

**HBV GENOTYPES AT THE GROOTE SCHUUR  
HOSPITAL LIVER CLINIC, CAPE TOWN:**

**Genotype And Subgenotype Prevalence And The Influence  
Of Ethnicity On Treatment Outcomes During Long Term  
Clinical Follow-Up Of Patients With Chronic HBV  
Infection**

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**In The Department Of Medicine**

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## ABBREVIATIONS

|              |                                            |
|--------------|--------------------------------------------|
| A            | Adenine                                    |
| ALB          | Albumin                                    |
| ALT          | Alanine Aminotransferase                   |
| anti-HBc IgM | HBV nucleocapsid antibody Immunoglobulin M |
| anti-HBe     | Hepatitis B envelope Antibodies            |
| anti-HBs     | HBV surface antibody                       |
| ARV          | antiretrovirals                            |
| AST          | Aspartate Aminotransferases                |
| C            | cytosine                                   |
| CHB          | Chronic Hepatitis B                        |
| CTL          | cytotoxic T lymphocyte                     |
| dATP         | dexyadenosine triphosphate                 |
| dCTP         | deoxycytidine triphosphate                 |
| ddNTPs       | dideoxyribonucleoside triphosphate         |
| dGTP         | deoxyguanosine triphosphate                |
| DNA          | Deoxyribonucleic Acid                      |
| dNTP's       | deoxyribonucleoside triphosphates          |
| dsDNA        | double stranded Deoxyribonucleic acid      |
| dTTP         | deoxythymidine triphosphate                |
| EPI          | Expanded Programme on Immunization         |
| EtBr         | Ethidium Bromide                           |
| FTC          | Entecavir and emtricitabine                |
| GHIS         | Guanine                                    |
| GHIS         | Gambia Hepatitis Intervention Study        |
| HAART        | Highly Active Antiretroviral Therapy       |
| HBcAg        | HBV nucleocapsid Antigens                  |
| HBeAb        | HBV envelope Antibodies                    |
| HBeAg        | HBV envelope Antigens                      |
| HBsAg        | Hepatitis B Surface Antigen                |
| HBV          | Hepatitis B Virus                          |
| HBx          | Hepatitis B X protein                      |

## **ABBREVIATIONS**

|                 |                                               |
|-----------------|-----------------------------------------------|
| HCC             | Hepatocellular Carcinoma                      |
| HCV             | Hepatitis C Virus                             |
| HCW             | Healthcare Workers                            |
| HIV             | Human Immunodeficiency Virus                  |
| HLA             | Human Lymphocyte Antigen                      |
| IFN- $\alpha$   | Interferon- $\alpha$                          |
| INR             | International Normalized Ratio                |
| LHBs            | Large HBsAg                                   |
| MHBs            | Middle HBsAg                                  |
| MHC             | Major Histocompatibility Complex              |
| n               | Number of patients                            |
| NCBI            | National Centre for Biotechnology Information |
| NK              | Natural Killer                                |
| ORF             | Open Reading Frame                            |
| PCR             | Polymerase Chain Reaction                     |
| RNA             | Ribonucleic Acid                              |
| S-gene          | Surface Gene                                  |
| SHBs            | small HBsAg                                   |
| ssDNA           | single stranded Deoxyribonucleic acid         |
| T               | Thymine                                       |
| TBE             | Tris/Borate/EDTA                              |
| TCR             | T cell receptor                               |
| TNF             | Tumor Necrosis Factor                         |
| Tris            | Tris(hydroxymethyl)aminomethane               |
| Tris-HCl        | Tris(hydroxymethyl)-methylammonium chloride   |
| UV              | Ultra Violet                                  |
| WHO             | World Health organisation                     |
| $\beta$ -globin | Beta-Globin                                   |

## ABSTRACT

In the developing world, hepatitis B virus (HBV) is an important cause of acute and chronic liver disease. Two billion people worldwide are infected with the virus and an estimated 350 million who have failed to clear the virus have chronic infection. In Africa, HBV is endemic with 98% of the inhabitants being infected with the virus at some point in their lives. Of these 10% fail to clear the virus. The emergence of HBV-HIV co-infected cases in sub-Saharan Africa adds a new dimension in the management of this disease and highlights the need to understand the pathogenesis and treatment of HBV. HBV genotype A and D have been shown to be present in southern Africa with genotype A generally being the predominant genotype documented in South Africa. This is based on studies carried out in the Gauteng and Kwa-Zulu Natal regions. There is very little prevalence data from the Western Cape and other provinces and there is no data on the pre-dominant HBV genotype seen in this region. This study investigates the predominant HBV genotype and subgenotypes seen at the Groote Schuur Liver Clinic, Cape Town and the possible correlations between the HBV genotype, race and disease severity. In addition early work investigating the possible presence of lamivudine resistant strains in lamivudine naïve patients are reported. This is important as the increase in incidence of lamivudine use in HBV-HIV co-infected patients may have potential implications in lamivudine treatment management programmes.

169 chronic HBV patients from all races and age groups were recruited into the study. The HBV DNA levels, HBeAg and HBsAg status were determined as part of their standard management at the Liver Clinic. Two methods were used in combination to identify the HBV genotype of the patient using the conserved S-gene on the HBV genome. The detection of lamivudine resistant mutations on the YMDD motif was determined using nucleotide sequence analysis. The possible correlations between race, HBV genotype, interferon- $\alpha$  (IFN- $\alpha$ ) treatment, virological and biochemical parameters were analysed.

In this study, 73% of the patients were identified as genotype D, 27% as genotype A with one patient being genotype B2. The subgenotypes D3 and D4 were also identified in the

cohort. Although genotype D predominates in the Mixed race this did not reach statistical significance in this study. The genotype B2 patient was an immigrant from Taiwan, in keeping with published data from Asia. The Caucasians were older compared to the other races while the non-Caucasoids presented at an older age and with more severe disease. The Asians showed the best response to IFN- $\alpha$  treatment while the Black Africans had significantly lower ALB levels at presentation compared to the other races. As noted in published literature, high ALT and low HBV DNA levels correlated with a better response to IFN- $\alpha$  treatment. No lamivudine resistant strains were found in the lamivudine naïve patients but one YVDD mutation was found in a patient who was on lamivudine therapy.

This study shows that genotype D and its subgenotype, D3, is the predominant genotype seen in this urban clinical cohort of patients. The unexpected presence of subgenotype D4 is documented. This interesting observation may be explained by the historically well documented population migration from the Indian and Asian Pacific islands into the Cape Town region. No lamivudine resistant strains present within the lamivudine naïve patients in the clinic indicates that these strains are not yet circulating as there have been reports of the presence of these resistant strains in lamivudine naïve patients in South Africa. Gene polymorphisms and socio-economic factors may explain the racial difference with respect to age at HBV diagnosis. The success of IFN- $\alpha$  treatment may be related to the varied genetics of the population.

# **CHAPTER 1:**

## **LITERATURE REVIEW**

### **INTRODUCTION**

Hepatitis B virus (HBV) is an important cause of acute and chronic liver diseases that include acute fulminant hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Hasegawa et al., 2004). Two billion people worldwide are infected with the virus and an estimated 350 million people who have failed to clear the virus have chronic infection. It is estimated that about 20-30% of those with chronic infection will die as a result of HBV related diseases (Osiowy and Giles, 2003). The understanding of the pathogenesis, prevention and treatment of HBV associated liver disease is therefore of crucial importance, particularly in the developing world.

#### **1.1 CLASSIFICATION AND STRUCTURE OF THE HEPATITIS B VIRUS**

HBV is a member of the *Hepadnaviridae* family. It has a partially double stranded deoxyribonucleic acid (DNA) genome that is circular, compact and approximately 3.2kb in length. The genome includes four overlapping open reading frames (ORF's) that encode the envelope, core, polymerase and X proteins. Although *hepadnaviruses* have a strong preference for infecting hepatocytes, minute amounts of HBV viral DNA can be found in the kidney, pancreas and mononuclear cells (Marion, 1988).

HBV virions, also known as Dane particles, are double shelled particles and most are approximately 40-42nm in diameter (Dane et al., 1970). The outer lipoprotein envelope contains three related envelope surface antigens (HBsAg) (Ganem, 1991). Within the envelope is the nucleocapsid that contains the core particle. The viral genome and DNA polymerase are contained within the core particle (Robinson and Lutwick, 1976).

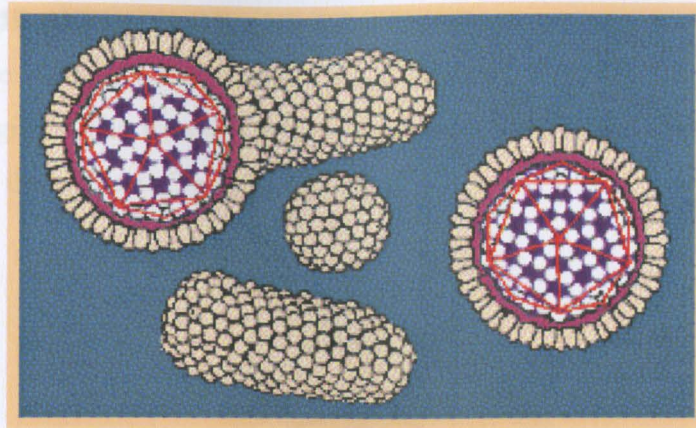


Figure 1.1: Schematic diagram of HBV virion and surface antigen components (Stannard, L. M, 1995)

Apart from the Dane particles, HBV infected cells produce two distinct non-infectious sub-viral particles; sphere and filamentous particles both 20nm in diameter. These sub-viral particles are thought to be excess HBsAg and contain only glycoproteins and host derived lipids and interestingly outnumber Dane particles (Ganem and Prince, 2004).

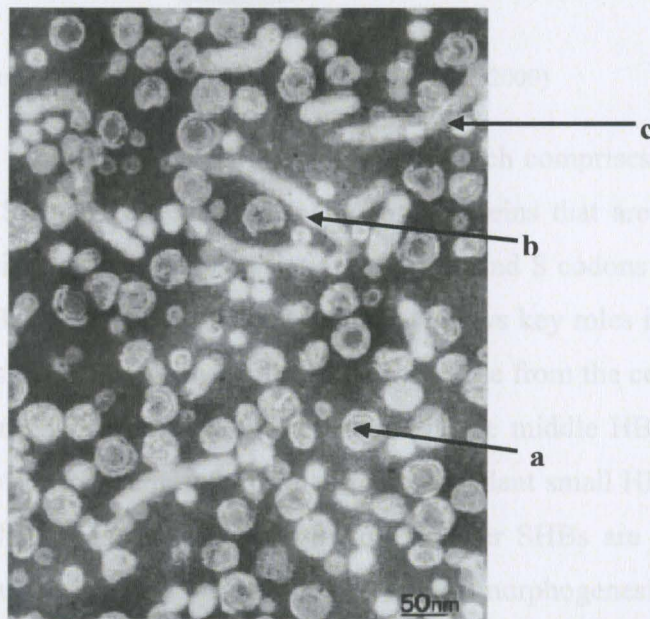


Figure 1.2: Electron micrograph of (a) HBV Dane particles; 42nm,(b)filaments and (c) spheres, 20-22nm, visualised in the serum sample of a patient (Ganem and Prince, 2004).

## 1.2 VIRAL GENOME STRUCTURE AND PROTEINS

The HBV genome has four ORF's namely the S (surface), C (core), P (polymerase) and X (HBx protein) regions. The P region covers more than 70% of the complete genome and partially overlaps the entire S, C and X regions.

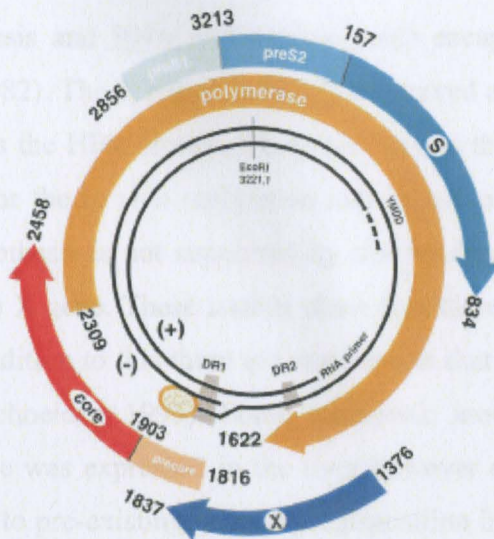


Figure 1.3: The HBV genome structure (Hunt et al., 2000)

The S region is elongated at the 5' end by the pre-S region which comprises of the preS1 and preS2 domains. The S region encodes three envelope proteins that are translated at three different in-frame initiation codons. Translation of preS1 and S codons results in the expression of the large HBsAg (LHBs) protein. This protein plays key roles in binding the virus to host-cell receptors, assembly of the virion and its release from the cell (Bruss and Ganem, 1991). Translation of preS2 and S codons results in the middle HBsAg (MHBs) protein while expression of the S codon only results in the abundant small HBsAg (SHBs) protein. The function of MHBs is relatively unknown, however SHBs are known to be major proteins of the viral surface and are essential for viral morphogenesis (Bruss and Ganem, 1991).

The C region is also elongated at the 5' end by the preC region. The preC-C region encodes the core protein and the e antigen (HBeAg). The two proteins are expressed by translation of two in-frame AUG codons (Ganem, 1996). The second or internal AUG that encodes the

core protein is 21kDa in size. It is involved in the encapsulation of the viral genome. The upstream AUG codon expresses the e antigen precursor, which is later cleaved by cellular proteases into the HBeAg, which is secreted into the blood. HBeAg plays no role in viral morphogenesis or infectivity and hence its function is unclear (Takahashi et al., 1983).

The viral polymerase is encoded by the P region. This protein is multifunctional as it is involved in DNA synthesis and RNA (ribonucleic acid) encapsulation in infected cells (Summers and Mason, 1982). The X region is highly conserved amongst the different HBV viral isolates and encodes the HBx protein. There are reports that suggest that the protein appears to be essential for the *in vivo* replication and spread of the virus (Zoulim et al., 1994), however this hypothesis is not supported by studies done on transfected cell lines with the knock out of the X gene. These studies show that there is only a moderate effect on viral replication. In addition to this there are also reports that suggest that the X gene is oncogenic (Benn and Schneider, 1995). Some transgenic mouse lines developed liver tumours when the X gene was expressed in the liver however other mouse lines did not. This is thought to be due to pre-existing genetic predisposition in mouse lines in which the X gene facilitated tumour formation. The exact role of the HBV X gene is yet to be determined as functions such as transcriptional activation, cell transformation, apoptosis sensitization and induction of cell cycle progression based on studies with cell lines and transgenic mice have not yielded any substantial observations that would indeed define an exact role of the HBV X gene (Benn and Schneider, 1995, Seeger, 1997).

### 1.3 LIFECYCLE AND REPLICATION OF HBV

The main characteristic of the *hepadnaviridae* replication cycle is the replication of the DNA genome by reverse transcription of an RNA intermediate (Summers and Mason, 1982). Infectious HBV virions bind via the preS1-domain of the LHBs protein to as yet unknown surface cell receptors and fuse with the cell membrane of the target cells. Once fusion has occurred the core is released into the cytosol and transported into the hepatocyte nucleus. The DNA genome is then converted to a closed circular form (cccDNA) which is the transcriptional template for the host RNA polymerase II. The cccDNA is transcribed into viral RNA and is then transported into the cytoplasm. Translation of the viral DNA

yields the viral envelope, core protein, polymerase, X and preC polypeptides which are packaged into nucleocapsids (Pollack and Ganem, 1994). Also incorporated into the nucleocapsid is some genomic viral RNA that is reverse transcribed into viral DNA (Pollack and Ganem, 1994). The synthesis of the viral DNA strands is sequential whereby the first DNA strand is synthesized from the packaged RNA template that is degraded and thereafter synthesis of the second strand proceeds with the first DNA strand acting as a template (Summers and Mason, 1982, Will et al., 1987, Wang, 1993). The resulting core particles with the mature genome are either transported back into the nucleus where the DNA genomes are converted back into cccDNA to maintain a stable pool of templates (Tuttleman et al., 1986) or bud into the endoplasmic reticulum to be enveloped and exported from the cell.

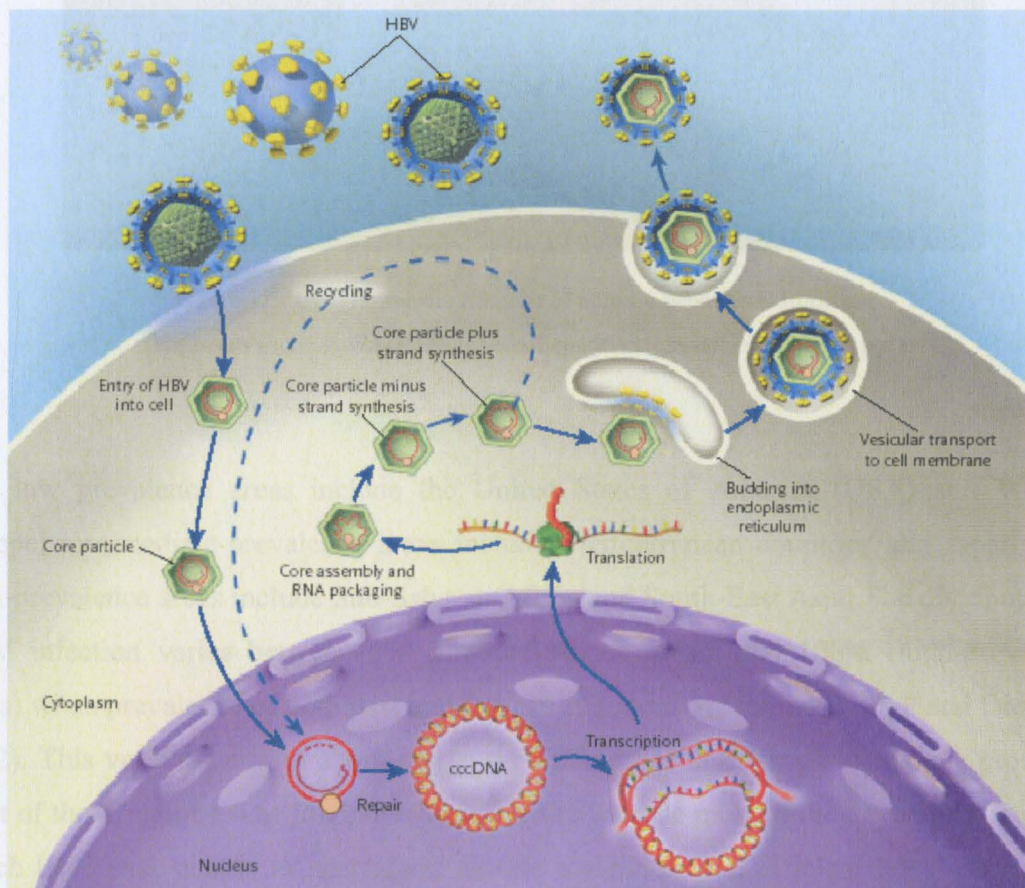


Figure 1.4: The HBV replication cycle (Ganem and Prince, 2004)

#### 1.4 PREVALENCE AND MODE OF TRANSMISSION

There is dramatic variation in the distribution of HBV infection globally. There are low, intermediate and high prevalence areas.

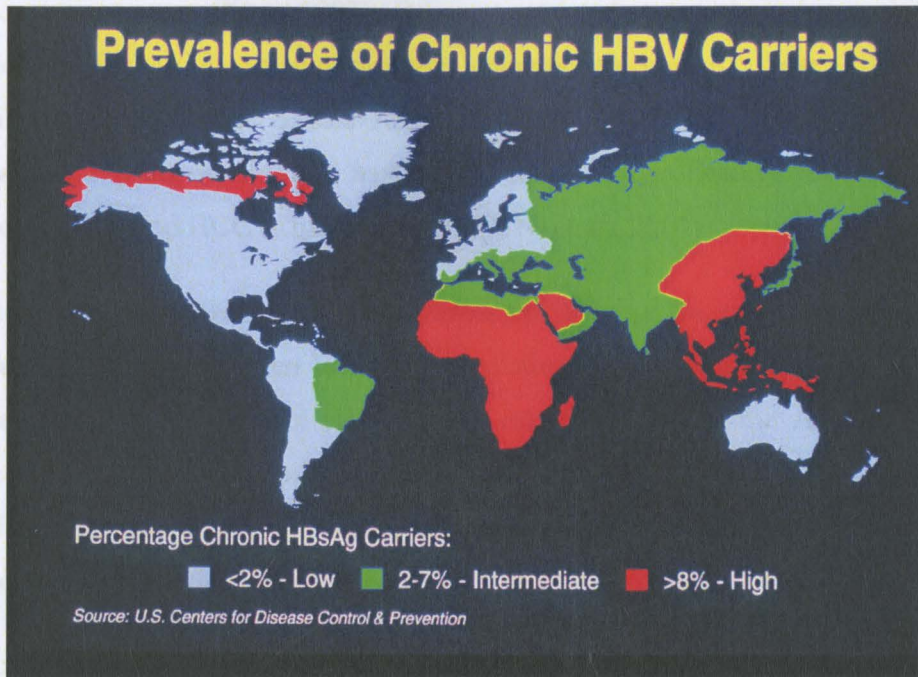


Figure 1.5: Geographic distribution of chronic hepatitis B infection  
([http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep\\_b/hep\\_b.ppt](http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_b/hep_b.ppt))

The low prevalence areas include the United States of America (USA) and Western Europe; intermediate-prevalence areas include Mediterranean countries and Japan while high-prevalence areas include Sub-Saharan Africa and South-East Asia. The distribution of HBV infection varies between 5% (low-prevalence areas) and 100% (high-prevalence areas) when prevalence of both past and current infections are tallied (Farrell and Dienstag, 2002). This variation comes about due to the differences in the socio-economic profile of most of these regions. The low prevalence regions include most of the developed countries which have easy access to healthcare, disease awareness and efficient HBV vaccination programmes. Developing countries fall under the high prevalence areas and due to economic constraints, the implementation of integrated HBV prevention programmes is

limited. These programmes include healthcare education campaigns and primary prevention and improvement of hygienic and sanitary conditions. On the other hand some of these developing countries such as Zambia are undergoing a change in their prevalence profile. Zambia is going into intermediate endemicity due to effective hepatitis B prevention programmes (Kew, 1997). Other countries that are observing a shift in their prevalence profile (high to intermediate endemicity) due to these effective HBV vaccination programmes are Saudia Arabia (Toukan, 1997) and Taiwan. In Taiwan the carrier rate of HBsAg in children and adolescents has significantly reduced as has the incidence of childhood HCC (Chang et al., 1997, Ni et al., 2001).

The variation in prevalence is not only restricted to large geographical areas but HBV carrier rates also vary between different population groups within communities. In the USA, Caucasians have a lower HBV carrier rate compared to the African-Americans, Hispanic-American and Asian-Americans (Alter and Mast, 1994). This trend is also observed in Australia where the Australian Aborigines have higher HBV infection rates compared to the other ethnic groups (Farrell and Dienstag, 2002).

A distinct relationship is observed between the mode of transmission and prevalence rates. In low-prevalence areas most of the HBV infections are acquired in early adult life when there are behavioural changes that facilitate the spread of blood borne infections. Examples of such behaviour are unprotected sexual intercourse, tattooing and intravenous drug use. In high-prevalence areas, mother to child (perinatal) and child to child (horizontal) transmission account for most infections (Lee, 1997). The mode of transmission and age of infection are also intimately linked. The age of infection has a significant impact on the clinical expression and outcome of HBV infection and hence contributes to variation in prevalence rates. For example perinatally acquired HBV infection leads to chronic HBV in infants due to immune tolerance, while those infected as adults have clinically recognizable acute HBV infection (Farrell and Dienstag, 2002).

There are various ways through which the HBV infection can be transmitted. The major modes of transmission are perinatal, sexual, percutaneous and horizontal. The most common mode of transmission tends to differ by geographical location due to differences

in prevalence rates. Perinatal transmission is common in South East Asia (high prevalence area) as most mothers are HBeAg positive and have high viral loads. In contrast, the rate of perinatal transmission is quite low in areas of low endemicity such as the USA, as intravenous and sexual transmissions are the most common routes of disease transmission (Farrell and Dienstag, 2002).

Sexual transmission is the most frequent route for HBV acquisition in the USA and other developed countries. The risk of sexual transmission is directly related to the number of lifetime sexual partners, homosexual or heterosexual preference, education level attained, occurrence of paid sex and prior history of sexually transmitted diseases (Farrell and Dienstag, 2002).

The most common form of percutaneous transmission is intravenous drug use however the re-use of contaminated needles for tattoos, ear piercing and acupuncture are also important but under emphasized risks. Any activity involving the puncture of the cutaneous or mucous- membrane with a blood contaminated instrument is a potential source of HBV infection (Farrell and Dienstag, 2002).

Horizontal transmission occurs between children due to body contact. This leads to transfer of infected blood or serum from cuts, abrasions and lesions via minor skin breaks. Oral transmission of HBV (e.g. kissing) hardly occurs despite the presence of HBV DNA in the saliva of some carriers (Farrell and Dienstag, 2002).

### 1.5 HBV SUBTYPES AND GENOTYPES

HBV has so far been classified into antigenic subtypes and genotypes that are based on viral nucleotide sequence divergence. The HBsAg has the main group specific determinant *a* that resides together with either one of these two mutually exclusive determinants; *d* and *y* (Le Bouvier, 1971) and *w* and *r* (Bancroft et al., 1972). Hence four major subtypes of HBsAg are generated; *adw*, *adr*, *ayw* and *ayr* that can be used to characterize each HBV strain. An additional nine serological subtypes were further identified namely; *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q-*, *adrq+* and *adrq-*. The molecular basis for the d/y and w/r

variations were shown to depend on Lysine/Arginine substitutions at residues 122 and 160 on the S gene on the HBV genome (Okamoto et al., 1988). Residue 127 was found to be important for the *w1-w4* variation. Strains *w1/w2*, *w3* and *w4* encoded Proline, Threonine and Leucine respectively at position 127 on the S gene. The *w1* subtype is also dependent on Arginine at position 122, Phenylalanine at position 134 and/or Alanine at position 159 (Norder et al., 1992). The determinant designated *q* is expressed by most HBV strains except strains encoding *adw4* and *adr*. The residues important for *q* expression are residue 177 and 178 (Norder et al., 1992). These subtypes have a distinct pattern in geographical distribution around the world. (Courouce-Pauty et al., 1983).

HBV genotypes are classified into eight groups, designated A-H, and based on about 92% similarity in complete HBV nucleotide sequence (Okamoto et al., 1988). This classification has been validated through sequencing and phylogenetic analysis (Norder et al., 1994, Mizokami et al., 1999). The genotypes show characteristic geographical distributions with some genotypes being more prevalent in certain geographical areas.

Genotype A is found in North America, Northern Europe and Sub-Saharan Africa (Magnius and Norder, 1995). Genotypes B and C are confined to Asia (Okamoto et al., 1988) while genotype D is distributed world wide but is most prevalent in the Mediterranean area. Genotype E is mostly found in West Africa (Odemuyiwa et al., 2001) while genotype F is found amongst the indigenous Aborigines of the Americas (Arauz-Ruiz et al., 1997). Genotype G is prevalent in the USA and France (Stuyver et al., 2000) and the recently discovered genotype H is found in the Amerindian populations of Central America (Arauz-Ruiz et al., 2002).

A correlation between subtypes and HBV genotypes exists although several subtypes may be encoded by more than one genotype (Norder et al., 1992). The *adw2* subtype occurs mainly in genotype A, B and G but may also be present in genotype C and D, however genotype A and B may in addition also specify *ayw1*. Genotype C specifies *adr* and *ayr* while strains specifying *adw4q*- occur only in genotype F and H. The genotype D specifies *ayw2* and *ayw3* while those strains specifying *ayw4* occur in genotype E (Norder et al., 2004).

Table 1.1: Genotypes, subtypes and geographical distributions of HBV (Norder et al., 2004).

| Genotypes | Subgenotypes | Subtypes | Geographical Location    |
|-----------|--------------|----------|--------------------------|
| A         | A1           | adw2     | North America            |
|           | A2           | ayw1     | Northern Europe          |
|           |              |          | Sub-Saharan Africa       |
| B         | B1           | ayw1     | Asia-                    |
|           | B2           | adw2     | Japan,Taiwan,China etc   |
| C         | C1           | adr      | Far East Asia, Thailand, |
|           | C2           | ayr      | Japan, Korea             |
|           | C3           |          |                          |
|           | C4           |          |                          |
| D         | D1           | ayw2     | Mediterranean            |
|           | D2           | ayw3     | North Africa             |
|           | D3           |          | South Africa, East Asia  |
|           | D4           |          | Australia                |
| E         | -            | ayw4     | West Africa              |
| F         | F1           | adw4q-   | Central and South        |
|           | F2           |          | America                  |
| G         | -            | adw2     | USA and France           |
| H         | -            | adw4q-   | Central America          |

Recent work has shown that HBV genotypes A, B, C, D and F can be further divided into subgenotypes. The genotypes and subgenotypes differ in their clinical manifestations and virological characteristics. HBV genotype A is divided into subgenotype A1 which is found in Africa and Asia (Liu et al., 2005, Kramvis et al., 2002) and A2 which is found in Europe and North America. Genotype A appears to induce chronic liver disease more frequently (Mayerat et al., 1999) and may be associated with a better response to interferon than genotype D (Zhang et al., 1996). In addition, genotype A appears to have sustained

remission and a higher frequency of clearance of HBV-DNA compared to genotype D in Caucasian patients treated with interferon. Subgenotype A1 is associated with reduced serum levels of HBV DNA and HBeAg (Kramvis et al., 1998, Kramvis et al., 1997). Patients with this subgenotype appear to seroconvert to anti-HBe more frequently and only 5% of them will possess HBeAg in their serum when they reach adulthood (Dusheiko et al., 1985). In addition, the subgenotype is also associated with the high prevalence of HCC in Africa (Attia, 1998) and also encodes HBsAg of serotype *adw* and *ayw* unlike the original genotype A that mainly encodes serotype *adw* (Bowyer et al., 1997, Sugauchi et al., 2003). A new subgenotype A3 was recently discovered in Cameroon. It has a high nucleotide divergence of 3.9% indicating that it has a long natural history in the Cameroonian population. Apart from unique amino acid/nucleotide substitutions being observed, one of the A3 strains studied revealed the presence of recombination between HBV/E and HBV/A in the short non-overlapping fragment of the RT polymerase domain (Kurbanov et al., 2005). Nonetheless further studies on virological and clinical characteristics as well as the recombinant form have to be carried out on this strain to establish its frequency, clinical relevance and impact in the Cameroonian population.

The HBV genotype B is classified into two subgenotypes: one with a recombination with genotype C over the pre-core and core region and the other without. The subgenotype with the recombination is known as subgenotype B2 and the subgenotype without the recombination is known as B1. The B1 isolates are found to be restricted to Japan while the B2 isolates are found in other Asian countries such as China, Hong Kong, Taiwan and Indonesia (Sugauchi et al., 2002). How the recombination occurs and why it has such a distinct geographical distribution is unknown. It has been noted though that when these two subgenotypes are compared, clinical differences can be observed. A difference in response to antiviral therapies and the prevalence of HBeAg among patients with chronic liver disease and infected with HBV/B2 and HBV/B1 is observed (Akuta et al., 2003, Sugauchi et al., 2003). Patients with the subgenotype HBV/B2 possessed HBeAg in the serum more frequently than those infected with subgenotype B1 (Kobayashi et al., 2005) and the response to lamivudine was poorer in patients infected with subgenotype B2 than B1 (Akuta et al., 2003). HBV/B2 is found to increase the rate of progression to cirrhosis

compared to subgenotype B1 and it is postulated that the recombination with genotype C may increase its capacity to induce severe liver disease (Sugauchi et al., 2002).

There are a number of studies that have shown that with genotype C infection, there is more severe liver disease and a longer duration of HBeAg positivity in the serum with delayed seroconversion to anti-HBe compared to genotype B (Kao et al., 2002, Lindh et al., 1999). The genomic characterization of genotype C resulted in the identification of at least two subgenotypes; C1 and C2. HBV/C1 is found in south East Asia only (Vietnam and Thailand) and HBV/C2 is found in Far East Asia (Japan, Korea and China) (Huy et al., 2004). Further analysis revealed two more subgenotypes; C3 and C4. HBV/C3 is mainly found in the aboriginal populations of New Zealand and the Polynesian islands (Norder et al., 2004). The subgenotype C4 was found mainly within the Aborigines of Northeast Australia (Sugauchi et al., 2001). Although genotype C causes more serious liver disease than genotype B, there have been no studies done to confirm whether these two subgenotypes have different virulence in causing chronic liver disease including a higher incidence of hepatocellular carcinoma.

Phylogenetic analysis of 33 complete HBV genomes distinguished four subgenotypes, D1-D4 which was supported by 74-100% bootstrap (Norder et al., 2004). Strains found in the Middle East belonged mainly to the D1 genotype, those from South Africa and Alaska mainly to D3. Those from Oceania and Somalia were mainly found to be D4 however subgenotype D1 and D2 could not be assigned a distinct geographical distribution possibly because the genotype D geographical distribution is less restricted when compared to genotype A, B and C (Norder et al., 2004).

Genotype F strains can be divided into subgenotype F1 and F2. Each is characterized by specific substitutions in the S gene product at position 45. Leucine and Threonine present at residue 45 encode for F1 and F2 respectively (Kato et al., 2005, Arauz-Ruiz et al., 1997). Subgenotype F1 is mainly found in strains from Central America and F2 mainly from South America (Norder et al., 2004).

## 1.6 VIRAL PATHOGENESIS AND NATURAL HISTORY OF INFECTION

HBV is itself not cytotoxic to the cells that it infects. It is the host's immune response to the virus that causes the cellular damage and determines the eventual outcome of infection. The severity of the host's immune attack on the HBV infected hepatocytes is responsible for liver injury (Lee, 1997). The degree of liver injury varies greatly from person to person. Some infected individuals contain the infection and clear the virus completely from their system. This could occur without clinically evident liver disease or with an acute inflammatory response of the liver resulting in acute hepatitis B. Some infected individuals are unable to clear the virus and will develop chronic hepatitis. The most important factor that determines the outcome of infection is the age at which the individual is infected. In immunocompetent adults the rate of chronic infection is less than 5%. However over 90% of perinatally infected neonates and 20-30% of infected children between the ages of 1-5 years develop chronic infection resulting in different degrees of chronic liver disease (Villeneuve, 2005).

Following acute infection with HBV, there is an incubation phase of 2 to 6 weeks which is then followed by the production of detectable HBsAg and HBV core IgM antibodies (anti-HBc IgM). High levels of serum aminotransferases are not observed until the infection is well established. This reflects the time needed to generate the T-cell mediated immune response that triggers liver injury (Ganem and Prince, 2004). Thereafter there is normalization of liver function tests as titres of the virus in liver and blood begin to drop but the HBsAg persists in the system until it is cleared. This can occur in a few months or after several years. Low levels of the HBV genome persist in the liver for life in the form of cccDNA. In the event of any form of immunosuppression, HBV infection may be reactivated from this cccDNA (Prince, 2001).

Early in chronic infection there is an immune tolerance phase during which there are high HBV viral loads and the HBeAg is positive but aminotransferase levels are normal. The immune tolerance phase is analogous to the incubation phase but differs in that it may last for decades (Villeneuve, 2005).

The immune tolerance phase is followed by an immune clearance phase. This phase is characterized by the presence of HBeAg, high HBV DNA levels, abnormal aminotransferase levels and progressive liver damage that may lead to cirrhosis and its complications. The occurrence of abnormal levels of serum aminotransferases (flares) is due to immune mediated lysis of infected hepatocytes (Tsai et al., 1992, Chu and Liaw, 1987). These flares may precede HBeAg seroconversion which is a key event in the evolution of chronic HBV infection. Higher rates of spontaneous HBeAg seroconversion are associated with various factors such as older age, high ALT levels, ethnicity and HBV genotypes (Yim and Lok, 2006, Villeneuve, 2005).

The third phase is the inactive carrier stage which is characterized by HBeAg negativity, presence of anti-HBe, undetectable HBV DNA levels, normal alanine aminotransferase (ALT) levels and minimal liver inflammation (Yim and Lok, 2006). Inactive cirrhosis though can be observed in patients who had severe liver injury during the immune clearance phase. At this stage the possible outcomes of infection are HBeAg reversion or sustained remission (Villeneuve, 2005).

There is another form of HBeAg negative chronic hepatitis that is caused by the selection of HBV variants. This is due to mutations in the pre-core and core promoter region of the HBV genome that lead to the loss of HBeAg expression. These HBeAg mutants can be present with high levels of ALT, HBV DNA, and chronic liver damage (Yim and Lok, 2006, Villeneuve, 2005).

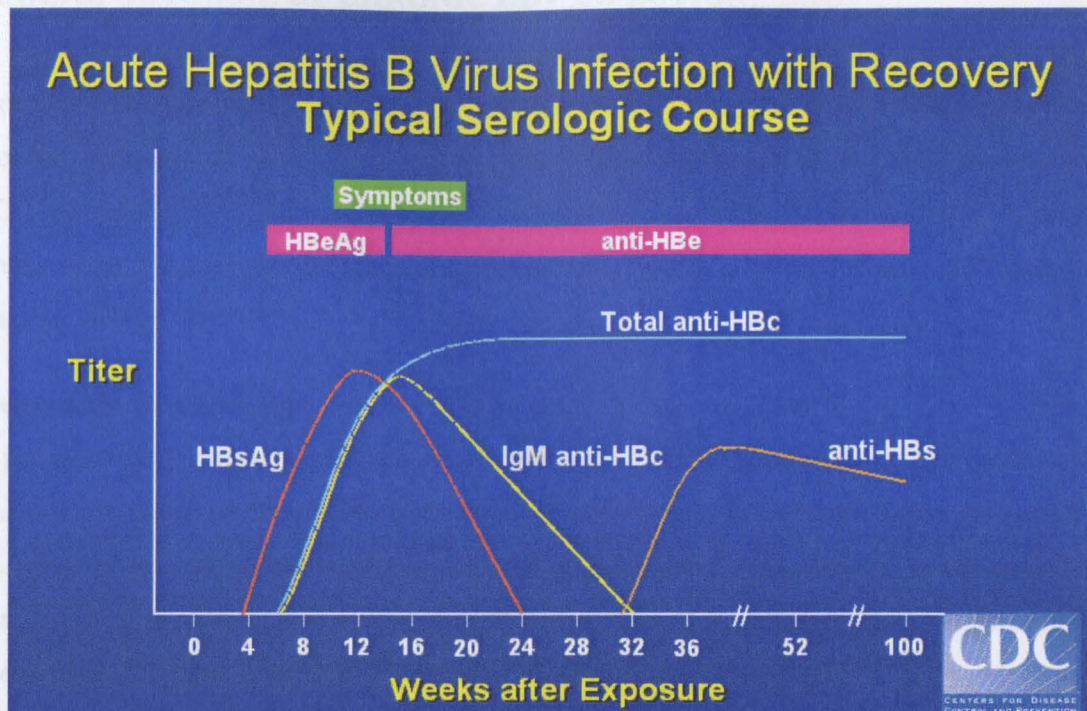


Figure 1.6: Serological course of acute HBV infection leading to immunity (Hunt, 2007).

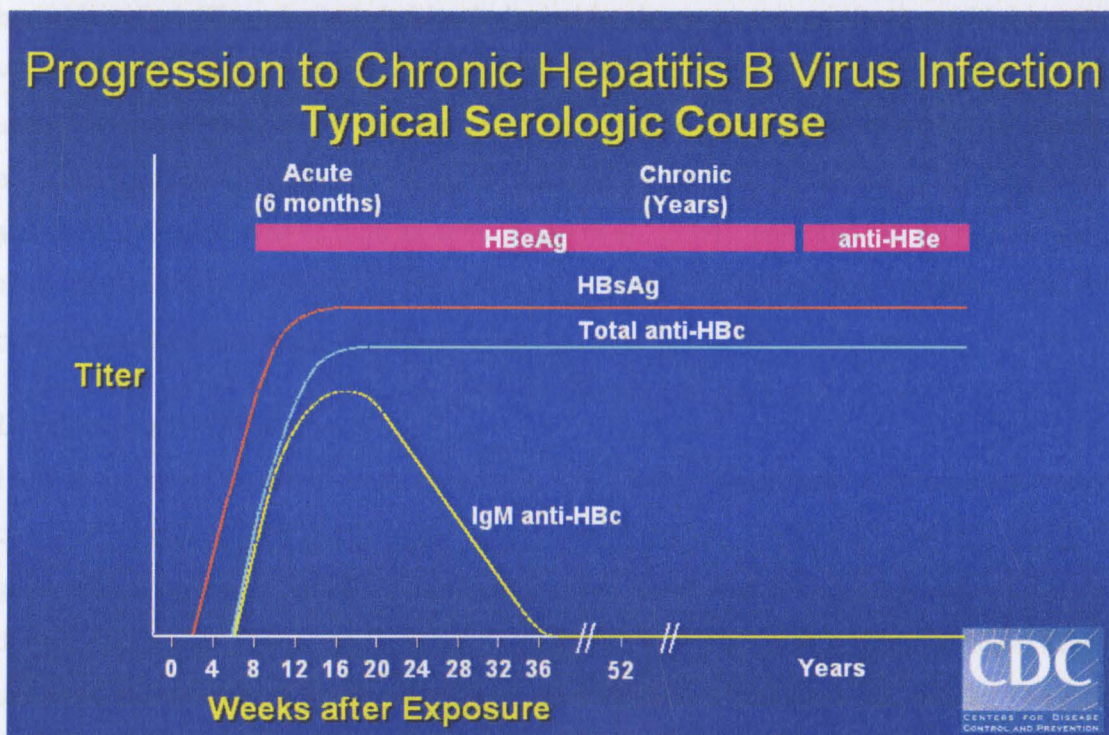


Figure 1.7: Serological course of progression to chronic HBV infection (Hunt, 2007).

## **1.7 CLINICAL OUTCOME OF CHRONIC HBV INFECTION**

The ultimate outcome of chronic HBV infection depends on the duration of the immune clearance phase and the severity of liver damage during this phase (Chu, 2000). The most important sequelae of HBV infection is cirrhosis and subsequently the development of HCC. Approximately 15-20% of patients who acquire HBV infection in adulthood develop cirrhosis with a higher percentage ultimately developing HCC when infected at childhood (Beasley, 1988).

### **1.7(a) Development Of Cirrhosis**

The progression of cirrhosis occurs at an annual rate of 2-5% in HBeAg positive patients. It appears to be faster in patients who are HBeAg negative with reported annual rates of 8-10% (Liaw et al., 1988, Fattovich et al., 1988, Fattovich, 2003). The higher rate of cirrhosis among HBeAg negative patients is related to older age and more advanced liver disease at presentation (Brunetto et al., 1989, Fattovich, 2003).

In the past few years additional factors associated with the progression to cirrhosis have been identified. These include habitual alcohol intake, concurrent infection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) (Liaw et al., 2004, Thio et al., 2002) and HBV genotype C which has more influence than genotype B (Kao et al., 2000, Sumi et al., 2003). A recent study in Taiwan revealed that cirrhosis in patients with chronic HBV and HCV infection was as high as 48% (Liaw et al., 2004). HIV and HBV co-infection has also been shown to increase the risk of developing cirrhosis compared with HBV monoinfection (Thio et al., 2002).

### **1.7(b) Development Of HCC**

HCC is the major cause of death in patients with liver cirrhosis and the third most common cause of cancer related death in the world (Parkin et al., 2001, Llovet et al., 2003). The geographic distribution of HCC varies around the world with 80% of the cases occurring in developing countries such as Far East Asia, South Asia, and Sub-Saharan Africa. These are the same regions where the prevalence of HBV is highest (Parkin et al., 1984). The risk of HCC in cirrhotic patients with chronic HBV infection is over 2% a year with a cumulative incidence risk of 15-20% (Fattovich et al., 1995).

At least two mechanisms are thought to have important roles in the induction of liver cancer by HBV. HBV may induce HCC indirectly by repeated liver damage resulting in necroinflammation and the regeneration of hepatocytes. Malignant transformations may occur due to these continuous and recurrent cycles of hepatocyte necrosis and regeneration. The high rate of cell turn over may act as a tumour promoter due to the accumulation of spontaneous DNA mutations. These mutations result in an increased selective growth advantage for the transformed cells (Wang, 1985). Also chronic necroinflammation may induce malignant transformation by generating mutagenic reactive oxygen species during the inflammatory period (Freeman and Crapo, 1982).

Another mechanism involved in the development of HCC is its oncogenic property. HBV is now officially recognised as a human carcinogen by the WHO. The HBV-DNA integrates into the hepatocyte DNA producing cellular oncogenes (Chisari et al., 1989, Brechot et al., 2000). The integration of viral DNA increases the production of the transactivator protein, HBx antigen, which may induce malignant transformation of hepatocytes. In addition it can bind to the p53 tumour suppressor gene leading to function disruption (Kim et al., 1991, Henkler and Koshy, 1996, Fietelson et al., 1993) and allowing for the growth of tumours.

A relationship also exists between viral replication and HCC development. Recent studies have shown that the risk of HCC development can be as high as 87% in patients between the ages of 30-70 years and positive for HBsAg and HBeAg. Those patients who are HBsAg positive have a 12% risk compared to a 1% risk in those who are HBsAg negative (You et al., 2004). Even though HBeAg seroconversion usually confers a favourable outcome, patients with the HBV e antigen negative mutant still develop cirrhosis which is a prerequisite for the development of HCC (Hsu et al., 2002).

Other factors that may increase the risk of HCC are HBV genotype, specific viral mutations of the enhancer II/core promoter/precore regions and HBV DNA levels (Yuen et al., 2008). Most studies examine HBV genotype B and C in relation to HCC as these are the two main genotypes in Asia which accounts for 75% of the world's Chronic HBV (CHB) population. Some studies suggest that genotype C has a higher risk of HCC

development, however this has not been substantiated by other groups (Chan et al., 2004). A large study conducted in Taiwan showed that genotype B is more common in patients in whom HCC developed at a young age, while in Caucasian and Indian/Asian populations genotype D is associated with a higher risk of HCC development compared to genotype A (Kao et al., 2000).

The core/precore promoter mutations of A to T at nucleotide 1762 and G to A at nucleotide 1764 which are commonly found in HBV genotype C has been associated with an increased risk of HCC development. These mutations result in changes in the overlapping X gene. The HBx protein is a known potent transactivator, it may activate host genes including oncogenes leading to tumourgenesis (Caselmann, 1995). These observations have not been substantiated in other studies because the predominant genotypes are different between Asia, Europe and USA. In addition to these common mutations, two other mutations have been associated with an increase in HCC development. These mutations are C to T at 1653 in the enhancer II region and T to C/A/G at 1753 in the core promoter region (Takahashi et al., 1999, Tanaka et al., 2006).

The most recent and largest study done by Yuen and colleagues examined the individual role as well as possible interacting effects of HBV genotype, specific viral mutations of enhancer II/core promoter/precore regions and HBV DNA levels. The large number of patients studied allowed for any possible links between these factors to be defined more explicitly. The study showed that there was a higher risk of HCC in patients with genotype C than genotype B. This was consistent with previous studies done (Chan et al., 2004). However genotype C was not found to be an independent factor for HCC. Core promoter mutations, T1653 mutations, high DNA levels and the presence of cirrhosis were found to be independent risk factors for HCC. This finding was not unusual as there is a strong association of genotype C with core promoter mutations (89.8%) and genotype B with precore mutations (72.9%) (Yuen et al., 2007).

Yuen and colleagues also showed that the risk of HCC was increased by 22.2 fold in patients with core promoter mutations and liver cirrhosis compared to those with core promoter wild type and without cirrhosis. Patients with core promoter mutations and HBV

DNA levels of  $>4\log_{10}$  copies/ml had a 7.2 fold increase risk of HCC compared to those with wild type core promoter and HBV DNA levels of  $<4\log_{10}$  copies/ml. Due to the large number of patients studied, it was confirmed that T1653 was an independent risk factor for HCC irrespective of HBV genotypes (Yuen et al., 2007).

## 1.8 HOST IMMUNE RESPONSE

The immune response to HBV antigens is responsible for viral clearance. Immediately after infection, the innate immune response plays a major role in limiting the spread of the pathogen while initiating the development of an adaptive immune response. The innate host response is characterized by the production of type-1 interferon (IFN)  $\alpha/\beta$  cytokines and the activation of natural killer (NK) cells. Type-1 IFNs production is triggered directly by virus replication through cellular mechanisms that detect the presence of viral RNA and DNA (Lund et al., 2003, Heil et al., 2004) while NK cells are activated by the recognition of major viral peptides presented on histocompatibility complex (MHC)-class 1 molecules on the surface of infected cells (Moretta et al., 2005).

In mouse models of acute viral infection, the viral kinetics observed is that of initial fast viral spread with subsequent rapid activation of the innate immune system. In human HBV infection a different pattern is observed. There is delayed replication and spread of the virus with HBV DNA and viral antigens being detectable in the serum or liver only at 4-7 weeks post-infection (Thimme et al., 2003). Thereafter HBV replication expands at a logarithmic rate (Whalley et al., 2001) that can be detected in the serum and result in the infection of most hepatocytes (Thimme et al., 2003). Previous studies have shown that HBV does not induce any IFN genes in the early phase of infection. No genes are up or down regulated in the lag phase of infection or log phase of viral spread. This indicates that HBV does not induce intrahepatic innate immune responses (Wieland 2004). The lack of induction may mean that the HBV DNA genome replicating within the nucleocapsid might be shielded from cellular immune response that would normally sense double stranded RNA (Samuel 2001) or that extracellular HBV particles or products are not being detected by toll-like receptors (Herzog et al., 2003). The inability to detect type-1 IFN production

during the early phase of HBV infection is an indication of HBV being a stealth virus, as it is able to sneak through the first line of the host defence mechanisms (Bertoletti and Gehring, 2006). However during the viral clearance phase, T-cell derived IFN- $\gamma$  regulated genes are induced in large numbers. This reflects the significant contribution the adaptive T-cell response has in inhibiting viral replication and terminating infection (Wieland et al., 2004)

The immunological events that lead to acute self-limited HBV infection suggests that, an initial containment of viral replication and the maturation of the adaptive immune response are both linked to the early production of IFN- $\gamma$  and TNF- $\alpha$  within the liver (Guidottu et al., 1999, Nakamra et al., 2001). Production of IFN- $\gamma$  and TNF- $\alpha$  activates the adaptive immune responses causing lysis of the liver cells containing HBV in low quantities and resulting in control of HBV replication (Menne et al., 2002).

The adaptive immune response consists of a web of effector cell types that have important key roles in the development of immunity to HBV. Helper T-cells, also known as CD4 T-cells produce cytokines that are required for the efficient development of effector cytotoxic CD8 T-cells and B cell antibody production. CD8 T-cells are involved in the clearance of HBV infected hepatocytes through cytolytic and non-cytolytic mechanisms (Guidotti and Chisari, 1996). This leads to the reduction in the levels of circulating virus. B cells produce antibodies that neutralize free viral particles and prevent the re-infection of hepatocytes (Alberti et al., 1978).

Patients with chronic or acute HBV infection have distinct differences in their adaptive immunity. Previous studies postulate that helper T-cells and cytotoxic responses are stronger in patients with acute HBV compared to those with chronic HBV infection. Patients with chronic infection usually have weak or undetectable virus specific T-cell responses. These findings suggest that the strength and multispecificity of the T-cell response is the principal determinant of viral clearance during HBV infection.

The humoral response to the viral envelope protein antigens leads to the clearance of circulating viral particles. The cellular immune response to the envelope, nucleocapsid and polymerase antigens results in the elimination of the infected cells. The integrated

activation of both responses allows the host to control infection. Both immune responses are so interconnected that the failure of one of them affects the expansion and protective efficacy of the other. The lack of CD4 T-cell help can impair CD8 T-cell activity and antibody production (Kalams and Walker, 1998) while the lack of CD8 T-cell response results in high levels of circulating virus that can not be cleared by antibodies alone (Ciurea et al., 2001).

### **1.8(a) Antibody Response**

The antibody response to HBV envelope antigens is T-cell dependant. The anti-envelope antibodies (anti-HBe) are detectable in patients with resolved HBV infection but undetectable in patients with chronic HBV infection. Antibodies play a crucial role in viral clearance by complexing with free viral particles and removing them from circulation or by preventing their attachment and uptake by susceptible cells (Milich and McLachlan, 1986).

The antibody response to HBV nucleocapsid antigens (HBcAg) is not clear. They are present in high titres in both acute and chronic HBV infection hence it is suggested that they may not neutralize viral infectivity (Chisari and Ferrari, 1995). The T-cell response to HBeAg and HBcAg in patients with acute HBV infection is quite strong while weak in patients with chronic infection. The fact that there is a high prevalence of a strong antibody response to HBcAg in chronically infected patients, may be due to that fact that HBcAg may function as both a T- cell independent and a T-cell dependent antigen (Milich and McLachlan, 1986).

### **1.8(b) CD4/Helper T-Cell Response**

A vigorous HLA-class II response is characteristic of patients with self limited acute hepatitis (Penna et al., 1991). The response is directed mainly at multiple epitopes within the nucleocapsid proteins (HBcAg and HBeAg). The HBcAg and HBeAg epitope recognized is located between core residues 50-69 and is most commonly recognized in patients with acute infections regardless of their HLA profile (Ferrari et al., 1991). In contrast, the HBV envelope proteins do not elicit a strong HLA-class II response during HBV infection (Ferrari et al., 1990, Bocher et al., 1999). The reason for the difference in the strength of the immunogenic response between these two antigens, nucleocapsid and

envelope antigens, is not clearly understood. T-cell responses to HBV envelope antigens may occur during the pre-clinical incubation phase of the disease (Vento et al., 1987). It can be hypothesised that HBV envelope specific T-cells may be exhausted or paralysed due to continuous stimulation by the high concentrations of envelope antigens that occur when the early immune responses fail to clear the infection completely (Chisari and Ferrari, 1995). However the HBV envelope protein can elicit a strong helper T-cell response in a good proportion of vaccine recipients who have been immunized by plasma derived or recombinant HBsAg vaccines (Celis et al., 1988, Ferrari et al., 1989, Bocher et al., 1999). This suggests that differences in antigen presentation or different regulatory networks may influence the immune responses in these two groups.

### **1.8(c) Cytotoxic T-Cell Response**

The cytotoxic T lymphocyte (CTL) response is believed to play a major role in the eradication of viral infection by identifying and killing virus infected cells through the recognition of viral peptides presented by HLA class I molecules. The study of HLA class I restricted CD8 T-cell responses to HBV has been difficult due to the restricted host of HBV and the inability to propagate HBV in cell culture (Chisari and Ferrari, 1995). A new experimental strategy to study the CTL response to HBV has been developed in which short synthetic peptides that mimic processed antigen fragments are combined with eukaryotic expression vectors that direct HBV antigen synthesis in human cells. This leads to the processing and presentation of the viral antigens on HLA class I molecules (Bertoletti et al., 1991, Penna et al., 1991). These studies have demonstrated that the CD8 T-cell response is stronger in self-limited than chronic infection. The response may last for decades after recovery from acute infection and it can also be observed after the resolution of chronic HBV infection (Rehermann et al., 1996).

Patients with self-limited acute hepatitis develop a vigorous, polyclonal HLA class-I restricted CTL response against multiple epitopes in the HBV envelope, nucleocapsid and polymerase proteins. Studies on self-limiting hepatitis B have mostly been done using peptides that bind specifically to HLA-A2 molecules. Hence most of the CTL epitopes identified are HLA-A2 restricted, although HBV specific cytotoxic epitopes restricted to

different HLA-class 1 molecules have also been identified (Missale et al., 1993, Sobao et al., 2001, Thimme et al., 2001).

An epitope hierarchy exists within the HBV-specific CD8 T-cell responses that can be altered by viral persistence. The dominant response among A2 restricted epitopes in patients with acute hepatitis is the core 18-27-specific CD8 T-cells response. This response is not absolute as in some patients Pol 455-463, Env 183-191 or Env 335-343 specific CD8 T-cells have been found to quantitatively dominate the CD8 T-cell response (Webster et al., 2000, Webster et al., 2004). When immunodominance is defined as the most common response amongst different patients, the overall dominance of these three responses among the different HLA-A2 restricted epitopes is maintained. The immunodominance coded by these sequences is due to their strong binding affinity for the HLA-A2 molecule. The reason for the dominance is thought to be that some HLA-class I epitopes are within helper T-cell epitopes. Helper T-cells are necessary for the maintenance of functional CD8 T-cells and the link between helper and cytotoxic epitopes have been shown to be important for the activation of CTL responses (Kalams and Walker, 1998).

In chronic hepatitis B patients, the functional fate of epitope specificities greatly differs from that found in patients with acute hepatitis B infection. Core 18-27 specific CD8 T-cells, which are immunodominant in acute hepatitis, can not be detected in circulation when HBV DNA levels are  $>10^7$  copies/ml. The inability to detect core 18-27 specific CD8 T-cells within the circulatory compartment is due to the fact that these cells within the liver are inversely proportional to the level of HBV replication (Webster et al., 2004). The only specificities that are observed in chronic hepatitis B is the envelope and polymerase specific CD8 T-cells. Their ability to persist within high levels of HBV replication ( $>10^7$  copies/ml) is apparently associated with the inability to exert antiviral function. Envelope specific CD8 T-cells are characterised by their tolerance to the fluctuations of HBV DNA levels whereas polymerase specific CD8 T-cells are persistent due to the low quantity of polymerase epitopes expressed by infected hepatocytes (Kakimi et al., 2002).

## 1.9 MECHANISMS OF VIRAL PERSISTENCE

For HBV to persist in the system it must be able to evade immune surveillance by either inducing an ineffective antiviral immune response or evolving strategies to escape an efficient immune response. The difference between the strength and multiplicity of the antiviral T-cell response in patients with acute hepatitis and those with CHB infection indicates that, this is the main determinant of viral clearance or persistence in this disease. The activation of an ineffective immune response to HBV can lead to the selection of viral mutants. This can change the virus's visibility to the immune system and make the process irreversible however the molecular and cellular reasons for the difference in the strength of the response are not well defined (Chisari and Ferrari, 1995).

### 1.9(a) Neonatal Tolerance

The chronic hepatitis B infection that develops in neonates born to infected mothers is thought to be due to transplacental infection of the developing foetus or transplacental passage of viral antigens. Neonates born to HBeAg positive mothers are successfully protected against HBV infection when immunized with HBsAg vaccine. This is inspite of their immune system having been exposed to HBV envelope antigens during maturation (Beasley et al., 1983). This indicates that neonates are immunocompetent with respect to this antigen and that HBV envelope-specific T and B cells are present and functioning at birth. This suggests that tolerance to HBV envelope antigens is not induced and that exposure to viral proteins during foetal life doesn't result in a permanent state of tolerance as nonresponsiveness can be reversed by HBsAg immunization (Jamieson and Ahmed, 1988). It is also evident that HBsAg immunization induces T-cell responses in these infants that can clear an ongoing infection. This is consistent with reports that human and murine HBsAg vaccine recipients produce class I-restricted cytotoxic T-cell responses to HBsAg and that accelerated viral clearance is achieved in chronically infected adults who have been immunized with the same HBsAg vaccine (Pol et al., 1993).

### 1.9(b) Tolerance in Infected Adults

In adults, immunological tolerance may be caused by the exhaustion of the antiviral CTL response due to high viral loads (Moskophidis et al., 1993). It could also be due to the induction of apoptosis during vigorous proliferative responses to antigens (Webb et al.,

1990). Alternatively exhaustion can be due to infection with low doses of virus or following weak proliferative responses (Marrack et al., 1993). It has been shown that some patients with acute hepatitis B display strong proliferative T-cell responses to HBV nucleocapsid antigens. This vigorous T-cell response is associated with the clearance of serum HBV envelope antigens. In mouse with lymphocytic choriomeningitis infection, this response can be exhausted resulting in a failure to eradicate infection (Moskophidis et al., 1993).

#### **1.9(c) Infection of the Immunologically Privileged Sites**

Infection of immune privileged tissue is ignored by the immune system as these sites are inaccessible to lymphocytes. This is also the case with the exposed viral antigens on cell types that do not express MHC molecules. Through this mechanism, viruses can evade recognition at the site of replication and hence avoid immunopathology of infected tissues. Virions released from immunologically protected sites can infect liver cells and stimulate memory T-cells hence increasing the risk of chronic liver disease (Chisari and Ferrari, 1995).

#### **1.9(d) Modification of Recognition Molecules on the Surface of Infected Cells**

Studies have revealed that viruses can influence the expression of HLA and other accessory molecules leading to the lack of recognition of infected cells by CTL and also virus escape from immune clearance (Oldstone, 1989). There is no evidence that directly links HBV or any possible influence on HLA expression and its accessory molecules processing pathways. However recent findings that HBcAg can suppress the transcription of the  $\beta$ -interferon gene (Whitten et al., 1991) and that the HBV polymerase protein can inhibit cellular responses to IFN  $\alpha$  and  $\beta$  suggest that HBV may have the capacity to down regulate these immunoregulatory molecules (Foster et al., 1991). Viruses can also evade the immune system by down regulating the expression of their own proteins (Oldstone, 1989) or through extensive viral genomic rearrangement that reduces its visibility to the immune system (Chisari and Ferrari, 1995).

**1.9(e) Inhibition of the Viral Gene Expression by the Immune Response**

The ability of the CTL immune response to suppress HBV gene expression can work against the host and subsequently contribute to viral persistence. This can result in a weak immune response that leads to viral persistence through sufficiently reducing the expression of viral antigens to a level low enough for most infected cells to escape immune recognition. The disease can then be maintained through the destruction of a set of partially downregulated hepatocytes, that express enough antigens to remain visible to the CTL (Chisari and Ferrari, 1995).

It may be possible that some HBV isolates are more or less sensitive to the regulatory effects of some of the inflammatory cytokines. Mutations with recognized viral elements that respond to these cytokines could influence the outcome of the infection profoundly because a single mutation could theoretically abrogate the antiviral effect of the entire immune response. However to date the extent to which this mechanism contributes to the development of viral persistence remains to be demonstrated.

**1.9(f) T-Cell Receptor Antagonism: Inhibition of Epitope Recognition by Mutant Viruses**

Amino-acid substitutions of T-cell receptor (TCR) contact sites can influence CTL responses by creating analogue peptides that can interact with the T receptor, but do not create strong immune stimulation to deliver a full signal hence acting as antagonists. This phenomenon is still largely undefined but it is possible that low affinity interactions between specific TCRs and variant peptide/HLA complexes that preclude stable and efficient TCR multimerization could inhibit proper signal transduction (Brown et al., 1993).

A recent study has demonstrated the presence of natural variants of the HBcAg 18-27 CTL epitope that interfere with the wild type epitope and act as T-cell receptor antagonists (Bertoletti et al., 1994). This property could give the variant a selective survival advantage over the wild type variant if the CTL response in these patients is solely focused on this epitope. As individual HBV infected cells contain many HBV-DNA copies, a single cell could simply express both variant and wild type peptides on the surface. This would lead to antagonism as the cell containing the mutant would be effectively protected from immune

elimination by CTL specific for the wild type epitope. Subsequently the selective expansion of the variant viral population due to survival of the infected cells could occur as these cells are not efficiently destroyed by CTL with other specificities. Under these conditions this mechanism could contribute to persistent infection by HBV or other viruses such as HIV and HCV that also have high mutation rates (Bertoletti et al., 1994).

#### **1.10 MANAGEMENT AND TREATMENT OF CHRONIC HBV INFECTION**

HBV is a preventable disease but the seriousness of infection and ease of transmission can have a profound impact on the health of the community (Thompson, 2002). The prevention and reduction of HBV infection rates can be effected by simple health measures.

In healthcare workers (HCWs), avoiding blood and body fluid contact during invasive procedures and practising safe injection techniques can aid in the prevention of HBV transmission from the infected patients and vice-versa. HBV is a major health hazard in renal dialysis units for both staff and patients. The dialysis units have guidelines that help to reduce transmission rates. These guidelines include the dialysing of HBV positive patients in a separate area and on a dedicated machine. This leads to a dramatic reduction in infection transmission rates although HBV transmission can still occur due to a number of other factors such as the contamination of equipment and environmental surfaces, inadequate sterilization of reusable dialyser needles and failure to identify HBsAg positive patients through regular testing (Thompson, 2002). Access to proper sanitation, screening blood products for the hepatitis B virus and also creating disease awareness amongst the public can also contain the spread of HBV in a community.

Passive and active immunisations against HBV have been used to combat the global transmission of the virus. Passive immunisation with hepatitis B immunoglobulin was used to prevent perinatal infection in infants (Chang et al., 1997). The introduction of an effective low cost HBV vaccine has enabled the introduction of immunization programmes that can control the transmission of HBV in both developed and developing countries. In addition to this, collaborative efforts between healthcare workers, policy makers and the

public is still needed to provide the necessary pressure needed to establish and maintain more national programmes in developing countries.

As most infections occur in the first few years of life, the most effective global vaccination programme is one that commences at birth. As most developing countries have a high prevalence of HBV, the whole community is at an increased risk of infection. A dramatic reduction in childhood HBV carrier rates has been observed in countries where universal hepatitis B vaccination has been implemented. In Taiwan in 1984, the first nationwide universal programme was launched. For the first two years the programme only covered neonates whose mothers were HBsAg carriers. Thereafter it was extended to cover all infants followed by children and finally adults. This resulted in a decrease in both perinatal and horizontal transmission and a 10-fold decrease in carrier rates (Chang et al., 1997). Due to these findings the World Health organisation (WHO) called for all countries to add the HBV vaccine to their national immunisation programmes (Kane, 1998). Since then reductions in carrier prevalence rates in Asia have been dramatic. In Thailand, the carrier rates are down to 0.8% from 5.4% and in five provinces of China, a decrease from 14.6% to 1.4% has been noted. In Africa and the Mediterranean a drop in childhood carrier rates have also been observed. In the Gambia, there has been a drop from 12% to 0.9%, Senegal to 2% from 12% and in Saudia Arabia, 6.7% down to 0.3% (Thompson, 2002).

A 'high risk strategy' was adopted by some countries with a low prevalence of HBV infection. The authorities targeted those who were at greatest risk due to their occupation, ethnic background or high risk behaviour. This however depended on these people being readily identified and seeking vaccination. The social stigma attached to high-risk behaviour deterred some individuals from disclosing their risk to HCWs and there was recognition that there were some cases that could not be prevented by targeted vaccination (Thompson, 2002). This was one of the reasons why implementing the targeted vaccination programmes was difficult in these low prevalence countries which have now mostly adopted the universal approach to hepatitis B control.

The main aim of treatment of patients with chronic hepatitis B virus is the prevention or decrease of the risk of cirrhosis and HCC development. This is achieved through the

reduction of viral replication and elimination of the virus which would finally improve patient survival (Pramoolsinsup, 2002). The treatment of these patients requires follow-up and monitoring. In most clinical trials, the standard therapeutic end points have been the loss of HBeAg and achieving undetectable HBV DNA levels. Viral suppression is indicated by the loss of HBeAg and the reduction of serum HBV DNA to levels undetectable by hybridization-based assays. The secondary endpoint is usually improved liver histology. The reduction in liver damage is indicated by normalization of ALT levels and an improvement in the histology of liver biopsy. The total eradication of the virus is rarely achieved during treatment and is therefore not used as a therapeutic end-point (Lai et al., 2002).

Agents that are currently in use or are being investigated for the treatment of chronic HBV infection are divided into two categories; nucleoside and nucleotide analogs and immunomodulators.

#### **1.10(a) Treatment Agent: Nucleoside and Nucleotide Analogues**

Nucleoside and nucleotide analogues suppress HBV replication through the inhibition of the viral polymerase thus blocking reinfection of healthy hepatocytes (Colacino and Staschke, 1998). To induce the complete elimination of HBV infection, the cccDNA pool in the nuclei has to be eradicated. cccDNA is minimally affected by nucleoside analogues as it is not duplicated by semi-conservative replication but by replenishment from the cytoplasmic pool of actively replicating genomes (Lai et al., 2002). Many nucleoside analogues have been tested for antiviral efficacy in chronic HBV patients and most were found to substantially inhibit HBV replication with minimal side effects. These nucleoside analogues are namely Lamivudine, Adefovir dipivoxil, Entecavir, Emtricitabine, Famciclovir-penciclovir, Clevudine, Tenofovir, Telbivudine and Ganciclovir (Chang et al., 2006, Lai et al., 2002). The most commonly used nucleoside analogue is Lamivudine and will be discussed further.

##### **1.10a(i) Lamivudine**

Lamivudine,  $(-)\text{-}\beta\text{-L-2',3'-dideoxy-3'-thiocytidine}$ , is a cytosine analogue that has a potent effect on the RNA dependent DNA polymerase of HBV and HIV (Pramoolsinsup, 2002). It is anabolized in the hepatocytes and triphosphorylated into the active form lamivudine 5'-

triphosphate. Lamivudine has minimal activity against mammalian DNA polymerase gamma, the enzyme responsible for mitochondrial DNA synthesis, and is not incorporated into the mitochondrial DNA (Chang et al., 1992). Hence it has no major effect on bone marrow, hepatocytes, muscle tissue and the nerves (Lai and Yuen, 2000b).

The active form of lamivudine has two modes of viral suppression. It mimics deoxycytidine triphosphate and is incorporated into growing DNA chains where it acts as a chain terminator (Ben-Ari et al., 1997, Johnson et al., 1999). Secondly, it demonstrates competitive inhibition of both the viral DNA dependent and RNA dependent DNA polymerase activity (i.e. reverse transcriptase activity). There are four possible sites of action for lamivudine: the most obvious site of action is during reverse transcription of pre-genomic RNA into the nascent (-)-strand DNA (pathway 1) (Seifer, 1998, Galliard, 1998). The second site of action hinders the formation of the (+)-strand DNA from the nascent (-)-strand DNA (pathway 2) (Xiong et al., 1998, Condreay, 1999). This is dependent on viral DNA polymerase activity. Lamivudine may also exert its effect by blocking the completion of the synthesis of the double-stranded DNA thus reducing cccDNA formation (pathway 3). cccDNA formation may also be interrupted both during the initial infection (pathway 4) and at the subsequent amplification steps (Lai and Yuen, 2000a).

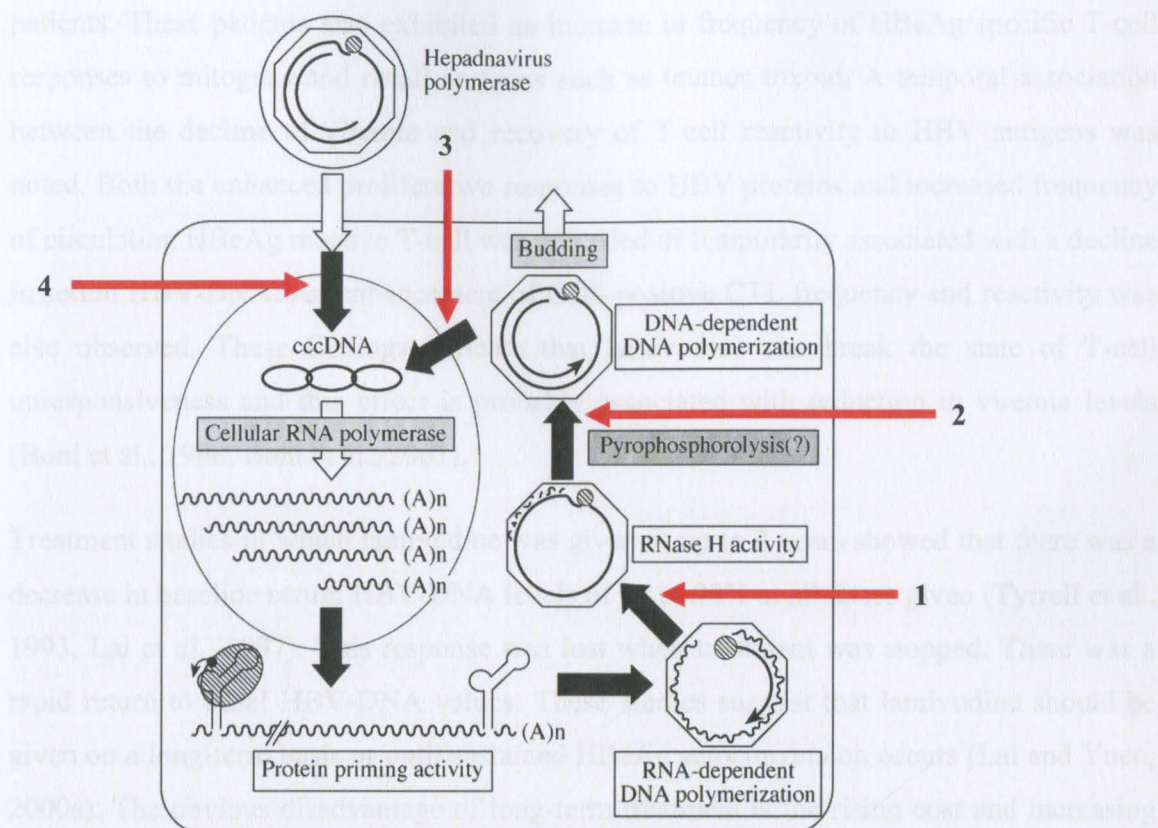


Figure 1.8: Adopted illustration of the four possible sites of action for lamivudine (Kamiya, 2003)

A crucial determinant of viral persistence and the ability to respond to therapy is T-cell hyporesponsiveness (Boni et al., 1998). The high viral load in CHB patients is thought to be the major driver in this phenomenon of immune tolerance. Lamivudine treatment has been found to reduce immune tolerance through reversing the T-cell hyporesponsiveness to HBV antigens. The importance of these two factors in the immune response to HBV infection was investigated by Boni and colleagues. They used the reduction of viral and antigen load by lamivudine as an experimental model to conduct the investigation (Boni et al., 1998). Twelve patients with chronic HBeAg positive hepatitis received lamivudine, 100mg/day, for twelve months and thereafter followed-up for six months. Before treatment the T-cell response to HBcAg and HBeAg was low but during lamivudine therapy the response to both antigens showed a significant and rapid increase that persisted throughout this therapy. After two weeks of lamivudine therapy, a significant enhancement of proliferative CD4 mediated response to HBeAg and HBcAg was observed in 83% of the

patients. These patients also exhibited an increase in frequency of HBeAg specific T-cell responses to mitogens and recall antigens such as tetanus toxoid. A temporal association between the decline of viremia and recovery of T-cell reactivity to HBV antigens was noted. Both the enhanced proliferative responses to HBV proteins and increased frequency of circulating HBeAg reactive T-cell was preceded or temporarily associated with a decline in serum HBV-DNA. An enhancement of CD8-positive CTL frequency and reactivity was also observed. These findings indicate that lamivudine can break the state of T-cell unresponsiveness and this effect is probably associated with reduction in viremia levels (Boni et al., 1998, Boni et al., 2001).

Treatment studies in which lamivudine was given to up to 2 years showed that there was a decrease in baseline serum HBV-DNA levels of up to 98% at all doses given (Tyrrell et al., 1993, Lai et al., 1997). This response was lost when treatment was stopped. There was a rapid return to basal HBV-DNA values. These studies suggest that lamivudine should be given on a long-term basis or until sustained HBeAg seroconversion occurs (Lai and Yuen, 2000a). The obvious disadvantage of long-term treatment is the rising cost and increasing development of drug resistance.

Lamivudine resistant HBV viruses emerge after 9 to 10 months of therapy (Liaw, 2001). These viruses have a characteristic mutation at the methionine residue in the tyrosine-methionine-aspartate-aspartate (YMDD) motif. This highly conserved motif is present in the RNA dependent DNA polymerase (Balzarini et al., 1996) and is shared by all hepadnaviruses as well as retroviruses. The mutation is an amino-acid substitution, where methionine is replaced by an isoleucine or a valine at codon 552 (YMDD to YIDD/M552I or YVDD/M552V). There is also a compensatory mutation at codon 528 where leucine is replaced by methionine (L528M) and is almost always found in association with M522I (Fu and Cheng, 1998). Many other mutants have been reported (Hussain and Lok, 1999) but it is only these three that have been shown to confer resistance to lamivudine treatment.

The alteration of the wild-type YMDD motif leads to a decline in the inhibitory effect of lamivudine and a reduction in effective replication in YMDD variants. This is most likely due to a reduced affinity of the viral polymerase for its nucleotide substrates (Allen et al.,

1998, Melegari et al., 1998, Ling and Harrison, 1999, Ono et al., 2001). *In-vitro* studies have shown that these strains can confer up to 10000 fold resistance suggesting that a clinical response can not be restored by increasing the dose of lamivudine (Allen et al., 1998). The clinical manifestations of these mutant strains are variable. In most patients with breakthrough viral replication, serum HBV-DNA and ALT levels remain below the pre-treatment values, there is significant improvement in liver histology and in some cases HBeAg seroconversion in the wild type. This suggests that there may be continued suppression by lamivudine on the wild-type virus. The beneficial effects seen in patients with the resistant mutants and continued lamivudine treatment may be related to the impaired replication capacity of the mutants and the rapid outgrowth by the wild type virus. Flares in hepatitis owing to re-emergence of the wild type virus can occur following the withdrawal of lamivudine therapy. The current treatment guidelines recommend that lamivudine and another agent should be added in this situation.

#### **1.10(b) Treatment Agent: Immunomodulating Agents**

Immunomodulators act by enhancing the host immune response to HBV antigens presented on the surface of the hepatocytes which results in the death of infected hepatocytes. Examples of immunomodulators include IFN- $\alpha$  and more recently pegylated IFN- $\alpha$ , thymosin- $\alpha$  and therapeutic vaccines (Lai et al., 2002). Patients with poor hepatic reserves due to cirrhosis should be treated with caution due to risk of hepatic decompensation.

##### **1.10b(i) Interferon- $\alpha$ (IFN- $\alpha$ )**

IFN- $\alpha$  was licensed in 1992 for the treatment of chronic HBV in most countries. IFN- $\alpha$  inhibits viral RNA pre-genomic packaging into core particles and also enhance expression of HBsAg by HLA molecules on hepatocytes (Lau et al., 1991). Transcriptional inhibition of the viral RNA blocks HBV replication as HBV utilizes reverse transcription and the plus RNA strand as the template for the first step in its replication i.e. synthesis of the minus DNA strand. IFN- $\alpha$  inhibits transcription by affecting the HBV enhancer gene on the genome. The IFN- $\alpha$  effect on the enhancer gene may be *cis* whereby IFN- $\alpha$  is internalized and binds to regulatory sequences in the HBV genome and hence blocking constitutive transcriptional enhancer activity. It may also act in *trans*, where it induces the production and modification of intracellular proteins which act as transrepressors. There is a lack of

evidence for the *cis* effect of IFN-a and so it is hypothesised that the inhibition usually acts via the *trans* effect (Tur-Kaspa et al.,1990).

IFN-a has been demonstrated by various studies to be quite an effective treatment for chronic HBV. A 4 month course of treatment resulted in a virological response in 30-40% of patients. These patients showed a significant reduction in serum HBV-DNA, ALT normalization, loss of HBeAg and in addition seroconversion of HBeAg to anti-HBe occurred in 15-20% of treated patients(Niederau et al., 1996, Wong et al., 1993, Cohard et al., 1994). As this therapy is expensive and potentially toxic, the potential beneficial response should be estimated before initiating therapy. The most important predictors of a favourable response to IFN-a are elevated serum ALT levels and low serum HBV-DNA levels. Other predictors of favourable response are shown in Table 1.2.

Table 1.2: Patient characteristics associated with favourable and unfavourable outcomes to IFN-a therapy (Pramoolsinsup, 2002)

| Favourable outcomes      | Unfavourable outcomes                                              |
|--------------------------|--------------------------------------------------------------------|
| HBeAg positive           | HBeAg negative                                                     |
| Low serum HBV-DNA level  | High serum HBV-DNA level (>100pg/ml)                               |
| Elevated serum ALT level | low to normal serum ALT level                                      |
| Active liver histology   | Normal liver histology                                             |
| Recent HBV infection     | Long-duration of HBV infection                                     |
| Non-cirrhotic            | Liver cirrhosis                                                    |
| HIV negative             | HIV Positive                                                       |
| Female Caucasian adults  | Male Asian                                                         |
|                          | Pre-core mutations that result in HBeAg negative chronic hepatitis |

The major side effects can be severe and are listed in Table 1.3. Most of the adverse effects are dose related and can be managed through timed dose reduction while the other side-effects may necessitate the cessation of therapy. IFN-a may also induce autoimmune

hepatitis in genetically predisposed patients. The presence of high titres of IFN-neutralising antibodies can counteract the effects of IFN-a but low levels of antibodies have no clinical significance (Lai et al., 2002).

Long-term follow-up data shows that most of the responders (80-90%) have sustained HBeAg seroconversion. Complete eradication of HBV is rare as most of the responders continue to have detectable HBV-DNA when using sensitive PCR assays unless the treated patient becomes HBsAg negative. 80% of patients who lose HBsAg within a decade have delayed seroconversion months to years after completion of treatment (Lai et al., 2002).

Table 1.3: Side effects of IFN-a (Lai et al., 2002) .

| Adverse effects                                |
|------------------------------------------------|
| Influenza like syndrome                        |
| Severe depression                              |
| Induction of auto antibodies                   |
| Induction of IFN neutralizing antibodies       |
| Fatigue, anorexia, hair loss ,nausea           |
| Bone marrow suppression                        |
| In patients with advanced hepatitis/cirrhosis: |
| ➤ susceptibility to cirrhosis                  |
| ➤ worsening of liver function                  |

### 1.10(c) Combination Therapy

Theoretically, combination antiviral therapy has some potential major advantages over monotherapy. Doses of the individual drugs used to provide the desired effect could be lower when used together and this may reduce undesirable side effects. The duration of treatment required to reach a particular end point could be shortened and the antiviral efficacy increased as the sites of action of the individual drugs are usually different or

complementary. An important advantage is that the incidence of drug resistance is reduced. The ideal combination should be an antiviral agent such as a nucleoside analogue with an immunomodulator. The antiviral agent will be used to prevent re-infection of healthy hepatocytes and decrease viral load while the immunomodulator will be used to achieve immune clearance of infected hepatocytes. However randomised trials of lamivudine and IFN have shown that this combination has no added clinical advantage (Lai et al., 2002).

Combination treatment of nucleoside and nucleotide analogues is increasingly being investigated due to the disappointing results of monotherapy. There is little doubt that combination therapy for the treatment of CHB infection is the logical progression for future management, however more ongoing clinical studies are needed to establish the efficacy of these drugs in combination (Lai et al., 2002).

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## **CHAPTER 2:**

### **HEPATITIS B VIRUS INFECTION IN AFRICA**

#### **2.1 PREVALENCE**

Although there is little or no accurate data on the prevalence of HBV infection in most parts of Africa, the disease is undoubtedly highly endemic in the region. The data available in 1992 showed that 98% of the 470 million African inhabitants had been infected with the virus at some point during their lives and 10% remained infected (Kew, 1992). In addition, HBV induced HCC causes approximately 230,000 deaths in Africans annually (Kew, 1992).

The size and diversity of the African continent, frequent lack of resources and distribution of the population among vast rural areas makes accurate determination of HBV carrier rates difficult. Nevertheless the epidemiology of HBV infection in some parts of Africa has been investigated in some detail. Available data shows that most of Africa falls within the highly endemic category. HBsAg positive “carrier rates” range from 5.7% in Morocco to 20.6% in the Democratic Republic of Congo (DRC) (Kiire, 1996, Kew, 1996). The HBV exposure rates are determined by the presence of HBsAb and core IgG, and range from 56.2% in Kenya to 98% in Namibia (Kew, 1996, Kiire, 1996).

Table 2.1: Summary of Prevalence of Hepatitis B Virus infection in the Adult Population of Africa (Kiire, 1996, Kew, 1996)

|                        | Carrier Rate (%)<br>HBsAg +ve | Total Exposure (%)<br>HBsAb, core IgG +ve |
|------------------------|-------------------------------|-------------------------------------------|
| <b>North Africa</b>    |                               |                                           |
| Tunisia                | 6.5                           | 60-64                                     |
| <b>West Africa</b>     |                               |                                           |
| Mali                   | 17.6                          | 96                                        |
| Liberia                | 7.6                           | 61                                        |
| <b>East Africa</b>     |                               |                                           |
| Ethiopia               | 11                            | 79                                        |
| Kenya                  | 11.4                          | 56.2                                      |
| <b>Central Africa</b>  |                               |                                           |
| Zaire                  | 20.6                          | 78.9                                      |
| <b>Southern Africa</b> |                               |                                           |
| South Africa           | 9.6                           | 76                                        |
| Zambia                 | 7.5                           | 62                                        |

## 2.2 ETHNIC AND RACIAL DIFFERENCES

HBV prevalence rates vary between tribes and racial groups within a country. For example, in a study done on blood donors from northern Nigeria, the HBV infection rates ranged from 2.9% in the Nupe to 21.4% in the Gwari tribe (Kulkarni et al., 1986).

The black ethnic population in Africa have a higher HBV prevalence rate compared to the other racial groups living in Africa. 50-70% of the nearly 3 million cases of acute viral hepatitis due to HBV occur in black Africans annually (Ayoola, 1988). HCC is also more commonly found in Black Africans living in Southern Africa (Kew, 1992). In 1986, a study that involved 29,312 black males from different parts of the continent employed on a South African gold mine revealed that the prevalence of HBsAg positivity ranged from 5.5% to 14% in different ethnic groups (Dusheiko et al., 1989). There are a number of possible explanations for these varied rates. These include the lack of disease awareness and access to vaccination programmes, co-infection with HIV which may lead to reactivation and reinfection of HBV in previously exposed individuals, socio-

economic issues and traditional practices such as scarification and circumcision under unhygienic conditions.

The largest population groups of Asians and Caucasians living in Africa are mainly found in South Africa. As a result the most complete information on HBV prevalence within these population groups comes from South Africa. The Caucasian population has a HBV carrier rate of only 0.2% and exposure rate of 5% (Kew et al., 1976). This is based on a study in Caucasian patients with diabetes mellitus. Thus the rates presented may not be an accurate representation of the true prevalence of HBV within the whole Caucasian population in South Africa. The Mixed race population have frequencies intermediate between those of Black Africans and Caucasians. A Johannesburg based cohort of Mixed race school children showed that the HBV carriage and exposure rates varied between 0.4-3% and 18-25% respectively (Kew et al., 1989).

In 1988, Song reported a study of the South African Chinese community which consisted of original Chinese settlers and their South African born descendants. These settlers migrated from the hepatitis B virus endemic mainland province of Guangdong in China. The South African Chinese had an overall HBV carrier rate of 5.3% which was highest in the 30-39 year old age group. Infection rates increased progressively with age and were as high as 50% in adults aged 60-69 years old. There is a decreasing trend in prevalence of HBV infection in South African Chinese and this may be due to improved hygiene and socio-economic conditions in comparison with those in Guangdong. These improvements may also have led to less horizontal transmission of the disease (Song et al., 1988).

### **2.3 RURAL-URBAN DIFFERENCES**

A marked difference in prevalence rates between rural and urban populations has been documented in a number of African countries. In most of the studies, a higher prevalence of HBV infection is found in the rural compared to urban inhabitants however there are studies that have shown no significant difference between the two geographical locations. In Senegal, despite a high average exposure rate of 91%, no significant difference in frequency of HBV infection between the rural and urban inhabitants was observed

(Coursaget et al., 1984). The rural-urban differences have been well documented in Southern Africa and East Africa. In Nairobi, the Kenyan capital city, the carrier rate is 5.3% compared to 12% in rural Kenyans (Bowry, 1983). In South Africa, the prevalence of chronic carriers in rural Eastern Cape is 15.5% while that in urban areas such as Durban and Soweto is lower at 7.4% and 1.3% respectively (Vos et al., 1984). In 1988, Karim and colleagues estimated that HBV exposure rates in Kwa-Zulu Natal's Black African children (6-14 years) from urban and rural areas were 31.4% and 62.1% respectively. Black African children from a children's institution were also included in the study and it was determined that 72% of these children had been exposed to HBV. In infants to 6 years olds, the HBV exposure rate was 2.5% and 7.1% for urban children and 53.1% and 70.3% in institutionalised children (Abdool Karim et al., 1988). A number of factors may contribute to a high prevalence of HBV infection in rural areas. These include poor hygiene, use of unsterile needles, HBV transmission via skin abrasions, unhygienic tribal scarification and insect bites (Abdool Karim et al., 1988, Kiire, 1996).

## **2.4 AGE AT ACQUISITION AND ROUTES OF TRANSMISSION**

### **2.4(a) Age at Acquisition**

HBV infection in Black Africans is largely established in early childhood with most of the individuals being infected between the ages of 1 to 5 years (Dumpis et al., 2001). Several studies in Africa have demonstrated that infection rates in children peak during the latter half of the first year of life and high carrier rates were present by 3-5 years of age (Barin et al., 1981, Whittle et al., 1983, Prozesky et al., 1983b, Botha et al., 1984). This pattern is also seen in HBV infected children in the Far East (Kew, 1996). In addition, baseline epidemiological studies done in Southern Africa revealed that the most infective reservoir is children below the age of 5 years as they have HBeAg positivity rates of up to 56.5% (Prozesky et al., 1983b).

### **2.4(b) Horizontal Transmission**

In Africa, most HBV infections are acquired through horizontal transmission of the virus in early childhood. This is in contrast with the Far East where vertical transmission is the predominant route of infection. Studies done in the early 1980's by Whittle (1983), Botha

(1984) and Tabor (1985) showed that horizontal transmission was common in Africa. In 1983, Whittle studied two villages in Gambia and showed a large variation in HBV prevalence between the two villages and clustered families with transmission between siblings being of major importance. It was found that in a village called Manduar, 62% of children aged 2-4 years were infected with HBV while only 27% of this age group in Keneba village were infected. In addition Whittle estimated that the chances of a child being an HBsAg carrier were approximately 42% if an elder sibling carried the antigen, 27% if either mother or father was a carrier and 15% if neither parent was a carrier (Whittle et al., 1983).

In Namibia, a study done by Botha and colleagues found that in children, only 1% of children less than 6 months of age were HBsAg positive compared with 13% of those over 1 year. Only 4% of HBsAg positive children were under 6 months old and 27% of HBsAg positive mothers had HBsAg positive children. This study confirmed that horizontal transmission of HBV was more important than perinatal transmission in Namibia (Botha et al., 1984).

In Zambia, Tabor and colleagues conducted a longitudinal study in five villages in which 620 residents were studied over a period of 5 years. During the study period, 24 new HBV infections were distributed among 20 households and were consistent with a pattern of horizontal transmission of infection (Tabor et al., 1985).

In 2001, Dumpis and colleagues investigated the main route of HBV transmission in Gambia. They showed that in horizontal transmission the sources of infection are from within the village; intravillage; between families in the same compound; intracompound or within the family unit; intrafamilial (Dumpis et al., 2001). Phylogenetic tree analysis of entire sequences of the pre-core/core region of the HBV genome showed distinct and significant clustering defined at the family and village levels. Intrafamilial and intracompound transmissions were found to be common routes of HBV infection. However in at least two-thirds of the families studied, intrafamilial transmission was observed. This provided strong evidence for higher intrafamilial transmission frequency than intracompound (Dumpis et al., 2001).

High risk behaviour in children may also contribute to high rates of horizontal transmission. Such behaviour includes the sharing of dental cleaning materials, bath towels, chewing gum, candies and chewing finger nails (Martinson et al., 1998). Other possible ways of horizontal transmission maybe via skin scarification by witchdoctors (Kew et al., 1973) circumcision under unhygienic conditions and bloodsucking insects such as mosquitoes and bedbugs (Vall et al., 1990).

#### **2.4(c) Vertical Transmission**

Vertical transmission is less frequent in Africa compared to the Far East due to the lower frequency of HBeAg positivity in African mothers. In Taiwan, 40% of HBsAg positive mothers are also HBeAg positive while in Zimbabwe only 5-15% of mothers who are HBsAg positive are HBeAg positive. The result is that Taiwanese mothers have high viral loads and are therefore very infectious compared to their Zimbabwean counterparts (Kiire, 1990).

The differences in vertical transmission are thought to be influenced by genetics, HBeAg status and HBeAb status. There is evidence that the expression of HBeAg is different in diverse populations and this maybe genetically determined (Ghendon, 1987). It is also postulated that not only is the frequency of HBeAg positivity in mothers genetically determined but also the susceptibility of infants to vertical infection. In Saudia-Arabia, a two year study of 50 HBsAg positive mothers and their new born infants did not reveal any evidence for maternal-foetal transmission vertically, perinatally or postnatally. In addition twelve mothers who were HBeAg positive during pregnancy also did not transmit the virus to their new borns. These infants remained free of infection for up to 2 years compared to Chinese babies who acquired infection at 6 weeks to 3 months of life (Basalamah et al., 1984, Beasley et al., 1983). Concealed vertical infection, with HBV infection manifesting later, can not be discounted. It is hypothesized that infants can be infected with HBV during delivery and remain persistently infected but for some genetically determined reasons have no discernible manifestation of infection for many years. The delay in infection could be due to the presence of maternal HBeAb in the child. However this can only account for the lack of infection in the very early months

before the maturation of the child's own immune system. The virus could then be subsequently reactivated (Kiire, 1996).

## **2.5 HBV AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) CO-INFECTION IN SUB-SAHARAN AFRICA**

### **2.5(a) Epidemiology**

In sub-Saharan Africa, HIV and HBV are endemic and little is known about the burden of co-infection and the interaction between these two viruses. In Africa, HIV is predominantly sexually transmitted whereas HBV is mainly transmitted horizontally with most children being infected by HBV at the age of 5 years. A large population of these children become chronic carriers of HBV who in adulthood may acquire HIV infection and then transmit both viruses sexually (Burnett et al., 2005).

Most of the information on HIV and HBV co-infection is not from Africa. The studies are from countries with low HBV endemicity (Europe and Australia) and where the majority of the population was not protected by antibodies to natural HBV infection by the time they reached sexual maturity (Burnett et al., 2005). Hence the shared route of transmission of HBV and HIV infection resulted in specific adolescent and adult risk groups being exposed to both viruses simultaneously (Burnett et al., 2005).

A number of the studies found that the prevalence of HBV infection among HIV-positive individuals is significantly higher than in HIV-negative individuals. This is thought to be due to the shared risk factors for viral acquisition (Taylor et al., 1988, Hess et al., 1989, Herrero Martinez, 2001, Scharschmidt et al., 1992). Individuals who are already HIV-positive by the time they are exposed to HBV for the first time are at a higher risk of becoming HBV carriers (Taylor et al., 1988, Hess et al., 1989, Herrero Martinez, 2001, Scharschmidt et al., 1992, Bodsworth et al., 1991, Hadler et al., 1991, Sinicco et al., 1997). In addition there may be an increase of newly acquired HBV infections in HIV-positive individuals with prior resolved HBV infections (Horvath and Raffanti, 1994, Gilson et al., 1989). HBV pre-exposed individuals who are then exposed to HIV have been shown to have reactivation of recovered as well as 'silent' chronic infection

(Horvath and Raffanti, 1994, Gilson et al., 1989, Vento et al., 1989). This may be due to immunosuppression brought about by HIV.

Most of the studies done in sub-Saharan Africa have so far not shown an increased prevalence of HBV in HIV-positive individuals as seen in Europe. However a few studies have shown both an increase and decrease of HBV prevalence in HIV-positive individuals. Studies from South Africa, Tanzania and Uganda, which were done when the HIV pandemic had reached high levels or included large numbers of HIV-positive subjects, showed an increased prevalence of HBV in HIV infected individuals (Lodenyo et al., 2000, Shao et al., 1993, Nakwagala and Kagimu, 2002). In contrast, a lower prevalence of chronic hepatitis B in HIV-positive patients compared to HIV-negative individuals was shown in a study conducted in the Central African Republic (Kashala et al., 1994). The reasons for this difference in prevalence are not immediately clear but it could be related to a greater mortality in those who have HBV-HIV co-infection (Hoffmann and Thio, 2007) .

Various studies that included large numbers of HIV-positive subjects were conducted in Cote d'Ivoire, Malawi and Tanzania (Rouet et al., 2004, Sutcliffe et al., 2002, Menendez et al., 1999, Matee et al., 2006). These studies did not show a significant difference in chronic hepatitis B prevalence between HIV-negative (6.0-14.4%) and positive (9.0-16.9%) individuals. With regard to HBV exposure, studies done in Tanzania and Uganda showed similar population wide exposure to HBV regardless of HIV status with 51-76% of HIV-negative and 61-73% HIV-positive individuals having serological evidence of exposure (de Lalla et al., 1990, Shao et al., 1993). Two studies done in pregnant women in the DRC and Zambia (Kashala et al., 1994, Oshitani et al., 1996) found no increased prevalence in exposure to HBV in HIV-positive individuals but raised the concern that HIV infection would have an impact on HBV epidemiology due to changes in HBV serology. Despite a similar prevalence of HBV exposure in HIV-positive and negative pregnant women there was a significant increase in HIV infection. 11.1% of the HIV-positive pregnant women compared to 2.7% of the HIV-negative women were positive for all three HBV markers (HBsAg, anti-HBs and anti-HBc). These results suggested that reinfection or reactivation of the HBV was occurring in the HIV-positive women. The

same study showed an increase in all three HBV markers in 15.9% of the HIV-positive patients compared to 5.9% of the HIV-negative blood donors (Kashala et al., 1994). In Zambia, a study conducted on pregnant women found no significant increase in HBsAg positive status in the HIV-positive women but there was a high prevalence of HBeAg positivity in HBsAg positive women suggesting a much higher rate of infectivity (Oshitani et al., 1996).

There are several possible explanations for the differences observed in HBV-HIV co-infection prevalence across the sub-Saharan continent. The HBV genotype found in a particular region or the genetic profile of a population group from a particular region may play a role in influencing the rate and prevalence of HIV-HBV co-infection. Some of the studies had significant weaknesses. These include research conducted on small sample groups (Menendez et al., 1999, Pawlotsky et al., 1995, Ogutu et al., 1990, Ahmed et al., 1998) with different age groups at different stages of HIV infection or done at a time when HIV was relatively low in a population (Candotti et al., 2001, Menendez et al., 1999). In view of these study weaknesses, new work is needed to better describe the epidemiology of HBV-HIV co-infection in areas of high HBV endemicity such as Africa.

## **2.5(b) Changes in the Natural History of Chronic Hepatitis B Virus (HBV) Disease**

### **Progression**

The natural history of chronic HBV infection is modified by co-infection with HIV (Puoti et al., 2002). The course of HBV infection in HIV-positive individuals has been best studied in cohorts of homosexual men. The majority of studies indicate that after the initial HBV infection, the development and persistence of HBV infection is greater in patients with prior HIV infection (Bodsworth et al., 1991, Gilson et al., 1997).

Before the era of the highly active antiretroviral therapy (HAART), HIV co-infection in patients with HBV was associated with a higher incidence of developing chronic HBV infection (Eskild et al., 1992, Bodsworth et al., 1989, Krogsgaard et al., 1987, Koblin et al., 1992). High levels of HBV replication which is consistent with loss of immune control of viral replication was observed (Perillo et al., 1986, Bodsworth et al., 1989, Hadler et al., 1991, Goldin et al., 1990, Thio et al., 2004). Depletion of CD4 + cells is

observed and this is mainly in those with spontaneous of interferon induced anti-HBe seroconversion. In addition, low ALT levels which indicate mild necro-inflammatory liver lesions and a low rate of seroconversion to anti-HBe and anti-HBs were features of HBV-HIV co-infection seen in patients (Taylor et al., 1988, Perillo et al., 1986, Eskild et al., 1992, Weller et al., 1986).

Table 2.2: Summary of the changes in the natural history of HBV related disease due to HIV co-infection before the HAART era (Puoti et al., 2002).

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**HIV Influence on Natural History of HBV Infection**

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- High rate of chronic HBV infection
  - High HBV replication level with lower ALT levels
  - Lower rates of sero-conversion
  - High rate of sero-reversion as disease advances and CD4+ cell counts drop
- 

There is conflicting data on the degree of necro-inflammatory activity in co-infected patients. Studies of HBV-HIV positive homosexual males in Europe and USA showed mild necro-inflammatory activity in HIV co-infected patients (Bodsworth et al., 1989, Perillo et al., 1986, Mills et al., 1990). This seems to be consistent with the model of immunopathogenic injury of HBV. In contrast, a study performed in French cohorts that included drug users showed increased necro-inflammatory activity in HIV-positive subjects (Bonacini et al., 1991, Housset et al., 1992).

Data on the impact of HIV on hepatitis B progression towards cirrhosis is contradictory. Three French studies have shown an accelerated progression towards cirrhosis in HBV-HIV co- infected individuals (Colin et al., 1999, Housset et al., 1992, Di Martino et al., 2001) and other studies performed in the USA and northern Europe did not reveal any critical impact (Gilson et al., 1997, Krogsgaard et al., 1987, Perillo et al., 1986). These discrepancies could be related to differences in the prevalence of infecting HBV genotypes and mutant HBeAg defective HBV strains, the degree of immune suppression

or the prevalence of different co-factors associated with liver injury e.g. alcohol (Puoti et al., 2002).

HAART increases the life expectancy of HIV-infected individuals through improved CD4 counts and preventing opportunistic infections and tumours (Fauci and Lane, 2001). The prolonged survival of HIV-positive individuals allows a longer time for cirrhosis to develop. As a result the number of liver related deaths has been rising (Rosenthal et al., 2003). HAART may have an impact on HBV co-infection through immune reconstitution and a decrease in the abnormal activation of the immune system (Fauci and Lane, 2001, Benhamou, 2004). Three antiretrovirals (ARVs) that are used in HAART regimes; lamivudine, tenofovir and emtricitabine are inhibitors of HBV replication (Nunez et al., 2003) and they influence the natural history of CHB.

Lamivudine, tenofovir and emtricitabine are able to suppress HBV replication. However depending on the patient's immune status complete suppression of HBV replication may not be attained and there could be persistent residual HBV replication (Wit et al., 2002, Bessesen et al., 1999). Prolonged inhibition of HBV replication leads to improved liver histology and a significant decrease or normalization of ALT levels. In addition it prevents disease progression to cirrhosis and end stage liver disease (Benhamou, 2004, Nunez et al., 2003). Reactivation of HBV infection with flares of ALT levels and hepatic decompensation has been associated with the withdrawal of these drugs (Haverkamp et al., 2003, Fang et al., 2003). Reactivation is also observed when lamivudine inhibition of HBV replication is lost due to the occurrence of lamivudine resistance mutations on the HBV genome (Cooley et al., 2003).

There are contradictory reports on the ability of HAART in restoring the adaptive immune response in HBV infected patients. Some reports suggest an induction of HBV seroconversion and hence better control of HBV replication with or without ALT flares (Carr and Cooper, 1997, Rouanet et al., 2003, Lascar et al., 2003) but in some studies, ALT flares have been reported without modification of the HBV markers and HBV replication levels (Manegold et al., 2001, Drake et al., 2004). To clarify these complex

interactions more work is needed in well selected cohorts who are matched for disease stage and HAART regiments.

Table 2.3: Summary of the changes in the natural history of HBV related disease due to HIV co-infection during the HAART era (Puoti et al., 2006).

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**Influence of HAART on Natural History of chronic HBV Infection**

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- Inhibition of HBV replication due to ARVs with dual activity
  - Reactivation of HBV replication after ARV withdrawal
  - Restoration of adaptive HBV specific and innate non-specific immune responses
  - Reduction of abnormal activation of the immune system
  - Prevention of acute HBV infection in patients using ARVs with dual action
- 

**2.5(c) Therapy for HBV and HIV Co-infection**

Lamivudine has been the drug of choice in HIV and HBV co-infections as HIV-positive individuals do not respond well to IFN- $\alpha$  therapy (Hess et al., 1989, Herrero Martinez, 2001). Lamivudine is a nucleoside analogue that has activity against both HBV and HIV (Herrero Martinez, 2001). It is included in most HAART regimes for HBV and HIV co-infection. The efficacy of lamivudine in HBV-HIV co-infected and HBV mono-infected patients was found to be equivalent (Benhamou et al., 1996, Dore et al., 1999). ARV treatment for HIV is slowly becoming widely available in most African countries as manufacturers are now reducing their prices. Many countries in Africa already have ARV therapy programmes for HIV that include lamivudine. The main drawback with long-term use of lamivudine is that it causes resistance in HBV (Conjeevaram and Lok, 2003). A recent study from South Africa investigated lamivudine resistance in HBV infected patients with or without HIV co-infection. The study determined the presence of lamivudine resistant strains in lamivudine naïve patients as well as HBV-HIV co-infected

patients in (Selabe et al., 2007). This has serious implications in sub-Saharan Africa where the majority of HBV chronic carriers are likely to be HIV-positive.

Updated international treatment recommendations for the management of HBV-HIV co-infected individuals were proposed in 2002 at a consensus conference on chronic viral hepatitis and HIV infection held in Spain (Soriano et al., 2004). All HBV-HIV co-infected patients should be considered candidates for anti-HBV therapy. Treatment with lamivudine and/or tenofovir should be considered in patients who need ARV therapy. So far the best results have been achieved with tenofovir due to its ability to lower HBV DNA levels by as much as mean log 4 within 6 months. In addition the response lasts for months and has so far not been associated with any significant development of resistance strains (Nelson et al., 2003). It is recommended that interferon (IFN) should be considered in HBV-HIV infected patients not on ARV therapy and having CD4 counts >350 cells/ $\mu$ l. Unfortunately the response to IFN in HBV-HIV patients is poor (Di Martino et al., 2002).

Tenofovir and lamivudine in combination should be considered as a first choice in patients who have never been exposed to ARV therapy but a third drug with anti-HIV activity such as abacavir should be included (Soriano et al., 2004).

Entecavir and emtricitabine (FTC) should be used in combination. FTC is similar to lamivudine and so this combination could be used as long as no lamivudine resistance has been selected by either HIV or HBV as these compounds share cross-resistance (Papatheodoridis et al., 2002).

Liver damage is thought to result from an increased number of cytotoxic T cells after immune reconstitution by HAART (Thio et al., 2002) or direct liver toxicity caused by protease inhibitors (Sulkowski et al., 2002). HBV reactivation may occur independent of lamivudine resistance or from lamivudine withdrawal (Manegold et al., 2001). It is therefore important to monitor HBV-HIV co-infected patients when administering HAART (Burnett et al., 2005). The data from sub-Saharan Africa is still incomplete but there are ongoing studies looking at this important problem.

**2.5(d) Occult HBV in Co-infected Patients**

Occult HBV infection, which is the presence of HBV DNA in the absence of HBsAg, is a well defined clinical entity. It has been increasingly detected in HIV-positive individuals throughout the world (Laure et al., 1985, Hofer et al., 1998, Piroth et al., 2002) including sub-Saharan Africa (Mphahlele and Moloto, 2002). The mechanisms that result in occult HBV infection and the burden of these infections in sub-Saharan Africa are largely undefined. In one study from South Africa, HBV DNA was detected in specimens that were negative for HBsAg, anti-HBc, anti-HBs, HBeAg and anti-HBe (Mphahlele and Moloto, 2002). In another study, HBV DNA was detectable in patients with only anti-HBs present in the serum (Owiredu et al., 2001).

Due to very little data available on the impact of HIV on occult HBV, Mphahlele and colleagues investigated the difference in the detection rate of HBsAg between HIV-positive and HIV-negative patients in South Africa. The study showed that there was an increase in the prevalence of occult hepatitis B infection in HIV-positive patients compared to HIV-negative patients (22.1% versus 2.4%). These findings strongly supported that HIV infection is one of the risk factors for a false negative HBsAg test (Mphahlele et al., 2006).

In Africa and other regions severely affected by the HIV pandemic, the increased prevalence of occult HBV infections caused by HIV may have a negative impact on the prevention and control of HBV. The challenge lies not only in effective laboratory diagnosis and management of HBV in HIV co-infected patients but also in the total control of the burden of HBV infections in HIV endemic regions (Burnett et al., 2005).

**2.6 DISTRIBUTION OF HBV GENOTYPES IN AFRICA**

There are few studies that describe the distribution of HBV genotypes in African countries with large parts of Africa still not documented. To date three HBV genotypes have been identified. These are genotypes A, D and E with genotype A predominating (Kew et al., 2005). The available data suggests that the genotype A is predominant in

Central Africa, Southern Africa and East Africa while the HBV genotype E is predominant in West Africa. Genotype D is predominantly found in North Africa.

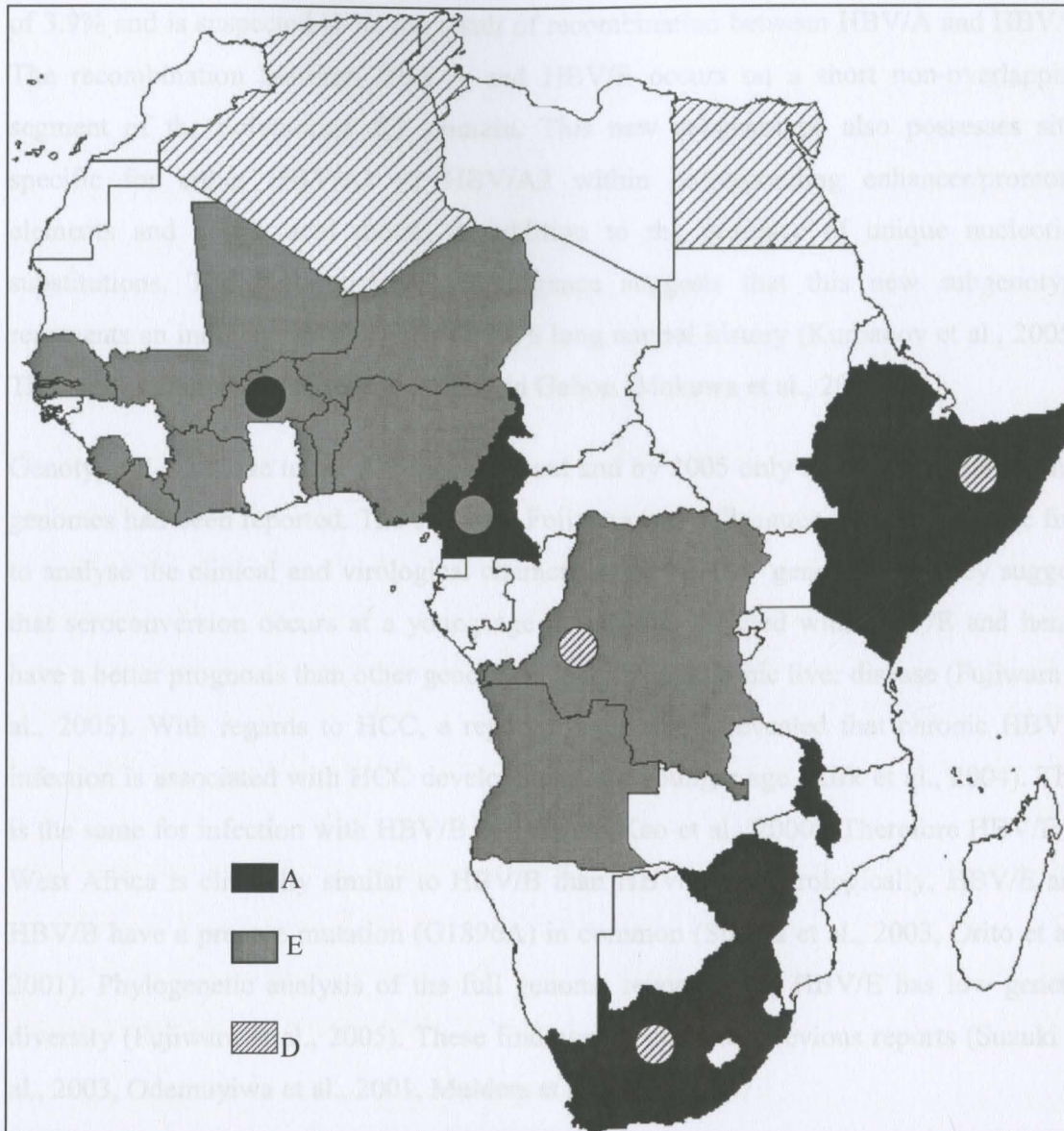


Figure 2.1: HBV genotype distribution across the African Continent (Mulders et al., 2004).

The molecular and virological characteristics of genotype A and D were discussed in detail in Chapter 1 section 1.5. These characteristics are also seen in genotype A and D found in African countries but with some minor variations.

A study from Cameroon by Kurbanov and colleagues showed the presence of a new genotype A; subtype A3. In this population HBV/A and HBV/E are distributed equally amongst the native population. This new subgenotype has a high nucleotide divergence of 3.9% and is suspected to be the result of recombination between HBV/A and HBV/E. The recombination between HBV/A and HBV/E occurs on a short non-overlapping segment of the polymerase RT domain. This new subgenotype also possesses sites specific for either HBV/A1 or HBV/A2 within corresponding enhancer/promoter elements and amino acid motifs in addition to the presence of unique nucleotide substitutions. The high nucleotide divergence suggests that this new subgenotype represents an indigenous HBV strain with a long natural history (Kurbanov et al., 2005). This same strain was also recently found in Gabon (Makuwa et al., 2006).

Genotype E is unique to the African continent and by 2005 only four human HBV/E full genomes had been reported. The study by Fujiwara and colleagues in Benin was the first to analyse the clinical and virological characteristics of HBV genotype E. They suggest that seroconversion occurs at a young age in subjects infected with HBV/E and hence have a better prognosis than other genotypes in terms of chronic liver disease (Fujiwara et al., 2005). With regards to HCC, a report from Gambia revealed that chronic HBV/E infection is associated with HCC development at a younger age (Kirk et al., 2004). This is the same for infection with HBV/B in Taiwan (Kao et al., 2000). Therefore HBV/E in West Africa is clinically similar to HBV/B than HBV/C and virologically, HBV/E and HBV/B have a precore mutation (G1896A) in common (Suzuki et al., 2003, Orito et al., 2001). Phylogenetic analysis of the full genome revealed that HBV/E has low genetic diversity (Fujiwara et al., 2005). These findings concur with previous reports (Suzuki et al., 2003, Odemuyiwa et al., 2001, Mulders et al., 2004).

From the limited data available it is clear that more work needs to be done on the distribution and molecular characterisation of HBV genotypes in Africa. This will have an impact on patient management and also in deciding what treatment strategy is best for the patients given that some genotypes appear to be more pathogenic than others.

## **2.7 HBV PREVENTION, CONTROL AND ERADICATION PROGRAMS IN SUB-SAHARAN AFRICA**

### **2.7(a) HBV Vaccination Studies in sub-Saharan Africa**

Due to the magnitude of the HBV problem in sub-Saharan Africa, a number of vaccination studies have been done in this region. The initial studies done were to determine the efficacy of the vaccines (Cancio-Bello et al., 1982, Prozesky et al., 1983a). An inactivated plasma derived vaccine (HevacB) was given to Senegalese infants less than 1 month old. 95% of these neonates showed a specific antibody response to HBsAg which was similar to that seen in older children (Cancio-Bello et al., 1982). The vaccine was without side effects although the immune responses were impaired by the maternal anti-HBs. Another study was carried out in South Africa where there were three groups of babies: new borns, 3 months and 6 months. 93% of the babies in each age group seroconverted to anti-HBs. The presence of the maternal anti-HBs did not interfere with immunization and this in addition to the seroconversion clearly established the immunogenicity of the vaccine in young babies (Prozesky et al., 1983a). Subsequently a study from Senegal revealed the need for a booster injection every 5- years so as to maintain persistent anti-HBs levels in the body (Yvonnet et al., 1987). In Nigeria, Ayoola and colleagues looked at the efficacy and immunogenicity of Hevac B given intradermally to HBV marker negative subjects aged between 1-45 years. One month after the booster vaccination, a positive response was maintained in all initial responders for the 24 month duration of the study. No significant side effects were reported (Ayoola et al., 1986). The study indicated that there was a possibility of protecting the population at risk in developing countries through mass vaccination.

The best known hepatitis B vaccine study is the Gambia Hepatitis Intervention Study (GHIS) (Hall et al., 1989). This long-term study was designed to evaluate the effectiveness of hepatitis B vaccination for the prevention of chronic liver disease and HCC. The study comprised of three overlapping phases: phase 1, a vaccination programme which took 5 years; phase 2, a longitudinal and cross-sectional study of HBV events in selected groups over 10 years; and phase 3, a long-term follow up to monitor the incidence and prevalence of HBV related chronic liver disease over 40 years. The

Gambia was chosen for this study due to a number of reasons: The Expanded Programme on Immunization (EPI) in the Gambia is one of the most successful programmes carried out in Africa. The country is small, has a high population density and a good road network with access to rural areas. In addition, HBV infection is highly endemic in the country with >90% of the population infected by age 15. The study was started in 1986 and within a year successful integration of hepatitis B into the EPI was achieved. After three years, approximately 96% of the children had hepatitis B immunisation induced protective antibody levels with the non-responders constituting only 4%. The vaccination was effective in reducing the numbers of persistently infected children at one year of age. Total national childhood immunisation coverage was achieved over a period of four years as no major logistical problems were encountered (Hall et al., 1989).

The International Task Force on Hepatitis B Immunisation recommended the establishment of model immunisation programmes within specific regions in a country before expansion nationwide. In these programmes innovative approaches for incorporating hepatitis B vaccination into EPI can be evaluated, vaccinators retained, and new educational and motivational material developed. Initially these models were introduced in Kenya and Cameroon and since then other countries such as South Africa, Botswana, Zimbabwe and Mauritius have taken these programmes as part of their national EPI programmes (Kiire, 1996).

### **2.7(b) Controlling Hepatitis B in Africa**

The eradication of HBV is one of the most important tasks facing public health authorities in Africa. This is due to the high incidence of HBV related liver disease that culminates in the development of HCC. There are important measures that could be used to control and eradicate HBV in an area. Public health measures such as the availability of sanitation, clean water and screening of HBV in blood banks should not be overlooked. Public awareness and education of the disease and availability of clinical services to those who are affected will enable the control of HBV in Africa. Immunisation is the single most important factor in any national hepatitis B control programme, as it has the potential to eradicate hepatitis B and reduce the incidence of chronic liver disease and cancer in Africa (Kiire, 1996).

Kiire argues that in sub-Saharan Africa the concept of high risk groups such as health care workers, homosexuals and dialysis patients is irrelevant as epidemiological studies have shown that the majority of infections are acquired in early childhood and hence all children should be regarded as a high risk group. As a result there have been two basic approaches to vaccination programmes in Africa. These are mass vaccination of all infants without screening of HBsAg and immunisation only of infants born to a HBsAg positive mother. The second approach can only reduce HBV chronic carrier rate by a small percentage as horizontal transmission predominates in Africa. In addition the limited resources available in Africa means that large scale testing of pregnant women is not feasible. Consequently Kiire argued that mass vaccination of all infants would be the best approach to hepatitis B prevention and control in sub-Saharan Africa (Kiire, 1996).

The success of taking this approach was seen in Taiwan as up to 15 to 20% of the general population were chronic carriers of HBsAg (Sung et al., 1984, Chen and Sung, 1977, Chen et al., 1987). Taiwan launched the world's first universal vaccination programme in 1984 (Chen et al., 1987). Initially only infants born to HBsAg positive mothers were vaccinated. However, over the years, the vaccination programme was extended to include all new born infants, children of pre-school age, school children, teenagers and finally adults. A study conducted 15 years after this mass vaccination revealed that there was a tremendous reduction in total HBV carrier and infection rates (Ni et al., 2001). This was reflected by a decreased prevalence of HBsAg and anti-HBc positivity. The prevalence of HBsAg among individuals younger than 15 years decreased from 9.8% in 1984 to 0.7% in 1999 while in the 15 to 20 year old cohort the HBsAg prevalence in 1999 was 7% (Hsu et al., 1986, Ni et al., 2001). Seropositivity for anti-HBc was found in 2.9% of individuals younger than 15 year old and in 20.6% of persons between 15 to 20 years. The rate of anti-HBs seropositivity was 75.8% in persons younger than 15 years and 70.7% in individuals between 15 to 20 years (Ni et al., 2001). Little was known about the effect of this vaccination programme among cohorts older than 18 years of age hence a similar study was conducted from 2000 to 2003. This study was conducted on university students who were born before and after the initiation of the HBV vaccination programme (Chen et al., 2007). The study demonstrated that the HBsAg carrier rate and HBV infection rates amongst these university students decreased from 11.4% to 5.5% and 34.4% to 15.2%

respectively. The HBsAg carrier rate and HBV infection rate decreased from 10.1% to 3.6% amongst university students born after the start of the vaccination programme (Chen et al., 2007).

Following this success, to date more than 110 countries have implemented the mass vaccination programmes for HBV and it is anticipated that these efforts will lead to a greater worldwide control of HBV in the 21<sup>st</sup> century. The main challenges will be to minimize vaccine failures and to deal with the potential emergence of vaccine escape mutants.

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## **CHAPTER 3:**

### **RESEARCH QUESTIONS, AIMS AND OBJECTIVES**

#### **3.1 SUMMARY OF PREVIOUS STUDIES AND RESEARCH QUESTIONS**

A clinical audit done at the Groote Schuur Hospital Liver Clinic in 1999 showed that 33% of the clinic workload was from patients infected with HBV, 11% with HCV, 34% with autoimmune liver disease and 8% with drug induced hepatitis. 68% of these HBV infected patients were male and 43% were HBeAg positive at the time of diagnosis. On average the HBV patients were younger than the HCV infected patients with a mean age of 35 years and 55 years respectively. The HBV patients had more severe disease with 55% having cirrhosis on liver biopsy compared to 46% in the autoimmune liver disease patients. The racial demographics of the patients in the study showed that the different race groups seen in South Africa were well represented. 41% of the patients were Mixed race, 31% Black African, 24% Caucasian and 4% Asian (Hairwadzi et al., 1999). The latest population census carried out in South Africa in 2001 showed that the Mixed race are largely represented at 61.1% in the Western Cape. They are followed by the Caucasians at 19.4%, the Asian at 4% and lastly the Black Africans at 3.4% (<http://www.statssa.gov.za/census01/html/WCPPrimary.pdf>).

*The Groote Schuur Hospital Liver Clinic was the ideal place to conduct this study due to the availability of a large cohort of patients with detailed and regularly updated clinical records as well as being representative of the different age groups and races seen in South Africa.*

As reviewed in Chapter 2, HBV is a major public health problem, particularly in Sub-Saharan Africa where the infection and carrier rates are much higher than those seen in other regions of the world. In Africa, HBV infection is acquired primarily through horizontal transmission of the virus during early childhood compared to Asia where the predominant route of transmission is vertical. In Africa, a difference in HBV prevalence rates is also observed between different ethnic groups. In South Africa, the highest carrier

and exposure rates are found amongst the Black Africans at 14% (Kew, 1992, Dusheiko et al., 1989) followed by the Mixed race (Kew et al., 1989) and Asians (Chinese) (Song et al., 1988) while the Caucasians have the lowest prevalence rates at only 0.2% (Kew et al., 1976). The geographic location of the individual also has an impact on HBV exposure. Rural-urban differences have an influence in the rates of HBV infection. The prevalence rates of chronic carriers in rural South Africa, such as the Eastern Cape, is higher than that in urban Durban and Soweto (Vos et al., 1984) with urban Black African children having a lower HBV carrier rate compared to the rural Black African children.

*This data may suggest that racial differences in HBV prevalence rates may be linked to differences in the genetic profile. This may contribute to one race being able to handle the HBV infection better than the other. In addition, the difference in prevalence rates within the same race but in different locations may suggest that the lack of access to health, good sanitary conditions and hygiene practices are factors that may contribute to these differences. Cultural practices such as scarification and circumcision may also be factors that contribute to a higher HBV prevalence rate in rural areas compared to the urban areas.*

In South Africa, the HBV vaccine was introduced into the EPI programme in 1995. During the first five years of the programme, a study was carried out to assess the impact of the vaccine in reducing HBsAg carriage. The HBV vaccine was shown to be highly effective and had a positive impact on the reduction of HBsAg in children aged less than 5 years (Tsebe et al., 2001).

*In spite of all these successes, HBV is still a major chronic disease in South Africa and the world at large. More research is needed to help address the problems encountered in the uptake and implementation of health programmes in different communities.*

By 1992, Schoub and colleagues estimated that there were 1.45 million HBV carriers in South Africa alone. Of these 122,951 were co-infected with HBV and HIV (Schoub, 1992). The HIV pandemic has increased the number of HBV-HIV co-infection cases and this problem has become endemic in sub-Saharan Africa as these two diseases share the same transmission routes (Burnett et al., 2005). The introduction of lamivudine as part of the HAART regimen has the potential to increase the prevalence of HBV lamivudine

resistant strains (Hussain and Lok, 1999). This has potentially serious implications on the control of viral HBV infection as most chronic carriers in sub-Saharan Africa are most likely to be HIV positive and have lamivudine as part of their HIV treatment.

*The frequency with which lamivudine resistant HBV strains occur could rise significantly as a result of frequent exposure to the treatment. The incidence of YMDD mutations in the HBV population and HBV-HIV co-infected population needs to be researched as these may have important implications on treatment strategies.*

To date most of the literature on HBV in Southern Africa indicates that genotype A and D co-exist with genotype A pre-dominating. Studies from South Africa suggest that genotype A is the predominant genotype compared to D (Kimbi et al., 2004). These local studies on HBV have been conducted primarily in the Gauteng and Kwa-Zulu Natal provinces (Kramvis and Kew, 2007a, Kramvis and Kew, 2007b, Abdool Karim et al., 1988). There is very little prevalence data from the Western Cape and other provinces and there is no data on the pre-dominant HBV genotype seen in these regions.

*These limitations in the scientific literature on HBV in sub-Saharan Africa highlights the need for more work to be done in defining the HBV genotypes found in the region. The possible influence that the genotypes might have on the age of infection, treatment outcome and ethnic differences in HBV infected patients also needed to be investigated.*

### 3.2 AIMS

The aims of the study were:

- To investigate the predominant HBV genotype found at the Groote Schuur Liver Clinic, Cape Town using two different methods
- To screen for the presence of YMDD mutations in patients with chronic hepatitis B infection within the Groote Schuur Liver Clinic, Cape Town

### **3.3 OBJECTIVES**

The objectives of the study were:

- To investigate possible correlations between HBV genotypes and race
- To assess possible correlations between HBV genotype and disease severity
- To evaluate the HBV genotypes in related cases in the cohort
- To evaluate for the possible presence of YMDD mutations in patients at the Liver Clinic that could influence the use of lamivudine treatment and management programmes of HBV and HIV infections.

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## **CHAPTER 4:**

### **MATERIALS AND METHODS**

#### **BACKGROUND TO EXPERIMENTAL PROCEDURES**

##### **4.1 POLYMERASE CHAIN REACTION**

The Polymerase Chain Reaction (PCR) is a rapid procedure used for *in vitro* enzymatic amplification of a specific segment of DNA. The theoretical basis of PCR was probably first described in 1971 by Kleppe and colleagues during their studies on polynucleotides (Kleppe et al., 1971). This technique did not generate much scientific interest until the mid-1980s when Mullis and co-workers developed the technique into one that could be used to generate large amounts of single-copy genes from genomic DNA (Mullis et al., 1986). Since then, PCR has enabled the amplification of genes resulting in a multitude of experiments that were previously impossible.

In the procedure, there are three nucleic acid segments; the segment of double stranded DNA (dsDNA) to be amplified and two single-stranded oligonucleotide primers flanking it, which are complimentary to a specific sequence within the target gene. Additionally there is a protein component (*Taq* DNA polymerase), deoxyribonucleoside triphosphates (dNTP's), a buffer (with or without  $MgCl_2$ ) and salts. The dNTP's used are deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP). The 10x PCR buffer used is made up of 100mM Tris-HCl, 500mM KCl, 15mM  $MgCl_2$  (Roche Applied Science, Germany).

The PCR technique consists of three basic reactions: denaturation, annealing and extension that can be repeated 30–40 times. This is done in an automated thermocycler that can heat and cool the tubes with the reaction mixture in a short time. The denaturation step involves separating the dsDNA into single stranded DNA (ssDNA) at a very high temperature (95°C). This leads to the second step that entails lowering the temperature to approximately 54°C to allow the primers to hybridize via hydrogen bonds

to the complimentary target sequence on both the ssDNA. Low annealing temperatures ( $<45^{\circ}\text{C}$ ) can result in non-specific amplification while high temperatures can inhibit the primers from annealing. A temperature gradient should therefore be performed to determine the appropriate annealing temperature for amplification of the desired product. Once annealed, these primers are finally extended by the *Taq* DNA polymerase with incorporation of dNTP's in the 5'-3' direction. The amplified product can be held at  $4^{\circ}\text{C}$  in the thermocycler for an indefinite period of time until one is ready to use the PCR product. At this temperature the amplified product will not degrade (Coen, 2001). Figure 4.1 below illustrates the PCR process.

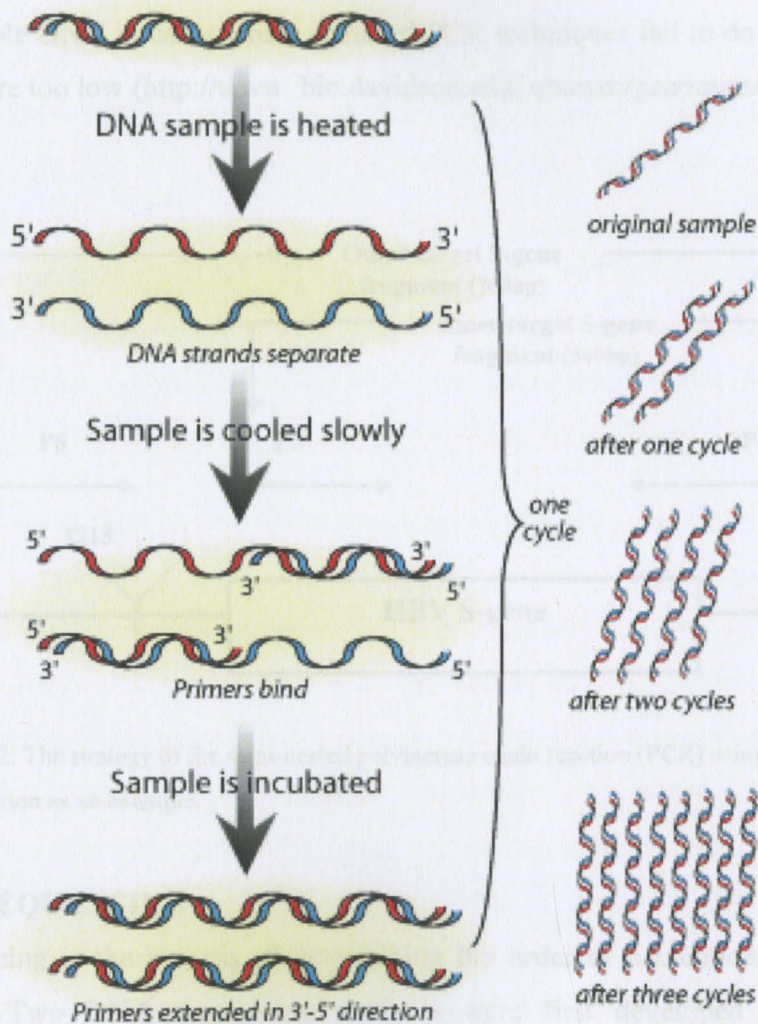


Figure 4.1: A schematic representation of the PCR strategy.

[www.scq.ubc.ca/wp-content/pcr1.GIF.gif](http://www.scq.ubc.ca/wp-content/pcr1.GIF.gif)

Several adaptations of PCR have been developed; these include nested and semi-nested PCR. In a nested PCR, two PCR runs are done in series, each with a pair of primers flanking the same sequence. The first PCR amplifies the sequence as seen in any standard PCR experiment. The PCR product generated in this reaction is used as input DNA in the second nested PCR with primers internal to the first set of primers. A semi-nested PCR is similar to a nested PCR but differs in that in the second PCR, one of the primers is a primer that has been used in the first PCR. The second pair of primers (nested primers) for the second PCR, bind within the first PCR product and produce a second PCR product that is shorter than the first one. The semi-nested technique was used in this study due to its greater specificity and because it uses more than two specific primers and yields detectable DNA in cases where standard PCR techniques fail to do so if the target DNA levels are too low (<http://www.bio.davidson.edu/courses/genomics/method/NestedPCR.html>).

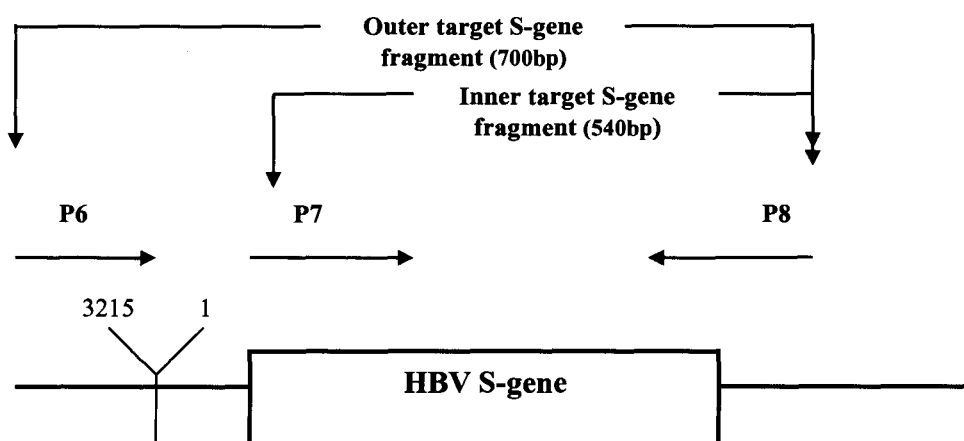


Figure 4.2: The strategy of the semi-nested polymerase chain reaction (PCR) using the HBV S-gene amplification as an example.

## 4.2 DNA SEQUENCING

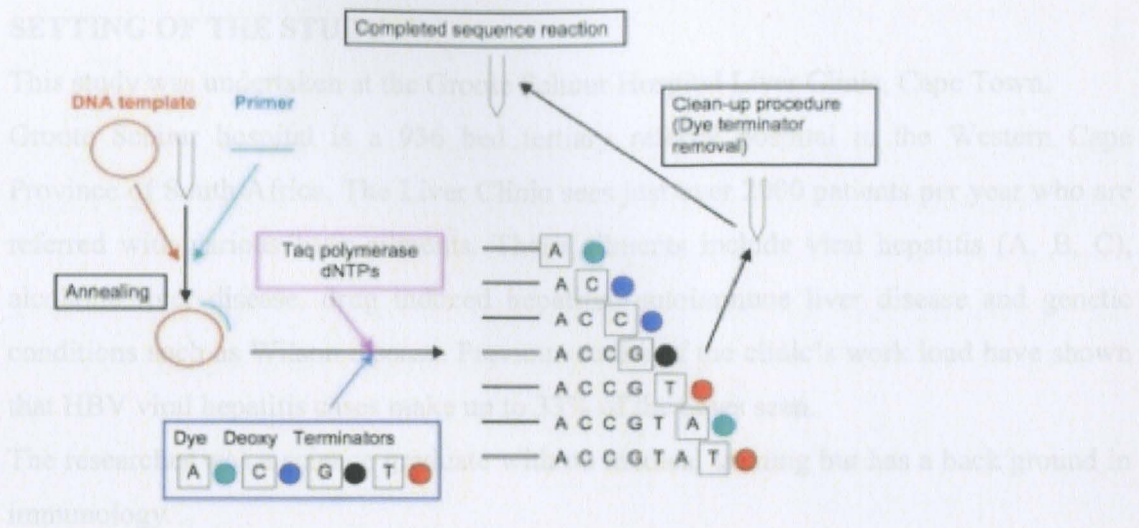
Sequencing is the process of determining the order of nucleotide bases along a DNA strand. Two DNA sequencing methods were first developed in 1977; the chain degradation method by Maxam and Gilbert, and the chain termination method by F. Sanger. The chain degradation method involves the modification and cleavage of specific

nucleotides and separating them by electrophoresis on a high-resolution denaturing acrylamide gel (Maxam and Gilbert, 1977, Maxam and Gilbert, 1980). The chain termination method is most commonly used and based on the principle that ssDNA molecules that differ in length by just a nucleotide can be separated from one another using polyacrylamide gel electrophoresis. The last base on these DNA molecules can be identified through a unique fluorescent label whose signal can be detected and the base identified (Sanger et al., 1977). The cycling sequencing method is a variation of this method that has been developed for automated sequencing machines. This method was used in this study as it is the most frequently used method in automated machines and it is reliable.

In cycle sequencing, a small amount of template DNA molecules are repetitively utilized to generate a sequencing ladder. A dideoxy sequencing reaction mixture consisting of template, primer, dNTPs, ddNTPs and thermostable DNA polymerase is subjected to repeated rounds of denaturation, annealing and elongation, similar to PCR. However the extension is terminated by the incorporation of a ddNTP. Linear amplification of the sequencing products enables the use of less template DNA (Ausubel et al., 2001). There are two different cycle sequencing strategies; the difference is in the way the fluorescent label is incorporated into the sequencing products, either through the primer or ddNTPs.

The automated sequencing machine automates the gel electrophoresis step, detection of DNA and analysis of bands. After the cycle sequencing reaction has been completed, the products are loaded onto an autosampler tray and electrophoresed on a urea-acrylamide gel. Detection occurs at a point near the bottom edge of the gel. The fluorescent dye labels on the DNA fragments are excited by a laser and the resultant energy emission is detected and recorded. All automated sequencers possess data collection and analysis software which interprets the results by detecting the bases from the fluorescence intensity at each data point. The four bases (A, C, G, T) are identified by four distinct emission colours; green, blue, black and yellow respectively. The sequence is then represented as an electropherogram (Ausubel et al., 2001).

## Automated Dye Terminator Sequencing



A The study was approved by the University of Cape Town Health Sciences Faculty Ethics committee; ethics approval number 417/2005 (see Appendix A).

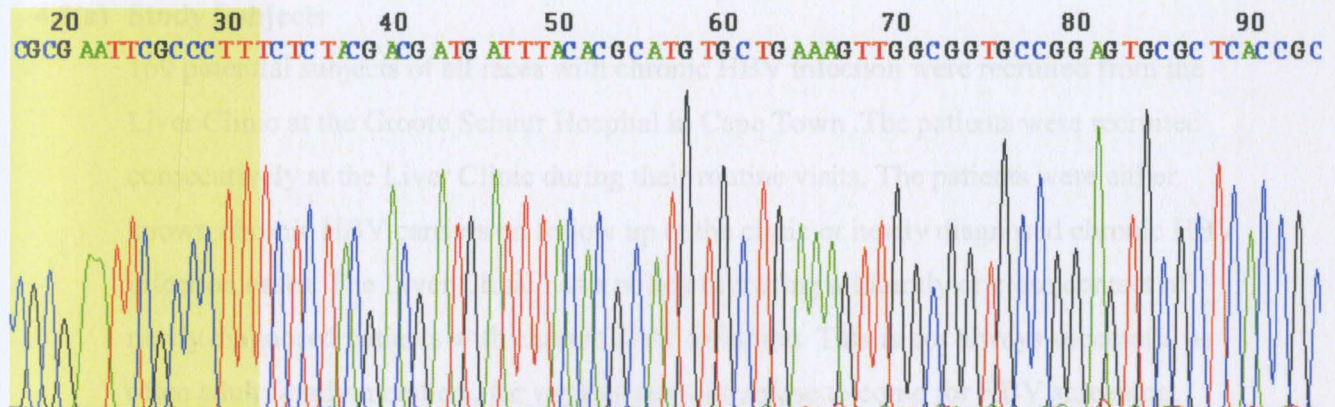


Figure 4.3: Illustration of cycle sequencing reaction using dye-labelled terminators (ddNTP) (A); an example of an electropherogram (B).

A: [http://www.udel.edu/dnasequence/UDSGC/35E4CCB4-35F3-4469-AB80-94D82B76759A\\_files/droppedImage.jpg](http://www.udel.edu/dnasequence/UDSGC/35E4CCB4-35F3-4469-AB80-94D82B76759A_files/droppedImage.jpg)

B: [http://dnasequencing.files.wordpress.com/2007/10/sanger\\_sequencing\\_read\\_display.gif](http://dnasequencing.files.wordpress.com/2007/10/sanger_sequencing_read_display.gif)

## EXPERIMENTAL PROCEDURES

### 4.3 SETTING OF THE STUDY

This study was undertaken at the Groote Schuur Hospital Liver Clinic, Cape Town.

Groote Schuur hospital is a 936 bed tertiary referral hospital in the Western Cape Province of South Africa. The Liver Clinic sees just over 2000 patients per year who are referred with various liver ailments. These ailments include viral hepatitis (A, B, C), alcoholic liver disease, drug induced hepatitis, autoimmune liver disease and genetic conditions such as Wilson disease. Previous audits of the clinic's work load have shown that HBV viral hepatitis cases make up to 33% of the cases seen.

The researcher was a science graduate with no medical training but has a back ground in immunology.

The study was approved by the University of Cape Town Health Sciences Faculty Ethics committee; ethics approval number 417/2005 (see Appendix A).

#### 4.3(a) Study Subjects

169 potential subjects of all races with chronic HBV infection were recruited from the Liver Clinic at the Groote Schuur Hospital in Cape Town .The patients were recruited consecutively at the Liver Clinic during their routine visits. The patients were either known chronic HBV carriers on follow up at the clinic or newly diagnosed chronic HBV infection cases. The Liver Clinic has a policy of tracing all family or close contact of newly diagnosed patients with chronic HBV infection. This is not always successful as often adult family members, for various reasons, refuse to come for HBV screening. However, as a result of this policy, the cohort recruited included close contacts who are members of the same family. This group has been called the "family contacts" for the purposes of future sub-group analysis. These family contacts will be used in future work that would investigate possible genetic influences in the immune response to HBV infection.

All the patients were known to be HBsAg positive for at least 6 months and so by definition had chronic HBV infection with either a positive or negative HBeAg. The

patients in this study were recruited over a period of 20 months ending June 2007. There is ongoing recruitment of this cohort as part of a long-term study with in the clinic. See Appendix B for the patient consent form and Appendix C for the patient information sheet that was used in this study.

Only those patients who were HBeAg positive and had abnormal liver tests with a high alanine aminotransferase (ALT) level above the reference range of 40 IU/L received IFN- $\alpha$  treatment. The IFN- $\alpha$  was given at a dose of 3million IU subcutaneously three times a week for a minimum of twelve weeks. There was dose monitoring for possible side effects such a bone marrow suppression and depression.

#### **4.4 DATA CAPTURE AND STATISTICAL ANALYSIS**

The data collected at recruitment was recorded on a data capture form before transfer onto an access data base. The data was analysed by a consultant statistician using SPSS Version 9 (SPSS Inc, Chicago Illinois).

The assessment of association between independent categorical variables such as race, gender was performed with the Pearson Chi-squared test ( $\chi^2$ ). A p value  $<0.05$  was considered significant and  $<0.0001$  was considered highly significant. Fishers exact test was used to assess for association between non-parametric categorical variables such as race, HBeAg and HBsAg loss. Multiple correlations between more than two groups of independent observations such as log HBV DNA, age and ALT levels was performed with the one way ANOVA.

#### **4.5 HAEMATOLOGY, BIOCHEMISTRY, VIRAL SEROLOGY AND HBV DNA**

Patients attending the Liver Clinic have liver tests, viral serology and HBV DNA quantification done as part of their standard clinical assessment. The results of the initial assessment were recorded and for those who received interferon therapy, the results prior to interferon therapy have been used in their analysis for uniformity as treatment was given based on liver test abnormalities.

In the clinic setting, liver synthetic function was assessed by the reduced ability of the liver to produce clotting factors and proteins as measured by the international normalized ratio (INR) and albumin (ALB) respectively. The degree of hepatocellular necrosis or inflammation was assessed using the alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

For the purposes of this study and taking into account of the background of the researcher, the degree of disease severity was assessed using the INR to observe synthetic functions and ALT to observe inflammatory activity in the liver. As few patients in this cohort had liver biopsies done, the liver histology was not included as part of the assessment of disease activity. The data on bilirubin, alkaline phosphatase,  $\gamma$  glutamyl-transpeptidase, a fetal protein and haematology were not included in statistical analysis results as they were not contributory.

Quantitative detection of HBV DNA and qualitative HBeAg and HBsAg tests were performed at the National Health Laboratory Service (NHLS) Virology Laboratory, Groote Schuur Hospital. Due to cost limitations, not all patients had their HBV DNA levels assayed during the early phase of recruitment. The HBV DNA quantification was done using the Artus HBV LC PCR Kit (Qiagen, Hamburg Germany) according to the manufacturer's recommendations. The HBV DNA results were reported in IU/ml with the  $\text{Log}_{10}$  conversion of the result being used in the statistical analysis of the data. HBeAg was determined by Elecsys HBeAg Kit (Roche Diagnostics, Mannheim Germany) and HBsAg by AxSYM HBsAg (V2) (Abbott Diagnostics, Germany).

The clinical description such as liver biochemistry, sex and age of the patients that participated in this study is described in Appendix D.

### **4.6 SERUM COLLECTION, DNA ISOLATION AND STORAGE**

Blood samples were collected and indexed from patients at the time of their visit to the Liver Clinic. The patient index was used in the study so that the researcher was blinded to the clinical and laboratory data of each case. To determine that consistent results were obtained, a group of samples comprised duplicate specimens from the same patient. The

#### CHAPTER 4: MATERIALS AND METHODS

negative control group were those patients without HBV. The blood was centrifuged at 3000rpm for 5 minutes and serum collected and stored at -80°C until use.

Genomic DNA was isolated from 200µl of serum using the Roche High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) as per the manufacturer's protocol (see Appendix E). The working solutions provided in the kit were prepared beforehand. The lyophilized proteinase K was dissolved into 4.5ml double distilled water and 200µl aliquots of the solution were stored at -15 to -25°C until needed. 20ml of absolute ethanol (Saarchem, Gauteng, South Africa) was added to the inhibitor removal buffer while 80ml of absolute ethanol was added to the wash buffer. Both were stored at +15 to +25°C until needed.

200µl of the sample serum, 200µl binding buffer and 40µl of reconstituted proteinase K was added to a nuclease free 1.5ml microcentrifuge tube (Axygen Scientific, Union City, USA) and mixed immediately using a Mistral mixer vortex (Labline-Instruments, Melrose Park, USA). The proteinase K was added to lyse the cells in the sample serum and inactivate endogenous DNase. This mixture was incubated at 70°C for 10 minutes in a water bath (Memmert, Lasec, South Africa). Thereafter 100µl of isopropanol (Sigma-Aldrich GmbH, Steinheim, Germany) was added to the mixture using a 100µl pipette (Gilson Medical Electronics, Villiers-le-Bel, France) to precipitate the DNA. The sample mixture was mixed thoroughly by pipetting the mixture up and down at least 6 times.

A high filter tube was inserted into a collection tube to create a high pure assembly; these tubes were both provided in the kit. The sample mixture was pipetted into the upper buffer reservoir of the filter tube using a 1000µl Gilson Pipetman (Gilson Medical Electronics, Villiers-le-Bel, France). The entire filter tube assembly was centrifuged in a microcentrifuge (Labnet International, Inc, USA) at 8000rpm for 1 minute. After centrifugation, the filter tube was removed from the collection tube and the flow through and collection tube were both discarded. The filter tube was then combined with a new collection tube and 500µl of inhibitor removal buffer was added to the upper reservoir of the filter tube.

The inhibitor removal buffer contained absolute ethanol which is used to remove salts and small inorganic molecules that may act as inhibitors. This mixture was centrifuged at 8000rpm for 1 minute. The flow through liquid and collection tube were discarded after centrifugation and a new collection tube was again combined with the filter tube. 500µl of wash buffer was added to the filter tube and centrifuged at 8000rpm for 1 minute and the flow through discarded thereafter. This wash process was repeated twice and in the second instance the high pure assembly was centrifuged for a further 10 seconds to ensure removal of residual wash buffer and contaminating salts. The filter tube was then inserted into a clean, sterile 1.5ml microcentrifuge tube and 100µl of pre-warmed elution buffer was added. This assembly was centrifuged at 8000rpm for 1 minute. The isolated DNA was eluted in 100µl instead of 200µl as per the manufacturer's protocol in order to increase the DNA concentration.

The eluted DNA was quantified using the Gene Quant RNA/DNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the DNA was determined by the optical density ratio (OD)  $_{260/280}$  obtained. Samples with an OD ratio less than 1.5 were discarded and the DNA extraction process was repeated. The eluted DNA was stored at 4°C until needed.

#### 4.7 AMPLIFICATION OF THE B-GLOBIN GENE

Globins are protein products of a large and ancient multigene family that includes the alpha (α) and beta (β) globins of haemoglobin. They are largely expressed in the bone marrow and their most important function is to bind oxygen ([http:// whozoo.org/mac/Music/ bglobin.htm](http://whozoo.org/mac/Music/bglobin.htm)).

In this study, amplification of the β-globin gene was used to determine the quality of each DNA sample for PCR. The β-globin gene was amplified using the forward (PC04) and reverse (gH20) primers adopted from a study by Bell, D.A (Bell et al., 1993) (Refer to Table 4.3). High quality DNA samples produced a bright 268bp amplicon. Good quality DNA samples were selected for use in the HBV genotyping and YMDD mutation analysis studies.

To prevent cross contamination, the PCR master mix for the amplification of the  $\beta$ -globin gene was made up whilst working with gloves and using sterile filter pipette tips (Whitehead scientific, Cape Town, South Africa) that were changed between reagents.

The master PCR mix was made up on ice to prevent degradation of the reagents.

In a sterile 1.5ml Eppendorf tube (Axygen Scientific, Union City, USA), the PCR master mix was made up using the recipe outlined in the table below:

Table 4.1:  $\beta$ -globin PCR master reagent recipe

| Reagents              | Stock solution    | Final conc.       | Volume( $\mu$ l) |
|-----------------------|-------------------|-------------------|------------------|
| PCR buffer plus       | 10x buffer; 15mM  | 1x buffer, 1.5mM  | 5                |
| MgCl <sub>2</sub>     | MgCl <sub>2</sub> | MgCl <sub>2</sub> |                  |
| dNTP                  | 10mM each         | 0.2mM             | 1                |
| pCO4                  | 25mM              | 0.5mM             | 1                |
| gH <sub>2</sub> O     | 25mM              | 0.5mM             | 1                |
| <i>Taq</i> polymerase | 1U/ $\mu$ l       | 1U                | 1                |
| dH <sub>2</sub> O     | -                 | -                 | 31               |

The master mix was vortexed briefly and 40 $\mu$ l aliquots were added to 0.5ml sterile Eppendorf tubes (Axygen Scientific, Union City, USA). 10 $\mu$ l of genomic DNA was added to these aliquots but to one reaction mixture in an Eppendorf tube, 10 $\mu$ l of distilled water was added instead of genomic DNA. This sample acted as the negative control to check for possible contamination. The samples were then vortexed briefly and put into a Robocycler Gradient 40 thermocycler (Stratagene, Los Angeles, USA) with the hot lid switched on to prevent evaporation of the sample. The following PCR parameters were set for the amplification of the  $\beta$ -globin gene:

Table 4.2:  $\beta$ -globin PCR reaction parameters

| Steps | Reaction     | Temperature (°C) | Time       | No. of cycles |
|-------|--------------|------------------|------------|---------------|
| 1     | Denaturation | 95               | 2 minutes  | 1 cycle       |
| 2     | Denaturation | 95               | 20 seconds | 40 cycles     |
|       | Annealing    | 57               | 30 seconds |               |
|       | Elongation   | 72               | 45 seconds |               |
| 3     | Elongation   | 72               | 7 minutes  | 1 cycle       |
| 4     |              | 6                | 2 minutes  | 1 cycle       |

After amplification the PCR products were subjected to 1.7% agarose gel electrophoresis to separate the amplicons. These were visualised with Ethidium Bromide (EtBr) staining of the DNA under UV illumination. The good quality DNA samples were stored at 4°C until further use.

#### 4.8 AMPLIFICATION OF THE S-GENE OF THE HBV GENOME

The S-gene region is a variable region and is often used as a reliable measure of HBV genetic variation enables identification of different HBV genotypes. The eight known HBV genotypes (A-H) are based on nucleotide differences of 4% or greater within the S-gene (Okamoto et al., 1988).

##### 4.8(a) Primer Design For the S-Gene Region

Two PCR primer sequences specific for the S-gene region (P7 and P8) were acquired from a publication by (Kew et al., 2005) while an additional third primer (P6) was designed for this study. The third primer sequence was designed based on the sequences of the S- gene of the HBV genome (GenBank accession number AY233274). The primer sequence information is detailed in Table 4.3.

# CHAPTER 4: MATERIALS AND METHODS

Table 4.3: Primers used for  $\beta$ -globin, S-gene and polymerase gene amplification

| PCR for                       | Primer   | Sequence                         | T <sub>m</sub><br>(°C) | %GC<br>content | product size(bp)                                                           |
|-------------------------------|----------|----------------------------------|------------------------|----------------|----------------------------------------------------------------------------|
| B-globin<br>gene <sup>a</sup> | PC04 (+) | 5'-CAACTTCATCCACGTTCAACC-3'      | 53.9                   | 50             | 268bp                                                                      |
|                               | GH20 (-) | 5'-GAAGAGCCAAGGACAGGTAC-3'       | 55                     | 55             |                                                                            |
| S-gene                        | P6(+)    | 5'-CCTGCTGGTGGCTCCAGTT-3'        | 60.3                   | 63.1           | Semi-nested PCR:<br>Reaction 1 (P6+P8) 700bp<br>Reaction 2 (P7+P8) 540bp   |
|                               | P7(+)*   | 5'-TGGTGGACTTCTCTCAATTTTC-3'     | 53.8                   | 43.4           |                                                                            |
|                               | P8(-)*   | 5'-CGGTAWAAAGGGACTCAMGAT-3'      | 53.1                   | 45.2           |                                                                            |
| Polymerase<br>gene<br>YMDD    | S1(+)    | 5'-GCAC(C/T)TGTATTCCCATCCCATC-3' | 57.7                   | 54.5           | Semi-nested PCR:<br>Reaction 1 (S1+AS1) 268bp<br>Reaction 2 (S2+AS1) 218bp |
|                               | S2(+)    | 5'-CATCATC(C/T)TGGGCTT TCG-3'    | 53.4                   | 55.6           |                                                                            |
|                               | AS1(-)   | 5'-GTATACCCAAAGACAAAAGA-3'       | 47.2                   | 35             |                                                                            |

(+) forward primer, (-) reverse primer, \* Kew et al., 2005, <sup>a</sup> Bell et al., 1993, bp-base pairs

## CHAPTER 4: MATERIALS AND METHODS

The location of the Surface (S) and polymerase gene primers sequences on the HBV genome are illustrated in Figure 4.4.

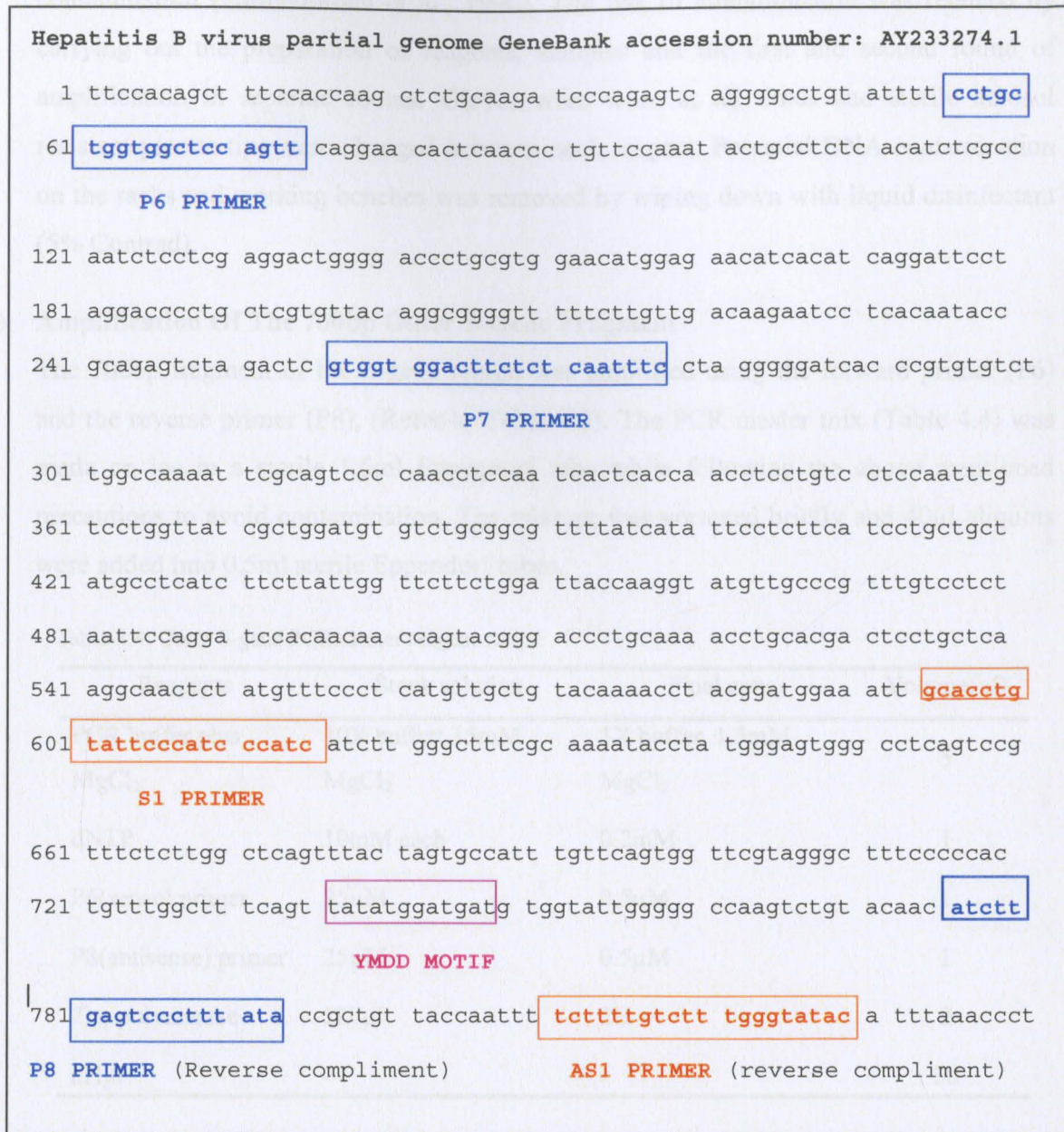


Figure 4.4: Location of the forward and reverse S-gene primers, Polymerase gene primers and the YMDD motif within the polymerase gene. The S-gene primers are indicated in blue; Polymerase gene primers are indicated in orange.

**4.8(b) Semi-Nested Polymerase Chain Reaction of The S-Gene**

Nested and semi-nested PCRs are known to be prone to error due to random contamination (Porter-Jordan et al., 1990). The risk of contamination was reduced by carrying out the preparation of reagents, samples and the first and second round of amplification in separate rooms. Gloves were worn at all times and sterile aerosol resistant pipette tips were changed between each reagent. Potential DNA contamination on the racks and working benches was removed by wiping down with liquid disinfectant (5% Contrad).

**4.8b(i) Amplification Of The 700bp Outer S-Gene Fragment**

The 700bp fragment of the S-gene region was amplified using the forward primer (P6) and the reverse primer (P8), (Refer to Table 4.3). The PCR master mix (Table 4.4) was made on ice in a sterile 1.5ml Eppendorf tube while following the above mentioned precautions to avoid contamination. The mixture was vortexed briefly and 40µl aliquots were added into 0.5ml sterile Eppendorf tubes.

Table 4.4: Outer S-gene PCR master reagent recipe

| Reagents              | Stock solution    | Final conc.       | Volume(µl) |
|-----------------------|-------------------|-------------------|------------|
| PCR buffer plus       | 10X buffer; 15mM  | 1X buffer, 1.5mM  | 5          |
| MgCl <sub>2</sub>     | MgCl <sub>2</sub> | MgCl <sub>2</sub> |            |
| dNTP                  | 10mM each         | 0.2mM             | 1          |
| P6(sense) primer      | 25µM              | 0.5µM             | 1          |
| P8(antisense) primer  | 25µM              | 0.5µM             | 1          |
| <i>Taq</i> polymerase | 1U/µl             | 2U                | 2          |
| dH <sub>2</sub> O     | -                 | -                 | 30         |

10µl of genomic DNA was added into each 40µl aliquot of PCR mix while one aliquot had 10µl of distilled water added instead of genomic DNA. This aliquot served as a negative control to check for contamination. These PCR samples were vortexed briefly and amplified on a Robocycler Gradient 40 thermal cycler (Stratagene, Los Angeles, USA) with the hot lid switched on to prevent evaporation of the sample.

The following PCR reaction parameters are performed on the samples:

Table 4.5: Outer S-gene PCR reaction parameters

| Steps | Reaction     | Temperature (°C) | Time       | No. of cycles |
|-------|--------------|------------------|------------|---------------|
| 1     | Denaturation | 95               | 2 minutes  | 1 cycle       |
| 2     | Denaturation | 95               | 20 seconds | 40 cycles     |
|       | Annealing    | 57               | 30 seconds |               |
|       | Elongation   | 72               | 45 seconds |               |
| 3     | Elongation   | 72               | 7 minutes  | 1 cycle       |
| 4     |              | 6                | 2 minutes  | 1 cycle       |

Presence of the outer S-gene fragment was confirmed by agarose gel electrophoresis as described in section 4.9. In this study the outer S-gene fragment was not used as the template for the inner S-gene fragment. This was due to the high chance of contamination and the amplification of unwanted fragments. The outer S-gene negative PCR samples were used as the templates because of the low levels of outer S-gene fragments which would limit contamination and also lead to the amplification of the desired 540bp inner S-gene fragment.

#### 4.8b(ii) Amplification Of The 540bp Inner S-Gene Fragment

The same PCR equipment, reagents and methods mentioned in section 4.8(b) were used but the PCR master reagent recipe, aliquot and reaction parameters were changed. The changes were as follows: an increase to 35µl from 30µl of distilled water, the forward primer was changed to P7 (Refer to Table 4.3), the PCR mix aliquots were increased from 40µl to 45µl and 5µl of the outer PCR was used as template.

Table 4.6: Inner S-gene PCR master reagent recipe

| Reagents              | Stock solution    | Final conc.       | Volume( $\mu$ l) |
|-----------------------|-------------------|-------------------|------------------|
| PCR buffer plus       | 10X buffer; 15mM  | 1X buffer, 1.5mM  | 5                |
| MgCl <sub>2</sub>     | MgCl <sub>2</sub> | MgCl <sub>2</sub> |                  |
| dNTP                  | 10mM each         | 0.2mM             | 1                |
| P7(sense) primer      | 25 $\mu$ M        | 0.5 $\mu$ M       | 1                |
| P8(antisense) primer  | 25 $\mu$ M        | 0.5 $\mu$ M       | 1                |
| <i>Taq</i> polymerase | 1U/ $\mu$ l       | 2U                | 2                |
| dH <sub>2</sub> O     | -                 | -                 | 30               |

The PCR samples were then subjected to the following PCR reaction parameters:

Table 4.7: Inner S-gene PCR reaction parameters

| Steps | Reaction     | Temperature (°C) | Time       | No. of cycles |
|-------|--------------|------------------|------------|---------------|
| 1     | Denaturation | 95               | 2 minutes  | 1 cycle       |
| 2     | Denaturation | 95               | 15 seconds | 40 cycles     |
|       | Annealing    | 53               | 30 seconds |               |
|       | Elongation   | 72               | 40 seconds |               |
| 3     | Elongation   | 72               | 7 minutes  | 1 cycle       |
| 4     |              | 6                | 2 minutes  | 1 cycle       |

The presence of the inner S-gene fragment PCR product was confirmed by agarose gel electrophoresis as described in section 4.9.

## 4.9 AGAROSE GEL ELECTROPHORESIS

This is a simple yet highly effective method for separating, identifying and purifying DNA fragments. The protocol is divided into three stages. Initially the gel is prepared with an agarose at a concentration appropriate for the size of DNA to be separated and the DNA samples are then loaded into the sample wells. The gel is then run at a voltage for a time period that would achieve maximum separation of the DNA fragments. The gel

is then stained for visualization or if ethidium bromide has been incorporated into the gel and electrophoresis buffer, it is visualized directly upon illumination with UV light (Moore, 2000).

Multipurpose agarose is a high strength agarose. The high gel strength permits the use of low agarose concentration in electrophoretic gels and reduces separation times for large DNA molecules while maintaining high resolution. The agarose matrix of the gel made with low concentrations of multipurpose agarose allows for rapid migration of high molecular weight molecules without any significant restriction (Moore, 2000).

Gel electrophoresis was carried out in a 1.7% multi purpose agarose gel (Roche Applied Science, Germany, Appendix E) dissolved in a 1x TBE buffer (Appendix E). 20µl of Ethidium Bromide (EtBr) (100µg/ml) was added to 100ml of 1.7% agarose solution. The EtBr is used to visualise the DNA under UV light. The dye intercalates between stacked bases of nucleic acid and fluoresces red-orange when illuminated with UV light (260-360nm) (Moore, 2000). The solution was poured into a casting tray and a gel comb was inserted making sure that there were no bubbles trapped underneath the combs and all bubbles on the surface of the agarose were removed before the gel set. After the gel had hardened, the gel comb was withdrawn carefully so as not to tear the sample wells and the set gel was placed in the electrophoresis tank. Sufficient 1x TBE electrophoresis buffer was added to cover the gel to a depth of about 1mm.

10µl of DNA sample was mixed with 1µl of loading buffer and loaded into the sample well with a pipette. 5µl of DNA molecular weight marker (Roche Applied Sciences, Germany, Appendix G) was included but loaded into a separate well. The leads were attached so that the DNA would migrate toward the anode lead when the voltage was set at 100V. The power supply was turned off when the bromophenol blue dye from the 6x loading buffer (Appendix E) had migrated a distance judged to be sufficient for the separation of DNA fragments. The DNA fragments were then visualized and photographed directly using a UV transilluminator (UVtec, Cambridge, U.K.).

**4.10 DIRECT SEQUENCING OF THE PURIFIED S-GENE PCR PRODUCT**

The PCR samples that contained the desired S-gene fragment were identified and prepared for direct sequencing. These fragments were purified from the PCR reaction solution using the GFX-PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, U.K, Appendix F) according to the manufacturer's protocol. For each purification, a GFX column was placed into a collection tube and 500µl of capture buffer added. Approximately 40µl of DNA solution was transferred into the GFX column and the mixture was mixed thoroughly by pipetting the sample up and down 4-6 times.

It was centrifuged in a microcentrifuge at 14,000rpm for 30 seconds. The flow through was discarded by emptying the collection tube which was placed back into the GFX column. 500µl of wash buffer was then added to the column and centrifuged at 14,000rpm for 30 seconds. The collection tube was discarded and the GFX column transferred to a fresh 1.5ml microcentrifuge tube. 50µl of 10mM Tris-HCl pH 8.0 (Appendix E) was applied directly to the top of the glass fiber matrix in the GFX column and incubated at room temperature for 1 minute. The column was then centrifuged at 14000rpm to recover the purified DNA. Each purified sample was quantified using the GeneQuant RNA/DNA calculator.

The University of Stellenbosch Genetics Department was used for sequencing and as per their specifications, the purified DNA samples had to be diluted to 6ng/µl while the forward and reverse primers were diluted to 1.1pmole/µl. The purified DNA was sequenced in both sense and anti-sense directions using an ABI 3130XL DNA automatic sequencer using the Big Dye Terminator cycle sequencing kit. The S-gene nucleotide sequence data was validated after visual inspection and analysed by using Bioedit Sequence Alignment Editor version 7.0 (Tom Hall, Ibis Therapeutics, a division of Isis Pharmaceuticals, USA) and Clustal X 1.83 ([www-igmmbc.u-strasbg.fr/BioInfo/clustalx/](http://www-igmmbc.u-strasbg.fr/BioInfo/clustalx/)).

#### **4.11 HBV GENOTYPING**

The patient HBV genotype was determined using the NCBI web-based genotyping tool and the phylogenetic analysis method. This phylogenetic analysis was used to confirm the genotype obtained from the NCBI genotyping tool and to further determine the sub-genotype of the sample.

##### **4.11(a) The National Centre For Biotechnology Information (NCBI) Web-Based Genotyping Tool**

To identify the HBV genotype of each patient, the NCBI genotyping tool was one of the methods used. The web-based program is available on the NCBI website; <http://www.ncbi.nih.gov/projects/genotypeing/formpage.cgi> and identifies the genotype or subtype of recombinant and non-recombinant viral nucleotide sequences. It uses BLAST analysis to compare a query sequence to a set of reference sequences of known genotypes. The predefined HBV reference sequences consist of complete genomes of genotypes A-H. This reference set was used to genotype each sample. Each patient's genotype was queried by entering the S-gene nucleotide sequence into the form page. The nucleotide sequence was submitted and each sequence segment compared to the reference set using BLAST analysis. The reference sequence genotype matching the query with the highest similarity was assigned to the query sequence and the result displayed graphically.

**NCBI** **Genotyping**

PubMed Nucleotide Protein Genome Taxonomy Structure PopSet

**Resources:**  
Retroviruses

**Reference sets:**  
Retroviruses  
Hepatitis B  
Hepatitis C  
Poliovirus

**Help**

**Tools:**  
Subtype Alignment

**External links:**  
Division of AIDS at NIAID  
LANL HIV

This tool helps identify the genotype of a viral sequence. A window is slid along the query sequence and each window is compared by BLAST to each of the reference sequences for a particular virus. This approach is especially useful for the analysis of recombinant sequences. (Try an [example](#)).

**Select:** Human immunodeficiency virus 1, (HIV-1), Retroviridae

**Add/remove reference sequences as Accession/subtype:**

- AF004885 | A
- AF069670 | A
- M62320 | A
- U51190 | A
- M26727 | B
- U63632 | B
- M17451 | B

**Enter local reference sequences as Accession/GI or FASTA:**

**Enter your query sequence as Accession/GI or FASTA**

AY308760

**Subtype** **Reset** **Clear**

**Enter window size:** 300 **Select background color:** White

**Enter increment step:** 100 **Similarity threshold (%):** 30

**Table output only:** ☐

Figure 4.5: Web-based NCBI genotyping tool form page. The query sequence is pasted into the query box and one has the option of selecting the type of reference sequence set. In this example the HIV sequence set has been chosen (Rozanov et al., 2004).

#### 4.11(b) Subgenotyping Using Phylogenetic Analysis

Phylogenetic analysis was also used to identify and sub-genotype the HBV sequence. The HBV S-gene nucleotide sequences were aligned using clustal X and genotypes by comparing the unknown sequence with 41 reference sequences of the HBV genome from GenBank database. These reference sequences represented HBV genomic isolates from the HBV genotypes A-H. Phylogenetic trees were drawn using Treecon for windows,

Version 1.3b (Yves Van de Peer, Department of Biology, University of Konstanz, D-78457, Konstanz, Germany) to confirm and sub-genotype the patient's HBV genotype. The Kimura-2-parameter was used to estimate the tree distance and the Neighbour-Joining method was used to draw the tree using the Treecon programme. Bootstrap values were calculated to indicate uniform support. High boot strap values indicate uniform support.

A comparison was made between the two genotyping methods to determine whether the genotypes obtained from the NCBI web-based genotyping tool concurred with those obtained from the phylogenetic analysis method.

#### **4.12 DETECTION OF YMDD MUTANTS (YIDD AND YVDD) IN THE PATIENT COHORT**

The YMDD motif of the HBV polymerase gene is highly conserved (Bartholomew et al., 1997). Long-term treatment of chronic HBV infection with lamivudine may induce the emergence of mutations in the YMDD motif which results in lamivudine resistant HBV strains: YIDD, YVDD, YSDD amongst others (Hussain and Lok, 1999). In this study, 29 patients who were HIV negative were screened for the presence of YMDD mutations.

##### **4.12(a) Amplification Of The Polymerase Gene Region Using Semi-Nested PCR**

The semi-nested method used was the same as that described in section 4.8. The primers used were designed by Dr. Smuts and Dr. Di Hardie at the NHLS Virology Laboratory, Groote Schuur Hospital (Refer to Table 4.3).

Semi-nested PCR reactions were performed for the polymerase gene region following the same procedure and protocol as described in section 4.8(b) but the PCR master mix recipe and PCR parameters were different in relation to the primers and annealing temperatures used.

**2a(i) Amplification Of The 268bp Outer Polymerase Gene Fragment**

The following PCR master mix recipe was used for the amplification of the outer polymerase gene fragment:

Table 4.8: Outer polymerase gene PCR master reagent recipe

| Reagents              | Stock solution    | Final conc.       | Volume( $\mu$ l) |
|-----------------------|-------------------|-------------------|------------------|
| PCR buffer plus       | 10X buffer; 15mM  | 1X buffer, 1.5mM  | 5                |
| MgCl <sub>2</sub>     | MgCl <sub>2</sub> | MgCl <sub>2</sub> |                  |
| dNTP                  | 10mM each         | 0.2mM             | 1                |
| S1(sense) primer      | 50 $\mu$ M        | 1 $\mu$ M         | 1                |
| AS1(antisense) primer | 50 $\mu$ M        | 1 $\mu$ M         | 1                |
| Taq polymerase        | 1U/ $\mu$ l       | 2U                | 2                |
| dH <sub>2</sub> O     | -                 | -                 | 30               |

The following PCR parameters were performed on the samples:

Table 4.9: Outer polymerase gene PCR reaction parameters

| Steps | Reaction     | Temperature (°C) | Time       | No. of cycles |
|-------|--------------|------------------|------------|---------------|
| 1     | Denaturation | 95               | 2 minutes  | 1 cycle       |
| 2     | Denaturation | 95               | 20 seconds | 40 cycles     |
|       | Annealing    | 50               | 30 seconds |               |
|       | Elongation   | 72               | 45 seconds |               |
| 3     | Elongation   | 72               | 7 minutes  | 1 cycle       |
| 4     |              | 6                | 2 minutes  | 1 cycle       |

**1.12a(ii) Amplification Of The 218bp Inner Polymerase Gene Fragment**

The same PCR equipment, reagents and methods mentioned above were used but the PCR master reagent recipe, aliquot and reaction parameters were changed. The changes were as follows: an increase to 35 $\mu$ l from 30 $\mu$ l of distilled water, the forward primer was

changed to S2, the PCR mix aliquots were increased from 40 $\mu$ l to 45 $\mu$ l and 5 $\mu$ l of the outer polymerase negative PCR samples were used as templates.

The following PCR master mix recipe and parameters were used for the outer polymerase gene fragment:

Table 4.10: Inner polymerase gene PCR master reagent recipe

| Reagents                 | Stock solution    | Final conc.       | Volume( $\mu$ l) |
|--------------------------|-------------------|-------------------|------------------|
| PCR buffer plus          | 10X buffer; 15mM  | 1X buffer, 1.5mM  | 5                |
| MgCl <sub>2</sub>        | MgCl <sub>2</sub> | MgCl <sub>2</sub> |                  |
| dNTP                     | 10mM each         | 0.2mM             | 1                |
| S2(sense) primer         | 50 $\mu$ M        | 1 $\mu$ M         | 1                |
| AS1(antisense)<br>primer | 50 $\mu$ M        | 1 $\mu$ M         | 1                |
| Taq polymerase           | 1U/ $\mu$ l       | 2U                | 2                |
| dH <sub>2</sub> O        | -                 | -                 | 30               |

Table 4.11: Inner polymerase gene PCR reaction parameters

| Steps | Reaction     | Temperature (°C) | Time       | No. of cycles |
|-------|--------------|------------------|------------|---------------|
| 1     | Denaturation | 95               | 2 minutes  | 1 cycle       |
| 2     | Denaturation | 95               | 20 seconds | 40 cycles     |
|       | Annealing    | 58               | 30 seconds |               |
|       | Elongation   | 72               | 45 seconds |               |
| 3     | Elongation   | 72               | 7 minutes  | 1 cycle       |
| 4     |              | 6                | 2 minutes  | 1 cycle       |

After each PCR the products were subjected to electrophoresis in a 1.7 % multipurpose agarose gel (Roche Applied Science, Germany,) to check for the desired amplified polymerase gene products (see section 4.9).

**2(b) Purification Of The Polymerase Gene PCR Products For Direct Sequencing**

The outer and inner polymerase gene PCR products were subjected to the same purification and sequencing procedures mentioned in section 4.10. Some of the polymerase gene PCR bands had to be excised from the agarose gel due to the appearance of other unwanted DNA bands with the desired PCR band in the same lane. This was done by running the total PCR reaction volume on 3% nusieve agarose gel (Appendix E). Nuseive agarose gel was used to resolve small DNA molecules at a high resolution. A space was left between each lane so that there was no risk of cross contamination when excising the desired DNA band. The excision was done under a low UV source of light using a sterile surgical blade for each sample to avoid contamination. Once excised the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK) was used to extract and purify the DNA from the 3% nusieve agarose gel in 1x TBE buffer.

Empty 1.5ml Eppendorf tubes were weighed to the nearest 10mg and the weight recorded. The gel slices were transferred to the pre-weighed 1.5ml Eppendorf tubes and the weighed again. The weight of the slice was determined by subtracting the weight of the empty Eppendorf tube from this weight. To each gel slice 10 $\mu$ l of capture buffer was added for each 10mg of gel slice. The tube was closed and mixed by vortexing vigorously and incubated at 60°C in a water bath until the agarose had completely dissolved. During the incubation, one GFX column was placed in a collection tube for each purification that was to be performed.

After the agarose was completely dissolved, it was centrifuged briefly to collect the sample at bottom of the tube. The sample was transferred to the GFX column and incubated at room temperature for 1 minute. After incubation the tubes were centrifuged in a microcentrifuge at 14,000rpm for 30 seconds. The flow-through was discarded by emptying the collection tube which was then placed back into the GFX column. 500 $\mu$ l of wash buffer was added to the column and centrifuge at 14,000rpm for 30 seconds. The collection tube was discarded and the GFX column transferred to a fresh 1.5ml Eppendorf tube. 50 $\mu$ l of 10mM Tris-HCl pH8.0 (elution buffer) was applied directly to the top of the glass fiber matrix in the GFX column and incubated at room temperature

for 1 minute. The sample was then centrifuge at 14,000rpm for 1 minute to recover purified DNA.

**2(c) Screening Of YMDD Wild Type and Mutants By Nucleotide Sequence Analysis**

The viral DNA sequences were aligned and edited using Bioedit and Clustal X. The presence of mutations in the YMDD motif were analysed by comparing these nucleotide sequences with three HBV polymerase gene sequences from the GenBank database (Accession no.AB033558, AF297623 and x51970).

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## CHAPTER 5:

### RESULTS

#### 5.1 OUTLINE OF CASE RECRUITMENT AND INVESTIGATION

To date, 169 patients were recruited at the Liver Clinic of which 146 were infected with HBV. Control patients were added to the study cohort and comprised of 14 patients who were duplicate HBV infected cases and 9 who were non-HBV.

#### 5.2 DESCRIPTIVE STATISTICS

##### 5.2(a) Race Demographics

The ethnic groups represented within the study cohort were the Caucasians, Mixed race, Asian and Black Africans. The Mixed race constituted the largest ethnic group in the study cohort, followed by the Black Africans, Asian and Caucasians. This is illustrated in Figure 5.1 below.

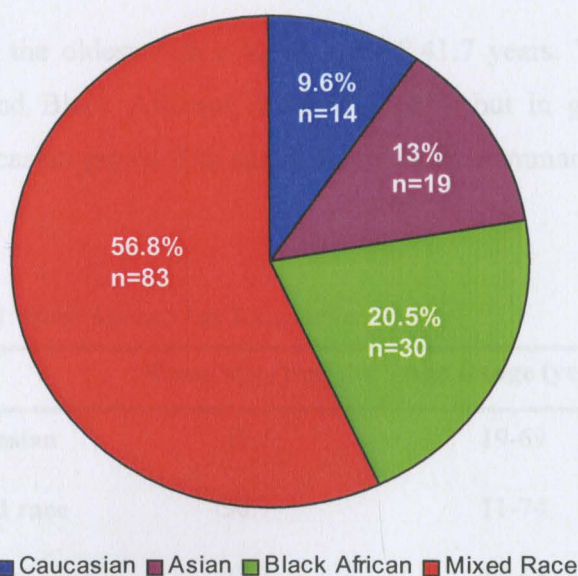


Figure 5.1: Pie Chart demonstrating total race distribution within the study cohort (n=number of patients).

5.2(b) Sex Distribution

The Mixed race had the highest number of females and the Caucasians had the lowest number of males in the cohort. This is summarized in Figure 5.2.

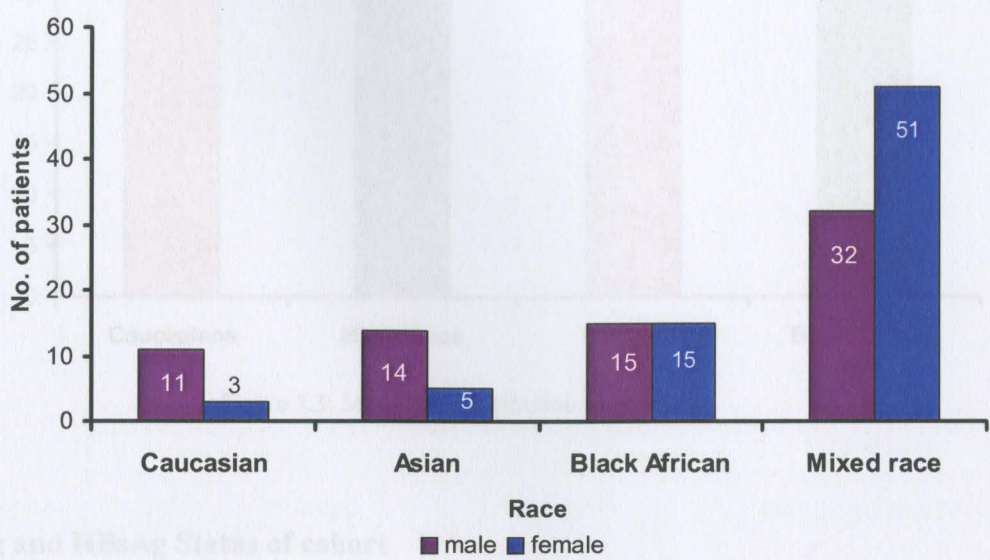


Figure 5.2: Sex distribution amongst the races in the study cohort.

5.2(c) Age Demographics

The Caucasians were the oldest with a mean age of 41.7 years. The mean age of the Mixed race, Asian and Black Africans differed slightly but in general they were all younger than the Caucasian group. The age differences are summarized in Table 5.1 and Figure 5.3.

Table 5.1: Mean Age and Age Range across Races

| Race          | Mean Age (years) | Age Range (years) |
|---------------|------------------|-------------------|
| Caucasian     | 41.7             | 19-69             |
| Mixed race    | 30.7             | 11-74             |
| Asian         | 33.3             | 11-63             |
| Black African | 32.3             | 17-56             |
| Study cohort  | 32.5             | 11-74             |

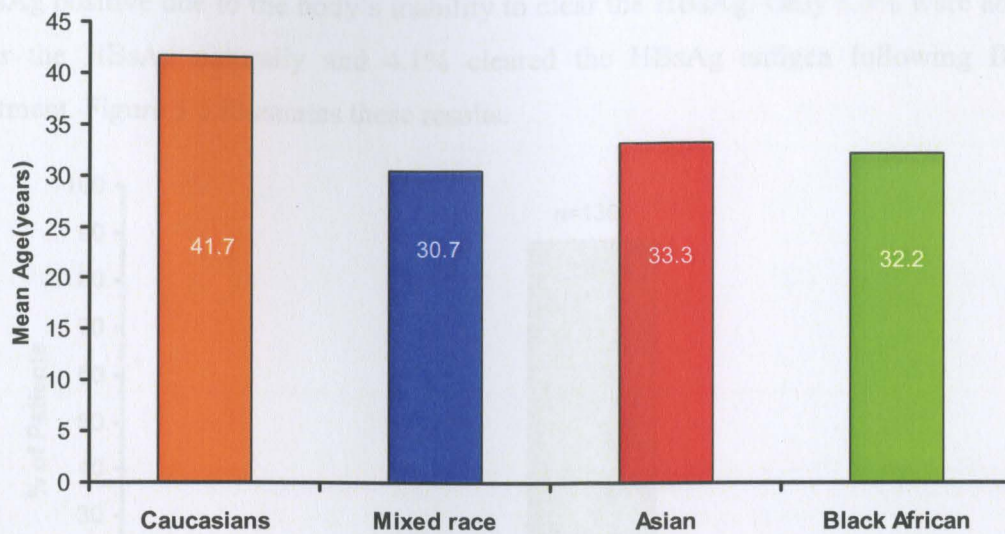


Figure 5.3: Mean Age distribution across races

#### 5.2(d) HBeAg and HBsAg Status of cohort

At the time of recruitment, 23.3% of the patients were HBeAg positive, 67.1% had achieved natural HBeAg loss and 9.6% were HBeAg negative following IFN- $\alpha$  treatment. These results are illustrated in Figure 5.4.

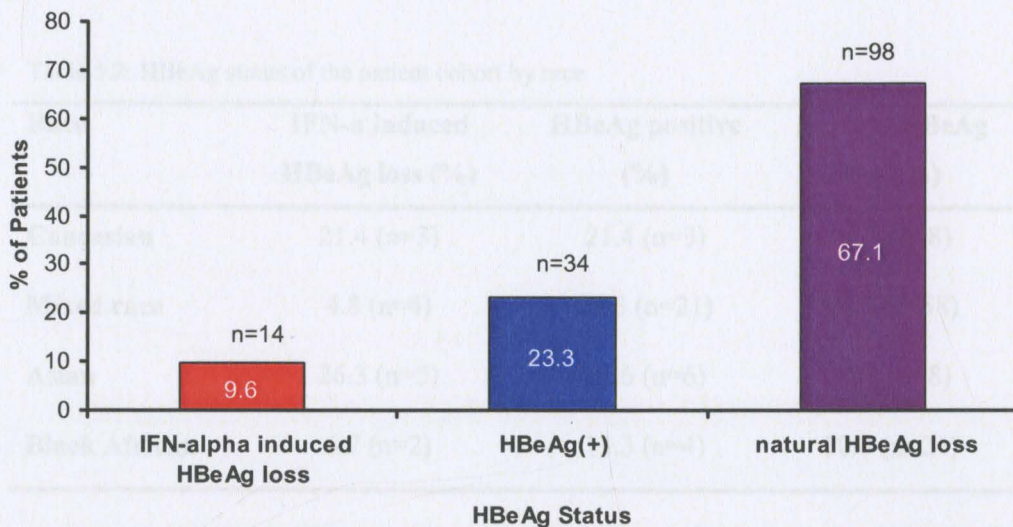


Figure 5.4: Graph illustrating the HBeAg status of the patients in the Liver Clinic. HBeAg negativity in the patients came about by either natural loss or with the aid of IFN- $\alpha$ .

At the end of 2 years of follow up, 89%, which is the majority of the patients were HBsAg positive due to the body's inability to clear the HBsAg. Only 6.8% were able to clear the HBsAg naturally and 4.1% cleared the HBsAg antigen following IFN-a treatment. Figure 5.5 illustrates these results.

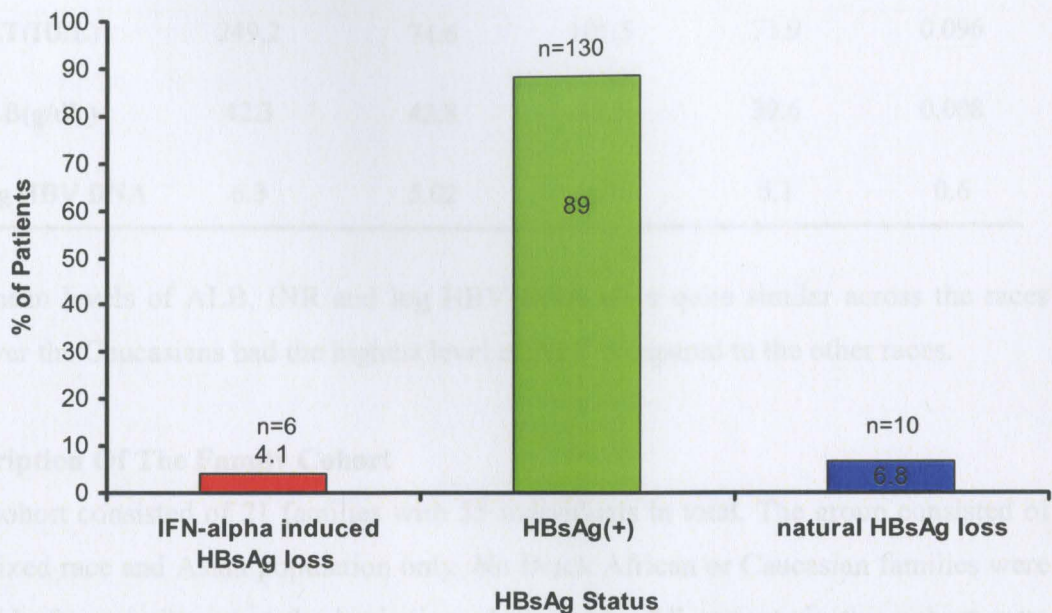


Figure 5.5: A graphical representation of the HBsAg status of the patients in the Liver Clinic at the end of follow up.

The following table shows the HBeAg status by race at the end of follow-up.

Table 5.2: HBeAg status of the patient cohort by race

| Race          | IFN-a induced<br>HBeAg loss (%) | HBeAg positive<br>(%) | Natural HBeAg<br>loss (%) |
|---------------|---------------------------------|-----------------------|---------------------------|
| Caucasian     | 21.4 (n=3)                      | 21.4 (n=3)            | 57.1 (n=8)                |
| Mixed race    | 4.8 (n=4)                       | 25.3 (n=21)           | 69.9 (n=58)               |
| Asian         | 26.3 (n=5)                      | 31.6 (n=6)            | 42.1 (n=8)                |
| Black African | 6.7 (n=2)                       | 13.3 (n=4)            | 80.0 (n=24)               |

The Asians had the highest percentage of patients achieving HBeAg clearance following IFN-a treatment while the Black Africans had the highest percentage of patients with natural HBeAg loss.

**5.2(e) Biochemical and Virological Description of the Patients**

Table 5.3: Mean ALT, ALB, and log HBV DNA levels in each race

|                    | Caucasians | Mixed race | Asian | Black African | P value |
|--------------------|------------|------------|-------|---------------|---------|
| <b>ALT(IU/L)</b>   | 249.2      | 74.6       | 101.5 | 71.9          | 0.096   |
| <b>ALB(g/dL)</b>   | 42.3       | 42.8       | 42.5  | 39.6          | 0.008   |
| <b>Log HBV DNA</b> | 6.3        | 5.02       | 4.7   | 5.1           | 0.6     |

The mean levels of ALB, INR and log HBV DNA were quite similar across the races however the Caucasians had the highest level of ALT compared to the other races.

**5.2(f) Description Of The Family Cohort**

This cohort consisted of 21 families with 55 individuals in total. The group consisted of the Mixed race and Asian population only. No Black African or Caucasian families were available for recruitment at the beginning of the study. All patients in this cohort were infected with HBV. 29.1% of the patients were HBeAg positive while 70.9% were HBeAg negative. Table 5.4 describes the mean age, log HBV DNA level, ALT, ALB and INR levels of the 21 families.

Table 5.4 Mean estimates of the family cohort

|                    | Mean estimate | 95% Confidence Interval |
|--------------------|---------------|-------------------------|
| <b>Age</b>         | 30.3          | 24.1-36.5               |
| <b>Log HBV DNA</b> | 4.8           | 3.6-6.1                 |
| <b>ALT(IU/L)</b>   | 129.2         | -3.0-261.3              |
| <b>ALB(g/dL)</b>   | 42.2          | 40.1-44.3               |
| <b>INR</b>         | 1.5           | 0.6-2.4                 |

Table 5.5 shows the percentage distribution of the races, HBeAg and HBsAg status of the family cohort. There was a higher percentage of patients who were HBsAg positive compared to HBeAg

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positive. A higher percentage of patients cleared HBsAg compared to HBeAg following IFN-a treatment while more patients cleared HBeAg naturally compared to HBsAg.

Table 5.5: Distribution of Race, HBeAg and HBsAg Loss in the Family Group

| <b>Race</b>              | <b>%</b> | <b>95% Confidence Interval</b> |
|--------------------------|----------|--------------------------------|
| Mixed race male          | 18       | 7.6-28.7                       |
| Mixed race female        | 61.8     | 48.5-75                        |
| Asian male               | 14.5     | 4.9-24                         |
| Asian female             | 5.5      | -0.7-11.6                      |
| <b>HBeAg loss</b>        |          |                                |
| IFN-a induced HBeAg loss | 9        | 12.4-16.9                      |
| HBeAg (+)                | 21.8     | 10.5-33                        |
| Natural HBeAg loss       | 69       | 56.4-81.7                      |
| <b>HBsAg loss</b>        |          |                                |
| IFN-a induced HBsAg loss | 36.3     | -1.4-8.7                       |
| HBsAg (+)                | 85.4     | 75-95                          |
| Natural HBsAg loss       | 10.9     | 2.4-19.4                       |

### 5.3 INFERENCE STATISTICS

As discussed in the objectives of the study (Chapter 3, Section 3.3), the possible correlations between genotype, race and clinical markers were investigated using the  $\chi^2$  test. A p value <0.05 was considered significant and a p value <0.0001 was considered highly significant.

#### 5.3(a) Correlations with Race

- **Race and Age**

At the time of HBV diagnosis, the Caucasians at 41.7 years were significantly older than the Mixed race at 30.7 years (p value=0.003) and Black African at 32.2 years (p value=0.034), (see Figure 5.3). The Mixed race and Black African presented with active HBV infection at a younger age than the Caucasians.

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### • Race, HBeAg and HBsAg loss

The difference between HBeAg loss due to IFN- $\alpha$  treatment and natural loss in the Mixed race and Asians was highly significant (p value = 0.006, refer to Table 5.6). 69.9% of the Mixed race compared to 42.1% of the Asian race had natural clearance of HBeAg. However, the Asians had a higher success rate with IFN- $\alpha$  treatment as 26.3% were able to clear the HBeAg with treatment compared to 4.8% of the Mixed race. When the Black Africans were compared to the other races, there was no statistical significance in the ability to clear HBeAg.

No statistically significant difference in the ability to clear HBsAg between the races was observed.

Table 5.6: The Racial Comparisons for HBeAg Clearance

| Racial Comparisons           | P value |
|------------------------------|---------|
| Mixed race vs. Asians        | 0.006   |
| Caucasians vs. Black African | 0.233   |
| Mixed race vs. Black African | 0.039   |
| Asian vs. Black African      | 0.2     |
| Caucasians vs. Asian         | 0.68    |
| Caucasians vs. Black African | 0.785   |

### • Race and Biochemistry (ALB and ALT levels)

The difference in ALB levels was highly significant between the Mixed race and Black African (p value= 0.001). The 95% confidence interval was 1.37 to 5.07. The Mixed race had higher levels of ALB than the Black African (refer to Table 5.3). There was no significant correlation between race and ALT levels (p=0.096).

## 5.3(b) Correlations with Log HBV DNA Levels

### • log HBV DNA, HBeAg and HBsAg loss

A statistically significant difference was observed between low log HBV DNA levels and the ability to clear HBeAg (p=0.0001). The 95% confidence interval was 1.73 to 4.2.

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Patients who cleared HBeAg naturally had a lower log HBV DNA level compared to those who cleared HBeAg following IFN-a treatment or those who failed to clear it.

No statistical significant difference between the loss of HBsAg and log HBV DNA levels was observed ( $p=0.567$ ). The confidence interval was -2.6 to +4.7.

- **log HBV DNA and Race**

There was no statistically significant difference in the log HBV DNA levels across the races ( $p=0.625$ )

### 5.3(c) Correlations with IFN-a Treatment

- **IFN-a treatment, log HBV DNA, HBeAg and HBsAg loss**

The IFN-a treated patients had a higher rate of HBeAg clearance than those who cleared naturally ( $p=0.0001$ ). A pre-treatment HBV DNA level of less than log 3.9 was predictive of HBeAg clearance following IFN-a treatment.

A statistical significance was observed in the rate of clearance in IFN-a treated patients and those who cleared HBeAg naturally ( $p=0.0001$ ).

The rate of HBsAg loss did not differ significantly between IFN-a treated patients and those who cleared naturally ( $p=0.567$ ).

- **IFN-a treatment and ALT levels**

Patients with ALT levels higher than 60 IU/L (1.5x normal) at time of IFN-a treatment were more likely to clear HBeAg ( $p=0.0001$ ) compared to those with ALT levels less than 60 IU/L. The 95% confidence interval was 2.64 to 2.83.

There was no statistically significant difference in HBsAg loss between patients with 1.5x normal ALT level and those with ALT levels less than 60 IU/L ( $p=0.440$ ).

### 5.4 ISOLATION OF GENOMIC DNA AND AMPLIFICATION OF B-GLOBIN GENE

73% of the patients had high quality DNA as indicated by the success of  $\beta$ -globin gene amplification by PCR. These samples were used for HBV genotyping and YMDD mutation screening. Some patients did not have their HBV DNA quantified due to cost limitations and this was the case in 44 patients who were  $\beta$ -globin positive. 58% of the  $\beta$ -globin positive patients had the HBV DNA assay done with 51% being HBV DNA positive. 57.4% HBV DNA positive and  $\beta$ -globin positive patients plus 6 patients who

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did not have their HBV DNA assayed but were  $\beta$ -globin positive had their HBV DNA genotyped. Therefore a total of 37 patients out of the initial 146 patients infected with HBV were genotyped. This is summarized in the Figure 5.6 below.

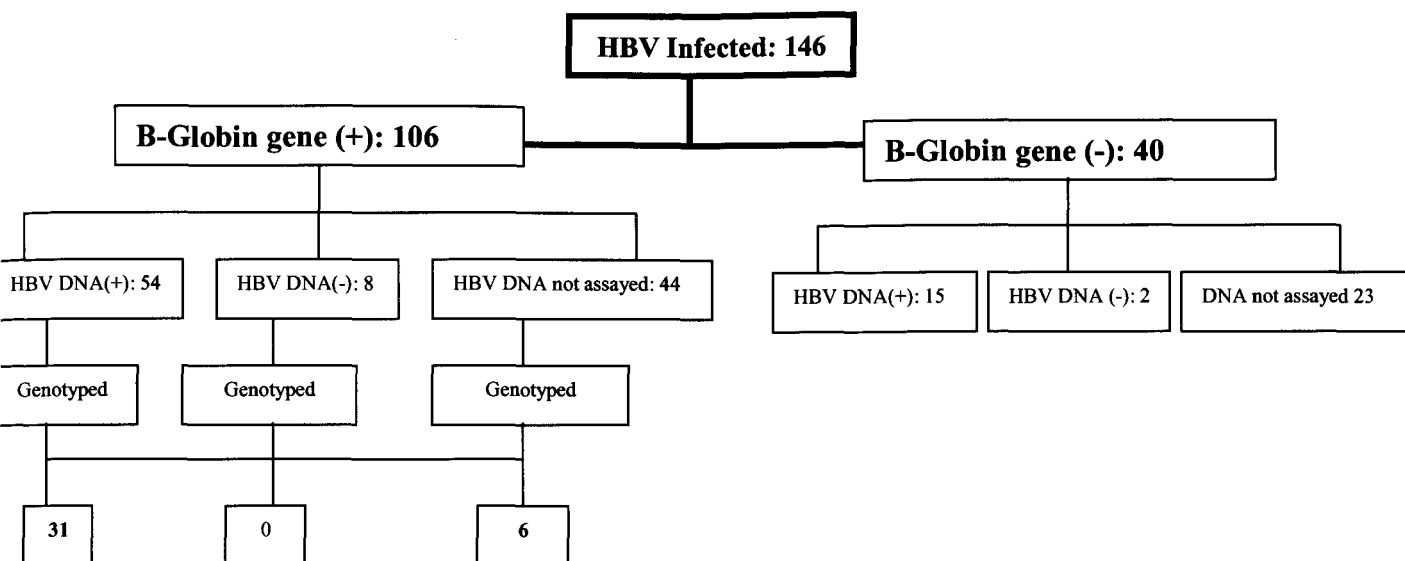


Figure 5.6: Outline of case recruitment and investigation  
 $\beta$ -globin(+):  $\beta$ -globin positive;  $\beta$ -globin(-):  $\beta$ -globin negative

Genomic DNA was isolated from 200 $\mu$ l of blood serum from the patients. The DNA yield ranged from 20-330 ng/ $\mu$ l. DNA samples with an (OD)<sub>260/280</sub> ratio of less than 1.2 were discarded and DNA extraction was redone.

To ascertain whether the genomic DNA was of good quality these samples were subjected to PCR amplification of the  $\beta$ -globin gene. There was successful amplification of this gene in 73% of the 146 samples. 40 DNA samples that were  $\beta$ -globin negative were not used in the study as this indicated that the DNA quality was not good enough for PCR.

Figure 5.7(a) and 5.7(b) illustrate the  $\beta$ -globin gene fragments obtained in 20 of the patients from the cohort. These illustrations are a representation of the results of 106 patient samples.

5.5 AMPLIFICATION OF THE  $\beta$ -GLOBIN GENE USING SEMI-NESTED PCR

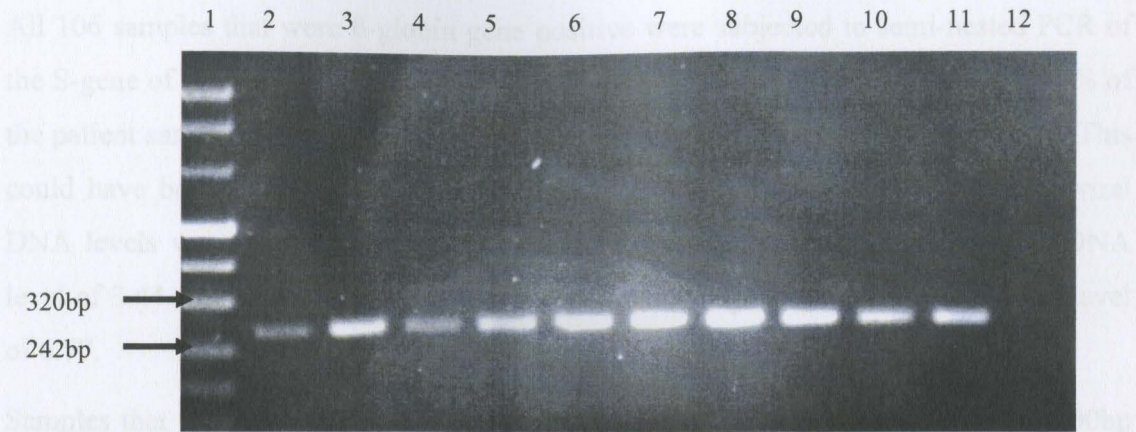


Figure 5.7(a): 1.7% ethidium bromide stained agarose gel of the  $\beta$ -globin gene PCR products. Lane 1 contains a DNA molecular marker and lane 12 contains a clear negative control. The negative control indicates that there was no contamination of samples during PCR. Lanes 2-11 demonstrate the 268bp  $\beta$ -globin gene PCR product of 10 patients from the study cohort. The brighter the fragment the better the quality of DNA extracted.

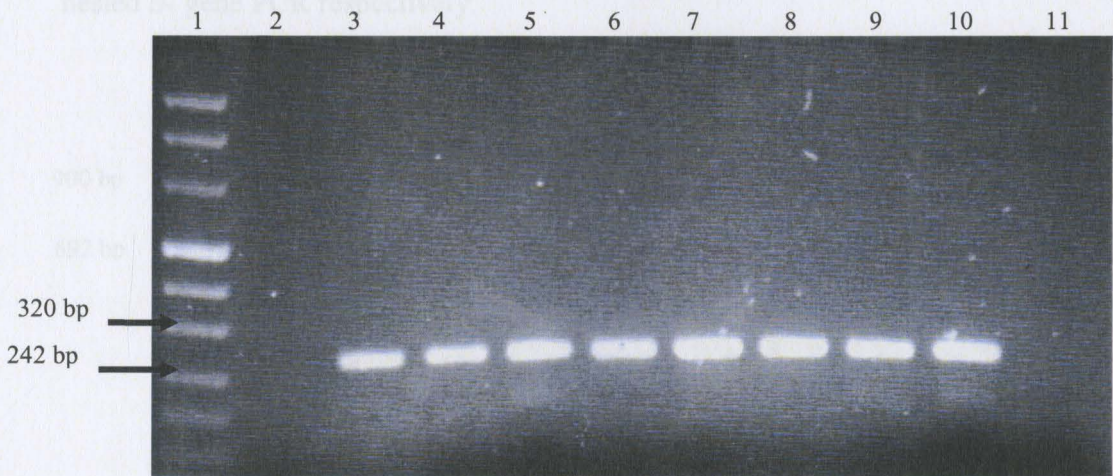


Figure 5.7(b): 1.7% agarose gel of the  $\beta$ -globin gene PCR products. Lane 1 contains a DNA molecular marker and lane 11 contains a negative control. Lane 2 represents a PCR sample that is  $\beta$ -globin negative indicating low DNA quality. Lanes 3-10 represent  $\beta$ -globin gene positive PCR samples from 9 patients from the study cohort. The PCR product size corresponds to 268bp.

### 5.5 AMPLIFICATION OF THE S-GENE USING SEMI-NESTED PCR.

All 106 samples that were  $\beta$ -globin gene positive were subjected to semi-nested PCR of the S-gene of the HBV genome. The S-gene region was successfully amplified in 35% of the patient samples while no DNA PCR product could be obtained from 69 samples. This could have been due to low viral DNA levels in the unsuccessful samples. Low viral DNA levels were found in the HBeAg negative samples with a mean log HBV DNA level of 3.44 while the HBeAg positive samples had a higher mean log HBV DNA level of 6.77.

Samples that were successfully amplified in the first round of PCR produced the 700bp outer S-gene fragment. Some samples were only successfully amplified in the second PCR round and these produced the shorter 540bp inner S-gene fragment. Hence both the inner and outer S-gene PCR products were used for genotyping.

Figure 5.8(a) and 5.8(b) illustrate the products of the first and second round of semi-nested S- gene PCR respectively.

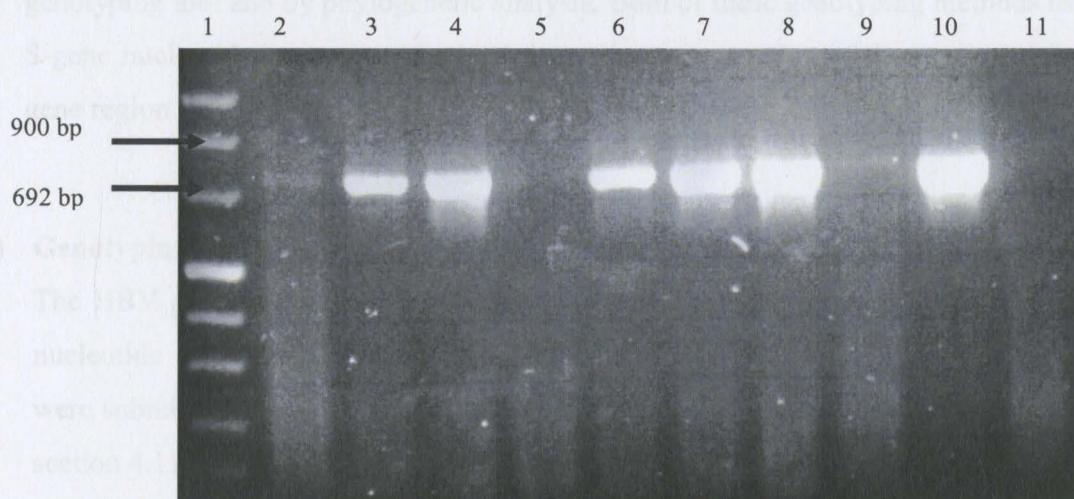


Figure 5.8(a): 1.7% agarose gel of the first round of semi-nested S-gene PCR. Lane 1 contains a DNA molecular marker and lane 11 contains a negative control. Lanes 2, 3, 4, 6, 7, 8 and 10 show the presence of outer S-gene PCR products from 7 patients in the cohort. The DNA band size corresponds to approximately 700bp. Lanes 5 and 9 represent samples of 2 patients that were negative for the outer S-gene PCR product. These negative samples were used in the second round of PCR.

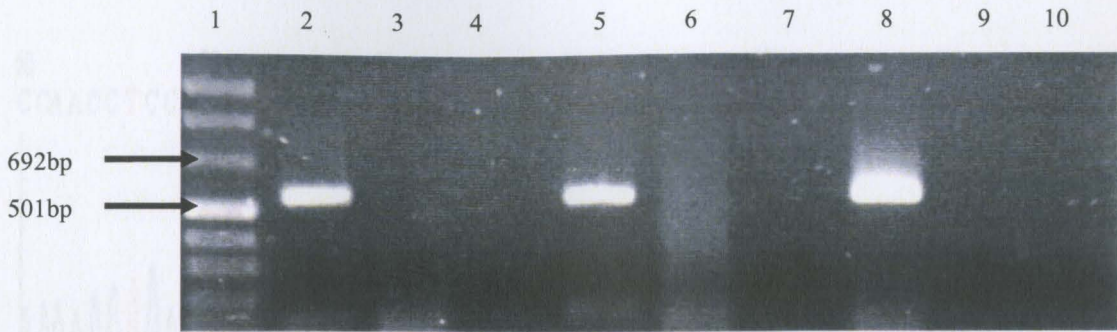


Figure 5.8(b): **Second round of the semi-nested S- gene PCR represented on a 1.7% agarose gel:** Lane 1 contains a DNA molecular marker and lane 10 contains a clear negative control. Lanes 2, 5 and 8 show the inner S- gene product while lanes 3,4,6,7 and 9 show patient samples negative for the inner S-gene product. The illustrated inner S- gene fragment corresponds to approximately 540bp.

## 5.6 HBV GENOTYPING

Patient HBV genotyping was performed using two methods; the NCBI web-based genotyping tool and by phylogenetic analysis. Both of these genotyping methods used the S-gene nucleotide sequences obtained from direct sequencing of the outer and inner S-gene region.

### 5.6(a) Genotyping Using the NCBI Web-Based Genotyping Tool

The HBV genotype was determined in 37 out of the 106 patient samples. The S-gene nucleotide sequences obtained from Stellenbosch University DNA sequencing facility were submitted for genotyping using the NCBI genotyping tool mentioned in Chapter 4, section 4.11(a).

An electropherogram of patients A5a S-gene nucleotide sequence is illustrated in Figure 5.9(a).

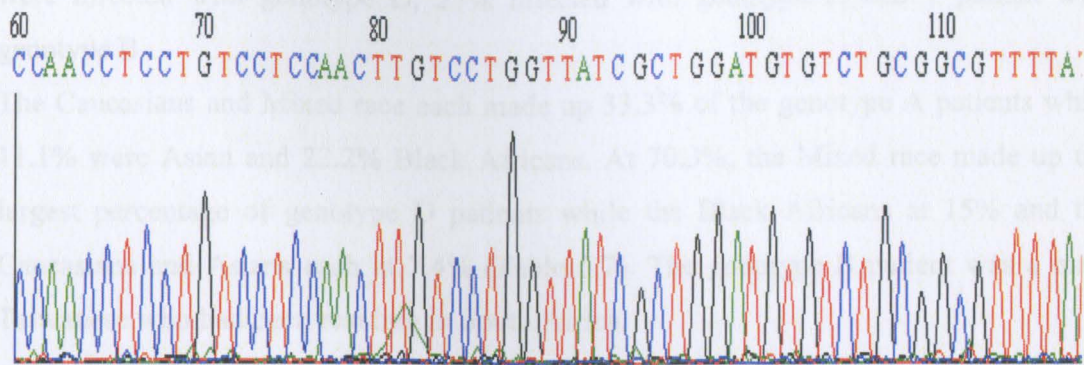


Figure 5.9(a): A representative electropherogram showing the S-gene nucleotide sequence of patient A5a. This nucleotide sequence is submitted into the NCBI genotyping tool form page.

Patient A5a HBV genotype result represented graphically by the NCBI genotyping tool is illustrated in Figure 5.9(b).

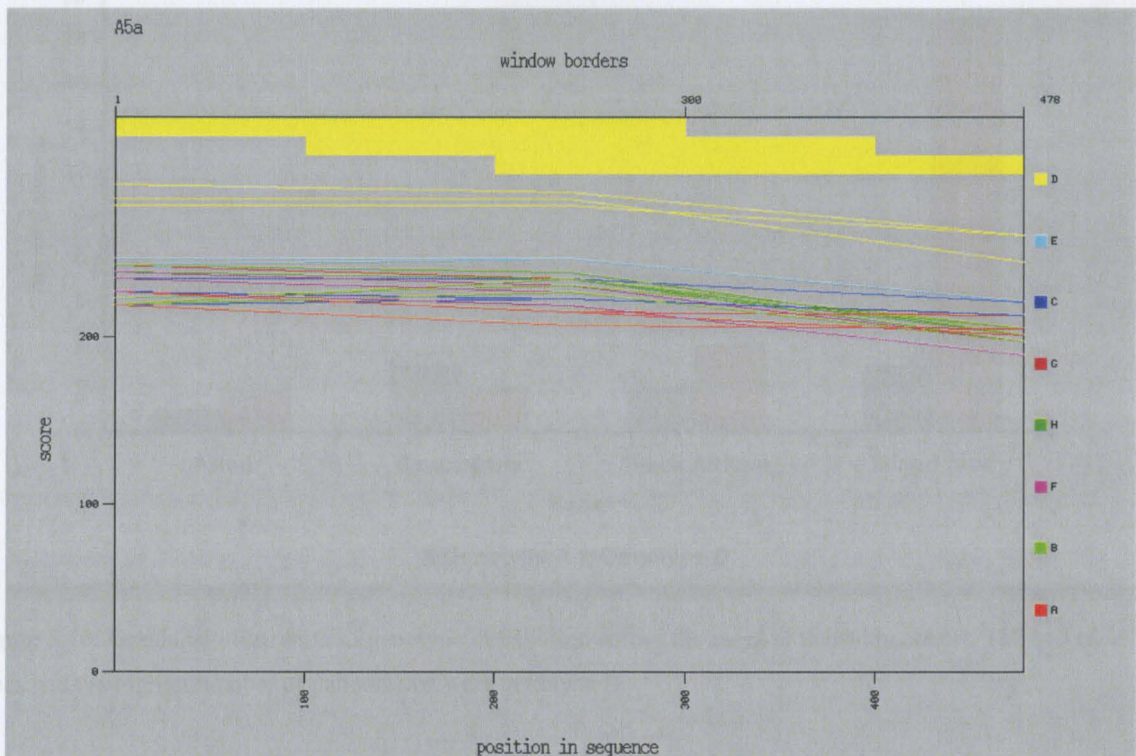


Figure 5.9(b): A representative graph depicting the HBV genotype result of patient A5a. The patient has been identified as genotype D as the yellow colour is dominant on the horizontal bar top.

Using predefined HBV reference sequences, it was determined that 73% of the patients were infected with genotype D, 27% infected with genotype A and 1 patient with genotype B.

The Caucasians and Mixed race each made up 33.3% of the genotype A patients while 11.1% were Asian and 22.2% Black Africans. At 70.3%, the Mixed race made up the largest percentage of genotype D patients while the Black Africans at 15% and the Caucasians and Asians each at 7.4% (Table 5.7). The genotype B patient was a male Taiwanese who had been residing in South Africa.

### 5.6(b) Genotype Distribution in the Cohort

Genotype A and D were found in the study. The Mixed race group predominantly had genotype D while genotype A was equally distributed across all races (Figure 5.10).

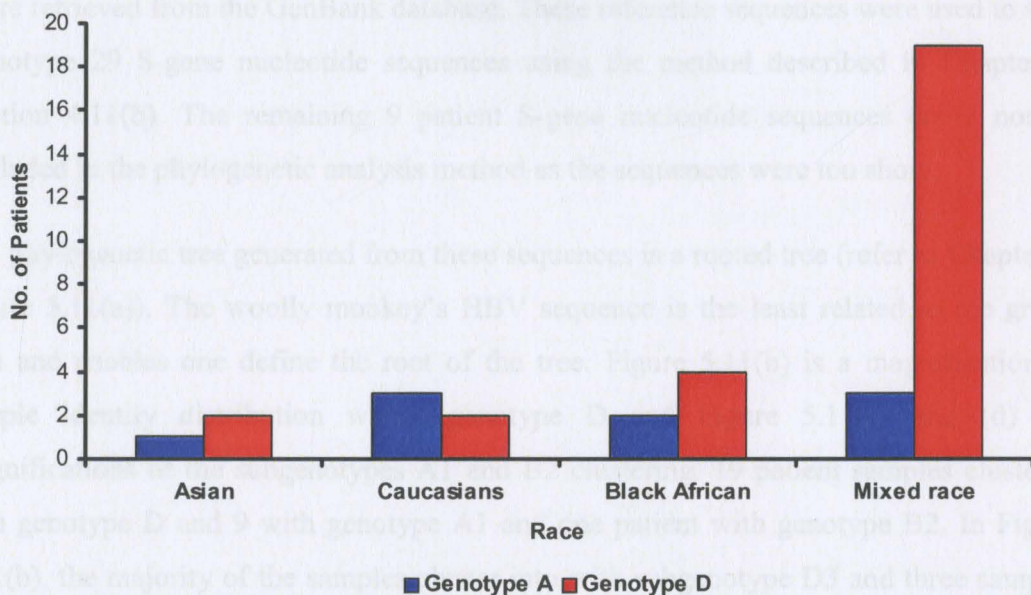


Figure 5.10: Graphical illustration of genotype distribution across the races in the study cohort. The Mixed races had the highest number of patients that were genotype D.

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Table 5.7: Web-based Genotype Distribution across the races

|                      | Genotype A (%) | Genotype D (%) |
|----------------------|----------------|----------------|
| <b>Caucasians</b>    | 33.3 (n=3)     | 7.4 (n=2)      |
| <b>Mixed race</b>    | 33.3 (n=3)     | 70.3 (n=19)    |
| <b>Asian</b>         | 11.1 (n=1)     | 7.4 (n=2)      |
| <b>Black African</b> | 22.2(n=2)      | 15 (n=4)       |

The p value= 0.164. The genotype B2 patient was excluded from the statistical analysis.

### 5.6(c) Subgenotyping Using Phylogenetic Analysis

41 HBV reference sequences representing the various genotypes and sub-genotypes A-H were retrieved from the GenBank database. These reference sequences were used to sub-genotype 29 S-gene nucleotide sequences using the method described in Chapter 4, section 4.11(b). The remaining 9 patient S-gene nucleotide sequences could not be included in the phylogenetic analysis method as the sequences were too short.

The phylogenetic tree generated from these sequences is a rooted tree (refer to Chapter 5, Figure 5.11(a)). The woolly monkey's HBV sequence is the least related to the group taxa and enables one define the root of the tree. Figure 5.11(b) is a magnification of sample identity distribution within genotype D and Figure 5.11(c) and (d) are magnifications of the subgenotypes A1 and B2 clustering. 19 patient samples clustered with genotype D and 9 with genotype A1 and one patient with genotype B2. In Figure 5.11(b), the majority of the samples cluster into with subgenotype D3 and three samples cluster with D4. There are four samples from the family cohort that were successfully genotyped and are shown in Figure 5.11(b). The samples fG1a and fG2a are siblings and are both subgenotype D3. Samples fE2a and fE3b, both subgenotype D4, are members of another family and are siblings.

Genotype A1 consisted of 4 Mixed race patients, 2 Caucasians, 2 Black Africans and 1 Asian. Genotype D was made up of 12 Mixed race patients, 3 Black Africans, 2 Caucasians and 2 Asians.

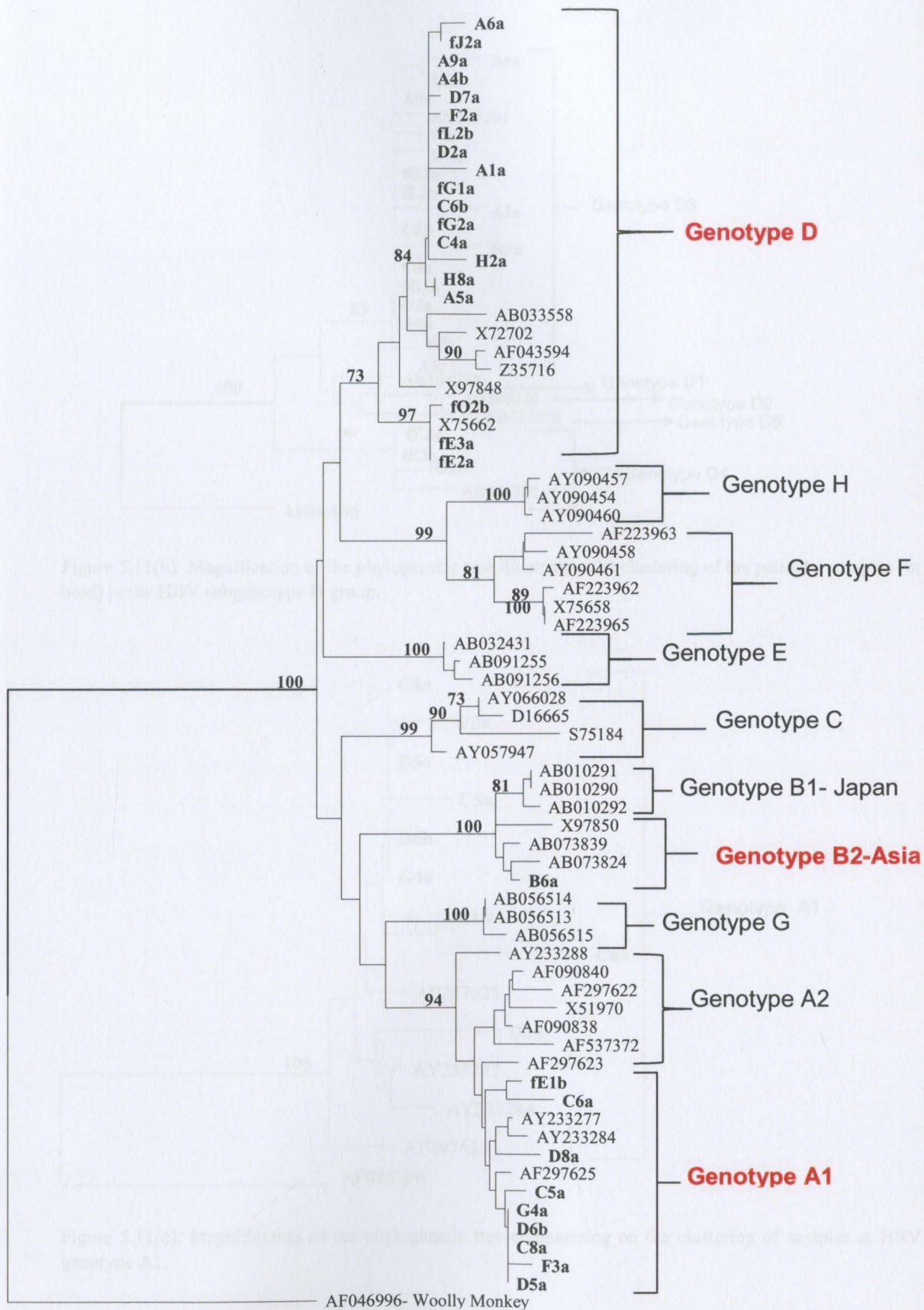


Figure 5.11(a): Phylogenetic tree constructed using 29 HBV S-gene nucleotide sequences determined in this study (in bold). The accession numbers of the HBV reference sequences and bootstrap values are given.

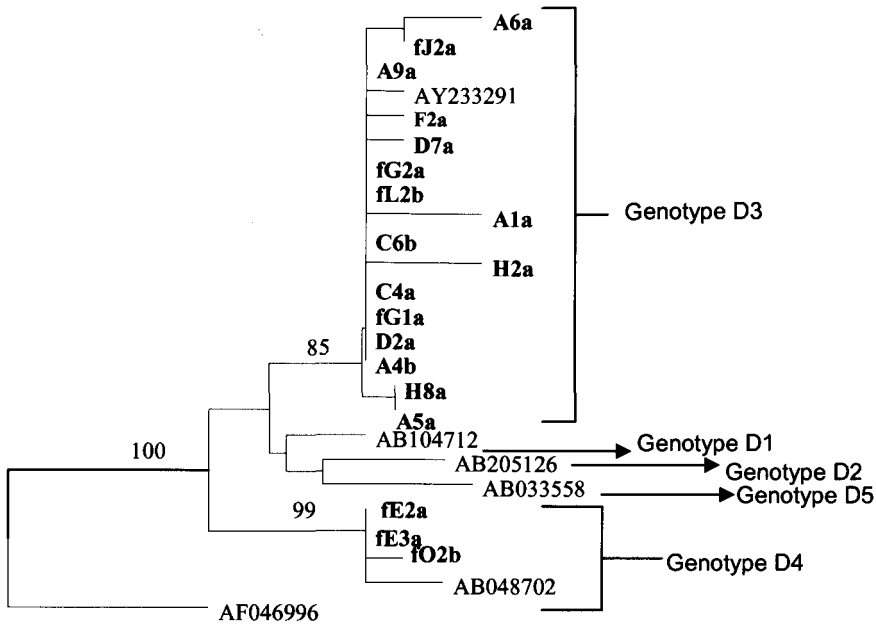


Figure 5.11(b): Magnification of the phylogenetic tree illustrating the clustering of the patients' samples (in bold) in the HBV subgenotype D group.

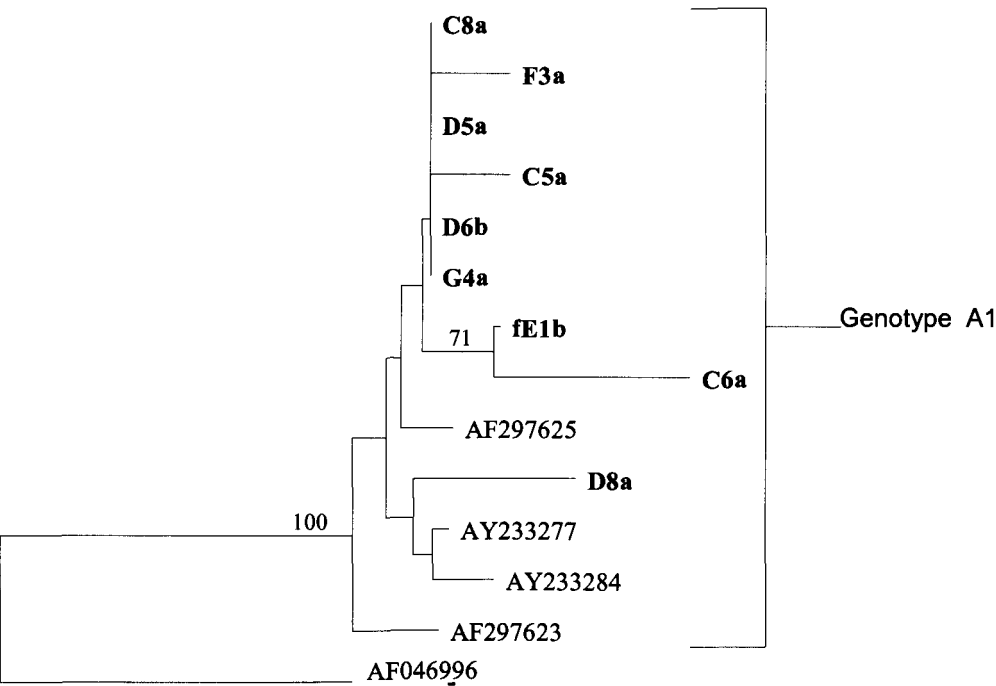


Figure 5.11(c): Magnification of the phylogenetic tree emphasizing on the clustering of samples at HBV genotype A1.

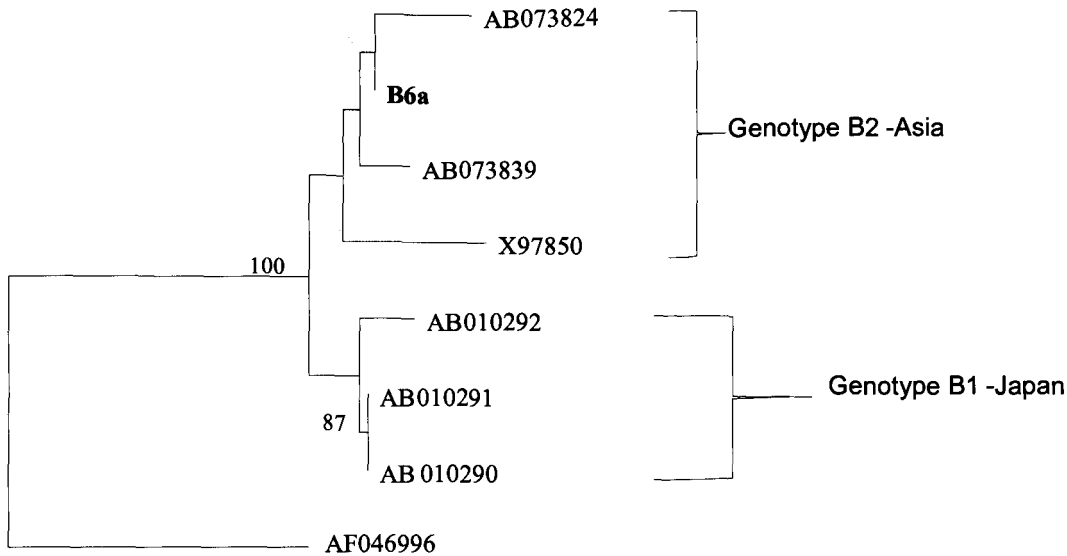


Figure 5.11(d): Phylogenetic tree illustrating the patient sample B6a clustering at HBV genotype B2.

**5.6(d) Comparison of the NCBI Web-Based and Phylogenetic HBV Genotyping Methods**

Both the HBV genotype results from the NCBI web-based genotyping tool and the phylogenetic analysis were compared. It was determined that the results from the NCBI web-based genotyping tool concurred 100% with those from the phylogenetic analysis. The main advantage of phylogenetic analysis is that it further determines the sub-genotype of the patient. Table 5.8 shows the HBV genotypes obtained using the two methods.

## CHAPTER 5: RESULTS

Table 5.8: Comparison between the HBV genotypes obtained from the NCBI genotyping tool and phylogenetic analysis.

| Patient Index | NCBI genotyping tool | Phylogenetic analysis |
|---------------|----------------------|-----------------------|
| A1a           | D                    | D                     |
| A2a           | A                    | -                     |
| A5a           | D                    | D                     |
| A6a           | D                    | D                     |
| A9a           | D                    | D                     |
| B6a           | B                    | B2                    |
| C2a           | D                    | -                     |
| C3a           | D                    | -                     |
| C4a           | D                    | D                     |
| C5a           | A                    | A1                    |
| C6a           | A                    | A1                    |
| C8a           | A                    | A1                    |
| D1a           | D                    | -                     |
| D2a           | D                    | D                     |
| D5a           | A                    | A1                    |
| D7a           | D                    | D                     |
| D8a           | A                    | A1                    |
| F2a           | D                    | D                     |
| F3a           | A                    | A1                    |
| G4a           | A                    | A1                    |
| H2a           | D                    | D                     |
| H3a           | D                    | -                     |
| H5a           | D                    | -                     |
| H8a           | D                    | D                     |
| A2b           | D                    | -                     |
| A4b           | D                    | D                     |

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|      |   |    |
|------|---|----|
| C6b  | D | D  |
| D6b  | A | A1 |
| D7b  | D | -  |
| fE2a | D | D  |
| fE3a | D | D  |
| fG1a | D | D  |
| fG2a | D | D  |
| fJ2a | D | D  |
| fE1b | A | A1 |
| fL2b | D | D  |
| FO2b | D | D  |

(-): S-gene nucleotide sequences that could not be used for phylogenetic analysis.

### 5.6(e) Correlations with HBV Genotypes

- **log HBV DNA and HBV Genotype**

There was no statistically significant difference between log HBV DNA levels and genotype A or D ( $p=0.784$ ).

- **HBV Genotype and Race**

HBV genotype A and D were present across all races with genotype D being most predominant in the Mixed race population (Refer to Figure 5.10 and Table 5.7). Interestingly this did not reach statistical significance ( $p=0.164$ ).

- **HBV Genotype, HBeAg and HBsAg loss**

The ability to clear HBeAg ( $p=0.598$ ) and HBsAg ( $p=0.75$ ) was not influenced by genotype.

- **HBV Genotype and ALT levels**

The ALT level between the genotypes was not statistically significant.

## 5.7 DETECTION OF YMDD MUTANTS (YIDD AND YVDD) IN THE PATIENT COHORT

Long-term use of lamivudine treatment of chronic HBV infection leads to the emergence of lamivudine resistant HBV YMDD mutant strains. The predominant mutants are the YVDD and YIDD. 29 patients who were HIV negative were used in the investigation of the presence of YMDD mutations.

### 5.7(a) Amplification of the Polymerase Gene Using the Semi-Nested PCR.

The polymerase gene region was successfully amplified in 29 patient serum samples. Two PCR products were obtained and used for DNA sequencing; the inner 218bp and outer 286bp amplicons.

Figure 5.12(a) and 5.12(b) demonstrate the first and second round products obtained in the semi-nested PCR of the polymerase gene.

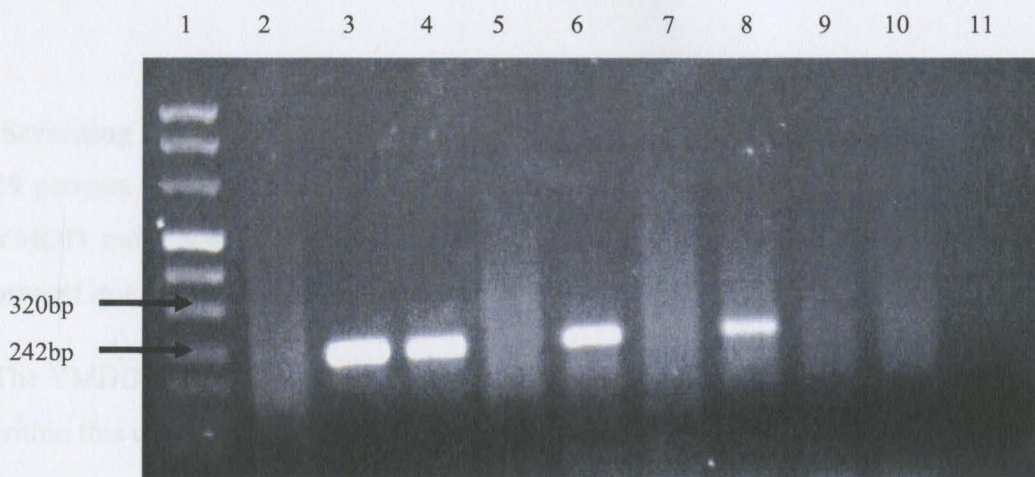


Fig 5.12(a): First round of semi-nested polymerase gene PCR represented on a 1.7% agarose gel stained with ethidium bromide: Lane 1 contains a DNA molecular marker and lane 11 is a negative control. Lane 3,4,6,8 show the outer polymerase gene product (268bp) obtained from patient samples. Lane 2,5,7,9 and 10 represent patient samples that are negative for the outer polymerase gene product.

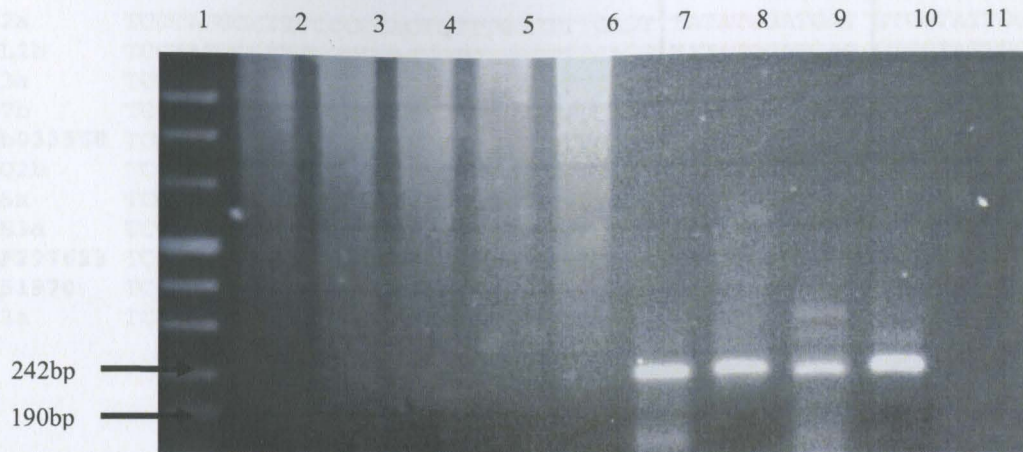


Fig 5.12(b): Second round of semi-nested polymerase gene PCR represented on a 1.7% agarose gel stained with ethidium bromide: Lane 1 contains a DNA molecular marker and lane 11 is a negative control. Lanes 7, 8, 9, 10 show the inner polymerase gene product (218bp) obtained from patient samples. Lanes 2, 3, 4, 5, 6, represent patient samples negative for the inner polymerase gene product. The inner polymerase gene product in lane 9 and 10 had to be excised out of the agarose gel as there is another DNA band present in the same lane.

### 5.7(b) Screening For YMDD Wild Type and Mutants by Nucleotide Sequence Analysis

29 patients were used in this analysis and three reference sequences with the wild type YMDD motif were obtained from the GenBank database. The YVDD mutation was present in 1 patient while the remaining 28 patients were YMDD wild type.

The YMDD wild type has an ATG codon in which mutations result in nucleotide changes within this codon (see Figure 5.13).

The YVDD mutation results in the amino acid methionine being replaced by isoleucine due to the change within the ATG codon to either ATT, ATC or ATA. The highlighted nucleotide 'G' is a degenerate nucleotide base in a wobble position. This nucleotide change is not considered as it is only the first two nucleotide bases on the codon that have to base-pair with the anti-codon on the tRNA so that the correct amino acid is added during translation.

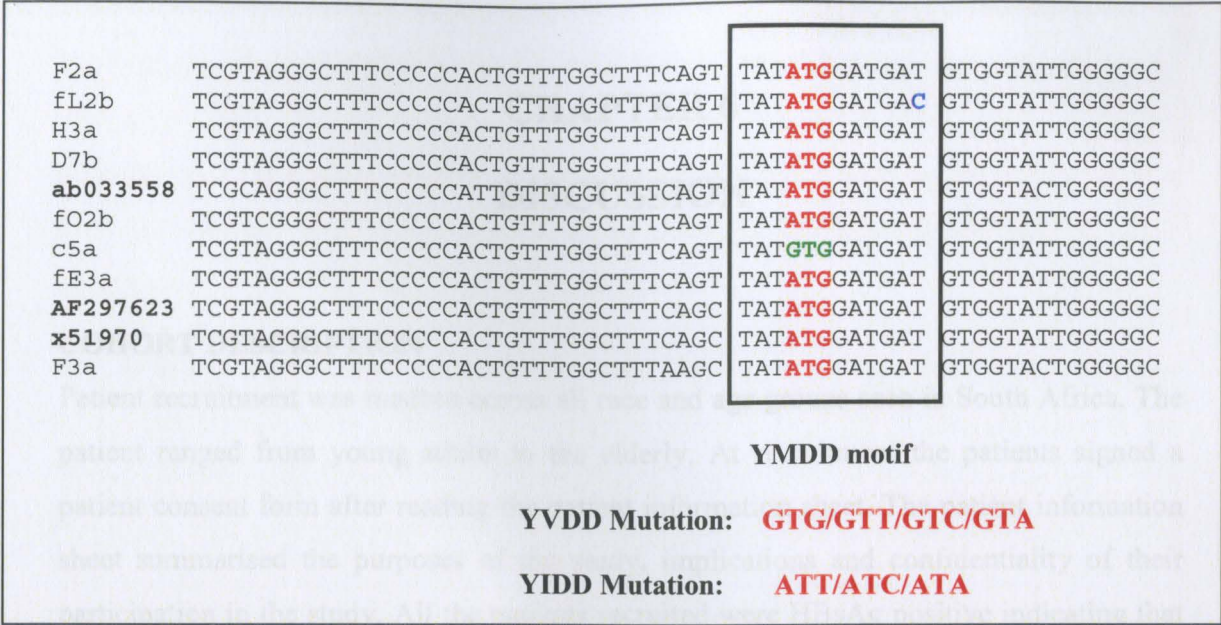


Fig 5.13: Section of the polymerase gene nucleotide sequence alignment showing the YMDD motif. Three database sequences (GenBank) are represented by their accession numbers in bold. Database sequences represent the wild type YMDD motif. Patient sequences analyzed for YMDD mutations are F2a, fL2b, H3a, D7b, fO2a, C5a, fE3a and F3a. YVDD and YIDD mutation are indicated in red. Either of the codons could replace the wild type **ATG codon**. YVDD mutation is present in patient C5a indicated in green. The highlighted **C** indicates a wobble position.

In the YVDD mutation, the changes with in the ATG codon are either GTG, GTT, GTC or GTA. The initial amino acid coded for by ATG is methionine and during translation this is changed to valine due to these nucleotide changes. One patient had the YVDD mutation present with the GTG codon replacing the ATG codon. According to the patient’s record he was on lamivudine therapy which resulted in the occurrence of the YVDD mutation. The remaining 28 patients were lamivudine naïve therefore no mutations were present.

The YIDD mutation results in the amino acid methionine being replaced by isoleucine due to the changes within the ATG codon to either ATT, ATC or ATA. The highlighted nucleotide ‘C’ is a degenerate nucleotide base in a wobble position. This nucleotide change is inconsequential as it is only the first two nucleotide bases on the codon that have to have precise pairing with the anti-codon on the t-RNA so that the correct amino acid is formed during translation.

## CHAPTER 6

### DISCUSSION

#### 6.1 COHORT DESCRIPTION

Patient recruitment was random across all race and age groups seen in South Africa. The patient ranged from young adults to the elderly. At recruitment the patients signed a patient consent form after reading the patient information sheet. The patient information sheet summarised the purposes of the study, implications and confidentiality of their participation in the study. All the patients recruited were HBsAg positive indicating that they had chronic HBV infection. This analysis was done over a two year period of follow-up.

#### 6.2 DESCRIPTIVE ANALYSIS

The Mixed race population was predominant and had the highest percentage of females in the study. The Caucasians had the lowest representation at 9.6% and the lowest number of males. It had been expected that the Mixed race would be highly represented as they are the predominant race in the Western Cape comprising 61.1% of the total population (<http://www.statssa.gov.za/census01/html/WCPrimary.pdf>). This finding supports that patient recruitment was at random.

All patients had chronic HBV infection at the time of recruitment, being HBsAg positive and either HBeAg positive or negative. The HBeAg negative patients were those entering or who had entered the low replicative phase. HBeAg seroconversion in this phase is characterised by low levels of circulating HBV DNA. The low levels of HBV DNA in these patients could explain the high percentage of patients (65%) with unsuccessful amplification of the S-gene on the HBV genome. 7% of the patients became HBsAg negative during follow-up. The natural ability of these patients to clear HBV surface and 'e' antigen could, in part, be explained by their genetic profile as 80% of those who achieved natural HBeAg clearance were Black Africans.

The patients who were HBeAg positive had active HBV viral replication as shown by their high mean DNA levels (log 6.77) and were possibly in the early phase of chronic hepatitis B infection. The majority of the patients remained HBsAg positive at 24 months of follow-up and this is a characteristic of the serological pattern of chronic HBV infection as HBsAg is much harder to clear.

The ability to clear HBeAg was linked to low HBV DNA and high ALT levels. In those who were treated with IFN-a, 9.6% became HBeAg negative while 4% cleared the HBsAg. More patients were able to clear the HBeAg compared to HBsAg during IFN-a treatment. This was associated with the presence of high ALT and low HBV DNA levels. The association is well established in the literature. The association of low HBV DNA and high ALT levels with the ability to clear HBeAg following IFN-a is supported by studies that showed that high ALT and low HBV DNA levels are the primary predictors of a favourable response to IFN-a treatment (Hoofnagle and di Bisceglie, 1997). Successful IFN-a treatment has been shown to result in the significant clinical improvement of the patient (Niederau et al., 1996, Wong et al., 1993).

The cohort of patients who were related to each other, 'family group', is still small. The Mixed race females in the family group made up a significant proportion of the family cohort compared to the Mixed race males and Asian males and females. No statistical correlations could be done on these related patients as the numbers of those genotyped successfully were too small and in addition not all races were represented in the cohort by the end of the study. However there is on going recruitment of family contacts and investigations for possible genetic influences on the immune response to HBV as all members in the family cohort are infected with HBV.

### **6.3 STATISTICAL ANALYSIS OF CORRELATIONS**

#### **6.3(a) Racial Correlations:**

At HBV diagnosis the Mixed race and Black Africans were significantly younger than the Caucasians. The young age at presentation was interesting. This could have been due

## CHAPTER 6: DISCUSSION

to differences in their genetic profile that resulted in these races being more susceptible to the hepatitis B virus or having more severe disease at a young age. The Black Africans and Mixed race may possess a gene that predisposes them to more severe and aggressive disease compared to the Caucasians and therefore they present earlier. A similar trend has also been documented in the Liver Clinic's cohort of patients with autoimmune hepatitis. The Black Africans presented at a younger age with more cirrhosis compared to the Caucasians who presented at an older age and with less cirrhosis (Hairwadzi et al., 2002). Recent work on associations between disease severity and genetics has shown a correlation between the ICAM-1 gene and decompensated cirrhosis in Chinese patients (Zhang et al., 2008). Genetic association with disease susceptibility has been documented in West Africa where the Fulani tribe are less susceptible to malaria than other local tribes. This is due to differences in the expression levels of immune response such as the T-helper 1 and T-helper 2 related genes (Torcia et al., 2008). A tribal difference is also documented in a study from northern Nigeria where the HBV prevalence rates differ between the Nupe and Gwari tribe (Kulkarni et al., 1986). Similar genetic polymorphisms may be involved in the immune response to HBV infection.

The fact that Caucasians presented at an older age could also be due to the route of HBV infection acquisition. Certain high risk adult behaviour such as tattooing, piercing and drug abuse could result in infection at an older age. Locally, horizontal transmission at an early age is the most common route of viral acquisition in the non-Caucasoids (Tabor, 1985) which could lead to disease presentation at an early age

A significant difference was observed in the rate of natural HBeAg loss and HBeAg loss following IFN-a treatment between the Mixed race and Asians. A large percentage of the Mixed race cleared HBeAg naturally compared to Asians while more Asians were able to clear HBeAg following IFN-a treatment. These findings may be explained by genetic factors that may have contributed to a better response to IFN-a treatment in the Asians compared to the Mixed race. The IFN-a response gene in the Asians may have been better up regulated during treatment exposure resulting in HBeAg loss. Further investigations are however needed to support this hypothesis.

ALB levels between the Mixed race and Black Africans differed significantly. The Black Africans had a lower level of ALB suggesting that they presented at the Liver Clinic with more severe liver disease compared to the Mixed race. This could, in part, be explained by socio-economic factors. The Black Africans generally have a lower level of education compared to the other races in the Western Cape with only 5.2% of individuals older than 20 years having a tertiary level of education ([http:// www. statssa.gov. za/census01/html/WCPPrimary.pdf](http://www.statssa.gov.za/census01/html/WCPPrimary.pdf)). The majority of the Black African population live in informal settlements with poor sanitation and very limited access to healthcare facilities. The lower education levels would lead to a reduced ability to access healthcare information which is mostly in the written form. Differences in the genetic profile between these two races may also explain the observed difference in that the Mixed race may have genes that result in less severe disease as defined by ALB levels compared to the Black Africans. These patients may possess polymorphisms associated with disease severity. An example of such a polymorphism has been shown in a recent study where there is an association between polymorphisms in the ICAM-I gene and active decompensated cirrhosis in Chinese chronic HBV carriers (Zhang et al., 2008).

### **6.3(b) Log HBV DNA correlations:**

The HBV DNA levels were not influenced by HBV genotype, race or ability to clear HBsAg. Low HBV DNA levels correlated with the ability to clear HBeAg following IFN- $\alpha$  treatment. The HBeAg negative IFN- $\alpha$  treated patients had low levels of HBV DNA. Low HBV DNA levels indicated a favourable response to treatment. In addition natural clearance of HBeAg (seroconversion to anti-HBe) in patients with long-term infection leads to a corresponding decline in HBV DNA levels indicating that the patient is in the immune clearance phase. The genotype B2 patient who wasn't included in the analysis had high HBV DNA levels and failed to clear HBeAg following IFN- $\alpha$  treatment.

### **6.3(c) IFN-a treatment Correlations:**

The difference in HBeAg clearance due to IFN-a is discussed in section 6.6. Low HBV DNA levels and ALT levels were significantly associated with the ability to clear HBeAg following IFN-a treatment. The high ALT and low levels of circulating HBV DNA usually occurs in patients who are in the immune elimination phase of the disease and the addition of a treatment increases the likelihood of HBeAg clearance.

The difference in HBeAg clearance in patients who failed to respond to IFN-a treatment and those who naturally cleared HBeAg was significant. This difference is being investigated in the long term cohort but a possible explanation could be genetic. Patients who cleared HBeAg naturally may have a gene that enhances their natural immune response to the virus and therefore the ability to clear HBeAg.

The results of this study show that HBV genotype and race do not play a role in the ability to clear HBeAg in these patients however a larger cohort would be needed to address this observation. However there is data suggesting that HBV genotype may have an influence on response to treatment as studies have shown that genotype A has a better response to IFN-a than genotype D (Zhang et al., 1996, Kobayashi et al., 2005, Palumbo, 2007). An interesting observation was that 80% of those who cleared HBeAg naturally were Black Africans which is probably due to the mode and age of HBV infection. Most Black Africans are infected horizontally at a young age (Tabor, 1985) and so would have cleared HBeAg by the time they present at the Liver Clinic. Although the numbers were too small for further interracial comparisons, this finding may suggest that race may be a factor.

### **6.4 ISOLATION OF GENOMIC DNA AND B-GLOBIN GENE AMPLIFICATION**

At the time of this analysis, 146 patients with chronic HBV infection had been recruited but only 106 had DNA extracted from serum that was suitable for PCR analysis as indicated by the successful amplification of the  $\beta$ -globin gene. In chapter 5, figure 5.6, the flow diagram showed that the other 40 samples had poor quality DNA for the purposes of this study. 15 samples were HBV DNA positive but  $\beta$ -globin negative. The HBV DNA quantification assays were done at a different diagnostic laboratory. The

extraction methods and samples used for these assays were different from the methods and samples used in this project. In addition, the samples were immediately sent off for HBV DNA quantification while the samples used in this project had been stored for a long period of time and may have been thawed and frozen several times. This would have contributed to poor quality and degradation of DNA

### **6.5 AMPLIFICATION OF THE S-GENE ON THE HBV GENOME USING SEMI-NESTED PCR**

Despite repeating the S-gene semi-nested PCR on 69 samples that were  $\beta$ -globin positive and increasing the volume of input DNA in the PCR mix, no S-gene amplicon was obtained. This may have been due to the presence of low levels of viral DNA despite the presence of good quality DNA. The levels of HBV DNA may have been very low as most of these samples were HBeAg negative. The HBeAg negative samples had a lower viral load compared to the HBeAg positive patients therefore there was a low rate of success in S-gene amplification.

Some samples had successful amplification of the larger fragment of the S-gene (700bp) and these fragments were used for direct sequencing. Other samples were successful only in the amplification of the smaller S-gene fragment (540bp) in the second round of the semi-nested PCR. Success in the first round of PCR is largely dependent on the HBV DNA concentration added to the PCR mix. The concentration of the DNA in each eluted sample would have varied between patients resulting in different concentrations being added to each PCR mix. This would explain the differences in the success of the outer S-gene fragment amplification in the first round of the semi-nested PCR with those that were unsuccessful being the cases that may have had low concentrations of HBV DNA present.

Another reason that the amplification of the outer S-gene amplification was not successful is that the size of the target sequence in the first round may have been too large. The synthesis efficiency decreases with an increase in target sequence length (Jeffreys et al., 1988). This, combined with low levels of viral DNA, may have resulted in very low levels of amplification occurring in the first round. The levels of DNA

amplified may have been below the detection limit of the agarose gel electrophoresis used. However when using the outer S-gene negative PCR samples as template, these samples were successfully amplified in the second round as the target sequence is smaller and the primers used in this round may have been more sensitive to the presence of some target DNA. When the amplification of the outer 700bp amplicon was successful, this was not used as the template for the second round of PCR. This is because of the presence of high concentrations of DNA fragments would have resulted in possible contamination of the subsequent PCR mix and amplification of non-specific products.

It was also later discovered that the *Taq* polymerase (Roche Applied Science, Germany) has been improved upon and therefore could have contributed to some problems encountered in the  $\beta$ -globin, S-gene and polymerase semi-nested PCR reactions such as amplification of multiple fragments, target length, sensitivity and yield of amplified DNA. The new and improved *Taq* polymerase (Faststart *Taq* DNA polymerase, dNTPack, Roche Applied Science, Germany) is now recommended for use in such work by the company as it has been optimised and improved to address most of the problems that were encountered. However this product was released when the project was closing and therefore could not be used. The new *Taq* polymerase will however be used in the on going work that is being done on stored samples.

### 6.6 HBV GENOTYPING

The HBV genotype results obtained from the NCBI genotyping tool and phylogenetic analysis were compared. The genotype results from the NCBI genotyping tool concurred 100% with those obtained from the phylogenetic analysis (refer to Chapter 5, Table 5.8). The added advantage of the phylogenetic analysis was that it confirmed the HBV genotypes obtained from the NCBI genotyping tool and further identified the subgenotype of the HBV strain. The combination of these two methods may be used as a reliable way of HBV genotyping and strain identification.

Using the web-based genotyping method, 37 S-gene nucleotides were genotyped. It was determined that a large percentage of the patients (73%) were genotype D compared to

## CHAPTER 6: DISCUSSION

only 27% genotype A with one patient being genotype B. Further analysis was used to confirm the genotypes and also determine the subgenotype of the patients.

29 of the 37 S-gene nucleotide sequences obtained from the patients were subgenotyped using phylogenetic analysis. The remaining 8 nucleotide sequences obtained were too short for successful analysis. If these 8 sequences had been used, large amounts of significant information would have been lost resulting in incorrect clustering of the S-gene nucleotide sequences and giving inaccurate genotyping data. 16 of the S-gene nucleotide sequences clustered with subgenotype D3, 3 with subgenotype D4, 9 with subgenotype A1 and one with subgenotype B2 (See Figure 6.1). These S-gene nucleotide sequences clustered with a certain HBV genotype due to close similarity in nucleotide sequences.

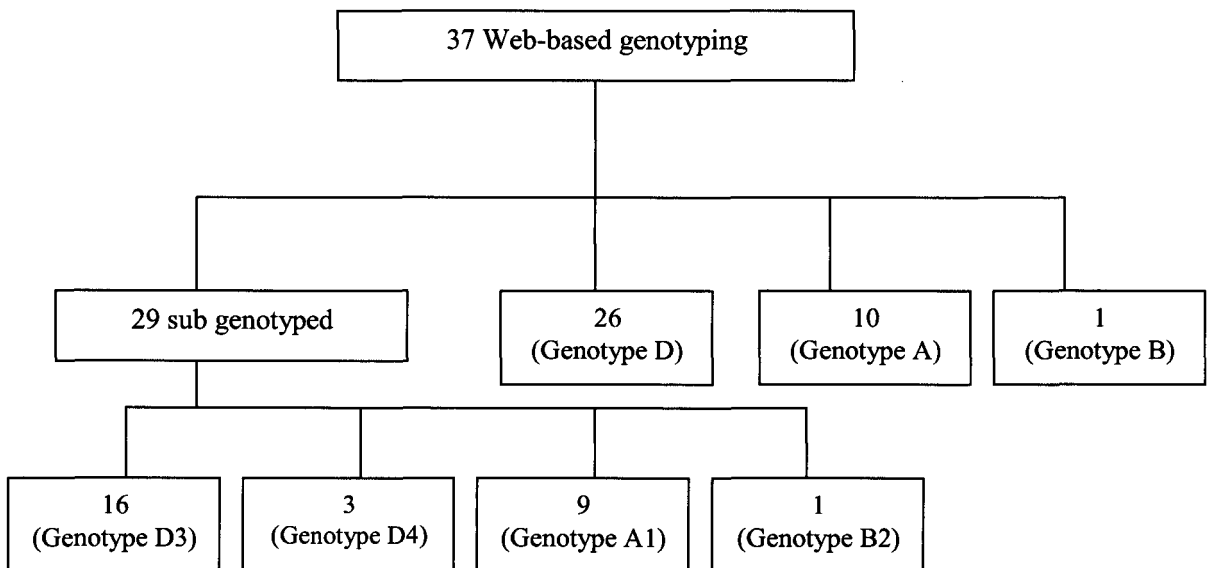


Figure 6.1: Summary of S-gene nucleotide samples used in the phylogenetic analysis

According to this study genotype D and A1 are both present in the Cape region, however genotype D is the most predominant genotype in the region and amongst the Mixed race. The fact that genotype D was most frequent in the Mixed race group did not reach statistical significance but as mentioned before a trend is observed and with further

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investigations and an increase in numbers a statistically significant difference may be established.

The presence of genotype D and A1 in South Africa is supported by studies conducted by various research groups. However these studies which were conducted in Gauteng and Kwa-Zulu Natal showed that genotype A1 is the predominant genotype in South Africa (Owiredo et al., 2001, Kimbi et al., 2004, Kramvis et al., 2002, Bowyer et al., 1997). This study showed that genotype D was predominant in the Cape region. Our results indicate that there is regional variation in genotype distribution in South Africa. Thus further investigations are needed to determine genotype distributions in other regions of South Africa.

Studies have shown that genotype D can be divided into five subgenotypes D1-D5 (Schaefer, 2006). The presence of subgenotype D3 and D4 in the cohort was very interesting. The presence of subgenotype D3 has been documented in East India and South Africa (Banarjee et al., 2006, Kimbi et al., 2004). There is very little data on subgenotype D3 in South Africa and so far only one research group has documented its presence in Black African labourers working in the Gauteng region of South Africa (Kimbi et al., 2004). In this study most of the genotype D samples were subgenotyped into subgenotype D3. This could indicate that this subgenotype is the most prevalent in the Western Cape region as it was equally distributed across all the races. More investigations are needed to define this subgenotype and its prevalence in South Africa.

Subgenotype D4 was first documented by Okamoto and colleagues in 1988. It was isolated from a healthy blood donor in Papua New Guinea. Subsequently, in 2001, Sugauchi and colleagues found three strains from the Australian aboriginal population that clustered with the strain reported previously by Okamoto in 1988 (accession number AB033559). Three samples from this study clustered with this same strain and indicating that the patients had subgenotype D4. All the samples were from the Mixed race population. The presence of subgenotype D4 in this cohort needs to be investigated further as migration may play an important part.

One patient was found to have HBV genotype B2 which is found in Asian countries such as China, Taiwan and Indonesia (Hasegawa et al., 2004). The patient's records revealed

## CHAPTER 6: DISCUSSION

that he was an immigrant from Taiwan who had been residing in South Africa for a number of years. This supported the hypothesis that he could have been infected with HBV while still living in Taiwan. However no medical records prior to his arrival in South Africa were available to confirm this.

The presence of genotype B2 and D4 in the cohort highlights the possible influence migration could have on the genotype prevalence in South Africa. Migration due to economic reasons may result in the introduction of genotypes B, C and D from Asia and the Mediterranean while refugees from West African countries could introduce genotype E in the region. Historically, the Cape Asian and Mixed race populations are descendents of migrants from the Indian and Asia Pacific region and this may explain the presence of subgenotype D4. Rural-urban migration within South Africa could affect the prevalence of genotype A and D in different regions of South Africa.

Two families were genotyped and it was determined that both members of the same family had the same HBV genotype but different subgenotypes. This was a very interesting observation as it suggests that the siblings may have acquired it perinatally or horizontally. However it is more likely that acquisition was horizontal as this is the common mode of HBV transmission locally. There is data from Africa that shows that horizontal transmission can occur within the family unit (intrafamilial) and this occurs at a higher frequency than between families in the same compound (intracompound) (Dumpis et al., 2001). Further investigations using the family cohort will help to determine the rate of intrafamilial horizontal transmission in South Africa.

HBV genotype did not correlate with ALT levels and the HBeAg and HBsAg status of the patients.

### **6.7 DETECTION OF YMDD MUTANTS IN THE PATIENT COHORT**

The inner and outer polymerase gene amplicons were used for direct sequencing. As in the case with the S-gene, some samples were successfully amplified only in the second round of the semi-nested PCR. This could have been due to the same reasons as discussed in section 6.5.

## CHAPTER 6: DISCUSSION

The polymerase gene was sequenced in 29 samples and only 1 sample had a YMDD mutation, YVDD, while the remaining 28 had no mutations present. According to the medical records, the patient with the YVDD mutation was on lamivudine therapy while the rest had not been treated nor previously exposed to lamivudine. However the presence of YMDD motif mutants in some lamivudine naïve HBV carriers have been observed in various studies (Zhang et al., 2003, Kobayashi et al., 2001). All these patients were HIV negative and not on HAART. The lack of YMDD mutation in these 28 patients might indicate that the mutation is not as yet a major clinical problem in locally infected patients being treated with lamivudine and in lamivudine naïve patients. However to confirm this, a larger cohort of patients is needed.

In addition, the patient with the YVDD mutation was genotype A and this concurs with published data that shows that the rate of resistance to lamivudine is higher in genotype A than D (Palumbo, 2007). It is therefore clinically important to investigate the different responses genotypes have to lamivudine as this also impacts on the therapeutic management of the patients.

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## **CHAPTER 7**

### **CONCLUSIONS**

This study forms part of a long-term project at the Groote Schuur Liver Clinic. Patient recruitment for the purposes of this MSc. thesis stopped after 2 years and these patients were studied over this two year period. This is the period over which the researcher was able to work at the MRC-UCT Liver Research Centre.

The patient cohort was well recruited. It represented all races seen in South Africa, both genders and all ages. It is representative of the population statistics of the Western Cape of South Africa.

The data shows no HBV genotype association with race, ALT levels and the ability to clear HBeAg and HBsAg. The current numbers are still small for a firm conclusion with respect to this result. However, it is interesting to observe that race seems to have an influence on the age at HBV diagnosis, ALB levels and ability to clear HBeAg following IFN-a treatment. This raises a possible association between genetics, disease severity and ability to respond to biological therapies such as IFN-a treatment. Further work that will investigate the genes involved in inflammation in the different ethnic groups is underway to answer these questions. The findings on the influence of HBV DNA and ALT levels on IFN-a response are established in the literature.

At this stage of follow up, it is too early to comment on racial correlations fully. This is being addressed in an ongoing parallel study. Any changes in the HBeAg and HBsAg status of these patients and the time it takes to clear HBeAg and HBsAg naturally and with IFN-a are being investigated. In addition the development of cirrhosis and HCC in these patients and associated mortality are being documented.

Two methods were used in combination to determine the patient's genotype. The predominant HBV genotype in this patient cohort was genotype D at 73% compared to

genotype A at 27%. In addition two subgenotypes, D3 and D4, were identified in the cohort. This is an interesting finding as to date, the few studies done in South Africa show that genotype A is predominant while there are very few studies on genotype D subgenotypes in sub-Saharan Africa. Genotype D was found to be most common in the Mixed race compared to the other races. In addition, the unexpected presence of subgenotype B2 and D4 indicate the possible influence of migration on genotype prevalence within a region. An enlargement of the cohort and studies to be done at satellite clinics in rural and urban areas will help to determine the true genotype that is prevalent in the Western Cape. Such studies would help to determine the influence of migration on genotype incidence in the Western Cape.

No YMDD mutations were found in the 28 lamivudine naïve patients in the clinic cohort. However one patient who was on lamivudine therapy had the YVDD mutation present. On going surveillance is needed as HBV-HIV co-infection cases are increasing and more patients will be treated with lamivudine. This would contribute to document and understand the prevalence of these mutations. In addition, it is planned that the on going recruited patients would be routinely screened for these mutations so as to manage the emergence of these mutations.

At present the number of patients genotyped and screened for the YMDD mutations is still low. Most patients are HBeAg negative and so have low HBV DNA levels. This contributed to a low success rate of  $\beta$ -globin, S-gene and polymerase gene amplification. The samples that were not successful have been stored for future studies using the new and improved Faststart *Taq* DNA polymerase, dNTPack (Roche Applied Science, Germany).

The data from the latest South African census in 2001 shows that the Western Cape is a very unequal society with respect to socio-economic variables. The influences of these socio-economic factors on the age of HBV infection and diagnosis need to be investigated. This will result in better planning of health programmes, access to treatment by all ethnic groups and a better understanding of the socio-genetic interactions in HBV infection.

## **APPENDIX A**



UNIVERSITY OF CAPE TOWN

**Health Sciences Faculty  
Research Ethics Committee**

**Room E52-24 Groote Schuur Hospital Old Main Building  
Observatory 7925**

**Telephone [021] 406 6338 • Facsimile [021] 406 6411  
e-mail: preaward@curie.uct.ac.za**

09 May 2006

**REC REF: 417/2005**

Dr HN Hairwadzi  
Senior Specialist  
MRC/UCT Liver Research Centre  
GSH

Dear Dr Hairwadzi

**PROJECT TITLE: THE INFLUENCE OF HBV GENOTYPE AND ETHNICITY ON TREATMENT  
OUTCOME DURING FOLLOW-UP OF PATIENTS WITH CHRONIC HBV INFECTION IN THE  
WESTERN CAPE**

Thank you for your letter to the Research Ethics Committee.

It is a pleasure to inform you that the Ethics Committee has approved the patient information and informed consent form for the above study.

**Please quote the REC. REF in all your correspondence.**

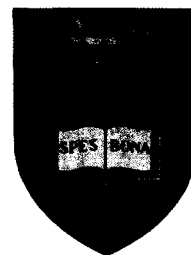
Yours sincerely

**DR. M. BLOCKMAN**  
**CHAIRPERSON, HSF HUMAN ETHICS**

## **APPENDIX B**



MRC - UCT Liver Research Centre  
Department of Medicine  
Division of Hepatology  
K47 Old Main Building . Groote Schuur Hospital .  
Observatory 7925 . Cape Town . South Africa  
Telephone: (021) 406 6394 Fax: (021) 447 9670



## CONSENT FORM

University of Cape Town Health Sciences Faculty Research Ethics Committee  
Reference Number: **417/2005**

**Patient hospital number:**

**Title of project:**

The influence of HBV genotype and ethnicity on the clinical outcomes of patients with chronic HBV infection who are managed at the MRC-UCT Liver Clinic, Groote Schuur Hospital.

**Name of researchers:** Dr HN Hairwadzi, Ms Michelle Nderu

I confirm that I have read and understand the information sheet dated May 2006, version 1 for the above study and have had the opportunity to ask questions

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected

I understand that sections of any of my medical notes may be looked at by responsible individuals from the Liver Research Centre who are participants in this study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

- Name of Patient.....

Signature..... Date.....

- Name of person taking consent .....

Signature..... Date.....

- Name of Researcher.....

Signature..... Date.....

**NB:** Three copies should be signed, for (1) patient, (2) researcher, (3) hospital notes

**"Our mission is to be an outstanding teaching and research university,  
educating for life and addressing the challenges facing our society."**

## Patient Information Sheet For The Hepatitis B Virus Genotypes Study

### Study title:

The influence of the different strains of hepatitis B virus on the progression of liver disease due to chronic hepatitis B virus infection

### What is hepatitis B virus and what does the infection result in?

The hepatitis B virus causes infection and chronic injury in the liver. It is a very common infection in the developing world. It is transmitted through direct contact and inoculation of infected blood during events such as blood transfusions, sexual contact, childbirth, tattooing and skin cuts. Infection can cause ongoing damage to the liver that results in the development of scarring, leading to cirrhosis. Over time, this can result in liver failure or cancer. There are several strains (genotypes) of the virus. They appear to have different abilities to cause long-term liver related complications.

### What is the purpose of the study?

Many factors are known to influence the progression of viral hepatitis B infection. These include the regular use of alcohol, iron overload states and the presence of other liver diseases. This study aims to investigate whether the different HBV genotypes influence the progression of disease and its clinical outcome in individual patients. This is a long-term study that is expected run over several years.

### Why have I been chosen?

You have been chosen to be part of the study because of the presence of hepatitis B virus in your blood. As a regular visitor to the Liver Clinic, you are in a position to be observed over many years as the disease progresses or is treated. This gives us the opportunity to correlate the behaviour and type of hepatitis B virus that you are carrying with the final outcome of your disease.

### Do I have to take part?

Participation in this study is voluntary and you are free to withdraw at any time without giving us any reasons. Your withdrawal will not affect the standard of care that you will receive from the Liver Clinic. As this is a long-term study, you can enter the study at any given time in the future as long as you continue to be a patient of the Liver Clinic. If you decide to take part, you will be asked to sign a consent form.

### What will happen to me if I take part?

Taking part in this study will not affect your usual Liver Clinic visits. Your usual routine clinic visits will be used to collect the study data. You will be required to give a blood sample that will be used for the extraction of the hepatitis B virus that you carry. Your usual treatment **will not** be changed in anyway.

### What do I have to do?

You will be required to give a blood sample during one of your clinic visits. No lifestyle changes will be required of you and your medication will not be changed.

### What are the side effects of taking part?

No side effects are expected, as you **will not** be receiving any new or experimental medications.

### What are the possible disadvantages and risks of taking part?

There are no risks or disadvantages involved as your usual treatment **will not** be changed in any way.

## APPENDIX C

### **What are the possible benefits of taking part?**

Other than getting to know the HBV genotype that you carry, no possible immediate benefits will be accrued to the patients involved in this study. However the doctors will gain information that could be used in the future to improve the management of patients with chronic HBV infection. Your care will not differ in any way from that given to patients who are not participating in the study.

### **What if new information becomes available?**

You will be informed of any new information regarding HBV genotypes and treatment outcome that may become available during the course of this study and has implications on how you should be treated.

### **Will my taking part in this study be kept confidential?**

Your participation in this study is confidential. Access to your medical records is given only to the researchers involved in the study. The access is restricted to the collection of information relevant to the study and this will be kept strictly confidential. There will be no use of your name in the handling of the data.

### **What will happen to the results of the research study?**

The results obtained will be used in the thesis of post-graduate students. The information will also be communicated to other doctors at seminars and conferences for educational purposes and published in medical journals. Your personal details will not be identified in any such reports or publications.

### **Who is organising and funding the research?**

The Medical Research Council of South Africa, the Polio Research Foundation of South Africa and the University of Cape Town fund this research. The researchers involved are not being paid any incentives to include patients in the study.

### **Who has reviewed the study?**

The University of Cape Town Health Sciences Faculty Research Ethics Committee has reviewed and approved this study.

### **Contact for further information**

Further information regarding the study can be obtained from Dr HN Hairwadzi at the Liver Clinic on the following numbers:

1. 021 406 6394
2. 021 406 6190

Should you agree to take part in this study, you will be given a copy of this information sheet and a consent form that you will be required to sign.

**Thank you for agreeing to participate in this study.**

Prepared by: Dr HN Hairwadzi and Ms Michelle Nderu

Adapted from the Bulletin of Medical Ethics, guidelines for researchers 1999

Reference: [http://ourworld.compuserve.com/homepages/Bulletin\\_of\\_Medical\\_Ethics/offmay99.htm](http://ourworld.compuserve.com/homepages/Bulletin_of_Medical_Ethics/offmay99.htm)

## APPENDIX D: PATIENT CLINICAL DESCRIPTION

| PATIENT INDEX | SEX    | RACE          | AGE  | DNA (IU/ml) | log DNA | ALT | ALB | INR  |
|---------------|--------|---------------|------|-------------|---------|-----|-----|------|
| A1a           | Female | Black African | 23   | ND          |         | 20  | 38  | 1    |
| A2a           | Female | Mixed race    | 21   | 145583286   | 8.2     | 27  | 41  | 1.22 |
| A3a           | Female | Black African | 26   | ND          |         | 20  | 43  | 1.2  |
| A4a           | Male   | Mixed race    | 34   | ND          |         | 76  | 44  | 1.28 |
| A5a           | Male   | Mixed race    | 36   | ND          |         | 18  | 39  | 0.8  |
| A6a           | Female | Asian         | 36.3 | 294         | 2.5     | 153 | 31  | 1.3  |
| A7a           | Male   | Black African | 54   | 10996571    | 7       | 40  | 40  | 1.83 |
| A8a           | Male   | Mixed race    | 24   | 26          | 1.4     | 61  | 43  | 1.23 |
| A9a           | Male   | Asian         | 34   | 17141714    | 7.2     | 97  | 38  | 1.11 |
| A1b           | Male   | Asian         | 29   | 0           | 0       | 86  | 45  | 1.11 |
| A2b           | Female | Mixed race    | 41   | 133131286   | 8.1     | 18  | 36  | 1.01 |
| A3b           | Female | Black African | 56   |             |         | 19  | 40  | 0.99 |
| A4b           | Male   | Mixed race    | 26   | 205781429   | 8.3     | 54  | 46  | 1.35 |
| A5b           | Female | Mixed race    | 36   |             |         | 21  | 39  | 1.03 |
| A6b           | Male   | Mixed race    | 38   |             |         | 30  | 46  | 1.02 |
| A7b           | Female | Mixed race    | 42   |             |         | 13  | 43  | 0.91 |
| A8b           | Female | Caucasian     | 32   | 109         | 2       | 22  | 46  | 0.96 |
| A9b           | Male   | Caucasian     | 48   |             |         | 37  | 37  | 1    |
| B1a           | Female | Asian         | 46   | ND          |         | 35  | 40  | 0.98 |
| B2a           | Female | Mixed race    | 33   | ND          |         | 12  | 36  | 1.01 |
| B3a           | Male   | Black African | 48   | ND          |         | 24  | 40  | 1.19 |
| B4a           | Female | Mixed race    | 19   | ND          |         | 499 | 42  | 1.06 |
| B5a           | Male   | Mixed race    | 23   | ND          |         | 41  | 49  | 1.03 |
| B6a           | Male   | Asian         | 43   | 6509000     | 6.8     | 38  | 43  | 0.95 |
| B7a           | Male   | Black African | 29   | 161714      | 5.2     | 177 | 24  | 1.93 |
| B8a           | Male   | Mixed race    | 20   | ND          |         | 23  | 46  | 1.16 |
| B9a           | Female | Caucasian     | 24   | ND          |         | 33  | 48  | 0.98 |
| B1b           | Male   | Mixed Race    | 22   | 0           | 0       | 51  | 43  | 1.02 |
| B2b           | Male   | Mixed race    | 69   |             |         | 37  | 43  | 0.95 |
| B3b           | Female | Mixed race    | 74   |             |         | 190 | 43  | 1.2  |
| B4b           | Female | Black African | 43   | 1148        | 3.1     | 16  | 45  | 1.11 |
| B5b           | Male   | Mixed Race    | 23   |             |         | 41  | 49  | 1.03 |
| B6b           | Male   | Caucasian     | 63   |             |         | 13  | 43  | 0.91 |
| B7b           | Female | Caucasian     | 69   |             |         | 67  | 44  | 0.89 |
| B8b           | Male   | Mixed Race    | 26   | 118         | 2.1     | 87  | 46  | 1.06 |
| B9b           | Male   | Mixed Race    | 22   |             |         | 37  | 44  | 0.98 |
| C1a           | Male   | Mixed race    | 23   | 283000000   | 8.5     | 58  | 43  | 1.28 |
| C2a           | female | Black African | 38   | ND          |         | 21  | 47  | 0.8  |
| C3a           | female | Mixed race    | 32   | 8.6         | 1.9     | 24  | 48  | 0.98 |
| C4a           | Male   | Black African | 17.3 | ND          |         | 33  | 41  | 1.45 |
| C5a           | Male   | Caucasian     | 19   | 76976000    | 7.9     | 49  | 45  | 0.99 |

## APPENDIX D: PATIENT CLINICAL DESCRIPTION

|     |        |               |      |            |      |      |    |      |
|-----|--------|---------------|------|------------|------|------|----|------|
| C6a | Male   | Black African | 23   | 15079857   | 7.2  | 99   | 36 | 1    |
| C7a | Female | Black African | 17   | ND         |      | 23   | 42 | 1.09 |
| C8a | Male   | Caucasian     | 48   | 152254000  | 8.2  | 92   | 40 | 1.13 |
| C9a | Male   | Black African | 31   | 145        | 2.2  | 19   | 37 | 1.31 |
| C1b | Male   | Black African | 29   | 145        | 2.2  | 19   | 37 | 1.31 |
| C2b | Male   | Mixed Race    | 36   | 73         | 1.9  | 37   | 45 | 1.08 |
| C3b | Female | Mixed Race    | 27   |            |      | 28   | 46 | 1.05 |
| C4b | Male   | Mixed Race    | 46   |            |      | 21   | 49 | 1.05 |
| C5b | Female | Mixed Race    | 60   |            |      | 12   | 45 | 1.07 |
| C6b | Male   | Mixed race    | 26   | 44010000   | 7.6  | 85   | 43 | 1.03 |
| C7b | Female | Mixed Race    | 41   |            |      | 22   | 40 | 1.16 |
| C8b | Male   | Caucasian     | 55   |            |      | 2469 | 39 | 0.98 |
| C9b | Male   | Mixed Race    | 35   | 960        | 3    | 22   | 39 | 1.01 |
| D1a | Female | Mixed race    | 26   | 157994857  | 8.2  | 271  | 49 | 1.08 |
| D2a | Male   | Caucasian     | 41   | ND         |      | 23   | 48 | 1.03 |
| D3a | Male   | Black African | 36.5 | ND         |      | 27   | 35 | 1.18 |
| D4a | Male   | Asian         | 46   | 290700000  | 5.5  | 21   | 43 | 1.4  |
| D5a | Male   | Mixed race    | 36.3 | 1046200    | 6    | 252  | 42 | 1.05 |
| D6a | Male   | Caucasian     | 35   | ND         |      | 45   | 43 | 1.05 |
| D7a | Female | Black African | 43   | 79         | 1.9  | 16   | 45 | 1.1  |
| D8a | Male   | Caucasian     | 34   | 29820      | 4.5  | 74   | 45 | 0.94 |
| D9a | Male   | Black African | 21   | 357        | 2.6  | 55   | 31 | 1.49 |
| D1b | Female | Mixed race    | 44   | 3274714    | 6.5  | 93   | 37 | 1    |
| D2b | Male   | Caucasian     | 49   |            |      | 11   | 45 | 0.99 |
| D3b | Male   | Mixed Race    | 34   | 0          | 0    | 68   | 41 | 1.09 |
| D4b | Male   | Mixed Race    | 18   | 714400000  | 8.85 | 235  | 45 | 1.17 |
| D5b | Female | Mixed Race    | 53   | 78         |      | 18   | 39 | 1.07 |
| D6b | Female | Black African | 44   | 1.9        |      | 18   | 42 | 1.35 |
| D7b | Female | Mixed Race    | 17   | 100 000000 | 8.3  | 16   | 48 | 1.01 |
| D8b | Female | Mixed Race    | 40   | 1031       | 3.1  | 21   | 40 | 0.85 |
| D9b | Female | Mixed race    | 29   | 337        | 2.5  | 31   | 43 | 1.01 |
| E1a | Female | Mixed race    | 40   | ND         |      | 22   | 43 | 0.91 |
| E2a | Male   | Mixed race    | 33   | 2427       | 3.4  | 54   | 39 | 0.98 |
| E3a | Male   | Caucasian     | 22   | 157823857  | 8.2  | 18   | 49 | 0.88 |
| E4a | Male   | Caucasian     | 66   | ND         |      | 26   | 44 | 1.06 |
| E1b | Male   | Black African | 34   | 30020      | 4.5  | 426  | 34 | 1.28 |
| E2b | Male   | Black African | 26   | 357        | 2.6  | 43   | 27 | 1.36 |
| E4b | Male   | Asian         | 60   | 423900     | 5.6  | 24   | 38 | 1.2  |
| E5a | Female | Black African | 2    | ND         |      | 24   | 4  | 1.19 |
| E6a | Male   | Caucasian     | 59   | ND         |      | 24   | 33 | 1.01 |
| E7a | Female | Black African | 24   | ND         |      | 769  | 42 | 0.98 |
| E8a | Male   | Black African | 31   | ND         |      | 38   | 30 | 1.33 |
| E9  | Female | Mixed race    | 32   | ND         |      | 22   | 40 | 1.01 |

## APPENDIX D: PATIENT CLINICAL DESCRIPTION

|      |        |               |       |            |     |     |    |      |
|------|--------|---------------|-------|------------|-----|-----|----|------|
| F1a  | Female | Black African | 38    | ND         |     | 10  | 39 | 0.96 |
| F2a  | Male   | Mixed race    | 26    | 100000000  | 8.3 | 60  | 46 | 1.17 |
| F3a  | Male   | Asian         | 19    | 156500     | 5.2 | 337 | 42 | 1    |
| F4a  | Male   | Black African | 19    | ND         |     | 26  | 47 | 1.04 |
| F5a  | Female | Mixed race    | 31    | ND         |     | 81  | 44 | 0.98 |
| F6a  | Male   | Mixed race    | 51    | 574        | 2.7 | 15  | 46 | 0.88 |
| F7a  | Male   | Caucasian     | 19    | ND         |     | 49  | 45 | 0.99 |
| F8a  | Male   | Mixed race    | 19    | ND         |     | 31  | 44 | 0.98 |
| F9a  | Male   | Black African | 27    | ND         |     | 17  | 43 | 0.99 |
| G1a  | Female | Black African | 39    | ND         |     | 21  | 42 | 0.93 |
| G2a  | Male   | Black African | 25    | ND         |     | 17  | 43 | 1.08 |
| G3a  | Male   | Asian         | 34    | ND         |     | 97  | 38 | 1.11 |
| G4a  | Male   | Mixed race    | 20    | ND         |     | 45  | 45 | 1.04 |
| G5a  | Female | Black African | 35    | ND         |     | 101 | 41 | 1    |
| G6a  | Female | Black African | 19    | ND         |     | 12  | 42 | 1.11 |
| G7a  | Male   | Mixed race    | 48    | ND         |     | 47  | 41 | 0.97 |
| G8a  | Male   | Black African | 30    | ND         |     | 26  | 44 | 1.17 |
| G9a  | Male   | Black African | 33    | 24700      | 4.4 | 15  | 35 | 1.37 |
| H1a  | Female | Black African | 56    | ND         |     | 18  | 40 | 1.03 |
| H2a  | Female | Mixed race    | 41    | 1940       | 3.3 | 23  | 48 | 0.97 |
| H3a  | Female | Mixed race    | 31.3  | 150636857  | 8.2 | 114 | 34 | 0.98 |
| H4a  | Female | Black African | 29    | ND         |     | 26  | 45 | 1.16 |
| H5a  | Male   | Caucasian     | 19    | 566000     | 5.7 | 132 | 42 | 0.99 |
| H6a  | Male   | Asian         | 22    | ND         |     | 25  | 54 |      |
| H7a  | Female | Asian         | 26    | 22680429   | 7.4 | 109 | 47 | 1.4  |
| H8a  | Female | Caucasian     | 33    | 485143     | 5.7 | 27  | 48 | 1.03 |
| fA1a | Female | Asian         | 19    | 491600     | 5.7 | 20  | 41 | 0.87 |
| fA2a | Male   | Asian         | 21    |            |     | 40  | 45 | 0.93 |
| fB1a | Male   | Asian         | 25    | 100 000000 | 8.5 | 44  | 46 | 1.18 |
| fB2a | Male   | Asian         | 23    | 0          | 0   | 39  | 48 | 1    |
| fC1a | Female | Mixed Race    | 11    |            |     | 12  | 44 | 0.98 |
| fC2a | female | Mixed race    | 44.25 |            |     | 32  | 42 | 0.92 |
| fC3a | Female | Mixed Race    | 18    |            |     | 18  | 45 | 0.89 |
| fD1a | Male   | Mixed Race    | 50    | 727714     | 5.8 | 245 | 40 | 1.1  |
| fD2a | Female | Mixed Race    | 22.3  | 40429      | 4.6 | 38  | 42 | 1.03 |
| fE1a | Female | Mixed Race    | 43.5  |            |     | 16  | 36 | 0.96 |
| fE2a | Female | Mixed Race    | 11.5  |            |     | 33  | 38 |      |
| fE3a | Female | Mixed race    | 16.26 |            |     | 13  | 41 |      |
| fF1a | Male   | Asian         | 60    |            |     | 190 | 36 | 1.1  |
| fF2a | Male   | Asian         | 55    | 3557714    | 6.6 | 33  | 38 | 1.2  |
| fF3a | Male   | Asian         | 31    | 40429      | 4.6 | 47  | 42 | 1    |
| fG1a | Female | Mixed race    | 21.75 | 100 000000 | 8.3 | 25  | 41 | 1    |
| fG2a | Female | Mixed race    | 16    | 100 000000 | 8.3 | 27  | 43 | 1.03 |

## APPENDIX D: PATIENT CLINICAL DESCRIPTION

|      |        |            |      |           |     |      |    |      |
|------|--------|------------|------|-----------|-----|------|----|------|
| fH1a | Female | Asian      | 63   | 28650     | 4.5 | 16   | 38 | 1    |
| fH2a | Male   | Asian      | 18   | 240       | 2.4 | 41   | 48 | 1.04 |
| fI1a | Female | Mixed Race | 48.5 | 40429     | 4.6 | 11   | 45 | 1.02 |
| fI2a | Male   | Mixed Race | 22   |           |     | 27   | 46 | 1.07 |
| fJ1a | Female | Mixed race | 37.3 |           |     | 46   | 47 | 0.98 |
| fJ2a | Male   | Mixed Race | 39   | 126258429 | 8.1 | 80   | 41 | 0.84 |
| fA1b | Male   | Mixed Race | 23   |           |     | 43   | 49 | 1.1  |
| fA2b | Female | Mixed Race | 54   |           |     | 125  | 41 | 0.88 |
| fB1b | Female | Mixed race | 18   |           |     | 13   | 43 | 1.05 |
| fB2b | Female | Mixed Race | 36   | 572       | 2.8 | 33   | 47 | 1.1  |
| fC1b | Male   | Mixed Race | 49   |           |     | 21   | 40 | 0.96 |
| fC2b | Female | Mixed Race | 25   | 0         |     | 21   | 43 | 1.01 |
| fD1b | Female | Mixed Race | 39   |           |     | 22   | 43 | 1    |
| fD2b | Female | Mixed race | 31   |           |     | 29   | 43 | 1.04 |
| fE1b | Female | Mixed Race | 45   |           |     | 17   | 44 |      |
| fE2b | Female | Mixed Race | 32   | 54840     | 4.7 | 17   | 44 | 0.82 |
| fF1b | Male   | Asian      | 35   |           |     | 45   | 43 | 1.05 |
| fF2b | Female | Asian      | 28   | 14109572  | 7.2 | 73   | 44 | 1    |
| fH1b | Female | Mixed Race | 17   | 70        | 1.8 | 13   | 42 | 1.08 |
| fH2b | Female | Mixed Race | 35   |           |     | 19   | 42 | 0.9  |
| fH3b | Female | Mixed Race | 17   | 70        | 1.8 | 13   | 42 | 1.08 |
| fI1b | Female | Mixed Race | 27   | 15        | 1.2 | 25   | 44 | 1.01 |
| fI2b | Female | Mixed Race | 20   |           |     | 18   | 45 | 1.03 |
| fJ1b | Female | Mixed Race | 51   |           |     | 15   | 46 |      |
| fJ2b | Female | Mixed Race | 26   |           |     | 61   | 44 | 0.98 |
| fJ3b | Male   | Mixed Race | 18   | 169395714 | 8.3 | 67   | 44 | 1.05 |
| fJ4b | Male   | Mixed Race | 34   |           |     | 54   | 48 |      |
| fJ5b | Female | Mixed Race | 25   |           |     | 21   | 45 | 1.04 |
| fK1b | Female | Mixed Race | 44   |           |     | 22   | 38 | 0.96 |
| fK2b | Male   | Mixed Race | 33   |           |     |      | 40 | 0.87 |
| fK3b | Female | Mixed Race | 20   | 0         | 0   | 1347 | 30 | 10   |
| fL1b | Female | Mixed Race | 24   |           |     | 23   | 38 | 1.01 |
| fL2b | Female | Mixed Race | 18   |           |     | 33   | 39 | 1.19 |
| fL3b | Female | Mixed Race | 24   |           |     | 28   | 45 | 0.88 |
| fM1b | male   | Mixed Race | 41   |           |     | 36   | 45 | 0.92 |
| fM2b | Female | Mixed Race | 27   | 0         |     | 28   | 46 | 1.05 |
| fN1b | Male   | Mixed Race | 18   |           |     | 32   | 47 | 1.12 |
| fN2b | Male   | Mixed Race | 49   |           |     | 14   | 44 | 1.18 |
| fO1b | Female | Mixed Race | 27   | 211       | 2.3 | 16   | 49 | 1    |
| fO2b | Female | Mixed Race | 17   | 124358286 | 8.1 | 62   | 36 | 1.09 |

## **APPENDIX E**

### **GELS AND BUFFERS**

#### **1. 10mM Tris-HCl, pH 8.0**

- Make a standard solution of 1M Tris-HCl pH 8.0 by dissolving 12.114g Tris-HCl into 70ml distilled water.
- Adjust pH to 8.0 using NaOH and make up to final volume of 100ml with distilled water.
- Autoclave at 121°C for 20 minutes at 15 pounds of pressure.
- To dilute the standard solution to 10mM, add 0.5ml from the standard solution to 49.5ml distilled water.

#### **2. 6x Loading buffer**

- Dissolve 12.5mg bromophenol blue, 2g sucrose and add 0.2μl of 0.5M pH 8.0 EDTA to 4.8ml distilled water.
- Aliquot and store at 4°C.

#### **3. 1x TBE Electrophoresis buffer**

- To make 5x stock solution dissolve 54g Tris base, 27.5g Boric acid and add 20ml of 0.5M pH 8.0 EDTA to 980ml distilled water.
- Autoclave at 121°C for 20 minutes at 15 pounds pressure.
- Dilute stock solution to 1x working solution by adding 200ml stock solution to 800ml distilled water.

#### **4. 1.7% Multipurpose Agarose gel**

- Use a flask that is 2 to 4 times the volume of the solution being prepared.
- Add 1.7g of dry agarose to 100ml 1x TBE electrophoresis buffer.
- Heat the slurry in a microwave oven until it starts to boil.
- Allow solution to boil for 1 minute or until all the particles have dissolved.

- Remove the flask gently from the microwave oven and gently mix the solution by swirling the flask.
- Cool the solution to approximately 60°C in a water bath before pouring and add 20ul of ethidium bromide solution.

**5. 3% Nusieve Gel**

- Use a flask that is 2 to 4 times the volume of the solution being prepared.
- Add 3g dry nusieve agarose to 100ml 1x TBE electrophoresis buffer.
- Heat the slurry in a microwave oven until it starts to boil.
- Allow solution to boil for 1 minute or until all the particles have dissolved.
- Remove the flask gently from the microwave oven and gently mix the solution by swirling the flask.
- Cool the solution to approximately 60°C in a water bath before pouring and add 20ul ethidium bromide solution.

## APPENDIX F

### SOLUTIONS AND KIT REAGENTS

1. **0.5M EDTA pH 8.0 (100ml)**

- Dissolve 18.612g EDTA into 50ml distilled water.
- Adjust the pH to 8.0 using 10N NaOH and make up to 100ml with distilled water.

2. **PCR Reagents (Roche Applied Science, Germany).**

- PCR Nucleotide Mix Deoxynucleotide Triphosphates ( 10mM of each dNTP)
- Forward and reverse primers (GH20 and PC04; See Appendix D)
- 10x PCR Buffer with MgCl<sub>2</sub> (100mM Tris-HCl, 500mM KCl, 15mM MgCl<sub>2</sub> )
- *Taq* DNA polymerase (1Unit/μl )

3. **GFX PCR DNA and Gel Band Purification Kit; Reagents and materials provided with kit (Amersham Biosciences, UK):**

- Capture buffer
- Wash buffer
- GFX MicroSpin™ columns
- Collection tubes

4. **High Pure PCR Template preparation Kit; Reagents and materials provided with kit (Roche Applied Science, Germany):**

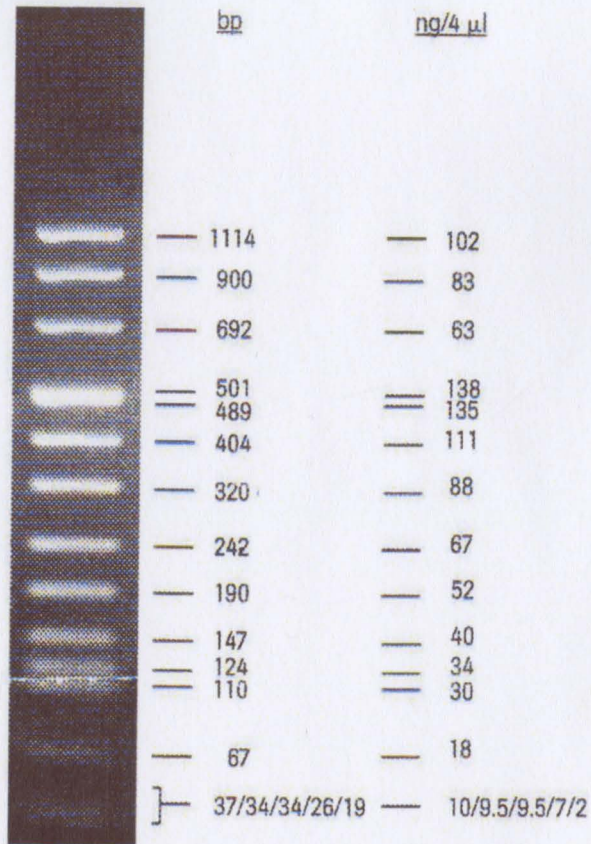
- Proteinase K, recombinant PCR grade
- Binding Buffer [6M guanidine-HCl, 10mM urea, 10mM Tris-HCl, 20% Triton X-100(v/v), pH 4.4 (25°C)]
- Inhibitor Removal Buffer [5M guanidine-HCl, 20mM Tris-HCl, pH 6.6 (25°C) final conc. after 20ml ethanol addition]

- Wash Buffer [20mM NaCl, 2mM Tris-HCl, pH 7.5 (25°C) final conc. after 80ml addition of ethanol]
- Elution Buffer [10mM Tris-HCl, pH 8.5 (25°C)]
- High Pure Filter Tubes
- Collection Tubes

## APPENDIX G

### DNA MOLECULAR WEIGHT MARKER VIII (0.019-1.11 kbp) Roche Applied Science, Germany.

**VIII**



1.8 %  
Agarose  
MP Gel