Genetic Variability of Hepatitis B Virus

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Summary

Screening of blood bank sera for occult hepatitis B infection need standardized highly sensitive PCRs with a lower limit of detection of < 10 IU/ml for HBV DNA and >0.1 ng/mL for HBsAg. Upon our validation of eight different PCRs covering the S, X, and C genes, only two assays were reliable below 10^3 copies/ml. One PCR of the S gene, and the precore (PC) PCR showed a sensitivity of 1.9 x 10^2 IU/ml (equivalent to 1.0 x 10^3 copies/ml). We detected HBV-DNA in two out of 15 (13.3%) archival liver biopsies from Saudi HCC patients.

Our real-time quantitative assay of HBV by SYBR green was practical, more accurate and has detection range from 10^2 to 10^{10} copies/ml. Comparative analysis of our SYBR green versus Amplicor assay, the difference in HBV DNA measurement between the two methods was 0.595 with a 95% CI of 0.10-1.094. The Amplicor test showed less concordance at higher HBV DNA levels of plasmid standards than our optimized method. The melting temperature varied 80.5 - 81.7 ^{o}C in different genotypes.

Mean HBV DNA level was significantly higher in UK than Saudi hepatitis B patients at 5.09 (95% CI 1.43-8.75) and 4.52 (95% CI 0.14-8.90) $\log_{10}$ copies/ml, respectively (p: 0.007). Mean HBV DNA in patients with HBeAg was higher at 7.18 and 6.44 $\log_{10}$ copies/ml in UK and Saudi patients, respectively. Within the liver disease group, 53/92 (57.6%) Saudi and 42/66 (63.6%) UK patients have high HBV DNA of >5 $\log_{10}$, whereas a much smaller proportion show moderate or low HBV DNA levels

Among genotyping PCRs, multiplex PCR was has lower detection limit (10^4 copies/ml) than RFLP PCR. HBV genotypes A, B, C and D were mostly detected in UK patients, A and D in Saudi patients. In UK patients, genotypes C and D showed higher mean HBV DNA than genotypes A or B. Saudi patients had higher mean HBV DNA in genotypes D than genotypes A or B. Within genotype D, UK patients had even higher HBV DNA levels than Saudi patients at 6.10 and 5.10 $\log_{10}$ IU/ml, respectively. The HBV genotypes of HBsAg blood donors were genotype A 6/25 (24%), C 1/25 (4%) and D 18/25 (72%). 5 were ungenotypable. Phylogenetic analysis showed that all UK HBV genotype A had subgenotype Ae, genotype B had subgenotype Ba, and genotype C subgenotype C1 (Cs). HBV detected in Saudi Samples had HBV subgenotype D1, while UK had subgenotype D2 mostly in Asian patients.

Core promoter region/X gene sequences showed clear correlation to HBV genotypes and subgenotypesunlike the conserved precore region. Sequencing of the S gene, Pre S1 region, Pre S2 and beginning as well as the end of S gene overlapping the YMDD region of the reverse transcriptase part of pol gene showed accurate discrimination between genotypes, and genotype-related sequence variations. In contrast, the more conserved regions at the `a` determinant and the rt part of the Pol gene. That can contribute to differences in fibronectin binding site to liver sinusoids, and the expression efficiency of HBsAg between different genotypes. Furthermore, the genotype-specific contribution of preS1 epitopes favours vaccination using the large HBsAg of the prevalent HBV genotypes in specific geographical areas. We found Single nucleotide polymorphism

(SNPs) at S/pol gene that are genotype-specific, and can be of benefit in new HBV molecular assays and DNA arrays.

BCP mutants 1762-1764 were seen in 19/26 (73%) Saudi patients compared to 9/23 (39%) UK patients (p: 0.003). In Saudi patients, BCP mutants were highest in genotype A and genotype D at 7/9 (78%) and 17/20 (85%), respectively. All possibilities were seen a change of AGG to TGG in 5 patients, 1762/1764 A/A, and the double mutant T/A were seen in 2 and 7 patients, respectively. In addition, two patients had G1762/T1764, one had C1764, one T1764 and one case showed 12 bp-deletion in BCP region. In UK patients, one BCP variants 1762-1764 had AGT. A/A, and the double mutant T/A were also seen in 3 and 5 patients, respectively. C1766T, T1768A and C1773T C1799G and C1858 were present. All patients had chronic hepatitis and one case presented with HCC

Significant proportion of Saudi patients 37/56 (66.1%) had precore (PC) mutants (p: 0.004), especially in genotype D 35/48 (73%),. Of those, codon 28 mutant (G1896A) was present in 30/37 (81.1%), resulting in a change of tryptophan to a stop codon. Double mutants in both codons 28 and 29 were seen in 15/37 (40.5%) of total mutants. G1899A mutation, which alters codon 29 from glycine to aspargine, was detected mostly in Saudi genotype D patients 22/37 (61%). In contrast, UK patients, precore mutations found in only 5/23 (21.7%), of HBV genotypes A, B and D. Their implications need to be verified in detailed studies. 4 out of 6 HBeAg-positive Saudi patients at had PC mutants. The mean HBV DNA of wild type in both BCP and PC was higher than mutants. Coexisted BCP and PC mutants occurred mainly in Saudi patients 21/25 (84%) versus only one UK patient. In this study, HBV X gene mutations lysine to methionine (K130M) was seen in a total of 6/22 UK and 11/25 Saudi patients. Valine has changed to isoleucine (V131I) present in 8/22 UK patients and 10/25 Saudi patients. Other X-gene variants; I127N and F132Y were seen. F132Y was evident in two genotype A out of 10 (20%) patients, and 6/20 (30%) genotype D patients.

Clinically, BCP mutants were present in liver disease patients were much higher in Saudi patients 18/21 (90%) than in UK patients 8/21 (38%). Saudi patients with inactive hepatitis had comparable rates of PC mutants 9/13 (69%) to liver disease 28/43 (65%) (P: 0.019); as acute hepatitis 4/5 and chronic hepatitis 17/29 (59%).

Contents Page

List of Graphs

Page

List of Tables Page

List of Figures Page

Abbreviation List

Ab Antibody
AFP Alfa fetoprotein
ALT Alanine aminotransferase
a.a. Amino acids
Anti-HBs Hepatitis B surface antibody
Anti-HBe Hepatitis B envelope antibody
Anti-HBc Hepatitis B core antibody
bp Base pair
cDNA Copy DNA
CAH Chronic active hepatitis
CMI Cell mediated immunity
CTL Cytotoxic T lymphocytes
CV Coefficient of variation
DC Dendritic cells
DMSO Dimethylsulfoxide
DNA Deoxyribonucleic acid
dNTP Deoxyribonucleotide triphosphate
ddNTP Dideoxy nucleotie triphosphate
EIA Enzyme immunososorbent assay
ER Endoplasmic retictulum
ExoI Exonuclease I
FITC Fluorescein isothiocyanate
FRET Fluorescent energy transfer probes
GBV-B GB- Virus B
HAV Hepatitis A Virus
HBV Hepatitis B Virus
HBsAg Hepatitis B surface antigen
HBeAg Hepatitis B envelope antigen
HCC Hepatocellular carcinoma
HCV Hepatitis C Virus
HIV Human Immunodeficiency Virus
HLA Human Leukocyte antigen
hTERT Human telomerase l; reverse transcriptase
HVR Hypervariable region
IFN Interferon
Ig Immunoglobin
IL Interleukin
IP Incubation period
IRES Internal ribosomal entry site
IVDU Intravenous drug user
kDa Kilo Dalton
LB Luria-Bertani
LXR Liver X recptor
MAbs Monoclonal antibodies
MHC Major histocompatibility
MHL of HBsAg
ml Milli liter
mRNA Messenger ribonucleic acid
N/A Not applicable
NANBH Non-A, Non-B hepatitis
NAT Nucleic acid testing
NCR Non-coding region
NICE National Institute for Clinical Excellence
NK Natural killer
NS Non-structural
ORF Open reading frame
PBMC Peripheral Blood Mononuclear Cell
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PEG Polyethylene glycol
PHSA Polymerised human serum albumin binding site
RE Restriction endonuclease
RFLP Restriction fragment length polymorphism
RNA Ribonucleic acid
SAP Shrimp alkaline phosphatase
SNP Single nucleotide polymorphism
S-S bridges Sulphur-Sulphur bridges in cysteine aminoacids
SYBR
TMA Transcription Mediated Amplification

Acknowledgements

This work is presented to the sole of my mother, to my father, my husband and children for their enormous support during the whole project.

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Chapter One: Introduction

1.1. Hepatitis B virus

1.1.1. History

Viral hepatitis was first described by Lurman in 1885, who reported the development of jaundice in 15% of 1,289 shipyard workers in Bremen after their vaccination with human derived smallpox vaccine (Fields Virology). Blumberg detected Australia Antigen, in 1963 in serum from an Australian aborigine that reacted with an antibody in the serum of an American haemophiliac. Electron microscopy of sera with Australia antigen showed Dane particles, which are hepatitis B virus (HBV) and its surface component; hepatitis B surface antigen (HBsAg) (Dane et al., 1970).

1.1.2. Classification of Hepadna Viruses

The Hepadnaviridae family are DNA viruses infecting humans and other primates, rodents and avian hosts targeting the liver (fig.1-1, table1-1) (Kidd- Ljunggren; 2002).

1.1.3. Definition of Hepatitis

Hepatitis is inflammation of the liver tissues. This can be caused by a variety of aetiologies, including metabolic, toxic as alcohol, and viral infections. Several systemic viral infections may affect the liver; such as cytomegalovirus, haemorrhagic fevers, and hepatotropic viruses; hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, hepatitis G virus HGV/GBV-C, and TTV (table 1-2).

1.1.4. Hepatitis B Virus Structure

Hepatitis B virus (HBV) is the prototype virus for the Hepadnaviridae family. HBV is a 42 nm double-shelled particle that consists of an outer envelope of HBsAg and an inner core antigen (HBcAg) and DNA polymerase enzyme (fig.1-2).

1.1.5. HBV Genome

The genome consists of circular partially double stranded DNA. Hepatitis B viral genome has four open reading frames S, Core, Pol and X-genes, and four promoters; S1, S2, Core promoter, and X-promoter. There are two enhancer elements; Enhancer I and II (Kreutz, 2002). The preS region is divided into Pre-S1 and Pre-S2 (fig.1-3).

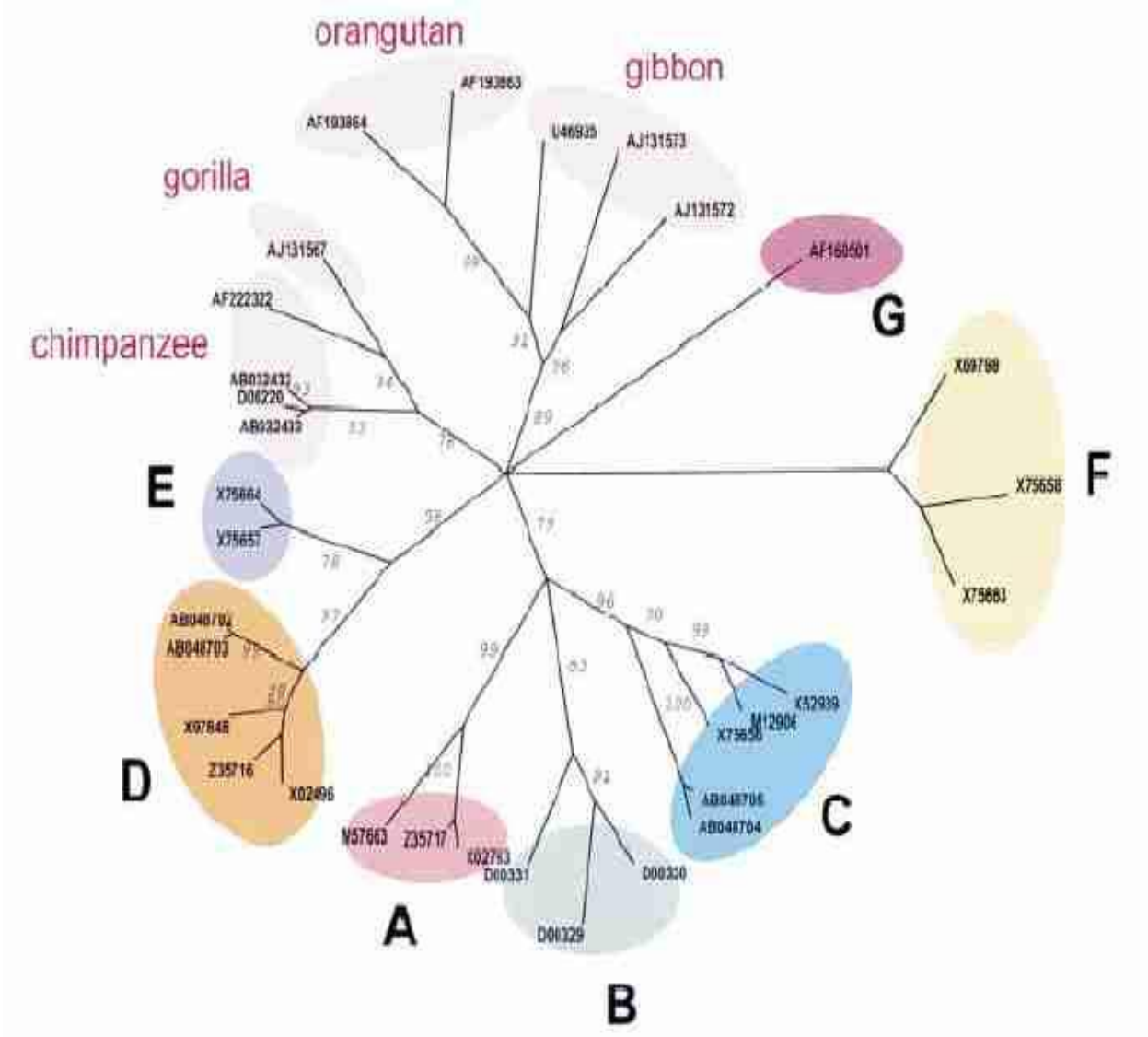


Figure1-1: Phylogenetic tree of different human and simian HBV genotypes. HBV genotypes A to G, and animal hepatitis viruses infecting chimpanzee, gorilla, orangutan, and gibbon (Kidd- Ljunggren; 2002).

Table1-1: Members of the Hepadnaviridae family; infecting humans, primates as chimpanzee, gorilla, gibbon, wood-chuck, ground and arctic squirrel, wooley monkey, avian Hepadnaviridae, duck, heron and Stork hepatitis B virus.

Virus	Host	Identity to HBV	Genome Size (bp)	Remarks	Surface Proteins	X-gene
I-Orthohepadnaviridae Hepatitis B virus HBV Human genotypes A-H Primate strains	Humans Primates: Chimpanzee, Gorilla, orangutan (Pongo pygmeaeus)	100% 90%	3215 Partially double stranded	Cirrhosis, HCC. Replicates in liver, kidney, pancreas and WBC	3	Present
Gibbon HBV	Gibbon	> 80%			3	Present
Woodchuck hepatitis virus WHV	Woodchucks Marmota monax	70%	3300	High incidence of HCC	3	Present
Ground squirrel hepatitis virus GSHV	Ground squirrels (Spermophilus beecheyi), Woodchucks, chipmunks	55%	3300	More related to WHV (80%), HCC	3	Present
Arctic squirrel hepatitis virus ASHV	Spermophilus parryi kannicotti			84% identity to GSHV and WHV	3	Present
Wooley monkey B hepatitis virus WMHBV	Lagothrix lagotricha			hepatitis	3	Present
II-Avian Hepadnaviridae Duck hepatitis B virus DHBV	Geese, Pekin ducks (Anas domesticus)	40%	3000 Fully double stranded		2: PreS1, PreS2	Absent
Heron hepatitis B virus HHBV	German grey herons (Ardea cinerea)			78% related to DHBV	1 PreS	Absent
Stork hepatitis B virus SHBV	Stork			Related to HHBV	1 PreS	Absent

The precore region (nt. 1814–1900) precedes the core region (nt. 1901-2449). The basal core promoter (BCP) (nt. 1742-1813) and regulatory sequences are essential for the synthesis of the pregenomic and precore mRNA, which is the template for HBV genome replication and control synthesis of the core protein.

1.1.6. Structural Protein

1.1.6.1. Hepatitis B surface antigen

Envelope polypeptides are encoded by the combination of the pre S and S gene regions. The major protein of hepatitis B surface antigen (HBsAg) particles is the smallest gene product (SHBs) (fig.1-4). The middle protein (MHBs) contains the pre-S2/S component. The large surface protein (LHBs) contains pre-S1, pre-S2 and HBsAg, and is incorporated in intact virus particles (Reifenberg et al., 2006). In viraemic carriers, MHBs and SHBs products predominate in the liver, whereas in non-viraemic carriers, LHBs products predominate. LHBs show direct toxic or immunomodulatory effect and interaction with cytokines (Ayada et al., 2006) which may result in massive hepatocellular necrosis, regeneration and the eventual development of HCC.

HBsAg (fig.1-2) is produced in excess amounts that circulate in the blood as empty 22 nm spherical, tubular and filamentous sub viral particles. S protein and pre-S1 of HBsAg binds to lymphocytes and human liver cell plasma membranes associated endonexin II affecting virus infectivity (de Bruin et al., 1996). Pre-S2 sequences bind to polymerised human serum albumin (PHSA) receptors correlate with the presence of virus in serum and maintenance of chronic infection (Kondo et al., 2002). The `a` determinant is part of the major hydrophilic region (MHR) of HBsAg, comprising positions 100-169. The cysteine web model of MHR accounts for S-S bridges (positions 124-147) (fig.1-5) (Francois et al., 2001).

Table 1-2: Comparison of different hepatotropic viruses; hepatitis A, B, C, D, E, GBV, TTV and SEN viruses.

Virus	Family	Nucleic acid	Distribution	Structural Proteins	Transmission	I.P*	Acute Disease	Chronicity	Mortality
Hepatitis A virus	Picorna virus Hepavirus	ssRNA 1ORF	Developing countries	VP1-4	Faecal-oral	15-60d	<5% children >20% adults	No	0.1-2%
Hepatitis B virus	Hepadnaviridae	Incomplete dsDNA 4 ORFs	Worldwide	HBcAg HBsAg	Parenteral vertical Sexual	45-120 d	<5% children >20% adults	>70% children <10% adults	Acute1% Chronic 1-10%
Hepatitis C virus	Flaviviridae Hepacivirus	ssRNA 1ORF	Worldwide	E1 (gp35) E2 (gp70)	Parenteral Vertical	2-52 wk	Rare	up to 80 %	1-10% long term
Hepatitis D virus	Viroid	ssRNA 1ORF	Mediterranian, Amazon	HDAg (HBsAg)	Parenteral	3-13 wk	Common	High	2-20%
Hepatitis E virus	Caliciviridae	ssRNA 3ORFs	Indian sub-continent	ORF2 (ORF3) products	Faecal-oral	3-9 wk	Common	No	1-2% 20-30% in pregnancy
GB virus C (GBV-C)	enveloped Flaviviridae family	positive-sense ssRNA	Worldwide. 6genotypes: 1:West Africa, 2 : Europe, America 3: Asia, 4: Southwest Asia, 5 : South Africa 6: Indonesia.		Parenteral		No	common	none
TTV	Circinoviridae	DNA	Japan, worldwide		Parenteral		No	common	none
SEN virus	2variants :SENV-D, SENV-H	DNA	worldwide China		Parenteral		No		none

*IP: Incubation period

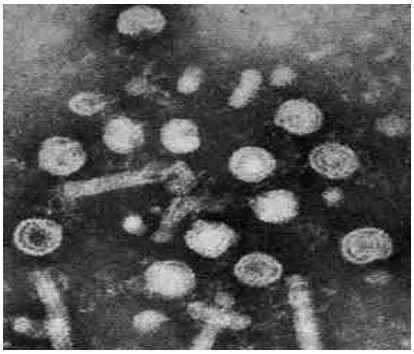


Figure 1-2: Electron micrograph of hepatitis B virus (HBV)
showing the double-shelled virion (Dane particles), and excess HBsAg in tubular and spherical forms.

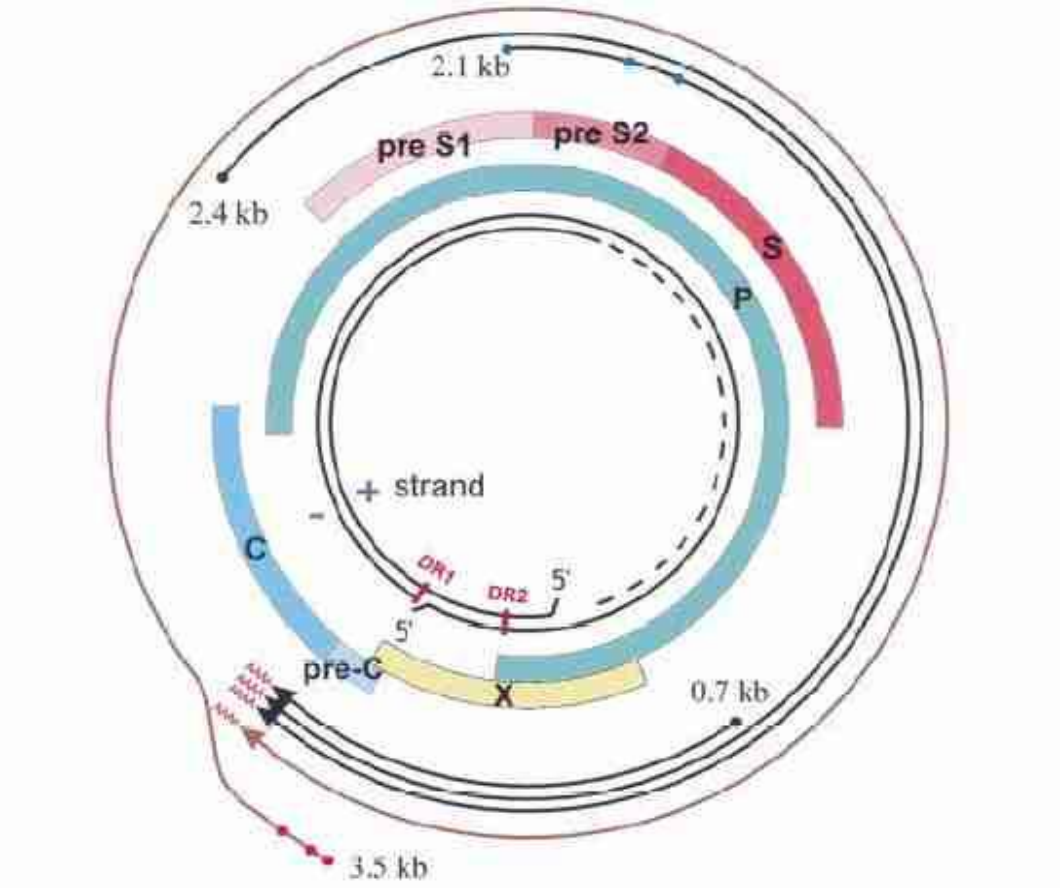


Figure 1-3: HBV genome organization;
Circular HBV genome partially double-stranded dsDNA indicated in black lines .The overlapping four open reading frames (ORFs) Pre-S/S(Pink line), PreC/Core (blue), Pol (blue), and X-genes(Beige line)(Kidd-Ljunggren et al., 2002).

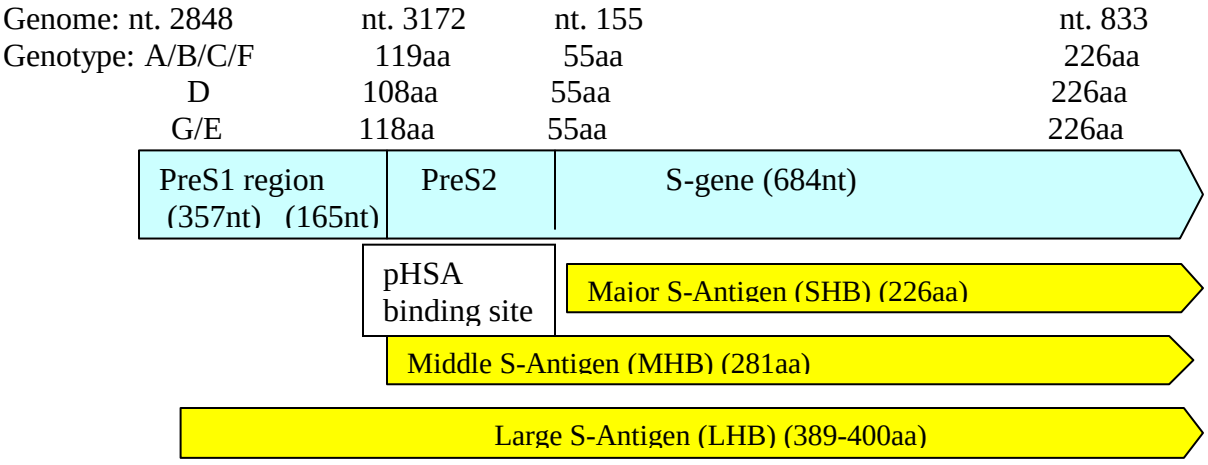


Figure 1-4: Pre-S/S-gene and its products;

HBV surface gene encoding the three hepatitis B surface antigen (HBsAg) proteins shown; large HBsAg (LHB), middle HBsAg (MHB) and small HBsAg (SHB). Amino acid numbering restarts at the first methionine of each the subparts as shown in each HBV genotype. The number of amino acids (aa) of Pre S1 is constant at 119 in genotypes A, B, C and F, but is 108 aa in genotype D and 118 in genotypes E and G. The number of amino acids in Pre S2 and S-gene is constant in all HBV genotypes at 55 and 226, respectively. PHSA: polymerized human serum albumin binding site.

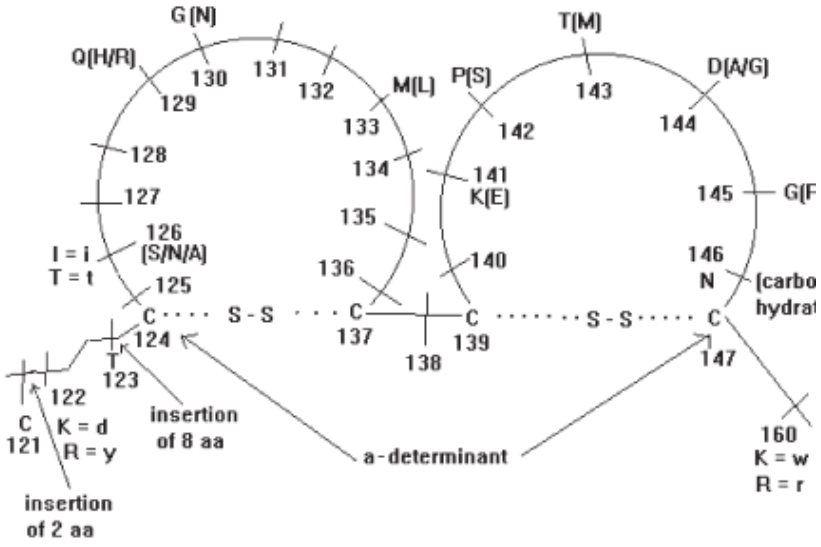


Figure 1-5: The double loop structure of `a`determinant of HBsAg (Kao and Chen, 2005); including the cysteines at positions 124, 137, 139 and 147 with S-S bridges (dotted lines). The amino acids 122 and 160 determine the HBsAg serotype as d or y and w or r respectively.

1.1.6.2. Core Proteins

The hepatitis B core antigen (HBcAg) (25 kD) is assembled into the capsid, which is essential for viral packaging. Its synthesis is restricted to liver tissues, and is an important target for immune recognition in chronic infection. Soluble Hepatitis B e antigen (HBeAg) (21kD) peptide is released into the circulation, and is a reliable marker for actively replicating virus, and hence, for high infectivity (fig.1-6) (Wu et al., 2007). Seroconversion from HBeAg to anti-HBe is commonly associated with the clearance of wild type (wt) HBV and the resolution of acute liver disease.

1.1.7. Nonstructural Proteins

1.1.7. 1. HBV Polymerase Enzyme

The HBV polymerase is a 56 to 70kD polypeptide. HBV polymerase has the following domains; the amino-terminal region; terminal protein (tp) which acts as a prime promoter for synthesis of the minus strand cDNA, spacer domain, the RNA-dependent DNA polymerase (reverse transcriptase), and the RNase H domain at the carboxy-terminus (fig.1-7). Mutation of polymerase affecting its activity will affect the amount of virus produced, as well as the number of templates available to encode viral proteins.

1.1.7.2. X-gene

The X gene protein (154 amino acids (aa.)) encoded by the X gene (nt. 1372-1834), exhibits numerous activities affecting intracellular signal transmission, gene transcription, cell proliferation, DNA repair, and apoptosis (Francois et al., 2001). HBx trans-activates and upregulates viral and cellular genes as the transcriptional expression of human telomerase l; reverse transcriptase (hTERT) (Qu et al., 2005), through activation of transcription factors, modulation of cell signalling pathways, RNA stabilization, and alteration of nucleocytoplasmic translocation and inhibition of serine protease activity (aa. 67-69 and 135-138 (Blackberg and Kidd-Ljunggren; 2003) (fig.1-7).

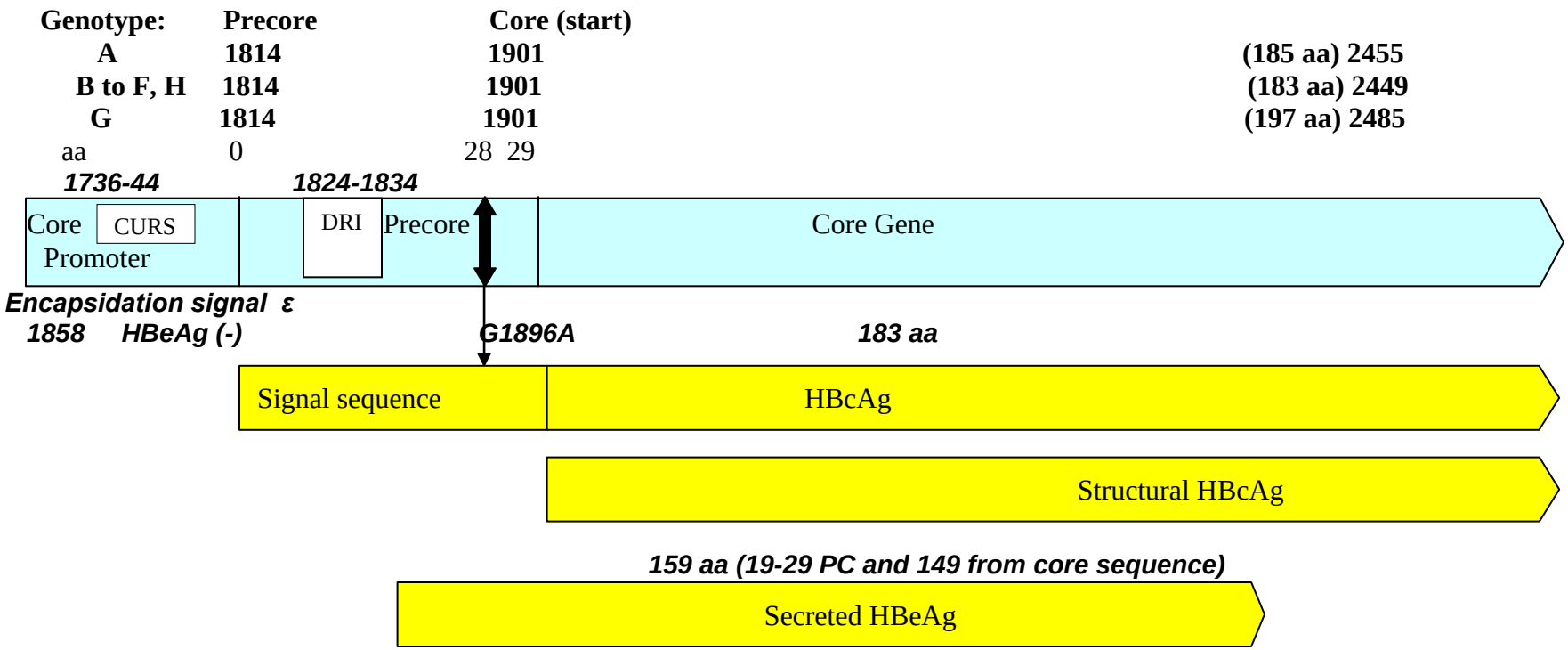


Figure 1-6: The organization of the HBV core gene in different HBV genotypes.

Starting with the core promoter (nt. 1742-1849), then the precore (PC) region (nt. 1814-1900) and the core region (1901-2449). The Direct Repeat (DR1) (nt. 1824-1834) is the origin of replication of the virus overlapping the precore region. The core genome length varies in different HBV genotypes in the core region only, but neither in the core promoter nor the precore regions. Precore mutants have stop codon (AUG) at G1896A in codon 28, results in HBeAg-negative phenotype (as shown by the bold arrow) CURS: Core upstream regulatory sequence (1645-1744) (modified from Stuyver et al., 2000).

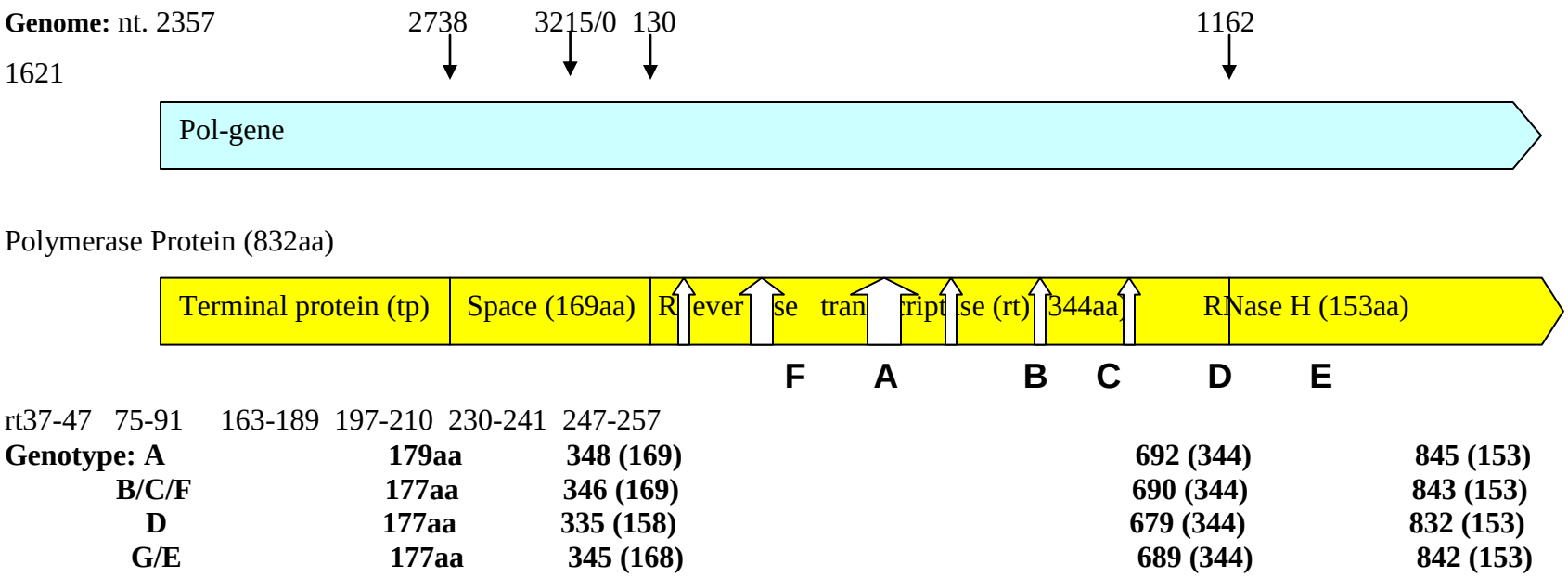


Figure 1-7: HBV Pol-gene (nt. 2357-1621). The polymerase protein has four domains;

Terminal protein (tp), spacer region, reverse transcriptase (rt) (RNA-dependent DNA polymerase), and ribonuclease H (RNase H). The currently used numbering system of HBV polymerase for each genotype i.e. the total length of the protein is outlined at each domain, and the standardized numbering system with three starting points at tp, spacer domain and rt. The domain`s amino acids (aa) number are indicated between brackets (modified from Stuyver; 2001). The rt and the RNAase H domains of the polymerase are constant in length in all genotypes mounting to 344, and 153 amino acids respectively. Within RT, six catalytic functional units are characterized; A, B, C, D, E and F are shown according to the RT numbering.

1.1.8. HBV replication

During infection, HBV penetrates into the cells after surface binding, then the virus is transported into the nucleus without processing, where replication starts by unwinding circular DNA which is converted into a covalently closed circular DNA (cccDNA) that acts as a template for transcription of HBV pregenomic, and messenger RNAs (Beck and Nassal, 2007).

Transcription starts from the core promoter to yield the 3.5 kb pregenomic RNA, which is packaged with polymerase into immature core particles, and then serves as a template for reverse transcription and negative strand DNA synthesis. The incomplete positive strand DNA is then synthesized. The mature core particles are packed into HBsAg and pre-S proteins in the endoplasmic reticulum then are exported from the cell (fig.1-8) (more on replication).

1.1.9. Immune Responses in HBV Infections

1.1.9.1. Humoral Immunity

The `a`determinant epitopes in HBsAg triggers protective neutralizing antibodies that prevent viral spread and subsequently eliminate circulating virus. The presence of anti-envelope antibodies denotes the resolution of acute hepatitis B (Jung and Pape; 2001). Their biological effects are dependent on the conformation (fig.1-5). The excessive production of HBsAg and the high viral replication induce immunological anergy or tolerance by the exhaustion of antiviral antibodies and CTLs. The virus also affects the immune system directly by infecting the lymphocytes.

1.1.9.2. Cell Mediated Immunity

Cell Mediated Immunity (CMI) is triggered by acute HBV infection yielding polyclonal specific cellular response (Jung and Pape; 2001) (fig.1-9). HBcAg contains also helper T cell epitopes (table 1-3), which might result from the diverse distribution of HLA antigens in different geographical areas (Jazayeri et al., 2004). HBcAg is an immunologic target of cytotoxic T lymphocytes (CTL), and immune recognition and elimination of

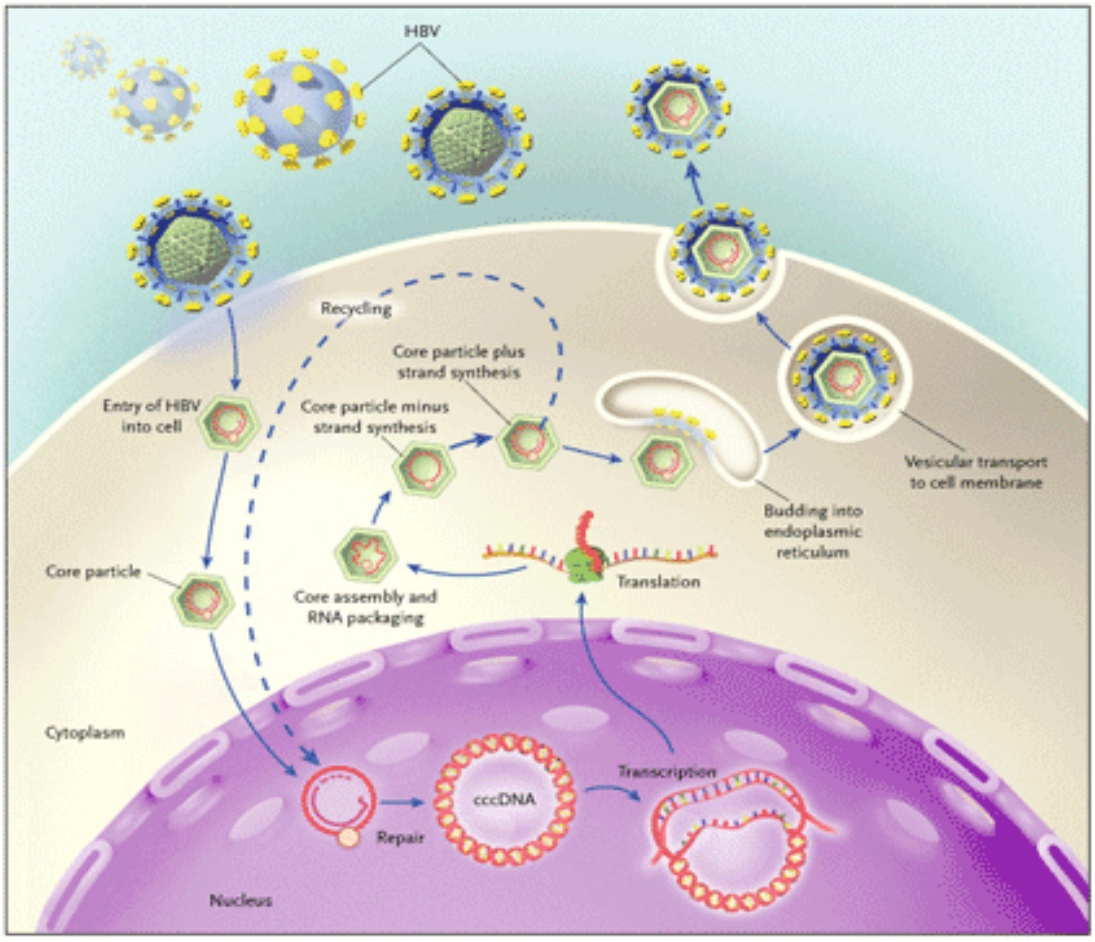


Figure 1-8: Hepatitis B virus life-cycle

The enveloped hepatitis B viral particle infects the cell by binding to host cell via envelope protein HBsAg interaction with host receptors. Uncoating of the envelope occur and entry into cell releasing the nucleocapsid into the cytoplasm. HBV DNA is transported to the nucleus, Replication inside the nucleus proceeds via cccDNA intermediate. Transcription of cccDNA by RNA polymerase II produces mRNAs and pregenomic RNA. Translation of viral proteins occurs in the cytoplasm and assembly of the core. Envelope forms during budding through the endoplasmic reticulum and virus release. Pregenomic RNA is encapsidated, together with P protein, and reverse transcribed inside the nucleocapsid. Part of these capsids is recycled to amplify the pool of intracellular cccDNA and the others are enveloped and released as virions. (www.infekt.ch/updown/ images/hbv_cycl.gif).

replicating HBV in chronic infection, as cytotoxicity that enhance viral clearance, and inhibit HBV gene expression through the secretion of antiviral cytokines. Remarkably, the CTLs also purge HBV replicative intermediates from the liver by secreting type 1

inflammatory cytokines thereby limiting virus spread to uninfected cells and reducing the degree of immunopathology required to terminate the infection (Chisari et al., 2010). HBeAg is strictly T-cell dependent (fig.1-9). Because HBcAg and HBeAg have cross-reactive T-cell epitopes, the production of HBeAg may partially or totally block the cellular immune responses against HBcAg and promote virus persistence (Jung and Pape; 2001) (table 1-3).

Persistent HBV infection is characterized by a weak adaptive immune response, thought to be due to inefficient CD4+ T cell priming early in the infection and subsequent development of a quantitatively and qualitatively ineffective CD8+ T cell response. Other factors that could contribute to viral persistence are immunological tolerance, mutational epitope inactivation, T cell receptor antagonism, incomplete down-regulation of viral replication and infection of immunologically privileged tissues. However, these pathways become apparent only in the setting of an ineffective immune response, which is, therefore, the fundamental underlying cause. Persistent infection is characterized by chronic liver cell injury, regeneration, inflammation, widespread DNA damage, and insertional deregulation of cellular growth control genes, which collectively lead to cirrhosis of the liver and hepatocellular carcinoma (Chisari et al., 2010).

HBV flourishes in an immunologically deprived haven. Hepatocytes present antigens poorly, as they do not express HLA class I and II molecules, except during inflammation. T-cells induce anergy upon exposure to viral antigens due to the absence of costimulation (Chen et al., 2005). Most activated lymphocytes die within the liver, thereby inactivating an essential arm of the immune response. In perinatal infections, the soluble HBeAg and HBxAg may cross the placenta, tolerize the immature neonatal immune system including the T-cells and favour the establishment of chronic HBV carrier state in 90% of cases. Such individuals may have high level of viraemia or clinical manifestations of chronic active hepatitis (CAH). Mature efficient T-cells are required to prevent chronic carrier state (Cacciola et al., 2002).

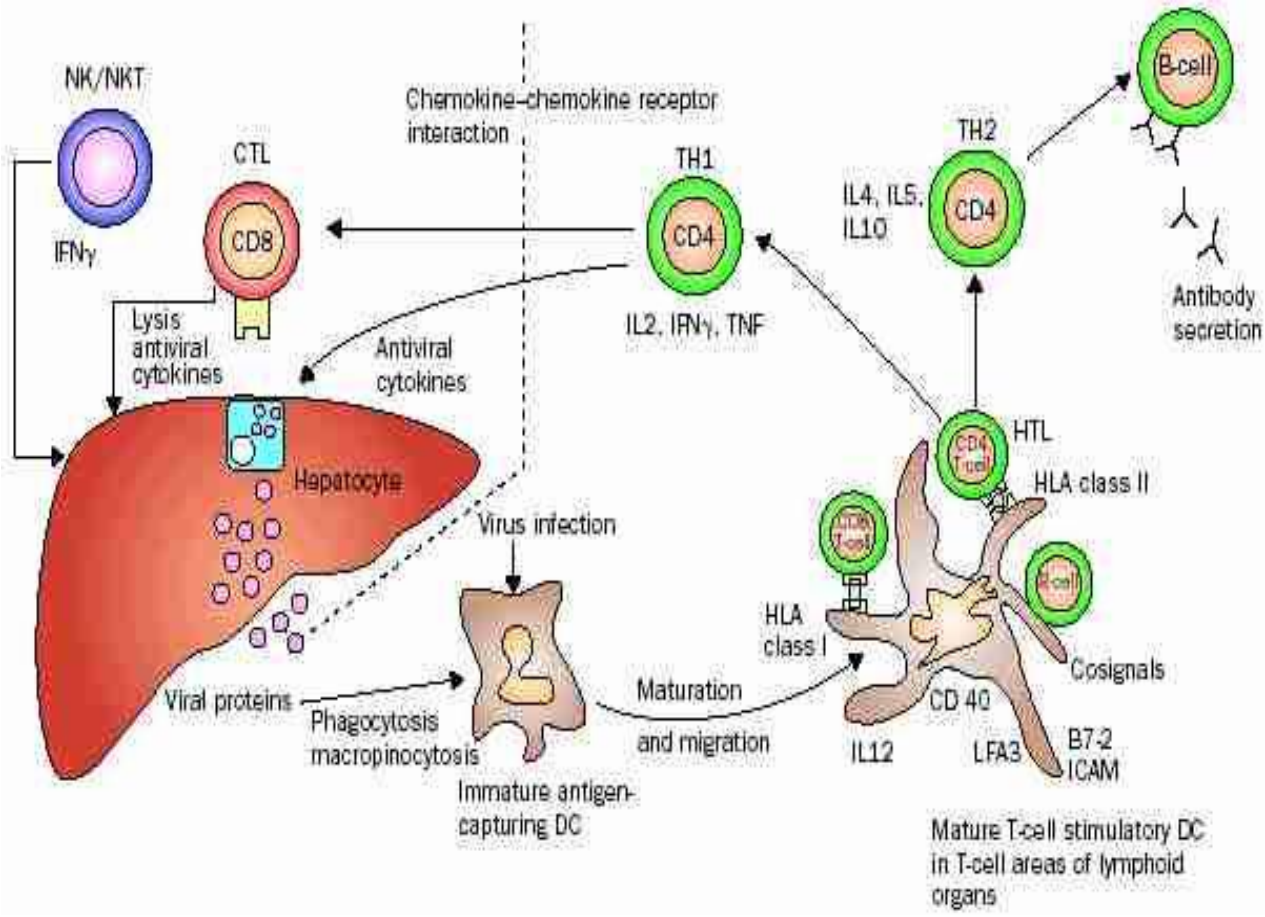


Figure 1-9: Interaction of the immune system against HBV infection

(NK: natural killer cells; CTL: cytotoxic lymphocytes, CD4: T-helper cells and macrophages) (Jung and Pape; 2001). Hepatocellular injury starts with antigen recognition by class I restricted HBV- specific cytotoxic T- lymphocytes (CTL) with induction of apoptosis in infected hepatocytes. CTL then recruit nonspecific inflammatory cells as macrophages, neutrophils, and lymphocytes, thereby forming necroinflammatory hepatocellular damage.

Table1-3: Immunological B-cell and T-cell epitopes within HBV proteins and the Human leucocyte antigens (HLA) activated by them (Caselmann and Koshy; 1998) (Kreutz, 2002) (Jazayeri et al., 2004).

ORF	Nucleotide	Residues LS no. ()	Properties	HLA Class	HLA Type
Pre S1	2878	10-17	Cytotoxic T cells	Class I	A11
	2878	10-19	CD4+ cells	Class II	DQ5
		58-100	Viral clearance		
Pre S2		109-123(1-10)	Cytotoxic T cells	Class I	A3
		109-123 (1-10)	CD4+ cells	Class II	DR2
HBsAg		182-191 (20-29)		Class II	DPw4
Polymerase		182-196 (20-33)		Class II	DR2w15
		215-260 (41-49)	Cytotoxic T cells	Class I	
		250-258 (88-96)		Class I	A2
		260-269 (97-106)		Class I	A2
		335-343 (172-180)		Class I	A2
		348-357 (185-194)		Class I	A2
		455-463 (rt 107-115)		Class I	A2
		551-559 (rt 203-211)		Class I	A2
		575-583 (rt 227-235)		Class I	A2
		655-663 (rt 307-363)		Class I	A2
		773-782 (rt 425-434)		Class I	A2
		816-824 (rt 468-576)		Class I	A2
	1901- 1960	1-20	CD4+ cells	Class II	ND
	1954-1981	18-27	Cytotoxic T cells Conserved	Class I	A2
	1984-2041	28-47	CD4+ cells	Class II	DR7, DP0402 / DP0201
	2050-2107	48-69		Class II	ND
Core	2122-2152	74-83	HBeAg/conformation determinant of HBc antigenicity	B cell	
	2128-2167	76-89	Linear determinant of HBe antigenicity	B cell	
	2155-2203	84-101	CD8/Cytotoxic T cells / deletions	Class I	Riedl et al., 2009)
	2221-2254	107-118	Determinant of HBc antigenicity		
	2251-2293	117-131	Conserved	Class II	DRw52/ DR6
	2260-2317	120-139	conformation determinant of HBe antigenicity	Class II	DR1
	2278-2305	126-135	HBeAg	B cell	
	2323-2353	141-151	Cytotoxic T cells	Class I	A31/ Aw68

ORF: Open Reading Frame *The amino acid numbers of the large S protein are shown; while the amino acid numbers of the corresponding segment appear between brackets. nt. : Nucleotide

Mutant HBV induces more severe immune disorders in host, resulting in the activation of lymphocyte and release of cytokines; TNF-alpha, IFN-gamma, IL-6 and IL-8 were higher in patients with severe than those in acute hepatitis B. The ratio of CD4+/CD8+ was higher in mutant than in wild-type HBV. Ultimately liver damage is more prominent (Wang and Liu; 2003). The cellular over expression of the immunogenic HBcAg in precore mutants during virus replication may have direct cytopathic effect, and trigger vigorous T cell-mediated immune response to core that contributes to the development of fulminant hepatitis (Chen et al., 2003).

1.1.10. Pathogenesis

The interaction between viral factors as infectivity, viral replication and mutations with host factors as immunity, results in different disease severity and outcome (table 1-4). HBV is not cytopathic, hepatocellular inflammation and damage occurs, as evidenced by the development of clinical hepatitis and elevated aminotransferases.

HBV viral genome integrates into multiple sites of host DNA, which may be accompanied by mutation in viral (and cellular) genes. The integrated template produces S and pre-S polypeptide that displays trans-activation properties, which may have a role in hepatocarcinogenesis mediated by topoisomerase (Brechot et al., 2004).

Viral integrants may contain cis-acting sequences other than viral promoters, such as enhancers and glucocorticoid-responsive elements (nt. 364-374). HBV can integrate into retinoic acid receptors gene and cyclin A gene thereby influencing their expression. X-gene products and other proteins provide transcriptional transactivating function, which interferes with cellular DNA repair processes of cells genotoxically damaged (fig.1-10) by environmental agents like aflatoxin. At the same time, the inhibition of p53 by HBx would allow regeneration of liver cells and inhibit apoptosis of DNA damaged cells. In this way, mutations would be allowed to accumulate, eventually leading to malignant transformation of the hepatocytes. HCV carcinogenesis develops through different mechanisms (fig.1-10).

1.1.11. Clinical Picture

Hepatitis B virus can cause a variety of liver diseases including acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

1.1.11.1. Acute infections:

After an incubation period of six weeks to six months, which is inversely proportional to the infective dose of the virus. The spectrum of the acute infection varies from mild to severe attacks. Clinically, acute infections manifest by fever, anorexia, nausea, malaise, vomiting, jaundice, dark urine, clay coloured stools, and abdominal pain. 1 to 2 % of acute disease results in fulminant hepatitis, with a case fatality ratio of 63 to 93 % (Bracho et al., 2006). Viraemia may reach up to 1010 virions per ml. HBV replicates in extrahepatic tissues, and particularly in peripheral blood mononuclear cells (PBMCs), which may serve as a reservoir for the maintenance of infection (Mazet-Wagner et al., 2006). Extra-hepatic manifestations of HBV infection occur in acute cases, and less frequently in chronic mild infections. The symptoms include fever, symmetric arthralgia, arthritis, urticaria, haematological abnormalities as lymphocytosis, mild or aplastic anaemia, and thrombocytopenia, as well as bradycadia, hypertension, pleural effusions, and respiratory infections, vasculitis, polyarteritis and glomerulonephritis. Less frequently neuropsychatric manifestations are encountered as depression, insomnia and headache (Fileds Virology; 2005). Acute exacerbations of infection may develop in chronically infected patients (table 1-5).

1.1.11.2. Chronic Hepatitis B:

Chronic HBV infection (CHB) may present in one of the four phases of infection: immune tolerance, immune clearance HBeAg-positive chronic hepatitis B, inactive carrier state, and reactivation (HBeAg-negative CHB). Chronic HBV carriers as defined by the persistence of HBsAg for more than six months or the absence of anti-HBc IgM antibodies. Chronic liver inflammation may occur with abnormal levels of liver enzymes. Such carriers have initial high levels of viraemia that decline gradually become undetectable after years or decades (fig.1-11).

Figure 1-4: Hepatitis B virus virulence factors and host factors, their interaction affect the clinical presentation, progression and outcome of infection.

Viral Virulence Factors	Host Factors
Immune escape mutants:	Age at infection
Vaccine escape mutants	Immune status
Altered surface protein, T-cell epitopes and CTL- epitopes	Tolerance
Precore mutants	Coinfection with HCV, HIV etc.
Viral genotypes	Nutrition
HBsAg serotypes	Alcohol and hepatotoxins
Viral persistence	
Viral integration into host genome	
Occult or silent infection.	

Table 1-5: Causes of acute flares and reactivation in chronic hepatitis (Kao; 2002).

Aetiology of Acute Flares in Chronic Hepatitis B
Spontaneous reactivation of chronic hepatitis B
Reactivated hepatitis due to immunosuppressive medications
Cancer chemotherapy
Antirejection drugs
Corticosteroids
Resulting from antiviral therapy
Interferon
Nucleoside analogues
Corticosteroid withdrawal
Induced by HBV genotypic variation
Precore mutant
Core promoter mutant
HBV DNA polymerase mutant
Due to superimposed infections with other hepatotropic viruses
Hepatitis A virus
Hepatitis C virus
Hepatitis delta virus
Caused by interaction with HIV infection
Reactivated hepatitis
Effect of immune reconstition therapy

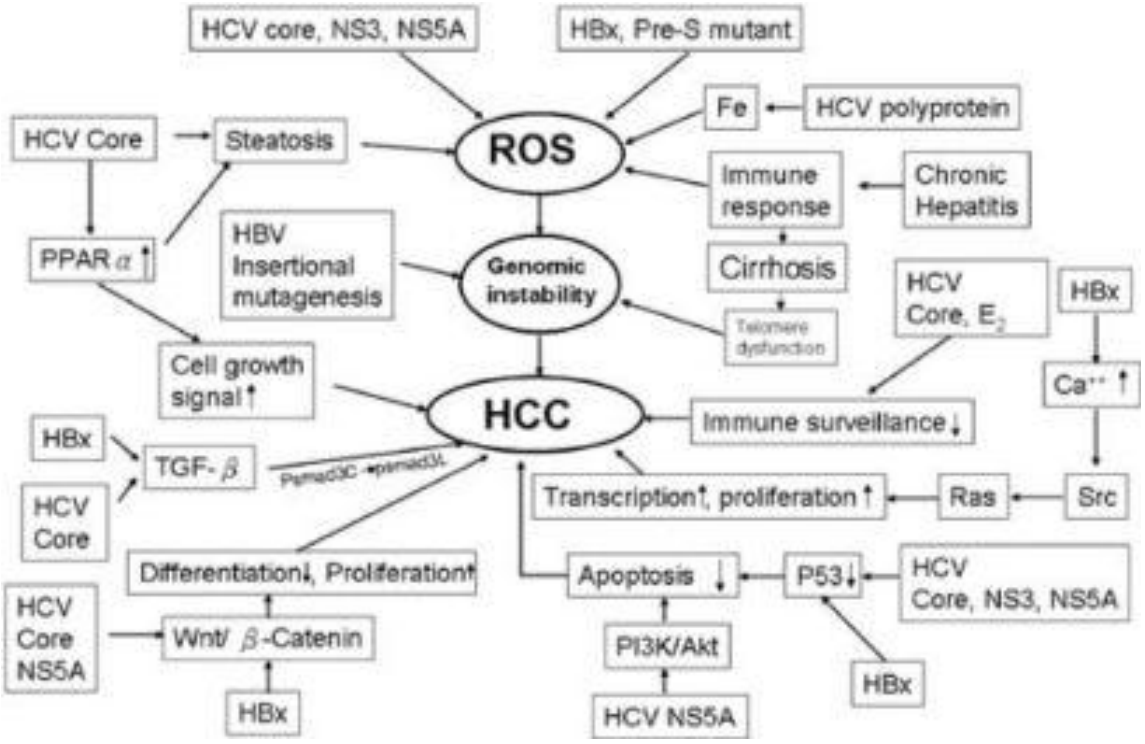


Figure 1-10: Mechanism of HBV and HCV-mediated carcinogenesis.

In the cytoplasm, HBx transactivates several cytoplasmic signaling pathways. Which include protein kinase C (PKC), JAK/STAT, PI3K, stress-activated protein kinase (SAPK)/c-Jun NH2-terminalkinase (JNK), ras-raf-MAPK, and reactive oxidative intermediate (ROI)-dependent mechanisms to stimulate gene expression through activator protein-1 (AP1), AP-2, nuclear factor-κB (NF-κB), Smad and Wnt, and by binding to nuclear transcription factors, including cAMP response element binding (CREB), activating transcription factor 2 (ATF-2), Oct-1 and TATA box binding protein (T BP). UGT1A1, UGT2B10 and GPX2 expression was preferentially repressed in HBV-HCCs, but not HCV-HCCs. E-cadherin and GSTP1 were preferentially methylated in HBV-HCC compared with HCV-HCC (Su et al., 2007). TNF-α-863A genotype, IL-1B-511C allele, C-A haplotype of IL-10 gene andXRCC1 Gln allele at codon 399, −509C→T polymorphism in the TGF-β1 gene promoter are all associated with HCC in chronic HBV carriers. HCV increased expression of genes encoding phase I enzymes CYP2E, AKR1C4, EPHX1 and FMO3 exclusively in HCV positive HCCs (Tsai and Chung, 2010)

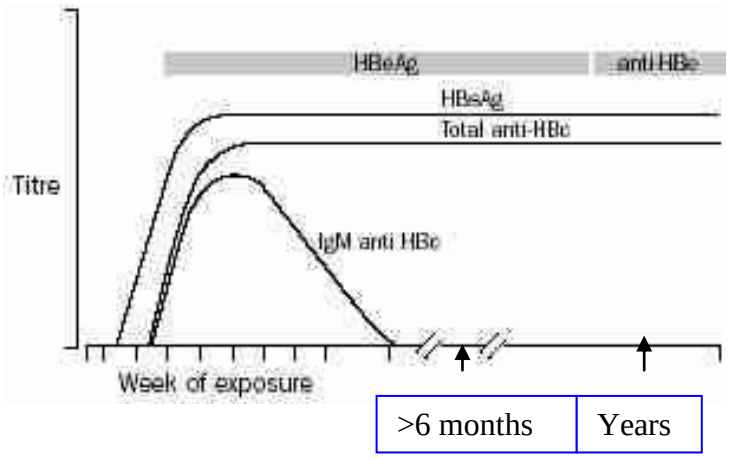


Figure 1-11: Serological markers in chronic HBV infection.

After the incubation period of 4 to 6 weeks, HBsAg and HBeAg start to increase and persist for more than 6 months. HBeAg seroconversion to anti-HBe occurs later during the course of disease.

Table1-6: Risk groups for HBV infections

Individuals at increased risk of HBV infection
Family contacts of HBV infected individuals
Infants born to HBV infected mothers
Institutionalised individuals (e.g., prisoners, mentally handicapped)
Health care and public safety workers
Recipients of multiple blood transfusions
Haemodialysis patients
Infants and children of immigrants from areas with high rates of HBV infection
Individuals with multiple sexual partners
Sexual contacts of infected person
Homosexual males
Parenteral drug abusers

The risk of developing chronic infection varies inversely with age and is highest (up to 90%) in infants infected in the perinatal period. Between 25 and 50% of children infected between 1 and 5 years, and 6 to 10% of acutely infected older children and adults become chronically infected and 33 to 50% of adults are symptomatic. The risk of developing chronic infections is increased by host factors as renal failure, HIV infection and diabetes (Mphahlele et al., 2006).

Chronic hepatitis B infection substantially predisposes to complications such as chronic liver disease, cirrhosis and hepatocellular carcinoma (fig.1-12). Death occurs in 15-25 % of chronically infected persons. Up to 25% of persons acquiring HBV as infants and young children develop either cirrhosis or HCC, compared to 15% of young adults who acquire chronic HBV infection. Histologically, liver biopsy shows chronic persistent hepatitis, chronic active hepatitis, and cirrhosis (Ahn et al., 2005). Approximately 0-10% of infected individuals become chronic carriers of HBV. Chronic carriers are symptomless, but may have histological evidence of hepatocellular damage from mild inflammation to cirrhosis and hepatocellular carcinoma HCC.

1.1.11.3. Hepatocellular Carcinoma:

Hepatocellular carcinoma (HCC) is one of the 10 most common cancers in man. The annual incidence is 250 000 worldwide. Risk factors are; HBV and HCV infections especially those acquired early in life or after prolonged course, cirrhosis, male sex, aflatoxin and smoking, peak incidence in 30-50 year age group. The relative risk of developing HCC is over 200-fold for HBsAg carriers over matched controls. Whilst most HCC arise in cirrhotic liver, this is not always the case (Liu et al., 2006).

1.1.12. Epidemiology

Approximately 5 % of the world`s population reaching 350 millions, have chronic HBV infection, which is the leading cause of chronic hepatitis, cirrhosis and HCC worldwide. It is estimated that 500, 000- to 1000, 000 persons die annually from HBV related liver disease (Hou et al., 2005). Most infections occur at birth or during early childhood. Infections usually cluster in households of chronically infected patients.

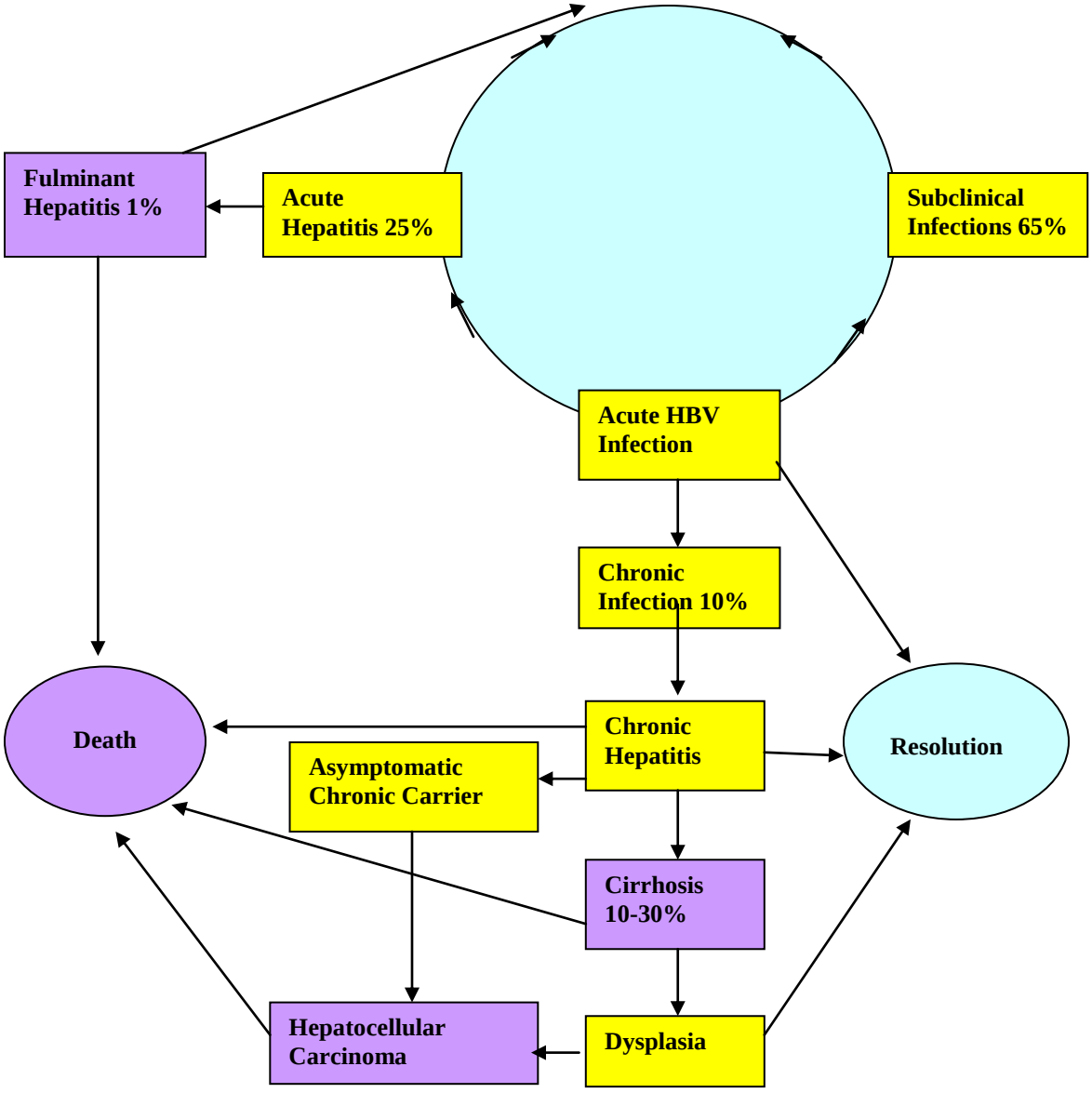


Figure 1-12: Clinical outcome of HBV infection

1.1.12.1. Geographical Distribution

Areas of high endemicity where prevalence is > 8% are China, Indian subcontinent and Africa. Intermediate endemicity areas show prevalence of 2 to 7 % in North Africa, India and Russia. Low endemicity < 2 % seen in Western Europe and North America (Fig. 4-3). In areas of high endemicity, the lifetime risk of HBV infection is > 60 % .

1.1.12.2. Routes of Transmission

Transmission of HBV is by percutaneous route or mucosal exposure to infectious body fluids, sexual contact or perinatally from infected mothers. HBV is highly infectious, especially in HBeAg positive individuals, up to 50 to 100 times more infectious than HIV. Risk groups for contracting HBV are listed in table 1-6.

1.1.12.3. Vertical Transmission

HBeAg among pregnant women increases the risk of perinatal infections up to 70 to 90% in the absence of immunoprophylaxis, whereas only 5 to 20 % of infants born to HBsAg positive and HBeAg negative mothers become infected (table 1-6). Postnatal early childhood transmission is also common in endemic areas.

1.1.13. Diagnosis

The development of sensitive and specific tests to detect HBV infections allowed the study of its natural history and epidemiology to prevent its transmission.

1.1.13.1. Serological Tests

Serological tests are the main stay of diagnosing and differentiating the various viruses causing hepatitis, and for blood bank screening, as they are quick, cheap, and detect HBsAg carriers. Acute HBV is characterized by the presence of HBsAg in serum and the development of IgM core antibodies (anti-HBc IgM), which may be the only marker in active hepatitis, and it correlates with inflammatory activity. In the convalescent stage, HBsAg and HBeAg are cleared with the development of anti-HBs, anti-HBe and anti-HBc antibodies. Anti-HBs is also elicited by vaccine.

In chronic HBV infections, HBsAg generally persists for life. Total anti-HBc tests for both IgM and IgG antibodies to HBV core protein, they indicate current or past infection

by HBV respectively. IgM anti-HBc disappears six months after the acute infection. The IgG anti-HBc appears shortly after HBsAg in acute disease and persists for life (fig.1-13). Different methods exist for detection of HBsAg as immunodiffusion, reverse passive haemagglutination assays, and the more sensitive enzyme linked immunoassays ELISA and radioimmunoassays with detection limit of = > 0.1 ng /ml of HBsAg.

Ordinary serological tests may not detect mutant HBsAg and would therefore give rise to false negative results. New immunoassays are designed for the detection of hepatitis B surface escape mutants, and are specifically useful in the monitoring of liver transplant recipients on HBIG prophylaxis (Ijaz et al., 2001).

1.1.13.2. Molecular Biology Techniques

Different molecular techniques have been used to detect HBV DNA (table 1-7). HBV DNA is detectable in serum by slot or dot blot hybridization assays (Shao et al., 2007) with detection limit of 1.5 pg per ml (4.0 $x10^5$ genomes/ml). PCR detects 103 pg /ml (approximately 100 to 1000 genomes). However, the high sensitivity of PCR is limited by the increased risk of false positive results. Clinical significance of HBV PCR is the same as detection of HBsAg and indicates current HBV infection. HBV DNA monitoring and quantitative PCR are essential in determining the response and follow up of chronic HBV infection to treatment. Nucleic acid sequence analysis is used to identify genetic variants of the virus, and to epidemiologically type nosocomial transmission of HBV (Gunson et al., 2006).

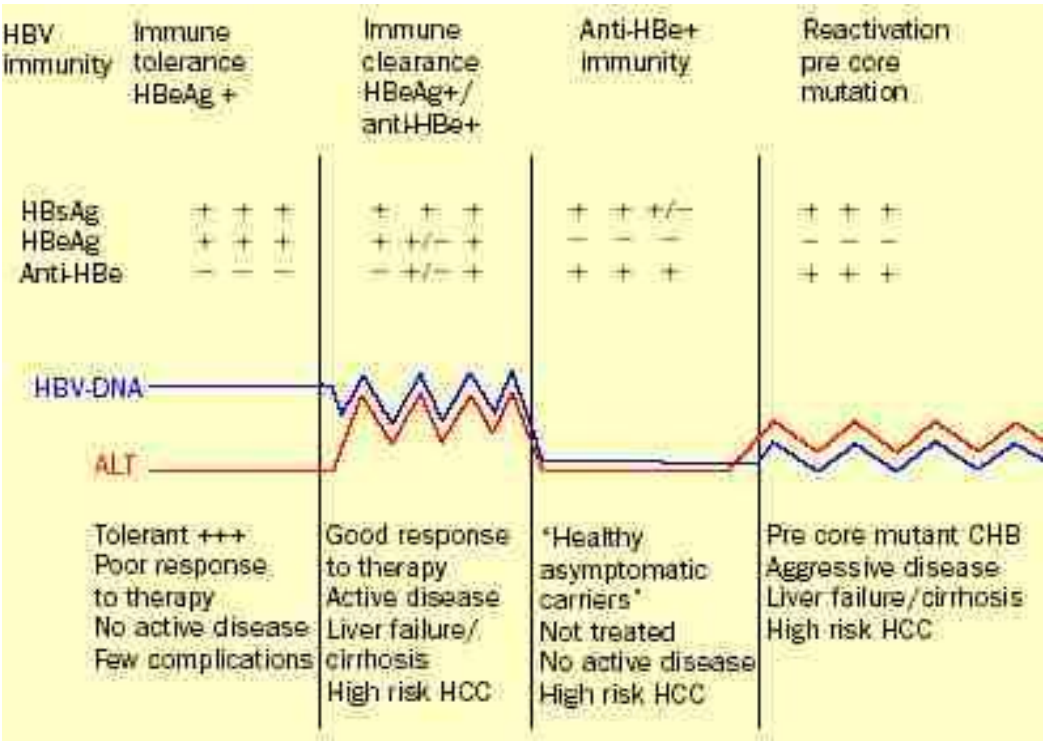


Figure 1-13: Serological, virological and clinical patterns of chronic hepatitis B infection (Jung and Pape; 2002).

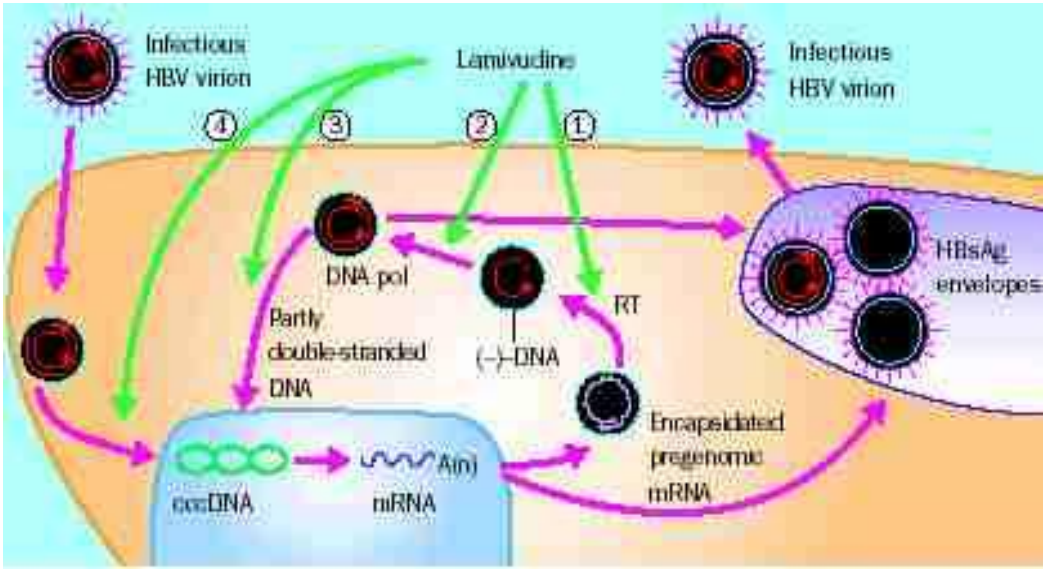


Figure 1-14: Four possible sites of action of lamivudine in HBV replicative cycle; (1) inhibit reverse transcriptase enzyme, (2) inhibits DNA polymerase, (3 and 4) inhibits cccDNA formation cell entry and in replication (Yuen and Lai, 2001).

1.1.14. Treatment

1.1.14.1. Clinical Management

Supportive and symptomatic therapy is the mainstay of management of acute HBV infections. Interferon is an immunomodulatory cytokine that increases the expression of HLA class-I molecules, thereby increasing the cytotoxic lymphocytes (CTL) elimination of virus infected cells. It aims to clear markers of HBV replication, and to improve the liver disease by normalization of alanine aminotransferase (ALT) levels and liver histologic changes (Hannoun et al., 2002).

Several drugs are used in different combination to treat hepatitis B infections. As in cases of active viral replication, high viral load, and HBeAg positivity, elevations in serum aminotransferases, persistent HBsAg, chronic hepatitis and compensated liver disease (table 1-9). The mainstay of therapy in chronic hepatitis B is inhibition of the replicative cycle of HBV in hepatocytes, aiming at complete suppression of serum HBV DNA using reverse transcriptase inhibitors as lamivudine (3`-thiocytidine) (fig.1-15). (Yuen and Lai 2001). Viral breakthrough, defined as an abrupt or continuous increase in serum HBV DNA levels after a period of persistent suppression, occurs with non compliance to therapy or lamivudine resistant mutants (Kennedy et al., 2008).

1.1.14.2. Immunoglobulins

Blood with anti-HBs was used for the preparation of hepatitis B immune globulin (HBIG), which was effective in preventing or modifying the course of HBV infections (table 1-9), and as post-exposure prophylaxis (table 1-10).

1.1.15. Prevention

1.1.15.1. Virus Inactivation

The immunogenicity and antigenicity of HBsAg are retained after exposure to ether, acid, and heat, while the virus is inactivated by boiling for two minutes, autoclaving at 121^{o}C for 20 minutes, or dry heat at 160^{o}C for one hour as well as sodium hypochlorite 0.5% for 10 minutes, 0.1 to 2% gluteraldehyde, and 70% isopropyl alcohol for 2 minutes.

Table 1-7: Comparison of molecular techniques for the diagnosis of HBV

Dot blot hybridisation	Branched DNA assay (bDNA)	Polymerase chain reaction (PCR)	Quantitative real-time PCR
Monitoring antiviral therapy	Easy for bulk testing	Monitoring antiviral therapy	Monitoring antiviral therapy
Less sensitive than PCR	Less sensitive than PCR	Little correlation with disease activity	Little correlation with disease activity
Detect 10^5 copies/ml (0.1-1pg)	Detect 2.5-3.5x10^5 copies/ml	Detect 10^2 copies/ml	Detect 12IU/ml
Qualitative	Semiquantitative	Prone to contamination	Less contamination

Table 1-8: Current or proposed therapeutic agents for chronic HBV infection (Yuen and Lai 2001).

Category	Agents
Immunomodulators	Interferon alfa (recombinant, pegylated)
	Thymosin alfa1
	Levamisol
Passive Immunity	hepatitis B immunoglobulins HBIG
Antiviral Agents A. Nucleoside/nucleotide Analogues	Lamivudine
	Adefovir dipivoxil
	Tenofovir
	Entacavir
	Emtricitabine
	Beta-L-2`-deoxythymidine
	Famciclovir
B. Glycosylation Inhibitors	Inhibit viral assembly
C. Gene Therapy	Antisense, ribozyme, targeted drug delivery, etc.
IV. Combination Therapy	Lamivudine plus Interferon alfa
	Lamivudine plus therapeutic vaccines
	Steroid priming followed by Lamivudine or Interferon alfa
	Lamivudine plus famciclovir or adefovir dipivoxil
V. Therapeutic Vaccines	PreS/S vaccines (preS2, preS1)
	Core lipopeptide
	HBs-DNA vaccine
	HBsAg-anti-HBs antibody complex
	CTL epitope vaccine
	Cell based: Adoptive immune transfer

Table 1-9: Strategies to prevent HBV transmission:

Extended program of childhood immunization (EPI)
HBV vaccination of risk groups and health care workers
Standard precautions against blood and body fluids
Proper use and disposal of sharps
Screening of blood and organ donors
Monitoring of haemodialysis patients
Screening of pregnant mothers
HBIG and vaccination at birth to infants of infected mothers
Avoid sharing of personal items as razors and tooth brushes
Care with tattoo and body piercing as potential source of infection
Avoid sharing of needles in IV drug users
Use of condoms and safe sex practice

Table1-10: Indications of Hepatitis B immunoglobulins (HBIG)

Infants of HBsAg positive mothers
Percutaneous or mucus membrane exposure to HBsAg positive blood or body fluids
Sexual exposure to HBsAg positive person
Protect patients from severe recurrent HBV infection after liver transplantation

Table 1-11: HBV Prophylaxis for Reported Exposure Incidents

HBV status of person exposed	Significant Exposure (Source Patient)			Non- significant Exposure	
	HBsAg* Positive	Unknown source	HBsAg negative	Continued risk	No further risk
=<1dose HBV vaccine pre-exposure	Accelerated HBV vaccination**, HBIG x 1	Accelerated vaccination	Initiate HBV vaccination	Initiate HBV vaccination	No HBV prophylaxis Reassure
=<1dose HBV vaccine pre-exposure (anti-HBs not known)	Two doses of vaccine one month apart	One dose of vaccine	Finish course of vaccine	Finish course of vaccine	No HBV prophylaxis Reassure
Known responder to HBV vaccine (anti-HBs>10miU/ml)	Consider booster dose of vaccine	Consider booster dose of vaccine	Consider booster dose of vaccine	Consider booster dose of vaccine	No HBV prophylaxis Reassure
Known non-responder to two full courses of vaccine (anti-HBs>10mIU/ml) 1-2 months post immunization	HBIG x2 one month apart	HBIG x2 one month apart	No HBIG	No HBIG	No HBV prophylaxis Reassure
Known non-responder to one full course of vaccine (anti-HBs>10miU/ml) 1-2 months post immunization	HBIG x 1, Start the second full course of vaccine Exclude HBV infection as the cause of non-responsiveness	HBIG x 1, Start the second full course of vaccine	No HBIG Start the second full course of vaccine	No HBIG Start the second full course of vaccine	No HBV prophylaxis Reassure, consider a second full course of vaccine

* Accelerated HBV vaccination consists of doses spaced at 0, 1, 2, and 12 months
Modified from CDR Review 1992:2; R97-R101, PHLS Hepatitis Subcommittee (CDC; 2001).

HBV retains its infectivity when stored at 4oC for six months and when frozen at –20^{o}C for 15 years (Fields Virology; 2005)

1.1.15.2. Infection Control

Nosocomial transmission of HBV has occurred in several situations in medical care settings (Kramer et al., 2006). Characterization of HBV strains by subtype or genotype has been used to detect the prevalent strains, and to investigate chains of nosocomial infection. Haemodialysis is a recognized risk procedure for nosocomial HBV transmission (Kondili et al., 2006), although this problem has been largely controlled by the introduction of prolonged HBV vaccination schedule to haemodialysis patients. Guidelines recommend the institution of infection control practices specifically designed for the haemodialysis setting, including routine serologic testing, surveillance and education and training of staff to reduce the opportunities for patient-to-patient transmission of infectious agents directly or indirectly via contaminated devices, equipments and supplies, environmental surfaces and hands of personnel (CDC; 2001).

1.1.15.3. Immunoprophylaxis

Passive immunization with neutralizing anti-HBs may provide protection from acute clinical hepatitis B and chronic HBV infection if given soon after the exposure. HBIG is prepared by Cohn Onely fractionation procedure from serum containing high titres of anti-HBs, and is standardized to 100, 000 IU of anti-HBs per ml. HBIG has several indications (table 1-11), and is used in combination with HBV vaccine to prevent perinatal infection in infants born to HBsAg positive mothers.

1.1.16. Accidental Exposure/Needle Stick Injury

In health care settings, significant exposure to HBV infected or suspected source carries a risk of transmission of the infection. Several guidelines have been published to handle such situations and to minimize the possibility of transmitting the infection (table 1-12). (Guidance for Clinical HCW prophylaxis in UK: Protection against Infection with blood borne viruses. www.open.gov.uk/doh/chcguid1.htm)

Table 1-12: HBV Prophylaxis for Reported Exposure Incidents Groups for whom hepatitis B immunization is recommended

A. Professional Risk:	
I- Staff in:	Renal dialysis facilities
	Clinical laboratories
	Mental handicap hospitals
	Infectious diseases units
	Sexually transmitted diseases units
	Oral surgery departments
	Accidents and emergency departments
	Operation rooms, delivery rooms
	Ambulance/police /prisons personnel
	General hospitals: offered to all medical/ nursing disciplines dealing with hazard situations, e.g. needle stick exposure
B. Contact Risks:	
I-Patients	In adult institutions
	In mental handicap hospitals
	Before haemodialysis programmes
	Before organ transplantation
	Before multiple transfusion of blood or blood products
	Household and sexual contacts of known HBsAg patients
	Household and sexual contacts of known HBsAg carriers
	Intravenous drug abusers
II-Infants	Of infected mothers, particularly where these women are HBeAg positive
	All infants in high risk areas
III- Individuals	International travellers to endemic areas
	Refugees

1.1.17. Vaccines

HBV vaccine is safe, immunogenic even in neonates, highly effective, and is the first vaccine that prevents cancer. HBV vaccine contains highly purified HBsAg, generated from either plasma or recombinant plasmids containing the S gene with or without the pre-S1 and/or preS2 gene.

The development of anti-HBs antibodies: titres of >10 mIU per ml are 100% protective against clinical illness and chronic infection. Two-dose vaccine is introduced by the WHO to facilitate adolescent's compliance. Other host factors affecting the response to vaccine are age, smoking, obesity, chronic disease, dialysis, and HIV infection. Vaccination against HBV is recommended to several risk groups preferably before exposure to the virus (table 1-13). A booster dose of vaccination and doubling of vaccine dose might be required in the second decade after vaccination, especially in immunosuppresed, haemodialysis and liver transplant patients (Gunther, 2006).

The new DNA vaccines provide a vaccination strategy that is less expensive and easier to produce than antigenic proteins. In addition DNA immunization induces a strong CTL as well as T-helper and antibody response to the expressed proteins (Mancini-Bourgine et al., 2006), which is directed against expressed and processed viral epitopes.

Chapter Two: Occult HBV Infection

2.1. Introduction

Occult hepatitis B infection (OBI) has been referred to as `inapparent hepatitis B virus (HBV) infection` and `serologically silent hepatitis B`(Raimondo et al., 2007). In 2008, the European Association for the study of the liver (EASL) defined OBI as the "presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing HBsAg-negative by currently available assays" with or without the presence of anti core (anti-HBc) and/or anti-HBs antibodies (Ocana et al., 2011) (fig.2-1, table 2-1).

OBI may have underlying liver disease indicating that viral replication may result in hepatocellular injury. OBI is associated with low HBV DNA levels and higher immune responses (Allain et al., 2009). HBV reactivation may appear upon immunosuppression or exposure to chemotherapy (Leaw et al., 2004), and can transmit the virus through liver transplant (Shetty et al., 2008).

Several possibilities were hypothesized as the mechanisms of OBI infection including mutations or integration of HBV DNA into host's chromosomes or as episomal DNA, formation of immune complex containing HBV, altered host immune response or interaction of HBV with other viruses. The mean percentage of HBV-infected hepatocytes was significantly lower in patients with OBI than in HBsAg chronic carriers. Patients with chronic hepatitis B have HBV DNA in their peripheral blood mononuclear cells (PBMCs) while this occurred less in cases with OBI (Mazet-Wagner et al., 2006).

Different clinical situations have been associated with OBI (table 2-1). OBI may follow recovery from infection, displaying anti-HBs and persistent low-level viraemia, escape mutants undetected by the HBsAg assays, or healthy carriage with anti-HBe and anti-HBc. Over time, in the latter situation, anti-HBe and, later, anti-HBc may become undetectable, but in most cases the explanation is probably the low level of viral replication (Jeantet et al., 2004).

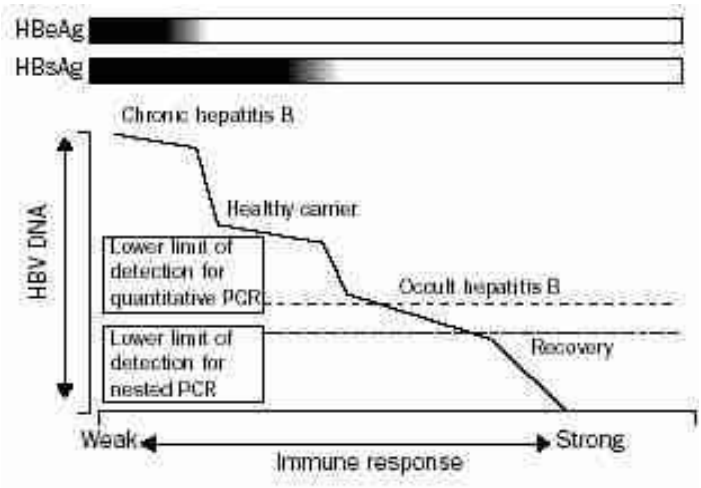


Figure 2-1: Serological profile and viral DNA in occult HBV infections (Thorbinson and Thomas; 2002).

Table 2-1: Anti-HBc states
Different clinical presentations showing their hepatitis markers and HBV viral loads (Torbenson and Thomas, 2002).

Clinical State	Anti-HBs	Anti-HBe	Anti-HBc	HBsAg	HBeAg	HBV DNA
Early window phase	-	-	+ IgM	+	-/+	+
Late HBV immunity	+	+	+	-	-	-
Unresolved HBV infection	-	-	+	+	+	+, $>10^5$ copies/ml
Low grade chronic infection	-	-/+	+	+	+/-	+, $>10^5$ copies/ml
Asymptomatic carriers	-	+	+	+/-	-	+, $<10^5$ copies/ml
OBI	-/+	-/+	-/+	-	-/+	+, $<10^3$ copies/ml

Table 2-2: Clinical groups at risk for occult hepatitis B (Torbenson and Thomas, 2002)

Clinical group	OBI prevalence	DNA concentration
Chronic HCV with hepatocellular carcinoma	High >50%	
Liver transplant recipients from core antibody positive donors	High >50%	
Chronic hepatitis C, positive for anti-HBc	Moderate 10-49%	
Cryptogenic cirrhosis/advanced fibrosis	Moderate 10-49%	
Intravenous drug users	Moderate 10-49%	
HIV, positive for hepatitis B core antibody	Moderate 10-49%	
Routine blood donors	Low <10%	2.2pg/ml
HBsAg-negative mutants		

OBIs carry the potential risk of HBV transmission in immunocompromised individuals as HIV (Nebbia et al., 2007), haemodialysis (Jain and Nijhawan; 2008), cancer chemotherapy and liver transplantation, or bone marrow transplant recipients (Shetty et al., 2008). In immunocompetent recipients, there is no evidence that anti-HBc antibody positive blood with anti-HBs antibody even at low titre, are infectious. Second, it may serve as the cause of cryptogenic liver disease, and contribute to acute exacerbation of chronic hepatitis B or chronic HCV. Third, it is associated with development of hepatocellular carcinoma. Fourth, OBI may affect disease progression and treatment response of chronic hepatitis C (Raimondo et al., 2007).

Clinicians should consider testing for OBI in certain situations for diagnosing and management of such cases as flares of OBI can occur and are treatable (table 2-2). OBI should be suspected in patients with risk factors in whom immunosuppression is expected, refugee and pregnant women in endemic areas. Also in patients with unexplained active liver disease; either unexplained elevations in liver enzymes or cirrhosis or with isolated anti-HBc in whom the presence of OBI would affect the clinical decisions such as suitability for organ donation. Other risk groups of OBI are HBV vaccination non-responders and patients with HCV resistant to interferon or ribavirin therapy or with rapid progression of liver disease (Rodriguez-Torres et al., 2007). OBI may contribute to the unfavourable IFN response in chronic HCV by down regulation of IFN receptor gene expression in the liver (Fukuda et al., 2001). Patients with anti-HBc antibodies might develop immune tolerance against HCV, with less advanced hepatolysis and necroinflammation even in the presence of HCV viraemia with sustained ALT normalization.

Prevalence of OBI depends on the prevalence of HBV in a population, and the prevalence of immunosupressed subjects particularly among individuals coinfected with HCV or HIV (Mphahlele et al., 2006). Screening of HIV-infected patients for OBI may have implications in terms of antiretroviral therapy and risk of immune reconstitution-associated flares (Shire et al., 2004).

The frequency of OBI diagnosis depends on the relative sensitivity of both HBsAg and accuracy of HBV DNA assays. It also depends on the prevalence of HBV infection in the

population. Anti-HBc antibodies estimation is recommended before HBV vaccination, in blood donors, organ donations especially Liver donors, and in relevant clinical situations. Confirmation of OBI is by HBV DNA testing, and monitoring of ALT elevation (Li et al., 2007).

2.2. Aim

The aim of occult HBV study is:

To detect the presence and prevalence of HBV DNA in anti-HBc positive blood donors from Saudi Arabia, and to evaluate the safety of anti-HBc-positive blood.

To check whether OBI is related to specific HBV genotypes or is host determined

To evaluate HBV infection in asymptomatic individuals as a control group to the liver disease group studied in next chapters.

To evaluate the prevalence of OBI in hepatitis C virus (HCV) patients in Saudi Arabia.

To investigate the prevalence of OBI in hepatocellular carcinomas in Saudi Arabia.

2.3. Patients and Blood Donors

2.3.1. Patients

5 groups of Saudi individuals were studied

Group-I :consisted of sera collected from 199 voluntary Saudi blood donors HBsAg-negative and anti-HBc IgG antibodies positive tested during the time period March 2002 to June 2005. Donors filled questionnaires for history of liver disease, previous tattooing, transfusions or drug addiction, to exclude risk factors for blood-borne infections. Those were divided into two subgroups

Ia : 104 anti-HBs antibody positive

Ib : 95 anti-HBs antibody negative

Group-II: 30 HBsAg-positive and anti-HBc IgG positive Saudi blood donors

Group-III: 250 Saudi blood donors negative for HBsAg, anti-HBc IgG and anti-HBs as control.

Group-IV: Hepatitis C patients. Serum samples were collected from 25 Saudi cases with positive HCV antibodies, anti-HBc antibodies positive and HBsAg negative. Inclusion criteria were the absence of other causes of chronic liver disease or alcohol consumption.

Group-V: Hepatocellular carcinoma: Liver biopsies were obtained from 15 Saudi patients with hepatocellular carcinoma to test for HBV DNA. All were HBsAg negative, four were anti-HBc positive.

2.3.2. Serological Tests

Serum samples were tested for HBsAg, total anti-HBc antibody, antibody to hepatitis C virus, HIV antibodies and P24, HTLV antibodies by ELISA technique using Murex kits. Negative and positive controls were included. Cutoff levels were calculated according to kits instructions.RPR was also done. All the HBsAg negative with anti-HBc positive units were checked for anti-HBsAg antibodies.

2.3.3. DNA Extraction from Sera

DNA was extracted from 200µl serum by QIAamp kits (Qiagen) into 100 µl sterile water (Sigma).

2.3.4. DNA Extraction from archival material

Liver biopsy archival specimens were cut into 3mm thick sections collected from each paraffin wax block in a microtube. A small section (not more than 25mg) of paraffin-embedded tissue was placed in a 2ml micro-centrifuge tube containing 1200µl of xylene, and vigorously vortexed. The tissue was then pelleted by centrifugation at 13000 rpm (10000 x g) for 5 minutes at room temperature. The supernatant was removed by pipetting, and the pellet washed twice with 1200µl absolute ethanol (100%) to remove any residual xylene. The pellet was then incubated at 37°C for 15 minutes in order to evaporate the ethanol. DNA was extracted with the QIAmp DNA tissue kit (Qiagen) according to the manufacturer's instructions. DNA was measured by spectrophotometry.

2.3.5. Amplification of Precore/core Gene

Amplification of the core promoter region of genomic HBV DNA by the Polymerase Chain Reaction (PCR), using primers PC1 and PC3 (De Castro et al., 2001) (table 2-3). The standard PCR mixture contains 0.15µl of hot start Taq polymerase (Qiagen), 5pmolof each primer (MWG-Biotech Ltd), 2.5µl 10x buffer (containing 15mMMgCl2, Qiagen), 100nM of each of four deoxyribonucleoside triphosphates (dNTPs) (Roche) and 2µl DNA template giving a total volume of 25µl in sterile distilled water (Sigma).

Positive and at least 3 negative controls were included in each run. The amplification started with 95oC for 10 minutes; cycle conditions were shown in table: 2-4 (PT-200 Peltier Thermal Cycler). 4µl of PCR products (296 bp) were loaded into a 2% agarose gel (Helena Biosciences). Electrophoresis was done at 90 Volts, then stained with ethidium bromide and visualized under UV light.

2.3.6. Nested Core PCR

Amplification of core gene was done using a modified semi-nested PCR. The outer primers were C1 and C2 (Kurosaki et al., 1996). The standard PCR reaction mix was as described in precore PCR. Primer sequences are shown in table 2-3. Product size was 374 bp. The second PCR was done using the inner primers: C3 (sense) and C2 (antisense) and 2µl of the first reaction products per tube under the conditions specified in table 2-4; the product size was 364 bp.

2.3.7. Extended Core PCR

Amplification of core gene was done using a modified PCR. The outer primers were C1 (Kurosaki et al., 1996) and PC3. The standard PCR reaction mix was the same, under the following conditions: 95oC for 10 minutes, then 50 cycles of 94oC for 1 minute, 60oC for 1 minute, 72oC for 3 minute. The product size was 475 bp.

2.3.8. Amplification of Surface Gene

The surface gene (S-ORF) from genomic HBV DNA was amplified by nested PCR (Fagan and Harrison; 1994) using the following outer primers: HBV-S1and HBV-S2 under the following conditions; 95oC for 10 minutes, then 40 cycles of 94oC for 1.5 minutes, 47oC for 1.5 minutes and72oC for 3 minutes. A final extension was done for 7 minutes at 72oC. The product size was 359 bp.

The second PCR was done using the inner primers: HBV-S3 and HBV-S4. Standard reaction mix have the same other components as first round PCR and 2µl first round PCR product giving a total volume of 25µl. The PCR conditions were as follows; 95oC for 10 minutes, then 40 cycles of 94oC for 1.5 minutes, 45oC for 1.5 minutes, 72oC for 3 minutes. The product size was 294 bp.

2.3.9. Short PCR of S-gene

Amplification of the S-gene was done as previously described (Brechtbuehl et al., 2001). The 25 µl PCR mixture contains 5 pmol of each primer HBVfr (sense nt. 376-395) and HBVrv (antisense nt. 656-637) (primers from MWG-Biotech Ltd) and 2µl DNA. The PCR conditions were shown in table 2-4. These primers were also used for real-time PCR. The product size was 281 bp.

2.3.10. RFLPs PCR

Using PCR to amplify conserved region in the S-gene, a seminested PCR (Mizokami et al., 1999) was performed with the first-round sense primer F1 and antisense primer R2, in standard master mix. The thermal cycler program was 95oC for 10 minutes; followed by 40 cycles of 94oC for 15 seconds (denaturation), 60oC for 45 seconds (annealing) 72oC for 45 seconds (extension); followed by further extension at 72oC for 7 minutes. The product size was 676 bp. The second round inner sense primer F3, and the antisense primer R2were amplified under the same conditions. The product size was 492 bp.

2.3.11 HBV Multiplex Genotyping PCR

See genotyping chapter.

2.3.12. Optimization for Occult HBV detection

4µl of template DNA were used instead of 2µl in all the above PCRs. Total reaction volume was 25µl by decreasing sterile water volume from 18.5µl to 16.5µl per tube.

Table 2-3: List of different HBV PCR used, their primers and their thermal cycle conditions

PCR	Primers Pairs	Nucleotide Position (nt.)	Product (bp)	Primer Sequence	Ref.	PCR cycles Conditions (temperature/time)			
						Denaturation /94°C	Annealing	Extension 72°C	Number
Core Gene: Pre-core (PC) PCR	PC1 PC3	1781-1803 2075-2056	296	5`-GGCTGTAGGCAT AAATTGGTCTG-3` 5`- TTGCCTGAGTGC AGTATGGT-3`	(De Castro et al., 2001)	45 sec	55 x 1min	3 min	50
X gene/Extended core (EC)	C1 PC3	1601-1620 2075-2056)	475	5`-ACGTCGCATGG AGACCACCG-3` 5`- TTGCCTGAGTG CAGTATGGT-3`	Kurosaki et al., 1996	1 min	60 x 1 min	3 min	50
Nested Core : first round C1	C1 C2	1601-1620 1974-1954	374	5`-ACGTCGCATGG AGACCACCG-3` 5`-GGAAAGAAGTC AGAAGGCAAA-3`	Kurosaki et al., 1996	1 min	55 x 1 min	3 min	40
Nested core second round C2	C3 C2	1611-1630 1974-1954	364	5`-GAGACCACCG TGAACGCCCA-3` 5`-GGAAAGAAGT CAGAAGGCAAA-3`		1 min	60 x 1 min	1 min	40
S-gene: Mx-S PCR*	HBVFr HBVRv	376-395 656-637	2.	5`-GATGTGTCTGC GGCGTTTTA-3` 5`-CTGAGGCCCAC TCCCATAGG-3`	Brechtbuehl et al., 2001	30 sec	55 x 1:30 min	3 min	40
Nested S-PCR first round S1	S1 S2	418-433 776-761	359	5`-GCGCTGCAGCT ATGCCTCATCTTC-3` 5`-GCGAAGCTTGC TGTACAGACTTGG-3`	Fagan and Harrison; 1994)	1:30 min	47 x 1:30 min	3 min	40
Second round S2	S3 S4	455-470 748-734	294	5`-GCGCTGCAGCA AGGTATGTTGCCCG-3` 5`-GCGAAGCTTCAT CATCCATATAGC-3`		1 min	45 x 1:30 min	3 min	40
Nested RFLP PCR First round R1	F1 R2	nt. 57-77 nt. 732-711	676	5`-Y (C/T)CCTGCTGG TGGCTCCAGTTC-3`, 5`-AAGCCANACA R (A/G)TGGGGGAAAGC-3`	Mizokami et al., 1999	15 sec	60 x 1 min	3 min	40
Second round R2	F3 R2	nt. 245-270) nt. 732-711	492	5`-GTCTAGACTCG TGGTGGACTTCTCTC-3` 5`-AAGCCANACARTGGGGGAAAGC-3`					
Genotyping nested P1	P1 S2		1097	5`-TCACCATATTCTTGGGAACAAGA3` 5`-CGAACCACTGAACAAATGGC-3`	Naito et al., 2001	30 sec	55 x 1 min	3 min	40
2nd round (Mix A) multiplex	B3N A4, R5, C6 (antisenses)		178, 389, 230	#		20 sec 20 sec	58 x 20 sec 60 x 20 sec	1 min 1 min	25 then at 30 additional cycles
2nd round (Mix B) multiplex	D7, E8, F9 (sense) R11 (antisense)		200, 225, 147	#					

* :Those primers were also used for real-time QPCR. Y: C or T, R: A or G, N: A or G or C or T.
# See table 4-2.

2.4. Results:

2.4.1 Evaluation of Different HBV PCRs

2.4.1.1 Optimized extended core PCR

Optimization was done using different combination of primers C1 and PC3, C3 and PC3 to cover this important region of the core promoter, precore and the beginning of the core gene. Variation of primers and PCR conditions led to the development of X gene/Extended core PCR (EC) with product size 475 bp. Primers C1 and PC3 were used.

2.4.1.2. Comparison of the detection limits and sensitivities of eight different PCRs of HBV core and S/pol genes

To test for occult HBV infections, first the most sensitive PCR method must be specified for two different genes, the core gene and the S-genes of HBV to test sera with low HBV DNA levels. The detection limits of each of different HBV PCRs were assessed by testing serial 10-fold dilution of the WHO standard and another four specimens whose HBV DNA level was low, around 10^4/ml as previously determined using Amplicor kits (table 2-4). These specimens were tested using eight different HBV PCRs. At first, the concentrations of 10^4, 10^3 and 10^2 copies/ml were entered into the different PCR reactions. Some were nested as core C2, S-gene PCR S-2, RFLPs PCR and the multiplex nested genotyping assay. In the nested PCR, both first and second round products were examined by gel electrophoresis. The precore (PC) PCR showed a sensitivity of 1.9 x 102 IU/ml (equivalent to 1.0 x 10^3 copies/ml) (fig.2-2).

X gene/Extended core (EC) PCR showed less sensitivity as only 3 out of 4 specimens with 10^3 copies per ml were seen, two with faint bands. Nested core PCR (C2) detected also 3/4 specimens but their bands were more intense. The single round precore (PC) PCR was the most sensitive assay of the core gene, of equal sensitivity to the nested core PCR. The extended core PCR was less sensitive

For S-gene, short S (Mx) PCR showed 4/4 positive of 103 copies/ml (fig.2-3). Unlike RFLPs nested PCR which were much less sensitive and gave only 2/4 positives (fig.2-3). The nested S-gene PCR (S2) gave 4/4 positive of our panel at 10^3 copies/ml, thus its

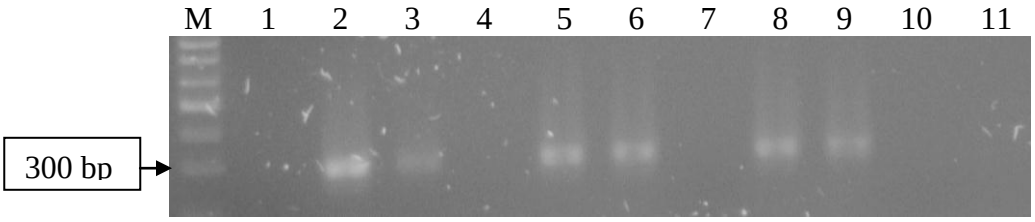


Figure 2-2: Precore (PC) PCR dilution

2% Agarose gel electrophoresis showing PCR products visualized under UV light; M: 100 bp DNA ladder, lane 1: Negative control. Lanes 2, 3 and 4:WHO standard; lane 2: 10^4 IU/ml, lane 3: 10^3 IU/ml, lane 4: 10^2 IU/ml, lanes 5, 6, and 7; Specimen N14, lane 5: 10^4 copies per ml, lane 6: 10^3 copies per ml, lane 7: 10^2 copies per ml, lanes 8, 9, and 10: specimen N17; lane 8: 10^4 copies per ml, lane 9: 10^3 copies per ml, lane 10: 10^2 copies per ml, lane 11: negative control.

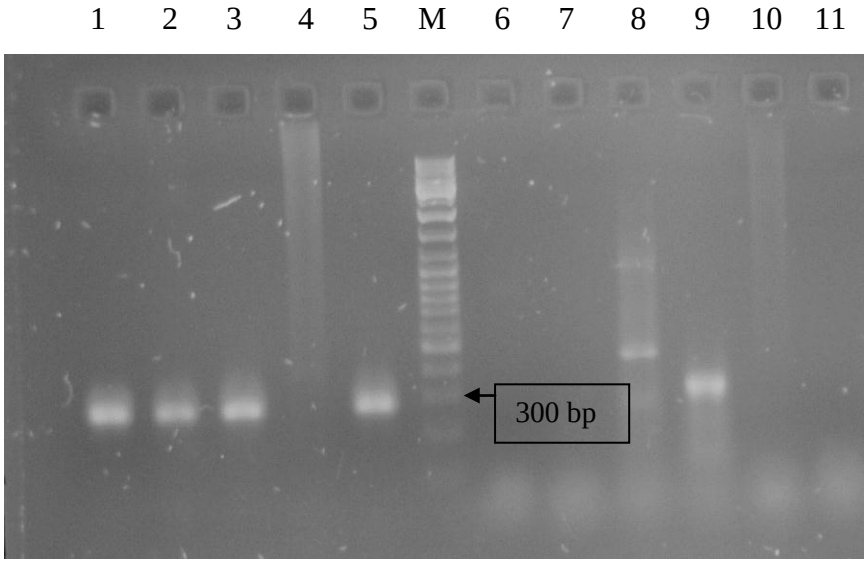


Figure 2-3: S-gene PCR, and RFLPs (R2) PCR

S-gene PCR:lanes 1-5; Lane 1; WHO standard 1:100 dilution (10^4 IU/ml), Lane 2; N14 1:10 (10^3 copies/ml), Lane 3; N17 1:10 (10^3 copies/ml), Lane 4; 1-50 1:100 (10^2 copies/ml) Lane 5; 1-56 1:10 (10^3 copies/ml), Lane 6; negative control. **Nested RFLPs (R2) PCR:** lanes 7-11; Lane 7; WHO standard 1:100 dilution (10^4 IU/ml), Lane 8; N14 1:10 (10^3 copies/ml) showing bands of both first and second round PCR, Lane 9; N17 1:10 (10^3 copies/ml), Lane10; 1-50 1:100 (10^2 copies/ml) Lane 11; 1-56 1:10 (10^3 copies/ml).

Table 2-4: Sensitivity and detection limit of different HBV PCRs in copies/ml.

The number of positive PCR is indicated in relation to the panel tested of dilutions of WHO standard and samples with known HBV DNA level by Cobas Amplicor quantitative PCR.

PCR	WHO standard			Sample N14			Sample N17			Sample 1-56		
	10^4	10^3	10^2	10^4	10^3	10^2	10^4	10^3	10^2	10^4	10^3	10^2
Core Gene: Pre-core (PC)	4/4	4/4	2/4	4/4	4/4	3/4	4/4	4/4	3/4	4/4	4/4	2/4
X gene/Extended core (EC)	4/4	2/4	0/4	4/4	2/4	0/4	4/4	2/4	0/4	4/4	2/4	0/4
Nested Core : first round C1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Nested core second round C2	4/4	2/4	0/4	4/4	3/4	0/4	4/4	2/4	0/4	4/4	2/4	0/4
S-gene: Short-S PCR	4/4	4/4	2/4	4/4	4/4	2/4	4/4	4/4	2/4	4/4	4/4	2/4
Nested S-PCR first round S1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	04	0/4
second round S2	4/4	2/4	0/4	4/4	2/4	0/4	4/4	3/4	0/4	4/4	2/4	0/4
Nested RFLP first round R1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
2nd round R2	1/4	1/4	0/4	¼	1/4	0/4	2/4	1/4	0/4	2/4	1/4	0/4
Genotyping first round P1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
2nd round multiplex	4/4	2/4	0/4	4/4	2/4	0/4	4/4	2/4	0/4	4/4	2/4	0/4

Table 2-5: Results of HBV PCR in different blood donors and patient groups

Groups		Hepatitis Markers	Number	HBV DNA positive (%)	P value
Blood Donors	Ia	HBsAg -negative anti-HBc +/ anti-HBs +	104	6 (5.8%)	0.004
	Ib	HBsAg -negative anti-HBc +/ anti-HBs -ve	95	22 (23.2%)	0.004
	II	HBsAg +	30	25 (83.3%)	
	III	HBsAg -negative anti-HBc -ve/ anti-HBs-ve	250	1 (0.4%)	0.001
Chronic Liver Disease Patients		HCV coinfected HBsAg -negative anti-HBc +/ HCV-Ab +	25	4 (16%)	
Liver Biopsy of Hepatocellular Carcinoma (HCC) patients		HBsAg -negative / anti-HBc +*	15	2 (13.3%)	0.15

***Only 4 had anti HBc-antibodies**

sensitivity is comparable to Mx PCR. The Mx PCR was the most sensitive PCR of the HBV S-gene. The RFLPs PCR showed the least detection level of 10^4 copies/ml. The multiplex genotyping PCR was performed on the same sera and results showed 4/4 positives.

2.4.1.3. Screening of Anti-HBc only Blood Donors for occult HBV infection

Sera from blood bank were screened by highly sensitive PCRs spanning two different genes of the HBV genome; precore PCR, which amplify a 268 bp region of the core gene, and S-gene (Mx) PCR that amplify a different region spanning the S-gene. Only twenty eight cases out of 199 (14.07%) blood donors showed positive HBV DNA. Most of them had a faint band of the expected size. This suggests a low viral load as expected. In addition, a third PCR; the extended core PCR with detection limit of more than 10^3 HBV DNA copies per ml, showed only one positive case (no.1-71), confirming the low HBV DNA of 10^3 or lower in most OBI cases. The real time quantitative PCR further confirmed that these cases have low HBV DNA level of = or < 10^3. The alanine aminotransferase (ALT) level in our cases with OBI was normal in most of blood donors with mean of 48 IU/ml. Abnormal ALT was seen in 16 cases from 52 to 128 IU/ml.

HBV DNA was detected in two out of 15 (13.3%) cases of HCC by precore PCR and S-gene (MX) PCR. The HBV genotypes of HBsAg blood donors were genotype A 6/25 (24%), C 1/25 (4%) and D 18/25 (72%).5 were ungenotypable.

2.5. Discussion

The choice of a highly sensitive, quantitative molecular test of HBV DNA at low viral loads is crucial for the diagnosis of OBI. The frequency and detection efficiency of OBI not only depends on the relative sensitivity of both HBsAg and HBV DNA assays, but also depends on the prevalence of HBV infection in the population (Allain; 2006). Hence, the type of assay used and its sensitivity must be specified (Said et al., 2009). As HBV DNA level in OBI is below 104 copies/ml or less (Valsamakis, 2007), the yield of PCR in OBI depends on the detection limit of the used test and thereby explains the variation in their detection rates. It also depends on the efficiency of DNA extraction, the amount of input DNA, the PCR primers, and assay conditions (Banerjee et al., 2007). In sensitive PCR assays, there is pronounced risk of false positive results from contamination or amplification of non-HBV DNA targets. We prefer single round PCR to decrease the possibility of contamination, though it has lower detection limit drawback of the test compared to nested PCRs.

Different regions of the HBV genome were amplified using two independent PCRs targeting the surface gene and the precore/core region of HBV. Amplifying different regions spanning the HBV genome serves as an independent double check of the HBV DNA positivity and to confirm the reliability of the results (Kew et al., 2008). That was recommended by other workers to confirm the results, and to rule out contamination by PCR products (Sagnelli et al., 2008). In our results the precore PCR was more sensitive than the S-gene PCRs except when the S-gene PCR amplifying a short segment of the S-gene was equally sensitive. Nevertheless, both tests must have comparable detection limits to enable proper evaluation. The detection limit was estimated at 200 genome copies/ml for both assays using samples with previously known HBV DNA levels. Our results were in agreement with other workers (Fabris et al., 2004) who found that PCR of the HBV core gene was more sensitive than the S-gene in serum and in the liver tissues. Possibly, due to better relaxing the double stranded HBV DNA at the core region near to the origin of replication. In contradiction, other groups experienced more sensitive PCRs of the S gene (Kew et al., 2008).

In our assay, the input 4µl template of the 100µl of DNA that was originally extracted from 200µl of serum. Therefore, when we can detect 1-2 copies of HBV per reaction,

amounting to about 125-250 copies of HBV DNA/ml of serum. This detection limit still needs to be improved to below 100 copies of HBV DNA/ml; to avoid the undesired risk of permitting infectious dose of 5x104 HBV copies per unit of 500 ml transfused blood. Some workers claim the detection of up to 10copies per ml of serum by real-time PCR (Allice et al., 2007), which is equivalent to 3500-5000 HBV copies in a unit of 500 ml transfused blood. In a study by Torbenson et al., (2004), semi-nested PCR (detection limit, 15 HBV copies per ml) detected HBV DNA in 81 of 180 (45%) of anti-HBc positive individuals using. The other implication of the very low viral load in OBI is the importance of validating a suitable molecular test to detect OBI in blood transfusion services, as HBV nucleic acid amplification technology (NAT). There is no standardized method to detect OBI, and assays vary in their sensitivities, ranging from 1-3 viral particles/ml of serum by nested PCR and 50 copies/ml in single round PCR coupled with hybridisation (De Maria et al., 2000) to 10 IU per ml in TaqMan assay (Allice et al., 2007). Whereas the specificity of the results is checked by sequencing of the amplicon, or hybridization with labelled DNA probes. Both rule out sample contamination, (Sagnelli et al., 2008).

In most Saudi cases, the HBV DNA was mostly at or below 103 copies/ml. At these levels, NAT is likely to be ineffective and its cost-effectiveness is questionable, particularly when used with plasma pools, and cause false sense of security by relying on a test of inadequate detection limit. HBV NAT needs either extreme sensitivity or use on individual donations to eliminate HBV DNA-containing units, although some groups recommended the exclusion of anti-HBc positive units (Alhababi et al., 2003). As in OBI, HBV DNA is usually below 104 and can be less than 351 IU/ml (Katsoulidou et al., 2009), making testing of plasma pools unsuitable unless the sensitivity of NAT significantly increases by genome enrichment or test improvement. In addition, such plasma pools must be screened first to exclude HBsAg positive units, because units with high HBV DNA levels can give misleading results. As HBV DNA is detected in up to 81.8% (18/22) of HBsAg positive blood donors, with a mean level of 3.67 (+/-1.77) log10 copies/ml (Hasegawa et al., 2006).

Generally, there is an increasing NAT positivity with increasing anti-HBc titres from 26 to 212 . As prevalence of anti-HBc decreases below the age of 40, an alarming increase

of HBV DNA occurs in this age group (75.8% vs.21.5%) (Yugi et al., 2006). . An increasing incidence of OBI is expected with the implementation of massive HBV vaccination (Chang; 2006). Thus, the need for highly sensitive DNA testing of donated blood will be increasing by time.

The main purpose of studying Saudi blood donor group is to check the prevalence of positive HBV DNA in the donated blood in an endemic area as Saudi Arabia. Our results showed the presence of HBV DNA in 14.1% of anti-HBc positive, HBsAg negative Saudi blood donors. Although HBVDNA ratio is not very high, it is still more than described from other parts of the world (Alhababi et al., 2003). This can be explained by the higher prevalence of HBV infections in Saudi Arabia, as it is an endemic area with an incidence of 1.9%, 4.1 % to 5.1% of HBsAg in the general population in Eastern, Central and Southern provinces, respectively (Panhotra et al., 2005) (Al Traif et al., 2004) (Ayoola et al., 2003). Clearly these rates are significantly lower than the HBsAg prevalence of 12% reported in 1985, and much lower HBsAg in vaccinated children 2 of 229 (0.9%), indicating a major decline even from the rate of 8.8% observed early in this decade (Ayoola et al., 2003).

Rates of isolated anti-HBc positive, anti-HBs negative in Saudi blood donors reached 853/26, 606 (3.2%) (Zekri et al., 2002). Taking into consideration that the prevalence of anti-HBc / anti-HBs positive 271/2687 (10.1%) to 194/1051 (18.46%) reported in Eastern and Western province, respectively (Panhotra et al., 2005) (Zekri et al., 2002). Donations from the first group are discarded according to blood bank regulations. While the anti-HBc /antiHBs positive group reaching 17.1% of total donations, may have positive HBVDNA in 5.8%. HBsAg-negative Anti-HBc prevalence varies in other neighboring countries ranging from 20.5% in Oman, (Kaminski et al., 2006), 17.28% in Pakistan (Bhatti et al., 2007), 10.96% in Egypt (El-Zayadi et al., 2008), and 7.1% in Lebanon (Ramia et al., 2008).

According to our results, the estimated prevalence of HBVDNA to 0.19 to 0.99% of total blood donations, which comprise a risk of HBV transmission from blood and consequently considered infectious. As HBV DNA was present in 5.8% of anti-HBc/antiHBs positive donors, donations carrying anti-HBc antibodies only and HBV

DNA can be infectious and this removes a threat where anti-HBc is not screened. Anti-HBc screening identifies most OBI but not all. Despite that serological loss of viral antigens and appearance of anti-HBs is generally believed to signify viral clearance. Our data agree with Fabris et al., 2004) that the seroconversion to anti-HBs is not always accompanied by the complete elimination of the virus. In another study of anti-HBc positive blood donors from Saudi Arabia, HBVDNA was detected in much lower rate at only 1.25% of sera (Zekri et al., 2002), possibly due to difference in detection limit of the tests used. Allain (2004) has pointed that carriers of anti-HBs or anti-HBc only were mainly infectious in immunosuppressed organ or bone marrow transplant recipients. In such cases, the risk of transmitting HBV justifies a specific prophylaxis in liver recipients. Up to 55% of recipients presented some degree of immunodeficiency including age. Under these circumstances, it is possible that the threshold of infectivity of antibody-complexed virions is significantly decreased (Allain 2006). In immunocompetent recipients, there is little evidence that anti-HBs-containing components are infectious.

The critical question is whether OBI is infectious by transfusion. While most infectious blood units are removed by screening for HBsAg, there is clear evidence that transmission by HBsAg-negative blood occurs, in part, during the serologically negative window period, but more so during the late stages of infection with isolated detection of anti-HBc, but positive for HBVDNA, with or without the presence of anti-HBs antibodies (Allain et al., 2004). However, HBV presents a much higher residual risk of transmission by transfusion than HCV or HIV in endemic areas (Velati et al., 2008).

Acute post transfusion HBV was not reported in our Saudi patients, although one case of the liver disease group has documented blood transfusion as the source of infection. The calculated window period reflects the incidence in the donor population (Kleinman and Busch; 2006).Our results agree with Yugi et al., (2006) that anti-HBc positive blood may still contain low level of HBVDNA. Therefore, the ideal strategy to prevent the residual risk of transfusion transmitted HBV infection relies mostly on serological screening of blood donors for anti-HBc by highly sensitive tests, and to eliminate all the positive donations. Preferably in conjunction with NAT to detect donors in the window period of

HBV infection. Despite the significant decline in anti-HBc positivity over the last decade, isolated anti-HBc positive leads to high rejection rate of collected blood units without completely covering the residual risk of HBV transmission by transfusion (Panhotra et al., 2005).

We found one case with positive HBV DNA despite completely negative HBV serology. This can be explained by either early infection or OBI following an old infection with negative serology. Low level of HBV DNA remains detectable in serum (Arase et al., 2007) and liver tissue in some patients who cleared HBsAg from either acute self-limited or chronic HBV infection or even after a successful anti-HBV therapy (Said et al., 2009). Rare cases of HBVDNA without any serological HBV marker has been reported, and can be associated with infectivity as the virus persists in the liver and is accompanied by abnormal liver histology for a decade after complete clinical recovery (Yuki et al., 2003).

The HBV genotypes of HBsAg blood donors were genotype A 6/25 (24%), C 1/25 (4%) and D 18/25 (72%). 5 were ungenotypable. HBV genotype D was common in Saudi Arabia (Al Traif et al., 2004) (Abdo et al., 2006). The predominant anti-HBe serology needs to be considered. The other cases were ungenotypable because of their low viral load characteristic of most OBI infections. The genotype of occult infection reflects the geographical distribution of HBV genotypes. In a study by (Alhababi et al., 2003), HBV DNA of genotypes A, E and G was detected in 6/151 (4%), all of which had HBV DNA<400 copies per ml.

The alanine aminotransferase (ALT) level in our cases with OBI was mostly normal. Blendis et al., (2003), found no association between serum aspartate aminotransferase (AST), activity grade, or stage of liver disease and the presence of OBI. Serum ALT levels were slightly higher in patients without OBI (46 vs. 35 IU/L).

In Saudi Arabia, an intermediate HBV endemic country, some patients may have persistent viral infection after the loss of HBsAg (Al Traif et al., 2004). OBI with positive HBV DNA was detected in 3 of 25 (12%) liver disease patients with anti-HBc only (Helmy and Al-Sebayel; 2006). OBI cannot be explained totally by mutations in the

HBV "a" determinant. However, it is appropriate to monitor the emergence of HBsAg negative mutants in vaccinated individuals (Chang; 2006).

Although OBI cases with HBV DNA in serum in the absence of HBsAg is common in chronic hepatitis C, the prevalence and the impact of OBI in patients with chronic hepatitis C infection is still a matter of some controversy. In Saudi patients with chronic HCV infection, anti-HBc alone was frequently reported, and is significantly more common in those with CAH than those with cirrhosis at 71 (64.5%) and 14 (23.7%) respectively (Helmy and Al-Sebayel; 2006). A proper follow up of HCV patients is needed to evaluate the effect of OBI on the progress of their disease and the outcome of therapy in different HBV genotypes.

Our results showed the presence of HBV DNA in only 4/25 (16 %) of HCV patients, who have both anti-HCV antibodies and anti-HBc antibodies. Our low level of HBV DNA in patients coinfected with HCV is consistent with the results of Kao et al., (2002) who found 31 of the 210 patients (14.8%) had HBV DNA in their sera, as did 15 of the 100 HBsAg-negative healthy controls (15%). In contrast, our rates of OBI in HCV patients are much higher than that found by Fabris et al., (2004), where he detected HBV DNA in only one of 51 (1.9%) serum samples. Higher prevalences of OBI in Greek HCV patients were reported by Georgiadou et al., (2004), with HBV DNA reaching 26.2% of 187 HCV-infected patients. HBV DNA was not associated with HBV markers, nor with the clinical status of HCV and non-HCV patients. This rate of coinfection suggests a common source of infection in a high-risk group as IV drug users. Another factor affecting the prevalence of OBI is significant increase in those who are HCV-RNA positive rather than negative (Said et al., 2009). Despite a reciprocal inhibition between both viruses leading to fluctuating HBV viraemia, HBV and HCV frequently coexist in liver tissue (Coppola et al., 2008).

HBV DNA was detected in two out of 15 (13%) Saudi cases of HCC, lower than 15/51 (29.4%) of liver tissue specimens from chronic HCV cases reported by Fabris et al., (2004), possibly integrated or have low level replication. The level of HBV DNA may not be sufficient to cause progressive liver disease (Hui et al., 2006). Ayoola and Gadour (2004) reported that the prevalence of HBsAg in 118 HCC Saudi patients (67%) was significantly higher than the rate (6.7%) in the controls. Dual HBV and HCV infections

occurred in only 3.4% and were associated with only a moderate increase in the risk of HCC. In 24.6% of the cases no virus was identified as the etiologic factor. Suggesting that HBV constitutes a major risk factor and HCV contributes a less significant role in the development of HCC in Saudi patients. A tight association of HBV and HCC was demonstrated with or without HCV (Geo et al., 2005, Ohaba et al., 2004).

2.6. Conclusion:

Anti-HBc/anti-HBs positive blood is of questionable safety in HBV endemic areas. Policy for checking the collected blood unit by 3 tests for anti-HBc, anti-HBs and HBsAg should be re-evaluated, in favour of HBV DNA testing by PCR, to possibly achieve the zero risk goal of transfusion transmitted HBV infection.

2.7. Future Direction:

We need to investigate the prevalence of OBI in UK blood donors with positive anti - HBc antibodies, and in HCV patients in UK and its association of with pathological or clinical findings. To investigate OBI in HIV coinfected Saudi individuals. And to test for HBV transcripts integration by covalently closed circular HBV (cccDNA) genomes.

Chapter Three: Real-Time Quantitative PCR of HBV Genotypes

3.1 Introduction

The laboratory diagnosis of hepatitis B virus (HBV) infection is based traditionally on serological assays. Yet the detection and quantitation of viral DNA is necessary when addressing directly the question of infectivity. Serial measurements of HBV DNA are required to assess non-responsiveness to antiviral drugs (Kennedy et al., 2008). Viral breakthrough occurs with inadequate dosage or noncompliance to therapy and in resistant mutants. Adefovir add-on therapy used for lamivudine resistant mutants, treatment algorithms are likely to consist of entecavir and tenofovir (Kennedy et al., 2008). Real-time assays are used for large-scale nucleic acid testing (NAT) for validation of blood products (Yugi et al., 2006). Multiplex real-time quantitative PCR assays were developed for simultaneous detection, genotyping and quantification of HBV (Liu et al., 2006). Quantitation of cccDNA evaluates replication and pathogenesis of the virus (Takkenberg et al., 2009).

Different methods are used to quantify HBV DNA e.g. competitive PCR (Payan et al., 1997), hybridization with labelled probes in the Amplicor monitor (Roche)(Yuan et al., 2004), branched-chain DNA signal amplification assay (bDNA) (Dai et al., 2004), and transcription mediated amplification (TMA). Standard hybridization assays allow for exact quantitation, but their sensitivity is limited from 103 to106 viral genomes per ml of serum. Real-time assays include minor groove binders and fluorescent energy transfer probes (FRET) for single nucleotide polymorphism (SNP) detection (Aliyu et al., 2004), intercalating dyes as SYBR green and the dual labelled TaqManTM probes which combines the sensitivity of PCR amplification and the quantitation of hybridization tests (table 3-1) (Allice et al., 2007). Fluorescent labelled primers as Scorpion primersTM, or molecular beacons, which are single-stranded, highly specific fluorophore-labeled nucleic acid probes that generate a fluorescent signal only in the presence of target, allowed quantitative real-time multiplex detection of PCR products (Gunson et al., 2006).

Quantitative methods based on real-time continuous monitoring of the exponential phase of PCR amplification are rapid, more accurate with greater dynamic range than those based on end determination. The end assays are influenced by limiting the concentration

of reagents, small differences in reaction components, or cycling conditions. In addition, they are unable to quantify the whole dynamic range over which HBV DNA should be measured, requiring serum dilutions for estimation of high HBV DNA levels (Gunson et al., 2006).

Most standardized technologies show divergent results with high inter-assay variations. This necessitates serial measurements using the same assay to achieve proper interpretation of results (Gunson et al., 2006). High specificity depends on selection of primers, probes and assay conditions. There are different detection limits of different quantitative techniques. The enhanced sensitivity and specificity are valuable for both diagnostic and epidemiological work. Different strategies have been attempted to optimise QPCR for detection of low copy number target DNA and to decrease primer dimer formation e.g. bovine serum albumin, HDL, RNase, tRNA, anti-Tag antibody and glycerol (Teo et al., 2002).

3.2. Hypothesis

We need quantitative HBV assay to give an insight about HBV infections and the viral activity. HBV genotypes may have different replication efficiencies and consequently can affect disease progression. The relation of the HBV DNA quantity to the genotype present can show this.

3.3. Aim

The aims of this study are:

To establish the reliability of SYBR Green I real-time PCR method in comparison to Amplicor kits.

To compare the performance of different HBV PCRs with the quantity of HBV DNA present.

To measure the amount of HBV DNA in Saudi and UK specimens.

To assess the relation of HBV DNA level to the HBeAg, and liver disease status.

To explore the natural history of infection with different HBV genotypes, and which genotype is associated with high viral replication.

Table 3-1: Comparison of quantitative molecular techniques for DNA and RNA viruses (Valsamakis 2007)

Features	Limiting dilution PCR	Branched DNA (bDNA)	NASBA /TMA	Co-amplification of reference template PCR	Competitive PCR QcPCR	Real-time Detection			
						Fluorogenic Dye:SYBR Green I	TaqMan (5`nuclease assay	Molecular beacons	Labelled primers Scorpions Sunrise
Detection method	Serial dilutions PCR in duplicates	Chemilumine-scence Signal Amplification	Electrochemilumines cence for RNA viruses, IS	Gel electrophoresis or colorimetric 1 IS single copy cellular gene or transcript	Colorimetric Ratio of product to competitor IS	Monitoring Fluorescence upon binding to dsDNA Use on large regions	Fluorescent generated on degradation of probes	Hairpin-shaped oligos fluoresce on binding to target	Stem loop tail with integral tail
Dynamic range (copies/ml)	100-100, 000	500-10^6 (version2.0). 50-50, 000000 (version 3.0)	80-10^7		400-750, 000 (standard) 50-50, 000 ultrasensitive)	100-10^{10}	10-10^{10}		
Intra-assay SD (log_{10} copies/ml)	High	0.12-0.32	0.13-0.23	variables affect both templates	0.15-0.33				
Contamination	Intermediate	Low	Low	Low	low	Low	low	low	low
Specificity		High			High	Intermediate	Highest	Highest	
Comment	Impractical, low precision. large no. of PCR /sample	Low sensitivity			Internal standard have similar primer binding, size, and base composition	Primer-dimer Variable melting peaks High sensitivity	Less false negatives in probe mismatch High sensitivity	False negatives in probe mismatch	Primer-dimer formation Strict design criteria

NASBA: Nucleic acid based amplification. TMA: Transcription mediated amplification, IS: Internal standard.

No. : Number.

3.4. Principle of Real-time QPCR Using SYBR Green

In real-time quantitative PCR, amplification of DNA with continuous detection of the product is achieved by monitoring the fluorescence emitted from SYBR Green ITM, which is activated by binding to the minor groove in double stranded DNA. Fluorescence ceases when the dsDNA dissociates and thereby depends on the melting temperature (Tm) of the target product; this is measured in the dissociation curves. The initial amount of HBV DNA can be measured during real-time PCR based on the cycle threshold (Ct), which is inversely proportional to the log of the initial copy number. Ct is defined as the cycle at which fluorescence is determined to be statistically significant above the background. Simultaneous monitoring of significant photon emission of both fluorophores SYBR Green I dye (FAM) and the control dye (ROX) is then correlated with the input copy number (-dF/dT versus T) (Wilkening et al., 2004).

3.5. Materials and Methods

3.5.1 Patients and Blood Donors:

Quantitative PCR was done on sera from 85 UK patients, 127 Saudi patients. Patients were not vaccinated for HBV. Liver function tests as ALT levels and serological hepatitis B markers tests were done by Murex ELISA kits. Liver ultrasonography and liver biopsy. Liver disease include Inactive hepatitis B, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).

3.5.1.1. UK Patients Information

The UK patients were mostly Caucasians, one Live in Taiwan. Other nationalities were 7 Chinese, 3 Pakistani, one of Bangladesh, Philippines, Vietnamese, Thai, Nigeria and Bulgaria. 19 patients were from the Virus reference laboratory, Colindale (London). The mean age of UK was 37 years. History of tattoo, ear and body piercing. One nurse. There were two cases presenting with acute hepatitis, one case had HCC with elevated AFP492 and HDV1991. Inactive hepatitis and liver disease in 2 and 21 UK patients. All have HBsAg. Liver biopsy showed 4 minimal activity, 5 mild portal fibrosis, 3 Bridging fibrosis, 2 Fibrosis score 3, Steato hepatitis

3.5.1.2. Saudi Patients Information

Saudi patients were attending gastroenterology, renal and surgical clinics and 10 Saudi blood donors. The mean age of was 43 years. HBsAg was positive; their clinical data include liver disease and other clinical diagnoses. ALT levels and serological tests as HBeAg and anti-HBe were done on most of those patients. HBsAg positive blood donors, asymptomatic carrier, inactive hepatitis and liver disease in 13 and 43 Saudi patients, respectively. Acute hepatitis, 3 HBeAg conversion, chronic carrier. 4 ENT patients; 2 Chronic sinusitis, otitis media, mastoiditis, one had history of blood transfusion. 3 rheumatic heard disease, 2 congestive heart failure, 2 cerebrovascular accidents, 1 SLE, 3 tuberculosis-one died. 4 Dental, 4 diabetics. 3 chronic renal failure patients, two kidney transplant, one nephrotic syndrome, one renal stone.

4 Orthopedic patients: 2 fracture, Head injury, laminectomy. General surgical patients; appendicitis, gastritis, cholecystectomy, one Gilbert`s disease.

Piles, perianal abcess, 2 addicts, 1 septicaemia, one VDRL positive, 2 detected in screening for marriage, pregnancy. Diabetes mellitus, breast carcinoma, carcinoma of glottis, pancreatic carcinoma, obstructive jaundice, lymphoma-diffuse B-cell. One had sickle cell disease, whose father died with HBV. 2 hepatitis D virus (HDV)

Upper gastrointestinal bleeding, 2 jaundice, Helicobacter pylori infection.

Liver biopsy showed CHILD classification 8 chronic hepatitis; Albumin 2.8. Fibrosis in 2/4 chronic hepatitis 2/4, chronic hepatitis grade1, chronic hepatitis 2/4 stage 0/4, 2/4 stage 0/4, stage 3 grade4, Grade4 and CAH-piecemeal fibrosis. 4 patients were treated by lamivudine, 2 by adefovir.

Every serum was aliquoted then stored at -80 oC. All sera were positive for HBsAg. DNA was extracted from 200µl serum by QIA amp kits (Qiagen) and their HBV DNA levels were estimated by two different assays using an MX 4000 machine (Stratagene). HBV multiplex genotyping PCR was done on 90 Saudi and 82 UK patients that were tested by quantitative PCR assays.

3.5.2. Preparation of HBV Plasmid Standards

HBV standards were prepared from plasmid encoded HBV S-gene. PCR products using the above primers were cloned in TOP10 Escherichia coli using TOPO cloning Kit (Invitrogen, Leek, Netherland). The plasmid`s DNA concentration was determined by fluorimetric method, then the copy number of the plasmid was calculated according to the standard equation.

Mass of 1 plasmid in ng = (vector size bp + insert size bp) x 662 x1.6605655x10-15ng (Avogadro`s constant).

Plasmid copies per µl= concentration of plasmid in ng /µl

mass of 1 plasmid in ng

3.5.3. Real-time PCR

Amplification of the S-gene was done, the PCR product size is 281 bp. Each real-time PCR reaction mixture contained 12.5 µl of the Brilliant® Strategene® SYBR Green QPCR Master Mix (no.600548), 0.75 µl of each of the forward primer (HBVfr) and reverse primer (HBVrv)(5pmol/µl) (MWG-Biotech) (Brechtbuehl et al., 2001) (table 2-3). 0.325 µl of a control dye (ROX) (1:500 dilution), 9.635 water and 1µl DNA template, were added to final volume of 25 µl.

The control dye (ROX) was added to each PCR reaction tube as a reference to calibrate the detection of fluorescence by the machine. ROX has excitation and emission maxima of 584 nm and 612 nm respectively. SYBR Green I dye (FAM) has different excitation and emission maxima of 497 nm and 520 nm, respectively. Serial dilutions of cloned plasmid encoded HBV S-gene segment (see fig. 3-4). Standards were added to each run in duplicates.

Our assay was evaluated using a 10-fold dilution series of a quantified WHO standard HBV DNA from the National Institute for Biological Standards and Controls (NIBSC, Hertfordschire, UK. code 98/780) and the sensitivity compared with Amplicor Monitor kits. Several negative controls were included to check the background fluorescence, which may be present in the reagents.

3.5.4. Real-time PCR Thermal Cycling Conditions

The real-time machine; Stratagene Mx 4000 machine (Stratagene, La Jolla, CA) was calibrated initially to the following PCR conditions; 95oC for 15 minutes (Hot start), then 40 cycles of denaturation at 94oC for 30 seconds, annealing at 55oC for 30 seconds, and extension at 72oC for 30 seconds, and a final ascent from 55oC to 95oC at 30 seconds per degree (dissociation curve), followed by a cold soak at 4oC.

3.5.5. Optimized real-time PCR conditions

Later the real-time PCR conditions were modified to increase the sensitivity of the test by increasing the standards and template DNA to 2µl, and increasing the annealing time to 1:30 minutes and the extension time to 2 minutes.

3.5.6. The HBV MONITOR assay

The HBV MONITOR assay (Roche) has a quantitative dynamic range from 200 to 2 x 10^5 copies/ml. A 104- bp fragment of the precore and core gene is amplified together with an internal standards (IS) DNA. Followed by hybridization to enzyme labelled probes. The signal was detected by ELISA. The amount of HBV DNA is calculated from the ratio of the HBV-specific well to the IS-specific well according to a standard curve.

3.6. Quantitative real-time PCR Results

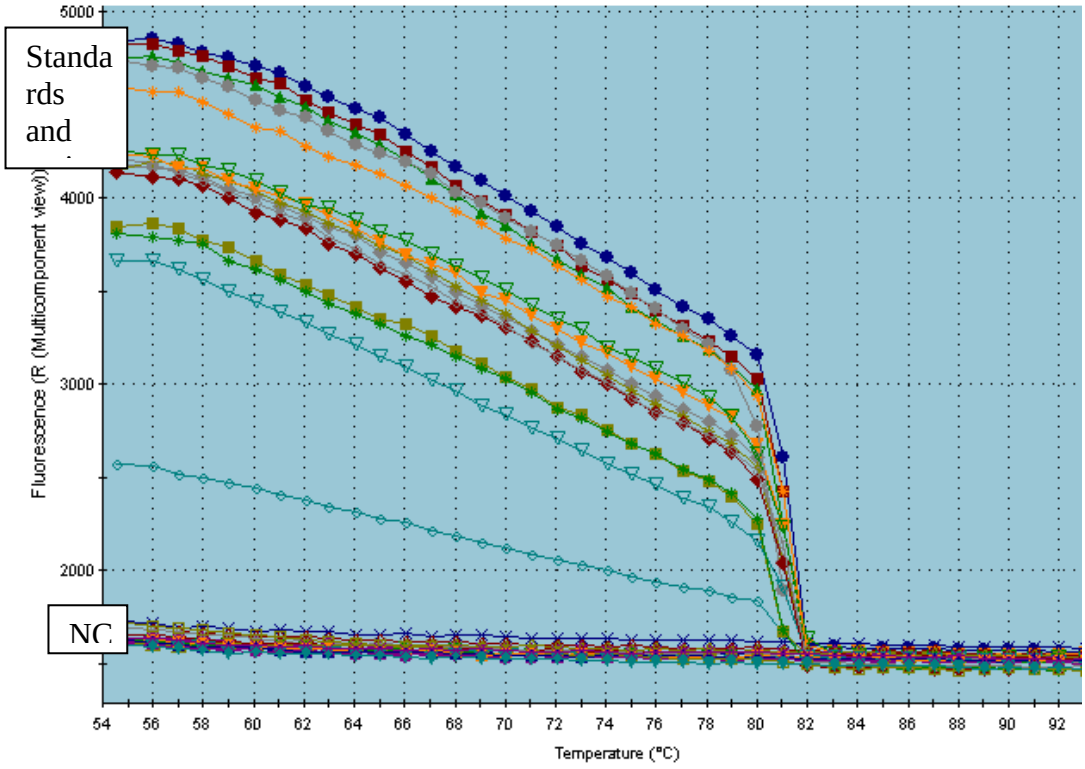
3.6.1. In-house quantitative Real-time PCR Results

Real-time QPCR was done the HBV S-gene. Verification of the size of the PCR product was achieved by gel electrophoresis. Each of the plasmid standards amplified as expected. Several negative control sera and two water blanks were included in each run, to measure the background fluorescence above which threshold is calculated. Background fluorescence varies from -100 to +100 R (T), but it is negligible as fluorescence of true positives reached 950 to 1200 R (T). Non-template negative controls did not produce fluorescence that breaks the threshold.

The amplification curve measures a different parameter of fluorescence, the corrected normalized fluorescence (dRn) against the cycles of real-time PCR (Graph.3-2). The threshold fluorescence cut-off is above the base-line fluorescence, the latter was calculated from background fluorescence emitted from the negative specimens. Cycle threshold (Ct) of standards and specimens is the cycle number at which the fluorescence exceeded the fluorescence threshold. **Analytical specificity** was determined by the inclusion of several negative controls. The overall specificity was 99% .

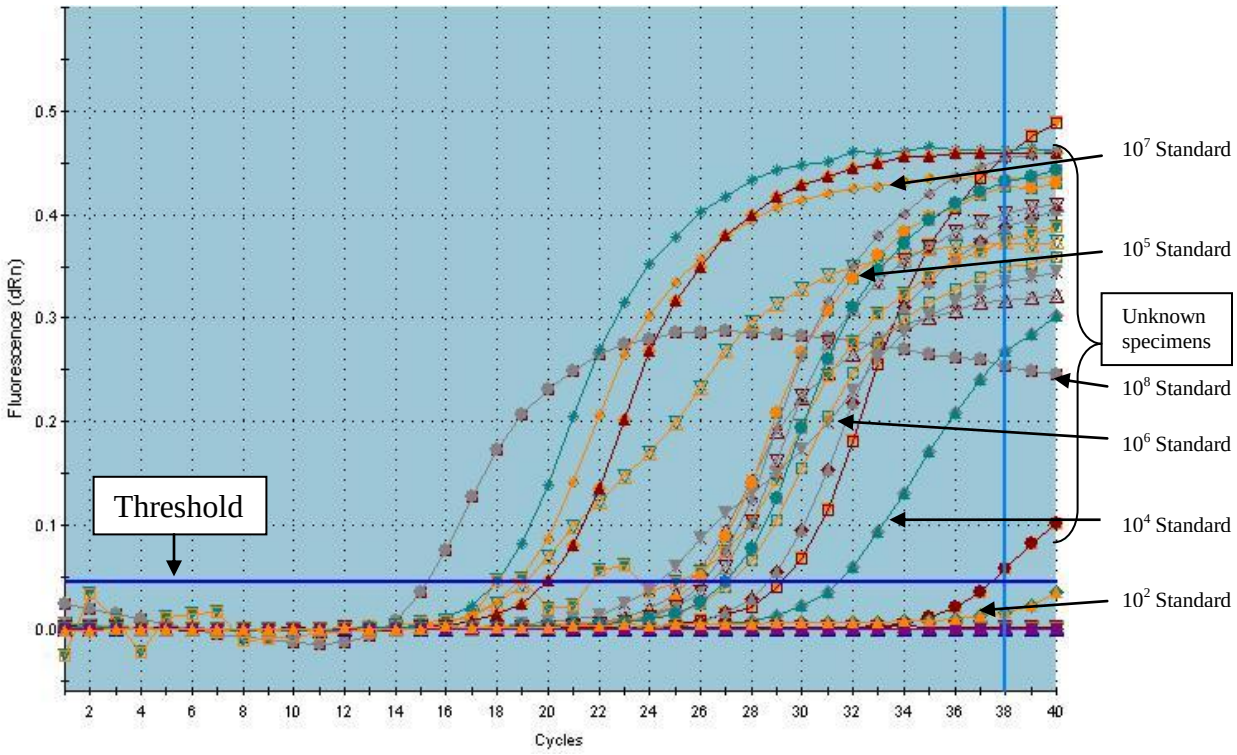
The SYBR green I dissociation curve on raw fluorescence reading data (Graph 3-1), and on processed data (Graph 3-3) plotted against the temperature showed clear peaks at 80.5-81.7^{o}C. The peak correlates with melting temperature (Tm). Single peak signifies the presence of a single type of PCR product. Primer dimers are not present in this graph, they usually generate low level fluorescence represented by smaller peaks, but their melting temperature is below 75^{o}C.

A standard graph was established, in which the cycle threshold (Ct) of serial dilutions of known quantity of cloned HBV S-gene was plotted against the concentration of the virus in each dilution (Graph 3-4). Duplicates of each concentration of the following: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 HBV S gene plasmid copies per ml were tested. The line of `best fit` was drawn according to these measurements to correlate the HBV plasmid concentration with the cycle thresholds (Ct). All the negative controls (NC) showed complete absence of fluorescence thereby confirming the absence of contamination in this PCR reaction. Quantitative range of SYBR green method as defined by the lowest and highest concentrations of HBV DNA was detectable with acceptable accuracy and precision.



Graph 3-1: The dissociation curves of plasmid-cloned HBV S-gene DNA standards and DNA extracted from patients sera.

A separate line on the dissociation curve denotes the fluorescence generated from each PCR reaction, fluorescence is monitored against different temperatures. Y-axis: Fluorescence; where R is the fluorescence reading against the temperature variation within the reaction. Background fluorescence is constant in the negative controls (NC); this baseline is also reached by the dissociated double stranded (ds) DNA of the amplicons into single stranded DNA in the denaturation step of the PCR after heating over 82^{o}C.



Graph 3-2**: A graph to show amplification curves of MX4000 real-time quantitative PCR using plasmid-cloned HBV DNA standards and DNA extracted from patient`s sera.**

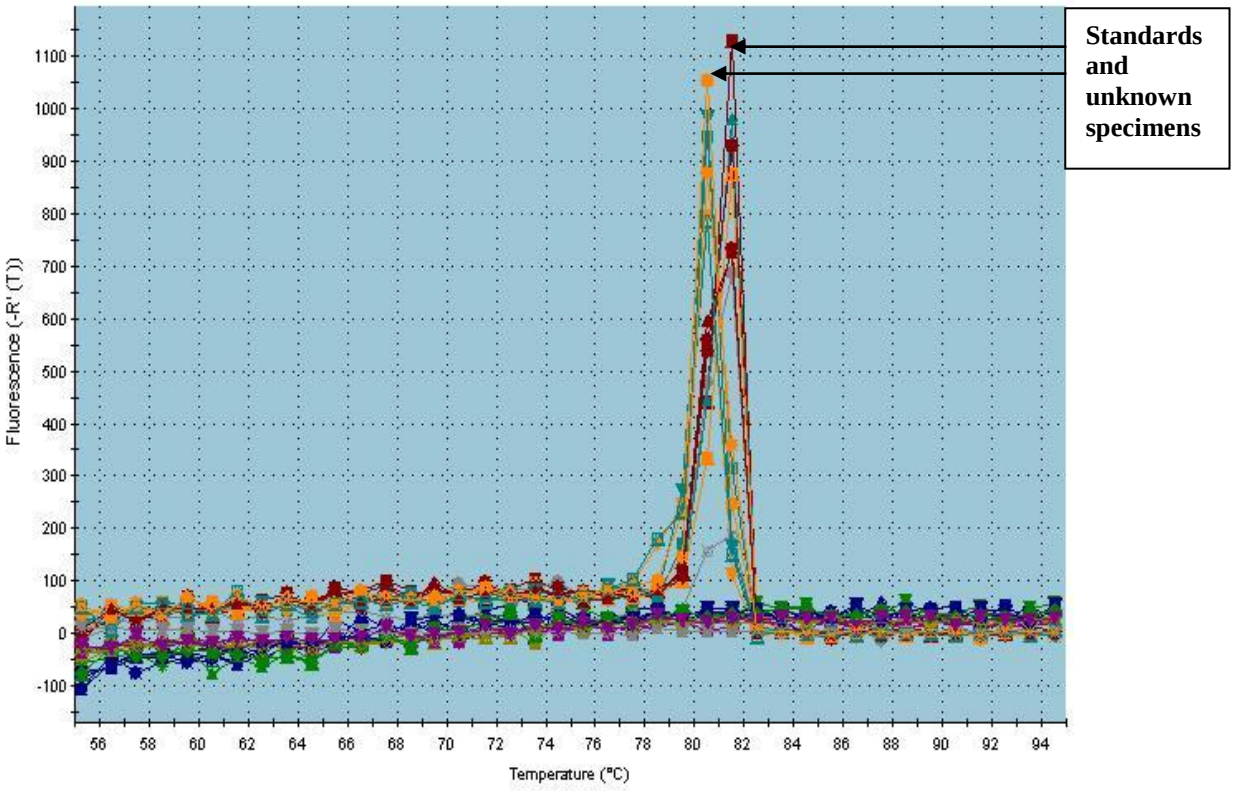
The x-axis denotes the number of the PCR cycles at which the fluorescence was detected plotted against baseline corrected normalized fluorescence (dRn) in they-axis. Fluorescence from each PCR reaction is represented by a separate line where amplified amplicon from either a plasmid-cloned HBV DNA standard (STD), a clinical specimen, or a negative template control (NTC). The threshold is the line above the negative reactions` base-line fluorescence, above which the reaction is considered positive. The cycle threshold (Ct) is the cycle at which fluorescence is determined to be statistically significant above the background.

Some specimens showed a steep rise in fluorescence starting at 78^{o}C, indicating gradual build up of dsDNA with the increase in temperature due to primer annealing and formation of the new DNA amplicons. No smaller peaks were noticed at lower temperatures, denoting the absence of primer dimers that can give low-grade fluorescence by their binding to SYBR green I in some occasions.

Analytical accuracy (linearity) which is the expected values versus observed $\log_{10}$ copies/ml mean quantitative values for each QC/standard panel were observed. The linear regression plot had a slope of Y= -3.589 log (X) +44.64 with a correlation coefficient (R2) of 0.99. Thus, good linearity was observed throughout the quantitative range of 2 to 8 $\log_{10}$ copies/ml. The quantitative efficiency was near 100% which further validate the results.

The HBV DNA was measured for each specimen in duplicates, and the mean was calculated and compared to the results generated using the light cycler. The World Health Organization (WHO) standard with HBV concentration of 1000, 000 IU (equivalent to 5.2 x 10^5 copies/ml) per ml gave a mean concentration of $7.07x10^5$ copies/ml. The sensitivity of the assay was assessed by comparing the observed and the expected Ct of plasmid standards. The mode of standard deviation of the cycle thresholds (Ct) of plasmid-cloned standards and specimens was 2.73. The standard deviation of cycle threshold (Ct) of specimens showed a small range between 0.06 and 0.68. Therefore, analytical precision (reproducibility) was high as measured by low % CV within run and between runs.

The validity and accuracy of this test in the lower ranges of HBV DNA was verified by testing serial dilutions of both the WHO standard, and dilutions of samples containing



Graph 3-3: The dissociation curves of plasmid-cloned HBV DNA standards dilutions and DNA extracted from patient's sera. A separate line on the dissociation curve denotes the fluorescence generated from each PCR reaction, where detected fluorescence is monitored against different temperatures. Y-axis: Fluorescence; where –Rn` (T) is the first derivative of the normalized fluorescence reading multiplied by –1. Background –Rn` (T) (-100 to+100) is constant in the negative controls (NC), this baseline is also reached by the dissociated dsDNA of the amplicons after heating over 82^{o}C. Those thermal denaturation profiles which showed peak fluorescence at 80.5 - 81.7^{o}C (one arrow for each temperature) due to melting of the target PCR products, belonging to two different genotypes.

High HBV DNAs. As well as repeated testing of samples with low concentrations of HBV DNA, using the optimized method showed better low detection limit. Accordingly, we doubled the reaction volume to 2µl, the PCR conditions were also modified (see methods). HBV DNA was measured by two methods, Amplicor and SYBR green QPCR method.

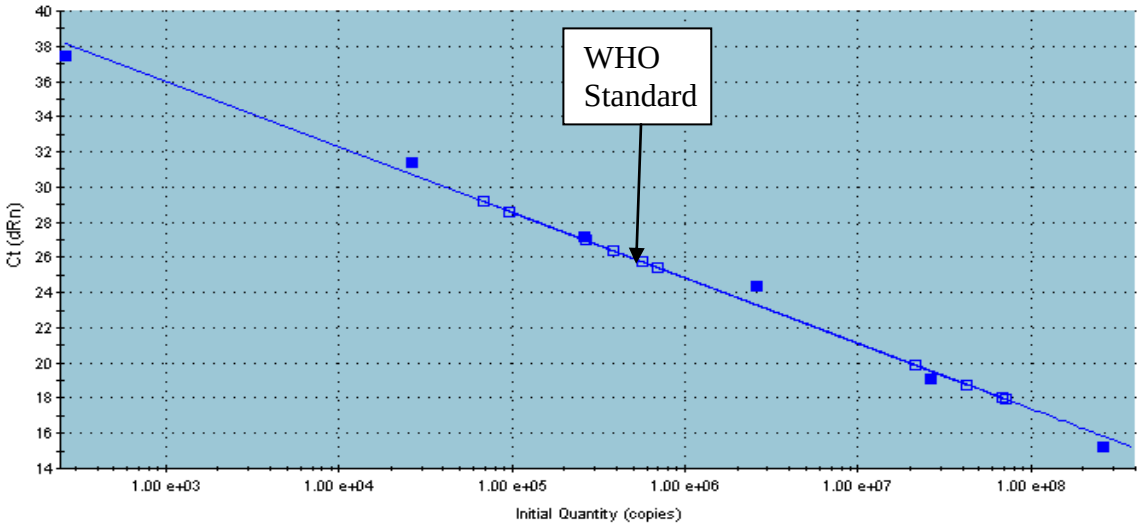
Comparison between HBV DNA quantitative PCR results done by both Amplicor and SYBR green QPCR methods.

The mean measured by SYBR green method which was slightly lower than by the Amplicor at 4.58 and 5.18 $\log_{10}$ copies/ml, respectively. SYBR green method showed less variation as measured by the standard deviation and the standard error of the mean (table 3-2).

Comparative analysis of the quantitative values of plasmid standards and sera from SYBR green versus Amplicor results. The difference in HBV DNA measurement between the two methods was 0.595 with a 95% CI of 0.10-1.094 (table 3-2). The slope was slightly different from a value of 1 indicating a less linear relationship between the two assays.

After the observation of many negative results in sera with low HBV DNA by SYBR green methods, we introduced modification (see methods). Optimization showed significantly better correlation with Amplicor at low HBV DNA levels (table 3-4), and even better detected the
WHO standard diluted at 10^4 IU per ml (equivalent to 1.9×10^3 copies per ml) as 1.53×10^3 compared to the manufacturer`s value. The Amplicor test showed less concordance at higher HBV DNA levels of plasmid standards than the optimized method. Analytical sensitivity, which is number of replicate samples with HBV DNA quantitative values above the detection cut-off, was determined.

Mean HBV DNA level was significantly higher in UK than Saudi patients at 5.09 (95% CI 1.43-8.75) and 4.52 (95% CI 0.14-8.90) $\log_{10}$ copies/ml, respectively (p: 0.007) (table



Graph 3-4: Standard curve graph in copies per ml, which is acceptable. Plot of MX4000 real-time quantitative PCR using plasmid-cloned HBV DNA standards and 10clinical specimens.

Quantitative PCR standard curve is a plot of the initial template HBV DNA concentration added to standard wells in copies per ml on the x-axis versus the cycle threshold (Ct) at the end of each cycle on they-axis. Each reaction is indicated by a square on the graph; the WHO standard (10^6IU/ml) is also shown. The full squares indicate the plasmid-encoded standards using the SYBR Green I (FAM) fluorescent dye. The empty squares indicate unknown clinical specimens where their HBV DNA levels are extrapolated from the standard curve plot according to their Ct values. dRn is the baseline corrected normalized fluorescence. The slope of the curve was calculated as: Y= -3.589 log (X) +44.64

Table 3-2: Paired differences of HBV DNA results done by comparing both Amplicor and SYBR green quantitative PCR methods t: 2.451, p: 0.021

Method	SYBR Green QPCR assay	Cobas Amplicor Kits	Mean difference between the two methods
Mean HBV DNA level (log_{10} copies/ml)	4.58	5.18	0.6
Standard deviation (SD)	2.15	2.79	0.64 1.26
Standard Error of the Mean	0.41	0.54	0.13 0.243
95% confidence interval of the difference			0.10-1.094

3-3). Mean HBV DNA in patients with HBeAg was 7.18 and 6.44 $\log_{10}$ copies/ml, while anti-HBe positive patients had 3.84 and 4.11 $\log_{10}$ copies/ml in UK and Saudi patients, respectively. That was 2.4 to 3.3 $\log_{10}$ less than the HBeAg positive group (table 3-4). Saudi HBeAg-negative patients had higher HBV DNA than UK patients.

3.6.2. Clinical presentation of HBV

Inactive hepatitis in Saudi patients was associated with low HBV DNA (table 3-5), have very low or undetectable HBV DNA $=<10^3$ copies/ml in their sera. On the other hand, active liver disease was associated with low, intermediate and high HBV DNA levels. Within the liver disease group, 53/92 (57.6%) Saudi and 42/66 (63.6%) UK patients have high HBV DNA of >5 $\log_{10,}$ whereas a much smaller proportion show moderate or low HBV DNA levels.

The correlations of HBV DNA levels with liver disease as acute hepatitis, chronic hepatitis, and cirrhosis and hepatocellular carcinoma in UK and Saudi patients are shown in table 3-4.

Table 3-3: Concordance between HBV DNA quantitative PCR by Amplicor and our in-house real-time assay before and after optimization (p0.014)

Cobas Amplicor results	In-house Q-PCR	Optimized in-house Q-PCR
<=3 log_{10} copies/ml (concordance within group)	5/0	4/6
3-<5 log_{10} copies/ml (concordance within group)	0/5	6/7
=>5 log_{10} copies/ml (concordance within group)	9/11 (81.8%)	3/6
Total concordance	9/16 (56.3%)	13/19 (68.5%)

Table 3-4: Mean HBV DNA in UK and Saudi patients and correlation between HBV DNA and serum HBeAg status, and the clinical presentation of age matched patients.

Clinical Manifestations	No. of Saudi Patients in log_{10} copies/ml				Mean HBV DNA (no. of patients)	No. of UK Patients In log_{10} copies/ml				Mean HBV DNA (no. of patients)
	<=3	>3-<5	=>5	Total		<=3	>3-<5	=>5	Total	
HBeAg	0	4	17	21	6.44 (21)	0	1	30	31	7.18 (31)
Anti-HBe	32	32	42	106	4.11 (106)	13	29	12	54	3.84 (54)
Mean (no. of patients) 95% CI					4.52 (127) 0.14-8.90					5.09 (85) 1.43-8.75
Inactive Hepatitis	15	12	-		2.8 (27)	7	11	-		2.43 (18)
Liver Disease	16	23	53		4.9 (92)	6	19	42		5.8 (66)
-Acute hepatitis	-	1	7		6.9 (8)	-	1	2		6.4 (3)
-Chronic hepatitis	11	17	40		4.9 (68)	4	15	34		6 (52)
-cirrhosis	5	4	2		4 (13)	1	3	6		5.3 (10)
-HCC		1	4		5.8 (5)	1	-	-		2 (1)
Total	31	35	53			13	30	42	85	

3.7. Discussion

Real-time PCR is increasingly used as a molecular diagnostic procedure within clinical laboratories, not only because it better evaluates viral activity and replication, but for its rapidity, reliability, and low contamination rates.

The use of fresh specimens of known HBV marker status was essential to avoid deterioration of viral count by repeated freeze and thaw cycles, to ensure proper comparison with other quantitative methods used simultaneously, and to exclude preanalytical causes of inter-test variations (Gunson et al., 2006). Specimens containing low viral load produce false negative or unreliable genotyping results. This can be proved by quantitation of HBV DNA using a sensitive real-time assay, with lower limit of detection range below 500copies per ml.

Our use of SYBR green standardized master mix for real-time PCR leaves the input DNA as the only unknown factor, thereby increasing the accuracy of the reaction. The inclusion of internal markers, and selection of appropriate sample preparation devices and methods further increase reliability and limit susceptibility to contamination, interference, or inhibitory factors than conventional HBV DNA quantification method (Gunson et al., 2006) (Olioso et al., 2007).

In an amplification plot (fig.3-2) of specimens and standards with high HBV DNA levels have lower threshold cycle (Ct) of 14-15, and consume their reagents quickly, so end point measurements will miss the true initial concentration, and will give rise to inaccuracies. Lower viral concentrations showed higher Ct values, but the amount of fluorescence (dRn), and possibly the amount of PCR product can reach higher magnitude by the end of the 38-40 amplification cycles. Because of the higher availability of reagents giving the robustness of the late starting reaction despite low initial template concentration, this would further confuse the end point measurements. All such variables are conveniently monitored and controlled by the real-time technology. This amplification plot proves the inherent accuracy and precision of the method, as well as

obviating the need for a separate detection step using gels or ELISA. Thus, further increasing their speed and throughput, removing possible interference by other variables.

This assay was evaluated for its specificity, efficacy, reproducibility and accuracy. Positive signals of HBV DNA were observed in sera from chronic hepatitis B patients tested but not in individuals negative for HBV markers. Quantitative measuring of sequential levels of HBV DNA in plasmid standards and in serum specimens was done in duplicates.

There were two melting temperatures (Tm) of 80.5 and 81.7^{o}C in the dissociation curve of HBV genotype A (including specimens and plasmid standards) and genotype B respectively (Graph3-3). Which are clear-cut for each reaction on its own, indicating the presence of pure product. But possibly small differences in the product sequences due genotype related variations, or to mutations, resulting in the observed slight differences in the Tm. Sequencing of any aberrant PCR product will verify the presence of different size product and will show the mutations, recombination, deletion, or insertion if present. Gel electrophoresis of these PCR reaction products did not show any variation in their sizes. Payungporn *et al.,* (2004) demonstrated significant differences in the melting temperature (Tm) of HBV genotype B lower than genotype C. The GC content of HBV genotypes A, B, C and D were 56.61, 58.24, 55.86 and 57.24, respectively. Genotype D has a 33- bp deletion in the PreS1 that may also affect the Tm. The Tm was used as a method to determine HBV genotype (Liu et al., 2006).

Regarding the dynamic range, a linear standard curve was obtained between 10^2 and 10^8 DNA copies/reaction. Thereby this test is capable of quantifying serum HBVDNA, covering the range of naturally replicating virus as well as occult hepatitis B infections. The standard deviation of cycle threshold (Ct) of specimens showed a small range between 0.06 and 0.68. The coefficient of variation (CV) both within and between experiments indicated remarkable reproducibility. CV was lowest in the middle of the standard curve at 10^5-10^6, denoting the area of highest precision. Lower precision was seen at both extremes of 10^2 and 10^8 copies/ml. Other studies of HBV quantitation

demonstrated a median coefficient of variation of inter experimental variability of 1.5% to 26.2% and 1.8% to 5.5% for Cobas Monitor and Cobas TaqMan assays, respectively (Laperche et al., 2006), while b-DNA tests showed total CV variation from 10.6 to 26.5% over the dynamic range (Yao et al., 2004). Differences in amplification efficiency and lower reproducibility will therefore affect more the weaker samples (Brechtbuehl et al., 2001). The inclusion of serial dilution of standardized quality control material is essential to validate our assay in the lower detection range. Accuracy achieved was tested by the accurate detection of the WHO standard to control reliability and validity of the test.

The mean difference between the SYBR green and the Amplicor measurements was significantly 0.595 $\log_{10}$ copies/ml lower than the SYBR green method (p: 0.021) (table 3-3), with a range of 0.10 to 1.094. This is not a big difference, as a minimum of 2 $\log_{10}$ copies/ml is required to define a clinically significant change of HBV DNA measurements. Nevertheless, necessitates using the same method for serial measurement of HBV DNA levels.

However, in samples at the lower part of the range i.e. below 10^3, larger differences between duplicate measurements were seen. The lower limit of 10^2 was not as accurate as was claimed by the test (Ho et al., 2003). Because its Ct of 34.4 cycles was so low as the final limit of the test was 38 cycles, after which non-specific results can occur from negative controls. This is to be expected since more amplification cycles are required before the threshold is crossed for these samples.

In our assay, the actual lower detection limit is one to two copies of HBV DNA in the minute volume of template per reaction. The low sensitivity for the HBV concentration can be explained by the very low amount of DNA; 0.1 copy in the 10^2 standard, which can be inaccurate, and its measurement is unreliable. The input DNA was later increased from 1 µl to 2µl out of100 µl DNA extracted from 200 ml of serum, which is equivalent to 500 and 250 HBV DNA copies/ml, respectively. To improve the test detection limit, a minimum of 10 to 20 µl of the template DNA can be added to the reaction containing 1-2 copies of the HBVDNA, as used in some real-time methods. Although some studies have

claimed the detection limit of 1 HBV DNA copy/reaction (Paraskevis et al., 2002), even 10 HBV DNA copies per ml detected in the more sensitive TaqMan assay (Allice *et al.*, 2007). However, in the latter assay, input DNA extracted from whole 1ml plasma.

Meanwhile, major drawback of increasing the template volume can be the production of excessive amount of fluorescence from DNA in the higher concentration standards of 10^6 to 10^8 copies/ml, thereby compromising the upper detection limits in an effort to improve the lower detection cut-off. The alternative is to tailor the test according to individual requirement with second nested PCR run to confirm low HBV DNA cases as described by Brechtbuehl et al., (2001). Another strategy is to repeat testing of all sera with low HBV DNA to confirm the specificity of assays with wide detection range (Gunson et al., 2006).

This can be balanced depending on the clinical diagnostic priorities. Low HBV DNA below 10^3 copies/ml, is important for determining response to therapy, and deciding to stop treatment without risking relapse for false negative HBV DNA assay. Patients with higher HBV DNA may also need specification, to determine changes of 1-3 $\log_{10}$ as a measure of clinical responsiveness to therapy.

Clinical presentation and implications HBV viral loads

Mean HBV DNA level was significantly higher in UK than Saudi patients at 5.09 (95% CI 1.43-8.75) and 4.52 (95% CI 0.14-8.90) $\log_{10}$ copies/ml, respectively (p: 0.007) (table 3-2). This difference reflected the lower viral replication of HBV in Saudi patients, which may be related to viral genotype or mutants present.

Hepatitis markers may correlate with and influence the interpretation of HBV DNA measurements in relation to the clinical information. Significantly higher HBV DNA levels were detected in patients with HBeAg positivity 7.18 and 6.44 $\log_{10}$ copies/ml in UK than Saudi patients, respectively. Conversely, HBeAg-negative UK patients had lower HBV DNA than Saudi patients at 3.84 and 4.11 $\log_{10}$ copies/ml, respectively (table 3-3 and graph 3-4). Possibly reflecting a more acute presentation of HBV in UK patients

as compared to vertical transmission or more chronic disease in Saudi patients with or without HBeAg-negative mutants (see core chapter). Is the mode of transmission or genotype responsible solely for this variation in the HBV epidemiology and biological behaviour, or are there differences in HBV replication and immunogenicity.

Generally, patients with HBeAg positivity had HBV DNA level above10^5 copies/ml. While those with negative HBeAg had around 10^4copies/ml, that is 10^2 to 10^3 copies/ml less than the HBeAg positive group, which is consistent with the knowledge that HBeAg positivity is associated with higher viral replication and infectivity. A significant drop in HBV DNA is required for HBeAg seroconversion (Chan; et al., 2003). In contrast, Saudi HBeAg-negative patients had higher HBV DNA than UK patients. Here, anti-HBe positive sera were associated with full range from undetectable to high HBV DNA levels. Possibly due to HBeAg-negative mutants. Hence, HBeAg-negative state does not exclude active HBV replication, and therefore serological monitoring cannot be relied upon in the management of HBV patients in Saudi Arabia.

Clinically, the HBV DNA cut-off level of below 4 $\log_{10}$ copies/ml was associated with anti-HBe antibody positivity, and previously was indicative of inactive liver disease in HBeAg negative or seroconversion patients (Martinot-Peignoux *et al.*, 2002). While HBV DNA levels of => 5 $\log_{10}$ copies/ml in HBeAg positive patients were consistent with the results of Chan et al., (2003) who described that HBV DNA levels of 10^4 to 10^5 copies/ml was a grey zone to predict disease activity because of the overlap in DNA levels between patients with remission and those with reversion of HBeAg. This further necessitates accurate monitoring of the proposed cut-off level of 10^4 instead of 10^5 and correlating it to a wider range of anti-HBe positive patients either during the natural history of the disease or after manipulation by therapy especially in Saudi patients. In support to our findings, recent guidelines have approved the limits of 2000-20000 IU/ml for tailoring therapy (Keeffe et al., 2008) (Kennedy et al., 2008).

HBV Saudi patients with active liver disease had low, intermediate and high HBV DNA levels. Hence, liver disease patients may have low or undetectable HBV DNA. This

indicates that factors other than HBV DNA level and active replication are associated with the degree of liver damage (Wiegand et al., 2008). Inactive hepatitis was associated with low HBV DNA in most patients (table 3-4). The low viral replication in chronic HBV infection in Saudi patients. Inactive hepatitis or carrier status is consistent with such DNA levels and low infectivity. These results indicate that in Saudi asymptomatic carriers, up to 19% can have high HBV DNA levels. Some patients have high HBV DNA levels despite the absence of both HBeAg and ALT elevation (Chan et al., 2002). On the other hand, Gandhe *et al.,* (2003) reported that HBV DNA PCR positivity among asymptomatic HBsAg-positive carriers was lower than in chronic hepatitis B. Although HBV DNA levels were reported to decrease in patients with cirrhosis or HCC (Payungporn et al., 2004).

High HBV DNA was a major factor for the development of cirrhotic complications, which continued to develop in 24.5% of patients with HBV DNA levels below 10^4 copies/ml (Yuan *et al.,* 2004). However, persistent active viral replication is strongly associated with the progression of fibrosis in HBeAg-negative chronic hepatitis (Kumar *et al.,* 2008).Our patients with high HBV DNA of more than $5\log_{10}$copies/ml are associated with more severe liver disease, and a much more prone to hazardous complications as cirrhosis and HCC. This necessitates greater care and more aggressive therapy for such patients, with clear aims of either suppressing the HBV DNA levels completely, or at least decreasing it to the moderate level of 3-5 $\log_{10}$ copies/ml to hinder the progression of liver disease (Payungporn et al., 2004).

Quantitative determination of serum HBV DNA concentration is the most important measure for an estimation of the infectivity of an HBV positive carrier as health care worker and the basis for the decision whether he or she is allowed to perform exposure prone procedures (EPP) (Buster *et al.,* 2007). Thus, questions are raised on how reliable quantitative assays are at or below 10^3 copies per ml, and how stable is the HBV DNA concentration is in a healthy chronic HBV carrier. Regular follow up is essential, and should be interpreted with some caution as they fluctuate widely in chronic carriers (Chen *et al.,* 2009). Over repetition of QPCR in short intervals can be time consuming, and wasteful of resources. Proper designing of the frequency of QPCR monitoring needs

to be standardized in clinical guidelines for patients on therapy after proper clinical studies on HBV viral kinetics and chronology of the development of drug resistance (Punia et al., 2004). In a study by Ide et al., (2003), response to therapy depends on the pre-treatment HBV DNA levels. Virological breakthroughs, were observed at 31 to 67 weeks in most patients, and in at 107 and 115 weeks in patients with low HBV DNA (< 2.6 $\log_{10}$ copies/ml).

3.8. Conclusion

The use of SYBR green I kits (Stratagene) showed high sensitivity, accuracy, and reproducibility in quantitative HBV DNA assay. This method had a dynamic range from $2.5x10^2$ to 10^8 HBV DNA copies/ml. However, we found out that improvement is required to increase sensitivity and accuracy at lower detection limit and to increase the QPCR yield at lower viral concentrations. Therefore, it was generally suitable for quantitative PCR assays, and provides the required test range for monitoring HBV infected patients. Titres obtained with this method show good correlation and wider dynamic range than those obtained with the AMPLICOR HBV MONITOR test.

Mean HBV DNA level was significantly higher in UK than Saudi patients at 5.09 (95% CI 1.43-8.75) and 4.52 (95% CI 0.14-8.90) $\log_{10}$ copies/ml, respectively (p: 0.007). Mean HBV DNA in patients with HBeAg was 7.18 and 6.44 $\log_{10}$ copies/ml. Saudi HBeAg-negative patients had higher HBV DNA than UK patients at 4.11 $\log_{10}$ and 3.84 copies/ml, respectively.

3.9. Future Directions

To study the HBV DNA quantity as a measure of replication in various HBV genotypes at different clinical stages of the infection.

To determine the HBV DNA levels in different mutations in relation to HBV genotypes as a measure of viral replication and activity.

To develop a real time assay to genotype HBV and another one to detect important mutations in the S, Pol and core genes, in order to conduct large molecular epidemiology study.

Chapter Four: Hepatitis B Genotypes

4.1. Introduction

4.1.1. HBsAg Serological Subtypes

Serological subtypes of HBsAg depend upon the antigenic structure of `a`determinant which is located within the amino acids 120 -147 domain, containing a double loop structure projecting from the surface of the virus (Fig.4-1). Bouvier (1975) first detected HBsAg subtype determinants d and y in 1971, then w and r. All combinations of these determinants exist in nine subtypes; ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq- and adrq- (Liu et al., 2002), which are specified by amino acid lysine or argnine at positions 122 and 160 of HBsAg (Kao and Chen, 2005). Other subtype determinants were mapped to amino acid positions 127, 144, 145, 158, 159, 177 and 178(Kramvis et al., 2008). Serotyping of HBV was used for epidemiological purposes, or to predict disease progression. The adw strains had a 20-fold higher risk of lamivudine resistance more than ayw strains (Zollner et al., 2004).

4.1.2. HBV Genotypes

4.1.2.1. HBV genotypes A to H

Genotypes of HBV are defined as strains that had DNA sequences with more than 8% divergence between the groups (Okamoto et al., 1988). Eight genotypes (A to H) of HBV have been described (Arauz-Ruiz et al., 2002). The rate of genomic nucleotide substitutions of HBV is 10^5 to 10^4 per position per year. Accordingly, genotypes diverged less than 600 years ago (Fares and Holmes; 2002). Changes occur from evolutionary drift, recombination, or as a consequence of a long-term adaptation of HBV to genetic determinants of specific host populations (Gunther; 2006). Genotyping is a more appropriate epidemiological tool to track common sources of infection and to investigate viral evolution. The correlation between different genotypes and related serotypes is shown in table 4-1 (Kramvis et al., 2008) (Kidd-Ljunggren et al., 2002).

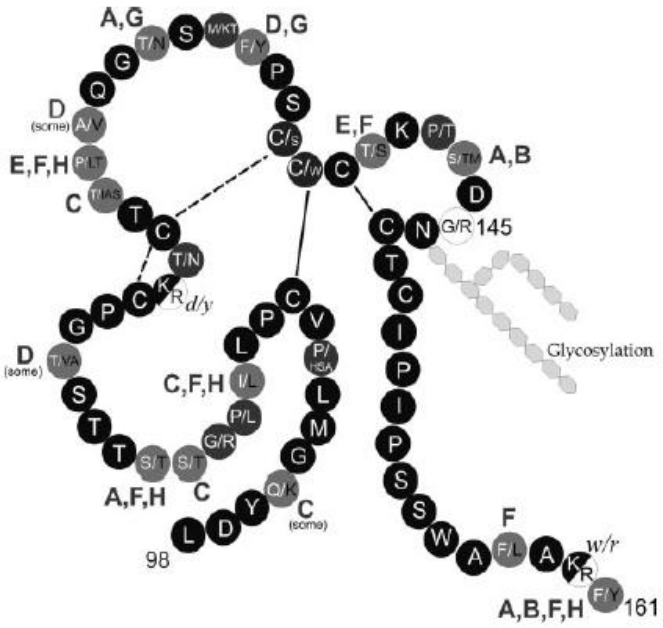


Figure 4-1: Genotype related variations within HBsAg.

Hypothetical model of the amino acids substitutions in the HBsAg including the `a`determinant in different HBV genotypes A to H. Conserved amino acid residues are shown in black. Non-conserved residues are shown in grey. If residues are varying subtype specially the minor genotype is indicated in bold beside of the amino acid. The position of the frequently described variant G145- R is shown in white. Residue 122 and 160 which confer subtype change from d/y and r, respectively, are indicated in black and white. W4- reactivity is determined by L127;I126 abolishes reactivity with w-antisera and is linked to the r- subdeterminant. The figure is based on an alignment of 166 SHBs- sequences translated from 166 wild type-HBV genomes from GenBank (Schaefer; 2005).

4.1.2.2. Relationship between HBV genotypes

There are structural, functional, infectivity and clinical differences between genotypes. Such differences include prognosis, progression of disease, complications as cirrhosis and hepatocellular carcinoma, as well as response to antiviral therapy. Structurally, HBV genotypes differ in the length of their genomes. The numbering of HBV genome from the EcoRI site leads to difficulties in comparing nucleotide positions between genotypes, unlike the numbering system, which is based on Bartholomeusz and Schaefer (2004). Functionally, the preS region that is important for virus attachment and cell entry, show significant differences between genotypes. Genotype A differs mainly in sequence of the

preS2 region, and has insertion of six nucleotides in the terminal protein portion of the polymerase gene overlapping the core gene, and shares some structural features with genotype F. Genotype D genome is most similar to Genotype E, especially in the X-gene. Differences in RNA splicing folding between genotypes could be predicted (van Hemert et al., 2008).

4.1.2.3. HBV Genotypes, recombinations and co-infection

Infection by multiple HBV genotypes or reinfection can exist, especially in high-risk individuals such as IV drug users (Olinger et al., 2007). In addition, recombination between genotypes has been reported as genotypes A with D (Sugauchi et al., 2004) (Schaefer, 2007), A with C and B with C (Sugauchi et al., 2003). The latter recombinant HBV (Ba) is a chimera in which the majority of the genetic framework is genotype B (fig.4-2), except for the basal core promoter (BCP), precore and core regions which correspond to genotype C. Coinfection with genotypes A and G exist (Zaaijer et al., 2011). Such events can increase as different genotypes circulate in the same region.

4.1.2.4. Geographical Distribution of HBV genotypes

There is a defined geographical distribution of different HBV genotypes (table: 4-1, fig.4-4), that reflect the evolution of the disease and is related to patterns of travel which lead to interaction between HBV genotypes. Genotype A in Northwestern Europe possibly reflects the immigration to North America and South Africa especially over the last century. In New Zealand and Australia genotype A, D and C are contributed by immigration from Southeast Asia and the Pacific Islands (Bartholomeusz and Schaefer; 2004). Genotypes A and D predominate in UK (Dervisevic et al., 2007). Although genotype D is the most widely distributed universally, with highest prevalence in a belt stretching from Southern Europe and Africa to India (see fig.4-3). Behavioural patterns also change the prevailing genotype as homosexual men co-infected with HIV harbour genotype A instead of C or B in Japan (Koibuchi et al., 2001), genotype E in West and South Africa, and Genotype G in Caucasians males(Bartholomeusz and Schaefer, 2004). Studies are needed to examine possible long-term changes in HBV genotypes within any region.

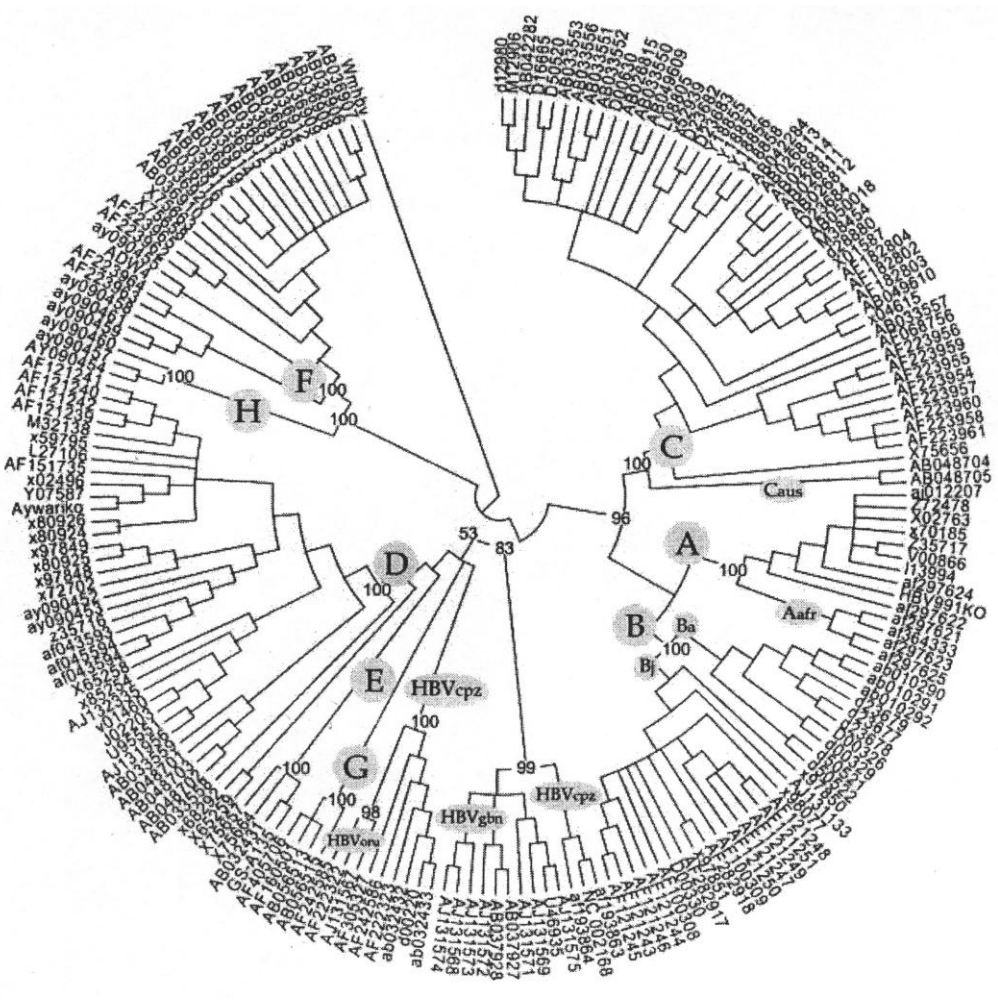


Figure 4-2: Phylogenetic analysis of HBV genotypes and sub genotypes.

Phylogenetic tree of 188 complete HBV genomes from Gene Bank aligned using Clustal W in the program DNASTAR. The alignment was further analyzed by boot-strapping using the neighbor-joining method contained in MEGA version 2.1. HBV genotypes A to H and sub genotypes A-Africa (A afr), chimeric genotype B and C (Ba), B-Japan (Bj), C-Australia (C aus), Chimpanzee strain (HBV cpz), Orgutan strain (HBV org), Gibbon strain (HBVgbn) (Bartholomeusz and Schaefer; 2004)

Figure 4-3: Geographical distribution of HBV genotypes and subgenotypes A, A Africa (A_{afr}), B, C, C Australia (C_{aus}), D, E, F, G and H (Bartholomeusz and Schaefer; 2004)

Table 4-1: Relationship between HBV genotypes and serotypes and their geographical distribution

HBV serotypes are designated according to the antigenic constitution of HBsAg determinants d or y and r or w. Different HBV genotypes can have the same HBsAg serotype, while a single genotype can be associated with several HBsAg serotypes. Differences in HBeAg seroconversion rate and age are also shown (Weber; 2005) (Allain; 2006).

Geno - type	Associated Serological Subtypes	Distribution	Main mode of transmission	% Chronic infection	% HBeAg	Median age of HBe seroconversion	% Viral load $>10^4$ copies/ml
A	Adw2, ayw1	North Western Europe, USA, Argentina, Japan, Philippines, Hong Kong, Australia, Central and South Africa, and India	Sexual IVDU	<1	25-80	20	40.3
A`		South Africa (Bowyer et al., 1997)					
B1	Adw, adw2,	Vietnam	Vertical	1-12	12-53	40	64.3
B2	ayw1	Southeast Asia					
C	adw2, adrq+, adrq-, ayr, adr	East Asia, China, Japan, Korea, Vietnam, USA Polynesia, Southeast Asia, south Pacific islands and Australia	Vertical	1-10	50-69	30	64.3
D	Ayw2, ayw3, ayw4	Mediterranean area, Middle East, Southern Europe, Russia, Australia, India, Africa, USA, and in IVDU worldwide	Vertical, sexual, nosocomial	<1-5	33-58	20	
E	ayw4 (adw2)	West and South Africa	Horizontal, nosocomial	3-25	21.5-29	<10	27.5
F	adw4q, adw2, ayw4	South and Central America, West and South Africa, Polynesia	Sexual, vertical	1	90	unknown	
G	adw2	Europe, U.S.A. Central America	homosexuals				
H	adw4	Central and South America (Arauz-Ruiz et al., 2002)					
I							
J							

IVDU: Intravenous drug users

HBV genotypes have distinct sub genotypes according to their geographical distribution and rates of progression. Genotype A has Aa (Africa/Asia) and Ae (Europe) (Sugauchi et al., 2004) (A1, A2). Genotype B is divided into B1 to B4. There is also chimeric genotype B and C; Ba and genotype Bj for Japanese strains (Norder *et al.*, 2004). Huy *et al.*, (2004) divided genotype C into C1 (Cs) from Southeast Asia including Vietnam, and Thailand, and genotype C2 (Ce) from Far East Asia including Japan, Korea and China. Sub genotypes C3, C4 and D1-D4 had been described (Norder et al., 2004). Genotype C has an Australian strain, C_{aus} as well (Bartholomeusz and Schaefer, 2004). Other recombinations between different HBV genotypes have been described (Schaefer, 2005) (Kramvis et al., 2008).

4.1.2.5. Clinical Characteristics

The clinical course of HBV infection varies depending on the patient`s age, immunological response, and viral genotype. HBV genotypes may evolve from a research tool into an essential diagnostic test, as HCV genotyping did. Specific genotypes of HBV are associated with particular clinical presentations and viral mutations. Chronic hepatitis in genotype A is more prevalent than in genotype D, whereas the opposite situation is found in patients with acute hepatitis and drug addicts. On the other hand, Genotype A patients were less likely to develop HBV recurrence or die after liver transplant compared with genotype D patients (Girlanda et al., 2004), biochemical remission, clearance of HBV DNA and clearance of HBsAg are more common in genotype A than D (Payungporn et al., 2004). In addition, genotype A infections had a better prognosis than the more aggressive genotype C. Fulminant hepatitis occurs in genotype B (ayw and adw2), sub genotype Bj (41%) (Sugauchi *et al.*, 2006). Genotype B may have motifs that bind to HLA class I molecules, thereby activating stronger immune response (Imamura et al., 2003). Genotype C (subtype adr) is more common in cirrhotic patients and hepatocellular carcinoma (HCC) patients older than 50 years (Chen et al., 2004). Genotype Care more resistant to interferon therapy than genotype A (Suzuki et al., 2004) or genotype B. Although subgenotype Ba shows a poorer response to therapy than Bj (Akuta and Kumada, 2005). However, vaccination with HBsAg from different HBV

sero/genotypes can prime cross-reactive, specific CD8 (+) T-cell immunity that breaks tolerance to HBsAg (Schirmbeck *et al.,* 2003).

4.1.2.6. Genotyping Methods

Sequencing of specific genes or even the whole genome is the gold standard for HBV genotyping. Genotyping is also done by restriction endonuclease digestion of HBV PCR products of the S gene, i.e. restriction fragment length polymorphism (RFLPs) (Mizokami et al., 1999) (Lindh et al., 1998); Post-PCR hybridization or line probe assays (Grandjacques et al., 2000); Multiplex PCR (Naito et al., 2001) using genotype specific primers; Simultaneous HBV genotyping and quantitation by real-time PCR (Payungporn et al., 2004). Usuda *et al.,* (1999) raised genotype-specific monoclonal antibodies. Cloning can assist in the study of different HBV genotypes and mutants especially in patients with coinfection and mixed viral populations.

4.2. Hypothesis

Saudi Arabia is an HBV endemic area. Whether HBV genotypes influence viral characteristics and clinical presentation. The natural history of HBV disease, as well as factors affecting disease progression remains to be explored. HBV patients from UK, a low incidence country were included for comparison.

Certain sequence differences occur between genotypes that determine the specificity the primer binding sites, and hence validate the genotyping PCR.

4.3. Aim

The aim is to gain insight into the molecular epidemiology, prevalence and distribution of HBV genotypes in Saudi Arabia and UK.

To analyze genotype associated HBeAg seroconversion, clinical presentations and liver disease.

To validate the genotype specificity of the primers used in genotyping assay.

To clone different HBV genotypes to provide high concentration of each genotype as reliable pure controls for both the multiplex genotyping PCR and the RFLPs genotyping assay.

To verify infections with multiple genotypes, to confirm whether these had more than one viral genotype.

To assess whether HBV DNA level interferes with the outcome of HBV genotyping methods.

4.4. Patients and Methods

4.4.1. Patients Details (see chapter 3; 3.5.1.1.and 3.5.1.2.)

DNA was extracted from sera of 130 Saudi and 105 UK patients.

4.4.2. HBV Multiplex Genotyping PCR

A nested multiplex PCR to detect conserved regions of the pre-S1 and S-gene was established for the purpose of genotyping HBV in clinical samples (Naito et al., 2001); the first-round PCR using outer pair universal primers P1 (sense) and S2 (antisense) (table 4-2 and fig.4-4) product size (1,097 bases). The reaction mixture contains 0.125µl of hot start Taq polymerase (Qiagen), 5pmol of each primer (MWG-Biotech Ltd), 2.5µl 10x buffer (containing 15mM Mg Cl_2, Qiagen), 100 nM of each of four deoxynucleoside triphosphate (dNTPs) (Roche) and 2µl DNA template giving a total volume of 25µl in sterile distilled water (Sigma). The amplification program had a hot start at 95^{o}C for 15 minutes, followed by 40 cycles of 94^{o}C for 20 seconds (denaturation), 55^{o}C for 20 seconds (annealing) 72^{o}C for 1 minute (extension); followed by further extension at 72^{o}C for 10 minutes, after that the product was kept at 4^{o}C. The second-round PCRs were performed for each specimen using primers (inner pairs) in two different combinations; mix A for genotypes A, B, and C; using universal primer B3 (sense) and A4 (genotype A-specific, antisense), R5 (genotype B-specific, antisense), and C6 (genotype C specific, antisense). Mix B for genotypes D, E, and F, using primer R10 (antisense) and D7 (genotype D-specific, sense), E8 (genotype E specific, sense, and F9 (genotype F-specific, sense). 2µl of the first PCR product was added to each mix and the same components as the first reaction. These were amplified for 40 cycles with the following

parameters: mixture contains 0.125µl of hot start Taq polymerase (Qiagen), 5 pmol of each primer (MWG-Biotech Ltd), 2.5µl 10x buffer (containing 15mM $MgCl_2$, Qiagen), 100nM of each of four deoxyribonucleoside triphosphates (dNTPs) (Roche) and 2µl DNA template giving a total volume of 25µl in sterile distilled water (Sigma). The thermal cycler program starts with 95^{o}C for 15 minutes, followed by 20 cycles of 94^{o}C for 20 seconds (denaturation), 58^{o}C for 20 seconds (annealing) 72^{o}C for 30 seconds (extension). Then an additional 20 cycles of 94^{o}C for 20 seconds, 60^{o}C for 20 seconds, 72^{o}C for 30 seconds; followed by further extension at 72^{o}C for 10 minutes (PT-200 Peltier Thermal Cycler). The PCR product was kept at 4^{o}C. 9µl of the products of this second round PCR were electrophoresed on 2% agarose gel, and are differentiated by the size of genotype-specific bands compared to DNA marker (Roche molecular weight Marker XIV(100 bp ladder)(11721933001).

4.4.2.1. Optimization of the Multiplex Genotyping PCR

Many factors can influence the efficiency of a PCR reaction; these include the concentration of the DNA, the magnesium concentration, the number of cycles in the reaction and primer design, which includes related factors such as annealing temperature. Cycle numbers were modified in some of the PCR programs to obtain perfect amplification products from PCR reaction. Optimization of PCR conditions was performed by the modification of some of these factors to achieve best amplicon yield.

4.4.2.1.1. Optimization of the first round PCR

The PCR conditions were altered to improve final PCR bands as follows: annealing at 55^{o}C for 1 minute instead of 20 seconds, extension at 72^{o}C for 3 minutes instead of 1 minute

4.4.2.1. 2. Optimization of the second round genotyping PCR

Q-solution (QIAGEN) was added 5µl per reaction to increase the stringency of the PCR. The amplification cycle was modified to 25 cycles of 94^{o}C for 20 seconds (denaturation), 58^{o}C for 20 seconds (annealing) 72^{o}C for 1 minute (extension); and an additional 30 cycles of 94^{o}C for 20 seconds, 60^{o}C for 20 seconds, 72^{o}C for 1 minute; followed by further extension at 72^{o}C for 10 minutes.

Table 4-2: Primers of multiplex PCR for HBV genotyping (Naito et al., 2001).

Primer	Sequence	Position	Polarity	Specificity	Product size	New product size	Melting Temp.Tm
First round PCR P1	5`-TCACCATATTCTTGGGAACAAGA3`	nt. 2823-2845	Sense	universal	1097 bp		57.1°C
S2	5`-CGAACCACTGAACAAATGGC-3`	nt. 704-685	Antisense	universal			57.3°C
B22	5`-CAAT (A/T)C (G/T) (C/T) TGACA (A/G/C/T) ACTTTCCAAT-3`	nt. 996-977	Antisense	Universal	1389 bp (P1 and B22)		64.2°C
Second round PCR: Mix A B3	5`-GGCTCM (A/C)AGTTCM (A/C)GGAACAGT-3`	nt. 67-86	Sense	Universal			59.4°C
A4	5`-CTCGCGGAGATTGACGAGATGT-3`	nt. 134-113	Antisense	Genotype A-specific	68 bp	178 bp	62.1°C
R5	5`-CAGGTTGGTGAGTGACTGGAGA-3`	nt. 345-324	Antisense	Genotype B-specific	279 bp	389 bp	61.8°C
C6	5`-GGTCCTAGGAATCCTGATGTTG-3`	nt186-165	Antisense	Genotype C-specific	120 bp	230 bp	60.3°C
Mix B D7	5`-GCCAACAAGGTAGGAGCT-3`	nt. 2979-2996	Sense	Genotype D-specific	119 bp	200 bp	56.0°C
E8	5`-CACCAGAAATCCAGATTGGGACCA-3`	nt. 2955-2978	Sense	Genotype E-specific	143 bp	224 bp	62.7°C
F9	5`-GYTACGGTCCAGGGTTACCA-3`	nt. 3032-3051	Sense	Genotype F-specific	66 bp	147 bp	60.4°C
R10	5`-GGAGGCGGAT (C/T)TGCTGGCAA-3`	nt. 3097-3078	Antisense	Universal	276 bp	357 bp	62.4°C
New Primers l B13N	5`-AGGAAGR (A/G) M (A/C) AGCCTACK (G/T) CCC –3`	nt. 3139-3156	Sense	A-C Universal			59.9°C
R11	5`-TGCAT (G/T) GCCTG (A/T) GGATG (A/T) GTGT-3`	nt. 3197- 3176	antisense	Universal D-F			61.2°C

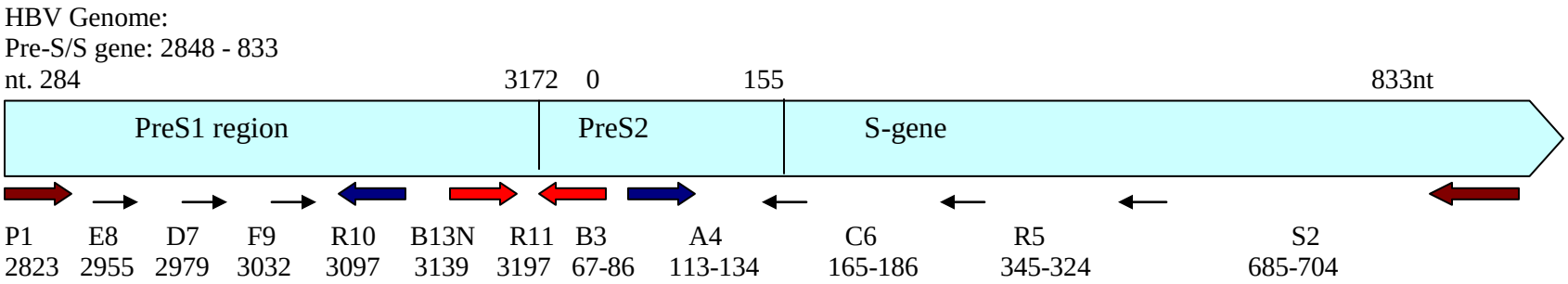


Figure 4-4: A diagram to show the binding sites of different multiplex PCR primers

First round primers are P1 (sense), and S2 (antisense). Second round primers in two different mixes; Mix A: primersB3 (common antisense primer), B13N (new common sense primer), A4 (antisense; genotype A-specific), R5 (antisense; genotype B-specific), C6 (antisense; genotype C-specific). Mix B: primers D7 (sense; genotype D-specific), E8 (sense; genotype E-specific), F9 (sense; genotype E-specific), R10 (common antisense primer), R11 (new common antisense primer).

4.4.2.1.3. Optimization of the first round product for sequencing

The PCR conditions were altered to give visible product of the first round PCR by undergoing a second run with the same primers

4.4.3. HBV Genotyping by TRUGENE Kits

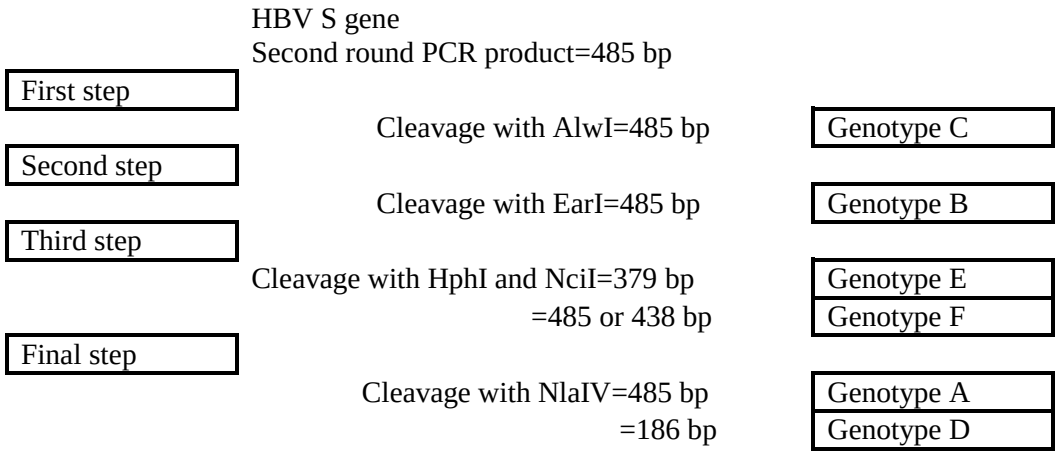
The TRUGENE HBV genotyping kit with Open Gene^{TM} Sequencing System (Visible Genetics) was used according to manufacturer's instructions. The principle is to amplify and sequence part of HBV S gene in gel electrophoresis tower. Then sequences are compared to stored database on established genotypes and mutations.

4.4.4. Genotyping by restriction Fragment Length Polymorphism

Using PCR to amplify conserved sequences that are genotype-specific, a nested PCR (Mizokami et al., 1999) was performed with the first-round sense primer F1, and antisense primer R2. The second round inner sense primer F3 and the antisense primer R2 (table 2-3). The reaction mix contains 0.125µl of hot start Taq polymerase (Qiagen), 5pmol of each primer (MWG-Biotech Ltd), 2.5µl 10x buffer (containing 15 mM $MgCl_2$, Qiagen), 100 nM of each of four deoxyribonucleoside triphosphates (dNTPs) (Roche) and 2µl DNA template giving a total volume of 25 µl in sterile distilled water (Sigma). The thermal cycler program was 95^{o}C for 15 minutes; followed by 40 cycles of 94^{o}C for 15 seconds (denaturation), 60^{o}C for 45 seconds (annealing) 72^{o}C for 45 seconds (extension). An additional 20 cycles of 94^{o}C for 20 seconds, 60^{o}C for 20 seconds, 72^{o}C for 30 seconds, followed by further extension at 72^{o}C for 7 minutes, after that the product was kept at 4^{o}C.

Restriction digestions (RFLP) were carried out with 5µl of the second-round PCR product for 3.5 hours after adjustment with 10x enzyme reaction buffer according to manufacturer`s recommendations. Reactions were carried out with 10 units of AlwI, HphI, NciI, NlaIV, or EarI (New England Biolabs) and 1% bovine serum albumin (BSA), at 37^{o}C, according to the scheme on graph4-2. The digested PCR products were electrophoresed on 3% agarose gel in 1xTBE buffer (134 mM Tris-HCl, pH 10; 68 mM

Figure 4-5: Scheme for restriction enzymes digestion for genotyping of HBV



Boric acid, 2.5 mM EDTA) containing 500 ng ethidium bromide per ml. The RFLPs pattern was then visualized under ultraviolet light.

4.4.5. Quantitative HBV PCR:

See Chapter 3.

4.4.6. Primer Alignment Method:

Clustal X alignment of each of the 11 primers of the multiplex genotyping PCR; P1, S2, B3, newB3, A4, R5, C6, R10, D7, E8, and F9, was performed with two distant isolates of each genotype from Gene Bank as follows: genotype A; Z35717 and X51970, genotype B; X97850 and M54923, genotype C; X75665 and D50517, genotype D; X80925 and U95551, genotype E; X75657 and X75664, genotype F; X69798 and X75658, genotype G; AF 160501, genotype H; AY090457 and AY0900460.

4.4.7. HBV Genotypes Cloning:

4.4.7.1. PCR amplification of S-gene

Single round of PCR of a 1397 bp region using primers P1 (sense nt. 2823-2845) and B22 (anti-sense nt. 996-977) yielded sufficient quantities of amplification products for

cloning of genotypes A, B, C, and D. The genotype E control sample gave a visible band only after two rounds of PCR using the same primers. The PCR reaction mixture contained 200 µM of each primer, dNTP mix 200 µM of each, 2 µL template DNA, buffer with $MgCl_2$ and Taq polymerase 0.125µL per tube. Sterile water was added to final volume of 25µL. The PCR reaction conditions were 10 minutes at 94^{o}C, then 55 cycles of 45sec at 94^{o}C, 1 minute at 55^{o}C, and 3 minutes at 72^{o}C. A final extension step at 72oC for 7 minutes was done, followed by soak at 4 ^{o}C. After gel electorphoresis, some bands were excised and gel was purified using QIAquick PCR purification Kit (QIAGEN).

4.4.7.2. Quantitation of PCR product DNA

The relative amount of DNA in 5µl of PCR products was estimated by gel electrophoresis with quantitative 1kb DNA ladder (Biolabs, N3232S). 1µl of the ladder mixed with 4µl water and 1µl 6x gel loading buffer. The intensity of bands was compared to the known quantities of the ladder bands that range from 33 to 125 ng.

4.4.7.3. Ligation.

Amplified S-gene products PCR products were cloned using the pCR3.1 (30ng/µl) vector by TA overhang cloning kit (Invitrogen®). This vector does not require restriction enzyme cleavage for subcloning. It also contains a T7 and BGH priming sites that facilitate the screening for incorporated DNA (inserts). Quantity of the PCR products used ranged from 1-2µl, with an average insert length of 1300 bp, gave a ratio of 1:2 (vector-insert) offering the best ligation. Ligation mixture also containing T4 ligase (4 units/µl), (10x Ligation buffer), distilled water was added to a final volume 10µl and incubated at 16^{o}C overnight. This vector contains T7 promoter/priming site, BGH reverse priming site, ampicillin resistance gene for selection and maintenance of the insert in E.coli, thereby facilitating the screening for the presence and orientation of the insert.

4.4.7.4. Transformation

Ligated plasmids with the S-gene insert of each genotype were used to transform Escherichia coli TOP10F cells (competent cells). 2µl of each ligation mixture was aliquoted into 25µl of TOP10F cells; the vials were incubated on ice for 30 minutes, heat shock for 30 seconds in 42^{o}C water bath and immediately placed on ice. 125µl of SOC medium (Invitrogen®) was added at room temperature and incubated in a shaking incubator at 37^{o}C for 1 hour at 225rpm. Two different volumes of each transformation (100 and 25µl) were plated on Luria-Bertani agar (LB) (Invitrogen®) containing ampicillin 50mg/µl), to allow for selection of bacteria harbouring the pCR3.1 since the ampicillin resistance gene is located on the vector. These plates were incubated at 37^{o}C overnight.

4.4.7.5. Screening for insert

Individual colonies were screened for the presence of the S-gene insert, using the vector primers T7 (sense nt. 638-657) (0.5µM) (5`-TAATACGACTCACTATAGGG-3`) and BGH (antisense nt. 831-813) (5`-TAGAAGGCACAGTCGAGGCT-3`). PCR reaction volumes are as follows; 200µM of each primer, dNTP mix 200µM of each, buffer with $MgCl_2$ 1.5mM and 2.5 units Hotstar Taq polymerase (QIAGEN) per tube. Sterile water was added to final volume of 24µL. Individual colonies were picked and added to PCR tubes. The PCR reaction conditions were 10 minutes at 94^{o}C, then 35 cycles of 1 minute at 94^{o}C, 1 minute at 50^{o}C, and 2 minutes at 72^{o}C. A final extension step at 72^{o}C for 7 minutes, followed by soak at 4^{o}C.

The direction of inserts was verified for the correct orientation. The vector sense primer T7 and the insert specific antisense primer B22, the second reaction using vector reverse primer BGH and primer B22 for genotypes A, B, and C. For genotypes D and E primers T7 and R11 (common antisense primer for genotypes D, E and F), and BGH and R11 were used. PCR reaction volumes are as follows; 200 µM of each primer, dNTP mix 200 µM of each, buffer with $MgCl_2$ 1.5mM and 2.5 units Hotstar Taq polymerase per tube. Sterile water was added to final volume of 24µL. Individual colonies were picked and added to PCR tubes. The PCR reaction conditions were 10 minutes at 94^{o}C, then 50

cycles of 45sec at 94^{o}C, 45sec at 50^{o}C, and 3 minutes at 72^{o}C. A final extension step was done at 72^{o}C for 7 minutes, followed by soak at 4^{o}C.

4.4.7.6. Plasmid extraction

Plasmid extraction from bacteria was done using QIA prep Kits (QIAGEN).

4.4.7.7. Quantitation of plasmid DNA

4.4.7.7.1. Fluorometry

Measurement of plasmid DNA using Hoechst 33258 fluorochrom was used for an accurate estimation of the amounts of DNA. In the presence of DNA, the fluorometer had an excitation spectrum that peaks at 365nm and an emission spectrum that peaks at 458nm. Calf thymus DNA 200ng/µl (Sigma) was used as standard. All measurements were made in TNE buffer (10mM Tris pH 7.4, 1mM NaCl, 100mM EDTA with 1ug/ml Hoechst-33258 dye)

4.4.7.7.2. Nanodrop machine

The amount of DNA was measured using the Nanodrop® NDd-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Inc.) according to absorbance at wavelength 260/280 (UV light).

4.4.7.8. Calculation of plasmid copies per microLiter

The PCR insert is 1397 bp length, and the pCR3.1 vector size is 3100 bp, both give a total of 4481 bp. Each base pair (bp) weighs 662 daltons.

A dalton weighs 1.6605655 x 10^{-24}grams or 10^{-15}ng (Avogadro`s constant).

Mass of 1 plasmid in ng = (vector size bp + insert size bp) x 662 x1.6605655 x10^{-15} ng

$$\text{Plasmid copies per µl} = \frac{\text{concentration of plasmid in ng/µl}}{\text{mass of 1 plasmid in ng}}$$

4.5. HBV Genotyping Results

4.5.1. Studying the Alignment of the Multiplex PCR Primers

There are several considerations to be fulfilled in primer design. Each primer/probe set of the multiplex must amplify well by itself. A complementarity matrix was run to ensure that the primers do not interfere with each other, and tested for primer dimer formation as well. Also melting temperature (Tm) and Mg^{++} concentration was matched. The sequence of the 12 primer binding sites from all HBV genotypes was analyzed (Table s 4-4, 4-5).

4.5.1.1. The first round PCR

Primers P1 (sense nt. 2823-2845) and S2 (antisense nt. 704-685) align fully with all genotypes A-H at the 3`end. Nevertheless, 1 to 2 nucleotides discrepancy occurs in P1 with genotypes B, D, E, G, and H. Only two nucleotides discrepancy was observed in genotypes F and H with primer S2. In conclusion, first round PCR amplify a 1097 bp segment from all genotypes, due to their annealing to highly conserved sequences.

4.5.1.2. The second round PCR

Mix A

Primer B3: Genotypes A-C common primer

The old B3 primer aligns completely to genotypes A, B, C and E. Discrepancy occurs in genotypes D, F, G and H, thus increasing the specificity of the assay (table 4-3). The main reason to design the new B13N primer was that the size of the genotype-A specific band of 68 bp was very small, which is mixed up with primer dimers common to multiplex and other PCRs, giving false positive and false negative results owing to the size of the product.

Primer B13N: Genotypes A-C common primer

The new B13N primer aligned completely to genotypes A, B, and C. At the 3`end, only one discrepancy was found in genotypes D, E, F and H, which decreases the chances of priming to those genotypes, and increases this primer specificity. Although genotype G had 1 discrepant nucleotide, it is complementary to B13N primer 3`end. Hence, the new B3 primer amplifies genotypes A, B, C, G and H. This highly conserved segment aligned

also to the sequences of our HBV cases (see appendix A). The genotype-related variation G to A was also accounted for in this redundant primer.

Primer A4 Genotype –A specific Primer

Primer A4 anneals completely with genotype A sequences, and has discrepancies with all other genotypes in the 3`end (table 4-4). In conclusion, A4 is a reliable highly specific primer with remote possibility of alignment with any other genotype.

Primer R5: Genotype-B specific Primer

Primer R5 aligns completely with one genotype-B sequence X97850, but had one discrepant nucleotide with other strain; M54923, 2nt. before the 5`end. This primer had discrepancies with other genotypes A, C, D, E, F and G. There is a remote possibility of annealing with genotype H.

Primer C6: Genotype-C specific Primer

Primer C6 anneals completely to genotype C sequences. It shows discordance with genotypes A, B, D, E, F, G, and H (table 4-4). Therefore it is highly specific for genotype C detection. Our genotype C cases show at one nucleotide before the 3` end with A to C change, although the primer did amplify those cases (N16, N19, and N45), it is accounted for by the redundant base A/C.

Mix B: Primer R10: Genotypes D-F common primer

Primer R10 shows complete alignment with genotypes D, E, F, and G. But one genotype E strain; X75664 had one discrepant nucleotide in the middle of the sequence. Discordance occurs with genotypes A, B, C, and H. Hence, primer R10 can amplify genotypes D, E, F, as well as genotype G (table 4-4).

Primer R11: Genotypes D-F common primer

Primer R11 shows complete alignment with genotypes B, D, E, F, and H. Only one nucleotide was discrepant in genotypes A, C and G.

Primer D7: Genotype-D specific Primer

Primer D7 had complete alignment only with genotype D. It shows discordance with all other genotypes at its3`end. This primer is highly specific for genotype D detection.

Primer E8: Genotype-E specific Primer (sense nt. 2955-2978) 24 bp

Primer E8 aligns completely with genotype E sequences. It shows marked discrepancies with all other genotypes as genotype A, B, C, D, G, and H at the 3`end of those genotypes. There is the very slight difference in size of genotype D from genotype E bands reaching 24 bp only.

Primer F9: Genotype-F specific Primer (sense nt. 3032-3051) 20 bp
Complete alignment is observed with genotype F sequences, whereas it shows marked discordance with all other genotypes. Therefore, although this primer is specific for genotype F, its alignment to genotype H is possible.

Generally, the specificity of all those primers was high in relation to Saudi and UK samples as proved after sequencing HBV isolates from UK and Saudi patients. Primers were also examined by software for secondary structure and for specificity and for primer-dimer formation.

4.5.2. Analysis of DNA sequences

DNA sequences were further analyzed using SeqMan 2 of DNA Star software package (Chapter 6). Phylogenetic analysis was performed to define the HBV genotypes and subgroups based on >8% and >4% heterogeneity of the entire HBV genome, respectively.

Table 4-3: Discrepancy of each genotype with the second round genotyping PCR common primers.

The sequence of the 6 primer binding sites with two distant isolates from each genotype; the number of nucleotides discrepancy of each sequence was calculated, with their percentage in relation to the total number of nucleotides in the relevant primer. Discrepancies at the 3`end of the primers were also analyzed, which mostly determine the specificity and their annealing to viral genome.

Genotype	Discrepancy							
	Primer B3 (A-C-specific) (20 bp)		Primer B13N (A-C-specific) (19 bp)		Primer R10 (D-F-specific) (20 bp)		Primer R11 (D-F specific) (20 bp)	
	3`	Total (%)	3`	Total (%)	3`	Total (%)	3`	Total (%)
A	0	0 (0)	0	0 (0)	1	5 (25)	1	1 (5)
B	0	0 (0)	0	0 (0)	1	3-4 (15-20)	0	0 (0)
C	0	0 (0)	0	0 (0)	1	4-5 (20-25)	1	1 (5)
D	0-1	0-1 (0-5)	1	2 (10)	0	0 (0)	0	0 (0)
E	0	0 (0)	1	2 (10)	0	0-1 (0-5)	0	0 (0)
F	2-3	3-4 (15-20)	1	5 (26)	0	0 (0)	0	0 (0)
G	0	2 (10)	0	1 (5)	0	0 (0)	1	1 (5)
H	2	3 (15)	1	3-4 (16-21)	1*	3-4 (15-20)	0	0 (0)

* One discrepant nt. before the 3`end.

Table 4-4: Discrepancy of each genotype with the second round PCR genotype-specific primers: Mix A and B

Genotype	Discrepancy											
	Primer A4 (A-specific) (20 bp)		Primer R5 (B-specific) (19 bp)		Primer C6 (C-specific) (20 bp)		Primer D7 (D-specific) (20 bp)		Primer E8 (E-specific) (19 bp)		Primer F9 (F-specific) (20 bp)	
	3`	Total (%)	3`	Total (%)	3`	Total (%)	3`	Total (%)	3`	Total (%)	3`	Total (%)
A	0	0	1	3 (14)	2	2 (9)	2	3 (17)	2	6 (25)	1	5-6 (25-30)
B	1	5-6 (23-27)	0	0-1 (5)	3	4-5 (15-20)	2	3-4 (17-22)	1	5-6 (20-25)	1	5-7 (25-35)
C	1	4 (18)	1	3 (14)	0	0 (0)	1	4-5 (22-28)	2	6-7 (25-29)	1	5 (25)
D	1	4-5 (18-23)	1	3 (14)	2	2-4 (9-14)	0	0 (0)	2	4-5 (17-20)	1	8 (40)
E	1	3 (14)	1	2-3 (9-14)	2	2 (9)	2	2 (11)	0	0 (0)	1	5-6 (25-30)
F	1	6-7 (27-32)	1	4 (18)	2	3 (14)	2	3 (17)	2	7-8 (29-33)	0	0 (0)
G	1	4 (18)	1	4 (18)	2	2 (9)	2	2 (11)	2	5 (20)	1	5 (25)
H	1	7 (32)	0	3 (14)	2	3-4 (14-18)	2	3 (17)	2	7 (29)	0	1-2 (5-10)

4.5.3. Comparison of Different Genotyping methods

39 out of 232 sera had low or undetectable HBV DNA not permitting genotyping by any method. Some of the patients were genotyped by more than one assay to confirm the results of our multiplex PCR. 83 patients were genotyped by VISGEN kits. The multiplex PCR had genotyped a total of 188 out of 232 patients (81%). Only two patients had false negative genotype and another two patients had different genotype than S-gene sequencing. The VISGEN genotyped 70 out of 86 (81.4%). Virus from two patients did not genotype by this method. One case was not genotyped by both methods, due to very low HBV DNA as measured by real-time PCR (table 4-5). Using the results obtained by direct sequencing and phylogenetic analysis of the S-gene as the reference method, the accuracy and specificity of HBV genotyping by multiplex PCR was high at 31/33 (94%).

The levels of concordance of the multiplex PCR with the results obtained by single genotype-specific primers, were 98% for single infection, 100% of multiple genotypes from the same as mix A for genotypes A, B, and C, and for double infections from different mixes A for genotypes A, B, and C, or mix B for genotypes D, E, and F. The multiplex PCR was superior to all other methods as sequencing and Visgen Kits in detecting double or multiple HBV genotypes infections as expected.

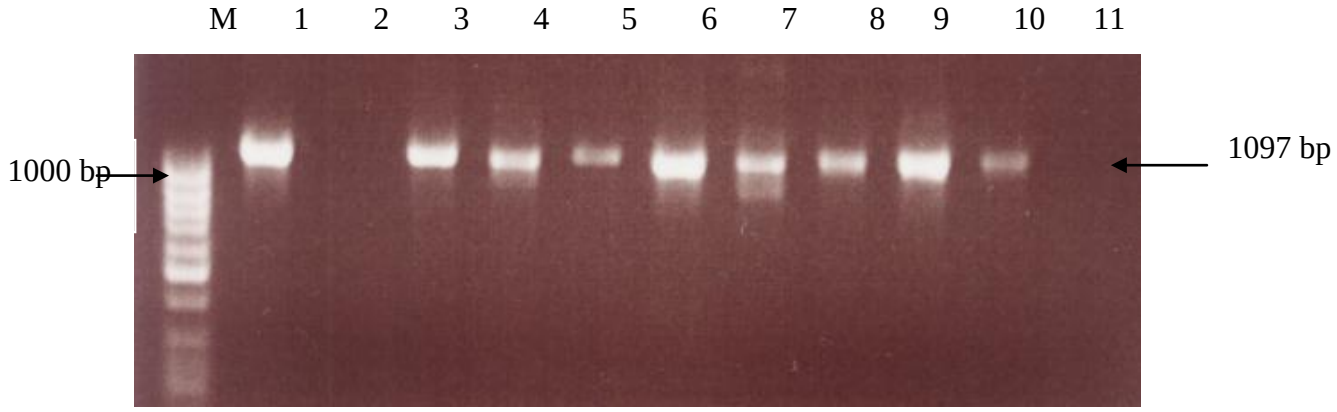


Figure 4-6: First round of Multiplex nested genotyping PCR:

Lane M: 100 bp DNA ladder, UK specimens, lane 1: specimen N1, lane 2: N2, lane3: N3, lane 4: N4, lane 5: N5, lane 6: N6, lane 7: N7, lane 8: N8, lane 9: N9, lane 10: N10, lane 11: Negative control.

Table 4-5: Detection by different HBV genotyping methods in sera from UK patients.
Concordance of HBV genotyping results between multiplex PCR, S-gene sequencing and core gene sequencing results with Visgen (p: 0.01).

Genotype	Multiplex PCR	S-gene sequencing	Core promoter sequencing	Visgen Kits	RFLPs
	(concordance with Visgen)				
A	^ 17/18	17/17	11/13	17/18*	6
B	11/11	6/6	7/9	11/11	2
C	6/8	6/6	5/7	*6/8	3
D	14/14	7/7	7/8	13/14	7
E	4/4	3/3	2/2	4/4	1
Multiple genotypes	2/0	0	0	0	0
Ungenotypable	9/11	3/3	3/3	9/11	0
Not done	0	31	27	0	0
Total	64	64	64	64	19

Note: Multiple genotypes were also counted with single genotype patients

*One case not genotyped by Visgen.

^Two patients showed bigger bands by multiplex PCR (p: 0.01)

Table 4-6: Comparison of genotyping techniques for HBV

Features	Multiplex PCR	S-gene sequencing	Core gene sequencing	Visgen Kits	RFLPs	Probe Hybridization
Principle	Genotype specific primers	Phylogenetic analysis	Phylogenetic analysis	Sequencing	Genotype specific restriction sites	Hybridization to genotype specific probes
Detection method	PCR	PCR, sequencing	PCR, sequencing	PCR, sequencing	PCR, RFLP	PCR, hybridization to labelled probes
Sensitivity	95.7%	80%	94 %	97.1%	94 %	99.3%
Specificity	98.5%	100%	91.7%	97.8%	100% *	100%
Detection limit	10^3 copies/ml	10^3 -10^4 copies/ml	10^3 copies/ml	10^3	10^4 copies/ml	10^4 copies/ml
Multiple genotypes	Detected	No	No	No	No	Detected
Comment	Rapid	Standard need experience	Tedious, need experience	Reliable, expensive	Need high HBV DNA level	Reliable, expensive

*Only 19 cases done

Another 19 patients were genotyped by a restriction fragment length polymorphism (RFLP) based method (Colindale). 14/19 (73 %) gave the correct genotype by multiplex PCR, another 4 patients were genotyped correctly after increasing the input DNA to 4µl giving a total yield of 18/19 (94.7%). On the other hand, our multiplex PCR correctly identified 3 patients infected with double genotypes and one case with triple HBV genotypes, which were not detected by the RFLPs-based assay.

4.5.3.1. Optimization of the multiplex PCR.

The multiplex genotyping PCR was done, but several problems were encountered at the beginning; the first round product of the PreS/S gene size 1097 bp using universal primers P1 and S2 (fig.4-6), was also used for sequencing of the S gene and phylogenetic analysis to confirm the HBV genotype and to verify the specificity and consistency of the primer-binding sites (Chapter 6).

The second round PCR bands were very faint to be visualized by gel electrophoresis despite the loading of 9 µl of the PCR products in each well. The PCR conditions were altered to give visible product, this further increased the band intensity, and improved the sensitivity of the reaction. Several nonspecific bands were seen in the second round with variable sizes and those could be due to multiple non-specific priming sites. The addition of Q-solution (QIAGEN) 5 µl per reaction had increased the stringency of the PCR, and diminished those nonspecific bands considerably.

The common primers B3 for mix A: genotypes A, B and C, and primer R10 for mix B: D, E, F respectively, were initially employed in the second round PCR. The band of genotype A was very small 68 bp and was easily missed because it was faint, and can be dismissed as primer dimer (fig.3-9), or the other way round giving false positive results as genotype A were interpreted in negative samples.

Consequently we modified the PCR products by altering the common A-C sense B3 primer to primer B13N (sense nt. 3139-3157), which solved the problem and significantly

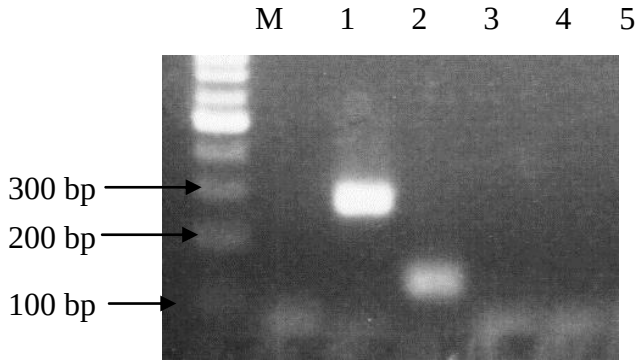


Figure 4-7: Multiplex genotype PCR; old primers:

M: 100- bp DNA ladder, lane1: genotype A, lane 2: genotype B, lane 3: genotype C, lanes 4 : mix B, 5: Negative control.

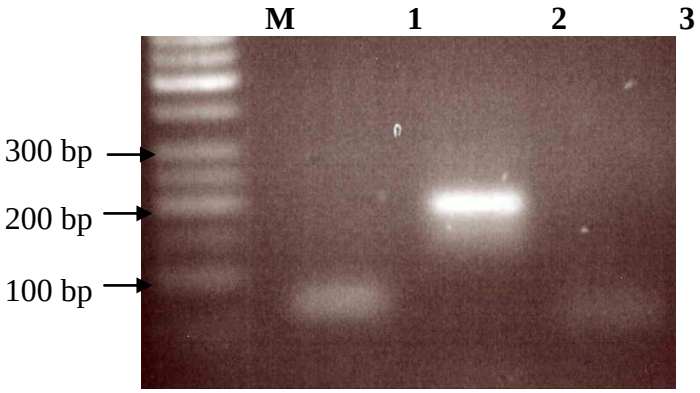


Figure 4-8: Multiplex genotyping PCR using new common primer B13N.

Lane M: 50- bp DNA ladder, lane 1: Genotype A, Mix A using primer old B3 primer, lane 2: genotype A control, Mix A using new B13N primer, lane 3: genotype A in Mix B showing primer dimers indistinguishable from the old 67- bp genotype A band.

improved the detection of genotype A by eliminating false positive results (fig.4-8). Although the drawback was the smaller difference between genotype A and C bands which increase to 178 bp and 230 bp respectively, necessitating the presence of both controls in each gel. Replacement of the common anti-sense primer R10 with primer R11 (anti-sense) had changed the proposed size of genotype F band is 66 bp which can go unnoticeable to 147 bp. Alternatively, genotype F–specific primer can be changed to (AGCACCTCTCTCAACGACA nt. 2840-2856) to better detect this genotype. However, we neither had a control genotype F stain, nor detected any case having this genotype.

To verify the avidity of genotype-specific primers in multiplex mixtures, pure plasmid genotype controls (cloning section) were used. No difference in the genotyping results of the multiplex PCR was found using single, different combinations or multiplex primer pairs together.

Originally, the detection limit of the genotyping PCR was 10^4HBV DNA copies/ml, but that was improved to10^3 copies/ml after optimization of the first round PCR conditions as mentioned in the methods chapter. The test was done on serial dilutions of the WHO HBV standard and plasmid preparations with known HBV DNA concentrations (see Chapter 2: Detection limits of different HBV PCRs).

Genotyping results of UK and Saudi specimens:

Genotyping 105 specimens from UK patients and 130 Saudi patients was done (fig.4-9), results are shown in tables4-8, 4-9.The first round PCR product covering the S gene was visible only in specimens with high HBV DNA measurment. Confirmation of genotypes was done by sequencing of the S-gene, and by Visgen HBV genotyping kits. Results showed the following fig4-8 and 4-9, table 4-7; No samples were detected in genotypes F, H, or G. Sample N2 was cloned into plasmid pCR 3.1 giving clone pEn2 whose sequencing shown genotype E. Overall, the high concordance between these results and sequencing results proved the reliability of our optimized multiplex genotyping PCR. Genotyping by VISGEN Kit had further confirmed our multiplex PCR results.

Some UK specimens showed an aberrant genotype A band (fig.4-8, lane 5 in mix A), that was reproducible as samples N5 and N13 come from the same patient.

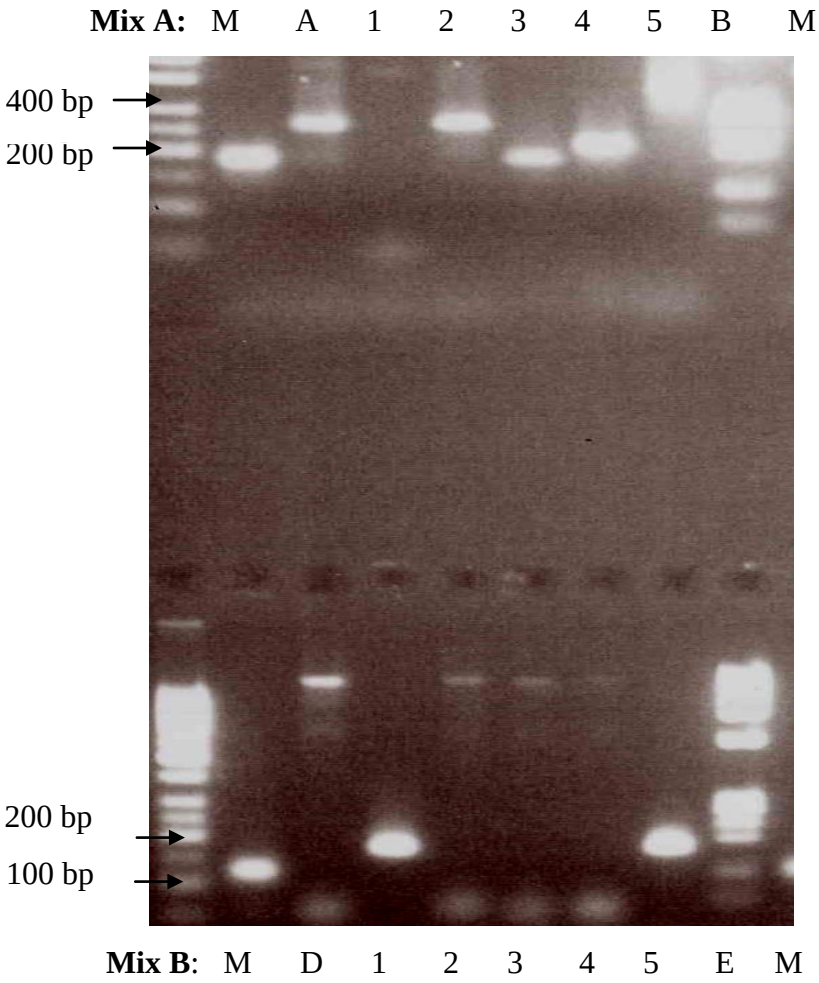


Figure 4-9: Modified multiplex HBV genotyping PCR on UK specimens

M:100 bp DNA ladder, Genotype controls A: genotype A, B: genotype B, D: genotype D, and E: genotype E. Specimens in mix A (above) and mix B (below) lane 1: N1: genotype C (230 bp mix A), lane 2: N2: genotype E (143 bp mix B), lane 3: N3: genotype C, lane 4: N4: genotype A (178 bp mix A), lane 5: N5: genotype A with slightly bigger sized band (200 bp mix A)**.**

Figure 4-10: 2% Gel electrophoresis of multiplex genotyping PCR of specimen N9 genotype B

Mix A: showing double bands 389 bp and 400 bp were separated.

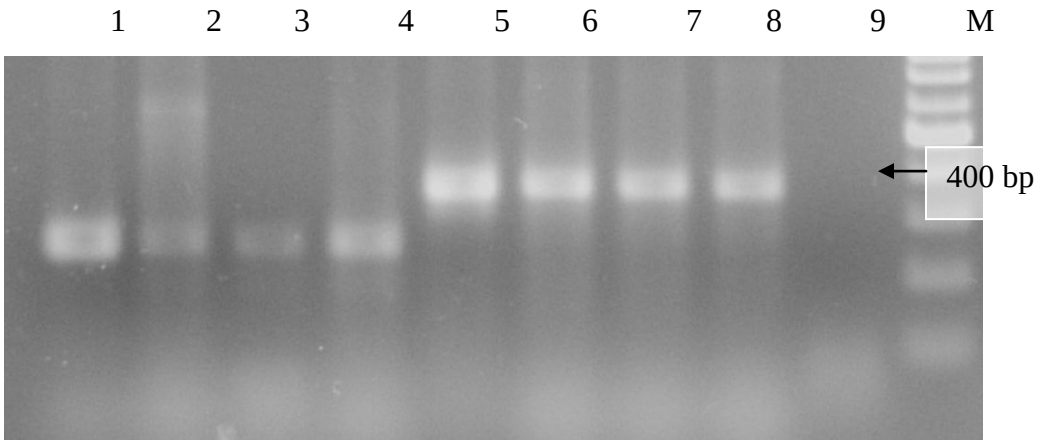


Figure 4-11: Cloning of specimen N9; giving two different clones;

Lanes 1-4 dilutions of plasmid pB10 bands sized 389 bp, and Lanes 5-8 dilutions of plasmid pB11 bands sized 400 bp, Lane 9: negative control, M: 100 bp-DNA ladder.

The band exhibited a size around 200 bp, between that of genotype A (178 bp) and genotype C (230 bp), sequencing did not show any aberrant primer binding site, and the phylogenetic analysis of the S-gene aligned it to genotype A. That was also confirmed by VISGEN Kit.

Another case had double genotype B bands, that consistently showed two different genotype B bands in all the three specimens collected from the same patient (N8, N9, and N10), thereby confirming the reproducibility of these results. Bands were excised and gel was purified using QIAquick PCR purification Kit (QIAGEN). One band was specific for genotype B (389 bp), while the other exhibited a size around 400 bp. Those bands were separated by prolonged gel electrophoresis (fig.4-10) and cloned into plasmids pB10 and pB11 (fig.4-11). Each clone had different genotype B band on genotyping PCR. The sequence of both pB10 and pB11 showed complete alignment with the original strain (N9) with genotype B. and both were genotype B.The reason for this is not clear. A possible explanation is that either there is another non-specific binding site for the primer, or the presence of single stranded DNA because the common sense primer B13N was a redundant primer, as we doubled its amount in the mix A reaction to provide sufficient quantity of each variety of this primer. A change in restriction site by polymorphism was shown upon computer analysis of one Gene Bank HBV isolate.

4.5.3.2. Double and multiple genotypes

Patients with double genotypes were not revealed by direct sequencing as expected. Several genotyping results of UK and patients showed multiple bands (4-12). Other genotyping methods did not reveal any case with multiple genotypes.

4.5.3.3. HBV genotypes in UK and Saudi patients

In UK, HBV genotype A was the most common, genotype D was less frequent followed by genotypes B and C (table 4-7). Genotype E was rare. Some genotypes B and C in UK patients had Chinese ethnicity, while most genotype D patients were Asian. In Saudi patients, genotype A and D were most frequent genotypes. Genotypes B, C and E had low frequency.

Table 4-7: The distribution of HBV genotypes in UK and Saudi populations. HBeAg status of different HBV genotypes in UK (p: 0.003) and Saudi patients (p: 0.001) including age of patients (p: 0.07).

Genotypes	UK				Saudi			
	HBeAg Positive	HBeAg Negative	Total (% within nationality)	Mean Age (years)	HBeAg Positive	HBeAg Negative	Total (% within nationality)	Age (mean in years)
	(% within genotype)				(% within genotype)			
A	8 (27%)	22 (%)	30 (30%)	45	2 (9%)	20 (%)	22 (22%)	42
B	8 (42%)	11 (%)	19 (19%)	38	0 (0%)	1 (%)	1 (1%)	42
C	5 (38%)	8 (%)	13 (13%)	31	1 (25%)	3 (%)	4 (4%)	34
D	6 (22%)	21 (%)	27 (27%)	31	12 (19%)	50 (%)	62 (62%)	41
E	0 (0%)	6 (%)	6 (5.5%)	46	0 (0%)	6 (%)	6 (6%)	47
Multiple genotypes	0 (0%)	6 (%)	6 (5.5%)	44	0 (0%)	5 (%)	5 (5%)	47
Ungenotypable	0 (0%)	4 (%)	4	52	0 (0%)	30 (%)	30	42
Total (valid %)	27 (26.7%)	78 (%)	105	40	15 (11.5%)	115 (%)	130	41

Table 4-8: HBV DNA level (by quantitative real-time PCR) of different HBV genotypes by multiplex PCR in all Saudi and UK patients (p: 0.001).

HBV Genotype	No. of Saudi Patients in each viral load group (log_{10} copies/ml)			Mean HBV DNA (patients no.)	No. of UK Patients in each HBV DNA group (log_{10} copies/ml)			Mean HBV DNA (patients no.)
	<=3	>3-<5	=>5		<=3	>3-<5	=>5	
A	3	5	6	4.22 (14)	-	8	14	5.53 (22)
B	-	-	-	-	2	3	13	5.65 (18)
C	1	-	2	5.01 (3)	-	4	6	6.13 (10)
D	8	11	28	5.10 (47)	3	4	10	6.10 (17)
E	1	2	3	6.09 (6)	-	3	1	4.69 (4)
Multiple genotypes	-	1	1	4.73 (2)	-	-	-	-
Ungenotyp-able	13	5	-	2.38 (18)	9	2	-	1.33 (11)
Total	26(29%)	24(27%)	40 (44%)	(90)	14 (17%)	24 (29%)	44 (54%)	(82)

Table 4-9: The correlation of HBV genotypes with clinical presentation of liver disease in UK and Saudi patients P: 0.192.

	Genotype	Inactive Hepatitis	Total Liver Disease	Acute Hepatitis	Chronic Hepatitis	Cirrhosis	HCC
UK patients	A	3	16 (84%)	1	15		
	B	3	12 (80%)	1	11		
	C	2	12 (86%)		8	4	
	D	3	17 (85%)	3	10	4	
	E	1	3 (75%)		3		
	Multiple	1	0 (0%)				
	Ungenotyp able	7	2 (22%)			1	1
Saudi patients	A	12	12 (50%)	2	9		1
	B	0	0 (%)		0		
	C	2	2 (100%)		2		
	D	12	50 (81%)	13	33	6	2
	E	2	5 (71%)		2	3	
	Multiple	1	2 (67%)				2
	Ungenotyp able	8	13 (62%)		10	2	1

In UK, HBeAg positivity is more in genotypes B, C than genotypes A and D, 42% , 38%, and 27% , 22% , respectively (table 4-7). Saudi genotype D had comparable number of patients with HBeAg to UK genotype D patients. Generally, HBeAg is higher in UK than in Saudi HBV patients at 26.7% and 11.5%, respectively.

High HBV DNA was seen in HBV genotypes A, B, C and D in UK patients, A and D in Saudi patients (table 4-8). Low HBV DNAs were mostly ungenotypable. In UK patients, genotypes C and D showed higher mean HBV DNA than genotypes A or B. Saudi cases had also higher mean

HBV DNA in genotypes D than genotypes A, but only 6 genotype E showed the highest mean HBV DNA. Genotype D had higher HBV DNAs in UK patients than in Saudi patients at 6.10 and 5.10 $\log_{10}$ IU/ml, respectively. Patients with HBV genotype A had low and intermediate HBV DNAs (table 4-8). Although most genotype A patients with high HBV DNAs were from UK.

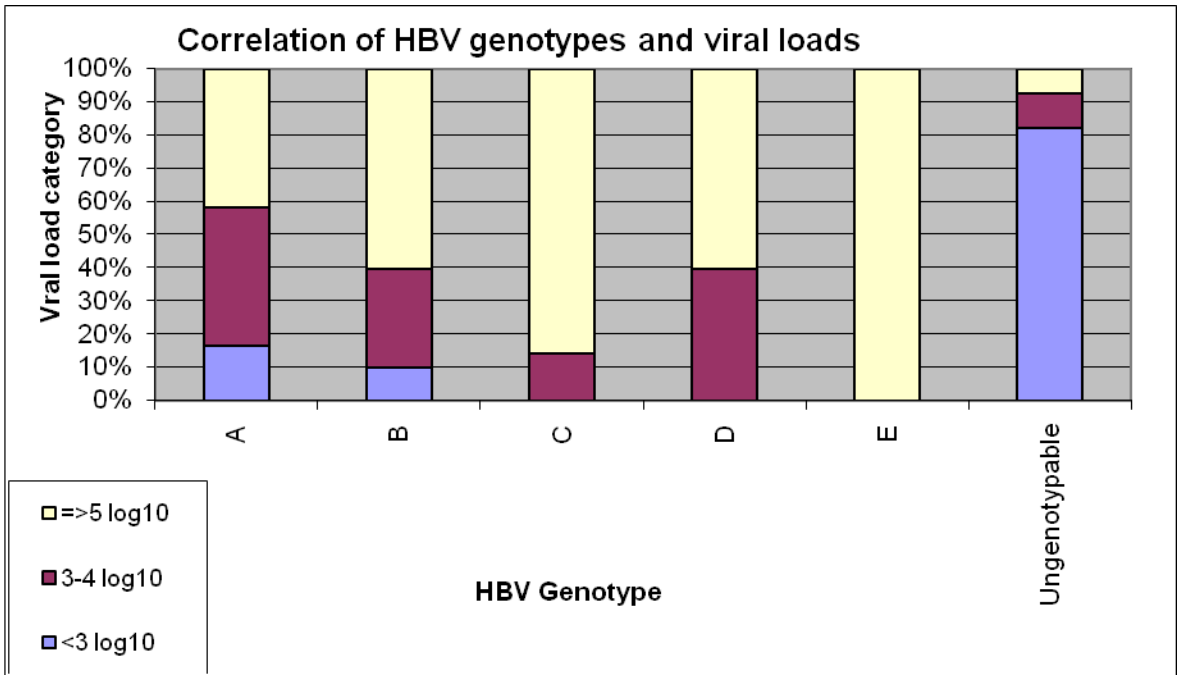
Regarding the clinical presentation of HBV in UK patients, genotypes A and D had equal proportion of inactive hepatitis and liver disease (table 4-9). Patients with acute hepatitis had genotypes A and D. Cirrhosis is present in genotypes C and D patients.

Saudi liver disease patients showed all spectrum of liver disease from acute hepatitis to HCC and had mainly HBV genotype D, and less proportion of genotype A.

4.5.3.4. Cloning of HBV genotypes A to E

4.5.3.5. PCR amplification of S-gene for cloning

The amplification of the S/pol -gene with primers P1 and B22 mapping nt. 2823 to nt. 996 (1389 bp) using control strains for genotypes A, B, C, D and E was done (fig.4-11). Single band was essential for cloning, because the smaller, unwanted products are easier to be incorporated into the vector. Genotype E showed visible band only after a second round of the same PCR. This PCR product covers the target sequences of both the multiplex genotyping PCR, and the RFLPs genotyping assay to provide high quality genotype controls for both tests. Quantitation of PCR product was done before cloning



Graph 4-1: Viral load of different HBV genotypes in all Saudi and UK patients

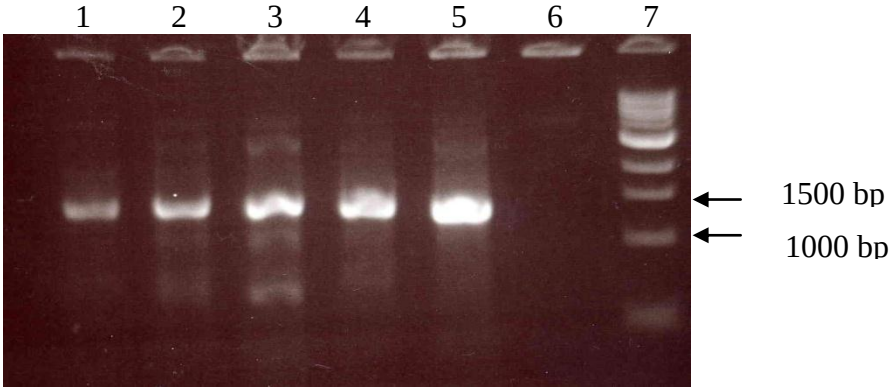


Figure 4-12: Quantitation of the extended S/pol gene PCR amplified using primers P1 (nt. 2823-2845) and B22 (nt. 996-977).

Product size 1389 bp. Using patients specimens, Lane 1: genotype A (WHO), lane2: genotype B (N9), Lane 3: genotype C, Lane 4: genotype D (3-38), Lane 5: genotype E (N2) (2nd round PCR), Lane 6: Negative control, and 7: quantitative 1kb DNA ladder (BioLabs: N3232S). Genotypes B, C and D showed good bands. Genotype E showed visible band only after a second round of the same PCR.

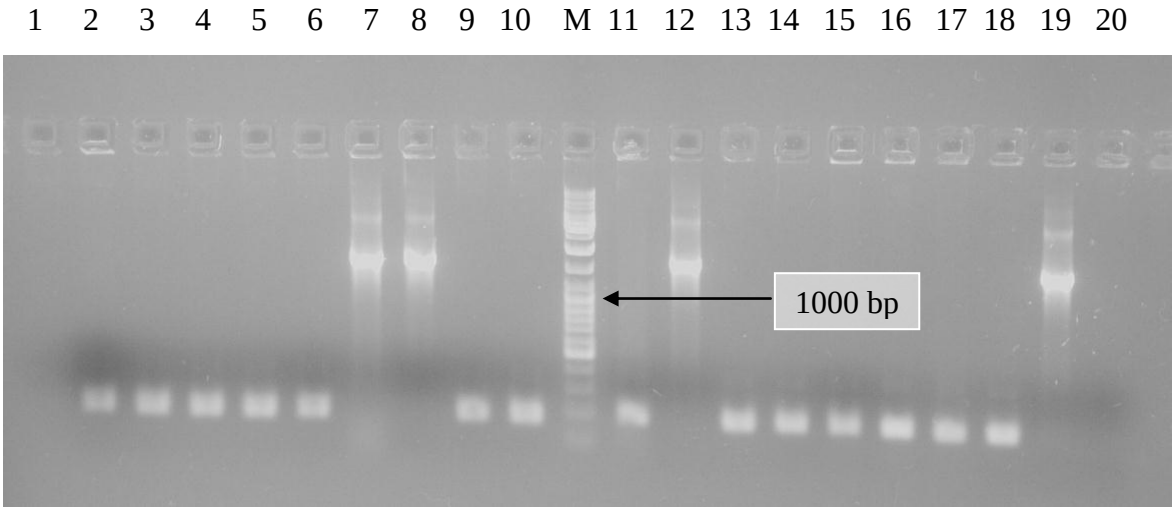


Figure 4-13: Gel electrophoresis of T7, BGH screening of genotype B and genotype C clones.

Lane 1:Negative control, lane 2: clone B5, lane 3: clone B6, Lane 4: clone B7, Lane 5: clone B8, Lane 6: clone B9, Lane 7: clone B10, Lane 8: clone: B11, Lane 9: cloneB12, Lane 10:cloneB13, M: DNA marker (100 bp ladder), Lane 11: clone C5, Lane 12:clone C6, Lane13: clone C7, Lane 14: clone C8, Lane15: clone C9, Lane 16: clone C10, Lane 17: cloneC11, Lane 18: clone C12, Lane 19: clone C13, Lane 20: Negative control. Only clones in lanes 6, 7, 11 and 18 show the appropriate sized inserts (1600 bp). The rest of clones show no inserts as evidenced by the small size (200 bp) of the PCR product.

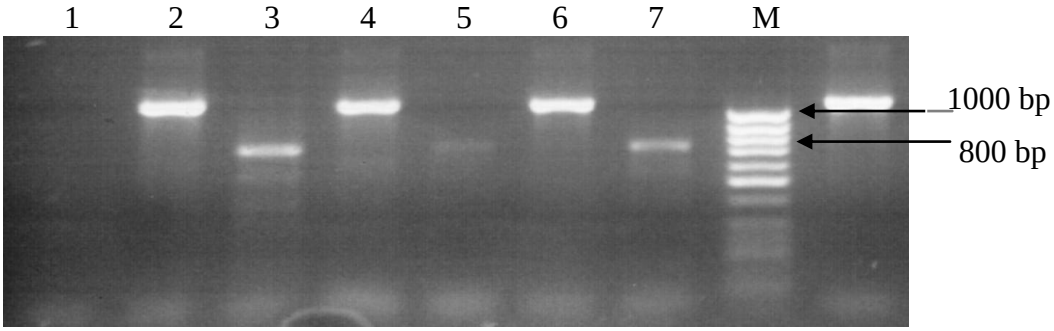


Figure 4-14: Orientation of plasmid inserts

Plasmid preparations were screened by PCR using plasmid primer T7 and the internal primer B13N (T), and another PCR using primers B13N and BGH (B). Lane1: negative control, Lane2: pW6-T, Lane 3: pW6-B, Lane 4: pW7-T, Lane 5: pW7-B, Lane 6: pB11-T, Lane 7: pB11-B, M: 100- bp DNA ladder. The expected product ~ 1180 bp, which is shown in lanes 2, 4 and 6 from antisense1400 bp insert (T7 and B13N primers).

using DNA marker with known concentrations (Roche molecular weight Marker XIV (100 bp ladder) (11721933001).

4.5.3.6. Ligation and transfection of PCR product

The PCR products were ligated to plasmid pCR3.1, then transfected into competent Top10 E. coli. After overnight incubation, the colonies were screened for the presence of the appropriate insert using vector specific T7 and BGH primers with bands about 1600 bp (fig.4-12). The transformation yield was low, as only two clones were positive out of the 10-15 clones screened from plasmids for each HBV genotype.

Plasmids were sequenced to confirm their identification and genotypes, and were further analyzed using SeqMan 3 of DNA Star software package.

4.5.3.7. Determination of insert orientation within plasmids:

After all plasmid preparations had a confirmatory screen for the correct size (1600 bp) inserts using vector specific primers T7 and BGH (fig.4-13). Several clones were screened to obtain the correct ones. The orientation of each insert inside plasmid was examined, using T7 and B13N, then B13N and BGH primers. The expected product ~ 1180 bp which is shown in lanes 2, 4 and 6 from antisense 1400 bp insert (T7 and B13N primers). The second product is ~ 780 bp from a 1000 bp insert (fig.4-14). Possible explanation is a second priming site for B13N or BGH within the amplified S gene of HBV.

4.5.3.8. Single primer verification of individual genotype clones

The genotype of each plasmid preparation was verified using the corresponding primer mix (fig.4-15). Six single primer pairs were also used separately to verify the reliability of the multiplex PCR with each genotype (fig.4-16). All plasmids gave positive bands in both the multiplex PCR and the corresponding genotype specific single primer pair. In addition, negative results were seen with all other genotypes to further prove the specificity of the genotyping.

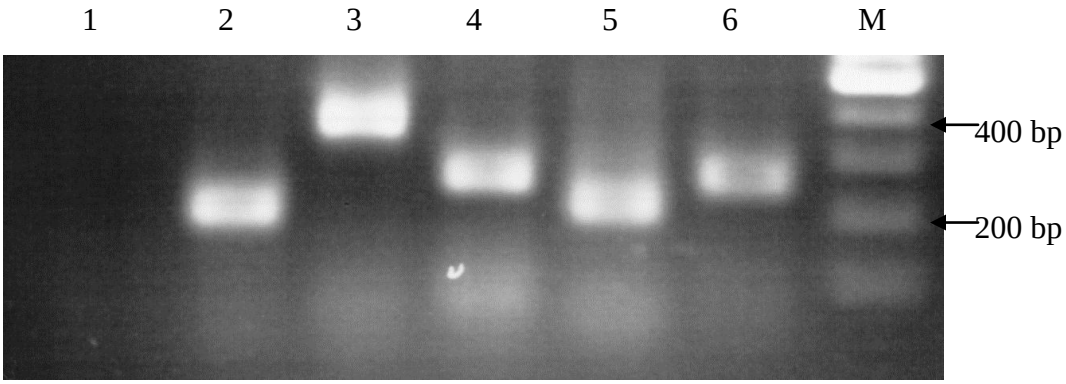


Figure 4-15: Verification of plasmid preparations genotypes:
Using multiplex genotyping PCR to confirm the genotype of each plasmid; lane 1: negative control, Mix A: lane 2: pW7 genotype A, lane 3: pB11 genotype B, lane 4: pC6 genotype C. Mix B: Lane 5: pD5 genotype D, Lane 6: pEn2 genotype E, M: 100 bp DNA ladder.

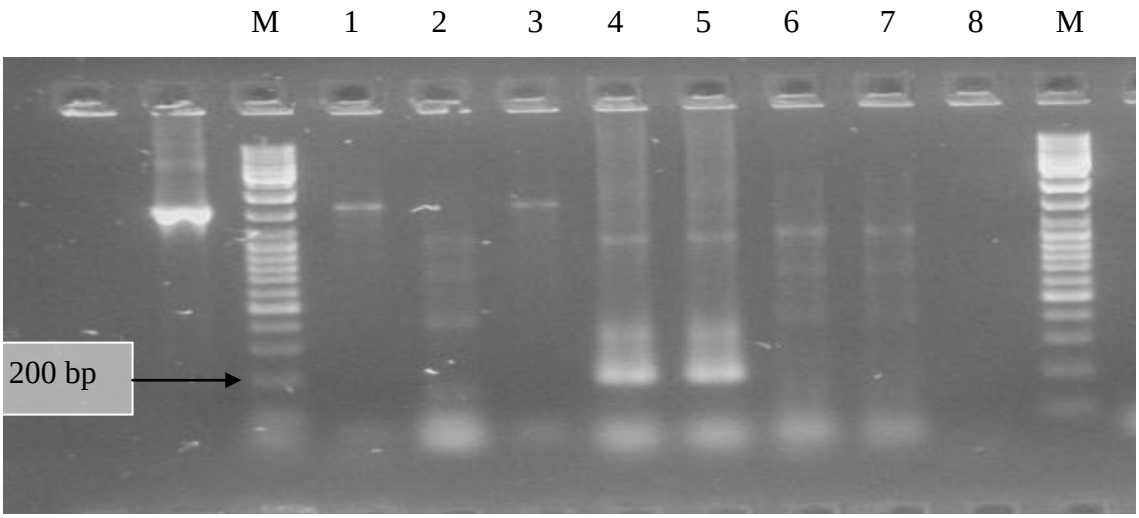


Figure 4-16: HBV Genotype D PCR using single primers

Lane M:DNA ladder, lane 1: genotype A-specific primerA4 and B13N, Lane 2: genotype B-specific primer B5 and B13N, Lane3: genotype C-specific primer C6 and B13N, Lane 4: multiplex PCR mix B, Lane 5: genotype D-specific primer D7 and R11, Lane6: genotype D-specific primer E8 and R11, Lane 7: genotype D-specific primer F9 and R11, Lane 8: Negative control.

4.6. Discussion

In molecular approach, the presence of pathogen or product cannot be demonstrated without searching specifically for it, unlike general virology methods as cell culture or electron microscopy. The concept of multiplexing molecular tests as PCR originated from the need to screen for large number of different viruses, genotypes or sub genotypes together. Multiplex PCR provides a simple, precise, sensitive, easy to use, and practical method to identify the different genotypes of HBV. Verification of the specificity of these primers was essential. Based on the alignment of complete nucleotide sequence of different genotypes, the specific sequence of each genotype was found. The six primers used were one for each of the six genotypes according to genotype-specific sequences. Primer pairs were used separately and in multiplex PCR. Several multiplex-PCR assays are described, that uses genotype-specific primer pairs for HBV genotypes A-F (Kirschberg, 2004). These primer pairs specifically amplified HBV DNA of the respective genotype, either in single genotype PCR or in multiplex.

Non-specific binding of primers can occur because of the minimal difference between genotypes, which may cause non-specific products of multiplex PCR as well as false genotyping. This assumption can be verified by comparing the primer binding sites of each genotype, and by analyzing the PCR products and sequencing the isolates, or by using different genotyping method as a confirmatory assay for the multiplex PCR results (Gunther, 2006).

Detection limit

This modified multiplex genotyping PCR had a high percentage of valid assay runs (95.4%). No false-positive results detected. Sensitivity was increased after optimization. In multiplex PCR, careful selection of primers is needed to avoid primer interactions that interfere with the sensitivity of the reaction. Each separate PCR was optimized singly on single and mixed genotype plasmid controls, before being assessed in optimized multiplex PCR (data not shown). Thus, further assessing the sensitivity of the multiplex assay for simultaneous detection of mixed infections and low copy targets in high copy

backgrounds as recommended by Gunson *et al.,* (2006). The number of cycles required for optimum amplification varies depending on the quantity of the starting materials and the efficiency of each amplification step.

Many of our specimens up to 30 specimens out of 160 (18.8%) (UK and Saudi sera) were non-genotypable by any method. The HBV DNA assay confirmed their low $<10^3$ copies/ml or negative HBV DNA in the serum (table 4-8). Many of those had very low or negative DNA, and therefore their genotyping was not feasible. Some specimens gave results only after increasing the input DNA to 3µl, but this also increased the non-specific bands. Due to the variability of HBV, or their detection limit, some diagnostic procedures risk giving false-negative results as reported in other studies (Bartholomeusz and Schaefer; 2004), thus influencing the yield of this method.

Co infection

Different methods were used to detect co infection with more than one HBV genotype. The drawback of multiplex PCR is the possible interference or masking of one component of test with the other products as found in mixing experiments (data not shown). It easy to detect double genotypes if they were in separate mixes; mix A (genotypes A, B and C) or mix B (genotypes D, E and F). Within the same mix, multiple genotypes are suggested by multiple bands, which then can be confirmed by single-genotype primer pair PCRs used separately. We used cloning and sequencing to confirm double infection. Genotype-specific probes assay GSPA, better identified co-infection with HBV genotypes than PCR-RFLP on the S region, PCR-RFLP on the pre-S region (Lim et al., 2007), and 28 (10.9%) by genotyping using hybridization with type-specific probes, while it was suggested by ELISA in only 2 (0.8%) sera (Kato et al., 2003). Coinfection seems to be more frequently detected by these methods. Different genotypes can co-exist, for example G and A or D and A, and the predominant genotype may shift during infection (Gunther, 2006). Sequencing cannot detect mixed genotype infections. Discrepant as well as indeterminate results can occur between the different methods (Osiowy et al., 2003) (Bartholomeusz and Schaefer; 2004).

Contradicting genotype results raises the possibility of recombination. Recombinations involving genotypes A/C, A/D, A/E, B/C, and B/D have been described (Kurbanov et al., 2005). Sequencing of suspected regions of HBV genome will exclude such a possibility.

Genotyping by RFLPs

Among the existing HBV genotyping methods, restriction fragment length polymorphism (RFLP) based methods are used widely, but HBV genetic variability may lead to wrong results. Comparison between RFLP and the multiplex PCR described gives 4% of discordance results, no untypable samples by RFLP.

We had done HBV genotyping using RFLP method (Mizokami et al., 1999), but that was unsuccessful for several reasons; first, the detection rate of the test was very low that is more than 4 $\log_{10}$ copies/ml as found in the cut-off experiments (data not shown). This did not suit our samples as a high proportion had low HBV DNA that was below this detection limit, giving no PCR product or faint band to begin with. Secondly, high amount of RFLP-PCR product was needed to cover the four different RFLPs digestions and an undigested control. Thirdly, the RFLPs scheme used was designed primarily to detect HBV genotypes B and C, which were not the main genotypes prevalent in UK or in Saudi patients. Lastly, the digestion into several bands was difficult to interpret, unlike RFLPs reactions, which digest into limited products whose sizes are distinct from the original starting band. In addition, a change in restriction site by polymorphism was shown upon computer analysis of one Gene Bank HBV isolate. Several RFLPs genotyping assays had been described, including a single RFLP assay (Rodriguez-Novoa et al., 2004) for both genotyping and lamivudine resistance. Other groups pointed out the limitations of RFLPs techniques, which need PCR conditions of sufficient specificity to produce a clean product that is digested and unambiguously analyzed. Incomplete digestion of the amplicon can further complicate the interpretation (Payungporn et al., 2004). A change in the sequences can abolish a restriction site or introduce a new one.

Cloning is used in HBV genotypes for several purposes. Known concentrations of cloned plasmid was essential both to determine the detection limit of each PCR assay, and to serve as control for quantitative real time PCR, and for all genotyping experiments as

PCR and RFLPs. Validation of reliable genotyping multiplex PCR required pure single genotype controls that give reproducible results with single primers. Also used for confirmation of patients with double genotypes by sequencing of all strains. Cloning was the only way for providing good controls to validate the specificity of the primers used, not only because of the higher concentration that can be achieved by plasmid encoded HBV, but also because of the purity of plasmid preparations.

Our cloning vector was TA pCR 3.1 that was generally used for rapid cloning of PCR products without the need for restriction enzyme step before cloning. We successfully cloned the S-gene and part of the overlapping Pol gene of five HBV genotypes A to E, for the above reasons, and as a prerequisite to study differences in proteins encoded by different HBV genotypes and mutants. The HBV preS domain encodes hydrophilic polypeptides that had been implicated in the binding of the virus to hepatocytes, and in the induction of virus-neutralizing antibodies. Vaccine-escape mutants may cause alteration of antigenicity of HBsAg and binding to anti-S antibodies. Cloning and protein expression has been also used to study the pathogenesis, assess the replication capacity of clinical HBV isolates and to analyze drug susceptibility (Cheng et al., 2003). Site-directed mutagenesis has been used to study the effects of point mutations in cloned HBV strains. The majority of cloned HBV genomes is suitable for further phenotypic characterization and can be used in resistance surveillance, cross-resistance analyses, and novel drug-discovery as adefovir susceptibility (Yang et al., 2004).

HBV genotypes in UK and Saudi patients

Eight genotypes A-H of HBV are known with variations in nucleotide sequences greater than 8%, while intra-genotype differences are =<4% (Gunther; 2006). HBV genotypes may account for the heterogeneity in disease manifestations among patients with chronic HBV infection. Large epidemiological studies will require the availability of a practical system that is rapid and reliable.

In Saudi, genotypes D, A, and UK patients genotypes A, D and B were more prevalent. The genotype distribution of UK patients is more of genotype A and D than the less

common genotypes B, C and E. Patients from Nottingham had genotypes A and B more than C, while the samples from London (Colindale) had mainly genotypes A and D. Possibly reflecting a more cosmopolitan population in the London area. Another study from UK detected prevalence of HBV genotypes A 41% , B 12% , C 5% , D 30% and 12% for mixed-genotype infections(Davidson et al., 2005). Non-Caucasian genotypes are present in the UK blood-donating population and will therefore affect the demographics of HBV infection. An example of changing molecular epidemiology of HBV is the widespread dissemination of a regional HBV strain in England, which is primarily associated with injection drug use (Hallett et al., 2004). A strong correlation was found between HBV genotypes and ethnicity. Similarly, in a study from USA, HBV genotype A was prevalent among white and black patients, whereas genotypes B and C were most common among Chinese patients (Chu et al., 2003).

On the other hand, Saudi patients had higher proportions of genotype D (62%) than A (22%), and lower frequency of genotypes B, C and E. None of our patients had HBV genotypes F, G or H. Abdo *et al.,* (2006) demonstrated higher prevalence of HBV genotype D (81.4%) and lower prevalence of genotype A (1.4%) but comparable rates of genotypes C (1.4%) and E (5.7%) from a tertiary center in Saudi Arabia. Genotype D is the most prevalent in the Middle East (Saudy et al., 2003).

Differences in the natural history of infection with different genotypes may have affected the prevailing transmission route of the virus. The mean age of UK patients with genotypes C and D was younger at 31 years. Saudi patients have mean age of 41 to 42 in most genotypes. Genotype B patients were reported to be significantly younger than genotype C patients (mean age 29vs.37years) (Kao et al., 2004) possible due to more acute presentation of the disease at young age.

HBV genotypes, HBsAg and HBeAg

Excretion of HBsAg was most abundant for Ae followed by Aa, Ba, Bj/C and remotely by D, which was consistent with mRNA levels (Sugiyama et al., 2006). In UK patients, the frequency of HBeAg was much higher than the Saudi group, reaching 26.7% versus

11.5% (genotype), respectively. On the other hand, HBeAg-negative phenotype is predominant in Saudi patients. That reflects more recent acquisition, or earlier detection of HBV in young UK patients. In UK, HBeAg is higher in genotypes B, C than genotypes A and D, 42%, 38%, and 27%, 22%, respectively (table 4-7). This suggests that genotypes B and C run more active course, or trigger less vigorous immune responses against them by eliciting an immunotolerant state than genotypes A and D.
The proportion of anti-HBe antibodies in Saudi genotypes A and D was 9% and 19%, which can also be explained by the high chronic carriage of HBV in Saudi Arabia. This is in agreement with Ganne-Carrie *et al.,* (2006) who demonstrated an increased prevalence of anti-HBe antibodies in patients infected with genotype D. The differences in HBV genotype prevalences and, or the HLA types and immune responses may also contribute to this discrepancy of anti-HBe prevalence between the two populations.

Several studies have demonstrated differences in HBeAg seroconversion in different HBV genotypes that could be due differences in viral replication, expression of HBeAg and severity of liver disease. In Karachi, genotype D chronic hepatitis B patients 71/109 (65%) were HBeAg negative (Abbas et al., 2006). HBeAg was significantly higher in genotype C than in genotype D (Duong *et al.,* 2004). HBeAg-seroconversion occurred one decade earlier in genotype B patients with a rate of 47% vs.27% compared with genotype C patients (Kao *et al.,* 2004). Within genotype B, HBeAg is more prevalent in subgenotype-Ba 16/62 (26%) than subgenotype-Bj 5/66 (8%) (Sugauchi et al., 2003).

HBV genotypes and viral loads

Our results showed higher HBV DNA levels in genotype D than genotypes A (table 4-8). Genotype D showed higher HBV DNA than genotype A in UK and Saudi patients, at mean. Many of patients with inactive hepatitis were ungenotypable, perhaps because of their low or undetectable HBV DNA. HBeAg correlates differently with viral load in different genotypes; as Lindh *et al.,* (2000) found that HBeAg positive genotype B have higher DNA level than genotype C at 9.1 and 7.6 $\log_{10}$ copies/ml. Conversely, anti-HBe positive genotype B has lower HBV DNA than genotype C at 4.5 and 5.5 $\log_{10}$ copies/ml,

respectively. Similarly, HBV DNA levels were the lowest for sub genotype Aa (Asia/Africa), followed by Bj/Ba and D/Ae, and the highest for genotype C (Sugiyama et al., 2006). HBV DNA levels in sub genotype Aa carriers were significantly lower than Ae or genotype D (median, 3.46, 6.09 and 5.48 log copies/mL), respectively (Tanaka et al., 2004).

Clinical presentation of HBV genotypes

In our patients, genotype D was predominant in Saudi asymptomatic carriers with inactive hepatitis, which is consistent with Doung *et al.,* (2004), where most of the 413 carriers with HBV genotype D were asymptomatic carriers (84.2%). Acute hepatitis had predominantly HBV genotype D both in UK and Saudi Arabia (see table 4-9). No fulminant hepatitis patients detected in both UK and Saudi group. In Japan, the occurrence of HBV genotype B in patients with acute hepatitis was significantly greater than in those with chronic liver disease (39.3% v 11.7%, respectively (Imamura et al., 2003). ALT levels were lower and HBV DNA titres were significantly higher (5.90 vs. 5.13$\log_{10}$copies/ml, and HBsAg persisted longer with milder clinical course in patients infected with genotype A than C (Yotsuyanagi et al., 2005).

The proportion of HBV genotypes differs with the various clinical features of HBV infection. The clinical presentation of our genotype D patients ranged from inactive hepatitis, acute hepatitis, chronic hepatitis, cirrhosis to HCC. Saudi patients with advanced liver disease had high prevalence of the HBV genotype D (table 4-9), compared to patients with inactive hepatitis who had genotype A more than D. UK patients with genotype C all had HBeAg positive as a reflection of active liver disease. Researchers demonstrated more severe liver fibrosis and higher serum transaminases in patients infected with genotype A and D (Ganne-Carrie *et al.,* (2006), while others did not (Yalcin et al., 2004). This can be explained by the prevalence patterns of HBV genotype rather than their effect on liver disease progression. Genotypes B and C reported to be more prevalent in cirrhosis and HCC patients (Sakamoto et al., 2006), especially subgenotype C2 (Kim *et al.,* 2009). HBV Genotype B patients exhibited more severe hepatic lobular

necroinflammation, less portal inflammation and fibrosis than genotype C patients did (Watanabe et al., 2005).

Although the clinical presentation of Saudi patients showed association of the HBeAg-positivity with active liver disease (table 4-9). HBeAg-negative states showed no significant correlation with liver disease activity. Thus, parameters other than HBeAg are needed to evaluate HBV patients as viral load in HBeAg-negative patients. Our results showed the association of genotype A with inactive HBV carriage and genotype D and to lesser extent genotypes A and E with chronic liver disease. HBV genotypes may be related to severity of liver disease. Generally, large-scale longitudinal studies are needed to find the relationship of HBV genotypes to liver disease severity and clinical outcomes.

4.7. Conclusion

This multiplex PCR genotyping method is suitable for rapid, large-scale epidemiological screening and clinical diagnosis of those virus infections occurring alone or in combination, but is also rapid and cost-effective. Virological differences among HBV genotypes were demonstrated that might influence HBV infections in clinical and epidemiological settings. HBV genotypes A and D are prevalent in UK, less frequently are genotypes B, C. HBV genotypes D followed by D are common in Saudi HBV patients.

4.8. Future Direction

Comparative studies using panels of sera from individuals infected with different HBV genotypes need to be performed to assess the influence of genotype on HBsAg, and anti-HBs determinations.

Protein expression of genotype-specific proteins large HBsAg and HBxAg, and studying the variation in their antigenicity and cellular interactions in different HBV genotypes

Study of disease progression and response to therapy in patients infected with different HBV genotypes.

Chapter Five : S gene and Pol-gene

5.1. Introduction

5.1.1. Genotype-related variations in S/Pol gene:

In HBV S-gene, HBsAg functional parts (transmembrane, extracellular, major hydrophilic regions and antigenic variants) are nearly identical for all genotypes (Stuyver et al., 2001). Only the pre-S1 region has length polymorphism. Because the efficiency of the backwards scanning mechanism depends on the distance between the 3`end of the core ORF and the initiation of the polymerase ORF, which is 143 nucleotides for genotype B to G, 149 for genotype A, these length polymorphism might help control the translation of the polymerase protein from pregenomic mRNA in a genotype-dependent manner.

Regarding pol gene, the amino terminal part of the terminal protein (tp) of genotype A has two additional amino acids (tpG17 and tpT18) (fig.1-7). This affects the overlapping ORF for HBxAg (cD153 and cR154), as this length polymorphism is located immediately at the carboxy terminal of the HBeAg processing site (Stuyver et al., 2001). According to the old continuous numbering system of the HBV polymerase (fig.1-2), the tyrosine-methionine-aspartate (YMDD) motif, which is related to lamivudine resistance, change from methionine to valine or isoleucine is genotype dependent. Thus, for genotype A, it is M552V/I; for genotype B, C and F, it is M550V/I; for genotype D, it is M539V/I; and for genotypes E and G, it is M549V/I. Corresponding to (rt) 203-206 in the old numbering system. Yano *et al.*, (2006) demonstrated that the incidence of YMDD mutants was slightly higher in those who were younger, had BCP, higher HBV DNA titres, lower ALT levels and genotype C.

5.1.2. S Gene Mutations

Mutations that alter the ratio of LHBs, MHBs and SHBs during infection variants may affect the pathogenesis of infection. High levels of LHBs or Pre S region mutations may inhibit particle secretion. Very few mutations within the pre S-1 residues 21-47 alter HBV binding to human hepatocytes (Huang et al., 2004). Deletion at the 3’ end of the pre

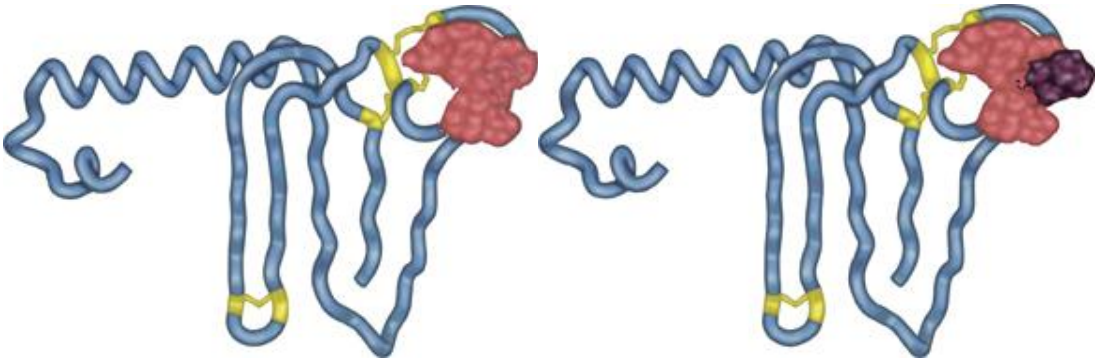


Figure 5-1: Wild type and mutant HBsAg (gly-145-arg)

Molecular models of HBsAg showing the "a" determinant site (pink) in wild type and HBsAg mutation where an arginine substituted for a glycine at amino acid position 145 (purple) is the most common (Adapted from Chen et al., 1996) National Academy of Sciences, USA. (Norder et al., 2004)

S-1 region, at the S gene promoter, results in lower S gene expression and lower virus production, that potentially lead to impaired viral clearance without affecting HBV attachment to hepatocytes and their subsequent penetration, and therefore contribute to the development of chronic hepatitis (Huang et al., 2004).

Pre S-2 determinants are targets for virus neutralization and are much more immunogenic than the S region of HBsAg (Kreutz, 2002). B-cell epitope within residues 14-24, the amino terminal 25-30 residues and an adjacent T-cell recognition sites within pre S-2 residues 30-55 indicate that mutations within these regions may compromise the host ability to eliminate acute infection. However, pre S-2 deletions are clustered around immunodominant epitope, indicating their selection by the immune responses against wild type HBsAg (Kreutz, 2002). In acute hepatitis B, recombination within the pre-S region emerged transiently and the pre-S deletion mutant was finally cleared. However, patients with progressive liver diseases have a higher frequency of pre-S deletion (Chen et al., 2006). PreS mutation accounts for virus retention and misassembly. Abnormal expression of HBV proteins can result in cytotoxicity and ground-glass appearance of

liver cells, complicated eventually by cirrhosis, fibrosing cholestatic cholangitis and HCC (Weber; 2005).

Mutation or deletion of sequences 48 and 27 bases upstream from the pre S-2 translation initiation site or lack of the pre S2 initiation codon, resulted in lower levels of MHBs and SHBs compared with LHBs, suggesting that preS2 including the PHSA binding site (preS-2 amino acid residues 17-29) (Kondo et al., 2002) may not be crucial for viral persistence and replication. In some cases, the LHBs component has aberrant electrophoretic mobility as well. Mutations preventing pre S expression were associated with the accumulation blocked virus secretion (Hartmann-Stuhler; 2001).

Mutations with frame shift, microdeletions or point mutations. Mutations preventing pre S expression were associated with the accumulation of supercoiled DNA from cytoplasmic core particles intervene by different mechanisms at the translational or post-translational level, including conformational or hydrophobic changes, insertion of basic residues, and reduced synthesis or secretion of HBsAg. Consequently, mutations may account for altered immune recognition, not recognized by vaccine-induced antibodies or interferes with detection by immunoassays (Weber; 2005).Lee et al., (2003) demonstrated that in HBsAg mutant proteins, the two N-linked glycans at Asn15 and Asn123 result in low titres of pre-S1 and S2-neutralizing antibodies. Several other point mutations are summarized in (tables 5-1 and 5-2). Mutation at nt. 551 of the HBV (adr) genome from A to G, leads to a substitution of methionine (Met) to valine (Val) at position 133 in the "a" determinant of HBsAg. Compared to the wild-type HBsAg, the binding activity of the mutant HBsAg to monoclonal antibodies (A6, A11 and S17) and to vaccine-raised human anti-HBs antibody decreased significantly (Chen et al., 2003).

S gene mutations affect immunogenicity and infectivity as well, although mutant HBsAg can still induce protective humoral immune responses (Ge et al., 2004). The wide spread vaccination in the WHO extended program of childhood vaccination, and other group vaccinations may increase the chances of development of HBV escape mutant, though the benefit of the vaccine far outweighs the risks. Exploring the antigenic differences

between HBV genotypes may influence the improvement of current vaccines. Vaccine escape mutants also occur in liver transplant patients immunized, and or treated with prophylactic hyperimmune gamma globulin. Mutations in the `a`epitope were correlated with the absence of detectable anti-HBsAg (Huang et al., 2004) (fig.5-1). Acute HBV infection may also occur in the presence of protective levels of anti-HBs (Gerlich, 2006). Detailed studies and enhanced surveillance to monitor the emergence of these variants are essential in evaluating the current immunization strategies.

HBsAg may co-exist with anti-HBs during the transition from acute to chronic infections. The virus continues to replicate at low levels, as evidenced by detectable HBV DNA in up to 27.2% of anti-HBs positive individuals (Chaudhuri et al., 2003). Here HBsAg may not be neutralized fully by the anti-HBs present, implying the existence of HBsAg variants with altered antigenicity. Hence, not all anti-HBs positive infections are associated with virus elimination. Mutants are also increased in amount by the selective pressure of therapy.

Mutation occurred at codons123, 124, 126, 129, 131, 133, 137, 140, 142 134, 143, 144 and /or 145 (Tsatsralt-Od et al., 2007), result in 1-2 amino acid differences at the `a`determinant in SHBs , reaching43% in occult hepatitis B infections including Q101K, T115A, K122N, T123A, T126N, Q129N, G130R, T131I, M133T, F134L, C138Y, K141E, P142S, D144E with or wothout145 glycine to arginine (G145R), N146S, and C147F/R alone or in combinations (Hou et al., 2001). Point mutation in and around the "a" determinant of HBsAg; allow continued replication of these variants at wild type levels and persistence in susceptible populations. In infants of HBsAg positive mothers, the incidence of HBV S-gene mutants reach 23.1 % of vaccine recipients (Hsu et al., 2004), to 51% of infants with chronic HBV infection acquired after post exposure prophylaxis (Nainan et al., 2002).

5.1.3. Natural HBsAg negative HBV infections

It is not clear whether HBsAg negative infections (fig.5-2) are due to loss of the HBsAg or are due to HBsAg negative variants. Most of these low titer defective replication

variants have low pathogenicity encouraging chronicity of atypical infections. Nevertheless, some HBsAg-negative infections are highly pathogenic. The role of HBV mutants in HBsAg negative liver disease, hepatocellular carcinoma and fulminant hepatitis have to be elucidated. Screening of transfused blood for anti-HBc prevents post transfusion hepatitis and detects HBV variants negative for HBsAg. The presence of HBV DNA in serum and liver of HBsAg-negative or in HCV patients (Tsatsralt-Od et al., 2007) suggests the presence of these or related variants as well. The main threat of HBsAg-negative mutants lies in the safety of blood transfusion system. In endemic areas, it is preferable to test blood donors for anti-HBc antibodies, which are positive in more than 95% of HBsAg-negative cases (Hsu et al., 1999). Monoclonal antibody binding tests revealed decreased immunoreactivity in 6 variants of HBsAg (Hou et al., 2001).

The ability of polyclonal and monoclonal antibody-based HBsAg assays to detect HBV mutants in viraemic patients was examined by combination of such diagnostic assays. It is expected that their proportion may increase with higher vaccine coverage rates, but they are unlikely to compromise vaccination programs. Serological assays were developed for the detection of HBsAg-negative variants (Ijaz et al., 2001).

5.1.4. HBV Polymerase mutations:

HBV polymerase has several functions related to viral replication including DNA polymerase, reverse transcriptase and RNase H activity as well as having critical domains required for virus replication, and packaging of pregenomic RNA into core particles. The 5'end of HBV Pol gene encoding sequences that prime minus strand synthesis during replication. Mutation of polymerase gene altering its activity will affect the amount of virus produced as well as the number of templates available to encode virus proteins to elicit appropriate and timely immune response. Multiple pol-gene mutations occur (Kreutz, 2002) alone or with co-existing mutations in other regions of the genome.

Though some polymerase mutations are lethal. Several lamivudine resistant polymerase mutations are described that evolve during therapy (Iwao et al., 2006), most commonly

the YMDD (tyrosine, methionine, aspartate, aspartate) mutants in the C-domain at position 552 (rt204). Interestingly, S gene encoding the `a` determinant of HBsAg is overlapped by the variable linker region between the A (nt. 410-426) and B domains (nt. 498-528) of the rt part of pol-gene. The YMDD mutations are unlikely to affect the critical antigenic sites of HBsAg. In addition, valine YVDD and isoleucine YIDD mutants have been described, and the linked mutation FLMA (phenyl alanine, leucine, methionine, and alanine) of the B-region of the pol-gene, resulting in leucine to methionine L528M (Kim et al., 2009).

Another substitutions at rt204 (ATG to AGT; T to G and G to T) lead to a methionine to serine change (YSDD) are associated with the rtL180M mutation can also confer lamivudine resistance (Bozdayi et al., 2003). The YMDD mutation confers resistance against a range of other cytosine analogues as FTC, and ddC, as well as the thymidine analogue azidovudine. In Lamivudine non-responders, replication became undetectable after tenofovir introduction. 'Vaccine escape' mutants exhibited rtV173L plus rtL180M plus M204V mutations (Roque-Afonso et al., 2003).

Pol-gene mutations abolish both encapsidation and replication, while mutations of the RT or RNAase H domains markedly decrease DNA synthesis without affecting RNA packaging; this favours the reversion to wild type upon cessation of adefovir therapy (Werle et al., 2004). Surprisingly both replication competent and replication or packaging deficient variants are maintained. Complementary mutants rescue each other as core positive polymerase negative clone rescues core negative, polymerase positive virus in co-transfected cells, this mechanism may restore the wild type virus again. Replication efficiency of YMDD mutant is less than that of the wild type virus and, after cessation of therapy, the wt virus re-overtakes the mutant. However, lamivudine-resistant viruses remain functional and pathogenic (Besisik et al., 2003).

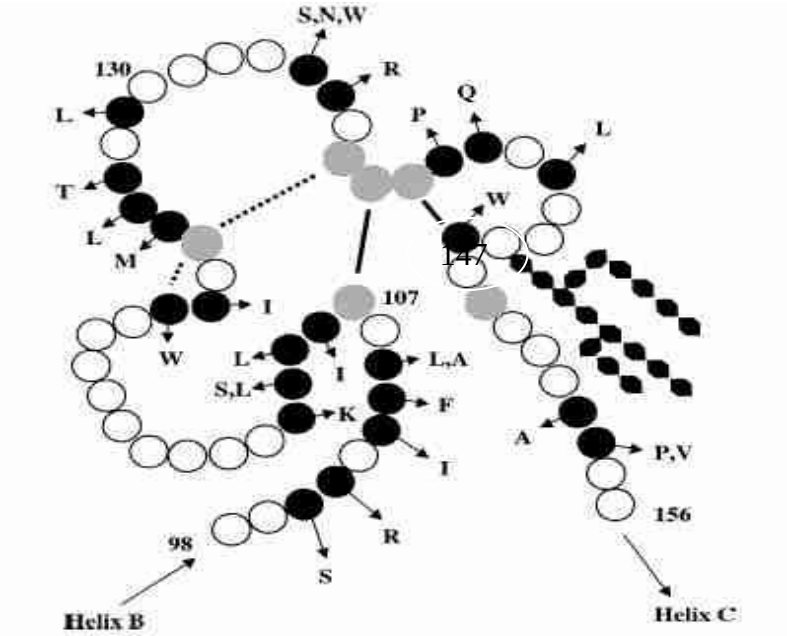


Figure 5-2: Amino acid changes of HBsAg-negative mutants. Amino acid chain in the MHL of HBsAg (aa 98-156): Showing the S-S bridges. The complex N-linked glycan bound to 146N.

Table 5-1: Types and clinical relevance of HBV mutants

HBV element	Mutation	Mollecular biology	Clinical association	References
Pre-S/S	pre-S2 Pre-S1/ /S-promotor	Misassembly	Fibrosing cholestatic hepatitis	
	PreS1	(aa 21-47) Hepatocytes binding region	Viral persistence Impaired viral clearance	
	pre-S2	Fibronectin–binding site (N-terminus) Viral attachment to liver sinusoides	Acute hepatitis	
	S	Alteration of B- and T-cell epitopes Decreased viral replication.	HBsAg-negative infections. Vaccine-escape infection. Diagnostic false negative HBsAg. Occult hepatitis	
Pol	Pol	Replication- deficieny Viral persistence	Viral latency and chronic hepatitis	
	YMDD motif	Resistance to antivirals Viral persistence	Lamivudine therapy escape	

Clinically, the majority of patients with severe hepatitis exacerbations due to YMDD mutants had uneventful course. YMDD variants are common in occult HBV infection in haemodialysis patients with chronic HCV infection, and were identified in six of 12 (50%) patients with occult HBV infection (Besisik et al., 2003).

Missense mutations in the initiation codon of pol gene prevent viral replication. However, mutations at codon 652 serine to proline, and at nt. 2798 proline to threonine of the terminal protein (TP) segment causes diminished replication (Wen et al., 2003). Finally, a terminal protein sequence may decrease the targeting to pregenomic RNA, thereby decrease viral replication.

5.1.5. Methods of detection of HBV mutants

Currently, HBV mutations can be determined by several methods (table 5-3). The variant detection varies as they are usually mixed with wild type in different proportions. Template-directed dye-terminator incorporation reaction (TDI-FP) have been shown to be more sensitive than sequencing and could detect variants as YMDD present in as little as 10% , 1% , 0.1% and 0.1% respectively of the virus mixture (Bai et al., 2003).

Table 5-2: Correlation of amino acid substitutions in HBV Pol and S ORFs

(Wakil et al., 2002)

Pol-ORF mutations	rt Position	Effect on S-ORF	HBeAg +/- %
T476I	128	P120S	14/17
N487D	139	G130G (silent mutations)	0/10
L528M	180	S171S (silent mutations)	29/10
V539I	191	W182stop	14/0
F549F (silent mutations)	201	S193L	14/0
M552V	204	W196L I195M	43/20
M552I	204	W196stop	
Q563S	215	S207R	14/10
F575F (silent mutations)	227	F219S	14/0
L577G	229	F220L and C221G	0/10

Table 5-3: Methods of HBV variant Detection

Method	Detection limit	Reference
High-density DNA chip	Detect large number of mutations, deletions and insertion	Tran et al., 2006)
Real-time detection by labelled probes		
Affigene HBV mutant VL Kits	Detect mixed mutants/wt	Zampino et al., 2005
5' nuclease technology		Chan et al., 2006
Competitively differentiated PCR.		Peng et al., 2005
Single nucleotide polymorhism (SNPs)		Park et al., ; 2004
Amplification refractory mutation system (ARMS)		Punia et al., 2004
Limiting dilution cloning PCR (LDC-PCR)	0.1%	Nainan et al., 2002
Sequence-specific PCR products (SP-PCR)	1%	Nainan et al., 2002
Line probe assay	2.5%	Wilson et al., 2003
Ligase chain reaction assay	2.9%	Osiowy et al., 2002
Cloning and sequencing	5%	Nainan et al., 2002
Direct sequencing	25% , Failed to identify 70% of wild-type/mutant mix	Davidson et al., 2005
Restriction fragment length polymorphism of PCR products (PCR-RFLP)		Hadziyannis et al., 2000
Colorimetric point mutation assay		Ballard and Boxal 1997

5.2. Hypothesis

HBV genotype sequences variations, especially regions encoding proteins, can be the cause of differences in biological behaviour and clinical outcome between HBV genotypes infections.

5.3. Aim

To verify the accuracy and specificity of the multiplex genotyping PCR assay

To confirm the HBV genotypes detected by multiplex assay by sequencing and phylogenetic analysis.

To determine HBV genotypes and subgenotypes present in Saudi and UK patients and whether apparent results in multiplex genotyping had a single variant or recombinations from different genotypes.

To check the patterns of mutations of HBV S-gene including the "a" determinant in Saudi and UK patients.

To detect the frequency of YMDD amino acid motif of the HBV Polymerase and related mutations with possible lamivudine resistance in this setting.

To analyze genotype specific variations present within HBV S and pol genes.

To check the genotype related differences in sequences used in HBV vaccinations.

To confirm the sequence and genotypes of the cloned plasmids and their validity as controls for genotyping assays.

5.4. Patients and Methods

5.4.1. Sequencing of the S-gene

Sera from 36 chronic hepatitis patients from UK and 20 Saudi patients were used. DNA was extracted by QIAAmp (blood) kits (Qiagen). PreS1/PreS2/ S gene of HBV was amplified by polymerase chain reaction (PCR). The first-round PCR using universal primers P1:5`-TCACCATATTCTTGGG AACAAGA-3` nt2823-2845 (sense) and S2: 5`-CGAACCACTGAACAAATGGC-3` nt. 685-704 (antisense), outer pair (1, 063 bases). The first-round product of the multiplex PCR was used to for sequencing, to detect regions of the pre-S/S-gene (Naito *et al.,* 2001).

Sequencing was done using ABI prism protocol. Sequencing primers are P1, B3: 5`-GGCTCMAGTTCMGGAACAGT-3 nt. 67-86 sense, B13N (nt. 3139-3156) and R10 (nt. 3097-3078) 5`-GGAGGCGGAT Y(C/T) TGCTGGCAA-3`anti-sense. The R10 was used for sequencing reverse sequences. Primer concentration was 0.25 pmol in a reaction using ``Big Dye`` fluorescent dye-terminator chemistry (Perkin Elmer) containing 4µl Big Dye mix and 2µl template to a final volume of 10µl undergone sequencing PCR at 94^{o}C for 10 sec, 50^{o}C for 5 sec, 60^{o}C for 4 minutes for 35 cycles. Sequencing templates were cleaned-up and purified by removing unincorporated primers and nucleotides from the PCR products using 1µl ExonucleaseI; which catalyzes the removal of nucleotides from single-stranded DNA in the 3` to5` direction, and 1µl shrimp alkaline phosphatase preparations (Amersham International, UK), which catalyses 5` dephosphorylation of nucleotides, respectively, to yield a clean PCR product for proper sequencing. The PCR product was incubated at 37^{o}C for 30 minutes, followed by heat inactivation at 70^{o}C for 15 minutes. A final volume of 30µl for each sample was prepared by adding 17µl water and 3µl Sodium acetate (pH 5.2). The DNA was precipitated from this solution by adding 100µl 100% ethanol, centrifuging at 15,000g for 20 minutes and aspirating the supernatant, leaving the pelleted DNA. Two further washes with 70% ethanol were performed. The residual ethanol was removed and the DNA pellets were air dried at room temperature for 20 minutes. The DNA was sequenced in the immunology department, University of Nottingham.

5.4.2. Phylogenetic analysis

Phylogenetic analysis and phylogenetic trees were done by bootstrap and neighbour joining methods. Comparison between complete genomes from Gene Bank and our sequenced strains using Chromas and Clustal X packages was used for method testing.

5.4.3. Genotype related variations

Intragenotypic and intergenotype variations of different parts of the HBV genome including the S-pol gene were determined using Mega3 packages. In addition, translation of HBsAg and the HBV polymerase proteins were done using the same program.

5.5. Results of S/Pol Sequencing

5.5.1. HBV Genotyping by Phylogenetic Analysis of S gene Sequences

Phylogenetic analysis of HBV S/Pol sequences of PCR products was done to define genotypes and subgroups on the basis of 8% and >4% heterogeneity of the entire HBV genome, respectively. Different areas were analysed of the S gene: preS1, pre S2, beginning of S gene encoding small HBsAg, the `a` determinant, and end of S/Pol gene (tables 5-4, 5-5, 5-6). Generally, there was high concordance between our multiplex genotyping assay and sequencing of the S gene. PreS2 region differences were not conclusive for genotyping comparison (table 5-4).

Pre S1 region, Pre S2and beginning as well as the end of S gene overlapping the YMDD region of the reverse transcriptase part of pol gene showed accurate discrimination between genotypes. Pre S1, Pre S2, and to a lesser extent the beginning of small HBsAg, carried more variation between different HBV genotypes than the more conserved regions at the `a` determinant and the rt part of the Pol gene. According to calculations of genetic distances by Kimura 2-parameter method, this further confirmed the differences between different regions of the S and Pol genes (table 5-5).

Discrimination was demonstrated between different HBV genotypes, although the latter region shows the YMDD motive for the detection of antiviral drug resistance as well. The phylogenetic analysis different regions of the S-gene has shown genotype-related variations (fig.5-7 to 5-11)

All UK HBV genotype A had subgenotype Ae, genotype B had subgenotype Ba, and genotype C subgenotype C1 (Cs). HBV subgenotypes detected in Saudi Samples had HBV subgenotype D1, while UK had subgenotype D2 mostly in Asian patients. All plasmids sequences confirmed the S-gene of relevant HBV genotypes.

Table 5-4: Concordance of HBV genotyping by phylogenetic analysis of different regions of S/ pol gene of Saudi and UK HBV isolates using Mega software.

Genotype	PreS1 nt. 2815 - 3084	PreS2 nt. 3085-154	S-gene nt. 155- 424	`a` determinant nt. 425 – 694	RT-pol nt. 695- 964
A	83% (5/6)	33% (2/6)	100% (6/6)	89% (8/9)	100% (7/7)
B	100% (4/4)	50% (2/4)	100% (4/4)	100% (4/4)	100% (5/5)
C	100% (6/6)	33% (2/6)	100% (6/6)	100% (4/4)	100% (7/7)
D	100% (16/16)	75% (9/12)	100% (18/18)	91% (21/23)	100% (7/7)
E	100% (1/1)	100% (1/1)	100% (2/2)	100% (3/3)	100% (2/2)

Table 5-5: Calculated mean of nucleotides distances within each genotype in 5 different regions of the S/pol gene by Kimura 2-parameter method

Genotype	PreS1	PreS2	HBsAg	`a` determinant	RT	Average
A	0.070	0.060	0.039	0.046	0.021	0.047
B	0.026	0.034	0.025	0.072	0.007	0.033
C	0.070	0.074	0.045	0.042	0.024	0.051
D	0.034	0.039	0.023	0.050	0.022	0.034
E	0.178	0.025	0.012	0.047	0.032	0.058
Average	0.073	0.047	0.028	0.051	0.021	0.044

Table 5-6: Genetic distance calculation by average difference between HBV genotype A and other genotypes in nucleotides (upper line) by Kimura 2-parameter, including transitions and transversions. Also, distance of translated amino acids between genotypes by Dayhoff Matrix Model for homogenous /uniform rates

Genotype	PreS1	PreS2	HBsAg	`a` determinant	RT
B nt.	0.244 / 0.543	0.171 / 0.348	0.062 / 0.138	0.102 / 0.201	0.048 / 0.069
C	0.154/ 0.325	0.140 / 0.287	0.055 / 0.120	0.121 / 0.225	0.062 / 0.097
D	0.326 / 0.651	0.185 / 0.418	0.033/ 0.072	0.105 / 0.210	0.066 / 0.107
E	0.242 / 0.515	0.159 / 0.351	0.053/ 0.118	0.107 / 0.229	0.054/0.066

5.5.2. Genotype-specific variations in PreS1 region

The pre-S1 together with pre-S2 and S-gene encode the large HBsAg protein. Within the pre-S region, many nucleotide exchanges are observed. These are partly correlated to the HBV genotypes. Genotype D exhibits several variations. All the sequenced cases (9/9) with genotype D, showed deletion of the naturally occurring 11 amino acid codons at the beginning of preS1 region as did the genotype D Gene Bank controls. Consequently, the preS1 starts at codon 12 as compared to genotypes B, C, F, and H. Genotype E and G show one aa. codon deletion at the beginning of preS1 segment. Resulting in a total of 108 aa in pre S1 instead of the 119 aa seen in genotypes B, C and F. Other genotype specific variations were observed in the beginning of the preS1 gene including P19S (CCC to AGC) (15/17), codons 88-91 LPAN motif found in all genotype D strains (appendix A).

Transcription activating sequence / Hepatocyte binding sites (codon 21-47)

L30V found in 3 genotype C cases (3/5), both have genotype C, unlike Gene Bank controls. Genotype-related variations were seen at positions 35, 38 and 39. All our UK and Saudi cases showed no deletions or insertions in PreS/S gene.

5.5.3. S2-promoter (nt. 3045-3180)

S2 promoter area primes the translation of preS2. HBV genotypes also show several consistent variations as follows; V90A found in subgenotype C-Australia and in N16, N45, while V90S seen in N19. N114D found in one genotype D Gene Bank strain X65258 and one Saudi case 4-27.

5.5.4. Mutations on PreS1

Several isolated point mutations were seen; one genotype D case (S5) has mutation at the initiation codon of pre S1, which could interfere with the production of large HBsAg. AF 62/63 SL in genotype C cases (N19 and N16) (2/5).

5.5.5. Genotype-specific variations in pre S2 region

Within the polymerised human serum albumin (PHSA) binding site (preS-2 aa 17-29), F22L was observed in A-Europe, genotype Bj subgenotypes, but all our genotype A cases (5/5) showed L22F unlike Gene Bank controls. V53A and T54P/L was different in

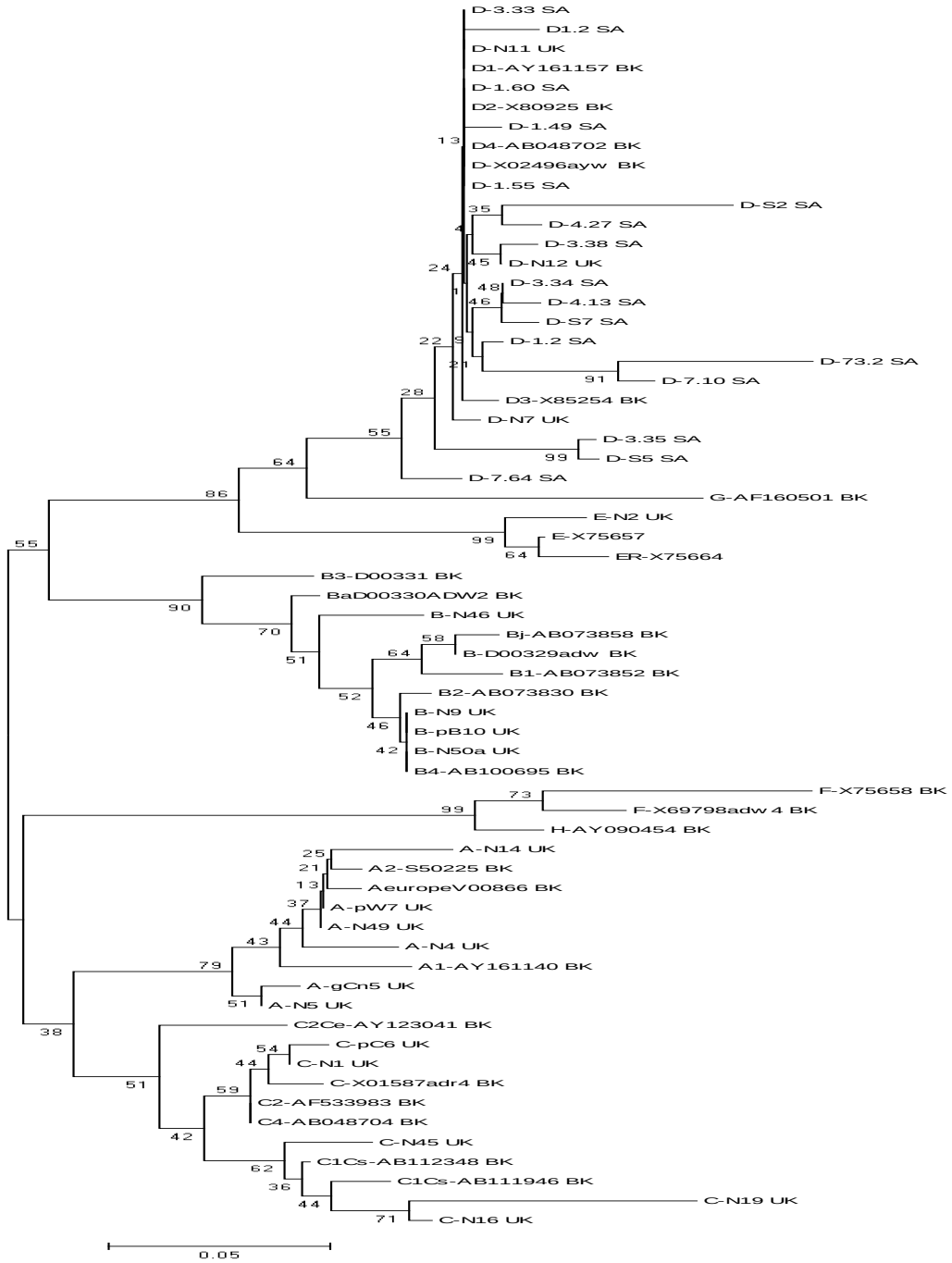


Figure 5-3: The phylogenetic tree of the beginning of Pre S1 gene nt. 2815-3084 showing genotypes and subgenotypes of UK (suffix UK) and Saudi (suffix SA) HBV isolates. Gene Bank (suffix_BK) sequences: A1-AY161140, A2-S50225HBsAg-, A-europV00866adw, A-afric-AF297625, Ba-D00330adw2, Bj-AB073858, B1-AB073852, B2-AB073830, B3-D00331, B4-AB100695, C1-AF411412, C1Cs-AB111946, C2Ce-AF533983, C3-X75656, C4aus-AB048704ayw, D1-AY161157, D2-X80925, D3-X85254, D4-AB048702, E-X75664, F-X69798adw4, H-AY090454, G-AF160501.

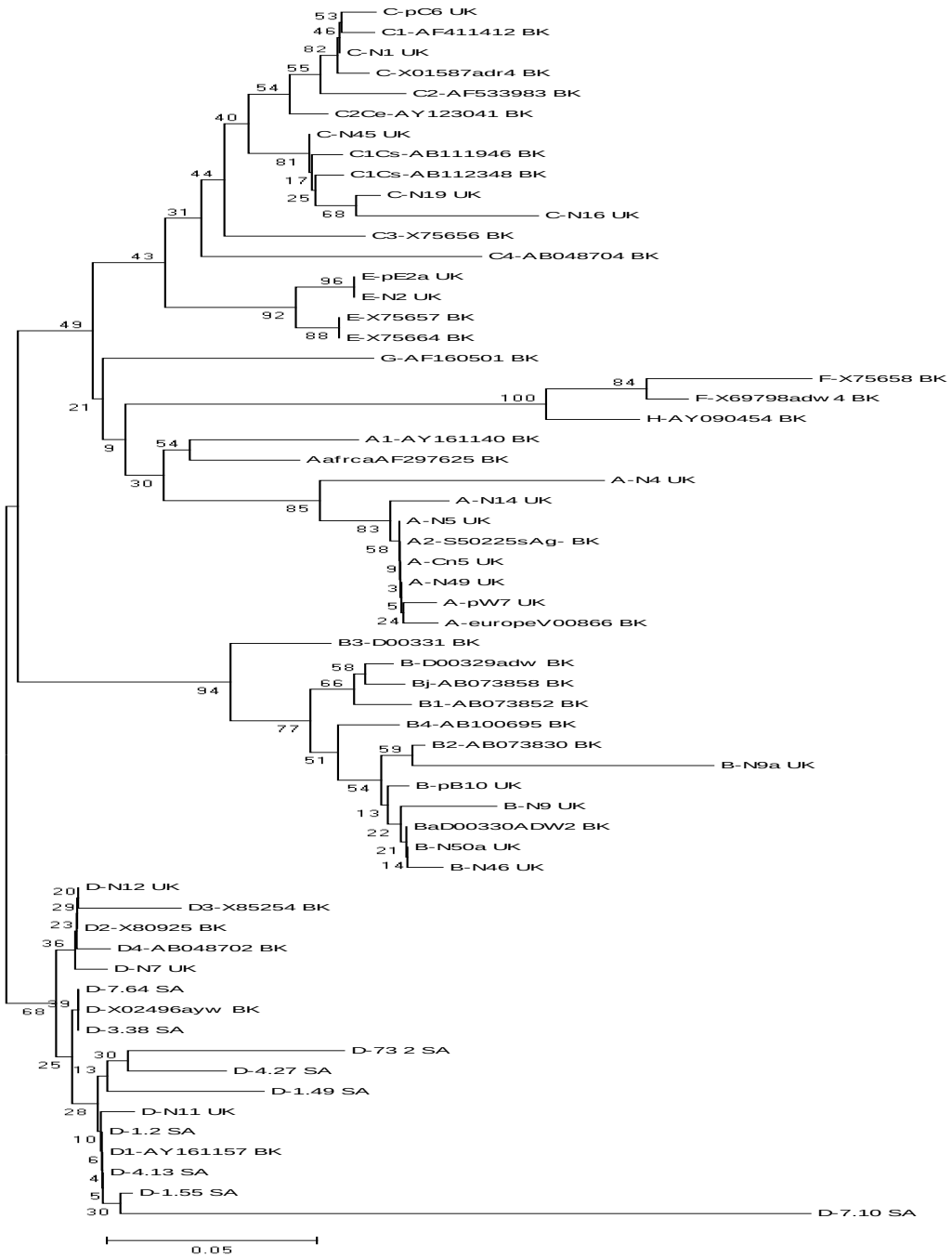


Figure 5-4: The phylogenetic tree of Pre S2 gene nt. 3085-154 showing genotypes of UK (suffix UK) and Saudi (suffix SA) HBV isolates. Gene Bank (suffix_BK) sequences: A1-AY161140, A2-S50225HBsAg-, A-europV00866adw, A-afric-AF297625, Ba-D00330adw2, Bj-AB073858, B1-AB073852, B2-AB073830, B3-D00331, B4-AB100695, C1-AF411412, C1Cs-AB111946, C2Ce-AF533983, C3-X75656, C4aus-AB048704ayw, D1-AY161157, D2-X80925, D3-X85254, D4-AB048702, E-X75664, F-X69798adw4, H-AY090454, G-AF160501.

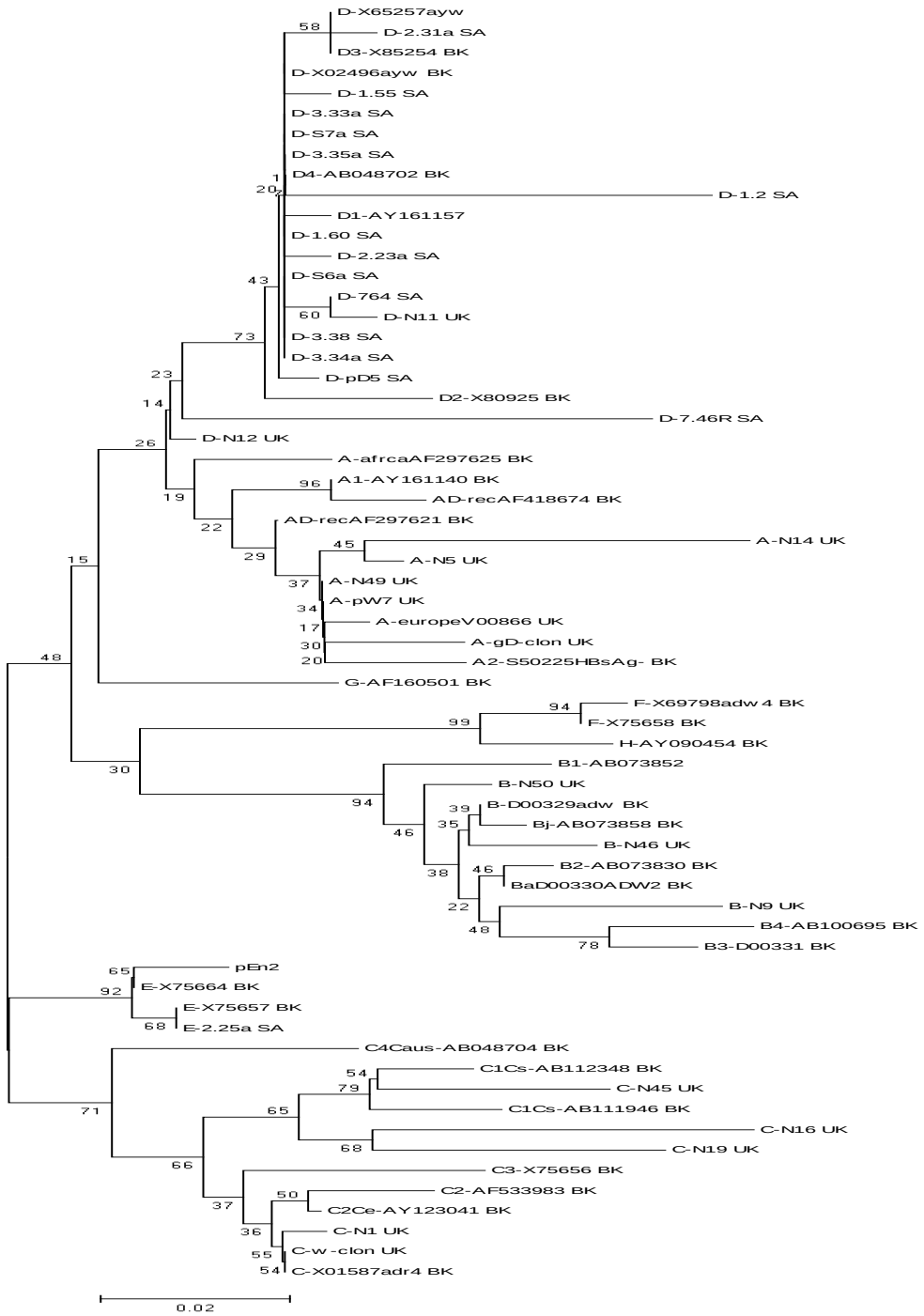


Figure 5-5: The phylogenetic tree of the beginning of small S-gene encoding HBsAg nt. 155-424. UK (suffix UK) and Saudi (suffix SA) HBV isolates. Gene Bank (suffix_BK) sequences: A1-AY161140, A2-S50225HBsAg-, A-europV00866adw, A-afric-AF297625, Ba-D00330adw2, Bj-AB073858, B1-AB073852, B2-AB073830, B3-D00331, B4-AB100695, C1-AF411412, C1Cs-AB111946, C2Ce-AF533983, C3-X75656, C4aus-AB048704ayw, D1-AY161157, D2-X80925, D3-X85254, D4-AB048702, E-X75664, F-X69798adw4, H-AY090454, G-AF160501.

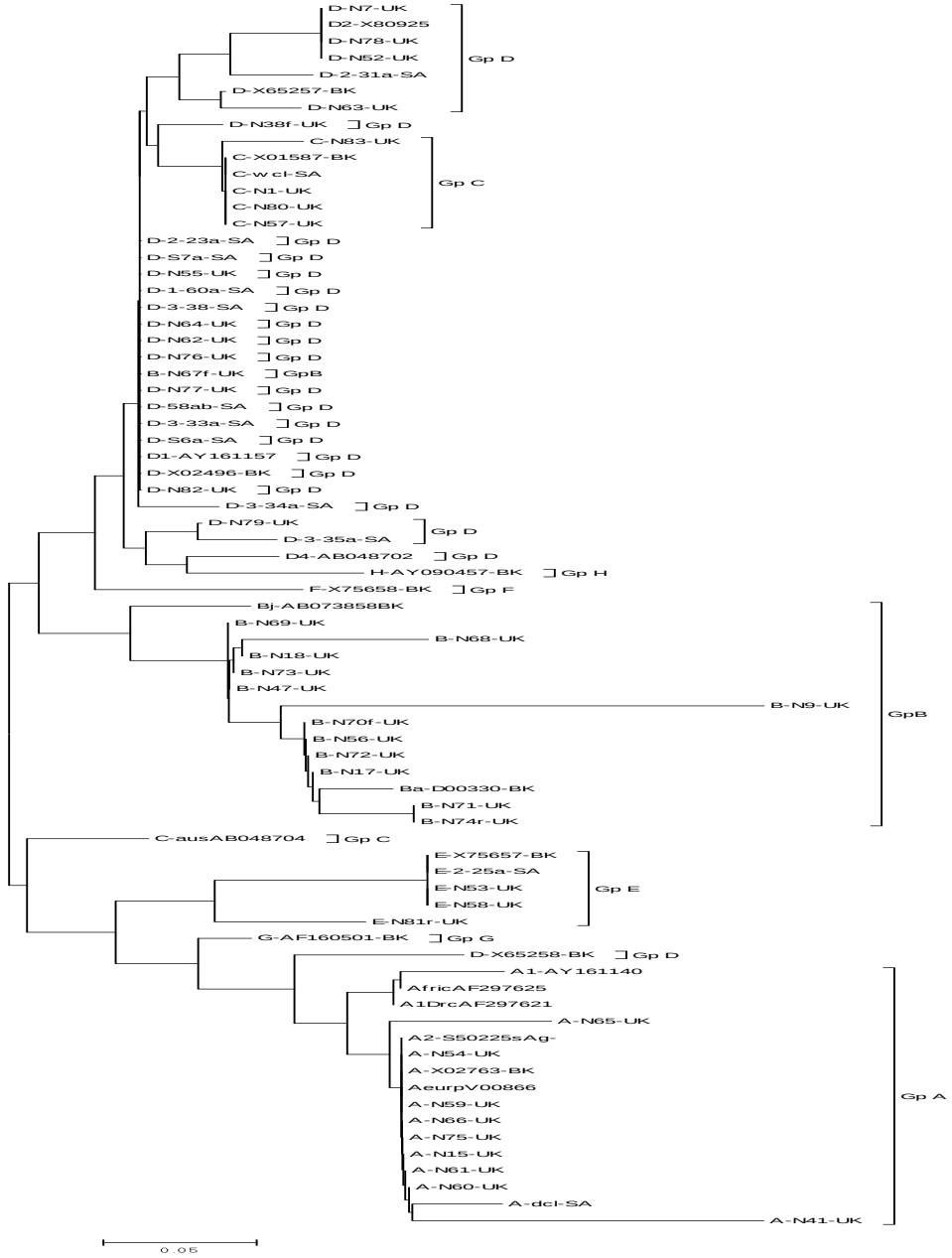


Figure 5-6: The phylogenetic tree of the S/Pol gene: nt. 425 – 694 encoding HBsAg `a`determinant. However, subgenotypes are not well differentiated in this tree. UK (suffix UK) and Saudi (suffix SA) HBV isolates. Gene Bank (suffix_BK) sequences: A1-AY161140, A2-S50225HBsAg-, A-europV00866adw, A-afric-AF297625, Ba-D00330adw2, Bj-AB073858, B1-AB073852, B2-AB073830, B3-D00331, B4-AB100695, C1-AF411412, C1Cs-AB111946, C2Ce-AF533983, C3-X75656, C4aus-AB048704ayw, D1-AY161157, D2-X80925, D3-X85254, D4-AB048702, E-X75664, F-X69798adw4, H-AY090454, G-AF160501.

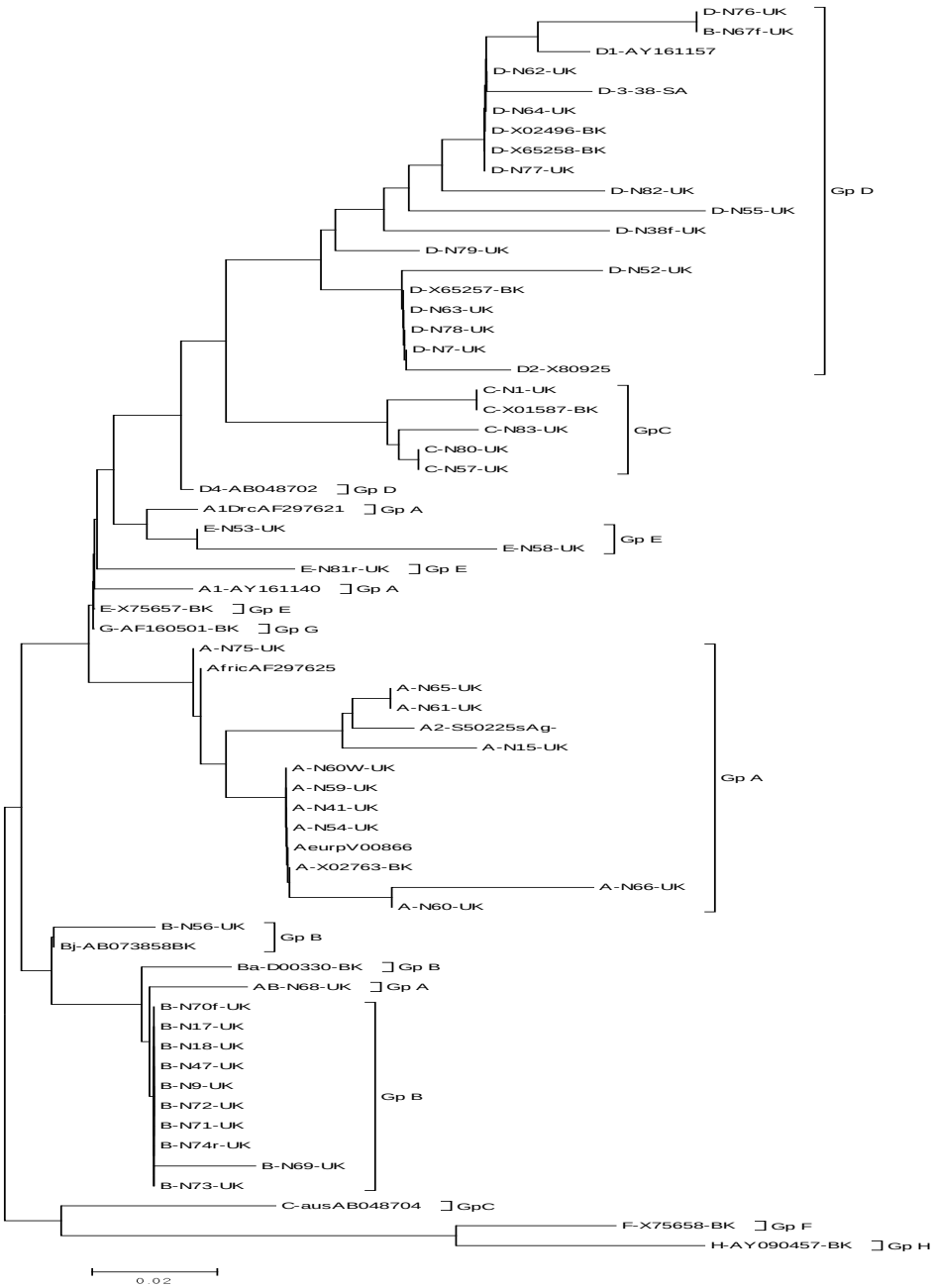


Figure 5-7: The phylogenetic tree of the reverse transcriptase region of the S/Pol gene nt. 695- 964. UK (suffix UK) and Saudi (suffix SA) HBV isolates. Gene Bank (suffix_BK) sequences: A1-AY161140, A2-S50225HBsAg-, A-europV00866adw, A-afric-AF297625, Ba-D00330adw2, Bj-AB073858, B1-AB073852, B2-AB073830, B3-D00331, B4-AB100695, C1-AF411412, C1Cs-AB111946, C2Ce-AF533983, C3-X75656, C4aus-AB048704ayw, D1-AY161157, D2-X80925, D3-X85254, D4-AB048702, E-X75664, F-X69798adw4, H-AY090454, G-AF160501.

different genotypes. This area lies in N-terminus of the PreS2 in the fibronectin binding site to liver sinusoids.

5.5.6. Mutations in PreS2

Only few point mutations were seen in preS2 gene. The most significant was the loss of initiation codon M1I in case N9 and the plasmid constructed from it pB10.

5.5.7. S-gene (encoding small HBsAg)

Sequencing of the S- gene was done, within the S region. Several genotype-associated variations were present; these are partly correlated to the serological hepatitis B surface antigen subtypes. No deletions or insertions were found. Two cases (N16, N19) had premature stop codon at codon 34 giving a truncated HBsAg.

The `a` determinant (aa. 124-147)

The main two loop structure of the `a` determinant is maintained by four cysteine residues at positions 124, 137, 139 and 147. All of them were unchanged in any of Gene Bank HBV genotypes controls, nor in our cases. Main genotype related variations are T126I was seen in genotype C, T131N in genotype A, F134Y found in genotype D. P127L and T140S in genotype E and S143T in genotypes A and C.

T-Lymphocytes epitopes in S/pol gene

PreS1 epitopes at aa.21-28 (Class I) and aa.21-30 (Class II) (table 1-3) did not show significant genotype specific variations. In the pre S1 epitope associated with HBV virus clearance (58-100). Codon A60 found in genotypes C controls and only two cases (2/5). Most our genotype C cases (3/5) have A60V. E86T was seen in genotypes A, B and C, but E86Q in all our genotype D cases and in only one Gene Bank control X65257. Pre S2 epitope aa.1-11 (Class I and II) shows genotype specific variation at codons 7, 11 and 13. Codon 7 is a SNP candidate as explained below.

The overlapping epitopes in small HBsAg aa. 20-29 and aa. 20-33 (Class I and II) show the following changes; R24K (AGA to AAA) found in genotypes B and E. Epitope aa.41-49 (Class I) Codons 45 and 47 show several genotype related changes as explained in single nucleotide polymorphism (SNPs) section. No specific variation was seen in epitopes aa. 88-96 and aa. 97-106 (both Class I). Epitope aa.185-194 (Class I) V194A seen in genotypes A (7/7).

In the pol gene, epitope rt107-115 (ClassI) has only L115V in genotype H and as point mutation L115V in N41 and N53. Epitope rt 203-211 (ClassI) This important epitope in the catalytic center of reverse transcriptase has genotype specific variations at codon rt207, immediately after the YMDD motif. V207L and epitope rt227-235 (Class I) L231V were not seen in our samples.

In general, minimal genotype related variations are seen in T-cell epitopes within the S gene. Their effect on lymphocyte activation has to be sought in a detailed study.

5.5.8. Mutations in S-gene

Only one case (N4) showed 21 bp-deletions in nt. 34. Point mutations (PM) were present in F20V (TTC to GTC) in 1-2 and 1-49. S61L (TCA to TTA) found in some genotype C cases (3/ 6). L95M seen in cases N7 and N62 and stop at codon 95 in N64. P127L found in 3/16 genotype D cases, A128V in cases N7 and N52 (genotype D) and in N59 (genotype A). M133L was observed in case N41 (A) as well as HBsAg-negative Gene Bank case (S0225) and in genotype B; Bj subgenotype. Early stop at codon 34 was seen in genotype C cases N16 and N19. TT45AP found in genotype D cases N7, N12.

5.5.9. Analysis of the translated HBV pol gene sequences into polymerase protein

Case N9 with genotype B and N41 (genotype A) have TCA to GCA in rt codon 109 S to A mutation.rtS78A was seen in N14, N16, N19, and 1-55 (genotype D). No UK patient showed mutation in the YMDD motive of rt 204 of the polymerase gene, nor is the associated L 180 (pol528) present. The only Saudi case (3-38) sequenced (and cloned) in this region, showed isolated M204I mutation (genotype D). rt H13N in genotype C

controls and some of our cases (3/6), R13L in our genotype B cases (3/5). I16T (ATT to ACT) seen in genotype A (4/7). I91L found in subgenotypes C-Australia and our genotype C cases (3/5). We found two UK cases with YVDD.

In pol-gene rt codon 103 GTT to GAT resulting V103D in UK genotype D cases (N7 and N62, N65) are of Asian origin. Codon L115V (TTG to GTG) found in N63 (genotype D), N53 (genotype E), and N41 (genotype A). H126R was seen in N7 and N52 (both are genotype D), M129L in N65 and 3-35. Q130P found in N63 and 2-31, S135Y in N7, N52, and 2-31a (all are genotype D). V266I was seen in N15 and in subgenotype A-Africa control strain from Gene Bank.

5.5.10. Genotype-specific variations in Pol gene

Genotype D specific variation in the pol gene comprising the deletion of 11aa was seen in our genotype D cases (17/17) and of 7 aa in genotype A (6/6), 1amino acid in genotypes E (1/1) and G. Pol codon A262P was found in all our genotype C cases (4/4), unlike Gene Bank controls. Pol S274T was seen in N1 and Gene Bank genotype C control while Pol T274A was found in our genotype C cases N16, N19, and N45.

Pol-gene codons L345I and L348R show genotype specific variability. In rt I16T in N45 (genotypes C). As well as in 2-31 (genotype D). T38A was seen in N9 (genotypes B), N16 (C).Pol-gene codon N288H found in genotype A L348R (6/6). 308Q found in cases N9 and pB10 (B).

5.5.11. Single nucleotide polymorphism (SNPs) candidates

Although there is 92% similarity in HBV genotypes, we found some point at S/pol gene that show considerable differences between HBV genotypes (table 5-7) and these are presented in the following section; 10Q (CAA) was seen in genotype C and cases (3/5). N39E (GAA) in genotype B controls and cases (3/4), 39H (CAC) occurred in genotype C controls, but our cases (6/6) showed H39N (CAC to AAC) as did the Australian subgenotype of genotype C.

Codon H51Q (CAA) found in genotype C (4/5) of our patients unlike control. A54E (GCG) found in genotype C controls and 2/5 of our cases. 84V found in genotype C Gene Bank controls, but V84I was found in all our genotype C cases (5/5). I84L was seen in Asian subgenotype of genotype B (Ba), as well as in most of our genotype B cases (3/4). A 91 was present in genotype C controls and some of our genotype C cases (3/5), while V91A observed in subgenotype C-Australia and our genotype C cases N16 and N45.

In preS2 segment, 7/8 AL was seen in genotypes A Europe and A Africa and case N7 (genotype D), while A7T found in both genotype B and C controls and cases 2/4 and 2/4, respectively. Point mutation T7N and T7I observed in genotype B cases N9, pB10 and N16, N19 (genotype C), respectively. P41H was seen in only one genotype D controlX65257 and most our cases (6/9).

Only two SNPs candidates were observed in the S-gene codons 45 and 47, whether this is the cause of false positive B genotype. SNPs candidates in the Pol gene include codons 267/268, 270, 273 and 348. L348I in cases N11, 1-49, and 4-13 of genotype D. rt **7**A (GAC), 53/54 and 122. F122L was seen in one genotype D Gene Bank control; X65267 and cases N63 and 2-31.

5.5.12. Verification of multiplex genotyping PCR Primer binding sites

Samples and plasmids sequences confirmed the S-gene of relevant HBV genotypes (See genotyping chapter).

Table 5-7: Amino acid changes deduced from single nucleotide polymorphism (SNPs) candidates in HBV S/pol gene

Genotype	A	A Africa	Ba	Bj	C	C australia	D	E	F	H	G
PreS1 Codon 35	G	G	K	K	G	G	R	R	R	R	R
39	T ACA	T	E GAA	E	H	N AAC	A GCA	R	S AGC	S AGC	N
51	H	H	N	N	H	H	T	H	S/T ACT/ AGT	N	P
54	A GCA	Q CAA	D	D	E	E GCG	D	E	M ATG	M	E
91	I	I V	A	A	A	A	N	D	D	D	D
Pre S2 41	H	H	A	A	P	H	H	L	L & ADrc	L	H
54	T	T	P	K	P	S	L	P	M	M	P
S gene 45	S	S	T	T	A	A	T	A	L	P	V
Pol gene 267	I	I	T	T	I	I	T	A	V/S	S	N
268	D	D	H	H	D	D	T	K	N	D	N
270	S	S	C	C	R	S	V/F/R	T	L	L	F
273	N		S	S	S	S	K	R	R	R	R
rt7	D	V	T	A	T	I	A	T	Y	Y	T
rt271	H	H	M	L	Q	H	E	D	E	D	Q

5.6. Discussion

Limitations of sequencing data

We used direct sequencing to detect mutants. Different methods have been used to detect HBsAg variants alone as direct sequencing, or in mixed populations as cloning and sequencing, restriction fragment length polymorphism of PCR products (PCR-RFLP) (Lim et al., 2007).

Differences in the nucleic acid sequences may be due to PCR artefacts, or reflect heterogeneity of the viral population, or by point mutations (PM). PM caused by polymerase itself ranges between $1x10^{-4}$ and $6x10^{-5}$ depending on the fidelity of the enzyme used. In addition, fluctuations in the intracellular [dTTP]/[dCTP] pools might give rise to a G to A hypermutation, which in some cases results in several Gs that are replaced by As throughout the entire genome (Gunther, 2006). PCR errors are most frequently A to G and T to C exchanges due to the rising melting temperature. The probability of PCR error is less likely the more clones or specimens that show the same mutation in parallel (Ruiz-Tachiquin et al., 2007).

Phylogenetic analysis of HBV genotypes and subgenotypes

We investigated the genotype specific variations and the prevalence of the HBV variant with the pre-S mutant using sera from Saudi Arabia and UK, where high and low levels of endemic HBV infection exists, respectively. Phylogenetic analysis of the sequences and correlation with multiplex and Visgen genotyping results was done (HBV Genotyping chapter 4). All UK HBV genotype A had subgenotype Ae, genotype B had subgenotype Ba, and genotype C subgenotype C1 (Cs). In genotype D, Saudi Samples had HBV subgenotype D1 while UK had subgenotype D2 (cases N6, N12) patients with Asian ethnicity. Currently, genotypes are subdivided into several subgenotypes: A1 to A3, B1 to B4, C1 to C4, F1 and F2 (Norder et al., 2004). The phylogenetic analysis of S-gene of UK patients (some of whom have Chinese origin) showed HBV genotype B2 / Ba analogy. This was in concordance with the results by Sugauchi et al., (2003) where subgenotype-Ba (recombination with genotype C) occurred in all 177 HBV carriers from countries of an Asian ethnicity except in Japan, whereas subgenotype-Bj was detected in

93% of the 97 carriers from Japan. Within HBV genotype C, Cs subgroup predominantly found in Southeast Asia (Vietnam, Thailand, Myanmar, and southern China) including HCC patients (Chan et al., 2006), while Ce subgroup predominantly found in the Far East (Korea, Japan, and northern China) (Chan et al., 2005). Thus, genotypes can contribute to the monitoring of infection by new virus strains from immigrants or as an interaction between circulating genotypes.

Comparison of different parts of the S-gene

Upon comparison of different areas of the S-gene; Pre S1, Pre S2, and to a lesser extent the `a` determinant were discriminative between HBV genotypes than the beginning of small HBsAg or the rt part of the Pol gene. A high degree of genetic stability is seen in the stem of the HBsAg encapsidation signal (Norder et al., 2003). However, phylogenetic analysis of the region including the YMDD motif of the polymerase was used for both HBV genotype discrimination and detection of lamivudine resistant mutants (Rodriguez-Novoa et al., 2004). The percentage of intra-genotypic distance among Afghan isolates was 1.05% and inter-genotypic distance with the other genotype D was 2.87% and with other genotypes was 7.50% -11.1% (Amini-Bavil-Olyaee et al., 2006)Our results showed highest distance in preS1 region between genotype A and genotypes B 0.244 / 0.543, C 0.154/ 0.325, D 0.326 / 0.651 and E 0.242 / 0.515 (table 5-6).

Properties of the Pre-S1 gene

Analysis of our preS1 sequences showed that we have subgenotype Ba in UK genotype B cases. Our genotype C cases exhibit resemblance to both reference genotype C control, as well as areas of analogy to subgenotype C-Australia. Further analysis of the whole genome is needed to establish whether this is a recombinant strain, or a new subgenotype .of genotype C. In preS1 codon 39, the resemblance to Australian subgenotype can arise from common source of infection either in Australian population or from Asian ethnicity in both countries.

Pre S1 contains two important epitopes; one is the hepatocyte binding region (aa 21-47), and the other (aa.58-100) is speculated to be the epitope recognized by neutralizing antibodies involved in viral clearance (Park et al., 2005). The first region also claimed to

have transcription activating function as well. Our results showed several differences between genotypes in this region, mainly in codons 35 and 39. Whether genotype related variation within the hepatocyte binding site affect the binding of different HBV genotypes to liver cells has to be explored by cell binding assays.

Viral proteins such as the truncated middle S protein and the X protein have been also implicated as transactivators. The transcription activating sequence was localized to the 21 to 47 amino acids of Pre S1 protein (Park et al., 2005). The differences in the S region may also change between HBV genotypes, thereby affecting the replication ability or oncogenic potentials of HBV genotypes. Analysis of their effects on the transactivation properties of HBV can be explored by further research. Blackberg and Kidd-Ljunggren (2003) showed that up to 50% of the patients with HCC carried HBV mutants with deletions or insertions in the N-terminal half of the pre-S2 region or had a point mutation in the start codon of pre-S2 compared to 21% of those with chronic HBV infection...

We detected several point mutations, including abolition of initiation codon of preS2. Similarly, pre-S2 starting codon was observed in 19 (26.7%) cases. The HBV mutants were more frequent in patients with hepatocellular carcinoma and in genotype B (25%) and genotype C (24.5%) (Huy *et al.*, (2003). The pre-S2 is highly immunogenic, it is not necessary for the HBV life cycle. Loss of the preS2 protein is a mechanism to escape the immune system. Amino acids 41 and 52 of the pre-S2 region are essential for transactivation. To generate the transactivating forms of the middle surface protein (pre-S2/S), a deletion of at least 87 C-terminal amino acids is required (Blackberg and Kidd-Ljunggren; 2003).

The PHSA binding site (preS-2 aa 17-29) showed minimal changes in codon 22, which may be involved in the presence or intensity of HBV viraemia, binding to circulating serum albumin in addition to the persistence of HBsAg in circulation. In a study by Huy *et al.,* (2003) HBV pre-S mutants were detected in 71 (18.3%) of 387 serum samples from Vietnam (36%), Nepal (27.3%), China (22.4%), more than Japan (7.7%), and Ghana (4.3%). Among the HBV deletion mutations, 15.5% (11 of 71) occurred in the pre-S1 and 46.5% (33 of 71) in the pre-S2 regions. Eight (11.3%) cases had a mutation in

both the pre-S1 and pre-S2 regions, in HCC and genotype B (25%) and genotype C (24.5%) (Huy *et al.,* (2003).

Single nucleotide polymorphism (SNPs) is gaining popularity as a specific, rapid method for genotyping using certain discriminative alleles to differentiate between different genotypes. Our analysis (table 5-6) showed several SNPs candidates that not only differentiate HBV genotypes, but also may reflect areas of crucial genotype-related changes in functional or structural proteins encoded by them.

S-gene mutations and HBsAg negative phenotype

The sequences encoding the `a`determinant of HBsAg were highly conserved among our HBV genotypes. We found minimal changes in the HBsAg. Our cases were HBsAg-positive therefore; comparison is difficult with HBsAg-negative variants. Some mutations can abolish the two loop structure of the `a` determinant by changing the hydrophilicity, the electric charge or the acidity of the loop or altering N-glycosylation site (Kreutz,2002). The triple mutation V519L/L526M/M550V causes the concomitant amino acid substitution E164D/I195M in the overlapping S protein. This has a reduced affinity to anti-HBs antibodies, similar to the vaccine escape mutant G145R (Sucupira et al., 2006). Consequently, there is an accumulation of multiple sequence variants (sequence evolution), some of which are selected during antiviral therapy.

There is a need for complete epidemiological data on the prevalence of HBsAg mutants and screening strategies need to be developed and evaluated. Detection of HBsAg needs to be improved by the introduction of HBsAg assays able to recognize S-gene mutants and escape variants with high sensitivity and has to detect smallest amounts of HBsAg in low level carriers.

Vaccine escape mutants

The difference within HBV virus clearance (58-100) epitope points to a D1 subgenotype of genotype D to be present in Saudi cases. Vaccine escape mutant were reported in genotypes B and C, and these also were found to be related to the severity of liver disease and sensitivity to therapy (Huy, 2003) (Abe, 2004).

Vaccine-induced mutants may cause alteration of antigenicity of HBsAg and binding to anti-S antibodies. Hence, preS/S vaccines will be more immunogenic than small HBsAg vaccines, but the former show high genotype-specific motifs that demand tailoring the vaccine according the pattern of HBV genotypes prevalent in certain geographical regions. Whether we need a complete vaccine containing PreS1-large HBsAg protein in Saudi Arabia with genotype D remain to be verified. Further epitope mapping and immunological studies are needed to define the exact epitopes within this area, and to determine the target cells as natural killer cells or cytotoxic T lymphocytes. Their effect on antibody binding and lymphocyte activation has to be sought in a detailed study.

Mutations of HBV polymerase and antiviral drug resistance

We found several mutations and genotype specific-changes in the spacer region of the polymerase gene. The spacer region of the polymerase enzyme is not functional, so genotype-specific variations probably merely reflect changes in the overlapping S-gene. Regarding the reverse transcriptase region of the pol-gene, we found two UK cases with YVDD, and only one HBeAg-negative cirrhotic genotype D Saudi case with YIDD mutation, and had high HBV viral load of 10^8 copies/ml. A stop codon created by the M552I causing termination of the major surface protein at amino acid 196 from its wild type to tryptophan. No YMDD mutants were found in genotypes A, B, C, and E in UK or Saudi isolates sequenced. The mutation in the C domain V204I causes a convergence in the corresponding site in SORF codon 182 from tryptophan to a stop codon, as observed in our YIDD case. High rates of lamivudine resistant mutants reported ranging from 9% (Bai et al., 2003), to 43% and 28% (Wakil et al., 2002) of HBeAg positive patients developing the YM552I/VDD and L528M mutations, respectively. YVDD and YIDD type tended to have the opposite background with regard to age, histology, and viral load.

Clinical or other virological factors may determine the rapidity of the viral breakthrough during therapy. In our case no 528 (rt180) mutation was present. Famciclovir resistant mutants are less common than lamivudine resistant mutants, and have L528M, V521L, V555I.The latter mutation also causes premature termination of the overlapping envelope protein. Nevertheless, lamivudine resistant mutants remain sensitive to adefovir and lobucavir.

5.7. Conclusion

In conclusion, the hepatocyte binding sites show considerable HBV genotype specific variations mainly in codons 35, 38 and 39. This might explain the differences in the hepatocyte binding rates contribution to pathogenicity, and consequently the intensity of the infection and the rate of infected hepatocytes with possible effect on the clinical consequences and severity of the disease.

The design of HBsAg assays require high sensitivity and should include diagnosis of escape mutants and variant sequences. PreS2 nt. 3085-154 and the `a`dereminant are the most variable regions within the PreS/S gene between HBV genotypes. This should be considered in vaccination programs in areas where the genotype is different from that of the recombinant vaccine.

5.8. Future Directions

Check genotype-specific variations in the rest of HBV genome, including the end of pol gene nt. 900-1500 (polymerase codons 655-824), which overlaps the beginning of HBV X- gene. Epitope mapping of virus clearance epitope (preS1 aa.58-100) can be explored for other cytotoxic or natural killer cells epitopes present.

Further studies are needed to analyze the effect of genotypic variations of preS1 and preS2 T-cell epitopes codons 7 and 11, and small S-gene codons 20-28, 185-194 (genotype A) for the immunogenicity in HBV vaccines, and whether this have any implications on current HBV vaccines, especially in populations with HBV genotypes different from the vaccine. In addition to comparison with HBsAg-negative occult HBV patients with groups of vaccinated individuals

To express the large HBsAg protein (LHB) from plasmid pB10 constructed from case N9, which lacks preS2 initiation codon and to check the binding affinity of this variant HBsAg to different antibodies and receptors

Perform expression experiments on plasmid pD5 constructed from case 3-38 carrying YIDD mutation, to examine the antigen-antibody binding ability of the truncated HBsAg resulting from this mutation.

Hepatocyte-binding assays can be useful to exploit genotype related differences in the hepatocyte binding sites on initial and continuous HBV infection, and to explore their contribution to pathogenicity, and their effect on the intensity of the infection as reflected by the number of infected hepatocytes.

Chapter Six: Genetic Variations of HBV Basal Core Promoter, Precore/Core and Overlapping X-gene

6.1. Introduction

6.1.1. Core promoter/ Precore/core gene mutations

Hepadnavirus genome have high mutation rate of 2 $x10^{4}$ base substitutions/site/year. HBV mutations arise spontaneously during replication due to infidelity of the viral replication machinery by reverse transcription, RNA pregenome synthesis by RNA polymerase, editing of viral DNA by cellular cytidine deaminases resulting in G to A and C to T substitutions, or recombination and cleavage/ligation of DNA by topoisomerase I. In addition, deletions or insertions can be generated by splicing of the pregenomic RNA (Gunther; 2006). Mutations within the core can be a mechanism to avoid the host immune response, thereby maintaining viral persistence during chronic infection. Alterations in the epitopes in the form of amino acid (aa) substitutions are mostly restricted to a small segment located in the middle of the core region (nt. 2063 to 2365, 303 bp).

The stem-loop structure of the core promoter is recognized by the viral polymerase. Disruption of the conformational structure by mutations interferes with encapsidation and binding of pregenomic RNA via the arginine–rich C-terminal domain (aa 67-262) of the core protein, resulting in defective replication. Compensatory mutations tend to re-establish base pairing in the hairpin loop stem and regain the secondary structure to permit RNA packaging (Francois et al., 2001). 1762-1764 mutations have importance at the level of transcription by providing four primer-binding nucleotides in DRI (fig.6b) rather than three (fig.6a). Further mutations at nt. 1751 to 1755 would enable the basal stem structure to open more easily for reverse transcription (Kidd-Lijunggren et al., 1997). Replication could also be influenced by a point mutation at nt. 1746 in the BCP that is responsible for the correct initiation of precore and pregenomic message (see table 6-1).

6.1.2. Mutations and HBeAg

Double BCP mutation (T1762/A1764) is increased in the HBeAg-seroconverters than in the non-converters (48% vs. 28%). Seroconversion occurred in 50% of those patients within 1 year, 88% within 2 years, and 93% within 5 years (Pang et al., 2004).

The wild type virus is predominant in HBeAg-positive stage. Prognostically, precore (PC) mutants mark the sustained HBeAg seroconversion, indicating that immune responses, specifically against HBeAg select precore stop mutants. G1896A started to increase 1 year before and became significantly higher at seroconversion (23 vs. 3%) than that in the non-converters (Yamaura et al., 2003). The precore G1896A /UGA stop codon aborts the translation of the soluble HBeAg, thereby causes HBeAg-negative mutants (Gunther; 2006). Impaired expression of HBeAg frequently arises at the chronic stage of infection, as exemplified by PC and BCP mutants. However, anti-HBe antibody develops possibly due to cryptic HBeAg that is exposed during HBcAg degradation and turnover in infection.

Sometimes precore (PC) mutations were associated with increased viral replication, so it contains regulatory polypeptide suppressing viral replication. The C-terminal of the PC region contains most mutation sites. A1838G changing the amino acid isoleucine (ATC) to valine (GTC) (Gandhe *et al.,* 2003). Such mutation may change the binding efficacy of transcription factors to the core promoter, as well as the production of precore and core messages. The hydrophobic domain of the precore peptide functions as a leader sequence to anchor the peptide to the membrane of the endoplasmic reticulum. The stability of the secondary structure formed by the precore pregenomic RNA depends on strict base-pairing, forming two copies of the stem-loop structure; one in the 5`forming the encapsidation signal ε (fig.6-2), which directs the packaging of pregenomic RNA into immature core particles during replication (Schaefer; 2005).

The frequency of anti-HBe positive chronic hepatitis B is determined by a C/U exchange at position 1858. An exchange of C1858 to U1858 favors the mutation of G1896 to A1896. This double mutation can form a stable base pairing between these two

nucleotides in the stem loop of the packaging signal of the hepatitis B virus pregenome. A1896 change codon 28 of preC to a stop codon which prevents the translation of HBeAg. For genotypes C and F strains have been described which show a different C/U1858 phenotype than indicated (see fig.6-1).

Deletion and /or insertions within the DR1 region (precore codon 6-7) are replication deficient and multiply by the assistance of another virus strains with complementary mutations or by a co-existing wt virus through alternative priming of DNA synthesis (Laperche *et al.*, 2006).

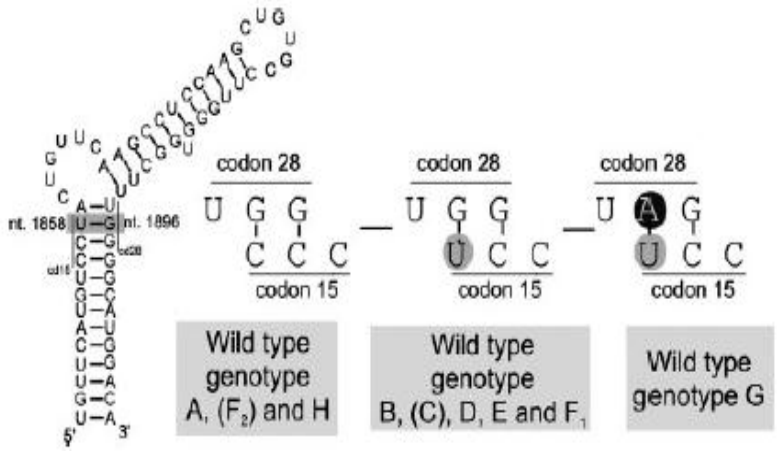


Figure 6-1: Precore encapsidation signal ε

Loop structure formed by folding of pregenomic HBV RNA. A U at 1850 and an exchange C1858U favoursthe mutation G1896A, thus forming a stable base pairing in the stem of the ε encapsidation signal as in wild type genotypes B, C strain, D, E and F1 (Schaefer,2005).

6.1.3. Core gene mutations:

Core mutants may also inhibit replication by a dominant negative effect up to 95%. Since disulfide bond maintains the secondary structure of HBeAg. Substitution of each cysteine especially C-7, rendered HBeAg nearly undetectable. Replacement of C61 with arginine, but not alanine, aspartic acid, phenylalanine, or serine, blocked virion secretion, which could be overcomed by co-expression of wild type core protein, generating viable HBeAg-negative variants (Bang *et al.*, 2005). The truncated C-terminal core protein was always accompanied by a point mutation at nt. 2112. Mutations A 2189 C and 2086C

HBV adr subtype genome core mutant (L97 and V60) exhibited HBV DNA replication activity (Chen *et al.*, 2005). Point mutation at nt. 2414 in one of the three SPRRR motifs, are important for phosphorylation and nuclear localization. Deletions lead to an impairment of HBV capsid assembly from core protein dimers (Francois et al., 2001).

6.1.4. X gene mutation

X-gene mutations overlap the core promoter/precore or ENII region. In addition, DR2 can down-regulate gene expression and thereby suppress HBV protein secretion. Mutations in X gene are related to progression to chronic disease as a consequence of the rescission of antiproliferative and apoptotic effects, which might produce uncontrolled growth and contribute to multistep hepatocarcinogenesis (Leon et al., 2005). A HBV mutant with fused X-C ORF caused by a single nucleotide insertion in the overlapping region has been identified (Francois et al., 2001).

The integrated HBV genome may activate cellular genes to promote growth of liver cells. Various viral factors are associated with HCC development as BCP mutations, high viral load and HBV genotype. Chen et al., (2005) found an insert mutation AGGCCC at position 204, that co-exist with G260A and G/C/T264A variants. Both were associated with the nuclear localization of HBx protein (Preikschat et al., 2002). Whilst the serine to alanine mutation at codon 31 was significantly more prevalent in cancer patients than in asymptomatic carriers (Chan et al., 2006). Thus, the low frequency of mutations reflects the absence of antiviral pressure and indicating a degree of association with disease progression.

6.1.5. Genotype differences

Genotype differences correlate to HBeAg-seroconversion and can be associated with mutation (tables6-2 and 6-3); HBV subgenotype Cs has a higher tendency than Ce to develop BCP and overlapping X-gene mutations (80% vs. 50%) (Chan et al., 2005). Characteristically, HBV genotype A have a 6-nucleotide insertion at the 3`end of the Core gene. Genotype G has a 36-nucleotide insertion at the 5`end of the core gene, and consequently expresses a 24-KD core protein with 12 extra amino acids at the C-terminus

and stop at precore codons 2 and 28 (Alvarado-Esquivel et al., 2006). Hasegawa *et al.*, (2006) identified T1809/T1812 ("Kozak" sequence) and A/T1888 (encapsidation signal) in 18/18 (100%) and 78%, respectively/subgenotype Aa. T1809/T1812 immediately upstream of the precore initiation codon, which would interfere with the translation of HBeAg (Tanaka et al., 2004). The nucleotide polymorphism C2733A differentiates HBV subgenotypes Cs (Southeast Asia) from Ce (Far East) (Chan et al., 2006).

Table 6-1: Common mutations in the HBV core promoter and precore and X gene

ORF*	DNA Position	Codon	Genotype/Association	Reference
Core Promoter 1742-1813	1762-1764 GGG to AGG or TGA (double BCP mutant	7, 8	Anti-HBe phenotype. Chronic HBeAg-negative hepatitis. Severe Liver damage, FH, HCC. Affecting transcriptional regulation and translation of precore gene. Enhanced replication and core expression. Interfere with binding of liver specific factors, X protein and hepatocyte nuclear factor 1.	Chen et al., 2005
	1766-1768 CTT to TTA	9	Advancing liver diseases. Increased transcription and encapsidation of pregenomic RNA. Carriers and chronic hepatitis B. Associated with T1762 and or A1764.	Gandhe *et al.*, 2003
	increased C1753, decreased T or G1752, T1773, A1775G G1799	4 12 17	HBeAg negativity advancing liver diseases. Fulminant hepatitis	Chen et al., 2005 Imamura *et al.*, 2003
Precore 1814-1899	1814, T1815C	1	HBeAg negativity. Initiation codon mutation	Gandhe *et al.*, 2003
	C1817T	2	Gln to Stop codon	De Castro *et al.*, 2001
	G1862T Missense mutation/ frame shift	17	HBeAg negativity Chronic hepatitis B HBV carriers, G1862A. T1858C	Gandhe *et al.*, 2003
	G1888A	25	increase stability of upper stem of the encapsidation signal in pregenomic RNA. T1858C. Chronic hepatitis B	Gandhe *et al.*, 2003
	G1896A	28	Trp to Stop mutation Loss or decrease of HBeAg associated with genotype B and D more than A or C. Severe HBeAg-negative hepatitis.	
	G1899A	29	Gly to Asp. Enhance the pregenomic RNA transcription	Gandhe *et al.*, 2003 De Castro 2001
Enhancer I			Decreased replication, Chronic hepatitis	Baumert and Blum; 2000
Core			Alteration of T-cell epitopes. Inhibit viral replication. Impaired capsid assembly, decrease pregenomic RNA binding. Viral persistence	
	A2339G/G2345A		Increased replication efficiency and accumulation of the full-length core protein without cleavage by furin-like protease.	Sugiyama et al., 2007)
X gene	L5M, Q8K, T12A, S29P, S31P, S33P, V37I, P40S, D48N, R87W, R103W, T106P, K130M/V131I. D110E,Stop codon 135aa		Transcriptional transactivation	Leon et al., 2005
	1768-1775 8 bp- deletion	9-11	Frame shift and truncation of X protein	Fischer et al., 2006,Chan *et al.*, 2006

*ORF: Open reading frame

6.1.6. Clinical Implications of Precore/core gene mutations:

Precore (PC) mutants are more associated with chronic than acute infections. Amino acid substitution rates peaks in exacerbation of hepatitis than those before and after exacerbations, and switching over to PC mutant was beneficial in maintaining disease free status (Gandhe et al., 2003). HBeAg-seroconversion was confirmed to occur earlier than core promoter or precore mutations in genotype D, and occur frequently in late teens in HBV genotype E from West Africa with very low genetic diversity (nucleotide homology 96.7-99.2%)(Fujiwara et al., 2005).In genotype D, severe disease is associated with single or double precore mutations (G1896A/G1899A) (Fujiwara et al., 2005). In patients with severe hepatitis-B, the mutation rate was 52.5% (12/23) (Wang and Liu, 2003).

BCP and PC mutations, higher age, elevated ALT and anti-HBe positivity are risk factors for severe acute hepatitis (Yotsuyanagi *et al.,* 2005), and fulminant than acute self-limited hepatitis (9% vs. 53% and 17% vs. 50%). Risk factors include Age 34 years or older, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and HBV genotype D (Wai et al., 2005) or Bj subgenotype (Ozasa et al., 2006).

On the other hand, chronically infected carriers with anti-HBe have increased viral replication at high levels, they are associated with PC mutants, enhanced pathogenicity, and increased core protein synthesis that eventually progress to severe chronic active hepatitis (CAH). However, other groups found no association between PC mutations and Hepatitis Activity Index (HAI) scores (Yuen et al., 2005).

Hepatitis activity was significantly inhibited in patients with initial PC rather than initial BCP mutation (Hagiwara et al., 2006). BCP mutations were significantly associated with higher Hepatitis Activity Index (HAI) and more severe necroinflammation, but not fibrosis score (Yuen et al., 2005). In HCC, 62.1% of patients with BCP mutations, genotype C and age > or =35 years had liver cirrhosis (Chen et al., 2005), and HBV load > or = 10^5 copies/ml (Liu et al., 2006).

Table 6- 2: Genotype specific sequences within the precore/core gene

ORF	DNA Position	Codon	Genotype/Association	References
X-gene	C1653T Core upstream regulatory sequence (CURS)		B, C, FH	
	1726- 1730 CTGAG		B	Lindh et al., 2000
	1744		B, C (adr) Liver damage	*
Core Promoter	G1752A G1752C		Genotype B, D Genotype A	Lindh et al., 2000
	increased 1762T/1764A, C1753, T1766, A1768, A1896 and decreased T or G1752, T1773, G1799, and C1858		Genotype C, advancing liver diseases	Chen et al., 2005
	G1757A		D, E, FH	De Castro et al., 2001
	C1773T		D	
	A1775G		C	
	C1802T G1803T		B, C, F, H	Lindh et al., 2000
Precore	A1850T	13	D, F Chronic hepatitis B, T1858C	De Castro et al., 2001, Gandhe et al., 2003
	T1858C	15	Genotype A	
	C1858T	15	Non A Genotypes : B, D, E, F HBeAg-negative Chronic hepatitis, encapsidation signal (ε)	De Castro et al., 2001
	G1896A	28	Genotype B > C, genotype D > A	
Core	2020A 2020G 2020T 2020-1AT	40 40-41	A, D B, C D, E, F, H G	Inoue et al., 2008

BCP/PC and antiviral therapy

Prognostically, BCP mutants predict the response to interferon. Lamivudine therapy resulted in a further increase in BCP mutants in complete responders (Chen *et al.*, 2006). PC stop mutation is associated with spontaneous or interferon-induced HBeAg-seroconversion (Kuwahara *et al.*, 2004), while low serum HBV DNA level have low breakthrough rate following lamivudine therapy (Shin et al., 2005). Initial ALT level of 300 U/L or more, G1896A mutant (Chen *et al.*, 2006), and genotype B infected patients treated with lamivudine (Huang et al., 2003). In liver transplant recipients, recurrent graft infection by PC/BCP variants may result in fibrosing cholestatic hepatitis (Lo *et al.*, 2004). In addition, other mutations in nucleotides 1752, 1773, 1802, 1803, 1845, 1850, and 1858 was found in the non-responders during lamivudine therapy, that might correlate with HBeAg persistence (Chen *et al.*, 2006).

6.2 Aim

The aim of this study was:

To characterize mutations present in HBV core promoter /precore gene, and to compare their prevalence in patients in Saudi Arabia, and UK; including the HBeAg-negative mutants.

To explore the relationship between different mutations and patient demographics, serum HBV DNA levels, and severity of liver disease.

To analyze the frequency of mutations in different HBV genotypes

To check the effect of genotypes on natural HBV sequences, and inferred core and X-proteins structure.

6.3 Hypothesis

Saudi HBV infected patients are usually HBeAg-negative, and generally have low viral load (chapter 3). The possibility of HBV mutants exists, particularly the HBeAg-negative mutants. The effect of HBV genotype among other undetermined factors may contribute to chronicity and favour the development of mutations.

6.4. Patients and Methods

Patients Details (see chapter 3; 3.5.1.1. and 3.5.1.2.)

DNA was extracted from HBsAg positive sera of 23 UK patients and 56 Saudi patients.

PCR of precore (PC) was performed on all 79 sera and basal core promoter (BCP) regions was done on only 26 from UK and 23 sera from Saudi hepatitis B patients.

DNA was extracted by QIAAmp (blood) kits (Qiagen).

Amplification of Core Promoter /Precore Gene(See Chapter 2)

Basal Core Promoter PCR

Optimization was done using combination of primers; C1 and PC3, or C3 nt. 1611-1630 5`-GAGACCACCGTGAAC GCCCA-3`and PC3 to cover this important region of the gene. Variation of primers and PCR conditions led to the development of the Xgene/basal core promoter PCR (EC) PCR using primers C1 and PC3 with product size 475 bp (data not shown) (See Chapter 2).

Quantitative PCR (See Chapter 3).

Nucleotide Sequencing Method:

Sequencing the products of precore PCR reaction (nt. 1781-2075; 286 bp), and Basal core promoter PCR product (nt. 1601-2075; 475 bp) with ABI prism protocol (see chapter 5). Sequencing primers are PC1, PC3 and C1. Sequencing was done in the immunology department, University of Nottingham.

Computer Assisted Sequence Analysis

DNA sequences were edited and aligned with reference sequences from the Gene Bank using Clustal X program. Sequences of the PCR products were compared and analyzed. Phylogenetic relationships between sequences were estimated by Neighbor-joining method (Saitou and Nei; 1987) applied to pairwise distances by estimated by the 2-parameter method (Kimura; 1980). Sites at which there was a gap in any sequence in the alignment were excluded from all comparisons. The reliability of the phylogenetic results was assessed by re-sampling using 1000 bootstrap replicates (Felsenstein; 1985). Associations with a bootstrap value of greater than 70% were deemed significant. These analyses were performed using the MEGA3 package.

6.5. Results:

PCR Amplification and sequencing of HBV Pre Core and Core Promoter Regions

The core gene was amplified by different PCR reactions spanning different regions of the gene. First amplification of the pre core region of genomic HBV DNA by PCR was done by precore polymerase chain reaction (PC PCR) primers PC1 and PC3 (fig.6-3). The sensitivity of precore PCR in detecting specimen with 2 $\log_{10}$copies/ml was higher than other PCR reactions, as Basal core promoter PCR (chapter 2) (data not shown). Being a single round, PC-PCR decreases the likelihood of contamination compared to nested reactions.

Basal Core Promoter PCR

X gene/Basal core promoter (EC) PR was done to cover basal core promoter area in addition to the PC region (fig.6-4).

Sequencing of the Core Promoter region of HBV genome:

Sequencing of basal core promoter PCR products gave an acceptable coverage of both, core promoter and precore regions as well as the beginning of the core gene. The results (table 6-4) showed basal core promoter (BCP) variants 1762-1764 were seen in 19/26 (73%) Saudi patients compared to 9/23 (39%) in UK patients (p:0.003). The first possibility of BCP 1762-1764 a change of AGG to TGG, was seen in five Saudi patients. While the other 2 variations; 1762/1764 A/A, and the double mutant T/A were seen in two and seven patients, respectively. Other types of BCP variation were seen only in five Saudi patients; two patients had G1762/T1764, one had C1764, 1 T1764 and one case showed 12 bp-deletions in BCP region. All had chronic hepatitis and one case presented with HCC.

Of the basal core promoter (BCP) variants 1762-1764 present in 9 out of 23 (39%) UK patients, one of them had AGT. 1762/1764 A/A, and the double mutant T/A were also seen in three and five patients, respectively. Some cases showed both wild type and mutants as the case N15, 1-50 and 1-48 (fig.6-5). C1766T, T1768A and C1773T found in 5/8, 2/8 and 8/8 Genotype A from UK, 4/17, 4/17 and 13/17 Genotype D Saudi patients, respectively. C1799G was present in all genotype B patients 7/7 and in 3/17 Genotype D Saudi patients. C1812T in only one genotype A case from UK.

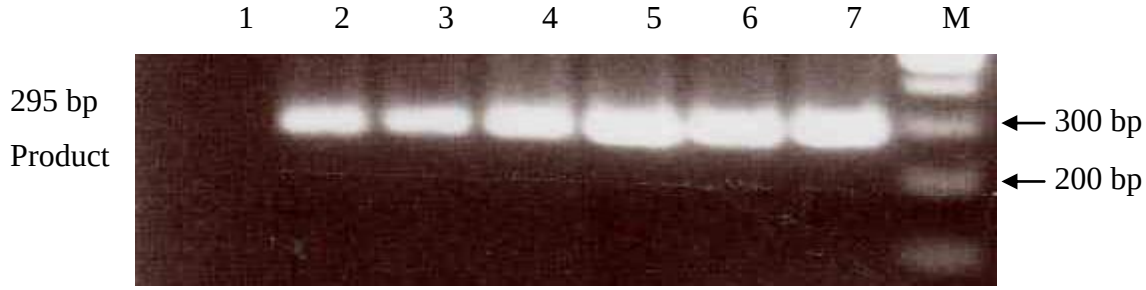


Figure 6-3: HBV Pre core PCR

Lane 1: negative control, lanes 2-7: UK specimens, 2: N1, 3: N2, 4: N3, l5: N4, 6: N5, 7:N6, M:100 bp DNA ladder.

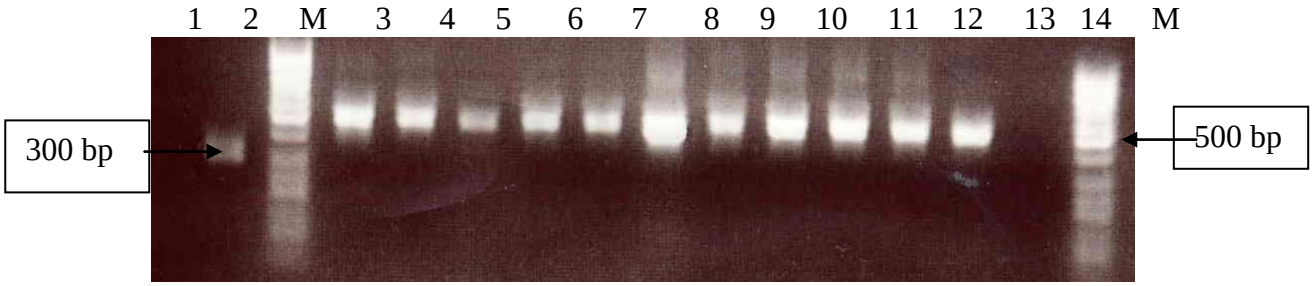
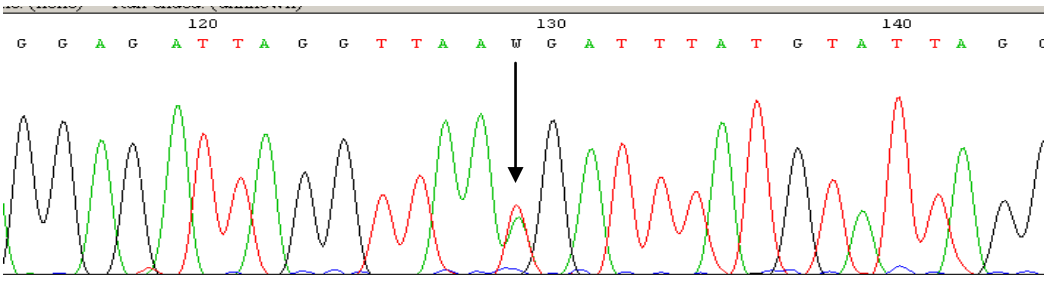


Figure 6-4: Pre core and Basal core promoter PCRs

lanes 1: Negative control, 2: precore PCR product, M: 100 bp DNA ladder, Lanes 3 -13: X gene/Basal core promoter (EC) PCR specimens N1-N11 respectively. 14: negative control.

Figure 6-5: Basal core promoter mutation

Sample N15 showing 1762-1764AGA and TGA double mutant. W: double peak of nucleotides A and T.



Sequencing of the Precore region of HBV genome:

Upon sequencing of the HBV precore region and the beginning of the core gene (nt. 1781 to 2075), significant proportion of Saudi patients 37/56 (66.1%) had precore mutants (p: 0.004) (table 6-4). Of those, codon 28 mutant (G1896A) was present in 30/37 (81.1%), resulting in a change of tryptophan to a stop codon. Double mutants in both codons 28 and 29 were seen in 15/37 (40.5%) of total mutants. G1899A mutation, which alters codon 29 from glycine to aspargine, was detected mostly in Saudi genotype D patients 22/37 (61%) (table 6-4). In contrast, UK patients showed a much lower prevalence of G1896A 5/23 (21.7%), of HBV genotypes A, B and D (appendix A).

Nt. C1858 was present predominantly in genotype A patients 7/9 UK and 8/9 Saudi genotype A. All genotype C 3/3 UK patients had TCC in nt. 1856-1858. All Saudi and UK patients with genotypes B and D and E had T at position 1858 regardless of their nt. 1896 status. Other point mutations were observed. Our genotype D had G1757A in 15/20 Saudi and 3/3 UK patients. In patients with double mutations at codons 28 and 29; eight patients had also mutations at position 1940 G to C. 32/40 Saudi genotype D patients (82%) had C1912T. This mutation was also present in genotype D1 Gene Bank control AY161157, and X02496. It is a non-coding mutation; thereby it does not change the amino acid aspargine (Asp). Nine patients from UK had T1850A, and seven patients had A to T at nt. 1934, which is present in genotypes Ba, C and E. None of the patients had A1838G, or G1888A mutation.

HBeAg status of core promoter/precore mutants

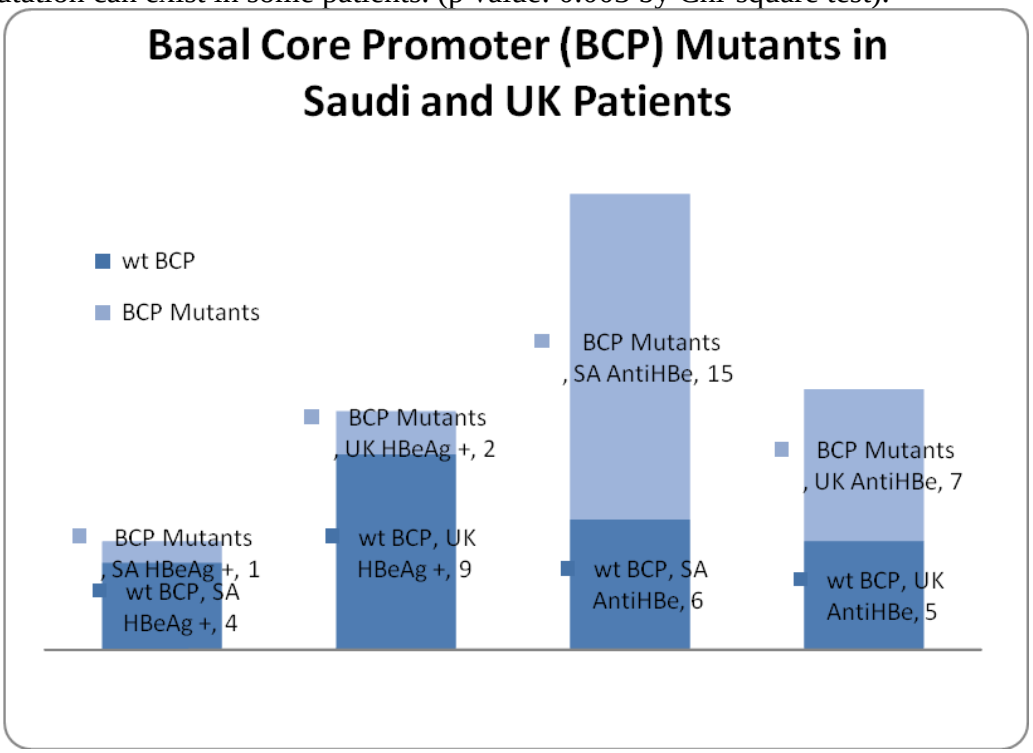
BCP/PC variants were analyzed for correlation with HBeAg, HBV viral load and genotype. Within HBeAg-positive, UK patients had mostly wild type BCP 9/11 (88%).Comparably, BCP mutations were present in 4 out of 5 (80%) Saudi cases. HBeAg-negative had predominantly BCP mutants, however, less in UK than in Saudi patients at 7/12 (58.3%) and 15/21 (71.4%), respectively (table 6-4).

Graph 6-1A: HBeAg status of HBV core promoter (BCP) (p value: 0.003).

Graph 6-1B: HBeAg status of HBV precore (PC) mutants in UK and Saudi patients.

Note predominantly wild type precore in UK patients compared to the high prevalence of precore mutants in Saudi specimens (p value by Chi-square test). More than one type of mutation can exist in some patients. (p value: 0.003 by Chi-square test).

A



B

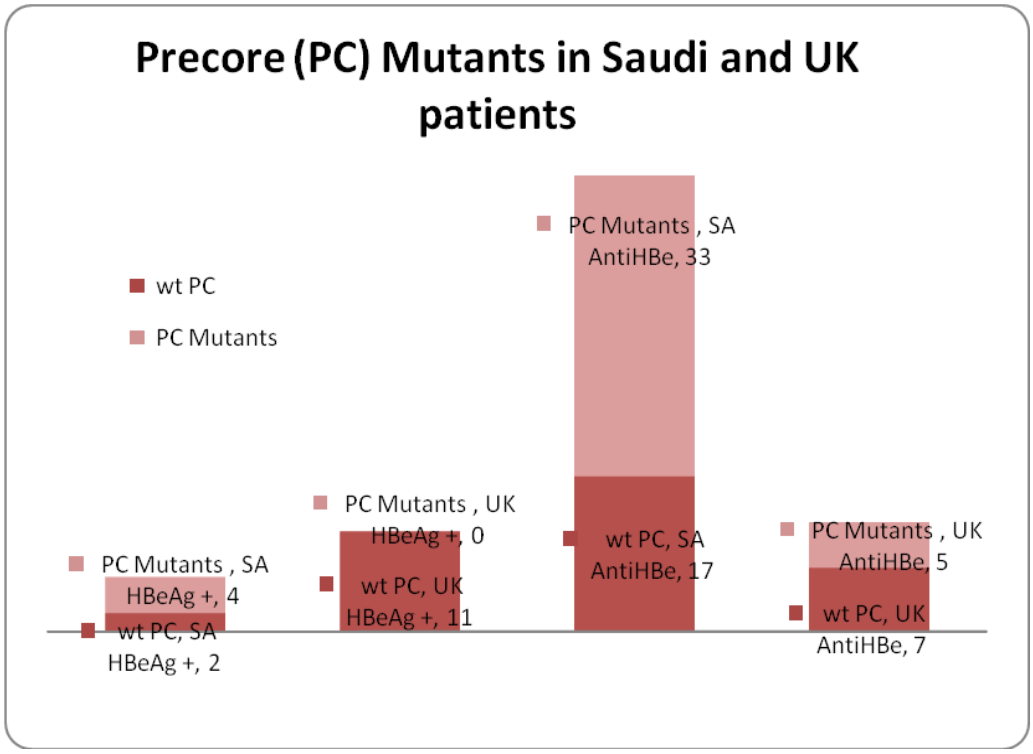


Table 6-3: HBeAg status of HBV core promoter (BCP) and precore (PC) mutants in UK and Saudi patients. (p value by Chi-square test).

	UK Patients			SA Patients		
	HBeAg +	AntiHBe	Total	HBeAg +	AntiHBe	Total
BCP Mutants P: 0.003	2/11 (22%)	7/12 (58.3%)	9/23 (39.1%)	4/5 (80.0%)	15/21 (71.4%)	19/26 (73.1%)
PC mutants p: 0.004	0/11 (0%)	5/12 (41.7%)	5/23 (21.7%)	4/6* (66.7%)	33/50 (66.0%)	37/56 (66.1%)

* Two had G1899A and two had G1896A/G1899A

Table 6-4: HBV core promoter (BCP) and precore (PC) mutants in UK and Saudi patients and their average viral loads. Different HBV X-gene mutants in Saudi and UK patients. More than one type of mutation can exist in some patients. PC mutations detected in either or both codons 28 and 29 corresponding to nt. 1896 and 1899, respectively. Note predominantly wild type precore in UK patients compared to the high prevalence of precore mutants in Saudi specimens (p value by Chi-square test).

BCP/PC status	UK Patients		SA Patients		p Value of mutation rates
		Mean HBV DNA ¥		Mean HBV DNA ¥	
Wild type BCP	14/23 (60.9%)	6.1	7/26 (26.9%)	4.7	0.003
Total BCP mutants (corresponding HBxAg):	9/23 (39.1%)	6	19/26 (73.1 %)	4.2	
- A1762T alone (K130M)	1/9 (11%)	6	5/19 (26%)	4.9	
- G1764A alone (V131I)	2/9 (22%)		2/19 (11%)		
- A1762T/G1764A (M130/I131)	5/9 (56%)	5.1	7/19 (37%)	4.2	
- Other BCP mutants	1/9≠ (11%)	6.6	5/19 (26.3%)*∞	3.9	
- (I127N)	4/9 (44%)		0/19 (0%)		
- (F132Y)	2/9 (22%)		7/19 (36.8%)		
Wildtype PC	18/23 (78.3%)	6.1	19/56 (33.9%)	4.6	0.004
Total precore mutants:	5/23 (21.7%)	5.9	37/56 (66.1%)	4.3	
- G1896A alone	3/5 (60%)	5.2	15/37 (40.5%)	3.9	
- G1899A alone	0/5 (0%)		7/37 (18.9%)	4.6	
- G1896A /G1899A	2/5 (40%)	4.9	15/37 (40.5%)	4.4	

* Other types of BCP mutations two patients had G1762/T1764 (K130R), one had C1764, one T1764.

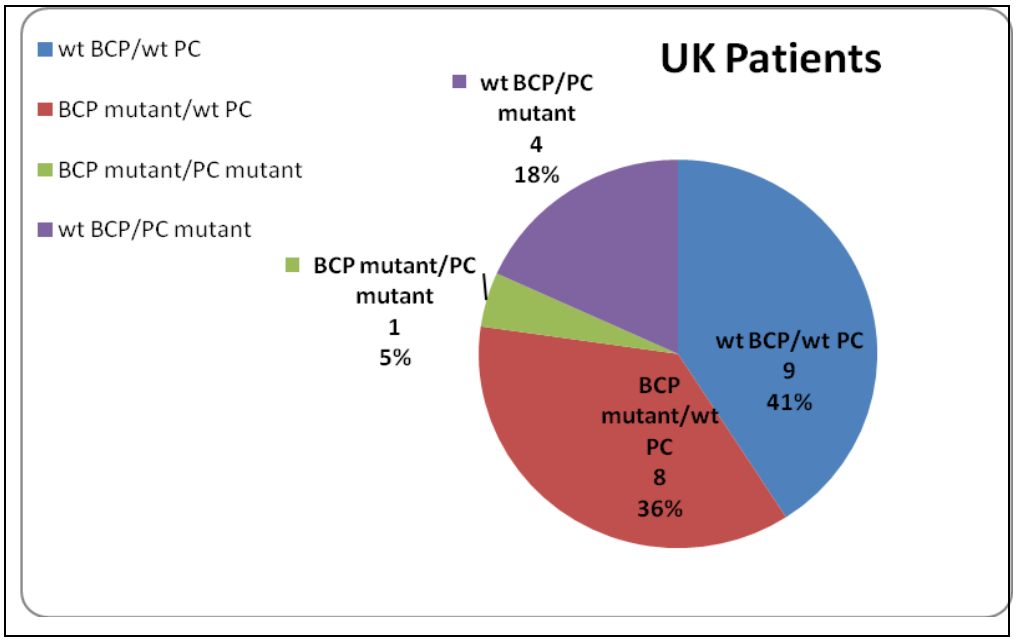
∞ One case had 12 bp-Deletion (nt. 1752 to 1763).

≠ A1762/T1764

¥ p value of mean HBV DNA is 0.155

Graph 6-2 A: Coexistence of HBV basal core promoter (BCP) mutants and precore (PC) mutants in UK patients. (p value: 0.003)
Note predominantly wild type precore present in UK patients with or without BCP mutants.
Graph 6-2 B: Coexistence of HBV basal core promoter (BCP) mutants and precore (PC) mutants in Saudi patients. In contrast, high rate of both mutants was found in Saudi patients.

A



B

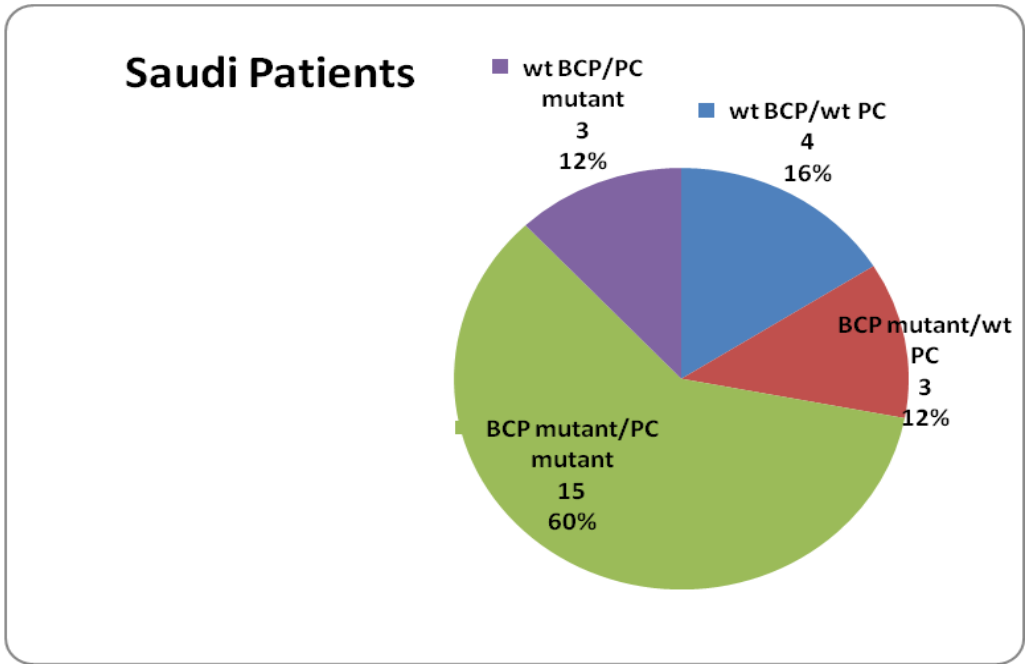


Table 6-5: Other identified core promoter and precore gene mutations

Open Reading Frame ORF	DNA Position	Nucleotide Change	Codon	UK patients	Saudi patients	Association
Core Promoter 1742-1814	1766-1768	CTT to TTT or TTA		5 genotype A	4-19, 3-6	
Precore 1814-1899	1858	C	15	7 genotype A 3 genotype C	None	In genotype A; 1858C prevent PC :TAG
	1858	T	15	6 genotype B 2 genotype D	9 Genotype D	B, C, D, E, F
	1862	G to T Missense mutation/frame shift	17	14 (A)	None	

Many UK patients had wild type in both BCP and PC 9/22 (40.9%) (Graph6-1). Wild type (normal) precore was seen in all HBeAg-positive UK patients 10/10 (100%), with genotypes A, B, C, D. HBeAg-negative Saudi had significantly more precore mutants than UK patients at 33/50 (66%) and 5/12 (41.7%), respectively. Either core promoter and/or precore mutants were found in most of the Saudi patients. On the other hand, coexisted BCP and PC mutants occurred mainly in Saudi patients 21/25 (84%) versus only one UK case 1/22 (4.5%) (Graph 6-1). BCP and PC coexisted mostly in Saudi genotypes D (10 cases).

Among other core promoter mutations, four out of 20 Saudi genotype D patients had T1753A, two had chronic hepatitis B and one cirrhotic and one HCC. All 3 UK genotype C patients had G1721A and /or A1775G. Two UK patients both were genotype A (N15, N4), and four Saudi patients 4/25 had C1653T, all were genotype D. Another observation is G1719T G1in the 3 genotype D UK and Saudi patients (17/40). In all our cases nt. 1838A was present from all genotypes including genotype B (subgenotype Ba). G1862 T was seen in only one genotype A case from UK (N14) with viral load of 4.49 $\log_{10}$ copies/ml clinically presented with cirrhosis (table 6-6).

HBV viral load and BCP/PC status:

The mean HBV DNA of wild type in both BCP and PC was higher than that of BCP or PC mutants (table s 6-5); double mutants had lower HBV DNA than single BCP or PC

mutants (table 6-4). The new BCP mutants from Saudi patients have even lower viral load. Both Saudi and UK PC mutants were associated with lower HBV DNA than their wild type PC.G1896A alone has lower mean viral load at 3.9 than wild type and G1899A both had 4.6 log_{10} copies/ml.

The correlation of BCP with PC status showed that within BCP mutants, the mean HBV DNA decreases in mutant BCP/wt PC, then mutant in both BCP and PC. While the less frequent wt BCP/ mutant PC showed the lowest viral load (Grph6-1).

UK BCP mutants were present more in the intermediate (=>3-<5 log_{10} copies/ml) viral load group double the rate in high viral load group at 3/4 (75%) and 6/17 (35%), respectively (graph 6-3). However, Saudi BCP mutants were predominant both in the intermediate and high HBV DNA levels at 8/11 (73%) and 8/10 (80%), respectively. Core promoter and precore mutants were associated with different serum HBV DNA levels in HBeAg-negative patients.

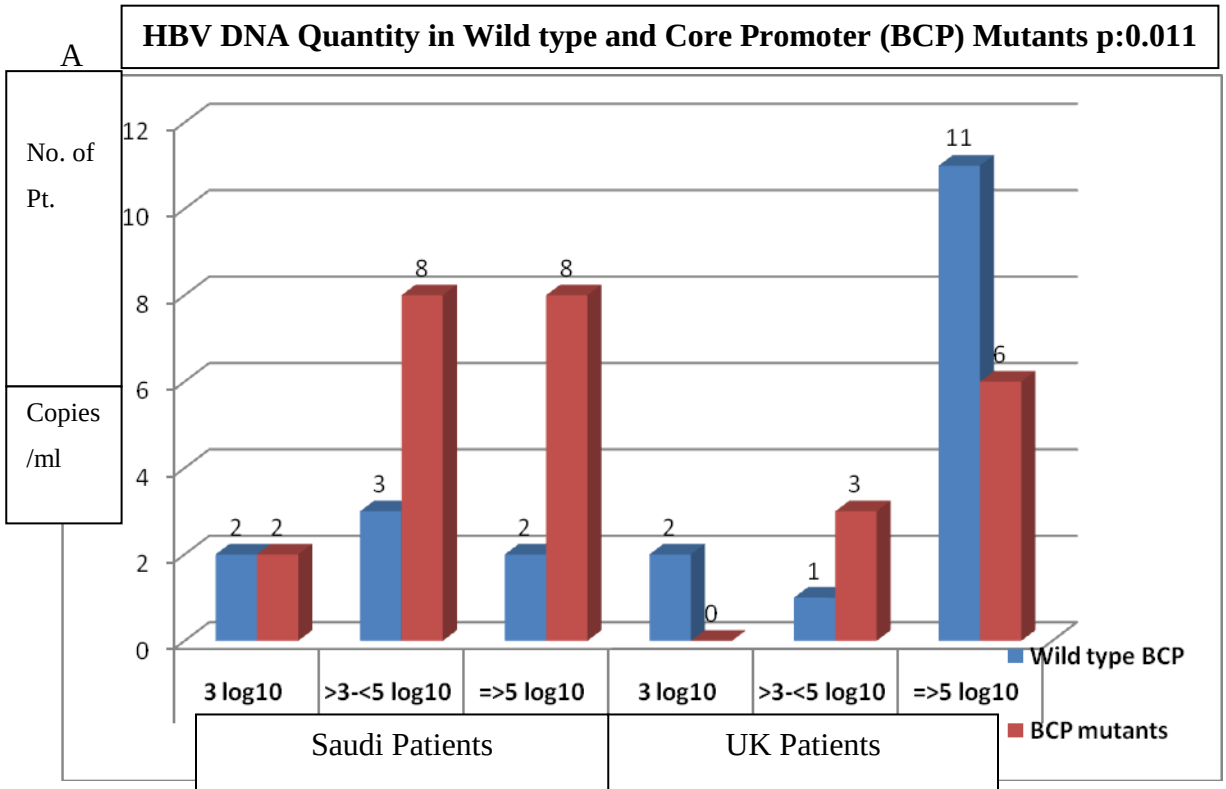
Saudi PC mutants were found at low HBV DNA levels of =<3log10 copies/ml more than>3 -<5, and =>5 log_{10} copies/ml reaching 8/10 (80%), 15/24 (63%), and 14/22 (64%), respectively. Some of them had very high HBV viral load (=> 7 log_{10} copies/ml). The rates of UK PC mutants were lower in both>3 -<5, and =>5 log_{10} copies/ml at1/4 (25%), and 3/17 (18%), respectively (table 6-6).

HBV genotype and BCP/PC status:

Core promoter mutants were highest in genotype A and genotype D at 7/9 (78%) and 17/20 (85%), respectively (table 6-10). Genotypes B showed less BCP mutants, but their numbers were too small for a solid conclusion. Double basal core promoter (BCP) mutations were seen in genotype A and less frequently in genotypes B, D and E patients at high HBV DNA levels. In contrast, genotype D predominated at intermediate and low viral loads (p: 0.121).

Graph 6-3 A: HBV viral load of basal core promoter (BCP) mutants in UK and Saudi patients (p: 0.011)

Graph 6-3 B: HBV viral load of precore PC mutants in UK and Saudi patients (p: 0.019)



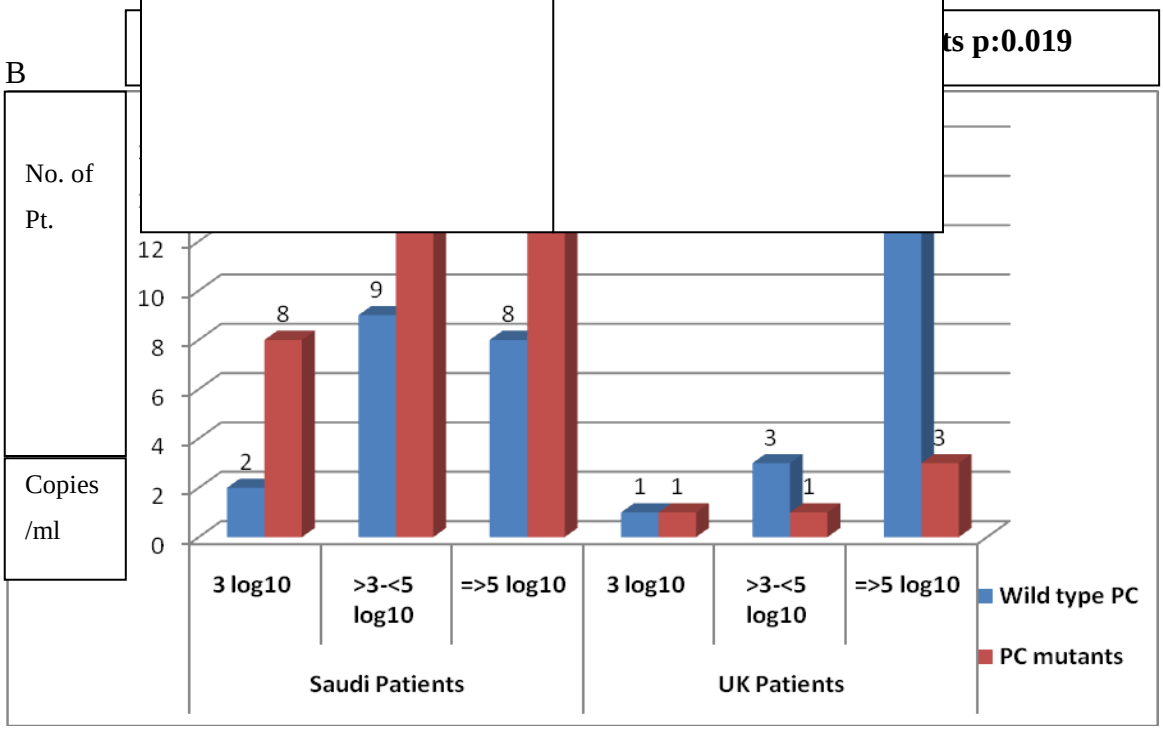


Table 6-6 : Correlation of HBV genotypes and basal core promoter (BCP) and precore (PC) mutants in UK and Saudi patients.

HBV Genotype		A	B	C	D	E	Multiple genotypes	Untypable
BCP mutants	UK	7/9 (78%)	2/7 (29%)	0/3 (0%)	0/3 (0%)	-		-
	SA	1/1 (100%)	-	-	17/23 (74%)	2/2 (100%)		-
PC mutants	UK	2/9 (22%)	2/7 (29%)	0/3 (0%)	1/3 (33%)	-		-
	SA	0/2 (0%)	-	0/1 (0%)	35/48 (73%)	2/2 (100%)	0/2	0 /1

Table 6-7: **Correlation of the clinical status and core promoter mutants (p: 0.033) and precore mutants (p: 0.019).**

Clinical condition	BCP Mutant		Precore Mutant	
	UK	SA	UK	SA
Inactive hepatitis	1/2 (50%)	0/2 (0%)	1/2 (50%)	9/13 (69%)
Liver disease	8/21 (38%)	19/24 (79%)	4/21 (19%)	28/43 (65%)
- Acute hepatitis	0/1 (0%)	2/2 (100%)	0/1 (0%)	4/5 (80%)
- Chronic hepatitis	6/18 (33%)	10/14 (71%)	4/18 (22%)	17/29 (59%)
- Cirrhosis	2/2 (100%)	4/5 (80%)	0/2 (0%)	5/6 (83%)
- HCC	-	3/3 (100%)	-	2/3 (67%)
Total	9/23 (39%)	19/26 (73%)	5/23 (22%)	37/56 (66%)

Precore mutants are predominant in Saudi HBV patients especially in genotype D 35/48 (73%) (table 6-10), one of the three UK genotype D patients had PC mutants. In contrast, PC mutations were less frequent in other HBV genotypes A, B, C and E. Interestingly, Saudi genotype D patients possessed high incidence of codon 29 mutant (G1899A) either alone or as double precore mutants reaching 21/48 (44%) compared to non genotype D cases..

HBV genotype and X/core gene sequence variation:

According to the phylogenetic analysis of the core promoter region (fig.6-7), sequences were grouped into six major clusters. Four of these were occupied by genotypes A-D. A majority of strains were homogenous in both their S and C gene trees (Genotyping chapter 4). Genotype E strains were the nearest to genotype D strains. Core promoter region sequences showed clear correlation to HBV genotypes and subgenotypes. In contrast, the precore region was highly conserved between genotypes and show little correlation if any to HBV genotypes (fig.6-6 and 6-7), the latter tree showed a large cluster containing genotypes A, B, C, D, E, H and G together. In addition, the short segment of nucleotides (200 bp) used was inconclusive to genotypes by phylogenetic analysis.

No significant differences were found in T cell epitope encoding regions as class I (aa18-27) nor class II (aa 1-20, 28-47)core epitopes between different HBV genotypes except V27I in genotypes B and C. Genotype A (adw2) and D (ayw2) sequences had amino acid substitutions at residues 12 and 27 which were not present in other strains.

HBV mutations and clinical presentation of HBV infection

Regarding clinical presentation, core promoter mutants (table 6-12) were present in patients with liver disease at 8/21 (38%) UK and 18/21 (90%) Saudi patients. Most Saudi patients with cirrhosis and HCC had BCP mutations. Saudi patients with cirrhosis and HCC had viral loads of 2 to 10.7 $\log_{10}$ copies/ml.

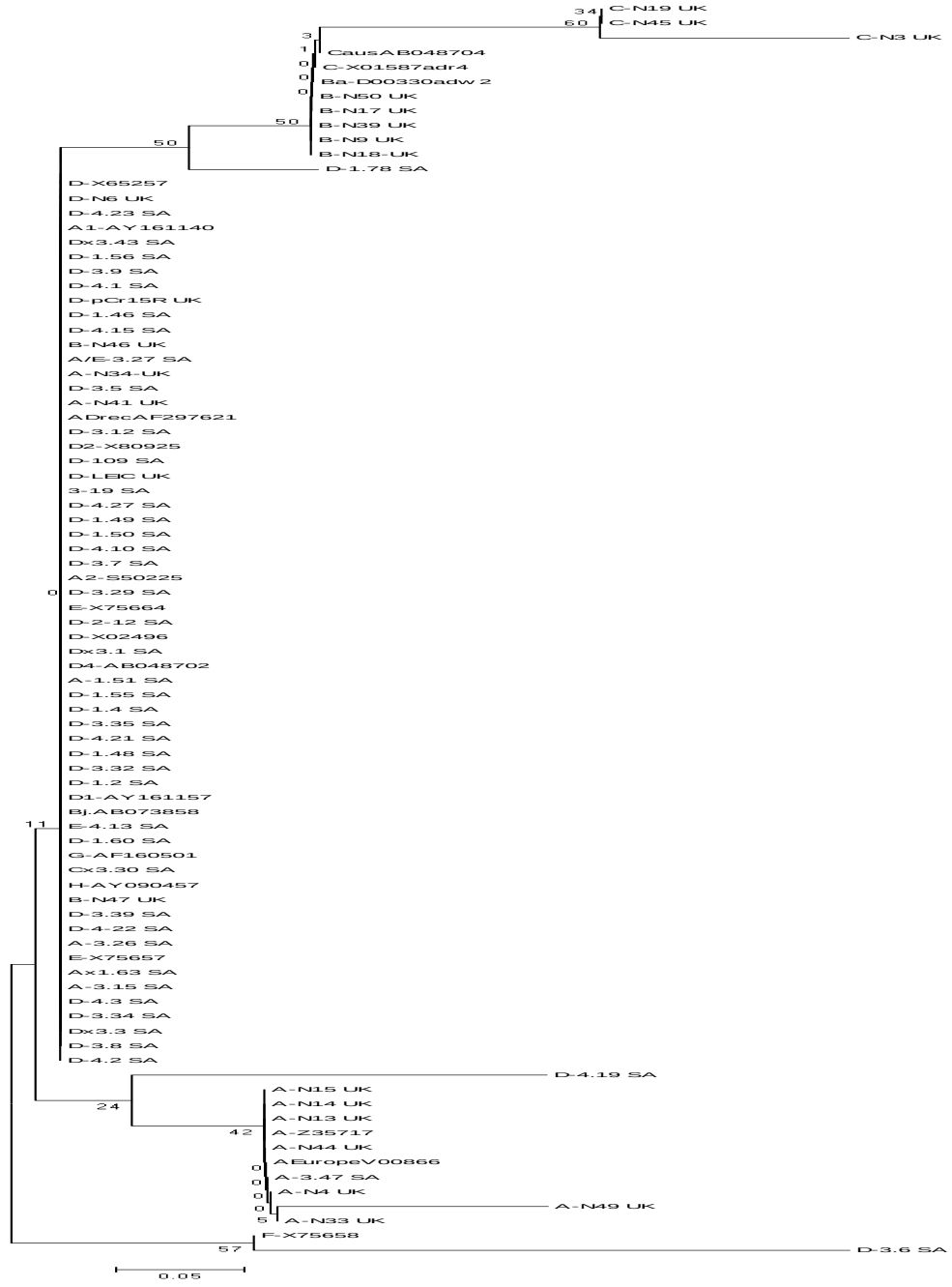


Figure 6-5: Phylogenetic tree of precore region/core nt. 1781-2075 showing absence of correlation to HBV genotypes. Gene Bank HBV genotype reference sequences; Z35717, V00866, AF297625, AB073858, X01587, X02496, X80925, X75657, X75664, AY090457, AF160501. UK and Saudi sequences marked with suffix UK and SA, respectively.

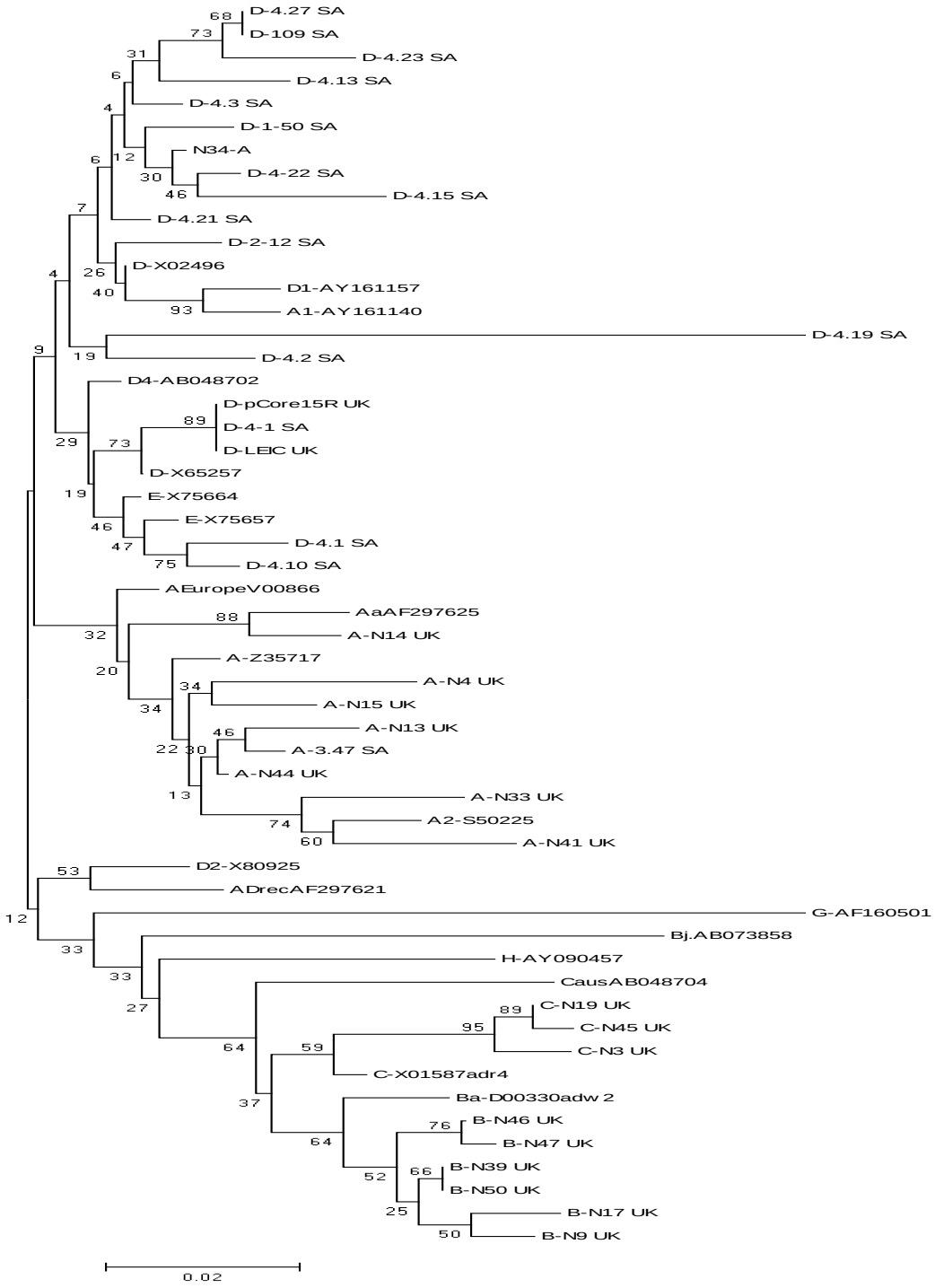


Figure 6-6: Phylogenetic tree of core promoter and precore regions nt. 1600-2075 showing correlation to HBV genotypes using bootstrap method 1000 replicates by Mega software. Gene Bank HBV genotype reference sequences; Z35717, V00866, AF297625, Doo330, AB073858, X01587, X02496, X80925, X75657, X75664, AY090457, AF160501 . UK and Saudi sequences marked with suffix UK and SA, respectively.

HBV X gene mutations

In this study, lysine to methionine (K130M) was seen in a total of 6/22 UK and 11/25 Saudi patients. Valine has changed to isoleucine (V131I) present in 8/22 UK patients and 10/25 Saudi patients (table 6-12). This overlaps the core promoter mutation 1762 and 1764. K130M/V131I were found in genotype A 4/7 from UK and 1/1 Saudi patients. Other X-gene variants; I127N and F132Y were seen.

Saudi patients with inactive hepatitis had comparable rates of PC mutants 9/13 (69%) to liver disease 28/43 (65%) (P: 0.019); as acute hepatitis 4/5 and chronic hepatitis 17/29 (59%) (table 6-7). Inactive hepatitis patients had low HBV DNA at or below 3.2 $\log_{10}$ copies/ml. Liver disease patients as chronic hepatitis B, cirrhosis with BCP/PC mutations had predominantly genotype D.

HBV X gene and genotypes

F132Y was evident in two genotype A out of 10 (20%) patients, and 6/20 (30%) genotype D patients.

6.6. Discussion

A comparative study like ours provides new information about HBV biology and shows important differences in the molecular epidemiology of the virus between Saudi and UK hepatitis B patients as regards to genotype, prevalence of mutations and clinical status. HBV is endemic in Saudi Arabia with a prevalence of 4.1% HBsAg carriers in the general population (Al Tarif *et al.,* 2004). However, there are very few studies on the local molecular epidemiology and genetic variation. Analyses studies suggest that mutations contribute to viral latency, pathogenesis of liver disease, immune escape, and resistance to antiviral therapy (Buti et al., 2005).

The prevalence of HBV basal core promoter (BCP) mutants in Saudi was nearly double the rate in UK patients at 19/26 (73%) and 9/23 (39%), respectively (table 6-4). In chronic hepatitis B patients from UK, BCP existed in wild type as variants AA 2/9 (22%), TG 1/9 (11%), and the double mutant TA 5/9 (56%). On the other hand, in USA, Hussain *et al.,* (2003) reported BCP AA mutant alone or with wild type (wt) in 16/384 (4%), TA alone 148/384 (38%) and in combination with wt in 186/384 (48%) of mutants. In Saudi HBV genotype D patients, all possibilities of core promoter mutations at 1762, 1764 exist with AA 3/19 (16%), TG 4/19 (21%), TA 7/19 (37%), GT 2/19 (11%), AC 1/19 (5%), AT 1/19 (5%) and deletion 1/19 (5%). These types of BCP mutants were not reported before. Their amino acid translation is 1762-1764 RL(G/T), KL(A/T), MT(A/A) and KM. Such new BCP mutants were observed at even lower viral load (table 6-5). Similarly, Amini-Bavil-Olyaee et al., 2006) detected a G1764T mutation in the core promoter.

C1766T, T1768A detected in 5/8, 2/8 genotype A from UK and 4/19, 4/19 genotype D Saudi patients, respectively. Also C1766T and T1768A are usually associated with the double T1762/A1764 mutations (Hussain et al., 2003). The combination of T1764/G1766 BCP mutation was common (29.2%) and was frequent in HBeAg-negative patients (39.3%), and mutants exhibited lower HBV-DNA and HBV core antigen (HBcAg) levels than wt BCP (Elkady et al., 2008). T1766 and/or A1768 mutants and pre-

S deletions were significantly associated with cirrhosis in HBeAg-negative patients (Chen *et al.*, 2007). While other researchers (Tsai et al., 2009) blame C1766T and T1753C, core promoter mutations for enhanced replication and suppressed HBeAg.
There is a striking high prevalence of precore mutants in the Saudi HBV patients where the rate of 37/56(66.1%) was significantly more than their incidence in UK patients 5/23 (21.7%) (p:0.002). G1896A was 30/37 (81%) of Saudi PC mutants, this mutation changed codon 28 from TGG to TAG whose translation was changed from tryptophan to a stop codon leading to premature termination of HBeAg.

Multiple peaks were seen at individual nucleotide sites. This was obvious in our cases N15 where AGA had an equal peak with TGA (fig.6-5). While in the case 1-50, nt.1762-1764 AGA was present despite of majority of wt. AGG, indicating continuous evolution of mutations and the existence of mixture of wild type and mutant in virus population. In addition, case 1-48 had both wild type precore and 1896 mutant strains together as well.
Mixed mutant strains can be separated by line probe method (Husain et al., 2003), but not by sequencing method (Candiotti et al., 2006). Other studied reported mutations in mixture with wild type virus (Ayed et al., 2007) (Hussain et al., 2003). In addition, mean viral load of our different BCP mutants further illustrates that mutation production is a dynamic process that occurs in sequential steps. The presence of G1898A only mutation was seen in a UK case (N45), which does not cause precore stop codon, but can cause indeterminate results in hybridization assays as reported by Chu *et al.*, (2003) G1897A mutant gave indeterminate InnoLipa results.

BCP/PC mutations and HBeAg

HBeAg correlates significantly with BCP mutants frequencies as found in 2/11(22%) of UK HBeAg-positive HBV patients (table6-5). HBeAg-negative Saudi patients had higher rate of BCP mutants than UK patients at 15/21(71.4%) and 7/12 (58.3%), respectively. Rates similar to the UK results were reported in Scottish blood donors, where the frequencies of mutations in the 23 HBeAg-positive were 22% each for mutations 1762 and 1764, increasing to 26% and 35% in the 56 anti-HBe-positive, respectively (Davidson et al., 2005). PC variant was commonly in both HBeAg-negative and HBeAg-

positive Tunisian patients at (94.5% vs. 87.8%), respectively (Ayed et al., 2007). Viral sequence diversity is 2.4-fold higher before a reduction in HBV DNA and HBeAg seroconversion, which could either be related to occurrence of stochastic mutations that lead to a break in immune tolerance or to increased immune reactivity that drives escape mutations. After seroconversion, there was a striking 10-fold increase in substitution rate and viral diversity (Lim et al., 2007).

As BCP mutants express less HBeAg (Hui and Lau, 2007), most infections in the community are HBeAg-negative and inactive. Whether HBeAg seroconversion is the cause or the result of BCP mutations remains to be verified. HBeAg Seroconversion is associated with more double BCP mutation T1762/A1764 than in the non-converters (48% vs. 28%), and occurred in 50% of patients within 1 year, 88% within 2 years, and 93% within 5 years (Pang et al., 2004). In Tunisia, HBeAg-positive patients had BCP mutants in 18.2% versus 65.4% in HBeAg-negative patients (Ayed et al., 2007). Consequently, BCP mutations greatly enhanced genome replication and suppressed HBeAg expression. These effects are mediated by transcriptional up regulation of pregenomic RNA and suppression of precore RNA (Tsai et al., 2009). Thereby such mutation may decrease the translation of HBeAg than wild type level (Qin et al., 2009).

No precore G1896A mutants were detected in the HBeAg-positive UK patients (table6-4). The high frequency of PC mutations in HBeAg-negative Saudi patients 33/50 (66%) far exceeds the rate in our small group of UK patients 5/12 (41.7%). As Saudi HBV infected patients are usually HBeAg-negative, and generally have low viral load, which further raises the possibility that HBV mutants exist, and contribute to hepatitis chronicity and pathogenesis. PC mutants totally stop HBeAg or express truncated products. HBeAg-seroconversion is caused mainly by a decrease in HBeAg production in the active replication group, and by a decrease in viral replication in the inactive replication carriers (Misawa et al., 2006). However, HBeAg negativity did not always correspond to the presence of precore mutants (Cheong et al., 2008).

Ballard and Boxall (2000) from UK, however, reported G1896A in 3/79(4%) with mixed wild /mutant PC population during seroconversion, raised to 44/82 (54%) in HBeAg-negative patients. This G1896A was much higher than that of our UK results, but similar to the prevalence of Saudi PC stop mutants. Such difference may be explained by difference in the clinical presentation, genotype distribution, or bigger size of the studied groups.

G1862T mutation was seen in only one antiHBe-positive case from UK (N14) with viral load of 4.49 log10copies/ml. Chauhan et al., (2006) detected G1862T more often in HBeAg-positive than HBeAg-negative (37% vs. 11%) patients. This mutation could affect HBeAg expression at two levels. It occurs close to the signal peptide cleavage site from the precursor of HBeAg in the endoplasmic reticulum, and might therefore abrogate HBeAg synthesis. Alternatively, this mutation might interfere with reverse transcription of pregenomic RNA through inhibition of priming of polymerase by binding to the bulge of ε at 1862, 1864, and 1865 (Francois et al., 2001), resulting in greatly impaired viral replication and low viraemia (Tong et al., 2005). However, several mutations can modulate HBeAg translation initiation, proteolytic cleavage, and secondary structure maintenance.

BCP/PC mutations and T or B cell epitopes

In our results, no significant differences were found between HBV genotypes in HLA class I (aa18-27) nor class II (aa 1-20, 28-47) core epitopes, as those have highly conserved sequences except V27I in genotypes B and C. Different HLA restricted CTL/T helper epitopes within the core gene as reflected by variation of amino acids within the core antigen, are capable of inducing significant T-cell responses. HBV genotypes as well follow a geographical pattern, which may be influenced by ethnic background or HLA type. Variations of B cell epitopes may affect their antigenicity causing changes in HBV serological markers or their affinities (Jazayeri et al., 2004). Patients with PC mutants or genotype B also had a significantly greater T helper (Th1) response and higher number of IFN-gamma producing cells with hepatitis B core antigen (HBcAg) stimulation, and

lesser Th2 response lower number of IL-10 producing cells with HBeAg stimulation compared to wt PC or genotype C (Yuen et al., 2007).

Viral load and BCP/PC mutants

Core promoter BCP mutants predominate in the intermediate (>3-<5 log10 copies/ml) viral load group in both Saudi and UK patients at 8/11 (73%) and 3/4 (75%), respectively. (tables 6-8 and 6-9). BCP mutants were significantly associated with high HBV DNA levels (Tsai et al., 2009), particularly those with high-affinity hepatocyte nuclear factor I (HNF I) motifs due to their enhanced replication capacity (Parekh et al., 2003). Conversely, reduced BCP activity and viral replication or secretion lower viral loads, due to missense mutations in the envelope gene or BCP transcription factor binding sites (Tong et al., 2005). Also genotype C has a predisposition to acquire BCP mutations that continuously activate virus replication after sero-conversion (Kaijima et al., 2009).

The other new BCP mutants G1762/T1764, AC and AT from Saudi patients have lowest viral load 3.9 log10copies/ml (Graph 6-3), suggesting their late appearance in the course of HBV infection. There is significantly less BCP mutations (42.5% vs. 71.4%), and HBV DNA levels (5.61vs. 6.18 log10 copies/ml) in HBV genotypes B than C (Du et al., 2007).

Some precore mutants were seen in UK patients 3/17(18%) with high viral load (>5 log10 copies/ml) (table 6-9). On the other hand, G1896A alone has marginally higher viral load than G1899A at 3.9 and 4.6 $\log_{10}$ copies/ml, respectively. This is partly explained by either increasing duration of infection or associated genotype D, where most G1899A exist. Few Saudi patients with PC mutants 14/22 (64%) had high viral loads, some of them had very high HBV viral load (=> 7 log10 copies/ml). Thus, confirming the high replication ability of codon 28 stop mutations as inferred from their occurrence in HBeAg-negative patients with high viral load. Alternatively, acute or severe infection can possibly be caused by PC mutants.

Cis-acting elements within the precore (PC) region are highly conserved; hence, only selected mutations can be viable. Interestingly, Saudi HBV PC mutants were seen in all levels of viral load. In intermediate viral loads, PC mutants could be encouraged in alternating balance between active viral replication and the counteracting immune response, as opposed to latent virus with low viral loads that preserve their wild type proportion. These findings were obtained by studies demonstrated that the quantity of PC mutant was negatively correlated with ALT and HBV DNA levels (Gandhe et al., 2003) (Nakashima et al., 2004), both before and after HBeAg seroconversion (Pang et al., 2004); but others did not (Candotti et al., 2006). Our number of patients with low viral load is too small to evaluate PC mutants at low viral loads.

Wild type in both BCP and PC was rare in Saudi HBV patients reaching only 1/26 (5%) compared with UK HBV patients 10/22 (45.5%). In contrast, coexisted BCP and PC mutants occurred in most of Saudi patients 10/20 (50%) versus only 1/22 (5%) in UK patients (table: 6-6). Coexistence was generally more in HBeAg-negative (19%) than HBeAg-positive (4%) patients (Chu et al., 2003). Peng et al., (2005) reported coexistence of PC and BCP mutants in 64/165 (38.8%) of total patients, in 64/83 (77.1%) out of sera with double BCP mutations, and in 64/128 (50.0%) out of sera with G1896A mutation. Those rates were comparable to results of the Saudi HBV patients, possibly due to longer duration of chronic infection.

Analysis of both BCP and PC status gives a clearer view, as it demonstrates the sequential event decreasing viral loads in mutant BCP with wt PC, then the less frequent wt BCP with mutant PC, finally mutant in both BCP and PC showed the lowest viral load. The mean viral load in coexisting BCP and PC mutation was 4.3 and 5.1log10 copies/ml in Saudi and UK patients, respectively (tables 6- and 6-9). That was comparable to other studies where single BCP variants have significantly higher viral load above 5 log10, while those associated with PC stop mutants, have viral load below 5 log10 (Fang et al., 2002)(Chih et al., (2002). As either the BCP or the PC mutants, can influence the synthesis of HBeAg. The impact of the BCP mutation on the replication of

HBV can also be modulated by the emergence of the PC stop codon mutations, which further complicates the analysis and implications of each mutation.

BCP/PC and ethnic origin

Our UK patients with BCP variants were mostly Caucasians and some Asian and Chinese ethnic origin. BCP variants were present equally in Caucasians and Asians and rare in Blacks (Wai et al., 2005). A contrasting high prevalence of PC mutants was seen in Saudi 37/56 (66.1%) compared to low prevalence in UK patients 4/22 (18%). The prevalence of PC mutation was similar in Saudi to Mediterranean, African, and Southern Europe prevalence reaching up to 50% of HBV patients (Chu and Lok. 2002). PC mutants were more in Asians than Caucasians, and rare in Blacks (Chu et al., 2003), which could be related to their ethnic origin as well as to viral genotype.

Viral Genotype and BCP/PC mutants

Our results suggest a strong correlation between HBV genotypes and BCP as well as PC variants. In UK patients, BCP mutants were high in genotype A subtype Ae at 7/9 (78%) (table 6-11). Single BCP mutants occurred in genotypes A and D only. Lower prevalence were reported where BCP alone or with PC variants in genotypes A (41%) and D (40%), more in genotype C (60%) and lowest in genotype B (26%) in UK (Davidson et al., 2005). Also in Spain, BCP mutations were similar in genotypes A and D (Jardi et al., 2004), while Tanaka et al., (2004) shown their existence in both subgenotype Aa (50%) and Ae (44%) more than in genotype D isolates (25%). Patients with genotype D were more likely to have persistent HBV infection with PC mutants (Kar et al., 2007). It can be concluded from this study that the pattern of BCP and PC mutations derived from UK patients was contradictory in some aspects to those described by De Castro et al., (2001). A high frequency of BCP (78%) (table 6-10) and lower rate of PC mutants 2/9(22%) was noted among UK genotype A isolates. Even more in Saudi genotype D who had 17/23 (74%) BCP or PC35/48 (73%),

Our UK genotype B (subtype Ba) patients had double BCP variant in both patients with mutation 2/7(28.6%), that was in agreement with a case-control study (Sugauchi et al., 2003), where the double BCP mutation and HBeAg were significantly more in 80 carriers each of subgenotype Ba than Bj (33% vs. 15% and 35% vs. 18%), respectively. Lim et

al., (20076) detected BCP mutants in genotypes B 24/77 (31.2%), and C 11/77 (14.3%). Genotype alone cannot be relied upon in interpreting mutation rates. However, the number of our patients of HBV genotypes B, C and E was too small to draw a conclusion. Higher frequencies of BCP mutants, PC mutants, genotype C, and higher HBV DNA levels were more frequently detected in HCC patients than that in chronic carriers (Tong et al., 2007). Genotype B had lower BCP mutants (41.7% versus 89.2%) and viral loads (3.6 +/- 0.9 versus 4.6 +/- 1.6compared with genotype C. Whereas HBeAg-negative genotype C patients, PC mutants had significantly higher viral loads and ALT levels than those with wt PC (Kawabe et al., 2009).

The high incidence of BCP 17/23(74%) and PC 35/48(73%) mutants in Saudi Arabia could result from the predominance of genotype D in this population, similar to the rates of 87% reported by Ganne-Carrie et al., (2006) in anti-HBe positive patients that were associated with genotype D and severe liver fibrosis. The occurrence of 1896A was higher in genotype B (77.27%) than that in genotype C (32.26%) (Pan et al., 2007) (table 6-11). On the other hand, UK genotypes A and B exhibited less PC variants at 2/9(22%), 2/7(29%), respectively. Our study demonstrated that PC mutants occurred preferentially in genotypes D in contradiction to De Castro et al., (2001) which relates PC stop mutant to genotypes B, C, D and E. Usually PC mutations were most common in genotype D than genotype A (82 vs. 40%)(Jardi et al., 2004). This can be a reflection of chronicity or mere predisposition of viral genotype to mutation. Patients with genotype D were more likely to have persistent HBV infection with PC mutants (Kar et al., 2007).

On the other hand, genotypes A, B and C subtype C1(Cs) exhibited less PC variants in our UK patients. In contrast, Scottish blood donors had less PC mutations in genotypes A and C than genotypes B and D at 10%, 25% and 88%, 74%, respectively (Davidson et al., 2005). Wild type PC was linked strongly to genotype A (Dal Molin et al., 2006), while PC mutations were significantly higher in genotype B than genotype C at (69 vs. 34%) with HBeAg-seroconversion (Hagiwara et al., 2006). Lower prevalence of G1896A were seen in subgenotype Cs than Ce (5% vs. 50%) (Chan et al., 2005). HCC-related mutations C1165T, A1762T and G1764A, T2712C/A/G, and A/T2525C were associated with

genotype B; T31C, T53C, and A1499G with subgenotype Ce, and G1613A, G1899A, T2170C/G, and T2441C with subgenotype Cs (Sung et al., 2008).

Among Saudi patients, codon 29 mutant (G1899A) was seen in 22/56(59.4%), fifteen of them had associated G1896A. Interestingly, Saudi genotype D patients possessed the highest prevalence of codon 29 mutant (G1899A) 19/48(39.6%) compared to other genotypes, namely genotype A and C. The high incidence of Saudi codon 29 mutants not found in the UK patients, could be attributed to the predominance of subgenotype D1 in Saudi cases (table 6-10). Conversely, in Yemen, a lower incidence of G1896A/G1899A variants was less frequently present at 2/12 (17%) of genotype D PC mutants (Salam et al., 2002). Other researcher demonstrated the presence of G1899A/G1896A in genotype E (Sitnik et al., 2007), genotype B patients and G1896A alone in genotype C with exacerbations of chronic hepatitis (Ozasa et al., 2006). Therefore the prevalence of both PC variants is dependent on HBV genotypes. Clinically, G1899A were detected in acute liver failure patients (Wai et al., 2005), and with T1753C/A in liver cirrhosis (Song et al., 2006) and/or T1754C/G and/or A1762T/G1764A were also significantly associated with fulminant hepatitis (FH) (Sainokami et al., 2007).

Genotype specific areas of BCP/PC

Molecular characterization can shed light on viral properties. Although the amino acid sequence of the core region is relatively conserved as compared to the surface gene, substitutions are observed which are largely linked to the clinical or serological picture. This contributes to the interpretation of disease progress, and may aid in the design of immunotherapies specific for HBV genotype or ethnic origin (Jazayeri et al., 2004). Moreover, construction of phylogenetic trees of Saudi and UK HBV sequences showed an almost identical pattern of HBV genotype distribution. The validity of our data was strengthened by comparison with international gene bank databases. The genotype specificity of the BCP region demonstrated in fig.6-3 might have implications on both the core promoter and replication efficiency of different HBV genotypes. In addition, the differences in the overlapping X gene and consequently the transactivation function and carcinogenesis of HBV among different genotypes. That was consistent with those in

Jazayeri`s study (2004) that areas of the core gene that allows allocation of a sequence to specific genotype and subtype, through combinations of 24 amino acid substitutions at nine residues. Phylogenetic analysis of the distal-X/PC region (Chen et al., 2007), and the relatively conserved HBsAg gene sequences were used, but showed a smaller phylogenetic signal than pre-core/core sequences (Clemente et al., 2009). Similarly, variations within the BCP/PC region (1653-1900), allowed genotyping of HBV with 97% sensitivity and 99% specificity (Kramvis et al., 2008). Although being more conserved region, phylogenetic studies of our precore/core region was not conclusive of HBV genotypes as BCP region did.

Analysis of our core gene sequences with those in gene bank verified that amino acid/nucleotide specific nucleotide/amino acid substitutions and motifs correlate with HBV genotype/subtype. Cheng et al., (2006) also found that of the 216 new mutations, some were genotype-specific. Kramvis et al., 2008) demonstrated that 1802-1803CG is characteristic of genotypes A, D, and E whereas 1802-1803TT are characteristic of genotypes B, C, and F, and might have an association with liver damage (Lindh et al., 1999). The Haploplot method revealed high linkage between loci 1858 and 1896, but strong evidence of recombination between loci 1862 and 1896. 1888A was positively associated with subgenotype A1 and TAA at 1817 with genotype G. Loci 1809-1812, 1862, and 1888 may have co-evolved together (Kramvis et al., 2008).

According to our results, nt.C1858 was present predominantly in genotype A UK patients 7/9, as well as in genotype C 3/3 patients. Most of those patients 5/7 (71.4%) had wild type (wt) PC, which forms a stable G-C pair with the second nucleotide G1896 of codon 28 (UGG) resulting in the wt PC. 1858C is positively associated with genotypes A, F, and H and subgenotypes C1, F2/F3 (Kramvis et al., 2008). T1858C may thus contribute to the rarity of HBeAg-negative mutants in Europe and North America, where genotype A is more prevalent. However, the unstable G1896A in genotype A reduce the replication efficiency and favors the evolution towards inactive hepatitis (Weber, 2005). Double BCP mutation was low (20%) among isolates with C1858 (Banerjee et al., 2005).

In contrast, in non-genotype A strains as genotypes B, U1858 of codon 15 (CCU) forms a wobble U-G pair with G1896 in the stem of the pregenomic RNA encapsidation signal ε. This facilitates the shift from G to A in nt.1896 to form a stable U-A pair, resulting in enhanced replication and packaging signal to initiate DNA synthesis, and favoring the development of HBeAg-negative state in genotypes B, D, E and G (Watanabe et al., 2005) and subgenotypes C2, F1/F4 (Kramvis et al., 2008). Up to 39/44 (89%) with genotype C and genotype D had T1858 (Banerjee et al., 2005).

All our genotype C subtype C1(Cs) 3/3 UK patients had TCC in nt.1856-1858, causing P15S change. Chan et al., 2006) observed that all 214 genotype C patients except one HBV strain with TCC at nucleotides 1856 to 1858 belonged to subgroup Cs, and was associated with the HBeAg (58% versus 36%), G1898A mutation (64%) and liver cirrhosis than in CCC (18% versus 5%). However, Sakurai et al., (2004) failed to demonstrate significant difference in clinical characteristics between C1858 and T1858 variants.

We demonstrated that some areas of the core promoter have been found to be strongly linked to genotypes; nt.1726-1727/1730 CT/G are specific for genotype B and C. In all our 7 genotype B (Ba) patients from UK, nt.1838A was present. That was in concordance with Sugauchi et al., (2003) who found that all the 41 subgenotype Ba) possessed G1838A in contrast to 1838G in all 29 of subtype Bj. It also existed regardless of the HBeAg positivity or disease activity as chronic or inactive hepatitis patients.

C1773T found in 8/8 Genotype A from UK and 13/17 Genotype D Saudi patients, respectively. C1799G was seen in all genotype B patients and in 3/17 Genotype D Saudi patients. Consensus genotype A has C1461, T1773, and A1850, while consensus genotype D had C1773, C1850 (Hannoun et al., 2002), and G1757A. Moreover, C1773T, C1802, G1803, T1846, A1850 mutations might have significant correlation with HBeAg (Chen et al., 2006). Clinically, in HBeAg-positive 328 carriers, mutation V1753 was predictive for HCC among C1/Cs carriers, and T1653 among C2/Ce-carriers, and T1653 or V1753 or double BCP mutants among HBeAg-negative C2/Ce carriers (Tanaka et al.,

2006). Such HBV variants are genotype specific and related to viraemia levels or binding of several liver specific factors. However, in view of HBV heterogeneity, single substitutions can represent naturally occurring variants or merely an immune-selected mutation in particular patient. Only one of our patients (N34) has mutations at G1740 T and G1745T and G1746T. Replication could also be influenced by a BCP mutation at nt.1746 which is responsible for the correct initiation of precore and pregenomic message (Francois et al., 2001). In 183 chronic genotype C, substitutions at nt. 1762, 1768, 1846, 1896, 2134, 2288, 2441 was of determinants of early non-detectable HBV-DNA, but did not suppress hepatocarcinogenesis in lamivudine-resistant patients with on adefovir (Akuta et al., 2008).

This study demonstrated G1721A in 3/3 genotype C patients from UK. Our genotype D had G1757A frequently in 15/20 Saudi and 3/3 UK patients. G1757A was observed in genotype E (Fujiwara et al., 2005), in addition to C1772, T1858 and A1757 (Sitnik et al., 2007). In genotype D, A1679 (86.96%), T1762 (39.13%), A1764 (30.43%), and T1915 (100%) variants were detected (Ahmed et al., 2009).

Laoi and Crowley (2008) 4 out of 11 patients with severe acute HBV infection harboured the G1896A stop codon mutation, while the remaining 21 had self-limiting illness. A triple mutation, T1753C/A1762T/G1764A was identified in one severe infection. The latter also had a mutation, A2339G, in the core gene, not previously reported in severe acute infection caused by genotype D (Chen et al., 2007) In HBV genotype Ba had earlier A1896 only before HBeAg seroconversion, while genotype C showed significantly earlier and persistently higher T1762/A1764 mutant than the A1896 mutant, compared to genotype Ba. Furthermore, HBV genotype C was associated with the development of G or C1753 and T1766/A1768 mutations and the reactivation of hepatitis after HBeAg seroconversion. In addition, the T1846 variant was an independent factor associated with HBeAg seroconversion.

Finally, G1862 T mutation was seen in a genotype A case (N14) clinically presented with cirrhosis. G1862T mutant is Genotype A-specific but is not always associated with

HBeAg. Subgenotype Aa (African/Asian) contains unique mutations upstream of the precore ATG codon of genotype Aa suppressed HBeAg expression (Tong et al., 2005). However, G1862A mutation has been reported both in asymptomatic HBV carriers (Gandhe et al., 2003), and in patients with chronic hepatitis, cirrhosis, HCC, or fulminant hepatitis (Francois et al., 2001).

One of our samples had discrepant genotype allocation based on analysis of two genes core and S gene. These discrepancies were not surprising. Recombination events between different genotypes have been described between A and C from Tibet (Cui et al., 2002) and between B and C from South-east Asia (Sugushi et al., 2002). Comparisons of our data with core gene sequences in databases strains were almost entirely consistent. Several variable residues have been described throughout the core gene. HBcAg codon V27I, T67N, N/V/G/T74s, D83E, T91V as well as F97I contained most discrepancy between genotypes B and C with other genotypes.

Clinical Presentation of HBV patients with BCP/PC mutations

The evolution of HBV mutations depends on the stage and progression of the disease. Thus, molecular markers could predict the clinical outcomes of HBV patients. In chronic hepatitis, BCP mutants existed in 11/13(85%) Saudi, and lower rates of 6/18 (33%) in UK patients. Considering the natural course of HBV, the early phase of HBeAg-positive chronic hepatitis is due to wild type HBV; while the late phase chronic hepatitis is due to PC or/and BCP variants. BCP and PC mutants increase starting with HBeAg-positive asymptomatic carrier at (30%) and 5%, inactive HBsAg carrier 65.7% and 22.5%, then chronic hepatitis 95% and 32.5%, and liver cirrhosis 90% and 50%, respectively Song et al., (2006).

In the liver disease group, PC mutations were frequently present in both inactive carriers and chronic hepatitis at 9/13 (69%), 28/43(65%) of Saudi patients, respectively. Conversely, PC mutants were seen in only 4/21(19%) of UK chronic hepatitis patients. This suggested that the severity of liver disease was not correlated with PC mutations. Similar to Saudi HBV cases, Jardi et al., (2004) demonstrated high frequency of PC mutations in inactive carriers (65%) and even higher (80%) in chronic hepatitis patients

with associated elevated ALT and highest HBV DNA, regardless of BCP mutations. PC variants were associated with viral persistence in anti-HBe positive patients with ongoing chronic hepatitis. Cumulative viral mutations in response to the host immunological selective pressure enable the virus for continuous survival and pathogenicity. Whether those associations are solely caused by PC mutations, chronic infection or are due to other coexisting variations remains to be established.

In addition, our results showed PC mutants with anti-HBe positive patients at all levels of viral loads especially =>5 log 10 copies/ml (table 6-7). This is consistent with the clinical correlation of PC variants and anti-HBe-positive chronic hepatitis. Yuan et al., (2005) found that within HBeAg-positive cirrhotic patients, complicated patients had significantly higher frequency of BCP mutations. In contrast, anti-HBe-positive patients with complications had significantly higher ALT and HBV DNA levels, but similar frequency of BCP and PC mutations. Cheong et al., (2008) demonstrated that lamivudine therapy induced initial reversion from PC mutants to wild-type virus, but mutants reappeared later. Conversely, wt PC was replaced by PC mutants, resulting in a flare-up of hepatitis after cessation of lamivudine.

Some groups demonstrated increased prevalence of BCP and PC mutants in CLD, cirrhosis or HCC (Livingston et al., 2007) (Song et al., 2005), while others did not (Liu et al., 2006) (Tsai et al., 2009). All Saudi patients with cirrhosis and HCC had BCP mutations and 60-67% had PC mutations with viral loads of 2 to 10.7log10 copies/ml (table 6-12). However, the number of our patients is too small to draw a solid conclusion. Liu et al., 2006) demonstrated that double BCP mutation, and viral load >or=105 copies/ml were independently associated with the risk of carcinogenesis in noncirrhotic as well as cirrhotic HCC. T1762/A1764 mutation was detected in 75.0% of HCC patients with high viral load (> or =5 log copies/mL). A1896 was detected in (70.8%) of HBV/D-infected patients (Elkady et al., 2008). At HBV DNA level of at least 104 copies/mL, HCC incidence was higher for those with the wt precore than for those with the G1896A variant (955.5 vs 269.4) per 100 000 person-years, and for those with the A1762T/G1764A than for those with wt BCP (1149.2 vs 358.7). The hazard ratio of

developing HCC was less for G1896A vs wt precore 0.34 , and more for A1762T/G1764A 1.73 vs wild type (Yang et al., 2008), or nine single nucleotide polymorphisms in the EnhII/BCP regions (six of which were genotype C HBV related) that was evident 9 years or more before diagnosis (Chou et al., 2008).

Our liver disease patients including chronic hepatitis B, cirrhosis with BCP/PC mutations had genotypes D and less frequently A. Genotypes A, C and D seem to be associated with more severe hepatic disease. In Karachi, within genotype D chronic hepatitis B patients, PC and BCP mutations were detected in 44/71 (62%) HBeAg-negative compared to only 9/38 (24%) HBeAg-positive (Abbas et al., 2006). Hence, HBV genotype D correlates with both PC stop mutation and liver disease, including severe liver fibrosis (Ganne-Carrie et al., 2006). In our study, BCP and PC coexisted in Saudi genotypes D and less frequently A and E. In early or severe patients, both the proportion of HBeAg-positive patients and ALT level were higher in the patients with the mutant/Wt pattern than mutants in both the BCP/PC regions (Hagiwara et al., 2006). Up to 50% of genotype D had coexistence of BCP and PC mutants (Bahri et al., 2006) and 71.0% in genotype C (Nakashima et al., (2004). Coexistence also correlate with high levels of HBV DNA and progressive liver diseases than PC mutant only (Peng et al., 2005), reflecting their higher pathogenicity or appearance in later stages of the disease after HBeAg seroconversion.

Conversely, we detected C1653T variants can occur in genotypes A and D. So this position is neither genotype-specific, nor associated with fulminant hepatitis as previously reported (Francois et al., 2001). Li et al., (2002) found C1653T and T1753C more frequently in hepatic failure patients with liver cirrhosis than with chronic hepatitis (100% vs 50%). Kim et al., (2009) demonstrated that the prevalence of the T1653 mutation in the box alpha region among HBeAg-negative patients, the A1689 mutation in between the box alpha and beta regions, and higher in coexisting T1762/A1764 BCP mutations was significantly higher in the HCC group compared to the non-HCC group (8.9% vs. 2.2%; 19.3% vs. 4.4%, and 60.7% vs. 22.2%).

Four of our 17genotype D patients had T1753A, two had chronic hepatitis B and one cirrhotic and one HCC. Salam et al., (2002) found 1753C in 8/14 (57%) of genotype D mostly associated with double BCP mutant. C1752 and/or V (not T) 1753 mutation was significantly prevalent in HCC patients (HCC vs CLD; 52.2% vs 20%) (Elkady et al., 2008). HBeAg-negative patients had lower frequency of A1752G mutation rate and higher frequency of mutations at A1775G, G1799C and nt.1846 than HBeAg-positive patients (Ni et al., 2004) did. In advancing liver diseases, C1753, T1766 and A1768, and PC mutations were increased, and the frequencies of T or G1752, T1773, G1799, and C1858 mutations decreased (Chen et al., 2005). The T1850A found in 8/10 (80%) genotype A UK patients had been linked by Gandhe and colleagues (2003) to chronic hepatitis patients in association with double PC variant G1896A-G1899A.

HBV-X gene mutations

V131I 2/9(22%) was seen in UK patients as well as M130K in 4/19(24%) and V131I 3/19(18%) in Saudi patients. M130K and V131I together in 5/9(59%) and 7/19(37%) UK and Saudi patients, respectively (table 6-12). Our results showed increased prevalence within genotype A of K130M/V131I in 4/7 patients, and single mutant V131I in another 3/7 patients. Higher frequencies of X-gene mutations K130M/V131I were reported in patients with HCC than in asymptomatic carriers (Song et al., 2005), more in genotypes B but not C (Lin et al., 2005). Conversely, K130M/V131I mutants alone or with F132Y showed lower activities in genotype B only. In genotype C, K130M/V131I with F132Y mutant showed higher anti-proliferative activity. The sequence of the X gene showed A1678T and G1759A in subtype ayw and C1792T for adw (Venard et al., 2000). Moreover, compared with the HBsAg-positive HCC controls, occult HBV-infected (OBI) HCC patients had higher frequencies of M1I and Q2K in pre-S2 gene, G185R and S210N in surface gene, A36T and A44L in X gene, and G1721A in enhancer II gene, and had lower rates of pre-S deletions and A1762T/G1764A, A1846T, G1896A and G1899A in BCP/PC genes (Chen et al., 2009).

/Other X gene mutations may exist in patients with chronic liver disease include V92L, H94Y, K113T I127L/N/T, F132S (Kurosaki et al., 1996). As I127T/N, T153V were detected in the carboxy functional region of X protein, which might be associated with

the transactivating function of the X protein as well as phenylalanine-132, cycteine-137 and histidine-139, which are essential for transactivation (Kidd-Lijunggren et al., 1997). Tang et al., 2005) demonstrated that regions between aa 52 to 65 and 88 to 154 are important for the augmentation function of HBx in HBV transcription and replication as a HBV mutant genome with defective X gene led to decreased levels of HBV RNA and HBV replication intermediates.

Only one Saudi HBeAg-negative chronic hepatitis case 2-12 showed a 12bp-deletion 1752-1765 resulting in frame shift in HBxAg and had high viral load, but with wild type precore. The whole BCP is absent in this unusual mutation and the regulatory sequences located therein. 12-bp deletion at nt.1754-1765 was described by Hussein et al., (2003). Core promoter deletions may causes decrease in DNA replication, limited transcription of precore mRNA and thereby decrease HBeAg synthesis and enhance transcription of core mRNA and HBcAg (Fang et al., 2009). They also may cause premature termination of the overlapping X ORF. Such deletion mutants highlight the functional potentials of viral sequences activated by mutations and may be of relevance for viral persistence.

6.7. Conclusion

BCP is common in UK HBV patients (41%) and are associated with moderately high serum HBV viral loads of =>5 $\log_{10}$ copies/ml and with anti-HBe antibody positivity, unlike PC mutants which were uncommon. In Saudi HBV patients, precore mutations are common reaching up to 64.8%, including either G1896A stop mutation, G1899A or both. Such mutations might explain the intermediate HBV levels in infected Saudi population. These results indicate that core promoter and/or precore mutants may be under-reported. Physicians should be aware of the existence of these HBV mutants and the clinical condition of "HBeAg-negative" chronic hepatitis in such population.

There is significant association of precore mutants with HBV genotype D more than other genotypes, and of genotype A with BCP mutations. Follow up studies and analysis of associated factors as viral loads and genotype are needed to investigate whether precore variants may predispose either singly or interacting with other factors for the

development of severe liver disease. Further study of the rest of the core gene is recommended to check for variations associated with codon 28 stop mutant.

Genotype specific sequences occur in the HBV core promoter region and the core gene. Whether these genotype-specific variations are related to viral replication or X-gene function and consequently the progression of liver disease and oncogenesis remain to be explored.

Basal core promoter and enhancer region C1653T Rapid disease progression and HCC AS A1753T Rapid disease progressionT1762A, T1764C HCC, C1766T Fulminant hepatitis, X K130 M and V131I HCC development. While Pre-S Deletions has potential association with HCC (Pujol et al., 2009).

6.8. Future Direction

Further studies are needed:

To determine whether the diversity of HBV core promoter and the overlapping X-gene mutations has clinical significance as disease progression and possibly carcinogensis.

A bigger number of patients need to be tested for the possible correlation between different HBV genotypes and the frequency of core promoter/precore mutations, and what are the characteristics of coexisted core promoter and precore variants.

- Full epidemiological studies in UK and Saudi Arabia are needed, to identify HBV genotypes, precore mutants and viral load in specimens representative of different geographical areas.
- Extend the study to check the rest of the core gene nt. 1960-2400 (codons 20-151) for genotype specific differences and mutations especially in T-cell and B-cell epitopes.

Chapter Seven : Update and Conclusion

Technical issues in HBV assays

Validation of molecular assays for hepatitis B virus is essential at low HBV DNA levels. Upon our assessment of eight different PCRs covering the S, X, and C genes, only two assays were reliable below 10^3 copies/ml. One PCR of the S gene, and one of the C gene, The precore (PC) PCR showed a sensitivity of 1.9 x 10^2 IU/ml (equivalent to 1.0 x 10^3 copies/ml) (fig.2-2).

Another factor affecting the sensitivity of the assay is the length of the PCR product. The S gene PCR product was 281 bp, while the C gene PCR product size was 296 bp. The number of cycles needed for HBV PCR is also higher than standard 35 to 38 cycles. We demonstrated better yield at higher cycle number. Polymerase and master mix quality must be validated to fit these conditions as high fidelity polymerase. Similarly most new tests as Cobas Taqman run 60 cycles, which further prove our finding. Single round precore PCR was the most sensitive assay of the core gene, of equal sensitivity to the nested core PCR.

Among genotyping PCRs, multiplex PCR was has lower detection limit than RFLP PCR. But still the lowest detection is at 10^4 copies/ml . Recently, WHO standards are available in different HBV genotypes (Chudy et al., 2012)

Our real-time detection of HBV by SYBR green was practical, more accurate and has detection range from 10^2 to 10^{10} copies/ml. This wide detection range is needed for monitoring of HBV as this virus can reach very high levels in clinical specimens. During comparative analysis of the quantitative results of standards and sera from our SYBR green versus Amplicor assay, the difference in HBV DNA measurement between the two methods was 0.595 with a 95% CI of 0.10-1.094. The slope was slightly different from a value of one indicating a less linear relationship between the two assays. The Amplicor test showed less concordance at higher HBV DNA levels of plasmid standards than our optimized method. Methods differences causes such limitations of the former assay .

Our thermal denaturation profiles which showed peak fluorescence at 80.5 - 81.7 ^{o}C due to melting of the target PCR products, belonging to two different genotypes.That was also proved later to be due to genotype differences in HBV genome (Liu et al., 2006), and mutations (Yang et al., 2008).

Occult HBV infection

Screening of blood bank sera for occult hepatitis B infection need highly sensitive PCRs as low viral load is expected. The real time quantitative PCR further confirmed that these cases have low HBV DNA level of = or < 10^3 copies/ml.

Detection of OBI requires standardized assays of the highest sensitivity and specificity with a lower limit of detection < 10 IU/mL for HBV DNA and >0.1 ng/mL for HBsAg (Ocana, et al., 2011). Some HBsAg tests have high analytical sensitivity of <0·13 IU/ml (Nick and Scheiblauer; 2007). OBI detection also depends technically on the efficiency of DNA extraction, genome enrichment, amount of input DNA, PCR primers and assay conditions (Banerjee et al., 2007). On the other hand, sensitive PCR assays may lead to false positive results from contamination or amplification of non-HBV-DNA targets (Kew et al., 2008). However, there is no standardized method to detect OBI that permit objective comparison of results (Fryer and Minor, 2009).

It is essential to validate suitable molecular tests to detect OBI in blood transfusion services, such as HBV nucleic acid amplification technology (NAT) (Fryer and Minor; 2009). Our Saudi HBV-DNA rate is still higher than that described from other endemic areas as 37.7 per 10^6 in Italy (Velati et al., 2008) and 158 of 15,721(1.01%) repository tubes in Japan (Satake et al., 2007). This highlights the threat where donor screening for anti-HBc antibodies is not implemented, though it identifies most but not all OBIs. Therefore, the ideal strategy is to exclude all anti-HBc positive unit (El-Zayadi et al., 2008) by highly sensitive serological screening, although this will result in high discard rate of collected blood units in endemic areas without completely covering the risk of transfusion transmitted HBV (Dhawan et al., 2008). The NAT aids in prevention by detection of donors in window period of HBV infection and OBI (Velati et al., 2008). The former reflects the incidence of new HBV infection. The prevalence of anti-HBc

decreases below the age of 40 due to the implementation of massive HBV vaccination, with an alarming increase of HBV-DNA than older age (75.8% vs. 21.5%) (Yugi et al., 2006).

Loss of HBsAg after acute HBV infection and the appearance of anti-HBs antibodies signify viral clearance. In this case, the anti-HBs is neither fully neutralizing nor completely protective. Our data that the seroconversion to anti-HBs is not always accompanied by the complete elimination of HBV virus, agree with Velati, et al., (2008), who demonstrated HBV DNA in 68% of donors with Anti-HBs antibody, despite that 74.5 % had concentrations considered protective (> or =10 mIU/mL). It is appropriate to monitor the emergence of HBsAg negative mutants in such cases.

The HBV genotypes of HBsAg blood donors were genotype A 6/25 (24%), C 1/25 (4%) and D 18/25 (72%). 5 were ungenotypable. There is a predilection for OBI in HBV genotype D than genotype A2 (Candotti et al., 2008), and in subgenotypes D3 (Chandra et al., 2009), D5 and Aa (Banerjee et al., 2007). The reduced sensitivity and false negative results of some HBsAg tests for genotypes D/*ayw3*, E/*ayw4* and F/*adw4* (Scheiblauer; 2010), together with the higher rates of HBsAg-negative precore mutants (data not shown) can explain this trend. Further analysis of immunogenic epitopes is needed to elucidate the reasons of such differences in OBI between different HBV genotypes.

Meta analysis showed highly variable prevalence of OBI in chronic HCV patients ranging from 0% to 52% (Levast et al., 2010), with serum HBV-DNA in the absence of HBsAg. Proper follow up is needed to evaluate the effect of OBI on the progress of disease and outcome of therapy. Coinfection suggests a common source of infection in a high risk group as dialysis patients (Jain and Nijhawan; 2008), and is characterized by the dominance of HCV, as HCV core protein decreases HBV replication capacity (Chen et al., 2003). Another contributing factor to OBI is the significant increase in those who are HCV-RNA positive rather than negative (Said et al., 2009), with decreased emergence of

HBV pre-C variants (De Mitri et al., 2007), or HIV co-infection (Rodríguez-Torres et al., 2007).

In our study, HBV-DNA was detected in two out of 15 (13.3%) archival liver biopsies from Saudi HCC patients. Similarly, Obika, et al., (2008) found HBV DNA in the liver tissue in 25 of 167 (15.0%) HCV patients with CLD, suggesting that most infection was contracted during childhood and superinfection by HCV may have occurred thereafter. However, it is not clear if HCC is the result of HBV or HCV or to their synergistic insult. HCC was reported in 12 of 167 HCV patients (7.2%), more in those with high amount of HBV DNA (Obika et al., 2008). OBI also had significantly higher prevalence in HCC patients compared with controls without HCC, at 90/222 (40.5%), and 24/300 (8%) respectively (Chen et al., 2009). Although almost half the patients in the HALT-C Trial had serological evidence of previous HBV infection there was no difference in the prevalence of anti-HBc in serum or HBV DNA in liver between patients who did or did not develop HCC (Lok et al., 2011).

OBI was reported in peripheral blood mononuclear cells (PBMCs), and in liver biopsy of 6/82 (7.3%) anti-HBc-negative much less than 10/16 (62.5%) in anti-HBc positive individuals (Raimondo et al., 2008). Low level of HBV-DNA remains detectable in the serum and liver tissue in some patients who cleared HBsAg from either acute self-limited or chronic HBV infection or even after a successful anti-HBV therapy (Said et al., 2009). Examination of liver DNA extracts seems to be the more reliable methodological approach than serum for OBI detection (Raimondo et al., 2008; Sagnelli et al., 2008), but is difficult to apply in clinical practice.

Quantitative HBV PCR

Mean HBV DNA level was significantly higher in UK than Saudi patients at 5.09 (95% CI 1.43-8.75) and 4.52 (95% CI 0.14-8.90) $\log_{10}$ copies/ml, respectively (p: 0.007). Mean HBV DNA in patients with HBeAg was higher at 7.18 and 6.44 $\log_{10}$ copies/ml, while anti-HBe positive patients was lower at 3.84 and 4.11 $\log_{10}$ copies/ml in UK and Saudi

patients, respectively. Saudi HBeAg-negative patients had higher HBV DNA than UK patient.

Inactive hepatitis in Saudi patients was associated with low HBV DNA, have very low or undetectable HBV DNA =<10^3 copies/ml in their sera. On the other hand, active liver disease was associated with wide range of low, intermediate and high HBV DNA levels. Within the liver disease group, 53/92 (57.6%) Saudi and 42/66 (63.6%) UK patients have high HBV DNA of >5 $\log_{10,}$ whereas a much smaller proportion show moderate or low HBV DNA levels. Their mean HBV DNA in Saudi and UK patients was 4.9 and 5.09 $\log_{10}$ copies/ml., respectively.

HBV Genotyping

HBV genotypes A, B, C and D were detected in UK patients, A and D in Saudi patients. Low HBV DNAs were mostly ungenotypable. In UK patients, genotypes C and D showed higher mean HBV DNA than genotypes A or B. Saudi cases had also higher mean HBV DNA in genotypes D than genotypes A or B. Within genotype D, UK patients had even higher HBV DNA levels than Saudi patients at 6.10 and 5.10 $\log_{10}$ IU/ml, respectively. Saudi patients with HBV genotype A had low and intermediate HBV DNA levels. In contrast, most genotype A patients from UK had high HBV DNA levels.

Regarding the clinical presentation of HBV in UK patients, genotypes A and D had equal proportion of inactive hepatitis and liver disease. Similarly, patients with acute hepatitis had genotypes A and D. Cirrhosis patients had genotypes C and D patients.

Interestingly, phylogenetic analysis showed that all UK HBV genotype A had subgenotype Ae, genotype B had subgenotype Ba, and genotype C subgenotype C1 (Cs). HBV subgenotypes detected in Saudi Samples had HBV subgenotype D1, while UK had subgenotype D2 mostly in Asian patients. All plasmids sequences confirmed the S-gene of relevant HBV genotypes .

Phylogenetic Analysis of S gene Sequences

Phylogenetic analysis of HBV S/Pol sequences of PCR products was done to define genotypes and subgenotypes on the basis of 8% and >4% heterogeneity of the entire HBV genome, respectively. Generally, there was high concordance between results of our multiplex genotyping assay and sequencing of the S gene.

Specifically, Pre S1 region, Pre S2 and beginning as well as the end of S gene overlapping the YMDD region of the reverse transcriptase part of pol gene showed accurate discrimination between genotypes, and showed genotype-related sequence variations. In contrast, the more conserved regions at the `a` determinant and the rt part of the Pol gene were inconclusive as regard to HBV genotypes. According to calculations of genetic distances by Kimura 2-parameter method, that further confirmed the differences between different regions of the S and Pol genes. The phylogenetic analysis of different regions of the S-gene have shown genotype-related variations as well. The YMDD motive for the detection of antiviral drug resistance as well.

The pre-S1 together with pre-S2 and S-gene encode the large HBsAg protein. Within the pre-S region, many nucleotide exchanges are observed. Clear genotype related variations are observed in the S gene. Genotype D exhibits several variations. All the sequenced cases (9/9) with genotype D, showed deletion of the naturally occurring 11 amino acid codons at the beginning of preS1 region as did the genotype D Gene Bank controls. Consequently the preS1 starts at codon 12 as compared to genotypes B, C, F, and H. Genotype E and G show one aa. codon deletion at the beginning of preS1 segment. Resulting in a total of 108 aa in pre S1 instead of the 119 aa seen in genotypes B, C and F. Other genotype specific variations were observed in the beginning of the preS1 gene including P19S (CCC to AGC) (15/17), codons 88-91 LPAN motif found in all genotype D strains (appendix A).

S2 promoter area primes the translation of preS2. HBV genotypes also show several consistent variations as follows; V90A found in subgenotype C-Australia and in N16, N45, while V90S seen in N19. N114D found in one genotype D Gene Bank strain

X65258 and one Saudi case 4-27. Possibly contributing to differences in the expression efficiency of HBsAg between different genotypes.

Several isolated point mutations were seen; one genotype D case (S5) has mutation at the initiation codon of pre S1, which could interfere with the production of large HBsAg. AF 62/63 SL in genotype C cases (N19 and N16) (2/5) were found.

Genotype-specific variations were also seen in pre S2 region. Within the polymerised human serum albumin (PHSA) binding site (preS2 aa 17-29), F22L was observed in A-Europe, genotype Bj subgenotypes, but all our genotype A cases (5/5) showed L22F unlike Gene Bank controls. V53A and T54P/L was different in different genotypes. This area lies in N-terminus of the PreS2 in the fibronectin binding site to liver sinusoids. This can be related to pathogenesis of HBV infections.

Regarding PreS1 regions encoding epitopes at aa. 21-28 (Class I) and aa.21-30 (Class II) did not show significant genotype specific variations. In the pre S1 epitope associated with HBV virus clearance (58-100), codon A60 found in genotypes C controls and two cases (2/5). Our genotype C cases (3/5) have A60V. E86T was seen in genotypes A, B and C, but E86Q in all our genotype D cases and in only one Gene Bank control X65257. Pre S2 epitope aa.1-11 (Class I and II) shows genotype specific variation at codons 7, 11 and 13. Codon 7 is a SNP candidate as explained below.

Furthermore, the genotype-specific contribution of preS1 epitopes favours vaccination using the complete HBsAg (PreS1, preS2 and small HBsAg) of the prevalent HBV genotypes in specific geographical areas.

In general, minimal genotype related variations are seen in T-cell epitopes within the S gene. Their effect on lymphocyte activation, the degree of inflammatory responses and viral clearance contributing to the pathogenesis of liver disease has to be sought in detailed studies.

In the Pol gene, we found two UK cases with YVDD and one YMDD mutation.

Single nucleotide polymorphism (SNPs) candidates

Although there is 92% similarity in HBV genotypes, we found some point at S/pol gene that show considerable differences between HBV genotypes and these are presented in table 5-7, and can be of benefit in new HBV molecular assays and DNA arrays.

Sequencing of basal core promoter PCR products gave an acceptable coverage of both, core promoter and precore regions as well as the beginning of the core gene. Basal core promote (BCP) variants 1762-1764 were seen in 19/26 (73%) Saudi patients compared to 9/23 (39%) in UK patients. (p: 0.003). All possibilities of BCP (1762-1764) mutations were detected. First a change of AGG to TGG, was seen in 5 Saudi patients. While the other two variations; 1762/1764 A/A, and the double mutant T/A were seen in 2 and 7 patients, respectively. Other types of BCP variation were seen only in 5 Saudi patients; two patients had G1762/T1764, one had C1764, one T1764 and one case showed 12 bp-deletion in BCP region. All patients had chronic hepatitis and one case presented with HCC.

In UK patients, basal core promoter (BCP) variants 1762-1764 present in 9 out of 23 (39%), one of them had AGT. 1762/1764 A/A, and the double mutant T/A were also seen in 3 and 5 patients, respectively. Some cases showed both wild type and mutants as the case N15, 1-50 and 1-48 (fig.6-5).

C1766T, T1768A and C1773T found in 5/8, 2/8 and 8/8 Genotype A from UK, 4/17, 4/17 and 13/17 Genotype D Saudi patients, respectively. C1799G was present in all genotype B patients 7/7 and in 3/17 Genotype D Saudi patients. C1812T in only one genotype A case from UK.

Sequencing of the Precore region of HBV genome:

Upon sequencing of the HBV precore region and the beginning of the core gene (nt. 1781 to 2075). Significant proportion of Saudi patients 37/56 (66.1%) had precore mutants (p:

0.004). Of those, codon 28 mutant (G1896A) was present in 30/37 (81.1%), resulting in a change of tryptophan to a stop codon. Double mutants in both codons 28 and 29 were seen in 15/37 (40.5%) of total mutants. G1899A mutation, which alters codon 29 from glycine to aspargine, was detected mostly in Saudi genotype D patients 22/37 (61%) . In contrast, UK patients showed a much lower prevalence of G1896A 5/23 (21.7%), of HBV genotypes A, B and D (appendix A). The implications of codon 29 mutations need to be verified in detailed studies.

Nt. C1858 was present predominantly in genotype A patients 7/9 UK and 8/9 Saudi genotype A. All genotype C 3/3 UK patients had TCC in nt. 1856-1858. All Saudi and UK patients with genotypes B and D and E had T at position 1858 regardless of their nt. 1896 status. Other point mutations were observed. Our genotype D had G1757A in 15/20 Saudi and 3/3 UK patients. In patients with double mutations at codons 28 and 29; eight patients had also mutations at position 1940 G to C. 32/40 Saudi genotype D patients (82%) had C1912T. This mutation was also present in genotype D1 Gene Bank control AY161157, and X02496. It is a non-coding mutation; thereby it does not change the amino acid aspargine (Asp). Nine patients from UK had T1850A, and seven patients had A to T at nt. 1934, which is present in genotypes Ba, C and E. None of the patients had A1838G, or G1888A mutation.

HBeAg status of core promoter/precore mutants

BCP/PC variants were analyzed for correlation with HBeAg, HBV viral load and genotype. Within HBeAg-positive, UK patients had mostly wild type BCP 9/11 (88%). Comparably, BCP mutations were present in 4 out of 5 (80%) Saudi cases. HBeAg-negative had predominantly BCP mutants, however, less in UK than in Saudi patients at 7/12 (58.3%) and 15/21 (71.4%), respectively.

Many UK patients had wild type in both BCP and PC 9/22 (40.9%) (Graph 6-1). Wild type (normal) precore was seen in all HBeAg-positive UK patients 10/10 (100%), with genotypes A, B, C, D. HBeAg-negative Saudi had significantly more precore mutants than UK patients at 33/50 (66%) and 5/12 (41.7%), respectively. Either core promoter

and/or precore mutants were found in most of the Saudi patients. On the other hand, coexisted BCP and PC mutants occurred mainly in Saudi patients 21/25 (84%) versus only one UK patient. There was predominance of wild type precore in UK patients with or without BCP mutants.

Among other core promoter mutations, four out of 20 Saudi genotype D patients had T1753A, two had chronic hepatitis B and one cirrhotic and one HCC. All 3 UK genotype C patients had G1721A and /or A1775G. Two UK patients both were genotype A (N15, N4), and four Saudi patients 4/25 had C1653T, all were genotype D. Another observation is G1719T G1in the 3 genotype D UK and Saudi patients (17/40). In all our cases nt. 1838A was present from all genotypes including genotype B (subgenotype Ba). G1862 T was seen in only one genotype A case from UK (N14) with viral load of 4.49 $\log_{10}$ copies/ml clinically presented with cirrhosis

HBV viral load and BCP/PC status:
The mean HBV DNA of wild type in both BCP and PC was higher than that of either BCP or PC mutants, double mutants had even lower HBV DNA than single BCP or PC mutants. The new BCP mutants from Saudi patients have even lower viral load. G1896A alone has lower mean viral load at 3.9 than wild type and G1899A both had 4.6 $\log_{10}$ copies/ml.

The correlation of BCP with PC status showed that within BCP mutants. There is a sequential HBV DNA decrease in mutant BCP/wt PC, then mutant in both BCP and PC. While the less frequent wt BCP/ mutant PC showed the lowest viral load (Graph 6-1).

UK BCP mutants were present more in the intermediate (=>3 -<5 $\log_{10}$ copies/ml) viral load group double the rate in high viral load group (=>5 $\log_{10}$ copies/ml) at 3/4 (75%) and 6/17 (35%), respectively (graph 6-3). However, Saudi BCP mutants were comparably predominant in both intermediate and high HBV DNA levels at 8/11 (73%) and 8/10 (80%), respectively. Core promoter and precore mutants were associated with different serum HBV DNA levels in HBeAg-negative patients.

Saudi PC mutants were found at low HBV DNA levels of =<3 log10 copies/ml more than >3 -<5, and =>5 log_{10} copies/ml reaching 8/10 (80%), 15/24 (63%), and 14/22 (64%), respectively. Some of them had very high HBV viral load (=> 7 log_{10} copies/ml). The HBV DNA levels of UK PC mutants were lower in both >3 -<5, and =>5 log_{10} copies/ml at 1/4 (25%), and 3/17 (18%), respectively .

HBV genotype and BCP/PC status:

Core promoter mutants were highest in genotype A and genotype D at 7/9 (78%) and 17/20 (85%), respectively. Genotypes B showed less BCP mutants, but their numbers were too small for a solid conclusion. Double basal core promoter (BCP) mutations were seen in genotype A and less frequently in genotypes B, D and E patients at high HBV DNA levels. In contrast, genotype D predominated at intermediate and low viral loads (p: 0.121).

Precore mutants are predominant in Saudi HBV patients especially in genotype D 35/48 (73%) , one of the three UK genotype D patients had PC mutants. In contrast, PC mutations were less frequent in other HBV genotypes A, B, C and E. Interestingly, Saudi genotype D patients possessed high incidence of codon 29 mutant (G1899A) either alone or as double precore mutants reaching 21/48 (44%) compared to non genotype D cases..

HBV genotype and X/core gene sequence variation:

According to the phylogenetic analysis of the core promoter region (fig.6-7), sequences were grouped into six major clusters. Four of these were occupied by genotypes A-D. The majority of strains were homogenous in both their S and C gene trees (Genotyping chapter 4). Genotype E strains were the nearest to genotype D strains. Core promoter region sequences showed clear correlation to HBV genotypes and subgenotypes. In contrast, the precore region was highly conserved between genotypes and show little correlation if any to HBV genotypes (fig.6-6 and 6-7). In addition, the short segment of nucleotides (200 bp) used was inconclusive to genotypes by phylogenetic analysis.

No significant differences were found in T cell epitope encoding regions as class I (aa18-27) nor class II (aa 1-20, 28-47) core epitopes between different HBV genotypes except

V27I in genotypes B and C. Genotype A (adw2) and D (ayw2) sequences had amino acid substitutions at residues 12 and 27 which were not present in other strains.

HBV mutations and clinical presentation of HBV infection

Regarding clinical presentation, core promoter mutants were present in patients with liver disease were much higher in 18/21 (90%) Saudi patients than in 8/21 (38%) UK patients. Most Saudi patients with cirrhosis and HCC had BCP mutations, and showed wide range of HBV DNA from 2 to 10.7 $\log_{10}$ copies/ml.

Precore mutations

Saudi patients with inactive hepatitis had comparable rates of PC mutants 9/13 (69%) to liver disease 28/43 (65%) (P: 0.019); as acute hepatitis 4/5 and chronic hepatitis 17/29 (59%). Inactive hepatitis patients had low HBV DNA at or below 3.2 $\log_{10}$ copies/ml. Liver disease patients as chronic hepatitis B, cirrhosis with BCP/PC mutations had predominantly genotype D.

HBV X gene mutations

In this study, lysine to methionine (K130M) was seen in a total of 6/22 UK and 11/25 Saudi patients. Valine has changed to isoleucine (V131I) present in 8/22 UK patients and 10/25 Saudi patients. This overlaps the core promoter mutation 1762 and 1764. K130M/V131I were found in genotype A 4/7 from UK and 1/1 Saudi patients. Other X-gene variants; I127N and F132Y were seen. Correlation to oncogenic potential of X gene in different genotypes need to be explored in future research.

HBV X gene and genotypes

F132Y was evident in two genotype A out of 10 (20%) patients, and 6/20 (30%) genotype D patients.

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Appendix A

S/Pol-gene Sequences

S-Promoter nt3045-3180

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HBsAg1start PreS112        Hepatocyte binding site (21-47)   51      Viral Clearance Epitope (58-100) 88
T-cell Epitopes
A1-AY161140_BK   YIMGGWSSKP RKGMGTNLSV PNPLGFFPDH QLDPAFKANS NNPDWDLNPI KDHWPQANQV GVGAFGPGFT PPHGGVLGWS PQAQGILATV  [ 90]
A2-S50225_BK     .S...GT... .......... .......... ......G... ......F... .....A.... ........L. .....I.... .......T..  [ 90]
AeuropeV00866_BK H.L.NK.YSI .......... ......L... ......G... T.....F... .....A.... ........L. .....I.... .......T..  [ 90]
AafrcaAF297625   -.L.NK.YSI .......... .......... ......G... T.....F... .......... ........HS .......... .......T..  [ 90]
A-N4_UK          --....P... .......... R......... ......G... ......F... .....A.... .......... ....SL.... .......T..  [ 90]
A-gCn5_UK        --......... .Q........ .......... ......G... ......F... .....A.... ........L. .....I.... .......T..  [ 90]
A-N5_UK          --......... .......... .......... ......G... ......F... .....A.... ........L. .....I.... .......T..  [ 90]
A-pW7_UK         --......... .......... .......... ......G... ......F... .....A.... ........L. .....I.... .......T..  [ 90]
A-N14_UK         --..RRP.... .......... .......... ......G... ......FYR. .....AS... ........L. .....I.... .H.....T..  [ 90]
A-N49_UK         --.....C.. ......K... .......... ......G... ......F... .....A.... ........L. .....I.... .......T..  [ 90]
BaD00330ADW2_BK  .S......... .......... .......... .......... E........N ..N..D..K. .......... .....L.... .....L.T..  [ 90]
Bj-AB073858_BK   .S......... .......... .......... .......... E........H ..N..D.HK. ......S... .....L.... .......TS.  [ 90]
B1-AB073852_BK   .S......... .......... .......... .......... E........H ..N..D.RK. .......... .....L.... .......TS.  [ 90]
B2-AB073830_BK   .S......... .......... .......... .......... E........H ..N..D..K. .........I .....L.... .......T..  [ 90]
B3-D00331_BK     .S......... .Q........ .......... .......... D........H ..N..DS.K. .......... .....L.... .......T..  [ 90]
B4-AB100695_BK   .S......... .......... .......... .......... E........H ..N..D..K. .......... .....L.... .......T..  [ 90]
B-N9_UK          --......... .......... .......... .......... E........H ..N..D..K. .......... .....L.... .....L.T..  [ 90]
B-pB10_UK        --......... .......... .......... .......... E........H ..N..D..K. .......... .....L.... .....L.T..  [ 90]
B-N50a_UK        --......... .......... .......... .......... E........H ..N..D..K. .......... .....L.... .....L.T..  [ 90]
B-N46_UK         --......... .......... ......L... ......G... .........H ..N..D..K. .........I .....L.... .......T..  [ 90]
C1Cs-AB111946_BK .S......... .Q........ .......... ......G... ......F..N .....A.... ...S...... .....L.... .L.....T..  [ 90]
C2Ce-AF533983_BK .S......... .Q........ .......... ......G... ......F..N .....E.... .A........ .....L.... .......T..  [ 90]
C4-AB048704_BK   .S.......H .......... .......... ......G... ......F..N .....E.... .A........ .....F.... .......T..  [ 90]
C-pC6_UK         --......... .Q........ .......... ......G... ......F..N ..Q..E.... .A........ .....L...N .......T..  [ 90]
C-N1_UK          --......... .Q........ .......... ......G... ......F..N ..Q..E.... .A........ .....L.... .......T..  [ 90]
C-N19_UK         --......... .T........ ...Q..Y... EVNQT.E... ......F..N ..Q..A.... ...SL.H... ....NL.... .......T..  [ 90]
C-N16_UK         ------.... .Q........ .......... ....L.R... SS....F..N ..Q..A.... ...SL..... ...SNL.... .......T..  [ 90]
C-N45_UK         --.......C .......... .......... .V....E... .........N ..P..A.... .......... ....NL.... .......T..  [ 90]
D-X02496ayw_BK   YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... .......E.L  [ 90]
D1-AY161157_BK   YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D2-X80925_BK     YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... ......IQ.L  [ 90]
D3-X85254_BK     YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... .......L.L  [ 90]
D4-AB048702_BK   YS----------..Q...T S......... ......R..T ......F..N ..T..D..K. .A....L... .....L.... ......MQ.L  [ 90]
D-3.33_SA        YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L.V. .QL..A.... RL--------  [ 90]
D-S2_SA          YS----------..Q...T S......... ......R.DT AS.G.VFT.H ..T..D..K. .A....---- ---------- ----------  [ 90]
D-S7_SA          YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... ..L....... RL--------  [ 90]
D-3.34_SA        YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L...- ----------  [ 90]
D-1.60_SA        YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L...- ----------  [ 90]
D-3.35_SA        YS----------..Q...T S......... ......R..T A.....F..N ..T..D..K. .A..S.L... ..PA.NQ.-- ----------  [ 90]
D-S5_SA          YS----------I.Q...T S......... ......R..T A.....F..N ..T..D..K. .A..S.L... ..L..Y---- ----------  [ 90]
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D-1.2_SA         YS----------R.Q...A S......... ......R..T AK....F..N ..T..D..K. .A....L.L. .....L...- ----------  [ 90]
D-1.55_SA        ---------- ---..Q...T S......... .........T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-N11_UK         ---------- ---..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-4.13_SA        ---------- ---..QT..T S.......V. ....S.R..T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-73.2_SA        ---------- ---..HH..S DP.P.....D ....S.R.DT A.....F..N ..T..D..K. .A....L.IS ---------. .......Q.*  [ 90]
D-4.27_SA        ---------- ---..QI..T S......R.. ......R..T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-3.38_SA        ---------- ---..K...T T......... ......R..T A....VF..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-1.49_SA        ---------- -------..T S......... P.....R..T A.....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-N7_UK          ---------- ---..Q...T S......... ......R..T A.....F..N ..T..D..K. .A....L... .....L.... ......IQ.L  [ 90]
D-N12_UK         ---------- ---..SY..T T......... ......R..T A.....F..N ..T..D..K. .A....L... ....L.... ......IQ.L  [ 90]
D1.2_SA          ---------- ------...T S......... ......R..T A.....F..N *.T..D..K. .A....L... ..N..L.... .......Q.L  [ 90]
D-7.64_SA        ---------- ---..HH..T S.....L... ......R..T ......F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
D-7.10_SA        ---------- ---..Q...S DH........ ..-------- --....F..N ..T..D..K. .A....L... .....L.... .......Q.L  [ 90]
E-X75664_BK      ....-L.WTV PLEW.K.I.T T......... ......R..T R.....H..N ....TE..K. .......... .....L.... .....M.K.L  [ 90]
E-N2_UK          --..-L.WTV PLEW.K.H.T T......... ......R..T R...G.H..N ....TE..K. .......... .....L.... .....M.K.L  [ 90]
F-X69798adw4_BK  --..APL.TT .R...Q.... .......... ......R..T A.....F.TN ..S..M..K. ...GY..... .....L.... .....V.T.L  [ 90]
H-AY090454_BK    .S..APL.TA .R...Q.... .......... ....L.R... SS....F.TN ..N..M..K. ...G..... .....L.... .......T.S  [ 90]
G-AF160501_BK    .S..-L.WTV PLEW.K...A S.....L... ......R..T ......F..K ..P..E..K. ....Y..... .....L.... ..S..T.T.L  [ 90]

79                   99                  Start PreS2Fibronectin Binding Site to Liver sinusoids
A1-AY161140_BK    PQAQGILATV PAVPPPASTN RQSGRQPTPI SPPLRDSHPQ AMQWNSTAFH QALQDPRVRG LYSPAGGSSS GTLNPVPIIA PLISSISSRIGDP [93]
A2-S50225sAg-_BK  .......T.. STI....... .......... .....E.... .......... .......... ..L....... ..V..A.N.. SH.....A.T... [93]
A-europeV00866_BK .......T.. STI....... .......... .......... ........L. .......... ..L....... ..V..A.N.. SH.....A.T... [93]
AafrcaAF297625_BK .......T.. .T...T.... .......... .......... ........L. .......... ..F....... .......NT. SH.......T... [93]
A-N5_UK           .......T.. STI....... .......... .......... .......... .......... ..F....... ..V..A.N.. SH.....A.T... [93]
A-Cn5_UK          .......T.. STI....... .......... .......... .......... .......... ..F....... ..V..A.N.. SH.....A.T... [93]
A-N49_UK          .......T.. STI....... .......... .......... .......... .......... ..F....... ..V..A.N.. SH.....A.T... [93]
A-pW7_UK          .......T.. STI....... .......... .......... .......... .......... ..F....... ..V..A.N.. SH.....A.T... [93]
A-N14_UK          .H.....T.. STI....... .....HT... .......... .......... .........R ..F....... ..V..A.N.. SH...T.A.T... [93]
A-N4_UK           .......T.. STI....... T......... .......... .P*.....VR .....T---- ..F....... ..V..ATN.P SH.....V.T... [93]
BaD00330ADW2_BK   .....L.T.. ..A....... .........L ......T... .......T.. .T....G..A ..F....... ..VS.AQNTV SA....L.KT... [93]
Bj-AB073858_BK    .......TS. ..A....... .........L ......T... .......T.. .T.......A ..L....... ..VS.AQNTV SA....L.KT... [93]
B1-AB073852_BK    .......TS. ..A....... .......... ......T... .......T.. .T.......A ..F....... ..VS.AQNTV STT...L.KT... [93]
B2-AB073830_BK    .......T.. ..A....... .........L ......T... .......T.. .T.......A ..F....... ..VS.AQNTV SA....L.KT... [93]
B3-D00331_BK      .......T.. .TA....... ..L..K...L ......T... .......T.. .T.......A ..F....... ..V...QNT. SS....L.TT... [93]
B4-AB100695_BK    .......T.. ..A....... .........L ......T... .......T.. .T.......A ..F....... ..VS.AQNT. SA...TF.KT... [93]
B-N9_UK           .....L.T.. ..A....... .........L ......T... .I.S..AN.. .T.......A ..F....... ..VS.DQNTV SA....L.NT. .. [93]
B-pB10_UK         .....L.T.. ..A....... .........L ......T... .I....AN.. .T.......A ..F....... ..VS.AQNTV SA....L.TT... [93]
B-N9a_UK          ---------- ---------- ---------- ---------- ---------- -P.R.T-SS. P.RT...... ..VS.AQNTV SA....L.KT.Y. [93]
B-N46_UK          .......T.. ..A....... .........L ......T... .......T.. .T.......A ..F....... ..VS.AQNTV SA....L.KT..L [93]
B-N50a_UK         .....L.T.. ..A....... .........L ......T... .......T.. .T.......A ..F....... ..VS.AQNTV SA....L.KT... [93]
C1Cs-AB111946_BK  .......T.. ..A....... .......... ........S. ......ST.. ...L..K... ..L....... ..V...QTT. SP....F..T... [93]
C2Ce-AF533983_BK  .......T.. .VA....... .......... .S........ .......T.. ...L...... ..F....... ..V....TT. SP....F..T.G. [93]
C3-X75656_BK      ---------- ---------- ---------- ---------- -----..T.Q ...L...... ..F....... ..V....TT. S.....F..T... [93]
C4-AB048704_BK    .......T.. ..A....... .......... ......T... .......... .......... ..F....... ..V....NTV SH....FTKT... [93]
C-N1_UK           .......T.. .V........ .......... .......... .......T.. ...L...... ..F....... ..V....TT. SP....F..T... [93]
C-N16_UK          .......T.. ..A....C.. .HP....... .......... ......SI.. ...L.S.... ..F..A...A ..V....TT. SP....F..TV.R [93]
C-N19_UK          .......T.. .SA....... .......... .......... ......SI.. ...L.S.... ..F....... ..V....TT. SP....F..T... [93]
C-N45_UK          .......T.. ..A....... .......... .......... ......ST.. ...L...... ..L....... ..V....TT. SP....F..T... [93]
C-pC6_UK          .......T.. .V........ ......A... P......... .......T.. ...L...... ..F....... ..V....TT. SP..T.F..T... [93]
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D1-AY161157_BK    .......Q.L ..N....... .........L .....NT. .. .......T.. .T........ ..F....... ..V....TTV SH....F...... [93]
D2-X80925_BK      ......IQ.L ..N....... .........L .....NT. .. .......T.. .T........ ..F....... ..V....TT. SP....F...... [93]
D3-X85254_BK      .......L.L ..N....... .........L T....NT. .. .......T.Q HT......K. ..L....... ..V....TT. SPL...F...... [93]
D4-AB048702_BK    ......MQ.L ..N....... .........L .....TT... .......T.. .T........ ..L....... ..V....TT. SP....F.....L [93]
D-N11_UK          .......Q.L ..N....... .........L .....NT. .. .......T.. .T........ ..F....... ..VS...TTV SH....F...... [93]
D-1.2_SA          .......Q.L ..N....... .........L .....NT. .. .......T.. .T........ ..F....... ..V....TTV SH....F...... [93]
D-1.55_SA         .......Q.L ..N...V... .........L .....NT. .. .......T.. .T......K. ..F....... ..V....TTV SH....F....V. [93]
D-4.13_SA         .......Q.L ..N....A.. .P.......L .....NT. .. .....T.T.. .T........ .FF....... ..V....TTV SH....F...... [93]
D-73_2_SA         .......Q.* ..DT...... .*L......L .....NT. .L ..H....T.* .I.L...--- ---....... E.V....TTV SH....F.....L [93]
D-4.27_SA         .......Q.L ..N....... .........L ......T... .......T.. .T........ ..F....... E.V..A.TTV SH....F...... [93]
D-7.10_SA         .......Q.L ..NT. ..... .........L ......T... .......T.. .T....E... ..FSV...C. ..VHL.TTTV SH..*.FT...RS [93]
D-1.49_SA         .......Q.L ..N....... .E.......V .....NT. .. .......T.. .T........ .DF....... ..VK.A.TTV SH....F..T..T [93]
D-7.64_SA         .......Q.L .TN....... .........L .....NT. .. .......T.. .T........ ..F....... ..V....TTV SP....F...... [93]
D-3.38_SA         .......Q.L ..N....F.. .........L .....NT. .. .......T.. .T........ ..F....... ..V....TTV SP....F...... [93]
D-N12_UK          ......IQ.L ..N....... .........L .....T... .......T.. .T........ ..F....... ..V....TT. SP....F...... [93]
D-N7_UK           ......IQ.L ..N.....A. .........L .....NT. .. .......... .T........ ..F....... ..V....TT. SP..*.F...... [93]
E-X75664_BK       .....M.K.L ..D....... .......... T.....T... .......T.. .......... ..F....... ..V....TT. S.....F...... [93]
E-N2_UK           .....M.K.L ..D....... .......... T.....T... .......T.. .......... ..L....... ..V....TTV S.....F...... [93]
E-pE2a_UK         ---------- ---------- ---------- ---------- -......T.. .......... ..L....... ..V....TTV S.....F...... [93]
F-X69798adw4_BK   .....V.T.L ..D....... .R...K...V ......T... .......Q.. ...L.....A ..F....... ..Q..A.T.. S.T...F.KT.G. [93]
H-AY090454_BK     .......T.S .PD....... .R...K...V ......T... .......Q.. ...L...... ..L....... E.Q....T.. S.T...F.KT... [93]
G-AF160501_BK     ..S..T.T.L ..D....... .......... .......... .......... ....N.K... ..F....... .IV....T.. SH....F...... [93]
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1Start Small HBsAg
A1-AY161140_BK     APNMENITSG FLGPLLVLQA GFFLLTRILT IPQSIDSWWT SLNFLGGSPV CLGQNSQSPT SNHSPTSCAP ICTGYRWMCL RRFIIFLFILL  [91]
A2-S50225HBsAg-_BK VT..... ..... ..S..... ....L..... ..... ..... .....P. ..P..... .....  [91]
A-europeV00866_UK  VTI..... ..... ..... ....L..... ..... ..... .....P. ..P..... .....  [91]
A-afrcaAF297625_BK .L..... ..... ..... ....L..... ..... .....Y.. .....P. ..P..... .....  [91]
AD-recAF297621_BK  V..... ..... ..... ....L..... ..... ..... .....P. ..P..... .....  [91]
A-pW7_UK           VT..... ..... ..... ....L..... ..... ..... .....P. ..P..... .....  [91]
A-N49_UK           VT..... ..... ..... ....L..... ..... ..... .....P. ..P..----- -----  [91]
A-N14_UK           VT.....*. L..... ..... ....L..... ..... .V....E... ..Y.T.C.P. .WP..... .....L--  [91]
A-gCn5_UK          VT..... L..... ..... ....L..... ...------- ---------- ---------- ---------- -----  [91]
A-N4_UK            V..... L.....L... ..... ....L..... .....AL. .....--- ---------- ---------- -----  [91]
A-N5_UK            VT..... L..... ..... ....L..... ..... ..... ...L....P. ..P..... .....  [91]
AD-recAF418674_BK  ..... .H..... ..... ..... ..... ..... ..... ..... ..... .....  [91]
A-gD-clon_UK       VTE..... ..... ..... ....L..... ..... ..... .....P. ..P..... .....  [91]
BaD00330ADW2_BK    V......A.. L..... ......K... ....L..... .....T.. .....QI .S....C.P. ..P..... .....C...  [91]
Bj-AB073858_BK     V.K....A.. L..... ......K... ....L..... .....T.. .....QI .S....C.P. ..P.....Y. .....C...  [91]
B1-AB073852        V......A.. L..... ......K... ....L..... .....E.P.. .P......QI .S....C.P. ..P..... .....C...  [91]
B2-AB073830_BK     V......A.. L..... ......K... ....L..... .....T.. .....QI .S....C.P. ..P..... .....C...  [91]
B3-D00331_BK       V......A.. L..... ...S..K... ..L.L..... .....ET.. .....QI .S....C.P. ..P..... .....C...  [91]
B4-AB100695_BK     V......A.. L..... ...S..K... ....L..L.. .....ET.. .....QI .S..L.C.P. ..P..... .....C...  [91]
B-N9_UK            V......S.. L..... ......K... ..H.L..... .....KT.. .....QI .S....**-- ---------- -----  [91]
B-N46_UK           V......A.. L..... ......K..I ....L..... ..S....T.. .....QI .S....W.P. ..P..... .....C...  [91]
B-pB10_UK          V......S.. L..... ......K... ....L...-- ---------- ---------- ---------- ---------- -----  [91]
B-N50_UK           V......A.. L..... ......K... ....L..... .....T.A .....I .S....C.P. ..P..... .----------  [91]
C1Cs-AB112348_BK   .....ST... ..... ..... ....L..... .....A.T .SV..L.... .....P. ..P..... .....  [91]
C1Cs-AB111946_BK   .....ST... ..... ..... ....L..... .....A.T .P...L.... .....P. ..P..... .....  [91]
C2-AF533983_BK     .....ST... ..... ..... V...L..R.. .....A.T .P..... .....P. ..P....T.. .....  [91]
C2Ce-AY123041_BK   .....ST... ..... ..... ....L..... .....A.T .P..... .....P. ..P..... .....  [91]
C3-X75656_BK       VT....T... ..... ..... ....L..G.. .....EA.R .P..... .....P. ..P..... .....  [91]
C4Caus-AB048704_BK .S...ST... ..... ..... ....L..... .....A.G .I......Q. .....P. T.P..... .....  [91]
C-pC6n             ......T..* ..... ....------ ---------- ---------- ---------- ---------- ---------- -----  [91]
C-N1_UK            ......T... ..... ..... ....L..... .....A.T .P..... ...L....P. ..P..... ..L.....  [91]
C-w-clon_UK        ......T... ..... ..... ....L..... .....A.T .P..... .....P. ..P..... .....  [91]
C-N16_UK           .....ST... ........L. ..... ....L.*... .V.....A.T .P...W.... F.......P. .WP..... P.LM.LR....  [91]
C-N3_UK            .....RR... L.EAV....T ..L**..... ..K.L..... .V.....A-- ---------- ---------- ---------- -----  [91]
C-N19_UK           V....ST... ..... ..... .R..L.*... CV.S...ATM .P...W.... .....P. .VP..... ...MM..I---  [91]
C-N45_UK           .....ST... ..... ..... ....L..... .....A.T .P...LE... ...LA.F.P. ..P..... .G.....  [91]
D1-AY161157        .L..... ..... ..... ....L..... .....TT. .........A .....P. T.P..... .....  [91]
D2-X80925_BK       .L.......D .......S.. ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D3-X85254_BK       .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D4-AB048702_BK     .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P.....F. .....  [91]
D-1.2_SA           .L..... L..... ..V..... ....L..... .....TT. ..... .S....C.P. T--------- -----  [91]
D-1.55_SA          .L..... ..... ..... ....L..... .....TT. ..... F.......P. TWP..... .....  [91]
D-1.60             .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....---  [91]
D-2.23a_SA         .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-2.31a_SA         .L..... L..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-S7a_SA           .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-3.33a_SA         .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
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D-S6a_SA          .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-pD5_SA          .L..... ....M..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-73_2_SA         .L..... ..... .--------- ---------- ---------- ---------- ---------- ----------  [91]
D-1.49_SA         .L......L. ......E... .YV.M....S ---------- ---------- ---------- ---------- ----------  [91]
D-N7_UK           .L..... ..... ..... ....L..... ...C...A.. .I.....--- ---------- ---------- ----------  [91]
D-3.38_SA         .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-7.64_SA         .L..... ..... ..... ....L..... .....TT. ..... ...L....P. ..-------- ----------  [91]
D-7.46R_SA        VL..... ....V..... .....K ....L..... ..S.V..QQ. ..... .....P. T.P.....Y. .....  [91]
D-4.27_SA         .L..... ..... ..... ....L..... .--------- ---------- ---------- ----------  [91]
D-3.34a_SA        .L..... ..... ..... ....L..... .....TT. ..... .....P. T.P..... .....  [91]
D-N11_UK          .L..... ..... ..... ....L..... .....TT. ..... ...L....P. T.P..... .....  [91]
D-3.35a_SA        .L..... ..... ..... ....L..... .....TT. ..... .....P. T*P..... .....  [91]
D-N12_UK          .L..... ..... ..... ....L..... .....A.. ..... .....P. T.P..... ..Y.-.....  [91]
D-4.13_SA         .L..... ..... ..... ....L..--- ---------- ---------- ---------- ----------  [91]
D-7.10_SA         .LH.AY.L.. ..R.....HW QSI...---- ---------- ---------- ---------- ---------- ----------  [91]
E-X75664_BK       .....S.... ..... ......K... ....L..... .....A.. ..... .....P. ..P..... .....  [91]
E-2.25a_SA        .....S.... ..... ......K... ....L..... .....A.. ..... .S.....P. ..P..... .....  [91]
E-N2_UK           .....G.... ..... ......K... ....L..... .....A.. ---------- ---------- ---------- ----------  [91]
pEn2              .....G.... ..... ......K... ....L..... .....A.. .....I ..... .....P. ..P..... .....  [91]
F-X69798adw4_BK   .M..D..... L..... VC....K... ....L..... .....L.G .P..... ...L....P. T.P..... .....  [91]
H-AY090454_BK     .M..... L..... VC....K... ....L..... .....VP.G .P.....I ...L....P. T.P..... .....  [91]
G-AF160501_BK     ..... ..... ..... ....L..... .....V.. .P.L..... .....I..P. T.P..... .....  [91]
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                   89                                  `a`determinant (124-147)
A1-AY161140_BK    LCLIFLLVLL DYQGMLPVCP LIPGSTTTST GPCKTCTTPA QGNSMFPSCC CTKPTDGNRT CIPIPSSWAF AKYLWEWASV RFSWLSLLVP [90]
A2-S50225HBsAg-_BK ..... ..... I..... ..... ....L..... .....C. ..... ......R... ..... [90]
A-europV00866adw_BK ..... ..... ..... ..... ....K..... .....C. ..... ..... ..... [90]
A-africAF297625_BK ..... ..... ..... ..... ..... ..... .....C. ..... ..... ..... [90]
A-pW7_UK          ..... ..... ..... ..... ..... ..... .....C. ..... ..... ..... [90]
A-Dclon_UK        ..... ..... ..... ..... ..... ..... .....C. ..... ..-------- ---------- [90]
A-N5_UK           .....A.. E.....W. -YS.TN.RP- TA.....- ---------- ---------- ---------- ---------- ---------- [90]
A-N66-UK          ---------. ..... ..... ..... ..... ..... .....C. ..... ..... ..... [90]
A-N60-UK          ---------. ..... ..... ..... ..... ..... .....C. ..... V..... ..... [90]
A-N75-UK          ---------- ---------- -----.... ..... ..... ..... .....C. ..... ..... ..... [90]
Ba-D00330adw2_BK  ..... ..... .....S.... ..... ..T..... ..... .....C. ..... ..... ..... [90]
Bj-AB073858_BK    ..... ..... .....S.... ..... ..T.L..... ..... .....C. ..... ..... ..... [90]
B1-AB073852_BK    ..... ..... .....S.... ..... ..T..... ..... .....C. ..... ..... ..... [90]
B2-AB073830_BK    ..... ..... .....S.... .....T. ..T..... ..... .....C. ..... ..... ..... [90]
B3-D00331_BK      ..... ..... .....S.... ..... ..T..... ..... .....C. ..... ..... ..... [90]
B4-AB100695_BK    ..... ..... .....S.... ...R..... ..T..... ..... .....C. ..... ..... ..... [90]
B-N47-UK          ---------- ---------- --...S.... ..... ..T..... ..... .....C. ..... ..F..... ..... [90]
B-N69_UK          --ILIS.... -..... .....S.... ..... ..T..... ..... .....C. ..... ..... ..... [90]
B-N70f_UK         ---------- ---------- -----S.... ..... ..T..... ..... .....C. ..... ..... ..... [90]
B-N71-UK          -.....V... N..... .....S.... ..... ..T...T... .I..... .....C. ..... ..... ..... [90]
B-N72f_UK         ---------- ---------- -------... ..... ..T..... ..... .....C. ..... ..... ..... [90]
B-N74r-UK         -.....V... N..... .....S.... ..... ..T...T... .I..... .....C. ..... ..... ..... [90]
B-N73-UK          ---------- ---------- -------... ...R..... ..T..... ..... .....C. ..... ..... ..... [90]
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B-N72-UK            -......... .......... .....S.... .......... ..T....... ........C. .......... .......... .......... [90]
AB-N68-UK           -......... .......... .....S.... .......... .......... ........C. .......... .......... .......... [90]
C1-AF411412_BK      ......W... .......... .L..TS.... .......I.. ..T....... ....S...C. .......... .RF....... .......... [90]
C1Cs-AB111946_BK    .......... .......... .L..TS...M .......... ..T....... ....S...C. .......... .RF....... .......... [90]
C2-AF533983_BK      .......... .......... .L..TS.... .......I.. ..T....... ....S...C. .......... .RF....... .......... [90]
C3-X75656_BK        .......... .......... .L..TS.... .......I.. ..T....... ....S...C. .......... VRF....... ........A. [90]
C4aus-AB048704ayw_BK.......... .......... .L........ ...R...IT. ..T....... ....S...C. ........G. ..F....... .......... [90]
C-N1-UK             -......... .......... .L..TS.... .......I.. ..T....... ....S...C. .......... .RF....... .......... [90]
C-N83-UK            -......... ..H....... .L..TS.... .......I.. ..TY...... ....S...C. .......... .RF....... .......... [90]
C-N80-UK            -......... .......... .L...S.... .......I.. ..T....... ....S...C. .......... .RF....... .......... [90]
C-wclon_UK          .......... .......... .L..TS.... .......I.. ..T....... ....S...C. .......... .RF....... .......... [90]
C-N1_UK             .......... ........W. -YS.TNS.T- AA.......- ---------- ---------- ---------- ---------- ---------- [90]
C-N16_UK            ....CSQ... .......... .LA.TS..D- AA-------- ---------- ---------- ---------- ---------- ---------- [90]
D1-AY161157_BK      .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D2-X80925_BK        .......... .......... .....S...A ...R....T. ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D3-X85254_BK        .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D4-AB048702_BK      .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-1.60a_SA          .......... .......... .....S.... ...R...... ..I..Y.... ....S...C. .......... G.F......A .......... [90]
D-2.23a_SA          .......... ......R... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-S7a_SA            .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... GNF......A .......... [90]
D-3.33a_SA          .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-S6a_SA            .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-3.35a_SA          .......... .......... .V...S.... ...R...... ..T..N.... ....S...C. .......... G.F......A .......... [90]
D-3.34a_SA          .......... .......... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-N36_UK            ---------- ---------- -----S.... .T.R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-N38f_UK           ---------- ---------- -----S.... .T.R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-N82_UK            ---------- --*....... .....S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-N78_UK            ---------- ---------- -----S...V ...R....TV ..T..Y.... ....S...C. .......... G.F......A .......... [90]
D-N79_UK            -.....M... .......... .....S.... ...R...... ..T..N.... ....S...C. .......... G.F......A .......... [90]
D-N76_UK            -.....M... .......... .L...S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A ......S... [90]
D-N77_UK            -......... .......... .L...S.... ...R...... ..T..Y.... ....S...C. .......... G.F......A ......S... [90]
D-3.38_SA           .......... .......... .....S.... ...R...... ..I..Y.... ....S...C. .......... G.F......A .......... [90]
D-pD5_SA            .......... .......... .....S.... ...R...... ..I..Y.... ....S...C. .......... G.F......A .......... [90]
D-N12_UK            .......... ......A.W. .....-S.-- ---------- ---------- ---------- ---------- ---------- ---------- [90]
D-N11_UK            .......... .......... .....Y.-.. ......---- ---------- ---------- ---------- ---------- ---------- [90]
D-7.46R_SA          ......*.I. .......... .....S.... .A.R....RT ..T..Y...- ---------- ---------- ---------- ---------- [90]
E-X75664_BK         .......... .......... .....S.... ...R..M.L. ..T....... .S..S...C. .......... G.F......A .......... [90]
D-2.31a_SA          .......... .......... .....S.... ...R..M.T. ..T..Y.... ....S...C. .......... G.F......A .......... [90]
E-2.25a_SA          .......... .......... .....S.... ...R....L. ..T....... .S..S...C. .......... G.F......A .......... [90]
E-N81r-UK           ---------- --........ .....S.... ...R...... ..T....... ........C. .......... G.F......A .......... [90]
E-En2_UK            .......... .......... .....S.... ...R....L. ..T....... .S..S.A.C. .......... G.F......A .......... [90]
F-X69798adw4_BK     .......... .......... .L........ ........L. ..T....... .S..S...C. .........L G........A .........Q [90]
H-AY090454_BK       .......... .......... .L........ ........L. ..T....... ....S...C. .......... G........A .........Q [90]
G-AF160501_BK       .......... .......... .....S.... .......... .....Y.... ....S...C. .......... .......... .......... [90]
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                  183                                  End of HBsAg (226)
A1-AY161140       FVQWFVGLSP TVWLSAIWMM WYWGPNLYNI LSPFIPLLPI FFCLWVYI*T LTKRRDGVIP *ISWVM*LEV GDISHRIILY KTTNTVLENF [90]
A2-S50225HBsAg-_BK ..........  ....L.....  .....S..S. VR........  ..........  ...QK.....  .T...TE...  .EHC...T..  .RS.......  [90]
A-europV00866adw_BK ..........  ..........  .....S..S. V.........  ..........  ...QK.....  .T..AT....  .ELC......  .RS.......  [90]
A-afric-AF297625_BK ..........  .....V....  .....S....  V.........  ..........  ...QK...T.  .T..D.....  .VHC......  .KS.......  [90]
A-N60-UK          .....A....  ..........  .....S....  V.........  ..........  ...QK.....  .T...T....  .ELC......  .RS.......  [90]
A-N66-UK          .....A....  I........I  L....R..T.  V.........  ..........  ...QK.....  .T...T....  .ELC......  .RS.......  [90]
A-N75-UK          ..........  ..........  .....S..S.  V.........  ..........  ...QK.....  .T.........  .GHC....--  ----------  [90]
A-pW7_UK          ..........  .........I  .....S..S.  V.........  ..--------  ----------  ----------  ----------  ----------  [90]
Ba-D00330adw2_BK  ..........  .....V...I  .F...S....  ....M.....  ..........  ...QK..D..  LT..D...GA  .AHC..N...  .KSKCG.G..  [90]
Bj-AB073858_BK    ..........  .....V....  .....S....  ..........  ..........  ...QK..D..  LT..D...G.  .AHC..N..H  .KL.Y..G..  [90]
B1-AB073852_BK    ..........  .....V....  ..........  ....M.....  ..........  ...QK..D..  LT..D..SG.  .GHC..N...  .KS.N.SG..  [90]
B2-AB073830_BK    ..........  .....V....  .....S....  S.........  ..........  .I.QK..D..  L...D...G.  .AHC..N...  .KSKC..G..  [90]
B3-D00331_BK      ..........  .....V....  .F...S....  ....M.....  ..........  .R.QK..AT.  LT..G.....  .GPYPKN..C  *KS.N..G..  [90]
B4-AB100695_BK    ..........  .....V....  .....S....  ....M.....  ..........  ...QK..D..  L...D...G.  .VPY..N..D  .KS.C..G..  [90]
B-N71-UK          ..........  .....V....  .....S...T  ....M.....  ..........  ...QK..D..  LT..D...G.  .AHC..N...  .KSKC..G..  [90]
B-N72f_UK         ..........  .....V....  .F...S....  ....M.....  ..........  ...QK..D..  LT..D...G.  .ARC..N.V.  .KL.------  [90]
B-N70f_UK         ..........  .....V....  .F...S....  ....M.....  ..........  ....K..D..  LT..D...G.  .AHC..N...  .KSKC..G--  [90]
B-N69_UK          ..........  .....V....  .F...S....  ....M.....  ..........  ...QK..D..  LT..D...G.  .AHC.KN...  .KSKR..G..  [90]
B-N74r-UK         ..........  .....V....  .....S...T  ....M.....  ..........  ...QK..D..  LT..D...G.  .AHC..N---  ----------  [90]
B-N47-UK          ..........  .....V....  .F...S....  ....M.....  ..........  ...QN..D..  LT..D...G.  .AHC..N---  ----------  [90]
B-N73-UK          ..........  .....V....  .....S....  ....M.....  ..........  ...QK..D..  L.......V.  .---------  ----------  [90]
AB-N68-UK         .....A....  ..........  .....S..S.  V...M.....  ..........  ...QK..D..  .T..D.....  .ARC......  .RS.......  [90]
C1-AF411412_BK    ..........  .....V....  ....RS....  ....L.....  ..........  .I.PNV..T.  LT..D.....  .VLYRKN...  .KSSN.F..C  [90]
C1Cs-AB111946_BK  ..........  .....V....  ....RS....  .N..L.....  ..........  .L.PNV..T.  LT..D.....  .APY.KN...  TKS.N.FG..  [90]
C2Ce-AF533983_BK  ..........  .....V....  .....S....  ....L.....  ..........  .I.PNV.AT.  LT..D.....  .VLYRKN...  *NLSN.F..C  [90]
C3-X75656_BK      ..........  .....V....  .....S....  ..........  ..........  .IRPK..A..  LT..A.....  .VPY.KN...  *KS.N.F...  [90]
C4aus-AB048704ayw_BK.....A....  .....V...I  .....S....  ....L.....  .L.......I  P..QK.....  STL.D..SG.  .EAYRKS...  TNS.......  [90]
C-N1-UK           ..........  .....V....  .....S....  ....L.....  ..........  .I.PNV.AT.  L...D...D.  .VLYRKN...  *KSSN.F..C  [90]
C-N80-UK          .....A....  .....V....  .....S....  ....L.....  ..........  .I.PSV.AT.  LT..D.....  .VLY..N..C  *KSSN..QKS  [90]
C-N83-UK          ..........  .....V....  .....S....  ....L.....  ..........  .I.PNV.AT.  FTL.D.....  .VLYR.N...  .NSSN.F..C  [90]
D3-X85254_BK      ..........  .....V....  .....S..S.  ....L..*..  ..........  ...Q....TL  ..L.GIS.D.  MGPC.KNTS.  .KSKN..G..  [90]
D-N36_UK          ..........  .....VM...  .....S..S.  ....L.....  ..F.......  ...QK...TL  Y...A.S...  MGHC.-----  ----------  [90]
D-N38f_UK         ..........  .....VM...  .....S..S.  ....L.....  ..F.......  ...QK...TL  Y...A.S...  MGHC.-----  ----------  [90]
D1-AY161157_BK    ..........  .....V....  .....RM.S.  ....L.....  ..........  ...QK...TL  Y...A.S.D.  MGHC.K.TS.  .KSKN.....  [90]
D2-X80925_BK      ..........  .....L....  .....S..S.  ....L.....  ..........  ...LK.....  ....A.S.A.  MGPC.K.TS.  RKSKN..G..  [90]
D4-AB048702_BK    ..........  .....V....  .....S..ST  ....L.....  ..........  ...QK....L  ....A.S...  ..HC.K.TS.  TKSKN....S  [90]
D-N76_UK          ..........  .....V....  .....R..S.  ....LL....  .....A....  ...QK....L  Y...A.S.D.  MGHC.K.TS.  RKSKN.....  [90]
D-N79_UK          ..........  .....V....  .....S..S.  ..........  ..........  ...QK...TL  Y...A.S.D.  MGHC.KSTS.  RKSKN.....  [90]
D-N78_UK          ..........  .....V....  .....S..S.  ....L.....  ..........  ...LK...TL  ....A.S.D.  MGH..K----  ----------  [90]
D-N77_UK          ..........  I....V....  .....S..S.  ....L.....  ..........  ...QK.....  Y...A.S.D.  MGHC...TS.  RKSKN.....  [90]
D-N82_UK          ..........  .A...V....  .....S..S.  ....L.....  ..........  ...QKG..TL  Y.L.A.S.D.  WGH-------  ----------  [90]
D-3.38_SA         ..........  .....V.L..  .....S..S.  ....L.....  ..........  ...QK...--  ----------  ----------  ----------  [90]
D-pD5_SA          ..........  .....V.L..  .....S..S.  ....L.....  ..--------  ----------  ----------  ----------  [90]
E-X75664_BK       .....A....  .....V....  .....S..D.  ..........  .........I  P..QK..D..  ..........  .GHY..NTS.  .KS......S  [90]
E-N81r-UK         .....A....  .....V....  .....S....  ........A.  .L.......I  ...QK..D..  ..L.D.....  .GHY..----  ----------  [90]
E-En2_UK          .....A....  .....V....  .....S....  ..........  .....A...I  P..QK..D..  ........V.  .GHY.KDTSD  *KSK.-----  [90]
F-X69798adw4_BK   ....C.....  ....LV...I  .......CS.  ..........  .CY...S..I  PL.QK...T.  Y.L...S.V.  ..HYPK.T..  TKSRNAF..C  [90]
H-AY090454_BK     ....C.....  ....LV...I  .......CS.  ..........  .CY..AS...  QL.QNG....  YTL..IS.G.  .GHCL.N..C  .KSKIAFA..  [90]
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G-AF160501_BK       .......... .......... .......... .......... .......... ...QK..... ..L.D..... .VLC.KNTSH RKLSN.FG.S [90
Pol ORF
      Polymerase 182
A1-AY161140_BK   HHGRLVIKTS QRHGDEPFCS QPSGILSRSS VGPCIQSQFK QSRLGPQPHK GPLATSQPGR SGSIRARVHS PTRRCFGVEP SGSGHIGHSA  [90]
A2-S50225_BK     Q...WD.... ......S... .......... .....R..L. .....L...Q ....S....G .......A.P S...Y..... ...R..D..V  [90]
AeuropeV00866_BK YSWEQELQH. ......S... .....P.... .....R..LN K....L...Q ....S..... .......A.P S...Y..... ......D..V  [90]
AafrcaAF297625   YSWEQELQH. ......S... .......... .....R...N K....L...Q .......... .....P.ALP S......... ......DY..  [90]
A-N4_UK          --...A.Q.. ......S... .......... .....R..L. .....F...Q ....S..S.. .........P S..QS..... ......D..V  [90]
A-gCn5_UK        --.....Q.. T.....S.G. .......... .....R..L. .....L.S.Q ....S..... .......A.P S...Y..... ......D..V  [90]
A-N5_UK          --.....Q.. ......S.G. .........P ....VR..L. .....L.S.Q ....S..... .......A.P S...Y..... ......D..V  [90]
A-pW7_UK         --........ ......S... .......... .....R..L. .....L...Q ....S..... .......A.P S...Y..... ......D..V  [90]
A-N14_UK         --.*EA.E.. ......S... .......... .....R..L. .....LL..Q ....SI.... .......A.P S...Y..... .C....D..V  [90]
A-N49_UK         --....MQ.. ......A... .......... .....R..L. .....L...Q ....S..... .......A.P S...Y..... ......D..V  [90]
BaD00330ADW2_BK  Q.....FQ.. K....KS..P .S....P... ........LR K.......EQ .Q..GR.Q.G .........P SPWGTV.... ....PTHNC.  [90]
Bj-AB073858_BK   Q.....LQ.. K....K...P .SP...P... ........LR K.......TQ .Q..GRPQ.G .....V...P SPWGTV.... .....THFC.  [90]
B1-AB073852_BK   Q.....FQ.. K....KSV.P .SP....... ........LR K.......TQ .Q..GRPQ.G .........P SSWGTV.... .....THIC.  [90]
B2-AB073830_BK   Q.....FQ.. K....KS..P .SP...P... ........LR K.......AQ .Q..GR.Q.G .R.......P SPWGTV.... .....THNC.  [90]
B3-D00331_BK     Q.....LQ.. T....KS.RP .S.......P ........LR ........TQ .Q..GL.Q.G ......GI.. TPWGTV.... .S...THNC.  [90]
B4-AB100695_BK   QY....FQ.. K....KS..P .SP...P... ........LR K.......AQ .Q..GR.Q.G .........P SPWGPV.... .....THNC.  [90]
B-N9_UK          --....FQ.. K....KS..P .SP...P... ........LR K.......AQ .Q..GR.Q.G .........P SPWGTV.... ....PTHNC.  [90]
B-pB10_UK        --....FQ.. K....KS..P .SP...P... ........LR K.......AQ .Q..GR.Q.G .........P SPWGTV.... ....PTHNC.  [90]
B-N50a_UK        --....FQ.. K....KS..P .SP...P... ........LR K.......AQ .Q..GR.Q.G .........P SPWGTV.... ....PTHNC.  [90]
B-N46_UK         --....FQ.. K....K...P .S...P.... A....R.... ........AQ .Q..GR.Q.G .R........ APWGTV.... .....THNC.  [90]
C1Cs-AB111946_BK Q.....FQ.. T.....S... .S........ ...G.R.... .....L..QQ .S..SGK... ..I..... T...S..... ......DNCT  [90]
C2-AF533983_BK   Q.....FQ.. T....KS... .S.......P ...SVR..L. .....L..QQ .S..RGKS.. .........P T..WS..... .....TDN..  [90]
C4-AB048704_BK   Q.....FQ.. E.....S... .S.......P ....VR..L. .....L..QQ .S..RGKS.. .........P T...F..... A.P...DN..  [90]
C-pC6_UK         --....FQ.. T.....S... .S........ ...G.R.... .....L..QQ .SM.RGKS.. .........P T...S..... ......DN..  [90]
C-N1_UK          --....FQ.. T.....S... .S.......P ....VR..L. .....L..QQ .SM.RGKS.. .........P T...S..... ......DN..  [90]
C-N19_UK         --....FQ.. Y.....S... .S.....G.R GE.D.R.... .....L..QQ .SM.SGK... ..I..... T..QS..... ......DYC.  [90]
C-N16_UK         -----.LQ.. T......... .S......PP ..ST...K.Q .....L..QQ .SM.SGK... ..I...G... T.QQS..... ......DNC.  [90]
C-N45_UK         --....FQM. K.....S... .S.......P G..G.R.... ........QQ .SM.SGK... ......G... T..QS..... ......DN.T  [90]
D1-AY161157_BK   ------WEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TS..P..... .....TTNL.  [90]
D2-X80925_BK     ------WEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA..P..... .....NTNF.  [90]
D3-X85254_BK     ------WEQD LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RC.Q.. .W....GF.P TA..P..M.. .....TTNF.  [90]
D4-AB048702_BK   ------WEQE LQ..A.S.HQ .S......PP ..SSL..KHQ .....L.SQQ .H..RR.Q.. .W.......P TA..P..... .....NANL.  [90]
D-3.33_SA        ---PYSWEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GS.P TA..R..... .---------  [90]
D-S2_SA          ---PYSWEQE LQ..A.S.HQ .S......PP ..SSL..RHS *.G..LHSPQ .H..RR.Q.. .WG...---- ---------- ----------  [90]
D-S7_SA          ---PYSWEQE LQ..A.S.HQ .S......PP ..SSL..KHR K.G..L.SQQ .H..GR.Q.. .W.....I.P TA..RS.... .---------  [90]
D-3.34_SA        ---PYSWEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..GR.Q.. .W....GI.P TA..P....- ----------  [90]
D-1.60_SA        ---P.FWEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA..P...-- ----------  [90]
D-3.35_SA        ---PYSWEQE LQ..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. ...FGTGI.P TAGWQP.--- ----------  [90]
D-S5_SA          ---PYSWLQE LQ.RA.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. ...FGSGI.P TA..L----- ----------  [90]
D-1.2_SA         ----YSWEQE LQQ.T.S.RQ .S......PP ..SSL..KHS KA...L.SQQ .H..RR.Q.. .W....GINP TS..P...-- ----------  [90]
D-1.55_SA        ---------- --..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA..P..... .....TTNL.  [90]
D-N11_UK         ---------- --..A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P T...P..... .....TTNL.  [90]
D-4.13_SA        ---------- --..ADS.HQ .S......PP ..SIL..KHR K....L.SQQ .H..GR.Q.. .W....GI.P TA..P..... .....TTNF.  [90]
D-73.2_SA        ---------- ---.ASS.LR SS......RP ..SLL..RHR K....L.SQQ .H..GR.Q.. .W....GNL- ---------. ....YTTNL.  [90]
D-4.27_SA        ---------- --..ADS.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA..P..... .....TTNF.  [90]
D-3.38_SA        ---------- ---.E.S.HH .S......PP ..SSL..KHS KP...L.SQQ .H..RR.Q.. .W....GF.P TA..P..... .....TTNL.  [90]
D-1.49_SA        ---------- -------.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA..P..... .....TTNL.  [90]
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D-N7_UK          ---------- ---.A.S.HQ .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .R....GI.P TA..P..... .....NTNL.  [90]
D-N12_UK         ---------- ---.VIS.HH .S......PP ..SSL..KHR K....L.SQQ .H..RR.Q.. .W....GI.P TA.P..... .....NTNL.  [90]
D1.2_SA          ---------- ------S.HQ .S......PP A.SSL..KHR K....L.SQL .H..RR.Q.. .W....GI.P T*..P..... .....TTNL.  [90]
D-7.64_SA        ---------- ---.ASS.HQ .S.R.P...P ...S...KYQ .....L.SQQ .H..RR.Q.. .W....GI.P TA..P..... .....TTNL.  [90]
D-7.10_SA        ---------- ---.A.S.LR PS......PP .--------- --...L.SQQ .H..RR.Q.. .W...TGI.P TA..P..... .....TTNP.  [90]
E-X75664_BK      ...--FLDGP S.M.E.Y.HH .S...F..PP ..SS...KHQ K......SQQ R..DG..Q.. ...L..G... ....P..... ...R.AKNI.  [90]
E-N2_UK          --.--FLDGP S.M.E.S.HH .S...F..PP ..SS...KHQ K..W...SQQ R..DR..Q.. ......W... ....P..... ...R.AKNI.  [90]
F-X69798adw4_BK  --.STSLNDT K...T.SL.A .S......PP ..SSL..KHR K....L.HKQ .Q..NGKQ.. ..RL.S...T ...WPA.... .STRCVNNL.  [90]
H-AY090454_BK    Q..STSLNGE KG..T.S..A .S......PP ..ST...K.Q .....L.HKQ .Q..NGKQ.. ..RLWS...T ...WPS.... ..T..SDNL.  [90]
G-AF160501_BK    Q..--FLDGP S.V.K...RQ .S.R.P...P ...S...KYQ .....L.SQ. ....RG.Q.. .W.LWT...P S...P..... .V...TNNF.  [90]
                aa262                                                                    Reverse Transcriptase (RT)1
A1-AY161140_BK    SGSGHIGHSA SSASSCLHQS AVRKAAYSHL STSKRQSSSG HAVEFHSFPP SSARSQSQGP VFSCWWLQFR NTQPCSNYCP SHLVNLL**EDW**  [90]
A2-S50225sAg-_BK  ...R..D..V NNS..... .......... ...E.K.... ......CL.. ...G.....S .S........ .SK...E..L ......R...  [90]
A-europeV00866_BK ......D..V NNS..... .......... .......... ......CLA. ...G.....S .S........ .SK...E..L ......R...  [90]
AafrcaAF297625_BK ......DY.. N.S.H..... .......... .......... ........A. .......... .......... ......Q..L ..........  [90]
A-N5_UK           ......D..V NNS..... .......... .......... ......CL.. ...G.....S .......... .SK...E..L ......R...  [90]
A-Cn5_UK          ......D..V NNS..... .......... .......... ......CL.. ...G.....S .......... .SK...E..L ......R...  [90]
A-N49_UK          ......D..V NNS..... .......... .......... ......CL.. ...G.....S .......... .SK...E..L ......R...  [90]
A-pW7_UK          ......D..V NNS..... .......... .......... ......CL.. ..TG..G..S .......... .SK...E..L ......R...  [90]
A-N14_UK          .C....D..V NNS..... .....Y.... .......... ......CL.. ...G....AS .......... .SK...E..L ..F...R...  [90]
A-N4_UK           ......D..V NNS......Y .........F .......... PL....CR.T ....H----S .......... .S*..YE.SL ....Y.R...  [90]
BaD00330ADW2_BK   ....PTHNC. ..S..... ........LI P...GH.... ....L.H... N.S..R.... .L........ .SE...E..L C.I...I...  [90]
Bj-AB073858_BK    .....THFC. ..S..... ........LI ....GH.... ....L.H... N.S......S .P........ .SK...E..L C.I...I...  [90]
B1-AB073852_BK    .....THIC. ..S..... .........I ....GHA... ....L.H... N.S......S .L........ .SK...E..L Y.I...I...  [90]
B2-AB073830_BK    .....THNC. ..S..... ........LI ....GH.... ....L.HV.. N.SG...... .L........ .SE...E..L C.I...I...  [90]
B3-D00331_BK      .S...THNC. N.S..... ....E...PV .....H.... N...L.HV.. N.S......S .L........ .SK...EH.L F.I...ID..  [90]
B4-AB100695_BK    .....THNC. ..T..... ........LI ....GH.... ....L.H..S N.S......S .LP....... .SE...E..L C.I.......  [90]
B-N9_UK           ....PTHNC. ..S..... ........LI ....GH.... .TE*LRQ... N.S....... .L....I... .SE.*.E..L C.I...I.Y.  [90]
B-pB10_UK         ....PTHNC. ..S..... ...T....LI ....GH.... .T..LRQ... N.S....... .L........ .SE...E..L C.I...ID..  [90]
B-N9a_UK          ---------- ---------- ---------- ---------- ---------- T.KGH-ILR. IQD..... .SE...E..L C.I...I...  [90]
B-N46_UK          .....THNC. ..S..... ........LI ....GH.... ....L.H... N.S....... .L........ .SE...E..L C.I...I...  [90]
B-N50a_UK         ....PTHNC. ..S..... ........LI ....GH.... ....L.H... N.S....... .L........ .SE...E..L C.I...I...  [90]
C1Cs-AB111946_BK  A.....DNCT .......... ....T.H... .......F.. N...LKHI.. .......E.. IP........ .SK...D..L ..I.......  [90]
C2-AF533983_BK    A....TDN.. .GT...F... ....T..... F......... ....L.NI.. ....P..E.. IL........ .SK...D..L T.I.......  [90]
C3-X75656_BK      ---------- ---------- ---------- ---------- ----L.NI.T .......E.. I......... .SK...D..L ..F.......  [90]
C4-AB048704_BK    A.P...DN.. ..S...I... ....TTH... T.AQ.H.P.. .......I.. ...G...K.S .......... .SK...E..L ...I..H...  [90]
C-N1_UK           ......DN.. ..T..... ...TT..... .......... ....L.NI.. ....P..E.. IL........ .SK...D..L T.I.......  [90]
C-N16_UK          ......DNC. .......... .S..T..... .......... ....LQHI.. ....F..E.. IL..C...C. .SK...D..L ..I......C  [90]
C-N19_UK          ......DYC. I......... ....T..... .......... ....LQHI.. ....F..E.. IL........ .SK...D..L ..I.......  [90]
C-N45_UK          ......DN.T .......... ....T..... .......... ....LQHI.. .......E.. IP........ .SK...D..L ..I.......  [90]
C-pC6_UK          ......DN.. ..T..... ...TTG...P .......... ....L.NI.. ....P..E.. IL........ .SK...D..L T.ID......  [90]
D1-AY161157_BK    .....TTNL. .KSA...Y.. P......PSV ..FEKH.... ....L.NL.. N......ER. ..P..... .SK...D..L ..I.......  [90]
D2-X80925_BK      .....NTNF. .KSA..IY.. P......PAV ..FEKH.... ....L.N... N.....GER. .LP..... .SK...D..L ..I.......  [90]
D3-X85254_BK      .....TTNF. .KSA...... P..Q...PAD ..FEKH.... ....L.NL.T H......ER. .SP..... .SK...DH.L .LI.......  [90]
D4-AB048702_BK    .....NANL. .KSA...Y.. T..T...PAV A..ENH.... ....L.NL.. N......ER. .SP..... .SK...D..L ..I.......  [90]
D-N11_UK          .....TTNL. .KSA...Y.. P......PSV ..FEKH.... ....L.NL.. N......ER. ..P..... .SK...D..L ..I.......  [90]
D-1.2_SA          .....TTNL. .KSA...Y.. P......PAV ..FEKH.... ....L.NL.. N......ER. ..P..... .SK...D..L ..I.......  [90]
D-1.55_SA         .....TTNL. .KSA...D.. P......PSV ..FEKH.... ....L.NL.. N......ER. ..P..... .SK...D..L ..I.......  [90]
D-4.13_SA         .....TTNF. .KSA..R... P......PSV ..FEKH.... ....H.N... N......ER. ..P..... .SK...D..L ..I.......  [90]
D-73_2_SA         ....YTTNL. .RYA...... L......PSV ..FEEY.... Y.L.LYY.LA N.....---- ---..... .SK...D..L ..I....Q..  [90]
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D-4.27_SA          .....TTNF. .KSA...Y.. P.......SV ..FE.HA... ....L.NL.. N......ER. ..P....... .SK...D..I ..I.......  [90]
D-7.10_SA          .....TTNP. .KYA...... ..G....TSV ...E.H.... ....L.NI.. N....R.ERS ..L....L.. YST..YD..L ..I...H...  [90]
D-1.49_SA          .....TTNL. .KSA...Y.. G......PCV ..FEKH.... ....L.NL.. N......ER. G....R.... .SKA..D..L ..I.......  [90]
D-7.64_SA          .....TTNL. NKSA...... P......PSV ..FEKH.... ....L.NL.. N......ER. ..P....... .SK...D..L ..I.......  [90]
D-3.38_SA          .....TTNL. .KSA...... P......PSV ..FEKH.... ....L.NL.. N......ER. ..P....... .SK...D..L ..I.......  [90]
D-N12_UK           .....NTNL. .KSA..IY.. P......PAV ..FEK.... ....L.NL.. N.....GER. .......... .SK...D..L ..I.......  [90]
D-N7_UK            .....NTNL. .KS...IC.. P......PAV ..FEKH.... ....L..L.. N.....GER. .......... .SK...D..L ..I.......  [90]
E-X75664_BK        ...R.AKNI. .RPA...... .......PNH ..FE.H.... ....L.NISS ...G...KR. .......... .SE...D..L T.........  [90]
E-N2_UK            ...R.AKNI. .RSA...... .......PNH ..FE.H.... ..M...NI.. ...G...KR. .S........ .SK...D..L T.........  [90]
E-pE2a_UK          ---------- ---------- ---------- ---------- -.M...NI.. ...G...KR. .S........ .SK...D..L T.........  [90]
F-X69798adw4_BK    .STRCVNNL. .RSA..F... ...EK.NPS. .....HT.T. N...LNPV.. ..VG..GK.S .LP....... D.E...D..L ..II......  [90]
H-AY090454_BK      ..T..SDNL. TRST.RF... E...ETNPS. ....GHT.T. ....LNTV.. .TVG.E.K.S .S........ ..E...D..L ..II......  [90]
G-AF160501_BK      .V...TNNF. .RSA...... S..E...... ..T....... ....LY.I.. ..TK...... .......... DSE...D..L .......Q..  [90]
                RT 6
A1-AY161140_BK     CAEHGEHHIR IPRTPARVTG GVFLVDKNPH NAEYRLVVD FSQTFSRGITR VSWPKFAVPN LQSLTNLLCS NLYWLSLDVS AAFYHIPLHP  [90]
A2-S50225HBsAg-_BK .D........ .......... .......... ....S..... .......V.. .P........ ........S. ..S....... ..........  [90]
A-europeV00866_UK  .DD....... .......... .......... ....S..... .......... .......... ........S. ..S....... ..........  [90]
A-afrcaAF297625_BK .V........ .......... .......... ....S..... .......L.. .......... ........S. ..S....... ..........  [90]
A-pW7_UK           .D........ .......... .......... ....S..... .......... .......... ........S. ..S....... ..........  [90]
A-N49_UK           .D........ .......... .......... ....S..... .......... .......... ........S. ..S...---- ----------  [90]
A-N14_UK           .D......M. T...A..... .......... ....S..... .......... .C....G.A. ..L.N...S. ..A....... ........P.  [90]
A-gCn5_UK          .D........ T......... .......... ....S..... ...------- ---------- ---------- ---------- ----------  [90]
A-N4_UK            .T........ T...A.I... .......... ....S..... .......S.. L.......-- ---------- ---------- ----------  [90]
A-N5_UK            .D........ T......... .......... ....S..... .......... .......... ........S. ..S....... .....L....  [90]
AD-recAF418674_BK  .......... ..W....... .......... .......... .......... .......... .......... .......... ..........  [90]
A-gD-clon_UK       .DGD...... .......... .......... ....S..... .........S .......... ........S. ..S....... ..........  [90]
BaD00330ADW2_BK    .T.....R.. T......... .......... ..T.S..... .......N.. .......... ........S. ..S....... .....L....  [90]
Bj-AB073858_BK     ...D...R.. T......... .......... ..T.S..... .......N.. .......... ........S. ..S....... ..........  [90]
B1-AB073852        .......R.. T......... .......... ....S..... .......T.. .......... ........S. ..S....... .....L....  [90]
B2-AB073830_BK     .T.....R.. T......... ....I..... ..T.S..... .......N.. .......... ........S. ..S....... .....L....  [90]
B3-D00331_BK       .......R.. T......... .......... ..S.S..... .......N.. .......... ........S. D.S....... .....L....  [90]
B4-AB100695_BK     .T.....R.. T......... .......... ..T.S..... .......N.. .......... ........S. ..S....... .....L....  [90]
B-N9_UK            .T.....L.. T......... .......... ..T*S..... ......EN.. .......... .......MT- ---------- ----------  [90]
B-N46_UK           .......R.. T......... .......... ..T.S..... .......N.. .......... .......VS. ..S....... .....L....  [90]
B-pB10_UK          .T.....L.. T......... .......... ..T.S...-- ---------- ---------- ---------- ---------- ----------  [90]
B-N50_UK           .T.....R.. T......... .......... ..T.S..... .......N.. .......... ........S. ..S....... .---------  [90]
C1Cs-AB111946_BK   .T.Y...... ...S...... .......... ..T.S..... .....G.SPH .......... ........S. ..S....... ..........  [90]
C2-AF533983_BK     .P.....N.. .......... .......... S.T.S..A.. .......S.H .......... ........S. ..S....... ..........  [90]
C3-X75656_BK       .N.....N.. .......... .......... ..T.S..G.. .......S.K .......... ........S. ..S....... .....L....  [90]
C4Caus-AB048704_BK .I.....N.. .......... .......... ..S.S..... .......SS. .Y........ ........S. ..S....... .....L....  [90]
C-pC6n             .T.....N.M .......... ....------ ---------- ---------- ---------- ---------- ---------- ----------  [90]
C-N1_UK            .T.....N.. .......... .......... ..T.S..... .......S.H .......... ........S. ..S......L ...N......  [90]
C-w-clon_UK        .T.....N.. .......... .......... ..T.S..... .......S.H .......... ........S. ..S....... ..........  [90]
C-N16_UK           .T*Y...... .......... .IL....... ....F..... .C.....S.H .....L.... ........S. ..A....... ..INDLT...  [90]
C-N3_UK            .T....KN.. .A.SR....D RILI.....N ..E.S..... .......S-- ---------- ---------- ---------- ----------  [90]
C-N19_UK           .T.Y...... .......... .......... ..T.S..... LC..F..SNH .....L.... ........S. .CA....... ..I.DV.HP-  [90]
C-N45_UK           .T.Y...... .......... .......... ..T.SG.... .......S.H ......G... ....S...S. ..S....... .R...L....  [90]
D1-AY161157        .......... .......... .......... ....S..... .......NY. .........S ........S. ..S....... .....L....  [90]
D2-X80925_BK       .......... L......... .......... ....S..... .......NH. .......... ........S. ..S....... ..........  [90]
D3-X85254_BK       .......... .....S.... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
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D4-AB048702_BK     .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-1.2_SA           .......... .V........ ..C....DS. ....S..... .......NY. ........S. .*......S. .--------- ----------  [90]
D-1.55_SA          .......... .......... .......... ....S..... .......NY. .......... ........S. ..A....... .....L....  [90]
D-1.60             .......... .......... .......... ....S..... .......NY. .......... ........S. ..S.I..... .....L..--  [90]
D-2.23a_SA         .......... .......... .......... ..S.S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-2.31a_SA         .......... T....S.... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-S7a_SA           .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-3.33a_SA         .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-S6a_SA           .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-pD5_SA           .......... ....H..... .......... ....S..... .......NY. .......... ........S. ..S.I..... ..........  [90]
D-73_2_SA          ...Y..Y... ....T..... .--------- ---------- ---------- ---------- ---------- ---------- ----------  [90]
D-1.49_SA          .......... .......... ..C.D....L ---------- ---------- ---------- ---------- ---------- ----------  [90]
D-N7_UK            .......... ....S..... .......... ....S..... ...L...S.. .Y.....--- ---------- ---------- ----------  [90]
D-3.38_SA          .......... .......... .......... ....S..... .......NY. .......... ........S. ..S.I..... ..........  [90]
D-7.64_SA          .......... .......... .......... ....S..... .......NY. .......... ........S. ..-------- ----------  [90]
D-7.46R_SA         .......... ....R..... .........Q ....S..... ....C.RTAG .......... ........S. ..S....... .....L....  [90]
D-4.27_SA          .......N.. .......... .......... ....S..... ..-------- ---------- ---------- ---------- ----------  [90]
D-3.34a_SA         .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... ..........  [90]
D-N11_UK           .......... .......... .......... ....S..... .......NY. .......... ........S. ..S....... .....L....  [90]
D-3.35a_SA         .......... .......... .......... ....S..... .......NY. .......... ........S. ..T....... .....L....  [90]
D-N12_UK           .......... .......... .......... ....S..... .......S.. .......... ........S. ..S....... ..L.--....  [90]
D-4.13_SA          .......... .......... .......... ....S..--- ---------- ---------- ---------- ---------- ----------  [90]
D-7.10_SA          ..A..I.T.. L.Q....L.L A.Y...---- ---------- ---------- ---------- ---------- ---------- ----------  [90]
E-X75664_BK        .T...K.... .......... .......... ....S..... .......SS. .......... ........S. ..S....... .....L....  [90]
E-2.25a_SA         .T...K.... .......... .......... ....S..... .......SS. .......... ........S. ..S....... .....L....  [90]
E-N2_UK            .T...R.... .......... .......... ....S..... .......SS. ---------- ---------- ---------- ----------  [90]
pEn2               .T...R.... .......... .......... ....S..... .......SS. .......... ........S. ..S....... .....L....  [90]
F-X69798adw4_BK    .Y...Q.Y.. T......... .......... ..T.S..... .......T.. .......... ........S. ..S....... .....L....  [90]
H-AY090454_BK      .Y........ T....S.... .......... ..T.S..... .......T.. .......... ........S. ..S....... .....L....  [90]
G-AF160501_BK      .T........ .......... .......... ....S..... .......SA. .......... ........S. ..S....... ..........  [90]

RT 97
A1-AY161140_BK     AMPHLLIGSS GLSRYVACLS SNSRINNNQY GTLQNLHDSC SRQLYVSLML LYKTYGWKSH LYSHPIILGF RKIPMGVGLS PFLLAQFTSAI [91]
A2-S50225HBsAg-_BK .......... .......R.. Y......... ..M....... ....F..... ........L. ......V... ......A... ..........  [91]
A-europV00866adw_BK .......... .......R.. .......... ..M....... ....*..... ........L. ......V... ..........  [91]
A-africAF297625_BK .......... .......R.. .....H.... .......... .......... ........L. .......... ..........  [91]
A-pW7_UK           .......... .......R.. .......... ..M....... .......... ........L. ......V... ..........  [91]
A-Dclon_UK         .......... .......R.. .......... ..M....... .......... ........L. ......V... .E--------- ----------  [91]
A-N5_UK            .......... .I.....R.A --F.Y*HAA- NCM....... ---------- ---------- ---------- ----------  [91]
A-N66-UK           ---------. R......R.. .......... ..M....... .......... ........L. ......V... ..........  [91]
A-N60-UK           ---------. .......R.. .........H ..M....... .......... ........L. ......V... ..........  [91]
A-N75-UK           ---------- ---------- -----..... ..M....... .......... ........L. ......V... ..........  [91]
Ba-D00330adw2_BK   ......V... .......R.. .....I...H R.M....N.. ..N....... ........L. .......... ..........  [91]
Bj-AB073858_BK     .......... .......R.. .....I.H.H ..M.D..N.. ..N.F..... ......R.L. .......... ..........  [91]
B1-AB073852_BK     ......V... .......R.. .....I.H.H ..M.D..N.. ..N....... ......R.L. .......... ..........  [91]
B2-AB073830_BK     ......V... .......R.. .....I...H R.M....NY. ..N....... ......R.L. .......... ..........  [91]
B3-D00331_BK       ......V... .......R.. .....I.H.H R.M....... ..N....... ......R.L. .......... ..........  [91]
B4-AB100695_BK     ......V... .......R.. .....I.H.H R.M....... ..N....... ......R.L. .......... ..........  [91]
B-N47-UK           ---------- ---------- ---..I...H R.M....... ..N....... ......R.L. .......... ..........  [91]
B-N69_UK           --HPH.V... -......R.. .....I...H R.M....... ..N....... ......R.L. .......... ..........  [91]
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B-N70f_UK           ---------- ---------- -----I...H R.M....N.. ..N.....  ......R.L. .......... .......... ..........  [91]
B-N71-UK            -....PS... E......R.. .....I...H R.M....N.. ..N...Y...  ......R.L. .......... .......... ..........  [91]
B-N72f_UK           ---------- ---------- --------.H R.M....N.. ..N.....  ......R.L. .......... .......... ..........  [91]
B-N74r-UK           -....PS... E......R.. .....I...H R.M....N.. ..N...Y...  ......R.L. .......... .......... ..........  [91]
B-N73-UK            ---------- ---------- --------.H R.M.....  ..N.....  ......R.L. .......... .......... .........V  [91]
B-N72-UK            -.....V... .......R.. .....I...H R.M....N.. ..N.....  ......R.L. .......... .......... ..........  [91]
AB-N68-UK           -.....V... .......R.. .....I...H R.M.....  ..........  ......R.L. ......V... .......... ..........  [91]
C1-AF411412_BK      ......V... ..P....R.. .T..NIDY.H ..M.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C1Cs-AB111946_BK    ......V... ..P....R.. .T..NI.Y.H ..M.D...T.  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
C2-AF533983_BK      ......V... ..P....R.. .T..NI.Y.H ..M.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C3-X75656_BK        ......V... ..P....R.. .T..NI.Y.H ..M.D.....  ..N.....  ....F.R.L. .......... .......... ..........  [91]
C4-AB048704_BK      ......V... .......R.. .T....DH.H ........H.  ..N.....  ....F.R.L. .......... .......... ..........  [91]
C4aus-AB048704ayw_B ......V... .......R.. .T....DH.H ........H.  ..N.....  ....F.R.L. .......... .......... ..........  [91]
C-N1-UK             -.....V... ..P....R.. .T..NI.Y.H ..M.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C-N83-UK            -.....V... ..P....R.. .T..NI.H.H .AM.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C-N80-UK            -.....V... ..P....R.. .T...V.Y.H ..M.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C-wclon_UK          ......V... ..P....R.. .T..NI.Y.H ..M.D.....  ..N.....L.  ....F.R.L. .......... .......... ..........  [91]
C-N1_UK             ......V... ..P....R.A --F.N*L.D- SCM.......  ----------  ----------  ----------  ----------  ----------  [91]
C-N16_UK            ......A... .......R.. .TC.NIY.G- .C--------  ----------  ----------  ----------  ----------  ----------  [91]
D1-AY161157_BK      ......V... .......R.. .....F.Y.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D2-X80925_BK        ......V... .......R.. .....L.H.R ..M.....Y.  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D3-X85254_BK        ......V... .......R.. .....L.H.H ..MPH.....  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D4-AB048702_BK      ......V... .......R.. .....I.H.H ..........  ..N.....L.  ....F...L. .......... .......... ..........  [91]
D-1.60a_SA          ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-2.23a_SA          ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-S7a_SA            ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. ..........  ..L.......  .V........  [91]
D-3.33a_SA          ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-S6a_SA            ......V... .......R.. .....V.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-3.35a_SA          ......V... .......R.. .S...F.H.H ..........  ..N..E..L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-3.34a_SA          ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......M.. .......... ..........  [91]
D-N36_UK            ---------- ---------- ------...H .NM.......  ..N.....L.  ..Q.F.R.L. .......... .......... ...M......  [91]
D-N38f_UK           ---------- ---------- ------...H .NM.......  ..N.....L.  ..Q.F.R.L. .......... .......... ...M......  [91]
D-N82_UK            ---------- ---....R.. .....F.Y.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-N78_UK            ---------- ---------- -----F.H.R ..M.....Y.  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-N79_UK            -.....D... .......R.. .....F.H.H ..M.......  ..N..E..L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-N76_UK            -.....D... .......R.. .T...F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-N77_UK            -.....V... .......R.. .T...F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-3.38_SA           ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-pD5_SA            ......V... .......R.. .....F.H.H ..M.......  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
D-N12_UK            ......V..P ......GR.A .....--.-- ----------  ----------  ----------  ----------  ----------  ----------  [91]
D-N11_UK            ......V... .......R.. .....LHH-H ..M.D.----  ----------  ----------  ----------  ----------  ----------  [91]
D-7.46R_SA          ......V.Y. .......R.. .....V.H*H .SM......Y  ..H......-  ----------  ----------  ----------  ----------  [91]
E-X75664_BK         ......V... .......R.. .....I.H.H ...P......  ..N.......  .F..F.R.L. .......M.. .......... ..........  [91]
D-2.31a_SA          ......V... .......R.. .....L.H.H ..MP....Y.  ..N.....L.  ..*.F.R.L. .......... .......... ..........  [91]
E-2.25a_SA          ......V... .......R.. .....I.H.. ...P......  ..N.......  .F..F.R.L. .......M.. .......... ..........  [91]
E-N81r-UK           ---------- ---....R.. .....I.... R.........  ..N.......  ......R.L. .......M.. .......... ..........  [91]
E-En2_UK            ......V... .......R.. .....I.H.. ...P......  ..N.......  .F..F.R.L. .......M.. .......... ..........  [91]
F-X69798adw4_BK     ......V... .......R.. .T...HDH.H .......N..  T.N.....L.  .FQ.L.R.L. .......... .......... ..........  [91]
H-AY090454_BK       ......V... .......RV. .T...Y.H.H .S.....H..  ..N.....L.  ..Q.F.R.L. .......... .......... ..........  [91]
G-AF160501_BK       ......V... .......R.. .D...LDH..  ..........  ..........  ....F.R.L. .......... .......... ..........  [91]
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RT 188204YMDD
A1-AY161140          CSVVRRAFPH CLAFSYMDDV VLGAKSVQHL ESLYTAVTNF LLSLGIHLNP NKTKRWGYSL NFMGYVIGSR GHKPQDHIVQ NNKHCFRKLP ANRPIDWKV [99]
A2-S50225HBsAg-_BK   .......... .......... .........R .A........ .......... .......... .....R...W .TL....... KI........ V......... [99]
A-europV00866adw_BK  .......... .......... .........R .......... .......... .......... .....I...W .TL....... KI........ V......... [99]
A-afric-AF297625_BK  .......... .......... .........R .......... .......... .......... .........W .TL.....I. KI........ V......... [99]
A-N60-UK             .......... .......... .........R .......... .......... .......... .....I...W .TL....... KI........ V.......-- [99]
A-N66-UK             .......... .........I .....T.H.R .......... .......... .......... .....I...W .TL....... KI........ V..A.S.-- [99]
A-N75-UK             .......... .......... .........R .......... .......... .......... .........W .TL....--- ---------- --------- [99]
A-pW7_UK             .......... .........M .........R .......... .--------- ---------- ---------- --------- ---------- --------- [99]
Ba-D00330adw2_BK     .......... .........M .......... ....A..... .......... H......... .........W .TL..E.... KI.MW..... V......... [99]
Bj-AB073858_BK       .......... .......... .......... .......... .......... H......... .........W .TL..E..T. KI.L...... V......... [99]
B1-AB073852_BK       .......... .......... .......... ....A..... .......... H......... .........W .TL..E.... KI.Q...... V......... [99]
B2-AB073830_BK       .......... .......... .......... .......... .......... H......... .........CW .TL..E.... KI.M...... V......... [99]
B3-D00331_BK         .......... .......... .......... ....A..... .......... Q......... .........W .TL..E...L KI.Q...... V......... [99]
B4-AB100695_BK       .......... .......... .......... ....A..... .......... H......... .........W .SL..E..G. KI.L...... V......... [99]
B-N71-UK             .......... .......... .......... ....A..... .......... H......... .........W .TL..E.... KI.M...... V......... [99]
B-N72f_UK            .......... .......... .......... ....A..... .......... H......... .........W .TL..E.S.. KI.------- ---------- [99]
B-N70f_UK            .......... .......... .......... ....A..... .......... H......... .........W .TL..E.... KI.M....-- ---------- [99]
B-N69_UK             .......... .......... .......... ..I.A...H. .......... H......... .........W .TL..E.... KI.T...... V.-..VR-- [99]
B-N74r-UK            .......... .......... .......... ....A..... .......... H......... .........W .TL..E---- ---------- ---------- [99]
B-N47-UK             .......... .......... .......... ....A..... .......... H......... .........W .TL..E---- ---------- ---------- [99]
B-N73-UK             .......... .......... .......... ....A..... .......... H......... .........W ---------- ---------- ---------- [99]
AB-N68-UK            .......... .......... .........R ....A..... .......... H......... .........W .TL....... KI........ V......... [99]
C-X01587adr4_BK      .......... .......... .......... ...F.SI... .......... .......... ........CW .TL..E...L KI.Q...... ---------- [99]
C1Cs-AB111946_BK     .......... .......... .......... ...F...... .....V.... T......... .........W .TL..E...H KI.Q...... L......... [99]
C2Ce-AF533983_BK     .......... .......... .......... ...F.SI... .......... .......... .........W .TL..E...L KL.Q...... V......... [99]
C3-X75656_BK         .......... .......... .......... .....SI... .......... .......... .........W .TL..E...L KI.Q...... V......... [99]
C4aus-AB048704ayw_BK.......... .........L .......... ...F...... .M........ H......... .........W .SL..E...H KL.........  V......... [99]
C-N1-UK              .......... .......... .......... ...F.SI... .......... .......... ........CW .TL..E...L KI.Q.......  V.....LE- [99]
C-N80-UK             .......... .......... .......... ...F.SI... .......... .......... .........W .TL..E...L KI.Q..A.VV  LSNCT-*-- [99]
C-N83-UK             .......... .......... .......... ...F.SI... .......... .......... H........W .SL..E.... KL.Q.......  V.......-- [99]
D1-AY161157_BK       .......... .......... .....N.... ...F...... .......... .......... H........CY .SL.....I. KI.E......  V......... [99]
D2-X80925_BK         .......... ....T..... .......... ...F...... .......... .......... ........CY .SL.....I. KI.E......  V......... [99]
D3-X85254_BK         .......... .......... .......... ...F...... .......... .......... .....I..CH .SL..E.... KI.E......  I......... [99]
D4-AB048702_BK       .......... .......... .......... ...F...... .......... .......... .........W .SL.....IH KI.E......  VH........ [99]
D-N36_UK             .......... ......V... .......... ...F...... .F........ .......... H........Y .SL..----- ----------  --------- [99]
D-N38f_UK            .......... ......V... .......... ...F...... .F........ .......... H........Y .SL..----- ----------  --------- [99]
D-N76_UK             .......... .......... .....T.... ...F...... .........T .......... H.......CY .SL.....I. KI.E......  V......... [99]
D-N79_UK             .......... .......... .......... .......... .......... .......... H.......CY .SL..E..I. KI.E......  V..T....- [99]
D-X02496ayw_BK       .......... .......... .......... ...F...... .......... .......... H........CY .S-------- ----------  --------- [99]
D-N78_UK             .......... .......... .......... ...F...... .......... .......... ........CY .SFA.----- ----------  --------- [99]
D-N77_UK             .......... .......... .......... ...F...... .......... .......... H.......CY .SL.....I. KI.E......  V.......- [99]
D-N82_UK             .......... .......... .......... ...F...... .......... .......... H.......CM .SL------- ----------  --------- [99]
D-3.38_SA            .......... ......I... .......... ...F...... .......... .......--- ---------- ---------- ----------  --------- [99]
D-pD5_SA             .......... ......I... .......... ...F...... .--------- ---------- ---------- ---------- ----------  --------- [99]
E-X75664_BK          .......... .......... .......R.. .....S.... .......... .......... .........W .SL..E..I. KI....G...  V......... [99]
E-N81r-UK            .......... .......... .......... .......S.. .V........ .......... .........W .SL...---- ----------  --------- [99]
E-En2_UK             .......... .......... .......... .....S.... .......... .......... .........W .SL..G..RL KI.D------  --------- [99]
F-X69798adw4_BK      .......... .........L .......... .......... ...V.....T S......... H........W .SL......H KI.E......  V......... [99]
H-AY090454_BK        .......... .........L .......... .......... ...V.....T A...W..... H....I...W .TL..E.... KI.D......  V......... [99]
G-AF160501_BK        .......... .......... .......... .......... .......... .......... .........W .TL..E..T. KI.Q......  V......... [99]
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Appendix B
Basal core promoter (BCP)/X-gene
HBx protein (HbxAg)

Alignment of last 77 amino acids sequences of HBx protein which shows genotype/subtype identification of 42 sera using Clustal X software. Amino acid residues presented in single-letter code. Genotype control stains from Gene Bank are included. Sequences name starting with capital letter indicating the genotype and suffix SA for Saudi strains and UK for UK strains.

```
Amino acid          79         90        100        110        120        130        140        150     End
AeurpeV00866_GB  MATTVNAHQI LPKVLHKRTL GLPAMSTTDL EAYFKDCVFK DWEELGEEIR LMIFVLGGCR HKLVCAPAPC NFFTSA* [ 77]
AaAF297625_GB    .E........ .......... .......... .......... .......... .KV....... ....F..SS. ....... [ 77]
A1-AY161140_GB   .E.......V ..R....... ..S....... .......L.R .......... .KV....... .......... ....... [ 77]
A-N13_UK         ---------- .......... .......... .......... ........T. .MI....... .......... ....... [ 77]
A-3.47_SA        -----..... .......... .......... .......... .......... .MT....... .......... ....... [ 77]
A-N44_UK         ---------- .......... .......... .......... .......... .KV....... .......... ....... [ 77]
A2-S50225_GB     .E.......S .......... .......... .......... .......... .MI....... .......... ....... [ 77]
A-N33_UK         ---------- .......... .......... .......... .......... .KI....... .......... ....... [ 77]
A-N41_UK         --------.. .......... .......... .......... .......... .MV....... .......... ....... [ 77]
A-Z35717_GB      .......... .......... .......... .......... .......... .MI....... .......... ....... [ 77]
A-N4_UK          -------... .....Y.... .......... .......... .......... .KIY...... .......V.. ....... [ 77]
A-N15_UK         --------.. .....Y.... .......... .......... .......... .MIY...... .......... ....... [ 77]
A-N49_UK         ---------- .......... .......... .......... .......... .KI......M ...A...P.. ....... [ 77]
A-N14_UK         ---------- .......... ......M... .......... .......... .MI....... .......SS. ....... [ 77]
B1-AB073852_GB   .E......RN .......... ..S....... .........T E...M...T. .MI....... .....S.... ....... [ 77]
B2-AB073830_GB   .E......WN .......... ..S....... .........T E.......... .KV....... .....S.... ....... [ 77]
Ba-D00330adw2_GB .E......GN .......... ..S....... .........N E.......V. .KV....... .....S.... ....... [ 77]
Bj-AB073858_GB   .E......WN .....Y.... ..S....... .........N E.......... .KIY...... .....S.T.. ....... [ 77]
B3-D00331_GB     .E......RN .......... ..S....... ..H......T E.......... .KV....... .....S.... ....... [ 77]
B4-AB100695_GB   .E......R. .S.D.RTM.. E.SGL..S.. ......R..T E.......... .MI......T .T.LFS.S.. ..LN... [ 77]
B-N17_UK         --------GN .......... ..S....... .........T E.......V. .MI....... ....RS.... ....... [ 77]
B-N9_UK          ---------- .......... ..S....... .........T E.......V. .MI....... ....RS.... ....... [ 77]
B-N39_UK         ---------- ...A...... ..S....... .........N E.......V. .KV....... .....S.... ....... [ 77]
B-N50_UK         ---------- .......... ..S....... .........N E.......V. .KV....... .....S.... ....... [ 77]
B-N47_UK         ---------- .......... ..S....... .........T E.......V. .KV....... .....S.... ....... [ 77]
B-N46_UK         ---------- .......... ..S....... .........T E.......M. .KV....... .....S.... ....... [ 77]
B-N18-UK         .E....T.GN .......... ..S....... .........N E.......V. .KV....... .....S.... ....... [ 77]
C-7.48_SA        -----...SF .......... ..A.....E. .......L.. .......... .QV....... .....S.... ....... [ 77]
C-N19_UK         ---------- .......... ..S....... .......... .......... .KV....... .....S.... ....... [ 77]
C-N3_UK          ----...R.V .......... ..S....... .......... .........T .KV....... .....S.... ....... [ 77]
C-N45_UK         ---------- .......... ..S....... .......... .......... .KV....... .....S.... ....... [ 77]
C-X01587adr4_GB  .E.......V .......... ..S....... .......L.. .......... .KV....... .....S.... ....... [ 77]
C1Cs-AB111946_GB .E.....R.V .......... ..S....... .......... ........T. .MI....... .....S.... ....... [ 77]
C2Ce-AF533983_GB .E.......V .......... ..S....... .......L.. .......... .MI....... .....S.... ....... [ 77]
C3-X75656_GB     .E......MV .......... ..S....... .......... .......... .KV....... .......... ....... [ 77]
C4ausAB048704_GB .E....T.M. .......... .......I.. .......L.. .......... .KV....... .....S.... ....... [ 77]
D-X65257_GB      .E........ .......... ..S....... .......L.. .......... .KV....... .......... ....... [ 77]
D-X02496_GB      .E...K...F .......... ..SV...... .......L.. .......... .KV....... .......... ....... [ 77]
```

```
D1-AY161157_GB   .E.......F .......... ..SV...... .......L.R .......... .KV....... .......... ....... [ 77]
D2-X80925_GB     .E......H. .........A. .......... .......... .......... .KV....... .......... ....... [ 77]
D3-X85254_GB     .E........ .......... ..S....... .......L.. .........W .KV....... .......... D...... [ 77]
D4-AB048702_GB   .E.......F .......... ..S....... .......L.. .......... .KV....... .......... ....... [ 77]
D-7.80_SA        ---------F .......... ..S....... ....T..L.. ........T. .MI....... .......... ....... [ 77]
D-3.34_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-N22_UK         ---------- ---------- ---------- --------.. .......... .KV....... .......... ....... [ 77]
D-3.12_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.7_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.205_SA       ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-2.11_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -...... [ 77]
D-3.3_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.1_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.43_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-4.27_SA        ---------- -......... ..SV...... .......L.. .......... .RL....... .......... ....... [ 77]
D-109_SA         ---------- -......... ..SV...... .......L.. .......... .RL....... .......... ....... [ 77]
D-1.46_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -...... [ 77]
D-3.18_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----... [ 77]
D-3.8_SA         ---------- ---------- ---------- ---------- ---------- ---------- -------... ....... [ 77]
D-4.7_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-4.23_SA        ---------C .......... ..SV...... .......L.. .......... .KM....... .......V.. ....... [ 77]
D-4.13_SA        --------.F .......... ..SV...... .......L.. E......... .KL....... .....V.... ....... [ 77]
D-4.3_SA         --------HF ......N... ..SG...... .......L.. .......... .KV....... .......... ....... [ 77]
D-4-22_SA        ---------- ---------- ----...... .......L.. .......... .KI....... .......... ....... [ 77]
D-4.2_SA         -----...HC .......... ..CV..M... .......L.. ........N. .MVY...... .......... ....... [ 77]
D-1.50_SA        ---------- .....Y.... ..SV...... .......L.. .......... .MI....... .......... ....... [ 77]
D-4.15_SA        ---------F .....Y.... ..SV...... .......L.. .......... .KL....... .......... ....... [ 77]
D-4.21_SA        ---------- .........I ..SV...... .......L.. .......... .KV....... .......... ....... [ 77]
D-7.84_SA        -------DHF .....LN... ..SV...... .......L.. .......... .KV....... .......... ....... [ 77]
D-3.26_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.36_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.55_SA        ---------- ---------- ---------- ---------- ---------- ---------- ------.... ....... [ 77]
DA-1.56_SA       ---------- ---------- ---------- ---------- ---------- ---------- --------.. ....... [ 77]
D-2.12_SA        ------...F .......... ..SV...... .......L.. ........-- ---....... .......... ....... [ 77]
D-3.32_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.5_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.29_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----... [ 77]
D-1.106_SA       ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-3.9_SA         ---------- ---------- ---------- ---------- ---------- ---------- --------S. ....... [ 77]
D-3.39_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.60_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.49_SA        ---------- ---------- ---------- ---------- ---------- ---------- --------.. ....... [ 77]
D-LEIC_UK        ---------- -......... ..S....... .......L.. ........L. .KV....... .......... ....... [ 77]
D-4-1_SA         ---------- .......... ..S....... .......L.. ........L. .KV....... .......... ....... [ 77]
D-p15R_UK        .E........ ...I...... ..S....... .......L.. ........L. .KV....... .......... ....... [ 77]
D1.48_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------- [ 77]
D-1.78_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------. [ 77]
D-3.19_SA        ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.2_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
D-1.4_SA         ---------- ---------- ---------- ---------- ---------- ---------- ---------. ....... [ 77]
```

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D-N12_UK          -----D...F .......... ..SV.P.... .......L.. .......... .KV.------ ---------- ------- [ 77]
D-N6_UK           ------..HF .......... ..SV...... .......L.. .......... .KV..S.... .......... ....... [ 77]
D-118_SA          --------.F .......... ..SV...... .......L.. ........N. .MVY...... .......... ....... [ 77]
D-3.35_SA         --------.F .......... ..SV...... .......L.. ........N. .MVY...... .......... ....... [ 77]
D-7.59_SA         -------DSC .....L.... ..SV...... .......L.. .......... .KVY...... ........T. ...P... [ 77]
D-7.86_SA         -------EPF .......... ..SV...... .......L.R ........N. .MI....... .......... ....... [ 77]
D-7.40_SA         -------DP. .....LM..S ..SV...... .......L.. .......... .KV....... .......... ....... [ 77]
D-7.5_SA          --------PF .......... ..SV...... .......L.. ........T. .MI....... .......... ....... [ 77]
D-4.19_SA         .E.......F .......... ..SV...... .......L.. ........N. .MVY.F.... .......... ....... [ 77]
D-3.6_SA          ---------- .......... ..SV...... .......L.. ........N. .MVY...... .......... ....... [ 77]
E-X75664_GB       .E........ .......... ..S....... .......L.. .......... .KV....... .......... ....... [ 77]
E-X75657_GB       .E........ .......... ..S....... .......L.. ....S..... .KV....... .......... ....... [ 77]
E-2.25_SA         ---------- .....Y.... ..S....... .......L.. ....S..... .MIY...... .......... ....... [ 77]
E-4.10_SA         ---------. .....Y.... ..S....... .......L.. ....S..... .MI....... .......... ....... [ 77]
F-X75658_GB       .E.....PWS ..T......I ..SGR.M.WI .E.I...... .......... .KV....... .......... ....... [ 77]
G-AF160501_GB     .E.AM.TSHH ..RQ.Y.W.. ..FV....GV .K.......A E.....N.S. .MT....... .......... ....... [ 77]
H-AY090457_GB     --------.S ..TT...... ..SPR...WI .E.I...... ....S...L. .KV....... .....S.... ....... [ 77]

1start HBeAg (3rd ORF)          1Start HBcAg
AeurpeV00866_GB   MQLFHLCLII SCTCPTVQAS KLCLGWLWGM DIDPYKEFGA TVELLSFLPS DFFPSVRDLL DTASALYR [136]
AaAF297625_GB     .......... .......... .......... .......... .......... .......... ........ [136]
A1-AY161140_GB    .......... ..S....... .......... ....H..... .......... .....A.... ........ [136]
A2-S50225_GB      .......... ..S.L..... .......*.. .......... S........H .......... ........ [136]
ADrecAF297621_GB  .......... ..S....... .......*.. .........S .......... .......... ........ [136]
A-N13_UK          .......... .......... .......... .......... .A........ ......K... ......F. [136]
A-3.47_SA         .......... .......... .......... .......... .......... .......... ........ [136]
A-N44_UK          .......... .......... .......... .......... .......... .......... ........ [136]
A-N33_UK          .*........ .......... .......... .......... S........H ..Y....... ........ [136]
A-N41_UK          .......... ..S....... .......... ...L...... S........H .....I.... ......H. [136]
A-N4_UK           .......... .......... .......... .......... S........ .......... ........ [136]
A-N15_UK          .......... .......... .......*.. .......... .......... .......... ........ [136]
A-N49_UK          .......... .......... N...V..... ---------- ---------- ---------- -------- [136]
A-N14_UK          .......... ......F... .......... .......... .......... .......... ........ [136]
Ba-D00330adw2_GB  .......... ..S....... .......... .......... S........ .......... ........ [136]
Bj-AB073858_GB    ........V. ..S....... .......*.. .......... .A.......A ..Y....... ...T.... [136]
B1-AB073852_GB    ........V. ..S....... .......*D. .......... ....I..... .......... ...T.... [136]
B2-AB073830_GB    .......... ..S....... .......*.. ...T...... S........ .....I.... ........ [136]
B3-D00331_GB      .......... ..S....... .......... .......... S........ .....I.... ........ [136]
B4-AB100695_GB    ....EF.... ..S....... .......... .......... S........ .......E.. ........ [136]
B-N17_UK          .......... ..S....... .......... .......... S........ .....I.... ........ [136]
B-N9_UK           .......... ..S....... .......... ...T...... S........ .......... ........ [136]
B-N39_UK          .......... ..S....... .......... .......... S........ .....I.... ........ [136]
B-N50_UK          .......... ..S....... .......... .......... S........ .....I.... ........ [136]
B-N47_UK          .......... ..S....... .......*.. ...T...... S........ .....I.... ........ [136]
B-N46_UK          .......... ..S....... .......*.. ...T...... S........ .......... ........ [136]
B-N18-UK          .......... ..S....... .......... .........D S......--- ---------- -------- [136]
C-7.48_SA         .......... ..S....... .......... ...S....-- ---------- ---------- -------- [136]
C-N19_UK          .......... ..S.S..... .......... .......... S........ .....I.... ........ [136]
C-N3_UK           .......... *...S..... .......... .......... S........ .....I.... ........ [136]
C-N45_UK          .......... ..S.S..... ........S. .......... S........ .....I.... ........ [136]
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C1Cs-AB111946_GB .......... ..S....... .......... .......... S......... ....NI.... ......F. [136]
C2Ce-AF533983_GB .......... ..S....... .......... .......... S......... .....I.... ......... [136]
C3-X75656_GB     .......... ..S....... .......... .......... S......... .....I.... ......... [136]
C4ausAB048704_GB .......... ..S....... .......*D. .......... S......... ....NI.... ......... [136]
D-7.80_SA        .......... ..S...F... .......*D. .......... S......... .......... ...A.... [136]
D-3.34_SA        .......... ..S....... ..WP...*D. .......... S......... ......P... ..P..... [136]
D-N22_UK         .......... ..S....... .......*D. .......... S......... .......... ......... [136]
D-3.12_SA        .......... ..S....... .......*D. .......... S......... .......... ......... [136]
D-3.7_SA         .......... ..S....... .......*D. .......... S.Q......T .......... ...T.... [136]
D-1.205_SA       .......... ..S....... .......*D. .......... S.Q......T .......... ...T.... [136]
D-2.11_SA        ---........ ..S....... .......*D. .......... S.Q....... .......... ...T.... [136]
D-3.3_SA         .......... ..S....... .......*D. .......... S.Q....... .......... ...T.... [136]
D-3.1_SA         .......... ..S....... .......*D. .......... S.Q....... .......... ...A.... [136]
D-3.43_SA        .......... ..S....... .......*D. .......... ..Q......A .......... ......... [136]
D-4.27_SA        .......... ..S....... .......*.. .......... S......... .......... ......... [136]
D-109_SA         .......... ..S....... .......*.. .......... S......... .......... ......... [136]
D-1.46_SA        ---........ ..S....... .......*.. .......... S......... .......... ......... [136]
D-3.18_SA        ------.... ..S....... .......*.. .......... S......... .......... ......... [136]
D-3.8_SA         .......... ..S....... .......*.. .......... .........T .......... ......F. [136]
D-4.7_SA         .......... ..S....... .......*.. .....N.... .........G .......... ......... [136]
D-4.23_SA        .......... ..S....... .......... .......... S.P....... .......... .......G [136]
D-4.13_SA        .......... ..S....... .......*.. .......... S......... .......... ......... [136]
D-4.3_SA         .......... ..S....... .......*.. .......... S......... .......... ...T.... [136]
D-4-22_SA        .......... ..S....... .......*D. .......... S......... .......... ......... [136]
D-4.2_SA         .......... ..S....... ........D. .......... .......... .......... ......... [136]
D-1.50_SA        .......... ..S....... .......*D. .......... ..Q....... .......... ...T.... [136]
D-4.15_SA        .......... ..S....... .......*D. .......... S........T ..Y.N..... ......F. [136]
D-4.21_SA        .......... ..S....... .......*.. .......... .......... .......... ......... [136]
D-X02496_GB      .......... ..S....... .......... .......... .......... .......... .N...... [136]
D-7.84_SA        .......... ..S....... .......... .......... .......... .......... ......... [136]
D-3.26_SA        .......... ..S....... .......... .......... .......... .......... ......... [136]
D-3.36_SA        .......... ..S...D... .......... .......... .......... .......... ......... [136]
D1-AY161157_GB   ...L...... ..S....... .......... .T........ .......... .......... ......... [136]
D-1.55_SA        .......... ..S....... .......... .......... .......... .......... ......... [136]
DA-1.56_SA       .......... ..S....... .......... .......... .......... .......... ......... [136]
D-2.12_SA        .......... ..S....... .......... .......... .......... .....L.... ......... [136]
D-3.32_SA        .......... ..S....... .......... .......... .......... .......... ......... [136]
D-3.5_SA         .......... ..S....... .......... .......... .......... .......... ......... [136]
D-3.29_SA        ------.... ..S....... ........D. .......... .A........ .......... ......... [136]
D-1.106_SA       ..F....... ..S....... .......*D. .......... .......... .......... ......... [136]
D-3.9_SA         .......... ..S....... ........D. .......... .......... .......... .......G [136]
D-3.39_SA        .......... ..S....... ........D. .......... ...S...... .......... ......... [136]
D-1.60_SA        .......... ..S....... ........D. .......... .......... .......... ......... [136]
D-1.49_SA        .......... ..S....... ........D. .......... .......... .......... ......... [136]
D-LEIC_UK        .......... ..S....... .......... .......... .......... .......... ......... [136]
D-4-1_SA         .......... ..S....... .......... .......... .......... .......... ......... [136]
D-p15R_UK        .......... ..S....... .......... .......... .......... .......... ......C. [136]
D1.48_SA         ---------- -.S....... .......... .......... .......... .......... ......... [136]
D-X65257_GB      .......... ..S....... .......... .......... .......... .......... ......... [136]
D4-AB048702_GB   .......... ..S....... .......... .......... .......... .......... ...A.... [136]
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D3-X85254_GB     .R........ ..S....... ....R..*.. .......... S......... .......... ........ [136]
D-1.78_SA        -------... ..S....... .......D. .......... S......... .......... ........ [136]
D-3.19_SA        .......... ..S....... .......... ...H...... .......... .......... ........ [136]
D-1.2_SA         .......... ..S....... .......... .......... .......... .......... ........ [136]
D-1.4_SA         .......... ..S....... .......... .......... .......... .......... ........ [136]
D2-X80925_GB     .......... ..S....... .......... .......... .......... .......... ........ [136]
D-N12_UK         ---------- ---------- ---------- ---------- ---------- ---------- -------- [136]
D-N6_UK          .......... ..S....... ....AR.... .T........ S--------- ---------- -------- [136]
D-118_SA         .......... .WS..I.... R......*.. ..-------- ---------- ---------- -------- [136]
D-3.35_SA        .......... ..S.------ ---------- ---------- ---------- ---------- -------- [136]
D-7.59_SA        ....P..... CV........ ........R. ..V.....-- ---------- ---------- -------- [136]
D-7.86_SA        .......... ..S....P.. ........D. .T-------- ---------- ---------- -------- [136]
D-7.40_SA        .......... ..S....... .......*.. ........-- ---------- ---------- -------- [136]
D-7.5_SA         .......... ..S...F... .......*D. .......... ---------- ---------- -------- [136]
D-4.19_SA        .......... ..Q.LI.... R....L.*.. ..V....... .......W.. ..L.....-- -------- [136]
D-3.6_SA         .......... .WF.LI.... R....L.*.. ..V......V I......... .S...----- -------- [136]
E-X75664_GB      .......... ..S....... .......... .......... .......... .......... ........ [136]
E-X75657_GB      .......... ..S....... .......... .......... .......... .......... ........ [136]
E-2.25_SA        .......... ..S....... .......*.. .......... S......... .......... ........ [136]
E-4.10_SA        .......... ..S....... .......*.. .......... .L........ .......... ........ [136]
F-X75658_GB      .......... ..L....... .......... .......... S...F..... .....I.... ........ [136]
H-AY090457_GB    .......... F.S....... .......... .......... S......... .......... ........ [136]
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AeurpeV00866_GB  EALESPEHCS PHHTALRQAI LCW [159]
AaAF297625_GB    .......... .......ET. ... [159]
A1-AY161140_GB   .......... .......... ... [159]
A2-S50225_GB     .......... .........V ... [159]
ADrecAF297621_GB .......... .......... ... [159]
A-N13_UK         D......... .....----- --- [159]
A-3.47_SA        .......... ....R...-- --- [159]
A-N44_UK         .......... .....----- --- [159]
A-N33_UK         .......... ........-- --- [159]
A-N41_UK         .........T .......--- --- [159]
A-N4_UK          .......... ........-- --- [159]
A-N15_UK         .......... .N......-- --- [159]
A-N49_UK         ---------- ---------- --- [159]
A-N14_UK         .......... ........-- --- [159]
Ba-D00330adw2_GB .......... .......... ... [159]
Bj-AB073858_GB   .........T A......... ... [159]
B1-AB073852_GB   D........T .........V ... [159]
B2-AB073830_GB   .......... .......... ... [159]
B3-D00331_GB     .......... .......... ... [159]
B4-AB100695_GB   .......... .......... ... [159]
B-N17_UK         .......... ........-- --- [159]
B-N9_UK          .......... ....------ --- [159]
B-N39_UK         .......... ......---- --- [159]
B-N50_UK         .......... .....----- --- [159]
B-N47_UK         .......... .....----- --- [159]
B-N46_UK         .......... .....----- --- [159]
B-N18-UK         ---------- ---------- --- [159]
C-X01587adr4_GB  .......... .......... ... [159]
C1Cs-AB111946_GB .........T .....I.... ... [159]
C2Ce-AF533983_GB .......... .........V ... [159]
C3-X75656_GB     .......... .........V ... [159]
C4ausAB048704_GB .......... .......... ... [159]
C-N3_UK          .......... ........-- --- [159]
C-N19_UK         .......... .....----- --- [159]
C-N45_UK         .......... ........-- --- [159]
C-7.48_SA        ---------- ---------- --- [159]
D-7.80_SA        D....S.... A.......S- --- [159]
D-3.34_SA        D..R.....T ....TF..-- --- [159]
D-N22_UK         .......... .........Y --- [159]
D-3.12_SA        .......... ........-- --- [159]
D-3.7_SA         ..*....... ........-- --- [159]
D-1.205_SA       ..*....... ........-- --- [159]
D-2.11_SA        .......... ........-- --- [159]
D-3.3_SA         .T........ ........-- --- [159]
D-3.1_SA         .......... ........-- --- [159]
D-3.43_SA        ........W. ........-- --- [159]
D-4.27_SA        .......... ........-- --- [159]
D-109_SA         .......... ........-- --- [159]
D-1.46_SA        .......... ........-- --- [159]
D-3.18_SA        .......... ........-- --- [159]
D-3.8_SA         .......... ........-- --- [159]
D-4.7_SA         Q......... ........-- --- [159]
D-4.23_SA        .......... ....------ --- [159]
D-4.13_SA        D......... ....------ --- [159]
D-4.3_SA         D....S.... ....------ --- [159]
D-4-22_SA        .......... .PR.------ --- [159]
D-4.2_SA         .......... ....------ --- [159]
D-1.50_SA        .......... ....------ --- [159]
D-4.15_SA        .......... ........-- --- [159]
D-4.21_SA        .........T .......--- --- [159]
D-X02496_GB      .......... .......... ... [159]
D-X65257_GB      D......... .......... ... [159]
D1-AY161157_GB   .......... .......... ... [159]
D2-X80925_GB     D......... .......... ... [159]
D3-X85254_GB     .........T .......... ... [159]
D4-AB048702_GB   D......... .......... ... [159]
D-7.84_SA        .......... ........-. --- [159]
D-3.26_SA        .......... ........-- --- [159]
D-3.36_SA        .......... --......-- --- [159]
D-1.55_SA        .......... ........-- --- [159]
DA-1.56_SA       .......... ........-- --- [159]
D-2.12_SA        .......... .......... ... [159]
D-3.32_SA        .......... ........-- --- [159]
D-3.5_SA         .......... ........-- --- [159]
D-3.29_SA        .......... ........-- --- [159]
D-1.106_SA       .......... ........-- --- [159]
D-3.9_SA         .......... ........-- --- [159]
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D-3.39_SA        .......... .........-- --- [159]
D-1.60_SA        .......... .........-- --- [159]
D-1.49_SA        .......... .........-- --- [159]
D-LEIC_UK        .......... .......... ... [159]
D-4-1_SA         .......... .......... ... [159]
D-p15R_UK        .......... .........E FHH [159]
D1.48_SA         .......... .........-- --- [159]
D-1.78_SA        .......... .........-- --- [159]
D-3.19_SA        D......... .........-- --- [159]
D-1.2_SA         .......... .........-- --- [159]
D-1.4_SA         .......... .........-- --- [159]
D-N12_UK         ---------- ---------- --- [159]
D-N6_UK          ---------- ---------- --- [159]
D-118_SA         ---------- ---------- --- [159]
D-3.35_SA        ---------- ---------- --- [159]
D-7.59_SA        ---------- ---------- --- [159]
D-7.86_SA        ---------- ---------- --- [159]
D-7.40_SA        ---------- ---------- --- [159]
D-7.5_SA         ---------- ---------- --- [159]
D-4.19_SA        ---------- ---------- --- [159]
D-3.6_SA         ---------- ---------- --- [159]
E-X75664_GB      D......... .......... ... [159]
E-X75657_GB      D......... .......... ... [159]
E-2.25_SA        D......... ---------- --- [159]
E-4.10_SA        D......... ....D.---- --- [159]
F-X75658_GB      D........T .N........ ... [159]
H-AY090457_GB    D........T .N...----- --- [159]
*:HBeAg stop codon
T-cell Epitopes
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HBV Basal Core Promoter/Precore

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nt. 1762          1896
                  AGGTCTT       GGGGC ATG
A1-AY161140_GB    A.G....       ....
A2-S50225_GB      T.A....       .A..
AeurpeV00866_GB   T.A....       ....
AaAF297625_GB     A.G....       ....
A-N13_UK          T.A....       ....
A-3.47_SA         T.AC...       ....
A-N44_UK          A.G....       ....
A-N33_UK          A.A.T..       ....
A-N41_UK          T.G.T..       ....
A-N4_UK           A.T.T.A       ....
A-N15_UK          T.A.T.A       A...
A-N49_UK          A.A....       ....
A-N14_UK          T.A.T..       ....
Ba-D00330adw2_GB  A.G....       ....
Bj-AB073858_GB    A.A.T.A       A...
B1-AB073852_GB    T.A....       A..A
B2-AB073830_GB    A.G....       A...
B3-D00331_GB      A.G....       ....
B4-AB100695_GB    T.A....       ....
B-N17_UK          T.A....       ....
B-N9_UK           T.A....       ....
B-N39_UK          A.G....       ....
B-N50_UK          A.G....       ....
B-N47_UK          A.G....       A...
B-N46_UK          A.G....       A...
B-N18-UK          A.G....       ....
C1Cs-AB112348_GB  A.G....       ....
C2Ce-AY123041_GB  A.G....       ....
C3-X75656_GB      A.G....       ....
C4aus-AB048704_GB A.G....       A..A
C-7.48_SA         A.G....       ....
C-N19_UK          A.G....       ....
C-N3_UK           A.G....       ....
C-N45_UK          A.G....       ..A.
D1-AY161157_GB    A.G....       ....
D2-X80925_GB      A.G....       ....
D3-X85254_GB      A.G....       A...
D4-AB048702_GB    A.G....       ....
D-7.80_SA         T.A....       A..A
D-N22_UK          A.G....       A..A
D-4.27_SA         G.T.G..       A...
D-109_SA          G.T.G..       A...
D-4.23_SA         A.A.G..       ....
D-4.13_SA         A.C.G..       A...
D-4.3_SA          A.G....       A...
D-4-22_SA         A.A....       A..A
D-4.2_SA          T.G.T.A       ...A
D-1.50_SA         T.A....       A..A
D-4.15_SA         A.T.G..       A..A
D-4.21_SA         A.G....       A...
D-7.84_SA         A.G....       ....
D-LEIC_UK         A.G....       ....
D-4-1_SA          A.G....       ....
D-p15R_UK         A.G....       ....
D-N12_UK          A.G....
D-N6_UK           A.G....       ....
D-118_SA          T.G.T.A       A...
D-3.35_SA         T.G.T.A
D-7.59_SA         A.G.T.A       ..C.
D-7.86_SA         T.A....       ...A
D-7.40_SA         A.G....       A...
D-7.5_SA          T.A....       A..A
D-4.19_SA         T.G.T.A       A...
D-3.6_SA          T.G.T.A       A...
D-2.12_SA         ***....       ....
D-3.34_SA                       A..A
D-3.12_SA                       A..A
D-3.7_SA                        A..A
D-1.205_SA                      A..A
D-2.11_SA                       A..A
D-3.3_SA                        A..A
D-3.1_SA                        A..A
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D-3.43_SA                       A..A
D-1.46_SA                       A...
D-3.18_SA                       A...
D-3.8_SA                        A...
D-4.7_SA                        A...
D-3.26_SA                       ....
D-3.36_SA                       ....
D-1.55_SA                       ....
DA-1.56_SA                      ....
D-3.32_SA                       ....
D-3.5_SA                        ....
D-3.29_SA                       ...A
D-1.106_SA                      A..A
D-3.9_SA                        ...A
D-3.39_SA                       ...A
D-1.60_SA                       ...A
D-1.49_SA                       ...A
D-1.48_SA                       ....
D-1.78_SA                       A..A
D-3.19_SA                       ....
D-1.2_SA                        ....
D-1.4_SA                        ....
E-X75664_GB      A.G....        ....
E-2.25_SA        T.A...A        A...
E-4.10_SA        T.A....        A...
F-X75658_GB      A.G....        ....
H-AY090457_GB    A.G....        ....
G-AF160501_GB    T.AC...        A...
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