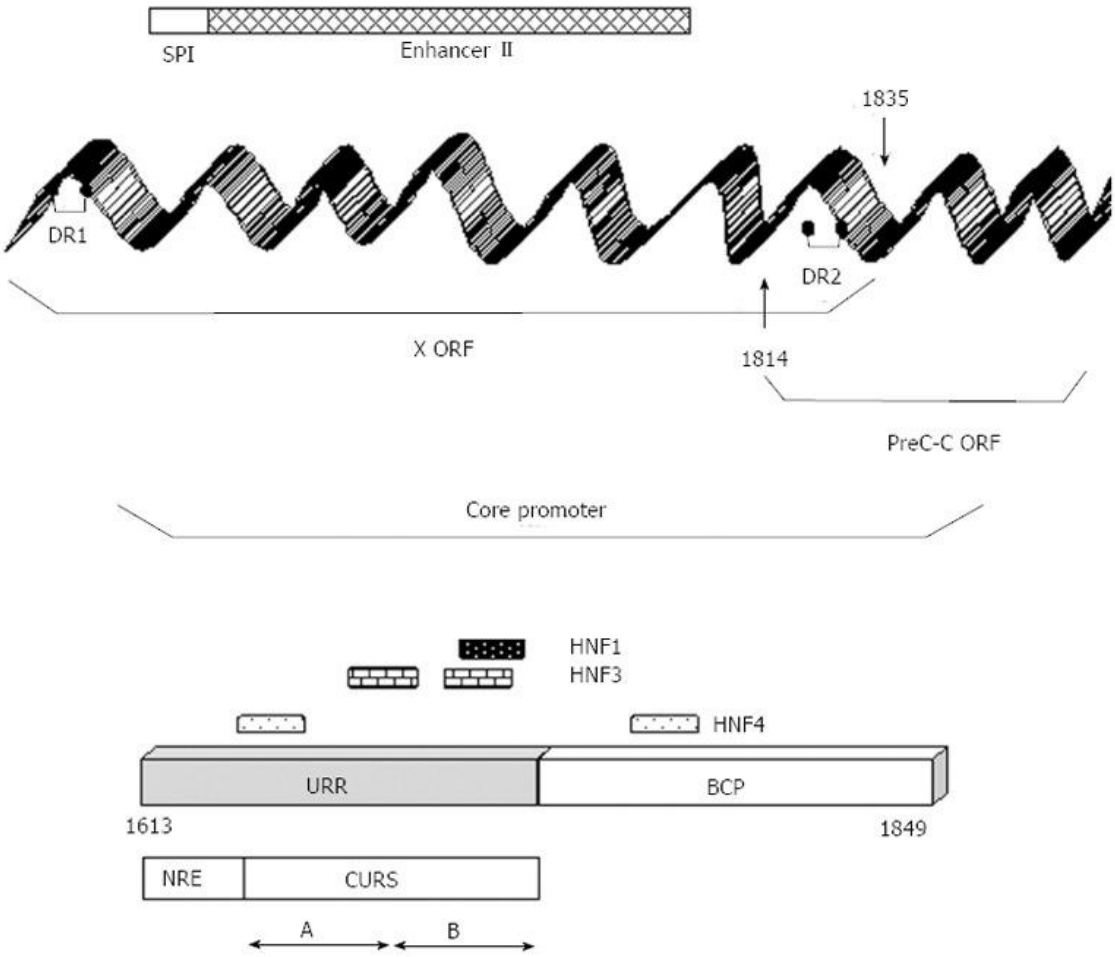


Figure 2.5: Molecular structure of the HBV core promoter. The core promoter (CP) (nt 1613-1849) encloses two domains namely the upper regulatory region (URR) and the basic core promoter (BCP). The URR comprises a negative regulatory element (NRE) and a core upstream regulatory sequence (CURS). The CURS is further divided in CURS-A and CURS-B domains. The enhancer II and binding sites hepatocyte nuclear factor 1, 3 and 4 (HNF1, HNF3, HNF4) are shown. SPI: S promoter; DR: direct repeat **(Quarleri, 2014)**. Reproduced with permission, Baishideng Publishing Group.

Mutations in the X gene

Figure 2.5 above shows overlapping of the CP with the 3'end of the X gene. Therefore, changes in the CP may affect the overlapping sequence in the X gene; as presented in Figure 2.6. As an example, the well-described A1762T/G1764A double BCP mutation leads to the amino acid changes K130M and V131I in the X gene. As most other viral genomic mutations described so far, the presence of HBx mutants has also been related to hepatocarcinogenesis **(Kim et al., 2016)**. Among these mutants, K130M, V131I, H94Y (BCP mutant C1653T), and I127L,T,N,S (BCP mutant T1753V) have been frequently reported **(Kim et al., 2016)**.

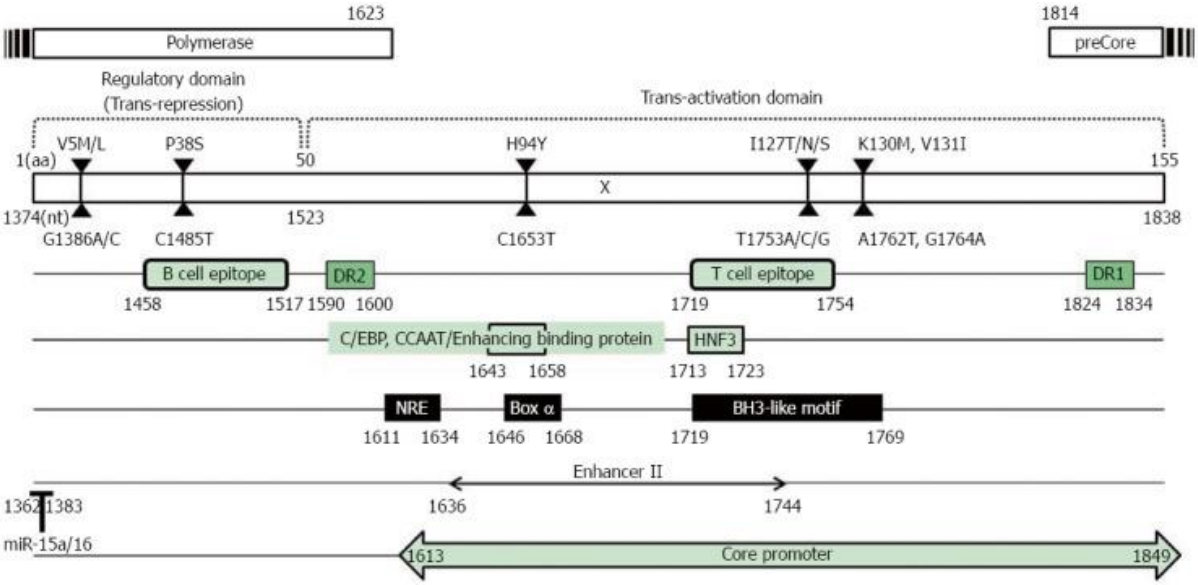


Figure 2.6: Molecular structure of the X gene. The X gene is 465 bp long (nt 1374 -1838) and comprises two domains: the regulatory domain (nt 1374-1523) and the transactivation domain (nt 1524-1838). The overlaps with the core promoter (nt1362-1383), enhancer II (1636-1744) and the microRNA-binding region (nt 1362 to 1383) are shown. The most frequent viral variants are shown too **(Kim et al., 2016)**. Reproduced with permission, Baishideng Publishing Group.

2.2 Epidemiology of HBV infection in sub-Saharan Africa

According to recent estimates on the global burden of viral hepatitis from the World Health Organization (WHO), about 257 million individuals are affected by HBV; translating to a global prevalence of 3.5% **(WHO, 2017a)**. The HBV burden is distributed unevenly across the world and between different population groups.

After the West Pacific region, Africa is the second most affected area with chronic hepatitis B (CHB) infection. The regional prevalence of the infection is estimated at 6.1% (95% uncertainty interval: 4.6% – 8.5%). This translates to 60 million chronic carriers at high risk of developing end-stage liver diseases. These cases are not spread evenly across the African continent **(WHO, 2017a)**. Prevalence rates up to 15% are reported in West Africa **(Schweitzer et al., 2015)**. In a recent population screening in Southern Nigeria, the authors described an HBsAg seroprevalence of 8.8% among 1365 adults **(Okonkwo et al., 2017)**. This is lower than the 2013 national prevalence reported at 13.6% (95% confidence interval [CI]: 11.5% - 15.7%) **(Musa et al., 2015)**. A 9.8% prevalence was observed among 2207 individuals screened in Ouagadougou, Burkina Faso **(Diarra et al., 2017)**. An even higher prevalence, 13.7%, was observed in Sierra Leone **(Ansumana et al., 2018)**. Studies in Central Africa have reported infection rates of 10.6%, 9.3% and 10.6% in Cameroon **(Bigna et al., 2017)**, Angola **(Guimarães Nebenzahl et al., 2013)** and rural Gabon **(Komas et al., 2013)**, respectively. A

recent study in South West Chad reported a significantly higher seroprevalence of HBsAg among 1309 individuals (22.9%) **(Suesstrunk & Djongali, 2017)**. Southern Africa is not spared. Interestingly, HBV epidemiology has been investigated predominantly in the HIV-infected population, most probably owing to the high burden of both viruses in this region. HIV/HBV co-infection rates of 6.6%, 11.3%, and 7.6% have been lately reported in Tanzania **(Kilonzo et al., 2017)**, Zambia **(Wandeler et al., 2016)**, and Mozambique **(Wandeler et al., 2016)**, respectively. While HBV statistics are widely available from other parts of Southern Africa, the burden of the infection in Namibia is not well known. Available HBsAg seroprevalence figures originate from earlier studies conducted in the northern part of the country. These include a 1983 research study among the general population living in the Kavango province, where the authors detected a 13.6% HBsAg carrier state **(Joubert et al., 1985)**. In Eastern Caprivi, Joubert et al. (1991) noticed an 18.95% (90/475) HBs antigenemia in males as compared to 10.71% (45/419) found in females **(Joubert et al., 1991)**. However, current national data are lacking.

2.2.1 Epidemiology of HBV infection in pregnant women

Rates of HBV infection have been studied in pregnant women in SSA. This population has been extensively studied in Nigeria; where a national seroprevalence of 14.1% within this population was observed **(Musa et al., 2015)**. This is slightly higher than the 13.1% pooled seroprevalence described in Ghana **(Ofori-Asenso & Agyeman, 2016)**. Other West African countries reported HBsAg seropositivity rate of 8.0%, 10.7% and 15.2% in Mali **(MacLean et al., 2012)**, Mauritania **(Mansour et al., 2012)** and Burkina Faso **(Sanou et al., 2018)**, respectively. Rates in Ethiopia range from 3.8% in the northern part of the country **(Zenebe et al., 2014)** to 7.8% in southern Ethiopia **(Metaferia, Dessie & Amsalu, 2016)**. Similar rates have been described in Kenya (3.8%, **Ngaira et al., 2016**), Rwanda (3.7%, **Mutagoma et al., 2017**) and in Tanzania (8%, **Manyahi et al., 2017**). In Cape Town, South Africa, a 4.5% HBsAg seroprevalence was described among 134 HIV-uninfected pregnant women **(Chotun et al., 2017)**. This rate was similar to the 2.9% described in 2013 among HIV-uninfected pregnant women in the Western Cape Province, South Africa **(Andersson et al., 2013)**. On the other hand, these rates are significantly higher than the 0.8% (18/2368) reported in the Tshwane district, South Africa **(Diale et al., 2016)**; suggesting heterogeneity between provinces in the country. A similar provincial heterogeneity of the prevalence of the infection is seen in Namibia. While a pooled prevalence of HBsAg of 7.3% was described in 2013 within the

national obstetrics population, rates as high as 10.1% and as low as 5.6% were described in provinces of the country **(Mhata et al., 2017)**. It is important to note that the retrospective study conducted by Mhata et al. (2017) is the only one, to date, investigating HBV infection among pregnant women in Namibia.

While appreciating that these statistics are already quite high in the HIV-uninfected antenatal population, higher rates are found among HIV-infected pregnant women. Chasela et al. (2014) found a 5% HBV infection rate among HIV-infected pregnant women in Malawi **(Chasela et al., 2014)**. A comparable HIV/HBV co-infection rate of 4.1% was described in Rwanda **(Mutagoma et al., 2017)**. Among 1543 HIV-infected pregnant women screened in the Western Cape Province of South Africa, 3.4% (53) tested HBsAg positive **(Andersson et al., 2013)**. In the province of KwaZulu-Natal, South Africa, a higher HBsAg prevalence was found in HIV-infected (7.4%; 16/215) as compared to HIV-uninfected (14/294; 4.8%) **(Thumbiran et al., 2014)**. Looking at Central Africa, of 650 pregnant women tested in Yaoundé, Cameroon, 7.85% (51) tested positive for HBsAg; among whom 28/301 (9.3%) were HIV/HBV co-infected, and 23/349 (6.59%) were HBV mono-infected **(Kfutwah, Tejiokem & Njouom, 2012)**. Despite this high burden of HBV infection within the antenatal population, routine screening of pregnant women for HBV is not offered in most parts of SSA. The high prevalence of HBV infection in pregnant women also raises concerns regarding the rates of vertical transmission of HBV to infants in SSA.

2.2.2 Epidemiology of HBV infection in children

HBV epidemiological data in the pediatric population are limited. A cross-sectional study conducted in the Central African Republic, Cameroon and Senegal in 2009-2010 among hospitalized children between the ages of three months and six years described HBV prevalence rates of 5.1%, 0.7%, and 0.2%, respectively **(Rey-Cuille et al., 2013)**. On the other hand, a national survey conducted in Nigeria found HBs antigenemia in 9.8% (4/41) of children below the age of 10 years **(Olayinka et al., 2016)**. This figure is quite high in comparison to those of South Africa, where rates of HBsAg range between 0.4% **(Chotun et al., 2015)** and 0.8% **(Jooste et al., 2016)**; the higher bound represented the prevalence among HIV-infected children. HBV seems to be relatively common among HIV-infected children in SSA. A recent seroprevalence study in Malawi found a low HBsAg positivity status of 2.2% (2/91) among HIV-infected children aged between three months and less than 15 years **(Varo et al., 2016)**. Pediatric HIV/HBV co-infection rates described in other Southern African countries such as

Tanzania **(Muro et al., 2013**) and Zambia **(Peebles et al., 2015)** are higher, with a prevalence of 7.0% and 10.4%, respectively. In the Zambian study, the authors distinguished the prevalence of HBsAg between children and adolescents born prior and post-implementation of HBV immunization starting at six weeks: 11.8% compared to 6.6% (p = 0.24), respectively **(Peebles et al., 2015)**. Data from Namibia revealed an 8.7% prevalence of HBV in 1057 HIV-infected children and adolescents (older than18 years). Children below the age of one year showed an approximate 6.5% HBsAg seropositivity rate **(Brandt et al., 2012)**. These statistics are indicative of the presence of a reservoir of chronic HBV infections in SSA.

2.3 Natural history and pathogenesis of HBV infection

As mentioned earlier, primary infection with HBV may cause an acute transient infection leading to acute or in severe cases of fulminant hepatitis. The infection may thereafter persist, leading to a chronic state; the risk for this depends on the age at which the infection is acquired.

2.3.1 Acute hepatitis B infection

Acute hepatitis B is characterized by self-limited inflammation of the liver cells ending with the formation of lasting immunity (Figure 2.7). The infection can be asymptomatic or symptomatic; the former occurs more often than the latter. Asymptomatic hepatitis is commonly seen in adults, while only about 10% of children between the age of one and five years old develop a symptomatic infection **(Ganem & Prince, 2004)**. Following a one to four months incubation period, from primary infection with the virus, HBsAg is produced. Soon after, HBeAg and HBV DNA can be detected. Antibodies to HBcAg (anti-HBc), of the IgM isotype (IgM anti-HBc), follow approximately one to two weeks later and remain detectable for up to six months post clearance of circulating HBsAg. As the infection progresses, HBsAg and IgM anti-HBc are cleared, leaving antibodies against HBsAg (anti-HBs) and total antibodies to anti-HBc (anti-HBc (total)). Anti-HBc (total) persists for life and is often used as a serological marker to determine past HBV infection. Anti-HBs, in contrast, tends to wane over time. This marker is used to determine immunity against the virus and usually develops following active immunization against HBV. Although short-lived in approximately 95% of adult cases, acute HBV infection may carry on in about 5% of adult cases, developing to persistent infection **(Hyams, 1995; Liaw & Chu, 2009)**.

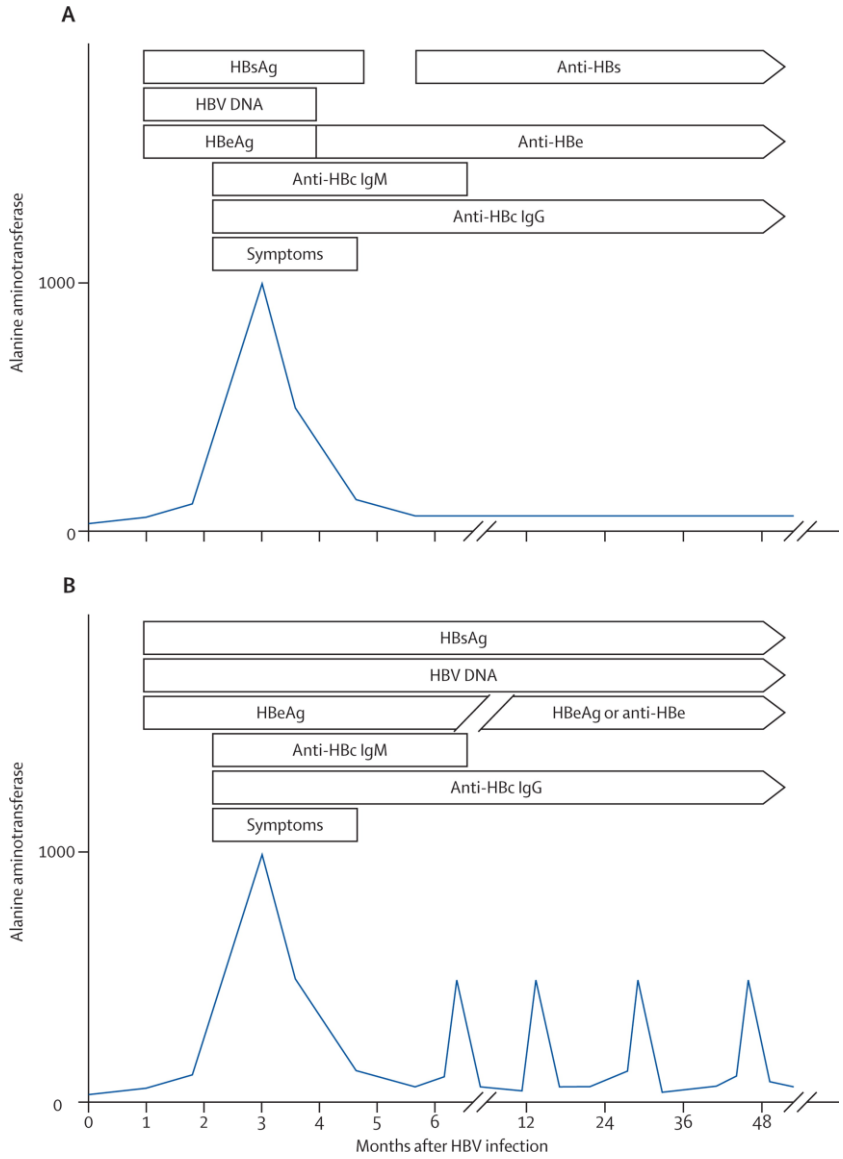


Figure 2.7: Clinical course of acute hepatitis B infection. A: HBV markers changes in acute HBV infection. From 3-4 weeks of acquisition of the virus, HBsAg can be detected. The production of HBeAg and HBV DNA follows. Within approximately two months of the infection, anti-HBc IgM and anti-HBc IgG can be detected. As the levels of anti-HBc IgM decline, anti-HBs are produced by the immune system and persist for life. Flares of ALT are detected following the appearance of HBc antibodies too. B: Transition of acute to chronic HBV infection. It is characterized by the persistence of HBsAg and HBV DNA **(Trepo, Chan & Lok, 2014)**. Reproduced with permission from Trepo, Chan & Lok, 2014.

2.3.2 Chronic hepatitis B infection

Chronic hepatitis B (CHB) infection is defined as the persistence of HBsAg in serum for more than six months, following an acute infection. HBV DNA viremia and HBe antigenemia persist too, and anti-HBs do not become detectable. CHB develops in about 90% of neonates born to HBsAg/HBeAg positive mothers and 30% of neonates of HBsAg positive/HBeAg negative mothers **(Beasley et al., 1977)**. Persistent HBV infection occurs in 30% to 50% of childhood infections acquired between the age of one and six years old **(Hyams, 1995)**. The immaturity

of the immune system of these infants and children may be responsible for the high rate of chronicity of HBV seen among this young age group. The immune tolerance developed by the fetus, as a result of the maternal transplacental transfer of HBeAg, also plays a role in the development of chronicity in neonates exposed to HBeAg *in utero* **(Milich & Liang, 2003)**. Based on the host immune response towards the hepatic inflammation and HBV replication level, the natural course of CHB is broken down to at least five major clinical phases, see Figure 2.8.

(1) The initial high replicative, low inflammatory phase

This phase clinically presents with high viremia (HBV DNA levels above 10^8 IU/ml), a seropositive status for HBeAg, minimal or no necroinflammation of the liver, and normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Failure of the host immune system to recognize the virus as foreign has been attributed to the exhaustion of the HBV-specific T-cells in the presence of high levels of HBeAg; hence the term "immune tolerant" to describe this phase **(Hadziyannis, 2011; Yang & Kao, 2016)**. The duration of the immunotolerance phase varies according to the mode of acquisition of the infection. In vertically-acquired infections, immune tolerance may last for more than three decades, while it is short-lived and difficult to recognize in children infected through horizontal transmission **(Yim & Lok, 2006)**. However, in the absence of HBeAg, brought about by the selection of viruses harboring mutations in the pre-C or CP regions of the pre-C/C gene, the immunotolerance is reduced. Then, the immune system slowly starts to recognize the foreign antigens, resulting in a strong immune response against the HBV-infected hepatocytes **(Milich & Liang, 2003)**. HBeAg-negative infection may occur as the disease progresses or may result from perinatal transmission of an HBeAg negative virus. The former may be used as an indicator of entry of the patient in the "immune clearance" phase of CHB. On the other hand, materno-fetal transmission of HBeAg negative virus is a known cause of fulminant hepatitis in neonates **(Chang et al., 1987; Beath et al., 1992; Liaw & Chu, 2009)**.

(2) The immune clearance phase

The mechanisms prompting the transition from the initial immune tolerance phase to this reactive phase remain unknown. The phase is marked by seroconversion from HBeAg to anti-HBe. Fluctuations of ALT levels, ranging from 100 U/mL to more than 1000 U/mL, are observed; representing changes during the inflammation. The stronger the immune response, the higher the degree of inflammation and ALT levels; high ALT levels are associated with

increased liver injury **(Liaw & Chu, 2009)**. A decrease of HBV DNA levels and seroconversion of HBeAg to anti-HBe eventually follow **(Gish et al., 2015; Yang & Kao, 2016)**. The duration of this phase with subsequent loss of HBeAg varies depending on age, the HBV genotype, the maternal HBsAg status, the mode of acquisition of the infection, and the geographic location **(Yim & Lok, 2006)**. HBeAg clearance is rare in children. Spontaneous seroconversion to anti-HBe in perinatally-infected children younger than three years old occurs at a rate below 2% **(Chang et al., 1989; Chang et al., 1995)**, and increases to 4% - 5% in children older than three years old in South-East Asia; where genotypes B and C predominate **(Chang et al., 1989)**. On the other hand, a higher annual rate of 14% to 16% is recorded in the Euro-Mediterranean and African region; where genotypes A and D viruses are found, and horizontal transmission commonly happens **(Iorio et al., 2007; Kramvis, 2016)**. A small proportion of patients would also experience a loss of HBsAg. However, the rate is as low as 0.1% to 0.8% in children and increases to 0.4% - 2% in adults **(Liu et al., 2010; Shimakawa et al., 2016a; Kramvis, 2016)**.

It is noteworthy that the difference in the duration of the immune clearance phase between South-East Asia, Euro-Mediterranean and African countries may contribute to the different rates of liver cirrhosis and HCC between these regions. Delayed HBeAg seroconversion, as it is seen in south-East Asia, may lengthen liver inflammation, thereby prolonging the liver damage **(Yim & Lok, 2006)**. Then again, following HBeAg seroconversion, the prognosis of the infection may not always be good but is dependent on the severity of the liver injury caused.

(3) The HBeAg-negative chronic hepatitis phase

During this phase, hepatic necroinflammation continues. It is characterized by fluctuating ALT levels and high to moderate viral loads, progressively leading to liver disease **(Gish et al., 2015; Kramvis, 2016)**. Abortion of the production of HBeAg, with no effects on viral replication from the selection of pre-C mutants such as the pre-C G1896A stop codon, may be attributed to this state of the infection **(Hadziyannis, 2011)**.

(4) The low replicative phase

Previously referred to as the "inactive HBsAg carrier state''. In this phase, patients present with normalization of ALT levels, low levels of serum HBV DNA (< 2000 IU/ mL), absence of HBeAg and the presence of anti-HBe **(Gish et al., 2015; Kramvis, 2016; Yang & Kao, 2016)**. Patients often remain in this inactive state of the infection for a lifetime. Still, about 5% of them

could experience seroreversion to HBeAg. About 10% to 20% could revert to the HBeAg-negative chronic hepatitis phase and may require antiviral treatment **(Yang & Kao, 2016)**.

(5) The reactivation phase

Recurrence of viremia, reversion to HBeAg and ALT flares are seen at this stage of the chronic infection; resulting in an increased risk of developing liver cirrhosis, fibrosis and HCC **(Kramvis, 2016)**. Immunosuppression brought by chemotherapy or any other immunosuppressive therapy as well as immunosuppressive infections may account for this viral rebound. As the immune system deteriorates, it fails to control the low level viral replication **(McMahon, 2009)**.

The rate of disease progression and the severity of the liver disease vary between individuals, depending on both viral and host factors. The former include HBV genotypes and mutations, as outlined in sections 2.1.4 and 2.1.5. Host factors such as gender and genetic variants have also been implicated in the pathogenesis of the disease. For instance, it is well established that men are more likely to develop CHB compared to women **(Blumberg et al., 1972)**. Similarly, a higher prevalence of HBV-related end-stage liver diseases has been reported among men compared to women **(Yuen et al., 2009; Stroffolini et al., 2015; Sun et al., 2017)**, due to the opposing effects of the sex hormones androgen and estrogen on the liver **(Liu & Liu, 2014; Wang, Chen & Yeh, 2015)**. Polymorphisms in the human leukocyte antigen (HLA) classes II allele (HLA-II), especially HLA-DP, HLA-DQ, and HLA-DR, have also been associated with persistent HBV infection **(Wong et al., 2013; Chang et al., 2014; Akgöllü et al., 2017)**, and with protection against CHB **(Nishida et al., 2012; Trinks et al., 2017)**.

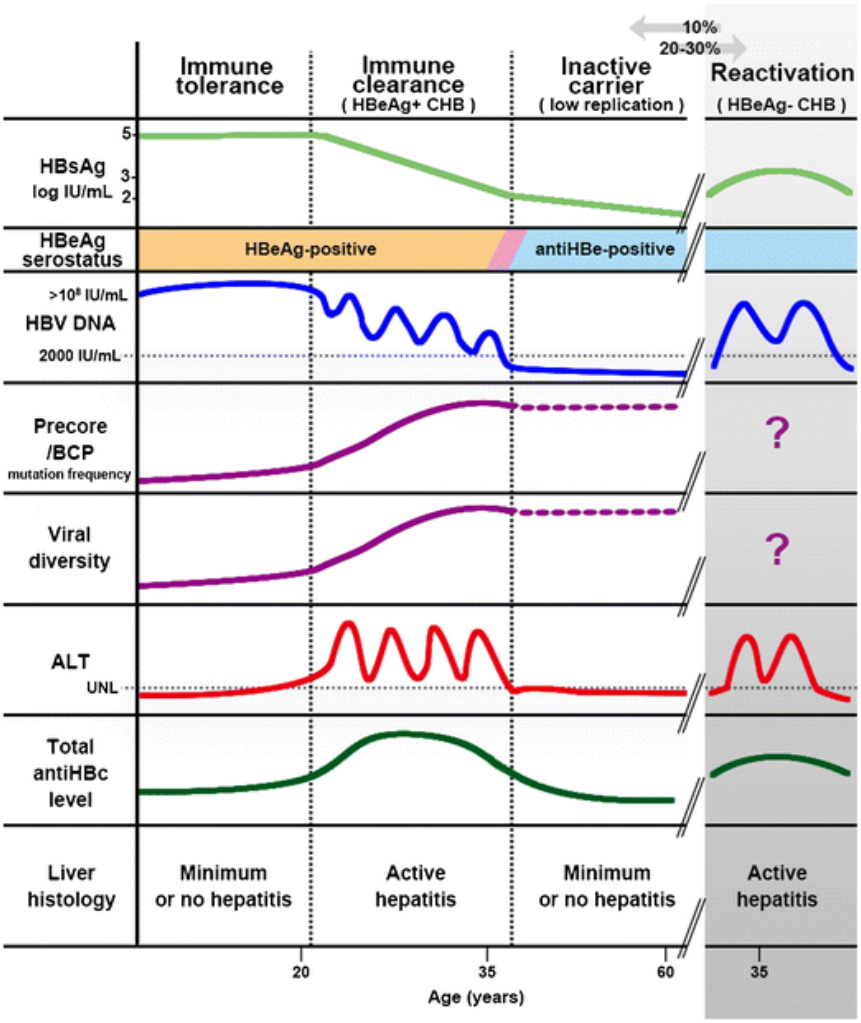


Figure 2.8: Clinical phases of chronic hepatitis infection. This figure depicts the clinical course of chronic hepatitis B (CHB) over the lifetime of the infected individual. Four phases are depicted on this figure namely (1) the immune tolerance, referred in the test as the initial high replicative, low inflammatory phase, (2) the immune clearance phase, (3) the inactive state, and (4) the reactivation phase. The selection of viral mutants over time is also displayed. As the infection progresses, viral genomic mutations may arise and may worsen liver damage **(Yang & Kao, 2016)**. Reproduced with permission.

2.4 Diagnostic testing for HBV infection

Testing and diagnosis of HBV infections allow access to preventive measures and treatment services available, which are a key component in the control of HBV. Historically, diagnostic technology has influenced where testing could be performed. With technological improvements, testing can be decentralized from the laboratory setting to the point-of-care level.

2.4.1 Laboratory-based testing of HBV

Laboratory assays remain the reference method for diagnostics worldwide, mainly due to their diagnostic and analytical accuracy. Current laboratory diagnostic technologies for HBV include both immune-based assays and nucleic acid-based assays.

Immune-based assays

HBV immune-based assays, also known as serological assays, are used to determine the presence or absence of viral antigens, such as HBsAg and HBeAg, and to assess the host immune response through measurement of host antibodies produced against the viral proteins (anti-HBs, anti-HBeAg, and anti-HBc). These assays are commonly found in the form of enzyme immunoassays (EIAs), chemiluminescence immunoassays (CLIAs) and electrochemiluminescence immunoassays (ECLIAs). These immunoassays differ by the modes of detection of immune complexes. EIAs are based on a calorimetric principle whereby the intensity of a color change, produced by a catalytic reaction between an enzyme bound to the antigen-antibody complexes present in the reaction and its substrate, is measured **(Koivunen & Krogsrud, 2006)**. Results are recorded as optical density (OD) values. CLIAs and ECLIAs, on the other hand, measure a light signal emitted on antigen-antibody complexes bound to a chemiluminescent or electrochemiluminescent compound. The signal is normalized in relation to a signal to cut-off (S/CO) value. The OD and S/CO values obtained for the samples tested are compared to a cut-off value. This cut-off value is usually determined by calibration of the assay and specifies whether the analyzed specimen is considered reactive or non-reactive for the analyte sought.

Although many of these assays offer excellent accuracy and allow a high-throughput of samples, they rely on the use of sophisticated equipment, a continuous supply of electricity, a high level of infrastructure (cold-storage, a temperature controlled-environment, precision pipettes, centrifuges, and computers), and can only be operated by skilled medical technologists **(Villar et al., 2015; Peeling et al., 2017)**.

Nucleic acid-based assays

Nucleic acid testing (NAT) includes qualitative and quantitative methodologies for confirmation of the presence or absence of viral nucleic acid, and for measurement of the levels of viral DNA, respectively. The assay is most frequently performed using the polymerase chain reaction (PCR)-based technology. Alternative nucleic acid amplification technologies include loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification

(NASBA), ligase chain reaction (LCR), rolling circle amplification (RCA), and many more. All, but LCR which is similar to PCR, are isothermal amplification assays performed at a single temperature without the need of a thermal cycler **(Fakruddin et al., 2013; Datta, Chatterjee & Veer, 2014)**. NAT is clinically performed to assess viral replication in HBV chronic patients, to guide treatment initiation and to monitor treatment response **(Peeling et al., 2017)**. Antiviral drug resistance can also be detected through qualitative NAT, followed by viral genome sequencing in case of a positive result and viral genotyping **(Villar et al., 2015)**. NAT assays are usually highly sensitive, with a possible limit of detection as low as six international units (IU)/ml **(Villar et al., 2015)**. Similarly to laboratory-based immunoassays, considerable infrastructure and skilled staff are required, too. Owing to the high costs of these tests, they are not readily available in many resource-limited settings (RLS).

2.4.2 Point-of-care testing of HBV

Point-of-care (POC) testing (POCT) has traditionally been defined as rapid diagnostic testing performed at the level of patient care rather than in centrally localized laboratories **(Kost et al., 1999)**. While this was not previously feasible, today's technologic advancements have facilitated the integration of traditional laboratory-based diagnostic assays performed on large and complex analyzers onto simplified, miniaturized and portable devices known as rapid diagnostic tests (RDTs). RDTs represent a potential diagnostic approach to improve the control and management of infectious diseases in many RLS, where advanced diagnostic resources are lacking. Unlike laboratory-based assays, they present with a simple format allowing for easy manipulation by trained lay providers or healthcare workers, low labor, low sample volume and rapid turnaround time **(Kost et al., 1999; Hanafiah, Garcia & Anderson, 2013; Chevaliez & Pawlotsky, 2018)**. The majority of RDTs can be performed with capillary blood collected via finger-prick, thereby eliminating the need for venipuncture at the POC level. Many can also make use of venous blood, serum, plasma or oral fluid samples **(Chevaliez & Pawlotsky, 2018)**.

HBV RDTs exist in the form of lateral flow, flow through or agglutination immunochromatographic devices **(Amini et al., 2017)**. The results are commonly displayed in the form of visible lines, appearing with different intensities that can be gauged by the naked eye. Several rapid tests exist for the qualitative assessment of HBsAg. To date, only a few of these assays are European Conformity (CE)-marked and WHO-prequalified **(WHO, 2017c)**. Currently available and commercialized HBsAg rapid tests have been reported as reliable

diagnostic tools for HBV diagnosis in a few SSA countries including The Gambia **(Njai et al., 2015)**, South Africa **(Chotun et al., 2017)** and Zambia **(Chisenga et al., 2018)** using capillary blood, and in Tanzania, using EDTA whole blood samples **(Franzeck et al., 2013)**. Results regarding the diagnostic sensitivity of these tests in HIV-infected individuals are somewhat conflicting. Previous laboratory based-studies reported an unsatisfactory sensitivity of 55.9% in Malawi **(Nyirenda et al., 2008)** and of 69.3% in Ghana **(Geretti et al., 2010)** of the Alere Determine™ HBsAg test (Alere Inc., Massachusetts, USA). However, a recent Zambian study observed an 87.9% sensitivity of the Determine™ HBsAg test in HIV-infected patients **(Chisenga et al., 2018)**. Low HBV DNA viral load and low levels of HBsAg observed in patients with false-negative test results have been suggested as possible reasons for the poorer performance of RDTs among HIV-infected patients **(Geretti et al., 2010)**. A recent systematic review and meta-analysis revealed an excellent pooled specificity of 99.5% (95% CI: 99.4% – 99.5%), and pooled diagnostic sensitivity of 90% (95% CI: 89.1% – 90.8%) of 33 brands of HBsAg RDTs compared to HBsAg laboratory-based immunoassays **(Amini et al., 2017)**. It is important to note that the diagnostic accuracy of RDTs varied widely among brands. The Determine™ HBsAg test and the Vikia HBsAg assay (BioMerieux, Basingstoke, UK) showed the highest diagnostic accuracy (Sensitivity 90.8% and specificity 99.1% compared to sensitivity 82.5% and specificity 99.9%, respectively) **(Amini et al., 2017)**.

Rapid tests for the detection of other HBV serological markers, such as HBeAg and anti-HBs have also been developed. An earlier systematic review and meta-analysis of studies performed between 1980 and 2010 reported a pooled sensitivity of 95.5% (95% CI: 88.9% - 99.4%), and a pooled specificity of 99.8% (95% CI: 99.3% - 100%) of the Binax dual HBsAg/HBeAg test (Binax Inc., Portland, Maine), the only HBeAg rapid test reported in the analysis. Anti-HBs RDTs presented with a pooled sensitivity of 93.2% (95% CI: 85.1% - 98.5%) and pooled specificity of 93.1% (95% CI: 81.9% - 99.9%) **(Shivkumar et al., 2012)**. Updated statistics on the performance of current commercially available rapid tests for anti-HBs **(Bottero et al., 2013; Poiteau et al., 2017)** and HBeAg **(Seck et al., 2018)** are limited.

2.4.3 Algorithm for HBV testing

The presence of an HBV infection is conventionally assessed by HBsAg screening, as a first-line assessment, using a quality-assured immune-based assay. The recent WHO guidelines on HBV testing recommend the use of a single serological assay, laboratory-based immunoassay or RDT, in regions where the prevalence of HBsAg is above 0.4%. In settings with a prevalence

of HBsAg below 0.4%, a positive HBsAg result should be confirmed, with a different assay of a similar precision or with neutralization test if using a first-line laboratory-based immunoassay **(WHO, 2017d; Peeling et al., 2017)**. The choice of immunoassay technology is dependent on the type and the capacity of infrastructures and resources available. Where RDT testing is the technique of choice, the diagnostic accuracy and the quality of the rapid test should be considered.

The presence of other serological markers of HBV may also be used to differentiate acute from chronic infection. Acute hepatitis is characterized by an IgM anti-HBc immune response, which disappears approximately six months post-HBsAg seroconversion. Although the marker is also seen in case of reactivation of the infection, an IgM anti-HBc negative result would indicate the absence of a recent new infection or the reactivation of an established infection **(Centers for Disease Control, 2011; Krajden, McNabb & Petric, 2005)**. As chronic hepatitis establishes, HBeAg persists along with HBsAg. The persistence of these two antigens would indicate the early phase of CHB and the presence of viral replication. Seroconversion from HBeAg to anti-HBe is often seen in the late phase of CHB. The presence of anti-HBe and anti-HBc IgM would be indicative of an acute infection. Where the serological profile of CHB is confirmed (HBsAg positive, HBeAg positive and anti-HBc IgM negative), the testing process follows to distinguish between the inactive and active stage of the chronic infection using NAT to quantify viremia and to guide and monitor treatment **(WHO, 2017d; Peeling et al., 2017)**. However, in many RLS, NAT may not always be feasible due to the high costs of HBV viral load tests.

2.4.4 Blood sampling approaches for laboratory testing

Serum and plasma samples, prepared from whole blood specimens collected by venipuncture, have traditionally been used as biological substrates for diagnostic testing. However, they can be logistically challenging in rural settings or remote areas in SSA with regards to transport and storage (following collection and during transportation). In contrast, dried blood spots (DBS) specimens do not require venipuncture, can be kept at room temperature and offer a lower biohazard risk. They can easily be transported from remote areas to central laboratories. They are prepared by applying capillary blood collected by finger or heel punctures directly onto the filter paper card, alternatively using venous blood collected via venipuncture **(Mei et al., 2001; Snijdewind et al., 2012)**. This sample type has been increasingly and successively used for HIV NAT, as well as early infant HIV diagnosis in many RLS **(Erba et al., 2015;**

Mavedzenge et al., 2015; Dowling et al., 2018). Only a few studies have reported on its reliability and precision for molecular testing of HBV. A recent systematic review and meta-analysis analyzed the pooled diagnostic accuracy and the analytical sensitivities of HBV viral load testing using DBS samples, in studies published between 1970 and 2015. The meta-analysis showed a pooled sensitivity of 96% (95% CI: 90% –98%) and pooled specificity of 99% (95% CI: 55% – 100%) of HBV DNA viral load testing using DBS. The limit of detection (LOD) of the assays incorporated in this analysis ranged from 10 to 100 IU/ml using serum or plasma samples **(Lange et al., 2017)**. Among these five studies, two reported on the LOD using DBS. These were 914 IU/ml **(Mohamed et al., 2013)** and 1000 IU/ml **(Vinikoor et al., 2015)** for the COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 (Roche Diagnostics, Basel, Switzerland), and 500 IU/ml for the rt2000 real-time PCR instrument (Abbott Molecular, DesMoines, IL, USA) **(Stene-Johansen et al., 2016)**. Vinikoor et al. (2015) also assessed the diagnostic accuracy of diluted samples at different cut-off values. At a cut-off value of 200 IU/ml, the test could not pick up 13.8% (95% CI: 7.7% – 23.7%) of DBS samples whose paired plasma were detected. This probability dropped to 1.8% (95% CI: 0.5% – 6.6%) at a 2,000 IU/ml cut-off and 0.2% (95% CI: 0.03% – 1.7%) at 20,000 IU/ml **(Vinikoor et al., 2015)**. All authors were in agreement for the use of DBS, as an alternative sampling for HBV DNA testing. The LODs observed in these reports, ranging from 500 to 1000 IU/ml – below the WHO recommended 20 000 IU/ml threshold for treatment initiation **(WHO, 2015)**, suggest DBS testing as a potential diagnostic tool to identify HBV chronically infected patients in need of antiviral treatment. However, further assessment of the use of DBS for HBV viral load measurement in SSA is required.

2.5 Transmission of HBV in sub-Saharan Africa

As a blood-borne pathogen, HBV is most commonly transmitted through percutaneous or mucosal contact with blood from infected individuals. The virus is also found in other bodily fluids including saliva, breast milk, semen, menstrual and vaginal discharge. Contact with any of these infected bodily fluids could result in the transmission of HBV. Transmission may take place horizontally or vertically. The virus can also remain stable outside of the human body for at least seven days **(Bond et al., 1981)**, and is known 100 times more infectious than HIV **(Alter, 2006)**.

Horizontal transmission

Horizontal transmission has been regarded as the main driver of the epidemic of HBV in SSA, and the main route of transmission of childhood-acquired HBV infections. From early epidemiological data in SSA, children older than two years compared to newborns were the most afflicted by HBV. In Namibia, Botha et al. (1984) noted a 1% (4/314) prevalence of HBsAg in children below the age of six months compared to a 13% (81/604) prevalence in those above the age of one year **(Botha et al., 1984)**. HBsAg prevalence in two Gambian villages was of 7.26% (9/124) in children one year old and younger compared to a 20.37% (33/162) in those between the age of two and four years **(Whittle et al., 1983)**. Pediatric infections were associated with contact with other infected children or infected siblings, and with infected parents within the same household **(Davis, Weber & Lemon, 1989; Whittle et al., 1983, 1990; Dumpis et al., 2001)**. Sharing bath towels, eaten candies or dental cleaning equipment, being bitten by a carrier, biting of fingernails, back scratching of a carrier, scarification, and injection by traditional healers represented other possible sources of transmission of the virus among children **(Martinson et al., 1998)**. Adulthood infections were, and remain, associated with a number of behavioral risk factors such as unprotected homo- and heterosexual intercourse with multiple sexual partners, and unsafe injections. Nosocomial infections through contaminated medical and surgical instrumentation, blood transfusions, and organ transplantations also occur **(Trepo, Chan & Lok, 2014)**. Scale-up of blood donation screening for HBsAg **(Apata et al., 2014; WHO, 2017e)** resulted in a substantial reduction of nosocomial HBV infections. However, a residual risk is left; depending on the screening strategies in place for blood donations **(Candotti & Laperche, 2018)**.

Vertical transmission

Commonly known as MTCT or perinatal transmission, vertical transmission remains the primary cause of chronic HBV infection in children worldwide; especially in East Asia where 40% to 50% of new cases of HBV infection is a direct consequence of MTCT **(Xu, 2013)**. Perinatal HBV infection is defined as the presence of HBsAg or HBV DNA in the serum of an infant born to an HBV-infected mother, within the first year of life **(Edmunds et al., 1996)**. Transmission may take place *in utero* (intrauterine transmission) or during delivery (intrapartum transmission). Intrauterine transmission of the virus from the mother's blood to the placenta could result following damage to the placenta **(Xu et al., 2001; Chen et al., 2013a),** or through transmission of maternal HBV-infected peripheral blood mononuclear cells (PBMCs) to the fetal circulation **(Xu et al., 2015)**. Intrapartum transmission may occur from

maternal-fetal microtransfusion, or the swallowing of infective fluids in the genital tract by the newborns during delivery **(Lee, Ip & Wong, 1978; Mavilia & Wu, 2017)**. Although HBV is detected in breast milk, current evidence does not suggest that breastfeeding contributes to HBV MTCT under recommended immunization **(Chen et al., 2013b; Zhang et al., 2014; Wang et al., 2015b)**.

Historically, perinatal transmission of HBV has been of little concern in SSA. The risk of MTCT is mainly dependent on the maternal HBeAg serostatus **(Okada et al., 1976; Beasley et al., 1977)** and the maternal HBV viral load **(Zhang, Han & Yue, 1998)**; both are markers of HBV infectivity. While HBV DNA positivity is poorly documented among HBV-infected pregnant women in SSA, HBeAg seropositivity rates have been increasingly described. HBeAg seroprevalence rates range from 16.6% (1/6) **(Chotun et al., 2017)** to 17.1% (7/41) **(Andersson et al., 2013)** among HBV mono-infected pregnant women in the Western Cape Province of South Africa. East African women have shown lower seropositivity rates 14.9% (7/47) in Uganda **(Bayo et al., 2014)** and 3.8% (8/211) in Tanzania **(Mirambo et al., 2016)**. Central and West Africa rates are as high as 22.7% (59/259) in Cameroon **(Ducancelle et al., 2013)** and 36.4% (4/11) in Nigeria **(Adegbesan-Omilabu et al., 2015)**. These data reflect the risk of HBV MTCT in different parts of SSA. In the context of HIV, a higher proportion of HBeAg positive pregnant women and increased viral replication are noted **(Andersson et al., 2013; Diale et al., 2016; Thumbiran et al., 2014)**; thereby increasing the risk of maternal transmission of HBV **(Chasela et al., 2014; Hoffmann et al., 2014)**.

It is extensively quoted that HBeAg positive pregnant women present with a 70% to 90% risk of transmitting HBV to their infants globally **(Thio et al., 2015; Mavilia & Wu, 2017)**. Yet, a recent systematic review and meta-analysis revealed that the rate of MTCT is 39% in SSA **(Keane et al., 2016)** and thus substantially lower. However, the robustness of this estimate is questionable owing to the small number of studies with small sample sizes used in that meta-analysis. Interestingly, the same meta-analysis also showed a higher risk of MTCT of HBeAg positive mothers from West Africa as opposed to East Africa: 66.4% vs. 21.2% ($p = 0.3$), respectively **(Keane et al., 2016)**. Although this difference was not statistically significant and inferred from a small sample size, it may suggest a geographical difference in the rate of MTCT in SSA and may be related to the different viral strains circulating in different parts of the region. Keane et al. (2016) also estimated that about 367 250 newborns acquire HBV at birth from their infected mothers every year in SSA **(Keane et al., 2016)**. These children become part of the reservoir of chronic HBV infections in SSA: HBV-infected female children may

become at risk of transmitting the virus to their infants, and HBV-infected male children may transmit to their sexual partners later in life; thereby perpetuating the cycle of continuous perinatal and horizontal transmission of HBV within communities. A shift of thinking, with regards to the role of MTCT in SSA, is needed to control the regional HBV epidemic.

2.6 Prevention of HBV MTCT

The annual number of perinatal HBV infections (n = 367 250) occurring in SSA **(Keane et al., 2016)** demonstrates the need for preventing HBV MTCT regionally. The WHO standard strategy for the prevention of HBV MTCT includes active HBV immunization, commencing at birth **(WHO, 2017b)**. Adding passive immunization, with hepatitis B immunoglobulin (HBIG), to active immunization at birth may increase protection to HBV-exposed newborns **(Wong et al., 1984; WHO, 2017b)**. This would, however, require identifying HBV-exposed pregnancies as HBIG is scarce, expensive and requires substantial infrastructure.

2.6.1 Pediatric active HBV immunoprophylaxis

Active immunization with the HBV vaccine is the cornerstone of strategies against HBV infection. The vaccine is safe and effective and has been available for nearly four decades. Commercially launched in 1982, the first generation of the vaccine was made from plasma-derived HBsAg proteins, harvested from the plasma of chronically infected individuals. A recombinant form of the vaccine was introduced in 1986, prepared from HBsAg synthesized in yeast or mammalian cells in which the HBsAg gene (HBsAg/pre-HBsAg genes) has been inserted **(Stevens et al., 2017)**.

Recognizing the global burden of HBV, the WHO Global Strategic Advisory Group of Experts on Immunization recommended including the HBV vaccine in the Expanded Programme on Immunization (EPI) in 1991 **(WHO, 1992)**. The planned schedule for the implementation and delivery of the vaccine was based on the epidemiology of the infection in countries: countries with an HBV prevalence of 8% or above were required to integrate the policy by 1995, while 1997 was the target date for countries with lower prevalence **(WHO, 1992)**. The HBV vaccine is available as a monovalent formulation, as well as a fixed combination with other vaccines. As a preventive measure against HBV MTCT, vaccination against HBV should commence at birth, ideally within 24-hours of birth, given in the form of the monovalent formulation **(WHO, 2004, 2009, 2017b)**. The BD vaccine should be followed by two additional doses given at six and fourteen weeks, to coincide with the first and third diphtheria–tetanus–pertussis (DTP)

vaccination. A four-dose schedule may be used too, with three additional doses administered at six, ten and fourteen weeks. These subsequent doses may be administered as a monovalent vaccine or as part of multivalent vaccine formulations **(WHO, 2004, 2009, 2017b)**. A large body of clinical trials has proven the effectiveness of this strategy **(Lee et al., 2006, 2007)**. From its recommendation in 1992, the global coverage of the pediatric HBV vaccination peaked from 1% to 84% in 2015. The BD vaccine coverage, on the other hand, remains low (39%) **(WHO, 2017b)**.

Universal HBV vaccination has been introduced in all 47 member states of the WHO African region. In 2015, about 76% of children in SSA were vaccinated against HBV, but only 10% received the BD vaccine **(Casey et al., 2016)**. BD vaccination is only provided in eleven countries (23.4%); of which nine have introduced universal BD vaccination, as presented in Table 2.3 **(Breakwell et al., 2017; Tamandjou et al., 2017)**. Mauritius and Sao Tome and Principe offer birth vaccination only to babies whose mothers are HBsAg positive **(Breakwell et al., 2017)**. Implementation of this policy remains under consideration in seven countries namely Benin, Cameroon, the Republic of Congo, Côte d'Ivoire, Ethiopia, Ghana, and Sierra Leone **(Spearman et al., 2017)**.

Table 2.3: Hepatitis B vaccine birth dose (BD) coverage in the WHO African Region. Adapted from Breakwell et al. (2017) **(Breakwell et al., 2017)** and WHO UNICEF Joint Reporting, 2017.

Country	Year of introduction[1]	Coverage (%)[1]			
		2013	2014	2015	2016
Algeria	2004	99	99	99	99
Angola	2015	-	-	19	-
Botswana	Pre 2000	-	-	-	-
Cabo Verde	2002	94	99	93	96
The Gambia	1990	-	-	-	-
Mauritania	2013	-	-	51	51
Mauritius[2]	NA	-	-	-	-
Namibia	2014	-	1	87	87
Nigeria	2004	32	32	32	32
Sao Tome and Principe[2]	2002	-	-	-	-
Senegal	2016	-	-	-	58

[1]Coverage estimates are derived from WHO UNICEF Joint Reporting, updated in July 2017. Available at: http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tswucoveragehepb_bd.html. Accessed on July 11, 2018. [2]BD vaccination is only provided to HBV-exposed babies.

2.6.2 Pediatric passive HBV immunoprophylaxis

Passive HBV immunization involves providing temporary immunity against HBV, using inoculation of immune globulin derived from donors with high titers of anti-HB, referred to as HBIG. Early clinical trials of HBIG in newborns born to HBsAg/HBeAg positive mothers, conducted in Southeast Asia, described a reduced rate of chronic HBV infection in children receiving HBIG compared to the controls: 54% in infants given a single dose of HBIG and only 26% with three doses of HBIG (at birth, three months, and six months), compared to 92% in controls **(Beasley et al., 1981, 1983a)**. During the same time period, European data suggested that a single dose of HBIG, immediately after birth, may provide enough protection to babies born to HBsAg/HBeAg positive mothers **(Rosendahl et al., 1983)**. However, passive antibodies provided by the doses of HBIG disappear three to four months later, leaving these children susceptible to HBV; and some infants became infected after their first year of life **(Beasley & Hwang, 1983)**. These results encouraged the theory that combining HBIG with the HBV vaccine would further decrease the risk of persistent HBs antigenemia in HBV-exposed babies while providing a lasting immunity against the infection. Indeed, children receiving both HBIG and HBV vaccine were significantly less likely to become carriers compared to those to whom neither HBIG nor vaccine had been given: 6% compared to 88%, respectively. Moreover, increased effectiveness of HBIG in conjunction with the HBV vaccine of 94% compared to 71% with HBIG alone and 75% with the HBV vaccine alone was observed **(Beasley et al., 1983b)**. Subsequent clinical trials corroborated findings from Beasley et al. (1983b): passive combined with active immunization is more effective than either on its own in children born to HBsAg/HBeAg positive mothers, regardless of the type of vaccine (recombinant or plasma-derived) **(Lee et al., 2006, 2007)**. HBeAg negative/anti-HBe positive mothers present a lower risk of MTCT, on the other hand **(Rosendahl et al., 1983; Lee et al., 2006)**. A recent Chinese study observed no difference in the occurrence of perinatal transmission between HBeAg negative/anti-HBe positive mothers whose babies received HBIG + vaccine and those receiving vaccine alone (0.1% (1/752) vs. 0.0% (0/132), p = 1.000) **(Lu et al., 2017)**; suggesting that vaccine alone may be enough for preventing perinatal HBV infection in infants born to HBeAg negative mothers **(Yang et al., 2003; Chen et al., 2012; Lu et al., 2017)**.

While the use of active immunization starting at birth in combination with HBIG for babies born to HBeAg positive mothers is routine practice in many parts of Southeast Asia and in high-income countries, this is not the case in SSA. There is no published documentation of

SSA countries with a formal policy of passive and active immunization for HBV MTCT. The high cost associated with HBIG is an important barrier **(Tamandjou et al., 2017)**, leaving neonates of HBeAg positive mothers still at risk of perinatal HBV infection. Still, passive and active immunization may also not be enough to eliminate the risk of maternal transmission of HBV.

2.6.3 Maternal antiviral therapy against HBV

A growing body of evidence has emerged describing the added benefits of maternal antiviral therapy against HBV MTCT; such attempts are driven by breakthrough HBV infections despite standard passive-active immunoprophylaxis in neonates born to HBeAg positive mothers **(Ngui et al., 1998; Wiseman et al., 2009; Wen et al., 2013; Foaud et al., 2015; Sellier et al., 2015)**. Maternal viral loads above 200 000 IU/ml and maternal HBeAg serostatus were established as the main predictors of immunoprophylaxis failure in neonates **(Song et al., 2007; Zou et al., 2012; Yin et al., 2013; Liu et al., 2015)**.

Among the seven oral nucleos(t)ide analogs agents approved by the FDA for the treatment of CHB (refer to section 2.1.5), lamivudine **(Xu et al., 2009)**, telbivudine **(Tan et al., 2016)** and TDF **(Pan et al., 2012; Celen et al., 2013)** have been reasonably well investigated for the prevention of perinatal transmission of HBV. A systematic review and meta-analysis of the data produced from these clinical research studies showed that the use of any of these three drugs in conjunction with passive-active immunization demonstrated a reduction of MTCT in HBV infectious women, compared to the use of immunization alone; with no adverse outcomes for the women nor the newborns **(Brown et al., 2016)**. The efficacy of this intervention is dependent on the type of antiviral drug used and the viral load of the mother. Lamivudine showed a 71% risk reduction, and telbivudine offered a 77% risk reduction **(Brown et al., 2016)**. Unlike TDF, lamivudine and telbivudine possess a low barrier to resistance, raising the issue of the emergence of resistance with the use of any of these two drugs. The efficacy of TDF for HBV MTCT was demonstrated in five geographic locations of China, through a randomized controlled trial. The trial included 200 HBeAg positive pregnant women with HBV DNA levels above 200 000 IU/ml (at risk of maternal transmission despite pediatric active-passive immunoprophylaxis), who were randomly assigned to a treatment group and a no treatment group. Women received TDF 300 mg daily from gestational age 30–32 week to four weeks postpartum. Passive and active immunoprophylaxis were provided to all neonates at birth. At postpartum week 28, significantly fewer perinatal HBV infections were diagnosed in

neonates born to TDF-treated mothers than in those born to untreated mothers: 0% vs. 7% (p = 0·01) by per-protocol analysis and 5% vs. 18% (p = 0·007) by intention-to-treat analysis **(Pan et al., 2016)**. A meta-analysis of this data along with non-randomized controlled trials demonstrated a 77% risk reduction of perinatal transmission of HBV in TDF-treated women versus untreated women. No serious adverse effects were observed **(Hyun et al., 2017)**.

As encouraging as these data are, no formal recommendation for routine maternal antiviral therapy for the prevention of HBV MTCT has been made by the WHO so far. In contrast, the three most prominent international liver organizations, namely American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL) and Asian Pacific Association for the Study of the Liver (APASL), suggest the initiation of TDF 300 mg daily at 28–32 weeks of pregnancy in those with an HBV DNA concentration equal to or above 200 000 IU/mL **(Sarin et al., 2016; EASL, 2017; Terrault et al., 2016, 2018)**. Although most clinical trials discontinued maternal antiviral therapy at birth or three months postpartum, EASL supports therapy discontinuation at 12 weeks postpartum **(EASL, 2017)**. These women should be monitored for ALT flares every three months for six months, following therapy discontinuation **(Terrault et al., 2018)**.

While extensive data about the efficiency of combining maternal antiviral prophylaxis with TDF and passive-active immunization have been published from Southeast Asia, where genotypes B and C are found, very little is known about this strategy in SSA. A relatively small prospective study investigated the efficacy of TDF and active immunization at birth in South Africa. A total of 134 women were enrolled in the study and were screened for HBV by means of a rapid test. Six of these women were found HBsAg positive, among whom two had high HBV DNA concentrations of 23 000 IU/ml and 767 000 IU/ml. A 300 mg daily dose of TDF was initiated to these two women at a gestational age of 28 – 26 weeks and interrupted one month postpartum. At follow up, after birth and seven months postpartum, none of the babies born to these two TDF-treated women had contracted HBV infection **(Chotun et al., 2017)**. Results from that study suggest that maternal antiviral therapy may be effective against HBV MTCT in Africa, too, possibly obviating the need for HBIG.

2.6.4 Routine HBV antenatal screening

Passive immunoprophylaxis and providing maternal antiviral therapy would certainly require identifying HBV-infected pregnant women, especially the HBeAg positive ones with high HBV DNA levels. Consequently, a starting point in reducing perinatal transmission of HBV

would involve antenatal HBV screening, as endorsed by many academics **(Andersson et al., 2015; Shimakawa et al., 2016b; Spearman et al., 2017)**. Antenatal screening for HBsAg has been endorsed by WHO, for settings with a ≥ 2% seroprevalence of HBsAg in the general population. The aim is to increase CHB case detection and to prevent further transmission of the virus within communities **(WHO, 2017d)**. However, many countries in SSA do not offer routine HBsAg screening to pregnant women.

From a public health point of view, screening allows the implementation of adequate safety measures to prevent the disease from spreading to the rest of the community. In the context of HBV MTCT, this would be to prevent transmission of the virus from the infected mother to the infant. Ideally, this should be done during the first trimester of pregnancy; thereby allowing adequate documentation of HBV-exposed babies in need of further care at birth, and linkage to maternal care and treatment services available. Screening programmes are required to adhere to the Wilson-Jungner criteria. Firstly, the disease should be an important public health problem, with treatment available for patients infected. The natural history of the disease should be adequately understood, and the latent or early symptomatic stage of the disease should be recognized. The infrastructures required for diagnosis and treatment of the disease should also be available. A suitable test or examination should be available, and acceptable to the population. Furthermore, there should be guidelines in place on whom to treat as patients, and the cost of case-finding that include diagnosis and treatment of patients should be economically balanced in relation to possible expenditure on medical care as a whole. Finally, the case-finding should be a continuing process rather than a one-off project **(Wilson & Jungner, 1968; Andermann et al., 2008)**. An antenatal screening programme for HBV would certainly fulfill most of the Wilson-Jungner criteria **(Chotun et al., 2017)**. The cost of screening pregnant women, on the other hand, remains poorly explored in SSA and requires attention **(Shimakawa et al., 2016b)**. Cost-effective screening strategies may need to be identified and validated for use in the region **(Shimakawa et al., 2018)**.

2.7 Management of pediatric chronic hepatitis B virus infection

Failure to prevent maternal or horizontal transmission of HBV to infants and children would almost certainly lead to childhood CHB. As described in section 2.3.2, many will remain in the immunotolerant phase with minimal or no necroinflammation of the liver. The absence of histological disease during this phase of the infection does not warrant treatment for reasons of drug safety in children, and the potential risk of antiviral resistance from prolonged exposure

to anti-HBV drugs. Where severe and sustained hepatic necroinflammation is detected, treatment with antiviral drugs may be required. However, the choice of drug is quite limited and age-dependent. As mentioned above, seven oral anti-HBV nucleos(t)ide analogs drugs have been FDA approved, along with the interferon-based therapy. While all can be used for the treatment of CHB in adults, only five have been evaluated and approved for use in children. These are interferon alpha (IFN-α), adefovir, entecavir, lamivudine, and TDF.

IFN-α is the most studied and the very first drug used for the treatment of CHB in children. While treatment with the drug leads to early HBeAg clearance or early HBeAg seroconversion, as well as HBsAg seroconversion, similar outcomes were observed in untreated patients at long-term **(Sokal et al., 1998; Bortolotti et al., 2000; Diem et al., 2005)**. A significant reduction of the risk of developing cirrhosis or HCC was not observed **(Jonas et al., 2016a)**. IFN-α based therapy offers the advantage of eliminating the risk of drug-resistance viral genomic mutations developing; but it requires multiple subcutaneous injections, leads to frequent undesired effects and is costly **(Della Corte et al., 2014)**. Lamivudine and entecavir are nucleoside analogs which, upon phosphorylation, act as chain terminators through competition with natural nucleotides during viral DNA synthesis, thereby inhibiting viral DNA replication **(Menéndez-Arias, Alvarez & Pacheco, 2014)**. While lamivudine only inhibits synthesis of the HBV DNA (+) strand, entecavir also hinders the DNA polymerase priming reaction **(Seifer et al., 1998; Jones et al., 2013)** and the reverse transcription of the minus strand from the mRNA **(Seifer et al., 1998)**. Both drugs can be prescribed from the age of two years in children. Lamivudine-based therapy is associated with a higher rate of selection of drug-resistance mutations **(Jonas et al., 2002)** as compared to entecavir **(Jonas et al., 2016b)**. Adefovir and TDF are nucleotide analogs. Both drugs have been approved for use in children above 12 years of age with CHB **(WHO, 2015; Terrault et al., 2018)**. This was based on results of a large multicenter randomized controlled trial showing significant antiviral efficacy in adefovir treated adolescents between the age of 12 to 17 years old, but not in those below 12 years old **(Jonas et al., 2008)**. TDF has only been studied in children ≥ 12 years old **(Murray et al., 2012)**. Thus, treatment options for children below the age of 12 years are quite limited. Drug-resistance mutations from therapy with either of these two antiviral agents, adefovir and TDF, in children have not been reported to date. It is noteworthy that efficacy and safety data of the antiviral agents used for the treatment of CHB in children were based on comparisons between treatment and placebo or no treatment, leaving gaps on how these analogs compared

with one another in children. Based on the comparison of these drugs performed in adults, entecavir and TDF are considered the drugs of choice to treat CHB **(WHO, 2015)**.

With regards to HIV co-infection, entecavir, lamivudine, and TDF possess anti-HIV activity too. However, monotherapy with entecavir in HIV/HBV co-infected patients can result to the selection of the HIV drug resistance mutation M184V **(Sasadeusz et al., 2008)**; thus, the drug cannot be used as monotherapy in the presence of HIV infection. Lamivudine is currently part of the preferred first-line ART regimen for the treatment of HIV/HBV co-infection in children below ten years old, as the only drug against HBV **(WHO, 2015)**. However, due to its low barrier to HBV resistance, children on a lamivudine-based therapy are at high risk of mounting HBV viral resistance. TDF, despite its high potency against HIV and its high barrier to HBV resistance, has been associated with decreased bone mineral density in children co-infected with HIV and HBV **(Purdy et al., 2008)**; and it is part of alternative first-line ART regimens in HIV/HBV co-infected children between the age of three and ten years **(WHO, 2015)**.

2.8 Health economics and prevention of HBV MTCT in SSA

Resource availability and costs are important considerations for implementing health interventions. Various programmes against HBV MTCT could be assessed using economic evaluations to inform decision-making with regards to the allocations of finite resources such as facilities, space, personnel and time to maximize health benefits in the society.

2.8.1 Principles and forms of economic evaluation

According to Drummond et al. (2015), an economic evaluation may be defined as the "comparative analysis of alternative courses of action regarding both their costs and consequences" **(Drummond et al., 2015)**. This definition embraces two fundamental concepts namely (1) the comparison of alternative options, and (2) their evaluations regarding both their costs and their health benefits. From the viewpoint of this definition, an economic evaluation in healthcare assists with an informed allocation of resources to one use instead of another to maximize social health benefits. As such, all costs and consequences related to the alternatives considered in the analysis should be identified, measured, valued and compared **(Drummond et al., 2015)**.

A shared feature of all economic evaluations is the method for measuring and valuing costs, referred to as a cost-analysis. Costs represent the value of the resources used for a particular health intervention. These resources may derive from:

- The health sector: drugs, medical equipment, hospital stays,
- Other public sectors: public agencies or voluntary agencies,
- Patient/family: transportation, child care, patient care; or
- Production losses or gains: loss of time at work, lost productivity while at work

The range of costs included in a cost-analysis is decided based on the perspective taken by the economic evaluation; which could be from a provider, patient or societal point of view. From a provider's perspective, health costs would involve direct medical costs: capital costs and recurrent costs. The latter include personnel costs, consumables costs, and overhead/operating costs. From the perspective of a patient or society, these costs would be predominantly patient related such as transportation, productivity losses due to the disease, and other public non-health care sectors associated with the disease. The costs included in a cost-analysis also reflect opportunity costs. These represent the lost opportunity to invest the same resources in another health intervention that could have return greater health benefits **(Shiell et al., 2002; Drummond et al., 2015)**.

In contrast to cost-analysis, the approach for measuring health benefits may differ depending on the nature of the outputs (health benefits) stemming from the considered alternatives. Three types of economic evaluations are commonly performed. These are cost-effectiveness analysis (CEA), cost-utility analysis (CUA) and cost-benefit analysis (CBA). The use of one type over the other is dependent on the question addressed in the economic evaluation. In a CEA, the common outcomes to the considered alternatives are measured in naturally occurring units such as life years (LYs) gained, cases detected by a screening programme, or cases averted by a treatment programme. The costs associated with this common outcome may differ in magnitude and are compared. Results from a CEA may be expressed as cost per unit of effect, or as effect per unit of cost. An incremental cost-effectiveness ratio (ICER) is calculated to determine the extra cost per unit of outcome obtained from one alternative compared to the other **(Drummond et al., 2015)**. A CEA is particularly useful where limited options are available for a limited budget **(Donaldson & Shackley, 1997)**. In a CUA, a broader unit of measure known as utility is used. Utility focuses on the quality of the health outcome produced or forgone by the considered health interventions; therefore is subjective to individuals or society's preferences for any particular set of health outcomes. The use of utilities enables a broader comparison of costs and outcomes across different programmes; thus is often seen as more useful than a CEA. Utility can be expressed in various quality of life scales such as quality-adjusted life years (QALYs), disability-adjusted life years (DALYs) **(Tan-Torres**

Edejer et al. 2003), or healthy years equivalents (HYEs) **(Mehrez & Gafni, 1989)**. Consequently, results from a CUA are typically stated in terms of the cost per QALY gained or cost per DALY averted **(Robinson, 1993; Shiell et al., 2002; Drummond et al., 2015)**. In contrast to CEA and CUA, both the costs and the health outcomes of alternative health programmes considered in a CBA are measured in monetary terms **(Drummond et al., 2015)**. This form of economic evaluation is typically used to identify whether or not the extra costs of obtaining a specific health outcome is worth it. The CBA provides the absolute and relative benefits of the healthcare programmes considered. Although assigning a monetary value to a health benefit may be challenging, a CBA enables comparison of health interventions across public sectors **(Shiell et al., 2002)**. The output of the analysis may be expressed in terms of a ratio of costs to benefits, or as a sum representing the net benefit or loss of one alternative over another **(Drummond et al., 2015)**.

Often, the costs and consequences of healthcare interventions may present with different time profiles. For instance, although an antenatal screening programme for HBV infection may incur immediate costs, the effects of avoiding liver cirrhosis caused by vertically-acquired CHB would only take place in the future. The comparison of this alternative to another would require adjustment of both costs and consequences to the present values through discounting, using a constant rate. This rate may either be predetermined by national health authorities or extracted from the literature **(Cairns, 2001; Drummond et al., 2015)**.

Furthermore, results from an economic evaluation, usually in the form of ICER, is compared to a benchmark or threshold to determine whether the assessed health intervention is cost-effective or not. If the ICER is lower than the threshold, the intervention is said to be cost-effective, because it yields greater benefits at a low cost. In contrast, if the ICER falls above the threshold, the intervention is not considered cost-effective. This benchmark represents what society is willing to pay for a healthcare service for health gains, and as such is known as the willingness-to-pay (WTP) threshold **(Drummond et al., 2015; Thokala et al., 2018)**. Various WTP thresholds have been suggested. According to the World Bank, ICERs ranging from $150 to $200 per DALYs averted are considered cost-effective in middle-income countries. In lower-income countries, an ICER below $50 per DALY averted is considered highly cost-effective **(World Bank, 1993)**. As per the WHO's Choosing Interventions that are Cost–Effective (WHO-CHOICE) project, health programmes whose ICERs fall between one to three times a country's gross domestic product (GDP) per capita per DALY averted are considered cost-effective. If the ICER is below the country's GDP, the intervention is concluded highly cost-

effective **(WHO, 2002; Hutubessy, Chisholm &Edejer, 2003)**. The WTP threshold of one to three times GDP per capita has been highly used in economic evaluations of health interventions in low- and middle-income countries **(Leech et al., 2018)**. However, Leech et al. (2018) argue that rather than relying on generic economic thresholds, countries should make use of country-specific cost-effectiveness thresholds (CET) that reflect local preferences, to determine the cost-effectiveness of health interventions **(Leech et al., 2018)**. Countries are called to generate WTP thresholds in the context of their resources; to help in determining the cost-effectiveness of new health interventions.

2.8.2 Economic evaluations of interventions against HBV MTCT

A few economic evaluations have examined and compared various strategies for the prevention of MTCT (PMTCT) of HBV. The majority have focused on routine newborn HBV vaccination. This strategy remained consistently cost-effective in both SSA and other low- and middle-income countries **(Aggarwal, Ghohal & Naik, 2003; Griffiths, Hutton & Pascoal, 2005; Vimolket & Poovorawan, 2005; Chen et al., 2013c; Lu et al., 2013)** except in areas of low HBV endemicity **(Lee & Park, 2016)**. The provision of universal newborn HBV vaccination in The Gambia was shown to produce an extra cost of US$28 per DALY averted from a societal perspective, or US$47 per DALY averted from a payer's perspective. Both ICERS fell below the 2002 country's GDP per capita; thus, the intervention was considered highly cost-effective **(Kim, Salomon & Goldie, 2007)**. Subsequent economic evaluations in SSA produced similar observations. In Mozambique, an additional BD of the HBV vaccine was compared to vaccination starting from six weeks of age of the infants, the status quo of the country. An ICER of US$250.95 per DALY averted was obtained from the CEA. Birth vaccination was also considered highly cost-effective as the ICER was lower than the WTP threshold of US$441, the 2008 GDP per capita of Mozambique **(Klingler, Thoumi & Mrithinjayam, 2012)**. Recent work in South Africa reported an ICER of US$329 per DALY averted, in comparison to vaccination from six weeks. Using a CET of US$3 810, which represents half per capita GDP of the country, the strategy was deemed highly cost-effective too **(Hecht et al., 2018)**. The high cost-effectiveness of this intervention remains, using the number of pediatric infections averted as the measure of outcome of the intervention. The recent analytic model from Cameroon also revealed that universal HBV vaccination with BD was the most cost-effective strategy for reducing pediatric HBV infections, as opposed to universal vaccination from six weeks, at a WTP threshold of US$150. Maternal HBsAg screening followed by

targeted HBV birth vaccination was also analyzed in the model. The intervention appeared to be the least costly alternative but did not show a greater decrease in pediatric HBV infections in comparison to the universal birth vaccination strategy **(Anderson et al., 2018)**. The costs and health benefits of maternal HBsAg screening has been predominantly studied along with universal HBV birth vaccination. In high incomes countries, mixed cost-effectiveness results have been reported. Compared to a do-nothing strategy, routine screening of pregnant women for HBsAg was shown to be cost-effective in the United States of America (USA), at an HBsAg prevalence of 0.2%. From the authors' analysis, the policy was found cost-saving **(Arevalo & Washington, 1988)**. The same policy, however, was not cost-saving in the Belgian context, with an HBsAg prevalence of 0.67% within the antenatal population **(Tormans et al., 1993)**. Whether this policy is cost-effective in SSA or not remains unknown. Other interventions, including the provision of HBIG to HBsAg-exposed babies in combination with universal birth vaccination, or maternal antiviral prophylaxis have also been economically assessed. In fact, Chen et al. (2016) demonstrated that universal HBV birth vaccination combined with HBIG could produce both direct and societal cost savings (US$11.68 billion and US$ 45.14 billion, respectively), while averting more than 12 million new HBV infections, and 576 209 cases of HBV related deaths **(Chen et al., 2016)**. Maternal antiviral prophylaxis with lamivudine from the third trimester of pregnancy, in addition to HBIG, produced an additional 0.0024 QALYs gain and averted 0.23 acute infections per birth in Taiwan, at a WTP threshold of US$20 000 **(Hung & Chen, 2011)**. The cost-effectiveness of these two interventions is unknown in SSA.

CHAPTER 3: A study of hepatitis B virus infection in HIV-infected children in Namibia

This chapter describes the molecular characterization of HBV infection in HIV/HBV co-infected children in Windhoek, Namibia, exposed to antiretroviral therapy (ART) containing lamivudine. The presence of any biochemical evidence of liver disease among these children is also reported.

3.1 Introduction

As in many countries in SSA, little is known of the burden of HBV infection in Namibian children in the era of HIV. To our knowledge, the only prevalence data available on HIV/HBV co-infection within this population date from a retrospective study conducted in Northern Namibia. An HBsAg seropositivity rate of 8.7% was found. All children were on ART therapy for HIV **(Brandt et al., 2012)**.

According to the 2016 WHO HIV treatment guidelines, the preferred first-line ART therapy for the management of HIV infection includes lamivudine alone as an antiviral agent with activity against HBV in children less than 10 years old, and a combination of TDF and lamivudine (or emtricitabine) in individuals/children of 10 years old and above against HBV **(WHO, 2016)**. Despite its potency against both HIV and HBV, prolonged exposure to lamivudine only against HBV is well known to be associated with the development of HBV resistance in infected individuals. Resistance to the drug is brought by the selection of viral mutants in the pol gene of the HBV genome **(Sokal et al., 2006)**, and leads to uncontrolled HBV infection. The uncontrolled infection is characterized by persistent high viral replication; it is a significant risk factor for advanced liver disease **(Beasley et al., 1981; Shimakawa et al., 2013)**. The selection of other genomic mutations with increased viral replication may also occur, thereby exacerbating liver damage.

3.2 Study aims

Primary aim

This study primarily aimed to characterize HBV in Namibian children with HIV/HBV co-infection who had been exposed to a lamivudine-based ART regimen.

Secondary aim

The study also sought to determine the role of HBV MTCT in the HBV infections described in these HIV/HBV co-infected children.

3.3 Study objectives

The aims of the study were achieved through the following objectives:

- Determine the rate of active HBV infection among HIV/HBV-infected children.
- Determine the circulating HBV genotypes and the prevalence of HBV mutants.
- Determine liver health, by AST platelet ratio index (APRI), in children who are co-infected with HIV and HBV.

3.4 Materials and Methods

3.4.1 Ethical considerations

The full protocol of this study was submitted to the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, Stellenbosch University, and to the Biomedical Research Ethics Committee (BREC) and Research Management Committee (RMC) at the Namibian Ministry of Health and Social Services (MoHSS) for ethical review. Ethical approval was obtained from both bodies with the HREC reference number N13/02/022. Approval letters from the HREC are found in Appendix A.

According to HREC guidelines, children participating in a research study should be given the opportunity to agree for participation in a research study through assent. This is an agreement given by an individual who is unable to give legal consent for participating in a research activity. Assent from the child should be accompanied by consent from the parent or legal guardian. As per these guidelines, assent was sought from children attending the study site for enrolment in the study, and consent was sought from the legal guardians too. Informed consent was also required from mothers attending with these children for their own participation in the study.

3.4.2 Study design

This was a cross-sectional study conducted at a pediatric HIV ART clinic at the Intermediate Katutura hospital, a state hospital in Katutura, a township of Windhoek.

3.4.3 Study setting

The Republic of Namibia is a country in the southwestern part of Africa covering a total area of 825 615 km^2 inhabited by 2 534 000 people **(United Nations, 2017)**. The country is divided into 14 regions: Kunene, Omusati, Oshana, Ohangwena, Oshikoto, Kavango West, Kavango East, Zambezi, Erongo, Otjozondjupa, Omaheke, Khomas, Hardap and Karas (Figure 3.1); further sub-divided in constituencies, localities, and villages.

The capital and largest city of Namibia is Windhoek, with 325 858 inhabitants as per the last 2011 census **(Namibia Statistics Agency, 2011)**. The city is located in the Khomas region, in the centre of the country, bordered by the Erongo region to the West, by Otjozondjupa to the North and by Omaheke to the East. Healthcare-wise, the regional health directorate comprises two hospitals: Windhoek Central hospital and Intermediate Katutura hospital, three health centers (Katutura, Okuryangava, and Khomasdal Health Centres) and eight primary healthcare clinics **(MoHSS, 2018)**.

Intermediate Katutura hospital is a teaching hospital with a bed capacity of 830. The pediatric HIV clinic opened its doors in 2010 for the care, support, and management of children and adolescents living with HIV. The clinic delivers integrated services including ART, voluntary counseling and testing (VCT), treatment adherence counseling, prevention and treatment of opportunistic infections, HIV disclosure as well as health education to caregivers and adolescent health services.

Figure 3.1: Geographic location of Namibia. The country is bordered by Zambia and Angola to the North, Botswana to the East and South Africa to the South and East. The map was produced using the ArcGIS® Online Software by Esri© 2018 (www.esri.com/software/arcgis/arcgisonline). Namibia province distribution shapefile was obtained from map library (www.maplibrary.com).

3.4.4 Study population

HIV-infected children aged less than 18 years old, exposed to lamivudine as part of their HIV infection treatment and thus inadvertently on monotherapy against HBV, attending the Katutura pediatric HIV ART clinic in Windhoek, Khomas, were considered for enrolment in the study. Current Namibian national guidelines recommend HBsAg screening for all individuals at HIV diagnosis **(MoHSS, 2014a)**. A repeat HBsAg test is done six months following the initial positive test. If the repeat test is negative, it is concluded that the patient is not a chronic carrier of HBV. If the repeat test is positive, the patient has chronic HBV infection and is initiated on ART including abacavir/lamivudine, if weighing less than 35 kg or TDF/lamivudine/efavirenz if weighing at least 35 kg. Children initiated on abacavir/lamivudine should be switched to TDF/3TC as soon as they weigh at least 35 kg. Children who already weigh at least 35 kg should initiate ART with TDF/lamivudine/efavirenz **(MoHSS, 2014a).** HBV screening is performed by sending samples collected at the clinic to the Namibia Institute of Pathology laboratory for testing. Consequently, HIV/HBV co-infected children are known to the clinic. Using convenience sampling, children identified as HBsAg

positive were informed and their assent was sought for participation in the study following the below-mentioned inclusion criteria.

- HBV infection diagnosed by an HBsAg test at the laboratory.
- HIV infection confirmed and diagnosed by:
    - Children 18 months or older at time of diagnosis: a positive rapid HIV antibody testing confirmed by a second rapid HIV antibody test,
    - Children younger than 18 months: a positive virologic test (HIV DNA PCR) for HIV or its components confirmed by a second virologic or antibody test performed on a separately obtained sample.
- On ART for ≥ 18 months. This criterion was applied based on the incidence of mutations associated with lamivudine resistance, which is known to increase with the duration of lamivudine monotherapy: 20% after 12 months of treatment, reaching 90% after 48 months **(Benhamou, 1999)**.

Recruitment took place at the 6-monthly routine follow-up appointment. HIV/HBV co-infected children referred from other provincial or local clinics to the study clinic were recruited at the initial contact. These children were known HIV/HBV co-infected based on laboratory records, available in both the patient's health booklet and in the laboratory system at health facilities in Namibia. Mothers attending with these children were invited to take part in the study.

3.4.5 Sample size

Given that this was an observational study to determine the molecular character of HBV infection in co-infected children in the presence of inadvertent exposure to lamivudine monotherapy against HBV, we planned to recruit about 20 HIV/HBV co-infected children using convenience sampling.

3.4.6 Sample collection

Five dried blood spots (DBS) were collected, through finger prick, on a Whatman perforated filter paper card (5 spots/card) (Lasec, Cape Town, South Africa). A total volume of 10 ml of blood was drawn in a serum separator tube (SST) and in an ethylenediamine tetraacetic acid (EDTA) blood tube from enrolled children ≥ two years old and adolescents. DBS were used for HBV characterization, while SST blood samples were of use for liver biochemistry and

HBV serological testing. EDTA blood samples were used for full blood count analysis. A DBS card, only, was collected from the consenting mothers attending with their HBV-infected children for HBV molecular characterization.

3.4.7 Data collection

Children/adolescents variables recorded include contact details, sex, gestation (preterm/term), mode of delivery, birth weight, HBV vaccination history (verbal history from mother and confirmation from patient care booklet), past history of liver disease, details of opportunistic infections, baseline CD4/ nadir CD4, date of commencing ARVs, type of current and past ART regimens. This information was collected from the patient care booklets. The maternal variables collected from their care booklets include: age, parity, region of birth, HBV status and HBV markers (where available), family history of liver disease, date of commencing ARVs, previous ART, current ART, CD4 count, HIV viral load (where available).

3.4.8 Sample handling

HBV viral load, HBV genotyping, HBeAg, anti-HBe, and anti-HBc are not routine tests offered by the national laboratory in Namibia. These tests were performed at the Division of Medical Virology, Stellenbosch University.

DBS and serum samples were stored at -20°C at the Namibia Institute of Pathology following collection. DBS were thawed at room temperature prior to shipment (for a minimum of 30 minutes), repackaged and sent at ambient temperature to the Division of Medical Virology laboratory at Stellenbosch University, South Africa, whilst residual serum samples were sent on dry ice. At the Division of Medical Virology, all samples (DBS and serum samples) were stored at -20°C and thawed at room temperature in a biosafety cabinet prior to analysis. Laboratory analyses performed on these samples are summarized in Figure 3.2.

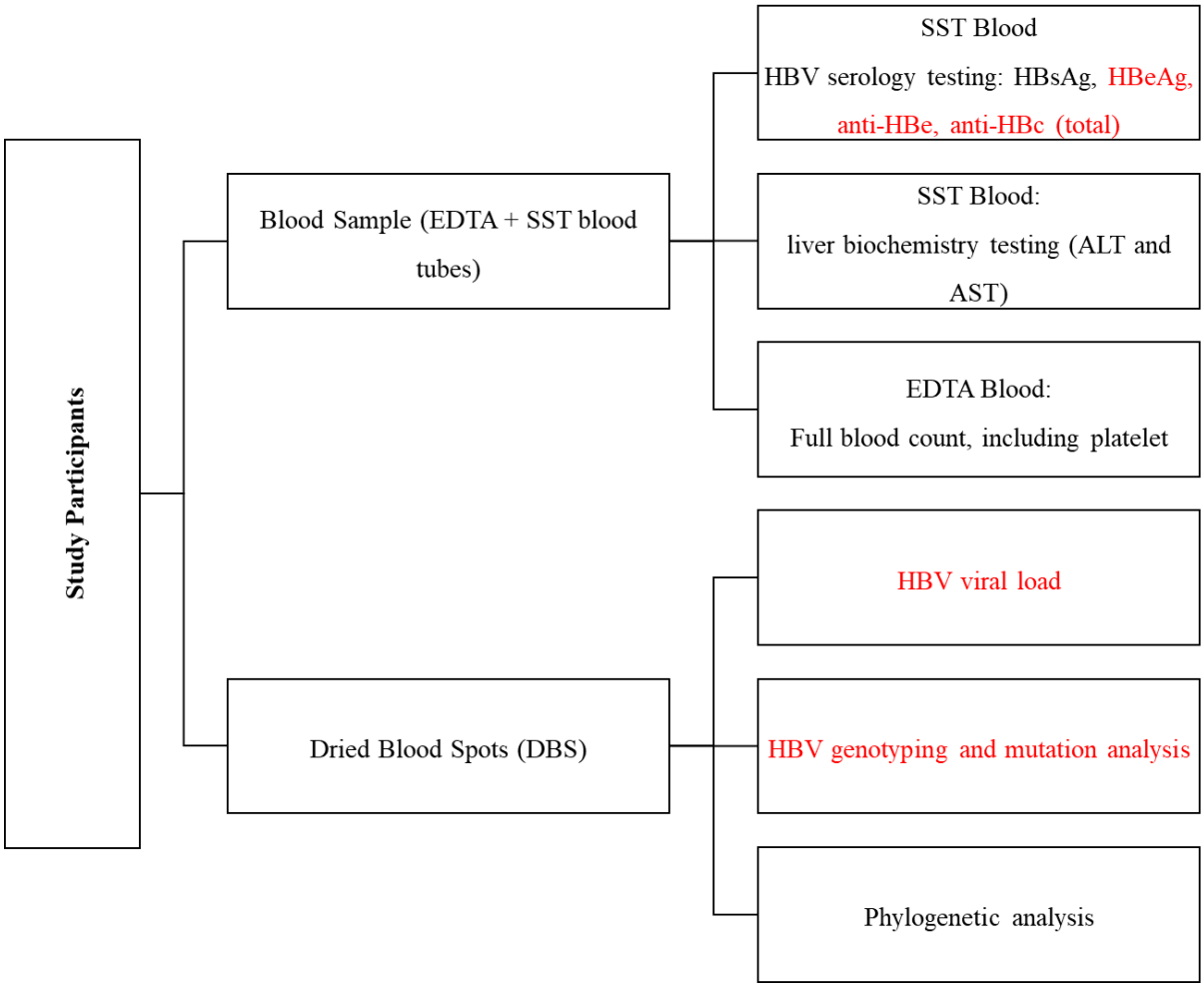


Figure 3.2: A flow diagram of laboratory analysis of samples collected from study participants. This diagram illustrates all the laboratory testing performed on blood samples and DBS samples collected from children enrolled in the study. DBS only were collected from the mothers attending with these children and were used for HBV molecular testing as illustrated in the second branch of the flow diagram. The laboratory testing highlighted in red were conducted at our laboratory while the others were conducted at the Namibian laboratory**.** ALT: alanine transaminase; AST: aspartate transaminase; anti-HBc: antibodies against hepatitis B core antigen, anti-HBe: antibodies against hepatitis B e antigen; DBS: dried blood spots; EDTA: ethylenediamine tetraacetic acid; HBeAg: hepatitis B e antigen, HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; IgM: immunoglobulin M; SST: Serum Separator Tube.

3.4.9 Hepatitis B virus serology assays

Residual serum samples received from the Namibia Institute of Pathology were tested for HBsAg and anti-HBc using the Murex Version 3 GE 34/36 Surface Antigen and the Murex anti-HBc and assays (Diasorin, Saluggia, Italy). HBeAg and anti-HBe were detected by the HBeAg/anti-HBe Diasorin assays (Diasorin).

3.4.9.1 HBsAg assay

Principle of the assay: The Murex HBsAg Version 3 GE 34/36 qualitatively measures the presence of HBsAg in human serum or plasma using the principle of a sandwich ELISA (Diasorin Murex version 3 kit insert).

Assay procedure: Detection of the HBs antigen was performed using 75 µl of the neat sample, following the manufacturer's instructions, as briefly explained henceforth. A volume of 75 µl of each sample and controls (two replicates of negative control and one replicate of positive control, provided in the kit) were incubated with 25 µl of sample diluent for a period of 60 minutes at 37ºC in a thermostatically-controlled incubator, the Auto Flow IR Direct Heat CO_2 Incubator – 37°C, 5% CO_2 standard requirements (Nuaire, Plymouth, USA). A 50 µl volume of the conjugate was added to the mix and incubated for another 30 minutes at 37ºC. After the 30-minute incubation period, the plate was washed five times with a maximum volume of 350 µl of wash buffer for each well using an automatic plate washer, the MW-12A Microplate Washer (Mindray, Shenzhen, China). Following the washing step, 100 µl of the substrate solution was added to the plate and incubated for 30 minutes at 37ºC; leading to a purple change in color where HBsAg was present. Then, 50 µl of stop solution (0.5M H_2SO_4) was added to each well, converting the purple color to a yellow color in reactive wells. The color intensity was measured at 450 nanometers (nm) and 630 nm as reference wavelengths using the dual wavelength ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc, Winooski, Vermont, USA).

Interpretation of results: As per the manufacturer's directives, the absorbance values obtained from the spectrophotometer were compared to a cut-off value. This cut-off value was calculated by adding 0.05 to the mean absorbance of the replicates of the negative control (NC). Samples with an OD value above the cut-off value, specific to each run, were considered reactive for HBsAg.

Quality control: Results of the assay were considered valid if:

(1) The mean absorbance of the NC was less than 0.15.

(2) The mean absorbance of the positive control (PC) was above 0.8.

3.4.9.2 HBeAg assay

Principle of the assay: The qualitative detection of this marker was achieved with the ETI-EBK PLUS kit, a direct non-competitive ELISA assay whereby mouse monoclonal antibody,

pre-coated on the microwells of the polystyrene microtitre plate (provided with the kit), reacts directly with the HBeAg present in the tested sample (DiaSorin ETI-EBK PLUS kit insert).

Assay procedure: Following the manufacturer's instructions, 50 µl of incubation buffer plus 100 µl of calibrator (three replicates), negative and positive controls and samples were dispensed in the microwells of the ELISA plate and incubated at 37°C in the Auto Flow IR Direct Heat CO_2 Incubator. The first microwell of the ELISA plate is left empty, referred here as the blank well. Following a 2-hour incubation period, a 5-cycle wash was done with a maximum volume of 350 µl of wash buffer on the MW-12A Microplate Washer (Mindray, Shenzhen, China). Then, 100 µL of enzyme tracer was added in the microwells of the plate, with the exception of the blank well. The reaction was left in the Auto Flow IR Direct Heat CO_2 Incubator for an hour at 37°C. Unbound enzyme was removed through a 5-cycle wash, and 100 µl of chromogen/substrate was pipetted in all wells of the reaction plate. Following a 30-minute incubation time at room temperature, away from direct light, 100 µl of 0.5M H_2SO_4 was added in all wells; converting the blue color in reactive wells to a yellow color. Non-reactive wells remained colorless. The color intensity of each microwell was measured with a spectrophotometer, the dual wavelength ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc, Winooski, Vermont, USA) at 450 nm.

Interpretation of results: The HBeAg reactivity of the samples were measured by comparing their absorbance values to a cut-off value. This cut-off was calculated by adding 0.060 to the mean absorbance of the three calibrators used during the assay. Sample positive for HBeAg required to have an OD value above the calculated cut-off value.

Quality control: Results of this assay were valid if the following criteria were met:

(1) The blank well OD ranged between 0.000 and 0.150. This well contained the substrate only and was used to blank the Microreader Plate.

(2) The mean absorbance for the calibrator was above -0.020, but less than 0.120.

(3) The OD of the NC ranged between -0.020 and 0.500 and the OD of the PC was between 0.120 and 2.500.

(4) The difference between the absorbance value of the positive control and the negative control was higher than 0.450.

3.4.9.3 Anti-HBe assay

Principle of the assay: The ETI-AB-EBK PLUS kit qualitatively determines the presence of anti-HBe in human serum or plasma. This is a competitive assay. Anti-HBe present in the sample together with monoclonal anti-HBe pre-coated microwells of the ELISA plate (provided with the kit) compete for binding to recombinant HBeAg (Diasorin ETI-AB-EBK PLUS kit insert).

Assay procedure: The assay was conducted according to the manufacturer's guidance. Accordingly, 50 µl incubation buffer was pipetted in the microwells of the plate (depending on the number of samples being tested); the first one was left empty and is referred to as the blank well. Then, 50 µl calibrator (three replicates), negative and positive controls and samples were dispensed in their respective microwells. A change in color to green or dark blue was observed. A volume of 50 µl of neutralizing solution was added to the microwells, apart from the blank well, and incubated for two hours at 37°C. A 5-cycle wash followed and 100 µl working enzyme tracer solution was pipetted in the wells in use, with the exception of the blank well. The reaction was placed in the Auto Flow IR Direct Heat CO_2 Incubator (Nuaire, Plymouth, USA) for an hour at 37°C. Another 5-cycle wash followed and 100 µl chromogen/substrate solution was dispensed in all wells in use, including the blank well. The plate was placed away from direct light at room temperature; allowing the colorimetric reaction to take place. The colorimetric reaction was stopped past 30 minutes with the addition of 100 µl of 0.5M H_2SO_4, the stop solution, in each well. Within an hour of adding the stop solution, the plate was read on the ELx800 Universal Microplate Reader, to determine the absorbance values of each used well.

Calculation of results: The presence of anti-HBe in the tested samples was determined by comparing the absorbance readings to a cut-off value. This cut-off value was determined by multiplying 0.500 by the mean absorbance of the three calibrators. As this is a competitive assay, samples with an absorbance value above the cut-off value were considered negative for anti-HBe.

Quality control: The following criteria were used to validate the results obtained from the assay:

(1) The absorbance for the blank well ranged between 0.000 and 0.150.

(2) The mean absorbance for the calibrator was above 0.500, but less than 2.500; with each calibrator within this range too.

(3) The absorbance for the NC was higher than 0.500 and lesser than 2.500, and the absorbance of the PC was between 0.050 and 0.300.

(4) The difference between the absorbance value of the NC and the PC was higher than 0.250.

3.4.9.4 Anti-HBc (total) assay

Principle of the assay: The Murex anti-HBc (total) qualitatively tests for the presence of total antibodies against HBc antigen (HBcAg) in human serum or plasma, following the principle of a competitive enzyme immunoassay too (Diasorin Murex anti-HBc (total) kit insert).

Assay procedure: Briefly, a mix of 50 µl of sample diluent and 50 µl of sample and control (two replicates of the positive controls and two replicates of the negative control) was pipetted in each well of the ELISA plate and incubated for 30 minutes at 37°C. The wells were washed five times, with a maximum volume of 350 µl of wash buffer per well. Then, 50 µl of the conjugate was dispensed in each well and once more placed in the incubator for 30 minutes at 37°C. A second 5-times washing cycle followed, and 100 µl of substrate solution was immediately dispensed in the sample's wells. A final incubation step at 37°C for 30 minutes was done, and finally 50 µl of the stop solution (0.5M H_2SO_4) was added in the wells of the plate. The intensity of the color (orange) developed was inversely proportional to the concentration of anti-HBc present in the tested samples. Within 15 minutes from adding the stop solution, the absorbance in the sample's wells were measured using the Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 630 nm as the reference wavelength.

Calculation of the results: Results were interpreted based on a cut-off value, resulting from an addition of the mean absorbances of the PC and the NC, divided by two. A result was considered positive where the absorbance value was lower than the cut-off value, whilst an absorbance value lower than the cut-off was concluded reactive for the tested analyte, anti-HBc.

Quality control: The interpretation of results was considered if set criteria were met. These include:

(1) The NC minus the PC was higher than 0.5 but less than 2.2.

(2) The mean absorbance of the PC was lower than 0.24.

3.4.9.5 Safety and quality measures for serology tests

All assays were performed under a class II biological laminar flow cabinet Bioflow-II cabinet (Labotec, South Africa), protecting the user from biohazardous substances handled in the cabinet and the reaction (kits and samples) from unsterile contaminants. The surface of the cabinet was disinfected with 10% bleach and 70% ethanol before and after every procedure. Pipettes were also cleaned before and after use with 10% bleach and 70% ethanol. Samples were centrifuged for one minute at 6000 x g (G-force), using the Centrifuge 5415D (Eppendorf, Hamburg, Germany) before being opened to remove droplets inside the lid of the sample tubes that may potentially cause cross-contamination between samples. Filtered tips were used and changed for each sample and between pipetting exercises performed during the testing procedures.

3.4.10 Hepatitis B virus molecular assays

3.4.10.1 Viral DNA quantification

Levels of HBV DNA present in patient's samples were quantified using DBS on the automated COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The test was completed at the National Health Laboratory Services (NHLS), Division of Medical Virology, Tygerberg. According to the manufacturer, the assay can detect HBV DNA levels ranging from 20 IU/ml to 170 000 000 IU/ml in serum or plasma specimens.

Principle of the assay: The instrument executes two major processes: HBV DNA isolation through the COBAS AmpliPrep, and subsequently simultaneous PCR amplification of the target DNA and HBV Quantitation Standard (QS) DNA coupled with detection of the dual-labeled probe specific to the target through the COBAS TaqMan analyzer. HBV QS DNA is a non-infectious DNA construct with similar primer binding sites as the HBV target DNA and a unique probe binding region allowing a distinction between the target HBV amplicon in the specimen and the HBV QS amplicon during amplification and quantitation.

Making use of generic silica-based capture technology, the COBAS AmpliPrep processes 500 µl of the specimen for DNA isolation. The sample is incubated with a known copy number of

HBV QS DNA, protease, chaotropic lysis buffer and magnetic glass particles at elevated temperatures. This incubation allows the lysis of HBV viral particles and the release of HBV nucleic acids. Released DNA and HBV QS DNA bind to the surface of the glass particles, and a washing step follows. During the washing step, unbound substances (salts, proteins, and other impurities) are removed from the reaction. An aqueous solution is used for nucleic acids elution (both target HBV DNA and HBV QS DNA). Eluted nucleic acids are added to the amplification mixture and transferred to the COBAS TaqMan analyzer equipped with a thermal cycler. The latter heats the reaction mixture allowing denaturation of the double-stranded DNA, followed by primers annealing to the target DNA as the mixture cools down. In the presence of manganese (Mn^{2+}) and excess deoxynucleotide triphosphates (dNTPs) such as deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine (in place of thymidine), the annealed primers are elongated along the target template forming a double-stranded DNA molecule (the amplicon) through the activity of the thermostable Thermus species Z05 DNA Polymerase. This process is repeated through a required number of cycles, each doubling the amount of DNA amplicons. These amplicons are detected in real-time, as they accumulate, using dual-labeled fluorescent probes. During each amplification process, the probes bind to a target sequence and are cleaved by the 5' → 3' nuclease activity of the Z05 DNA polymerase. The fluorescent lights emitted during each amplification cycle are measured at different wavelengths for target HBV DNA and HBV QS DNA.

Sample processing: Briefly, one DBS was eluted in a 1000 µl volume of sample pre-extraction (SPEX) buffer (30 mM sodium citrate dihydrate, 42.5% guanidine thiocyanate, 4% polydocanol and 2% dithiothreitol), provided in the AmpliPrep/COBAS TaqMan HBV test Version 2.0 kit. Elution took place overnight on a slow shaker, The Coulter Mixer, (Coulter Electronics Limited, Luton Bedfordshire, UK) at room temperature. The eluate was used for both viral DNA extraction and amplification by the analyzer.

Dilution factor between DBS and plasma: Plasma forms 60% of whole blood. In an approximate volume of 50 µl of capillary blood applied on a DBS, theoretically, 30 µl of plasma was eluted in the 1000 µl of SPEX buffer. Of this, 650 µl is used for the assay corresponding to an input sample volume of 20 µl in the assay. Given that HBV DNA viral load is reported by the analyzer as the number of international units in a 1000 µl, a dilution factor of 50 was used to calculate the viral loads.

Quality control: Three controls including the HBV High Positive Control (HPC), the HBV Low Positive Control (LPC) and the HBV Negative Control (human plasma) are provided in

the AmpliPrep/COBAS TaqMan HBV test V2.0 kit. These controls were included during each test batch on the AmpliPrep/COBAS TaqMan to validate the results. The HBV DNA IU/ml result obtained for each positive control was verified to fall within the ranges provided by the manufacturer (LPC range: $10^2 – 10^3$ IU/ml, HPC range: 3.21 x 10^5 – 3.20 x 10^6 IU/ml) and the negative control must give a "Target not detected (TND)" result.

Interpretation of results: An in-house validation for the use of DBS specimen for HBV viral load was previously completed at the NHLS laboratory, Tygerberg Hospital, South Africa. The assay showed a limit of quantification (LoQ) of 843 IU/ml (2.93 $\log_{10}$ IU/ml) (95% CI: 479 IU/ml – 2600 IU/ml) **(Claassen et al., unpublished)** (Appendix B). Where the assay recorded a viral load < 20 IU/ml, the result was reported as < 843 IU/ml. A "TND" output was recorded as "lower than the detection limit (LDL)". Where a value within the range of the assay was obtained, this was multiplied by 50, the dilution factor, and recorded as the HBV DNA concentration of the analyzed sample. Further molecular analysis was performed on samples with detectable levels of HBV DNA, described as follow.

3.4.10.2 Manual viral DNA extraction

Viral DNA was manually extracted from samples with detectable levels of DNA for genome sequencing using the QIAamp® MinElute® Virus Spin kit (QIAGEN, Hilden, Germany). Prior to viral nucleic acid extraction, DBS were eluted following a previously published protocol **(Mössner et al., 2015)**, with slight modifications. Briefly, one DBS (per patient) was pushed out from the perforated card in a clean 1.5 ml microcentrifuge tube (Thermo Fisher Scientific Inc, California, USA) using a clean tip. Tips were changed between samples. A 500 µl volume of 1xPBS/0.05% Tween 20/0.08% sodium azide (NaN_3) elution buffer was pipetted in the microcentrifuge tubes. The samples were gently shaken on a microtube foam adapter inserted on the Vortex Genie 2 (Scientific Industries Inc, New York, USA) for overnight elution at room temperature. Viral DNA was extracted, as per the manufacturer's instructions using 200 µl of eluates (QIAamp® MinElute® Virus Spin Handbook).

The viral nucleic acid extraction procedure follows four main steps namely: lysis, binding, washing, and elution. During the lysis step, 200 µl of each eluate is added to 25 µl of QIAGEN protease solution (reconstituted in buffer AVE) in a 2 ml microcentrifuge tube. A 200 µl volume of buffer AL mix, containing 5.6 µg of carrier RNA and 3.5 µl of murine cytomegalovirus (mCMV) extract, was added to the sample. The reaction was mixed by pulse-vortexing for 15 seconds and incubated at 56ºC for 15 minutes on a heating block (Omega

Scientific Pte, Ltd, Thailand). The mCMV extract, here considered as the internal control, was received from the Blood Borne Virus Unit (BBVU), PHE (Colindale, UK). After the 15-minutes incubation period, the reaction tubes were briefly centrifuged in the Eppendorf centrifuge 5415 D (Merck, New Jersey, USA) and 250 µl of 100% ethanol (Merck, USA) was added in the reactions. The lysates were thoroughly mixed by pulse-vortexing, and a five-minute incubation followed at room temperature. Following another brief spin of the microcentrifuge tubes, each lysate was pipetted onto the QIAamp Mini spin column and centrifuged for a minute at 6000 x g; allowing the viral DNA to bind to the silica membrane of the columns. Bound viral DNA was purified through two subsequent washing cycles, performed using 500 µl buffer AW1 and 500 µl buffer AW2. Centrifugation at 6000 x g for a minute followed each washing cycle. Final DNA purification was completed by adding 500 µl of 100% ethanol to the membrane of the QIAamp mini columns. The columns were centrifuged at 6000 x g for three minutes. Ethanol carry over that may be present in the columns was removed through additional centrifugation at 20 000 x g, and the columns were incubated at 56ºC for three minutes with caps opened for complete evaporation of remaining ethanol. To end, 60 µl of buffer AVE (the elution buffer) was pipetted to the center of the silica membrane of the mini columns, and DNA elution was completed at 20 000 x g for a minute, following a five minute incubation period.

Extracted HBV DNA was subjected to Sanger sequencing for HBV genotyping, and to determine the presence of drug-associated or vaccine-escape mutants as well as mutants that have been previously associated with the development of HCC.

3.4.10.3 Nucleotide sequencing of the polymerase/surface and X/core genes

Sequencing of these two genes was performed using protocols developed at the BBVU, PHE (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684). These protocols have been transferred to and are now established in our laboratory.

Genomic sequences were obtained through DNA amplification by means of nested PCR to generate specific DNA for the sequencing reaction, gel electrophoresis, DNA template purification, cycle sequencing PCR and capillary electrophoresis. Details of each step are provided below.

3.4.10.3.1 DNA Amplification of the polymerase/surface (pol/surf) gene

First round of amplification (Pre-nested PCR)

Two primers (Table 3.1) were used to amplify the pol/surf region, including domains A to E of the RT region, of the viral genome. A master mix was prepared in a 2 ml microcentrifuge tube as per Table 3.2 and pipetted in volumes of 20 µl in 0.2 ml PCR tubes while working on ice. Five microlitres of DNA was added in their respective PCR tubes, and the reaction was carried on in the GeneAmp PCR system 9700 (Applied Biosystems (ABI)) following cycling conditions tabulated in Table 3.3.

Table 3.1: Oligonucleotides used for pre-nested PCR amplification of the pol/surf gene

Oligonucleotide name[#]	5'→3' Sequence	Position on HBV genome*
HBV Z – Forward	AGC CCT CAG GCT CAG GGC ATA	1179 - 1199
HBV 3 – Reverse	CGT TGC CKD GCA ACS GGG TAA AGG	2478 - 2455

[#]http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684. *Numbered according to Pugh et al. (1986) **(Pugh et al., 1986)**.

Table 3.2: Master Mix for pre-nested PCR amplification of the pol/surf gene

Reagents	Stock concentration	Volume (µl) per 1 rxn	Final concentration
PCR buffer	10X	2.50	1 x
$MgCl_2$	50mM	0.75	1.5mM
dNTPs	10mM	0.50	0.2mM
Taq Polymerase		0.10	
HBV 3	20pmol/µl	0.50	0.4pmol/µl
HBV Z	20pmol/µl	0.50	0.4pmol/µl
NFW	n/a	15.15	n/a
Total volume µl	n/a	20.00	n/a
Combine 5 µl of DNA with 20 µl of Master Mix			

PCR buffer (Invitrogen, California); deoxynucleoside triphosphate (dNTP) mix (Bioline, London); magnesium chloride ($MgCl_2$) (Invitrogen, California); *Thermus aquaticus (Taq)* Polymerase (Invitrogen, California); NFW: Nuclease free water; mM: millimolar; pmol/µl: picomole per microlitres; n/a: not applicable; Rxn: reaction.

Table 3.3: Cycle conditions for pre-nested PCR amplification of the pol/surf gene

Steps	Cycling parameters	Cycles	Temperature	Time
1	Initial denaturation	1	95ºC	5 minutes
2	Denaturation	34	94ºC	30 seconds
3	Annealing	34	55ºc	30 seconds
4	Extension	34	72ºC	1minute
5	Final extension	1	72ºC	2 minutes

Steps 2, 3 and 4 were repeated 34 times.

Second round of amplification (Nested PCR)

The second round of amplification, using the pre-nested PCR products as templates and a different set of primers (see Table 3.4), was performed. Each reaction was prepared by combining one µl of the pre-nested PCR product with 49 µl of a master mix, prepared as per Table 3.5. DNA amplification was performed in the GeneAmp PCR system 9700 (ABI) at cycling conditions tabulated in Table 3.6. DNA products acquired from the nested PCR were visualized by gel electrophoresis (see below), allowing confirmation of the correct size of the amplicons for pol/surf genomic sequencing.

Table 3.4: Oligonucleotides used for Nested PCR amplification of the pol/surf gene

Oligonucleotide name[#]	5'→3' Sequence	Position on HBV genome*
HBV P – Forward	TCA TCC TCA GGC CAT GCA GT	1292 – 1311
HBV M – Reverse	GAC ACA CTT TCC AAT CAA TNG G	2306 – 2287

[#]http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684. *Numbered according to Pugh et al. (1986) **(Pugh et al., 1986)**.

Table 3.5: Master Mix for nested PCR amplification of the pol/surf gene

Reagents	Stock concentration	Volume (µl) per 1 rxn	Final concentration
PCR buffer	10X	5.0	1 x
$MgCl_2$	50mM	1.5	1.5mM
dNTPs	10mM	1.0	0.2mM
Taq Polymerase		0.2	
HBV P	20pmol/µl	1.0	0.4pmol/µl
HBV M	20pmol/µl	1.0	0.4pmol/µl
NFW	n/a	39.3	n/a
Total volume µl	n/a	49.0	n/a
Combine 1 µl of pre-nested DNA product with 49 µl of Master Mix			

PCR buffer (Invitrogen, California); deoxynucleoside triphosphate (dNTP) mix (Bioline, London); magnesium chloride ($MgCl_2$) (Invitrogen, California); *Thermus aquaticus (Taq)* Polymerase (Invitrogen, California); NFW: Nuclease free water; mM: millimolar; pmol/µl: picomole per microlitres; n/a: not applicable; Rxn: reaction.

Table 3.6: Cycle conditions for nested PCR amplification of the pol/surf gene

Steps	Cycling parameters	Cycles	Temperature	Time
1	Initial denaturation	1	95ºC	5 minutes
2	Denaturation	34	94ºC	30 seconds
3	Annealing	34	50ºc	30 seconds
4	Extension	34	72ºC	1 minute
5	Final extension	1	72ºC	7 minutes

Steps 2, 3 and 4 were repeated 34 times.

3.4.10.3.2 DNA Amplification of the X/core gene

As performed for the amplification of the pol/surf gene in section 3.3.9.3.1, DNA templates of the X/core gene were produced through two rounds of amplification too.

First round of amplification

The pre-nested PCR reaction was prepared using two primers (Table 3.7) pipetted in a master mix, as detailed in Table 3.8, to make a total volume of 20 µl. Five microlitres of the DNA template was pipetted in 0.2 ml PCR tubes with the master mix. The PCR was completed in the GeneAmp PCR system 9700 (ABI) using cycling conditions presented in Table 3.9.

Table 3.7: Pre-nested PCR oligonucleotides for HBV X/core gene amplification

Oligonucleotide name[#]	5'→3' Sequence	Position on HBV genome*
H4072 – Forward	TCT TGC CCA AGG TCT TAC AT	1602 – 1621
Outer core – Reverse	TCC CAC CTT ATG AGT CCA AG	2468 - 2449

[#]**(Dervisevic et al., 2007)**. *Numbered according to Pugh et al. (1986) **(Pugh et al., 1986)**.

Table 3.8: Master Mix for pre-nested PCR amplification of the X/core gene

Reagents	Stock concentration	Volume (µl) per 1 rxn	Final concentration
PCR buffer	10X	2.50	1 x
$MgCl_2$	50mM	0.75	1.5mM
dNTPs	10mM	0.50	0.2mM
Taq Polymerase		0.10	
H4072	20pmol/µl	0.50	0.4pmol/µl
Outer Core	20pmol/µl	0.50	0.4pmol/µl
NFW	n/a	15.15	n/a
Total volume µl	n/a	20.00	n/a
Combine 5 µl of pre-nested DNA product with 20 µl of Master Mix			

PCR buffer (Invitrogen, California); deoxynucleoside triphosphate (dNTP) mix (Bioline, London); magnesium chloride ($MgCl_2$) (Invitrogen, California); *Thermus aquaticus (Taq)* Polymerase (Invitrogen, California); NFW: Nuclease free water; mM: millimolar; pmol/µl: picomole per microlitres; n/a: not applicable; Rxn: reaction.

Table 3.9 Cycling conditions for pre-nested PCR amplification of the X/core gene

Steps	Cycling parameters	Cycles	Temperature	Time
1	Initial denaturation	1	94ºC	2 minutes
2	Denaturation	35	94ºC	30 seconds
3	Annealing	35	55ºc	30 seconds
4	Extension	35	72ºC	1minute
5	Final extension	1	72ºC	2 minutes

Steps 2, 3 and 4 were repeated 34 times.

Second round of amplification

This was completed using a 50 µl reaction combining 49 µl of a prepared Master Mix (Table 3.11) and one µl of pre-nested PCR DNA product. The primers used are listed in Table 3.10. DNA amplification took place in the GeneAmp PCR system 9700 (ABI) using cycling parameters similar to the first round of amplification; mentioned above (Table 3.9). Next, DNA amplicons were analyzed by gel electrophoresis.

Table 3.10 Nested PCR oligonucleotide for nested PCR amplification of the X/core gene

Oligonucleotide name#	**5'→3' Sequence**	**Position on HBV genome***
H4072 – Forward	TCTTGCCCAAGGTCTTACAT	1602 – 1621
Inner core – Reverse	CAGCGAGGCGAGGGAGTTCTTCTT	2422 – 2445

#**(Dervisevic et al., 2007)**.*Numbered according to Pugh et al. (1986) **(Pugh et al., 1986)**.

Table 3.11 Master Mix for nested PCR amplification of the X/core gene

Reagents	**Stock concentration**	**Volume (µl) per 1 rxn**	**Final concentration**
PCR buffer	10X	5.0	1x
$MgCl_2$	50mM	1.5	1.5mM
dNTPs	10mM	1.0	0.2mM
Taq Polymerase		0.2	
H4072	20pmol/µl	1.0	0.4pmol/µl
Inner core	20pmol/µl	1.0	0.4pmol/µl
NFW	n/a	39.3	n/a
Total volume µl	n/a	49.0	n/a
Combine 1 µl of pre-nested DNA product with 49 µl of Master Mix			

PCR buffer (Invitrogen, California); deoxynucleoside triphosphate (dNTP) mix (Bioline, London); magnesium chloride ($MgCl_2$) (Invitrogen, California); *Thermus aquaticus (Taq)* Polymerase (Invitrogen, California); NFW: Nuclease free water; mM: millimolar; pmol/µl: picomole per microlitres; n/a: not applicable; Rxn: reaction.

3.4.10.3.3 Gel electrophoresis

This method is used for the separation of nucleic acids (DNA or RNA) and proteins molecules according to their molecular size. During the gel electrophoresis, the molecules are pulled to move through the pores of the gel by an electric field; the smaller molecules move faster and migrate farther than the larger ones, forming different size bands on the gel.

Agarose gel electrophoresis was used. The concentration of agarose used is dependent on the size of the DNA fragments to be separated. For DNA fragments of approximately 1kb, a 1.5% gel is often used. A 1.5% agarose gel was prepared by heat-dissolving 1.5 g of powder

SeaKem® LE agarose (LONZA, Base, Switzerland) in 100 ml of 1× tris-acetate-ethylene diamine tetra acetic acid (TAE) buffer for two minutes in a microwave. The 1×TAE buffer was prepared from a 50×TAE stock solution, prepared by dissolving 242 g Tris base (Boehringer, Mannheim, Germany) in 57.1 ml of glacial acid (Merck Chemicals, Germany), 100 ml of 0.5 EDTA of pH = 8.0 and distilled water. The melted agarose was allowed to cool down at room temperature and poured in a gel cast, whose dimensions were dependent on the number of samples analyzed during each experiment. A comb was immediately placed in the geld mold to create wells for the loading of samples. Once set, the comb was removed, and the gel was submerged in 1×TAE buffer for loading of DNA. Five microlitres of each nested PCR product was mixed with one µl of 6X Novel Juice (GeneDireX Inc, Taiwan), the loading dye, and loaded in each well of the gel. Novel Juice is a fluorescent dye providing instant visualization of DNA bands upon applying blue light or UV light to the agarose gel. The buffer contains three tracking dyes namely Bromophenol Blue (dark blue color) migrating with larger DNA fragments, Xylene Cyanol FF (light blue color) co-migrating smaller fragments and, Orange G (light orange) migrating ahead of Bromophenol Blue; thereby enabling visual tracking of DNA fragments migrating through the gel during gel electrophoresis (https://www.genedirex.com/novel-juice/). A one kb DNA ladder (GeneRuler™, Fermentas, Massachusetts, USA) was used as a molecular marker to confirm the molecular weight of the amplified DNA products. The DNA ladder was stained with Novel Juice and pipetted at one end of the gel. Electrophoresis was conducted at 80Volts for 30 minutes. The gel slab was visualized on the UVItec Prochemi (UVItec, Cambridge, UK) image acquisition system, after being exposed to ultraviolet (UV) light at 245 nm wavelength. Images acquired were edited using the UVIband-1D gel analysis software (Cambridge, UK) and stored for interpretation.

An approximate DNA size of 1000 bp and 750 bp was expected for the pol/surf and the X/Core genes, respectively. DNA products of the correct size were purified for further molecular analysis.

3.4.10.3.4 Purification of nested PCR products and DNA concentration quantification

Purification of DNA products seen on the gel slab was done by means of the Isolate II PCR and Gel Kit (Bioline, London, UK). The kit allows both purification and concentration of DNA through the principle of DNA capture - DNA binding - washing and elution.

As per the manufacturer's instructions, one volume (45 µl) of each sample was mixed in two volumes (90 µl) of binding buffer CB in labeled Isolate II PCR and gel columns, provided in

the kit. The columns were centrifuged for 30 seconds at 11 000 x g, using the Prism™ Microcentrifuge (Labnet International Inc, Edison, New Jersey, USA) and the flow through was discarded. A 700 µl volume of wash buffer CW was pipetted in the columns. These columns were subsequently centrifuged at 11 000 x g for another 30 seconds. The flow-through was discarded, and the column placed back in the collection tube. Another centrifugation step followed for 60 seconds at 11 000 x g, to remove residual ethanol present in the wash buffer. Finally, the columns were placed in clean 1.5 ml microcentrifuge tubes and 15 µl of elution buffer C was directly applied onto the silica membrane of the columns and left to incubate for 60 seconds at room temperature. Centrifugation followed at 11 000 x g for 60 seconds for DNA elution.

The purified DNA products were quantified spectrophotometrically by means of the NanoDrop® ND-1000 (Thermo Fisher Scientific, USA). This device makes use of a patented retention technology operating with surface tension alone to hold the sample in place. This technology eliminates the need for cuvettes and other sample containment devices and allows for rapid cleaning between samples during measurements. A blanking cycle was performed at first: one µl of the elution buffer C was measured to confirm that the instrument is working as expected. One µl of each purified product was pipetted onto the instrument, and the concentration and purity of DNA present were automatically determined with the "Nucleic Acid" Application module of the NanoDrop Software Version 3.1.0. DNA purity was determined as per the absorbance 260/280 ratio ($A_{260/280nm}$ ratio); a ratio of approximately 1.8 is accepted as "pure". Absorbance was measured at a 260 nm wavelength, and nucleic acid concentration (in ng/µl) was calculated. The pedestal was cleaned up between samples to avoid cross-contamination. Results were printed and analyzed. Samples with a DNA concentration above 20ng/µl were diluted to the recommended range (5-20ng/µl) for the sequencing PCR reaction.

3.4.10.3.5 Cycle sequencing PCR using purified nested DNA products

The entire HBsAg gene region, which includes domains A to E of the overlapping HBV pol region, was investigated for HBV genotyping. Mutations associated with vaccine escape and antiviral drug resistance were of interest in this region, while the BCP and pre-C nucleotide sequences were of interest in the core gene. Sequencing of these viral genes was performed through Sanger sequencing, also known as the chain-termination method. This methodology is based on the selective incorporation of chain-terminating modified dNTPs known as

dideoxynucleotide triphosphates (ddNTPs). ddNTPs do not have the 3'-hydroxyl (OH) group required for the formation of a phosphodiester bond between two nucleotides (nt), thereby causing termination of DNA elongation when incorporated. During this reaction, when a dNTP (A, C, G, or T) is added to the 3′ end of the single-stranded DNA template, chain extension carries on. And, when a ddNTP (ddA, ddC, ddG, or ddT) is added to the 3´ end of the primer, DNA synthesis ends.

The reaction was prepared through a fluorescent-based cycle sequencing PCR using the BigDye® Terminator V3.1 Cycle Sequencing Ready Reaction kit (ABI, California, USA), a pre-mixed format to which the template, purified nested DNA products, and primers specific to the region of interest (listed in Table 3.12 and Table 3.13) were added in. Fluorescent-labeled dNTPs are incorporated by a DNA polymerase for DNA elongation, and whenever a ddNTP is added, the DNA polymerase ceases elongation; resulting in the formation of DNA fragments of various lengths with ddNTPs at the 3′ -OH end.

The number of reactions for each sample was dependent on the number of primers required; thus four reactions were prepared for each sample, as four primers (two forward primers and two reverse primers) were needed for each gene. A 10 µl reaction, per sample, was prepared as outlined in Table 3.14 and pipetted in a 96-well plate **(**ThermoFisher Scientific, Massachusetts, USA**)**. The cycle sequencing PCR was executed on the GeneAmp PCR system 9700 (ABI) through cycling conditions presented in Table 3.15. Sequencing products acquired were purified.

Table 3.12: Oligonucleotides used for sequencing the HBV pol/surf gene

Oligonucleotide name	**5'→3' Sequence**	**Position on HBV genome***
HBV P – Forward	TCA TCC TCA GGC CAT GCA GT	1292 - 1311
HBV M – Reverse	GAC ACA CTT TCC AAT CAA TNG G	2306 - 2287
HBV H – Forward	TAT CAA GGA ATT CTG CCC GTT TGT CCT	1767 - 1793
HBV N - Reverse	ACT GAG CCA GGA GAA ACG GAC TGA GGC	1991 - 1965

*Numbered according to Pugh et al. (1986) **(Pugh et al., 1986)**.

Table 3.13: Oligonucleotide used for sequencing the HBV X/core gene

Oligonucleotide name	5'→3' Sequence	Position on HBV genome*
H4072 - Forward	TCT TGC CCA AGG TCT TAC AT	1602 – 1621
Inner Core– Reverse	CAG CGA GGC GAG GGA GTT CTT CTT	2422 – 2445
CSEQR – Reverse	GGA GGA GTG CGA ATC CAC ACT	2314 – 2334
RSP – Forward	GTT CAA GCC TCC AAG	1830 – 1844

*Numbered according to Pugh et al. (1986) **(Pugh et al., 1986).**

Table 3.14: Sequencing PCR reaction mix

Reagents	Volume (µl) per 1 rxn
Terminator ready reaction mix	1 µl
ABI sequencing buffer	3 µl
Nuclease-free water	4 µl
Total Volume	8 µl
Combine 8 µl of this mater mix with 1 µl of each primer per well and 1 µl of DNA amplicons	

Rxn: reaction

Table 3.15: Cycling conditions for sequencing pol/surf and X/core genes

Steps	Cycling parameters	Cycles	Temperature	Time
1	Denaturation	30	96ºC	20 seconds
2	Annealing	30	55ºC	20 seconds
3	Extension	30	60ºC	4 minutes

These three steps were repeated 30 times

3.4.10.3.6 Purification of cycle sequencing products

Purification of the sequencing reaction products entails removal of unincorporated ddNTPs used during the sequencing reaction and salts that might interfere with base calling during capillary electrophoresis, using the BigDye® Xterminator Purification Kit (ABI, Foster City, California, USA). The XTerminator® solution gets rid of unincorporated dye terminators and free salts, while the SAM™ solution enhances the XTerminator performance and stabilizes the samples after purification.

A master mix was pre-made, composed of 49.5 µL SAM solution and 11 µL XTerminator solution per sample. Fifty-five µl of the mix was pipetted in each well of the 96-well plate containing the sequencing products. The plate was closed with a MicroAmp Optical sheet (ABI, California, USA) and vortexed on the Multi-microplate Genie microplate shaker (Scientific Industries, New York) for 30 minutes at 2000 x g. Centrifugation of the plate for

two minutes at 1000 x g followed to collect all the liquids at the bottom of the wells, and the sequences were read on the ABI Prism 3130xl genetic analyzer (ABI, California, USA).

3.4.10.4 Sequence and phylogenetic analysis

Sequence chromatograms acquired from the DNA sequencing analysis software on the 3130xl genetic analyzer were uploaded and analyzed on Geneious Version 10.2.3 (Biomatters, New Zealand). The raw sequences were trimmed and edited, where necessary, and contigs were assembled for each sample. The consensus sequences were fed in the Basic Local Alignment Sequence Tool (BLAST) on the NCBI website to confirm that the sequences acquired were truly the targeted regions of HBV. Pol/surf consensus sequences were submitted to the National Library of Medicine HBV Genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) for genotyping, and to the Max Planck Institute for Informatics HBV genotyping tool (Geno2Pheno) (http://hbv.geno2pheno.org/index.php) and the HBV Sequencing tool from Stanford University (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) for mutation analysis.

Viral pol/surf gene sequences matching our query sequences on Genbank, based on the nucleotide similarities and geographical location similarities, were downloaded and fed in Geneious Version 10.2.3. The ClustalW algorithm was used for sequence alignment in Geneious. The aligned sequences were imported in Molecular Evolutionary Genetics Analysis (MEGA) Version 7 **(Kumar et al., 2016)**. The phylogenetic relationship between sequences in our dataset was inferred through a neighbor-joining tree analysis **(Saitou & Nei, 1987)**, performed using the p-distance model. A bootstrap test (1000 replicates) **(Felsenstein, 1985)** was included to validate the accuracy of the tree. The p-distance model calculated the proportion of nucleotide sites at which a pair of sequences being compared differs. A best-fit substitution model analysis was run in the JModelTest Version 2.1.3 software **(Posada 2008)**. As the name suggests, the analysis is aimed to determine the most suitable model for use to infer the evolutionary relationship between the sequences in our dataset. The programme compares different nucleotide substitution models and chooses among them the most appropriate one for analysis of the dataset using five strategies namely hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC), and a decision theory method (DT). The best-fitted model, chosen using AIC and BIC scores, was applied to our dataset to complete a maximum-likelihood analysis **(Felsenstein, 1981)** in MEGA7.

The URR (nt 1653 to nt 1741), BCP (nt 1742–1849) and pre-C (nt 1814–1900) regions of the HBV core sequences were manually extracted using Geneious Version 10.2.3 and variants were manually detected using BioEdit Version 7.2.5. **(Hall, 1999)**, using the wild-type gene sequences as references. These gene sequence references were provided by the BBVU at PHE (Colindale, UK).

3.4.10.5 Quality assurance for molecular assays

All molecular assays were performed in dedicated rooms, under a class II biological laminar flow cabinet (Bioflow-II cabinet). Clean gloves were always worn when completing the different procedures. The surfaces of the biosafety cabinets and of the laboratory benches were disinfected with 10% bleach and 70% ethanol before and after each procedure. Pipettes were also cleaned before and after use with 10% bleach and 70% ethanol.

Sample tubes were briefly centrifuged to eliminate sample-glove contact. An NC, normal human plasma (NHP) previously tested negative for all HBV markers was used for each extraction procedure and during each PCR procedure to detect cross-contamination during sample handling. A PC and a No Template Control (NTC) were added in each PCR procedure. The NTC (nuclease-free water) was used to exclude contamination from the reagents used in the assays, and the PC served to confirm true positive results. The PC was extracted from a dilution (4 $\log_{10}$ IU/ml) of an in-house HBV control, with a known viral load of 8 $\log^{10}$ IU/ml, made in NHP. The in-house HBV control was previously calibrated against the second WHO HBV DNA standard for NAT assays obtained from the National Institute for Biological Standards and Control (NIBSC) (NIBSC code: 97/750).The NIBSC standard had an HBV DNA viral load of 6 $\log_{10}$ IU/ml.

3.4.11 Liver health evaluation

All HIV/HBV co-infected children were evaluated for laboratory evidence of liver disease. This entailed a laboratory assessment including liver biochemistry, full blood count and the AST platelet ratio index (APRI). AST, ALT and platelet count testing were performed at the Namibia Institute of Pathology in Windhoek. These measurements were used for the calculation of the APRI score following the formula: APRI = (AST level/Upper normal limit) X100/platelet count. An AST upper limit of normal (ULN) value of 40 was used to calculate the APRI score, and an APRI > 0.5 was considered a sign of liver fibrosis **(McGoogan et al., 2010)**.

3.5 Results

3.5.1 Patient recruitment

Between September 2014 and May 2015, 21 participants were enrolled in the study: 15 children/adolescents and six mothers attending with their children. The mothers of six participants were deceased, and the other two mothers did not attend the clinic with their children at the time of recruitment. Demographics and baseline clinical characteristics of participants are represented in Table 3.16. At enrolment, three children were following a TDF-containing ART regimen, and 11 were on lamivudine therapy as the only agent against HBV. One child (Child 2C) previously on a TDF-based ART had been switched to lamivudine therapy. Treatment history was unknown for one child (14C). A summary of pediatric ARV therapy regimen is provided below in Table 3.17. All mothers enrolled were following a TDF-containing ART regimen for the treatment of their HIV-1 infection.

Table 3.16: Demographics and clinical characteristics of study participants

Characteristic	Children	Mothers
Sample number	15 n/N; %	6 n/N; %
Age at sample collection		
Median	14 years	41.5 years
Range (min – max)	8 – 19	32 – 46
Sex		
Female	7/15 ; 46.7%	6/6; 100%
Male	8/15; 53.3%	NA
HBV vaccination		
Yes	0/15; 0.0%	NA
No	13/15; 86.7%	NA
Unknown	2/15; 13.3%	NA
Past liver disease		
Yes	1/15; 6.7%	NA
No	12/15; 80.0%	NA
Unknown	2/15; 13.3%	NA
Baseline AST[¥] (IU/ml)		
Median	27	Not Available
Range (min – max)	27 – 27	Not Available
Baseline ALT[$] (IU/ml)		
Median	38	35
Range (min – max)	13 – 359	20 – 59
CD4 count[$] (most recent at enrollment)		
Median	628	405
Range (min – max)	447 – 1887	120 – 1312

[¥]Baseline AST level was available for patient 9C only. [$]Baseline ALT levels and the most recent CD4 count from the date of enrollment were available for 13 patients. ALT: alanine aminotransferase; AST: aspartate aminotransferase; HBV: hepatitis B virus; IU/ml: international units per milliliter; Kg: kilogram; NA: Not Applicable.

Table 3.17: Antiretroviral therapy regimen history of children and adolescents enrolled

Patient SID	HAART regimen at enrolment	Start date	Previous HAART regimen	Start date
1C*	ABC/3TC/EFV	08/04/2014	D4T/3TC/EFV	02/09/2008
2C	AZT/3TC/NVP	07/03/2014	TDF/3TC/NVP	26/01/2011
3C	AZT/3TC/NVP	08/12/2005	AZT/3TC/NVP	08/12/2005
4C	AZT/3TC/EFV	22/07/2011	AZT/3TC/EFV	22/07/2011
5C	TDF/3TC/NVP	15/09/2010	AZT/3TC/NVP	03/10/2008
6C	AZT/3TC/NVP	20/02/2014	D4T/3TC/NVP	15/09/2005
7C	AZT/3TC/LPV/r	23/01/2014	D4T/3TC/Lpv/r	11/05/2007
8C	AZT/3TC/ABC/LPV/r	02/04/2014	AZT/3TC/NVP	09/09/2004
9C	AZT/3TC/TDF	22/08/2011	AZT/3TC/NVP	29/04/2004
10C	AZT/3TC/NVP	28/07/2005	AZT/3TC/NVP	28/07/2005
12C	AZT/3TC/LPV/r	06/07/2006	AZT/3TC/Lpv/r	06/07/2006
13C	AZT/3TC/EFV	30/03/2005	AZT/3TC/EFV	30/03/2005
14C	Unknown	Unknown	Unknown	Unknown
16C	TDF/3TC/EFV	16/04/2014	AZT/3TC/NVP	28/07/2005
17C	AZT/3TC/EFV	30/01/2014	D4T/3TC/EFV	07/01/2011

*Child 1C had followed two other HAART regimen prior 2008-09-02. These were: AZT/3TC/NVP in 04/11/2008 and AZT/3TC/EFV in 29/04/2008. 3TC: Lamivudine; ABC: Abacavir; AZT: Stavudine; C: Child; EFV: Efavirenz; HAART: Highly Active Antiretroviral Therapy; LPV/r: Ritonavir-boosted Lopinavir; NVP: Nevirapine; SID: Study Identificant; TDF: Tenofovir. In red are marked children receiving a TDF-based ART for the treatment of their HIV infection.

3.5.2 Sample collection

DBS and blood samples were successfully collected from all recruited patients. These were 21 DBS samples (15 pediatric and six maternal samples) and 15 blood samples from the children and adolescents. All 21 DBS samples (15 pediatric and six maternal samples) and 10 residual pediatric serum samples were shipped to and received at the Division of Medical Virology for further HBV laboratory testing. The other five pediatric serum samples could not be traced for retrieval at the Namibian laboratory and were not forwarded to the Division of Medical Virology at Stellenbosch University, South Africa. Serological testing was performed using serum samples, while DBS were used for molecular testing.

3.5.3 Hepatitis B virus serological profiles

3.5.3.1 HBsAg assay results

All 10 samples received from the Namibian laboratory were re-tested for HBsAg. HBsAg reactivity was confirmed in all 10 samples.

3.5.3.2 HBeAg assay results

HBeAg seropositivity was noted in seven samples (7/10; 70%). The other three samples (3/10; 30%) were found non-reactive for HBeAg.

3.5.3.3 Anti-HBe assay results

Eight samples tested non-reactive for anti-HBe (8/10; 80%); of which one (sample 5C) had an OD value falling within the ±10% of the cut-off value thus was re-tested. A repeat testing of the sample confirmed non-reactivity for anti-HBe. Two samples tested positive for anti-HBe; these samples were HBeAg negative.

3.5.3.4 Anti-HBc (total) assay results

All samples reacted positive for anti-HBc (total).

All quality control criteria were met, thereby validating the results obtained in these assays. These results are summarized in Table 3.18.

Table 3.18: HBV markers profiles of HIV/HBV co-infected children

Patient ID	HBsAg	HBeAg	Anti-HBe	Anti-HBc (total)
1C	+	-	+	+
2C	+	+	-	+
3C	+	+	-	+
4C	+	-	+	+
5C	+	-	-	+
6C	+	+	-	+
7C	+	+	-	+
8C	+	+	-	+
10C	+	+	-	+
13C	+	+	-	+

Anti-HBc: antibodies against hepatitis B core antigen; anti-HBe: antibodies against hepatitis B e antigen; HBsAg: hepatitis B surface antigen; ID: identificant.

3.5.4 Hepatitis B virus molecular profiles

3.5.4.1 HBV DNA viral load

HBV DNA viral level measurements were performed for all 21 participants, 15 children, and six mothers. Eight of the children tested positive for HBV DNA (8/15; 53.3%), while none of the mothers had detectable levels of HBV DNA. HBV DNA levels of positive patients ranged from 6.23 $\log_{10}$ IU/ml to 7.64 $\log_{10}$ IU/ml (median: 7.20 $\log_{10}$ IU/ml); tabulated in Table 3.18. Of these eight HBV DNA positive children, seven were HBsAg and HBeAg positive, anti-HBe negative. The eight child was not tested for these serological markers, as we did not receive a serum sample to do so. Of the other seven children with undetectable levels of DNA, three were tested for further HBV serological markers. Of these three children, two were HBeAg negative/anti-HBe positive, and one was HBeAg negative/anti-HBe negative. Viral nucleic acid was successfully extracted from the eight HBV DNA positive samples for sequencing of the pol/surf and X/core genes.

Table 3.19: HBV DNA viral load of HBV DNA positive children.

Patient ID	HBV viral load* (IU/ml)	HBV viral load $\log_{10}$ (IU/ml)	HBsAg	HBeAg	Anti-HBe
2C	12 850 000	7.11	+	+	-
3C	30 400 000	7.48	+	+	-
6C	7 200 000	6.86	+	+	-
7C	1 695 000	6.23	+	+	-
8C	19 850 000	7.30	+	+	-
10C	44 000 000	7.64	+	+	-
13C	19 700 000	7.29	+	+	-
17C	4 755 000	6.68	ND	ND	ND
Median HBV viral load: 7.20 $\log_{10}$ IU/ml					

*These values were obtained from multiplying the results obtained by the COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 by the dilution factor between DBS and plasma (50). Anti-HBe: antibodies against hepatitis B e antigen; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; ID: identificant; IU/ml: international units per millilitre; ND: Not done.

3.5.4.2 Nucleotide sequencing of the pol/surf gene

DNA template specific for the pol/surf gene was successfully obtained for sequencing of the gene from all eight HBV DNA positive pediatric samples. Visualization of the nested PCR products is shown in Figure 3.3.

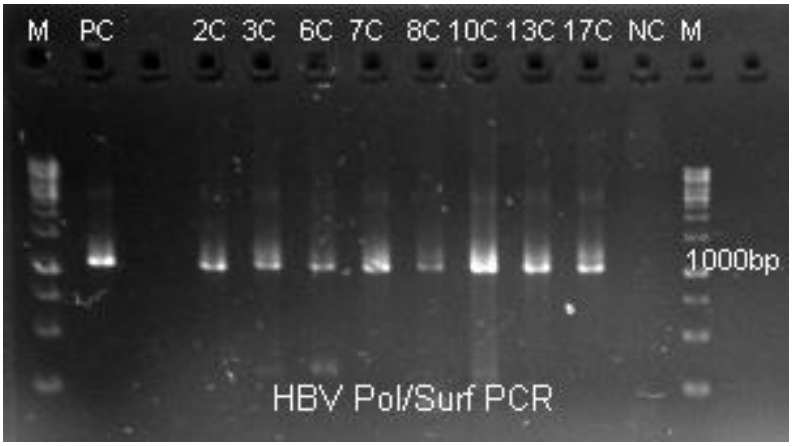


Figure 3.3: Nested pol/surf PCR DNA products. This gel slab shows results from the nested PCR DNA amplification for seven HBV DNA positive children. The bands indicate the presence of DNA products of 1000 bp size; the expected size of the target pol/surf region containing the RT region of the gene. No band is observed on the NTC and NC lanes suggesting that cross-contamination did not occur during the handling of reagents and samples. The PC lane shows a band of 1000bp too; validating the DNA size obtained for our query samples. C: Child; M: Marker of 1kb molecular weight; NTC: No Template Control; NC: Negative Control; PC: Positive Control.

DNA products visualized on this gel slab were purified and spectrophotometrically analyzed to assess the purity and to determine the concentration of DNA present in our samples. Results from the spectrophotometric analysis, presented in Table 3.20, show that DNA present in our samples was "pure" with concentration above the recommended range for sequencing (5-20ng/µl).

Table 3.20: DNA concentration and purity of nested PCR products for pol/surf gene sequencing

Sample ID	DNA concentration (ng/uL)	Absorbance (A_{260})	DNA purity ($A_{260/280}$)
2C	58.48	1.17	1.8
3C	61.34	1.227	1.79
6C	55.59	1.112	1.74
7C	70.94	1.419	1.72
8C	48.96	0.979	1.78
10C	75.93	1.519	1.75
13C	56.23	1.125	1.84
17C	66.09	1.322	1.80

A: Absorbance; ID: Identificant, ng/µL: nanogram per microlitres

Samples were diluted to a 20ng/µl DNA concentration using nuclease-free water for sequencing, and the PC used during the HBV DNA amplification reactions was included in the sequencing reaction. Products from the sequencing reaction were purified and analyzed on the ABI Prism 3130xl genetic analyzer. Resulting chromatograms were edited and trimmed, where

necessary, forming contiguous sequences of approximately 980bp long. No sequence was acquired from the NC, suggesting that cross-contamination did not take place.

HBV genotyping

NCBI HBV Genotyping tool revealed that query strains belonged to genotype E (6/8; 75%) and genotype D (2/8; 25%). The Geno2pheno analysis further described the genotype D sequences as sub-genotype D3.

Mutation analysis

Mutation analysis of these sequences through Geno2Pheno revealed the presence of amino acid (aa) substitutions including leucine (L) to isoleucine (I) at position 80 (rtL80I), valine (V) to leucine (L) at position 173 (rtV173L), valine (V) to isoleucine (I) at position 191 (rtV191I) and methionine (M) to valine (V) or isoleucine (I) at position 204 (rtM204V/I) in the RT region of the pol gene; all known as mutations associated with lamivudine drug resistance. Owing to the overlap between the pol and surf genes, changes in the RT region led to nucleotide substitutions in the overlapping SHB region. These included sE164D, sW182*, sI195M and sW196LS representing a change of glutamic acid (E) to aspartate (D) at position 164, tryptophan (W) to a stop codon at position 182, isoleucine (I) to methionine (M) at position 195, and tryptophan (W) to leucine/serine (L/S) at position 196 of the surface gene, respectively. SHB changes related to RT variations are tabulated in Table 3.21.

These aa changes, associated with lamivudine resistance were observed in patterns including rtV173L+rtL180M+rtM204V+sE164D+sI195M (4/8; 50%), rtL80I+rtV173L+rtL180M+rtM204I+ sE164D+ sW196LS (1/8; 12.5%), rtL180M+rtM204V+ sI195M (2/8; 25%) and rtL80I+rtM204I+sW196L (1/8; 12.5%).

Table 3.21: HBsAg changes associated with reverse transcriptase mutations

HBV RT variants	Corresponding changes in HBsAg
V173L	E164D
L180M	None
V191I	W182*
M204V	I195M
M204I	W196*/S/L

HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; RT: reverse transcriptase.

Phylogenetic analysis

The neighbor-joining analysis, presented in Figure 3.4, indicates that sequences obtained from children 2C, 3C, 6C, 7C, 10C, and 13C clustered together. These sequences formed a cluster with other genotype E sequences originating from Namibia **(Botha et al., 1984; Kramvis et al., 2005)**. 8C and 17C formed a cluster with genotype D sequences and were closely related to genotype D3 sequences from South Africa and Botswana. This analysis confirmed the genotyping analysis performed with the NCBI HBV online genotyping tool (https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

The maximum likelihood model phylogenetic analysis was completed using the General Time Reversible substitution model with gamma distributed rate heterogeneity and a proportion of invariant sites (GTR+I+G) **(Tavaré, 1986)** in MEGA7, based on the AIC and BIC criteria; as calculated by the best-fit substitution model analysis ran through the JModelTest 2.1.3 software. A similar cluster pattern of our query sequences is observed in this analysis; see Figure 3.5.

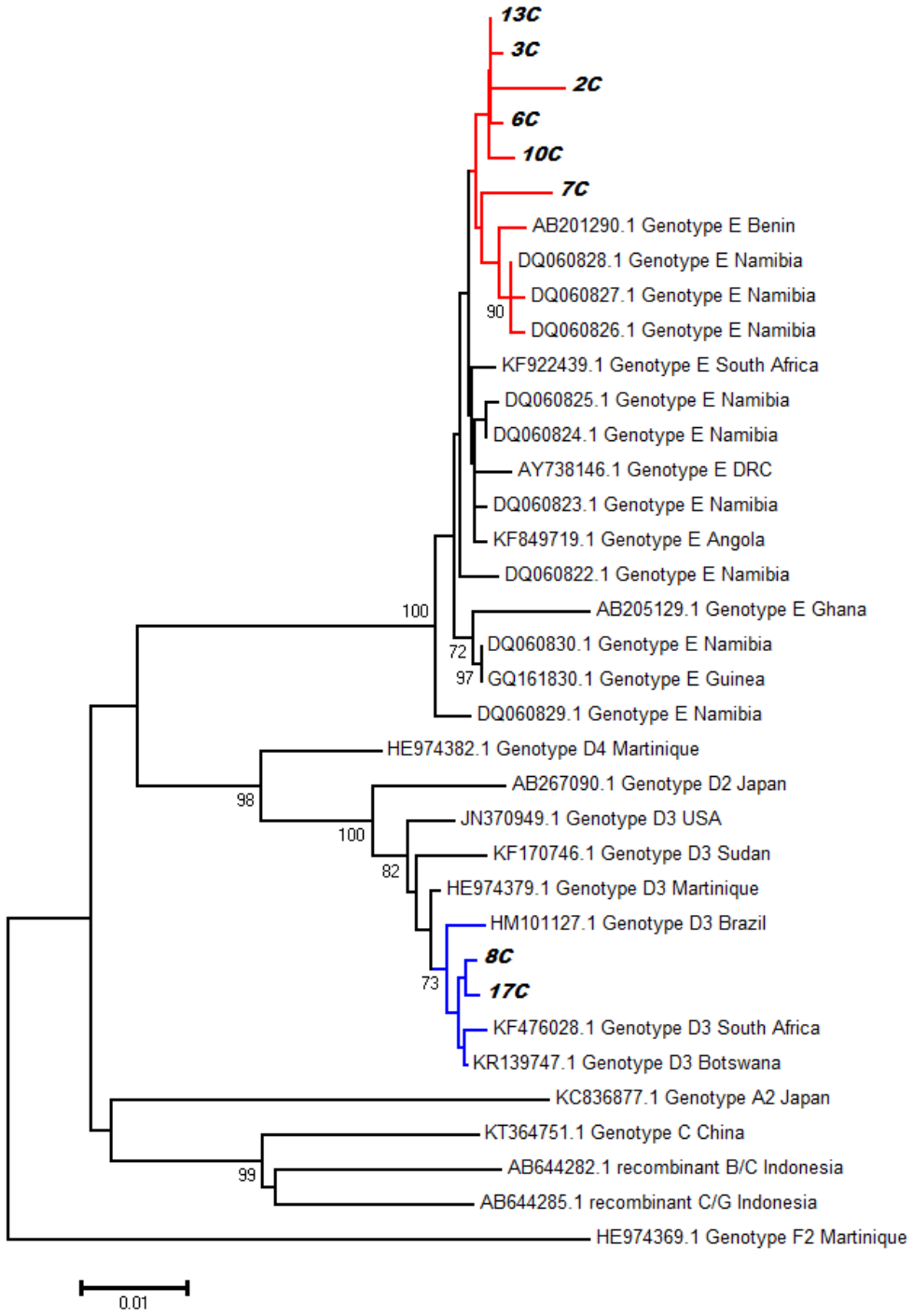


Figure 3.4: Phylogenetic analysis of HBV strains identified in the study based on the pol/surf region of the HBV genome using the Neighbor-Joining method. The 980bp pol/surf genomic fragment was used to infer the evolutionary relationship between our query sequences, using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Distance values at the branch nodes lower than 70% were excluded from the tree. The evolutionary distances were computed using the p-distance method. Sequences obtained in this study are highlighted in **bold**. The red branches represent the close cluster between our sequences and those previously obtained in Namibia. The blue branches represent the cluster formed by the study query sequences and other closest sub-genotype D3 sequences.

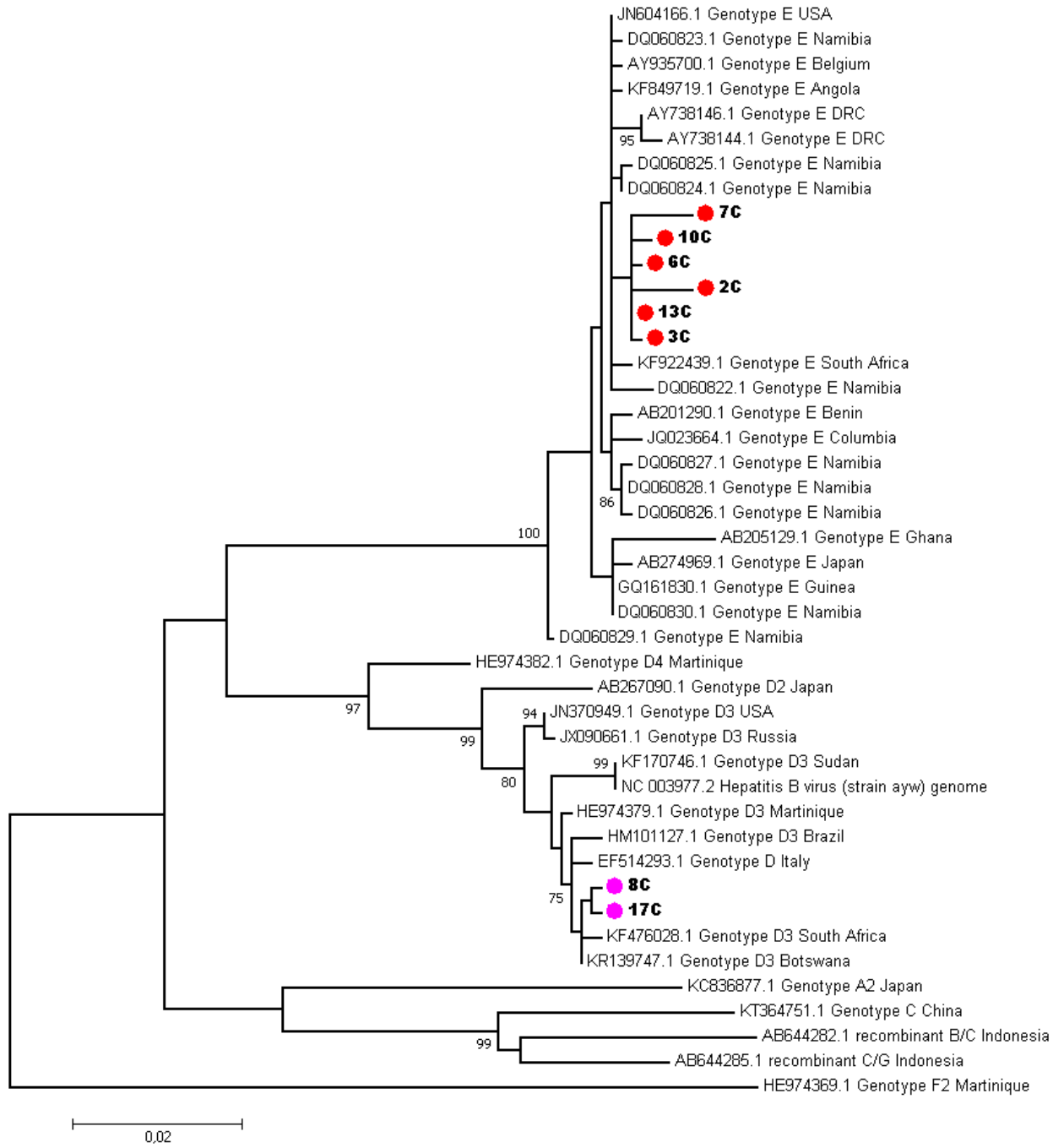


Figure 3.5: Phylogenetic analysis of HBV sequences identified in the study using the Maximum Likelihood method. This analysis was based on the General Time Reversible model with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Distance values at the branch nodes lower than 70% were excluded from the tree. A discrete Gamma distribution was used to model evolutionary rate differences among sites. There was a total of 860 positions in the final dataset. Positions containing gaps and missing data were eliminated. Study sequences are highlighted in **bold** with colored bullets: red for Genotype E sequences and pink for Genotype D sequences.

3.5.4.3 Nucleotide sequencing of the X/core gene

DNA amplification of the X/core gene was successful; as depicted on the agarose gel below, Figure 3.6. Nested PCR products were purified, and the DNA concentration present in each sample was measured spectrophotometrically. Results are represented below in Table 3.22. All samples had a DNA concentration above the required 20ng/µl for DNA sequencing and were diluted to a final concentration of 20ng/µl using nuclease-free water.

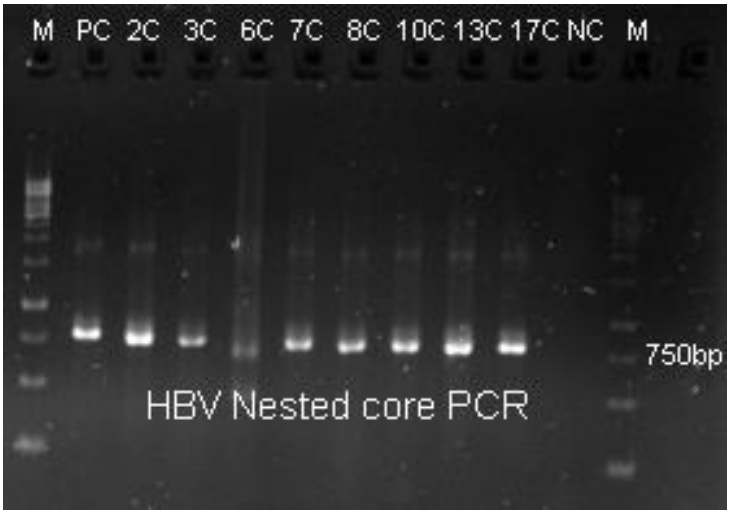


Figure 3.6: Nested X/core PCR DNA products. On this gel, results from the nested X/core PCR reaction are appreciated for eight HBV DNA positive samples. The bands represented indicate the presence of target DNA of 750 bp size; the expected size of the HBV core gene containing the pre-core and basal core promoter regions. The NC shows no bands hence we could conclude that cross-contamination did not occur. The PC lane shows a band at the same level as our samples; validating our results. C: child; M: Marker of 1kb DNA ladder; NTC: No Template Control; NC: Negative control.

Table 3.22: DNA concentration and purity of nested PCR products for X/core gene sequencing

Sample ID	DNA concentration (ng/µL)	Absorbance (A_{260})	DNA purity ($A_{260/280}$)
2C	104.07	2.081	1.9
3C	91.61	1.832	1.91
6C	95.8	1.916	1.88
7C	99.43	1.989	1.75
8C	98.47	1.969	1.92
10C	102.76	2.055	1.9
13C	110.05	2.201	1.92
17C	63.39	1.268	1.87

A: absorbance; ID: Identificant; ng/µL: nanogram per microlitres

The diluted products were used to perform the sequencing reaction, and sequencing products were analyzed on the ABI Prism 3130xl genetic analyzer. Resulting sequences from each

primer and each sample were edited and trimmed (where necessary) and assembled; forming contiguous sequences of approximately 730bp. The pre-C and BCP regions of the core sequences were extracted and manually analyzed for the presence of viral genomic mutations.

A portion of the X gene covering nt 1653 to nt 1835 (aa 94 to 154) was analyzed for the presence of viral genomic mutations. This region overlapped with the CP from nt 1653 to nt 1835 (aa 94 to 154). The complete pre-C region (nt 1814 to nt 1900; aa 1 to 29) was also analyzed.

Pre-C mutation analysis

Substitution of valine (V) to phenylalanine (F) at position 17 (V17F) corresponding to the G1862T Pre-C mutant was found in patient 2C. A mixed population of the wild-type virus and the Pre-C stop codon W28*/W (G1896A/G) was observed in children 3C and 6C; represented in Figure 3.7.

Figure 3.7: Snapshot of the multiple alignment of query sequences and the HBV Pre-Core reference sequence represented on Geneious V10.2.3. 2C, 3C, 6C, 7C, 8C, 10C, 13C, and 17C represent the pre-Core sequences obtained from the HBV DNA positive children being analyzed. Dissimilarity is observed at position V17F (2C); highlighted in beige. The mixed population W28*/W is shown too; highlighted in grey for 3C and 6C sequences. * represents a stop codon. C: child.

Core promoter (CP) mutation analysis

This genomic region includes the URR (nt 1653 to nt 1741; aa 94 to 122) and the BCP (nt 1742 to nt 1835; aa 123 to 154). Substitutions were observed in three children: aspartate (D) to asparagine (N) at position 119 (D119N) and valine (V) to methionine (M) at position 131 (V131M) in child 2C, phenylalanine to isoleucine at position 132 (F132I) in child 6C and, alanine to valine at position 102 (A102V) in child 7C, as represented on the aa alignment in Figure 3.8. These variants are represented as C1678T (A102V) on the overlapping CP region (aa 94 to 122), G1728A (D119N), G1764T/C1766G (V131M) and T1768A (F132I) on the overlapping BCP region.

Figure 3.8: Multiple alignment of the query sequences and the HBV X gene reference. The amino acid sequence of this region of the X gene is numbered from 94 to 154. The dots show where amino acids are similar to that of the reference gene (X NA ref). 2C, 3C, 6C, 7C, 8C, 10C, 13C, and 17C represent the X gene sequences obtained from the HBV DNA positive children being analyzed. Dissimilarities are observed at position D119N, V131M (Child 2C), F132I (Child 6C) and A102V (Child 7C). C: child.

3.5.5 Liver health

Liver enzymes measurements were completed for nine children: five with high levels of HBV DNA and four with undetectable levels of HBV DNA, see Table 3.23. The median levels of AST and ALT were higher in HBV DNA positive children as compared to HBV DNA negative children. These were: median ALT = 50.0 IU/L (Interquartile range: 41 - 57) compared to 23.0 IU/L (Interquartile range: 14.5 – 34.5) (p=0.063), and median AST = 45.0 IU/L (Interquartile range: 36.0 – 46.0) compared to 30.0 IU/L (Interquartile range: 26.5 – 38.0) (p=0.190), respectively. Median platelet counts among these children were 280 x 10^9/L. Eight children (8/9; 88.9%) showed a normal APRI score (< 0.5); of whom four were HBV DNA positive and four were HBV DNA negative. An elevated APRI score of 0.713, suggesting liver fibrosis (> 0.5), was detected in one HBV DNA positive child (1/9; 11.1%).

Table 3.23: Liver biomarkers of HBV positive children

Patient ID	HBV viral load log_{10} (IU/ml)	Platelets (10^9/L)	ALT (IU/L)	AST (IU/L)	APRI
1C	LDL	289	31	43	0,372
2C	7.11	263	105	75	0.713
3C	7.48	234	50	45	0.481
4C	LDL	213	14	26	0,305
5C	LDL	287	38	33	0,287
6C	6.86	349	57	46	0,33
7C	6.23	280	41	36	0,321
9C	LDL	333	15	27	0,203
10C	7.64	261	16	26	0.249
Median ALT: 38 IU/l; Median AST: 36 IU/l; Median platelets: 280 x 10^9/L					

ALT: Alanine transaminase; APRI: AST platelet Ratio index; AST: Aspartate transaminase; HBV: hepatitis B; ID: Identificant; IU/L: International units per liter, LDL: lower than the detection limit. In red is marked the child with an APRI score > 0.5. Upper limits of normal were 40 IU/L for both AST and ALT, and 400 x 10^9/L for platelets.

3.6 Summary of findings

Active HBV infection was found in more than half of the children included in this study (8/15; 53.3%); all of them were HBeAg positive. HBV strains were classified as genotype E (6/8; 75%) and sub-genotype D3 (2/8; 25%). Following sequencing of the RT region of the pol gene, eight children harbored genomic mutations associated with resistance to lamivudine. These RT changes led to changes in the SHB of the surf gene, overlapping with the RT region. Genomic mutations in the X/core gene were also detected in some of these children. Liver biochemistry assessments revealed that HBV DNA positive children had higher transaminases as compared to those with undetectable viral load; one child presented with an APRI score above 0.5. The epidemiological and clinical characteristics acquired from HBV DNA positive children are summarized in Table 3.24, below.

Table 3. 24: Serology and molecular analysis data of HBV DNA positive children

Patient ID	2C	3C	6C	7C	8C	10C	13C	17C
Age at recruitment (years)	17	14	10	8	14	11	19	17
Gender	Male	Male	Female	Female	Female	Male	Female	Male
ART regimen at recruitment	AZT/3TC/NVP	AZT/3TC/NVP	AZT/3TC/NVP	AZT/3TC/LPV/r	AZT/3TC/ABC/LPV/r	AZT/3TC/NVP	AZT/3TC/EFV	AZT/3TC/EFV
Duration of lamivudine-only therapy (years)	3	9	9	7	10	9	9	4
ALT (IU/l)	105	50	57	41	Not Available	16	Not Available	Not Available
AST (IU/l)	75	45	46	36	Not Available	26	Not Available	Not Available
Platelets (10^9/l)	263	234	349	280	Not Available	261	Not Available	Not Available
APRI	0.713	0.481	0.388	0.378	Not Available	0.249	Not available	Not Available
HBsAg	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Not Tested
HBeAg	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Not Tested
Anti-HBe	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Not Tested
Anti-HBc (total)	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Not Tested
HBV DNA (log_{10} IU/ml)	7.11	7.48	6.86	6.23	7.10	7.64	7.29	6.68
HBV genotype	E	E	E	E	D3	E	E	D3
RT mutations	L180M, V191I, M204V	V173L, L180M, M204V	V173L, L180M, M204V	L180M, M204V	L80I, V173L, L180M, M204I	V173L, L180M, M204V	V173LV, L180M, M204V	L80I, M204I
SHB mutations	W182*, I195M	E164D, I195M	E164D, I195M	I195M	E164D	E164D, I195M	E164D, I195M	W196L
Pre-core mutations	V17F (G1862T)	W28*W (G1896A/G)	None	None	None	None	None	None
BCP and URR mutations	D119N (G1728A), V131M (G1764T/C1766G)	None	F132I (T1768A)	A102V (C1678T)	None	None	None	None

3TC: Lamivudine; ABC: Abacavir; ALT: aspartate liver transaminase (reference 10 – 40 IU/l), Anti-HBc (total): Total Antibody to hepatitis B core antigen; Anti-HBe: Antibody to hepatitis B e antigen; APRI: AST to Platelet Ratio Index; ART: Antiretroviral therapy; AST: aspartate liver transaminase (reference 15 – 41 IU/l). AZT: Zidovudine; BCP: basal core promoter; EFV: Efavirenz; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; ID: Identificant; IU: international units; LPVr: Ritonavir-boosted Lopinavir; ml: millilitres; NVP: Nevirapine; RT: reverse transcriptase, SHB: small hepatitis B surface antigen, URR: upper regulatory region.

3.7 Interpretation of findings

3.7.1 Active HBV infection in HIV-infected children: clinical implications

Eight of the sixteen (8/15, 53.3%) HIV/HBV co-infected children enrolled in this study had active HBV infection; characterized by high levels of HBV DNA, above 20 000 IU/ml, and HBeAg seropositivity. Many of the studies reporting on HBV among HIV-infected children in Africa have focused on assessing the presence of the virus through serological testing, but few describe HBV viral DNA levels. Published in 2016, Jooste et al. (2016) observed active HBV infection in five out of 625 (0.8%) HIV-infected children in Bloemfontein, South Africa. Four of those five children were on a lamivudine-containing ART regimen; two presented with DNA levels above 5 $\log_{10}$ IU/ml and tested HBeAg positive **(Jooste et al., 2016)**. High viral replication and HBeAg seropositivity are known indicators of the high replicative phase of CHB. In HBV mono-infected children, this phase can last for three decades if the infection was acquired vertically but is short-lived if acquired horizontally **(Hoffmann et al., 2007; Kramvis, 2016)**. Although the present data suggest that the risk of developing severe liver diseases is low in mono-infected individuals during this phase **(Chu et al., 2004)**, the opposite may be seen in the presence of co-infection with HIV. Through immune-suppression, HIV is known to cause a delay in seroconversion to anti-HBe and increased replication of HBV among HIV/HBV co-infected adults **(Thio, 2009)**. However, very little is known in HIV/HBV co-infected children; who require further investigations. Extrapolating from studies in adults, immune-suppression caused by HIV may lengthen the high replicative phase, characterized by high DNA levels and HBeAg seropositivity, in which children in this study presented. Elevated HBV DNA levels and persistent HBeAg positivity are known as independent risk factors for the development of liver cirrhosis and HCC **(Chen et al., 2006a; Iloeje et al., 2006)**. Viral replication may also be maintained through the selection of viral genomic mutations that have been associated with an increased risk of liver cirrhosis and HCC. The presence and possible clinical implications of these mutants within this cohort are thoroughly discussed in sections 3.7.4.2 and 3.7.4.3, below.

These data highlight the need for adequately controlling HBV infection in the HIV-infected pediatric population. The presence of active HBV infection observed among eight children demonstrates failure of lamivudine treatment, a potent antiretroviral drug with activity against both HIV and HBV. As children are initiated on lamivudine against HIV infection, if infection occurs during infancy, they are not systematically screened for HBV. Many co-infected with HBV go undetected and may grow up to develop liver damage that could have been controlled

at an earlier stage. There is thus a need for screening HIV-infected children for HBV, prior to initiating lamivudine therapy. Identifying cases of HBV infection in HIV-infected children allows for optimization of their antiviral treatment, where necessary. Control of these HBV pediatric infections would also prevent horizontal transmission of the virus to other susceptible children or adolescents within communities.

3.7.2 Liver health in HIV-infected children with HBV infection

Non-invasive evaluation of liver health was completed for 9 out of the 15 patients enrolled in this study; based on the APRI score, as the preferred non-invasive test to assess liver disease in resource-limited settings **(WHO, 2015)**. This was done in nine children, of whom four had undetectable levels of HBV DNA, and five had a high HBV load. One of these nine children had an APRI score above 0.5 (0.713). This abnormal APRI score indicated the possible presence of liver fibrosis. Elevated APRI scores amongst HIV-infected children have been previously documented elsewhere. An investigation of liver disease in Polish HIV-infected children and adolescents revealed four of seventy-nine (4/79; 5%) HIV-infected children with an APRI score > 0.5. All of these children were co-infected with HBV or HCV. Through a multiple regression analysis, the authors showed a positive correlation between an elevated APRI score and HBV or HCV co-infection (p-value = 0.0001), as a predictor of liver disease in HBV-infected children **(Pokorska-Śpiewak et al., 2017)**. Although liver biopsy remains the gold standard for evaluating liver damage, the use of APRI has been previously validated against liver biopsy results as a measure of liver fibrosis in children with chronic viral hepatitis **(McGoogan et al., 2010)**. The study by McGoogan et al. (2010) showed a reasonable performance of the APRI score for predicting fibrosis in children with HCV or HBV, but a relatively poor performance for predicting cirrhosis **(McGoogan et al., 2010)**. Although a high APRI score does not ascertain the presence of liver fibrosis, it may warrant close monitoring of children showing elevated scores, especially where high viremia is noted. Still, further research is needed to identify reliable non-invasive markers for the evaluation of liver disease in children.

3.7.3 Transmission of HBV infection in children

Similarly to the rest of SSA, horizontal transmission has played a key role in the HBV epidemic in Namibia. This observation was made by three main studies, conducted more than two decades ago in the country **(Botha et al., 1984; Joubert et al., 1991; Aspinall et al., 1994)**.

Of these three studies, the one by Botha et al. (1984) alone investigated HBV infection in mother-child pairs. The authors noted that children born to HBsAg positive mothers were more likely to be HBsAg positive too, but hypothesized that the infection might have occurred at a stage beyond the perinatal period **(Botha et al., 1984)**.

This study investigated the role of MTCT as a possible source of HBV infection in HIV/HBV co-infected children, using HBV DNA testing of mothers of HIV/HBV co-infected children enrolled in the study. Six mothers of the 15 children participating in the study were successfully recruited. Seven mothers were deceased, and the other two did not attend the clinic with their children at the time of recruitment. All mothers were HIV-infected and were following TDF-containing ART regimens when enrolled. They consequently had undetectable viral loads upon HBV DNA testing. Regarding HBsAg serological status, one mother (6M) was reported HBsAg positive, three had an unknown HBsAg status, one had an indeterminate HBsAg result, and one other mother was noted HBsAg negative (17M). Owing to the unavailability of venous blood samples from these mothers, these HBsAg statuses could not be confirmed. Previous exposure to the virus could also not be confirmed by anti-HBc testing. For these reasons, HBV MTCT as a possible source of HBV infection among these children could not be confirmed. Given the reported HBsAg seronegative status of mother 17M, the role of HBV MTCT from this mother to her child was debatable. Although rare, HBsAg loss has been recorded among HIV/HBV co-infected patients during treatment with TDF **(Zoutendijk et al., 2012; Boyd et al., 2015)**. This may be accountable to the HBsAg negative status of mother 17M. On the other hand, the HBsAg seropositive status of both mother 6M and her child (6C) suggested possible HBV MTCT. This child was born in 2004, prior to the implementation of HBV vaccination at six weeks after birth in 2009 and at birth in 2014. Thus, pediatric exposure to HBV could have occurred either during the perinatal period, from the mother, or postpartum possibly from the mother still.

All children enrolled in this study were born before universal HBV vaccination from six weeks of age, and at birth, were implemented in Namibia. Therefore, the possibility that these children could have acquired HBV infection during early childhood **(Joubert et al., 1991)** could not be eliminated. The role of MTCT in this pediatric population could also not be entirely eliminated.

3.7.4 Molecular characterization of HBV

3.7.4.1 HBV genotyping and liver disease progression

HBV genotyping revealed a predominance of genotype E in 75% (6/8) of viral strains isolated in this pediatric cohort. Phylogenetic analysis showed close clustering of genotype E strains identified with other genotype E sequences described in Namibia **(Kramvis et al., 2005)**, thereby confirming the predominance of this genotype in the country.

Sub-genotype D3 was uncovered among two isolates (2/8, 27%). From the phylogenetic analysis, these sequences grouped with strains from South Africa and Botswana. Namibia is a neighbor to Botswana from the East and to South Africa to the South and East. The presence of this sub-genotype in Namibia may reflect the effect of human movements across borders in Southern Africa.

In terms of clinical pathogenesis, the natural history and severity of the HBV infection may be influenced by the viral genetic differences. For instance, studies in India have shown a more severe course of chronic liver disease with genotype D (sub-genotype D1) as compared to genotype A **(Thakur et al., 2002)**. In South Africa, where sub-genotype D3 is found, genotype A is associated with more severe liver damage as compared to genotype D **(Kew et al., 2005)**. The contradicting observation between these two geographic locations, with regards to the association between genetic variations in the virus and the progression of liver disease, is mainly due to the pathogenic differences between D1 and D3 viruses. The increased risk of hepatocarcinogenesis of sub-genotype D1 compared to sub-genotype D3 was demonstrated in a recent functional study. Sub-genotype D3 was mostly associated with apoptosis, leading to fibrosis. Sub-genotype D1 showed a higher oncogenic potential, characterized by its increased ability to induce cell proliferation and higher expression of epithelial-mesenchymal transition markers associated with tumor progression in relation to D3 viruses **(Khatun et al., 2018)**. Results from Kathun et al. (2018) suggest that children infected with the D3 strains may be at higher risk of developing liver fibrosis. With regards to genotype E, a large longitudinal study in The Gambia conducted among 405 chronic carriers of HBV revealed an HCC incidence of 55.5/100 000 carrier-years; 95% of these carriers were infected with genotype E virus and 5% with genotype A **(Shimakawa et al., 2016a)**. Children infected with genotype E may thus be at a higher risk of developing HCC later in life. Longitudinal follow-up of these children would be required to confirm these hypotheses.

In agreement with a study performed in Sudanese liver disease patients and HBV asymptomatic carriers **(Yousif et al., 2013)**, our results also showed that patients infected with genotype D presented with lower viral load and a higher rate of HBeAg seroconversion compared to patients infected with genotype E. The distinct virological manifestation between these two genotypes may be explained by the prevalence of pre-C and BCP mutations associated with the production of HBeAg and viral replication, respectively. The effects of these mutations on the virological characteristics of patients are further discussed in section 3.7.4.3, below.

3.7.4.2 Pol/surf gene mutations

RT variants

The primary M204V/I change, characteristic of lamivudine drug resistance **(Allen et al., 1998; Ono-Nita et al., 1999)**, was observed in all children with active HBV infection. Selection of this mutant has been associated with decreased viral fitness **(Allen et al., 1998)**, subsequently leading to the emergence of compensatory changes such as L180M, V173L, L80I/V to partially restore viral fitness **(Allen et al., 1998; Delaney et al., 2003; Warner et al., 2007)**. Four children harbored the triple mutant V173L+L180M+M204V, one had the quadruple mutant L80I+V173L+L180M+M204I, another displayed the L80I+M204I, and two presented with L180M+M204V mutations. As observed in this study, the *rt*L80I mutant often occurs in the presence of *rt*M204I than *rt*M204V **(Warner et al., 2007; Lei et al., 2013)**, and is most commonly described among genotype D strains **(Warner et al., 2007)**. Furthermore, a decreased susceptibility to entecavir was observed in the presence of the M204V variant and L180M mutant **(Mukaide et al., 2010)** Drawing from studies in adults, a higher dose of entecavir (1mg daily dose) has been proven effective in the HBV DNA suppression in lamivudine-exposed patient harboring the M204V and L180M mutants **(Pessôa et al., 2008)**.

It is well known that the longer the exposure to lamivudine, the higher the incidence of resistant HBV **(Benhamou et al., 1999; Sokal et al., 2006)**. Although TDF is recommended from 10 years of age for the management of HIV infection in children **(WHO, 2016)**, many remain on lamivudine therapy for their HIV infection for more than 10 years; owing to the lack of pediatric systematic screening for HBV. Seven of these eight children were older than 10 years old, and as such qualified for ART regimen including TDF. Yet, they were still on lamivudine therapy when enrolled in the study. Unlike lamivudine, TDF carries a very low risk of drug resistance, with significant activity against lamivudine-induced resistant strains **(Patterson et al., 2011)**. TDF could be used as rescue therapy in lamivudine drug-resistant patients.

However, resistance to this drug in the presence of multi-site lamivudine mutants including *rt*L180M and *rt*M204V/I has been previously reported **(Lee et al., 2014)**. This report raised concerns regarding the efficacy of TDF in HIV/HBV co-infected children previously exposed to lamivudine. Given that the current dose of TDF is associated with bone mineral density in children and nephrotoxicity in HIV-infected children below the age of 10 years old, the need for a lower dose of TDF as first-line therapy for the control of HBV-infection in these children is emphasized. The use of TAF as an alternative for TDF, for the treatment of pediatric HBV infection in HIV/HBV co-infected chldren, is attractive. Recent data from clinical trials have demonstrated the effectiveness, tolerability and safety of TAF co-formulated with elvitegravir, cobicistat, and emtricitabine (E/C/F/TAF) in HIV-infected children **(Giacomet et al., 2018)**. The drug is FDA approved for the use in children from six years of age.

Three children at the time of enrolment were following a TDF-containing regimen subsequent to previous exposure to lamivudine and showed no signs of HBV drug resistance during this analysis. Samples prior to TDF initiation were not available to assess the prior existence of resistance to lamivudine.

Other than lamivudine drug-resistant mutants, the *rt*V191I substitution was also observed. This gene variant has been previously described among both nucleotide reverse-transcriptase inhibitor (NRTIs) experienced and non-NRTIs experienced patients **(Margeridon-Thermet et al., 2009)**; suggesting that its selection may not be involved in drug resistance but may be a naturally occurring mutation **(Colledge et al., 2017)**.

Small surface (SHB) variants

Amino acid substitutions downstream of the "a" determinant of the SHB were also observed amongst the drug-resistant HIV/HBV co-infected children. The most commonly found variants were *s*E164D and *s*I195M, which corresponded to the *rt*V173L and *rt*M204V mutants in the RT region, respectively. The presence of these two variants has been associated with minimal antigen-antibody binding, a characteristic of viral immune evasion **(Torresi et al., 2002)**. Sloan et al. (2008) revealed that immune evasion is brought on by these changes through disruption of the "a" determinant **(Sloan et al., 2008)**. The *s*W196L point mutation was also observed in this study. This mutation was selected by the lamivudine-resistant *rt*M204I mutant. Looking at the structure of the SHB protein, the two alpha helices of the carboxyl-terminal end of the protein are held together by a loop spanning aa 192 to aa 200. Subsequent changes in this region such as *s*I195M and *s*W196L may impact on the integrity of the protein, without necessarily

affecting viral assembly and secretion **(Prange et al., 1995)**, while leading to reduced antigen-antibody binding **(Torresi et al., 2002)**. Other than point mutations associated with immune escape, a stop codon was also detected. The *s*W182*, observed in a genotype E sequence, was produced by the overlapping *rt*V191I nucleotide substitution in the RT region. The presence of this variant causes termination in the SHB protein. Termination in this viral protein subsequently leads to reduced extracellular secretion of HBV viral particles, which accumulate intracellularly **(Colledge et al., 2017)**. Prolonged intracellular accumulation of HBsAg protein has been previously associated with the development of HCC by causing cellular stress **(Ringelhan & Protzer, 2015)**. Colledge et al. (2017) also showed increased apoptosis of hepatocytes in the presence of *s*W182* in comparison to those infected with the wild-type virus; this may play an indirect role in hepatocarcinogenesis **(Colledge et al., 2017)**. This observation further emphasizes the plausible increased risk of HCC among genotype E infected children.

In view of the current data, children harboring the *s*W182* may be at an increased risk of liver damage if active viral replication is not controlled. Where rescue therapy is initiated, and viral replication is suppressed, other viral factors such as BCP and core mutations may play a role in carcinogenesis. There may also be potential transmission of immune-escape viruses from these HIV/HBV co-infected children to vaccinated individuals, a potential problem that requires more exploration.

3.7.4.3 X and core gene mutations

Pre-C mutations

Expression of the HBeAg protein is prompted by the translation of the upstream initiation codon in the pre-C domain of the core gene; thus, changes in this region may affect the production of the protein **(Locarnini, 2004)**. The well documented G1896A nucleic acid mutation, corresponding to the W28* stop codon, was detected in two HIV/HBV co-infected children. Located in the encapsidation signal (ε) structure of the pre-C, the selection of this stop codon abolishes the synthesis of the HBeAg, hence its association with an HBeAg-seronegative status **(Locarnini, 2004; Jammeh et al., 2008)**. However, serum samples of these children were positive for HBeAg. HBeAg seropositivity in these children was explained by the detection of a mixed viral population of the variant and the wild-type viruses (W28*/W). The seropositive status of these two children is suggestive of the dominance of the wild-type species over the mutant species. The pre-C sequence of another HIV/HBV co-infected sample (Child

2C) carried the G1862T pre-C mutant. This mutant mostly occurs amongst HBV genotype A-infected patients, especially sub-genotype A1, as opposed to genotype D patients **(Tanaka et al., 2004; Chandra et al., 2007; Saha et al., 2014)**. *In vitro* translational experiments have previously demonstrated a reduced production of HBeAg, at the post-translational level, in the presence of the G1862T pre-C mutant **(Hou et al., 2002)**. Still, child 2C tested positive for HBeAg. Nevertheless, the occurrence of this mutant has been described in both HBeAg positive and anti-HBe positive patients **(Chandra et al., 2007; Saha et al., 2014)**. The authors indicated that the G1862 pre-C mutant might be a naturally occurring variant change **(Chandra et al., 2007; Saha et al., 2014)**.

Core promoter (CP) mutations

The CP is composed of two important functional domains namely the URR and the BCP. Both domains play an important role in the transcription of the pre-C mRNA, and the pgRNA for the synthesis of HBeAg, HBcAg and the polymerase respectively and are thus of critical importance for viral replication **(Kramvis & Kew, 1999)**. Viral mutations in the BCP have been extensively described and studied amongst genotype B and genotype C infected patients **(Hu et al., 2015; Wei et al., 2017)**. On the other hand, there is minimal data on the prevalence of mutations in the URR region and their role in the progression of liver damage.

Analysis of these two domains revealed the presence of four aa substitutions among three drug-resistant HIV/HBV co-infected children. Two of these mutants, namely C1678T and G1728A, occurred in the core upstream regulatory sequence (CURS) of the URR. The CURS is involved in the positive regulation of the BCP activity **(Yuh et al., 1992)**. The other two changes included the G1764T/C1766G BCP double mutation and the T1768A variant. The former was induced by substitution of the aa V131M of the *X* gene and has been predominantly described among cirrhotic patients as compared to chronic patients **(Poustchi et al., 2008; Salarneia et al., 2016)**. The G1764T/C1766G BCP double mutation has also been associated with a decreased viral replication and HBeAg negativity in patients infected with genotype D **(Elkady et al., 2008)**. T1768A has been associated with increased HBV replication, but reduced HBeAg expression in genotype C strains **(Jammeh et al., 2008)**. The two children presenting with these two mutations were HBeAg positive, had a high viral load and were infected with genotype E viruses. Whilst our observation did not match the one made by Elkady et al. (2008) **(Elkady et al., 2008)**, the high viral load noted in child 6C (carrier of the T1768A mutant) was in agreement with the observations made by Jammeh et al. (2008) **(Jammeh et al., 2008)**. The discrepancy between results from Elkady et al. (2008) **(Elkady et al., 2008)** and ours may be

due to the difference in the genotype of the virus. Functional characterization of the G1764T/C1766G BCP variant in genotype E viruses would assist in clarifying this observation.

It is worth mentioning that the viral CP mutants observed in this cohort occurred among genotype E viral strains only. Unlike genotypes A – D, genotype E has not been well studied. The pathogenesis and clinical impact of viral mutants occurring in HBV E viruses are not well understood, but call for thorough investigations for a better understanding of HBV-related liver diseases among carriers of this viral strain.

3.8 Strengths and limitations of the study

Through this study, children who required optimization of their antiviral treatment, from the lamivudine monotherapy to a TDF-based regimen, for the control of their HBV infection were identified.

Limitations of the study included the unavailability of serum samples to confirm the HBV status of the mothers. Also, given that all of the enrolled mothers were HIV-infected and following a TDF-based treatment against HIV, all had undetectable levels of HBV DNA. For that reason, HBV could not be sequenced to determine if MTCT could be the route of transmission of HBV in these children and adolescents. Furthermore, the natural history of HIV/HBV co-infection in these children could not be established due to the cross-sectional design of the study.

CHAPTER 4: A study for the prevention of mother-to-child transmission of hepatitis B virus in Windhoek, Namibia

In this chapter, the feasibility of a screen-treat-vaccinate health intervention for the prevention of HBV MTCT in Namibia is described. The prevalence of the infection among pregnant women attending ANC in Windhoek and the possible risk factors associated with HBV infection within this population are also documented.

4.1 Introduction

MTCT of HBV is currently regarded as a primary cause of the ongoing transmission of hepatitis B in many parts of SSA **(Keane et al., 2016)**. The WHO supports HBV vaccination commencing at birth for the prevention of HBV MTCT, especially in high endemic regions. The recommendation also makes a note of providing temporary immunity with HBIG for babies born to HBeAg positive mothers **(WHO, 2017b)**. Yet, vaccination is provided from six weeks of age in all but nine countries in SSA **(WHO, 2017a)**; and HBIG is of limited access to SSA owing to its high costs. Although vaccination starting at six weeks has been effective in preventing infections occurring during infancy **(Amponsah-Dacosta et al., 2014)**, HBV infections in babies vaccinated according to this regimen are not uncommon **(Chasela et al., 2014; Hoffman et al., 2014; Chotun et al., 2015)**. Combined immunoprophylaxis reduces the risk of perinatal transmission; but it may not prevent MTCT where the pregnant woman is highly infectious, defined as being seropositive for HBeAg with HBV DNA levels above 200 000 IU/ml **(Wen et al., 2013; Foad et al., 2015; Sellier et al., 2015)**. Identifying these highly infectious women provides further opportunities to establish additional prophylactic measures such as antiviral prophylaxis to the mother starting from the third trimester of pregnancy, preferably with TDF for its high barrier to resistance **(Hyun et al., 2017)**. The optimal preventive measure against HBV MTCT in SSA would include routine antenatal HBV screening combined with maternal antiviral prophylaxis and pediatric immune prophylaxis.

The published literature on the rate of HBsAg seropositivity within the antenatal population is relatively extensive in many parts of SSA, but Namibia. Recent Namibian published data, dating from 2013, reported national prevalence of 7.3% in pregnant women **(Mhata et al., 2017)**; which indicated a large proportion of women with a potential risk of transmitting HBV to their newborns. Preventive measures against HBV MTCT in Namibia consist of routine antenatal HBsAg screening, followed by the provision of HBIG to HBV-exposed babies

(MoHSS, 2014a). The national EPI schedule includes HBV vaccination starting from birth, followed with three additional doses of the vaccine at six, ten and fourteen weeks **(WHO, 2018)**. Still, averting immunoprophylaxis failure with maternal antiviral prophylaxis may be required for mothers with HBV DNA levels above 200 000 IU/ml.

4.2 Primary and secondary aims

4.2.1 Primary aim

This study sought to determine the feasibility of rapid-test based maternal screening and HBV treatment (where necessary) combined with pediatric immunoprophylaxis to prevent HBV MTCT, defined here as the number of perinatal HBV infections detected in mother-child pairs.

4.2.2 Secondary aims

The secondary aims of the study included:

- Measuring the prevalence of HBV infection among pregnant women in Windhoek, Namibia.
- Identifying risk factors associated with HBsAg positivity among pregnant women in Windhoek, Namibia.

4.3 Objectives

Six objectives were set to achieve the study aims. These were:

- Perform voluntary screening of pregnant women by means of rapid testing.
- Determine the diagnostic accuracy of the Determine HBsAg rapid test (Alere Inc., Massachusetts, USA)
- Assess the acceptance of the HBsAg rapid test from the perspective of healthcare workers
- Determine the association between possible risk factors and HBsAg seropositivity in the studied antenatal population.
- Establish pathways for continuum of care, including clinical work-up, laboratory tests, treatment (where applicable), and referral for women identified as HBV-infected.
- Determine the rate of MTCT of HBV in mother-child pairs.

4.4 Materials and Methodology

4.4.1 Ethical considerations

Ethical clearance was obtained from the HREC at Stellenbosch University, South Africa (Reference number: **S17/01/001**), the BREC and RMC at MoHSS (Reference number: **17/3/3**), and from the Research Ethics committee of the Namibia Institute of Pathology in Namibia (no reference number was provided). These approval letters are attached on Appendices C, D and E; respectively.

Written authorization was also obtained from the medical superintendents at the Intermediate Katutura hospital and Windhoek Central hospital for conducting the study at the antenatal clinics (ANCs) of the hospitals. Verbal approval was also provided by the senior registered nurses in charge of the ANCs.

Written informed consent (Appendix F) was provided by all study participants for inclusion in the study. Participation in the study entailed HBV rapid testing, sociodemographic data collection, clinical data collection, HBV risk factors data collection, blood sample collection and HBV screening for their babies' post-delivery, where necessary. Participants were also given the option to decide on storage of their blood samples after completion of the study for further use in viral hepatitis and related fields of scientific research. The storage and use of patient's biological samples are made in the strictest confidentiality and in respect of personal rights. Study participants were only identified by a Study Identification Number (SID) to ensure the confidentiality of the participants' information.

4.4.2 Study design

This was a longitudinal prospective study conducted at ANCs at the Intermediate Katutura hospital and Windhoek Central hospital, two state hospitals located in Katutura, Windhoek. A brief overview of the study methodology is outlined below in Figure 4.1.

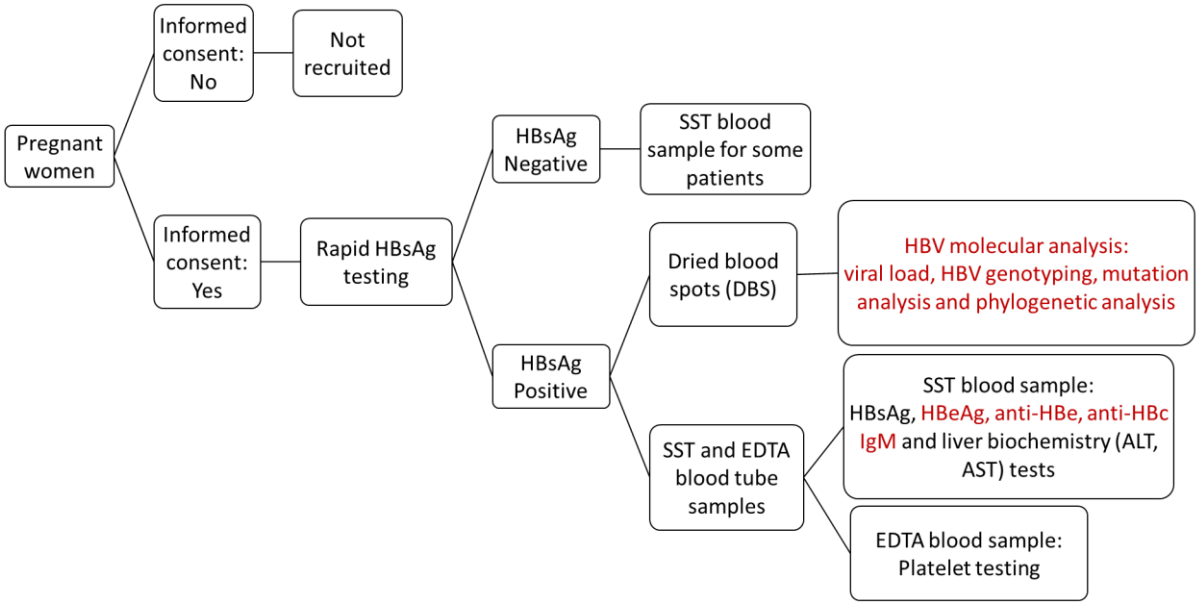


Figure 4.1: Flow diagram of the study methodology at both study sites. Informed consent was sought from the pregnant women prior to enrolment. Once given, the women were tested for HBsAg using a rapid test, and epidemiological and clinical data were recorded. Where the rapid test was positive, a DBS and a blood sample (EDTA and SST blood tubes) were drawn for further molecular and serological testing, respectively. The laboratory tests highlighted in red were conducted at the Division of Medical Virology, while the others were conducted at the Namibia Institute of Pathology. An SST blood sample was collected from a subset of HBsAg negative patients, referred to as "some patients" on the diagram, as part of quality assurance. ALT: alanine transaminase; AST: aspartate transaminase; anti-HBc: antibodies against hepatitis B core antigen, anti-HBe: antibodies against hepatitis B e antigen; DBS: dried blood spots; EDTA: ethylenediamine tetraacetic acid; HBeAg: hepatitis B e antigen, HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; IgM: immunoglobulin M; SST: Serum Separator Tube.

4.4.3 Study setting

The Windhoek Central hospital, situated 3.7 km away from Intermediate Katutura hospital, is an 855 bed capacity referral and teaching hospital in Windhoek. The hospital ANC operates Monday to Friday; the first ANC visits take place on Wednesdays and Thursdays, follow-up ANC visits on Mondays and Tuesdays, and postnatal care is given on Fridays. Here, first ANC is provided to pregnant women referred from other healthcare facilities in Windhoek due to pregnancy complications such as hypertension, diabetes and previous miscarriages or previous multiple C-sections. The Windhoek Central hospital ANC is visited by 15 to 20 pregnant women every Wednesday and Thursday for first ANC. The Intermediate Katutura hospital clinic caters for first ANC visits on Tuesdays and Wednesdays for more than 20 pregnant women per day. Antenatal follow-up visits happen on Mondays and Thursdays, and postnatal care is delivered on Fridays.

Both hospital ANCs provide routine antenatal screening services at the first ANC visit, such as HIV rapid testing, HBsAg laboratory screening, full blood count, Rhesus factor testing, syphilis testing, hemoglobin determination and urinalysis for glucose. In the morning of the first ANC visit day, a health education session is offered to the patients, covering topics of HIV and AIDS (HIV test, significance of the rapid test results, ART), family planning (injectable, pills, intra-uterine devices, condoms, tubal ligation, vasectomy and arm implants), breastfeeding for both HIV-infected and HIV-uninfected women, danger signs in pregnancy, travel and dress code during pregnancy, sexually transmitted infections (STIs), antenatal screening tests (significance of the blood tubes and importance of the tests) and the purpose of physical examination **(MoHSS, 2011)**.

4.4.4 Sample size estimation

The sample size calculation was done to estimate the prevalence of HBV among pregnant women in Windhoek, Namibia. Owing to the lack of national and regional epidemiological studies among pregnant women by the time of the design of the study, the estimation was made using the prevalence of HBV in the Namibian general population. An HBsAg seroprevalence of 8%, inferred from studies conducted in the 1970s and 1980s in Namibia **(Schweitzer et al., 2015)**, was used for the calculation of the study sample size. The minimum number of pregnant women required for the study was calculated at 453, with a 95% CI width of 0.05 (5%) and a precision of 0.025 (5%), using the Stata 14 software **(StataCorp, 2015)**. Consecutive recruitment of pregnant women in the study was set at a target of 500 pregnant women, from both study sites.

4.4.5 Study population and enrollment

All pregnant women attending for first ANC at the Intermediate Katutura hospital and Windhoek Central hospital were considered and approached for inclusion in the study, as they presented at the clinics. These pregnant women were informed on the study during the morning health education session. A careful explanation of the concept of the study was provided to them using a study guide. A nurse also explained the study in the local languages, when it was needed. The women were enrolled in the study according to the following inclusion criteria:

- Pregnant women ≥ 18 years old,
- Written informed consent given by the pregnant woman for her and her baby's participation in the study.

Participation in the study was finalized once written consent was provided by the pregnant woman. Informed assent for their babies' participation in the study was given when signing the informed consent form. The form was designed to be relevant for the participation of both the pregnant woman and her unborn baby. Women below 18 years of age and patients who did not give consent for participation were not enrolled in the study.

4.4.6 Data collection

Once informed written consent was obtained from each participant, sociodemographic, clinical data were collected using a case record form. Information regarding potential risk factors associated with HBV in pregnancy was collected using a structured questionnaire. The collection of the data was completed by the PMTCT counselor/testers or myself through face to face interviews. Sociodemographic information included age, place and area of birth, level of education, marital status, employment status, parity (the number of live births) and gravidity (the number of pregnancies). These were also assessed as risk factors for HBV infection (HBsAg positive serostatus). Other potential risk factors considered included: syphilis exposure, HIV infection, invasive mode of delivery during previous pregnancy(ies), previous blood transfusion, previous surgical procedures, family member with HBV infection or liver disease, past history of liver disease, previous abortion, tattooing, body piercing, and unprotected sexual intercourse.

Gestational age was estimated using three methods: (1) direct calculation using the date of last menstruation period (where the woman could remember), (2) measuring height of fundus, and/or (3) obstetric ultrasound. Ultrasonography was performed on the same day as the first ANC visit at the Intermediate Katutura hospital ANC, while deferred to the following week (Wednesday and Thursday) at the Windhoek Central hospital ANC.

4.4.7 HIV rapid testing

Pregnant women attending the ANCs and enrolled in the study were eligible for HIV testing using the SURE CHECK® HIV 1/2 test (Chembio Diagnostic Systems Inc, Medford, New York, USA). The SURE CHECK® HIV 1/2 test is an immunochromatographic test for the detection of antibodies to HIV-1 and HIV-2 in whole blood collected through finger-prick, venous whole blood, serum, or plasma specimens. As per the manufacturer's instructions, whole blood was collected from finger-prick in the capillary tip of the sampler of the test device until the tip was full. The device was firmly pressed in the buffer cap of the test, and the results

were visually read after a 15-minute incubation period. In the presence of the control line only, the patient was considered negative for both HIV-1 and HIV-2. Where both the control and the patient lines were noted, the result was interpreted as positive for either virus.

A second rapid test was performed for patients who tested HIV positive by the SURE CHECK® HIV 1/2, using the Alere Determine™ HIV-1/2 kit (Alere Inc., Massachusetts, USA) following the manufacturer's directives. A volume of 50 µL finger-prick whole blood was collected from the patient and transferred onto the sample pad of the test strip. One drop of chase buffer was immediately dropped onto the sample pad. Following an incubation period of 15 minutes, the results were visually read. The interpretation of these results was similar to the SURE CHECK® HIV 1/2 test results interpretation. A patient positive by both the SURE CHECK® HIV 1/2 test and the Determine™ HIV-1/2 test was concluded and reported positive for HIV. In case of a positive SURE CHECK® HIV 1/2 test result and a negative Determine™ HIV-1/2 test result, whole blood was collected from the patient for confirmation at the laboratory. Patients whose HIV status were known, or who were tested within the last three months prior to their visits at the ANCs were not re-tested.

4.4.8 HBsAg rapid testing and quality management

4.4.8.1 The Alere Determine™ HBsAg test

The Alere Determine™ HBsAg test (Alere Inc., Massachusetts, USA) is a qualitative, visually read immunochromatographic assay for the detection of HBsAg in human serum, plasma or whole blood. A positive HBsAg result indicates an HBV infection. The manufacturer reported a 98.36% sensitivity and a 100% specificity using whole blood, and a 94.64% sensitivity and 99.85% specificity using serum.

4.4.8.2 Rapid testing procedure

Enrolled pregnant women were tested for HBsAg by means of the Alere Determine™ HBsAg assay, following the procedure outlined in Figure 4.2. Similarly to the Determine™ HIV-1/2 rapid testing procedure, 50 µL of whole blood was collected from the patient through finger-prick and pipetted out onto the sample pad of the strip. One drop of chase buffer was immediately added to the sample pad, and the results were visually read after 15 minutes. The results were interpreted by the nurse(s)/counsellor(s) performing the test or by myself. In the presence of two red bands at the control and patient window, the test was considered and recorded as a positive test result (Figure 4.3). Only one band on the control line indicated a

negative test result (Figure 4.4). Where an indeterminate result was observed, defined as the absence of a band on the control line, the test was repeated.

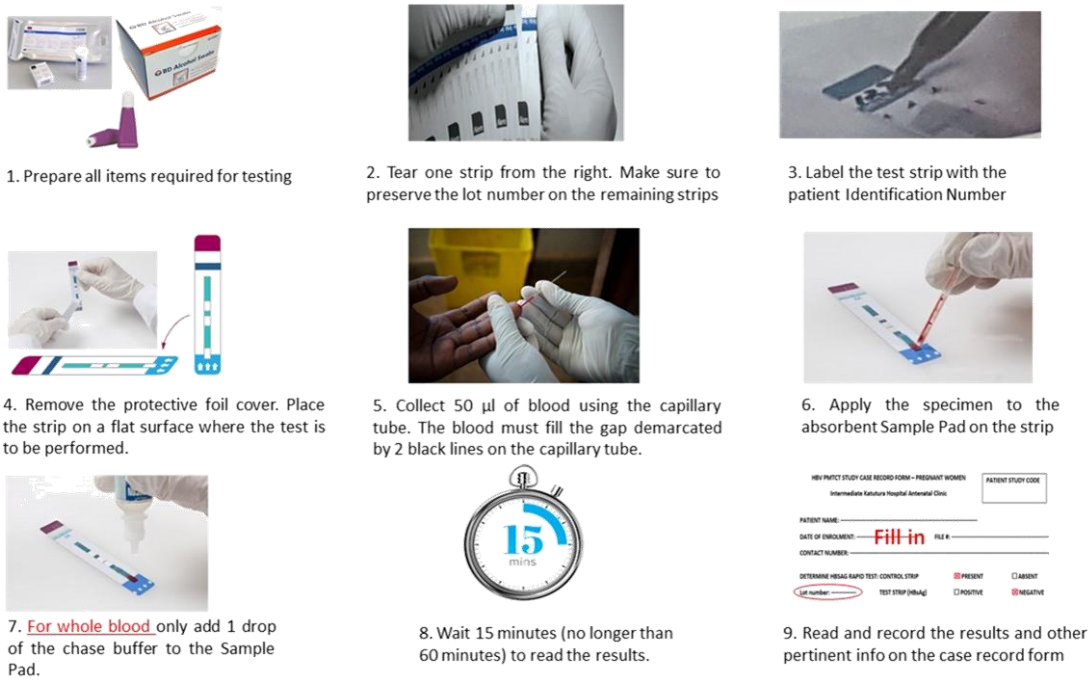


Figure 4.2: Hepatitis B rapid testing procedure using the Alere Determine™ HBsAg test (Alere Inc., Massachusetts, USA). Consented participants were tested for HBsAg at the antenatal clinics by trained PMTCT nurses and health assistants.

Figure 4.3: A representation of a negative Determine HBsAg rapid test using finger-prick blood. Only the control line is observed, and no band is seen on the patient window, indicating a negative HBsAg rapid test result.

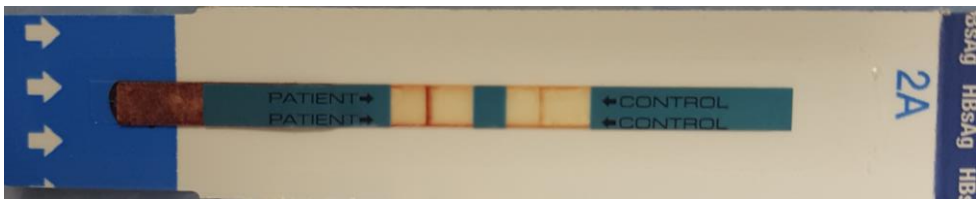


Figure 4.4: A representation of a positive Determine HBsAg rapid test using finger-prick blood. Both the control and the patient lines are observed, indicating a positive HBsAg rapid test result.

4.4.8.3 Rapid testing counseling

Pre-test and post-test counseling were provided to study participants for both HIV and HBV at the same time. Pre-test counseling was done in groups through health education. During health education, the topics of HBV and its modes of transmission, MTCT, preventive measures (vaccination), antenatal HBV testing, and the interpretation of a negative and a positive HBsAg result were discussed.

Post-test counseling was adapted as per the rapid test results. For those who tested negative for HBsAg, the counselor reiterated the meaning of the result, advised HBV vaccination and educated the patient on preventive measures for their families and sexual partners (HBV testing and HBV vaccination if testing negative for the virus). Where the patient tested HBsAg positive, the counsellor clarified the significance of the result and emphasized the need for further laboratory testing (liver function tests, repeat of HBsAg testing at the laboratory and HBV DNA viral load) to distinguish between active and inactive infection, and to assess the need for ART to prevent MTCT of the virus. The counselor also provided information on preventing transmission such as vaccinating non-immune family members and sexual partners against HBV, in case they test negative for the virus, and more importantly HBV birth vaccination of the baby and administration of HBIG if available at birth. Furthermore, the women were reminded to bring back their babies for a follow-up at six weeks and 36 weeks of age, when they come for the baby's immunization at the clinic. The aim of the follow-ups was to evaluate the HBV status of these babies and is discussed in section 4.4.17.

4.4.8.4 Quality assurance of HBV rapid testing

A study-specific quality assurance (QA) scheme for HBV rapid testing at the participating ANCs was established. This QA was adapted from the QA programme already established in Namibia for HIV RDT. This scheme consisted of two components namely internal QA and external QA.

Internal quality assurance (QA)

This involved weekly testing of HBsAg positive and HBsAg negative sera controls at the clinics, prior to patient testing. Quality control samples are not provided in the Alere Determine HBsAg rapid kit. A negative control and positive control were prepared at the Division of Medical Virology, Stellenbosch University.

The negative control refers to serum, plasma or a whole blood sample negative for the tested analyte; in this case, it is HBsAg. This control was produced using normal human plasma (NHP) provided by the Western Cape Province Blood Transfusion Service (WPBTS). This plasma was negative for HIV-1, HIV-2, HBV, and HCV on serological and molecular diagnostic assays. The NHP was tested in duplicate on the Determine HBsAg rapid test to confirm its HBsAg seronegative status on the rapid test. Sixty aliquots were prepared in 2 ml cryovials; each contained a 300 µl volume of the NHP.

A positive control is a plasma, serum or whole blood sample with a low concentration of the tested analyte. This control sample was prepared using an in-house HBsAg positive control, with a known HBsAg concentration.

A series of 1 in 20 dilutions of the in-house HBsAg serum control was performed, using NHP as a diluent, to prepare an HBsAg positive serum sample with a final HBsAg concentration of 20 IU/ml. The limit of detection of the rapid test lies around 3.4 IU/ml **(Geretti et al., 2010)**. The prepared sample was tested in duplicates on the rapid test to confirm its HBsAg seropositive status on the rapid test. Once confirmed, 300 µl volume aliquots were prepared in 60 2 ml cryovials.

Our study-specific sera controls were shipped on dry ice to the Namibia Institute of Pathology Quality assurance department situated at their reference laboratory at Windhoek Central Hospital, for local distribution to the participating ANCs. The controls were stored at -20°C in the laboratory.

Packaged by the Namibia Institute of Pathology, 30 negative controls and 30 positive controls were dispatched to Windhoek Central hospital ANC and Intermediate Katutura hospital ANC for weekly HBV rapid testing using the Determine HBsAg rapid test. The controls were kept in temperature-monitored freezers placed at the ANCs.

A comprehensive test result log, adapted from the HIV rapid test result logging sheet currently available at the participating clinics, was designed and provided to the testers to login HBV rapid test results every week for the duration of the recruitment. The sheets were checked every week, before testing, by myself to ensure that the QA was done and to verify the results.

The presence of a colored band at the control window was also used to further validate the results obtained by the rapid test. As mentioned in section 4.4.8.2, the absence of a control line was considered an invalid result and the test had to be repeated until a valid result (presence of the control line) was obtained.

External quality assurance

The external QA included a repeat of every fifth sample with an HBsAg negative result obtained by the rapid test, during the recruitment, period using the ARCHITECT HBsAg test on the automated analyzer ARCHITECT i2000SR (Abbott Laboratories, Abbott Park, Illinois, USA). This methodology was based on results from a previous field-based HBsAg screening intervention performed in the Gambia. An 11.5% chance of false negatives was reported in the study **(Njai et al., 2015)**. This false negativity rate equated to approximately 58 false negative results that may have been observed in this study. Here a 20% margin of error (i.e. 80% sensitivity) was used, taking into account human errors by the HCWs at the ANCs. This would ensure that more than 58 negative results are retested thereby reducing the chance of missing infected patients. Every patient testing HBsAg positive by the rapid test was also re-tested at the Namibia Institute of Pathology using the ARCHITECT HBsAg test for confirmation, and to exclude false positives.

PMTCT nurses and HIV counselors were trained to use the rapid test with the help of the technical team/representative at Alere Healthcare before the study commenced. Protocols and Standard Operating Procedures (SOPs), such as flowcharts for HBV testing, were designed and provided at the testing sites to ensure consistent, reliable, good quality testing.

4.4.8.5 Other quality assurance of HBV rapid testing

As it is performed for any diagnostic procedure, quality control measures were also put in place to maintain the accuracy and reliability of the results.

Rapid test storage

The manufacturers recommend storage of the kits at 2-30°C. Owing to the high temperatures in Namibia, the rapid test strips and the chase buffers were kept in refrigerators, while the capillary tubes remained at room temperature. The temperature in the testing rooms and the refrigerators, where the kits were stored, were monitored daily and logged onto temperature charts. These charts were checked daily to ensure that all storage conditions were optimum for the rapid tests.

Good testing practices

Rapid testing was performed in dedicated rooms at each hospital. The surface where the testing was completed was covered with linen savers. These were regularly changed in-between patient testing. Protective equipment, such as gloves and aprons, were worn at all times

throughout the procedure. A new pair of gloves were worn between patient testing to prevent cross-contamination.

4.4.9 Blood sample collection, handling, and shipping

Blood sample collection

Approximately 10 ml of venous blood (5 ml of EDTA blood sample and 5 ml of SST blood sample) were drawn from each pregnant woman testing HBsAg positive at the ANC for further laboratory tests. The EDTA sample was collected for platelet count testing, and the SST tube was required for HBsAg laboratory confirmation, for testing other HBV serological markers (HBeAg, anti-HBe, and anti-HBc IgM), and for liver function tests (AST and ALT). One dried blood spot (DBS) card, five spots per card, was also collected from these patients for HBV DNA viral load quantification and other molecular testing.

As per the external QA established, 5 ml of SST blood sample was collected from a subset of pregnant women testing HBsAg negative to confirm the negative result obtained by the rapid test at the laboratory.

Sample handling

Blood samples collected from the patients were packaged and sent to the Namibia Institute of Pathology, as per the protocol established at the ANCs. In brief, the samples were packaged in individual Namibia Institute of Pathology sample bags, each containing a requisition form filled with the patient details, the doctor's name and the requested tests to be performed on the samples. DBS collected were left on a drying rack overnight to dry and were packaged the next morning, according to guidelines established at the ANCs. Porters positioned at Central Hospital would make two to four rounds per day at the ANC to collect samples, and would drop them by the Namibia Institute of Pathology main laboratory situated at the hospital. The samples collected at Intermediate Katutura hospital ANC were transported on ice by Katutura Namibia Institute of Pathology to the main laboratory at Central Hospital to ensure central testing of our patients.

DBS samples were batched and sent to the Division of Medical Virology for HBV DNA viral load measurements, while recruitment was taking place in Windhoek. The results were used to inform on the need for ART against HBV to prevent transmission of HBV from the infected mother to her baby.

Serum was separated from SST blood samples at the Namibia Institute of Pathology for HBsAg and liver biochemistry markers (ALT and AST) testing. Whole EDTA blood samples were used for platelet count testing, while DBS were stored at -20°C. Residual serum samples were transferred in cryovials labeled with the sample requisition number or Patient Study ID and stored at -20°C in a sample box labeled "Stellenbosch University – HBV PMTCT Study". Import permits were obtained from the South African Department of Health for shipping samples collected from the study participants to the Division of Medical Virology at Stellenbosch University, South Africa.

Sample shipping

DBS were batched, packaged as per Namibia Institute of Pathology DBS packaging guidelines, and shipped at ambient temperature to the Division of Medical Virology, Stellenbosch University. Prior to shipment, the DBS were equilibrated to room temperature (minimum of 30 minutes) and re-packaged for shipping at ambient temperature. Three batches were made during the recruitment period and sent to the Division of Medical Virology. Stored serum samples were shipped on dry ice to the Division of Medical Virology at the end of the recruitment period for serological tests (HBeAg, anti-HBe and anti-HBc IgM) unavailable at the Namibia Institute of Pathology.

4.4.10 Hepatitis B virus laboratory serological investigations

All serology laboratory testing was performed using the automated analyzer ARCHITECT i2000SR (Abbott Laboratories, Abbott Park, Illinois, USA). This analyzer is positioned at both the Namibia Institute of Pathology and the NHLS Virology laboratory, Tygerberg Hospital.

4.4.10.1 HBsAg testing

Principle: The ARCHITECT HBsAg Qualitative assay is a chemiluminescent microparticle immunoassay (CMIA) used to determine the presence of HBsAg in human serum and plasma. Anti-HBs coated paramagnetic microparticles, and anti-HBs acridinium-labeled conjugate are incubated with the sample, for the binding of HBsAg present in the specimen to the anti-HBs. Unbound particles are washed off. The addition of two substrates, namely the pre-trigger (Hydrogen peroxide) and trigger (Sodium hydroxide) solutions, to the reaction triggers a chemiluminescent signal measured as relative light units (RLUs). The chemiluminescent signal is compared to an S/CO value determined from an active ARCHITECT HBsAg calibration. A

signal lower than the cut-off value represents a negative HBsAg result while a signal equal to or higher than the cut-off value is interpreted as a positive HBsAg result.

Assay calibration and quality control: Prior to patient's sample testing, HBsAg Qualitative calibrators (Calibrator 1 and Calibrator 2) were run in triplicates to calibrate the assay every time a new kit was used. The mean S/CO values from the calibrators replicates is calculated by the system of the machine and is used to calculate the S/CO values of the samples. HBsAg Qualitative controls (Negative Control and Positive Control) were tested in single replicate, each testing day, as per the manufacturer's directions and the quality control procedures established at the Namibia Institute of Pathology. The assay was deemed calibrated in the case when each control result was within the range specified in the package insert of the controls.

Testing procedure: Serum samples collected from the SST blood samples, registered with a requisition number generated by the Namibia Institute of Pathology laboratory sample logging system, were inserted in the ARCHITECT i2000SR machine for testing. The machine was operated by a trained laboratory technician.

Interpretation of results: An S/CO value ≥ 1 was considered positive while an S/CO < 1 was considered negative. Results obtained were signed off by clinical consultants at the Namibia Institute of Pathology and were updated onto their system for access at the healthcare facilities. These results were also printed and kept for the study records.

4.4.10.2 HBeAg testing

Principle: The ARCHITECT HBeAg assay is a CMIA for the qualitative detection of HBeAg in human serum and plasma. The assay is completed in two steps. At first, sample, assay diluent, and anti-HBe (mouse, monoclonal) coated paramagnetic microparticles are mixed; allowing the HBeAg present in the sample to bind to the anti-HBe coated microparticles. A wash step, removing unbound microparticles, follows and the acridinium-labeled anti-HBe conjugate is added. This is followed by another washing step and the addition of the pre-trigger and trigger solutions to the reaction. The resulting chemiluminescent signal is directly proportional to the amount of HBeAg in the sample. The emitted chemiluminescent signal is compared to the S/CO determined from an ARCHITECT HBeAg calibration. The tested sample is considered reactive for HBeAg if the chemiluminescent signal of the reaction is equal to or above the cut-off signal.

4.4.10.3 Anti-HBe testing

Principle: The ARCHITECT Anti-HBe assay is a competitive two-step CMIA. The anti-HBe present in the patient's sample competes with anti-HBe (mouse, monoclonal) coated paramagnetic microparticles for binding with recombinant HBeAg present in a neutralizing reagent added in the reaction. The resulting chemiluminescent signal detected is inversely proportional to the amount of anti-HBe present in the sample. The sample is considered non-reactive for anti-HBe if the S/CO obtained is greater than the cutoff signal.

4.4.10.4 Anti-HBc IgM testing

Anti-HBc IgM serves as a measure of an acute HBV infection; that is an infection of less than six months, in the presence of an HBsAg positive result. This testing served to differentiate between acute and chronic HBV infection.

Principle: This is a two-step CMIA for the qualitative detection of anti-HBc IgM in human serum and plasma using the ARCHITECT i2000SR. During the assay, the sample is incubated with anti-human IgM (mouse monoclonal) coated paramagnetic microparticles; allowing human IgM present in the specimen to bind to mouse monoclonal anti-human IgM coated microparticles. A washing step is completed to remove unbound microparticles, and the acridinium-labeled rHBcAg conjugate is added in the reaction. The resulting chemiluminescent signal, following the addition of the Pre-Trigger and Trigger solutions, is proportional to the amount of anti-HBc IgM in the sample. A positive anti-HBc IgM result is obtained should the chemiluminescent signal of the reaction be greater than or equal to the cutoff signal.

HBeAg, anti-HBe, and anti-HBc IgM assay Calibration and quality control: Following the manufacturer's directives, HBeAg Assay calibrators (Calibrators 1 and 2), anti-HBe calibrator 1 only, anti-HBc IgM Calibrator 1 and Calibrator 2 were run in replicates of three whenever a new kit was opened. A single replicate of the controls (Negative Control and Positive Control) were run prior to patient's sample testing to validate the assays calibration. The calibration was considered valid providing that each control result was within the ranges specified by the manufacturer. These ranges may vary between lot numbers of the kit.

The control range values for each test, as per the lots used for these assays, are as follow:

- HBeAg negative control range (0.0 PEI U/mL): 0.000 – 0.800 S/CO
- HBeAg positive control range (0.7 PEI U/mL): 2.026 – 6.077 S/CO
- Anti-HBe negative control (0.0 PEI U/mL): 1.30 – 2.70 S/CO

- Anti-HBe positive control range (0.38 PEI U/mL): 0.21 – 0.80 S/CO
- Anti-HBc IgM negative control range (0.0 PEI U/mL): 0 – 0.25 S/CO
- Anti-HBc IgM positive control range (150 PEI U/mL): 1.608 – 4.825 S.CO

HBeAg, anti-HBe, and anti-HBc IgM testing procedure: Following successful calibration of the assays, residual HBsAg positive serum samples received from the Namibia Institute of Pathology were loaded onto the ARCHITECT i2000SR machine at the NHLS Virology laboratory at Tygerberg. The HBeAg, anti-HBe and anti-HBc IgM statuses of these samples were assessed.

Interpretation of results: S/CO ≥ 1 results were considered positive for HBeAg or anti-HBc IgM and negative for anti-HBe. S/CO < 1 results were deemed positive for anti-HBe, and negative for HBeAg or anti-HBc IgM.

4.4.11 Hepatitis B virus laboratory molecular investigations

4.4.11.1 HBV DNA viral load quantification

HBV DNA viral load was determined using both DBS and serum samples, tested on the automated COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0. DBS testing was adapted following a previously published methodology **(Vinikoor et al., 2015)** and the DBS viral load assay described in Chapter 3 (section 3.4.9.1). Briefly, two DBS were eluted in a 1000 µl volume of SPEX buffer. Following the same principle of dilution explained in section 3.4.9.1 of Chapter 3, 60 µl of plasma (30 µl per spot) is diluted in a 1000 µl of SPEX buffer, corresponding to an approximate 39 µl of input sample in the total volume of 650 µl used for the assay. The dilution factor between DBS and plasma here was 25.64. Elution took place overnight on The Coulter Mixer (Coulter Electronics Limited, Luton Bedfordshire, UK) at room temperature. The eluates were used for both viral DNA extraction and amplification, completed on the automated COBAS AmpliPrep / COBAS TaqMan system. The rationale behind the use of two spots instead of one spot was to increase the volume of plasma sample from 30 µl in one spot of whole blood to 60 µl with two spots, to potentially improve the LoQ of the assay. Elution was completed at room temperature to mimic conditions in laboratories with limited access to specialized equipment. The agreement between serum and DBS measurements was assessed by the Bland-Altman analysis, using $\log_{10}$ transformed data **(Bland & Altman, 1986)**. DBS measurements ($\log_{10}$-transformed data) were plotted against serum samples measurements to determine the linearity and correlation (R^2 value) between the

two sample types (linear regression analysis). For the purpose of these two analyses, viral load measurements with < 20 IU/ml (< 1.30 log_{10} IU/ml) results were recorded as 10 IU/ml (1 log_{10} IU/ml); serum samples viral load with >170 000 000 IU/ml (> 8.3 log_{10} IU/ml) were recorded as 8.3 log_{10} IU/ml. Samples with an undetectable viral load in serum were removed from the analyses.

The LoQ of this DBS assay was assessed using dilutions of the third WHO HBV international standard for NAT assays (NIBSC code 10/264). The standard was reconstituted in 500 µl of nuclease-free water to obtain an HBV viral load of 850 000 IU/ml (5.93 log_{10} IU/mL), as per the manufacturer's instructions, prior to use. The reconstituted material was further diluted using whole blood that was negative for HBV serological markers and HBV DNA. DBS cards (50 µl per spot) of the dilutions were prepared with Whatman filter paper card (five perforated spots/card) (Lasec, Cape Town, South Africa) and left to dry overnight under a biosafety cabinet. Eluates were run in triplicates for each concentration during two different days, on the automated COBAS AmpliPrep / COBAS TaqMan system. Due to the limited volume of the WHO standard, matched DBS and NHP dilutions could not be tested in parallel. The Probit regression analysis was completed to infer the LoQ of the assay, as performed elsewhere **(Weiss et al., 2004; Dowling et al., 2018)**, with the Statistical Package for Social Sciences (SPSS) software Version 25 (IBM Corp., Armonk, New York, USA).

4.4.11.2 Manual viral DNA extraction

HBV DNA was manually extracted from samples with detectable levels of DNA using the QIAamp® MinElute® Virus Spin kit (QIAGEN, Hilden, Germany). This methodology was previously described in Chapter 3, section 3.4.9.2.

4.4.11.3 Nucleotide sequencing of the pol/surf and X/core genes

HBV pol/surf and X/core genes sequencing were performed using viral DNA extracted from HBV DNA positive samples. These assays were performed as described in Chapter 3, section 3.4.9.3 with slight modifications, described as follow. Gel electrophoresis was completed using the Enduro™ Gel XL Electrophoresis System (Labnet International Inc, Woodbridge, New Jersey, USA) at a voltage of 100V for 45 minutes. Cycle sequencing products were sent to the DNA sequencing Unit of Central Analytical Facilities, situated at Stellenbosch University, for capillary electrophoresis.

4.4.11.4 Nucleotide sequence analysis and phylogenetic analysis

Pol/surf and X/core DNA chromatograms obtained from the DNA sequencing Unit of Central Analytical Facilities at Stellenbosch University were analyzed as described in Chapter 3, section 3.4.9.4. As per the described protocol, viral pol/surf consensus sequences generated were aligned with similar sequences acquired on Genbank using the ClustalW algorithm on Geneious Version 10.2.3. The alignment was imported in MEGA 7 for a neighbor-joining tree analysis, assessing the distances between the sequences in the dataset. A best-fit substitution model analysis followed and was completed using JModelTest 2.1.3 to identify the best fitted model for maximum likelihood phylogenetic analysis. The suggested nucleotide substitution model was subsequently used for executing maximum likelihood phylogenetic analysis. Core gene sequences were analyzed using the BioEdit software.

4.4.11.5 Quality assurance for molecular assays

This has been described in detail in Chapter 3, section 3.4.10.5. Briefly, assays were performed in dedicated rooms under a class II biological laminar flow cabinet. An NC, NTC, and PC were included in each PCR reaction for each gene amplification and sequencing. A dedicated set of pipettes and filtered tips were available for use in each room where the assays were completed. Lastly, the surfaces of the biosafety cabinets, the laboratory benches and the pipettes used were disinfected before and after use with 10% bleach and 70% ethanol.

4.4.12 Syphilis laboratory testing

Antenatal syphilis screening was performed at the Namibia Institute of Pathology through a two-step process. The first line screening test is done for the detection of non-specific antibodies against lipoidal and lipoprotein-like materials released by the host cells damaged by the bacterium Treponema pallidum, the causative agent of syphilis. The assay is performed using the rapid plasma reagin (RPR) Carbon Antigen Kit (Biorex Diagnostics, Antrim, UK) for the detection of reagin antibodies, as per the manufacturer's instructions. Briefly, a drop of the antigen, provided in the kit, is mixed with 50 µl of neat patient's sample. The antigen, made of particulate carbon suspension coated with lipid complexes, binds to reagin antibodies present in the sample tested forming clumps. These clumps, viewed macroscopically, indicated the presence of reagin antibodies in the sample tested. The absence of clumps indicated the absence of reagin antibodies in the sample tested, and the patient was reported negative for syphilis. A positive RPR sample was further tested to determine the presence of specific antibodies against

the bacterium using the Treponema pallidum haemagglutination (TPHA) kit (Biorex Diagnostics, Antrim, UK). The assay was completed following the manufacturer's directives. Briefly, a 1 in 20 diluted sample is incubated with Trepanoma pallidum sensitized avian erythrocytes cells for a minimum period of 45 minutes, away from direct sunlight and heat. In the presence of antibodies against the bacterium in the sample tested, an agglutination reaction takes place between antibodies and antigen, forming a characteristic pattern of cells. The sample is thus recorded positive for syphilis.

4.4.13 Liver health assessment

All pregnant women testing HBsAg positive by the rapid test were evaluated for biochemical evidence of liver disease using the APRI. ALT, AST, and platelet count testing were performed at the Namibia Institute of Pathology. Results from these measurements were used to calculate the APRI score using the formula outlined in Chapter 3, section 3.4.10. An APRI score >1.5 was considered abnormal.

4.4.14 Evaluation of antiviral therapy

Eligibility for maternal antiviral prophylaxis against HBV MTCT was determined as per the 2016 American Association for the Study of Liver Diseases (AALSD) guidelines **(Terrault et al., 2016)**. That is: HBsAg positive pregnant women with HBV DNA levels above 200 000 IU/ml were eligible for treatment with TDF, 300 mg oral daily dose. On the basis that access to HBIG was uncertain for most women, pregnant women with an HBsAg seropositive status with HBV DNA levels above 100 000 IU/ml were reviewed for TDF treatment by a clinician collaborating on the study. The risks and benefits of ART were discussed with each woman. Where applicable, the HBV-infected pregnant woman was initiated on TDF from 28 weeks of pregnancy until one month postpartum for HBV DNA viral load suppression.

4.4.15 Analysis of risk factors associated with HBsAg seropositivity

Risk factors associated with an HBsAg positive serostatus were grouped into the categories of sociodemographic, social behaviors and nosocomial exposure. The sociodemographic variables considered were age, marital status, the area of birth, employment status and educational level. Factors related to social behaviors included HIV-infection, syphilis status, body tattooing, body piercing, and unprotected sexual activity. Parity, gravidity, route of delivery (history of C-section), history of blood transfusions, surgical procedures and abortions

represented factors related to nosocomial exposure. The previous history of liver disease, as well as the presence of family members with previous/current HBV infection or liver disease, were also included in the analysis. The association between these factors and an HBsAg seropositive status was investigated through univariate logistic regression. For the purpose of this analysis, some variables were categorized in groups: parity (nulliparous, primiparous, multiparous), marital status (unmarried, married), unprotected sexual activity (yes or no, the "sometimes" responses were counted in as "No" for the purpose of the analysis). With regards to the educational level, women with a secondary level of education or less were classified as "low", and "high" for those with a tertiary level of education or enrolled in a tertiary level programme. The logistic regression was completed using the SPSS software Version 25 (IBM Corp., Armonk, New York, USA). Odds ratio and their 95% CI were recorded and served to establish the association between the considered factors and HBsAg seropositivity. Multivariate logistic regression analysis was performed including the risk factors with $p \leq 0.25$ in the univariate regression analysis. The significance level was set at $p < 0.05$ in the multivariate logistic regression analysis, to demonstrate the true association between the risk factors and HBsAg seropositivity.

4.4.16 Feasibility of rapid testing analysis

The feasibility of integrating rapid testing for HBV in existing HIV PMTCT services was assessed at both study sites. The concept of feasibility was broken down to the diagnostic performance, and the acceptance and usability of the rapid test by the HCWs at the ANCs. The perspectives of these HCWs concerning HBV rapid testing was measured using a user-appraisal questionnaire presented in Appendix G. Copies of this questionnaire were distributed to the PMTCT nurses and HIV counselors performing both the HBV and HIV rapid testing during the study period. The questionnaire was designed to evaluate the minimal training needs, ease of use and user acceptability of the HBV rapid test. It included both closed questions, and open-ended questions prompting qualitative responses. The data were collected and entered in an Excel sheet for analysis. HBsAg results obtained with the rapid test using finger-prick blood were compared to ARCHITECT HBsAg results obtained from venipuncture serum samples at the laboratory. This served as determining the diagnostic accuracy (diagnostic sensitivity and diagnostic specificity) of the rapid test for field use. A 2x2 contingency table, below, compares the diagnostic accuracy criteria between the candidate assay (Determine HBsAg test) against the reference diagnostic assay (ARCHITECT i2000SR HBsAg assay). The diagnostic

sensitivity measures the ability of the rapid test to correctly identify HBV-infected patients, whilst the diagnostic specificity measures the ability of the test to identify those who aren't infected with the virus. The formulas outlined below were used to compute the diagnostic sensitivity and specificity of the rapid test used for antenatal HBsAg screening at the ANCs.

		Architect i2000SR "Gold standard"		**Total**
		Positive	Negative	
Determine HBsAg rapid test	Positive	**# true positive (TP)**	**# false positive (FP)**	**TP+FP**
	Negative	**# false negative (FN)**	**# true negative (TN)**	**FN+TN**
Total		**TP+FN**	**FP+TN**	

HBsAg: hepatitis B surface antigen

- The estimated diagnostic sensitivity (true positive rate) = 100 x [TP/(TP+FN)]
- The estimated diagnostic specificity (true negative rate) = 100 x [TN/(FP+TN)]

4.4.17 HBV-exposed babies testing

Based on the estimated gestational age of the HBsAg positive women identified during the recruitment period, the estimated due date of delivery of each woman was calculated. These women were traced and telephonically contacted for the six-week follow-up of their babies. At this follow-up, an SST pediatric blood sample was collected for HBsAg testing at the Namibia Institute of Pathology laboratory, and a case record form was completed. The case record form served to document the personal details, HBV vaccination history, and the HIV clinical history, where applicable, of the HBV-exposed babies.

4.5 Results

4.5.1 Patient recruitment and sociodemographic characteristics

Between September 29 and November 16 2017, a total of 515 pregnant women were recruited in the study: 273 (53%) from Intermediate Katutura hospital ANC and 242 (47%) from Windhoek Central hospital ANC. Study participants were of a median age of 28 years old (Range: 18 – 45 years old), with a median number of live births of one (Range: 0 – 9 children) and a median number of pregnancies of two (Range: 1 – 10 pregnancies). The median pregnancy age was 24 weeks (Range: 9 – 39 weeks).

The majority of these women were unmarried (433/515; 84.1%) and had a secondary level of education (389/515; 75.5%) while only 19.4% (100/515) reported either having some tertiary level degree or being enrolled at a tertiary institution. In terms of employment status, 50.7% (261/515) women reported having informal or formal employment while 38.6% (199/515) were unemployed, and 10.7% (55/515) were students (Table 4.1). Two hundred and ninety-five (295/515; 57.3%) women were born in urban areas while the remainder were born in rural areas (220/515; 42.7%). Geographically, the majority were born in the Khomas province (133/515; 25.8%), followed by Omusati (77/515; 15%), and Ohangwena (64/515; 12.4%) provinces (Figure 4.5). Some pregnant women were from other African countries including: Angola (14/515; 2.7%), Botswana (2/515; 0.4%), Kenya (1/515; 0.2%), South Africa (1/515; 0.2%), Tanzania (2/515; 0.4%), Zambia (1/515; 0.2%) and Zimbabwe (4/515; 0.8%).

A significant difference was noted with regards to the level of education ($p < 0.001$) and employment status ($p = 0.006$) between pregnant women attending these two ANCs. Pregnant women attending Windhoek Central hospital ANC had a higher level of education and more likely to be employed as compared to those attending Intermediate Katutura hospital ANC.

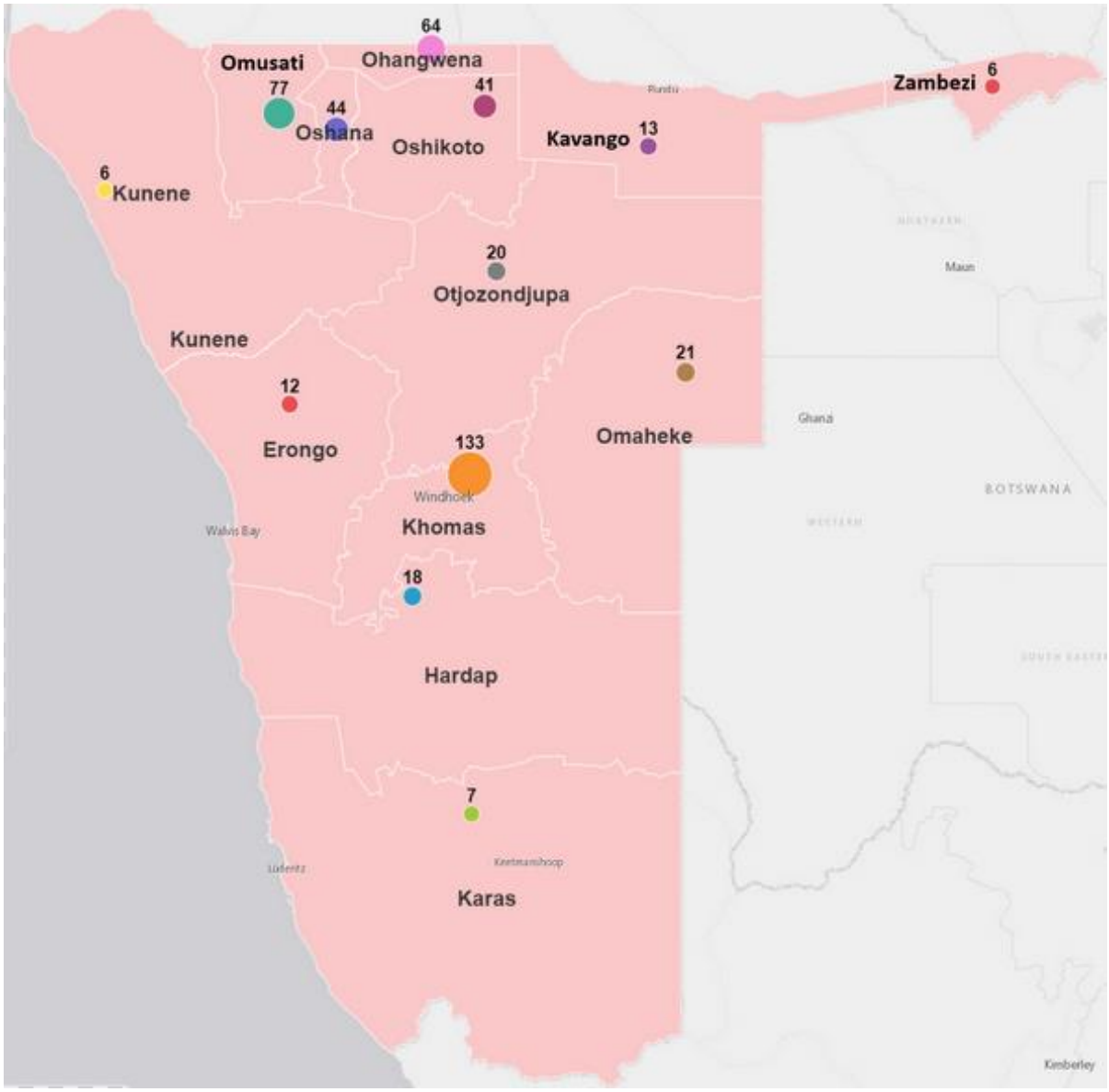


Figure 4.5: Geographic distribution of the study population enrolled in the study in Namibia. Study participants originating from neighboring countries of Namibia are not represented on this graph. The map was produced using the ArcGIS® Online Software by Esri© 2018 (www.esri.com/software/arcgis/arcgisonline). Namibia province distribution shapefile was obtained from map library (www.maplibrary.com).

Table 4.1: Sociodemographic characteristics of enrolled pregnant women

Variable	Total N (%)	IKH N (%)	WCH N (%)	p-value
Patient Count	N = 515	273	242	
Age				
≤24[1]	142 (27.6%)	75 (27.5%)	67 (27.7%)	0.94
25-34	261 (50.7%)	137 (50.2%)	124 (51.2%)	
≥35	112 (21.7%)	61 (22.3%)	51 (21.1%)	
Area of birth				0.518
Urban	295 (57.3%)	160 (58.6%)	135 (55.8%)	
Rural	220 (42.7%)	113 (41.4%)	107 (44.2%)	
Marital status				0.221
Unmarried	433 (84.1%)	236 (86.4%)	197 (81.4%)	
Married	80 (15.3%)	36 (13.2%)	44 (18.2%)	
Divorced	2 (0.4%)	1 (0.4%)	1 (0.4%)	
Level of education				< 0.001*
None	8 (1.6%)	8 (2.9%)	0 (0.0%)	
Primary	18 (3.5%)	18 (6.6%)	0 (0.0%)	
Secondary	389 (75.5%)	213 (78.0%)	176 (72.7%)	
Tertiary	100 (19.4%)	34 (12.5%)	66 (27.3%)	
Employment status				0.004*
Employed[#]	261 (50.68%)	123 (45.05%)	138 (57.02%)	
Unemployed	199 (38.6%)	124 (45.4%)	75 (31.0%)	
Student	55 (10.7%)	26 (9.5%)	29 (12.0%)	
Gravidity				0.057
Primigravida	117 (22.7%)	53 (19.4%)	64 (26.4%)	
Multigravida	398 (77.3%)	220 (80.6%)	178 (73.6%)	
Parity				0.054
0	130 (25.2%)	59 (21.6%)	71 (29.3%)	
1-3	347 (67.4%)	189 (69.3%)	158 (65.3%)	
> 3	38 (7.4%)	25 (9.2%)	13 (5.4%)	

[1]include patients from the age of 18 years old. [#]Include formal and informal employment. *p-value < 0.05 showing statistically significant differences between variables. Primigravida = first pregnancy; multigravida = have had more than one pregnancy. ANC: antenatal clinic; IKH: Intermediate Katutura hospital; WCH: Windhoek Central hospital. Statistical significance was computed using the Pearson chi-square test, except for the variables Marital status and Level of education which were analyzed using the Fisher's exact test.

4.5.2 Seroprevalence of HIV infection

The HIV infection was detected in 66 women with a median age of 33 years (Range: 18 – 45 years), a median number of live births of two (Range: 0 – 6 children), and a median number of pregnancies of three (Range: 1 – 9 pregnancies). This gave an overall HIV infection seroprevalence of 12.8% (66/515). Of these 66 HIV-infected women, 20 (30.3%; 20/66) were

newly diagnosed patients during the study period, and 46 (69.7%; 46/66) were known cases of HIV infection. The median age within these two groups was 26.5 years (Range: 20 – 39 years) for newly HIV-infected women and 34 years (Range: 18 – 45 years) for known HIV-infected patients.

Looking at the distribution of these HIV infections between the two study sites, a 7% (17/242) and 17.9% (49/273) prevalence was found at Windhoek Central hospital ANC and Intermediate Katutura hospital ANC, respectively. This difference was found to be statistically significant ($p < 0.001$); as seen in Table 4.2. Among these new HIV infections, 65% (13/20) were found at the Intermediate Katutura hospital ANC, while only 35% (7/20) were detected at the Windhoek Central hospital ANC. Thirty-six (36/46; 78.3%) of the known HIV infections were identified at Intermediate Katutura hospital ANC, while ten (21.8%) were from Windhoek Central hospital ANC.

Table 4.2: Sociodemographic characteristics of enrolled HIV-infected pregnant women

Variable	Total	HIV status of pregnant women		
		Negative n (%)	Positive n (%)	P-value
	515	449	66	
Age				
≤24[1]	142	132 (29.4%)	10 (15.2%)	0.001*
25-34	261	230 (51.2%)	31 (47.0%)	
≥35	112	87 (19.4%)	25 (37.9%)	
Clinic				< 0.001*
IKH ANC	273	224 (49.9%)	49 (74.2%)	
WCH ANC	242	225 (50.1%)	17 (25.8%)	
Area of Birth				0.630
Urban	220	190 (42.3%)	30 (45.5%)	
Rural	295	259 (57.7%)	36 (54.5%)	
Marital status				1.000
Divorced	2	2 (0.4 %)	0 (0%)	
Married	80	70 (15.6%)	10 (15.2%)	
Unmarried	433	377 (84.0%)	56 (84.8%)	
Level of education				
None	8	7 (1.6%)	1 (1.5%)	<0.001*
Primary	18	12 (2.7%)	6 (9.1%)	
Secondary	389	333 (74.2%)	56 (84.8%)	
Tertiary	100	97 (21.6%)	3 (4.5%)	
Employment status				< 0.001*
Employed[#]	261	238 (53.0%)	23 (34.8%)	
Student	55	53 (11.8%	2 (3.0%)	
Unemployed	199	158 (35.2%)	41 (62.1%)	
Parity				<0.001*
0	130	126 (28.1%)	4 (6.1%)	
1 – 3	347	292 (65.0%)	55 (83.3%)	
> 3	38	31 (6.9%)	7 (10.6%)	
Gravidity				< 0.001*
Primigravida	117	114 (25.4%)	3 (4.5%)	
Multigravida	398	335 (74.6%)	63 (95.5%)	

Primigravida = first pregnancy; multigravida = have had more than one pregnancy. [#]Include formal and informal employment. *p-value < 0.05 showing statistically significant differences between variables. ANC: antenatal clinic; IKH: Intermediate Katutura hospital; WCH: Windhoek Central hospital. Statistical significance was computed using the Pearson chi-square test. The Fisher's exact test was performed for the variables: Age, Marital status, Level of education, Employment status, Parity and Gravidity.

4.5.3 Seroprevalence of syphilis

Seven women reacted positive to the RPR test (7/515; 1.4%); of whom five (5/7; 71.4%) tested TPHA positive. The proportion of TPHA positive women translated to a 1% (5/515)

seroprevalence of syphilis in our study population. The median age of these infected women was 32 years (Range: 30 – 41 years). The median number of live births was two (Range: 1 – 3 children), and the median number of pregnancies was four (Range: 2 – 5 pregnancies). Four of these five TPHA positive women were HIV positive ($p < 0.001$), and none were co-infected with HBV.

Table 4.3: Sociodemographic characteristics of enrolled syphilis-infected pregnant women

Patient ID	Age*	HIV status	Gravidity	Parity	Area of birth	Marital status	Level of education	Employment status
PG133	33	+	4	3	Urban	Unmarried	Secondary	Employed
PG137	30	+	3	2	Urban	Unmarried	Secondary	Unemployed
PG211	41	+	5	3	Urban	Unmarried	Secondary	Unemployed
M234	32	-	4	2	Urban	Married	Secondary	Unemployed
M251	30	+	2	1	Urban	Unmarried	Secondary	Unemployed

*age in years. PG ID patients were enrolled at Intermediate Katutura hospital ANC. M ID patients were from Windhoek Central hospital ANC. ANC: antenatal clinic, ID: Identificant, PG: Pregnant Woman, M: Mother.

4.5.4 HBsAg rapid testing results

4.5.4.1 HBsAg seroprevalence

Twenty-eight pregnant women were found positive for HBsAg. This is an HBsAg of 5.4% (28/515) (95% CI: 3.71% - 7.86%). The median age was 30.5 years [Range: 19 – 41 years], a median gestational age of 26 weeks [Range: 15 – 39 weeks], a median gravidity of three [Range: 1 – 6 pregnancies] and a median parity of 2 [Range: 0 – 5 children]. Many were from rural areas (18/28; 64.3%), and the majority was unmarried (24/28; 85.8%) and possessed a secondary level of education (18/28; 64.3%). Seven (7/28; 25%) stated holding a tertiary level qualification or pursuing a tertiary level study programme; of whom three were students, three were employed, and one was unemployed. Geographically, the majority of these positive women originated from the northern part of the country: Ohangwena and Omusati provinces (Figure 4.6). The seroprevalence of HBsAg was similar at both ANCs: 5.5% (15/273) at Intermediate Katutura hospital ANC and 5.4% (13/242) at Windhoek Central hospital ANC.

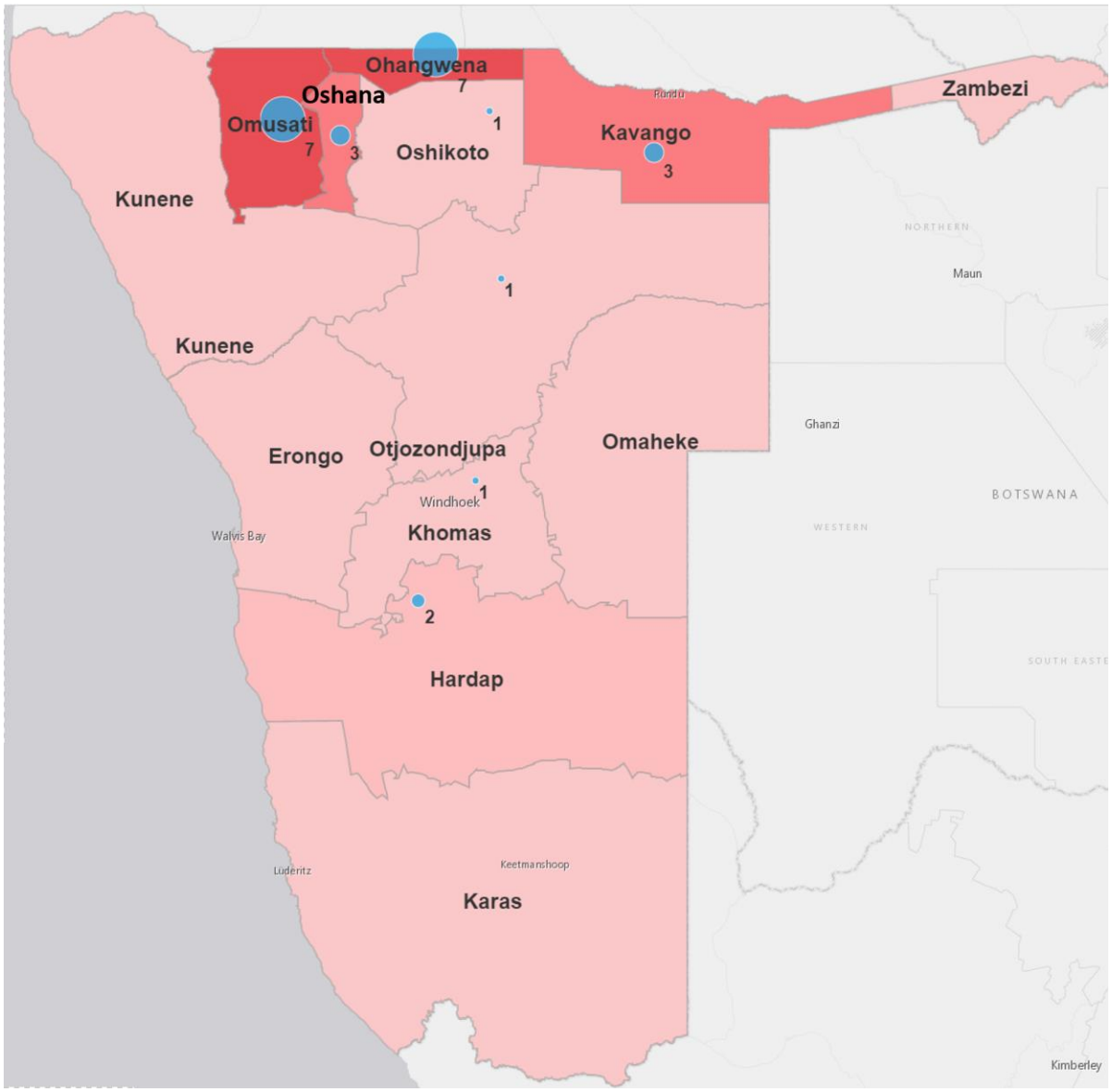


Figure 4.6: Geographic distribution of HBV positive across Namibia. The majority of HBV-infected pregnant women identified in the study were originally from Omusati and Ohangwena, close to the border with Angola. The map was produced using the ArcGIS® Online Software by Esri© 2018 (www.esri.com/software/arcgis/arcgisonline). Namibia province distribution shapefile was obtained from map library (www.maplibrary.com).

Furthermore, 25 of these HBsAg positive women (89.3%) were HBV mono-infected, while three were co-infected with HIV, giving a 0.6% (3/515) overall HIV/HBV co-infection seroprevalence. None tested reactive for syphilis. Table 4.4 illustrates the sociodemographic and clinical characteristics of these HBsAg positive patients.

Table 4.4: Sociodemographic and clinical characteristics of HBsAg positive pregnant women

Patient ID	Age (years)	Gestational age (weeks)	HIV status	Syphilis status	Gravidity	Area of birth	Marital status	Level of education	Employment status
PG16	38	27	+	-	5	Urban	Married	Primary	Unemployed
PG22	30	18	-	-	3	Urban	Unmarried	Tertiary	Student
PG39	21	38	-	-	2	Rural	Unmarried	Secondary	Employed
PG62	32	39	-	-	4	Urban	Unmarried	Primary	Unemployed
PG74	26	19	-	-	3	Urban	Unmarried	Secondary	Employed
PG87	27	29	-	-	1	Rural	Unmarried	Secondary	Employed
PG108	30	25	-	-	3	Rural	Unmarried	Secondary	Unemployed
PG119	19	31	-	-	1	Rural	Unmarried	Secondary	Unemployed
PG127	23	28	-	-	2	Rural	Unmarried	Tertiary	Employed
PG162	31	19	-	-	1	Rural	Married	Secondary	Employed
PG194	37	25	-	-	5	Urban	Unmarried	Secondary	Unemployed
PG196	33	23	-	-	3	Urban	Married	Primary	Unemployed
PG225	33	29	-	-	6	Rural	Unmarried	Secondary	Employed
PG246	24	22	-	-	2	Rural	Unmarried	Secondary	Employed
PG272	25	29	-	-	4	Rural	Unmarried	Secondary	Unemployed
M26	36	15	-	-	3	Urban	Married	Tertiary	Student
M66	28	24	-	-	2	Rural	Unmarried	Tertiary	Student
M81	24	28	-	-	1	Rural	Unmarried	Secondary	Student

M99	22	27	-	-	1	Urban	Unmarried	Secondary	Unemployed
M148	41	21	-	-	4	Rural	Unmarried	Secondary	Employed
M151	34	26	-	-	3	Rural	Unmarried	Tertiary	Employed
M170	23	26	+	-	3	Rural	Unmarried	Secondary	Unemployed
M174	28	22	-	-	3	Urban	Unmarried	Secondary	Employed
M179	34	27	-	-	2	Rural	Unmarried	Secondary	Unemployed
M180	31	21	-	-	3	Rural	Unmarried	Tertiary	Employed
M190	32	23	-	-	1	Rural	Unmarried	Tertiary	Unemployed
M244	33	32	+	-	5	Rural	Unmarried	Secondary	Unemployed
M245	34	36	-	-	2	Urban	Unmarried	Secondary	Unemployed

PG ID patients were enrolled at Intermediate Katutura hospital ANC. M ID patients were from Windhoek Central hospital ANC. ID: Identificant, PG: Pregnant Woman, M: Mother.

4.5.4.2 Quality assurance results

Internal quality assurance

The HBV positive controls and negative controls, prepared at the Division of Medical Virology as previously explained (section 4.4.8.4), were successfully tested at the ANCs every week, prior to the patient's testing. All positive controls remained positive on the rapid test, and all negative controls remained negative on the rapid test, throughout the testing period. The results from this exercise are attached in Appendix H and Appendix I for Intermediate Katutura hospital ANC and Windhoek Central hospital ANC, respectively.

External quality assurance

Every fifth HBsAg negative rapid test result attained at the ANCs was planned to be repeated at the laboratory. This corresponded to every fifth of 487 HBsAg negative results and equated to 97 samples to repeat at the laboratory. However, 268 HBsAg negative results obtained at the ANCs were repeated at the laboratory. A seventh of HBsAg negative patients identified by the rapid test at Intermediate Katutura hospital ANC (n = 39/258), and all patients testing HBsAg negative by the rapid test at Windhoek Central hospital ANC (n = 229) were re-tested for HBsAg using the ARCHITECT HBsAg CLIA assay at the Namibia Institute of Pathology. The 28 HBsAg positive patients detected using the Determine HBsAg rapid test were successfully re-tested at the laboratory for HBsAg too.

4.5.5 Sample collection and shipping

A total of 28 DBS and blood samples were collected from HBsAg positive patients on the day of their testing and sent to the Namibia Institute of Pathology laboratory. All 28 DBS were successfully shipped to and received at the Division of Medical Virology for HBV molecular testing. However, six of the serum samples collected could not be traced for retrieval at the laboratory, and 22 out of the 28 serum samples were shipped to and received at the Division of Medical Virology for further HBV serological markers testing. The other six samples were re-collected post-partum but were still included in our analysis.

4.5.6 Hepatitis B virus laboratory serological results

4.5.6.1 HBsAg testing

All HBsAg positive patients (28) detected by the rapid test at the ANCs, repeated at the Namibian laboratory, and remained HBsAg positive on the Architect i2000SR. All HBsAg

negative samples (39 from Intermediate Katutura hospital ANC and 229 from Windhoek Central hospital ANC) re-tested, as part of EQA, remained HBsAg negative too. These results are schematically presented in Figure 4.7.

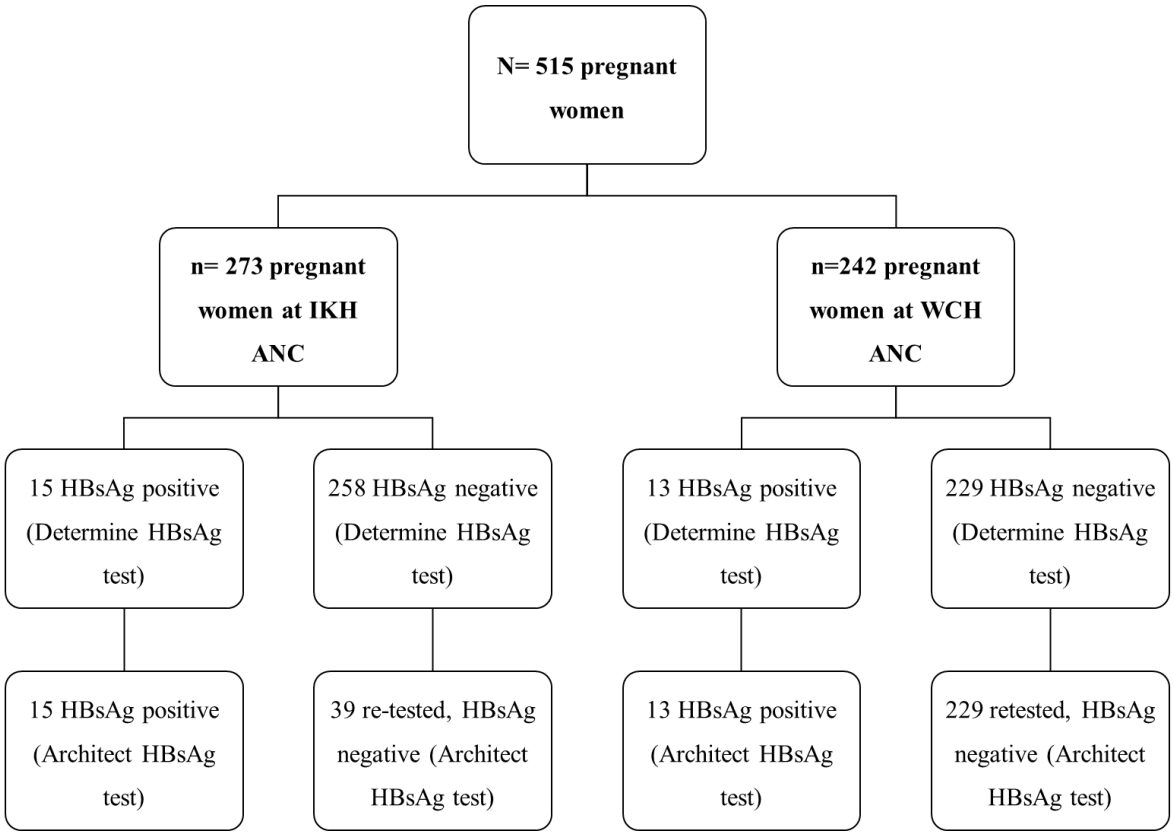


Figure 4.7: A flowchart representation of HBsAg testing using the Determine HBsAg rapid test and the Architect HBsAg laboratory test. ANC: antenatal clinic; HBsAg: hepatitis B surface antigen; IKH: intermediate Katutura hospital; WCH: Windhoek Central hospital.

4.5.6.2 Seroprevalence of HBeAg among HBsAg positive

All 28 serum samples were available for HBeAg testing. Three specimens were HBeAg reactive (3/28; 10.7%), whilst the rest were non-reactive (25/28; 89.3%).

4.5.6.3 Seroprevalence of anti-HBe among HBsAg positive

Of the 28 HBsAg positive samples, 24 were anti-HBe reactive (24/28; 85.7%); all were HBeAg negative. Four samples were anti-HBe non-reactive (4/28; 14.3%): three were HBeAg positive, and the other was HBeAg negative.

4.5.6.4 Seroprevalence of anti-HBc IgM among HBsAg positive

All 28 samples were also tested for anti-HBc IgM. They tested nonreactive for this marker.

4.5.7 Hepatitis B virus laboratory molecular results

4.5.7.1 Viral DNA quantification

Limit of quantification of the two DBS assay

Results of the repeat testing of DBS from dilutions of the third HBV WHO international standard for NAT assays (NIBSC 10/264) are shown in Table 4.5. As tabulated in Table 4.6, the level at which 50% of the reactions were quantified was 3.70 $\log_{10}$ IU/ml (5000 IU/ml). At a minimum 95% quantification rate, determined by the Probit analysis, the lower limit of quantification of the COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 was approximately 7110 IU/ml (3.85 $\log_{10}$ IU/ml) using two DBS (Appendix J).

Table 4. 5: HBV DNA measurements in two DBS using dilutions of the NIBSC 10/264

NIBSC HBV DNA levels (IU/ml)	NIBSC HBV DNA levels ($\log_{10}$ IU/ml)	2 DBS HBV DNA levels* ($\log_{10}$)	Difference*
2000	3.30	< 20	Not computed
5000	3.70	2.85[#]	0.85
10 000	4.00	3.26	0.74
15 000	4.18	3.47	0.70
20 000	4.30	3.62	0.68
30 000	4.48	3.69	0.79
35 000	4.54	3.70	0.85
200 000	5.30	4.43	0.87

*Computed from six replicates of each dilution. [#]Three out of six replicates were successfully quantified. DBS: dried blood spot; HBV: hepatitis B virus; NIBSC: National Institute for Biological Standards and Control.

Table 4. 6: Limit of quantification in two DBS of the COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0 assay

NIBSC HBV DNA levels (IU/ml)	NIBSC HBV DNA levels ($\log_{10}$ IU/ml)	Number of replicates	Number quantified	Rate of quantification
2000	3.30	6	0	0%
5000	3.70	6	3	50%
10 000	4.00	6	6	100%
15 000	4.18	6	6	100%
20 000	4.30	6	6	100%
30 000	4.48	6	6	100%
35 000	4.54	6	6	100%
200 000	5.30	6	6	100%
PROBIT 95% hit rate: 7110 IU/ml (3.85 $\log_{10}$ IU/ml)				

DBS: dried blood spot; HBV: hepatitis B virus; NIBSC: National Institute for Biological Standards and Control.

Viral load measurements in patient's samples

HBV DNA was quantifiable in six DBS samples (6/28; 21.43%), with a median HBV viral load of 3.52 log_{10} IU/ml (Range: 3.12 – 7.43 log_{10} IU/ml). Of these six samples, HBV DNA levels above 100 000 IU/ml (5 log_{10} IU/ml), the threshold for maternal TDF treatment initiation in the study, were noted in two samples: 6.91 log_{10} IU/ml and 7.43 log_{10} IU/ml. These two patients were HBeAg positive and anti-HBe negative. HBV DNA levels in their corresponding plasma samples were above > 8.23 log_{10} IU/ml, the upper limit of quantification of the assay using serum or plasma samples. The median viral load in the corresponding plasma samples of the other four patients with quantifiable DNA in DBS was 2.99 log10 IU/ml (Range: 2.82 – 3.95 log_{10} IU/ml).

HBV DNA was detected, but not quantifiable, in seven DBS samples (7/28; 25%). The matching serum samples had a median viral load of 3.17 log_{10} IU/ml (Range: 2.73 – 3.42 log_{10} IU/ml). HBV DNA was undetectable in 15 DBS samples (15/28; 53.57%); of which three belonged to HIV co-infected patients on a TDF-based ART (TDF/FTC/EFV fixed-dose combination). Similar results were noted in the corresponding serum samples of five of these 15 DBS samples; two of the five samples were HIV/HBV co-infected. Of the remaining 10 corresponding serum samples, two had a viral load < 20 IU/ml (one was co-infected with HIV), and the other eight had a median viral load of 1.66 log_{10} IU/ml (Range: 1.36 log_{10} – 2.62 log_{10} IU/ml), below the level of quantification of the DBS assay. These results are summarized in Table 4.7.

Correlation, linearity, and agreement between DBS and serum sample measurements

Twenty-two matched DBS and serum samples collected during the antepartum period of these patients were available to assess the agreement between viral measurements of these two sample types. Four samples with undetectable viral loads in both serum and DBS were excluded, and the remainder 18 were further analyzed. DBS viral loads were on average 1.05 log_{10} IU/ml (95% CI: 0.68 - 1.42 log_{10} IU/ml) lower than their matching serum samples ($p < 0.001$). The Bland-Altman plot, illustrating the agreement between the two measurements, showed that the differences in viral load between these two sample types fell within the 95% limits of agreement (-0.40 to 2.50 log_{10} IU/ml) of the average difference (Figure 4.8).

Correlation and linearity between viral load measurements obtained with these two sample types were assessed using 10 matched samples, with detectable viral loads in both 2 DBS and

serum samples. As per Figure 4.9, the $R^2 = 0.89$ suggested strong correlation between HBV DNA levels attained with the two paired sample types (Figure 4.9).

Table 4.7: HBV viral copies and HBV serological status of HBsAg positive patients

Patient ID	HIV status	Serum HBV DNA log_{10} (IU/ml)	DBS HBV DNA log_{10} (IU/ml)	HBsAg	HBeAg	Anti-HBe	Anti-HBc IgM
PG16[¥]	+	LDL	LDL	+	-	+	-
PG22	-	2.87	LQL	+	-	+	-
PG39	-	LDL	LDL	+	-	+	-
PG62	-	3.42	LQL	+	-	+	-
PG74	-	LQL	LDL	+	-	+	-
PG87	-	1.63	LDL	+	-	+	-
M26	-	2.62	LDL	+	-	+	-
PG108[#]	-	LDL	LDL	+	-	+	-
M66	-	3.35	LQL	+	-	+	-
M81	-	1.36	LDL	+	-	+	-
PG119	-	3.45	LQL	+	-	+	-
PG127	-	> 8.23	6.90	+	+	-	-
PG162	-	LDL	LDL	+	-	+	-
M99	-	2.82	3.53	+	-	+	-
M148*	-	1.49	LDL	+	-	+	-
M151*	-	1.69	LDL	+	-	+	-
M170	+	LQL	LDL	+	-	+	-
M174*	-	2.98	3.11	+	-	+	-
PG194*	-	> 8.23	7.41	+	+	-	-
PG196*	-	3.00	3.48	+	-	+	-
PG225	-	1.98	LDL	+	-	+	-
M179	-	2.73	LQL	+	-	+	-
M180	-	1.49	LDL	+	-	+	-
M190	-	3.95	3.33	+	-	+	-
M244*	+	LDL	LDL	+	+	-	-
M245	-	1.71	LDL	+	-	-	-
PG246	-	2.76	LQL	+	-	+	-
PG272	-	3.17	LQL	+	-	+	-

[¥]Result from a ½ dilution, due to insufficient sample volume for the assay. [#]Result from a 1/10 dilution, due to insufficient sample volume for the assay. *Blood samples collected from these patients at enrollment were lost at the Namibian laboratory and were re-collected postpartum. Anti-HBc IgM: antibodies Immunoglobulin M against hepatitis B core antigen; anti-HBe: antibodies against hepatitis B e antigen; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; IU/ml: International Units per millilitres; LDL: lower than the detection limit; LQL: lower than the quantification limit (< 20 IU/ml with serum and 3.85 IU/ml with DBS); M: Mother; NT: Not Tested; PG: Pregnant woman; TND: Target Not Detected. In red are HIV/HBV co-infected patients.

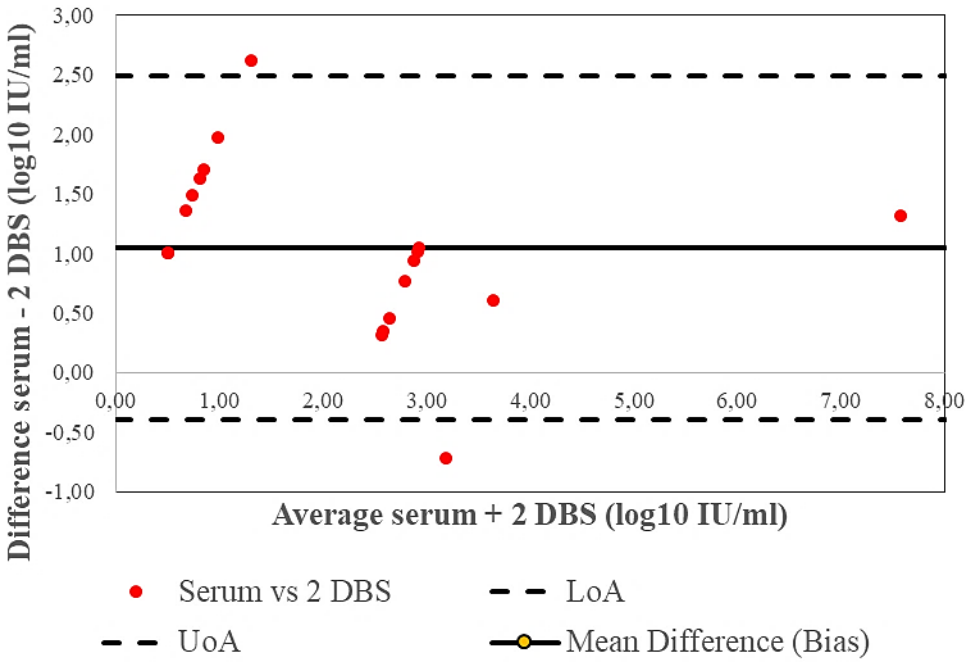


Figure 4.8: Bland-Altman plot of agreement between HBV DNA viral loads of 18 paired serum samples and DBS samples. The solid line represents the mean difference between the serum and DBS viral load measurements. The dashed lines represent the upper and lower limit of agreements (±1.96 standard deviations) of the differences between the measurements of both samples' types. DBS: Dried blood spots; LoA: lower limit of agreement; UoA: upper limit of agreement.

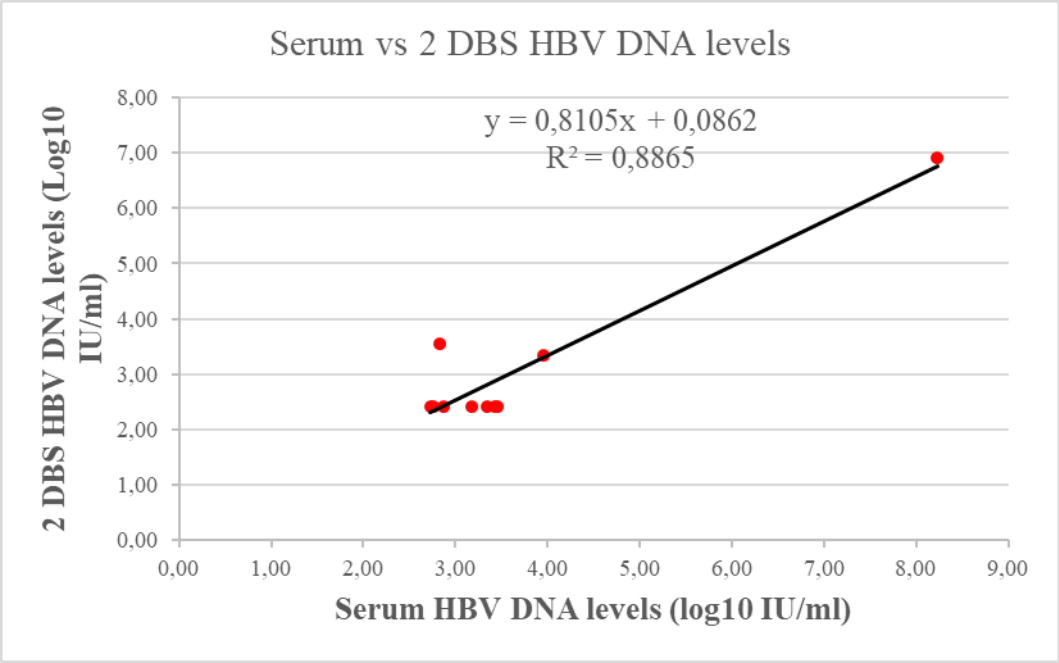


Figure 4.9: Linear regression analysis HBV DNA levels of 10 paired DBS and serum samples. The graph shows a good correlation (R^2 = 0.89) between measurements attained in two DBS and serum samples. HBV: hepatitis B virus.

4.5.7.2 Nucleotide sequencing of the pol/surf gene

Viral DNA extraction was completed on all HBsAg positive patients with detectable levels of DNA and pol/surf gene amplification was completed. HBV DNA was successfully amplified for 11 out of the 13 samples (11/13; 84.6%) with detectable DNA.

Nested PCR products were purified and used for cycle sequencing. Purified sequencing products went through capillary electrophoresis. Chromatograms were obtained from the 11 samples with detectable HBV DNA, and consensus sequences of approximately 900 bp long were formed. These were further analyzed for genotyping and the presence of genomic mutations.

HBV genotyping

Viral sequences were fed in the NCBI HBV Genotyping tool. Genotype E was identified in eight samples (8/11; 73%). The other three viral strains belonged to Genotype D and were further described as sub-genotype D1 through Geno2Pheno analysis.

HBV Reverse transcriptase mutation analysis

The RT region of the pol gene was explored for the presence of drug-associated mutations. Twenty-seven polymorphisms were found in the analyzed sequences; of which one was found (V27I) in both genotypes while the others were genotype-specific. However, none were associated with resistance to any nucleos(t)ide analogs drugs used for the treatment of HBV. A list of these genetic variants is shown in Table 4.8.

Table 4.8: Distribution of mutations in the RT region of the polymerase gene

Sample ID	Genotype	RT amino acid substitutions
PG22	E	V27I, R110S, K154EK
PG119	E	V27I
PG127	E	M267L, R274IR
M99	E	M267L
M174	E	K11R, N76D, M267L, D271E, N279H
PG194	E	K11R, I122T, Y126H, W257F, M267L
PG196	E	M267L, D271E
PG272	E	M267L
PG62	D1	V27IV, Y54HY, N118T, N123D, Y135S, L164M, I233V, N248H, I266R, Q267H
M179	D1	A38E, Y54H, N123D, Y135S, L164M, I233V, N238HN, K241R, Y245H, N248H, I266R, Q267H
M190	D1	N53KN, L115M, N123D, Y135S, L164M, I233V, N248H, C256S, I266R, Q267H

Substitutions, highlighted in green, were found in both genotype E and sub-genotype D1. The other substitutions appeared genotype-specific. ID: identificant; RT: reverse transcriptase.

HBV surf gene mutation analysis

Due to the overlap between the pol and the surf reading frames, the small surface antigen (SHB) overlapping with the RT region was also examined for possible genetic variations. Twenty-one amino acid polymorphisms were observed in ten samples; each had more than one variant. None were associated with vaccine escape when analyzed with the geno2pheno web-based genotyping tool. One strain harbored a premature stop codon at aa L216*. Moreover, mixed viral populations were observed in two sub-genotype D1 samples. These samples showed a mixture of wild-type and mutant viruses. Table 4.9 shows the list of variants observed in the SHB region of our dataset and their genotypes.

Interestingly, the genotype E strain harboring the stop codon L216* also presented with a 15 bp deletion in the pre-S2 region, spanning from nt 51 to nt 65. This nucleotide deletion corresponds to the deletion of aa 137 to aa 141.

Table 4.9: Distribution of mutations in the surface antigen region.

Sample ID	Genotype	surface antigen amino acid substitutions
PG22	E	N3S, L13R, F20S, Q51L, T57I, G102A, S136L, A184V
PG119	E	N3S, T57I, P67L, L215R, L216*, aa 137-141 deletion
PG127	E	N3S, T57I, L127P
M99	E	N3S, T57I
M174	E	N3G, T57I
PG194	E	N3G, T57I, S114P
PG196	E	N3S, T57I
PG272	E	N3S, T57I, T189I
PG62	D1	F8S, I110L, P203R, Y206CY, S207N, P214LP
M179	D1	Q30K, S174N
M190	D1	T45NT

Substitutions, highlighted in green, represent mixed viral populations observed. ID: identificant.

Phylogenetic analysis

A neighbor-joining analysis was completed to assess the distances between the query sequences and those obtained in Genbank through BLAST. The phylogenetic tree constructed (Figure 4.10) showed close clustering of HBV genotype E sequences from Namibia with Genotype E strains identified in the study: PG196, M99, M174, PG194, and PG127. These sequences also appeared to be closely related to other HBV Genotype E sequences identified in Chapter 3 (section 3.5.4.2). HBV sequences from Chapter 3 are represented in the tree as 2C, 3C, 6C, 7C, 10C, and 13C. On the other hand, genotype D sequences identified in these two studies showed no relationship. As reported in Chapter 3 (section 3.7.4.2), HBV sequences from 8C and 17C clustered with sub-genotype D3 sequences from Botswana and South Africa; while sequences from PG62, M179, and M190 clustered with sub-genotype D1 and D2 sequences.

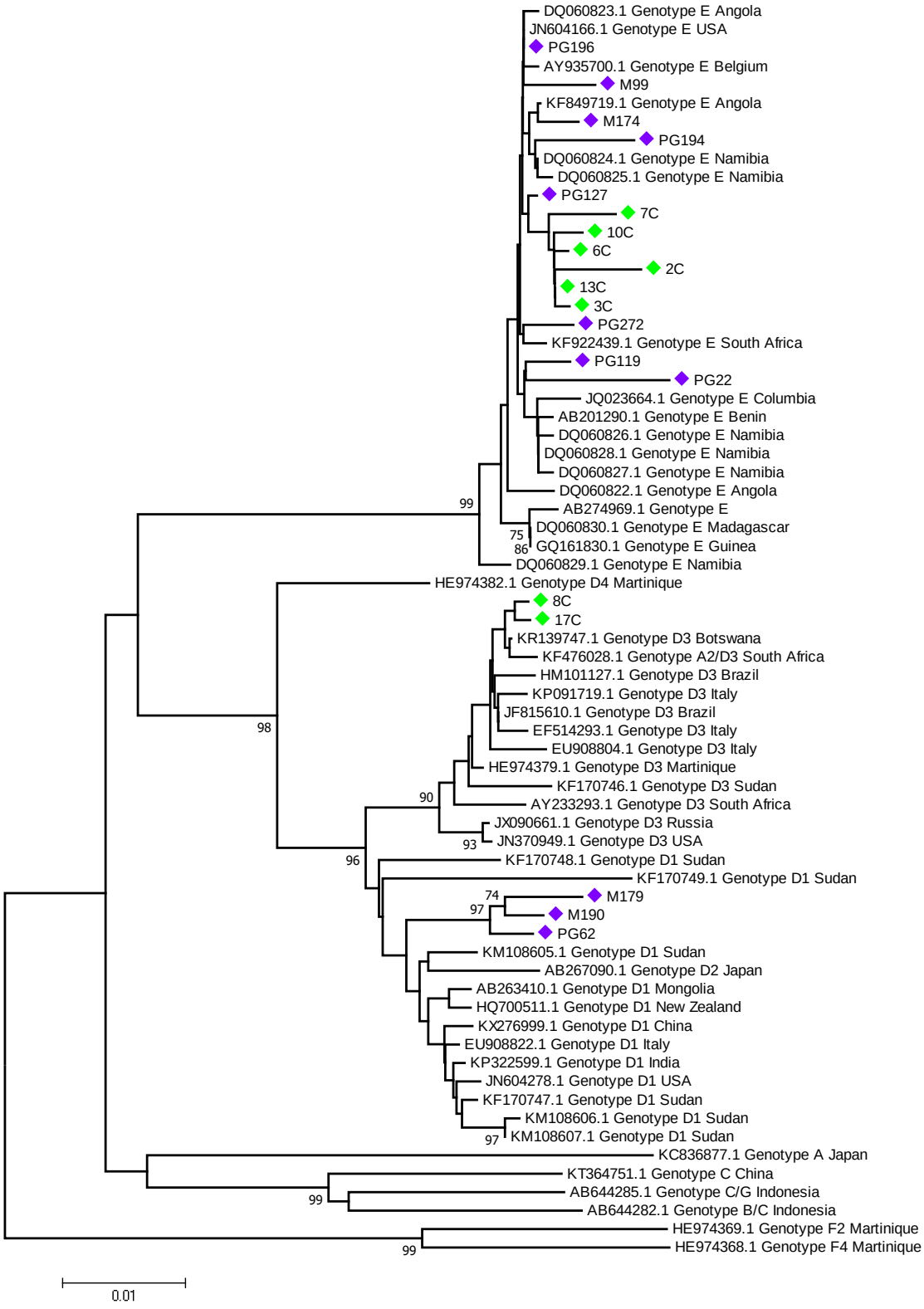


Figure 4.10: Molecular phylogenetic analysis of HBV strains identified in the study based on the pol/surf region of the HBV genome using Neighbor-joining model. The evolutionary relationships between the study query sequences were inferred using the pol/surf genomic fragment. The evolutionary distances were computed using the p-distance method **(Nei & Kumar, 2000)**, and the consensus tree was inferred from 1000 bootstrap replicates. Distances values at the branch nodes lower than 70% are not shown. The antenatal sequences are represented with purple pentagons, and the pediatric sequences (Chapter 3) are represented with bright green pentagons.

Maximum likelihood phylogenetic analysis was completed using the GTR+I+G model, as per the JModelTest 2.13 test results. A similar cluster of sequences was observed on this phylogenetic tree, Figure 4.11.

Pairwise comparison of sub-genotype D1 sequences showed a close similarity between the query sequences (PG62, M179, and M190) and sub-genotype D1 sequences from Sudan (KM108605.1), India (KP322599.1) and New Zealand (HQ700511.1). All genotype E sequences were closely similar.

The close clustering of HBV genotype E sequences identified in the dataset (pediatric and antenatal HBV DNA positive samples) prompted further phylogenetic molecular analysis. Genotype E sequences from other parts of South West Africa and from West and Central Africa were retrieved from Genbank. These sequences were aligned with the HBV DNA positive pediatric and antenatal sequences, and the multiple alignment was fed in MEGA 7 for maximum likelihood phylogenetic analysis using the GTR+I+G model (Figure 4.12). PG22 and PG119, only, appeared closely related to other Genotype E sequences from Namibia, while the other study Genotype E sequences grouped with sequences from Angola. While these strains are part of one clade (Genotype E), they appear as two lineages on the phylogenetic tree: isolates from West and Central Africa (Cameroon, Central African Republic (CAR), Cote d'Ivoire, Ghana, Guinea, Niger, and Nigeria) grouped together, while strains from the Southern part of Africa (Namibia, Angola and the Democratic Republic of Congo (DRC)) formed another group.

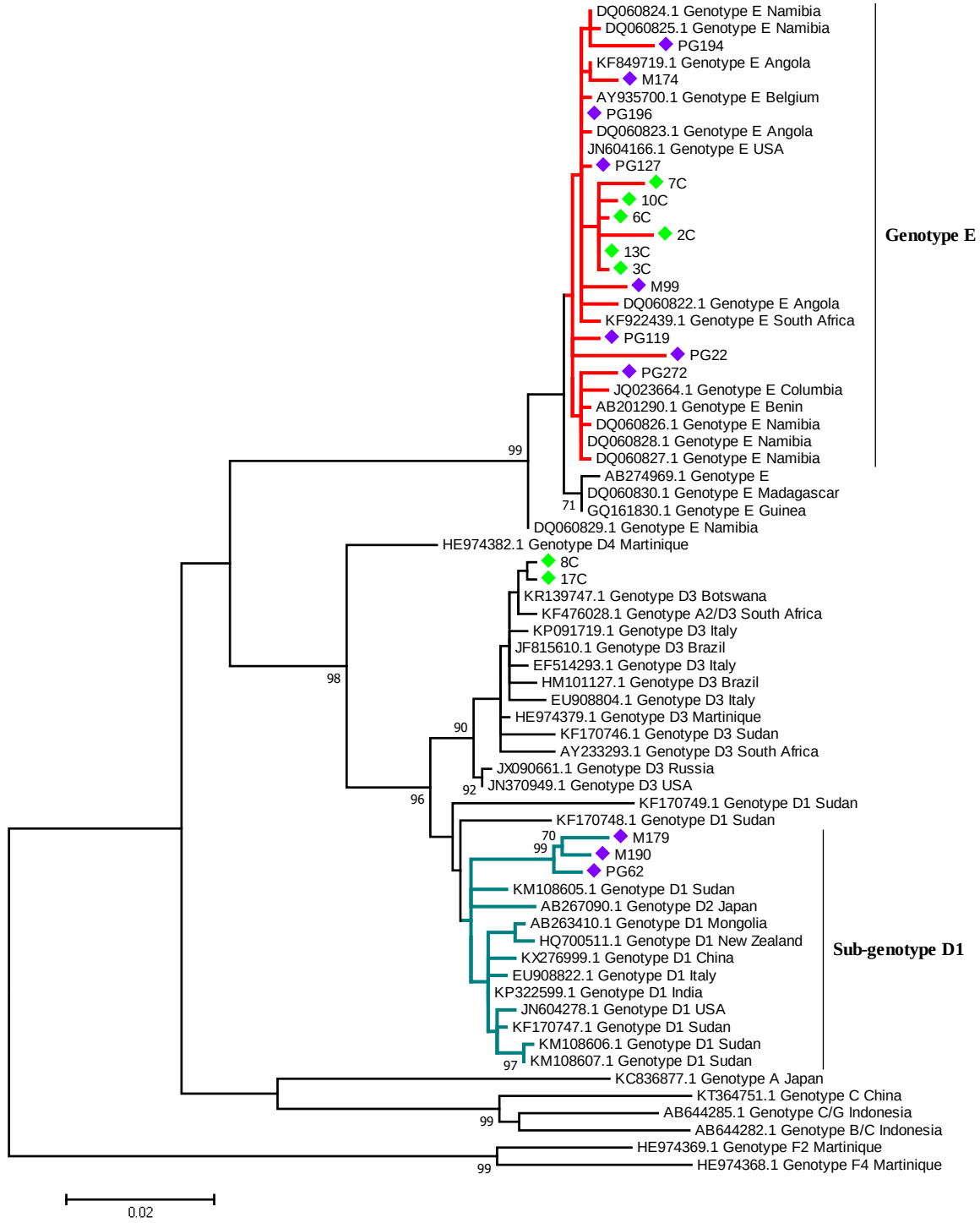


Figure 4.11: Molecular phylogenetic analysis of HBV strains identified in the study based on the pol/surf region of the HBV genome using maximum-likelihood model. This evolutionary history was inferred based on the General Time Reversible model. Bootstrap statistical analysis was performed using 1000 replicates. Distance values at the branch nodes lower than 70% were excluded from the tree. Antenatal and pediatric query sequences are represented with purple and bright green pentagons, respectively. The red lines show the relationship between Genotype E sequences and lines highlighted in teal represent D1 sequences.

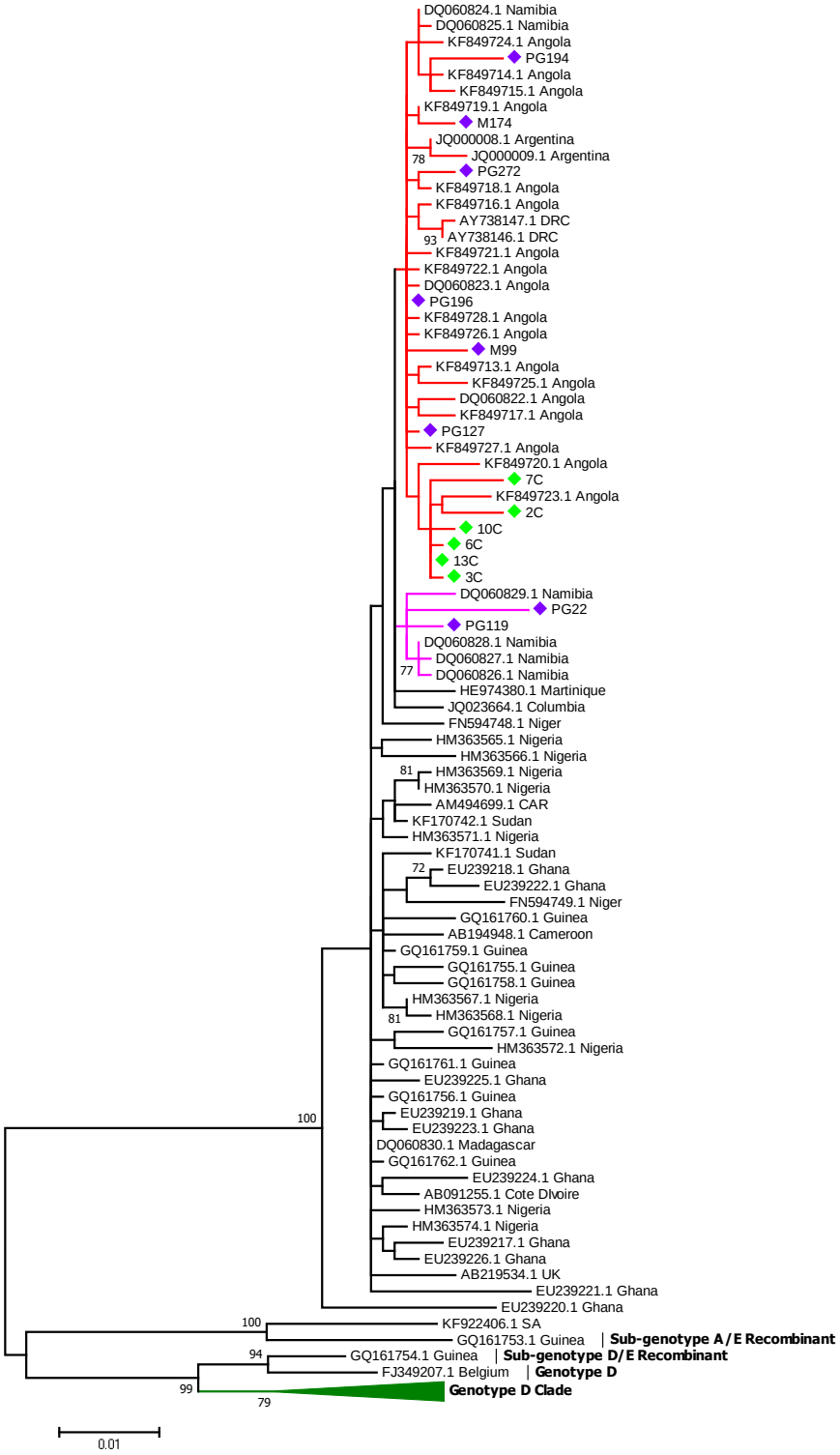


Figure 4.12: Molecular phylogenetic analysis of Study HBV Genotype E strains with other African HBV genotype E strains. Evolutionary distances were inferred using the maximum likelihood method, based on the General Time Reversible model, using 1000 bootstrap replicates. Distance values at the branch nodes lower than 70% were excluded from the tree. The query sequences are annotated with colored pentagons: in purple are strains from HBV-infected pregnant women (Chapter 4) and in bright green are strains from HIV/HBV-infected children (Chapter 3). The lines in blue show the cluster formed by query sequences and those from Angola. The pink lines denote the relationship between the query sequences and those from Namibia. The uncolored part of the tree shows Genotype E sequences from Cameroon, Central African Republic (CAR), Cote d'Ivoire, Ghana, Guinea, Niger, and Nigeria. The tree was rooted with Genotype D sequences annotated in dark green at the root of the tree.

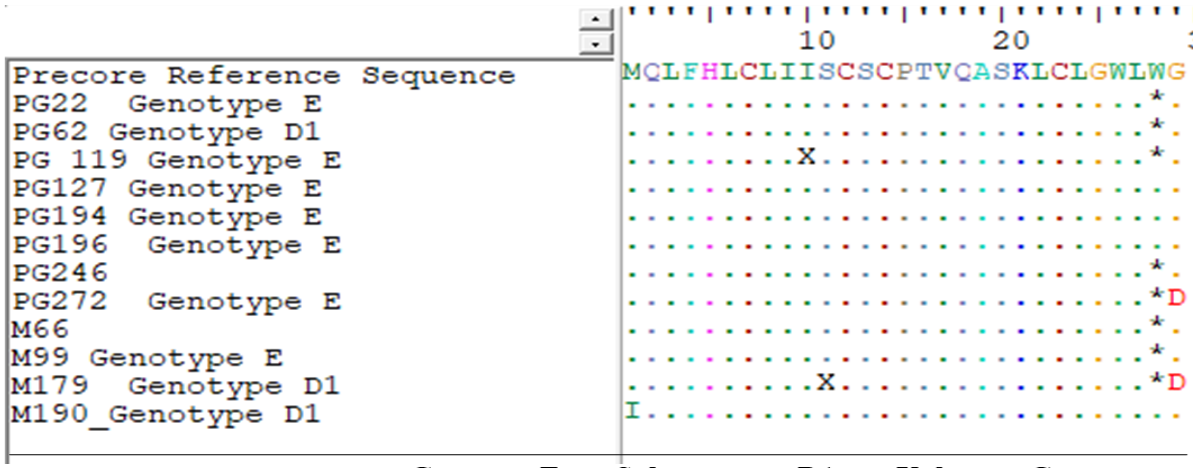
4.5.7.3 Nucleotide sequencing of the X/core gene

Thirteen samples with detectable HBV DNA levels went through cycle sequencing for the X/core gene.

These PCR products were purified and analyzed via spectrophotometry to assess the purity and measure the concentration of DNA present. All DNA products, but M99, were diluted to a final DNA concentration of 20 ng/µl using nuclease-free water and were sequenced. The sequencing products were sent to the DNA sequencing Unit of Central Analytical Facilities at Stellenbosch University for capillary electrophoresis, and the resulting chromatograms were analyzed. Consensus sequences were successfully obtained for all 13 samples with detectable HBV DNA.

Pre-C mutation analysis

Pre-C genomic mutations were observed in all, but three patients (PG127, PG194, and PG196) Figure 4.13. Eight HBV viral strains (8/13; 61.5%) harbored the pre-C stop codon W28*, corresponding to the G1896A nucleic acid mutation. In two of these strains, PG272 - Genotype E) and M179 - sub-Genotype D1), W28* were accompanied by the G29D mutant corresponding to a G to A exchange at nt1899 (G1899A). The point mutation G to T at position 1816 (G1816T), the pre-C start codon, was also observed in patient M190. The mutation led to the M1I amino acid substitution. Lastly, patient M174 – genotype E, showed an insertion of an adenine between nt1839 and nt1840. This insertion was confirmed by additional independent experiments and is depicted in Figure 4.14.



Pre-Core Mutations	Genotype E n=8	Sub-genotype D1 n=3	Unknown Genotype n=2
Stop codon G1896A (W28*)	4	2	2
G1899A (G29D)	1	1	0
G1816T (M1I)	0	1	0

Figure 4.13: Graphic representation of pre-C mutations on BioEdit V7.2.5 and their frequency in the study dataset. The amino acid sequence of this region is numbered from 1 to 29. PG119, PG272, PG246, PG196, PG127, PG62, PG22, M190, M179, and M66 represent our query pre-C sequences. The W28* mutant is shown for patients PG22, PG62, PG119, PG246, PG272, M66, M99, and M179; * representing a stop codon. G29D mutant is seen in patients PG272 and M179 too. The Pre-C initiation codon point mutation M1I is observed in M190 pre-C sequences. X represent ambiguities in the nucleic acid sequence. The dots show where amino acid similarities are observed between query sequences and the pre-C reference sequence. Unknown genotypes are samples whose pol/surf sequencing could not be completed due to low HBV viral load. M: mother; PG: pregnant woman.

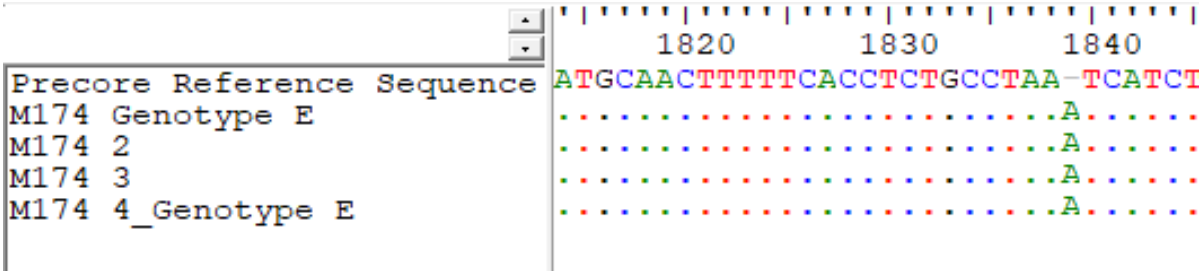


Figure 4.14: A snapshot of the multiple alignment of M174 and the pre-C reference nucleotide sequences. The nucleotide sequence of this region is numbered from 1814 to 1900. Cycle sequencing of the X/core gene for sample M174 was repeated to obtain more than one consensus sequence confirming the insertion of an adenine between nt 1839 and nt 1840. The dots represent similarities between the sequences. M: mother; nt: nucleotide.

Core promoter (CP) mutation analysis

Amino acid substitutions in the X gene, overlapping with the BCP region, were observed in eight sequences. The most prevalent aa substitution was at position 127; found in four samples namely: PG62, PG196, M99, and M179. These were a change of isoleucine to thymine (I127T), corresponding to a T to C nucleotide change at position 1753 in samples PG62, PG196, and M179. On the other hand, M99 had a substitution of isoleucine to serine (I127S), corresponding to the double A1752T/T1753C nucleic acid mutation. The double A1762T/G1764A (K130M/V131I) BCP mutant was detected in the viral sequences of three samples: M66, M99, and PG196. M99 and PG196 were also harboring the I127T and I127S changes in their viral sequences, respectively. The V131I (G1764A) amino acid change was found alone in the viral sequence of patient PG246, who also had the W28* stop codon and G29D (G1899A) pre-C mutations. The pre-C start codon mutation (M1I) noted in patient M190 was observed in the BCP region as C148F (G1816T), where the overlap between the BCP and the pre-C regions starts. This viral strain belonged to sub-genotype D1. Lastly, one genotype E showed an adenine (A) insertion between nucleotides 1765 and 1766. These genomic changes are graphically represented in Figure 4.15.

Changes in the URR were observed in seven samples. Sequences from these samples harbored pre-C mutants too, Figure 4.15. The most prevalent URR mutant observed was the adenine to valine at position 102 (A102V), translating to the C1678T amino acid substitution. The mutation was found in six samples. Three were genotype E, two sub-genotype D1, and one was of unknown genotype. A one bp deletion at position 1686 was found in the URR sequence from sample M190. This deletion is represented in Figure 4.16 along with the BCP nucleotide insertion. The distribution of these amino acid substitutions is listed in Table 4.10

Table 4.10: Distribution of CP mutations among HBV positive women

Sample ID	Genotype	Nucleotide (amino acid) substitutions
PG22	E	C1678T (A102V)
PG62	D1	C1678T (A102V), T1753C (I127T)
M66	Unknown	A1762T/G1764A (K130M/V131I)
M99	E	A1752T/T1753C(I127S), A1762T/G1764A (K130M/V131I)
PG196	E	A1762T/G1764A (K130M/V131I), T1753C (I127T)
PG119	E	C1678T (A102V)
M174	E	C1678T (A102V)
M179	D1	C1678T (A102V), T1753C (I127T)
M190	D1	G1816T (C148F), 1 bp deletion at position 1686
PG246	Unknown	C1678T (A102V), G1764A (V131I)
PG194	E	1 bp insertion between nucleotides 1765 and 1766

Bp: base pair; M: mother; ID: identificant; PG: pregnant woman.

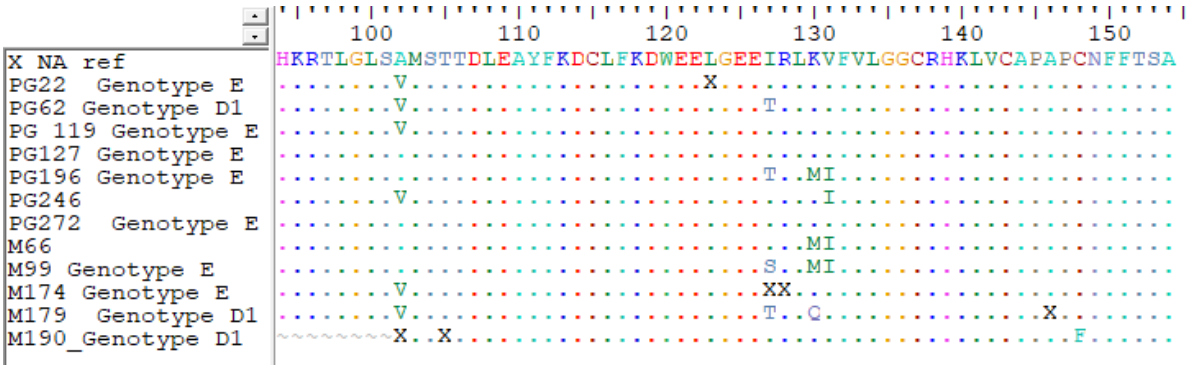


Figure 4.15: Multiple alignment of the query sequences and the HBV X gene reference represented on BioEdit V7.2.5. The amino acid sequence of this region is numbered from 94 to 154 (nt 1653 to nt 1835). PG119, PG272, PG246, PG196, PG127, PG62, PG22, M190, M179, and M66 represent our query BCP sequences. The A102V amino acid change is found in PG22, PG62, PG119, PG246, M174, and M179. Amino acid substitution at position 127 is seen for PG62, PG196, M179 (I127T), and M99 (I127S). PG196, M66, and M99 harbor the double K130M/V131I mutation.V131I only is found in PG246. M190 shows a C148F amino acid substitution. X represent ambiguities in the nucleic acid sequence. The dots show amino acid similarities between query sequences and the pre-C reference sequence. BCP: basal core promoter; M: mother; nt: nucleotide; PG: Pregnant woman.

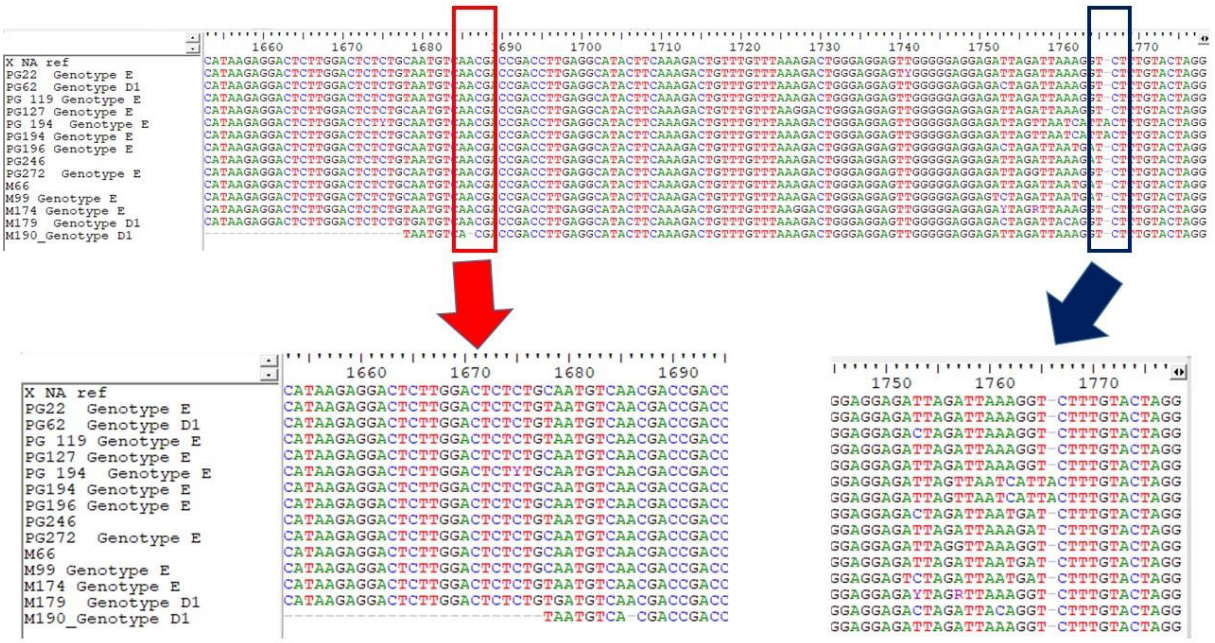


Figure 4.16: Multiple alignment of M190 and PG194 with the X gene reference nucleic acid sequences, from nt1653 to 1778. A gap/deletion is observed at position 1686 in the X gene sequence of patient M190 (in the red rectangle) while an adenine (A) is added between nucleotides 1765 and 1766 and in the X gene sequence of patient PG194 (in the blue rectangle). M: mother; PG: pregnant woman.

Core gene mutation analysis

Scrutiny of the rest of the core gene (nt 1901 to nt 2452) sequences revealed an 89 bp deletion in a genotype E viral strain. This deletion spanned from nt 2168 to nt 2257, corresponding to the deletion of 30 aa (aa 89 to aa 119) Figure 4.17.

Figure 4.17: A snapshot of the multiple alignment of PG194 and other HBV genotype E whole genome sequences from Namibia downloaded from Genbank. These whole genome sequences are numbered from 1 to 3215; the core region is spanning from nucleotide 1901 to 2452. The two repeats core gene sequences of PG194 show 89 bp deletions from nucleotide 2168 to nucleotide 2257. NC003977.21 represents the NCBI HBV reference sequence. Sequences starting with DQ are other HBV genotype E whole genome sequences downloaded in Genbank. Bp: base pairs; PG: pregnant woman.

Six samples harbored genetic variations in more than one region of the X/core gene. These samples are summarized in Table 4.11.

Table 4.11: Summary of samples with combined pre-C and BCP mutations

Patient ID	Genotype	HBeAg	Anti-HBe	pre-C mutants	BCP mutants
PG62	D1	-	+	G1896A (W28*)	T1753C (I127T)
M66	Unknown	-	+	G1896A (W28*)	A1762T/G1764A (K130M/V131I)
M99	E	-	+	G1896A (W28*)	A1752T/T1753C(I127S), A1762T/G1764A (K130M/V131I)
M179	D1	-	+	G1896A (W28*), G1899A (G29D)	T1753C (I127T)
M190	D1	-	+	G1816T (M1I)	G1816T (C148F)
PG246	Unknown	-	+	G1896A (W28*)	G1764A (V131I)

Unknown are samples whose pol/surface sequencing failed owing to low HBV DNA. Anti-HBe: antibodies against HBeAg; BCP: basal core promoter; HBeAg: hepatitis B e antigen; ID: identificant; M: mother; PG: Pregnant woman; pre-C: pre-core.

4.5.8 Liver health assessment results

AST, ALT, and platelet count measurements were successfully completed for all HBsAg positive pregnant women identified during the testing period (Table 4.12). Median liver transaminases were slightly higher in patients with HBV DNA viral load > 5 $\log_{10}$ IU/ml in comparison to those with a viral load < 3.6 $\log_{10}$ IU/ml in DBS. The median ALT in both groups was 16.5 IU/l compared to 13 IU/l, respectively. Median AST was 21.5 IU/l compared to 19 IU/l, respectively. On the other hand, these levels were comparable between patients with HBV DNA levels > 5 $\log_{10}$ IU/ml and those with undetectable or unquantifiable HBV DNA levels: median ALT between the two groups was 16.5 IU/l vs. 16 IU/l, respectively; and median AST was 21.5 IU/l vs. 20 IU/l, respectively.

An AST range of 15 to 41 IU/l is used as the AST reference range at the Namibia Institute of Pathology. The ULN AST value of 41 was used in the APRI score calculation. The APRI scores of this cohort did not suggest any evidence of liver cirrhosis.

Table 4.12: Liver transaminases, platelets and APRI results of HBsAg positive women

Patient ID	DBS HBV DNA log10 (IU/ml)*	Platelets ($x10^9$/L)	ALT (IU/l)	AST (IU/l)	APRI
PG16	LDL	181	59	71	0.957
PG22	LQL	192	11	16	0.203
PG39	LDL	276	9	17	0.15
PG62	LQL	249	14	20	0.196
PG74	LDL	353	20	19	0.131
PG87	LDL	177	28	48	0.661
M26	LDL	275	10	14	0.124
PG108	LDL	287	16	15	0.127
M66	LQL	218	13	19	0.213
M81	LDL	205	19	23	0.274
PG119	LQL	231	10	18	0.19
PG127	6.90	271	18	24	0.216
PG162	LDL	222	18	20	0.22
M99	3.53	240	13	23	0.234
M148	LDL	225	17	20	0.217
M151	LDL	243	16	19	0.191
M170	LDL	229	18	22	0.234
M174	3.11	116	13	14	0.294
PG194	7.41	209	15	19	0.222
PG196	3.48	212	16	23	0.265
PG225	LDL	308	13	21	0.166
M179	LQL	257	16	19	0.18
M180	LDL	220	14	20	0.222
M190	3.33	226	11	14	0.151
M244	LDL	275	16	21	0.186
M245	LDL	193	16	21	0.265
PG246	LQL	204	17	16	0.191
PG272	LQL	218	17	20	0.224

*DBS measurements. ALT: Alanine transaminase; APRI: AST platelet Ratio index; AST: Aspartate transaminase; ID: Identificant; IU/l: International units per liter; LDL: lower than the detection limit; LQL: lower than quantitation limit (3.92 $\log_{10}$ IU/ml – 8422 IU/ml); M: Mother; PG: pregnant woman.

4.5.9 Antiviral therapy evaluation results

Two HBsAg positive pregnant women (PG127 and PG194) presented with HBV DNA levels in DBS of 6.90 $\log_{10}$ IU/ml and 7.41 $\log_{10}$ IU/ml, respectively. They were reviewed for ART against HBV with TDF. PG127 was started on a 300 mg daily dose of TDF on October 27 2017, for three

months; she was 29 weeks and 03 days pregnant. Although patient PG194 was offered TDF, this was not provided to her. This woman was enrolled in the study on October 25, 2017; she was 29 weeks and 01 day pregnant. Following her HBV viral load result, she was reviewed on November 15 2017, to assess kidney functions, prior TDF initiation. Her gestational age was 32 weeks and 01 day and her estimated date of delivery was on January 01 2018.

4.5.10 Analysis of risk factors associated with HBsAg seropositivity

Results from the univariate logistic regression analyses are depicted in Table 4.13. Area of birth was statistically associated with HBsAg seropositivity. Pregnant women born in rural areas had 2.54 times greater odds of being HBsAg positive than those born in urban areas (COR: 2.541, 95% CI: 1.148 - 5.617, p = 0.021). None of the other sociodemographic variables included in this analysis showed a statistically significant association with an HBsAg positive serostatus. HIV infection (COR: 0.808, 95% CI: 0.237 - 2.753, p = 0.733) and syphilis infection (p = 0.99) were also not identified as statistically significant risk factors with an HBsAg seropositive status.

A total of 109 (21.2%) pregnant women had undergone surgical procedures, 75 women (75/515; 14.6%) had previously undergone caesarian sections, and 26 (5.0%) had a history of abortion. Twenty-eight women reported a history of blood transfusion (5.4%). About 29.5% (152/515) of these pregnant women reported being involved in unprotected sexual activities. Body tattoos and piercing were found in 71 (71/515; 13.8%) and 486 (486/515; 94.4%) pregnant women, respectively. None of these risk factors were associated with an HBsAg seropositive status.

Women with a history of liver disease (2/515; 0.4%) had 19 times greater odds of being HBsAg positive than those without a history of liver disease (COR: 19.783, 95% CI: 1.199 - 326.387, p = 0.037). The majority of the patients had no knowledge of any family member who had or is still suffering from HBV infection (343/515; 66.6%) or liver disease (235/515; 45.6%). Among the remainder, 26 (26/280; 9.3%) and eight (8/172; 4.7%) had a family history of liver disease or HBV infection, respectively. Pregnant women with a family history of liver disease were 3.67 times more likely (COR: 3.667, 95% CI: 1.090 - 12.331, p = 0.036) to be HBsAg positive as compared to those without, while no statistically significant association was found between HBsAg seropositivity and a family history of HBV infection (COR: 2.200, 95% CI: 0.246 - 19.672, p = 0.481).

Variables with a p-value ≤ 0.25 including the area of birth, abortion history, body tattooing, body piercing, patient's history of liver disease and a family member with a previous liver disease were selected for multivariate regression analysis. The analysis was performed, using the backward stepwise (likelihood ratio) model, to determine which of these variables were independently associated with HBsAg seropositivity. As represented in Table 4.13, the multivariate regression model showed that none of the variables, included in the model, was an independent risk factor for an HBsAg seropositive status.

Table 4.13: Association between studied risk factors and HBsAg positivity among pregnant women attending Intermediate Katutura hospital and Windhoek Central hospital ANCs

Variable	Total	HBsAg negative n (%)	HBsAg positive n (%)	COR (95% CI)	p value	AOR (95%CI)	p value
	515	487	28				
Age							
≤24	142	135 (27.7%)	7 (25.0%)	1 (reference)			
25-34	261	244 (50.1%)	17 (60.7%)	1.344 (0.544 - 3.321)	0.522		
≥35	112	108 (22.2%)	4 (14.3%)	0.714 (0.204 - 2.504)	0.599		
Gravidity							
Primigravida	117	111 (22.8%)	6 (21.4%)	1 (reference)			
Multigravida	398	376 (77.2%)	22 (78.6%)	1.082 (0.428 - 2.736)	0.867		
Parity							
Nulliparous	131	125 (25.7%)	6 (21.4%)	0.72 (0.27 - 1.93)	0.52		
Primiparous	159	151 (31.0%)	8 (28.6%)	0.80 (0.33 -1.95)	0.62		
Multiparous	225	211 (43.3%)	14 (50.0%)	1 (reference)			
Marital status							
Unmarried[1]	435	411 (84.4%)	24 (85.7%)	1.109 (0.374 - 3.288)	0.851		
Married	80	76 (15.6%)	4 (14.3%)	1 (reference)			
Area of birth							
Rural	220	202 (41.5%)	18 (64.3%)	2.540 (1.148 - 5.617)	0.021*	2.130 (0.941 - 4.819)	0.070
Urban	295	285 (58.5%)	10 (35.7%)	1 (reference)		1 (reference)	
Educational level							
Low[2]	415	394 (80.9%)	21 (75.0%)	0.708 (0.292 - 1.715)	0.445		
High[3]	100	93 (19.1%)	7 (25.0%)	1 (reference)			
Employment status[4]							
Employed	261	250 (51.3%)	11 (39.3%)	1 (reference)			
Student	55	51 (10.5%)	4 (14.3%)	1.783 (0.546-5.820)	0.338		

Unemployed	199	186 (38.2%)	13 (46.4%)	1.588 (0.696-3.625)	0.272		
HIV status							
Negative	449	424 (87.1%)	25 (89.3%)	1 (reference)			
Positive	66	63 (12.9%)	3 (10.7%)	0.808 (0.237 - 2.753)	0.733		
Syphilis status							
Negative	510	482 (99.0%)	28 (100.0%)	1 (reference)			
Positive	5	5 (1.0%)	0 (0%)	0.000	0.999		
Previous C-section							
No[5]	440	415 (85.2%)	25 (89.3%)	1 (reference)			
Yes	75	72 (14.8%)	3 (10.7%)	0.692 (0.204 - 2.351)	0.555		
Abortion history							
No	489	464 (95.3%)	25 (89.3%)	1 (reference)		1 (reference)	
Yes	26	23 (4.7%)	3 (10.7%)	2.421 (0.681- 8.609)	0.172	1.715 (0.427 - 7.892)	0.447
Blood transfusion history							
No	485	458 (94.1%)	27 (96.4%)	1 (reference)			
Yes	28	27 (5.5%)	1 (3.6%)	0.628 (0.082 - 4.800)	0.654		
Don't know	2	2 (0.4%)	0 (0%)	0.000	0.999		
Surgical history[6]							
No	405	382 (78.4%)	23 (82.1%)	1 (reference)			
Yes	109	104 (21.4%)	5 (17.9%)	0.798 (0.296 - 2.151)	0.656		
Unprotected sexual activity							
No	363	341 (70.0%)	22 (78.6%)	1 (reference)			
Yes	152	146 (30.0%)	6 (21.4%)	0.637 (0.253 - 1.604)	0.338		
Body tattooing							
No	444	417 (85.6%)	27 (96.4%)	1 (reference)		1 (reference)	
Yes	71	70 (14.4%)	1 (3.6%)	0.221 (0.030 - 1.650)	0.141	0.289 (0.038 - 2.215)	0.232

Body piercing							
No	29	26 (5.3%)	3 (10.7%)	1 (reference)		1 (reference)	
Yes	486	461 (94.7%)	25 (89.3%)	0.470 (0.133 - 1.659)	0.241	0.480 (0.132 - 1.741)	0.264
History of liver disease							
No	478	455 (93.4%)	23 (82.1%)	1 (reference)		1 (reference)	
Yes	2	1 (0.2%)	1 (3.6%)	19.783 (1.199 - 326.387)	0.037*	4.430 (0.198 - 98.932)	0.348
Don't know	35	31 (6.4%)	4 (14.3%)	2.553 (0.831 - 7.842)	0.102	2.632 (0.803 - 8.634)	0.110
Family member with previous HBV infection[9]							
No	164	154 (31.6%)	10 (35.7%)	1 (reference)			
Yes	8	7 (1.4%)	1 (3.6%)	2.200 (0.246 - 19.672)	0.481		
Don't know	343	326 (67.0%)	17 (60.7%)	0.803 (0.359 - 1.795)	0.593		
Family member with previous liver disease							
No	254	242 (49.7%)	12 (42.9%)	1 (reference)		1 (reference)	
Yes	26	22 (4.5%)	4 (14.3%)	3.667 (1.090 - 12.331)	0.036*	2.419 (0.610 - 9.592)	0.209
Don't know	235	223 (45.8%)	12 (42.9%)	1.085 (0.478 - 2.465)	0.845	0.905 (0.382 - 2.146)	0.821

[1]two divorced patients were counted as unmarried in the analysis and were HBsAg negative. [2]include eight patients with no formal education, 18 with primary and 389 with secondary level of education. [3]include all patients with a tertiary level of education, of whom 37 are enrolled in a tertiary level programme. [4] Include formal and non-formal employment. [5]include 126 Primigravida pregnant women. [6]Include patients with previous cesarean sections. AOR: adjusted odds ratio; CI: Confidence interval; COR: crude odds ratio. *p-value < 0.05 showing statistically significant association with HBsAg seropositivity.

4.5.11 Feasibility analysis of HBsAg rapid testing

Qualitative assessment

Six HCWs directly involved in the study, including four health assistants and two registered PMTCT nurses, completed a structured questionnaire that assessed their perceptions of the ease of use and user acceptability of the rapid test. Table 4.14 shows the HCWs responses to the questionnaire questions.

All HCWs (100%) had previously performed rapid testing, found the instructions of use of the kit very easy to understand, and none (0%) found any steps of the testing difficult to perform. Many HCWs commented about the "easy" and "simple" nature of the testing steps. One PMTCT nurse mentioned, *"Because it's the same steps that I am using when I perform the HIV rapid test"* as a reason for the ease of use of the rapid test. The majority (83.3%) found the rapid test very convenient to perform and were very confident that they had well performed the test. All (100%) trusted the results obtained from the rapid tests, suggesting that this is because the kits are unexpired, quality controls and confirmation procedures are in place for the rapid testing: *"Because all the test kits was not expired and we ran the controls every week and for positive results we collect venous blood for confirmation"* - Counsellor. A PMTCT nurse stated *"The test is instantly done and results are available after 15 minutes, hence there is no contamination of blood on devices"*, as another reason for the trust in the results of the rapid test. The HCWs (100%) also thought that it is useful for the patients to know their HBV result within 15 minutes and would support the use of the test at the ANCs for antenatal care. Reported reasons for these responses included that this method of testing is cheaper, quicker and more accessible, can facilitate early detection and treatment, and that the immediate nature of the results may reduce patient anxiety. A PMTCT nurse commented *"It is cheaper and reliable. In ± 15 minutes the client will know her result"*. A counselor stated, *"It is very much helpful to the mommies, for knowing their HB status for early detection"*.

These HCWs (100%) deemed that the test was acceptable to the patient, justified by the fact that the majority of patients approached at the ANCs consented to be tested and were happy and eager to receive their results after 15 minutes: *"Clients were happy to know their results in 15 minutes time"* – PMTCT counselor. However, the HCWs also indicated concerns about patients' reactions

to the test, with 33.3% and 66.7% of HCWs reporting that patients were very anxious or a little anxious, respectively, with regards to the rapid test.

Quantitative assessment – Diagnostic performance of the Determine HBsAg rapid test

The diagnostic accuracy of the Determine HBsAg rapid test was measured using HBsAg results obtained from the subset of patients (296) who were tested using both the Determine HBsAg rapid test and the ARCHITECT HBsAg CLIA test. As described in section 4.5.4.2, all HBsAg positive and negative samples re-tested with the laboratory-based CLIA HBsAg test matched HBsAg results attained with the rapid test. These results translated to a sensitivity of 100% and a specificity of 100%.

Table 4.14: Assessment of training needs, ease of use and user acceptability of the Determine HBsAg rapid test among HCWs at Intermediate Katutura hospital and Windhoek Central hospital ANCs.

Response	N (%)	Justification
1. Have you previously perform rapid test?		
Yes	6 (100)	-
No	0 (0)	
2. Did you find the instructions of use of the kit easy to understand?		
Very easy	6 (100)	-
Easy	0 (0)	
Little difficult	0 (0)	
Very difficult	0 (0)	
3. How convenient was it to perform the rapid test?		
Very convenient	5 (83.3)	-
Convenient	1 (16.7)	
Little inconvenient	0 (0)	
Inconvenient	0 (0)	
4. Which steps of the kit did you find difficult?		
Preparing the kit	0 (0)	*"Because I found no difficulties in doing the test and preparations of the kit."*
Taking the sample	0 (0)	*"All the way to use the kit it was easy."*
Adding the buffer	0 (0)	*"To me everything is simple and easy to perform."*
Reading the results	0 (0)	*"All the methods used in the kit were easy to perform."*
None	6 (100)	*"Because it's the same steps that I am using when I perform the HIV rapid test."*
6. How confident are you that you have done the HBV rapid test correctly?		
Very confident	5 (83.3)	-
Confident	1 (16.7)	
Little unconfident	0 (0)	
Very unconfident	0 (0)	

7. Do you trust the result of the HBV rapid test?		
Yes	6 (100)	*"Because all the test kits was not expired and we ran the controls every week and for positive results we collect venous blood for confirmation."*
No	0 (0)	*"Yes, I do because the HBV is done by drawing the Elisa. So far the results were the same"*
		"Because after 15 minutes, the results are clear and visible very well."
		"The test is instantly done and results are available after 15 minutes, hence there is no contamination of blood on devices."
		"Because it is according to the algorithm and also confirm by DBS, venous blood, (sALT, AST, HBsAg)"
9. How did the patient react to the HBV rapid test?		
Little anxious	4 (66.7)	-
Very anxious	2 (33.3)	
Not anxious	0 (0)	
10. Do you think that knowing the results in 15 minutes is useful for the patient?		
Yes	6 (100)	*"Very much useful because it is for early detection and for early treatment."*
No	0 (0)	*"The sooner the better for treatment and prevention."*
		"Because all the patient who tested for HBV just waiting for their result after they were tested."
		"Because in 15 minutes result is clear and you can see them well."
		"Yes, because it is cheaper and fast, even accessible."
		"Because the results can be confirmed within 15minutes, and to reduce anxiety of the client."
12. Would you recommend the use of this test at the clinic for antenatal care?		
Yes	6 (100)	*"It is very much helpful to the mommies, for knowing their HB status for early detection."*
No	0 (0)	

		"I wouldn't say much since the HBV tube is included in the process of ANC." *"Because to know your result on the same day is very understandable than waiting for few days."* *"Because it is fast and the patient is just getting her result in the same day."* *"It is cheaper and reliable. In ± 15 minutes the client will know her result."* *"Because there were many patients who were tested positive."*
14. Was the test acceptable by the patient?		
Yes	6 (100)	*"They had no problem of testing and as well as receiving their results."* *"Majority, although some didn't much agree."* *"Because most of the patients sign the consent forms."* *"Yes, because the kit is clearly showing the result just after 15 minutes."* *"Clients were happy to know their results in 15 minutes time."* *"Because most of the patients were eager to know their HBV results."*
No	0 (0)	
16. Any additional comments		
"Thank you so much for this study. It was really helpful to our nation and community."		
"HBV must be introduced in all facilities for the people to do it as a rapid test."		
"It must be introduced in all ante-natal care, so that the client can be evaluated."		

AST: Aspartate aminotransferase; DBS: Dried blood spots; HB: hepatitis B; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; sALT: Serum alanine aminotransferase

4.5.12 HBV-exposed babies testing

A total of 25 of 28 (89.3%) HBV-exposed babies were successfully traced and called back to the ANCs for HBsAg testing: 13 from Intermediate Katutura hospital ANC and 12 from Windhoek Central hospital ANC. Three babies, born to mothers M170, PG225 and PG108, were lost to follow-up. These mothers had traveled outside of Windhoek. The 25 babies were tested within the neonatal period 5 – 20 weeks after birth. Fourteen (14/25; 56%) were males, and eleven were females (44%); all were non-reactive for HBsAg. The characteristics of the tested babies are given in Table 4.15, below.

Table 4.15: Characteristics of HBV-exposed babies tested for HBsAg

Characteristics	Number, n/N (%)
Sample number	25/28 (89.3%)
Age at sample collection (weeks)	
Median	7
Range (min – max)	5.57 – 20.29
Sex	
Male	14/25 (56%)
Female	11/25 (44%)
HBV BD vaccine	
Yes*	25/25 (100%)
HBIG at birth	
Yes	15/25 (60%)
No[#]	4/25 (16%)
No record	6/25 (24%)
HBV vaccination at 06 weeks	
Yes	25/25 (100%)
No	0/25 (0%)
HIV status	
No	2/25 (8%)
Not Applicable	23/25 (92%)

*21 babies were vaccinated within 24 hours of birth, three babies were vaccinated two days postpartum, and one baby was vaccinated four days postpartum. [#]two babies did not receive HBIG due to a shortage of stock, and two babies were not considered HBV-exposed as the mothers were not recorded HBsAg positive at the labor wards. ANC: antenatal clinic; BD: birth dose; HBV: hepatitis B virus; HIBG: hepatitis B immunoglobulin; HIV: human immunodeficiency virus.

4.6 Summary of findings

A 5.4% HBsAg seroprevalence (28/515) was observed amongst 515 pregnant women tested at ANCs in Windhoek, Namibia. None of the predisposing risk factors considered in the study was independently associated with HBsAg seropositivity. Among these HBsAg positive women, two had DNA levels above 100 000 IU/ml (2/28; 7.1%) and were eligible for ART with TDF, as per the study cut-off for initiation of antiviral prophylaxis against HBV MTCT. These two women were also HBeAg positive, thus, were at high risk of infecting their newborns. HBV viral strains within the infected population predominantly grouped with genotype E virus (8/11; 72.7%), followed by sub-genotype D1 viruses (3/11; 27.3%). Postpartum, 25 babies born to these 28 HBV-infected pregnant women (25/28; 89.3%) were followed-up. All infants received a BD of the HBV

vaccine, and 15 (60%) were administered HBIG. None of these babies presented with acute HBV infection, including the babies born to the two pregnant women (PG127 and PG194) with HBV DNA levels above 100 000 IU/ml.

4.7 Interpretation of findings

4.7.1 HBsAg seroprevalence

In the present study, a cohort of 515 pregnant women was enrolled in Windhoek, Namibia. Among these women, an HBsAg seroprevalence of 5.4% (28/515) was found. This seroprevalence was lower than the national 7.3% seroprevalence that was previously reported amongst 11 390 pregnant women screened nationwide for HBV in Namibia during the year 2013 **(Mhata et al., 2017)**. According to the WHO classification of HBV endemicity **(Previsani & Lavanchy, 2002)**, the seroprevalence reported by Mhata et al. (2017) suggested an intermediate to high seroprevalence of HBV in the Namibian antenatal population.

The study by Mhata et al. (2017) also investigated the seroprevalence of the virus across provinces in Namibia. Among 6235 pregnant women tested for HBV in the Khomas province, in which Windhoek is situated, 350 (5.6%) had tested HBsAg reactive **(Mhata et al., 2017)**; a seroprevalence comparable to the 5.4% described within our cohort. The similarity in the rate of HBsAg seroprevalence observed in 2013 **(Mhata et al., 2017)** and in this study, 5.6% vs. 5.4%, respectively may indicate the existence of an HBV reservoir in the country. This similarity could be attributed to both (1) the historical trend of horizontal transmission of the virus during childhood, as is the case in most SSA countries, and to (2) residual MTCT. Differentiating between acute and chronic HBV infection within the adult population may be informative in terms of understanding the current dynamics of transmission of the virus within this population, as well as the general population. A higher rate of chronic infection, as compared to acute infection, may further strengthen the hypothesis of the presence of an HBV reservoir, while a higher prevalence of acute infections may reflect on current behaviors associated with continuous horizontal transmission of the virus within communities; bearing in mind that 5% to 10% of adulthood acquired infections progress to chronic infection in the presence of immune-suppression **(McMahon et al., 1985; Hyams, 1995)**. These HBsAg positive women were negative for anti-HBc IgM, a marker of recent infection or re-infection; which indicated that it is unlikely that these

women were newly infected by the virus. Noting that universal HBV vaccination (from six weeks of age) was introduced in Namibia in 2009, the majority of these women (median age of 30.5 years) were not vaccinated; suggesting that these infections may have been acquired during early childhood or infancy.

Furthermore, the seroprevalence of HBsAg among women enrolled appeared heterogeneous, depending on the province of birth. The majority of HBsAg seropositive pregnant women were born in the Omusati (7/28) and Ohangwena (7/28) provinces. In relation to the number of patients enrolled from these two regions, this translated to a 9.1% (7/77) and 10.9% (7/64) HBsAg seroprevalence, respectively. The seroprevalence observed in Ohangwena (10.9%) is comparable to the 10.1% described among 4096 pregnant women tested for HBV in that province, before **(Mhata et al., 2017)**. On the other hand, the rate seen among women from the Omusati region (9.1%) suggested an increasing trend of the prevalence of the infection, in comparison to the earlier report of 6.8% (48/705) in the same province in 2013 **(Mhata et al., 2017)**. These statistics are indicative of high endemicity of HBV in these two Namibian provinces. Omusati and Ohangwena provinces are situated in the northern part of the country, neighboring Angola; where HBV infections were previously recorded at a rate of 9.3% **(Guimarães Nebenzahl et al., 2013)** and 12.4% **(Schweitzer et al., 2015)**. The proximity between Angola and Northern Namibia facilitates easy migration between the two regions. The constant movements between the two countries may lead to a shared burden of HBV infections. Interestingly, the high seroprevalence of HBsAg noted among women born in Northern Namibia (9.1% in Omusati and 10.9% in Ohangwena) in the present study is in accordance with reports from earlier studies conducted in this part of the country. Botha et al. (1984) observed an HBsAg carrier state of 11% among mothers in Ovamboland **(Botha et al., 1984)**. Ovamboland regroups four provinces namely Omusati, Ohangwena, Oshana, and Oshikoto in Northern Namibia. In another province of Northern Namibia, Eastern Caprivi (now called Zambezi), Joubert et al. (1991) described a 10.7% prevalence among women within the general population **(Joubert et al., 1991)**. There is little known about the endemicity of the virus in other parts, and provinces of Namibia. Still, migrations between provinces in the country could have possibly led to a spill-over of HBV infection from the northern part of the country to the provinces situated inland. This is illustrated by the heterogeneity of patients enrolled in this study. The majority of enrolled women were born in the Khomas province (133/515; 25.8%) followed by the Omusati (77/515; 15%) and Ohangwena

(64/515; 12.4%) provinces. Yet, only one out of 133 patients (1/133; 0.75%) of Khomas origin was HBsAg positive compared to the 9.1% (7/77) and 10.9% (7/64) seroprevalence observed in women born in Omusati and Ohangwena, respectively. The heterogeneity of HBsAg prevalence seen between provinces is not unique to Namibia. Similar trends are seen elsewhere in SSA. A 4.5 % HBsAg seroprevalence was recently noted among 134 pregnant women in the Western Cape Province of South Africa **(Chotun et al., 2017)**, while a 0.8% (18/2368) seropositivity rate had been reported in the Tshwane district of Gauteng province, in South Africa still **(Diale et al., 2016)**.

To date, the literature counts only one study reporting on the prevalence of HBV infection among pregnant women in Namibia **(Mhata et al., 2017)**. The study was a retrospective analysis of a nationwide laboratory dataset of antenatal HBV results and only reported on the seroprevalence of HBsAg. Earlier studies had investigated women within the general population in Northern Namibia **(Botha et al., 1984; Joubert et al., 1991)**. The present study thereby provides updated statistics on the burden of HBV infection within the Namibian antenatal population. These data may be used for the planning of strategies against MTCT of HBV, and for the elimination of HBV in the country.

4.7.2 Seroprevalence of HIV infection

As per national policies, all pregnant women enrolled in our study were tested for HIV. A 12.8% (66/515) seroprevalence was found within the present cohort. This prevalence was slightly less than the pooled 16.6% (54/236) prevalence observed during the last 2016 biennial National HIV Sentinel Survey (NHSS) conducted in Windhoek **(MoHSS, 2016)**, based on sampling performed at Windhoek Central hospital and Intermediate Katutura hospital ANCs. Patients enrolled in the NHSS were tested for HIV using the Abbott ARCHITECT HIV Ag/Ab combo assay (Abbott Diagnostics, USA) to detect HIV-1/2 antibodies. Positive results acquired with the ARCHITECT HIV-1/2 assay were confirmed using the DXI 800 (Beckman-Coulter, USA), testing for the p24 antigen and HIV antibodies. Positive samples recorded by both assays were documented as positive for HIV. During the 2016 NHSS, 80 women were enrolled at Windhoek Central hospital ANC; five of whom were HIV positive (6.2%). At Intermediate Katutura hospital ANC, 246 women were enrolled, and 49 were found HIV positive (19.9%) **(MoHSS, 2016)**. The lower seropositive rate of HIV observed in our study as compared to the 2016 NHSS data (16.6% in 2016

compared to 12.8% in 2017) may represent a cohort effect; with fewer HIV-infected pregnant women attending ANC visits rather than a decrease in the HIV prevalence.

Considering the rate at each ANC, a 19.9% rate of infection was noted at Intermediate Katutura hospital during this 2016 NHSS compared to 17.9% in 2017, described in this study. At Windhoek Central hospital, a 6.2% prevalence rate was reported in 2016, while a 7% was noted in this study. The statistical difference (7% vs. 17.9%, $p < 0.001$) of the HIV positivity rate between the two ANCs observed in our study was statistically significant. This substantial difference appeared historical **(MoHSS, 2016)**, and may be an artifact of the sociodemographic of the patients attending these two health facilities. Patients attending Intermediate Katutura hospital ANC were found less educated and showed a lower employment status compared to those attending Windhoek Central hospital ANC. These two variables were statistically significant between the HIV-infected group and the HIV-uninfected group (level of education: $p = 0.001$ and employment status: $p < 0.001$), which puts forward the role of socioeconomic inequality and education in this disparity. The literature shows conflicting results with regards to the association between education and HIV in SSA. A higher prevalence of HIV amongst higher educated individuals has been previously noted in Ethiopia and Malawi; whilst lower educated individuals were the most afflicted by the epidemic in Lesotho, Kenya, and Zimbabwe **(Hargreaves et al., 2015)**. However, earlier data suggested a positive correlation between the low level of education and HIV infection **(Hargreaves et al., 2008)**. Similarly, while poverty is mostly assumed to be a driver of promiscuity, thereby predisposing individuals to HIV, a growing body of evidence has been challenging that connection. Higher HIV seropositivity rates have been described among wealthier countries in SSA, and wealthier individuals in SSA countries **(Fox, 2010; Hajizadeh et al., 2014)**. In the context of Namibia, earlier data showed a decreasing prevalence of the infection as educational attainment increased **(Gustafsson-Wright et al., 2011)**. The same data portrayed a higher rate of the infection amongst employed individuals compared to the unemployed ones: 11.1% (117/1053) vs. 7.8% (65/838), respectively **(Gustafsson-Wright et al., 2011)**. The last national demographic health survey, dating from 2014, also revealed that wealthier individuals were less likely to be HIV-infected **(MoHSS, 2014b)**. The positive correlation between HIV infection and a low level of education captured in our study is in agreement with data from Gustafsson-Wright et al. (2011) **(Gustafsson-Wright et al., 2011)**. However, unlike Gustafsson-Wright et al. (2011), a higher rate of HIV infection was noted among unemployed pregnant women

compared to employed pregnant women: 41/66 (62.1%) vs. 23/66 (34.8%) ($p<0.001$). This discrepancy may be due to the difference in the groups of population studied between the two studies. Anthropological investigations may be required for a better understanding of the societal determinants of HIV within the Namibian antenatal population.

4.7.3 Risk factors associated with HBV infection

The HBsAg seropositivity rate presented in this study was lower than the rates recorded in parts of West Africa, such as in Nigeria (14%) **(Musa et al., 2015)**, but higher than reports in Southern Africa (4.5% in South Africa **(Chotun et al., 2017)**. These epidemiological differences may be attributable to sociodemographic or social behaviors associated with the risk of acquiring the infection. A few known predisposing factors to HBV infection, in the categories of sociodemographic, nosocomial exposure and social behaviors, were analyzed as potential risk factors of HBV infection within this cohort of women.

4.7.3.1 Sociodemographic variables

HBsAg positive women and HBsAg negative women were compared with regards to sociodemographic characteristics such as age, marital status, area of birth (urban or rural), employment status, gravidity, parity and educational level using univariate logistic regression. Among these variables, area of birth only appeared to be statistically associated with being HBV infected (HBsAg positive). Pregnant women born in rural areas were more likely than those born in urban areas to be HBV infected (COR: 2.541, 95% CI: 1.148 - 5.617, $p = 0.021$). As mentioned in section 4.7.1, the majority of patients participating in this study were born in Northern Namibia and had the highest HBsAg seropositivity rates (10.9% in Ohangwena and 9.1% in Omusati); the region is predominantly rural. Earlier investigations of HBV in this region of the country had also noted a high prevalence of HBV among both males and females of the general population. Various factors including cultural practices, such as ritual scarification **(Joubert et al., 1991)** and tick vectors such as the hut tampan tick (*Ornithodoros moubata*) **(Joubert et al., 1985)** were hypothesized as possible routes of horizontal transmission of the virus within communities. Extrapolating from these studies, being born in a rural area could be a confounder variable rather than a risk factor of HBV infection within this population. This interpretation was confirmed in the multivariate logistic regression model, which showed that being born in a rural area was not

independently associated with a HBsAg seropositive status (Adjusted OR [AOR]: 2.103, 95% CI: 0.932 - 4.745, p =0.074). These observations prompt for further anthropological studies in Northern Namibia. These studies may be important to understand the reason why the prevalence of HBV remains high in this region in comparison to other parts of the country.

A high proportion of pregnant women within the age groups of 25 to 34 years old (17/28; 60.7%) were HBV-infected. However, a statistically significant difference in the distribution of HBV in different age groups ($p > 0.05$) was not observed. This finding is similar to recent data from a case-control study conducted in Namibia **(Mwaningange, 2018)**. The study investigated the risk factors associated with HBV infection among pregnant women in the Kunene province of the country. The author noted a statistically significant difference in the distribution of HBV in different age groups ($p = 0.02$) in the univariate logistic regression; but the observation was not confirmed with a multivariate logistic regression model **(Mwaningange, 2018)**. These results concur with findings from other African countries such as Tanzania **(Rashid, Kilewo & Aboud, 2014)**, Ethiopia **(Yohanes, Zerdo & Chufamo, 2016)**, and Cameroon **(Noubiap et al., 2015)**. In contrast, a study in Uganda noted a significant association between HBV infection and the age group below 20 years old. The authors hypothesized that this significant association might have been related to the high vulnerability of these young women to STIs **(Bayo et al., 2014)**. Similarly to our findings, the prevalence of HBV infection in the case-control study in Kunene was higher within the age group of 20 to 35 years old **(Mwaningange, 2018)**. The author attributed this observation to the fact that the majority of study participants were within that age group, as it was the case in our study group too. The population of pregnant women within this age group may also represent a subset of the reservoir of chronic HBV in Namibia. However, this hypothesis requires confirmation through further research.

With regards to marital status, no association was found between HBV infection (HBsAg positive) and not being married. Yet, a high proportion of unmarried pregnant women carried the virus. These results are also in accordance with findings from Uganda **(Bayo et al., 2014)**, Tanzania **(Rashid, Kilewo & Aboud, 2014)** and Cameroon **(Noubiap et al., 2015)**. In contrast, polygamous marriages appeared as a significant risk factor for HBV acquisition in the Kunene region **(Mwaningange, 2018)**; this significant association suggested that sexual transmission of the virus may be occurring among pregnant women in Kunene.

HBV infection was mostly detected among multigravida (22/28; 78.6%) and multiparous (14/28; 50%) women. Yet, there was no significant association between multiple pregnancies and HBsAg seropositivity. This observation was in agreement with a report from the Eastern region of Ghana **(Cho et al., 2012)**, and data from the Kunene region of Namibia too **(Mwaningange, 2018)**. Multiple pregnancies often indicate engagement in multiple unprotected sexual activities. Although these sexual activities may not necessarily take place with multiple sexual partners, the susceptibility to STIs including HBV infection would be increased. The apparent lack of significant association between these two variables may suggest the limited role of sexual transmission in the spread of HBV within this population. This hypothesis does not infer the complete absence of sexually transmitted HBV infections within this community.

The majority of HBsAg positive pregnant women were unemployed (13/28, 46.4%). Among them, many (11/13; 84.6%) were unmarried and had a primary (3/13; 23.1%) or secondary (9/13; 69.2%) level of education. These observations indicated a low socio-economic status within our cohort. Poverty has been previously associated with risky socio-behaviors such as having multiple sexual partners as a means for accessing basic needs **(Buot et al., 2014)**, and unprotected sexual activity is often seen among individuals with a low level of education **(Lagarde et al., 2001)**. The lack of a significant association between both unemployment and a low level of education observed in this cohort could mean that a low socio-economic status in Windhoek may not be a predisposing risk factor for acquiring HBV infection; as it has been observed in other parts of SSA **(Rabiu et al., 2010; Rashid, Kilewo & Aboud, 2014**; **Noubiap et al., 2015**; **Yohanes, Zerdo & Chufamo, 2016)**.

4.7.3.2 Risk of nosocomial HBV infection

Possible sources of HBV infection also include the nosocomial route through the instrumentation used during abortion and other surgical procedures such as caesarian section, or blood products obtained through blood transfusions. In this study, history of abortion, blood transfusion, caesarian section, and surgery were investigated as potential risk factors for HBsAg seropositivity. Surprisingly, these variables showed no significant association with HBsAg seropositivity. Although these findings are in agreement with studies conducted elsewhere **(Rabiu et al., 2010; Alegbeleye et al., 2013; Rashid, Kilewo & Aboud, 2014; Metaferia, Dessie & Amsalu, 2016)**,

other African studies have found opposing results with regards to abortion **(Ngaira et al., 2016; Yohanes, Zerdo & Chufamo, 2016)**, blood transfusions and surgery **(Zenebe et al., 2014)**.

The lack of association between HBsAg positive serostatus and blood transfusion noted in this study was also found in the Kunene region **(Mwaningange, 2018)**. These findings reflect the strength of national policy on the universal screening of all blood donations for transfusion-transmitted infections, including HBV infection, in Namibia **(Mavenyengwa et al., 2014)**. In contrast, abortion appeared to be a significant risk factor (AOR: 2.91; 95% CI: 1.38-6.16; p-value: 0.00) of HBV infection within the Kunene cohort **(Mwaningange, 2018)**. The absent correlation between a history of caesarian section or any other surgery and HBsAg positivity may indicate compliances with safe practices and the use of sterile equipment for patient's care at healthcare facilities in Namibia. Yet, dental procedures were significantly associated with HBV infection within the antenatal population studied in Kunene **(Mwaningange, 2018)**. The difference seen with regards to these three variables (history of abortion, caesarian section, and surgical procedures) between these two Namibian cohorts may be a reflection of different risk factors of the infection in different regions of the country.

4.7.3.3 Social behaviors and patient's clinical characteristics

The role of sexual transmission as a source of HBV amongst these pregnant women was further explored through data associated with risky social behaviors such as unprotected sexual activity, HIV infection, and syphilis. Unprotected sexual activity did not show any significant association with an HBsAg positive serostatus. Similarly, HIV and syphilis infections also showed no significant association with HBV infection (HBsAg positive). In contrast, a positive correlation has been previously observed between syphilis infection and HBsAg seropositivity **(Rabiu et al., 2010)** or HIV infection and HBsAg seropositivity **(Noubiap et al., 2015)** in Nigeria and Cameroon, respectively. The difference seen with regards to these two infections (HIV and syphilis) may be explained by the absence of HBV-syphilis co-infection, and the proportionate distribution of HIV infection among HBV-infected (10.71%; 3/28) and HBV-uninfected pregnant women (12.9% 63/424) recorded in this cohort. This observation further emphasizes the hypothesis regarding the minor role of sexual transmission as the source of HBV among these pregnant women (section 4.7.3.1).

The risk of acquiring blood transmissible infections such as HBV using unsterile needles for body tattooing or body piercing is also not negligible. In fact, both body tattooing and body piercing/scarification were found to be significant risk factors for HBV infection among pregnant women in Kunene **(Mwaningange, 2018)**. A similar observation was made in pregnant women studied in Bahir Dar city, Northwest Ethiopia **(Zenebe et al., 2014)**. Using multivariate logistic regression analysis, Zenebe et al. (2014) revealed that pregnant women with body tattoos were 5.7 times more likely to be HBsAg positive (Adjusted odds ratio = 5.7, 95% CI, 1.24-26.50) **(Zenebe et al., 2014)**. However, this observation was not confirmed in this study, suggesting once more a difference in the risk factors between regions of Namibia and outside of Namibia.

Given that horizontal transmission during childhood has been identified as the major mode of transmission of HBV in Namibia **(Botha et al., 1984)**, contact with family members infected with HBV was expected to statistically correlate with HBsAg seropositivity. On the contrary, it did not. Still, pregnant women in contact with a family member with HBV infection remained 2.2 times more likely to test HBsAg positive (COR: 2.200, 95% CI: 0.246 - 19.672, p = 0.481). HBV is well known as a silent killer. Patients present at a late stage of the disease with either cirrhosis or HCC and are diagnosed then with HBV-related liver disease. Thus, it is not unexpected that many of both HBsAg negative and HBsAg positive patients were unaware of their family history of HBV infection, or believed that the infection was not present within their family (10/28; 35.7%). Furthermore, having a family member with previous liver disease was significantly associated with HBV infection (COR: 3.667, 95% CI: 1.090 - 12.331, p = 0.036), as was a medical history of liver disease of the patient (COR: 19.783, 95% CI: 1.199 - 326.387, p = 0.037); when they were analyzed separately. However, in the multivariate analysis, there was no significant association between HBsAg seropositivity and these two variables. Where a history of liver disease within the family was reported, alcohol was pointed out as the cause of the disease. Two women (0.4%) reported previously suffering from liver disease; one of whom was HBsAg positive. Both women remembered being told of having a liver problem when they were younger but had no further details with regards to the cause of the health problem. The association found between these two factors (family member with previous liver disease and history of liver disease of the patient) and HBV infection in the univariate analysis may, therefore, be due to confounding. Although alcohol was predominantly cited as the cause of liver disease of family members, HBV infection is a known risk factor for liver disease. Drawing from the historical trend of horizontal transmission

of HBV in Namibia, contact with a family member with HBV-related liver disease would be a predisposing risk factor for HBV acquisition, which in turn would lead to live disease in the patient. Given that these two variables are important predisposing risk factors for HBV infection, further exploration is required to ascertain their roles in the risk of HBV infection within this population. It is also important to mention that the large 95% CI of the ORs attached to the likelihood of pregnant women with history of liver disease to be HBsAg positive was indicative of an imprecise measure of the association between this variable and HBV infection. The imprecision of this measure was due to the low number of HBsAg positive patients with a history of liver disease and re-emphasized the need for further research for a precise measure of the association between this variable and HBV infection.

The absence of a significant association between HBsAg seropositivity and the behavioral risk factors studied here further supports the possibility that these women may have acquired HBV infection in early childhood. It is noteworthy that 75% of these infected women reported the occasional use of physical contraceptive; thus, represented a risk of transmission of the virus to their partners. Although 90% of HBV infections acquired during adulthood resolve, a 10% risk of developing CHB remains if the patient is immunocompromised; as it is the case with HIV-infection. In fact, the high prevalence of HIV (11.5%) documented among the general population aged 15 to 49 years old, during the 2017 Namibia Population-Based HIV Impact Assessment (NAMPHIA) survey **(MoHSS, 2018)**, warrants HBV control within the Namibian adult population for the prevention of new cases of HBV infections within the HIV-infected population. Interventions such as education and catch-up vaccination programmes would be required for disease control within the adult population.

Overall, none of the risk factors considered in this study were independently associated with HBV infection within this population. However, they may well be significant risk factors in other parts of the country as was seen in the Province of Kunene **(Mwaningange, 2018)**. The differences observed between this cohort and the one from Kunene may also be an artifact of the study types. The present study was of cross-sectional nature. Studies of this nature are often applied to measure prevalence. Case-control studies, on the other hand, are predominantly conducted for the investigation of a possible relation between previous exposures and an outcome **(Stephenson & Babiker, 2000)**; this study type was applied in Kunene. Still, results from this cross-sectional study

may serve to direct case-control studies in Windhoek or the wider Khomas province, for future investigations of risk factors associated with HBV infection in this region. It is also important to bear in mind that association is not causation. From a sociological perspective, there is no certainty that individuals testing positive or negative for HBsAg are so because they are or aren't part of a larger pool of individuals exposed to certain factors **(Buot et al., 2014)**.

4.7.4 Risk of mother-to-child transmission of HBV

The risk of HBV MTCT can be assessed through two viral markers namely: HBeAg and levels of HBV DNA. The former suggests viral replication and the latter hints at the number of viral particles present in the HBV-infected patient. In the presence of HBeAg and high levels of HBV DNA, transmission of HBV from the mother to her newborn is high.

The presence of both HBV infectivity markers was investigated within the HBV-infected obstetrics population described in this study. Three women (3/28; 10.7%) tested HBeAg reactive. Thirteen women (13/28; 46.4%) had detectable levels of HBV DNA, based on DBS testing. Among these 13 HBV DNA positive patients, two (2/13; 15.4%) had a viral load of 6.90 $\log_{10}$ IU/ml (PG127) and 7.41 $\log_{10}$ IU/ml (PG194). PG127 and PG194 were HBeAg positive too and had an 87.5% risk of transmitting HBV to their newborns **(Edmunds et al., 1996)**. The other 11 patients with detectable levels of DNA were HBeAg negative, thus at a reduced risk of 13.2% **(Edmunds et al., 1996)**.

Vertical transmission in Namibia was explored more than three decades ago by Botha et al. (1984); the authors investigated HBV among mothers and their children in Ovamboland, northern Namibia. HBsAg was found in 11% (137/1272) of the tested mothers, and 15% (20/136) of these HBV-infected mothers were HBeAg positive. Among the HBeAg positive women, 12 (12/19; 63%) had HBsAg positive children, while 16/92 (17%) HBsAg positive/anti-HBe positive and 6/16 (38%) HBsAg positive/ anti-HBeAg negative women had HBsAg positive children **(Botha et al., 1984)**. Botha et al. (1984) reported horizontal transmission as the main mode of transmission of HBV during early childhood within the Namibian HBV epidemic. The authors also recognized the role of vertical transmission among HBV-infected children, and supported administering HBIG and HBV vaccination shortly after birth for children born to HBV-infected pregnant women,

provided that antenatal screening of HBsAg is in place to identify these infected mothers **(Botha et al., 1984)**.

4.7.4.1 Infant active and passive immunoprophylaxis

Unlike many SSA countries, WHO recommendations regarding HBV BD vaccination and routine antenatal HBV screening **(WHO, 2017b, d)** are currently part of Namibian policies **(MoHSS, 2011; MoHSS, 2014a)**. As per these policies, every pregnant woman presenting for her first ANC should be tested for HIV together with HBV. Where the woman tests positive for HBsAg, HBIG should be administered to her baby at birth. HBV BD vaccination at birth is given, as per the national EPI, irrespective of the maternal HBV status.

Immunization data were successfully retrieved from 25 out of the 28 babies (25/28; 89.3%) born to HBV-infected pregnant women identified in this study. The 25 babies received a BD of the HBV vaccine. Six babies (6/25; 24%) received it within 24 hours of birth, while the rest (19/25; 76%) were vaccinated within a day to four days postpartum. Babies vaccinated at one day old may have been born at night and immunized the next morning or afternoon. With regards to HBIG, 15/25 (60%) of these babies were passively immunized, while 4/25 (16%) were not: HBIG was out of stock in two cases, and two babies were not identified as HBV-exposed because the mothers were not recorded as HBV-infected. No record of HBIG administration in the baby's health booklet was noted in six other cases (6/25; 24%).

The discrepancy between the nationally recommended immunization timing and the immunization timing noted in this study portrayed the real-world situation observed in many resource-limited settings. Gaps between national recommendations and routine practices with regards to immunoprophylaxis in infants born to HBV positive mothers have been revealed elsewhere. A provincial population-based study in China reported that out of 298 HBV-exposed babies, only 37.6% (112) received HBIG, and 84.9% (253/298) received a timely BD vaccine **(Hu et al., 2012)**. Hu et al. (2012) raised the issue of insufficient knowledge concerning standard prophylaxis among HCWs **(Hu et al., 2012)**. Personal communication with the HCWs in our study revealed that many did not know the importance of administering HBV active and passive immunoprophylaxis within 24-hours of birth; which may also explain the late administration of the HBV BD vaccine in this study. Poor knowledge and awareness of HBV among HCWs has been raised before in Namibia **(Mhata, Small & Hunter, 2017)**. However, they are not unique to Namibia. A recent Ghanaian

study also revealed that 87.3% (110/126) of midwives and physicians were unaware of the availability of a vaccine that when combined with HBIG and administered to HBV-exposed babies could prevent HBV MTCT **(Adjei et al., 2016)**. The experienced unavailability of HBIG observed during the study period was not surprising, as HBIG is costly. Moreover, the two HBV-exposed babies missed for the administration of HBIG was apparently due to the oversight of the HCWs at the ANCs, and probably reflect the limited human resources seen in many resource-limited settings too.

4.7.4.2 Maternal antiviral prophylaxis

Two HBV-infected pregnant women (PG127 and PG194) were at high risk of transmitting HBV to their newborns, as per their positive HBeAg serostatus and high viral loads. Babies born to these two women received the BD of the HBV vaccine within 24 hours of birth. Delivery of HBIG was documented in one (PG194) of these two babies. Assuming that both babies received passive immunization, a 3% to 10% risk of MTCT remained due to maternal HBV viral load above 5.3 $\log_{10}$ IU/ml (200 000 IU/ml) **(Wiseman et al., 2009; Sellier et al., 2015)**. As per the AASLD recommendations **(Terrault et al., 2016, 2018)**, these two patients were reviewed for antiviral prophylaxis with a 300 mg oral daily dose of TDF, starting from the third trimester of pregnancy. While PG127 was successfully put on TDF treatment from 29 weeks of gestation, antiviral prophylaxis was offered but not provided to PG194. Again, this scenario reflected a real-world situation.

Failure to provide treatment to patient PG194 may be given many reasons. First is the lack of transport money from the patient to the clinic or hospital to collect the prescription or for the follow-up visit. As highlighted in section 4.7.3.1, many of these infected women were unemployed. Thus, money for taxi fares may be difficult to attain for some of them. Second, the issue of HCWs' knowledge and awareness is re-emphasized. Considering that the evidence around maternal antiviral prophylaxis to prevent HBV MTCT is relatively new, the knowledge of this prophylactic measure may also be low among HCWs; as recently raised in a Canadian study **(Van Ommen et al., 2017)**. Thus, if the nurses had knowledge of the high risk of HBV MTCT associated with a high HBV viral load for an HBsAg positive pregnant woman and had recognized the need for a referral to the doctor for treatment against HBV, treatment may had been provided to this patient (PG194). Third, the reference system in place may not be optimal for the care of HBsAg

pregnant women. Nurses are the first point of contact of all pregnant women in Windhoek, and refer patients to specialized care where the need is recognized. However, the need may not always be recognized or may be missed. Taking the case of Burkina Faso as an example, HBsAg positive women go through a number of primary caregivers, such as auxiliary midwives and midwives to get to a gastroenterologist for care. However, Guingané et al. (2014) observed that only 38% (45/119) of Burkinabe HCWs had referred HBV-infected pregnant women for care to the hepatologists **(Guingané et al., 2014)**. Fourth is the issue of behavior or attitude of HCWs towards patients. A number of negative attitudes and behaviors of maternal health care providers such as poor communication and verbal abuse has been documented **(Mannava et al., 2015)**. Although these behaviors may have not been common among HCWs at the study sites, patients may have been victims of these negative behaviors/attitudes at other health facilities. Exposure to these negative attitudes may have created the fear of speaking up to seek care.

The reasons outlined above should be addressed in future studies in resource-limited settings to ensure adequate care of HBV-infected pregnant women and for policy implementation. These observations also prove the need for empowering pregnant women to demand care where necessary, through education. Similarly, education of HCWs is critical to ensure adequate provision of interventions against HBV MTCT in SSA.

4.7.4.3 The rate of HBV MTCT

Although HBV MTCT is defined as the presence of HBsAg in infants born to HBV-infected mothers from six months of age, HBV infection was assessed in 25 out of the 28 (89.3%) HBV-exposed babies six weeks postpartum through HBsAg testing. All infants tested non-reactive, indicating the absence of acute perinatal HBV infection. These findings are in agreement with recent data from South Africa **(Chotun et al., 2017)**. The South African study described the rate of HBV MTCT among 134 pregnant women in the Western Cape Province, South Africa. HBsAg seropositivity was found in six women (6/134, 4.5%; 95% CI 0.99%–8.01%); of whom two had high HBV viral loads of 23 000 IU/ml (4.36 $\log_{10}$ IU/ml) and 767 000 IU/ml (5.88 $\log_{10}$ IU/ml) and were offered TDF at 36 and 28 weeks of pregnancy, respectively, till one month postpartum. Babies born to the six HBV-infected mothers were vaccinated at birth but did not receive HBIG. Four of these six babies were followed-up at a mean age of 97 days (range: 34–151 days) and at seven months of age. At both time points, the babies were negative for HBV, including the babies

born to the mothers with high HBV viral loads. These two follow-ups demonstrated the absence of both acute and persisting HBV infections in babies who were at relatively high risk of infection **(Chotun et al., 2017)**. Nine-month follow-ups of the HBV-exposed identified in this study are currently on-going and will determine the rate of chronic HBV infections amongst these babies.

4.7.5 Feasibility of implementing HBsAg rapid screening

The current national protocol for routine screening of HBV in pregnant women in Namibia includes testing for HBsAg at the laboratory **(MoHSS, 2011)**. However, recent data suggest low coverage of this policy in many parts of the country **(Mhata et al., 2017)**. This study described the feasibility of HBsAg screening at the POC level in Windhoek using RDT as an alternative for routine antenatal screening of HBsAg in Namibia.

4.7.5.1 Diagnostic performance of the HBsAg rapid testing

Results from this analysis showed that all 28 positive results attained by the Determine HBsAg rapid test at the ANCs were confirmed true positives at the laboratory. More than half of the negative results (55%; 268/487) recorded by the rapid test were re-tested and confirmed true negatives at the laboratory. Based on the number of samples (296 samples) tested at both the POC level and at the laboratory level, this data translates to a 100% sensitivity and a 100% specificity. This is the very first study in Namibia and the fourth in Africa reporting on the use of this rapid test at the POC level, operated by HCWs rather than laboratory technologists. In The Gambia, the test showed a diagnostic sensitivity and specificity of 88.5% and 100%, respectively during community-screening **(Njai et al., 2015)**. In South Africa, the rapid test was used for the screening of HIV-uninfected pregnant women and showed 100% sensitivity. Negative patients were not confirmed at the laboratory; this did not allow for measuring the diagnostic specificity of the test **(Chotun et al., 2017)**. A sensitivity of 87.9% and a specificity of 99.7% were observed among HIV-infected individuals tested in Zambia **(Chisenga et al., 2018)**.

The possibility that false negative results may have occurred during this study cannot be excluded. These were reported in The Gambia **(Njai et al., 2015)** and in Zambia **(Chisenga et al., 2018)**. Low levels of HBsAg (median: 1.2 IU/ml; range: 0.8-25.5 IU/ml) **(Njai et al., 2015)**, and low to undetectable viral load **(Chisenga et al., 2018)** were associated with the false negatives results described in these two studies. False negative results may also derive from poor compliance to the

recommended testing protocol. Inconsistencies in the use of capillary tube for finger-prick blood collection, the immediate application of the chase buffer to the sample pad of the test, and the recommended incubation period of the test were described as potential reasons for the low sensitivity of HIV rapid tests at the POC level in a previous South African study **(Wolpaw et al., 2010)**. A recent Zambian study also noted low accuracy levels among non-laboratory personnel such as lay counselors and nurses, during two HIV proficiency testing (PT) exercises using HIV rapid test as a possible cause of false RDT results **(Mwangala et al., 2016)**. Occurrences of false negative, false positive and indeterminate results were higher amongst the non-laboratory personnel during both PT exercises. The authors noted an improvement in the accuracy level between the two PT exercises among the non-laboratory testers; the improvement was positively associated with HIV rapid testing training and adherence to the nationally recommended testing algorithm **(Mwangala et al., 2016)**. The risk of obtaining false negative results from procedure errors was eliminated during this study by training the counselors prior to the commencement of the study and repeating a subset of the HBsAg negative rapid test results. Furthermore, HBV rapid testing at the ANCs was performed by HCWs and counselors who are experienced with HIV rapid testing using the Determine™ HIV-1/2 rapid test, and continuously go for HIV rapid test training as part of the PT exercises in place in Namibia. These data showed that where rapid testing is commonly used, training of the testers is vital for good accuracy level and reliability of the results communicated to the patients. Rapid testing training should be a continuous process rather than a once-off event.

4.7.5.2 Qualitative assessment of HBsAg rapid screening

Understanding the perspective and perception of HCWs towards the use of a new RDT, and the potential implementation of a new screening programme is also important to consider in the planning for an effective strategy for HBV screening. Using a structured questionnaire, this study also examined the opinion, ease of use and acceptability of the Determine™ HBsAg rapid test amongst the community counselors and nurses involved in the HIV PMTCT programme at the ANCs. Overall, these HCWs showed a positive response to the use of the rapid test. All felt confident in their use of the test and found similarities with the rapid test they use for HIV screening. The trust in regard to the results from the rapid tests was justified by the quality control programme and confirmation procedures that were established for the rapid testing during the

study. More importantly, they recognized the advantage of rapid testing for HBV over laboratory testing for they believed this method would be of low cost and would facilitate early detection and treatment of HBV for these pregnant women. These findings are similar to data from Zambia by Ansbro et al. (2015) **(Ansbro et al., 2015)**. The authors looked at the feasibility of antenatal syphilis rapid testing by comparing the experiences from HCWs during a pilot study and following roll-out of the programme in four national districts. Across the two phases, implementing RDT for syphilis was accepted and encouraged by the HCWs. Good usability of the rapid test was also noted **(Ansbro et al., 2015)**.

Although the positive response of HCWs towards HBV rapid testing noted in this study is encouraging, it may not be generalized to all PMTCT nurses or community counselors in Windhoek, or across the country, owing to the small number of HCWs and ANCs examined. The integration of this programme in the existing antenatal services also need attention. Looking at the distribution of tasks at the ANCs participating to this study, the counselor and PMTCT nurses were only involved in the HIV PMTCT programme. This programme entails health education/group pre-test counseling, rapid testing and post-test counseling, ART therapy prescription and follow-ups for HIV. Amongst other ANC services offered, the other ANC nurses were in charge of the collection of blood samples for the laboratory HBV test. Offering HBV RDT at the ANCs would lead to task shifting among the HCWs at the POC level. HBV PMTCT would be added to the HIV PMTCT programme that is currently being carried out by the PMTCT nurses and counselors at the ANCs. Adding HBV rapid testing may subsequently have an impact on the number of patients seen at the ANCs and lead to difficulties in integrating this service in the clinic's workflow. Integration of HBV RDT in the provided ANC services would, thus, require political buy-in, the employment of more HCWs, and constant training to ensure effective scale-up and provision of this antenatal service for the benefits of HBV PMTCT.

With regards to the end-user acceptability, four HCWs (4/6; 66.7%) reported a little anxiety, and two (2/6; 33.3%) noted high anxiety amongst patients towards receiving HBV rapid testing. Still, many patients were eager to receive their results and were happy to receive them after 15 minutes; as reported by the HCWs. For these reasons, added to the high rate of acceptance for participation in the study from the patients, the HCWs deemed this practice acceptable to the patient. Although the acceptance rate for HBV rapid testing was not quantified in this study, previous studies have

noted a high acceptability rate for this service at the POC level. A 93% rate of acceptance was reported in a recent antenatal South African study **(Chotun et al., 2017),** and a 68.9% acceptability rate was recorded in The Gambia among the general population **(Lemoine et al., 2016)**. Buy-in from the patients is essential in the success of a screening programme. Additional investigations of the attitudes and perceptions of pregnant women towards HBV RDT would be useful to drive implementation of routine HBV antenatal screening in Namibia. Education may also serve as a powerful tool to empower these women and to drive policy-change with regards to rapid test-based antenatal screening nationally. RDTs are required to fulfill a set of criteria, known as ASSURED, for the implementation of an effective screening programme. The acronym stands for Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable to end-users **(Peeling et al., 2006)**. The Determine HBsAg rapid test showed a diagnostic sensitivity and specificity of 100%. Its ability to differentiate HBV-infected pregnant women from those who aren't provided the opportunity to deliver adequate care to these pregnant women. The data collected from the HCWs informed on the ease of use of the rapid test and its delivery to the end-user, in this case, the pregnant women. No extra equipment was needed to perform the rapid test during the study, and the 15-minute incubation period recommended by the manufacturers coincided with the incubation of the HIV rapid test in use at the ANCs. These results are suggestive of the compliance of the Determine HBsAg rapid test with the ASSURED criteria; which indicates its candidacy as an RDT for HBsAg screening at the POC level in Namibia and across SSA.

4.7.6 Molecular characterization of HBV

4.7.6.1 HBV DNA quantification using DBS samples

The use of DBS as an alternative sampling approach offers the advantages of minimal infrastructure for storage and transport and stability at room temperature over blood sampling. This specimen type is particularly beneficial for pediatric testing, as it is currently done for neonatal HIV screening, and for scale-up of HBV diagnostic testing in remote areas with difficult access to centralized laboratories **(Peeling et al., 2017; Chevaliez & Pawlotsky, 2018)**.

DBS viral load testing was also assessed in this study, as a tool to determine the need for maternal ART for HBV PMTCT. Viral load quantification was performed using both DBS samples and serum samples of HBsAg positive pregnant women. Viral load measurements from antepartum

paired DBS and serum samples were compared. Of the 22 pairs available for analysis, six with undetectable HBV DNA in both serum and DBS were excluded. The mean viral load in the 18 DBS samples analyzed was approximately 1.05 $\log_{10}$ IU/ml (95% CI: 0.68 - 1.42 $\log_{10}$ IU/ml) lower compared to viral loads in their matching serum samples. Although this difference was statistically significant, the difference in measurements between the two samples types remained within the limit of agreements of the mean average (-0.40 to 2.50 $\log_{10}$ IU/ml). The R2 = 0.89 inferred from the linear regression analysis suggested a strong correlation between HBV DNA levels attained with the two paired sample types. The trendline was inferred using five out of the 10 data points included in the analysis, which suggested a linear relationship between measurements attained with 2 DBS and serum, with HBV DNA levels in serum above 10^3 IU/ml. However, a larger dataset is needed to draw firm conclusions.

The difference in HBV DNA levels observed between serum and DBS was not unexpected, considering the smaller input volumes of plasma in DBS (60 µl) in comparison to 650 µl used in plasma or serum testing. This phenomenon has been observed in other studies too, with the use of one spot **(Mohammed et al., 2013; Stene-Johansen et al., 2016)** or two spots **(Vinikoor et al., 2015)**. Aside from the low volume of plasma present in DBS samples, efficient elution of nucleic acid from the spots may also explain this difference in viral load and impacts the LoQ of the assay. Existing protocols suggest elution of DBS overnight at room temperature **(Grüner, Stambouli & Ross, 2015; Mössner et al., 2015; Claassen et al., unpublished)** or at high temperatures ranging from 56°C to 95°C for a duration of 10 minutes to 45 minutes **(Mohammed et al., 2013; Vinikoor et al., 2015; Stene-Johansen et al., 2016)** with continuous agitation. Through elution of one DBS with or without using high temperatures, the LoQ of the one DBS assay on the COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 has been estimated at around 914 IU/ml (2.96 $\log_{10}$ IU/ml) **(Mohamed et al., 2013)** and 843 IU/ml (2.93 $\log_{10}$ IU/ml) **(Claassen et al., unpublished)**, respectively. Yet, while Vinikoor et al. (2015) described an LoQ of about 3 $\log_{10}$ IU/ml (1000 IU/ml) using two DBS eluted at a high temperature, an increased LoQ of 3.85 $\log_{10}$ IU/ml (7110 IU/ml) was observed in this study, as determined by the Probit analysis (95% hit rate). The increased LoQ of the assay using two DBS observed in this study was not expected considering that heating and incubation at room temperature with one spot has produced a comparable LoQ **(Mohamed et al., 2013; Claassen et al., unpublished)**. Although the reduced analytical sensitivity (LoQ) of the assay did not impede on the ability to determine treatment

eligibility to avert HBV MTCT, the reasons behind this reduced sensitivity are worth further investigations. It should be noted that a low volume (500 µl) of the NIBSC 10/264 WHO standard was available, hence why the validation only included six replicates per concentration of the standard assessed. Given this relatively low number of replicates per concentration assessed, a 95% CI of the LoQ of the assay could not be calculated in the Probit analysis.

Translating to the clinical setting, these results showed that the two DBS assay could potentially assist in evaluating the need for antepartum antiviral prophylaxis against HBV MTCT at a viral load cut-off of 5.3 $\log_{10}$ IU/ml (200 000 IU/ml). With regards to non-pregnant women CHB patients, the assay also has the ability to assist in decision-making for treatment initiation using the WHO recommended threshold of 4.30 $\log_{10}$ IU/ml (20 000 IU/ml) **(WHO, 2015)**. This level of viremia was consistently detected and quantified with dilutions of the NIBSC 10/261 HBV DNA standard, as represented in Table 4.5 above. It is worth mentioning that the WHO viral load threshold (4.30 $\log_{10}$ IU/ml - 20 000 IU/ml) was also used in the South African study by Chotun et al. (2017) **(Chotun et al., 2017)** to initiate maternal antiviral treatment against HBV MTCT, given that babies born to highly viremic mothers had no access to HBIG. Should this HBV DNA threshold be potentially used in future studies or in real-world settings, this DBS assay may also be useful in assessing the need for antenatal antiviral prophylaxis against HBV MTCT in SSA. Given that levels of HBV DNA of 3.30 $\log_{10}$ IU/ml (2000 IU/ml) could not be accurately quantified using two DBS, this assay would not be suitable if using viral load of 3.30 $\log_{10}$ IU/ml - 2000 IU/ml as the threshold to determine HBV treatment eligibility in HBeAg-negative CHB patients, as per EASL guidelines **(EASL, 2017)**.

In view of these findings, it appears that HBV DNA quantification with one DBS is potentially a suitable alternative for serum or plasma HBV viral load testing. However, possibilities to increase the LoQ of DBS assays may be worth investigating. The utility of the COBAS plasma separation cards (Roche Diagnostics, Risch-Rotkreuz, Switzerland), recently launched for HIV diagnostic testing, may be explored as a substitute for HBV DBS testing to achieve lower LoQs.

4.7.6.2 HBV genotyping and phylogenetic analysis: effects on HBV MTCT

The HBV E viral strain was largely described in this study; representing 73% (8/11) of the strains identified in this HBV-infected antenatal cohort. Phylogenetic analysis showed close clustering of genotype E strains detected within this cohort with those isolated within the pediatric cohort

(Chapter 3), and other genotype E sequences described in Namibia and Angola. Further analysis of the evolutionary relationship between these E strains with those from West and Central Africa revealed the close proximity of the majority (12/14; 85.7%) of the study-specific sequences (six from the pediatric cohort and eight from this antenatal cohort) with isolates from Angola. While only two (2/14; 14.3%) appeared closely related to previous sequences from Namibia. The close grouping of E Namibian strains with Angolan strains may be interpreted as cross-contamination of the virus between Namibia and Angola. The virus may have been introduced within the Namibian population by Angolans moving in the country or by Namibians traveling between Angola and Namibia. Further work is needed to confirm this hypothesis.

Interestingly, genotype E sequences from Namibia, Angola and the DRC clustered separately from genotype E strains isolated in seven other African countries namely Cameroon, CAR, Cote d'Ivoire, Ghana, Guinea, Niger, and Nigeria; situated in West/Central Africa. These two clusters are subsequently referred to as the "South-West African lineage" and the "West African lineage"; respectively. A similar observation has been previously noted, when comparing partial and full-length genomic sequences of HBV genotype E viruses originating from the "genotype E crescent" **(Hübschen, Andernach & Muller, 2008; Lago et al., 2014)**. Hübschen et al. (2008) detected a higher genetic diversity among E sequences from the "West African lineage" than compared to those from the "South-West African lineage"; suggesting that this genotype may have emerged in West Africa and spread towards the Southern part of the genotype crescent **(Hübschen, Andernach & Muller, 2008)**.

As opposed to the pediatric cohort studied in Chapter 3, where sub-genotype D3 viral strains were found, sub-genotype D1 viruses instead were isolated in the present antenatal cohort. These sequences fell within a cluster of D1 sequences isolated from different parts of the world but were more closely related to those from Sudan and India. Despite the wide global distribution of genotype D, D1 viruses are predominantly found in the Middle East and in Central Asia **(Kramvis, 2014)**, as well as in the northern part of Africa **(Pourkarim et al., 2014)**. The D1 viruses found in this study demonstrated yet again the introduction of new genotypes in the region, possibly as a result of human migration. To our knowledge, this is the first description of these viral strains in Namibia. Increased migration in Africa may lead to the introduction of new genotypes from other parts of the region, creating a shift of the regional viral diversity. A clear example of this

phenomenon is the uncovering of genotype E in some European countries, introduced by immigrants originating from West Africa **(Palumbo et al., 2007; Rivas et al., 2013)**.

Similarly to the HBV-infected children described in chapter 3, pregnant women infected with genotype D presented with lower viral load and many were anti-HBe positive compared to women infected with genotype E. Bringing these observations to MTCT, a higher HBeAg seropositivity rate and viral load among pregnant women infected with genotype E may imply a higher risk of transmitting HBV to their infants compared to women infected with genotype D. This is further demonstrated by the recent systematic and meta-analysis by Keane et al. (2016). In their heterogeneity analysis, the authors observed a higher risk of HBV MTCT in West Africa, where genotype E predominates, as opposed to East Africa (66.4% vs. 21.2%; respectively) where genotypes A and D are found **(Keane et al., 2016)**. However, this hypothesis needs further investigation.

4.7.6.3 Surface gene mutations

The small surface L216* stop codon was detected in a genotype E infected pregnant woman. This protein change has been observed in the past amongst NRTIs treated and NRTIs naïve patients **(Margeridon-Thermet et al., 2009)**, caused by silent mutations in the overlapping RT region. Its clinical significance is unknown and requires further exploration. In addition to the L216* stop codon, a 15 bp deletion in the pre-S2 region spanning from aa 137 to aa 141 was detected in this genotype E virus. A similar deletion pattern has been observed among HCC patients infected with genotype E viral strain in Sudan **(Yousif et al., 2013)**. A positive correlation between the presence of pre-S deletions with HCC has been demonstrated amongst CHB patients infected with either genotype B or C in numerous Asian studies **(Chen et al., 2006b; Fang et al., 2008; Qu et al., 2014a; Zhang, 2017)**. The deletion found in this E viral strain infected patient spans part of the B cell epitope situated in the pre-S2 region (aa 120-145). Selection of this deletion may form part of the viral mechanism for immune evasion, thereby causing accumulation of pre-S2 defective viruses in the endoplasmic reticulum of type II ground glass hepatocytes **(Wang et al., 2003)**. Accumulation of these defective proteins in the endoplasmic reticulum is known to induce oxidative stress that subsequently activates a number of signaling pathways associated with the development of HCC **(Pollicino et al., 2014)**. The deletion also spans part of the transactivator domain in the pre-S2 region (aa 120 to aa 172) **(Chen et al., 2006b)**; which may also play a role

in hepatocarcinogenesis. While there is limited data on the clinical impact of pre-S deletions and mutations in HBV chronically infected patients in SSA, findings from Asian studies suggest that patients with viral polymorphisms in that genomic region may be at a higher risk of developing HCC. Additional studies are required in SSA to establish the prevalence, pathogenesis and clinical impact of these mutants among African chronic carriers of HBV.

4.7.6.4 X and core gene mutations

Pre-C mutations

The G1896A (W28*) stop codon was detected in 61.5% (8/13) of the antenatal HBV cases. All the pregnant women with pre-C sequences carrying this mutant were HBeAg negative; and its presence in conjunction with other core mutants may be a risk factor for hepatocarcinogenesis **(Jang et al., 2012; Lyu et al., 2013; Park et al., 2014)**. This hypothesis was further explored by Malik et al. (2016) among a spectrum of liver diseases including acute HBV infection, fulminant hepatitis, CHB, liver cirrhosis, and HCC. The authors noted a higher rate of the G1896A mutant among HCC patients (17/18; 94.4%) and those with fulminant hepatitis (18/21; 85.7%); demonstrating that this mutant may be a risk factor for the development of severe liver disease **(Malik et al., 2016)**. Furthermore, two isolates harboring the G1896A mutant were accompanied by the G1899A nucleic acid mutation, corresponding to the G29D aa change. The combined presence of these two variants did not appear genotype-specific. One sub-genotype D1 virus (M190) harbored the start codon mutation G1816T that induced the translation of the isoleucine instead of the methionine start codon (M1I). Pre-C start codon mutations have been predominantly described amongst HBV A1 infected patients **(Li et al., 1993; Makondo et al., 2012; Mayaphi et al., 2013)**, but rarely in those infected with non-A genotypes **(Sendi et al., 2005; Flichman et al., 2009)**; suggesting that its presence may be the main contributing factor to the HBeAg negative infection in patients infected with A1 viruses **(Mayaphi et al., 2013)**. In agreement with these studies, the presence of this mutant may explain the negative HBeAg serostatus of this pregnant patient (M190). Other than point mutations, a single nucleotide insertion was also detected in a genotype E HBeAg negative strain (M174). An adenine (A) was inserted between nucleotide 1839 and nucleotide 1840. This insert has been previously described in liver tissue samples of HBeAg negative chronic carriers of HBV **(Dienes et al., 1995)** and among HBeAg negative HCC patients as well **(Kramvis, Kew & Bukofzer, 1998)**. However, these two studies did not mention the

genotypes in which the insert was identified, nor discussed the potential clinical impact of this insert. The lack of information regarding this nucleotide insert may warrant additional investigations.

Core promoter (CP) mutations

The point URR mutation C1678T occurred in 46% (6/13) of the antenatal HBV cases described in this study. Another sample presented with one bp deletion in the CURS. The deletion abrogated translation of the aa threonine (T) at position 105. Given the scarcity of data on the pathogenesis of viral changes in the URR, the impact of this deletion on viral replication is unknown.

In the BCP region, the most known double A1762T/G1764A BCP mutation happened in 14% (3/13) of the HBV antenatal patients. Functional characterization of these viral mutants has demonstrated its association with enhanced viral replication **(Hussain et al., 2009; Khatun et al., 2018)**. In a number of instances, A1762T/G1764A has been observed at higher rates among HCC patients compared to chronic HBV carriers **(Baptista, Kramvis & Kew, 1999; Tong et al., 2007; Yang et al., 2016)**; suggesting that persistence of the mutant may play a role in liver damage. Interestingly, the T1753C mutant occurred as a single mutation amongst D1 viruses. In sequences from genotype E strains, the mutant was found in combination with other BCP mutation such as the double A1762T/G1764A BCP mutant in patient PG196, and both A1752T (A1752T/T1753C) and A1762T/G1764A BCP mutants in patient M99. BCP mutations were recently investigated across four clinical phases of HBV infection namely: inactive HBV carriers, active HBV carriers, liver, cirrhosis and HCC patients by a Saudi Arabian study. Seventy-nine percent (79%) of the patients included in the study were infected with genotype D. The authors found an increased frequency of the triple T1753C/A1762T/G1764A as the clinical phase of the infection progresses. The mutant appeared in 46.4% (13/28) of the HCC cases compared to 11.5% (3/26) in liver cirrhosis patients, 9.4% (23/245) of inactive HBV carriers and 8.8% (11/125) in active HBV carriers **(Al-Qahtani et al., 2017)**. Findings from Al-Qahtani et al. (2017) are suggestive of an increasing selection of BCP mutants as CHB progresses to HCC, and are similar to results from genotypes B and C Asian studies. Extrapolating from those studies, a similar trend may happen amongst genotype E infected patients. In addition to point mutations, insertion of an adenine (A) at nt 1766 happened in another E strain. Point mutations at this site are well described, and

deletions of a nucleotide at this position were recently reported **(Salarnia et al., 2017)**. Interestingly, this sequence also harbored an internal core deletion, as described below.

Core gene mutations

A single in-frame deletion of the HBV core protein was observed in one pregnant woman, infected with a genotype E viral strain. The deletion truncated the gene by 30 aas, spanning from aa 89 to aa 119. The HBV core gene contains a number of epitopes involved in the human immune response against HBV such as the CD4+ T cell, cytotoxic T lymphocyte (CTL) and B cell recognition epitopes, situated within the first 155 aa of the gene **(Vanlandschoot, Cao & Leroux-Roels, 2003)**. As described elsewhere **(Zhu et al., 2012; Xia, Zou & Liu, 2016)**, the truncated fragment of the core gene observed in this patient affected both the B cell epitope (aa 107 to aa 118), and the CTL epitope (aa 88 to aa 96) found in the sequence of the core gene. Previous data have shown that hepatocytolysis could be triggered through recognition of the CTL epitope by HLA-A11-restricted CD8+ T-cells **(Tsai et al., 1996)**. Where these epitopes are not expressed, viral immune evasion may occur, and lead to immune tolerance. Moreover, truncation of the B-cell epitope region may result in ineffective recognition of HBcAg by the B-cells, resulting in on-going infection. These findings suggested that deletions in the core gene may play a role in viral immune escape, and be a contributing factor to on-going chronic infection. Functional characterization of this deletion pattern is required to confirm these hypotheses.

4.8 Strengths and limitations of the study

This is the first study in Namibia looking at the effectiveness of preventing HBV MTCT. The large sample size of pregnant women available for HBV screening enabled generating updated statistics on the prevalence of HBV infection in the Namibian antenatal population and provided feasibility data for the implementation of rapid-test based screening for HBV in Namibia and possibly across the rest of SSA. This large research study was conducted at primary healthcare facilities and involved local HCWs rather than a dedicated research workforce. Involving the local HCWs enabled us to assess the feasibility of implementing rapid-test based HBsAg screening in the current health system, and to identify gaps related to HBV antenatal care in this health system. The study combined medical virology and epidemiology to answer the important public health problem that HBV MTCT is in SSA. And, through this project, interdisciplinary knowledge was generated.

The study also presents with a few limitations that could have influenced the interpretations of its findings. The use of HBsAg seropositivity, instead of anti-HBc (total) seropositivity, as case definition for HBV infection may have not been ideal for the accurate measure of risk factors associated with HBV infection in pregnant women. Using anti-HBc (total) seropositivity as case definition for HBV infection would have enabled us to take into consideration both current and resolved HBV infections in this analysis. However, given that resolved HBV infections do not impact on HBV MTCT, anti-HBc (total) testing was not performed for HBsAg negative pregnant women. Moreover, the small sample size of HCWs questioned for the qualitative assessment of rapid-test based HBsAg screening may not allow for the generalizability of these findings to other primary healthcare facilities across Namibia. The challenges associated with logistics are also highlighted. For instance, given that HBV viral load and HBeAg testing were not available in Namibia, samples needed to be sent to the Division of Medical Virology for testing. Due to the high costs associated with the shipping of biological samples, these samples were sent in batches and brought a delay in the turnaround time for the assessment of maternal antiviral treatment eligibility.

CHAPTER 5: A cost-effectiveness analysis of strategies for the prevention of mother-to-child transmission of HBV in Namibia

This chapter reports on the economic evaluation of preventive strategies against HBV MTCT in the context of Namibia. These strategies include combinations of different preventive measures such as screening, immunization, and maternal antiviral prophylaxis.

5.1. Introduction

Prevention of HBV MTCT in SSA may require a combination of various health interventions. The feasibility of these interventions would depend on the local endemicity of the infection, and more importantly on the availability of resources (costs) and the health outcomes associated with these interventions. Costs and health outcomes are examined through economic evaluations. These evaluations provide a framework for the allocation of scarce resources to health programmes. As explained in Chapter 2, economic evaluations compare the costs and health benefits of two or more health interventions, usually in terms of an incremental cost-effectiveness ratio (ICER). The ICER is considered as the price of health benefits gained through an intervention, and as such is compared to a threshold (the WTP threshold) to determine whether or not the intervention is cost-effective. A number of thresholds have been suggested and include the WHO-CHOICE-recommended one to three times country's GDP per capita **(WHO, 2002; Hutubessy, Chisholm &Edejer, 2003)**, and the World bank-recommended threshold of $150 to $200 per DALYs averted **(World Bank, 1993)**. ICERs below the WTP threshold are considered cost-effective, while those above the threshold are not **(Drummond et al., 2015)**.

There is limited knowledge on economic evaluations of interventions against HBV MTCT in SSA. Previous economic evaluations have mainly focused on the implementation of universal infant vaccination starting at birth in comparison to infant vaccination starting at six weeks after birth **(Griffiths, Hutton & Pascoal, 2005; Kim, Salomon & Goldie, 2007; Klingler, Thoumi & Mrithinjayam, 2012; Anderson et al., 2018; Hecht et al., 2018)**. These economic analyses are all in agreement regarding the low costs and substantial benefits of universal BD vaccination for preventing HBV perinatal transmission in SSA, as opposed to the current practice of vaccination from six weeks. Drawing from the Asian experience and results from a number of clinical trials in that region, BD vaccination alone may not be enough given the increase in effectiveness when it

is combined with HBIG **(Beasley et al., 1983b, Lee et al., 2006, 2007)**. The combination of these two immunoprophylactic measures, alongside routine antenatal screening, is the current standard of care in many high-income countries. This strategy has been shown to be cost-effective in those settings **(Chen et al., 2013c; Barbosa et al., 2014; Chen et al., 2016; Fan et al., 2014)**. In low-income countries, the issue of costs and cold chain storage limit access to both the BD vaccine and HBIG. As a high HBV endemic region with an HBsAg prevalence ≥ 6% **(WHO, 2017a)**, routine serological testing of HBsAg of pregnant women has been supported by the WHO **(WHO, 2017d)**. Yet, this practice is mainly offered in high-income countries, where the HBV endemicity is lower than in SSA. Screening of HBsAg of the general population was found cost-effective in The Gambia **(Nayagam et al., 2016a)**. However, the cost-effectiveness of this practice within the antenatal population in SSA is unknown. The costs and health outcomes associated with the implementation of BD vaccination combined with HBIG in SSA are also unknown. Babies born to mothers with high levels of HBV DNA are still at risk of acquiring HBV perinatal infection, despite timely administration of the HBV BD vaccine and HBIG **(Wiseman et al., 2009; Sellier et al., 2015)**. The addition of maternal antiviral prophylaxis to active and passive immunization to eliminate this residual risk of transmission has been studied, and has been considered cost-effective **(Hung & Chen, 2011; Fan et al., 2016; Lee, Shin & Park, 2018)**. The value of this strategy (both costs and health benefits) has also not been explored in SSA.

5.2. Study aims

5.2.1. Primary aim

The primary aim of this study was to assess the costs and effectiveness of rapid test-based HBsAg antenatal screening combined with maternal ART for HBV-infected pregnant women presenting with high HBV viral load, and pediatric active-passive immunization for the prevention of HBV MTCT in Namibia.

5.2.2. Secondary aim

The secondary aim of the study was to evaluate the costs and health benefits of universal BD vaccination, and the combination of BD vaccination and HBIG in the Namibian antenatal population.

5.2.3. Objectives

These study aims were attained through completion of the following objectives:

- Estimating the costs and outcomes of each strategy considered in this analysis,
- Estimating the ICER of these strategies,
- Interpreting the calculated ICERs for decision-making.

5.3. Materials and methodology

5.3.1. Target population and study setting

This economic evaluation was conducted alongside the antenatal study described in Chapter 4. Briefly, pregnant women attending for their first ANC visit were enrolled in the study at two public primary healthcare ANCs in Windhoek, Namibia. Antenatal care provided to pregnant women at their first ANC visit includes testing for HBV. Venipuncture is applied for sample collection. The HBV test is performed at the national laboratory with a diagnostic assay. HBV-exposed babies receive active immunization in the form of a vaccine, and passive immunization using HBIG at birth **(MoHSS, 2014a)**. Babies born to HBV negative mothers receive a BD of the HBV vaccine, only, as per the current childhood EPI schedule **(WHO, 2018)**. All medical expenses throughout this process are covered by the Namibian Ministry of Health and Social Services (MoHSS).

5.3.2. Comparator strategies

Based on the current health strategy against MTCT of HBV documented as a policy in Namibia, four health strategies were considered in this analysis. All interventions included timely BD vaccination. These strategies are summarized in Figure 5.1 and are detailed as follow:

Strategy 1: Universal HBV BD vaccination

All infants are vaccinated at birth against HBV. Vaccination is completed, regardless of maternal HBsAg status.

Strategy 2: Universal BD vaccination and targeted HBIG

Pregnant women attending their first ANC visit are screened for HBsAg, using the Determine^{TM} HBsAg rapid test. Infants born to HBsAg positive mothers receive HBIG and the HBV vaccine at birth. Infants born to HBV-uninfected pregnant women receive the HBV BD vaccine alone.

Strategy 3: Universal BD vaccination, HBV viral load testing, maternal antiviral prophylaxis and targeted HBIG

In this strategy, pregnant women receive antenatal screening for HBsAg via rapid testing at the ANC, and a blood sample is collected from the HBsAg positive patients for HBV viral load testing at the laboratory. The viral load test serves to assess both the risk of perinatal transmission and the need for maternal ART against HBV to prevent HBV MTCT. According to the 2016 AASLD guidelines on the management of chronic HBV infection **(Terrault et al., 2016)**, pregnant women with a viral load from 200 000 IU/ml are eligible for ART with a 300 mg daily dose of TDF, starting from the third trimester of pregnancy. Antiviral prophylaxis is, thus, initiated from the third trimester (26 – 28 weeks) of pregnancy to women presenting with HBV DNA levels ≥ 200 000 IU/ml at the ANCs. Treatment is stopped at one month postpartum; as previously described **(Celen et al., 2013; Chen et al., 2015; Pan et al., 2016)**. Pediatric post-exposure prophylaxis includes HBIG and BD vaccination. Babies born to HBV-uninfected pregnant women receive birth vaccination against HBV alone.

Strategy 4: Universal BD vaccination, HBeAg testing, maternal antiviral prophylaxis, and targeted HBIG

Following HBsAg screening with the Determine HBsAg rapid test at the clinic, a serum sample is collected from HBsAg positive pregnant women for HBeAg testing at the laboratory. HBeAg is used here as a surrogate marker to assess viral replication in the infected pregnant women. Those testing positive for HBeAg are offered ART with a 300 mg daily dose of TDF for four months; as per the schedule described in strategy 3. HBIG and HBV BD vaccination are administered to HBV-exposed babies, whilst BD vaccination alone is provided to HBV-unexposed babies.

The rapid test-based screening methodology, instead of laboratory testing for HBsAg, was used in the testing strategies because it was assumed that parts of Namibia and many settings in SSA would not be able to provide and sustain routine laboratory testing for HBsAg. These settings would be

able to implement HBsAg rapid testing through the infrastructures that are already in place for HIV rapid testing. Furthermore, the use of plasma samples, as the standard biological specimen for HBV viral load testing at the laboratory was included in this analysis.

Strategy 1 - Universal BD vaccination

- Prenatal HBV screen: No
- Maternal antiviral prophylaxis: No
- Universal birth dose vaccination

Strategy 2 – Universal BD vaccination + targeted HBIG

- Prenatal HBV screen: HBsAg rapid test
- Maternal antiviral prophylaxis: No
- Paediatric immunization
  - HBV-exposed babies: HBIG + BD vaccine
  - HBV-unexposed babies: birth vaccine only

Strategy 3 - Universal BD vaccination + HBV viral load testing + maternal antiviral prophylaxis + targeted HBIG

- Prenatal HBV screen: HBsAg rapid test + HBV viral load testing of HBsAg positive women
- Maternal antiviral therapy: Yes, indicated for high HBV viral load
- Paediatric imunization
  - HBV-exposed babies: HBIG + BD vaccine
  - HBV-unexposed babies: birth vaccine only

Strategy 4 - Universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis + targeted HBIG

- Prenatal HBV screen: HBsAg rapid test + HBeAg testing of HBsAg positive women
- Maternal antiviral therapy: Yes, indicated for HBeAg positive
- Paediatric imunization
  - HBV-exposed babies: HBIG + BD vaccine
  - HBV-unexposed babies: birth vaccine only

Figure 5.1: Study strategies for the prevention of mother-to-child transmission of HBV. BD: birth dose; HBIG: hepatitis B immunoglobulin; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; TDF: tenofovir disoproxil fumarate.

5.3.3. Perspective and time horizon

This CEA was performed from the perspective of the Namibian MoHSS, the healthcare service provider. Thus, only the direct medical costs involved in the study strategies were taken into consideration. The time horizon for the analysis was one year, during which all activities were performed and health outcomes measured.

5.3.4. Costing methodology

Costs were estimated based on information collected at both Windhoek Central hospital ANC and Intermediate Katutura hospital ANC for the financial year April 2016 to March 2017. The cost-analysis was completed using a mixed methodology consisting of micro-costing and gross-costing; as performed in previous economic evaluations **(Hendricks et al., 2014; Cunnama et al., 2016)**. Micro-costing, also referred to as ingredient costing, was applied to identify, measure and value all inputs (and their usage) used for providing HBsAg rapid testing at the POC level and maternal antiviral prophylaxis with TDF. Conversely, building space, furniture, and clinic overheads were computed using gross costing; whereby total expenditures were taken and allocated to a unit cost based on allocation factors **(Conteh & Walker, 2004)**. Resource usage was collected prospectively through observation and timing of processes during the recruitment period of the antenatal study (Chapter 4).

The costs of assessing infant HBV status postpartum were not taken into consideration, as this practice is unlikely to be incorporated as part of pediatric care for infants born to HBV-infected mothers. Intervention start-up costs related to rapid test training, the shipping of materials from Cape Town to Windhoek, and the design of materials for the rapid testing QA were excluded from this analysis. These start-up activities were performed during the antenatal study described in Chapter 4 and may vary according to national standards. The future costs of managing or treating the sequelae of CHB were also not taken into consideration.

Costs incurred in each study alternative were classified, depending on the duration of usage of the resources used in service delivery. Resources used up in less than a year are referred to as recurrent items, while those that last for more than a year are known as capital items. Capital and recurrent items involved in the activities and included in the considered strategies were identified, measured and valued. The various types of costs considered in this analysis are presented in Table 5.1.

Table 5.1: Classification of costs by activities performed in health interventions

Rapid-test based HBsAg screening	
A. Capital costs	B. Recurrent costs
Clinic - Space - Furniture	Consumables - HBV rapid test kit - Capillary tubes - Chase buffer - Finger prick consumables Clinic human resources - HBV pre-test and post-test counseling time (donated)* - Community counselor or PMTCT registered nurse time for the RDT (donated)* Overheads (utilities)
Laboratory HBV diagnostic assessment	
A. Capital costs	B. Recurrent costs
Clinic - Space - Furniture	Blood collection consumables HBV laboratory diagnostic tests: HBeAg, and HBV viral load Clinic human resources -Registered PMTCT nurse time – blood sample collection (donated)* Overheads (utilities)
Maternal antiviral prophylaxis	
A. Capital costs	B. Recurrent costs
Clinic - Space - Furniture	Laboratory tests: Creatinine, full blood count, ALT, and AST HBV treatment: TDF Clinic human resource - Consultation with the Medical Officer (donated)* Overheads (utilities)
Pediatric immunoprophylaxis	
A. Capital costs	B. Recurrent costs
NA	HBIG BD HBV vaccine

*The time donated by these HCWs was costed for the purpose of this economic evaluation. ALT: alanine transaminase; AST: aspartate transaminase; BD: birth dose; HBIG: hepatitis B immunoglobulin; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; NA: not applicable; PMTCT: prevention of mother-to-child transmission; RDT: rapid diagnostic testing; TDF: Tenofovir.

5.3.4.1. Measurement and valuation of capital costs

The capital resources used to perform screening of HBsAg using rapid testing, to collect blood for HBV laboratory confirmation (HBeAg and HBV viral load), and to provide maternal antiviral prophylaxis were identified at the healthcare facility level. These included the furniture and the space used in each of these activities at the ANCs.

Rapid testing was broken down in three sub-activities: pre-test counseling (also known as health education), rapid testing, and post-test counseling. Each of these sub-activities was performed in three different rooms. At both ANCs, the testing room was also used to collect blood samples for HBV laboratory testing. A consultation with a medical officer was required for the prescription of maternal antiviral prophylaxis. At Intermediate Katutura hospital ANC, the consultation with a medical officer took place in a separate room, while the post-test counseling room was also used for this additional activity at Windhoek Central hospital ANC. The length and width of these four rooms (pre-test counseling room, rapid testing room, post-test counseling room, and consultation room) were measured. The cost of each room was inferred by multiplying the area (m^2) of each space by the cost per m^2. The cost/m^2 was obtained from a construction company (WV Construction (Pty) Ltd, Windhoek, Namibia). The furniture in each room was noted down, and replacement prices were obtained from furniture companies, such as Waltons and Officeconomix, in Windhoek, Namibia. These capital costs were annuitized using a 7% discount rate per year, allowing the depreciation aspect and the opportunity cost aspects of these items to be incorporated into their costs. The annuitization discount rate (7%) represented the average interest rate between April 2016 and March 2017 in Namibia. Buildings were expected to have a life expectancy of 30 years and equipment 2 to 15 years. The life expectancy and the discount rate (7%) were used to determine the annuitization factor of each item, using the discount table by Drummond et al. (2015) **(Drummond et al., 2015)**. The formula of this calculation is presented in Box 5.1 below.

The unit cost of each activity, in terms of space and furniture, was estimated based on allocation factors. All first ANC patients were assumed to receive pre-test counseling. The annual number of first ANC visits was obtained from each ANC and was used for the allocation of pre-test counseling capital costs (both space and furniture). As an example, cost/pre-test counselling = (total costs of furniture + total costs of space) / total number of first ANC visits. Post-test counseling capital costs were also allocated based on the annual number of first ANCs at

Intermediate Katutura hospital ANC. The space and furniture in the post-test counseling room at Windhoek Central hospital ANC were shared between post-test counseling and for consultations with HBsAg pregnant women who required ART against HBV. Time was used as an allocation factor for these two activities at Windhoek Central hospital ANC. The total costs of furniture of this shared space were divided by the combined time spent on both activities, annually, to estimate a cost per minute of usage of the room. This cost was multiplied by the average time usage per each activity to calculate a cost per consultation and a cost per post-test counseling session. The same methodology was applied to allocate space costs of this room to these two activities. The two unit costs, in terms of furniture and space, were summed to obtain the capital cost per minute for the post-test counseling activity at the Windhoek Central ANC (See Box 5.1). Similarly, because the testing room was also shared for both HIV and HBV rapid testing and phlebotomy at both clinics, time was also used as an allocation factor. The allocation was performed following the methodology used to allocate the costs of the post-test counseling room at Windhoek Central hospital ANC, following the equations represented in Box 5.1. It is noteworthy that the annual time spent in the shared testing room at both clinics and the shared post-test counseling room at Windhoek Central hospital ANC would be different for the different strategies included in this economic evaluation. In strategies 1 and 2, the annual time usage of the testing room would be an addition of the total time to perform HIV and HBV rapid testing, annually. In strategies 3 and 4, this would be an addition of the total annual time spent on HIV and HBV rapid testing plus the time usage for collecting blood samples for HBV viral load or HBeAg laboratory tests. Similarly, the yearly time usage of the post-test counseling room at Windhoek Central hospital ANC in strategies 1 and 2 would only represent the annual time spent on post-test counseling for both HBV and HIV. In strategies 3 and 4, the time usage would be an addition of the annual time spent on post-test counseling for HBV and HIV with the annual time spent for consultation for maternal antiviral prophylaxis. These consultations took place in a dedicated room at the Intermediate Katutura hospital ANC. The consultation room at the Intermediate Katutura hospital ANC was also used for many other activities. Given that the details regarding the types and schedule of these other activities were unknown, the total number of minutes during which this room is used in a year was estimated. It equated to the annual number of minutes during which the clinic is open. The room was assumed to be used for six hours (360 minutes) of a day for 247.25 days, which represented the annual total number of working days (number of days in the year – number of

weekends – annual number of public holidays) – equating to 89 010 minutes in a year. The total costs of space and furniture of this area were divided by the annual time usage to infer a cost per minute for the usage of this area. This cost was multiplied by the average time of consultation for HBV ART to estimate the unit cost of this activity at the Intermediate Katutura hospital ANC (Refer to Box 5.1).

Box 5.1: Formulae for capital annuitization and capital costs allocations

A. Formulae for capital annuitization

Annuitization factor = $\frac{1-(1+r)^{-n}}{r}$. Where *n* is the useful life of the capital item, *r* is the interest rate (7%)

EAC = R / annuitization factor. Where, EAC is equivalent annual cost of each capital item; R is the replacement price of the capital item.

B. Formulae for allocating shared costs

(1) Post-test counselling room at Windhoek Central Hospital ANC

- $\text{Cost/minute}_{\text{room}} = \frac{\text{Total EACs of furniture + Total EACs of space}}{\text{Annual time post-test counselling + Annual time consultations}}$

- Cost/medical consultation = $\text{Cost/minute}_{\text{room}}$ X average time per consultation

- Cost/post-test counselling = $\text{Cost/minute}_{\text{room}}$ X average time per post-test counselling

(2) Rapid testing room at both ANCs

- $\text{Cost/minute}_{\text{room}} = \frac{\text{Total EACs of furniture + Total EACs of space}}{\text{Annual time HBV rapid test + Annual time HIV rapid test+Annual time phlebotomy}}$

- Cost/HBV rapid test = $\text{Cost/minute}_{\text{room}}$ X average time per rapid test

- Cost/Phlebotomy = $\text{Cost/minute}_{\text{room}}$ X average time per phlebotomy

(3) Consultation room at Intermediate Katutura ANC

$\text{Cost/minute}_{\text{consultation room}}$ = (total costs of space + total costs of furniture) / 89010. Where 89010 represents the total number of minutes during which the room was used in a year.

- Cost/medical consultation = $\text{Cost/minute}_{\text{consultation room}}$ X average time of consultation

5.3.4.2. Measurement and valuation of recurrent costs

Recurrent items included consumables and human resources involved in the activities of all strategies. The quantification of resource usage was documented through timing and observation of processes, in the context of the antenatal study.

HBsAg screening

Recurrent resource use related to HBsAg screening included the rapid test kits, the chase buffer, the capillary tubes, other miscellaneous disposable materials used for finger-prick, and the healthcare workers (HCWs) time.

The prices of the rapid test kits, the chase buffer, and the capillary tubes were obtained from Analytical Technology & Chemical Supply Pty (Ltd) (Windhoek, Namibia), the supplier of the Determine^{TM} HBsAg rapid test in Namibia. The prices of miscellaneous disposable items such as latex gloves, alcohol swabs, absorbent workspace covers, and biohazard waste-disposal bags were obtained from quotes supplied by biomedical and medical companies in Windhoek. With regards to personnel costs, the level of qualification and the type of HCWs performing counseling (pre- and post-test) for HBV rapid testing and the testing itself were noted. The type of HCWs involved in this activity included community counselors and the registered HIV PMTCT nurses at the ANCs. Hands-on time for each component of the HBV screening (pre-test counseling, testing, post-test counseling for HBV negative and HBV positive patients) was observed and recorded during the recruitment period of the antenatal study at the ANCs. Personnel costs were calculated based on (1) the average time estimates of each sub-activity of the rapid testing procedure, and (2) the average public sector annual wage in Namibia, per personnel type. The average time spent on HBV rapid testing was presumed the same at both clinics due to the lack of substantial time and motion data from Windhoek Central hospital ANC. The HCWs average annual wages were obtained from the health facilities where the antenatal study took place. The annual wages of each personnel type were divided by the yearly average patient-contact time (in minutes) to estimate the average hourly pay (or pay per minute) of each personnel type. These were multiplied by the average time spent on each component of the rapid testing procedure (pre-test counseling, testing, and post-test counseling). The costs of each component were added to estimate the cost of performing HBsAg rapid testing, for each personnel type involved in this activity.

Laboratory HBV confirmatory diagnostic testing

These costs were measured at both the ANC and laboratory levels. Recurrent items used at the ANC level included blood collection consumables such as phlebotomy equipment (needles, blood tubes), and the personnel time (the registered nurse collecting blood) for blood sample collection.

The time to collect blood from HBsAg positive patients at the clinic for HBV viral load or HBeAg test was assumed to take approximately 10 minutes. The duration of this activity also took into consideration the filling of forms and sample packaging. This time estimate was assumed owing to the lack of time and motion data. Time costs were calculated as described above for the HBsAg screening activity.

At the laboratory level, the prices charged by the Namibia Institute of Pathology for the HBeAg test and the HBV viral load test were used.

Maternal antiviral prophylaxis

Resources incurred here included (1) the HCW time for assessing eligibility for treatment with TDF, (2) the laboratory tests completed while initiating the eligible patient on TDF, and (3) the four-month course of the ART drug (TDF).

The laboratory tests performed during initiation of TDF were creatinine, full blood count, ALT, and AST. The creatinine test served to assess the kidney function, while full blood count, ALT, and AST tests were used as non-invasive markers to assess liver fibrosis. These prices were obtained from the Namibia Institute of Pathology.

The consultation with the medical officer, the HCW involved in this activity, was presumed to take an average time of 20 minutes. This average time was multiplied by the average public sector salary of a medical officer in Namibia.

The price of the four-month course of TDF (30 tablets for a month, each tablet is a 300 mg dose) was obtained by multiplying the unit cost of a month's prescription (public sector charges) by the total number of months (four) of the pregnant woman's treatment.

Pediatric immunoprophylaxis

The prices of a dose of HBIG and of the HBV BD vaccine were used for costing, based on the Namibian public sector charges. An ingredient costing of the HCW's time was not completed due to the lack of time and motion data. These data were not recorded given the absence of a researcher on site during deliveries of HBV-exposed babies. The HBV vaccination series is completed with three subsequent doses of the HBV vaccine at six, 10 and 14 weeks of the infants, given as a combined vaccine. These costs, common to all strategies, were not included in the analysis.

5.3.4.3. Measurement and valuation of overhead costs

Overheads included items such as electricity, water usage, cleaning, security, telephone usage, transport, waste disposal, repairs, and maintenance. These annual expenses were collected from each hospital. This costing was completed through a top-down approach whereby overall expenditures at a central level were allocated to each ANC patient. The total number of patient day equivalent outpatient (PDE-outpatient) visits at each hospital was used as the allocation factor. PDE-outpatient visits were calculated using the total number of outpatient visits and total inpatient days during the 2016/2017 financial year, following the formula presented in Box 5.2. The overhead items included in the analysis differed from each hospital but were based on actual spending incurred at each health facility. For each hospital, the costs of each overhead item were summed and divided by the total number of PDE-outpatients to obtain a cost per ANC visit (Refer to Box 5.2).

As mentioned above, all costs were inferred using the financial year 2016/2017 as the base year. Costs obtained during the year 2018 were deflated to 2017 using the 2016/2017 average consumer price index (CPI); by dividing the 2018 prices by the decimal value of the CPI. The 2016/2017 average CPI was calculated by adding CPIs of April 2016 through March 2017, and the sum was divided by 12. The decimal value of the CPI was inferred by dividing the nominal value by 100. These calculations are summarized in Box 5.2.

Costs were collected in Namibian Dollars (NAD) but are presented in US dollars (US$) using an average exchange rate of US$1= NAD14.05 (NAD1 = US$0.07), the mean exchange rate of April 2016 to March 2017 (http://fxtop.com/en/historical-exchange-rates.php, accessed on June 13,

2018). Total costs were discounted at a 0% rate as they were incurred in a one year period **(Drummond et al., 2015)**.

Box 5. 2: Formulae for the allocation of overhead costs and deflation of costs

A. Formulae for allocating overhead costs

- $PDE_{outpatients}$ = (annual inpatient days $\times$ 3) + (annual outpatient visits)

- Cost/ANC visit = $\frac{\sum Costs\ of\ overhead\ items}{PDE_{outpatients}}$

B. Formulae for deflating costs

Decimal $CPI_{2016/2017}$ = $\frac{\sum CPI_{APRIL2016,\ MARCH\ 2017}}{12}$/100

EC = $\frac{R}{CPI_{2016/2017}}$. Where EC is the deflated equivalent cost of the recurrent item; R is the replacement price of the recurrent item, and $CPI_{2016/2017}$ represents the decimal value of the CPI.

5.3.5. Heath outcome measures

The effectiveness measure used in this analysis was the number of perinatal HBV infections averted. This was defined as a negative HBsAg serostatus at 9 – 12 months of age in a baby born to an HBV-infected mother. Since the presence of anti-HBc from passive transfer of maternal antibodies usually persists in the infant for 12 months or more and the long-term complications of anti-HBc positivity in these children are negligible, this marker was not used as a criterion for perinatal HBV infection in the model. The long-term outcomes of perinatal HBV infections were not included. These outcomes were not discounted, given that they are measured in a one year period.

5.3.6. Model parameters

The literature available in the English language was searched in the Scopus and Medline databases to obtain HBV epidemiological parameters (Table 5.2) required to assess the effectiveness of each study strategy. The search combined the medical subject headings (Mesh) terms “Hepatitis B virus” AND “pregnant women”, “Namibia”, OR “Africa” in combination (AND) with each of the following words: “prevalence”, “vertical transmission”, “mother-to-child transmission”, “maternal

viral load", and "tenofovir". The bibliographies of the identified sources were also searched for additional articles.

5.3.6.1. HBV epidemiological parameters

The risk of perinatal infection is associated with HBeAg serostatus and high HBV viremia. Consequently, the prevalence of HBV infection among pregnant women was differentiated in three parts according to (1) the seroprevalence of HBsAg, (2) the prevalence of HBeAg, and (3) the prevalence of high viral load (≥ 200 000 IU/ml) among HBsAg positive women. The 200 000 IU/ml cut-off value for high HBV DNA levels was based on previous studies that have reported pediatric breakthrough infections in babies born to HBV-infected mothers presenting with viral load from this level of viremia **(Ngui et al., 1998; Wiseman et al., 2009; Zou et al., 2012; Wen et al., 2013; Foad et al., 2015; Sellier et al., 2015)**. This level of viremia is the cut-off value for maternal antiviral prophylaxis initiation in the AASLD guidelines **(Terrault et al., 2016)**.

A 7.3% prevalence of HBsAg among pregnant women was used as a base case value; it represented the seroprevalence of HBsAg observed in a previous retrospective Namibian study **(Mhata et al., 2017)**. The lower and upper values of the range of this variable were set at 5.44% (data from our antenatal study) and 10.10%, respectively. The upper value of this range represented the maximum prevalence of HBsAg reported in one of the 14 Namibian provinces **(Mhata et al., 2017)**. Prevalence data for HBeAg were estimated from an earlier study by Botha et al. (1984) and the antenatal study (Chapter 4). In the antenatal study, three HBsAg pregnant women, among 28, were HBeAg positive. One of these three women was co-infected with HIV. Given that this analysis focused on the HBV mono-infected antenatal population, an HBeAg seroprevalence of 7.14% (2/28) was considered. Botha et al. (1984) observed an HBeAg prevalence of 14.71% (20/136) among HBsAg positive women whose children were HBV-infected, in Ovamboland **(Botha et al., 1984)**. A pooled estimate of 13.73% from the two studies was calculated using a DerSimonian-Laird random-effects meta-analysis **(DerSimonian & Laird, 1986)**. The meta-analysis was completed with the MedCalc Statistical Software Version 18.9 **(MedCalc Software, Ostend, Belgium)**. The software uses a Freeman-Tukey transformation **(Freeman & Tukey, 1950)** to calculate weighted summary proportions. The lower rate of 7.14% and upper rate of 14.71% constituted the lower and upper bound of the range of this parameter.

Namibia-specific data for HBV viral load among HBV-infected pregnant were not available. Looking at other parts of SSA, HBV viral load above 200 000 IU/ml among HBV-infected pregnant women has been reported in Cameroon **(Ducancelle et al., 2013)**, Ghana **(Candotti, Danso & Allain, 2007)**, Senegal **(Seck et al., 2018)** and in South Africa **(Andersson et al., 2013; Chotun et al., 2017)**. Ducancelle et al. (2013) did not quantify HBV DNA levels in all HBsAg positive pregnant women. Viral load levels in the Andersson et al. (2013) study were reported in ranges and median. All pregnant women's samples used in the study by Seck et al. (2018) had a high viremia (≥ 200 000 IU/ml). A pooled proportion of HBV viral load among HBsAg positive pregnant women could not be estimated from these studies. In the Ghanaian **(Candotti, Danso & Allain, 2007)** and South African **(Chotun et al., 2017)** studies, all HBsAg positive women with an HBeAg seropositive status harbored HBV DNA levels above 200 000 IU/ml. Similarly, all HBV mono-infected HBeAg positive pregnant women within the antenatal study (Chapter 4) had HBV viral loads above 200 000 IU/ml. Therefore, HBeAg positive women in our analysis were assumed to carry HBV DNA levels ≥ 200 000 IU/ml. Consequently, the prevalence rates for these two variables, among HBsAg positive women were considered the same in the analysis.

5.3.6.2. Perinatal transmission rates

The rate of perinatal transmission of HBV varied according to the type of prophylaxis provided in the studied strategies: (1) timely BD vaccine, (2) timely BD vaccine + targeted HBIG, and (3) antiviral prophylaxis during pregnancy + timely BD vaccine + targeted HBIG. This rate was defined as the proportions of infants positive for HBsAg divided by the total number of babies/mothers adhering to the use of each prophylactic measure. Due to the limited availability of data on MTCT rates in SSA, these estimates were derived from studies performed elsewhere and were pooled if reported by more than one study.

BD vaccination

The rate of MTCT of HBV from HBeAg positive pregnant women was extracted from the following literature. Among sixteen babies immunized at birth, one month, and six months old with a plasma-derived vaccine, Halliday et al. (1992) observed breakthrough infections in eight (8/16; 50%) children **(Halliday et al., 1992)**. Similarly, Xu et al. (1995) observed persistence of HBsAg among 12 children (21.82%; 12/55) born to HBeAg positive mothers, despite birth

vaccination with a plasma-derived vaccine **(Xu et al., 1995)**. In Thailand, Lolekha et al. (2002) reported a 12.4% (12/97) rate of HBsAg chronic carriage in children vaccinated with a recombinant vaccine **(Lolekha et al., 2002)**. The pooled value of these proportions was estimated at 24.68% (95% CI: 10.17% - 43.00%) and was used as the base rate of transmission of HBV with BD vaccination from HBeAg positive mothers in the model.

With regards to HBeAg negative pregnant women, Xu et al. (1995) did not observe any HBV infections among vaccinated babies born to these mothers (0/56; 0%), using a plasma-derived vaccine **(Xu et al., 1995)**. Similar observations were made by Esteban et al. (0/30; 0%), Lee et al. (2016) (0/21; 0%), and Lu et al. (2017) (0/132; 0%) using a recombinant vaccine **(Esteban et al., 1986; Lee et al., 2016; Lu et al., 2017)**. On the other hand, Halliday et al. (1992) noted four infections among 33 children (12.12%; 4/33) **(Halliday et al., 1992)**. Chen et al. (2012) reported three HBV-infected children out of 1050 vaccinated children (0.29%; 3/1050) **(Chen et al., 2012)**. Results from the meta-analysis produced a pooled value of 1.02% (95% CI: 0.08% - 3.02%).

BD vaccination plus HBIG

Rates of MTCT ranging from 1.90% to 18% have been described in previous randomized controlled trials (RCTs) from HBeAg positive mothers, with the addition of HBIG to BD vaccination. The lowest rate (1.98%) was described by Hieu et al. (2002) among 101 immunized children **(Hieu et al., 2002)**. An earlier RCT by Xu et al. (1995) demonstrated a 6.25% (1/16) transmission rate in the study group who received 1 mL of HBIG (250 IU/mL) at birth plus plasma-derived vaccine at birth and at one month and six months of age **(Xu et al., 1995)**. Recent RCTs by Pan et al. (2016) and Jourdain et al. (2018) reported 6.82% (6/88) and 2.04% (3/147) MTCT rates, respectively **(Pan et al., 2016; Jourdain et al., 2018)**. Looking at other study types, two cases of perinatal transmission were described among 24 babies (2/24; 8.33%) born to HBeAg positive pregnant women with HBV DNA levels ≥ 200 000 IU/ml in Turkey **(Celen et al., 2013)**. Greenup et al. (2014) reported two cases of breakthrough pediatric infections in 10 (20%; 2/10) immunized babies born to HBeAg positive mothers with HBV DNA levels above 10^7 IU/ml **(Greenup et al., 2014)**. A 10.71% (6/56) MTCT rate was described from mothers with HBV viral load > $10^{7.5}$ IU/ml in Taiwan **(Chen et al., 2015)**. Results from these studies generated a pooled rate of transmission of 6.90% (95% CI: 3.86% - 10.73%).

HBeAg negative mothers presented with a lower risk of transmitting the virus to their babies with BD and HBIG. Xu et al. (1995), as well as Esteban et al. (1986), did not record any HBV infection events in babies born to HBeAg negative mothers **(Esteban et al., 1986; Xu et al., 1995)**. Chen et al. (2012) noted an HBsAg positivity rate of 0.14% (1/723) in children born to HBeAg negative mothers, despite receiving BD and HBIG at birth in Taiwan **(Chen et al., 2012)**. A similar rate (1/752; 0.13%) was observed in China **(Lu et al., 2017)**. In Singapore, perinatal transmission occurred in two out of 117 (1.71%) children born to HBeAg negative mothers despite receiving HBIG **(Lee et al., 2016)**. Pooling of these studies suggested a transmission rate of 0.34% (95% CI: 0.07% - 0.82%) from HBeAg negative mothers, following pediatric active and passive immunization.

BD vaccination, plus HBIG and maternal antiviral prophylaxis with TDF

The rate of transmission of HBV using this preventive strategy was extracted from five clinical trials performed on HBeAg positive pregnant women with HBV DNA levels ≥ 200 000 IU/ml. The non-randomized controlled trial (NRCT) by Celen et al. (2013) showed absence of HBV infection in children (0/21; 0%) born to HBeAg positive mothers who were treated with TDF during pregnancy, added to pediatric BD vaccination and HBIG **(Celen et al., 2013).** Findings from two RCTs corroborated those findings **(Pan et al., 2016; Jourdain et al., 2018)**. Two other NRCTs described a 2.27% (1/44) and 1.54% (1/65) residual rate of transmission **(Greenup et al., 2014; Chen et al., 2015)**. Pooling these data together, a residual rate of transmission of 0.73% (95% CI: 0.10% - 1.93%) was estimated.

5.3.6.3. Diagnostic accuracy of the HBsAg screening tool

The diagnostic sensitivity and specificity of the Alere Determine HBsAg rapid test were extracted from the latest systematic review and meta-analysis of the diagnostic accuracy of HBsAg RDTs **(Amini et al., 2017)**. Based on 10 studies, the pooled sensitivity and specificity of the Determine HBsAg rapid test was estimated at 90.80% (95% CI: 88.90% - 92.40%), and 99.10% (95% CI: 98.90% - 99.40%), respectively. A summary of these estimates is found in Table 5.2.

Table 5.2: Estimates of HBV prevalence in pregnant women and rates of maternal transmission

Variables	Base value	Range values	Further description	Data sources
Epidemiological assumptions				
Prevalence of HBsAg	7.30%	5.44% - 10.10%	The lower bound is the prevalence described in Chapter 4, and the higher bound represents the highest prevalence described in ref [1].	[1]
Prevalence of HBeAg/high VL[$]	13.73%	7.14% - 14.71%	The lowest value of the range is the prevalence described in Chapter 4, and the highest value was extracted from ref [2].	[2]
Rate of MTCT with TBD				
HBeAg negative women[$]	1.02%	0.08% - 3.02%	Range represents the 95% CI	[3 – 8]
HBeAg positive women[$]	24.68%	10.17% - 43.00%	Range represents the 95% CI	[4, 5, 9]
Rate of MTCT with TBD and HBIG				
HBeAg negative women[$]	0.34%	0.07% - 0.82%	Range represents the 95% CI	[3, 5 – 8]
HBeAg positive women[$]	6.90%	3.86% - 10.73%	Range represents the 95% CI	[5, 6, 10 – 15]
Rate of MTCT with maternal TDF treatment, TBD and HBIG				
HBeAg positive women[$]	0.73%	0.10% - 1.93%	Range represents the 95% CI	[11 - 15]
HBsAg rapid test performance				
Diagnostic sensitivity	90.80%	88.90% - 92.40%	A full diagnostic assessment including HBV viral load or HBeAg would be performed for patients with false positive patients. Range values represent 95% CI.	[16]
Diagnostic specificity	99.10%	98.90% - 99.40%	Patients with a false HBsAg negative results would not be detected at the screening stage. Range values represent 95% CI.	[16]

[$]The analysis considered that HBeAg negative women would have a viral load < 200 000 IU/ml and HBeAg positive women would present with a viral load ≥ 200 000 IU/ml; *Maternal ART with TDF is given from 3rd trimester of pregnancy to 4 weeks postpartum. **[1] Mhata et al., 2017; [2] Botha et al., 1984; [3] Esteban et al., 1986; [4] Halliday et al., 1992; [5] Xu et al., 1995; [6] Chen et al., 2012; [7] Lee et al., 2016; [8] Lu et al., 2017; [9] Lolekha et al., 2002; [10] Hieu et al., 2002; [11] Celen et al., 2013; [12] Greenup et al., 2014; [13] Chen et al., 2015; [14] Pan et al., 2016; [15] Jourdain et al., 2018; [16] Amini et al., 2017**. CI: confidence interval; HBIG: hepatitis B immunoglobulin; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; MTCT: mother-to-child transmission; TBD: timely birth dose; TDF: Tenofovir disoproxil fumarate.

5.3.7. Decision analytic model

A decision tree model was built using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA) to evaluate the costs and health outcomes of each study strategy, using a hypothetical cohort of 10 000 women. Identified as the appropriate modeling approach for this evaluation, decision trees are the simplest forms of analytical modeling in economic evaluations **(Karnon & Brown, 1998; Petrou & Gray, 2011)**. Figures 5.2, 5.3 and 5.4 show the branches of the decision tree for all strategies. Each node of the tree represents an epidemiological circumstance of HBV, including the prevalence of HBsAg among pregnant women, the prevalence of HBeAg positive mothers with a viral load above 200 000 IU/ml among HBsAg positive pregnant women, and the rates of transmission with each prophylaxis measure. The alternatives branching out from each node are mutually exclusive, and their probabilities add up to one. The endpoints of each branch represent an event that reflects the outcome measure of the evaluation; in this instance a pediatric HBV infection. The costs incurred through each pathway are summed.

Briefly, women enter the decision tree model with a probability of being HBV-infected given by the HBsAg prevalence of 7.3%. In strategy 1, 13.73% of the HBsAg positive women are positive for HBeAg, and the remaining women are HBeAg negative. All babies receive the BD vaccine and are at a 24.68% or 1.02 % risk of acquiring HBV perinatal infection if born to an HBeAg positive or HBeAg negative mother, respectively. In strategies 2, 3 and 4, women are tested for HBsAg using a rapid test, and the results are either positive or negative for HBsAg. Due to the diagnostic sensitivity (90.80%) and specificity (99.10%) of the rapid test used for HBsAg screening, some disease-free women test reactive for HBsAg (false positives), and some infected women test negative for HBsAg (false negatives).

Given that false HBsAg negative results produced by the Determine HBsAg rapid test have been attributed to low HBsAg levels and low viral DNA levels **(Njai et al., 2015; Chisenga et al., 2018)**, the model takes in consideration that the full proportion of HBeAg positive pregnant women is found among true HBsAg positive patients (N x prevalence x RDT diagnostic sensitivity). As such, a new proportion of HBeAg positive women/women with a viral load above 200 000 IU/ml is recalculated at 15.12% in the model, based on a 90.80% sensitivity of the rapid test. This re-calculation is applied to strategies 2, 3, and 4. Consequently, as the sensitivity of the rapid test changes, this proportion is automatically re-calculated in the model. In the base analysis, 15.12% of the true HBsAg positive patients are HBeAg positive, and the remainder are HBeAg negative. Following active and passive immunization in strategy 2, the

residual rate of transmission remains at 6.90% and 0.34% for babies born to an HBeAg positive or HBeAg negative mother, respectively. In strategies 3 and 4, the rate of perinatal transmission of HBV from HBeAg positive mothers is reduced to 0.73%, whilst the rate of transmission from HBeAg negative mothers remains 0.34%.

False HBsAg negative mothers are all considered negative for HBeAg, given the low levels of viremia reported among false HBsAg negative patients **(Chisenga et al., 2018)**. These women are not detected as actual carriers of the virus. Babies born to these mothers are only vaccinated at birth, and at a 1.02% risk of acquiring HBV infection from their HBeAg negative mothers. False HBsAg positive women are treated at the clinic as HBsAg positive patients; both BD vaccination and HBIG are administered to their babies. These babies are not at risk of acquiring HBV infection through MTCT.

MTCT is considered the only route of acquisition of HBV infection among infants born to the women included in the analysis, and complete compliance with routine HBsAg antenatal screening is assumed in the model. The model also presumes that all pregnant women eligible for antiviral prophylaxis would not refuse treatment with TDF and would adhere to the full course of the treatment. Full compliance with the Namibian immunization policy was also assumed in the model. That is all babies will be vaccinated at birth, and HBIG will be administered to HBV-exposed babies in strategies 2, 3, and 4. Subsequent doses of the HBV vaccine follow at 6, 10 and 14 weeks of the infants, this was not included in this analysis.

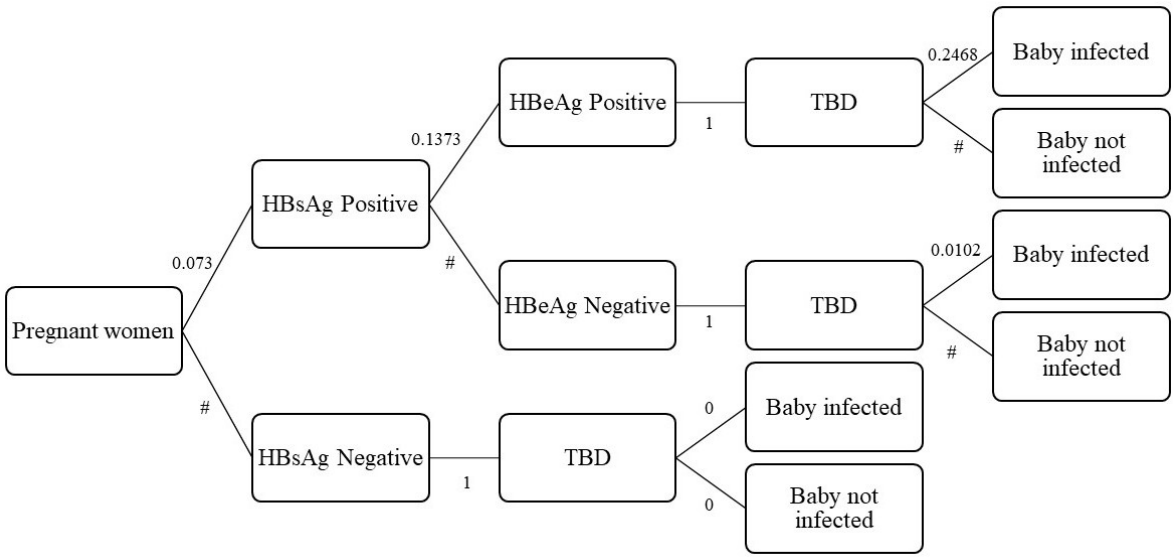


Figure 5.2: Decision tree structure for the universal HBV vaccination strategy (Strategy 1). HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; TBD: timely birth dose. # refers to the remaining number of patients found in the next branch of the tree, branching out of a node.

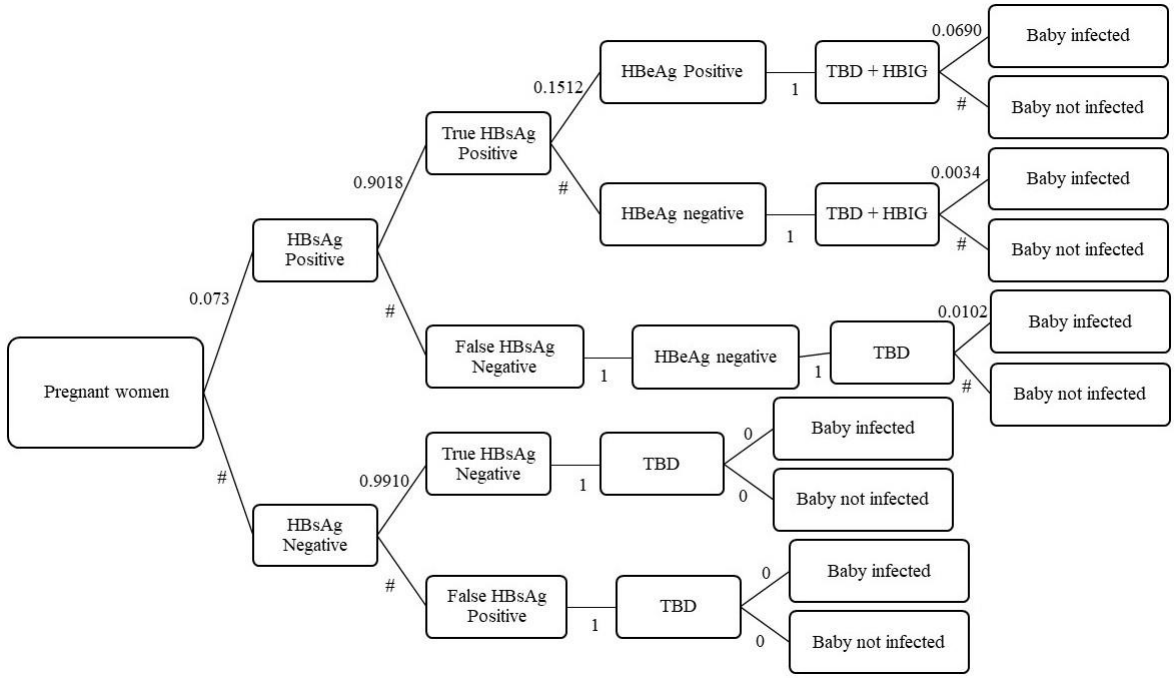


Figure 5.3: Decision tree structure for the universal HBV vaccination and targeted HBIG strategy (strategy 2). HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; TBD: timely birth dose. # refers to the remaining number of patients found in the next branch of the tree, branching out of a node.

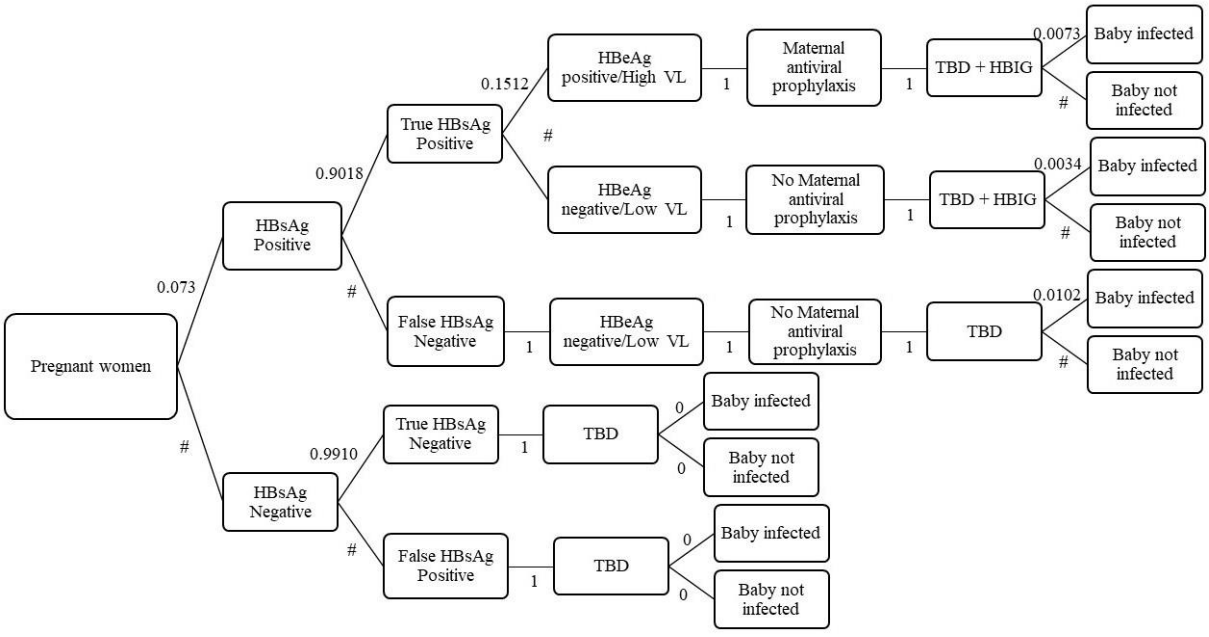


Figure 5.4: Decision tree structure for maternal treatment with TDF, universal HBV vaccination and targeted HBIG strategies (strategy 3 and strategy 4). # refers to the remaining number of patients found in the next branch of the tree, branching out of a node. HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; TBD: timely birth dose. TDF: tenofovir disoproxil fumarate.

5.3.8. Cost-effectiveness analyses

The total costs and the total number of perinatal infections averted in each strategy were calculated, based on the decision-tree model analysis. The strategies were arranged from least to the most costly. The incremental costs over the incremental outcomes, known as the ICER, was calculated using the formula: $(Cost_B - Cost_A) / (Effectiveness_B - Effectiveness_A)$**.** This formula represents the difference in costs divided by the difference in effectiveness (expressed in the number of pediatric HBV infections averted) between each more costly strategy and the previous non-dominated less costly strategy **(Drummond et al., 2015)**. Any interventions with extended or absolute dominance were removed from the analysis. Extended dominance is seen where a strategy is more costly and more effective, but produces an ICER higher than the next most expensive strategy. Absolute dominance is whereby an intervention is of high cost but equally effective than the previous less expensive intervention. The final ICER was interpreted as the incremental cost to avert an incremental perinatal HBV infection.

5.3.9. Sensitivity analysis

A sensitivity analysis allows for identifying the impact of uncertainties of the parameters used in the decision-tree model. The main parameters of the model are changed to assess their impact on the ICER. This analysis also helps to determine the parameters that drive the change in the ICER and which would require more attention during the data collection or decision-making.

A series of one-way sensitivity analysis was performed to assess the robustness of the ICERs produced by the base analysis **(Briggs, 1995)**, by altering all epidemiological parameters and four cost estimates used in the model. The altered cost estimates were the prices of HBIG, TDF, the HBeAg test and the viral load test. The range values of the epidemiological parameters, low and high, used in the sensitivity analysis are presented in Table 5.2. The impact of these changes to the incremental costs, the incremental effectiveness, and the ICERs were noted. The most influential variables identified in the one-way sensitivity analysis were further explored in two-way sensitivity analyses; in which two parameters were changed at a time.

5.4. Results

5.4.1. Direct medical costs

The direct costs involved in each strategy were estimated and are presented in Table 5.3. Based on the average costs and the rate of utilization of resource from patients in each strategy (Table 5.4), four unit costs related to HBV testing were calculated: (1) cost per negative RDT test, (2) cost per true positive RDT result, (3) cost per HBV positive patient with a low viral load/HBeAg negative, and (4) the cost per HBV positive patient with a high viral load (≥ 200 000 IU/ml)/HBeAg positive. The cost per false positive RDT test was equivalent to the cost per HBV positive patient with a low viral load/HBeAg negative, in the analysis. These costs are listed in Table 5.5.

Table 5.3: Costs estimates per categories and activities included in study strategies

		IKH	WCH	Average cost
Category	**Measure**	**Cost (US$)**	**Cost (US$)**	**Cost (US$)**
Staff costs				
Pre-test counseling	Cost per session	$0.15	$0.09	$0.12
Rapid test	Cost per test	$0.22	$0.22	$0.22
Post-test counseling HBV positive	Cost per session	$0.74	$0.74	$0.74
Post-test counseling HBV negative	Cost per session	$0.28	$0.28	$0.28
Drawing blood – HBeAg/HBV VL tests	Cost per session	$1,84	$1,84	$1,84
MO consultation for TDF patients	Cost per session	$4.38	$4.38	$4.38
Patient-specific costs				
Rapid test	Cost per test	$2.30	$2.29	$2.30
HBV viral load test[1]	Cost per test	$143.11	$143.10	$143.11
HBeAg test[1]	Cost per test	$42.37	$42.35	$42.36
TDF orientated lab tests[2]	Cost per assessment	$33.93	$33.93	$33.93
Maternal ART with TDF[3]	Cost per 4-month course	$16.16	$16.16	$16.16
HBIG[4]	Cost per HBIG	$54.96	$54.96	$54.96
HBV BD vaccination	Cost per dose	$1.48	$1.48	$1.48
Overheads				
Facility overhead costs	Cost per visit	$2.62	$1.67	$2.14
Capital costs - furniture and space				
Pre-test counseling room	Cost per session	$0.86	$0.36	$0.61
Rapid test room – testing only	Cost per session	$0.07	$0.08	$0.08
Rapid test room – testing + phlebotomy for HBeAg/HBV VL tests	Cost per session	$0.06	$0.07	$0.07

Post-test counseling room – IKH ANC[5]	Cost per session	$0.10	NA	NA
Post-test counseling room– no consultation for TDF[6]	Cost per session	NA	$0.06	$0.08*
Post-test counseling room – with consultation for TDF[6]	Cost per session	NA	$0.06	$0.08#
Phlebotomy for HBeAg/HBV VL tests	Cost per session	$0.18	$0.21	$0.19
MO consultation room for TDF treatment	Cost per session	$0.04	$0.18	$0.11

[1]The costs of the viral load test and the HBeAg test differ between the two ANCs due to the difference in the usage of consumables between the two ANCs.[2]Counts creatinine, FBC, ALT and AST tests. [3]Cost of the drug only. [4]cost of a single vial. [5]includes both HBV positive and HBV negative patients. [6]Post-test counseling costs at WCH ANC include HBV positive and HBV negative. *Average of the cost of post-test counseling at IKH and WCH (without consultation for TDF treatment in this area - applicable to strategies 1 and 2). #Average of post-test counseling at IKH and WCH ANC (with consultation for TDF treatment in this area, - applicable to strategies 3 and 4). ALT: alanine transaminase; AST: aspartate transaminase; ANC: antenatal clinic; ART: antiretroviral therapy; BD: birth dose; FBC: full blood count; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HBIG: hepatitis B immunoglobulin; IKH: Intermediate Katutura hospital; Lab: laboratory; MO: medical officer; NA: Not applicable; TDF: tenofovir disoproxil fumarate; VL: viral load; WCH: Windhoek Central hospital.

Table 5.4: Utilization of medical resources in each strategy

	Universal BD vaccination		**Universal BD vaccination + targeted HBIG**		**Universal BD vaccination + HBV viral load testing + maternal antiviral prophylaxis + targeted HBIG**			**Universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis + targeted HBIG**		
Resources	HBV Neg	HBV Pos	HBV Neg	HBV Pos	HBV Neg	non-TDF patient	TDF patient	HBV Neg	non-TDF patient	TDF patient
ANC	1	1	1	1	1	1	2*	1	1	2*
HBV RDT	1	1	1	1	1	1	1	1	1	1
HBV VL test	-	-	-	-	-	1	1	-	-	-
HBeAg test	-	-	-	-	-	-	-	-	1	1
TDF orientated tests	-	-	-	-	-	-	1	-	-	1
Maternal ART	-	-	-	-	-	-	1	-	-	1
BD vaccine	1	1	1	1	1	1	1	1	1	1
Infant HBIG	-	-	-	1	-	1	1	-	1	1

*These included the first ANC visit when the patient is screened for HBV, and a second visit whereby blood is drawn for the TDF-orientated tests and the TDF prescription is provided to the patients. ART: antiretroviral therapy; BD: birth dose; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HBIG: hepatitis B immunoglobulin; Neg: negative; Pos: positive; RDT: rapid diagnostic test; VL: viral load; TDF: tenofovir disoproxil fumarate.

Table 5.5: Costs of each HBV status in each strategy. Costs are expressed in USD.

	Universal BD vaccination	Universal BD vaccination + targeted HBIG	Universal BD vaccination + HBV viral load testing + maternal antiviral prophylaxis + targeted HBIG	Universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis + targeted HBIG
Testing per HBV status				
HBV RDT negative[1]	-	$5.82	$5.81	$5.81
HBV RDT positive	-	$6.28	-	-
HBeAg negative/low viral load[2]	-	-	$151.42	$50.67
HBeAg positive/high viral load	-	-	$208.14	$107.39
Pediatric immunization per HBV status				
True HBV negative	$1.48	$1.48	$1.48	$1.48
False HBV negative	$1.48	$1.48	$1.48	$1.48
True HBV positive	$1.48	$56.43	$56.43	$56.43
False HBV positive	$1.48	$56.43*	$56.43[#]	$56.43[#]

[1]These costs applied to both false and true negative patients. [2]These costs applied to both true positive patients with a low viremia/HBeAg negative status and false HBsAg positive patients to whom HBeAg or HBV DNA testing is performed. *The mothers of these babies aren't ruled out as false positives given that HBsAg positive samples are not confirmed at the laboratory. [#]Mothers of these babies would test negative for HBV but are still given HBIG based on the positive HBsAg result. BD: birth dose; HBV: hepatitis B virus; RDT: rapid diagnostic testing.

5.4.2. Cost-effectiveness analysis

Listed from the least expensive to the most expensive, strategy 1 including universal BD vaccination was the cheapest option, costing US$15 827.22; followed by strategy 2 (US$114 325.96), strategy 4 (US$153 053.55), and strategy 3 (US$228 238.62). These were equivalent to US$1.58, US$11.43, US$ 15.31, and US$22.82 per pregnant woman presenting at the ANC, respectively. The considerable variation in costs between the universal BD vaccination strategy and the other interventions was attributed to the cost of additional HBIG, and of the HBV viral load and HBeAg tests. On the other hand, the lowest effectiveness was attributed to universal BD vaccination; which produced 31 perinatal HBV infections. These were reduced to 10 infections with BD vaccine + HBIG, and to three infections with the use of TDF in addition to BD vaccine and targeted HBIG (strategies 3 and 4).

Compared to the universal HBV BD vaccination strategy, the current Namibian strategy (BD vaccine + HBIG) averted an extra 22 cases of perinatal infections; thereby producing an ICER of US$4 550.34/pediatric HBV infection averted. Six additional cases were averted in the TDF strategy using HBeAg testing, in comparison to the BD vaccine + HBIG strategy; and produced an ICER of US$6 262.42/pediatric infection averted. The TDF strategy using HBV viral load testing was excluded through absolute dominance; it costed more than using HBeAg testing but produced the same effectiveness. A summary of these results is found in Table 5.6.

Table 5.6: Cost-effectiveness results by prevention strategy*. The ICER is expressed as US$ per incremental pediatric HBV infection averted. Costs are expressed in US$.

Strategies	Costs	Incremental costs	Total infections	Infections averted	ICER
Universal BD vaccination	$15 827.22	-	31	-	-
Universal BD vaccination + targeted HBIG	$114 325.96	$98 498.74	10	22	4 550.34
Universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis + targeted HBIG	$153 053.55	$38 727.59	3	6	6 262.42
Universal BD vaccination + HBV viral load testing + maternal antiviral prophylaxis + targeted HBIG	$228 238.62	$75 185.07	3	0	Absolute dominance

*Based on a hypothetical cohort of 10000 women. Both costs and outcomes were not discounted. ART: antiretroviral therapy; BD: birth dose; HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBV: hepatitis B virus; ICER: Incremental cost-effectiveness ratio; TBD: Timely birth dose.

5.4.3. Sensitivity analysis

5.4.3.1. One-way sensitivity analysis

Univariate sensitivity analyses were performed with all epidemiological parameters used in the model. Costs with a potential influence on the ICERs of the comparative strategies were also explored. Results from these analyses for strategies 2 and 3 are graphically presented in Figure 5.5 and Figure 5.6, respectively. The impact of this analysis on the incremental costs and incremental infections averted is tabulated in Table 5.7.

Strategy 2: Universal BD vaccination and targeted HBIG

Two parameters, related to transmission, had the most significant impact on the ICER of this intervention. These were (1) the rate of MTCT with BD vaccine from HBeAg positive women, and (2) the rate of MTCT with BD vaccine and HBIG from HBeAg positive women. At the low rate of 10.17% of HBV MTCT from HBeAg positive women with BD vaccine, a sharp increase of the base value of the ICER from US$4 550.34 to US$13 866.73 was noted. This intervention was eliminated due to extended dominance; the produced ICER of US$13 866.73 was higher than the ICER of the TDF strategy (US$6 262.42). Extended dominance of the BD + HBIG strategy by the TDF strategy was also observed when the high value (10.73%) for the rate of HBV MTCT from HBeAg positive women with BD and HBIG was used in the model. The ICER of the BD + HBIG strategy decreased to US$5 531.24, and so did the ICER of the TDF strategy (US$3 863.91).

A change in the epidemiology of HBV, both HBsAg and HBeAg/high VL prevalence, had a moderate impact on the ICER of this strategy. It rose from the base value of US$4 550.34 to US$ 5 550.89 at the lowest HBsAg prevalence of 5.44%, and to US$7 339.60 at the lowest HBeAg/high VL prevalence of 7.14%. At the highest value of 10.10% and 14.71% of these two parameters, the ICER decreased to US$3 740.89 and US$4 306.93, respectively. The sensitivity analysis also indicated that a 50% change in the cost of HBIG only led to a 21% change in the ICER, in both directions. The rate of transmission of HBV from HBeAg negative pregnant women also had a moderate influence on the ICER; as portrayed in Figure 5.5. The diagnostic accuracy of the HBsAg rapid test had a minimal impact on the costs and effectiveness of this intervention.

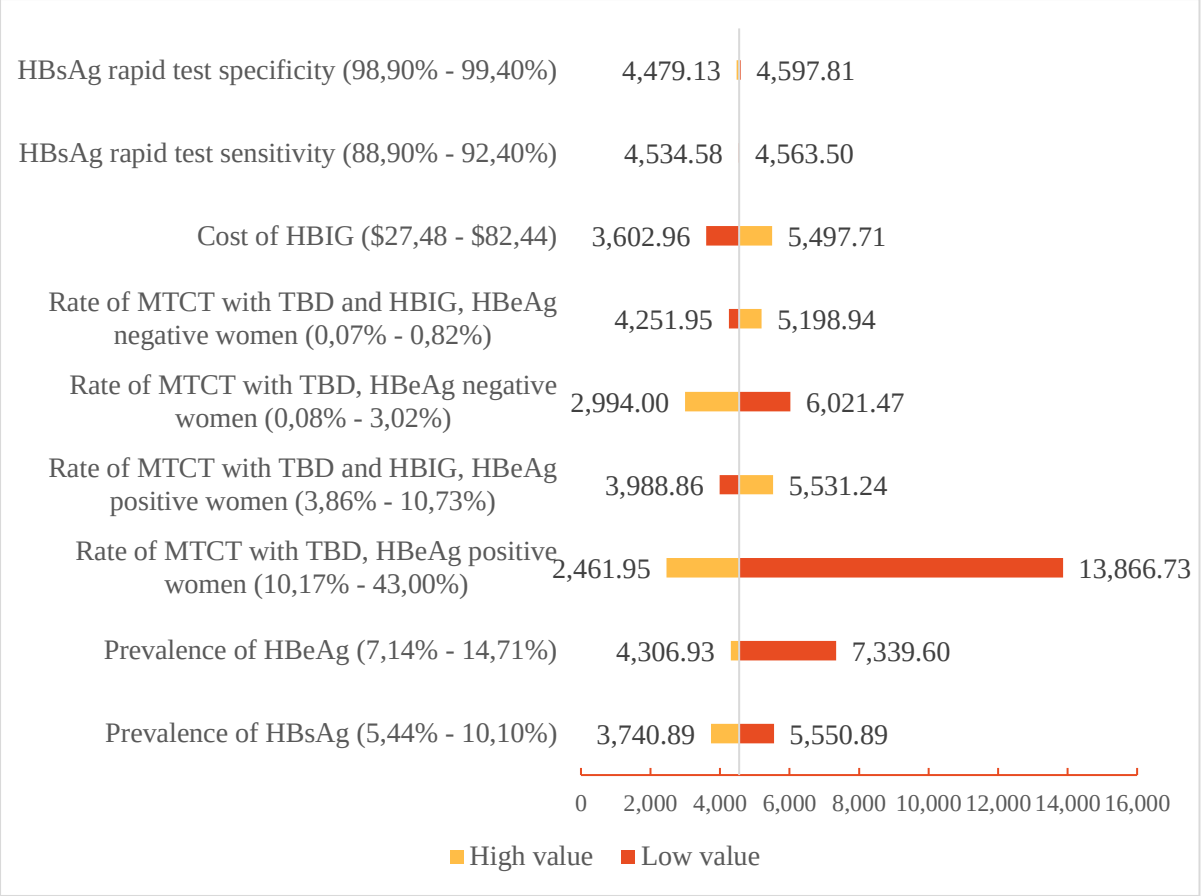


Figure 5.5: Tornado diagram presenting results from one-way sensitivity analyses: universal BD vaccination + targeted HBIG vs. universal BD vaccination only. Both strategies include antenatal HBsAg screening. The base ICER of this strategy is US$4 550.34/pediatric infection averted. ART: antiretroviral therapy; BD: birth dose; TBD: timely birth dose; HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; MTCT: mother-to-child transmission.

Strategy 4: Universal BD vaccination, HBeAg testing, maternal antiviral prophylaxis, and targeted HBIG

Three variables had the most significant influence on this health intervention: (1) the prevalence of HBeAg, (2) the rate of MTCT from these women with maternal TDF, BD vaccine and HBIG, and (3) the costs of the HBeAg test. As the prevalence of HBeAg positive women/high VL decreased from the base value of 13.73% to 7.14%, the ICER of this intervention increased from US$6 262.42 to US$11 193.93/pediatric HBV infection averted (Figure 5.6). This implies that the intervention is unlikely to be cost-effective at lower prevalence levels of HBeAg positive women/high VL. Based on the significant effect of this parameter on the ICER, and taking in consideration that this proportion may vary among HBV-infected pregnant women across different parts of Namibia, the effect of further variations of

the parameter was explored. A two-fold (27.46%) and a 2.5 increase (34.33%) of the prevalence of HBeAg positive women/high VL from the base value of 13.73% decreased the ICER to US$3 590.87 and US$3 056.25 for every additional pediatric HBV infection prevented by the intervention, respectively. By altering the variables associated with the rate of MTCT from HBeAg positive mothers treated with TDF during pregnancy to the lowest value of 0.10% and highest value of 1.93%, the ICER decreased to US$5 682.22 but increased to US$7 774.47 per additional pediatric infection averted, respectively.

As mentioned above, when the lowest rate (10.17%) of HBV MTCT with BD vaccine and the highest rate (10.73%) of HBV MTCT with BD vaccine + HBIG from HBeAg positive women was used in the model, separately, the universal BD vaccination + HBIG strategy was removed due to extended dominance. In these two scenarios, the antiviral prophylaxis strategy was compared to the universal BD vaccination strategy and produced two new ICERs of US$10 327.57 and US$4 930.77, respectively.

At a 50% decrease of the price of the HBeAg test (US$20.86), this intervention produced an ICER (US$4 371.46) lower than the universal BD vaccination + HBIG strategy's one (ICER = US$4 550.34). The influence of the price of this test on the ICER of this intervention was further explored. It was noted that a 35% decrease of the original price of the HBeAg test to US$27.12 was the threshold at which the antiviral prophylaxis strategy eliminated the universal BD vaccination + HBIG strategy through extended dominance. At a US$27.12 cost of the HBsAg test, the antiviral prophylaxis strategy was compared to the universal BD vaccination intervention; and a new ICER of US$4 539.25 was produced.

A reduction of the cost of TDF by half reduced the ICER to US$6 131.45/incremental infection averted. When the cost of TDF was increased to US$69 per 4-month course, calculated as per the 2016 pharmaceutical price of the TDF offered in SSA (US$207 per person per year) **(Médecins Sans Frontières, 2016)**, the ICER rose to US$7 118.79/incremental infection averted.

The tornado diagram representing the results of this analysis is illustrated in Figure 5.6. The diagram also shows that the diagnostic accuracy of the HBsAg rapid test had a minimal impact on this intervention; in terms of both costs and effectiveness. The change of the cost of HBIG and the rates of HBV MTCT with BD vaccine and BD vaccine + HBIG from HBeAg negative women did not influence the ICER of this strategy.

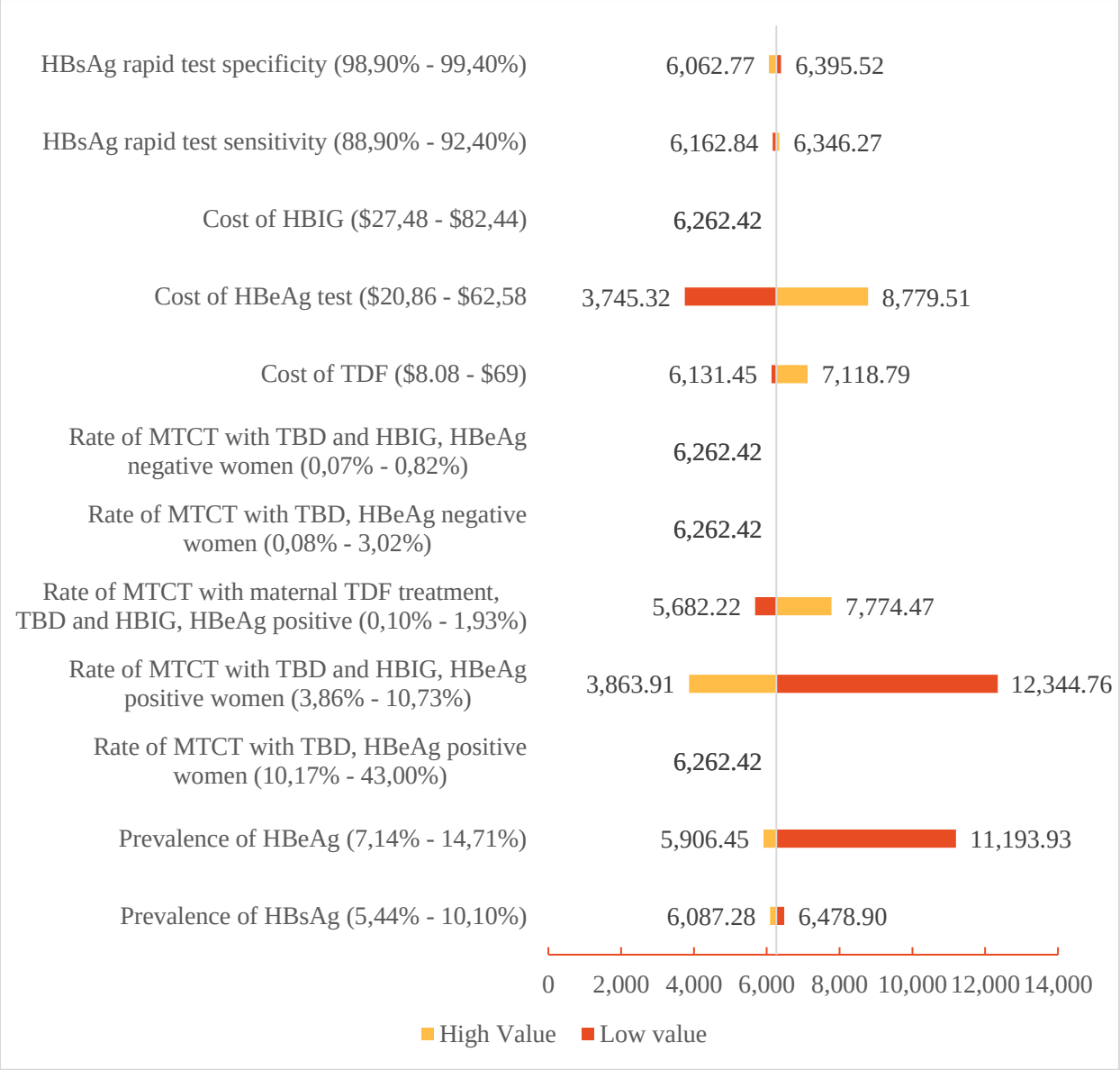


Figure 5.6: Tornado diagram presenting results from one-way sensitivity analyses: HBeAg testing + maternal antiviral prophylaxis + active-passive immunization vs. BD vaccination + targeted HBIG. The base ICER is US$6 262.42/pediatric infection averted. Three parameters are seen to have a high impact on the base ICER. These are (1) the risk of MTCT from HBeAg positive pregnant women with a high viral load whose babies receive both HBIG and BD vaccine, (2) the proportion of HBeAg positive pregnant women, and (3) the cost of the HBV viral load test. ART: antiretroviral therapy; BD: birth dose; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; ICER: incremental cost-effectiveness ratio; MTCT: mother-to-child transmission.

Strategy 3: Universal BD vaccination, HBV viral load testing, maternal antiviral prophylaxis, and targeted HBIG

Variations of the cost of the HBV viral load were completed to determine the cost at which viral load testing would be comparable to HBeAg testing to determine maternal antiviral prophylaxis eligibility. At a viral load test cost of US$41.72, which represented the price of the HBeAg test, HBV viral load testing was found cheaper than HBeAg testing by US$43.67.

Table 5.7: Results of the one-way sensitivity analysis

Model parameters	Base value	Range values	Incremental Costs	Infections averted	ICER*	Base ICER*	ICER* 2
Universal BD vaccination + targeted HBIG						**4 550,34**	
Prevalence of HBsAg	7.30%	5.44%	$89 490.61	16	5 550.89		
		10.10%	$112 036.74	30			
Prevalence of HBeAg/high VL	13.73%	7.14%	$98 498.74	13	7 339.60		
		14.71%	$98 498.74	23	4 306.93		
		27.46%	$98 498.74	39	2 539.57		
		34.33%	$98 498.74	47	2 079.72		
Rate of MTCT with TBD from HBeAg positive women	24.68%	10.17%	$98 498.74	7	13 866.73		Extended dominance
		43.00%	$98 498.74	40	2 461.95		
Rate of MTCT with TBD + HBIG from HBeAg positive women	6.90%	3.86%	$98 498.74	25	3 988.86		
		10.73%	$98 498.74	18	5 531.24		Extended dominance
Rate of MTCT with TBD from HBeAg negative women	1.02%	0.08%	$98 49874	16	6 021.47		
		3.02%	$98 498.74	33	2 994.00		
Rate of MTCT with TBD + HBIG from HBeAg negative women	0.34%	0.07%	$98 498.74	23	4 251.95		
		0.82%	$98 498.74	19	5 198.94		
Cost of HBIG	$54.96	$27.48	$77 991.41	22	3 602.96		
		$82.44	$119 006.07	22	5 497.71		
Rapid test sensitivity	90.80%	88.90%	$97 730.03	22	4 534.58		
		92.40%	$99 146.08	22	4 563.50		
Rapid test specificity	99.10%	98.90%	$99 526.28	22	4 597.81		
		99.40%	$96 957.43	22	4 479.13		

Universal BD vaccination + HBV viral load testing + maternal antiviral prophylaxis+ targeted HBIG						**Absolute dominance**
Cost HBV viral load test	$142.52	$71.26	$22 004,54	0	NA	
		$44.85	$11 368.49	0	NA	
		$42.76	$732.43	0	NA	
		$41.72[¥]	-$43.67	0	NA	
Universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis+ targeted HBIG						**6 262.42**
Prevalence of HBsAg	7.30%	5.44%	$29 840.61	5	6 478.90	
		10.10%	$52 083.54	9	6 087.28	
Prevalence of HBeAg/high VL	13.73%	7.14%	$35 998.86	3	11 193.93	
		14.71%	$39 133.38	7	5 906.45	
		27.46%	$44 412.79	12	3 590.87	
		34.33%	$47 257.46	15	3 056.25	
Rate of MTCT with TBD from HBeAg positive women	24.68%	10.17%	$38 727.59	6	6 262.42	4 539.25
		43.00%	$38 727.59	6	6 262.42	
Rate of MTCT with TBD + HBIG from HBeAg positive women	6.90%	3.86%	$38 727.59	3	12 344.76	
		10.73%	$38 727.59	10	3 863.91	4 930.77
Rate of MTCT with maternal TDF, TBD, and HBIG from HBeAg positive women	0.073%	0.10%	$38 727.59	7	5 682,22	
		1.93%	$38 727.59	5	7 774,47	

Rate of MTCT with TBD from HBeAg negative women	1.02%	0.08%	$38 727.59	6	6 262.42	
		3.02%	$38 727.59	6	6 262.42	
Rate of MTCT with TBD + HBIG from HBeAg negative women	0.34%	0.07%	$38 727.59	6	6 262.42	
		0.82%	$38 727.59	6	6 262.42	
Cost of TDF	$16.16	$8.08	$37 917.66	6	6 131.45	
		$24.24	$39 537.53	6	6 393.39	
Cost of HBeAg test	$41.72	$27.12	$27 831.37	6	4 500.45	4 539,25
		$62.58	$54 293.63	6	8 779.51	
Cost of HBIG	$54.96	$27.48	$38 727.59	6	6 262.42	
		$82.44	$38 727.59	6	6 262.42	
Rapid test sensitivity	90.80%	88.90%	$38 111.82	6	6 162.84	
		92.40%	$39 246.14	6	6 346.27	
Rapid test specificity	99.10%	98.90%	$39 550.69	6	6 395.52	
		99.40%	$37 492.95	6	6 062.77	

*ICER is expressed in US$ per pediatric infection averted. [¥]price of the HBeAg test; [#]35% decrease of the price of the HBeAg test. In red are values at which the universal BD vaccination + targeted HBIG strategy is dominated by the universal BD vaccination + HBeAg testing + maternal antiviral prophylaxis+ targeted HBIG strategy (strategy 4), and a new ICER for strategy 4 is calculated in comparison to the universal BD vaccination strategy. ART: antiretroviral therapy; BD: birth dose; TBD: timely bird dose; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; ICER: incremental cost-effectiveness ratio; MTCT: mother-to-child transmission.

5.4.3.2. Multi-way sensitivity analysis

A series of two-way sensitivity analysis was performed, using variables with the highest influence on the ICERs of strategies 2 and 4 in the one-way sensitivity analyses.

Strategy 2: Universal BD vaccination and targeted HBIG

A critical change of the ICER of this intervention was observed using variations of the following parameter combinations (1) the prevalence of HBsAg and the prevalence of HBeAg/high VL, (2) the cost of HBIG and the prevalence of HBeAg/high VL, (3) the prevalence of HBeAg/high VL and the rate of HBV MTCT with BD + HBIG from HBeAg positive/highly viremic women. The pairing was dependent on the impact caused on the ICER by the epidemiology of HBeAg/high VL pregnant women who have the highest risk of HBV MTCT, and on the effectiveness of this strategy in this population of high-risk women.

In the first combination, the lowest ICER of US$1 709.77 was found at a 10.10% prevalence of HBsAg and 34.33% prevalence of HBeAg (Table 5.8). At the lowest values of 5.44% and 7.14% prevalence of HBsAg and HBeAg/high VL, respectively, the ICER rose to US$8 953.47. A 50% reduction of the cost of HBIG in combination with a high prevalence of HBeAg/high VL (34.33%) decreased the ICER by nearly 64% to US$1 646.73. But a 28% increase of the ICER to US$5 811.50 was observed at low prevalence of 7.14% of HBeAg/high VL, even at the low HBIG cost of US$27.48 (Table 5.8). A 34.44% prevalence of HBeAg/high VL combined to the lowest rate of HBV MTCT from highly viremic HBeAg positive women using BD + HBIG resulted in a lower ICER too (US$1 791.54).

Table 5.8: Most influential variables included in two-way sensitivity analyses of universal BD vaccination and targeted HBIG compared to universal BD vaccination only.

		Prevalence of HBsAg		
		5.44%	7.30%	10.10%
Prevalence of HBeAg/high VL	7.14%	8 953.47	7 339.60	6 033.98
	13.73%	5 550.89	4 550.34	3 740.89
	14.71%	5 253.96	4 306.93	3 540.78
	27.46%	3 097.98	2 539.57	2 087.81
	34.33%	2 537.02	2 079.72	1 709.77
		Cost of HBIG		
		$27.48	$54.96	$82.44
Prevalence of HBeAg/high VL	7.14%	5 811.51	7 339.60	8 867.70
	13.73%	3 602.96	4 550.34	5 497.71
	14.71%	3 410.23	4 306.93	5 203.63
	27.46%	2 010.83	2 539.57	3 068.30
	34.33%	1 646.73	2 079.72	2 512.72
		Rate of transmission with TBD + HBIG from HBeAg positive		
		3.86%	6.90%	10.73%
Prevalence of HBeAg/high VL	7.14%	6 564.53	7 339.60	8 622.16
	13.73%	3 988.86	4 550.34	5 531.24
	14.71%	3 768.95	4 306.93	5 251.30
	27.46%	2 194.74	2 539.57	3 166.34
	34.33%	1 791.54	2 079.72	2 608.33

Values in blue represent the base ICER of the strategy. Values in red represent the lowest ICER in all combinations. ICERs are expressed in cost/incremental pediatric HBV infection averted. BD: birth dose; HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; VL: viral load.

Strategy 4: Universal BD vaccination, HBeAg testing, maternal antiviral prophylaxis, and targeted HBIG

In section 5.4.3.1, it is shown that the ICER of this strategy was sensitive to variations of five parameters. These parameters were further explored as pairs in the following combinations: (1) cost of HBeAg test and the prevalence of HBeAg/high VL, (2) prevalence of HBsAg and prevalence of HBeAg/high VL, (3) the rate of transmission with TBD + HBIG and with TDF + TBD + HBIG from highly viremic HBeAg positive. The implementation of this strategy would be

dependent on the proportion of HBeAg positive/high HBV VL women in need of TDF, the cost of the HBeAg test used to determine antiviral prophylaxis eligibility, and of the rate of MTCT from these women if TDF is added to BD and HBIG. These reasons guided the combination of parameters used in these two-way sensitivity analyses of this strategy.

In agreement with the one-way sensitivity analysis, the lower the cost of the HBeAg test became, accompanied by an increase in the proportion of HBsAg positive women requiring antiviral prophylaxis (HBeAg positive women/high HBV VL), the lower was the ICER. The lowest value of US$1 453.06 per additional pediatric infection averted was obtained when considering an HBeAg cost of US$8.50, the price of the HBeAg test at the NHLS in South Africa, and prevalence of HBeAg/high VL of 34.33%. Furthermore, at 5.44% HBsAg prevalence and 7.14% prevalence of HBeAg/high VL, the ICER went up to US$11 610.22; and decreased to US$2 986.20 at 10.10% and 34.33% prevalence of HBsAg and HBeAg/high VL, respectively.

When the rate of HBV MTCT with antiviral prophylaxis (TDF) + BD + HBIG from HBeAg positive decreased to 0.10%, and the rate of HBV MTCT from HBeAg positive pregnant women/high HBV VL with the current Namibian strategy (BD + HBIG) decreased to 3.86%, the ICER of the antiviral prophylaxis strategy raised to US$10 276.36/pediatric infection averted. The ICER decreased to US$3 634.91 when the rate of HBV MTCT from HBeAg positive pregnant women/high HBV VL with BD + HBIG increased to 10.73%, while the rate of HBV MTCT using antiviral prophylaxis (TDF) remained at 0.10%. A summary of these results is presented in Table 5.9 below.

Table 5.9: Most influential variables included in two-way sensitivity analyses of maternal antiviral prophylaxis using HBeAg testing, universal BD vaccination and targeted HBIG compared to universal BD vaccination targeted HBIG.

		Cost of HBeAg test						
		$41.72	$29.20	$27.12	$25.03	$20.86	$16.69	$8.50
Prevalence of HBeAg/high VL	7.14%	11 193.93	8 289.75	7 805.72	7 321.69	6 353.64	5 385.58	3 485.59
	13.73%	6 262.42	4 752.16	4 500.45	4 248.74	3 745.32	3 241.90	2 253.86
	14.71%	5 906.45	4 496.81	4 261.87	4 026.93	3 557.05	3 087.17	2 164.95
	27.46%	3 590.87	2 835.74	2 709.89	2 584.03	2 332.32	2 080.61	1 586.59
	34.33%	3 056.25	2 452.23	2 351.56	2 250.90	2 049.56	1 848.22	1 453.06
		Prevalence of HBsAg						
		5.44%		7.30%		10.10%		
Prevalence of HBeAg/high VL	7.14%	11 610.22		11 193.93		10 857.15		
	13.73%	6 478.90		6 262.42		6 087.28		
	14.71%	6 108.51		5 906.45		5 742.98		
	27.46%	3 699.11		3 590.87		3 503.30		
	34.33%	3 142.83		3 056.25		2 986.20		
		Rate of transmission with TBD + HBIG from HBeAg positive						
		3.86%		6.90%		10.73%		
Rate of MTCT with TDF +TBD + HBIG	0.10%	10 276.36		5 682.22		3 634.91		
	0.73%	12 344.76		6 262.42		3 863.91		
	1.93%	20 020.26		7 774.47		4 390.81		

Values in blue represent the base ICER of the strategy. Values in red represent the lowest ICER in all combinations. ICERs are expressed in cost/incremental pediatric HBV infection averted. BD: birth dose; HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; MTCT: mother-to-child transmission; TBD: timely birth dose; VL: viral load.

5.5. Summary of findings

In the analysis presented in this study, universal HBV BD vaccination and targeted HBIG appeared to be more effective compared to universal HBV BD vaccination against HBV MTCT; in a cohort of 10 000 births, 22 extra cases of perinatal HBV infections (ICER: US$4 550.34/pediatric HBV infection averted) were averted in the HBV BD vaccination and targeted HBIG intervention. Providing maternal antiviral prophylaxis with TDF had the highest effectiveness; the intervention averted six extra cases of perinatal HBV infection in comparison to universal HBV BD vaccination and targeted HBIG. However, the costs of this intervention were high: $153 053.55 with HBeAg testing (ICER: US$6 262.42/pediatric HBV infection averted) and $228 238.62 with HBV viral load testing (ICER: Absolute dominance). Still, the sensitivity analyses demonstrated that adding maternal antiviral prophylaxis with sequential HBeAg testing to the current universal HBV BD vaccination and targeted HBIG strategy may be a cost-effective intervention against HBV MTCT in Namibia.

5.6. Interpretation of findings

5.6.1. Universal HBV BD vaccination and targeted HBIG compared to universal HBV BD vaccination only

BD vaccination has been and remains an essential tool for preventing perinatal HBV infections globally. Consistent with the current economic literature **(Griffiths, Hutton & Pascoal, 2005; Kim, Salomon & Goldie, 2007; Klingler, Thoumi & Mrithinjayam, 2012; Anderson et al., 2018)**, this strategy was the least expensive measure for preventing HBV MTCT, with a total cost of US$15 827.22. Although inexpensive, 31 pediatric HBV infections were recorded still in a hypothetical cohort of 10 000 women, of whom 730 were HBsAg positive. Still, the need for implementing this intervention is not debatable. In Cameroon, where this immunoprophylaxis measure is not offered, a recent decision analytical model showed that 813 infections, including infections acquired through horizontal transmission, are still recorded per 10 000 children with pediatric vaccination from six weeks of age. With the delivery of the BD vaccine, an additional 384 HBV infections were averted in the model at an incremental cost of US$130.41 per additional

infection averted **(Anderson et al., 2018)**. The model by Anderson et al. (2018) also revealed a residual 430 infections (including both perinatally-acquired and horizontally-acquired infection), despite universal BD vaccination **(Anderson et al., 2018)**. These data merely indicated that BD vaccination is highly effective and low cost in the SSA context, but may not be sufficient if provided alone to eliminate HBV MTCT in the region.

Combining BD vaccination with passive immunity from HBIG has shown higher effectiveness in comparison to vaccination alone in Asia **(Lee et al., 2006, 2007)**. In our model, providing HBIG to HBsAg-exposed babies in addition to the BD vaccine was at an extra cost of US$98 498.74, but prevented an additional 10 infections. This equated to an incremental US$4 550.34 per pediatric HBV infection averted (ICER). At an intermediate HBV endemicity of 5% in Taiwan, a model by Chen et al. (2013c) showed that combining BD vaccination with HBIG reduced the number of pediatric HBV infections attained with BD vaccination alone from 738 to 225 (incremental infections averted = 98) at an incremental costs of US$385 700, in a cohort of 100 000 people. The calculated ICER of the strategy was US$3 956 per infection averted **(Chen et al., 2013c)**. In Thailand, the same strategy had an ICER of US$716.78 per infection averted compared to universal BD vaccination **(Devine et al., 2017)**. Two main parameters differed between the model of this study and those two Asian models. These were the cost of HBIG and the prevalence of HBeAg; both parameters had a substantial impact on our ICER. At a high HBeAg prevalence of 34.33%, which is similar to the prevalence seen in Asia, a 54% decrease of the ICER to US$2 079.72 was noted. Decreasing the cost of HBIG by half reduced the ICER by 21% (US$3 602.96). Using simultaneous variations of these two parameters, the lowest ICER of US$1 646.73 per pediatric infection averted was produced. These changes of the ICER of the strategy indicated that more efforts are needed to reduce the price of HBIG. They also showed that adding HBIG to BD vaccination is of value in settings where a large proportion of HBsAg positive pregnant women are also positive for HBeAg. Similarly, the higher is the prevalence of HBsAg, the lower the ICER would be; as seen in the two-way sensitivity analysis and in Taiwan too **(Chen et al., 2013c)**. In the Taiwanese analytical model, a gradual increase of the ICER of the strategy including HBIG and BD vaccine from US$3 234 to US$3 956 was observed as the HBsAg carrier rate decreased from 25% to 5%, and reached US$ 8 783 at a lower HBsAg carrier rate of 1% **(Chen**

et al., 2013c). As such, a lower ICER for the HBIG + BD strategy would be observed in the northern part of Namibia where prevalence of HBsAg and HBeAg as high as 10.10% **(Mhata et al., 2017)** and 14.71% **(Botha et al., 1984)**, respectively, have been reported. At these HBV epidemiological rates, the intervention costed US$3 540.70 per infection averted; this was equivalent to a 22% decrease of the ICER from its base value of US$4 550.34.

In the presence of a high burden of pregnant women at the highest risk of transmitting HBV to their babies, the effectiveness of the intervention against HBV MTCT also plays a big role in the decision-making process. As per the two-way sensitivity analysis, the lower was the rate of HBV MTCT from HBeAg positive women with the use of BD and HBIG in a large population of HBeAg positive pregnant women, the lower the ICER was too. At a 14.71% prevalence of HBeAg among pregnant women in Ovamboland combined with a 3.86% rate of HBV MTCT if using this strategy, 26 extra pediatric infections would be prevented in that region at an incremental cost of US$98 498.74 (ICER = US$3 768.95 per infection averted); in comparison to BD vaccine alone. However, in Windhoek, where an HBeAg prevalence of 7.14% was found in our antenatal study, another 15 pediatric infections would be averted; thereby costing US$6 564.53 per infection averted (ICER) in the region. Given that the prevalence of both HBsAg and HBeAg vary across parts of the country, results from the two-way sensitivity analysis has shown that the use of the strategy may be of high costs in some parts of the country, but low cost in other parts. Thus, the choice of an optimal HBV immunization strategy against HBV MTCT is dependent on the HBsAg carrier rate and the availability of resources in settings. Updated statistics of the epidemiology of HBV in Namibia would be valuable for a choice between BD vaccination alone and BD vaccination plus targeted HBIG for preventing HBV MTCT.

5.6.2. Maternal antiviral prophylaxis: HBeAg testing and HBV viral load testing

From the recent growing body of evidence, providing maternal antiviral prophylaxis may eliminate the residual risk of HBV MTCT where active-passive immunization fails, in babies born to highly viremic HBV-infected mothers **(Brown et al., 2016; Pan et al., 2016; Jourdain et al., 2018)**. The recommendation of treating HBeAg positive women presenting with a viral load from 200 000

IU/mL has been made by international liver organizations **(Sarin et al., 2016; EASL, 2017; Terrault et al., 2016, 2018)**.

Treating HBeAg positive women with a viral load ≥ 200 000 IU/ml with TDF during pregnancy (from third trimester) in addition to pediatric active-passive immunization, led to the least number of HBV pediatric infections (three infections) at high total costs: US$153 053.55 with HBeAg testing, and US$228 238.62 with HBV viral load testing. The average cost per pregnant woman was US$15.31 and US$22.82 using HBeAg testing and HBV viral load testing, respectively, to prevent HBV MTCT. The difference in costs between these two interventions was primarily driven by the prices of the two diagnostic tests: US$41.72 for the HBeAg test and US$142.52 for the viral load test. In the one-way sensitivity analysis, when the price of the HBV viral load test was similar to the price of the HBeAg test, using HBV viral load testing to inform on maternal treatment was cheaper by US$43.67. The US$43.67 difference in costs was due to the prices of the consumables used for blood collection for these two tests (EDTA blood tubes for the viral load test and SST blood tubes for the HBeAg test).

The purpose of testing for HBeAg and HBV viral load is to identify highly infectious HBsAg pregnant women, whose babies have the highest risk of acquiring HBV through perinatal transmission. Although HBeAg is often used as a surrogate marker to assess viral replication, viral load testing remains the most accurate tool to determine the infectivity of HBV-infected individuals. The majority of HBeAg positive women **(Andersson et al., 2013; Ducancelle et al., 2013; Seck et al., 2018)** or all HBeAg positive women **(Candotti, Danso & Allain, 2007; Chotun et al., 2017)** would carry levels of HBV DNA ≥ 200 000 IU/ml. A small number of HBeAg negative patients may also present with similar viremia. For instance, in a recent Chinese study, 24 out of 758 (3.2%) HBeAg negative pregnant women had a high viral load of ≥ 200 000 IU/mL **(Lu et al., 2017)**. Similar findings were previously reported in Indonesia **(Fujiko et al., 2015)**. In the context of SSA where genotypes A, D, and E are found, Seck et al. (2018) described 13 out of 28 (46.4%) highly viremic (HBV DNA levels ≥ 200 000 IU/ml) Senegalese pregnant women with an HBeAg seronegative status **(Seck et al., 2018)**. High viremia in HBeAg negative pregnant women may be explained by the presence of BCP and pre-C mutations, as discussed in section 4.7.6.4. The selection of mutations in the BCP and PC region of the viral genome would abolish

or reduce the production of HBeAg, without affecting viral replication **(Thompson et al., 2010; Fujiko et al., 2015)**. Consequently, with an antiviral prophylaxis strategy in which HBeAg testing is used to inform on the need for HBV treatment, a number of highly viremic HBeAg negative pregnant women with a high risk of transmitting HBV to their babies would be missed.

Assuming that all HBeAg positive women would carry levels of HBV DNA ≥ 200 000 IU/ml, using HBeAg testing was the preferred option as opposed to viral load testing to inform on the need for maternal antiviral prophylaxis in this study. The maternal antiviral prophylaxis strategy involving HBV viral load testing was removed from the analysis due to absolute dominance: it costed more than using HBeAg testing but had the same effectiveness. The scenario may have been different with reliable SSA data regarding the proportion of HBsAg positive pregnant women, including both HBeAg positive and HBeAg negative, with HBV DNA levels ≥ 200 000 IU/ml to use in the model. To further elucidate the cost-effectiveness of an antiviral prophylaxis strategy using sequential HBV viral load testing, additional studies reporting HBV DNA viremia in HBsAg positive pregnant women, both HBeAg positive and HBeAg negative, in SSA are warranted.

Both HBV DNA quantification and HBeAg diagnostic assays are currently only available at the laboratory and are performed on sophisticated, expensive instruments operated by skilled laboratory technologists **(Villar et al., 2015; Peeling et al., 2017)**. They make use of a large number of costly resources; hence their high costs. It is worth mentioning that despite the availability of the instrumentation required for these two tests at the local Namibian laboratory, these assays are not performed locally. Samples are shipped elsewhere for testing. The high costs associated with shipping blood samples may explain the high costs of the HBeAg and HBV viral load tests in Namibia. Implementation of these diagnostic tests locally may reduce their costs and render the strategy less costly in this setting. The need for inexpensive, RDTs for either of these two viral markers is also strongly highlighted in these results. Although a few HBeAg RDTs are commercially available, recent data indicated poor diagnostic sensitivity and analytical sensitivity of these RDTs **(Seck et al., 2018)**. Currently, there is only one POC assay for HBV viral load testing: the newly developed GeneXpert HBV DNA viral load POC diagnostic technology

(Cepheid Inc, Sunnyvale, California, USA). There is no data regarding the performance of the assay as yet.

5.6.3. Maternal antiviral prophylaxis with TDF compared to universal HBV BD vaccination and targeted HBIG only

When compared to active-passive immunization, adding maternal treatment with TDF starting at the third trimester of pregnancy using sequential HBeAg testing, was at an incremental cost of US$38 727.59 for averting six more pediatric infections – equating to US$6 262.42 per pediatric HBV infection averted (ICER). Judgement about whether the health benefit from this intervention, in comparison to the BD vaccination + HBIG strategy (ICER = US$4 550.34), is worth the money spent should ideally be made by comparing this ICER to a WTP threshold **(Drummond et al., 2015)** or CET **(Leech et al., 2018; Thokala et al., 2018)**. The CET should be country-specific, reflecting local preferences and resource availabilities **(Leech et al., 2018)**. To our knowledge, a CET does not exist in Namibia. In the absence of country-specific thresholds, the common practice has been to compare the ICER to multiples – one to three times – of the GDP per capita of the country in which the economic evaluation is performed **(Leech et al., 2018)**. Given the current criticism attached to this practice, recent work suggests that the interpretation of ICERs in relation to less than half of per capita GDP are likely to be more representative of the cost-effectiveness of health interventions **(Woods et al., 2016)**. In Namibia, this would be US$2 613.60 (Namibia's 2017 one times GDP per capita = $5 227.20) **(World Bank, 2018)**. However, this comparison can only be made when the ICER is expressed using a generic measure of health such as cost/QALY gained or per DALY averted. For instance, in South Korea, providing maternal antiviral prophylaxis was deemed cost-effective (ICER = US$16 159/QALY gained) at a WTP threshold of US$29 000/QALY gained; the GDP per capita of the country in 2014 **(Lee, Shin & Park, 2018)**. Interestingly, in the USA, introducing antiviral prophylaxis with lamivudine for pregnant women dominated their current strategy that includes universal BD vaccination + HBIG; it led to more QALYs gained at lesser costs. The authors reported an ICER of US$6 376/QALY saved for the antiviral prophylaxis strategy, in comparison to universal HBV vaccination **(Fan et al., 2016)**. ICERs expressed in disease-specific natural units, as is ours, have been compared to the country's one times GDP per capita in some studies. Devine et al. (2017) deemed universal BD vaccination

+ HBIG cost-effective at a WTP threshold of US$1 200, the GDP per capita of Myanmar; the ICER of the strategy (US$716.78) was lower than US$1 200 **(Devine et al., 2017)**. However, the validity of this practice is unclear.

Although our ICER may not be compared to multiples of Namibia's GDP per capita to inform on the value for money of the intervention, in comparison to the universal BD vaccination + HBIG strategy, it is reasonable to believe that averting six more pediatric HBV infections would avert more than six DALYs or lead to more than six QALYs gained. For example, in a recent South Korea model, the provision of antiviral prophylaxis with lamivudine averted 34 cases of pediatric CHB per 100 000 births and led to 82 QALYs saved; in comparison to universal BD vaccination + HBIG **(Lee, Shin & Park, 2018)**. The same intervention in the USA averted 489 pediatric chronic infections and saved 800 QALYs; based on a hypothetical 2010 birth cohort of four million infants **(Fan et al., 2016)**.

Further exploration of the ICER of this intervention in the sensitivity analysis revealed scenarios in which providing antiviral prophylaxis appeared as the preferred option, in comparison to universal BD vaccination + HBIG. By lowering the cost of the HBeAg test alone from US$41.72 to US$27.12, representing a 35% decrease of the base cost, the ICER of this strategy became lower than the ICER of the universal BD vaccination + HBIG strategy. When compared to universal HBV BD vaccination alone, the antiviral prophylaxis intervention prevented 28 extra pediatric infections at an incremental cost of US$126 330.11 – equating to an ICER of US$4 539.25 per infection averted. This ICER was still lower than the original ICER (US$4 550.34/infection averted) of the universal BD vaccination + HBIG strategy. The impact of a low-cost HBeAg test on the ICER of this intervention was further reiterated at a higher prevalence of HBeAg within the antenatal population; when used in combination in the two-way sensitivity analysis. As the proportion of pregnant women requiring antiviral prophylaxis increased, the ICER of the antiviral prophylaxis strategy decreased. Furthermore, as the prevalence of HBeAg/high HBV viral load among HBsAg positive pregnant women increased, a decrease of the cost of HBeAg was warranted to keep the intervention cheaper and more effective than the universal BD vaccination + HBIG strategy. What was indicated by these results was that by decreasing the cost of the HBeAg test alone, adding maternal antiviral prophylaxis to pediatric active-passive immunization would be

cheaper than pediatric active-passive immunization only, and more effective in Namibia. To achieve this goal, implementation of HBeAg testing locally would be required to reduce the cost of the HBeAg test. In doing so, extra costly activities such as shipping biological samples internationally would be eliminated.

A large proportion of the costs of this intervention was driven by the cost of the HBeAg test and of HBIG. The treatment of highly viremic HBeAg pregnant women with TDF, on the contrary, had little weight on the total costs of this intervention. The generic price of TDF used in the base analysis was US$16.16 for a 4-month course. This is lower than the price of US$207 per person per year (US$69 per 4-month course) offered to SSA countries by Gilead in 2016 **(Médecins Sans Frontières, 2016)**. If Namibia had to access TDF at a price of US$69 per 4-month course for the treatment of highly viremic mothers, the intervention would become more expensive. The availability of generic formulations of TDF has had a tremendous impact on access to treatment for HIV-infected pregnant women across SSA, for HIV PMTCT. As seen in this model, extending it to HBV mono-infected pregnant women would bring more benefits to prevent HBV MTCT.

5.6.4. HBsAg rapid testing

Unlike the universal BD vaccination strategy, provision of HBIG to HBsAg-exposed babies or TDF to HBeAg-exposed babies requires routine antenatal HBsAg screening. Screening may be performed at the laboratory or at the POC level. As opposed to laboratory testing, POC testing offers a number of advantages including easy manipulation, the need for fewer infrastructures, rapid turn-around time for results, and delivery of screening in remote areas **(Kost et al., 1999; Hanafiah, Garcia & Anderson, 2013; Chevaliez & Pawlotsky, 2018)**. For these benefits, the use of rapid testing at the POC level was used as the methodology for HBsAg screening in the model described in this study. Screening costs per patient tested at the clinic came to about US$6. This cost is much lower than the approximate cost of US$20 of an HBsAg laboratory-based EIA or CLIA test. Still, HBsAg testing at the POC level would require additional resources such as trained counselors, constant training of these counselors, and a robust QA scheme to ensure validity and accuracy of the results communicated to patients. These activities may have an impact on the costs of this practice. Our model presumed a health system with these infrastructures already available. In many SSA countries, rapid test-based HIV screening is provided routinely at ANCs

for HIV PMTCT. The model is merely suggesting the use of infrastructures that are already in place for HIV rapid testing to provide HBV rapid testing to pregnant women, rather than separating the two. During the antenatal study described in chapter 4, the QA scheme established for HBsAg screening was built on the scheme that was already in place for HIV. The packaging and dispatch of the HBV controls were performed through the same route used for the HIV controls, and testing of the controls on the rapid test strips were performed at the same time for both viruses. In doing so, HBsAg rapid testing remains low cost. However, in settings where HIV rapid testing is not available yet, these specific costs need further exploration prior to implementation.

The Determine HBsAg rapid test used to identify HBsAg positive pregnant women in the model carried a false positive rate of 0.9%; meaning that some children would receive HBIG when they do not need it in the maternal antiviral prophylaxis + universal BD vaccination + HBIG and in the universal BD vaccination + HBIG strategies. The higher is the false positivity rate of the rapid test; the more money would be spent to provide HBIG or to perform sequential HBeAg testing when it is not required. Although adverse effects of HBIG are rare, anaphylaxis has been previously documented **(Bulbul et al., 2010)**. Eliminating the unnecessary costs of providing HBIG to babies who do not need it, or to perform sequential HBeAg testing when it is not warranted, and the possibility of adverse effects in newborns due to HBIG would require continuous training of testers to limit false positive events. With regards to the diagnostic sensitivity of the test, a false negative rate of 9.2% was attached to the Determine^{TM} HBsAg rapid test. Although false negative results have been documented in patients with low levels of HBsAg and low viremia **(Njai et al., 2015; Chisenga et al., 2018)**, who would be at very low risk of transmitting HBV in the case of MTCT, the use of a sensitive rapid test remains vital. HBsAg rapid tests with poor diagnostic accuracy have been reported in the literature **(Shivkumar et al., 2012; Amini et al., 2017)**. Care must be taken in the choice of the rapid test for scale-up of HBV screening, depending on the purpose of the screening.

5.7. Strengths and limitations of the study

To our knowledge, this is the first economic evaluation for the prevention of HBV MTCT conducted in Namibia. It is also the first describing the costs and health outcomes associated with

the provision of HBIG against HBV MTCT in SSA. Important insights towards driving policy implementations and changes in Namibia are drawn out of the analysis. These include the low costs associated with routine antenatal rapid test-based HBsAg screening and the health benefits attached to the current national strategy for preventing HBV MTCT. As per the rationale of an economic evaluation, the analysis also suggests ways for better use of the local resources available. These include implementing the HBeAg test at the local laboratory to enable provision of maternal antiviral prophylaxis; which would generate higher health benefits at lesser costs compared to the current national BD vaccination and HBIG strategy in place.

This CEA also has a few limitations to consider. First, the long-term costs and effects associated with the severe sequelae of pediatric HBV infections were not evaluated in this analytical model. This limited our ability to appreciate the full value of preventing perinatal HBV infections. The ICERs were expressed in a disease-specific natural unit rather than generic units such as DALYs and QALYs. Thus they could not be compared to a CET/WTP threshold. The comparison of the ICERs to either of these two benchmarks would have enabled a more definitive assessment of the value for money of the interventions included in this analysis. Another limitation was the scanty availability of HBV epidemiological data from other provinces of Namibia. It led to uncertainties around the proportion of HBsAg positive pregnant women carrying levels of HBV DNA $\geq$ 200 000 IU/ml and the prevalence of HBeAg among pregnant women. The data available in Windhoek, from our study (Chapter 4), could not be extrapolated to other provinces given that the prevalence of the infection varies across the country. Other data limitations include uncertainty around transmission rates, especially from mothers receiving antepartum antiviral prophylaxis but whose babies do not receive HBIG or BD vaccine or both at birth. The unavailability of this data led to assumptions around coverage (100%) of the BD vaccine, HBIG and clinic delivery in Namibia. These assumptions may have led to an overestimation the ICER. Still, by assuming a 100% coverage of these practices, the calculated ICER portrays the true costs of scaled-up interventions for maximal benefits, in an ideal setting for the elimination of HBV MTCT.

CHAPTER 6: Discussion and conclusion

This chapter brings together the findings of the studied described in Chapters 3, 4 and 5 in the context of prevention of HBV MTCT in Namibia and possibly across the SSA region. The significance and originality of this study are also highlighted, and topics that deserve further investigation are brought forward.

6.1 General discussion

Horizontal transmission has been the main route of acquisition of HBV infections in children in SSA, leaving MTCT neglected in the region. The successful implementation and scale-up of infant HBV vaccination in the region, given from six weeks of age, has had a significant impact on the prevalence of HBV infection in the pediatric population. HBV vaccination at six weeks does not prevent HBV MTCT to neonates. As a result, MTCT has emerged as the current primary cause of pediatric CHB infections in the region **(Keane et al., 2016)**.

6.1.1 Chronic HBV infection in children

Chronic carriage of HBV in children is a serious health issue, yet to be fully controlled. Where treatment is indicated, options are limited, especially for children below the age of 12 years. Given that HBV-infected children typically present in the immunotolerant phase of the infection, treatment is usually postponed until the late phases of the disease, during adulthood. In the context of co-infection with HIV and HBV, characteristics of the immunotolerant phase of CHB were observed in eight out of 15 HIV/HBV (8/15; 53.3%) co-infected children and adolescents presented in Chapter 3; these children were treated with lamivudine for their HIV infection. Uncontrolled HBV infection in these children was due to the presence of viral quasispecies resistant to lamivudine, caused by prolonged exposure to the drug. Treatment of HIV/HBV co-infection ideally should be with TDF, given its high barrier to HBV resistance in comparison to lamivudine. However, with the current TDF dosage of 300 mg, bone toxicity and nephrotoxicity are a concern in children below the age of 10 years; and raises the issue of treatment of CHB in children too. Past the age of 10 years, a change of ART with TDF would be required for the control

of both HIV and HBV infections. Alternatively, E/C/F/TAF may be used; where it is available. Still, routine screening of pediatric HIV patients, before initiating HIV ART, in SSA is highly recommended to control both viral infections in these children.

With regards to liver disease progression, the course of CHB in the pediatric HIV population is not well established. Drawing from data generated in adults **(Berretta et al., 2011; Vinikoor et al., 2016; Boyd et al., 2017)**, it would be reasonable to assume that progression to liver disease may be hastened due to the immunosuppression brought by HIV. Severe liver diseases may develop at a younger age. Interestingly, despite suppressive therapy, the risk of liver disease in HIV/HBV co-infected children is not nullified. Seers et al. (2017) recently described a case of HCC in an HIV/HBV co-infected 20 years old male, whose HIV and HBV infections were both acquired through MTCT **(Seers et al., 2017)**. Co-infection with both viruses was diagnosed when the patient was six years old, and ART with TDF was initiated on compassionate grounds (prior pediatric licensing in 2012) due to his active HBV infection and the profound immunosuppression caused by HIV. At seven years of age, advanced fibrosis was observed on biopsy and HCC developed thirteen years later, despite being HIV and HBV virologically suppressed **(Seers et al., 2017)**. Drawing from the case report by Seers et al. (2017) HBV and HIV virologically suppressed HIV/HBV co-infected children reported in Chapter 3 are still at risk of severe liver damage and should also be closely monitored. The need for longitudinal follow-up data in African HIV/HBV co-infected children is warranted for a better understanding of the natural history of CHB in the context of HIV, and to improve clinical management of this key population. While results from the pediatric study (Chapter 3) are in agreement with the literature, they also add emphasis to the critical importance of interrupting, even eliminating, HBV MTCT.

6.1.2 Elimination of HBV MTCT

The toolbox for the elimination of HBV MTCT includes universal BD vaccination, HBIG, and more recently added, the use of antiviral drugs in highly viremic pregnant women. The last two interventions (HBIG and maternal treatment) require routine antenatal HBsAg screening. Many challenges exist with each of these tools **(Tamandjou et al., 2017; Dionne-Odom, Njei & Tita, 2018)**.

Routine antenatal HBsAg screening

Given, the expense and infrastructure required for HBsAg diagnostic testing, offering routine antenatal screening is a challenge in SSA. In the antenatal study (Chapter 4), implementing POC HBV rapid testing as an alternative of laboratory testing was found feasible, in terms of both the performance of the rapid test used and the perceptions of the testers. The Determine^{TM} HBsAg rapid test showed a good accuracy with regards to its ability to differentiate between HBV-infected and HBV-uninfected patients, in a rapid turnaround time. The cost of rapid testing ranged from US$5.81 to US$6.28 for an HBsAg negative RDT result and an HBsAg positive RDT result, respectively. With regards to implementation at health facilities, HCWs did not raise any concerns with the procedures of the test and recognized the advantages of this practice over laboratory HBV testing as an ANC service. Considering that HBV screening is taken away from the controlled environment that the laboratory offers and brought to the uncontrolled POC level, QA is a significant component to add in the implementation of HBV rapid testing. The QA structure would be dependent on resource availability, and the diagnostic accuracy of the rapid test of choice; thus, its structure would vary between settings. Constant training of the HCWs or lay counselors performing the tests would be necessary too.

In addition to accessing preventive measures against HBV MTCT, HBV screening also empowers these women to seek care for their own health, knowing their HBV serostatus. Linkage to care of HBV-infected pregnant women is warranted to avert future complications of the infection and the risk of HBV MTCT in future pregnancies. Monitoring for post-hepatic flares every three months for a period of six months, following discontinuation of therapy in women treated during pregnancy, is recommended **(Terrault et al., 2016, 2018)**. Extending ART, as HBV MTCT prevention, beyond delivery has shown no effect on the rate nor the severity of these flares **(Nguyen et al., 2014)**. Continued monitoring of disease progression, for both treated and untreated women postpartum, is also required to determine the need for future long-term treatment **(WHO, 2015)**. Although follow-up rates are unknown in SSA, data from elsewhere have shown that these rates are low **(Chang et al., 2015, 2016b)**. According to data by Chang et al. (2015), 47% (137/291) of HBV-infected women identified during pregnancy in Massachusetts, USA, had received HBV specialized care post-partum. Only 19% of these HBV-infected women (55/291)

had been appropriately tested to monitor the progression of the infection (HBeAg and HBV DNA levels) and liver health (ALT), within a year of their HBV diagnosis **(Chang et al., 2015)**. Reasons for the low rate of follow-ups may include the absence or the lack of knowledge of a referral system, and the suboptimal knowledge of CHB infection diagnosis among pregnancy-specialized HCWs to recognize the need for referrals **(Kwong et al., 2018)**. Shortage of liver health or infectious disease specialists and adherence of patients to follow-ups could also impact follow-up rates. Decentralization of HBV follow-up care services to the primary healthcare level may be required, as it is currently done for the prescription and management of ART in HIV PMTCT programmes **(Zuber et al., 2014)**. Firm evidence on the challenges associated with low or poor linkage-to-care of HBV-infected pregnant women in SSA needs to be identified and tackled.

HBV BD vaccination

The critical role of BD vaccination for the prevention of HBV MTCT cannot be overstated. Nonetheless, the cold chain requirements and home births have impeded its implementation and scale-up in SSA. Drawing from the experience in Windhoek, a high proportion of births took place at the health facilities, including HBsAg-exposed births. All 25 HBsAg-exposed babies followed-up were delivered at the healthcare facilities and were vaccinated against HBV at birth. These observations re-emphasized the role of healthcare facilities-based births for successful scale-up of HBV BD vaccination in SSA. In settings with a high proportion of outside-health-facilities deliveries, alternative delivery strategies of the HBV BD vaccine, such as the controlled temperature chain (CTC), may be considered. The CTC strategy has been endorsed by the WHO, provided the availability of monovalent vaccines licensed for use in the CTC **(WHO, 2017b)**. This strategy would be cost-saving by $1 920 (95% CI: US$1 540 – US$2 140) per 1000 births and would avert 167 DALYs (95% CI: 136 – 198) per 1000 births, in comparison to no CTC in SSA **(Scott et al., 2018)**. It should be noted that estimates calculated by Scott et al. (2018) were derived from SSA countries, including Namibia, where routine BD vaccination already exists. The efficacy attributed to vaccine timing for preventing MTCT, used in the model by Scott et al. (2018), was based on estimates extracted from a study **(Marion et al., 1994)** in which HBIG was provided to neonates at birth. Thus, care must be taken during the interpretation of Scott et al. (2018) findings **(Scott et al., 2018)**. Education of pregnant women during ANC visits is also a critical approach to

consider. These antenatal care visits are opportunities to encourage pregnant women to bring their newborn shortly after birth for BD vaccination. These visits may also be used to bring awareness of HBV in the antenatal population and of prevention of HBV MTCT, and to raise the importance of HBV BD vaccination to prevent HBV MTCT. A growing body of evidence has described poor knowledge of HBV among pregnant women. Many have never heard of HBV infection **(Shah et al., 2015)**, and are not aware of the risk of transmission of the virus to their newborns **(Adeyemi et al., 2013; Abdulai et al., 2016; Dun-Dery et al., 2017; Chaquisse et al., 2018)**, in comparison to HIV **(Chaquisse et al., 2018)**.

As demonstrated in the health economics component of this study (chapter 5), BD vaccination is of low cost (US$15 827.22) and evidently effective. Therefore, scale-up of this intervention must be the forefront element in strategies implemented against HBV MTCT in SSA.

Hepatitis B immunoglobulin (HBIG)

Our model also showed that the sole use of HBV BD vaccination might not be enough for the elimination of HBV MTCT. Added to the constraints of cold chain requirements, similarly to BD vaccine, the feasibility of offering HBIG in SSA is challenging given its high cost and restrained supply **(Spearman et al., 2017; Dionne-Odom et al., 2018)**. Where it is available, constant availability would be challenging. Fifteen out of the 25 babies followed-up in the antenatal study (Chapter 4) had received HBIG while the rest did not, for various reasons that include stock availability and oversight from the HCWs. While the cost and supply of HBIG are important issues to consider, the effectiveness of the combined use of these two immunoprophylaxis measures cannot be overlooked. In the base analysis of this study's decision analytic model, 22 extra pediatric infections were averted with the addition of HBIG to BD vaccination. Although this intervention is the current status quo in Namibia, coverage is unknown. Therefore, the full benefits of this strategy, for preventing HBV MTCT in Namibia, are unknown. Still, the model indicated that a step closer to the elimination of HBV MTCT would be achieved in this setting with full coverage of this intervention.

Maternal antiviral prophylaxis

The residual number of pediatric infections noted with the BD vaccine + HBIG strategy, mostly from highly viremic mothers, would be eliminated with maternal antiviral prophylaxis. The use of antiviral drugs in HBV-infected pregnant women with high viral load (HBV DNA levels ≥ 200 000 IU/ml) requires HBV DNA testing, which is not accessible in many SSA settings **(Spearman et al., 2017; Dionne-Odom et al., 2018)**. Where it is available, the high costs associated with the test do not permit routine testing. Thus, HBeAg remains the only measure of infectiousness in these women, and tool to determine treatment eligibility **(Dionne-Odom et al., 2018)**. Certainly, the intervention including HBsAg rapid testing, BD vaccination, HBIG and the provision of maternal ART using sequential HBeAg testing was expensive (ICER = US$6 262.42 per additional infection averted). However, better use of the resources that are already available in Namibia, such as performing HBeAg testing locally with the platform that is currently used for HBsAg testing at the laboratory, would render this intervention cheaper than the use of BD vaccination and HBIG alone. For these reasons, the interpretation of the ICERs recorded in the economic evaluation presented in this study must not stop at the monetary value attached to the prevention of a pediatric infection. The parameters, such as the prevalence of HBsAg and HBeAg represented into these ICERs, also need consideration for decision making. Consequently, additional efforts are required to generate additional HBV prevalence data in this population for a better understanding of the epidemiology of the infection within the Namibian antenatal population. This intervention (HBeAg testing and maternal antiviral prophylaxis) could also form part of gateways to establish or improve linkage to care in the country.

HBsAg screening, maternal antiviral prophylaxis and HBV immunization

The high costs of the screen-treat-vaccinate intervention, using sequential HBeAg testing, investigated in this study was also driven by the cost of HBIG. The high cost of this immunoprophylactic agent questions its scale-up in SSA. The idea of a screen-treat-BD vaccine alone for SSA, thus, becomes attractive. This intervention was recently investigated in South Africa and produced an ICER of US$26 241/DALY averted. At a CET equals to half per South Africa's GDP per capita (South Africa's 2015 GDP per capita = US$7 620), the strategy was not cost-effective **(Hecht et al., 2018)**. The analysis conducted by Hecht et al. (2018) made use of a

theoretical model developed by Nayagam et al. (2016b), who had previously investigated this strategy (screen-treat-BD vaccine) at a global scale **(Nayagam et al., 2016b)**. The cost-analysis component of the model developed by Nayagam et al. (2016b) included HBsAg rapid test-based screening at the ANCs, treatment with TDF, and the human resources to deliver both BD vaccine and treatment at healthcare facilities. The cost of consumables and human resources associated with the delivery of treatment amounted to 80% of the total drug management for person per year and was the most expensive component of this intervention **(Nayagam et al., 2016b)**. It is likely that these costs also had the biggest impact on the ICER reported by Hecht et al. (2018); especially if only a small proportion of HBV mono-infected, highly infectious, pregnant women would require treatment. In terms of effectiveness, the model assumed a 0.5% rate of HBV MTCT from treated-HBeAg positive women with BD vaccination provided to their babies; a rate similar to HBeAg negative pregnant women whose babies receive BD vaccination at birth. As pointed by Nayagam et al. (2016b), effectiveness data on this strategy from clinical trials are currently lacking. The assumption takes into consideration that a high viremia is the only cause of breakthrough infections in babies born to highly infectious mothers. Yet, the immune-tolerogen characteristic of HBeAg may also play a role in immunoprophylaxis failure in these babies and may need to be accounted for.

Sequential HBV viral load or HBeAg testing are required to identify women with the need for treatment for the prevention of HBV MTCT. It must be acknowledged that the majority of SSA does not have access to both diagnostic tests. The alternative would be to provide antiviral prophylaxis with TDF to all HBsAg positive pregnant women. This health intervention was recently put forward by Spearman et al. (2017) **(Spearman et al., 2017)**, and present with a few advantages. Eliminating the need to perform costly HBV viral load and HBeAg testing may potentially reduce the costs of preventing HBV MTCT. The delayed turnaround time associated with HBV viral load or HBeAg testing often leads to loss of follow-up of patients, who may end up not starting treatment. Treating all HBsAg positive pregnant women could serve as a safety net to catch all women with the highest risk to transmit HBV to their infants. The health benefits associated with the intervention would be maximized, provided that treatment is initiated on the day of HBV diagnosis as it is currently done for HIV PMTCT. A few limitations remain. Maternal

treatment is at present recommended for up to three months after birth for the purpose of HBV PMTCT **(EASL, 2017)**. Multiple pregnancies in HBsAg positive pregnant women may lead to multiple on and off exposure to the drug and repeated post-treatment hepatic flares, especially for women who may not necessarily need treatment. The long-term consequences of these flares and the risks of developing antiviral drug resistance following disrupted treatment are unknown. Adherence rate of pregnant women to therapy during pregnancy and the effectiveness of this intervention with low adherence to treatment, are also not known. Consequently, the health benefits and risks attached to this strategy require further investigations for recommendations.

6.2 Significance and originality of this research project

The findings of this research project provide evidence-based data, which were previously not available, for policy-making against HBV MTCT in Namibia and may possibly be translated to the rest of SSA. To our knowledge, this is the first investigation of the health impact of BD vaccination in combination with HBIG and maternal antiviral prophylaxis for the prevention of HBV MTCT in Namibia and in SSA. The results of the study strengthen the call for implementing universal HBV BD vaccination in the region. They also demonstrate that routine rapid HBV testing as an add-on to existing HIV POCT infrastructures is feasible in Namibia and possibly in other SSA countries where HIV rapid testing is already in place. The challenges associated with the integration of this practice and to implement provision of maternal antiviral prophylaxis for HBV PMTCT into existing health systems are also highlighted. These stumbling blocks are important considerations for the roll-out of these health interventions. HBV awareness and the importance of HBV PMTCT were indirectly raised among HCWs through the completion of the study and may have improved their attitudes towards HBV PMTCT.

This is also the first economic evaluation of a screen-treat-vaccination intervention, with HBIG, against HBV MTCT in SSA. This data add to the limited economic literature available in the region. The results of this economic evaluation confirmed the low costs associated with BD vaccination. Routine antenatal HBsAg screening was proven cheap and should be implemented for HBV PMTCT in SSA countries. The investigation of four HBV PMTCT strategies provide

assistance for decision-making and may be used as a guide for future economic evaluations in Namibia and other SSA countries.

The originality of this research study lies in its inter-disciplinary nature. Epidemiology, virology, social science, and health economics were used to address a global public health issue. The epidemiology component of the study provided updated HBV statistics, including HBeAg seroprevalence and HBV viral loads, in the Namibian obstetric population. The need for robust and extensive HBV epidemiological data, for a better understanding of the epidemic and for policy planning, cannot be overly emphasized. The virology aspect of the study demonstrated the diversity of viral strains circulating in Namibia. The last report on the molecular characterization of HBV strains in the country date from an investigation conducted more than a decade ago **(Kramvis et al., 2005)**. The investigation by Kramvis et al. (2005) focused on the characterization of genotype E viruses only, making the present study the first report on the diversity of HBV strains circulating in Namibia. Social science methodologies were used to determine the feasibility of offering rapid test-based HBsAg screening. The lack of resources has been and remains a stumbling block for the implementation of health intervention in SSA. As such, the principles and the fundamentals of economic evaluation were used to generate the first costs and health benefits associated to interventions including HBIG and antenatal antiviral prophylaxis to prevent HBV MTCT, from an SSA perspective.

6.3 Future research focus

Interesting opportunities for further investigations are drawn from this study. The long-term impact of HIV/HBV co-infection in children, with controlled and uncontrolled HBV infection, deserves further research. The clinical health impact and the economic impact of alternative HBV PMTCT strategies such as maternal antiviral prophylaxis for all HBsAg positive pregnant women or HBeAg positive women only, with vaccination starting at birth or from six weeks also warrant further investigations. Additional work is required to determine follow-up rates of HBV-infected women identified during pregnancy and to assess effective strategies for linkage to care in this key population. Large-scale studies are warranted to measure the costs and the feasibility of

implementing HBV public health education programmes. The impact of these programmes on HBV PMTCT in SSA is worth exploring too.

6.4 Concluding remarks

In Namibia, elimination of HBV MTCT is achievable using a screen-treat-vaccinate (BD vaccine + HBIG) strategy at a relatively high cost. As was illustrated in this research project, the cost of this strategy can potentially be reduced by lowering the cost of the diagnostic test used to determine treatment eligibility for women at highest risk of HBV MTCT. Alternatively, treating HBV-infected pregnant women with high risk of transmission in addition to universal BD vaccination only may also be sufficient to achieve elimination. Effectiveness data for a screen-treat-birth dose vaccination strategy are required in order to confirm this hypothesis and to update currently available cost-effectiveness models for informed policy-making.

With regards to the rest of SSA, intervention strategies against HBV MTCT must be customized for each setting. The epidemiology of HBV infection, the proportion of HBeAg positive or highly viremic pregnant women with the highest risk of HBV MTCT, and the costs of the resource needed to implement these interventions must be considered. To this aim, regional efforts are required to generate additional robust epidemiological, clinical and economic data on HBV. Additional initiatives to create awareness and improve HBV knowledge among the general public, policy-makers and HCWs are also needed. Establishing new collaboration and strengthening existing ones are also essential to improve intervention strategies and build innovative approaches to obtain maximum health benefits.

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Appendices

Appendix A - Chapter 3 Study Stellenbosch University HREC original approval letter

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Approval Notice
Response to Modifications- (New Application)

21-May-2013
Andersson, Monique MI

Ethics Refernce #: N13/02/022
Title: **A pilot study of Hepatitis B Virus infection in HIV infected children in Namibia**

Dear Doctor Monique Andersson,

The **Response to Modifications - *(New Application)*** received on **16-Apr-2013**, was reviewed by members of **Health Research Ethics Committee 2** via Expedited review procedures on **17-May-2013** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **20-May-2013 -20-May-2014**

Please remember to use your **protocol number** (**N13/02/022**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Included Documents:
APPLIC FORM
DEC LETTER RUKATO
SYNOPSIS
DEC LETTER2
DEC LETTER 5
COV LETTER
CV3
DEC LETTER 4
IC ASSENT
PROTOCOL

Appendix B – In-house validation of the COBAS AmpliPrep/COBAS TaqMan HBV test Version 2.0 using one DBS specimen for HBV viral load

B.1. Aim

The aim of this evaluation was to assess the use of DBS for quantifying HBV with the COBAS AmpliPrep/COBAS TaqMan HBV Version 2.0 quantitative assay for use in the diagnosis and treatment of HBV infected patients.

B.2. Methodology

B.2.1. Limit of detection (LoD) and limit of quantification (LoQ) of the one DBS assay

Repeat testing of DBS prepared with dilutions of the third HBV WHO international standard for NAT assays (NIBSC 10/264) were used to infer both the LoD and LoQ of the assay. The linearity of the assay was also examined. Dilutions of the NIBSC were made in a known HBV negative whole blood sample, and 50 µl was spotted on Whatman 903 DBS cards. One DBS for each dilution was tested in triplicates once and thereafter in one replicate over six different days. A linear regression analysis was also performed to determine the levels of linearity of the assay. The Probit analysis was completed, with SPSS Version 25, to determine the LoQ of the assay.

B.2.2. Correlation and linearity between DBS and serum sample measurements

A total of 55 plasma were retrieved at the NHLS Virology laboratory for this evaluation. A volume of 50 µl of each whole blood sample was spotted on Whatman 903 DBS cards, and left to dry overnight. Plasma were collected from the residual whole blood samples. Paired DBS and plasma samples were tested with the COBAS AmpliPrep/COBAS TaqMan HBV Version 2.0. Prior testing, elution of the DBS was performed as described in Chapter 3, Section 3.4.9.1. The correlation between these two measurements was analyzed using a Bland-Altman plot, and linear regression analysis. Plasma samples with < 20 IU/ml (< 1.30 $\log_{10}$ IU/ml) or > 170 000 000 IU/ml (> 8.3$\log_{10}$ IU/ml) results were recorded as 10 IU/ml (1 $\log_{10}$ IU/ml) and as 8.3$\log_{10}$ IU/ml, respectively. Plasma samples with an undetectable viral load were removed from the analyses.

B.3. Results

B.3.1. Limit of detection (LoD) and limit of quantification (LoQ) of the one DBS assay

Table B.1 below summarizes the average values detected at the different dilutions. As observed in Table B.1, viral loads were undetectable below 2 $\log_{10}$ IU/ml. The LoD of the one DBS assay was, thus, concluded at 100 IU/ml (2 $\log_{10}$ IU/ml). The Probit analysis was completed using the data tabulated in Table B.2. The LoQ of the assay by the Probit analysis at the 95% quantification rate was at approximately 843 IU/ml (2.93 $\log_{10}$ IU/ml) (95% CI: 479 IU/ml – 2600 IU/ml) (Table B.3). The linear range of detection was concluded between 5 $\log_{10}$ IU/ml and 3 $\log_{10}$ IU/ml, as illustrated in Figure B.1.

Table B.1: HBV DNA levels measurements in one DBS using dilutions of the NIBSC 10/264.

NIBSC HBV DNA levels*	NIBSC HBV DNA levels ($\log_{10}$)	DBS HBV DNA levels	DBS HBV DNA levels[#] ($\log_{10}$)	X dilution factor 50	X dilution factor 50 ($\log_{10}$)
0	-	LDL	-	-	-
1	-	LDL	-	-	-
10	1.00	LDL	-	-	-
100	2.00	< 20	< 1.30	-	-
1000	3.00	60	1.78	2983	3.47
10 000	4.00	572	2.76	28 600	4.46
100 000	5.00	4824	3.68	241 222	5.38

*HBV DNA levels are expressed in IU/ml. [#]Calculated from nine replicates of each dilution. DBS: dried blood spot; HBV: hepatitis B virus; NIBSC: National Institute for Biological Standards and Control.

Table B.2: Limit of quantification in one DBS of the COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0 assay.

NIBSC HBV DNA levels (IU/ml)	NIBSC HBV DNA levels ($\log_{10}$ IU/ml)	Number of replicates	Number quantified	Positivity rate
10	1,00	9	0	0%
100	2,00	9	0	0%
1000	3,00	9	9	100%
10 000	4,00	9	9	100%
100 000	5,00	9	9	100%
Probit* 95% hit rate: 843 IU/ml (95% CI: 479 IU/ml – 2600 IU/ml)				

*The results of this analysis are detailed in Table B.3 below. DBS: dried blood spot; HBV: hepatitis B virus; NIBSC: National Institute for Biological Standards and Control.

Table B.3: Outputs of the Probit analysis and confidence intervals.

	95% Confidence Limits for NIBSC HBV DNA concentrations		
Probability	**Estimate**	**Lower Bound**	**Upper Bound**
0.010	124.829	-1416.304	489.180
0.020	174.107	-1196.720	544.593
0.030	205.372	-1060.809	583.158
0.040	228.892	-960.675	614.277
0.050	248.023	-880.750	641.115
0.060	264.307	-813.919	665.156
0.070	278.585	-756.310	687.225
0.080	291.369	-705.573	707.829
0.090	302.996	-660.167	727.305
0.100	313.698	-619.028	745.891
0.150	358.008	-456.106	830.243
0.200	393.224	-336.179	906.842
0.250	423.437	-240.813	980.078
0.300	450.569	-161.437	1052.111
0.350	475.710	-93.279	1124.256
0.400	499.567	-33.360	1197.472
0.450	522.649	20.343	1272.578
0.500	545.365	69.300	1350.388
0.550	568.081	114.650	1431.805
0.600	591.163	157.330	1517.933
0.650	615.020	198.177	1610.221
0.700	640.162	238.010	1710.691
0.750	667.293	277.738	1822.373
0.800	697.506	318.533	1950.180
0.850	732.722	362.208	2103.030
0.900	777.032	412.310	2300.203
0.910	787.735	423.725	2348.512
0.920	799.361	435.859	2401.260
0.930	812.145	448.899	2459.562
0.940	826.423	463.113	2525.025
0.950	842.707	478.909	2600.102
0.960	861.838	496.946	2688.828

0.970	885.358	518.425	2798.601	
0.980		916.623	545.926	2945.577
0.990		965.901	587.210	3179.290

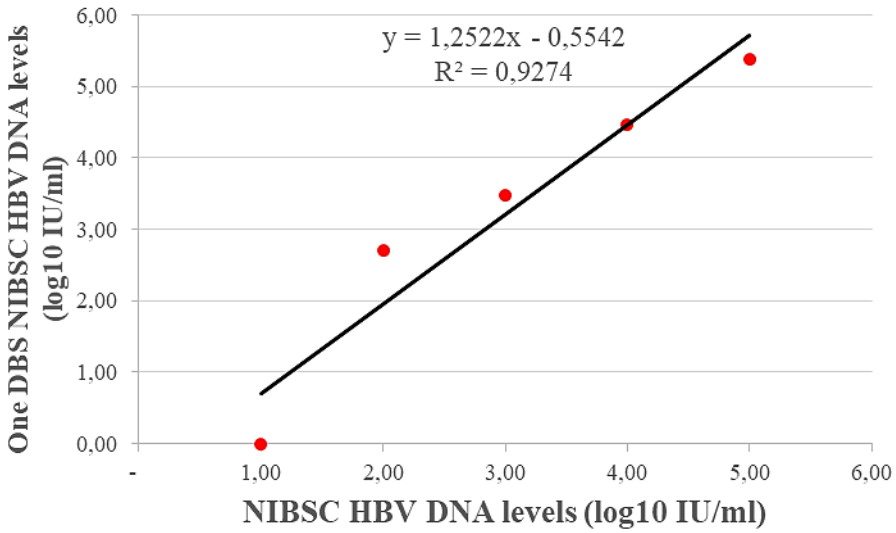

Figure B.1: Linearity of the one DBS assay using the NIBSC 10/264 WHO standard. The linearity of the assay starts from 1000 IU/ml (3 log_{10} IU/ml). DBS: dried blood spots; HBV: hepatitis B virus; NIBSC: National Institute for Biological Standards and Control.

B.3.2. Correlation, linearity, agreement between DBS and serum sample measurements

Thirty four out of the 55 samples (34/55; 61.8%) and 22 (22/55; 40.0%) had detectable HBV DNA in plasma (Range: (< 1.30 log_{10} IU/ml - > 8.3log_{10} IU/ml) and in one DBS (Range: 1 log_{10} IU/ml – 6.80 log_{10} IU/ml, without dilution factor adjustment), respectively (Table B.4). The mean difference between HBV DNA levels measurements using plasma and one DBS samples (with dilution factor adjustment) was -0.37 log_{10} IU/ml (95% CI: -0.62 log_{10} IU/ml – 0.12 log_{10} IU/ml; p = 0.005). These differences fell within the 95% limits of agreement (-1.03 to 1.77 log_{10} IU/ml) of the mean difference, except for one (Figure B.2). HBV DNA viral loads between the two sample types correlated well (R^2 = 0.93) and good linearity was observed at viral loads from 3 log_{10} IU/ml (Figure B.3), as previously observed in Figure B.1.

Table B.4: HBD DNA levels in paired plasma and DBS clinical samples. HBV DNA levels are expressed in IU/ml.

Plasma HBV DNA levels	Plasma HBV DNA levels (log_{10})	DBS HBV DNA levels	DBS HBV DNA levels (log_{10})	X dilution factor (50)	X dilution factor (50) (log_{10})
2810	3.45	< 20*	-	-	-
939	2.97	< 20	-	-	-
658	2.82	< 20	-	-	-
1060	3.03	< 20	-	-	-
450	2.65	< 20	-	-	-
2000	3.30	24,5	1.39	1225	3.09
4180	3.62	78,8	1.90	3940	3.60
12300	4.09	138	2.14	6900	3.84
3160	3.50	140	2.15	7000	3.85
9030	3.96	269	2.43	13450	4.13
3980	3.60	309	2.49	15450	4.19
63000	4.80	588	2.77	29400	4.47
54500	4.74	2380	3.38	119000	5.08
99900000	8.00	46900	4.67	2345000	6.37
10300000	7.01	81100	4.91	4055000	6.61
> 170000000[#]	> 8.23	95200	4.98	4760000	6.68
> 170000000	> 8.23	368000	5.57	18400000	7.26
24800000	7.39	408000	5.61	20400000	7.31
> 170000000	> 8.23	1550000	6.19	77500000	7.89
> 170000000	> 8.23	6240000	6.80	312000000	8.49
148	2.17	< 20	-	-	-
217	2.34	< 20	-	-	-
24,9	1.40	LDL[$]	-	-	-
29	1.46	LDL	-	-	-
< 20*	< 1.30	LDL	-	-	-
< 20	< 1.30	LDL	-	-	-
< 20	< 1.30	LDL	-	-	-
< 20	< 1.30	LDL	-	-	-
37	1.57	LDL	-	-	-
< 20	< 1.30	LDL	-	-	-
42,8	1.63	LDL	-	-	-
< 20	< 1.30	LDL	-	-	-

< 20	< 1.30	LDL	-	-	-
100	2.00	LDL	-	-	-

*Samples with a < 20 IU/ml viral load result were assigned a viral load of 10 IU/ml for the purpose of the analysis. #These samples were assigned a viral load of 170 000 000 IU/ml. $These viral loads were assigned a 0 IU/ml value in the analyses. HBV: hepatitis B virus. LDL: lower than the detection limit.

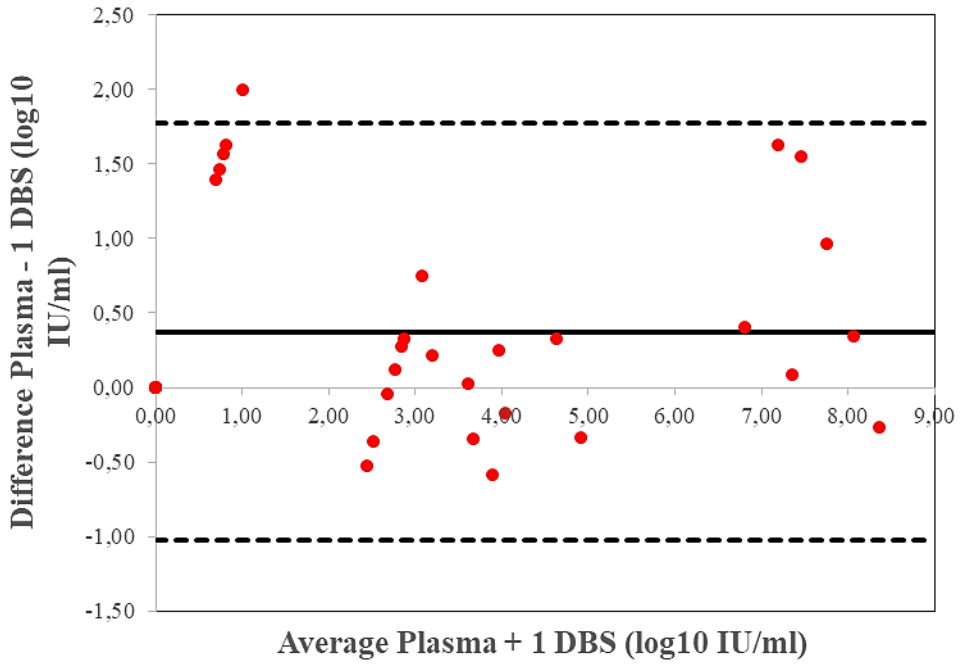

Figure B.2: Bland-Altman plot of agreement between HBV DNA viral loads in 34 paired plasma samples and DBS samples. The solid line represents the mean difference between the plasma and DBS viral load measurements. The dashed lines represent the upper and lower limit of agreements (±1.96 standard deviations) of the differences between the measurements of both samples types. DBS: Dried blood spots; LoA: lower limit of agreement; UoA: upper limit of agreement.

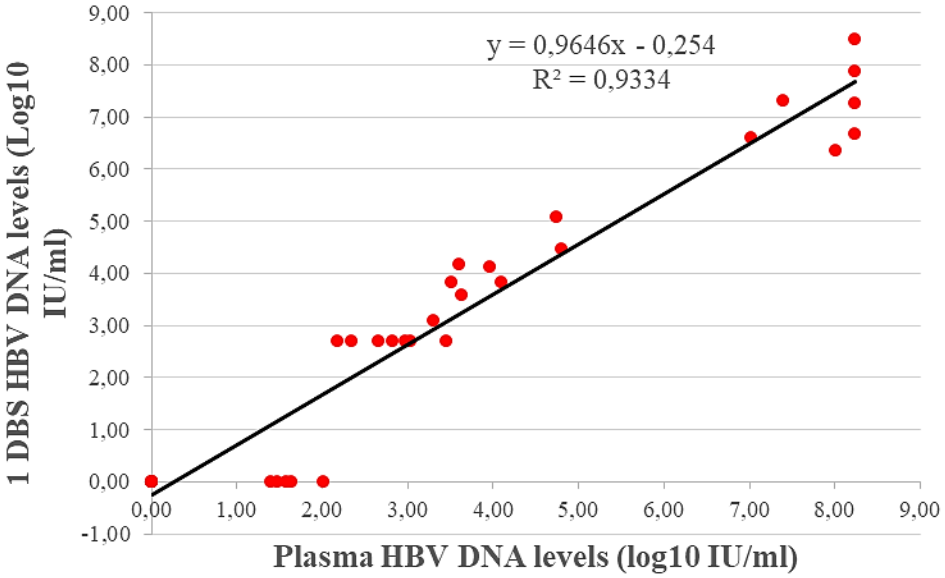

Figure B.3: Linear regression analysis of HBV DNA levels in 34 paired DBS and plasma samples. The graph shows a good correlation (R^2 = 0.93) between measurements attained in one DBS and plasma samples. DBS: dried blood spots; HBV: hepatitis B virus.

Appendix C – Chapter 4 Study Stellenbosch University HREC original approval letter

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Approved with Stipulations
New Application

09-Feb-2017
Tamandjou, Cynthia CR

Ethics Reference #: S17/01/001

Title: **A study on the feasibility and cost analysis of rapid test-based routine screening for hepatitis B virus in pregnancy for the prevention of mother-to-child transmission in Namibia**

Dear Miss Cynthia Tamandjou,

The **New Application** received on **26-Oct-2016**, was reviewed by members of **Health Research Ethics Committee 1** via Expedited review procedures on **03-Feb-2017**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **09-Feb-2017 -08-Feb-2018**

The Stipulations of your ethics approval are as follows:
This study can only begin once the Namibian authorities have granted ethical approval consequent to this HREC approval.

Please remember to use your **protocol number** (**S17/01/001**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Appendix D - Chapter 4 Study Ministry of Health and Social Sciences approval letter

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 – 203 2562
Fax: 061 – 222558
E-mail: hnangombe@gmail.com

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3
Enquiries: Dr. H. Nangombe

Date: 08 June 2017

Ms. Cynthia R. Tamandjou
Stellenbosch University
Division of Medical Virology
16959531@sun.ac.za

Dear Ms. Tamandjou

Re: A study of the prevention of mother-to-child transmission of hepatitis B virus in Windhoek Namibia

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

3. Kindly be informed that permission to conduct the study has been granted under the following conditions:

3.1 The data to be collected must only be used for academic purpose;

3.2 No other data should be collected other than the data stated in the proposal;

3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;

3.4 A quarterly report to be submitted to the Ministry's Research Unit;

3.5 Preliminary findings to be submitted upon completion of the study;

3.6 Final report to be submitted upon completion of the study;

3.7 Separate permission should be sought from the Ministry for the publication of the findings.

Yours sincerely,

Andreas Mwoombola (Dr)
Permanent Secretary

PERMANENT SECRETARY
MINISTRY OF HEALTH & SOCIAL SERVICES

"Health for All"

Appendix E - Chapter 4 Study Namibia Institute of Pathology approval letter

NAMIBIA INSTITUTE OF PATHOLOGY LIMITED
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OFFICE OF THE CHIEF OPERATIONS OFFICER

Enquiries: Mr Boniface Makumbi; Tel.: 061-295 4210

Date: 07 June 2017

Ms Cynthia Raissa Tamandjou
Stellenbosch University
Division of Medical Virology
Faculty of Medicine and Health Sciences
Cape Town
South Africa

Dear Ms Tamandjou

RE: A Study on the feasibility and cost analysis of rapid testing based routine screening for hepatitis B virus in pregnancy for the prevention of mother-to-child transmission in Namibia.

1. The above mentioned research proposal was referred to the Research/Ethics Committee of the Namibia Institute of Pathology Limited for review.
2. It is a pleasure to inform you that that approval was granted for you to proceed with the research on condition that the following be complied with:

2.1 No data should be collected other than the data stated in the research proposal.

2.2 Observe and adhere to all ethical considerations and confidentiality to protect patient information by delinking patient identification from the specimens, and let it be clear in the consent form.

2.3 There be an appointed Co-investigator from the Namibia Institute of Pathology Limited.

2.4 That at least three rapid test kits from the WHO approved list be evaluated/validated at NIP so that a rapid test kit which is highly specific and sensitive for our settings be selected.

2.5 Please clarify which results will be used for patient management, if any, if so we suggest that only the laboratory confirmed results be used, and this should be clearly stated in this research proposal.

2.6 Under section 3.3, bullet four, should rather read, Informed "consent" from mothers for their babies.

2.7 The budget for the tests to be done at NIP should be reviewed taking into consideration any additional specimen collection vacutainer tubes.

2.8 Final report to be shared with the Namibia Institute of Pathology Limited.

Yours Sincerely

Harold T Kaura

Chief Operations Officer

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Appendix F – Chapter 4 Study Written inform consent form

PARTICIPANT CONSENT FORM

TITLE OF THE RESEARCH PROJECT: A study of the prevention of mother-to-child transmission of hepatitis B virus in Windhoek, Namibia

REFERENCE NUMBER: S17/01/001

PRINCIPAL INVESTIGATOR: Cynthia Raissa Tamandjou

ADDRESS: Division of Medical Virology, Department of Pathology

Faculty of Medicine and Health Sciences, Stellenbosch University

Cape Town, South Africa

SUB-INVESTIGATOR IN NAMIBIA: Dr. Josef Mufenda

CONTACT NUMBER IN SOUTH AFRICA: +21 (0)21 938 9360 / +21 (0)78 227 7522

CONTACT NUMBER IN NAMIBIA: +264 81 223 6177

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the nurse or doctor any questions about any part that you do not fully understand.

This research project has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to ethical guidelines and principles of the international Declaration of Helsinki 2008, South African Guidelines for Good Clinical Practice, the Medical Research Council (MRC) Ethical Guidelines for Research, the Biomedical Research Ethics Committee (BREC) and Research Management Committee (RMC) of Namibia and the Namibia Institute of Pathology (NIP) Ethical Guidelines for Research.

1. What is this research study all about?

We want to test 500 pregnant women to find out how many of them have hepatitis B and are at risk of transmitting the infection to their babies. Those at risk of transmission may be put on antiviral treatment for a short duration to prevent transmission of the infection to their babies. We also want to test the baby of those who have hepatitis B after birth to find out if the woman has transmitted the infection to her baby.

2. Why have you been invited to participate?

Because you might have hepatitis B and be at risk of transmitting the infection to your baby. Babies who acquire the infection from their mums at the time of birth may develop chronic hepatitis B and be at risk of developing severe liver problems later in life.

3. What will you have to do?

If you agree to take part in this study, **we will ask you a few questions so that the researcher can have a better understanding of the disease.**

The nurse/health counsellor will also prick your finger and use a few drops of your blood for a rapid test for hepatitis B. This test is quick and the nurse will inform you of your result after 15 minutes.

- **If your result is negative:** The nurse/counsellor will inform you of your result and your participation in the study is complete. No further actions will be required from your side but your data will be recorded as part of the project data for analysis
- **If your result is positive:**
  - **We will collect a blood spot which is collected on a small piece of blotting paper and an extra 10ml of your blood to perform some tests in the laboratory.** These tests will help the doctor (gynaecologist) decide if you need treatment or not. These results will be communicated to you by the nurse at the clinic. **If you need treatment, we will contact you for an appointment with the doctor.**
  - **After you have given birth, your baby will be vaccinated against hepatitis B. We will also test your baby for hepatitis B at the age of 6 weeks and 9 months.** At 6 weeks, we will collect 1ml of blood from your baby and test for hepatitis B markers only.
    - If your baby tests negative at 6 weeks, we will inform you of the result. We will test your baby again at 9 months to confirm that your baby truly does not have the disease. If the result is negative, we will inform you of the result and the participation of your baby to the study will be complete. **If your baby tests positive for hepatitis B markers and viral levels at any point, we will arrange an appointment for your baby with a doctor (pediatrician).** The doctor will use the results of the tests to determine how to take care of your baby.
    - If your baby tests positive at 6 weeks, we will test for hepatitis B viral levels using the same blood sample we took. The doctor will use these results to determine how to take care of your baby. We will not test your baby at 9 months for hepatitis B anymore.

4. Will you benefit from taking part in this research?

- **The test is free and you will know if you have hepatitis B or not, and if at risk of transmitting this disease to your baby.**
- **If you have the infection, we will direct you to the care of a specialist. We will also give you advices to prevent the spread of the infection in your home and to any future children.**
- **We will determine if your baby is free of the disease or not. If he/she carries the disease, we will refer you to a specialist who will advise on how to take care of your baby.**

5. Are there any risks involved in your taking part in this research?

- You may experience some minor bruising and discomfort during rapid testing and blood collection, as a result of the finger prick or needle stick.
- **To avoid causing pain to your baby during blood collection, we will apply an anti-pain cream on the area where the needle stick will be administered.**

6. If you do not agree to take part, what alternatives do you have?

You will continue to be seen in the clinic. Your care will not be harmed in any way. Your participation is **entirely voluntary**. You are also free to withdraw from the study at any point, even if you do agree to take part.

7. Who will have access to your medical records?

The only people who will have access to your information and those of your baby will be your doctor and the scientists working on the study.

8. Will you be paid to take part in this study and are there any costs involved?

- You will not be paid to take part in the study but all the tests associated with this study will be free.
- Your transport costs will be covered for each follow-up visit associated with the study.

9. Is there anything else that you should know or do?

- You can contact Dr Josef Mufenda at tel +264 81 223 6177 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 0027 21 938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- **You will receive a copy of this information and consent form for your own records**.

10. If your blood and that of your baby is to be stored is there a chance that it will be used for other research?

Your blood and that of your baby will only be used for research that is directly related to hepatitis B virus. Also if the researchers wish to use your stored blood for **additional research in this or a related field** they will be required to apply for permission to do so from the Health Research Ethics Committee at Stellenbosch University and the Biomedical Research Ethics Committee (BREC) and Research Management Committee (RMC) at the Namibian Ministry of Health and Social Services.

If you do not wish your blood specimen and/or the blood of your baby to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled "A study of the prevention of mother-to-child transmission of hepatitis B virus in Windhoek, Namibia".

I declare that:

- I have read or someone had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part. I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Tick the option you choose:

☐ I agree that my blood or tissue sample and that of my baby can be stored **for 5 years from the end of this project,** but I can choose to request at any time that these stored samples be destroyed. My sample and that of my baby will be identified with a special study code that will remain linked to our names and my contact details. I have the right to receive confirmation that my request has been carried out.

☐ I agree that my blood or tissue sample and that of my baby can be stored **for 5 years** after the project is completed but that it is anonymized with all possible links to our identity removed, and that the researchers may then use it for additional research in this or a related field. Once our samples are anonymised, our rights to those samples are waivered. Our samples may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

☐ Please destroy my blood sample and that of my baby as soon as the current research project has been completed.

Signed at (place).....…..……. on (date) ……...…….. 2017.

....

Signature of Participant **Signature of witness**

6.4.1 Declaration by investigator

I (name) **Cynthia Raissa Tamandjou** declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (If an interpreter is used then the interpreter must sign the declaration below.

Signed at (place).....….....…….. on (date) ……...…….. 2017.

....

Signature of investigator **Signature of witness**

6.5 Declaration by interpreter

I (name) ……..…… declare that:

- I assisted the investigator (name) …**Cynthia Raissa Tamandjou**……. to explain the information in this document to (name of participant) ……..…….. using the native language of the patient.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place).....….....…….. on (date) ……...……..2017.

....

Signature of interpreter **Signature of witness**

Appendix G – HBV rapid test-based screening questionnaire

HBV RAPID TEST-BASED SCREENING ASSESSMENT FOR NURSE

PMTCT NURSE NAME: ----------

CLINIC NAME: ----------

DEMOGRAPHIC DATA

☐ Registered Nurse ☐ Enrolled Nurse

Years after qualifications: ----------

Please marks with a cross (☒). Only one choice, unless specified.		
1. Have you previously perform rapid test?	☐ Yes	☐ No
2. Did you find the instructions of use of the kit easy to understand?	☐ Very easy ☐ A little difficult	☐ Easy ☐ Very difficult
3. How convenient was it to perform the rapid test?	☐ Very convenient ☐ A little inconvenient	☐ Convenient ☐ Inconvenient
4. Which steps of the kit did you find difficult?	☐ Preparing the kit ☐ Adding the buffer	☐ Taking the sample ☐ Reading the results ☐ None
5. Please, justify your answer to Question 4		
6. How confident are you that you have done the HBV rapid test correctly?	☐ Very confident ☐ A little unconfident	☐ Confident ☐ Very unconfident
7. Do you trust the result of the HBV rapid test?	☐ Yes	☐ No

8. Please justify your answer to Question 7		
9. How did the patient react to the HBV rapid test?	☐ A little anxious ☐ Not anxious	☐ Very anxious
10. Do you think that knowing the results in 15 minutes is useful for the patient?	☐ Yes	☐ No
11. Please justify your answer to Question 10		
12. Would you recommend the use of this test at the clinic for antenatal care?	☐ Yes	☐ No
13. Please justify your answer to Question 12		
14. Was the test acceptable by the patient?	☐ Yes	☐ No
15. Please justify your answer to Question 14		
16. Any additional comments:		

Appendix H – HBV rapid test quality control record sheet: Intermediate Katutura hospital ANC

Stellenbosch University HBV Rapid Testing Forms

HBV RT. Quality Control Record Sheet – PMTCT STUDY

TESTING SITE: Intermediate Katutura Hospital MONTH: September - November 2017

Date	NEGATIVE CONTROL		POSITIVE CONTROL		Name of Tester	Comments
	NEG QC Batch Nr.*	Determine HBsAg	POS QC Batch Nr.*	Determine HBsAg		
26.09.17	NEG Con 12 25.07 2017	Negative	Pos Con 26 07/08/17	Positive	Albertina Nghiyalwa	New stock
02.10.17	Neg: Con 12 25.07.17	Negative	Pos Con 26 07/08/17	Positive	Albertina Nghiyalwa	Weekly Control
10.10.17	Neg Con 12 25.07.17	Negative	Pos Con 26 07/08/17	positive	Julia Hashikutuva	Weekly Control
16.10.17	Neg 25.07.17	Negative	Pos Con 12 07/08/17	Positive	Albertina Nghiyalwa	weekly control
20.10.17	25.07.17 Neg	Negative	07/08/17 Pos	Positive	Julia Hashikutuva	weekly control
30.10.17	25.07.17 Neg	Negative	07/08/17 Pos	Positive	Jennifer Kamwaa	weekly control
06.11.17	25.07.17 N	Negative	07/08/17 Pos	Positive	Albertina Nghiyalwa	weekly Control

*Name of control written on the tube. Example: Neg Con 1, Pos Con 2

Kit name	Lot no.	Expiry Date
Alere Determine HBsAg	83589K100A	2018-12-01
Alere Determine HBsAg		
Alere Determine HBsAg		
Alere Determine HBsAg		

Appendix I – HBV rapid test quality control record sheet: Windhoek Central hospital ANC

Stellenbosch University HBV Rapid Testing Forms

HBV RT. Quality Control Record Sheet – PMTCT STUDY

TESTING SITE: Windhoek Central Hospital MONTH: October 2017 - November 2017

Date	NEGATIVE CONTROL		POSITIVE CONTROL		Name of Tester	Comments
	NEG QC Batch Nr.*	Determine HBsAg	POS QC Batch Nr.*	Determine HBsAg		new week
04/10/17	NEG CON 34 25/07/2017	Negative	POS CON 50 07/08/2017	Positive	Cynthia Tamandjou	new week + new Lot #
11/10/17	NEG CON 34 25/07/2017	Negative	POS CON 50 07/08/2017	Positive	Cynthia Tamandjou	new week
18/10/17	NEG Con 19	Negative	POS CON 49	Positive	Cynthia Tamandjou	new week
25/10/17	NEG Con 19	Negative	Pos Con 49	Positive	Cynthia Tamandjou	new week
01/11/17	NEG Con 42	Negative	Pos Con 55	Positive	Cynthia Tamandjou	new week
15/11/17	Neg Con 42	Negative	Pos Con 55	Positive	Cynthia Tamandjou	new week

*Name of control written on the tube. Example: Neg Con 1, Pos Con 2

Kit name	Lot no.	Expiry Date
Alere Determine HBsAg	83589K100A	2018-12-01
Alere Determine HBsAg		
Alere Determine HBsAg		
Alere Determine HBsAg		

Appendix J – Outputs of the Probit analysis

Parameters estimates

	Parameter	Estimate	Std. Error	Z	Significance	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	HBV DNA Conc (IU/ml)	0.001	0.001	1.317	0.188	0.000	0.002
	Intercept	-4.006	2.923	-1.371	.171	-6.929	-1.083

[a]PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square tests

		Chi-Square	df[b]	Significance
PROBIT	Pearson Goodness-of-Fit Test	0.052	6	1.000[a]

[a]Since the significance level is greater than 0.150, no heterogeneity factor is used in the calculation of confidence limits. [b]Statistics based on individual cases differ from statistics based on aggregated cases.

Cell counts and residuals

	Number	HBV DNA Conc (IU/ml)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
PROBIT	1	2000.000	6	0	.047	-0.047	0.008
	2	5000.000	6	3	2.924	0.076	0.487
	3	10 000.000	6	6	6.000	0.000	1.000
	4	15 000.000	6	6	6.000	0.000	1.000
	5	20 000.000	6	6	6.000	0.000	1.000
	6	30 000.000	6	6	6.000	0.000	1.000
	7	35 000.000	6	6	6.000	0.000	1.000
	8	200 000.000	6	6	6.000	0.000	1.000

Confidence limits

Probability	95% Confidence Limits for HBV DNA Conc (IU/ml)		
	Estimate	Lower Bound	Upper Bound
0.010	2113.363	.	.
0.020	2456.328	.	.
0.030	2673.929	.	.
0.040	2837.621	.	.
0.050	2970.772	.	.
0.060	3084.105	.	.
0.070	3183.475	.	.
0.080	3272.450	.	.
0.090	3353.368	.	.
0.100	3427.854	.	.
0.150	3736.245	.	.
0.200	3981.344	.	.
0.250	4191.617	.	.
0.300	4380.449	.	.
0.350	4555.430	.	.
0.400	4721.470	.	.
0.450	4882.116	.	.
0.500	5040.214	.	.
0.550	5198.312	.	.
0.600	5358.958	.	.
0.650	5524.998	.	.
0.700	5699.979	.	.
0.750	5888.811	.	.
0.800	6099.084	.	.
0.850	6344.183	.	.
0.900	6652.574	.	.
0.910	6727.060	.	.
0.920	6807.978	.	.
0.930	6896.953	.	.
0.940	6996.323	.	.
0.950	7109.656	.	.
0.960	7242.807	.	.

0.970	7406.499	.	.
0.980	7624.100	.	.
0.990	7967.065	.	.