Hepatitis B Virus Genotypic Variation: Implications for Global Immunisation Programme

Uma Devi
MB BS, FRACP

Submitted in total fulfillment of the requirements of the degree of
Doctor of Philosophy

October 2015

Department of Microbiology and Immunology, University of Melbourne
and
Victorian Infectious Diseases Reference Laboratory
I would like to dedicate this thesis to:

Bhagavan Sri Sathya Sai Baba

and my parents

Permal and Deoyani Swamy
Abstract

Hepatitis B virus (HBV) infection can be prevented through vaccination and is achieved by the production of anti-HBs. However, ‘a’ determinant, the major neutralizing domain of hepatitis B virus (HBV) has a conformational dependent structure and under positive selection pressure and vaccine-derived anti-HBs has been shown to fail neutralisation; mainly because of the emergence of vaccine escape mutants (VEM). Most HBV vaccines have been derived from genotype A2 adw and vaccine failure has been noted in non-A2 adw HBV, despite adequate anti-HBs. Variability of HBsAg has been reported for the different HBV genotypes, HBV variants either naturally occurring or due to immune escape from vaccination and/or hepatitis B immunoglobulin and variants related to antiviral drug resistance. Currently, there are no phenotypic assays that can identify the differences in HBsAg or features of HBsAg associated with vaccine failure.

In this study, 19 monoclonal antibodies (mAb) directed to the C terminal, Loop 1, 2, conformation dependant and N-terminal regions of HBsAg were utilized to map the ‘a’ determinant using the BioPlex immunoassay platform. The initial analysis was performed for the in-vitro generated HBsAg and included those derived from different HBV genotypes, variants implicated in vaccine escape including the canonical vaccine escape mutant (VEM) sG145R and HBV with an altered envelope due to antiviral drug resistance. BioPlex analysis revealed nine changes in mAb binding in HBsAg expressing the substitution sG145R, and showed an increase in binding to mAb 17, a Loop 2 mAb designed to identify vaccine escape mutants. Genotypes E, F and HBV variants sD144E/sG145R, sM133L, sS132A, sT126N, sP120T and antiviral drug resistance associated variants, sE164D and sW196S also showed increased binding to mAb 17. However, for HB Ig associated variants sD144A and sD144E, mAb 17 binding remained unaltered.

BioPlex changes seen in in-vitro derived HBsAg constructs were compared to serum HBsAg from four different clinical groups of patients. Group A consisted of 10 serum samples with sG145R, determined by ultradeep pyrosequencing (UDPS). Analysis of the in-vitro construct of sG145R showed 9 changes. However, only one clinical sample, which had high relative proportion (almost 100%) of sG145R population on UDPS exhibited all of these nine changes. Loss of mAb 12 binding was observed in all samples (10/10), followed by mAb 8 (9/10) and mAb 16 (7/10) and these were Loop 2 mAbs. Group B consisted of clinical samples with substitutions claimed as vaccine escape and
those associated with diagnostics failures. “Diagnostic failures” are samples known to be HBV DNA positive, but HBsAg negative on serological testing. In this group, there was a poor correlation between the in-vitro constructs and the clinical samples. Group C consisted of samples with HBV expressing antiviral drug associated substitution sD164E/I195M, and was compared to in-vitro construct of sG145R; overall there was a poor correlation between the clinical and reference constructs. Group D comprised of samples from seven pregnant women in their third trimester with high HBV loads (>10^8 copies/mL) and samples from their babies’ at 9 months of age. The phylogenetic studies showed both the mother and baby pairs were from the same respective clades. Serum HBsAg in five out of seven babies exhibited loss of mAb 8. However there was no specific BioPlex feature associated with mother to baby transmission.

In conclusion, BioPlex analysis of in-vitro constructs showed that there were measurable phenotypic changes in HBsAg between the HBV genotypes. Although various profiles were identified for variants associated with vaccine escape, it was not possible to determine a signature phenotypic profile, which could be used categorically to classify a potential VEM. The utility of HBsAg phenotypic profiling in clinical setting needs further prospective studies and evaluation in conjunction with population-based sequencing and next generation sequencing so that laboratory based phenotypic profile can be established and used in surveillance studies.
Declaration

I declare that this thesis comprises of my original work towards Doctor of Philosophy, except where acknowledged and indicated in the text, in accordance with the guidelines of University of Melbourne. The thesis is less than 100,000 words, exclusive of figures, tables and bibliography.
Acknowledgement

I would like to thank Prof Stephen Locarnini for presenting the idea for this research and for his invaluable help as my supervisor. I would like to express my great appreciation to Prof Scott Bowden for proofreading this thesis and his advise during my study.

I would like to thank National Health and Medical Research Council for my Ph.D scholarship and my Ph.D committee members for their input during my candidature.

Many thanks to Dr Renae Walsh for her help with BioPlex, Dr Lilly Yuen, Dr Margaret Littlejohn and Ms Rosalind Edward for sequencing and mutational analysis and Dr Nadia Warner for her help with cloning. I am also very grateful to the assistance provided by the staff of Molecular Research and Development Laboratory: Ms Danni College, Ms Maragret Nash, Ms Kathy Jackson, Ms Vtina Sozzi, Dr Peter Revill, Ms Rachael Hammond, Dr Liz Vincan, Dr Bayliss, Mr Bang Tran, Dr Lucy Lim and Dr Liz Bannister.

I am grateful to my parents for their support, would like to thank my sisters, nephews and niece for their help and optimism and appreciate the support and the support of my colleagues and friends.

Finally, I would like to express my deepest gratitude to Bhagwan Sri Sathya Sai Baba for His blessings and guidance, forever.
List of Abbreviations

ADAPVEMs - Antiviral drug associated potential vaccine escape mutants
ALT - Alanine amino transaminase
AST - Aspartate transaminase
bp - Base pairs
CHB - Chronic hepatitis B
CTL - Cytotoxic T-Cell
DMEM - Dulbecco's Modified Eagle Medium
dNTP - Deoxyribonucleotide triphosphate
DMSO - Dimethyl sulfoxide
ECL - enhanced chemiluminescence
EIA - Enzyme immunoassay
EPI - Extended Programme for Immunization
ER - Endoplasmic reticulum
ETV - Entecavir
FCS - Fetal Calf Serum
HBIG - Hepatitis B immunoglobulin
HBV - Hepatitis B Virus
HBCAg - Hepatitis B core-antigen
HBeAg - Hepatitis B e-antigen
HCC - Hepatocellular carcinoma
HCV - Hepatitis C virus
HDV - Hepatitis D virus
HIV - Human immunodeficiency virus
IDU - Injecting drug user
LMV - Lamivudine
LdT - Telbivudine
TDF - Tenofovir
mAb - Monoclonal antibody
NaCl - Sodium chloride
NFW - Nuclease free water
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
pHSA - polymerized human serum albumin
pgRNA - Pregenomic RNA
rc - Relaxed circular
rt - Reverse transcriptase
RFHBs - Royal Free Hospital antibody
TFV - Tenofovir
TLR-2 - Toll-like receptor 2
UDPS - ultradeep pyrosequencing
ULN - Upper limit of normal
VEM - Vaccine escape mutant
WHO - World Health Organisation
YMDD - tyrosine-methionine-aspartate
Table of Contents

Title i
Dedication ii
Abstract iii
Declaration v
Acknowledgement vi
List of Abbreviation vii

Chapter 1: Literature Review
1.1 Introduction 1
1.2 Epidemiology 1
1.3 Transmission 1
   1.31 Modes of Transmission 1
   1.32 Perinatal Transmission 2
   1.33 Early Horizontal Transmission 3
1.4 Global Burden of Hepatitis B by WHO Region 3
   1.4.1 Asia 4
   1.4.2 Americas 4
   1.4.3 Western Pacific 5
   1.4.4 Eastern Mediterranean 5
   1.4.5 Europe 5
   1.4.6 Africa 6
1.5 Clinical Manifestation and Outcome of Hepatitis B Infection 6
   1.5.1 Overview of Hepatitis B Infection 6
   1.5.2 Acute Hepatitis B Infection 6
   1.5.3 Serological Events 6
   1.5.4 Phases of Chronic Hepatitis B 8
   1.5.5 Immune Tolerance 9
   1.5.6 Immune Clearance 10
   1.5.7 Immune Control 11
   1.5.8 Immune Escape 12
1.6 Hepatitis B Vaccinations 12
   1.6.1 Overview of Hepatitis B Vaccination 12
   1.6.2 Passive Immunisation 13
   1.6.3 Impact of Vaccination in Prevalence and Complications of CHB
1.7 Treatment of Chronic Hepatitis 14

viii
1.7.1 Immunomodulators
1.7.2 Direct Acting Anti-viral
1.7.2A L-Neucleoside analogue group
1.7.2B Acyclic phosphonate group
1.7.2C D-Cyclopentane group

1.8 Complications of Chronic Hepatitis B

1.9 Overview HBV Structure
1.9.1 Overview of HBV Structure
1.9.2 Hepatitis B Virus Genome
1.9.3 Hepatitis B Viral Replication
1.9.4 Encapsidation and Reverse Transcription
1.9.5 Translation Products of Hepatitis B Virus

1.10 The Hepatitis B Surface Protein
1.10.1A) Serotypes
1.10.1B) SHBs and the ‘a’ determinant
1.10.2 MHBs
1.10.3 LHBs

1.11 Genotypes and Variants
1.11.1 Genotypes
1.11.2 Hepatitis B Virus Variants
1.11.3 Naturally Selected HBV S-Gene Variants
1.11.4 Vaccine and Immunoglobulin Escape Variants

1.12 Hepatitis B Vaccine Escape Mutants Overview
1.12.1 ‘a’ Determinant amino acid 100-124
1.12.2 ‘a’ Determinant amino acid 125-138
1.12.3 ‘a’ Determinant amino acid 139-148
1.12.4 Changes Outside ‘a’ determinant

1.13. Anti-viral Associated S-gene Variants

1.14 Aims of this study
1.15 Hypothesis
1.16 Objective
1.17 Specific Aims

**Chapter 2: Methodology**

2.1A Cell Culture
2.1B H166
2.2 Cloning S-gene
2.2.1 Vector and S-gene
2.2.2 Amplification of the S-gene
2.2.3 Digestion of PCR product and Plasmid, Extraction and Ligation
2.2.4 Transformation in *E. coli*
2.2.5 Mini Prep
2.2.6 Sequencing of S-gene
2.2.7 Maxi-Prep
2.2.8 Pairwise Analysis
2.2.9 Transfection

2.3 Protein Expression
2.3.1 Western Blotting
2.3.2 Quantifying HBsAg
2.3.3 Cell Culture Modification to Increase HBsAg

2.4 Generation of S-gene Variants
2.4.1 Site-Directed Mutagenesis and expression of HBsAg

2.5 BioPlex Methodology
2.5.1 HBsAg Epitope Mapping
2.5.2 Bioinformatics Analysis
2.5.3 Data Presentation

2.6 BioPlex of *In-vitro* constructs

Chapter 3: Cloning and Protein Expression
3.1 Background
3.2 Specific Aims
3.3 Materials and Method
3.4 Results
3.4.1 Reference Genotype Serotype Panel
3.4.2 Pairwise analysis
3.4.3 Protein Expression
3.4.4 Cell Culture Modifications
3.4.5 Generation of S-gene variants 61
3.5 Discussion 66

Chapter 4: BioPlex Analysis of In-Vivo HBsAg 70
4.1 Introduction 70
4.2 Aims 75
4.3 Methods, Material and Analysis 75
4.4 Results 78
  4.4.1A Control 78
  4.4.1B Genotype/Serotype Profile 79
  4.4.1C Vaccine/HBlg/Drug Resistant Profile 89
4.5 Discussion 108

Chapter 5: BioPlex Analysis of Ex-Vivo HBsAg 120
5.1 Introduction 120
5.2 Aims 122
5.3 Methods, Material and Analysis 123
5.4 Results 131
  5.4.1 Group A 131
  5.4.2 Group B 143
  5.4.3 Group C 158
  5.4.4 Group D 167
5.5 Discussion 185

Chapter 6: Conclusion and Future Directions 200
6.1 Conclusion 200
6.2 Future Directions 205
Chapter 1:
Literature Review

1.1 Introduction

Chronic hepatitis B virus (HBV) infection is a worldwide public health problem and is associated with significant morbidity and mortality, predominantly from the development of cirrhosis and hepatocellular carcinoma (HCC) [1-4]. In developing countries there is also a considerable economic burden, with chronic infection placing demands on limited health resources.

An effective vaccine was first licensed for hepatitis B in the early 1980s, with the original plasma-derived vaccine being soon replaced by recombinant versions[5, 6]. Since the vaccine's introduction, it has played an integral role in the control of HBV infection and was one of the major public health successes of the twentieth century [7-9]. Nonetheless, there are a small number of vaccine non-responders, a failure to achieve complete protection from perinatal transmission of newborns and the identification of possible vaccine escape mutants (VEM) [10-14], which has raised concerns that the current vaccination programs can be compromised.

1.2 Epidemiology

Approximately two billion people, which equates to one third of the world's population, have been infected with the HBV, with 240 million of these persistently infected [9, 15]. The most recent global burden of disease study estimates the total number of deaths related to viral hepatitis infection to be 1.29 million per year, twice as many as estimated previously, with 786,000 of these attributable to hepatitis B [16, 17]. The highest mortality rates are mainly found in endemic and resource poor settings[9].

1.3 Transmission

1.3.1 Modes of Transmission

In countries with high endemicity, there are two main modes of transmission of HBV, perinatal, (at birth, mother-to-baby) and early horizontal (in childhood) through close contacts. The estimated risk of HBV acquisition in these countries is close to 60% and approximately 45% of the global population lives in such areas [18, 19].
Exposure to HBV is 50 -100 times more likely to cause infection than with equivalent exposure to human immunodeficiency virus (HIV). HBV can be found in most body fluids, however, transmission has never been documented through exposure to saliva unless associated with trauma, such as a skin puncturing bite [20, 21]. All HBsAg positive individuals are considered infectious and the likelihood of transmission is generally proportional to viral load (HBV DNA). Hepatitis B e antigen (HBeAg) is considered a good marker of infectivity, and a HBeAg positive patient is more likely to transmit infection than a similar contact with HBeAg negative hepatitis [22, 23].

In developed countries, the main route of transmission is horizontal through unsafe sexual practices or injecting drug use (IDU). Living in a household with a HBsAg-positive individual can expose others to HBV via non-sexual activities such as sharing shaving razors or toothbrushes or being in contact with exudates from skin disorders. This may be one of the factors in transmission for individuals with an intellectual disability, living in long-term care facilities [19, 20, 22, 24, 25].

Healthcare related transmission of HBV remains high, with an estimated 21 million new infections occurring every year. The high transmission rates can be attributed to poor infection control practices and lack of appropriate vaccination in health care settings. Conversely transmission from HBsAg positive health care providers is rare, even when invasive procedures are undertaken [26].

Transmission of HBV in the setting of blood transfusion is extremely rare in most countries due to high quality HBsAg screening assays and the advent of nucleic acid testing for HBV DNA to further reduce the window period before development of HBsAg positivity [27, 28].

1.3.2) Perinatal Transmission
Perinatal or mother-to-baby transmission is the major source of infection in high seroprevalence countries where many women of childbearing age are HBeAg positive [29, 30]. The risk of transmission from a HBeAg positive mother to her baby can be as high as 90% in the absence of infant immunization, while that for HBeAg negative mothers is approximately 30% [31-33]. North Uganda reported HBsAg prevalence of 20% in pregnant women aged less than 20 years; alarmingly, vaccination starts at 6 weeks of age [30].
The mode of delivery (caesarian versus normal vaginal delivery) does not appear to influence the rates of infection. Active and passive prophylaxis at birth (active vaccination and passive hepatitis B immunoglobulin (HBlg)) has been found to be the most effective in preventing infection in the infant [32, 33]. The most important predictor for infant prophylaxis failure is maternal viral load [13, 34]. Antiviral therapy in the third trimester has been used to decrease high maternal viral load and further reduces the risk of HBV transmission [35, 36].

Breast-feeding has been investigated as a potential mode of HBV transmission but the risk has been shown to be negligible if infants are given HBV vaccine and/or HBlg at birth [11, 37, 38].

1.3.3. Early Horizontal Transmission

Early horizontal transmission is also one of the major risk factors for developing chronic HBV infection [2]. Studies in China have reported an estimated 80% of infected individuals acquired infection in early childhood; this could be due to close contact with the infected mother, other infected infants or iatrogenically via infected syringes and needles [39]. Studies in The Philippines and India showed variable prevalence of HBsAg, with the highest rates in 15-20 year old in Tamil Nadu, whilst in some regions of The Philippines the highest rates of HBsAg positivity are in children 2-9 years of age, suggesting early horizontal transmission [40, 41]. More recently, it has also been proposed that early horizontal transmission of HBV takes place in the Euro-Mediterranean countries and Africa in general, consistent with the different natural history of hepatitis B seen in South East Asia [42].

1.4) Global Burden of Hepatitis B Infection by WHO region

To classify the burden of chronic HBV infection around the world, the prevalence of infection has been categorized into three groups. For countries with HBsAg prevalence of less than 2%, prevalence is regarded as low, rates between 2%-7% as intermediate and greater than or equal to 8% as high, as shown in Figure 1.1 [15, 43, 44].

While these categories reflect the overall prevalence, the rates may be different in a particular population within the country. The World Health Organisation (WHO) classifies countries into regions, and these include the Americas, Europe, Eastern Mediterranean, Africa, South-East Asia and Western Pacific [1] along with the rates of chronic HBV infection by region. Additionally, within a region there may be substantial
differences in the rates of infection as well[1, 43]. Not surprisingly then, targeted prevention and management strategies require identifying high risks population. A summary of these findings follows.

A meta-analysis, involving a review of the literature for reports on HBV infection from around the world was conducted by Ott et al. Using a Bayesian hierarchical model and data analysis from the literature published between 1990-2005 on hepatitis B surface antigen (HBsAg) seroprevalence, they calculated the age-specific prevalence and endemicity of infection on a global level and for all the world's regions [15].

Figure 1.1 shows the distribution of HBV infection and its prevalence around the world. Adapted from reference [44]

1.4.1 Asia
Asian regions showed diverse prevalence patterns. East Asia had the highest HBsAg prevalence compared to the rest of the Asian region. From 1990 – 2005, there was an age-specific decline in HBsAg prevalence in those persons less than 19 years old from 8% to approximately 6%, whilst an increase in those aged >35 years. Central Asia had an intermediate seroprevalence range, while South Asia had a lower prevalence and strong decline prevalence in children reflecting the success of the immunization programme[15].

1.4.2 Americas
In the United States, the prevalence of HBV infection between 1986-1996 was 4.6% and 0.3% had evidence of active chronic infection. An increase in the vaccination rate of targeted populations such as infants of women with positive HBV serology in high-risk
adolescents and adults began in 1991, and a survey in 2007 showed a decrease in the HBsAg prevalence rate compared to the previous survey. Meanwhile, in Canada, the rate of HBV infection in persons born there was 0.1%, while in the Inuit and migrant population the rates reflect a high intermediate (7%) prevalence [15, 45]. In South America, the infection rate ranges from 0.5% - 8%, again depending on the population group sampled. The Indigenous populations of Brazil, Columbia, Peru and Venezuela have much higher rates, 8% or more with the commonest mode of transmission being horizontal, including intra-familial, sexual, tattooing and parenteral [15, 18, 43, 46].

1.4.3. Western Pacific
This region like most around the world had a decrease in HBsAg seroprevalence rates from that of a high endemic region (≥8%) in 1990 to low intermediate in 2005. Overall Australia and New Zealand have a low prevalence rate, however, the rates of infection in Indigenous and migrant population can be substantially higher[15, 47]. Modes of transmission are different across this region, for example in Taiwan the transmission is mainly via mother-to-baby, whilst in The Philippines, the mode of transmission is predominantly early horizontal[15, 18].

1.4.4. Eastern Mediterranean
Data on the prevalence HBsAg in this region is incomplete. However, according to a WHO bulletin, it ranges from <1% in Saudi Arabia to >8% in Somalia and Sudan. Furthermore, studies in Egypt and Oman show that most of acute hepatitis B occurs in individuals born prior to the implementation of the Extended Programme for Immunisation (EPI)[15].

1.4.5 Europe
Western Europe consistently had a low prevalence of HBV, < 2% from 1990 – 2005 while Central and Eastern Europe showed a reduction in the rates from 1990 – 2005, especially in the younger group (0-9 year old), from >8% to less than 4%[15].

1.4.6. Africa
Africa has the second highest rate of HBsAg seroprevalence in the world after Asia. The rates are particularly high in Western African regions and among rural African children. The Gambia and Senegal have a prevalence rate of approximately 20% amongst 10-20 year olds[15]. In Africa, horizontal transmission is the commonest mode of acquisition. In Western Africa the rates of HBV infection in a newborn are low. However, by 3 years of age approximately 20% of these children have evidence of infection and 15% go on to
develop chronic infection. Risk of horizontal transmission includes using contaminated needles, tribal scarification and skin abrasion and poor socioeconomic status leading to poor sanitation [15]. An alarming trend of HCC-related mortality has been seen in Western Africa. This region has the highest cancer related mortality identified in both male (25%) and females (16%) in 2012 [48].

1.5) Clinical Manifestation and Outcome of Hepatitis B Virus Infection

1.5.1. Overview of HBV Infection

The spectrum of disease following an acute infection depends upon the age at which an individual was infected. For children aged under 5 years, less than 10% are symptomatic while approximately 30-50% who acquire infection during adulthood are symptomatic. Progression to chronic infection also depends on the age of acquisition, for perinatal infection, 90% progress to chronic hepatitis B while 20% who acquire HBV in childhood go on to develop chronic infection. The rate of chronic B is much lower when acquired in adulthood at around 5% [49-51].

1.5.2. Acute Hepatitis B Infection

HBV is not directly cytopathic to the infected hepatocyte and it is the immunological response associated with infection that causes liver injury. Recovery from an acute infection is mediated by a plethora of different immune responses, particularly the cytotoxic T cell response (CTL) along with a vigorous Th-1 and B cell response [51-53].

Most individuals with acute hepatitis B infection have a mild, often subclinical illness or are asymptomatic. Others develop the classic hepatitis symptoms of nausea and fatigue with jaundice. Although rare, acute hepatitis B infection can lead to fulminant hepatic failure (FHF). During an acute infection, a rise in prothrombin index is an ominous sign, indicating a higher risk of progressing to FHF [54]. Several risk factors have been recognised for this devastating sequelae and these include an advanced age, presence of basal core promoter and/or precore mutations and core promoter mutations, HBeAg-negativity and co-infection with hepatitis C virus (HCV), hepatitis D virus (HDV) or HIV [55-58]. In the elderly population acute HBV infection can be devastating and has been associated with FHF resulting in death or requiring liver transplantation [59, 60].

1.5.3. Serological Events

Acute HBV infection is defined by WHO as a transient HBs antigenemia of less than 6 months duration whilst chronic infection is persistent HBs antigenemia for greater than 6 months [23, 61].
The incubation period of HBV infection averages 2–3 months but can range from 1-6 months, depending on a number of factors including the dose of inoculum, the infecting strain and host influences. HBsAg can be detected some 5-12 weeks after exposure and is often present weeks before the onset of symptoms or biochemical indications of hepatitis. HBeAg appears soon after and has been shown to be a useful marker of infectivity. As the titres of the antigens in the blood peaks, the level of the serum alanine aminotransferase (ALT) begins to rise and this is the period when symptoms may first become apparent (see Figure 1.2) [49, 62-64].

**Figure 1.2**

![Figure 1.2](image.png)

*Figure 1.2 shows the serological, viral and biochemical profile following an acute HBV infection and the onset of symptoms. Adapted from reference [65].*

Normally, antibodies to the antigens begin to appear during the symptomatic phase. The hepatitis B core antigen (HBcAg) is an extremely immunogenic protein and during an acute infection, it is the most dominant antigen that is recognised by the CD4+ T cells. This leads to the production of the first antibody, anti-HBc IgM, occurring approximately within a month of HBsAg development. In the symptomatic phase, anti-HBc IgM is found in high concentrations peaking in early convalescence and gradually declining over 3-12 months, more or less complementing the increase in titres of anti-HBc IgG. Even though
the presence of anti-HBc IgM is associated with an acute infection, in up to 20% of patients, it can persist for at least 2 years [66, 67]. Furthermore, it can also reappear during periods of acute exacerbation, such as hepatic flares in those chronically infected [5, 9, 67], thus limiting the diagnostic utility for acute hepatitis B.

One to two weeks after the appearance of anti-HBc IgM, the serum ALT levels rise and can range from 500 – 1200 IU/L. During this stage of acute infection, adult patients may have symptoms such as anorexia, nausea, dark urine, jaundice and right upper quadrant pain. Infants and children generally have minimal or even no symptoms [62, 67]. Other laboratory abnormalities include a raised serum aspartate transaminase (AST) level, however it is generally lower than the ALT level. Transaminase levels usually normalize within 1-4 months for those who recover and develop antibodies to HBsAg (anti–HBs). Meanwhile some patients may have persistently high levels of ALT for 6 months or longer, suggesting progression to chronic infection [62, 63, 68].

1.5.4) Phases of Chronic Hepatitis B Infection
Failure to eradicate HBV during an acute infection is the result of an inadequate innate and adaptive immune response [51]. In perinatally-acquired HBV infection, HBeAg status and maternal HBV DNA high load (greater than 10^8 copies/mL) are important predictors [11]. Furthermore, a poor CD4+ T cell and CTL response is often associated with failure to clear an acute infection amongst adult patients. Host factors such as certain Human Leukocyte Antigen (HLA, class I/II) have also been implicated with failure to eradicate the virus during an acute episode, resulting in chronic infection [52, 69].

Chronic HBV infection results from a complex interplay between host immunity, viral replication and the infected hepatocyte. Since the early 1990s, four sequential phases of chronic infection have been defined (see Figure 1.3), although patients do not necessarily experience all of them. The phases have been described as immune tolerance, immune clearance, immune control and immune escape. Each phase is associated with a characteristic virological, immunological and disease profile. [23, 49, 70].
1.3 shows the 4 phases of chronic Hepatitis B infection, Immune tolerance, Immune clearance, Non-replicative and Reactivation or Immune escape (shaded area). The highest risk of developing cirrhosis is in the immune clearance phase and re-activation. Adapted from reference [65].

1.5.5) Immune Tolerance

Perinatal or early horizontal acquired chronic HBV infection usually has a lengthy immunotolerant phase. It is characterized by the presence of very high levels of HBsAg, reaching 100-500μg/mL in the serum and high viral load, often in excess of $10^8$ IU/mL. Individuals in this phase are mainly young (women <19 and male <30), exclusively HBeAg–positive (wild type), have little or no virus mutant population and normal serum ALT levels. Liver biopsy reveals minimal fibrosis or necroinflammation but strong staining of HBeAg, especially in the nucleus [23, 70].

Despite a high viral load, the absence of liver disease in these patients is believed to be due to the unresponsiveness of the helper T-cells. During pregnancy, whilst the virus is unable to cross the placenta, the soluble HBeAg can pass through the placental barrier. Circulating hepatitis B e antigen (HBeAg) has been proposed to induce deletions of T-cell response as well as an impaired CTL response, which is a major determinant of a clearing immune response. HBeAg has also been shown to be involved in modulating the innate response by regulating the Toll-like receptor 2 (TLR-2). In the presence of HBeAg,
expression of TLR-2 is substantially reduced adding weight to the suggestion that HBeAg can act as a tolerogen. Upon establishment of chronic infection, the continuous secretion of HBeAg ensures minimum immunological response and therefore minimum liver damage[69, 71, 72].

The duration of the immune tolerance phase can be lengthy in perinatally acquired HBV but very short or even absent in those individuals who are infected as an adult [73, 74]. In a Taiwanese study of patients in this phase, the rates of progression to cirrhosis were low, at approximately 5% and there were no cases of HCC at approximately 10.5 year follow up[72].

1.5.6) Immune Clearance
Immune clearance is the second phase of chronic hepatitis B infection, which typically occurs between the ages of 20-40 years. The reason or ‘switch’ from immune tolerance to immune clearance is unknown and most patients are asymptomatic with fluctuating HBV DNA levels and high ALT. Histologically, liver biopsies reveal a decrease in nuclear and increase in cytosolic HBcAg level [23, 73]. Typical HBeAg seroconversion is preceded by an elevation in serum ALT, suggesting marked liver inflammation followed by a decrease in HBV DNA load. Following the drop in HBV DNA, the ALT normalizes, suggesting resolution of necroinflammation of the liver. In a minority of patients, at least 5%, seroconversion is not associated with normalization of ALT and these patients are classified as 'HBeAg negative chronic hepatitis B'. These are typically older patients[23, 73].

During the immune clearance phase, patients can also have episodes of hepatic flares, and the incidence of these can vary; it can be as high as 25% in the first 3-5 years. Features associated with flares include ALT levels of greater than 5 times the upper limit of normal (ULN), which is preceded by a raised HBV DNA level. Histologically, lobular hepatitis with bridging hepatic necrosis as seen with acute hepatitis B is also present during flares [19, 73, 75]. Furthermore, some patients may also have elevated levels of anti-HBc IgM, but at much lower levels compared to acute hepatitis B. Hepatic flares can be asymptomatic, however 3-5% can succumb to hepatic decompensation [76, 77].

The duration of immune clearance can last many years and eventually results in HBeAg seroconversion (HBeAg loss and production of anti-HBe with normalization of ALT). During the natural history of chronic HBV infection, the average incidence of HBeAg seroconversion is approximately 10% per year in untreated patients. Several factors
have been associated with HBeAg seroconversion and these include mode and age of transmission, gender, geographical region of origin, HBV genotype, baseline ALT levels and histological changes within the hepatocyte [72, 78-80].

Asian children infected by horizontal transmission have a higher rate of HBeAg seroconversion than non-Asian children with perinatal transmission [78, 81]. An Alaskan study found that the average age at seroconversion for non-genotype C HBV, which included A, B, D and F, was associated with an earlier seroconversion at around 20 years of age, whilst genotype C HBV infected persons had delayed seroconversion [45]. Furthermore, as described in a Taiwanese study, the level of ALT during seroconversion was significantly higher for genotype C compared to B, suggesting a more vigorous immune mediated hepatocyte inflammation associated with attempted viral clearance [82].

Other independent factors associated with earlier HBeAg seroclearance are lower levels of HBV DNA and the presence of the precore mutation G1896A. Patients undergoing flares have a 72% chance of seroclearance over 3 months if there had been a concomitant increase in alpha-fetoprotein levels to greater than 100ng/mL. Similarly a high baseline ALT level, at least 5 times ULN is associated with 17% rate of seroclearance over 18 months [83, 84].

Delayed seroconversion is associated with an increased rate of cirrhosis and HCC [85]. A German study also demonstrated in a group of untreated patients with liver related complications that hepatic decompensation and death were more common over a three year period in HBeAg seropositive individual compared to those patients who were HBeAg negative [86].

1.5.7) Immune Control
The immune control phase is also known as the inactive carrier state or non-replicative phase and is the result of successful HBeAg seroconversion. It is characterized by a combination of persistently normal serum ALT levels and HBV DNA level < 2000 IU/mL in HBeAg negative non-cirrhotic patients [87]. Recent studies examining the role of quantitative HBsAg titres in chronic hepatitis B have noted that for genotype D, HBsAg level of less than 1000 IU/mL and HBV DNA < than 2000 IU/mL be used to classify immune control phase. During this quiescent phase of HBV infection, liver biopsy reveals an absence of expressed intrahepatic HBCAg and minimum necroinflammation with little or no cirrhosis. There is also a marked reduction in annual cirrhosis and HCC
rates, 0.1% and 0.2% respectively [87-90], and HBeAg seroconversion before the age of 30 years is associated with a lower risk of complications [85].

1.5.8) Immune Escape
The immune escape phase is also known as HBeAg negative chronic hepatitis B or “reactivation” and is characterized by HBeAg negative, anti-HBe positive serostatus, but with elevated ALT and HBV DNA levels of > 2,000 IU/mL in an infected individual [91]. The incidence of HBeAg negative chronic hepatitis has a wide geographical variation with rates of 80-90% seen in the Mediterranean region, less than 10% in Northern America and Europe[42] and around 30-50% in Taiwan. Many patients transition to this phase of chronic infection after a prolonged period of being in the inactive carrier state, although approximately 5% may convert to this stage directly from the immune tolerant phase. In a substantial proportion of patients, HBeAg negative chronic hepatitis is caused by immune escape variants that encode changes that abrogate or reduce the production of HBeAg[79, 91, 92].

Several factors have been associated with this HBeAg negative chronic hepatitis phase and these include viral genotype and host immune status. Patients infected with HBV genotype C are more likely to develop HBeAg negative disease compared to those infected with genotype B, [82] while those infected with genotype D are also more likely to progress to HBeAg-negativity than those infected with genotype A [93]. HBeAg seroconversion before the age of 30 years was associated with a lowered risk of reactivation, while those who seroconvert after 40 years of age are at an increased risk. Similarly, a high ALT (>5 times the ULN) during immune clearance has been associated with increased risk of developing HBeAg negative chronic hepatitis. Finally, patients who are in the immune escape phase are at increased risk of cirrhosis and HCC compared to those who are in immune control [72, 74, 82, 91, 94, 95].

1.6 Hepatitis B Vaccination
1.6.1 Overview of Hepatitis B Vaccination
Vaccination revolutionized preventive medicine and continues to be an integral part of clinical practice. In 1991, WHO recommended that countries implement a policy of universal HBV vaccination by 1997 and the hepatitis B vaccine became part of the WHO Extended Programme for Immunization (EPI). Of WHO member states, data collected from 2008 shows that 92% (177/193) have now successfully initiated HBV vaccination programs. Since the discovery of HBsAg by Blumberg et al in the 1960s, efforts were made to use this as a potential substrate to generate a vaccine and prevent infection [5,
The 'first generation' vaccine was derived from the plasma of chronically infected persons; this was licensed for use in the USA in 1981. This vaccine constituted heat and/or chemical inactivated HBsAg sub-viral particles purified from the plasma of chronically infected individuals. Subsequently, advances in molecular biology allowed a recombinant 'second generation' version of the vaccine to be developed, using the S-gene of HBV cloned into an expression vector in Saccharomyces cerevisiae (Baker's yeast); this produced large amounts of the protein in a 'safe' form free from adventitious agents and was licensed for use in 1986[6, 102].

The HBsAg most commonly found in the vaccine is encoded from HBV genotype A2 serotype adw-2 [6]. The vaccination schedule consists of three doses of vaccine given intramuscularly at time points 0, 1 month and 6 months. The vaccine is believed to be effective in 95% of patients and levels of anti-HBs >10 IU/mL are considered to be protective against HBV infection. Once seroconversion has occurred with adequate anti-HBs levels (>10 IU/mL), a further booster dose is not recommended since subsequent exposure, even in the setting of low circulating anti-HBs (10 IU/mL) would result in an anamnestic response sufficient enough to provide protection [44, 63, 103].

The HBV vaccine was recommended for high risk populations such as men who have sex with men (MSM), persons who inject drugs and people in correctional facilities [99]. However, this targeted policy of prevention was not sufficient to reduce the prevalence of HBV infection in most countries and gave impetus to the WHO recommending incorporation of the HBV vaccine into the EPI or its equivalent programmes [61, 104].

1.6.2 Passive Immunisation
Hepatitis B immunoglobulin (HBIg), containing high levels of anti-HBs, can be administered as a temporary protection against HBV exposure in a non-vaccinated person. This manner of immunization, in conjunction with HBV vaccine, is also an essential component of preventing infection in a neonate born to a HBsAg positive mother, thereby reducing the risk of perinatal transmission. Using this combination, the rate of transmission in this setting has been reduced by over 90% and has become the recommended standard of care in most countries [31, 33, 105]. Unfortunately, in highly endemic countries such as Africa and some parts of Asia, the HBIg component is not administered due to cost issues [30, 105].
HBlg has also been used effectively in treating persons exposed to HBV, including sexual exposure [106] and needle stick injury and may be the only mode of the prevention of infection in individuals who do not respond to vaccine [107].

Another important group of HBlg recipients include those patients undergoing liver transplantation for end stage liver disease due to HBV infection. In these patients high doses of HBlg are administered to prevent the newly transplanted liver from becoming infected [108, 109]. Use in this situation has recently diminished due to the availability of suitable nucleos(t)ide analogues (NAs) to reduce viral load pre-transplant [110-112].

**1.6.3 Impact of Vaccination in Prevalence and Complications of Chronic HBV Infection**

The current HBV EPI worldwide prevents an estimated 64 million chronic HBV infections and 1.6 million deaths per year [7]. There has been a dramatic decline in acute hepatitis B infections in infants resulting in an impressive decline in the prevalence of HBsAg in children born in endemic countries [63, 113-115]. Furthermore, the HBV vaccine represents the first vaccine for cancer prevention and this has been substantiated by the decline in HCC cases in countries such as Taiwan and Alaska, where childhood HBV related HCC was high [45, 116, 117]. Since the instigation of infant vaccination, the rates have reduced to 0.36/100 000 from 0.76/100 000 [29].

**1.7) Treatment of Chronic Hepatitis B**

The principle goal of therapy for chronic HBV infection is to prevent cirrhosis, hepatic failure and HCC. With the advancement in knowledge about the pathogenesis and the natural history of HBV infection, the ability to manage this chronic infection has improved substantially over the last few decades [118, 119].

Many factors influence treatment regimens for chronic HBV. These include the disease phase, presence of other infections and co-morbidities, viral load, serum ALT levels and if available, liver histology. Therapy is also considered when there is evidence of active disease or high risk of progression to cirrhosis or HCC, for example a positive family history. More recently biomarkers have been identified that indicate likely progression of disease with increasing HBV DNA load [23, 120-122]. Other advances have been recognition of the importance of HBsAg titres, both in differentiating between some of the disease phases of chronic hepatitis B and as an ‘on-treatment predictor’ of the response to interferon and the rate of HCC [122-124].
Even though HBV was isolated and found to be the aetiological agent of hepatitis B more than 50 years ago, until recently treatment was restricted to interferon. Interferon α, which has both immunomodulatory and antiviral activity was the first preparation licensed for treatment of chronic HBV infection [96, 125]. The HIV epidemic in developed countries helped provide impetus for investigation of nucleos(t)ide analogues (NAs) as potential inhibitors of active replication by blocking reverse transcription. As HIV and HBV both employ reverse transcription steps, it was found that many of the NAs developed to treat HIV infection were also capable of inhibiting HBV replication [126, 127].

1.7.1 Immunomodulators
Interferon α was approved for the treatment of chronic hepatitis B infection in 1990 and later replaced by pegylated forms, enabling more consistent and pharmacologically appropriate weekly dosing. It gives patients a finite time of treatment and if successful, an effective and durable HBeAg seroconversion[86].

The aim of treatment of chronic hepatitis B infection is to achieve sustained virological response (SVR), defined as achieving low HBV replication (<2,000 IU/mL of HBV DNA) and ultimately anti-HBs seroconversion. In HBeAg negative patients, the aim of treatment is to achieve HBV viral load < 2,000 IU/mL and normalization of ALT at 6 months of treatment[23, 122, 128][129].

The overall response rate to pegylated interferon is poor for the HBeAg positive patient, with only up to 25% of individuals achieving HBeAg seroconversion with undetected HBV DNA levels. In HBeAg negative disease, the initial treatment response is much better, 63% will have undetected HBV DNA, however, by 4 years the rate is comparable to HBeAg positive patients with only 18% of patients having undetectable virus. The response to pegylated interferon is also influenced by viral factors, such as genotype [23, 122, 130].

The rate of HBsAg loss at 1 year is 4% and this doubles by 4 years and long term follow up shows a better outcome in initial responders. There is an improvement in the liver histology and reduction in the incidence of HCC and decompensated liver disease. So far, treatment with interferon has not been associated with the emergence of resistant variants [86, 131].
Treatment with interferon is associated with significant adverse effects, which may restrict its use. These include flu like symptoms, depression, bone marrow suppression that may require treatment with granulocyte stimulating factors and precipitation or exacerbation of autoimmune disorders. Cirrhotic patients have to be carefully evaluated whilst on treatment with immunomodulators as there is a high risk of hepatic flare and whilst this can be associated with seroconversion, it can also culminate in hepatic decompensation leading to death. [131].

1.7.2 Direct Acting Antiviral Agents
Currently, the only class of direct acting antiviral agents licensed for the treatment of chronic HBV infection is the NAs. Lamivudine was the first NA agent to gain approval [132, 133] and subsequently four other agents have been approved (see below). Treatment with these NA has led to a substantial decrease in HBV-related morbidity and mortality [119, 134, 135] but incomplete viral suppression can select for the emergence of drug-resistant HBV variants [136-140].

Based on their chemical structure, antiviral agents for can be divided into three major categories: the L-nucleoside analogues, the acyclic nucleoside phosphonates and cyclopentane-modified deoxyguanosine analogues[141, 142]. Resistance to a drug belonging to a particular structural category confers at least some degree of cross-resistance to other group members and may also affect sensitivity to agents from other categories, leading to the development of multi-drug resistance (MDR) [136]. Due to the overlapping reading frames of HBV genome, the selection of substitutions conferring antiviral resistance has the potential to lead to changes in the S-gene, including the selection of variants associated with vaccine escape [14, 143-145].

1.7.2A The L-nucleoside Analogues
Examples of this group of agents include lamivudine (LMV), a synthetic cytidine analogue and telbivudine (LdT), the ‘unnatural’ L-enantiomer of the natural (D-) thymidine [142]. These two agents have a low genetic barrier to resistance and an estimated 70% of patients treated will develop resistance after three years of therapy with LMV and approximately 29% with LdT after two years of therapy [146-148]. Resistance is conferred by mutations that result in substitution of the methionine at amino acid position 204 in the tyrosine-methionine-aspartate (YMDD) catalytic site motif of the reverse transcriptase (rt) by valine (rtM204V) or leucine (rtM204I). The rtM204I substitution can occur alone but rtM204V is usually found in association with other changes, notably rtL180M and rtV173L[149, 150]. Other primary mutations
which also confer LMV resistance include the substitutions rtA181T/V, and these changes are associated with resistance to LdT and cross-resistance to the acyclic phosphonate, adefovir (ADV) (See Table 1.1). [136, 139, 151, 152].

Table 1.1 Antiviral therapy related substitutions in HBV polymerase

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Mutations in Polymerase</th>
<th>LMV</th>
<th>LdT</th>
<th>ETV</th>
<th>ADV</th>
<th>TDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-nucleoside (LdT/LMV)</td>
<td>rtM204I/V</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Acyclic phosphonate (ADV/TFV)</td>
<td>rtN236T</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>Shared (L-NA and acyclic phosphonate)</td>
<td>rtA181T/V</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>Double (ADV and TFV)</td>
<td>rtA181T/V + rtN236T</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>D-cyclopentane (ETV)</td>
<td>rtL180M+M204V/I +/−I169+/−T184+/−S202+/−M250</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: R- resistant, I- Intermediate, S- sensitive
Shared refers to selection of mutation (rtA181T/V) by the acyclic phosphonates and L-nucleosides. Double pathway is due to the selection of substitutions decreasing tenofovir (TFV) efficacy. Adapted from reference [141]

1.7.2B The Acyclic Phosphonate group

ADV was the first drug from this class to be approved for the treatment of chronic HBV infection, followed by its congener, the more efficacious tenofovir (TFV). ADV and TFV are active against LMV-resistant HBV variants both in-vitro and in-vivo, but not if the substitution rtA181T is present (Table 1.1). [136, 153].

HBV resistance to ADV occurs less frequently than resistance to LMV, with a prevalence of around 2% after two years, 4% after three years, 18% after four years, and 29% after five years[153, 154]. There is minimum evidence for the occurrence of primary resistance to tenofovir, however, the substitutions associated with ADV resistance (rtA181T/V and/or rtN236T) can decrease the efficacy of TFV [142, 155, 156].

1.7.2C The D-Cyclopentane group

Entecavir is the prototype antiviral agent of this group and has both anti-HBV and anti-HIV activity [157]. The rate of resistance is less than 1% at 6 years of therapy in treatment naive patients. Entecavir resistance appears to require the initial presence of rtM204V/I (which confers primary resistance to the L-nucleosides) followed by the
acquisition of mutations that encode for at least one additional entecavir "signature" substitution at rtI169, rtT184, rtS202 and/or rtM250A. Profound reduction in the susceptibility to ETV, approximately 100-fold, is seen in HBV with rtL180M+rtM204V in combination with rtI169T or rtS184G or rtS202G/I or rtM250V. However, in the absence of rtL180M+rtM204V and with the single substitution rtM250V, there is only a 10-fold decrease in susceptibility [158-160]. Substitutions rtT184G and rtS202I changes alone have little effect on ETV efficacy[136] (see Table 1.1).

1.8) Complications of Chronic Hepatitis B Infection

Complications of chronic hepatitis B include cirrhosis and HCC. HBV was one of the first human viral carcinogens identified and despite the availability of a preventative vaccine and several treatment modalities, it is the second most common cause of cancer mortality in males and sixth in females worldwide. The majority of these deaths occur in developing countries [48] [1, 44].

While liver cirrhosis increases the risk of development of HCC, it is not an absolute prerequisite. Due to an increased risk of hepatic decompensation, cirrhosis by itself is also associated with significant morbidity and mortality. It is believed that HCC development in chronic HBV infection is due to multiple factors, including viral DNA integration, the effects of viral gene products such as the HBV X protein, a known transcriptional transactivator, the presence of precore and basal core promoter mutations and as well as the influence of external factors such as alcohol and aflatoxin [161-165].

HCC usually develops as a small nodule and has an average doubling time of 6 months, however, this can range from 1–19 months. The major factors influencing the overall survival include the severity of underlying liver disease and the size of tumour at the time of diagnosis. Tumour size of less than 5 cm has a survival rate of 81-100% at 1 year without treatment however, at 3 years the survival rate decreases to 21% or less without treatment, strongly suggesting instigation of treatment early in the diagnosis[166, 167] as well as effective screening programmes.

Management of HCC depends on the size, number of lesions and extent of metastasis. Hepatic resection is the treatment of choice for small lesions in a non- cirrhotic liver to minimize the risk of decompensation while in a cirrhotic patient, the degree of hepatic reserve should be properly assessed prior to resection. Liver transplantation would be an option for a patient with a lesion less than 5 cm or a maximum of three lesions,
measuring 3 cm in diameter or less. In these patients ongoing HBV replication is associated with poor prognosis, therefore suppressive antiviral therapy has become the cornerstone of effective management [110, 168, 169].

Non-surgical treatment of HCC includes percutaneous injection of alcohol, which causes necrosis of the lesion. This form of therapy is suited for a peripheral lesion, which is less than 3 cm in diameter. Transarterial chemoembolization (TACE) has been proven to be effective, especially for patients with adequate residual liver function, with a combination of lipoidol and a chemotherapeutic agent such as doxorubicin. Systemic chemotherapy, however, has not been shown to be as effective with reported success rates of only 10-15% with doxorubicin as a single agent [168, 169]. Results from the use of hormonal targeting agents such as tamoxifen, stilbestrol and flutamide for the treatment of HCC are also discouraging [166].

1.9 Structure and Replication of the Hepatitis B Virus

1.9.1 Overview of HBV Structure
The serum of a patient with CHB infection contains virions, spherical sub viral particles and tubular filaments. The HBV virion, also known as the Dane particle is infectious. It is approximately 42nm in diameter and an infected patient may have up to $10^{10}$ particles/ml (see Figure 1.4) [170, 171]. An infected patient can also have significant excess of spherical and filamentous subviral particles, which are approximately 20nm in diameter. These particles are composed of the viral surface proteins and some lipid, they lack nucleic acid and are therefore are non-infectious [171, 172].

The outer structure of the virion consists of an envelope protein that is approximately 8 nm in width and consists of protein, lipid and carbohydrate. Embedded in this envelope protein are the surface proteins, namely, large (L), medium (M) and small (S). Enclosed within this envelope is an inner icosahedral nucleocapsid, with a triangulation number of T=4, and this contains the viral partially double stranded DNA covalently attached to the viral DNA polymerase enzyme (see Figure 1.5)[171, 172].
Figure 1.4 shows Dane particle, filamentous and spherical particles, adapted from reference [171].

Figure 1.5 shows the structure of the HBV envelope proteins, small (SHBs), medium (MHBs) and large (LHBs). Within the capsid (HBC) is the polymerase (RT) with primase (pr) and reverse transcriptase domains linked to the HBV DNA genome. Adapted from reference [5]

1.9.2 Hepatitis B Virus Genome

The HBV genome is approximately 3200 nucleotides (nt) in length and is one of the smallest known for a human viral pathogen. Despite its small size, the functional capability of the HBV genome is substantial.
The genome is a partially double stranded, relaxed circular (rc) DNA to which an endogenous polymerase protein is covalently attached at its 5’ end. The rc DNA is made up of a minus sense or negative strand of DNA of fixed length with defined 5’ and 3’ ends and a terminal redundancy of 8-9 nt, and an incomplete plus sense strand with a variable 3’ end. The two linear DNA strands are held in a circular configuration by a 226 base pairs (bp), forming a cohesive overlap between the 5’ ends which contain two 11nt direct repeats called DR1 and DR2. At the 5’ end of the plus strand, there is a capped RNA, spanning approximately 18 nt; this acts as primer for the second strand DNA synthesis during replication. The minus strand of HBV encodes four major open reading frames (ORFs) which carry all the protein-coding capacity of the virus; they are frame shifted, partially overlapping and encode for the polymerase (P), surface (S), core (C) and HBx (X) proteins [171, 173].

1.9.3 Hepatitis B Viral Replication
Infection with HBV involves successful entry of the virus into the hepatocyte via the recently discovered sodium taurocholate cotransporting polypeptide (NTCP) receptor. In 2012, Yan et al demonstrated that a specific domain in the N-terminal portion of the preS1 region of the large protein binds to the NTCP receptor to mediate viral entry. Their studies also demonstrated that blocking the NTCP receptor led to inhibition of both HBV and HDV infection. [174].

Following entry, the virion uncoats and the core particle is transported to the nuclear pore where the viral DNA is released. Once HBV DNA has entered the nucleus of the hepatocyte, the ‘gap region’ of the partially double stranded rc HBV DNA is repaired by host cell enzymes to convert it to a fully double stranded DNA molecule (see Figure 1.6). From this replicative form, covalently closed circular DNA (cccDNA) is generated which binds with cellular histones to form a viral minichromosome. This remains in the cell nucleus and acts as the major transcriptional template for mRNA production using the host cell RNA polymerase II. Unlike cellular mRNA, unspliced HBV mRNA is then transported into the cytoplasm as the RNA is transcribed from the minus strand DNA. The mRNA is plus sense and has a 5’ cap and is polyadenylated at the 3’ end [173, 175, 176].
Figure 1.6 shows the HBV replication cycle, cccDNA is established as minichromosome in the hepatocyte, followed by retroviral replication. Adapted and modified from reference [2].

To regulate the transcription process, the HBV genome contains four promoters (PreC/C basal core promoter, S promoter, PreS1 promoter and HBx promoter), two enhancers (Enh I and Enh II) and a glucocorticoid responsive element. Several mRNA species are produced and can be classified into two groups, a subgenomic set of transcripts and a set of greater than genome length transcripts. The subgenomic transcripts encode for the envelope (PreS1, PreS2 and S) proteins and the X protein. The two 3.5 kilobase (kb) greater than genome length transcripts, designated PreC/C and pregenomic RNA (pgRNA) serve as the mRNA for the precore protein, the precursor of HBeAg, and for the translation of the core and polymerase protein, respectively. After encapsidation, the pgRNA also functions as the template for reverse transcription of the viral genomic DNA[171].
1.9.4 Encapsidation and Reverse Transcription

Encapsidation appears to be triggered by the binding of the polymerase to a critical stem loop structure, termed epsilon (ε) at the 5’ end of the pgRNA. Epsilon also plays a key role in the initiation of HBV DNA synthesis. HBV DNA replication begins soon after encapsidation with the synthesis of minus strand DNA by reverse transcription, a unique strategy as HBV is the only known double stranded DNA virus that uses reverse transcription to replicate its genome. The reverse transcriptase is multifunctional in that in its primase domain, a tyrosine residue is used as a substrate for the formation of a phosphodiester linkage with the first deoxynucleotide (dG). Priming occurs in the bulge region of the epsilon RNA generating a small oligonucleotide, which along with the reverse transcriptase is translocated to the complementary sequences of the DR1, located at the 3’ end of the pgRNA. Minus strand DNA synthesis ensues and terminates at the 5’ end of the pgRNA, generating a small terminal redundancy. After completion of synthesis of the minus strand, RNase H degrades the pgRNA except for the 5’ capped portion which acts as a primer for the plus strand after another translocation from the 5’ end to the 3’ end of the minus strand DNA. For reasons poorly understood, the plus sense strand synthesis terminates before completion, leaving up to 50% of the genomic DNA single stranded \[2, 175, 177, 178\].

Following DNA replication, the newly packaged synthesized DNA has two possible fates: the nucleocapsids can be enveloped and secreted as virions or they can recycle back to the nucleus as part of a regulatory pathway to maintain the cccDNA reservoir. The exact mechanism whereby the nucleocapsid moves from the cytosol to post endoplasmic reticulum (ER) and pre-Golgi membrane where the envelopment by surface protein occurs remains poorly understood \[171, 178, 179\].

1.9.5 Translation Products of Hepatitis B Virus

Of the four ORFs on the minus strand DNA, the longest encodes the viral polymerase (Pol). The envelope or PreS/S ORF (PreS1, PreS2 and S) is located entirely within the Pol ORF and the remaining two ORFs, PreC/C and X also partially overlap the Pol ORF. HBV is able to encode more than one protein from an ORF by using multiple internal start codons, creating nested sets of proteins with different N-termini. This gene overlap makes economical use of limited coding capacity but does place limitations on the mutation rates of HBV \[171\].
The Pol is translated from the pgRNA, producing a protein of approximately 90 kilodaltons, (kDa) which is multifunctional and comprises four recognized domains. The N-terminal portion has the terminal protein, which is involved in the protein priming of the minus DNA strand, followed by the spacer or tether region, which has no recognized function. The third domain has the reverse transcriptase (rt), which has both RNA-dependent and DNA-dependent DNA polymerase activities and the C-terminal region contains the RNase H activity\[171\].

The PreC/C ORF encodes for the 21kDa core protein, the major polypeptide of the core particle that expresses the HBcAg. Like the Pol, it too is translated from the greater than genome length pgRNA. Translation of the other slightly longer 3.5kb RNA transcript leads to the synthesis of the precore protein. The first 19 amino acids of the precore protein are a signal sequence that are cleaved off by a host cell signal peptidase in the ER leading to the eventual secretion of the HBeAg after further post-translational processing as it passes through the Golgi\[171\].

The X ORF produces a 0.7kb mRNA encoding the X protein of approximately 17 kDa. Although HBx appears not to be required for HBV DNA synthesis in-vitro, it is essential for virus infectivity in-vivo. It is capable of transactivating several viral and cellular promoters and is believed to be a cofactor in the development of HBV-related HCC \[180, 181\].

The PreS/S ORF can be divided into PreS1, PreS2 and S domains by three in-frame start codons. Transcription from the PreS/S ORF generates a 2.4kb mRNA encoding the large hepatitis B surface protein (LHBs or L) and a 2.1kb transcript encoding the medium (MHBs or M) and small (SHBs or S) proteins. The two larger proteins contain the SHBs domain plus N-terminal extensions generated by initiation at upstream start codons. The extra domain of MHBs is known as preS2, whilst the domain unique to LHBs is called preS1. These three envelope components are glycosylated, transmembrane proteins, which can form multimers and stabilized by disulfide bridges between cysteine residues present in the SHBs domain (see Figure 1.7) \[171, 182\].

1.10 The Hepatitis B Surface Proteins

The three envelope proteins are all found as components of the 42nm-diameter virion. The small SHBs protein is the major component of the virion envelope with the LHBs and MHBs proteins, present in approximately equal amounts, comprising the remaining 30%. The filamentous and spherical 22nm subviral particles that are secreted from
infected cells are also composed of the three envelope proteins, but in different ratios reflecting their morphological variations[170, 171].

1.10.1A) SHBs and the ‘a’ determinant
The synthesis and assembly of the viral envelope, the small particles and filaments occur in the ER with the resultant assembled viral proteins budding into the ER lumen. The SHBs is found in glycosylated (gp27) and non-glycosylated (p24) forms. Within the SHBs is a domain containing a high number of conserved disulphide bonded cysteine (Cys) residues (at least eight) that form the backbone of a highly conformational structure [182, 183]. This is the major antigenic determinant of the HBsAg (from amino acid residues 99 to 170), referred to as the ‘a’ determinant or the major hydrophilic region (MHR) (see Figure 1.7) The ‘a’ determinant is found in all HBV isolates and is considered the major neutralization domain and the binding site of anti-HBs [172, 182, 184]. The deduced structure of the ‘a’ determinant has a double loop conformation, the first and larger loop (Loop 1) is from amino acids 107–138 and includes a small projection formed by bonding between Cys 121-Cys 124, often referred to as the ‘mini-loop’ region. The second loop (Loop 2) is from amino acids 139–147. Several models of the ‘a’ determinant have been proposed with Loop 1 being stabilized by disulphide bridges between Cys-107 and Cys-138, while Loop 2 is stabilized by bonding between pairs Cys-139 to 147 and Cys-137 to Cys-149[183, 185, 186] (see Figure 1.7)[186].

**Figure 1.7**

*Figure 1.7 shows the ‘a’ determinant, Loops 1, 2 and mini Loop. The formation of disulphide bonds between 121 – 124, 137-149, 107-138 and 139-147 are indicated in red line. Figure adapted and modified from reference [187].*
1.10.2B) Serotypes

Additional subdeterminants have been identified and designated d/y and w/r, mutually exclusive, allowing for the classification of four major HBV serotypes; ayw, ayr, adw and adr (see Figure 1.8). It was subsequently discovered that these serotypic variations were due to amino acid differences at positions 122 and 160 within the ‘a’ determinant. Determinant ‘d’ has a lysine (K) at position 122 while ‘y’ is represented by an arginine (R) at this position; subtype ‘w’ has a lysine at position 160 and ‘r’ has an arginine (see Figure 1.8) [188, 189]. During HBV infection, antibodies against all the determinants are produced but only those against the ‘a’ determinant are protective against a challenge with any of the other subtypes [190].

Figure 1.8

![Figure 1.8](image)

Figure 1.8 shows the designation of d/y and w/r to define the serotypes of HBV at amino acid positions 122 and 160 respectively. The major serotypes are adw, adr, ayw and ayr.

1.10.2) MHBs

In addition to the glycosylation within the S domain at N146, MHBs has further glycosylation sites in the N-terminal, the 55 amino acid PreS2 region, which varies between HBV genotypes. The true function of MHBs is not completely understood, as it is not essential for virus assembly or release or even infectivity. The PreS2 portion is exposed on the surface of viral and subviral particles and is more immunogenic than SHBs. The central part of the PreS2 region carries a major antigenic epitope and this is preceded by a conformationally independent region between amino acids 3-16 that has the ability to bind polymerized human serum albumin (pHSA); the significance of this is unclear but it is possibly a co-factor in enhancing virus attachment and entry [170, 171, 181, 191].
1.10.3) LHBs
The LHBs has a further N-terminal extension (PreS1) to the MHBs and exists in both glycosylated (gp42) and unglycosylated (p39) forms, depending on genotype. LHBs is also modified by N-terminal myristylation, which is necessary for virus infectivity. LHBs can exist in two different topologies, with the PreS1 domain on the cytosolic side of the membrane playing a role in envelopment of mature core particles, and exposed on the cell surface as the key determinant for NTCP binding. LHBs has important immunogenic B and T cell epitopes and can elicit neutralizing antibodies[170, 172, 174].

1.11 HBV Genotypes and Variants
1.11.1) Genotypes
HBV has been classified into ten different genotypes, denoted A-J, based on nucleotide sequence divergence. A difference of 8% in the complete sequence categorizes HBV to a unique genotype while a 4% difference within a genotype defines a subgenotype [192, 193]. In general, the genotype/subgenotype has a distinct geographic distribution but immigration has changed virus genetic diversity within many regions. Other than a geographical association, genotypes have a number of important pathogenic and therapeutic differences [130, 194-196]. Furthermore, recent studies have implicated genotype divergence as a potential cause of vaccination failure [197-201].

Genotype A has three major subtypes, A1, A2 and A3 and is regarded as a pandemic genotype with an extensive distribution range compared to the other genotypes. A1 and A3 are found in central/Eastern and central/Western regions of sub-Saharan Africa respectively, while A2 is found in North American and Northern and Western Europe, predominantly in the Caucasian population[194, 202].

Genotype B can be divided into two groups, B Japan (Bj) and B Asia (Ba), but these can be discriminated into six major subgenotypes (B1-B6). Genotype B1 (Bj) is most common in Japan with B6 found in the Indigenous populations living in Arctic regions, including Alaska, Canada and Greenland [194]. Subgenotypes B2-5 (Ba) are recombinants between genotype B and C in the precore/core region and are found predominantly in East Asia along with new subtype B7 [203]. New subtypes B8 and B9 have also been recently described in Nusa Tengarra, in East Indonesia [204]. Compared to HBV genotype C, genotype B infected persons have has been associated with an earlier HBeAg seroconversion, lower risk of cirrhosis and HCC and better response to interferon α based therapy.[194, 205, 206]
Genotype C has been classified into many subtypes with C1-C5 the most common and these are mainly found in Asia, the Pacific Islands and Australia [207]. HBV C4 has been isolated exclusively from the Indigenous population of Australia and has surface gene sequence similar to HBV genotype J [208, 209]. HBeAg seroconversion in patients chronically infected with genotype C occurs almost a decade later compared to persons infected with HBV genotype B. Therefore, women infected with genotype C tend to be HBeAg positive during child bearing years which increases the risk of maternal transmission. HBV genotype C is considered to have the greatest oncogenic potential of all the genotypes [82, 205, 210].

Genotype D has been classified into eight subgenotypes, D1-D8 [211]. However, recent work has proposed to reclassify D6 as D3 as one subtype and D8 as recombinant of D/E [212]. Genotype D has an extensive worldwide distribution and includes the Mediterranean, Middle East, Indian subcontinent, Russia and Eastern Europe [207]. In terms of disease progression, patients infected with genotype D have a higher risk of cirrhosis and HCC compared to genotype A and the clinical response to interferon α is lower [93, 213].

Genotype E HBV is largely restricted to West Africa. Interestingly, despite a history of slave trade between Africa and Americas, the distribution of genotype E is localized mainly to the African subcontinent. HBV genotype E HBV does not have subtypes, suggesting a relatively recent origin [214-216].

Genotype F is one of the most diverse amongst all the genotypes and clusters into four subtypes (F1-F4) [192, 217]. It is mainly found in Amerindian populations of Central and South America, with some isolates coming from Alaska. There is some evidence that infection with HBV genotype F is associated with more aggressive liver disease and HCC [45, 205].

Genotype G is almost exclusively found in association with another co-infecting HBV, most commonly A2, presumably because it lacks the ability to produce HBeAg. Its geographical distribution includes USA, Europe and Australia. It does not have any subtypes. Any effect on disease severity is difficult to determine due to the confounding infection with other HBV genotypes[207, 218].

Genotype H is found in indigenous populations in Central America [219], whilst HBV genotype I was originally reported from Vietnam and Laos. Analysis of the full genome
of genotype I isolates indicate that it is a recombinant of genotypes A/C/G [220]. HBV genotype J has only been reported in a Japanese soldier who acquired the infection in Borneo during World War 2. The isolate clusters with Asian primate HBV isolates and there is some evidence of recombination having occurred between genotype C and gibbon HBV. The subject died from HBV related HCC suggesting a severe form of disease [221], but as this has been the only isolate of HBV genotype J, conclusions are difficult to draw.

1.11.2) Hepatitis B Virus Variants
HBV replication incorporates an error-prone reverse transcription step, generating a heterogeneous population of variants or quasispecies. Under selective pressure, such as that from the immune system or antiviral therapy, the fittest variant in the quasispecies can rapidly expand and predominate converting the quasispecies pool to one of selection-driven newly emerging viral variants. The HBV mutation frequency has been estimated to be between 10^4-10^5 nucleotide substitutions/site/year, around 10-fold higher than most DNA viruses and approaching that of some RNA viruses[171, 175, 222]. The magnitude and rate of virus replication are also important in the process of mutation generation, with viral load up to 10^{11} virions/ml. The high viral loads coupled with poor replication fidelity influence the depth of the HBV quasispecies pool. However, the ability of HBV to generate variants is also limited by constraints imposed by the ORFs in that a mutation in one reading frame may confer a selective advantage and have a deleterious impact in an alternative ORF [2, 223].

1.11.3) Naturally Selected HBV S-Gene Variants
The selection of a minor quasispecies and subsequent expansion of HBV S-gene variants can occur spontaneously during chronic hepatitis B infection under pressure from the host immune response. Sequence analysis of HBV from untreated chronically infected patients show that PreS deletions and other mutations in the S-gene were more frequent in those patients with progressive liver disease, including those with cirrhosis and HCC. This observation proposes that such changes may interfere with the regulation pathways of surface protein synthesis leading to intracellular retention of HBV proteins, resulting in liver damage [224-226]. New technology, such as Next Generation Sequencing has shown that S-gene variants are not uncommon in chronic hepatitis B with many stop codons detected at levels below that of traditional population-based sequencing [227, 228].
1.11.4) Vaccine and Immunoglobulin Escape Variants

Since the inclusion of the hepatitis B vaccine in the EPI, HBV surface variants have been reported from around the world, especially from regions of high endemicity [12, 229-233]. The selection of HBV S-gene mutants have been described in neonates given vaccine and HBIg to reduce maternal transmission as well as in cases of acute hepatitis B infection in children and adolescents.[31] The first report of a vaccine escape mutant (VEM) of HBV was in 1990 by Carman et al [12]. They described the selection of the HBV variant sG145R after the administration of HBIg and vaccine in a child in Southern Italy with apparently perinatally transmitted HBV from an HBeAg-positive mother. This child was followed for five years and sG145R HBV persisted during this period, indicating its relative fitness[12].

Since this first report, numerous studies have been conducted to evaluate the impact of vaccination on the prevalence of HBV infection and on the emergence of HBV surface gene variants [229, 231, 234-239]. Taiwan was one of the first countries to launch a nationwide hepatitis B vaccination program in 1984 and successfully decreased the country's HBsAg prevalence rates [100]. However, there have been reports of an increase in the number of HBV surface variants since its introduction. Risk factors for infection with these variants included age greater than 18 years and immunisation with three doses of vaccine during childhood [240]. Similarly other countries such as Singapore and China have reported an increase in these variants [231, 238]. China has reported an increase from 9.8% in 1992, at the commencement of HBV vaccination to approximately 15% in 2005 [238], although only a few of these variants could be considered true VEMs and not polymorphic variants [241].

The rise in HBV surface variants post-immunisation [183, 240], however, has not been seen in all countries. Whilst some endemic countries noted a rise in HBV surface variants, others such as South Africa, South Pacific and North America did not notice any changes to their incidence [242-244]. The reason for such a key difference has not been identified. However, genotype/serotype variation, variation in the timing of vaccination, especially the birth dose, and concurrent use of HBIg as well as the baseline prevalence of HBV may explain some of the disparities.

Other than primary prevention of chronic HBV infection, HBIg is also an effective mode of therapy for HBV related orthotopic liver transplantation (OLT). HBIg is often administered to patients with chronic HBV infection undergoing liver transplantation to reduce the chance of the new graft becoming infected. Long-term passive
immunoprophylaxis after OLT results in a 60–80% reduction of HBV recurrence [109]. The mechanism whereby HBV protects the liver graft from infection is poorly understood, although there is a dose-dependent response. Whilst early recurrence, defined as infection within eight weeks of transplantation is often due to wild type HBV and possibly the result of the inability of the HBV to neutralize the high HBV DNA load, infection later than eight weeks is typically due to S-gene variants. This delayed infection is consistent with the selection pressure of HBV and the immunosuppressive regime employed to reduce graft loss that can lead to an increase in the viral replication rate [245].

A number of mutations in the HBV genome associated with HBV therapy in OLT patients have been reported and these include the sG145R variant, seen in infected neonates given vaccine and HBV [12, 246, 247]. Persistence of sG145R and other immune escape variants/S-gene variants have been documented even five years after the withdrawal of HBV [12]. These variants were not detected prior to therapy and this could be due to the low sensitivity of the methods used because it is presumed that these variants pre-exist [248-250].

1.12 HBV Vaccine Escape Mutants

1.12.1 Overview

VEMs have the ability to avoid neutralization, despite adequate amounts of anti-HBs and there are some concerns over their potential impact on hepatitis B vaccination programmes [183, 240]. Currently, there is no consensus on what constitutes or defines a VEM. Many isolates derived from patients experiencing vaccine failure have been claimed as VEMs but no efforts have been made to discriminate between common polymorphisms or authentic selection-driven escape substitutions. Virological and clinical criteria need to be established in order to reach a consensus of the threat that VEMS could possibly represent. To date, only one reviewer has proposed any such criteria. Four criteria have been considered: (i) the mutant must be stable for transmission to occur, (ii) the altered antigenicity must be enough to prevent neutralization by vaccine generated anti-HBs, (iii) it must be capable of transmission and infection of an immunized individual and (iv) there must be virological and/or clinical evidence of acute or chronic infection[14]. At present, only variants encoding the sG145R change fulfill these criteria [251]. The most challenging criterion is the one requiring demonstration of the lack of neutralisation by vaccine derived anti-HBs. Infection of immunized chimpanzees has both ethical and cost issues and in-vitro assays, using primary human hepatocytes are difficult to standardize and not widely available.
Robust and reproducible in-vitro phenotypic assays need to be developed to measure anti-HBs neutralization.

1.12.2 The ‘a’ determinant
Amino acid substitutions, conferred by point mutations, insertions and deletions, have been identified throughout the HBV surface proteins, but those changes in the ‘a’ determinant (see Figure 1.7) of the SHBs are the cause of most concern [183, 229, 253, 254]. Neutralisation by anti-HBs is highly dependent on an intact conformation of the ‘a’ determinant. Amino acid changes which disrupt the two loop structure (see Figure 1.8) of the ‘a’ determinant can affect anti-HBs binding. Such effects include changes in the hydrophobicity/hydrophilicity [12], addition of possible extra N-glycosylation sites or changing the stability of the disulphide bridges such as the substitutions sC124Y and sC137Y. The ‘a’ determinant can be considered within three regions:

1.12.3 (i) ‘a’ determinant - amino acid 100-124 (region 1)
Amino acid region spanning positions 100-118 forms part of the major B-cell epitope of the ‘a’ determinant. However, substitutions in this region have not been associated with VEMs. Evaluation of various diagnostic assays for detection of HBsAg has not revealed any major change in sensitivity for substitutions in this region, suggesting that antigen-antibody binding is not greatly affected [255].

The region between 118 and 123 (mini – loop region) has long been recognized as a hot spot for insertions, deletions and mutations that affect the diagnosis of HBsAg [186, 253, 256, 257]. It is a highly immunogenic region and changes here can cause disruption of epitopes leading to immune escape. Furthermore, a study on the antigenicity of changes at residue 118 by substitution of threonine to alanine showed significant reduction in hydrophilicity [253]. Substitution of proline at position 120 of HBsAg has generated a lot of interest in studies for possible VEMs [258-260]. All HBV genotypes have proline at this position [259]; due to its cyclic side chain it has a remarkable conformational rigidity. Proline is commonly found in β turns necessary for secondary structure [261]. Substitution of proline to threonine (sP120T) has been associated with immune escape in several mother to baby transmission studies, presumably due to immunological pressure of vaccine, with or without HBlg, [13, 260] and with exposure to HBlg in the setting of liver transplantation[248]. Immunoassay studies using monoclonal antibodies confirmed that there was appreciable reduction in binding of HBsAg with sP120T and whilst this reduction was not as marked as sG145R, [262], this observation is consistent with the ability of these mutants to escape anti-HBs, vaccine induced or passively via
Other substitution observed at position 120 include sP120L/S/Q, and these too have been implicated with vaccine and HBlg therapy.[258]

The cysteine residues in the ‘a’ determinant are critical for maintaining its conformation. Two of these are found in the small hydrophilic region between residues 120-124 [264]. Site-directed mutagenesis studies involving substitution of Cys-121 and Cys-124 with serine or alanine results in a substantial reduction in binding titres of monoclonal and polyclonal antibodies directed to the ‘a’ determinant, consistent with the finding that amino acid changes at Cys-121 represents a diagnostic escape. Nonetheless, for these variants, the secretion of HBsAg and its ability to elicit anti-HBs response was similar to wild-type virus variant [265]. Despite in-vitro studies demonstrating the potential to escape neutralisation by anti-HBs, clinical cases of vaccine failure from substitution of these cysteines are rare. The lysine or arginine at position 122 is the subtype determinant ‘d’ or ‘y’ (see Figure 1.8) and as expected, this position is critical to the antigenicity of the HBsAg with substitutions shown to induce diagnostic escape [266, 267]. Similarly, another naturally occurring amino acid substitution of threonine at position 123 to asparagine (T123N) [268] can result in aberrant glycosylation, leading to a secretion defect due to reduced virion assembly.

1.12.4 (ii) ‘a’ determinant - amino acid 125-138 (region 2)

The amino acid region spanning from 125-138 constitutes the remainder of Loop 1, terminating with Cys-137 and Cys-138 (see Figure 1.7). The amino acid at position 126 has been proposed as another subtype determinant being either threonine or isoleucine (sI/T126) [269]. HBV variants having asparagine, serine or alanine at 126 have been found in patients with persistent HBsAg who are also seropositive, although this did not appear to influence detection of HBsAg by diagnostic assays [270]. Variants at positions 129 (glutamine to histidine/arginine/proline) and 133 (methionine to leucine, sM133L) of the ‘a’ determinant have been associated with escape from vaccine induced immunity and also identified in occult hepatitis B [254, 271, 272]. As they often occur in conjunction with other changes, it is difficult to determine their precise role. These variants are recognized by most commercial HBsAg diagnostic assays [266]. Substitution at amino position 132 (sS132A) has also been described as a ‘escape mutation’, [239] however, its occurrence is rare and lacks immunogenicity studies.
1.12. 5 (iii) ‘a’ determinant - amino acid 139-148 (region 3)

Loop 2, which protrudes from the viral membrane is stabilized by the cysteine pair Cys-139 and Cys-147 with further stability provided by the disulphide bridge between Cys-137 and Cys-149 (see Figure 1.7) [264]. Compared to the other domains across the ‘a’ determinant, Loop 2 changes have a stronger association with anti-HBs immune pressure. Variants associated with vaccine and HBIg therapy emerging in this region are frequent and include substitutions around the cysteine residues and point mutations conferring substitutions sS143L/W, D, sE144G/A, sG145R/A have all been reported. [183, 240, 248, 273, 274].

The amino acid region 139-148 has overlapping B and T cell epitopes. It is relatively conserved among the serotypes and shares 77% homology with non-human mammalian hepadnaviruses [275]. Substitutions in this region have been evaluated using a peptide replacement analysis to identify critical amino acids for anti-HBs binding; this revealed that the epitope between amino acids 141-145 was integral [186].

The HBV surface variant sG145R has been described from most regions around the world and is now considered the prototype VEM [12, 234, 276-278]. The body of literature supporting that its emergence is due to immune pressure from HBIg and vaccine is strong, yet it may also occur as a natural variant. HBV sG145R variants have been shown to cause liver disease, HCC and are capable of transmission amongst family members. Multiple studies have been performed to elucidate the effects of various HBV S-gene mutants on the binding to anti-HBs and sG145R is used as a benchmark for its vaccine escape potential [183, 237, 245, 273, 279].

The next most frequent variant found after the sG145R is at position 144, with substitution of aspartic acid to alanine (sD144A) [183, 260, 280, 281]. An early study by Protzer-Knolle et al evaluated HBs gene sequences in chronically infected patients undergoing OLT and identified variants post-liver transplant harboring sG144A and one patient with a glutamic acid substitution at this position (sG144E) [248]; it was concluded that these variants were selected under immune pressure generated by HBIg. Selection of sD144A has been reported in a previously immunized person with HIV co-infection. During a 5-year follow up, this patient developed biochemical and virological evidence of acute HBV infection with sG144A, despite having anti-HBs [282].

Combination of Loop 2 variants at position 144 and 145 have been studied using antibodies from vaccinee sera and has demonstrated reduction in reactivity with dual substitutions sD144E/sD145R, comparable to sG145R [187]. Losses of glycosylation

34
sites have also been associated with immune escape, *in-vitro* studies have shown substitution sN146A reduced binding of anti ‘a’ determinant antibodies [283].

1.12.6 Changes Outside ‘a’ determinant

Naturally occurring HBs variants outside the ‘a’ determinant have been reported and include mutations both upstream and downstream from this domain. In a study conducted in Singapore evaluating mother to baby transmission, variants at positions 183 and 184 of HBsAg were detected in serum from both the mother and baby, suggesting vertical transmission. These isolates showed decreased binding to diagnostic monoclonal and polyclonal anti-HBs directed to the ‘a’ determinant, suggesting an influence on its conformation [284].

1.13 Antiviral Associated S- Gene Variants

Unlike antiretroviral therapy (ART) for HIV infection where multiple drugs target different stages of the HIV replication cycle[285], virological targets for HBV infection are limited to the reverse transcriptase. As the HBV polymerase gene overlaps the envelope gene, changes conferring antiviral resistance can cause concomitant changes to the ORF of the envelope gene, which has the potential to cause a modification to the surface protein [14]. A change in an amino acid in the ‘a’ determinant may impair the ability of anti-HBs to neutralize the virus [187, 251].

As highlighted previously, the risk of resistance associated with NA therapy is greatest for agents with a low genetic barrier to resistance such as LMV. Whilst most international clinical practice guidelines discourage LMV, LdT or ADV as first line agents for therapy [23, 122, 128], they remain widely used in resource poor countries [286]. The risk of resistance with LMV therapy rises from 15% in the first year to approximately 67% at four years. Failure of LMV often leads to sequential NA exposure from alternative agents, increasing the risk of resistance and the development of HBV surface protein variants. Substitutions commonly associated with LMV resistance such as the rtM204V, result in a change at sI195M in the HBsAg, while rtM204I is linked to three possible HBsAg changes, sW196S, sW196L or a termination codon. In HIV/HBV co-infection, a resistance variant with triple substitutions rtV173L+rtL180M+ rtM204V, was commonly found when LMV was used as part of ART [287]. This can result in changes at sE164D + sI195M of the surface protein [136, 288]. The effect of these various changes have been examined *in-vitro* where the LMV resistant variants showed reduced binding to anti-HBs, suggesting an antigenically altered HBsAg [187]. HBV with substitutions at sE164D + sI195M reduced anti-HBs binding to a similar degree to that of the sG145R VEM and were capable of infecting vaccinated chimpanzees[251].
1.14 Aims of this study
It has been assumed that protective immunity generated by vaccination or recovery from natural infection is associated with neutralizing antibodies directed to the conserved immunodominant domain, the ‘a’ determinant. This contains a number of highly conformational epitopes, stabilized by disulphide bridges between pairs of cysteine residues. Substitution of amino acids in the ‘a’ determinant can lead to reduced immunogenicity, resulting in failure to bind neutralizing antibodies and escaping detection by diagnostic assays.

HBV variants generated by immune escape have been reported from all around the world. The current method of confirming the identification of these variants relies on a population based sequencing approach which shows mutations conferring amino acid substitutions. To be detected against a background of wild-type sequences, variants need to be present at greater than 20% of the total population. Thus, the identification of HBV variants relies largely on sequence analysis and the determination of whether it is associated with immune escape is deduced from the location of the amino acid substitution in the ‘a’ determinant. To further characterize the influence of such changes requires the development of a phenotypic assay.

1.15 Hypothesis
Hepatitis B variants, either naturally occurring or selected due to vaccine/HBIg or NA therapy, circulating in the population may compromise current vaccination programmes.

Objective
To develop a laboratory-based algorithm for establishing HBV VEM status.

Specific Aims
Aim 1: To generate in-vitro reference reagents for validating a high throughput phenotyping assay.
Aim 2: To establish an epitope profile of the different HBV genotype/serotype and common variants based on the phenotyping assay.
Aim 3: To correlate the phenotypic profile of in-vitro constructs to HBV from clinical groups.
Chapter 2:
Methodology

Methods and Materials
Outlined in this Chapter are the various materials and methods used for these studies, however, some specifics are provided in more detail in the different Chapters.

Abbreviations
DMEM - Dulbecco’s Modified Eagle Medium
dNTP – Deoxyribonucleotide triphosphate
FCS- Fetal calf serum
LB – Luria Bertani
mAb – Monoclonal antibody
NaCl – Sodium chloride
NFW – Nuclease free water
PBS – Phosphate buffered saline
PCR - Polymerase chain reaction

2.1A Cell Culture
The Huh-7 cell line was used in this study for the expression of HBsAg from the different S-gene constructs. This cell line is derived from hepatocellular carcinoma cells. The cells were maintained in DMEM with 10% (v/v) FCS and 0.5U/ml penicillin, 0.5µg/ml streptomycin and L-glutamine. The cells were passaged twice a week and re-suspended in 10% DMEM-FCS and seeded onto 100mm diameter tissue culture plates. The tissue culture plates were kept at 37°C in humidified incubators with 5% (v/v) CO₂, media was changed every 2-3 days and cells were examined daily under an inverted microscope.

2.1B H166
H166 is an IgM antibody, directed to amino acid position 121-124 of HBsAg [186] and used in Western Blotting assays described below. The antibody was provided by Dr Nadia Warner, VIDRL.

2.2 Cloning of the S-gene
2.2.1 Vector and S-gene
The S-gene DNA templates for genotype A2, B, C and F were previously generated at VIDRL and provided by Dr Peter Revill. For genotypes A1, E and F, DNA was extracted from patient sera and supplied by Dr Margaret Littlejohn. The S-gene for genotype D
was previously cloned at VIDRL into the pCI vector and used for HBsAg expression and *in-vitro* BioPlex studies.

2.2.2 Amplification of S-gene

PCR master-mix was set up for the HBV S-gene by using 1μl of template DNA, 5μl of 10 x buffer, 10μl of Q buffer, 0.5μl of DNTPs, 0.3 μl of Taq polymerase (Life Technologies, CA, USA), 1μl of forward primer (see Table 1.1, GeneWorks Pty Ltd) and 1μl of reverse primer (see Table 1.2, GeneWorks Pty Ltd) at 20 micromoles, for each genotype sample. The forward and reverse primer sequences are outlined in Table 2.1 and Table 2.2.

The cycling condition was set up as 94°C for 3 minutes, 40 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 90 seconds, followed by 72°C for 4 minutes and held at 4°C. Each sample was then visualized by performing gel electrophoresis, this included running at 100V in 0.7 % (vol/vol) agarose gel in ethidium bromide with molecular weight markers.

Each PCR product was partially purified using the PCR cleanup kit, (MO BIO Laboratories, CA, USA) as per manufactures' instruction. This involved adding spin buffer to the PCR product at a ratio of 5 to 1 followed by mixing and then transferring into a spin filter unit and then centrifuging at 13,000 rpm in a tabletop microcentrifuge. The flow-through was decanted and 300μl spin-clean buffer, which contained 80% ethanol (v/v), was added to the spin filter unit and centrifuged at 13,000 rpm for 30 seconds. The final product was dissolved in 50μl of nuclease free water (NFW).

**Table 2.1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sequence (startHind3gen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>TCGAAGCTTGCCACCATGGAAACATCACATCAGG</td>
</tr>
<tr>
<td>B</td>
<td>TCGAAGCTTGCCACCATGGAGAGGCATCGCATCAGG</td>
</tr>
<tr>
<td>C</td>
<td>TCGAAGCTTGCCACCATGGAGAGCACAACATCAGG</td>
</tr>
<tr>
<td>E</td>
<td>TCGAAGCTTGCCACCATGGAGAGCATCACATCAGG</td>
</tr>
<tr>
<td>F</td>
<td>TCGAAGCTTGCCACCATGGACAAACATCACATCAGGAGCTT</td>
</tr>
</tbody>
</table>

*Table 2.1 shows the forward primer sequence sequences used in the cloning of genotype A, B, C, E, F and G used in this study.*
Table 2.2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sequence (s_reverseNot1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2,B, C, E, G</td>
<td>GTGCGGCCCCCTTTAAATGTATACCAAGAC</td>
</tr>
<tr>
<td>F</td>
<td>TCGAAGGCTTGCCACATGGACAACATCACATCAGGAAGCTT</td>
</tr>
</tbody>
</table>

Table 2.2 shows the reverse primer sequences used in the cloning of genotype A, B, C, E, F and G used in this study.

2.2.3 Digestion of PCR product and Vector, Extraction and Ligation

The PCR product and plasmid was digested using restriction enzyme NotI (Promega, WI, USA) and HindIII (Promega, WI, USA), this involved addition of 10µl of DNA, 2µl of HindIII, 5µl of 10× buffer and 3µl NotI and NFW to make up to the volume of 50µl for each of the genotypes (A1, A2, B, C, E, F and G).

For the digestion of vector, 13µl of prcCMV, 2µl of NotI, 5µl of 10 × buffer 3 and 13µl nuclease free water was added. This was followed by digestion with HindIII as describe above. All digest mixtures, which included the DNA from PCR product and vector (prcCMV) (see Figure 2.1) were put into Eppendorf tubes and left on the heat block for 2 hours at 37°C.

Figure 2.1

*Figure 2.1 shows 5.5kb prc/CMV vector with restriction sites [289].

Entire products were run on agarose gels and bands of interest excised. The product was cleaned using the QIAquick kit by following the manufacturer's instructions (Qiagen, Hilden, Germany) and the digested PCR product was ligated into the vector.
(prcCMV) at a molar ratio of 1:3. This involved adding 1µl vector, 1µl ligase (Promega, WI, USA), 1µl 10 × buffer and 7µl of insert and leaving at 4°C overnight.

2.2.4 Transformation in E. coli
Competent E.coli DH5α™ cells (Invitrogen, Carlsbad, USA) were thawed on ice for 10 minutes and 15mL falcon polypropylene tubes were chilled. The reaction was set up in the falcon tube with 50µl competent cells and 5µl of ligation mix from all of the genotypes, described above. This was left on ice for 30 minutes, mixing frequently during this period and incubated for 45 seconds at 42°C in a water bath and then the transformation reaction was chilled in ice for 2 minutes.

LB broth media was previously prepared by mixing 1g bacto-tryptone (Becton Dickinson, Sparks, MD, USA), 0.5g bacto-yeast extract (Becton Dickinson, Sparks, MD, USA) and 1g NaCl for every 100mL of distilled water. The transformation reaction was incubated in 300µl SOB media at 37°C in a shaker for an hour and plated on agar plates with ampicillin at the concentration of 100µg/µl, and then incubated overnight at 37°C.

2.2.5 Mini prep
The plates were examined for colonies the following day and at least 20 different colonies per genotype were patched onto agar plates with ampicillin as described above. Each colony was inoculated in 2mL of LB broth and incubated at 37°C in a shaker overnight (12 hours). Miniprep DNA was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia) and suspended in 50µl NFW.

2.2.6 Sequencing
Each mini prep DNA product of the different HBV genotypes was sequenced using Seq 2 (forward primer) and TTA4 (reverse primer) (see Table 2.3). For each sample, 2µl of 5 × buffer, 4µl of primer at the concentration of 0.8µmol, 1µl BigDye® Terminator (Life technologies, TX, USA) and 3µl NFW was used to make 12µl. The PCR sequencing cycle was 30 cycles of 96°C for 10 sec, 55°C for 30 sec, 60°C for 4 minutes and maintained at 4°C[209, 290]
Table 2.3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTA4 (reverse)</td>
<td>GAA AAT TGG TAA CAG CGG</td>
</tr>
<tr>
<td>Seq 2 (forward)</td>
<td>TTG GCC AAA ATT CGC AGT C</td>
</tr>
</tbody>
</table>

Forward and reverse primer used for sequencing the S-gene of cloned HBV genotypes

The sequencing product was precipitated by adding 50μl of 96% (v/v) of ethanol, 2μl of sodium acetate and 8μl of reaction mix and left at room temperature for 20 minutes before centrifugation at 14,000g for 20 minutes. Following this, the supernatant was aspirated and washed by adding 180μl of 70% (v/v) ethanol and centrifuged again for 5 minutes. The supernatant was aspirated and dried on a heat block at 37°C until the ethanol had evaporated. The DNA sample was sent to Micromon, Monash University, for sequencing; the chromatogram and sequence were analysed using the Seqscape software program[291].

2.2.7 Maxi prep
Sequences of clones that were consistent with wild-type HBV S-gene for each genotype were identified on the patch-plate and a single colony was inoculated in 2mL of LB with 2μl ampicillin (100μg/μl) and left for 8 hours at 37°C. Cultures were set-up for 14 hours, overnight at 37°C on a shaker by inoculating 150μl of these cultures into 150mL of LB with 150μl of ampicillin. Plasmid DNA was isolated for each genotype the following morning by using the Plasmid Maxi kit (Qiagen) as per the manufacturer’s instruction. Final DNA product was eluted in 50μl of NFW and diluted at 1μg/μl, by measuring the concentration with a Nanodrop Spectrophotometer (Implen GmbH, Munich, Germany), and stored at -20°C. The extracted DNA was diluted at 1 to 20, digested using HindIII and NotI (Promega) and analysed by gel electrophoresis with molecular markers as described above and sequenced using primers Seq2 and TTA4 (see Table 2.3). The results of sequencing were analysed using Seqscape software program [291] and clustal alignment for the S-gene was carried out using the software program BioEdit.

2.2.8 Pairwise Analysis
Nucleotide difference within the ‘a’ determinant of the different HBV genotypes cloned in this study was performed by Dr Lilly Yuen, using Molecular Evolutionary Genetic Analysis (MEGA) software, version 4.

2.2.9 Transfection
Huh 7 cells were maintained by passaging twice a week at 1 in 6 dilution in 100mm dishes and washing with PBS. Transfection was performed 24 hours after cells were
passaged, incubated at 37°C in 5% CO₂ with monolayers 60-70% confluent. A transfection mix was prepared by adding 400μl of OptiMEM (Gibco, NY, USA) to 32μl of Fugene transfection reagent (Roche, Mannheim, Germany) and leaving for a 5 minute incubation. This mixture was then added to 5μg of cloned DNA, thoroughly mixed and incubated for 30 minutes. This was then added to each of the 100mm cell culture dishes of Huh 7 cells in a drop-wise fashion and incubated at 37°C in 5% CO₂. Media was changed the following day and on day 3 with supernatant harvested at day 5 to perform further studies for protein expression and phenotypic analysis, described below.

2.3 Protein Expression Study

2.3.1 Western Blotting

Western blotting studies were performed to confirm protein expression. However, ultracentrifugation through a sucrose cushion, was carried out initially to concentrate the supernatant from transfection studies above. This involved centrifuging 35mL of supernatant from the transfected cells and layering it below 20% (w/v) sucrose in PBS and then centrifuged at 25,000g for 6 hours at 4°C on a Beckman Coulter Ultracentrifugation using the rotor 110/18. The supernatant of the centrifuge product was aspirated and the pelleted HBsAg particles were resuspended in 50μl of PBS.

Western blotting assay was performed, by loading 5μl of Laemmli buffer with 20μl of concentrated HBsAg from sucrose cushion, boiling at 100°C for 1 minute before loading into polyacrylamide gels (Mini-Protein TGX, Biorad, CA, USA). These were run at 150 volts for 10 minutes and then 100 volts for approximately 30 minutes, until the dye-front reached the end of the gel. The protein was transferred to nitrocellulose membranes (Transblot-Turbo transfer pack, Biorad, CA, USA), blocked at 4°C by suspending the membrane into trays of 25ml of 0.1% PBS-tween solution containing 1.25g (or 1%) skim milk overnight.

To detect HBsAg, the membrane was suspended in H166 mouse anti-IgM antibody for an hour at a concentration 1 to 5,000. The membrane was washed with 0.1% PBS Tween for 10 minutes by placing it in a shaker; this was repeated 3 times. The membranes was then conjugated with secondary antibody, anti-mouse IgM antibody and again washed with 0.1% PBS Tween three times by placing it in the shaker at 10 minutes interval. The membrane was suspended in 1 mL of ECL white and 1ml brown of Western Lightning Plus ECL, which was added as per the manufacture’s instructions (Perkin Elmer, Waltham, MA, USA) directly into centre of the membrane and incubated for 1 minute before putting into the Biorad ChemiDoc MP Imaging System to capture the image.
2.3.2 Quantifying HBsAg

HBsAg concentration in the harvested supernatant from cell culture at Day 5 was quantified by using Elecsys HBsAg II quant (Roche Diagnostics) as per manufactures’ instructions [292].

2.3.3 Cell Culture Modification To Increase HBsAg

To increase HBsAg concentration, the media was modified by adding different concentration of FCS and DMSO and compared to standard media containing 10% FCS in DMEM. This experiment was based on a study by Gripon et al, showing increased intracellular HBV viral DNA by the addition of DMSO[293]. A subsequent study also demonstrated that there was a significant increase in concentration of HBV intermediates in the AD38 cell line in the presence of 1% DMSO[294]. However, no study has been performed with the Huh 7 cell line, which do not constitutively express HBV. The following media was prepared with variable concentrations of FCS and DMSO for the different wells. Outlined below are the concentrations of FCS and DMSO mixed with DMEM for the wells described in Table 2.4.

Well 1 – 10% FCS in 500mL of DMEM (10% FCS-DMEM), standard media for transfection studies at VIDRL

Well 2 – 5mL of 10% FCS-DMEM mixed with 100μl of DMSO, to make up 2% DMSO concentration

Well 3 – 5mL of 10% FCS-DMEM mixed with 50μl of DMSO to make 1% DMSO concentration

Well 4 – 5mL of 10% FCS-DMEM mixed with 25μl of DMSO to make 0.5% DMSO concentration

For Wells 5-8, 5% FCS – DMEM was prepared by adding 1.5mL of FCS to 30mL of DMEM.

Well 5 – 5% FCS-DMEM

Well 6 – 5mL of 5% FCS mixed with 100μl of DMSO, to make up 2% DMSO concentration

Well 7 – 5mL of 5% FCS-DMEM mixed with 50μl of DMSO, to make up 1% DMSO concentration

Well 8 - 5mL of 5% FCS – DMEM mixed with 25μl of DMSO, to make up 0.5% DMSO concentration

For Wells 9-12, 0.5% FCS-DMEM was used and kindly provided by Dr Nadia Warner

Well 9 - 5mL of 0.5% FCS – DMEM only

Well 10 – 5mL of 0.5% FCS-DMEM mixed with 100μl of DMSO, to make up 2% DMSO concentration
Well 11 - 5mL of 0.5% FCS-DMEM mixed with 50μl of DMSO, to make up 1% DMSO concentration

Well 12 - 5mL of 0.5% FCS-DMEM mixed with 25μl of DMSO, to make up 0.5% DMSO concentration

Set-up of plates

Table 2.4

<table>
<thead>
<tr>
<th></th>
<th>No DMSO</th>
<th>2% DMSO</th>
<th>1% DMSO</th>
<th>0.5% DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FCS-DMEM</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5% FCS-DMEM</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>2% FCS-DMEM</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2.4 shows the different concentrations of DMSO (0-2%) in DMEM with different concentration of FCS (2-10%). Well 1, marked in yellow represents the media concentration used for transfection and protein expression studies of HBV at Molecular Research and Development Laboratories, at VIDRL.

Two sets of 12-well plates were seeded with Huh 7 cells, at a 1 in 8 ratio (3mL of cell suspension in 27mL of media) and 1mL was seeded into each well. The cells were grown overnight and transfected with HBV genotype D ayw, as outlined previously. At Day 1 and 3, media was changed for each well using the different concentration for FCS-DMEM-DMSO media as outlined above and harvested for HBsAg at Day 5, and HBsAg was quantified, as described above.

2.4 Generation of Common S-gene variants

2.4.1 Site Directed Mutagenesis

To generate HBsAg variants associated with vaccine/HBIg escape, site directed mutagenesis (SDM) was performed using QuickChange II Site Directed Mutagenesis kit (Agilent Technologies, Stratagene) on HBV genotype A2 adw-2 and primer sequences are outlined in Table 2.5.

For each HBV variant, genotype A2 adw -2, generated previously on pCI vector was used as template, diluted 1 to 20 in NFW. Each reaction was set up using the 1 in 3 of forward and reverse primer diluted at a concentration of 0.1μg/μl. From the SDM kit (Agilent Technologies, Stratagene) 5μl of 10× buffer, 1μl of dNTP, 1μl of PfuUltra high fidelity DNA polymerase (Agilent Technologies,) and was made up to volume of 50μl by adding NFW. The PCR reaction was set-up in the PCR cycle 95°C for 1 minute, 95°C (30 seconds) for 18 cycles, 55°C for 1 minute, 68°C for 18 cycles and then maintained at 10°C.
Reaction for digestion was set up by taking 45μl of each sample and digested with 1μl of dpN1 for 2 hours. Following this, the bands were visualized by using pulse gel electrophoresis to check the band size of the mutagenized S-gene product, as outlined above (Section 2.2.2). Each sample was then transformed in *E.coli* and patch plated on agar with ampicillin as outlined above. Each clone was also sequenced and once the appropriate sequence was confirmed, a maxi prep was done as outlined previously to obtain DNA for transfection. Transfection studies were done in Huh 7 cells to express HBsAg and quantified as outlined above.
| Table 2.5 |
|-----------------|-----------------|
| sP120L_F:       | CAACACCTACGGAACATGCAAACCTGCACGACTCC |
| sP120L_R:       | GGAGTCGTACGGGTTCACAGTCGTAATGGTCTGTTTC |
| sP120T_F:       | CAACACCTACGGAACATGCAAACCTGCACGACTCC |
| sP120T_R:       | GGAGTCGTACGGGTTCACAGTCGTAATGGTCTGTTTC |
| sM133L_F:       | CCTGCTCAAGGGAACCTGACGGAAATTGCACCTGCACGACTCC |
| sM133L_R:       | GCAACATGAGGGAAACAGAGAGTTGCCTTGAGCAGG |
| sT126N_F:       | GCAACATGAGGGAAACAGAGAGTTGCCTTGAGCAGG |
| sT126N_R:       | GAGTTGCCTTGAGCAGGATTCGTGCAGGTTTTGC |
| sD144E_F:       | GCTGTACAAAACCTACGGAGGAAATTGCACCTGCACGACTCC |
| sD144E_R:       | CAGGTGCAATTTCCCTCCGTAGGTTTTGTACAGC |
| sD144A_F:       | GCTGTACAAAACCTACGGAGGAAATTGCACCTGCACGACTCC |
| sD144A_R:       | CAGGTGCAATTTCCCTCCGTAGGTTTTGTACAGC |
| sG145A_F:       | CAAAACCTACGGATGCAATTTGCACCTGATTTCC |
| sG145A_R:       | gGGAATACAGGTGCAATTTGcATCCGTAGGTTTTG |
| sN146A_F:       | CCTACGGATGCAATTTGCACCTGATTTCC |
| sN146A_R:       | GGGATGGGAAATACAGGTGCAATTTGcATCCGTAGGTTTTG |
| sD144A+G145A_F: | CAAAACCTACGGAGGAAATTGCACCTGATTTCC |
| sD144A+G145A_R: | GGAATACAGGTGCAATTTGcATCCGTAGGTTTTG |
| sS132A_F:       | CCTGCTCAAGGGAACGCTAGTGGTTTCCCTCATG |
| sS132A_R:       | CATGAGGGAAACATAGcGTTGCTTTGAGCAGG |

Table 2.5 shows the primer sequence, forward (F) and reverse (R) used for generating the HBsAg variant. Codons in coloured font show the changes of nucleotide sequence of the S-gene variants.

BioPlex Methodology

2.5.1 HBsAg Epitope Mapping

A multiplex bead-based flow cytometric platform was developed by Dr Renae Walsh at VIDRL and has been used to perform an HBsAg epitope fingerprinting assay and establish and map the HBsAg [295]. The HBsAg multiplex immunoassay was based on a multiplexed panel of 19 monoclonal antibodies (mAbs) directed against HBsAg ‘a’ determinant and C-terminal domain epitopes spanning residues 99-226 of the small HBsAg. The HBsAg epitopes are categorized into the following domains: N-terminal (mAb 1), Loop1 (mAbs 5, 6, 10), Loop2 (mAbs 7, 8, 11, 12, 16, 17, 19), Loop1/2 combinational (mAbs 13, 14, 15), C-terminal (mAbs 2, 3, 4), and conformational (mAbs 9, 18)(see Figure 2.2A and 2.2B.). The source of these mAbs and epitopes at which they interact were provided by the manufacturers (see Table 2.6).
This assay comprised fluorescently labeled microsphere beads covalently coupled to purified anti-IgG antibody, coupled indirectly to a single anti-HBs mAb (according to manufacturer's instructions, Bio-Rad Laboratories, see Figure 2.2), such that each uniquely identifiable bead/mAb set identifies a single HBsAg epitope. The anti-HBs labelled beads sets were prepared as multiplex panels at equal quantities (each at 500 beads/μl). For all of the in-vitro constructs, supernatant from transfected cell culture was harvested at Day 5 and prepared for analysis, whilst serum samples were diluted from the stored sera. The multiplexed beads were incubated overnight with HBsAg-positive samples (sera and cell culture supernatant) at a dilution series of 8, 16, and 32 IU/well HBsAg, as determined by the Roche Elecsys diagnostic assay. Following plate washing (3x) in PBS-T (PBS + 0.05% Tween-20), RPE-conjugated polyclonal anti-HBs secondary antibody was added and incubated for 2 hours, the plate wash repeated and samples resuspended in 100ul/well BioPlex staining buffer (Bio-Rad Laboratories) prior to analysis on a BioPlex200 instrument. Output was recorded in relative fluorescence units (RFU) based on the intensity of RPE (secondary antibody) associated with captured HBsAg for each bead/mAb (anti-HBs epitope), and measured for 100 beads of each mAb specificity.

The epitope recognition profile was determined by the RFU intensity data collected for each sample across 19 epitopes of HBsAg which were then processed through an in-house developed bioinformatics models (see below), established against extensive control data panels specific for wild-type reference HBV strains, confirmed by sequencing. Bioinformatics processing for each sample consists of three stages: 1) Correction of potential batch variation between bead-anti-HBs coupling; 2) Identification of the optimal data set from the HBsAg dilution series (8, 16, 32 IU/well); 3) Normalization of each sample data set using both control anti-HBs/bead sets which are stable epitopes across HBV strains, and against the control data set of the relevant HBV strain backbone to control for HBV genotype variation in anti-HBs epitope display [295, 347-349]

2.5.2 Bioinformatics
The first step in the bioinformatics data process was to correct for bead/mAb labelling batch variation required determination at each epitope of the multiplication factor to correct potential inter-assay variation. This was achieved by averaging RFU intensity at each epitope for control A2 adw-2 reference sera data sets for an experiment, with normalization to the pooled control A2 adw2 reference data panel. The second step for
bioinformatics processing selected the optimal dilution data set from the three HBsAg dilutions analysed, based on fluorescent intensity within the assays dynamic range, across several anti-HBs epitopes. Sample dilution range was analysed to account for potential inaccuracy of the diagnostic kit reported HBsAg concentration (IU/mL), which could potentially have translated as an over or under estimation of HBsAg (IU/well) in the HBsAg profile assay thereby corresponding to fluorescence intensity outside (above or below) the assays dynamic range at each epitope. The final bioinformatics processing step normalized RFU data sets for each sample against stable epitopes, and to the appropriate HBV strain backbone. The fluorescence intensity recorded at each epitope for a sample was normalized by the average RFU at two control or stable epitopes (mAbs 3 and 15) and natural log transformed. The sample data was then normalized at each epitope to the appropriate HBV strain (i.e. genotype and serotype backbone) to similarly processed data from the established control data sets of reference HBV strain panels.

2.5.3 Data Presentation
The final processed and corrected data was expressed as a fold change of epitope recognition of the HBsAg sample when compared to the reference backbone at each HBsAg epitope. The normal range of variation in epitope recognition was initially established based on panels of wildtype reference sera including HBV genotype A-D strains. The 19plex epitope recognition data collected from multiple analysis of these clinical diagnostic reference panels, expressed as fold change was used to determine the the 95% confidence interval (CI) of epitope recognition. A range of +0.5 fold to -0.5 fold variation in binding averaged across the 19plex anti-HBs mAbs and the HBV strains (range 0.44 fold to 0.58 fold) were identified. Epitope recognition outside of the 95% CI (+/- 0.5 fold change) was identified as significantly different to the wildtype backbone/reference strain. Results of positive fold change (> 0.5 fold) were indicative of gain of epitope recognition (better epitope display), whilst negative fold change (<-0.5 fold) results corresponded to a loss or reduction of epitope binding [279, 348-350].
Figure 2.2A

Figure 2.2B

<table>
<thead>
<tr>
<th>N-term</th>
<th>Loop 1</th>
<th>Loop 2</th>
<th>Combination</th>
<th>Conformation</th>
<th>C-term</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 1</td>
<td>mAbs 5, 6, 10</td>
<td>mAbs 7, 8, 11, 12, 16, 17, 19</td>
<td>mAbs 13, 14, 15</td>
<td>mAbs 9, 18</td>
<td>mAbs 2, 3, 4</td>
</tr>
</tbody>
</table>

*Figure 2.2A shows the interaction sites of the 19 mAb used in the BioPlex analysis as provided by Dr Renae Walsh.*

*Figure 2.2B shows the summary of mAb interaction sites in relation to the ‘a’ determinant.*
Table 2.6

<table>
<thead>
<tr>
<th>mAb</th>
<th>Source</th>
<th>Species</th>
<th>Region &quot;a&quot; determinant</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>N-terminal</td>
<td>aa 101-105</td>
</tr>
<tr>
<td>5</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>Loop 1</td>
<td>aa 121-137</td>
</tr>
<tr>
<td>6</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>Loop 1</td>
<td>aa 121-137</td>
</tr>
<tr>
<td>10</td>
<td>Abbott</td>
<td>Mouse</td>
<td>Loop 1</td>
<td>aa 121-124</td>
</tr>
<tr>
<td>7</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>aa 139-147</td>
</tr>
<tr>
<td>8</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>aa 139-147</td>
</tr>
<tr>
<td>11</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>12</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>16</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>17</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>19</td>
<td>XTL Bio 17</td>
<td>Human Fc</td>
<td>Loop 2</td>
<td>S-linear</td>
</tr>
<tr>
<td>13</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Combination</td>
<td>aa 120-125, 134-157</td>
</tr>
<tr>
<td>14</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Combination</td>
<td>aa 120-125, 134-157</td>
</tr>
<tr>
<td>15</td>
<td>Abbott Diagnostics</td>
<td>Mouse</td>
<td>Combination</td>
<td>aa 120-125, 144-149, 214-219</td>
</tr>
<tr>
<td>2</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>Mouse</td>
<td>C-terminal</td>
<td>aa 175-189</td>
</tr>
<tr>
<td>3</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>Mouse</td>
<td>C-terminal</td>
<td>aa 175-189</td>
</tr>
<tr>
<td>4</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>Mouse</td>
<td>C-terminal</td>
<td>Aa 199-208</td>
</tr>
<tr>
<td>9</td>
<td>Prof H. Thomas*</td>
<td>Mouse</td>
<td>Conformation</td>
<td>conformational</td>
</tr>
<tr>
<td>18</td>
<td>XTL Bio 17</td>
<td>Human Fc</td>
<td>Conformation</td>
<td>conformational</td>
</tr>
</tbody>
</table>

Abbreviation - * Prof Howard Thomas, Imperial College, London, provided these antibodies.
Table 2.6 outlines the mAbs used in the phenotypic analysis of HBsAg on the BioPlex immunoassay platform. The epitopes are described, as per the sources of mAbs.
2.6 BioPlex Analysis of In-Vitro Constructs
The supernatant harvested at Day 5 of transfection was diluted at three different concentrations; 8, 16 and 32IU/well for BioPlex analysis described above.

2.7 Clinical Cohorts For HBsAg Phenotypic Study
BioPlex analysis was performed on serum samples in the following groups, identified by Dr Lilly Yuen and Dr Margaret Littlejohn by the sequencing results from samples sent to VIDRL for assessment of potential vaccine escape mutant. The samples were identified by accessing HBV sequencing database at VIDRL. The sera was accessed under the following clause: “The use of serum samples for assay validation and QC without identifying data is within the terms of reference (remit) of VIDRL as the Victorian State Reference Laboratory, and as such, a formal ethics application is not required.”

This was a descriptive study of HBsAg phenotype, analysed on BioPlex and each sample was normalized to the genotype of the sample plotted individually or co-plotted with the in-vitro constructs for comparison. Four clinical groups were set up and samples were retrieved from -20°C storage at VIDRL.

Clinical Groups
2.7.1A Group A – sG145R
This group constituted 10 serum samples with HBV containing the S-gene variant sG145R, identified by population-based sequencing performed previously by Dr Margaret Littlejohn, Ms Rosalind Edwards and Dr Lilly Yuen. The region sequenced was the S-gene and mutational analysis identified S-gene substitution, genotype and serotype of each sample using ABI program SeqScape as described previously [291]. HBsAg was quantified using Elecysis HBsAg II quantitative assay, as per manufacturer’s instructions [292] and each sample was diluted into three different concentrations: 8IU, 16IU and 32IU/well for BioPlex analysis, outlined above. The phenotypic profile was compared to the profile of in-vitro construct of cloned HBV construct of sG145R, to identify if there were similar patterns of mAb losses or gains in both samples.

2.7.1B Ultra Deep Pyrosequencing
Nine out of 10 samples had ultra deep pyrosequencing (UDPS) performed on GS-FLX platform (Roche) by Dr Julianne Bayliss and Ms Kathy Jackson. This included amplification of DNA extracted previously, using forward and reverse primers that covered amino acid positions 40 – 160 of the S-gene. Amplicon sequence analysis was performed using an in-house bioinformatics pipeline developed by Dr Lilly Yuen
(VIDRL). Substitutions in the S-gene were identified and quantified as relative proportions of HBV variants and reported as percentages (%).
2.7.2 Group B - Claimed Vaccine Escape Variants
The second group of clinical samples comprised of clinical samples of serum with HBV containing S-gene mutations associated with vaccine failure. These included sD144E, sD144A, sP120T and sM133L and samples referred to VIDRL for diagnostic work-up after failing detection of HBsAg at the regional laboratories. These three samples had substitutions within the ‘a’ determinant that included sG145A/sP120Q, sT123S and sT126I. Population-based sequencing and BioPlex analysis was performed as described previously. Each BioPlex analysis of the clinical sample was compared to the in-vitro analysis of the same HBV S-gene variant or to sG145R, to identify patterns of mAb loss.

2.7.3 Group C - Antiviral Resistance Associated Variants
This group comprised of nine samples from HIV/HBV co-infected patients and patients infected with HBV with viral breakthrough. Samples were identified from VIDRL database for patients who had antiviral associated mutations sE164D and sI195M. Data on S-gene mutations was provided by Dr Margaret Littlejohn. HBsAg was quantified for each sample and diluted at 8, 16 and 32IU/mL and analysed on BioPlex. Each sample was compared to the in-vitro HBV construct, with sG145R and presented as a bar graph.

2.7.4A Group D- Mother to Baby Transmission
This group comprised seven mother-baby pairs; enrolled between 2007-2010 for a study by Wiseman et al, to identify virological features associated with HBV transmission in pregnancy at Liverpool Hospital, New South Wales, Australia [247]. These mothers had HBV viral loads of >10^8IU/mL and were HBeAg positive at 30 weeks of gestation. They had declined treatment and comprised the control group for the study. Babies were diagnosed with HBV at 9 months of age and all except one baby received HBV vaccine and HBlg at birth, as per clinical guideline[296]. Viral loads were performed at Liverpool Hospital and HBV sequencing was performed at VIDRL as described[247]. S-gene changes were provided by Dr Margaret Littlejohn.

As described previously, HBsAg was quantified for each sample, followed by BioPlex analysis as described above. UDPS, as described above (see Section 2.7.1B) was performed for six out seven mother-baby pair.
2.7.4B Phylogenetic Analysis

Phylogenetic analysis was carried out by Dr Margaret Littlejohn, using software program MEGA 5. The analysis involved 153 nucleotide sequences with a total of 591 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated next to the branches. The evolutionary distance were computed using the Maximum Composite Likelihood method \[297\] and are in units of the number of base substitutions per site and rate variation among sites was modeled with gamma distribution (shape parameter=4)\[298\].
Chapter 3:
Cloning and Protein Expression

3.1 Background
The small hepatitis B surface antigen (HBsAg) is derived from the translation of the S-gene (Figure 3.1) and exists as a highly conformational protein with multiple immunogenic epitopes. Nucleotide substitutions that confer amino acid changes can alter this complex structure, which may result in reduced epitope binding and potential HBsAg diagnostic escape. Such changes have been attributed to multiple factors. There are naturally occurring different genotypic/serotypic HBV isolates; indeed these were originally designated as different serotypes based on specific antibody reactivity to the ‘a’ determinant. Variants have also been reported, being selected under immune pressure after administration of the HBV vaccine or hepatitis immunoglobulin (HBlg). In addition, naturally occurring surface variants have been described in individuals who have not been immunized or treated with HBlg. Over the last two decades, multiple studies have attempted to characterize these genotypic changes phenotypically using enzyme immunoassay binding assays [187, 299, 300]. More recently, employing four distinct anti-HBs monoclonal antibodies (mAb) to interrogate HBsAg structure using a new platform, Luminex® (Biorad), altered antigenicity by epitope loss within the ‘a’ determinant could be assigned to respective substitutions [301]. However, differentiating the epitope profile of wild-type HBV, commonly claimed vaccine/HBlg escape variants and antiviral therapy-associated mutations affecting the HBsAg, is still lacking, as is the development of a high throughput assay with the ability to map multiple epitopes across HBsAg.

Figure 3.1

Figure 3.1 shows the overlapping polymerase/surface gene and location of the ‘a’ determinant (grey).
Clinical samples from patients with CHB typically contain quasispecies of HBV variants [139, 227]. Direct PCR (population-based) sequencing only identifies those variants present at greater than 20% in the circulating viral population [227, 302]. The minority species of less than 20% on sequence analysis may also comprise multiple variants that can contribute to the data generated from a phenotypic assay. In order to validate, optimize and evaluate the phenotype assay described in this thesis, the HBsAg encoded by various S-gene clones was used to correlate phenotype with genotype. These reference reagents will be used for the next step of evaluating quasispecies differences that may have relevance in context of phenotypic variants, in particular those associated with vaccine escape.

Thus, the overall aim of the experimental work described in this Chapter was to generate a ‘reference’ set of HBsAg expressed from in-vitro constructs of the various HBV genotypes and serotypes as well as commonly claimed VEMs. These reagents would then be used to characterize phenotypic changes by the phenotype assay, which will be explored in more detail in Chapter 4 and 5.

### 3.2 Specific Aims

#### 3.1) To establish wild-type HBsAg gene constructs of genotypes A1, A2, B, C, D, E, F and G, express HBsAg, and then determine the serotypes of the constructs based on the amino acids at codon 122 and 160 using sequence alignments.

#### 3.2) To compare S-gene changes of the HBV constructs at the nucleotide level using pairwise comparison.

#### 3.3) To optimize HBsAg expression and production in a cell culture model for subsequent phenotypic analysis.

#### 3.4) To generate a panel of HBsAg variants commonly associated with vaccine/HBlg escape and antiviral drug resistance and to express and compare HBsAg by phenotypic analysis.

### 3.3 Materials and Method

The S-gene of HBV genotypes A1, A2, B, C, E, F and G was cloned into the prc/CMV vector (see Chapter 2, section 2.2) and sequenced to confirm the genotype. Clustal alignment using the BioEdit software programme was performed to compare changes in the amino acid sequence between the genotypes, and pairwise analysis was carried out to identify
any differences in the 'a' determinant. In this study, the HBV genotype D construct used was previously cloned at VIDRL into a pCI vector, and the nucleotide sequence and deduced amino acid sequence were provided by Dr Nadia Warner at VIDRL.

Plasmids containing the respective eight reference HBV genotype S-gene clones were then transfected into Huh 7 cells. The supernatant was harvested at Day 5 post-transfection, as per protocol, and HBsAg sub-viral particles concentrated after centrifugation through a sucrose cushion as described in Chapter 2, section 2.3.1. The resulting pellet was resuspended in Laemmli buffer and a Western blot assay performed as described in Chapter 2. Monoclonal antibody (mAb) H166 was used to detect HBsAg employing enhanced chemiluminescence (ECL) technology to improve sensitivity (refer to Chapter 2 for details).

3.4 Results
Aim 3.1
3.4.1 Reference Genotype and Serotype Panel
Clustal alignment of the different HBsAg sequences was carried out using the BioEdit software programme. The cloning and sequencing of HBV genotype C4 ayw and J ayw were performed by Dr Margaret Littlejohn from VIDRL. The selection of the genotypes was based on obtaining a representative set of HBV from high prevalence countries. Genotypes A1 (adw) and E (ayw) are the predominant strains in the African subcontinent, B (adw) and C (adr) from Asia, A2 (adw) and D (ayw) from Europe and F (adw) from Central and South America [221]. Genotype A2 (adw) is an important construct for this study, since the current vaccine associated immunity (anti-HBs) is derived from immunization with recombinant HBsAg predominantly from this genotype[6, 303]; this was used as the main comparator. Genotype C4 (ayw), the predominant HBV in the Northern Territory and genotype J (ayw) have a similar S-gene sequence and were also aligned in the clustal to evaluate the amino acid changes with respect to A2 (adw)(see Figure 3.2).
Figure 3.2 shows the clustal alignment of the HBV genotype (A1-G) cloned, as described in Chapter 2. HBV C4 ayw and J ayw were previously cloned onto vectors prc/CMV and pCI respectively.

Figure 3.2 shows the amino acid sequence alignment of S-gene constructs of the HBV genotypes/subtypes from codons 1 - 226. To determine the serotypes of each of the genotypes cloned, the amino acid at position 122 and 160, highlighted in Figure 3.2 by the red box, was assessed. The following serotypes were determined for genotypes A1, A2, B, F and G and this was based on the presence of either amino acid lysine (K) or arginine (R) at position 122 and 160. For the reference genotype C, the serotype was ‘adr’ due to arginine at position 160 (see Figure 1.8 and Figure 3.9. For the remaining genotypes D, C4 and E, the serotype was ‘ayw’ because of the arginine at position 122
and lysine at position 160. Other features noted on the sequence included four extra cysteine (C) residues at position 19, 183, 206 and 220 for genotype F adw and leucine (L) at position 158 instead of phenylalanine and glutamine instead of proline (P) at position 178.

Aim 3.2

3.4.2 Pairwise Analysis

The HBsAg 'a' determinant region, which forms the major neutralization domain region of HBV, is highly conserved with 89% homology when genotypes A, B, C, D, E and F were compared[192]. Phenotypic similarities between the genotypes have not been assessed for the 'a' determinant and to correlate the phenotypic profile of the different genotype cloned, a pairwise analysis was done using the MEGA 4 software programme (see Chapter 2, section 2.2.8).

Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>A1 adw</th>
<th>A2 adw</th>
<th>B adw</th>
<th>C adr</th>
<th>D ayw</th>
<th>E ayw</th>
<th>F adw</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B adw</td>
<td>5%</td>
<td>5.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adr</td>
<td>9%</td>
<td>8.6%</td>
<td>5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D ayw</td>
<td>8.6%</td>
<td>8.6%</td>
<td>6.6%</td>
<td>7.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E ayw</td>
<td>7.8%</td>
<td>8.2%</td>
<td>7.4%</td>
<td>7.8%</td>
<td>6.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F adw</td>
<td>7.8%</td>
<td>9%*</td>
<td>6.1%</td>
<td>9%*</td>
<td>8.2%</td>
<td>6.1%</td>
<td></td>
</tr>
<tr>
<td>G adr</td>
<td>4.1%</td>
<td>6.1%</td>
<td>6.1%</td>
<td>8.6%</td>
<td>7.8%</td>
<td>7%</td>
<td>7%</td>
</tr>
</tbody>
</table>

Comparisons of the nucleotide sequences of 'a' determinant of the constructs genotypes shows the highest divergence between A2 adw and genotype F adw and genotype C adr, marked as *.

The result of this pairwise analysis is shown in Table 3.1 and reveals that the least divergence is between genotype A1 and A2 (2%), consistent with their overall similar genotype/serotype backbone, whilst most differences were greater than 4%. Genotype F adw had the highest divergence when compared to the vaccine derived strain A2 adw (9%), while B adw had the least (5.8%). For genotype F adw, the lowest divergence was with genotype E ayw and B adw; both were 6.1%. Genotype B adw had the least divergent profile (<8%) when compared to other genotypes, whilst genotype E ayw had amongst the highest divergence (8.2%).
3.4.3 Protein Expression

The aim of HBV S-gene cloning was to express adequate amounts of HBsAg from the various genotypes, as well as the variants claimed to be associated with vaccine or HBIG failure, for further phenotypic study. Huh 7 cells have been shown to be an optimal cell line for HBsAg expression [304] and were used in this study. Following transfection, the HBsAg was quantified [292], using a modified HBsAg assay (Roche Diagnostics, Germany) on the Elecsys® instrument (see Figure 3.3) and concentrated by performing ultracentrifugation through a sucrose cushion. Subsequent Western blots were performed, as shown in Figure 3.4.

**Figure 3.3**

![Figure 3.3](image)

*Figure 3.3 shows the HBsAg levels of the cell culture supernatants (HBsAg in IU/ml on the Y-axis and the genotypes on the X-axis).*

Each transfection reaction was repeated 3 times and the concentration of HBsAg was quantified. Mean HBsAg concentration was computed using excel spreadsheet and standard error of mean was calculated, as indicated by the error bars shown in Figure 3.3. The different HBV S-gene constructs had variable levels of HBsAg expression. Genotype A1 possessed the lowest levels (see Figure 3.3), approximately 10 IU/mL and the highest level was seen with genotype D (>120 IU/ml). All isolates except genotype D were cloned in the prc/CMV vector. Genotype D was cloned previously at VIDRL in the vector pCI (see Chapter 2 for details).
Western blotting of HBsAg generated from the genotypes tested revealed both glycosylated (gp27) and un-glycosylated (p24) forms (see Figure 3.4). HBsAg derived from genotypes B and C had roughly equimolar amounts of each form, whilst the remainder appeared to have slightly less of the glycosylated form. Despite concentrating the subviral particles via centrifugation through a sucrose cushion, Western blots of HBsAg from genotype F and A1 showed poor intensity bands, consistent with the quantification data (Figure 3.3).

Aim 3.3

3.4.4 Cell Culture Modifications

The levels of HBsAg expressed from the cloned HBV S-gene showed lower levels of HBsAg titres for genotypes A1 and F. Studies have demonstrated that addition of 1% dimethyl sulfoxide (DMSO) increases both secreted HBV DNA (likely virions) and HBsAg levels in the Hep AD38 cell line [293]. The current protocol at VIDRL uses 10% fetal calf serum (FCS) without DMSO for transient transfections in the Huh 7 cell line. To further optimize the transfection efficiency, variable levels of DMSO and FCS were added to cultures and supernatants evaluated by quantification of HBsAg levels at Day 5 to determine any changes in the expression levels.
Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>No DMSO</th>
<th>2% (vol/vol)</th>
<th>1% (vol/vol)</th>
<th>0.5% (vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM+10% FCS</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>DMEM+5% FCS</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>DMEM+2% FCS</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 3.2 shows the wells (1-12) with the different concentrations of FCS and DMSO. Well 1 represents the current protocol for HBV transfection studies at VIDRL.

Figure 3.5

Figure 3.5 shows the graph of HBsAg, expressed in IU/mL (y-axis), with different concentrations of FCS and DMSO in the wells 1-12 (x-axis). The highest level of HBsAg was observed in well 4 whilst well 10 had the lowest.

Figure 3.5 showed that well 4 (10% FCS and 0.5% DMSO) gave the highest level of expressed HBsAg (18 IU/mL). However, this was only marginally greater compared to the standard protocol (10% FCS and no DMSO), shown in well 1 (16 IU/mL). Addition of DMSO did not greatly increase HBsAg levels when used with the lower concentrations of FCS; in fact for the lower concentrations of FCS the highest levels were found in those cultures not treated with DMSO.

Aim 3.4

3.4.5 Generation of HBV s-gene variants

Since the introduction of the prophylactic hepatitis B vaccine, several S-gene mutations have been identified and/or claimed as vaccine escape mutants (VEM). Of these, sG145R, was the first to be described [12] and was associated with transmission of HBV within household members [237]. Infected persons can become chronically infected with the virus, persisting for at least 10 years. The sG145R variant has been regarded as the prototype VEM [12, 143, 305]. Several studies since then have claimed HBV variants with changes in the ‘a’ determinant around Loop 1, Loop 2 and at position 120 as possible VEMs (see Figure 3.6). Unfortunately, there is no consensus on what constitutes
a VEM and its impact on the vaccination programme[241]. Current and future evaluation of what constitutes a VEM, including a consensus on a definition is critically important.

**Figure 3.6**

*Figure 3.6 shows the 'a' determinant of HBsAg, with Loop 1 and Loop 2. Adapted and modified from [187,251]*

In addition to the variants associated with HBlg and vaccination and naturally occurring S-gene variants, HBsAg changes can be indirectly brought about by anti-viral therapy. The S-gene, encoding HBsAg, is contained with the P-gene in an alternate reading frame. This gene overlap is important because the mutations encoding the reverse transcriptase substitutions associated with lamivudine resistance (such as rtV173L+rtL180M+rtM204V), can result in concomitant nucleotide changes encoding significant changes in HBsAg (sE164D+sI195M). These have been shown to substantially reduce anti-HBs (vaccine-associated) binding *in vitro* and even evasion of vaccine induced immunity in chimpanzee models of HBV infection.[251]

To characterise the features of a number of these variants, the reference HBV wild-type construct of genotype A2 *adw* (vaccine strain) was modified using site-directed mutagenesis as described in Chapter 2, subsection 2.3. A number of variants, selected from a review of international literature on the commonly claimed VEMs, were constructed (Table 3.3). The sequences of these variants were confirmed as outlined in Chapter 2, and Clustal Alignment performed using BioEdit (Figure 3.7 and Figure 3.8).
Confirmed variants were transfected into Huh 7 cells, with supernatant collected at Day 5 and any HBsAg quantified (Table 3.3).

HBV variants associated with vaccine or HBlg escape, or escape as a consequence of antiviral therapy, are not restricted or limited to a particular genotype. For this study all the variants were generated on genotype A2 adw backbone in order to eliminate backbone related phenotypic changes and to allow comparison of the direct immunity derived from the existing vaccine.

**Table 3.3**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A2 adw</th>
<th>S-gene change</th>
<th>HBsAg IU/mL</th>
<th>Selection Pressure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 adw</td>
<td>Wild-type</td>
<td>90</td>
<td>Nil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>E164D§</td>
<td>238</td>
<td>Antiviral drugs</td>
<td>[187]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>G145R*</td>
<td>29</td>
<td>Vaccine/HBlg</td>
<td>[12, 144, 263, 271]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>G145A*</td>
<td>128</td>
<td>Vaccine/HBlg</td>
<td>[233, 271]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>D144A*</td>
<td>43</td>
<td>Predominantly HBlg/Vaccine</td>
<td>[229]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>W196S§</td>
<td>189</td>
<td>Antiviral drugs</td>
<td>[187]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>D144E/G145R*</td>
<td>42</td>
<td>HBlg</td>
<td>[248]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>S132A*</td>
<td>232</td>
<td>Vaccine/HBlg</td>
<td>[239]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>P120T*</td>
<td>205</td>
<td>Vaccine/HBlg</td>
<td>[263]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>P120L*</td>
<td>62</td>
<td>Vaccine/HBlg</td>
<td>[258, 263]a</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>N146A*</td>
<td>66</td>
<td>Vaccine</td>
<td>[283]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>T126N*</td>
<td>61</td>
<td>Vaccine/HBlg</td>
<td>[271]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>M133L*</td>
<td>68</td>
<td>Vaccine/HBlg</td>
<td>[228]</td>
<td></td>
</tr>
<tr>
<td>A2 adw</td>
<td>sD144E*</td>
<td>56</td>
<td>Predominantly HBlg/Vaccine</td>
<td>[248]</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.3 shows the genotype backbone and the levels of HBsAg. Symbol * denotes selection pressure of vaccine and/or HBlg and § denotes antiviral associated changes in the S-gene. Note all variants are on HBV genotype A2 adw backbone.*
therapy, however, the number of cases reported or in

Figure 3.7

Wild type genotype A2 adw sequence of the S-gene corresponding to codons 99-198, aligned with mutagenized HBV variants commonly associated with vaccine/HBIg escape. The second sequence shows sG145R, a commonly claimed vaccine escape mutation and for all the sequences.

Figure 3.8

Wild type genotype A2 adw sequence of the ‘a’ determinant (99-198) corresponding to codons 99-198, aligned with further HBV variants typically claimed to be associated with vaccine/HBIg escape. The last two variants, sE164D, and I196S are associated with the emergence of resistance to antiviral therapy and the changes seen are outside the ‘a’ determinant. The second sequence is that of sG145R, a commonly claimed vaccine escape mutation and for all the sequences. The other sequences are those seen with vaccine/HBIg therapy, however, the number of cases reported or in-vitro studies are limited.
3.5 Discussion

Ten HBV genotypes, distinguished by nucleotide divergence of greater than 8%, have been shown to have a distinct geographical distribution. Each genotype can be further classified into sub-genotype, based on nucleotide difference of greater than 4% but less than 8%. In this study, seven genotypes (A-G), as well as two subtypes of genotype A (A1 and A2), representing HBV from various parts of the world, had their S-genes cloned into expression vectors. Since the current vaccine is derived from genotype A2 adw, this study incorporated cloning and expression of this strain as a reference point and direct comparator. Prior to the availability of sequencing technology, HBV was classified based on serotyping with the common “a” determinant and mutually exclusive sub-antigenic determinants w and r, and d and y, respectively, leading to four major serotypes adr, adw, ayr and ayw, as shown in Figure 3.9[189].

**Figure 3.9**

*Figure 3.9 shows the amino acid at codon 122 and 160 of the S-gene, with the derivation of the 4 major serotypes adw, adr, ayw and ayr [189].*

Whilst genotypes have been associated with disease outcome and response to treatment[306], the earlier serotyping technique was predominantly used to map the worldwide distribution of HBsAg subtypes and was not used to define any particular pathological feature of CHB infection[221, 307, 308]. As sequence data of HBV genomes accumulated, the relationship between HBV genotype and serotype was determined. Genotypes A and B expresses serotype adw, genotype C expresses adw, adr and ayr whilst D and E express serotype ayw, and genotype F and G express adw [221]. The S-
gene sequence for genotype C4, previously described as a divergent strain amongst other subgenotype C, displays the serotype ayw. This strain of HBV has been found only in Indigenous Australians and molecular analysis has shown that whilst the other genes are consistent with genotype C, the S-gene sequence is closely related to genotype J ayw [209], indicating a likely recombination event. Interestingly, only one patient with genotype J has been diagnosed with CHB infection, a Japanese male soldier who presumably acquired HBV in Borneo during World War II, and who subsequently developed HCC [221](Prof Mizokami Personal communication).

The study of HBsAg has been limited by the lack of a protein crystal structure, hence the variation in deduced amino acid sequence between the different genotypes has been used to ascertain and interpret possible structural or conformational differences. In the study described in this section, a representative range of HBV genotype S-genes was constructed and the sequences aligned to evaluate changes in the surface protein determinant. As described in previous studies, genotype F adw is characterized by four extra cysteines in HBsAg; this raft of cysteines at positions 19, 183, 206 and 220 is presumably crucial in establishing cross-linking disulphide bonds, making for what would be a different phenotype when compared to other HBV genotypes (see Figure 3.2 and 3.6).

A comparison of the 226 amino acid sequence of the S-gene of HBV genotypes A-F was performed by Norder et al and compared to other regions. The S-gene sequence was found to be the most conserved with 82% conservation across the different genotypes[192]. In Table 3.1 a pairwise analysis of the nucleotide sequence of the ‘a’ determinant was performed. The ‘a’ determinant is the major neutralizing domain of HBV, and part of the S-gene from codon 99 – 170. The pairwise analysis revealed the highest homology was between genotype A1 and A2 adw as expected, whilst genotype F had the most sequence variation (9%) compared to genotype A2 adw.

Genotype E ayw, a predominant strain in West Africa has been shown to have an overall low genetic diversity compared to other genotypes and thus has no subtype. This is believed to be due to its possible recent introduction in Africa and subsequent transmission to humans [214, 216]. Sequence analysis of the S-gene revealed serine instead of threonine at 140, a key part of Loop 2 of the ‘a’ determinant and at position 127 a threonine was identified instead of proline, changes also seen in genotype F (see Figure 3.2). These changes may facilitate spread and transmission. Proline maintains the tertiary structure of protein by forming links and generating perpendicular type turns.
Thus a change in genotype E ayw of proline to threonine would potentially disrupt the 'a' determinant structure and impair anti-HBs binding, suggesting lack of neutralization of HBV.

Studies of HBV have been hampered by the lack of availability of a suitable animal model, which can be naturally infected. In-vitro HBV study models have proved useful, increasing our knowledge of HBV replication, infectivity and anti-viral susceptibility. The Huh 7 cell line was initially isolated from a Japanese patient with HCC in 1982 and has subsequently been established as a major cell culture model for HBV replication. Despite some limitations of transient transfection for studies of replication and secretion [309], HBV genotypes successfully cloned (some had been previously cloned at VIDRL, including C4 ayw, D ayw and J ayw) were shown to express HBsAg in both glycosylated and un-glycosylated forms. However, for the phenotypic studies planned, adequate quantities of HBsAg were required and in order to increase HBsAg levels, modifications to the cell culture system were investigated. The lower values of HBsAg observed (see Figure 3.3) for genotypes A1 and F are unclear. Possible explanations include differences in the amino acid structure leading to poor secretion or post-translational handling of HBsAg in Huh 7 cell culture system. There are no published data on the levels of HBsAg in patients with CHB infection with genotypes A1 and F and given the discrepancies observed in this study, clinical studies could be undertaken to evaluate this. Furthermore, different cell culture systems could be used to compare the levels of HBsAg secretion for the different HBV genotypes.

Studies describing the incorporation of low concentrations of DMSO into cell culture medium have shown an enhancement of virus yield [310]. HBV replication has also shown to be increased by corticosteroids and DMSO in a transfected cell clone of HepG2 cells [293] and increased susceptibility of infection by HBV in HepaRG hepatoma-derived cell line [311]. Unfortunately, modifying the level of FCS with various concentrations of DMSO in this study showed no substantial increase in HBsAg secretion compared to the standard protocol.

Emergence of S-gene variants has been described subsequent to the incorporation of hepatitis B vaccine into the Extended Programme of Immunization (EPI) or the equivalent childhood immunisation programme[240]. Similarly, variants have arisen after additional administration of HBIg to prevent mother to baby transmission or infection post liver transplant [281]. These changes are typically in the ‘a’ determinant and have been associated with reduced anti-HBs binding to both polyclonal and
monoclonal antibodies in commercial assays for HBsAg, occasionally leading to misdiagnosis[266]. In this Chapter, variants such as M133L, sT126N, sG145R/A, sD144E/A, sP120T/L and the combination of mutations sD144E/G145R were examined, all of which have been associated with diagnostic escape and, in some cases, with virus transmission. In this study HBsAg titres of the variants were determined using the HBsAg electrochemiluminescent (ECL) immunoassay on the Roche Elecsys® platform, and these variants were detected and quantified; suggesting the sensitivity of diagnostics assays depends largely on anti-HBs used [292].

Whilst most variants constructed for this study (see Table 3.3) are associated with vaccine/HBIg escape or indirect anti-viral drug-related vaccine escape, variants such as N146S, are rarely associated with immune escape. Codon sN146 is the glycosylation site of the surface protein, which normally occurs when the S-region is exposed to endoplasmic reticulum (ER)/Golgi compartment, and substitution at this region is associated with impaired HBsAg secretion [272, 312] In this study, there was adequate levels of HBsAg expression of sN146S after transfecting it in Huh 7 cells, (66 IU/mL), however currently HBsAg phenotypic profile of this variant is unknown.

In conclusion, S-gene clones of seven HBV genotypes A-G, and two sub-genotypes were successfully cloned and with the exception of genotype A1 (based on immunoblot), adequate HBsAg was expressed. Western blots of concentrated HBsAg showed two bands, representing the glycosylated and un-glycosylated protein forms. To assess the phenotypic profile of commonly claimed HBV S-gene variants, site-directed mutagenesis was performed on HBV genotype A2 adw, and these variants successfully expressed HBsAg. Generation of HBsAg from the different HBV genotypes and HBV S-gene variants described in this Chapter will enable characterization of their phenotypic profiles using a novel immunoassay platform (Chapter 4 and 5).
Chapter 4:
BioPlex Analysis In-Vitro Constructs of HBsAg

4.1 Introduction

The first indication of the viral nature of hepatitis B came about in 1965 as the result of a chance finding by Baruch Blumberg and colleagues [313]. Blumberg's laboratory had a major interest in human immunological variation. Using an agar diffusion method to test the antigenicity of serum protein variants from geographically diverse populations, an atypical precipitin line was observed between the serum of an Australian aboriginal person and that of a hemophiliac. This “Australia antigen” was later shown to be the excess surface protein of HBV and became a key marker for defining acute and chronic infection. The case definition of chronic hepatitis B is ‘HBsAg positive for at least 6 months’ whilst for acute hepatitis B, it is transient HBs antigenemia [23, 314]. The HBsAg is found predominantly in blood as spherical and filamentous 22nm sub-viral particles. A sub-unit vaccine based on the 22nm forms of HBsAg was approved by the Food and Drug Administration in 1981, and it is now the recombinant form of the HBsAg that is used in current vaccination programmes [303]. Recent developments in the treatment of chronic hepatitis B have led to interest in quantitative serology for HBsAg, predicting treatment response [315].

Despite its multiple applications in the diagnostic landscape, disease monitoring and in prevention, the fine molecular and antigenic configuration of HBsAg remains unclear because of a lack of a crystal structure. Impediments may be due to its strong membrane association (at least four putative trans-membrane domains) as well the structure contains an inordinate number of cysteine and proline residues between codon 99-170, also known as the ‘a’ determinant (see Figure 4.1)[264]. Likewise, the phenotypic properties associated with HBsAg, such as diagnostic escape or even vaccine escape of HBV, have been derived indirectly through immunoassay studies using monoclonal antibodies (mAb) directed against one of the many epitopes identified so far within the ‘a’ determinant [186].

As discussed in Chapter 1, the ‘a’ determinant is the dominant antigenic or immunogenic region of HBsAg and represents the major neutralisation domain of HBV. It spans from amino acid 99-170 of the S protein and is a hydrophilic, conformationally complex structure (see Figure 4.1). Enzyme immunoassays (EIA) have used a battery of mAbs targeting this region to identify and map particular epitopes, and filamentous phage peptide libraries have been generated to confirm its exceptional conformational structure [186]. It has been established that HBsAg has a number of putative trans-
membrane helices and a major hydrophilic region (MHR) that is exposed on the external surface, which is responsible for eliciting the protective anti-HBs response [186, 280].

**Figure 4.1**

Figure 4.1 illustrates the sequence of S-gene encoding amino acids 99-226, showing Loops 1 and 2 of the ‘a’ determinant. The assumed disulphide bonds between 107-138, 121-124, 137-149 and 139-147 are shown. Marked in red is codon 145, and when glycine (G) is substituted for arginine (R), this is the canonical vaccine escape mutant (VEM)[316].

**Overview**

The hepatitis B vaccine consists of recombinant HBsAg, synthesized in yeast and typically derived from genotype A2 adw-2 genotype/serotype. Since the introduction of the vaccination programme for HBV, emergence of possible ‘escape’ variants has been widely reported [274]. The prototype variant that exhibits the key features of vaccine escape, which is defined by its ability to evade vaccine induced immunity and cause infection[14], is the sG145R variant (see Figure 4.1). Since the first report of this vaccine escape mutant (VEM) from Italy [12], this virus variant has been isolated from around the world and linked to and identified from persons who have failed HBV vaccination[183, 208, 237, 239, 277].

Whilst sG145R was initially identified in Italy in a child born to a mother with chronic hepatitis B, other HBV variants with changes within the ‘a’ determinant have been detected and associated with vaccine failure (see Table 4.1, in particular sD144, sP120, sN146, sM133, sS132 and sT126) and therefore claimed to be or classified as VEM [254, 259, 263, 317, 318]. However, no consensus exists to verify such claims or classification[241]. There is a clear need for the development of such a statement or
consensus, based on strong scientific evidence of vaccine escape [14]. These features could include a lack of neutralization of the virus by vaccine associated anti-HBs in an *in-vitro* cell culture infection model or an equivalent study *in-vivo* to block super-infected immunized animals, such as the chimpanzee [251]. Other than the immunological pressure for selection of S-gene variants associated with vaccination and/or HB Ig usage (see Chapter 1), antiviral drug associated changes in the HBsAg due to the overlapping reading frames of the pol/envelope regions, can also lead to changes in the surface protein (see Table 4.2). Since these variants have public health implications, common antiviral associated drug resistant HBV variants have also been investigated and shown to have the potential to breakthrough vaccine derived anti-HBs [251]. Thus, antiviral drug associated variants such as sE164D and sW196S should also be included in validation studies of what constitutes a VEM. This study has 2 variants sD144A and sD144E, both associated with HB Ig therapy and used as negative control for VEM (see Table 4.1); the phenotypic profile of these were compared to the canonical VEM sG145R.

### Table 4.1

<table>
<thead>
<tr>
<th>S-gene variants associated with vaccine escape</th>
<th>Selection pressure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sG145R</td>
<td>Vaccine/HB Ig Escape</td>
<td>[229, 263, 271, 319]</td>
</tr>
<tr>
<td>sG145A</td>
<td>Vaccine/HB Ig Escape</td>
<td>[233, 271]</td>
</tr>
<tr>
<td>sD144A</td>
<td>HB Ig Escape (predominantly)/Vaccine escape</td>
<td>[229, 263, 320]</td>
</tr>
<tr>
<td>sD144E</td>
<td>HB Ig Escape (predominantly)/Vaccine escape</td>
<td>[34, 248]</td>
</tr>
<tr>
<td>sD144E/G145R</td>
<td>Vaccine/HB Ig Escape</td>
<td>[248]</td>
</tr>
<tr>
<td>sP120T</td>
<td>Vaccine/HB Ig Escape</td>
<td>[34, 263]</td>
</tr>
<tr>
<td>sP120L</td>
<td>Vaccine/HB Ig Escape</td>
<td>[258, 263]</td>
</tr>
<tr>
<td>sN146A</td>
<td>Vaccine</td>
<td>[283]</td>
</tr>
<tr>
<td>sM133L</td>
<td>Vaccine/HB Ig Escape</td>
<td>[228]</td>
</tr>
<tr>
<td>sS132A</td>
<td>Vaccine/HB Ig Escape</td>
<td>[239]</td>
</tr>
<tr>
<td>sT126N</td>
<td>Vaccine/HB Ig Escape</td>
<td>[271]</td>
</tr>
<tr>
<td>sE164D§</td>
<td>Antiviral Escape</td>
<td>[187, 263]</td>
</tr>
<tr>
<td>sW196S§</td>
<td>Antiviral Escape</td>
<td>[187]</td>
</tr>
</tbody>
</table>

Table 4.1 lists the common HBV variants associated with HB Ig/vaccine escape. Variants marked with ‘§’ are those associated with antiviral therapy where mutations in the polymerase impact on the overlapping surface gene.
Table 4.2, provided by Prof Stephen Locarnini, modified from [136] shows the changes in particular nucleotides in the polymerase (leading to anti-viral resistance mutations) causing concomitant changes to amino acids in HBsAg, associated with particular antiviral agents.
To classify HBsAg and its variants, many studies have employed EIAs to map particular epitopes of the ‘a’ determinant and these have included evaluation of potential polymorphic differences between the genotypes/serotypes [321] as well as various specific mutations including vaccine/HB1g escape and antiviral drug resistance associated mutations [144, 299]. Most of these studies have used anti-HBs from vaccinee sera, or in-vitro mAbs made for diagnostic and therapeutic applications or research uses for mapping the ‘a’ determinant [187, 322]. All of these approaches have been limited by the number of samples or epitopes that can be analysed. Whilst EIAs are cheap and easy to perform, they are generally used to detect one antigen at a time and for a complex region such as the ‘a’ determinant of HBsAg where multiple epitopes can be affected by a single substitution, a comprehensive high-throughput immunoassay with the ability to interrogate multiple epitopes simultaneously would offer a significant advantage.

With the advancement in diagnostic applications, multiplex platforms such as Luminex® and BioPlex, have been successfully used to simultaneously detect and quantify chemokines and cytokines in inflammatory conditions and also to detect different viruses associated with outbreaks such as gastroenteritis and influenza [323, 324]. These immunoassays use capture antibody-coupled beads, incubated in an analyte and subsequently coupled with a capture antibody with phycoerythrin (PE) and then sorted by dual laser technology [325].

Recently, Ijaz et al used the Luminex® platform to study HBsAg. They performed epitope mapping with four mAbs to phenotype HBsAg from patients’ sera [301]. They demonstrated that loss of mAb binding coincided with the site of mutation in the corresponding mapped epitopes, however, these changes in mAb binding became unpredictable if more than one substitution was present. In that study, the mAbs were specific for only a limited number of ‘a’ determinant epitopes and baseline differences in mAb binding for different HBV genotypes were not examined.
4.2 Aims

4.1) To define epitope binding profiles and potential changes of HBsAg derived from a range of clones representing different HBV genotypes and compare those to the vaccine A2 adw-2 HBsAg.

4.2) To define epitope binding profiles and potential changes of HBsAg associated with HBV serotypes adw and ayw in the setting of the major genotypes of HBV, then compare these to adw-2 of genotype A2.

4.3) To define the epitope binding profile and changes associated with vaccine escape (sG145R) /HB Ig escape (sD144E, D144A) and antiviral drug resistance-associated variants (sE164D) on the HBsAg 'a' determinant and compare these changes to the classical vaccine escape variant sG145R and reference A2 adw-2 profile.

4.3 Methods, Materials and Analysis

a) BioPlex Multiplex Platform

The HBsAg multiplex immunoassay is based on individually identifiable fluorescently-labelled beads conjugated to a panel of 19 mAbs directed against epitopes in the 'a' determinant (see Figure 4.1). The assay was principally designed by Dr Renae Walsh at VIDRL. Following incubation of HBsAg-containing samples and the multiplex anti-HBs mAb panel, the interaction of antigen and antibody is measured on the BioPlex platform and the output reported in relative fluorescent units (RFU) intensity. The RFU collected for each mAb across 19 epitopes of HBsAg, as outlined in Table 4.3, was processed by Dr Renae Walsh whilst Dr Lilly Yuen (VIDRL) designed a high-throughput bioinformatics pipeline capable of analyzing the large amount of raw data in the bioinformatics programme[295]. This established an extensive data panel of wild-type reference HBV strains from the major genotypes. The major steps involving the bioinformatics data process included correction of potential inter-assay variation, selection of optimum dilution of the supernatant (8, 16 or 32 IU/well) based on fluorescent intensity within the assays dynamic range and finally normalization to the genetic backbone of the relevant HBV genotype. Output data was log₂ transformed and then expressed as fold-change across the 19 mAbs normalized to the relevant reference genetic backbone, with a ±0.5 fold difference within the 95% confidence interval (CI) of this immunoassay, denoting significant variation from the reference genotype. A more detailed description
of this BioPlex methodology, including bioinformatics analysis, can be found in Chapter 2.

Table 4.3

<table>
<thead>
<tr>
<th>mAb</th>
<th>Source</th>
<th>Region &quot;a&quot; determinant</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prof Howard Thomas*</td>
<td>N-terminal</td>
<td>aa 101-105</td>
</tr>
<tr>
<td>5</td>
<td>Prof Howard Thomas*</td>
<td>Loop 1</td>
<td>aa 121-137</td>
</tr>
<tr>
<td>6</td>
<td>Prof Howard Thomas*</td>
<td>Loop 1</td>
<td>aa 121-137</td>
</tr>
<tr>
<td>10</td>
<td>Abbott Diagnostics</td>
<td>Loop 1</td>
<td>aa 121 - 124</td>
</tr>
<tr>
<td>7</td>
<td>Prof Howard Thomas*</td>
<td>Loop 2</td>
<td>aa 139-147</td>
</tr>
<tr>
<td>8</td>
<td>Prof Howard Thomas*</td>
<td>Loop 2</td>
<td>aa 139-147</td>
</tr>
<tr>
<td>11</td>
<td>Abbott Diagnostics</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>12</td>
<td>Abbott Diagnostics</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>16</td>
<td>Abbott Diagnostics</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope</td>
</tr>
<tr>
<td>17</td>
<td>Abbott Diagnostics</td>
<td>Loop 2</td>
<td>Loop 2: unspecified epitope ((sG145R))</td>
</tr>
<tr>
<td>19</td>
<td>XTL-19</td>
<td>Loop 2</td>
<td>S - linear</td>
</tr>
<tr>
<td>13</td>
<td>Abbott Diagnostics</td>
<td>Loop 1 &amp; 2</td>
<td>aa 120-125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aa 134-157</td>
</tr>
<tr>
<td>14</td>
<td>Abbott Diagnostics</td>
<td>Loop 1 &amp; 2</td>
<td>aa 120-125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aa 134-157</td>
</tr>
<tr>
<td>15</td>
<td>Abbott Diagnostics</td>
<td>Loop 1 &amp; 2</td>
<td>aa 120-125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aa 144-149</td>
</tr>
<tr>
<td>2</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>C-terminal</td>
<td>aa 214-219</td>
</tr>
<tr>
<td>3</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>C-terminal</td>
<td>aa 175-189</td>
</tr>
<tr>
<td>4</td>
<td>bioMerieux Clinical Diagnostic</td>
<td>C-terminal</td>
<td>aa 199-208</td>
</tr>
<tr>
<td>9</td>
<td>Prof Howard Thomas*</td>
<td>Conformation</td>
<td>conformational</td>
</tr>
<tr>
<td>18</td>
<td>XTL-17</td>
<td>Conformation</td>
<td>conformational</td>
</tr>
</tbody>
</table>

Table 4.3 shows the mAb number, the location of epitope of HBsAg to which it is directed and the sources of mAbs.

* These mAbs were kindly provided by Prof Howard Thomas, Imperial College, London, and were made when he was at Royal Free Hospital.
**Experimental Design**

The experimental approach was based on taking the recombinant HBsAg preparation from the different HBV variants, described in Chapter 3 and testing the cell culture supernatant fluids on the BioPlex platform. This requires transfecting the HBsAg expressing clones, described in Chapter 2, section 2.2.9, into the Huh 7 cell culture line, harvesting the supernatant and analysing the epitope profile at three different concentrations, 8, 16 and 32 IU/well on the BioPlex platform as described above.

**Data Presentation**

The following Figures in this data set are arranged in a similar format which typically shows for each isolate or variant the multiple amino acid sequence (clustal) alignment (codon 99 – 226) as Panel 1, then in Panel 2 the BioPlex profiles, whilst Panel 3 shows the location of the mAb used for this study and the site at which it is thought to interact with HBsAg.

More specifically Panel 2 shows the BioPlex results for each sample and is presented as a graph with the particular mAb antibodies on the x-axis and the log difference compared to the reference genotype, which in this Chapter is of genotype A2 serotype adw-2, on the y-axis. Significant ‘shape changes’, as described above and in detail in Chapter 2, have been marked by red lines, and values above (gain in mAb binding) or below (reduction in mAb binding) with 0.5 fold difference (95% CI) defined as a significant change. This assay was validated for 0.5 log change for consistency across all analysis, including the clinical samples.

**Statistical Evaluation**

Statistical evaluation of data presented here is problematic. These are correlated data points and are not independently associated, so a standard statistical analysis was not performed. Further work would be needed to perform a formal statistical evaluation, which would be complex in nature and require substantial input from an expert in the field.
4.4 Results

4.4.1 A) Control testing
The first analysis, shown in Figure 4.2A shows the normalized values of the BioPlex profile for genotype A2 _adw_ that was then used as a reference for comparison of the other HBV genotypes and serotypes. Values across the 19 mAbs have been normalized to 0, as shown in Figure 4.2A, Panel 2.

4.4.1B) Genotype/Serotype Profile
The full genotype/serotype-testing result profiles are shown in Table 4.4. In the first analysis (Figure 4.2B), genotype B1 and genotype G were analysed because both have the same serotype (_adw_). The graph (see Figure 4.2B, Panel 2) is normalized to the reference A2 _adw_. Panel 3 is the reference model of the ‘a’ determinant with the highlighted interaction site of the particular mAb (1-19) The only finding of note in this analysis is a gain in the reactivity of mAb 8 (1/19 mAb change, see Table 4.4), that interacts at Loop 2 as shown in Panel 3 (>0.5 fold change). Panel 1 shows the alignment of the sequences compared to A2 _adw_ and both genotypes B1 _adw_ and G _adw_, has a serine (S) instead of tyrosine (Y) at position 114 and alanine (A) instead of valine (V) at position 159, denoted by the blue arrow. It is worth noting that the pairwise analysis showed a difference of 5.8% between A2 _adw_ and B _adw_ and 6.1% between A2 _adw_ and G _adw_ as shown in Chapter 3 Table 3.1.

Figure 4.3 Panel 2 shows the BioPlex profile of genotype C2 _adr_ and C4 _ayw_ 3, and the plots are normalized to A2 _adw_, with Panel 1 showing the clustal amino acid alignment and Panel 3 showing the mAb binding sites. The BioPlex profile shows more changes for genotype C2 _adr_ across all the epitopes compared to the C4 _ayw_. Despite both of the samples sharing the same genotype, the only change common to these two samples is the gain of mAb 8 reactivity acting on Loop 2 (see Panel 2 and 3). Interestingly, whilst both isolates have significant changes for mAb 7 (Loop 2) (red box), C4 _ayw_ shows a loss of epitope binding, whilst C2 _adr_ shows a gain. Other changes for C2 _adr_ include gains with mAb 1 (N-terminus), and mAb 12 (Loop 2). The clustal alignment in Figure 4.2, Panel 1 shows amino acid threonine (T) for C2 _adr_ instead of S (serine) at position 113 (blue arrow), whilst genotype C4 has T instead of proline (P) at position 127 and both C2 and C4 have isoleucine (I) at 126 instead of threonine, a substitution associated with antigenic structural change [268]. For genotype C2 _adr_, codon 122 has lysine (K) whilst C4 _ayw-3_ has arginine (R) at the same codon. This last change is part of what defines _ad_ from _ay_ serotype [207] [209].
In the third analysis, Figure 4.4, genotype J ayw3 was compared to C4 ayw. Panel 2 of this figure shows the BioPlex profile of these two genotypes, where similar changes across the ‘a’ determinant and the C-terminal domains are seen for both genotypes, when the graph is normalized to A2 adw. The only difference in BioPlex profile between these two genotypes was found at mAb 12 in Loop 2, where genotype J shows a significant gain (red arrow), not seen with genotype C4 ayw (orange box). These two genotypes share the same serotype and have only a very small difference (3.57%) in their S-gene sequence [209]. In the clustal alignment, Figure 4.4, Panel 1, genotype J ayw has an A instead of a V at position 177 and 183 (double red arrow) and both genotypes have an R instead of S at position 210. However, it is unclear whether these downstream changes from the ‘a’ determinant cause the changes in mAb binding that result in the significant epitope shift read-outs.

Figure 4.5, Panel 2 shows genotype D ayw on a graph normalised to A2 adw-2. In this analysis, there is a gain with mAb 8, also seen with other genotypes and loss of mAb 6 (Loop 1) and 7 (Loop 2) seen with genotypes C4 ayw, marked with blue arrows (Figure 4.4 Panel 2). The loss with mAb 11, a Loop 2 mAb (see Panel 3), is unique to genotype D ayw and E ayw (discussed below) and not seen with other genotypes analysed in this study. Panel 1 shows the clustal alignment, for genotype D ayw. At position 127 there is a T instead of a P (A2 adw).

Figure 4.6 Panel 2 shows the BioPlex profile for genotype C, D, E and J, all serotype ayw. The profiles are normalized to A2 adw. Overall, there are five changes across the ‘a’ determinant for genotype E ayw and most changes are around the Loop 2 mAbs. These include loss of 6, 7 and 11 and gain in binding for mAb 8, as indicated in Panel 3. The gain in mAb 8 reactivity is similar to that observed with all the other genotypes in the BioPlex profile. However, the gain with mAb 17 (red box) has only been observed with genotype E ayw in this analysis. Changes seen for serotype ayw for these genotypes were loss of binding for mAb 7 (Loop 2) and 6 (Loop 1) (indicated by black arrows), as seen in Panel 3. These may reflect serotype related changes.

Figure 4.7, Panel 2 shows the BioPlex profile of genotype F adw, normalized to A2 adw. In total, there are 13 changes in the mAb set across all domains (see Table 4.4). Genotype F is the most divergent genotype in the wild type HBV genotype analysis. Loss of reactivity was seen for mAbs 6 (Loop 1), 11 (Loop 2) and 3 (C-terminal). Gain of epitope binding was observed with mAb 1 (C terminal), 5 (Loop 1), 16 and 17 (Loop 2), 13 (combination mAbs), 2 (C-terminal) and 18 (conformation dependent mAb). This

79
difference in the BioPlex profile was consistent with the pairwise analysis (see Chapter 3), where the difference between A2 adw and F adw was 9%, the highest found when comparing all genotypes phylogenetically to A2 adw.

Figure 4.7 also includes the BioPlex profile for genotype G adw, B adw and F adw normalized A2 adw. All of the constructs have serotype adw and interestingly, unlike serotype ayw, there was no specific mAb change associated with this, presumably due to the graph being normalized to adw serotype. However, a gain in reactivity with mAb 17 was noted for genotype F adw (red box), not exhibited by either G adw or B adw, and the only other genotype that demonstrated this change was E ayw.

In summary (Table 4.4), HBsAg from wild type HBV but representing eight different genotypes and the two different serotypes (ad versus ay) typically showed a gain for mAb 8, a Loop 2 mAb for all the genotypes, when compared to A2 adw, whilst HBV isolates expressing serotype ayw were shown to result in the loss of mAb 6 (Loop 1) and mAb 7 (Loop 2). The pattern of a gain of epitope for mAb 17 and loss of mAb 6 was seen for genotype E ayw and F adw with both genotypes showing the loss of P at codon 127 and a substitution at 140 to serine.

In this study, there were four different genotypes (D, E, C4 and J) with serotype ayw, and when compared to A2 adw, loss mAb 7 binding was associated with this serotype, whilst the gain shown with mAb 8 was seen in all the other genotypes and was not specific for the serotypic changes examined. The other serotype examined in this study was adw, however, no single mAb change was associated with adw serotype that did not share a change with another serotype. Of the 19 mAb profiles studied, mAb 10 (Loop 1), mAb 19 (Loop 2), mAb 9 (conformation dependent), mAb 15 (combination of Loop 1 and 2) and mAb 9 (conformation) was unaltered for all the genotypes or serotypes studied (see Table 4.4).
Figure 4.2A
Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

Normalized values of genotype A2 adw. All the mAb values have been set to 0, to allow for subsequent comparisons.

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.2B

Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

BioPlex HBsAg profile derived from in-vitro Genotype B adw and G adw. The epitope recognition profiles have been normalized to the A2 adw backbone.

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.3

Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

BioPlex HBsAg derived from in-vitro Genotype C2 adr and C4 ayw. The epitope recognition profiles have been normalized to the A2 adw backbone.

Panel 3

Sites of monoclonal antibody interaction in the ‘a’ determinant and C-terminus
Figure 4.4

Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

BioPlex HBsAg derived from in-vitro Genotype C4 and J ayw. The epitope recognition profiles have been normalized to the A2 adw backbone.

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.5
Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

BioPlex HBsAg derived from in-vitro Genotype D ayw. The epitope recognition profiles have been normalized to the A2 adw backbone.

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.6
Panel 1

Clustal amino acid alignment of all genotypes

Panel 2

BioPlex HBsAg derived from in-vitro Genotype D, E, J and C4 ayw. The epitope recognition profiles have been normalized to the A2 adw backbone.

Panel 3

Sites of monoclonal antibody interaction in the ‘a’ determinant and C-terminus
**Figure 4.7**

**Panel 1**

Clustal amino acid alignment of all genotypes

**Panel 2**

BioPlex HBsAg derived from in-vitro with genotype B, F and G, serotype adw. The epitope recognition profiles have been normalized to the A2 adw backbone.

**Panel 3**

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Summary of BioPlex Findings
Table 4.4

<table>
<thead>
<tr>
<th>Site of mAb</th>
<th>B adw</th>
<th>C adr</th>
<th>C4 ayw</th>
<th>D ayw</th>
<th>E ayw</th>
<th>F adw</th>
<th>G adw</th>
<th>J ayw</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Global Changes | 1 | 4 | 3 | 4 | 5 | 12 | 1 | 4 |

Table 4.4 shows the summary of BioPlex findings of HBsAg derived from wild type HBV in-vitro constructs, compared to genotype A2 adw. Abbreviations: ↑ indicates an increase in mAb binding, ↓ indicate a reduction of binding. Genotype F adw shows the most number of global changes (12/19), whilst all genotypes increased mAbs 8. mAb 6 decreases with all serotype ayw and both genotypes E ayw and F showed a gain in mAb 17.
4.4.1C) Vaccine/ HBlg / Drug Resistant HBV Profiles

The first HBsAg variant to be analysed, generated by site-directed mutagenesis on the HBV genotype A2 adw backbone, was sG145R (from genotype A2 adw-2). This variant was constructed by a single nucleotide change (G to A) at codon 145. The BioPlex profile (Figure 4.8 Panel 2) was normalized to the A2 adw backbone as with the previous analyses. The BioPlex analysis revealed a number of changes in epitope reactivity: nine out of 19 mAbs (see Table 4.5) across the ‘a’ determinant of sG145R gave significantly different profiles from the reference A2 adw, including the N-terminal, Loop 1, Loop 2, C-terminal and conformation-dependent mAbs (see Panel 3). Whilst the N-terminal and C-terminal mAb reduction was marginally greater than 0.5 fold change (0.58 and 0.59 respectively), the reduction of mAbs 5 and 6 (loop 1), 8, 12 and 16 (Loop 2) and mAb 9 (conformation dependent antibody) was much greater at 2.5 fold difference. The loss seen for mAb 8 in this analysis was uniquely different, even when compared to other genotypes, which typically show a gain in binding at this epitope (see Table 4.5). Interestingly, the only mAb that showed increased binding was mAb17 (Loop 2) was also the case with wild-type genotype E and F.

Figure 4.9 shows the analysis for HBsAg derived from HBV variants expressing either the sG145R and sG145A substitutions with the profiles normalized to HBV wild-type on A2 adw-2 backbone. The loss of epitope recognition observed with sG145A was similar to sG145R for mAbs 1, 6, 8 and 12, as well the gain of reactivity seen with mAb 17 (red box). No changes were seen at mAb 5 (Loop 1) or mAb 2 (C-terminal), as indicated in Panel 3 for sG145A, when compared to sG145R. Despite substitution at the same codon 145, the R variant (sG145R) conferred more changes (9/19 mAbs, see Table 4.5) in the epitope profile compared to the A variant (sG145A) with only 5/19 mAbs. Similar changes were seen for sG145R and sG145A with mAbs 6, 8, 12 and 17. However, the loss or gain for sG145R was more pronounced than that observed for sG145A, specifically, a loss of greater than 2.5 fold change in mAb 8 and 12 but also the gain in mAb17 (see Table 4.5).

Figure 4.10, Panel 2 shows the HBsAg BioPlex profile of sD144E and sG145R on the A2 adw backbone and plotted separately, the graph has been normalized to wild type HBV A2 adw. Both HBV variants have six similar changes for mAbs 5 and 6 (Loop 1), 8, 12 and 16 (Loop 2) and mAb 9 (conformation dependent). Changes unique to sD144E included loss of binding for mAb 7 and 19, both Loop 2 mAbs (marked by the green arrow, see Panel 2). The increased binding of mAb 17 observed with sG145R was not seen for
sD144E. No changes were observed with the N-terminal and C-terminal mAbs for sD144E (see Table 4.5).

Figure 4.11 Panel 2 shows the BioPlex analysis of sG145R and sD144A separately plotted and normalized to wild type HBV on genotype A2 adw backbone. There were five mAb reactivity changes common to sG145R and sD144A (blue arrows): mAb 5 and 6 (Loop 1), mAb 8 and 12 (Loop 2) and mAb 9 (conformation dependent) although the loss of reactivity was less profound for mAbs 6, 8 and 12. A small but significant binding loss was detected for mAb 11 which appeared to be unique to sD144A. There were no changes in mAb 17 nor the C- and N-terminal reacting mAbs, similar to what was observed for sD144E (indicated by arrows.) Overall, there were fewer mAb changes with sD144A (6/19) compared to sD144E (8/19, see Table 4.5). The gain of reactivity for mAb 17 observed with sG145R and sG145A was not observed with sG144A.

In the next analysis, shown in Figure 4.12, combinations of substitutions were tested. Panel 2 shows sD144E+sG145R compared to sG145R alone, with the profiles normalized to genotypes A2 adw. The combination of substitutions showed a similar profile to sG145R alone, including gains for mAb 17 (red box). The exception was the substantial loss of reactivity at mAb 19 (red box), also seen to a lesser degree with sD144E alone (see Table 4.5). This combination of substitutions exhibited the most number of changes seen in the study of HBV variants, 14/19 (see Table 4.5), and included reduced binding for mAb 13 and 14 (Loop 1 and 2 mAbs) and changes in all of the Loop 2 mAbs.

Figure 4.13 Panel 2 shows the BioPlex profile of sP120T and sG145R (in the A2 adw backbone), the graph is normalized to HBsAg derived wild type A2 adw. There were three changes common to both variants, reduced binding of mAb 5 and 6 (Loop 1) and gain in mAb 17 (marked by red box). Changes unique to sP120T were loss of mAb 7 (Loop 2) and 15 (combination mAb, see Panel 3), the latter epitope loss is (marked by arrow) interesting because it has only been seen with changes around the amino acid 120 region. In comparison to sG145R, sP120T had 5/19 mAb changes, whilst sG145R had 9/19 (see Table 4.5).

Figure 4.14 Panel 2 shows the BioPlex profile of sP120L and sG145R (on the A2 adw backbone) plotted separately, the graph is normalized to HBsAg derived from wild-type A2 adw. The sP120L variant conferred the most number of epitope changes (11 out of 19) for any HBV variant with a single amino acid change in the ‘a’ determinant (see
Table 4.5). These included changes also observed with sG145R which were binding reductions for mAb 1 (N terminal), mAb 5 and 6 (Loop 1), mAb 8 and 16 (Loop 2) and mAb 2 (C-terminal) (6/9, shown in Panel 2). Changes unique to sP120L compared to sG145R included loss of binding for mAb 7 (Loop 2), mAbs 13, 14, and 15 (combination) and mAb 4 (C-terminal). Importantly, gain of mAb reactivity for mAb 17, seen with both sP120T and sG145R, was not observed with sP120L. The loss of reactivity for mAb 15 was also seen with sP120T (see Table 4.5). The loss of binding of the combination loop mAb (13 and 14) was also seen with the variant with the double mutation sG144E/G145R, (see Table 4.5 and Figure 4.12), a feature not seen with any other variant studied.

Figure 4.15 shows the BioPlex analysis of sT126N (Loop 1 variant) and sG145R plotted separately and the graph has been normalized to wild type HBV, genotype and serotype A2 adw. The BioPlex profile was little changed from the normalized data but showed a gain in binding with mAb 8 also seen with wild-type genotypes B1, C2, C4, D3, E, F and G (see Table 4.4). Notably, the gain in binding observed with sG145R for mAb 17 was also seen with the sT126N variant (see Table 4.6).

Figure 4.16 shows the BioPlex analysis of HBsAg derived from the variant sM133L and compared to sG145R in a genotype A2 adw backbone. The graph is normalized to wild type A2 adw. Compared to wild type A2 adw, there were two changes with a greater than a 0.5 fold increase for sM133L derived HBsAg: mAb 17 (Loop 2) consistent with the sG145R and mAb 9 (conformation dependent) which contrasted the loss of binding with sG145R (see Table 4.6).

Figure 4.17 shows the BioPlex HBsAg profile of sS132A (Loop 1 variant) compared to sG145R on genotype A2 adw backbone plotted individually with the graph normalized to wild-type genotype A2 adw. Significant epitope changes for sS132A includes a gain of binding for mAb 8, as seen with sT126N and wild type genotypes B, C, C4, D, E, F and G and gain of mAb 17 binding seen with wild-type genotype E and F (see Table 4.4) and variants sG145R, sG145A, sT126N, sP120T, and sM133L (see Table 4.5 and Table 4.6).

In Figure 4.18 Panel 2, the BioPlex profile of sN146A (a Loop 2 variant) is compared to sG145R plotted individually and normalized against the wild type A2 adw backbone. This analysis shows a reduced binding for sN146A with Loop 1 mAbs 5 and 6 and Loop 2 mAbs 12 and 16 consistent with that of sG145R along with reduced binding for mAbs 7 (Loop2) and 14 for the sN146A variant alone. There was a gain in binding for mAb 8
(Loop 2) when compared to genotype A2 adw and this was in contrast to the reduction observed with sG145R for mAb 8 (see Table 4.5 and 4.6). Of note, no gain in binding for mAb 17 was observed for sN146A.

D) Antiviral Drug Resistant Variant Profiles

Common mutations encoding the substitutions causing lamivudine resistance (such as rtV173L+rtL180M+rtM204V or rtM204I +/- rtL180M, can result in concomitant nucleotide changes encoding important and significant substitution in the overlapping HBsAg. Figure 4.19 Panel 2 shows the BioPlex profile of sE164D (which can arise from the change rtV173L). The profile for sG145R is also plotted separately and the graph is normalized to wild-type A2 adw. Several changes from the normalized A2 adw plot can be observed with HBsAg from sE164D. Significant epitope changes for sE164D included the important gain in mAb 17 (red box), as seen with sG145R, sG145A, sT126N, sP120T and sM133T as well as wild type genotypes E and F. There is a gain in binding of mAb 8, as seen with wild-type genotypes B, C2, C4, D, E, F and G (see Table 4.4, 4.5 and 4.6).

Figure 4.20 Panel 2 shows HBsAg derived from sW196S compared to sG145R on genotype A2 adw backbone, plotted separately and the graphs are normalized to wild-type A2 adw. There is an increased binding with gain of mAb 8, as seen with other genotypes (see Table 4.4, 4.5 and 4.6) such as B adw, C2 adr, C4 ayw, D ayw, E ayw, F adr and G and gain in mAb 17 (red box), as seen with sG145R. Interestingly despite the presence of this substitution being downstream from the ‘a’ determinant, Loop 2 mAbs are altered (mAbs 8 and 17, see Panel 3 map) whilst there are no changes in the C-terminal mAbs (2, 3 and 4).

In summary (see Table 4.5 and 4.6), the changes in BioPlex immunoassay for the canonical VEM sG145R include changes in mAb binding across five domains, N-terminal, Loop 1, Loop 2, combination and conformation dependent mAbs. Whilst other substitutions in HBsAg show some of these changes, none of the other variants except, sP120L exhibit changes as substantial and/or as extensive as with the sG145R. Combination of mutations at position 144 and 145 show changes similar to sG145R, with the addition to the loss of mAb 19 binding. Mutations sT126N, s132A, sE164D and sW196S had similar changes in the epitope profile; gain of mAb 8 and 17, and of all the HBV variants, with single substitutions studied, sP120L had the most divergent profile (11/19 mAbs), and shared seven of these changes with sG145R.
Figure 4.8

Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and the graph is compared to and normalized wild type A2 adw

Panel 3

Sites of monoclonal antibody interaction in the ‘a’ determinant and C-terminus
Figure 4.9

Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and sG145A compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.10

Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and sD144E compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.11

Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and sD144A compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.12

Panel 1

<table>
<thead>
<tr>
<th>A2</th>
<th>sG145R</th>
</tr>
</thead>
<tbody>
<tr>
<td>D144E+G145R.</td>
<td></td>
</tr>
<tr>
<td>S132A</td>
<td></td>
</tr>
<tr>
<td>E164D</td>
<td></td>
</tr>
<tr>
<td>W196S</td>
<td></td>
</tr>
</tbody>
</table>

Clustal amino acid alignment of variants and variant combinations

Panel 2

Graph of sG145R and sD144E/G145R compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
**Figure 4.13**

**Panel 1**

*Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants*

**Panel 2**

*Graph of sG145R and sP120T compared and normalized to wild type on genotype A2 adw backbone*

**Panel 3**

*Sites of monoclonal antibody interaction in the ‘a’ determinant and C-terminus*
Figure 4.14
Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and P120L compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.15
Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG14R and P126N compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.16
Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and sM133L compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.18

Panel 1

Clustal amino acid alignment of genotype A2 adw and in-vitro derived variants

Panel 2

Graph of sG145R and sN146A compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.19

Panel 1

Clustal amino acid alignment of variants and variant combinations

Panel 2

Graph of sG145R and sE164D compared and normalized to wild type on genotype A2 adw backbone

Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Figure 4.20
Panel 1

Clustal amino acid alignment of variants and variant combinations
Panel 2

Graph of sG145R and W196S compared and normalized to wild type on genotype A2 adw backbone
Panel 3

Sites of monoclonal antibody interaction in the 'a' determinant and C-terminus
Table 4.5

<table>
<thead>
<tr>
<th></th>
<th>G145R</th>
<th>G145A</th>
<th>D144E</th>
<th>D144A</th>
<th>D144E</th>
<th>P120T</th>
<th>P120L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N-terminal</td>
<td>▼</td>
<td>▼</td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>5</td>
<td>Loop 1</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>6</td>
<td>Loop 1</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>10</td>
<td>Loop 1</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>7</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>8</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>11</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>12</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>16</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>17</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>19</td>
<td>Loop 2</td>
<td>▼</td>
<td>▼</td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>13</td>
<td>Loop 1+2</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>14</td>
<td>Loop 1+2</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>15</td>
<td>Loop 1+2</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>2</td>
<td>C-terminal</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>3</td>
<td>C-terminal</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>4</td>
<td>C-terminal</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>9</td>
<td>Conformation</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>18</td>
<td>Conformation</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Global Changes</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>14</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 4.5 shows the BioPlex findings of the commonly claimed HB Ig/vaccine variants. Column 1 shows changes in canonical vaccine escape variant, sG145R. Abbreviations: ▼ indicates an increase in mAb, ▼ indicate a loss of binding. Of note, all the variants exhibited some changes in the BioPlex profile. Most changes seen were for combination sD144E and sG145R, global change of 12/19. Gain in mAb 17 was seen in all of the variants except sG144E and sG144A and sP120L.
Table 4.6 shows the BioPlex profile of putative HB Ig/vaccine escape variants and the last two columns show antiviral drug associated changes. Abbreviations: \( \uparrow \) indicates an increase in mAb, \( \downarrow \) indicate a loss of binding. The BioPlex findings of the commonly claimed HB Ig/vaccine variants. Column 1 shows changes in canonical vaccine escape variant, sG145R. Notable finding is the gain of mAb 17 seen with all of the HBV variants displayed in this table except sN146A. Other BioPlex findings of site directed mutagenesis variants are shown in Table 4.7.

<table>
<thead>
<tr>
<th>Site of mAb</th>
<th>G145R</th>
<th>M133L</th>
<th>S132A</th>
<th>T126N</th>
<th>N146A</th>
<th>E164D</th>
<th>W196S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 C- terminal</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Loop 1</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Loop 1</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Loop 2</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>11 Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 Loop 2</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>19 Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 C-terminal</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 C-terminal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 C-terminal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Conformation</td>
<td>↓</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 Conformation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Global Changes | 9 | 2 | 2 | 2 | 7 | 2 | 2 |
4.5 Discussion

In this study, a novel immunoassay was used to identify differences in HBsAg phenotype at the genotype and serotype level as well as for commonly claimed HBV variants associated with vaccine escape. Of the 19 mAbs used to discern these differences, a gain in mAb 8 reactivity was found across all the genotypes studied, compared to genotype A2 adw, whilst serotype ayw showed loss of epitope binding with mAb 6 and 7. A gain in mAb 17 reactivity was observed in a number of variants claimed to be associated with vaccine/HBIg escape including sG145R and sP120T (see Table 4.5) and was also observed for genotypes E ayw and F adw (see Table 4.4 and 4.5), both genotypes associated with cases of vaccine failure despite the presence of vaccine derived anti-HBs[197-199, 201]. Other variants with a gain at mAb 17 included sG145A, sT126N, sM133L, sS132A, sE164D and sW196S (see Table 4.5 and 4.6). These results provide for the first time, possible phenotypic biomarkers for a reliable definition of VEMs of HBV using as a reference point the key profile of sG145R, the classical VEM. These studies were performed with recombinant HBsAg preparation and the next step would be evaluation of these proposed criteria in clinical samples (see Chapter 5).

There are 10 different HBV genotypes worldwide, defined by a nucleotide divergence of greater than 8% of full-length genomes. However, prior to Sanger (population based) sequencing, serological differences were used to “classify” or “type” HBV isolates and these were based on particular antigenic determinants detected and measured initially using immunodiffusion studies [211, 307, 308, 313]. The HBsAg contains the ‘a’ determinant (see Figure 4.1), regarded as the major neutralization domain of HBV. The current hepatitis B vaccine comprises recombinant 22nm HBsAg particles containing the S protein that elicits anti-HBs, generally resulting in robust immunity against HBV infection. Whilst the S-gene encoding HBsAg is relatively conserved, the structure itself has not been crystallized. A number of different studies have established that it is a conformationally dependent antigen [186], making it possible that anti-HBs derived by vaccination with one genotype (A2 adw) may not provide the same level of immunity against other HBV genotypes [190]. The studies described in this Chapter attempted to further investigate this possibility.

The first part of this study evaluated potential epitope differences in HBsAg phenotype of the two main serotypes (adw versus ayw) in the context of eight of the HBV genotypes. A novel immunoassay using the BioPlex platform, which utilised 19 different mAbs, permitted the epitope mapping of the different regions of the ‘a’ determinant, including its conformational structure, in order to show differences when comparing
isolates of HBV, but in particular to the ‘reference’ vaccine genotype A2 adw. One of the most interesting findings of this study was the lack of mAb binding uniformity across the various genotypes when compared to A2 adw. All other genotypes showed an increase in binding for mAb 8 (see Table 4.4), which is directed against Loop 2 of the ‘a’ determinant. This suggests genotypic differences can affect this structure and of the 19 different mAbs, the gain in mAb 8 reactivity could be a discriminator between A2 adw and the other genotypes. All genotypes expressing the serotype ayw showed loss of reactivity for mAb 6 (Loop 1) and mAb 7 (Loop 2), although the former change was also seen with genotype F adw. This finding indicates that mAb 6 and 7 could be potential discriminators between serotype ayw and genotypes adr and adw. The defining difference between ‘ad’ and ‘ay’ is which amino acid is expressed at codon 122; if lysine (K) it results in ‘ad’ specificity whilst if arginine (R), it results in ‘ay’ [193]. With mAb 6 binding to the Loop 1 region containing sK/R122, it is understandable that this can discriminate between serotypes, although it is a paradox why HBV genotype F adw also shows a loss of binding. The additional loss of binding reactivity for mAb 7, which binds well away from Loop 1, suggests further significant conformational differences between subtypes.

In the genotype analysis presented here, genotype E ayw was found to have the most diverse phenotypic profile compared to the other genotypes. Genotype E ayw is the predominant strain in West Africa (e.g The Gambia) and at the amino acid level there were a number of changes across Loop 2 of the ‘a’ determinant compared to A2 adw. Importantly this virus has been associated with high vaccine failure rates despite the standard recommended three doses of HBV vaccine during infancy [197, 198] Epidemiological studies have shown that between 18-27% of adolescents subsequently tested positive for anti-HBc, indicating infection, despite a full course of vaccination. Other studies involving blood donor screening have also reported acute HBV infections with non-A2 adw genotypes, regardless of having adequate levels of circulating anti-HBs derived from vaccination. [199, 326]. The BioPlex profile of genotype E ayw (see Figure 4.5 panel 2) revealed four changes in Loop 2 mAbs and one change in Loop 1. This represented the most number of changes for all genotypes with serotype ayw. The clustal amino acid alignment showed a change from proline (P) to leucine (L) at codon 127 (Loop 1). The biochemical properties of P include strongly defining the secondary structure of α-helices and holding the protein in a specific conformation by forming turns. Therefore, replacement by L could lead to changes to both structure and conformation (see Figure 4.1).
Genotype F adw is the predominant HBV genotype in Central and South America and has been associated with multiple and severe complications of chronic HBV infection. A previous study involving the sequencing of the S-gene of genotype F has found it to be one of the most divergent compared to other genotypes [192] and the BioPlex immunoassay confirmed this. Pairwise analysis of the nucleotide and amino acid sequence of the ‘a’ determinant of the S-gene performed in this study also revealed significant variation, including a 9% difference between genotype F adw and A2 adw (see Chapter 3, Table 3.1).

The BioPlex profile of genotype F revealed changes in a number of domains that affected Loop 2, C-terminal and N-terminal binding mAbs, indicating pressure not only for both antigenic loops but also for the binding of those mAbs associated with the conformational nature of HBsAg (see Figure 4.7.) The divergent nucleotide sequence of the S-gene for genotype F adw encodes three additional cysteine residues compared to genotype A2 adw (see Figure 4.7 Panel 1 highlighted in red), presumably associated with formation of even further disulphide bonds. These are located upstream from the ‘a’ determinant. A number of other codon changes were noted for this genotype, including the L substitution at position 158 instead of phenylalanine (F) and glutamine instead of P at 178. Again the latter amino acid is crucial in the maintenance of the secondary protein structure for genotype F, as noted above for genotype E and sP127L change. Analysis of genotype F also revealed a gain in mAb 17, and consistent with the hypothesis that this could be a possible phenotypic biomarkers for HBV VEM, vaccine failure has been described in a previously A2 adw vaccinated patient, super-infected with wild type genotype F HBV [201]. Whilst Central and South America has seen an overall decline in CHB infection as a result of universal hepatitis B vaccination[15], further surveillance studies should include screening for anti-HBc in previously vaccinated individuals to assess vaccine efficacy.

Genotype C2 adr is predominantly found in Asia whilst C4 ayw is exclusively found in the Indigenous Australian population in the Top End[209]. The C4 subtype is a recombinant HBV and shares S-gene sequence with genotype J ayw [211]. The BioPlex profile of genotype J ayw and C4 ayw were similar, reflecting the homogeneity of this sequence. Whilst both C2 adr and C4 ayw share the same genotype, sub-genotypic and serotypic differences were evident in the BioPlex profiles with more changes seen for C2 adr then C4 ayw.
Historically, substitutions in HBsAg have raised concerns regarding vaccine efficacy and their public health significance has been the subject of much discussion and controversy. The first variant identified that was associated with vaccine failure, sG145R, was described by Carman and colleagues, and has been subsequently described from most parts of the world [183]. This variant has shown comparable replication fitness to wild-type HBV in *in-vitro* studies [327] as well as the ability to be transmitted within family members and close contacts [237]. Thus, not surprisingly sG145R is regarded as a prototype for VEM “status” [14].

The biochemical properties of the amino acids define the properties of protein structure and after the identification of the first sG145R isolate extensive studies including assessment of the amino acids 139 -147 spanning the ‘a’ determinant of the sG145R variant were undertaken. Kyte Doolittle plots as well as Hopps and Woods curves have been generated and the analysis demonstrated that the HBsAg of the sG145R variant is more hydrophilic than the wild type HBV [280].

In the analysis described in this thesis, many significant changes in the BioPlex profile of sG145R were noted across the five mAb interaction domains within the ‘a’ determinant. Loss of reactivity to C-terminal, N-terminal mAbs, Loop 2 mAbs 8, 12 and 16 and conformation dependent mAbs was observed. There were nine out of nineteen changes in the mAb profile. Whilst an overall loss of mAb reactivity was generally seen with the sG145R variant, mAb 17 showed a gain in reactivity. The change in BioPlex profile reflects the change in the structural properties of the protein by this single substitution and is consistent with the reported change in hydrophilicity. In wild type HBV at codon 145, G is a small nonpolar, hydrophobic amino acid that stabilizes the protein by its hydrophobic properties. This is replaced in the VEM sG145R by the positively charged amino acid, R (see Figure 4.21). The resultant structural and biophysical change in protein presumably leads to greater exposure of the epitope that is recognised by mAb 17, resulting in the gain in reactivity observed.
Panel A shows the chemical structure of amino acid glycine, located at codon 145 of HBsAg and Panel B is the chemical structure of arginine. The notable differences are in size and charge. Glycine is a smaller amino acid compared to arginine and the area marked by the red box denotes the methyl [R] group of each amino acid, which determines the properties of amino acid, whilst the region outside the red box is common to all amino acid. Arginine is positively charged and polar, whilst glycine is small and non-polar hydrophilic amino acid. Figure adapted and modified from [261].

The importance of these biochemical properties of particular amino acids changing the epitope profile was further demonstrated by the BioPlex profile of sG145A, (see Figure 4.9). In contrast to the sG145R, substitution at this site by alanine (sG145A) was associated with fewer epitopes losses. However once again, the gain of mAb 17 reactivity for sG145A strongly suggests that there had been significant changes in the epitope overall and neutralization in particular, allowing for a greater shape changing/shifting and hence gain of binding of mAb 17. This study allowed an assessment of the impact of codon specific changes such as sG145R and sG145A on the BioPlex readouts common to both of these variants. These changes included loss of mAb 1 (N-terminal), mAb 6 (Loop 1), mAb 8 and 12 (Loop 2) and gain of mAb 17 (Loop2). Whilst some of these changes are shared across HBV variants (see Table 4.5 and 4.6), loss of mAb 1 (N-terminal) was seen with sG145R and sG145A and with the double mutations sD144E and sG145R (see Table 4.5). mAb 17 detects conformational epitope as a consequence of the sG145R substitution. The VEM mechanism is considered to be a consequence of the type of conformational change caused by sG145R. Thus, HBV variants which exhibited a gain of epitope with mAb17, presumably have a conformation similar to that of sG145R. Optimization of mAb 17 so that it always detects VEM was beyond the scope of this thesis.
Table 4.7 shows the BioPlex changes seen for codon specific changes for sP120, sD144 and sG145. Of note gain of mAb 17 was only seen for sG145 and sP120T, whilst loss of mAb 6 was seen for all three variant HBsAg. Of all the variants studied in this study, loss of mAb 15 was only seen with sP120 variants.

In clinical studies, emergence of both variants sD144A and sD144E have been typically associated with HBVg administration [34, 266, 281]. However, when the HBV variant sD144A was evaluated using the Kyte Doolittle plot approach, the overall hydrophilicity profile was unchanged [34]. Conversely, in-vitro studies have shown reduced mAb binding with D144E [305]. Interestingly, the BioPlex profile showed loss of mAbs 5, 6, 8, 12 and 9 for sD144A and sD144E. This was consistent with those seen with sG145R (see Table 4.5), indicating that whilst the changes in the BioPlex profile do not necessarily match entirely, there are common changes for all three variants that might show the potential for such an isolate to behave as VEM. Furthermore, codon specific changes seen for sD144E and sD144A included loss of mAb 6 (Loop 1), mAb 8 and 12 (Loop 2) also seen with codon specific changes at position 145 and loss of mAb 9 (conformation dependent) (see Table 4.7). However, unlike sG145R, there was no gain of epitope reactivity with mAb 17.

Using enzyme immunoassays with anti-HBs derived from pooled human vaccinee sera, Torresi et al demonstrated in-vitro an appreciable loss of binding with the double substitution sD144E/sG145R (see Table 4.5) [144]. However, the loss of binding of anti-HBs (see Table 4.7) was not as marked as for sG145R. In the analysis described in this thesis, the combination of substitutions sD144E/sG145R was analysed on the BioPlex
platform to gain further insight and compare the differences in epitope profiles. The combination of sD144E/sG145R showed extensive loss of epitopes, including mAbs 14 and 11, exhibiting more changes than the prototype VEM, sG145R. The profound loss of binding observed with sD144E/sG145R (Figure 4.12) was not consistent with the data described in the earlier study by Torreisi et al [144](see Table 4.8). This may be due to the differences in the assays themselves; the immunoassay used in previous studies showed the 'overall binding’, which was saturable (>55.0μg/mL), rather than the discrete changes for specific epitopes or shapes, whereby a complete loss of epitope may be compensated by an increase or gain in another HBsAg epitope (Table 4.3).

Table 4.8

<table>
<thead>
<tr>
<th>Envelope Mutants</th>
<th>Polymerase Mutant</th>
<th>Ag-Ab Binding [IC50 (μg/ml)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>1.09</td>
</tr>
<tr>
<td><strong>HBIg-Escape</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sG145R</td>
<td>rtW153Q</td>
<td>&gt;55.0</td>
</tr>
<tr>
<td>sD144E/sG145R</td>
<td>rtG153E</td>
<td>&gt;55.0</td>
</tr>
<tr>
<td><strong>Drug Resistant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sF158Y</td>
<td>rtF166L</td>
<td>1.86</td>
</tr>
<tr>
<td>sE164D</td>
<td>rtV173L</td>
<td>14.86</td>
</tr>
<tr>
<td>sW196S</td>
<td>rtM204I</td>
<td>8.29</td>
</tr>
<tr>
<td>sI195M</td>
<td>rt204V</td>
<td>5.26</td>
</tr>
<tr>
<td>sM198I</td>
<td>rtV207I</td>
<td>12.5</td>
</tr>
<tr>
<td>sE164D/I195M</td>
<td>rtV173/rtL180M/rtM204V</td>
<td>54.53</td>
</tr>
</tbody>
</table>

*Table 4.8 adapted and modified from Torreisi et al [144] shows the amount of protein required to be added to decrease the binding between wild-type HBsAg and anti-HBs antibody in a pooled vaccinee sera.*

Clinical isolates of sP120T and sP120L have been observed in situations where immunological pressures such as vaccination and HBIg administration have been operating, and in-vitro studies using immunoassays have demonstrated altered mAb binding [265, 276]. Again, as discussed above, P is an amino acid that can influence substantial structural effects including changes of folding and conformation, since the side chains can result in taking a cis configuration and thereby forming tight turns. Interestingly codon 120 falls just outside the Loop 1, defined as spanning from amino acid 124–137 and Loop 2 is located upstream from 139–147, [183, 186]. However, for a conformational dependent structure such as the 'a' determinant, a change of this P would be expected to typically result in significant changes in the BioPlex profile. Such
changes do mean re-consideration of the folding of the ‘a’ determinant in terms of possible models (see Figure 4.1).

In this study sP120L also showed a number of mAb reactivity changes (see Table 4.5), seen with 11 out of the 19 mAbs. Unlike sG145R and sP120T, there was no gain at mAb 17. Similarly substitution of P by threonine (T) at position 120 showed changes in Loop 1, Loop 2, including a gain in mAb 17, and combination mAbs. However, the C-terminal mAbs were not affected. Comparison of codon specific changes for sP120T and sP120L included loss of mAb 5 (Loop 1), mAb 6 and 7 (Loop 2), mAb 15 (combination of Loop 1 and 2). Loss of mAb 15 (combination Loop 1 and 2) was only seen with these two variants (see Table 4.5).

In terms of the biochemical properties of these amino acids, both T and P are polar and have an uncharged methyl group [261] but as discussed above, a gain or loss of P would significantly affect folding and conformation. In contrast, a change to L (sP120L) is a conservative change in size but is non polar with an aliphatic methyl group, resulting in minor effects on shape and folding.

In the BioPlex analysis, changes in mAb reactivity have been noted in association with various substitutions. For example, a change unique to substitutions at codon 120 has been the loss of mAb 15, on genotype A2 adw backbone. mAbs 13, 14 and 15 bind to epitopes of both Loops 1 and 2, and whilst changes have been noted for mAb 13 and 14 with sD144E/G145R, genotypes E ayw and genotype F adw (see Table 4.5), mAb 15 remained consistently unaffected.

Mutations around the ‘a’ determinant have been associated with impaired HBsAg secretion or increases in secretion in the presence of another substitution such as sM133T. In-vitro studies have demonstrated an enhanced secretion phenotype, especially in the presence of vaccine escape mutants, such as sG145R [312]. The sM133L substitution has been associated with vaccination failure, and whilst HBsAg structure has not been resolved by crystallization, Le et al used the Jameson-Wolf algorithm to predict an antigenic index [254]. In that study various HBsAg mutants, which included sT123A, sT123N, sM133L, sM133I, sM133T, sM133V, sT143M and sT143L, were constructed and tested for antigenic index. Of these, sM133L was noted to have the least antigenic change. However, in the BioPlex profile shown in this thesis (see Figure 4.16), there were changes in reactivity for two important mAbs, gains in 17 (Loop 2) and 9 (conformation dependent mAbs), but with fewer changes across the panel compared to
other variants. This is presumably due to the similarity of the two amino acids
methionine and leucine, both being non-polar with an aliphatic methyl group[261]

Threonine has been associated with stabilization of the disulphide bonds [268] and the
BioPlex profile of HBV variant sT126N, showed a minimum number of changes,
confirming again that with increases in mAb 8 and 17, changes similar to M133L and
sS132A, that a gain in mAb 17 maybe a potential marker for VEM.

N-linked glycosylation is essential for virion secretion and virus recognition ([283]
However, the phenotypic profile of HBV variants with loss of the glycosylation site has
not been studied in the context of vaccine failure. For the non-glycosylated variant
sN146A, the phenotypic profile on BioPlex demonstrated seven shape changes. However
mAb17 reactivity was not affected. Therefore the role of this variant, in the presence of
vaccine induced anti-HBs to fully evade neutralization, requires further evaluation.

Antiviral therapy for CHB targets the polymerase and mutations associated with therapy
can cause corresponding S-gene changes (see Table 4.1 and 4.2). Such variants are
particularly relevant in limited resource countries, where drugs with a low genetic
barrier to resistance such as lamivudine are still the initial treatment of choice for CHB
because of cheap pricing [328]. Another group where patients have been exposed to
these a drugs are the HIV/HBV co-infected patients where lamivudine was part of
antiretroviral treatment for HIV. Recent modelling studies have shown that this group of
patients are at an increased risk of carrying HBV with S-gene mutations and this can
potentially lead to a number of antiviral associated potential vaccine escape mutation
(ADAPVEM) (Lacombe 2013, Clements). A number of in-vitro and in-vivo studies have
shown that the HBV lamivudine resistant variant rtV173L+rtM204V+rtL180M,
producing double variant sE164D/sI195M changes either alone or in combination in the
overlapping HBsAg, can result in reduced mAb binding in HBsAg immunoassays,
comparable to the effect of sG145R [187] (see Table 4.7). This observation has led to
further in-vivo studies to assess the protection status of standard HBV vaccine
immunized chimpanzees with high levels of protective anti-HBs in challenge
experiments with these drug resistant variants[251]. Animals were subsequently
inoculated with HBV expressing the sE164D/sI195M HBsAg changes. This resulted in
infection in the animals, confirmed by both virological and immunological assays,
suggesting that these antiviral therapy associated changes in the S-gene can lead to a
phenotype of vaccine escape [187, 251]. The BioPlex studies of variant sE164D showed
gains in mAb 8 and most importantly mAb 17. Interestingly, these two changes were
also consistent with HBsAg variants associated with potential vaccine escape variants sT126N and sS132A, as seen in this study (see Table 4.6). Furthermore, the profile of the substitutions sE164D/sI195M (rtV173L+rtM204V+rtL180M) also suggests that these particular changes have a net similar effect to that observed with double mutations involving codons 144/145 consistent with breakthrough infections seen in in-vivo and clinical case reports of vaccine failure [201, 251].

Despite being downstream from the 'a' determinant, the sW196S variant also showed an increase in mAb 8 and mAb 17 reactivity, similar to that observed with sE164D. It has been established previously, that due to the conformational nature of the HBsAg protein[186], changes outside the 'a' determinant can alter the HBsAg phenotype of “neutralisation”. A possible explanation for this change in the BioPlex profile can be explained by examining changes in the biochemical profiles of sW196S; substitution of tryptophan by serine is a significant change and so could potentially alter the HBsAg structure, altering more epitopes resulting in gains of Loop 2 mAbs (8 and 17).

This study identified a combination of mAb binding changes using the BioPlex platform that can be used to identify a potential VEM. These changes include loss of C-terminal mAb (1), Loop 1 (5 and 6), Loop 2 (12 and/or 16) and at least one change targeting C-terminal mAbs (2, 3 or 4), one of the conformation mAbs (9 and/or 18) and loss of one or more combination mAbs (13, 14, 15) combined with an increase in mAb 17. Most commonly claimed vaccine escape variants exhibited an increase in mAb 17 (sG145R, sD145A, sP120T, sT126N, S132A and M133L) and antiviral associated HBsAg variants (sE164D and sW196S). Interestingly HBIg associated variants sD144A and sD144E did not show this gain in mAb 17, suggesting that it could be a feature of VEM and ADAPVEM. However, these findings need to be further extended with this variant in different genotypic backbones and clinical samples. The availability of an in-vitro natural infection system for HBV using the sodium taurocholate cotransporting polypeptide (NTCP)–Hep G2 system [174] may permit the testing of these possible VEM profiles in future virus neutralisation of infectivity studies.

Gains or losses of reactivity to particular mAbs, regardless of the sites of known interaction within the HBsAg, were identified in this study. Monoclonal antibody 8, which interacts with Loop 2 and specifically at amino acids 139-147 (see Chapter 2, Table 3), showed a gain of reactivity for all non–A2 adw genotypes (see Table 4.4), whilst loss of binding of mAb 7, which interacts between aa 139-147 (Loop 2) was seen with all ayw serotype. This suggests that despite the designation of specific epitope
interaction sites of these mAb, there are overall structural changes of HBsAg that leads to the gain or loss of binding that are not necessarily readily predictable. The loss of mAb 7 reactivity with serotype ayw is interesting since serotypic differences are identified by the presence of either K or R at amino acid position 122 and K or R at amino acid position 160 and neither of these sites appear to be mapped by mAb 7 (see Table 4.3 and 4.4). Loss of mAb 15 was seen with HBsAg variants with substitutions at codon 120 (sP120T and sP120L) which interacts between amino acids 120-125 (Loop 1) and amino acids 144-149 (Loop 2). However, there were no changes in mAbs 13 and 14 binding despite interacting at the same Loop 1 epitope (120-125) but with different Loop 2 epitope (aa134 -157) residues (see Table 4.4). These findings suggest that the loss of mAb 15 reactivity, as seen with HBsAg derived from sP120T and sP120L (see Table 4.7), is presumably due to resultant conformational changes at the Loop 2 region (aa 144-149), despite these variants having a substitution at Loop 1 (sP120). As outlined in Table 4.3, mAb 17 targets Loop 2 of the ‘a’ determinant, this study revealed the increase in binding for sG145R, a VEM, and other variant commonly associated with HBIG/vaccine escape and antiviral-associated variants. The inclusion of this mAb enabled detection of putative VEMs (see Table 4.5), including a gain for Genotype E ayw and F adw (see Table 4.4), both associated with breakthrough infections despite adequate anti-HBs levels [198, 199, 201]. Whilst the exact interaction site of mAb 17 or the other Loop 2 mAbs (7, 8, 12 and 16) is not known (see Table 4.3), it is reasonable to assume that none of the latter mAbs interact at the same epitope as mAb 17, since none of these display an increase in binding. Whilst the gain in mAb 17 seen with other variants such as sM133L, sS132A, sT126N, sE164D, sW195S has not been extensively as sG145R. Therefore, given the overall complexity of the ‘a’ determinant of HBsAg, the next step could be site-directed mutagenesis at the particular epitopes in order to better understand the conformational nature. The changes in amino acid at these sites presumably results in changes in the tertiary structure of HBsAg resulting in exposure of epitopes and an increase in mAb 17; the feature of mAb 17 that recognizes this phenomenon was outside the original scope of this thesis.

In conclusion, none of the HBV genotypes studied in this project exhibited all the features of the classical sG145R VEM. However, genotype E ayw and F adw both showed an increase in mAb 17, suggestive of partial VEM features. This finding could account for the high vaccine failure rates seen in The Gambia and with genotype F HBV infection in previously immunized people [198, 199, 201]. This study is the first to demonstrate the differences in HBsAg epitope profile of different genotypes, serotypes and those with substitutions both upstream and downstream of the ‘a’ determinant. This assay also
identified an increase in binding with mAb 17 as a potential marker for VEMs. By incorporating other BioPlex features such as concomitant losses at mAbs interacting with Loop 2 and the conformational region, along with changes in binding for mAb 17, the identification and classification of the vaccine escape features of HBsAg, may provide an important tool for future surveillance studies.
Chapter 5:

BioPlex Analysis of Ex-vivo HBsAg

5.1 Introduction
The BioPlex in-vitro phenotypic assay of HBsAg from HBV genotypes and variants described in Chapter 4 revealed differences in the binding of the panel of 19 mAbs, presumably secondary to alterations in the folding of the envelope protein. The genotypes evaluated were compared to the HBsAg derived from HBV genotype A2 adw-2, used in most currently approved vaccines worldwide [6, 303]. These changes were observed across the ‘a’ determinant and seen as a gain or loss of binding of mAbs directed against the ‘a’ determinant and C-terminal region (see Figure 5.1).

Figure 5.1A

Figure 5.1 shows a model of the ‘a’ determinant of the HBsAg. In this model, the cysteine at codon 137 forms a disulphide bond with codon 149 and the cysteine at codon 107 forms a disulphide bond with codon 138, generating the two loops of the ‘a’ determinant. Labelled over the ‘a’ determinant are the binding positions of the monoclonal antibodies used in the BioPlex assay, Figure 5.1 is adapted and modified from [144].
To define the phenotypic features of commonly claimed vaccine escape mutants (VEMs), HBV variants were generated by site-directed mutagenesis using the same genotype and serotype A2 adw-2 backbone in order to control for inter-genotypic differences. The BioPlex analysis showed recognizable and reproducible patterns of mAb (epitope) loss and gain (see Chapter 4). These phenotypic changes were initially analysed for the canonical VEM, sG145R, which has been previously shown to cause acute infection despite adequate levels of circulating vaccine derived anti-HBs; infection has been found to persist in the host for at least eight years [12, 183, 237]. Other HBsAg S-gene variants studied, described in Chapter 4, included those that had been implicated in vaccine failure and these variants also displayed some BioPlex patterns similar to the case definition that was developed for sG145R, the classical VEM (summary Tables 4.5 and 4.6).

During the study of the variants associated with vaccine failure, the sG145R BioPlex features of the HBsAg derived from in-vitro studies revealed an overall gain in the binding with mAb 17 as well as loss of mAb binding around the C- and N-terminal region and the binding of Loop 1, 2 and the conformation dependent mAbs (see Chapter 5, Figure 5.1A). Of all of these changes, the gain of mAb 17 was considered a possible ‘biomarker’ for a VEM since this mAb was generated specifically against sG145R (Dr Tom Leary, Abbott Laboratories, USA - personal communication). Since a concomitant loss of epitope binding was observed with other Loop 2 mAbs such as 8, 12 and 16, it also seemed reasonable to propose their role as potential but less reliable biomarkers for VEMs, more likely due to other variations in the HBsAg.

The in-vitro generated HBsAg (data in Chapter 4 constituted 100% of the viral population, yielding the observed phenotype. However, in a patient with CHB, due to an error-prone mechanism of replication of HBV, viral quasispecies exists. The main aim of this Chapter was to determine if the in-vitro findings correlated with those from clinical samples. This was undertaken by performing BioPlex assays on serum samples from various groups of patients with CHB infection with particular variations in the HBsAg, ascertained by population-based sequencing and ultradeep pyrosequencing, (UDPS) on GS-FLX platform (Roche).

Samples from four groups (A-D) of patients were studied in this Chapter. The first, Group A comprised of patients with CHB infected with the sG145R variant. The second group (B) included persons infected with commonly claimed VEMs and variants associated with diagnostic failure. The third group (C), included those patients with
antiviral associated potential vaccine escape mutants (ADAPVEM), with changes in the polymerase that were known to impact on the overlapping S-gene and cause concomitant changes in HBsAg. The fourth group (D) constituted samples of pregnant women with CHB infection with high viral load (>10^7 IU/mL) who declined treatment with antiviral agents in the third trimester of pregnancy and were known to have transmitted HBV to their offspring; samples were also available from the babies at nine months age.

The aims of this Chapter are as follows:

5.2 Aims

Aim 5.1 – To define epitope profiles and changes of HBsAg in patients with CHB who were infected with sG145R defined by population-based sequencing. To correlate these findings with the other changes observed within the ‘a’ determinant in the context of population-based sequencing and compare relative variant populations (RP) identified by ultra-deep pyrosequencing. Finally, to correlate these clinical profiles against in-vitro generated profiles.

Aim 5.2 - To define the epitope profiles and changes of HBsAg in serum samples of patients with CHB who were infected with HBsAg variants associated with vaccine/HBlg escape (but not sG145R) or infected with diagnostic escape variants, and correlate these findings with that exhibited by the in-vitro cloned variants.

Aim 5.3 – To define the epitope profiles and changes of HBsAg serum samples of patient with CHB infected with antiviral drug resistant variants, which altered the HBsAg and possible neutralisation phenotype for vaccine-associated immunity.

Aim 5.4 – To measure the HBsAg epitope profile in a cohort of pregnant women and their babies, with the women known to have transmitted HBV to their offspring in spite of active (vaccine) and passive (HBlg) prophylaxis, administered at birth according to standard clinical practice (at birth, the vaccine two months, four months and six months of age).
5.3 Methodology and Patient Cohorts

For each of the serum samples used in this study, the sequence analysis had been performed previously at the Division of Research and Molecular Development at VIDRL. This typically involved extracting HBV DNA from serum samples of patients with CHB, followed by population-based sequencing of the pol/rt gene and of the overlapping S–gene and then aligned using the Seqscape® software programme. Mutational analysis was performed using SeqHepB software programme [329]. Dr Margaret Littlejohn and Dr Lilly Yuen at VIDRL provided these sequence data. Sequences are classified as ‘filtered’ which excluded any polymorphism found in the sequence, whilst ‘unfiltered’ were all the substitutions identified via population based sequencing, as analysed through SeqHepB.

For each sample, the level of HBsAg was measured using Elecsys HBsAg II quant assay (Roche Diagnostics) as per manufacturer’s instruction. Each sample was diluted to yield 8, 16 and in 32 IU/well of HBsAg. The serum sample was analysed on BioPlex by Dr Renae Walsh, as described in Chapter 2, Section 2.5. The bioinformatics analysis was set-up and performed by Dr Lilly Yuen at VIDRL. Each sample was normalized to its own wild type genotype and respective serotype and then the data was log transformed so that a change of ±0.5 fold difference outside the 95% confidence interval of this immunoassay denoted significant variation from the reference genotype. The 0.5 fold change was validated for the BioPlex assay and was not changed for any of the clinical groups. A descriptive analysis of each sample was done for all the groups.

Samples from two groups, Group A (sG145R) and Group B (Mother-to-Baby transmission), had UDPS performed using the GS-FLX platform (Roche), (see Chapter 2) This was carried out by Dr Julianne Bayliss and Ms Kathy Jackson. The initial step of UDPS included amplifying HBV DNA with primers covering codon 40–160 of the S-gene Amplicon sequence analysis was done using a Bioinformatics pipeline developed by Dr Lilly Yuen (VIDRL), and the relative proportions of HBV variants were reported as percentages (%). The study samples of HBsAg were included from four different groups. Phylogenetic analysis was performed by Dr Margaret Littlejohn which included using the Neighbor-Joining method [297] and the optimal tree is shown as Figure 5.1B. The analysis was done using MEGA5 (see Chapter 2, section 2.7.4B)
Clinical Groups of Special Populations

Group A) Canonical Vaccine Escape Mutant – sG145R (Table 5.1)

For the clinical sample analysis, ten serum samples from patients with CHB exhibiting the substitution sG145R, or a mixed population of sG145G/R, were selected from the VIDRL panel. In the BioPlex testing, each sample was normalized to its own particular genotypic and serotypic wild type BioPlex classification and tested as per previous methodology developed by Dr Renae Walsh and Dr Lilly Yuen at VIDRL, and then compared to in-vitro generated HBsAg with the sG145R variant generated and analysed previously (see Chapter 3 and Chapter 4). The phenotypic findings of BioPlex was next correlated to all of the changes in the ‘a’ determinant identified by population-based sequencing, as provided by Dr Margaret Littlejohn whilst the UDPS permitted an assessment of the relative proportion of all HBsAg identified from codon 30 up to codon 160 of the “a” determinant, as described in Chapter 2.
Table 5.1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype Serotype</th>
<th>Sanger (population-based) sequencing</th>
<th>Ultradeep Pyrosequencing (UDPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All S-gene Changes</td>
<td>Filtered ** Changes</td>
</tr>
<tr>
<td>2</td>
<td>C adr</td>
<td>sT126I/T131T/N, sM133T/M, G145R/G, a184V, S210N</td>
<td>sM133T/M, G145R/G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C adr</td>
<td>sL104L/V, I126N/I, sG145R,G, A184V, s210N</td>
<td>sG145R,G, sL104L/V, I126N/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>C adr</td>
<td>sG145R,G, A184V, S210N</td>
<td>sG145R/G</td>
</tr>
<tr>
<td>8</td>
<td>B ayw</td>
<td>sG145R,G, A184V, F200Y</td>
<td>sG145R/G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation - ** Filtered sequence refers to the sequence, where all polymorphisms has been excluded.

Table 5.1 shows the sample (1-10), genotypes/serotypes, S-gene variants identified by population-based sequencing and ultradeep pyrosequencing with the relative proportion of these variants. Most samples were of genotype C and serotype adr. The highest relative proportion of sG145R was seen in sample 3 (99.7%). Note the amplicon analysed for UDPS covered amino acid positions 40-160 of the HBsAg, whilst for population-based sequencing, the amplicon spanned amino acid 1 – 226.
Group B) Commonly Claimed VEM and Diagnostic Escape Variants (Table 5.2)

The second group comprised eight patients with chronic HBV infection and CHB, with substitutions in the S-gene (not sG145R) claimed by various investigators to be associated with vaccine escape. Population-based sequencing was carried out as part of the virological work-up for these patients and it showed substitutions such as sD144E, sP120S/T, /sY134N/Y, sD144A and sM133L, as outlined in Table 5.2. This group also included three serum samples referred to VIDRL from regional laboratories following failure to detect HBsAg on screening assays in patients strongly suspected of HBV infection. Diagnostic work-up of these samples included population-based sequencing and analysis with the BioPlex assay. Each sample was normalized to the genotypic backbone and compared to in-vitro samples when the HBV variant was available if previously cloned (Chapter 3 and 4) or to sG145R when the in-vitro clone was not available. Dr Margaret Littlejohn and Dr Lilly Yuen provided the amino sequences of these samples.

Table 5.2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype/serotype</th>
<th>S-gene changes (Population-based sequencing)</th>
<th>Reason for inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>B ayw</td>
<td>sD144E</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V2</td>
<td>A ayw</td>
<td>sD144E</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V3</td>
<td>A ayw</td>
<td>sD144E</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V4</td>
<td>B ayw</td>
<td>sD144E/D</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V5</td>
<td>B ayw</td>
<td>sD144A</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V6</td>
<td>A adw</td>
<td>sP120T</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V7</td>
<td>D ayw</td>
<td>sG145A/P120Q</td>
<td>Diagnostic failure</td>
</tr>
<tr>
<td>V8</td>
<td>D ayw</td>
<td>sP120S/P, Y134N/Y</td>
<td>Claimed VEM</td>
</tr>
<tr>
<td>V9</td>
<td>B adw</td>
<td>sM133L</td>
<td>Claimed VEM,</td>
</tr>
<tr>
<td>V10</td>
<td>D ayw</td>
<td>sT123S</td>
<td>Diagnostic failure</td>
</tr>
<tr>
<td>V11</td>
<td>C adr</td>
<td>sT126I</td>
<td>Diagnostic failure</td>
</tr>
</tbody>
</table>

Table 5.2 shows genotype/serotype and mutations in the S-gene, identified by population-based sequencing. There are 11 clinical samples, three samples were referred to VIDRL after failing detection of HBsAg by standard diagnostic assay and eight samples were sequenced from patients previously referred for diagnostic work-up.
Group C) Antiviral associated Variants (Table 5.3)

The third group of patients comprised patients with CHB with antiviral associated mutations sE164E/sI195M, as a consequence of previous lamivudine therapy. These samples had population-based sequencing performed as part of their clinical work-up. Lamivudine therapy was instituted for the treatment of CHB infection in five patients with CHB mono-infection, whilst four patients were co-infected with HIV. Table 5.3 summarizes the genotypes, serotypes, S-gene mutations and mono versus co-infection with HIV status. The sequences of the HBV in clinical samples (SR1 –SR9) were provided by Dr Margaret Littlejohn at VIDRL. BioPlex analysis was performed as previously described which included normalizing each sample to the genotype and comparing to the in-vitro construct of sG145R, as shown (see Figure 5.2) by co-plotting the two graphs together.

Table 5.3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype/Serotype</th>
<th>Antiviral Mutation</th>
<th>Clinical Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR1</td>
<td>A adw</td>
<td>sE164E/D, sI195M, sS210R</td>
<td>HIV/HBV co-infected</td>
</tr>
<tr>
<td>SR2</td>
<td>D ayw</td>
<td>sE164E/D, sI195M</td>
<td>HBV mono-infected</td>
</tr>
<tr>
<td>SR3</td>
<td>A adw</td>
<td>sE164E/D, sI195M</td>
<td>HBV mono-infected</td>
</tr>
<tr>
<td>SR4</td>
<td>A adw</td>
<td>sE164E, sI195M</td>
<td>HIV/HBV co-infected</td>
</tr>
<tr>
<td>SR5</td>
<td>A adw</td>
<td>sE164E/D, sI195M</td>
<td>HIV/HBV co-infected</td>
</tr>
<tr>
<td>SR6</td>
<td>C adr</td>
<td>sE164D, sI195M, sF2195/F</td>
<td>HIV/HBV co-infected</td>
</tr>
<tr>
<td>SR7</td>
<td>C adr</td>
<td>sE164D, sW172W/C, sL173V, sL186L/R, sI195M</td>
<td>HBV mono-infected</td>
</tr>
<tr>
<td>SR8</td>
<td>C adr</td>
<td>sT123P, sL173V, sI195M</td>
<td>sE164D, HBV mono-infected</td>
</tr>
<tr>
<td>SR9</td>
<td>A adw</td>
<td>sE164D, sI195M, sM133I</td>
<td>HBV mono-infected</td>
</tr>
</tbody>
</table>

Table 5.3 shows the sequences of patient samples (SR1-SR9) with antiviral resistance associated mutations sE164E and sI195M, and other relevant S-gene changes identified by population-based sequencing. These patients were either mono-infected with HBV or co-infected with HIV.

127
Group D) Mother to Baby Transmission (Table 5.4)

The fourth group in the study comprised clinical samples of mothers during their third trimester of pregnancy who transmitted HBV to their babies as well as clinical samples of the babies at nine month of age. There were seven mothers who were HBsAg and HBeAg positive with viral load of >10^8 copies/mL at 30 weeks of gestation. These mothers declined treatment with antiviral therapy and were included as the control group in a study by Wiseman et al studying perinatal transmission of HBV [247]. All except one baby (B4) received HB Ig/HBV vaccine per standard practice guidelines, as outlined in Table 5.4; Baby 4 (B4) missed HB Ig administration. At nine months of age, these babies tested positive for HBV infection (HBsAg serum profile) and sequencing was performed [247] as outlined in Table 5.4. All the babies had the same HBV genotype/serotype as their mother, and the transmission of the infection was presumed to be from mothers. The S-gene sequence results and S-gene changes were provided by Dr Margaret Littlejohn. Dr Lilly Yuen and Dr Renae Walsh provided the results of UDPS (see Table 5.4).

HBsAg phenotypic analysis using the BioPlex was performed on both the mother (third trimester of pregnancy) and baby samples (nine month age) to identify phenotypic changes. These findings were correlated to population-based sequencing and UDPS, as described for Group A (sG145R) in this Chapter. In cases where there was a known mutation in the samples, in-vitro generated samples described in Chapter 4 were co-plotted with the BioPlex results of the clinical samples in order to look for similar or common features. BioPlex results of HBV from the mothers were analysed to identify potential biomarkers associated with transmission of virus.

Phylogenetic analysis was performed to determine the clade relationships of both mother and babies and this included using the Neighbor-Joining method and the optimal tree is shown as Figure 5.1B. The analysis was performed by Dr Margaret Littlejohn and done using MEGA 5. This analysis showed the HBV of mother-baby pairs were from the same clade (blue boxes).
### Table 5.4

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Genotype/Serotype</th>
<th>HBV S-gene population-based Sequencing (VIDRL)</th>
<th>Ultradeep Pyrosequencing (UDPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All changes in S-gene</td>
<td>Filtered** Changes</td>
</tr>
<tr>
<td>M1</td>
<td>B2 adw</td>
<td>sY161F, I198M, F200Y</td>
<td>Nil</td>
</tr>
<tr>
<td>B1</td>
<td>B2 adw</td>
<td>sY161F, I198M, F200Y</td>
<td>Nil</td>
</tr>
<tr>
<td>M2</td>
<td>D1 ayw</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>
| B2 §        | D1 ayw            | Nil                     | Nil                       | **sP127S - 10.56%**  
|             |                   |                          |                            | **sD144A - 2.3%**  
|             |                   |                          |                            | sG130E - 4.94% |
| M3          | B4 ayw            | Nil                     | Nil                       | sP127S - 1.63% |
| B3          | B4 ayw            | Nil                     | Nil                       | Nil |
| M4          | B4 ayw            | sF200Y, D144E/D         | sD144E/D                  | sD144E - 55.59%  
|             |                   |                          |                            | sG145A - 19.72%  
|             |                   |                          |                            | sQ101H - 6.83% |
| B4 *        | B4 ayw            | sF200Y, D144E/D         | sD144E/D                  | Insufficient specimen |
| M5          | B4 ayw            | sF200Y                  | Nil                       | Nil |
| B5          | B4 ayw            | sF200Y                  | Nil                       | Nil |
| M6          | C1 adr            | A184V, S210N, Y221C    | Nil                       | Nil |
| B6 §        | C1 adr            | Q101H, D144A, A184V, S210N, Y221C | sQ101H, D144A | **sD144A -98.33%**  
|             |                   |                          |                            | **sG145R - 1.49%**  
|             |                   |                          |                            | sQ101H - 98.56% |
| M7          | D1 ayw            | Nil                     | Nil                       | Insufficient specimen |
| B7          | D1 ayw            | Nil                     | Nil                       | Insufficient specimen |

**Abbreviation** – B – Baby, M – Mother, ★ – B4 did not receive HBV Ig at birth § – sample that showed S-gene changes associated with VEM (e.g. sD144A and sG145R) on UDPS, however, not present in the mother’s sample. The substitutions are highlighted with a red box.

**“Filtered changes”** excludes all the polymorphism, unfiltered are all the changes identified on sequencing.

Table 5.4 shows the S-gene genotype, serotype and S-gene sequence by population based sequencing, which divided into all changes seen and filtered changes. UDPS was performed for mother-baby pair shown. Of note is the presence of sD144A (B2) and sG145R and sD144A (B6) not identified in mothers (M2 and M6) in either population-based sequencing or UDPS, marked by a red box.
Figure 5.1B. Phylogenetic analysis of HBV sequences of seven mother/baby pairs, showing the same clade arrangement for each mother and baby. Analysis provided by Dr M Littlejohn and Dr L Yuen. Genotype C show 16 subtypes [346].
5.4 Results

5.4.1 A - sG145R: Canonical VEM (see Table 5.1)

Figure 5.2 shows the BioPlex profile of the in-vitro reference sample of HBsAg derived from cloned sG145R, presented in Chapter 4 (see Figure 4.8 and Table 4.5). The graph is normalized to the genetic backbone A2 adw-2. There are nine changes in the BioPlex profile, these changes included loss of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), loss of binding for mAb 8, 12 and 16 and gain of mAb 17 (Loop 2), loss of binding of mAb 2 (C-terminal) and mAb 9 (conformation dependent mAb). The relative proportion of HBsAg in this in-vitro sample can be assumed to be 100% of the sG145R genotype and therefore the VEM phenotype. For all of the following clinical samples, the BioPlex phenotype of this in-vitro sample will be co-plotted for comparison.

Figure 5.3 shows clinical sample 1, HBV genotype C adr (orange bars) co-plotted with the reference in-vitro HBsAg profile (blue) of sG145R. Overall there are significant changes but only four changes consistent with the in-vitro VEM reference sample. These changes in the BioPlex profile included loss of mAb 1 (N-term), mAb 8 and 12 (Loop 2) and mAb 9 (conformation dependent). In addition to these, there were other changes in the clinical sample 1 that were not observed with in-vitro sG145R reference cloned sample, including loss of binding at mAb 17 and 4, Loop 2 and C-terminal mAbs, respectively. These findings were then compared to the sequence changes in the ‘a’ determinant identified by population-based sequencing. The main ‘filtered’ variants were sG145R/G and sW172* (Table 5.1). Upstream changes at sA184V, sS210N and sY221C were also observed, however these three substitutions have not been implicated with vaccine failure, whilst sW172* (as a consequence of rtA181T) has been implicated with a virus secretion defect and antiviral drug resistance[330, 331]. Ultra-deep Pyrosequencing (see Table 5.1) of this sample revealed the relative proportion of sG145R to be 17.84%. Therefore, since the cloned HBsAg sample contained 100% of HBsAg with the same sequence, a lower relative proportion (17.84%) would be expected to show fewer changes, (4/9, see Table 5.5) and might possibly explain the opposite effect on mAb 17.

Figure 5.4 shows the BioPlex findings of sample 2 (mixed population of sG145R/G), HBV genotype C adr (orange) (see Table 5.1) and co-plotted against in-vitro (reference) derived HBsAg (blue), as reference sample with the substitution sG145R. This clinical sample is genotype C adr and has a mixed population of sG145R/G and the sM133T change shown on population-based sequencing as the major variants (Table 5.1).
BioPlex analysis showed six changes compared to the nine changes consistent with in-vitro cloned reference sG145R (see Table 5.5) and these included loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), mAbs 8 and 12 (Loop 2) and mAb 9 (conformation). Whilst population-based sequencing showed a mixed population of sG145R/G, UDPS identified the relative proportion of sG145R to be 36.56%. Whilst this is still less than 50%, the mAb profile of sample 2 was more consistent with the reference sample of sG145R (blue bars in Figure 5.3). This could possibly indicate that other ‘a’ determinant changes identified by population based sequencing such as sQ101K, T131N, M133T (see Table 5.1 and Table 5.5) may have contributed to the profile. Furthermore, the BioPlex profile of sample 2 also showed three changes; losses of mAb 10 (Loop 1), mAb7 and 17 (Loop 2) that were not consistent with the in-vitro reference sG145R profile. These three changes may be due to other S-gene changes identified by population-based sequencing and UDPS (sM133T, sT131N, sQ101K). Interestingly, sM133T has also been associated with vaccine failure and had the highest relative proportion of 62.9% in sample 2 as revealed by UDPS.

Figure 5.5 shows the BioPlex profile of sample 3, (sG145R, dominant) HBV genotype C adr (orange), co-plotted against the reference in-vitro derived HBsAg with substitution sG145R (blue), as reference sample. This clinical sample 3 exhibited all the epitope profile features (9, see Table 5.5) seen in the reference sample sG145R, including a gain in mAb 17 (red box). These findings were consistent with the relative proportion of sG145R of 99.72% as analysed by UDPS (see Table 5.5). In addition to these findings, this sample had 5 other epitope changes in the BioPlex not seen with in-vitro sG145R, and these included losses at mAb 7, 11, 19 (Loop 2), mAb 14 (Combination) and mAb 4 (C-terminal). Population-based sequence analysis revealed sW182*/W and UDPS showed HBV variants sS136Y and sK141R with relative proportion of 81.26% and 18% respectively (see Table 5.5). Whilst both of these variants may explain the differences in the BioPlex profile, the sS136Y had a higher relative proportion, implying that it is more likely to result in these changes observed on the BioPlex analysis. The sS136Y, is immediately before the first cysteine of the sC137 of the cysteine raft (see Figure 5.1) and a change from serine (S) to tryptophan (T), one of the largest amino acids, could be the cause of a significant change or shift in mAb binding.

Figure 5.6 shows the BioPlex findings of clinical sample 4 HBV genotype C adr (orange), co-plotted against the reference in-vitro derived reference sample of sG145R. There are eight out of nine changes (see Table 5.5) in sample 3 that coincide with the reference sample sG145R. These include loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop
mAb 8, 12 and 16 (Loop 2) mAb 2 (C-terminal) and mAb 9 (Conformation dependent mAb). The only change not observed in the BioPlex analysis that could ‘define’ a VEM for this sample was the gain in mAb 17. Due to insufficient DNA remaining in the sample, UDPS could not be performed. However, population based sequencing showed sG145R, without any evidence of a mixed population (Table 5.1) and other changes identified in the ‘a’ determinant included sS136Y, sL192F and sI195M which may have contributed towards changes observed in BioPlex not consistent with the *in-vitro* reference sG145R. These changes were gain of mAb 10 (Loop 1) mAb 7, 11, 19 (Loop 2), mAb 13 and 14 (Combination) and mAb 4 (C-terminal) however, this cannot be fully interpreted in the absence of reference sample of sS136Y as a comparator.

Figure 5.7 shows the BioPlex analysis of sample 5 (sG145R/G, mixed population), HBV genotype C *adr*, co-plotted against the reference *in-vitro* derived reference sample of sG145R. This variant showed three out of the nine characteristic changes (Table 5.5) found in the reference *in-vitro* sample G145R. These changes were loss of mAb 8 and 12 (Loop 2) and mAb 9 (conformation dependent). The presence of only 3/9 (see Table 5.5) changes in common with *the in-vitro* sG145R may reflect the lower relative proportion of sG145R of 21.11% (see Table 5.1) as revealed by UDPS. Interestingly there were no changes in the BioPlex analysis of the sample that were not consistent with the reference sample of sG145R. Furthermore, UDPS did not identify any additional changes in the S-gene. Population-based sequencing identified sW182*/W (Table 5.1), a change to a mixture of stop codon and wild-type sequence. The impact of such a change observed in the BioPlex is unclear at this stage and like sS136Y presented above, would require construction of an *in-vitro* reference sample.

Figure 5.8 shows the BioPlex profile of sample 6 (mixed population of (sG145R/G) on HBV genotype C *adr* backbone, co-plotted against the reference *in-vitro* derived reference sample of sG145R. There were three out of nine changes consistent with the reference variant (see Table 5.5). These changes were all around Loop 2 mAbs and included loss of mAb 8, 12 and 16. The relative proportion of sG145R on UDPS was 46.53%. Other changes in the ‘a’ determinant (see Table 5.1) included the introduction of possible N-glycosylation sites at codons 115 (sT115N) at 21.01%, codon 123 (sT123N) at 18.21% and codon 126 (sI126N) at 23.56%. Despite these changes, there were no other changes in the phenotypic profile except for those that are typically associated with the reference sample sG145R. There was no significant change in mAb 17.
Figure 5.9 shows the BioPlex analysis of sample 7 of HBV (sG145R/G mixed population) and genotype C adr (orange) compared to the reference (in-vitro derived) sample sG145R (blue). This sample had only 2/9 mAb changes (see Table 5.5) that were found against the reference sG145R and these included loss of binding mAb 12 and 16, both Loop 2 mAbs. This was the only sample in this group that did not show loss of mAb 8 and a lower relative proportion of sG145R on UDPS, of 20.75%. Other changes observed included loss of mAb 11, 17 and 19 (Loop 2) and gain of mAb 5 (Loop 1), these changes are not seen with the sG145R reference isolate. Population-based sequencing showed changes in the S-gene and these included sA184V also seen with other samples (see Table 5.1).

Figure 5.10 shows the BioPlex analysis of sample 8 of HBV variant sG145R/G, genotype B ayw backbone (orange) compared to the reference in-vitro derived sG145R (blue). There were three changes common with sG145R and included loss of binding of mAbs 8, 12 and 16, all of which were Loop 2 mAbs. The relative proportion of sG145R was 23.9% (see Table 5.2). There were four changes that were not consistent with sG145R, loss of mAb 10 (Loop 2), 17 and 19 (Loop 2) and gain of mAb 7 (Loop 2). Population-based sequencing showed a mixed population of sG145R/G and did not show other changes in the ‘a’ determinant. However the HBV sample did have changes upstream from the ‘a’ determinant, these were sA184V and sequence from the sF200Y, whilst UDPS did not show any HBV variants with S-gene mutation, reflecting the smaller amplicon from 40-160.

Figure 5.11 shows the BioPlex profile for sample 9 with HBV variant sG145R/G, genotype C adr (orange) co-plotted against the reference in-vitro derived sample sG145R. There were eight out of nine changes (see Table 5.5) in sample 9 consistent with reference sample sG145R. These included loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), mAb 8, 12 and 16 (Loop 2), mAb 2 (C-terminal) and mAb 9 (conformation dependent mAb). The only change not observed in the BioPlex analysis of this sample was the gain in mAb 17. The relative ratio of sG145R on UDPS was 74.32%. Population-based sequencing also confirmed the mixed population at codon 145 (G/R), 130 (sG130N/R/S/E/D) and sT116N. UDPS did not detect the changes at codon 130 detected by population-based sequencing (sG145R/G). However, it did identify sT116N with a relative ratio of 39.3% (see Table 5.1). Other changes identified were sY100C (14.54%), sT155N (15.96%), sT115N (4.09%) sT115A (10.83%) and sT116N/T (39.31%). S-gene changes identified by population-based sequencing and UDPS may explain six of the changes seen in sample 9 that were not consistent with the reference
sample and these included loss of mAb 7, 11, 17 and 19 (Loop 2), mAb 14 (combination of both loops) and mAb 4 (C-terminal).

Figure 5.12 shows the BioPlex profile of the reference in-vitro derived sample of sG145R (blue) co-plotted against clinical sample 10, HBV genotype C adr (orange). There were three out of nine changes (see Table 5.5) in sample 10 that were co-incident with the reference sample sG145R. These changes were around Loop 2 - mAbs 8, 12 and 16. The presence of fewer changes (3/9) compared to the sG145R could be again due to the relative lower proportion of sG145R of 28.27% identified by UDPS (see Table 5.1). Changes not consistent with reference sample sG145R included loss of reactivity with mAb 17 and 19 (Loop 2). However, the lower relative proportion of sQ101K (1.45%) and sT123N (1.45%) would not be expected to explain these changes. Interestingly population-based sequencing identified sW172*/W, sA184V, sS210N and sY221C and these substitutions could have resulted in the BioPlex changes in Loop 2. However, this was not validated in this study.

In summary (see Table 5.5), there was a reasonable correlation between BioPlex profiles seen with HBsAg derived by cloned HBV S-gene variant (sG145R) and those of serum samples from patients with CHB infected with sG145R but this appeared to only correlate directly when the relative proportion detected by UDPS was well over 90%. The number of changes seen in individual serum sample tended to correlate with the relative population of in-vitro generated sG145R population. Populations of sG145R greater than 70% tended to exhibit almost all the nine characteristic changes of the sG145R VEM (sample 3 and 9), whilst this decreased if the sample contained sG145R in a relative proportion of less than 30% (samples 1, 5, 7, 8 and 10). Of all the BioPlex changes associated with the reference sample of sG145R, gain in mAb 17 was only seen in one sample where the relative population was 99.7%. Thus for clinical isolates, gain in mAb 17 would not appear to be a useful screening biomarker for sG145R/VEM. In contrast, loss of mAb 12 binding was found in all the samples tested and further clinical studies on this epitope should be explored. Similarly, mAb 8 (nine samples) and mAb 16 (seven samples) could also prove useful in helping to define VEM phenotype, both alone and in combination with mAb 12. Other than sG145R, presence of HBV variant sM133T appeared to have contributed to changes similar to sG145R (sample 2), the latter has also been implicated as a VEM but the published evidence is not as strong as for sG145R. Thus, two phenotype clusters were identified: gain of mAb 17 and loss of mAb 8, 12 and 16. The latter profile occurred alone or in combination.
Figure 5.2

Figure 5.2. BioPlex analysis from the in-vitro generated HBsAg variant sG145R, as shown in Chapter 4 (see Figure 4.8). The graph is normalized to the genetic backbone A2-adw-2. There are nine changes; loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), mAb 8, 12 and 16 (Loop 2), mAb 2 (C-terminal) and mAb 9 (conformation). There is one gain in reactivity, mAb 17 (Loop 2).

Figure 5.3

Figure 5.3 shows the BioPlex profile of clinical sample 1 (orange), genotype C adr and co-plotted with sG145R (blue). There are four changes in sample 1 that are consistent with the reference sample, gain of binding in mAb 1 (N-terminal), 8 and 12 (Loop 2) and 9 (conformation). There were changes in the clinical sample not consistent with the reference sample, these included loss of binding of mAb 17 and mAb 4 (C-terminal). The UDPS showed relative population sG145R of 17.8%, explaining the fewer changes.
Figure 5.4 shows the BioPlex profile of clinical sample 2 (orange), genotype C adr and sG145R compared to reference sample sG145R (blue). The relative proportion of sG145R variant in sample 2 was 36.56%, other variants were sQ101K = 2.65%, sT131N = 55.35%, sM133T = 62.96%. There were six changes out of nine in sample 3, which were consistent with changes in the reference sample. These included loss of mAb 1 (N-terminal), mAbs 5 and 6 (Loop 1), mAb 8 and 12 (Loop 2) and mAb 9 (conformation dependent).

Figure 5.5 shows the BioPlex analysis of reference sG145R (blue) and clinical sample 3 (orange). All the nine changes seen in the reference sample were also seen in sample 3 and this in concordance with the relative ratio of 99.72% of sG145R seen on UDPS. This sample also exhibited a gain of mAb 17 (red box) seen with the sG145R reference sample and other commonly claimed VEMs. In addition to these changes, there were five changes not consistent with sG145R and these included gain in mAb 10 (Loop 1) and loss of mAb 7, 11 and 19 (Loop 2) and mAb 4. These additional changes may be due to high relative ratio of sS136Y (81.26%), whilst there was a lower proportion of sK141R (18%) seen on UDPS.
Figure 5.6 shows the BioPlex changes of clinical sample 4 (orange) compared to reference sample of sG145R (blue). There were 8/9 changes in sample 4, consistent with reference sample and these changes were loss of mAb 1 (N-terminal), 5, 6 (Loop 1), 8, 12 and 16 (Loop 2), 2 (C-terminal) and 9 (conformation dependent). There were five changes not consistent with sG145R and these included gain in mAb 10 (Loop 1) and loss of mAb 7, 11 and 19 (Loop 2) and mAb 4. UDPS was not performed for this sample, however, population-based sequencing showed evidence of variants sS136Y, V177Y, A184V, L192F, I195M, S210N and Y221C.

Figure 5.7 shows the BioPlex analysis of clinical sample 5, compared to the reference sample of sG145R. There were 3/9 changes in the sample consistent with the reference sample. These changes were loss of mAb 8 and 12 (Loop 2) and mAb 9 (conformation dependent). UDPS showed a relatively lower proportion of sG145R in sample 5 of 21.11%. There were no other changes in the BioPlex profile and these were in keeping with lack of variants seen on UDPS.
Figure 5.8 shows sample 6, genotype C adr compared to reference sample of sG145R. There are three changes out of a potential nine changes in sample 3 consistent with the reference sample. There were no changes in this sample except those associated with sG145R. UDPS showed the relative proportion of sG145R to be 46.53%. There were minor population of other variants sL1104F = 21.01%, sT115N = 17.5%, sT123N = 18.21%, sI126N= 23.56%.

Figure 5.9 shows the BioPlex profile of clinical sample 7, genotype C adr compared to reference sample sG145R. There were 2/9 changes that were consistent with sG145R and included loss of mAb 12 and 16. There were two changes not consistent with sG145R and these were loss of mAb 17 and 19 (Loop 2). Fewer changes were seen in this sample possibly due to lower relatively lower sG145R proportion on UDPS of 20.75%.
Figure 5.10 shows clinical sample 8 genotype B and serotype ayw compared to reference sample sG145R. There were three out of a potential nine changes in this sample consistent with the reference sample sG145R. UDPS showed a lower relative proportion of sG145R (23.94%). There were no changes in the BioPlex profile other than those associated with sG145R. There was loss of mAb 10 (Loop 7), 17 and 19 (Loop 2) that were not consistent with sG145R.

Figure 5.11 shows clinical sample 9, genotype/serotype C adr compared to the reference sample sG145R. There were 8/9 changes in the sample consistent with sG145R which may reflect the higher relative ratio of sG145R at 74.32%. There were six changes in the BioPlex profile that was not consistent with sG145R, loss of mAb 7, 11, 17 and 19 (Loop 2), mAb 14 (combination) and mAb 4 (C-terminal). UDPS showed other changes that may explain these non sG145R changes and these variants were sY100C = 14.54%, sT155N = 15.96%, sT115N = 4.09%, sT115A= 10.83%, sT116N = 39.31%.
Figure 5.12 shows the BioPlex analysis of clinical sample 10, compared to the reference sample. Out of nine possible changes possible seen in sG145R reference sample, sample 10 shows three changes, these include mAb 8, 12 and 16 (Loop 2). There were two other changes in sample 3, not seen in reference sample 3 and included loss of reactivity to mAb 17 and 19 (Loop 2). UDPS showed the relative proportion of sG145R was 28.27%, other changes were sQ101K (1.45%) and sT123N (1.45%).
Table 5.5

<table>
<thead>
<tr>
<th>Sample</th>
<th>RP of sG145R</th>
<th>N-term mAb</th>
<th>Loop 1 mAb</th>
<th>Loop 2 mAb</th>
<th>C-term mAb</th>
<th>Conform mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Ref sG145R</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>99.7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>74.32%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>46.53%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>28.27%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>23.94%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>20.75%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17.82%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Insufficient Specimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: sn – supernatant, RP – relative proportion, Ref – reference, sn- supernatant loss of mAb binding, gain in mAb binding.

Table 5.5. Summary of BioPlex findings consistent with sG145R from the reference sample, analysed in Chapter 4 and the findings incorporated in this Table. The samples have been arranged in the order of descending relative proportion of sG145R, from ultradepth pyrosequencing (UDPS). RP values of greater than 74% showed eight or more BioPlex changes consistent with the reference sample of sG145R, whilst those samples less than 50% had two or more changes. The most consistent finding was the loss of reactivity of mAb 12, found in all samples, followed by loss of mAb 8 found in 9/10 samples and loss of mAb 16 was found in 7/10 samples. The least common finding was the gain of mAb 17, exhibited by one sample (sample 3) and RP of sG145R was >99%. Sample 4 could be assumed to have a RP of sG145R of >80%, based on correlations seen with other samples.
5.4.2 Group B) Vaccine/HBlg and Diagnostic Escape Variants (see Table 5.2)

Figure 5.13 shows the BioPlex analysis of sample V1, HBV genotype B ayw with the substitution sD144E (see Table 5.2), co-plotted with *in-vitro* generated reference sD144E on genotype A2 adw backbone, as analysed previously in Chapter 4. Both samples have been normalized to their respective genotypes. The *in-vitro* generated sample has eight phenotypic changes, including loss of binding of mAb 5 and 6 (Loop 1), mAb 7, 8, 12, 16 and 19 (Loop 2) and mAb 9 (conformation dependent). Out of these eight changes, clinical sample V1 had three changes consistent with the *in-vitro* sample which included loss in binding of mAb 6 (Loop1) and mAbs 8 and 19 (Loop 2) as indicated by blue boxes.

Figure 5.14 shows the BioPlex analysis of sample V2, HBV genotype A ayw with the substitution sD144E (see Table 5.2), co-plotted with *in-vitro* generated reference sD144E on genotype A2 adw backbone, as analysed previously in Chapter 4. Both samples have been normalized to their respective genotypes. The *in-vitro* generated sample has eight phenotypic changes, as described above. From these eight changes, clinical sample V2 had one change consistent with the *in-vitro* sample, loss in binding mAb 6, marked by a blue box. An unexpected finding in the clinical specimen V2 was that of a gain in mAb 17 (Loop 2), marked by a red box and as previously shown in Chapter 4, this change was seen in genotypes E ayw, F adw, sG145R, sG145A, double mutation sD144E and sG145R, sM133L, sS132A, sT126N, sE164D and sW196S (see Chapter 4, Table 4.5 and Table 4.6). Other changes in clinical sample V2 included gain of mAb 10 (Loop 1), mAb 12 (Loop 2) and loss of binding of mAb 11 (Loop 2).

Figure 5.15 shows the BioPlex analysis of sample V3, HBV genotype A ayw with change sD144E (see Table 5.2), co-plotted with *in-vitro* generated reference sD144E on genotype A2 adw backbone as analysed previously in Chapter 4. Both samples have been normalized to their respective genotypes. The BioPlex profile of *in-vitro* generated sample has eight phenotypic changes, as previously described. Clinical sample V3 shows only one change consistent with sD144E, loss of mAb 19 (conformation dependent mAb). There were a number of changes in V3 not exhibited by the *in-vitro* construct and these included gain of binding at mAb 5, 6 and 10 (Loop 1), mAb 7, 8, 12 and 17 (loop 2) and mAb 9 (conformation dependent). As observed with clinical sample V2 (Figure 5.14), the gain of mAb 17 was also apparent for V3 (red box) and has been previously seen with genotypes E ayw, F adw, sG145R, sG145A, double mutation sD144E and sG145R, sM133L, sS132A, sT126N, sE164D and sW196S (see Chapter 4, Table 4.5 and Table 4.6).
Figure 5.16 shows the BioPlex analysis of sample V4, HBV genotype B ayw with a mixed variant population sD144E/D (see Table 5.2), co-plotted with in-vitro generated reference sD144E on genotype A2 adw backbone as analysed previously in Chapter 4. Both samples have been normalized to their respective genotypes. The BioPlex profile of in-vitro generated sample sD144E, has 8 phenotypic changes, these include loss of binding of mAb 5 and 6 (Loop 1), mAb 7, 8, 12, 16 and 19 (Loop 2) and mAb 9 (conformation dependent). Clinical sample V4 has mixed population of sD144E/D and exhibited two changes consistent with the reference in-vitro generated sample and these included loss of mAb 12 and 19, Loop 2 mAbs. Other changes for V4 were gain in mAb 9 (conformation) and mAb 8 (Loop 2).

Figure 5.17 shows the BioPlex profile of clinical profile V5, HBV genotype B ayw (purple) with the substitution sD144A compared to in-vitro generated sD144A (green). Both samples have been normalized to their respective genotype. There were six changes in the in-vitro cloned sD144A, and these include loss of binding of mAb 5 and 6 (Loop 1), mAb 8, 11 and 12 (Loop 2) and mAb 9 (conformation dependent). Population-based sequencing of clinical sample V5 showed the presence of sD144A but there were no BioPlex changes consistent with the BioPlex profile of the synthetic sD144A. However, there were 10 changes in the BioPlex profile of V5 and these included gain in binding of mAb 5 and 6 (Loop 1), mAb 7, 8 and 17 (Loop 2), mAb 13 (Loop 1 and 2), mAb 2 and 4 (C-terminal), MAb 9 and 18 (conformation). Interestingly, gain of mAb 17 (Loop 2), exhibited by genotypes E ayw, F adw, sG145R, sG145A, double mutation sD144E and sG145R, sM133L, sS132A, sT126N, sE164D and sW196S was also noted in this sample (red box). In addition, gain of mAb 13 (Loop 1 and 2, combination) (blue arrow) has only been seen with genotype F adw (see Chapter 4, Table 4.4).

Figure 5.18 shows the BioPlex profile of clinical sample V6, HBV genotype A adw with the substitutions sP120T, co-plotted with in-vitro generated sP120T. There are five BioPlex changes in the in-vitro sample and these include loss of binding of mAb 5 and 6 (Loop 1), mAb 7 (Loop 2) and mAb 15 (combination) and gain of binding at mAb 17 (Loop 2). Clinical sample V6 had four changes consistent with BioPlex findings of the in-vitro construct. These phenotypic changes were loss of binding at mAb 5 and 6 (Loop 1), mAb 7 (Loop 2) and mAb 15 (combination). There were four other changes in the clinical sample V6 and these included loss of binding at mAb 10 (Loop 1), mAb 8 and 17 (Loop 2) and mAb 9 (conformation).
Figure 5.19 shows the BioPlex profile of clinical sample V7, a diagnostic escape isolate, a diagnostic escape isolate, HBV genotype D ayw, and with substitutions sG145A and sP120Q, co-plotted with *in-vitro* construct of sG145A. Clinical sample V7 was referred to VIDRL because the sample initially tested negative for HBsAg. There were five phenotypic changes in the BioPlex profile of sG145A in the *in-vitro* sample, and these include loss of binding at mAb 1 (N-terminal), mAb 6 (Loop 1), mAb 8 and 12 (Loop 2) and gain of binding at mAb 17. V7, the clinical specimen exhibited two changes consistent with the *in-vitro* construct, loss of mAb 1 (blue box) and gain in mAb 17 (red box). There were 10 other changes in V7, not seen with the *in-vitro* construct, including gain for mAb 5 and 6 (Loop 1), mAb 7 (Loop 2), mAb 3 (C-terminal) and mAb 9 and 18 (conformation), and loss of binding of mAb 16 and 19 (Loop 2), mAb 13 and 15 (combination). Since the clinical sample V7 had sP120Q and had an altered phenotypic profile (12/19 mAb changes), this sample was compared to other *in-vitro* constructs, sP120T and sP120L.

Figure 5.20 shows the BioPlex profile of clinical sample V7, HBV genotype D ayw, with substitutions sG145A and sP120Q, co-plotted with *in-vitro* construct of sP120T. HBsAg derived from *in-vitro* construct sP120T has five changes in the phenotypic profile, loss of binding at mAb 5 and 6 (Loop 1), loss of binding at mAb 7 and gain at mAb 17 (Loop 2) and loss of mAb 15 (combination). A common change observed with the both *in-vitro* constructs sP120T and sG145A with V7 was a gain of mAb 17 (red box, Figure 5.19 and 5.20). An interesting finding of the HBV variants studied in Chapter 4 (see Chapter 4, Table 4.5) was loss of binding of mAb 15 (combination) seen with substitutions at surface amino acid position 120; this was also observed with V7 (sG145A and sP120Q), marked with a blue box.

Figure 5.21 shows the BioPlex profile of V7, HBV genotype D ayw and double mutations sG145A and sP120Q, co-plotted with *in-vitro* generated sP120L. There were 11/19 changes in the cloned sP120L, these include loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), mAb 7, 8 and 16 (Loop 2), all combination mAbs, 13, 14 and 15, mAb 2 and 4 (C-terminal). Compared to previously analysis of sG145A (Figure 5.19) and sP120T (see Figure 5.21), clinical sample V7 had the most number of consistent changes compared to the reference sample sP120L shown in this analysis. These changes were loss of binding at mAb 1 (N-terminal), mAb 16 (Loop 2), mAb 13 and 15 (combination) and mAb 2 (C-terminal). In this analysis, and that done in Chapter 4 (see Table 4.4 and 4.5), the loss of binding of 2/3 combination mAbs (13 and 15) has only been seen with
sP120L and V7, presumably due to the change of sP120Q, whilst sP120T showed loss of mAb 15 (combination) only.

Figure 5.22 shows the BioPlex analysis of clinical sample V8, HBV genotype D ayw with substitutions sP120P/S and sY134N/Y, co-plotted with in-vitro generated sP120T. There were five BioPlex changes in the in-vitro sample and these include loss of binding of mAb 5 and 6 (Loop 1), mAb 7 (Loop 2) and mAb 15 (combination) and gain of binding at mAb 17 (Loop 2). There were three changes in the BioPlex profile of V8 consistent with sP120T, mAb 5 and 6 (Loop 1) and mAb 7 (Loop 8). There were, however, six other changes exhibited by V8. These were gain mAb 1 (N-terminal), mAb 16 (Loop 2), mAb 14 (Combination) and mAb 18 (conformation) and loss of binding at mAb 8 (Loop 2) and mAb 9 (conformation).

Figure 5.23 shows the BioPlex analysis of clinical sample V8, HBV genotype D ayw with substitutions sP120P/S and sY134N/Y, co-plotted with in-vitro generated sP120L. There were 11 changes observed with sP120L, these include loss of binding of mAb 1 (N-terminal), mAb 5 and 6 (Loop 1), mAb 7, 8 and 16 (Loop 2), all of combination mAbs, 13, 14 and 15, mAb 2 and 4 (C-terminal). There were four changes seen in the profiles of both samples, loss of binding of mAb 5 and 6 (Loop 1), and mAb 7 and 8 (Loop 2). Compared to sP120T (see Figure 5.22), V8 had only one extra change consistent with sP120L, however, loss of mAb 15 (combination) seen with in-vitro sample sP120T and sP120L and clinical sample V7 (sG145A and sP120Q) was not observed for V8, despite the presence of a mixed population of sP120P/S. Other significant changes in V8 were gain in binding in mAb 1 (N-terminal), mAb 16 (Loop 2), mAb 14 (combination) and mAb 18 (conformation) and loss of binding mAb 9 (conformation).

Figure 5.24 shows the BioPlex profile of V8, HBV genotype A adw, with substitutions sP120P/S and sY134Y/N. These substitutions have been associated with vaccine escape profile and in this analysis it is co-plotted with in-vitro generated sG145R. There were nine changes in the in-vitro generated sample, and these included loss of mAb 1 (N-terminal) mAb 5 and 6 (Loop 1), loss of binding for mAb 8, 12 and 16 and gain of mAb 17 (Loop 2), loss of binding of mAb 2 (C-terminal) and mAb 9 (conformation dependent mAb). Clinical sample V8 showed four common changes (blue boxes) and these were loss of binding of mAb 5 and 6 (Loop 1), mAb 8 (Loop 2) and mAb 9 (conformation).

Figure 5.25 showed the BioPlex profile of clinical sample V9, HBV genotype B adw and in-vitro generated HBsAg construct with mutation sM133L, both samples have been normalized to its respective genotype. In-vitro generated shows 2 changes, gain in
binding of mAb 17 (Loop 2) and mAb 9 (conformation). The clinical sample showed one change, gain in binding of mAb 17 (Loop 2), there are, however, other changes in the epitope profile and these include loss of binding at mAb 6 (Loop 1) and mAb 8 (Loop 2).

Figure 5.26 shows the BioPlex profile of clinical sample V10, referred to VIDRL for further studies due to diagnostic failure (see Table 5.2). Population-based sequencing showed HBV genotype D ayw and substitution sT123S. The BioPlex profile of V10 is compared to the in-vitro construct sG145R, and both samples are normalized to their respective genotype. Clinical sample V10 had six changes in the BioPlex profile, however, only 2 changes were in common with sG145R, and these included gain in binding of mAb 17(red box) (Loop 2) and loss mAb 8 (Loop 2) (blue boxes). Other changes displayed by V10 included loss of binding for mAb 10 (Loop 1), gain of mAb 11, 12 and 19 (Loop 2).

Figure 5.27 shows the BioPlex profile of clinical sample V11, HBV genotype C adr with the substitution sT126I. The sample was referred to VIDRL for diagnostic work-up after failure to be detected by the diagnostic assay. Clinical sample V11 was compared to in-vitro construct of sG145R, to evaluate if there were any similarities in the profiles, however, there were no changes common to both. Sample V11 did display a number of changes in the phenotypic profile, these included gain in binding of mAb 1 (N-terminal), mAb 5 (Loop 1), mAb 2(Loop 2), mAb 13 (combination), mAb 2 and 4 (C-terminal) and loss of mAb 19 (Loop 2).

In summary (see Table 5.6), commonly claimed VEMs studied in this group were sD144E, sD144E, sP120T, sP120L, sP120P/S and sM133L and those associated with diagnostic failure, which included sT123S, sT126I and the double mutation G145A and sl123T. There was little correlation between the in-vitro constructs and the clinical samples. However, in-vitro generated HBsAg variants such as sP120T demonstrated loss of mAb 15 (combination) also observed with the clinical samples (V6) with the substitution sP120T as determined by population-based sequencing. The variants associated with diagnostic failure had an altered phenotype, exhibiting six or more changes which were distributed across the various epitopes of the ‘a’ determinant and C-terminus. However, no characteristic pattern could be resolved in the BioPlex profiles. The two phenotypic profile changes seen with the clinical sample sG145R were gain in mAb 17, observed in six clinical samples in this group and the combination of loss in mAb 8 was seen in five samples and loss of mAb 12 and 16 was seen in 1 sample (see Table 5.6 and 5.7).
Figure 5.13 shows the BioPlex profile of clinical specimen V1, genotype B ayw with the substitution sD144E (purple), compared to HBsAg of in-vitro cloned HBV-S-gene variant, sD144E derived by site-directed mutagenesis (green). There were three changes in V1 consistent with the reference sample sD144E (blue boxes), these are loss of binding of mAb 6 (Loop 1), mAb 8 and 19 (Loop 2).

Figure 5.14 shows the BioPlex profile of clinical sample V2 (genotype A ayw and sD144E) and the in-vitro generated sD144E. There was one change consistent in both of the profiles, loss of mAb 6 (Loop 1), marked by the blue box. Clinical sample V2 also showed a gain of mAb 17 (red box), not associated with sD144E.
Figure 5.15 shows the BioPlex profile of clinical sample V3, genotype A ayw containing sD144E, and the in-vitro generated sD144E (genotype A2 adw), both samples have been normalized to their respective genotype. There was one change seen in both these samples, loss of binding of mAb 19 (blue box). There was gain in binding of mAb 17, (red box) which is not consistent with the profile of in-vitro generated sD144E, however, this was also seen in sample V2.

Figure 5.16 shows the BioPlex profile of clinical sample V4 with mixed population of sD144E/D, genotype B ayw. There were no changes consistent in V4 that was consistent with the reference sample sD144E, however, of note was the gain in mAb 17 (Loop 2), marked by the red box.
Figure 5.17 shows the BioPlex profile of clinical sample V5, genotype B ayw with the mutation sD144A, co-plotted with in-vitro generated sD144A. There were no changes in V5 consistent with sD144A, however there was a gain in binding of mAb 17 (red box), generally associated with VEM profile, and gain in mAb 13 (blue arrow), previously seen only with genotype F adw.

Figure 5.18 shows the BioPlex profile of clinical sample V6, genotype A adw with sP120T and in-vitro construct of sP120T. There were four changes in the clinical sample that exhibit the same changes as that of the in-vitro construct (blue box). Other phenotypic changes include gain of binding at mAb 1 and loss of binding at mAbs 17 and 9.
Figure 5.19 shows the BioPlex profile of clinical sample V7, genotype D ayw, with the substitutions sG145A and sP120Q, compared to the in-vitro construct sG145A. There were 11 phenotypic changes in V7 and two of these changes were consistent with reference sample, including loss of binding of mAb 1 (N-terminal) and a gain in mAb 17 (Loop 2).

Figure 5.20 shows the BioPlex profile of V7 genotype D ayw (sG145A and sP120Q), compared to the in-vitro construct sP120T. Changes in the profile seen for both samples included a gain in binding of mAb 17 (Loop 2) and loss of mAb 15 (combination).
Figure 5.21

Figure 5.21 shows the BioPlex analysis of V7 (genotype D ayw, sG145A and sP120Q), compared to sP120L. There were four changes seen in both the samples, loss of binding of mAb 1 (N-terminal), mAb 16 (Loop 2), mAb 13 and 15 (Combination) and mAb 3 (C-terminal), marked by blue boxes.

Figure 5.22

Figure 5.22 shows the BioPlex profile of clinical sample V8, genotype A adw with substitutions sP120P/S and sY134Y/N, compared to sP120T. There were two changes consistent with in-vitro generated sP120T, loss of mAbs 5 and 6 (Loop 1), shown in blue boxes.
Figure 5.23 shows the BioPlex profile of clinical sample V8 (genotype D ayw, sP120P/S and sY134N/Y), compared to in-vitro generated sP120L. There were four changes seen with both samples, loss of binding of mAb 5 and 6 (Loop 1) and mAb 7 and 8 (Loop 2).

Figure 5.24 shows the BioPlex profile of sample V8 genotype A adw with substitutions sP120P/S and sY134Y/N compared to in-vitro generated sG145R. There were four changes in the phenotypic profile seen in both samples, loss of binding of mAb 5 and 6 (Loop 1), and mAb 8 (Loop 2) and mAb 9 (conformation), marked by blue boxes.
Figure 5.25 shows the epitope profile of clinical sample V9, genotype B adw, sM133L and in-vitro construct of sM133L. There is one change seen in both samples, gain of mAb 17 (Loop 2), marked by red box.

Figure 5.26 shows the BioPlex profile of clinical sample V10, genotype D ayw, sT123S, associated with diagnostic failure, compared to the in-vitro construct of sG145R. There were three changes in the phenotypic profile of V10, matching that of sG145R and this included loss of binding of mAb 1 (N-terminal) and mAb 8 (Loop 2), marked by blue boxes and a gain in mAb 17 (Loop2), marked by a red box.
Figure 5.27 shows the BioPlex profile of V11, genotype C adr, sT126I, compared to in-vitro construct sG145R. There were no BioPlex changes common to either of these samples.
Table 5.6

<table>
<thead>
<tr>
<th>Sample</th>
<th>N-term</th>
<th>Loop 1</th>
<th>Loop 2</th>
<th>Combination</th>
<th>C-term</th>
<th>Conform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  5  6 10  7  8  11  12  16  17  19  13  14  15  2  3  4  9  18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref sD144E</td>
<td>▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1 (B ayw sD144E)</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2 A ayw sD144E</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3 A ayw sD144E</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V4 B ayw sD144E</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref sD144A</td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V5 B ayw sD144A</td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref sP120T</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td>▼ ▼ ▼</td>
<td></td>
</tr>
<tr>
<td>Ref sP120L</td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
</tr>
<tr>
<td>Ref sG145R</td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
</tr>
<tr>
<td>V6 A ayw sP120T</td>
<td>▼ ▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td>▼ ▼ ▼ ▼ ▼</td>
<td></td>
</tr>
<tr>
<td>V8 D ayw sP120P/S sY134N/Y</td>
<td>▼ ▼ ▼ ▼</td>
<td>▼ ▼ ▼ ▼</td>
<td>▼ ▼ ▼ ▼</td>
<td>▼ ▼ ▼ ▼</td>
<td>▼ ▼ ▼ ▼</td>
<td>▼ ▼ ▼ ▼</td>
</tr>
<tr>
<td>Ref sM133L</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V9 B adw sM133L</td>
<td>▼ ▼ ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ref – Reference strain, ▼ gain in binding of mAb, ▼ loss of mAb binding
Table 5.6 shows the summary of BioPlex findings of commonly claimed VEM as outlined in Table 5.2. Each sample is compared to the in-vitro construct of HBsAg variants (red arrows) presented in Chapters 3 and 4.
<table>
<thead>
<tr>
<th>Sample</th>
<th>N-term</th>
<th>Loop 1</th>
<th>Loop 2</th>
<th>Combination</th>
<th>C-term</th>
<th>Conform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref sG145A</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref sP120T</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Ref sP120L</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>V7 D ayw sG145A sP120Q</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Ref sG145R</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>V10 D ayw sT123S</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>V11 C adr sT126I</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ref – Reference strain, ↓ loss in binding of mAb, ↑ gain of mAb binding

Table 5.7 shows the summary of BioPlex findings of the clinical samples associated with diagnostic failure. These samples were compared to the in-vitro constructs of HBsAg variants or sG145R, to evaluate for e features that is may be identifiable to a VEM phenotype.
5.4.3 Group C) Antiviral Associated Variants (see Table 5.3)

Figure 5.28 shows the BioPlex graph of clinical sample SR1, HBV genotype A adw with mixed populations as shown by population-based sequencing of sE164D/E, sI195M and sS210R, co-plotted with in-vitro generated sG145R. There were nine changes in the BioPlex profile of the in-vitro generated sG145R and these included loss of mAb 1 (N-terminal) mAb 5 and 6 (Loop 1), loss of binding for mAb 8, 12 and 16 and gain of mAb 17 (Loop 2), loss of binding of mAb 2 (C-terminal) and mAb 9 (conformation dependent mAb). Clinical sample SR1 had one similar change with sG145R, loss of mAb 9 (conformation), marked by the green box. Loss of binding of mAb 17 was seen in SR1, which was the opposite of what was observed with the reference sG145R phenotypic profile.

Figure 5.29 shows the HBsAg BioPlex profile of clinical sample SR2 that has HBV genotype D ayw, and population-based sequencing revealed mixed population of sE164D/E and sI195M, co-plotted with in-vitro generated sG145R. There were nine changes in the BioPlex profile of the in-vitro generated sG145R as described above. There were two changes in SR2, gain in mAb 6 (Loop 1) and mAb 7 (Loop 2), and neither of these changes were evident with the reference sG145R construct (see Table 5.8)

Figure 5.30 shows the BioPlex profile of HBsAg from SR3. Genotypic analysis of the infecting strain of HBV showed it to have genotype A adw and population-based sequencing revealed variants sE164E/D and sI195M. The graph is co-plotted with in-vitro construct of sG145R. Of the nine changes in the BioPlex profile of sG145R, there was one change in the BioPlex profile of SR3 consistent with the in-vitro construct, loss of mAb 8 (Loop 2), marked by a green box (see Table 5.8)

Figure 5.31 shows the BioPlex profile of HBsAg of SR4, a sample from a patient with CHB and co-infected with HIV. The HBV genotype in this patient sample was A adw and carries the antiviral resistance associated substitutions sE164D and sI195M. The BioPlex profile is co-plotted with in-vitro construct of sG145R. Of the nine changes associated with sG145R, SR4 shares three features and these include loss of mAb 5 (Loop 1), gain of binding for mAb 17 (Loop 2) and loss of binding of mAb 9 (conformation), marked by green and red boxes respectively (see Table 5.8).

Figure 5.32 shows the BioPlex profile of HBsAg from SR5, a sample from a patient with CHB, co-infected with HIV. The HBV genotype in this patient sample was A adw and has sE164E/D, sI195M and sS210R changes revealed by population-based sequencing. The profile is co-plotted with the in-vitro construct of sG145R. The BioPlex profile of SR5 did
not have any features consistent with sG145R, with two changes seen with SR5, loss of reactivity to mAb 17 and 19 (Loop 2) (see Table 5.8).

Figure 5.33 shows the BioPlex profile of HBsAg from SR6, a sample from a HBV/HIV co-infected patient. The HBV genotype/serotype in this patient was C adr, and had sE164D/sI195M and sF219S/F variants identified by on population-based sequencing. The profile was compared to sG145R, in-vitro construct. There were nine changes in the reference sG145R, however, there were only two changes in clinical sample SR6 similar to the reference strain, namely, loss of binding at mAb 12 (green box) and gain at mAb 17 (red box). Interestingly, gain of mAb 17 (Loop 2) as also seen with SR4 (see Figure 5.31 and Table 5.8), was exhibited by genotypes E ayw, F adw, sG145A, double variant sD144E and sG145R, sP120T, sM133L, sS132A, sT126N, sE164D and sW196S in the analysis described in Chapter 4 (see Chapter 4, Table 4.4 and 4.5).

Figure 5.34 shows the phenotypic profile of HBsAg from SR7, from the BioPlex platform. The HBV genotype of this sample was C adr, with substitutions sE164D, sW172W/C, sL173V, sL186L/R and sI195M. This graph is co-plotted with in-vitro construct sG145R. There were five changes in the epitope profile of SR7 and these include loss of binding at mAb 10 (Loop 1), mAb 12, 17 and 19 (Loop 2) and gain of mAb 7 (Loop 2). However, only one of these changes, loss of mAb 12 (Loop 2) overlapped with the in-vitro reference sample of sG145R.

Figure 5.35 shows the BioPlex profile of HBsAg from SR8, HBV genotype C adr with changes sT123P, sE164D, sL173V and sI195M, co-plotted with in-vitro generated sG145R. There were eleven changes in the phenotypic profile of this sample and include loss of binding of mAb 6 and 10 (Loop 1), mAb 7, 12, 16 and 17 (Loop 2), mAb 15 (Loop 1 and 2) and mAb 9 (conformation), gain of binding of mAb 3 and 4 (C-terminal) and mAb 18. Of these changes, loss of binding of mAb 6 (Loop 1), mAb 12 and 16 (Loop 2) and mAb 9 (conformation) were also seen in the BioPlex profile from the in-vitro sG145R construct. However, it is to be noted that whilst substitutions at sE164D, sL173V and sI195M are associated with antiviral drug resistance, the HBV DNA sample of SR8 also has sT123P, not associated with antiviral therapy. Furthermore, SR8 showed a loss of mAb 15 (blue arrow), so far only seen with in-vitro construct of sP120T and sP120L (Chapter 4, Table 4.5) and clinical sample of sP120T and sP120Q (see Figure 5.19 and Figure 5.20).

Figure 5.36 shows the BioPlex profile of HBsAg from clinical sample SR9, co-plotted with in-vitro construct of sG145R. HBV genotype for SR9 is A adw with substitutions sE164D,
sI195M and sM133I identified by population-based sequencing. There were only two changes seen in this clinical sample, a gain in binding of mAb 10 (Loop 1) and mAb 8 (Loop 2) and none of these features were consistent with the nine BioPlex changes seen with the 

In summary, clinical samples with HBV antiviral resistance were evaluated for epitope changes. When compared to the BioPlex profile of the VEM sG145R, no readily identifiable or consistent phenotypic marker was observed with the clinical samples. The phenotypic profile observed with the clinical samples of sG145R and implicated as potential features of VEM includes gain in mAb 17, or loss of mAb 12, mAb 8 and 16, either individually or in combination. In this group, loss of mAb 12 was seen in three samples (SR6, SR7 and SR8) and loss of mAb 8 was observed in one sample (SR 8), whilst gain of mAb 17 was seen in two other samples (SR 4 and SR6) (see Table 5.8). The greatest overall number of phenotypic changes was seen with SR8, presumably due to concomitant substitution of sT123P.
Figure 5.28 shows the BioPlex profile of the antiviral associated variant SR1, genotype A adw with sE164D/E, sI195M and sS210R co-plotted with in-vitro generated sG145R. There were two significant changes in the BioPlex profile of SR1, loss of mAb 9 (conformation) (green box), which is consistent with sG145R profile and loss mAb 17, not seen with the reference HBsAg phenotype.

Figure 5.29 shows the BioPlex profile of SR2, genotype D ayw (sE164E/D and sI195M), compared to in-vitro construct of sG145R. There were no matching phenotypic changes in either of the samples.
Figure 5.30

Figure 5.30 shows the BioPlex profile of SR3, genotype A adw with sE164E/D and sI195M, co-plotted with the in-vitro construct of sG145R. There was one change common to both samples, loss of mAb 8 (Loop 2), shown in the green box.

Figure 5.31

Figure 5.31 shows the BioPlex profile of HBsAg of a sample from a HBV/HIV co-infected patient. SR4 was genotype A adw, with sE165E/sI195M. There were three features in SR4 consistent with in-vitro generated sG145R, loss of binding for mAb 5 (Loop 1) and mAb 9 (conformation), marked by green boxes and gain of mAb 17, Loop 2 (red box).
**Figure 5.32**

Figure 5.32 shows the BioPlex of HBsAg, genotype A adw with sE164E/D, sI195M and sS210R. There were no changes in sample SR5 consistent with the BioPlex profile of the VEM sG145R.

**Figure 5.33**

Figure 5.33 shows the BioPlex profile of SR6, genotype C adr, with sE164E/sI195M and sF219S/F, co-plotted with in-vitro construct sG145R. There were two changes seen in both sample profiles, gain of mAb 17 (red box) and loss of mAb 12 (green box).
Figure 5.34

Figure 5.34 shows the BioPlex profile of HBsAg from SR7, HBV genotype C adr with sE164D, sW172W/C, sL173V, sL186L/R and sI195M, co-plotted with in-vitro construct sG145R. There is one change seen with both samples, loss of mAb 12 (Loop 2), marked by the green box.

Figure 5.35

Figure 5.35 shows the BioPlex profile of HBsAg from clinical sample SR8, genotype sT123P, sE164D, sL173V and sI195M, compared to HBsAg derived from the in-vitro construct sG145R. There were 11 changes in the phenotypic profile of SR8, with four shared with in-vitro construct sG145R (green boxes) and loss of binding at mAb 15 (blue arrow).
Figure 5.36 shows the HBsAg BioPlex profile of clinical sample SR9, genotype A adw, with sE164D, sI195M and sM133I, co-plotted with in-vitro derived sG145R. There were no common features in either of these samples.
### Table 5.8

<table>
<thead>
<tr>
<th>Sample</th>
<th>N-term</th>
<th>Loop 1</th>
<th>Loop 2</th>
<th>Combination</th>
<th>C-term</th>
<th>Conform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref sG145R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR1 A adw sE164E/D, sI195M, sS210R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR2 D awv sE164E/D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR3 A adw sE164E/D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR4 A adw sE164D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR5 A adw sE164E/D, sI195M, sS210R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR6 C adr sE164D, sI195M, sF219S/F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR7 C adr sE164D, W172W/C, sL173V, L186L/R, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR8 C adr sT123P, sE164D, sL173V, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR9 A adw sE164D, sI195M, sM133I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ref – Reference strain, ↑↑ gain in binding of mAb, ✔ loss of mAb binding

Table 5.8 shows the BioPlex profile of HBsAg from clinical samples (SR1–9) with antiviral therapy associated S-gene changes. These samples were compared to in-vitro generated HBsAg derived from the HBV construct of sG145R (red). Two samples, SR4 and SR6 have a gain in mAb 17, whilst SR8 exhibited the most number of phenotypic changes.
5.4.4 Group D) Mother to Baby Transmission (see Table 5.4)

Figure 5.37 shows the BioPlex profile of the HBsAg from samples of mother 1 (green) and baby 1 (pink) (M1/B1). The mother and baby profile are similar phenotypically. The HBV from both samples were genotype B2 adw, and the graph has been normalized to the respective genotype. There were two significant and near identical changes in both M1 and B1 profile, namely loss of mAb 8 and 17 (Loop 2), indicated by the blue box (see Table 5.11 and 5.12). Population-based sequencing showed the sY161F, I198M and F200Y changes (see Table 5.4) encoded by the S-gene, ‘filtered sequences analysed by SeqHepB software programme, did not indicate that these changes were significant. No changes were identified by UDPS (see Table 5.4). The significance of these two BioPlex changes is unclear, and it is possible that sY161F, sI198M and sF200Y may have contributed, however, as previously shown with the profile of canonical VEM sG145R, co-existence of these two BioPlex changes does not fit the VEM profile. The phylogenetic studies revealed HBV from both M1 and B1 are of the same clade, (see Figure 5.1B) indicating significant relatedness.

Figure 5.38 shows the BioPlex profile of HBsAg from samples of mother 2 (green) and baby 2 (pink) (M2/B2). Interestingly, in contrast to M1/B1, the mother and baby profiles are not similar phenotypically. The HBV from both samples was genotype D1 ayw, the graph has been normalized to that genotype. Phylogenetic studies show HBV from both M2 and B2 are from the same clade (see Figure 5.1B) Population-based sequencing did not reveal any S-gene changes in either M2 or B2. However UDPS showed B2 was infected with HBV which contained sP127S=10.56%, sG144A= 2.30% and sG130E=4.94%, whilst M2 did not have any substitution observed (see Table 5.4). M2 showed 2 changes in the BioPlex profile, gain of mAb 6 (Loop 1) and mAb 7 (Loop 2) (see table 5.11). Significant BioPlex differences for B2 were observed with five changes and these included gain in binding of mAb 5 (Loop 1), mAb 14 (combination), mAb 2 and 4 (C-terminal) and gain in mAb 17 (Loop 2) (see Table 5.12). These changes may be due to the cumulative presence of the minority species/population of sG144A, sG130E and sP127S. Given there are at least three different variants identified by UDPS, the individual or combination of S-gene changes could affect the profile of B2. Based on the earlier studies, if the relative proportions of each of these HBsAg variants were less than 20%, such minor species by themselves could have little effect on the BioPlex profile. Of note here though is the substitution at position 127 of a proline to serine. Physiochemical characteristics of proline show it can be associated with the formation of bends or ‘kinks’ and ‘turns’ in the secondary structure of the α- helix backbone and substitution by serine may alter the folding of the HBsAg and so may help explain the
changes in phenotype observed in B2. An interesting observation was noted when the UDPS results of M2 was compared to B2 (see Table 5.4), the presence and potential selection of sG144A in B2 (see Table 5.4), a commonly claimed VEM. This presumably was the result of HBV vaccine and HBIg administered at the time of birth and persisted at nine months of age, when the sample was tested and sequenced.

Figure 5.39 shows the BioPlex profile of B2 (D1 ayw) with relative proportions of sP127S=10.56%, sG144A=2.30% and sG130E=4.94% detected by UDPS (Table 5.4) and co-plotted with in-vitro BioPlex profile of sD144A (blue bars). This analysis was performed to identify any similar changes seen in both samples to potentially delineate phenotypic features of sD144A in the B2 sample. There were five BioPlex changes in B2 (Table 5.10) as discussed in Figure 5.38 above, whilst the in-vitro construct of sD144A had four changes. However, none of the changes seen in these profiles overlapped, suggesting the epitope changes seen in B2 were due to either sP127S or sG130E or both (see Table 5.12). Therefore it is possible that failure to cause phenotypic changes by sD144A may have been due to a relatively lower proportion of sD144A (2.3%) and this was also evident in the previous analysis of the clinical samples of sG145R (Table 5.5) where the number of changes exhibited by the clinical samples was consistent with the in-vitro construct of sG145R, which was directly proportional to the relative proportion of the variant.

Figure 5.40 shows the BioPlex profile of HBsAg from samples of mother 3 (green) and baby 3 (pink) (M3/B3) and summarized in Table 5.10. The mother and baby profile overall are similar phenotypically (see table 5.10), like M1/B1. The HBV DNA from both samples was genotyped as B4 ayw and the graph has been normalized to that genotype. Phylogenetic studies show HBV from both M3 and B3 are from the same clade (see Figure 5.1B). Population-based sequencing did not identify changes in the S-gene sequence of either M3 or B3. There were no significant changes observed in the BioPlex profile of M3, (see Table 5.11) and this was consistent with wild-type sequence of the S-gene (see Table 5.4). The BioPlex profile of B3 revealed one change, (see Table 5.12), loss of binding at mAb 8 (Loop 2) and UDPS showed relative proportion of sP127S = 1.63%.

Figure 5.41 shows the BioPlex profile of HBsAg from samples of mother 4 (green) and baby 4 (pink) (M4/B4) and summarized in Table 5.10. The mother and baby profile are not similar phenotypically. The HBV in both samples is genotype B4 ayw and the graph has been normalized to that genotype. At birth, administration of HBIg was inadvertently missed for B4 (Dr Miriam Levy, personal communication). Population-
based sequencing of HBV for M4 revealed a mixed population at position sD144D/E whilst UDPS showed RP of sD144E = 55.59%, sG145A = 19.72% and sQ101H = 6.83% (see Table 5.4). Due to inadequate recover DNA from the sample, UDPS could not be performed for B4. The phenotypic profile of M4 showed two changes, loss of mAb 8, also evident in B4 (blue box) and gain of mAb 17 (Loop 2), (red box). However these two changes were not seen with B4. The phenotypic profile of B4 revealed six changes, loss of binding of mAb 1 and 6 (N-term), mAb 8, 16 and 19 (Loop 2) and mAb 9 (Conformation), however, population-based sequencing showed sF200Y and mixed population sD144E/D (see Table 5.12). Given the altered HBsAg, phenotype of B4 comprising of 5/19 mAb changes, it would be reasonable to assume that a number of S-gene changes were present, despite only the mixed population of sD144E/D being observed.

Figure 5.42 show the BioPlex profile of M4, genotype B4 ayw (as seen in Figure 5.41) and summarized in Table 5.10. Results of sequence analysis of M4 by UDPS revealed sD144E, sG145A and sQ101H in the proportions described above (see Table 5.4). The BioPlex profile of M4 was co-plotted with the in-vitro construct of sD144E and sG145A to identify any similar changes in the epitope profile. There is only one change, gain in binding of mAb 17 (Loop 2) marked by the red box, found in M4 that is similar to in-vitro construct of sG145A, and this is despite a low RP of sG145A = 19.72% (see Table 5.11). In contrast to the previous analysis in the first group of clinical samples comparing the VEM sG145R, (see Table 5.1), the gain in mAb 17 was only seen when the RP of sG145R in the clinical sample was well over 90%. This sample, (M4), also contained HBV with a substitution sQ101H, however, at a lower RP (6.83%), and it is unclear if this substitution can result in a gain in binding of mAb 17 and therefore making a contribution towards this change. Similarly, the presence of sF200Y upstream from the ‘a’ determinant detected by population-based sequencing on the phenotypic profile of these two samples is unclear, whilst loss of mAb 8 was observed in all three samples (Loop 2), marked by the black box and is the only common phenotypic change observed in M4.

Figure 5.43 shows the BioPlex profile of HBsAg of mother/baby pair 5 (M5–green/B5-pink). The mother and the baby profile are not similar phenotypically (see Table 5.10). Both samples are infected with HBV genotype B4 ayw. Population-based sequencing of both indicated the presence of the variant sF200Y. UDPS did not show any changes since the substitution identified by population-based sequencing was outside the amplicon range. There were three significant changes seen in M5 (see Table 5.10), gain in binding at mAb 6 (Loop 1), mAb 7 (Loop 2) and mAb 9 (conformation). In contrast, B5 had only
one change (see Table 5.12), loss of binding of mAb 8 (Loop). Surprisingly, despite the similarity in S-gene sequence seen on population-based sequencing (presence of sF200Y), the phenotypic changes are quite distinct between in M5/B5, with M5 exhibiting more changes and affecting a greater HBsAg region, namely the Loop 1, 2 and conformational sites. Phylogenetic studies revealed that the HBV from both M5 and B5 are of the same clade (see Figure 5.1B).

Figure 5.44 shows the BioPlex profile of mother (green)/baby (pink) pair 6 (M6/B6). The mother and baby profile are not similar phenotypically. Both are infected with HBV genotype C1 ayw and the graph has been normalized to that genotype. Phylogenetic studies show HBV from both M6 and B6 are from the same clade (see Figure 5.1B). Population-based sequencing showed a number of variants in the ‘a’ determinant as well as upstream and downstream from the ‘a’ determinant in B6 and these included sQ101H, sD144A, A184V, sS210N and sY221C whilst UDPS identified S-gene changes encoding sD144A=98.33%, sG145R=1.49% and sQ101H=98.56% (see Table 5.4). Phenotypic changes for B6 included loss of binding of mAb 6 (Loop 1), mAb 8, 12 and 18 (Loop 2) and mAb 9 (conformation). M6 had no significant changes identified through population-based sequencing, however, ‘unfiltered’ changes included sA184V, s210N and sY221C. The UDPS did not identify any changes within the ‘a’ determinant, and the substitution identified by population-based sequencing (see Table 5.4) was outside the amplicon range for UDPS codons (40-160). Interesting, despite the lack of changes in the ‘a’ determinant, M6 revealed a number of phenotypic changes and these included gain in binding of mAb 6 and 10 (Loop 1), mAb 7 and 17 (Loop 2).

Figure 5.45 shows the BioPlex profile of M6 (green bars), infected with HBV genotype C1 ayw, shown previously (see Figure 5.45), compared to the reference in-vitro construct of sG145R and sD144A. Sequence analysis by UDPS of the ‘a’ determinant did not show any significant variants (see Table 5.4), however, there were five significant changes in the BioPlex profile which included gain of mAb 5, 6 and 10 (Loop 1), mAb 7 (Loop 2), but more interestingly, gain of binding in mAb 17 (Loop2) (see Table 5.11). The latter change has been observed in genotypes E ayw, F adw, sG145R, sG145A, double mutation sD144E and sG145R, sM133L, sS132A, sT126N, sE164D and sW196S (see Chapter 4, Table 4.5 and Table 4.6), however, none of these substitutions were present in M6, analysed by both UDPS and population-based sequencing (see Table 5.4). In this analysis, the epitope profile of M6 was compared to the in-vitro construct of sD144A. This substitution was absent in M6 but present in B6, presumably selected due to active and passive immunization. Overall, the HBsAg genotypic profile of M6 was not
consistent with the phenotypic profile, and the gain of binding of mAb 17 (red box) was the only feature consistent with the in-vitro reference sG145R (see Table 5.11).

Figure 5.46 shows the BioPlex HBsAg profile of B6, infected with HBV genotype C1 ayw, compared to in-vitro construct of sG145R and sD144A. Sequence analysis of B6 by UDPs of the ‘a’ determinant showed RP of sD144A=98.33%, sG145R=1.49% and sQ101H=98.56%. In this analysis, B6 has the most number of changes (six- marked by pink box) consistent with sG145R, which included mAb 6 (Loop 1), mAb 8, 12, 19 and 16 (Loop 2) and mAb 9 (conformation). Four of these changes overlap with sD144A and these include mAb 6 (Loop 1), mAb 8 and 12 (Loop 2) and mAb 9 (conformation).

Figure 5.47 shows the BioPlex HBsAg profile of B6, infected with HBV genotype C1 ayw, compared to the in-vitro construct of sD144E. Of the seven changes in the reference in-vitro construct sD144E BioPlex profile, B6 shared six of them. These included loss of binding of mAb 6 (Loop 1), mAb 8, 12, 16 and 19 (Loop 2) and mAb 9 (conformation). Interestingly B6 exhibited loss in binding of mAb 19, not observed in the in-vitro constructs sD144A and sG145R (see Figure 5.46 and Table 5.12). However, in the analysis of in-vitro clones of HBV S-gene variants studied in Chapter 4, it had been only sD144E and double mutation sD144E+sG145R that had exhibited a similar loss at mAb 9 (see Chapter 4 Table 4.6). Of note though, UDPs of HBV from B6 showed high RP of sQ101H (98.56%), and this loss of mAb 9 may be attributable to this substitution.

Figure 5.48 shows the BioPlex analysis of HBsAg derived from mother (green)/baby (pink) pair 7 (M7/B7), normalized to their respective genotype and summarized in Table 5.10. The mother and baby profile are not similar phenotypically. Samples M7 and B7 are of genotype B1 ayw and population–based sequencing did not show changes in the S-gene in either sample. UDPs for both M7 and B7 was not possible due to insufficient DNA sample. Interestingly, despite wild-type S-gene sequences, both samples exhibited epitope changes, M7 showed a gain in binding of mAb 6 (Loop 1), whilst B7 shows loss of binding for mAb 17 (Loop 2) and mAb 9 (conformation). In the absence of UDPs, and failure to detect S-gene changes by population-based sequencing, it was not possible to determine the reason for the lack of phenotypic similarity but the analysis suggests potential minor genotypic differences may be responsible.

In summary, despite the HBV in both mother and baby pairs being grouped in the same respective clades phylogenetically (Figure 5.1B), there was not a strong phenotypic correlation between the samples when their corresponding HBsAg was analysed on the
BioPlex (see Table 5.10). The only similarity was observed in the M1/B1 pair, where HBsAg profiles of both samples had identical changes. This overall phenotypic discordance could be due to the differences in the S-gene sequences identified by UDPS. One of the profiles associated with VEM phenotype observed in clinical samples of sG145R included a gain in Ab 17 and this was seen with two mother samples, M4 and M6, however, none of the baby samples exhibited this change. The second phenotypic profile associated with VEM was loss of mAb 8, 12 or 16. One sample did exhibit loss of mAb 12 (B6) and loss of mAb 16 was seen with two samples (B4 and M6). The loss of mAb 8 (5/7) was the commonest finding in this mother-baby transmission analysis (see Table 5.12).

There was no obvious BioPlex biomarker for the maternal transmission of HBV, although M6 had a partially altered profile (loss of mAb 8 and gain of mAb 17), typically seen in in-vitro constructs of sG145R and sG145A (see Table 5.4). Paradoxically, these variants were not detected in the clinical sample of M6 using either population-based sequencing or UDPS. These findings only suggest that an altered phenotype of a gain in binding of mAb 17, in the setting of pregnancy with high viral load, despite no changes in S-gene sequence can potentially identify VEMs. However, further studies are clearly needed to accurately define these associations.
Figure 5.37 shows the BioPlex profile of HBsAg from M1 (green)/B1 (pink). The HBV genotype was B2 adw. There were no significant mutations detected in the S-gene by population-based sequencing and UDPS, however, there were two common changes observed with their BioPlex profiles (blue box).
Figure 5.38 shows the BioPlex sample of HBsAg for M2 (green)/B2 (pink), both HBV samples are of genotype D1 ayw. UDPs showed B2 contained sP127S=10.56%, sG144A=2.30% and sG130E=4.94%, whilst M2 did not show any significant variants (see Table 4.5). There were two BioPlex changes for M2, whilst B2 had five changes, consistent with the multiple substitutions, including sG144A.
Figure 5.39 shows the BioPlex profile of B2 (pink), compared to the in-vitro construct of sD144A in order to verify consistent changes identified by UDPS (sD144A=2.30%). It is to be noted that other substitutions identified by UDPS for B2 were sP127S = 10.56% and sG130E = 4.94%. There were no common changes in either of the two samples tested, possibly related to lower RP of sD144A.

Figure 5.40 shows the BioPlex profile of HBsAg from M3 (green) and B3 (pink). Both HBV samples are of genotype B4 ayw. The only variant identified was sP127S (1.63%) and there was one significant BioPlex change (loss of mAb 8).
Figure 5.41 shows the BioPlex profile of HBsAg from M4 (green) and B4 (pink). The sequence of M4 encoded the variant sD144D/E and UDPS showed RP of sD144E = 55.59% sG145A =19.72% and sQ101H = 6.83%. Significant changes in the BioPlex profile were loss of binding at mAb 8 (blue box) and gain in mAb 17 (red box). B4 showed mixed population of sD144D/E and UDPS could not be performed due to insufficient sample.
Figure 5.42 shows the BioPlex profile of HBsAg from M4. The HBV sample is genotype B4 ayw. UDPS showed sD144E=55.59%, sG145A=19.72% and sQ101H=6.83%. This sample was compared to the in-vitro constructs of sG145A and sD144E. Loss of mAb 8 was shown in all three samples, whilst gain of binding of mAb 17 (Loop 2) was observed in M4 and sG145R.
Figure 5.43 shows HBsAg profile of M5 (green) and B5 (pink). HBV of both samples was genotype B4 ayw, and filtered sequence analysis did not show any changes. The phenotypic profiles of both samples are dissimilar, showing more changes in M5. These changes cannot be explained by the sequences.
Figure 5.44 shows the BioPlex profile of HBsAg samples from M6 (green)/B6 (pink). Both samples are C1 adr. Filtered S-gene changes in B6 encoded sQ101H, sD144A, sa184V, sS210N and Y221C, whilst UDPS showed sD144A=98.33%, sG145R=1.49% and sQ101H=98.56%. There were five epitope changes in B6. M6 had sA184V, sS210N and sY221C whilst UDPS did not show changes in the ‘a’ determinant. Despite the lack of S-gene changes in the ‘a’ determinant there were four changes in the BioPlex epitope profile, including a gain in mAb 17 (Loop 2).

Figure 5.45 shows BioPlex changes of M6, genotype C1 ayw, unfiltered S-gene sequence analysis indicated the presence of sa184V, s210N, y221C, however, there were no changes in the ‘a’ determinant by UDPS. The phenotypic profile showed an increase in mAb 17(Loop 2), indicated by the red box, consistent with sG145R.
Figure 5.46 shows the BioPlex changes of B6, with HBV genotype C1 ayw, with RP of sD144A=98.33%, sG145R=1.49% and sQ101H=98.56% determined by UDPS, co-plotted with in-vitro construct of sG145R and sD144A. There were five changes in B6 shared with sG145R (pink box) and five with sD144A. There was loss of mAb 19, not observed with the in-vitro constructs.

Figure 5.47 shows the BioPlex profile of B6, with HBV genotype C1 ayw co-plotted with in-vitro construct of sD144E. UDPS showed RP of sD144A=98.33%, sG145R=1.49% and sQ101H=98.56%. There were six changes in B6 in common with the seven changes for sD144E (blue box).
Figure 5.48 shows the BioPlex profile of HBsAg from M7 (green)/B7 (pink). Both samples are genotype B1 ayw. Due to insufficient sample, UDPS could not be performed, however, population-based sequencing did not show any S-gene changes. M7 had one change, gain in binding of mAb 6 (Loop 1) whilst B7 had two changes, loss of mAb 17 (Loop 2) and mAb 9 (conformation).
Table 5.10 shows a summary of the BioPlex profiles of HBsAg from mother–baby pairs. Overall there were differences in the phenotypic profiles, except for mother–baby pair 1, whilst the rest are not.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>B1</th>
<th>M2</th>
<th>B2</th>
<th>M3</th>
<th>B3</th>
<th>M4</th>
<th>B4</th>
<th>M5</th>
<th>B5</th>
<th>M6</th>
<th>B6</th>
<th>M7</th>
<th>B7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Loop 1</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Loop 1</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Loop 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Loop 2</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C-term</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C-term</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Confor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Confor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.11

<table>
<thead>
<tr>
<th></th>
<th>Ref sG145R</th>
<th>Ref sD144E</th>
<th>Ref sG145A</th>
<th>M1 sY161F, 198M, F200Y</th>
<th>M2 NC</th>
<th>M3 sP127S</th>
<th>M4 sD144E, sG145A, sQ101H</th>
<th>M5 NC</th>
<th>M6 sA184V, sS210N sY221C</th>
<th>M7§ NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-term</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Loop 1</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>6</td>
<td>Loop 1</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>10</td>
<td>Loop 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>8</td>
<td>Loop 2</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>11</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Loop 2</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Loop 2</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>19</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>13</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C-term</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Confor</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>18</td>
<td>Confor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations - NC – No changes in S-gene sequence, § – UDPS was not done due to insufficient sample

Table 5.11 shows the HBsAg BioPlex profile of M1-M7, S-gene changes identified by UDPS and/or population-based sequencing. BioPlex findings for in-vitro constructs of sG145R, sG145A and sD144E are presented as reference (red arrows). Overall, there was no pattern or specific epitope changes associated with transmission of HBV from mother-to-baby, however, changes in binding were noted for M1, M2, M5, M6 and M7 despite absence of S-gene changes seen by either population-based sequencing or UDPS. The phenotypic profile of M6 is interesting, it has the most number of changes seen in this group including a gain in binding of mAb 17, which had been associated with sG145R and sG145A; the change was not observed with the corresponding baby sample, B6 (see Table 5.10 and 5.12).
### Table 5.12

<table>
<thead>
<tr>
<th></th>
<th>Ref sG145R</th>
<th>Ref sD144A</th>
<th>Ref sD144E</th>
<th>B1 sY161F l198M, F200Y</th>
<th>B2 sP127S sD144A sG130E</th>
<th>B3 NC</th>
<th>B4 $D144E/A$</th>
<th>B5 NC</th>
<th>B6 sD144A sG145R sQ101H sA184V, sS210N, sY221C</th>
<th>B7 NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Loop 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Loop 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Loop 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Loop 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Loop 1+2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C-ter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Conform</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Conform</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations** - NC = No changes in S-gene sequence, § – UDPS was not performed due to insufficient sample

Table 5.12 shows the HBsAg BioPlex profile of B1-B7, S-gene changes identified by UDPS and/or population-based sequencing. BioPlex findings of in-vitro constructs of sG145R, sG144A and sD144E are presented as references (red arrows). Overall, there were more BioPlex changes seen in the baby samples, presumably due to the selection of variants by the administration of active and passive immunisation (in all cases except B4, where HBV vaccine only was given).
5.5 Discussion

This study evaluated the phenotype of HBsAg epitopes in samples from patients with CHB infection from four different clinical groups, with any S-gene variants previously identified by population-based sequencing and UDPS. The main aim of this Chapter was to correlate the sequence data with the BioPlex results derived from the clinical samples and compare to the BioPlex profiles of relevant in-vitro constructs described in Chapter 4. The BioPlex findings for the first group (A), the canonical VEM sG145R samples revealed that there were similar phenotypic changes in the clinical samples and the in-vitro constructs of HBsAg. However, this was dependent on the relative proportion (RP) of the variant (sG145R) in the clinical samples. If the RP of sG145R was greater than 99% in the clinical sample, all the nine phenotypic features of the in-vitro construct were observed (see Table 5.5). Other than RP of the variant in serum sample, other factors that could lead to the differences in the phenotype of in-vitro generated HBsAg construct and clinical sample includes post translation modification, albumin binding and presences of other anti-HBs. Group B, comprised of clinical samples with HBV S-gene variants claimed by various investigators as possible VEMs as well as clinical samples associated with HBsAg diagnostic failure. In this group there was one sample (V6), which contained the substitution sP120T as identified by population-based sequencing and the BioPlex changes shown for this sample included loss of mAb 15 binding, previously observed only for the in-vitro constructs of sP120T and sP120L, as described in Chapter 4 (see Chapter 4, Table 4.5). Unfortunately, overall there was a poor correlation between serum samples with HBV variants of sD144E and sD144A with the in-vitro constructs, whilst those samples associated with diagnostic failure had a number of phenotypic changes (6-12 mAb changes) on HBsAg profile, suggesting a complex and altered HBsAg. The multiple changes in the phenotype seen in the samples with diagnostic failure (see Table 5. 7) may indicate diverse structural differences compared to the studied wild-type reference HBsAg, thereby evading detection of HBsAg in standard monoclonal antibody based diagnostic assays. Group C, the third group comprised of HBV variants with antiviral drug resistance-associated changes, which had resulted in important substitutions in the overlapping HBsAg and these samples were compared to the 'gold-standard' VEM profile of the in-vitro construct of sG145R. There were no obvious patterns of loss or gain in the binding of mAbs typical of sG145R profile (9/19). However, as with group B, more BioPlex changes were seen when there were more substitutions encoded by the HBV S-gene in these samples (see Table 5.8). The last group (D) of clinical samples came from HBV-infected pregnant women with high viral loads (>10^8 copies/mL) who were in their third trimester of pregnancy; a blood sample was also available from their infected baby at nine months of
Despite the obvious similarity in the HBV S-gene sequences identified by population-based sequencing of these mother/baby pairs, which was confirmed by phylogenetics (see Figure 5.1B), there were striking differences on the BioPlex profiles in six of the seven respective mother-baby samples. However, UDPS identified other S-gene changes within the ‘a’ determinant and it is possible that these substitutions would have led to the complex phenotypic changes observed. Whilst there were no obvious BioPlex biomarkers indicating likelihood of transmission, sample M6 showed gain of mAb 17 (see Figure 5.45). This change had been seen for in-vitro constructs of commonly claimed VEM previously studied (see Chapter 4, Table 4.4). This finding further indicates the complexity of the ‘a’ determinant and its folding and antibody binding properties thus, at this stage at least, a potential role for using BioPlex as a screening diagnostic assay for pregnant women with high viral load for the risk assessment of HBV transmission would appear premature.

The first clinical group (A) studied comprised ten samples referred to VIDRL as possible vaccine breakthrough infections and contained HBV with the sG145R substitution. Correlation of their BioPlex profiles to the in-vitro sG145R construct may have enabled the identification of crucial mAbs that could be used as potential phenotypic biomarkers for high throughput screening of VEMs. Sequencing results, filtered and unfiltered (see Table 5.1) identified all changes encoded by the S-gene that may have affected the epitope profile, whilst UDPS results allowed in-depth and quantitative correlation between the RP and overall phenotypic changes observed in the BioPlex profile. The most consistent finding for these samples was the loss of binding of mAb 12 (Loop 2), present in all ten clinical samples with sG145R and mixed populations of sG145G/R (see Table 5.5). This change was neither dependent on the RP of sG145R nor the mixed population of sG145G/R (see Table 5.1) and appeared to be an independent marker. Loss of binding of mAb 8 (Loop 2) was noted in 9/10 samples and the next most frequent finding, was the loss of binding for mAb 16 (7/10). The loss of mAb 8 was also independent of RP of the variant, as observed with loss of mAb 16. This combination of loss of binding (mAb 12, 8 +/- 16) may suggest a VEM profile (see Table 5.16), however, this profile is not specific or unique for sG145R since similar patterns of loss had been seen with in-vitro constructs, sG145A, sG144E and sD144A (see Chapter 4, Table 4.5). Thus, this loss of binding may reflect the clinical presentation of vaccine breakthrough or failure, but would require independent validation in a "infectivity neutralization assay, see Chapter 6")
The *in-vitro* reference construct of sG145R has 100% identical S-gene sequence and exhibited nine mAb changes in the BioPlex assay (see Figure 5.2). Outlined in Table 5.13 is the location of the various epitopes in the HBsAg with the site of mAb interaction and associated BioPlex changes seen for the sG145R reference strain. The site of binding for mAb 8 is within the Loop 2 of the ‘a’ determinant from amino-acid position 139–147, however, the interaction site for mAb 12 and 16 (Loop 2 mAbs) has not been specifically mapped by the sponsor/supplier (see Table 5.13). As expected, the HBsAg profile of the cloned sG145R revealed more changes directed to Loop 2 mAbs. Previous mAb binding studies comparing HBsAg derived from sG145R to wild-type has also shown reduction in binding to mAbs directed to the Loop 2 region [183, 332]. Possibly due to the conformational structure of HBsAg, changes were not restricted to Loop 2 mAbs, and include reduced binding to mAb 1, which maps to positions 101–105 (Loop 1), and mAb 2, which interacts with the C-terminal region. In the study by Waters et al, the binding of mAbs to HBsAg from clinical samples with sG145R substitutions to those with no changes was evaluated. Immunoassay was performed using three mAbs, two mAbs (mAb 7 and 8) were directed to position 139–147, whilst the third one (mAb 5) targeted position 127 – 137. As expected, mAb 7 and 8, both showed loss of binding, however, mAb 5 that targets the region downstream from position 145 (position 121-137), also showed loss of binding, indicating the complexity of HBsAg structure and a single substitution, sG145R, can potentially alter its phenotype[184, 333].

Interestingly, the RFHBs antibodies were developed at Royal Free Hospital by Professor Howard Thomas, (outlined in Table 5.13) who also explored their therapeutic potential. Specifically, the therapeutic outcome of RFHBs mAb to treat CHB infection has been demonstrated in patients with hypogammaglobulinaemia, a disorder characterized by an intact cell-mediated immunity but defective B lymphocytes resulting in low levels of antibodies [334] In such patients, infection with HBV can progress rapidly to hepatic failure [335]. However, two such patients with severe chronic liver disease secondary to CHB infection have been successfully treated with RFHBs 1 (mAb 5), presumably by neutralizing and then degrading HBV. Both patients were HBeAg positive and had clinical, biochemical and histological evidence of severe liver disease. After treatment, transaminases normalized, one patient had adequate sample to be tested for DNA and viral load was undetectable, and both patients seroconverted to anti-HBe but did remain HBsAg positive for the duration of study, 1-2 years[336].

In this study, loss of Loop 1 mAbs, (mAb 5 and 6) was observed with the *in-vitro* reference strain. However, in the clinical samples, these were only affected with higher
RP (>47%) of sG145R (see Table 5.5). In the clinical samples, with the exception of sample 2 (36.5%), the loss of reactivity with these two mAbs was only observed when the RP was >50%. This finding suggests that the tertiary structure of HBsAg with the sG145R substitution not only affects Loop 2 of the ‘a’ determinant, but also Loop 1 and the phenotypic response is dictated by the RP of the variant. These observations do have implications for models of the ‘a’ determinant as shown in Figure 5.1. For example, the cysteine residues in Loop 1 may very well also interact with cysteines in Loop 2. This certainly seems to be the case for the sP120T substitution, resulting in an unexpected or unpredictable folding.
Table 5.13

<table>
<thead>
<tr>
<th>mAb</th>
<th>Source</th>
<th>Region &quot;a&quot; determinant</th>
<th>Epitope</th>
<th>BioPlex change in reference sG145R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biomerieux</td>
<td>N-terminal</td>
<td>aa 101-105</td>
<td>↓</td>
</tr>
</tbody>
</table>
| 5   | Prof Howard Thomas
Imperial College, London | Loop 1                 | aa 121-137    | ↓                                 |
|      | (RFHBs 1)                           |                        |               |                                   |
| 6   | Prof Howard Thomas
Imperial College, London | Loop 1                 | aa 121-137    | ↓                                 |
|      | (RFHBs 2)                           |                        |               |                                   |
| 8   | Prof Howard Thomas
Imperial College, London | Loop 2                 | aa 139-147    | ↓                                 |
|      | (RFHBs 7)                           |                        |               |                                   |
| 12  | Abbott                              | Loop 2                 | Loop 2: unspecified epitope | ↓                                 |
| 16  | Abbott                              | Loop 2                 | Loop 2: unspecified epitope | ↓                                 |
| 17  | Abbott                              | Loop 2                 | Loop 2: unspecified epitope but included sG145R in immunogen | ↑                                 |
| 2   | Biomerieux                          | C-terminal             | aa 214-219    | ↓                                 |
| 9   | Prof Howard Thomas
Imperial College, London | Conformation           | Conformational | ↓                                 |
|      | (RFHBs 18)                          |                        |               |                                   |

Table 5.13 shows the sites of interaction of the nine mAbs as provided by the manufacturer/supplier. BioPlex changes for the in-vitro construct of sG145R, compared with wild-type HBV, are shown by the arrows. Abbreviation ¶ - RFHBs were kindly provided by Professor Howard Thomas and were developed at the Royal Free Hospital.
One of the interesting isolates in the first group (A) of the study was clinical sample 2 (see Table 5.1) comprising of HBV genotype C adr. This sample had a lower RP of sG145R (36.5%), however, 6/9 BioPlex changes were observed consistent with the reference sG145R. Other substitutions identified by UDPS included sM133T (62%), sT131N (55.3%) and the one substitution, sQ101K, with a low RP of 2.65%. A study by Sloan and colleagues using ELISAs to evaluate reactivity of HBsAg with mAb D2H5, which targets positions 142–147 and so is similar to mAb 8, showed almost complete loss of reactivity of HBsAg from HBV genotype C with substitutions sM133T and sD144A, suggesting that sM133T in combination with other Loop 2 substitutions can potentially alter the HBsAg phenotype[322]. However, it should be noted that in sample 2, the six changes which were consistent with the sG145R profile may also had been due to sT131N and sQ101N, both of which may introduce novel glycosylation patterns. Further specific SDM studies are required to resolve these observations.

A very interesting aspect of the study of the first group (A) with the VEMs was to determine the role of mAb 17 as a potential biomarker. As described in Chapter 4, the in-vitro construct of genotype E ayw and F adw, both genotypes associated with high vaccine failure rates [197, 198, 201] showed a gain in binding of mAb 17. Furthermore, as seen with in-vitro constructs commonly claimed VEMs such as sG145A, double mutant sD144E and sG145R, and sM133L, sS132A, sT126N, sE164D and sW196S also exhibited a gain in mAb 17 binding. However, the only clinical sample that showed this gain was sample 3, with a RP of sG145R of almost 100%, as shown by UDPS. Therefore, it is highly unlikely that mAb 17 can be used as a screening or a ‘sensitive biomarker’ for VEM, as the remaining clinical samples had mixed populations of sG145R. The role of mAb 17 as a ‘specific biomarker’ however, should be further evaluated. These studies can be done using clinical samples of HBsAg with these different HBV mutations (sG145A, double mutation sD144E and sM133L, sS132A, sT126N, sE164D and sW196S) in conjunction with UDPS, so that the RP of each substitution can be quantified and correlated with BioPlex findings. A direct correlation between the gain in binding of mAb 17 to the RP of these variants would more strongly suggest that it can play a role as a potential specific biomarker and become an important tool for population-based surveillance studies.

In the second group (B) studied, the potential VEMs and diagnostic failures, 11 clinical samples were analysed, out of which eight specimens had HBsAg variants commonly claimed as VEMs. Three samples had the sD144E substitution and one had mixed sD144D/E on population-based sequencing and these were compared to in-vitro
generated reference sample of sD144E, as outlined in Chapter 4. There were eight phenotypic changes associated with the reference sD144E (see Table 5.6), but none of the four samples (V1–V4), displayed all the changes consistent with sD144E. These samples did have a number of other phenotypic changes with two of the samples (V2, and V3) showing an increase in mAb 17 binding (see Table 5.17) which warrants further investigation. UDPS may be required to ascertain the reasons for these variations. Other samples studied in this group included V5 which had the variant sD144A detected by population-based sequencing and, there were no phenotypic similarities between the sample and the in-vitro reference sample. Sample V9, with the HBsAg variant sM133L, when compared to the in-vitro generated reference sample, had two changes but only shared one (gain in binding of mAb 17) with the clinical sample. Loss of mAb 15, associated with in-vitro constructs sP120T and sP120L was also observed in two of the clinical samples, V6 and V7, and interestingly, whilst V6 did have the substitution sP120T, V7 had a combination sG145A and sP120Q. The in-vitro construct of sG145A did not result in changes to mAb 15, (see Table 5.7) therefore it is possible that similar to sP120T and sP120L, sP120Q can also result in the loss of mAb 15 binding. For the samples associated with diagnostic failure (V7, V10 and V11), there were no typical features that indicated a characteristic phenotypic profile. However, V7 and V10 had a number of changes across the ‘a’ determinant, affecting mAbs 8 and 12 respectively (see Table 5.7). Furthermore, gain in mAb 17 was seen in V7, presumably due to the presence of sG145A (see Table 5.7), and although not studied previously, HBV variant sT123S, also appears to exhibit this change.

Group C in this study comprised of nine clinical serum samples containing HBV expressing sE164D/I195M as a result of concomitant changes in the S-gene caused by lamivudine-associated resistance. The relevance of these mutations with breakthrough infection, despite adequate levels of circulating anti-HBs has been demonstrated by in-vivo studies, where successfully HBV vaccinated chimpanzees had virological and immunological evidence of infection when challenged with HBV encoding these two substitutions sE164D/I195M [251]. Previous in-vitro studies have also demonstrated that HBV variants with sE164D/I195M had a reduction in mAb binding to HBsAg[144]. A review published in the Bulletin of WHO on the effects of these HBV variants has described them as antiviral associated vaccine escape mutation (ADAPVEM) [14].To evaluate the prevalence of these variants, multiple studies evaluated HBV sequences especially in HIV/HBV co-infected cohorts [145, 337], where modeling has shown that the cumulative incidence of these variants could be up to 5.5/100 person years[145]. Of the nine clinical samples studied, and compared to in-vitro generated sG145R, none
exhibited all the nine features of sG145R (see Table 5.8). Two samples (SR4 and SR6) showed an increase in mAb 17 binding, and whilst SR6 had loss of mAb 12, SR4 lacked the other phenotypic features indicative of the sG145R profile (see Table 5.8). The predominant finding in the first clinical group (A) (VEM with sG145R, see Table 5.5) was loss of mAb 12 with losses of either mAb 8 or 16 or both. However, none of these samples (SR 1-9) evaluated in this third clinical group (see Table 5.8) had the combination of all of these losses. The most number of phenotypic changes (11/19) observed was for the sample SR 8, however, the HBV in this sample encoded other substitutions, including sT123P and sL173V, that may have contributed towards this profile.

The fourth group (D) comprised of clinical samples of HBV from seven pregnant women who were HBsAg positive, HBeAg positive with viral load of >10^8 copies/mL at 30 weeks of gestation and who had declined antiviral treatment [247]. These individuals transmitted HBV to their babies and samples were available from nine months of age when the diagnosis of perinatal transmission was confirmed. HBV from both the mother and corresponding baby sample was analysed by population-based sequencing and UDPS. Comparisons were made between the sequences and phenotypic findings of the BioPlex analysis.

Analysis of six of the mother/baby pairs revealed that there was no relationship between the BioPlex profile of wild-type ‘a’ determinant sequence, despite HBV isolated from each pair was from the same clade (see Figure 5.1B). The S-gene sequence in M1/B1 as well as M5/B5 matched (see Table 5.4), yet the BioPlex profile did not reflect this concordance; with M1/B1 showing a similar profile whilst M5/B5 did not. Likewise M6, showed six epitope changes in binding at Loop 1 (gain for mAb 5, 6 and 10) and Loop 2 (gain in mAb 17 and 8 and loss of mAb 16), however, there was no S-gene changes that could fully explain these findings. There were polymorphisms, or ‘unfiltered changes’ which included sA184V, S210N, Y221C detected by population-based sequencing (see Table, 5.4) but none within the ‘a’ determinant itself. Changes including loss of mAb 16 and gain 17 (Loop 2), were both features found in sG145R (see Table 5.19). It is possible that the altered HBsAg phenotypic profile may indicate there is a higher chance of transmission and commonly claimed VEMs sD144A and canonical VEM sG145R seen in the sample from B6. It was interesting to observe that despite the absence of these two variants in the mother’s (M6) sample (see Table 5.4), B6 had selected these variants, which may be attributed to HBlg administered at birth. Surveillance studies show that HBlg administration can cause selection pressure.
enabling expansion of such variants; this has also been shown for liver transplant recipients [5, 248, 280]. In-vitro studies have shown that HBs specific immunoglobulin (IgG) can reduce the secretion of wild-type HBsAg, but not if the HBV has changes such as sG145R [279]. Similarly B2, despite absence of HBV S-gene sequence changes in M2, selected out sD144A, also associated with vaccine failure (see Table 5.4).

Transmission of HBV from a mother who is HBeAg positive to her baby carries a risk of 70-90%, but the risk is reduced to 5-15% if active and passive vaccines are administered at birth [338]. This risk is further reduced to <2% by administration of antiviral agents such as tenofovir, lamivudine or telbivudine in highly viremic pregnant women (viral loads >10^7IU/mL) in the third trimester [11, 339, 340]. Whilst current treatment strategies are based on viral loads and HBeAg status, the clinical implications of an altered phenotype with features such as an increase mAb 17 may be important in this setting. As seen with M6 and transmission of HBV to B6 where the HBV variant sD144A was selected, phenotypic analysis can be an important “forensic” tool for assessing the risk of the selection of variants associated with immunisation and HBIG administration and would suggest a greater need to administer antiviral agents in this setting.

Due to the cross-sectional nature of this study, one of the limitations was that there were no controls such as pregnant women with HBV viral load <10^8 copies/mL, to compare the phenotypic profile of HBsAg in the third trimester for mothers who have a reduced chance of transmitting HBV to their babies.

One of the overall aims of this Chapter was to compare clinical samples shown to have HBV S-gene variants associated with vaccine failure, to in-vitro construct of the same variants. One of the mothers, M4, was infected with HBV encoding sD144E (RP of 55.59%) and sG145A (RP of 19.72%). The in-vitro constructs of these variants were shown to have a number of phenotypic changes (see Table 5.11), namely loss of mAb 8 binding, seen with both in-vitro constructs and gain of mAb 17 (Loop 2), observed in the sG145A construct only. B2 had sD144A, but did not show any phenotypic features consistent with the in-vitro construct of sD144A. This could be possibly due to a low RP of sD144A, quantified by UDPS (2.3%), similar to what was seen with the first group (A) of sG145R clinical samples. However, B6 provided an interesting insight to the concordance between clinical samples and in-vitro constructs and the RP of variants. In this sample, two S-gene variants were selected presumably due to the administration of HBIG and vaccine and included sD144A (RP=98.33%) and sG145R (RP=1.49%). There were five epitope changes for this clinical sample, and four of these changes, including
loss of mAb 9 (conformation), were in common with the in-vitro reference sD144E (see Figure 5.47). Interestingly though, sD144E was not found in the clinical sample and a possible explanation that dual substitution of sG145R and sD144A results in the combination of loss of mAb 9 (conformation), and mAb 19 (Loop 2), only seen with the in-vitro construct of sD144E and combination of sD144E/sG145R (Chapter 4, Table 4.5).

The key findings for the first group (A) of clinical samples with HBV encoding sG145R were loss of mAb 12 binding observed in all samples (10/10), followed by loss of mAb 8 (9/10) and then mAb 16 (7/10) (see 5.5 and 5.16). Gain in mAb 17 was observed in 9/13 in-vitro constructs (see Table 5.14). By incorporating these findings (loss of mAb 12, 8 and 16 and gain of mAb 17), summary tables for all the in-vitro constructs and the clinical samples from the four groups were compiled (see Tables 5.14, 5.15, 5.16, 5.17, 5.18 and 5.19). This allows comparison of the similarity between sG145R and the clinical samples for their relative reactivity to these four mAbs (mAb 12, 8, 16 and 17). The in-vitro constructs of the various genotypes (see Table 5.15) did not reveal any changes except for gain in mAb 17 for genotype E ayw and F adw compared to A2 adw. The least similarity was observed in Group C, ‘antiviral resistance associated changes’ (see Table 5.18), where only two or less changes were seen in 5/9 samples (see Table 5.18). However, it is noteworthy that two of these samples exhibited gain in mAb 17 and whilst none of these samples showed all the four changes, vaccine failure has been observed in in-vivo models with HBV harboring sE164D/I195M substitutions [251]. Interestingly, gain of mAb 17, which was observed in 9/13 in-vitro constructs (see Table 5.14) including sG145R and commonly claimed VEM constructs, was also the predominant finding (6/11) in Group C which constituted the ‘commonly claimed VEM and diagnostic escape samples’, suggesting that whilst it may not be predominant finding in clinical samples of sG145R, it may have a role in identifying potential VEM in clinical samples. Overall, loss of mAb 12, observed in all clinical samples of HBV with sG145R mutation was not the predominant finding in in-vitro constructs or in the other three clinical groups and suggests that it cannot be used as a sole biomarker for VEM (see Table 5.5, 5.16, 5.17, 5.18 and 5.19). Similarly loss of mAb 8 observed in 9/10 clinical samples with HBV sG145R was not the predominant finding in any of the other clinical cohort, except the mother–baby transmission group, where 5/7 babies had loss of mAb 8 and 1/7 had sG145R in combination sD144A and sQ101H (see Table 5.19).

In conclusion, this study evaluated four groups of clinical samples including classical VEM sG145R, commonly claimed VEM and diagnostic escape variants of HBV, anti-viral associated resistance surface substitutions and clinical samples of treatment naïve CHB
mother/baby pairs where there had been maternal transmission. The number of phenotypic changes in sG145R was related to the RP of variants, identified by UDPS. The commonest marker was the loss of binding of mAb 12 (Loop 2), found in all samples. In the second group, there was no correlation between the phenotypic profile of the in-vitro generated commonly claimed VEM and clinical samples, except for the loss of mAb 15 (Loop 1 and 2), binding associated with sP120T, seen in both clinical and in-vitro samples. A similar loss was also seen in the clinical sample with substitution ssP120Q, suggesting that this loss may be unique to position 120. The phenotypic profile of HBV variants with antiviral associated changes (sE164D/sI195M) did not show any pattern of changes consistent with VEM when compared to in-vitro constructs of sG145R. The analysis of BioPlex profiles for the final group, comprising mother/baby pairs, did not identify any clear biomarker for likelihood of transmission. However, in this group one mother’s sample (M6) was identified with multiple phenotypic changes on the BioPlex, despite no apparent changes in the ‘a’ determinant; HBV was transmitted to her baby with the baby’s virus encoding S-gene substitutions sG144A and sG145R, both associated with vaccine failure. The combination of mAb changes which included loss of mAb 12, 8, 16 and gain of mAb 17, was observed in the clinical group of HBV with sG145R, whilst gain of mAb 17, not a common feature of the first group, was observed more frequently in Group B comprising of HBV variants associated with vaccine failure.

Table 5.14

<table>
<thead>
<tr>
<th>Reference</th>
<th>mAb 12</th>
<th>mAb 8</th>
<th>mAb 16</th>
<th>mAb 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>sG145A</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sD144E</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>sD144E</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sD144A</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>sD144E/G145R</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>sP120T</td>
<td></td>
<td>↓</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>sP120L</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sM133L</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sS132A</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sT126N</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164D</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sW196S</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total mAb loss</strong></td>
<td><strong>7/13</strong></td>
<td><strong>6/13</strong></td>
<td><strong>5/13</strong></td>
<td><strong>9/13</strong></td>
</tr>
</tbody>
</table>

Table 5.14 shows the reference in-vitro constructs of commonly claimed VEMs compared to losses associated with sG145R for four mAbs, 12, 8, 16 and 17. The only variant that has all of these changes was sD144E/sG145R.
Table 5.15

<table>
<thead>
<tr>
<th></th>
<th>mAb 12</th>
<th>mAb 8</th>
<th>mAb 16</th>
<th>mAb 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>sG145R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4 ayw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D ayw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E ayw</td>
<td></td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>F adw</td>
<td></td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>G adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mAb loss</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2/7</td>
</tr>
</tbody>
</table>

Table 5.15 shows the changes for the four mAbs associated with the VEM profile, for HBsAg from cloned genotypes. The genotypes E ayw and F adw were the only two genotypes that had a change in gain for mAb 17.

Table 5.16

<table>
<thead>
<tr>
<th></th>
<th>mAb 12</th>
<th>mAb 8</th>
<th>mAb 16</th>
<th>mAb 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>sG145R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (sG145R/G,W172*)</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (sG145R/G/M133T)</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (sG145R,L192F, I195M)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>4 (sG145R, L192F, I195M)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>5 (sG145R/G,W182*)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>6 (G145R/G, L104L/V, I126N/l)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>7 (sG145R/G)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>8 (sG145R/G)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>9 (sT116N/T, G145R/G, G130K/N/R/S)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>10 (G145R/G, W172*)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Number of samples with changes</td>
<td>10/10</td>
<td>9/10</td>
<td>7/10</td>
<td>2/10</td>
</tr>
</tbody>
</table>

Table 5.16 shows the four changes associated with VEM profile as seen in the reference sG145R profile marked in red and the changes seen for clinical samples of HBV with sG145R substitution (1-10). All samples had loss of mAb 12, whilst only two had a gain in binding of mAb 17.
<table>
<thead>
<tr>
<th>Reference sG145R</th>
<th>mAb 12</th>
<th>mAb 8</th>
<th>mAb 16</th>
<th>mAb 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1 (sD144E)</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2 (sD144E)</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3 (sD144E)</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V4 (sD144E)</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V5 (sD144A)</td>
<td></td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>V6 (sP120T)</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V7 (G145A/P120Q)</td>
<td></td>
<td></td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>V8 (P120P/S, Y134N/Y)</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V9 (M133L)</td>
<td>↓</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>V10 (T123S)</td>
<td>↓</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>V11 (T126I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Number of samples with changes | 1/11 | 5/11 | 2/11 | 6/11 |

Table 5.17 shows the four changes in the reference sample of sG145R, associated with VEM profile (red) and the HBsAg profile of clinical group, commonly claimed VEM. The most common finding in this group was a gain in binding of mAb 17, which is different from the clinical samples of sG145R (see Table 5.14).
Table 5.18

<table>
<thead>
<tr>
<th></th>
<th>mAb 12</th>
<th>mAb 8</th>
<th>mAb 16</th>
<th>mAb 17</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sG145R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164E/D, sI195M, sS210R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D ayw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164E/D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164E/D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164D, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164E/D, sI195M, sS210R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164D, sI195M, sF219S/F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164D, W172W/C, sL173V, L186L/R, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sT123P, sE164D, sL173V, sI195M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A adw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE164D, sI195M, sM133I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of samples with changes</strong></td>
<td>3/9</td>
<td>1/9</td>
<td>1/9</td>
<td>2/9</td>
</tr>
</tbody>
</table>

Table 5.18 shows the BioPlex profile of clinical samples with antiviral drug resistance mutation and the four changes seen with VEM profile.
Table 5.19 shows the BioPlex profile of mother–baby pairs with the four mAbs associated with the VEM profile. Substitutions associated with vaccine failure as identified by UDPS and population-based sequencing are listed for this group.
Chapter 6:
Conclusion and Future Directions

6.1 Conclusion

Genotypic differences within ‘a’ determinant of HBsAg and Protein Expression

There are 10 major genotypes of HBV and four commonly described serotypes. A nucleotide difference of >8% characterizes a different genotype, and >4% but <8% as a different sub-genotype. Genotypic analysis of the ‘a’ determinant of the eight different HBV genotypes cloned in this study (A1, A2 adw, B adw, C adr, D adw, E ayw and F adw) showed that genotype F adw had four extra cysteine residues encoded at positions 19, 183, 206 and 220 of the S-gene. The cysteine residues form a major component in the folding of HBsAg by forming disulphide bonds, which can then impact on the structural differences between the various genotypes. Pairwise analysis of the nucleotide sequence of the ‘a’ determinant of the various cloned genotype/serotypes, when compared to A2 adw of the cloned HBV constructs, showed the greatest differences between A1 and C adr (9%), A2 and F adw (9%) and C adr and F adw (9%). In-vitro expression of HBsAg from the different serotypes using transient transfection of Huh 7 cells showed reduced levels for genotypes A1 and F adw and modifications to the transfection system by adding DMSO did not increase the yield.

Phenotypic analysis of in-vitro constructs of HBsAg of different genotypes compared to A2 adw

A novel immunoassay, based on the BioPlex platform was used to map HBsAg, and this identified differences between the different HBV genotypes and serotypes, compared to genotype A2 adw-2, the genotype used for the generation of the most widely used HBV vaccine. The gain in mAb binding suggests unmasking of the epitopes secondary to ‘shape shifts’, allowing exposure and interaction between the mAb and ‘a’ determinant, whilst loss of mAb binding suggests lack of epitope exposure and interaction. All the genotypes showed gain of mAb 8, a Loop 2 antibody, compared to A2 adw-2. Genotypes B adw and G adw had a similar phenotypic profile. However, pairwise analysis of the ‘a’ determinant did not show such similarity, and exhibited a difference of 6.1% in the nucleotide sequence. Genotype J ayw, the most recently described HBV genotype and so far only isolated from one Japanese patient with CHB infection [221], had S-gene sequences that closely resembled that of genotype C4 ayw the predominant genotype in the Indigenous population of Australia. The nucleotide difference in the S-gene of these two genotypes was 3.57%, however, when compared to the whole genome, the divergence was 11.4% [209]. This finding was reflected in the BioPlex immunoassay
when these two genotypes were analysed and subsequently compared, genotype J ayw had four mAb changes and three of these changes were observed in C4 ayw. The most phenotypically divergent HBV genotype was F adw exhibiting 13/19 epitope changes, when compared to A2 adw. This finding is consistent with breakthrough infections seen with genotype F, despite adequate circulating anti-HBs, derived from vaccine [201] and concerns raised by previous investigators regarding vaccine efficacy for persons exposed to HBV of genotypes E and F[192]. One of the antibodies, mAb 17, a Loop 2 mAb, was specifically constructed by Abbott Diagnostics to identify the canonical vaccine escape mutant (VEM) sG145R (personal communication Dr Tom Leary, Abbott Diagnostics). Of the eight genotypes (B adw, C3 adr, C4 ayw, D ayw, E ayw F adw, G adw and J ayw) studied and compared to A2 adw, two of the genotypes, E ayw and F adw, exhibited an increase in binding for mAb 17, and interestingly, both of these genotypes have been implicated in vaccine failure. This observation suggests a potential role for population-based screening using the phenotypic assessment of HBsAg for vaccine escape profiles.

**Phenotypic analysis of in-vitro HBsAg constructs of serotypes**

Serotypes of HBV are determined by the presence of lysine or arginine at positions 122 and 160 of the S-gene and is within the ‘a’ determinant of HBsAg[188]. The current HBV vaccine is derived from adw serotype in an A2 genetic backbone, therefore mismatches between the serotypes can lead to inadequate protection[190]. Phenotypic differences were observed between adw and ayw. Loss of mAb 7 (Loop 2), was present in all the genotypes with ayw serotypes (D, E, C4 and J), but not seen with serotype adw, suggesting that the epitope profile are different within these two serotypes. However, further studies need to be undertaken to characterize the differences in the phenotype due to serotypic differences.

**Phenotypic analysis of HBsAg derived from sG145R construct and other commonly claimed Vaccine Escape Mutant**

HBV variant sG145R was the first described VEM and phenotypic analysis of the in-vitro construct identified nine changes in the ‘a’ determinant and C-terminal region. These phenotypic losses involved mAb interacting with N-terminal, Loop 1, Loop 2, C-terminal and conformation dependent mAbs, suggesting that the variant evades neutralisation by altering multiple epitopes. Furthermore, the gain of mAb 17 observed suggests that it can identify VEM and be utilised as a potential biomarker. Analysis of other HBV variants implicated as a VEM revealed that sG145A, sD144E/sG145R, sP120T, s132A sM133L, sT126N, and antiviral drug associated variants sE164D and sW196S exhibited
an increase in binding of mAb 17, suggesting that these substitutions alter the HBsAg structure in a similar manner to sG145R, which could lead to vaccine escape. HBV variants sD144A and sD144E, has been widely claimed as VEM, and comparatively there is a paucity of information regarding sN146A and its role as a VEM. All three of these variants did not show an increase in mAb 17, however, sD144A and sD144E did show changes in the conformation mAb 9, and this change, in conjunction with binding differences for Loop 1 and Loop 2 mAbs would suggest significant change in the overall structure of HBsAg and potential concern as VEMs.

Clinical correlation of in-vitro constructs to serum samples of sG145R
Utilization of an assay as a diagnostic tool requires correlation of phenotypic findings to in-vitro derived supernatant to serum sample. This study was the first to identify the phenotypic changes associated with the canonical VEM sG145R in in-vitro constructs and compare these with the clinical samples in conjunction with the relative proportions (RP) by ultradeep pyrosequencing (UDPS). All 10 serum samples of HBsAg with the sG145R mutation showed binding loss of mAb 12, which is directed towards Loop 2 of the ‘a’ determinant. The second most common finding was binding loss of mAb 8 (loop 2) exhibited by 9/10 samples. There was a direct correlation between the RP of sG145R in the sample, quantified by UDPS and the number of epitope change identified by BioPlex. The nine BioPlex changes seen with the in-vitro construct were present in serum samples when the RP was close to 100%. The gain in mAb 17, which was seen in HBsAg generated by site-generated mutagenesis of sG145R was only observed in the serum sample (sample 3) when the RP was almost 100%, and suggests that this cannot be used as a screening tool. However, a gain in mAb 17 may suggest that HBsAg has a RP close to 100%. However, further prospective studies should be undertaken to evaluate the extent of BioPlex changes and with various relative proportion of sG145R, and these could be performed in comparison to HBsAg without sG145R or other substitutions associated with a VEM profile. These studies could identify positive predictive value of the changes in the different mAbs associated with sG145R and at a different RP. This study in conjunction with the profiles established by the in-vitro constructs identified two possible profiles of VEM. The first profile included an increase in mAb 17 and the second profile showed loss in mAb 12, 8 and 16, individually or in combination. However, as discussed previously, these profiles should be further evaluated with more clinical samples and in-vitro constructs with different genetic backbone and serotypes.
**Phenotypic changes associated with commonly claimed VEM and correlation with *in-vitro* constructs**

Evaluation of serum samples of HBV with sD144E, a variant associated with HB Ig/vaccine escape, had poor correlation with *in-vitro* construct sD144E. There were eight epitope changes in the *in-vitro* construct. However, out of the four samples with sD144E, only one sample had the most changes (3/8) that was consistent with the *in-vitro* reference construct, and these included loss of mAb 6 (Loop 1), mAb 8 and 19 (Loop 2). Interestingly, there were other changes observed in samples with sD144E that included a gain in mAb 17 (Loop 2), identified in three samples. However, one of the limitations of this study was the lack of UDPS and quantified RP of the variants and identification of other S-gene variants that may have contributed towards this change. Gain of mAb 17 has been associated as a VEM profile, discussed previously, therefore further evaluation of these clinical samples at a genotypic level with UDPS should be considered. Similarly, there was a poor correlation of sD144A and sD145A with the *in-vitro* construct of the same substitution. As observed with clinical samples of sD144E, gain in mAb 17 was also observed with serum sample of sD144A, however, this was not seen in the *in-vitro* construct. Overall, the number of phenotypic changes for serum samples appeared to reflect the number of S-gene changes identified by population-based sequencing in this group. However, these findings should be investigated by analysing more HBsAg harboring mutations claimed as VEM and those associated with diagnostic failure. The VEM profile seen with the serum sample of sG145R included gain in mAb 17 or loss of mAb 12, 8 or 16, individually or in combination. In this group, seven out of 11 samples exhibited a gain in mAb 17 and two samples, V9 (sM133L) and V10 (sT123S) also had a loss of mAb 8 and one sample (V10) had loss of mAb 16. There were more samples in this group that exhibited a gain in mAb 17 compared to sG145R, this may be due to more S-gene changes, other than those identified by population-based sequencing. Therefore, further studies evaluating features of VEM in these HBV variants should be done in conjunction with UDPS, S-gene changes can be identified and quantified.

**Comparison of phenotypic changes associated with antiviral drug resistance associated mutation and sG145R**

Several studies of both *in-vitro* constructs and *in-vivo* has demonstrated the potential of antiviral drug resistance associated mutations, such as sE164D/I195M to display structural changes to HBsAg leading to evasion of vaccine derived anti-HBs and causing infection[14, 187]. The double substitution sE164D/I195M did not reveal extensive phenotypic changes that were exhibited by sG145R. However, more epitope changes
were noted when other substitutions such as sT123P, sW173V and sW172W/C which were concomitantly present. The two possible VEM profile established by analysing serum samples with sG145R and the in-vitro constructs included gain in mAb 17 or loss of mAb 12, 8 or 16, individually or in combination. Two patients exhibited a gain in mAb 17, and one (SR 6) had a concomitant change in loss of mAb 12, suggesting that there may be an altered HBsAg phenotype. Three samples had a single loss of either mAb 12 or 16, and indicative of an altered phenotype. However, further studies with in-vitro constructs of sE164D/I195M need to be performed to establish a primary profile of sE164D/I195M.

**Phenotypic analysis of HBV in the setting of transmission from mothers to baby**

The risk factors of transmission of HBV include HBeAg positivity in a mother and high viral load (>10⁷ IU/mL) [33, 247]. The phenotypic features of HBsAg in cases of maternal transmission have never been studied. Selection of VEM in several studies has been associated with exogenous immunological pressure such as HBV vaccine and HBlg. Despite all of the HBV in babies’ being in the same clade as their mothers, five out of seven babies showed phenotypic features of HBsAg not consistent with the maternal HBV, suggesting that population-based sequencing does not reflect potential phenotypic changes. Whilst there were no features suggestive of transmission of HBV from mothers to babies, altered HBsAg phenotypes, such as gain of mAb 17 was observed in one mother, despite no S-gene changes evaluated by both population-based sequencing and UDPS. In this setting, BioPlex analysis of maternal HBsAg should warrant administration of antiviral drugs to prevent transmission of HBV. One of the limitations of this retrospective study was the absence of a control group, and further studies should be done including evaluation of HBsAg of pregnant women with CHB and viral loads <10⁷ IU/mL, would allow comparison of HBsAg phenotype in a low risk of transmission but with standard clinical practice of vaccine and HBlg administration. This study demonstrated that the selection of sG145R and sD144A, post administration of vaccine and HBlg occurred in babies, despite its absence in the maternal HBV. Out of the seven babies with CHB infection, one baby selected a HBV variant with sD144A and another both sD144A and sG145R. This data indicates that reduction in maternal HBV load by administration of an antiviral agent in the third trimester of pregnancy in the setting of high viral load (>10⁷) should be a strong consideration. Furthermore, population-based sequencing had not identified these substitutions, therefore HBV S-gene variants may be under reported in perinatal transmission of HBV and further studies involving HBV transmission and selection of VEMs should include UDPS. The VEM profile seen with the serum sample of sG145R included gain in mAb 17 or loss of mAb 12, 8 and 16,
individually or in combination. Comparison of these phenotypic profiles to mothers and babies in this study showed loss of mAb 8 in most babies (5) and gain in mAb 17 was seen in two mothers, one had sG145A which can show this change, and the second sample did not show any genotypic changes that lead to this change. Four samples had two changes consistent with the VEM profile, two samples had S-gene changes that could explain the phenotypic changes, while two samples did not exhibit any S-gene changes when analysed by both population-based and UDPs. However, since the sample size in this group was small and did not include appropriate control subjects, further prospective studies should be performed to identify the frequency of these phenotypic changes.

6.2 Future Directions
Recent studies into viral infections have led to insights into the complex immunological mechanisms responsible for elimination, control and eventual outcome of the host-pathogen relationship. Study of humoral immunity has identified production of neutralizing antibodies and this utilizes several mechanisms such as inhibition of receptor binding and fusion, ultimately preventing viral replication[341] [342] with such studies impacting on next - generation vaccination design. In HBV infection, the primary target for anti-HBs, derived from vaccine or HBIG is the ‘a’ determinant within the HBsAg sub-viral particles. However, the drawback of this mode of neutralization for viruses such as HBV is that it replicates by reverse transcription and a large pool of quasispecies is generated, exhibiting heterogenous surface protein. As seen in the group with HBV transmission from HBV infected mother-to-baby (Chapter 5), these variants have the potential to escape neutralization and thus establish infection.

Immunological studies of HIV and other viruses have shown humoral response and emergence of neutralizing and non-neutralizing antibody during the course of infections. The role of virus specific non-neutralizing antibodies has been shown to assist in clearing virus particle as well as infected cells. Extensive studies involving HIV infection have also shown that during an infection, only a fraction of the antibodies produced bears a virus neutralizing effect. However, the non-neutralizing antibodies are equally essential since they are involved in viral clearance by phagocytosis of immune complexed virus and lysis of infected cells by activating antibody-dependent cellular cytotoxicity (ADCC) [342, 343]. HIV infected patients classified as “long-term slow progressors” maintain a relatively normal immunological response and have a broader repertoire of ADCC response compared to those who are not [344]. Other functions of non-neutralising antibodies associated with HIV also include induction of
conformational changes in the epitopes thereby allowing better accessibility to the neutralizing antibody [345]. These findings suggest that whilst neutralization of HBV virus primarily targets the ‘a’ determinant of HBsAg, other factors are essential for viral clearance and prevention of chronic infection.

The current study identified differences in the phenotype of HBsAg from various genotypes, serotypes and S-gene variants in several clinical scenarios. Whilst the current HBV vaccine is effective, it lacks a broadly neutralizing effect that would enable clearance of the virus with substitutions in the ‘a’ determinant, including sG145R and also be efficacious against all the genotypes, sub-genotype and serotypes. To achieve this, it is essential to understand HBV neutralisation and clearance and the roles of various antibodies produced during both acute and CHB infection. Future studies should be undertaken in collaboration with immunologists to characterize the immune response of HBV in patients who anti-HBs seroconvert, whilst on antiviral therapy and during the natural history to HBV infection. Studies of HBV until recently were restricted due to lack of an in-vitro infectious models. However, the recent identification of HBV receptor has led to cell-culture models using the NTCP-receptor [174] which should enable neutralization assays to be performed using different antibodies and will provide further insight into effective neutralization process. Marrying the phenotype derived from epitope studies described in this thesis directly to virus neutralization would be a logical next step in this process.


[54] Tillman HL, Zachou K, Dalekos G. Management of severe acute to fulminant hepatitis B: to treat or not to treat or when to treat? Liver Int 2012; 32:544-553.


[64] Alexopoulou A, Karayiannis P. HBeAg negative variants and their role in the natural history of chronic hepatitis B virus infection. World J Gastroenterol 2014; 20:7644-7652.


[82] Chu CM, Liaw Y, F. Genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B: a longitudinal study of hepatitis B e-positive patients with normal aminotransferase levels at baseline. J Hepat 2005; 43:411-417.


[187] Torresi J, Earnest-Silveira L, Deliyannis G et al. Reduced antigenicity of the hepatitis B virus HBSAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. Virology 2002; 293:305-313.


Chen CH, Hung CH, Lee CM et al. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. Gastroenterology 2007; 133:1466-1474.


[240] Lai MW, Lin TY, Tsao KC et al. Increased seroprevalence of HBV DNA with mutations in the s gene among individuals greater than 18 years old after complete vaccination. Gastroenterology 2012; 143:400-407.


[269] Ohnuma H, Okamoto H, Tsuda F. Allelic subtypic determinants of hepatitis B surface antigen (i and t) that are distinct from d/y or w/r. J Virol 1993; 67:927-932.


[295] Lim L, Yuen L, Hammond R, et al. Mapping the HBsAg immune phenotype to predict HBsAg loss or decline in chronic hepatitis B in patients receiving nucleot(s)ide analogue therapy. Hepatology 60, supplement (1) Abstract 1623. 2014; .


[326] Seed C, Jone NT, Pickworth A. Two cases of asymptomatic HBV "vaccine breakthrough" infection detected in blood donors screened for HBV DNA. Medical Journal of Australia 2012; 196:651-652.


[348] Lim L, Yuen L, Hammond R, et al. Mapping the HBsAg immune phenotype to predict HBsAg loss or decline in chronic hepatitis B in patients receiving nucleot(s)ide analogue therapy. Hepatology. 2014;60(4 (Suppl)):980A.
