Investigating drug and vaccine resistance as a challenge for hepatitis B virus elimination in Africa

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A thesis submitted for the degree of Doctor of Philosophy

Michaelmas Term 2020



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For my father, mother and siblings

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Acknowledgements

I want to express my sincere gratitude to everyone who directly or indirectly supported me throughout this journey.

I am eternally grateful to you my supervisors, Philippa Matthews and Eleanor Barnes. Working with you these past three years has made me a better person in so many ways. I am very grateful for your mentorship, and for the knowledge, skills and experience I have gain by working with you. Thank you for all the support you have given me that has made me excel academically and progress in my career. I am very grateful to you for guiding me through my uncertainties, fear and anxieties with kindness, and for helping me find my way. Thank you for caring about my well-being throughout this journey.

To my colleagues, thank you for your patience, and support during this learning period.

Richard Walker, thank you for believing in me and for the opportunities you gave me while I was pursuing my Nursing degree in Kenya; these have contributed immensely to who I am today. I sincerely appreciate all the support you have given me so far.

Helena Kuivaniemi, thank you for your mentorship, love, support and kindness. You have been my guiding light since we met in South Africa.

Jacob McKnight, I sincerely appreciate your friendship, mentorship and support throughout this journey.

Kevin Marsh, thank you for your mentorship and for the support you have given me which has helped me advance in my career.

To my friends, I appreciate your company, support, love and kindness throughout this journey. Oluwatomisin, you have never failed me, and you have been there with me in every crucial moment. Thank you for coming through for me as you did – I will never forget.

Dad and Mum, thank you for everything you have given and sacrificed so that I can have the best and become everything I am meant to be. You are the world's greatest, you are my heroes and inspiration, and I celebrate you every single day. To my siblings: Frida, Wycliffe, Linda, Druscilla and Brenda, and your respective families, you have all been my rock. Frida, thank you sharing your home and family with me, and thank you for holding my hand when I was still finding my way. Thank you for setting the pace as our first born. Wycliffe and Milka, thank you for always encouraging me, for your generosity and for helping me start life in the UK. Thank you for showing me how to be bold, resilient and successful in a foreign land. Linda, your ability to envision and plan for the future has taught me how to organise my work and plan for my future. Thank you for showing me what consistency and hardwork looks like. Druscilla, your free-spirited nature has helped me believe that I am not limited, and your resilience has taught me that there is nothing I cannot overcome regardless of how challenging. Thank you for your generosity. Brenda, your fearless attitude has taught me how to reach for the stars and pursue what I desire with boldness. Thank you for always reminding me to embrace the present moment and for not allowing me to give up when things seemed impossible. Thank you for fighting for me and ensuring that I have all that I need to be happy. My family, thank you for your unconditional love, support, inspiration, generosity, prayers and encouragement which have guided and held me throughout this journey. Thank you all for believing in me. This is our victory as a family and I dedicate this major achievement to you my family.

And lastly, to God Almighty, in you I live, and move and have my being. Great is your faithfulness!

Abstract

Background

The United Nations Sustainable Development Goals set out to eliminate viral hepatitis by the year 2030. However, hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) and/or vaccine escape mutations (VEMs) are potential barriers to the achievement of this target. I have set out to describe the prevalence, distribution and impact of RAMs and VEMs in African populations infected with HBV, with a focus on cohorts in South Africa.

Methods

Through systematic literature reviews, analysis of published sequence data downloaded from GenBank, as well as sequence data generated from individuals infected with chronic HBV infection (CHB) recruited from South Africa, I have assessed the frequency, co-occurrence, distribution, evolution and transmission of HBV RAMs and VEMs. In addition, I have assessed the impact of HBV RAMs on HBV prevention of mother-to-child transmission (PMTCT) interventions through a cost-effectiveness analysis.

Results

In Africa, the most common RAM was M204I/V, occurring either alone or in combination with compensatory mutations, present among HIV/HBV coinfected and HBV monoinfected patients, and identified in both reportedly treatment-naïve and treatment-experienced adults. I also report the first case from Africa with strong evidence for HBV resistance to tenofovir (TFV), on the basis of high HBV DNA viral load (VL) despite 60 months of therapy, with a suppressed HIV VL, and a combination of sequence polymorphisms that have been reported in association with TFV resistance. Although screening and treatment of all women with CHB with TFV during pregnancy may be a cost-effective strategy for HBV PMTCT in Africa, increased selection of TFV RAMs could increase health-care costs.

Discussion

There is emerging evidence for polymorphisms that may reduce susceptibility to TFV. In order to drive progress towards 2030 elimination targets, there is a need for enhanced surveillance,

scale-up of HBV prevention and treatment, and new therapeutic strategies that can bring about cure.

Chapter one: Introduction

1.1 United Nations Sustainable Development Goals

Chronic hepatitis B virus (HBV) infection is a huge public health problem. Globally, approximately two billion people have been exposed to HBV and about 290 million people are chronically infected, causing more than 800,000 deaths each year as a result of complications including liver cirrhosis and liver cancer (Nayagam et al. 2016; WHO, 2017a).

The United Nations sustainable development goals have set a target aiming to combat viral hepatitis as a public health threat by the year 2030 by upscaling prevention and treatment strategies which include ensuring: (i) 90% coverage of universal hepatitis B immunisation of infants; (ii) 90% coverage of hepatitis B birth dose vaccine; (iii) 90% coverage in diagnosis of HBV and HCV; (iv) 80% coverage in treatment of HBV and HCV; (v) 100% coverage in screening of blood donations in a quality-assured manner; (vi) 90% coverage of injections given with safety-engineered devices; and (vi) distribution of at least 300 sterile needles and syringes to each person who injects drugs every year (WHO, 2016a). The focus of my thesis is HBV infection, addressing questions that are pertinent to both prevention and treatment, providing data that are relevant to aims (i), (ii) and (iv) in this list.

1.2 Discovery of hepatitis B virus (HBV)

Although not yet discovered at the time, there were several outbreaks of HBV infection in the mid 20th century following smallpox and yellow fever vaccination (Gerlich, 2013). During this period, human serum was included in vaccines for stabilisation and therefore contributed to infectious disease transmission (Gerlich, 2013). ‘Australia antigen’ (AuAg) was discovered in 1963 by Blumberg and colleagues when studying antigenic properties of serum obtained from individuals who received blood transfusions (Blumberg, 1977; Blumberg, 2002; Gerlich, 2013). In 1971, using transmission electron microscopy, Dane and colleagues discovered HBV virions composed of the core antigen which was surrounded by surface antigen envelope, and

at this stage it was clear that AuAg was the hepatitis B surface antigen (HBsAg) (Gerlich, 2013). In 1974, William Robinson discovered HBV DNA having detected endogenous DNA polymerase activity within the virus (Gerlich, 2013).

1.3 Structure of HBV

HBV is a DNA virus that affects the liver causing acute or chronic liver disease (WHO, 2018a). It is a member of the *Hepadnaviridae* family; these are hepatotropic DNA viruses that affect mammals such as woodchucks, ground squirrels, bats and primates (genus: *Orthohepdnaviridae*), and birds including ducks, herons, storks, geese and parrots (genus: *Avihepadnavirus*) (Locarnini and Roggendorf, 2013; Karayiannis, 2017). There are two types of HBV viral particles usually identified in the serum of an infected individual: sub-viral particles and infectious virions (Liang, 2009; Venkatakrishnan and Zlotnick, 2016; Karayiannis, 2017). Sub-viral particles are made of an outer glycosylated envelope containing surface antigen and are usually non-infectious as they lack viral genome (Karayiannis, 2017). A mature infectious virion is composed of an outer glycosylated envelope containing surface antigen which surrounds a nucleocapsid enclosing the viral genome and a copy of the viral polymerase (Liang, 2009; Venkatakrishnan and Zlotnick, 2016). The viral genome is made up of partially double-stranded DNA of approximately 3200 base pairs and is organised into partially or wholly overlapping open reading frames (ORF) which encode the viral proteins which include polymerase, surface, structural core and non-structural pre-core (secreted e-antigen), and X protein (Glebe and Bremer, 2013). Nucleotide substitution in one ORF can result in non-synonymous nucleotide substitution in the overlapping ORF (Cento et al. 2013). Within these ORF also lie regulatory elements such as enhancers (Enh1, Enh2), promoters (core, S1, S2 and X) and signals that drive polyadenylation, encapsidation (ε) and replication (DR1, DR2) (Karayiannis, 2017) **(Figure 1.1).**

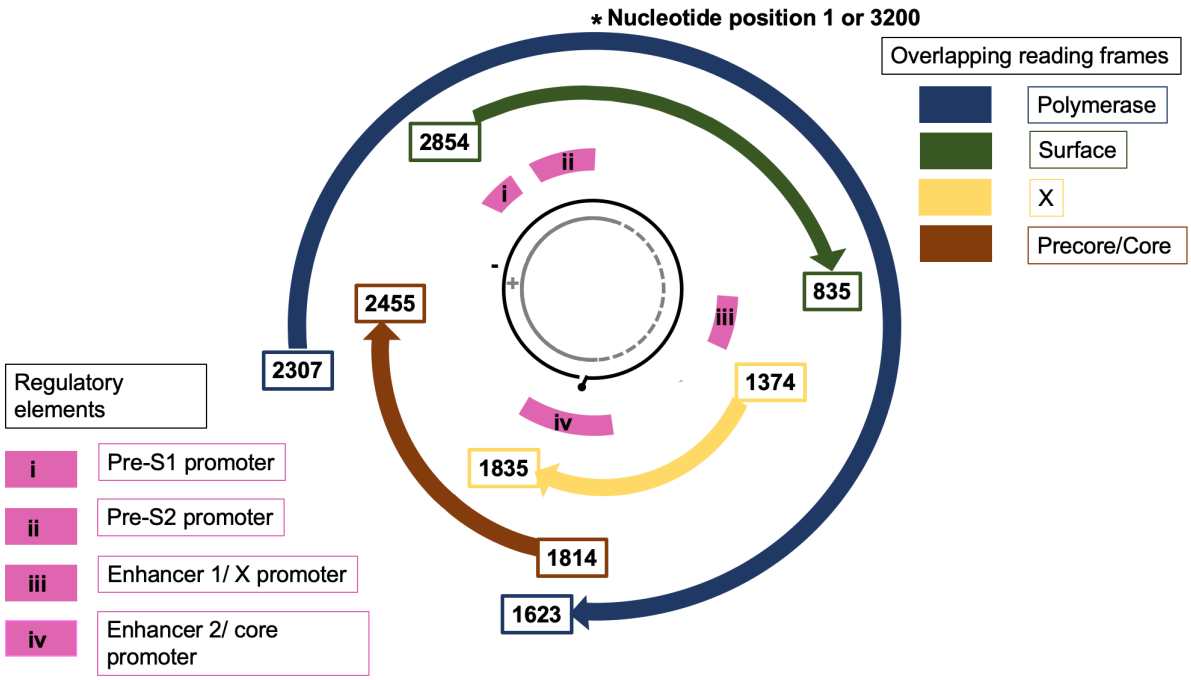


Figure 1.1: Structural organisation of hepatitis B virus (HBV) genome. The genome is composed of a partially double-stranded DNA; the incomplete positive inner strand (coloured grey) is of variable length (missing region indicated by dotted lines), whereas the negative outer strand (coloured black) has a definite 5' and 3'-end near nucleotide position 1800 (He et al. 2002). The genome is organised into four overlapping reading frames: the polymerase protein (coloured dark blue) spans nucleotide positions 2307–3200 and 0–1600 ; surface protein (coloured green) spans nucleotide positions 2853–3200 and 1–835; X protein (coloured yellow) spans nucleotide positions 1374–1835; and Precore/Core protein (coloured brown) spans nucleotide positions 1814–2455 (McNaughton et al. 2019a). Regulatory elements (coloured in pink) are as follows: Pre-S1 promoter spans nucleotide positions 2710–2800; Pre-S2 promoter spans nucleotide positions 2960–3180; Enhancer 1 (X promoter) spans nucleotide positions 1074–1234; and Enhancer 2 (core promoter) spans nucleotide positions (1575–1849) (Moolla, Kew and Arbuthnot, 2002). Sequence numbering shown here and in subsequent figures is based on HBV genotype A reference sequence (FJ692557) (McNaughton et al. 2020a) which was obtained from publicly available HBV Database (Hayer et al. 2013). *Nucleotide position 1 or 3200, indicates the start or end of viral genome.

1.4 Viral proteins

1.4.1 Pre-core/Core protein

HBV *core* gene codes for both pre-core and core proteins (Schödel et al. 1993; Datta et al. 2012). The core protein (HBcAg) forms a ~20 kDa icosahedral nucleocapsid that encloses HBV genomic DNA and self-assembles to form different sizes of the shell which include T=3

(180 subunits) or T=4 (240 subunits) icosahedral asymmetry (Venkatakrishnan and Zlotnick, 2016; Wynne, Crowther and Leslie, 1999). HBcAg is made up of a total of 183 or 185 amino acids (depending on the genotype) and it is divided into three regions: an N-terminal domain (amino acid residues 1–140), a linker peptide (amino acid residue 141–149) and a C-terminal domain (amino acid residue 150–183/185) (Schödel et al. 1993; Zlotnick et al. 2015; Venkatakrishnan and Zlotnick, 2016; Liu et al. 2018) **(Figure 1.2).** The N-terminal domain and linker peptide are required for capsid assembly whereas the C-terminal domain is required for pre-genomic RNA (pgRNA) packaging and reverse transcription (Liu et al. 2018). The pre-core protein has 29 amino acids preceding the N-terminal domain of the core protein which serve as signal peptides directing pre-core protein to the endoplasmic reticulum for secretion (Schödel et al. 1993; Venkatakrishnan and Zlotnick, 2016). Ten pre-core amino acids and the N-terminal domain of the core protein form the e-antigen (HBeAg) (Zlotnick et al. 2015). While a mature HBeAg is not required for viral replication, it plays a role in reducing the effect of host immune responses, therefore resulting in viral persistence and development of chronic HBV infection (CHB) (Walsh and Locarnini, 2012).

Approximately 15–30% of manufactured HBeAg are not secreted; these cytoplasmic HBeAg inhibit innate immunity by suppressing Toll-like receptor signalling pathways, and also contribute to host immune exhaustion through formation of empty capsids which are enveloped and secreted (Milich and Liang, 2003; Datta et al. 2012). On the other hand, secreted HBeAg deletes inflammatory TH1 cells, impairs CD8+ cytotoxic T lymphocytes and preferentially induces non-inflammatory TH2 cells (Milich and Liang, 2003).

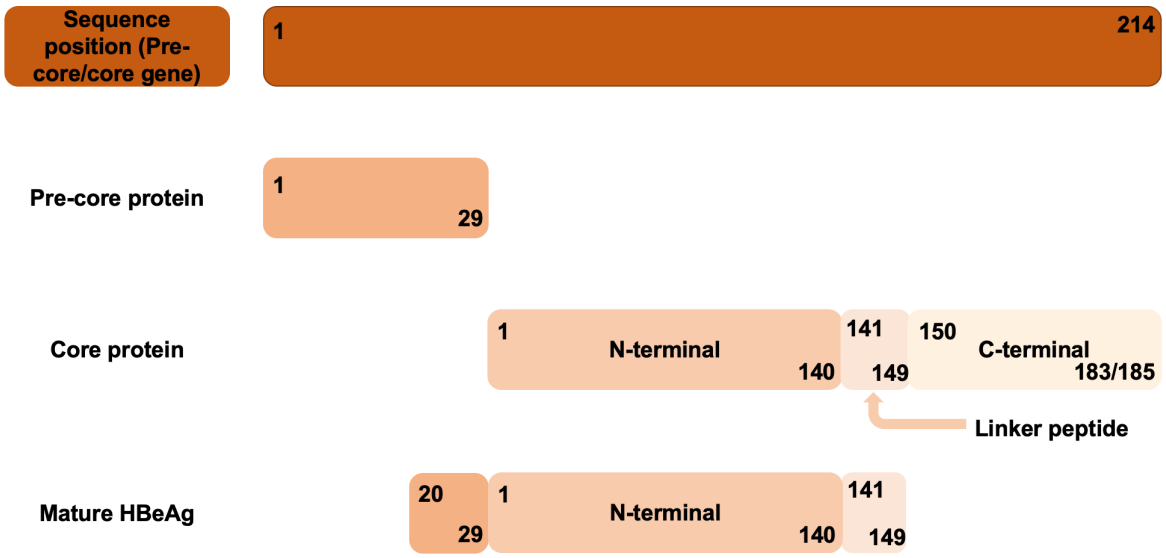


Figure 1.2: Sequence structure of hepatitis B virus pre-core/core protein. Pre-core/Core protein contains 214 amino acids (coloured dark brown bar). Pre-core protein contains 29 amino acids. Core protein contains 183/185 amino acids depending on genotype (genotype A has 183) and it is divided into three regions: N-terminal which has 140 amino acids; linker peptide which has nine amino acid; and C-terminal which has 33/35 amino acid. Mature HBeAg contains nine amino acids from the end of the pre-core protein and 148 amino acids from the core protein [N-terminal (140 amino acids) and Linker peptide (nine amino acids)] (Schödel et al. 1993; Zlotnick et al. 2015; Venkatakrishnan and Zlotnick, 2016; Liu et al. 2018).

1.4.2 X protein

HBV X protein (HBx) is coded by the *X* gene. Its name was derived due to the lack of identification of a similar protein in both host and other viral proteins, although similar proteins were later identified in ground squirrel and woodchuck hepatitis virus (Murakami, 1999). HBx is made up of 154 amino acids encoded by 462 nucleotides (Kew, 2011). It is a highly conserved protein and overlaps with polymerase and pre-core proteins as well as enhancer 1 (X promoter) and enhancer 2 (core promoter) regulatory elements (Kew, 2011; Geng et al. 2015) **(Figure 1.1 section 1.3).** The solved crystal structure of HBx demonstrated that mutations including deletions in HBx interfere with viral replication and transcriptional regulation (Geng et al. 2015). HBx does not bind directly to DNA, however, it plays a role in transcriptional activation which is essential for viral replication, cell cycle progression,

modulation of growth regulators, protein degradation, apoptosis, genetic stability through interaction with host factors, and it is strongly associated with hepatocellular carcinogenesis (Kew, 2011; Ma et al. 2011; Geng et al. 2015).

1.4.3 Surface protein

The HBV surface (S) ORF, divided into pre-S1, pre-S2 and S domains, is transcribed into two sub-genomic mRNA: preS1 mRNA (2.4 kb) and preS2/S mRNA (2.1 kb) (Glebe and Bremer, 2013; Churin, Roderfeld and Roeb, 2015). In the endoplasmic reticulum, preS1 mRNA is translated into large protein (LHBs) and preS2/S mRNA is translated into small (SHBs) and middle (MHBs) proteins; these three proteins represent HBV surface antigen (HBsAg) which forms the viral envelope (Glebe and Bremer, 2013; Churin, Roderfeld and Roeb, 2015; Cornberg et al. 2017) **(Figure 1.3).** Two thirds of HBV envelope, in both virions and sub-viral particles, is made up of SHBs and the remaining one third is equally represented by MHBs and LHBs (Locarnini and Bowden, 2012; Cornberg et al. 2017). SHBs (226 aa long) carries the major antigenic determinant (a-determinant; aa 99-169) and has a low affinity binding site for heparan sulphate proteoglycan which is required for infectivity (Glebe and Bremer, 2013; Churin, Roderfeld and Roeb, 2015; Cornberg et al. 2017). MHBs (281 aa long) is highly conserved, however its function is not yet understood (Churin, Roderfeld and Roeb, 2015; Cornberg et al. 2017). LHBs (between 389–400 aa long) have a high affinity to sodium taurocholate receptor (NTCP) and it is required in the envelopment of mature core particles and therefore essential in HBV infectivity (Churin, Roderfeld and Roeb, 2015; Cornberg et al. 2017).

The second-generation vaccine contains only SHBs which is able to induce humoral immune response to all three envelope proteins (Shouval, 2003). Third generation HBV vaccine containing pre-S antigens, has the ability to induce both B- and T-cell mediated immune responses (Shouval, 2003; Shouval, Roggendorf and Roggendorf, 2015). A crystal structure has been solved for a very small chunk of the pre-S2 region (aa 123–145) which is referred to

'adrl23' (Chi et al. 2006). This region which is included in 3rd generation vaccines, has been shown to be highly immunogenic and appears to be more stable than pre-S2 containing all the 55 amino acids (Shouval, 2003; Xu et al. 2014).

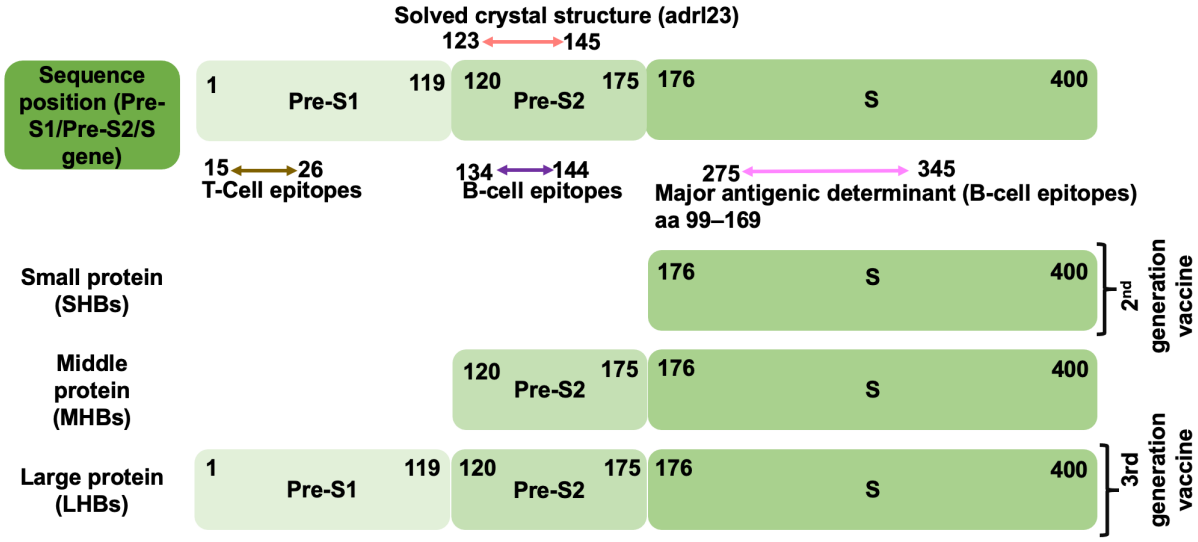


Figure 1.3: Sequence structure of hepatitis B virus surface protein. *Pre-S1/Pre-S2/S* gene encodes for Pre-S1, Pre-S2 and S proteins which contain 180, 55 and 224 amino acids, respectively (Churin, Roderfeld and Roeb, 2015; Cornberg et al. 2017)**.** Small protein (SHBs) contains surface protein. Middle protein (MHBs) contains Pre-S2 and S proteins. Large proteins (LHBs) contain Pre-S1, Pre-S2 and S proteins. 2nd generation vaccine contains SHBs and 3rd generation vaccine contains LHBs.

1.4.4 Polymerase protein

HBV polymerase protein is composed of four domains [terminal, spacer, reverse transcriptase (RT) and RNase H]; this protein is enzymatically active and plays an essential role in viral replication (Jones et al. 2014; Clark and Hu, 2015a) **(Figure 1.4).** The terminal domain (181 aa long) has three roles: it initiates DNA synthesis by facilitating attachment of the first nucleotide of the viral minus-strand DNA to polymerase enzyme, it facilitates binding and packaging of pgRNA into nucleocapsid, and is also essential for protein priming (Clark and Hu, 2015a). The spacer domain (165 aa long) has been previously considered non-essential for viral replication competency (Jones et al. 2014). However, it has been proven to be a highly variable site serving as a flexible spot that allows the virus to adapt to conformational changes as a response to immune selective pressure given its overlap with pre-S domain (Chen et al.

2013). The RT domain (344 aa long) forms the catalytic core of the enzyme and is targeted by nucleos(t)ide analogues (NAs) to inhibit viral replication (Clark and Hu, 2015a). RT is classified into subdomains (termed 'finger', 'palm', and 'thumb') which are further divided into conserved regions annotated A–G (Clark and Hu, 2015a) **(Figure 1.4).** Functions of the conserved regions A–G are as follows (Bartholomeusz, Tehan and Chalmers, 2004; Wang et al. 2012; Clark and Hu, 2015a):

i. **A** contains a phenylalanine residue which discriminates against deoxyribonucleotide triphosphate (dNTP) and nucleoside triphosphate (NTP), and then interacts with regions B and G to form dNTP binding pocket;
ii. **B** pairs the primer and template strand;
iii. **C** contains the tyrosine-methionine-aspartate-aspartate ('YMDD') active site;
iv. **D** has no role in dNTP binding;
v. **E** aids in polymerase - RNA epsilon binding and DNA synthesis;
vi. **F** is essential for enabling interaction between dNTP and template nucleotide.

Similar to the terminal domain, the RNase H domain (153 aa long) has three functions: it contains the aspartic acid-glutamic acid-aspartic acid-aspartic acid ('DEDD') motif which coordinates metal ion binding which is essential for viral replication; it degrades RNA strand of RNA-DNA heteroduplexes formed during replication; and it is essential for pgRNA packaging (Cheng et al. 2003; Jones and Hu, 2013; Clark and Hu, 2015a). There are no solved crystal structures for any of the polymerase protein domains.

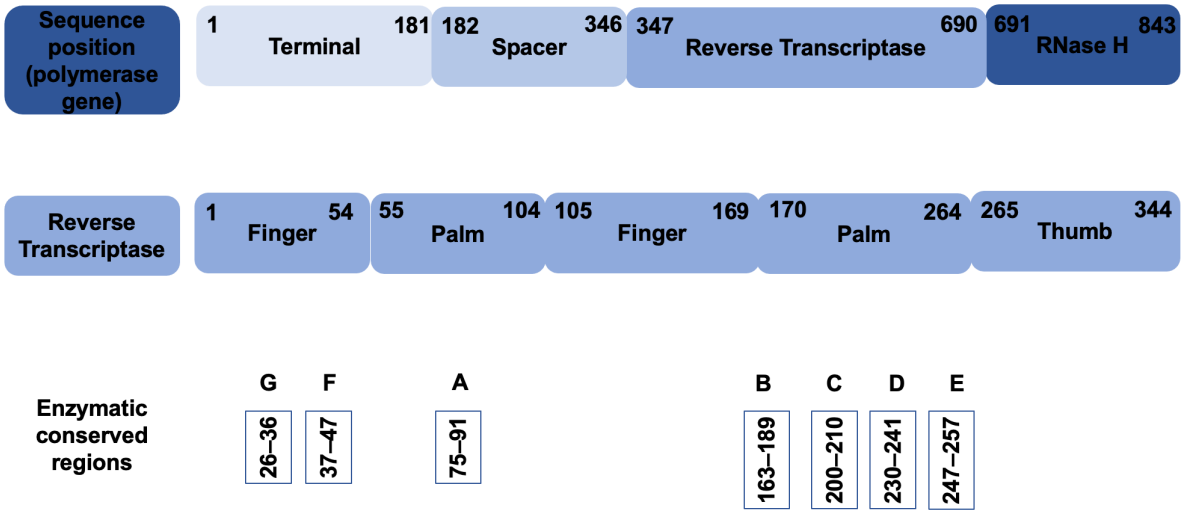


Figure 1.4: Sequence structure of hepatitis B virus polymerase protein. *Polymerase* gene encodes for polymerase protein which is divided into four regions: terminal which has 180 amino acids; spacer which has 165 amino acids; reverse transcriptase (RT) which has 343 amino acids and; RNase H which has 152 amino acids. RT protein is further classified into sub-domains: Finger, Palm, and Thumb which are further classified into seven enzymatic conserved regions annotated A–G (Bartholomeusz, Tehan and Chalmers, 2004; Wang et al. 2012; Clark and Hu, 2015a).

1.5 HBV replication cycle

During infection, HBV attaches onto the hepatocyte via the NTCP receptor, then releases its genome into the cell cytoplasm (Li, 2015). The partially double-stranded DNA (rcDNA) is then transported into the cell nucleus where it is repaired to a complete double-stranded DNA and then converted to a covalently closed circular structure (cccDNA) by both viral and cellular enzymes (Grimm, Thimme and Blum, 2011). cccDNA molecules exist as 'minichromosomes' and form the transcriptional template for viral mRNA and pgRNA which are necessary for production of viral proteins and viral replication, respectively (Revill and Locarnini, 2016; Allweiss and Dandri, 2017). In the cytoplasm, mRNA is translated to viral proteins that are organised into four overlapping reading frames, whereas the pgRNA is reverse transcribed via the viral polymerase enzyme to form rcDNA, which is either imported back into the nucleus to replenish the cccDNA pool, or is secreted via the endoplasmic reticulum to infect other hepatocytes. Incomplete degradation of pgRNA and mispriming during viral replication results in the formation of double-stranded linear (dsl) DNA which is the precursor of HBV DNA integration into host DNA (Beck and Nassal, 2007; Revill and Locarnini, 2016) **(Figure 1.5).**

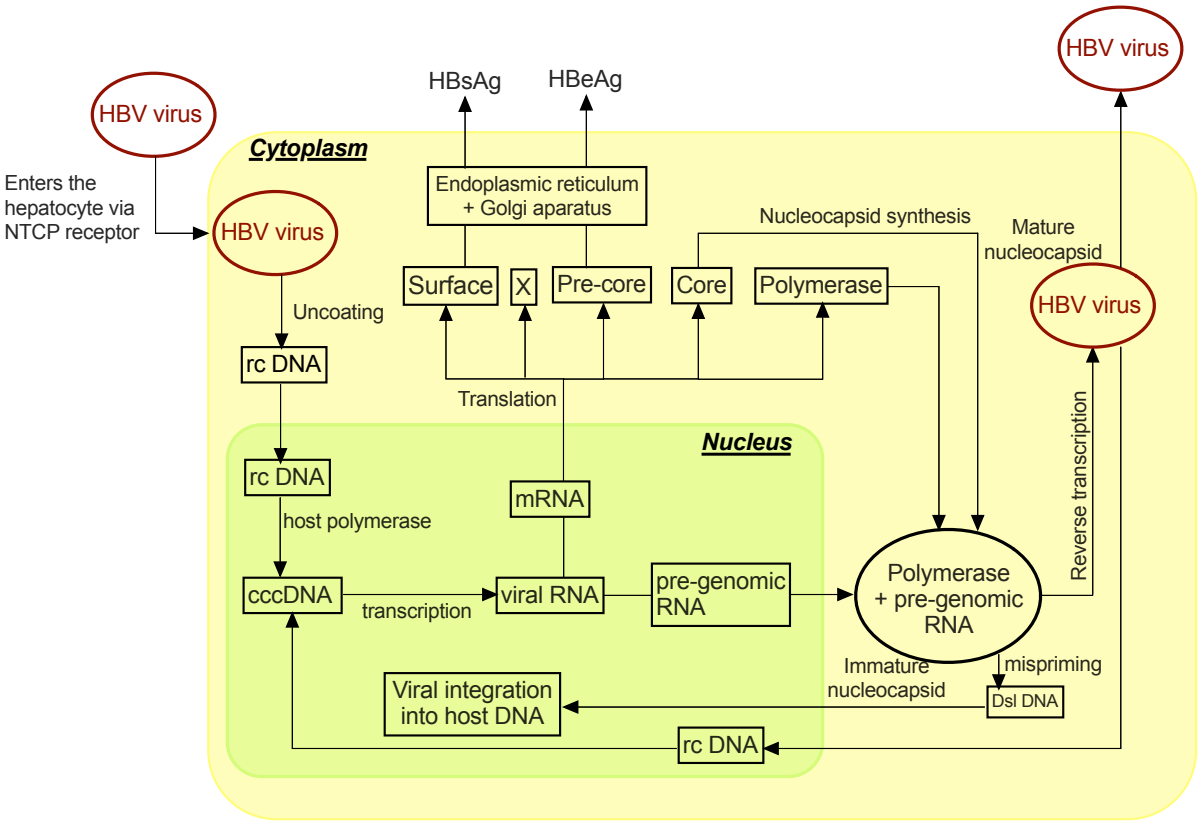


Figure 1.5: Hepatitis B virus replication cycle. Yellow rectangle represents the hepatocyte. The green inner rectangle represents nucleus.

1.6 HBV diversity and evolution

The HBV polymerase enzyme lacks a proofreading function which allows for incorrect nucleotide incorporation during replication, with an estimated mutation rate of between 7.9 x 10^{-5} and 3.2 x 10^{-4} substitutions per site per year; this is higher than that estimated for other DNA viruses but lower than for most RNA viruses (Osiowy et al. 2006; Littlejohn, Locarnini and Yuen, 2016). In addition to having an error-prone RT enzyme, HBV has a high replication rate of approximately 10^{12} virions per day (Beloukas and Geretti, 2017); these two factors result in the occurrence of genetically distinct variants called genotypes and sub-genotypes, and in the development of drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) (Osiowy et al. 2006; Littlejohn, Locarnini and Yuen, 2016; Beloukas and Geretti, 2017). Ten genotypes have been identified, annotated A–J, and genotypes A, B, C, D and F are further classified into sub-genotypes (Sunbul, 2014; Lin and Kao, 2015). Based on complete genome sequences, the nucleotide difference between genotypes is >8% whereas

nucleotide difference between sub-genotypes is between 4–8% (Sunbul, 2014; Lin and Kao, 2015). Genotypes and sub-genotypes are distributed across different geographical regions and can be associated with differences in disease progression, severity and prognosis, and also response to treatment (Sunbul et al. 2014; Revill et al. 2020) **(Table 1.1).**

Genotype-specific differences in mutation rates and host population dynamics have an influence on virus evolutionary rates, which may directly affect the probability of appearance of RAMs/VEMs (Osiowy et al. 2006; Bouckaert, Alvarado-Mora and Pinho, 2013). In addition, in genotypes in which the wild type amino acid at a specific position is part of a sequence motif associated with drug or vaccine resistance, the barrier to resistance is likely to be inherently lower. This phenomenon has been described in hepatitis C virus (HCV) infection, explaining why some sub-genotypes are intrinsically resistant to the most widely used direct acting antiviral drugs (DAAs) (Rose et al. 2013; Davis et al. 2019; Fourati et al. 2019).

Table 1.1: Distribution of hepatitis B virus genotypes across geographical locations and their clinical associations.

Genotype	Sub-genotypes	Geographical location	Clinical implication
A	1–4	Africa, Europe and Asia (India)	• Higher rates of spontaneous HBsAg seroconversion compared to other genotypes (Ganem and Prince, 2004; Flink et al. 2006; Hadziyannis and Papatheodoridis, 2006) • Higher HBeAg clearance compared to genotypes B, C and D when on pegylated interferon (Janssen et al. 2005) • High frequency of PC mutations (A1896) (Sunbul, 2014)
B	1–6	Asia, North America and Greenland	• Lower prevalence of HBeAg positivity and higher rates of HBeAg seroconversion (Hadziyannis and Papatheodoridis, 2006) • Higher prevalence of PC mutation (1896A) compared to genotype C (Watanabe et al. 2005) • Earlier HCC development among younger patients and those with non-cirrhotic HCC (Kao et al. 2000; Yin et al. 2008) • Better response to pegylated interferon compared to individuals infected with genotype C (Kong et al. 2014)
C	1–11	Asia and Australia	• Associated with chronicity in Chinese individuals (Zhang et al. 2008) • Higher HBeAg seropositivity among children infected with genotype C compared to those infected with genotype B (Ni et al. 2004) • Longer periods of high HBV DNA replication among patients with genotype C, who experience delayed seroconversion of HBeAg (Chan et al. 2003; Sumi, 2003; Ni et al. 2004; Yuen et al. 2004; Watanabe et al. 2005) • Higher risk of HCC development and severe form of liver disease compared to genotype B (Watanabe et al. 2005; McMahon, 2009) • Frequent double mutation (1762T/1764A) in the BCP in genotype C than genotype B (Watanabe et al. 2005; Yu et al. 2005; Yang et al. 2008; Papatheodoridis et al. 2010) • Frequent pre-S/S deletions in genotype C than genotype B (Sugauchi et al. 2003)
D	1–6	Africa, Europe and Asia	• High frequency of PC mutations (A1896) (Sunbul, 2014) • High risk of developing HCC and liver cirrhosis (Sunbul, 2014) • Associated with acute liver failure (Wai et al. 2005)
E	N/A	Africa (mainly found in the Gulf of Guinea)	• High viral loads with consequent higher rates of perinatal transmission and high rates of HBeAg positivity (Sonderup, 2019) • Associated with both precore and BCP mutations (Sonderup, 2019)
F	1–4	Central and South America	• High risk of developing HCC and liver cirrhosis (Sunbul, 2014)
G	N/A	Europe and North America	• Low response to pegylated interferon (Erhardt et al. 2009) • Might be difficult to make serological diagnosis due to impaired production of HBsAg (Zaaijer et al. 2011)
H	N/A	Central America	None described
I & J	N/A	Asia	There are very few sequences described that determining outcomes and treatment response has not been possible to date

HBsAg: Hepatitis B surface antigen. PC: Precore. HCC: Hepatocellular carcinoma. BCP: Basal Core Promoter.
S: Surface.

1.7 HBV epidemiology

The prevalence of HBV varies across different geographical regions, classified as high at >8%, intermediate at 2–7% and low at <2% (Nayagam et al. 2016). **Figure 1.6** shows the prevalence of HBV across different regions.

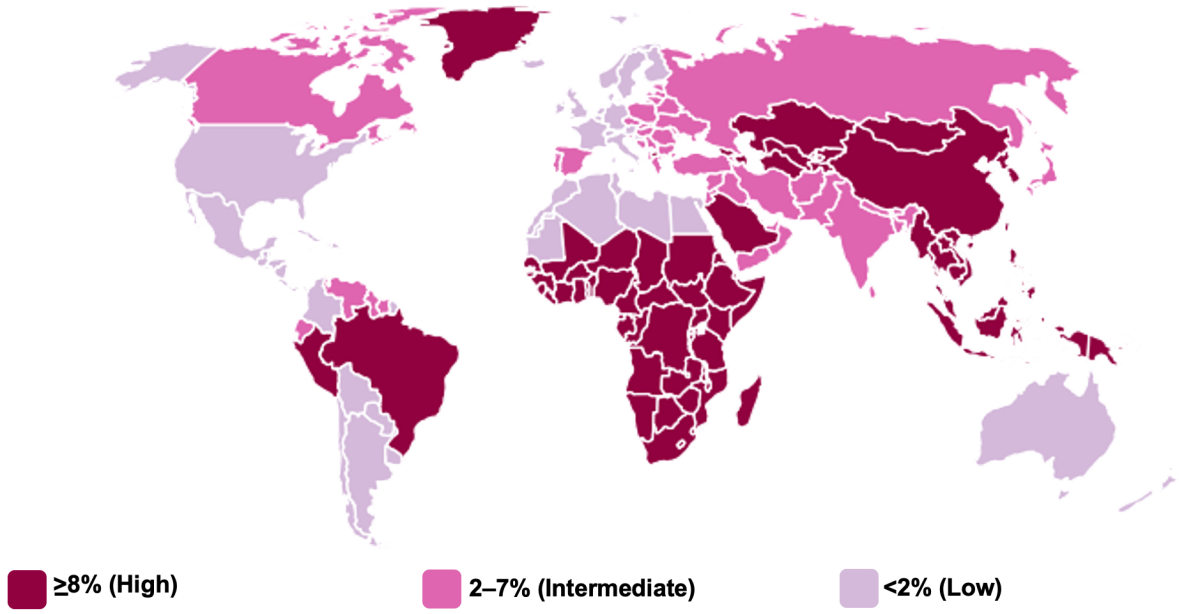


Figure 1.6: Geographic distribution of hepatitis B virus infection. High prevalence ≥8% areas include Southeast Asia, China, Pacific Islands and sub-Saharan Africa (sSA); intermediate prevalence 2–7% areas include Eastern Europe, Mediterranean basin, Middle East, Central and South Asia, Japan, Central and South America; and low prevalence <2% areas include North America, Western Europe, Australia and New Zealand (Hou, Liu and Gu, 2005; WHO, 2017a). I made this figure using a free publicly available interactive map (https://simplemaps.com/resources/).

1.8 Coinfections

HCV, Hepatitis D virus (HDV) and Human Immunodeficiency Virus (HIV) are blood-borne viruses with potentially shared routes of transmission which can occur either simultaneously and/or as a superinfection (infection occurring in someone who already has HBV infection) (WHO, 2015).

1.8.1 HBV and Human Immunodeficiency Virus (HIV)

Among HIV infected individuals, the global prevalence of CHB is estimated at 7.4% with the highest burden in sub-Saharan Africa (sSA) (WHO, 2015; WHO, 2017a). It is believed that

HBV infection typically precedes HIV infection in high endemic areas with the predominant mode of HBV transmission happening perinatally or in early childhood (WHO, 2015; WHO, 2017a). There are conflicting data regarding the clinical impact of HIV/HBV coinfection; on one hand, it has been reported that HIV coinfection worsens the disease process in HBV as people who are dually coinfected are more likely to develop chronicity, have higher HBV replication rates, are less likely to achieve spontaneous clearance and seroconversion of HBsAg and HBeAg, and are more likely to progress to cirrhosis and liver cancer (WHO, 2015). On the other hand, a recent study that I have contributed to as a coauthor, demonstrated that compared to individuals who are HIV/HBV coinfected, HBV monoinfected individuals have worse clinical outcomes due to limited access to treatment and clinical care (Maponga et al. 2020).

There is some degree of homology between the sequence, structure and function of HIV and HBV RT enzymes, explaining why certain NAs are active against both viruses (Das et al. 2001). Although no crystal structure has been resolved for HBV RT, some studies have modelled this enzyme based on the HIV crystal structure (Das et al. 2001; Ghany and Liang, 2007; Daga, Duan and Doerksen, 2010), suggesting that insights into HBV drug resistance mechanisms might be inferred from what is known about HIV.

1.8.2 HBV and Hepatitis C Virus (HCV)

It is estimated that 1–15% of the world's population has HBV/HCV coinfection with variations across different geographical regions (Mavilia and Wu, 2018). Although data from Africa is limited, the prevalence of coinfection may be low in most African communities given that the overall seroprevalence of HCV in sSA is estimated at 2.98% (Rao et al. 2015). In HCV/HBV coinfection, there is an increased progression of liver disease and increased risk of developing liver cancer (WHO, 2015). HBV DNA viral load (VL) is usually low or undetectable during coinfection, with HCV being responsible for chronic infection (WHO, 2015). While DAAs can cure HCV (Holmes, Rutledhe and Chung, 2019), monitoring of HBV DNA after HCV clearance is essential as there is a potential risk of HBV reactivation (WHO, 2015).

1.8.3 HBV and Hepatitis D Virus (HDV)

Globally, it is estimated the 5% of those with CHB are coinfected with HDV, and HDV coinfection is estimated to be associated with approximately 20% of HCC associated with CHB (Stockdale et al. 2020). HDV infection 'dominates' HBV infection, with low or undetectable HBV DNA VL typically seen during coinfection (Stockdale et al. 2020). HDV lacks a virus specific polymerase enzyme which makes it rather a challenging therapeutic target (Yurdaydin et al. 2019). Pegylated interferon alpha (PegIFNa) is the only treatment option available for HDV as NAs do not have activity against HDV replication (Heidrich, Manns and Wedemeyer, 2013; WHO, 2015). Other new treatment options for HDV infection that target various steps of HBV and HDV replication cycle are currently in development (Yurdaydin et al. 2019). Myrcludex B which inhibits entry of HDV into the hepatocyte by blocking the NTCP receptor has been tested in several phase II clinical trials (Yurdaydin et al. 2019). Other compounds in development include prenylations inhibitor, nucleic acid polymers and interferon lambda (Yurdaydin et al. 2019).

1.9 Mode of transmission

HBV can be transmitted through exposure of infected blood and body fluids such as saliva, blood, and sexual transmission (Croagh, 2014; WHO, 2017a) **(Table 1.2).**

Table 1.2: High risk population and mode of transmission of hepatitis B virus infection.

High risk population	Predominant mode of transmission
Infants born to infected mothers	Mother-to-child transmission, contaminated medical and/or surgical equipment
Infants/children	Horizontal transmission (Interfamilial, child-to-child e.g. through open wound/ cuts)
Adults	Sexual transmission, horizontal transmission through open wound/ cuts
People who inject drugs, people in prisons and closed settings	Contaminated injecting equipment, sexual transmission
Unvaccinated health care professionals	Contaminated sharps, body fluids, and medical waste
Persons exposed in health-care settings	Contaminated medical and/or surgical equipment during medical, surgical and dental procedures
Persons exposed via other invasive procedures	Tattooing, piercing, scarification, circumcision

1.10 HBV prevention

HBV prevention can be achieved through immunisation (passive or active) and/or ensuring safe practices such as screening of blood products and body organs, sterilisation of medical equipment and sharps, as well as avoiding risky behaviours (Chang, Chen and Chen, 2015). In addition, testing and treating with NAs results in diminishing the population reservoir of HBV infection and therefore decreases transmission risk (Pan et al. 2016; McNaughton et al. 2020b).

1.10.1 Passive immunisation

Hepatitis B immunoglobulin (HBIg) is a purified solution obtained from human plasma that contains high titres of antibody to HBsAg, providing passive immunity if administered shortly before exposure or as post exposure prophylaxis (Zuckerman, 2007). HBIg provides protection immediately, but its effects last only three to six months (Zuckerman, 2007). HBIg is mainly used in combination with HBV vaccine to prevent mother-to-child transmission (MTCT); three doses of HBIg is more effective in prevention compared to one dose (Chang, Chen and Chen, 2015). HBIg is not widely available in many settings due to high costs, dependency on a robust cold chain, and short-term effectiveness (Zuckerman, 2007; Chang, Chen and Chen, 2015).

1.10.2 Active immunisation

HBV vaccine provides active immunity against HBV infection and its complications including liver cirrhosis and liver cancer (Chang, Chen and Chen, 2015). In the late 1970s, first generation HBV vaccine containing purified HBsAg obtained from serum of chronically infected individuals was developed in USA (containing 22 nm of SHBs of HBsAg) and France (containing SHBs of HBsAg and small parts of pre-S) (Shouval, 2003; Shouval, Roggendorf and Roggendorf, 2015). In the 1980s, a second-generation HBV vaccine produced in yeast transfected with HBV DNA was developed using advances in recombinant DNA technology due to concerns regarding safety of using antigens obtained from blood products (Shouval, 2003; Shouval, Roggendorf and Roggendorf, 2015). This second-generation vaccine contains SHBs of the surface gene and is widely used as a component of the WHO Expanded Programme on Immunization (EPI) of infants (Shouval, 2003; Shouval, Roggendorf and Roggendorf, 2015). A third-generation HBV vaccine constructed in transfected mammalian cells has been developed in Germany, Korea, Israel and France (Shouval, 2003; Shouval, Roggendorf and Roggendorf, 2015).

HBV second generation vaccine is available as both monovalent and combined formulations (Lavanchy, 2012). The combined formation (pentavalent or hexavalent) contains immunisations against HBV, diphtheria, tetanus, pertussis and Haemophilus influenzae type b (Hib) with or without polio vaccine, and is given to infants at the ages of six, 10 and 14 weeks as part of the WHO EPI (Lavanchy, 2012; Nayagam, Shimakawa and Maud, 2020), aiming to interrupt horizontal transmission (Nayagam, Shimakawa and Maud, 2020). HBV monovalent vaccine should be administered to all infants within 24 hours of birth, especially those born in high endemic areas (WHO, 2015). Three doses of this monovalent formulation should also be administered to adults in high risk groups, including health care professionals, people requiring frequent blood or blood products, people injecting drugs, prisoners, household and sexual contacts of people with CHB and travellers to endemic areas (Lavanchy, 2012; Chang, Chen and Chen, 2015; WHO, 2018a).

1.10.3 HBV prevention of mother-to-child transmission (PMTCT)

The current guidelines recommend screening antenatal women for infection using HBsAg, and risk-stratification of vertical transmission based on further laboratory tests for HBeAg and/or HBV DNA VL. Prophylactic antiviral therapy is recommended for women with high HBV DNA VL or HBeAg positivity (Ayoub and Cohen, 2016; EASL, 2017). PMTCT guidelines suggest administering HBV birth dose vaccine within 24 hours of birth as well as three doses of HBV vaccine provided from the age of six weeks, as well as administering HBIg to high risk babies in the first day of life (Ayoub and Cohen, 2016; EASL, 2017). Together, these strategies reduce the rate of vertical transmission by 85–95% (Nelson, Jamieson and Murphy, 2014), representing a crucial component of efforts towards HBV elimination (WHO, 2015; O'Hara et al. 2017). However, breakthrough transmission can occur, especially among mothers with high HBV VL (Chang, 2010), in settings where the first dose of vaccine is delayed, and where HBIg is not available.

As of 2015, the global coverage of third dose and birth dose HBV vaccine among infants was estimated at 84% and 39%, respectively, with certain regions including Europe, Eastern Mediterranean and Africa facing coverage gaps compared to other settings (WHO, 2017b). As of 2016, 46% of infants received HBV birth dose vaccination; 13% of infants born to mothers with CHB had received HBV birth dose vaccine, HBIG, a complete series of HBV vaccinations; and <1% of pregnant women with high VL had received antiviral treatment (Razavi-Shearer, 2018). By 2019, only ten countries in Africa (an estimated coverage of 6%) had adopted birth dose HBV vaccination as part of the routine infant immunisation schedule (WHO, 2019).

1.10.4 Vaccine escape mutations (VEMs)

Changes in the S protein, specifically within the major antigenic determinant **(Figure 1.3)**, can result in conformation changes leading to: (i) evasion of vaccine-induced immunity and HBIg therapy due to VEMs (Cooreman, Leroux-Roels and Paulij, 2001; Caligiuri et al. 2016;

Lazarevic et al. 2019); (ii) false negative HBsAg testing (occult HBV infection) by commercial assays due to diagnostic escape mutations (Caligiuri et al. 2016; Lazarevic et al. 2019). Mutations in HBV polymerase can also lead to amino acid changes in the S protein due to ORFs in the genome (Caligiuri et al. 2016). The surface protein mutation G145R is a well-known VEM which on its own can be responsible for vaccine escape; experimentally, it fails to bind to HBsAg antibodies (HBsAb) and has also been isolated from vaccinated children who become infected (Steward et al. 1993; Carman, 1997; Lazarevic et al. 2019). In addition, the mutation K141E has been reported to have reduced binding to HBsAb and has also been isolated from children vaccinated children who became HBV core antibody positive (a marker of infection) (Steward et al. 1993; Carman, 1997). Recently several other mutations in S protein have also been associated with vaccine/HBIg resistance (Lazarevic, 2014; Lazarevic et al. 2019). There are very limited data for VEMs in Africa, but in other settings of high endemicity, VEMs can be common, as evidenced by a reported prevalence of 28% in vaccinated HBV-infected children in Taiwan (Hsu et al. 1999).

1.11 Natural course of HBV infection and diagnosis

The natural course of acute or CHB is determined by a complex interplay of viral factors (genotype, mutations and VL), host factors (age, gender, genetics, immune response and family history) and environmental factors (liver toxins, alcohol, aflatoxins, and coinfection such as HIV, HCV, HDV, and schistosomiasis) (Croagh, 2014). For example, following acute infection, 90% of neonates develop chronic infection whereas 20–60% of children under the age of five years and 5% of adults develop chronic infection (WHO, 2017a).

Mode of transmission also plays a role in the natural history of CHB infection (Nayagam, Shimakawa and Maud, 2020). Individuals who have acquired HBV infection through MTCT have a higher risk of persistent viral replication, lower possibility of spontaneous HBeAg seroconversion and higher severity of liver disease with a higher risk of HCC compared to individuals who acquired HBV infection through horizontal transmission (Chiu, 2014;

Shimakawa, 2015; Shimakawa, 2016). Asia has been considered the continent where MTCT predominates, with an estimated transmission rate of 40% compared to 10% in Africa during the pre-vaccination era (Edmunds, 1996; Nayagam, Shimakawa and Maud, 2020). A greater proportion of transmissions in Africa are horizontal transmissions occurring between the ages 2–10 years, although the mechanisms are poorly understood (Kao, 2002; Lemoine and Thursz, 2017). HBV genotype differences in these continents have been proposed as a reason for these differences in modes of transmission (Kao, 2002). Other proposed theories include varying HBV DNA VL and differences in obstetric complications in these continents (Keane, Funk and Shimakawa, 2016). However, as HBV infant vaccination becomes increasingly available, epidemiology of transmission may change with all regions having a similar risk of perinatal transmission (Nayagam et al. 2016).

1.11.1 Acute HBV infection

Acute infection may be self-limiting, or may proceed to chronic infection (EASL, 2017; WHO, 2017a), as shown in **Figure 1.7.**

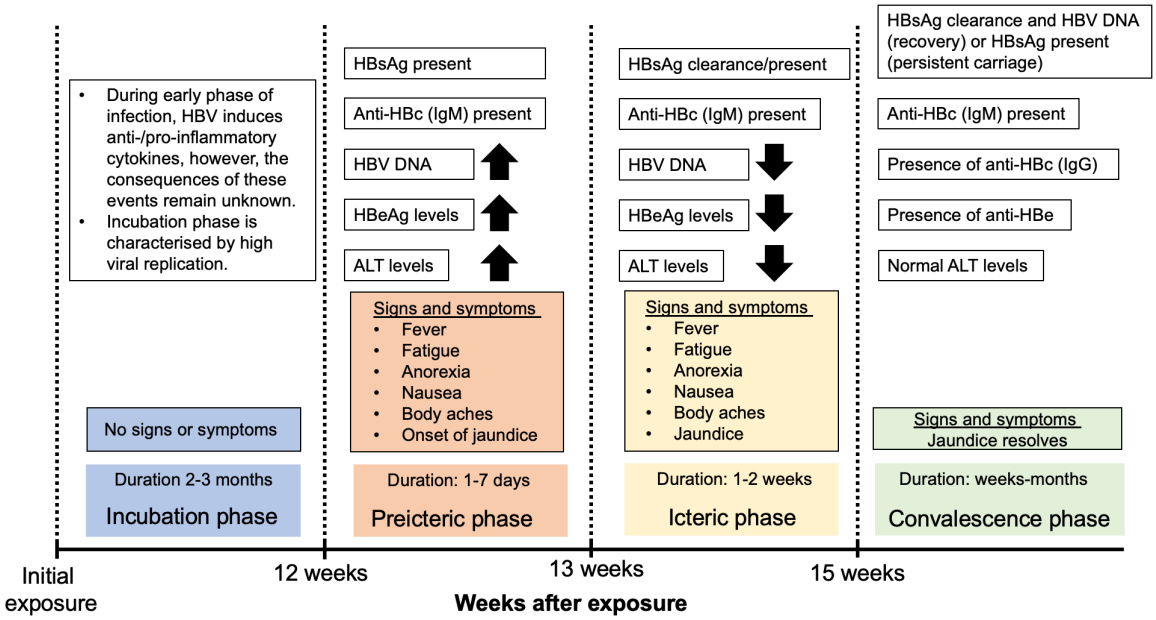


Figure 1.7: Clinical course and serological profile of acute hepatitis B virus infection (Liang, 2009; Liu and Zhang, 2015). HBsAg: HBV surface antigen; Anti-HBc: antibody to HBV core antigen; HBeAg: HBV e antigen; ALT: Alanine Aminotransferase; Anti-HBe: antibody to HBV e antigen.

The innate immune response in humans may be responsible for early viral containment through the action of natural killer, Kupffer and dendritic cells which produce cytokines that inhibit viral replication and kill infected hepatocytes (Tang et al. 2018). The cytokines can also activate the adaptive immune responses (Tan, Koh and Bertoletti, 2015). Dendritic cells, monocytes and macrophages are antigen presenting cells and therefore present internalised HBV epitopes to T cell receptors (TCR) of CD4+ T cells via major histocompatibility (MHC) class II molecules (Bertoletti and Ferrari, 2016). CD4+ T cells produce cytokines which maintain CD8+ T cells and B cells (Bertoletti and Ferrari, 2016). CD8+ T cells are necessary in the control of CHB infection (Matthews et al. 2016; Lumley et al. 2016; Lumley et al. 2018) (**Figure 1.8).**

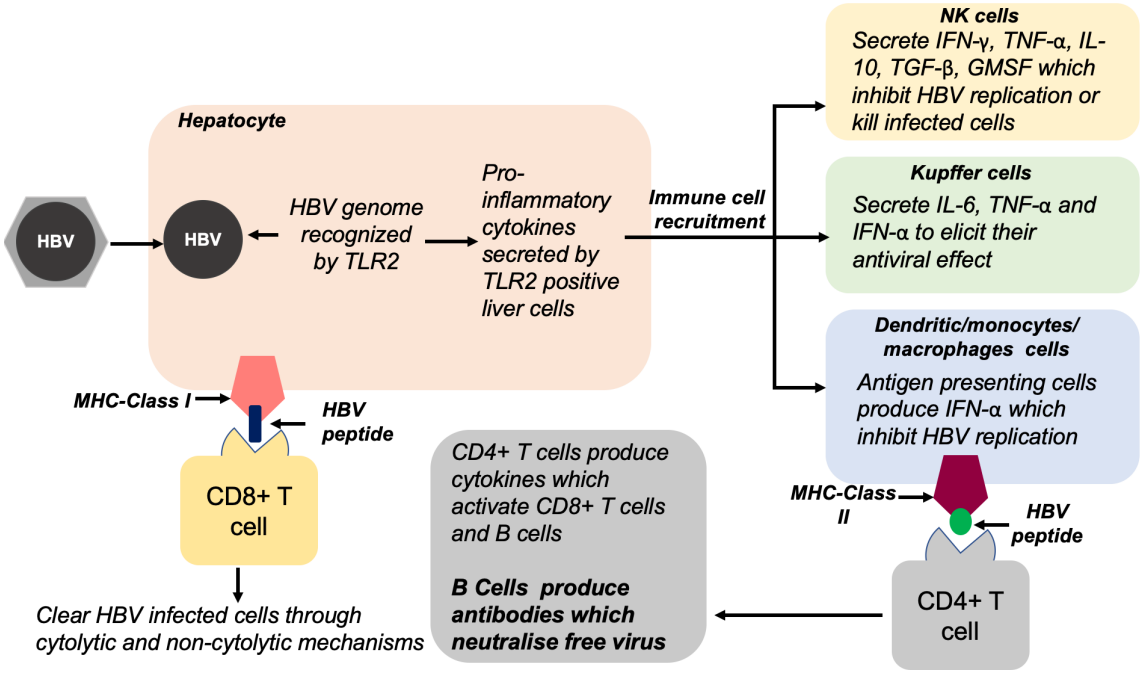


Figure 1.8: Immune responses to hepatitis B virus infection (Bertoletti and Ferrari, 2016; Tang et al. 2018). TLR2: Toll like receptors. NK: natural killer cells. IFN-γ: interferon gamma. TNF-α: tumor necrosis factor alpha. IL-10: interleukin-10. GMSF: granulocyte-macrophage stimulating factor. TGF-β: transforming growth factor beta. IL-6: interleukin-6. TNF-α: tumor necrosis factor alpha. IFN-α: interferon alpha. MHC: major histocompatibility. TCR: T cell receptors.

1.11.2 Chronic HBV infection

CHB is a dynamic process based on the interplay between the host immune system and viral replication. Outcomes vary between development of chronic inflammatory liver disease, cirrhosis, and/or hepatocellular carcinoma, but many of those infected remain asymptomatic for life (Tan, Koh and Bertoletti, 2015; Seto et al. 2018). European Association for the Study of the Liver (EASL) 2017 guidelines describe CHB in four phases with different biochemical and virological profiles (**Table 1.3)**.

Table 1.3: Clinical course and serological profile of hepatitis B virus infection.

Phase	Characteristics
Phase 1 (HBeAg positive chronic infection)	• High HBsAg • HBeAg positive • HBV DNA >7 log_{10} IU/ml • Normal ALT • None/minimal liver disease
Phase 2 (HBeAg positive chronic hepatitis)	• High/intermediate HBsAg • HBeAg positive • HBV DNA between 4–7 log_{10} IU/ml • Elevated ALT • Moderate/severe liver disease
Phase 3 (HBeAg negative chronic infection)	• Low HBsAg • HBeAg negative • HBV DNA between <2,000–20,000 IU/ml • Normal ALT • No liver disease
Phase 4 (HBeAg negative chronic hepatitis)	• Intermediate HBsAg • HBeAg negative • HBV DNA between >2,000 IU/ml • Elevated ALT • Moderate/severe liver disease
Phase 5 (HBsAg negative phase: Occult HBV infection)	• HBsAg negative • Antibodies to core antigen +/- antibodies to HBsAg • Undetectable HBV DNA • Normal ALT • HBsAg loss before cirrhosis is associated with minimal risk of cirrhosis

HBsAg: HBV surface antigen. HBeAg: HBV e antigen. ALT: Alanine Aminotransferase.

1.12 HBV treatment

The primary purpose of treatment in CHB is to reduce liver related morbidity and mortality, and to improve the quality of life and survival among those infected (Ward et al. 2016; EASL, 2017). HBV treatment can also have a population level impact, by reducing the reservoir of virus for transmission (Pan et al. 2016; McNaughton et al. 2020b). The current treatments for HBV inhibit viral replication, however, they do not typically lead to sustained remission or cure, and therefore require long term administration (Ward et al. 2016; EASL, 2017).

1.12.1 Treatment strategies

There are two main treatment options for CHB:

1.12.1.1 Pegylated interferon alpha (PegIFNa)

PegIFNa was developed in 2005 for CHB treatment (Woo, Kwok and Ahmed, 2017). PegIFNa is administered through subcutaneous injection once a week for a period of 24 weeks; it is

thought to increase host cell mediated immunity against HBV and also target various parts of the HBV life cycle including decreasing HBV RNA transcription thus inhibiting viral replication (EASL, 2017; Woo, Kwok and Ahmed, 2017). Despite the finite duration of treatment and its potential ability to induce long-term immunological control, only about 30% of individuals achieve a sustained off treatment response (Woo, Kwok and Ahmed, 2017). Other disadvantages of PegIFNa include its adverse side effect profile, such that it cannot be used in infants below one year of age, pregnant women and individuals with decompensated cirrhosis, hypersplenism, thyroid disease, autoimmune diseases, heart diseases, kidney diseases, psychiatric illness, retinopathy, and blood disorders, and it is also limited by cost (WHO, 2015; EASL, 2017; Woo, Kwok and Ahmed, 2017).

1.12.1.2 Nucleos(t)ide analogues (NAs)

NAs that can be used for HBV treatment include lamivudine (3TC), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TFV) which is formulated either as tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF).

1.12.1.2.1 Lamivudine

3TC was initially developed and widely used for HIV infection, and in 1998 the FDA approved it for treatment of CHB (De Clercq et al. 2010; Lingala et al. 2016; Luo, Jiang and Ren, 2018).

3TC is a nucleoside analogue which inhibits HBV viral replication by acting as a chain terminator when incorporated into the growing first strand of DNA synthesis thus preventing RNA-DNA transcription (De Clercq et al. 2010). It is administered orally in its inactive form and then transported into the cell where it is converted to its active metabolite, lamivudine triphosphate (3TC-TP) by intracellular kinases (Else et al. 2012). 3TC is considered safe for use among both children and adults, including during pregnancy (De Clercq et al. 2010). Although successful in HBV treatment, resulting in increased rates of HBeAg seroconversion and improved liver function and disease outcomes among both HBeAg-positive and -negative individuals (Sokal 2005; De Clercq et al. 2010; Lingala et al. 2016), its use is currently

discouraged given the high rates of resistance (De Clercq et al. 2010; Lingala et al. 2016). The best recognised 3TC RAM arises at position 204 of the RT protein, representing the second position of the YMDD domain C of the viral polymerase and is commonly associated with compensatory mutations located in positions 173, 169, 180, 184, 202 and 215 in polymerase domains A, B and in the B–C interdomain (Warner and Locarnini, 2014; Caligiuri et al. 2016; Beloukas and Geretti, 2017).

It is estimated that 20% of HBV monoinfected individuals develop 3TC resistance within the first year of treatment while 90% of HIV/HBV coinfected individuals develop resistance within five years of treatment (Fischer, Gutfreund and Tyrrell, 2001). However, where ETV and TFV are not available and in instances where it is included in antiretroviral therapy (ART) regimen as a second drug with anti-HBV activity, 3TC can still be considered for HBV treatment (De Clercq, 2015). 3TC is also used in prophylaxis to prevent HBV reactivation in HBc-Ab positive individuals undergoing immunosuppressive treatment and may be used in a combination therapy (Pattullo, 2016).

1.12.1.2.2 Adefovir dipivoxil

ADV was approved for treatment of CHB in 2002 (Lingala et al. 2016). It is a nucleotide analogue administered orally as adefovir which is then converted to ADV in plasma and then converted to its active metabolite, adefovir diphosphate (ADV-DP) intracellularly (Marcellin et al. 2003). ADV-DP inhibits viral replication by competing with the natural substrate deoxyadenosine 5' triphosphate for incorporation into viral DNA thus acting as chain terminator (Marcellin et al. 2003). Although ADV is considered effective in inhibiting both wildtype and YMDD mutant HBV variants, its use has been discouraged due to it weak antiviral activity, nephrotoxicity and increasing association with resistance (Villamil and Cairo, 2013).

1.12.1.2.3 Telbivudine

LdT was approved for treatment of CHB in 2006 (Long et al. 2017). LdT is a nucleoside analogue of thymidine which is administered orally and then converted to its active form,

telbivudine 5'-triphosphate intracellularly by cellular kinases (Amarapurkar, 2007). Its active metabolite, 5-triphosphate of β-L-2-deoxynucleosides, is incorporated into the second strand of the growing DNA (DNA-to-DNA transcription) thus inhibiting replication (Amarapurkar, 2007). Although it has superior rates of viral suppression compared to 3TC with protective properties against nephrotoxicity, it has been shown to have high rates of resistance, with mutations especially within the YMDD region (Amarapurkar, 2007; Burke and Marks, 2017).

1.12.1.2.4 Entecavir

ETV was approved for treatment of CHB in 2005 (Lingala et al. 2016). ETV is administered orally then converted to its active metabolite, entecavir triphosphate (ETV-TP) intracellularly (Yan et al. 2006). ETV is carbocyclic analogue of 2' deoxyguanosine which inserts into the first and second strands of the viral DNA inhibiting both RNA-to-DNA and DNA-to-DNA transcription, and also prevents replication by inhibiting the unique protein-linked priming activity (Dimou, Papadimitropoulos and Hadziyannis, 2007; Langley et al. 2007). ETV is considered to have potent antiviral activity with a high genetic barrier to resistance (Villamil and Cairo, 2013). However, resistance in ETV arises more commonly in the context of prior 3TC exposure, in the presence of RAMs M204I/V and L180M, whereby 51% of individuals exposed to 3TC develop resistance to ETV after five years of treatment compared to only 1.2% of treatment naïve individuals (Fung et al. 2011; Locarnini, 2017). ETV treatment is therefore recommended among patients who are treatment naïve (De Clercq, 2015). ETV has no activity against HIV and cannot be used during pregnancy due to its carcinogenic potential shown in animal studies (Lin et al. 2008; Aslam et al. 2018). While ETV can be procured in some low and middle income countries, it is expensive at cost of 427 United States dollars (USD) per person per year of treatment, and therefore not available in practice in many countries in sSA (Hill et al. 2015).

1.12.1.2.5 Tenofovir

TDF was approved for treatment of CHB in 2008 (Lingala et al. 2016). It is an acyclic nucleotide analogue that was initially developed and is widely used for HIV treatment (Lovett et al. 2017). It is administered orally as TDF which is then converted to TFV in plasma and finally to its active ingredient tenofovir diphosphate (TFV-DP) intracellularly (Kearney, Flaherty and Shah, 2004; Abdul Basit et al. 2017; Byrne, Carey and Agarwal, 2018) **(Figure 1.9)**.

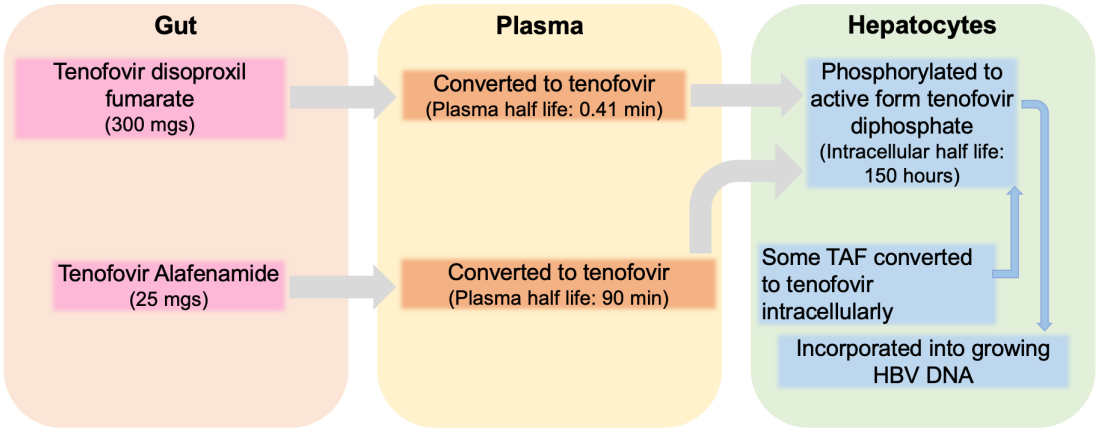


Figure 1.9: Pharmacokinetics of tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF). Orange rectangle represents the gut, showing amount of drug absorbed in milligrams (mg); yellow rectangle represents plasma, in which TDF and TAF are converted to tenofovir. Green rectangle represents hepatocytes where tenofovir is converted to its active form (tenofovir diphosphate) which is then incorporated into growing HBV DNA to stop viral replication.

TFV-DP inhibits viral replication by competing with the natural substrate nucleotide deoxyadenosine 5´triphosphate for incorporation into the viral DNA and therefore terminating DNA chain elongation (Kearney, Flaherty and Shah, 2004; Abdul Basit et al. 2017; Byrne, Carey and Agarwal, 2018). TDF can be safely used in pregnancy (WHO, 2016b) and is included in some guidelines for HBV PMTCT (EASL, 2017; Terrault et al. 2018). Approved in 2015, TAF a phosphonate prodrug of TFV, was developed to mitigate the risk of renal and bone toxicities associated with TDF (De Clercq, 2016; Byrne, Carey and Agarwal, 2018) since it can be at administered at a lower dose (25 mgs) and has high plasma stability compared to TDF (Abdul Basit et al. 2017; Byrne, Carey and Agarwal, 2018; Childs-Kean, Egelund and Jourjy, 2018) **(Figure 1.9)**. Given that TDF and TAF are both used for CHB management, I

refer to TFV throughout the thesis to represent either form of the drug. HBV resistance to TFV is not well characterised, but there are emerging data from *in vitro* studies associating RT mutations A194T and N236T with decreased susceptibility (Sheldon et al. 2005; Fung et al. 2011). Nevertheless, TAF and TDF have a high genetic barrier to resistance and together with ETV, are the current recommended treatment options for CHB (WHO, 2015; EASL, 2017).

1.12.2 Treatment guidelines

Currently there are five published treatment guidelines for HBV management which represent the optimal management of HBV infection. These include the European Association for the Study of the liver (EASL, 2017), American Association for the Study of Liver Diseases (AASLD) (Terrault et al. 2018), Asian-Pacific Association for the Study of the Liver (APASL) (Sarin et al. 2015), South Africa guideline for the management of CHB (Spearman et al. 2013), and WHO guidelines for the prevention, care and treatment of persons with CHB (WHO, 2015), summarised in **Table 1.4.**

Table 1.4: Summary of published hepatitis B virus treatment guidelines.

	EASL 2017	AASLD 2018	APASL 2015	South African 2013	WHO 2015
Who should be treated?	• Immune reactive HBeAg positive or HBeAg negative CHB (positive/negative HBeAg status, >2,000 IU/ml HBV DNA and elevated ALT levels) • Cirrhosis (compensated or decompensated) • Immune tolerant or immune reactive HBeAg positive and >30 years old	• Immune-active chronic HBV (HBeAg positive/negative) • Immune tolerant and >40 years	• Cirrhosis (compensated or decompensated) with detectable HBV DNA • Immune reactive HBeAg positive	• Cirrhosis (compensated or decompensated) with detectable HBV DNA • Immune suppressed individuals with chronic HBV infection	• Cirrhosis (compensated or decompensated) • >30 years, have persistently abnormal ALT levels and >20,000 IU/ml HBV DNA
Recommended NAs	**TDF:** 245 mg/day **TAF:** 25 mg/day **ETV:** 0.5 mg/day	**TDF:** 300 mg/day **TAF:** 25 mg/day **ETV:** 0.5 mg/day	**TDF:** 300 mg/day **TAF:** 25 mg/day **ETV:** 0.5 mg/day	**TDF:** 300 mg/day **ETV:** 0.5 mg/day if 3TC naïve; 1 mg/day if exposed to 3TC	**TDF:** 245 mg/day **ETV:** 0.5 mg/day for adult with compensated liver disease and 3TC naive; 1 mg once daily for adult with decompensated liver disease
Duration of treatment	Long term until loss of HBsAg	• Indefinite for HBeAg positive individuals with cirrhosis or HBeAg negative individuals with immune-active CHB infection • Long term until seroconversion to anti-HBe for HBeAg positive individuals without cirrhosis	• Indefinite for HBeAg positive individuals with cirrhosis or HBeAg negative individuals with immune-active chronic HBV infection • Long term until seroconversion to anti-HBe for HBeAg positive individuals without cirrhosis	• Long term until anti-HBe seroconversion and undetectable HBV DNA levels in HBeAg positive • Indefinite HBeAg negative individuals and those with cirrhosis	Indefinite for all persons with cirrhosis
Estimated level of HBV DNA at 48 or 52 weeks of treatment (HBeAg positive)	**TDF:** 76% achieve <60–80 IU/ml **TAF:** 76% achieve <60–80 IU/ml **ETV:** 67% achieve <60–80 IU/ml	**TDF:** 76% achieve <60 IU/ml **TAF:** 73% achieve <29 IU/ml **ETV:** 61% achieve <50–60 IU/ml	None described	None described	None described
Estimated level of HBV DNA at 48 or 52 weeks of treatment (HBeAg negative)	**TDF:** 93% achieve <60–80 IU/ml **TAF:** 93% achieve <60–80 IU/ml **ETV:** 90% achieve <60–80 IU/ml	**TDF:** 93% achieve <60 IU/ml **TAF:** 90% achieve <29 IU/ml **ETV:** 90–91% achieve <50–60 IU/ml	None described	None described	None described

Potential side effects	**TDF:** • Nephrotoxicity • Osteomalacia **TAF:** Not recommended in individuals with creatinine clearance <15 mL/min and not receiving haemodialysis **ETV:** Lactic acidosis	**TDF:** • Nephropathy • Fanconi syndrome • Osteomalacia • Lactic acidosis **TAF:** Lactic acidosis Dose adjustment required if creatinine clearance is <15 mL/min **ETV:** Lactic acidosis	None described	**NAs:** • Nephrotoxicity • Bone toxicity	**NAs:** • Nephrotoxicity • Bone toxicity

CHB: Chronic hepatitis B virus. TDF: Tenofovir disoproxil fumarate. TAF: Tenofovir alafenamide. ETV: Entecavir. 3TC: Lamivudine. NAs: Nucleos(t)ide Analogues. HBsAg: HBV surface antigen. Anti-HBc: antibody to HBV core antigen. HBeAg: HBV e antigen. ALT: Alanine Aminotransferase. Anti-HBe: antibody to HBV e antigen.

1.13 Treatment failure in chronic HBV infection (CHB)

Treatment failure is defined by the presence of detectable serum HBV DNA while on therapy for a defined period of time. Treatment non-responders can be classified into several categories (Spearman et al. 2013; Sarin et al. 2015; WHO, 2015; EASL, 2017; Terrault et al. 2018), as follows:

i. Primary non-responders: <1 $\log_{10}$ decrease of serum HBV DNA after three months of therapy. Possible causes include treatment non-compliance and/or drug resistance.

ii. Partial non-responders: >1 $\log_{10}$ IU/ml decrease of serum HBV DNA but still detectable at least 12 months of therapy. Possible causes include insufficient intracellular drug levels, high pre-treatment HBV viral load (VL) and/or drug resistance

iii. Virological breakthrough: increase in HBV DNA level >1 $\log_{10}$ IU/ml compared to the lowest value of HBV DNA assessed during therapy. Possible causes include treatment non-compliance, insufficient intracellular drug levels and/or drug resistance.

Treatment failure in CHB can be due to several factors including drug resistance, treatment non-compliance and drug-drug interaction.

1.13.1 Drug resistance

Section 1.12.1.2 describes NAs that have or are currently being used to manage CHB as well as RAMs associated with these drugs. HBV RAMs occur in the context of high viral replication in combination with high mutation rates arising as a result of the error prone RT enzyme, and

with the availability of replication space (hepatocyte turnover), variants with these RAMs become dominant as a result of exposure to drugs (Bartholomeusz and Locarnini, 2006; Zoulim, 2011) **(Figure 1.10)**.

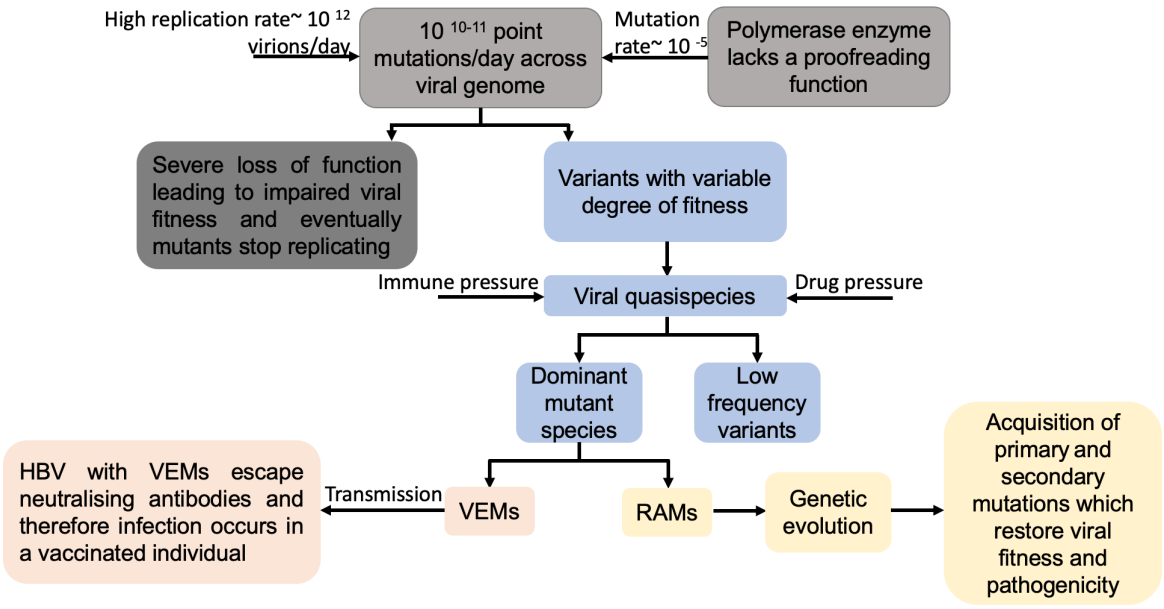


Figure 1.10: Mechanism of resistance in hepatitis B virus. Mutations associated with drug or vaccine resistance occur as a result of high viral replication. Some mutations can lead to severe loss of function thus impairing viral fitness and replication capacity, whereas other mutations can lead to the development of variants which can become dominant with exposure to drug or vaccine. These variants evolve to acquire compensatory mutations which restore their replication capacity. Drug resistance-associated mutations (RAMs) can continue to replicate in the presence of therapy (yellow rectangle). Vaccine escape mutations (VEMs) can be transmitted to a vaccinated individual causing infection (orange rectangle).

1.13.2 Treatment compliance

Treatment non-compliance can contribute to virological breakthrough (Boyd et al. 2014). Treatment compliance can sometimes be ascertained with confidence by taking a careful history from the patient, who may report that they are not taking medications consistently, for a variety of reasons. In some situations, this can be difficult to assess clearly partly because self-reporting of compliance may be inaccurate. Testing for drug levels in plasma, peripheral blood mononuclear cells (PBMCs), red blood cells (RBCs), hair and urine can provide an objective way of assessing treatment compliance (Koss et al. 2018; Murnane et al. 2019; Cressey et al. 2020; Drain et al. 2020). Pharmacologically active TFV-DP has a longer

intracellular half-live compared to TFV in plasma (**Figure 1.9**), and therefore, drug levels assessment in PBMCs or RBCs may provide long-term information on treatment compliance (Castillo-Mancilla et al. 2013). The window of treatment of exposure represented in plasma and PBMCs is typically 2–7 days and 7–14 days, respectively (Castillo-Mancilla et al. 2013; Louissaint et al. 2013; Baxi et al. 2015). Hair samples can also inform on long-term exposure as they reflect drug uptake from systemic circulation over weeks to months (Beumer, Bosman and Maes, 2001; Gandhi et al. 2015).

There are few studies that have assessed for TFV (Marcellin et al. 2008; Boyd et al. 2014; Kitrinos et al 2014; Wong et al. 2016), and ETV drug levels in HBV (Boglione et al. 2016). However, there is a gap in the literature and in published treatment guidelines regarding appropriate thresholds to confirm treatment compliance and appropriate time, relative to the dose that has been or should be taken, to collect samples for drug levels assessment in CHB.

A summary of pharmacokinetics and drug levels of TFV and ETV assessed in samples obtained from healthy individuals (without HIV and HBV infection) is provided in **Table 1.5**.

Table 1.5. Summary of pharmacokinetics and drug levels of tenofovir (TFV) and entecavir (ETV) across different samples obtained from adult not infected with hepatitis B virus (HBV) and reported to be fully compliant with treatment. There were no studies that recruited individuals infected with HBV.

NAs	Sample type	Form of drug	Half-life	Type of study	Study participants	Duration of Rx	Time of sample collection	Median (IQR/SD) drug concentration in sample)	Reference
TFV	Plasma	TFV	17 hours	Randomised pharmacokinetic study	Healthy male (ages 18–49 years), n=28	6 weeks	Beginning of weeks 1, 3, 5 and 7 of treatment immediately before dosing (trough level)	41.0 (26.0–52.0) ng/ml	Drain et al. 2020
	PBMCs	TDF-DP	4.2 days	Longitudinal	HIV negative individuals* (ages 22–47 years), n=17	30 days	Day 1, 3, 7, 20 and 30 of treatment (steady state)	100 (90–200) fmol/10^6 cells	Castillo-Mancilla et al. 2013
	RBCs	TDF-DP	17 days	Longitudinal	HIV negative individuals* (ages 22–47 years), n=17	30 days	Day 1, 3, 7, 20 and 30 of treatment (steady state)	130(104–170) fmol/10^6 cells	Castillo-Mancilla et al. 2013
	Hair	TFV	No data	Randomised controlled trial	Healthy male and female individuals (ages >18 years), n=28	6 weeks	collected at endpoint visit (6th week)	0.038 (0.021–0.053) ng/ml	Liu et al. 2014
	Urine	TFV	No data	Randomised controlled trial	Healthy male (ages 18–49 years), n=28	6 weeks	Beginning of weeks 1, 3, 5 and 7 of treatment immediately before dosing (trough level)	6480 (3940–14300) ng/ml	Drain et al. 2020
ETV	Plasma	ETV	128–149 hours	Randomised controlled trial	Healthy male, n=6	28 days	Days 1, 7, and 14 immediately before dosing (trough level)	4.23 (9) ng/ml	Yan et al. 2006
	Urine	ETV	No data	Randomised controlled trial	Healthy male, n=6	28 days	Days 1, 7, and 14 immediately before dosing (trough level)	Urinary recovery mean (SD) %: 65.22 (16.27)	Yan et al. 2006

NAs: Nucleos(t)ide analogues. PBMCs: Peripheral blood mononuclear cells. RBCs: Red blood cells.
Rx: Treatment. TFV: Tenofovir. TFV-DP: Tenofovir diphosphate. ETV: Entecavir. ETV-TP: Entecavir triphosphate.
Healthy: means both HIV and HBV negative. *HBV status not reported.
IQR: Interquartile range. SD: Standard deviation
ng/ml: nanograms/mole. fmol/10^6cells: femtomole/one million cells.
Healthy volunteers have primarily been used as the study population in studies investigating pharmacokinetics and pharmacodynamics of drugs, since the main goals are to determine the mechanism by the which the drug is absorbed, metabolised and excreted, as well as to characterise its safety and tolerability across a range of doses (Karakunnel et al. 2018; Pasqualetti et al. 2010). Both ETV and TFV reach a steady-state exposure between seven and 14 days after initiation of once-daily dosing (Markowitz et al. 2014; Yan et al. 2006).

A randomised pharmacokinetic study for the measurement of TFV in plasma carried out among healthy males by Drain et al 2020, reported a median steady-state trough concentration of TFV in plasma at 41.0 [Interquartile range (IQR), 26.0–52.0] ng/ml in the perfect adherence arm (taking 300mg of TFV tablet once daily); 16.0 (IQR, 14.0–19.0) in the moderate adherence arm (taking 300mg of TFV tablet four time a week); and 4.0 (IQR, 3.0–5.1) ng/ml in the low adherence arm (taking 300mg of TFV tablet twice a week). These levels may help inform on thresholds to confirm TFV treatment compliance; with levels >26.0 ng/ml suggesting compliance, levels <26.0 and >5.1 ng/ml suggesting partial compliance, and levels <5.1 ng/ml suggesting minimal or no treatment being taken.

1.13.3 Drug-drug interactions

Use of other drugs with NAs or co-administrating NAs may affect their plasma levels, thus interfering with treatment response. Pharmacokinetics of ETV are not altered when co-administered with TFV, 3TC, ADV or LdT (accessdata.fda.gov). Use of Tipranavir, a protease inhibitor used to treat resistant HIV infection, interacts with TFV resulting in reduced TFV plasma levels and thus may contribute to delayed treatment response (drugs.com). **Table 1.6** shows drug-drug interaction of ETV/TFV with other drugs**.**

Table 1.6. Summary of drugs that may decrease plasma levels of entecavir (ETV) or tenofovir (TFV) (drugs.com and hiv-druginteractions.org).

NA	Drug interactions with ETV/TFV	Indications
ETV	Orlistat	Used to treat obesity
TFV	Tipranavir	Resistant HIV infection (PI)
	Orlistat	Used to treat obesity
	African potato (medicinal plant in the *Hypoxidaceae* family)	Used to treat urinary tract infections, prostate problems, prostate cancer, other cancers and lung disease
	Carbamazepine	Used to treat epilepsy
	Oxcarbazepine	Used to treat seizures
	Phenobarbitone	Used to treat seizures
	St John's Wort (medicinal plant in the *Hypericaceae* family)	Used to treat mild to moderate depression and to manage menopause symptoms

DAA: Direct acting antiviral. NNRTI: Non-nucleoside reverse transcriptase inhibitor. NA: Nucleos(t)ide analogue. NRTI: Nucleoside reverse transcriptase inhibitor. PI: Protease inhibitor.

1.14 HBV sequencing

HBV whole genome sequencing offers the opportunity to explore mutations that are associated with drug and vaccine resistance, diagnostic failure and different clinical outcomes, and also provides the opportunity to study viral transmission and evolution (Gunther et al. 1995; Chook et al. 2015; Wang et al. 2017; McNaughton et al. 2019c). Sanger sequencing has also provided insights into HBV genetic sequence, HBV diversity and its role in pathogenesis (Gunther et al. 1995). However, this method generates only a consensus-level sequence and is therefore unable to capture information on HBV quasispecies (McNaughton et al. 2019c). PCR amplification of sub-genomic fragments has been widely used to study the genetic structure of HBV. However, this method does not allow for the analysis of the whole genome and therefore the impact of mutations within a single genomic molecule on different parts of the genes has not been studied. A single step whole genome PCR amplification has been developed, but this approach requires samples with high HBV DNA VL levels of >4 $\log_{10}$ IU/ml (Gunther et al. 1995). Nested PCR methods, generating overlapping sub-genomic fragments, have been developed to improve sequencing sensitivity for low VL samples (Vincenti et al. 2009; Sun et al. 2012; Taha et al. 2013; Chook et al. 2015). However, these approaches require multiple rounds of amplification with an increased risk of bias and of generating recombinant genomes from the use of multiple PCR primers (Martel et al. 2013).

An approach to amplify serum HBV DNA encompassing a combination of three techniques [*in vitro* completion ligation to convert rcDNA to dsDNA molecule; rolling circle amplification (RCA) which polymerises more than 70,000 nucleotides without detaching from the original DNA template; and genomic PCR], has been suggested to lower the risk of PCR bias (Margeridon et al. 2008; Martel et al. 2013). However, the completion ligation step generates low level of circularised rcDNA thus compromising yield and overall amplification success (Martel et al. 2013).

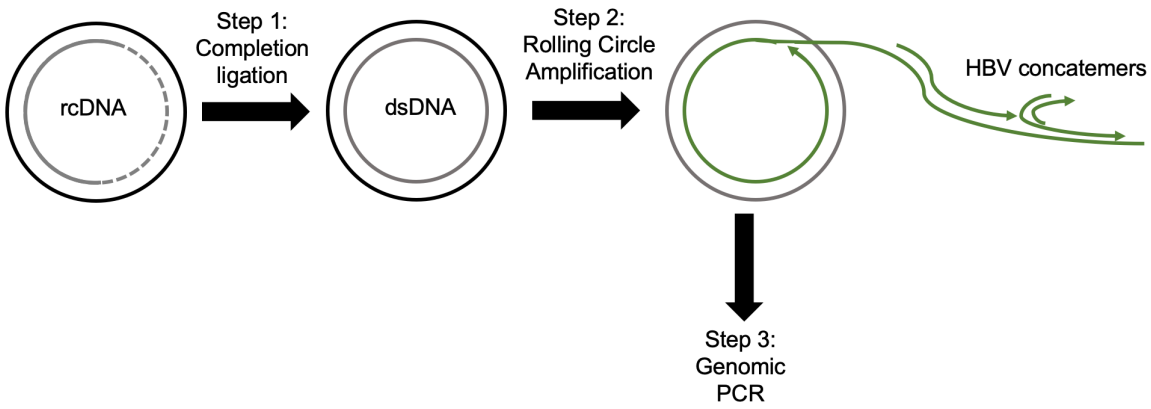


Figure 1.11: Amplification of serum hepatitis B virus DNA using rolling circle amplification. The first step involves converting the relaxed circular double-stranded DNA into a completely double-stranded DNA (dsDNA). Using the dsDNA, rolling circle amplification generates multiple full-length copies of HBV genome (HBV concatemers; shown in green). The generated HBV genomes are then sequenced using Sanger sequencing (Margeridon et al. 2008; Martel et al. 2013).

Next generation sequencing (NGS) platforms, such as Illumina and Nanopore, can reveal the full landscape of HBV quasispecies in individual patients, allowing a more detailed analysis of within- and between-host distribution of resistant mutations (McNaughton et al. 2019a; McNaughton et al. 2019c). Nanopore directly sequences single molecules of amplified PCR products, producing genome-length reads that can reveal mutations contained within a single viral particle (Sauvage et al. 2018; McNaughton et al. 2019c). However, its use to date has been limited due to high error rate in raw reads of ~5–10% (Sauvage et al. 2018; McNaughton et al. 2019c). Illumina is considered the most robust sequencing platform with the ability to capture within sample diversity with high accuracy (McNaughton et al. 2019c). However, it generates short viral reads and it therefore remains challenging to link polymorphisms contained within a single viral particle (McNaughton et al. 2019c). Both Illumina and Nanopore have poor sequencing sensitivity for VL of <3.5 $\log_{10}$ IU/ml (McNaughton et al. 2019c). To mitigate this challenge, one study has used amplicons generated from nested PCR to improve NGS sensitivity in low VL samples; this approach has been reported to sequence samples with VL of as low as 3 $\log_{10}$ IU/ml (Wang et al. 2017).

In most populations, median HBV DNA VL is ~ 4 $\log_{10}$ IU/ml. A study carried out in Abuja showed that 46.8% (312/666) of the patients had VL levels between 20 and 2 × 10^3 IU/ml (Iregbu and Nwajiobi-Princewill, 2016), the median HBV VL among 564 chronic patients recruited in Brazil was 2351 copies/ml (~ 690 IU/ml) (Nita et al. 2009), and the mean HBV VL among 2293 patients recruited in Thailand was 2.2 $\log_{10}$ IU/ml (Leuangarun and Sriprayoon, 2012). Similarly, 351 and 600 serum samples from individuals infected with CHB in Oxford and South Africa, respectively, had a mean HBV VL of 3.4 $\log_{10}$ IU/ml (Downs et al. 2020). The majority of these samples may therefore not be amenable to successful sequencing by Sanger, Illumina or Nanopore (McNaughton et al. 2019c).

1.15 Challenges facing HBV elimination and management globally

Challenges facing HBV elimination are complex despite the availability of effective vaccine and treatment. The HBV vaccine has no impact on existing infection, and changes in the HBV surface protein can result in the development of VEMs (Cooreman, Leroux-Roels and Paulij, 2001; Caligiuri et al. 2016), as well as diagnostic escape mutations which result in false negative HBsAg testing (Caligiuri et al. 2016). Antiviral therapies do not cure HBV infection, requiring long term administration with the attendant risk of drug resistance (Beloukas and Geretti, 2017). There are gaps in diagnosis, vaccine and treatment coverage, with only 9% of those infected globally being diagnosed and only 16% of those diagnosed being treated and linked to care (O'Hara et al. 2017; Revill et al. 2019). In addition, PegIFNa and HBIg are expensive and therefore unavailable in many settings globally.

1.16 Challenges facing HBV elimination and management for sub-Saharan Africa (sSA)

1.16.1 High prevalence of HBV infection

There is a high prevalence of HBV infection in sSA **(Figure 1.6)**; it is estimated that between 60 and 100 million people are chronically infected with HBV in sSA, with an estimated death

rate of 90,000 individuals per year, mainly from cirrhosis and liver cancer (Lemoine, Eholié and Lacombe, 2015; Spearman et al. 2017). Transmission in this region is believed to occur mainly in early childhood with the highest prevalence observed among males and disproportionately affecting those residing in rural areas where resources are limited (Lemoine and Thursz, 2017; Spearman et al. 2017).

1.16.2 High risk of HBV related hepatocellular carcinoma (HCC)

CHB is the major cause of HCC, and HCC is a leading cause of death in Africa (Lemoine and Thursz, 2017). The prognosis of HCC is poor in this continent as the majority of those infected present to hospital at a late stage, where effective treatment would be challenging for any healthcare system (Lemoine and Thursz, 2017). Male sex, HBV perinatal transmission, HBeAg seropositivity, high HBV DNA levels, HBV genotype A and environmental toxins such as aflatoxins have been associated with HCC development in Africa (Kew et al. 2005; Parkin et al. 2008; Liu et al. 2012; Jemal et al. 2012; Shimakawa et al. 2015; Shimakawa et al. 2016). However, good quality seroprevalence data from different African countries are scarce, limiting the accurate quantification of the burden and risk factors associated with HCC development (Lemoine and Thursz, 2017).

1.16.3 HIV coinfection and antiretroviral therapy (ART)

The preferred first line ART regimen for HIV for adolescents and adults include: TDF + 3TC [or emtricitabine (FTC) + efavirenz] (WHO, 2016b). The widespread use of ART incorporating NAs that also have activity against HBV, may have an impact on HBV through improved rates of viraemic suppression, but also potentially by driving the selection of RAMs. The WHO recommends screening for HBsAg in all HIV-1 infected individuals prior to ART initiation, and for all pregnant women during antenatal visits, to improve the clinical outcomes of people living with CHB and to enhance interventions that reduce the incidence of new cases (WHO, 2015). However, screening of HBsAg is not routinely performed in many settings in Africa, with lack of implementation at least partially driven by cost and lack of programmes for HBV treatment

outside the setting of HIV coinfection. HBV infected patients either remain untreated (most typical in the setting of monoinfection), or are exposed to antiviral drugs without proper monitoring and often intermittently, putting them at risk of developing RAMs (more likely in the setting of HIV coinfection) (Kim et al. 2011; Day et al. 2013; Hamers et al. 2013; Beloukas and Geretti, 2017; O'Hara et al. 2017).

Although the cost of TFV has substantially reduced to 32 USD per person per year of treatment, it is mainly accessible as part of combination therapy for HIV/HBV coinfected individuals as it is the first line ART treatment regimens for HIV in sSA (WHO, 2016b). It remains the case that 3TC is used as the only HBV-active agent in some settings (Day et al. 2013; Beloukas and Geretti, 2017), as well as in second line regimens, exemplified by South Africa where second-line ART substitutes Zidovudine (AZT) for TFV leaving only 3TC coverage for HBV (WHO, 2016b). Among HIV/HBV coinfected children in South Africa treated with regimens including 3TC and/or TFV, HBV viraemia has been demonstrated, highlighting potential underlying HBV drug resistance (Jooste et al. 2016).

1.16.4 High risk of mother-to-child transmission (MTCT)

There is a high risk of MTCT of HBV in sSA with an estimated 1% of babies born to mothers with CHB being infected with HBV (Keane, Funk and Shimakawa, 2016). Timely administration of HBV birth dose vaccine followed by three dose of HBV vaccine to infants reduces the risk of HBV MTCT to 10–30 % in HBeAg-positive mothers and <0.5% in HBeAg-negative mothers (Spearman et al. 2017; Shimakawa et al. 2018; Nayagam, Shimakawa and Lemoine, 2020). Despite the effectiveness of HBV birth dose vaccination, its coverage in Africa is still low due to the high cost of HBV monovalent vaccine for countries in terms of infrastructure and systems for delivery, a high number of out-of-hospital births, absence of weekend vaccination services, poor vaccine cold chain infrastructure especially in rural health facilities, and low awareness among healthcare workers (Moturi et al. 2018; Nayagam, Shimakawa and Lemoine, 2020).

In addition, screening of HBV during pregnancy is not routinely done in many countries in sSA, HBIg is expensive and not readily available in most primary healthcare facilities, and prophylactic antiviral therapy during the third trimester is mostly only available to women who are HIV/HBV coinfected (HBV monoinfected have limited access to diagnosis and linkage to care). Providing antiviral treatment for HBV PMTCT relies on HBV diagnosis, but currently there is no widespread access to laboratory-based assays for HBsAg, HBeAg or HBV DNA VL, and many antenatal programmes do not provide routine HBV screening (Wilson et al. 2018). VL test assays are expensive with majority of individuals lacking health insurance to cover the costs of diagnosis (Lemoine, Eholié and Lacombe, 2015). Furthermore, cheap point-of-care tests (POCT) for HBV DNA, HBeAg and HBcrAg (which strongly correlates with high HBV VL), have either poor diagnostic sensitivity and specificity, or have not been validated in a larger field context (Gani et al. 2019; Nayagam, Shimakawa and Lemoine, 2020).

1.16.5 Stigma in HBV infection

Stigma and discrimination have been recognised in the setting of chronic viral hepatitis infections which can be barriers to prevention, diagnosis and treatment, but there is very limited research to determine the nature and impact of stigma, and this is especially true for Africa (Mokaya et al. 2018a). Although not specifically presented in this thesis, during my D.Phil studies I engaged in a collaboration with a social sciences team based in Uganda to investigate awareness, beliefs and behaviour associated with HBV infection in a rural population. We found no specific word for HBV infection in local languages and there was limited knowledge on the cause, mode of transmission and treatment of HBV infection in this population (Mugisha et al. 2019).

1.17 D.Phil project rationale

In order to achieve the United Nations sustainable development goals aiming to eliminate HBV as a public health threat by the year 2030, more people will need to be vaccinated, tested and treated in the coming years resulting in an increased exposure to HBV vaccine and NAs. My

project focuses on the barriers to elimination strategies, with a special interest on the extent to which RAMs and VEMs circulate in African populations. An understanding of the nature, prevalence and clinical consequences of RAMs (especially those associated with TFV - given its role in driving elimination) and VEMs in Africa is essential to improving HBV therapy and prevention in patients with and without HIV coinfection. I therefore set out to address the following research aims:

1.17.1 Aim 1: To review the epidemiology of HBV resistance associated mutations (RAMs) and VEMs in Africa

i. **Objective:** To carry out a systematic literature review which describes the frequency, co-occurrence and distribution of HBV RAMs and VEMs in Africa, and to determine whether changes are needed in recommendations for laboratory diagnostics and/or approaches to drug therapy or vaccine deployment.

   Rationale: To date, no systematic review has assessed the geography and prevalence of HBV RAMs and VEMs in Africa

   Hypothesis: Given that HBV infection is endemic in Africa, HBV RAMs and VEMs may be common in this continent.

ii. **Objective:** To assimilate the current evidence for the development of clinical or virological HBV breakthrough during TFV therapy through a systematic literature review and to perform structural analysis to understand mechanisms of HBV resistance to TFV.

   Rationale: The distribution and clinical significance of HBV genetic signatures that underpin TVF resistance are not well understood. Unlike other NAs, TFV has a high genetic barrier to resistance (Beloukas and Geretti, 2017), corroborated by studies that report no resistance after many years of treatment (Lee and Lee, 2017). However, there are emerging reports of a wider range of amino acid substitutions that are associated with reduced TFV sensitivity, described in both treatment-experienced and treatment-naïve individuals with CHB (Cho et al. 2018; Park et al. 2019). A better

understanding of the role of TFV therapy in driving HBV elimination at a population level is crucial to underpin efforts to move towards international targets for elimination by the year 2030.

Hypothesis: In populations in which HIV and HBV are both endemic, as exemplified by many settings in sSA, drug resistance in HBV may be frequently selected, given the widespread population exposure to TFV as a component of first-line ART for HIV.

1.17.2 Aim 2: To describe the phylodynamics of HBV RAMs and VEMs

Objective: To assimilate data on the global prevalence and distribution of HBV RAMs and VEMs from public sequence databases, and to explore the evolution and genetic relatedness of viruses bearing these mutations.

Rationale: A number of studies have reported the frequencies of RAMs/VEMs in HBV from different populations (Zhang et al. 2015; Hermans et al. 2016; Meng et al. 2017; Mokaya et al. 2018b), however, the global prevalence, geographic distribution, and their association with different HBV genotypes remain unknown. Furthermore, there is uncertainty as to whether RAMs arise without exposure to antivirals or are exclusively a result of prior drug exposure.

Hypothesis: Given that HBV has a higher prevalence in Africa, HBV RAMs and VEMs may be more common in Africa compared to other continents, as well as in genotype A sequences since it is one of the predominant genotypes in Africa. In addition, RAMs and/or VEMs are being selected independently in individual hosts and therefore will be randomly distributed across branches of phylogenetic trees.

1.17.3 Aim 3: To optimise HBV amplification for sequencing in low VL samples

Objective: To optimise selected published nested PCR assays to improve their sensitivity for low VL samples while ensuring full coverage of both the polymerase and surface HBV proteins, in order to allow for sequencing and therefore assessment of HBV RAMs/VEMs in low VL samples.

Rationale: >50% of HBV serum samples have VL of < 4 $\log_{10}$ IU/ml and sequencing sensitivity decreases in low VL samples (see **section 1.14**).

Hypothesis: HBV RAMs and VEMs are present in low VL samples.

1.17.4 Aim 4: To describe the epidemiology of HBV RAMs and VEMs in South Africa and the UK

Objective: To describe the epidemiology of HBV RAMs and VEMs in South Africa and the UK by analysing sequence data generated from serum samples of individuals infected with CHB recruited from clinic centres in these countries. I specifically set out to:

1. Determine the prevalence of HBV RAMs and VEMs in adults recruited from South Africa and the UK;
2. Identify any host and viral factors that are associated with HBV RAMs and VEMs, analysing sequences at both consensus and deep sequence level;
3. Identify factors associated with persistent viraemia while on TFV therapy by:
    - describing virological suppression while on therapy with TFV in the presence of HBV RAMs
    - quantifying TFV drug levels in plasma/PBMCs samples of individuals with varying HBV VL to assess for treatment compliance

Rationale: The differences in HBV prevalence, risk of HCC development, HBV genotypes and treatment exposure between Africa and other continents, may differently impact on the development of HBV RAMs/VEMs.

Hypothesis: Given that HBV is endemic in Africa and the widespread population exposure to ART, the prevalence of HBV RAMs and VEMs might be higher among individuals with CHB infection recruited from clinic centres in South Africa compared to those recruited from clinic centres in the UK.

1.17.5 Aim 5: To consolidate an approach to defining and managing HBV drug resistance in clinical practice

Objective: To develop an algorithm which provides an up-to-date and a comprehensive step-by-step approach to managing treatment failure in CHB in a clinical setting, considering other contributing factors alongside drug resistance.

Rationale: Currently there are no robust universal guidelines for managing HBV drug resistance in clinical practice.

1.17.6 Aim 6: To evaluate the cost-effectiveness of TFV for HBV PMTCT

Objective: To evaluate the cost-effectiveness of antenatal screening for HBV infection using standard laboratory assays for HBsAg and treating HBsAg-positive women with TFV in sSA and to determine the impact of drug resistance on the cost effectiveness of HBV PMTCT interventions.

Rationale: Given the high risk of HBV MTCT in Africa due to limited deployment of HBV screening assays, as well as limited availability and accessibility of HBV PMTCT interventions, an important priority for this continent is to have affordable, accessible and sustainable PMTCT programmes. Modelling the cost-effectiveness of HBV PMTCT interventions allows for a combined analysis of clinical outcomes and potential budget impact, which is fundamental to inform financial investment in HBV elimination programmes in sSA countries. The impact of drug resistance on this intervention has not previously been modelled.

Hypothesis: Use of TFV as an antiviral prophylaxis among pregnant women with CHB infection in sSA is cost-effective for HBV PMTCT.

Chapters two–seven: Experimental Chapters

Each of the six chapters addresses a specific research aim. Chapter-specific methods precede the results in each individual chapter; these chapters are structured as follows: a brief background, methods, results and discussion.

Chapter two: The epidemiology of HBV RAMs and VEMs in Africa

This chapter is divided into two sections:

Section 2.1 addresses **aim 1 objective (i)**. It describes the frequency, co-occurrence and distribution of HBV RAMs and VEMs in Africa through a systematic literature review [published in PLoS Neglected Tropical Diseases (**Mokaya et al. 2018b**)].

Section 2.2 addresses **aim 1 objective (ii)**. It assimilates the current evidence for the development of clinical or virological HBV breakthrough during TFV therapy through a systematic literature review and structural analysis [currently under review at Wellcome Open Research (**Mokaya et al. 2020a**)].

2.1 Frequency, co-occurrence and distribution of HBV RAMs and VEMs in Africa

2.1.1 Background

Given that HBV infection is endemic in Africa, HBV RAMs and VEMs may be common in this continent. I have therefore carried out a systematic literature review which describes the frequency, co-occurrence and distribution of HBV RAMs and VEMs in Africa, and suggest whether changes are needed in recommendations for laboratory diagnostics and/or approaches to drug therapy or vaccine deployment. This work has been published in PLoS Neglected Tropical Diseases (**Mokaya et al. 2018b**).

2.1.2 Methods

2.1.2.1 Literature search strategy

I searched published literature, in MEDLINE, SCOPUS and EMBASE between October 2017 and January 2018 (and an updated second search was carried out in August 2020) using terms detailed in **Table 2.1.1** and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria illustrated in **Figure 2.1.1**. Searches were done for individual groups using the Boolean operator 'OR' and a combined search was done for groups A to D using the 'AND' operator. PRISMA checklist is provided as **Appendix Table 2.1.1.**

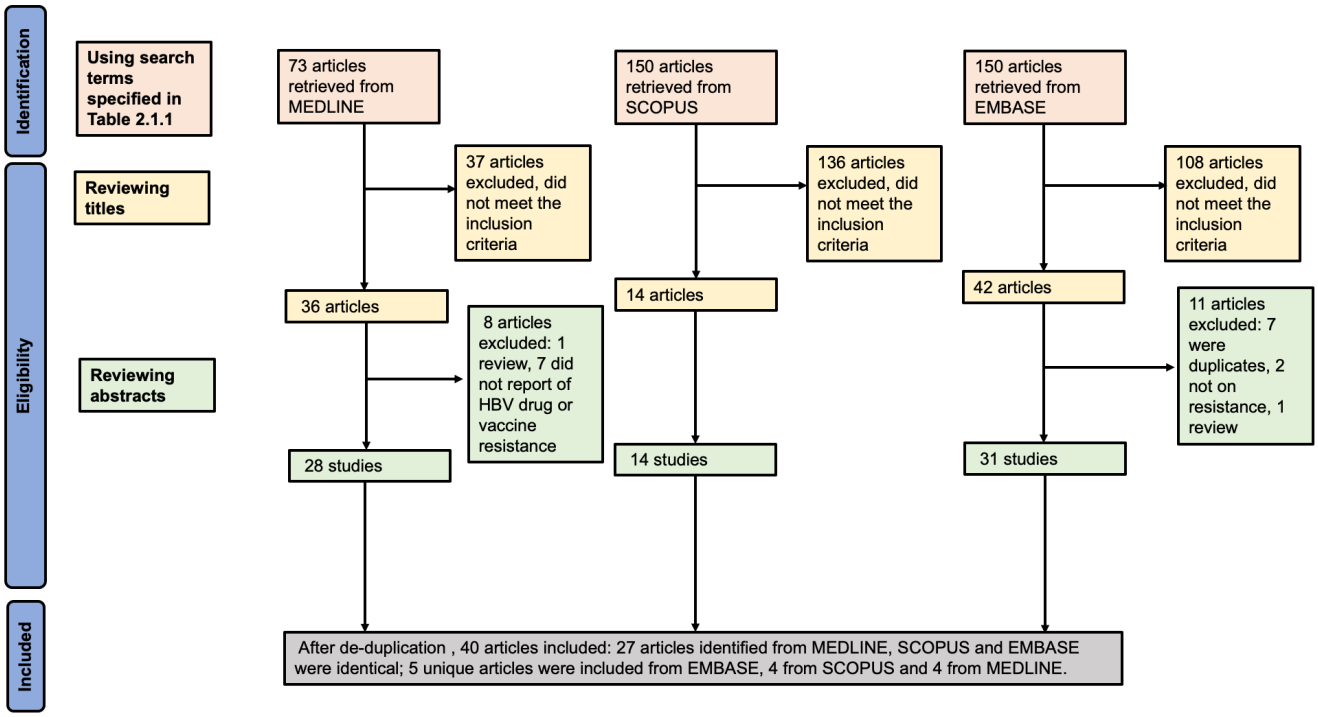


Figure 2.1.1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram illustrating identification and inclusion of studies for a systematic review of drug resistance-associated mutations and vaccine escape mutations in Africa.

Table 2.1.1: Details of search strategy used to identify studies on hepatitis B virus drug resistance-associated mutations and vaccine escape mutations conducted in Africa, from PubMed, Scopus and Embase database.

Group A: Problem (#1)	Group B: Region (#2)	Group C: Intervention (#3)	Group D: Outcome (#4)
Hepatitis B virus [Mesh] *OR* "hepatitis b" *OR* HBV	Africa[MeSH] *OR* africa[tiab] *OR* "sub Saharan africa"[tiab] *OR* "sub-Saharan Africa"[tiab] *OR* Angola[tiab] *OR* Benin[tiab] *OR* Botswana[tiab] *OR* "Burkina Faso"[tiab] *OR* Burundi[tiab] *OR* Cameroon[tiab] *OR* "Cape Verde"[tiab] *OR* "Central African Republic"[tiab] *OR* Chad[tiab] *OR* Comoros[tiab] *OR* "Republic of the Congo"[tiab] *OR* "Democratic Republic of the Congo"[tiab] *OR* "Cote d'Ivoire"[tiab] *OR* Djibouti[tiab] *OR* "Equatorial Guinea"[tiab] *OR* Eritrea[tiab] *OR* Ethiopia[tiab] *OR* Gabon[tiab] *OR* "The Gambia"[tiab] *OR* Ghana[tiab] *OR* Guinea[tiab] *OR* "Guinea-Bissau"[tiab] *OR* Kenya[tiab] *OR* Lesotho[tiab] *OR* Liberia[tiab] *OR* Madagascar[tiab] *OR* Malawi[tiab] *OR* Mali[tiab] *OR* Mauritania[tiab] *OR* Mauritius[tiab] *OR* Mozambique[tiab] *OR* Namibia[tiab] *OR* Niger[tiab] *OR* Nigeria[tiab] *OR* Rwanda[tiab] *OR* "Sao Tome and Principe"[tiab] *OR* Senegal[tiab] *OR* Seychelles[tiab] *OR* "Sierra Leone"[tiab] *OR* "South Africa"[tiab] *OR* "South Sudan"[tiab] *OR* Sudan[tiab] *OR* Swaziland[tiab] *OR* Tanzania[tiab] *OR* Togo[tiab] *OR* Uganda[tiab] *OR* Zambia[tiab] *OR* Zimbabwe[tiab]	antiviral* *OR* lamivudine *OR* LAM *OR* 3TC *OR* adefovir *OR* ADV *OR* telbivudine *OR* LdT *OR* entecavir *OR* ETV *OR* Tenofovir *OR* TDF	resista* *OR* drug muta* *OR* DRMs *OR* RAMs OR vaccine escap* *OR* vaccine mut* *OR* VEMs *OR* escap*

Mesh: Medical subject headings. tiab: Collecting free text terms.

I reviewed the titles and abstracts matching the search terms and only included those relating to drug or vaccine resistance in HBV infection, including only those that presented original data and had undergone peer review. All retrieved articles were in English, therefore no exclusion in relation to language was required.

For each publication, I recorded reference, publication year, study design, sample size, study population, proportion of participants who tested HBsAg positive or HBV DNA positive, country, year(s) of specimen collection, genotype identified, antiviral treatment, sequencing method, gene sequenced, number of sequenced samples, participant recruitment site and sequence accession number. Data were curated using MS Excel software (Microsoft, Redmond, WA).

2.1.2.2 Quality appraisal

I used the BMJ adapted Quality Assessment Tool for Quantitative Studies (gh.bmj.com) to assess the quality of the included studies.

2.1.2.3 Analysis

I reported the prevalence of RAMs and VEMs in individual studies using the following approach:

$$Prevalence = \frac{\begin{array}{c}total\ number\ of\ HBV\ positive\ patients \\ with\ the\ specified\ mutation\ as\ reported\ in\ published\ studies\end{array}}{total\ number\ of\ HBV\ positive\ patients\ as\ reported\ in\ published\ studies}$$

I also pooled data by country in order to provide regional estimates.

$$Prevalence = \frac{\begin{array}{c}total\ number\ of\ HBV\ positive\ \ patients \\ with\ the\ specified\ mutation\ assimilated \\ from\ all\ published\ studies\ of\ a\ given\ country\end{array}}{\begin{array}{c}total\ number\ of\ HBV\ positive\ patients \\ assimilated\ from\ all\ published\ studies\ of\ a\ given\ country\end{array}}$$

2.1.3 Results

2.1.3.1 Characteristics of studies identified for a systematic literature review of HBV RAMs and VEMs in Africa

The initial search yielded 73 articles in MEDLINE, 150 in SCOPUS and 150 in EMBASE. Of these, 37, 136 and 108 were excluded from search results of MEDLINE, SCOPUS and EMBASE, respectively, as they did not meet the inclusion criteria. After de-duplication, 40 articles were included. 27 articles identified from the three data sources were identical; five unique articles were included from EMBASE, four from SCOPUS and four from MEDLINE. A total of 40 articles were downloaded in full. **Table 2.1.2** provides a summary of the characteristics of studies included. **Metadata of all 40 studies are provided in Appendix Table 2.1.2.**

Table 2.1.2: Summary of studies included in a systematic literature review to collate evidence for the prevalence and distribution of hepatitis B virus drug resistance-associated mutations and vaccine escape mutations in Africa.

	Number of studies
Study design	
Cross-sectional	34
Longitudinal	6
Regions represented	
Southern Africa	16
East Africa	10
West Africa	9
Central Africa	5
Study population	
HBV/HIV coinfected patients	30
HBV infected with or without HIV coinfection	9
Chronic HBV monoinfection	1
Antiviral treatment	
Treatment-naïve	8
3TC-based regimen only	11
TVF-based regimen only	1
Regimens including 3TC or TFV	5
Mixed regimen where some received 3TC, others TVF, while others left untreated	9
Treatment regimen not specified	6
HBV protein sequenced	
Polymerase only	12
Surface only	3
Polymerase and surface	15
Polymerase, surface and PC/BCP	4
Surface and PC/BCP	3
Whole genome	2
Sequencing	
Consensus	27
Deep	1
Genotype	
Specified genotype	36

3TC: Lamivudine; TFV: Tenofovir. PC: Precore. BCP: Basal Core Promoter.

2.1.3.2 Quality appraisal

I undertook a formal quality appraisal using the BMJ adapted Quality Assessment Tool for Quantitative Studies. The overall quality rating for all the 40 studies included was moderate given that all studies were observational in nature. In all studies, inclusion criteria were reported, however, there was typically not a clear description of the denominator population, there was between-study heterogeneity, and the majority of these studies did not perform a truly systematic assessment with most recruiting only high-risk groups, particularly those with HIV/HBV coinfection (**Appendix Table 2.1.3).**

2.1.3.3 Prevalence of HBsAg, HBeAg and HDV coinfection in Africa

The prevalence of HBsAg in these study cohorts ranged from 3–100 % **(Table 2.1.3)**; however, the populations included were highly selected and therefore not necessarily representative of the general population, particularly as a result of a strong bias towards HIV-infection. A total of 16 studies reported prevalence of HBeAg, which ranged between 14–89% (**Table 2.1.3)**. Only three studies included in this review reported on HDV prevalence: two studies did not detect any HDV antibodies (Andersson et al. 2013; Matthews et al. 2015), whereas the other study reported a HDV prevalence of 25% among adults with CHB in Guinea-Bissau (Hønge et al. 2014).

Table 2.1.3: Prevalence of hepatitis B surface and e antigen reported in 40 studies included in a systematic literature review on hepatitis B virus drug and vaccine resistance in Africa.

Region	Study (Reference)	Country	HIV coinfection status of cohort[a]	HBV prevalence in this cohort (reported as HBsAg prevalence unless otherwise specified)	HBeAg prevalence among HBV-positive individuals
East Africa	Deressa et al. 2017	Ethiopia	+	17/308 (6%)	Not reported
	Hundie et al. 2016	Ethiopia	±	391/391 (100%)	Not reported
	Day et al. 2013	Kenya	+	11/159 (7%)	6/11 (55%)
	Kim et al. 2011	Kenya	+	27/389 (7%)	24/27 (89%)
	Mabeya et al. 2017	Kenya	+	29/400 (7%)	Not reported
	Auodjane et al. 2014	Malawi	+	133/1117 (12%)	67/133 (50%)
	Galluzzo et al. 2012	Malawi	+	21/21 (100%)	7/21 (33%)
	Mahgoub et al. 2011	Sudan	±	16/404 (4%)	Not reported
	Yousif et al. 2014	Sudan	+	96/358 (27%) [b]	32/ 50 (64%)
	Calisti et al. 2015	Uganda	+	109/2820 (4%)	Not reported
West Africa	Boyd et al. 2015	Cote d'Ivoire	+	259/ 2465 (11%)	39/168 (23%)
	Archampong et al. 2017	Ghana	+	235/235 (100%)	Not reported
	Chadwick et al. 2012 [d]	Ghana	+	143/371 (39%)	Not reported
	Geretti et al. 2010 [d]	Ghana	+	140/838 (17%)	37/140 (26%)
	Ndow et al. 2017	Gambia	+	23/187 (12%)	Not reported
	Stewart et al. 2011	Gambia	+	70/ 570 (12%)	6/21 (29%)
	Hønge et al. 2014	Guinea Bissau	+	94/576 (16%)	16/94 (17%), HDV prevalence: 18/72 (25%)
	Akanbi et al. 2019	Nigeria	+	81/81 (100%)	Not reported
	Faleye et al. 2015	Nigeria	±	15/272 (6%)	Not reported
Central Africa	Gachara et al. 2017	Cameroon	+	20/337 (6%) [c]	Not reported
	Kouanfack et al. 2012	Cameroon	+	54/552 (10%)	Not reported
	Magoro et al. 2016	Cameroon	+	116/445 (26%)	16/102 (16%)
	Bivigou-Mboumba et al. 2016	Gabon	+	71/762 (9%)	Not reported
	Bivigou-Mboumba et al. 2018	Gabon	+	43/487 (9%)	Not reported
Southern Africa	Anderson et al. 2015a	Botswana	+	81/81 (100%)	Not reported
	Matthews et al. 2015*	Botswana	±	17/443 (4%)	16/60 (27%); HDV: negative
		South Africa	±	49/507 (10%)	Not reported; HDV: negative
	Chambal et al. 2017	Mozambique	+	47/518 (9%)	Not reported
	Wandeler et al. 2016	Mozambique	+	78/1032 (8%)	24/168 (14%)
		Zambia	+	90/797 (11%)	24/168 (14%)
	Andersson et al. 2013	South Africa	±	97/3089 (3%)	17/94 (18%); HDV: negative
	Amponsah-Dacosta et al. 2015	South Africa	±	33/201 (16%)	Not reported
	Amponsah-Dacosta et al. 2016	South Africa	+	5/5 (100%)	5/5 (100%)
	Baxter et al. 2019	South Africa	Unknown	37/889 (4%)	Not reported
	Hamers et al. 2013*	South Africa	+	37/175 (21%)	Not reported
		Zambia	+	55/523 (11%)	Not reported
	Gededzha et al. 2016	South Africa	±	8/9 (89%)	Not reported

	Lukhwareni et al. 2020	South Africa	+	56/56 (100%)	Not reported
	Makondo et al. 2012	South Africa	+	71/298 (24%) [b]	Not reported
	Powell et al. 2015	South Africa	+	37/394 (9%)	Not reported
	Selabe et al. 2007	South Africa	±	35/35 (100%)	Not reported
	Selabe et al. 2009	South Africa	-	17/17 (100)	9/17 (53%)
	Baudi et al. 2017	Zimbabwe	+	19/176 (11%)	Not reported

HBsAg and HBeAg prevalence was determined from 37 studies (Treatment naïve: n= 8 studies, 589 individuals with HBsAg; Treatment experienced: n= 17 studies, 1179 individuals with HBsAg; Mixed regimen where some were treatment experienced, naïve or treatment status not specified: n= 15 studies, 1421 individuals with HBsAg). Studies were identified by a systematic literature search of HBV resistance associated mutations (RAMs) and vaccine escape mutations (VEMs) from African cohorts published between 2007 and August 2020.

[a] HIV status is designated '+' whole cohort HIV-positive; '±' some of cohort HIV-positive; '-'none of cohort HIV-positive.

[b] HBV prevalence in these cohorts was reported using HBV DNA detection rather than HBsAg

[c] Occult HBV prevalence reported in these cohorts

[d] These two studies recruited from the same overall cohort in Ghana.

2.1.3.4 Occurrence and distribution of HBV RAMs and VEMs across African countries

The co-occurrence and distribution of HBV RAMs and VEMs are summarised according to the region where they were identified **(Figure 2.1.2)**. Most studies reported both HBV RAMs and VEMs (n=28) compared to those that only reported RAMs (seven studies) or VEMs (four studies). This illustrates the patchy and limited data that are available, with South Africa, Ghana and Cameroon best represented, but with large areas (especially in northern and central Africa) not represented at all in the literature.

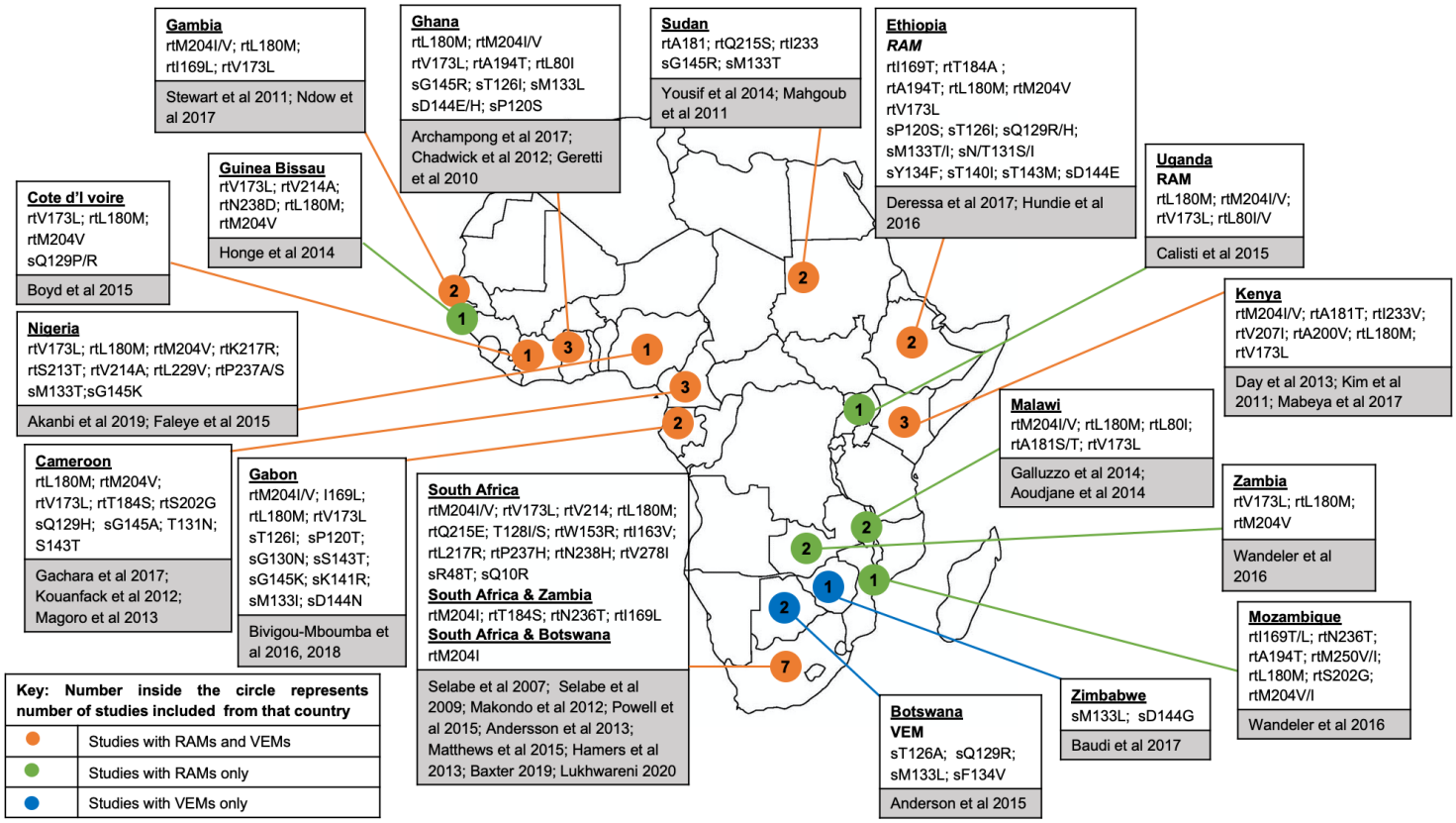


Figure 2.1.2: Annotated map to summarise hepatitis B virus drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs). RAMs/VEMs identified from 37 studies of African cohorts published between January 2007 and August 2020. Three studies included in this review are not represented here as they did not report any RAMs/VEMs. Figure reproduced from (Mokaya et al. 2018b) published in PLoS Neglected Tropical Diseases, under the terms of the Creative Commons Attribution (CC BY) license. RT: Reverse Transcriptase. S: Surface.

2.1.3.5 Occurrence and distribution of drug RAMs across genotypes reported from Africa

It was possible to group HBV RAMs and VEMs according to genotype in 14 included studies (**Figure 2.1.3)**. The remaining 23 studies did not specifically state the genotype of HBV within which RAMs were identified. RAMs were reported mainly in genotypes A and E; this pattern is a reflection of the predominant HBV genotypes within Africa (Sunbul, 2014) [see **chapter 1: section 1.6 (Table 1.1)**]. Interpreting RAMs according to sub-genotypes was difficult since most studies did not specify sub-genotype.

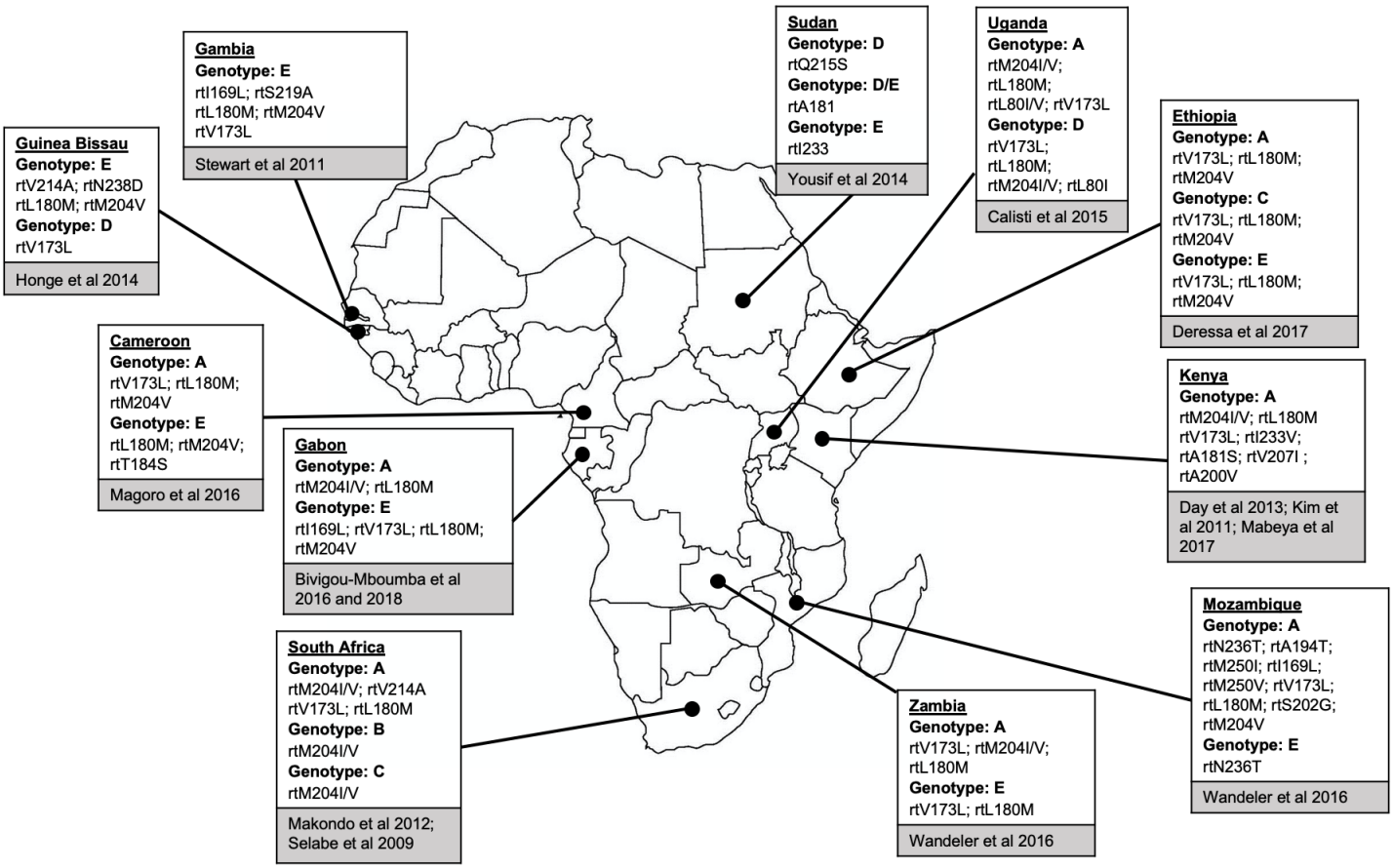


Figure 2.1.3: Hepatitis B virus drug resistance-associated mutations (RAMs) grouped according to genotypes and geographical location. RAMs identified from 14 studies of African cohorts published between January 2007 and August 2020.

2.1.3.6 Prevalence of drug RAMs in Africa

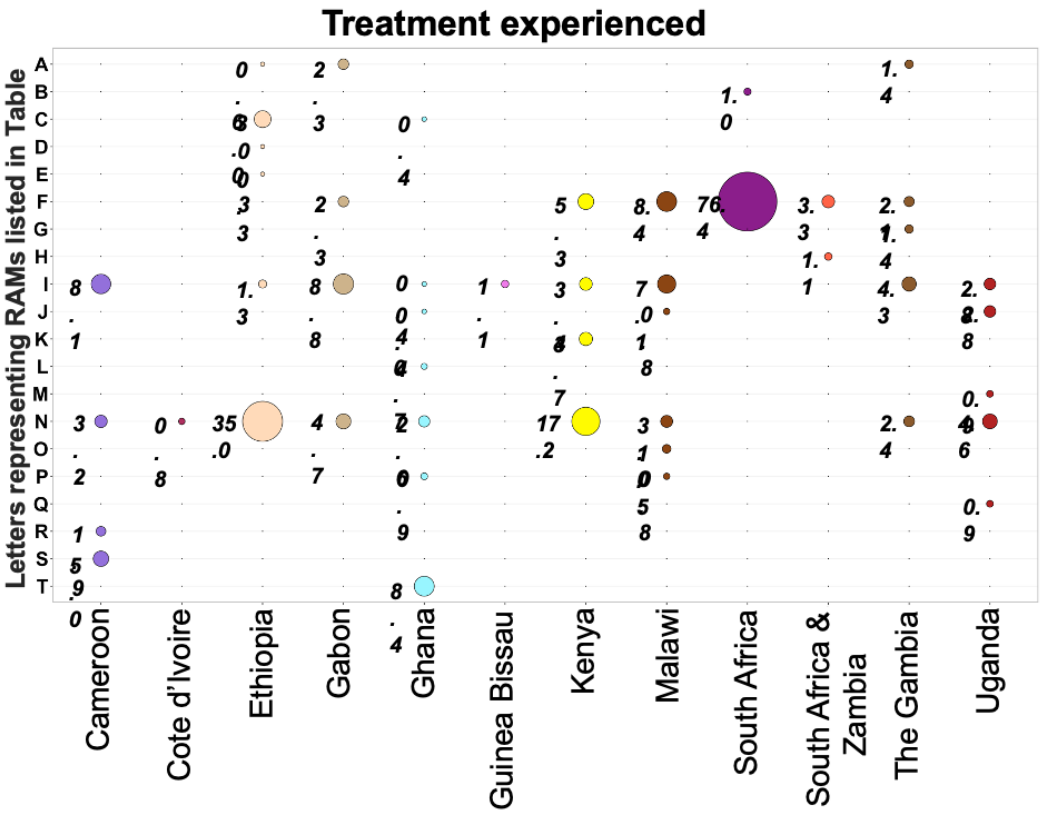
Overall, the most prevalent RAM was M204V/I in both treatment experienced and treatment naïve individuals, occurring either alone or in combination with other polymorphisms L80I/V, V173L, L180M, A181S, T184S, A200V and/or S202S (**Figure 2.1.4** and **Figure 2.1.5)**; mutations among individuals with and without exposure to HBV therapy are listed in **(Appendix Table 2.1.4** and **Appendix Table 2.1.5).** Across all cohorts, the M204V/I mutation was present in 208/2569 (8%) of all individuals represented, but this varied widely between populations [from 0.4% in Ghana (Archampong et al. 2017) to 76% in South Africa (Selabe et al. 2009)].

M204V/I, without a combination of any other polymorphisms, was most prevalent in South Africa; on pooling data for three studies from this setting, it was present in both treatment experienced and treatment naïve patients [n=13/17, 76% (Selabe et al. 2009) and n=16/72,

22% (Selabe et al. 2007; Powell et al. 2015), respectively]. This mutation was also frequent in Malawi among treatment experienced patients [n=13/154, 8.4% (Aoudjane et al. 2014; Galluzzo et al. 2012)] (**Figure 2.1.4)**, and in genotype non-A infection: the mutation was frequent in genotype B infection (n=3/17, 18%) (Selabe et al. 2009).

TFV has a high genetic barrier to resistance, and is associated with reliable suppression of HBV viraemia (Sheldon et al. 2005; Zoulim, 2011; Hamers et al. 2013; Beloukas and Geretti, 2017). However, mutation N236T, which have been linked with resistance to both TFV and ADV (Beloukas and Geretti, 2017), have been identified in Southern Africa in both treatment naïve (Wandeler et al. 2016) and treatment experienced (Hamers et al. 2013) patients **(Figure 2.1.4** and **Figure 2.1.5).**

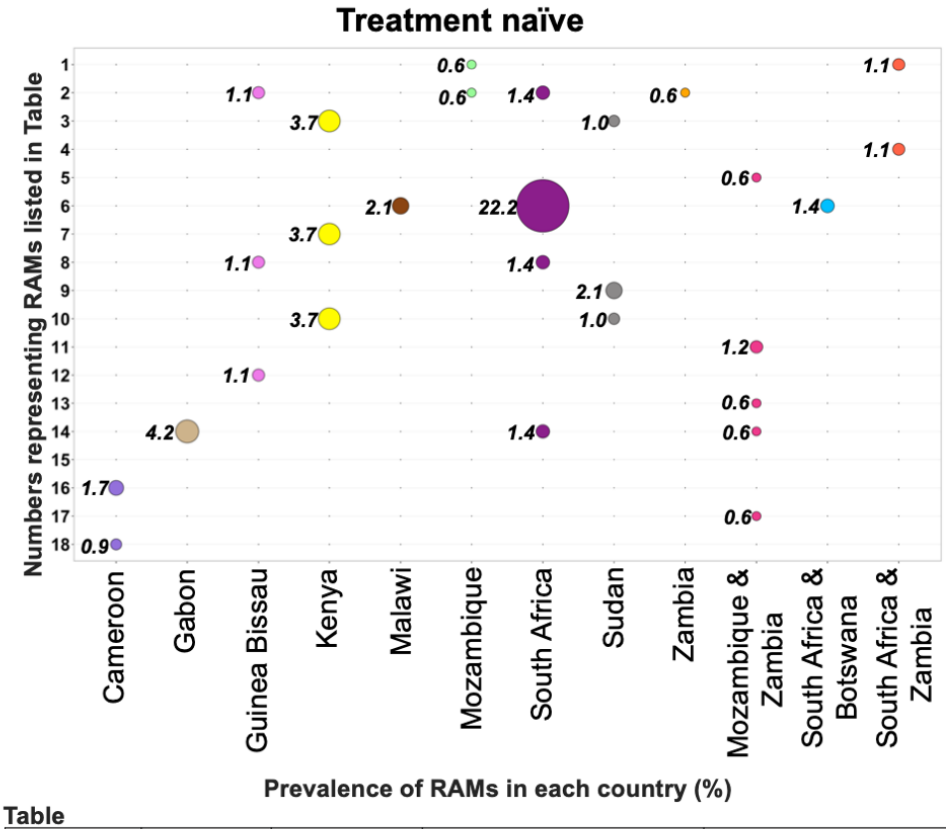
Of concern is the detection of RAMs even in reportedly treatment naïve individuals **(Figure 2.1.5** and **Appendix Table 2.1.5),** suggesting that RAMs are being transmitted. A study in South Africa that recruited 3TC-naïve HBV infected adults with or without HIV, reported M204I in 13/35 (37%) individuals (Selabe et al. 2007) **(Appendix Table 2.1.5).**



Table

A: I169L	**E:** A194T	**I:** M204I/V+L180M	**M:** M204I+V173L	**Q:** M204I+L80I +L80V
B: V173L	**F:** M204I/V	**J:** M204I/V+L80I	**N:** M204I/V+V173L +L180M	**R:** L180M+S202G+M204V
C: L180M	**G:** S219A	**K:** M204I/V+A200V	**O:** M204V+V173L +A181S	**S:** L180M+M204I+T184S
D: T184A/S	**H:** N236T	**L:** V173L+A194T	**P:** M204V+L80I +L180M	**T:**M204I/V+/-L80I+/-V173L+/-L180M

Figure 2.1.4: Prevalence of hepatitis B virus (HBV) resistance-associated mutations (RAMs) in polymerase/reverse transcriptase protein among treatment experienced HBV infected patients in Africa. These data are derived from 23 studies of HBV drug resistance in Africa published between January 2007 and August 2020. A detailed summary of RAMs identified from each study is presented in **Figure 2.1.2** and **(Appendix Table 2.1.4).** Prevalence of RAMs for a specific country was determined by grouping all studies from that country that reported a specific mutation. I used all individuals who tested HBsAg positive to generate a denominator in order to provide a conservative estimate of RAM prevalence, and the numerator was the total number of individuals with that specific mutation from these studies. Figure reproduced from (Mokaya et al. 2018b) published in PLoS Neglected Tropical Diseases, under the terms of the Creative Commons Attribution (CC BY) license.



Table

1: I169L	**5:** A194T	**9:** Q215S	**13:** M250I/V	**17:** L180M+S202G+M204V
2: V173L	**6:** M204I/V	**10:** I233V	**14:** M204I/V+L180M	**18:** L180M+M204I+T184S
3: A181S	**7:** V207I	**11:** N236T	**15:** V173L+L180M	
4:T184A/S	**8:**V214A	**12:** N238D	**16:** M204I/V+V173L +L180M	

Figure 2.1.5: Prevalence of hepatitis B virus (HBV) resistance-associated mutations (RAMs) in polymerase/reverse transcriptase protein among treatment naïve HBV infected patients in Africa. These data are derived from 12 studies of HBV drug resistance in Africa published between January 2007 and August 2020. A detailed summary of RAMs identified from each study is presented in **Figure 2.1.2** and **(Appendix Table 2.1.5).** Prevalence of RAMs for a specific country was determined by grouping all studies from that country that reported a specific mutation. I used all individuals who tested HBsAg positive to generate a denominator in order to provide a conservative estimate of RAM prevalence, and the numerator was the total number of individuals with that specific mutation from these studies. Figure reproduced from (Mokaya et al. 2018b) published in PLoS Neglected Tropical Diseases, under the terms of the Creative Commons Attribution (CC BY) license.

WHO guidelines recommend a first-line regimen including TFV in HIV/HBV coinfected patients (WHO, 2015), and the South African Department of Health HIV/AIDS treatment guideline included TFV as first-line regimen from 2010 (WHO, 2010), however I found a minority of studies (9/37, 24%) reporting TFV-containing regimens for HIV/HBV coinfected individuals. As anticipated, most of the studies that did use TFV were carried out after 2010, whereas those that used 3TC were generally earlier **(Appendix Table 2.1.2).**

2.1.3.7 Prevalence of VEMs in Africa

VEMs were identified in Central, East, West and Southern Africa **(Figure 2.1.2).** However, it was not possible to ascertain whether individuals harbouring these mutations had been vaccinated against HBV infection. The most common VEM was the triple mutation V173L+L180M+M204I/V, found in the polymerase protein. This suite of mutations was identified in 13 studies at a pooled prevalence of 3% (45/1319) **(Figure 2.1.2; Appendix Table 2.1.4** and **Appendix Table 2.1.5)**. Other VEMs G145K/R and M133L/T, located within the surface protein, were identified in six and seven studies, respectively **(Figure 2.1.2)**.

2.1.3.8 Data visualisation

We developed an R package (gene.alignment.tables) for the visualisation of the sequence data in this study. The package development was supported by University of Oxford's Interactive Data Network and a specific instance of the visualisation is hosted as a Shiny app which can be viewed here: (https://livedataoxford.shinyapps.io/1510659619-3Xkoe2NKkKJ7Drg/). **Figure 2.1.6** shows a screenshot of how data are displayed in the Shiny app.

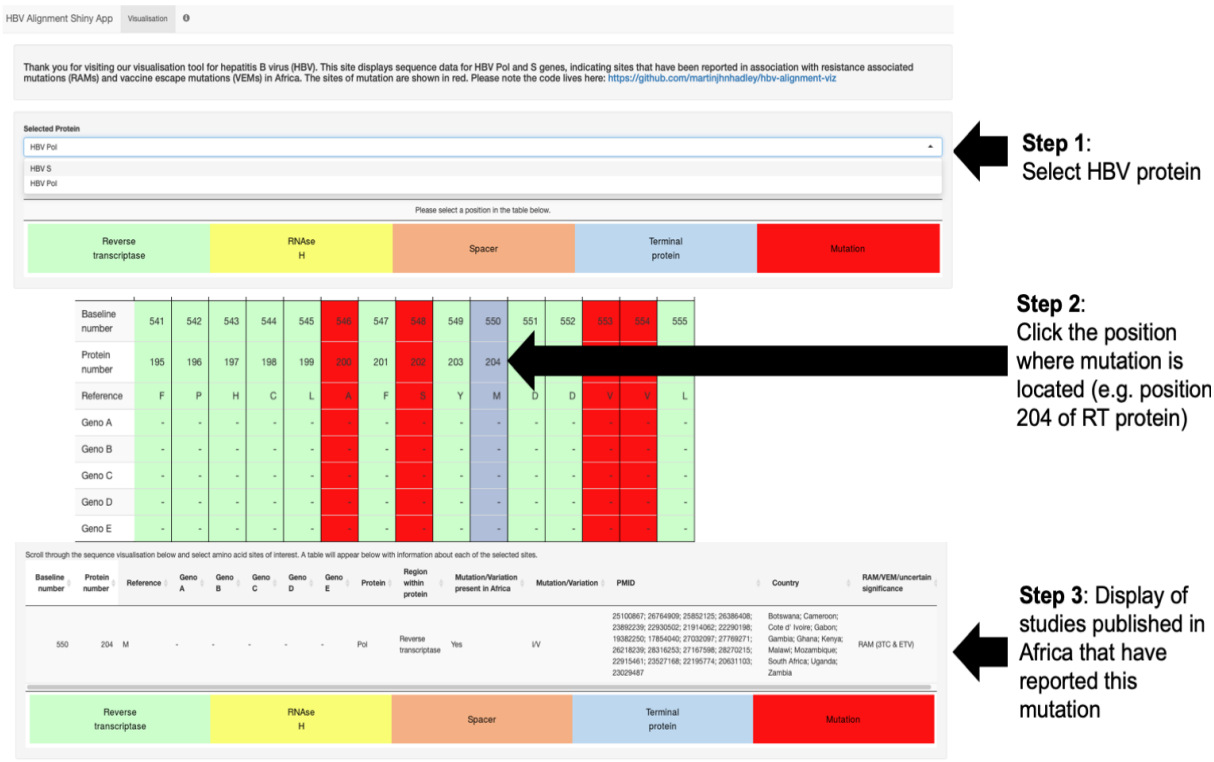


Figure 2.1.6: A screenshot of the online visualisation tool which presents hepatitis B virus drug resistance-associated mutations and vaccine escape mutations assimilated from sub-Saharan Africa through a systematic literature review and analysis of published sequence data (Mokaya et al. 2018b). This visualisation tool was developed in collaboration with Martin Hadley in Oxford Academic IT.

2.1.4 Discussion

This work provides the first overview of occurrence and distribution of HBV RAMs and VEMs in Africa. The high rates of HBV infection among HIV infected individuals in some locations including Cameroon (Magoro et al. 2016) and South Africa (Hamers et al. 2013) could be an indication that HBV infection has been previously under-reported, possibly due to lack of routine screening, poor awareness, stigma, high costs and limited clinical and laboratory infrastructure (Kim et al. 2011; Chadwick et al. 2012; Galluzzo et al. 2012; Day et al. 2013; Hamers et al. 2013; O'Hara et al. 2017). This is also true given that there was only one study that recruited HBV monoinfected individuals, which may indicate that majority of HBV monoinfected individuals in Africa remain undiagnosed and therefore not linked to care,

reinforcing the observation that HBV is indeed neglected as a public health problem (O'Hara et al. 2017; Maponga et al. 2020).

The literature suggests a widespread exposure of the HIV-infected population to 3TC-based treatment. This may be changing over time in line with current ART treatment recommendations [regimens for Africa summarised in **(Appendix Table 2.1.6)**], however, introduction of TFV-based regimens for HIV treatment has been inconsistent, and TFV monotherapy is not consistently available for HBV infection in the absence of HIV (O'Hara et al. 2017; Maponga et al. 2020).

2.1.4.1 Summary of HBV RAMs in Africa

The most common RAM identified was M204I/V, either alone or in combination with compensatory mutations L180M±V173L. Of concern, M204V/I was seen in 76% of treatment experienced patients (Selabe et al. 2009) and 22% of treatment naïve patients (Powell et al. 2015; Selabe et al. 2007) in South Africa. A review of worldwide incidence of RAMs among treatment naïve patients also described RAM M204V/I as the most frequent, but with a much lower prevalence of 5% (Zhang et al. 2015). The contribution of unreported or undocumented 3TC exposure in reportedly treatment-naïve populations remains to be determined. A European study demonstrated that the most frequent primary mutation was M204V/I, found in 49% of treatment experienced patients (Hermans et al. 2016), while in China M204I, N236T and L180M+M204V+V173L/S202G were also the most prevalent RAMs (Meng et al. 2017).

The triple mutation M204V+L180M+V173L has been identified in East, West and Central Africa (Geretti et al. 2010; Stewart et al. 2011; Chadwick et al. 2012; Kouanfack et al. 2012; Aoudjane et al. 2014; Boyd et al. 2015; Calisti et al. 2015). This combination of polymorphisms is associated with both vaccine escape and resistance to 3TC and other L-nucleoside analogues (Geretti et al. 2010; Stewart et al. 2011; Kouanfack et al. 2012; Aoudjane et al. 2014; Calisti et al. 2015; Magoro et al. 2016).

Apart from the nature of drug being used for HBV treatment, other predictors of HBV drug resistance include HBV VL, HBV intra host heterogeneity, HBeAg status, host body mass index and serum alanine aminotransferase (ALT) activity (Zoulim and Locarnini, 2009; Khudyakov, 2010; Aoudjane et al. 2014). Individuals with M204V/I plus compensatory mutations typically exhibit high HBV DNA levels (Aoudjane et al. 2014) and are therefore highly infectious to others. The spread of RAMs may lead to a rise in drug resistance in treatment naïve CHB, representing a substantial challenge for Africa and highlighting an imperative to ensure routine use of TFV in preference to, or in combination with, 3TC-based therapy.

2.1.4.2 Summary of HBV VEMs in Africa

VEMs have been reported in ten African countries representing East, West, Central and Southern Africa. Whether viral isolates with these mutations were obtained from individuals who were previously vaccinated against HBV is uncertain. However, two strands of evidence suggest significant population exposure to HBV vaccination. First, vaccination has been progressively rolled out in most countries in sSA since the mid-1990's with the third dose and birth dose HBV vaccine coverage having reached approximately 75% and 10%, respectively, as of 2015 (WHO, 2017b) (see **chapter 1: section 1.10.2**); secondly, most HBsAg mutations reported by these studies are located within the common immunodominant B cell epitope (aa 99–169) [see **chapter 1: section 1.4.3 (Figure 1.3)**] in which selection of polymorphisms is associated with HBV vaccination (Chang, 2010; Hudu et al. 2014).

VEM have been more robustly reported from Asia, in settings where the HBV infant vaccination programme is well established; for example, in Taiwan, VEM prevalence among vaccinated children increased from 7.8% to 28.1% within the first 10 years of the launch of the universal vaccine program and then decreased to 23.1% at year 15; this decline in VEM prevalence thereafter may be partly related to a smaller HBV carrier pool (Chang, 2010). The decline in the prevalence of VEM after the launch of universal vaccine program, may indicate that VEMs play a minor role in ongoing vertical transmission. Furthermore, findings from

another study carried out Taiwan in 2004 reported that 1% of children had breakthrough infections after vaccinations which could also be due to other factors such as high maternal viral load, intrauterine infection, immunosuppression, hypo-responsiveness to vaccine due to host genetics and delayed vaccination (Ni et al. 2007). Birth dose vaccination would help prevent transmissions occurring during delivery or shortly after birth HBV MTCT, especially in the case of high maternal viral load, and consequently the *de novo* selection of escape mutations from vaccine-induced immune responses, particularly in the setting of delayed vaccination (Chang, 2010; Hudu et al. 2014). Only 10 African countries recommend the first HBV vaccine dose at birth, in contrast to the majority of African countries in which HBV vaccination is delayed until six weeks of age (Wilson et al. 2018). It is likely that this delay not only provides a window of infection but also increases the possibility of transmitted VEM and/or emergence of new escape mutations. It therefore remains essential to advocate for increased coverage of birth dose vaccination especially in areas with high HBV prevalence.

High maternal HBV VL and immunosuppression are other risk factors associated with VEM among infants (Chang, 2010); both of these are pertinent for emergence of VEMs in Africa given that HBV VL testing is not routinely available, and HIV is highly prevalent in some populations. Effective PMTCT strategies in Africa, including screening and treating antenatal women, increasing access to VL monitoring, and introducing HBV birth dose vaccine will help to decrease the prevalence of VEM (O'Hara et al. 2017; Wilson et al. 2018; McNaughton et al. 2019b).

2.1.4.3 TFV resistance in Africa

The identification of mutations associated with reduced TFV susceptibility is of concern, as this suggests the potential for increasing prevalence of polymorphisms that confer partial or complete viral escape from a drug that to date has not been widely associated with resistance. There is now potential for increasing selection of TFV resistance as this drug becomes more widely used. However, as a new first line single tablet option incorporating 3TC, TFV and Dolutegravir (DTG) (triple therapy abbreviated to 'LTD') emerges as a recommended option

for HIV treatment in Africa, surveillance is needed to determine the clinical outcomes for HBV (Cohen, 2017). I go on to explore this in **chapter 5.**

If clinically significant, TFV resistance mutations may still represent a particular problem for many African settings, as resource constraints make it unrealistic to provide baseline screening for RAMs, or to monitor patients on treatment with serial VL measurements. Despite these potential concerns, it has been shown that TFV is effective even in the presence of RAMs and that there is comparable efficacy among 3TC-experienced and NA-naïve patients (Baran et al. 2013).

2.1.4.4 Impact of coinfection on drug and vaccine resistance

2.1.4.4.1 HIV/HBV coinfection

In studies that reported RAMs among treatment naïve individuals, the literature suggests that sequence analysis was performed prior to ART initiation. However, the possibility that some of these participants had prior ART exposure cannot be excluded. Due to the nature of the cohorts that have been studied, most of the RAMs identified were from HIV/HBV coinfected individuals. It is possible that HIV increases the risk of HBV RAMs both in terms of drug exposure, and also as a function of increased HBV VL. A study from Malawi demonstrated the rapid emergence of 3TC resistance in HIV coinfection, with virtually all treatment naïve HBeAg positive individuals starting antiviral treatment showing emergence of M204I by six months (Aoudjane et al. 2014). Likewise, a study carried out in Italy revealed that patients with HIV coinfection were more likely to harbour the M204V mutation and to show multiple mutations compared to HBV monoinfected patients (Iacomi et al. 2009). It would be worth further exploration of this observation in Africa, as there are currently very limited data.

2.1.4.4.2 HDV/HBV coinfection

One study from the literature review reported a high HDV prevalence of 25%; however, in this cohort, RAMs occurred in individuals with HBV monoinfection (Hønge et al. 2014). HDV is characteristically associated with decreased HBV replication (Negro, 2014) (see **chapter 1;**

section 1.8.3). It is possible that emergence of HBV RAMs may be altered in HDV/HBV. A study reporting prevalence and characteristics of HBV drug resistance in Europe found no significant association between presence of HBV drug resistant mutations and HDV (Hermans et al. 2016). However, as the true prevalence and impact of HDV in sSA is not known (Stockdale et al. 2017), further studies are needed to determine the impact of HDV coinfection on HBV RAMs.

2.1.4.5 Caveats and limitations

2.1.4.5.1 Limited data

There is very limited robust epidemiological data on HBV research from Africa; a total of 40 studies representing 16 African countries were included. This made it difficult to determine the true prevalence of resistance mutations from pooled data and draw up robust conclusions.

Representation of resistant mutation identified from the studies included from Africa could not be ascertained as this can be skewed depending on the study design; in cross-sectional studies, there is a possibility of overrepresentation if screening is undertaken in patients with HIV coinfection, who are more at risk of advanced disease and prolonged drug exposure or underrepresentation if screening is undertaken only at baseline. Altogether, 34 studies undertook a cross-sectional approach and most of these studies recruited individuals from hospital settings, which raises the possibility of overrepresentation (**Table 2.1.2**). Only six studies undertook a longitudinal approach to detection of drug resistance which gives a less biased overview of the prevalence of these mutations (**Table 2.1.2**).

2.1.4.5.2 Sequencing approach

Mutations across the whole genome might be relevant in determining resistance (Betz-Stablein et al. 2016). However, most of the studies included from Africa analysed only defined genes from within the HBV genome; only two sequenced the whole genome (Amponsah-Dacosta et al. 2016; Gededzha et al. 2016), and these determined consensus sequence. This potentially results in an under-representation of RAMs and VEMs that may be present as low

numbers of quasispecies, but could become significant if selected out by exposure to drug or vaccine.

2.2 Evidence of HBV resistance to TFV

2.2.1 Background

Given the widespread population exposure to TFV as a component of first-line ART for HIV and as the recommended therapy in driving HBV elimination which will result in many more people being treated, understanding HBV resistance to TFV remains essential (**see chapter 1; section 1.12.1.2.5 and section 1.16.3**). The distribution and clinical significance of HBV genetic signatures that underpin TVF resistance are not well understood. I have therefore undertaken a systematic approach to assimilate the current evidence for the development of clinical or virological HBV breakthrough during TFV therapy. This work is currently under review at Wellcome Open Research (**Mokaya et al. 2020a**).

2.2.2 Methods

2.2.2.1 Literature search strategy

Using the terms ("*Hepatitis B virus" [Mesh] OR "hepatitis b" OR HBV*) AND (*Tenofovir OR TDF* OR *TAF* OR *"Tenofovir alafenamide"* OR "*Tenofovir Disoproxil Fumarate*") *AND* (*resista* OR drug muta* OR DRMs OR RAMs OR viremia OR replica**), I searched published literature, in MEDLINE and SCOPUS in February 2019 (and an updated second search was carried out in August 2020), using PRISMA criteria illustrated in **Figure 2.2.1.** PRISMA checklist provided as **Appendix Table 2.2.1.**

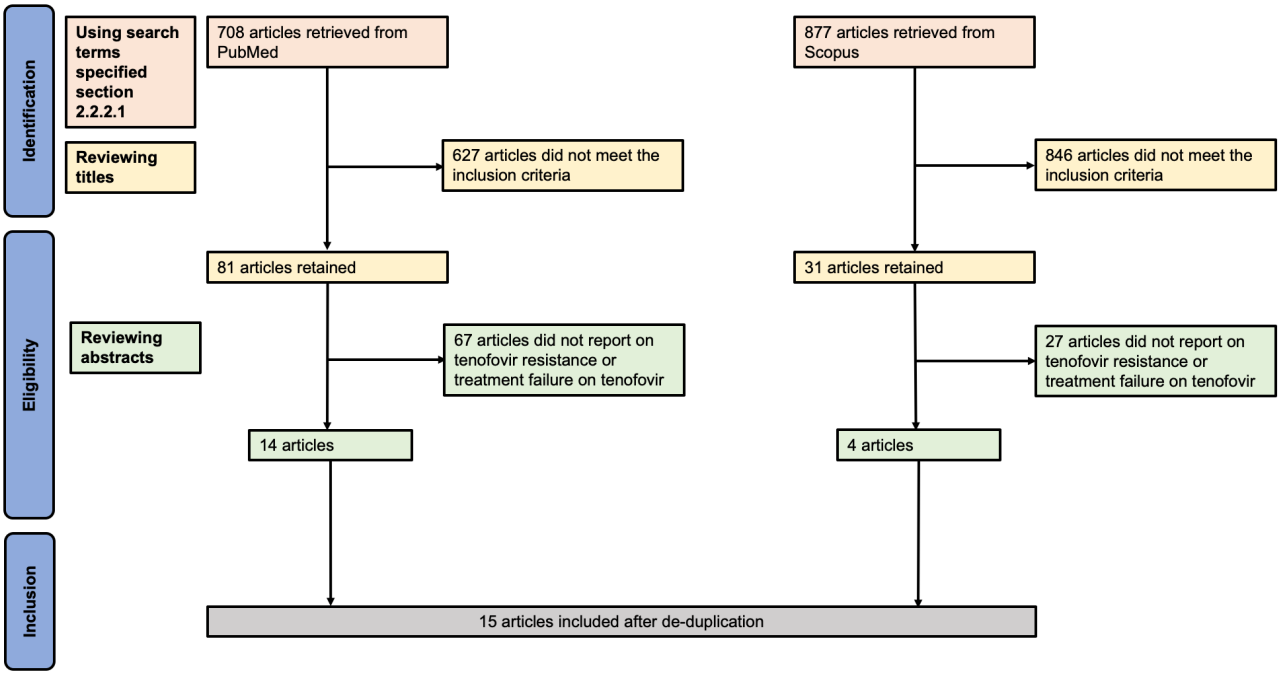


Figure 2.2.1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram to illustrate the identification of studies for inclusion in a systematic review of tenofovir resistance in hepatitis B virus infection.

I reviewed the titles and abstracts matching the search terms and included those reporting virological HBV breakthrough after exposure to TFV, only including studies that presented original data and had undergone peer review. All retrieved articles were published in English. For each study, I extracted information on type of study, characteristics of study participants, sequencing method, genotype, HBV treatment used, mutations associated with TFV resistance and method used to define TFV resistance.

2.2.2.2 Quality appraisal

I used the Joanna Briggs Institute Critical Appraisal tool checklist (joannabriggs.org) to assess quality of case reports and the BMJ adapted Quality Assessment Tool for Quantitative Studies (gh.bmj.com) to assess the quality of quantitative studies.

2.2.2.3 Sequence analysis

To assess similarity between HIV and HBV RT, I downloaded HIV (HXB2: K03455), and HBV reference sequences (Genotype A: FJ692557, Genotype B: GU815637, Genotype C: GQ377617,

Genotype D: KC875277, Genotype E: GQ161817) from publicly available repositories: HIV sequence database (https://www.hiv.lanl.gov/content/index) and HBV Database (Hayer et al. 2013). I aligned amino acid RT sequences using MAFFT version 7 (Katoh, Rozewicki and Yamada, 2019). The alignment illustrates regions of similarity and differences between HIV and HBV RT. I obtained RAMs associated with HIV resistance to TFV published in the Stanford University HIV drug resistance database under drug resistance summaries for nucleoside RT inhibitors (Soo-Yon et al. 2003).

2.2.2.4 Structural analysis

The crystal structure for HBV RT has not been solved. However, the enzyme is homologous to HIV RT explaining why certain NAs are active against both viruses (Das et al. 2001). To consider evidence for a mechanistic influence, we mapped HBV RAMs onto a previously solved crystal structure of HIV RT [Protein Data Bank (PDB) code 3dlk] (Sarafianos et al. 2009) using ICM-Pro platform which provides a direct link to the PDB (http://www.molsoft.com/icm_pro.html). Structural analysis was performed by Dr. Brian Marsden affiliated with Structural Genomics Consortium at the University of Oxford.

2.2.3 Results

2.2.3.1 Nature of the evidence identified

I identified 15 studies reporting on evidence of HBV resistance to TFV. **Table 2.2.1** gives a summary of the characteristics of studies included. Metadata for individual studies are provided in **Appendix Table 2.2.2.**

Table 2.2.1: Characteristic of 15 studies included in a systematic literature review for the evidence of tenofovir resistance to hepatitis B virus (HBV). Metadata of all 15 studies are provided in Appendix Table 2.2.2.

	Number of studies
Type of studies	
Case reports	7
In vitro studies	4
Longitudinal studies	4
Countries represented	
Asia	5
Australia	1
Europe	6
USA	3
Study population	
HBV/HIV coinfected patients	5
Chronic HBV monoinfection/ coinfection status not specified	6
In vitro studies	4
Antiviral treatment	
TDF monotherapy	7
TDF-based regimen	6
TAF monotherapy	2
HBV protein sequenced	
Polymerase only	14
Whole genome	1
Sequencing	
Consensus	14
Deep	1
Genotype	
Reported HBV genotype	8

TDF: Tenofovir disoproxil fumarate. TAF: Tenofovir alafenamide.

2.2.3.2 Quality appraisal

Among the case reports, three were of high quality as they clearly described patients' characteristics, clinical history, current clinical condition, diagnostic tests or assessment methods, interventions or treatment procedures, post-intervention clinical condition, and concluded with take away lessons (Lee et al. 2014; Shirvani-Dastgerdi et al. 2017; Lu et al. 2018). Three further case reports did not describe diagnostic test or assessment methods used (Schirmer, Winters and Holodniy, 2011; Mikulska et al. 2012; Cho et al. 2018), and one study (Park et al. 2019) did not describe post-intervention clinical condition of the participant.

The overall quality rating for four cohort studies was strong because individuals selected represented the target population, characteristics of participants were clearly described, there was a clear hypothesis for the study, and inclusion/exclusion criteria were specified (Sheldon et al. 2005; Lada et al. 2012; van Bommel et al. 2012; Audsley et al. 2016). Two studies had a weak

rating because there was no description of the characteristics of study participants and it was not clear whether participants were representative of the target population (Liu et al. 2014; Liu et al. 2017). Two natural experimental studies were not rated because the quality assessment questions were not applicable (Amini-Bavil-Olyaee et al. 2009; Qin et al. 2013). A detailed quality assessment of each individual reference is included in **Appendix Table 2.2.3** and **Appendix Table 2.2.4.**

2.2.3.3 Approach to defining resistance

Resistance was studied based on exposure to TDF in 13 studies and TAF in two studies; I therefore use the more inclusive acronym, TFV, throughout the results section. TFV resistance was determined using a range of strategies, which can be summarised as follows:

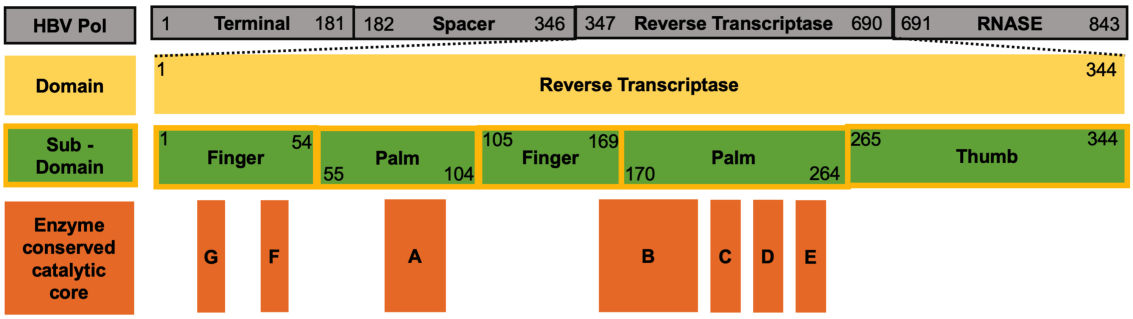
i. A sequencing approach to identifying possible RAMs in HBV sequence isolated from individuals in whom viraemia was not suppressed by TFV therapy, undertaken in seven studies (Schirmer, Winters and Holodniy, 2011; van Bommel et al. 2012; Mikulska et al. 2012; Lee et al. 2014; Audsley et al. 2016; Cho et al. 2018; Lu et al. 2018);
ii. *In vitro* assays to measure the effect of TFV on viral replication in cell lines, reported by three studies (Amini-Bavil-Olyaee et al. 2009; Liu et al. 2014; Liu et al. 2017);
iii. Approaches (i) and (ii) in combination, applied in four studies (Sheldon et al. 2005; Lada et al. 2012; Shirvani-Dastgerdi et al. 2017; Park et al. 2019);
iv. Approach (ii) combined with an animal model, described by one study (Qin et al. 2013).

In 12/15 studies, HBV mutations were reported in association with TFV resistance or reduced TFV susceptibility (**Appendix table 2.2.5).** In the remaining three studies, persistent viraemia was reported among individuals with CHB while on TFV but either no RAMs were identified (Schirmer, Winters and Holodniy, 2011; Audsley et al. 2016) or viral sequencing was not performed due to low VL (Lu et al. 2018).

2.2.3.4 RAMs occurring at consensus level

Recognising that there is sparse and varied evidence to support TFV RAMs, I divided the assimilated data into two categories, classified as a 'long list' (full list of all RAMs) and a 'short list' (RAMs supported by the highest quality evidence).

The 'long list' of all polymorphisms that have been reported in association with TFV resistance, and sets out to summarise all the available data in the most inclusive way. The polymorphisms included in this list were present both within (n=15) and outside (n=22) enzymatically active sites in RT **(Figure 2.2.2; Appendix Table 2.2.5).**



Enzyme conserved catalytic core	RAMs associated with TDF/TAF located within active sites
A (rt75 – rt91)	S78T; L80M;L91I
B (rt163 – rt189)	I63V; V173L; P177G; **L180M**; **A181T/V**; T184A/L
C (rt200 – rt210)	A200V; **M204I/V**; V207L
D (rt230 – rt241)	**N236T**
E (rt247 – rt257)	F249A; C256S
F(rt37 – rt47)	Nil known
G (rt26 – rt36) &	Nil known

Enzyme domains	RAMs associated with TDF/TAF located within active sites
Finger (rt1 – 54)	Y9H
Palm (rt55 – 104)	**S106C/G**
Finger (rt105 – 169)	T118C/G; F122L; H126Y; Q130S; D134E; **R153W/Q**
Palm (rt170 – 264)	V191I; R192P; **A194T**; L217R; F221Y; S223A; L229V/W; D263E
Thumb (rt265-344)	Q267L; **I269L**; V278I/V; A317S; K333Q; N337H

Figure 2.2.2: Mutations associated with tenofovir (TFV) resistance located within and outside the active sites of the hepatitis B virus reverse transcriptase (RT) enzyme. Polymerase numbering shown in the grey bars is based on genotype A sequence (accession number X02763). Yellow bar represents RT; green bars represent subdomains which are designated finger, palm and thumb; orange rectangles represent active sites of the enzyme referred to as regions A–G. Mutations associated with TFV resistance (n=37 sites) are listed according to their location within active sites of the enzyme (orange table) or outside active sites (green table). The sites shown in bold represent the nine mutations in the short-list with best literature support (evidence summarised in **Table 2.2.2**). Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥2 polymorphisms. Figure reproduced from (Mokaya et al. 2020a) under review in Wellcome Open Research, under the terms of the Creative Commons Attribution (CC BY) license.

HBV mutations outside active sites of the enzyme occurred in combination with RAMs located within active sites, with the exception of A194T. Only two studies reported TFV resistance arising from the selection of a single mutation, S78T and A194T (Amini-Bavil-Olyaee et al. 2009; Shirvani-Dastgerdi et al. 2017). S78T was defined by sequencing HBV from two individuals in whom viraemia was not suppressed by TFV, combined with *in vitro* assays (Shirvani-Dastgerdi et al. 2017), while A194T was only defined *in vitro* (Amini-Bavil-Olyaee et al. 2009). In all other studies, ≥2 RAMs were required to confer TFV resistance [two RAMs in four studies (van Bommel et al. 2012; Qin et al. 2013; Liu et al. 2014; Liu et al 2017), three RAMs in one study (Sheldon et al. 2005), five RAMs in one study (Park et al. 2019), and ≥8 RAMs in a further four studies (Lada et al. 2012; Mikulska et al. 2012; Lee et al. 2014; Cho et al. 2018)]. This pattern supports the high genetic barrier to selection of TFV resistance.

I then refined the 'long list' of 37 polymorphisms into a 'short list' including just those sites supported by the most robust evidence, based on reporting in ≥2 studies and a combination of *in vivo* and *in vitro* evidence (nine sites; **Table 2.2.2)**.

Table 2.2.2: Mutations most commonly reported in association with tenofovir resistance in hepatitis B virus (HBV), identified from a systematic literature review. This table contains a 'short-list' of mutations identified by two or more different studies. The asterisks indicates the method(s) used to determine drug resistance. All positions are in HBV RT, listed in numerical order. The 'long-list' of 37 mutations identified in 15 included studies is reported in **Appendix Table 2.2.5.**

Mutations	Park et al. 2019	Cho et al. 2018	Lee et al. 2014	Mikulska et al. 2012	Liu et al. 2014	Amini-Bavil-Olyaee et al. 2009	Van Bommel et al. 2012	Lada et al. 2012	Sheldon et al. 2005	Liu et al. 2017	Total number of studies	Associated with 3TC resistance	Associated with ETV resistance
S106C/G	***	*									2	No	No
R153W/Q			*					***			2	No	No
V173L				*				***			2	Yes	Yes
L180M			*	*				***	***		4	Yes	Yes
A181T/V					**		*	***		**	4	Yes	Yes
A194T						**			***		2	No	No
M204I/V	***		*	*				***	***		5	Yes	Yes
N236T					**		*			**	3	No	No
I269L	***	*									2	No	Yes

KEY:	Method used to identify TFV resistance
*	Sequencing HBV sequence from individuals in whom viraemia was not suppressed by TFV
**	In vitro assays to measure the effect of TFV on viral replication in cell lines
***	Approaches (i) and (ii) in combination as described in **section 2.2.3.3**

The most frequently described RAMs were L180M, A181T/V, M204I/V, and N236T, which were all identified through sequencing and tested in *in vitro* assays to measure the effect of TFV on viral replication in cell lines **(Table 2.2.2).** As I showed in **section 2.1.3.6 (Figure 2.1.4** and **Figure 2.1.5)**, mutations at position 204 are the most prevalent across Africa. While these have generally been reported in association with 3TC resistance, the mutation is also a component of TFV resistance, commonly arising in combination with substitutions at positions V173, L180 and A181, while N236 substitutions appear to be more specifically associated with reduced susceptibility to ADV and TFV (Zoulim and Locarnini, 2009). Mutations at sites 177, 194 and 249 may also be more specific to TFV resistance, having been less clearly reported in association with resistance to other agents (Kim et al. 2017; Mokaya et al. 2018b). Polymorphisms at positions 80, 173 and

184 have been described as compensatory changes to allow the virus to accommodate the primary drug escape substitution (Zoulim and Locarnini, 2009), (see **chapter one: section 1.12.1.2.1)**. These mutations on their own may not be sufficient to mediate TVF resistance but may be a necessary compensatory contribution to combinations of mutations that underpin resistance. One study assessed the replication competence and susceptibility to TFV of mutated HBV clones *in vitro* and *in vivo* using mouse models. The introduction of P177G and F249A mutations (substitutions in active sites of the RT enzyme) in HBV clones resulted in a reduction in their susceptibility to TFV (Qin et al. 2013).

2.2.3.5 RAMs occurring as minor quasispecies

There is limited evidence for the significance of TFV RAMs occurring as minor quasispecies. One study that performed ultra-deep pyrosequencing enrolled HIV/HBV coinfected individuals on (or about to start) TFV-containing ART, reporting minor variants present at <20% with mutations (V173, L180M, A181T/V and M204V) in 2/50 TFV naïve samples and 1/14 sample obtained from a TFV-experienced individual (Audsley et al. 2016). One other study performed deep sequencing using Illumina on HBV clones (Park et al. 2019), revealing that RAMs S106C, H126Y, D134E, M204I/V and I269L were predominant. Only one study reported sequencing the whole HBV genome, but this was undertaken following *in vitro* introduction of RAMs into a clinically isolated virus (Liu et al. 2017), so it does not provide any evidence of the association between TFV RAMs and other polymorphisms that might arise on the same viral haplotype.

2.2.3.6 Assessing treatment compliance

Five studies reported the duration for which individuals were on treatment prior to treatment failure, with virological breakthrough occurring between 48 weeks and 48 months of TFV therapy [48 weeks (Lee et al. 2014), 18 months (Lu et al. 2018), 20 months (Lada et al. 2012), 26 months (Park et al. 2019), and 48 months (Shirvani-Dastgerdi et al. 2017; Cho et al. 2018)]. Treatment compliance was assessed in six studies (Sheldon et al. 2005; Lada et al. 2012; Mikulska et al. 2012; Lee et al. 2014; Lu et al. 2018; Park et al. 2019), using chromatography plasma

quantification (Lada et al. 2012) and measurement of serum concentration of TFV, which showed appropriate serum concentration (130 ng/ml and 190 ng/ml) associated with good compliance (Park et al. 2019). Virological breakthrough despite good treatment compliance was reported in five studies (Sheldon et al. 2005; Lada et al. 2012; Lee et al. 2014; Lu et al. 2018; Park et al. 2019). Only one study reported concerns with compliance (Mikulska et al. 2012).

2.2.3.7 Understanding the mechanism of TFV resistance

2.2.3.7.1 Comparison between HIV and HBV RT

Alignment of sequences of HBV and HIV RT demonstrates 25–27% homology between HBV and the HXB2 HIV reference sequence (**Figure 2.2.3)**.

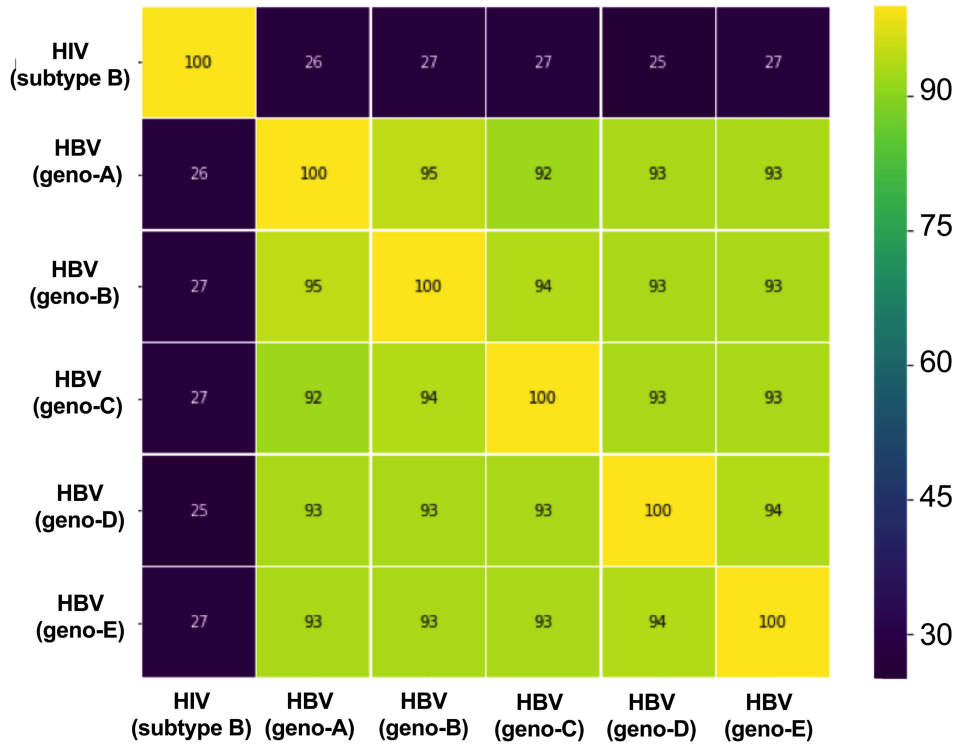


Figure 2.2.3: Heatmap showing identity comparison matrix of reference sequence alignment of hepatitis B virus (HBV) reverse transcriptase (RT) and human immunodeficiency virus (HIV) RT. Chart shows a comparison based on sequences downloaded from HIV sequence database (https://www.hiv.lanl.gov/) and HBV Database (Hamer et al. 2013) and aligned using MAFFT version 7. HIV reference sequence is HIV HXB2 (K03455). HBV reference sequences are Genotype A: FJ692557, Genotype B: GU815637, Genotype C: GQ377617, Genotype D: KC875277, Genotype E: GQ161817. Figure reproduced from (Mokaya et al. 2020a) under review in Wellcome Open Research under the terms of the Creative Commons Attribution (CC BY) license. *This figure was generated by Dr. Armand Bester based at University of the Free State, Bloemfontein, South Africa.*

I compared sites that have been reported in association with drug resistance in HBV vs HIV **(Figure 2.2.4, Appendix Table 2.2.6),** and found that among the long-list of 37 HBV RAMs, two sites had identical substitutions in HIV RT [M204 and L229 in HBV (Das et al. 2001; Daga, Duan and Doerksen, 2010), corresponding to M184 and L210 in HIV, respectively (https://hivdb.stanford.edu/dr-summary/resistance-notes/NRTI/)] (**Figure 2.2.4).** Other sites reporting an association with TVF resistance in HBV have substitutions that overlap with HIV RAMs, but not all of these are associated with TFV resistance (**Appendix Table 2.2.6)**.

HBV SEQUENCE POSITION	201	202	203	204	205	206	207	208	209	210	211	212		213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233
HBV_Geno_A1	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	T	A	V	T	N	F	L	L	S	L	G	I
HBV_Geno_B2	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	A	A	V	T	N	F	L	L	S	L	G	I
HBV_Geno_C2	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	F	T	S	I	T	N	F	L	L	S	L	G	I
HBV_Geno_D1	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	F	T	A	V	T	N	F	L	L	S	L	G	I
HBV_Geno_E	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	T	S	V	T	N	F	L	L	S	L	G	I
HBV TDF mutation hotspots				M204I/V			V207L											L217R				F221Y		S223A						L229V/W				
HIV SEQUENCE POSITION	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214
HIV_Subtype_B	Y	Q	Y	M	D	D	L	Y	V	G	S	D	L	E	I	G	Q	H	R	T	K	I	E	E	L	R	Q	H	L	L	R	W	G	L
HIV TDF mutation hotspots				M184I/V																										L210W				
Sites with same AA when comparing HIV and HBV RT after alignment			*	*	*	*				*																			*	*			*	

Figure 2.2.4: A section of the reference sequence alignment of hepatitis B virus (HBV) reverse transcriptase (RT) and human immunodeficiency virus (HIV) RT. Sequences downloaded from HIV sequence database (https://www.hiv.lanl.gov/) and HBV Database (Hamers et al. 2013). Sequences were aligned using MAFFT version 7. HIV subtype B reference sequence is shown in light green (accession number K03455). HBV reference sequences are shown in yellow (Genotype A: FJ692557; Genotype B: GU815637; Genotype C: GQ377617; Genotype D: KC875277; Genotype E: GQ161817). Sites of TFV resistance are highlighted in red, based on the data assimilated in this study. HIV TFV RAMs were obtained from the online Stanford Database (https://hivdb.stanford.edu/dr-summary/resistance-notes/NRTI/). Sites marked * have the same amino acid in HIV and HBV RT after alignment, and those coloured blue also share TFV resistance mutations. This section is shown as it contains the only two homologous TFV RAMs that we have identified using this approach. Sequence alignments and RAMs throughout the whole RT protein is shown in (**Appendix Table 2.2.6)**. Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥2 polymorphisms. Figure reproduced from (Mokaya et al. 2020a) under review in Wellcome Open Research under the terms of the Creative Commons Attribution (CC BY) license.

Six established TFV RAMs in HIV RT (M41L, K65R, K70E, Y115F, Q151M and T215F/Y) (Soo-Yon et al. 2003) do not correspond to an equivalent mutation in HBV RT, although three of these HIV RAMs have an HBV RAM within three amino acids up- or down-stream in the equivalent sequence, suggesting there may be homology in the mechanism through which drug resistance is mediated.

2.2.3.7.2 Structural analysis of drug resistance mechanisms

We mapped HBV RAMs onto the crystal structure of the structurally-related HIV RT (pdb code 3dlk) in order to visualise their approximate 3D locations and infer possible functional consequences **(Figure 2.2.5; Appendix Table 2.2.7).**

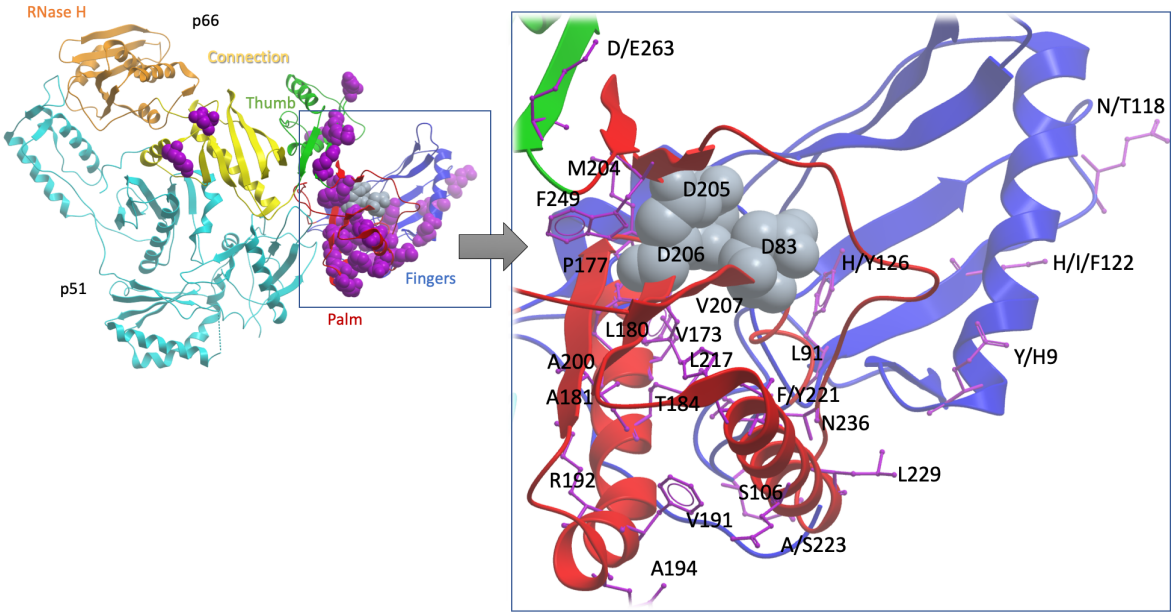


Figure 2.2.5: Cartoon to show the sites of tenofovir (TFV) drug resistance polymorphisms, using the homologous crystal structure of human immunodeficiency virus (HIV) reverse transcriptase (RT) as a model. The sequence alignment of HBV was extended with HIV RT's p66 domain and then projected onto a high-resolution HIV RT structure (pdb code 3dlk). Sub-domains of the HIV RT are coloured and annotated. Positions associated with resistance are scattered primarily throughout the finger and palm subdomains of the p66 domain (purple space-filled representations, left whole-molecule view, purple stick representation on the zoomed in view on the right). Three aspartate residues, D83, D205 and D206 (indicated by grey space-filled representation) form the catalytic triad of the enzyme and are shown as a point of reference. Of the 37 sites identified as potential TFV RAMs, 24 residues which are visible in the structure are labelled (using HBV numbering). This excludes seven putative HBV mutations at sites which do not have a homologous site in the HIV structure (sites 78, 80, 130, 134, 153, 163 and 256), and six sites which are beyond the end of the sequence of the solved crystal HIV structure (267, 269, 278, 317, 333 and 337). Figure produced using the ICM platform (http://www.molsoft.com/icm_browser.html). Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥2 polymorphisms. Figure reproduced from (Mokaya et al. 2020a) under review in Wellcome Open Research under the terms of the Creative Commons Attribution (CC BY) license. *This figure was generated by Dr. Brian Marsden based at the Structural Genomics Consortium, University of Oxford.*

The RAMs are primarily located within the 'fingers', 'palm', 'thumb' and 'connection' subdomains of the p66 polymerase domain of HIV RT, with the majority within the 'palm'. A number of RAMs (e.g. V207, M204, F249) are spatially adjacent to the catalytically critical (and highly conserved) residues D110, D185 and D186 in HIV RT (D83, D204 and D205 in HBV), suggesting that these RAMs are highly likely to affected catalytic competency. Twenty-two HBV RAMs map to HIV RT residue positions which, if mutated, are likely to cause polymerase structure destabilisation, suggesting that many of these RAMs are likely to impact resistance.

2.2.4 Discussion

2.2.4.1 Summary of key findings

TFV is a safe and effective treatment choice for CHB in the majority of cases, and large case series have not raised significant concerns about clinically significant drug resistance. However, it is important to consider the potential for the emergence of resistance, demonstrated by persistent viraemia on therapy and/or reduced virologic suppression *in vitro*. Based on existing evidence, TFV resistance seems likely to depend on the selection of suites of mutations (most commonly including L180M, A181V/T, M204I/V and/or N236T), overlapping with RAMs that allow escape from other NA drugs. There is also a suggestion that, rarely, single mutations can confer TFV resistance, best demonstrated for S78T.

Notably, the literature to date is limited and heterogenous, and there remains a lack of evidence about the frequency and likely impact of proposed TFV RAMs either within individual patients or at population level. To tackle this uncertainty, I classified RAMs associated with TFV resistance have been divided into a 'long-list' (all reported RAMs) and a 'short-list' (RAMs with the best evidence-base of support). This will be a useful resource for future efforts in identifying and describing TFV resistance in HBV, and has been the foundation for subsequent analyses presented later in **chapters 3, 5 and 7.**

2.2.4.2 Overlap between TFV RAMs with RAMs to other NA agents

RAMs L180M, M204I/V and A181T/V have been associated with resistance to 3TC, LdT and ETV (Yatsuji et al. 2006; Shaw, Bartholomeusz and Locarnini, 2006; Hermans et al. 2016; Meng et al. 2017; Mokaya et al. 2018b); their reported association with TFV resistance is of concern in suggesting that prior NA exposure can increase the likelihood of cross-resistance to TFV. A study of HIV/HBV coinfected individuals demonstrated a decreased likelihood of HBV DNA suppression with TFV among individuals exposed to prolonged 3TC treatment, possibly due to accumulation of such mutations (Kim et al. 2014). A large study in China reported A181 and/or N236 substitutions in 11% of the population (Meng et al. 2017), which may underpin reduced susceptibility to TFV. The structural similarities between ADV and TFV, and similar interaction with HBV polymerase (Delaney et al. 2006; Beloukas and Geretti, 2017) explain why the ADV RAMs A181T/V and N236T are also reported to confer resistance to TFV (Nidhi et al. 2014; Beloukas and Geretti, 2017).

Although TFV has been considered effective in the context of resistance to other NAs (Lim, 2017), the current evidence suggests that there may be common pathways to resistance (Hemert, Berkhout and Zaaijer, 2014). There is some evidence showing co-location of RAMs conferring resistance to different antiviral agents on the same viral haplotype (Yim et al. 2006). These findings suggesting cross-resistance are of concern, especially for settings in which there has been widespread use of NA therapy as a component of ART for HIV (Mokaya et al. 2018b).

2.2.4.3 Sites of TFV RAMs in HBV RT

Resistance to TFV can be explained by RAMs both within and outside the active site of the RT enzyme, some of which may have similar mechanisms to those described in HIV (Das et al. 2001; Clark and Hu, 2015b). The mechanism of resistance in most of these polymorphisms remains unknown but may interfere with drug access to sites of activity through steric hindrance. Mutations within active sites of the enzyme may be associated with a higher fitness cost to the virus than mutations at other locations in the RT sequence, as they are more likely to interfere with the RT

function. Some polymorphisms listed as RAMs may in fact represent compensatory mutations, that are co-selected in the presence of primary RAMs. For example, substitution at position 269 has been previously described as a compensatory mutation that restores impairments to RT function (Ahn et al. 2015).

2.2.4.4 Other factors associated with persistent viraemia while on treatment with TFV

In addition to RAMs, there are other explanations for incomplete suppression of HBV viraemia on therapy, supported by reports of phenotypic resistance in the absence of any known RAMs (Schirmer, Winters and Holodniy, 2011; Audsley et al. 2016). Factors associated with persistent HBV DNA viraemia on TFV treatment include a higher baseline HBV DNA level, positive baseline HBeAg status, prior history of 3TC exposure, a lower nadir CD4+ T cell count in the context of HIV coinfection, and high serum HBV RNA levels (Matthews et al. 2013; Kim et al. 2014; Hu and Liu, 2017). These diverse phenomena make it difficult to be certain of a clinical (phenotypic) definition of drug resistance and may confound studies that set out to identify viral sequence (genotypic) variants that are genuinely associated with altered TFV susceptibility.

2.2.4.5 Treatment compliance

Incomplete adherence to drug therapy can also contribute to virological breakthrough (see **chapter 1: section 1.13.2**). Evidence of potential TFV resistance may emerge when individuals with HIV/HBV coinfection are adherent to TFV-containing regimen leading to suppression of HIV but with sustained HBV vireamia (Mokaya et al. 2020b; see **chapter 5: section 5.3.6**).

2.2.4.6 Slow response to treatment

The time to virological breakthrough on TFV treatment ranged from 48 weeks to 48 months in the studies I identified. It has been reported to take three years for 90% of HBV infected individuals to reach viraemic suppression on therapy (Price et al. 2013), in contrast to HIV, in which 88% of patients suppress the virus within the first year of TFV-based treatment (WHO, 2018b). This raise concerns on the effectiveness of NAs for HBV treatment as these drugs (TFV and 3TC) were

initially developed for HIV treatment (De Clercq et al. 2010; Lingala et al. 2016; Lovett et al. 2017; Luo, Jiang and Ren, 2018). In addition, HBV DNA is inhibited in a dose-dependent manner (Delaney et al. 2006), and therefore possible that insufficient drug delivery to the infected hepatocyte could be the cause of persistent viraemia even in the absence of specific RAMs.

2.2.4.7 Caveats and limitations

There is sparse literature on HBV resistance to TFV, and studies are of varying quality. While there is a high genetic barrier to selection of TFV resistance, it is likely that there is under-reporting of cases of resistance, particularly in low/middle income settings in which routine monitoring of HBV VL on treatment is not undertaken. It can be difficult to infer the impact of common polymorphisms on drug resistance phenotype; for example, it is plausible that M204I/V may be enriched among TFV resistant strains simply as a 'footprint' of prior exposure to 3TC.

Most studies to date have used Sanger sequencing, and it is possible that significant minority variants may be under-represented, as suggested by one report in which phenotypic TFV resistance was associated with RAMs in <20% of minor variants (Audsley et al. 2016). To be able to undertake an appropriate haplotypes analysis, datasets with robustly phenotyped patients (displaying clinical evidence of drug resistance), together with full length viral sequence data, would be required; such datasets have not been generated to date but are an important long-term aim.

There are limitations of drawing direct comparisons between HIV and HBV RT, given the limited (<30%) sequence homology between the two enzymes, and the finding that only 2/37 sites associated with TVF resistance in HBV are homologous RAMs in HIV. This highlights a need for future work to solve the crystal structure of HBV RT.

Chapter three: The phylodynamics of HBV RAMs and VEMs

3.1.1 Background

There are a number of studies that have reported the frequencies of RAMs/VEMs in HBV from different populations, among those exposed and unexposed to HBV vaccine/treatment (Zhang et al. 2015; Hermans et al. 2016; Meng et al. 2017; Mokaya et al. 2018b). However, the global prevalence, geographic distribution, time of origins, and their association with different HBV genotypes remain unknown (see **chapter 1: section 1.17.2**). Using sequence data obtained from a publicly available database, I have assimilated data on the global prevalence and distribution of HBV RAMs and VEMs and explored the genetic relatedness of viruses bearing these mutations. This work is currently under review at Virus Evolution (**Mokaya et al. 2020c**).

3.2.1 Methods

3.2.1.1 HBV sequences curation process

I analysed sequences downloaded from the HBV Database (Hayer et al. 2013), accessed on 20th November 2018. I downloaded a total of 6219 full length genome sequences **(Figure 3.1)**. Using MEGA7 software (Kumar, Stecher and Tamura, 2016), I generated neighbour joining phylogenetic trees to validate HBV genotype assignment, discarding sequences that had been incorrectly classified. I then generated pairwise distances for aligned sequences in each genotype using dist.alignment function of the R package seqinr (http://seqinr.r-forge.r-project.org/), and excluded sequences with >99.5% similarity in order to remove closely related isolates for instance duplicates and/or isolates derived from the same individual. For the remaining sequences, I obtained sample collection date and sampling country from GenBank. A total of 2938 sequences had geographical data and 2167 had both sample collection date and geographical data.

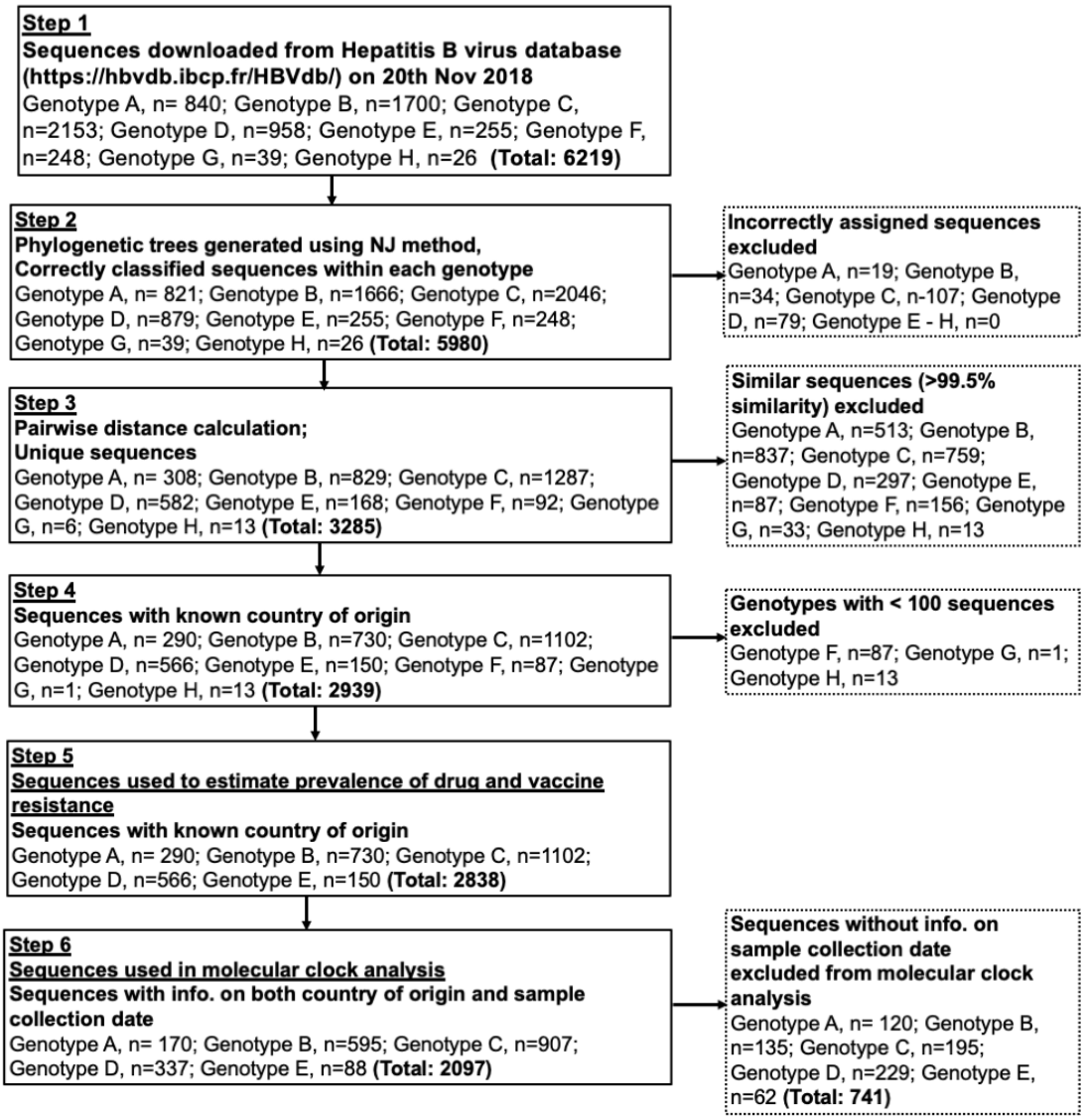


Figure 3.1: Flow diagram showing data curation process of sequences downloaded from a public database (Hayer et al. 2013) included in the analysis of the global prevalence and evolution of hepatitis B virus drug resistance-associated mutation and vaccine escape mutations.

3.2.1.2 Identification of HBV RAMs

I used a catalogue of pre-existing drug RAMs identified from published studies (Zhang et al. 2015; Caligiuri et al. 2016; Beloukas and Geretti, 2017; Choi, Lee and Kim, 2018; Mokaya et al. 2018b; Mokaya et al. 2020a) (**Table 3.1**), and stratified according to the NA to which they cause resistance as described below:

a. I classified RAMs associated with 3TC into three categories: (i) primary RAMs, which are well known to cause resistance to 3TC in isolation; (ii) compensatory RAMs, which by themselves do not confer resistance but when combined with primary RAMs enhance resistance and viral functional capacity (Beloukas and Geretti, 2017); and (iii) putative RAMs for which there is limited clinical/*in vitro* evidence supporting 3TC resistance.
b. Two or more amino acid substitutions are required across the HBV RT protein to confer resistance to ETV which could occur as a combination of M204I/V with one or more of the following substitutions L80I/V, I163V, I169T, V173L, L180M, A181S/T/V, T184X, A186T,S202C/G/I/R, M250I/V and/or C256S/G (Fung et al. 2011; Beloukas and Geretti, 2017; Locarnini, 2017).
c. I classified RAMs associated with TFV into three categories: those with both clinical and *in vitro* evidence; those with only clinical evidence; and those with only *in vitro/ in vivo* evidence.

Classification of RAMs associated with TFV were informed by the systematic literature review described in **(chapter 2: section 2.2)** (Mokaya et al. 2020a).

Table 3.1: Hepatitis B virus drug resistance-associated mutations. Data obtained from published systematic reviews (Zhang et al. 2015; Caligiuri et al. 2016; Beloukas and Geretti, 2017; Choi, Lee and Kim, 2018; Mokaya et al. 2018b; Mokaya et al. 2020a).

RAMs	3TC			ETV	TFV		
	Primary	Compensatory	Putative		Clinical and *in vitro* evidence	Only clinical evidence	Only *in vitro* evidence
1. H/Y9H						✓	
2. V/N/S/T53N			✓				
3. S78T					✓		
4. L80I/M/V		✓		✓		✓	
5. L82M			✓				
6. I/L91I			✓			✓	
7. S106C/G					✓		
8. T118C/G						✓	
9. I/F/H/L/N/Y122L						✓	
10. H/Y126Y					✓		
11. T128A/I/N			✓				
12. Q/P130S						✓	
13. D/H/N134E					✓		
14. N/Q139D/E			✓				
15. Q/R/W153Q/W			✓		✓		
16. I163V				✓	✓		
17. F166L			✓				
18. I169L/T		✓		✓			
19. V173L		✓		✓	✓		
20. P177G							✓
21. L180M		✓		✓	✓		
22. A181T/V	✓			✓	✓		
23. T184A/C/F/G/I/L/M/S		✓		✓		✓	
24. A186T				✓			
25. V191I			✓			✓	
26. R192P						✓	
27. A194T					✓		
28. A200V			✓			✓	
29. S202C/G/I		✓		✓			
30. M204I/V/S/Q	✓			✓	✓		
31. V/L207I/L			✓			✓	
32. S213T			✓	✓			
33. Q215E/H/P/S			✓				
34. L217R					✓		
35. F/Y221Y						✓	
36. A/S223A						✓	
37. L229G/F/V/W			✓		✓		
38. N236T					✓		
39. F249A							✓
40. M250I/L/V				✓			
41. C/S256G/S			✓	✓		✓	
42. E/D263E						✓	
43. H/L/M/Q267L						✓	
44. I269L					✓		
45. V278I						✓	
46. A/S317S						✓	
47. K/Q/T333Q						✓	
48. N337H						✓	

RAMs common to 3TC & ETV	I169L/T; S202C/G/I; S213T
RAMs common to 3TC & TFV	I/L91I/L; Q/R/W153Q/W; V191I; A200V; V/L207I/L; L229G/F/V/W
RAMs common to ETV & TFV	I163V
RAMs common to 3TC & ETV & TFV	L80I/V/M; V173L; L180M; A181T/V; T184A/C/F/G/I/L/M; M204I/V/S/Q; C/S256G/S

3.2.1.3 Identification of HBV VEMs

All pre-existing VEMs included in this study were identified from published studies (Waters et al. 1992; Steward et al. 1993; Howard, 1995; Carman, 1997; Ngui et al. 1997; Protzer-Knolle et al. 1998; Roznovsky et al. 2000; Chang, 2010; Coppola et al. 2015; Lazarevic, 2014; Colagrossi et al. 2018; Mokaya et al. 2018b) **(Table 3.2).** G145A/R and K141E/I/R have the strongest evidence base of clinical and *in vitro* data to support HBV vaccine resistance (Carman, 1997), while other VEMs are considered putative, as they are supported by less robust data.

Table 3.2: Hepatitis B virus vaccine escape mutations. Data obtained from published studies (Waters et al. 1992; Steward et al. 1993; Howard, 1995; Carman, 1997; Ngui et al. 1997; Protzer-Knolle et al. 1998; Roznovsky et al. 2000; Chang, 2010; Coppola et al. 2015; Lazarevic, 2014; Colagrossi et al. 2018; Mokaya et al. 2018b).

VEMs	HBIG	VEMs	Immune escape
1. Q101K			✓
2. V106A			✓
3. I110L			✓
4. S113T			✓
5. S/T114F/R/T	✓		✓
6. T116A/N		✓	
7. T118A/R/V	✓		
8. P120A/E/N/Q/S/T	✓	✓	✓
9. R/K122S			
10. T123A/N	✓		✓
11. C124R/Y	✓		
12. I/T126A/H/I/N/R/S	✓	✓	✓
13. P/T127L/P		✓	✓
14. A128V			✓
15. Q129H/N/R/P	✓	✓	✓
16. G130N/R	✓		✓
17. N/T131I/N/S	✓	✓	✓
18. M133I/L/T	✓	✓	✓
19. F/Y134I/L	✓	✓	✓
20. C137R/Y	✓		
21. C138Y			✓
22. C139S	✓		
23. S/T140I	✓		✓
24. K141E/I/R	✓	✓	
25. P142S	✓	✓	✓
26. S/T143M/L		✓	
27. D144A/E/G/H/N	✓	✓	✓
28. G145A/K/R	✓	✓	✓
29. N146S	✓		✓
30. C147S	✓		
31. K160R			✓

VEMs with both phenotypic and experimental evidence	K141E/I/R; G145A/K/R

3.2.1.4 Prevalence of HBV RAMs

For the global prevalence analysis, I included published sequences with known country of origin from genotypes A–E; I excluded genotypes F, G & H from this analysis because of low sample size (<100), resulting in a total of 2838 sequences (**Figure 3.1**). For all polymorphisms that have been reported in association with resistance listed in **Tables 3.1** and **3.2**, I calculated the prevalence as total number of sequences with a specified mutation as a proportion of the total number of sequences in each genotype/continent.

I carried out prevalence analysis reporting confidence intervals (CI) and p-values for individual RAMs common to 3TC, ETV and TFV, for individual or combined RAMs associated with ETV and TFV resistance, and for individual VEMs. I calculated 95% CI using an online software Epitools (http://epitools.ausvet.com.au). I used Chi square test to compare the prevalence of RAMs/VEMs between different genotypes and between different continents

I used GraphPad Prism v7.0 and R package ggplot2 (https://github.com/tidyverse/ggplot2) for data visualisation and statistical analyses.

3.2.1.5 Distribution of selected HBV RAMs and VEMs on maximum likelihood phylogenetic trees

I generated maximum likelihood (ML) phylogenetic trees for genotype A (n=290), B (n=730), C (n=1102), D (n=565) and E (n=150) sequences for which geographic information was available. I used the general time reversible nucleotide substitution model with among-site rate variation (GTR + G) in IQ-TREE (Nguyen et al. 2015). I chose this model as it incorporates different rates for every nucleotide change and different nucleotide frequencies, thus allowing for most flexibility, and allowing me to avoid a model-selection step (Abadi et al. 2019). I rooted the trees using the mid.point function of the R package phangorn (https://github.com/KlausVigo/phangorn).

For this analysis, I considered a total of 12 RAMs (S106C/G, D134E, R153W/Q, V173L, L180M, A181T/V, A194T, A200V, M204I/V, L217R, L229V/W, I269L). These RAMs were selected because they are primary RAMs or have robust evidence in causing resistance to 3TC, ETV and/or TFV (Fung et al. 2011; Beloukas and Geretti, 2017; Locarnini, 2017; Mokaya et al. 2020a). I also considered eight VEMs (C139S, S/T140I, P142S, S/T143L/M, D144A/E/G/N, G145A/E/R, K141A/I/R and C147S) which are located within the epitope (aa139–147) which is a neutralising epitope for HBV vaccine (see **chapter 1: Figure 1.3**).

I used treeBreaker (Ansari and Didelot, 2016) to test if sequences with individual mutations were randomly distributed across the branches of the phylogenetic trees reconstructed for each genotype. The program calculates per-branch posterior probability of having a change in the distribution of a discrete character and gives a Bayes factor to show the strength of this evidence (Ansari and Didelot, 2016). A Bayes factor of >30 indicates a strong evidence that sequences with RAMs/VEMs are not randomly distributed on a phylogenetic tree. I performed this analysis for each mutation separately.

3.2.1.6 Phylogenetic dating

I performed molecular clock phylogenetic analyses to estimate the times of emergence of mutations of interest, focused on RAMs V173L, L180M and M204I/V as they have been commonly identified in HBV resistance to 3TC, ETV and TFV [Beloukas and Geretti, 2017; Mokaya et al. 2018b; Mokaya et al. 2020a (see **chapter 2: section 2.2.3.4**)], and VEMs G145A/R and K141E/I/R as they have been best described to cause HBV vaccine resistance (Zanetti et al. 1988; Ahmed and Abdelwahab, 2015; Romanò et al. 2015). In this analysis I included genotypes that had >50 sequences with associated sampling date information: genotype A (n=170), B (n=594), C (n=906), D (n=336) and E (n=88). I manually inspected sequences for misalignments in AliView program (Larsson, 2014) and then excluded codon positions associated with resistance (I excluded all sites listed in **Table 3.1** and **Table 3.2**) to ensure that parallel evolution of RAMs/VEMs does not affect the phylogeny (Matthews et al. 2009). I used ML phylogenetic trees generated for each genotype as described above and then dated these phylogenetic trees using

IQ-TREE v2.0.3 (Thu-Hien et al. 2016). I resampled the trees 100 times and chose lognormal relaxed molecular clock model because it has performed best in other studies of HBV evolution (Zehender et al. 2015; Mozhgani et al. 2018). I used TempEst to estimate the molecular clock signal in my datasets by regressing the root-to-tip genetic distance of each sequence in the tree and its sampling date (Rambaut et al. 2016). Based on application of TempEst, I estimated the correlation between the dates of the tips of the sequences and the divergence from the root to be 7.8×10^{-2}, 3.9×10^{-1} , 4.3×10^{-2} , 2.3×10^{-2} and 2.1×10^{-1} for genotypes A, B, C, D and E, respectively. Due to the lack of correlation in this dataset, I used the substitution rate estimated before and applied in phylodynamic analyses of HBV (Osiowy et al. 2006; Littlejohn, Locarnini and Yuen, 2016), and therefore I fixed the mean substitution rate to 5.0×10^{-5} (SD 4.12×10^{-6}) subs/site/year for all genotypes in all subsequent analyses. I reported the time to most recent common ancestor (TMRCA) of two or more sequences that clustered together having the same mutation as this TMRCA likely corresponds to the lower bound of the age of the mutation.

I also performed molecular clock phylogenetic analyses using Bayesian Evolutionary Analysis Sampling Trees (BEAST). I have described this method in the **Appendix Method 3.1.**

3.2.3 Results

3.2.3.1 Global prevalence of HBV RAMs

3.2.3.1.1 All HBV RAMs

I set out to investigate the prevalence of polymorphisms associated with drug resistance across 48 different sites within RT protein in a total of 2838 full length HBV sequences. The complete list of mutations is provided in **Table 3.1**.

90% (43/48) of the sites had polymorphisms associated with drug resistance **(Figure 3.2).** 60% (26/43) and 63% (27/43) of the sites had polymorphisms associated with drug resistance occurring at the prevalence of <9% in all genotypes and all continents, respectively. Genotypes

A and C, as well as Europe had the highest number of sites (nine sites for genotype A and C, and 11 sites for Europe) with polymorphisms associated with drug resistance occurring at a prevalence of >20%. RAM H/Y9H is wildtype in genotypes A–E; this mutation is most likely to add to resistance as flexible position in the protein, in which compensatory change is easily incorporated (**Figure 3.2)**.

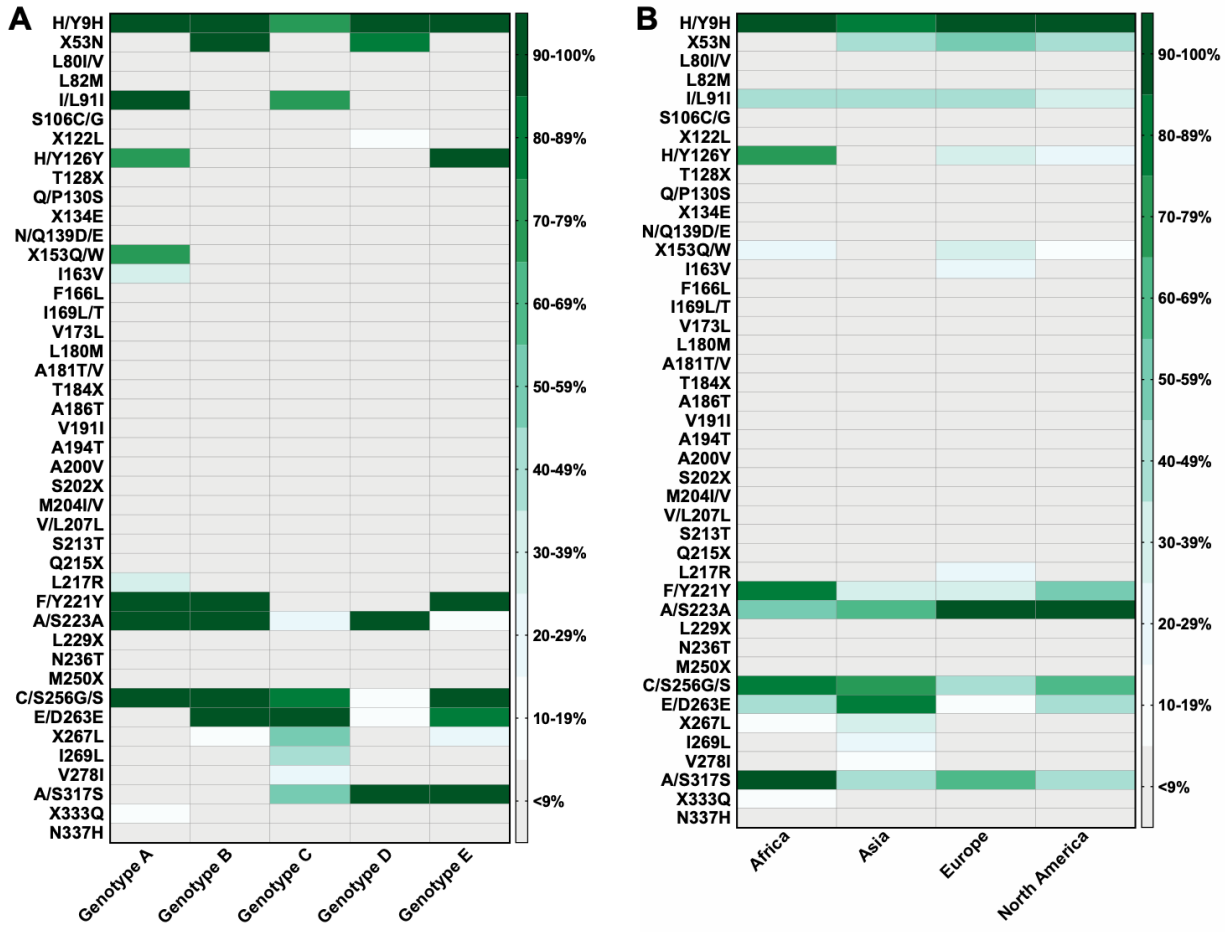


Figure 3.2: Global prevalence of hepatitis B virus (HBV) drug resistance-associated mutations obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (Hamers et al. 2013). A. Prevalence of polymorphisms across genotypes. **B.** Prevalence of polymorphisms across continents. X53N represents V/N/S/T53N. X122L represents I/F/H/L/N/Y122L. T128X represents T128A/I/N. X134E represents D/H/N134E. X153Q/W represents Q/R/W153Q/W. T184X represents T184A/C/F/G/I/L/M/S. S202X represents S202C/G/I. Q215X represents Q215E/H/P/S. L229X represents L229G/F/V/W. M250X represents M250I/L/V. X267L represents H/L/M/Q267L.

3.2.3.1.2 RAMs common to 3TC, ETV and/or TFV

RAMs L80I/M/V, V173L, L180M, A181T/V, T184X are common to 3TC, ETV and/or TFV. M204I/V had the highest prevalence at 3.8% (109/2838) **(Figure 3.3A).** Genotype C had the highest prevalence of all of these mutations, apart from L80I/M/V which was most common in Genotype D (although not statistically significant) **(Figure 3.3B).** L180M and M204I/V were both present in all genotypes and continents analysed in this manuscript **(Figure 3.3B** and **Figure 3.3C).** However, there were no significant differences in prevalence of these RAMs across continents.

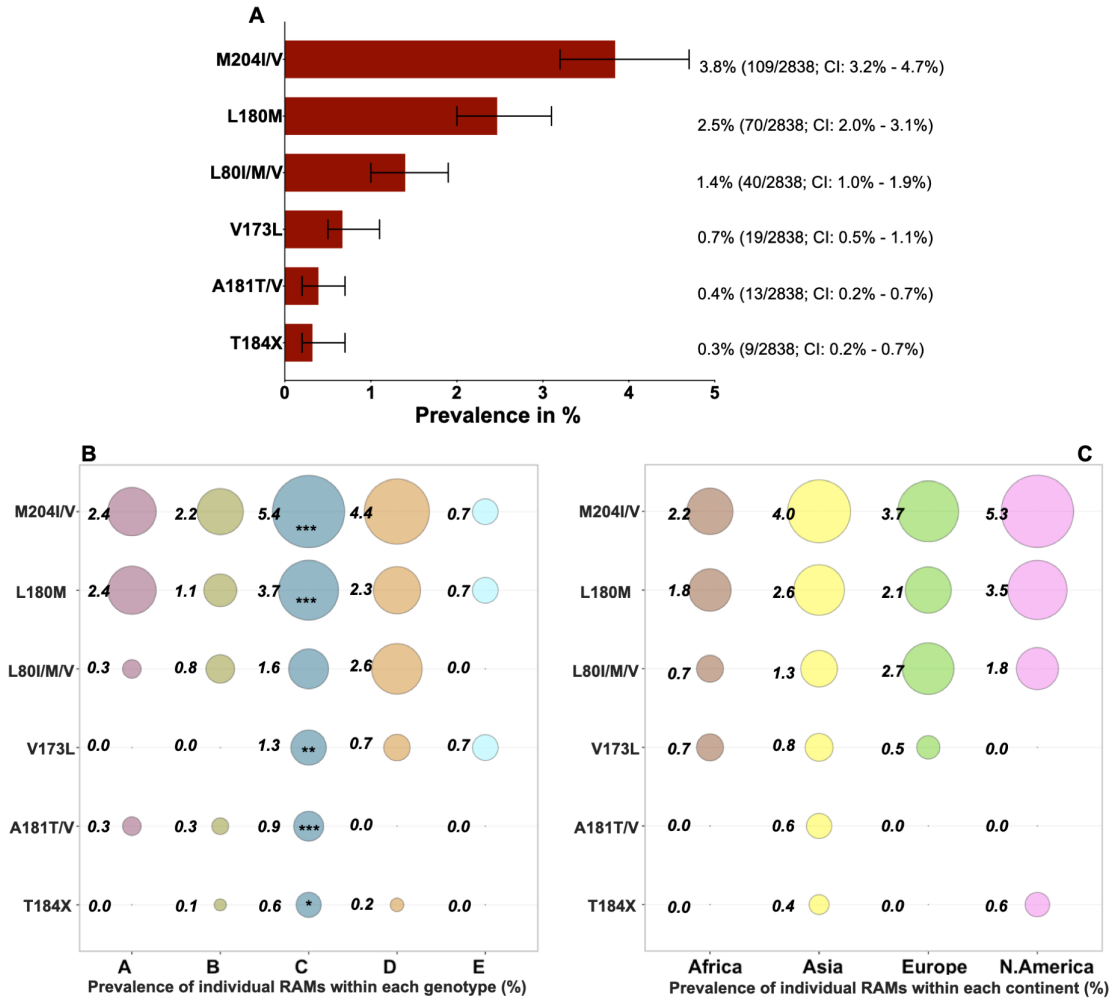


Figure 3.3: Global prevalence of hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (https://hbvdb.ibcp.fr/HBVdb/). A. Overall prevalence of RAMs common to 3TC, ETV and TFV. **B.** A bubble plot showing the overall prevalence of RAMs common to 3TC, ETV and TFV within each genotype (genotype A, n=290; Genotype B, n=730; Genotype C, n=1102; Genotype D, n=566; Genotype E, n=150). **C.** A bubble plot showing the overall prevalence of RAMs common to 3TC, ETV and TFV within each continent (Africa, n=277; Asia, n=2109; Europe, n=187; North America, n=170). Numbers next to the circles are prevalence (%) of individual RAMs in each genotype/continent. The asterisks (***/**/*) within certain circles indicate RAMs that have a higher prevalence within the specified genotype/continent compared to the prevalence of that RAM in other genotypes/continents and is statistically significant. *** p value <0.001; **p value < 0.005; *p value <0.05. Bars show 95% confidence intervals. T184X represents T184A/C/F/G/I/L/M/S.

3.2.3.1.3 RAMs associated with ETV resistance

The overall prevalence of ETV resistance in this dataset, determined by the presence of RAMs M204I/V+L180M, was 2.4% (67/2838); other combinations of ETV drug resistant mutations were uncommon (all <0.6%); **Figure 3.4**. As previously, the most common resistance mutations were seen in genotype C at 3.5% (39/1102 vs 28/1736 in other genotypes; p=0.001).

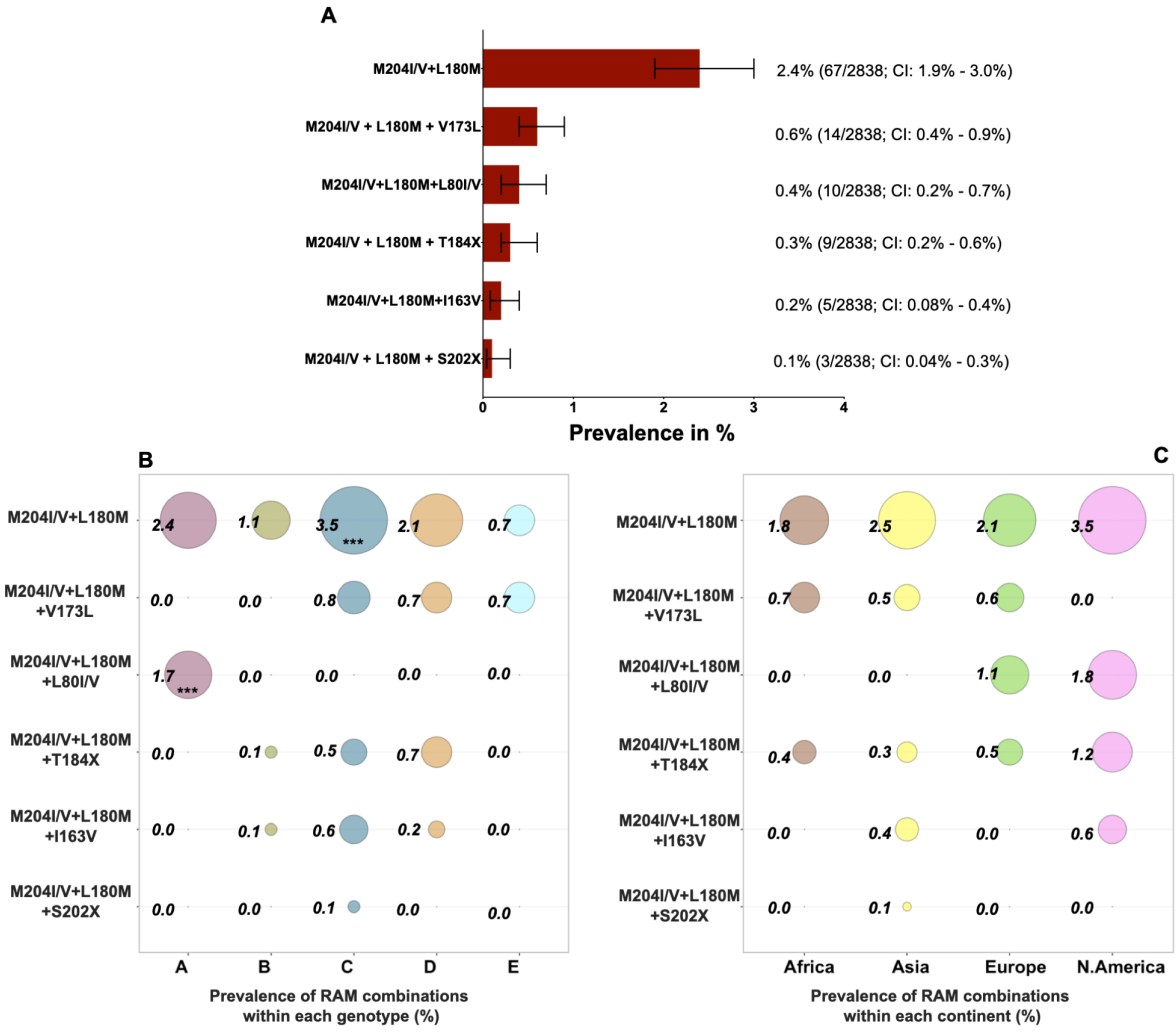


Figure 3.4: Global prevalence of hepatitis B virus (HBV) entecavir (ETV) resistance-associated mutations (RAMs) obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (https://hbvdb.ibcp.fr/HBVdb/). A. Overall prevalence of ETV RAMs. **B.** A bubble plot showing the prevalence of ETV RAMs within each genotype (genotype A, n=290; Genotype B, n=730; Genotype C, n=1102; Genotype D, n=566 and Genotype E, n=150). **C.** A bubble plot showing the prevalence of ETV RAMs within each continent (Africa, n=277; Asia, n=2109; Europe, n=187 and North America, n=170). Numbers next to the circles are prevalence (%) of individual RAMs in each genotype/continent. The asterisks (***/**/*) within certain circles indicate RAMs that have a higher prevalence within the specified genotype/continent compared to the prevalence of that RAM in other genotypes/continents and is statistically significant. *** p value <0.001; **p value < 0.005; *p value <0.05. Bars show 95% confidence intervals. T184X represents T184A/C/F/G/I/L/M/S and S202X represents S202C/G/I/R.

3.2.3.1.4 RAMs associated with TFV resistance

The prevalence of individual mutations that have been associated with TFV resistance ranged between 0.2–19.5%. Compared to all other genotypes, genotype C had the highest prevalence

of individual RAMs S106C/G, DH/N134E and I269L; and Asia had the highest prevalence of these individual RAMs S106C/G, DH/N134E and I269L compared to other continents **(Figure 3.5).**

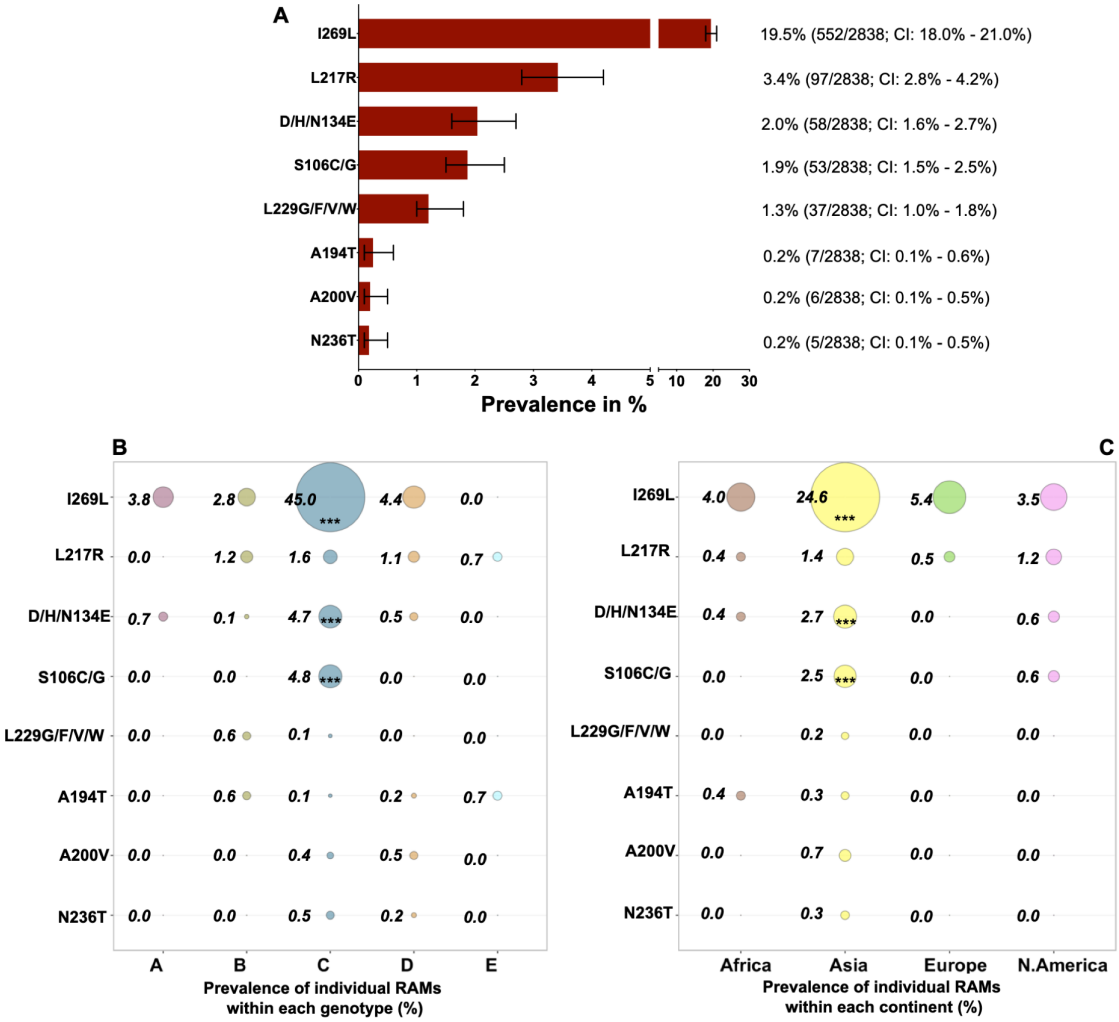


Figure 3.5: Global prevalence of hepatitis B virus (HBV) tenofovir (TFV) resistance-associated mutations (RAMs) obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (https://hbvdb.ibcp.fr/HBVdb/). A. Overall prevalence of TFV RAMs. **B.** A bubble plot showing the overall prevalence of TFV RAMs within each genotype (genotype A, n=290; Genotype B, n=730; Genotype C, n=1102; Genotype D, n=566 and Genotype E, n=150). **C.** A bubble plot showing the overall prevalence of TFV RAMs within each continent (Africa, n=277; Asia, n=2,109; Europe, n=187 and North America, n=170). Numbers next to the circles are prevalence (%) of individual RAMs in each genotype/continent. The asterisks (***/**/*) within certain circles indicate RAMs that have a higher prevalence within the specified genotype/continent compared to the prevalence of that RAM in other genotypes/continents and is statistically significant. *** p value <0.001; **p value < 0.005; *p value <0.05. Bars show 95% confidence intervals.

Sequences with certain combinations of RAMs are likely to have the highest probability of TFV resistance [Mokaya et al. 2020a; **(Chapter 2: section 2.2.3.4 (Table 2.2.2** and **Appendix Table 2.2.5**]. I therefore sought evidence of these combinations of mutations in the sequence database (n=2838). In each case, I only identified between one and three sequences with each combination of RAMs giving an overall prevalence of between 0.04–0.1% (**Table 3.3**), suggesting these arise infrequently and are unlikely to be of significance at a population level. The majority of sequences carrying these drug resistance motifs were again in genotype C.

Table 3.3: Description of sequences with individual or combination of hepatitis B virus drug resistance-associated mutations (RAMs) that are highly likely to cause resistance to tenofovir (TFV) obtained from analysing 2838 HBV sequences (Hamers et al. 2013). These RAMs combination include ≥ 1 RAMs from the 'short list' in combination with ≥ 3 other RAMs from the 'long list' as described in **chapter 2: section 2.2.3.4 (Table 2.2.2 and Appendix Table 2.2.5).**

Sequence ID	Individual and combination of mutations that are highly likely to cause resistance to TFV	No. of sequences with RAM (%)	Continent and number of sequences	Genotype and number of sequences
GQ358144–7; GQ377536; KT366495; GQ161771	A194T*	7 (0.2)	Asia n=6; Africa n=1	B, n=4; C, n=1; D, n=1, E, n=1,
FJ386681	A181T/V*+N236T	1 (0.04)	Asia n=1	B n=1
KJ803809	S106C*+D134E*+Q267L+I269L*+K333Q	1 (0.04)	Asia n=1	C n=1
FJ386579; FJ787470; FJ787471	S106C*+V173L*+L180M*+M204I/V*+Q267L	3 (0.1)	Asia n=3	C n=3
EU939588	S106C*+L180M*+A200V+M204I*+Q267L	1 (0.04)	Asia n=1	C n=1
FJ386623	S106C*+L180M*+ M204I/V*+Q267L	1 (0.04)	Asia n=1	C n=1
FJ386620; JX026877	S106C*+L180M*+ M204I/V*+I269L	2 (0.07)	Asia n=2	C n=2
AB182589	S106C*+D134E*+Q267L+I269L*+K333Q+N337H	1 (0.04)	Asia n=1	C n=1
JQ040132	D134E*+L180M*+M204I*+I267L+N337H	1 (0.04)	Asia n=1	C n=1
AY641561	D134E*+I267*L+K333Q+N337H	1 (0.04)	Asia n=1	C n=1
EU560439	D134E*+Q267L+I269L*+K333Q	1 (0.04)	Asia n=1	C n=1
JN827423	R153Q*+V173L*+L180M*+M204V*+I269L*+V278I	1 (0.04)	Asia n=1	C n=1
JQ707346	R153W*+L180M*+M204V*+V207L+L217R*	1 (0.04)	North America n=1	A n=1
FJ899789	R153Q*+Q267L+I269L*+V173L*+N337H	1 (0.04)	Asia n=1	C n=1
MF772345	R153W*+L217R*+V278I+K333Q	1 (0.04)	Africa n=1	A n=1
JN257203	R153Q*+F122L+V278I+K333Q	1 (0.04)	Africa n=1	D n=1
KX357637	V173L*+L180M*+M204V*+V207L	1 (0.04)	Asia n=1	D n=1
FJ032355	V137L+L180M+M204V+Q267L+I269L*	1 (0.04)	Asia n=1	C n=1
JN827418; JN827421; MF925409	V137L*+L180M*+M204V*+I269L*+V278I	3 (0.1)	Asia n=3	C n=3
AB697490	V137L*+L180M*+M204V*+N337H	1 (0.04)	Asia n=1	C n=1
FJ787453	V137L*+ M204V*+ Q267L+I269L*	1 (0.04)	Asia n=1	C n=1
FJ386604	L180M*+A181V*+M204V*+I269L*	1 (0.04)	Asia n=1	C n=1
JF828921	L180M*+T184L+M204V*+L229V*+Q267L+K333Q+N337H	1 (0.04)	Asia n=1	C n=1
JF828923; JF828937	L180M*+T184A/L+M204V*+ Q267L+K333Q+N337H	2 (0.07)	Asia n=2	C n=2
EU939564;	L180M*+A200V+M204I*+Q267L	1 (0.04)	Asia n=1	C n=1
FJ386653; FJ787455; FJ787456	L180M*+M204I/V*+L229V*+Q267L	3 (0.1)	Asia n=3	C n=2
JN827422; JN827424	L180M*+M204I/V*+I269L*+V278I	2 (0.07)	Asia n=2	C n=2
DQ246215	L180M*+M204I*+V278I+N337H	1 (0.04)	Asia n=1	C n=1

* RAMs provided in 'short list' described in **chapter 2: section 2.2.3.4 (Table 2.2.2)**; these RAMs are supported by the highest quality evidence (i.e. isolated from treatment compliant individuals in whom viraemia was not suppressed by TFV and these RAMs were also tested in *in vitro* assays to measure the effect of TFV on viral replication in cell lines.

3.2.3.2 Global prevalence of HBV VEMs

I assessed for the prevalence of polymorphisms associated with vaccine/immune escape across 31 different sites within surface protein in a total of 2838 full length HBV sequences; I used a list of VEMs provided in **Table 3.2**. In this dataset, 74% (23/31) of the sites had polymorphisms associated with vaccine/immune escape (**Figure 3.6).**

61% (14/23) of the sites had polymorphisms associated with vaccine escape occurring at the prevalence of <9% in all genotypes and all continents. Genotype C and Asia had the highest number of sites with polymorphisms associated with vaccine escape occurring at the prevalence of >20%, compared to other genotypes and continents **(Figure 3.6)**.

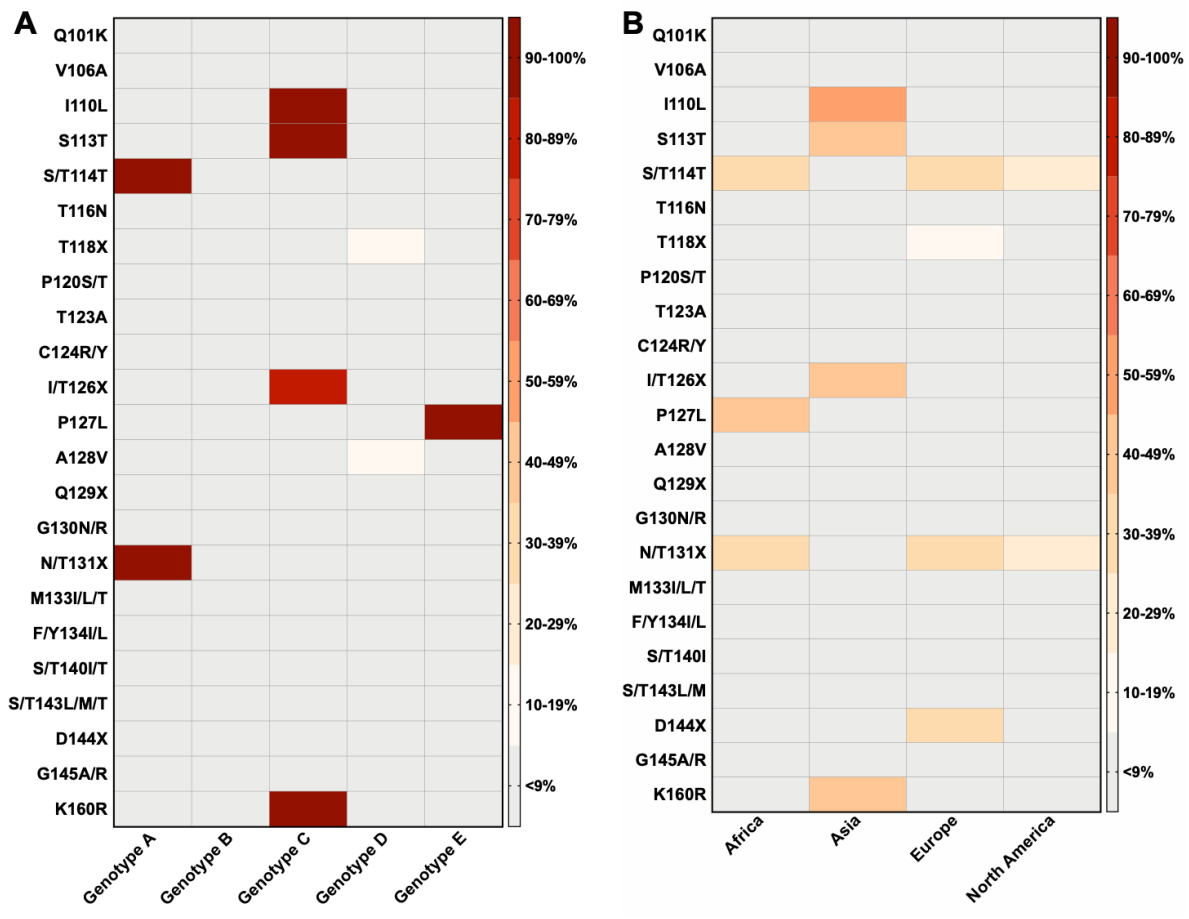


Figure 3.6: Global prevalence of hepatitis B virus (HBV) vaccine escape mutations (VEMs) across genotypes, obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (Hamers et al. 2013). A. Prevalence of polymorphisms across genotypes. **B.** Prevalence of polymorphisms across continents.

VEM K141E/I/R was not present in our dataset. G145A/R had an overall prevalence of 1.3% (37/2838) and had the highest prevalence in genotype C at 2.2% (24/1102; p=0.002), and in Asia at 1.6% (34/2109; p=0.009); this is the best recognised VEM (**Figure 3.7A).** Other VEMs that had an overall prevalence of >1% were T118A/R/V, M133I/L/T, A128V, Q129H/N/R, G145A/R, P120S/T and S/T143L/M (**Figure 3.7A).** T118A/R/V, A128V and S/T143L/M had the highest prevalence in genotype D and in Europe, being present >3% of the sequences; whereas VEMs M133I/L/T and Q129H/N/R had the highest prevalence in genotype B and M133I/L/T had the highest prevalence in Asia, also being present in >3% of the sequences **(Figure 3.7B** and **Figure 3.7C).**

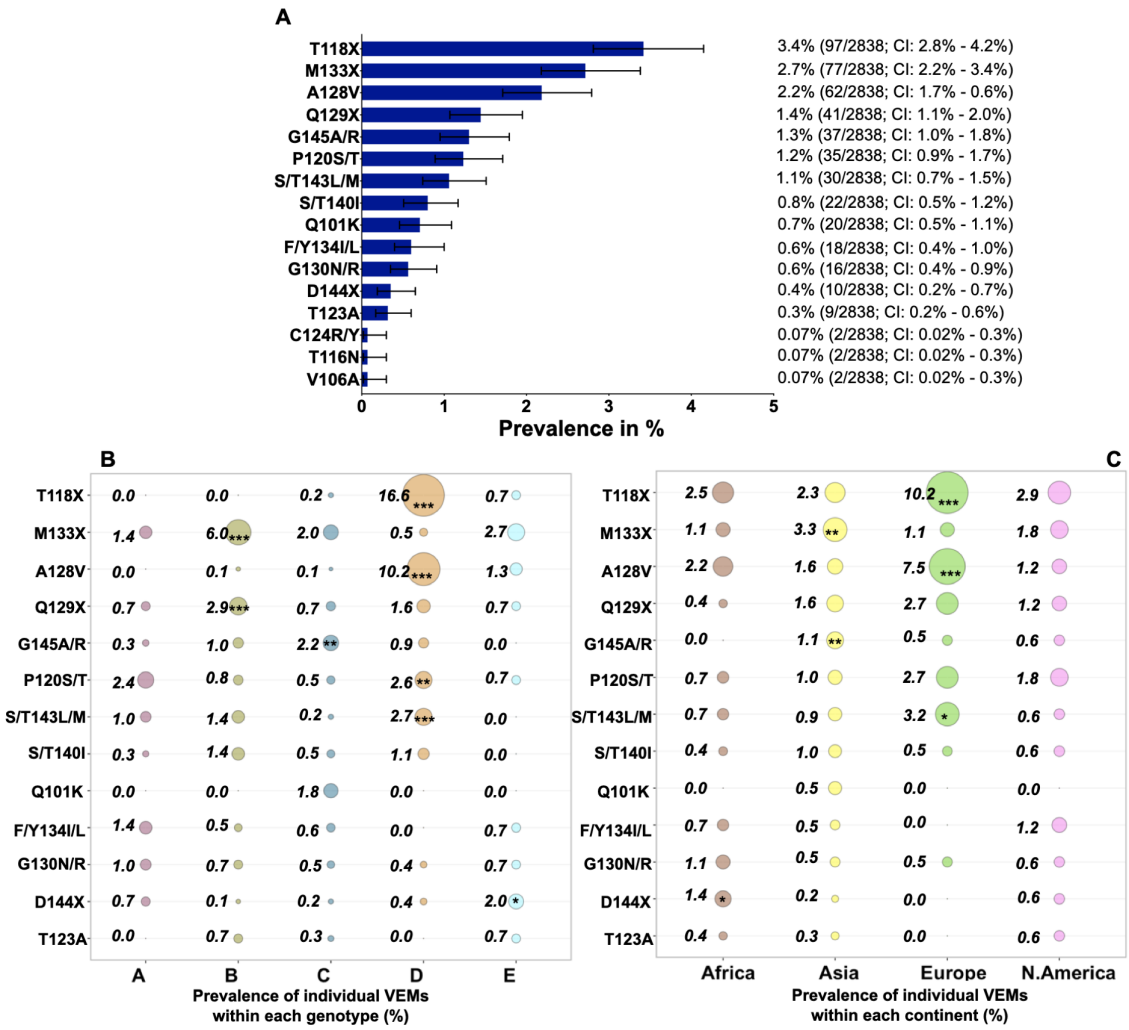


Figure 3.7: Global prevalence of hepatitis B virus (HBV) vaccine escape mutations (VEMs) obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (https://hbvdb.ibcp.fr/HBVdb/). A. Overall prevalence of putative VEMs and/or VEMs with only clinical or *in vitro* evidence. **B.** A bubble plot showing the overall prevalence of putative VEMs and/or VEMs with only clinical or *in vitro* evidence within each genotype (genotype A, n=290; Genotype B, n=730; Genotype C, n=1102; Genotype D, n=566 and Genotype E, n=150), with a prevalence of >0.1%. **C.** A bubble plot showing the overall prevalence of putative VEMs and/or VEMs with only clinical or *in vitro* evidence within each continent (Africa, n=277; Asia, n=2109; Europe, n=187 and North America, n=170), with a prevalence of >0.1%. Numbers next to the circles are prevalence (%) of individual RAMs in each genotype/continent. The asterisks (***/**/*) within certain circles indicate RAMs that have a higher prevalence within the specified genotype/continent compared to the prevalence of that RAM in other genotypes/continents and is statistically significant. *** p value <0.001; **p value <0.005; *p value< 0.05. T118X represents T118A/R/V. M133X represents M133I/L/T. Q129X represents Q129A/R. D144X represents D144A/E/G/N.

3.2.3.3 RAMs and VEMs present as wildtype amino acid in reference sequences of genotypes A–E

Determining the clinical significance of individual RAMs/VEMs in HBV sequences is difficult because some of the mutations that have been described occur at consensus level in reference sequences of some genotypes**.** For example, 12 polymorphisms associated with drug resistance and nine polymorphisms associated with vaccine escape had a prevalence of >50% in ≥ 1 genotype (s), **Table 3.4** and **Table 3.5.** RAMs H126Y and R/W153W, which contribute to TFV resistance when combined with ≥ 3 other RAMs [Mokaya et al. 2020a; **chapter 2: section 2.2.3.4 (Appendix Table 2.2.5)**], are both wildtype in genotype A. This shows that resistance to different drugs or HBV vaccine might be more easily selected in certain populations or regions, based on the global distribution of HBV genotypes.

Table 3.4: Global prevalence of hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) that are wildtype amino acid. Prevalence rates were obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (Hamers et al. 2013). A. Identification of RAMs as wildtype in certain genotypes using HBV reference sequences for genotypes A–J. **B.** Prevalence of RAMs that are wildtype amino acid across genotypes. **C.** Prevalence of RAMs that are wildtype amino acid across continents. HBV reference sequences were obtained from a published manuscript (McNaughton et al. 2020a).

			HBV RAMs that are wildtype in certain genotypes											
	Genotype	**Reference sequence accession number**	**H/Y9H**	**X53N**	**I/L91I**	**H/Y126Y**	**X153X**	**F/Y221Y**	**A/S223S**	**C/S256S**	**E/D263E**	**X267L**	**A/S317S**	**X333Q**
A	A	FJ692557	H	-	I	Y	W	Y	A	S	-	-	-	-
	B	GU815637	H	N	-	-	-	Y	A	S	E	-	-	-
	C	GQ377617	H	-	I	-	-	-	-	S	E	L	S	-
	D	KC875277	H	N	-	-	-	-	A	-	-	-	S	-
	E	GQ161817	H	-	-	Y	-	Y	-	S	E	-	S	-
	F	HM585194	H	N	-	-	-	Y	A	S	-	-	-	Q
	G	AB056513	H	-	I	Y	-	Y	A	S	E	-	-	-
	H	FJ356715	H	-	-	-	-	Y	A	S	E	-	-	-
	I	AB562463	H	-	-	Y	Q	Y	A	S	-	-	-	Q
	J	AB486012	-	-	-	-	-	Y	A	S	-	-	-	-

Genotypes

B	Genotype A												
	Genotype B												
	Genotype C												
	Genotype D												
	Genotype E												

Continents

C	Africa												
	Asia												
	Europe												
	North America												

Key

81–100%	
61–80%	
41–60%	
21–40%	
0–20%	

X153Q/W represents Q/R/W153Q/W. X53N represents V/N/S/T53N. X333Q represents K/Q/T333Q.

Table 3.5: Global prevalence of hepatitis B virus (HBV) vaccine escape mutations (VEMs) that are wildtype amino acid. Prevalence rates were obtained from analysing 2838 HBV sequences with information on country of origin, downloaded from a public database (Hamers et al. 2013). **A.** Identification of VEMs as wildtype in certain genotypes using HBV reference sequences for genotypes A–J. **B.** Prevalence of VEMs that are wildtype amino acid across genotypes. **C.** Prevalence of VEMs that are wildtype amino acid across continents. HBV reference sequences were obtained from a published manuscript (McNaughton et al. 2020a).

HBV RAMs that are wildtype in certain genotypes

	Genotype	Reference sequence accession number	I110L	S113T	S/T114T	I/T126I	P/L127L	N/T131N	S/T140T	S/T143T	X160R
A	A	FJ692557	I	-	T	-	-	N	T	T	-
	B	GU815637	I	-	-	-	-	-	T	T	-
	C	GQ377617	L	T	-	I	-	-	T	-	R
	D	KC875277	I	-	-	-	-	-	T	-	-
	E	GQ161817	I	-	-	-	L	-	-	-	-
	F	HM585194	L	-	T	-	L	-	-	-	-
	G	AB056513	I	-	-	-	-	N	T	-	-
	H	FJ356715	L	-	T	-	L	-	T	-	-
	I	AB562463	I	-	-	-	-	N	T	-	-
	J	AB486012	L	-	T	I	-	-	T	-	-

Genotypes

B	Genotype A									
	Genotype B									
	Genotype C									
	Genotype D									
	Genotype E									

Continents

C	Africa									
	Asia									
	Europe									
	North America									

Key

81–100%	
61–80%	
41–60%	
21–40%	
0–20%	

X160R represents K/R160R.

3.2.3.4 Distribution of selected RAMs and VEMs on maximum likelihood phylogenetic trees

I considered the distribution of 12 RAMs (S106C/G, D134E, R153W/Q, V173L, L180M, A181T/V, A194T, A200V, M204I/V, L217R, L229V/W, I269L) and eight VEMs (C139S, S/T140I, P142S, S/T143L/M, D144A/E/G/N, G145A/E/R, K141A/I/R and C147S) across the branches of ML phylogenetic trees. Most of these RAMs and all VEMs were randomly distributed across the branches of phylogenetic trees reconstructed from genotypes A–E sequences, suggesting parallel evolution.

However, there were several RAMs that clustered within genotype B, C and D sequences (**Figure 3.8)**. In genotype B, all sequences containing the A194T variant clustered together (Bayes factor, BF, support >100; n=4 sequences). Sequences with this RAM were all from Indonesia, reported by a study exploring HBV genetic diversity (Thedja et al. 2011). Some sequences containing both M204V and L180M formed a cluster in genotype B (BF = 54.99, n=4 sequences) and some with M204I formed a cluster in genotype D (BF >100, n=3 sequences). In genotype C, there were clusters of RAMs S106C (BF >100, n=5 sequences), R153Q (BF >100, n=3 sequences), and I129L (BF >100, n=34 sequences). Clustering of sequences with RAMs might suggest an emerging sublineage of treatment resistant virus.

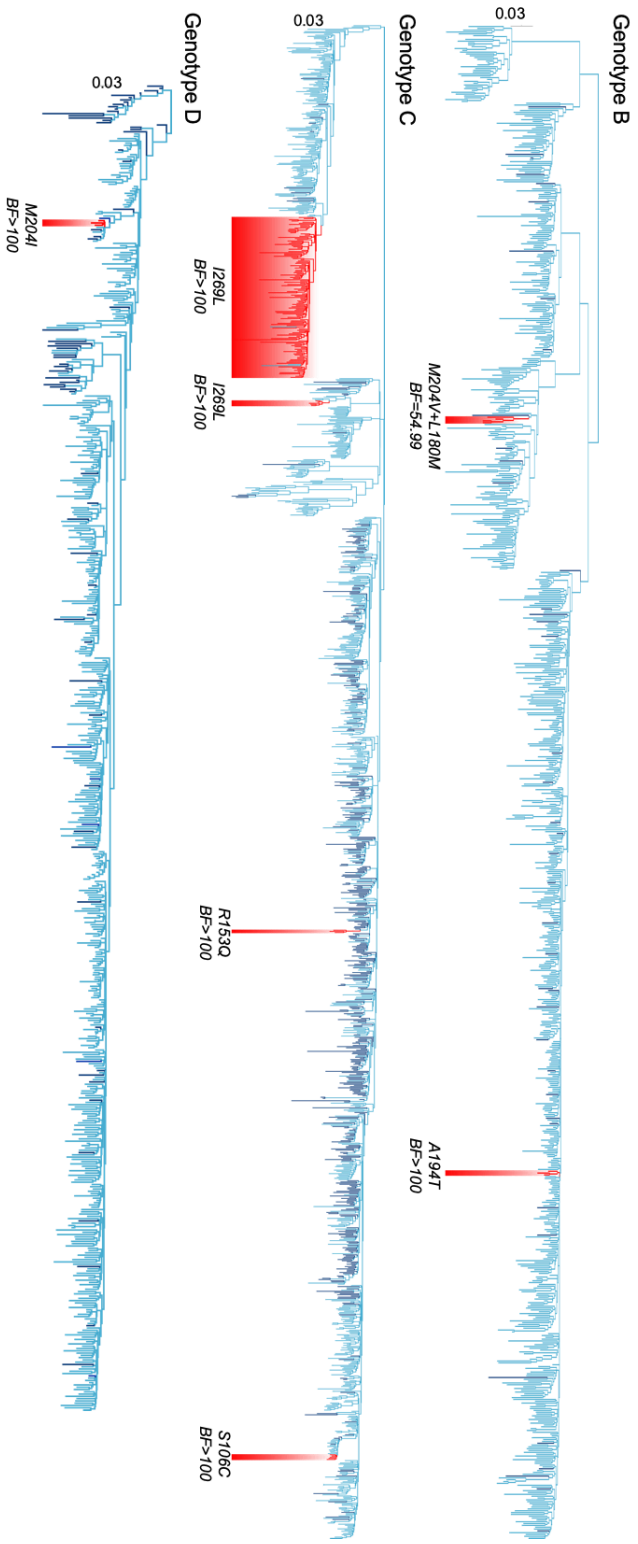


Figure 3.8: Hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) distribution on rooted maximum likelihood phylogenetic trees for genotypes B, C and D. Branches in dark blue represent sequences with one or more RAMs/VEMs. Branches in light blue have no specified RAMs/VEMs. Branches highlighted in red indicate clustered sequences with a RAM with Bayes factor of >30, suggesting strong evidence of clustering. ML trees for genotype A and E were not displayed because they had no sequences with specified RAM/VEM which formed clusters. Phylogenetic trees were rooted in FigTree v.1.4.4 (Suchard et al. 2018).

3.2.3.5 Evolution of sequences with HBV RAMs and/or VEMs

Most sequences with RAMs/VEMs in this analysis were published after the approval of NAs/HBV vaccine, as a result of widespread improvements and availability of sequencing that have arisen in parallel with roll out of drugs and vaccine. However, four sequences (KF214668, KF214671, KF214673 and KF214676) with RAM I269L and one sequence (KF214659) with VEM S/T143M were sampled from Asia in 1963, and one sequence (HQ700441) with RAM L180M was sampled from Oceania in 1984, demonstrating that mutations can arise without exposure to treatment or vaccination.

I performed ML molecular clock analysis for full datasets of sequences of genotypes A–E. However, only genotype C had clusters that had at least two isolates with the same resistant mutations with a single common ancestor as shown in **Table 3.6**. The estimated time of emergence of branches with RAMs M204V+L180M was around year 1945 [95% highest posterior density (HPD) 1897 - 1971]; and branches with VEM G145R was estimated to emerge around year 1930 (95% HPD 1866 - 1958). Importantly, in both cases the higher bound of the 95% HPD interval of the TMRCA of these clusters, which likely correspond to the lower bound of the estimate of the age of these mutations, precedes the years NAs and the HBV vaccine were introduced; these were 1998 (for 3TC), 2005 (for ETV), 2008 (for TFV) and 1981 (for HBV vaccine). The results I obtained from ML molecular clock analysis and BEAST analysis were consistent.

Table 3.6: Estimated time of the most common recent ancestor (TMRCAs) and [95% highest posterior density (HPD)] of branches with specific hepatitis B virus drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) on molecular clock trees. Only genotype C is displayed because it had at least two isolates with the same RAM with a single common ancestor.

Genotype	RAMs/VEMs	Cluster of isolates with specified RAMs/VEMs	Estimated TMRCAs (95%HPD)
C	M204V+L180M	FJ032355	1945 (1897 - 1971)
		FJ386620	
	G145R	KU964229	1930 (1866 - 1958)
		KU964230	

3.2.4 Discussion

3.2.4.1 Novel findings and comparison with previous literature

I describe the global prevalence of drug and vaccine resistance in HBV across genotypes and geographical regions and explore the evolution of these mutations using phylogenetic analysis, in order to provide a high-resolution picture of the origins and distribution of drug and vaccine resistance. From this analysis, HBV drug and vaccine resistance are not common, with the highest frequency of individual or combined mutations that are well known to cause resistance, being ~4% and the majority being <1%. These mutations are distributed across various continents and genotypes, with the most frequent RAMs/VEMs identified in genotype C, concordant with previous studies from China (Li et al. 2017; Zhang et al. 2019). I show that these mutations are not only driven by exposure to drug or vaccine but are likely to have been present in some sequences for hundreds of years. More studies representing all genotypes are needed, alongside careful correlation with clinical evidence of drug resistance.

M204I/V is one of the best recognised drug resistance motifs in HBV and had the highest overall prevalence of 3.8% within this whole sequence database. A previous meta-analysis estimated the prevalence of M204I/V as 4.9% among >12000 treatment-naïve individuals (Zhang et al. 2015), and another review reported a prevalence of M204I/V of 5.9% among 8435

treatment-naïve individuals (Choi, Lee and Kim, 2018). These reviews reported prevalence as the proportion of individuals with mutations within the total number of treatment naïve individuals, without accounting for closely related sequences (thus may include multiple sequences from a single individual). In contrast, I used full length HBV sequences and excluded identical sequences, which might explain the lower prevalence I report.

I reported the prevalence of ETV resistance as 2.4%, which is slightly higher than the prevalence of 1.7% reported from a large survey carried out in China among 1223 treatment experienced patients and also a prevalence of 1.2% reported from a longitudinal study that followed 108 HBV infected treatment naïve individuals for five years (Tenney et al. 2009; Meng et al. 2017). Unlike these two studies, I took a lenient approach by reporting the overall prevalence of ETV resistance considering sequences with RAMs M204I/V+L180M, with or without an additional compensatory mutation. These two are always present in ETV resistant variants and are the main ETV RAMs reported in published HBV treatment guidelines (WHO, 2015; EASL, 2017).

I estimated the overall prevalence of TFV resistance to be between 0.04–0.2%. There have been few studies that have reported on TFV resistance (Mokaya et al. 2020a; **chapter 2: section 2.2**) and more robust data are still needed to define HBV resistance in order to guide better estimation of the prevalence of relevant RAMs.

The global prevalence of the VEM G145A/R in this analysis data was 1.3%, which is comparable to that of 0.3–1% previously reported across genotypes A–F (Q, Ma and Wang, 2012). A study carried out in Italy reported a higher prevalence of 3.1%, in a cohort dominated by genotype D infection (Sticchi et al. 2013). Regional differences might explain the difference in prevalence. However, the majority of individuals with this mutation from the Italian study were immunocompromised and it was not clear if they had been vaccinated prior to becoming infected.

3.2.4.2 Relationship between genotype and HBV drug and/or vaccine resistance

The prevalence of RAMs/VEMs across different regions is influenced by the predominant genotype, but may also relate to different patterns of drug or vaccine exposure in the population. For example, T118A/R/V, A128V and D144A/E/G/N variants are more common in Europe **(Figure 3.7),** which may relate to better vaccine coverage (WHO, 2017b) that drives the selection of resistant variants.

Currently, HBV genotyping is not routinely undertaken in clinical practice, so it is difficult to amass data for any potential relationship between resistance and viral genotype. However, some polymorphisms that have been described in association with resistance are wildtype in certain genotypes, which might indicate that these genotypes may be more susceptible to the development of clinically significant drug resistance. For example, TFV resistance might be selected more easily in genotype A given that TFV RAMs H126Y and R/W153W are wildtype in this genotype (**Table 3.4**). The identification of Y9H and C256S as a TFV RAMs (see **chapter 2: Appendix Table 2.2.5**), should be viewed with caution as H9 is frequently the wildtype residue in all genotypes except in genotype J and S256 is frequently the wildtype residue in all genotypes except in genotype D (**Table 3.4**). There is a pressing need for more clinical data combined with *in vitro* data from assays that can confirm a resistant phenotype.

3.2.4.3 Phylogenetic analysis of selected RAMs and VEMs

I provide evidence that RAMs can arise without exposure to treatment/vaccine, showing that certain RAMs emerged prior to the NAs and vaccine era. Using molecular clock analysis, I estimate that RAMs M204V and L180M, and VEM G145R were already present around the mid 20th century. Although these estimates have wide confidence intervals, their upper bounds precede the time of introduction of NAs and the HBV vaccine. A previous study estimated the origin date of HBV genotype D in Iran as 1894 (95% HPD 1701 - 1957) (Mozhgani et al. 2018), and the root age of genotype A polymerase sequences is estimated as the year 955 (95% HPD 381 - 1482) (Zehender et al. 2015). A study that analysed 167 full length genotype E sequences,

estimated the TMRCA to be 174 years (95% HPD 36 - 441) (Andernach, Hunewald and Muller, 2013). Similar to my analysis, these studies used an uncorrelated relaxed lognormal clock which is reported to the best fitting clock (Andernach, Hunewald and Muller, 2013; Zehender et al. 2015; Mozhgani et al. 2018). However, given the differences in the evolutionary rates and substitution models, with some studies using sequences for just a single gene, direct comparison of the estimated TMRCA generated by these studies and with estimates from this analysis is challenging.

3.2.4.4 Selection vs transmission of HBV RAMs

Most RAMs were randomly distributed across the branches of HBV phylogenetic trees, which suggests that these polymorphisms are being selected independently in individual hosts [parallel evolution (Gutierrez, Escalera-Zamudio and Pybus, 2019)] rather than becoming fixed and disseminated from a founder strain. The high viral replication and mutation rate of HBV can result in amino acid substitutions at sites of resistance, leading to the stochastic emergence of drug RAMs even in individuals who have not been exposed to treatment (Zoulim and Locarnini, 2009; Khudyakov, 2010; Thai et al. 2012). Individuals can also be infected with HBV strains containing drug RAMs which could significantly comprise virological response to therapy, as has been shown in HIV (Hong et al. 2011).

3.2.4.5 Caveats and limitations

The major constraint in this work is the relative lack of HBV sequence data; given the huge global burden of infection there is a striking lack of high-quality sequence data available in the public domain. I obtained sequences for this analysis from GenBank and metadata on individual characteristics and treatment exposure were not available. These analyses may not be representative, given the biased nature of sequence data that are available, disproportionately representing certain populations and regions, and samples containing high VL (McNaughton et al. 2019c). Drug resistant sequences may be over-represented, given that virus suppressed by

drug therapy is not accessible for sequencing and individuals with break-through viraemia on treatment are more likely to have samples submitted for sequence analysis.

Molecular clock analysis in HBV is challenging. Its ORFs raises controversies around its evolution rates. HBV sequences lack temporal signal thus making it challenging to reliably date HBV evolution using molecular clock methods. In addition, estimation of TMRCA uses sample collection dates obtained from GenBank, which may not be accurate.

While Asia and Africa are known to have the highest prevalence of CHB infection worldwide at 6.2% and 6.1%, respectively (WHO, 2016a), 74% (2109/2838) of sequences included in this analysis were from Asia and only 10% (277/2838) published from Africa. This low representation of sequences from Africa highlights HBV as a neglected disease, with very few individuals diagnosed and linked to care (WHO, 2016a; O'Hara et al. 2017). In addition, the influence of the widespread use of ART containing 3TC and TFV, on suppression and/or emergence of drug resistance is not yet understood.

For evolutionary analysis, I received supervision input from Dr. Tetyana Vasyleva, Dr. Azim Ansari and Prof. Oliver Pybus.

Chapter four: Optimisation of HBV amplification for sequencing in low VL samples

4.1 Background

Our research group is developing NGS approaches for HBV sequencing, however, these approaches have proven challenging for low VL samples. Furthermore, more than 50% of serum samples have HBV DNA VL <4 $\log_{10}$ IU/ml (Downs et al. 2020) (see **chapter 1: section 1.14**). I have therefore set out to optimise selected published nested PCR assays to improve their sensitivity for low VL samples, while ensuring full coverage of both the polymerase and surface HBV proteins, which were my focus of interest as the locations in which RAMs and VEMs most commonly arise.

4.2 Methods

4.2.1 Samples: Oxford viral hepatitis project

Adults with CHB have been recruited into a cross-sectional cohort based at the Oxford University Hospitals NHS Foundation Trust, UK. Ethics approval for this project was provided by the University of Oxford Tropical Research Ethics Committee (ref: OXTREC 01-18) and NIHR Oxford Biomedical Research Centre Ethics Committee (ref: BRC REC 09/H0604/20).

Samples for sequencing were selected in May 2019, at which time, 295 individuals had been recruited. Their pseudonymised descriptive and clinical data were stored in a database (LabKey). Serum samples obtained from the study participants were processed and stored at -80°C freezers at laboratories located at Peter Medawar Building.

I selected 22 samples that were used to optimise Sanger sequencing sensitivity. I chose these samples to represent a range of VL levels and because adequate plasma volume was available.

However, the VL levels of three of these samples were unknown; I included them because they had adequate plasma volume and I later quantified their VL levels as described in **section 4.2.3.**

To these 22 samples, I added another one sample (Khep002) obtained from a HIV/HBV coinfected child recruited from a study that set out to investigate HBV prevalence in an HIV-positive paediatric cohort in Kimberley Hospital, South Africa. This study was approved under the terms of the Ethics Committee of the Faculty of Health Science, University of Free State, Bloemfontein, South Africa (ref. ETOVS Nr 08/09).

HBV genotypes of these 23 samples were unknown.

4.2.2 HBV DNA extraction

I extracted HBV DNA from these 23 samples using NucliSENS magnetic extraction system (BioMérieux). For each of the 23 samples, HBV DNA was extracted from 1 ml of serum sample and eluted in 25 ul of elution buffer.

4.2.3 Quantifying HBV VL levels

Of the 23 samples selected for optimising Sanger sequencing, 19 samples had HBV VL levels quantified at the clinical diagnostic laboratory at Oxford University Hospitals NHS Foundation Trust, whereas HBV VL of four samples (1291, 1220, 1346 and KHep002) were unknown. I used a published protocol (Ghosh et al. 2016) to quantify the VL of these samples using an in-house quantitative real time polymerase chain reaction (qPCR). **Table 4.1** provides qPCR protocol used to quantify HBV DNA VL.

Table 4.1: In-house quantitative real time polymerase chain reaction assay protocol obtained from published study (Ghosh et al. 2016).

In-house quantitative real time polymerase chain reaction assay protocol	
Primers (5' – 3')	Sense: AGGAGTTCCGCAGTATGG Antisense: GCTGACGCAACCCCCACT N/B: These primers are specific to HBV polymerase gene
20-ul Reaction mixture	• 1 ul of 10uM forward primer • 1 ul of 10uM reverse primer • 10 ul of SYBR Green mix • 6 ul of Nuclease free water • 2 ul of DNA template
Reaction profile in LightCycler 480 Real-Time PCR system	• Step 1: 95°C for 10 minutes • Step 2: 95°C for 15 seconds • Step 3: 60°C for 60 seconds • Repeat steps 2 and 3, 45 times • Acquisition temperature set at 60°C

A: Adenine. C: Cytosine. G: Guanine. T: Thymine. PCR: Polymerase Chain Reaction.

I used the 4th WHO International Standard for HBV which had a VL of 955,000 IU/ml (5 $\log_{10}$) as a control. I extracted DNA from this control using NucliSENS magnetic extraction system (BioMérieux) and then diluted the extracted DNA to form a dilution series of 4 $\log_{10;}$ 3 $\log_{10;}$ and 2 $\log_{10.}$

I performed qPCR on the control dilution series and on HBV samples using the protocol detailed in **Table 4.1** to obtain Cycle threshold (Ct) values. Ct values of the controls (including the dilutions) and their estimated VLs were used to formulate the equation (y = 5E+11e-0.585x), which was then used to calculate the HBV DNA VL for the four samples (1291, 1220, 1346 and KHep002) as shown in **Figure 4.1.**

A

Samples	Ct value obtained from in-house qPCR assay	Estimated HBV VL (IU/ml)
Control (5 $\log_{10}$)	22.6	955,000
Control (4 $\log_{10}$)	26.4	95,500
Control (3 $\log_{10}$)	30.4	9,550
Control (2 $\log_{10}$)	34.4	950
1291	40.0	34.39
1220	16.2	38296686.5 (7.58 $\log_{10}$)
1346	19.87	4474583.96 (6.65 $\log_{10}$)
KHep002	19.9	5563962.63 (6.75 $\log_{10}$)

B

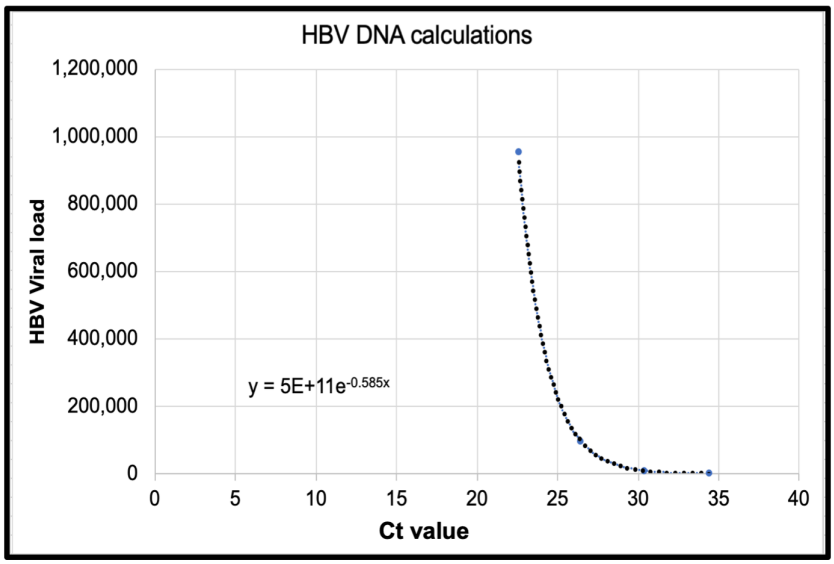


Figure 4.1: Hepatitis B virus (HBV) viral load (VL) quantification using in-house quantitative polymerase chain reaction (qPCR) assay and WHO International control standard for HBV DNA. A. Cycle threshold (Ct) values for the 4th WHO International control standard for HBV DNA (including its dilution series: 4 $\log_{10}$, 3 $\log_{10}$ and 2 $\log_{10}$) and four HBV samples [1291, 1220 and 1346 (from the Oxford viral hepatitis project)], [KHep002 (from Kimberley hospital in South Africa)], obtained from performing qPCR using an in-house assay. **B.** Ct values of the controls and their estimated VL were used to formulate the equation (y = 5E+11e-0.585x), which was then used to estimate VL for samples (1291, 1220, 1346 and KHep002).

Table 4.2: Hepatitis B virus (HBV) viral load (VL) levels of a bank of serum samples, from which selected samples were used for optimising Sanger sequencing sensitivity. HBV samples were obtained from 23 individuals infected with chronic HBV (CHB) recruited in Oxford (22 samples) and South Africa (one sample).

Sample ID (PMB)	HBV VL (IU/ml)	HBV VL in log scale (IU/ml)	Recruitment site	Ethnicity
462	32	1.50	Oxford, UK	Not known
1291	34	1.53	Oxford, UK	Not known
1402	36	1.56	Oxford, UK	Not known
1176	298	2.47	Oxford, UK	Black / African / Caribbean / Black British - African
1223	120	2.08	Oxford, UK	Black / African / Caribbean / Black British - African
1226	328	2.52	Oxford, UK	Arab
1242	426	2.63	Oxford, UK	Not known
1219	892	2.95	Oxford, UK	Black / African / Caribbean / Black British - African
1249	1291	3.11	Oxford, UK	Not known
1325	3839	3.58	Oxford, UK	White - Any Other White background
1337	5872	3.77	Oxford, UK	Not known
1243	6219	3.79	Oxford, UK	Asian / Asian British - Any other Asian background
1363	15106	4.18	Oxford, UK	Black / African / Caribbean / Black British - African
1365	15675	4.20	Oxford, UK	Asian / Asian British - Any other Asian background
1318	353288	5.55	Oxford, UK	Asian / Asian British - Chinese
1399	1209030	6.08	Oxford, UK	Asian / Asian British - Chinese
1346	4474583	6.65	Oxford, UK	Asian / Asian British - Chinese
KHep002	5563962	6.75	Kimberley, South Africa	Black / African / Caribbean / Black British - African
1220	38296686	7.58	Oxford, UK	Asian / Asian British - Chinese
715	170000000	8.23	Oxford, UK	Asian / Asian British - Chinese
1332	170000000	8.23	Oxford, UK	Black / African / Caribbean / Black British - African
1331	170000000	8.23	Oxford, UK	Not known
1348	170000000	8.23	Oxford, UK	Not known

PMB: Peter Medawar Building.

For Sanger sequencing optimisation, I used a selection of these samples based on the availability of sufficient volume.

4.2.4 Whole genome nested polymerase chain reaction (PCR) assays

I selected two published whole genome nested PCR assays (Chook et al. 2015; Wang et al. 2017), hereafter defined as assay one and two, to test on the selected HBV DNA samples. I selected these nested PCR assays as they have been reported to successfully amplify DNA from samples with HBV VL as low as 3 $\log_{10}$ IU/ml.

Table 4.3: Summary of two published whole genome nested polymerase chain reaction assays tested on selected Hepatitis B virus (HBV) samples and modified to improve sequencing of low viral load (VL) samples.

Nested PCR assay	Title of manuscript	Number of primer pairs	Number of fragments generated	Range of HBV DNA VL tested	Reference
Assay one	Universal Primers for Detection and Sequencing of Hepatitis B Virus Genomes across Genotypes A to G	4 primer pairs (1st PCR reaction) 6 primer pairs (2nd PCR reaction)	Six	3.8–6.1 log_{10} IU/ml.	Chook et al. 2015
Assay two	Integrating Nested PCR with High-Throughput Sequencing to characterise Mutations of Hepatitis B Virus Genome in Low Viral Load Samples	2 primer pairs (1st PCR reaction) 2 primer pairs (2nd PCR reaction)	Two	3.0–6.0 log_{10} IU/ml	Wang et al. 2017

a. Assay one

A nested PCR assay using universal primers (designed using sequences of 5154 HBV genomes of genotypes A to I) (Chook et al. 2015) were tested on nine HBV DNA samples (1331, 1332, 1399, 1365, 1348, 1346, 1402, 1291 and Khep002). This assay generates six overlapping fragments covering the entire HBV genome as shown in **Figure 4.2. Table 4.4** provides details on primers, reaction mixture and thermal cycle profile for this assay.

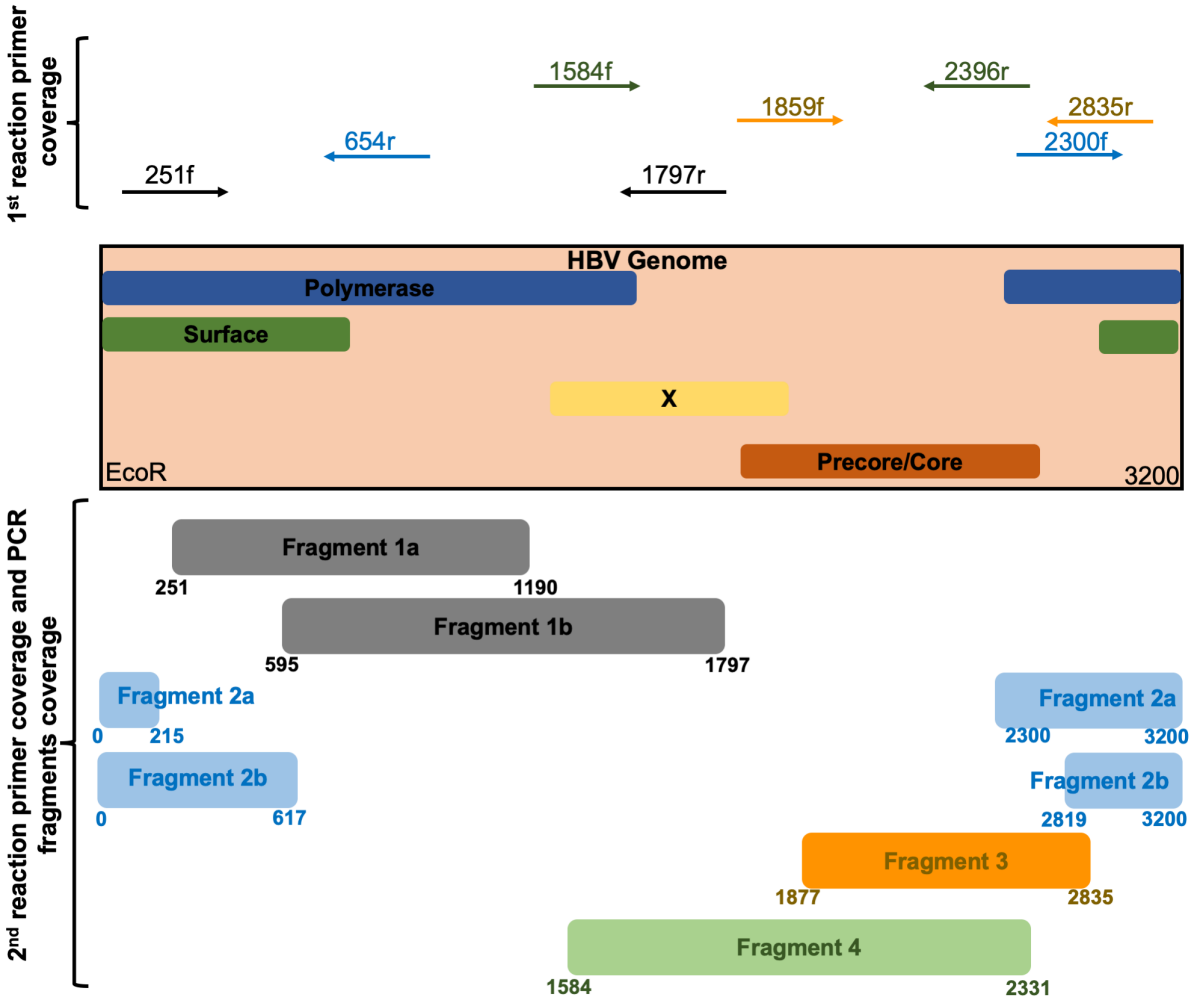


Figure 4.2: Primers and polymerase chain reaction (PCR) fragment coverage across hepatitis B virus genome. Primers were obtained from a published nested PCR assay (Chook et al. 2015). 1st PCR reaction generates four outer fragments using four pairs of primers: set 1 (251f & 1797r), set 2 (2300f & 654r), set 3 (1859f & 2835r) and set 4 (1584f & 2396r). 2nd PCR reaction uses outer fragments generated in 1st reaction to generate six inner fragments using six pairs of primers. Set 1 in the 1st reaction generates fragment 1a using primer set (251f & 1190r) and fragment 1b using primer set (595f & 1797r). Set 2 in the 1st reaction generates fragments 2a using primer set (2300f & 215r) and 2b using primer set (2819f & 617r). Set 3 in the 1st reaction generates fragment 3 using primer set (1877f & 2835r). Set 4 in the 1st reaction generates fragment 4 using primer set (1584f & 2331r).

Table 4.4: Primers, reaction mixture and thermal cycle profile for assay one (published by Chook et al. 2015).

1st PCR reaction generating four fragments using four pairs of primers		
Primers (5' – 3')	1st pair *54°C	251f: *GAC TYG TGG TGG ACT TCT C* 1797r: *CCA ATT TMT GCY TAC AGC CTC*
	2nd pair *50°C	2300f: *CCA CMW AAT GCC CCT ATC* 654r: *GSC CCA MBC CCA TAG G*
	3rd pair *52°C	1859f: *ACT NTT CAA GCC TCC RAG CTG* 2835r: *GTT CCC AVG WAT AWG GTG AYC C*
	4th pair *57°C	1584f: *ACT TCG MBT CAC CTC TGC ACG T* 2396r: *GTC KGC GAG GYG AGG GAG TT*
20-ul Reaction mixture	• 10 ul of a premix containing MyFi 2x enzyme and 3 mM MgCl2 • 0.8 ul of 10 uM forward primer • 0.8 ul of 10 uM reverse primer • 3.4 ul of Nuclease free water • 5 ul of DNA template	
PCR thermal profile	• Initiation: 95°C for 3 minutes • Denaturation: 97°C for 30 seconds • Annealing: 1 minute; temperature* • Elongation: 72°C for 3 minutes • Repeat cycle (Denaturation: 97°c for 30 seconds) 35 times • Final elongation: 72°C for 10 minutes	
2nd PCR reaction generating six overlapping fragments from four fragments generated from the 1st PCR reaction		
Primers (5' – 3')	1st pair (1a) *54°C	251f: *GAC TYG TGG TGG ACT TCT C* 1190r: *TCA GCA AAY ACT YGG CA*
	1st pair (1b) *56°C	595f: *CAC HTG TAT TCC CAT CCC A* 1797r: *CCA ATT TMT GCY TAC AGC CTC*
	2nd pair (2a) *50°C	2300f: *CCA CMW AAT GCC CCT ATC* 215r: *AGR AAM ACM CCG CCT GT*
	2nd pair (2b) *50°C	2819f: *ACC WTA TWC YTG GGA ACA A* 617r: *GAY GAY GGG ATG GGA ATA CA*
	3rd pair *52°C	1877f: *CTG TGC CTT GGR TGG CTT* 2835r: *GTT CCC AVG WAT AWG GTG AYC C*
	4th pair *57°C	1584f: *ACT TCG MBT CAC CTC TGC ACG T* 2331r: *GGA AGY GTK GAY ARG ATA GGG GCA TT*
20-ul Reaction mixture	• 10 ul of a premix containing MyFi 2x enzyme and 3 mM MgCl2 • 0.8 ul of 10 uM forward primer • 0.8 ul of 10 uM reverse primer • 5.4 ul of Nuclease free water • 3 ul of DNA template	
PCR thermal profile	• Initiation: 95°C for 3 minutes • Denaturation: 97°C for 30 seconds • Annealing: 1 minute; temperature* • Elongation: 72°C for 3 minutes • Repeat cycle (Denaturation: 97°C for 30 seconds) 31 times • Final elongation: 72°C for 10 minutes	

A: Adenine. C: Cytosine. G: Guanine. T: Thymine.
B represents: C (Cytosine) or G (Guanine) or T (Thymine).
M represents: A (Adenine) or C (Cytosine).
R represents: A (Adenine) or G (Guanine).
S represents: C (Cytosine) or G (Guanine).
V represents: A (Adenine) or C (Cytosine) or G (Guanine).
W represents: A (Adenine) or T (Thymine). Y represents: C (Cytosine) or T (Thymine).
MgCl2: Magnesium chloride. ul: microlitre. uM: micromolar.

b. Assay two

I tested and optimised a nested PCR assay, which uses two sets of primers in each reaction, on 15 HBV DNA samples with adequate volume. This assay generates two fragments (A and B) covering the entire HBV genome as shown in **Figure 4.3** (Wang et al. 2017). To generate fragment A, primer pair (P3 & AR1) was used in the 1st PCR reaction followed by primer pair (P3 & AR2) in the second reaction. To generate fragment B, primer pair (P4 & AF1) was used in the 1st PCR reaction followed by primer pair (P4 & AF2) in the second reaction. **Table 4.5** provides details on primers, reaction mixture and thermal cycle profile.

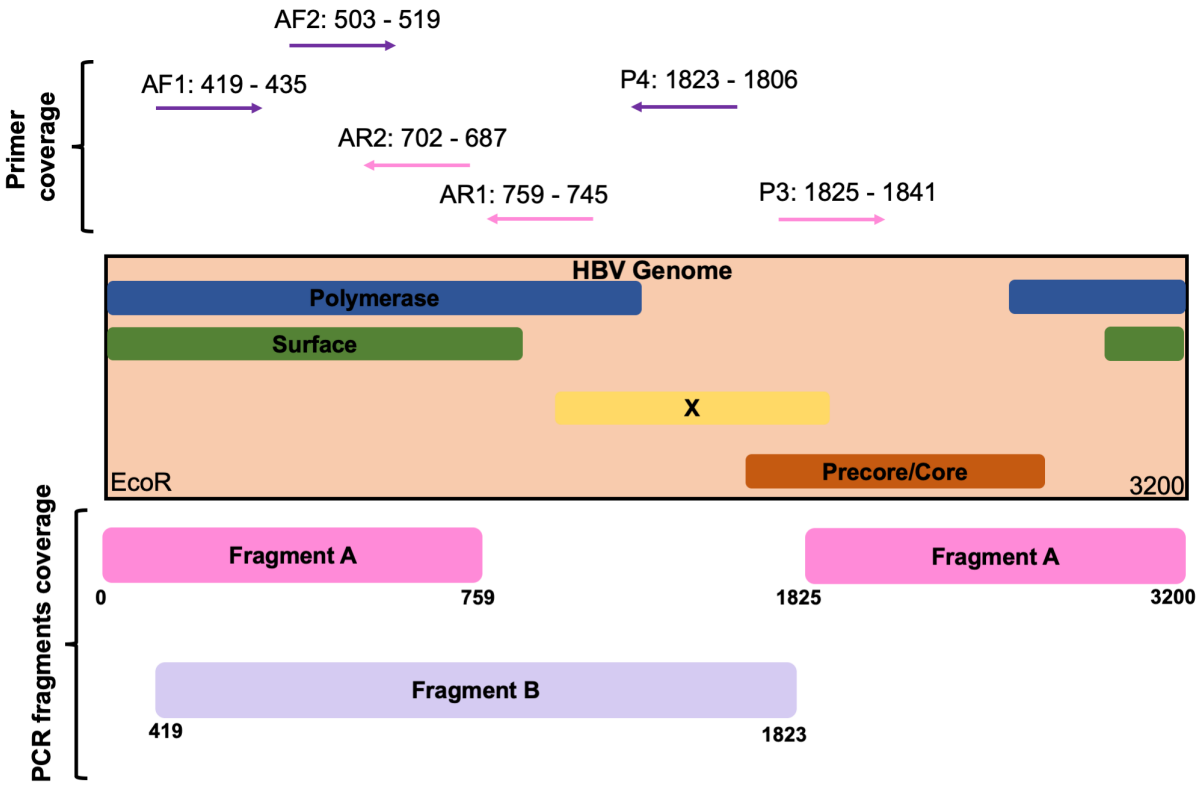


Figure 4.3: Primers and polymerase chain reaction (PCR) fragment coverage across hepatitis B virus genome. Primers obtained from a published nested PCR assay (Wang et al. 2017). The 1st PCR reaction generates fragment A using primer pair (P3 & AR1) and fragment B using primer pair (P4 & AF1). The 2nd PCR reaction uses fragments generated in the 1st reaction; generation of fragment A is completed using primer pair (P3 & AR2) and generation of fragment B is completed using primer pair (P4 & AF2).

Table 4.5: Primers, reaction mixture and thermal cycle profile for assay two (published by Wang et al. 2017).

1st PCR reaction generating two fragments using two pairs of primers		
Primers (5' – 3')	1st pair *58°C	P3: *TTTTTCACCTCTGCCTAATCA* AR1: *GCTTTCCCCCACTGT*
	2nd pair *58°C	P4: *CCAGCACCATGCAACTTTTTGG* AF1: *GTCTGCGGCGTTTTATC*
25-ul Reaction mixture	• 0.5 ul of 10 uM forward primer • 0.5 ul of 10 uM forward primer • 2.5 ul of La Taq buffer • 4 ul of dNTP • 2.5 ul 25 mM $MgCl^2$ • 0.25 ul Takara La Taq enzyme • 2 ul of DNA template • 12.75 ul of Nuclease free water	
PCR thermal profile	• Initiation: 94°C for 5 minutes • Denaturation: 94°C for 30 seconds • Annealing: 30 seconds; temperature* • Elongation: 72°C for 3 minutes • Repeat cycle (Denaturation: 94°C for 30 seconds) 35 times • Final elongation: 72°C for 10 minutes	
2nd PCR reaction generating two fragments using two pairs of primers		
Primers (5' – 3')	1st pair *58°C	P3: *TTTTTCACCTCTGCCTAATCA* AR2: *GCCTCAGYCCGTTTCT*
	2nd pair *58°C	P4: *CCAGCACCATGCAACTTTTTGG* AF2: *TGCCCGTTTGTCCTCTA*
25-ul Reaction mixture	• 0.5 ul of 10uM forward primer • 0.5 ul of 10uM forward primer • 2.5 ul of La Taq buffer • 4 ul of dNTP • 2.5 ul 25mM $MgCl^2$ • 0.25 ul Takara La Taq enzyme • 2 ul of DNA template • 12.75 ul of Nuclease free water	
PCR thermal profile	• Initiation: 94°C for 5 minutes • Denaturation: 94°C for 30 seconds • Annealing: 30 seconds; temperature* • Elongation: 72°C for 3 minutes • Repeat cycle (Denaturation: 94°C for 30 seconds) 35 times • Final elongation: 72°C for 10 minutes	

A: Adenine. C: Cytosine. G: Guanine. T: Thymine.
B represents: C (Cytosine) or G (Guanine) or T (Thymine).
M represents: A (Adenine) or C (Cytosine).
R represents: A (Adenine) or G (Guanine).
S represents: C (Cytosine) or G (Guanine).
V represents: A (Adenine) or C (Cytosine) or G (Guanine).
W represents: A (Adenine) or T (Thymine).
Y represents: C (Cytosine) or T (Thymine).
dNTP: deoxyribonucleotide triphosphate. MgCl2: Magnesium chloride. ul: microlitre. uM: micromolar.

4.2.5 Sanger sequencing

I stained the amplified PCR fragments obtained from **assay one** and **assay two** with ethidium bromide and verified successful amplification using agarose gel electrophoresis under UV light. Direct sequencing was performed on the generated fragments from both assays using ABI 3730 / 3730xl capillary sequencer (University of Oxford, Department of Zoology). I used sequencing primers from **assay one** (Chook et al. 2015) **(Table 4.6),** to sequence amplified HBV fragments generated from both assays.

Table 4.6: Sequencing primers used on fragments generated from assay one and assay two.

Nested PCR assays	Fragment	Sequencing primers	Source of primers
Assay one	Fragment 1a	251f: *GAC TYG TGG TGG ACT TCT C*	**Primers from assay one**
	Fragment 1b	595f: *CAC HTG TAT TCC CAT CCC A*	
	Fragment 2a	215r: *AGR AAM ACM CCG CCT GT*	
	Fragment 2b	617r: *GAY GAY GGG ATG GGA ATA CA*	
	Fragment 3	2835r: *GTT CCC AVG WAT AWG GTG AYC C*	
	Fragment 4	2331r: *GGA AGY GTK GAY ARG ATA GGG GCA TT*	
Assay two	Fragment A	215r: *AGR AAM ACM CCG CCT GT* 251f: *GAC TYG TGG TGG ACT TCT C* 617r: *GAY GAY GGG ATG GGA ATA CA* 1859f: *ACT NTT CAA GCC TCC RAG CTG* 1877f: *CTG TGC CTT GGR TGG CTT* 2819f: *ACC WTA TWC YTG GGA ACA A* 2300f: *CCA CMW AAT GCC CCT ATC* 2835r: *GTT CCC AVG WAT AWG GTG AYC C*	**Primers from assay one**
	Fragment B	595f: *CAC HTG TAT TCC CAT CCC A* 1190r: *TCA GCA AAY ACT YGG CA* 1797r: *CCA ATT TMT GCY TAC AGC CTC*	

A: Adenine. C: Cytosine. G: Guanine. T: Thymine.
B represents: C (Cytosine) or G (Guanine) or T (Thymine).
M represents: A (Adenine) or C (Cytosine).
R represents: A (Adenine) or G (Guanine).
S represents: C (Cytosine) or G (Guanine).
V represents: A (Adenine) or C (Cytosine) or G (Guanine).
W represents: A (Adenine) or T (Thymine).
Y represents: C (Cytosine) or T (Thymine).
dNTP: deoxyribonucleotide triphosphate.

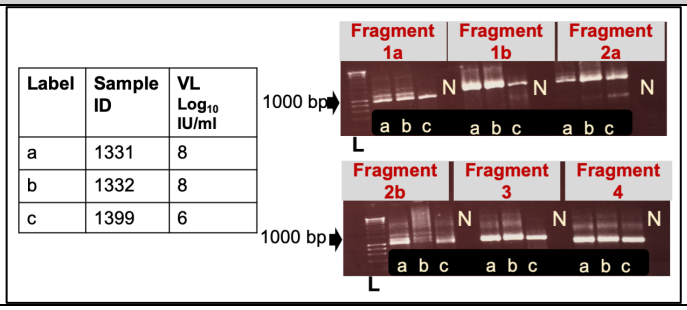
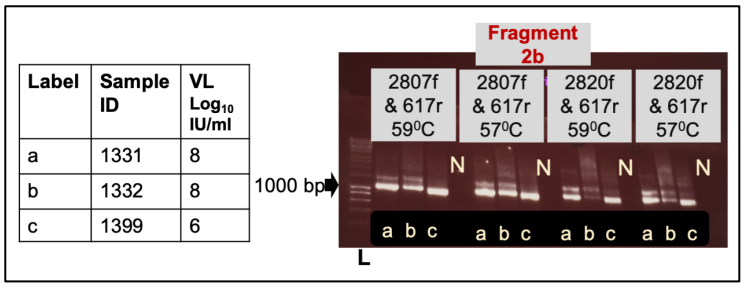
4.2.6 Sanger sequence analysis

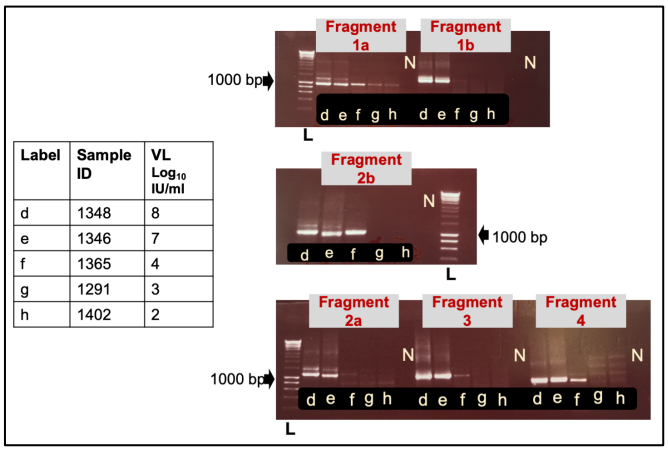
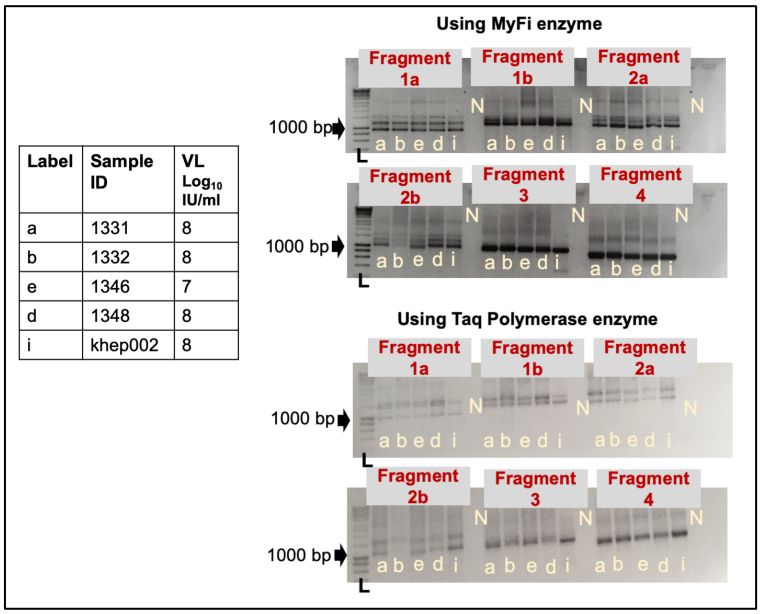
I inspected and edited raw DNA data (AB1 files) generated from Sanger Sequencing using Benchling programme (https://www.benchling.com), and merged the fragments to form a single consensus sequence using a combine fasta online programme (Stothard, 2000). I performed sequence alignment using AliView program (Larsson, 2014). To determine HBV genotypes of the generated sequences, I aligned the generated consensus sequences with HBV reference sequences obtained from GenBank (McNaughton et al. 2020a), generated ML phylogenetic trees in IQ-TREE using the general time reversible nucleotide substitution model with among-site rate variation (GTR + G) (Nguyen et al. 2015), and looked for clustering of generated sequences with HBV reference strains of the closest similarity, using FigTree v.1.4.4 (Suchard et al. 2018).

4.3 Results

4.3.1 Assay one optimisation steps

Table 4.7: Summary of the optimisation steps used for assay one (published by Chook et al. 2015).

Optimisation steps	Agarose gels with amplified fragments 1a, 1b, 2a, 2b, 3 and/or 4	Description and outcomes
Step 1: Initial amplification without modification		I initially tested this nested PCR assay (without modifications) on three samples (1331, 1332 & 1399). These samples were selected because they had a high VL. All PCR fragments were successfully amplified, except fragment 2b for sample 1332.
Step 2: Primer modification		To improve sensitivity for fragment 2b, I modified primer **2819f: *ACC WTA TWC YTG GGA ACA A*** to: 2820f: *GRTCACCWTATWCBTGGGAAC* (salted adjusted melting temperature of between 57.5°C – 61.2°C) and; 2807f: *CGCHTCATTTTGYGGGTCAC* (salted adjusted melting temperature of between 58.4°C – 62.5°C) I modified the primer by including more G and C bases because these bases have a stronger bonding compared to A and T bases, and therefore this could help promote specific binding. I performed nested PCR on the selected three samples (1331, 1332 & 1399) using the new modified primers pairs (2820f & 617r) and (2807f & 617r) to generate fragment 2b. I tested two different annealing temperatures of 57°C and 59°C for both primer pairs. Primer pair (2807f & 617r) performed better at both annealing temperatures of 57°C and 59°C compared to primer pair (2820f & 617r).

Step 3: Amplification using modified primers	 **Label** / **Sample ID** / **VL Log_{10} IU/ml**: d 1348 8; e 1346 7; f 1365 4; g 1291 3; h 1402 2	I tested the performance of this assay on five different samples (1348, 1346, 1365, 1291 and 1402) with varying VLs, having replaced the primer pair (2819f & 617r) with new modified primer pair (2807f & 617r) for fragment 2b in the 2nd PCR reaction. Amplification of fragment 1a was successful for all samples, whereas amplification of fragment 1b and 2a was successful for samples 1348 (8 log_{10} IU/ml) and 1346 (7 log_{10} IU/ml), and amplification of fragment 2b, 3 and 4 was successful for samples 1348 (8 log_{10} IU/ml), 1346 (7 log_{10} IU/ml) and 1365 (4 log_{10} IU/ml), although fragment 3 for sample 1365 had a faint band.
Step 4: Comparing different enzymes	 **Label** / **Sample ID** / **VL Log_{10} IU/ml**: a 1331 8; b 1332 8; e 1346 7; d 1348 8; i khep002 8	I compared the performance of nested PCR assay using the enzyme from the published protocol (MyFi) and a different enzyme (Taq polymerase) on five samples (1331, 1332, 1346, 1348 and KHep002). There was no difference in amplification when using MyFi enzyme or Taq polymerase. The difference in the boldness of the bands is because the agarose gels used for MyFi enzyme was of better quality than that used to assess Taq Polymerase enzyme (this can be seen by the boldness of 3 kb DNA ladder). Due to limited sample volume, it was not possible to repeat the experiment.

Alphabetical letters used to identify bands for each sample. **N** represents negative control (nuclease free water). **L** represents 3 kb DNA ladder. Underlined primers in step 2 show the changes I made from the origin primer.
A: Adenine. C: Cytosine. G: Guanine. T: Thymine.
B represents: C (Cytosine) or G (Guanine) or T (Thymine).
H represents: A (Adenine) or C (Cytosine) or T (Thymine).
W represents: A (Adenine) or T (Thymine).
Y represents: C (Cytosine) or T (Thymine).
Y represents: C (Cytosine) or T (Thymine).

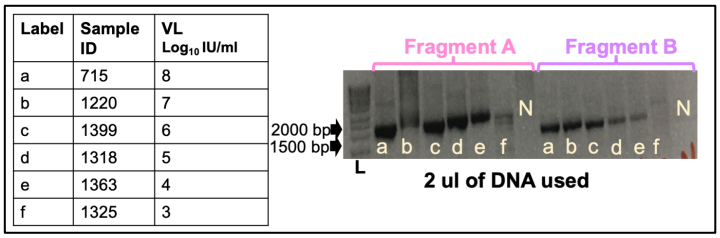
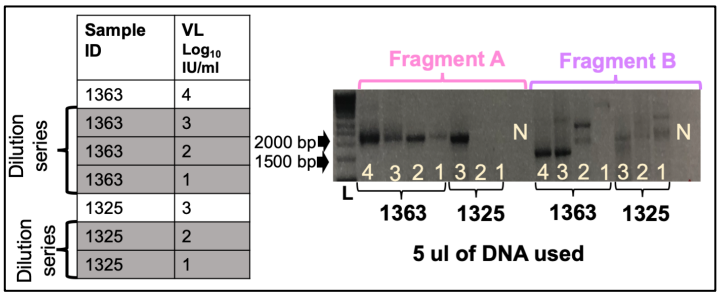
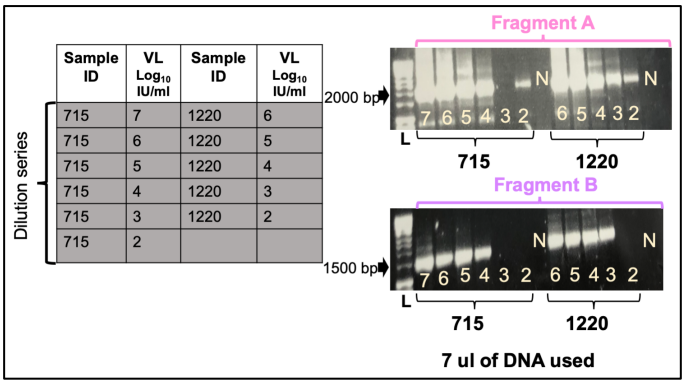
4.3.2 Amplification summary of assay one

Assay one was able to successfully amplify HBV DNA samples with VL levels of >6 log_{10} IU/ml generating six fragments (1a, 1b, 2a, 2b, 3 and 4) for each sample, having modified the primer for fragment 2b to (2807f & 617r) to improve amplification. Amplification of samples having a VL of <6 log_{10} IU/ml was possible for few samples although this was not consistent as amplification was successful for only certain fragments (1a, 2b, 3 and/or 4). There was no difference in amplification when a different enzyme was tried.

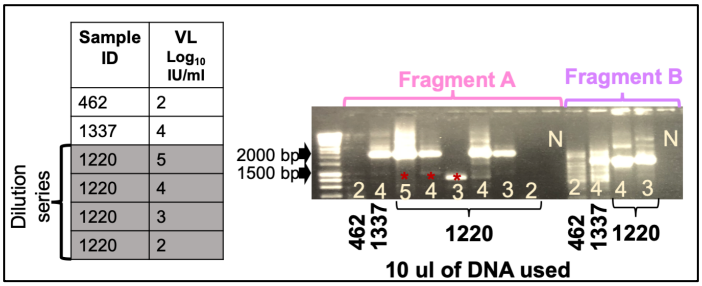
4.3.3 Assay two optimisation steps

Table 4.8: Summary of the optimisation steps used for assay two (published by Wang et al. 2017).

I used a different set of samples for this assay from those used in assay one because of insufficient plasma volume.

Optimisation steps	Agarose gels with amplified fragments A and/or B	Description and outcomes
Step 1: Initial amplification without modification		I tested this assay (without modifications) on six samples (715, 1220, 1399, 1318, 1363 and 1325) with varying VL levels. Amplification was successful for both fragments A and B in samples (715, 1220, 1399, 1318 and 1363) which had VL levels of >4 log_{10} IU/ml, whereas sample (1325) which had a VL of 3 log_{10} IU/ml, only fragment A was successfully amplified.
Step 2: Sample DNA concentration adjustment (using 5 ul of DNA)		Given that the published study where this assay was obtained from was able to amplify samples with VL of as low as 3 log_{10} IU/ml, I wanted to get it to consistently amplify samples with VL of ≤ 3 log_{10} IU/ml. I therefore diluted samples 1363 and 1325 which had VL levels of 4 log_{10} IU/ml and 3 log_{10} IU/ml respectively, into a series of tenfold dilutions, and performed nested PCR amplification on these dilution series having increased the DNA template input to 5 ul from 2 ul. For sample 1363, amplification of fragment A was successful for all dilution series whereas amplification of fragment B was successful for dilution series up to 2 log_{10} IU/ml. For sample 1325, amplification was only successful for fragment A for VL level of 3 log_{10} IU/ml.
Step 3: Sample DNA concentration adjustment (using 7ul of DNA)		I diluted samples 715 and 1220 which had VL levels of 8 log_{10} IU/ml and 7 log_{10} IU/ml, respectively, into a series of tenfold dilutions, and performed nested PCR amplification, having increased DNA template input to 7ul. For sample 715, amplification of fragment A was successful for dilution series as low as 2 log_{10} IU/ml (although no band was visualised at 3 log_{10} IU/ml), whereas amplification of fragment B was successful for dilution series up to 4 log_{10} IU/ml. For sample 1220, amplification of fragment A was successful for dilution series up to 2 log_{10} IU/ml whereas amplification of fragment B was successful for dilution series up to 3 log_{10} IU/ml.

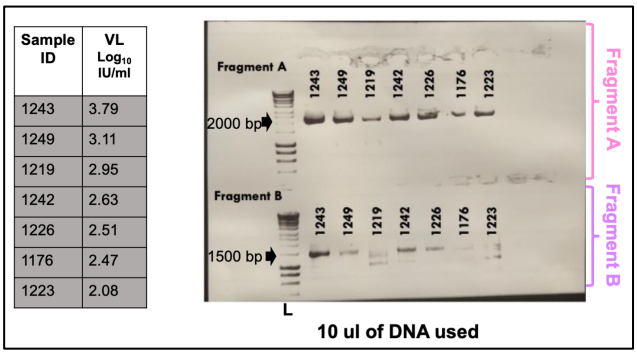
Step 4: Sample DNA concentration adjustment (using 10 ul of DNA)



Different dilution of sample 1220 (diluted on a different day)

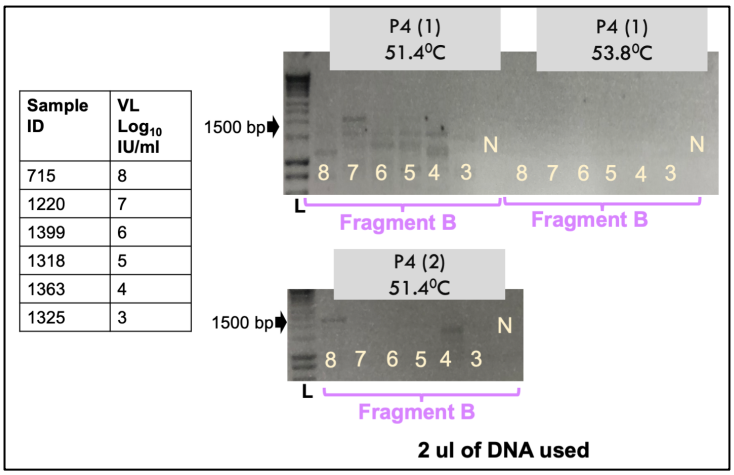
I further tested the performance of this assay having increased the DNA template input to 10 ul. I used samples 462 and 1337 with VL of 2 log_{10} IU/ml and 4 log_{10} IU/ml, respectively, and dilution series of sample 1220. Amplification for fragments A and B was successful for sample 1337 and the dilution series of sample 1220 (5 log_{10} IU/ml, 4 log_{10} IU/ml and 3 log_{10} IU/ml).

Step 5: Sample DNA concentration adjustment (using 10 ul of DNA) of freshly extracted DNA samples



I retested this assay using an increased DNA template of 10 ul on seven freshly extracted HBV DNA samples with varying VL between 2–3 log_{10} IU/ml. Amplification for fragments A and B for all the samples was successful although fragment B had faint bands.

Step 6: Amplification using modified primers



I used VL levels to label samples on gel

In the attempt to improve sensitivity for fragment B, I replaced the published primer **P4: *CCAGCACCATGCAACTTTTTGG*** with two new primers:
P4 (1): *CTG CCT ART CAT CTY WTG* (salted adjusted melting temperature of between 51.4^{0}C – 53.8^{0}C) and;
P4 (2): *TCA CCT CTG CCT AAT CAT* (salted adjusted melting temperature of 51.4^{0}C).

I replaced primer P4 with the new primers P4 (1)/P4 (2) because P4 is located at nucleotide positions (1805–1827), whereas the two new primers P4 (1) and P4 (2) are located at nucleotide positions (1831–1848) and (1824–1842), respectively. These new primers might perform better since they are located a little further away from the breakpoint of the negative strand of partially double-stranded HBV genome located at nucleotide position 1800, and therefore, there would be less interference of the polymerase enzyme during the elongation process

I performed nested PCR on six selected samples to generate fragment B using the new primers as follows: [P4 (1) & AF1/AF2] and [P4 (2) & AF1/AF2]. For primer set [P4 (1) & AF1/AF2], I tested two different annealing temperatures of 51.4^{0}c and 53.8^{0}c, and for primer set [P4 (2) & AF1/AF2], I used annealing temperature of 51.4^{0}C. These primer modifications did not successfully amplify HBV DNA.

N represents negative control (nuclease free water). **L** represents 3kb DNA ladder.
A: Adenine. C: Cytosine. G: Guanine. T: Thymine.
R represents: A (Adenine) or G (Guanine).
W represents: A (Adenine) or T (Thymine).
Y represents: C (Cytosine) or T (Thymine).

4.3.4 Amplification summary of assay two

Using the original published primers, **assay two** was able to successfully amplify HBV DNA samples with VL levels down to 2 $\log_{10}$ IU/ml generating fragments A and B for each sample after modifying the protocol to increase the DNA template input to 10 ul.

4.3.5 Sanger sequencing

Successfully amplified fragments of 17/23 samples that were used for optimisation, were submitted for Sanger sequencing. 6/23 samples which were not submitted for sequencing were not successfully amplified due to low VL levels. I merged the sequenced fragments (six from **assay one** and two from **assay two**) for each sample to form a single consensus sequence using a combine fasta online programme (Stothard, 2000).

From **assay one** amplification, samples 1331 (8 $\log_{10}$ IU/ml) and 1332 (8 $\log_{10}$ IU/ml) had complete genome coverage after sequencing, whereas samples 1399 (8 $\log_{10}$ IU/ml), 1348 (8 $\log_{10}$ IU/ml), and 1346 (7 $\log_{10}$ IU/ml) and KHep002 (8 $\log_{10}$ IU/ml) had gaps in >1 position across the genome despite having high VL levels (**Figure 4.4).**

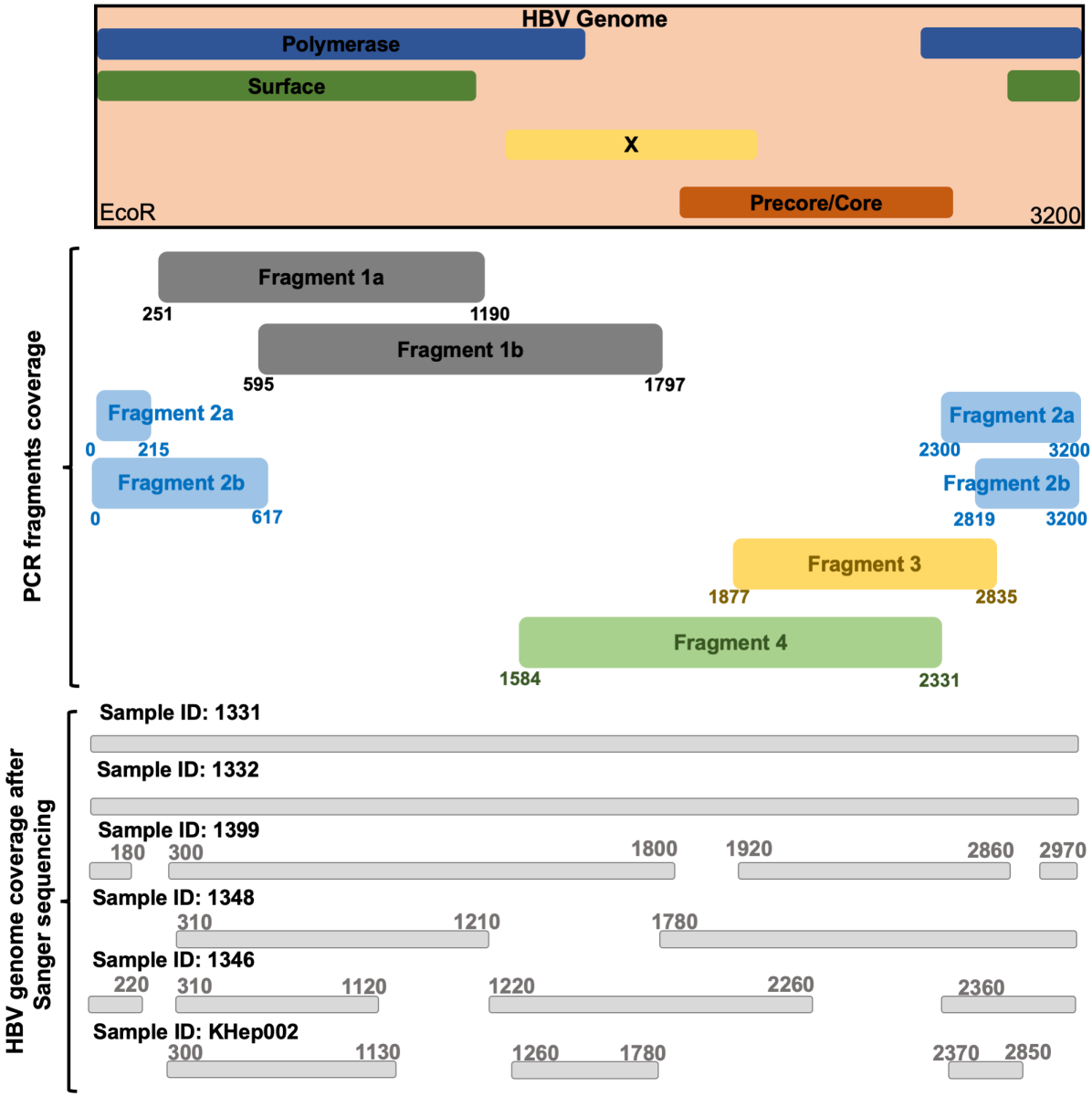


Figure 4.4: Hepatitis B virus genome coverage after Sanger sequencing of fragments 1a, 1b, 2a, 2b, 3 and/or 4 generated from a nested polymerase chain reaction assay (assay one) for six samples 1331, 1332, 1399, 1348, 1346 and KHep002.

From **assay two**, sequencing coverage was obtained for all samples with VL levels of between 2–8 $\log_{10}$ IU/ml after Sanger sequencing. However, there was a consistent gap of approximately 100 bp between nucleotide positions 1830–1930; these nucleotide positions are located within the X and Precore/core proteins of the HBV genome **(Figure 4.5).**

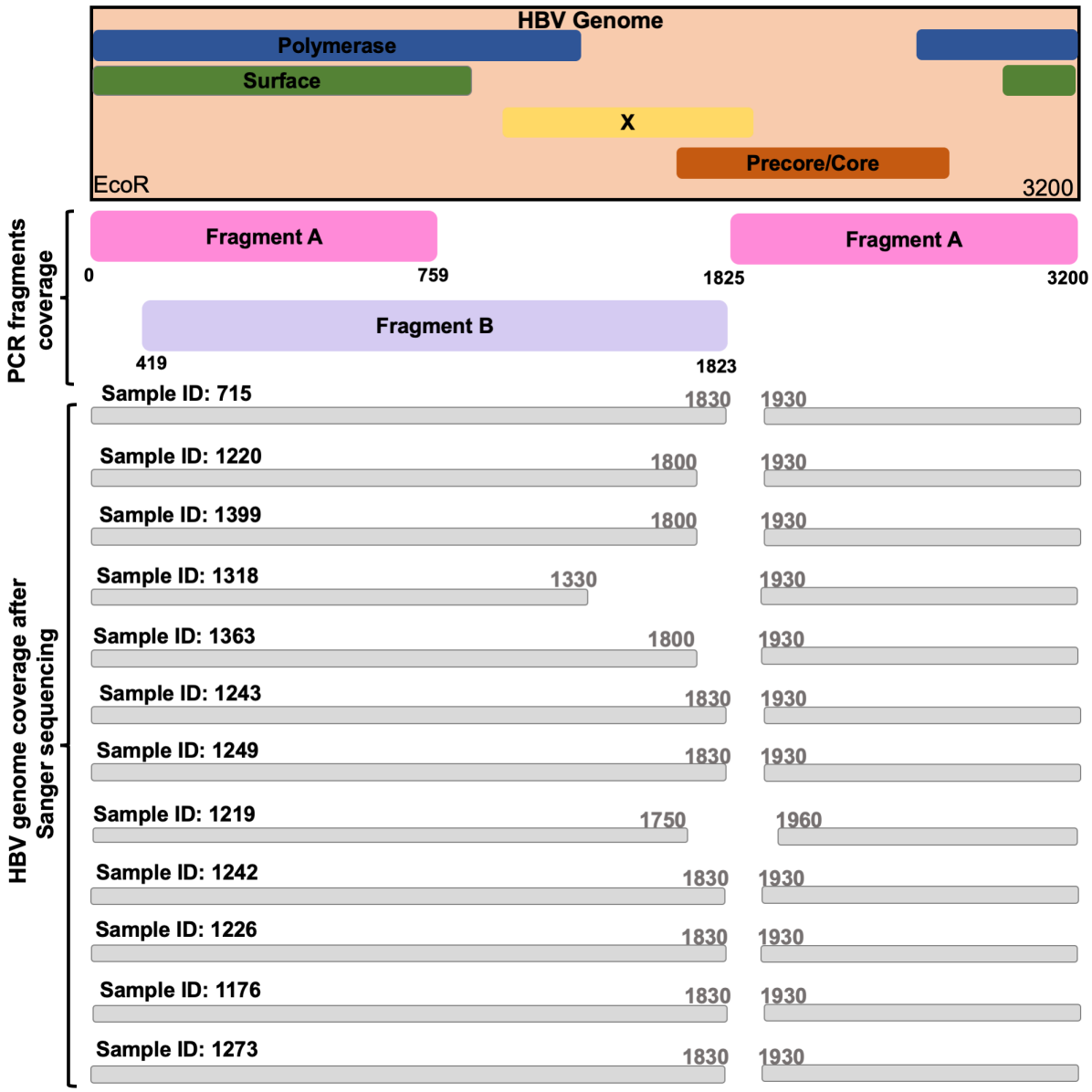


Figure 4.5: Hepatitis B virus genome coverage after Sanger sequencing of fragments A and B generated from a nested polymerase chain reaction assay (assay two) for 12 samples 715, 1220, 1399, 1318, 1363, 1243, 1249, 1219, 1242, 1226, 1176 and 1223.

4.3.6 Comparing sequences generated by Sanger and Illumina sequencing platforms

The 8/17 samples that were sequenced using Sanger, also had sequence data generated by Illumina, all with VL >3.5 $\log_{10}$. A detailed description of Illumina methodology is provided in **chapter 5: section 5.2.2.** Compared to Sanger, sequence coverage by Illumina was better given that it generated whole genome sequence for all samples except for sample 1346 **(Table 4.9).**

Table 4.9: Sequence coverage comparison of sequences of eight samples generated by Sanger and Illumina sequencing platforms.

Sample ID (PMB)	HBV VL log10 (IU/ml)	Nested PCR Amplification Assay	Sanger sequencing coverage	Sanger sequencing complete coverage of Pol & S proteins	Illumina sequencing coverage	Illumina sequencing complete coverage of Pol & S proteins
1243	3.79	Assay 2	1–1830 1930–3200	Yes	1–3200	Yes
1363	4.18	Assay 2	1–1800 1930–3200	Yes	1–3200	Yes
1318	5.55	Assay 2	1–1330 1930–3200	Yes	1–3200	Yes
1399	6.08	Assay 2	1–1800 1930–3200	Yes	1–3200	Yes
1346	6.65	Assay 1	1–220;310–1120; 1220–2260; 2360–3200	Gaps in surface and polymerase	1–1550 1630–3200	Gap in the polymerase
1220	7.58	Assay 2	1–1800 1930–3200	Yes	1–3200	Yes
1332	8.23	Assay 1	1–3200	Yes	1–3200	Yes
1331	8.23	Assay 1	1–3200	Yes	1–3200	Yes

PMB: Peter Medawar Building. VL: Viral load. PCR: Polymerase chain reaction. Pol: Polymerase. S: Surface.

4.3.7 Genotype classification

I aligned the consensus sequences of the 17 samples with HBV reference sequences obtained from GenBank (https://www.benchling.com) using AliView program (Larsson, 2014). I then generated ML phylogenetic trees in IQ-TREE (Nguyen et al. 2015) using the aligned consensus sequences (with or without gaps), and visualised using FigTree v.1.4.4 (Suchard et al. 2018) (**Figure 4.6).**

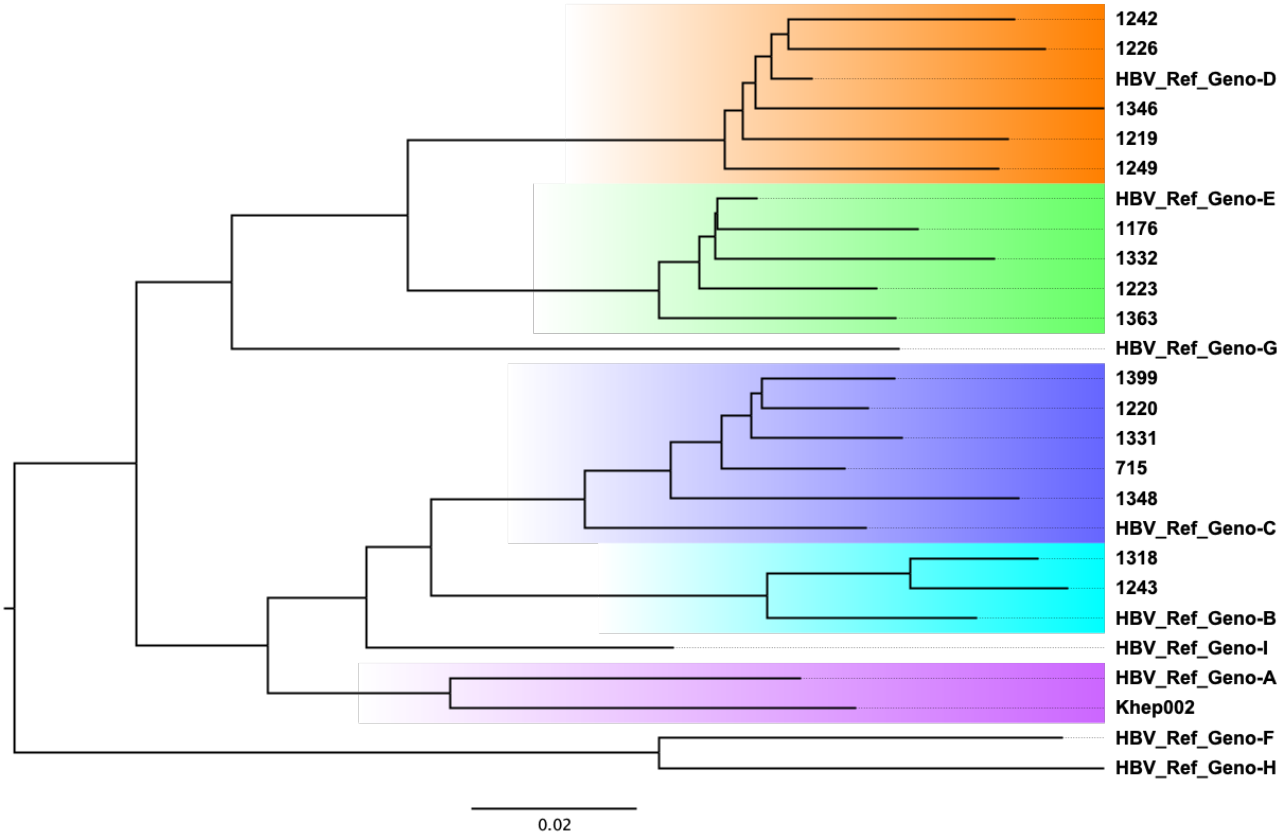


Figure 4.6: Rooted maximum likelihood phylogenetic tree showing genotype classification of 17 hepatitis B virus samples sequenced using Sanger sequencing. Coloured blocks indicate different genotypes. Phylogenetic trees drawn in FigTree v.1.4.4 (Sunchard et al. 2018).

4.4 Discussion

Nested PCR assay two (Wang et al. 2017) was superior and less laborious compared to nested PCR assay one (Chook et al. 2015). Assay two was able to amplify HBV DNA samples representing VL levels of between 2–8 $\log_{10}$ IU/ml and genotypes B, C, D and E in two fragments, with complete coverage of both polymerase and surface proteins (**Figure 4.5** and **Table 4.10**). On the other hand, assay one was only able to amplify HBV DNA samples with VL >6 $\log_{10}$ IU/ml in six fragments with complete coverage of polymerase and surface protein for 2/6 sequenced samples (**Figure 4.4** and **Table 4.10**).

Table 4.10: Summary of performance of two published nested polymerase chain reaction (PCR) assays tested on selected hepatitis B virus samples and modified to improve sequencing of low viral load (VL) samples.

Nested PCR assay	Samples tested	VL in log_{10} IU/ml	Successfully amplified	Sequenced	Genotype	Complete coverage of polymerase protein [0–1623 & 2307–3200]	Complete coverage of surface protein [0–835 & 2854–3200]
Assay one (Chook et al. 2015)	1331	8	Yes	Yes	C	Yes	Yes
	1332	8	Yes	Yes	E	Yes	Yes
	1348	8	Yes	Yes	C	No	No
	KHep002	8	Yes	Yes	A	No	No
	1346	7	Yes	Yes	D	No	No
	1399	6	Yes	Yes	C	No	No
	1365	4	No	No	Not available	Not available	Not available
	1291	3	No	No	Not available	Not available	Not available
	1402	2	No	No	Not available	Not available	Not available
Assay two Wang et al. 2017)	715	8	Yes	Yes	C	Yes	Yes
	1220	7	Yes	Yes	C	Yes	Yes
	1399	6	Yes	Yes	C	Yes	Yes
	1318	5	Yes	Yes	B	Yes	Yes
	1363	4	Yes	Yes	E	Yes	Yes
	1325	3	No	No	Not available	Not available	Not available
	1243	3.8	Yes	Yes	B	Yes	Yes
	1249	3.1	Yes	Yes	D	Yes	Yes
	1337	3.7	Yes	No	Not available	Not available	Not available
	1325	3	Yes	Yes	Not available	Not available	Not available
	1219	2.9	Yes	Yes	D	Yes	Yes
	1242	2.6	Yes	Yes	D	Yes	Yes
	1226	2.5	Yes	Yes	D	Yes	Yes
	1176	2.4	Yes	Yes	E	Yes	Yes
	1223	2.0	Yes	Yes	E	Yes	Yes
	462	2	No	No	Not available	Not available	Not available

To improve assay sensitivity on low VL samples, assay two (Wang et al. 2017) was slightly modified to use 10 ul of HBV DNA input. Addition of excessive amounts of DNA template into a PCR reaction can prematurely saturate primers or dNTPs before enough copies of the target region is sufficiently covered (Jansson and Hedman, 2019). However, successful amplification was possible due to the very low concentration of DNA copies within a 10 ul volume and therefore, addition of more template volume did not seem to exhaust primers and/or dNTPs.

All sequences from assay two had a 100 bp gap occurring consistently at nucleotide position (1830–1930) in the core and X protein. This gap can be explained by insufficient primer coverage for this region given that fragment A starts at nucleotide 1825 and fragment B ends at nucleotide 1823. In addition, the HBV viral genome is partially double-stranded with two breakpoints at

nucleotide position 1600 in positive strand and 1800 in the negative stand (He et al. 2002; Chook et al. 2015) **(chapter 1: Figure 1.1).** This structural arrangement can also interfere with polymerase enzyme extension along the viral genome resulting in these gaps.

While HBV genetic diversity may also interfere with successful amplification due to incompatibility with primers, assay two (Wang et al. 2017) was able to amplify HBV DNA samples of different genotypes although this assay was developed using samples obtained from individuals infected with CHB recruited from China (a country in Asia where HBV genotype B is predominant; see [**chapter 1: section 1.6 (Table 1.1)**].

In summary, nested PCR assay two (Wang et al. 2017) is a better and an efficient option for amplification for sequencing (for HBV RAM and VEM detection) in low VL samples, as it was able to amplify HBV DNA samples as low as 2 $\log_{10}$ IU/ml in only two fragments, able to amplify different HBV DNA samples representing different genotypes, and with complete coverage of both polymerase and surface proteins.

Chapter five: The epidemiology of HBV RAMs and VEMs in South Africa and the UK

5.1 Background

Given that HBV is endemic in Africa and the widespread population exposure to ART, the prevalence of HBV RAMs and VEMs might be higher among individuals with CHB infection recruited from clinic centres in South Africa compared to those recruited from clinic centres in the UK. This chapter describes the prevalence and risk factors associated with HBV RAMs and VEMs among individuals infected with CHB recruited in South Africa and the UK, and also explores the impact of HBV RAMs and the role of treatment compliance in persistent HBV viraemia.

I have described and published the first evidence of HBV resistance to TFV from Africa from analysing the sequence data generated from the cohort recruited from South Africa [published in the Journal of Clinical Virology **(Mokaya et al. 2020b)**].

5.2 Methods

5.2.1 Study design, study population and data collection

Data used in these analyses were obtained from adults (≥18 years of age) with CHB from four different cohorts recruited in South Africa and the UK as detailed in **Table 5.1.**

Table 5.1: Description of cohorts of adults from four different settings used in the analysis of occurrence and factors associated with drug resistance-associated mutations and vaccine escape mutations in chronic hepatitis B virus (HBV) infection.

Project name	Tygerberg HCC cohort	OxSAHep project	Oxford viral hepatitis project	London HBV cohort
Country of recruitment	South Africa	South Africa	UK	UK
Site of recruitment	Teaching hospitals in Western Cape and Gauteng provinces	Tygerberg Hospital in Cape Town and Bloemfontein Hospital in Free state	Oxford University Hospitals NHS	Hospital clinics in London
Diagnosis	Chronic HBV, with or without HCC	Chronic HBV, with or without HIV coinfection	Chronic HBV infection	Chronic HBV infection
Principal Investigators	Dr. Tongai Maponga	Prof. Philippa Matthews	Prof. Ellie Barnes and Prof. Philippa Matthews	Prof. Mala Maini
Ethics Approval	Health REC at Stellenbosch University (S13/04/072 and N11/09/284).	University of Oxford Tropical REC (ref. OXTREC 01-18).	NIHR Oxford Biomedical Research Centre REC (ref 09/H0604/20).	11/LO/0421

HCC: Hepatocellular carcinoma. REC: Research Ethics Committee. National Institute for Health Research: NIHR. Oxford-South Africa Hepatitis Cohort, 'OxSA-Hep'.
All participants provided a written informed consent in accordance with the Declaration of Helsinki.

5.2.1.1 Tygerberg HCC cohort

A total of 161 South African adults infected with CHB, with or without hepatocellular carcinoma (HCC), were enrolled into a cross-sectional study recruited from teaching hospitals in the Western Cape and Gauteng provinces, between December 2012 and October 2015 by Dr. Tongai Maponga.

Participants were classified as having HCC (n=68) if lesions in the liver were detected by either abdominal ultrasound scan or contrast-enhanced computed tomography in addition to having serum alpha feto-protein levels of >400 ug/L. All 161 participants, with or without HCC, had liver stiffness assessed using transient elastography [Fibroscan 402 (Echosens, France)].

Pseudonymised descriptive and clinical data were recorded. Serum samples obtained from recruited participants (n=161) were used to assess the presence of HBsAg and HBeAg using DiaSorin ETI-EBK PLUS assay (DiaSorin, Saluggia, Italy). Quantification of HBV VL was done using Cobas Ampliprep/Taqman tests (Roche Molecular Diagnostics, the Netherlands). HBV DNA was extracted from serum samples of 34 individuals with HBV VL >4 $\log_{10}$ IU/ml (HCC, n=20; non-

HCC, n=14) using NucliSENS magnetic extraction system (BioMérieux) and shipped to Oxford for sequencing.

Recruitment, clinical assessment, routine clinical data collection and laboratory testing were carried out by our collaborators in teaching hospitals in the Western Cape and Gauteng provinces in South Africa.

5.2.1.2 OxSAHep project

The OxSAHep project (Oxford-South Africa Hepatitis Cohort, 'OxSA-Hep') is a collaborative project involving researchers based at the University of Oxford, UK and Stellenbosch University, South Africa, with the overall aim of defining host and viral factors that are associated with HBV control and clearance among African adults. This project has prospectively recruited adults with CHB, with or without HIV infection, attending routine clinical follow-up in hepatology and infectious diseases outpatient clinics in Tygerberg Hospital (Western Cape) and Bloemfontein University Hospital (Free State) since July 2018 (Maponga et al. 2020). As of the 29th November 2019, a total of 195 individuals had been recruited. Demographic, clinical and laboratory data were entered into a central database (LabKey).

Serum samples from the study participants were used to measure HBsAg, HBeAg, and HIV antibodies, and liver function tests [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] using the Cobas 6000 series e601 module analyzer (Roche Diagnostics GmbH, Germany). HBV DNA was extracted from serum samples using NucliSENS magnetic extraction system (BioMérieux). Quantification of HBV-DNA was done using Cobas Ampliprep/Taqman tests (Roche Molecular Diagnostics, the Netherlands).

Participant recruitment and data collection were carried out by our collaborators in South Africa. A total of 15 HBV DNA samples with a viral load of >4 $\log_{10}$ IU/ml were shipped to Oxford, UK for sequencing.

5.2.1.3 Oxford viral hepatitis project

a. Samples used for sequencing

The description of this cohort has been provided in **section 4.2.1**. From this cohort, a total of 22 samples were used for optimising Sanger, of which 16 were successfully sequenced by Sanger methods. 8/16 samples were also sequenced using Illumina. Among 8/16 samples only sequenced using Sanger, 7/8 had VL levels <3.5 log $_{10}$ IU/ml which is too low for Illumina sequencing. 16 new samples (not used to optimise Sanger sequencing) were only sequenced using Illumina. These samples were selected as they had HBV VL >3.5 $\log_{10}$ IU/ml and had sufficient plasma volume. Thus, a total of 32 HBV DNA samples were sequenced **(Table 5.2).**

Table 5.2: Summary of samples sequenced from the Oxford viral hepatitis project.

Sequencing platform	Oxford viral hepatitis project Sequenced samples
Sanger only	8
Illumina and Sanger	8
Illumina only	16
Total no. of samples sequenced	**32**

b. Samples used to assess TFV drug levels

I also selected serum samples from 12 individuals recruited in this cohort to assess the levels of TFV in plasma, of whom three also had PBMCs assessed for TFV levels. These 12 individuals were selected as they were on treatment with TFV and their plasma samples represented a range of HBV viraemia [high level viraemia (HLV), n=1; low level viraemia (LLV), n=1; and 10 individuals had undetectable VL]. Serum from the individual with HLV was among the 16 samples that were sequenced using Illumina.

5.2.1.4 London HBV cohort

Our research group received serum samples of 41 individuals infected with CHB infection recruited from hospital clinics in London, UK, together with their pseudonymised demographic, clinical and laboratory data. Cross-sectional data were available for 37/41 individuals, and 4/41 individuals had longitudinal data from between 2–6 time points. HBV DNA was extracted from serum samples using NucliSENS magnetic extraction system (BioMérieux). (I performed HBV

extraction for 14 samples). 42 HBV DNA samples (one sample from 34 participants, two samples from one participant and six samples from one participant) were prepared for sequencing.

5.2.2 Illumina Sequencing

Table 5.3 shows the number of samples sequenced from each cohort and the sequencing platform used.

Table 5.3: Summary of samples sequenced, and the sequencing platform used.

Sequencing platform	Tygerberg HCC cohort	OxSAHep project	Oxford viral hepatitis project	London HBV cohort	Total
Illumina only*	34	15	16	42	107
Sanger only	0	0	8	0	8
Both Illumina and Sanger	0	0	8	0	8
Total no. of samples sequenced	**34**	**15**	**32**	**42**	**123**

* 107 samples sequenced by Illumina only due to limited sample volume. HCC: Hepatocellular carcinoma. HBV: Hepatitis B virus.

HBV sequencing using Illumina entails four broad steps shown in **Figure 5.1**.

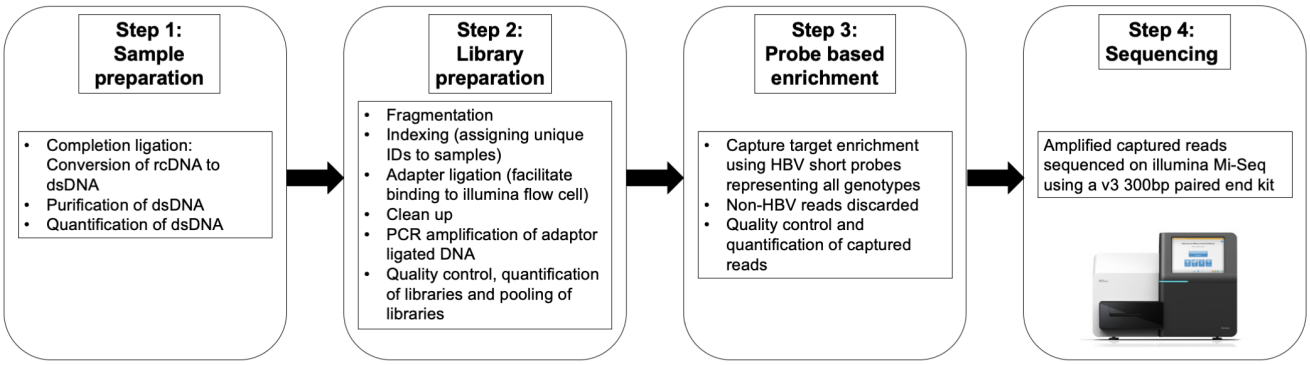


Figure 5.1: Summary of hepatitis B virus sequencing process using Illumina.

I have described each of these steps in detail in **Table 5.4.**

Table 5.4: A step by step summary of hepatitis B virus (HBV) sequencing process using Illumina.

Step 1: Sample preparation	
1.1: Completion ligation	HBV genomes were converted into fully double-stranded (ds)DNA genomes using a completion-ligation approach based on a published protocol (Martel et al. 2013). In summary: a. 7.4 ul of HBV DNA was added to a mixture containing: 1 ul of 1x ligase buffer, 0.1 ul of bovine serum albumin (0.1 ug/ml), 0.5 ul of TA DNA polymerase (1.5 U) (New England Biolabs, Ipswich, MA, USA), 0.5ul of TA DNA ligase (200 U) (New England Biolabs, Ipswich, MA, USA), and 0.5ul of dNTPs (0.5 mM each) to form a final volume of 10 ul. b. The reaction was carried out at 30°C for 45 minutes followed by 75°C for 20 minutes to inactivate the enzymes.
1.2: Purification of the formed dsDNA	The dsDNA was purified with Agencourt RNAClean XP beads (Beckman Coulter) to remove salts and enzymes from the completion ligation step. This was done as follows: a. 28 ul of Agencourt RNAClean XP beads (Beckman Coulter) was added to 10 ul of HBV DNA. b. This was gently mixed and incubated for 10 minutes at room temperature, and then placed on a magnetic stand for three minutes. c. Supernatant was carefully discarded without disturbing beads containing DNA targets. d. 200 ul of 80% ethanol was used to wash the beads which were then left to air dry for 10–15 minutes after the second ethanol wash. e. 12 ul of nuclease free water was added to the beads and gently mixed until completely resuspended. f. This mixture was then placed on a magnetic stand for three minutes and supernatant containing DNA was carefully removed and placed in new PCR tubes.
1.3: Quantification of HBV dsDNA	The purified HBV dsDNA was quantified using Qubit dsDNA broad range (BR) Assay Kit (ThermoFisher Scientific) as follows: a. In a thin walled clear 0.5 ml tube, 2 ul of HBV DNA was added to 198 ul of working solution (197 ul broad range buffer and 0.99 ul of broad range reagent). b. This mixture was incubated for two minutes at room temperature and then placed in a qubit fluorometer to quantify DNA in ng/ul.
Step 2: Library preparation using NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs).	
2.1: Fragmentation	Fragmentation involves chopping of DNA input to fragment size of between 150–300 base pairs (bp). This was done as follows: a. In a 0.2 ml thin wall PCR tube, 26 ul of purified HBV dsDNA (100 pg–100 ng) was added to 7 ul NEBNext Ultra II FS Reaction Buffer and 2 ul of NEBNext Ultra II FS Enzyme Mix to form a total volume of 35 ul. b. This was mixed and incubated for 30 minutes at 37^{0}C.
2.2: Adapter Ligation	a. 35 ul of fragmented DNA solution was mixed with 30 ul of NEBNext Ultra II Ligation Master Mix, 1 ul of NEBNext Ligation Enhancer and 2.5 ul of NEBNext Adaptor for Illumina, forming a total volume of 68.5 ul. b. This formulation was incubated at 20^{0}C for 15 minutes. 3 ul of USER Enzyme was added to the ligation mixture, mixed well and incubated at 37^{0}C for 15 minutes.
2.3: Clean up	a. 57 ul (0.8X) of resuspended NEBNext Sample Purification Beads was added to the Adaptor Ligation reaction, mixed well, and incubated at room temperature for five minutes. b. Tubes containing the mixture were then placed on a magnetic stand to separate the beads from the supernatant. c. The supernatant was carefully discarded without disturbing beads containing DNA targets. 200 ul of 80% ethanol was used to wash the magnetic beads containing DNA targets.

	d. The beads were then left to air dry for five minutes after the second ethanol wash. 17 ul of 0.1X TE (10 mM Tris pH 8.0, 1 mM EDTA) was added to the beads, gently mixed until completely resuspended and then incubated for two minutes at room temperature. e. This mixture was then placed on a magnetic stand for five minutes and supernatant containing DNA was carefully removed and placed in new PCR tubes.
2.4: PCR amplification of Adaptor-ligated DNA	a. 15 ul of adaptor ligated DNA fragments was mixed with 25 ul of NEBNext Ultra II Q5 Master Mix and 10 ul of universal primer. b. The reaction was carried out at 98^0C for 30 minutes followed by three cycles of denaturation at 98^0C for 10 seconds and annealing at 65^0c for 75 seconds. Final extension was then done at 65^0C for five minutes. c. 45 ul (0.9X) of resuspended NEBNext Sample Purification Beads was added to the PCR reaction mix, mixed well and incubated for at room temperature for five minutes. d. Cleaning up of amplified DNA was done as described in **step 2.3 (b-e).**
2.5: Quality control, quantification of libraries and pooling of libraries	a. Quantification of adaptor ligated mixture was done as described in **step 1.3.** Quality control and pooling of libraries was done as follows: b. 2 ul of amplified adaptor ligated mixture and 2 ul of high sensitivity D1000 sample buffer was mixed in a new tube. Another 2 ul of high sensitivity D1000 sample buffer and 2 ul of high sensitivity D1000 ladder were put in a separate tube. Formations in these tubes were mixed and then loaded into the Agilent 4200 TapeStation instrument system to visualise the size distribution on libraries in base pairs using electropherogram analysis. c. Libraries for different samples were then pooled according to their molar concentration to generate an equimolar library pool. d. Molar calculation was done as follows: Molar concentration (nM)= [(ng/ul [obtained from qubit measurement])/ (size of base pair [obtained from TapeStation instrument] * 660 g/ml)] * 1,000,000.
Step 3: Capture target enrichment	a. Pooled libraries included in each sequence capture experiment had an absolute amount of between 300–1000ngs. As an example, for a volume containing 500 ng, this was calculated as follows:500 ng * total volume of the library pool (ul)/Total amount of DNA in the library pool (ng) b. Pooled libraries were enriched using short probes covering all HBV genotypes based on SEQCAP EZ protocol designed by Dr. Azim Ansari. Captured DNA library was amplified using PCR and then quantified and diluted to a concentration of 4 nM.
Step 4: Sequencing	The quantified amplified captured library pool was sequenced on an Illumina Mi-Seq using a v3 300bp paired end kit.

ml: millilitre. ul: microlitre. ng: nanogram. bp: base pairs. mM: millimole. g: gram. nM: nanomole.
Dr. Anna McNaughton and Dr. Louise Downs helped perform the completion-ligation and HBV dsDNA purification for some of the samples. These experiments were done at containment level 2 (CTL 2) laboratory at the Peter Medawar Building. Library preparation and sequencing was performed by Dr. Mariateresa de Cesare at the Wellcome Centre for Human Genetics, Old Road Campus.

5.2.3 TFV drug levels quantification

Assessment of TFV drug levels was performed using liquid chromatography at the Cambridge Clinical laboratories (https://www.camclinlabs.co.uk).

5.2.4 Analyses

5.2.4.1 Illumina sequence analysis

For **Tygerberg HCC cohort**, generated reads pairs and adapter sequences were de-multiplexed using QUASR v7.01 and CutAdapt v1.7.1, respectively (Martin, 2011). Reads mapping to the human reference sequence and reads with less than 50 base pairs (bp) length were discarded. This was done using Bowtie v2.2.4 (Langmead and Salzberg, 2012). The remaining reads were mapped to HBV reference sequences for genotypes A–J using BWA mem v0.7.10 (Li and Durbin, 2009).

For samples from **OxSAHep project, Oxford viral hepatitis project** and **London HBV cohort**, bioinformatic analysis was performed using Tanoti v1.3 (https://bioinformatics.cvr.ac.uk/software/tanoti/), with genotyping using the HBV-GLUE resource (http://hbv-glue.cvr.gla.ac.uk/).

These analyses were supported by Dr. Azim Ansari, Dr. Joshua Singer, Dr. Vattipally Sreenu and Dr. Anna McNaughton.

5.2.4.2 Identification of HBV RAMs and VEMs

I looked for HBV RAMs and VEMs in consensus sequences generated by both Sanger and Illumina, and also within minor variants generated by Illumina. I used a list of HBV RAMs and VEMs I assimilated from published literature; these are provided in **chapter 3: section 3.2.1.2 (Table 3.1)** and **section 3.2.1.3 (Table 3.2).**

5.2.4.3 Statistical analysis

a. Sequence data statistical analysis

Using the sequence data generated, I set out to describe the prevalence and risk factors associated with HBV RAMs and VEMs among adults with CHB recruited from South Africa and the UK.

I grouped sequences from South Africa into one group (n=50) and those from the UK into a second group (n=73), and summarised characteristics of participants in each group using proportions for categorical/binary variables and medians and inter-quartile ranges (IQR) for continuous variables. I used Fisher's exact test to compare categorical/binary variables and Mann-Whitney U test to compare continuous variables.

I assessed for the presence of polymorphisms that have been reported in association with resistance, listed in **chapter 3: section 3.2.1.2 (Table 3.1)** and **section 3.2.1.3 (Table 3.2),** in consensus and deep sequence data.

For consensus sequence analysis, I calculated the prevalence of individual mutations for each region as follows:

$$Prevalence = \frac{Total\ number\ of\ sequences\ with\ a\ specified\ mutation}{Total\ number\ of\ sequences\ obtained\ from\ individuals\ infected\ with\ CHB\ recruited\ from\ South\ Africa\ or\ the\ UK}$$

I reported 95% CI and p-values from Fisher's exact text comparing the proportions of each RAM/VEM present in consensus sequences for each region. I calculated 95% CI using an online software Epitools (http://epitools.ausvet.com.au).

I grouped consensus sequences into two groups: sequences without any RAMs/VEMs; and sequences with ≥1 RAM/VEM. I used logistic regression, reporting odds ratio (OR) and 95% CI for the association between RAMs/VEMs and specific host and viral factors such as age, sex, HCC status, HIV status, HBeAg status, HBV DNA VL, treatment exposure, genotype, median coverage by Illumina sequencing and region of recruitment.

For deep sequence analysis, I focused on sequences with >1000 median coverage by Illumina sequencing to ensure analysis of good quality reads. I assessed for the prevalence of individual mutations in HBV variants generated from each sample. Considering HBV variants from all samples together, I grouped these variants into three groups according to the presence of

RAMs/VEMs as follows: (i) variants with no RAMs/VEMs present; (ii) minor variants with ≥1 RAMs/VEMs (occurring at <20% of the viral population); and (iii) variants with RAMs/VEMs occurring at >20% of the viral population. I used multinomial logistic regression reporting beta coefficients and 95% CI for the association between presence of RAMs/VEMs and specific host and viral factors.

I set the initial threshold for significance at $p < 0.05$. However, to account for multiple comparisons, I used a Bonferroni approach, dividing the significance threshold of 0.05 by the total number of comparisons (i.e. 0.05/11 variable comparisons = 0.005). I therefore used $p < 0.005$ as the final threshold for significance. I used GraphPad Prism v7·0 and Stata version 13.1 for data visualisation and statistical analyses.

b. Statistical analysis for TFV drug levels

I described TFV drug levels in plasma/PBMCs samples of individuals with varying HBV VL to determine treatment compliance and assessed for associated risk factors. I used linear regression to assess for the association between TFV plasma levels with specific patient and viral attributes such as HBV VL, duration of treatment, duration of sample storage, and age. I used Mann-Whitney U test to assess for the association between TFV plasma levels and sex.

I set the threshold for significance at 0.05 and I used GraphPad Prism v7·0 for data visualisation and statistical analyses.

5.3 Results

5.3.1 Summary of sequenced samples obtained from individuals recruited from South Africa and the UK

In order to generate HBV sequence data representing South Africa and the UK, I worked with samples from adults with CHB recruited through four clinical cohorts. Sequences were generated

by Sanger and Illumina methods, as described **chapter 4: section 4.2** and **chapter 5: section 5.2.2,** respectively.

A total of 123 HBV DNA samples that were available based on recruitment of adults in hepatitis clinic in South Africa and the UK were submitted for sequencing, from which I was able to generate 120/123 full length HBV sequences. Characteristics of the 120 sequenced sample are provided in **Table 5.5**.

Table 5.5: Summary of samples sequenced from each cohort.

Sequencing platform	Region	Total no. of samples submitted for sequencing	Total no. of samples successfully sequenced
Tygerberg HCC cohort	**South Africa**	**34**	**34**
OxSAHep project	**South Africa**	**15**	**13**
Oxford viral hepatitis project	**UK**	**32**	**32**
London HBV cohort	**UK**	**42**	**41**
Total	**-**	**123**	**120**

HCC: hepatocellular carcinoma. HBV: Hepatitis B virus.

Compared to samples obtained from the UK, samples obtained from South Africa had more genotype A sequences ($p<0.001$) and a higher median coverage by Illumina sequencing ($p<0.001$). Individuals with a mixed-race ethnicity, and individuals who were HIV and HCC positive were only present in the South African cohort, whereas individuals with Asian and Caucasian ethnicity, and samples with genotype B and C sequences were only present in the UK cohort **(Table 5.6).**

Table 5.6: Characteristics of 120 individuals with chronic hepatitis B virus (HBV) infection recruited from South Africa and the UK, and summary of their HBV sequence data.

		South Africa	UK	P value
Successfully sequenced, n (%)		47/50 (94.0)	73/73 (100)	0.16
Male sex, n (%)		36/47 (76.6)	31/73 (42.5)	0.01
Ethnicity	**African/Black**	7/47 (14.9)	10/73 (13.7)	>0.99
	Asian	0	24/73 (32.9)	**<0.001**
	Caucasian	0	12/73 (16.4)	**0.003**
	Mixed Race	6/47 (12.8)	0	**<0.001**
	Other/unknown	34/47 (72.3)	27/73 (35.6)	**<0.001**
Median Age in years, median (IQR)		41 (33 – 50)	41 (37 – 46)	0.71
HIV-positive, n (%)		17/47 (36.2)	0	**<0.001**
HBeAg-positive, n (%)		23/47 (48.9)	17/73 (23.3)	0.04
HCC present, n (%)		21/47 (44.7)	0	**<0.001**
On treatment, n (%)		17/47 (36.2)	19/73 (26.0)	0.54
Median HBV viral load (VL) IU/ml (log_{10})		5.71 (4.70 – 7.16)	4.85 (3.84 – 6.19)	0.03
Genotype	**A, n (%)**	34/47 (72.3)	17/73 (23.3)	**<0.001**
	B, n (%)	0	8/73 (11.0)	0.02
	C, n (%)	0	15/73 (20.5)	**<0.001**
	D, n (%)	9/47 (19.1)	21/73 (28.8)	0.28
	E, n (%)	4/47 (8.5)	12/73 (16.4)	0.28
Median coverage/site by Illumina sequencing (log_{10})		3.69 (2.68 – 4.86)	2.42 (1.80 – 3.22)	**<0.001**

Fisher's exact test was used to compare categorical/binary variables. Mann-Whitney U test was used to compare continuous variables. I set the threshold for significance set at 0.003 to account for multiple comparisons by dividing the significance threshold of 0.05 by the total number of variables (19 variables) performed on the dependent variable (i.e. 0.05/19=0.003). There was a positive correlation between high median coverage by Illumina sequencing and high HBV VL levels (r=0.64, p<0.001).

5.3.2 Prevalence of HBV RAMs and VEMs at consensus level

120 HBV consensus sequences were generated from serum samples of individuals recruited from South Africa (n=47) and the UK (n=73). Using the list of HBV RAMs and VEMs provided in **chapter 3: section 3.2.1.2 (Table 3.1)** and **section 3.2.1.3 (Table 3.2),** I assessed for polymorphisms associated with drug resistance at 48 sites across the HBV RT protein and for polymorphisms associated with vaccine resistance at 31 sites across the HBV S protein. I identified RAMs at 69% (33/48) of the sites across the HBV RT protein and VEMs at 65% (20/31) of the sites across the HBV S protein **(Figure 5.2).**

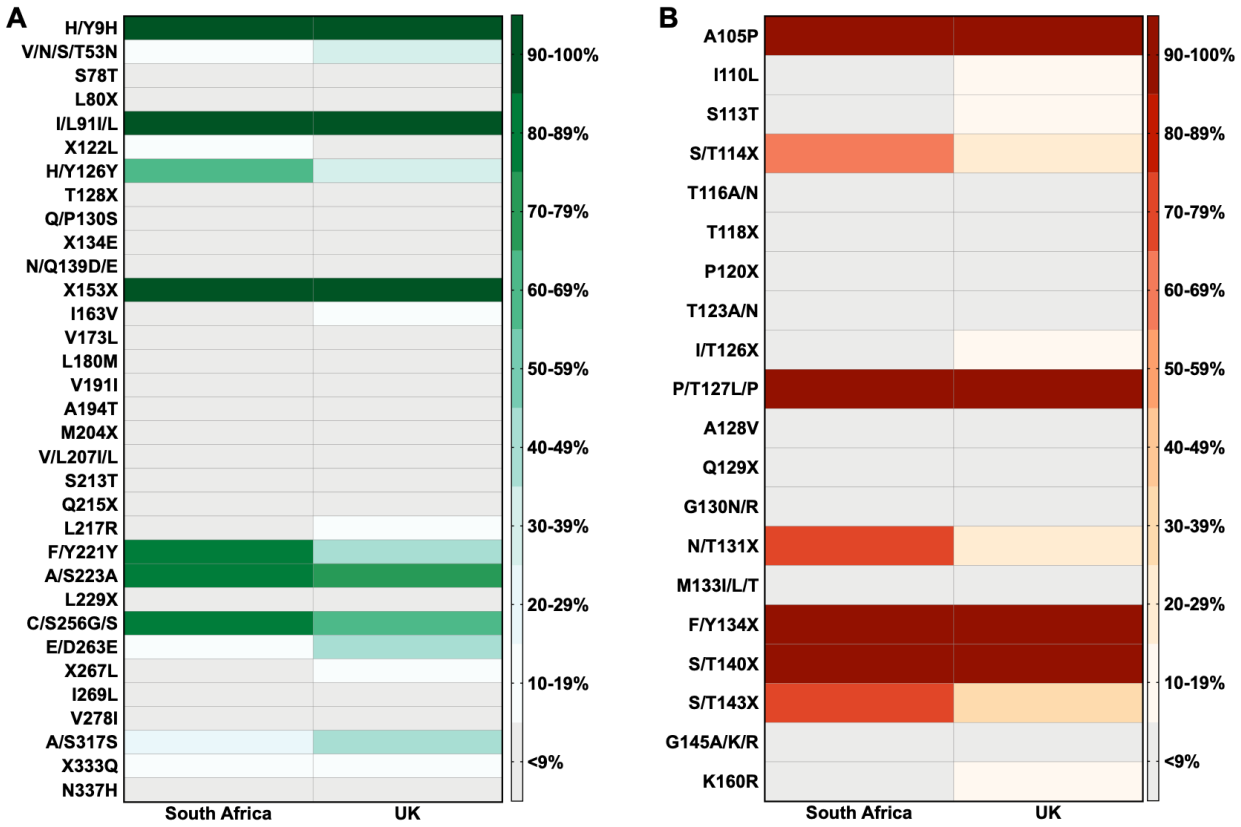


Figure 5.2: Prevalence of individual hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) obtained from analysing consensus sequences isolated from 120 individuals infected with chronic HBV infection recruited in South Africa (n=47) and the UK (n=73). A. Showing the prevalence of RAMs in 33 sites across the HBV RT protein. **B.** Showing prevalence of VEMs in 20 sites across the HBV S protein**.** L80X represents L80I/M/V. X1227 represents I/F/H/L/N/Y122L. T128X represents T128A/I/N. X134E represents D/H/N134E. X153Q/W represents Q/R/W153W/Q. M204X represents M204I/V/S/Q. Q215X represents Q215E/H/P/S. L229X represents L229G/FV/W. X267L represents H/L/M/Q267L. X333Q represents K/Q/T333Q. S/T114X represents S/T114F/RT. T118X represents T118A/R/V. P120X represents P120A/E/N/Q/S/T. I/T126X represents I/T126A/H/I/N/R/S. Q129X represents Q129H/N/R/P. N/T131X represents N/T131I/N/S. M133X represents M133I/L/T. F/Y134X represents F/Y134F/I/L/Y. S/T140X represents S/T140I/S/T. K141X represents K141E/I/R. S/T143X represents S/T143M/L/T. D144X represents D144A/E/G/H/N.

Polymorphisms in 10/33 sites within the HBV RT protein and 7/20 sites within the S protein were wildtype amino acid in reference sequences of some genotypes see **chapter 3: section 3.2.3.3 (Table 3.4** and **Table 3.5).** I therefore excluded these polymorphisms in the subsequent analysis, focusing only on RAMs/VEMs that were not present as wildtype amino acid in any reference sequences of genotypes A–E.

The prevalence of individual RAMs across 23/33 sites ranged between 0–15% in sequences from both South Africa and the UK **(Figure 5.3A)**, whereas prevalence of individual VEMs across 13/20 sites ranged between 0–8% and 0–4% in sequences from South Africa and the UK, respectively (**Figure 5.3B)**. There were no differences in prevalence of individual RAMs or VEMs between South Africa and UK (>0.005).

RAM M204I/V which is best described to cause resistance to 3TC, ETV and TFV (individually or synergistically)(see **chapter 2**), had a prevalence of 8% and 1% in South Africa and the UK, respectively, whereas VEM G145A/R which has been best described to cause HBV vaccine resistance (Carman, 1997), had a prevalence of 0% and 3% in South Africa and the UK, respectively (**Figure 5.3A** and **Figure 5.3B)**.

43% (52/120) of the sequences had RAMs in ≥1 of these 23 sites; 46% (22/47) and 41% (30/73) of these sequences were obtained from South Africa and the UK, respectively. 15% (18/120) of the sequences had VEMs in ≥1 of these 13 sites; 19% (9/47) and 12% (9/73) of these sequences were obtained from South Africa and the UK, respectively. There were no differences in the total number of sequences with RAMs or VEMs between South Africa and the UK (**Figure 5.3C** and **Figure 5.3D)**.

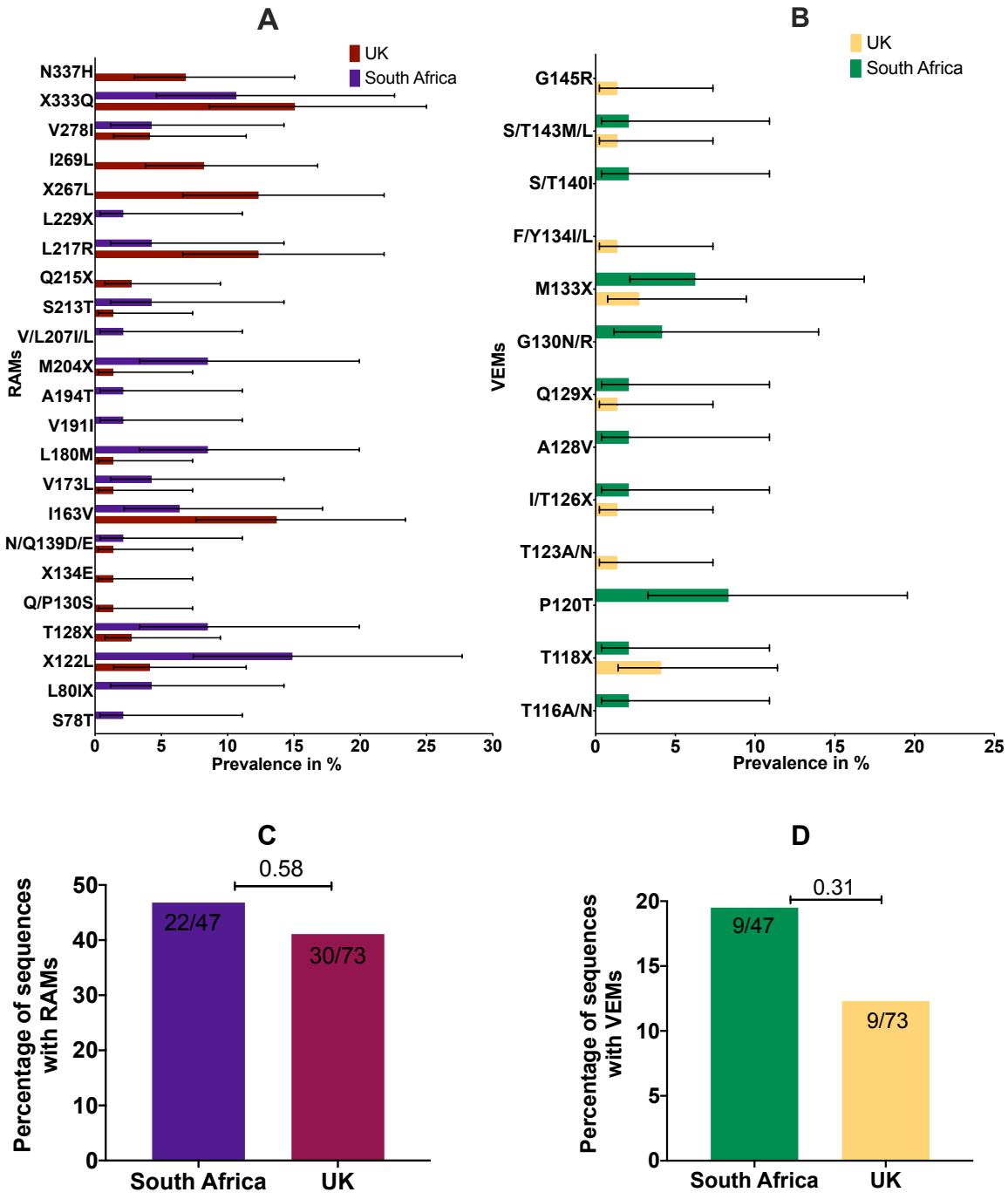


Figure 5.3: Prevalence of hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) obtained from analysing consensus sequences isolated from 120 individuals infected with chronic HBV infection recruited in South Africa (n=47) and UK (n=73). A. Compares the prevalence of individual RAMs across 23/33 sites with HBV RT protein in sequences obtained from South Africa and the UK (these RAMs were not present as wildtype amino acid in HBV reference sequences A–E). **B.** Compares the prevalence of individual VEMs across 13/20 sites with HBV S protein in sequences obtained from South Africa and the UK (these VEMs were not present as wildtype amino acid in HBV reference sequences A–E). **C.** Compares the percentage of sequences with RAMs between South Africa and the UK. **D.** Compares the percentage of sequences with VEMs between South

Africa and the UK**.** Fisher's exact test was used to compare the prevalence of RAMs or VEMs between South Africa and the UK. I set the threshold for significance set at 0.002 to account for multiple comparisons by dividing the significance threshold of 0.05 by the total number of variables (23 RAMs) performed on the dependent variable (i.e. 0.05/23=0.002); I used these values to both RAMs and VEMs. L80X represents L80I/M/V. X1227 represents I/F/H/L/N/Y122L. T128X represents T128A/I/N. X134E represents D/H/N134E. M204X represents M204I/V/S/Q. Q215X represents Q215E/H/P/S. L229X represents L229G/FV/W. X267L represents H/L/M/Q267L. X333Q represents K/Q/T333Q. T118X represents T118A/R/V. I/T126X represents I/T126A/H/I/N/R/S. Q129X represents Q129H/N/R/P. M133Xrepresents M133I/L/T.

Certain RAMs (either alone or in combination with other RAMs) have a higher probability of clinically significant TFV resistance [**chapter 2: section 2.2.3.4 (Table 2.2.2** and **Appendix Table 2.2.5)**]. I therefore sought evidence of these combinations of mutations in this dataset; 9% (4/47) and 3% (2/73) of these sequences were obtained from South Africa and the UK, respectively. Patterns of drug resistance to TFV are varied, which makes it difficult to derive clear definitions for TFV resistant variants (**Table 5.7).**

Table 5.7: Summary of hepatitis B virus (HBV) sequence data with drug resistance-associated mutations (RAMs) combination suggesting possible resistance to tenofovir (TFV) from analysing consensus sequences isolated from a total of 120 individuals infected with chronic HBV infection recruited in South Africa (n=47) and UK (n=73).

Participant ID	**HS89.3**	**209**	**9**	**7502**	**25**	**HEP-0712**
Combination of RAMs suggesting possible resistance to ETV, and/or TFV	V173L* + L180M* + M204I/V* + V278I	X153Q/W* + V173L* + L180M* + M204I/V*	L80I/V + X153Q/W* + L180M* + M204I/V* + X333Q	L80I/V + X153Q/W* + L180M* + A194T* + M204I/V* + X333Q	**S78T** + X122L + V278I	X267L + I269L* + V278I + X333Q + N337H
Region	UK	SA	SA	SA	SA	UK
Sex	Male	Male	Female	Female	Male	Male
HCC Status	Negative	Negative	Positive	Positive	Negative	Negative
HIV status	n/a	Positive	Positive	Positive	Positive	Negative
HBeAg status	Positive	Positive	Positive	Negative	Positive	Positive
Genotype	D	A	A	A	D	C
On treatment	No	Yes	No	Yes	Yes	Yes
Duration of treatment in months	N/A	63	N/A	N/A	N/A	N/A
HBV viral load IU/ml (log_{10})	4	8	8.2	4.8	5.2	8.2

X1227 represents I/F/H/L/N/Y122L; X153Q/W represents Q/R/W153W/Q; X267L represents H/L/M/Q267L; X333Q represents K/Q/T333Q. ETV: Entecavir. TFV: Tenofovir. HCC: Hepatocellular carcinoma. HBeAg: Hepatitis B virus e antigen. UK: United Kingdom. SA: South Africa.

* RAMs provided in 'short list' described in **chapter 2: section 2.2.3.4 (Table 2.2.2)**; these RAMs are supported by the highest quality evidence (i.e. isolated from treatment compliant individuals in whom viraemia was not suppressed by TFV and these RAMs were also tested in *in vitro* assays to measure the effect of TFV on viral replication in cell lines) RAM shown in bold has the ability to cause resistance to TFV in isolation (Shirvani-Dastgerdi et al. 2017).

5.3.3 Risk factors associated with HBV RAMs and VEMs at consensus level

In univariate analysis, there were no significant risk factors associated with sequences having ≥1 RAMs, however, genotype C sequences were 6.15 times more likely to have ≥1 VEMs compared to genotype A sequences, [OR: 6.15 (1.77, 21.31); p=0.004], **Table 5.8.** Upon adjusting for all other variables, there were no significant risk factors associated with sequences having ≥1 VEMs.

Table 5.8: Univariate analysis for risk factors associated with sequences having ≥1 drug resistance-associated mutations (RAMs) or vaccine escape mutations (VEMs) from analysing consensus sequences isolated from a total of 120 individuals infected with chronic hepatitis B virus infection recruited in South Africa (n=47) and the UK (n=73).

Sequences with ≥1 RAMs		Sequences with ≥1 VEMs	
	OR (95% CI)		OR (95% CI)
Sex		**Sex**	
Female	Ref	Female	Ref
Male	1.98 (0.90, 4.37)	Male	1.01 (0.42, 2.43)
Age[a]		**Age[a]**	
	0.99 (0.95, 1.03)		0.99 (0.94, 1.04)
Ethnicity		**Ethnicity**	
African	Ref	African	Ref
Asian	2.00 (0.52, 7.63)	Asian	7.47 (0.82, 68.11)
Caucasian	3.20 (0.72, 14.15)	Caucasian	1.23 (0.07, 21.64)
Mixed Race	2.40 (0.36, 16.21)	Mixed Race	1.20e-06 (0, Inf)
Unknown/other	1.71 (0.36, 8.08)	Unknown/other	11.43 (1.12, 116.71)
HCC		**HCC**	
Negative	Ref	Negative	Ref
Positive	3.42 (1.19, 9.85)	Positive	1.72 (0.60, 4.97)
HIV status		**HIV status**	
Negative	Ref	Negative	Ref
Positive	1.42 (0.48, 4.16)	Positive	0.68 (0.20, 2.36)
HBeAg status		**HBeAg status**	
Negative	Ref	Negative	Ref
Positive	1.23 (0.56, 2.70)	Positive	0.98 (0.40, 2.42)
Median HBV viral load IU/ml (log_{10}) [a]		**Median HBV viral load IU/ml (log_{10}) [a]**	
	1.09 (0.87, 1.37)		1.16 (0.89, 1.50)
On treatment		**On treatment**	
Yes	Ref	Yes	Ref
No	1.04 (0.46, 2.37)	No	0.91 (0.36, 2.29)
Genotype		**Genotype**	
A	Ref	A	Ref
B	0.13 (0.01, 1.11)	B	1.34 (0.24, 7.81)
C	2.44 (0.69, 8.70)	C	6.15 (1.77, 21.31) *
D	0.44 (0.17, 1.13)	D	1.25 (0.42, 3.72)
E	0.21 (0.05, 0.81)	E	0.27 (0.03, 2.32)
Region		**Region**	
UK	Ref	UK	Ref
South Africa	1.26 (0.60, 2.64)	South Africa	1.36 (0.58, 3.17)
Median coverage from Illumina sequencing (log_{10}) [a]		**Median coverage from Illumina sequencing (log_{10}) [a]**	
	1.08 (0.81, 1.44)		0.96 (0.68, 1.37)

*p value = 0.005. OR: Odds ratio. 95% CI: 95% confidence interval. Inf: Infinity.
I set the threshold for significance set at 0.005 to account for multiple comparisons by dividing the significance threshold of 0.05 by the total number of variables (11 variables) performed on the dependent variable.
[a] Represent continuous variables.
HCC: Hepatocellular carcinoma. HBeAg: Hepatitis B virus e antigen.

5.3.4 Prevalence of HBV RAMs and VEMs at deep sequence level

I analysed deep sequence data generated from 46 samples; these samples were selected because they had a median coverage of >1000 by Illumina sequencing. RAMs V191I and A194T were only present within minor variants (<20%) in four and seven samples, respectively, whereas RAMs V173L and L180M were only present in >20% of the viral population in two and one sample, respectively **(Figure 5.4A)**. RAMs L80I/M/V, X1227, Q/P130S, X267L and X333Q where not present in any of these selected sequences. All VEMs were present within minor variants (<20%) and VEMs T118X, P120T, I/T126X, G130N/R and M133X were also present in >20% of the viral population on some of the samples **(Figure 5.4B)**.

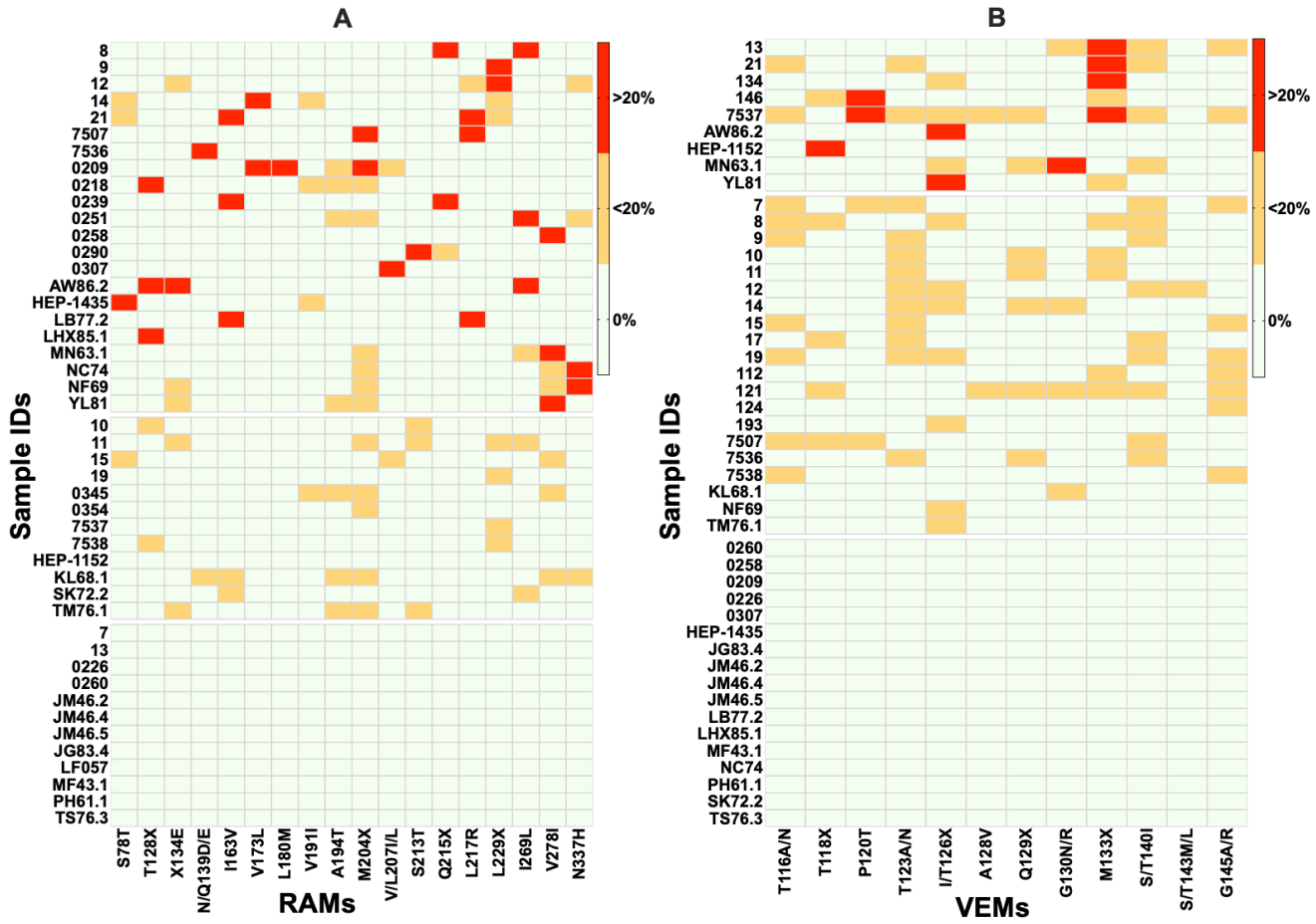


Figure 5.4: Frequency of hepatitis B virus (HBV) drug resistance-associated mutations (RAMs) and vaccine escape mutations (VEMs) obtained from analysing deep sequences isolated from 120 individuals infected with chronic HBV infection recruited in South Africa (n=47) and UK (n=73). A. Frequency of RAMs. **B.** Frequency of VEMs. Samples were selected because they had a median coverage of >1000. T128X represents T128A/I/N. X134E represents D/H/N134E. M204X represents M204I/V/S/Q. Q215X represents Q215E/H/P/S. L229X represents L229G/FV/W. T118X represents T118A/R/V. I/T126X represents I/T126A/H/I/N/R/S. Q129X represents Q129H/N/R/P. M133Xrepresents M133I/L/T.

5.3.5 Risk factors associated with HBV RAMs and VEMs at deep sequence level

Using multinomial logistic regression, I compared patient and viral attributes between three groups of sequences: (i) sequences with no RAMs/VEMs, (ii) sequences with RAMs/VEMs occurring as minor variants (<20%), and (iii) sequences with RAMs/VEMs present in >20% of the viral population. There were no significant risk factors associated with presence or absence of RAMs in any of these group of sequences (**Table 5.9**). VEMs were more likely to present within minor variants in samples obtained from individuals recruited from South Africa compared to samples obtained from individuals recruited from the UK [ß: 2.61 (1.00, 4.22); p=0.001] **(Table 5.10).**

Table 5.9: Analysis for risk factors associated of hepatitis B variants with or without specified drug resistance-associated mutations (RAMs) from analysing deep sequence data obtained from 46 individuals infected with chronic hepatitis B virus infection recruited in South Africa (n=27) and UK (n=19). The group of variants without any RAMs was used as the base outcome in multinomial logistic regression.

	≥1 RAMs present in minor variants (<20%)	**≥1 RAMs in variants occurring in >20% of the viral population**
	ß-coefficient (95% CI)	**ß-coefficient (95% CI)**
Sex		
Female	Ref	Ref
Male	-0.18 (-1.90, 1.53)	0.77 (-0.80, 2.35)
Age		
	0.02 (-0.08, 0.12)	0.04 (-0.05, 0.13)
Ethnicity		
African	Ref	Ref
Asian	-1.79 (-5.09, 1.51)	-0.47 (-3.21, 2.27)
Caucasian	-18.49 (-11757.31, 11720.32)	-17.99 (-7932.30, 7896.31)
Mixed Race	-1.10 (-4.68, 2.48)	-0.69 (-3.94, 2.56)
Unknown/other	-18.49 (-11757.31, 11720.32)	-17.99 (-7932, 7896.31)
HCC		
Negative	Ref	Ref
Positive	0.92 (-1.23, 3.06)	0.69 (-1.30, 2.69)
HIV status		
Negative	Ref	Ref
Positive	-1.95 (-4.58, 0.69)	-0.59 (-2.53, 1.35)
HBeAg status		
Negative	Ref	Ref
Positive	-1.44 (-3.40, 0.53)	-1.15 (-3.00, 0.66)
HBV DNA		
	-0.42 (-1.04, 0.19)	-0.24 (-0.79, 0.31)
On treatment		
No	Ref	Ref
Yes	-1.81 (-3.81, 0.18)	-1.35 (-3.00, 0.27)
Genotype		
A	Ref	Ref
B	-15.50 (-3459.97, 3428.79)	-1.01 (-4.01, 1.99)
C	-0.56 (-3.39, 2.47)	0.37 (-2.10, 2.85)
D	-1.48 (-3.52, 0.57)	-1.52 (-3.35, 0.31)
E	-1.25 (-3.95, 1.44)	-1.70 (-4.36, 0.95)
Region		
UK	Ref	Ref
South Africa	1.97 (0.08, 3.87)	1.09 (-0.36, 2.54)
Median coverage from Illumina sequencing (log_{10})		
	-0.13 (-1.10, 0.83)	0.24 (-0.55, 1.02)

*p value = 0.005. 95% CI: 95% confidence interval. ß-coefficient: Beta coefficient.
I set the threshold for significance set at 0.005 to account for multiple comparisons by dividing the significance threshold of 0.05 by the total number of variables (11 variables) performed on the dependent variable.
HCC: Hepatocellular carcinoma. HBeAg: Hepatitis B virus e antigen.

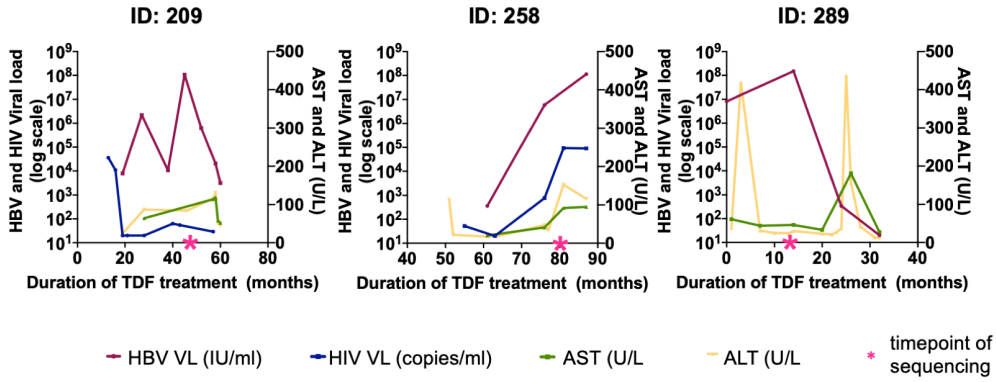
Table 5.10: Analysis for risk factors associated of hepatitis B variants with or without specified vaccine escape mutations (VEMs) from analysing deep sequence data obtained from 46 individuals infected with chronic hepatitis B virus infection recruited in South Africa (n=27) and UK (n=19). The group of variants without any VEMs was used as the base outcome in multinomial logistic regression.

	≥1 VEMs present in minor variants (<20%)	**≥1 VEMs in variants occurring in >20% of the viral population**
	ß-coefficient (95% CI)	**ß-coefficient (95% CI)**
Sex		
Female	Ref	Ref
Male	-0.06 (-1.50, 1.37)	-0.25 (-1.97, 1.48)
Age		
	-0.01 (-0.08, 0.06)	-0.03 (-0.12, 0.07)
Ethnicity		
African	Ref	Ref
Asian	-0.92 (-3.21, 1.38)	-0.51 (-2.94, 1.92)
Caucasian	-16.78 (-6110.85, 6077.30)	-16.71 (-7227.31, 7193.90)
Mixed Race	-0.00 (-2.53, 2.53)	-15.58 (-4116.78, 4085.62)
Unknown/other	-0.54 (-45555.07, 45554)	19.51 (-27631.04, 27670.07)
HCC		
Negative	Ref	Ref
Positive	18.02 (-4838.63, 4874.69)	17.42 (-4839.24, 4874.08)
HIV status		
Negative	Ref	Ref
Positive	-0.79 (-2.71, 1.13)	-1.61 (-4.29, 1.07)
HBeAg status		
Negative	Ref	Ref
Positive	-1.10 (-2.67, 0.47)	-1.32 (-3.17, 0.53)
HBV DNA		
	-0.23 (-0.73, 0.27)	-0.47 (-1.08, 0.15)
On treatment		
No	Ref	Ref
Yes	-0.96 (-2.49, 0.58)	-0.29 (-2.16, 1.59)
Genotype		
A	Ref	Ref
B	-17.53 (-5745.07, 5710.01)	-17.63 (-8.555.75, 8520.48)
C	-1.97 (-4.47, 0.52)	-0.59 (-2.73, 1.56)
D	-1.57 (-3.30, 0.17)	-1.97 (-4.40, 0.45)
E	0.51 (-1.92, 2.93)	-15.35 (-3862.21, 3831.52)
Region		
UK	Ref	Ref
South Africa	2.61 (1.00, 4.22) *	1.10 (-0.58, 2.78)
Median coverage from Illumina sequencing		
	-0.20 (-0.90, 0.51)	-0.74 (-1.83, 0.34)

*p value = 0.005. 95% CI: 95% confidence interval. ß-coefficient: Beta coefficient.
I set the threshold for significance set at 0.005 to account for multiple comparisons by dividing the significance threshold of 0.05 by the total number of variables (11 variables) performed on the dependent variable.
HCC: Hepatocellular carcinoma. HBeAg: Hepatitis B virus e antigen.

5.3.6 Assessment of HBV virological suppression while on therapy with TFV in the presence of HBV RAMs

Among the 47 samples obtained from individuals with CHB recruited from South Africa which were successfully sequenced, samples from three individuals had HBV DNA VL of ≥20,000 IU/ml despite being on therapy with TFV for ≥12 months **(Figure 5.5).**



Participant ID	209	258	289
Age at recruitment (yrs)	49	42	70
Sex	Male	Male	Male
HIV status	+	+	-
Antiviral therapy	TDF+3TC+FTC	TDF+3TC+FTC	TDF
Elastography score (kPa)	6.9	5.1	9.1
HBV DNA viral load, IU/ml (log 10) at time of sequencing	8	7	8
HIV RNA viral load, copies/ml (log 10) at time of sequencing	2	5	N/A
Median coverage/site	4262	1601	14
HBV genotype	A	D	A
HBV reverse transcriptase polymorphisms potentially associate with TFV resistance	Y9H L91I H126Y R153W* V173L*† L180M*† M204V*† S223A C256S	Y9H F122L S223A D263E V278I A317S	Y9H L91I H126Y S223A C256S

Figure 5.5: Longitudinal viral load and liver function tests for three adults with detectable hepatitis B virus (HBV) viraemia >20,000 IU/ml despite ≥12 months prescribed tenofovir (TFV) therapy, with the corresponding clinical data for each patient. Underlined polymorphisms have been reported as RAMs but occur as wildtype amino acid in reference sequence of certain genotypes [see **chapter 3: section 3.2.3.3 (Table 3.4)**] *These RAMs have been identified based on a combination of both *in vivo* and *in vitro* data and are reported in ≥2 independent peer-reviewed sources, giving them the most stringent evidence base of all the polymorphisms listed (Sheldon et al. 2005; Lada et al. 2012; Park et al. 2019) (**Appendix Table 2.2.5**). †These RAMs cause HBV resistance to 3TC (Warner and Locarnini, 2014; Caligiuri et al. 2016; Beloukas and Geretti, 2017). 3TC: Lamivudine; FTC: Emtricitabine. TFV: Tenofovir.

Individual 209 had nine RAMs associated with TFV resistance (**Figure 5.5**), of which four RAMs (R153W, V173L, L180M, and M204V) have been identified based on the most stringent combination of evidence, reflecting both *in vivo* and *in vitro* data, and reported in ≥2 independent peer-reviewed sources (Sheldon et al. 2005; Lada et al. 2012; Park et al. 2019) (see **chapter 2: section 2.2.3.4**). This individual was also HIV coinfected however, his HIV RNA was well suppressed, thus providing a good evidence for adequate adherence to antiviral therapy increasing the probability that HBV drug resistance is genuinely present.

Individual 258 was also HIV coinfected and had six putative TFV RAMs (**Figure 5.5**). However, both HIV and HBV viraemia remained detectable suggesting a possible impact of incomplete adherence to therapy, with or without drug resistance in one or both viruses**.**

Individual 289 was HBV mono-infected with five putative TFV RAMs (**Figure 5.5**). HBV viral suppression occurred eventually after a cumulative 30 months on treatment indicating a possibility of slow VL suppression (see **chapter 2: section 2.2.4.6**).

5.3.7 Quantification of TFV drug levels in plasma/PBMCs to assess for treatment compliance

The previous sections describe the prevalence and risk factors associated with drug resistance (**sections 5.3.2 and 5.3.3**), and also explore presence of drug resistance-associated mutations as a possible explanation for detectable HBV viraemia in an individual on treatment (**section 5.3.6**). However, an alternative is that individuals may not adhere to prescribed therapy. In order to develop a better understanding of the relationship between HBV VL and drug levels, I measured TFV levels in plasma samples of 12 individuals recruited into the Oxford Viral hepatitis project. 3/12 of these individuals also had PBMCs assessed for TFV-DP levels. These 12 individuals were selected as they had been prescribed TFV for at least six months, and their plasma samples represented a range of HBV VL grouped as follows: [high level viraemia (HLV) HBV VL>2,000 IU/ml, n=1]; [low level viraemia (LLV) HBV VL <2,000 & >100 IU/ml, n=1]; and 10 individuals had undetectable VL HBV VL <100 IU/ml.

Table 5.11. Summary of assessment of tenofovir (TFV) drug levels in 12 hepatitis B virus (HBV) samples obtained from HBV mono-infected individuals recruited from the UK.

ID	HBV VL (IU/ml)	Duration of sample storage (years)	Age (years)	Sex	HBeAg status	Duration of Rx (months)	TFV Plasma levels (ng/ml)	TFV-DP intracellular levels (ng/ml)
HEP-1089*	15675	5	33	Female	Positive	21	155	n/a
HEP-1109	616	8	29	Unknown	Negative	70	Undetected	Undetected
HEP-0708	20	10	58	Male	Negative	71	230	n/a
HEP-0884	20	5	53	Male	Negative	27	Undetected	Undetected
HEP-0892	20	11	41	Female	Negative	108	Undetected	n/a
HEP-0905	20	4	51	Female	Negative	21	49	n/a
HEP-1044	20	5	41	Female	Negative	27	63	n/a
HEP-1054	20	3	40	Male	Negative	36	33	n/a
HEP-1071	20	6	57	Male	Negative	38	81	43.6
HEP-1076	20	7	40	Female	Negative	57	60	n/a
HEP-1230	20	4	33	Male	Negative	17	69	n/a
HEP-1493	20	3	37	Female	Negative	6	326	n/a

TFV: Tenofovir. TFV-DP: Tenofovir diphosphate. Rx: Treatment. RAMs: Resistant associated mutations. VL: Viral load. * The only sample sequenced, and no RAMs were detected which makes compliance a more likely explanation for persistent VL levels. Sequencing of other samples was not possible because very low VL levels.

9/12 individuals (HLV, n=1 and undetectable HBV VL, n=8) had detectable TFV plasma level; the one individual with HLV also had sequence data available, however, no RAMs were detected **(Table 5.11).** These nine individuals had TFV plasma levels of >26 ng/ml; this value was the lower bound of the 95% CI interval of the median TFV plasma level reported in a randomised pharmacokinetic study carried out among healthy males on treatment with TFV for six weeks and reported to be fully compliant (Drain et al. 2020) **(chapter one: section 1.13.2)**, thus suggesting likely compliance among these nine individuals.

TFV-DP levels were assessed in PBMC samples from three individuals **(Table 5.11)**, and despite all the three individuals being on treatment for ≥ 27 months, only one had detectable levels of TFV-DP at 43.6 ng/ml which is lower than that reported by published study at a median concentration of 100 (90 – 200) fmol/10^6cells among HIV negative individuals on TFV therapy for 30 days (Castillo-Mancilla et al. 2013).

There was no association between TFV levels with HBV VL, duration of treatment, duration of sample storage, as well as with age and sex of the participants (**Figure 5.6**). TFV-DP level was only available for one sample and therefore any further comparisons were not possible.

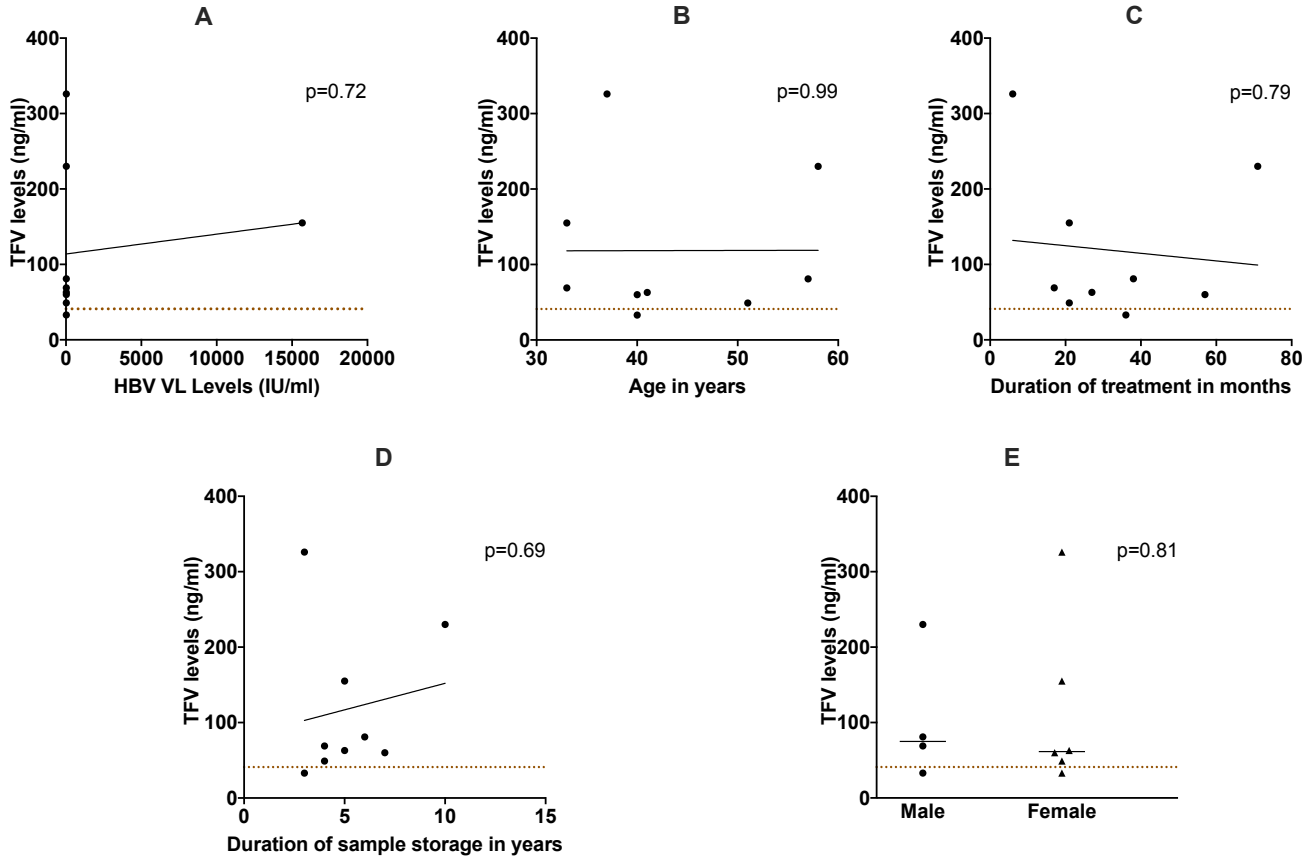


Figure 5.6: Comparison of tenofovir (TFV) plasma levels with patients' attributes of nine individuals with chronic hepatitis B virus (HBV) infection recruited from Oxford viral hepatitis project. A. Comparison of TFV plasma levels with HBV viral load (VL). **B.** Comparison of TFV plasma levels with participant's age. **C.** Comparison of TFV plasma levels with duration of treatment. **D.** Comparison of TFV plasma levels with duration of sample storage. **E.** Comparison of TFV plasma levels according to patient sex. The brown horizontal dashed line shows the median threshold of compliance which is 41 ng/ml (Drain et al. 2020) [see **chapter 1: section 1.13.2 (Table 1.5)**].

5.4 Discussion

5.4.1 Summary of findings

I describe the prevalence and risk factors associated with HBV drug RAMs and VEMs from analysing 120 sequences obtained from individuals infected with CHB recruited from South Africa and the UK. In South Africa, the prevalence of individual RAMs and VEMs ranged

between 0–15% and 0–8%, respectively, whereas in the UK, the prevalence of individual VEMs ranged between 0–15% and 0–4%, respectively. 5% (6/120) of the sequences had mutations suggesting possible ETV and/or TFV resistance; sequences with these mutations were identified in both South Africa and the UK. There were no differences in prevalence of RAMs and VEMs between these two settings and there were no risk factors associated with RAMs at both consensus and deep sequence level, although factors such as high VL levels and previous treatment exposure have been associated with the development of RAMs (Torresi et al. 2002; Bartholomeusz and Locarnini, 2006) (see **chapter 1: section 1.13**).

From this analysis, VEMs were more likely to be present in genotype C sequences; this finding corroborates that also reported in **chapter 3: section 3.2.3.2** for which genotype C had the highest prevalence of VEM G145A/R from analysing HBV sequence data downloaded from GenBank. Similarly, a recent analysis of HBV surface protein sequences obtained from a public database also reported a slightly higher percentage of VEMs in genotype C compared to other genotypes (Raheel, Choga and Blackard, 2020). Genotype C is predominant in Asia and Australia [**Chapter 1: section 1.6 (Table 1.1)**], and given that HBV birth dose vaccine coverage is highest in these regions at 84% compared to other regions (WHO, 2017b), the widespread exposure to HBV vaccine could result in the selection of dominant HBV variants with VEMs and therefore explain the higher likelihood of these mutations within genotype C sequences.

I also showed that VEMs were more likely to be present within minor variants in sequences obtained from individuals recruited from South Africa. High viral replication in combination with high mutation rates would explain the development of these mutations (see **chapter 1: section 1.13.1**) and given that HBV birth dose vaccine coverage is quite low in Africa estimated at 6% (WHO, 2019), the chance of these variants evolving to become dominant species may be be less likely due to low immune pressures due to majority of individuals being unvaccinated (see **chapter 1: section 1.10.3**).

5.4.2 Evidence of TFV resistance in Africa

I present the first potential case of TFV resistance in an adult being treated for CHB in Africa, having combined phenotypic observations of a TFV resistant phenotype with viral sequencing data. In particular, individual 209 recruited from South Africa, represents a strong evidence for HBV resistance to TFV, on the basis of high HBV DNA VL (>10^8 IU/ml) despite 60 months of therapy, with a suppressed HIV VL, and a combination of sequence polymorphisms that have been reported in association with TFV resistance. This individual was treated with TFV, 3TC and FTC, making it likely that exposure to 3TC resulted in the development of RAMs V173L, L180M and M204V, possibly setting the scene for the subsequent development of cross-resistance to TFV. Although FTC is not part of the recommended treatment options for CHB, it is widely used for HIV and is active against HBV. Combination of TFV and FTC has been shown to have better viral suppression compared to TFV monotherapy among treatment naïve individuals with CHB (Cui, Xu and Diao, 2015), suggesting these mutations confer resistance to both drugs.

In addition, some RAMs identified in this individual (Y9H, L91I, H126Y, S223A and C256S) are all present as wildtype amino acid in genotype A **[Chapter 3: section 3.2.3.3 (Table 3.4)**] and therefore the precise contribution of these polymorphisms to resistance remains to be determined with certainty. Identifying putative RAMs, and ascertaining their significance to clinical phenotype, is more difficult when these polymorphisms represent the 'wild type' sequence in certain genotypes (as described in **chapter 3: section 3.2.3.3** and **section 3.2.4.2**).

5.4.3 TFV plasma levels

I assessed 12 adults with CHB monoinfection displaying varying HBV VL levels, all of whom had been prescribed TFV therapy for at least six months.

Given that there were no RAMs to explain persistent viraemia and assuming good treatment compliance, detection of TFV plasma levels in the one individual with HLV might be explained

by slow treatment response possibly due to insufficient treatment delivery into the hepatocyte as explained in **chapter 2: section 2.2.4.6.** While drug levels in the hepatocyte can be assessed by measuring TFV-DP in PBMCs, PBMC sample from this individual was not available to carry out this assessment. Another possible explanation for persistent viraemia despite detectable TFV plasma levels in this individual could be due to 'white coat adherence' in which the individual took treatment shortly before the sample was taken, masking previous inconsistencies in taking medication as prescribed (Podsadecki et al. 2008; Baxi et al. 2014). Taking a detailed medical history from an individual on treatment might help pick up these inconsistencies, however an objective approach to assess drug levels would be ideal.

The three individuals (HEP-1109, HEP-0884 and HEP-1071) whose PBMCs I assessed for TFV-DP levels, aliquots of viable cells from these individuals were stored in liquid nitrogen for a period of 417, 260 and 312 weeks, respectively (**Table 5.11**). While it is possible that loss of TFV-DP might have occurred due to prolonged storage as reported in some studies whereby TFV-DP levels were lower in viable cryopreserved PBMCs compared to freshly processed PBMCs (Kiser et al. 2008; Baheti et al. 2011), another study demonstrated no loss of TFV-DP concentration in viable cells cryopreserved in liquid nitrogen for a period of 119 weeks (Grant et al. 2010).

TFV is eliminated primarily by renal excretion, and therefore, age, sex, ethnicity and body mass index may also have an impact on TFV and TFV-DP pharmcokinetics as these covariates have been associated with renal funtion (Baheti et al. 2011; Naim et al. 2011). One study reported a lower plasma TFV concentration but increased intracellular TFV-DP concentration among HIV-1 children and young person compared to adults who were all receiving the usual dose of 300mg of TFV once daily (Baheti et al. 2013). A lower plasma TFV concentration among these children was thought to be due higher plasma clearance rate possibly because children/young people have a larger kidney size relative to body size compared to adults; and the increased intracellular TFV-DP concentration in younger people was thought to be because they are more susceptible to the formation of TFV-DP from a given

level of exposure to TFV or have a slower intracellular elimination compared to adults (Baheti et al. 2013). In my dataset, the assessment of these associations were limited to due to small sample size.

This preliminary assessment shows the need for more robust longitudinal studies that will assess treatment compliance by measuring drug levels in samples (such as plasma, PBMCs, hair and urine) obtained from HBV monoinfected individuals and the associated risk factors, as well as assess the impact of time at which samples should be collected with respect to the treatment dose, as there still remains a major gap in literature and published guidelines on the appropriate threshold to confirm treatment compliance and appropriate time to collect samples for drug levels assessment (see **chapter 1: section 1.13.2**). Furthermore, given that drug levels are likely to be related to the timing of collection of serum sample with respect to most recent drug dose, a standardise approach that ensures accurate assessment of when the last dose was taken and information on processing and storage for HBV samples are necessary for HBV therapeutic drug monitoring.

5.4.4 Caveats and limitations

The data presented here were obtained from individuals recruited from different studies which increases heterogeneity and therefore this inhibits generalisation of these findings to other populations. Although this study analysed all available data obtained from our recruited participants, this study was underpowered given that the sample sizes were small. A number of sequences had limited sequence coverage depth by Illumina sequencing. However, to ensure analysis of authentic minor variants, I only included sequences with >1000 median deep coverage although this approach decreases the sample size. While I identified three individuals with persistent HBV viraemia despite being on treatment for >12 months with presence of HBV RAMs possibly explaining the persistent viraemia, incomplete treatment adherence can also result in virological breakthrough, and therefore assessment of drug levels in plasma and/ or PBMCs would be necessary to provide a more objective information on treatment exposure, especially in HBV monoinfected patients in whom suppression of HIV

cannot be used as a surrogate measure of treatment compliance. While I was able to assess for therapeutic drug levels in 12 serum samples and three PBMC samples obtained from individuals with CHB recruited in the UK, the cost of assessing drug levels is high, estimated at £100/sample which can be a limiting factor in the planning of large longitudinal studies which would be more informative.

Chapter six: A clinical algorithm for defining and managing HBV drug resistance

6.1 Background

Although current guidelines provide brief recommendations about drug resistance, there is no unified tool to summarise the approach to a patient in whom there is clinical concern about drug resistance, based on detection of viraemia despite NA therapy (manuscript in preparation).

6.2 Methods and Results

Informed by the data described in the previous chapters of this thesis, I have developed an algorithm **(Figure 6.1**) which provides a step-by-step approach to managing treatment failure in CHB infection in the clinic, which considers treatment compliance, use of sequence data to determine whether relevant RAMs are present, and assessment for other potential causes of treatment failure such as drug-drug interactions.

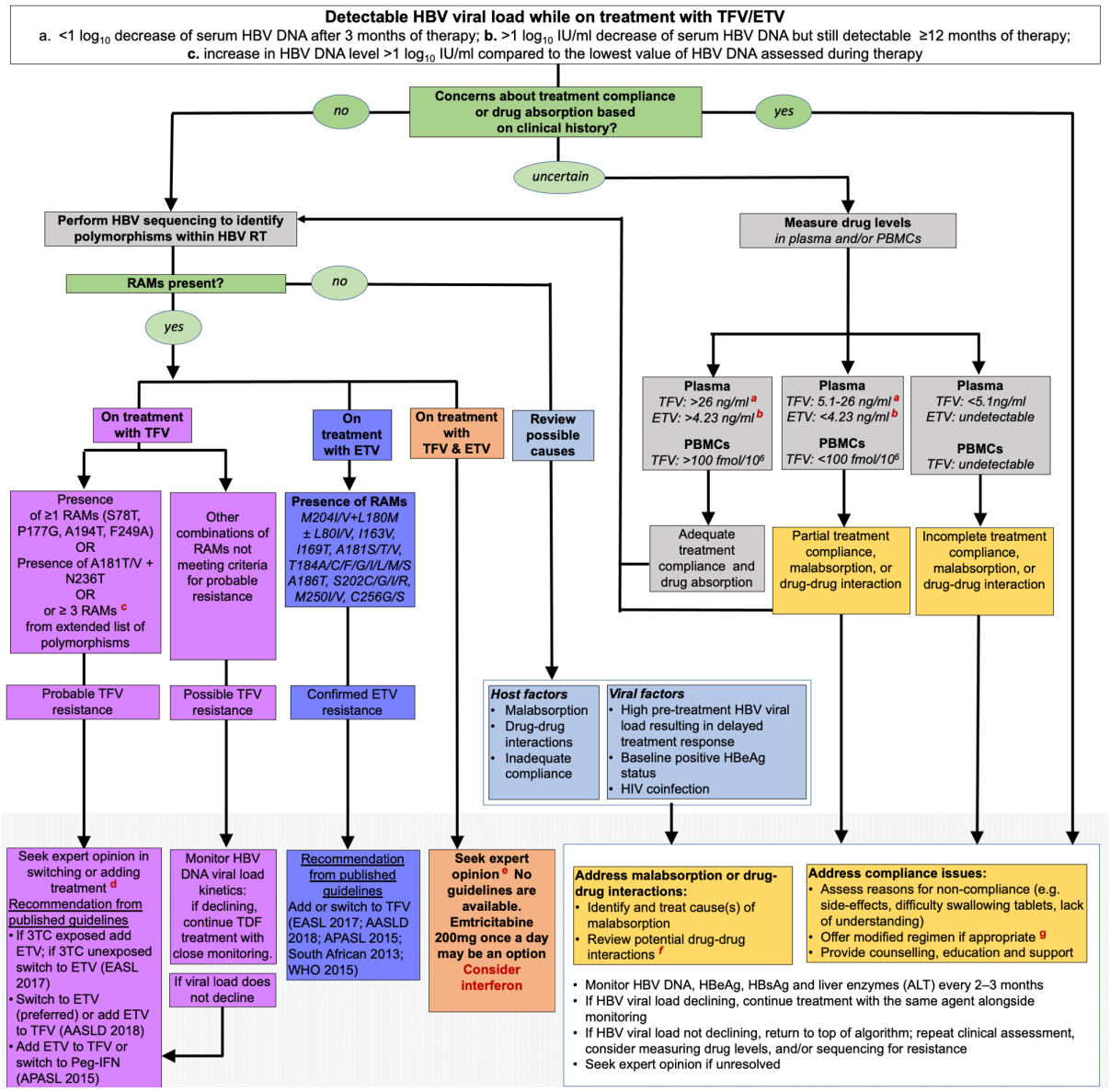


Figure 6.1: Algorithm for defining hepatitis B virus (HBV) resistance to tenofovir (TFV) and entecavir (ETV).

[a]41(IQR 26–52) ng/ml is the median TFV plasma level reported by a randomised controlled trial assessing TFV plasma levels among healthy males on TFV treatment for six weeks and reported to be fully compliant (Drain et al. 2020) [see **Chapter 1: section 1.13.2 (Table 1.5)**].

[b] 4.23 (SD 9) ng/ml is the median ETV plasma level reported by a randomised controlled trial assessing ETV plasma levels among healthy males on ETV treatment for 28 days and reported to be fully compliant (Yan et al. 2006) [see **Chapter 1: section 1.13.2 (Table 1.5)**].

[c] See **chapter 2: section 2.2.3.4 (Table 2.2.2 and Appendix Table 2.2.5)** for RAMs associated with TFV resistance.

[d] and [e] Therapeutic failure of TFV – whether in the presence or absence of known RAMs – should lead to an expert clinical decision about switching therapy or combining agents, as long as adherence has been optimised and supported. The presence of recognised RAMs, especially when in combination,

may support a change of therapy, considering ETV or combination therapy, although guidance and options are currently limited.

[f] See **chapter 1: section 1.13.3 (Table 1.6)** for drugs that can reduce TFV/ETV plasma levels and **chapter 2: section 2.2.4.6** on slow response to treatment.

[g] For individuals who find it difficult to swallow tablets, TFV is also available in granules/powder form which can be mixed with soft food and ingested immediately.

6.3 Discussion

6.3.1 What this algorithm adds

HBV RAMs used to determine HBV resistance to ETV and TFV in this algorithm are up-to-date and catalogued based on the quality of evidence of studies from which they were obtained (Mokaya et al. 2020a). In addition, this algorithm includes threshold for drug levels in plasma and PBMCs which might help inform on treatment compliance, including details that are not captured by existing treatment guidelines. Similarly, commercially available tools such as 'geno2pheno hbv' and TRUGENE HBV genotyping assay (Neumann-Fraune et al. 2014), only identify RAMs in HBV sequences using a list of RAMs which may not be up-to-date and do not include guidance around treatment compliance or other cause of treatment failure.

6.3.2 Limitations

This algorithm has been developed based on information obtained from published literature which are sparse and heterogenous. While it provides a framework for assessment, clinicians would need to be mindful that management would vary depending on the individual patient, for example considering age, renal function, other co-morbid conditions, pill burden, engagement/compliance with therapy, and patient preference. Furthermore, the application of this algorithm may be limited to high-income settings where healthcare costs are publicly funded; measuring of HBV DNA and testing of drug levels in plasma/PBMCs is expensive (see **chapter 5: section 5.4.4**), and might be inaccessible to individuals paying out-of-pocket, as is the case in many low-income countries (Chang et al 2019). However, drug level assessment

assays have been widely used for HIV care and might represent an opportunity for linkage with HBV care.

Treatment failure is a relatively rare event and therefore the practical evaluation of this algorithm would require a collaborative effort between multiple settings.

Chapter seven: Impact of drug resistance on the cost-effectiveness of TFV for HBV PMTCT

7.1 Background

Although the current practice guidelines for HBV PMTCT recommend screening antenatal women for infection using HBsAg, and risk-stratification based on further laboratory tests for HBeAg and/or HBV DNA VL, which can be used to stratify the risk of vertical transmission (see **chapter 1: section 1.10.3**), these are not routinely performed in many settings in Africa. This chapter evaluates the cost-effectiveness of antenatal screening for HBV infection using standard laboratory assays for HBsAg and treating HBsAg-positive women with TFV in sSA, and also evaluates the impact of HBV drug resistance on the cost-effectiveness of HBV PMTCT interventions. An important priority for Africa is to have affordable, accessible and sustainable PMTCT programmes given the risk of HBV PMTCT in this continent as described in **chapter 1: section 1.16.4**. This work has been published, describing application of TFV therapy for PMTCT in South Africa [published in BMC Public health (**Mokaya et al. 2019**)]. In this thesis I also present an expanded (unpublished) analysis to explore the application of this approach in other African countries.

7.2 Methods

7.2.1 Modelling the cost-effectiveness of HBV PMTCT

7.2.1.1 Target population and study perspective

I used a hypothetical cohort of 10,000 pregnant African women to evaluate the cost-effectiveness of three different strategies for antiviral therapy with TFV for HBV PMTCT:

a. **Strategy 1 (S1): No TFV prophylaxis.**

No pregnant woman is screened for HBsAg and therefore no HBV treatment is given perinatally. To date, this has been the situation in many resource-limited settings in sSA (**chapter 1: section 1.16.4)**.

b. Strategy 2 (S2): Screening and TFV prophylaxis for all women who test HBsAg positive.

All pregnant women are screened for HBsAg; those who test positive are treated with TFV from 28 weeks' gestation to four weeks post-partum.

c. Strategy 3 (S3): Screening and TFV prophylaxis for women who test HBsAg and HBeAg positive.

All pregnant women are screened for HBsAg; those who test positive are then screened for HBeAg. Only those who are HBeAg positive are treated with TFV from 28 weeks' gestation to four weeks post-partum. This is the current recommended practice (see **chapter 1: section 1.10.3).**

I populated this model with data using a healthcare system perspective and therefore considered costs that would be directly incurred by the Ministries of Health in four African Countries: Kenya, Nigeria, Cameroon and South Africa to represent East Africa, West Africa, Central Africa and Southern Africa, respectively. These countries were selected because data on prevalence of HBV infection among pregnant women and costs of treatment and diagnosis of HBV were available, and also to represent different regions in Africa. These costs relate to screening and treatment of HBV infection during pregnancy. Individual patient information was not included in this analysis and therefore ethics approval was not required for this study.

7.2.1.2 Assumptions

For the purposes of this analysis, I made a number of modelling assumptions:

- All pregnancies are singleton and result in the delivery of a live infant;

- All infants receive HBV vaccine at six, 10 and 14 weeks, as per current standard practice in Kenya, Nigeria, Cameroon and South Africa (Government of Kenya, 2013; Bigna et al. 2017; Chotun et al. 2017). Even for babies born to mothers who are HBsAg-positive, birth dose vaccination is not offered within 24 hours (due to lack of maternal screening in many sSA countries);
- HBV screening uptake is 100% among antenatal women;
- HBsAg and HBeAg are tested using laboratory assays which are 100% sensitive and specific;
- Infants born to mothers who were HBsAg-negative were assumed also to be HBsAg-negative;
- I made assumptions around point estimates due to lack of data.

7.2.1.3 Theoretic modelling approach

A decision tree providing a framework for comparison of the three strategies is shown in **Figure 7.1.** The decision tree considers the period covering weeks 28 through 40 weeks of gestation up to six months post-partum. Model structure was the same for all strategies. HBsAg-positive mothers had a defined probability of being HBeAg-positive or -negative; these probabilities were the same for all strategies. Children whose mothers were HBsAg-positive had a probability of being HBsAg-positive themselves (i.e. a case of MTCT). This probability depended on whether the mother was also HBeAg-positive and whether antiviral prophylaxis was received by the mother during pregnancy, which varied between strategies.

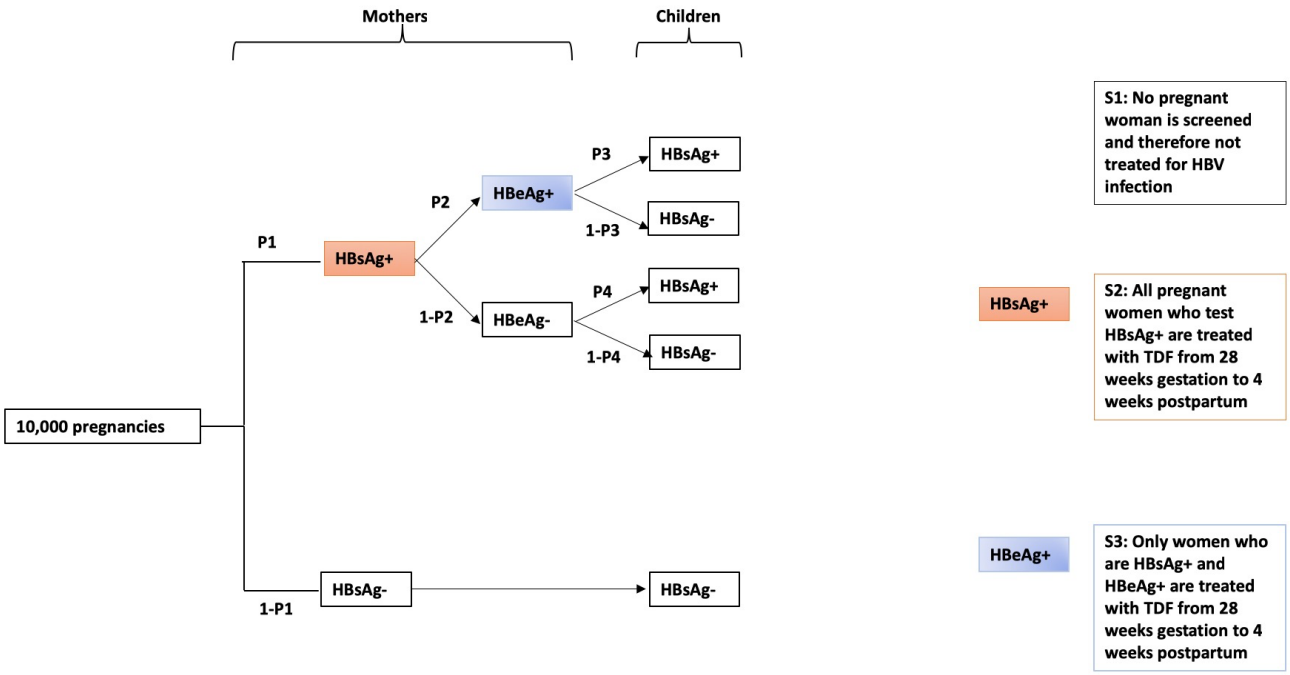


Figure 7.1: Decision tree showing possible approaches to evaluation of hepatitis B virus (HBV) infection in a simulated cohort of 10,000 antenatal women, and in their infants at age six months. P1 is the probability of women testing positive for HBsAg. P2 is the probability of women who are positive for HBsAg also testing positive for HBeAg. P3 is the probability of infants born to HBsAg-positive/HBeAg-positive mothers testing HBsAg positive at six months of age. P4 is the probability of infants born to HBsAg-positive/HBeAg-negative mothers testing HBsAg positive at six months of age. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; TDF: Tenofovir disoproxil fumarate. HBsAg: Hepatitis B surface antigen. HBeAg: Hepatitis B e antigen.

7.2.1.4 Measurement of effectiveness

I obtained prevalence of HBsAg and HBeAg among pregnant women for East Africa, West Africa, Central Africa and Southern Africa from a published systematic literature review and meta-analysis (Bigna et al. 2019). For each region, I used the HBeAg prevalence which was derived from the same meta-analysis (Bigna et al. 2019)**;** the same prevalence is used for all four regions because not all studies included in this review reported the prevalence of HBeAg among pregnant women.

I derived point estimates for perinatal transmission with and without antiviral therapy for HBV during pregnancy from a published systematic review and a meta-analysis (Keane, Funk and

Shimakawa, 2016; Hyun et al. 2017). In the absence of intervention (HBV vaccination, HBIg and/or antiviral therapy), the perinatal transmission rate for HBsAg-positive/HBeAg-positive mothers in sSA was estimated at 38%, and for HBsAg-positive/HBeAg-negative mothers was 4.8% (Keane, Funk and Shimakawa, 2016). Due to lack of data, I used these estimates in my model, although in my model I assumed that infants were vaccinated starting at the age of six weeks. I accounted for this uncertainty by including 95% CI around these estimates.

There is only one randomised control trial, which was carried out in China, that examines the efficacy of TFV on HBV PMTCT, in which perinatal TFV reduced the risk of infant HBsAg seropositivity by 71% (Pan et al. 2016). Data from a systematic review and meta-analysis are comparable, in estimating this value as 77% (Hyun et al. 2017). To obtain a point estimate for perinatal transmission rate for those receiving TFV peripartum prophylaxis, I therefore reduced the perinatal transmission rate for women receiving no TFV (HBeAg positive 38.3% (CI:7.0% - 74.4%); and HBeAg negative women 4.8% (0.1% - 13.3%) by 71%; these rates were therefore estimated at 11.1% (2.0% - 21.6%) for HBeAg-positive women and 1.4% (0.02% - 3.9%) for HBeAg-negative women.

Due to lack of data on HBV antiviral adherence rates among pregnant women in sSA, I used HIV antiretroviral adherence rate among women during and after pregnancy, obtained from a published systematic literature review (Nachega et al. 2012); this is estimated at 73.5%.

I incorporated an expected adherence of 73.5% into the model, assuming that those mothers who did not adhere to prophylaxis would incur the full cost of treatment but would have the same probability of transmission as untreated women.

Table 7.1: Parameters used for input into a model of cost-effectiveness of tenofovir for hepatitis B virus prevention of mother-to-child transmission in sub-Saharan Africa, based on a simulated cohort of 10,000 antenatal women and their babies.

Parameter	Southern Africa (South Africa)	East Africa (Kenya)	West Africa (Nigeria)	Central Africa (Cameroon)	Source (references)
	Point estimate Uncertainty (lower bound - upper bound)	**Point estimate** Uncertainty (lower bound - upper bound)	**Point estimate** Uncertainty (lower bound - upper bound)	**Point estimate** Uncertainty (lower bound - upper bound)	
P1: Probability of mother being HBsAg-positive	3.8% (2.0% - 6.0%)	5.5% (4.4% - 6.7%)	8.3% (7.1% - 9.5%)	9.7% (6.2% - 13.9%)	Bigna et al. 2019
P2: Probability of mother who is HBsAg-positive being HBeAg-positive	19.0 (14.6% - 23.8%)	19.0 (14.6% - 23.8%)	19.0 (14.6% - 23.8%)	19.0 (14.6% - 23.8%)	Bigna et al. 2019
P3: Probability of mother who is HBsAg-positive and HBeAg-positive having child who is HBsAg-positive (no PMTCT)	38.3% (7.0% - 74.4%)	38.3% (7.0% - 74.4%)	38.3% (7.0% - 74.4%)	38.3% (7.0% - 74.4%)	Keane, Funk, and Shimakawa, 2016
P4: Probability of mother who is HBsAg-positive and HBeAg-negative having child who is HBsAg-positive (no PMTCT)	4.8% (0.1% - 13.3%)	4.8% (0.1% - 13.3%)	4.8% (0.1% - 13.3%)	4.8% (0.1 - 13.3%)	Keane, Funk, and Shimakawa, 2016
Relative risk reduction (efficacy) of TFV	71% (26% - 89%)	71% (26% - 89%)	71% (26% - 89%)	71% (26% - 89%)	Pan et al. 2016
Antiviral adherence	73.5% (69.3% - 77.5%)	73.5% (69.3% - 77.5%)	73.5% (69.3% - 77.5%)	73.5% (69.3% - 77.5%)	Nachega et al. 2012
S2: Cost of diagnostics: laboratory test for HBsAg	8.96 USD per mother	29.29 USD per mother	2.60 USD per mother	17.00 USD per mother	See Table 7.2
S3: Cost of diagnostics: laboratory test for HBsAg and HBeAg	8.96 USD per mother (HBsAg test) + 8.96 USD for HBsAg-positive mother (HBeAg test)	29.29 USD per mother (HBsAg test) + 29.29 USD for HBsAg-positive mother (HBeAg test)	2.60 USD per mother (HBsAg test) + 6.50 USD for HBsAg-positive mother (HBeAg test)	17.00 USD per mother (HBsAg test) + 17.00 USD for HBsAg-positive mother (HBeAg test)	See Table 7.2
Treatment cost: monthly cost of TFV, applied to all HBsAg-positive women (S2) or only to HBsAg-positive/HBeAg-positive women (S3)	2.48 [a] USD per month	55.86[a] USD per month	26.00[a] USD per month	8.50[a] USD per month	See Table 7.2
Estimated relative risk reduction (efficacy) of TFV when combined with birth dose vaccine and HBIg	90% (85% - 95%)	90% (85% - 95%)	90% (85% - 95%)	90% (85% - 95%)	Nelson, Jamieson, and Murphy, 2014
Prevalence of TFV resistance	0.3%	0.3%	0.3%	0.3%	Hayer et al. 2013

[a]To cover the cost of TFV treatment from 28 weeks' gestation to four weeks post-delivery, I multiplied the cost for one month by four. A triangular distribution allows for uncertainty in all probabilities (except costs which I assume are fixed such as costs for HBsAg and HBeAg).

The uncertainty values for P1, P2, P3, P4 relative risk reduction for TFV (independently or when combined with birth dose vaccine and HBIg), antiviral adherence and TFV resistance were from 95% confidence intervals around the mean value.

USD: United States dollars. TFV: Tenofovir. HBsAg: Hepatitis B surface antigen. HBeAg: Hepatitis B e antigen. PMTCT: Prevention of mother-to-child transmission. HBIg: Hepatitis B virus immunoglobulin.

7.2.1.5 Outcomes

The outcome of this model was the cost per HBV infection avoided among infants at the age of six months. This outcome was selected because it is quantifiable, clinically relevant and could be easily compared between strategies.

7.2.1.6 Estimating costs

HBsAg screening cost applied to all women in **S2** and **S3**; HBeAg screening costs applied to women who tested positive for HBsAg in **S3**. TFV costs applied to all women who tested positive for HBsAg in **S2**, and women who tested positive for both HBsAg and HBeAg in **S3**.

Table 7.2: Summary of costs for laboratory assays and tenofovir used to model cost-effectiveness of tenofovir (TFV) for hepatitis B virus prevention of mother-to-child transmission in South Africa, Kenya, Nigeria and Cameroon.

Variables	South Africa to represent Southern Africa	Kenya to represent East Africa	Nigeria to represent West Africa	Cameroon to represent Central Africa
HBsAg laboratory assay costs	R 108.86	KES 3,149	N 1,000	CFA 10,000
HBeAg laboratory assay costs	R 108.86	KES 3,149	N 2,500	CFA 10,000
HBV DNA test costs	R 1173.32	KES 15,349	N 80,000	Not available
TFV costs	2.48 USD for 30 tablets	KES 6,000 for 30 tablets	N 10,000 for 30 tablets	CFA 5,000 for 30 tablets
Laboratory assay costs obtained from	National Health Laboratory Services (NHLS) and Department of Health (DOH)	2020 price list produced by pathologists Lancet Kenya	Website (https://nigerianprice.com/cost-of-treating-hepatitis-b-in-nigeria/).	Ministry of Health of Cameroon
TFV costs obtained from	International Medical products price guide for year 2015	Wholesale pharmacy in Kenya (http://www.kampharmacy.com)	Website (https://nigerianprice.com/cost-of-treating-hepatitis-b-in-nigeria/)	Ministry of Health of Cameroon
Exchange rate to USD	1 Rand = 0.083 USD	1 KES = 0.0093 USD	N 1 = 0.0026 USD	CFA 1 = 0.0017 USD
Exchange rate website and access date	www.xe.com website accessed on the 1st May 2018	www.xe.com website accessed on the 13th July 2020	www.xe.com website accessed on the 13th July 2020	www.xe.com website accessed on the 13th July 2020

R: Rand. KES: Kenya shillings. N: Naira. CFA: Cameroon franc. USD: United States dollars.

Dr. Dominique Goedhals (University of the Free State), Dr. Adrian Omanga (Kenyatta National Hospital), Dr. Lausdeus Otitochukwu (Ministry of Health Nigeria) and Dr. Feteh Fambombi Vitalis (Ministry of Health Cameroon), provided support in obtaining costs of TFV and diagnosis of HBsAg and HBeAg in South Africa, Kenya, Nigeria and Cameroon, respectively.

7.2.2 Scenario analysis

I assessed the impact of three additional scenarios, estimating the number of HBV MTCT cases, costs and incremental cost per infection avoided in each case.

7.2.2.1 Antenatal screening for HBV infection using a rapid point of care test (POCT) in South Africa

I derived costs for point of care test (POCT) for HBsAg from a 2015 price list produced by NHLS and DOH, South Africa. I converted the price of HBsAg POCT (R18) from South African Rand to USD; 1 Rand = 0.083 USD as at 1st May 2018.

I estimated the cost-effectiveness of using a POCT for HBsAg screening, based on a POCT sensitivity of 97.6% and specificity of 100%, compared to laboratory ELISA HBsAg testing as the gold standard (Chotun et al. 2017). I incorporated this into the model by reassigning the proportion of women expected to test falsely HBsAg-negative using a POCT into the untreated group and removing the cost of TFV treatment for these individuals.

I did not model cost-effectiveness for PMTCT using POCT for other regions (East, West and Central Africa) due to lack of data on the cost of POCT for HBsAg.

Table 7.3: Parameters used for point-of-care (POCT) input into a model of cost-effectiveness of tenofovir hepatitis B virus prevention of mother-to-child transmission in South Africa, based on a simulated cohort of 10,000 antenatal women and their babies.

Parameter	Point estimate	Source (references)
S2: Cost of diagnostics: POCT for HBsAg	2 USD per mother	NHLS and DOH (South Africa)
S3: Cost of diagnostics: POCT for HBsAg-positive and laboratory test for HBeAg-positive	2 USD for all mothers (HBsAg test) + 8.96 USD for all HBsAg+ mothers (HBeAg test)	NHLS and DOH (South Africa)
POCT sensitivity	97.6%	Chotun et al. 2017

NHLS represents National Health Laboratory Services. DOH represents Department of Health (South Africa). USD: United States dollars. HBsAg: Hepatitis B surface antigen. HBeAg: Hepatitis B e antigen.

7.2.2.2 Recommended PMTCT guidelines: Universal birth dose vaccination and HBIg

To assess the cost-effectiveness of antenatal screening and treating of HBsAg-positive women with TFV in combination with universal birth dose vaccine and HBIg, I estimated that perinatal transmission rate will be reduced by 90% (Nelson, Jamieson and Murphy, 2014). To incorporate the impact of TFV in this cohort, I therefore reduced the perinatal transmission rate by 90%; MTCT rates were thus estimated for HBsAg positive women who are HBeAg positive and HBeAg negative at 3.8% and 0.48%, respectively.

7.2.2.3 HBV resistance to TFV

In order to consider the possible impact of drug resistance on the PMTCT strategies outlined here, I estimated the prevalence of TFV resistance based on HBV sequences retrieved from the HBV database (Hayer et al. 2013) accessed on the 20th November 2018. These sequences were curated as described **Chapter 3: section 3.2.1.1.** For prevalence calculation, the numerator was the total number of sequences with mutations within the polymerase protein associated with reduced sensitivity/resistance to TFV (A194T and/or N236T+A181T/V) (Beloukas and Geretti, 2017; Shirvani-Dastgerdi et al. 2017) and the denominator was the total number of sequences available in the database (8/2838, 0.3%). The prevalence of these individual mutations has been described in **Chapter 3: section 3.2.3.1 (Figure 3.3** and **Figure 3.5).** This prevalence was incorporated into the model by re-assigning 0.3% of those receiving TFV into the untreated category (as although they receive the drug, resistance would render this functionally equivalent to no treatment).

7.2.3 Analytic methods and definitions

I used the following definitions:

- A strategy was ‘dominated’ if it had a higher expected cost and more predicted cases of HBV MTCT compared to an alternative strategy.

- The incremental cost per HBV infection avoided was determined by dividing the difference in cost between two strategies over the difference in number of infections (Jakubiak-Lasocka and Jakubczyk, 2014).

I compared the strategies by first applying decision rules to eliminate any strategies which were dominated by others, and then estimated the incremental cost per infection avoided for the remaining strategies.

The combined effect of uncertainty in probability estimates was assessed using probabilistic sensitivity analysis, with 1000 Monte Carlo simulations run. I used triangular distributions to approximate Beta distributions for probabilities. Triangular distributions required a lower and upper bound, with the peak taken as the expected (mean) value. The impact of uncertainty is shown on a cost-effectiveness plane, where sets of incremental costs and number of infections avoided are plotted for **S2** and **S3**, relative to **S1**.

7.2.4 Standardised criteria

This analysis conforms to the standardised criteria for economic evaluations of health interventions. The Consolidated Health Economic Evaluation Reporting Standards (CHEERS) checklist is available as **Appendix Table 7.1.**

7.3 Results

7.3.1 Cost-effectiveness of strategies for HBV PMTCT under base case assumptions

In four different countries within sSA, (South Africa, Kenya, Nigeria and Cameroon), I modelled three different strategies for peri-partum TFV prophylaxis: **S1**: no screening or antiviral therapy; **S2:** screening and antiviral prophylaxis for all women who test HBsAg positive; **S3:**

screening for HBsAg, followed by HBeAg testing and antiviral prophylaxis for women who are HBsAg positive and HBeAg positive **(Figure 7.1)**.

Based on the simulated scenario of 10,000 singleton pregnancies in each country, the following are the results for each region **(Table 7.1; Figure 7.1)**:

a. South Africa (Southern Africa)

With no antiviral therapy for HBV PMTCT **(S1)** and using a laboratory-based assay for HBsAg detection, this model predicts 42 infants would be infected with HBV at the age of six months (incidence 0.42%). If antiviral prophylaxis interventions are employed based on **S2** and **S3**, this would be reduced to 20 and 28 infants, (incidence 0.20% and 0.28%), respectively, at the cost of deploying the intervention for the whole population. Compared to **S1**, cost per infection avoided was 4,244 USD for **S2**, lower than for **S3** which would be more costly and avoid a lower number of infections **(Table 7.4; Figure 7.2A).**

b. Kenya (Eastern Africa)

With no antiviral therapy for HBV PMTCT **(S1)** and using a laboratory-based assay for HBsAg detection, this model predicts 61 infants would be infected with HBV at the age of six months (incidence 0.61%). If antiviral prophylaxis interventions are employed based on **S2** and **S3**, this would be reduced to 29 and 41 infants, (incidence 0.29% and 0.41%), respectively, at the cost of deploying the intervention for the whole population. Although **S2** was more costly than **S3**, **S2** was still the most cost-effective strategy because it had a lower cost per infection avoided at 12,993 USD when compared to **S1**, whereas the cost per infection avoided for **S3** was estimated at 16,617 USD when compared to **S1 (Table 7.4; Figure 7.2B).**

c. Nigeria (Western Africa)

With no antiviral therapy for HBV PMTCT **(S1)** and using a laboratory-based assay for HBsAg detection, this model predicts 93 infants would be infected with HBV at the age of six months (incidence 0.93%). If antiviral prophylaxis interventions are employed based on **S2** and **S3**, this would be reduced to 44 and 61 infants, (incidence 0.44% and 0.61%), respectively, at the

cost of deploying the intervention for the whole population. **S3** was the most cost-effective strategy because it had a lower cost per infection avoided at 1,493 USD when compared to **S1**, whereas the cost per infection avoided for **S2** was estimated at 2,292 USD when compared to **S1 (Table 7.4; Figure 7.2C).**

d. Cameroon (Central Africa)

With no antiviral therapy for HBV PMTCT **(S1)** and using a laboratory-based assay for HBsAg detection, this model predicts 108 infants would be infected with HBV at the age of six months (incidence 1.08%). If antiviral prophylaxis interventions are employed based on **S2** and **S3**, this would be reduced to 52 and 71 infants, (incidence 0.52% and 0.71%), respectively, at the cost of deploying the intervention for the whole population. Like Kenya, **S2** was the most cost-effective strategy because it had a lower cost per infection avoided at 3,624 USD when compared to **S1**, whereas the cost per infection avoided for **S3** was estimated at 5,209 USD when compared to **S1 (Table 7.4; Figure 7.2D).**

Table 7.4: Cost-effectiveness results under base case assumptions for screening for hepatitis B surface antigen using laboratory-based assay on a simulated birth cohort of 10,000 live singleton infants to African women infected with chronic hepatitis B virus infection.

Countries (Regions)	Strategy	Number of infant HBV infections[a] (95% CI)	Cost of deploying the intervention for the whole population[b] (n=10,000) in USD (95% CI)	Incremental cost per infection avoided (USD)
South Africa	**S1**	42 (23 - 81)	0	-
	S2	20 (11 - 49)	93,370 (93,101 - 93,708)	**4,244.09** (compared to S1)
	S3	28 (15 - 59)	93,721 (93,194 - 94,433)	6,694.36 (compared to S1) *Dominated[c] (by S2)*
Kenya	**S1**	61 (35 - 110)	0	-
	S2	29 (18 - 67)	415,792 (412,320 - 419,892)	**12,993.50** (compared to S1)
	S3	41 (22 - 82)	332,359 (326,675 -339,394)	16,617.95 (compared to S1)
Nigeria	**S1**	93 (49 - 160)	0	-
	S2	44 (25 - 97)	112,320 (110,434 -114,165)	2,292.24 (compared to S1)
	S3	61 (33 - 121)	47,796 (44,463 - 51,742)	**1,493.63** (compared to S1)
Cameroon	**S1**	108 (58 - 205)	0	-
	S2	52 (28 - 118)	202,980 (201,155 - 205,101)	**3,624.64** (compared to S1)
	S3	71 (39 - 151)	192,756 (190,027 - 196,525)	5,209.62 (compared to S1)

[a] World Health Organisation (WHO) criteria for HBV elimination states an aim of 90% reduction in new chronic infection (WHO 2016a).
[b] Price of TFV estimated at 2.48 USD/month (South Africa), 55.86 USD/month (Kenya), 26.00 USD/month (Nigeria), and 8.50 USD/month (Cameroon), for strategies S2 and S3. Price of TFV obtained from (International Medical Products Price Guide, 2016; http://www.kampharmacy.com/; Cost of Treating Hepatitis B in Nigeria, 2020; and Ministry of Health Cameroon).
[c] S3 is dominated due to both higher costs and higher infections compared to S2.
Incremental cost per infection avoided shown in bold represents the most cost-effective strategy in each country.
CI = confidence interval.
USD: United States dollars.

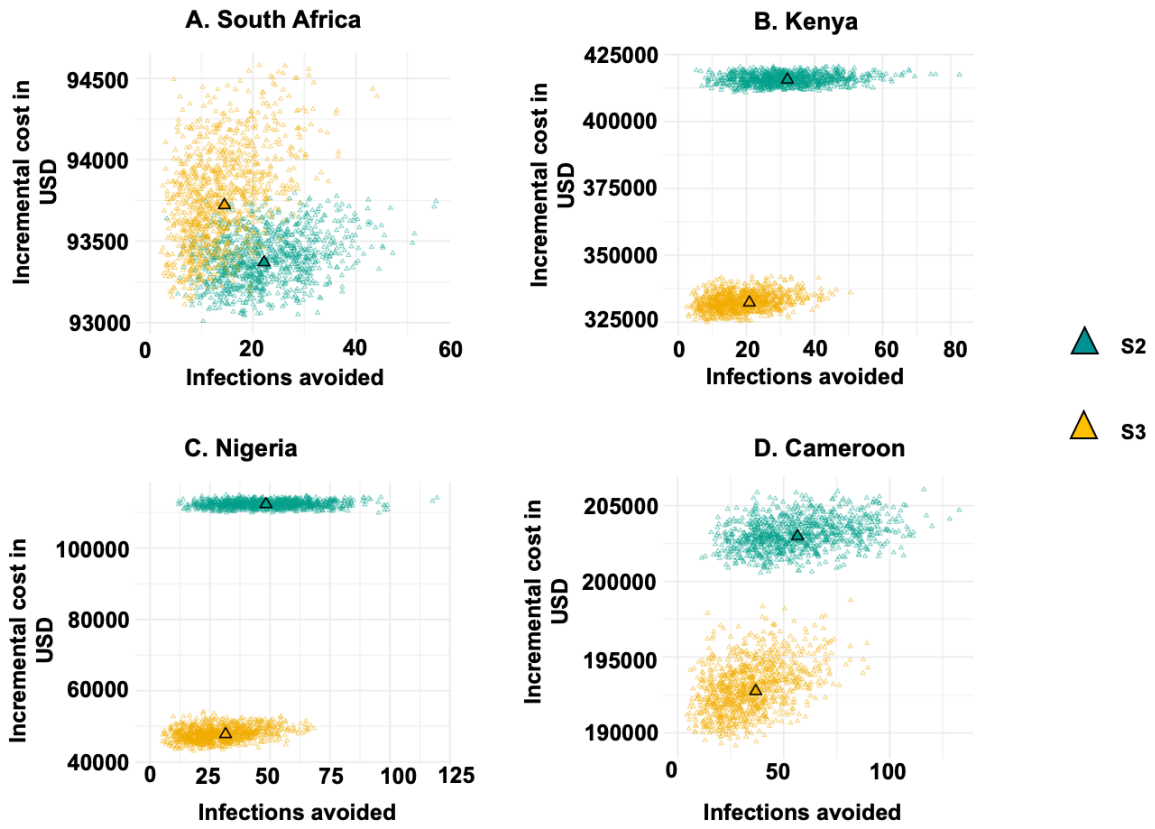


Figure 7.2: Cost-effectiveness (CE) plane for hepatitis B surface antigen (HBsAg) using laboratory assay in a simulated cohort of 10,000 antenatal African women.

S2: All pregnant women are screened for HBsAg; those who test positive are treated with TFV from 28 weeks' gestation to four weeks post-partum.

S3: All pregnant women are screened for HBsAg, those who test positive are screened for HBeAg. Only those who are HBeAg positive are treated with TFV from 28 weeks' gestation to four weeks post-partum.

TFV: Tenofovir. USD: United States dollars.

The incremental cost difference between **S2** and **S3** in South Africa, Kenya, Nigeria and Cameroon was 351 USD, 83,433 USD, 64,524 USD, and 10,224 USD, respectively. These incremental costs differences explain the gap differences between **S2** and **S3** in the graphs for each country **(Figure 7.2).** Higher costs of diagnosis of HBsAg and HBeAg in Kenya and Cameroon, compared to South Africa and Nigeria, explains the higher incremental costs in these setting. Whereas the higher cost of TFV in Kenya, Nigeria and Cameroon, compared to South Africa, explains the higher incremental cost of **S2** compared to **S3** in these three settings.

7.3.2 Cost-effectiveness of strategies for HBV PMTCT under scenario analyses

7.3.2.1 Antenatal screening for HBV infection using a rapid POCT

Use of POCT for HBsAg test was assessed for South Africa due to the availability of data for this test for this population.

Using the simulated cohort of 10,000 pregnant women, POCT for HBsAg was cheaper in comparison to laboratory based HBsAg assay (for **S2**: POCT 23,679 USD vs lab test 93,370 USD and for **S3**: POCT 24,022 USD vs lab test 93,721 USD); **(Table 7.4** and **Table 7.5)**.

Based on evidence for the sensitivity of HBsAg detection via laboratory ELISA vs POCT [100% and 97.6%, respectively (Chotun et al. 2017)], in this simulated cohort, laboratory testing would detect all maternal HBV infections (n=380 in the hypothetical South African cohort of 10,000), while the POCT for HBsAg approach would detect 370 and miss ten cases of infection. This loss of sensitivity translates into a marginal decrease in cost-effectiveness: the incremental cost per infection avoided is estimated at 1,127 USD compared to 1,080 USD if the test had 100% sensitivity.

Table 7.5: Cost-effectiveness results under scenario analysis for screening for hepatitis B surface antigen (HBsAg) using a rapid point-of-care test on a simulated birth cohort of 10,000 live singleton infants to South African women infected with chronic hepatitis B virus infection.

Strategy	Number of infant HBV infections[a] (95% CI)	Cost of deploying the intervention for the whole population[b] (n=10,000) in USD (95% CI)	Incremental cost per infection avoided (USD)
S1	42 (22 - 82)	0	-
S2	21 (11 - 48)	23,679 (23,422 - 24,005)	**1,127.57** (compared to strategy 1)
S3	28 (15 - 61)	24,074 (23,585 - 24,739)	1,719.57 (compared to strategy 1) *Dominated[c] (by strategy 2)*

[a] World Health Organisation (WHO) criteria for HBV elimination states an aim of 90% reduction in new chronic infection (WHO 2016a).
[b] Price of TFV estimated at 2.48 USD/month (South Africa)
[c] S3 is dominated due to both higher costs and higher infections compared to S2.
Incremental cost per infection avoided shown in bold represents the most cost-effective strategy.
CI = confidence interval. USD: United States dollars.

7.3.2.2 Recommended PMTCT guidelines: Universal birth dose vaccination and HBIg

Incorporating universal birth dose vaccination and HBIg into this model resulted in a further reduction in the number of infant infected at six months (**Table 7.6**), compared to when PMTCT interventions include only TFV and HBV vaccination beginning at six weeks (**Table 7.4**).

However, the cost per infection avoided were much higher, increasing from 4,244 USD to 46,685 USD for **S2** in South Africa; from 12,993 USD to 138,597 USD for **S2** in Kenya; from 3,624 USD to 33,830 USD for **S2** in Cameroon; and from 1,493 USD to 14,853 USD for **S3** in Nigeria (**Tables 7.4 and 7.6**).

Table 7.6: Cost-effectiveness results under scenario analysis (universal birth dose vaccination) on a simulated birth cohort of 10,000 live singleton infants to African women infected with chronic hepatitis B virus infection.

Countries	Strategy	Number of infant HBV infections[a] (95% CI)	Cost of deploying the intervention for the whole population[b] (n=10,000) in USD (95% CI)	Incremental cost per infection avoided (USD)
South Africa	**S1**	4 (2 - 8)	0	-
	S2	2 (1 - 5)	93,370 (93,114 - 93,710)	**46,685.00** (compared to S1)
	S3	3 (2 - 6)	93,721 (93,227 - 94,425)	93,721.00 (compared to S1) *Dominated[c] (by S2)*
Kenya	**S1**	6 (3 - 11)	0	-
	S2	3 (2 - 7)	415,792 (412,132 - 419,781)	**138,597.33** (compared to strategy S1)
	S3	4 (2 - 8)	332,359 (326,701 - 338,909)	166,179.50 (compared to strategy S1)
Nigeria	**S1**	9 (5 - 17)	0	-
	S2	4 (3 - 10)	112,320 (110,512 - 114,137)	22,464.00 (compared to S1)
	S3	6 (3 - 12)	44,559 (40,993 - 48,666)	**14,853.00** (compared to S1)
Cameroon	**S1**	11 (6 - 21)	0	-
	S2	5 (3 - 12)	202,980 (201,311 - 205,171)	**33,830.00** (compared to strategy S1)
	S3	7 (4 - 15)	192,756 (190,062 - 196,579)	48,189.00 (compared to strategy S1)

[a] World Health Organisation (WHO) criteria for HBV elimination states an aim of 90% reduction in new chronic infection (WHO 2016a).
[b] Price of TFV estimated at 2.48 USD/month (South Africa), 55.86 USD/month (Kenya), 26.00 USD/month (Nigeria), and 8.50 USD/month (Cameroon), for strategies S2 and S3. Price of TFV obtained from (International Medical Products Price Guide, 2016; http://www.kampharmacy.com/; Cost of Treating Hepatitis B in Nigeria, 2020; and Ministry of Health Cameroon).
[c] S3 is dominated due to both higher costs and higher infections compared to S2.
Incremental cost per infection avoided shown in bold represents the most cost-effective strategy in each country.
CI = confidence interval. USD: United States dollars.

7.3.2.3 HBV resistance to TFV

Incorporating potential HBV resistance to TFV, based on a global prevalence of drug resistance estimated at 0.3%, predictably resulted in an increase in incremental cost per infection avoided from 4,244 USD to 6,224 USD for **S2** in South Africa; 12,993 USD to 18,899 USD for **S2** in Kenya; 3,624 USD to 5,204 USD for **S2** in Cameroon; and from 1,493 USD to 2,172 USD for **S3** in Nigeria (**Tables 7.4 and 7.7**).

Table 7.7: Cost-effectiveness results under scenario analysis [incorporating estimated tenofovir resistance (0.3%)] on a simulated birth cohort of 10,000 live singleton infants to South African women infected with chronic hepatitis B virus infection.

Countries	Strategy	Number of infant HBV infections[a] (95% CI)	Cost of deploying the intervention for the whole population[b] (n=10,000) in USD (95% CI)	Incremental cost per infection avoided (USD)
South Africa	**S1**	42 (23 - 80)	0	-
	S2	27 (16 - 58)	93,370 (93,102 - 93,698)	**6,224.67** (compared to S1)
	S3	32 (19 - 65)	93,721 (93,230 - 94,387)	9,372.10 (compared to S1) *Dominated[c] (by S2)*
Kenya	**S1**	61 (33 - 109)	0	-
	S2	39 (23 - 78)	415,792 (412,063 - 420,011)	**18,899.64** (compared to S1)
	S3	47 (27 - 89)	332,359 (326,915 - 339,786)	23,739.93 (compared to S1)
Nigeria	**S1**	93 (53 - 165)	0	-
	S2	59 (35 - 117)	112,320 (110,432 - 114,289)	3,303.53 (compared to S1)
	S3	71 (40 - 133)	47,796 (44,315 - 52,068)	**2,172.55** (compared to S1)
Cameroon	**S1**	108 (58 - 197)	0	-
	S2	69 (39 - 142)	202,980 (201,234 - 205,091)	**5,204.62** (compared to S1)
	S3	83 (47 - 164)	192,756 (190,001 - 196,420)	7,710.24 (compared to S1)

[a] World Health Organisation (WHO) criteria for HBV elimination states an aim of 90% reduction in new chronic infection (WHO 2016a).
[b] Price of TFV estimated at 2.48 USD/month (South Africa), 55.86 USD/month (Kenya), 26.00 USD/month (Nigeria), and 8.50 USD/month (Cameroon), for strategies S2 and S3. Price of TFV obtained from (International Medical Products Price Guide, 2016; http://www.kampharmacy.com/; Cost of Treating Hepatitis B in Nigeria, 2020; and Ministry of Health Cameroon).
[c] S3 is dominated due to both higher costs and higher infections compared to S2.
Incremental cost per infection avoided shown in bold represents the most cost-effective strategy in each country.
CI = confidence interval. USD: United States dollars.

7.3.3 Summary

A summary of infections and costs have been provided in **Table 7.8**.

Table 7.8: Summary of cost-effectiveness results under base case assumptions for screening for hepatitis B surface antigen (HBsAg) using a laboratory-based assay, and under scenario analysis (universal birth dose vaccination & incorporating tenofovir resistance) on a simulated birth cohort of 10,000 live singleton infants born to South African, Kenyan, Nigerian or Cameroonian women infected with chronic hepatitis B virus infection.

				Using laboratory-based assay		Incorporating universal birth dose vaccination and HBIg		Incorporating potential HBV resistance to TFV	
Country	**Prevalence of HBsAg in pregnancy**	**Cost of HBsAg in USD (cost of HBeAg in USD)**	**Cost of TFV/month in USD**	**Number of infant HBV infections**	**Cost-effective strategy**	**Number of infant HBV infections**	**Cost-effective strategy**	**Number of infant HBV infections**	**Cost-effective strategy**
South Africa	3.8%	8.96 (8.96)	2.48	**S1**: 42 **S2**: 20 **S3**: 28	**S2**: 4,244.09	**S1**: 4 **S2**: 2 **S3**: 3	**S2**: 46,685.00	**S1**: 42 **S2**: 20 **S3**: 28	**S2**: 6,224.67
Kenya	5.5%	29.29 (29.29)	55.86	**S1**: 61 **S2**: 29 **S3**: 41	**S2**: 12,993.50	**S1**: 6 **S2**: 3 **S3**: 4	**S2**: 138,597.33	**S1**: 61 **S2**: 29 **S3**: 41	**S2**: 18,899.64
Nigeria	8.3%	2.60 (6.50)	26.00	**S1**: 93 **S2**: 44 **S3**: 61	**S3**: 1,493.63	**S1**: 9 **S2**: 4 **S3**: 6	**S3**: 14,853.00	**S1**: 93 **S2**: 44 **S3**: 61	**S3**: 2,172.55
Cameroon	9.7%	17.00 (17.00)	8.50	**S1**: 108 **S2**: 52 **S3**: 71	**S2**: 3,624.64	**S1**: 11 **S2**: 5 **S3**: 7	**S2**: 33,830.00	**S1**: 108 **S2**: 52 **S3**: 72	**S2**: 5,204.62

HBV: Hepatitis B virus. HBIg: Hepatitis B immunoglobulin. TFV: Tenofovir. HBeAg: Hepatitis B e antigen. HBsAg: Hepatitis B surface antigen.

The higher the prevalence of CHB infection among pregnant women, the higher the number of infants infected at six months, as shown in Cameroon compared to South Africa. The higher the costs of diagnosis or treatment, the higher the incremental cost per infection avoided, as shown in Kenya compared to South Africa.

Screening pregnant women for CHB and treating those infected as described in strategies (**S2** or **S3**) resulted in an overall reduction in the number of infants infected at six months compared to when no PMTCT intervention was employed (**S1**). **S2** was the most cost-effective strategy

in South Africa, Kenya and Cameroon. Whereas in Nigeria, **S3** was the most cost-effective strategy.

Use of both universal birth dose vaccination and HBIg resulted in a much lower number of infants infected at six months. However, the incremental cost per infection avoided was far higher when screening and treatment strategies (**S2**, and **S3**) were factored into the model.

When potential TFV resistance was incorporated into the model, there was an increase in incremental cost per infection avoided in all the four countries.

7.4 Discussion

7.4.1 Summary of findings and comparison with previous literature

Antenatal screening and treating of pregnant women for HBV is not routinely performed in many settings in sSA, despite the high population prevalence of HBV (Andersson et al. 2012; Andersson et al. 2013; Hoffmann et al. 2014; Chotun et al. 2017; O'Hara et al. 2017). In resource-limited settings, screening and providing prophylactic TFV for all pregnant women who are HBsAg positive may be the most efficient strategy, especially when using a POCT for HBsAg. These results are derived from a theoretical model, and careful consideration is needed for application to different real-world settings.

Based on existing data for South Africa, Kenya, Nigeria and Cameroon, screening and treating women with TFV from 28 weeks of pregnancy to four weeks post-delivery reduces the number of infants with HBV at six months. Previous studies have also reported on the safety and effectiveness of TFV for HBV PMTCT (Chen et al. 2017; Hyun et al. 2017). There is evidence for the cost-effectiveness of combining antiviral therapy during pregnancy with HBV immunoprophylaxis, as highlighted by a systematic review and meta-analysis carried out in China (Wang et al. 2016) and a study conducted in North America (Fan et al. 2016).

Use of universal birth dose vaccine and HBIg resulted in fewer infant infections, however, the cost of intervention was much higher when using maternal TFV for PMTCT as described in **S2** or **S3 (Table 7.6)**. A study in Thailand reported no significant benefit of maternal TFV for PMTCT (Jourdain et al. 2018). The lack of benefit from TFV in this case may be because all infants in the Thai study received birth dose of both HBIg and HBV vaccine, followed by four additional doses of HBV vaccine; this enhanced immunoprophylaxis probably accounts for the low rate of HBV transmission even among mothers who did not receive TFV. However, it is difficult to apply any of these findings to sSA, which differs in having limited (or no) access to HBIg, gaps in vaccine coverage, and delays in the first dose of HBV vaccine (frequently until

age six weeks) (Andersson et al. 2015b; Zampino et al. 2015).

This study provides a head-to-head economic analysis of simulated screening for HBsAg alone vs. use of HBsAg in combination with risk stratification using HBeAg. Limited resources and infrastructure have impeded antenatal diagnosis and treatment of HBV (O'Hara et al. 2017), and stratification of HBsAg-positive antenatal women with HBV DNA level and/or HBeAg to determine eligibility for TFV incurs further cost. Given that TFV is becoming more accessible for HBV treatment in Africa (WHO, 2015), and has a well-established safety record as a result of antenatal use in HIV infection, treating all HBsAg positive women should be safe and practical, as well as cost-effective. As this option is simple to implement, it is therefore also most likely to be successful. However, careful consideration is required for different settings given the differences in prevalence of CHB, and costs of treatment and diagnosis. For example, in Nigeria where the cost of treatment was much higher than the cost of diagnosis, the current recommended strategy of treating only women at a higher risk of vertical transmission was cost-effective.

Given the high prevalence of HBV in Africa (O'Hara et al. 2017), POCT for HBsAg is an appealing route to increasing diagnosis and therefore access to treatment. Although POCT for HBsAg proves advantageous on cost grounds, it is less sensitive than laboratory assays. Furthermore, laboratory support remains key for monitoring response to treatment, identifying and monitoring drug resistance, and evaluating prognosis.

There is a potential risk that increasing population exposure to TFV may increase selection of resistance [Mokaya et al. 2018b (see **chapter 2: section 2.1.4.3)**]. However, the genetic barrier to TFV resistance is high, and more data are needed to determine the prevalence and clinical significance of putative drug resistance mutations. When accounting for an estimated background rate of HBV resistance to TFV, there was an increase in the cost per infection avoided in all the four regions. With increasing evidence of TFV resistance, this pattern may

be an indication that a further increase in the prevalence of TFV resistance can lead to an increase in the number of infections and the overall costs of interventions for PMTCT of HBV infection.

Despite the potential for increased risk of HBV MTCT in HIV/HBV coinfection (Spearman et al. 2017), there is evidence to show that HIV has very little effect on HBV interventions (McNaughton et al. 2019b). Since HIV guidelines now recommend commencement of ART as soon as HIV diagnosis is made (WHO, 2020), the impact of HIV on HBV PMTCT is further reduced.

7.4.2 Caveats and limitations

This was a simple model with only a single outcome measure, thus overlooking other possible risks/benefits of antiviral therapy, including side-effects, drug interactions, and rebound hepatitis after treatment cessation. However, extensive experience has led to the inclusion of TFV in first-line regimens for HIV, including in pregnant women. Base values for this model were obtained from published literature, but there are limited data for HBV epidemiology and transmission for most African populations; the lack of certainty around certain parameters has been acknowledged by including confidence intervals.

High HBV DNA levels that exceed 200,000 IU/ml have been closely related with a higher risk of HBV MTCT. While HBV DNA testing is the gold standard approach to HBV diagnosis, it is often not available in resource-limited settings. I therefore used HBeAg status as a surrogate marker to represent infectivity and high VL and assumed 100% sensitivity for laboratory-based assays for HBsAg and HBeAg; this does not account for occult infection and other false negatives and may lead to an over-estimation of the cost-effectiveness of maternal TFV. A cheaper alternative to the standard HBV DNA measurement is the recently developed HBV DNA GenXpert cartridges, which has an estimated cost of 14 USD, however it needs to be validated in wider field context (Nagayam, Shimakawa and Lemoine, 2020).

Although administering TFV is effective for HBV PMTCT (Chen et al. 2017; Hyun et al. 2017), the clinical impact of short-term use of TFV (between 28 weeks' gestation to four weeks postpartum) and administering it over multiple gestations to mothers with CHB is unknown and requires further investigation (Joshi and Coffin, 2020).

The assumption that the first dose of HBV vaccine is delayed until age six weeks is an over-simplification: if birth dose vaccine is given more widely or targeted for infants born to HBsAg-positive mothers, more new infections will be averted.

The findings of this analysis are applicable to settings where the cost of screening and treatment is publicly funded; in situations where individuals meet the cost of their own screening and treatment, these results cannot be applied to informing public health strategy. In addition, this analysis did not consider the need for healthcare and laboratory infrastructure to support proposed interventions which include costs of monitoring pregnant women during TFV treatment, and costs for clinical visits and counselling for mothers testing HBsAg positive, leading to an underestimation of the total costs included in providing robust PMTCT. However, this is offset by the potentially very substantial lifelong costs of CHB, both to individuals and to society, arising from morbidity and mortality typically affecting young and middle-aged adults. This is difficult to quantify but leads to a substantial burden of chronic disease with economic consequences for the health-care system, as well as imposing financial consequences on families and society (Lemoine and Thursz, 2017; Wilson et al. 2018).

Chapter eight: General Discussion

8.1 Summary of what this thesis adds to the field

My D.Phil work contributes knowledge and insights regarding the molecular epidemiology of HBV in South Africa as well as the cost-effectiveness of using TFV for HBV PMTCT in sSA.

The United Nations Sustainable Development Goals have set out to eliminate viral hepatitis by the year 2030. However, HBV is neglected, with many challenges that are potentially major barriers to achievement of this target; these include a large pool of undiagnosed cases, limited epidemiological data especially from Africa, increasing drug RAMs and VEMs, gaps in vaccine coverage, limited provision of diagnostic tests and treatment, and stigma, poverty, and lack of knowledge.

My starting point was therefore to undertake a systematic literature review to assimilate existing evidence on drug and vaccine resistance in Africa and to identify gaps where more efforts are needed. The majority of the data from Africa were from HIV/HBV coinfected cohorts, and the commonest RAM identified in both reportedly treatment-naïve and treatment-experienced adults was M204I/V occurring either alone or in combination with compensatory mutations, with the suite of mutations M204V/I+L180M+V173L, that has been associated with vaccine escape, being identified in over 1/3 of cohorts. Overall, there is an urgent need for improved diagnostic screening, enhanced laboratory assessment of HBV before and during therapy, and sustained roll out of TFV in preference to 3TC alone.

I have also done detailed work, through a systematic literature review, to assimilate and catalogue HBV RAMs associated with TFV, which is especially important given its role in driving HBV elimination. The selection of clinically significant resistance to TFV remains controversial as HBV is strongly considered to have a high genetic barrier to resistance (Beloukas and Geretti, 2017; Lee and Lee, 2017). However, there is emerging evidence of

HBV RAMs associated with TFV resistance, although the literature to date is limited and heterogenous. I therefore classified the assimilated RAMs into two groups: a 'long-list' of all reported RAMs, and a 'short-list' of RAMs with the best evidence of support. This analysis demonstrated that if clinically significant resistance to TFV arises, it is most likely to be in the context of suites of multiple RAMs. However, the patterns of mutations reported in association with TFV resistance are varied, and the consensus viral sequence varies according to genotype, which makes it difficult to derive clear definitions for TFV resistant variants.

Despite identifying HBV RAMs and VEMs in African countries among HIV/HBV coinfected and HBV monoinfected patients, and before and during treatment (through the systematic literature review), the data were insufficient to form a clear picture of the prevalence and distribution of these mutations. I therefore analysed full-length HBV sequence data obtained from GenBank to understand to global prevalence, distribution and evolution of HBV RAMs and VEMs, and their association with genotypes. I estimated the global prevalence of HBV RAMs and VEMs at 4% with a significantly higher prevalence in genotype C sequences. These mutations are being selected independently in individual hosts through parallel evolution, and can arise in the presence or absence of treatment/vaccine exposure.

I also analysed sequence data obtained from individuals infected with CHB recruited from clinics in South Africa and the UK. In South Africa, the prevalence of individual HBV RAMs and VEMs range between 0–15% and 0–8%, respectively, whereas in the UK, the prevalence of individual HBV RAMs and VEMs range between 0–15% and 0–4%, respectively. Except for VEMs which were more likely to be present in genotype C sequences, there were no risk factors associated with RAMs/VEMs that could be ascertained in the cohort analysed to date. Recruitment of greater numbers will add more power for the detection of potential associations in future studies. 9% of the sequences obtained from 47 individuals recruited from South Africa had mutations suggesting possible TFV resistance, and from this population I have reported a first case from Africa with strong evidence for HBV resistance to TFV on the basis

of high HBV DNA VL despite 60 months of therapy, with a suppressed HIV VL, and a combination of sequence polymorphisms that have been reported in association with TFV resistance.

8.2 Implication of HBV RAMs for patient management

I have developed an algorithm for defining HBV resistance to TFV and ETV as a guide to the clinical management of individuals infected with CHB not suppressing HBV viraemia on therapy. However, there is currently insufficient data about TFV RAMs to underpin robust universal guidelines for clinical practice. However, the evidence that I have gathered in this thesis can underpin some practical recommendations:

i. There is an urgent need for more HBV sequencing data, together with contemporaneous VL measurements and clinical metadata to advance understanding of the relationship between viral sequence and treatment outcomes. Sequence repositories, databases and tools for sequence analysis should regularly review the evidence for TFV RAMs in order to highlight all sites that may be significant in mediating resistance.

ii. In the context of failure of viraemic suppression in a patient prescribed therapy, assessing and supporting drug compliance is crucial, ideally together with viral sequencing. In HIV/HBV coinfected patients, persistent HBV viraemia in the context of HIV suppression could be regarded as an indicator or 'red flag' for TFV resistance.

iii. Therapeutic failure of TFV – whether in the presence or absence of known RAMs – should lead to an expert clinical decision about switching therapy or combining agents, as long as adherence has been optimised and supported. The presence of recognised RAMs, especially when in combination, may support a change of therapy, considering ETV or combination therapy, although guidance and options are currently limited.

iv. If there is an ongoing emergence of data to suggest TFV resistance, there will be a need for expert guidelines to include practical recommendations in order to unify

clinical approaches. HIV guidelines should take an active stance on incorporating recommendations for those with HBV coinfection, particularly if dual therapy regimens are adopted.

v. Evidence of TFV resistance highlights the need for enhanced studies representing larger numbers of patients, tracking longitudinal viral sequence changes, and monitoring viral suppression over time are needed. In addition, the evolution of better *in vitro* models will support experiments to investigate the effect of individual and combined RAMs. In order to optimise the use of NA therapy as a tool in driving advancements towards elimination at a population level, improved insights into drug resistance are essential. If resistance emerges as a substantial clinical problem, there will be a need for consideration of synergistic drug regimens, new agents that inhibit a target other than viral RT, and for the development of new therapeutic strategies that can bring about cure.

Although there is emerging evidence of TFV resistance which calls for more scrutiny, treatment failure on TFV is likely to be a relatively rare event given that up to 90% of individuals with CHB are unaware of their diagnosis and therefore not linked to care (O'Hara et al 2017). While more data is required to define TFV resistance, in the meantime, it remains paramount to keep advocating for diagnosis and scale-up of treatment, with the current recommended antiviral therapies, to reduce liver related morbidity and mortality.

8.3 Tenofovir for HBV PMTCT

Prophylactic antiviral therapy during pregnancy for women with CHB, with administration of HBV birth dose vaccination reduces the risk of infant HBsAg seropositivity by 71% (Pan et al. 2016). The current guidelines recommend prophylactic antiviral therapy restricted to women with high HBV DNA VL or HBeAg positivity (EASL, 2017; Terrault et al. 2018). However, administration of HBV birth dose vaccine is not a routine practice in many settings in Africa,

there are gaps in HBV antenatal screening due to high costs and lack of diagnostic assays, and <1% of pregnant women with high VL have accessed antiviral treatment (Nayagam, Shimakawa and Maud, 2020). Given that TFV is cheap, safe to use during pregnancy and widely available in Africa, I have demonstrated that screening and treating all women with CHB, regardless of HBV VL/HBeAg status, with TFV from 28 weeks' gestation to four weeks postpartum is a cost-effective strategy for HBV PMTCT intervention, and may be an appropriate approach in some African countries where the risk of MTCT remain high. However, an increase in exposure to antiviral therapy could also lead to increased selection of resistant mutations that could potentially impact on the cost-effectiveness of this strategy in the long run. At present, the prevalence of drug resistance is too low to make a significant impact, but prospective surveillance will be important, and this modelled intervention ideally requires practical evaluation in different settings before guidelines are updated.

8.4 Sequencing method for low VL samples

I have optimised Sanger sequencing for low HBV VL samples of up to 2 $\log_{10}$ IU/ml, however, I have not had the opportunity to apply this method widely due to limited sample availability. Given that recruitment of individuals with CHB is currently ongoing within our research group, this method can be used to sequence low VL samples as they become available.

8.5 Challenges and opportunities for Africa

8.5.1 Public health significance of RAMs/VEMs

My analyses have shown that data from Africa on HBV RAMs and VEMs are currently insufficient to form a clear picture of the prevalence, distribution or clinical significance of these mutations. Furthermore, there are no recommendations to stipulate any specific prevalence threshold above which HBV RAMs/VEMs represent a significant barrier to successful prevention or treatment at a population level. For HIV, surveillance for transmission of RAMs is based on screening recently infected, treatment naïve individuals, and classifies drug

resistance using thresholds of <5%, 5–15%, and >15% to stratify the risk to public health (WHO, 2013). Similar thresholds and recommendations for HBV could help to underpin the assimilation of epidemiological data and to unify treatment approaches.

8.5.2 Screening of HBV RAMs/VEMs

Screening for HBV infection is not routinely performed in many African settings and therefore the true prevalence and characteristics of HBV infection are not known (Beloukas and Geretti, 2017; O'hara et al. 2017; Nayagam, Shimakawa and Maud, 2020). Although the WHO recommends monitoring for the development of drug resistance once on therapy (WHO, 2015), implementation remains challenging as VL monitoring and sequencing are both rarely available (Beloukas and Geretti, 2017; O'hara et al. 2017; Nayagam, Shimakawa and Maud, 2020); despite the advancement and availability of HIV testing and monitoring, in many settings it remains uncommon to monitor HIV VL after ART initiation (Ellman et al. 2017; Manoto, 2018). Affordable, accessible and sustainable platforms for quantifying both HIV and HBV VL remain an important priority for many settings in Africa, given the lack of on-treatment monitoring in many settings. Given the simplicity and relative ease of collection, preparation and transport of dried-blood-spot (DBS) samples (Lange et al. 2017), adopting DBS testing could improve access to HBV diagnosis, VL monitoring and linkage to care, especially in areas with limited access to laboratory facilities.

Development of a cheap, rapid test for the detection of the most frequently observed RAMs and VEMs should be considered as a potentially cost-effective strategy for Africa. Proof of principle for a rapid test for diagnosis and detection of resistance has been demonstrated by the GeneXpert MTB/RIF assay for *Mycobacterium tuberculosis* (MTB) (Zeka, Tasbakan and Cavusoglu, 2011). A similar approach has been applied for HBV through use of a multiplex ligation-dependent probe real time PCR (MLP-RT-PCR) (Shuangrong et al. 2014). Although this assay is able to detect RAMs quickly and cheaply, there are still limitations as the test requires high VL samples, is based on detection of known RAMs from within discrete regions

of the genome and may not identify RAMs that are present as minor quasispecies. Nanopore technology also has the potential to develop into an efficient point of care test that could detect viral infection and coinfection, as well as determining the presence of RAMs and VEMs, but is currently limited by cost, concerns about high error rates and decreased sensitivity on low VL samples (McNaughton et al. 2019c)

Existing infrastructure for diagnosis, clinical monitoring and drug therapy for HIV represents an opportunity for linkage with HBV care. Particularly in settings of limited resource, joining up services for screening and management of blood-borne virus infection could be a cost-effective pathway to service improvements.

8.5.3 HBV PMTCT

There remains an urgent need for more high-quality data to underpin the relevant epidemiology, risks of MTCT, and relative benefits of different interventions in settings across sSA. Despite the effectiveness of HBV birth dose vaccination, its coverage in Africa is still low and there is a lack of high-quality data from Africa which makes it challenging to make accurate policy and public health planning for HBV PMTCT (Nayagam, Shimakawa and Maud, 2020). The majority of data that inform HBV PMTCT models are obtained from non-African countries, and HBV prevalence data among pregnant women reported from many African countries are not nationally representative (Nayagam, Shimakawa and Maud, 2020).

8.5.4 Stigma in HBV infection

Stigma, poverty, and lack of knowledge are barriers to prevention, diagnosis and treatment of HBV infection. I have contributed to a systematic literature review which describes the evidence for the nature and impact of stigma associated with HBV infection (Mokaya et al. 2018a). This work highlights the lack of research in this area with only two studies published from Africa (Mkandawire et al. 2013; Adjei, Naab and Donkor, 2017). I have also contributed to a study that investigated how HBV is understood and described in communities in Uganda

and whether there is evidence of potential stigma (Mugisha et al. 2019). Findings from these three studies published from Africa demonstrate limited knowledge regarding the cause, mode of transmission, and treatment of HBV infection, an inability to define the disease with no specific word for HBV infection in local languages, confusion between HBV and other infections, including malaria, yellow fever and HIV, and the association of HBV symptoms with witchcraft or poisoning (Mkandawire et al. 2013; Adjei, Naab and Donkor, 2017; Mugisha et al. 2019).

Lack of knowledge or awareness can lead to misinformation that feeds stigma and discrimination. Stigma and discrimination may have a wide-reaching influence on physical health (for example inhibiting interaction with clinical services and reducing treatment adherence), psychological well-being (through increasing isolation, anxiety and depression), and interactions with family and the wider society (by limiting relationships, social interactions and employment opportunities). Recognising, understanding and tackling the issue of this stigma in African populations, with careful dissemination of accurate information that promotes acceptance of interventions for prevention, diagnosis, and treatment, could be a valuable tool to improve population health and to underpin advances towards elimination strategies proposed for the year 2030. Education plays a very important role in reducing discrimination and stigma. There is also a pressing need for more research in this area, to identify and evaluate interventions that can be used effectively to tackle stigma in HBV, and for collaborative efforts between policy makers, health care workers, traditional healers, religious leaders, charity organisations and support groups, to improve awareness and tackle stigma in HBV in Africa.

8.5.5 Impact of COVID-19 on HBV infection

While there is no clinical evidence of the impact of COVID-19 disease course on chronic viral hepatitis infection (Boettler et al. 2020), public health efforts towards upscaling of prevention,

diagnosis and treatment interventions that are needed for the elimination of viral hepatitis, may be significantly disrupted by the COVID-19 pandemic which has resulted in a major shift in government and public health investment priorities, as well as transformation of healthcare systems with redeployed of staff and suspension/closure of centralised clinical services or healthcare facilities (The Lancet Gastroenterology and Hepatology editorial, 2020). Social distancing stay at home policies and fear of contracting COVID-19 from healthcare facilities may further impact on health seeking behaviours resulting in fewer people being able to access testing services, barriers to being linked to care and accessing treatment, more babies being born outside healthcare settings, and fewer infants having access to HBV vaccination (Boettler et al. 2020; Tapper and Asrani, 2020). Nevertheless, the approaches that are being used to control the pandemic such as decentralised elimination efforts, mass testing, contact tracing and emphasis on the importance of vaccination, can provide lessons that can help inform HBV management strategies (The Lancet Gastroenterology and Hepatology editorial, 2020). In addition, this pandemic has demonstrated the urgent need of anticipating and planning for uncertainties (Boettler et al. 2020; Tapper and Asrani, 2020; The Lancet Gastroenterology and Hepatology editorial, 2020).

8.6 Future work

8.6.1 HBV RAMs and VEMs in Africa

This thesis has shown that there is very limited, patchy and non-nationally representative data for HBV RAMs and VEMs that are available from Africa. Multi-national collaboration is needed to gather longitudinal HBV sequence data obtained from different populations within Africa, ideally supported by demographic, clinical and treatment metadata, particularly representing the HBV monoinfected population, in order to develop an understanding of the distribution, nature and significance of drug and vaccine resistance at an individual and population level. Evaluating viral sequence changes before and after treatment exposure, and monitoring viral

and host attributes over time will help inform on the impact of treatment on the development of RAMs, as well as the impact of RAMs on specific clinical outcomes.

8.6.2 Drug levels assessment in clinical samples

There are potential influences of other viral and host factors such as treatment compliance that can contribute to persistent viraemia on therapy, in the presence or absence of RAMs. Given that there is very limited literature to inform on the appropriate threshold for drug levels to confirm treatment compliance in HBV infection, there is a need for longitudinal studies that will assess treatment compliance by measuring TFV levels in samples (such as plasma/serum, PBMCs, hair and urine) obtained from HBV monoinfected and HIV/HBV coinfected individuals. In addition, assessing for the appropriate time at which samples should be collected for drug levels assessment, relative to the dose that has been or should be taken, is needed.

8.6.3 Drugs in development for HBV

In order to end the HBV endemic, there is need for effective therapies for CHB given that the approved NAs are not curative, they do not commonly result in the clearance of HBsAg, and their indefinite use can result in the emergence of RAMs which are potentially significant concern for their effectiveness. The ultimate goal for future therapy would be to attain sterilising cure which results in the complete removal of all cccDNA and integrated virus within the host (Gish et al. 2015). Achieving a functional cure may be possible in the near future given that there are currently several novel agents in pre-clinical or phase I/II trials, whose goal is to result in the loss of HBsAg which would allow for treatment discontinuation (Dawood et al. 2017). These agents inhibit viral replication by either interfering with a specific step in viral replication (DAAs) or modify host cell function (host-targeting agents) (Dawood et al. 2017). Among the DAA in development include RNA interference therapies, cccDNA formation and transcription inhibitors, core/capsid inhibitors, RT inhibitors, HBsAg release inhibitors,

antisense oligonucleotides, and helioxanthin analogues. Among the host-targeting agents in development include entry inhibitors (Myrcludex-B), cyclophilin inhibitors, and multiple immunomodulatory agents (such as Toll-like receptor agonists, immune checkpoint inhibitors, therapeutic vaccines, engineered T cells, and cytokine agents [such as recombinant human interleukin-7 (CYT107) and SB 9200] (Lin and Kao, 2016; Dawood et al. 2017).

8.7 Impact of published work from my thesis

During my D.Phil, I have published six papers (and two currently in review) as a first author in peer review journals which form the backbone of results presented in this thesis. My most recent publication describes the first evidence of HBV resistance to TFV in Africa (Mokaya et al. 2020b**)**. I have also published a systematic literature review that characterises the prevalence and distribution of mutations in HBV that are associated with drug resistance in Africa, which has been cited 26 times since 2018 (Mokaya et al. 2018b**)**, a systematic literature review that has assimilated evidence of HBV resistance to TFV globally (currently in review); a study that has described the global prevalence and phylogeny of HBV RAMs and VEMs (currently in review); a study evaluating the cost-effectiveness of TFV on HBV PMTCT in South Africa (Mokaya et al. 2019), a systematic literature review on stigma in HBV infection (Mokaya et al. 2018a), a study exploring the nature of the stigma in HBV among individuals chronically infected in Uganda (Mugisha et al. 2019), and a study that has characterised liver diseases in a rural population in Uganda specifically examining liver function tests and liver fibrosis scores (O'Hara et al. 2020**)**.

I have also contributed as a co-author to five other published manuscripts that are pertinent to driving forward an understanding of the clinical and molecular epidemiology of HBV in African populations. I described the situation for patients and healthcare workers in Kenya as part of a contribution to an article describing HBV as a neglected tropical disease which has been cited 49 times (O'Hara et al. 2017); other manuscripts I have contributed to describe the

dynamics of HBV surface antigen clearance (Downs et al. 2019); use of different sequencing techniques to derive full length genome HBV sequences (McNaughton et al. 2019c); the impact of diagnosis and treatment compared to catch-up vaccination as elimination tools for HBV (McNaughton et al. 2019b); and the impact of therapy among individuals infected with CHB infection in South Africa (Maponga, 2020).

8.8 Concluding remarks

In order to drive progress towards 2030 elimination targets, sustainable long-term investment is required to drive improvements in clinical services that include screening of infection, appropriate targeted clinical monitoring for treated patients with monitoring of drug resistance, provide universal access to antenatal screening, expand consistent drug and vaccine supply, improve education of the public and health-care workers on HBV prevention, diagnosis and treatment, and underpin robust deployment of PMTCT interventions including vaccination and TFV therapy. If drug resistance emerges as a substantial clinical problem, there will be a need for consideration of synergistic drug regimens, new agents that inhibit a target other than viral RT, and for the development of new therapeutic strategies that can bring about cure.

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Appendix

Chapter 2

Section 2.1

Appendix Table 2.1.1: PRISMA criteria for frequency, co-occurrence and distribution of hepatitis B drug and vaccine resistant mutations in Africa systematic literature review.

	#	Checklist item	Section reported in manuscript
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Title of the published manuscript
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Provided in the published manuscript
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Provided in the introduction of the published manuscript and thesis (chapter 1, section 1.17.1)
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Provided in the introduction of the published manuscript and thesis (chapter 1, section 1.17.1)
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	N/A
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Provided as S1 Table in the published manuscript and in methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Provided as S2 Table in the published manuscript and in methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Provided as S2 Table in the published manuscript and in methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.1.2)

Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	N/A
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.1.3)
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.1.3)
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.1.2)
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.1.3)
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.1.3)
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.1.3)
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	See point 12, 15 and 19.
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.1.4)
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.1.4)
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.1.4)
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Provided in the published manuscript

Appendix Table 2.1.2: Full details of 40 studies identified by a systematic literature search of hepatitis B virus (HBV) resistance associated mutations (RAMs) and vaccine escape mutations (VEMs) from African cohorts published between 2007 and August 2020.

First author and year	Country	Total sample size	HIV co-infection status of cohort	Proportion of participants who tested HBsAg + or HBV DNA+	Characteristics of study population	HBeAg prevalence among HBV-positive individuals	Genotypes identified	Drug treatments	Sequencing method	Gene loci sequenced	Participant recruitment site	Accession Number
Anderson et al 2015a	Botswana	81	+	N=81/81 (100%)	Stored plasma samples of HIV/HBV co-infected individuals collected from studies conducted in a Research Institution	Not reported	Geno A-86%; Geno D-13%; Geno E- 1%	Treatment naive	Sanger	Pol and S	Not specified	GenBank: KR139680; KR139749
Gachara et al 2017	Cameroon	455	+	N=20/455 (4.4%)	Patients attending outpatient ART health centre	Not reported	Geno A-10%; Geno E-90%	N/A	Sanger	Pol and S	Health facility	Not specified
Kouanfack et al 2012	Cameroon	552	+	N=54/552 (9.8%)	Patients attending outpatient ART clinic at tertiary hospitals	Not reported	Geno A-58%; Geno E-42%	3TC-containing ART	Sanger	Pol	Health facility	Not specified
Magoro et al 2016	Cameroon	455	+	N=116/455 (25.5%)	Patients attending outpatient ART health centre	16/102 (16%)	Geno A-30%; Geno E-70%	Some patients ART naive, some were on 3TC-containing ART	Sanger	Pol and S	Health facility	GenBank: KU900150-KU900195

Boyd et al 2015	Cote d'Ivoire	2465	+	N=259/2465 (10.5%)	Individuals enrolled in randomised multi centre trials of benefits and risks of early ART initiation	39/168 (23%)	Geno E-100%	3TC containing ART, n=82 & TDF/FTC containing ART, n=86	Sanger	Pol and S	Health facility	Not specified
Deressa et al 2017	Ethiopia	308	+	N=17/308 (5.5%)	Patients attending outpatient ART clinic at tertiary referral university hospital	Not reported	Geno A-41%; Geno C- 6%; Geno D-12%; Geno E-12%; Geno G- 6%; Undetermined-23%	3TC-containing ART, n=12; TDF + 3TC-containing ART, n=5	Sanger	Pol, S and Core	Health Facility	GenBank: pending
Hundie et al 2016	Ethiopia	391	±	N=391/391 (100%)	Stored plasma samples from HBV infected blood donors obtained from blood bank centres	Not reported	Geno A – 78%; Geno D – 22%	Not specified	Sanger	S	Community (blood donors)	GenBank: KP310929; KP311299
Bivigou-Mboumba et al 2016	Gabon	762	+	N=71/762 (9%)	Patients attending outpatient ART clinic	Not reported	Geno A-68%; Geno E-32%	3TC-containing ART	Sanger	Pol, S and Core	Health facility	GenBank: KM983561-KM983588
Bivigou-Mboumba et al 2018	Gabon	487	+	N=43/487 (8.8%)	All HIV patients attending HIV care centers during study period	Not reported	Geno A – 60%; Geno E – 40%	3TC-containing ART, 24%; TDF - containing ART, 70%; Not specified, 24%	Sanger	S	Health Facility	GenBank: KY271377; KY271392
Ndow et al 2017	Gambia	870	+	N=94/870 (10.8%)	Individuals attendi	Not reported	Geno A- 1%; Geno E-99%	some were on ART:	Not specified	Pol	Health facility	Not specified

					ng HIV clinic			3TC-containing ART n=54; TDF-containing ART, n=7				
Stewart et al 2011	Gambia	570	+	N=70/570 (12.3%)	Individuals receiving HAART; recruitment site not specified	6/21 (29%)	Geno E-100%	3TC-containing ART	Sanger	Pol	Not specified	X75664; AM410963
Archampong et al 2017	Ghana	235	+	N=235/235 (100%)	Serum samples from HBV-HIV co-infected patients collected at tertiary referral university hospital	Not reported	Geno A-6%; Geno D-2%; Geno E-92%	Some were treatment naïve; some on 3TC, n=27; TDF containing ART, n=2	Not specified	Pol (RT)	Health facility	GenBank: KU711604-KU711666
Chadwick et al 2012	Ghana	551	+	N=143/551 (26%)	Stored sera from all adult patients attending the HIV clinic at a tertiary referral university hospital	Not reported	N/A	3TC-containing ART	Sanger	Pol (RT)	Health facility	Not specified
Geretti et al 2010	Ghana	838	+	N=140/838 (16.7%)	Paired serum and plasma samples collected from HIV-infected patients attending a tertiary referral university hospital	37/140 (26%)	Geno A-5%; Geno E-95%	Some ART status not known (2/3), some were on 3TC-containing ART (1/3)	Sanger	Pol (RT) and S	Health facility	Not specified

Langhoff Hønge et al 2014	Guinea-Bissau	576	+	N=94/576 (16.3%)	Patients attending outpatient ART clinic at tertiary referral university hospital	16/94 (17%), HDV prevalence: 18/72 (25%)	Geno E-99%; Geno D- 1%	some were on 3TC-containing ART	Sanger	Pol	Health facility	Not specified
Day et al 2013	Kenya	159	+	N=11/159 (6.9%)	Longitudinal cohort study of female sex workers in an urban setting	6/11 (55%)	Geno A-100%	3TC-containing ART	Not specified	Pol (YMDD)	Community (Female sex workers)	Not specified
Kim et al 2011	Kenya	389	+	N=27/389 (6.9%)	Individuals from an urban centre enrolled in randomised controlled trial of adherence to ART	24/27 (89%)	Geno A-100%	3TC-containing ART	Sanger	Pol	Health facility	Not specified
Mabeya et al 2017	Kenya	400	+	N=29/400 (7.3%)	Individuals seeking treatment at the comprehensive HIV Clinic at tertiary referral university hospital	Not reported	Geno A-100%	some on 3TC-containing ART, n= 55%; TDF-containing ART, n= 45%	Sanger	Pol (RT)	Health facility	Not specified
Auodjane et al 2014	Malawi	1117	+	N=133/1117 (11.9%)	Individuals starting ART treatment at a tertiary referral university hospital	67/133 (50%)	Geno A-99%; Geno E-1%	3TC-containing ART	Sanger and Deep sequencing by Illumina	Pol (RT)	Health facility	Not specified
Galluzzo et al 2012	Malawi	21	+	N=21/21 (100%	Pregnant women enrolle	7/21 (33%)	Geno A-99%; Geno E-1%	3TC-containing ART	Not specified	Pol and S	Not specified	Not specified

					d in a PMTCT study on safety and pharmacokinetics of antiretroviral drugs							
Chambal et al 2017	Mozambique	518	+	N=47/518 (9.1%)	Patients attending outpatient ART health centre	Not reported	Geno A-93%; Geno E-7%	Treatment naive	Sanger	Pol (RT) and S	Health facility	Not specified
Wandeler et al 2016	Mozambique and Zambia	1032	+	N=168/1032 (16.3%)	Individuals starting ART treatment at urban clinic in Mozambique and rural clinic in Zambia	24/168 (14%)	Geno A-59%; Geno E-38%; Geno A/E-1%	Treatment naive	Sanger	Pol	Health facility	Not specified
Akanbi et al 2019	Nigeria	81	±	N=81/81	HIV/HBV co-infected individuals	Not reported	Geno E-100%	3TC-containing ART; some treatment naïve	Sanger	Pol and S	Health facility	Not specified
Faleye et al 2015	Nigeria	272	±	N=15/272 (5.5%)	Pregnant women attending antenatal clinics from two tertiary university hospitals	Not reported	Geno E- 71%	Not specified	Not specified	S	Health Facility	GenBank: KM225621-KM225627
Amponsah-Dacosta et al 2015	South Africa	201	±	N=33/201 (16.4%)	Stored serum of individuals exposed to HBV participating in a	Not reported	Geno A1-92%; Geno A2-5%; Geno D4- 3%	Not specified	Not specified	S	Not specified	Not specified

					health facility-based hepatitis B serosurvey conducted at a provincial level.							
Amponsah-Dacosta et al 2016	South Africa	5	+	N=5/5 (100%)	Individuals due to HAART initiation enrolled in longitudinal study	5/5 (100%)	Geno A-100%	3TC-containing ART, n=4	Sanger	Complete genome	Not specified	Not specified
Andersson et al 2013	South Africa	3099	±	N=97/3099 (3.1%)	Stored serum of women infected with HIV enrolled in an Antenatal Sentinel HIV and Syphilis Prevalence Survey	17/94 (18%); HDV: negative	Geno A-93%; Geno D- 7%	Some were on 3TC-containing ART; some on TDF-containing ART	Not specified	S	Health facility	Not specified
Baxter et al 2019	South Africa	889	Unknown	N=37/889	Women	Not reported	Geno A-100%	TDF	Sanger	Pol	Health facility	GenBank: X02763
Gededzha et al 2016	South Africa	9	±	N=9/9 (100%)	Stored sera from HBV infected individuals attending a tertiary referral university hospital	Not reported	Geno A-56%; Geno C-22%; Geno D-22%	some were on 3TC-containing ART	Sanger	Complete genome	Health facility	GenBank: KT347087-KT347092; GQ184323; GQ184326; GQ167301
Lukhwareni et al 2020	South Africa	56	±	N=56/56 (100%)	HIV/HBV co-infected individuals	Not reported	Geno A-48%; Geno D- 4%; Geno G-2%	3TC-containing ART	Sanger	Pol	Health facility	GenBank:MH431627-MH431693
Makondo et al 2012	South Africa	298	+	N=71/298 (23.8%)	Stored sera from HIV infected	Not reported	Geno A-99%; Geno D-1%	Treatment naive	Sanger	S and Core	Not specified	GenBank/EMBL/DDBJ: JX1442

					individuals prior to ART initiation, recruitment site not specified							70-JX144323
Powell et al 2015	South Africa	394	+	N=37/394 (9.4%)	Stored serum samples of individuals infected with HIV receiving care at a tertiary university hospital	Not reported	Geno A1-24%; Geno A2-69%; Geno D4- 6%	Not specified	Not specified	Pol and S	Health facility	GenBank: KF475982; KF476030
Selabe et al 2007	South Africa	35	±	N=35/35 (100%)	Individuals infected with HBV admitted at tertiary University hospital	Not reported	N/A	Treatment naive	Sanger	Pol (YMDD)	Health facility	GenBank: DQ529242-DQ529244
Selabe et al 2009	South Africa	17	-	N=17/17 (100%)	Individuals infected with HBV admitted at tertiary University hospital	9/17 (53%)	Geno A-59%; Geno B-23%; Geno C-12%; Geno E-6%	3TC-containing ART	Sanger	Pol, S and Core	Health facility	Not specified
Matthews et al 2015	South Africa & Botswana	1022	±	N=72/1022 (7%)	Women attending antenatal and paediatric clinics	16/60 (27%); HDV: negative	Geno A-88%; Geno D-12%	Treatment naive	Sanger	Pol (RT)	Health facility	Not specified
Hamers et al 2013	South Africa & Zambia	1087	+	N=92/1087 (8.5%)	Individuals enrolled in a multicentre prospective study of ART resistance	Not reported	Geno A-76%; Geno D-2%; Geno E-22%	3TC-containing ART, n=54; TDF-containing ART, n=48	Not specified	Pol and S	Not specified	Not specified

					monitoring							
Yousif et al 2014	Sudan	358	+	N=96/358 (26.8%)	Individuals seeking treatment at a AIDS care unit and HIV treatment centre	32/ 50 (64%)	Geno A-19%; Geno D-46%; Geno E-22%; Geno D/E-14%	Treatment naive	Not specified	Pol, S and Core	Not specified	GenBank: KM108588; KM108626
Mahgoub et al 2011	Sudan	404	±	N=16/404 (4%)	Plasma samples from blood donors from capital city in Sudan	Not reported	Geno A2-2%; Geno D-41%; Geno E- 56%	Not specified	Not specified	S and Core	Community (blood donors)	GenBank: HQ385227
Calisti et al 2015	Uganda	2820	+	N=109/2820 (3.9%)	All HIV patients attending a regional referral hospital	Not reported	Geno A-83%; Geno D-17%	3TC-containing ART, 96%; TDF +3TC-containing ART, 4%	Sanger	Pol (RT) and S	Health facility	Not specified
Baudi et al 2017	Zimbabwe	176	+	N=19/176 (10.8%)	Stored plasma samples of individuals attending HIV support clinic	Not reported	Geno A-100%	Treatment naive	Sanger	S and Core	Health facility	GenBank: KX648543; KX648713

Pol: Polymerase. RT: Reverse Transcriptase. S: Surface.
HIV status is designated '+' whole cohort HIV-positive; '±' some of cohort HIV-positive; '-'none of cohort HIV-positive.

Appendix Table 2.1.3: Quality appraisal using the BMJ adapted Quality Assessment Tool for Quantitative Studies for 40 studies identified for a systematic literature review of drug and vaccine resistance in Africa.

Quantitative studies quality assessment												
	Selection bias			Study design		Confounders		Withdrawals and drop-outs	Reporting			Overall rating
Quantitative studies included	Are the individuals selected to participate in the study likely to be representative of the target population	What percentage of selected individuals agreed /able to participate?	Rating selection bias	Indicate the study design	Rating study design	Were analyses appropriately adjusted for confounders	Rating confounding	Were withdrawals and drop-outs reported in terms of numbers and/or reasons per group?	Are the hypothesis/aim/objective of the study clearly described?	Were inclusion/exclusion criteria specified and number of exclusions reported	Rating reporting	
Anderson et al 2015a	Not likely	Can't tell	Weak	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Inclusion criteria reported	Moderate	Moderate
Gachara et al 2017	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Kouanfack et al 2012	Very likely	Can't tell	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Magoro et al 2016	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Boyd et al 2015	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions	Strong	Moderate

										reported		
Deressa et al 2017	Very likely	Can't tell	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Inclusion criteria reported	Moderate	Moderate
Hundie et al 2016												
Bivigou-Mboumba et al 2016	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Inclusion criteria reported	Strong	Moderate
Bivigou-Mboumba et al 2018	Very likely	Can't tell	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Inclusion criteria reported	Moderate	Moderate
Ndow et al et al 2017	Very likely	Can't tell	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Inclusion criteria reported	Moderate	Moderate
Stewart et al 2011	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Archampong et al 2017	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Chadwick et al 2012.	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Geretti et al 2010	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Hønge et al 2014	Very likely	Can't tell	Moderate	Observational	Moderate	Participants characteristics	Strong	Not applicable	Yes	Inclusion criteria	Moderate	Moderate

						clearly described				reported		
Day et al 2013	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Kim et al 2011	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Mabeya et al 2017.	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Aoudjane et al 2014	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Galluzzo et al 2012	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Chambal et al 2017	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Wandeler et al 2016	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Faleye et al 2015.	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate

Amponsah-Dacosta et al 2015	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Amponsah-Dacosta et al 2016	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Anderssson et al 2013	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Gededzha et al 2016	Not likely	Not applicable	Weak	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Makondo et al 2012	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Powell et al 2015	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Selabe et al 2007	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Selabe et al 2009	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Matthews et al 2015	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly	Strong	Not applicable	Yes	Criteria or number of not exclusi	Moderate	Moderate

						described				ons reported		
Hamers et al 2013	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Yousif et al 2014	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Mahgoub et al 2011	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Moderate
Calisti et al 2015	Very likely	Not applicable	Strong	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate
Baudi et al 2017	Somewhat likely	Not applicable	Moderate	Observational	Moderate	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria or number of not exclusions reported	Moderate	Moderate

Appendix Table 2.1.4: Number of samples with specific polymerase/reverse transcriptase mutations among treatment experienced hepatitis B virus (HBV) infected patients in Africa, from 23 studies published between 2009 and August 2020.

Pol/RT mutation	Treatment experienced			
	Country and number of HBV positive study participants	Author, year	HIV co-infection status of cohort[a]	Number (%) of individuals with mutation
I169L	Gambia (n=70)	Stewart et al 2011	+	1/70 (1.4%)
	Gabon (n=43)	Bivigou-Mboumba et al 2018	+	1/43 (2.3%)
	Ethiopia (n=391) #	Hundie et al 2016	±	1/391 (0.3%)
V173L	South Africa (n=97)	Andersson et al 2013	±	1/97 (1.0%)
L180M	Ethiopia (n=17)	Deressa et al 2017	+	1/17 (6%)
	Ghana (n=235)	Archampong et al 2017	+	1/235 (0.4%)
T184A/S	Ethiopia (n=391) #	Hundie et al 2016	±	1/391 (0.3%)
A194T	Ethiopia (n=391) #	Hundie et al 2016	±	1/391 (0.3%)
M204I/V	Kenya (n=11)	Day et al 2013	+	1/11 (9.1%)
	Kenya (n=27)	Kim et al 2011	+	1/27 (3.7%)
	Malawi (n=133)	Auodjane et al 2014	+	8/133 (6.0%)
	Malawi (n=21)	Galluzzo et al 2012	+	5/21 (23.8%)
	Gabon (n=43)	Bivigou-Mboumba et al 2018	+	1/ 43 (2.3%)
	Gambia (n=94)	Ndow et al 2017	+	2/94 (2.1%)
	South Africa (n=17)	Selabe et al 2009	-	13/17 (76.4%)
	Zambia & South Africa (n=92)	Hamers et al 2013	+	3/92 (3.3%)
S219A	Gambia (n=70)	Stewart et al 2011	+	1/70 (1.4%)
N236T	Zambia & South Africa (n=92)	Hamers et al 2013	+	1/92 (1.1%)
M204/IV+L80I	Malawi (n=133)	Auodjane et al 2014	+	1/133 (0.8%)
	Ghana (n=235)	Archampong et al 2017	+	1/235 (0.4%)
	Uganda (n=109)	Calisti et al 2015	+	3/109 (2.8%)
A200V+M204I	Kenya (n=27)	Kim et al 2011	+	1/27 (3.7%)
V173L+A194T	Ghana (n=140)	Geretti et al 2010 [d]	+	1/140 (0.7%)
L180M+M204I	Ghana (n=235)	Archampong et al 2017	+	1/235 (0.4%)
M204I/V+L180M	Cameroon (n=20)	Gachara et al 2017	+	1/20 (5%)
	Cameroon (n=54)	Kouanfack et al 2012	+	5/54 (9.3%)
	Gabon (43)	Bivigou-Mboumba et al 2018	+	1/43 (2.3%)
	Gabon (n=71)	Bivigou-Mboumba et al 2016	+	9/71 (12.7%)
	Gambia (n=70)	Stewart et al 2011	+	2/70 (2.9%)
	Gambia (n=94)	Ndow et al 2017	+	5/94 (5.3%)
	Ghana (n=235)	Archampong et al 2017	+	1/235 (0.4%)
	Ethiopia (n=391) #	Hundie et al 2016	±	5/391 (1.3%)
	Kenya (n=29)	Mabeya et al 2017	+	1/29 (3.4%)
	Malawi (n=133)	Auodjane et al 2014	+	10/133 (7.5%)
	Malawi (n=21)	Galluzzo et al 2012	+	1/21 (4.8%)
	Uganda (n=109)	Calisti et al 2015	+	3/109 (2.8%)
	Guinea-Bissau (n=94)	Langhoff Honge et al 2014	+	1/94 (1.1%)
M204I+V173L	Uganda (n=109)	Calisti et al 2015	+	1/109 (0.9%)
M204I/V+V173L+L180M	Cameroon (n=20)	Gachara et al 2017	+	1/20 (5%)
	Cameroon (n=54)	Kouanfack et al 2012	+	1/54 (1.9%)
	Cameroon (n=116)	Magoro et al 2016	+	4/116 (3.4%)
	Cote d'Ivoire (n=259)	Boyd et al 2015	+	2/259 (0.8%)
	Gabon (n=43)	Bivigou-Mboumba et al 2018	+	2/43 (4.7%)
	Ethiopia (n=17)	Deressa et al 2017	+	6/17 (35%)
	Gambia (n=94)	Ndow et al 2017	+	3/94 (3.2%)

	Gambia (n=70)	Stewart et al 2011	+	1/70 (1.4%)
	Ghana (n=140)	Geretti et al 2010 [d]	+	3/140 (2.1%)
	Ghana (n=235)	Archampong et al 2017	+	6/235 (2.6%)
	Kenya (n=29)	Mabeya et al 2017	+	5/29 (17.2%)
	Malawi (n=133)	Auodjane et al 2014	+	4/133 (3.0%)
	Uganda (n=109)	Calisti et al 2015	+	5/109 (4.6%)
M204V+L180M+A181S	Malawi (n=133)	Auodjane et al 2014	+	2/133 (1.5%)
M204V+L80I+L180M	Ghana (n=235)	Archampong et al 2017	+	2/235 (0.9%)
	Malawi (n=133)	Auodjane et al 2014	+	1/133 (0.8%)
M204I+L80I+L80V	Uganda (n=109)	Calisti et al 2015	+	1/109 (0.9%)
L180M+S202G+M204V	Cameroon (n=54)	Kouanfack et al 2012	+	1/54 (1.9%)
L180M+M204I+T184S	Cameroon (n=20)	Gachara et al 2017	+	1/20 (5%)
M204I/V+/-L80I+/-V173L+/-L180M	Ghana (n=143)	Chadwick et al 2012 [d]	+	12/143 (8.4%)

* We have reported mutations within a specified HBV gene as in the index studies. However, it is possible that mutations in the polymerase gene could influence the sequence of more than one protein due to the overlapping reading frames (ORFs) in the HBV genome.

[a] HIV status is designated '+' whole cohort HIV-positive; '±' some of cohort HIV-positive; '-'none of cohort HIV-positive.

Study did not specify if patients were on treatment or not.

Appendix Table 2.1.5: Number of samples with specific polymerase/reverse transcriptase mutations among treatment naïve HBV infected patients in Africa, from 12 studies published between 2009 and August 2020.

Pol/RT mutation	Treatment naïve			
	Country and number of HBV positive study participants	Author, year	HIV co-infection status of cohort[a]	Number (%) of individuals with mutation
I169L	South Africa & Zambia (n=92)	Hamers et al 2013	+	1/92 (1.1%)
	Mozambique (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
V173L	Mozambique (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
	South Africa (n=71)	Makondo et al 2012	+	1/71 (1.4%)
	Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
	Guinea-Bissau (n=94)	Langhoff Honge et al 2014	+	1/94 (1.1%)
A181/S	Kenya (n=27)	Kim et al 2011	+	1/27 (3.7%)
	Sudan (n=96)	Yousif et al 2014	+	1/96 (1.0%)
T184A/S	South Africa & Zambia (n=92)	Hamers et al 2013	+	1/92 (1.1%)
A194T	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
M204I/V	Malawi (n=47)	Galluzzo et al 2012	+	1/47 (2.1%)
	South Africa (n=37)	Powell et al 2015	+	3/37 (8.1%)
	South Africa (n=35)	Selabe et al 2007	±	13/35 (37.1%)
	South Africa & Botswana (n=72)	Matthews et al 2015	±	1/72 (1.4%)
V207I	Kenya (n=27)	Kim et al 2011	+	1/27 (3.7%)
V214A	South Africa (n=71)	Makondo et al 2012	+	1/71 (1.4%)
	Guinea-Bissau (n=94)	Langhoff Honge et al 2014	+	1/94 (1.1%)
Q215S	Sudan (n=96)	Yousif et al 2014	+	2/96 (2.1%)
I233/V	Kenya (n=27)	Kim et al 2011	+	1/27 (3.7%)
	Sudan (n=96)	Yousif et al 2014	+	1/96 (1.0%)
N236T	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	2/168 (1.2%)
N238D	Guinea-Bissau (n=94)	Langhoff Honge et al 2014	+	1/94 (1.1%)
M250I/V	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
M204V/I+L180M	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
	South Africa (n=71)	Makondo et al 2012	+	1/71 (1.4%)
	Gabon (n=71)	Bivigou-Mboumba et al 2016	+	3/71 (4.2%)
V173L+L180M	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
M204I/V+V173L+L180M	Cameroon (n=116)	Magoro et al 2016	+	2/116 (1.7%)
L180M+S202G+M204V	Mozambique & Zambia (n=168)	Wandeler et al 2016	+	1/168 (0.6%)
L180M+M204I+T184S	Cameroon (n=116)	Magoro et al 2016	+	1/116 (0.9%)

* We have reported mutations within a specified HBV gene as in the index studies. However, it is possible that mutations in the polymerase gene could influence the sequence of more than one protein due to the overlapping reading frames (ORFs) in the HBV genome.

[a] HIV status is designated '+' whole cohort HIV-positive; '±' some of cohort HIV-positive; '-'none of cohort HIV-positive.

Study did not specify if patients were on treatment or not.

Appendix Table 2.1.6: First line antiretroviral (ART) regimen for adults in Africa and overlap with hepatitis B virus (HBV) therapy. Information derived from published ART guidelines in all cases where these are available in the public domain.

Country	1st line ARV regimen	1st line ART regimen appropriate for HBV treatment	Reference
Algeria	TDF+FTC/3TC+EFV	Yes	World Health Organisation - Algeria HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Algeria_v2.pdf
Angola	AZT+3TC+NVP or TDF+3TC/FTC+EFV	Yes	World Health Organisation - Angola HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Angola.pdf
Benin	TDF+3TC+EFV	Yes	World Health Organisation – Benin HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Angola.pdf
Botswana	TDF/FTC+DTG	Yes	World Health Organisation - Botswana HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Botswana.pdf
Burkina Faso	AZT+3TC+NVP or TDF+3TC/FTC+EFV	Yes	World Health Organisation – Burkina Faso HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Burkina_Faso.pdf
Burundi	AZT+3TC+NVP or TDF+3TC+EFV	Yes	World Health Organisation - Burundi HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Burundi.pdf
Cameroon	AZT+3TC+NVP or TDF+3TC+EFV	Yes	World Health Organisation - Cameroon HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Cameroon.pdf
Chad	AZT+3TC+NVP or TDF+FTC+EFV	Yes	World Health Organisation - Chad HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Chad.pdf
Comoros	TDF+3TC+EFV	Yes	World Health Organisation - Comoros HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Comoros.pdf
Democratic Republic of Congo	AZT+3TC+NVP or TDF+FTC/3TC+EFV	Yes	World Health Organisation - Congo HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Congo.pdf
Eritrea	AZT+3TC+NVP or TDF+FTC+EFV	Yes	World Health Organisation - Eritrea HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Eritrea.pdf
Eswatini (Swaziland)	TDF+3TC+ETV	Yes	World Health Organisation - Swaziland HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Swaziland.pdf
Ethiopia	TDF/AZT+3TC+EFV	Yes	National Guidelines for Comprehensive HIV Prevention, Care and Treatmenthttps://aidsfree.usaid.go

			v/sites/default/files/ethiopia_art_guidelines_2017.pdf
Gambia	AZT+3TC+NVP	No	World Health Organisation - Gambia HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Gambia.pdf
Ghana	AZT+3TC+NVP or TDF+FTC/3TC+EFV	Yes	World Health Organisation - Ghana HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Ghana.pdf
Guinea	AZT+3TC+NVP or TDF+FTC+EFV	Yes	World Health Organisation - Guinea HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Guinea.pdf
Guinea-Bissau	TDF+FTC+ETV	Yes	World Health Organisation – Guinea-Bissau HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_ Guinea-Bissau.pdf
Kenya	TDF+3TC+EFV	Yes	Guidelines on use of Antiretroviral Drugs for Treating and Preventing HIV infection in Kenya https://aidsfree.usaid.gov/sites/default/files/kenya_art_2016.pdf
Lesotho	TDF+3TC+EFV	Yes	World Health Organisation – Lesotho HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Lesotho.pdf
Liberia	TDF+3TC/FTC+EFV	Yes	2nd Edition National Standard Therapeutic Guidelines and Essential Medicine List Liberia 2017 http://moh.gov.lr/wp-content/uploads/2017/12/Liberia-NSTG-EML_2nd-Edition_2017.pdf
Madagascar	TDF+3TC+EFV	Yes	World Health Organisation – Madagascar HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Madagascar.pdf
Malawi	TDF+3TC+EFV	Yes	World Health Organisation – Malawi HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Malawi.pdf
Mozambique	TDF+3TC+EFV	Yes	World Health Organisation – Mozambique HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Mozambique.pdf
Namibia	TDF+FTC/3TC+EFV	Yes	National Guidelines for Antiretroviral therapy; 5th Edition 2016 https://aidsfree.usaid.gov/sites/default/files/na_national_guidelines_art.pdf
Niger	TDF+3TC/FTC+EFV	Yes	World Health Organisation – Niger HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Niger.pdf
Nigeria	AZT+3TC+NVP or TDF+FTC/3TC+EFV	Yes	World Health Organisation – Nigeria HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Nigeria.pdf
Rwanda	TDF+3TC+EFV	Yes	World Health Organisation – Rwanda HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Rwanda.pdf
Senegal	TDF+3TC+EFV	Yes	World Health Organisation –

			Senegal HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Senegal.pdf
Sierra Leone	AZT+3TC+NVP	No	World Health Organisation – Sierra Leone HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Sierra_Leone.pdf
South Africa	AZT+3TC+NVP or TDF+FTC/3TC+EFV	Yes	World Health Organisation – South Africa HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_South_Africa.pdf
South Sudan	AZT+3TC+NVP or TDF+3TC+EFV	Yes	World Health Organisation – South Sudan HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_South_Sudan.pdf
Sudan	TDF+3TC+EFV	Yes	Guidelines for the use of Antiretroviral Drugs for HIV Prevention and Treatment http://preventcrypto.org/wp-content/uploads/2015/10/sudanartguidelines20141413103428.Pdf
Tanzania	TDF+3TC+EFV	Yes	National Guidelines for the management of HIV/AIDS: The United Republic of Tanzania https://aidsfree.usaid.gov/sites/default/files/04_11_2016.tanzania_national_guideline_for_management_hiv_and_aids_may_2015._tagged.pdf
Togo	TDF+3TC/FTC+EFV	Yes	World Health Organisation – Togo HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Togo.pdf
Uganda	TDF/FTC+DTG	Yes	World Health Organisation – Uganda HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Uganda.pdf
Zambia	AZT+3TC+NVP or TDF+FTC/3TC+EFV	Yes	World Health Organisation – Zambia HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Zambia.pdf
Zimbabwe	AZT+3TC+NVP or TDF+3TC+EFV	Yes	World Health Organisation – Zimbabwe HIV Country Profile: 2016 http://www.who.int/hiv/data/Country_profile_Zimbabwe.pdf

I was unable to find published guidelines for the following countries: Cape Verde, Central Africa Republic, Ivory Coast, Djibouti, Egypt, Equatorial Guinea, Gabon, Libya, Mai, Mauritania, Mauritius, Morocco, São Tomé and Príncipe, Seychelles and Tunisia. Abbreviations: AZT: Zidovudine. DTG: Dolutegravir. EFV: Efavirenz. FTC: Emtricitabine; 3TC: Lamivudine. NVP: Nevirapine. TDF: Tenofovir.

Section 2.2

Appendix Table 2.2.1: PRISMA criteria for evidence of hepatitis B virus resistance to tenofovir.

Section/topic	#	Checklist item	Reported
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Title of the published manuscript
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Provided in the published manuscript
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Provided in the introduction of the published manuscript and thesis (chapter 1, section 1.17.1)
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	No review protocol for this study.
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.2.3)
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	N/A
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Provided in the results section of the published manuscript

			and thesis (chapter 2, section 2.2.3)
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Provided in the methods section of the published manuscript and thesis (chapter 2, section 2.2.2)
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.2.3)
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.2.3)
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Provided in the results section of the published manuscript and thesis (chapter 2, section 2.2.3)
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	See point 12, 15 and 19.
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.2.4)
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.2.4)
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Provided in the discussion section of the published manuscript and thesis (chapter 2, section 2.2.4)
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Provided in the published manuscript

Appendix Table 2.2.2: Full details of 15 studies identified by a systematic literature search of evidence of hepatitis B virus resistance to tenofovir.

Reference	Type of study	Country	Characteristics of study participants	Sequencing method	Genotype	Treatment used	Mutations	Approach to defining HBV resistance to TFV
Bommel et al 2012	Longitudinal	Germany	10 patients with viral breakthrough associated with genotypic resistance to ADV monotherapy	Sanger	A & D	TDF for >6 months	rtN236T; rtA181T/V; rtL180M; rtM204Q	Clinically isolated
Lee et al 2014	Case report	Korea	54-year-old man with HBeAg positive chronic HBV infection	INNOLiPA HBV genotyping	C	3TC (rtL180M + rtM204V/I) ETV (rtT184A/L) 3TC+ADV TDF monotherapy	rtL80M, rtL180M, rtM204V/I, rtA200V, rtF221Y, rtS223A, rtT184A/L, rtR153Q, and rtV191I combined mutations without rtA194T mutation.	Clinically isolated
Shirvani-Dastgerdi et al 2017	Case report	Germany	• 53-year-old Caucasian man with chronic HBeAg negative HBV infection • 41-year-old Asian woman with chronic HBeAg positive HBV infection	Ultra-deep pyrosequencing and direct PCR	Not specified	ETV/TDF combined therapy	rtS78T/sC69*	• Clinically isolated • In vitro analyses
Lu et al 2018	Case report	China	• 60-year-old Chinese man with chronic HBeAg negative HBV infection	Not specified	Not specified	• Initially LdT and ADV (muscle soreness & elevated creatine kinase led to treatment change, TDF replaced LdT and ADV) • TDF (renal insufficiency & lack of full viral suppression led to	Not specified	Clinically isolated

						treatment change TDF to TAF) • TAF		
Lada et al 2012	Longitudinal	France	7 HIV infected individuals with HBeAg positive chronic HBV & delayed repsonders to TDF	Sanger	A/E/G	Add-on TDF plus 3TC	rtA181T (1 patient); rtR153W (3 patient3); ,rtI163V (5 patients); ,rtL217R (4 patients); rtL229V/W (3 patients); rtL180M,rtM204I/V, rtV173L	• Clinically isolated • In vitro analyses
Schirmer et al 2011	Case report	USA	55-year-old HIV infected African American man with chronic HBeAg positive HBV infection	INNOLiPA HBV genotyping	A	FTC & TDF	sF8L, sY100C, sK122R, sM133I, sD144A, sV168A, sF220C, & rtI16T, rtV142I	Clinically isolated
Liu et al 2017	Experimental	USA	11 drug resistant HBV constructs were created by site-directed mutagenesis of a full-length genotype D construct.	PCR	D	TAF	rtA181V+rtN236T	In vitro analyses
Audsley et al 2016	Longitudinal	Australia	Ninety-two HIV–HBV coinfected participants on, or about to commence, TDF-containing cART	Ultra-deep pyrosequencing	Not specified	TDF	Not specified	Clinically isolated
Mikulska et al 2012	Case report	Italy	22-year-old HIV infected man with chronic HBeAg positive HBV infection	Trugene sequencing	D	• 3TC (resistance developed treatment change TDF) • TDF (rebound occurred treatment changed to TDF/FTC) • TDF/FTC	rtF122L, rtQ130S, rtV173L, rtL180M, rtR192PR, rtM204V, rtV207L, rtC256S, rtD263E, rtV278IV sT125M, sP127T, sE164D, sV184LV, sI195M, sM198I	Clinically isolated
Amini-Bavil-Olyaee et al 2009	Experimental	Germany	Assessed the sensitivity against tenofovir in HBV con-structs	Not specified	Not specified	TDF,3TC, Ldt,ETV	rtA194T alone or in addition to 3TC RAMs (rt180M rtM204V),	In vitro analyses

			carrying the rtA194T mutation in vitro					
Choo et al 2018	Case report	Korea	37-year-old Korean man with chronic HBeAg positive HBV infection	Not specified	Not specified	TDF	rtY9H, rtL91I, rtS106C, rtS106G, rtT118C, rtT118G, rtQ267L, rtI269L, rtA317S, rtK333Q, and rtN337H	Clinically isolated
Qin et al 2013	Experimental	China/Germany	Assessed the replication competence and susceptibility to TDF of the mutated HBV clones both in vitro and in vivo.	Not specified	Not specified	TDF	rtP177G & rtF249A	In vitro and in vivo analysis
Liu et al 2013	Experimental	USA	Assessed TDF susceptibility of HBV genotype B, C and D clinical isolates with adefovir resistance-associated mutations (ADV-R).	Sanger	B, C,D	TDF	rtA181T, rtA181V, rtN236T, rtA181T + rtN236T and rtA181V + rtN236T	In vitro analyses
Sheldon et al 2005	Longitudinal	Germany	43 patients with chronic HBV infection and HIV coinfection serum assessed before and during TDF therapy.	Sanger	A, C,D,F	TDF	rtA194T + rtL180M + rtM204V	• Clinically isolated • In vitro analyses
Park et al 2019	Case report	Korea	• 57-year-old man with chronic HBV infection • 66-year-old man with chronic HBeAg postive HBV infection	Sanger	Not specified	TDF	rtS106C [C], rtH126Y [Y], rtD134E [E], rtM204I/V, and rtL269I [I]	• Clinically isolated • In vitro analyses

FTC: Emtricitabine. 3TC: Lamivudine. NVP: Nevirapine. TDF: Tenofovir disoproxil fumarate. TAF: Tenofovir alafenamide. RT: Reverse transcriptase. S: Surface.

Appendix Table 2.2.3: Quality appraisal using the Joanna Briggs Institute Critical Appraisal tool checklist for seven qualitative studies identified for a systematic literature review for the evidence of hepatitis B virus resistance to tenofovir.

Case-reports quality assessment								
Case-report studies included	Were patient's demographic characteristics clearly described?	Was the patient's history clearly described and presented as a timeline?	Was the current clinical condition of the patient on presentation clearly described?	Were diagnostic tests or assessment methods and the results clearly described?	Was the intervention(s) or treatment procedure(s) clearly described?	Was the post-intervention clinical condition clearly described?	Were adverse events (harms) or unanticipated events identified and described?	Does the case report provide takeaway lessons?
Shirvani-Dastgerdi et al 2017	Yes • 53-year-old Caucasian man with chronic HBeAg negative HBV infection • 41-year-old Asian woman with chronic HBeAg positive HBV infection	Yes 53-year-old Caucasian man with chronic HBeAg negative HBV infection 2009: baseline HBV VL of 6 x 105 copies/ml started on TDF treatment immediately, also diagnosed with HCC 2012: HBV VL Load increased after prior suppression while on TDF, ETV added to TDF treatment June 2012: there was insufficient suppression of HBV replication and recurrent HCC diagnosed 41-year-old Asian woman with chronic HBeAg positive HBV infection 2008: High baseline HBV VL, patient	Yes • 53-year-old Caucasian man with chronic HBeAg negative HBV infection presented to our outpatient clinic in August 2009 with a baseline HBV viral load of 6 × 105 copies/ml • 41-year-old Asian woman with chronic HBeAg positive HBV infection and a high viral load (baseline 2 × 109 copies/ml) presented to our outpatient clinic in January 2008.	Yes • Serum samples from two time points each were subjected to ultra-deep pyrosequencing11 and direct PCR analyses. • three replication-competent HBV constructs containing the rtS78T/sC69∗ mutation (M1), the preS1/S2 deletion (preS1/preS2del, M2) with an in-frame deletion of the nucleotides 1124 to 1385 as well as the combination of the two, rtS78T/sC69∗ + preS1/S2del (M3), were generated using site directed mutagenesis and recombination techniques	Yes 53-year-old Caucasian man with chronic HBeAg negative HBV infection • baseline HBV VL of 6 x 105 copies/ml started on TDF treatment immediately • HBV VL Load increased after prior suppression while on TDF, ETV added to TDF treatment 41-year-old Asian woman with chronic HBeAg positive HBV infection • High baseline HBV VL, patient started on ETV • Notable increase in HBV VL, TDF added to ETV	Yes 53-year-old Caucasian man with chronic HBeAg negative HBV infection • died from aggressive hepatocellular carcinoma (HCC). 41-year-old Asian woman with chronic HBeAg positive HBV infection • the patient stopped both drugs at her own decision. Her viral loads remain high due to cessation of treatment.	Not specified	• The rtS78T/sC69∗ HBV mutation, associated with enhanced replication and insufficient response to antiviral treatment, may favor long-term persistence of these isolates. This HBV mutation may predispose patients to carcinogenic effects.

		started on ETV 2009: Notable increase in HBV VL, TDF added to ETV 2013: Viral load not suppressed even with dual antiviral therapy		• Human hepatoma HuH7 cells were transiently transfected with the replication-competent HBV plasmid constructs, as described previously. • Hepatitis B surface antigen (HBsAg) and hepatitis B envelope antigen HBeAg were measured in the supernatant by commercially available kits (Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).				
Lu et al 2018	• 60-year-old Chinese man with chronic HBeAg negative HBV infection	Yes • Nov 2011: developed viral breakthrough, adefovir dipivoxil (ADV) was added as a rescue therapy • June 2015: due to renal toxicity, treated with telbivudine (LdT) and ADV • May 2016: suffered muscle soreness, treated with TDF • Developed viral breakthrough, treated with TAF	Yes: a 60-year-old individual suffering liver cirrhosis and renal dysfunction, and being infected with multidrug-resistant HBV	Yes • HBV DNA measured by Cobas TaqMan Test, Roche Diagnostic, Basel, Switzerland). • Ultrasonic tests showed liver cirrhosis and splenomegaly	Yes • Nov 2011: developed viral breakthrough, adefovir dipivoxil (ADV) was added as a rescue therapy • June 2015: due to renal toxicity, treated with telbivudine (LdT) and ADV • May 2016: suffered muscle soreness, treated with TDF • Developed viral breakthrough	Yes: HCC occurred during TAF treatment despite controlling the viral load.	Not specified	Yes: The risk of HCC could not be eliminated and should be monitored during TAF treatment.
Schirmer et al 2011	Yes: 55-year-old HIV infected African-American man with	Yes • Longstanding HIV infection • 2002: completed self-reported	Yes Case of acute hepatitis B virus genotype A vaccine	Partly • HBV antiviral resistance testing was performed using INNO-	Yes • Initially received lopinavir/ritonavir and tenofovir/emtricitabine for hic HIV-1 infection • Later ARVs were modified to include	Yes Unresponsive HBV infection to tenofovir/emtricitabine treatment	Not specified	Yes • Need for more routine testing of HBsAb and

	chronic HBeAg positive HBV infection	HBV vaccine series prior to 2002 with serial HBsAb+ results • Aug 2008: HBsAg- • April 2009: HBsAg+, HBV DNA noted • June 2009 – September 2009: HBsAb negative • Jan 2010: HBsAg+ • Feb 2010: HBeAg+, HBV DNA 30,500 copies/ml • Vaccine escape mutations identified	escape mutant infection with loss of HBV vaccine-induced seropositivity in a HIV-1 infected patient.	LiPA HBV DR v2 (Innogenetics, Gent, Belgium), • Diagnostic tools used to test for HBsAb, HBsAg, HBeAg, HBV DNA levels, hepatitis D virus (HDV) Ab and RNA were not mentioned.	darunavir/ritonavir, raltegravir and tenofovir/emtricitabine. • Other regular medications at that time included sulfamethoxazole/trimethoprim, alprazolam, loperamide, omeprazole, and metformin.	demonstrated by persistent viremia despite lacking known resistance mutations and while having an undetectable HIV-1 viral load.		HBsAg despite normal AST and ALT in the previously HBV vaccinated HIV-positive population. • Further longitudinal description and evaluation of HBV vaccine escape mutants in HIV–HBV co-infected patients is needed
Mikulska et al 2012	Yes: 22-year-old HIV infected man with chronic HBeAg positive HBV infection	Yes • Infected with HIV and HBV perinatally • On 3TC and TDF, developed resistance to 3TC • September 2004 – April 2006: TDF was included in the antiretroviral therapy, with a decrease in HBV-DNA from 1.7 × 106 to 1.8 × 103 IU/ml, but a rebound occurred when the patient decided to discontinue all medications. • July 2008: a new antiretroviral therapy was started with TDF/FTC (245/200 mg/daily), darunavir (1,200 mg/dai	Yes: A case of a 22-year-old patient with co-infection with HBV and HIV transmitted perinatally is reported. After prolonged and intermittent treatment of HIV with lamivudine and tenofovir, HBV became resistant to lamivudine. Subsequently, clinical resistance to tenofovir occurred, manifesting as HBV-DNA	Partly • Polymerase and S-gene sequencing of HBV was performed by Trugene® HBV Genotyping kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY), • Diagnostic tools used to test HBsAg, HBeAg, HBV DNA levels, were not mentioned.	Yes • On 3TC and TDF, developed resistance to 3TC • TDF was included in the antiretroviral therapy, with a decrease in HBV-DNA • A new antiretroviral therapy was started with TDF/FTC (245/200 mg/daily), darunavir (1,200 mg/daily), ritonavir (200 mg/daily), raltegravir (800 mg/daily), and enfuvirtide (180 mg/daily), which was successful in making the HIV-RNA undetectable.	Yes: At that point, the patient had HBeAg-positive chronic hepatitis B, an undetectable HIV-RNA for over 12 months, severe immunodeficiency with T-cell CD4+ of 56 cells/μl and normal aspartate aminotransferase (28 IU/L) and alanino aminotransferase (29 IU/L) levels.	Not specified	Yes: The rtR192PR, which is located in the B domain near the rtA194T, occurring in a context of a very complex substitutions patterns, might be associated with resistance to tenofovir.

		ly), ritonavir (200 mg/daily), raltegravir (800 mg/daily), and enfuvirtide (180 mg/daily), which was successful in making the HIV-RNA undetectable. • December 2008: a transient increase in HBV-DNA was observed due to a lack in compliance • April 2010: after counselling and good treatment compliance, the patient experienced a viral breakthrough with >2 log increase in HBV viral load, strongly suggesting TDF/FTC failure	breakthro ugh.					
Choo et al 2018	Yes: 37-year-old Korean man with chronic HBeAg positive HBV infection	Yes • Age 20: diagnosed with chronic hepatitis B • Age 37: started on Tenofovir disoproxil fumarate (TDF) 300 mg, one tablet daily. • After 16 months, the HBV DNA level was undetectable. • after 43 months of continuous treatment, HBV DNA had increased to 7.5 log10 copies/mL.	Yes: a 37-year-old Korean male who visited the clinic because of elevated liver enzymes.	Partly • HBV DNA titer was greater than 8.99 log10 copies/mL, as measured by the Amplicor™ Monitor PCR assay • Genotyping and diagnostic tools used to test HBsAg, HBeAg, HBV DNA levels, were not mentioned.	Yes • Age 20: diagnosed with chronic hepatitis B • Age 37: started on Tenofovir disoproxil fumarate (TDF) 300 mg, one tablet daily. • After 16 months, the HBV DNA level was undetectable. • after 43 months of continuous treatment, HBV DNA had increased to 7.5 log10 copies/mL.	Yes: We changed the therapy from tenofovir to entecavir with a regimen of 0.5 mg once daily. After 4 mo, the HBV DNA titer decreased to 267 copies/mL. The AST and ALT levels normalized to 32 IU/L and 26 IU/L, respectively (Figure 3). HBeAg seroconversion had not yet occurred.	Not specified	Yes: The results showed 9 mutation sites, most of which had not been well-known as mutation sites. Further in vitro study is needed to reveal whether the 9 mutation sites are associated with an increased TDF IC
Park et al 2019	Yes: • 57-year-old man with chronic	Yes: A 57-year-old woman with CHB	Yes: • 57-year-old man with chronic	Yes • Direct PCR-based DNA	Yes: A 57-year-old woman with CHB • 2007: started lamivudine	Partly • Not described for the 57-year-old woman with	Not specified	Yes: Our study reveals that the

	HBV infection • 66-year-old man with chronic HBeAg postive HBV infection "	• 2007: started lamivudine monotherapy • Aug 2009: adefovir dipivoxil (10 mg/day) was added to the regimen because of viral breakthrough • Dec 2012: the antiviral regimen was changed to lamivudine (100 mg/day) plus TDF (300 mg/day) due to another viral breakthrough . • Feb 2014: viral breakthrough re-occurred treatment was changed to entecavir (1 mg/day) and TDF (300 mg/day) • 2015: viral breakthrough developed again and reached 43,500,000 I U/ A 66-year-old man with CHB • 2006: took lamivudine treatment for 1 year • June 2007: treated again with entecavir (0.5 mg/day) for HBV e antigen (HBeAg)-positive • May 2009: developed entecavir-resistance • June 2009 – July 2012: underwent adefovir (10 mg/day) plus lamivudine	HBV infection • 66-year-old man with chronic HBeAg postive HBV infection "	sequencing was performed to identify genotypic resistance • The reverse transcriptase (RT) gene was amplified by PCR and cloned into the HBV 1.2-mer replicon and sequenced • 11 artificial HBV 1.2-mer replicons were generated by site-directed mutagenesis of the wild-type HBV 1.2-mer • Huh7 cells, a human hepatocellular carcinoma cell line, were seeded into 6-well plates and transfected with 2 µg of each replicon	monotherapy • Aug 2009: adefovir dipivoxil (10 mg/day) was added to the regimen because of viral breakthrough • Dec 2012: the antiviral regimen was changed to lamivudine (100 mg/day) plus TDF (300 mg/day) due to another viral breakthrough. • Feb 2014: viral breakthrough re-occurred treatment was changed to entecavir (1 mg/day) and TDF (300 mg/day) • 2015: viral breakthrough developed again and reached 43,500,000 IU/ A 66-year-old man with CHB • 2006: took lamivudine treatment for 1 year • June 2007: treated again with entecavir (0.5 mg/day) for HBV e antigen (HBeAg)-positive • May 2009: developed entecavir-resistance • June 2009 – July 2012: underwent adefovir (10 mg/day) plus lamivudine (100 mg/day) combination therapy, developed viral breakthrough again in July 2012 • July 2012: started TDF (300 mg/day) plus telbivudine (600 mg/day) combination treatment • June 2014: started TDF monotherapy and achieved complete viral suppression • June 2017: developed viral breakthrough • Sep 2017: died of hepatocellular carcinoma	CHB • The 66-year-old man with CHB died of hepatocellular carcinoma in September 2017.		quadruple (CYEI) mutation increases the amount of tenofovir required to inhibit HBV by 15.3-fold in IC50 and 26.3-fold in IC90. These results demonstrate that tenofovir-resistant HBV mutants can emerge, although the genetic barrier is high.

		(100 mg/day) combination therapy, developed viral breakthrough again in July 2012 • July 2012: started TDF (300 mg/day) plus telbivudine (600 mg/day) combination treatment • June 2014: started TDF monotherapy and achieved complete viral suppression • June 2017: developed viral breakthrough • Sep 2017: died of hepatocellular carcinoma						
Lee et al 2014	Yes: 54-year-old man with HBeAg positive chronic HBV infection	Yes	Yes: 54-year-old man with HBeAg positive chronic HBV infection and has experienced virological breakthrough on 3TC, ETV, ADV and TDF treatments	Yes: The serum HBV DNA level determined by the Amplicor™ Monitor PCR assay; HBV genotype was determined by INNOLiPA HBV Genotyping assay	Yes: was treated with entecavir (ETV) 1 mg/day following an initial unsuccessful lamivudine (LAM) treatment; The LAM and adefovir combination therapy was followed by virological breakthrough; The therapy had been switched to TDF monotherapy. However, this patient experienced virological breakthrough under TDF	Yes: The therapy had been switched to TDF monotherapy. However, this patient experienced virological breakthrough under TDF	Not specified	Yes: TDF resistance may emerge due to multi-site polymerase mutations rather than single-site polymerase mutation.

Appendix Table 2.2.4: Quality appraisal using the BMJ adapted Quality Assessment Tool for eight quantitative studies identified for a systematic literature review for the evidence of hepatitis B virus resistance to tenofovir.

Quantitative studies quality assessment												
	Selection bias			Study design		Confounders		Withdrawals and drop-outs	Reporting			Overall rating
Quantitative studies included	Are the individuals selected to participate in the study likely to be representative of the target population	What percentage of selected individuals agreed/able to participate?	Rating selection bias	Indicate the study design	Rating study design	Were analyses appropriately adjusted for confounders	Rating confounding	Were withdrawals and drop-outs reported in terms of numbers and/or reasons per group?	Are the hypothesis/aim/objective of the study clearly described?	Were inclusion/exclusion criteria specified and number of exclusions reported	Rating reporting	
Bommel et al 2012	Very likely	Not applicable	Strong	Did not state method	Weak	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Strong
Lada et al 2012	Very likely	Not applicable	Strong	Did not state method	Weak	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Strong
Liu et al 2017	Can't tell	Not applicable	Weak	Did not state method	Weak	Can't tell	Weak	Not applicable	Yes	Criteria and number of exclusions not reported	Moderate	Weak
Audsley et al 2016	Very likely	Not applicable	Strong	Did not state method	Weak	Participants characteristics clearly described	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Strong	Strong
Amini-Bavil-Olyaee et al 2009	Not applicable	Not applicable	No rating	Did not state method	Weak	Not applicable	No rating	Not applicable	Yes	Not applicable	No rating	No rating
Qin et al 2013	Not applicable	Not applicable	No rating	Did not state method	Weak	Not applicable	No rating	Not applicable	Yes	Not applicable	No rating	No rating
Liu et al 2013	Can't tell	Not applicable	Weak	Did not stat	Weak	Can't tell	Weak	Not applicable	Yes	Criteria and number of	Moderate	Weak

				e met hod						exclusions not reported		
Sheld on et al 2005	Very likely	Not applic able	Stro ng	Did not stat e met hod	We ak	Particip ants charact eristics clearly describ ed	Strong	Not applicable	Yes	Criteria and number of exclusions reported	Stro ng	Strong

Appendix Table 2.2.5: Complete list ('long list') of mutations associated with tenofovir (TFV) resistance obtained from studies reporting evidence of hepatitis B virus resistance to TFV.

RAMs	Approach to defining resistance	Isolated from individuals in whom treatment compliance confirmed	Associated with TFV resistance	Associated reduced TFV sensitivity	Isolated from an individual exposed to 3TC	Isolated from an individual exposed to ETV	Isolated from an individual exposed to ADV	Associated with TFV resistance/reduced TFV sensitivity in isolation	Associated with TFV resistance/reduced TFV sensitivity in combination with other RAMs	Corresponding changes in other proteins	Present in minor variants	References
Y9H		X	✓	✓	X	X	X	X	L91I+S106C/G+T118C/G+Q267L+I269L+A317S+K333Q+N337H	none	X	Choo et al. 2018
S78T		X	✓	✓	X	✓	X	✓	X	S78T creates a premature stop codon (C69) in the surface protein which deletes the entire small HBV surface protein.	X	Shirvani-Dastgerdi et al. 2017
L80M		✓	✓	✓	✓	✓	✓	X	L180M+M204I/V+ A200V+ F221Y+ S233A+ T184A/L+R153Q+V191I	none	X	Lee et al 2014
L91I		X	✓	✓	X	X	X	X	Y9H+S106C/G+T118C/G+Q267L+I269L+A317S+K333Q+N337H	none	X	Choo et al. 2018
S106C/G		✓	✓	✓	✓	✓	✓	X	H126Y+D134E+V173L+L180M+M204I/V+L269I	none	X	Choo et al 2018; Park et al 2019
T118C/G		X	✓	✓	X	X	X	X	Y9H+L91I+S106C/G+Q267L+I269L+A317S+K333Q+N337H	none	X	Choo et al. 2018
F122L		X	✓	✓	✓	✓	X	X	Q130S+V173L+L180M+R192P+M204V+V207L+C256S+D263E+V278I	T125M, P127T, E164D, V184LV, I195M, M198I	X	Mikulska et al 2012
H126Y		✓	✓	✓	✓	✓	✓	X	S106C/G+V173L+L180M+M204I/V+L269I	none	X	Mikulska et al 2012
Q130S		X	✓	✓	✓	✓	X	X	F122L+V173L+L180M+R192P+M204V+V207L+C256S+D263E+V278I	T125M, P127T, E164D, V184LV, I195M, M198I	X	Mikulska et al 2012
D134E		✓	✓	✓	✓	✓	✓	X	S106C/G+H126Y+V173L+L180M+M204I/V+L269I	I126S/N	X	Park et al 2019
R153W/Q		✓	✓	✓	✓	✓	✓	X	• L180M+M204V+V191L+S202I • L180M+M204I+V103I+I163V+R153W+L217R+V190E+S219F+L229W • L180M+M204V+I163V • V173L+L180M+M204V+I103V+N122H+W153R+A223S+L229V • L80M +L180M+M204I/V+ A200V+ F221Y+ S233A+ T184A/L+V191I	P120T; G145R; D144E	X	Lee et al 2014; Lada et al 2012
I163V		✓	X	✓	✓	✓	✓	X	• L180M+M204I+V103I+R153W+L217R+V190E+S219F+L229W	none	X	Lada et al 2012

									• L180M+M204V+R153W			
V173L		✓	✓	✓	✓	✓	X	X	• F122L+Q130S+L180M+R192P+M204V+V207L+C256S+D263E+V278I • L180M+M204V+I103V+N122H+W153R+A223S+L229V	E164D	X	Mikulska et al. 2012; Lada et al. 2012
P177G		X	X	✓	X	X	X	✓	X	sR169G- affects HBsAg expression	X	Qin et al. 2013
L180M		✓	✓	✓	✓	✓	✓	X	• L80M +M204I/V+ A200V+ F221Y+ S233A+ T184A/L+R153Q+V191I • F122L+Q130S+V173L+R192P+M204V+V207L+C256S+D263E+V278I • M204V+V191L+S202I • M204I+V103I+I163V+R153W+L217R+V190E+S219F+L229W • M204V+R153W+I163V • V173L+M204V+I103V+N122H+W153R+A223S+L299V	none	X	Lada et al 2012; Lee et al 2014; Mikulska et al 2012; Sheldon et al 2005
A181T/V		✓	✓	✓	X	X	✓	X	N236T	Creates a stop codon at (W172)	X	Liu et al 2014; van Bommel et al 2012; Lada et al 2012; Liu et al 2017
T184A/L		✓	✓	✓	✓	✓	✓	X	L80M+L180M+M204I/V+A200V+ F221Y+S233A+R153Q+V191I	none	X	Lee et al 2014
V191I		✓	✓	✓	✓	✓	✓	X	L80M+L180M+M204I/V+A200V+ F221Y+S233A+T184A/L+R153Q	Creates a stop codon at (W182)	X	Lee et al 2014
R192P		X	✓	✓	✓	✓	X	X	F122L+Q130S+V173L+L180M+M204V+V207L+C256S+D263E+V278I	T125M; P127T; V184LV	X	Mikulska et al 2012
A194T		✓	✓	✓	✓	✓	X	✓	L180M+M204V	Presence of BCP (A1762T/G1764A) and /or PC (C1858T/G1896A) increase the replication capacity of variants with A914T	X	Amini-Bavil-Olyaee et al 2009; Sheldon et al 2005
A200V		✓	✓	✓	✓	✓	✓	X	L80M +L180M+M204I/V+ F221Y+ S233A+ T184A/L+R153Q+V191I	L192F	X	Lee et al 2014
M204I/V		✓	✓	✓	✓	✓	✓	X	• L80M +L180M+A200V+ F221Y+ S233A+ T184A/L+R153Q+V191I • F122L+Q130S+V173L+L180M+R192P+V207L+C256S+D263E+V278I • L180M+V191L+S202I • L180M+V103I+I163V+R153W+L217R+V190E+S219F+L229W • L180M+R153W+I163V • V173L+L180M+I103V+N122H+W153R+A223S+L299V • S106C/G+H126Y+D134E+V173L+L180M+L269I	I195M; W196S/L Creates a stop codon at (W196)	X	Park et al 2019; Lada et al 2012; Lee et al 2014; Mikulska et al 2012; Sheldon et al 2005
V207L		X	✓	✓	✓	✓	X	X	F122L+Q130S+V173L+L180M+R192P+M204V+C256S+D263E+V278I	M198I; Creates a stop codon at (W199)	X	Mikulska et al 2012

L217R		✓	✓	✓	✓	✓	✓	X	L180M+M204I+V103I+I163V +R153W+V190E+S219F+L2 29W	S210I/T	X	Lada et al 2012
F221Y		✓	✓	✓	✓	✓	✓	X	L180M+M204I/V+ A200V+ F221Y+ S233A+ T184A/L+R153Q+V191I	none	X	Lee et al 2014
S223A		✓	✓	✓	✓	✓	✓	X	L180M+M204I/V+ A200V+ F221Y+ S233A+ T184A/L+R153Q+V191I	none	X	Lee et al 2014
L229V/W		✓	✓	✓	✓	✓	✓	X	• L180M+M204I+V103I+I163V +R153W+L217R+V190E+S2 19F • V173L+L180M+M204V+I103 V+N122H+W153R+A223S	C221L; F220L	X	Lada et al 2012
N236T		X	✓	✓	X	X	✓	X	N236T	none	X	Liu et al 2014; van Bommel et al 2012; Liu et al 2017
F249A		X	X	✓	X	X	X	✓	X	none	X	Qin et al 2013
C256S		X	✓	✓	✓	✓	X	X	F122L+Q130S+V173L+L180 M+R192P+M204V+V207L+D 263E+V278I	T125M; P127T; V184LV	X	Mikulska et al 2012
D263E		X	✓	✓	✓	✓	X	X	F122L+Q130S+V173L+L180 M+R192P+M204V+V207L+C 256S+V278I	T125M; P127T; V184LV	X	Mikulska et al 2012
Q267L		X	✓	✓	X	X	X	X	Y9H+L91I+S106C/G+T118C/ G+I269L+A317S+K333Q+N3 37H	none	X	Cho et al 2018
I269L		✓	✓	✓	✓	✓	✓	X	• Y9H+L91I+S106C/G+T118C/ G+Q267L+A317S+K333Q+N 337H • S106C/G+H126Y+D134E+V 173L+L180M+M204I/V+L269 I	none	X	Park et al 2019; Cho et al 2019
V278I/V		X	✓	✓	✓	✓	X	X	F122L+Q130S+V173L+L180 M+R192P+M204V+V207L+C 256S+D263E	T125M; P127T; V184LV	X	Mikulska et al 2012
A317S		X	✓	✓	X	X	X	X	Y9H+L91I+S106C/G+T118C/ G+Q267L+I269L+K333Q+N3 37H	none	X	Cho et al 2018
K333Q		X	✓	✓	X	X	X	X	Y9H+L91I+S106C/G+T118C/ G+Q267L+I269L+A317S+N3 37H	none	X	Cho et al 2018
N337H		X	✓	✓	X	X	X	X	Y9H+L91I+S106C/G+T118C/ G+Q267L+I269L+A317S+K3 33Q	none	X	Cho et al 2018

(i)	Sequencing HBV sequence from individuals in whom viraemia was not suppressed by TDF
(ii)	Sequencing HBV sequence from individuals in whom viraemia was not suppressed by TDF and *In vitro* assays to measure the effect of TFV on viral replication cell lines
(iii)	Approach (ii) combined with an animal model

✓: Yes X: No.

Appendix Table 2.2.6: Reference sequence alignment of hepatitis B virus reverse transcriptase (RT) and human immunodeficiency virus (HIV) RT.

HBV SEQUENCE POSITION																						1	2	3	4	5				
HBV_Geno_A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	D	W	G	P	-	-	-	-
HBV_Geno_B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	D	W	G	P	-	-	-	-
HBV_Geno_C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	D	W	G	P	-	-	-	-
HBV_Geno_D1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	D	W	G	P	-	-	-	-
HBV_Geno_E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	D	W	G	P	-	-	-	-
HBV TDF mutation hotspots																														
HIV SEQUENCE POSITION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		25	26	27	28	29
HIV_Subtype_B	P	I	S	P	I	E	T	V	P	V	K	L	K	P	G	M	D	G	P	K	V	K	Q	W	-	P	L	T	E	E
HIV TDF mutation hotspots																														
Sites with same AA when comparing HIV and HBV RT after alignment																														

HBV SEQUENCE POSITION									6	7	8				9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
HBV_Geno_A1	-	-	-	-	-	-	-	-	C	A	E	-	-	-	H	G	E	H	H	I	R	I	P	R	T	P	A	R	V	T
HBV_Geno_B2	-	-	-	-	-	-	-	-	C	T	E	-	-	-	H	G	E	H	R	I	R	T	P	R	T	P	A	R	V	T
HBV_Geno_C2	-	-	-	-	-	-	-	-	C	T	E	-	-	-	H	G	E	H	N	I	R	I	P	R	T	P	A	R	V	T
HBV_Geno_D1	-	-	-	-	-	-	-	-	C	A	E	-	-	-	H	G	E	H	H	I	R	I	P	R	T	P	A	R	V	T
HBV_Geno_E	-	-	-	-	-	-	-	-	C	T	E	-	-	-	H	G	K	H	H	I	R	I	P	R	T	P	A	R	V	T
HBV TDF mutation hotspots															Y9H															
HIV SEQUENCE POSITION	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
HIV_Subtype_B	K	I	K	A	L	V	E	I	C	T	E	M	E	K	E	G	K	I	S	K	I	G	P	E	N	P	Y	N	T	P
HIV TDF mutation hotspots												M41L																		
Sites with same AA when comparing HIV and HBV RT after alignment																														

HBV SEQUENCE POSITION	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
HBV_Geno_A1	G	G	V	F	L	V	D	K	N	P	H	N	T	A	E	S	R	L	V	V	D	F	S	Q	F	S	R	G	V	T
HBV_Geno_B2	G	G	V	F	L	V	D	K	N	P	H	N	T	T	E	S	R	L	V	V	D	F	S	Q	F	S	R	G	N	T
HBV_Geno_C2	G	G	V	F	L	V	D	K	N	P	H	N	T	T	E	S	R	L	V	V	D	F	S	Q	F	S	R	G	S	T
HBV_Geno_D1	G	G	V	F	L	V	D	K	N	P	H	N	T	A	E	S	R	L	V	V	D	F	S	Q	F	S	R	G	N	Y
HBV_Geno_E	G	G	V	F	L	V	D	K	N	P	H	N	T	A	E	S	R	L	V	V	D	F	S	Q	F	S	R	G	S	S
HBV TDF mutation hotspots																														
HIV SEQUENCE POSITION			60	61	62	63	64	65	66			67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85
HIV_Subtype_B	-	-	V	F	A	I	K	K	K	-	-	D	S	T	K	W	R	K	L	V	D	F	R	E	L	N	K	R	T	Q
HIV TDF mutation hotspots								K65R							K70E															
Sites with same AA when comparing HIV and HBV RT after alignment			*	*				*									*			*	*	*								

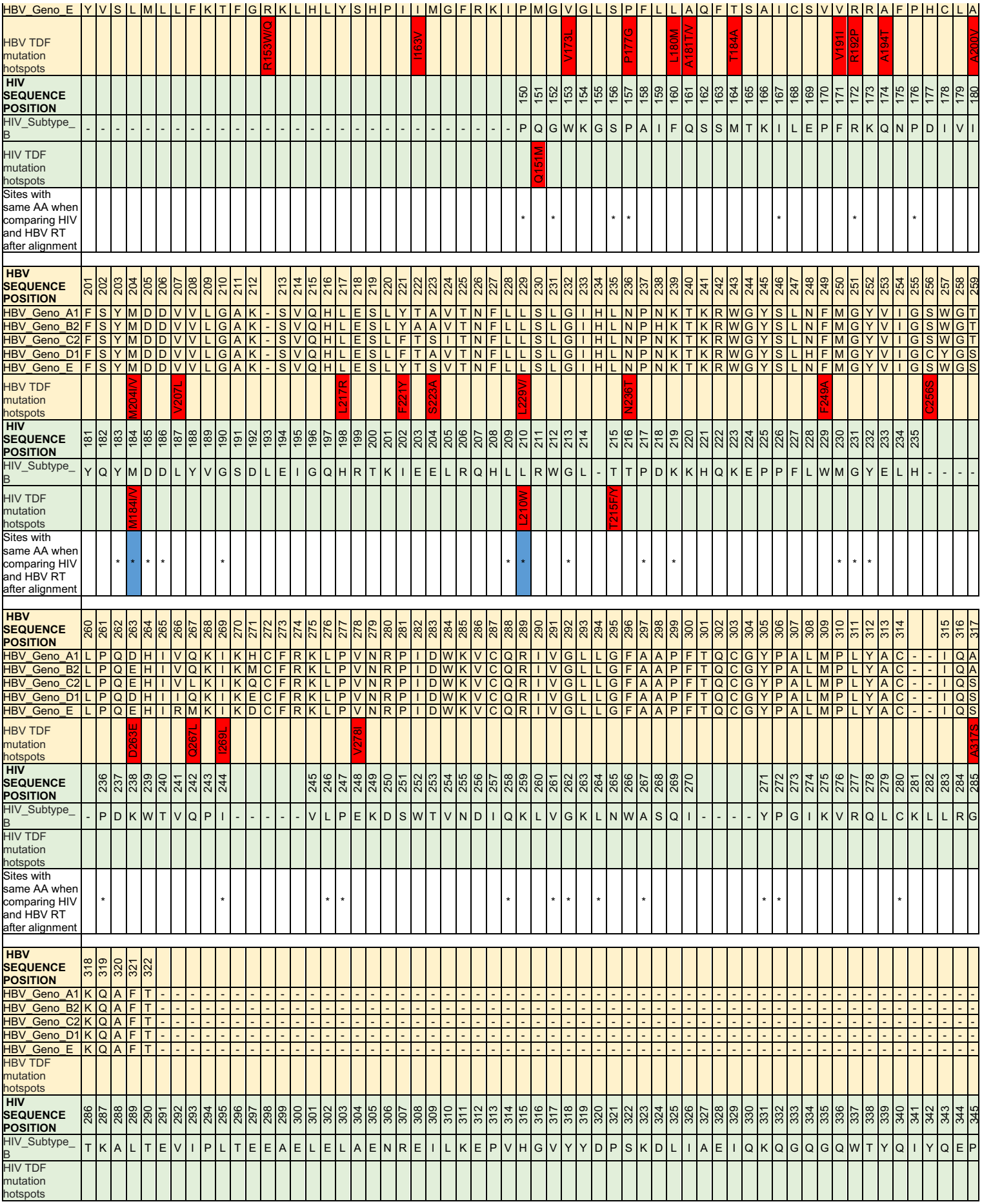
HBV SEQUENCE POSITION	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84
HBV_Geno_A1	R	V	S	W	P	K	F	A	V	P	N	L	Q	S	L	T	N	L	L	S	S	N	L	S	W	L	S	L	D	V
HBV_Geno_B2	R	V	S	W	P	K	F	A	V	P	N	L	Q	S	L	T	N	L	L	S	S	N	L	S	W	L	S	L	D	V
HBV_Geno_C2	H	V	S	W	P	K	F	A	V	P	N	L	Q	S	L	T	N	L	L	S	S	N	L	S	W	L	S	L	D	V
HBV_Geno_D1	R	V	S	W	P	K	F	A	V	P	N	L	Q	S	L	T	N	L	L	S	S	N	L	S	W	L	S	L	D	V
HBV_Geno_E	R	V	S	W	P	K	F	A	V	P	N	L	Q	S	L	T	N	L	L	S	S	N	L	S	W	L	S	L	D	V
HBV TDF mutation hotspots																								S78T		L80M				
HIV SEQUENCE POSITION	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108					109	110	111
HIV_Subtype_B	D	F	W	E	V	Q	L	G	I	P	H	P	A	G	L	K	K	K	K	S	V	T	V	-	-	-	-	L	D	V
HIV TDF mutation hotspots																														
Sites with same AA when comparing HIV and HBV RT after alignment										*					*					*								*	*	*

HBV SEQUENCE POSITION	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	
HBV_Geno_A1	S	A	A	F	Y	H	I	P	L	H	P	A	A	M	P	H	L	L	I	G	S	S	G	L	S	R	Y	V	A	-
HBV_Geno_B2	S	A	A	F	Y	H	L	P	L	H	P	A	A	M	P	H	L	L	V	G	S	S	G	L	S	R	Y	V	A	-
HBV_Geno_C2	S	A	A	F	Y	H	I	P	L	H	P	A	A	M	P	H	L	L	V	G	S	S	G	L	P	R	Y	V	A	-
HBV_Geno_D1	S	A	A	F	Y	H	L	P	L	H	P	A	A	M	P	H	L	L	V	G	S	S	G	L	S	R	Y	V	A	-
HBV_Geno_E	S	A	A	F	Y	H	L	P	L	H	P	A	A	M	P	H	L	L	V	G	S	S	G	L	S	R	Y	V	A	-
HBV TDF mutation hotspots							L91I															S106C/G								
HIV SEQUENCE POSITION	112	113	114	115	116	117	118	119	120												121	122	123	124	125	126	127	128	129	130
HIV_Subtype_B	G	D	A	Y	F	S	V	P	L	-	-	-	-	-	-	-	-	-	-	-	D	E	D	F	R	K	Y	T	A	F
HIV TDF mutation hotspots				Y115F																										
Sites with same AA when comparing HIV and HBV RT after alignment			*					*	*																		*		*	

HBV SEQUENCE POSITION				114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
HBV_Geno_A1	-	-	-	R	L	S	S	N	S	R	I	H	N	N	Q	Y	G	T	L	Q	N	L	H	D	S	C	S	R	Q	L
HBV_Geno_B2	-	-	-	R	L	S	S	N	S	R	I	I	N	N	Q	H	R	T	M	Q	N	L	H	D	S	C	S	R	N	L
HBV_Geno_C2	-	-	-	R	L	S	S	T	S	R	N	I	N	Y	Q	H	G	T	M	Q	D	L	H	D	S	C	S	R	N	L
HBV_Geno_D1	-	-	-	R	L	S	S	N	S	R	I	F	N	H	Q	H	G	T	M	Q	N	L	H	D	S	C	S	R	N	L
HBV_Geno_E	-	-	-	R	L	S	S	N	S	R	I	I	N	H	Q	Y	G	T	L	P	N	L	H	D	S	C	S	R	N	L
HBV TDF mutation hotspots								T118C/G				F122L				H126Y				Q130S				D134E						
HIV SEQUENCE POSITION	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149											
HIV_Subtype_B	T	I	P	S	I	N	N	E	T	P	G	I	R	Y	Q	Y	N	V	L	-	-	-	-	-	-	-	-	-	-	-
HIV TDF mutation hotspots																														
Sites with same AA when comparing HIV and HBV RT after alignment															*															

HBV SEQUENCE POSITION	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170
HBV_Geno_A1	Y	V	S	L	M	L	L	Y	K	T	Y	G	W	K	L	H	L	Y	S	H	P	I	I	L	G	F	R	K	I	P
HBV_Geno_B2	Y	V	S	L	M	L	L	Y	K	T	Y	G	R	K	L	H	L	Y	S	H	P	I	I	L	G	F	R	K	I	P
HBV_Geno_C2	Y	V	S	L	L	L	L	Y	K	T	F	G	R	K	L	H	L	Y	S	H	P	I	I	L	G	F	R	K	I	P
HBV_Geno_D1	Y	V	S	L	L	L	L	Y	Q	T	F	G	R	K	L	H	L	Y	S	H	P	I	I	L	G	F	R	K	I	P

HBV SEQUENCE POSITION	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
HBV_Geno_A1	M	G	V	G	L	S	P	F	L	L	A	Q	F	T	S	A	I	C	S	V	V	R	R	A	F	P	H	C	L	A
HBV_Geno_B2	M	G	V	G	L	S	P	F	L	L	A	Q	F	T	S	A	I	C	S	V	V	R	R	A	F	P	H	C	L	A
HBV_Geno_C2	M	G	V	G	L	S	P	F	L	L	A	Q	F	T	S	A	I	C	S	V	V	R	R	A	F	P	H	C	L	A
HBV_Geno_D1	M	G	V	G	L	S	P	F	L	L	A	Q	F	T	S	A	I	C	S	V	V	R	R	A	F	P	H	C	L	A


HBV_Geno_E
HBV TDF mutation hotspots
HIV SEQUENCE POSITION
HIV_Subtype_B
HIV TDF mutation hotspots
Sites with same AA when comparing HIV and HBV RT after alignment
R153W/Q
I163V
V173L
P177G
L180M
A181T/V
T184A
V191I
R192P
A194T
A200V
Q151M
HBV SEQUENCE POSITION
HBV_Geno_A1
HBV_Geno_B2
HBV_Geno_C2
HBV_Geno_D1
HBV_Geno_E
M204I/V
V207L
L217R
F221Y
S223A
L229V/I
N236T
F249A
C256S
HIV SEQUENCE POSITION
M184I/V
L210W
T215F/Y
HBV SEQUENCE POSITION
D263E
Q267L
I269L
V278I
A317S
HIV SEQUENCE POSITION
HBV SEQUENCE POSITION
HIV SEQUENCE POSITION

Sites with same AA when comparing HIV and HBV RT after alignment			*		*																																																						

HBV SEQUENCE POSITION																																								323	324	325	326	327	328	329	330	331	332	333										
HBV_Geno_A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	S	P	T	Y	K	A	F	L	S	K	-	-	-	-	-	-	-	-	-	-
HBV_Geno_B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	S	P	T	Y	K	A	F	L	S	K	-	-	-	-	-	-	-	-	-	-
HBV_Geno_C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	S	P	T	Y	K	A	F	L	C	K	-	-	-	-	-	-	-	-	-	-
HBV_Geno_D1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	S	P	T	Y	K	A	F	L	C	K	-	-	-	-	-	-	-	-	-	-
HBV_Geno_E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	S	P	T	Y	K	A	F	L	C	K	-	-	-	-	-	-	-	-	-	-
HBV TDF mutation hotspots																																																		K333Q										
HIV SEQUENCE POSITION	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405
HIV_Subtype_B	F	K	N	L	K	T	G	K	Y	A	R	M	R	G	A	H	T	N	D	V	K	Q	L	T	E	A	V	Q	K	I	T	T	E	S	I	V	I	W	G	K	T	P	K	F	K	L	P	I	Q	K	E	T	W	E	T	W	W	T	E	Y
HIV TDF mutation hotspots																																																												
Sites with same AA when comparing HIV and HBV RT after alignment																																										*			*					*										

HBV SEQUENCE POSITION										334	335	336	337	338	339	340	341	342	343	344																345
HBV_Geno_A1	-	-	-	-	-	-	-	-	-	Q	Y	M	N	L	Y	P	V	A	R	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
HBV_Geno_B2	-	-	-	-	-	-	-	-	-	Q	Y	L	N	L	Y	P	V	A	R	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
HBV_Geno_C2	-	-	-	-	-	-	-	-	-	Q	Y	L	N	L	Y	P	V	A	R	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
HBV_Geno_D1	-	-	-	-	-	-	-	-	-	Q	Y	L	N	L	Y	P	V	A	R	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
HBV_Geno_E	-	-	-	-	-	-	-	-	-	Q	Y	L	N	L	Y	P	V	A	R	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
HBV TDF mutation hotspots													N337H																							
HIV SEQUENCE POSITION	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441
HIV_Subtype_B	W	Q	A	T	W	I	P	E	W	E	F	V	N	T	P	P	L	V	K	L	W	Y	Q	L	E	K	E	P	I	V	G	A	E	T	F	Y
HIV TDF mutation hotspots																																				
Sites with same AA when comparing HIV and HBV RT after alignment													*			*																				

Sequences downloaded from HIV sequence database - https://www.hiv.lanl.gov/) and (Hepatitis B Virus Database - https://hbvdb.ibcp.fr/HBVdb/).

Alignment done using an online alignment program (MAFFT version 7).

TDF: Tenofovir disoproxil fumarate.

Appendix Table 2.2.7: Mapping of resistance associated mutations (RAMs) to the human immunodeficiency virus (HIV) reverse transcriptase (RT) structure. RAMs were mapped onto the high-resolution structure of HIV RT to infer their possible functional consequences in terms of structure.

HBV TDF mutation hotspot	HIV RT p66 residue	HIV RT p66 residue location	Interpretation
Y/H9	E44	Fingers: Solvent exposed	No impact on fold/function
S78		Fingers: Deletion, but probably buried	Destabilising
L80		Fingers: Deletion, but probably buried	Destabilising
L91	V118	Fingers: Core	Destabilising
S106	E122	Fingers: Solvent exposed	No obvious impact on fold/function
N/T118	E138	Fingers: Solvent exposed	No obvious impact on fold/function
H/I/F122	I142	Fingers: Partially buried	Probably destabilising
H/Y126	Y146	Fingers: Core	Destabilising
P/Q130		Fingers: Deletion	
D/N134		Fingers: Deletion	
R/W153		Fingers: Deletion	
I163		Palm: Deletion	
V173	W153	Palm: Core	Destabilising
P177	P157	Palm: Solvent exposed, but in helix	Possibly destabilising
L180	S160	Palm: Core	Destabilising
A181	Q161	Palm: Core	Salt-bridge, destabilising
T184	M164	Palm: Core	Destabilising
V191	F171	Palm: Core	Destabilising
R192	K172	Palm: Solvent exposed	No obvious impact on fold/function
A194	Q174	Palm: Solvent exposed	No obvious impact on fold/function
A200	I180	Palm: Core	Destabilising
M204	M184	Palm: Solvent exposed	Adjacent to catalytic site. Destabilising
V207	L187	Palm: Core	Adjacent to catalytic site. Destabilising
L217	H198	Palm: Core	Destabilising
F/Y221	I202	Palm: Core	Destabilising
A/S223	E204	Palm: Solvent exposed	No obvious impact on fold/function
L229	L210	Palm: Solvent exposed	No obvious impact on fold/function
N236	L214	Palm: Core	Destabilising
F249	W229	Palm: Core	Adjacent to catalytic site. Destabilising
C/S256		Palm: Deletion	
D/E263	K238	Palm: Solvent exposed, but possible chelation site	Probably no impact
Q267	Q242	Thumb: Solvent exposed, but possible salt bridge	Possibly destabilising
I/L269	I244	Thumb: Core	Destabilising
I/V278	E248	Thumb: Solvent exposed	No obvious impact on fold/function
A/S317	G285	Thumb: Loop turn	Destabilising
K333	K395	Connection: Partially exposed but pi cation	Possibly destabilising
N/T337	N418	Connection: Solvent exposed	No obvious impact on fold/function

Chapter 3

Appendix Methods 3.1: Phylogenetic dating using Bayesian Evolutionary Analysis Sampling Trees (BEAST).

I performed Bayesian Markov chain Monte Carlo (MCMC) analyses using BEAST v.1.10 (Suchard et al. 2018). I used a GTR+G nucleotide substitution model, a coalescent Bayesian Skygrid model with 50 points (Gill et al. 2013) and the uncorrelated lognormal relaxed molecular clock model. These models were selected because they have performed best in other studies estimating HBV evolution (Zehender et al. 2015; Mozhgani et al. 2018). I also used a mean substitution rate to 5.0×10^{-5} (SD 4.12×10^{-6}) and a mean standard deviation to 2.0×10^{-5} (SD 4.96×10^{-7}) subs/site/year, for all genotypes in all subsequent BEAST analyses, as this rate has been estimated before and applied in phylodynamic analyses of HBV (Osiowy et al. 2006; Littlejohn, Locarnini, and Yuen, 2016).

To avoid convergence issues, I selected smaller subsamples from each of the sequence alignments, depending on their size, to ensure that alignments used in BEAST analyses were <200 sequences. Thus, Genotype B (total n=564) was split into three subsamples, Genotype C (total n=906) into six subsamples and Genotype D (total n=336) into two subsamples. I used stratified random sampling, ensuring equal representation of sequences with mutations in each subsample. I ran one BEAST analysis for Genotypes A and E since their full alignments contained <200 sequences.

Two MCMC chains of 100×10^6 generations (10% burn-in) were run with sampling every $10{,}000^{th}$ generation for Genotypes A and E, and for each subsample of Genotypes B, C and D separately. The two MCMC chains for separate analyses were combined using LogCombiner v1.10.4 (Suchard et al. 2018). I inspected convergence of the MCMC analyses using Tracer v.1.7.1 (Rambaut et al. 2018) to ensure effective sample size (ESS) >200 for all model parameters. I inferred maximum clade credibility trees using TreeAnnotator v.1.10.0 (Suchard et al. 2018) and visualised them using FigTree v.1.4.4 (Suchard et al. 2018).

Chapter 7

Appendix Table 7.1: CHEERS checklist— Modelling cost-effectiveness of alternative strategies for prevention of mother to child transmission of hepatitis B virus infection in sub-Saharan Africa.

Section/item	Item No	Recommendation	Reported on page No/ line No
Title and abstract			
Title	1	Identify the study as an economic evaluation or use more specific terms such as "cost-effectiveness analysis", and describe the interventions compared.	Title of the published manuscript
Abstract	2	Provide a structured summary of objectives, perspective, setting, methods (including study design and inputs), results (including base case and uncertainty analyses), and conclusions.	Provided in the published manuscript
Introduction			
Background and objectives	3	Provide an explicit statement of the broader context for the study.	Provided in the introduction of the published manuscript and thesis (chapter 1, section 1.17.6)
		Present the study question and its relevance for health policy or practice decisions.	Provided in the introduction of the published manuscript and thesis (chapter 1, section 1.16.4)
Methods			
Target population and subgroups	4	Describe characteristics of the base case population and subgroups analysed, including why they were chosen.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Setting and location	5	State relevant aspects of the system(s) in which the decision(s) need(s) to be made.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Study perspective	6	Describe the perspective of the study and relate this to the costs being evaluated.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Comparators	7	Describe the interventions or strategies being compared and state why they were chosen.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Time horizon	8	State the time horizon(s) over which costs and consequences are being evaluated and say why appropriate.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Discount rate	9	Report the choice of discount rate(s) used for costs and outcomes and say why appropriate.	Discount rate not applicable Cost described in (chapter 7, section 7.2)
Choice of health outcomes	10	Describe what outcomes were used as the measure(s) of benefit in the evaluation and their relevance for the type of analysis performed.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Measurement of effectiveness	11a	*Single study-based estimates:* Describe fully the design features of the single effectiveness study and why the single study was a sufficient source of clinical effectiveness data.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
	11b	*Synthesis-based estimates*: Describe fully the methods used for identification of included studies and synthesis of clinical effectiveness data.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Measurement and valuation of preference-based outcomes	12	If applicable, describe the population and methods used to elicit preferences for outcomes.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)

Section/item	Item No	Recommendation	Reported on page No/ line No
Estimating resources and costs	13a	*Single study-based economic evaluation:*Describe approaches used to estimate resource use associated with the alternative interventions. Describe primary or secondary research methods for valuing each resource item in terms of its unit cost. Describe any adjustments made to approximate to opportunity costs.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
	13b	*Model-based economic evaluation:* Describe approaches and data sources used to estimate resource use associated with model health states. Describe primary or secondary research methods for valuing each resource item in terms of its unit cost. Describe any adjustments made to approximate to opportunity costs.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Currency, price date, and conversion	14	Report the dates of the estimated resource quantities and unit costs. Describe methods for adjusting estimated unit costs to the year of reported costs if necessary. Describe methods for converting costs into a common currency base and the exchange rate.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Choice of model	15	Describe and give reasons for the specific type of decision-analytical model used. Providing a figure to show model structure is strongly recommended.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Assumptions	16	Describe all structural or other assumptions underpinning the decision-analytical model.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Analytical methods	17	Describe all analytical methods supporting the evaluation. This could include methods for dealing with skewed, missing, or censored data; extrapolation methods; methods for pooling data; approaches to validate or make adjustments (such as half cycle corrections) to a model; and methods for handling population heterogeneity and uncertainty.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Results			
Study parameters	18	Report the values, ranges, references, and, if used, probability distributions for all parameters. Report reasons or sources for distributions used to represent uncertainty where appropriate. Providing a table to show the input values is strongly recommended.	Provided in the methods section of the published manuscript and thesis (chapter 7, section 7.2)
Incremental costs and outcomes	19	For each intervention, report mean values for the main categories of estimated costs and outcomes of interest, as well as mean differences between the comparator groups. If applicable, report incremental cost-effectiveness ratios.	Provided in the results section of the published manuscript and thesis (chapter 7, section 7.3)
Characterising uncertainty	20a	*Single study-based economic evaluation:* Describe the effects of sampling uncertainty for the estimated incremental cost and incremental effectiveness parameters, together with the impact of methodological assumptions (such as discount rate, study perspective).	N/A
	20b	*Model-based economic evaluation:* Describe the effects on the results of uncertainty for all input parameters, and uncertainty related to the structure of the model and assumptions.	Provided in the results section of the published manuscript and thesis (chapter 7, section 7.3)
Characterising heterogeneity	21	If applicable, report differences in costs, outcomes, or cost-effectiveness that can be explained by variations between subgroups of patients with different baseline characteristics or other observed variability in effects that are not reducible by more information.	Provided in the results section of the published manuscript and thesis (chapter 7, section 7.3)
Discussion			
Study findings, limitations, generalisability, and current knowledge	22	Summarise key study findings and describe how they support the conclusions reached. Discuss limitations and the generalisability of the findings and how the findings fit with current knowledge.	Provided in the results section of the published manuscript and thesis (chapter 7, section 7.4)
Other			
Source of funding	23	Describe how the study was funded and the role of the funder in the identification, design, conduct, and reporting of the analysis. Describe other non-monetary sources of support.	Provided in the published manuscript
Conflicts of interest	24	Describe any potential for conflict of interest of study contributors in accordance with journal policy. In the absence of a journal policy, we recommend authors comply with International Committee of Medical Journal Editors recommendations.	Provided in the published manuscript

For consistency, the CHEERS statement checklist format is based on the format of the CONSORT statement checklist.