

https://dspace.org/

The relationship between hepatitis b virus and apoptosis in humans and in a transgenic mouse model

Viana, Raquel Valongo

2012-01-17
http://hdl.handle.net/10539/10992

Downloaded from a DSpace repository. https://dspace.org/

THE RELATIONSHIP BETWEEN HEPATITIS B VIRUS AND APOPTOSIS IN HUMANS AND IN A TRANSGENIC MOUSE MODEL

Raquel Valongo Viana

Thesis submitted in compliance with the requirements for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand

Johannesburg, 2011

DECLARATION

I, Raquel Valongo Viana declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

_____ day of _____2011

DEDICATION

This thesis is dedicated to my parents, Alice and Eduardo Viana, who taught me from a young age to strive for excellence. It was through their example of sacrifice, persistence and hard work that I grew up with the notion that anything in life is attainable as long as you willing to put in the time and work to achieve it. I thank them for their unwavering love, support, and belief in me that I could achieve this monumental goal.

PUBLICATIONS AND PRESENTATIONS

The following articles emanated from techniques that were developed for the present study:

- Kew MC, Welschinger R, **Viana R**. J Gastroenterol Hepatol. 2008 Sep; 23(9): 1426 – 30. Occult hepatitis B virus infection in Southern African blacks with hepatocellular carcinoma.

- Firnhaber C, **Viana R,** Reyneke A, Schultze D, Malope B, Maskew M, Di Bisceglie A, MacPhail P, Sanne I, Kew M. Int J Infect Dis. 2009 July;13(4):488-92. Occult hepatitis B virus infection in patients with isolated core antibody and HIV co-infection in an urban clinic in Johannesburg, South Africa.

- **Viana R,** Wang R, Yu MC, Welschinger R, Chen CY, Kew MC. J Med Virol. 2009 Sept; 81(9):1525-30. Hepatitis B viral loads in Southern African blacks with hepatocellular carcinoma.

Presentations:

- **Viana R**, Meuleman P, Kew MC, Leroux-Roels G, Kramvis A. Functional characterization of subgenotype A1 isolates *in vivo* using a uPA-SCID transgenic mouse model. Joint Flemish/South African International Science Liaison Symposium: New insights in HBV diversity, pathogenesis, diagnosis and treatment. Het Pand, University of Ghent, Gent. December 13th – 14th 2007.

- **Viana, R**; Wang, RW; Yu, MC; Yuen, MF; Lai, CL; Kew, MC; Tanaka, Y; Mizokami, M; Kramvis, A. Apoptotic caspase activation in hepatocellular carcinoma patients and in asymptomatic carriers of the hepatitis B virus from Hong Kong, Japan and South Africa.19th Conference of the Asian Pacific Association for the Study of Liver.COEX Hong Kong 13th – 16th February 2009.

ACKNOWLEDGEMENTS

No study can be truly successful without input, assistance and encouragement from others. I would therefore like to acknowledge and thank the following individuals:

- First and foremost my supervisor, Professor Anna Kramvis, which is the ideal mentor and professional that I can only dream to aspire to be one day. Her ease and willingness to impart her expert knowledge have been fundamental in the completion of this thesis. I thank her for all the time she has invested in me and the patience she has shown me. I hope that I have made her proud and will strive to continue to do so in my future endeavors.

- I would also like to thank my co-supervisor, Professor Michael C Kew for all the advice and knowledge he has imparted to me. It has been an honour and privilege to have worked with the leading expert in hepatology.

- Thank you to all the members of the Hepatitis B Virus Diversity Research Programme (Formerly the Molecular Hepatology Research Unit) for all their support and encouragement. Some of my most memorable moments during this study took place hunched over the lab workbench debating with you all as to what had contributed to a failed experiment. I appreciate all the constructive criticism and encouragement you shared with me.

- I would like to acknowledge and thank Professor Geert Leroux-Roels and Dr Philip Meuleman from Ghent University in Belgium for welcoming me into their group and imparting all the specialized skills required for the use of the uPA-SCID mouse model.

- Thank you to Professor Mimi Yu, from the University of Minnesota, for the critical statistical analysis performed that was essential to the population based study.

- Thank you to Professors Masashi Mizokami and Yasuhito Tanaka, from Nagoya City University in Japan, and to Professor Man-Fung Yuen, from the University of Hong Kong, for the much needed samples required for the population based study.

- Lastly I would like to thank my family and friends for all their encouragement, patience and support during this doctorate.

ABSTRACT

Hepatitis B virus (HBV) has been found to be highly endemic in Africa and south east Asia. In southern Africa, subgenotype A1 and genotype D prevail while in south east Asia genotype B and C predominate. Infection with HBV can lead to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC). It has been shown that viral factors as well as a number of host and environmental factors can influence the course of HBV infection. Development and progression of various liver diseases are associated with either an increase or decrease in hepatocyte apoptosis. Dysregulated apoptosis itself may be a fundamental feature of most acute and chronic human liver diseases.

The purpose of this study was to characterise the subgenotype A1 and genotype D HBV infection, prevailing in South Africa. To control for the influence of host factors on HBV infection as well as to avoid the use of *in vitro* cell lines, such as Huh-7, that have defective apoptotic pathways, the *in vivo* urokinase plasminogen activator severe combined immunodeficient (uPA-SCID) transgenic mouse model was utilised. The HBV infection of the transgenic mice infected with HBV positive sera containing either subgenotype A1 wild-type, subgenotype A1 with the G1862T mutation, subgenotype A2 or genotype D, was compared.

For the first time, we were able to demonstrate the successful infection of the uPA-SCID transgenic mouse model with subgenotype A1 of HBV. The successful establishment of the *in vivo* HBV infection with different genotypes or subgenotypes in the uPA-SCID transgenic mice was demonstrated by the increase of HBV DNA levels, the presence of cccDNA and HBV transcripts as well as the detection of the core and/or surface HBV antigens in the liver tissue of the chimeric mice. Differences between the HBV infections with the various genotype/subgenotypes were observed. Subgenotype A1 with the G1862T mutation showed the earliest detection and therefore highest levels of cccDNA as well as the highest HBV DNA levels when compared to the other strains. The highest HBV DNA levels were recorded for the subgenotype A1 G1862T infected transgenic mouse followed by genotype D, subgenotype A2 and the lowest levels observed in the subgenotype A1 wild-type infected

transgenic mouse. HBsAg was also only detected in the livers of mice infected with subgenotype A1 with the G1862T mutation. HBcAg staining in the chimeric liver was positive when the mice were infected with genotype D, which concurs with previous observations that genotype D is characterised by high HBcAg expression. Subgenotype A1 with the 1862 mutant showed the highest levels of apoptosis as a result of the abnormal precore precursor protein accumulation shown to be associated with this 1862 missense mutation. Thus different genotypes and subgenotypes as well as variations within genotypes can influence HBV infection. Moreover, the results of these experiments in the immunocompromised chimeric mice, grafted with liver cells from a single donor, suggests that even when host and environmental risk factors are controlled for, the subgenotype or genotype can influence the course of infection.

The limitations of the uPA-SCID transgenic mouse model include the lack of an immune system and the short life-span of the animal; therefore a population based study was carried out to investigate the influence of host factors on HBV infection in various disease groups. The study cohort comprised 635 serum samples from South Africa, China and Japan. Of these samples, 564 were HBsAg-positive and the remaining 71 HBsAg-negative, HBV DNA negative controls. The study cohort included asymptomatic carriers; chronically infected HBV patients as well as patients with HBV associated HCC. Possible associations were determined between HBV genotype, HBV viral load, apoptosis levels, disease group and the age and gender of the patient where available. Apoptosis levels were quantified by the measurement of cleaved cytokeratin 18 (M30) in serum.

Patients infected with genotype C or subgenotype A1 were shown to possess a higher odds ratios of developing HCC compared to subgenotype B2 or genotype D, respectively. Significantly higher HBV viral loads were observed in genotype C compared to subgenotype B2. Among the Asian cohort, it was also shown that the male gender was positively associated with high viral loads in HCC patients. Moreover, a positive association between higher HBV viral load levels and HCC in the South African cohort was observed. Male gender, older age, HBV viral load, subgenotype A1 and the presence of the G1862T mutation were shown to be positively and significantly associated with higher levels of apoptosis. In this study it was discovered that the levels of cleaved cytokeratin 18 could potentially be used as a biomarker for the severity of HBV infection because a significant difference was

observed with the apoptosis levels between the asymptomatic and HCC patient disease groups.

We conclude that even when the influence of host and environmental factors is controlled for, as is the case in the chimeric mouse model, the HBV genotype can affect the progression of infection. Moreover, it was shown in the population based study that the effect of HBV genotype on the outcome of HBV infection can be influenced by host factors. The subgenotype A1 G1862T mutation was shown in both studies to affect both HBV infection and apoptosis. This suggests that HBV variants should be investigated to ascertain their potential impact on the course of HBV infection as it may differ from the wild-type. Apoptosis was shown to be associated with HBV infection in both studies and could possibly be an ideal marker of the progression of HBV infection.

These findings are important in helping us to understand factors influencing the course of HBV infection. We have therefore shown in both the studies that differences do exist between the South African subgenotype A1 and genotype D, and that these differences should be taken into consideration for the future evaluation of HBV infection and treatment of South African HBV infected patients. Moreover, cleaved cytokeratin 18 may provide an ideal surrogate marker for HBV disease progression and monitoring.

TABLE OF CONTENTS

	Page
Title Page……	i
Declaration…….	ii
Dedication…….	iii
Publications and Presentations……..	iv
Acknowledgement…….....	v
Abstract…….	vii
Table of contents……...	x
List of Figures……	xv
List of Tables…….	xviii
List of Abbreviations…….....	xix
CHAPTER 1: INTRODUCTION……	1
1.1. Hepatitis B virus classification, structure and replication…….	2
1.2. HBV molecular characterization……...	5
1.2.1. HBV genotypes and subgenotypes……..	6
1.2.1.1.Genotype A……..	6
1.2.1.2.Genotype B……..	7
1.2.1.3.Genotype C……..	7
1.2.1.4.Genotype D……..	8
1.2.1.5.Genotype E……...	8
1.2.1.6.Genotype F……	8
1.2.1.7.Genotype G……	8
1.2.1.8.Genotype H……	9
1.2.1.9.Genotype I…….	9
1.2.1.10.Putative Genotype J…….	9
1.2.2. Precore/core HBV mutants…….	10
1.3. HBV infection and disease…….	11
1.3.1 Acute Hepatitis…….	12
1.3.2. Chronic Hepatitis…….	13
1.3.3. Stages of HBV infection…….	15

Page

1.3.4. Consequences of long-term chronic hepatitis…… 16

1.3.4.1. Clinical relevance of HBV genotypes/subgenotypes……. 17

1.3.4.2. Clinical relevance of HBV PreS and S region mutations…….. 18

1.3.4.3. Clinical relevance of HBV EnhII / Basic core promoter (BCP) / Precore mutations……. 19

1.3.4.4. Role of hepatitis B virus X protein (HBx)…… 21

1.3.4.5. Clinical relevance of HBV viral load……. 22

1.4. Liver disease associated cell death…… 23

1.4.1. Apoptotic pathways linked to liver disease…… 24

1.4.1.1. Lysosomal pathway……. 25

1.4.1.2. Mitochondrial pathway…… 26

1.4.1.3. Death receptor-mediated pathways…….. 26

1.4.1.4. Keratins…… 27

1.5 HBV animal models…… 28

1.6. Objectives of the study…….. 33

CHAPTER 2: FUNCTIONAL CHARACTERISATION OF HBV SUBGENOTYPE A1 ISOLATES *IN VIVO*: USING A UROKINASE-TYPE PLASMINOGEN ACTIVATOR SEVERE COMBINED IMMUNODEFICIENCY (uPA-SCID) TRANSGENIC MOUSE MODEL.... 34

2.1. Introduction…… 34

2.2. Materials and methods…… 35

2.2.1. HBV DNA positive sera…….. 36

2.2.2. DNA extraction from HBV positive sera…… 36

2.2.3. Genotyping PCR and restriction fragment length polymorphism (RFLP) analysis…….. 36

2.2.3.1. Genotyping PCR…….. 36

2.2.3.2. RFLP analysis…….. 37

2.2.4. Subgenotypic PCR and RFLP analysis of genotype A isolates…….. 37

2.2.4.1. Subgenotyping PCR……. 37

2.2.4.2. RFLP analysis…….. 38

2.2.5. Amplification and sequencing of the HBV precore/core region……. 38

2.2.5.1. Precore/core nested PCR…….. 38

Page

2.2.5.2. Automated Sequencing……. 39
2.2.6. Cloning procedure…… 39
2.2.6.1. Full length PCR……. 39
2.2.6.2. A' overhang addition and PCR product purification……. 40
2.2.6.3. Cloning of full length HBV product into pCR XL-TOPO vector……. 40
2.2.6.4. Identification of positive clones…… 41
2.2.7. Genotyping and subgenotyping of clones…… 41
2.2.7.1. Genotyping PCR…… 41
2.2.7.2. Subgenotyping PCR…….. 42
2.2.8. Automated sequencing……. 42
2.2.9. Phylogenetic analysis…….. 42
2.2.10. Monitoring of HBV infection in the uPA-SCID transgenic mice…….. 43
2.2.10.1. Isolation of human hepatocytes and transfer into uPA-SCID mice…… 43
2.2.10.2. Inoculation of the uPA-SCID mice with HBV-positive sera…….. 43
2.2.10.3. Human Albumin Quantification…….. 43
2.2.10.4. HBV DNA Quantification……... 44
2.2.10.5. Quantification of apoptosis…….. 44
2.2.11. DNA extraction from uPA-SCID transgenic mice livers…… 46
2.2.12. HBV cccDNA detection by real-time PCR…… 46
2.2.13. Testing for the presence of the original HBV inoculum strain in the uPA-SCID transgenic mice livers…….. 47
2.2.14. Techniques used to confirm HBV transcription…… 47
2.2.14.1. RNA extraction from uPA-SCID transgenic mice livers…… 47
2.2.14.2. Reverse transcription PCR (RT-PCR)…… 48
2.2.14.3. Glyceraldehyde – 3- phosphate dehydrogenase (GAPDH) PCR…… 48
2.2.14.4. Precore/core nested PCR used for confirmation of HBV transcription activity…… 49
2.2.15. Immunohistochemical staining…… 49
2.3. Results…….. 53
2.3.1. Characterisation of inocula…… 53
2.3.1.1. Genotyping of HBV inocula……... 53
2.3.1.2. Cloning…… 53
2.3.1.3. Genotyping and subgenotyping of HBV clones…….. 54

Page

2.3.1.4. Sequencing and Phylogenetic analysis…… 55

2.3.2. Inoculation and monitoring of transgenic mice……. 57

2.3.3. End-point experiments performed after sacrifice of transgenic mice…… 59

2.3.3.1. HBV cccDNA……. 59

2.3.3.2. HBV Transcription…….. 60

2.3.3.3. Presence of HBV inoculum in transgenic mice livers…… 61

2.3.3.4. Immunohistochemical staining……. 63

2.4. Discussion…….. 65

CHAPTER 3: INVESTIGATION OF THE RELATIONHIP BETWEEN HBV INFECTION AND APOPTOSIS IN HUMANS…… 72

3.1. Introduction…… 72

3.2. Materials and methods…… 73

3.2.1. Study subjects…….. 74

3.2.2. Hepatitis B surface antigen (HBsAg) ELISA……. 75

3.2.3. DNA extraction……. 76

3.2.4. Genotyping PCR and restriction fragment length polymorphism (RFLP) analysis…… 76

3.1.4.1. Genotyping nested PCR…….. 76

3.1.4.2. RFLP analysis……. 77

3.2.5. Subgenotyping PCR and RFLP analysis…….. 77

3.1.5.1. Subgenotyping nested PCR for B1 versus B2……. 77

3.1.5.2. RFLP analysis…….. 77

3.2.6. Amplification and sequencing of the HBV Precore/core region…….. 78

3.2.7. Real-time PCR quantification of HBV DNA…… 78

3.2.8. Quantification of apoptosis…… 79

3.2.9. Statistical analysis…….. 79

3.3. Results……. 80

3.3.1. Distribution of genotypes and subgenotypes……... 80

3.3.2. HBV viral load……. 82

3.3.3. Apoptosis……. 84

3.4. Discussion……. 89

Page

3.4.1) Genotype/Subgenotype associations……... 89

3.4.2) HBV Viral Load associations…….. 90

3.4.3) Apoptosis associations…… 91

CHAPTER 4: CONCLUSION…….. 95

Appendix A – Listing of solutions…… 98

Appendix B – Ethics…….. 101

References…….. 102

LIST OF FIGURES

		Page
Figure 1.1	Schematic representation of a Dane particle, the infectious HBV particle, depicting its structural components……	2
Figure 1.2	The HBV Genome……	3
Figure 1.3	The Replication Cycle of HBV……	5
Figure 1.4	The clinical course and serological patterns observed during acute HBV infection……..	13
Figure 1.5	The clinical course and serological patterns observed during chronic HBV infection……..	14
Figure 1.6	Extrinsic and intrinsic pathways of hepatocyte apoptosis……	25
Figure 1.7	Cytokeratin 18 cleavage during apoptosis resulting in M30 antibody binding site formation……	27
Figure 1.8	Schematic representation of the production of uPA-SCID transgenic mice and their potential scientific research applications……..	32
Figure 2.1	Techniques used to monitor HBV infection in the uPA-SCID transgenic mouse model……..	35
Figure 2.2	M30-Apoptosense ELISA plate assay……	45
Figure 2.3	Relative position of the sequencing primers to the clone of the full length HBV amplicon……	52
Figure 2.4	Chromatograph with the HBV precore-core 1862 mutation….	53
Figure 2.5	Representative gel image of the full length HBV amplicon…..	54
Figure 2.6	Representative gel image of RFLP analysis using *Hinf*I and *Tsp509*I restriction enzymes for the HBV clone genotype determination……..	54
Figure 2.7	Representative gel image of RFLP analysis using *Stu*I restriction enzyme for genotype A subgenotyping of clones…..	55
Figure 2.8	Phylogenetic relationship of the complete genome of HBV isolates cloned from human sera to representative sequences belonging to genotype A - G, obtained from GenBank, established using neighbour-joining……	56

Page

Figure 2.9 Human albumin and HBV DNA (log transformed) over the twelve week post inoculation period…… 57

Figure 2.10 Human albumin and M30 apoptosis levels over the 12 week post inoculation period……. 58

Figure 2.11 M30 apoptosis and HBV DNA ratios relative to subgenotype A2 at twelve weeks post inoculation…… 58

Figure 2.12 Amplification graph of HBV cccDNA generated by real-time PCR…….. 59

Figure 2.13 RNA extracted from the uPA-SCID mice livers…….. 60

Figure 2.14 Resolution of the precore-core region of HBV and the housekeeping gene GAPDH using cDNA generated from the infected chimeric mice extracted RNA…….. 60

Figure 2.15 Phylogenetic relationship of a portion of the S region (nt 315 to 693 from *EcoR*I site) of HBV isolates from human inoculum sera, human inoculum sera-derived clones and mice sera, to the representative sequences of the HBV genotypes A-G, obtained from GenBank, established using neighbour-joining……. 62

Figure 2.16 Haematoxylin and eosin staining of chimeric liver…… 63

Figure 2.17 Representative image of HBV S-antigen cytoplasmic staining in the liver of the HBV subgenotype A1 1862 infected transgenic mouse……. 64

Figure 2.18 Representative image of HBV core antigen nuclear staining in the liver of the HBV genotype D infected transgenic mouse…… 64

Figure 3.1 Organogram of techniques used…….. 73

Figure 3.2 Number of subjects by disease category and country…… 74

Figure 3.3 The Monolisa Ag HBs PLUS ELISA plate assay…….. 75

Figure 3.4 Genotypes and subgenotypes from South Africa, Japan and China.. 80

Figure 3.5 Chi square test used for the evaluation of HBV genotype by age and gender in Asians……. 81

Figure 3.6 Analysis of covariance with log-transformed viral load as the dependent variable and the age and gender as independent variables……. 82

Page

Figure 3.7 Analysis of covariance with log-transformed M30 value as the dependent variable and age as the independent variable…… 85

Figure 3.8 Analysis of covariance with log-transformed M30 as the dependent variable and age and disease group as independent variables……. 85

Figure 3.9 Analysis of covariance with log-transformed M30 as the dependent variable and gender as the independent variable…….. 85

LIST OF TABLES

		Page
Table 1.1	Risk factors for HBV-related cirrhosis and HCC……..	16
Table 2.1	PCR Primers and Cycling Profiles used for amplification……	50
Table 2.2	Primers used for full length sequencing of HBV clones……	51
Table 2.3	Mean nucleotide divergence (%)* of complete genome sequences of HBV clones obtained using DAMBE……	55
Table 3.1	Relative risk and Odds Ratio of developing HCC among the different population groups……	81
Table 3.2	Age-sex-disease group adjusted geometric means of viral load (copies/ml) by HBV genotype in China and Japan……	83
Table 3.3	Crude geometric means of viral load (copies/ml) by HBV genotype in South Africans……	83
Table 3.4	Pearson correlation coefficients of log transformed M30 and log transformed viral load in Asians and South Africans……	86
Table 3.5	Age-sex adjusted geometric means* of M30 levels (U/l) by HBV genotype in Asians…….	86
Table 3.6	Crude geometric means of M30 levels (U/l) by HBV genotype in South Africans……	86
Table 3.7	Comparison of age-sex-adjusted geometric means* of M30 levels (U/l) between HCC cases and non-cases in Asians……	87
Table 3.8	Comparison of crude geometric means* of M30 levels (U/l) between HCC cases and non-cases in South Africans……	87
Table 3.9	Comparison of crude geometric means^ of M30 levels (U/l) between HCC cases and non-cases using total subjects…….	87
Table 3.10	Crude geometric means* of M30 levels (U/l) by 1862 nucleotide in South Africans……	88
Table 3.11	Summary of associations……	88

LIST OF ABBREVIATIONS

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDV	Hepatitis D virus
ORF/s	Open reading frame/s
HBsAg	S antigen or hepatitis B surface antigen
HBcAg	Core antigen or hepatitis B core antigen
HBeAg	e antigen
cccDNA	Covalently closed circular DNA
pgRNA	Pregenomic RNA
ε	Epsilon
mRNA	Messenger RNA
bp	Base pair
kb	Kilo base
G	Guanine
A	Adenosine
T	Thymidine
C	Cytosine
ERGIC	Endoplasmic Reticulum Golgi intermediate compartment
ALT	Alanine aminotransferese
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
DISC	Death-inducing signaling complex
TNFα	Tumor necrosis factor alpha
MOMP	Mitochondrial outer membrane permeabilization
SMAC	Second mitochondrial activator of caspase
DIABLO	Direct IAP binding protein with low pI
JNK	c-*jun* N-terminal kinase
Fas L	Fas ligand
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TNFR1	Tumor necrosis factor receptor 1
IF/s	intermediate filament/s
TUNEL	TdT –mediated dUTP nick-end labeling

WHV	Woodchuck hepatitis virus
DHBV	Duck hepatitis B virus
Alb-uPA	Urokinase-type plasminogen activator transgene controlled by an albumin promoter
SCID	Severe combined immune deficient
ccc	Covalently closed circular
TBS	Tris-buffered saline
G1862T	Guanine to thymine transversion at nucleotide position 1862
ANCOVA	Analysis of covariance
ASC	Asymptomatic carriers
UC	Uninfected controls
CH	Chronic carriers
Mt	Mutant
Wt	wild-type
HCV	Hepatitis C virus
TBE	Tris-borate-EDTA
TAE	Tris-acetic-EDTA
LB	Luria bertoni
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcription PCR
GAPDH	Glyceraldehyde – 3- phosphate dehydrogenase
uPA-SCID	Urokinase-type plasminogen activator severe combined immunodeficiency
EU	European Union
U.S.A.	United States of America
XAP3	Clone designated X-associated protein 3

CHAPTER 1

INTRODUCTION

Hepatitis B virus (HBV) is a virus that attacks the liver and can cause both acute and chronic disease. The virus is transmitted through contact with the blood or other body fluids of an infected individual. About 25 % of adults who become chronically infected during childhood later die from cirrhosis (scarring of the liver) or hepatocellular carcinoma (liver cancer). A vaccine against HBV has been available since 1982 and is 95 % effective in preventing HBV infection and its chronic consequences. This was the first vaccine developed and produced against a major human cancer, hepatocellular carcinoma (HCC), a sexually transmitted disease, and that protects against another viral infection, hepatitis D virus (HDV). Despite the existence of this effective vaccine, HBV infection remains a major health problem with about 2 billion people worldwide having been infected with the virus during the course of their life. Approximately 350 million of these are chronic carriers of the virus. An estimated 600 000 people die each year as a result of the acute or chronic consequences of the infection (WHO, August 2008). Epidemiological studies conducted in sub-Saharan Africa in the last three decades have clearly shown that hepatitis B virus (HBV) is highly endemic (≥8% HBV carriage, with carriage being defined as hepatitis B (HB) surface antigen [HBsAg] positivity for more than 6months) and is a major public health problem (Burnett et al., 2005, Kiire, 1996). It has been estimated that HBsAg chronic carriage in black South Africans is 9.6% (Kiire, 1996), with a much lower prevalence in whites and Indians (0.2%), and those of mixed descent (0.4–3.0%) (Kew, 1996). Also, rural black populations have a much higher HBsAg chronic carriage (e.g.: 15.5% in the rural Eastern Cape) than urban black populations (e.g.: 1.3% in Soweto) (Kew, 1996). The outcomes for chronic carriers in sub-Saharan Africa are often severe, with 25% being expected to die from liver disease (Kiire, 1996). In the region 20% of cirrhosis cases and 70% of hepatocellular cancers are thought to be caused by HBV infection (Kew, 1996). The major route of HBV transmission in this region is horizontal (i.e. transmission unrelated to recognised sexual, perinatal, or parenteral exposure (Davis et al., 1989)) in children under 5 years of age.

1.1. HEPATITIS B VIRUS CLASSIFICATION, STRUCTURE AND REPLICATION

HBV is the prototype member of the family *Hepadnaviridae*, which comprises hepatotropic DNA viruses. The host specificity of HBV is limited to humans and chimpanzees. HBV-infected cells are known to produce three distinct viral particles. Spherical viral particles with a diameter of 20 nm and noninfectious filaments of variable lengths with a width of 22 nm and composed of surface antigen and host-specific lipids (Gavilanes et al., 1982). The spherical double-shelled infectious virion, known as the Dane particle, is approximately 42 nm in diameter and consists of an outer lipoprotein envelope containing surface antigens (Ganem, 1991). Within the envelope is an inner nucleocapsid or core composed of core antigen complexed with the viral genome and polymerase (Summers et al., 1975) (Figure 1.1).

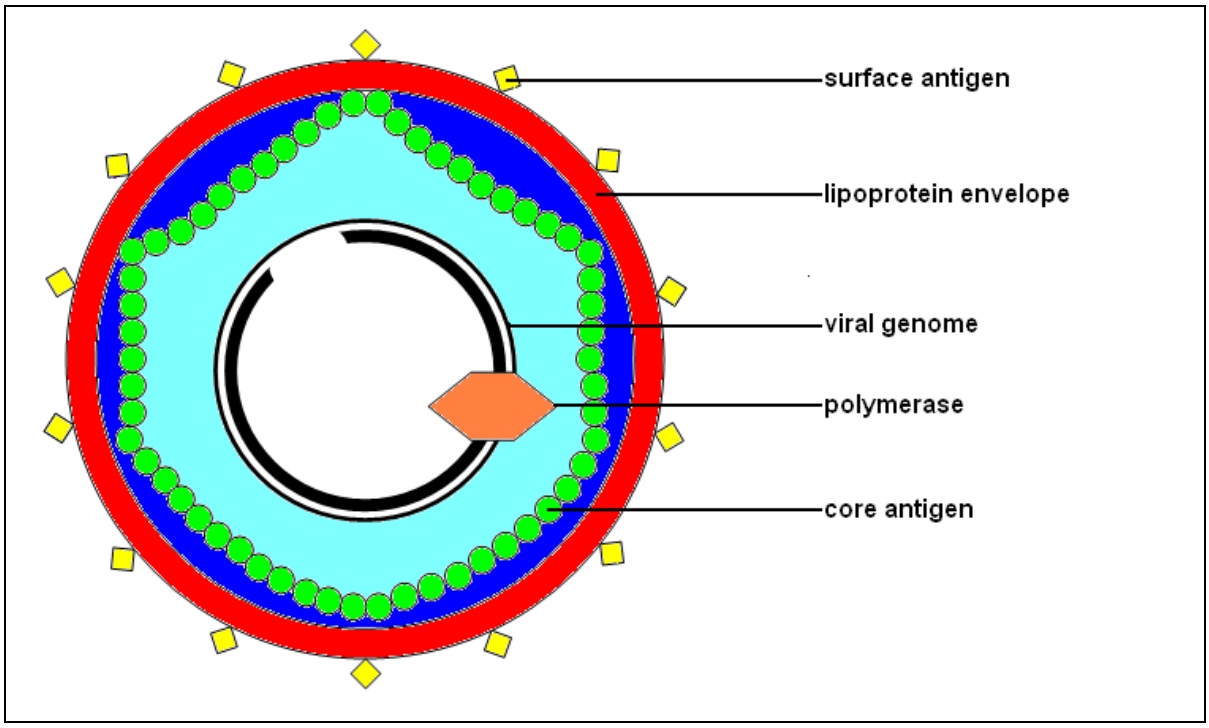


Figure 1.1. Schematic representation of a Dane particle, the infectious HBV particle, depicting its structural components.

The viral genome is a relaxed-circular, partially double-stranded DNA molecule of 3.2 kb in length, which contains four open reading frames (ORFs) (Figure 1.2). The S ORF consisting of the pre-S1, pre-S2 and S regions, encodes three viral surface envelope proteins: large, middle and small S antigens. The most abundant of these three proteins is the 24 kD S

antigen (HBsAg). The precore-core ORF encodes the core antigen (HBcAg) and e antigen (HBeAg) while the P ORF encodes the viral DNA polymerase involved in replication and RNA encapsidation. The X ORF encodes the X protein, which has been shown to be a potent transactivator of cellular and viral genes (Tiollais et al., 1985, Ganem and Varmus, 1987).

Two direct repeats, DR1 and DR2, present on the 5' ends of the viral genome plus strand are required for HBV replication (Seeger et al., 1986). Enhancer elements, EN1 and EN2 are required for liver-specific expression of the HBV viral gene products (Yee, 1989). Also identified within the genome are a glucocorticoid-responsive element sequence within the S gene (Tur-Kaspa et al., 1986), a polyadenylation signal within the core gene and a post-transcriptional regulatory element overlapping EN1 and part of the X gene (Huang and Liang, 1993).

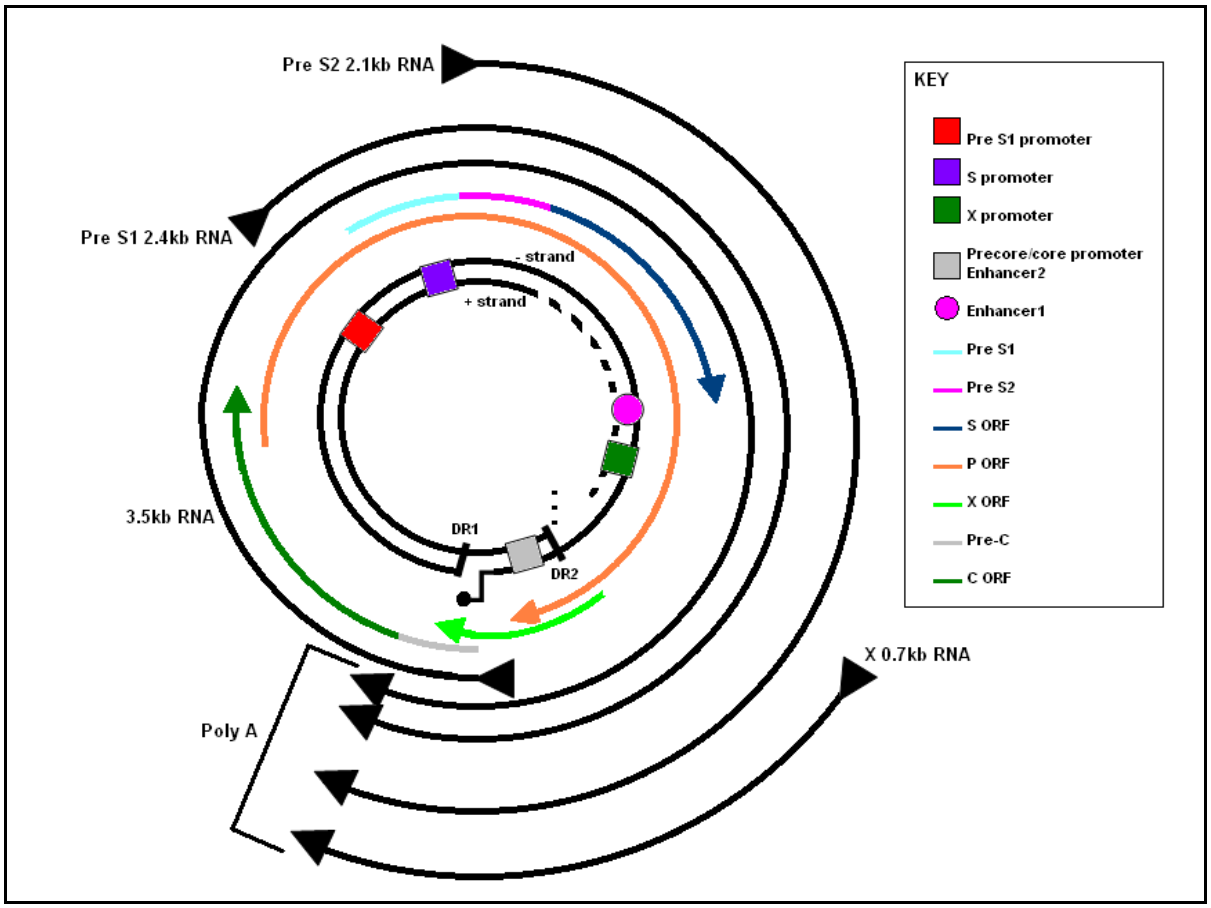


Figure 1.2. The HBV Genome. The genomic organization is shown with HBV RNA transcripts, gene products and several key regulatory elements.

HBV replication begins with the attachment of mature virions via the pre-S domain of the large surface protein (Klingmuller and Schaller, 1993), to the host cell membrane via receptors as yet unidentified (Figure 1.3). Only the core particle enters the cytoplasm and is transported to the nucleus via the process of membrane fusion. There the single-stranded gap region in the viral genome is repaired by the viral polymerase producing covalently closed circular DNA (cccDNA), which serves as a template for transcription of genomic and subgenomic transcripts (Kock and Schlicht, 1993). The transcripts produced are unspliced, polyadenylated and possess a 5' cap structure. They are all transported to the cytoplasm, where translation yields the viral envelope, precore, core, polymerase and X polypeptides. There are two species of 3.5 kb genomic transcripts, the pregenomic RNA and precore mRNA. The pregenomic RNA (pgRNA) is translated into core and polymerase after the pgRNA has been packaged, together with polymerase, into a nucleocapsid composed of core protein. For the pgRNA to be encapsidated, its 5' end is folded into a stem-loop structure known as the encapsidation signal or epsilon (ε), which is recognized by the terminal protein of polymerase (Pollack and Ganem, 1993). The polymerase is then activated by binding to epsilon and mediates the reverse transcription of the pgRNA to minus-strand DNA and subsequent positive-strand synthesis creating the circular DNA viral genome. Some nucleocapsid or core particles containing their newly formed genomes are transported back to the nucleus, where the genome can be converted once again into cccDNA to maintain a stable intranuclear pool of transcriptional templates (Nassal and Schaller, 1996). Most core particles then interact with the envelope proteins in the endoplasmic reticulum to assemble into mature virions, which are then secreted from the cell (Ganem and Prince, 2004).

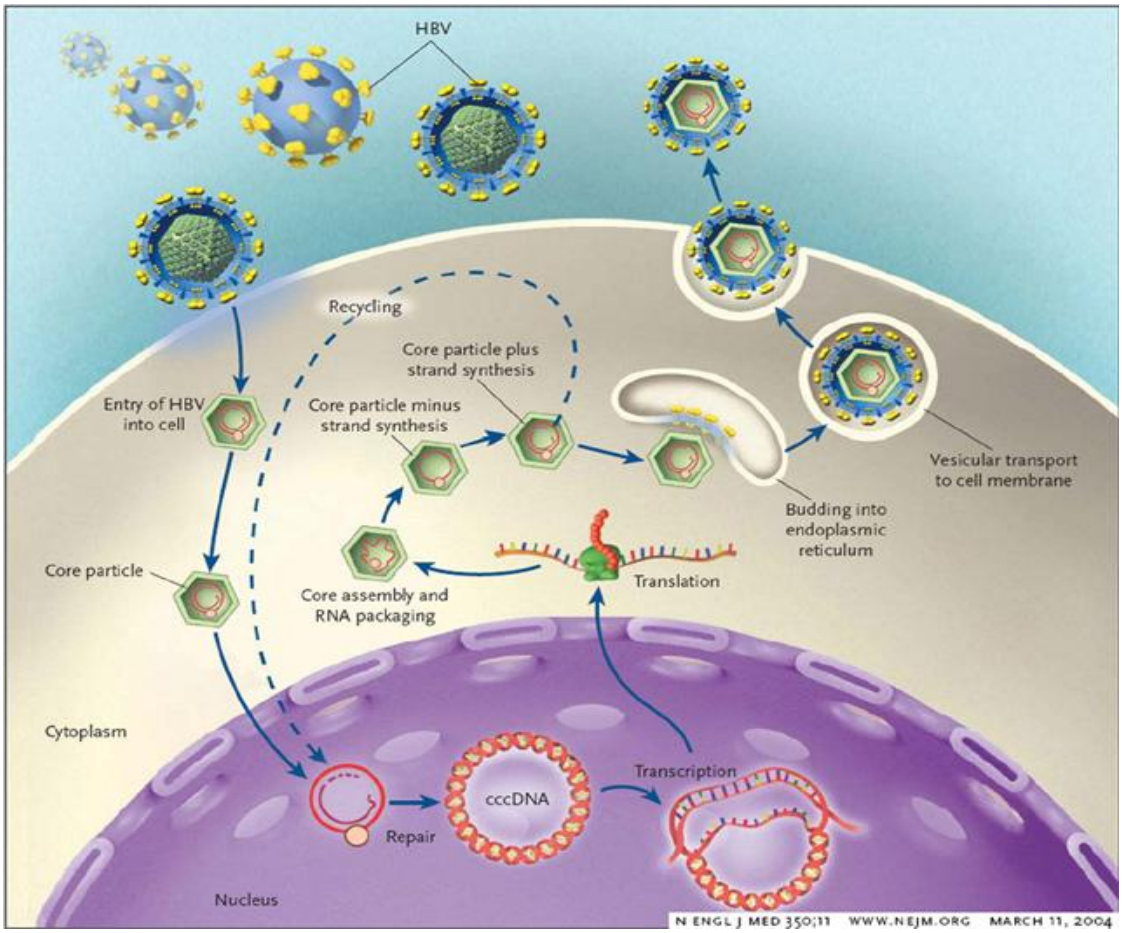


Figure 1.3. The Replication Cycle of HBV. HBV virions bind to surface receptors and are internalized. Viral core particles migrate to the hepatocyte nucleus, where their genomes are repaired to form a covalently closed circular DNA (cccDNA) that is the template for viral messenger RNA (mRNA) transcription. The viral mRNA that results is translated in the cytoplasm to produce the viral surface, core, polymerase, and X proteins. There, progeny viral capsids assemble, incorporating genomic viral RNA (RNA packaging). This RNA is reverse-transcribed into viral DNA. The resulting core paticles can either bud into the endoplasmic reticulum to be enveloped and exported from the cell or recycle their genomes into the nucleus for conversion to cccDNA. The small, peach-colored sphere inside the core particle is the viral DNA polymerase. Permission granted for reproduction (Ganem and Prince, 2004).

1.2. HBV MOLECULAR CHARACTERISATION

HBV replicates by reverse transcription of an RNA intermediate, pgRNA, mediated by a viral polymerase, which lacks proofreading ability, thus the virus is highly prone to mutations. The estimated mutation rate of HBV and other members of the family *Hepadnaviridae* is above 20 000 base substitutions per site per year (Chu and Lok, 2002). This is about 100 times higher than DNA viruses but 1000 times lower than RNA viruses (Chu et al., 2002b).

1.2.1. HBV GENOTYPES AND SUBGENOTYPES

Genotype is a term used to define the various groups of replication competent, stable HBV genomes circulating in various geographical regions (Francois et al., 2001). Currently eight HBV genotypes, A to H, have been recognized (Okamoto et al., 1988, Norder et al., 1994, Stuyver et al., 2000, Arauz-Ruiz et al., 2002) with a further two putative genotypes, I and J, being recently described (Huy et al., 2008, Tatematsu et al., 2009, Yu et al., 2010, Osiowy et al., 2010, Arankalle et al., 2010). Although differences based on an intergroup divergence of at least 8 % in the complete genome and 4 % in the S gene were originally used to differentiate genotypes, more recent analysis has shown that a 7.5 % or more intergroup divergence can be utilized for genotype assignment (Kramvis et al., 2008). The identification of recombination between genotypes often complicates this genotype assignment (Fang et al., 2011). Subgenotype groups have been identified within the genotypes and their separation is based on an intergroup divergence of between 4 % and 8 % across the complete genome of HBV (Norder et al., 2004, Kramvis et al., 2005, Kimbi et al., 2005). These genotypes/subgenotypes have been observed to have a distinct geographic distribution that may in time disappear as a result of increasing human mobility.

HBV can also be divided into one of nine serotypes (*ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adwq*, *adr*, *adrq*$^-$) based on the antigenic heterogeneity of HBsAg. Serotypes do not correspond closely to genotypes or subgenotypes and do not have a distinct geographic distribution (Francois et al., 2001).

1.2.1.1. *Genotype A*

Genotype A possesses a unique 6-nucleotide insert at the carboxyl terminus of the core gene. To date, six subgenotypes have been identified, A1 to A6, with a tentative A7 being found in isolates from Rwanda and Cameroon (Hubschen et al., 2010). Subgenotype A1 prevails in sub-Saharan Africa and South Asia (Kramvis and Kew, 2007) while subgenotype A2 is found mainly in Europe and America but has also been found in Africa (Kimbi et al., 2004). Subgenotype A3 was identified in Cameroon and Gabon isolates (Kurbanov et al., 2008). Subgenotype A4, A5 and A6 were reported from Mali, Nigeria (Olinger et al., 2006) and Haiti (Andernach et al., 2009b) isolates respectively. The most recently discovered, subgenotype A6, was isolated from Congolese and Rwandan patients (Pourkarim et al., 2010b). Pourkarim

et al recently re-analyzed the full length genome of isolates belonging to subgenotype A1 to A6 and suggested that genotype A subgenotypes be re-classified as "subgenotype A1, A2, quasi-subgenotype A3 and A4"(Pourkarim et al., 2010a, Pourkarim et al., 2011).

1.2.1.2. *Genotype B*

Subgenotypes B1 to B8 have been found within genotype B. Originally subgenotypes B1, from Japan, and B2, from continental South East Asia, were referred to as Bj and Ba, respectively (Sugauchi et al., 2002). Japanese subgenotype B1 and the more recently discovered subgenotype B6 isolates originating from the Arctic region (Sakamoto et al., 2007), are referred to as the "pure" genotype B isolates (Sugauchi et al., 2002). Subgenotypes B1 and B6 lack the genotype C recombinant region overlapping the precore/core ORF, which is characteristic of the remaining genotype B subgenotypes (Sugauchi et al., 2003). Formally known group Ba consists of four subgenotypes: B2 (China), B3 (Indonesia), B4 (Vietnam) and B5 (Philippines). Subgenotype B7 (referred to by the authors as a tentative new subgenotype "B6") is a recombinant of B3, B4 and B5 found in a southern province of China (Shen et al., 2009). The most recently discovered, subgenotype B8 originates from Indonesia (Mulyanto et al., 2009).

1.2.1.3. *Genotype C*

Genotype C is found throughout the eastern and southeastern regions of Asia and the Pacific Islands, as well as in Asian immigrants in the United States, Europe, Australia and New Zealand. Genotype C can be divided into several subgenotypes, the most established being C1 to C4. Subgenotype C1 represents isolates from Vietnam, Thailand and Myanmar while C2 strains are mainly derived from China, Korea and Japan (Huy et al., 2004, Ahn et al., 2009). Isolates from the Pacific Islands cluster into subgenotype C3, while subgenotype C4 is found in the Australian Aborigines. Subgenotypes C6 (Lusida et al., 2008) and C7 (Mulyanto et al., 2009) were discovered in Indonesia. Another tentative subgenotype designated "C9" was identified in Tibetans (Yin et al., 2010) while "C10" was observed in Indonesians (Mulyanto et al., 2010). A thymine to cysteine transversion as nucleotide 1858 exists in 10 to 25% of genotype C carriers in East Asia (Boner et al., 1995, Sugauchi et al., 2002a).

1.2.1.4. *Genotype D*

Genotype D is characterized by a 33 nucleotide deletion at the N terminus of the preS1 region. It is found world-wide and consists of subgenotypes D1 to D6 (Banerjee et al., 2006, Bozdayi et al., 2005, Norder et al., 2004, Schaefer, 2007). Subgenotype D1 isolates are derived from Europe, Middle East, Egypt, India and Asia while subgenotype D2 can also be found in Europe, India and Japan. Subgenotype D3 is also widespread with isolates identified from Europe, Asia, South Africa, India and the United States. Subgenotype D4 is specific to Australia, Japan and Papau New Guinea while subgenotype D5 has only been found in India and subgenotype D6 in Indonesia (Lusida et al., 2008).

1.2.1.5. *Genotype E*

Genotype E is found throughout West Africa, into Central Africa (Kramvis and Kew, 2007) and most recently in India (Singh et al., 2009) and Colombia (Alvarado Mora et al., 2010). It is similar to genotype D in the X and C ORFs but contains a unique three-nucleotide deletion at the amino terminus of the preS region (Norder et al., 1994). It has been suggested that genotype E has only recently been introduced into the human population and that its current high prevalence levels in Africa may be as a result of the use of unsafe needles (Andernach et al., 2009a).

1.2.1.6. *Genotype F*

Genotype F is divided into subgenotype F1 to F4. Isolates belonging to F1 are from South America while F2 to F4 can be found in South and Central America. Subgenotype F2 codes for a thymine to cysteine transversion at nucleotide 1858.

1.2.1.7. *Genotype G*

Genotype G is the most uncommon of all HBV genotypes. It has been found in a limited distribution of patients from France, Germany, Italy, United Kingdom, Brazil and the United States. It is almost exclusively found in patients co-infected with another HBV genotype, most commonly genotype A. A study showed that mono-infection with genotype G failed to yield HBV DNA indicating that the virus replicated poorly. However, when genotype A was

introduced it was rapidly replaced by genotype G (Sugiyama et al., 2007). Genotype G has a unique 36 base pair (bp) insert and shares the 3 bp deletion in the preS1 region found in genotype E. The precore/core region has 2 translational stop codons potentially affecting the expression of HBeAg (Stuyver et al., 2000, Kato et al., 2002).

1.2.1.8. *Genotype H*

Genotype H is found in Central America, mainly in Mexico and Nicaragua. It is most closely related to genotype F and likely evolved from this genotype in the New World (Arauz-Ruiz et al., 2002).

1.2.1.9. *Genotype I*

Genotype I was originally described in Vietnamese (Hannoun et al., 2000, Huy et al., 2008) and Laotian (Stuyver et al., 2000) isolates but failed to reach the 7.5 % nucleotide divergence from genotype C and was therefore not considered to be a separate genotype (Kurbanov et al., 2008). A recently discovered isolate from northwest China, related to these Vietnamese and Laotian strains, has been found to divert from genotype C by 8.4 % and therefore justifying separate genotype assignment (Yu et al., 2010). This genotype has also been identified in India (Arankalle et al., 2010) and in Canadians of Vietnamese origin (Osiowy et al., 2010).

1.2.1.10. *Putative Genotype J*

Genotype J has been identified in an elderly Japanese patient that resided in Borneo during World War II. It is 9.9 to 16.5% divergent from both human and ape genotypes, respectively. Based on the comparison of the four ORFs individually, it appears to be a recombinant of human subgenotype C4 and ape (gibbon and orangutan) strains. In a chimeric mouse study genotype J was shown to have efficient infection, replication and antigen expression (Tatematsu et al., 2009). More strains of this putative genotype require to be discovered before this 10^{th} genotype is recognized.

1.2.2. PRECORE/CORE HBV MUTANTS

One of the most predominant precore mutations involves a guanine (G) to adenosine (A) substitution at nucleotide 1896 (G1896A), converting the codon for tryptophan into a stop codon. This prevents the translation of the precore protein and abolishes HBeAg expression (Carman et al., 1989). This mutation is located in the highly conserved ε signal, which is essential for the encapsidation of the pgRNA during the viral replication cycle (Lok et al., 1994). The G1896A forms a base pair with nucleotide 1858 at the base of the stem loop. In genotypes B, D, E and some C strains, the nucleotide at position 1858 is a thymidine (T) therefore stabilizing the stem loop by binding to the adenosine at position 1896 (Lok et al., 1994). In contrast, the G1896A is rarely seen in genotypes A, F and some strains of C because cytosine (C) is the nucleotide at position 1858 resulting in wobble pairing and consequently decreased pgRNA encapsidation and thus decreased replication (Lok et al., 1994, Li et al., 1993).

Another mutation identified within the precore region, specifically in the bulge of the encapsidation signal, is a G to T transversion at nucleotide 1862 resulting in a valine to phenylalanine amino acid substitution at codon 17 (Santantonio et al., 1991, Kramvis et al., 1997, Kramvis and Kew, 1998, Valliammai et al., 1995). The presence of the aromatic phenylalanine residue at the -3 position of the signal peptide cleavage site is not tolerated (von Heijne, 1983) resulting in the accumulation of uncleaved mutant HBeAg in the Endoplasmic Reticulum Golgi intermediate compartment (ERGIC), the formation of aggresomes and ultimately in a decrease of secreted HBeAg (Chen et al., 2008). The G1862T mutation has been observed in several strains of HBV genotypes including genotype A and D (Kurbanov et al., 2008).

The basic core promoter region and the core upstream regulatory sequences are located upstream of the precore region and also play an important role in HBV replication and HBeAg production. Mutations in these regions downregulate precore mRNA transcription, and hence HBeAg production. The most common core promoter variant involves two point mutations: A to T at nucleotide 1762 and G to A at nucleotide 1764. This double mutation decreases precore mRNA production without affecting pgRNA transcription (Okamoto et al., 1994, Buckwold et al., 1997). It has been speculated that this is as a result of the conversion of a nuclear receptor binding site into a HNF-1 binding site (Li et al., 2002). This double

mutation is strongly associated with some strains of genotype B, C, F and G (Kurbanov et al., 2008). Less common but still strongly associated with subgenotype D1, is the T to C point mutation at nucleotide 1753 within the core promoter (Takahashi et al., 1999).

1.3. HBV INFECTION AND DISEASE

In the highly endemic areas, where the HBV carrier rate ranges from 8 % to 15 %, such as Africa and south east Asia, the main route of transmission in early childhood is horizontal and perinatal, respectively. In low endemic areas, where the carrier rates are less than 2%, such as the Western countries and Japan, HBV is predominantly a disease of adolescents and adults as a result of high risk sexual behaviour or intravenous drug abuse (Elgouhari et al., 2008).

Most cases of HBV infection acquired in adulthood resolve spontaneously within 6 months. In contrast most infections acquired at birth or in early childhood persist and become chronic (Elgouhari et al., 2008, Zanetti et al., 2008). Infection with HBV can lead to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and HCC.

HBV produces antigens that can be detected in the blood and that are cleared as the body's defense system produces antibodies against them. HBV DNA and surface antigen are often the first detectable markers of acute infection, appearing before the onset of symptoms or elevation of alanine aminotransferese (ALT) and aspartate aminotransferase (AST). By definition, HBV infection is considered chronic if HBsAg persists for longer than 6 months. HBeAg is considered a marker of HBV replication and infectivity (Liang and Ghany, 2002), however mutations in the precore-core region can result in decreased HBeAg expression without affecting viral replication (Kramvis and Kew, 1998, Carman et al., 1989). In chronic infection, HBeAg can persist for years. HBcAg is not secreted in serum but IgM antibodies and later IgG antibodies against HBcAg can be detected. These and other markers offer important clues as to the state and stage of disease.

1.3.1 ACUTE HEPATITIS

The incubation period of acute hepatitis B usually ranges from 2 to 3 months depending on the level of virus exposure (Barker and Murray, 1972). The majority of patients are asymptomatic with the infection being undetected but approximately one third of patients develop clinical symptoms of the infection (McMahon et al., 1985). The most common symptoms are fatigue, nausea, jaundice and rarely liver failure. HBV DNA followed by HBsAg and HBeAg are the first viral markers to be detected in the patient's serum (Figure 1.4). HBsAg can be detected as early as 1 to 2 weeks or as late as 11 to 12 weeks after exposure. The incubation period is followed by a preicteric phase, defined as preceding the appearance of jaundice, which typically lasts a few days to a week. ALT, AST, HBsAg and HBV DNA levels rise followed by the onset of jaundice (icterus) in some cases. The icteric phase follows for 1 to 2 weeks during which ALT levels normalize and the jaundice resolves with recovery. The HBsAg is cleared and the HBV DNA levels low because of early immune clearance. Anti-HBc IgM is detectable and declines as levels of anti-HBc IgG rise. Loss of HBeAg and the detection thereafter of anti-HBe is a favourable marker indicating recovery. Antibody to HBsAg (anti-HBs) rises during recovery and persists thereafter, being the marker associated with HBV immunity. However approximately 10 % to 15 % of patients who recover from HBV never develop detectable anti-HBs and therefore anti-HBc is the only marker of previous infection. Diagnosis of acute hepatitis is therefore made by the detection of IgM anti-HBc in the patient serum. HBV DNA detection has improved dramatically over the past few years with many real-time polymerase chain reaction assays being able to detect as low as 5 to 10 copies of HBV DNA copies/ml. Testing of HBV DNA is therefore becoming the preferred tool for the diagnosis and management of HBV infection (Kuhns and Busch, 2006).

Acute fulminant hepatitis occurs in 0.1 % to 0.5 % of patients and is characterized by signs of liver failure and low or undetectable HBV DNA levels. Acute liver failure has been found to occur in approximately 1 % of patients presenting with acute hepatitis B and jaundice (Berk and Popper, 1978).

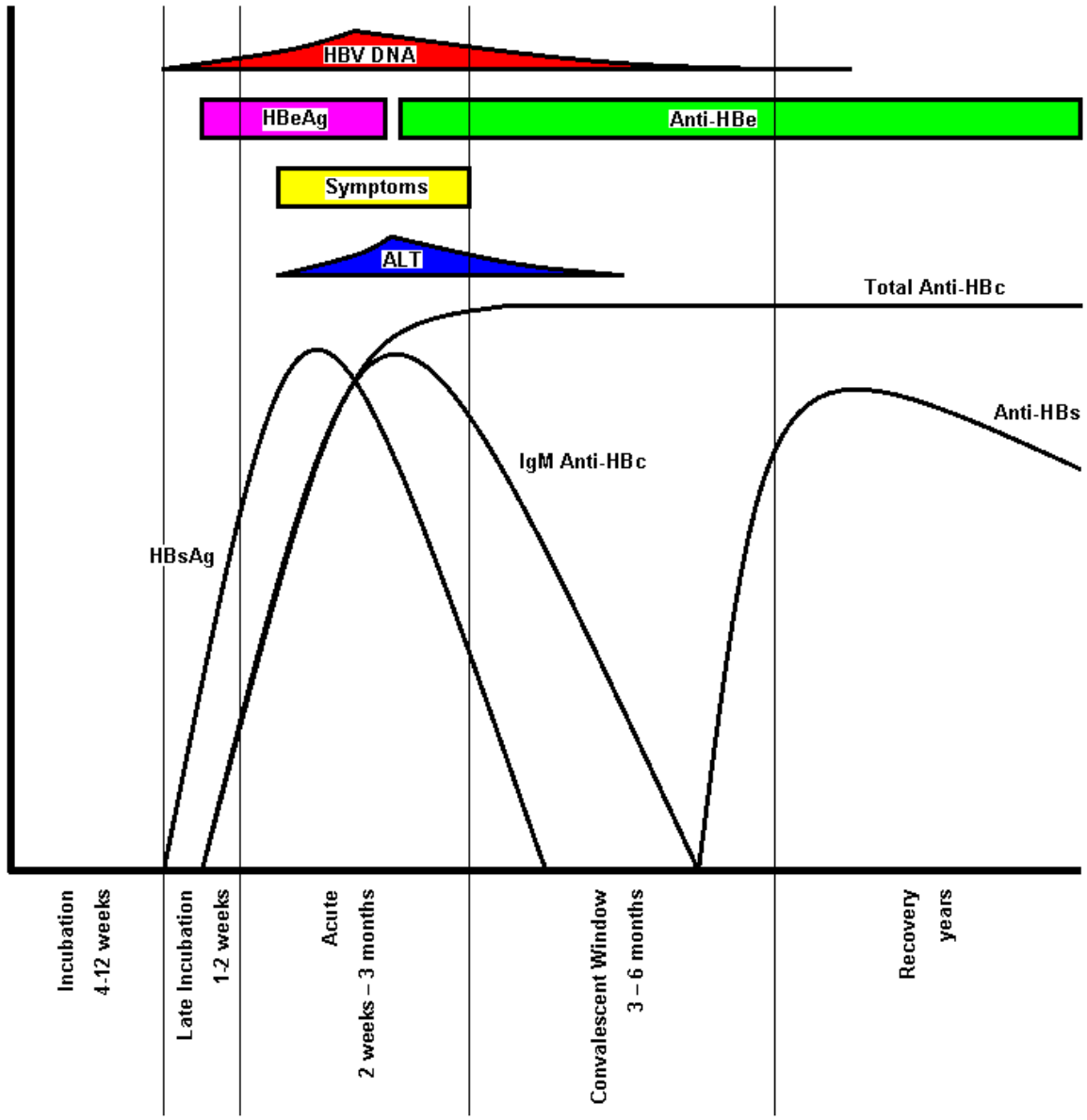


Figure 1.4. The clinical course and serological patterns observed during acute HBV infection.

1.3.2. CHRONIC HEPATITIS

Patients who develop chronic hepatitis B, initially have a similar pattern of infection to acute hepatitis but viral replication persists with HBsAg, HBeAg and HBV DNA being detectable in the serum (Figure 1.5). Chronic hepatitis B is usually diagnosed through a workup of abnormal liver function tests or as a result of screening high risk patients because carriage can be asymptomatic. The finding of HBsAg without IgM anti-HBc suggests the chronic hepatitis but a finding of the persistence of HBsAg for at least 6 months is required for confirmation (Perrillo et al., 1983). If diagnosis of HBV infection occurs during the acute phase,

monitoring of HBV DNA indicating persistently high levels following resolution of this phase may indicate the development chronic hepatitis (Loomba and Liang, 2007). In late cases, signs of cirrhosis can present. It should be noted that liver enzyme levels can be normal, even in patients with cirrhosis. The most characteristic histological feature of chronic HBV infection is the ground-glass hepatocyte, as a result of intracellular accumulation of HBV surface antigen.

Fewer than 5 % of immunocompetent adults infected with HBV remain chronically infected. On the other hand, 80 % to 90 % of infected infants and 20 % to 50 % of children 1 to 5 years old at the time of acute infection remain chronically infected (McMahon et al., 1985).

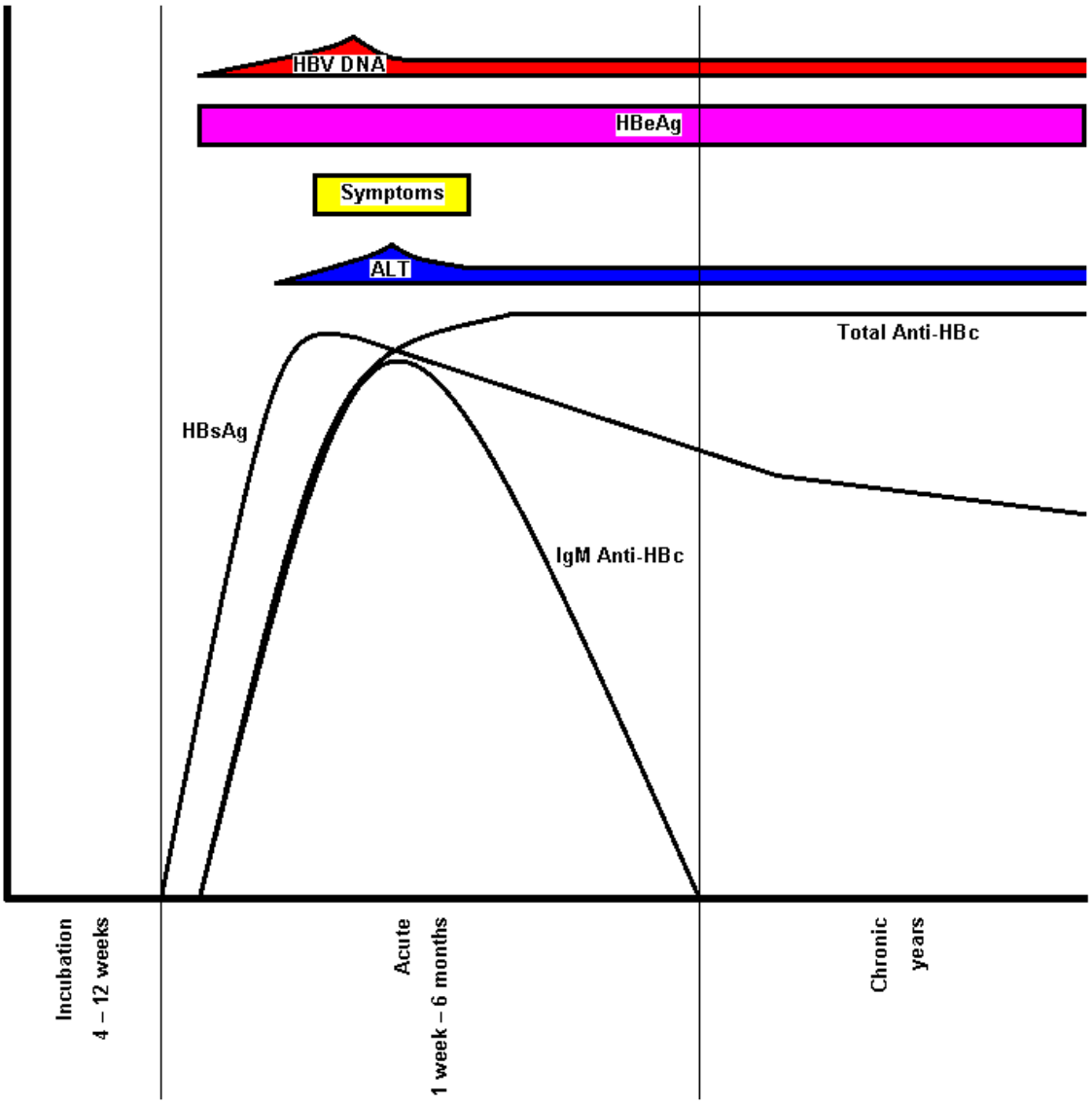


Figure 1.5. The clinical course and serological patterns observed during chronic HBV infection.

1.3.3. STAGES OF HBV INFECTION

There are three stages of HBV infection based on viral-host interaction: immune tolerant phase, immune clearance phase and the inactive carrier phase with or without reactivation. After acute infection some patients may remain HBeAg positive with high levels of HBV DNA, little or no symptoms, normal ALT levels and minimal histological activity in the liver, this is known as the immune tolerance phase. This phase is typical of infection in children and young adults. It usually lasts for 2 to 4 weeks but can last for years in those who acquired the infection during the perinatal period (Merican et al., 2000). Individuals in this group are highly contagious and can transmit HBV easily through body fluids. When the tolerogenic effect is lost, immune-mediated lysis of the infected liver cells occurs and the patients enter the second stage defined as the immune clearance phase characterized by decreasing HBV DNA levels and increasing ALT levels. The duration of this phase lasts from months to years. This is followed by the immune clearance phase, in which seroconversion of HBeAg to anti-HBe occurs, HBV DNA becomes non-detectable and ALT levels normal, reflecting very low or no replication of HBV and mild or no hepatic injury. The inactive carrier stage may last for years or undergo spontaneous reactivation of chronic hepatitis featuring elevated ALT, high levels of HBV DNA, moderate to severe liver histological activity and with or without HBeAg seroconversion.

A proportion of patients who undergo HBeAg seroconversion demonstrate a recurrence of high HBV DNA levels and intermittent or persistent ALT level elevations. These individuals have a naturally occurring mutant form of HBV that does not produce HBeAg due to a mutation in the precore core region. The most frequently occurring precore mutation is a guanine to alanine substitution at nucleotide 1896 which creates a stop codon and results in the loss of HBeAg synthesis. The most common core promoter mutation involves a 2 nucleotide substitution at nucleotides 1762 and 1764. HBeAg-negative carriers are a heterogenous group and may have either high or low viral DNA levels as well as relatively normal levels of ALT. Long-term prognosis is poorer among HBeAg-negative individuals who actively replicate the virus, compared with their counterparts, who are HBeAg-positive (Funk et al., 2002).

1.3.4. CONSEQUENCES OF LONG-TERM CHRONIC HEPATITIS

Following the diagnosis of chronic hepatitis B the survival rate is 100 % at 5 years however cirrhosis and HCC are two major long-term complications of chronic infection that significantly increase morbidity and mortality. The mortality rate at 5 years is 16 % for patients with compensated cirrhosis (symptomless) and 65 % to 86 % for decompensated cirrhosis (associated with complications) (Benvegnu et al., 2004). In untreated individuals with predominantly HBeAg positive chronic hepatitis B infection, the incidence of cirrhosis ranges from 2 % to 5.4 %, with 5 year cumulative incidence of cirrhosis of 8 % to 20 % (Fattovich, 2003). A higher rate of cirrhosis, 8 % to 9 %, has been reported in HBeAg-negative as compared to positive patients (Fattovich, 2003).

The development of HCC is one of the main causes of death from chronic hepatitis B infection. Chronically infected patients have a 100 times increased risk of HCC compared with non-carriers (Pungpapong et al., 2007). The annual rate of HCC has been estimated to be higher in patients with cirrhosis, 2.5 % to 3 %, than in non-cirrhotic carriers, 0.5 % to 1 % (Yim and Lok, 2006). The risk factors for cirrhosis and HCC are summarized in Table 1.1 (Lok and McMahon, 2007, Yim and Lok, 2006, Lok, 2004, Fattovich et al., 2004, Yu et al., 1997).

Table 1.1: Risk factors for HBV-related cirrhosis and HCC

CIRRHOSIS RISK FACTORS	**HCC RISK FACTORS**
Longer duration of infection	Longer duration of infection
High levels of HBV DNA	High levels of HBV DNA
HBV genotype and subgenotype	HBV genotype and subgenotype
Male sex	Male sex
Heavy Alcohol consumption	Heavy Alcohol consumption
Co-infection with HCV or HDV or HIV	Co-infection with HCV
Obesity	Obesity
Diabetes Mellitus	Diabetes Mellitus
	Race (African, Asian)
	Presence of cirrhosis
	Family history of HCC
	Cigarette smoking
	Aflatoxin exposure
	Reversion from HBe antibody to HBeAg
	HBV variants

1.3.4.1. *Clinical relevance of HBV genotypes/subgenotypes*

HBV genotypes and subgenotypes have been shown to differ with regard to clinical outcome, prognosis, and response to antiviral treatment. Infection with HBV genotype A is associated with high viral load which facilitates viral transmission. High replication rates of genotype A in adults lead to an increased risk of horizontal transmission of HBV by sexual activity because a high concentration of HBV DNA in serum is associated with high concentrations in semen and other body fluids of HBV carriers (Kidd-Ljunggren et al., 2006). Genotype A also tends to cause chronic infection following an acute course. This has been demonstrated in Japan where genotype A introduced from Europe has started to increase sharply in patients with acute infection since 1991, and gradually in those with chronic infection (Kobayashi et al., 2008). Spontaneous HBeAg seroclearance was significantly higher in genotype A carriers than in carriers of genotypes C, B, D, and F. After losing HBeAg, those with genotypes C and F were more likely to revert to the HBeAg-positive state (Livingston et al., 2007a). Infection with subgenotype B2 is associated with HCC or HCC recurrence in young, mostly noncirrhotic, patients in Mainland China and Taiwan (Kao et al., 2000, Yin et al., 2008, Ni et al., 2004), whereas infection with subgenotype B1 is frequently associated with fulminant hepatitis B in Japan (Kusakabe et al., 2009). Infection with HBV genotype C is associated with increased risks of liver cirhosis and HCC at an older age as compared with infection with the HBV genotype B (Yu et al., 2005, Chan et al., 2004, Chan et al., 2009a). Although HBV subgenotypes C1 and C2 are associated with the risk of HCC, only HBV subgenotype C2 is independently associated with an increased risk of HCC (Chan et al., 2008). Genotype B has recently been shown to be more likely to cause acute hepatitis B, while the serum viral load of asymptomatic carriers with genotype B is significantly higher than those infected with genotype C (Zhang et al., 2008). As compared with genotype C, HBV genotype B has been shown to be associated with earlier HBeAg seroconversion, and associated with better response to interferon therapy in HBeAg-positive chronic hepatitis (Chu et al., 2002a, Wai et al., 2002). Early HBeAg seroconversion typically confers a favorable outcome (Lin and Kao, 2008). In HBeAg-negative patients, detectable HBV DNA and HBV genotype C are associated with more severe liver damage (Chan et al., 2002). Thus, infection with HBV genotype C is associated with worse clinical outcome as compared with genotype B. With reference to the clinical characteristics of HBV genotype D infection and disease severity, the facts are contentious. Genotype D has been shown to be associated with severe liver disease, and a higher prevalence of genotype D than genotype A was observed in patients with HCC in

comparison with asymptomatic carriers (Thakur et al., 2002). In contrast, other reports indicated a lack of association between this genotype and a distinct clinical phenotype (Gandhe et al., 2003, Bahri et al., 2006). In the Caucasian and Indian populations, genotype D has been found to be associated with a greater risk for HCC than genotype A (Thakur et al., 2002). Data from India has shown that HBV subgenotype D1 is significantly associated with chronic liver disease, whereas HBV subgenotype D3 is significantly associated with occult HBV infection. No apparent clinical relevance was observed in those infected with HBV subgenotypes D2 and D5 (Chandra et al., 2009). There is very limited data on the association of genotype E with its clinical relevance. Population-based prospective cohort studies have found that HBV genotypes C and F are associated with the highest risk for HCC or liver cirrhosis (McMahon, 2009).

1.3.4.2. *Clinical relevance of HBV PreS and S region mutations*

HBV genomic variations in the PreS and S regions which are selected during the infection course are of clinical and public health importance. The HBV envelope is composed of 3 forms of HBsAg, the so-called large (L, coded for by the PreS1/S2/S gene), middle (M, the PreS2/S gene), and small (S, the S gene) proteins. The small or major peptide is 226 amino acids in length, and the M and L proteins are assembled by amino-terminal extension of 55 amino acids at the PreS2 domain and of 108-119 amino acids of the PreS1 domain. HBsAg is the main target for viral neutralization, either by natural or vaccine-induced anti-HBs. A central major hydrophilic region (approximately residues 103-173) exposed at the surface of viral particles. The major hydrophilic region itself is structured into five regions, including three central loops held together by disulphide bonds. The immunodominant "a" determinant (residues 124-147), against which most neutralizing antibodies are directed and which is the major target of HBsAg detection tests, is formed by loops 2 and 3 (Kay and Zoulim, 2007). HBV with mutations in the portion of the S gene coding the "a" determinant of hepatitis surface antigen, including a glycine to arginine substitution at position 145 (G145R) and other S gene mutations in the region of amino acids 120-147, can potentially evade neutralizing anti-HBs antibody and infect vaccinated people. G145R is by far the most common immune escape mutant, whereas the most important immune escape mutants with substitutions outside of the "a" determinant is P120S/T. Mutations in the S genes within "a" determinant (but not G145R) are partially responsible for occult HBV infection, which are characterized by the presence of HBV DNA in serum in the absence of detectable HBsAg, and could present a risk to blood safety (Tabor, 2006). Mutations

in PreS are also associated with occult HBV infection (Mu et al., 2009), probably due to the inactivation of the overlapping PreS2/S promoter which causes impaired HBsAg secretion.
The 5' flanking region of the S gene coding the PreS1 and PreS2 domains is overlapped by the region of the *P* gene coding the spacer domain of the viral polymerase. Genotype D and non-human primate isolates inherently have a 33 nucleotide deletion at or near the beginning of the PreS1 open reading frame (Schaefer, 2005, Kay and Zoulim, 2007). The PreS1 protein contains the hepatocyte binding site (amino acids 21-47) and is known to be essential for virion assembly and for the transporting of virions out of the hepatocyte (Shinkai et al., 2007). The PreS1 and PreS2 regions play an essential role in the interaction with immune responses because they contain several epitopes for T or B cells (Chen et al., 2007). There is little evidence supporting the idea that PreS mutants are transmissible, therefore, the PreS mutation might generate during the pathological process following the infection. The PreS mutations emerge in chronic infections, often in patients treated with interferon, and seem to represent desperate attempts to escape from host immune surveillance (Kay and Zoulim, 2007). Many of the mutations affecting the PreS domains of the envelope proteins are deletions. More recently, PreS deletions are frequently associated with an increased risk of HCC, especially in those infected with HBV genotype C (Chen et al., 2007, Mun et al., 2008, Chen et al., 2006a, Gao et al., 2007). Recent meta-analysis showed that the frequencies of the PreS deletion mutation consecutively increased during the progression of chronic HBV infection from asymptomatic carrier states to liver cirrhosis or HCC, while the frequencies of mutations at the promoter sites of PreS1 and PreS2 were significantly higher in the patients with HCC than in the patients without HCC (Liu et al., 2009). It is suggested that PreS deletion and nucleotide substitution mutations at the promoter sites of PreS1 and PreS2 may serve as useful biomarkers for predicting the clinical outcomes of HBV-infected patients, especially for predicting HCC (Cao, 2009).

1.3.4.3. *Clinical relevance of HBV EnhII / Basic core promoter (BCP) / Precore mutations*

The core promoter, positively and negatively regulated by Enhancer II (EnhII) and to some extent by Enhancer I, controls the transcription of precore mRNA and pregenomic RNA that can be the mRNA for both core protein and the viral polymerase and is the template for viral replication. HBeAg expression indicates active viral replication. There are two classes of mutants that affect HBeAg expression: BCP mutants and precore mutants. Although viral loads are generally several logs lower in HBeAg-negative patients than in HBeAg-positive patients and children born to HBeAg-positive mothers have a much higher risk of contracting

chronic HBV infection than children born to HBeAg-negative mothers (Kay and Zoulim, 2007), some combined mutations in the EnhII/BCP/Precore region like 1766/1768, 1762/1764/1766, 1753/1762/1764, and 1753/1762/1764/1766 mutations have been associated with high HBV DNA production in the *in vitro* transfection studies (Parekh et al., 2003, Jammeh et al., 2008). HBV core promoter mutations other than those at 1762/1764 appear to upregulate viral DNA replication and, at the same time, greatly reduce HBeAg production. Although expression of HBeAg has been associated with an increased risk of HCC in a prospective study (Yang et al., 2002), high viral load in HBeAg-negative patients is often associated with worse outcome of chronic HBV infection, especially in those with HBV carrying mutations at the PreS and EnhII/BCP/Precore regions (Chen et al., 2007, Wong et al., 2008, Tong et al., 2006, Yuen et al., 2009, Bahramali et al., 2008, Choi et al., 2009).

Several mutations at the EnhII/BCP/Precore region have been recently associated with an increased risk of HCC. These mutations include C1653T, T1753V, T1766/A1768, and A1762T/G1764A (Bahramali et al., 2008, Yuen et al., 2009, Yang et al., 2008, Guo et al., 2008, Dong et al., 2008, Fang et al., 2008, Sung et al., 2008, Ito et al., 2007). Recent data has shown that C1653T, T1753V, and A1762T/G1764A are each associated with an increased risk of HCC (Liu et al., 2009), whereas precore mutations G1896A and C1858T were found to not be associated with an increased risk of HCC, regardless of HBeAg status and HBV genotype (Liu et al., 2009).

Double mutataion, A1762T/G1764A has been shown to be a valuable biomarker for identifying a subset of male HBsAg carriers who are at extremely high risk of HCC in a prospective study (Fang et al., 2008). In a community-based prospective study, A1762T/G1764A and genotype C have been associated with an increased risk of HCC, whereas G1896A in the precore region has been associated with decreased risk of HCC (Dong et al., 2008). G1896A has been associated with fulminant hepatitis in Japan (Ozasa et al., 2006). Since the EnhII/BCP/Precore region overlaps with X gene in the HBV genome, mutations in the EnhII/BCP/Precore region should be included in evaluating the role of HBV X protein on the development of HCC. That is to say, the mutated X protein might be more carcinogenic than the wild-type X protein in HBV-induced hepatocarcinogenesis (Cao, 2009) .

C1653T, T1753V, and A1762T/G1764A are increasingly more prevalent as chronic HBV infection progresses from the asymptomatic HBsAg carrier state to liver cirrhosis or HCC, indicating that these mutations accumulate before the diagnosis of HCC (Liu et al., 2009). This finding suggests that these HBV mutations may serve as useful biomarkers for predicting clinical outcomes of the patients with chronic hepatitis, especially with regard to predicting whether they will develop HCC. Like the PreS mutants, HCC-associated HBV mutants in the EnhII/BCP/Precore region, e.g. A1762T/G1764A mutants, may not transmit *via* mother-to-child vertical transmission because the children whose mothers carrying HBV mutants were mostly found to be infected with the wild-type form of the same viruses (Cheng et al., 2009, Shen et al., 2008). These HBV mutations are likely generated during HBV-induced pathogenesis. A1762T/G1764A is frequently detected approximately 10 years before the diagnosis of HCC (Liu et al., 2009). It is therefore necessary to set up likely checkpoints for the examination of the HCC-associated HBV mutations in HBV-infected subjects.

Recently, several clinical scoring systems, or nomograms, consisting of previously confirmed independent risk predictors such as sex, age, family history of HCC, alcohol consumption, serum alanine aminotransferase (ALT) level, HBeAg status, serum HBV DNA level, and/or HBV genotype have been introduced (Yuen et al., 2009, Wong et al., 2010, Yang et al., 2010). These easy-to-use nomograms are based on noninvasive clinical characteristics and have been found to accurately predict HCC risk in either community- or hospital-based HBV-infected persons. Their use could facilitate communication between practicing physicians and patients in daily practice. However, it has been suggested that these predictive scoring systems need further validation in different populations across the world to be truly effective (Lin and Kao, 2011, Kao, 2011).

1.3.4.4. *Role of hepatitis B virus X protein (HBx)*

HBx, the nonstructural regulatory protein of HBV has been associated with the development of liver cancer in some HBx-transgenic mouse strains and with increased progression to liver cancer in other toxin-exposed HBx transgenic mouse strains (Bouchard and Schneider, 2004). HBx has been hypothesized to carry out various functions via undefined mechanisms and how these activities affect viral replication (and possibly liver cell transformation) remains undefined. The T1653 mutation resulting in a histidine-to-tyrosine amino acid substitution at codon 94 of the X protein (the center of the immunodominant antigenic domain of amino

acids 85–110) (Stemler et al., 1990) did not induce clonal outgrowth of cells and apoptosis as reported for wildtype HBx-expressing strains (Sirma et al., 1999). Indeed, codon 94 (nt 1653–1655) is within the functional domain of the X protein reported to play a central role in transactivation (Kumar et al., 1996). Cong et al, 1997 also reported that one of the activation domains of the X protein (amino acids 90–122) is required for XAP3 binding (protein kinase C-binding protein) (Cong et al., 1997). Protein kinase C is a large family of phospholipid-dependent kinases involved in cell growth, differentiation, and carcinogenesis. It is of interest whether the T1653 mutation affects protein-protein interactions between the X protein and XAP3. The X-ORF encoding the 154-amino acid HBx protein that possess transactivator properties that can affect various cellular and viral genes via cis- or transactivating elements (Kumar et al., 1996) has been reported to play a prominent role in the development of HCC (Kekule et al., 1993). Since the X-ORF overlaps the C-terminus of the polymerase gene and the N-terminus of the precore/core gene a mutation within the X-region could potentially affect three genes simultaneously (Li et al., 1999). In fact, mutations and deletions have been identified in integrated HBx sequences (Hsia et al., 1997, Takeuchi et al., 1997). In contrast to the mutations occurring in the immunogenic HBsAg protein characteristic of vaccine escape mutants (Chong-Jin et al., 1999), the effects of these HCC-integrated HBx mutants on hepatocarcinogenesis remain unclear, although some truncated HBx genes have been shown to retain biologically activity (Poussin et al., 1999). Some HBx mutations detected in HBV-infected patients have been linked to disease presentation and severity such as the dual mutations at position A1762T and G1764A in the BCP discussed in the above section. Although the molecular mechanisms mediated by HBV that result in liver cancer are not well defined, their correlation with HCC is well established. For example, a study using HBx transgenic mice developed hepatic adenoma and subsequently HCC (Kim et al., 1991). A different study demonstrated that although HBx was not sufficient for the induction of carcinogenesis it acted as a promoter for malignant transformation in hepatocytes (Zhu et al., 2004). These studies suggested that HBx is a multifunctional protein that plays various roles in HCC development.

1.3.4.5. *Clinical relevance of HBV viral load*

Many long term follow-up studies in Taiwan, China, and Japan have assessed the relationship between viral loads in chronic carriers of HBV and their subsequent chance of HCC development. These studies showed that high viral loads (defined as 10^5 viral copies per ml in

some studies (Harris et al., 2003, Chen et al., 2006b, Fung et al., 2007, Liu et al., 2008) or 10^4 viral copies per ml in others (Yang et al., 2002, Tang et al., 2004, Chan et al., 2008, Wu et al., 2008, Yuen et al., 2008)) predict an increased risk for the development of HCC. The effect of viral load on hepatocarcinogenesis is influenced by a number of variables, including male gender [Chen et al., 2006], HBeAg status (Chen et al., 2006b), genotype (Yu et al., 2005), and possibly age (Tsai et al., 2007). Significantly higher HBV viral loads in Black Africans with HCC compared to healthy Black carriers of the virus (Viana et al., 2009) confirms the finding in an earlier study of a small number of Senegalese patients (Tang et al., 2004), and is in keeping with the more extensive results in Taiwanese, Chinese and Japanese patients (Harris et al., 2003, Tang et al., 2004, Yu et al., 2005).

1.4. LIVER DISEASE ASSOCIATED CELL DEATH

Apoptosis or programmed cell death is a genetically conserved process that maintains tissue homeostasis and is essential for both normal development and some pathological processes. In the dying cell, a family of cysteine-proteases called caspases is responsible for apoptotic signaling, during which the cell undergoes several morphological and biochemical changes. In the early stages, the cell volume decreases and contact with the neighbouring cells is interrupted while the plasma membrane undergoes dynamic membrane blebbing (Kerr et al., 1972). The dying cell subsequently forms buds and fragments into apoptotic bodies that are phagocytosed by macrophages or neighbouring cells (Jacobson et al., 1997). The engulfment of the apoptotic bodies ensures that this form of cell death does not lead to inflammatory reactions.

Necrosis and apoptosis have long been viewed as morphologically and biochemically distinct forms of cell death. Necrosis results from acute metabolic disruption with adenosine triphosphate (ATP) depletion, ion deregulation, mitochondrial and cellular swelling, and activation of degradative enzymes culminating in the rupture of the plasma membrane and loss of intracellular proteins, metabolites and ions (Lemasters, 1999). Necrosis and apoptosis, however have been shown to occur simultaneously in tissues and cell cultures exposed to the same stimulus (Shimizu et al., 1996).

Necrapoptosis is used to describe the shared pathways leading to both forms of cell death. Pure apoptosis and pure necrosis represent extremes in the spectrum of necrapoptotic

responses. However, the more typical response of tissues and cells to injurious stresses and other death signals is a mixture of events associated with both forms (Lemasters, 1999). The contribution of cell death to liver diseases has been shown to be a consequence of necrapoptosis (Malhi et al., 2006).

Important to note is that a healthy liver is characterized by a certain degree of apoptosis. In fact, in recent years it has become obvious that development and progression of various liver diseases are associated with either an increase or decrease in hepatocyte apoptosis. Moreover, dysregulated apoptosis itself may be a fundamental feature of most acute and chronic human liver diseases (Patel et al., 1998, Malhi and Gores, 2008). A direct link has been suggested between upregulated apoptosis, the subsequent release of inflammatory mediators, and the development of fibrosis (Canbay et al., 2004). This link has been observed for ethanol-induced hepatitis, non-alcoholic fatty liver disease, cholestatic liver diseases, alpha-1-antitrypsin, Wilson's disease, viral hepatitis and ischemia-reperfusion injury (Galle and Krammer, 1998). Sustained, excessive apoptosis leading to fibrosis can culminate in cirrhosis and liver failure (Jaeschke et al., 2002). In contrast, the combination of altered apoptosis with increasing cell proliferation may promote the development of cancer (Guicciardi and Gores, 2005). Dysregulated apoptosis is thus associated with considerable morbidity and mortality in many liver diseases (Patel et al., 1998).

1.4.1. APOPTOTIC PATHWAYS LINKED TO LIVER DISEASE

Apoptosis in the liver may occur via the death receptor-mediated extrinsic pathway or the intracellular organelle-based intrinsic pathway (Figure 1.6). Regulation of apoptotic machinery in hepatocytes is complex and commonly triggered through activation of death receptors (Rust and Gores, 2000). The intracellular pathway can be initiated by several organelles involving one or more of the following: lysosomal permeabilisation, pathological alterations in the cellular storage and mobilization of calcium or in processes that are located in the endoplasmic reticulum. Intracellular DNA damage or mitochondrial dysfunction are known to trigger apoptosis (Hengartner, 2000).

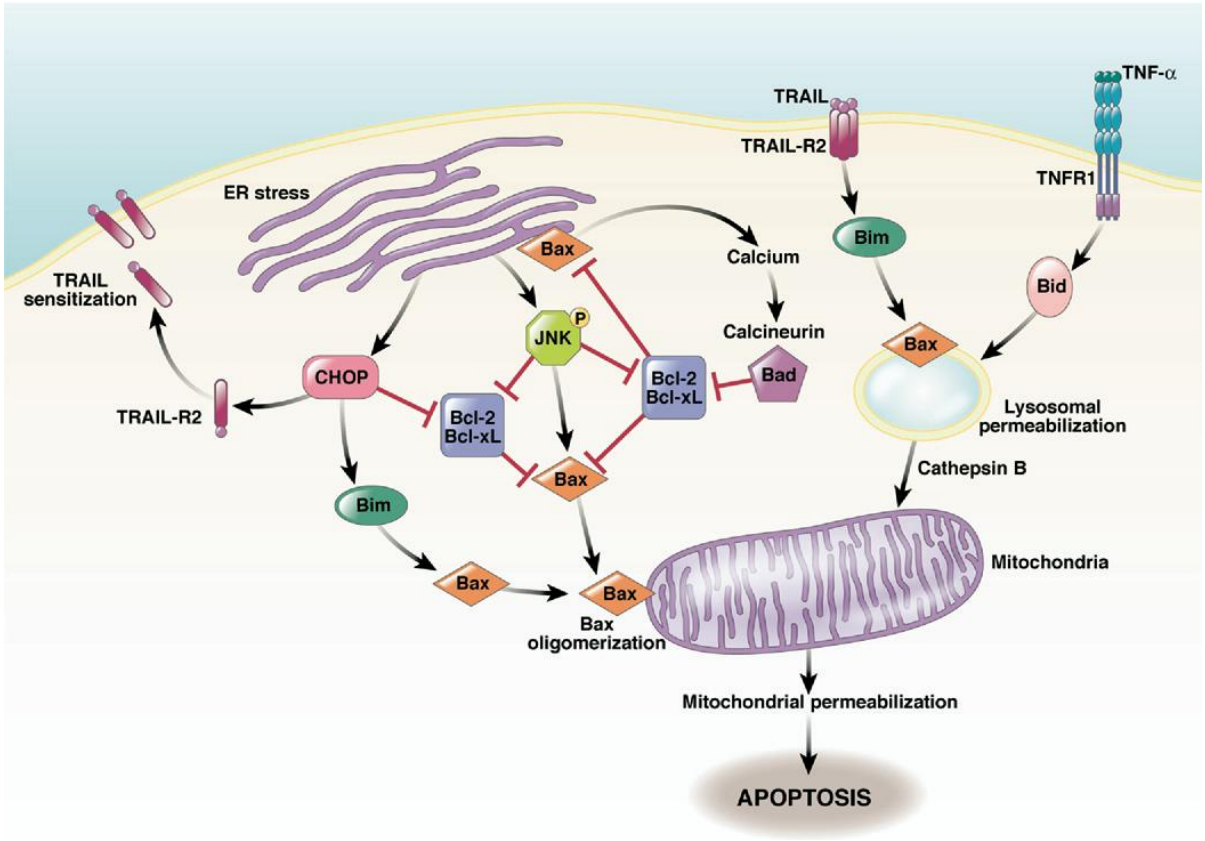


Figure 1.6. Extrinsic and intrinsic pathways of hepatocyte apoptosis. The extrinsic pathway is activated by death receptors. Fas or TRAIL (depicted here) bind to their cognate receptors, leading to the formation of the death-inducing signaling complex (DISC), with caspase 8 activation, Bid cleavage, and subsequent mitochondrial permeabilization. Bim activation can also occur downstream of death receptor signaling, leading to Bax activation and mitochondrial permeabilization. The TNF-α signaling pathway also leads to Bid cleavage with lysosomal permeabilization, leading to release of lysosomal contents and mitochondrial permeabilization. The intrinsic pathway of cell death can be initiated by myriad intracellular stressors that can activate the ER stress pathway, lysosomal permeabilization, or JNK activation. These cascades lead to inhibition of the antiapoptotic proteins (Bcl-xL, Bcl-2) and activation of the proapoptotic proteins (Bax, Bim, Bad, Bid). Mitochondrial permeabilization occurs eventually and is required for hepatocyte apoptosis. Permission obtained for copyright (Malhi and Gores, 2008).

1.4.1.1. *Lysosomal pathway*

Lysosomal involvement in cell death is an early event that occurs prior to mitochondrial permeabilization or caspase activation. Lysosomes can be activated by the extrinsic pathway or a myriad of intracellular stimuli such as reactive oxygen species (Guicciardi et al., 2004). The release of lysosomal proteases, called cathepsins, mediates downstream apoptotic effects. Cathepsin B has been linked to several models of liver injury (Kyaw et al., 1983). Lysosomal ultrastructural abnormalities are seen in many chronic liver disorders, while studies show that the lysosomal pathway of apoptosis is important in steatohepatitis, cholestatic liver injury and tumor necrosis factor alpha (TNFα) mediated liver injury (Guicciardi et al., 2000, Feldstein et al., 2004).

1.4.1.2. *Mitochondrial pathway*

Mitochondrial dysfunction is the commitment step in hepatocyte apoptosis and is dependent on it (Malhi et al., 2006). Mitochondrial inner and outer membranes isolate a number of proapoptotic proteins within the intermembrane space. Mitochondrial outer membrane permeabilization (MOMP) leads to the release of these proteins namely cytochrome c, second mitochondrial activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO), HtrA2/Omi, apoptosis-inducing factor and endonuclease G (Green and Kroemer, 2004). The protein release in turn activates caspases and initiates apoptotic nuclear changes (Bradham et al., 1998). MOMP occurs selectively, mediated via activated Bax / Bak or secondary to mitochondrial permeability transition (Green, 2005). It can also be activated downstream of death receptor-triggered signaling cascades, lysosomal permeabilization, endoplasmic reticulum stress pathways or activation of intracellular stress kinases such as c-*jun* N-terminal kinase (JNK).

1.4.1.3. *Death receptor-mediated pathways*

TNFα, Fas ligand (Fas L) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) are death ligands that signal via binding to their respective death receptors. Death receptors belong to the TNF/nerve growth factor superfamily and are essential for death ligand-mediated cell death. The death receptors Fas, tumor necrosis factor receptor 1 (TNFR1) and TRAIL receptors have recognized roles in liver injury (Mochizuki et al., 1996, Yamada et al., 1998, Schneider et al., 1997). The process of the ligand binding to the receptor leads to receptor trimerization and activation of intracellular signaling cascades. The cascade begins with the activation of the initiator caspase 8 followed by the cleavage of Bid to tBid, which in turn then translocates to the mitochondria causing mitochondrial permeabilization. Lysosomal permeabilisation can occur via TNFα-induced Bid-dependent and TRAIL-induced Bax-dependent pathways (Werneburg et al., 2007). Keratins 8 and 18 have been shown to modulate apoptosis induced by Fas/TNF family receptors (Caulin et al., 2000, Gilbert et al., 2001, Ku et al., 2003).

1.4.1.4. *Keratins*

The cytoskeleton consists of three distinct filament systems: intermediate filaments (IFs), actin-containing microfilaments and microtubules. There are five different types of intermediate filaments: types I-IV being cytoskeletal IFs and type V nucleoskeletal IFs (Helfand et al., 2004). The keratins represent the largest subgroup among the IF proteins and are differentially expressed as pairs of type I and type II IF proteins in epithelia (Hesse et al., 2001). Type I keratins include keratin 9 to keratin 23 (K9-K23) and type II keratins include keratins 1 to 8 (K1-K8). It has long been believed that the primary function of cytokeratins is as a structural scaffold that gives mechanical strength in cells. However, recent data indicates that IFs are remarkably dynamic rather than static and that IFs exhibit a complex array of motile activities (Helfand et al., 2004). Additional functions include their participation in cell signaling and in the resistance of stress and apoptosis (Gilbert et al., 2001).

In hepatocytes the IFs consist only of the K8/K18 pair. K18 is cleaved at two sites into three fragments during apoptosis by effector caspases (MacFarlane et al., 2000). The monoclonal antibody, M30, has been shown to recognize the neo-epitope of K18 exposed after caspase cleavage (Leers et al., 1999). The antibody detects only the cleaved fragment but not the native or intact K18. The epitope has been shown to map to the C terminus of the caspase cleavage site (amino acids 387-396) (Figure 1.7). This antibody has subsequently been shown to detect cells earlier in apoptosis than the popular standard TdT –mediated dUTP nick-end labeling (TUNEL) assay (Leers et al., 1999). The limited prevalence and localisation of cytoskeletons in epithelial cells makes the proteins a potentially useful tool for monitoring cancers arising in this tissue type (Barak et al., 2004).

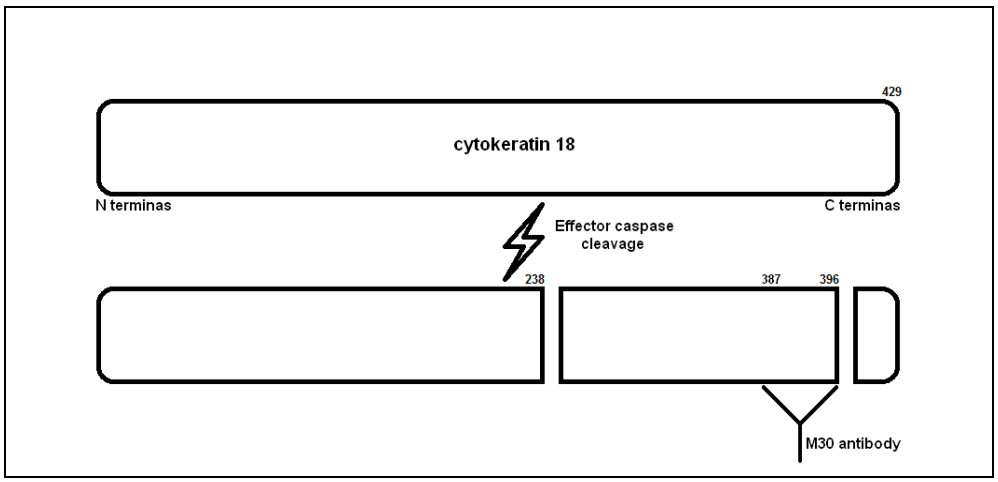


Figure 1.7. Cytokeratin 18 cleavage during apoptosis resulting in M30 antibody binding site formation.

1.5 HBV ANIMAL MODELS

Members of the family *Hepadnaviridae* are found in a wide variety of mammals and avians, belonging to the genera *Orthohepadnavirus* and *Avihepadnavirus*, respectively. The largest contribution in HBV research however is based on infection studies with the woodchuck and duck HBV (Fioravanti et al., 2011, Roggendorf et al., 2010, Kulkarni et al., 2007, Schultz et al., 2004, Chassot et al., 1993). The woodchuck hepatitis virus (WHV) was the first to be described after the discovery of HBV (Summers et al., 1978). WHV is classified as a member of the genus *Orthohepadnavirus* and therefore its genetic organization is similar to other mammalian hepadnaviruses including HBV. Woodchucks chronically infected with WHV progressively develop severe hepatitis and HCC, which present in a remarkably similar manner to the clinical manifestations of HBV infection in humans (Snyder et al., 1982). Chronic WHV carrier woodchucks have therefore become a valuable animal model for the preclinical evaluation of antiviral therapy for HBV infection, providing useful pharmacokinetics and pharmaco-dynamic results (Rajagopalan et al., 1996). Since the description of WHV, several other closely related hepadnaviruses within the genus *Orthohepadnavirus* have been described: california ground squirrel hepatitis virus, arctic ground squirrel hepatitis virus and the woolly monkey hepatitis B virus.

Several avian hepadnaviruses belonging to the genus *Avihepadnavirus* have been described: duck hepatitis B virus (DHBV), heron hepatitis B virus and snow goose hepatitis B virus. Much of what is known about the replication of hepadnaviruses has been learned using the DHBV model both *in vitro* and *in vivo* (Summers and Mason, 1982). The molecular structure (Newbold et al., 1995), role of cccDNA as the template for viral transcription (Summers, 1988), mechanism of cccDNA pool replenishment (Summers et al., 1991) and the control of this pathway by surface antigen (Summers et al., 1990) have all been investigated using DHBV. The duck animal model has also been invaluable in antiviral drug strategies (Bishop et al., 1990, Kern, 1996, Caselmann, 1994, Ying et al., 2010, Feng et al., 2010).

A limitation of the woodchuck and duck model is that neither develops cirrhosis as a result of a difference in the mechanism of pathogenesis compared to that of HBV. Therapeutic efficiency of immunological reagents, such as monoclonal antibodies, against HBV can therefore not be investigated within these animal models. Moreover, the DHBV lacks the X ORF found in HBV (Lin and Anderson, 2000).

The chimpanzee, although endangered in the wild and therefore restricted in its research use, has been invaluable in HBV research. Chimpanzees, and to a lesser extent rhesus monkeys, are the only animals that can be infected by human HBV and have been shown to develop circulating antibodies and elevated enzyme levels indicative of liver damage following inoculation (Barker et al., 1975). The chimpanzee animal model has played an important role in the development of HBV vaccines (Trepo et al., 1975), contributing to human welfare by preventing the development of HCC (Prince et al., 1997).

The development of transgenic mouse technology, which permits insertion of any gene of interest into fertilized mouse ova, has made it possible to construct mice expressing a target transgene so that its function and consequence can be studied *in vivo* (Palmiter and Brinster, 1985). This scientific advancement has provided an opportunity to create an ideal small laboratory animal model for the study of HBV.

In 1990 a group of scientists while trying to develop a model to study neonatal bleeding disorders, generated a novel murine line carrying a urokinase-type plasminogen activator transgene controlled by an albumin promoter (Alb-uPA) that when expressed was toxic to the mouse hepatocytes (Heckel et al., 1990). Demonstration of the ability to "rescue" the mice from liver failure with mouse hepatocyte transplants (Rhim et al., 1994) and subsequently rat hepatocytes were used as xenografts (Rhim et al., 1995). Soon thereafter human hepatocyte transplantation was successfully executed with modest repopulation of the livers and the support of HBV infection (Dandri et al., 2001). Meuleman *et al* then further morphologically and biochemically characterized uPA transgenic mice backcrossed with severe combined immune deficient (SCID) mice, repopulated with human hepatocytes (Meuleman et al., 2005). As a result of this work, a number of potential applications of this animal model were proposed (Figure 1.8) (Kneteman and Mercer, 2005) and this led to a number of HBV-related investigations (Okumura et al., 2007) (Sugiyama et al., 2006) (Turrini et al., 2006, Meuleman et al., 2006).

Dandri et al. were the first to successfully infect chimeric immune deficient uPA mice with HBV (Dandri et al., 2001). The human hepatocytes were transplanted into heterozygous uPA mice and not in homozygous uPA animals. The transfer of hepatocytes in heterozygous mice resulted in a repopulation grade with human cells of only 2 to 10%, meaning that most of the

liver parenchyma was occupied by healthy mouse hepatocytes derived from uPA revertants. A lethal effect of HBV infection could not be observed since the mice were capable of survival without a hepatocyte graft. The infected animals were sacrificed 8 weeks after transplantation, at which time the animals had high levels of HBV DNA and HBV proteins in their plasma and a strong nuclear expression of the HBV core protein in the liver, phenomena also observed by Meuleman et al (Meuleman et al., 2006). Levels of circulating HBV DNA with values exceeding 10^9 copies/ml are also common in immunosuppressed HBV patients. Tsuge et al. described long-term HBV infection in uPA-SCID mice (Tsuge et al., 2005). Interesting to note is that several weeks after infection, the concentration of human albumin decreased in these animals. It is not clear whether this is due to the HBV infection or to a spontaneous loss of chimerism. Obvious signs of a cytopathic effect were not reported, and a lethal effect is less likely in this setup because the low levels of human albumin suggest the presence of high amounts of regenerative red nodules. These nodules would rescue the animals in case of graft failure.

uPA/RAG2 mice transplanted with Tupaia hepatocytes have also been infected with HBV (Dandri et al., 2005) (10). In these animals the HBV infection persisted for 6 months, the experimental endpoint, and no signs of hepatocellular damage were reported. The main differences with the human liver-uPA model are that the onset of viremia was considerably delayed and plasma levels of HBV DNA significantly lower. This may be due to the nature of the Tupaia hepatocytes. Similarly, hepatocytes from a woodchuck infected with the woodchuck hepatitis virus were transplanted in uPA/RAG2 mice. These animals experienced high viremia for more than 10 months without obvious lesions in the liver. It might be that the woodchuck hepatitis virus is less cytopathic than HBV. Alternatively, this difference may also be related to the different nature of the woodchuck hepatocytes.

In 2006 Meuleman et al successfully infected nine uPA SCID mice with HBV genotype E and saw similar histology to patients with fibrosing cholestatic hepatitis (Meuleman et al., 2006). In the same year, Sugiyama et al, successfully infected uPA SCID mice with HBV genotypes/subgenotypes A2 and C and observed differences in HBV DNA levels, with the genotype C infected mice having a 2 log higher viral load compared to the subgenotype A2 infected mice (Sugiyama et al., 2006). The HBV cytopathic effect observed in Meuleman's study was not evident in Sugiyama's study suggesting that this effect was as a result of the HBV genotype used for inoculation and infection.

While the potential scope of utility is vast, the uPA-SCID model is not without limitations. Immunodeficiency may allow the engraftment of human hepatocytes but prevents the study of the immune system and therefore immunization. Promising developments include partial reconstitution with donor-specific immunity (Kneteman and Mercer, 2005). Breeding these transgenic mice can be challenging with many mice perishing as a result of the bleeding complications. Optimization of breeding protocols however can result in relatively high yields of mice. Finally, hepatocyte donor supply can be limiting (Kneteman and Mercer, 2005).

Factors affecting animal survival include engraftment efficacy, kinetics of repopulation and susceptibility to infection with HBV/HCV after transplantation of the human hepatocytes (Vanwolleghem et al., 2010). Engrafted human hepatocytes in the uPA transgenic liver have a proliferative advantage over the sick endogenous murine hepatocytes which leads to progressive humanization of the rodent liver (Meuleman et al., 2005, Mercer et al., 2001, Dandri et al., 2001). The donor cell type determines not only animal survival but also engraftment efficacy and susceptibility of the chimeric liver to infection with HBV or HCV. Interestingly, graft requirements for HBV infectivity are less stringent, leading to a 100% infection rate even in animals with human albumin levels of less than 1 mg/ml. Furthermore, in HBV and HCV co-infected animals no apparent viral interference has been noted. Both observations are important and independent confirmations of published data (Tsuge et al., 2005, Hiraga et al., 2009). In contrast to the reproducible rapid viral kinetics after HCV inoculation, slower and divergent HBV viral kinetics have been observed after HBV infection (Vanwolleghem et al., 2010). A similar lag phase before HBV reaches peak titers has been described after experimental HBV inoculations in chimpanzees, which is thought to reflect the slower spread of HBV throughout the liver (Wieland and Chisari, 2005). The superior survival rate, the higher engraftment efficacies and infection rates and the logistic constraints posed by isolating fresh hepatocytes, are all in favour of the use of commercially available cryopreserved cells for the humanization of the uPA transgenic mouse liver (Vanwolleghem et al., 2010).

Neither donor age nor donor cell viability predict engraftment potential however the underlying medical condition of the donor may have an impact on the proliferative capacity of

donor cells (Vanwolleghem et al., 2010). Furthermore, the freezing protocol itself influences the migratory and proliferative capacities of cryopreserved cells (Hengstler et al., 2000).

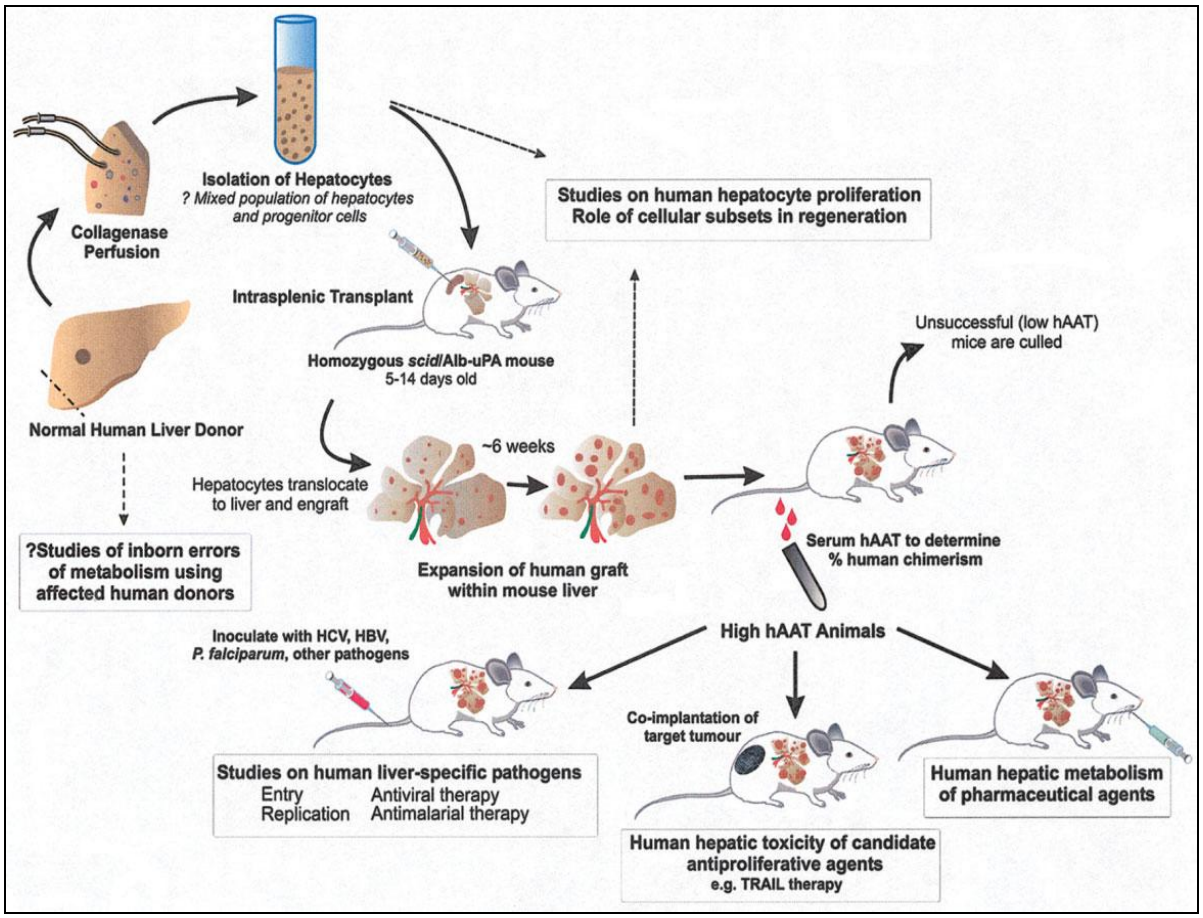


Figure 1.8. Schematic representation of the production of uPA-SCID transgenic mice and their potential scientific research applications. Permission obtained for copyright (Kneteman and Mercer, 2005).

1.6. OBJECTIVES OF THE STUDY

The main objective of the study was to functionally characterize subgenotype A1, which has unique characteristics including the G1862T mutation (Hou et al., 2002). It has been previously shown that the presence of the G1862T mutation, commonly present in South African subgenotype A1 isolates, in the precore/core region of HBV results in the accumulation of a precore/core mutant protein in the ERGIC leading to impaired secretion of HBeAg (Chen et al., 2008). Transfection of Huh-7 cells with constructs containing the G1862T mutation resulted in an increase in apoptosis probably as a result of the accumulation of the precore/core mutant protein in the ERGIC (Viana, 2005).

The objectives of the present study were therefore to compare the effect of subgenotype A1 with or without the mutation to other genotypes/ subgenotypes of HBV *in vivo* using:

- Infection of the uPA-SCID transgenic mouse model (Chapter 2)

- Population based study using sera from geographically diverse regions of the world from patients with different disease manifestations of HBV infection (Chapter 3).

CHAPTER 2

FUNCTIONAL CHARACTERISATION OF HBV SUBGENOTYPE A1 ISOLATES *IN VIVO*: USING A UROKINASE-TYPE PLASMINOGEN ACTIVATOR SEVERE COMBINED IMMUNODEFICIENCY (uPA-SCID) TRANSGENIC MOUSE MODEL

2.1. INTRODUCTION

The influence of HBV viral factors such as genotype/subgenotype, HBV DNA levels (Chan et al., 2009b) and mutations on disease progression and clinical outcome of HBV infection have been well documented (Taylor et al., 2009). Subgenotype A1 is the most common HBV strain found in South Africa (Kramvis et al., 2002). Unique to this subgenotype is the guanine to thymine 1862 mutation (Tanaka et al., 2004) that is present in 24% of HBeAg negative southern African carriers (Kramvis and Kew, 2007). Studies have shown that the G1862T mutation is associated with HCC in the southern African black population, particularly in HBeAg negative patients and may therefore play a part in the development of HCC (Kramvis et al., 1998).

In order to investigate the *in vivo* effect of HBV infection on cell death, a HBV susceptible animal model was used. The uPA-SCID transgenic mouse provides a small animal model that can be infected with HBV positive human sera.

The objectives of this part of the study were therefore to:

- Infect uPA-SCID transgenic mice with subgenotype A1 HBV and to follow the replication of this subgenotype in this animal model.

- Compare infection of the uPA-SCID transgenic mice infected with different genotypes and subgenotypes, namely: subgenotype A1, subgenotype A1 (G1862T mutant), subgenotype A2 and genotype D.

- Determine the effect of HBV infection on apoptosis in the human hepatocytes grafted in the uPA-SCID transgenic mice.

2.2. MATERIALS AND METHODS

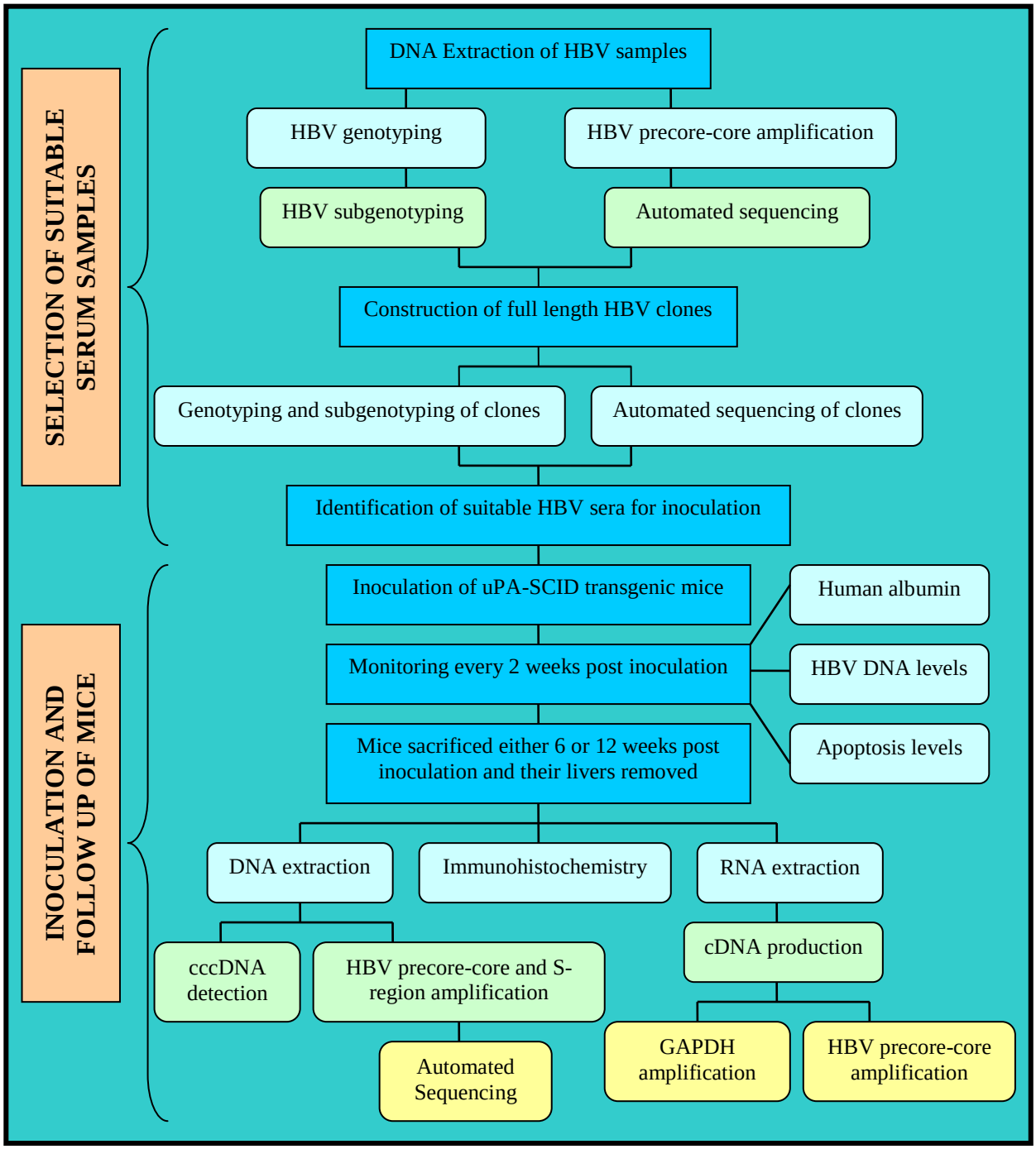


Figure 2.1. Techniques used to monitor HBV infection in the uPA-SCID transgenic mouse model. These are divided into two sections, one focusing on the techniques utilized for the selection of suitable serum samples for inoculation of the mouse model, the other focusing on the techniques used for uPA SCID mice inoculation and techniques used to follow up the HBV infection.

2.2.1. HBV DNA POSITIVE SERA

Stored HBV positive serum samples from acute hepatitis B patients were selected at random and screened for use as potential inocula of the transgenic mice. The requirement of relatively larger volumes of serum for the uPA SCID mice precluded the testing for eAg. Informed consent to use these sera for research purposes was obtained from the patients. The study was approved by the Human Ethics Committee of the University of the Witwatersrand (Appendix B).

2.2.2. DNA EXTRACTION FROM HBV POSITIVE SERA

Total DNA was extracted from the serum samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the blood and blood fluid spin protocol provided by the manufacturer. Briefly, 200 µl of serum was used and the cells lysed with proteinase K. The DNA was then bound to a QIAamp membrane before two stringent washes were performed. The DNA was finally eluted in 75 µl of nuclease-free sterile water (Fermentas, PureExtreme, EU) and stored at -20 ^{o} C. A blank (sterile water sample) reaction was performed in conjunction with a negative control.

2.2.3. GENOTYPING PCR AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

HBV genotype was determined using the Lindh technique (Lindh et al., 1997).

2.2.3.1. *Genotyping PCR*

The reaction mixture consisted of 10 µl of 10 X NH_4, 6 µl of 50 mM $MgCl_2$, 8 µl of 40 mM dNTP mix, 5 µl each of 20 nM primer P7 and P8 (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA polymerase (Bioline, London, England) and 1 µl of DNA made up to a final volume of 100 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was carried out using the Mastercycler Gradient (Eppendorf, Hamburg, Germany). A 10 µl aliquot of the PCR product was electrophoresed on a 1 % agarose gel (Appendix A1). The gel was run at 100 volts for 2 hours followed by visualization using an ultraviolet trans-illuminator. A 100 bp and/or 1 kb molecular weight markers (Promega, Madison, U.S.A) were also run to estimate the size of

the PCR product. The gel image was captured using the Gel Doc XR System (Bio-Rad, Milan, Italy).

2.2.3.2. *RFLP analysis*

Amplicons were digested with restriction enzymes to generate specific fragments characteristic of the different HBV genotypes. The digestion mixture consisted of 2 µl 10 X NE buffer (supplied with restriction enzyme), 0.5 µl restriction enzyme, 15 µl PCR product made up to a final volume of 20 µl with 2.5 µl of nuclease-free sterile water. Restriction enzymes *Hinf*I and *Tsp509*I (New England Biolabs, Ipswich, Massachusetts, U.S.A) were utilized in separate reactions. The reactions containing *Hinf*I were incubated at 37 ^{o} C while the *Tsp509*I reaction mixtures were incubated at 65 ^{o} C, for 3 hours. 5 µl of 6 X gel loading dye (Appendix A3) was added to end the restriction reaction. A 20 µl aliquot of each of the digests was electrophoresed on a 3 % composite agarose gel (Appendix A4). The gel was run at 30 V overnight to ensure maximum separation of the DNA fragments. The Gel Doc XR System was used to capture the gel image. The band patterns produced by both *Hinf*I and *Tsp509*I restriction enzymes were then analyzed according to Lindh *et al*, and the HBV genotype determined (Lindh et al., 1997).

2.2.4. SUBGENOTYPIC PCR AND RFLP ANALYSIS OF GENOTYPE A ISOLATES

2.2.4.1. *Subgenotyping PCR*

The reaction mixture consisted of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 1 µl each of 20 µM primer 522F and 1192R (Table 2.1), 0.4 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of viral DNA made up to a final volume of 50 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the Mastercycler Gradient. A 10 µl aliquot of the PCR product was electrophoresed at 100 volts for 2 hours on a 1 % agarose gel. A 100 bp and/or 1 kb molecular weight markers were also run to estimate the size of the PCR product. The gel image was then captured using the Gel Doc XR System.

2.2.4.2. *RFLP analysis*

PCR products were digested using *Stu*I restriction enzyme (New England Biolabs, Ipswich, Massachusetts, U.S.A). The digestion mixture consisted of 2 µl 10 X NE buffer, 0.5 µl of *Stu*I restriction enzyme, 10 µl of PCR product made up to a final volume of 20 µl with 7.5 µl of nuclease-free sterile water. The reaction mixture was incubated at 37 ^{o} C for 1 hour, followed by the addition of 5 µl of 6 X gel loading dye to terminate the restriction reaction. A 20 µl aliquot of the digested PCR product was electrophoresed on a 3 % composite agarose gel. The gel was run at 30 V overnight to ensure maximum separation of the DNA fragments before its image was captured using the Gel Doc XR System. The band patterns generated for each of the samples was evaluated to determine the subgenotype of A (Kew et al., 2005).

2.2.5. AMPLIFICATION AND SEQUENCING OF THE HBV PRECORE/CORE REGION

The HBV precore/core region of the samples was amplified and sequenced to determine the presence or absence of the 1862 mutation.

2.2.5.1. *Precore/core nested PCR*

The reaction mixture for the first round PCR consisted of 2.5 µl of 10 X NH_4, 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 40 mM dNTP mix, 1.25 µl each of 20 nM primer 1730F and 2043R (Table 2.1), 0.1 µl of 5 U/µl BioTaq DNA polymerase and 2.5 µl of DNA made up to a final volume of 25 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the Mastercycler Gradient. For the second round the reaction mixture was made up of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 2.5 µl each of 20 nM primer 1763F and 1966R (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of the first round PCR product made up to a final volume of 50 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the Mastercycler Gradient. A 10 µl aliquot of the PCR product was electrophoresed at 100 volts for 2 hours on a 1 % agarose gel. A 100 bp and/or 1 kb molecular weight markers were run to determine the size of the PCR product. The gel image was captured using the Gel Doc XR System.

2.2.5.2. *Automated Sequencing*

Products amplified by PCR were sequenced by Inqaba Biotechnical Industries Ltd (Pretoria, South Africa) using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA) and the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). The primers used for sequencing the PCR amplicons were the same as those used to generate the second round PCR products in the nested PCR. Chromatograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia).

2.2.6. CLONING PROCEDURE

HBV genomes that were sequenced and found to be one of the following: subgenotype A1, subgenotype A1 with the G1862T mutation, genotype D as well as subgenotype A2 were cloned.

2.2.6.1. *Full length PCR*

The complete genome of the virus was amplified using the method described previously (Gunther et al., 1995). The Expand High Fidelity System (Roche, Mannheim, Germany) was used. The reaction mixture consisted of 2 µl of 10 X buffer with 15 mM $MgCl_2$, 0.2 µl of 25 mM dNTP mix, 1.25 µl of 20 µM primer P1 and P2 (Table 2.1), and 5 µl of DNA made up to 20 µl with nuclease-free sterile water. The enzyme mix was made up of 0.5 µl of 10 X buffer, 0.75 µl of Expand enzyme (3.5 U/µl), and 3.75 µl of nuclease-free sterile water. The reaction mix was preheated to 94 ^{o} C for 2 minutes and then cooled to 58 ^{o} C at which point 5 µl of enzyme mix was added. This was followed by 40 cycles in the cycling profile shown in Table 2.1. The reaction was carried out on the MyCycler (Bio-Rad, U.S.A). A 5 µl aliquot of the PCR product was electrophoresed on a 1 % agarose gel. The gel was run at 80 volts for 2 hours followed by visualization using an ultraviolet trans-illuminator. A 100 bp and/or 1 kb molecular weight markers were also run to estimate the size of the PCR product. The gel image was captured using the Gel Doc XR System.

2.2.6.2. *A' overhang addition and PCR product purification*

Once the presence of the ±3.2 kb fragment was confirmed, 2 µl of Takara Ex Taq 5 U/µl (Takara Bio Inc, Shiga, Japan) was added to the remaining 15 µl full length PCR product and incubated at 72 ^{o}C for 15 minutes to allow for the addition of the A' overhang. This mixture was purified using the MinElute Reaction Cleanup Kit (Qiagen, Hilden Germany) as per the manufacturer's protocol and eluted in 10 µl of nuclease-free sterile water.

2.2.6.3. *Cloning of full length HBV product into pCR XL-TOPO vector*

The cloning of the purified full length HBV PCR product was carried out as per the TOPO-XL Cloning Kit (Invitrogen, California, U.S.A) manufacturer's instructions. Briefly, 10 µl of Crystal Violet Loading dye was added to the 10 µl purified PCR product and the mixture loaded into a 0.8 % Crystal Violet agarose gel (Appendix A5). A 1 kb molecular weight marker (Promega, Madison, U.S.A) was used to determine the size of the PCR product. The gel was run at 60 volts for 1 hour and then placed on a white light box to visualize the PCR fragment. The 3.2 kb PCR fragment was excised from the gel and incubated at 45 ^{o}C with sodium iodide until melted. At room temperature the binding buffer was added and the entire mixture placed in a SNAP purification column and centrifuged several times until the DNA was bound to the column. The column was washed and then dried by centrifugation. The DNA was eluted in 20 µl of TE buffer.

For the cloning reaction 10 µl of the DNA was added to 1 µl of the pCR-XL-TOPO vector and mixed gently. A negative vector-only control was also included to rule out contamination. After a 5 minute room temperature incubation, 2 µl of TOPO Cloning Stop solution was added. The entire mixture was transferred to a vial of One Shot cells and incubated on ice for 30 minutes followed by a heat-shock treatment at 42 ^{o}C for 30 seconds. The vial was immediately incubated on ice for 2 minutes. SOC medium was added and the vial incubated at 37 ^{o}C for 1 hour with horizontal shaking. Aliquots of 50 µl, 100 µl and 150 µl from each transformation were spread onto kanamycin plates (Appendix A7) and incubated at 37 ^{o}C overnight to allow for sufficient individual colony growth. Individual colonies were restreaked onto fresh kanamycin plates and incubated at 37 ^{o}C. 5 ml aliquots of kanamycin LB Broth (Appendix A8) were inoculated with cells from individual colonies and incubated

overnight at 37 ^{o}C in the Orbital Shaker Incubator LM-510 (YIH DER, Germany) at 150 rpm to allow for plasmid growth. Glycerol stocks of all of the clones were prepared by adding 500 µl of the clone culture to an equal volume of autoclaved glycerol and then stored at -70 ^{o}C. The plasmid DNA was then isolated using the Genelute Plasmid Mini-Prep Kit (Sigma, U.S.A) as per the manufacturer's instructions. The plasmid DNA was eluted in 50 µl of nuclease-free sterile water.

2.2.6.4. *Identification of positive clones*

In order to determine which clones contained the full length HBV amplicon, *Eco*RI enzyme (New England Biolabs, Ipswich, Massachusetts, U.S.A) digestions were performed. The reaction mixture consisted of 1 µl *Eco*RI enzyme, 1 µl NE buffer and 3 µl of plasmid DNA made up to 10 µl with nuclease-free sterile water. A drop of sterile mineral oil was added to the surface of the 10 µl mixture and the tube incubated at 37 ^{o}C for 2 hours. The enzymatic reaction was terminated by the addition of 4 µl of 6 X gel loading dye placed beneath the mineral oil. The entire 14 µl sample was then loaded onto a 1 % agarose gel and run at 30 volts overnight. A 100 bp and 1 kb molecular weight markers were also run to determine the size of the digestion-generated DNA fragments. The gel was visualized using an ultraviolet trans-illuminator and the image captured using the Gel Doc XR System. Clones were considered positive when two HBV fragments, approximately 1.8 kilobases and 1.4 kilobases in size, were detected. Clones that did not possess these HBV fragments were discarded. For each serum sample approximately 100 clones were genotyped and subgenotyped.

2.2.7. GENOTYPING AND SUBGENOTYPING OF CLONES

To determine whether more than one genotype or subgenotype was present in each of the selected serum samples, genotyping and subgenotyping using RFLP analysis was carried out on all of the positive clones as described in sections 2.2.2 and 2.2.3 except for the following modifications.

2.2.7.1. *Genotyping PCR*

The reaction mixture consisted of 10 µl of 10 X NH_4, 12 µl of 50 mM $MgCl_2$, 2 µl of 40 mM dNTP mix, 5 µl each of 20 nM primer P7 and P8 (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA

polymerase and 1 µl of clone DNA made up to a final volume of 100 µl with nuclease-free sterile water.

2.2.7.2. *Subgenotyping PCR*

The reaction mixture consisted of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 1 µl each of 20 µM primer 522F and 1192R (Table 2.1), 0.4 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of clone DNA made up to a final volume of 50 µl with nuclease-free sterile water.

2.2.8. AUTOMATED SEQUENCING

Once it had been determined that only one genotype and subgenotype existed per serum sample, ten clones from each serum sample were sent for full length HBV sequencing. The kanamycin plates containing the streaked clones were sent to and sequenced by Inqaba Biotechnical Industries Ltd (Pretoria, South Africa) using a SpectruMedix model SCE 2410 automated sequencer and the ABI Big Dye Terminator Cycle Sequencing kit version 3.1. Several primers (See Table 2.2 and Figure 2.3) were used to sequence the entire full length of HBV contained within each clone. Chromatograms of the sequences generated were inspected with Chromas software version 1.45. Serum samples which contained HBV closest to the relevant consensus sequence were selected as inocula for the uPA-SCID transgenic mice.

2.2.9. PHYLOGENETIC ANALYSIS

Complete HBV genome sequences were compared with corresponding sequences of HBV from GenBank. Multiple sequence alignments were carried out using Dambe (Xia and Xie, 2001). The complete alignments were edited manually in GeneDoc (Nicholas, 1997) and fed into PHYLIP (Phylogeny inference package) version 3.65(Felsestein, 1995). SEQBOOT, DNADIST and NEIGHBOR were used for bootstrapping of 1000 data sets while CONSENSE was used to compute a consensus tree. Trees were visualized using TreeView Win 32 (Page, 1996) software program.

2.2.10. MONITORING OF HBV INFECTION IN THE uPA-SCID TRANSGENIC MICE

2.2.10.1. *Isolation of human hepatocytes and transfer into uPA-SCID mice*

The uPA-SCID transgenic mice experiments were performed in Belgium. Human hepatocytes were isolated via a standard collagenase digestion from tumour-free liver fragments collected from a single patient undergoing a partial hepatectomy (Meuleman et al., 2005). Written informed consent was obtained from the patient from whom the liver biopsy was obtained and all experiments were approved by the Ethics Committee of the Ghent University Hospital. Ten homozygous uPA-SCID mice were transplanted with one million human hepatocytes two weeks after birth.

2.2.10.2. *Inoculation of the uPA-SCID mice with HBV-positive sera*

Five weeks later, the grafted mice were inoculated with HBV-positive serum. Each mouse was infected with 2 x 10^5 IU/ml of the relevant HBV serum sample via an intraperitoneal injection. Two mice were infected with HBV genotype D, four with wildtype subgenotype A1, three with subgenotype A1 with the G1862T mutation and one with subgenotype A2. At selected time intervals the animals were bled and the serum stored at -80 ^{o} C until analyzed. One of the subgenotype A1 wildtype and one of the subgenotype A1 (G1862T) mice perished during the course of the experiment and were therefore not included in the analysis.

2.2.10.3. *Human Albumin Quantification*

An in-house enzyme-linked immunosorbent assay for human albumin was used to monitor graft survival (Meuleman et al., 2005). Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with 2.5 µg/ml of goat anti-albumin antibodies (Bethyl Laboratories, Montgomery, Texas, U.S.A). The plates were blocked then washed several times before the diluted samples (1:100) and calibrators were added. The human albumin was bound to the plate and detected with a horse radish peroxidase (HRP)-conjugated goat-anti-human albumin antibody (10 ng/ml) (Bethyl Laboratories, Mongomery, Texas, U.S.A) at 450 nm on the Model 680 Microplate Reader (BioRad, U.S.A).

2.2.10.4. *HBV DNA Quantification*

HBV DNA levels were quantified every two weeks using the Cobas Amplicor HBV Monitor test (Roche Diagnostics, Mannheim, Germany) to monitor successful HBV infection.

2.2.10.5. *Quantification of apoptosis*

The M30-Apoptosense ELISA (Peviva, Bromma, Sweden) was used for the quantification of the apoptosis-associated cytokeratin 18Asp396 (M30) neo-epitope in the mice serum as per the manufacturer's instructions (Figure 2.2).

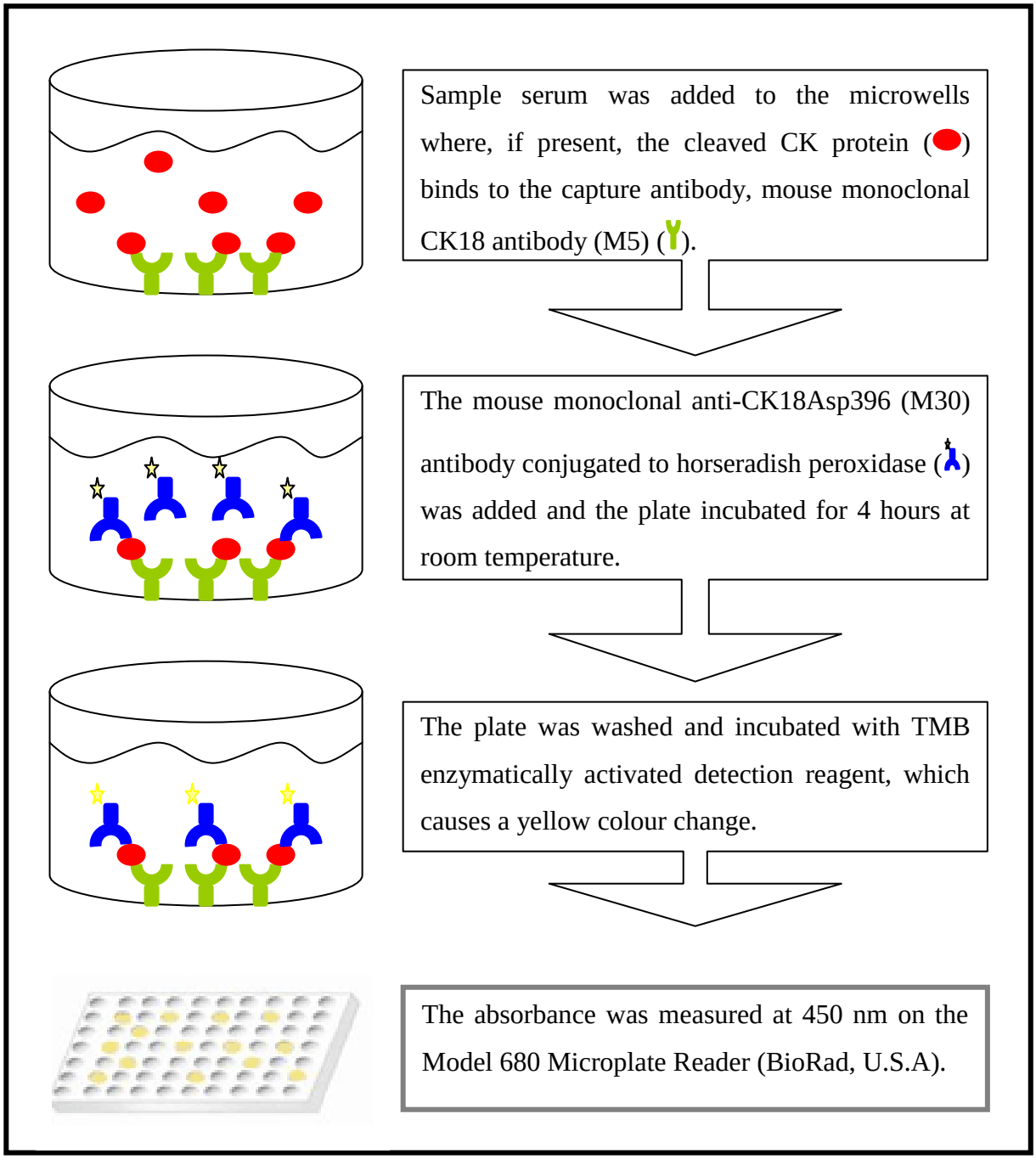


Figure 2.2. M30-Apoptosense ELISA plate assay.

The mice were sacrificed either at 6 or 12 weeks post inoculation and the livers stored at -80 ^{o}C for further analysis in South Africa.

2.2.11. DNA EXTRACTION FROM uPA-SCID TRANSGENIC MICE LIVERS

DNA was extracted from the 12 week infected transgenic mice livers as well as from controls, which included a non-infected uPA-SCID transgenic mouse liver and a HBV infected human liver sample, using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). The DNA was eluted in 50 µl of nuclease-free sterile water and stored at -20 ^{o}C. A blank (sterile water sample) reaction was performed in conjunction to rule out any contamination.

2.2.12. HBV cccDNA DETECTION BY REAL-TIME PCR

Real-time PCR was used to detect covalently closed circular (ccc) DNA, a key role player in the persistence of HBV infection, in the transgenic mice livers (Chen et al., 2004). To eliminate all linear double-stranded DNA the samples were treated with Plasmid-Safe ATP-dependent DNase (Epicentre Biotechnologies, Wisconsin, U.S.A) before real-time PCR was performed. The reaction mixture consisted of 1 µl of 25 mM ATP, 2.5 µl of 10 X reaction buffer, 2 µl of 40 U Plasmid-Safe DNase, and 10 µl of liver-extracted DNA made up to a final volume of 35 µl with nuclease-free sterile water. The mixture was placed at 37 ^{o}C for 1 hour to allow for enzymatic digestion followed by a 30 minute incubation at 70 ^{o}C to inactivate the DNase. The real-time PCR reaction mixture consisted of: 25 µl of 2 X TaqMan Universal PCR Master Mix (Applied Biosystems, New Jersey, U.S.A), 4.5 µl each of 10 µM primer CCCF1 (5' ACT CTT GGA CTC BCA GCA ATG 3') and CCCR1 (5' CTT TAT ACG GGT CAA TGT CCA 3'), 10 µM of CCCP FAM/TAMRA-labelled probe (5' CTT TTT CAC CTC TGC CTA ATC ATC TCW TGT TCA 3') and 5 µl of DNase-treated DNA made up to a final volume of 50 µl with nuclease-free sterile water. The thermal profile was performed using the ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Singapore) as follows: following 2 minutes at 50 ^{o}C and 10 minutes at 95 ^{o}C, there were 40 cycles at 95 ^{o}C for 15 seconds with data acquisition at the end of each cycle at 61.5 ^{o}C for 1 minute. A blank (sterile water) and positive HBV DNA sample were included as controls. A 15 µl aliquot of the real-time PCR product was electrophoresed on a 1 % agarose gel for visual confirmation of the real-time results. The gel was run at 100 volts for 2 hours followed by visualization using an ultraviolet trans-illuminator. A 100 bp molecular weight marker was included to

estimate the size of the PCR product. The gel image was then captured using the Gel Doc XR System.

2.2.13. TESTING FOR THE PRESCENCE OF THE ORIGINAL HBV INOCULUM STRAIN IN THE uPA-SCID TRANSGENIC MICE LIVERS

To confirm that the original HBV strain used to infect the transgenic mice was the replicating virus present in the mouse liver, the HBV precore/core region as well as the S region were amplified and sequenced. The precore/core PCR amplification and detection were performed as described in section 2.2.4. The S-region nested PCR was performed as follows:

The reaction mixture for the first round PCR consisted of 2.5 µl of 10 X NH_4, 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 40 mM dNTP mix, 1.25 µl each of 20 nM primer 231F and 801R (Table 2.1), 0.1 µl of 5 U/µl BioTaq DNA polymerase and 2.5 µl of DNA made up to a final volume of 25 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the MyCycler. For the second round the reaction mixture was made up of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 2.5 µl each of 20 nM primer P7 and P8 (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of the first round PCR product made up to a final volume of 50 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the MyCycler. A 10 µl aliquot of the PCR product was electrophoresed at 100 volts for 2 hours on a 1 % agarose gel. A 100 bp and/or 1 kb molecular weight markers were also run to estimate the size of the PCR product. The gel image was then captured using the Gel Doc XR System. The precore/core and S-region amplicons were sent to and sequenced by Inqaba Biotechnical Industries Ltd as explained in section 2.2.4.2.

2.2.14. TECHNIQUES USED TO CONFIRM HBV TRANSCRIPTION

2.2.14.1. *RNA extraction from uPA-SCID transgenic mice livers*

RNA was extracted from the 12 week infected transgenic mice as well as from controls, which included a non-infected uPA-SCID transgenic mouse and an HBV infected human liver sample, using the RNeasy Micro Kit (Qiagen, Hilden Germany) according to the tissues protocol provided by the manufacturer. The RNA was eluted in 14 µl of RNase-free water

and stored at -20 ^{o}C. A blank (sterile water sample) reaction was performed in conjunction to rule out any contamination. The quantity of RNA extracted from each liver sample was determined using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.) while the quality of the RNA was evaluated by running 1 µg RNA of each sample on a 1 % agarose gel. The gel was run at 110 volts for 30 minutes followed by visualization using an ultraviolet trans-illuminator. The gel image was then captured using the Gel Doc XR System.

2.2.14.2. *Reverse transcription PCR (RT-PCR)*

The RT-PCR master mix was made up of reagents from the GeneAmp RNA PCR Core Kit (Applied Biosystems, New Jersey, U.S.A). The reaction mixture consisted of 2 µl of 10 X PCR buffer II, 4 µl of 25 mM $MgCl_2$, 8 µl of 10 mM dNTP mix, 1 µl of 20 U/µl RNase Inhibitor, 1 µl of 50 µM Oligo d(T)16, 1 µl of 50 U/µl MuL V Reverse Transcriptase and 1 µg of liver-extracted RNA made up to a final volume of 20 µl with nuclease-free sterile water. The cycling profile of 22 ^{o}C for 12 minutes, 42 ^{o}C for 15 minutes and 99 ^{o}C for 5 minutes was performed using the MyCycler. Non-reverse transcribed negative controls were prepared in an identical manner except that nuclease-free sterile water was used in place of the MuL V Reverse Transcriptase enzyme.

2.2.14.3. *Glyceraldehyde – 3- phosphate dehydrogenase (GAPDH) PCR*

A fragment of the GAPDH housekeeping gene was amplified by PCR. The reaction mixture was made up of 2.5 µl of 10 X KCl buffer, 0.5 µl of 40 mM dNTP mix, 0.625 µl of each 20 µM GAPDHF and GAPDHR primer (Table 2.1), 0.1 µl of 5 U/µl BioTaq DNA polymerase and 4 µl of cDNA made up to a final volume of 25 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the MyCycler. A 10 µl aliquot of the PCR product was electrophoresed at 100 volts for 2 hours on a 1 % agarose gel. A 100 bp and/or 1 kb molecular weight markers were also run to estimate the size of the PCR product. The gel image was then captured using the Gel Doc XR System.

2.2.14.4. *Precore/core nested PCR used for confirmation of HBV transcription activity*

Amplification of the HBV precore/core region was performed as per section 2.2.4.1 except that cDNA was used as the template.

2.2.15. IMMUNOHISTOCHEMICAL STAINING

The immunohistochemical staining was performed under the supervision of Professor Patterson in the Department of Anatomical Pathology at the University of the Witwatersrand Medical School. In order to ensure unbiased results, samples were numbered randomly. The immunohistochemical staining was performed on 4 mm sections of formalin-fixed, paraffin-embedded tissue sections on 2 % APES coated microscope slides, using the DakoCytomation Autostainer (DakoCytomation, Denmark). Sections were deparaffinized and rehydrated through immersion in xylene and graded alcohol. Heat-induced antigen retrieval was performed using a microwave and a pressure cooker. This entailed heating 10 mM citrate buffer (pH6) in the pressure cooker and then using the microwave after its addition to the slides. After cooling, endogenous peroxidase was blocked through the incubation of slides in 3 % H_2O_2 in distilled water for 15 minutes. Slides were then thoroughly washed in Tris-buffered saline (TBS) (Dakocytomation, Denmark) (pH 7.6) containing 0.1 % Tween20, before blocking any non-specific antigen activity through the immersion of the slides in 5 % normal goat sera. Sections were subsequently exposed to the monoclonal antibodies (dilutions prepared in antibody diluent; DakoCytomation, Denmark) in a humidified atmosphere at room temperature for 1 hour. After rinsing thoroughly with TBS, slides were then treated with a peroxidase-conjugated polymer and antibody (i.e. secondary antibody) directed against rabbit and mouse immunoglobulin for 30 minutes (ChemMate Dako EnVision Detection kit, DakoCytomation, Denmark). Slides were then washed and incubated with 3,3'-diaminobenzidine (DAB) chromogen and H_2O_2 for 7 minutes. Sections were then washed in distilled water, counterstained with haematoxylin, dehydrated and mounted with a cover slip. The antigen-antibody dilution sources used were the rabbit polyclonal hepatitis B core antigen (HBcAg) 1:120 (Signet) and the mouse monoclonal hepatitis B surface antigen (HBsAg) (clone S1-210) 1:100 (Signet). Images were captured with the Olympus Soft Imaging Solutions, analySIS docu V 5.0 Build 1243, using an Olympus BX41 Microscope and ColorView1 digital camera attached to an Olympus 0.5 c mount adapter.

Table 2.1: PCR Primers and Cycling Profiles used for amplification

Primer	Position*	Sequence	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Reference
P7 P8	256-278 796-776	5' GTG GTG GAC TTC TCT CAA TTT TC 3' 5' CGG TAW AAA GGG ACT CAM GAT 3'	94 °C 3 mins	94 °C 45 secs	53 °C 60 secs	72 °C 90 secs	72 °C 7 mins	Lindh *et al*, 1997
522+ 1192-	522-541 1192-1173	5' CCT GCA CGA CTC CTG CTC AA 3' 5' CGT CAG CAA ACA CTT GGC AC 3'	94 °C 3 mins	94 °C 45 secs	60 °C 60 secs	72 °C 90 secs	72 °C 7 mins	Kew *et al*, 2005
1730+ 2043-	1730-1747 2043-2022	5' CTG GGA GGA GTT GGG GGA 3' 5' CAA TGC TCA GGA GAC TCT AAG G 3'		94 °C 30 secs	62 °C 50 secs	72 °C 50 secs	72 °C 7 mins	Kramvis *et al*, 1997
1763+ 1966-	1763-1783 1966-1946	5' GGT CTT TGT ACT ACG AGG ATG 3' 5' GTC AGA AGG CAA AAA CGA GAG 3'		94 °C 30 secs	58 °C 50 secs	72 °C 50 secs	72 °C 10 mins	Kramvis *et al*, 1997
P1 P2	1820-1841 1825-1806	5' CTT TTT CAC CTC TGC CTA ATC A 3' 5' AAA AAG TTG CAT GGT GCT GG 3'	94 °C 2 mins	94 °C 40 secs	57 °C 90 secs	^68 °C 180 secs		Gunther *et al*, 1995
231+ 801-	231-249 801-782	5' TCA CAA TAC CGC AGA GTC T 3' 5'AAC AGC GGT ATA AAG GGA CT 3'		94 °C 60 secs	53 °C 5 mins	72 °C 3 mins	72 °C 10 mins	Lindh *et al*,1995
PC1+ COR-	1813-1836 2285-2266	5' CAT GCA ACT TTT TCA CCT CTG CCT 3' 5' GAG TGC GAA TCC ACA CTC CA 3'	94 °C 4 mins	94 °C 60 secs	65 °C 5 mins	72 °C 3 mins	72 °C 10 mins	Sugauchi *et al*, 2004
PC2+ COR-	1861-1881 2285-2266	5' TGT TCA AGC CTC CAA GCT GTG 3' 5' GAG TGC GAA TCC ACA CTC CA 3'	94 °C 3 mins	94 °C 45 secs	64 °C 60 secs	72 °C 90 secs	72 °C 7 mins	Sugauchi *et al*, 2004
GAPDHF GAPDHR		5' CCC TTC ATT GAC CTC AAC TAC ATG 3' 5' CAT GCC AGT GAG CTT CCC GTT CAG 3'		¶94 °C 60 secs	52 °C 30 secs	72 °C 90 secs	72 °C 10 mins	Weinberg *et al*, 2000

(+), sense; (-), anti-sense.

*Represents the nucleotide position of HBV (GenBank accession number X70185) where the *EcoR*1 cleavage site is position 1.

^The Extension time was increased by 120 seconds for every 10 cycles.

¶All cycling profiles ran for 40 cycles except for the GAPDH amplification which only ran for 30 cycles.

Table 2.2: Primers used for full length sequencing of HBV clones

Map number	Primer	Position	Sequence	HBV Region covered
	M13R	TOPO Vector	5’ AGC GGA TAA CAA TTT CAC ACA GG 3’	1822-2322
1	1843 (+)	1843-1863	5’ CTT GTT CAT GTC CTA CTG TTC 3’	1843-2343
2	2230 (+)	2230-2250	5’ GGA AGA GAA ACC GTC ATA GAG 3’	2230-2730
3	2408 (+)	2408-2437	5’ TCT CAA TCG CCG CGT CGC AGA AGA TCT CAA 3’	2408-2908
4	2548 (+)	2548-2568	5’ CCT GAT ATT CAT TTG CAC CAG 3’	2548-3048
5	2807 (+)	2807-2827	5’ CAT CAT TTT GTG GGT CAC CAT 3’	2807-107
6	112 (+)	112-128	5’ TAT CGT CAA TCT TCT CG 3’	112-612
7	521 (+)	521-540	5’ CCT GCA CGA CTC CTG CTC AA 3’	521-1021
8	970 (+)	970-989	5’ TAT TGA TTG GAA AGT ATG TC 3’	980-1440
9	1373 (+)	1372-1392	5’ TGG CTG CTA GGC TGT GCT G 3’	1373-1822
10	1416 (+)	1416-1436	5’ CCT TTG TCT ACG TCC CGT CGG 3’	1406-1822
11	2498 (-)	2498-2477	5’ AAG CCC AGT AAA GTT TCC CAC C 3’	2000-2498
12	2532 (-)	2532-2513	5’ CCA GTT AGG ATT AAA GAC AG 3’	2032-2832
13	2880 (-)	2880-2862	5’ TCG AGA GGG ACC GTC CAA G 3’	2380-2880
14	268 (-)	268-245	5’ AGA GAA GTC CAC CAC GAG TCT AGA 3’	2962-268
15	472 (-)	472-453	5’ CAA ACG GGC AAC ATA CCT TG 3’	3100-455
16	1009 (-)	1009-975	5’ CAA AAG ACC CAC AAT TCT TTG ACA TAC TTT CCA AT 3’	500-1011
17	1327 (-)	1327-1301	5’ CGA TAA GTT TTG CTC CAG ACC GGC TGC 3’	827-1327
	T7	TOPO Vector	5’ TAA TAC GAC TCA CTA TAG GG 3’	1322-1822

(+), sense; (-), anti-sense.

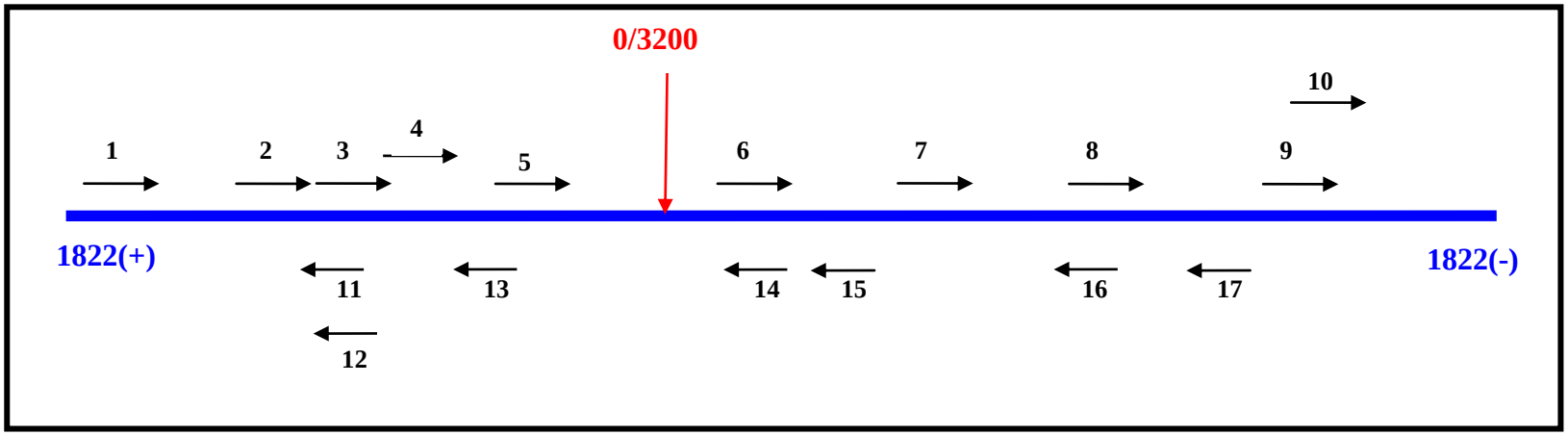


Figure 2.3. Relative position of the sequencing primers to the clone of the full length HBV amplicon.

2.3. RESULTS

2.3.1. CHARACTERISATION OF INOCULA

2.3.1.1. *Genotyping of HBV inocula*

Thirty HBV positive serum samples were randomly selected to be used as potential inocula for the mice. Using RFLP analysis twenty six of these sera were found to be infected with subgenotype A1, three with subgenotype A2 and one with genotype D. The precore/core region of all subgenotype A1 isolates was amplified and sequenced. Two of these samples were found to possess the guanine to thymine transversion at nucleotide position 1862 (G1862T) (Figure 2.4).

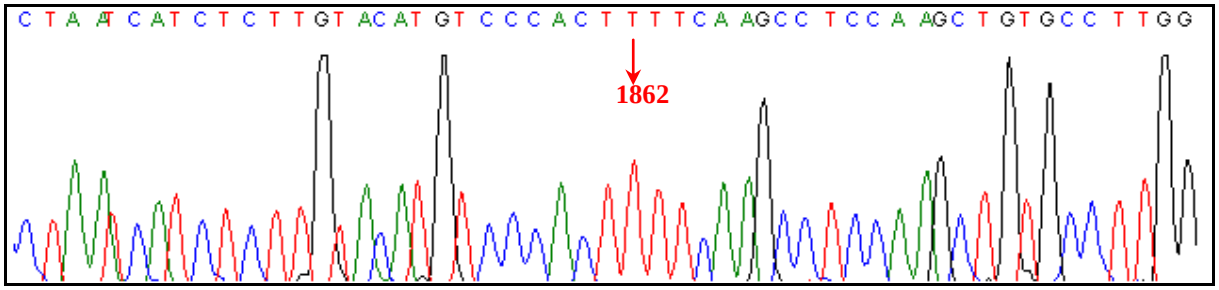


Figure 2.4. Chromatograph with the HBV precore-core 1862 mutation.

2.3.1.2. *Cloning*

A sample from each of the HBV groups: subgenotype A1 , subgenotype A1 with the 1862 mutation, subgenotype A2 and genotype D , was selected. The complete genome of HBV isolated from the serum samples was amplified (Figure 2.5) and cloned. Following *Eco*RI digestion, clones that contained the full length HBV genome produced two fragments, 1.8 and 1.4 kilobases. These clones were analysed using RFLP analysis to determine their genotype and subgenotype.

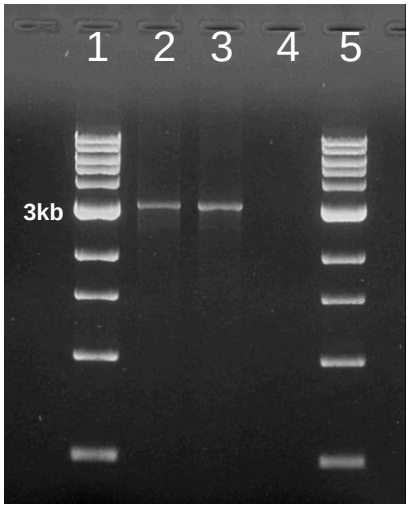


Figure 2.5. Representative gel image of the full length HBV amplicon. Lanes 1 and 5: 1 kb molecular weight marker. Lanes 2 and 3: 3.2 kb full length HBV amplicon. Lane 4: blank water control.

2.3.1.3. *Genotyping and subgenotyping of HBV clones*

One hundred HBV clones were analysed for each sample and found to belong to the same genotype (Figure 2.6) and subgenotype (Figure 2.7) identified originally in the serum, indicating that the major population of HBV belonged to a single genotype/subgenotype.

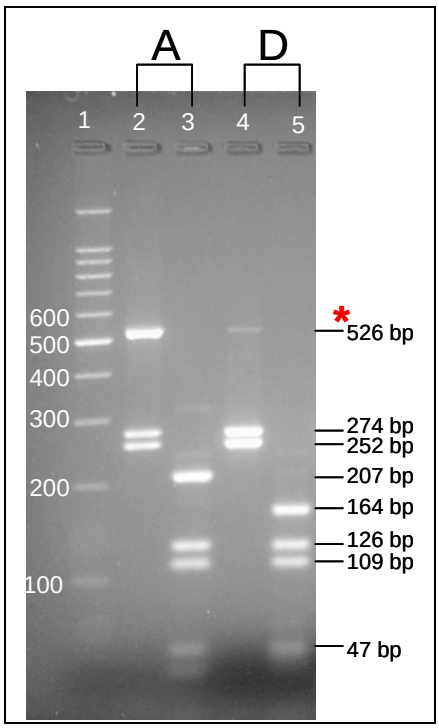


Figure 2.6. Representative gel image of RFLP analysis using *Hinf*I and *Tsp509*I restriction enzymes for the HBV clone genotype determination. Lane 1: 100 bp molecular weight marker. Lanes 2 and 4: *Hinf*I digestion mixtures. Lanes 3 and 5: *Tsp509*I digestion mixtures. Lanes 2-3 show the digestion pattern for genotype A while lanes 4-5 show a pattern for genotype D according to Lindh *et al*, 1995.
* residual unrestricted product

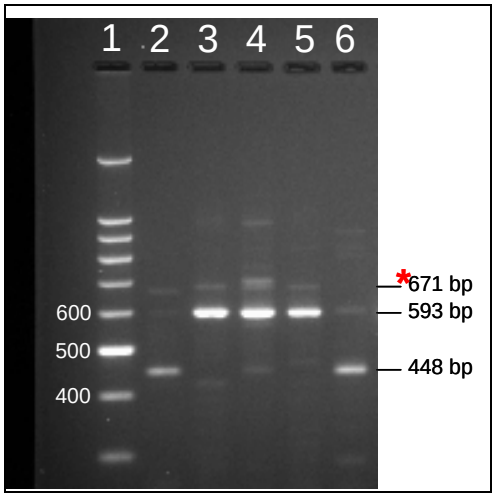


Figure 2.7. Representative gel image of RFLP analysis using *Stu*I restriction enzyme for genotype A subgenotyping of clones. Lane 1: 100 bp molecular weight marker. Lanes 2 and 6 have the subgenotype A2 samples and lanes 3 to 5 subgenotype A1 samples.
* residual unrestricted product

2.3.1.4. *Sequencing and Phylogenetic Analysis*

Eight to ten clones of each sample were selected at random and the complete HBV genome amplified and sequenced. The length of the complete genome for the subgenotype A1, subgenotype A1 1862 and subgenotype A2 isolates was 3 221 bp. Sequences revealed that all these isolates belonged to serotype *adw*2. The genotype D isolates were 3 182 bp in length and serotype *ayw*2 (Kramvis et al., 2005). The intragroup divergence of the clones from each serum sample was low indicating a relative homogenous viral population (Table 2.3). The intergroup divergence between subgenotype A1 and A1 with the 1862 mutation was found to be higher at 2.5 % but still within normal range for intragroup divergences (Kramvis et al., 2008). The complete genomes of the isolates were aligned with 27 sequences from GenBank and analysed phylogenetically (Figure 2.8). The clones clustered with the respective genotype/subgenotype and did not contain any unusual mutations, insertions or deletions when compared to the consensus sequence generated from sequences in GenBank.

Table 2.3: Mean nucleotide divergence (%)* of complete genome sequences of HBV clones obtained using DAMBE

	Intragroup divergence				**Intergroup divergence**
	A1	**A1 1862**	**A2**	**D**	**A1 vs A1 1862**
Nu. of clones	10	10	8	10	20
Complete Genome	0.09 ± 0.05 (0 - 0.18)	0.40 ± 0.11 (0.18 – 0.68)	0.02 ± 0.02 (0 – 0.62)	0.20 ± 0.07 (0.03 – 0.37)	2.50 ± 0.07 (2.38 – 2.72)

The sequences compared are the clones included in the phylogenetic analysis (ten A1, ten A1 1862, eight A2 and ten D). *The mean nucleotide divergence (%)± standard deviation (%) and the range in parenthesis.

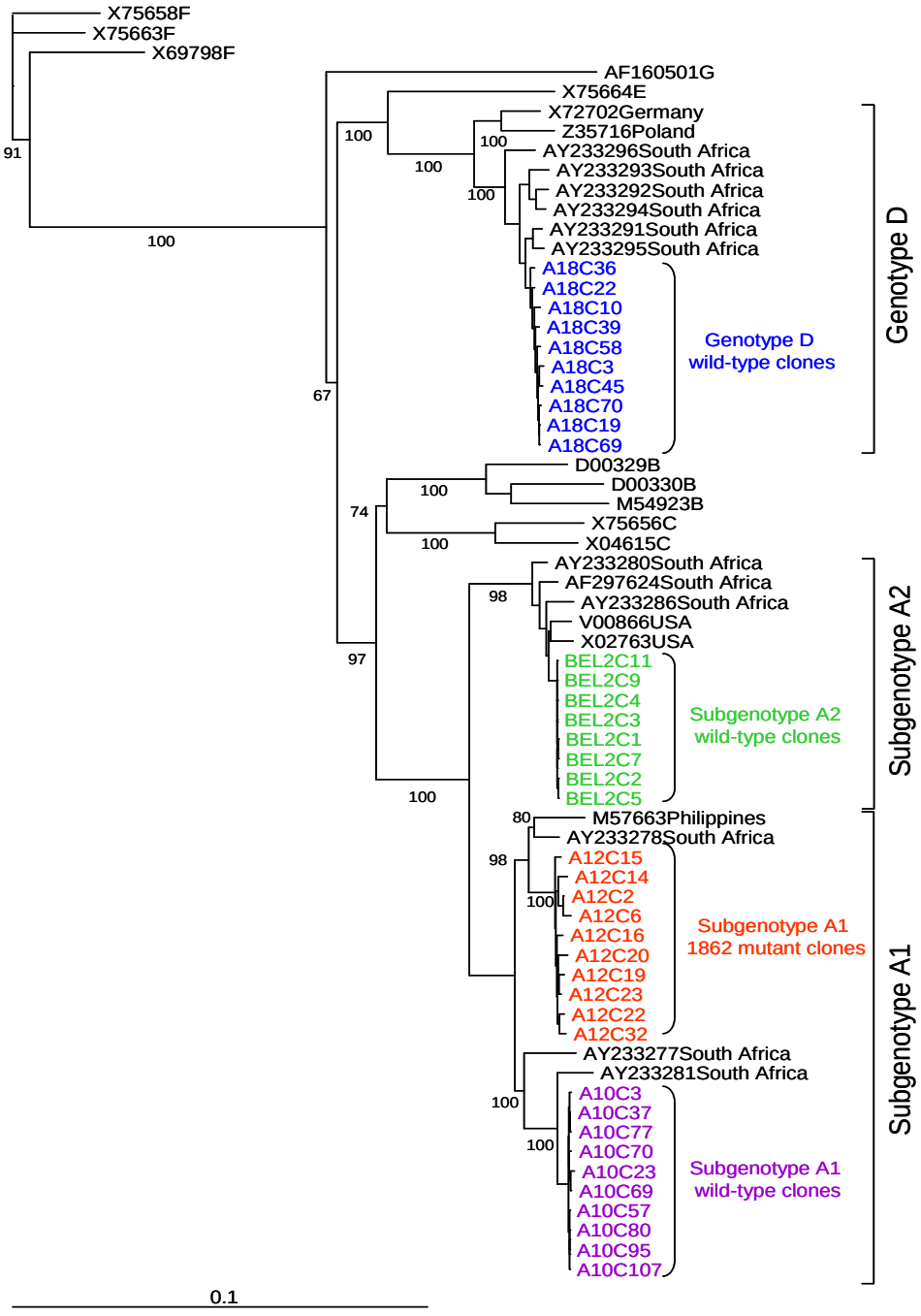


Figure 2.8: Phylogenetic relationship of the complete genome of HBV isolates cloned from human sera to representative sequences belonging to genotype A - G, obtained from GenBank, established using neighbour-joining. Bootstrap statistical analysis was performed using 1000 data sets and the numbers on the nodes indicate the percentage of occurrences.

2.3.2. INOCULATION AND MONITORING OF TRANSGENIC MICE

All the uPA-SCID mice were grafted with human hepatocytes from a single donor to ensure that no difference existed between the uPA-SCID transgenic mice utilised. The success of the graft was determined by human albumin detection. The uPA-SCID transgenic mice were inoculated with the serum samples from which the HBV was amplified and cloned. The mice sera were collected every 2 or 3 weeks post inoculation to monitor human albumin levels, indicative of the human hepatocyte graft survival, and HBV DNA levels to monitor the HBV infection. HBV DNA levels increased over time for all the infected mice. Mice infected with subgenotype A1 1862 mutant showed the highest levels of HBV DNA throughout the twelve weeks of infection (Figure 2.9). Mice infected with the other genotypes/subgenotypes showed similar HBV DNA levels. The HBV DNA levels were not associated with human albumin levels, which remained constant throughout the infection.

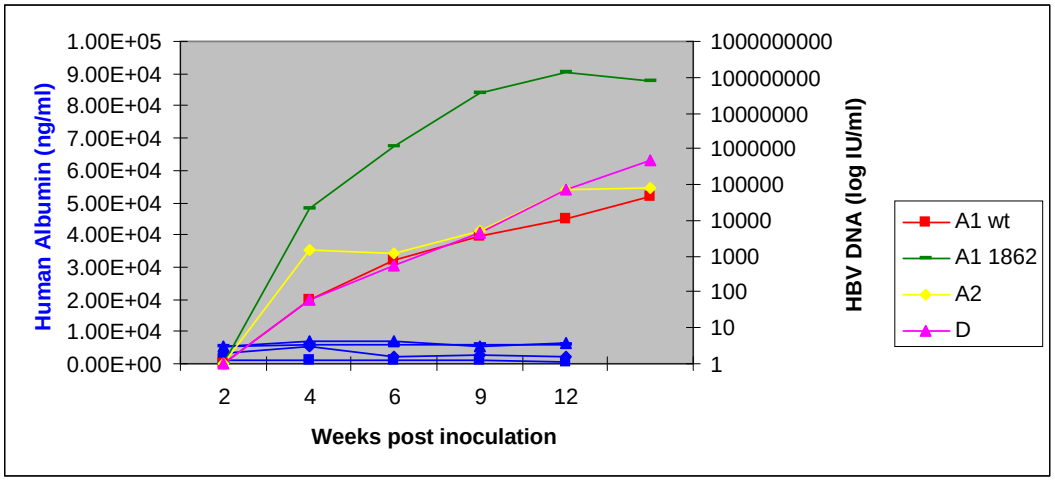


Figure 2.9. Mean human albumin and HBV DNA (log transformed) over the twelve week post inoculation period. Number of mice used for analysis: three for A1 wt, two for A1 1862, one for A2, and two for D.

M30 apoptosis levels were not associated with human albumin levels (Figure 2.10) i.e. an increase in apoptosis was not found to be dependent on the relative number of human hepatocytes. For the last three time points, subgenotype A1 1862 infected mice showed the highest apoptotic M30 levels.

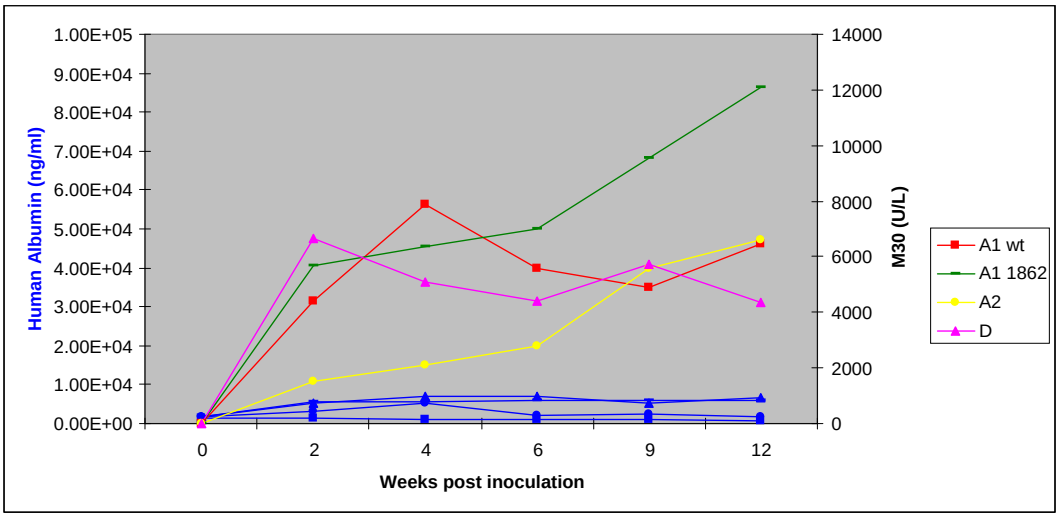


Figure 2.10. Human albumin and M30 apoptosis levels over the 12 week post inoculation period.

At twelve weeks post inoculation, mice infected with subgenotype A1 1862 showed the highest levels of HBV DNA and M30 (Figure 2.11). This relationship between M30 and HBV DNA levels however did not hold true for the mice infected with genotype D.

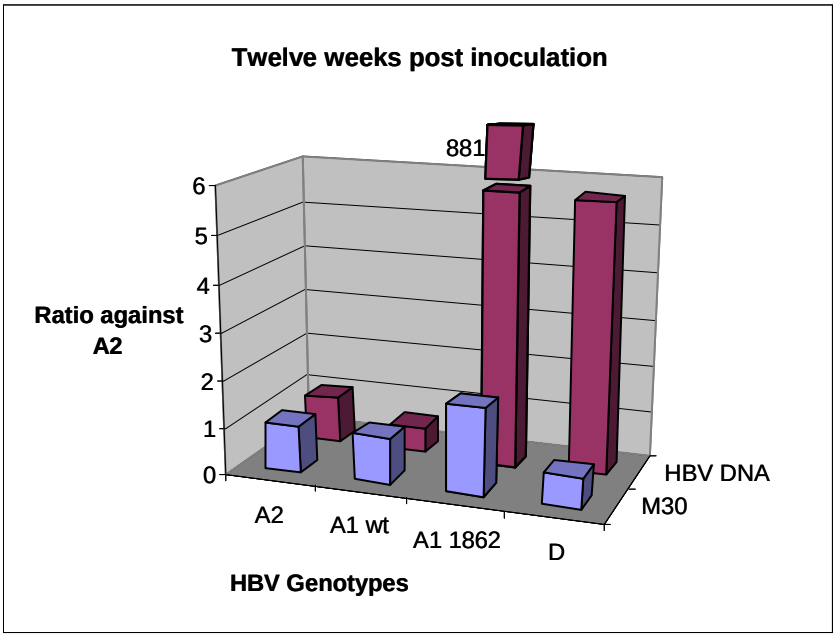


Figure 2.11. M30 apoptosis and HBV DNA ratios relative to subgenotype A2 at twelve weeks post inoculation

2.3.3. END-POINT EXPERIMENTS PERFORMED AFTER SACRIFICE OF TRANSGENIC MICE

2.3.3.1. *HBV cccDNA*

Closed covalently circular (ccc) DNA plays a key role in the persistence of HBV infection therefore the mice were sacrificed and their livers tested for the presence of cccDNA by real-time PCR. The mouse-derived subgenotype A1 HBV isolate with the 1862 mutation had the lowest critical threshold (Ct) value indicating that it possessed the highest levels of cccDNA, followed by the positive control (HBV-associated HCC liver tissue). The remainder of the mice infected with the other HBV genotypes/subgenotypes had similar Ct values (Figure 2.12).

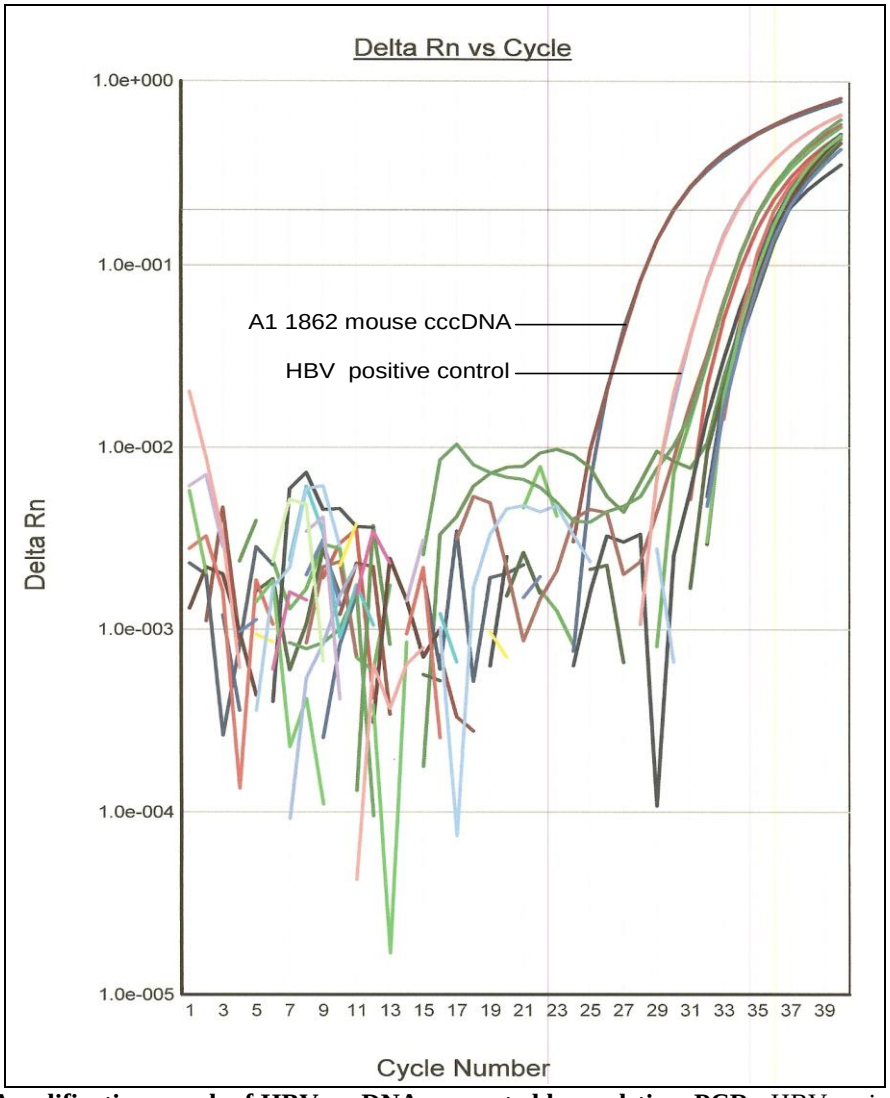


Figure 2.12. Amplification graph of HBV cccDNA generated by real-time PCR. HBV region amplified (nt 1661 to 1921 from *Eco*RI site) detected using a FAM/TAMRA probe.

2.3.3.2. *HBV Transcription*

In order to determine whether HBV transcription was occurring within the transgenic mice livers, RNA was extracted, quantified and found to be of good quality (Figure 2.13) before being used for cDNA production. All mice samples were positive for the amplification of the housekeeping GAPDH gene and the precore-core region of HBV (Figure 2.14), confirming that HBV transcription was occurring.

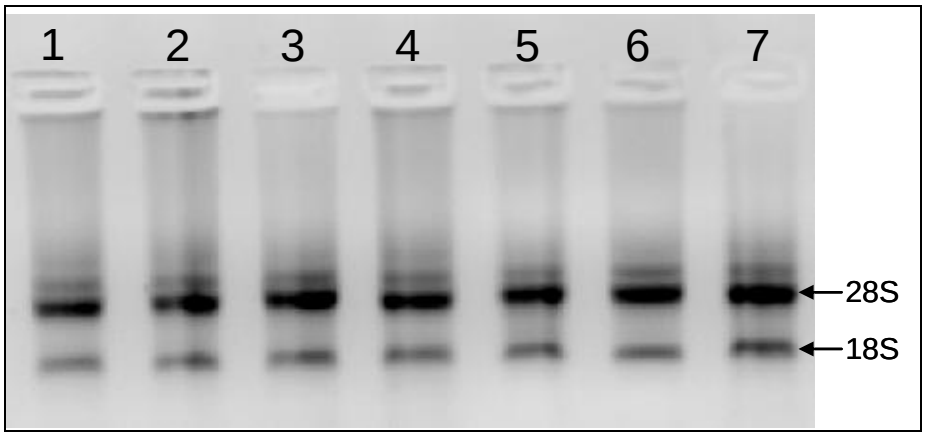


Figure 2.13. RNA extracted from the uPA-SCID mice livers. Lane 1: Genotype D-infected mouse. Lanes 2 and 3: Subgenotype A1-infected mice. Lanes 4 and 5: Subgenotype A1 with the 1862 mutation-infected mice. Lane 6: Subgenotype A2-infected mice. Lane 7: positive human HBV control.

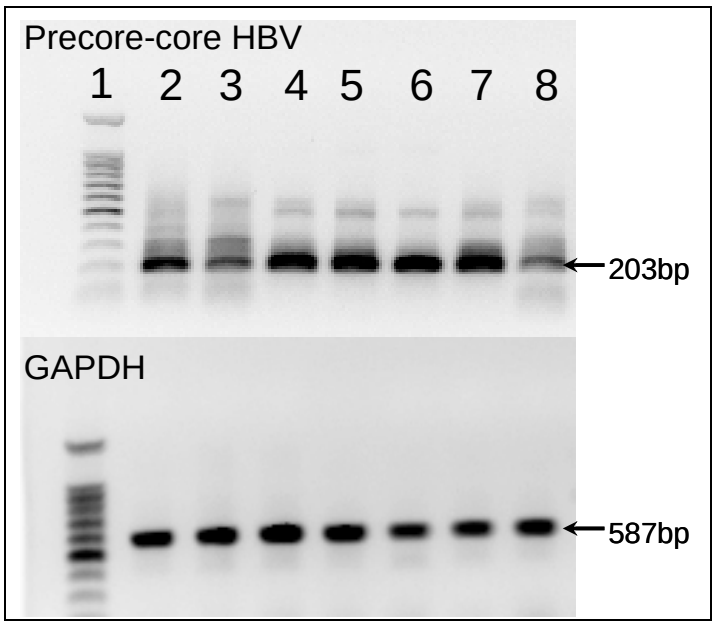


Figure 2.14. Resolution of the precore-core region of HBV and the housekeeping gene GAPDH using cDNA generated from the infected chimeric mice extracted RNA.
Lane 1: 100 bp molecular weight marker. Lane 2: Genotype D-infected mouse. Lanes 3 and 4: Subgenotype A1-infected mice. Lanes 5 and 6: Subgenotype A1 with the 1862 mutation- infected mice. Lane 7: Subgenotype A2-infected mice. Lane 8: positive human HBV control.

2.3.3.3. *Presence of HBV inoculum in transgenic mice livers*

To confirm that the original HBV inoculum strain was replicating in the livers of the sacrificed mice, the S and precore-core regions of HBV were amplified. Sequences obtained for these HBV regions were aligned with 27 sequences from GenBank and the original human HBV inoculum serum sequence for phylogenetic analysis. All the mice-derived sequences for both the S and precore-core region clustered with the original human serum-derived and clone sequences. This is illustrated by the figure for the S region (Figure 2.15). The intragroup divergence for the S region was between 0 and 0.56 $\pm$ 0.09 while the precore-core divergence range was between 0 and 2.18 $\pm$ 0.81, both of which fell within the normal range of 0 to 2.81 $\pm$ 0.85 (Kramvis et al., 2008, Kurbanov et al., 2008).

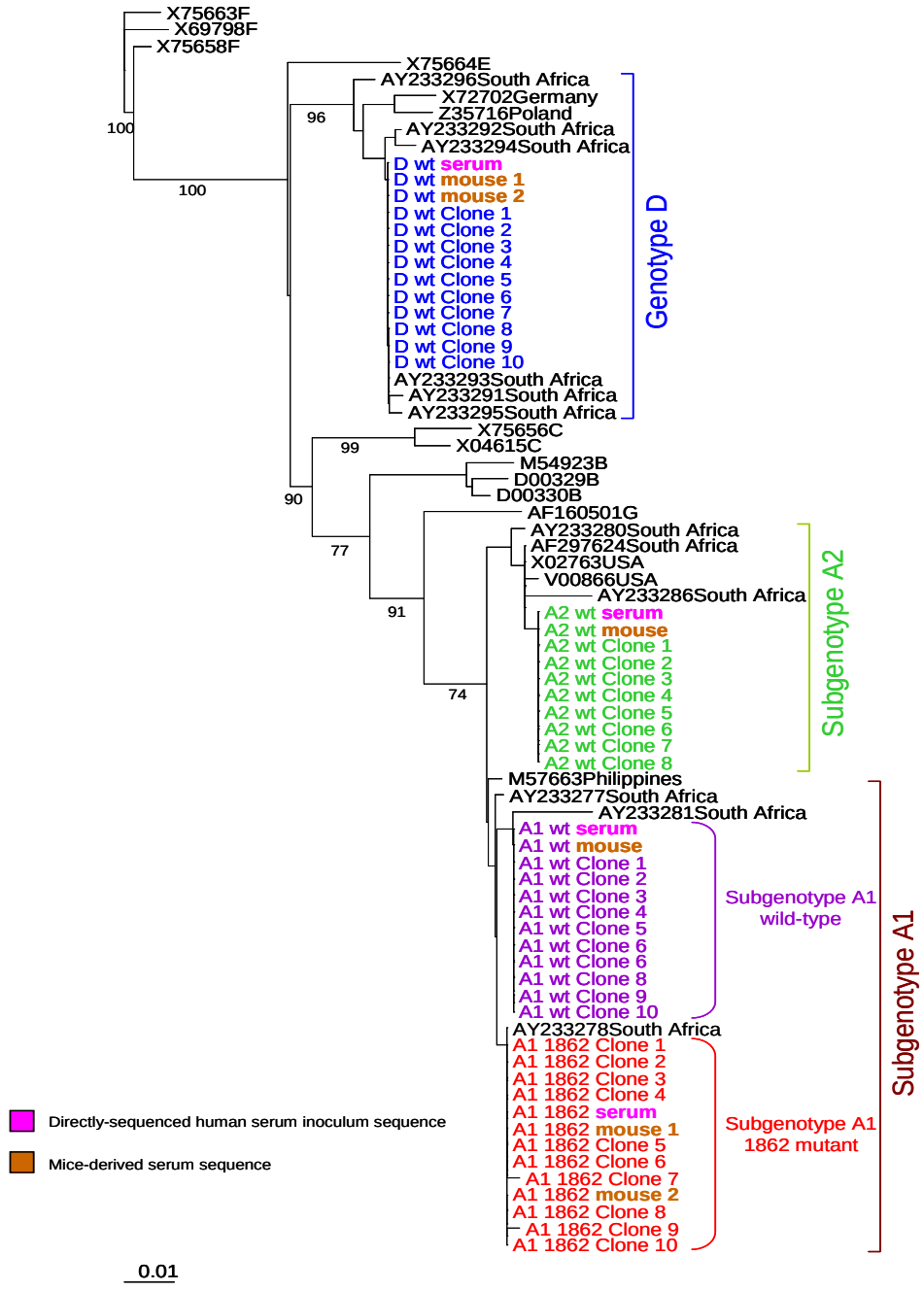


Figure 2.15. Phylogenetic relationship of a portion of the S region (nt 315 to 693 from *Eco*RI site) of HBV isolates from human inoculum sera, human inoculum sera-derived clones and mice sera, to the representative sequences of the HBV genotypes A-G, obtained from GenBank, established using neighbour-joining. Bootstrap statistical analysis was performed using 1000 data sets and the numbers on the nodes indicate the percentages of occurrences.

2.3.3.4. *Immunohistochemical staining*

Haematoxylin and eosin staining of the chimeric mice livers was used to examine the liver morphology. The cytoplasm of the human hepatocytes had an unusual pale and granular appearance, resembling the morphology of hepatocytes in glycogen storage diseases that made it easy to differentiate from the mouse parenchyma (Figure 2.16). To investigate the expression of HBV proteins in the mice liver, labelled antibodies targeting the HBsAg and HBc antigens were utilised. The mouse infected with subgenotype A1 1862 mutant stained positively for HBs (Figure 2.17) at twelve weeks while the remaining infected mice (subgenotype A1 , subgenotype A2 and genotype D) were negative. The mouse infected with genotype D had a positive core nuclear stain (Figure 2.18) at twelve weeks while the remaining infected mice (subgenotype A1 with and without the mutation and subgenotype A2) were negative for the core stain.

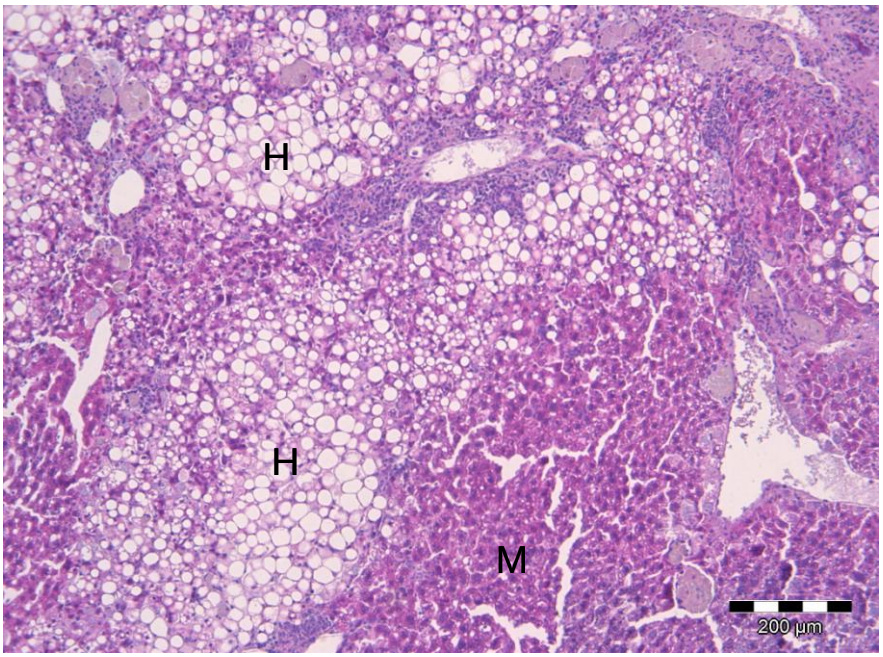


Figure 2.16. Haematoxylin and eosin staining of chimeric liver. The staining allows for the identification of the human hepatocytes (H) within the mouse (M) parenchyma.

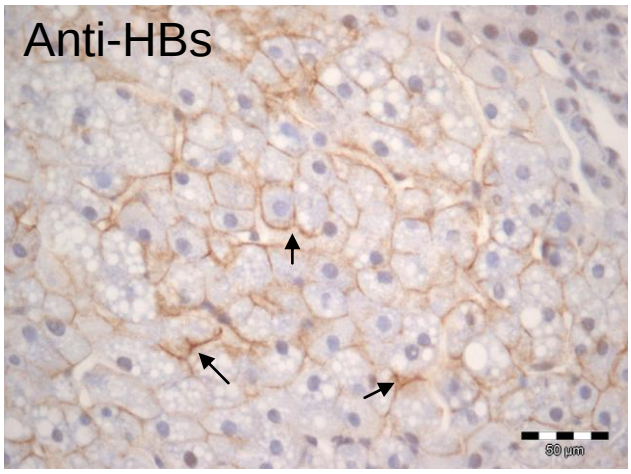


Figure 2.17. Representative image of HBV S-antigen cytoplasmic staining in the liver of the HBV subgenotype A1 1862 infected transgenic mouse.

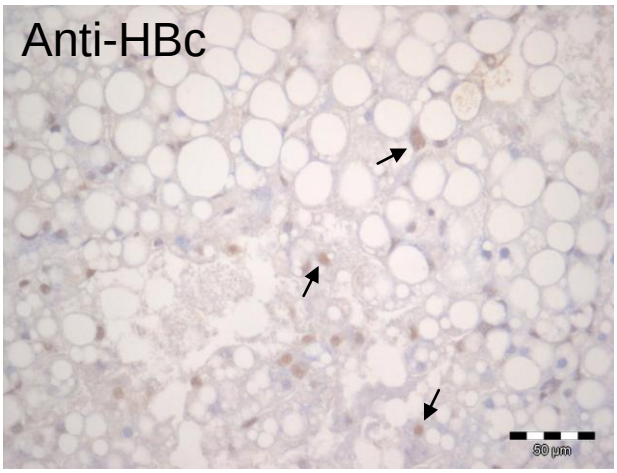


Figure 2.18. Representative image of HBV core antigen nuclear staining in the liver of the HBV genotype D infected transgenic mouse.

This is the first demonstration of *in vivo* infection of uPA-SCID mice using subgenotype A1 of HBV. These experiments showed that transgenic mice can be infected with HBV belonging to subgenotype A1 (with and without the 1862 mutation) as well as subgenotype A2 and genotype D. This was indicated by the increase in HBV DNA levels, presence of cccDNA, HBV transcription and the detection of HBV antigens. This led to the cleavage of cytokeratin-18, indicating apoptosis.

2.4. DISCUSSION

Diverse host and possible interfering environmental factors as well as working with a varied distribution of HBV genotypes/subgenotypes can influence the outcomes of large comparative population based studies. It is therefore beneficial in some cases to utilise an *in vitro* or *in vivo* experimental model to control for such variables. It has previously been shown *in vitro* that the precore/core 1862 mutation causes an increase in hepatocyte apoptosis as a result of the abnormal accumulation of the precore/core protein (Viana, 2005) in the Endoplasmic Reticulum Golgi Intermediate Compartment (Chen et al., 2008). A potential limitation of the previous *in vitro* studies include the use of transfection of hepatoma cell lines, such as the Huh7 cell line, which is known to have several defective cell death pathways, including the p53 pathway (Bressac et al., 1990). Moreover, the use of artificially constructed plasmids may not reflect what is happening in *in vivo* infections. Therefore in the present study uPA-SCID transgenic mice were inoculated with human HBV positive serum and the replication of different genotype/subgenotypes of HBV investigated together with their ability to cause apoptosis.

HBV commonly presents as a quasi-species, a term used to describe a cluster of variant viruses arising from mutations over time within an isolate (Domingo et al., 1978). The human HBV inoculum was therefore fully characterized by cloning and sequencing to confirm HBV mono-sub/genotype infection and to obtain serum containing limited quasi-species population to minimize the influence of alternate mutations, insertions or deletions on the study outcome. Clones from each sample containing one of the following: subgenotype A1 (wild-type or 1862 mutant strain), subgenotype A2, and genotype D were evaluated to determine the majority viral species present. Serum samples containing virus closest to the wild-type consensus for each genotype/subgenotype were selected as inocula. Intragroup divergence ranging from 0 % to 0.68 % was observed (Table 2.3) indicating a relative homogenous viral population and therefore minimal quasi-species presence. The intergroup divergence between the wild-type and 1862 mutant subgenotype A1 strains was higher at between 2.38 % and 2.72 % (Table 2.3), but still below the 4 % delineating subgenotypes (Kramvis et al., 2005). Despite these efforts, we are unable to rule out the possibility of the potential effect of the presence of uncharacterized variations on HBV infection.

In order to ensure standardization of the uPA-SCID mice HBV infection, all mice utilized were grafted with human hepatocytes from a single European donor and inoculated with serum containing identical HBV viral loads. Albumin, a protein of approximately 66000 Daltons, is synthesized by the liver and present in the blood at a very high concentration. In addition to maintaining blood osmotic pressure, it also plays a role in transporting fatty acids and a variety of other substances by forming complexes with them (Doweiko and Nompleggi, 1991). It therefore serves as the ideal indicator of human liver function (Whicher and Spence, 1987). In agreement with previous studies, human albumin levels were monitored in the current study as an indicator of graft success and percentage of human hepatocytes present (Dandri et al., 2001, Meuleman et al., 2005, Sugiyama et al., 2006). Levels of human albumin remained similar and constant throughout the lifespan of all the mice (Figure 2.9 and 2.10) indicating successful graft of the human hepatocytes in the individual mice, with similar human hepatocyte levels in the individual mice. Albumin levels as low as 0.5 mg/ml have been shown to be sufficient for the establishment of an HBV infection in the uPA-SCID transgenic mouse model (Vanwolleghem et al., 2010). The levels of human albumin in this study ranged from 0.5 to 7.94 mg/ml.

Despite the fact that ten homozygous uPA-SCID mice were inoculated with HBV, only 8 survived to study completion. Factors affecting animal survival include engraftment efficacy, kinetics of repopulation and susceptibility to infection with HBV after transplantation of human hepatocytes (Vanwolleghem et al., 2010).

Once inoculated, the successful establishment of the *in vivo* HBV infection within the uPA-SCID transgenic mice was demonstrated by the increase of HBV DNA levels, presence of cccDNA and HBV transcripts as well as the detection of the core and/or surface HBV antigens in the chimeric mice liver tissue. These HBV infection indicators were previously selected for evaluation in several transgenic mice studies (Meuleman et al., 2006, Dandri et al., 2001). Genotypes A2, C, D and E have been previously investigated (Sugiyama et al., 2006, Tsuge et al., 2005, Dandri et al., 2005, Meuleman et al., 2006), however this is the first demonstration of the subgenotype A1 *in vivo* infection and evaluation of apoptosis levels in the uPA-SCID transgenic mouse model infected with different genotypes/subgenotypes of HBV.

HBV cccDNA, the template of HBV replication in natural infection, was first described in a non-transgenic mouse model (Takehara et al., 2006), albeit at very low levels of between 1 to 4 copies. All the inoculated mice in the present study contained cccDNA essential for sustainable HBV replication and expression. A real-time qualitative assay was used to detect cccDNA because it has been shown that successful establishment of an HBV infection is not dependent on the copy number of cccDNA but merely its presence of as little as one copy (Werle-Lapostolle et al., 2004, Bourne et al., 2007, Chisari, 1996, Raney et al., 2001, Zhang et al., 2003). It is however interesting to note that the mouse infected with subgenotype A1 1862 showed the earliest detection and therefore lowest Ct value, indicating a greater number of cccDNA copies present. The cccDNA in the remaining mice infected with the other strains of HBV was detected at a relatively higher Ct value, indicative of lower cccDNA levels (Figure 2.12). The 1862 mutation has been shown to cause the abnormal retention of the precore core protein reducing its secretion, which may affect cccDNA production levels. Summers and colleagues were the first to demonstrate that by knocking out surface protein expression, and thus virion secretion, resulted in a drastic increase of cccDNA copy numbers (Summers et al., 1990, Summers et al., 1991). More recent studies have confirmed this observation (Gao and Hu, 2007, Guo et al., 2007) and suggested that a negative-feedback mechanism might involve the large surface protein suppressing cccDNA amplification. When nuclear cccDNA levels reach a specified number of copies, sufficient large surface protein is made to efficiently shut off the cccDNA amplification pathway and redirect mature nucleocapsids to envelopment and extracellular secretion. It has previously been shown that the nuclear cccDNA is organised into minichromosomes with HBV core protein being a component thereof (Bock et al., 2001). A similar mechanism could therefore be taking place, whereby the accumulation of precore/core protein in the ERGIC and interference with its expression results in an increase of cccDNA production. Further functional studies are required to investigate this possibility.

cccDNA serves as a template for transcription of genomic and subgenomic HBV transcripts. Efficient transcription of HBV genes requires a number of ubiquitous transcription factors such as nuclear factor-1 as well as several liver-enriched transcription factors such as hepatocyte nuclear factor 1, that are also key regulators of hepatocyte function and morphology (Kock and Schlicht, 1993). In order to determine that HBV transcription was occurring within the uPA-SCID transgenic mice, the precore/core coding region was amplified successfully from extracted mice liver-derived RNA (Figure 2.1.4).

HBV DNA levels were monitored regularly because quantification of HBV DNA is the gold standard in providing information regarding infectivity, prognosis and in clinical settings, the need for, response and required duration of therapy. We have shown in this *in vivo* study that by 12 weeks post inoculation the 1862- expressing mice demonstrate a 3 log increase in viral DNA compared to the wild-type virus suggesting perhaps that the presence of this mutation may increase the replication fitness of the subgenotype A1 strain (Figure 2.9). Previous *in vitro* studies have shown unchanged replication potential (Chen et al., 2008) while others have shown a decrease in replication associated with the presence of the 1862 mutation (Guarnieri et al., 2006). Both studies involved transfection with artificially constructed plasmids into cancerous cell lines which may not reflect what is occurring *in vivo*. It is important to note however that one of the pitfalls of using serum is the presence of quasispecies and the possibility of the occurrence of unknown sequence variations, in addition to 1862, that may affect HBV infection and expression. In future studies this limitation can be eliminated by infecting the transgenic mice with virus derived from cells transfected with a replication compentent clone containing only one strain of HBV that only differs at the 1862 mutation site compared to the wild-type replication competent clone. This approach would eliminate the quasispecies issue and utilizing virus derived from a stable cell line would allow for an endless supply of identical inoculum as a "pure" population.

The remaining HBV genotypes/subgenotypes, including the subgenotype A1 wild-type strain, showed similar HBV DNA levels throughout the 12 week infection period (Figure 2.9). Interestingly, in week 4 shortly following inoculation, subgenotype A2 showed only a 1 log difference compared to the 1862 mutant mice suggesting that perhaps the European donor hepatocytes may be predisposed to infection by the commonly occurring European subgenotype A2 strain suggesting the potential involvement of the donated host genetic factors (Frodsham, 2005) or possibly that HBV subgenotype A2 has evolved to infect these European host hepatocytes. At 12 weeks post inoculation, genotype D demonstrated a 1 log higher difference compared to the lowest levels observed for subgenotype A1 (wild-type) and A2. Higher viral loads following transfection with genotype D relative to subgenotype A1 and A2 has been previously observed *in vitro* (Sugiyama et al., 2006). Sugiyama *et al* (2006) compared the intra- and extracellular levels of HBV DNA for genotypes A, B, C and D after transfection of Huh7 cells. Intra- and extra-cellular expression of HBV DNA was found to be the highest for genotype C, followed by genotype B and further by D, with genotype A having

the lowest levels. In the present study, expression of HBV DNA was found to be the highest for subgenotype A1 with 1862, followed by genotype D and further by subgenotype A2, with wild-type genotype A1 having the lowest levels. Tanaka *et al* in 2004 also found a higher HBV DNA level associated with subgenotype A2 compared to A1 (Tanaka et al., 2004). It is of interest that the 1862 mutation only occurs in subgenotype A1, regarded as an "older" strain of HBV compared to the other subgenotypes/genotypes A2 and D. Perhaps the higher viral load associated with this mutation causes an increase in immune pressure resulting in its de-selection in the "newer" strains, A2 and D, of HBV.

Haemotoxylin and eosin staining allowed for the differentiation of human and mouse hepatocytes, visually confirming the successful grafting of human liver (Figure 2.16). The human hepatocytes are clearly distinguishable from the mouse parenchyma in that their morphology resembles that typically observed with glycogen storage disease. This abundant accumulation of glycogen has been previously observed (Meuleman et al., 2005). Meuleman *et al* (2005) suggested that the cause of the aberrant polysaccharide accumulation phenomenon is most likely attributable to inappropriate recognition of murine signals by human hepatocytes. This metabolic disturbance has a negative effect on the animal as a whole and the grafted hepatocytes, as suggested by the overall appearance of the chimeric mouse and the aspect of the liver cells in histology studies. It is plausible that there are other biochemical pathways that may not function optimally, possibly because of communication failure between mouse ligands or receptors and their human counterparts (Antonopoulos et al., 2011).

Tissue staining for the HBsAg was positive only in the 1862 expressing mouse liver, indicative of the high HBV replication levels and therefore proportionally increased production of HBsAg. On the other hand, HBcAg staining in the chimeric liver was positive when the mice were infected with genotype D. A possible explanation for this is that it has been shown *in vitro* that genotype D has a 3-fold higher core antigen expression compared to subgenotype A1 and A2 (Sugiyama et al, 2006). Sugiyama *et al* (2006) reported that intracellular levels of HBV DNA and core protein were higher for genotype D than A, which may increase the activity of liver disease and explain the increased resistance to interferon in patients infected with genotype D (Erhardt et al., 2005, Zhang et al., 1996). The lower replication levels of genotype A compared to D may result in an imbalance between the

syntheses of HBsAg and core protein; resulting in a bias in favour of HBsAg for genotype A, and core protein for genotype D. Sugiyama *et al* (2006) further postulated that the lowest replicative activity of genotype A may explain how genotype A can evade the immune pressure against it and persist in up to 10 % of all adulthood infection (Ozasa et al., 2006, Kobayashi et al., 2002). In the present study, however, only the mouse infected with mutant subgenotype A1 stained positive for HBsAg and not the wild-type A1 or subgenotype A2, suggesting that possible sequence variations or host factors may be influencing the expression of HBsAg by the different subgenotypes of A.

As was previously shown *in vitro*, transfection of Huh-7 cells with constructs containing the G1862T mutation resulted in an increase in apoptosis because of the accumulation of the precore/core mutant protein in the ERGIC (Chen et al., 2008, Viana, 2005). Similarly by 12 weeks post inoculation the 1862 expressing mice demonstrated almost double the amount of apoptosis compared to the other HBV strains (Figure 2.10). The extremely high levels of apoptosis observed within the 1862 expressing mouse is most likely as a result of the accumulation of the mutant precore/core protein within the hepatocytes, as has previously been described *in vitro* (Chen et al., 2008). This observation was found not to be directly associated with the increased viral load burden because genotype D was found to possess relatively low apoptosis levels but high viral load levels (Figure 2.11).

Phylogenetic analysis of the precore/core and S region of HBV revealed no significant sequence variation, confirming that the replicating virus was derived from the original infective inoculum strain (Figure 2.15). This observation is probably attributable to the lack of immune pressure in the severely immunodeficient mice (Meuleman and Leroux-Roels, 2008, Mason et al., 2010) and low mutation rate of the HBV genome (Orito et al., 1989, Osiowy et al., 2006).

To summarize, the present study was the first demonstration of the successful HBV infection of the uPA-SCID transgenic mice, with subgenotype A1 HBV with the detection of cccDNA, presence of HBV transcripts, increasing levels of HBV DNA, and HBV antigen production.

Care was taken at all levels to ensure standardisation and normalisation to be able to accurately compare the infection characteristics of subgenotype A1 with the 1862 mutant, subgenotype A1 wild-type, subgenotype A2 and genotype D, which are the genotypes

circulating in the South African population. This included the characterisation of inocula, the use of a single hepatocyte donor for the human hepatocyte engraftment into the uPA-SCID mice, inoculation with an identical viral copy number of HBV, fixed follow up periods and the confirmation of the presence of the original inoculum at the time of animal sacrifice. Subgenotype A1 with the 1862 mutant was found to demonstrate the highest levels of almost all markers of HBV infection except for the core antigen that was only observed in mice infected with genotype D.

This is also the first time that uPA-SCID transgenic mice have been used for the study of apoptosis, in relation to HBV infection. Apoptosis levels were not associated with HBV DNA levels, although the low number of mice infected and the absence of an immune response precludes us reaching any firm conclusions.

One of the potential limitations of the *in vivo* uPA-SCID transgenic mouse model is their limited lifespan and general poor health of the animal as a result of the absence of an immune system. Despite these limitations, the uPA-SCID transgenic mouse model has presented itself as an ideal platform for the study of HBV infection by using healthy human liver cells with intact apoptotic pathways reflecting an *in vivo* HBV infection as opposed to cancerous cell lines with defective pathways associated with *in vitro* studies. Because we found differences in the levels of apoptosis between the different genotypes and the viral load, we decided to investigate this further by looking at HBV infection and apoptosis in a population based study.

CHAPTER 3

INVESTIGATION OF THE RELATIONHIP BETWEEN HBV INFECTION AND THE M30 APOPTOSIS MARKER IN HUMANS

3.1. INTRODUCTION

In population-based cohort studies, a sample, or even the entirety, of a defined population is selected for longitudinal assessment of exposure-outcome relations. The study of a cohort, which is representative of a defined population, offers three advantages. 1) It allows the estimation of distributions and prevalence rates of relevant variables in the reference population (information on risk factors is used for the calculation of population attributable risks). 2) Risk factor distributions measured at baseline in a study involving periodic examinations of the cohort can be compared with distributions in future cross-sectional samples so as to assess risk factor trends over time. 3) A representative sample is the ideal setting in which to carry out unbiased evaluations of relations, not only of confounders to exposures and outcomes, but also among any other variables of interest, even those which were not specified in the original study hypotheses (Szklo, 1998).

The majority of data generated from population based studies of HBV has been primarily focused on genotypes B and C predominating in Asia. Population based data available for genotype A is collected mainly from European and American derived studies contributing to information on subgenotype A2 commonly circulating in these geographic regions. Conversely, very limited data is available for subgenotype A1, the predominating HBV strain in South Africa.

The purpose of this study was to determine whether:

- there was a difference in apoptosis levels between HBV asymptomatic carriers, chronic HBV patients, HBV associated HCC patients and healthy uninfected control subjects
- any associations exist between HBV genotype, age, apoptosis levels, gender and HBV viral load within and across the various disease groups.

3.2. MATERIALS AND METHODS

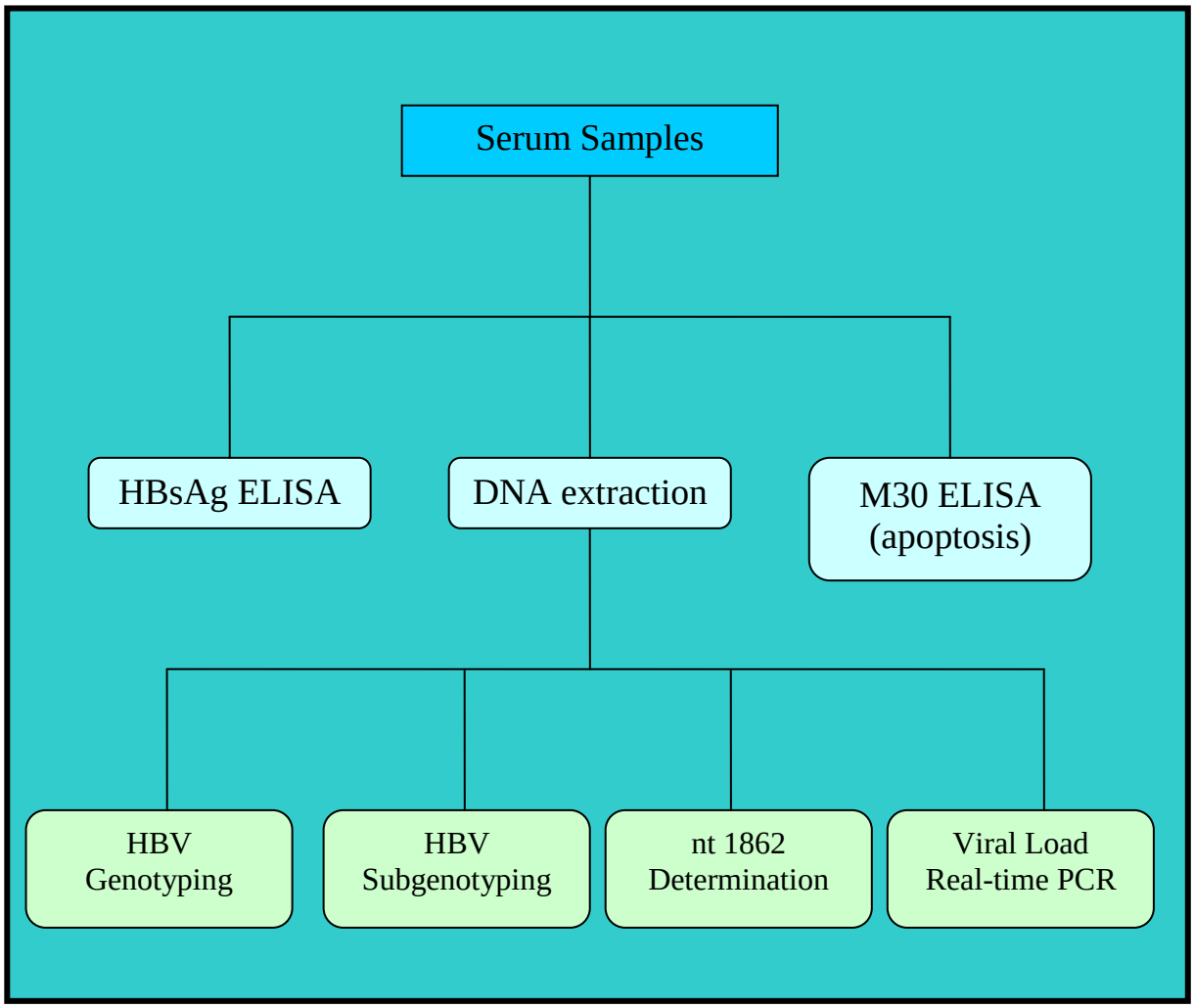


Figure 3.1. Organogram of techniques used.

3.2.1. STUDY SUBJECTS

Six hundred and thirty five (635) samples were obtained for evaluation with 564 of them being HBsAg-positive sera obtained from South African (346), Japanese (151) and Chinese (138) patients (from Hong Kong) and asymptomatic carriers of the HBV virus (Figure 3.2). Informed consent was obtained from each participant and the study had ethical clearance from each institution that provided the patient samples. This was presented to the ethics board of the University of the Witwatersrand and ethical clearance obtained for the project (Appendix B).

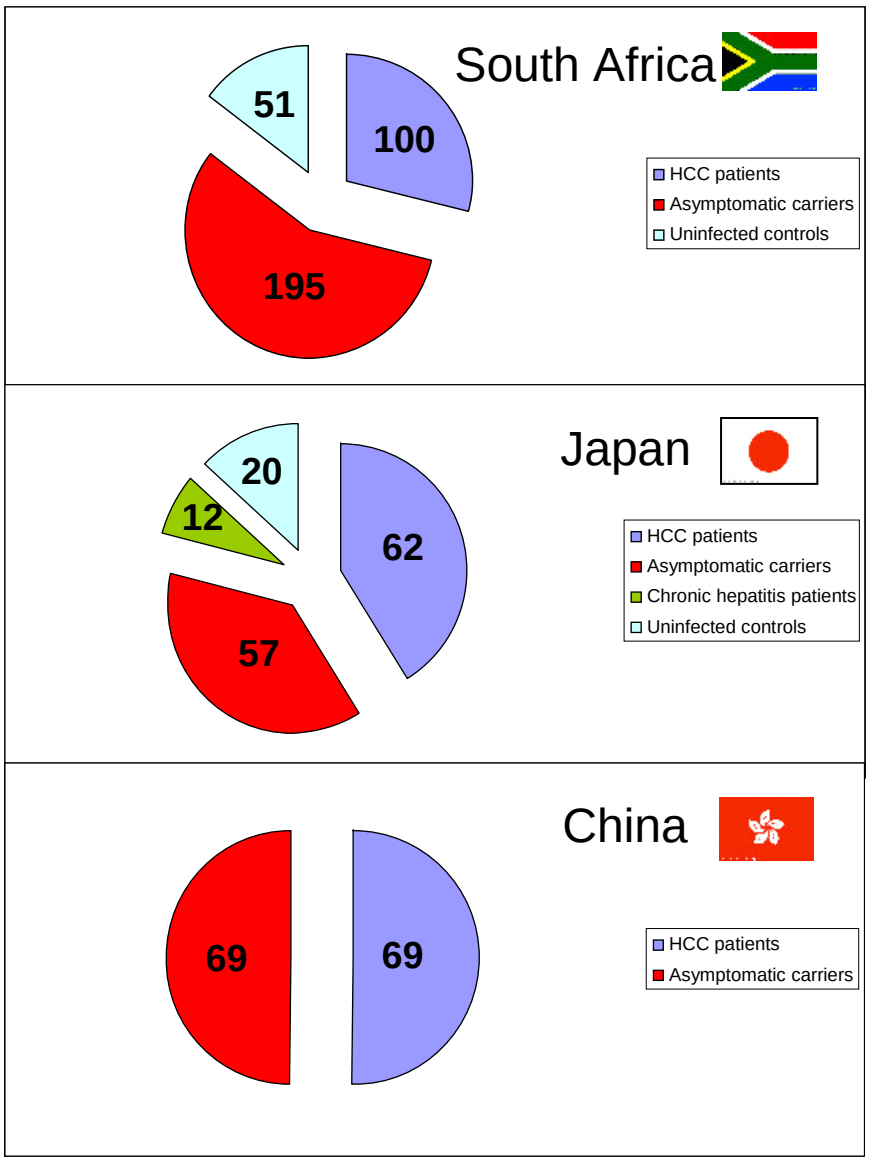


Figure 3.2. Number of subjects by disease category and country.

3.2.2. HEPATITIS B SURFACE ANTIGEN (HBsAg) ELISA

The Monolisa Ag HBs PLUS kit (BioRad, Steenvoorde, France) was used according to the manufacture's instructions to determine which samples were HBsAg-positive (Figure 3.3).

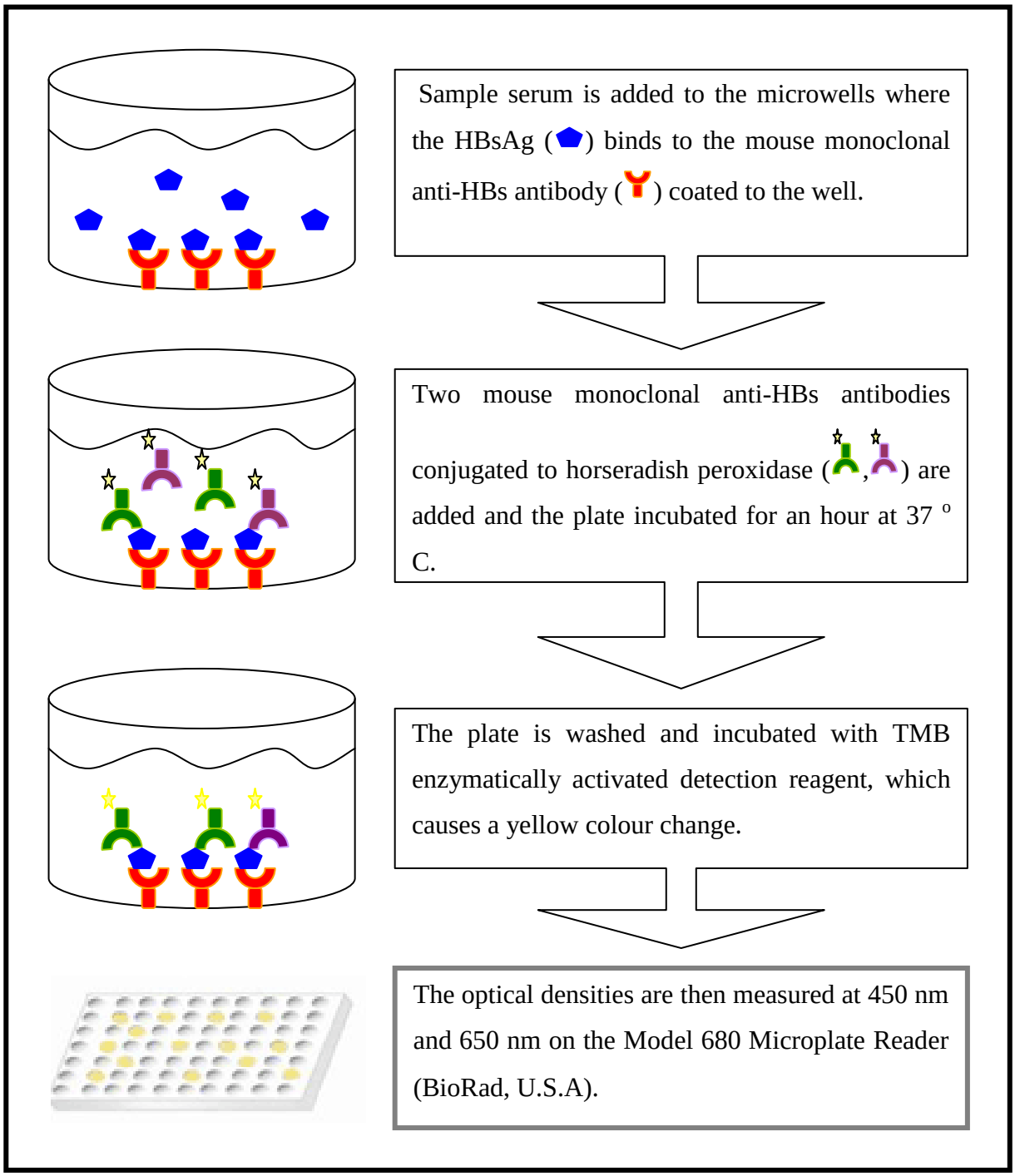


Figure 3.3. The Monolisa Ag HBs PLUS ELISA plate assay.

3.2.3. DNA EXTRACTION

DNA was extracted using the QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 50 µl of serum was added to 175 µl of 0.9 % sodium chloride and protease solution. The sample was then lysed by the addition of 200 µl of Buffer AL and incubation at 56 ^{o}C for 15 minutes. The DNA was precipitated by 250 µl of 100 % ethanol and the entire reaction mixture placed in a column and centrifuged to allow the DNA to bind to the column. Following two stringent washes the column was then dried by centrifugation. The DNA was finally eluted in 50 µl of nuclease-free sterile water and stored at -20 ^{o}C. A blank (sterile water sample) reaction was performed in parallel to rule out any contamination.

3.2.4.GENOTYPING PCR AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

RFLP analysis was used to genotype HBV isolated from South African and Chinese serum samples (Lindh et al., 1997) (Table 2.1). Negative controls included the HBsAg negative serum samples. The genotypes of the HBV from Japan sera were previously determined by our Japanese collaborators using the HBsAg Subtype EIA genotyping kit (Institute of Immunology, Tokyo, Japan)(Tanaka et al., 2009).

3.2.4.1. *Genotyping nested PCR*

The reaction mixture for the first round PCR consisted of 2.5 µl of 10 X NH_4, 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 40 mM dNTP mix, 1.25 µl each of 20 µM primer 231F and 801R (Table 2.1), 0.1 µl of 5 U/µl BioTaq DNA polymerase (Bioline, London, England) and 2.5 µl of DNA made up to a final volume of 25 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the Mastercycler Gradient (Eppendorf, Hamburg, Germany).

For the second round the reaction mixture consisted of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 2.5 µl each of 20 µM primer P7 and P8 (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of the first round PCR product made up to a final

volume of 50 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the Mastercycler Gradient.

3.2.4.2. *RFLP analysis*

Amplicons were then digested in separate reactions with restriction enzymes, *Hinf*I and *Tsp509*I, as previously detailed in section 2.2.3.2 and the genotype determined (Lindh et al., 1997).

3.2.5. SUBGENOTYPING PCR AND RFLP ANALYSIS

Genotype A isolates were subgenotyped using the method described by Kew *et al* (Kew et al., 2005) as detailed in section 2.2.3 in order to differentiate between subgenotype A1 and A2. Genotype B HBV isolates were subgenotyped using the method of Sugauchi *et al*, 2004 (Sugauchi et al., 2004).

3.2.5.1. *Subgenotyping nested PCR for B1 versus B2*

The reaction mixture for the first round PCR consisted of 2.5 µl of 10 X NH_4, 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 40 mM dNTP mix, 1.25 µl each of 20 µM primer PC1 and COR (Table 2.1), 0.1 µl of 5 U/µl BioTaq DNA polymerase and 2.5 µl of DNA made up to a final volume of 25 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the MyCycler (Bio-Rad, U.S.A).

For the second round the reaction mixture consisted of 5 µl of 10 X NH_4, 3 µl of 50 mM $MgCl_2$, 1 µl of 40 mM dNTP mix, 2.5 µl each of 20 µM primer PC2 and COR (Table 2.1), 0.2 µl of 5 U/µl BioTaq DNA polymerase and 5 µl of the first round PCR product made up to a final volume of 50 µl with nuclease-free sterile water. The cycling profile (Table 2.1) was performed using the MyCycler.

3.2.5.2. *RFLP analysis*

The amplicons were then digested with restriction enzymes to generate specific fragments characteristic of the subgenotypes of genotype B. The digestion mixture consisted of 2 µl 10

X NE buffer (supplied with restriction enzyme), 0.5 µl of restriction enzyme, 15 µl of PCR product made up to a final volume of 20 µl with 2.5 µl of nuclease-free sterile water. Restriction enzymes *Hpa*I and *Stu*I (New England Biolabs, Ipswich, Massachusetts, U.S.A) were utilized in separate reactions. Both enzyme mixtures were incubated at 37 ^{o} C for 3 hours followed by the addition of 5 µl of 6 X gel loading dye (Appendix A3) to end the digestion reaction. HBV isolates were subgenotyped according to the banding patterns described in Sugauchi *et al*, 2004(Sugauchi et al., 2004).

3.2.6. AMPLIFICATION AND SEQUENCING OF THE HBV PRECORE/CORE REGION

In order to determine which of the South African serum samples contained the G1862T mutation, the precore/core region was amplified and sequenced according to section 2.2.4.

3.2.7. REAL-TIME PCR QUANTIFICATION OF HBV DNA

An in-house real-time PCR was utilized to quantitate the HBV viral load of all the samples(Weinberg et al., 2000). The real-time PCR reaction mixture consisted of: 25 µl of 2 X TaqMan Universal PCR Master Mix (Applied Biosystems, New Jersey, U.S.A), 1.5 µl each of 10 µM primer Taq1 (5' CAA CCT CCA ATC ACT CAC CAA C 3') and Taq2 (5' ATA TGA TAA AAC GCC GCA GAC 3'), 10 µM of BS-1 FAM/TAMRA-labelled probe (5' TCC TCC AAT TTG TCC TGG TTA TCG CT 3') and 2 µl of DNA made up to a final volume of 50 µl with nuclease-free sterile water. The thermal profile was performed using the ABI Prism 7500 Real-Time PCR machine (Applied Biosystems) as follows: After 2 minutes at 50 ^{o} C and 10 minutes at 95 ^{o} C, there were 40 cycles at 95 ^{o} C for 15 seconds with data acquisition being collected at the end of each cycle at 60 ^{o} C for 1 minute.

A plasmid encoding a single genome of HBV DNA was run to obtain a linear standard curve with an observed range of 10^3 to 10^8 viral copies in agreement with previous reports for this primer/probe set (Weinberger et al., 2000). The second World Health Organization International Standard for HBV Nucleic Acid Amplification Techniques, product code 97/750, which has a final concentration of $1x10^6$ IU/ml was obtained from the National Institute for Biological Standards and Controls (NIBSC; Hertfordshire, United Kingdom) and used as a positive control as well as to calibrate and align the standard curve. The standard curve, blank, positive and negative controls and samples were all tested in duplicate. The

measured IU/ml for each reaction was calculated using the C_t value of each PCR interpolated against the linear regression of the standard curve. For each sample, the duplicate average value was multiplied by 4.7 to convert from IU/ml to copies/ml. The detection limit was found to be 10 IU/ml or 50 copies/ml.

3.2.8. QUANTIFICATION OF APOPTOSIS

The M30-Apoptosense ELISA (Peviva, Bromma, Sweden) was used as described in section 2.2.10.5 to measure apoptosis in human sera.

3.2.9. STATISTICAL ANALYSIS

The distributions of M30-antigen and HBV viral load levels in our study population were markedly skewed. Therefore, statistical analysis was conducted on logarithmically transformed values of M30 and viral load, and geometric (as opposed to arithmetic) mean values are presented. The analysis of covariance (ANCOVA) method (Winer, 1971) was used to compare M30 and viral load levels in study subjects, stratified by gender, age and race. The ANCOVA method also was used to compare levels of M30 between HCC patients and non-HCC control subjects, with control subjects variously defined as HBV carriers alone, uninfected controls alone, and HBV carriers and uninfected controls combined. We used the Pearson Correlation Coefficient (Winer, 1971) to examine the association between M30 and viral load values within individuals, separately for Asian HCC patients, Asian HBV carriers, South African HCC patients, and South African HBV carriers. Since the race- and disease-status-specific correlation coefficient values were comparable, correlation coefficients for all Asians, all South Africans, all HCC patients, all HBV carriers, and all study subjects combined were also computed and formally tested for statistically significance. We used the Chi-square test (Winer, 1971) to examine the distribution of HBV genotype across gender- and age-specific subgroups of Asian study subjects. All p values are two-sided and p values less than 5 % are considered statistically significant.

3.3. RESULTS

3.3.1. DISTRIBUTION OF GENOTYPES AND SUBGENOTYPES

RFLP analysis was used to genotype and subgenotype 295 South African, 131 Japanese and 138 Chinese HBV isolates. The expected geographical distribution of the genotypes/subgenotypes was found: the South African HBV isolates predominately subgenotype A1 followed by genotype D, Japanese primarily genotype C followed by subgenotype B1 and Chinese HBV isolates primarily genotype C followed by subgenotype B2 (Figure 3.4).

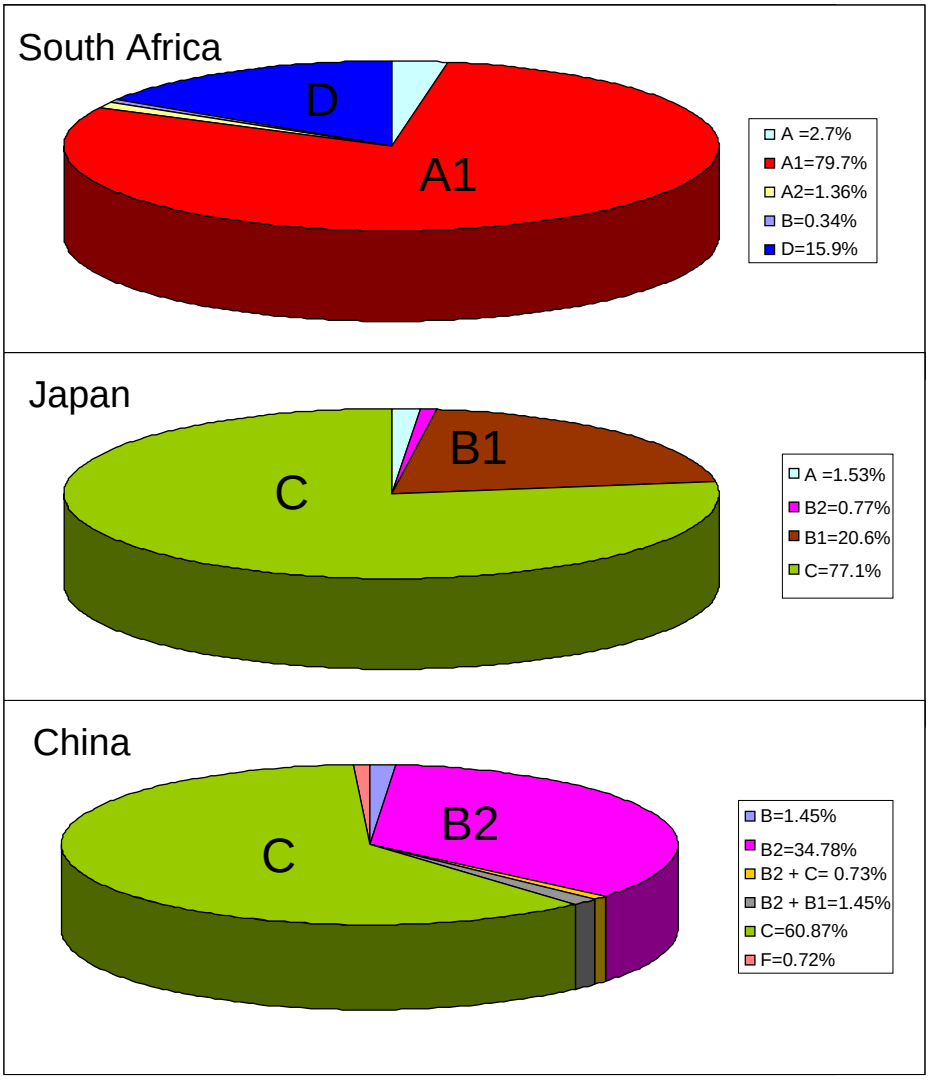


Figure 3.4. Genotypes and subgenotypes from South Africa, Japan and China.
Samples recorded as genotype A or B alone could not be subgenotyped.

When the Japanese and Chinese samples were analysed independently, the patients' age, gender and disease group (asymptomatic carriers versus HCC patients) did not affect the genotype/subgenotype distribution. However, when the Japanese and Chinese data were combined, a higher frequency of genotype B was found among older men (p = 0.053) (Figure 3.5). Chinese patients infected with genotype C had a 1.45 greater probability or 20 fold greater odds ratio of developing HCC compared to those infected with subgenotype B2 (Table 3.1). There was no difference in the probability or odds ratio of developing HCC between genotype C and B1 in Japanese patients. Among the South African patients the effect of age and gender could not be analysed because these parameters were not known which is a limiting factor. The genotype/subgenotype distribution was not affected by the disease condition of the South African patients. South African patients infected with subgenotype A1 had a 1.57 greater probability or 1.85 greater odds ratio of developing HCC than those infected with genotype D (Table 3.1).

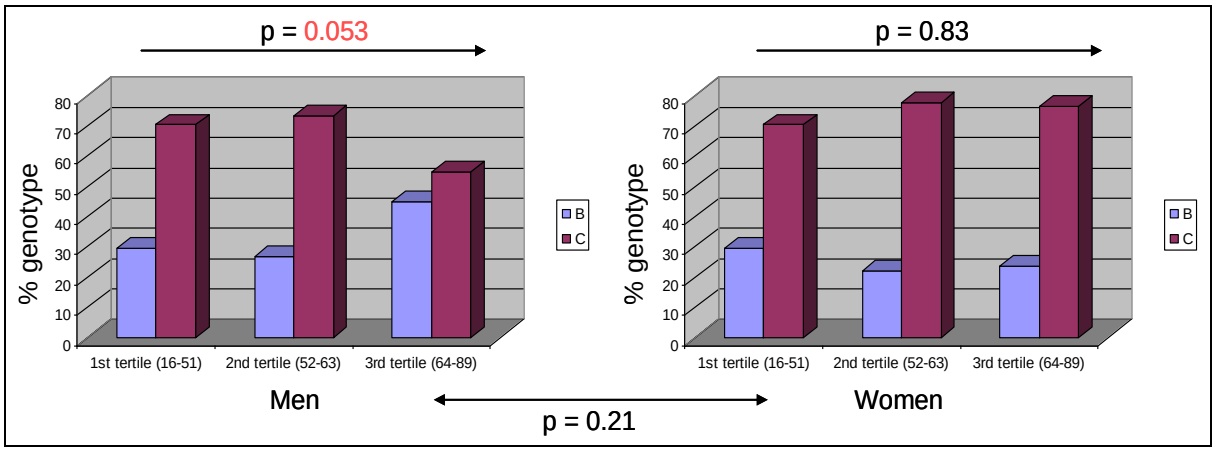


Figure 3.5. Chi square test used for the evaluation of HBV genotype by age and gender in Asians. No significant difference was found between Asian men and women in regards to the genotype/subgenotype distribution (p=0.21). There was also no significant difference observed among the various female age groups regarding the genotype/subgenotype distribution (p=0.83). However among the Asian men, a significantly higher frequency of genotype B was found in men aged between 64 and 89 (p=0.053).

Table 3.1: Relative risk and Odds Ratio of developing HCC among the different population groups

Population	South African	Japanese	Chinese
Sub/Genotype	Subgenotype A1 vs Genotype D	Genotype C vs Subgenotype B1	Genotype C vs Subgenotype B2
Relative Risk	1.57	0.98	1.45
Odds Ratio	1.85	0.96	**20.73**

The South African subgenotype A1 as well as the Chinese genotype C – infected patients had a greater probability or odds ratio of developing HCC.

3.3.2. HBV VIRAL LOAD

An in-house real-time PCR, using primers targeting the conserved HBV S-region of the HBV isolates, was utilized to quantify HBV DNA levels. Statistical analysis revealed that among the Asian asymptomatic carriers, HBV viral load levels were lower in men than women, while the opposite was observed among the HCC patients with higher levels in men than women (Figure 3.6). The HBV viral loads of the Asian men and women were found to be unrelated to their age. However, a significant association was observed between the HBV viral load and the HBV genotype/subgenotype. Patients infected with genotype C had significantly higher viral loads compared to those infected with genotype B ($p = 0.001$) (Table 3.2). Further analysis found that this association was derived from the Chinese population group i.e. patients infected with HBV genotype C had significantly higher viral loads than those infected with subgenotype B2 ($p < 0.001$) (Table 3.2). Among the Asian patients the HBV viral load was not affected by the disease condition. In South African patients no association was found between HBV viral load and HBV genotypes A and D. However, a significant difference was observed between the HBV viral load and disease groups, with HCC patients having higher viral loads compared to the asymptomatic carriers ($p < 0.001$) (Table 3.3).

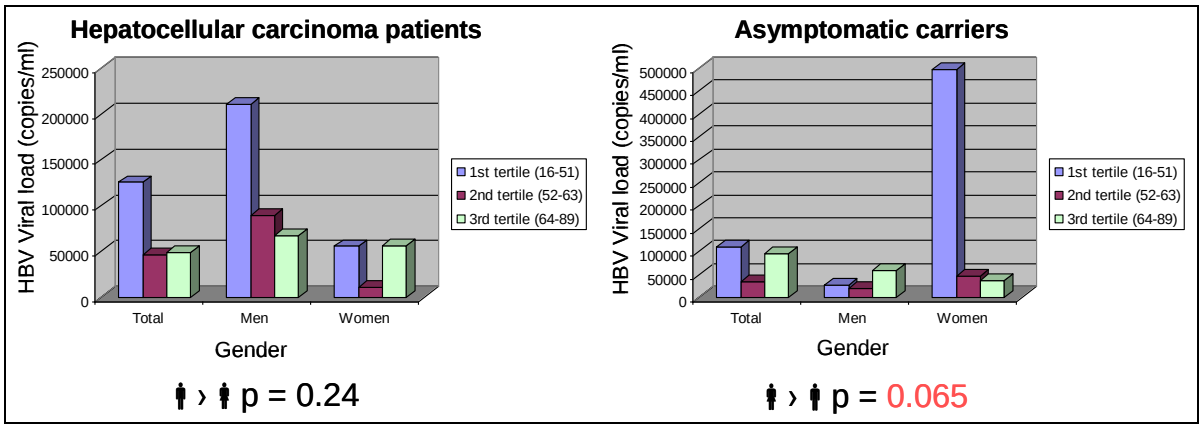


Figure 3.6. Analysis of covariance with log-transformed viral load as the dependent variable and the age and gender as independent variables. Among Asian asymptomatic carriers, women possessed higher HBV viral loads as opposed to men (p=0.065). The opposite was observed among Asian HCC patients.

Table 3.2: Age-sex-disease group adjusted geometric means of viral load (copies/ml) by HBV genotype in China and Japan

HBV genotype	Geometric mean of viral load					
	China		Japan		China + Japan	
	n	Viral load	n	Viral load	n	Viral load
Genotype B*	48	15427	17	40421	65	21636
Genotype C	83	216780	85	67488	168	137598
P (genotype)		**<0.001**		0.673		**0.001**
P (country)						0.083

* Genotype B2 for Chinese and B1 for Japanese.

Table 3.3: Crude geometric means of viral load (copies/ml) by HBV genotype in South Africans

HBV genotype	Geometric mean of viral load					
	HCC patients		Carriers		Total	
	n	Viral load	n	Viral load	n	Viral load
A	89	481620	158	79782	247	191238
B	0	---	1	855400	1	1923769
D	11	169518	36	110763	47	188311
P (genotype)						0.732
P (disease group)						**<0.001**

3.3.3. APOPTOSIS

The M30-Apoptosense ELISA was used for the quantification of the apoptosis via the detection of cytokeratin 18Asp396 (M30) neo-epitope. The relationship between the Asian patients' age and apoptosis M30 levels was found to be different in the various disease groups ($p = 0.027$) (Figure 3.7). In asymptomatic carriers, apoptosis M30 levels were significantly higher in patients 64 or older ($p = 0.001$), which was not observed with the HCC patients ($p = 0.668$) (Figure 3.8). In both disease groups, however a gender effect was observed with apoptosis M30 levels being significantly higher in men than in women, after the adjustment for age ($p = 0.004$) (Figure 3.9). This shows that it is important to take into account the age and gender impact on apoptosis and disease in all subsequent statistical analysis. This information was not available for the South African data.

Within the Asian and South African cohorts, apoptotic M30 levels were found to be positively and significantly associated with HBV viral loads (Table 3.4). There was no significant difference in M30 levels between Asians infected with genotype B and C, even when differentiating between the subgenotypes of B (Table 3.5). In contrast, there was a significant difference in South Africans infected with subgenotype A1 compared to those infected with genotype D, with M30 levels significantly higher in subgenotype A1 infected patients ($p = 0.001$) (Table 3.6).

There was no significant difference in apoptotic M30 levels between Asian HCC and chronic hepatitis patients ($p = 0.684$) (Table 3.7). However, the apoptotic M30 levels of all HCC patients were found to be significantly higher than in asymptomatic carriers or healthy controls ($p < 0.001$) (Table 3.8), even after a statistical adjustment for HBV viral load and HBV genotype ($p < 0.001$) (Table 3.8). Furthermore, significantly higher apoptotic M30 levels were observed in South African HCC patients infected with the 1862 mutant variant as opposed to asymptomatic carriers infected with the same mutant ($p < 0.001$) (Table 3.10).

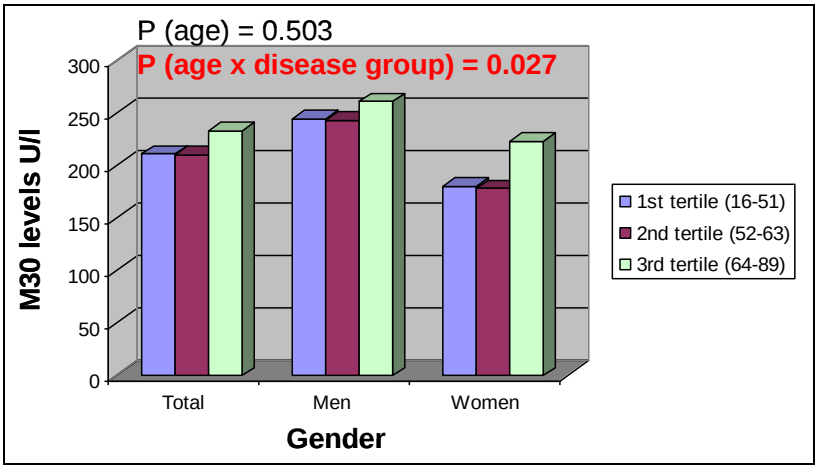


Figure 3.7. Analysis of covariance with log-transformed M30 value as the dependent variable and age as the independent variable. A significant difference was observed between the Asian patient's age and M30 levels in relation to the disease group (p = 0.027).

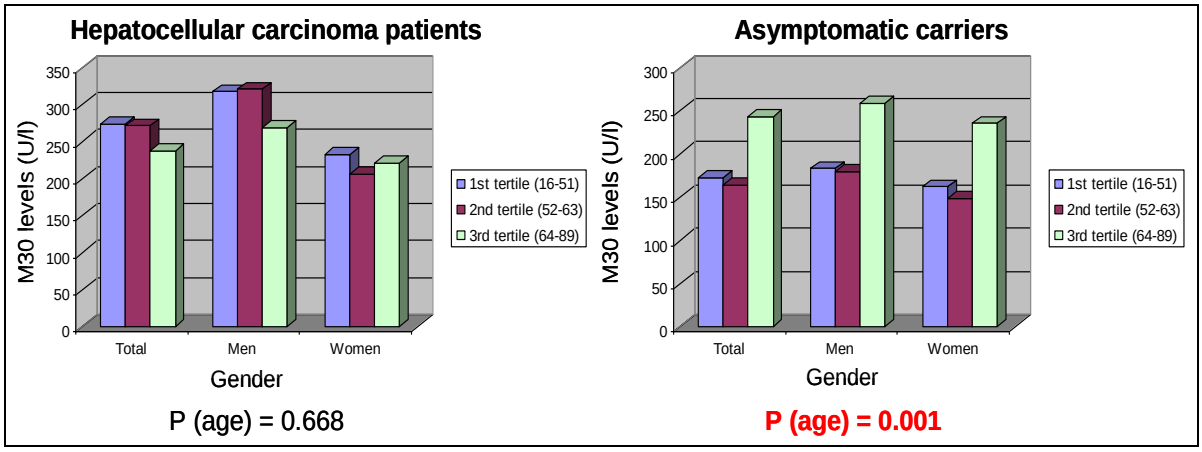


Figure 3.8. Analysis of covariance with log-transformed M30 as the dependent variable and age and disease group as independent variables. M30 levels are significantly higher in Asian asymptomatic carriers 64 and older (p = 0.001).

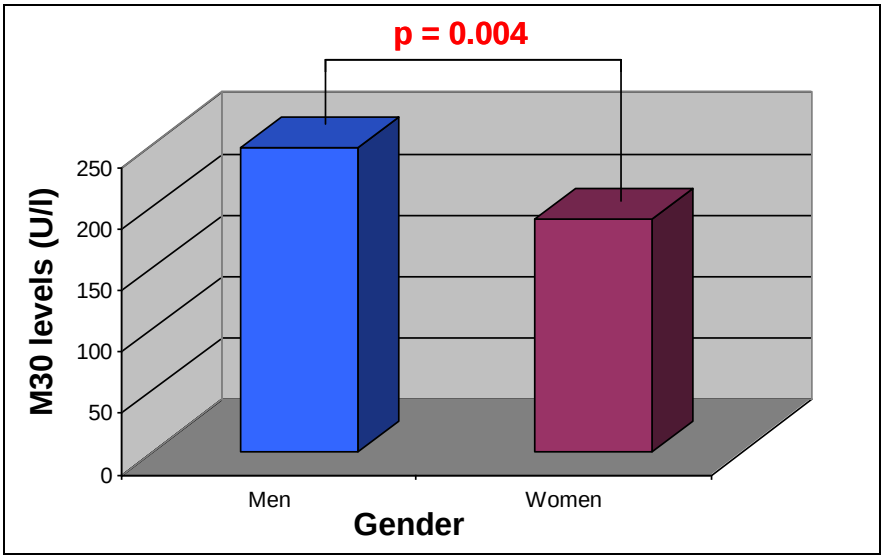


Figure 3.9. Analysis of covariance with log-transformed M30 as the dependent variable and gender as the independent variable. M30 levels are significantly higher in men compared to women.

Table 3.4: Pearson correlation coefficients of log transformed M30 and log transformed viral load in Asians and South Africans

Disease type	Asians		South Africans		Asians + South Africans	
	n	r	n	r	n	r
HCC patients	129	0.22 **(p=0.013)**	100	0.15 (p=0.128)	229	0.23 **(p<0.001)**
Carriers	126	0.19 **(p=0.029)**	195	0.29 **(p<0.001)**	321	0.18 **(p=0.001)**
Total	255	0.20 **(p=0.002)**	295	0.33 **(p<0.001)**	561	0.23 **(p<0.001)**

Table 3.5: Age-sex adjusted geometric means* of M30 levels (U/l) by HBV genotype in Asians

HBV genotype	HCC patients		Carriers		Total	
	n	M30	n	M30	n	M30
A	1	1235.7	1	155.3	2	445.4
B	30	223.9	40	191.9	70	208.0
C	85	261.2	84	196.4	169	222.8
P (genotype)						**0.260**
P (disease group)						**<0.001**

*One-way analysis of covariance with age and sex as the covariates and log-transformed M30 value as the dependent variable.

Table 3.6: Crude geometric means of M30 levels (U/l) by HBV genotype in South Africans

HBV genotype	HCC patients		Carriers		Total	
	n	M30	n	M30	n	M30
A	89	491.9	158	102.5	247	225.1
B	0	---	1	83.8	1	185.1
D	11	296.9	36	54.3	47	123.2
P (genotype)						**0.001**
P (disease group)						**<0.001**

Table 3.7: Comparison of age-sex-adjusted geometric means* of M30 levels (U/l) between HCC cases and non-cases in Asians

Disease category	No.	Geometric mean (95%CI)	P value^
HCC patients	116	265.8 (232.4, 304.0)	
Non-HCC controls	158	192.5 (173.0, 214.2)	**<0.001**
Carriers + uninfected controls	146	187.7 (168.3, 209.4)	**<0.001**
Carriers	126	186.2 (165.3, 209.7)	**<0.001**
Chronic hepatitis patients	12	288.2 (198.4, 418.7)	0.684
Uninfected controls	20	189.2 (141.9, 252.2)	**0.037**

*From one-way analysis of covariance with age and sex as the covariates and log-transformed M30 as the dependent variable.
^ Test for difference between HCC and other disease groups.

Table 3.8: Comparison of crude geometric means* of M30 levels (U/l) between HCC cases and non-cases in South Africans

Disease category	No.	Geometric mean (95%CI)	P value^
HCC patients	100	465.3 (382.6, 566.0)	
Non-HCC controls	246	81.5 (71.8, 92.5)	**<0.001**
Carriers	195	91.1 (79.1, 104.8)	**<0.001**
Uninfected controls	51	53.4 (40.6, 70.2)	**<0.001**

*From one-way analysis of covariance with log-transformed M30 value as the dependent variable and disease group as the independent variable.
^Test for difference between HCC and other disease groups.

Table 3.9: Comparison of crude geometric means^ of M30 levels (U/l) between HCC cases and non-cases using total subjects

	Geometric mean (95%CI)		
Disease type	Model I*	Model II**	Model III***
HCC patients	330.1 (277.2, 393.0)	322.7 (271.3, 383.7)	332.6 (221.6, 499.2)
Carriers	120.2 (99.8, 144.9)	123.3 (102.4, 148.6)	130.0 (86.6, 195.1)
Uninfected controls	76.8 (58.6, 100.5)	---	---
P value	**<0.001**	**<0.001**	**<0.001**

^One-way analysis of covariance with race, HBV viral load (log transformed) and HBV genotype as the covariates and log-transformed M30 value as the dependent variable.
*Adjusted for race.
** Further adjusted for HBV viral load (log transformed).
*** Further adjusted for HBV genotype.

Table 3.10: Crude geometric means* of M30 levels (U/l) by 1862 nucleotide in South Africans

1862nt	HCC patients		Carriers		All subjects	
	n	M30	n	M30	n	M30
Wild-type	70	390.1	168	90.8	238	192.8
Mutant	20	856.7	11	77.4	31	290.3
P (1862nt)						**0.044**
P (disease)						**<0.001**
P (1862nt x disease)						**0.021**

*From analysis of variance with log-transformed M30 value as the dependent variable and 1862nt and disease group as the independent variable.

The following table summarises the observations noted within this chapter:

Table 3.11: Summary of associations

	Genotypes/subgenotypes		HBV Viral load		M30 apoptosis	
Effect of	Asian	South African	Asian	South African	Asian	South African
Gender	↑ ♂ 64 years and older infected with genotype B	N/A	↓ ASC ♂ ↑ HCC ♂	N/A	↑ ♂	N/A
Age	None	N/A	None	N/A	↑ ASC 64 years or older	N/A
Genotype	N/A	N/A	↑ Chinese genotype C vs genotype B2	None	None	↑ genotype A vs genotype D
Disease	Genotype C 1.45 ↑ risk HCC vs B2	Subgenotype A1 1.57 ↑ risk HCC vs D	None	HCC > ASC	HCC > ASC / UC HCC ≡ CH	HCC > ASC / UC 1862 Mt > Wt
Viral load	N/A	N/A	N/A	N/A	Positively and significantly associated	Positively and significantly associated

Abbreviations: HCC = hepatocellular carcinoma, ASC = asymptomatic carriers, UC = uninfected controls, CH = chronic carriers, N/A = not applicable, Mt = mutant, Wt = wild-type , ↑ = more or higher, ↓ = lower, ♂ = male.

3.4. DISCUSSION

The influence of HBV viral factors such as genotype/subgenotype, HBV DNA levels (Chan et al., 2009b) and mutations on disease progression and clinical outcome of HBV infection have been well documented (Taylor et al., 2009, Kao et al., 2003). Subgenotype A1 is the most common HBV strain found in South Africa (Kramvis et al., 2002). Unique to this subgenotype is the guanine to thymine 1862 mutation that is present in 24% of HBeAg negative southern African carriers (Kramvis et al., 1997). Studies have shown that the 1862 mutation is associated with HCC in the southern African black population, particularly in HBeAg negative patients and may therefore play a part in the development of HCC (Kramvis et al., 1998). In addition to host factors, including an older age and male gender, viral factors such as being positive for HBV e antigen and having high viral HBV DNA levels, genotype and basal core promoter mutations have been suggested to be associated with a higher risk of HCC (Chan et al., 2008, Chen et al., 2006c, Kao et al., 2003, Yang et al., 2002).

The present study cohort comprised 635 serum samples from South Africa, China and Japan. Of these samples, 564 were HBsAg-positive and the remaining 71 HBsAg-negative, HBV DNA negative controls. Possible associations were determined between HBV genotype, viral load, apoptosis levels, disease group and the age and gender of the patient where available. The association of these factors and apoptosis had not been previously investigated.

3.4.1) Genotype/Subgenotype associations

The proportion of Asian men, older than 64 years that were infected with genotype B was higher compared to younger men (Figure 3.5). Asian patients infected with genotype C had a 1.45 greater probability of developing HCC compared to subgenotype B2 infected patients. A similar association has been observed in a large study from Hong Kong where it was found that genotype C was associated with a 1.5 greater rate of developing HCC (Chan et al., 2008) while several other studies have observed even greater odd risks associations of 5.85 (Chou et al., 2008) and 5.97 (Chou et al., 2008, Wu et al., 2008) (Table 3.1). In a study among Alaskan Native people, the odds of HCC were four times higher in adults infected with genotype A compared to genotype D (Livingston et al., 2007b). A similar association was observed amongst the present South African cohort, with subgenotype A1 having a 1.85 greater odd of HCC compared to genotype D. A higher odds risk of 4.5 was found in an

earlier South African study, which included rural participants and discussed the possibility of aflatoxin as a contributing factor (Kew, 2010).

A few other studies have compared genotypes A and D, without differentiating subgenotypes. Some studies suggested that genotype A is associated with more severe disease (Kobayashi et al., 2006, Livingston et al., 2007b) while other studies suggest that genotype D results in more severe liver disease (Sanchez-Tapias et al., 2002, Thakur et al., 2002). Possible explanations for the conflicting observations are differences in subgenotype distribution as well as host factors. When comparing subgenotype A1 and A2 strains, mutations were found to occur at different frequencies and mutations unique to each subgenotype have been identified (Kramvis et al., 2008).

3.4.2) HBV Viral Load associations

Among the Asian cohort, genotype C infected patients had significantly higher HBV viral loads compared to those infected with subgenotype B2 (Table 3.2). Moreover, HCC patients had higher viral loads compared to the asymptomatic carriers. Higher HBV viral load levels have been consistently associated with a poorer clinical outcome involving an increased risk of HCC development (Tong et al., 2006, Iloeje et al., 2006). Both genotype C and high HBV DNA levels combined have been shown previously to be associated with HCC development (Mahmood et al., 2005). In the present study, the South African HCC patients had significantly higher viral loads compared to the asymptomatic carriers regardless of whether they were infected with either genotype A or D (Table 3.3) confirming our previous findings (Viana et al., 2009). This observation re-affirms the accumulating evidence that high levels of HBV DNA are associated with increased risk of HCC and death (Chen et al., 2006b, Ikeda et al., 2005, Yu et al., 2005), regardless of the HBV genotype. Age and sex were not differentiated in the South African cohort.

Males and females tend to follow different clinical courses with HBV infection (Shiratori et al., 1995, Lee et al., 1999, Tsay et al., 2009). The relative risk of chronic HBV-related end-stage liver disease, HCC and liver disease-related death are consistently several fold higher in males than females (Evans et al., 2002, Chang et al., 2000, Taylor et al., 2009). This has been suggested to be attributable to several factors involved in the production of testosterone (Yu et al., 2000, Yu et al., 2001, Yeh et al., 2007). Among the Asian asymptomatic carriers, HBV viral load was significantly lower in men than in women, whereas among the HCC patients

the opposite was true; it was significantly higher in men than in women (Figure 3.6). This finding supports the observation discussed above that both viral load as well as male gender are associated with HCC.

3.4.3) Apoptosis associations

When the normal balance of populations of apoptotic and proliferating cells is disturbed, this can lead to the development of diseases like cancer and autoimmunity. In tumours compared to normal tissue, spontaneous apoptosis is increased and often associated with tumour cell turnover (Strater et al., 1995). Apoptosis levels were quantitated by the detection of cytokeratin 18Asp396 (M30) neo-epitope and associations determined between the host and viral factors. Host factors including older age (Figure 3.8) and male gender (Figure 3.9) were found to be associated with significantly higher levels of apoptosis. The HBV viral load was also found to be positively and significantly associated with apoptosis (Table 3.4). Relative levels of HBV DNA have been shown to reflect the degree of necroinflammatory activity in the liver, which itself reflects the host's immune response to the virus (Mommeja-Marin et al., 2003), it therefore can be expected that higher apoptosis levels are associated with heightened disease activity associated with a higher HBV viral load. Similarly, it has been shown that older age (Ito et al., 2010) and male gender are associated with an increased risk of liver disease and HCC (Taylor et al., 2009), therefore again it can be surmised that higher apoptosis levels would be expected to be associated with these increased disease risk factors.

Significantly higher apoptosis levels were found to be associated with subgenotype A1 compared to genotype D (Table 3.6). This supports data suggesting that genotype A is associated with more severe liver disease (Kobayashi et al., 2006, Livingston et al., 2007b) as well as the increased HCC risk association shown in the present and in an earlier study (Kew et al., 2005). A similar association between genotype C and B was not observed suggesting perhaps a different mode of cell death or liver disease generation. An *in vitro* study has previously shown an association with apoptosis and HBV genotypes however genotype B displayed a stronger pro-apoptotic effect compared to genotype A and C (Lu et al., 2007). Subgenotypes however were not distinguished in the *in vitro* study.

As apoptosis goes through various extrinsic and intrinsic pathways, with activation of caspases and cleavage of specific substrates, such as cytokeratin 18, HBV proteins can interfere with the various apoptotic processes. So far, five HBV proteins have been shown to

be involved with apoptosis: the large envelope protein, a truncated form of the middle envelope protein, the HBx protein, HBSP - a protein generated from spliced mRNA, and the precore protein (Pollicino et al., 1998, Liang et al., 2007, Lu and Chen, 2005, Lu et al., 2006, Assrir et al., 2010). Like the truncated middle envelope protein, the G1862T mutation causes the accumulation of unprocessed precore protein in the endoplasmic reticulum (Caselmann, 1995, Chen et al., 2008). In the current study, significantly higher levels of apoptosis were found to be associated with South African HCC patients infected with the G1862T mutant variant compared to the asymptomatic patients infected with the same variant. It is important to note, however that when comparing patients infected with the variant versus the wild-type, apoptosis levels were found to be significantly lower in asymptomatic patients infected with the variant. The opposite was observed within the HCC patient group, with significantly higher levels of apoptosis being associated with patients infected with the variant (Table 3.10). These observations suggest that the G1862T mutation has an effect on the levels of apoptosis however the effect on apoptosis may differ at different stages of HBV disease progression. Further investigation of the role of the G1862T mutation as a pro- or anti-apoptotic factor in the various stages of HBV infection could explain these differences.

When comparing the levels of apoptosis among the different disease groups, no significant difference was observed between the Asian HCC and chronic hepatitis patients (Table 3.7). The same observation was noted by McPartland *et al* in 2005 when comparing histologically four chronic HBV patients and seven HCC patients (McPartland et al., 2005). The number of chronically infected subjects investigated in the current study was also low (n=20) and further investigation with larger numbers of chronically infected HBV patients is suggested. Significantly higher levels of apoptosis however were found to be associated with the HCC patients compared to the asymptomatic carriers and uninfected control subjects, even after the adjustment for HBV viral load and genotype (Table 3.9). This significant observation suggests the potential use of cleaved cytokeratin 18 (M30) as a biomarker of HBV disease progression and potential presence and development of HCC.

Liver biopsy, the gold standard for liver injury and fibrosis staging, was widely recommended for the assessment of liver disease and hepatocellular carcinoma. However, the limitations of liver biopsy include its invasive nature and possible complications, inadequate biopsy size, intra- and inter-observer variability, tissue fragmentation, high cost, and its low acceptance by most patients (Cadranel et al., 2000, Bedossa et al., 2003, Regev et al., 2002). This has lead

to the development of noninvasive procedures to stage liver disease, which include either imaging methods or assays based on serum biomarkers (Saito et al., 2004, Castera et al., 2005). Serum alanine aminotransferase (ALT) levels are regarded as a reliable biomarker of hepatocyte damage; however discrepancies in serum ALT concentrations and histologic activity have been documented (Sanai et al., 2008). Bantel *et al* in 2004 reported an increase in the presence of cleaved cytokeratin 18 in the sera from hepatitis C virus (HCV) infected patients over healthy controls (Bantel et al., 2004). As in the case of HCV, cleaved cytokeratin 18 has also been shown to be an accurate biomarker for assessing and monitoring the histologic activity of nonalcoholic fatty liver disease (NAFLD) as well as alcoholic hepatitis (Tsutsui et al., 2010, Gonzalez-Quintela et al., 2009). Recently it was shown that cleaved cytokeratin 18 can be used in the monitoring of chronic hepatitis B treatment. Cleaved cytokeratin 18 levels were found to parallel the ALT, AST and HBV DNA levels observed during oral antiviral therapy (Giannousis et al., 2011).

In tumors compared to normal tissue, spontaneous apoptosis is increased and often associated with tumour cell turnover (Strater et al., 1995). Higher baseline apoptotic indices in untreated tumours are associated with undifferentiated malignancies and lower survival rates (Meggiato et al., 2000). Upon treatment of a tumour with a therapy that induces killing, tumour size regresses and this can be attributed to apoptosis or senescence and seems to be tumour type specific (Serrano, 2007). In a study of breast cancer patients who received neoadjuvant chemotherapy, total cytokeratin 18 sera levels correlated positively with treatment response and patient survival (Olofsson et al., 2007). A number of recent studies have shown that cleaved cytokeratin 18 can be used to monitor cancer therapy treatment response (Brandt et al., 2010, Ausch et al., 2009, Micha et al., 2008) and could therefore potentially be used as a biomarker for the monitoring of the development and treatment of HBV derived HCC.

To summarise, several associations were found between viral and host factors. Patients infected with genotype C or subgenotype A1 were shown to possess a higher odds ratios of developing HCC compared to subgenotype B2 and genotype D, respectively. As has previously been reported, significantly higher HBV viral loads were observed in genotype C compared to subgenotype B2. A positive association between higher HBV viral load levels and HCC in the South African cohort, where subgenotype A1 and genotype D prevail, was observed. Among the Asian cohort it was shown that the male gender was positively associated with high viral loads in HCC patients.

Male gender, older age, HBV viral load as well as subgenotype A1 have been shown in the present study to be positively and significantly associated with higher levels of apoptosis. The presence of the G1862T variant in HCC South African patients was also shown to be positively and significantly associated with apoptosis. Even in the absence of the G1862T variant, significantly higher apoptosis levels were observed in the HCC patient group compared to the other non-HCC patient groups suggesting the potential role of cleaved cytokeration 18 as a non-invasive marker for HBV disease progression and possibly the detection and monitoring of HCC.

Risk equations and risk functions are widely applied in patient management for predicting outcomes in liver diseases (Iacob et al., 2007, Kim et al., 2007). Risk equations are used for patient counseling/management, clinical diagnosis, risk stratification, treatment selection, and prognosis prediction. Identifying patients at risk of progressing to hepatocellular carcinoma may help in early management decisions and justifying health resource allocation. Nomograms derived from risk functions are easy-to-use graphs for predicting the risk of an individual developing an outcome over a specified time frame (McCormack et al., 1997, D'Agostino et al., 2008).

A risk score of hepatocellular carcinoma developed from a retrospectively assembled hospital population was been reported (Yuen et al., 2009). Yang *et al* recently developed a nomogram utilising the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer–Hepatitis B Virus (REVEAL-HBV) study data (Yang et al., 2010). This nomogram includes all the risk factors detailed in this chapter and could in future potentially incorporate cleaved cytokeration 18 levels as a predictor following further prospective studies.

CHAPTER 4

CONCLUSION

The main objective of this thesis was to functionally characterise the strains of HBV prevailing in South Africa, namely subgenotype A1 and D, relative to other genotypes, using both an animal model and in a population based study. To control for the influence of host factors on HBV infection as well as to avoid the use of *in vitro* cell lines, such as Huh-7, that have defective apoptotic pathways, the *in vivo* urokinase plasminogen activator severe combined immunodeficient (uPA-SCID) transgenic mouse model was utilised. Furthermore, a population based study was carried out to investigate the influence of host factors and immune pressure on HBV infection in various disease groups comparing genotypes prevailing in South Africa and south east Asia, the other region besides Africa, where HBV is hyperendemic.

This is the first study in which uPA-SCID transgenic mice grafted with human hepatocytes have been infected with subgenotype A1 of HBV. This is also the first study relating apoptosis to HBV infection in this transgenic mouse model. The successful establishment of the *in vivo* HBV infection with different genotypes or subgenotypes in the uPA-SCID transgenic mice was demonstrated by the increase of HBV DNA levels, the presence of cccDNA and HBV transcripts as well as the detection of the core and/or surface HBV antigens in the chimeric mice liver tissue. Differences between the HBV infections with the various genotype/subgenotypes were observed in both the uPA-SCID mice and population based study. Subgenotype A1 with the G1862T mutation showed the earliest detection and therefore highest levels of cccDNA as well as the highest HBV DNA levels when compared to the other strains. The highest HBV DNA levels were recorded for the subgenotype A1 G1862T infected transgenic mouse followed by genotype D, subgenotype A2 and the lowest levels observed in the subgenotype A1 wild-type infected transgenic mouse. When comparing HBV viral load levels of subgenotype A1 and genotype D in the population study no significant difference was observed. This suggests that host factors and immune pressure may play a role in the regulation of HBV DNA production.

HBsAg was only detected in the livers of mice infected with subgenotype A1 with the G1862T mutation, which was indicative of the high HBV replication levels. HBcAg staining

in the chimeric liver was positive when the mice were infected with genotype D, which concurs with previous observations that genotype D is characterised by high HBcAg expression (Sugiyama et al., 2006). Subgenotype A1 with the G1862T mutant showed the highest levels of apoptosis as a result of the abnormal precore precursor protein accumulation shown to be associated with this 1862 missense mutation (Chen et al., 2008, Hou et al., 2002). In the population based study a similar finding was observed altlhough apoptosis levels differed between the disease groups. Significantly higher apoptosis levels were observed in HCC patients infected with subgenotype A1 1862 mutant, while the opposite was observed in the asymptomatic group, with significantly lower apoptosis levels associated with patients infected with subgenotype A1 1862. These findings suggest that the G1862T variant influences HBV infection by affecting the levels of apoptosis at different stages of HBV disease progression. This may suggest a potential switch from anti- to pro- apoptosis depending on the progression of HBV disease and requires further investigation. Thus different genotypes and subgenotypes as well as variations within genotypes can influence the outcome of HBV infection, which can further be influenced by host factors.

In the population based study, associations were found between genotypes and host factors including age and gender. Moreover, subgenotypes or genotypes were found to be related to HBV viral load as well as to apoptosis and to HCC. Patients infected with genotype C or subgenotype A1 were shown to possess a higher odds ratios of developing HCC compared to subgenotype B2 or genotype D, respectively. In agreement with previous studies, significantly higher HBV viral loads were observed in genotype C compared to subgenotype B2. Among the Asian cohort, it was also shown that the male gender was positively associated with high viral loads in HCC patients. Moreover, a positive association between higher HBV viral load levels and HCC in the South African cohort was observed.

Male gender, older age, HBV viral load, subgenotype A1 and the presence of the G1862T mutation were shown to be positively and significantly associated with higher levels of apoptosis. Apoptosis was measured by the presence of cleaved cytokeratin 18 (M30) and shown to be significantly higher in the HCC disease group compared to the asymptomatic group regardless of ethnicity, viral load and genotype. This suggests the potential use of M30 as a surrogate marker for HBV disease progression.

When comparing the genotypes that prevail in South Africa, significantly higher apoptosis levels were found to be associated with subgenotype A1 compared to genotype D. This shows that even within a specific geographical area, the presence of multiple genotypes has an effect on HBV infection and disease.

We conclude that even when the influence of host and environmental factors is controlled for, as is the case in the chimeric mouse model, the HBV genotype can affect the progression of infection. Moreover, it was shown in the population based study that the effect of HBV genotype on the outcome of HBV infection can be influenced by host factors. The subgenotype A1 G1862T mutation was shown in both studies to affect both HBV infection and apoptosis. This suggests that HBV variants should be investigated to ascertain their potential impact on the course of HBV infection as it may differ from the wild-type. Apoptosis was shown to be associated with HBV infection in both studies and could possibly be an ideal marker of the progression of HBV infection.

These findings are important in helping us to understand factors influencing the course of HBV infection. We have therefore shown in both the studies that differences do exist between the South African subgenotype A1 and genotype D, and that these differences should be taken into consideration for the future evaluation of HBV infection and treatment of South African HBV infected patients. Moreover, cleaved cytokeratin 18 may provide an ideal surrogate marker for HBV disease progression and monitoring.

APPENDIX A – LISTING OF SOLUTIONS

A1) 1 % AGAROSE GEL

- 100 ml 1X TBE buffer (A2)
- 1 g of agarose
- 6 µl of 0.01 mg/µl ethidium bromide

A2) 1 X TRIS-BORATE-EDTA (TBE) BUFFER

- 10.8 g of Tris-base
- 0.93 g of EDTA
- 5.5 g Boric acid
- Dissolve in 1 ℓ distilled water

A3) 6 X GEL LOADING DYE

- 3 ml glycerol
- 25 mg bromophenol blue (Merck, Germany)

Made up to 10 ml with nuclease-free sterile water

A4) 3 % COMPOSITE AGAROSE GEL

- 100 ml 1 X TBE buffer (A2)
- 1 g of D1 agarose (Pronadisa, Madrid, Spain)
- 2 g of NuSieve GTG agarose (BioWhittacker Molecular Applications, Rockland, ME)
- 6 µl of 0.01 mg/µl ethidium bromide

A5) 0.8 % CRYSTAL VIOLET AGAROSE GEL

- 50 ml 1 X TAE buffer (A6)
- 0.4 g of agarose
- 40 µl of 2 mg / µl Crystal Violet solution

A6) 1 X TRIS-ACETIC-EDTA (TAE) BUFFER

- 4.84 g of Tris-base
- 0.74 g of EDTA
- 1.14 ml glacial acetic acid
- Dissolve in 1 ℓ distilled water

A7) LURIA BERTANI (LB) AGAR PLATES WITH KANAMYCIN

- 15 g agar
- 10 g/ℓ sodium chloride
- 10 g/ℓ tryptone
- 5 g/ℓ yeast extract
- Dissolve in 1 ℓ distilled water

} pH 7.4

- Autoclave for 20 minutes
- Cool to 55 ^{o}C and add 1 ml of kanamycin (50 mg/ml)
- Pour into 60 mm petri dishes and store at 4 ^{o}C

A8) LURIA BERTANI (LB) BROTH WITH KANAMYCIN

- 10 g/ℓ sodium chloride
- 10 g/ℓ tryptone
- 5 g/ℓ yeast extract
- Dissolve in 1ℓ Sabax water

} pH 7.4

- Autoclave for 20 minutes
- Cool to 55 ^{o}C and add 1 ml of kanamycin (50 mg/ml)
- Store at 4 ^{o}C

APPENDIX B – ETHICS

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Viana

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070904**
PROJECT	Functional Characterisation of Subgenotype A1 Isolates in Vivo using a Urokinase-Type Plasmingen Activator-Severe Combined Immunodeficiency (uPA-SCD) Transgenic....
INVESTIGATORS	Miss R Viana
DEPARTMENT	Department of Medicine
DATE CONSIDERED	07.09.28
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.10.01 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

UNIVERSITY OF THE WITWATERSRAND
PROF AJ WOODIWISS
Co - Chair
2007 -10- 05
REC (Medical)
JOHANNESBURG

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Kew

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

REFERENCES

AHN, S. H., YUEN, L. & REVILL, P. 2009. Clarification required for the definition of hepatitis B virus subgenotypes C1 and C2. *Intervirology,* 52**,** 321-2.

ALVARADO MORA, M. V., ROMANO, C. M., GOMES-GOUVEA, M. S., GUTIERREZ, M. F., CARRILHO, F. J. & PINHO, J. R. 2010. Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol,* 91**,** 501-8.

ANDERNACH, I. E., HUBSCHEN, J. M. & MULLER, C. P. 2009a. Hepatitis B virus: the genotype E puzzle. *Rev Med Virol,* 19**,** 231-40.

ANDERNACH, I. E., NOLTE, C., PAPE, J. W. & MULLER, C. P. 2009b. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis,* 15**,** 1222-8.

ANTONOPOULOS, A., NORTH, S. J., HASLAM, S. M. & DELL, A. 2011. Glycosylation of mouse and human immune cells: insights emerging from N-glycomics analyses. *Biochem Soc Trans,* 39**,** 1334-40.

ARANKALLE, V. A., GANDHE, S. S., BORKAKOTY, B. J., WALIMBE, A. M., BISWAS, D. & MAHANTA, J. 2010. A novel HBV recombinant (genotype I) similar to Vietnam/Laos in a primitive tribe in eastern India. *J Viral Hepat*.

ARAUZ-RUIZ, P., NORDER, H., ROBERTSON, B. H. & MAGNIUS, L. O. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol,* 83**,** 2059-73.

BAHRAMALI, G., SADEGHIZADEH, M., AMINI-BAVIL-OLYAEE, S., ALAVIAN, S. M., BEHZAD-BEHBAHANI, A., ADELI, A., AGHASADEGHI, M. R., AMINI, S. & MAHBOUDI, F. 2008. Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients. *World J Gastroenterol,* 14**,** 5448-53.

BAHRI, O., CHEIKH, I., HAJJI, N., DJEBBI, A., MAAMOURI, N., SADRAOUI, A., MAMI, N. B. & TRIKI, H. 2006. Hepatitis B genotypes, precore and core promoter mutants circulating in Tunisia. *J Med Virol,* 78**,** 353-7.

BARAK, V., GOIKE, H., PANARETAKIS, K. W. & EINARSSON, R. 2004. Clinical utility of cytokeratins as tumor markers. *Clin Biochem,* 37**,** 529-40.

BARKER, L. F., MAYNARD, J. E., PURCELL, R. H., HOOFNAGLE, J. H., BERQUIST, K. R. & LONDON, W. T. 1975. Viral hepatitis, type B, in experimental animals. *Am J Med Sci,* 270**,** 189-95.

BARKER, L. F. & MURRAY, R. 1972. Relationship of virus dose to incubation time of clinical hepatitis and time of appearance of hepatitis--associated antigen. *Am J Med Sci,* 263**,** 27-33.

BERK, P. D. & POPPER, H. 1978. Fulminant hepatic failure. *Am J Gastroenterol,* 69**,** 349-400.

BOCK, C. T., SCHWINN, S., LOCARNINI, S., FYFE, J., MANNS, M. P., TRAUTWEIN, C. & ZENTGRAF, H. 2001. Structural organization of the hepatitis B virus minichromosome. *J Mol Biol,* 307**,** 183-96.

BOUCHARD, M. J. & SCHNEIDER, R. J. 2004. The enigmatic X gene of hepatitis B virus. *J Virol,* 78**,** 12725-34.

BRESSAC, B., GALVIN, K. M., LIANG, T. J., ISSELBACHER, K. J., WANDS, J. R. & OZTURK, M. 1990. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc Natl Acad Sci U S A,* 87**,** 1973-7.

BUCKWOLD, V. E., XU, Z., YEN, T. S. & OU, J. H. 1997. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor

mutations on hepatitis B virus gene expression and replication. *J Gen Virol,* 78 (Pt 8)**,** 2055-65.
BURNETT, R. J., FRANCOIS, G., KEW, M. C., LEROUX-ROELS, G., MEHEUS, A., HOOSEN, A. A. & MPHAHLELE, M. J. 2005. Hepatitis B virus and human immunodeficiency virus co-infection in sub-Saharan Africa: a call for further investigation. *Liver Int,* 25**,** 201-13.
CANBAY, A., FRIEDMAN, S. & GORES, G. J. 2004. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology,* 39**,** 273-8.
CAO, G. W. 2009. Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J Gastroenterol,* 15**,** 5761-9.
CARMAN, W. F., JACYNA, M. R., HADZIYANNIS, S., KARAYIANNIS, P., MCGARVEY, M. J., MAKRIS, A. & THOMAS, H. C. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet,* 2**,** 588-91.
CHAN, H. L., HUI, A. Y., WONG, M. L., TSE, A. M., HUNG, L. C., WONG, V. W. & SUNG, J. J. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut,* 53**,** 1494-8.
CHAN, H. L., TSANG, S. W., LIEW, C. T., TSE, C. H., WONG, M. L., CHING, J. Y., LEUNG, N. W., TAM, J. S. & SUNG, J. J. 2002. Viral genotype and hepatitis B virus DNA levels are correlated with histological liver damage in HBeAg-negative chronic hepatitis B virus infection. *Am J Gastroenterol,* 97**,** 406-12.
CHAN, H. L., TSE, C. H., MO, F., KOH, J., WONG, V. W., WONG, G. L., LAM CHAN, S., YEO, W., SUNG, J. J. & MOK, T. S. 2008. High viral load and hepatitis B virus subgenotype ce are associated with increased risk of hepatocellular carcinoma. *J Clin Oncol,* 26**,** 177-82.
CHAN, H. L., WONG, G. L., TSE, C. H., CHIM, A. M., YIU, K. K., CHAN, H. Y., SUNG, J. J. & WONG, V. W. 2009a. Hepatitis B virus genotype C is associated with more severe liver fibrosis than genotype B. *Clin Gastroenterol Hepatol,* 7**,** 1361-6.
CHAN, H. L., WONG, V. W., WONG, G. L., CHIM, A. M., LAI, L. H. & SUNG, J. J. 2009b. Evaluation of impact of serial hepatitis B virus DNA levels on development of hepatocellular carcinoma. *J Clin Microbiol,* 47**,** 1830-6.
CHANDRA, P. K., BISWAS, A., DATTA, S., BANERJEE, A., PANIGRAHI, R., CHAKRABARTI, S., DE, B. K. & CHAKRAVARTY, R. 2009. Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. *J Viral Hepat,* 16**,** 749-56.
CHEN, B. F., LIU, C. J., JOW, G. M., CHEN, P. J., KAO, J. H. & CHEN, D. S. 2006a. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology,* 130**,** 1153-68.
CHEN, C. H., HUNG, C. H., LEE, C. M., HU, T. H., WANG, J. H., WANG, J. C., LU, S. N. & CHANGCHIEN, C. S. 2007. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. *Gastroenterology,* 133**,** 1466-74.
CHEN, C. Y., CROWTHER, C., KEW, M. C. & KRAMVIS, A. 2008. A valine to phenylalanine mutation in the precore region of hepatitis B virus causes intracellular retention and impaired secretion of HBe-antigen. *Hepatol Res,* 38**,** 580-92.
CHEN, G., LIN, W., SHEN, F., ILOEJE, U. H., LONDON, W. T. & EVANS, A. A. 2006b. Past HBV viral load as predictor of mortality and morbidity from HCC and chronic liver disease in a prospective study. *Am J Gastroenterol,* 101**,** 1797-803.

CHEN, Y., SZE, J. & HE, M. L. 2004. HBV cccDNA in patients' sera as an indicator for HBV reactivation and an early signal of liver damage. *World J Gastroenterol,* 10**,** 82-5.

CHENG, H., SU, H., WANG, S., SHAO, Z., MEN, K., LI, M., LI, S., ZHANG, J., XU, J., ZHANG, H., YAN, Y. & XU, D. 2009. Association between genomic heterogeneity of hepatitis B virus and intrauterine infection. *Virology,* 387**,** 168-75.

CHOI, C. S., CHO, E. Y., PARK, R., KIM, S. J., CHO, J. H. & KIM, H. C. 2009. X gene mutations in hepatitis B patients with cirrhosis, with and without hepatocellular carcinoma. *J Med Virol,* 81**,** 1721-5.

CHU, C. J., HUSSAIN, M. & LOK, A. S. 2002a. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology,* 122**,** 1756-62.

CHU, C. J., HUSSAIN, M. & LOK, A. S. 2002b. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology,* 36**,** 1408-15.

CHU, C. J. & LOK, A. S. 2002. Clinical significance of hepatitis B virus genotypes. *Hepatology,* 35**,** 1274-6.

CONG, Y. S., YAO, Y. L., YANG, W. M., KUZHANDAIVELU, N. & SETO, E. 1997. The hepatitis B virus X-associated protein, XAP3, is a protein kinase C-binding protein. *J Biol Chem,* 272**,** 16482-9.

D'AGOSTINO, R. B., SR., VASAN, R. S., PENCINA, M. J., WOLF, P. A., COBAIN, M., MASSARO, J. M. & KANNEL, W. B. 2008. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation,* 117**,** 743-53.

DANDRI, M., BURDA, M. R., TOROK, E., POLLOK, J. M., IWANSKA, A., SOMMER, G., ROGIERS, X., ROGLER, C. E., GUPTA, S., WILL, H., GRETEN, H. & PETERSEN, J. 2001. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology,* 33**,** 981-8.

DANDRI, M., BURDA, M. R., ZUCKERMAN, D. M., WURSTHORN, K., MATSCHL, U., POLLOK, J. M., ROGIERS, X., GOCHT, A., KOCK, J., BLUM, H. E., VON WEIZSACKER, F. & PETERSEN, J. 2005. Chronic infection with hepatitis B viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes. *J Hepatol,* 42**,** 54-60.

DAVIS, L. G., WEBER, D. J. & LEMON, S. M. 1989. Horizontal transmission of hepatitis B virus. *Lancet,* 1**,** 889-93.

DOMINGO, E., SABO, D., TANIGUCHI, T. & WEISSMANN, C. 1978. Nucleotide sequence heterogeneity of an RNA phage population. *Cell,* 13**,** 735-44.

DONG, Q., CHAN, H. L., LIU, Z., CHAN, D. P., ZHANG, B., CHEN, Y., KUNG, H. F., SUNG, J. J. & HE, M. L. 2008. A1762T/G1764A mutations of hepatitis B virus, associated with the increased risk of hepatocellular carcinoma, reduce basal core promoter activities. *Biochem Biophys Res Commun,* 374**,** 773-6.

DOWEIKO, J. P. & NOMPLEGGI, D. J. 1991. The role of albumin in human physiology and pathophysiology, Part III: Albumin and disease states. *JPEN J Parenter Enteral Nutr,* 15**,** 476-83.

ELGOUHARI, H. M., ABU-RAJAB TAMIMI, T. I. & CAREY, W. D. 2008. Hepatitis B virus infection: understanding its epidemiology, course, and diagnosis. *Cleve Clin J Med,* 75**,** 881-9.

FANG, Z. L., SABIN, C. A., DONG, B. Q., GE, L. Y., WEI, S. C., CHEN, Q. Y., FANG, K. X., YANG, J. Y., WANG, X. Y. & HARRISON, T. J. 2008. HBV A1762T, G1764A mutations are a valuable biomarker for identifying a subset of male HBsAg carriers at extremely high risk of hepatocellular carcinoma: a prospective study. *Am J Gastroenterol,* 103**,** 2254-62.

FATTOVICH, G. 2003. Natural history of hepatitis B. *J Hepatol,* 39 Suppl 1**,** S50-8.
FATTOVICH, G., STROFFOLINI, T., ZAGNI, I. & DONATO, F. 2004. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology,* 127**,** S35-50.
FELSESTEIN, J. 1995. PHYLIP (Phylogeny Inference Package). Department of Genetics, University of Washington, Seattle, Washington.
FRANCOIS, G., KEW, M., VAN DAMME, P., MPHAHLELE, M. J. & MEHEUS, A. 2001. Mutant hepatitis B viruses: a matter of academic interest only or a problem with far-reaching implications? *Vaccine,* 19**,** 3799-815.
FRODSHAM, A. J. 2005. Host genetics and the outcome of hepatitis B viral infection. *Transpl Immunol,* 14**,** 183-6.
FUNG, J., YUEN, M. F., YUEN, J. C., WONG, D. K. & LAI, C. L. 2007. Low serum HBV DNA levels and development of hepatocellular carcinoma in patients with chronic hepatitis B: a case-control study. *Aliment Pharmacol Ther,* 26**,** 377-82.
FUNK, M. L., ROSENBERG, D. M. & LOK, A. S. 2002. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. *J Viral Hepat,* 9**,** 52-61.
GALLE, P. R. & KRAMMER, P. H. 1998. CD95-induced apoptosis in human liver disease. *Semin Liver Dis,* 18**,** 141-51.
GANDHE, S. S., CHADHA, M. S. & ARANKALLE, V. A. 2003. Hepatitis B virus genotypes and serotypes in western India: lack of clinical significance. *J Med Virol,* 69**,** 324-30.
GANEM, D. 1991. Assembly of hepadnaviral virions and subviral particles. *Curr Top Microbiol Immunol,* 168**,** 61-83.
GANEM, D. & PRINCE, A. M. 2004. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med,* 350**,** 1118-29.
GANEM, D. & VARMUS, H. E. 1987. The molecular biology of the hepatitis B viruses. *Annu Rev Biochem,* 56**,** 651-93.
GAO, Z. Y., LI, T., WANG, J., DU, J. M., LI, Y. J., LI, J., LU, F. M. & ZHUANG, H. 2007. Mutations in preS genes of genotype C hepatitis B virus in patients with chronic hepatitis B and hepatocellular carcinoma. *J Gastroenterol,* 42**,** 761-8.
GAVILANES, F., GONZALEZ-ROS, J. M. & PETERSON, D. L. 1982. Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins. *J Biol Chem,* 257**,** 7770-7.
GIANNOUSIS, I. P., MANOLAKOPOULOS, S. G., HADZIYANNIS, E., GEORGIOU, A. & PAPATHEODORIDIS, G. V. 2011. Changes of serum levels of keratin-18 fragments in hepatitis B e antigen-negative chronic hepatitis B patients under oral antiviral therapy. *Antivir Ther,* 16**,** 505-14.
GILBERT, S., LORANGER, A., DAIGLE, N. & MARCEAU, N. 2001. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. *J Cell Biol,* 154**,** 763-73.
GREEN, D. R. 2005. Apoptotic pathways: ten minutes to dead. *Cell,* 121**,** 671-4.
GREEN, D. R. & KROEMER, G. 2004. The pathophysiology of mitochondrial cell death. *Science,* 305**,** 626-9.
GUARNIERI, M., KIM, K. H., BANG, G., LI, J., ZHOU, Y., TANG, X., WANDS, J. & TONG, S. 2006. Point mutations upstream of hepatitis B virus core gene affect DNA replication at the step of core protein expression. *J Virol,* 80**,** 587-95.
GUICCIARDI, M. E. & GORES, G. J. 2005. Apoptosis: a mechanism of acute and chronic liver injury. *Gut,* 54**,** 1024-33.
GUICCIARDI, M. E., LEIST, M. & GORES, G. J. 2004. Lysosomes in cell death. *Oncogene,* 23**,** 2881-90.

GUNTHER, S., LI, B. C., MISKA, S., KRUGER, D. H., MEISEL, H. & WILL, H. 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol,* 69**,** 5437-44.

GUO, X., JIN, Y., QIAN, G. & TU, H. 2008. Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qidong, China. *J Hepatol,* 49**,** 718-25.

HANNOUN, C., NORDER, H. & LINDH, M. 2000. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol,* 81**,** 2267-72.

HARRIS, R. A., CHEN, G., LIN, W. Y., SHEN, F. M., LONDON, W. T. & EVANS, A. A. 2003. Spontaneous clearance of high-titer serum HBV DNA and risk of hepatocellular carcinoma in a Chinese population. *Cancer Causes Control,* 14**,** 995-1000.

HELFAND, B. T., CHANG, L. & GOLDMAN, R. D. 2004. Intermediate filaments are dynamic and motile elements of cellular architecture. *J Cell Sci,* 117**,** 133-41.

HENGARTNER, M. O. 2000. The biochemistry of apoptosis. *Nature,* 407**,** 770-6.

HENGSTLER, J. G., UTESCH, D., STEINBERG, P., PLATT, K. L., DIENER, B., RINGEL, M., SWALES, N., FISCHER, T., BIEFANG, K., GERL, M., BOTTGER, T. & OESCH, F. 2000. Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. *Drug Metab Rev,* 32**,** 81-118.

HESSE, M., MAGIN, T. M. & WEBER, K. 2001. Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J Cell Sci,* 114**,** 2569-75.

HIRAGA, N., IMAMURA, M., HATAKEYAMA, T., KITAMURA, S., MITSUI, F., TANAKA, S., TSUGE, M., TAKAHASHI, S., ABE, H., MAEKAWA, T., OCHI, H., TATENO, C., YOSHIZATO, K., WAKITA, T. & CHAYAMA, K. 2009. Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice. *J Hepatol,* 51**,** 1046-54.

HUANG, J. & LIANG, T. J. 1993. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. *Mol Cell Biol,* 13**,** 7476-86.

HUBSCHEN, J. M., MBAH, P. O., FORBI, J. C., OTEGBAYO, J. A., OLINGER, C. M., CHARPENTIER, E. & MULLER, C. P. 2010. Detection of a new subgenotype of Hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria. *Clin Microbiol Infect.*

HUY, T. T., NGOC, T. T. & ABE, K. 2008. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *J Virol,* 82**,** 5657-63.

HUY, T. T., USHIJIMA, H., QUANG, V. X., WIN, K. M., LUENGROJANAKUL, P., KIKUCHI, K., SATA, T. & ABE, K. 2004. Genotype C of hepatitis B virus can be classified into at least two subgroups. *J Gen Virol,* 85**,** 283-92.

IACOB, S., CICINNATI, V. R., HILGARD, P., IACOB, R. A., GHEORGHE, L. S., POPESCU, I., FRILLING, A., MALAGO, M., GERKEN, G., BROELSCH, C. E. & BECKEBAUM, S. 2007. Predictors of graft and patient survival in hepatitis C virus (HCV) recipients: model to predict HCV cirrhosis after liver transplantation. *Transplantation,* 84**,** 56-63.

ITO, K., TANAKA, Y., KATO, M., FUJIWARA, K., SUGAUCHI, F., SAKAMOTO, T., SHINKAI, N., ORITO, E. & MIZOKAMI, M. 2007. Comparison of complete sequences of hepatitis B virus genotype C between inactive carriers and hepatocellular carcinoma patients before and after seroconversion. *J Gastroenterol,* 42**,** 837-44.

JACOBSON, M. D., WEIL, M. & RAFF, M. C. 1997. Programmed cell death in animal development. *Cell,* 88**,** 347-54.
JAESCHKE, H., GORES, G. J., CEDERBAUM, A. I., HINSON, J. A., PESSAYRE, D. & LEMASTERS, J. J. 2002. Mechanisms of hepatotoxicity. *Toxicol Sci,* 65**,** 166-76.
JAMMEH, S., TAVNER, F., WATSON, R., THOMAS, H. C. & KARAYIANNIS, P. 2008. Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. *J Gen Virol,* 89**,** 901-9.
KAO, J. H. 2011. Molecular epidemiology of hepatitis B virus. *Korean J Intern Med,* 26**,** 255-61.
KAO, J. H., CHEN, P. J., LAI, M. Y. & CHEN, D. S. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology,* 118**,** 554-9.
KATO, H., ORITO, E., GISH, R. G., BZOWEJ, N., NEWSOM, M., SUGAUCHI, F., SUZUKI, S., UEDA, R., MIYAKAWA, Y. & MIZOKAMI, M. 2002. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology,* 35**,** 922-9.
KAY, A. & ZOULIM, F. 2007. Hepatitis B virus genetic variability and evolution. *Virus Res,* 127**,** 164-76.
KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* 26**,** 239-57.
KEW, M. C. 1996. Progress towards the comprehensive control of hepatitis B in Africa: a view from South Africa. *Gut,* 38 Suppl 2**,** S31-6.
KEW, M. C. 2010. Hepatocellular carcinoma in African Blacks: Recent progress in etiology and pathogenesis. *World J Hepatol,* 2**,** 65-73.
KIDD-LJUNGGREN, K., HOLMBERG, A., BLACKBERG, J. & LINDQVIST, B. 2006. High levels of hepatitis B virus DNA in body fluids from chronic carriers. *J Hosp Infect,* 64**,** 352-7.
KIIRE, C. F. 1996. The epidemiology and prophylaxis of hepatitis B in sub-Saharan Africa: a view from tropical and subtropical Africa. *Gut,* 38 Suppl 2**,** S5-12.
KIM, B. K., KIM, S. A., PARK, Y. N., CHEONG, J. Y., KIM, H. S., PARK, J. Y., CHO, S. W., HAN, K. H., CHON, C. Y., MOON, Y. M. & AHN, S. H. 2007. Noninvasive models to predict liver cirrhosis in patients with chronic hepatitis B. *Liver Int,* 27**,** 969-76.
KIM, C. M., KOIKE, K., SAITO, I., MIYAMURA, T. & JAY, G. 1991. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature,* 351**,** 317-20.
KIMBI, G. C., KRAMVIS, A. & KEW, M. C. 2004. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol,* 85**,** 1211-20.
KIMBI, G. C., KRAMVIS, A. & KEW, M. C. 2005. Integration of hepatitis B virus DNA into chromosomal DNA during acute hepatitis B. *World J Gastroenterol,* 11**,** 6416-21.
KLINGMULLER, U. & SCHALLER, H. 1993. Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor. *J Virol,* 67**,** 7414-22.
KNETEMAN, N. M. & MERCER, D. F. 2005. Mice with chimeric human livers: who says supermodels have to be tall? *Hepatology,* 41**,** 703-6.
KOBAYASHI, M., IKEDA, K., ARASE, Y., SUZUKI, F., AKUTA, N., HOSAKA, T., SEZAKI, H., YATSUJI, H., SUZUKI, Y., WATAHIKI, S., MINETA, R., IWASAKI, S., MIYAKAWA, Y. & KUMADA, H. 2008. Change of hepatitis B virus genotypes in acute and chronic infections in Japan. *J Med Virol,* 80**,** 1880-4.

KOCK, J. & SCHLICHT, H. J. 1993. Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. *J Virol,* 67**,** 4867-74.
KRAMVIS, A., ARAKAWA, K., YU, M. C., NOGUEIRA, R., STRAM, D. O. & KEW, M. C. 2008. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *J Med Virol,* 80**,** 27-46.
KRAMVIS, A., BUKOFZER, S., KEW, M. C. & SONG, E. 1997. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. *Hepatology,* 25**,** 235-40.
KRAMVIS, A., KEW, M. & FRANCOIS, G. 2005. Hepatitis B virus genotypes. *Vaccine,* 23**,** 2409-23.
KRAMVIS, A. & KEW, M. C. 1998. Structure and function of the encapsidation signal of hepadnaviridae. *J Viral Hepat,* 5**,** 357-67.
KRAMVIS, A. & KEW, M. C. 2007. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res,* 37**,** S9-S19.
KRAMVIS, A., KEW, M. C. & BUKOFZER, S. 1998. Hepatitis B virus precore mutants in serum and liver of Southern African Blacks with hepatocellular carcinoma. *J Hepatol,* 28**,** 132-41.
KRAMVIS, A., WEITZMANN, L., OWIREDU, W. K. & KEW, M. C. 2002. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol,* 83**,** 835-9.
KUHNS, M. C. & BUSCH, M. P. 2006. New strategies for blood donor screening for hepatitis B virus: nucleic acid testing versus immunoassay methods. *Mol Diagn Ther,* 10**,** 77-91.
KURBANOV, F., TANAKA, Y., KRAMVIS, A., SIMMONDS, P. & MIZOKAMI, M. 2008. When should "I" consider a new hepatitis B virus genotype? *J Virol,* 82**,** 8241-2.
KUSAKABE, A., TANAKA, Y., MOCHIDA, S., NAKAYAMA, N., INOUE, K., SATA, M., ISODA, N., KANG, J. H., SUMINO, Y., YATSUHASHI, H., TAKIKAWA, Y., KANEKO, S., YAMADA, G., KARINO, Y., TANAKA, E., KATO, J., SAKAIDA, I., IZUMI, N., SUGAUCHI, F., NOJIRI, S., JOH, T., MIYAKAWA, Y. & MIZOKAMI, M. 2009. Case-control study for the identification of virological factors associated with fulminant hepatitis B. *Hepatol Res,* 39**,** 648-56.
KYAW, A., AUNG, T., HTUT, T., MYINT, H. & TIN, K. M. 1983. Lysosomal enzyme activities in normals and in patients with chronic liver diseases. *Clin Chim Acta,* 131**,** 317-23.
LEMASTERS, J. J. 1999. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *Am J Physiol,* 276**,** G1-6.
LI, J., XU, Z., ZHENG, Y., JOHNSON, D. L. & OU, J. H. 2002. Regulation of hepatocyte nuclear factor 1 activity by wild-type and mutant hepatitis B virus X proteins. *J Virol,* 76**,** 5875-81.
LI, J. S., TONG, S. P., WEN, Y. M., VITVITSKI, L., ZHANG, Q. & TREPO, C. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol,* 67**,** 5402-10.
LIANG, T. J. & GHANY, M. 2002. Hepatitis B e Antigen--the dangerous endgame of hepatitis B. *N Engl J Med,* 347**,** 208-10.
LIN, B. & ANDERSON, D. A. 2000. A vestigial X open reading frame in duck hepatitis B virus. *Intervirology,* 43**,** 185-90.
LIN, C. L. & KAO, J. H. 2008. Hepatitis B viral factors and clinical outcomes of chronic hepatitis B. *J Biomed Sci,* 15**,** 137-45.

LIN, C. L. & KAO, J. H. 2011. The clinical implications of hepatitis B virus genotype: Recent advances. *J Gastroenterol Hepatol,* 26 Suppl 1**,** 123-30.
LINDH, M., ANDERSSON, A. S. & GUSDAL, A. 1997. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus--large-scale analysis using a new genotyping method. *J Infect Dis,* 175**,** 1285-93.
LIU, S., ZHANG, H., GU, C., YIN, J., HE, Y., XIE, J. & CAO, G. 2009. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst,* 101**,** 1066-82.
LIU, T. T., FANG, Y., XIONG, H., CHEN, T. Y., NI, Z. P., LUO, J. F., ZHAO, N. Q. & SHEN, X. Z. 2008. A case-control study of the relationship between hepatitis B virus DNA level and risk of hepatocellular carcinoma in Qidong, China. *World J Gastroenterol,* 14**,** 3059-63.
LIVINGSTON, S. E., SIMONETTI, J. P., BULKOW, L. R., HOMAN, C. E., SNOWBALL, M. M., CAGLE, H. H., NEGUS, S. E. & MCMAHON, B. J. 2007a. Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. *Gastroenterology,* 133**,** 1452-7.
LIVINGSTON, S. E., SIMONETTI, J. P., MCMAHON, B. J., BULKOW, L. R., HURLBURT, K. J., HOMAN, C. E., SNOWBALL, M. M., CAGLE, H. H., WILLIAMS, J. L. & CHULANOV, V. P. 2007b. Hepatitis B virus genotypes in Alaska Native people with hepatocellular carcinoma: preponderance of genotype F. *J Infect Dis,* 195**,** 5-11.
LOK, A. S. 2004. Prevention of hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology,* 127**,** S303-9.
LOK, A. S., AKARCA, U. & GREENE, S. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci U S A,* 91**,** 4077-81.
LOK, A. S. & MCMAHON, B. J. 2007. Chronic hepatitis B. *Hepatology,* 45**,** 507-39.
LOOMBA, R. & LIANG, T. J. 2007. Treatment of chronic hepatitis B. *Antivir Ther,* 12 Suppl 3**,** H33-41.
LU, Y. W., TAN, T. L., ZHANG, J. & CHEN, W. N. 2007. Cellular apoptosis induced by replication of hepatitis B virus: possible link between viral genotype and clinical outcome. *Virol J,* 4**,** 117.
MALHI, H. & GORES, G. J. 2008. Cellular and molecular mechanisms of liver injury. *Gastroenterology,* 134**,** 1641-54.
MCCORMACK, J. P., LEVINE, M. & RANGNO, R. E. 1997. Primary prevention of heart disease and stroke: a simplified approach to estimating risk of events and making drug treatment decisions. *CMAJ,* 157**,** 422-8.
MCMAHON, B. J. 2009. The natural history of chronic hepatitis B virus infection. *Hepatology,* 49**,** S45-55.
MCMAHON, B. J., ALWARD, W. L., HALL, D. B., HEYWARD, W. L., BENDER, T. R., FRANCIS, D. P. & MAYNARD, J. E. 1985. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *J Infect Dis,* 151**,** 599-603.
MCPARTLAND, J. L., GUZAIL, M. A., KENDALL, C. H. & PRINGLE, J. H. 2005. Apoptosis in chronic viral hepatitis parallels histological activity: an immunohistochemical investigation using anti-activated caspase-3 and M30 cytodeath antibody. *Int J Exp Pathol,* 86**,** 19-24.
MEGGIATO, T., CALABRESE, F., VALENTE, M., FAVARETTO, E., BALIELLO, E. & DEL FAVERO, G. 2000. Spontaneous apoptosis and proliferation in human pancreatic cancer. *Pancreas,* 20**,** 117-22.

MERCER, D. F., SCHILLER, D. E., ELLIOTT, J. F., DOUGLAS, D. N., HAO, C., RINFRET, A., ADDISON, W. R., FISCHER, K. P., CHURCHILL, T. A., LAKEY, J. R., TYRRELL, D. L. & KNETEMAN, N. M. 2001. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med,* 7**,** 927-33.

MERICAN, I., GUAN, R., AMARAPUKA, D., ALEXANDER, M. J., CHUTAPUTTI, A., CHIEN, R. N., HASNIAN, S. S., LEUNG, N., LESMANA, L., PHIET, P. H., SJALFOELLAH NOER, H. M., SOLLANO, J., SUN, H. S. & XU, D. Z. 2000. Chronic hepatitis B virus infection in Asian countries. *J Gastroenterol Hepatol,* 15**,** 1356-61.

MEULEMAN, P., LIBBRECHT, L., DE VOS, R., DE HEMPTINNE, B., GEVAERT, K., VANDEKERCKHOVE, J., ROSKAMS, T. & LEROUX-ROELS, G. 2005. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology,* 41**,** 847-56.

MEULEMAN, P., LIBBRECHT, L., WIELAND, S., DE VOS, R., HABIB, N., KRAMVIS, A., ROSKAMS, T. & LEROUX-ROELS, G. 2006. Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus. *J Virol,* 80**,** 2797-807.

MOMMEJA-MARIN, H., MONDOU, E., BLUM, M. R. & ROUSSEAU, F. 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: analysis and review of the literature. *Hepatology,* 37**,** 1309-19.

MU, S. C., LIN, Y. M., JOW, G. M. & CHEN, B. F. 2009. Occult hepatitis B virus infection in hepatitis B vaccinated children in Taiwan. *J Hepatol,* 50**,** 264-72.

MULYANTO, DEPAMEDE, S. N., SURAYAH, K., TJAHYONO, A. A., JIRINTAI, NAGASHIMA, S., TAKAHASHI, M. & OKAMOTO, H. 2010. Identification and characterization of novel hepatitis B virus subgenotype C10 in Nusa Tenggara, Indonesia. *Arch Virol*.

MULYANTO, DEPAMEDE, S. N., SURAYAH, K., TSUDA, F., ICHIYAMA, K., TAKAHASHI, M. & OKAMOTO, H. 2009. A nationwide molecular epidemiological study on hepatitis B virus in Indonesia: identification of two novel subgenotypes, B8 and C7. *Arch Virol,* 154**,** 1047-59.

MUN, H. S., LEE, S. A., JEE, Y., KIM, H., PARK, J. H., SONG, B. C., YOON, J. H., KIM, Y. J., LEE, H. S., HYUN, J. W., HWANG, E. S., KOOK, Y. H. & KIM, B. J. 2008. The prevalence of hepatitis B virus preS deletions occurring naturally in Korean patients infected chronically with genotype C. *J Med Virol,* 80**,** 1189-94.

NASSAL, M. & SCHALLER, H. 1996. Hepatitis B virus replication--an update. *J Viral Hepat,* 3**,** 217-26.

NEWBOLD, J. E., XIN, H., TENCZA, M., SHERMAN, G., DEAN, J., BOWDEN, S. & LOCARNINI, S. 1995. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. *J Virol,* 69**,** 3350-7.

NI, Y. H., CHANG, M. H., WANG, K. J., HSU, H. Y., CHEN, H. L., KAO, J. H., YEH, S. H., JENG, Y. M., TSAI, K. S. & CHEN, D. S. 2004. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology,* 127**,** 1733-8.

NICHOLAS, K. N. H. 1997. GeneDoc: Analysis and Visualization of Genetic Variation.

NORDER, H., COUROUCE, A. M., COURSAGET, P., ECHEVARRIA, J. M., LEE, S. D., MUSHAHWAR, I. K., ROBERTSON, B. H., LOCARNINI, S. & MAGNIUS, L. O. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology,* 47**,** 289-309.

OKAMOTO, H., TSUDA, F., AKAHANE, Y., SUGAI, Y., YOSHIBA, M., MORIYAMA, K., TANAKA, T., MIYAKAWA, Y. & MAYUMI, M. 1994. Hepatitis B virus with

mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol,* 68**,** 8102-10.
OLINGER, C. M., VENARD, V., NJAYOU, M., OYEFOLU, A. O., MAIGA, I., KEMP, A. J., OMILABU, S. A., LE FAOU, A. & MULLER, C. P. 2006. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol,* 87**,** 1163-73.
OSIOWY, C., KAITA, K., SOLAR, K. & MENDOZA, K. 2010. Molecular characterization of hepatitis B virus and a 9-year clinical profile in a patient infected with genotype I. *J Med Virol,* 82**,** 942-8.
OZASA, A., TANAKA, Y., ORITO, E., SUGIYAMA, M., KANG, J. H., HIGE, S., KURAMITSU, T., SUZUKI, K., TANAKA, E., OKADA, S., TOKITA, H., ASAHINA, Y., INOUE, K., KAKUMU, S., OKANOUE, T., MURAWAKI, Y., HINO, K., ONJI, M., YATSUHASHI, H., SAKUGAWA, H., MIYAKAWA, Y., UEDA, R. & MIZOKAMI, M. 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology,* 44**,** 326-34.
PAGE, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci,* 12**,** 357-8.
PALMITER, R. D. & BRINSTER, R. L. 1985. Transgenic mice. *Cell,* 41**,** 343-5.
PAREKH, S., ZOULIM, F., AHN, S. H., TSAI, A., LI, J., KAWAI, S., KHAN, N., TREPO, C., WANDS, J. & TONG, S. 2003. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol,* 77**,** 6601-12.
PATEL, T., ROBERTS, L. R., JONES, B. A. & GORES, G. J. 1998. Dysregulation of apoptosis as a mechanism of liver disease: an overview. *Semin Liver Dis,* 18**,** 105-14.
PERRILLO, R. P., CHAU, K. H., OVERBY, L. R. & DECKER, R. H. 1983. Anti-hepatitis B core immunoglobulin M in the serologic evaluation of hepatitis B virus infection and simultaneous infection with type B, delta agent, and non-A, non-B viruses. *Gastroenterology,* 85**,** 163-7.
POLLACK, J. R. & GANEM, D. 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. *J Virol,* 67**,** 3254-63.
POURKARIM, M. R., AMINI-BAVIL-OLYAEE, S., LEMEY, P., MAES, P. & VAN RANST, M. 2010a. Are hepatitis B virus "subgenotypes" defined accurately? *J Clin Virol,* 47**,** 356-60.
POURKARIM, M. R., AMINI-BAVIL-OLYAEE, S., LEMEY, P., MAES, P. & VAN RANST, M. 2011. HBV subgenotype misclassification expands quasi-subgenotype A3. *Clin Microbiol Infect,* 17**,** 947-9.
POURKARIM, M. R., LEMEY, P., AMINI-BAVIL-OLYAEE, S., MAES, P. & VAN RANST, M. 2010b. Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *J Clin Virol,* 47**,** 93-6.
PRINCE, A. M., WHALEN, R. & BROTMAN, B. 1997. Successful nucleic acid based immunization of newborn chimpanzees against hepatitis B virus. *Vaccine,* 15**,** 916-9.
PUNGPAPONG, S., KIM, W. R. & POTERUCHA, J. J. 2007. Natural history of hepatitis B virus infection: an update for clinicians. *Mayo Clin Proc,* 82**,** 967-75.
RAJAGOPALAN, P., BOUDINOT, F. D., CHU, C. K., TENNANT, B. C., BALDWIN, B. H. & SCHINAZI, R. F. 1996. Pharmacokinetics of (-)-2'-3'-dideoxy-3'-thiacytidine in woodchucks. *Antimicrob Agents Chemother,* 40**,** 642-5.
RHIM, J. A., SANDGREN, E. P., DEGEN, J. L., PALMITER, R. D. & BRINSTER, R. L. 1994. Replacement of diseased mouse liver by hepatic cell transplantation. *Science,* 263**,** 1149-52.

RUST, C. & GORES, G. J. 2000. Apoptosis and liver disease. *Am J Med,* 108**,** 567-74.
SANTANTONIO, T., JUNG, M. C., MISKA, S., PASTORE, G., PAPE, G. R. & WILL, H. 1991. Prevalence and type of pre-C HBV mutants in anti-HBe positive carriers with chronic liver disease in a highly endemic area. *Virology,* 183**,** 840-4.
SCHAEFER, S. 2005. Hepatitis B virus: significance of genotypes. *J Viral Hepat,* 12**,** 111-24.
SEEGER, C., GANEM, D. & VARMUS, H. E. 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science,* 232**,** 477-84.
SERRANO, M. 2007. Cancer regression by senescence. *N Engl J Med,* 356**,** 1996-7.
SHEN, T., GAO, J. M., ZOU, Y. L., DONG, H. & YAN, X. M. 2009. Novel hepatitis B virus subgenotype in the southern Yunnan Province of China. *Intervirology,* 52**,** 340-6.
SHEN, T., YAN, X. M., ZOU, Y. L., GAO, J. M. & DONG, H. 2008. Virologic characteristics of hepatitis B virus in patients infected via maternal-fetal transmission. *World J Gastroenterol,* 14**,** 5674-82.
SHIMIZU, S., EGUCHI, Y., KAMIIKE, W., ITOH, Y., HASEGAWA, J., YAMABE, K., OTSUKI, Y., MATSUDA, H. & TSUJIMOTO, Y. 1996. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. *Cancer Res,* 56**,** 2161-6.
SHINKAI, N., TANAKA, Y., ITO, K., MUKAIDE, M., HASEGAWA, I., ASAHINA, Y., IZUMI, N., YATSUHASHI, H., ORITO, E., JOH, T. & MIZOKAMI, M. 2007. Influence of hepatitis B virus X and core promoter mutations on hepatocellular carcinoma among patients infected with subgenotype C2. *J Clin Microbiol,* 45**,** 3191-7.
SINGH, J., DICKENS, C., PAHAL, V., KUMAR, R., CHAUDHARY, R., KRAMVIS, A. & KEW, M. C. 2009. First report of genotype e of hepatitis B virus in an Indian population. *Intervirology,* 52**,** 235-8.
SNYDER, R. L., TYLER, G. & SUMMERS, J. 1982. Chronic hepatitis and hepatocellular carcinoma associated with woodchuck hepatitis virus. *Am J Pathol,* 107**,** 422-5.
STEMLER, M., WEIMER, T., TU, Z. X., WAN, D. F., LEVRERO, M., JUNG, C., PAPE, G. R. & WILL, H. 1990. Mapping of B-cell epitopes of the human hepatitis B virus X protein. *J Virol,* 64**,** 2802-9.
STRATER, J., KORETZ, K., GUNTHERT, A. R. & MOLLER, P. 1995. In situ detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis. *Gut,* 37**,** 819-25.
STUYVER, L., DE GENDT, S., VAN GEYT, C., ZOULIM, F., FRIED, M., SCHINAZI, R. F. & ROSSAU, R. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol,* 81**,** 67-74.
SUGAUCHI, F., ORITO, E., ICHIDA, T., KATO, H., SAKUGAWA, H., KAKUMU, S., ISHIDA, T., CHUTAPUTTI, A., LAI, C. L., GISH, R. G., UEDA, R., MIYAKAWA, Y. & MIZOKAMI, M. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology,* 124**,** 925-32.
SUGAUCHI, F., ORITO, E., ICHIDA, T., KATO, H., SAKUGAWA, H., KAKUMU, S., ISHIDA, T., CHUTAPUTTI, A., LAI, C. L., UEDA, R., MIYAKAWA, Y. & MIZOKAMI, M. 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol,* 76**,** 5985-92.
SUGIYAMA, M., TANAKA, Y., KATO, T., ORITO, E., ITO, K., ACHARYA, S. K., GISH, R. G., KRAMVIS, A., SHIMADA, T., IZUMI, N., KAITO, M., MIYAKAWA, Y. & MIZOKAMI, M. 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology,* 44**,** 915-24.
SUMMERS, J. 1988. The replication cycle of hepatitis B viruses. *Cancer,* 61**,** 1957-62.

SUMMERS, J. & MASON, W. S. 1982. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell,* 29**,** 403-15.
SUMMERS, J., O'CONNELL, A. & MILLMAN, I. 1975. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc Natl Acad Sci U S A,* 72**,** 4597-601.
SUMMERS, J., SMITH, P. M. & HORWICH, A. L. 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol,* 64**,** 2819-24.
SUMMERS, J., SMITH, P. M., HUANG, M. J. & YU, M. S. 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *J Virol,* 65**,** 1310-7.
SUMMERS, J., SMOLEC, J. M. & SNYDER, R. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc Natl Acad Sci U S A,* 75**,** 4533-7.
SUNG, J. J., TSUI, S. K., TSE, C. H., NG, E. Y., LEUNG, K. S., LEE, K. H., MOK, T. S., BARTHOLOMEUSZ, A., AU, T. C., TSOI, K. K., LOCARNINI, S. & CHAN, H. L. 2008. Genotype-specific genomic markers associated with primary hepatomas, based on complete genomic sequencing of hepatitis B virus. *J Virol,* 82**,** 3604-11.
SZKLO, M. 1998. Population-based cohort studies. *Epidemiol Rev,* 20**,** 81-90.
TABOR, E. 2006. Infections by hepatitis B surface antigen gene mutants in Europe and North America. *J Med Virol,* 78 Suppl 1**,** S43-7.
TAKAHASHI, K., OHTA, Y., KANAI, K., AKAHANE, Y., IWASA, Y., HINO, K., OHNO, N., YOSHIZAWA, H. & MISHIRO, S. 1999. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. *Arch Virol,* 144**,** 1299-308.
TANAKA, Y., SUGAUCHI, F., MATSUURAA, K., NAGANUMA, H., TATEMATSU, K., TAKAGI, K., HIRAMATSU, K., KANI, S., GOTOH, T., WAKIMOTO, Y. & MIZOKAMI, M. 2009. [Evaluation of hepatitis B virus genotyping EIA kit]. *Rinsho Byori,* 57**,** 42-7.
TANG, B., KRUGER, W. D., CHEN, G., SHEN, F., LIN, W. Y., MBOUP, S., LONDON, W. T. & EVANS, A. A. 2004. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic carriers. *J Med Virol,* 72**,** 35-40.
TATEMATSU, K., TANAKA, Y., KURBANOV, F., SUGAUCHI, F., MANO, S., MAESHIRO, T., NAKAYOSHI, T., WAKUTA, M., MIYAKAWA, Y. & MIZOKAMI, M. 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol,* 83**,** 10538-47.
TAYLOR, B. C., YUAN, J. M., SHAMLIYAN, T. A., SHAUKAT, A., KANE, R. L. & WILT, T. J. 2009. Clinical outcomes in adults with chronic hepatitis B in association with patient and viral characteristics: A systematic review of evidence. *Hepatology,* 49**,** S85-95.
THAKUR, V., GUPTAN, R. C., KAZIM, S. N., MALHOTRA, V. & SARIN, S. K. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol,* 17**,** 165-70.
TIOLLAIS, P., POURCEL, C. & DEJEAN, A. 1985. The hepatitis B virus. *Nature,* 317**,** 489-95.
TONG, M. J., BLATT, L. M., KAO, J. H., CHENG, J. T. & COREY, W. G. 2006. Precore/basal core promoter mutants and hepatitis B viral DNA levels as predictors for liver deaths and hepatocellular carcinoma. *World J Gastroenterol,* 12**,** 6620-6.

TREPO, C., VNEK, J. & PRINCE, A. M. 1975. Delayed hypersensitivity and Arthus reaction to purified hepatitis B surface antigen (HBsAg) in immunized chimpanzees. *Clin Immunol Immunopathol,* 4**,** 528-37.
TSAI, F. C., LIU, C. J., CHEN, C. L., CHEN, P. J., LAI, M. Y., KAO, J. H. & CHEN, D. S. 2007. Lower serum viral loads in young patients with hepatitis-B-virus-related hepatocellular carcinoma. *J Viral Hepat,* 14**,** 153-60.
TSUGE, M., HIRAGA, N., TAKAISHI, H., NOGUCHI, C., OGA, H., IMAMURA, M., TAKAHASHI, S., IWAO, E., FUJIMOTO, Y., OCHI, H., CHAYAMA, K., TATENO, C. & YOSHIZATO, K. 2005. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology,* 42**,** 1046-54.
TUR-KASPA, R., BURK, R. D., SHAUL, Y. & SHAFRITZ, D. A. 1986. Hepatitis B virus DNA contains a glucocorticoid-responsive element. *Proc Natl Acad Sci U S A,* 83**,** 1627-31.
VALLIAMMAI, T., THYAGARAJAN, S. P., ZUCKERMAN, A. J. & HARRISON, T. J. 1995. Precore and core mutations in HBV from individuals in India with chronic infection. *J Med Virol,* 45**,** 321-5.
VANWOLLEGHEM, T., LIBBRECHT, L., HANSEN, B. E., DESOMBERE, I., ROSKAMS, T., MEULEMAN, P. & LEROUX-ROELS, G. 2010. Factors determining successful engraftment of hepatocytes and susceptibility to hepatitis B and C virus infection in uPA-SCID mice. *J Hepatol,* 53**,** 468-76.
VIANA, R. 2005. *The effect of the accumulation of hepatitis B virus e-antigen precursor on cell viability.* Masters dissertation.
VIANA, R., WANG, R., YU, M. C., WELSCHINGER, R., CHEN, C. Y. & KEW, M. C. 2009. Hepatitis B viral loads in southern African Blacks with hepatocellular carcinoma. *J Med Virol,* 81**,** 1525-30.
VON HEIJNE, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem,* 133**,** 17-21.
WAI, C. T., CHU, C. J., HUSSAIN, M. & LOK, A. S. 2002. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology,* 36**,** 1425-30.
WEINBERG, M., PASSMAN, M., KEW, M. & ARBUTHNOT, P. 2000. Hammerhead ribozyme-mediated inhibition of hepatitis B virus X gene expression in cultured cells. *J Hepatol,* 33**,** 142-51.
WEINBERGER, K. M., WIEDENMANN, E., BOHM, S. & JILG, W. 2000. Sensitive and accurate quantitation of hepatitis B virus DNA using a kinetic fluorescence detection system (TaqMan PCR). *J Virol Methods,* 85**,** 75-82.
WHICHER, J. & SPENCE, C. 1987. When is serum albumin worth measuring? *Ann Clin Biochem,* 24 (Pt 6)**,** 572-80.
WHO. August 2008. *Hepatitis B* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs204/en/ [Accessed].
WIELAND, S. F. & CHISARI, F. V. 2005. Stealth and cunning: hepatitis B and hepatitis C viruses. *J Virol,* 79**,** 9369-80.
WILSON, P. W., D'AGOSTINO, R. B., LEVY, D., BELANGER, A. M., SILBERSHATZ, H. & KANNEL, W. B. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation,* 97**,** 1837-47.
WINER, B. 1971. *Statistical Principles in Experimental Design,* New York, McGraw-Hill.
WONG, G. L., WONG, V. W., CHOI, P. C., CHAN, A. W., CHIM, A. M., YIU, K. K., CHAN, H. Y., CHAN, F. K., SUNG, J. J. & CHAN, H. L. 2008. Evaluation of alanine transaminase and hepatitis B virus DNA to predict liver cirrhosis in hepatitis B e

antigen-negative chronic hepatitis B using transient elastography. *Am J Gastroenterol,* 103**,** 3071-81.

WONG, V. W., CHAN, S. L., MO, F., CHAN, T. C., LOONG, H. H., WONG, G. L., LUI, Y. Y., CHAN, A. T., SUNG, J. J., YEO, W., CHAN, H. L. & MOK, T. S. 2010. Clinical scoring system to predict hepatocellular carcinoma in chronic hepatitis B carriers. *J Clin Oncol,* 28**,** 1660-5.

WU, C. F., YU, M. W., LIN, C. L., LIU, C. J., SHIH, W. L., TSAI, K. S. & CHEN, C. J. 2008. Long-term tracking of hepatitis B viral load and the relationship with risk for hepatocellular carcinoma in men. *Carcinogenesis,* 29**,** 106-12.

XIA, X. & XIE, Z. 2001. DAMBE: software package for data analysis in molecular biology and evolution. *J Hered,* 92**,** 371-3.

YANG, H. I., LU, S. N., LIAW, Y. F., YOU, S. L., SUN, C. A., WANG, L. Y., HSIAO, C. K., CHEN, P. J., CHEN, D. S. & CHEN, C. J. 2002. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med,* 347**,** 168-74.

YANG, H. I., SHERMAN, M., SU, J., CHEN, P. J., LIAW, Y. F., ILOEJE, U. H. & CHEN, C. J. 2010. Nomograms for risk of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *J Clin Oncol,* 28**,** 2437-44.

YANG, H. I., YEH, S. H., CHEN, P. J., ILOEJE, U. H., JEN, C. L., SU, J., WANG, L. Y., LU, S. N., YOU, S. L., CHEN, D. S., LIAW, Y. F. & CHEN, C. J. 2008. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst,* 100**,** 1134-43.

YEE, J. K. 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. *Science,* 246**,** 658-61.

YIM, H. J. & LOK, A. S. 2006. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. *Hepatology,* 43**,** S173-81.

YIN, J., ZHANG, H., HE, Y., XIE, J., LIU, S., CHANG, W., TAN, X., GU, C., LU, W., WANG, H., BI, S., CUI, F., LIANG, X., SCHAEFER, S. & CAO, G. 2010. Distribution and hepatocellular carcinoma-related viral properties of hepatitis B virus genotypes in Mainland China: a community-based study. *Cancer Epidemiol Biomarkers Prev,* 19**,** 777-86.

YIN, J., ZHANG, H., LI, C., GAO, C., HE, Y., ZHAI, Y., ZHANG, P., XU, L., TAN, X., CHEN, J., CHENG, S., SCHAEFER, S. & CAO, G. 2008. Role of hepatitis B virus genotype mixture, subgenotypes C2 and B2 on hepatocellular carcinoma: compared with chronic hepatitis B and asymptomatic carrier state in the same area. *Carcinogenesis,* 29**,** 1685-91.

YU, H., YUAN, Q., GE, S. X., WANG, H. Y., ZHANG, Y. L., CHEN, Q. R., ZHANG, J., CHEN, P. J. & XIA, N. S. 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". *PLoS One,* 5**,** e9297.

YU, M. W., HSU, F. C., SHEEN, I. S., CHU, C. M., LIN, D. Y., CHEN, C. J. & LIAW, Y. F. 1997. Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers. *Am J Epidemiol,* 145**,** 1039-47.

YU, M. W., YEH, S. H., CHEN, P. J., LIAW, Y. F., LIN, C. L., LIU, C. J., SHIH, W. L., KAO, J. H., CHEN, D. S. & CHEN, C. J. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J Natl Cancer Inst,* 97**,** 265-72.

YUEN, M. F., TANAKA, Y., FONG, D. Y., FUNG, J., WONG, D. K., YUEN, J. C., BUT, D. Y., CHAN, A. O., WONG, B. C., MIZOKAMI, M. & LAI, C. L. 2009. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. *J Hepatol,* 50**,** 80-8.

YUEN, M. F., TANAKA, Y., SHINKAI, N., POON, R. T., BUT, D. Y., FONG, D. Y., FUNG, J., WONG, D. K., YUEN, J. C., MIZOKAMI, M. & LAI, C. L. 2008. Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels. *Gut,* 57**,** 98-102.

ZANETTI, A. R., VAN DAMME, P. & SHOUVAL, D. 2008. The global impact of vaccination against hepatitis B: a historical overview. *Vaccine,* 26**,** 6266-73.

ZHANG, H. W., YIN, J. H., LI, Y. T., LI, C. Z., REN, H., GU, C. Y., WU, H. Y., LIANG, X. S., ZHANG, P., ZHAO, J. F., TAN, X. J., LU, W., SCHAEFER, S. & CAO, G. W. 2008. Risk factors for acute hepatitis B and its progression to chronic hepatitis in Shanghai, China. *Gut,* 57**,** 1713-20.