ASPECTS OF HBV S GENE VARIATION

BY

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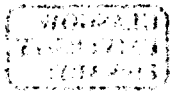

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SUMMARY

Surface (S) gene mutations are clinically important in both hepatitis B virus (HBV) prevention and diagnosis. Several HBV vaccination programmes in endemic countries have revealed their influence on failure of immunisation. Furthermore, sera harboring these variants can escape serological detection, but, remain detectable by HBV-DNA testing. According to these findings, the current work can be divided into two main parts. In the first, the impact of vaccination on the prevalence of S gene variants and, interestingly, how to use HBsAg variability as a marker of human population history, were studied. In the second, the influence of S gene variants on diagnostic failure, and also how to standardise and evaluate these variants in a novel tag system were investigated.

Initially (Chapter 3.1), four methods of DNA extraction have been compared to enable quick and efficient human genomic and viral DNA extraction from clotted blood. Two of these, a phenol based in-house method and Tripure isolation reagent (Roche), only achieved a low DNA yield. In contrast, QIAamp blood kit (Qiagen) and High Pure Viral Nucleic Acid kit (Roche Diagnostics) were equally efficient and sensitive.

The prevalence rate of hepatitis B in the south Pacific is amongst the highest in the world. Regional immunisation programmes were introduced in four Pacific islands in 1995: Vanuatu and Fiji in Melanesia, Tonga in Polynesia and Kiribati in Micronesia (Chapter 3.2). To assess the efficacy of these programmes, sera from infants and their mothers were tested. Following immunisation there was a dramatic fall in the seroprevalence of surface antigen, especially in Fiji to 0.7% of children. However, in Vanuatu and Tonga it was 3.0% and 3.8% respectively, and occurred mostly in babies of HBeAg positive mothers denoting the importance of this mode of transmission. On the other hand, the 3.8% HBsAg prevalence in Kiribati among children of non-carrier mothers indicated that most of these infections were horizontally transmitted. This relative failure to provide satisfactory protection could reflect poor health services or insufficient neutralisation. Moreover, although the opportunity for the emergence of vaccine escape variants in these populations was high due to the presence of a considerable amount of the virus with apparently incomplete protection, there were no "a" determinant variants discovered, suggesting that these variants are relatively insignificant in this population. Some other variants were noted, but the functional significance of these remains to be determined.

Geographic distribution of HBV genotypes is thought to reflect aspects of human population history. Hepatitis B virus surface antigen (HBsAg) variation from the four Pacific island locations has been analysed (Chapter 3.3). Samples were collected from unvaccinated children and adults and tested for HBsAg. At least 20 HBsAg positives from each island were amplified by PCR and sequenced. HBV isolates from C and D genotypes were identified, Genotype C predominated in Vanuatu, Fiji and Tonga while D was the dominant genotype in Kiribati. The diversity of the C genotype sample was significantly greater than that of D, consistent with a longer history of HBV infection in those islands. Strong geographic identity was evident in all populations except Tonga and Fiji, which were statistically indistinguishable. Analysis of HBV sequences from other locations will be required to fully interpret these data.

HBsAg negativity does not exclude hepatitis B viraemia and HBsAg variants can be responsible for such diagnostic failures. In Chapter 3.4, we cloned 13 different HBsAg variants. Variant protein then produced in a mammalian expression system and tested using seven commercial HBsAg diagnostic assays. Of 12 variants analysed, 6 samples displayed similar reactivity to the standard HBsAg sequence in most of the assays but 6 samples, containing various mutations throughout the entire major hydrophilic region (MHR), showed reduced reactivity. Loss of cysteine at aa124 in one sample was found to influence the secretion as well as the reactivity of HBsAg in the expression system. Finally, not all assays were equally able to detect HBsAg variants implying that, to attain an acceptable level of sensitivity, the antibody repertoire of the current assays should be extended.

Reduced reactivity might be due to antigenic changes or reduced particle production. To investigate the reason(s) for non-detection, supernatants derived from *in-vitro* expression of cloned HBsAg variants were used. We have developed an antibody capture system, using a non-HBV epitope, to standardize the amount of *in-vitro* expressed HBsAg protein (Chapter 3.5). Three tag systems were assessed. The successful one, influenza HA-tag, was inserted into the HBV S gene of control samples and 12 diagnostically important variants within different backbone subtypes: one *ayw1*, four *ayw2*, two *ayw3*, and five *adw2*. The amount of *in-vitro* expressed HBsAg was then equalised in an ELISA that recognises the tag. Subsequently, the immunoreactivity of each variant was compared using three commercial HBsAg assays. We were then able to precisely attribute the diagnostic failure of the investigated variants to antigenic non-recognition and/ or poor secretion of HBsAg protein. In

found that single tag epitopes of up to 15 aa could be inserted at either end of HBsAg protein without affecting HBsAg reactivity. However, insertion at both ends led to a major impact on HBsAg conformation.

Differentiation of second episodes of HBsAg positivity as reinfection or reactivation is a matter of debate. 5 patients who had a serological picture suggesting a second hepatitis B virus episode were studied compared to a control group of two patients who were HBsAg positive throughout with fluctuating HBeAg status. We suggest molecular criteria to distinguish between these two possibilities: number of nucleotide substitutions; number of amino acid substitutions; situation of aa changes; phylogenetic relatedness, co-incidence of mutation with immune or antiviral therapy; and genotype/ subtype shifts (Chapter 3.6). Interestingly, S gene variants were found in all 5 cases with unusual serology but in neither of the controls. Abnormal serology was therefore accompanied in all patients by rare sequences.

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2.2 METHODS

ABBREVIATIONS

A	Adenosine
Ab	Antibody
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
Ag	Antigen
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ASC	Asymptomatic carrier
ATP	Adenosine triphosphate
β-ME	β-mercaptoethanol
bp	Base pairs
Bio	Biotin
BSA	Bovine serum albumin
C	Cytosine
CAH	Chronic active hepatitis
ccc	Covalently-closed circular
C/EBP	CCAAT/ enhancer binding protein
CPH	Chronic persistent hepatitis
C terminal	Carboxy terminal end of protein
CTL	Cytotoxic T-lymphocyte
Da	Dalton
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2'3'-dideoxyadenosine 5'-triphosphate
ddCTP	2'3'-dideoxycytidine 5'-triphosphate
ddGTP	2'3'-dideoxyguanosine 5'-triphosphate
ddTTP	2'3'-dideoxythymidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DHBV	Duck hepatitis B virus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
ds	Double-stranded
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
ELISA	Enzyme-linked immuosorbant assay
Enh	Enhancer
ER	Endoplasmic reticulum
ESLD	End stage liver disease
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyante
G	Guanosine
gp	Glycoprotein
GSHV	Ground squirrel hepatitis B virus
HBcAg	Core antigen

HBeAg	e-antigen
HBsAg	Surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
HRP	Horse radish peroxidase
HSV	Herpes simplex virus 1
IFN	Interferon
LHBs	Large hepatitis B virus surface protein
kb	Kilobase pairs
mAb	Monoclonal antibody
MHBs	Middle hepatitis B virus surface protein
MHC	Major histocompatability complex
mRNA	Messenger RNA
NLS	Nuclear localisation signal
N terminal	Amino terminal end of protein
OD	Optical density
ORF	Open reading frame
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PgRNA	Pre-genomic RNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SHBs	Small hepatitis B virus surface protein
ss	Single stranded
T	Thymine
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	T-helper cell
TNF	Tumour necrosis factor
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
WHV	Woodchuck hepatitis B virus

ONE AND THREE LETTER AMINO ACID ABBREVIAATIONS

Amino acid	Three letter code	One letter code	Codons
Alanine	Ala	A	GCU GCC GCA GCG
Arginine	Arg	R	AGA AGG CGU CGC CGA CGG
Asparagine	Asn	N	AAU AAC
Aspartic acid	Asp	D	GAU GAC
Cysteine	Cys	C	UGU UGC
Glutamine	Gln	Q	CAA CAG
Glutamic acid	Glu	E	GAA GAG
Glycine	Gly	G	GGU GGC GGA GGG
Histidine	His	H	CAU CAC
Isoleucine	Ile	I	AUU AUC AUA
Leucine	Leu	L	UUA UUG CUU CUC CUA CUG
Lysine	lys	K	AAA AAG
Methionine	Met	M	AUG
Phenylalanine	Phe	F	UUU UUC
Proline	Pro	P	CCU CCC CCA CCG
Serine	Ser	S	AGU AGC UCU UCC UCA UCG
Threonine	Thr	T	ACU ACC ACA ACG
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAU UAC
Valine	Val	V	GUU GUC GUA GUG

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1.1 GENERAL INTRODUCTION

Although hepatitis as a clinical disease has been recognised from the very earliest times, the identification of its many viral causes, including hepatitis B virus (HBV), was a relatively recent event. The hepatitis B virus, firstly, came to light with the discovery of Australia Antigen (AuAg) by Blumberg and his colleagues in 1965 (Blumberg et al., 1965). Because they were interested in serum protein polymorphisms as genetic markers in the human population, they initially considered a hypothesis that the "AuAg" system was another serum protein polymorphism. Subsequent studies revealed that the antigen occurred more frequently among patients who received multiple transfusions and blood products (Blumberg et al., 1967). One year later, electron microscopy of the partially purified Ag, showed it to be organised into virus-like particles of approximately 22 nm in diameter (Bayer et al., 1968).

The direct relationship of AuAg to type B hepatitis was revealed on examination of serum samples collected from Willowbrook State School in New York. In this study, two forms of viral hepatitis were detected after human inoculation and cross-challenge experiments. Additionally, AuAg was found to be mostly associated with the long incubation period, parenterally transmissible form of the disease (Giles et al., 1969). Then, over the next 10 years, rapid progress was made in the structure and biological characterisation of the virus. With the advent of serological markers, the epidemiology and transmission of HBV was described in more detail (Blumberg, 1977; Hoofnagle, 1981). But, the narrow host range of the virus and inability to be propagated in cultured cells hampered early efforts to uncover the molecular details of viral replication (Ganem and Varmus, 1987).

HBV is a major problem in the world; it is endemic in Africa, South America and Asia. There are at least 350 million persons chronically infected with HBV, each of whom is potentially infectious. In hyperendemic regions, transmission is mainly from mother to baby or between children. In low incidence regions, such as Western Europe, transmission is by sex, intravenous drug usage and needle-stick exposure. This makes it a major health care problem even in these regions. Expensive and only moderately efficacious therapies are available, including liver transplantation. Consequently, WHO recommends that all countries should introduce universal infant vaccination. The vaccine simply consists of hepatitis B surface antigen (HBsAg), which contains neutralising epitopes that lead to the production of protective surface antibody (anti-HBs).

The emergence of variants of HBV that are not neutralised by vaccine-induced anti-HBs is also of major concern especially as several studies from different parts of the world have consistently detected them. If eradication of HBV is our goal, we need to assess the epidemiological and antigenic significance of these variants. But a number of things are unknown. First, what pressure does immunisation put on to the HBsAg population? Do new variants appear *ab initio* or do certain ones present in the pre-vaccination population become selected? Can they be transmitted to others? Are they truly neutralisation escape variants?

1.2 HBV STRUCTURE AND GENOMIC ORGANISATION

Electron microscopic examination of partially purified serum derived preparations of HBV revealed the presence of three kinds of particles. Complete infectious virions of about 42-nm in diameter, which consist of a 36-nm icosahedral nucleocapsid of 240 units of the hepatitis B virus core antigen (HBcAg) and a 7-nm lipoprotein bilayer derived from the endoplasmic reticulum (ER) membrane of the host (Dane et al., 1970; Patzer et al., 1986; Crowther et al., 1994). Three HBV surface proteins of varying sizes- large hepatitis B surface protein (LHBs), middle hepatitis B surface protein (MHBs) and small hepatitis B surface protein (SHBs)- are inserted into this lipoprotein bilayer (see section 1.4). The viral DNA, a virus encoded RNA-dependent DNA polymerase, a protein kinase which phosphorylates HBcAg and a genome-bound protein covalently linked to the negative strand of HBV DNA are all contained in the nucleocapsid (Albin & Robinson, 1980; Gerlich & Robinson, 1980; Figure 1.1).

Small spherical and filamentous forms of non-infectious subviral particles of 20-nm diameter are composed of SHBs with variable amounts of MHBs and LHBs in addition to the host derived lipids. Spherical forms contain very little LHBs while excessive amounts of LHBs enhance the formation of filaments (Peterson, 1981; Aggerbeck & Peterson, 1985; Chisari et al., 1986). Although they do not contain viral DNA or nucleocapsid, they are highly immunogenic and elicit a strong antibody response in most individuals which is capable of conferring immunity to reinfection *in vivo*. Therefore, these particles have been exploited as a subunit vaccine for HBV infection (Szmuness et al., 1980; Valenzuela et al., 1982; Mc Aleer et al., 1984). The concentration of subviral particles often exceeds the virion concentration by at least three fold.

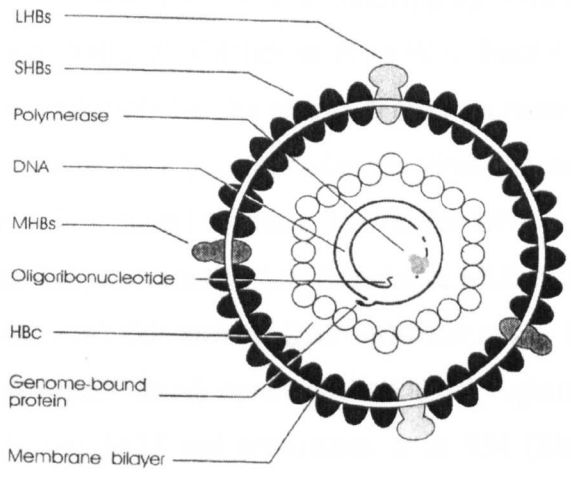


Figure 1.1: Schematic structure of HBV Dane particle

The infectious virion consists of an outer membrane containing the three surface gene products: SHBs (HBsAg), MHBs (preS2+HBsAg) and LHBs (preS1+PreS2+HBsAg). The internal nucleocapsid consists of core protein (HBc) dimers and encloses the partially double stranded circular DNA genome and the polymerase which is covalently linked to the minus strand DNA via its terminal protein. (Taken from Caselmann, 1996)

HBV is hepatotropic with a small, approximately 3.2 Kb in length, circular DNA which contains a single stranded region of different length in different molecules (Landers et al., 1977; Hruska et al., 1977). HBV DNA molecule has two remarkable asymmetries that make it unique from other viruses. Whilst the length asymmetry between its double strands constitute the first, the second occurs at their 5` termini. At this position, the long (minus) strand contains a protein that is covalently linked whereas the shorter (plus) strand has a small oligoribonucleotide (Gerlich & Robinson 1980; Will et al., 1987).

HBV DNA is highly organized with compact coding to efficiently use every nucleotide in its small-sized genome through the following strategies: every nucleotide in the genome is translated; 50% of the genome sequence can be read in more than one frame; there is differential initiation at several AUG codons within certain ORFs that are sometimes used in several genes; and all cis-acting regulatory elements (eg., transcripional enhancer and promotor elements) are also contained within the genomic sequences. Among these is an 11-nucleotide sequence that is represented twice, termed DR1 and DR2, and located near the 5` termini of the minus and plus strands respectively, where, they play important roles in directing the initiation of viral DNA synthesis (Seeger et al., 1986, Will et al., 1987).

The long minus strand carries five overlapping ORFs: preS/S (surface), C (core), P (polymerase), X and "ORF 5" (Miller et al., 1989). Four of these coding regions have been assigned to known viral proteins: the preS/S region encodes the envelope proteins; the C gene encodes the core protein; the P gene encodes the viral polymerase; and the X gene encodes the transactivator HBx (Ganem and Varmus, 1987). For ORF 5, which lies within the X gene, no protein product has been detected so far (Kaneko & Miller, 1988). On the other hand, the plus strand contains only one ORF, named ORF 6 (Miller et al., 1989). Two transcripts are derived from the HBV plus strand which contains the ORF 6 region; the first, about 0.7-kb in length, starts at nucleotide (nt) 1635 and terminates at nt 954 (Standring et al., 1983). The second transcript, 2.8-kb in length, extends between nt 2381 and 1861 and may serve as a promoter (Zelent et al., 1987). However, no ORF 6-specific protein has been detected (Figure 1.2).

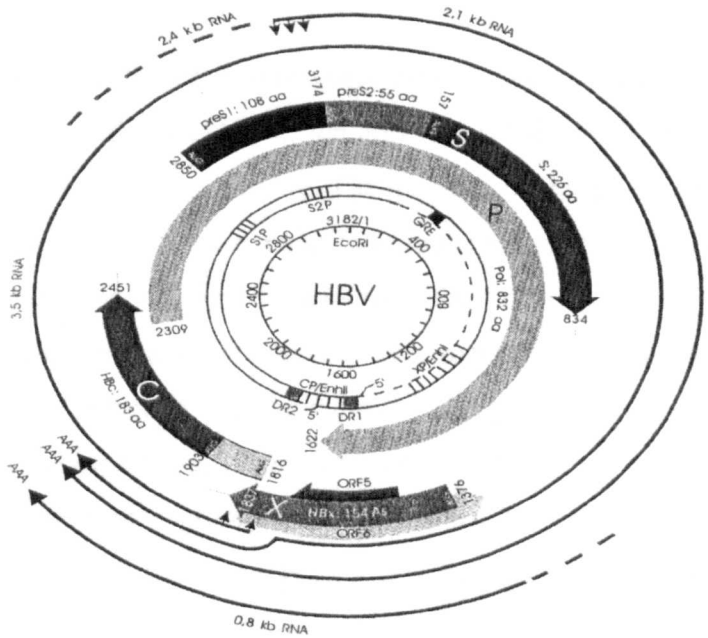


Figure 1.2: Genomic organization of HBV (subtype ayw)

The numbering system shown in the diagram is according to Gilbert et al., (1979). The outer lines represent the different classes of transcripts. The four major ORFs (S, P, C and X) are indicated in the center, and ORFs 5 and 6 are shown as well in relation to ORFX. The partially double stranded circle represent the viral genome, showing the promoters (S1P, S2P, XP and CP), the enhancers (EnhI and EnhII), and direct repeats (DR1 and DR2). (Taken from Caselmann, (1996))

Many recent reports have dealt with this region. For instance, Velhagen et al. (1995) showed that transcription of the 0.7-kb RNA molecule could be regulated by a promoter-like activity between nt 1885 and 1575. Later, direct evidence for the presence of an antisense promoter within the ORF 6 region of woodchuck hepatitis virus (WHV) was revealed by Shimoda et al. (1998). Several cis-regulatory elements have also been detected within the ORF 6 region and the corresponding X ORF. However, none of them have been shown to be involved in ORF 6 expression (Huang & Liang 1993; Donello et al., 1996; 1998). Recently a cis-regulatory element was shown to inhibit gene expression within the ORF 6 region in an orientation- and position- dependent manner, perhaps as a specific RNA-destabilizing element (Wagner et al., 1999).

1.3 HBV LIFE CYCLE

Little is known about the early molecular events such as virus-receptor interaction, viral uptake or uncoating, although a considerable knowledge of later events such as viral replication and virion release is available. The narrow host range of HBV and lack of suitable cell lines susceptible to infection are major limitations for such studies. Until recently, primary hepatocytes prepared from uninfected ducks were the only readily available cells known to be susceptible to infection by hepadnaviruses (Tuttleman et al., 1986; Pugh & Summers 1989). The low efficiency of infection and their limited availability has impeded the use of human primary hepatocytes (Gripon et al 1988; Ochiya et al., 1989).

However, successful production of HBV both *in vitro* and *in vivo* by rat hepatoma cells and rat hepatocytes respectively was reported after transfection with a construct containing the HBV DNA (Shih et al., 1989; Takahashi et al., 1995). Additionally, cross species replication of HBV has been shown in hepatocytes of HBV transgenic mice (Farza et al., 1988; Guidotti et al., 1995). Furthermore, primary tupaia hepatocytes (PTHs), as an alternative to primary human hepatocytes, were shown recently to support the complete HBV genomic replication cycle. Consequently, tupaias have been suggested as a useful animal model for HBV infection (Walter et al., 1996; Ren & Nassal et al., 2001). According to these findings, it was proposed that the species barrier for HBV infection was located at the initial stages of viral adsorption and/ or penetration. The steps of the HBV life cycle are shown in figure 1.3.

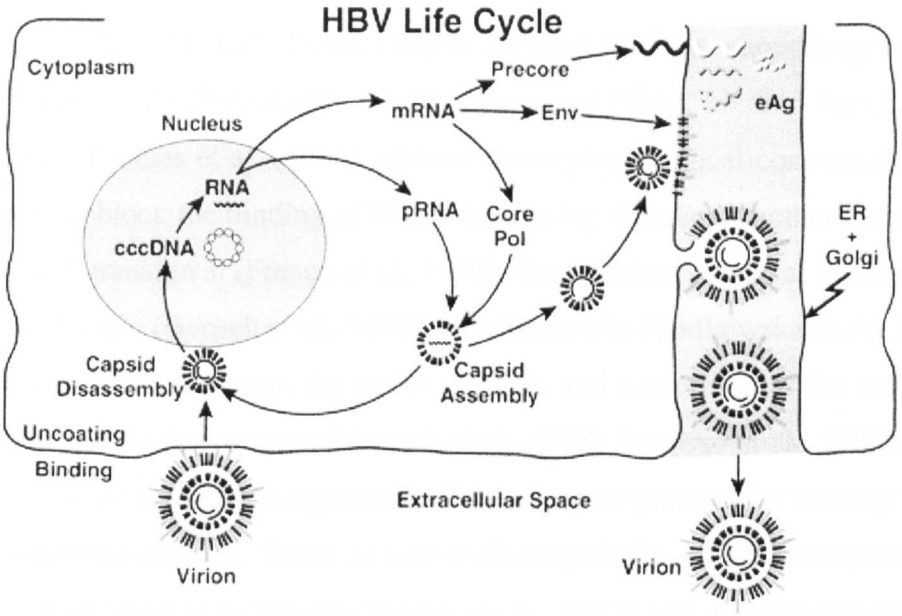


Figure 1.3: HBV life cycle

A diagrammatic representation of the HBV lifecycle, with the key stages shown. Binding to the receptor, penetration of the hepatocyte, uncoating and transport of the viral genome into the nucleus, formation of cccDNA, RNA synthesis and transport to the cytoplasm, translation of viral mRNAs, nucleocapsid assembly and encapsidation of PgRNA, reverse transcription and DNA synthesis, export of enveloped virions from the hepatocyte and retention of some genomes for the amplification of cccDNA. (Taken from Chisari, 2000)

1.3.1 Attachment to the cell membrane

The exact mechanism of attachment and penetration of HBV has not been fully identified. Binding of a viral particle to the human hepatocyte plasma membrane is an important step in viral entry and replication of HBV. In this interaction, the envelope proteins of HBV are thought to play a crucial role. Although there have been many reports during the last two decades, the identity of the HBV receptor is still controversial.

It was initially suggested that a preS2 domain could act as the attachment site to human hepatocytes via polymerised human serum albumin (PHSA) (Trevisan et al., 1982; Machida et al., 1983; Ishihara et al., 1987). However, this liver cell-PHSA interaction has been shown to be non-species-specific as rat liver cells also bind to PHSA (Wright et al., 1987). Nevertheless, natural HBsAg spheres or recombinant middle HBs protein, after treatment with PHSA, could bind to human liver plasma membranes (Pontisso et al., 1989a, b) and antiserum against a preS2 peptide (aa 1-25) was able to neutralise the virus and prevent HBV infection in challenged chimpanzees (Neurath et al., 1986). It was also noted that polymerised albumin of

other experimental animals, which are not susceptible to HBV infection, does not bind to HBsAg particles (Tuttleman et al., 1986). Despite all these findings supporting the hypothesis of preS2 domain as an attachment site, the importance of PHSA binding for HBV infection has been doubtful. Thomas et al. (1988) showed that a physiological concentration of native albumin was able to block the binding of PHSA to HBsAg. Candidates other than PHSA have also been proposed: transferrin (Franco et al., 1992), the N-linked glycan at the amino terminal end of the preS2 domain (Gerlich et al., 1993) and fibronectin (Budkowska et al., 1995).

Other observations have shown the preS1 domain, and not preS2, as the most important attachment site to human hepatocytes (Neurath et al., 1986; Pontisso et al., 1989b; Petit et al., 1991). However, as for preS2, the suggested cellular receptor proteins for binding to the preS1 domain have been controversial. These include immunoglobulin A (IgA) receptor (Pontisso et al., 1992), human Interleukin-6 (hIL-6) (Neurath et al., 1992) and asialoglycoprotein (ASGP) receptor (Treichel et al., 1994, 1997). Regardless of the controversial findings on the receptor molecules for the attachment of preS1, Neurath et al. (1989) further supported the importance of preS1 domain for the attachment to liver cells when they demonstrated HBV neutralisation in chimpanzees by using rabbit anti-sera against preS1.

Small HBs has also been reported to bind via apolipoprotein H (apo H) to human hepatocytes (Mehdi et al., 1994). Human annexin V (hA-V, previously named endonexin II), a member of Ca^{2+} dependent phospholipid binding proteins present on plasma membranes of human liver, has also been proposed as the HBV receptor (Hertogs et al., 1993). Anti-idiotypic anti-HBs antibodies have been demonstrated in rabbits immunised with hA-V but not in rabbits immunised with rat annexin V, despite more than 90% sequence homology between both annexins. This provided evidence for the "receptor-ligand" relationship between hA-V and HBsAg (Hertogs et al., 1994). Recent reports have further supported the role of hA-V in facilitating HBV entry and infection into host cells. Expression of the hA-V gene in rat hepatoma cells, whether due to the transfection of a construct containing hA-V gene or due to the administration of hA-V to primary cultures of rat hepatocytes, was found to confer susceptibility to HBV infection (Gong et al., 1999; De Meyer et al., 2000).

Difficulties in sustaining the currently available *in vitro* infection system for HBV (Gripon et al., 1993), have led to usage of the duck HBV (DHBV) model to investigate hepadnaviral entry. Early observations showed that non-infectious subviral particles (SVP) or recombinant particles containing only the L protein can inhibit the DHBV infection of primary duck

hepatocytes (Klingmüller & Schaller, 1993). A year later, Kuroki et al. (1994) detected a cellular glycoprotein of 180 kDa (gp180) which interacts with DHBV particles via the preS region. As anti-preS antibodies were shown to inhibit this binding, gp180 was suggested as a possible entry factor for DHBV. This host cell glycoprotein was shown later to be encoded by a member of the carboxypeptidase family (Kuroki et al., 1995), and termed carboxypeptidase D (CPD). The essential role of DHBV-preS (DpreS) for receptor recognition was further supported by mutational analysis of DpreS in which an extended sequence (aa 30-115) was identified as the receptor binding site of DHBV (Urban et al., 1998b).

The gp180/CPD, which is a trans-Golgi resident protein and cycles to the plasma membrane and back, has the characteristics for a DHBV receptor (Breiner et al., 1998; Breiner & Schaller, 2000). Indeed, CPD seems crucially involved in avian hepadnaviruses infection. Uptake of DpreS and viral particles by various cell lines was enhanced by their transfection with CPD-expressing plasmids (Breiner et al., 1998). Infection of duck hepatocytes with DHBV can be efficiently blocked with soluble duck CPD (sduCPD) (Urban et al., 1999). The binding site of CPD corresponds to the mapped receptor binding site within DHBV preS (Ishikawa et al., 1994; Breiner et al., 1998; Urban et al., 1998b). Levels of CPD are also greatly reduced upon DHBV infection, similar to the receptor down-regulation seen in classical retroviruses (Breiner et al., 2001). More precisely, the C-domain of duCPD (which has an extra two domains: A and B) was shown to be the virus binding domain (Eng et al., 1999; Urban et al., 2000). It has also been demonstrated that the structure required for binding and receptor recognition does tolerate a degree of variation. Clearly, this may enable the virus surface proteins to maintain the receptor affinity while simultaneously escaping immune surveillance by mutation (Urban et al., 2000).

1.3.2 Viral entry and uncoating

Although the exact post-binding steps are still unclear, several mechanisms have been suggested for viral entry. There is evidence from DHBV that entry proceeds by a pH-independent mechanism (Rigg & Schaller 1992). Endocytosis has also been described (Offensperger et al., 1991; de Bruin et al., 1995). Additionally, Lu et al. (1996) showed that HepG2 cells internalized HBV after proteolytic cleavage of the preS domain by V8 protease. This exposed a fusion domain within the envelope protein of HBV that enabled viral fusion with host cell membranes.

It is also not clear in what form the viral genome is transported into the cell nucleus since the diameter of core particles is at the maximal limit for transport through the nuclear pore (Feldherr et al., 1984). Firstly, nuclear localisation signals (NLS), which are contained within the C terminus of the HBV nucleocapsid, were suggested to allow nucleocapsid translocation into the nucleus (Eckhardt et al., 1990; Yeh et al., 1990). However, HBV nucleocapsid particles were not able to cross the nuclear membrane in either direction in transgenic mice expressing the C protein and only do so upon dissolution of the nuclear envelope (Guidotti et al., 1994). Thus the nucleocapsid itself might not normally traverse the nuclear pore. Alternatively, core particles may bind to the nuclear membrane and release their viral DNA into the nucleus or even disassemble in the cytoplasm before reaching the nuclear membrane (Bock et al., 1996; Kann et al., 1997; Qiao et al., 1999). Phosphorylation of the core protein C-terminal serine residues by protein-kinase C has been suggested to destabilise the core particles and thus allow virion DNA release giving further support to this model (Kann et al., 1993; Kann & Gerlich, 1994).

The HBV viral genome, once into the nucleus, is then repaired to its covalently closed circular (ccc) DNA form. This process requires the following modifications: the removal of its terminal structures (from both strands); repair of the single-stranded gap region (by completion of the plus strand synthesis); and covalent ligation of the DNA termini. Host cell enzymes are most likely responsible for all of these reactions (Köck and Schlicht, 1993).

1.3.3 Transcription

Nuclear ccc HBV DNA is transcribed by host RNA polymerase II. It creates an RNA template for HBV replication and leads to the synthesis of all viral mRNA transcripts. All major transcripts are unspliced, have negative strand polarity and terminate with a poly (A) tail of about 100 nt in length (Cattaneo et al., 1983, 1984).

1.3.3A Viral transcripts

Four extensively overlapping viral RNAs are produced predominantly (3.5, 2.4, 2.1, and 0.7-kb in length). These are exported into the cytoplasm where translation of viral proteins, assembly of viral particles and genome replication occurs.

a) 3.5-kb RNAs: they cover the entire genome and are terminally redundant (Seeger et al., 1991). The 3.5-kb transcript produces the polymerase, core and precore proteins and serves as

the pregenomic RNA template (PgRNA) that is reverse transcribed in viral genome replication (Chang et al., 1989; Schlicht et al., 1989).

b) 2.4-kb RNA: it is initiated approximately 38 nt upstream of the start codon of the preS/S gene (Will et al., 1987). It serves as the mRNA for LHBs and is transcribed only in hepatocytes as it is controlled from the S1 promoter (Chang et al., 1989).

c) 2.1-kb RNAs: these 2.1-kb RNAs transcripts are the templates for the synthesis of MHBs and SHBs proteins (Standring et al., 1984; Siddiqui et al., 1986). They are initiated at different initiation sites and their transcription is regulated by the S2 promoter which lacks a TATA box and tissue specificity (Cattaneo et al., 1983).

d) 0.7-0.9-kb RNA: this transcript functions as the mRNA for the X protein which has transcriptional transactivating potential (Siddiqui et al., 1987; Balsano et al., 1994).

1.3.3B Spliced transcripts

Although all HBV proteins identified to date have been encoded by unspliced RNAs, spliced HBV transcripts have been detected in HBV-transfected cell hepatoma cell lines, in HBV-transgenic mice and in HBV-infected liver (Chen et al., 1989; Su et al., 1989a, b; Suzuki et al., 1989, 1990; Choo et al., 1991; Wu et al., 1991). Spliced RNAs have also been detected in the related duck (DHBV) and woodchuck hepatitis viruses (WHV) albeit with variable splicing patterns (Ogston & Razman, 1992; Hantz et al., 1992; Obert et al., 1996). Single and double spliced forms of HBV PgRNAs have been described, which have different splice donor and acceptor sites and give rise to variable sizes of spliced transcripts (Chen et al., 1989; Su et al., 1989a, Suzuki et al., 1989; Wu et al., 1991).

Recently, Günther et al. (1997) could not detect any spliced sites downstream of nt 486 and upstream of nt 2067 and proposed that the efficiency and type of splicing, which was different from patient to patient in their study, might be HBV genotype dependent. Furthermore, an HBV spliced-generated protein (HBSP), 93 aa in length, was described as the encoded protein of a singly spliced RNA. In this study, HBSP protein and its anti-HBSP antibodies were detected in HBV-infected liver samples and sera collected from chronic HBV carriers respectively (Sousaan et al., 2000). It was also shown earlier that much of LHBs of DHBV is translated from a spliced transcript derived from the C pre-mRNA and not from preS1 as expected (Obert et al., 1996).

The precise involvement of spliced HBV RNAs in the HBV life cycle is still unclear. Spliced HBV transcripts are not essential as mutations of splice donor and acceptor sites do not influence viral replication (Su et al., 1989b; Wu et al., 1991; Caselmann et al., 1996). However, encapsidation of these singly spliced RNAs has been reported. These are most probably secreted as defective HBV particles through trans-complementation with a helper wild-type virus (Terré et al., 1991). These HBV defective particles have been detected in livers and sera from chronically infected patients (Terré et al., 1991; Rosmorduc et al., 1995; Günther et al., 1997). Thus, spliced HBV RNAs and their encoded proteins such as HBSP may play a role, whether direct or indirect, in pathogenesis and/ or persistence of HBV (Rosmorduc et al., 1995; Günther et al., 1997; Sousaan et al., 2000).

1.3.3C Transcription regulation

To date, four promoters (S1 promoter (S1P), S2 promoter (S2P), C promoter (CP), and X promoter (XP)) have been identified as responsible for the transcription of the HBV mRNAs (Siddiqui et al., 1986; Treinin & Laub 1987; Honigwachs et al., 1989). Of these four promoters, S2P (nt 3045-3180) is the least liver specific, being active in a wide range of mammalian cell lines (Dubios et al., 1980; Standring et al., 1984). However, it is most active, as for all HBV promoters, in liver cells (Chang & Ting, 1989; Seifer et al., 1990). A CCAAT box, which is located between nt 3105 and 3110 and shown to mediate NF-Y activation, has been described to regulate both S1P and S2P promoters as it enhances the S2P activity and down-regulates S1P transcript levels (Lu et al., 1995; Lu & Yen 1996).

On the other hand, S1P and CP are highly liver specific and cells in which CP is inactive cannot support HBV DNA replication, as the production of genomic RNA, which is the template for reverse transcription, is mostly under the control of this promoter (Seeger et al., 1989). Only the S1P located between nt 2710 and nt 2800 has a TATA-box; consequently, this determines a precise 5` end for the LHBs-encoding transcripts (Schaller & Fischer 1991). XP is partly overlapped with EnhI. In addition to the cellular factors that are important for X gene transcription, HBx was shown to have a role in enhancing XP activity by binding a 20bp element at its 5' end (Takada et al., 1996).

The promoter activities are regulated by two enhancers: enhancer I (EnhI, nt 970-1240) and enhancer II (EnhII, nt 1627-1774). EnhI is located between the S and X ORFs whereas EnhII is just upstream of the CP (Shaul et al., 1985; Yee, 1989). EnhI displays only a preference for

hepatocytes, whilst EnhII shows a highly hepatocyte-specific activity (Shaul et al., 1985; Tognoni et al., 1985; Yee, 1989; Wang et al., 1990b).

EnhI consists of three elements: a modulator element at the 5' terminus; a central domain; and the 3' end that partially overlaps with XP. Several nuclear proteins have been identified as able to bind to the central domain, which has at least four motifs. The external two, at the 5' and 3' terminals, bind nuclear factor-1 (NF-1) and rheumatoid factor-1 (RF-1) respectively. The inner two mediate the tissue-specific regulation of EnhI, and bind HNF3 and HNF4 (Garcia et al., 1993; Kosovsky et al., 1996). S2P, XP and CP are under the control of EnhI, which is regulated by a complex interaction of hepatocyte-specific and ubiquitous transcription factors (Yen 1993; Kosovsky et al., 1996). Recently, Bock et al. (2000) have shown that the decreased transcription activity associated with mutations of EnhI region, precisely the binding sites for HNF3 and HNF4, might result in chronic persistence of HBV by reducing replication and immunogenicity of the virus.

EnhII, which is located within the X ORF and partially overlaps CP, consists of two parts (A and B), with part B as the basal functional unit (Wang et al., 1990b). In a position- and orientation-independent manner, it enhances the transcription from S2P and XP and to a small extent from S1P (Yuh & Ting, 1990; Zhou & Yen 1990). It also positively regulates the basal CP, but in a position- and orientation-dependent way (Yuh et al., 1992; Yuh & Ting 1993). Hepatocyte transcription factors such as hepatocyte nuclear factor 1 (HNF1), HNF3, HNF4, SP1 and others, which are important for upregulating CP/EnhII activity, are responsible for the hepatocyte specificity of EnhII. Other negative regulatory elements, not identified yet, were also shown to down-regulate CP/EnhII by binding to its 5' terminal in non-hepatic cells (Guo et al., 1993; Zhang et al., 1993; Li et al., 1995; Raney et al., 1997; Wang et al., 1998).

A transcriptional termination signal is situated just after the pregenomic RNA-start site (Ganem & Varmus, 1987). However, it can only function as a terminator when it is more than 400bp away from the start of transcription (Cherrington et al., 1992), enabling RNA polymerase II to ignore it during the first pass from the 3.5kb RNA start point.

1.3.3D Post-transcriptional regulation

A post-transcriptional regulating element (PRE) within HBV transcripts, ~ 500 bases long and partially overlaping X ORF, was discovered by the observation that HBV S gene expression was reduced if the downstream region of ORF S was deleted from expression plasmids (Huang & Liang, 1993; Huang & Yen, 1994). Deletion studies of HBV PRE (HPRE) have shown that it consists of two independent sub-elements, PREα nt 1151-1412 and PREβ nt 1413-1684, which function in a co-operative manner (Donello et al., 1996). Distinct binding sites for cellular RNA binding proteins that mediate the function of PRE are most probably located within these sub-elements (Donello et al., 1996; Huang et al., 1996b). HPRE regulates the level of preS/S-specific transcripts by facilitating their transport from the nucleus to the cytoplasm (Huang & Yen, 1994). This HPRE-effect is most probably due to a positive role; prevention of splicing and activation of export (reviewed in Yen, 1998).

Further work from the Hope group on WHV PRE (WPRE) has revealed that WPRE consists of three sub-elements, WPRE α, β and γ, where the first two are similar to those of HPRE. This "tripartite" WPRE was also more active than the "bipartite" HPRE, most probably due to the additional cis-acting sequence in WPRE (Donello et al., 1998). Moreover, insertion of WPRE into HIV-derived vectors significantly improves their efficiency, suggesting its employment in gene therapy. The WPRE effect was only orientation-dependent but neither cell- nor promoter- dependent (Zufferey et al., 1999). In another elegant experiment from the same group, important RNA components were identified within HPRE α and β (HPRE stem-loop α nt 1292-1321 (HSLα) and HSLβ nt 1408-1433) which were essential for the full function of HPRE. Further analysis showed that smaller fragments containing these HSL α and β, rather than the whole HPRE α and β sub-elements, are sufficient for the HPRE function (Smith et al., 1998).

1.3.4 DNA replication

Summers and Mason (1982), using DHBV, established that hepadnaviral DNA replication is accomplished by reverse transcription of an RNA intermediate. In this model, encapsidation of RNA template with the viral polymerase into the core is followed by sequential synthesis of the two viral DNA strands. Minus-strand is made first from the RNA template, accompanied by degradation of the latter, followed by the plus-strand synthesis using the newly synthesised minus-strand as a template.

1.3.4A RNA encapsidation

Assembly and initiation of reverse transcription in hepadnaviruses are coupled reactions that depend on the formation of a ribonucleotide protein complex (RNP). Both viral pol (reverse transcriptase) and a sequence on PgRNA (ε) are required for the formation of this complex (Bartenschlager et al., 1990; Hirsch et al., 1990; Pollack & Ganem 1993; Wang & Seeger 1993). The incorporation of the RNA template with the viral pol protein into viral cores is a highly selective reaction as only viral RNAs of genomic length, not subgenomic or cellular RNAs, are efficiently incorporated into virions (Enders et al., 1987). Moreover, only the 5' copy of ε within the terminal redundancy of PgRNA serves as the functional encapsidation signal, however, it is still unclear how the packaging machinery accomplishes this discrimination (Hirsch et al., 1991; Pollack & Ganem 1993).

Specific ε recognition is a function of the pol protein, first suggested when ORF P-mutant viruses were shown to produce empty capsids (Hirsch et al., 1990; Bartenschlager et al., 1990). Site-specific binding of Pol to ε was later demonstrated *in vitro* (Pollack & Ganem 1994; Wang et al., 1994a). This reaction was shown to depend on host factors such as heat shock protein HSP90 and P23 (chaperone partner for HSP90) in addition to ATP hydrolysis. A multi-component chaperone complex in an energy-dependent process was therefore proposed to maintain the viral pol in a conformation permissive for RNA packaging (Hu & Seeger 1996; Hu et al., 1997). Recently, human HBV pol was also shown to form a complex with HSP90, as DHBV did, where C-terminal regions of the TP and RT domains interacted independently with HSP90 (Cho et al., 2000).

1.3.4B DNA synthesis

DNA replication starts with binding of the viral polymerase (Pol) to the ε bulge at the 5' end of PgRNA. The Pol then primes DNA synthesis, using a tyrosine in its own aminoterminal protein domain (TP) as a primer and ε as a template. After three or four nucleotides the DNA synthesis is arrested (Fig 1.4A). Further DNA synthesis requires three switches; one during minus-strand synthesis and two during plus-strand synthesis.

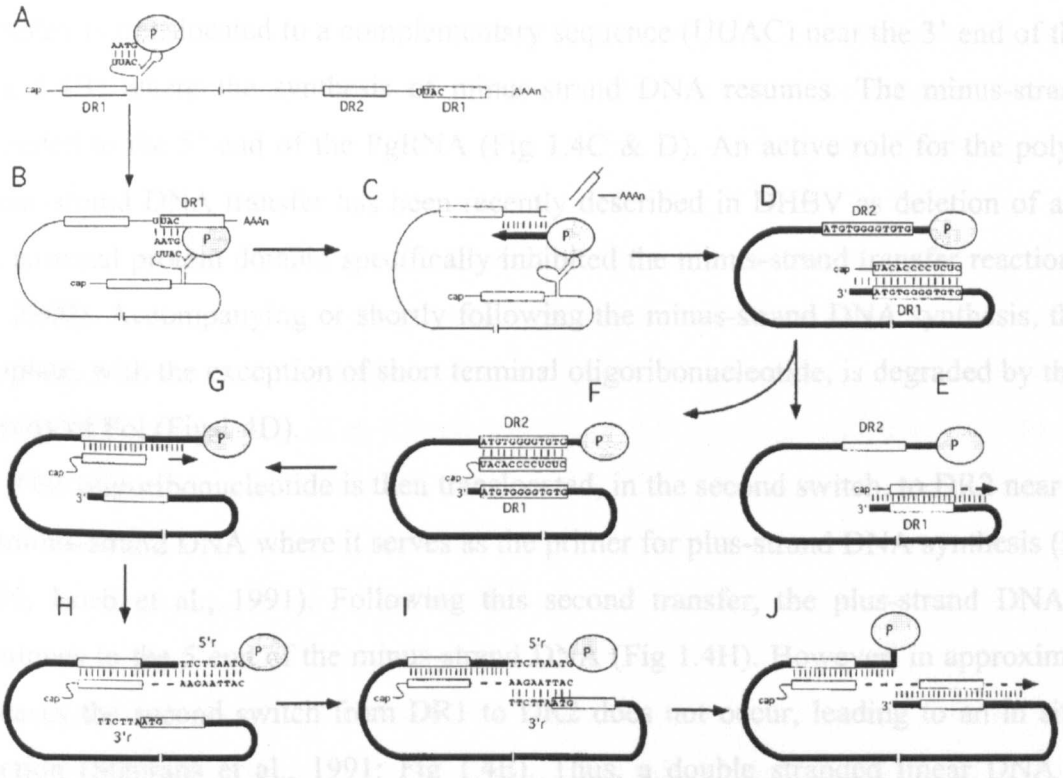


Figure 1.4: HBV genome replication

The thin line stands for the PgRNA, dashed line for the plus strand and bold line for the minus strand. The direct repeats are represented as boxes labeled DR1 and DR2. The UUAC involved in the minus strand template switch are shown. The shaded circle represents the viral P protein, which binds the ε bulge at the 5' end of PgRNA. The sequences of the terminal redundancy of minus strand DNA (shown as 5'r and 3'r). Basepairing is shown as hatch marks. (B) Minus strand template switch. (C) Elongation of the minus strand. (D) Completion of minus strand synthesis and generation of the plus strand primer. (E) Generation of duplex linear genome. (F) Plus strand primer translocation. (G) Initiation of plus strand synthesis. (H) Plus strand synthesis continues to the 5' terminus of the minus strand DNA. (I) Circularisation template switch. (J) Generation of a relaxed circular DNA genome. (Taken from Havert & Loeb, 1997)

The first template switch occurs shortly after the priming reaction. The primed Pol complex is translocated to a complementary sequence (UUAC) near the 3' end of the PgRNA (Fig 1.4B) where the synthesis of minus-strand DNA resumes. The minus-strand is then extended to the 5' end of the PgRNA (Fig 1.4C & D). An active role for the polymerase in minus-strand DNA transfer has been recently described in DHBV as deletion of aa 79-88 in the terminal protein domain specifically inhibited the minus-strand transfer reaction (Gong et al., 2000). Accompanying or shortly following the minus-strand DNA synthesis, the pgRNA template, with the exception of short terminal oligoribonucleotide, is degraded by the RNaseH activity of Pol (Fig 1.4D).

This oligoribonucleotide is then translocated, in the second switch, to DR2 near the 5' end of minus-strand DNA where it serves as the primer for plus-strand DNA synthesis (Lien et al., 1986; Loeb et al., 1991). Following this second transfer, the plus-strand DNA synthesis continues to the 5'end of the minus-strand DNA (Fig 1.4H). However, in approximately 10% of cases the second switch from DR1 to DR2 does not occur, leading to an in situ priming reaction (Staprans et al., 1991: Fig 1.4E). Thus, a double stranded linear DNA (dslDNA) genome, rather than a relaxed circular DNA (rcDNA), is produced.

A third template switch occurs once the plus-strand DNA synthesis reaches the 5' end of minus-strand DNA. The minus-strand DNA template contains a 7-9 nt terminal redundancy (r). This redundancy is required for the third template switch in which the 3' end of the nascent plus-strand is translocated to the 3' end of minus-strand (Loeb et al., 1997: Fig 1.4I) to circularize the genome and permit resumption of the plus-strand synthesis. The synthesis of plus-strand is only partially completed resulting in a noncovalently closed, partially double stranded, circular DNA genome (rcDNA).

It is noteworthy that other sequences, distinct from DR1, DR2, 5' and 3' copies of (r), have been shown to be required for the primer translocation and genome circularisation. These cis-acting sequences have been mapped to three different regions of the DHBV genome: 3E region, near the 3' end of minus-strand template; M region, near the middle of the minus strand; and 5E region which is located 3' of the DR2 on the minus strand. Although the exact mechanism remains unclear, these sequences have been proposed to establish a template conformation that is supportive for efficient template switching during plus-strand synthesis (Havert & Loeb, 1997). Moreover, changing the site of initiation of plus-strand DNA synthesis, using a new position relative to the 5' end of the template, has been shown to inhibit

the subsequent template switch and genome circularisation (Loeb et al., 1998). It seems therefore, that there are several poorly identified factors that could influence the mechanisms responsible for primer translocation and genome circularisation. These need to be elucidated.

1.3.4C Viral Assembly

Nucleocapsids containing rcDNA can be either enveloped at an internal cellular membrane and actively secreted as mature virions or transported back into the nucleus, where the genome is repaired to yield cccDNA to amplify the intracellular genome pool (Tuttleman et al., 1986). The destiny of nucleocapsids, either disintegration and release of the genome or envelopment, is regulated. In the DHBV model, it was demonstrated that disintegration and genome amplification prevail during the early phase of infection, whereas later, genome amplification ceases and envelopment of capsids dominate, yielding mature virions (Lenhof & Summers 1994).

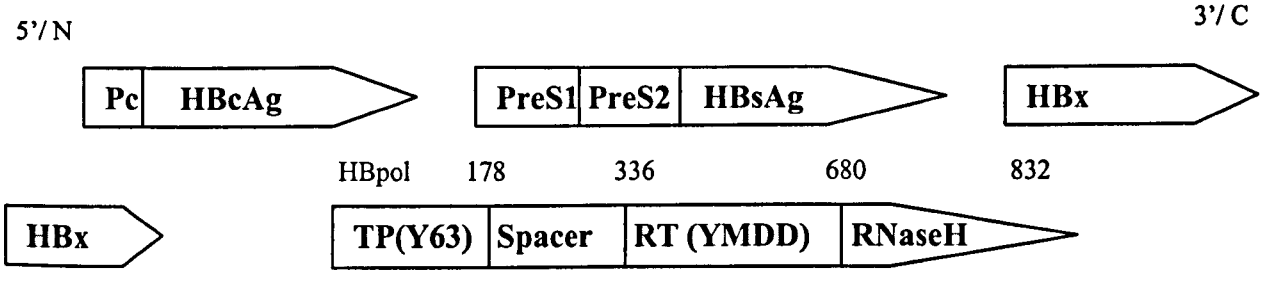
Moreover, the newly formed cytosolic-capsids were found incompetent for envelopment as they contain PgRNA and not the DNA genome. It is suggested that a maturation step or signal that is linked to the synthesis of the DNA genome makes the nucleocapsid ready for envelopment (Gerelsiakhan et al., 1996; Wei et al., 1996; Koschel et al., 2000). In contrast, empty and DNA-containing core particles were shown recently to bind equally to the envelope protein domains (Hourioux et al., 2000).

LHBs, in addition to SHBs, is required for the assembly and production of mature virions; however, most of pre-S1 region is dispensable for this process as demonstrated by experimental mutagenesis (Bruss & Ganem 1991a, b; Bruss & Thomssen, 1994). Poisson et al (1997) suggested the involvement of the first 8 N- terminal aa of pre-S2, in addition to the 13 C-terminal aa of pre-S1, in the envelope-nucleocapsid interaction, as a peptide corresponding to this region showed the greatest binding affinity to the nucleocapsid. This was further supported by le Seyec et al. (1998), who showed that the first 5 aa of pre-S2 were essential for virion export and the domain required for this process should be extended to the N-terminal of pre-S2. Furthermore, it has been suggested that the cytosolic S loop, aa 29- 79, mediates the contacts between the nucleocapsid protein and the envelope. A synergistic effect of both SHBs and LHBs proteins during HBV assembly has been proposed (Tan et al., 1999; Löffler-Mary et al., 2000).

HBV core gene mutations that may alter the capsid particle, which interact with surface proteins during envelopment, have also been reported to block the nucleocapsid envelopment (Koschel et al., 2000). Thus, there are several factors controlling the envelopment of core particles and the ones identified to date are the infection phase and core/ surface proteins-interactions.

1.4 HBV VIRAL PROTEINS

At least seven different proteins are synthesised by the cell which is infected by HBV: the L, M, S hepatitis B surface proteins, the hepatitis B core and e proteins, the RNA-dependent DNA polymerase and the X protein (Figure 1.5).



genotype	PreC	HBcAg	PreS1	PreS2	HBsAg	HBpol (YMDD)*	HBx
A	29	185	119	55	226	845 (M552)	154
B	29	183	119	55	226	843 (M550)	154
C	29	183	119	55	226	843 (M550)	154
D	29	183	108	55	226	832 (M539)	154
E	29	183	118	55	226	842 (M549)	154
F	29	183	119	55	226	843 (M550)	154
G	29†	195	118	55	226	842 (M549)	154

Figure 1.5: Overlapping and total length of HBV viral proteins in different genoptypes.

*: Positions are highlighted where variability is observed between different genotypes.

†: Genotype G may contain translational stop codons, affecting the precore region length.

1.4.1 Hepatitis B surface proteins

Only one ORF encodes for these proteins. LHBs covers the entire reading frame, MHBs initiates at an internal site and the 226 amino acid sequence of SHBs starts further downstream (Heermann et al., 1984). The sequence between the first and second start site is termed pre S1 and is one of the most variable regions of the genome; the sequence between the second and the third start site is termed preS2 (Peterson, 1981; Stibbe & Gerlich, 1983). Properties and functions of hepatitis B surface proteins are shown in Table (1.1). Surface proteins are believed to mediate the attachment of virions to the hepatocyte membrane and to regulate virus assembly and amplification of the supercoiled DNA (see section 1.3.1 & 1.3.4C). They are also used for the production of plasma-derived and recombinant vaccines (Krugman & Giles, 1973; McAleer et al., 1984) as they are highly antigenic and stimulate the production of virus-neutralising antibodies.

Table 1.1: Properties and functions of hepatitis B surface proteins

	SHBs	MHBs	LHBs
Genes	S gene	Pre S2 & S	Pre S1, Pre S2 & S
Amino acid Length	226	281	389- 400
Molecular weight	p24 & gp27	gp33 & gp36	p39 & gp42
Function	Immune target Receptor binding Virion assembly	Immune target Receptor binding? Virion assembly	Immune target Receptor binding Virion assembly Secretion

SHBs, which is 226 aa long, exists in two forms: p24, unglycosylated and gp27, monoglycosylated with a complex glycan residue at amino acid Asn-146. Both forms are equally represented in the viral envelope (Peterson 1981, 1987; Heerman et al., 1984). Both N and C termini of SHBs are believed to be oriented to the ER lumen (Eble et al., 1987; Bruss & Ganem, 1991a; see Figure 1.6a). Five regions, three hydrophobic and two hydrophilic, have been identified so far in SHBs (Prange & Streeck, 1995).

The first two hydrophobic regions constitute ER signal sequences (I, aa 11-28; II, aa 80-98) that initiate the translocation of N-terminal sequences and anchor the protein within the ER membrane. The second region has also been shown to be essential for functional organisation of the S protein (Bruss & Ganem, 1991a, b). The C-terminal region, aa 169-210, represents the third hydrophobic domain. In contrast to the extreme sensitivity of the N-terminus to deletion, the C-terminus seems to tolerate much larger deletions. An HBsAg protein with a 51 aa C-terminal deletion could be assembled and secreted (Prange et al., 1995). However, Bruss and Ganem, (1991a) found that the C-terminally truncated S protein (lacking 51 aa) could only be secreted when co-expressed with the wild type.

It is believed that the S region is essential for virus assembly, as cells that synthesise SHBs exclusively can form regular 22-nm particles (Liu et al., 1982). Secretion of 22-nm SHBs particles occurs via the Golgi apparatus after aggregation of SHBs molecules. The S proteins in secreted viral and subviral particles are extensively cross- linked by disulphide bridges; such cross-linking has been shown to occur concomitantly with the assembly and budding of the particles (Huovila et al., 1992). Of 14 cysteine residues presumably involved in this process, four are located in the first hydrophilic region and eight are located in the second hydrophilic region (Mandart et al., 1984; Norder et al., 1992a).

The first hydrophilic region, aa 29- 79, is on the inner cytoplasmic surface of the viral envelope and thus may facilitate core particle envelopment during maturation of the virion (Prange et al., 1995; Löffler-Mary et al., 2000). Deletion mutants reveal that this region tolerates only minor structural changes (Prange et al., 1992). Three of the four cysteine residues in this region, Cys 48, 65 and 69, are conserved among all hepadnaviruses and were shown to be essential for secretion. However, none of these essential cysteines are important for dimerisation as none of the single or multiple cysteine mutants were found to accumulate in a monomeric form (Mangold and Streeck 1993).

The second hydrophilic region (aa 99- 160) is exposed on the outer surface of secreted viral and subviral particles. It contains the major group and subtype-specific antigenic determinants (Le Bouvier, 1971; Bancroft et al., 1972; Norder et al., 1992a). This region has a highly complex structure and is very cysteine dense; eight of the 14 cysteine residues in HBsAg are located here and all of them are highly conserved among mammalian hepadnaviruses (Mandart et al., 1984; Norder et al., 1992a, 1993). Disulphide bonds between these residues, whether intra- or inter-molecular, are likely to contribute to a highly complex

structure. Antigenicity of HBsAg is dependent upon this complex structure and substitution of many of these cysteine residue results in loss of immunoreactivity (Mangold et al., 1995; Bruce & Murray 1995). However, not all cysteines are equally important for antigenicity and/ or secretion (Mangold and Streeck, 1993; Mangold et al., 1995) (Table 1.2).

Table 1.2: The effect of HBsAg cysteine-residues changes according to previous mutational analysis studies.

Cys/ mutant	Secretion *	Antigenicity†	Employed Ab	Reference
124 Cys/ Ser	ND	+	pAb	Ashton-Rickardt & Murray (1989)
147 Cys/ Ser	ND	+		
107 Cys/ Ala	+	-	mAb	Mangold & Streeck (1993)
121 Cys/ Ser	++	+		
121/ 124 Cys/ Ala	++	-		
137/138/139 Cys/ Ala	+	-		
147/ 149 Cys/ Ala	+	+		
107 Cys/ Ala	+	ND	mAb	Mangold et al., (1995)
137 Cys/ Ala	++	+		
138 Cys/ Ala	+	ND		
139 Cys/ Ala	++	+		
137/ 139 Cys/ Ala	-	ND		
147 Cys/ Ala	++	++		
149 Cys/ Ala	+	++		
137 Cys/ Ser	ND	-	pAb	Bruce & Murray (1995)
138 Cys/ Ser	ND	+		
139 Cys/ Ser	ND	-		
149 Cys/ Ser	ND	-		

* Secretion: ++, efficient; + reduced secretion; - mostly retained into the cells.

† Antigenicity: ++, reactive; +; reduced reactivity; -, non-reactive. ND, not done.

PAb: polyclonal antibody; mAb: monoclonal antibody.

Cys: cysteine; Ala: alanine; Ser: serine.

MHBs, which is encoded by the preS2 and S regions (see Figure 1.6b), is 281 aa long and can be found in two glycosylated forms: gp33, with Asn at aa-4 of the preS2 region and gp36, with Asn at aa-146 within the S region (Peterson, 1981). The preS2-glycosylation site is highly conserved, so it has been proposed that the N-linked glycan may play a role in virus adsorption to the hepatocyte surface (Pontisso et al., 1989a; Gerlich et al., 1993). Additional candidates for mediation of adsorption of preS2 region to human hepatocytes were discussed in section 1.3.1. The function of M protein is not well understood. Inhibition of M protein expression had no effect on viral morphogenesis (Bruss & Ganem 1991a). MHBs is unlikely to be involved in viral infectivity (Fernholz et al., 1993). However, the first 5- 8 aa at its N-terminal were shown recently to be involved in the envelope-nucleocapsid interaction (see section 1.3.4C).

LHBs is 389- 400 aa long due to a variable preS1 domain (108-119 aa), and this is dependent on viral genotype. PreS1 may hide the preS2 and S regions (Bruss et al., 1994; see Figure 1.6c). LHBs accounts for only 1-5% of surface proteins in subviral particles and exists in two forms, p39 and gp42. LHBs was shown to be modified by N-terminal myristylation (Persing et al., 1987). Myristylation is not required for efficient virion assembly but is required for viral infectivity and perhaps anchoring the LHBs in the viral-envelope lipoprotein bilayer (Kuroki et al., 1989; Gripon et al., 1995; Bruss et al., 1996). LHBs is co-secreted with nucleocapsids from the ER (Ueda et al., 1991). When LHBs is over-expressed compared to SHBs, it inhibits secretion of the latter (Chisari et al., 1986).

Amino acids 21-47 of the preS1 region allow specific binding of HBV to the human hepatocyte membrane (Petit et al., 1992), suggesting a potential receptor function for LHBs (see section 1.3.1). Besides its role in infection, LHBs is required for the assembly and production of mature virions (see section 1.3.4C). However, this function requires display of pre-S1 epitopes on the cytosolic side. As a consequence of this dual function, it has been suggested that a fraction of L polypeptides should keep the pre-S1 domain inside the virion (i-preS form). The remaining L polypeptides undergo a conformational shift (a post-translational translocation) to transfer the pre-S1 binding receptor region across the lipid bilayer to the exterior of the virus particle (e-preS form), displaying a topology similar to the M protein (Bruss et al., 1994). This transfer appears to occur as viruses mature during their passage along the secretory pathway (Prange & Streeck 1995; Guo & Pugh 1997).

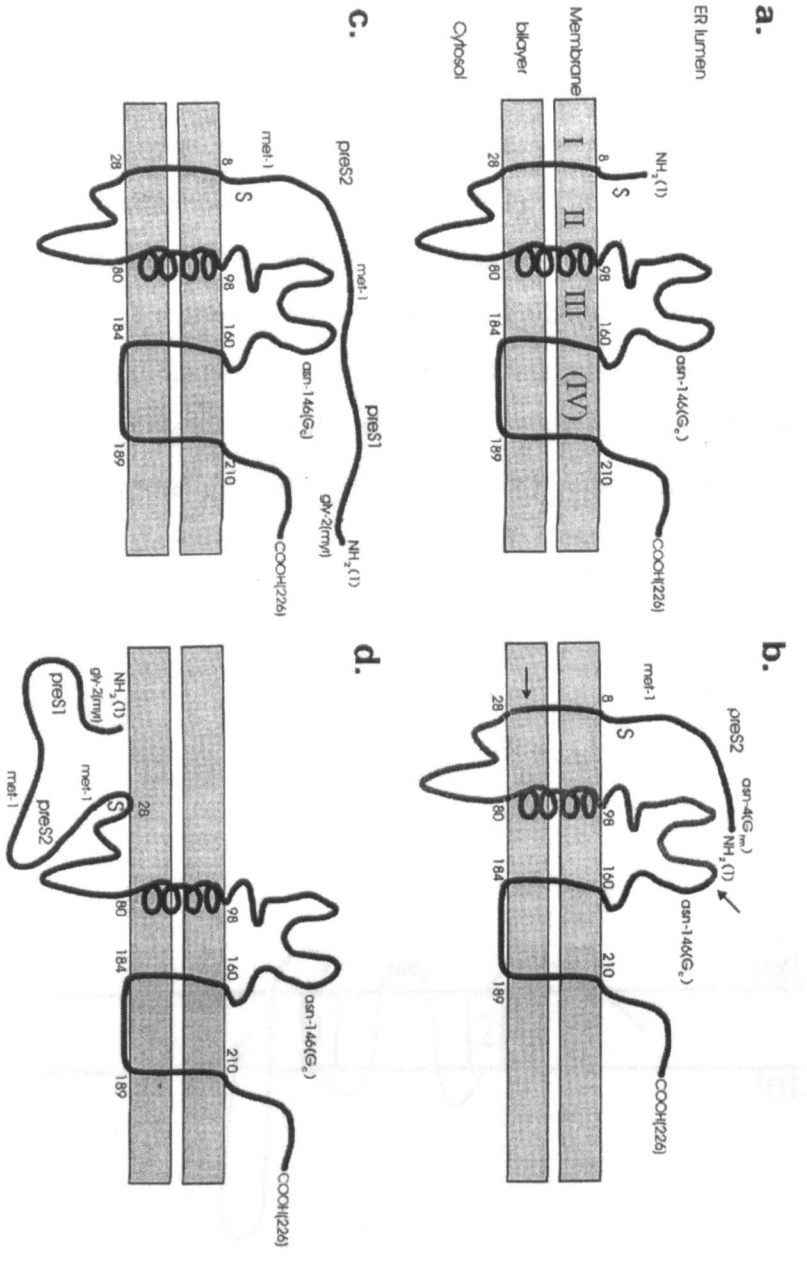


Figure 1.6: The two-dimensional model of HBV surface proteins.

(a) SHBs, (b) MHBs, (c & d) LHBs with alternative membrane topology of N terminus. Arabic numerals reflect amino acid positions and Roman numerals reflect transmembrane domains. Gly: glycine; met: methionine; asn: asparagine; myr: myristyl residue. (Taken from Caselmann, 1996)

The lack of glycosylation of LHBs at Asn-4 in the preS2 region gave further support to this model with the cytoplasmic location of the N terminus (Ostapchuk et al., 1994; Figure 1.6d). Recently, a partial translocated topology, as an intermediate configuration, has also been suggested in DHBV (Guo & Pugh, 1997). A model for pre-S translocation has been proposed based on the presence of an aqueous channel in the virus envelope. This model recalls the early postulation of Stirck et al. (1992). The channel would most probably be created by oligomerisation of transmembrane domains in the S region (Guo & Pugh 1997; Grgacic et al., 2000). Moreover, the cytoplasmic loop between transmembrane domains 1 and 2 (TM1 and TM2) was shown as membrane embedded and probably buds to the particle surface. Accordingly, a highly folded L molecule with five membrane-spanning domains has been suggested with a possible role for this 49 aa-long region, S aa 30-79, in the formation of the presumed preS translocation channel (Stirck et al., 1992; Grgacic et al., 2000; Figure 1.7).

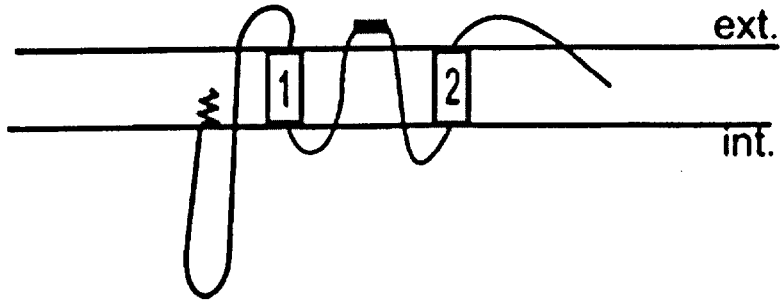


Figure 1.7: Topology of DHBV L protein with the loop region between TM1 and TM2.
The loop between TM1 and TM2 (equivalent to first hydrophilic region in HBV) is shown as membrane embedded with part of the loop, shown as a black bar, exposed to the particle surface. (Taken from Grgacic et al., 2000)

1.4.2 Hepatitis B virus core protein

The hepatitis B core protein (HBcAg; p21) is translated from the pregenomic mRNA and is 183 amino acid long in most genotypes. Two major domains exist within core protein: the assembly-domain, which includes the N-terminal up to aa 144; and the arginine-rich domain, located at the C-terminal, probably situated inside the particle (Seifer & Standaring 1994; Zlotnick et al., 1997).

The highly basic C-domain, starting at aa 150, acts as a binding domain which is required for RNA encapsidation and proper reverse transcription (Hatton et al., 1992; Nassal 1992). This C-terminal domain has been shown to be phosphorylated via an endogenous protein kinase (Albin & Robinson, 1980). The relevance of this phosphorylation has not been clarified. A possible role in nuclear transport of virion DNA after nucleocapsid disassembly or production of an essential undefined signal required for virion maturation have been suggested (Kann & Gerlich, 1994; Kann et al., 1997; Qiao et al., 1999: see Section 1.3.2).

Assembly of core proteins into icosahedral particles necessitates the formation of dimer subunits, which are the only detectable assembly intermediates (Zhou & Standring 1992; Chang et al., 1994). Functional analyses of core protein variants showed that two regions (aa 78- 117 and 113-143) are required for the dimerisation of core monomers and for the subsequent assembly into core particles (Böttcher et al., 1997; Conway et al., 1997; Zlotnick et al., 1997; Konig et al., 1998: Figure 1.8). In this model, core protein contains four α helices and each monomer should have at least two interfaces, one for dimerisation and the other to mediate the multimerisation. Cysteine cross-links at aa 48, 61 and 183, also have a role in the dimerisation and assembly process (Nassal, 1992; Zheng et al., 1992).

Two classes of core particle have been identified by cryoelectron microscopic analysis; the first class consists of 120 dimer subunits, which are assembled into a large shell with a diameter of 36-nm and a triangulation number of 4 (Crowther et al., 1994). A smaller shell containing 90 dimer subunits, with a triangulation number of 3, constitutes the second class; these are formed when core particles with small truncations at their C-terminus are expressed (Zlotnick et al., 1996). Core assembly can tolerate C-terminal truncations up to aa 144 or 140, however, no particles can be detected with further truncated and poorly secreted variants (Brinbaum & Nassal 1990; Zlotnick et al., 1996). Capsids containing full-length core proteins are mostly stable, probably due to the additional interactions between the basic C-

terminus and encapsidated RNA, while those made of truncated variants are unstable *in vitro* (Brinbaum & Nassal 1990; Wingfield et al., 1995).

Core particles are strongly immunogenic, as the HBcAg B cell immunodominant epitope (aa 74-89) is probably located at or near the spikes on the particle surface (Salfeld et al., 1989; Crowther et al., 1994; Conway et al., 1998). Antibodies against HBcAg appear early in acute infection (anti-HBc-IgM) and anti-HBc-IgG usually remains detectable for life (Cohen, 1978). The cellular immune response against HBcAg is considered a major pathogenic mechanism of HBV-induced liver damage (Ferrari et al., 1988) (see Section 1.6).

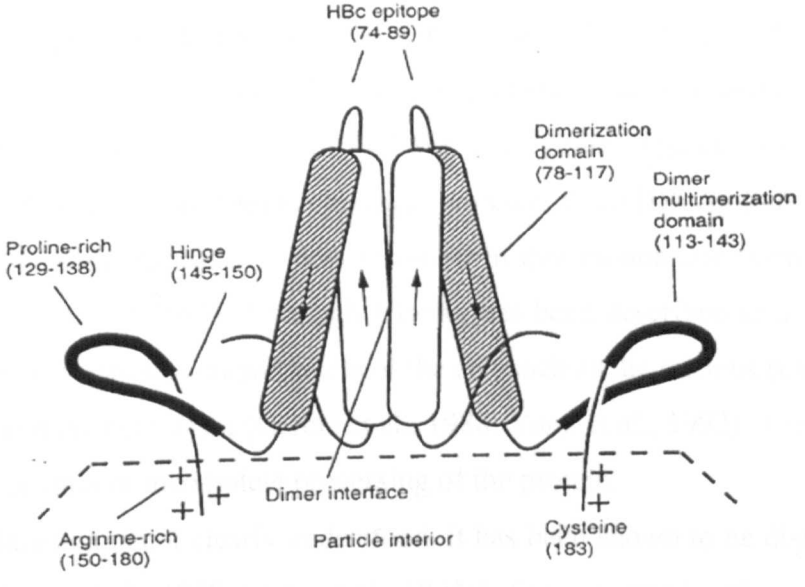


Figure 1.8: Three-dimensional model of the core protein dimer based on electron cryomicroscopy
Central α helices (boxes) and regions employed in core protein dimerisation (gray) and dimer multimerisation (black) are shown. The assignment of functional domains and amino acids positions is speculative. The folding of the C terminus within the interior of core particle is currently unclear. (Taken from Günther et al., 1999).

1.4.3 Hepatitis B virus eAg

The hepatitis B virus e antigen (HBeAg) is produced by proteolytic cleavage of a p25 precursor protein that is translated from the start codon of the preC region. The p25 protein contains an extra 29 amino acids at the N-terminus of core protein. The first 19 N-terminal aa of these 29 constitute a signal peptide sequence that directs the precursor protein into the ER (Ou et al., 1986; Carlier et al., 1995). An intermediate p22 protein is produced by the cleavage of this 19-aa signal peptide which is then either translocated into the ER lumen or released back into the cytoplasm (Garcia et al., 1988). The translocated p22 protein is further processed at its carboxyl terminus within the Golgi compartment to produce a soluble protein (p17) that is secreted and detected in the circulation as HBeAg (Takahashi et al., 1983; Wang et al., 1991a). HBeAg is present in serum during active infection and generally correlates with the degree of viraemia.

This soluble protein is found mostly as a monomer and is antigenically different from core protein (HBeAg and HBcAg), though they appear to share major T-cell epitopes (Bertoletti et al., 1993). A hydrophobic triad motif (WLW) and cysteine residue within the ten pre-core amino acids left after signal peptide cleavage, probably force HBeAg into a conformation that is incompatible with aggregation, thus resulting in this monomeric form (Wasenauer et al., 1992; Nassal & Rieger 1993). Although HBeAg has been described as a secretory protein, it can be detected in various compartments of the cell such as the nucleus (Ou et al., 1989; Yang et al., 1992) and the cytoplasm (Garcia et al., 1988; Yang et al., 1992). This is probably due to lack of translocation or incomplete processing of the protein.

HBeAg function is not clearly understood. It has been shown to be dispensable for *in vivo* infections (Chang et al., 1989; Chen et al., 1992a). Experiments in mice suggest that HBeAg, acting as an immunomodulatory protein, may cause depletion of Th1 helper cells, thereby suppressing the cytotoxic T-lymphocyte (CTL) response to the infected hepatocytes (Milich et al., 1990, 1998). Moreover, Scaglioni et al, (1997), showed that levels of HBV replication are suppressed by the HBeAg overexpression, probably due to formation of hybrid particles that are unable to support encapsidation. This was further supported by an early report of Lamberts et al. (1993), where high levels of viral replication were detected when a precore-minus genome was used in transfection assays. Similar findings have been reported in an HBV transgenic mouse model (Guidotti et al., 1996a).

1.4.4 Hepatitis B virus polymerase

The hepatitis B viral polymerase (pol) is translated from PgRNA (Ou et al., 1990). Pol has four domains which are arranged from the N to the C terminus as follows: terminal protein (TP) which is covalently linked to the 5' end of the minus-strand and serves as a primer for reverse transcription; a spacer region which can be deleted without loss of enzyme activity; DNA polymerase/ reverse transcriptase (pol/ RTase); and RNase H activity (Bösch et al., 1988; Radziwill et al., 1990; Lee et al., 1997b: see Figure 1.5). Pol/ RTase consists of two subdomains; the C-terminal one contains the reverse transcriptase activity as it has the conserved YMDD motif (Radziwill et al., 1990). Sequence analysis of the mammalian and avian Pol ORF revealed highly conserved regions among hepadnaviruses (Chen et al., 1992b, 1994). A change of Asp to His at residue 699, one of the conserved aa in RNase H region, substantially abrogated the activity of this domain (Lee et al., 1997b).

The fact that HBV P protein is difficult to express in an active form in a recombinant system has hampered its analysis for many years. Thus, DHBV has been the most frequently used model for the investigation of hepadnaviral replication. DHBV has been expressed using *in vitro* translation or the yeast retrotransposon TyI system (Tavis & Ganem, 1993; Wang & Seeger, 1992). Both systems produced pol that showed accurate protein priming and reverse transcriptase activity (Wang & Seeger, 1993; Tavis et al., 1994). However, for unknown reasons, these systems were not applicable to human HBV. Nevertheless, functional HBV pol has been expressed recently using the baculovirus expression system and rabbit reticulocyte lysate system (Lanford et al., 1995; Kim & Jung, 1999).

Expressing HBV pol using the baculovirus system, Urban et al, (1998a) showed metal ion preferences for both the protein priming and reverse transcription activities; protein priming was enhanced by manganese while reverse transcription was dependent on magnesium. In a recent study of the effect of deletion mutants, polymerase activity was still exhibited by a smaller region than the polymerase domain, and RNase H domain deletion was more deleterious than the deletion of TP or spacer on pol activity (Kim et al., 1999). Observations by Lee et al. (1997b) gave further support to these findings, as mutation of highly conserved aa in this domain was shown to diminish or even abrogate the Rnase H activity.

1.4.5 The hepatitis B virus X protein

The hepatitis B virus X protein (HBx), which has a mass of 16.5-kDa and is 154 aa long, is encoded by the HBV X gene which is well conserved among mammalian hepadnaviruses (Haruna et al., 1991; Wang et al., 1991b). Translation usually starts at the first of the three AUGs at the 5'end of the X mRNA. HBx is mostly cytoplasmic (Doria et al., 1995; Sirma et al., 1998). HBx has not been detected in patients' sera, but circulating anti-HBx antibodies in HBV-infected humans and naturally infected animals suggest its expression (Persing et al., 1986; Pfaff et al., 1987; Vitvitski et al., 1990). Anti-HBx antibodies were also detected in liver samples obtained from WHV-infected woodchucks (Dandri et al., 1996).

HBx is a multifunctional protein with a well-described activity affecting transcription (Andrisani & Barnabas 1999) cell growth (Benn & Schneider, 1995) and programmed cell death (Chirillo et al., 1997). As HBx does not directly bind to DNA, it is believed that its activity is mediated via protein-protein interactions. For instance, HBx has been shown to enhance transcription through AP-1 (Natoli et al., 1994). Several possible cellular targets have also been identified; these include members of the CREB/ATF family (Maguire et al., 1991; Williams & Andrisani, 1995), the TATA-binding protein (Qadri et al., 1995), the UV-damaged DNA-binding protein (Lee et al., 1995), and the proteasome complex (Huang et al., 1996a). HBx has also been described to interact with p53 and inhibit its function (Wang et al., 1994b: Truant et al., 1995).

In addition, HBx possess aa sequence homology to the functional domains of Kunitz-type serine proteases inhibitors and mutation of this putative motif inactivates the transactivation function of HBx (Arii et al., 1992). Using cDNA microarray analysis, Han et al. (2000) examined the effect of HBx on the transcriptional regulation of 588 cellular genes and showed its selective action in human liver cells. As the functional complex between HBx and cellular transcriptional machinery has not been determined *in vivo* and HBx activates a multitude of promoters, HBx has been suggested to act through an indirect mechanism which will alter the capacity of cellular transcription (Benn & Schneider, 1995; Zhang et al., 2000).

The importance of HBx in the life cycle of HBV is well described but the underlying molecular function of HBx remains unclear. X-defective virus is unable to initiate infection *in vivo* as C-terminal truncations in the related WHV have been shown to decrease viral replication *in vitro* and to inhibit the establishment of infection *in vivo* (Chen et al., 1993a; Zolium et al., 1994). A novel hepatitis B binding protein (XIP), which specifically complexes

with the C-terminus of HBx, was recently shown to negatively regulate the HBx and hence virus replication probably through the endogenous viral CP/enhancer elements (Melegari et al., 1998a). In agreement with these findings, the transcription of HBV CP was shown to be enhanced by the interaction of HBx and CCAAT/enhancer-binding protein α (C/EBPα) (Choi et al., 1999). Moreover, EnI/XP and EnII/CP were differentially regulated by the synergistic effect of HBx and C/EBPα. In contrast, the HBx and C/EBPβ interaction was reported to have no functional effect *in vivo* (Barnabas et al., 1997).

1.5 HBV VARIANTS

Extreme variability of some viruses may be a consequence of an unusually high mutation rate (Holland et al., 1982; Domingo et al., 1985). The relatively high mutation rate of the eukaryotic viruses is obviously important in generating a pool of mutant genomes from which new variants can emerge, however differences between viruses in their rate of variation also reflect differences in selective forces (Smith & Inglis, 1987). Variation in viral genomes is generally dependent upon rates of polymerase error and the relative influence of selection pressures. In HBV, variation probably arises due to the use of a non-proofreading reverse transcriptase enzyme in the replication cycle. However, other contributing factors have also been reported such as transcription from integrated DNA (Girones & Miller, 1989), spliced RNAs generated during viral replication (Rosmorduc et al., 1995) and interaction between normal and defective genomes (Obert et al., 1996).

HBV is a DNA virus and its genome is estimated to undergo nucleotide substitution at a rate of 1.4- 3.2 X 10^{-5} per site per year (Okamoto et al., 1987). This is much closer to RNA viruses than to DNA viruses which are relatively stable with an estimated rate of nucleotide substitution of 10^{-9} per site per year (Britten, 1986). This relatively high rate of HBV mutation may contribute to its persistence by generating a pool of variants, some of which have inactive viral epitopes and can escape the immune surveillance.

The persistence of HBV variants has been suspected for many years based on the finding of HBV DNA in serum and liver from HBsAg- negative patients (Wands et al., 1982). A Chinese study, using PCR in HBsAg-/anti-HBc+ people, showed that 3% of the Chinese general population fell into this category (Luo et al., 1991). Mutations could be responsible for the diminished rate of replication and consequently for the low-titre immunologically negative HBV infection (Preisler-Adams et al., 1993). Alternatively, they may affect the "a"

determinant, the major B cell epitope cluster in S protein, and therefore be responsible for antigenic change in the HBsAg resulting in failure to react in commercial HBsAg assays (Coleman et al., 1999).

Two classes of HBV variants can be generally identified: class I variants, which occur naturally and have been selected over years dependent on the genetic background of the host eg. subtype defining determinants. In contrast, class II variants have been selected by human intervention. Vaccination (Carman et al., 1990; Karthigesu et al., 1994; Hsu et al., 1999) and administration of immunoglobulin antibodies after liver transplant (Cariani et al., 1995; Hawkins et al., 1996; Protzer-Knolle et al., 1998) or during antiviral therapy (Tipples et al., 1996; Ling et al., 1996) have all been reported. This section will describe some of the important HBV variants along with any significance that has been attributed to them.

1.5.1 Small envelope protein variants

The "a" determinant was believed to be situated between aa 124-147 (Brown et al., 1984) but recent evidence, from observed natural and medically induced variants, suggests that this epitope cluster could be extended up and downstream to include the entire major hydrophilic region, MHR (Wallace & Carman, 1997; Figure 1.9).

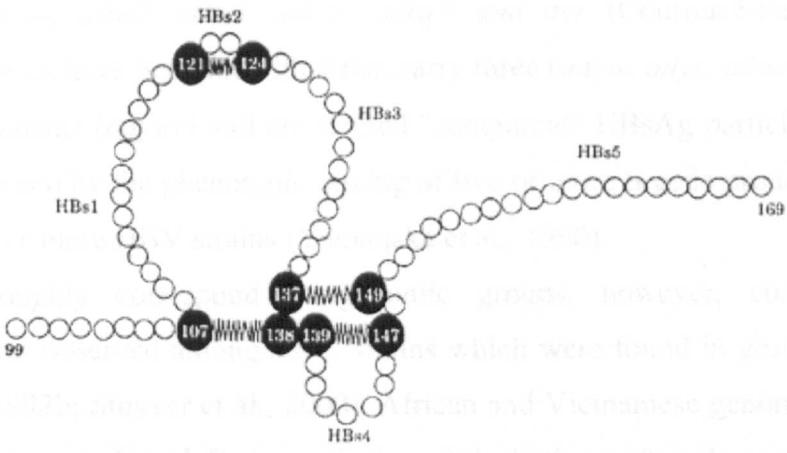


Figure 1.9: Proposed model of major hydrophilic region (MHR)
The five proposed antigenic regions are labeled HBs1 through HBs5. Cysteine to cysteine disulphide bridges are also shown. (Taken from Wallace & Carman 1997)

In this model, the suggested disulphide bridges included (C121 to C124), (C139 to C147), (C107 to C138) and (C137 to C149). Moreover, Wallace & Carman (1997) proposed that the epitopes of HBsAg MHR cluster into five regions: HBs1 upstream of aa 121; HBs2 between aa 121 and 124; HBs3 between aa 125 and 137; HBs4 between aa 139 and 147 or 149 and HBs5 from 148 or 150 downstream to 169. HBs2 and HBs4 can also be considered as a single antigenic complex as they are probably spatially close. Furthermore, variants of HBsAg are clinically relevant and can be detected in many scenarios: in samples that react poorly in diagnostic HBsAg assays (Carman et al., 1997b; Coleman et al., 1999); after or during monoclonal antibody or hyperimmune globulin (HBIG) (McMahon et al, 1992; Hawkins et al., 1996; Sterneck et al., 1997); after vaccination (Carman et al., 1990; Okamoto et al., 1992; Karthigesu et al., 1994; Hsu et al., 1997); and during chronic infection with or without immunosuppression (Moriyama et al., 1991; Kidd-Ljunggren et al., 1995).

1.5.1A Natural variants

Subtype defining determinants: HBsAg carries a group specific determinant "a", common to all subtypes and two sets of subtype determinants, *d/y* and *w/r*, which are mutually exclusive (Le Bouvier, 1971). Both *d* to *y* and *w* to *r* changes are dependent on lysine to arginine substitution at amino acids 122 and 160 respectively (Okamoto et al., 1987). Identification of *q* determinant (Magnius et al., 1975) and description of subdeterminants of *w* determinant, *w1-w4* (Couroucé et al., 1976), led to a nine member classification namely *ayw1, ayw2, ayw3, ayw4, adw2, adw4, adrq-, adrq+ and ayr* (Couroucé-Pauty et al., 1978). Additional subtypes have been identified that carry three (*adyw, adyr, adwr and aywr*) or four subtypic determinants (*adywr*) and are termed "compound" HBsAg particles. These particles are probably formed by the phenotypic mixing of two or more S gene products in hepatocytes infected by two or more HBV strains (Yamanaka et al., 1990).

Subtypes roughly correspond to genomic groups, however, considerable genetic heterogeneity was observed among *adw2* strains which were found in groups A, B, C and G (Norder et al., 1992b; Stuyver et al., 2000). African and Vietnamese genomes encoding *ayw1* were found in groups A and B respectively, while both *ayw2* and *ayw3* strains could be allocated to group D (Norder et al., 1992b). Strains expressing *r* have only been found in group C (Okamoto et al., 1988). On the other hand, *ayw4* and *adw4* strains, which differed to a

great extent from each other and from other groups, were allocated to groups E and F respectively (Norder et al., 1992b, 1993).

Norder et al. (1992a) have defined the molecular basis for the serological heterogeneity of HBV subtypes. Residue 127 is important for the subdeterminant *w;* Pro, Thr and Leu characterise *w1/w2, w3 and w4* respectively. Residues 134, 143, 159, 161 and 168 are important for the molecular difference between *ayw1* and *ayw2*. However, these substitutions were shared between *ayw1* and *adw2*, implying that Arg was also important for *w1* expression. Moreover, the absence of the *q* determinant in *adrq-* and *adw4q-* was found to differ at the amino acid level; 159 and 177 were responsible in the former, whereas, the adjacent positions at residues 158 and 178 were identified in the latter (Norder et al., 1992a, 1993; Table 1.3).

Table 1.3: HBV genotypes and subtypes*

Geno.	Subtype	Amino acid sequences					
		120					180
A	adw2	PCKTCTTPAQG	NSMFPSCCCT	KPTDGNCTCI	PIPSSWAFAK	YLWEWASVRF	SWLSLLVPFV
	ayw1	--R--------	----------	----------	----------	----------	----------
B	adw2	----------	T---------	----------	----------	----------	----------
	ayw1	--R--------	T---------	----------	----------	----------	----------
C	adr	------I----	T---------	--S-------	---------R	F---------	----------
	ayr	--R---I----	T---------	--S-------	---------R	F---------	----------
	adrq-	------I----	T---------	--S-------	--------VR	F---------	------A---
D	ayw2	--R--------	T--Y------	--S-------	--------G-	F------A--	----------
	ayw3	--R--M-T---	T--Y------	--S-------	--------G-	F------A--	----------
E	ayw4	--R----L---	T--------S	--S-------	--------G-	F------A--	----------
F	adw4q-	-------L---	T--------S	--S-------	-------LG-	-------A--	-------Q--
G	adw2	----------	---Y------	--S-------	----------	----------	----------

*Genotypes from A-G are shown with the allocated subtypes based on S gene typing. S gene sequences between 120 and 180 are shown. Dashed line represents the amino acids that are similar to the standard sequence.

Natural S gene variants: Insertions in the S gene have been described in a few studies. Yamamoto et al. (1994) studied six HBV carriers. Substitution of Ile/Thr to Ser/ Asn at residue 126 was detected in three of them (No. 1, 5 & 6). Two carriers, no. 2 & 3, showed G145R mutation. In contrast, none of 12 clones from the last carrier (no. 4) showed mutations of codon 126 or 145, but all possessed an in-phase insertion of eight amino acids between Thr 123 and Cys 124 which constituted the largest insertion detected to date. Carman et al. (1995a) showed a two aa insertion between 122 and 123 positions (along with G145R) in a patient with fulminant hepatitis from Indonesia. Hou et al. (1995) described two patients: one of them had an insertion of two aa between codons 122 and 123 and the other had a three aa insertion between codons 123 and 124.

Point mutations are also well described. In a study from Thailand of 34 HBsAg positive patients, Kidd-Ljunggren et al. (1995) sequenced S gene region from 18 chronic carrier. There were several non-conservative point mutations in the S gene; however, two samples (No. 8 & 14) had aa changes in the "a" determinant and both were anti-HBs negative. Sample no. 8 showed G145A, while sample no. 14 showed a mixture of Gly or Arg at position 145 which was suggested as the first step in changeover from Gly 145 to Arg. In contrast, none of the 16 variants defined by Carman et al. (1997b) were in the immunodominant region of the "a" epitope cluster (aa 139- 147) and were thus considered as non-neutralising escape mutants.

Mutations in S gene co-occurring with anti-HBs antibody in sera from chronic carriers have also been described (Kohno et al., 1996; Shinji et al., 1998). G130N and G145R mutations were detected by Kohno et al. (1996), while the T/I126S mutation was found in the two carrier patients in Shinji's study. Bahn et al. (1997) described HBV variants in chronically infected children after seroconversion from HBsAg to anti-HBs, where seven out of nine children (HBsAg-ve/ anti-HBs+ve) were found to have HBV DNA. The described mutations were in codons 122, 125, 127, 131, 134, 143, 159 and 161 of the S gene. Three of these patients showed a genotype change from A (serotype *adw*) to D (serotype *ayw*).

Recently, 10 out of 42 patients (24%) with chronic hepatitis revealed mutations within the "a" determinant region associated with the presence of anti-HBs in their sera (Ogura et al., 1999). Furthermore, "a" determinant variants have been reported frequently during chronic infection as well as in presence of anti-HBs antibodies, reflecting their exposure to a sustained immune pressure (see Table 1.4) (Günther et al., 1999).

Table 1.4: Prevalence of "a" determinant variants according to course and stage of HBV infection*

Course/ Stage[1]	Prevalence of a-determinant variants (%)	Patients analysed (n)	References[3]
Chronic			
HBsAg+, HBeAg+, ALT+/-	13	45	2, 3, 12, 24
HBsAg+, HBeAg-, ALT+/-	36	21	12, 24
HBsAg+, HBeAg+/-,	30	74	5-8, 12, 14, 17, 19, 22
(ESLD/ HCC)			
HBsAg-and/or anti-HBs+	80	50	3, 9, 11, 13, 15, 21, 23, 24
Fulminant[2]	5	21	1, 4, 10, 16, 18, 20

*: A compilation of 211 data sets.

[1] HBsAg+/-: HBsAg positive/ negative patients; HBeAg+/-: HBeAg positive/ negative patients; ALT+/-: ALT elevated/normal; ESLD/ HCC; patients who developed ESLD or HCC.

[2] Adults; the mutations of strains associated with common-source epidemics were included only once in the compilation and not according to the number of infected patients.

[3] Key to References: (1) Alexopoulou et al., (1996); (2) Arauz-Ruiz et al., (1997b), (3) Asahina et al., (1996a); (4) Asahina et al., (1996b); (5) Brind et al., (1997); (6) Cariani et al., (1995); (7) Carman et al., (1996); Ghany et al., (1998); Guptan et al., (1996); (10) Hasegawa et al., (1994); (11) Kato et al., (1996); (12) Kidd-Ljunggren et al., (1995); (13) Kohno et al., (1996); (14) McMahon et al., (1992); (15) Moriyama et al., (1991); (16) Ogata et al., (1993a); (17) Pollicino et al (1996); (18) Pollicino et al (1997); (19) Protzer-Knolle et al., (1998); (20) Sterneck et al., (1996); (21) Suzuki et al., (1995); (22) Tai et al., (1997); (23) Yamamoto et al., (1994); (24) Zhang et al., (1996). (modified from Günther et al., 1999)

It is likely, therefore, if these natural variants can escape neutralising anti-HBs, they may infect vaccinated individuals and become more common as vaccination coverage increases, raising potential problems for successful vaccination. However, follow-up of children infected with such variants arguescurrently against their expected significant spread (Oon et al., 1996; Hsu et al., 1997). Thus, at the moment their importance appears to be related to the efficacy of diagnostic assays (Carman et al., 1997b; Jongerius et al., 1998; Coleman et al., 1999).

1.5.1B Medically selected variants

Variants after vaccination, HBIG administration or antiviral therapy have been reported. Carman et al. (1990) described a child from Italy who developed HBV infection at birth by an Arg 145 mutant despite passive-active immunisation. It was proposed that vaccine escape was due to loss of the "a" determinant configuration because it is known that vaccine-induced anti-HBs is mainly directed towards this structure. Similar findings have been reported from Japan and a further mutation in codon 126 of the S gene was detected, with Thr/ Ile to Asn substitution (Okamoto et al., 1992).

In Singapore, a survey of vaccinated babies showed 11.9% of the vaccinees had breakthrough HBV infections, despite being given HBIG and vaccine at birth and a surprising number of Arg 145 mutants were described (Zuckerman et al., 1994; Oon et al., 1995). In the Gambia, 8.3% of vaccinated children had subclinical infections (positive anti-HBc). Of these, 37.3% had high levels of anti-HBs, which might indicate the ability of mutants to dominate in a population with high vaccination coverage (Karthigesu et al., 1994). In the USA, a study of children born to carrier mothers revealed similar results to those from Singapore. A number of amino acid changes were observed and Arg 145 was the most frequent (Nainan et al., 1997).

In China, Hsu et al. (1997) described that five of fifteen immunised infants born to HBsAg/ HBeAg-positive carrier mothers had S gene mutants. Three of them had the Arg 145 mutation. Matsumoto et al. (1997) reported that two cases out of 29 immunised infants born to HBeAg +ve carrier mothers were positive for HBV DNA. One of them had the wild type virus and became negative during follow up, 3 years after immunisation. The other had a mixed viral population, the mutant having P120G and G145R. Follow up indicated that this infant only had the mutant at six years after immunisation.

As regards HBIG therapy, Mc Mahon et al. (1992) described Arg 145 emergence in mAb-treated patients and the development of mutations in the "a" determinant was correlated with

the duration of the HBIG therapy. Another two studies by Cariani et al. (1995) and Hawkins et al. (1996) showed the presence of mixed populations of viruses in the serum of patients pre-transplant, including Arg 145. The emergence of a variant HBsAg as the dominant population was observed after HBIG administration in transplanted patients. These mutants do not commonly appear in patients who never received HBIG or when infection occurs after withdrawal of HBIG.

Similar findings have been reported by Carman et al. (1996). S gene mutations were found in five patients who experienced reinfection while receiving HBIG, while in those who experienced reinfection after termination of HBIG, no mutations were found in the S gene. Moreover, Sterneck et al. (1997), in a study to detect HBV sequence changes evolving in liver transplant recipients with fulminant hepatitis, reported that one of the three cases developed an Arg 145 mutant under prolonged HBIG treatment. Similar observations have been described in many recent reports (Protzer-Knolle et al., 1998; Terrault et al., 1998; Shields et al., 1999). One of the possible reasons for this selection/ reinfection in HBIG treated patients could be the source of HBIG, which can be sourced from acute self-limited hepatitis patients with a highly homogeneous viral population (Rodriguez-Frias et al., 1999).

Variants have also been noticed with antiviral therapy eg. lamivudine. As the surface ORF is overlapped entirely by the polymerase ORF, mutations in the polymerase domain (see section 1.5.4) may affect the expression and antigenicity of HBsAg. Ling et al. (1996) reported that the YMDD (tyrosine, methionine, aspartate, aspartate) motif mutations that appear with lamivudine therapy resulted in amino acid substitutions in HBsAg: Met for Ile at aa 195 in patient 1, and Ser for Trp at aa 196 in patient 2. However, these aa are placed in the lipid envelope and unlikely to affect antigenicity (Bruss et al., 1994). Ala to Asp substitution at aa 157 of HBsAg was noticed in patient 2 in association with Phe to Leu change at residue 512, upstream of YMDD. This may affect the *w/r* subdeterminant (Okamoto et al., 1987).

Tipples et al., (1996) showed other changes in the S gene sequence in association with the polymerase protein mutations during lamivudine therapy but the significance of these changes is not known yet. Two consecutive aa mutations located outside the "a" determinant, T115I and T116N, were also detected in a patient being treated with thymosin alpha1 (Tang et al., 1998). A stop codon at aa 199 of S gene in association with an overlapping V542I mutant of pol gene during long term famciclovir was recently reported. Transfection of this mutant virus into hepatoma cells showed that HBsAg was not produced (Pichoud et al., 1999).

1.5.2 Pre-S1 and Pre-S2 variation

Mutations of the pre-S region are mostly natural and have been reported in the form of deletions, point mutations and rearrangements which could lead to changes in immunogenicity of the viral particles and thus affect immune clearance (Tran et al., 1991; Santantonio et al., 1992; Yamamoto et al., 1994). Generally PreS1 deletion variants are in frame and express competent pol protein, because they are overlapped with the dispensable spacer domain (Radziwill et al., 1990). Mutations or deletions that prevent translation of pre-S2 (pre-S2 defect) have also been described as replication competent and infectious (Fernholz et al., 1993). PreS variants were reviewed in Günther et al. (1999).

Gerken et al. (1991) described viral deletions in the pre-S gene that eliminated the pre-S2 promoter region and B- and T- recognition sites but conserved the pre-S1 binding site. Consequently, such deletions would lead to impairment in viral clearance without affecting viral penetration in liver cells. This may explain why significant variants have not been described in the binding site of pre-S1 (aa 21- 47) although point mutations and deletions have been noted down-stream (Tran et al., 1991). In agreement with this observation, Yamamoto et al. (1994) detected a deletion in pre-S1 region in one carrier only while pre-S2, especially at the 5' terminus, was a favoured site for deletion as shown by clone sequencing from all carriers. Also, subviral particles that are isolated from patients infected with pre-S1 and pre-S2 variants lacked binding activity to preS1/preS2 specific monoclonal antibodies (Gerkin et al., 1991; Kohno et al., 1996).

In DHBV, selection of pre-S variants that escaped neutralisation has been described (Sunyach et al., 1997). MAb (900), which recognises epitope ^{83}IPQPQWTP^{90}, was used to generate immune pressure. Point mutations affecting only proline residues were detected (P90H within this epitope and/ or P5L upstream). P5L mutation reduces the mAb 900 mutant recognition twofold, while the substitution of both prolines (5 & 90) almost completely abolishes reactivity with the mAb. However, these proline substitutions did not affect either replication capacity or *in vivo* infectivity of the virus.

Pre-S2 variation is often associated with a pre-core mutant in anti-HBe positive chronic carriers with high viraemia. However, no substantial data are available to show whether pre-core and pre-S2 variants occur simultaneously or sequentially and thus their relative contributions to pathogenesis are unclear. Recently, sequential changes in the preS region before and after anti-HBe seroconversion, in patients who were infected in childhood, were

investigated. The effect of interferon (IFN) treatment on selection of mutants was also evaluated. Analysis of preS sequences before and during therapy did not show any nucleotide change, while numerous mutations were detected in both groups immediately after seroconversion. In addition to the selection of preS2 start codon mutants in 3 cases, an A to D genotype change was seen in 7 cases after seroconversion (Gerner et al., 1998).

1.5.3 Pre-C/C variants

The precore and core proteins, although initiated from two different start codons, are translated from the same ORF and have a common stop codon. There are certain epitopes for B and T cells that are common between these proteins while others are peculiar to one polypeptide (Ferrari et al., 1991; Bertoletti et al., 1991, 1993). Precore stop mutations have been detected in healthy chronic carriers as well as in patients with fulminant hepatitis or chronic active hepatitis (Laskus et al., 1995; Lindh et al., 1996). Therefore, it is likely that concomitant core gene mutations or other mutations are necessary to increase viral virulence (Akarca & Lok, 1995; Hur et al., 1996; Hunt et al., 2000).

1.5.3A Natural class:

Pre-C stop codon mutations may emerge during the natural course of HBV infection and are usually followed by a loss of HBeAg and the appearance of anti-HBe (Carman et al., 1989; Okamoto et al., 1990). People who have fluctuating HBe/ anti-HBe status usually retain the HBeAg-producing strain (Carman et al., 1992), however low levels of stop codon mutant strains have also been reported in HBeAg positive cases in the early seroconversion phase (Naoumov et al., 1992). Precore mutants were shown to be associated with fulminant hepatitis (Liang et al., 1991; Laskus et al., 1995), however it is difficult or even impossible to establish a causal relationship between the pre-C stop codon mutation and the clinical course of the disease. Pre-C stop codon mutants have different clinical presentations. Moreover, in any patient with chronic HBV infection, many mutants may coexist as a "quasispecies" and multiple mutations may be found in a single viral genome (Blum, 1993).

The most common precore mutation during seroconversion to anti-HBe is at nt 1896 (A_{1896}) from G to A, leading to a translational stop codon at codon 28. This may be related to the contiguous guanosines (Gs) in this area as each of the four Gs has been described as mutated to an A (Brunetto et al., 1989; Santantonio et al., 1991; Carman et al., 1992). A_{1899}

has been described in a number of studies (Carman et al., 1989; Brunetto et al., 1989) and is usually associated with a stop codon. Another precore mutation, without an obvious function, occurs at aa 15 where Pro has been replaced with Ser (Carman et al., 1992). However, simultaneous presence of A_{1896} and a Ser at aa 15 would lead to an unstable encapsidation signal which may explain why these two mutations have never been found together (Lok et al., 1994; Boner et al., 1995).

Different genotypes of HBV differ with regard to their association with precore variants depending on the substitutions at position 1858 in the encapsidation signal. If there is a T at 1858, the A_{1896} mutation will enhance the stability of the secondary structure of the encapsidation signal and consequently viral replication. In contrast, the presence of a C_{1858}, generally occurring in genotype A, prevents the G to A mutation at 1896 since it will destabilise the stem of the encapsidation signal (Li et al., 1993; Lok et al., 1994; Rodriguez-Frias et al., 1995).

On the basis of these base-pairing requirements, a high prevalence of precore variants has been observed in genotype B to E strains which have a T_{1858} (Li et al., 1993; Rodriguez-Frias et al., 1995; Lindh et al., 1996). For genotype F, there is a controversy about the substitution at position 1858. C_{1858} has been reported by Norder et al. (1993) and Naumann et al. (1993); however T_{1858} was found explaining the presence of A_{1896} by Arauz-Ruiz et al. (1997a) who found only one of 17 genotype F strains had C_{1858}.

Core protein contains B cell, T helper cell and cytotoxic T lymphocyte epitopes (Salfeld et al., 1989; Bertoletti et al., 1991; Ferrari et al., 1991). Variability in this gene has been reported in patients with ongoing disease, whether HBeAg or anti-HBe positive (Ehata et al., 1992 & 1993). A graduation of numbers of aa substitutions in the core protein has also been noticed, with the fewest being in HBeAg positive patients with minimal disease and the most being in anti-HBe positive patients (Carman et al., 1995b; Bozkaya et al., 1996).

A number of mutations were detected between residues 57 and 68 in the core protein of genotype F strains (Arauz-Ruiz et al., 1997a), which differ from previously described hot spots (Hur et al., 1996; Lee et al., 1996). However, Ehata et al., (1993) showed that the mutation clustering regions differ between genotypes. According to their classification, core genotype 1 (representing the core sequence of genotype C and most genotype B strains) had a clustering region between residues 84 and 99, while core genotype 2 (representing the core sequence of genotype A and D) had a clustering region between residues 48 and 68. Genotype

F strains may have a unique clustering region between residues 57 and 68 (Arauz-Ruiz et al., 1997a).

Some specific substitutions have been noted such as core T12S which is found more often in those with severe disease and only after preselection of the precore stop codon (Carman et al., 1992). Core aa 12 is within a CD4-restricted T helper epitope (aa 1-20) and may lead to non recognition by the appropriate T cell population (Ferrari et al., 1991). Core protein mutations in the major T helper epitope might thus allow immune escape and are more prevalent during clinical remission while those in B cell epitopes are more prevalent during progressive disease (Carman et al., 1997a). Mutations in the HBc epitope (aa 18-27) have also been described that result in a peptide antagonist to the T cell receptor and consequently ineffective CTL activity leading to persistent infection (Bertoletti et al., 1994). However, mutations in CTL epitopes occurs much less frequently than in B cell or T helper cell epitopes (Carman et al., 1995b). Deletions within the core protein sequence, often in-frame, have also been reported in patients with chronic disease with the possibility that core particles are still formed. However, even in-frame-deleted sequences do not appear to produce nucleocapsids (D. William, Ph D thesis 1997).

1.5.3B Medical class:

Fattovich et al. (1995) showed that fewer patients select a pre-core mutant on interferon (IFN) treatment than after natural remission. However, other studies showed either selection in almost all patients (Günther et al., 1992) or no selection at all in any patient after treatment (Xu et al., 1992; Lee et al., 1994). There is also controversy regarding pre-core mutants and prediction of interferon response (Takeda et al., 1990; Brunetto et al., 1993). Fattovich et al., (1995) found that the pre-core sequence has no influence on the outcome of IFN therapy in anti-HBe positive patients.

Tran et al. (1991) described two HBV DNA molecules in a chronic carrier after a combination course of acyclovir and interferon for 3 months (serum sample, 1988). One of them was identical to the pre-treatment sequence (wild type), whereas the mutant showed a stop codon in the pre-C region at positions 1897 to 1899, an in-frame 36 bp insertion located 6 bp after the initiation codon and a 6 bp deletion (2257-2262) in the C ORF. One year later, the pre-C/C region still contained both the wild and mutated molecules. Bhat et al. (1990) showed another in-frame 36 bp insertion in the C gene in an HIV-positive patient serologically

negative for anti-HBc, along with two point mutations in the pre-C/C gene. In contrast, in a study of anti-HBc-negative children with HBV infection undergoing chemotherapy for malignancies, no pre-C/C mutations were found (Melegari et al., 1991). As regards core gene variability and prediction of IFN response, it was found that core variants may affect the response to interferon therapy but do not influence the outcome of liver disease in adults (Fattovich et al., 1995; Naoumov et al., 1995). However, in children with chronic hepatitis B the presence of core variants does not seem to be involved either in outcome of infection or in the response to interferon therapy (Schepis et al., 1997) (see section 1.7.2).

1.5.4 Polymerase gene variants

Replication of HBV is dependent on reverse transcription, therefore nucleoside analogues, particularly reverse transcriptase inhibitors, have activity against HBV replication. Lamivudine (Dienstag et al., 1995; Lai et al., 1997) and famciclovir (Schalm et al., 1995) trials have shown them to be effective in reducing the viral load in chronic hepatitis B infection. Moreover, lamivudine prophylaxis against HBV reinfection in liver transplantation gives satisfactory results (Grellier et al., 1996). However, emergence of resistant strains with mutations in HBV polymerase gene made famciclovir (Aye et al., 1997; Seigneres et al., 2000) or lamivudine (Ling et al., 1996; Tipples et al., 1996) less effective in suppressing replication of such strains.

1.5.4A Natural class

Naturally occurring HBV variants with mutations affecting polymerase activity are not commonly reported. Blum et al., (1991a) reported a serologically immune patient with a latent HBV infection who had a viral genome with a point mutation in the TP region of polymerase gene terminating HBV replication through a loss of RNA encapsidation function. Another natural variant has been detected in the DHBV system where a point mutation in the C-terminus of the polymerase gene (encoding for RNase H activity) prevented viral packaging (Chen et al., 1992b). Recently, the polymerase RT domain and overlapping HBsAg "a" determinant were shown to be more variable in HBsAg-ve carriers than in HBsAg+ve controls. It has been suggested that there is a functionally defective RT domain which results in reduced or impaired replication. This would result in HBsAg which cannot be detected in serum by conventional tests (Weinberger et al., 2000).

1.5.4B Medical class

Ling et al. (1996) reported a mutation of Met to Val or Ile in the highly conserved YMDD motif of HBV polymerase gene in two cases after liver transplantation which conferred resistance to lamivudine. Thus, resistance of HBV to lamivudine is analogous to that of HIV (Tisdale et al., 1993). However, the significance of additional substitutions like Met for Leu at aa 528 upstream of the YMDD motif and Phe to Leu further upstream is still unclear (Ling et al., 1996). Bartholomew et al. (1997), analysing sequence variation in the HBV polymerase gene in three patients, showed resistance to lamivudine after OLT. Mutation at Met residue of YMDD was common to the three patients and was detected only in serum obtained after recurrence and not in pre-treatment serum. Ling et al. (1996) observed Leu to Met substitution at position 526 in association with Val (YVDD) in two patients, but not Ile (YIDD).

The development of resistance to lamivudine in these previous studies was thought to be related to the immunosuppression and associated high levels of viral replication. However, Honkoop et al., (1997) showed that immunocompetent patients with chronic hepatitis B infection can also develop lamivudine resistance during prolonged lamivudine monotherapy. An immunocompetent patient on long term famciclovir therapy developed similar resistance. A Val 542 Ile mutant in the C domain of viral pol was selected and led to viral persistence (Pichoud et al., 1999). Several similar results with resistant pol mutants on long-term famciclovir treatment have been reported. Aye et al. (1997) described a patient in whom resistance to famciclovir appeared to be due to mutations upstream of the YMDD motif. In 28 patients on long-term famciclovir, TP and RT domains of pol were sequenced. Again, the YMDD motif was conserved, however the RT domain was frequently mutated in non-responding patients and Leu 528 Met was selected in two patients plus 14 novel mutations in another 7 patients (Seigneres et al., 2000).

Sequential viral mutations in a liver transplant recipient reinfected with hepatitis B have been reported. Famciclovir therapy for 6 months failed to prevent graft infection. Ile 513 Leu change was detected while on HBIG prior to famciclovir treatment. A change to lamivudine therapy was associated with a good response and normal graft function. However, after 12 months of lamivudine therapy, HBV DNA rose again and the patient developed a severe acute hepatitis and subsequently died. Sequence analysis of HBV polymerase gene showed a mutation in close proximity to the YMDD motif which was considered to be a novel variant selected by lamivudine therapy (de Man et al., 1998). Similar findings were recently reported,

where rapid selection of resistant strains due to sequential antiviral therapy and limited efficacy of the second-line antiviral therapy has been observed (Seigneres et al., 2000; Mutimer et al., 2000).

The competence of mutants observed during lamivudine and famciclovir therapy has been investigated *in vitro*. Impaired replication of YI/VDD or F501L mutants (and to a lesser extent L515M mutant) in HEK 293 (after deoxynucleotide depletion) and HCC cells was detected (Melegari et al., 1998b). Several other groups have reported similar results (Allen et al., 1998; Ling & Harrison 1999). Thus, these *in vitro* data may explain the low viremia in breakthrough infections and reemergence of standard type soon after cessation of lamivudine (Buti et al., 1998; Niesters et al., 1998). Consequently, it is unlikely that these pol variants will spread in human populations. However, this down-regulation may be beneficial to the variant by allowing escape from immune surveillance (Blum et al., 1991a, b).

1.5.5 X Gene variants

X gene variants are associated with multiple outcomes. Deletions, insertions and point mutations in the X gene have been described in asymptomatic carriers, chronic persistent hepatitis (CPH) patients, some fulminant cases (Okamoto et al., 1994; Laskus et al., 1994) and in chronic HBV patients without the usual serological markers (Blum et al., 1991b; Preisler-Adams et al., 1993). Recently, X gene sequencing, from 26 patients with fulminant hepatitis (FHBV), showed that substitution of nucleotides in the X gene in the form of particular motifs was linked to FHBV pathogenesis. These specific variant motifs were associated with increased luciferase expression *in vitro* that correlated with rapid progression of the disease (M. Yasmin, Ph D thesis 1997).

Due to overlap, core promoter mutations often affect the structure and consequently the function of X protein. Most deletions/ insertions in the basal core promoter (BCP) shift the X gene frame producing truncated X proteins. These X proteins lack a highly conserved domain at their C-terminus (aa132- 140) that is essential for transactivation activity and mediates the interaction with some cellular proteins (Arii et al., 1992; Kumar et al., 1996; Huang et al., 1996a). This domain is also important for establishing infection in the related WHV (section 1.4.5). HBV containing core promoter mutants is usually complemented by HBV that can express full-length X protein (Günther et al., 1996). However, the 1768-1775 deletion variants were described in the absence of detectable full-length X gene strains suggesting their

exclusive expression (Fukuda et al., 1995). In contrast to the deletions/insertions in the core promoter, 1766T-C/ 1768A-T point mutations introduced changes in the X protein that did not affect its transactivation activity *in vitro* (Baumert et al., 1996).

Silent HBV infection associated with deletions of various lengths within X gene has also been reported (Preisler-Adams et al., 1993; Feitelson et al., 1994). An 8-nt deletion in the region encoding the X gene has been detected in serologically negative sera from patients with acute and chronic hepatitis (Uchida et al., 1994; Uchida et al., 1995). This mutation probably suppressed the replication and expression of HBV DNA resulting in negativity of the serological markers (Uchida et al., 1995; Fukuda et al., 1996). Low serum DNA levels, that are frequently seen in asymptomatic carriers containing X gene deletions, are consistent with this assumption (Horikita et al., 1994; Fukuda et al 1995).

Hepatitis C virus has been described as frequently associated with silent HBV infection (Sardo et al., 1994; Gonzalez et al., 1995). *In vitro* co-transfection of both viruses showed that silent HBV variant (containing 8-nt deletion in X region) probably promotes HCV replication, perhaps playing a role in hepatocarcinogenesis of chronic hepatitis C infection (Uchida et al., 1997). This assumption is supported by the high frequency of HBV DNA detected in HCC samples from patients with anti-HCV +ve/ anti-HBc+ve/ HBsAg-ve status (Koike et al., 1998).

Other forms of X gene mutants that do not suppress HBV replication have been described, such as the replication competent HBV genome with a pre-X open reading frame (Loncarevic et al., 1990). Also, insertions in the pre-C region, creating a fused X-C reading frame, were replication competent and expressed two types of X-core fusion protein (Kim et al., 1992; Preisler-Adams et al., 1993; Kim et al., 1994). Aye et al. (1997) reported that during almost 4 years of antiviral therapy (ganciclovir followed by famciclovir), there were no nucleotide-changes in the X gene compared to the pre-treatment sequence. Thus, it seems that X gene variants are mostly natural.

1.6 HBV INFECTION

Approximately one third of the world's population has already been infected with HBV. Of these 350 million people are chronic carriers and at least one million will eventually die from the sequelae of this infection (Kane 1996; André 2000). HBV infection has variable outcomes that range from subclinical infection to fulminant hepatitis. HBV infection is more common in males than in females (London & Drew 1977; Craxi et al., 1982). Moreover, chronic HBV infection varies inversely with age; only 5-10% of adults develop chronic infection, while up to 90% of children become chronically infected (McMahon et al., 1985; Margolis et al., 1991).

1.6.1 Epidemiology of HBV transmission

In highly endemic regions (HBsAg prevalence >7%), HBV infection has been identified in geographically remote and culturally isolated populations (eg. South Pacific Islanders and Alaskan Natives) as well as in large densely populated regions (eg. sub-Saharan Africa and Asia). HBV infection usually occurs early in life in high-prevalence areas as a consequence of maternal-neonatal transmission and horizontal spread among young children. HBV infection can be detected in most children by 10 years of age in these regions (Margolis et al., 1991). HBV infection during infancy and early childhood is usually followed by chronic infection, even in low endemicity areas, as chronic HBV infection is age-dependent (McMahon et al., 1985). Vertical HBV transmission in these high endemicity areas is correlated mostly with the mother's HBeAg status (Stevens et al., 1979; Beasley & Hwang 1983). In areas of high endemicity where the prevalence of HBeAg among mothers is low (Africa, South America and Middle East), early childhood transmission is the dominant mode of HBV infection (Marinier et al., 1985; Hyams et al., 1988; Toukan et al., 1990).

In intermediate endemicity regions (HBsAg prevalence 2-7%) such as India, Philippines, Korea and Taiwan, HBV infection occurs later in adolescence and young adulthood. However, high rates of chronic infection are still maintained by transmission during early childhood. More than 40% of world's population live in regions of intermediate endemicity with an expected lifetime risk of 20-60% of HBV infection (Mahoney, 1999).

In low prevalence populations (HBsAg prevalence <2%) such as United States and Western Europe, most HBV infections occur among adults. Sexual activity, IV drug use, occupationally acquired infection and use of multiple blood products constitute the principle

mechanisms of HBV transmission (Margolis et al., 1991). However, high rates of early childhood HBV transmission have also been reported among children of Pacific Islanders who reside in the United States (Tong et al., 1981; Hurie et al., 1992).

1.6.2 Pathogenesis of HBV infection

Most of our knowledge about HBV- host interactions has been unravelled over the last two decades. Outcome of HBV infection is known to be affected by host genetic factors, however the exact mechanism is unclear. Viral clearance is associated with a strong polyclonal multispecific T cell response to HBV in addition to the humoral response, but little is known about the factors determining the individual's ability to mount such T cell response. In contrast, ineffective immune responses were observed in patients who failed to clear the virus (Chisari & Ferrari 1995).

1.6.2A The antibody response

The humoral response involves the formation of antibodies against all proteins of HBV. Anti-HBe, anti-HBc and anti-HBs antibodies have all been extensively studied whilst anti-pol and anti-HBx antibodies, perhaps due to lack of proper assays, are not well described. Pol protein is quite immunogenic and anti-pol antibodies can be detected during acute and chronic infections (Yuki et al., 1990). Therefore, it has been suggested that these antibodies serve as markers of infection and ongoing viral replication (Weimer et al., 1990). Anti-HBx antibodies were also detected at high levels in chronically infected patients (Stemler et al., 1990).

The antibody response to HBV surface proteins is a T cell dependent process (Milich & McLachlan 1986). These antibodies are detectable in patients who clear the virus and recover from acute infection and usually are undetectable in chronic HBV infection. Anti-HBs antibodies play an important role in virus neutralisation as evaluated both *in vitro* and *in vivo* in chimpanzees (Pontisso et al., 1989a; Ogata et al., 1993b; Ryu et al., 1997). They are also believed to contribute, by forming immune complexes, to the pathogenesis of extrahepatic syndromes associated with the HBV infection (Alpert et al., 1971; Brzosko et al., 1974; Michalak 1978). Millard & Pilot (1998) recently showed that the "a" determinant contains at least three epitopes which are recognised by the human immune system and induce protection. One of them is well defined and recognised by five mAbs (mAb #1), while the other two are partially overlapping.

On the other hand, the role of antibody response to nucleocapsid antigens (anti-HBe and anti-HBc antibodies) in HBV pathogenesis is still unclear. Although an early report has shown that chimpanzees injected with anti-HBe are mostly protected against HBV infection (Stephan et al., 1984), both anti-HBe and anti-HBc are usually considered as non neutralising antibodies as they are present in high titres during both acute and chronic HBV infection. Cross reactivity of core and e proteins at the T cell level (Milich et al., 1987a; Bertoletti et al., 1993) and the ability of core protein to act as both a T cell-dependent and a T cell-independent antigen (Milich & Mclachlan 1986) are probably responsible for these high antibody levels.

1.6.2B Class II-restricted T Lymphocyte response

In acute hepatitis patients, strong HLA class II restricted $CD4^+$ responses to HBV nucleocapsid antigens and much weaker envelope-specific Th responses were detected (Chisari & Ferrari 1995). In contrast, a good percentage of vaccinees developed a vigorous envelope-specific Th response, suggesting antigen load, presentation or processing as possible factors to explain these response differences (Celis et al., 1988; Jin et al., 1988; Ferrari et al., 1989). On the other hand, a weak and ineffective HLA class II-restricted response to all HBV viral antigens has been observed during chronic HBV infection (Ferrari et al., 1990; Jung et al., 1991). Thus, a class II restricted nucleocapsid-specific T cell response is believed to play a major role in viral clearance most probably through induction of virus-specific CTL and an "intermolecular" help mechanism (Milich et al., 1987b; Penna et al., 1997).

Consistent with this view, chronically HBV infected subjects, although hyporesponsive to immunisation, showed a dose dependent-HBV specific CTL response after injection of the same vaccine (Theradigm-HBV vaccine), indicating that CTL precursors are present in HBV infected patients and can be activated (Heathcote et al., 1999). Further characterisation of the observed proliferative responses revealed Th0 or Th2 cytokine profile responses. Responses to the tetanus toxoid epitope (included within the vaccine as an universal helper T lymphocyte (HTL) epitope) were also reduced, suggesting altered HTL responses during chronic HBV infection (Livingston et al., 1999).

In transgenic mice, transfer of HBV-specific CD4+ Th1 cells to two inbred lineages (one expressing LHBs and the other expressing all viral proteins plus replicating virus in liver) has led to recognition of expressed viral antigens and transient liver injury. Cytokine release and subsequent suppression of viral replication were also observed, supporting a dual role for

CD4+ cells (Franco et al., 1997). Thus, the activation of appropriate T-helper cells, which secrete cytolytic cytokines and, most importantly, activate specific CTL responses, is quite important for the immune clearance of HBV. CTL also produce cytokines that may exert direct antiviral effects independent of their cytolytic activity (see section 1.6.2C; reviewed in Guidotti & Chisari, 2000).

Unlike the association between HLA class I alleles and outcome of HBV infection, which is not consistent, many reports have documented an association with HLA class II alleles such as HLA-DR13 & DRB1*1301- 02 (Thursz et al., 1995; Hohler et al., 1997; Diepolder et al., 1998). DRB1*1301 and DRB1*1302 were shown to be highly frequent in chronic HBV patients, who spontaneously cleared the virus, both in Africa and Europe (Thursz et al., 1995; Hohler et al., 1997). In addition, a vigorous HBV core-specific $CD4^+$ T cell response was found to be associated with HLA DR13 allele in those patients who successfully eliminated the virus in another study by Diepolder et al. (1998). In contrast, other HLA II alleles, such as DQA1*0501, DQB1*0301, and homozygosity of class I/ II were shown to be associated with persistence of HBV infection (Pollicino et al., 1996; Thio et al., 1999).

1.6.2C Class 1-restricted T Lymphocyte response

In transgenic mouse studies, adoptive transfer of CD8-positive HBsAg-specific CTL into mice which express HBsAg, caused a liver disease similar to acute viral hepatitis (Moriyama et al., 1990; Ando et al., 1993). This CTL-induced hepatitis was found to proceed in a stepwise order. The first step is apoptosis that occurs within 1 hour of CTL administration and is directly caused by CTL. The second step, which occurs between 4-12 hours, is due to accumulation of antigen nonspecific inflammatory cells such as macrophages and NK cells, and is characterised by focal necrosis. This necrosis limited to less than 5% of hepatocytes, was attributed to the low effector to target cell ratio (1/30-1/100) and the architectural constraints of the liver (Ando et al., 1994; Chisari & Ferrari 1995). However, in mice that overexpress HBsAg, so that their hepatocytes are sensitive to destruction by IFN-γ (Gilles et al., 1992), the process extends to fulminant hepatic failure, which is mostly attributed to antigen-nonspecific inflammatory cells and various cytokines such as IFN-γ and TNF-α (Ando et al., 1993).

In agreement with these results, TNF-α, produced by Kupffer cells, was shown to cause hepatic necrosis independent of macrophage activation (Orange et al., 1997). Also, TNF-α,

secreted by virus-specific CTL, was described as being able to amplify the damage to nearby non-infected cells during HCV infection (Ando et al., 1997). Recently, IFN-γ and TNF-α, produced by HBsAg-specific Th1 cells, were shown to be indispensable in the pathogenesis of liver injury (Ohta et al., (2000).

Although transgenic mouse models have contributed much to our understanding of HBV pathogenesis, there are two important limitations. First, the mice are not infectible by the virus, so observations related to viral entry and spread cannot be made. Second, the episomal ccc HBV DNA forms, purging of which is an indicator of viral clearance, cannot be produced by the mice (Guidotti et al., 1995). Chimpanzees, which are infectible by HBV, have a similar immune response to that seen in acutely infected humans and are ideal to test the non-cytopathic clearance of HBV during acute infection (Barker et al., 1975; Bertoni et al., 1998). Guidotti et al. (1999b) showed that disappearance of at least 90% of viral DNA (including the cccDNA) from the liver preceded the major influx of T cells, which was associated with liver damage, and coincided with induction of IFN-γ, suggesting early noncytopathic control of HBV in a "tissue-sparing process". This was attributed mostly to the early influx of non-T cells, perhaps natural killer cells. The remaining infected cells will be killed by antigen-specific CTL causing liver disease. Similar observations have been described during clearance of lymphocytic choriomeningitis virus (LCMV), as the virus was noncytopathically eliminated from hepatocytes (Guidotti et al., 1999a). Cytokine-mediated control of viral infection and its role in pathogenesis were reviewed in Guidotti & Chisari (2000).

Thus it appears that generation or maintenance of early strong cellular immune responses after viral infection is very important for viral clearance and those interactions between cellular and humoral immune responses will determine the outcome of infection.

1.7 PREVENTION AND TREATMENT

Vaccination is an important strategy to prevent HBV infection and consequently to decrease the risk of chronic HBV infection and its subsequent complications. Also, a wide range of antiviral and immunomodulatory therapies have been evaluated in the last 30 years, but very few have passed the challenge. In this section, I will briefly shed light on the important and recent concerns about the control of HBV infection.

1.7.1 Prevention

Although the key to control of HBV infection is immunoprophylaxis, general preventive measures (proper disinfective measures, appropriate screening of blood products for HBsAg, sexual health education and behavior modification) are important tools to reduce the rate of HBV infection (Minuk et al., 1987; Mast et al., 1999; Stroffolini et al., 2000).

Passive immunoprophylaxis is used predominantly in the following situations, usually in conjunction with vaccination: in neonates born to HBeAg-positive mothers (Reesink et al., 1979); after needle-stick exposure (Grady et al., 1978); after sexual exposure (Perrillo et al., 1984); and after liver transplantation (Müller et al., 1991; Samuel et al., 1991). In neonates, a dose of 0.5ml of HBIG should be given after delivery within 24 hours into the anterolateral muscle of the thigh, while in adults 0.05 to 0.07 ml/kg should be given, as soon as possible and within no more than 7 days (Grady et al., 1978).

HBV vaccine was initially recommended for individuals at high risk of exposure to HBV infection (health care workers, parenteral drug users, household contacts and infants of infected mothers). However this strategy was shown to have little effect on the incidence of new HBV infections. Therefore, universal infant vaccination was recommened by the WHO, especially in areas where hepatitis B is endemic (Kane 1996).

Two types of hepatitis vaccine have been widely used; plasma derived vaccine (consisting of HBsAg particles and small amounts of LHBs and MHBs) and yeast-derived recombinant vaccine, consisting of major S gene product. The latter is used more than the former (McAleer et al., 1984; Hollinger 1987). Unlike the plasma product, the yeast-derived vaccine contains nonglycosylated S polypeptides. However, both vaccines are equally efficacious (Andre 1989). New recombinant vaccines containing both preS and S antigens have been developed to circumvent non-response to conventional vaccines (Clements et al., 1994; Jones et al., 1998). To get the advantage of inducing cytotoxic T cells as well as neutralising antibodies, a

nucleic acid vaccine and a vaccine in a salmonella vector have also been described (Davis et al., 1993; Schödel et al., 1994).

Vaccine is given intramuscularly in three doses at 0, 1 and 6 months. An accelerated schedule for administering the 3 doses (at 0,1 and 2 months) will result in a quicker response but may reduce peak titers (Jilg et al., 1989; Hadler et al., 1989). In adults the conventional dose is 10- 20ug, however it varies according to age, immunologic status and in patients on dialysis, while infants may be vaccinated with lower doses. Protective immune serum response is defined as an anti-HBs titre of $\geq$ 10mIU/mL and can be elicited in 90%-95% of infants on combined HBIG- vaccine programs as well as in young healthy persons who receive full immunisation (Hollinger 1989; Andre & Zuckerman 1994). Follow up studies of immunised infants showed long-term protection against disease and the higher the anti-HBs levels after vaccination, the longer they persist (Hadler et al., 1986; Marion et al., 1994; Xu et al., 1995). When the anti-HBs titre falls below 10mIU/mL, HBV infections may occur but are mostly subclincal and usually without detectable HBsAg (Szmuness et al., 1981; Wainwright et al., 1989). Thus, protection against HBV disease remains even with a low anti-HBs titre. However, such low levels may favor the selection of escape mutants.

Reduced anti-HBs response or nonresponse has been shown to be as high as 10% (Craven et al., 1986; Alper 1995). Vaccine source, dose, patient age, body mass index, site of injection, immunosuppression, renal insufficiency, smoking and genetic factors all have been described as associated with impaired response to hepatitis B vaccine (Craven et al., 1986; Wood et al., 1993; Waters 1998; McDermott et al., 1998). Several strategies have been adopted to overcome this problem. Revaccination is the first option as approximately half of the people who did not respond after a three dose series will do so after additional doses (Hadler et al., 1986; Hadler & Margolis 1992). As different mechanisms of uptake determine the intracellular compartment to which antigens are delivered and may lead to generation of different T cell epitopes (Lanzavecchia et al., 1996), intradermal injection of hepatitis B vaccine has also been tried. Although higher seroconversion rates were detected in non-responding dialysis patients, comparable or less effective results to those with the IM injected vaccine have been observed in healthy nonresponders (Clarke et al., 1989; Heijtink et al., 1989; Chang et al., 1996; Fabrizi et al., 1997).

Another interesting approach is the use of preS containing vaccines following the early observations and recent encouraging results in animals (Milich et al., 1986; Jones et al., 1998).

Incorporation of the pre-S domains with the S antigen has been suggested to recruit pre-S specific T cells which would aid anti-HBs antibody production (Milich et al., 1986). Using this type of vaccine induce an anti-HBs antibody response in more than 70% of persistently non-responder individuals after a single dose. It was dose dependent (Zuckerman et al., 1997; McDermott et al., 1999). However, no difference was detected between pre-S containing vaccine and standard vaccine in another study for revaccination of healthy non-responders where only high doses of vaccine, 40ug, produced a statistically significant difference in development of protection (Bertino et al., 1997). Obviously, further studies are required that will not only assess the efficacy of these vaccines but also would facilitate greater understanding of individual variability in eliciting an immune response. Immunotherapy of chronic hepatitis B using anti-HBV vaccine is discussed in the next section (1.7.2).

Improved immune response has also been reported with the co-administration of IFNα or IL2 in renal patients (Grob et al., 1984; Meuer et al., 1989). There appear to be two different groupings in non-responders to vaccine. One group will develop satisfactory titres if they are simply given more doses. Those in the second fail to respond no matter how many doses they are given. Here the defect could be in the helper cells as well as in the antigen processing or transport of processed peptides to MHC molecules on antigen-presenting cells (Salazor et al., 1995; McDermott et al., 1999).

On the other hand, HBV vaccines are well tolerated and are among the safest available vaccines. The most commonly observed side effects are mild reactions at injection site and slight increase in body temperature (Stratton et al., 1994; Lakshmi et al., 2000). Anaphylaxis and symptoms of immediate hypersensitivity are rare but do occur (Stratton et al., 1994). Guillain-Barré syndrome and, recently, multiple sclerosis have been reported in vaccine recipients, but they do not appear to be any more common among vaccinees than among the general population and there was no evidence for any causal link (Stratton et al., 1994; Monteyne & André 2000). There is no harm in using the vaccine, but also not much benefit, in immunising HBV chronic carriers (Dienstag et al., 1982; Barin et al., 1983).

1.7. 2 Treatment of HBV infection

In acute hepatitis B, although symptomatic treatment may be required, no specific therapy is indicated as more than 90% of cases will spontaneously clear their infection (Gitlin 1997). However, a minority of these patients has to be transferred to hospital and liver transplant may

be even required if liver functions deteriorate rapidly with acute liver failure (O'Grady et al., 1989; Perillo & Mason 1993). On the other hand, no effective treatment of asymptomatic healthy HBsAg carriers is currently available, as administration of HBV vaccine has no influence on the carrier state (Dienstag et al., 1982; Barin et al., 1983) and treatment with interferon is not recommended (Rodriguez-Iñigo et al., 1997).

In chronic HBV infection, the main goal is eradication of the virus. In early stages of the disease, therapeutic approaches have been used to stop replication of the HBV aiming at viral clearance or at least to prevent or reduce complications of the disease. Patient stabilisation, prevention of fatal outcome and prevention of HBV reinfection of transplanted liver are the objectives in end stage cirrhotic patients. Until recently, alpha interferon, which is effective in about 30% to 40% of well-selected patients, was the only available therapy for chronic HBV infection. However, introduction of nucleoside analogues such as lamivudine and famciclovir into clinical trials has markedly improved the clinical outcome of HBV infection. Emergence of escape mutants due to aa changes within the reverse transcriptase enzyme (the YMDD mutant) leads to decreased antiviral potency and is a major limitation of therapy.

Interferon alpha

Although interferon alpha (IFN-α) has been used since 1976 (Greenberg et al., 1976) for chronic hepatitis B, it was only recently licensed for therapy. In addition to its antiviral activity, IFN-α acts principally as an immunomodulatory agent by stimulating the immune system, increasing natural killer cells and enhancing MHC class I display (Peters 1989; Goodbourn et al., 2000). The recommended regimen for IFN-α is 10 million units 3 times a week for 16-24 weeks by subcutaneous injection or 5 million units daily for the same period (Wong et al., 1995; Hoofnagle & Di Bisceglie 1997).

In about 30-40% of chronic HBV patients, IFN-α therapy will induce a long-term remission which is identified by the loss of HBeAg and HBV DNA, normalisation of serum aminotransferase levels and improvement in liver lesions (Perrillo, 1993; Gitlin, 1997). Indeed, IFN-α therapy improves the clinical outcome even in the presence of cirrhosis (Niederau et al., 1996). Several studies have shown that HBV-related decompensated cirrhosis might benefit from low-dose IFN-α therapy (Nevens et al., 1993; Perrillo et al., 1995). However, severe acute flares and life threatening side effects resulting from interferon therapy make it less likely to be beneficial (Hoofnagle et al., 1993). IFN-α may also induce remissions

in patients with extrahepatic disorders (eg. glomerulonephritis) associated with chronic HBV infection (Conjeevaram et al., 1995).

Some patients achieving HBeAg seroconversion also eventually lose HBsAg and this is associated with improved clinical outcome (Korenman et al., 1991; Niederau et al 1996; Fattovich et al., 1998). However, other studies reported no loss of HBsAg over prolonged follow up suggesting an effect of ethnic or racial variation (Lok et al., 1993; Lin et al., 1999). Genomic variation in precore and core CTL region (aa 18-27) is also one of the much debated issues in interferon response. Some studies showed enhanced response in the presence of the precore mutant A_{1986} (Takeda et al., 1990; Lok et al., 1995) while others contradicted this (Xu et al., 1992; Fattovich et al., 1995). Other authors have suggested that specific mutations in the core protein may impair the response to IFN therapy (Carman et al., 1995b; Naoumov et al., 1995), however, others have found that core gene mutations were unrelated to IFN response (Bozkaya et al., 1996; Shindo & Okuno 2000).

There are several criteria for increasing the likelihood of response to IFN therapy; these include low HBV DNA levels, high ALT levels, a short interval since the onset of HBV infection and female gender (Gitlin, 1997). Although IFN therapy is potentially successful and constitutes the mainstay of treatment for chronic hepatitis B, it has several disadvantages. For instance, in endemic regions most chronic HBV patients do not fit these selection criteria and thus have a reduced probability of response to IFN. Also, IFN-α is only successful in patients with an active immune response, making it ineffective in patients infected with HIV or immunocompromised patients. Furthermore, Asian patients, who constitute 75% of world carriers, respond poorly to IFN (Lok et al., 1993). Moreover, interferon treatment is expensive, administered by injection and poorly tolerated with side effects including flu like symptoms, injection-site reactions, anorexia, rash, neutropenia and thyroid disorders (Gitlin, 1997).

IFN-α therapy for children with hepatitis B has also been approved recently (Sokal et al., 1998). A successful response has been reported in 26-33% of children on interferon therapy, which is similar to that detected in adults (Torre & Tambini 1996; Sokal et al., 1998; Vajro et al., 1998). Therefore, IFN-α therapy is recommended in children who have chronic hepatitis B after being well selected, using similar criteria to those used in adults, to reduce the chance for the development of major complications (Roberts, 2000). However, Bortolotti et al. (2000) suggested that interferon therapy in children only speeded up a spontaneous event and their early observations in Caucasian children further supported this view (Bortolotti et al., 1998).

Nucleoside analogues

The development of nucleoside analogues, which block viral replication directly by inhibition of the HBV polymerase, greatly improved the outcome of hepatitis B treatment. To date, lamivudine is the only nucleoside analogue to have been approved for the treatment of chronic hepatitis B as other nucleoside analogues have been shown to either have less efficacy e.g. famiciclovir or to be poorly tolerated e.g. tobucavir (Dusheiko, 1999).

Early studies have shown that lamivudine rapidly reduces HBV replication and suppresses HBV DNA to undetectable levels after a few weeks of treatment (Dienstag et al., 1995; Lai et al., 1997). Furthermore, long-term trials in both Asian and Western patients have shown that lamivudine significantly reduced the progression of hepatic histopathological changes and normalized serum ALT levels (Lai et al., 1998; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000). Lamivudine has also many advantages over IFN-α such as better tolerability, oral administration and most importantly its global effectiveness irrespective of ethnicity, patients' sex or onset of infection (Lai et al., 1998; Maddrey, 2000).

Lamivudine thus offers a promising therapeutic option for chronic hepatitis B patients. However, emergence of resistant strains (see section 1.5.4) results in reduced efficacy of lamivudine (Tipples et al., 1996; Allen et al., 1998). Nevertheless, many reports indicate that patients on prolonged lamivudine therapy continue to receive benefit as shown by lower HBV DNA levels and improvements in serum ALT concentrations relative to pre-treatment values (Lai et al., 1998; Liaw et al., 2000). Moreover, it has been suggested that combination of lamivudine with other nucleoside analogues or with IFN-α might delay or prevent the emergence of viral resistance (Lee, 1997). One recent study has shown that the use of combination of lamivudine and IFN-α was more effective than either monotherapy (Schalm et al., 2000), however, more studies are needed to confirm the superiority of this combination and to investigate different regimens.

Other Approaches

As inadequate host immune response is believed to have a role in chronic hepatitis B infection (Chisari & Ferrari 1995), immunomodulatory agents such as thymosin α1 (Tα1), whether alone or in combination with IFN-α, have been tested in chronic HBV carriers. Early observations have shown that Tα1 therapy in chronic hepatitis B was associated with cessation of viral replication and clinical improvement (Mutchnick et al., 1991). However,

recent studies regarding the efficacy of Tα1 monotherapy are still controversial (Andreone et al., 1996; Chien et al., 1998; Mutchnick et al., 1999). A combination of Tα1 and low dose IFN-α was associated with promising results in chronic HBV patients with a sustained response in 60% (Rasi et al., 1996). Therefore, further trials are still needed to assess this combination.

Therapeutic vaccines are another interesting approach to stimulate the immune system against HBV. The mechanisms involved in the response to HBV vaccine therapy are still unclear. Post-infection vaccination might broaden the immune repertoire against the pathogen and hence bypass the inadequate immune response to natural infection. Additionally, differences in antigen presentation, processing, post-translational modification and recruitment of dendritic cells by intramuscular injection of the vaccine epitopes may induce modulations of the immune response. Further support for this approach comes from transgenic mice. Both T cell proliferation and anti-HBs production were induced by administration of the therapeutic vaccine via activation of dendritic cells, implying a key role in the antiviral response (Akbar et al., 1997, 1999). Clinical trials in chronic hepatitis B carriers have shown a reduction in serum HBV DNA levels or clearance of the virus in about 30% of patients (Pol et al., 1994, 2000). Induction of CD4+ T cell response and restoration of the specific B cell immune response were detected in another study during vaccine therapy (Couillin et al., 1999). PreS/ S vaccine was more frequently associated with disappearance of serum HBV DNA (7/35) in chronic HBsAg carriers than in those who received S vaccine only (1/21) or no vaccine (1/32) (Pol et al., 1998). In the same study, it was suggested that vaccination enhanced the efficacy of IFN-α therapy. Other vaccine strategies based on single-CTL epitope or HBV vaccine and anti-HBs complex have been also investigated (Wen et al., 1995; Heathcote et al., 1999). DNA based vaccines which induce both humoral and cellular immune responses, and also seem to bypass HLA restriction, showed optimistic results in mice, woodchucks and ducks (Geissler et al., 1997; Lu et al., 1999; Rollier et al., 1999). These approaches deserve further study and clinical trials, in particular in combination with nucleoside analogues, to define the optimal protocols for the treatment of chronic HBV patients.

Adoptive transfer of immunity, rather than its stimulation by vaccination, is another approach to treat chronic HBV carriers. Early observations have shown that adoptive transfer of immunity to HBV can be achieved by bone marrow transplantation (BMT) from immune competent donors (Lok et al., 1992; Ilan et al., 1993). The feasibility of adoptive immunity

transfer approaches to HBV in humans BMT recipients through transferring of HBV immune peripheral blood lymphocytes (PBLs) was also described (Shouval & Ilan 1995). Recent reports further supported the role of adoptive immunity transfer in clearance of HBV in BMT recipients whose donors were immune competent (Lau et al., 1997, 1998). Although the role of BMT as a treatment of chronic hepatitis B is limited by the high risk and cost associated with the procedure, use of nucleoside analogues in addition might allow design of better therapeutic strategies (Lau et al., 1998).

Liver transplantation

Liver transplantation is often the only therapeutic option for patients with acute or chronic liver failure caused by HBV infection (Todo et al., 1991; Perillo & Mason 1993). However, HBV reinfection of the liver graft is a major problem in those patients who receive immunosuppressive medication to prevent graft rejection. In these cases, retransplantation may be required due to the rapidly progressive course of the disease which often leads to graft failure and high mortality rates (O'Grady et al., 1992; Samuel et al., 1993). Therefore, prevention of HBV reinfection after liver transplantation is important and mainly entails the usage of anti-HBs (Lauchert et al., 1987).

Indeed, reduction of reinfection risk and better survival of liver transplant were described in patients on prophylactic anti-HBs (Muller et al., 1991; Samuel et al., 1991). However, escape mutants with frequent mutations within S gene, especially in the MHR, were observed in some patients who suffered reinfection (Carman et al., 1996; Protzer-Knolle et al., 1998). IFN-α has also been tried to prevent HBV reinfection in liver transplanted patients. Pre-transplantation treatment with IFNα did not prevent graft reinfection even if there was a response pre-transplant (Marcellin et al., 1994). Early studies showed similar results with IFN-α administration whether before or after transplantation (Rakela et al., 1989; Lavine et al., 1991). Thus IFN-α does not appear to prevent allograft infection after transplantation except perhaps in those who become PCR-negative before transplantation (Marcellin et al., 1994).

A newer option is the use of lamivudine or famciclovir; both have been shown to prevent or reduce HBV reinfection of the liver graft (Böker et al., 1994; Grellier et al., 1996; Aye et al., 1997). However, resistant viruses with mutations in the reverse transcriptase enzyme have emerged (Ling et al., 1996; Grellier et al., 1996). Nevertheless, other studies have reported that a combination of lamivudine and long term HBIG was highly effective in preventing

HBV recurrence, however at significant expense (Markowitz et al., 1998). Several recent reports showed similar successful results by using this combination, although in different regimens (Dodson et al., 2000; Angus et al., 2000). Reduction of the costs of this efficacious but very expensive strategy could be achieved either by reducing the dose of HBIG or replacing the HBIG by another effective antiviral agent in combination with lamivudine (Yao et al., 1999; Angus et al., 2000; Perrillo et al., 2000).

1.8 USAGE OF VIRUS-HETEROGENEITY AS A MARKER TO CHART HUMAN POPULATION MOVEMENTS

Regardless of the details of earliest origins of viruses on earth, it is generally accepted that viruses interact, and may even co-evolve with cellular nucleic acid. These cellular interactions with viruses play a major role in the evolution of both the host cell and infecting viruses. Evaluation of molecular properties of viruses such as nucleotide sequencing will thus determine how much of their evolution can be reconstructed, may reflect valuable information on human history and may even enable us to make predictions about the future development of viral diseases since the dissemination of viruses can be followed both locally and globally. Viruses that have the virtue of vertical transmission, medium mutation rate, and endemic distribution are good candidates for this purpose. Consequently, viral sequence variability has been proposed as a marker of human migration. Here, I briefy review of what has been described so far.

1.8.1 Human T-cell lymphotropic viruses

The human T-cell lymphotropic viruses HTLV-I and HTLV-II are retroviruses. HTLV-I is regarded as the causative agent of adult T-cell leukaemia (ATL) and HTLV-I-associated myelopathy/ tropical spastic paraparesis (HAM/TSP). On the other hand, HTLV-II is weakly correlated with HAM/TSP-like diseases (Yoshida et al., 1982; Murphy et al., 1993). STLV-I, which is the simian equivalent, infects most Old World primate species and is also associated with lymphoma and leukaemia (Watanabe et al., 1986; Tsujimoto et al., 1987). These human and simian viruses are known as primate T-cell lymphotropic viruses (PTLVs).

The rate of PTLVs transmission is remarkably low, as it requires close and frequent contacts such as breast-feeding, sexual intercourse and blood transfusion (Hino et al., 1985; Vitek et al., 1995). Unlike other retroviruses, which have a high mutation

rate leading to quasi-species because of high replication levels and lack of a proof-reading mechanism of the viral polymerase, PTLVs exhibit unusually low levels of diversity within individuals (Katz & Skulka 1990; Gessain et al., 1992). These low levels of diversity have been attributed to the clonal expansion of HTLV-infected cells, which is the dominant replication mode for such viruses after the initial period of active viral replication (Wattel et al., 1995; Cimarelli et al., 1996). The nucleotide substitution rate of HTLV-II is estimated to be $1.08x\ 10^{-4}$- $2.7x\ 10^{-5}$ per site per year. The HTLV-I evolutionary rate is slightly lower, at 0.4- $6.8x\ 10^{-7}$ (Liu et al., 1994; Salemi et al., 1998).

HTLV-I is distributed world-wide, but is endemic in Africa, Japan, South America, the Caribbean basin and the Melanesian region. Phylogenetically, HTLV-I has been classified into three major groups: the Cosmopolitan (HTLV-Ia); Central African (HTLV-Ib); and Melanesian (Gessain et al., 1992; Nerurkar et al., 1993). Recently, distinct HTLV-I variants from Central Africa have been identified and proposed as a fourth group (HTLV-Id) (Mahieux et al., 1997). As their names suggest, these phylogenetic groups are generally correlated with the geographic origins and ethnic backgrounds of the various carriers.

In contrast to HTLV-I, HTLV-II was originally thought to be a New World pathogen restricted to Amerindian tribes. However, the discovery of diverse strains of the virus in different tribes among African human ethnic groups contradicted the "New World exclusive" hypothesis. HTLV-II epidemiology has been changed recently to potential global distribution, as the virus has invaded new host populations of intravenous drug users (IVDU) in Europe and North America (reviewed in Slattery et al., 1999).

HTLV has been used as a genetic marker in endemic populations to trace the origin of the virus, the migration of ethnic groups, and the contact between populations (Gessain et al., 1992; Vandamme et al., 1998). It is generally believed that type-I viruses have become globally distributed by multiple episodes of interspecies transmission and successful invasion of new host populations (Mahieux et al., 1997; Vandamme et al., 1998). On the other hand, HTLV-II strains have a common ancient human ancestor virus and selection may be a factor in its mutation process relative to type I (Slattery et al., 1999).

1.8.2 Human papilloma viruses

Human papilloma viruses (HPVs), which can induce neoplastic proliferation of human epithelial cells, are a group of DNA tumour viruses with an 8-kb circular genome. More than 100 HPV types have been described with many new types still being characterised. An association with additional human cancers, such as non-melanoma skin cancers have been reported (deVilliers et al., 1997; Harwood et al., 2000). HPVs can be transmitted by several possible routes: close personal contact of most cutaneous warts particularly in presence of trauma at site of inoculation; neonatal infections (vertical transmission) which are acquired by passing through infected birth canal; fomites contaminated by HPV-infected cells; and sexual transmission (Ho et al., 1993; Tay SK, 1995).

Because of the high similarity between different isolates of HPV types (such as type 16 and 18), it was difficult to estimate the mutation rate of HPVs (Ho et al., 1993; Ong et al., 1993). Comparison of HPV type 16 variants from many geographical locations with different ethnic backgrounds showed that this type evolved along 5 main branches: two in Africa; two in Asia; and a dominant one in Europe and India (Ho et al., 1993). Furthermore, because of the similarity of HPV evolution pattern to that of human races, ancient coevolution of humans and papillomaviruses has been suggested as a likely possibility (Ho et al., 1993). Analysis of HPV type-18 diversity further supported the coevolution hypothesis and suggested a very slow evolution process; a single point mutation represents at least 12,000 years of evolution (Ong et al., 1993).

1.8.3 Human polyomavirus JC

Human polyomavirus JC (JCV) virus has a circular double-stranded DNA just over 5kb in length. Both JCV and HPVs are members of the Papovaviridae family. JCV was first isolated in 1971 and causes a fatal demyelinating disease known as progressive multifocal leukoencephalopthy (PML) (Padgett et al., 1971). It is a horizontally transmitted virus; however, it requires long cohabitation to be transmitted. Therefore, JCV is frequently transmitted from parents to children but rarely among human populations (Kunitake et al., 1995; Kato et al., 1997). After infection in childhood, it persists in the renal tissue for life. In adults, JCV DNA can be detected in urine (Tominaga et al., 1992; Kitamura et al., 1997). Infection with this virus appears to be widespread, but asymptomatic in the majority of patients.

Genotypes of JCV showed a distinctive geographical distribution. Type 1 (subtype EU) is found in Europe, type 2 (subtypes B1 and MY) in Asia and types 3 and 6 (subtypes Af1 and Af2) in Africa (Sugimoto et al., 1997; Jobes et al., 1998). Therefore, typing of JCV has been used as a marker of human migration and also to study the racial composition of China (Sugimoto et al., 1997; Agostini et al., 1997; Guo et al., 1998). JCV has a slow rate of mutation ($4x\ 10^{-7}$); this makes it a good witness only where there is a long history of evolution, but it is not sensitive for recent drift (Hatwell & Sharp 2000).

1.8.4 Human herpes virus-8

Human herpes virus-8 (HHV-8), which was discovered only a few years ago, has a large DNA genome (140-kb bounded by 40-kb of terminal tandem repeats) and belongs to the γ2 group of Gammaherpesvirinae (Chang et al., 1994; McGeoch & Davison 1998). HHV-8 causes Kaposi's sarcoma (KS) and other neoplastic disorders. Classic KS is common in certain geographic areas such as Middle East, certain parts of Africa and specific regions of the Mediterranean countries of Greece, Italy, and Turkey. On the other hand, a low incidence has been seen in Northern European countries such as Sweden and England (Grulich et al., 1992; Cottoni et al., 1996).

Four major subtypes have been determined: subtype A is found in USA, B is mostly confined to Africa, C in Middle East and Asia, and the rare D found in Pacific island patients (Zong et al., 1999). HHV-8 is spread sexually; however, nonsexual routes of transmission are likely to occur in HHV-8 endemic areas where the infection is acquired early in childhood (Schulz, 1998). The HHV-8 genome contains, at the left hand end, the ORF-K1 gene encoding a transmembrane protein that exhibits much more diversity than the rest of the viral genome (Zong et al., 1999; Cook et al., 1999). On phylogenetic analysis, a significant correlation between the clade patterns of this protein and the geographic or ethnic backgrounds of infected patients has been detected. Therefore, it has been concluded that such distribution patterns may reflect the migration of modern human populations (Zong et al., 1999; Hayward, 1999).

Clearly, the genetic relatedness of several viruses recovered from different geographical regions has shown that viruses may hold valuable information about ancient human population movements. It appears that there are still more viruses in the list that have not been discovered yet. There are uncertainties in virus data. First, there is the inherent effect of genetic drift.

Second, the initial colonisation of certain regions may precede the introduction of viruses. Third, the possibility exists that they may not have affected all ancient populations similarly. Nevertheless, virus analysis has some interesting aspects that human genome analysis does not. It provides a much greater range of diversity and creates greater power in the conclusions one can draw as an independent source of evidence. Furthermore, viral sequences have high mutation rates that allow variations to be determined progressively and consequently the timing of historical events can be estimated as long as the virus mutation rate is known.

Aims and Hypotheses

The overall aim of this work was to investigate aspects of HBV S gene variation in relation to virus infection and diagnosis.

Firstly, we hypothesised that HBV S gene "a" determinant variants will emerge in vaccinated infected children in the Pacific region. To assess this hypothesis, blood samples were collected from vaccinated children and their mothers from 4 different islands in this region. Serological markers of HBV infection were tested. PCR and sequencing were carried out on all children's sera with serological profiles that indicated past or present HBV infection. Sera from mothers of positive children were treated similarly. Maternal sequences allowed the incidence of natural variants to be assessed, while sequences from the children revealed the effect of vaccination. HBsAg positive samples from unvaccinated subjects were also used to define the background HBV sequence peculiar to the Pacific.

Secondly, we hypothesised that HBV variation can be employed to chart Pacific human migration. Based on reasonable non-virological evidence that the people of South East Asia migrated eastwards into Polynesia, we have chosen to study HBsAg variation from four Pacific islands which have different ethnic backgrounds. Kiribati represented Micronesia, Vanuatu and Fiji represented Melanesia and Tonga represented Polynesia. Using phylogenetic analysis of these Pacific HBsAg strains and database sequences isolated from other parts of the world, we attempted to show that specific sequences co-localised with ethnicity and that the evolutionary pattern of hepatitis B virus matched the proposed migration patterns of these people.

Thirdly, we hypothesised that HBsAg negativity in conventional diagnostic assays was sometimes due to variants that fail to bind to capture anti-HBs. As serum containing HBsAg particles is rarely available in volumes sufficient for testing against a multitude of capture

antibodies, we cloned variant HBsAg from 13 diagnostically relevant cases and tested cell culture supernatants in seven commercial diagnostic assays. As the project evolved, it became clear that standardisation of the amount of *in vitro* expressed HBsAg particles was necessary to allow a fair comparison of the reactivities of these variants. We therefore developed a tag system, by insertion of a non-HBV tag epitope into the S gene, to standardise the number of HBsAg particles before measuring reactivity.

Finally, we hypothesised that it may be possible to differentiate between HBV reactivation and reinfection by comparing sequences at two or more time points. Five patients who had a serological picture suggesting a second hepatitis B virus episode were studied compared to a control group of two patients who were HBsAg positive throughout with fluctuating HBeAg status. Though differentiation between HBV reactivation and reinfection might not add too much to our clinical understanding of HBV infection, it may provide some insights into the pathological events of HBV infection.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

a) DNA extraction from serum and blood clots

a1) In House procedure

Nucleic acid lysis mix	0.M NaCl, 10mM Tris, 2mM EDTA (pH 8.2)
Machine lysis buffer	Applied Biosystems, Cheshire, UK.
Proteinase K	10mg/ ml
Phenol/ Chloroform	25:24:1 with isoamyl alcohol
1x TE buffer	10mM Tris, 1mM EDTA (pH 7.5)

a2) TriPure™ Isolation Reagent (Roche Diagnostics, Lewes, East Sussex, UK)

TriPure Isolation Reagent is a monophasic solution of phenol and guanidine thiocyanate that allows the isolation of total RNA, DNA and protein from the same sample in a single-step liquid phase separation. Reagents required but not supplied: ethanol (96% and 75%), 8mM NaOH and 0.1M sodium citrate in 10% ethanol.

a3) High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Lewes, East Sussex, UK)

Binding buffer	6M guanidine HCl, 10mM Urea, 10mM Tris-HCl, 20% Triton® X-100 (v/v), pH 4.4
Poly(A) carrier RNA	0.2mg/40ul (after reconstitution)
Proteinase K	20mg/ml (after reconstitution)
Wash buffer	20mM NaCl, 2mM Tris-HCl (pH 7.5)
Elution buffer	Nuclease-free redistilled H_2O
High Pure filter tubes	Polypropylene tubes have two layers of glass fibre fleece and can hold up to 700ul of sample volume
Collection tubes	2ml Polypropylene tubes

a4) QIAamp Blood Kit (250) (QIAGEN Ltd., Crawley, West Sussex, UK).

It includes 250 QIAamp spin columns, Proteinase K, Buffers: AL for lysis; AW for wash; and AE for elution, and collection tubes (2ml). However, the ingredients of these materials are not provided by the manufacturer.

b) Synthetic oligonucleotides

Oligonucleotides were synthesised in house using a Cruachem PS250 automated synthesiser. Oligonucleotides used during this work are listed in table 2.1.

c) Enzymes

Taq DNA polymerase, restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics. RNase and Lysozyme were purchased from Sigma.

d) Reagents and buffers for PCR and cloning

10x PCR buffer	200mM Tris-HCl (pH 8.4), 500 mM KCl
10x dNTPs	100mM of each dATP, dCTP, dGTP, dTTP
TaqStart Antibody	1.1ug/ ul in storage buffer: 50mM KCl, 10mM Tris-HCl (pH 7.0), 50% glycerol
TaqStart dilution buffer	50mM KCl, 10mM Tris-HCl (pH 7.0)
10x TBE	89mM Tris HCl (pH 8.0), 89mM boric acid, 1mM EDTA
10x agarose gel loading buffer	1x TBE, 1% SDS, 50% sucrose, 1mg/ml bromophenol blue
Acrylamide gel elution buffer	0.5M ammonium acetate, 10mM $MgCl_2$, 0.1% SDS, 1mM EDTA
10x ligase buffer	250mM Tris HCl (pH 7.6), 50mM MgCl2, 5mM DTT, 5mM ATP, 25% PEG 8000
10x TAE	0.2 Tris, 50mM EDTA (pH adjusted to 8.0 with acetic acid)

e) Human β globin and HBV DNA PCR positive controls

Our positive β globin PCR control was the DNA extracted from primary human embryonic lung cells (MRC5) infected with the human cytomegalovirus (HCMV). The HBV DNA positive standard was at a titration of 10^{-6} (40 gev/ml).

f) Plasmid

PJI was used to express full-length surface genes (standard and variant types) in COS7 mammalian cells. PJI plasmid has a pUC backbone and contains a cytomegalovirus (CMV) promoter which allows very high expression of proteins in a variety of mammalian cells, a multiple cloning site (MCS) downstream of the promoter, for insertion of the target gene, a SV40 late poly (A) signal to direct proper processing and for increased stability of the PJI mRNAs (SV40pA), SV40 origin of replication for single stranded DNA production, and an ampicillin resistant gene (AMP^R) for prokaryotic selection.

g) Bacterial strain and growth media

DH5α	Escherichia coli. Genotype: ϕ80dlacZΔM 15, recA1, endA1, gyrA96, thi-1, hsdR17 (r_k^-, m_k^-), supE44, rel A1, deoR, Δ (lacZYA-argF) U169.
L-broth	Luria-Bertani liquid medium (10 g NaCl, 10 g Bacto Tryptone, 5 gm Bacto-yeast extract in one litre distelled water.
L-broth in agar	As above plus 10 g Bacto-agar.

h) COS7 culture system

These monkey kidney cells were originally derived from an African-monkey kidney cell line transformed by an origin defective mutant of SV-40. Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% bovine calf serum, 100 IU/ml penicillin/ 100 ug/ml streptomycin and 2mM glutamine. Trypsin (0.25% trypsin dissolved in Tris-saline) and Versene (600mM EDTA in PBS A, 0.0015% (w/v) phenol red) were used for splitting of the confluent cells.

i) Reagents and solutions for small scale plasmid preparation

Miniprep Solution I	25mM Tris HCl (pH 8.0), 50mM glucose, 10mM EDTA (pH 8.0)
Miniprep Solution II	0.2M NaOH, 1% SDS
Miniprep Solution III	3M pot. acetate, 5M acetic acid
Ethanol	100% and 70% (diluted with distilled water)

j) Reagents and solutions for large scale plasmid preparation using Midiprep QIAGEN Kit (Crawley, West Sussex, UK).

Buffer P1	50mM Tris HCl (pH 8.0), 10mM EDTA, 100ug/ml RNase A
Buffer P2	0.2M NaOH, 1% (w/v) SDS
Buffer P3	3M potassium acetate (pH 5.5)
Buffer QBT	750mM NaCl, 50mM MOPS (pH 7.0), 15% isopropanol, 0.15 % triton X-100
Buffer QC	1M NaCl, 50mM MOPS (pH 7.0), 15% isopropanol
Buffer QF	1.25 M NaCl, 50 mM Tris HCl (pH 8.5), 15% isopropanol
Buffer QN	1.6M NaCl, 50mM MOPS (pH 7.0), 15% isopropanol

k) Reagents and solutions for transfection

k1) $CaPO_4$ method reagents

$CaCl_2$	1M $CaCl_2$, filter-sterilised (0.22um), stored in 5ml aliquots at –20°C
2x HEBS buffer	280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 12mM Dextrose, 50mM HEPES, pH adjusted to 7.05 with NaOH, filter-sterilised and stored in aliquots as above.

K2) Lipofectase transfection Reagent

Lipofectase reagent was made "in house" using the following chemicals.

DDAB: Dimethyldioctadecyl-ammonium bromide; purchase from Sigma, D 2779.

DOPE: Dioleoyl L-α-phosphatidyl ethanolamine; purchased from Sigma, P0510.

K3) FuGENE™ 6 Transfection Reagent (Roche Diagnostics, Lewes, East Sussex, UK

FuGENE Reagent is a proprietary blend of lipids (non-liposomal formulation) and other compounds in 80% ethanol, filter-sterilised.

l) Reagents, solutions and requirements for ELISA

10xPBS	100mM phosphate, 1.5M NaCl (pH 7.2)
Dilution buffer	1xPBS
Blocking buffer	2% bovine serum albumin (BSA) in 1xPBS
Wash buffer	0.1% Tween® 20 (v/v) in 1xPBS
Substrate (ABTS, Peroxidase substrate system,	Purchased from Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA.
Flat bottomed Immulon wells	Dynatech laboratories Ltd, West Sussex, UK
ELISA plate reader	Anthos HT2 Version 1.21, Labtech International Limited, East Sussex, UK.

m) Antibodies for ELISA and immunofluorescence

Monoclonal anti-CMV Late Nuclear Protein antibody (anti-pp65 mouse)	Capricorn Products Inc. Scarborough, USA
Rabbit polyclonal antibody	A kind gift from Dr. H. Marsden (Virology Institute). Raised against a peptide from the N terminus of HMCV UL102 protein.
Protein A (used as anti-species anti-polyclonal antibody)	Purchased from Pharmacia.
High affinity anti-HA peroxidase (3F10)	MAb conjugated with peroxidase, used for the detection of HA-tagged recombinant proteins. (All anti-HA antibodies were purchased from Roche Diagnostics).
Biotinylated anti-HA	MAb conjugated with biotin, used for the capture of HA-tagged recombinant proteins.
FITC conjugated anti-HA	FITC conjugated MAb used for IF.
Polyclonal Goat anti-hepatitis B surface antigen (anti-HBs)	DAKO (Carpinteria, USA). Used at a working dilution of 1: 900 as the primary Ab for detection.
FITC conjugated rabbit anti-Goat IgG (whole molecule)	Sigma & used as secondary detection Ab at a working dilution of 1: 64.

n) Common Reagents

All reagents and chemicals were purchased from BDH Chemicals, (Poole, UK) or Sigma-Aldrich Co. (Poole, UK) unless otherwise stated in this section or in the methods section

Manufacturer	Chemicals
Beecham Research	Ampicillin
Bio-Rad	TEMED, Ammonium persulphate
Fluka	Formaldehyde
National Diagnostics	Sequagel 6 and Protogel pre-prepared acrylamide solutions
Oncor	Deionized formamide
Prolabo	Boric acid, chloroform, ethanol, glacial acetic acid, glycerol, isopropanol, methanol
Roche Diagnostics	Agarose, Tris base

Table 2.1: Primers used in this study

S gene Primers

Name	Primer sequence (5'>3')	Position	Type
S1	CCTGCTGGTGGCTCCAGTTC	56-75	Sense
S4	GTATGTTGCCCGTTTGTCCTC	459-479	~~
S6C	GCACACG*GAATTCCGAGGACTGGGGACCCTG	129-146	~~
S10	TCCTATGGGAGTGGGCCTCAG	636-656	~~
S2Na	CCACAATTCKTTGACATACTTTCCA (K=G/T)	1003-979	Anti-sense
S3	AATGGCACTAGTAAACTGAGCC	690-669	~~
S7D	GACACC†AAGCTTGGTTAGGGTTTAAATGTATACC	842-823	~~
S8	AGAAGATGAGGCATAGCAGC	434-415	~~

Pre S primers

Name	Primer sequence (5'>3')	Position	Type
F0	TGGGAACAAGAG/TCTAC	2835-2950	Sense
F7	AATCCA/CGATTGGGACT/CTCAA	2971-2990	~~
R4	TCCTG/AACTGG/CCGATTGGT	3159-3142	Anti-sense

C gene primers

Name	Primer sequence (5'>3')	Position	Type
C1	GGGAGGAGTTGGGGGAGGAGA	1732-1752	Sense
C3a	GA/GTCTWTGTAYTAGGAGGCTG (Y=C/T)	1763-1783	Sense
C4N	CCTTATGAGTCCAAGGRATA (R=G/A)	2478-1459	Anti-sense

S gene tagged Primers

Name	Sequence before the tag epitope including the restriction enzyme	Tag epitope	Sequence after the tag epitope
ABtag1	5' **GAG*GAATTC** ^{155} ATG	M-CMV	^{158} GAGAACATCACATCAGGA
ABtag2	5' **GAG*GAATTC** ^{155} ATG	P1-CMV	^{158} GAGAACATCACATCAGGA
ABtag3	5' **GAG*GAATTC** ^{155} ATG	P2-CMV	^{158} GAGAACATCACATCAGGA
ABtag4	5' **GAG*GAATTC** ^{155} ATG	Flu-HA	^{158} GAGAACATCACATCAGGA
BAtag1	5' **GACACC†AAGCTT** ^{838}GGTTTA	M-CMV	^{832} AATGTATACCCAGAG
BAtag2	5' **GACACC†AAGCTT** ^{838}GGTTTA	P1-CMV	^{832} AATGTATACCCAGAG
BAtag3	5' **GACACC†AAGCTT** ^{838}GGTTTA	P2-CMV	^{832} AATGTATACCCAGAG
BAtag4	5' **GACACC†AAGCTT** ^{838}GGTTTA	Flu-HA	^{832} AATGTATACCCAGAG

*: E coR1 restriction site (underlined).

†: Hind III restriction site (underlined).

Non-HBV sequence are written in bold and nucleotide position numbering are according to Okamoto et al., 1988.

2.2 METHODS

2.2.1 HBV DNA extraction, PCR, sequencing and Phylogenetic analysis

a) Extraction of human and HBV DNA from blood clots

a1) In house procedure

A pea sized blood clot was transferred to a sterile Eppendorf tube and the following reagents were added: 250 ul nucleic lysis mix, 250 ul "machine" lysis buffer (Applied Biosystems, Warrington, Cheshire, UK) and 50 ul proteinase K at 10 mg/ml. Samples were incubated at 55°C for 2-3 hours or overnight at 37°C followed by vortexing to dissolve the blood clot. Then, 500 ul of phenol chloroform was added and the DNA precipitated and washed with ethanol. The pellet was left to air dry and then resuspended in 50 ul 1x TE buffer.

a2) TriPure^{TM} Isolation Reagent (Roche Diagnostics, Lewes, East Sussex, UK).

1 ml TriPure isolation reagent was added to the pea sized blood clot in a sterile Eppendorf tube and the cells were lysed by repetitive pipetting. The samples were incubated for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes. 0.2 ml chloroform was added, the tube capped securely and shaken vigorously. Further incubation at room temperature for 2-15 min was carried out followed by centrifugation at 12,000 x g for 15 min to separate the solution into three phases. After centrifugation, the upper, aqueous, colourless phase containing RNA was carefully removed. DNA precipitation from the interphase and the red organic phase was performed with 96% ethanol. Samples were washed 3 times with 0.1 M sodium citrate in 10% ethanol and then once in 75% ethanol. The DNA pellet was air-dried and then resuspended in 50 ul 8 mM NAOH. Finally, the pH of the isolated DNA was adjusted to 8.4 using 0.1 M HEPES.

a3) High Pure Viral Nucleic Acid kit (Roche Diagnostics, Lewes, UK).

For the Roche kit, 200 ul of working solution (binding buffer supplemented with poly (A) carrier RNA) and subsequently 40 ul of 20 mg/ml proteinase K were added to the pea sized blood clot in a sterile Eppendorf tube, mixed and incubated for 10 min at 72°C. After the incubation, 100 ul of isopropanol was added. The filters and collection tubes were combined

and the samples pipetted into the upper reservoir followed by centrifugation for 1 min at 8,000 x g and the flowthrough discarded. The filter was washed twice with the wash buffer and the flowthrough discarded after each wash. Finally, centrifugation for 10 seconds at full speed removed all the residual wash buffer. Collection tubes were discarded and clean nuclease-free 1.5 ml tubes were used to collect the eluted DNA in 50 ul of elution buffer.

a4) QIAamp blood kit (Qiagen Ltd, Crawley, UK).

For the Qiagen kit, although Roche and Qiagen kits employ the same principle, the reagents are different. Buffer AL, Qiagen protease, ethanol, and buffer AW were used instead of the working solution, proteinase K, isopropanol, and wash buffer respectively that were employed in the Roche kit.

NB. HBV DNA were extracted from the sera using either High Pure Viral Nucleic Acid Roche Kit or the QIAamp blood kit from QIAGEN.

b) Preparation of oligodeoxyribonucleotides

All oligodeoxyribonucleotides used in this study were synthesised "in house" using a Cruachem PS250 oligonucleotide synthesiser. They were eluted from their synthetic columns with 1.5ml ammonia solution (high grade), deprotected at 55°C for 5 hours and dried under vacuum by spinning overnight. The precipitated pellets were resuspended in 100- 200 ul dH_2O and oligonucleotides concentration was determined by measuring the optical density (OD) at 260 and 280 nm wave lengths (where 260/ 280 reading ratio should be around 1.8, to rule out any contamination possibility). Aliquots were made and stored at –20°C.

For oligonucleotides over 45-50 bases further purification have been done by polyacrylamide gel electrophoresis (PAGE). 50ul of each resuspended oligonucleotide was mixed with an equal volume of deionized formamide and run on a 12% polyacrylamide gel (20ml of 30% protogel, 5ml 10x TBE, 24.4 dH2O, 40ul TEMED and 600ul ammonium persulphate). The oligonucleotides were run in separate wells (with a few wells space left in between them to prevent cross contamination). Gel loading buffer was loaded in a separate well to act as a molecular weight marker. Electrophoresis was performed at 400V/ 10mA for 2-3 hours in 1x TBE. Then, the gel was removed from the plates, wrapped in cling film and viewed by ultraviolet shadow- casting technique. The bands were cut with a sterile scalpel, diced and incubated overnight in 1ml acrylamide gel elution buffer at 37°C. After

centrifugation, the eluted oligonucleotides were removed, phenol: chloroform extracted and ethanol precipitated. The pellet was washed with 70% ethanol, dried under vacuum and finally resuspended in 30- 50 ul dH_2O.

c) PCR amplification of the extracted DNA

c1) β globin PCR

Five microliters of extracted DNA was amplified in 50ul master mix containing 1.25 U Taq polymerase (Gibco, Paisley, UK), 2.5 U TaqStart^{TM} antibody (Clontech Laboratories Inc, Palo Alto, CA, USA), 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 10X PCR buffer (supplied with Taq polymerase) and 20 pmol of each primer, Pco3 (5` ACACAACTGTGTTCACTAGC) and Pco4 (5` CAACTTCATCCACGTTCACC). The reaction mix was overlaid with 50ul mineral oil to prevent evaporation. The reactions were performed on a Biometra TRIO Thermoblock using the following program; five min at 94°C, followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min. The product is 110 bp.

c2) HBV DNA S gene PCR

Hot start PCR was performed using a nested protocol and antibody to Taq polymerase to amplify the surface (S) gene of HBV. Five microliters of extracted DNA was amplified in 50ul solution containing 1.25 U Taq polymerase (Gibco, Paisley, UK), 2.5 u TaqStart^{TM} antibody (Clontech Laboratories Inc., Palo Alto, CA, USA), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 10X PCR buffer, and 25pmol of each primer (S1: sense 5'-CCTGCTGGTGGCTCCAGTTC-3' and S2Na: antisense 5'-CCACAATTCKTTGACATACTTTCCA-3'; where K= G or T), for 5 cycles of 95°C for 1 min, 55°C for 1min, and 72°C for 90 sec followed by 35 cycles with the denaturation temperature reduced to 90°C. One microlitre of first round PCR product was then re-amplified in the same solution as above except for nested primers, (S6C: sense and S7D: anti sense, see Table 2.1). Conditions used were; 5 cycles of 95°C for 1 min, 55°C for 75 sec, and 72°C for 90 sec followed by 25 cycles with the denaturation temperature reduced to 90°C.

c3) HBV DNA Core/ PreS regions PCR

A hemi-nested protocol was used for the amplification of both regions. Primers C1 and C4 followed by C3a and C4 were used in core region amplification, whereas, primers F0 and R4 followed by F7 and R4 were used to amplify the Pre-S region. The reaction mix (except for

using $MgCl_2$ at 1.5 mM concentration in core amplification) and program cycles of Biometra TRIO Thermoblock, were similar to those used in S gene amplification. Amplification of these regions was employed to confirm the PCR or sequencing results of S gene.

d) Agarose gel electrophoresis

Agarose gel electrophoresis was used to confirm the right size of our amplified PCR products by visualizing the gel on an ultraviolet transilluminator. Also, it was used to check linearized plasmids (after enzyme cut and before ligation) and restriction digests of miniprep DNA. Gels were prepared by adding 1g agarose to 100ml 1X TBE buffer. The solution was boiled until dissolved and left to cool. Then, 50ul ethidium bromide (1mg/ ml) was added before pouring the gel. One ul of agarose gel loading buffer was added to each sample before loading, followed by running the gel at 80-90V in 1X TBE buffer for 25min.

e) Purification of the PCR product

e1) DNA purification using Geneclean Kit (Bio lab 101 Inc, CA, USA):

After running an adequate amount of the PCR product on the gel, DNA fragments of the expected size were located by visualizing the gel on an ultraviolet transilluminator. The correct bands were cut and placed in 1.5ml tubes with 3 volumes of sodium iodide and 0.5 volume of TBE gel modified buffer and incubated at 55°C for 10-15min. After complete melting of the gel, 5ul of glass milk was added to each tube. The tubes were vortexed and incubated at room temperature for 10min. The tubes were spun for 30 seconds at 13,000 rpm in a bench-top microfuge, and the resulting pellet washed twice with 0.5ml ice cold NEW wash (containing 14ml concentrate provided with the Geneclean kit, 280ml distilled water and 310ml 100% ethanol). The pellet was dried and the DNA eluted in 50ul of dH_2O by incubation at 55°C for 5min. Finally, the supernatant was collected after spinning the suspension at 13,000rpm for 2min.

e2) DNA purification by High Pure PCR Product purification kit
(Roche Diagnostics, Lewes, UK)

250ul of binding buffer was added to 50ul of PCR reaction product and mixed well. The mixture was poured onto the High Pure filter tube and centrifuged at 13,000rpm for 30sec.

The filter tube was washed twice. Finally, the filter was inserted in RNase free 1.5ml tube and dH_2O was applied to elute the DNA (higher volumes of dH_2O for elution are preferred as it increases the elution efficiency).

f) DNA sequencing

f1) DNA purification and sequencing

DNA was firstly purified by using one of the previously mentioned kits (2.2.1. e1, 2). Sequencing of the S gene was performed either directly from the purified PCR products or after S gene cloning using an automated sequencer (ABI Prism, 377 DNA sequencer, Applied Biosystem, Perkin Elmer) according to the manufacturer's instructions. The reaction mix was prepared by adding 1.6pmol of the primer to 20-30ng purified PCR product in a total volume of 6ul. For the cloned S gene, 200ng of the DNA were used instead in the reaction mix.

f2) Sequence data analysis

The Sequence Navigator software program (Applied Biosystem, Cheshire, UK) was used to analyse our sequence data. The sequences were aligned and the consensus sequence was determined for sequences that have the same subtype/ genotype. Moreover, other HBV S gene sequences from Gene bank, representing the different HBV genotypes, were retrieved and analysed with our studied sequences.

g) Phylogenetic analysis

Sequences were aligned using Clustal V. Phylogenetic trees were reconstructed and drawn using NEIGHBOR and DRAWGRAM programes from the PHYLIP package v3.5C (Felsentein 1993) and phylogenetic networks were constructed using NETWORK 2.0B (Bandelt et al., 1995).

g1) Phylogenetic tree construction

A big simple Neighbor-joining Tree was constructed using the S gene (681bp) nucleotide sequences from 102 isolates (Figure 3.4), consisting of 20 sequences chosen randomly from each island and 22 S gene sequences representing the different genotypes of HBV retrieved from Genbank. The aim was to reveal the overall picture of circulating HBV genotypes in the four Pacific islands.

NB** For a small part of the work, phylogenetic analysis was performed on a 477-bp fragment of the S gene bracketing nucleotides 82-558 using sequences obtained from GenBank and sequences from within the UK. The phylogenetic tree (which was performed by Siew Lin Ngui; Chapter 3.6, Fig 3.12) was constructed using Megalign from the Lasergene Navigator suite of programmes (DNASTAR, Madison, WI, USA).

g2) Phylogenetic network construction

Construction of a Genotype C Pacific Network

In order to resolve the ancestry of the Pacific C genotype sample in the context of world HBV C genotype variability, a phylogenetic network was constructed containing the 64 Pacific C genotype HBsAg sequences. This was augmented by the inclusion of a selection of 25 database sequences, with highest similarity in FASTA research to the sample sequences. The pooled dataset contained 89 sequences of 681nt, with 129 variable sites across the S gene sequence. Of these, 20 tri-and tetra-morphic sites were excluded as unsuitable for analysis using the reduced median network approach, which requires binary data. A further 13 dimorphic sites were initially excluded on the basis that they displayed a high degree of incompatibility with other sites in the data. This was taken as evidence that they had mutated several times in the history of the sample, and therefore would not be helpful in inferring the structure of the phylogeny.

Initially, separate networks were constructed for the database sequences and the Pacific sequences, including some key sequences in both datasets. Inspection of the initial network for Pacific sequences indicated that a further site (nt13) should be removed in order to reduce the amount of reticulation in the network. In the next stage, selected sequences central to each network were added to the alternate dataset to construct overlapping networks sharing key haplotypes. The networks were then obtained by superimposing these key haplotypes. Finally, each site which had previously been excluded was tested for re-inclusion, and was re-incorporated if parallel mutations at that site could be resolved into separate mutations in distinct clusters which were already apparent in the rest of the data. Thus, 5 out of the 14 excluded sites were re-introduced in this way (see Figure 3.5).

Construction of a Genotype D Pacific Network

The reconstruction of the D genotype phylogeny was simpler than that for the C genotype, because of the smaller sample size and consequent reduction in the amount of homoplasy, or site incompatibility, corresponding to evidence for multiple substitutions at some sites. A screen of the database was used to identify all distinct sequences that were within 7 mutational steps of any sequence in the sample, and the augmented dataset was used to construct a phylogenetic network. The dataset contained 50 sequences, with variation at 76 sites. There were 12 trimorphic sites, which were unambiguously resolved into separate characters before network construction. Of these 12, only one was trimorphic within the Pacific sample, which was indicative of the high degree of relatedness between Pacific D sequences.

h) Time estimates

The method of Morral (1994) was used to make relative estimates of the time since the respective putative ancestor of the C and D samples. The quantity rho; the average number of mutations along branches to the founder sequence, was calculated for each genotype (Table 2.2). V102, the central sequence of the main cluster was taken as the founding C genotype because the sequences V185, X75656 and X75665 appear to share an older common ancestor with the main cluster which may have existed before the main founding event (see Figure 3.6). The sequence defined by K232/T269 was taken as the founder sequence for the D sample and because it is uncertain whether the branch leading to K202, F148, F306 and T251is derived from the same colonisation event, calculations were done both with and without these sequences (see Figure 3.5).

For D, rho was calculated from the network for an arbitrarily chosen, likely tree, and other likely trees gave similar values. For C, because of the complicated nature of the final network, rho was calculated approximately from the full incidence matrix generated by resolving most incompatibilities into separate sites. The effect of the remaining ambiguity in the network structure was a slight inaccuracy of some branch lengths. This effect is unlikely to be serious where parallelisms in the network are small and confined to single branches, and this is demonstrated for D where the data was uncomplicated enough to apply both methods. The Saillard et al. (2000) estimator for var (rho) was used to decide whether differences in rho were statistically significant, and, as before the calculations were done explicitly for D and by approximation for C.

Table 2.2: Time estimates of HBV ancestor in Pacific islands*

genotype	C	D		D	
method	approx.	exact	approx.	exact	approx.
sequences	V102 cluster	all sequences	all sequences	without K202 branch	without K202 branch
rho	8.87 nt	3.08 nt	3.04 nt	2.19 nt	2.2 nt4
s.d.	1.44	0.52	0.54	0.39	0.43
Confidence Interval	6.0 – 11.8	2.0 – 4.1		1.4 – 3.0	
Age (1)	432 – 850 yr	144 – 295 yr		101 – 216 yr	
Age (2)	39 -77 yr	13 – 27 yr		9 – 20 yr	

*: Calculations were performed according to the mutation rates published in Hannoun et al. (2000).

Rho: The average number of mutations along branches to the founder sequence

(1): HBeAg positive rate; 2.04×10^{-5}

(2): HBeAg negative rate; 2.25×10^{-4}

2.2.2 Molecular cloning and transfection methods

a) Digestion of the vector/ insert by endonuclease digestion

Intially, PCR products of the amplified S genes (DNA insert) were purified by one of the described methods (2.2.1.e). Then, restriction enzyme digestion of both insert and vector was carried out with Hind III and EcoR I enzymes. Typically, a digestion mixture was composed of DNA, endonuclease enzyme (10 units of enzyme per 1ug of DNA) and the optimal buffer at the right concentration. Temperature and time of incubation were adjusted as specified by the manufacturers.

b) Purification and concentration estimation of digested DNA

Digested vector and insert were run on 1% agarose gel and visualised on an ultra-violet transilluminator. Then, appropriate bands were excised and purified which was followed by a

comparative gel, where different concentrations of both vector and insert were estimated with agarose gel electrophoresis in presence of molecular weight marker of known concentration.

c) Ligation

PCR products were ligated into mammalian expression vector PJI after digestion with Hind III and EcoR I. The appropriate vector: insert ratio was used. Ligation mixture was added to the vector insert mix, containing 1 unit T4 ligase/ ug DNA, 3ul T4 ligase buffer and dH2O to a final volume of 15. This was incubated for 3-4 hr at 16°C. Half of the ligated DNA was used immediately and the rest stored at –20°C.

d) Preparation of *E.* coli competent cells

10ul of DH5α glycerol stock was added to 10ml of LB broth and incubated overnight at 37°C in a shaking incubator. 0.5ml of the overnight culture transferred to 500ml of pre-warmed LB broth and incubated at 37°C for 2-3hr, until the OD_{600} of the culture was approximately 0.3. Cells were then transferred to 50ml Falcon tubes and spun at 2,800rpm for 10min at 4°C. The cells pellet was resuspended in 20ml cold sterile 100mM $CaCl_2$ and kept on ice for 2hr. This spin was repeated and the pellet resuspended in 2ml of cold 100mM $CaCl_2$ and left on ice for 30min. The resuspended cells were then kept overnight at 4°C before transformation to enhance their competency. For storage, 1ml aliquots of re-suspended cells, after adding glycerol at a concentration of 15%, were snap frozen in liquid nitrogen and stored at –70°C. Glycerol stocks were prepared by adding glycerol to a final concentration of 40%, snap frozen and stored as before.

e) Transformation of E. coli

7.5ul of the ligation reaction was added to 100ul of competent E. coli and incubated on ice for 1hr. The cells were then heat shocked in a 42°C water bath for 2min followed by 5min incubation on ice. One ml of RT-pre-warmed L-broth was added to the cells and incubated at 37°C for 1hr. In the meantime, LB agar plates containing ampicillin at 100ug/ml were prepared. The bacteria were spun for 10 seconds at 13,000 rpm followed by removal of the supernatant except for 100ul. These transformed bacteria, after gentle shaking, were plated onto the LB agar/ampicillin plates. 100ng of uncut plasmid, positive control, cut plasmid, negative control, were also transformed. 10ul of 10^{-6} competent E. coli were plated on LB

agar plates without ampicillin as a bacterial control. The plates were then incubated overnight at 37°C.

f) Small Scale Plasmid Preparation (Mini-prep)

Colonies of transformed bacteria were inoculated into 2-3ml of LB broth containing ampicillin at 100ug/ml and incubated in a shaker at 37°C overnight. One ml of the overnight culture was transferred to a 1.5ml eppendorf tube and centrifuged at 13,000rpm for 30sec. The pellet, after decanting the supernatant, was resuspended in 100ul of ice cold solution (I) and left at RT for 5min. 200ul of freshly prepared solution (II) was added, mixed gently and left on ice for 2-3min. 150ul of solution (III) were added, mixed by few inversions, left on ice for 2-3min. 150ul of phenol/chloroform was added to the mixture, vortexed and centrifuged at 13,000rpm for 3-5min. The aqueous phase was transferred to a fresh tube, 800ul of 100% ice cold ethanol was added to precipitate the plasmid DNA and centrifuged for 10min.

The pellet was then washed with 70% chilled ethanol, air dried and finally resuspended in 50ul dH_2O containing Rnase at 20ug/ml to remove any contaminating RNA.

g) Restriction enzyme digestion

Restriction digestion of plasmid DNA was carried out to confirm successful cloning. The standard mixture consisted of 1.5ul 10X restriction buffer (B), 0.2ul (2 units) of each restriction enzyme (EcoR1 & HindIII), 5ul plasmid DNA and dH_2O to a final volume of 15ul. The digest mixture was then incubated at 37°C for 2-3hr.

h) Large scale plasmid DNA preparation

Large scale plasmid preparations were performed to purify plasmid DNA, using the QIAGEN midi kit. 0.5ml of the overnight culture of confirmed clones was added to 50ml L-broth containing 100ug/ml ampicillin and incubated overnight at 37°C. The culture volume was divided into two equal parts which were spun at 3,000rpm for 15min at 4°C. One of the bacterial pellets was stored at -20°C, while the second was resuspended by vigorous vortexing in 4ml of buffer P1. 4ml of buffer P2 was added and mixed gently by several inversions. After 5min incubation at RT, 4ml of chilled buffer P3 was added and mixed gently. The lysate was poured into the QIAfilter cartridge with screw cap on and incubated at RT for 10min. In the meantime, QIAGEN-tip 100 was equilibrated with 4ml of buffer QBT. The lysate filtrate was

then transferred from QIAfilter to the equilibrated QIAGEN-tip and left to drip by gravity flow. After all the lysate filtrate had dripped through the QIAGEN-tip, the resin tip was washed twice with wash buffer QC. Elution of the plasmid DNA from the resin tip was finally achieved by addition of 5ml elution buffer QF. The plasmid DNA was precipitated by the addition of 3.5ml isopropanol followed by centrifugation at 11,000rpm (Sorvall SM24 rotor) for 30min at 4°C. The pellet was then washed with 70% ethanol, air dried and resuspended in 100ul dH_2O. Finally, the plasmid DNA concentration was determined by measuring the absorbance at 260 nm.

i) COS7 cell culture

COS7 cells were grown and passaged in sterile, disposable 175cm^2 flasks. From a confluent cell culture flask, media were poured off and cells washed twice with 20ml versene. The cells were then trypsinised with a solution of trypsin and Versene, at 1:1 ratio, gently swirled and poured off. After 5min incubation at 37°C, the flask was gently tapped to dislodge all the cells.

Cells were then resuspended in 10ml of DMEM containing 100IU/ml penicillin, 100ug/ml streptomycin, 2mM glutamine and 10% foetal calf serum (FCS). 1ml of harvested cells was used to seed a new flask containing 50ml medium, gassed with 70ml CO_2 and incubated at 37°C+5% CO_2. For storage, COS7 stock cells containing DMSO at a final concentration of 15% were kept in nitrogen liquid after overnight incubation at –20°C.

j) Transfection

j1) Calcium phosphate ($CaPO_4$) transfection

For 6cm diameter plates, cells were seeded on 13mm glass coverslips at 2x 10^6 cells per plate and incubated overnight at 37°C until they become 60-70% confluent. 250ul solution containing the plasmid DNA (10ug) and $CaCl_2$ (0.25M) were made and left at RT for 1-2hr. 250ul of 2x HEBS were then added to the DNA/$CaCL_2$ solution slowly and mixed gently to allow a fine precipitate of $CaPO_4$ to form. After 30min incubation at RT, the transfection mixture was added across the plate and incubated at 37°C for approximately 16hr. The medium containing the transfection mixture was then replaced with a 5ml of fresh DMEM and the incubation continued for a further 2-3 days. Two plasmids (pJI) containing standard HBV DNA surface gene sequence (both *adw* and *ayw* subtypes) were used as positive

controls for transfection and antigenic analysis. Negative transfection control, an uncut plasmid without gene sequences, was also used during each transfection. To confirm HBsAg protein expression, the supernatants were harvested after 3 days and immunofluorescent staining performed on the cell-monolayered coverslips.

j2) Liposomal Transfection

The plasmid, with its entire HBV surface gene insert, was transfected into subconfluent monolayers of COS7 cells on 13mm coverslips in 60mm petri-dishes using cationic liposomes made from dioleoyl L-α-phosphatidyl ethanolamine and dimethyldioctadecyl ammonium bromide (Sigma-Aldrich, Poole, UK). Briefly, 2μg plasmid was diluted in 200μl Optimem 1 reduced serum medium (Life Technologies, Paisley, UK) and, in a separate vial, 24μl of liposomes was added to 200μl of Optimem 1. The two solutions were mixed and allowed to stand for 15 min at room temperature, then further diluted to 2ml using Optimem 1 and added to pre-washed COS7 cells. The cells were incubated with the transfection mixture for 5h at 37°C in 5% CO_2 and then 3ml of COS7 medium added (DMEM with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamin (Life Technologies, Paisley, UK).

Cells were incubated for 16h at 37^0C in 5% CO_2 when the transfection mixture was removed and 5ml of fresh COS7 medium added. Transfection controls and HBsAg protein expression detection were done as described before in $CaPO_4$ method.

j3) FuGENE™ 6 transfection Reagent

The FuGENE™ 6 Transfection Reagent (Roche Diagnostics, Lewes, East Sussex, UK) has enabled us to use a smaller concentration of pasmid DNA, achieve a better transfection and, hence, protein expression. The DNA was transfected into subconfluent monolayers of COS7 cells on 13mm coverslips. 10ul of FuGENE was added to 95ul of Optimem 1 reduced-serum medium (Life Technologies, Paisley, UK), and incubated at room temperature for 5 min. Meanwhile, 3ug of DNA were added to a separate tube. The FuGENE/ Optimem mixture was added slowly to the DNA and incubated for 15-20 min at RT. Then, this mixture was added to the adherent subconfluent COS7 cells in a 60mm petri dish containing 5ml DMEM and incubated for 3 days at 37°C. Transfection controls and HBsAg protein expression detection were done as described before in $CaPO_4$ method.

k) Immunofluorescence

Transfected cell monolayers on cover slips were washed twice with PBS. The cells were then fixed with chilled methanol (at RT for 10min), washed three times with PBS and permeabilised with 0.5% triton X-100 in PBS. The permeabilised cells were rinsed twice with 0.05% Tween in PBS and incubated with goat anti-HBsAg polyclonal antibody (Dako Ltd, High Wycombe, UK) at 1: 900 dilution for 60 min at room temperature in a moist dark box. After washing, the cells were further incubated with anti-goat FITC labelled immunoglobulin (IgG) for 30 minutes (Sigma-Aldrich Company, Poole, UK) at 1: 64 dilution. After three washes, the cover slips was dried carefully and mounted on glass slides with a drop of Citifluor, a glycerol/ PBS solution. After 10-15min, cover slips were then examined using a Nikon Microphot-SA fuorescence microscope and pictures of fluorescing cells were taken. Additionally, FITC-conjugated monoclonal anti-HA (anti-HA FITC conjugated, Roche Diagnostics, Lewes, East Sussex, UK) was used for direct immunofluorescence. The same steps were followed as before with omitting the secondary detection antibody step.

2.2.3 Tag ELISA system

a) Epitope Tags and Approaches

M-CMV epitope, ERKTPRVTGG, is derived from pp65 matrix protein (McLauchlan et al., 1994).

P_1-CMV epitope, MTAQPPLHHRHHPYA, is derived from the first 15 aa of the N terminus of HCMV UL102 protein and is recognised by an in-house rabbit polyclonal serum (PAb 371).

P_2-CMV epitope, is the first 10 amino acids of P_1-CMV.

Flu-HA epitope, YPYDVPDYA, is derived from human influenza hemagglutinn protein.

Tag epitopes were first inserted into the standard S gene sequence to evaluate their effect on HBsAg reactivity. M-CMV was inserted at either the 5'(after start codon) or 3' terminus (before stop codon) of S gene (see Figure 3.7; Constructs Y tag & Y 67). Anti-CMV MAb (Capricorn Products, Scarborough, USA) was used for capture and anti-HBs (Murex Biotech Ltd, Dartford, UK) for detection (see Figure 3.8). Next, P-CMV epitopes (P_1 or P_2) were inserted at the opposite end to M-CMV in Y tag and Y67 constructs. This design would have allowed a sandwich ELISA to be designed without cross-reacting capture and detection antibodies. The epitope was previously defined as 15 aa long (H. Marsden, unpublished data)

but to avoid any length effect on HBsAg antigenicity, the first 10 aa was used in separate experiments. All four possible dual constructs were generated (T1, T2, T3 and T4). To assess the effect on HBsAg antigenicity of insertion of single P-CMV epitopes, all four constructs were also generated (P1, P2, P3 and P4). In the dual tag system, a MAb anti-CMV was used for capture and rabbit PAb followed by protein A for detection. Finally, we made a modified single tag system using HA-tag epitope at the 5'end of S gene. A biotinylated anti-HA antibody was used for capture on to avidin-coated plates and a peroxidase-labelled anti-HA for detection.

b) Primers design and PCR

A series of primers were constructed to insert the tags at the 5' or 3' end of HBsAg (Table 2.1). All primers were synthesized in house using a Cruachem PS250 oligonucleotide synthesizer. For single tag insertion, one tagged primer, BAtag, (anti-sense) or ABtag (sense), was used in conjunction with a previously described non-tagged primer, S6C (sense) or S7D (anti-sense) respectively. Both ABtag (sense) & BAtag (antisense) were employed for dual tag insertion.

Single round PCR was performed on plasmids previously constructed containing HBV DNA S gene of variant or standard type. Briefly, one microlitre of plasmid (100fg) was amplified in 50ul solution containing 1.25 U Taq polymerase (Life Technologies, Paisley, UK), 2.5 U TaqStart^{TM} antibody (Clontech Laboratories Inc., Palo Alto, CA, USA), 0.25 mM dNTPs (Pharmacia, St. Albans, UK), 2.5 $MgCl_2$, 10X PCR buffer, and 25 pmol of each primer for 5 cycles of 95°C for 1 min, 55°C for 75 sec, and 72°C for 90 sec followed by 25 cycles with the denaturation temperature reduced to 90°C.

c) Cloning, transfection & Immunofuorescence

PCR products were ligated into the mammalian expression vector pJI, after digestion with Hind III & EcoR I. Ligation and transformation were performed using standard methods (2.2.2. c & e). Plasmid was purified using Qiagen plasmid midi-kit (Qiagen Ltd, Crawley, West Sussex, UK). All constructs were confirmed by sequencing on an automated sequencer (ABI Prism, 377 DNA sequencer, Perkin Elmer). Constructs were transfected into subconfluent monolayers of COS7 cells on 13mm coverslips using the FuGENE^{TM} reagent (2.2.2. J). Immunofluorescence of the transfected cells was also performed as described before in section (2.2.2. k).

d) Cell lysate

Transfected cells, which were left in the plates after removal of cover slips, were washed with PBS and harvested with cell scrapers (Becton Dickinson, New Jersey, USA) into 1 ml PBS. The samples were centrifuged at 6.500rpm for 5min and the supernatant discarded. The cell pellets were lysed in 300ul of PBS containing 0.25 M Tris (pH 8.0) by freezing on dry ice and thawing in a 37°C water bath for two cycles. After centrifugation at 13,000rpm for 5min, the supernatants were collected.

e) ELISA and standardisation of HBsAg variants

I did not develop ELISA for the first two systems, M-CMV tag system and the dual tag, due to reagent costs and destroyed HBsAg antigenicity respectively (see Chapter 3.3). The HA-tag was inserted at 5' end of the surface gene into standard sequence subtypes *ayw*1, *ayw*2, *ayw*3, and *adw*2 in addition to 12 diagnostically important variants. The clinical background and aa changes of the variants used in this study are shown in Table 3.15.

Briefly, flat bottomed immulon wells (Dynatech Laboratories Ltd, Daux Road, West Sussex, UK) were coated with the capture Ab, biotinylated anti-HA, at 200 ng/well overnight at 4°C. This was followed by washing 5 times with the wash buffer.

Then, blocking with 2% bovine serum albumin (BSA) was carried out for 2 hours at 37°C followed by washing. HBsAg containing samples were incubated for 1 hour at 37°C followed by washing. Detection Ab, anti-HA labelled with peroxidase, was added at 100ng/ml, and incubated for 1 hour at 37°C followed by washing. Substrate (ABTS Microwell, peroxidase substrate system, Maryland, USA) was added and left for 15-30 min for colour development. Finally, the OD was determined at 405 by an ELISA plate reader (Anthos HT2 Version 1.21, Labtech International Limited, East Sussex, UK).

A yeast expressed, recombinant HA-tagged HBsAg protein was used as a positive control and non-tagged standard S gene was used as a negative control. Cut-off value was calculated by adding 0.05 to the mean of the negative control replicates (Mean Negative Control). Assay specificity was confirmed by substituting one component at a time; capture Ab, samples with different tag and detection Ab. The HA-tag assay was repeated at least five times, either from the same supernatant patch or from a different transfection-expression patch, to assess reproducibility of test results. I also defined the sensitivity of our tag ELISA by using decreasing concentrations of the positive control (a kind gift from Dr Ralph Gehrke). The

lowest limit of detection was 50pg/ml (see Figure 3.10). The mean value of L/S ratios was also statistically analysed (see below)

To standardise the expressed-tagged particles in the ELISA, various concentrations (up to five times) of the supernatants were generated using Vivaspin (Vivascience Ltd, Lincoln, UK). Each concentration was tested in the tag-ELISA and a dilution that gave a similar signal, 0.9 OD at 405nm, chosen for all constructs (variants and controls). After this standardisation, three commercial assays were used to compare HBsAg reactivity. IMX HBsAg (V2) semi-automated system (Abbott Laboratories Ltd., Maidenhead, UK) employs mouse MAb for capture and goat PAb for detection. Murex HBsAg GE14, (Murex Biotechnology Ltd., Dartford, UK) (2 hour procedure), uses goat PAb for capture and mouse MAb for detection. BioELISA HBsAg colour, (BIOKIT, Longfield, Kent, UK) (standard procedure) uses guinea pig PAb for capture and goat PAb for detection. A standard serum containing 0.5ng/ml HBsAg (working standard from National Institute for Biological Standards and Control, South Mimms, UK), was used as a sensitivity control. Reactivities were also expressed as a percentage of that found for the standard HBsAg (see Chapter 3.3).

f) Statistical analysis

MINITAB Program (1994 copyright, release 10.1) was used to analyse our data. One-way analysis of variance (ANOVA) was used to compare the values of variants L/S ratios to those of their cognate standard ratio. A P value of <0.05 was considered statistically significant.

g) HBsAg ELISA (Commercial assays)

The assays used in the study (see Chapters 3.4 and 3.5) were - [1] bioELISA HBsAg colour, BIOKIT, Longfield, Kent, UK (standard procedure); [2] AUSRIA II-125, Abbott Laboratories Ltd., Maidenhead, UK (overnight room temperature procedure); [3] VIDAS HBsAg, bioMerieux SA, Marcy-l'Etoile, France (long protocol); [4] Enzymun-Test HBsAg ES300, semi-automated system, Boehringer Mannheim GmbH, Mannheim, Germany; [5] IMX HBsAg (V2) semi-automated system, Abbott Laboratories Ltd.,Maidenhead, UK; [6] Murex HBsAg GE14, Murex Biotechnology Ltd., Dartford, UK (2 hour procedure) and [7] Enzygnost HBsAg Monoclonal II, Behring Diagnostics GmbH, Marburg, Germany (manual procedure). All the assays were used according to the manufacturer's instructions.

CHAPTER 3 RESULTS

3.1 Pacific study preparatory work

(An efficient extraction method from blood clots for studies requiring both host and viral DNA)

3.1.1 Introduction

The clot from blood is usually discarded after collection of the serum. Yet, it contains nucleated white blood cells and a substantial amount of serum. Moreover, HLA typing of individuals who have either cleared HBV infection, are chronic carriers or have no evidence of infection would allow us to study host immune factors that may influence the course of infection. Specific HLA class II alleles are associated with both hepatitis B and C viral clearance (Thursz et al., 1995; Diepolder et al., 1998; Cramp et al., 1998).

If testing for HLA and hepatitis viruses is required in addition to other serological markers, there is a need to minimize the blood volume used in laboratory testing, especially in young children as is the case with our Pacific study I (Chapter 3.2). Direct PCR amplification from whole blood without prior DNA isolation has been attempted (McCusker et al 1992), but sensitivity of viral DNA detection is low (Mercier et al., 1990), and the DNA cannot be stored for further investigation (Kanai et al., 1994).

Methods have been described in the literature for DNA extraction from whole liquid blood (Parzer & Mannhalter 1991; Scherczinger et al., 1997) as well as from clotted blood (Kanai et al., 1994; Garg et al., 1996). As clotted blood is usually discarded after collection of the serum, extraction of DNA from clots could be useful and efficient. Proteinase K, a powerful proteolytic enzyme with a broad target spectrum, has been used in nucleic acid isolation for more than 25 years (Gross et al., 1973).

In this study we compared four methods, three dependent on digestion with proteinase K, for extraction of both human (β globin) and viral (HBV) DNA from clotted blood (see Section 2.2.1). In the initial extraction, we amplified β globin from clots of HBV negative blood. To control the reaction, DNA extracted by a standard technique from primary human embryonic lung cells (MRC5) infected with the human cytomegalovirus (HCMV) was used. The HBV DNA source was blood from two HBV carrier patients with a low titre of viremia.

Subsequent precise analyses were done on a dilution series of HBV positive stock serum (our internal laboratory standard) diluted in normal blood negative for HBV. The dilution series was from 10^{-3} to 10^{-6}, equivalent to 4 x 10^4- 4x 10 gev/ ml (genome equivalent per ml). After leaving the blood to clot, serum and blood clots were separated by centrifugation and aliquoted. Our positive serum standard for HBV PCR at a dilution of 10^{-6} (40 gev/ml) was also extracted.

3.1.2 Results and Discussion

The kits and reagents were assessed for extraction of human DNA β globin on HBV negative blood and then on HBV carrier patients in order to measure the HBV DNA levels. Equal volumes of the PCR products were run on 1% agarose gels stained with ethidium bromide. The intensity of PCR bands, reflecting the quantity and perhaps the purity of the isolated DNA, was used to roughly estimate the amount of DNA isolated using each kit. Qiagen & Roche High Pure kits had obviously brighter DNA bands as shown in (Figure 3.1 & Table 3.1).

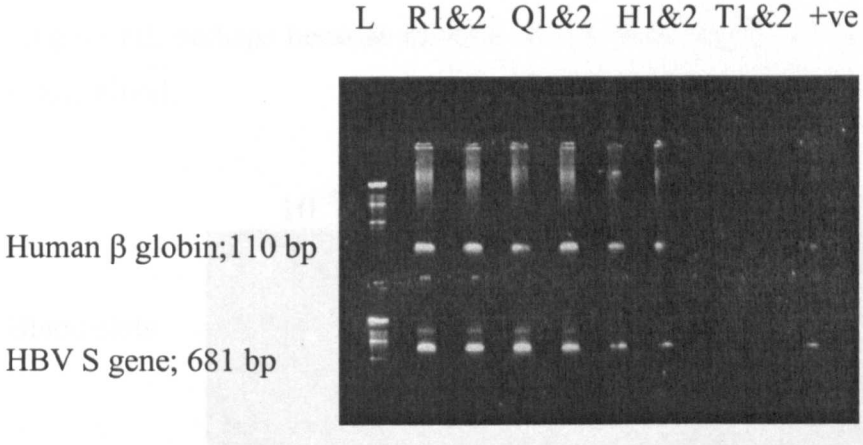


Figure 3.1: Comparison of PCR yield from two carrier patients with low viremia using four different extraction methods

R1&2: Extracted by Roche High Pure Viral kit; Q1&2: Extracted by Qiagen QIAamp blood kit

H1&2: Extracted by In house procedure; T1&2: Extracted by TriPure Isolation Reagent

+ve: positive control; L: 100 bp ladder

Table 3.1: Comparison of the PCR yield using four different extraction methods.

Method	Intensity of PCR bands*
QIAamp blood kit	+++
High Pure Viral Nucleic Acid kit	+++
In house procedure	++
TriPure^{TM} Isolation Reagent	-

Intensity of PCR bands*: +++: high, ++: moderate, -: not detected.

The Qiagen & Roche High Pure kits were then assessed for sensitivity using a dilution series of a positive serum containing HBV DNA of known concentration in negative blood. Both sera and blood clots of these dilutions were extracted and PCR carried out in the same run. For both sera and blood clots, the intensity of PCR bands was consistent between the two kits; HBV DNA was detected up to a dilution of 10^{-5} that was equivalent to 4 x 10^{2}gev/ ml (see Table 3.2; Figure 3.2). However, the positive control serum gave a positive result at 4 x 10 gev/ ml, perhaps because dilution of the stock serum is done in negative serum and not whole blood.

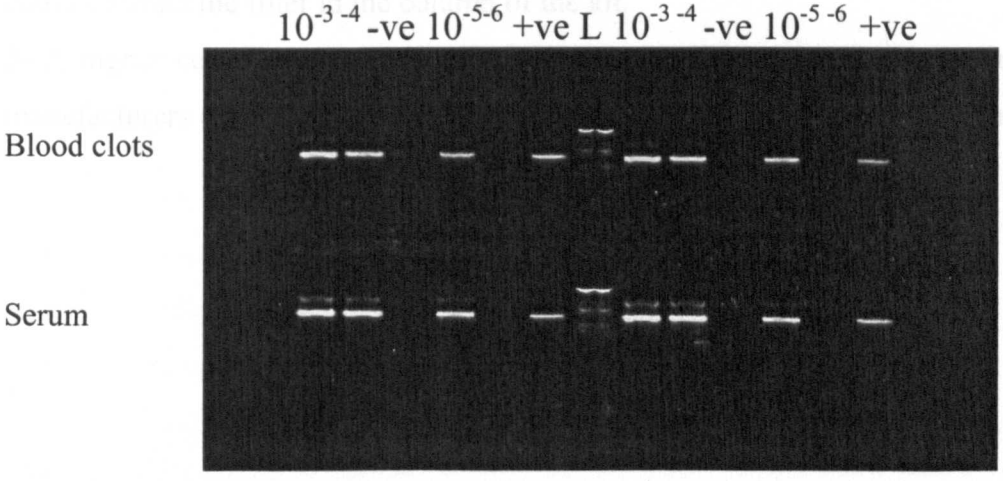


Figure 3.2: HBV DNA extraction sensitivity of Qiagen and Roche kits

PCR results of 10^{-3} up to 10^{-6} dilutions of HBV positive control extracted by Qiagen and Roche kits.

-ve& +ve: negative and positive controls, L: 100 bp ladder marker

Table 3.2: HBV PCR results of serum and blood clots extracted by Qiagen QIAamp blood and Roche High Pure Viral Nucleic acid kits*.

Sample dilution	QIAamp blood kit		High Pure Viral Nucleic Acid	
	serum	blood clot	serum	blood clot
10^{-3}	+	+	+	+
10^{-4}	+	+	+	+
10^{-5}	+	+	+	+
10^{-6}	-	-	-	-

*: Control serum was also amplified by PCR; titre was 10^{-6} (4x 10 gev/ ml).

+: detected; -: not detected.

Thus, both Qiagen and Roche High Pure kits were equally efficient and sensitive for extraction of DNA from clotted blood as well as simple to use and widely available. From a practical point of view, our experience with these methods revealed that:

1- blood clots do not have to be completely dissolved, as the incubation time with proteinase K is only 10 min.

2- Extreme care must be taken on transferring the digested blood to avoid any debris, which could obstruct the filter in the column of the kit.

3- A higher centrifugation speed (10,000 x g) is preferred to the recommended one by the manufacturers (8,000 x g) as the partially digested blood is heavier than serum.

3.2 Pacific study I

(Impact of regional infant immunisation in 4 Pacific Islands and prevalence of HBsAg variants)

3.2.1 Introduction

Hepatitis B infection is an important global health problem that needs collaboration of all world health organisations to achieve optimal prevention, control and hence its eradication. Approximately 350 million people are chronic carriers of HBV, of whom it is estimated that up to 30% will die of the consequences of their infection. HBV modes of transmission and their relative importance vary in different regions of the world. In highly endemic regions (> 7% HBsAg prevalence), transmission mainly occurs perinatally or in early childhood. By contrast, most infections are acquired during early adult life in low endemicity regions (< 2% HBsAg prevalence) (Mast et al., 1999; André et al., 2000). Therefore different immunisation strategies to prevent HBV transmission have been considered. For instance, infant vaccination will rapidly eliminate transmission in countries of highly endemic HBV infection, whereas vaccination for older children, adolescents and adults are preferred in countries with intermediate and low endemicity (Mast et al., 1999).

HBV infection during infancy and early childhood is usually followed by chronic infection, even in low endemicity areas, as chronic HBV infection is age-dependent (McMahon et al., 1985). Moreover, it has been shown that the risk of HBsAg positive mothers infecting their babies varies and is best correlated with HBeAg positivity (Stevens et al., 1975; Hwang et al., 1985; André and Zuckerman 1994). It has been postulated that early exposure to HBeAg may induce peripheral tolerance to the epitopes that are usually the target of CTL and therefore specific suppression of cell mediated immune response. Exhaustion of T cell response by the high viral load may be an additional mechanism. Alternatively, clonal deletion of HBV specific T cells may occur if this exposure was due to transplacental infection, during the developing fetus stage, where viral antigens will be recognized as self (Chisari, 2000). It was also suggested that transfer of maternal anti-HBc across the placenta may result in either modulation of HBcAg display or of the cell mediated immune response to this protein and lead to failure of clearance of HBV infected cells (Thomas & Lever, 1986).

Hepatitis B is highly endemic in most countries of the western Pacific and South East Asian regions except Australia, New Zealand and Japan, where the mean carrier rate is less than 2%. More than 75% of the world's chronic carriers are living in such densely populated region where carrier rates are relatively high (Maynard et al., 1989). The first universal childhood immunisation program in this region was initiated by the republic of Nauru in 1983 (Speed et al., 1989). Since that time, national programs have been introduced in many countries with varying degrees of success. As immunisation programs have been in place for a sufficient period in some countries, their impact has been evaluated. It was clear that most countries of the region achieved a considerable reduction in the carrier rate among their children (Ruff et al., 1995; Oon et al., 1995; Chen et al., 1996a). However, over the last few years several reports showed that a number of neonates and children have developed infection despite the presence of anti-HBs antibody. On sequencing of the amplified HBV DNA isolated from the sera of these children, mutations with amino acid changes within and outside the MHR were detected (Carman et al., 1990; Okamoto et al., 1992; Karthigesu et al., 1994; Oon et al., 1999).

Here we address certain points of major concern regarding HBV infection in these isolated islands. Firstly, to determine the infection rate after vaccination in different ethnic populations and to identify risk factors associated with failure of immunisation. Secondly, concerning the detection of S gene variants: a) their incidence after universal vaccination in ethnically diverse population; b) whether they were previously seen in their mothers or were selected *de novo*; and c) their prevalence in vaccinated populations compared to an unvaccinated control group. Finally, we wished to evaluate the horizontal transmission rate in a vaccinated background.

Subjects and study design

Hepatitis B immunisation programmes were launched in four Pacific islands in 1995; Kiribati (KK), Vanuatu (VV), Fiji (FF) and Tonga (TT). All infants were vaccinated with 3 doses of plasma-derived vaccine (Korean Green Cross vaccine; UNICEF) 10ug each at 0, 1 and 6 months of age. Unfortunately, we were unable to gain any information about the HBV strain included in this vaccine. After a regional immunisation programme, sera from pre-school children were collected from four Pacific islands (156 from KK, 132 from VV, 285 from FF and 211 from TT). Sera were then aliquoted and stored at –70C until tested further.

All samples were investigated for serological markers of HBV infection. Children were at least 3 years old before sample collection to allow a sufficient time for horizontal transmission to take place. So as not to miss any positive HBV DNA containing samples, we studied all children with serological marker profiles that are directly (HBsAg +ve, n= 19 plus one sample missing) or indirectly (anti-HBc+ve or anti-HBc+/ anti-HBs+, n=36) suggestive of current HBV infection. PCR was employed to detect and amplify the HBV DNA S gene, followed by direct sequencing of the detected amplicons (see Section 2.2.1). The mothers of these 55 children were screened for HBV serological markers and then sera amplified by PCR. To control for the natural background HBV sequence in unvaccinated subjects, 40 HBsAg positive unvaccinated samples (10 from each island) were used.

This study was a collaborative one between Glasgow, Melbourne and Brisbane. All serological assays were performed in Melbourne, DNA extraction in Brisbane, and PCR and HBsAg sequencing in Glasgow. Unfortunately, HLA typing has not been performed due to some financial and political problems.

3.2.2 Results

A total of 784 pre-school children, 285 from FF, 211 from TT, 132 from VV and 156 from KK, were screened in parallel with their mothers, for serological markers of HBV infection after immunisation (Table 3.3). Of these 784, 19 samples (1 from FF, 4 from VV, 8 from TT, and 6 from KK) were HBsAg positive. 68% of this HBsAg positive pre-school children (13/ 19) had HBsAg positive mothers. All HBsAg positive mothers who have HBsAg positive children were HBeAg positive, indicating the importance of HBeAg positivity as a major factor for failure of immunisation. On the other hand, there were some HBsAg positive children who were born to HBsAg negative mothers (1 in VV, 1 in TT and 4 in KK), implying that these infections may be horizontally transmitted.

The effect of immunisation on children was also evaluated. Low sero-conversion rates with significant variation between the four regions were observed, 77% in FF, 72% in VV, 60% in TT and 47% in KK (see Table 3.3). For ease of understanding, I will discuss the results from each island separately with especial concern to the PCR and sequencing results. A comparison of the immune induced variants to natural variants isolated from the same island is also shown in a separate section.

Table 3.3: Prevalence of hepatitis B markers in the Pacific islands†

Population	HBsAg		HBeAg		Anti-HBc		Anti-HBs		All markers‡	
	No.	%	No.	%	No	%	No	%	No	%
Fiji§										
Mothers	19/290	6.6	12/17	70.6	195/290	67.2	168/290	57.9	226/290	77.9
Students	20/288	6.9	-		141/288	49.0	115/288	39.9	160/288	55.6
Children	02/285	0.7	-		15/281	5.3	217/282	77.0	-	
Kiribati										
Mothers	27/179	15.1	11/23	47.8	160/179	89.4	087/176	49.4	169/179	94.4
Students	37/135	27.4	-		125/135	92.6	066/135	48.9	127/135	94.1
Children	06/156	3.8	-		019/149	12.8	068/145	46.9	-	
Tonga										
Mothers	40/215	18.6	19/40	47.5	183/216	84.7	130/215	60.5	193/216	89.4
Students	25/225	11.1	-		099/220	45.0	096/219	43.8	126/220	57.3
Children	08/211	3.8	-		025/203	12.3	121/202	59.9	-	
Vanuatu										
Mothers	16/130	12.3	08/16	50.0	085/130	65.4	076/129	58.9	109/130	83.8
Students	22/135	16.3	-		073/135	54.1	048/134	35.8	079/135	58.5
Children	04/132	3.0	-		012/129	9.3	089/123	72.4	-	
All countries										
Mothers	102/814	12.5	50/96	52.1	623/815	76.4	461/810	56.9	697/815	85.5
Students	103/783	13.2	-		438/778	56.3	325/776	41.9	496/782	63.4
Children	20/784	2.6	-		071/762	9.3	495/752	65.8	-	

†: Modified from Wilson et al., 2000
‡: Past infection in vaccinated children is indicated by the presence of HBsAg and/ or anti-HBc.
§: Melanesian population only (ie, Indo-Fijians were excluded)

Tonga Island

In Tonga (Table 3.4), samples were collected from two cities, namely Nukualofa and Kolavai. In Nukualofa, 13 children were studied. Four out of these 13 (C223, C22, C20 and C31) were PCR positive. All positives but one (C223) were positive for HBsAg. On the other hand, PCR was negative in one case (C57) that had HBsAg as the only detected marker. All PCR positive children were shown to have PCR positive mothers.

On sequencing, three children were found to have identical HBV S gene sequence to those amplified from their mothers (C22/M18, C20/M16 and C31/M226). One infant showed a completely different subtype/ genotype from that has been isolated from his mother (C223 *ayw3*/ M17 *adrq*-). On the other hand, one infant was protected against the infection from his mother (C33/M90).

In Kolavai, 7 children were studied. Of these, 4 were positive by PCR. All positive children had PCR positive mothers (C47/M15, C32/M11, C61/M2 and C39/M3). The first three infant mother pairs showed a complete sequence concordance on sequencing. Interestingly, the fourth infant mother pair (C39/M3) was identical to the sequences amplified from Betio City in KK. Similar to Nukualofa, one child (C23/M9) was successfully protected from catching the HBV infection from his mother who was negative for HBeAg; her S gene sequence was similar to the natural sequences circulating in the island.

Vanuatu Island

In Vanuatu (Table 3.5), samples were collected from Vila Central and Mele-Maat cities. Seven children were studied from Vila Central. Of these, four were PCR positive. All positives but one (C12) were positive for HBsAg. All the PCR positive children except one (C38) were shown to have PCR positive mothers. All PCR positive mothers but one (M13) were HBsAg positive. However, one mother (M6) who was positive for all HBV serological markers has a negative PCR result for unknown reasons.

On sequencing within and between families, complete sequence concordance was observed between two infant mother pairs (C48/M102 and C19/M101). On repeat, we got the same sequence from these two pairs. However, infection in a couple of children did not seem to be of maternal origin. The first has an HBV subtype that is different from his mother (C12/M13). The other most probably has an immunised mother because she has only anti-HBs

(C38/M96). On the other hand, one child was protected from contracting the infection from his mother who was positive for both HBsAg and HBeAg (C64/M74).

In Mele-Maat, 4 child mother pairs were studied. Of these, only one pair (C37/M75) was PCR positive. Serologically, this pair was positive for the HBsAg marker. On sequencing, identical S gene sequences were detected in the mother and child samples.

Fiji Island

In Fiji (Table 3.6), samples were collected from three cities, namely Valelevu, Raiwaqa and Navua. In Valelevu, 5 child mother pairs were studied. Of these, one pair (C58/ M10) and one mother (M78) were PCR positive. All PCR positive samples were HBsAg positive. On sequencing, both samples of the defined pair (C58/M10) were shown to be identical. The child of the PCR positive mother seems to be protected (C224/ M78).

In Navua and Raiwaqa, one and four pairs were studied respectively. None of the tested samples were PCR positive.

Kiribati Island

In Kiribati (Table 3.7), samples were collected from Bikenibeu and Betio cities.
In Bikenibeu, 7 child-mother pairs were studied. On PCR, three children (C91, C60 and C45) gave positive results. Of these, one was negative for HBsAg (C91). None of the positive PCR children had a positive PCR mother. On sequencing, C91 was of *awy3* subtype, whereas the other two (C60 and C45) was of *ayw2* subtype. However, C45 showed a sequence that was identical to those sequences amplified from Betio.

Seven child-mother pairs were studied from Betio. Of these, two pairs (C35/M97 and C41/M98) were PCR positive. Additionally, three infants (C21, C42 and C56) were positive by PCR while their respective mothers were PCR negative. All PCR positive samples but one (C21) were positive for HBsAg. On sequencing all positive samples, three children and two child mother pairs showed exactly the same sequence of subtype *ayw2*. On repeat the amplified sequences were the same. These samples have been repeated either directly by re-amplification of the extracted DNA material or by re-extraction and amplification from the original sera. The highly heterogeneous pre-S region was also identical on sequencing.

Table 3.4: Serology and PCR results of child mother pairs in Tonga

A) **Nukualofa City**

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
1200 (223/17)	-	+	36	+	+	+	+	-	+
1484	-	+	>100	-	-	-	+	>100	ND
1428	-	+	28	-	-	-	+	29	ND
1054 (57/14)	+	-	-	-†	+	-	+	-	-
1464 (22/18)	+	+	-	+	+	+	+	-	+
1542 (20/16)	+	+	-	+	+	+	+	-	+
1590 (31/226)	+	+	-	+	+	+	+	-	+
1592 (33/90)	-	+	>100	-	+	+	+	-	+
1598	-	+	>100	-	-	-	+	46	-
1224	-	+	>100	-	-	-	+	>100	ND
1182	-	+	51	-	-	-	+	25	ND
1490	-	+	99	-	-	-	+	-	ND
1540	-	+	52	-	-	-	+	-	ND

B) **Kolovai City**

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
1268 (23/9)	-	+	>100	-	+	+	+	-	-
1314	-	+	>100	-	-	-	+	76	ND
1386	-	+	>100	-	-	-	+	27	ND
1400 (47/15)	+	+	-	+	+	+	+	-	+
1392 (39/3)	+	+	-	+‡	+	+	+	-	+
1324 (32/11)	+	+	-	+	+	+	+	-	+
1364 (61/2)	+	+	-	+	+	+	+	-	+

Numbers between brackets: PCR samples number (child/ mother)

PCR*: S gene; †: Core gene was also tested; ‡: Pre S region was also tested

antiHBs§: anti-HBs serum levels in miu/ml; +: Positive; -: Negative; ND: not done

Table 3.5: Serology and PCR results of child mother pairs in Vanuatu

A) Vila Central City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	PCR* M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
3274	-	+	52	-	-	-	+	>100	
1762 (70/6)	-	+	15	-	-†‡	+	+	-	+
1756 (48/102)	+	+	12	+	+	+	+	-	+
1788 (38/96)	+	+	-	+	-	-	-	>100	ND
3272 (19/101)	+	+	-	+	+	+	+	-	+
1744 (12/13)	-	+	44	+	+	-	+	-	ND
3454 (64/74)	-	+	>100	-	+	+	+	-	+

B) Mele-Maat City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	PCR* M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
3386 (37/75)	+	+	-	+	+	+	+	-	+
3466	-	+	>100	-	-	-	+	>100	ND
3378	-	+	41	-	-	-	+	>100	ND
3412	-	+	>100	-	-	-	+	-	ND

Numbers between brackets: PCR samples number (child/ mother)

PCR*: S gene; †: Core gene was also tested; ‡: Pre S region was also tested

antiHBs§: anti-HBs serum levels in miu/ml

+: Positive; -: Negative; ND: not done

Table 3.6: Serology and PCR results of child mother pairs in Fiji

A) Valelevu City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
904	-	+	>100	-	-	-	+	>100	ND
842	-	+	>100	-	-	-	+	>100	ND
612	-	+	>100	-	-	-	+	-	ND
604 (224/78)	-	+	>100	-	+	+	+	-	+
770 (58/10)	+	+	-	+	+	+	+	-	+

B) Raiwaqa City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
380	-	+	>100	-	-	-	+	>100	ND
404	-	+	>100	-	-	-	+	89	ND
330	-	+	39	-	-	-	+	12	ND
340	-	+	36	-	-	-	+	44	ND

C) Navua City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
996	-	+	>100	-	-	-	+	-	ND

Numbers between brackets: PCR samples number (child/ mother)

PCR*: S gene; †: Core gene was also tested; ‡: Pre S region was also tested

antiHBs§: anti-HBs serum levels in miu/ml

+: Positive; -: Negative; ND: not done

Table 3.7: Serology and PCR results of child mother pairs in Kiribati

A) Bikenibeu City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
3234	-	+	13	-	-	-	+	>100	ND
3244	-	+	99	-	-	-	+	>100	ND
3246 (91/44)	-	+	33	+	-	-	+	-	ND
3218	-	+	>100	-	-	-	+	38	ND
3250	-	+	>100	-	-	-	+	>100	ND
3230 (60/104)	+	+	-	+	-	-	+	-	ND
3254 (45/106)	+	+	-	+‡	-	-	+	44	ND

B) Betio City

Code number	Child HBsAg/ anti-HBc/ anti-HBs§			PCR* C	M	Mother HBsAg/ anti-HBc/ anti-HBs/ HBeAg			
3062 (21/86)	-	+	65	+	-	-	+	-	ND
3076	-	+	62	-	-	-	+	17	ND
3088	-	+	19	-	-	-	+	74	ND
3092 (56/107)	+	+	-	+	-	-	+	32	ND
3144 (42/76)	+	+	-	+	-	-	+	13	ND
3162 (35/97)	+	+	-	+‡	+	+	+	-	+
3184 (41/98)	+	+	-	+‡	+	+	+	-	+

Numbers between brackets: PCR samples number (child/ mother)

PCR*: S gene; †: Core gene; ‡: Pre S region

antiHBs§: anti-HBs serum levels in miu/ml

+: Positive; -: Negative; ND: not done

HBsAg variants in immunised children and their mothers

HBsAg sequences of immunised children and their mothers from different islands were analysed for nucleotide and aa substitutions in HBsAg by their comparison to standard HBsAg sequences. Different strains of *adrq-* were considered, as we detected three different strains of subtype adrq-, GV, ER and EV (see Chapter 3.3). The distribution of detected substitutions within HBsAg is shown in Table 3.8. "a" determinant variants were not detected and also no particular site changes were restricted to vaccinated children compared to non-vaccinated people (either their mothers or background sequences; see Tables 3.8 and 3.14). 13 out of 15 child/ mother pairs have concordant sequences, while the remaining two were discordant, one from TT (*ayw3*C223/ *adrq*-M17) and the other from VV (*ayw2*C12/ *adrq*-M13). All these children have HBeAg positive mothers except the Vanuatan pair (C12/M13) where HBeAg was not done.

3.2.3 Discussion

Perinatal transmission is a crucial factor in maintaining the reservoir of infection, particularly in highly endemic regions such as southeast Asia. A positive feedback mechanism that relates the average age at infection, transmission rate and probability of carriers' development following infection has been recently suggested. A 'Catastrophic' model for dynamics of HBV infection and control was also proposed where the epidemiological outcome of infection is critically dependent on a threshold phenomenon (Medley et al., 2001). Although hepatitis B virus can infect the fetus *in utero*, HBeAg positive mothers mostly transmit the infection to their infants at the time of, or shortly after, birth. It has been suggested that the HBeAg protect the infected hepatocytes from the immune surveillance by developing tolerance to HBcAg and HBeAg (Milich et al., 1990). However, this mode of transmission is less important in infection among older children, 5-15 years, where infections are mainly horizontally transmitted.

South East Asia is one of the highly endemic areas for HBV infection; hepatitis B carriers are infected mainly during infancy or early childhood (Hsu et al., 1986). Although these high-risk infants can be protected from HBV infection by active and passive immunoprophylaxis soon after birth, there is still a high percentage of carriers rate, 5-10% (Lee et al., 1991). This failure of immunoprophylaxis could be due to many reasons that will be discussed later.

Table 3.8: HBsAg variants in immunised children and their mothers.

The table is classified into 5 parts (A to E) according to the prevalent subtype/ strain.

ER, GV and EV are the major strains of *adrq-* subtype prevalent in the Pacific islands.

TT: Tonga; FF: Fiji; VV: Vanuatu; KK: Kiribati.

C/M†: Sequencing results from 15 child/ mother pairs who are numbered from 1 to 15 (see previous tables 3.3- 3.6).

‡: 2 child/ mother pairs which have identical sequences.

Gray: According to the reference group the related sequence of the two discordant child/ mother pairs (VV C12/ M13 and TT C223/M17).is highlighted in gray

Yellow: silent sites; *: Important sites for subtyping of HBV.

ND: no HBV DNA detected in these samples (negative PCR).

§: These three underlined pairs (KK; C35/M97, C41/M98 and TT; C39/M3) and other *ayw2* samples from KK (C42, C45, C56; not shown) had identical sequences on comparison and did not show any mutation.

A) *adrq-* Reference (E/R)	2 GAG(E)	3 AAC(N)	4 ACA(T)	46 CCC(P)	113* ACA(T)	114* TCA(S)	128 GCT(A)	175 TTA(L)	177* GCG(A)	198 ATG(M)
1) TT C61/M2†				CCA		TCC		TCA(S)		
2) TT C22/M18	GAA	GGC(G)	ACC	CCA	ACC				GTG(V)	
3) TT C20/M16		AGC(S)		CCA						ATA(I)
4) TT C223/M17		AGC(S)		CCA		TCC				
5) VV C48/M102‡				CAA						
6) VV C19/M101‡				CAA						
7) FF C58/M10					ACC					
KK C21/ND		AGC(S)					GCA			

B) *adrq-* Reference (E/V)	5 ACA(T)	31 AGT(S)	45* GCA(A)	49 CCT(P)	63 ACC(T)	68* ATT(I)	100 TAC(Y)	94 TTG(L)	113* ACA(T)	116 ACC(T)
8) TT C31/M226	GCA(A)					ACT(T)		TTA	ACC	ACA
9) TT C47/M15	GCA(A)					ACT(T)	TAT	TTA	ACC	
10) TT C32/M11	GCA(A)	AAT(N)	CCA(P)	CAT(H)		ACT(T)	TAT	TTA	ACC	ACA
TT ND/M9	GCA(A)				ATC(I)	ACT(T)		TTA	ACC	ACA

C) *adrq-* Reference (G/V)	3 AAC(N)		71 GGC(G)	82 ATC(I)	100 TAC(Y)	122* AAG(K)	128 GCT(A)	160* AGA(R)	175 TTA(L)	181 CAG(Q)
11) VV C12/M13				ATA					TCA(S)	
VV C38/ND					TTC(F)				TCA(S)	
VV ND/M74	AGC(S)		GGT	ATA			GCA		TCA(S)	
12) VV C37/M75	AGC(S)		GGT				GCA		TCA(S)	
FF ND/M78	AGC(S)		GGT	ATA		AGG(R)		AAA(K)	TCA(S)	CAT(H)

D) *ayw2* Reference			44 GGG(G)			126 ACT(T)				204 AGT(S)
11) VV C12/M13			GGA			ACA				
13) TT C39/M3§										
14) KK C35/M97										
15) KK C41/M98										
---KK C60/ND										AAT(N)

E) *ayw3* Reference	12 CTG(L)		88 CTG(L)							207 AAC(N)
TT ND/M14	CTA									AGC(S)
KK C91/ND										
4) TT C223/M17			CTT							

3.2.3a Infection rate and implications

In this study, pre-school children showed the lowest HBsAg prevalence among the studied groups, mean 2.6% (see Table 3.3). The percentage of HBsAg infected students were significantly higher than that of the mothers (27.4% versus 15.1%) in KK. The reverse was almost correct in TT (18.6% for mothers versus 11.1% in students) and VV (16.3% versus 12.3). On the other hand, there was no significant difference in the prevalence of HBsAg between these two groups, students and mothers, in FF island (6.9% versus 6.6%). These data may have several implications.

Firstly, the obviously reduced infection rate in pre-school children is proof of the success of the immunization program. Secondly, the significantly different infection rates between the studied groups after launching the immunization program could reflect the main mode of transmission in these different age groups within each island in this region. For instance, vertical transmission could be expected as the main mode of transmission for preschool children infection, where it was successfully interrupted by infant immunization (Beasley et al., 1982, 1983). On the other hand, horizontal transmission from household members and transmission due to sexual contact with a carrier may be responsible to some extent for augmenting the infection rate in students and mothers groups.

Thirdly, the 13.7% risk of a child born to an HBsAg positive mother being HBsAg positive fell to 0.9% if the mother was HBsAg negative; all HBsAg positive mothers who had HBsAg positive infants were positive for HBeAg. HBeAg positivity is therefore of high importance in predicting the risk of infant infection as has been described earlier (Stevens et al., 1975; Hwang et al., 1985). Of these infants at risk, despite passive active immunization, the frequency of the carrier's state varies. In the Netherlands, R del Canho et al. (1994) reported 8/118 (6.8%) as the determined carrier rate, while Ip et al. (1989) detected 14/124 (11.3%) in Hong Kong. In India, higher percentages, 14.2% and 25%, were detected (Sehgal et al., 1992). These differences were attributed to the usage of different vaccine doses or different HBV DNA levels of the infected mothers. But there are other factors that have not been considered in this interpretation and could play a role such as genetic background and quality of vaccination services (McDermott et al., 1999; Streefland et al., 1999).

3.2.3b Horizontal transmission role

Vertical transmission is the main mode of transmission of HBV infection in Asia (Stevens et al., 1975; Beasley et al., 1982), while horizontal transmission is the prevalent one in Africa (Tabor et al., 1985; Tsega et al., 1988; Martinson et al., 1998). This continental preference has been mostly attributed to the differences in the prevalence of the HBeAg in these populations, which is much higher among Asians.

However, Tibbs et al. (1987) reported that mother to child transmission in Kiribati was not as important as in Taiwan and other parts of south east Asia. This conclusion was based on the low prevalence of HBeAg among mothers, higher concordance of HBsAg presence in sibling pairs than in child-mother pairs and detection of HBsAg and HBeAg in exudates of tropical ulcers that might represent an infectious source to uninfected people. Another report from Vanuatu showed that cross-infection between children is an important source of infection that mostly spread through skin sores (Maher et al., 1991). Furthermore, presence of HBsAg positive siblings and intra-familial interactions have been shown to increase the frequency of HBsAg carriers among Asians, whether born in their ancestral country or USA (Tong et al., 1981; Franks et al., 1989; Hurie et al., 1992).

All previous reports were from subjects in an unvaccinated background. Here, we have supportive data for the transmission of HBV horizontally among immunised children in Asian Pacific islands. Firstly, the significantly elevated HBsAg infection rate among unvaccinated students and mothers over that found in vaccinated children, interpreted previously as a result of vaccination, could be also in part due to horizontal transmission reflecting different degrees of exposure to HBV infection between these different groups (Table 3.3). Secondly, there was some molecular evidence among immunised children. By PCR, we detected infected children but non-infected mothers, as in KK (Bikenibeu: **C91**, C60 and C45; Betio: **C21**, C56 and C42) and VV (Vila central: C38). On sequencing of the circulating HBV recovered from some child-mother pairs, unrelated sequences were detected in TT (Nukualofa: **C223**/M17) and VV (Vila central: **C12**/M13).

Among the 9 children who seem to have contracted the infection horizontally, only four had anti-HBs; a titre of less than 100 miu/ml was detected (black and underlined). Determination of virus quantity has not been performed in mothers of these children due to

negativity of HBsAg, The only HBsAg/ HBeAg positive mother (M17), however, may have a low virus load as sequencing of her baby virus revealed a different strain.

Of significant interest, only one infant out of the nine cases had the *adrq-* subtype (genotype C); all the other cases were of genotype D (subtype *ayw2 and ayw3*). This might be a real selection of a certain strain over the other, *ayw2* (D)> *adrq-* (C). Alternatively, it could be selection bias due to the presence of six samples from KK, in which most infected people have genotype D (see Chapter 3.3). If it is the former, it may be of especial importance as the plasma-derived vaccine used in this study was derived from Korea (which mostly contains the *adr* subtype). Thus, insufficient neutralisation or weak cross protection by the induced antibodies against the different subtypes/ genotypes (*ayw2 & 3*/ D) is possible as both *adr* and *ayw*2/3 are different at several crucial residues within the MHR such as amino acids 113, 122, 126, 127 and 159 (see Chapter 4).

Among the six cases identified in Kiribati, four children had identical sequences (C45, C21, C56 and C42). The same sequences were recovered from another two child-mother pairs from Kiribati and, interestingly, one child/ mother pair from Tonga (39/3). To exclude contamination due to laboratory work, we have repeated the sequencing of these samples both from extracted DNA and original sera. All samples revealed the same results. Even the pre-S sequences, which are the most heterogeneous part of the HBV genome, were also identical. Although contamination at the site of collection cannot be totally ruled out, it seems unlikely due to detection of the same virus from different cities (within KK) or even islands (KK and TT). Alternatively, the isolated virus could be circulating highly among these people due to a common source of transmission. This unusual phenomenon in Kiribati might extend to the nearby islands upon inter-island migration. It might be that exudates from the fairly common ulcers caused by the rugged Kiribati reef-top environment may provide a highly infections source for uninfected people (Tibbs 1987). Perhaps we can consider those Kiribati children who have shown an anti-HBs response in association with anti-HBc (C91 & C21), as due to natural exposure rather than vaccination that has failed.

Nevertheless, it would appear that horizontal transmission plays a minor role in South East Asia and Pacific islands (except Kiribati as shown above); however, it cannot be ignored. In conclusion, strategies of immunisation for prevention and control of HBV infection should be tailored to the local transmission route.

3.2.3c S gene variants and Pacific islands

Coexistence of HBsAg and anti-HBs, samples with anti-HBc as the only HBV marker and samples with discordant serology (samples that react in one HBsAg assay but not the other) have all been found to be associated with S gene variants (Carman et al., 1997b; Oon et al., 1999; Hsu et al., 1999). These S gene variants with missense mutations, whether within or outside the MHR, have been described in infected infants despite the presence of vaccine induced anti-HBs antibodies (Carman et al., 1990; Okamoto et al., 1992; Karthigesu et al., 1994; Oon et al., 1999). Accordingly, it was suggested that such mutants escaped the immune pressure whose target was the standard virus.

In the Pacific islands, of the 55 children who showed markers suggestive of HBV infection, 19 had positive HBsAg (Group 1: G1) and 36 were positive for anti-HBc (Group 2: G2); 22 were positive on PCR. Only one HBsAg positive sample in G1 was negative by PCR; this might be a false positive as it was negative for other HBV markers. On the other hand, 4 out of the 36 negative HBsAg samples in G2 were positive by PCR. Except for 7 cases, six from Kiribati and one from Vanuatu, all the PCR positive children were shown to have PCR positive mothers (15 child mother pairs). On sequencing, 13 out of these 15 child/ mother pairs showed a complete concordance in their sequences whereas the remaining two pairs, TT (C223/M17) and VV (C12/M13), showed not only different sequences but also different subtype/ genotype (see Table 3.8).

Analysis of S gene recovered from the immunised children showed clearly that "a" determinant mutants were not detectable. Yet, mutations outside the "a" determinant were randomly distributed along the S gene, such as at residues 3, 5, 68, 175 and 177 (Table 3.8). Carman et al. (1997b) reported similar observations in vaccinated health care workers. Furthermore, alterations outside the "a" determinant have been detected recently in immunised infants from Singapore where decreased binding to the "a" determinant specific monoclonal antibody was observed to some of the identified variants (Oon et al., 1999). Thus, escaping neutralisation by antibodies elicited by vaccination due to the presence of S gene mutations is still a possibility as the functional analysis of the variants we discovered has not been performed.

Alternatively, breakthrough infections in these immunised infants might be due to additional factors other than the immune escape mutants. The most likely, but worrying

scenario, is the improper quality of heath services applied in these poor areas such as improper vaccine storage, incomplete dosage or improper administration whether in regard to timing or site of injection (Ruff et al., 1995; Streefland et al., 1999). High maternal viral load also plays a role, especially in HBeAg positive mothers (Lee et al., 1986; Ip et al., 1989). Failure to respond adequately to vaccine can be due to genetic or acquired causes (Carne et al., 1987; Alper et al., 1989; McDermott et al., 1999). HLA typing has not been performed in our study, due to financial and political problems, however immune responses to vaccination and outcomes of HBV infection are known to be influenced by the type of HLA alleles (Craven et al., 1986; Hsu et al., 1993; McDermott et al., 1999, Diepolder et al., 1998; Thio et al., 1999; Ahn et al., 2000). Although all these possibilities have their supporters, there is still some debate (Poovorawan et al., 1997; Tang et al., 1998; R del Canho et al., 1994). This controversy over the reasons for breakthrough infections in immunised individuals is complicated by several variables between the different studies, including geographical and ethnicity differences which are expected to play an important role (see Table 3.9).

Although it has been reported recently that universal immunisation has accelerated the accumulation of HBsAg "a" determinant mutants in vaccinated children (Lee et al., 1997a; Hsu et al., 1999), we did not find any significant difference between the immunised children and non-immunised control group, since the detected variants were similar both in site and frequency. This may indicate that these variants were mostly natural and not vaccine-induced. Geographical differences may play a role in the discrepancies between our results and those of others. Another reason could be the time interval that has elapsed since launching the immunisation program as has been shown recently by Hsu et al. (1999). In Taiwan, the prevalence of "a" determinant variants jumped from 7.8%, just before vaccination, to 28.1%, 10 years after introduction of universal vaccination (see section 4.2). Indeed, this interval may allow the virus to survive long enough to acquire mutations that may enhance viral replication and consequently variant eventual dominance. Additional factors could be which vaccination strategy is adopted and the HBV endemicity level, for example in England and Wales, 12% prevalence rate was seen due to selective vaccination in a low endemicity region (Nugi et al., 1997). Furthermore, different HBV strains, in different geographical regions, may be associated with specific nucleotide or amino acid changes that are more suitable for the viral fitness (see Section 4.2).

Table 3.9: Percentage of HBV infection and "a" determinant variants after infant immunoprophylaxis in different countries.

Country	HBV infection %*	"a" determinant variants	Immunisation	Reference
Singapore	41/345 (11.9%)	16/41 (39%)	HBIG/ vaccine	Oon et al., (1995)
Taiwan 1984	115/1200 (9.6%)	8/103 (7.8%)	Before vaccine	Hsu et al., (1999)
1989	52/1134 (4.6%)	10/51 (19.6%)	Vaccine	Hsu et al., (1999)
1994	20/1515 (1.3%)	9/32 (28.1%)	Vaccine	Hsu et al., (1999)
Taiwan	27/455 (5.9%)	6/27 (22%)	HBIG/ vaccine	Lee et al., (1997)
USA	94/1092 (8.6%)	22/94 (23.4%)	HBIG/ vaccine	Nainan et al., (1997)
Czech Republic	1/ 402	1/1 (100%)	HBIG/ vaccine	Roznovsky et al., (2000)
Japan**	2/29 (6.9)	1/ 2 (50%)	HBIG/ vaccine	Matsumoto et al., (1997)
China (Shanghi)	42/ 340 (12.4%)	4/ 24 (16.7%)	Vaccine	He et al., (1998)
England and Wales	20/321(6.2%)	2/17 (11.8%)	HBIG/ vaccine	Nugi et al., (1997)
Netherlands	8/705 (1.1%)	ND	HBIG/ vaccine	del Canho et al., (1994)
Gambia	32/358 (9%)	3/32 (DNA+ve) 1/3 (33.3%)	Vaccine	Fortuin et al., (1994)
Hong Kong	14/124 (11.3%)‡ 15/63 (23.8%)	ND ND	HBIG/ vaccine Vaccine	Ip et al., (1989)
Indonesia	24/1717 (1.4%)† 20/656 (3%)	ND ND	Vaccine (<7days) Vaccine (>7 days)	Ruff et al., (1995)
India	(25%) (14.2%)‡	ND ND	HBIG/ vaccine Vaccine	Sehgal et al., (1992)

HBV infection*: based on HBsAg positivity, except in the Gambia was based on anti-HBc positivity.

Japan**: small study for immunisation of high risk infants.

†: Different rates were due to different timing of the first vaccine dose (before or after 7 days).

‡: Different rates were due to the addition of HBIG.

3.3 Pacific study II

Use of HBsAg variability as a marker of human population history in the Pacific

3.3.1 introduction

Studying the genetic relationship between viruses can reveal information on the origin and geographical distribution of a particular strain, the routes of virus transmission and may be useful for the development of control measures. HBV is the prototype of the Hepadnaviridae family which includes avian viruses such as duck and heron hepatitis B viruses (Mason et al., 1980; Sprengel et al., 1988) and mammalian viruses. The latter comprise rodent members such as woodchuck, ground squirrel and tree squirrel viruses (Summers et al., 1978; Marion et al., 1980; Feitelson et al., 1986) and primate members that infect humans, gibbons, wooly monkeys, orangutans and chimpanzees (Norder et al., 1996; Lanford et al., 1998; Warren et a., 1999; Takahashi et al., 2000).

Phylogenetically, HBV has been classified into seven genotypes, denoted A to G, often with a distinct geographic association. Genotypes A and D are widely distributed in the old world, while genotypes B and C are confined to east Asia. Genotype E is mainly found in sub-saharan Africa, genotype F in the aboriginal population of the Americas and the recently discovered G has so far been detected in USA and France (Okamoto et al., 1988; Norder et al., 1992b; Stuyver et al., 2000). Therefore, it has been suggested that the divergence of viral genotypes may reflect the migration of human populations (Norder et al., 1994, 1996). There are also nine different HBV subtypes, defined serologically with a characteristic geographic patterns (Le Bouvier 1971; Bancroft et al., 1972; Couroucé-Pauty et al., 1983). Although some of these subtypes, such as *adw2,* are genetically heterogeneous there is an accepted degree of correspondence between the genetic and serological classification. By 1992, the molecular basis for the serologic heterogeneity of HBV subtypes had been defined and it thus became possible to determine the serotype by sequencing of the HBV S gene (Norder et al., 1992a). The validity of this approach was confirmed by sequencing the complete genome (Norder et al., 1994; Arauz-Ruiz et al., 1997).

Geographically, Pacific islands are divided into three regions; Micronesia, Melanesia and Polynesia. Polynesia occupies a large triangle in the eastern and central Pacific region extending from Hawaii in the north to Rapanui in the east and New Zealand in the west (Figure 3.3).

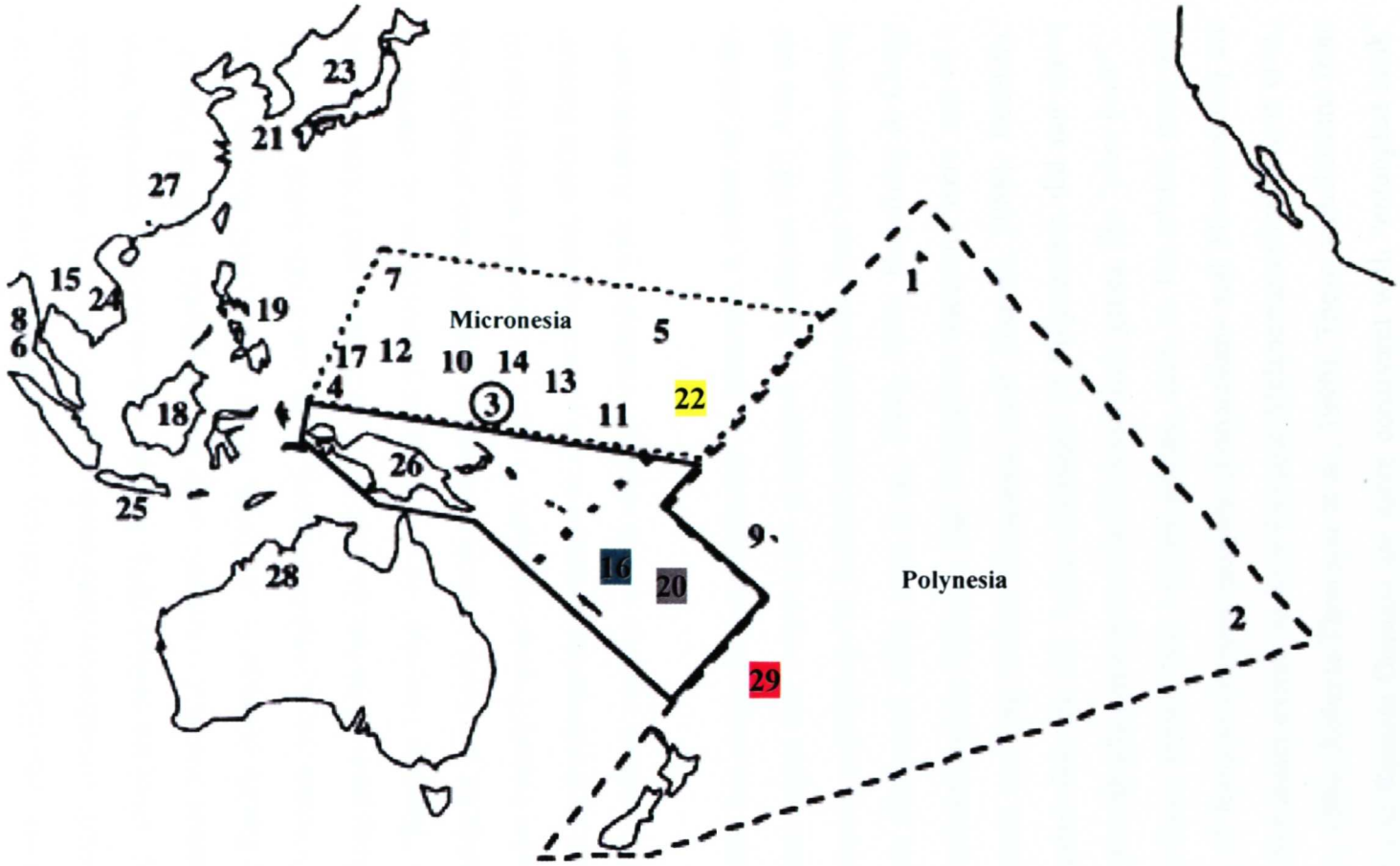


Figure 3.3: Pacific map showing boundaries of Micronesia, Polynesia and Melanesia (solid line), Pacific islands and surrounding countries. 1: Hawaii; 2: Rapanui; 3-5 and 10-14: small islands within Micronesia; 6: Moken; 7: Marianas; 8: Urak Lawoi; 9: Samoa; 15: Thialand; 16: vanuatu; 18: Borneo; 19: Philippines; 20: Fiji; 21: Korea; 22: Kiribati; 23: Japan; 24: Vietnam; 25: Java; 26: Papua New Guinea; 27 China; 28: Australia; 29: Tonga (modified from Lum et al., 1998).

Melanesia extends from New Guinea to Fiji and contains the western island chains that lie south of the equator. Micronesia includes the group of islands that lie west of Polynesia and north of Melanesia.

The origin of Pacific islanders has attracted the attention of many scholars over the last 30 years, and work on a variety of searches including archeology, linguistics and human genetics has led to major progress in our understanding of history of human settlement in this part of the world. Two waves of Pacific colonization have been proposed. The first, which is dated between 30 and 50,000 years before the present (BP), includes regions such as Australia, New Guinea and Northern Melanesia that are correlated with the geographic limit of Papuan-speaking populations in the Pacific (Groube et al., 1986; Allen et al., 1988; Roberts et al., 1990). The second relatively recent wave, which occurred in the last 3000- 4,000 years BP, includes Austronesian-speaking parts of Melanesia and all of Micronesia and Polynesia. The terms "Near Oceania" and "Remote Oceania" have also been proposed as an alternative classification of Pacific islands on the basis of these concordant linguistic and geographical patterns. Near Oceania includes mainly Papuan-speakers living in the internal antique regions in western Pacific in addition to Austronesian speakers in the coastal regions, while Remote Oceania includes the recently settled regions and is mainly inhabited with Austronesian-speakers (Kirch, 1997).

The ultimate origin of this last major human migration is, however, a matter of debate. One group, chiefly geneticists, argue that Polynesians originated in Southeast (SE) Asia and swept rapidly through Melanesia, explaining the limited admixture with Near Oceania, along their way to settle eastwards (Diamond 1988; Lum et al., 1994, 1998; Hagelberg & Clegg 1993). The other, chiefly archaeologists, believes that Polynesian ancestors were one of a number of diverse populations residing within Melanesia itself (Terrell, 1988). Recently, Kayser et al. (2000) suggested that the SE Asian ancestors of Polynesians did not move rapidly but extensively mixed with Melanesians and left behind their genes, the *"slow boat"*.

Nuclear genetic studies have shown that nuclear alleles, such as the globin gene and human leucocyte antigens, of Remote Oceanic peoples (Polynesians and Micronesians) are related to both Asians and, to a lesser extent, to Near Oceanic Melanesians (Serjeantson et al., 1982; O'Shaughnessy et al., 1990; Roberts-Thomson et al., 1996). These contributions from both Asia and Near Oceania to Remote Oceania are more consistent with "entangled bank" model of Terrell (1988) that assumed a complex pattern of gene flow as a result of long term

interactions among Near Oceanian and western Pacific populations without a second migration. An opposing view, supported by the accumulated mitochondrial DNA (mtDNA) analyses, see this admixture as the result of contact with the Polynesian ancestors who came from SE Asia and passed through Melanesia along their way to settle in Polynesia (Hertzberg et al., 1989; O'Shaughnessy et al., 1990; Lum et al., 1998; Hagelberg et al., 1999).

Linguistic patterns are in agreement with this latter model, termed the "express train" (Diamond 1988; Gray & Jordan 2000). However, as mtDNA is maternally inherited while Y-chromosomal microsatellites (short tandem repeats: STR) are bi-parentally inherited, discordant results during times predominated by male gene flow could be detected, as has been proposed by Lum et al. (1998).

A recent bottleneck has also been proposed as an alternative explanation for this discordance between mtDNA and nuclear variation patterns (Fay & Wu 1999). It is noteworthy that the amount of genetic diversity among certain populations is dependent on their migration rate, with more diversity expected among those groups with a higher migration rate (Tajima et al., 1990). Consistent with this hypothesis, mtDNA and STR diversity were greatly reduced or even lost in geographically isolated Remote Oceanic populations (Lum et al., 1998). On the other hand, under conditions of geographical isolation it is believed that both genetic and linguistic patterns are significantly correlated to reflect the same history while discordance between the two patterns suggests lack of such isolation and probably a recent colonization event.

Based on the evidence that the people of SE Asia migrated eastwards into Polynesia, we have chosen to study HBsAg variation from four Pacific islands which have different ethnic backgrounds. Kiribati represents Micronesia (KK), Vanuatu and Fiji represent Melanesia (VV & FF) and Tonga represents Polynesia (TT). By phylogenetic analysis of these Pacific HBsAg strains in addition to database sequences representing other parts of the world, hypothetically, we would be able to match the proposed migration patterns of these people. We believe HBV is ideally suited to this purpose as it is transmitted by intimate contact or from mother to child, chronic carriage is associated with easily detectable viremia and the viral sequence remains fairly constant during the early years of infection. We have also made the assumption that there is little intermarriage between these ethnic groups, so that characteristic HBV strains should remain relatively isolated.

Subjects and study design

HBsAg positive samples were collected from mothers and school children from four Pacific islands: KK, VV, FF and TT. The samples were divided into aliquots and stored at –20°C until use. Initially, we randomly chose and analysed 20 PCR positive samples from each island (study level I). Next, the two identified genotypes, C and D, were analysed separately (study level II). In addition to the four genotype C isolates detected in KK, we augmented the number of the studied samples representing this genotype from the other three islands. A total of 64 isolates of genotype C (four from KK and 20 from each of VV, FF and TT) were analysed. All identified genotype D isolates, being 16 from KK, 6 from FF and 4 from TT, were also processed.

All samples were investigated for serological markers of HBV infection. PCR was employed to detect and amplify the HBV DNA S gene followed by direct sequencing of the detected amplicons (see section 2.2.1). S gene sequences were determined using a variety of forward and backward primers to eliminate sequence variations caused by Taq polymerase possible errors during amplification. The nucleotide sequences were then edited and assembled using Sequence Navigator software program. To detect important nucleotide changes and motifs, Pacific HBsAg sequences from different islands were lined up and compared to identify any island specific differences. They were also compared with previously published HBsAg sequences from the Pacific region (Norder et al., 1993).

A simple Neighbor-joining Tree was first constructed using the S gene (681bp) nucleotide sequences from 102 isolates (Figure 3.4), consisting of the 20 sequences chosen randomly from each island and 22 S gene sequences representing the different genotypes of HBV retrieved from GenBank (Table 3.10). The aim was to reveal the overall picture of circulating HBV genotypes in the four Pacific islands. We were not only interested in the crude genotype distribution, but also wished to investigate whether sequence specific motifs could be identified on specific islands, which may support theories of inter-island migration at some time in the past. Therefore, in addition to the comparative analysis to detect specific island differences, separate genotype specific networks were constructed, one for genotype C and the other for genotype D, using the reduced median network approach (Figure 3.5 and 3.6: for phylogenetic analysis see Section 2.2.1). Phylogenetic network analysis was performed in Oxford by Rory Bowden; Department of Statistics and Institute of Biological Anthropology.

Table 3.10: Database sequences used in this study*

Accession	Origin	Sub/Geno	Accession	Origin	Sub/Geno
X75665	New Caled	adrq-/ C	Y07587	Germany	ayw2/ D
X75656	Polynesia	adrq-/ C	M32138	Turkey	ayw2/ D
X04615	Japan	ayr/ C	AB033558	Japan	ayw2/ D
X75667	Vietnam	ayr/ C	AB033559	New Guinea	ayw2/ D
AF068756	Thailand	adrq+/ C	AF061523	Germany	ayw2/ D
D23682	Japan	adrq+/ C	AF061528	Germany	ayw2/ D
M54892	China	adrq+/ C	AF065112	Germany	ayw2/ D
X14193	Korea	adrq+/ C	AF065118	Germany	ayw2/ D
D50518	Japan	adrq+/ C	AF121240	Turkey	ayw2/ D
AF074449	Thailand	adrq+/ C	AF151735	Turk/Greek	ayw2/ D
M23807	Japan	adrq+/ C	AF209398	Germany	ayw2/ D
M23809	Japan	adrq+/ C	AF214659	Tunisia	ayw2/ D
M12906	Japan	adrq+/ C	AF214660	Tunisia	ayw2/ D
X75792	France	adrq+/ C	AF214661	Tunisia	ayw2/ D
Ab031262	Vietnam	adrq+/ C	L27106	Israel	ayw2/ D
AF052576	China	adrq+/ C	A01865	Unknown	ayw3/ D
AB014399	Japan	adrq+/ C	Z35716	Poland	ayw3/ D
AB014374	Japan	adrq+/ C	U55227	Brazil	ayw3/ D
X01587	Japan	adrq+/ C	U55228	Brazil	ayw3/ D
Y18856	China	adrq+/ C	U87851	S Africa	ayw2/ D
AF209402	Germany	adrq+/ C	U91804	Costa Rica	ayw3/ D
D00630	Japan	adrq+/ C	U91832	Costa Rica	ayw3/ D
Y18855	China	adrq+/ C	V01460	France	ayw3/ D
D16666	Japan	adrq+/ C	X02496	Latvia	ayw2/ D
V00867	Japan	adrq+/ C	Bang110**	Bangladesh	ayw3/ D
J02202	France	ayw2/ D	Bang 171**	Bangladesh	ayw2/ D
X65257	Italy/patientC	ayw2/ D	Bang 49**	Bangladesh	ayw3/ D
X72702	Germany	ayw3/ D	IN0008**	India	ayw2/ D
X75662	France	ayw2/ D	IN0015**	India	ayw2/ D
X75668	France	ayw3/ D	IN0017**	India	ayw2/ D
X59795	Italy	ayw2/ D	IN0051**	India	ayw2/ D
X85254	Italy	ayw2/ D	IN0062**	India	ayw3/ D
X97848	Greek (Pat 2)	ayw2/ D	IN0083**	India	ayw3/ D
IN0134**	India	ayw3/ D	IN0091**	India	ayw3/ D

*: The database sequences used for this study were selected from amongst the Genotype C and D sequences in GenBank based on similarity to the sample sequences, or by geographic affinity.

**: unpublished sequences from previous studies performed in our Laboratory.

3.3.2 Results

Genotype and subtype prevalence in Pacific islands

Table 3.11 shows that, in Kiribati, 16 out of 20 sequenced isolates were genotype D while the remaining four had genotype C. In contrast, Vanuatu had only genotype C while Fiji and Tonga inhabitants were mainly (67% and 80% respectively) infected with HBV sequences of genotype C. Subtype *adrq*⁻ was the main subtype in genotype C occupied islands while ayw_2 was the prevalent one in Kiribati. Other subtypes, such as *ayw3, adrq+, ayr*, had a low prevalence and sporadic distribution (Table 3.11). Only one recombinant subtype was detected in Tonga (sample 286T).

Distribution of HBV strains within the Pacific islands

Based on residues at positions 44 and 47, in addition to other changes across the S gene, we were able to define three major strains of the *adrq-* subtype in Pacific islands, glycine/ valine (G/V), glutamic acid/ arginine (E/R) and glutamic acid/ valine (E/V) (Table 3.12). The last strain, having E/V, was novel. The prevalence of these strains of genotype C was island-specific, particularly in Vanuatu. 13 out of 20 sequences in Vanuatu had G/V. In contrast, only three isolates of this strain were found in Fiji and none in Tonga. E/R strain, though, detected in various percentages in all islands, was the dominant one in Fiji. E/V strain was seen in 12 of 20 Tongan sequences. In keeping with the hypothesised migration from West to East (Vanuatu, Fiji and Tonga), specific variants were identified in the Pacific island that either increased, or decreased, from West to East. For example, nucleotide changes at positions 13, 203, 282, 339, 348, 551 and 552 were mainly seen in Tonga. On the other hand, changes at 213, 384, 524 nucleotide positions were predominant in Vanuatu. However, no specific nucleotide changes were detected in Fiji (Table 3.12).

Furthermore, detailed analysis of the distribution of strains within islands revealed some intriguing features. For example, in Tonga, a clear selective distribution of E/V and E/R strains between the two cities was observed (Table 3.13). Twice as many E/V strains were observed in Kolovai compared to Nukualofa. In contrast, E/R strain was mainly prevalent in Nukualofa, with 6 isolates versus 2 from Kolovai. In Kiribati, with a predominance of genotype D, Betio, which is located in the west, was the only region where genotype C isolates were detected. Perhaps, this is because it is much closer to Papua New Guinea (PNG) where this genotype was previously isolated (Carman et al., 1997b).

Table 3.11: HBV genotype and subtype distribution within Pacific islands

HBV Typing	Kiribati	Vanuatu	Fiji	Tonga
Genotype C	4 (20%)	20 (100%)	14 (67%)	16 (80%)
Subtype adrq-	3	18	14	16
Subtype adrq+	1	1	-	-
Subtype ayr	-	1	-	-
Genotype D	16 (80%)	Nil	6 (33%)	4 (20%)
Subtype ayw2	15	-	4	2+1*
Subtype ayw3	1	-	2	1

*: Recombinant sample

Table 3.12: Important motif (44-47)* and nucleotide differences for differentiation between HBV prevalent strains in Fiji, Tonga and Vanuatu**

44-47 aa pair	Vanuatu	Fiji	Tonga
G/V	13	3	Nil
E/R	5	12	8
E/V	2	5	12

Nucleotide Position & change	Vanuatu	Fiji	Tonga
13. A>G	1	2	10
203. T>C	2	3	12
213. C>T (S)	12	2	nil
246. C>A (S)	4	1	nil
282. G>A (S)	1	3	11
339. A>C (S)	3	nil	13
348. C>A (S)	1	3	11
384. T>A (S)	13	2	nil
524. T>C	14	2	2
551. T>C	1	3	12
552. A>C (S)	1	3	10
594. G>T or A	5 (T)	nil	1(A)

*: The motif is from nt130 to nt141, and is flanked by 44 and 47 amino acids; G/V, E/R & E/V (highlighted).
**: Nuumber of isolates that has the identified changes is shown underneath each island.
S: Silent change, all the other changes are not silent.

Table 3.13: Distribution of genotype C *adrq-* strains and genotype D in specific cities

Island cities	G/V	E/V	E/R	Genotype D
Tonga				
Nukualofa	-	4	6	
Kolovai	-	8	2	
Kiribati				
Betio	1	1+1*	1	4
Bikenibeu (Tarawa)	-	-	-	6
Bairka	-	-	-	6

*: One isolate of adrq+ subtype.

Pacific islands versus database comparative analysis

Choosing the correct reference sequence is mandatory, especially in cross-sectional studies, otherwise overestimation of mutations can occur. The *adrq-* subtype is rarely found in Genbank; the four available isolates (HMA, Cha, Wan and Del), published by Norder et al. (1993), constituted our reference sequences.

There are a number of variants that were unique to the Pacific islands (Table 3.14). Firstly, there were single amino acid changes dispersed across the S gene. Aa 18 was always valine (V) in our adrq- sequences. None of our adrq-sequences from the four islands had lysine (K) at amino acid number 24, though previously described in the HMA isolate. Position 175 was occupied by serine (S) instead of leucine (L) in 18 Pacific isolates, 14 of them from Vanuatu. Valine (V) or alanine (A) was the amino acid usually detected at residue 184.

Secondly, some amino acid pairs were changed either together or separately. Of particular interest was the pair of aa 44 and 47, which constituted a motif within an important CTL epitope and showed three possibilities: G/V, E/R and E/V (Table 3.12). Residues 159 and 177, which are described to be responsible for q- determinant expression in this subtype, made up the second important pair. Valine instead of alanine at position 177 was detected in nine sequences (4FF, 2TT, 3VV). In addition to the change at the aa 177, alanine instead of valine at 159 position was detected in another two isolates (K271 and V185).

Phylogenetic analysis

Primary analysis of the prevalent pacific HBV genotypes (level I)

Figure 3.4 shows that the Pacific sequences grouped into 4 major clusters. The first is mainly occupied by Kiribati sequences (genotype D), with a few isolates from FF and TT. The second mainly contains Vanuatuan sequences (14VV, 1FF and 1KK). The third and fourth clusters, though more heterogeneous, mainly contain Fijian (10FF, 7TT, 4VV and 1KK) and Tongan (9TT, 3FF, 1VV and 1KK) sequences respectively.

After identifying the circulating genotypes in the four Pacific islands, a re-alignment was performed according to their predominant genotype. Among genotype C occupied islands, VV, FF and TT, characteristic nucleotide differences were determined (see Table 3.12). In contrast, no significant differences were observed between genotype D sequences that were isolated from KK, FF or TT. This indicates that genotype D indeed arrived in the Pacific as a relatively homogeneous population and has probably been distributed by admixing with stable human populations.

Genotype C Network

A major cluster containing about 18 sequences is centered on the haplotype defined by V102 (see Figure 3.5). 24 of the 64 sequences, and one of the database sequences (X75665), are within two mutational steps of this haplotype (distances measured discounting the excluded sites). The central cluster contains mostly Fijian and Tongan sequences. Most of the rest of the sequences are felt in two clusters, which centered 2 and 3 mutations respectively from the central haplotype. The first of these is predominantly Vanuatuan (13 out of 16 sequences) with 2 sequences from Fiji and one from Kiribati. On the other hand, all but one (Vanuatuan) sequence in the second cluster are from Tonga (11 sequences) and Fiji (6 sequences). 5 of the remaining sequences (V150, K271, T243, K240 and V235) are on relatively rare lineages derived from the central cluster. Another 3 observed sequences are intermediate on the lineages leading to the derived clusters (K136, V159 and T285).

The Pacific network can be rooted using the database sequences. All the Pacific sequences in the dataset, plus two sequences of Pacific origin from the database (X75665 & X75656), share a single link to the database sequences representing other parts of the world, indicating a common origin. Furthermore, V185 and the two Pacific database sequences are closer to the rest of the world than all other Pacific sequences.

Table 3.14: Amino acid changes across S gene in both genotype-C and D sequences

*: Important amino acid positions for subtyping and genotyping of HBV.

Amino acid changes were detected within **: E/V strain; †: G/V strain; ‡: E/R strain.

Red: amino acid changes within the major hydrophilic region that contain the major B cell epitopes.

Yellow: amino acids changes within CTL epitopes.

Between brackets is the no of isolates that have the variant.

Table 3.14A: Amino acid changes across S gene in genotype-C sequences

Ref.Seq	E	N	T	F	V	V	I	Q	S	S	N	G	E	A	P	V
	2	3	5**	8	14	18	25	30	31	34	40	43	44	45	46	47
VV		S(6)	A(1)	L(1)	G(1)	G(1)	V(1)						G(13)			R(5)
FF		S(4) G(3)	A(2)								S(2)	R(1)	G(3)			R(12)
TT		S(3) G(1)	A(10)					R(1)	N(1)	L(1)				P(1)	L(1)	R(8)
KK	D(1)	S(1)	A(1)													

Ref.Seq	P	S	Q	S	P	T	I*	V	Y	S*	K	I*	P*	Q	G	V*	R*
	49	53	54**	61	62	63	68**	96	100	114	122	126	127	129	130	159	160
VV	L(1)			L(1)			T(2)	A(2)	F(3)	T(4)		S(1) T(1)	S(1) T(1)			A(1)	
FF			P(3)				T(3)		F(1)		R(1)						K(1)
TT	H(1)			.L(2)	Q(1)	I(1)	T(12)					N(1)		R(1)	N(1)		
KK		L(1)					T(2)					T(1)				A(1)	

Refer. sequence	S 174	L 175†	A* 177‡	Q 181	V 184**	S 193	M 198†	P 203	S 204	N 207	S 210
VV		S(14)	V(4)		A(1)		I(5)		N(2)		
FF	N(1)	S(2)	V(4)	H(1)	A(3)			R(1) Q(1)	G(1)		
TT			V(1)		A(12)	T(1)	I(1)		N(1)	S(1)	R(1)
KK		S(1)	V(1)		A(1)						

3.14B: Amino acid changes across S gene in genotype-D sequences

Ref.Seq	N	R	Q	N	P	C	P	T	S	L	V	T	S	S	S
	3	24	30	40	70	76	127	131	143	175	184	189	193	207	210
KK		K(1)	K(1)	S(1)			T(1)	N(1)	L(1)	S(1)	A(1)	I(4)	L(1)	R(2)	
FF	T(1)				A(1)										
TT			K(1)			Y(1)								T(1)	R(1)

Figure 3.4: N-J phylogenetic tree of Pacific HBsAg isolates.

Phylogenetic analysis of HBV isolates was based on the nucleotide sequences of S gene. Genotypes of HBV are indicated from A to G.

VV: Vanuatu; FF: Fiji; TT: Tonga; KK: kiribati.

All the other sequences are retrieved from GenBank and are shown by accession numbers.

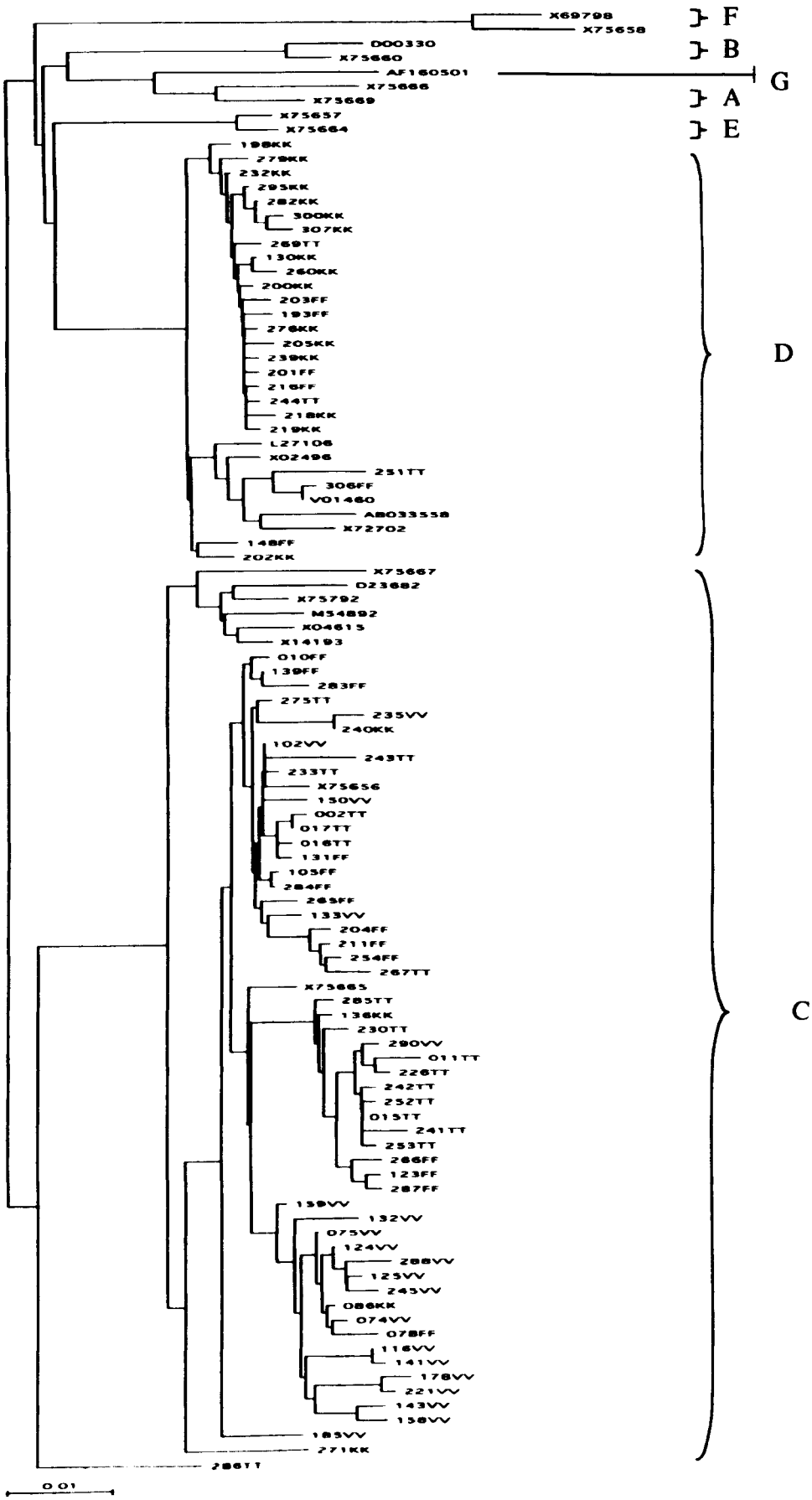

X69708
X75658
D00330
X75660
AF160501
X75666
X75669
X75657
X75664
198KK
279KK
232KK
295KK
282KK
300KK
307KK
269TT
130KK
260KK
200KK
203FF
193FF
276KK
205KK
239KK
201FF
216FF
244TT
218KK
219KK
L27106
X02496
251TT
306FF
V01460
AB033558
X72702
148FF
202KK
X75667
D23682
X75792
M54892
X04615
X14193
010FF
139FF
283FF
275TT
235VV
240KK
102VV
243TT
233TT
X75656
150VV
002TT
017TT
016TT
131FF
105FF
284FF
265FF
133VV
204FF
211FF
254FF
267TT
X75665
285TT
136KK
230TT
290VV
011TT
226TT
242TT
252TT
013TT
241TT
253TT
286FF
123FF
287FF
159VV
132VV
075VV
124VV
288VV
125VV
245VV
086KK
074VV
078FF
116VV
141VV
178VV
221VV
143VV
158VV
185VV
271KK
286TT
0.01
F
B
G
A
E
D
C

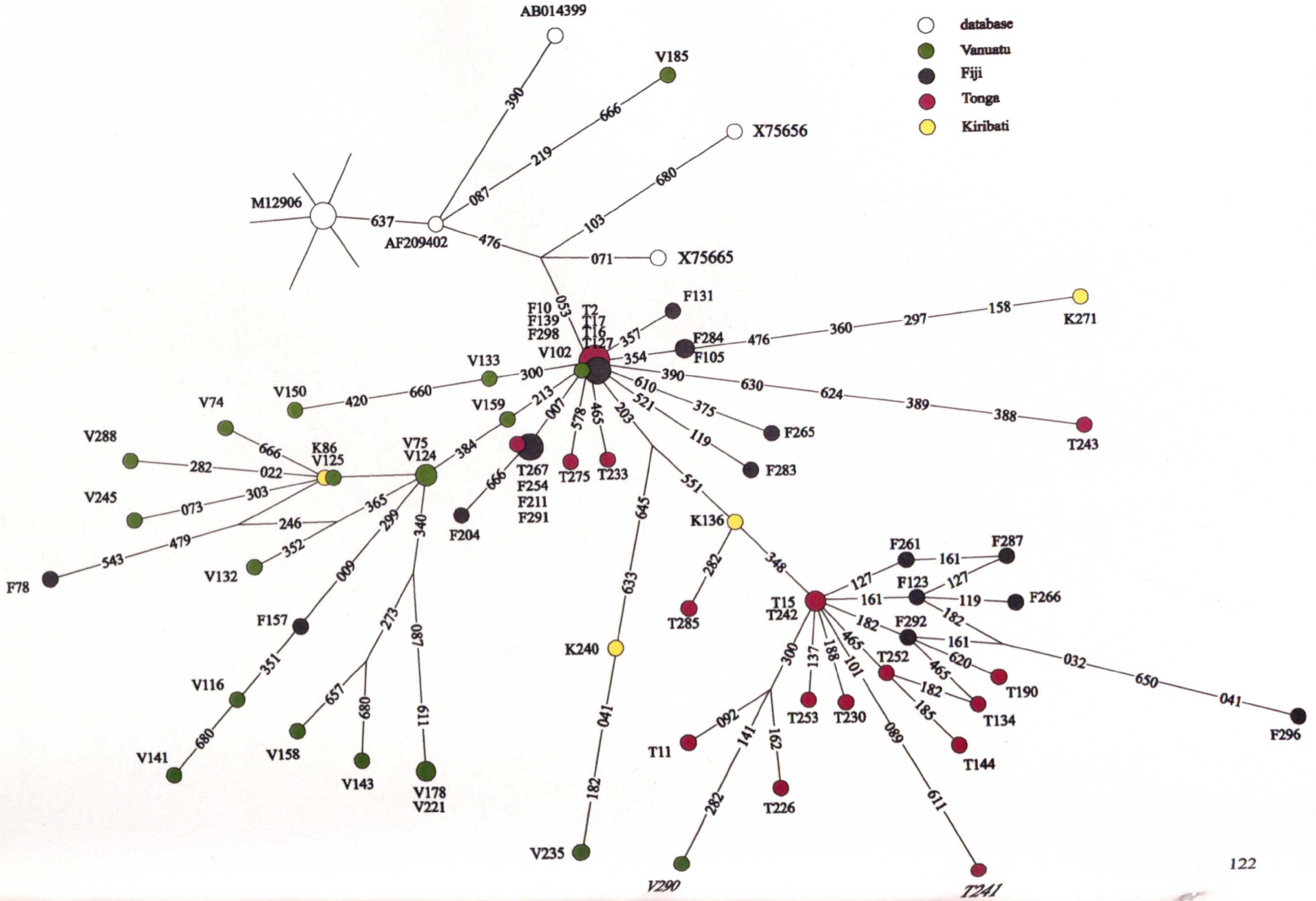
Figure 3.5: Phylogenetic Network for Genotype C Pacific HBsAg sequences†

The network classifying the Pacific HBsAg sequences can be summarised in the following way: A single lineage joins all the most similar database sequences to the Pacific sample. A major cluster is ancestral to all the Pacific sequences except V185 and two database Pacific HBV sequences, from Polynesia and New Caledonia. 25 sequences are within 2 mutational steps of the founder of the cluster, and 6 descendant lineages are derived from this founding type.

There are two further clusters, the first of which is predominantly (13 of 16 sequences) Vanuatuan, with 2 sequences from Fiji and one from Kiribati. The second cluster contains a single Vanuatuan sequence, with 11 Tongan and 6 Fijian sequences. The 4 Kiribati HBV genotype C sequences are scattered through the phylogeny, although all are descendants of the founding type. For clarity, only those database sequences closest to the Pacific sample are shown in the network.

†: The phylogenetic network analysis was based on the nucleotide sequences of S gene and distances along the lines are not drawn to scale.

Labels are F: Fiji; V: Vanuatu; T: Tonga; K: Kiribati.


database
Vanuatu
Fiji
Tonga
Kiribati
AB014399
V185
X75656
M12906
AF209402
X75665
F10
F139
F298
T2
T17
T16
T127
V102
F131
F284
F105
K271
V133
V150
V74
V159
F265
T243
V288
K86
V125
V75
V124
T267
F254
F211
F291
T275
T233
F283
V245
F204
K136
F78
V132
F157
V116
V141
V158
V143
V178
V221
K240
T285
F261
F287
F123
F266
T15
T242
F292
T252
T190
T134
T144
T253
T230
T11
T226
V235
V290
T241
F296

Genotype D Network

One of the sequences, T286, was clearly a C/D recombinant (C left part, D right part, breakpoint between 203 and 300). Therefore its position at the end of a long branch in the D network reflects the number of fixed differences between C and D genotypes in the first ~250bp of the HBsAg sequence. T286 was most closely related to V159 and X75656 amongst the known C sequences (5/250 mismatches in nt 1-250), and K130, K232 and AB03359 amongst known D sequences (1/431 mismatches in nt 251-681).

The final network for D contained several reticulations, or ambiguities, in the implied mutation order and tree topology (see Figure 3.6). The identification of nt 192 as a 'fast' site and T286 as a recombinant sequence allowed the tentative resolution of the parallelisms on the branch leading to K202, F148, F306 and T251. It seems clear that 21 of the 26 sequences are found in a cluster together with two database sequences, one from from Papua New Guinea (AB0335599) and the other from France (X75662). J02202, a third database sequence from France is on a derived, but probably not ancestral, branch. The main cluster is related to all other known sequences through a single branch, which therefore represents the presumptive ancestral lineage. A mutation at nt513 defines the main cluster, and separates it from the remaining Pacific sequences, K202 and F148, which share an ancestral branch.

The other most similar database sequences form a second cluster to the right of the figure, and originate from India. The data indicates a variable distribution of largely homogeneous D sequences across a wide area of the Pacific, including Papua New Guinea, consistent with a relatively recent origin and spread of D genotype. This is in contrast to C, where the variability seems to be older and more geographically specific

Time estimates science the first ancestor

Published estimates for the substitution rate for HBV vary with pathogenic status of the infection (Bozkaya et al., 1997). Family data from Hannoun et al. (2000) lead to point estimates of 2.04 e-5 and 2.25 e-4 $nt^{-1}yr^{-1}$, for HBeAg positive and negative patients respectively. These estimates were incorporated to give estimated dates for HBV colonisation of the Pacific (see Section 2.2.1h; Table 2.2). In our study, it is at least clear from historical information ruling out very recent introduction of HBV, that the HBV mutation rates observed are more consistent with HBeAg-positive infection rather than HBeAg negative status which are most frequent in highly endemic areas in Asia like the Pacific region.

Figure 3.6: Phylogenetic Network for Genotype D Pacific HBsAg sequences†

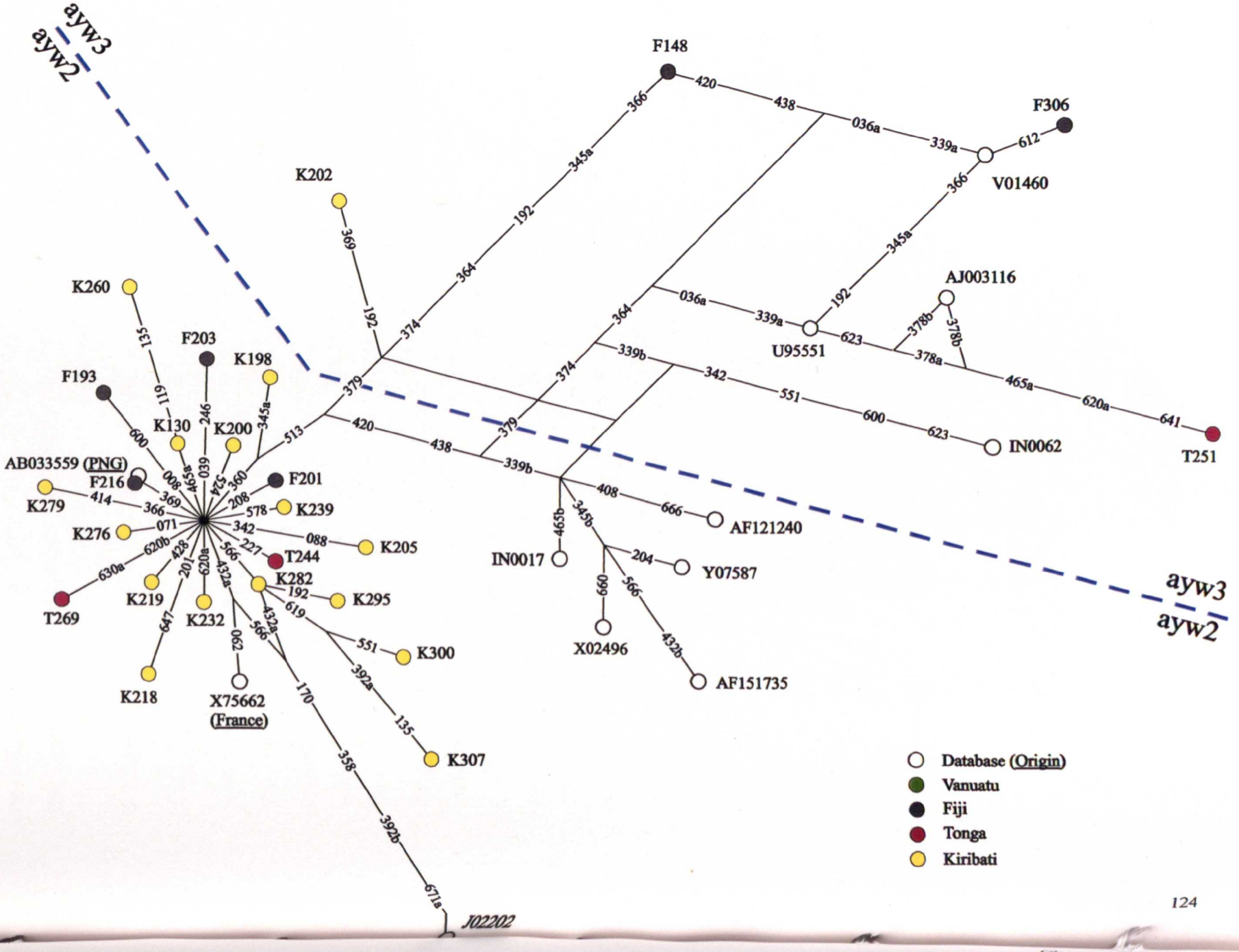
21 of the 26 Pacific sequences are found in a cluster together with two database sequences (AB0335599; from Papua New Guinea and X75662; from France). A third database sequence, J02202, is on a derived, but probably not ancestral, branch. The main cluster is related to all other known sequences through a single branch, which therefore represents the presumptive ancestral lineage.

A mutation at nt513 defines the main cluster, and separates it from the remaining Pacific sequences, K202 and F148, which share an ancestral branch. Blue dotted line passing through nt379, is shown dividing the data set into ayw2 and ayw3 subtypes.

The other most similar database sequences form a second cluster to the right of the figure, and originate from India. There are no published sequences which are more closely related to any of the Pacific sample D sequences than any of the database sequences shown on the network. For clarity, only those database sequences closest to the Pacific sample are shown in the network.

†: The phylogenetic network analysis was based on the nucleotide sequences of S gene and distances along the lines are not drawn to scale.

Labels are F: Fiji; V: Vanuatu; T: Tonga; K: Kiribati.


ayw3
ayw2
F148
F306
V01460
K202
AJ003116
U95551
K260
F203
K198
F193
K130
K200
AB033559 (PNG)
F216
F201
K279
K239
K276
K205
T244
K219
K282
K295
T269
K232
K300
K218
X75662
(France)
K307
IN0062
T251
AF121240
IN0017
Y07587
X02496
AF151735
J02202
ayw3
ayw2
Database (Origin)
Vanuatu
Fiji
Tonga
Kiribati

Moreover, assuming the same rate of nucleotide substitution in the two genotypes, it can be inferred from the calculations that the time elapsed since the major common ancestor for the C sample is 2– 4 times longer than that for the D genotype.

3.3.3 Discussion

80% of strains in Kiribati were genotype D, whilst genotype C was found in all strains from Vanuatu and between 67% and 80% of strains from other Melanesian/ Polynesian sites. We believe that this can be explained as result of inter-island movement after the main migration (Table 3.11). In genotype C dominated islands (Figure 3.5), the identified HBV isolates share a common origin, related most probably to the initial colonization of the region. The relationships between sequences suggest that varying degrees of isolation and migration are responsible for the spatial distribution differences. The absence of genotype D in Vanuatu supports this contention as Vanuatu has a net migration rate of 0 migrants/1000 population (1999 est.) and is mainly occupied by indigenous Melanesian people (94%), in addition to other ethnic minorities; French, Chinese, Vietnamese and other Pacific islanders, (CIA, 1999). On the other hand, recent Indian immigrants who usually have genotype D and constitute around 44% of total Fijian population might play a role in Fiji (CIA, 1999).

Adrq- and ayw2 subtypes are the predominant subtypes in genotype C and D dominated islands, respectively (Table 3.11). adrq+ is the prevalent subtype in South-East Asia, while adrq- is only found in Oceania. Geographical transition from Asian adrq+ to South American adw4q- through the adrq- subtype in Oceania has also been suggested (Couroucé-Pauty et al., 1983). Adrq+ and adrq- are consistently different at three sites, 159, 177 and 213 and there are additional non-consistent differences. Ayw2 and ayw3 (both genotype D) have a worldwide distribution; however, it is predominant in the Mediterranean area, through the middle East and extending into India (Norder et al., 1993).

Comparative analysis & important variants

Both simple sequence comparisons and the derived networks clearly identified island-specific nucleotide and amino acid sequences on genotype C dominated islands (Table 3.12; Figure 3.5). Two types of changes have been observed. Firstly, there were single aa changes across the S gene such as that observed at residues 5, 68, 175 or 184, that were island-specific

(Table 3.14). Other sites such as aa 18 and 24 were not island-specific. For instance, amino acid 18 was always Valine (V) in all identified sequences, while only one of the four adrq- isolates published by Norder et al. 1993 (Del isolate) has this aa, in addition to subtype adw4q-. Also, A, mainly in Tonga, or V was the amino acid detected at residue 184, but never D as has been previously described in Del isolate (Norder et al., 1993).

Secondly, two pairs of aa changes at two important regions have been determined. The first pair was at residues 44 and 47 and is located within a region containing the HLA class I-restricted CTL epitope, aa 38 to 47 (Nayersina et al., 1993). The larger region (aa 29-53) is found frequently mutated in chronic hepatitis and hepatocellular carcinoma patients who are under immune pressure from CTL, suggesting a potential role of CTL escape in HBV persistence (Chen & Oon 1999). Although there is much debate about this relationship (Rehermann et al., 1995), the accumulated data would appear to support such a link (Bertoletti et al., 1994; Tai et al., 1997; Chen & Oon 1999; Khakoo et al., 2000).

Consistent with this data, further changes at positions 175 (mainly in Vanuatu), 177 and 184 (mainly in Tonga) have also been observed in our Pacific study (see Table 3.14); these are contained within another two identified HLA class 1 restricted CTL epitopes (172- 180 & 175- 184: Nayersina et al., 1993). In contrast, mutations within the MHR, aa 99-160, containing the major B cell epitope cluster of HBsAg (Brown et al., 1984; Waters et al., 1992a) were less frequently detected and had no island linkage.

The second pair was at residues 159 and 177. Both or one of these residues were proposed to be responsible for abolishing the expression of q subdeterminant in adrq-, while the adjacent residues (aa 158 and 178) were described for the same effect in adw4q- subtype (Norder et al., 1992). V, instead of A, at position 177 was detected in nine Pacific sequences (4FF, 2TT, 3VV). However, A instead of V at position 159 was only detected in two isolates along with V at residue 177. These two isolates, K271 and V185, were in fact of adrq+ subtype. Thus, residue 159 seems to be more conserved in adrq- subtype and perhaps has an interaction with residue 177 to abolish q expression, as it was never changed in isolation. Consequently, G 159 in ayw2, ayw3 and ayw4 and A in the other q+ subtypes will have no effect on q expression, as has been previously noted (Norder et al., 1992a).

HBsAg as a marker to trace the migration of Pacific people

We set out to use the HBV sequence as a surrogate marker of the migratory patterns of Pacific people. The origin of Polynesians and the relationship among Pacific and Asian populations is a matter that has been long debated between geneticists and archaeologists (Diamond 1988; Terrell 1988). Employment of mitochondrial DNA (mtDNA) to infer the ethnic backgrounds of human subjects has been widely used in Pacific region (Merriwether et al., 1991; Sykes et al., 1995; Lum et al., 1998; Matisoo-Smith et al., 1998). Other tools such as language, archaeology and nuclear encoded loci have also been employed (O'Shaughnessy et al., 1990; Kirch, 1997; Hagelberg et al., 1999).

In fact HBV has been used to support population theory in the past. A report from 25 years ago showed a South-to-North gradient in the distribution of the r determinant in Japan and suggested employing HBsAg sequences to predict Japanese ancestors (Yamashita et al., 1975). Moreover, serological subtyping of HBV was recently employed in Indonesia to trace ethnic origins (Mulyanto et al., 1997).

There are several hypotheses for the origin of Polynesian people: "Express train", "entangled bank", "Melanesian origin", "eastern Indonesia" and recently "Slow boat" (Diamond 1988; Terrell 1988; Hagelberg & Clegg 1993; Redd et al., 1995; Kayser et al., 2000). Our results are most consistent with the first, which suggests that the Polynesian people were originally agriculturists from Southern China. Along their way, they spread to Taiwan, Philippines, eastern Indonesia and then Melanesia and Fiji by around 3,500 years ago and radiated across the Pacific to fill the Polynesian triangle by around 1,000 years ago (Diamond 1988; Bellwood 1991). On the other hand, Melanesians may have been earlier or later arrivals after this great migration in keeping with our closely related sequences from Tonga and Fiji, and previous reports (Hagelberg & Clegg 1993; Gibbons 1994). Additionally, or alternatively, back migration from Polynesia to Melanesia could be another factor responsible for these shared sequences between Tonga and Fiji (Hagelberg et al., 1999).

The existence of 3 Pacific sequences (V185, X75656 and X75665) along the implied ancestral lineage, rather than elsewhere amongst the remaining genotype C sequences, strongly suggests that further diversity exists within the Pacific region, and that these sequences are sampled from the pool (see Figure 3.5). This is still in agreement with the radiation phase of the previously described "Express train" hypothesis (Diamond 1988;

Bellwood 1991). Alternatively, this diversity pattern may reflect the influence of different migrations at different times or random genetic drift in these populations (Merriwether et al., 1999). Of interest is that the four KK genotype C isolates were extremely diverse, were found along three clusters in genotype C network, and are consequently consistent with recent introduction of derived C strains from other islands.

The situation for genotype D is quite different and unlike the pattern in genotype C dominated islands (Figure 3.6). The extreme lack of diversity suggests a history of sub-endemic or absent HBV followed by recent introduction of closely related D lineages. The few D sequences on other islands show a similar homogeneity indicating a recent contact between Kiribati and the genotype C dominated islands (see above). Consequently, the HBV strains identified on these islands is in keeping with a varied degree of contact and isolation among Pacific islanders as has been previously described (Mastisoo-Smith et al., 1998).

Pacific genotype D sequences are found in a cluster together with two database sequences one from PNG (AB0335599) and the other from France (X75662). A third database sequence, J02202, is on a derived, but probably not ancestral, branch. The main cluster is related to all other known sequences through a single branch, parallel to the line separating *ayw2* and *ayw3* subtypes (see Figure 3.6), which therefore represents the presumptive ancestral lineage. Analysis of HBV sequences from other locations will be required to interpret these data fully.

Times estimation for HBV in Pacific islands

Although it is helpful to estimate the depth of the history, this approach is by nature approximate. In Pacific region, the dates inferred using the lower mutation rates detected in HBeAg positive individuals in Hannoun et al. (2000) are at least within an order of magnitude of consistency with the hypothesis that HBV accompanied settlers in the major colonisation events (Diamond 1988; Bellwood 1991; see Table 2.2). However, it should be noted that there are several sources of bias (see below). Alternatively, these shallow dates might indicate that the virus entered the Pacific region at a later date (after the human colonisation) and then simply become isolated on each individual island along human movements (Eddie Holmes; personal communication).

The extreme heterogeneity of mutation rates across sites in HBV would have the effect of leading to underestimation of times. This is because events at fast sites are over-represented in short-term longitudinal studies used to estimate mean rates, but are hard to resolve on genealogies, with many mutational events remaining undetected. Another factor influencing the mutation rate (real and inferred), is the type of infection predominating (Lin et al., 1991;Carman et al., 1995b; Bozkaya et al., 1997; Hannoun et al., 2000). From the above, it should be clear that the average mutation rate clearly differs between a hyperendemic population and one in which infection is non or sub-endemic, perhaps due to differences in pathology and immune selection. Secondly, the effective population size, relating the number of infections and the rate and mode of transmission, will affect the accumulation of genetic variability in a way which may be harder to predict.

3.4 Diagnostic assay reactivity of HBsAg variants

3.4.1 Introduction

Antigenic variation of HBsAg is clinically significant and has been discussed in several reviews (Locarnini, 1998; Gunther et al., 1999; Hunt et al., 2000). HBsAg variants (hepatitis B viruses containing mutations in the surface gene) can be divided into 2 aetiological classes. The first class occurs naturally and includes subtype variation and amino acid (aa) changes which may be poorly detected in diagnostic assays (Yamamoto et al., 1994; Carman et al., 1995a). Class 2 variants are selected by medically induced immune pressure (Carman et al 1990; McMahon et al., 1992; Oon et al., 1995; Ghany et al., 1998; see Section 1.5.1). The MHR, an important external domain of HBsAg, contains clusters of epitopes within which are the proposed neutralising epitopes (Brown et al., 1984; Waters et al., 1992a, b). Although class 2 antigenic variation tends to occur within these neutralising epitopes, both classes show variation within the MHR.

Furthermore, it is possible that gene variation can affect the disease process. A study of 120 Asian Indians with chronic HBV revealed S gene variants in 10.8% of patients. These showed an unfavourable clinical course compared with the standard strain (Guptan et al., 1996). G145R and D144A variants were shown to be associated with a worse clinical outcome and longer persistence despite the termination of HBIG treatment (Protzer-Knolle et al., 1998). The increased frequency of association of variant compared to standard sequences has also been described in some cases of hepatocellular cancer (Zhong et al., 1999).

Although a range of commercial assays for HBsAg is widely available, not all are equally sensitive (Carman et al., 1997b; Coleman et al., 1999). The backbone, either subtype *d* or *y*, of the tested variant has a role in HBsAg detection efficacy (Wallace et al., 1994; Carman et al., 1997b). Moreover, the geographical distribution of HBsAg subtypes has to be considered (Couroucé-Pauty et al., 1983). On the other hand, serum containing HBsAg particles is rarely available in volumes sufficient for testing against a multitude of capture antibodies, therefore we cloned variant HBsAg from 13 diagnostically relevant cases and tested cell culture supernatants in seven commercial diagnostic assays.

3.4.2 Results

The mutations detected in each of the variant samples are shown (Table 3.15). Also listed is the clinical background and country of origin of the serum samples. As sample T5N gave uninterpretable results, this is dealt with separately (see below).

3.4.2a Immunofluorescence results

Immunofluorescence was performed to measure transfection efficiency (Table 3.16). Most samples (10/12) showed 40-60 % of cells expressing HBsAg. BA3.2 was lower at 20-30% but still within the lower range of the standard Gly Y and expected to produce detectable amounts of surface antigen.

3.4.2b Diagnostic assay reactivity

The reactivity of each variant is presented in two ways. First, it was calculated as an index (Table 3.16) i.e. reactivity of the variant divided by that of the NIBSC standard (0.5 IU/ml) for each assay. Secondly, they are expressed as a percentage of the reactivity of the standard HBV sequence of the same subtype, *ay* or *ad* (Table 3.17). These two analyses allow comparison both to a known amount of natural HBsAg and to an *in vitro* expressed standard sequence. Representing the data in these ways allows both inter- and intra-assay comparisons to be made. All assays performed within the sensitivity limits claimed by their manufacturers using two preparations of a standard serum (working standard and monitor sample; NIBSC).

Both standard sequences and seven of the variants (Arg145, 91-4696, HK188, AP3.1, SA4, SA6 and SA7) were detected by all assays although Arg145 was less reactive than the working standard in assays 3, 6 and 7, and only marginally above cut off level in 6 and 7. This group contained variants in HBs regions 1, 3, 4 and 5 of the MHR (see Figure 1.9). Three samples (1056Sp, BA 3.4 and BA 2.4) were detected by most of the assays, although some displayed only low level reactivity: 1056Sp was low in assay 7 and negative in assay 4; BA3.4 was negative in assays 3 and 5; and BA2.4 was negative in assay 4 and low in assays 3, 5 and 7. This group contained variants in HBs regions 1, 2 and 4 of the MHR.

In this chapter, the serological assays work was performed by Jacqueline Ireland, Barbara O'Donnell, and Joy Kean, whose efforts are very much appreciated.

Table 3.15. Expressed Hepatitis B Surface Antigen Variants.

Sample	Subtype	Mutations within the MHR*	HBs region of MHR where mutations are located (A)	Origin & reference	Clinical background
Gly Y	*ayw*	Standard sequence		Spain	Positive control
Gly D	*adw*	Standard sequence		Spain	Positive control
Arg145	*adw*	G145R	4	Italy (B)	Vaccinee
1056Sp.	*ayw*	P120S/S143L	2, 4	Spain (C)	Subtype study. IVDU†
M5	*ayw*	Y100S/T118V/R122K/M133I/Y134N /P142S/S143L/G145K	1- 4	Saudi Arabia	Discrepant serology. Renal transplant patient.
T5N	*ayw*	D99N/122NT123/G145R	1, 2, 4	Indonesian (D)	Discrepant serology, vaccinee
91-4696	*adw*	S113T/T143S	1, 4	South Africa	Diagnostic failure
HK188	*adr*	L98V/Q101R	1	Hong Kong	Diagnostic failure, bone marrow donor
BA3.2	*ayw*	T123N/C124R	2	Pakistan (E)	Liver transplant –HBIG‡ treated
BA2.4	*ayw*	Y100C/P120T	1,2	Pakistan (E)	Liver transplant -HBIG treated
BA3.4	*ayw*	T123N	2	Pakistan (E)	Liver transplant -HBIG treated
AP3.1	*adw*	D144A	4	UK (E)	Liver transplant -HBIG treated
SA4	*adw*	M133T/Y161F	3, 5	South Africa	Acute hepatitis B
SA6	*adw*	Q129R/G130N/A166V	3, 5	South Africa	Chronic Liver Disease
SA7	*ayw*	M133T	3	South Africa	Chronic Liver Disease

*: The convention chosen to describe the mutation is the amino acid (aa) position (numbered from the start of the surface gene) preceded by the usual aa and followed by the aa found in the variant sequence.

†: Intravenous drug user

‡: Hepatitis B hyperimmunoglobulin

(A): Based on a model proposed by Wallace & Carman (1997); (B): Carman et al., (1990); (C): Wallace et al., (1994); (D): Carman et al., (1995a); (E): Carman et al., (1999).

Table 3.16. Reactivity[1] of Expressed Hepatitis B Surface Antigen Variants as Determined by Seven Commercial Diagnostic Assays.

Sample	(1) bioELISA HBsAg colour	(2) AUSRIA II -125	(3) VIDAS HBsAg	(4) Enzymun -Test HBsAg ES300	(5) IMX HBsAg (V2)	(6) HBsAg GE14	(7) Enzygnost HBsAg Monoclonal II	Immunofluorescence (pAb) - % of positive cells per field at x10 magnification
Gly Y[2]	3.59	21.94	12.89	9.02	4.78	8.83	4.73	30-40
1056Sp.	3.05	11.88	1.36	0.52 Neg[3]	1.16	6.17	0.35 low[4]	40-50
BA2.4	3.24	7.07	0.69 low	0.37 Neg	0.76 low	6.66	0.33 low	40-50
BA3.4	2.20	4.08	0.029 Neg	5.67	0.27 Neg	2.20	2.47	40-50
SA7	2.79	5.20	9.59	4.27	4.59	8.04	5.59	40-50
M5	1.57	3.34	0.024 Neg	0.71 Neg	1.40	0.23 Neg	0.30 Neg	40-50
BA3.2	N/A[5]	N/A	N/A	N/A	N/A	N/A	N/A	20-30
Gly D[2]	3.50	33.40	13.22	18.78	7.01	8.70	4.15	50-60
Arg145	3.40	15.60	0.40 low	8.42	4.83	0.27 low	0.38 low	50-60
91-4696	3.66	29.88	12.97	14.50	5.76	8.35	5.11	40-50
HK188	3.64	22.40	12.94	32.97	11.52	8.51	5.01	40-50
AP3.1	3.31	17.10	11.60	15.46	5.18	6.93	6.71	40-50
SA4	1.89	2.91	3.46	6.06	4.58	2.31	1.38	40-50
SA6	2.70	1.43	8.21	9.52	5.02	4.45	3.77	30-40
pJI (plasmid)[6]	0.52 Neg	0.11 Neg	0.024 Neg	0.32 Neg	0.196 Neg	0.16 Neg	0.27 Neg	Neg
COS7 cells[6]	0.39 Neg	0.16 Neg	0.063 Neg	0.46 Neg	0.20 Neg	0.18 Neg	0.28 Neg	Neg
WorkingStandard[7]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	Not tested
Monitor Sample[7]	0.68	0.31	0.30	0.73 Neg	0.40	0.39	0.53	Not tested
Assay cut -off[8]	0.53	0.23	0.14	0.95	0.28	0.24	0.31	

1. Reactivity is expressed as an index = OD of sample / OD of working standard
2. Gly Y, Gly D are standard sequences of HBV subtype ay and ad respectively.
3. Neg: negative; reactivity below the kit cut off level.
4. Low: reactivity below that obtained for the working standard.
5. N/A: results not applicable. BA3.2 expressed antigen was later found to be retained within the cells (see Chapter 3.4 and 3.5).
6. Supernatants from COS7 cells alone and cells transfected with the plasmid pJI which certains no insert of hepatitis B surface gene represent the negative controls.
7. The working standard (0.5 IU/ml) and the monitor sample (0.125 IU/ml) are serum preparations that used as external controls (NIBSC).
8. Assay cut-off: the value prescribed by the assay above which a serum sample would be deemed to be reactive.

Table 3.17. Percentage reactivity[1] of HBsAg variants in seven assays compared to the positive control (100%).

Sample	(1) bioELISA HBsAg colour	(2) AUSRIA II -125	(3) VIDAS HBsAg	(4) Enzymun -Test HBsAg ES300	(5) IMX HBsAg (V2)	(6) HBsAg GE14	(7) Enzygnost HBsAg Monoclonal II
Gly Y[2]	100	100	100	100	100	100	100
1056Sp.	84.9	54.2	10.6	5.8	24.3	69.9	7.4
BA2.4	90.3	32.2	5.35	4.1	15.9	75.4	6.98
BA3.4	61.3	18.6	0.2	62.9	5.7	24.9	52.2
BA3.2	N/A[3]	N/A	N/A	N/A	N/A	N/A	N/A
SA7	77.7	23.7	74.4	47.3	96.0	91.0	118.2
M5	44.9	10.0	0.2	3.8	20.0	2.6	7.2
Gly D[2]	100	100	100	100	100	100	100
Arg145	97.1	46.7	3.0	44.8	68.9	3.2	9.2
91-4696	104.6	89.5	96.1	77.2	82.2	96.3	123.1
HK188	104.0	67.1	97.9	175.6	164.3	97.8	120.7
AP3.1	94.6	51.2	87.7	82.3	73.9	79.7	161.7
SA4	54.0	8.71	26.2	32.3	65.3	26.6	33.3
SA6	77.1	4.28	62.1	50.7	71.6	51.1	90.8
pJI (plasmid)[4]	14.5	0.51	0.2	3.6	4.1	1.8	5.8
COS7 cells [4]	10.9	0.73	0.5	5.1	4.2	2.0	6.0
Working Standard[5]	27.9	4.6	7.8	11.1	21.0	11.5	21.1
Monitor Sample[5]	18.9	1.4	2.3	8.1	8.4	4.5	11.2
Capture antibody	Guinea pig pAb[6]	Guinea pig pAb	Mouse mAb[7]	Mouse mAb	Mouse mAb	Goat pAb	Sheep pAb
Detection antibody	Goat pAb	Human pAb	Mouse mAb	Mouse mAb	Goat pAb	Mouse mAb	Mouse mAb

1. Reactivity: OD of sample / OD of standard sequence of same subtype x 100%.

2. Gly Y, Gly D: standard sequences of HBV subtype ay and ad respectively.

3. N/A: results not applicable. BA3.2 expressed antigen was later found to be retained within the cells (see Chapter 3.4 and 3.5).

4. Supernatants from COS7 cells alone and cells transfected with the plasmid pJI which certains no insert of hepatitis B surface gene represent the negative control.

5. The working standard (0.5 IU/ml) and the monitor sample (0.125 IU/ml) are serum preparations that used as external controls (NIBSC).

6. pAb: polyclonal antibody.

7. mAb: monoclonal antibod.

Sample M5, which had mutations in HBs regions 1- 4, was negative in assays 3, 4, 6 and 7. Moreover, sample BA 3.2, containing mutations in HBs region 2, could not be detected by any of the assays. To attribute non-reactivity of BA 3.2 to the mutations *per se*, we had to exclude intracellular retention of surface antigen particles. Therefore, cell lysates of BA3.2 and appropriate controls (cell lysates of Gly Y and standard sera) were tested using assays 1, 5 and 6. Positive results for BA3.2 were detected in all three assays, with a reactivity of approximately 20% of that of the standard HBsAg sequence. This indicates that the loss of cysteine at amino acid 124 has had a deleterious effect on secretion as is suggested by the low positivity found by immunoflourescence on the transfected cells (Table 3.16).

We also compared the assay performance of the variants that react at a level greater than or equal to 10% of the standard HBV sequence (Table 3.18). In general, the use of polyclonal antibody in the capture and/or detection phases was associated with higher detection rates: assays 1, 2, 5 and 6 detected 11, 9, 10 and 9 of the 11 variants respectively. The exception was assay 7 (detected seven variants) which used sheep pAb in the capture phase and mouse mAb for detection. Assays 3 and 4, which detected only seven and eight of the variants respectively, employed mAb in both capture and detection phases. Variants of HBV subtype *d* are also detected more readily than those of subtype *y* using this panel of assays (Table 3.18).

As a measure of expression efficiency, reproducibility of variant antigen reactivities was determined on supernatants from multiple transfection experiments using the IMX HBsAg (V2) assay (Table 3.19). The number of supernates tested for each antigen ranged from 4 to 11 and the range of reactivity was relatively consistent at a level of 0.5-1.6 times the mean value. Only sample M5 produced a wider range of values; 0.45-2.2 times the mean value.
Transfection efficiency was also evaluated by repeating six of the seven assays using a separate batch of supernatants from a different transfection experiment. Only M5 in assay 1 (bioELISA) gave an obviously different reactivity on repeat testing; 44% of standard sequence activity compared to 13% in a previous experiment. Four other samples of low reactivity (one in assay 3, Arg145, and three in assay 7, Arg145, 1056P and BA2,4) became either borderline positive, having initially been negative, or became negative having initially been borderline positive. Clearly, this is not considered significant. In fact, the reactivity of these samples compared to that of the appropriate standard GlyY/D (set at 100%) ranged from 6-12.6%, a level at which repeat testing would be required in a clinical or diagnostic setting.

Table 3.18. Number of Variants Detected by each Assay at a Level of ≥10% of the Standard HBsAg (GlyY/GlyD).

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7
ay subtypes	5/5	5/5	2/5	2/5	4/5	4/5	2/5
ad subtypes	6/6	4/6	5/6	6/6	6/6	5/6	5/6
Number of variants detected	11/11	9/11	7/11	8/11	10/11	9/11	7/11
Capture antibody	Guinea pig pAb*	Guinea pig pAb	Mouse mAb	Mouse mAb†	Mouse mAb	Goat pAb	Sheep pAb
Detection antibody	Goat pAb	Human pAb	Mouse mAb	Mouse mAb	Goat pAb	Mouse mAb	Mouse mAb

pAb*: polyclonal antibody.

mAb†: monoclonal antibody.

Table 3.19: Expressed surface antigen results of multiple transfection experiments using the IMX HBsAg (V2) Assay.

Sample	Mean index of reactivity* ± s.e† (number of transfection experiments	Range of reactivity	Range as a multiple of mean
Gly Y	4.80 ± 0.49 (10)	3.26-7.81	0.68-1.63
1056Sp.	1.43 ± 0.25 (6)	0.64-2.20	0.45-1.54
BA2.4	0.73 ± 0.21 (5)	0.24-1.43	0.33-1.96
BA3.4	0.23 ± 0.041 (4)	0.13-0.33	0.57-1.43
SA7	5.30 ± 0.37 (5)	4.38-6.47	0.83-1.22
M5	0.95 ± 0.22 (8)	0.43-2.07	0.45-2.18
Gly D	4.79 ± 0.36 (8)	3.50-6.32	0.73-1.32
Arg145	5.40 ± 1.10 (9)	1.59-10.38	0.3-1.92
91-4696	4.72 ± 0.84 (7)	1.96-8.04	0.42-1.70
HK188	8.58 ± 1.07 (10)	4.72-14.04	0.55-1.64
AP3.1	6.57 ± 1.33 (6)	1.93-10.72	0.30-1.63
SA4	4.88 ± 0.98 (4)	2.44-7.16	0.50-1.47
SA6	3.91 ± 0.64 (4)	2.51-5.24	0.64-1.34
PJI‡	0.14 ± 0.014 (11)	0.08-0.27	0.57-1.93
COS7 cells‡	0.15 ± 0.014 (11)	0.09-0.25	0.60-1.67

*: Reactivity = OD of sample / OD of NIBSC standard serum

†: Standard error.

‡: Negative controls are supernatants from COS7 cells alone and cells transfected with plasmid pJ1 without cloned HBV surface gene.

To assess intra-test variation, four of the assays (assays 1, 5, 6 and 7) were repeated using the original supernatants. The reactivities of the variants were comparable with the original results with only one borderline positive sample becoming negative on repeat and one negative sample becoming borderline positive in assay 7.

Finally, the variant T5N, containing a 2 amino acid insertion and mutations in HBs regions 1, 2 and 4, was not detected by any of the assays either in the supernatant or the cell lysate. This either implies gross antigenic diversity or a lack of production. The original serum containing this variant was found to be negative by monoclonal antibody-based Auszyme assay, but positive by the polyclonal radioimmunoassay AUSRIA II (Abbott Laboratories Ltd, Maidenhead, UK) (Carman et al., 1995a). Perhaps the AUSRIA II positivity was due to higher viral load in serum. However, we cannot exclude the possibility (albeit unlikely) of there being differences in secondary structure of expressed HBsAg compared with the native HBsAg which would affect reactivity in diagnostic assays. To confirm whether the variant was adequately expressed, plasmid sequences upstream of the cloning site were found to be identical to those seen in the parent plasmid pJI, so expression of cloned HBsAg was unlikely to have been affected by changes in the vector sequence. On immunofluorescence, T5N showed only 5-10% of cells fluorescing which indicates some binding by pAbs and therefore some degree of expression; however, it remains possible that the antigen level in the supernatant was insufficient for detection. It is not clear why the T5N variant was not detected by any of the seven assays used. Interestingly, one group has detected it *in vitro* recently (Coleman et al., 1999).

3.4.3 Discussion

The main observation in this work is that all assays are not equally able to detect expressed HBsAg variants, mainly due to employment of anti-HBs antibodies with variable specificities and sensitivities against different HBsAg epitopes. However different levels of HBsAg expression, whether *in vivo* or *in vitro*, could also play a role. Obviously, in any analysis of this nature, standardisation of HBsAg particles is required, but this presented difficulties. Electron microscopy was attempted but the particles were difficult to count because of clumping and an uneven distribution. Also, Bradford assay for total protein

determination proved unhelpful as HBsAg was masked by large quantities of foetal calf serum. We have finally developed an epitope tag system to standardise the quantity of HBsAg particles in the supernatants independently of HBsAg antigenicity (see Chapter 3.5).

A mammalian expression system was used in the study because the availability of sera containing HBsAg variants is usually limited. Additionally, we assume there is little or no alteration in the secondary structure of expressed HBsAg compared to the natural material since all of the post-translational modifications should occur in COS7 cells. However, expressed HBsAg is not absolutely ideal for characterising variation as measured reactivity depends both on antigenicity and on the total amount of protein. Due to the transient nature of expression and relatively low numbers of cells the assay reactivities are lower than would be expected in serum.

In this cohort of samples we observed the following points. Firstly, the samples which displayed similar reactivity to the standard sequence had variation in regions 1, 3, 4 and 5, while those with reduced reactivity all had variation within HBs region 2 (1056sp, BA 3.4 and BA 2.4 in regions 1, 2 and 4; M5 in regions 1- 4; BA 3.2 in region 2; and T5N in regions 1, 2 and 4) (see Table 3.15 and Figure 1.9). This region, either solely or discontinuously with other regions, clearly contributes to the loss of reactivity. There is also evidence that the 4 amino acids bounded by cysteines at aa 121 and 124, HBs region 2, form a distinct epitope on the tip of a loop (Chen et al., 1996b; Qiu et al., 1996). Cysteines within the MHR are responsible for the formation of intra- and inter-molecular disulphide bridges that give the HBsAg its highly complex structure. Antigenicity of HBsAg is dependent upon this structure and substitution of many of the cysteine residues results in either reduced or complete loss of immunoreactivity (Ashton-Rickardt & Murray 1989; Mangold & Streek 1993; Mangold et al., 1995). Alternatively or additionally, it could be that there are secondary effects on other regions up- or down-stream from the mutations (Bruce & Murray 1995; Wallace & Carman 1997).

Secondly, it was clear that there is no correlation between the number of mutations across S gene and altered antigenicity. It seems to be the site and not the number that is responsible for this reduced reactivity. For example, Arg145 and BA3.4 had single mutations and displayed <50% of standard reactivity in five and four assays respectively. Samples SA4 and BA 2.4, each with two mutations, showed <50% of standard reactivity in five assays. However AP3.1, with one mutation, HK188 and 91-4696, with two mutations, and SA6, with

three mutations, showed good levels of reactivity, with <50% in either one or no assays (see Table 3.17).

Thirdly, some poor reactivity is due to reduced secretion from cells. A comparison between samples BA3.4, which contains T123N and BA3.2, which had the additional C124R is instructive. The loss of cysteine at aa124 completely abrogated reactivity in all seven assays when the supernatant was used. When the cell lysate was tested in assays 1, 5 and 6, it displayed reactivities of 28%, 18% and 15% of that of the GlyY respectively. Since BA 3.2 cell lysate is still detected at lower levels compared to BA3.4, we can conclude, supported by the data presented in Chapter 3.5, that addition of C124R and T123N has a dual effect on both immunoreactivity and secretion.

Fourthly, *in vitro* results do not always confirm *in vivo* observations. Supernatants from samples HK188 and 91-4696 (see Chapter 3.6; patient 1), which were initially HBsAg negative in serum, surprisingly displayed similar reactivity to the positive control samples. The failure to detect serum HBsAg may have been due to the presence of a low level of HBsAg or, perhaps, due to complexes between anti-HBs and HBsAg preventing the antigen from being detected (Ackerman et al., 1994). Fifthly, it has previously been suggested that *ay* subtype samples react less than those with an *ad* background (Wallace et al., 1994; Carman et al., 1997b). This also appeared to be the case here, however this needs confirmation using samples with the same mutations in both subtype backgrounds.

Finally, it is obvious that the ability of an assay to detect a variant depends critically on the choice of anti-HBs used. In general these samples were best detected by assays which employed pAbs in the capture and/or detection phases (with the exception of assay 7; Table 3.18). Assays that contained MAbs for both phases of the assay appeared to perform less efficiently in detecting this set of variants.

3.5 A novel epitope tag system to standardise HBsAg variant particles after in vitro expression

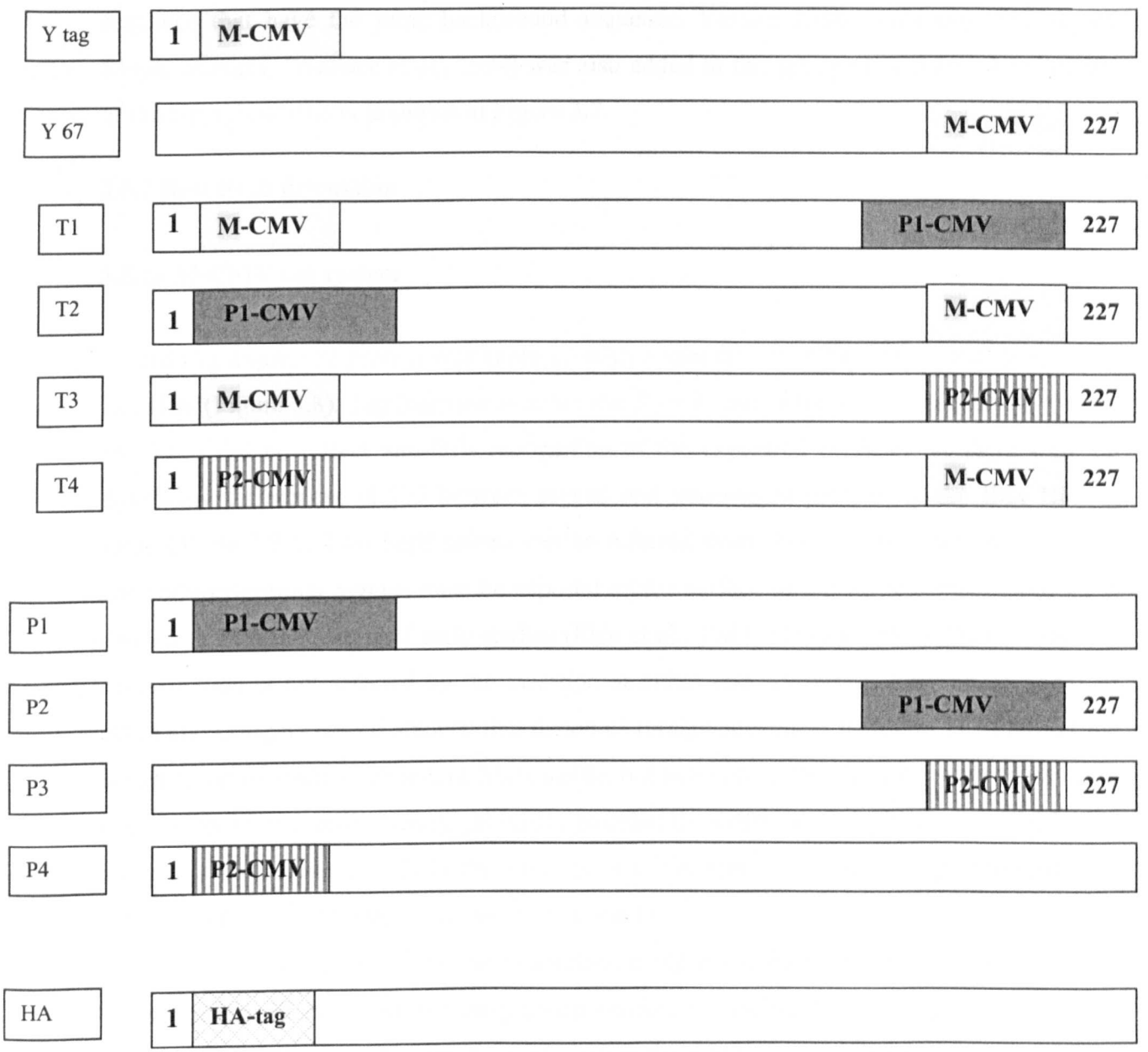
3.5.1 Introduction

The HBV genome is encapsidated along with a virus-encoded polymerase in a 32-nm diameter nucleocapsid surrounded by a host-derived lipid envelope bearing three viral surface proteins. These HBV surface proteins are translated from a single open reading frame of the viral genome using three different in-phase start codons. HBV surface proteins can be independently secreted from the infected hepatocytes as 22-nm diameter spherical or tubular non-infectious particles which are composed mainly of SHBs and host-derived lipids (Heermann et al., 1984; Peterson 1987).

Variants of the surface gene, which encodes HBsAg, are clinically relevant and can be detected in a number of situations (see Section 1.5.1). As sera containing HBsAg variants are rarely available in volumes sufficient for testing against a wide range of assays, *in-vitro* expressed supernatants of cloned HBsAg variants have been used instead in these assays to test their reactivity (Coleman et al., 1999; see Chapter 3.4). Reduced reactivity of such expressed variants could be due to either antigenic changes or reduced particle production. Consequently, standardisation of the number of expressed HBsAg particles is required before antigenic analysis. In a previous study, Chapter 3.4, both electron microscopy for counting particles and Bradford assay for total protein determination proved unhelpful. Such quantification has been recently attempted using Ausria II assay, which relies on polyclonal antibodies for both capture and detection, or the Hepanostika HBsAg Uni-form II plus which employs MAb for capture and PAb HRP-labelled anti-HBs for detection (Coleman et al., 1999; Cooreman et al., 1999). However, a conflict develops if particle number is assessed using methods that rely on antigenic recognition.

Here, an epitope tag system for standardising the number of particles, independent of HBsAg antigenicity, has been developed. Four epitopes, have been inserted stepwise into either or both termini of HBsAg (after the start codon and or before the stop codon): three were derived from cytomegalovirus (CMV) and the fourth was derived from influenza virus haemagglutinin (HA) protein (see Section 2.2.3).

Figure 3.7: Schematic figure of the tag constructs



1: HBsAg start codon; 227: HBsAg stop codon.

M-CMV: 10aa epitope derived from pp65 matrix protein; P1-CMV: 15aa epitope derived from N terminus of HCMV UL102 protein; P2-CMV: first 10aa of P1-CMV; HA-tag: 9 aa Flu-HA epitope.

M-CMV tag constructs included: 1) Y tag: M-CMV inserted at the 5'end of standard HBsAg (after the start codon); 2) Y 67: M-CMV inserted at the 3' end of standard HBsAg (before the stop codon).

M-CMV/P-CMV constructs included: 1) T1: 5'end M-CMV/ 3' end P1-CMV; 2) T2: 5' end P1-CMV 5'/ 3'end M-CMV; 3) T3: 5'end M-CMV/ 3' end P2-CMV; 4) T4: 5' end P2-CMV 5'/ 3'end M-CMV.

P-CMV tag constructs included: 1) P1: 5' end P1-CMV; 2) P2: 3' end P1-CMV; 3) P3: 5' end P2-CMV; 4) P4: 3' end P2-CMV.

HA-construct: HA-tag at the 5'end of HBsAg.

Variants used were the same panel that used in the previous study (chapter 3.4) except for T5N, due to unreliable results (see chapter 3.4), and HK188 due to unavailability of a standard sequence that have the same background sequence. Variant 2030, containing T127A and S143L changes, (Wallace et al., 1994) was also added to this group of samples. A schematic of the tagged constructs is shown in Figure 3.7.

3.5.2 Results & discussion

3.5.2a M-CMV tag system

HBsAg expressed protein was captured with a specific anti-CMV MAb and detected by anti-HBs (Figure 3.8). Tag insertion at either the 5' or 3' end of the HBsAg (Figure 3.7; Y tag and Y67) did not affect anti-HBs recognition of the expressed particles, as there were no significant differences in OD between tagged and non-tagged proteins in the IMx HBsAg assay (Table 3.20). Two implications can be inferred from these results. First, both amino- and carboxyterminal regions must be exposed on the surface of the particle; such topology is consistent with the results of early studies (Eble et al., 1987; Peterson 1987). Second, HBsAg conformation is not affected by the insertion at either end. However, early observations of Bruss and Ganem (1991a) showed that fusion of foreign sequences to codon 11 of pre-S2 led to formation of unstable chimeras. More stable, but non-secretable, chimeras were those fused with codon 11 of S gene. This might be due to either the difference in epitope size, 10-15 aa in our study versus 143-243 aa in the study of Bruss & Ganem, or the epitope insertion site, which was after the first codon rather than codon 11.

However, estimation of protein production using anti-HBs in this way is misleading as variants might affect the expression of group-specific determinants of HBsAg and most of the commercially used anti-HBs are directed against the major B cell epitope cluster of HBsAg between aa 124-147 (Brown et al., 1984; Waters et al., 1992a, b). For instance, Coleman et al. (1999) have recently quantified the *in-vitro* expressed HBsAg particles using the Ausria II, which depends on the hypothesised ability of polyclonal capture and detection to recognise a broad range of HBsAg variants. However, in our previous study, BA3.2 variant escaped detection and two other variants, SA4 and SA6, showed less than 10% reactivity with this assay (see Chapter 3.4).

Figure 3.8: Schematic figure showing the three tag systems used in this study

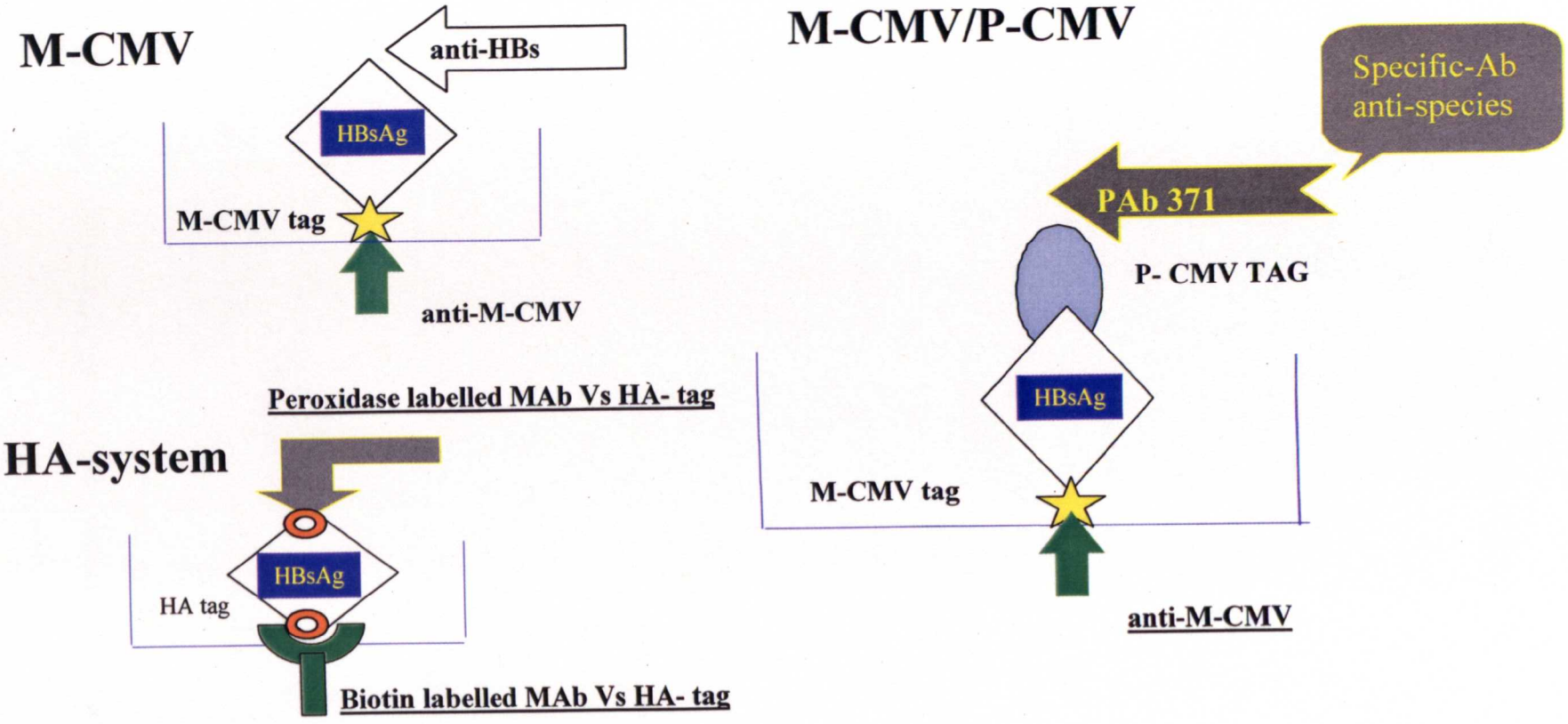


Table 3.20: IMX results* of M-CMV and P-CMV single tag systems and cell lysate results of dual tag system.

Sequence	Supernatant†	Lysate‡	Immunofluorescence§
Y tag	35.95	ND	++++
Y 67	40.48	ND	++++
P1	38.20	ND	++++
P2	39.75	ND	++++
P3	37.85	ND	++++
P4	40.25	ND	++++
T1	1.81	7.69	+
T2	5.69	37.16	++
T3	1.34	3.73	+
T4	1.55	7.42	+
Gly. D	43.45	49.11	++++
Gly. Y	40.25	47.50	++++
cells	1.36	2.00	-
PJI	1.36	1.82	-

*: All results in this table were obtained by the conventional $CaPo_4$ transfection method. Abbott IMx assay was employed to show that tag insertion did not significantly affect the antigenicity of HBsAg.

†: All supernatants were used as neat.

‡: In addition to the controls, (positive; Gly. D and Gly Y) and (negative; cells and PJI), lysates from transfected dual tag transfected cells were tested as the HBsAg was suspected to be retained within the cells.

§: The positive signals are reflecting the degree of fluoresence of each relative sequence. ++++: high; +++: moderate; ++: mild; +: minimal; -: negative.

Gly. Y and Gly. D are standard sequences of *adw* and *ayw* subtypes respectively and represent the positive controls.

Cells and PJI (plasmid containing no insert) represent the negative controls.

3.5.2b M-CMV/P-CMV system

To address the conflict between protein quantification and antigenicity of the M-CMV system, constructs were generated with different tags at either end, so that a sandwich ELISA could be designed. Either P1-CMV or P2-CMV, both recognised by a rabbit antiserum, was inserted into the 3' or 5' of the CMV-tagged protein at the opposite terminus (Figure 3.7; T1, T2, T3 and T4). Anti-CMV MAb was again used for capture, but instead of anti-HBs, PAb 371 (anti-P-CMV; see section 2.2.3) was used for detection (Figure 3.8).

However, these proteins were either intracellularly retained or poorly secreted (Table 3.20). As proteins with one M-CMV tag at either end have a normal biology, this effect should be due to a change of conformation, due either to tags being at both ends or to the P-CMV *per se*. Consequently, all four possible constructs (Figure 3.7; P1, P2, P3, P4) containing P-CMV only at either the 3' or 5' terminus were generated; all resulted in good signals in comparison to the non-tagged HBsAg in the IMx HBsAg assay (Table 3.20). Thus, inserting tags at both ends simultaneously was responsible for the system failure and the following effects on S protein structure could be responsible for this failure.

A direct effect on the neighbouring transmembrane helices and thus HBsAg assembly may have occurred, as interactions between S monomers during early assembly were shown to be grossly affected by the disposition of the N-terminus. Perhaps inserting both epitopes simultaneously at both ends led either to major displacement of nearby termini or to formation of unstable protein. Alternatively, the dual tag insertion might indirectly have deleterious effects on tertiary structure, due to displacement of important aggregation domains (Bruss & Ganem 1991a). Furthermore, if these tagged proteins overcame such impediments, they might be faced with a secretion challenge (see below).

HBsAg conformation is maintained by disulphide bridges and mutations of an essential cysteine (eg at aa, 48, 65, 69) can lead to intracellular retention (Prange et al., 1995). At least one of these cysteines seems to be essential for further oligomerisation, but none of them is required for dimerisation (Mangold and Streeck 1993). Dimers of the S protein are formed early in the ER, then sorted to a post-ER, pre-Golgi compartment where they are slowly converted into disulphide-linked oligomers (Huovila et al., 1992). The secretion-deficient variants that have the capacity for oligomerisation are most likely trapped in this intermediate

compartment and fail to extrude from the membrane (Prange et al., 1995). In our dual tag constructs, although the essential cysteines are not mutated, they still might form aberrant oligomeric structures due to shuffling of disulphide bonds which results from the misfolding or major displacement produced after dual tag insertion. Thus, aggregation and retention by the ER may then occur (Doms et al., 1993, Mangold et al., 1995).

All M-CMV/P-CMV dual constructs (except T2, which had a borderline positive signal) led to negative IMx HBsAg signals and showed minimal immunofluorescence in comparison to the standard sequence (Table 3.20). Consequently, cell lysates of all dual tag constructs in addition to the appropriate controls were tested for HBsAg reactivity using IMX HBsAg (V2) assay. Only T2 showed a significant signal, but repeat experiments were not consistent and the fluorescing cells looked abnormal where the low expressed tagged proteins stacked to the cell membrane rather than being located within the cytoplasm proper (Figure 3.9).

3.5.2c Flu HA tag system

To overcome these problems, another single tag system was developed. The HA tag (9 aa-epitope) was inserted at the 5' end of HBsAg (Figure 3.7; HA), although the M-CMV work indicated it could have been inserted at either terminus. The same MAb against the inserted tag was used for both capture and detection (but in different forms, biotin- and peroxidase-labelled: Figure 3.8). This modification unfortunately can not be applied to the first M-CMV tag system, as generating biotinylated or peroxidase-labelled anti-M-CMV was prohibitively expensive due to the price of anti-CMV antibody; 10mg/ml was required (100 vials: £110/ vial containing 100ug). However, the experience gained from this system allowed progress with the HA-tag system, as described below. An ELISA that recognises HA tag was then employed to standardise the amount of HBsAg particles after their expression. The sensitivity of this tag ELISA was confirmed by using decreasing concentrations of the positive control (a kind gift from Dr Ralph Gehrke). The lowest limit of detection was 50pg/ml (Figure 3.10). After standardisation (see Section 2.2.3), three commercial assays, IMX HBsAg (V2), Murex HBsAg GE14 and bioELISA HBsAg colour, were used to measure the HBsAg antigenicity.

Figure 3.9: Immunofuorescence pictures* of Cos7 cells transfected with different tag constructs.

M-CMV: this is included in Y tag and Y67 constructs.

P-CMV: this is included in P1, P2, P3 and P4.

Dual tag (M-CMV/P-CMV): these are included in T1, T2, T3 and T4.

HA-tag: this is included HA-tagged construct.

+ve control: cells transfected with plasmid containing Standard virus.

-ve control: cells transfected with plasmid only.

Pictures were taken by Nikon microphot-SA microscope (x400 magnification power)

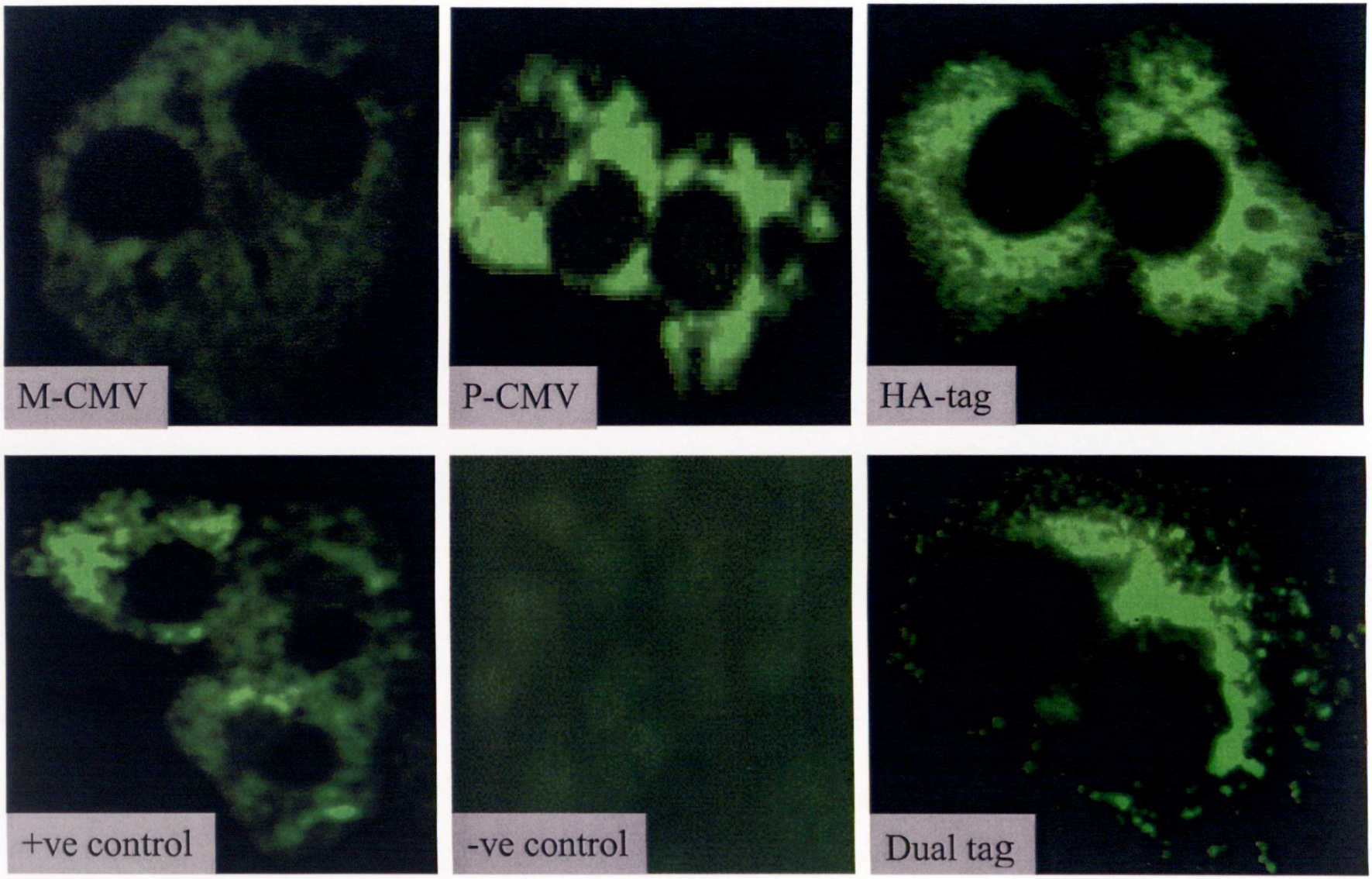
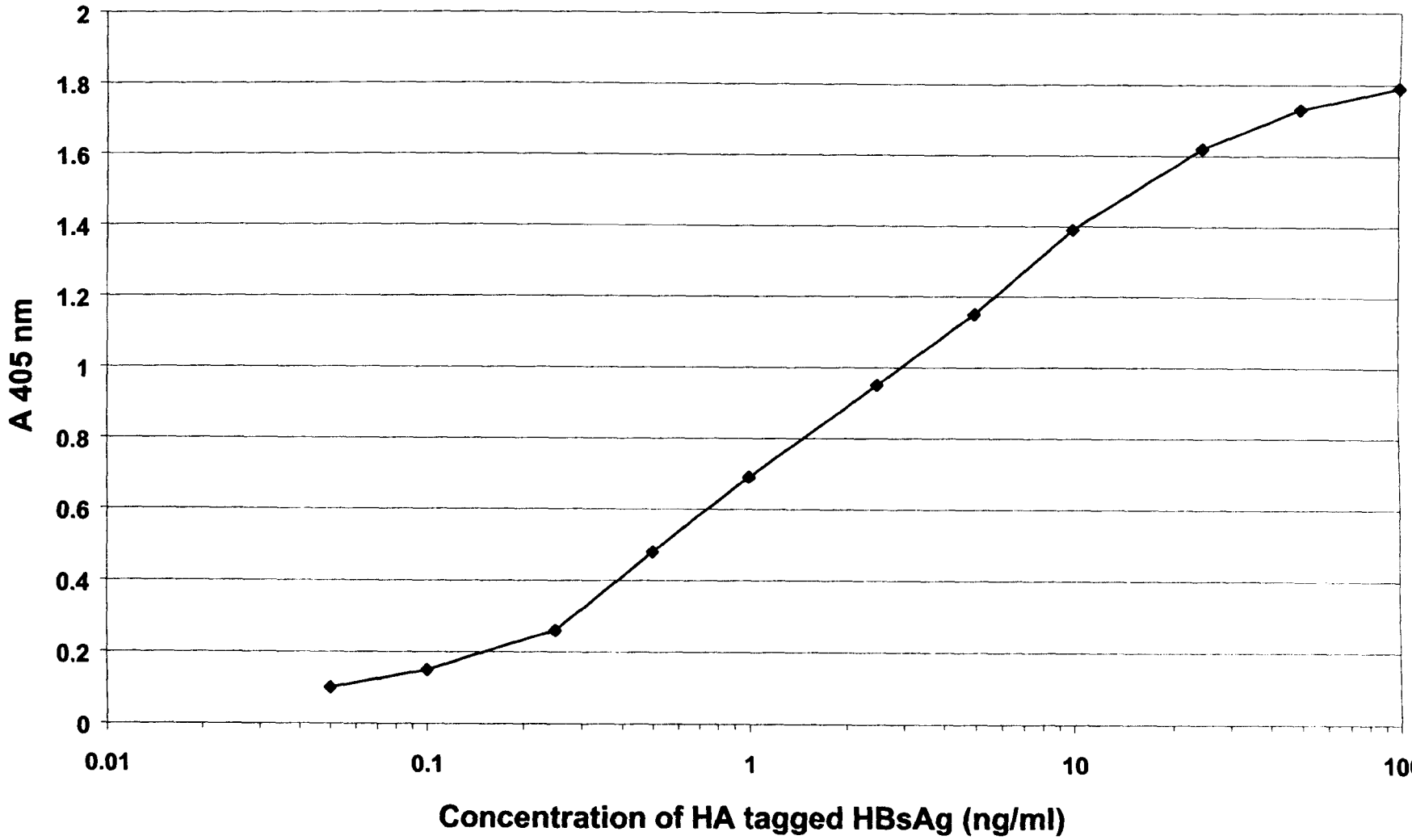

M-CMV
P-CMV
HA-tag
+ve control
-ve control
Dual tag

Figure 3.10 Sensitivity of the HA-tagged ELISA



Here, the subtype was taken into consideration (Norder et al., 1992a and 1993), as the backbone, either subtype *d* or *y* of the tested variant, has a role in HBsAg detection efficacy (Wallace et al., 1994, Carman et al., 1997b). Moreover, the HBsAg subtype can be affected by changes within the MHR (Ashton-Rickardt and Murray 1989). Therefore, the HA-Tag epitope was inserted into HBV DNA S gene of four standard sequences, one of each classical subtype, as well as 12 diagnostically important variants of different backbone subtypes (one *ayw1* (genotype A), four *ayw2*, two *ayw3* (genotype D), and five *adw2* (genotype A). The subtype of the backbone was as defined by Norder et al. (1993). Then, all the variants were compared to the standard sequence of that subtype.

ayw3 backbone variants

Subtype *ayw3* differs at least at two positions within the MHR from *ayw2,* yet both subtypes fall within genotype D (Norder et al., 1993). V1 and V8 have an *ayw3* backbone. After standardisation, V1 had 32%, 58% and 65% reactivity on IMX, Murex and bioElisa respectively. P120S and S143L, the variants found in V1, reduced the binding of monoclonal antibodies to the MHR (Wallace et al., 1994) and P120E escaped detection by two assays in another study (Coleman et al., 1999). Secretion efficiency was similar to the standard sequence, as the lysate/ supernatant (L/S) ratios for variant to standard sequence was 1.4/ 1.3 (Table 3.21; Figure 3.11). Clearly, the main explanation for poor detection of this variant is an antigenic effect.

On the other hand, V8, which has T127A and S143L, had similar reactivity to standard sequence after standardisation and there was no impact on secretion efficiency (ratios 1.25/ 1.3: Table 3.21). This is in agreement with other studies that showed both the site and nature of amino acid substitutions are critical to antigenicity (Chiou et al., 1997; Cooreman et al., 1999; Chapter 3.4) and that combined mutations may have an unpredictable impact on altering HBsAg conformational structure. Thus, P120S, solely or discontinuously with S143L, contributes the deleterious effect on HBsAg reactivity in this subtype backbone.

ayw2 backbone variants

V3, V5, V6 and V11 are included in this group. V5 (Y100C and P120T) reacted well after standardisation in the three assays (70%, 95% and 90%) (Table 3.22). The L/S ratios were 1.6/1.2 (Table 3.21; Figure 3.11), indicating a significant effect on secretion. This can be further supported from the results showed in chapter 3.4, whereas this variant (V5; BA2.4) revealed a lower reactivity in IMX HBsAg and Murex assays (see Table 3.17), 15.9% and 75.4 instead of 70% and 95% respectively, before being standardised. But, V1 (P120S and S143L) like V5, which contains a variant at aa 120, was not detected as well as V5, perhaps due to the different backbone or the different site and nature of the additional substitutions (Mangold et al., 1995; Chiou et al., 1997).

V3, which had 12 aa changes, had a dual effect. The L/S ratios were 2.06/ 1.20 (variant/ standard sequence) indicating a secretion defect. After standardisation, the variant was only detected at 50-55% compared to standard sequence or even escaped detection. Consistent with this view, IMX HBsAg and bioElsia assays in chapter 3.4 showed low reactivity signals for this variant (M5), 20% and 44.9% respectively (see Table 3.17). After the standardisation the variant was still detected at low levels, 50 and 55% respectively, or even escaped detection (Table 3.22).

V6 and V11 have T123N, but V6 has C124R in addition. Both variants showed negative or borderline positive reactivity in IMX HBsAg assay, even after standardisation. V11 could be detected with Murex and bioElisa at 45% and 71% respectively, but V6 always escaped detection (Tables 3.22). This poor reactivity can be partially attributed to reduced secretion, as L/S ratios were 2.85 and 2.45 respectively versus 1.20 for the standard sequence. In contrast, a double non-natural mutant, C121/124A, has previously shown secretion with wild-type efficiency (Mangold and Streeck 1993), perhaps due to the different substitution (Mangold et al., 1995; Chiou et al., 1997).

None of the HBsAg assays detected V6 after standardisation (Table 3.22) despite it giving a reasonable signal in the tag ELISA (more than five fold of the cut-off value), indicating significantly reduced antigenicity. Loss of cysteine at aa 124 has been previously shown to strongly reduce or even abrogate the reactivity of HBsAg (Mangold et al., 1995; Chapter 3.4).

Table 3.21: HA tag-based ELISA: results of HA-tagged HBsAg variants

Variant	Supernatant*	Lysate†	L/S ratio	P value‡	Immunofluores.
Vyw3 (ayw3)	**1.297**	**1.690**	**1.303**		+++++
V1 (1056SP)	1.225	1.715	1.40	0.584	+++
V8 (2030)	1.345	1.683	1.251	0.7	+++
Vyw2 (ayw2)	**1.321**	**1.590**	**1.203**		+++++
V3 (M5)	0.648	1.341	2.069	0.006	+++
V5 (BA2.4)	0.936	1.567	1.674	0.044	+++
V6 (BA3.2)	0.452	1.289	2.851	0.000	++
V11 (BA3.4)	0.589	1.445	2.453	0.002	++
Vyw1 (ayw1)	**1.351**	**1.709**	**1.264**		+++++
V13 (SA7)	1.419	1.765	1.243	0.904	+++++
Vdw2 (adw2)	**1.387**	**1.670**	**1.204**		+++++
V2 (145R)	1.485	1.695	1.141	0.675	+++++
V4 (SA6)	0.692	1.293	1.868	0.015	+++
V9 (91-4696)	1.637	1.848	1.128	0.620	+++++
V10 (AP3.1)	1.249	1.569	1.256	0.759	+++
V12 (SA4)	0.858	1.452	1.692	0.039	+++
-ve1	0.018	0.035			
-ve2	0.021	0.039			
-ve3	0.024	0.042			
Cut-off	0.071	0.089			

*: The supernatant of tagged variants was used neat.

†: The cell lysate was collected in 300ul per plate (60mm plates).

‡: P value of the difference in L/S ratios between tagged variants and their cognate standard were calculated; values less than 0.05 are considered statistically significant.

-ve 1: no capture Ab; -ve 2: no or different tagged construct; -ve 3: no detection Ab.

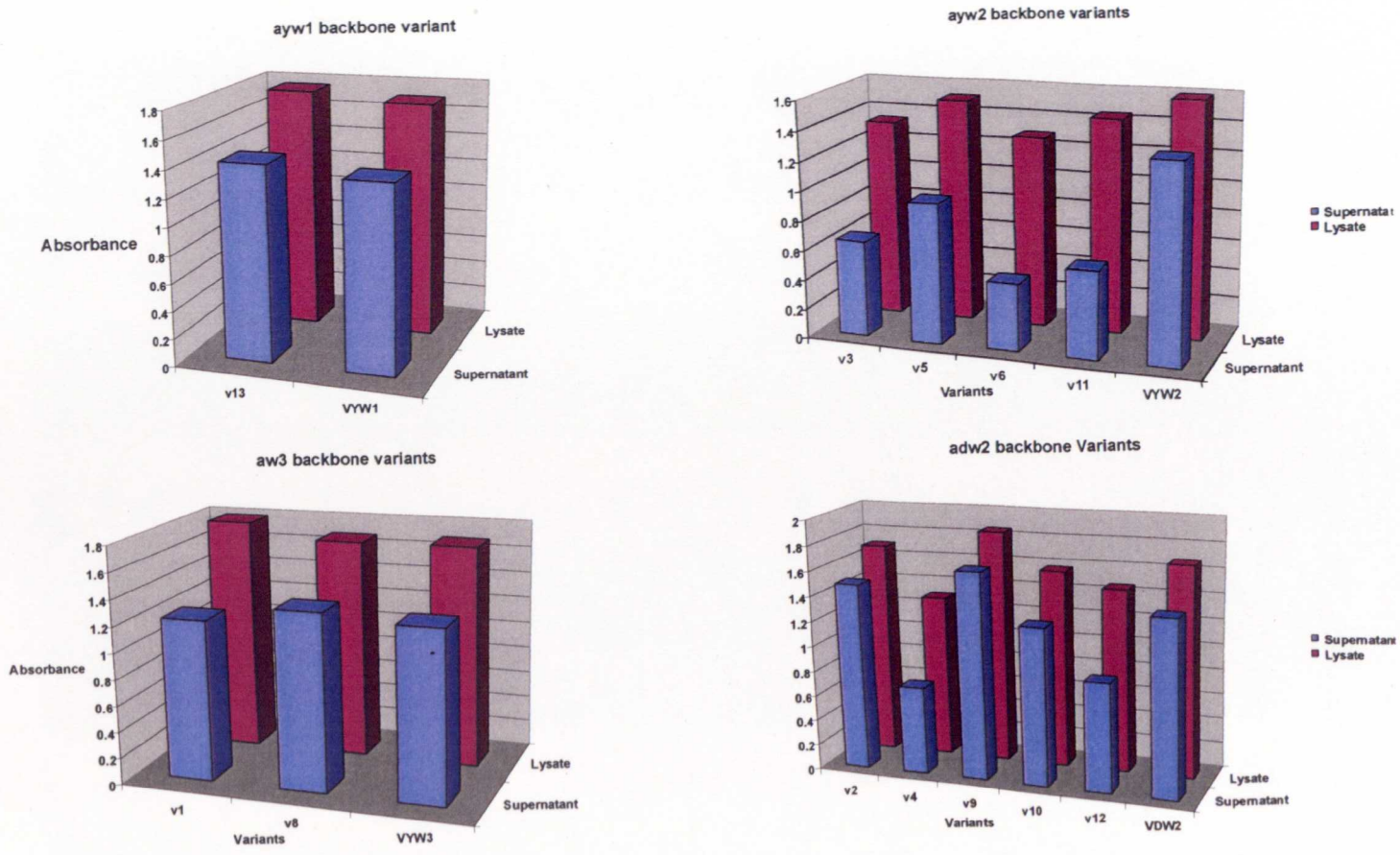


Figure 3.11: Chart showing the Lysate/ Supernatant ratios of HA-tagged variants (V1- V13) and their cognate subtype standard Vyw1, Vyw2, Vyw3 and Vdw2

Table 3.22: ELISA results of three commercial assays after the standardisation

Sequence	Vol/Conc*	IMX HBsAg	POR†	Murex	POR	bioELISA	POR
vyw3	30ul/Neat	78.90	100%	2.900	100%	2.600	100%
V1	30ul/Neat	25.30	32%	1.690	58%	1.700	65%
V8	28ul/Neat	57.94	73%	2.585	89%	2.320	89%
vyw2	30ul/Neat	80.75	100%	2.950	100%	2.650	100%
V3	50ul/2.5X	40.60	50%	0.116	NA	1.470	55%
V5	50ul/Neat	56.70	70%	2.795	95%	2.339	90%
V6	50ul/2.8X	1.73	NA	0.070	NA	0.132	NA
V11	50ul/2.8X	2.56	3%	1.340	45%	1.890	71%
vyw1	28ul/Neat	82.95	100%	2.941	100%	2.747	100%
V13	28ul/Neat	80.82	97%	2.985	101%	2.290	83%
vdw2	28ul/Neat	83.65	100%	2.992	100%	2.796	100%
V2	21ul/Neat	45.75	55%	0.093	NA	1.85	66%
V4	50ul/2.2X	83.50	100%	2.980	100%	2.745	98%
V9	14ul/Neat	65.55	78%	2.989	100%	2.628	94%
V10	28ul/Neat	56.20	67%	1.85	62%	2.05	73%
V12	50ul/2.3X	65.96	79%	2.398	80%	1.870	67%
Neg	50ul/Neat	<2.00		0.072		0.105	
WS	50ul/Neat	8.20		0.420		0.320	
Cut-off		2.00		0.122		0.145	

* : Volumes and Concentrations that are used after standardisation. All volumes made up to 50ul with phosphate buffered saline (PBS).

POR†: percentage of reactivity; rounded to remove the decimal figures.

NA: not applicable; Neg: negative control.

WS: a working serum standard of 0.5 IU/ml concentration (NIBSC) has been used in all assays.

ayw1 backbone variant

V13 (M133T) was the only variant studied in this group. Reactivities were similar to those of the standard control: 97%, 101% and 83%. The L/S ratios were also similar, (1.24/ 1.26) (Table 3.21). This is in agreement with a recent study, where only one out of 28 MAb showed reduced reactivity with this variant (Cooreman et al., 1999). Moreover, M133L was detected with equivalent signals to the standard antigen in nine commercial assays (Coleman et al., 1999). Thus, M133T change in this subtype backbone has little, or no, effect on HBsAg secretion or reactivity.

adw2 backbone variants

The five variants that had this backbone can be divided into two subgroups according to the site of aa changes: the first comprises V4 and V12; the second V2, V9 and V10.

V4 (Q129R, G130N and A166V), was the best example of reduced HBsAg reactivity being solely due to poor secretion as the L/S ratio was significantly higher in comparison to that of the standard, 1.86/ 1.20, (Table 3.21). After standardisation, HBsAg reactivity was 100% of the standard (Table 3.22). Results from this variant (SA6) in chapter 3.4 (see Table 3.17), are consistent with this view. This combination of aa changes was therefore tolerable in terms of the antigenic structure.

V12 (M133T and Y161F), although nearly affecting the same region of HBsAg as the V4, had a potential effect on antigenicity. The variant L/S ratio was also significantly higher than the standard L/S ratio: 1.69 compared to 1.20 (Table 3.21). After standardisation, none of the assays detected V12 with a similar sensitivity to that of standard HBsAg, indicating this low reactivity cannot be explained only by poor secretion. Considering that V13, although it has a different backbone, also had M133T, the potential effect on HBsAg antigenicity is likely to be due to the variant at 161, either solely or discontinuously with M133T.

Reactivity for two variants, V2 (G145R) and V9 (S113T/ T143S) was significantly higher in the tag ELISA than those for the standard; hence, they had to be tested at lower concentrations (75% and 50% respectively) in the standardisation assay. Initial results in chapter 3.4, showed that V9; 91-4696 variant was well recognised in most of the assays (see

Table 3.17), however, V2; Arg145, due to its major effect on the "a" determinant, revealed low reactivity in most of the assays or even escaped detection as in Murex assay. Their L/S ratios were not significantly different from the standard, 1.14 and 1.12 versus 1.20.

V9, with S113T and T143S, had a reactivity similar to the standard HBsAg after standardisation (Table 3.22). T143S has been observed previously in two *adw2* isolates from France. Two additional *adw* isolates, one from Japan and the other from Indonesia, had S113T (Norder et al., 1993). It appears that S113T and T143S can be tolerated by that subtype and consequently, have no significant effect on HBsAg antigenicity or secretion. Although V1 and V8 also have a variant at aa 143, the backbone is different (*ayw3*), and there were additional associated mutations at aa 120 and 127 respectively. On the other hand, both V2 and V10 were shown to have a major impact on HBsAg antigenicity, but normal secretion (L/S ratios, 1.14 and 1.25 versus 1.2 respectively) (Table 3.21). After standardisation, they showed low reactivity that did not exceed 66% and 73% of the standard.

Does this simply indicate that V2 and V9 variants were overproduced in the transient expression system used here, or might it be that there is additional selection? If the latter, the implication is serious (see Chapter 4), especially for V2 containing G145R, which escapes detection and can be selected from a previous variant in vaccinees (Carman et al., 1990). If these mutations become fixed, they may become further propagated (Blum, 1993).

In summary, single tag epitopes of up to 15 aa can be inserted at either end of HBsAg protein without affecting HBsAg reactivity. However, insertion at both ends led to a major impact on HBsAg conformation and hence its antigenicity. Furthermore, variants used in this study were found to have different strategies in affecting HBsAg reactivity. V1, V2 and V10 were the only class that showed a pure effect on altering HBsAg structure and hence its antigenicity, while a combined effect due to inefficient secretion and altering HBsAg structure was found to be responsible in other group containing V3, V6, V11 and V12. The effect on antigenicity was dominant in V3 and V6. On the other hand, the reduced reactivity of V4 and V5 was mainly due to poor secretion.

3.6 Reappearance of hepatitis B surface antigen: reinfection or reactivation ?

3.6.1 Introduction

HBsAg is frequently used as the primary marker of current hepatitis B virus infection, and thus infectivity. However, there are reports of serologically negative patients, both with and without disease, who have HBV DNA in serum, liver and/or mononuclear cells (Brechöt et al., 1985; Preisler-Adams et al., 1993; Cabrerizo et al., 2000). It is also well known that people who are positive for anti-HBc alone can be infectious (Larsen et al., 1990; Luo et al., 1991; Grob et al., 2000). Some of these people were described to be infected with variants of HBsAg that are not detectable in conventional assays (Carman et al., 1997b; Grethe et al., 1998). As both variants and standard viruses can be detected using nucleic acid amplification methods such as PCR, HBsAg can no longer be considered to be the "gold standard" marker of current infection (van Deursen et al., 1998).

Second episodes of hepatitis B, defined by the reappearance of HBsAg or HBV DNA in serum, are well described (Maeland et al., 1989; Gilson et al., 1989; Martin et al., 1995). A history of clinical recovery from acute hepatitis B followed by HBsAg loss may not always help in classifying a second episode of hepatitis B as reinfection, because persistent HBV infections occur in the absence of serological markers of active infection (Michalak et al., 1994; Yotsuyanagi et al., 1998). On the other hand, detection of low HBV DNA levels in such individuals may function as an immune trigger to stimulate long-lasting CTL responses (Rehermann et al., 1996; Penna et al., 1996). In addition, selected HBsAg variants can persist in the presence of anti-HBs (Asahina et al., 1996a; Kohno et al., 1996). Therefore, several tests may be required to differentiate between reinfection and reactivation as the cause for reappearance of HBsAg. Obviously, such differentiation would be beneficial to understand the pathogenesis during recurrence.

Although not as greatly informative as cloning for determining sequence changes, direct sequencing using PCR is reliable for monitoring HBV infection and assessing responses to antiviral therapies (Alexopoulou et al., 1997; Pawlotsky et al., 2000). Direct sequencing using PCR has been carried out successfully in several studies (for longitudinal and sequential analysis or tracing the route of infection); the HBV genome is mostly stable unless exposed to

host immune pressure (Zuckerman et al., 1995; Ashina et al., 1996a; Hannoun et al., 2000). For example, 4 point changes at the most were detected between samples taken over 5 years (Alexopoulou et al., 1997). Sequential analysis of HBV genomes before and after acute exacerbations associated with appearance of anti-HBe and anti-HBs showed that mutations were mainly located in the surface and pre-core/ core genes; at most, 15 nucleotide changes were detected across the entire genome (Ashina et al., 1996a). Similarly, about 20 mutations over a 20-35 year period were found across the whole genome on sequencing in an intrafamilial comparison study in HBeAg negative carriers (Hannoun et al., 2000).

Here, we describe five cases of second episodes of HBV activity that may have been reinfection or reactivation. The potential existence of HBsAg variants was also considered. Two continuously HBsAg-positive patients with fluctuations in their HBeAg status were used as a control group for serial sequence variation. We suggest molecular criteria to distinguish between these two possibilities. The criteria were: number of nucleotide substitutions; number of amino acid (aa) substitutions; situation of aa changes; phylogenetic relatedness, co-incidence of mutation with immune or antiviral therapy; and genotype/ subtype shifts. Using these criteria will not only reflect how far is the divergence between the sequential isolates from each patient but also will show if they were exposed to any immune pressure. For example, high proportion of non-silent changes, selection of important target eitopes as the site of aa change and usage of immune or antiviral therapy are more consistent with reactivation (Lok et al., 1991; Kato et al., 1996).

3.6.2 Results

3.6.2a PCR and sequencing results

Patient 1: HBV DNA was detected by PCR in samples 1991, 1996-a and 1996-b, but not in samples 1994-a and 1994-b (Table 3.23). The nucleotide and amino acid substitutions of PCR positive samples are shown in Table 3.28. There were 14 nucleotides changes between sequences 1991 and 1996-b, resulting in seven amino acid substitutions: F8Y, T113S, S143T, D144A, A194V, S207N and V209L. The percent similarity was 97.3% between amino acid sequences and both were genotype A. Nucleotide sequences remained the same throughout 1996 despite discrepant serology. There were two amino acid substitutions within the MHR in

sample 1991 as compared with the prototype sequence of the same subtype, S113T and T143S (*adw*) (Ono et al., 1983). Both have been reported in other *adw* sequences (Norder et al., 1993). There were also five amino acid substitutions in sample 1996-b as compared with the *adw* prototype : F8Y, D144A, A194V, S207N, and V209L.

Patient 2: Samples in 1987 and 1992 were both PCR positive (Table 3.24). The nucleotide and amino acid sequences revealed 3 nucleotide differences between sequences (Table 3.28), resulting in three amino acid substitutions: V96G, M103I, and K122R. The percent similarity was 99.1% between sequences. K122R is significant, being a subtype-specific change. The sequence in 1987 showed only one amino acid substitution (F219S) as compared with the *adw* prototype sequence, while the sequence in 1992 had 4 substitutions (V96G, M103I, K122R and F219S) in comparison with *adw*.

Patient 3: For the serological picture of 1987 and 1995 patient samples see Table 3.25. The nucleotide and amino acid sequences of the these two samples revealed 3 nucleotide differences resulting in three amino acid substitutions, Y161F, L193S and S210R (Table 3.28). The percent similarity was 99.1%, both sequences being genotype A. The sequence in 1987 showed 2 amino acid substitutions as compared with the *adw* prototype sequence (G44E and S193L) while the sequence in 1995 had 3 substitutions (G44E, Y161F and S210R) in comparison with *adw*.

Patient 4: Samples from 1989 and 1990 were PCR negative for both core and S genes, despite the patient being anti-HBc positive (Table 3.26). The S sequence of all positive PCR samples from 1991 and 1992 showed two amino acid substitutions, Q101H, D144E, as compared with the *ayw2* (genotype D) prototype sequence with the 1992 serum also having the additional change of S143L (Table 3.28). It should be noted that aa 143 and 144 are both within the immunodominant epitope of the HBsAg.

Patient 5: All samples were PCR negative for S gene except for the one from August 1997 (Table 3.27). The S sequence of this sample showed only one amino acid substitution as compared with the *adw* prototype sequence (E164V).

Table 3.23: HBV serology and PCR results for patient 1.

Date	Sample No	HBsAg	anti-HBs	anti-HBc	HBeAg	HBV DNA	PCR*
1990		-	+	+	ND	-	NA
1991		-	+ (41.2)	+	ND	-	+
1993		-	+ (27.7)	+	ND	-	NA
1994	1994-a	-	+ (40.8)	+	ND	ND	-
1994	1994-b	-	+ (59.7)	+	ND	-	-
1995		-	+ (68.5)	+	ND	ND	NA
1996	1996-a	-	+ (43.4)	+	ND	-	+
1996	1996-b	+	-	+	+	-	+
1996		+	-	+	ND	ND	NA

Table 3.24: HBV serology and PCR results for patient 2.

Date	HBsAg	anti-HBs	anti-HBc	HBeAg	anti-HBe	PCR*
1985	-	-	-	ND	ND	NA
1986	+	-	ND	+	-	NA
1987	+	-	ND	-	-	+
1988	-	-	+	ND	ND	NA
1992	+	-	ND	+	-	+
1994	+	-	+	+	ND	NA

Patient 1 has no vaccination history.
HBsAg, anti-HBs, anti-HBc, anti-HBe and HBeAg were tested by Abbott IMX.
HBV DNA was tested by the Digene HBV (Murex, Dartford, UK) assay.
Figures in brackets are in mIU/ml
NA, serum depleted; ND, not done
-: negative; +: positive
*: surface gene

Table 3.25: HBV serology and PCR results for patient 3.

Date	HBsAg		anti-HBc		HBeAg	anti-HBe	PCR *
	RIA	CIE	IgG	IgM			
1984	+	+	+	-	-	-	NA
1987	+	-	ND	ND	ND	+	+
1995	+	ND	+	+	-	ND	+

Table 3.26: HBV serology and PCR results for patient 4.

Date	HBsAg	anti-HBc		HBeAg	anti-HBs	PCR*
		IgG	IgM			
10/1989	-	+	ND	ND	(49)	- †
04/1990	-	+	ND	ND	(9)	- †
04/1991	-	+	ND	ND	(2)	+
10/1991	+	+	-	+	(1)	+
04/1992	ND	ND	±	+	ND	+
12/1992	ND	ND	-	+	ND	+

Table 3.27: HBV serology and PCR results for patient 5.

Date	HBsAg	anti-HBc	anti-HBs	PCR*
04/1995	-	+	ND	-
04/1995	-	+	ND	-
08/1996	-	+	(8)	-
12/1996	-	+	(11)	-
01/1997	-	+	(11)	-
08/1997‡	+	+	ND	+
06/1998	-	+	ND	-

HBsAg, anti-HBs, anti-HBc, anti-HBe and HBeAg were assayed by Abbott IMX.
RIA: radioimmunoassay; CIE: counterimmune electrophoresis
*: surface gene; †: core gene
‡: August 1997 sample was HBeAg and anti-HBcIgM positive
Figures in brackets are in mIU/ml
NA: serum depleted; ND: not done.
-: negative; +: positive; ±: borderline.

Table 3.28: Nucleotide and amino acid mutations following second episode of hepatitis B

Patient	Nucleotide// amino acid substitutions
Patient 1 (adw/ A)* N // aa change (from 1991 to 1996)	T23A// F8Y, A131C// S, G282A// S, A337T// T113S, C339A// S, T351C// S, A360C// S, T427C// S143T, A431A// D144A, G462A// S, C581T// A194V, G620A// S207N, G625T// V209L, C666T// S.
Patient 2 (adw/ A) N // aa change (from 1987 to 1992)	T287G// V96G, G309A// M103I, A365G// K122R.
Patient 3 (adw/ A) N // aa change (from 1987 to 1995)	A482T// Y161F, T578C// L193S, T630G// S210R
Patient 4 (ayw2/ D) N // aa change (from standard seq.)	A303C// Q101H, C428T// S143L†, C432A// D144E.
Patient 5 (adw/ A) N // aa change (from standard seq.)	A491T// E164V.

*: subtype and genotype.

N // aa change: the convention chosen to describe the mutation is the same as in table (3.15)

†: only in sample of 1992

//S: silent

Control group (two patients): Both isolates had subtype *ayw2* (genotype D) and showed nearly identical S gene sequence from all available samples for each patient (over 3 and 5 years respectively). Compared to the prototype *ayw2* sequence, patient 6 was identical, while patient 7 had two silent mutations at nucleotides 465 (C/T) and 660 (T/C).

3.6.2b Phylogenetic Analyses

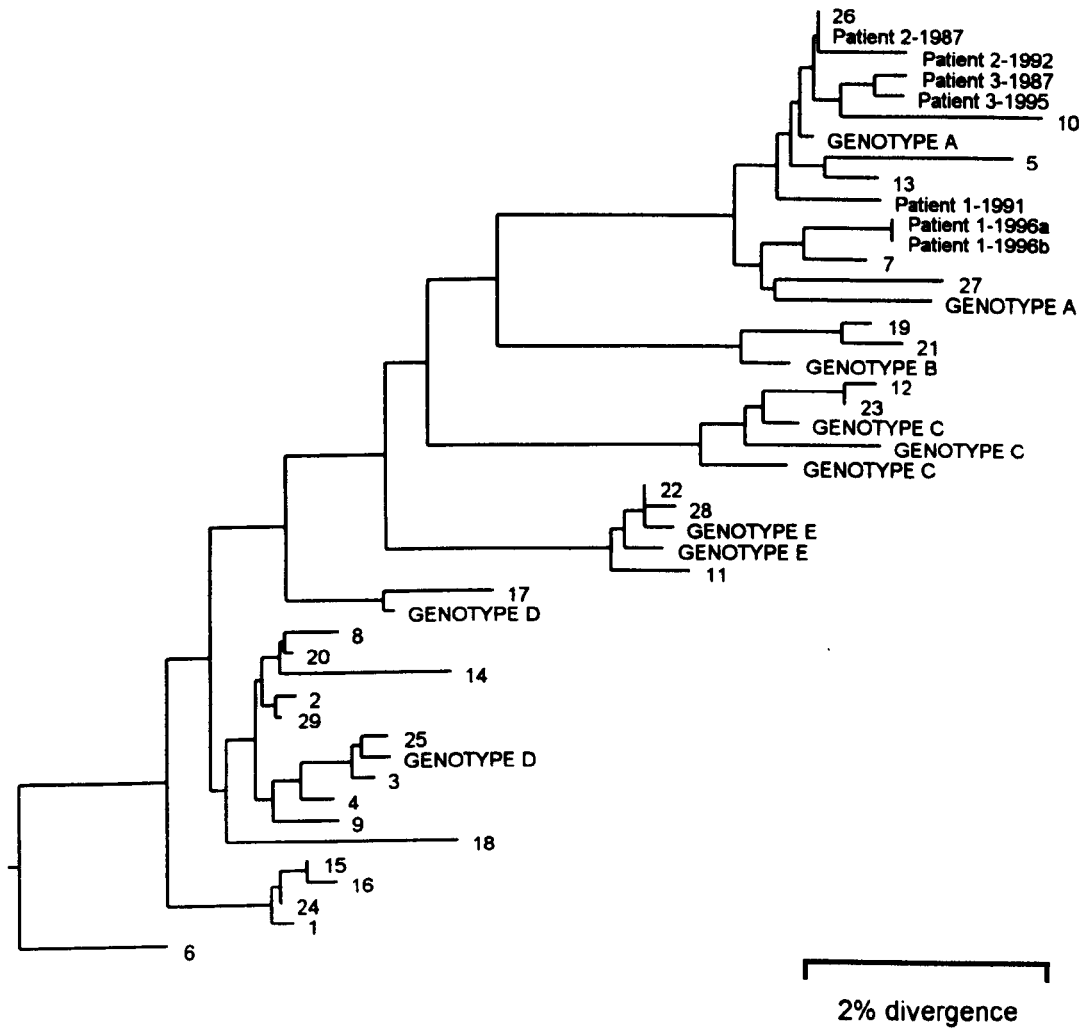
The phylogenetic analysis was performed on a 477-bp fragment of the S gene bracketing nucleotides 82-558 using sequences obtained from Genbank and sequences from within the UK. As only the sequence for the second episode is available for patient 4 and 5, they are not included (Figure 3.12). Isolated samples from patient 1 were greatly different, while those from patients 2 and 3 were highly related.

3.6.3 Discussion

HBsAg may reappear after becoming undetectable. Such a progression of events was found in patients 1, 2, 4 and 5. There are three possible explanations for this: maintenance of low level virus replication or "latency" of the virus followed by reactivation, emergence of an HBsAg antigenic variant, or reinfection. That these patients can be positive for HBV DNA by PCR concurrent with being negative for serum HBsAg demonstrates the value of PCR as a confirmatory test for current infection (van Deursen et al., 1998). Low levels of replication and significant variability due to selection of minor populations of the existing strains in subsequent sera were usually seen in anti-HBe positive subjects (Wright & Lau 1993; Alexopoulou et al., 1997). As all patients in this study were HBeAg positive, except patient 3 who was anti-HBe positive in 1987, the probability that their sera may contain heterogenous HBV populations is low. If these patients even had such assumed minor HBV strains, clinical history showed that none of them have been exposed to induced immune pressure.

Patient 1 may have selected an escape mutant, because there were two amino acid substitutions, S113T and T143S, within the MHR in the 1991 sample. Infection by an escape mutant would be compatible with the reappearance of HBsAg following a period of anti-HBs positivity. However, this variant (91-4696) surprisingly displayed similar reactivity to the positive control samples on antigenic analysis (see Chapter 3.4). The reason that this patient tested negative for HBsAg in 1991 may have been that the level of variant HBsAg present in the serum was below that detectable by the kit. There was also a discrepancy in HBV serology between the two 1996 samples in spite of their identical esquences.

Figure 3.12: N-J Phylogenetic tree of patients 1, 2, 3.



This is best explained by observations that the reactivity of variant HBsAg is determined not only by absolute (i.e. plus/minus) binding between anti-HBs in the kit and HBsAg, but also by the ability of the kit to detect low levels of variant antigen (van Deursen et al., 1998). This interpretation is supported by 1996-b sample being positive for HBeAg as well as HBsAg. Furthermore, there is long-standing evidence that previous infection with HBV and subsequent development of anti-HBs does not completely protect against later infection by a virus of a different HBsAg subtype (Koziol et al., 1976; Foutch et al., 1983).

Thus, the reappearance of HBsAg in 1996 is more likely to be due to reinfection than reactivation (Table 3.29). First, this patient received hemodialysis and was at risk of reinfection from other hemodialysis patients. Second, HBV DNA was not detected by PCR between 1991 and 1996. Third, 50% of the 14 nucleotide changes were silent, a high percentage that is unlikely to be due to escape from immune pressure. Fourth, amino acid subsitutions seen in 1996 were scattered throughout the S sequence, not concentrated within the MHR. Fifth, the phylogenetic distance between the two sequences isolated in 1991 and 1996 support reinfection rather than reactivation.

The K122R change seen in the second hepatitis episode in patient 2 appears to be more compatible with reactivation rather than reinfection although similar cases associated with HIV infection have been reported where reinfection with a different subtype presumably occurred (Maeland et al., 1989). This is because firstly, we have to differentiate between a different subtype and just a change at one of the subtype-identifying positions, which is the situation here. Secondly, the co-infection by different subtypic clones generated by point mutations, or dual infection of viruses with different subtypes, was also demonstrated (Yamanaka et al., 1990). Those observations implied that HBsAg with a different subtype-specific change might be detected in a second episode of hepatitis B as a result of reactivation. Moreover, only three nucleotide changes occurred in the whole S gene, all of which resulted in amino acid substitutions; which are more consistent with reactivation rather than reinfection (Table 3.29). Finally, the HBV sequence in 1992 was related to the sequence in 1987 by phylogenetic analysis. Therefore, we believe this second episode is most likely to be due to reactivation.

Anti-HBc IgM is well known to become detectable not only in acute hepatitis but also in persistent HBV infection in response to reactivation or exacerbation. In patient 3, sequences in

1987 and 1995 had a very close phylogenetic distance and all nucleotide changes in 1995, only three, resulted in amino acid substitutions. From these results, the second episode in patient 3 is likely to be due to reactivation.

Patient 4 and 5 represents a different situation from the other three cases because of the lack of early sequence; consequently, we were not able to classify them as reinfection or reactivation according to our proposed criteria. Nevertheless, the abnormal serology was accompanied in both patients, as well as the first three patients, by detection of variant sequences which shows the importance of variants in this situation (see Section 4.7). Thus, of the three analysable cases described here, we believe that the second episodes of hepatitis B were due to reinfection in one and reactivation in two and we suggest that our proposed criteria should be taken into consideration for the molecular differentiation between reinfection and reactivation.

Although chapter 3.6 was the earliest piece of work in this thesis, attempts to get sufficient samples that have the same criteria (reappearance of HBsAg) were not successful. Therefore, faithful differentiation between both re-infection and reactivation, although potentially achievable, was quite difficult. Larger number of patients would be helpful to tightening up these criteria and increasing the confidence in the interpretations.

Table 3.29: Proposed criteria for differentiation of second episodes of hepatitis B.

Criteria	Patient 1	Patient 2	Patient 3
Subtype/genotype change	–	+	–
Phylogenetic analysis	Different	related	Closely related
Number of nucleotide changes	14	3	3
Number of amino acid changes	7	3	3
Site of amino acid substitutions in S gene	Scattered	MHR*	Scattered
Therapy (immune or antiviral)	None given	None given	None given

Patients 4 and 5 are not included because of the unavailability of earlier sequences

MHR*: major hydrophilic region; –: negative; +: positive.

CHAPTER 4 DISCUSSION

4.1 Low and different seroconversion rates among the Pacific islands on vaccination

Using the same vaccine in Indonesia, a 68- 77% seroconversion rate was achieved by Ruff et al., (1995). Seroconversion rates in Fiji and Vanuatu were similar to that detected in Indonesia while those in Tonga and Kiribati were 20 to 30% less. On the other hand, higher seroconversion rates were seen in the Gambia, Alaska and Taiwan (Whittle et al., 1991; Tsen et al., 1991; Fortuin et al., 1993; Wainwright et al., 1997). This discrepancy could be due to the following possibilities: incompatibility of the vaccine strain with the circulating strains in the Pacific region; poor quality of provided health services such as vaccine storage, handling and administration; or geographical and genetic background differences. (Ruff et al., 1995; Streffland et al., 1999; Karthigesu et al., 1999; McDermott et al., 1999).

It appears important that the vaccine strain (s) should be of similar antigenicity to the prevalent strains in the target populations. There is long-standing evidence that previous infection with HBV and subsequent development of anti-HBs does not completely protect against later infection by a virus of a different HBsAg subtype (Koziol et al., 1976; Foutch et al., 1983). Kohno et al. (1996) showed that *adw*-specific antibodies and HBsAg of *adr* subtype, which do not have any mutations that affect MHR or HBsAg antigenicity, co-existed in the serum of one patient. Further analysis revealed that these adw-specific antibodies were not able to agglutinate cells coated with HBsAg of *adr* subtype. Also, there have been similar concerns raised in some Pacific countries regarding the immunogenicity of HBV vaccine (Milne et al., 1995). The same issue has been raised in the Gambia, where a major difference in antigenicity was found between the prevalent subtype, *ayw4*, and the plasma-derived HBsAg subtype, being *adw* (Karthigesu et al., 1999). Furthermore, 5 out 12 MAbs raised against natural HBsAg/*ayw2* were predominantly subtype specific and did not react with *adw2* subtype (Sobotta et al., 2000).

As regards the quality of health services, significant differences in seroconversion rates were observed among children served by different health centers in different villages and districts as has been reported in the Gambia and Venezuela (Hadler et al., 1989; Fortuin et al., 1993). Similar results were observed in our study between the Pacific islands. An example that may reflect the importance of the role of the health services is the discrepancy between two child/ mother pairs from different cities in Vanuatu (C64/M74 from Vila central and C37/M75

from Mele-Maat). Of these two pairs, both mothers were HBeAg positive and had similar HBsAg sequences, however, the first child (C64) seems protected (anti-HBs >100 mIU/ml) while the other had no detectable anti-HBs and became infected (see Tables 3.4 and 3.8).

Although this is the simplest explanation for this striking difference, it also seems the most likely if we assume that other variable parameters are absent as they are from the same island which has very little immigration. Ruff et al. (1995) showed that protection was better if the first vaccine dose was given within the first week after birth than later (the seroconversion rate was 77% versus 68% and HBsAg prevalence was 1.4% versus 3.0%). Additionally, the importance of general preventive non-immunogenic measures cannot be ignored.

Genetic modulation of immune responses to vaccination also has a potentially major role. The inflammatory process that results from intra-muscular injection of vaccine will result in high levels of local IL-1 and IL-12 production by macrophages. These mediators have the potential to recruit more inflammatory cells and to activate those cells to express effector functions. The antigen would then be presented to an MHC class II molecule of an antigen presenting cell (APC) (Steinman, 1991). The HLA class II antigen presenting cells will interact with, and activate, specific T-cells in specialised regions of the lymph node which then migrate to the B-cell follicles to provide help via cytokines for the production of anti-HBs antibody (Goodnow, 1997). According to this model, low doses of vaccine may be inadequate to induce the inflammatory response, due to personal or racial variation resulting from different HLA haplotypes; larger amounts of the antigen may be required to start this stimulatory cascade process (McDermott et al., 1999).

Furthermore, several studies showed that the count of T helper cells, T4/T8 ratio and percentage of T8 cells in peripheral blood have a role in immune response to vaccination or revaccination (Lee & Tong 1985; Nowicki et al., 1985; Keet et al., 1992; Rey et al., 2000). Hypo-responders have been associated with a high frequency of HLA DR7 and DR3 alleles in the Caucasian population; on the other hand, those from China were found to have HLA-DR14 and DR52. In the UK, such low response was correlated with the presence of homozygous HLA-DRB1*0701 and DQB1*0202 alleles (Craven et al., 1986; Hsu et al., 1993; McDermott et al., 1997, 1999). Several possibilities have been suggested to explain this relationship between HLA haplotype and anti-HBs response: different efficiencies in presenting antigenic fragments derived from the vaccine; linkage to deletion of vital T-cell

clones; and induction of periheral tolerance or "molecular mimicry" to a self antigen (Rocha & Von Boehmer 1991; Janeway, 1992; Kyburz et al., 1993; Schwartz, 1996).

Finally, we believe that this poor response in Pacific populations is to be further studied and evaluation of HBV vaccine in use is essential particularly in hyperendemic areas. Vaccines containing more than one genotype may be a more useful approach; it is noteworthy that the first escape mutant had a genotype D while the applied vaccine contained a genotype A (Carman et al., 1990). Weak or non-responses in healthy individuals are not well understood and a high percentage of apparent non-responders will respond to additional vaccination (Cheng et al., 1994; Belloni et al., 1998). Revaccination of these children who failed to respond on primary vaccination in the Pacific is thus recommended.

4.2 HBV S gene "a" determinant variants in vaccinated Pacific children

Norder et al. (1992b), based on dendograms derived from S gene sequences of 32 HBV genome, devised a scheme that showed a substantial correlation between antigenic subtypes and genotypes except for the heterogeneity of both *ayw1* and *adw2* subtypes. However, as more sequence data accumulate from different geographical regions, it is expected that more precise strain sequences reflecting their origin should be available and genotyping schemes will thus undergo refinement. Extended studies in wider geographical contexts are thus likely to contribute to the finer mapping of both the circulating regional strains and the relationship between genotypes and antigenic subtypes.

HBV vaccination programmes have significantly reduced both new HBV infections and the carrier rates in various endemic regions of the world. However, viruses with mutations in the "a" determinant of HBsAg have been found in several populations (Carman et al., 1990; Oon et al., 1995; Hsu et al., 1999). These mutants are described as vaccine-escape mutants that probably have altered expression of HBsAg "a" determinant epitopes which allows both infection in previously vaccinated individuals as well as lack of detection by the conventional immuno-based assays for HBV (Carman et al., 1990; Karthigesu et al., 1994; Carman et al., 1997b; Zuckerman & Zuckerman 1999).

Some vaccine escape viruses have mutations in other parts of HBsAg (outside "a" determinant) as has been shown in Japan (Miyake et al., 1996) and Singapore (Oon et al., 1999). These HBsAg mutants also showed an altered binding affinity to neutralizing antibodies (Carman et al., 1997b; Oon et al., 1999). Intriguingly, mutations in the "a"

determinant have been observed after using a PreS2-containing vaccine (Surya et al., 1996). In contrast, vaccination of chronic HBV carriers using a PreS2/S-containing vaccine did not reveal either "a" determinant mutants or any common hot spot mutations by comparison of the full envelope protein sequence before and six months after vaccination (Soussan et al., 2001). Perhaps this was due to the short period elapsed after vaccination (6 months) or the limited number of patients studied. This wide-variety of mutations, that could display several advantages to the virus, is clearly supporting the complexity of HBsAg structure.

No "a" determinant variants were discovered in the Pacific vaccinated children (Chapter 3.2). Further, they were less prevalent than these variants within HLA class I-restricted CTL epitopes in non-immunised individuals. There could be a number of explanations: different geographically defined basal prevalence of variants; duration of exposure to immune pressure; HBV endemicity level; and strength of the immune pressure.

Firstly, geography must be taken into consideration in interpretation of differences in the prevalence of HBsAg "a" determinant variants eg., Singapore, 39%, and Taiwan, 22%; see Table 3.9 (Oon et al., 1995; Lee et al., 1997a). The time interval that has elapsed since launching the immunisation program could be another factor, as has been shown recently by Hsu et al. (1999). The prevalence of "a" determinant variants jumped from 7.8% in 1984 (just before vaccination) to 19.6% in 1989 (5 years after applying vaccination) and then to 28.1% in 1994 (10 years after introduction of universal vaccination).

It is noteworthy that the basal prevalence of these variants was 7.8% at 1984, which reflect the presence of these mutants at a non-negligible percentage before the introduction of vaccination program. Consistent with this view, HBsAg "a" determinant-variants have been detected in Singapore in the random population (Oon et al., 1996). In Japan, variants of "a" determinant were also observed in 10 out of 42 patients (24%) with chronic hepatitis. However, the frequency of mutations at the hot spot codons 40 and 47 that coincide with HLA class I-restricted CTL epitopes were very low compared to those seen in Taiwan and Singapore (Tai et al., 1997; Ogura et al., 1999; Chen & Oon 1999).

A low prevalence of HBV mutants may reflect a weaker immune pressure on the virus, as possibly shown in England and Wales (Ngui et al., 1997). In this study, low immune pressure resulting from a selective vaccination strategy and low endemicity infection led to a 12% prevalence of "a" determinant variants. Consistent with this is the emergence of such variants in several patients who had received human monoclonal anti-HBs antibody or HBIG after

liver transplantation, and also in chronically HBV infected individuals (Moriyama et al., 1991; McMahon et al., 1992; Hawkins et al., 1994; Kidd-Ljunggren et al., 1995; Protzer-Knolle et al., 1998; see Section 1.5.1). However, an immunological selection process is not always required as naturally occurring HBV mutants may be already circulating as is the case in the Far East (Yamamoto et al., 1994; Hsu et al., 1995).

Thus, firstly, it seems that "a" determinant variants did not contribute to the cases of breakthrough infections in the Pacific children. Alternatively, in addition to vaccine non-responsiveness, other factors such as intrauterine infection and high maternal viral DNA load (see chapter 3.2) may be responsible for such infections. Other exposures such as ear piercing, tattooing and exposed ulcers may also have a role. Special attention should be therefore given to improve the preventive and educational measures especially in defined risk groups. These preventive non-immunologic measures, in combination with immunisation, proved very successful in Italy (Stroffolini et al., 2000).

Secondly, different HBV strains may be associated with specific nucleotide or amino acid changes in different geographical regions. These changes may result in greater viral fitness which is suitable for the challenge with HBIG therapy and anti-HBs antibodies elicited by vaccination. Monitoring of the circulating HBV strains in immunised populations from different ethnic backgrounds for a long time is thus essential as it will determine if the prevalent strains do have unique mutations. More epidemiological studies would be therefore of major importance for the control and eradication of HBV infection. Finally, though vaccination did not prevent all infections, it remains the most cost-effective mean to decrease HBV infection especially in endemic countries, at least by delaying the age of infection; this delay will greatly reduce the proportion of infections that become chronic. Therefore, further reductions of the cost of HBV vaccine are extremely important to help the people from hyperendemic regions with poor economies to control HBV infection.

4.3 HBV S gene variants within CTL epitopes

Over the last decade, S gene escape mutants, mainly within the MHR, have received a lot of interest (McMahon et al., 1992; Oon et al., 1995; Ghany et al., 1998). Regions of S gene outside this large domain have been little investigated so far (Oon et al., 1999).

In the present work (Chapter 3.3; Table 3.14), mutated residues were mainly located within regions coinciding with class I HLA-A2-restricted CTL epitopes: residues 38- 47, 172-

180 and 175- 184 (Nayersina et al., 1993). Interestingly, Paulij et al. (1999) showed that residues 178-186 of S gene constituted an important HBsAg epitope for human MAb 4-7B, and suggested the exposure of this region on the particle surface, in contrast to the classical model where this epitope is located within the lipid membrane (Stirk et al., 1992). Consistent with this recent view, Chen et al. (1996b) showed that HBsAg displays clusters of antigenic epitopes some of which are located in the region spanning aa 160- 207.

Variants within these CTL epitopes-containing regions have been recently described in several reports. In one study, 83% of patients (10/12) had mutations between S gene residues 40-47 while the mutations within the main B cell domain, 124-148, was found in 58% (7/12) of chronic carriers (Tai et al., 1997). The HBsAg region encompassing residues 29-53 was also shown to be frequently mutated in chronic hepatitis and hepatocellular carcinoma patients in comparison to the immunogenic "a" determinant (83% versus 25%), suggesting a potential role of CTL escape and thus HBV persistence (Chen & Oon 1999). In immunised Singaporean infants, S gene variants with aa substitutions outside the "a" determinant were also detected. Positions 183 and 184 were among these changes; in particular, F183C variant was associated with reduced binding to a MAb directed against the "a" determinant (Oon et al., 1999).

Residues 175-198 of HBsAg overlaps domain C of Pol gene, which is functionally important and notorious for the emergence of antiviral-induced resistant variants (S: aa 175-198/ P: aa 532-555) (Poch et al., 1989; Ling et al., 1996). Interestingly, none of the S gene changes in our Pacific-detected variants resulted in aa changes in the overlapping P gene, except for those at S aa 198 which led to V555L/S in the P gene, which is already known as a variable site (Poch et al., 1989). Thus, the S gene changes within this region are likely replication tolerable and may even enhance the replication efficiency of the virus.

It has been shown previously that viral mutations in the core 18-27 region inhibit CTL recognition and amino acid substitutions at positions 21, 22, 23 or 24 (major contact sites) reduce specific CTL activation (Bertoletti et al., 1997). Residues involved in HLA binding can also indirectly affect T cell receptor (TCR) recognition (Chen et al., 1993b). Consistent with this view, substitutions at HLA anchor residues (L19M substitution in core 18-27 epitope) showed an inhibitory effect on CTL specific proliferation (Bertoletti et al., 1997). Therefore, to have and maintain efficient CTL responses, the infected individual has to mountain a CTL response capable of recognizing the mutated epitope (Haanen et al., 1999).

In passing, an applied example for how serious the situation is in such cases can be inferred from similar observations in the core protein within one of the important Th epitopes, aa 50- 69 (Ferrari et al., 1991). Helper T cell epitopes are important for optimal CTL responses. For example, carriers of heterogeneous HBV populations are predisposed to transmit the non-dominant strains in perinatal and nosocomial transmitted infections (Von weizacker et al., 1995; Ngui et al., 2000). The basis for this selective transmission is still not clear. However, possession of "genetic determinants" which may give these minority strains priority to enter the new host and replicate easily have been suggested (Ngui et al., 2000). In this report, the authors showed that a variant which constitutes only 7% of HBV population in a surgeon, and has mutated residues at aa 57, 58 and 64 in the core gene, was transmitted to 3 patients. However, they did not highlight the immunological importance of the changes found in this strain. Interestingly, the mutated residues are located within the important Th epitope (50- 69 aa) which might explain virus non-recognition by new host immune surveillance and thus superiority in transmission and infection (Ferrari et al., 1991; Diepolder et al., 1996). Indeed, Th epitope aa 50-69 is one of the epitopes that have been described to elicit a strong immune response in patients with acute resolving hepatitis B (Ferrari et al., 1991). Although its relevance to IFN-α response is much debated, aa variation in this Th epitope (aa 50- 69) is thought to impair cellular clearance (Fattovich et al., 1995; Alexopoulou et al., 1998).

Unlike immune escape from the humoral immune response by variant virus, it is unclear if single aa substitutions in T cell epitopes can allow similar escape (Carman et al., 1990; Cariani et al., 1995; Protzer-Knolle et al., 1998). Ishikawa et al. (1998) believe that single aa changes in HBsAg CTL epitopes cannot abolish CTL recognition. Also, as the vigorous cellular immune response, the basis for clearance of HBV, is against multiple epitopes, a cellular escape in a similar manner to the immune escape from humoral immune responses was considered unlikely (Rehermann et al., 1995). However, a substitution of residues in dominant epitopes that affect its anchoring to HLA binding motif should prevent or at least weaken CTL recognition (Rosenberg, 1999). Likewise, peptides presented by HLA-B*4402 and HLA-B*4403 subtypes, which had a single aa difference (residue 146; Asp in B*4402 and Leu in B*4403) were recognised differently by CTL (Herman et al., 1999). It has been also demonstrated that once a CTL response against an epitope has been formed, the immune system may be resistant, or at least take a longer time, to develop a CTL response against the mutated epitope (Klenerman et al., 1998). Moreover, the accumulated data would appear to

support a relationship between mutations within CTL epitopes and weak CTL responses, whether as a cause or a result (Bertoletti et al., 1994; Tai et al., 1997; Chen & Oon 1999; Khakoo et al., 2000).

Several conditions have been similarly characterised where CD8+ T cell responses decrease or disappear. First, CTL-escape virus variants in vivo have been demonstrated not only for HBV, but also for HIV, HCV and LCMV viral infections (Pircher et al., 1990; McMichael & Phillips 1997). Second, unresponsiveness or exhaustion of CTL response may occur (Moskophidis et al., 1993; Zajac et al., 1998). Third, CTL responses may be initially low (Moskophidis et al., 1995), and consequently facilitating virus escape from neutralising Ab responses. Indeed, in mice, although neutralising antibodies efficiently controlled LCMV in the absence of CD8+ T cells, neutralization-resistant viral mutants emerged and escaped the established polyclonal Ab response during prolonged CTL absence (Ciurea et al., 2000)..

Taken together, a virus with mutations within residues 175-198 of S gene would get several benefits; escaping humoral and cellular immune responses, and also being highly replicative, may be reflected in an unfavourable outcome of HBV infection and resistance to antiviral therapy. Therefore, our strategies toward S gene variants should not be restricted to those variants within the MHR as others may be equally or even more important. Furthermore, optimal activation of both cellular and humoral immune responses is thus required not only for immune activation capable of mediating protection, but also to clear any settled infections and prevent emergence of viral escape variants.

4.4 How reliable is phylogenetic network analysis of S gene variability?

In recent years, powerful computerised tools for phylogenetic analysis of nucleotide and amino acid sequences have been produced, where the relationship between members of a given data set or evolutionary history of organisms and genes generated from sequence alignments can be presented in the form of "trees" or "networks".

In a tree, each terminal node (branch end) represents a single sequence from the alignment, while internal nodes (where branches meet) represent hypothetical ancestral sequences. Based on a range of philosophical and mathematical approaches, a wide variety of tree-building techniques have been developed. Phylogenetic techniques either examine a sample of all possible tree topologies looking for the best possible tree as defined by chosen criteria (searching methods; e.g., Maximum Parsimony (MP)), or they use an algorithm to

generate a tree starting with a few members of the data set and adding the rest one at a time (clustering methods; e.g., Neighbour Joining (NJ)) (Page & Holmes 1998). However, these techniques cannot take a count of recombination within the data set and were also unable to model the mutation process in the presence of an intrinsically variable rate of mutation (Bollyky et al., 1996; Bollyky & Holmes 1999); in such cases, a network may be more appropriate.

Networks have proved to be an effective way to represent ambiguity and try to explicitly place mutations on the branches of a tree. A network contains one or more cycles (a group of nodes where it is possible to trace a path that starts and ends at the same node without visiting any other node more than once), whereas trees do not. To date, two network models have been developed, reduced median (RM) and median joining (MJ). RM networks, briefly, display the principal character relationships present in the data and resolve likely parallel events while retaining character conflicts in the form of reticulations when ambiguity remains (Bandelt et al., 1995). In MJ networks, additionally, larger sets of data can be analysed and a faster speed can be achieved, however, MJ should be applied to a recombination-free data (Bandelt et al., 1999). A network approach is particularly useful in studying intra-specific data due to small phylogenetic distances (such as variation within human mitochondrial DNA (mtDNA) and virus genotypes as in our Pacific study; see chapter 3.3), where alternative potential evolutionary paths in the form of cycles can be displayed (Page & Holmes 1998).

HBV genotyping has been performed by two molecular techniques; direct sequencing of full-genome or restriction fragment length polymorphism (RFLP) analysis (Okamoto et al., 1988; Norder et al., 1994; Mizokami et al., 1999). Unlike PCR, RFLP is simple, less expensive and can detect variation across large genomic regions. RFLP was used earlier to relate HBV serotypes to certain restriction patterns (Shih et al., 1991), and recently for HBV genotyping based on the analysis of S gene region (Lindh et al., 1997; Mizokami et al., 1999). However, RFLP analysis detects only a small proportion of the total genetic variation; which probably limits its use in studying within-genotype variation or investigating and identifying transmission routes (Nei, 1987; Sugauchi et al., 2000). In contrast, direct sequencing yields more complete information about variation at the sequenced site, however, its greater expense and technical requirements limit its application to small genomic regions ($\leq$ 1000 bases) (Arens, 1999).

The molecular basis for the serological heterogeneity of HBsAg, which is encoded by the small S gene, has been defined (Norder et al., 1992a). HBV genotyping based on limited sequencing within S gene is also consistent with those based on sequencing of the full HBV genome (Norder et al., 1994). The validity of S gene sequencing-analysis for HBV genotyping has been further confirmed in several studies (Arauz-Ruiz et al., 1997; Mizokami et al., 1999). Taken together, the approach of phylogenetic network analysis for studying the S gene variability, based on direct sequencing, in Pacific islands to investigate the relation of such variability to the different Pacific islands and to trace the history of people movements in this part of the world is reliable.

4.5 HBsAg as a marker for tracing the migration pattern of Pacific people

Human genetic evidence, in addition to linguistic and archeological evidence, has been gathered in attempts to resolve the origin of Pacific people; however, a clear conclusion has not yet emerged. One hypothesis suggest that the whole cultural and genetic background of Polynesia came from South East Asia, and migrant populations passed through Melanesia without mixing appreciably with aboriginal populations, this is termed the "*Express Train*" (Diamond 1988). An opposing view holds that there was indeed a general push eastwards, originating in South East Asia, but that the degree of mixing between migrant and aboriginal populations was much greater, the "*Tangled Bank*" (Terrell 1988). Recently, using analysis of Y chromosomes, Kayser et al. (2000) suggested more complex migratory patterns for the origin of these people, the *"Slow Boat"*.

Genetic information from persistent viruses has long been recognised as a potential source of independent evidence concerning population history (Gessain et al., 1992; Ho et al., 1993). Variability of viral sequences has one major advantage over human genomic or mtDNA sequences that relates to their high mutation rates which allow variations to be determined progressively. Timing of historical events can also be calculated; as long as the virus mutation rate is known. For example, different genotypes of a virus may differ by several hundred nucleotides and their co-existence in a geographical region probably reflect different origins rather than recent viral evolution.

The ideal virus to trace the human-population movements would be transmitted perinatally so it acts as an inherited marker, would not be subjected to immune pressure till transmitted to the next generation and likely not lead to death before subsequent transmission so the carrier

will be treated as any community member. Finally, it should be a common virus so that "non carriers" would have antibodies and probably life long immunity, so they would rarely acquire the virus from other peoples. HBV in Asia and Pacific islands satisfies all these requirements.

Usage of HBV viral sequence variability has not been employed before to chart population movements, although it has long been known that viral serotypes and genotypes have a characteristic world geographic distribution (Couroucé-Pauty et al., 1981; Norder et al., 1993). It has been hypothesised that the origins of the genotypes are related to events in the formation of human populations; however, few data are available about patterns of within-genotype variation which might hold information about more recent virus and host population history. Therefore, we collected data on the variability of HBV in the Pacific in the expectation that it might contain information about the origins of the human populations in the region.

The dominant HBV genotype in Kiribati was D, while genotype C predominated in the other three island locations. Isolates that are exceptions to this general trend can be interpreted as the result of limited transfer of viruses between locations within the Pacific and, in the case of Fijian D isolates, acquisition of new strains by migration. This pattern, and the distribution of finer scale variation within genotypes across islands, is consistent with a single ancient HBV colonization event in the western Pacific, leading to the predominance of a single C lineage in Vanuatu, Fiji and Tonga, and a separate, more recent event colonizing Kiribati.

Thus, we believe that each population wave carried with it a specific subtype of HBV and that, within each subtype, only selected viral strains were carried on to the next island that then evolved independently. However, no strong evidence was found for Indian admixture in Fijian D genotype sequences, in spite of the >40% Indian origin of the Fijian population (est, 1999). This could be explained either by selective sampling (as only Melanesian Fijians and not Indo-Fijians were included), with a lack of transfer of HBV types between the two sub-populations, or by differences in the incidence of HBV in the two source- populations as the latter have much lower HBV infection rate. Moreover, although it is described that B genotype makes up a significant proportion of Asian HBV, this is not substantiated in our Pacific study. Therefore, further investigation of population samples from extra Pacific populations should help to clarify both the role and origin of B isolates in this region, and also the degree to which different source populations contributed to the settlement of various Pacific locations.

A question of current importance is whether it will be possible to use HBV data to help to distinguish between hypotheses concerning the origins of Pacific populations. Although uncertainties in virus data due to the inherently greater effects of genetic drift and the possibility that HBV may not have affected all the ancient populations similarly cannot be completely excluded, we think the data presented in Chapter 3.3 on HBV sequence variability have yielded useful information about the history of human populations in the Pacific region. Obviously, a cultural expansion with its origins in South East Asia was an important determining factor in the initial colonization of the Pacific islands. It is also reasonably clear from the data and interpretation (see Chapter 3.3) that HBsAg population variability contains patterns that are at least consistent with aspects of the known history of host populations. Furthermore, virus analysis has some interesting aspects that human genome analysis does not. Clearly, it provides much greater range of diversity and creates greater power in the conclusions one draws as an independent source of evidence. Finally, we believe that HBV is a good candidate that can be employed as a pseudo-genetic marker, at least in Asia and Pacific islands, in tracing human population history.

4.6 Tag system for evaluation of HBsAg variants: are they truly escape mutants? Do some variants have enhanced replication efficiency?

Failure to detect variant HBsAg could be due to the presence of aa substitutions that markedly change the conformation dependent antigenic structure of HBsAg or low serum levels of the variants that are below the detection limit of the employed diagnostic kits. Thus, it is essential to establish that failure of HBsAg variant detection is due to the antigenic changes and not due to variant low production. Therefore, we have developed an antibody capture system, using a non-HBV epitope, to standardise the amount of in-vitro expressed HBsAg protein. The amount of in-vitro expressed HBsAg was then equalised in ELISA that recognises the tag.

The advantage of this approach is that sufficient amounts of HBsAg can be generated and quantified to comparable and known concentrations. These antigens can then be evaluated in several HBsAg assays to determine their ability of detection of such antigens. The outcome of such testing would therefore reveal the mutations affecting diagnostically important epitopes for detection of HBsAg. According to the results presented in chapter 3.4 and 3.5, it appears that some variants can be detected by using the right combination of antibodies while others

still escape detection. Therefore, further improvements in our HBsAg assays design are still required to detect these HBsAg variants such as using different panel of antibodies developed to other epitopes outside MHR that are not influenced by the conformational change of HBsAg or by the introduction of nucleic DNA chip technology.

Recently, Karthigesu et al. (1999) showed enhanced antibody binding with L141E HBsAg variant to some monoclonal antibodies, which were raised against the standard antigen. These reactivity differences have been interpreted on the basis of antigenic differences between both strains. Additionally, or alternatively, this could be attributed to variant overproduction, as the expressed particles were not standardised. Quantification of serum HBV DNA in children infected with S gene variants, one of them G145R, revealed a relatively high concentration giving further support to our observations (Hsu et al., 1999). G145R and D144A, which are the main aa changes within V2 and V10 constructs respectively (see Chapter 3.5), have been associated with a worse clinical outcome and longer persistence despite the termination of HBIG treatment of infection of second and even third liver grafts (Protzer-Knolle et al., 1998). It thus appears that these variants could become the predominant strain.

Similar observations of enhanced replication have been reported with HBV core promoter deletion variants, although the deletions remove part of the core promoter that enhances the synthesis of the pregenomic RNA. Variants that have core promoter deletions can have slightly enhanced replication in cell culture. Consistent with these findings, high viremia has been detected in three patients infected by HBV with similar deletions in the core promoter region (Günther et al., 1996; Moriyama et al., 1997; Chen & Oon 2000).

Even though results of *in vitro* data cannot be fully applied to the *in vivo* situation, due to the complexity of reactions, the observations in Chapter 3.5 provide a clue that enhanced replication of some variants *in vitro* may lead to high viremia *in vivo*. However, the relevance of this observation to the outcome of infection warrants further investigations.

4.7 Abnormal or discrepant serology: does it mean presence of HBsAg variants?

HBsAg negativity usually reflects clinical situations such as the late phases of chronic and acute infection where suppression of HBV replication is known to occur and consequently disappearance of HBsAg (Fong et al., 1993; Michalak et al., 1994; Loriot et al., 1997). HCV or HDV co-infection can suppress HBV replication and also lead to HBsAg negativity (Fong et al., 1991; Sheen et al., 1992; Francisci et al., 1995; Jilg et al., 1995).

Mutations or deletions elsewhere in the genome, which theoretically down-regulate HBV replication, have also been reported to result in HBsAg negativity. These have been seen in the core promoter/ X- gene (deletion of 8 nucleotides), the S promoter region (selective down-regulation); and in the polymerase gene (showed to terminate HBV replication in vitro) (Blum et al., 1991a; Uchida et al., 1994; Fukuda et al., 1996; Melegari et al., 1997; Bock et al., 1997).

The presence of "a" determinant variants, in addition to low levels of HBsAg which are below the detection limits of the used assays, could however lead to a false negative HBsAg test (Jongerius et al., 1998; Grethe et al., 1998; chapter 3.4). Consistent with this view a very important finding of the work in Chapter 3.6 was the association between abnormal serology and S gene variant sequences in all patients. In patient 4, the S gene sequences of the later samples (1991 and 1992) were identical to each other and were different from the standard *ayw2* sequence at two positions (Q101H and D144E). These substitutions, particularly the latter one, have previously been shown to be antigenically important, as has the additional mutation S143L, seen in both samples from 1992 (Wallace et al., 1994). In patient 5, there was only one substitution (E164V), as compared to the standard sequence, which has not been described before. Thus, the association of abnormal serology by variant sequences in both patients shows the importance of variants in this situation. This conclusion is strongly supported by patients 6 and 7 (control group), where the sequential samples were not only identical but also did not contain any variant away from the prototype.

On the other hand, sensitivity of HBsAg assays could also has a crucial role and be responsible for this discrepancy as has been observed in patient 3 in this work, chapter 3.4 or previous reports (Carman et al., 1995a, 1997b; Grethe et al., 1998). For example, a polyclonal antibody-based radioimmunoassay was successful in detecting HBsAg variant from an Indonesian patient that escaped the detection by monoclonal antibody-based ELISA (Carman et al., 1995a). Furthermore, similar concern has been raised recently about the sensitivity of HBsAg assays and the resultant discordant serology of tested samples (Carman et al., 1997b).

Therefore, a diagnostic screening protocol of testing for both HBsAg and anti-HBc should at least be performed for each suspected infected individual. It is also most important that results of serological assays are carefully assessed. Additionally, test reagents have to be validated for use according to the epidemiological finding in a region, as there are significant antigenic differences between the circulating subtype strains in different parts of the world

(Karthigesu et al., 1999; Sobotta et al., 2000). Finally, as there are several reports of HBV DNA positive individuals with undetectable HBsAg, the possibility of new uncharacterised S gene mutants with little known about their replication competency, in addition to low-titer standard virus, supports the argument for PCR testing (van Deursen et al., 1998; Zuckerman & Zuckerman 1999). PCR testing will not only allow us to determine HBV DNA in HBsAg negative individuals but to detect any sequence variation under host selection and also might give an explanation for the variable outcome of HBV infection.

4.8 Conclusions

The data presented in this thesis focused on several important issues. First, efficient viral and human DNA extraction from blood clots. This enables good use of a material that is often thrown away. Second, the prevalence of "a" determinant variants in HBV endemic regions (Pacific islands) after applying universal vaccination. Emergence of vaccine escape variants in these populations was relatively insignificant. Consequently, failure of vaccination is not always due to emergence of HBsAg variants and other preventive public health measures are important, particularly in developing countries.

Third, it is useful to employ S gene variability to trace human population history in Asia and the Pacific islands. Indeed, looking at virus heterogenity of native populations gives a glimpse into ancient times and provides a good insight into how people have moved across the globe. Fourth, HBsAg variants react differently in various commercial assays. This has important implications for diagnosis and blood donor screening. Fifth, standardisation of HBsAg particle quantity is important. Insertion of an influenza tag into the end of HBsAg allows the antigenicity rather than expression efficiency of mutant HBsAg to be assessed. Sixth, abnormal or discrepant serological findings in HBV infected individuals may be an indicative marker for the presence of HBsAg variants.

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Reactivity of 13 *In Vitro* Expressed Hepatitis B Surface Antigen Variants in 7 Commercial Diagnostic Assays

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The primary marker of current hepatitis B infection is the surface antigen (HBsAg), however HBsAg negativity does not exclude hepatitis B viremia. HBsAg variants can be responsible for such diagnostic failures. Here 13 different HBsAg variants were cloned, variant protein produced in a mammalian expression system, and tested using 7 commercial HBsAg diagnostic assays. Of 12 variants analyzed, 6 samples displayed similar reactivity to the positive control (containing standard HBsAg sequence) in most of the assays, but 6 samples, containing various mutations throughout the entire major hydrophilic region (MHR), showed reduced reactivity. It was found that the loss of cysteine at amino acid (aa) 124 in 1 sample affected the secretion as well as the reactivity of HBsAg in the expression system. Thus, not all assays are equally able to detect HBsAg variants, implying that, to attain an acceptable level of sensitivity, the antibody repertoire of the current assays should be extended. (HEPATOLOGY 2000;31:1176-1182.)

Antigenic variation of hepatitis B surface antigen (HBsAg) is clinically significant and has been discussed in several reviews.[1,2] HBsAg variants (hepatitis B viruses containing mutations in the surface gene) can be considered as 2 etiological classes. The first class occurs naturally and includes subtype variation and amino acid (aa) changes that may be poorly detected in diagnostic assays.[3-5] Class 2 variants are selected by medically induced immune pressure, for example after vaccination and treatment with hepatitis B immunoglobulin or monoclonal antibody.[6-12] One external domain of HBsAg, referred to as the major hydrophilic region (MHR), contains clusters of interleaved epitopes within which are the proposed neutralizing epitopes.[1] Although class 2 antigenic variation tends to occur within these neutralizing epitopes, both classes show variation extensively within the MHR.

The clinical and epidemiological significance of HBsAg variants cannot be fully characterized until their true prevalence is known. Despite more than 50 reports (1992-1999) on the occurrence of surface (S) gene variants, very few large sero-epidemiological studies have been reported as yet. One such study[13] reported 63 variants out of 2,305 (3%) vaccinated children. Several other studies report the incidence of S gene mutations after immune prophylaxis in their study cohorts ranging from 5% to 22%.[7,14-16] This level suggests a risk of the spread of hepatitis B virus (HBV) variants due to immune pressure selecting against vaccine susceptible wild type HBV.[17] Large prevalence studies are currently under way around the world, which will hopefully answer this important question. However, because there are clear clinical consequences of diagnostic failure, it is important to the further development of commercial HBsAg assays. There have been at least 12 reports (1992-1999) of naturally occurring S gene variants leading to nondetection of blood and organ donors.[18,19] Further, it is possible that gene variation can affect the disease process. A study of 120 Asian Indians with chronic HBV reported S gene variants in 10.8% of patients. These showed an unfavorable course compared with the standard strain.[20] The increased frequency of association of variant compared with standard sequences in some cases of hepatocellular cancer[21] is also of clinical concern. Although a range of commercial assays for HBsAg are widely available, not all are equally sensitive, and it is thus imperative that the antibodies used for antigen capture in diagnostic assays are tested against a wide variety of variants to maximize their sensitivity and specificity.

Sera containing HBsAg variants are rarely available in volumes sufficient for testing against a multitude of capture antibodies, therefore we cloned variant HBsAg from 13 diagnostically relevant cases and tested cell culture supernatants in 7 commercial diagnostic assays. The observed substantial discrepancies in assay reactivity have serious implications for the design of commercial assays.

MATERIALS AND METHODS

Derivation of Variant Sequences

Surface gene sequences from 13 patients in different clinical and geographical settings were used in this study as detailed in Table 1. These particular examples were chosen from our bank of variants collected because of clinical relevance. In some cases they have been described in a number of reports; others were highly divergent and therefore of particular interest. HBV DNA was extracted from 50 μL

Abbreviations: HBsAg, hepatitis B surface antigen; aa, amino acid; MHR, major hydrophilic region; S gene, surface gene; HBV, hepatitis B virus; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; mAb, monoclonal antibody; pAb, polyclonal antibody.

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TABLE 1. Expressed Hepatitis B Surface Antigen Variants

Sample	Subtype	Mutations Within the MHR*	HBs Region of MHR Where Mutations Are Located†	Origin	Clinical Background
Gly Y	*ayw*	Standard sequence		Spain	Positive control
Gly D	*adw*	Standard sequence		Spain	Positive control
Arg145	*adw*	G145R	4	Italy[6]	Vaccinee
1056Sp.	*ayw*	P120S/S143L	2, 4	Spain[34]	Subtype study, IVDU
M5	*ayw*	Y100S/T118V/R122K/M133I/Y134N/P142S/S143L/G145K	1-4	Saudi Arabia	Discrepant serology, renal transplant patient
T5N	*ayw*	D99N/122NT123/G145R	1, 2, 4	Indonesia[4]	Discrepant serology, vaccinee
91-4696	*adw*	S113T/T143S	1, 4	South Africa	Diagnostic failure
HK188	*adr*	L98V/Q101R	1	Hong Kong	Diagnostic failure, bone marrow donor
BA3.2	*ayw*	T123N/C124R	2	Pakistan[10]	Liver transplant—HBIG treated
BA2.4	*ayw*	Y100C/P120T	1, 2	Pakistan[10]	Liver transplant—HBIG treated
BA3.4	*ayw*	T123N	2	Pakistan[10]	Liver transplant—HBIG treated
AP3.1	*adw*	D144A	4	UK[10]	Liver transplant—HBIG treated
SA4	*adw*	M133T/Y161F	3, 5	South Africa	Acute hepatitis B, diagnostic failure
SA6	*adw*	Q129R/G130N/A166V	3, 5	South Africa	Chronic liver disease, diagnostic failure
SA7	*ayw*	M133T	3	South Africa	Chronic liver disease, diagnostic failure

Abbreviations: IVDU, intravenous drug user; HBIG, hepatitis B immunoglobulin.

*The convention chosen to describe the mutation is the amino acid (aa) position (numbered from the start of the surface gene) preceded by the usual aa and followed by the aa found in the variant sequence.

of serum using the QIAamp Blood Kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. A hotstart, nested polymerase chain reaction (PCR) was performed to amplify the S gene. Five microliters of extracted DNA was amplified in a 50-μL solution containing 1 U Taq polymerase (Life Technologies, Paisley, UK), 1.4 μmol/L TaqStart antibody (Clontech Laboratories Inc., Palo Alto, CA), 0.25 mmol/L dNTPs (Pharmacia, St. Albans, UK), 2.5 mmol/L $MgCl_2$, 5 μL of 10 × PCR buffer, and 25 pmol/L of primers S1 (56F, 5′- CCTGCTGGTGGCTCCAGTTC-3′) and S2Na (1003R, 5′-CCACAATTCKTTGACATACTTTCCA-3′, where K= G or T), for 5 cycles of 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 90 seconds followed by 35 cycles with the denaturation temperature reduced to 90°C. All primers are numbered according to the system used by Okamoto et al.[22] One microliter of first round PCR product was reamplified as described above with the nested primers, S6C (129F, 5′-GCACACGGAATTCCGAGGACTGGGGACCCTG-3′) and S7D (842R, 5′-GACACCAAGCTTGGTTAGGGTTTAAATGTATACC-3′) for 5 cycles of 95°C for 60 seconds, 55°C for 75 seconds, and 72°C for 90 seconds followed by 25 cycles, with the denaturation temperature reduced to 90°C. DNA fragments of expected size, were extracted from 1% agarose gel using Geneclean II kit (Bio 101, La Jolla, CA).

Cloning and Surface Gene Sequencing

The S6C and S7D primers used in the nested PCR incorporate restriction sites for *Eco*RI and *Hin*dIII, respectively. The purified PCR product was ligated into the mammalian expression vector pJI and transformed into the *Escherichia coli* strain DH5α (Life Technologies). Plasmid DNA was purified using a Qiagen plasmid midi-kit (QIAGEN) and fluorescence-based sequencing of the whole surface gene was carried out using the ABI PRISM Ready Reaction dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Cheshire, UK) according to the manufacturer's instructions. The primers used for sequencing were S6C and S7D with the internal primers S3 (690R, 5′-AATGGCACTAGTAAACTGAGCC-3′), S4 (459F, 5′-GTATGTTGCCCGTTTGTCCTC-3′) and S8 (434R, 5′-AGAAGATGAGGCATAGCAGC-3′). Sequence analysis was performed with the GCG program (Wisconsin sequence analysis package, version 9.1. Genetics Computer Group, Madison, WI).

Expression of HBsAg

The plasmid, with its entire HBV surface gene insert, was transfected into subconfluent monolayers of COS7 cells on 16-mm coverslips in 60-mm petri dishes using cationic liposomes made from dioleoyl L-α-phosphatidyl ethanolamine and dimethyldioctadecyl ammonium bromide (Sigma-Aldrich, Dorset, UK).[23,24] Briefly, 2 μg plasmid was diluted in 200 μL Optimem 1 reduced serum medium (Life Technologies) and, in a separate vial, 24 μL of liposomes was added to 200 μL of Optimem 1. The two solutions were mixed and allowed to stand for 15 minutes at room temperature, then further diluted to 2 mL using Optimem 1 and added to prewashed COS7 cells. The cells were incubated with the transfection mixture for 5 hours at 37°C in 5% CO_2 and then 3 mL of COS7 medium was added (Dulbecco's modified Eagle's medium with 10% foetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine; Life Technologies). Cells were incubated for 16 hours at 37°C in 5% CO_2 when the transfection mixture was removed and 5 mL of fresh COS7 medium was added. Plasmid pJI containing standard HBV DNA S gene sequence (both *adw* and *ayw* subtypes) was used as a control for transfection and antigenic analysis. After 3 days, the culture medium was harvested and immunofluorescent staining was performed on the cell monolayered coverslips.

Preparation of Cell Lysates

To check that HBsAg was being expressed in samples with nonreactive supernatants, the cell lysates were also tested. Cells from 60-mm petri dishes were removed using sterile cell scrapers (Becton Dickinson, Paramus, NJ) in 1 mL phosphate-buffered saline (PBS). The samples were centrifuged at 6,500 rpm for 10 seconds and the supernatant was removed. The cell pellet was washed twice with PBS, the supernatant was removed, and the cells were resuspended in 250 μL PBS. The washed cells were frozen/thawed 3 times and finally spun at 13,000 rpm for 2 minutes. The supernatant, presumably containing HBsAg, was removed and tested using the IMX HBsAg (V2) assay (Abbott Laboratories, North Chicago, IL).

Antigenic Analysis of Expressed HBsAg

Diagnostic Assay Reactivity. Aliquots of culture supernatant were tested according to manufacturers' instructions in 7 commercial diagnostic assays, which use various monoclonal (mAb) and/or polyclonal (pAb) antibodies for either capture or detection (Table 2). The signal to cutoff ratio was obtained for each sample in each assay and divided by that of a standard serum containing 0.5 ng/mL HBsAg (called the "working standard" 0.5 IU/mL, National Institute

TABLE 2. Reactivity of Expressed Hepatitis B Surface Antigen Variants as Determined by 7 Commercial Diagnostic Assays

Sample	1 bioELISA HBsAg colour	2 AUSRIA II −125	3 VIDAS HBsAg	4 Enzymun-Test HBsAg ES300	5 IMX HBsAg (V2)	6 HBsAg GE14	7 Enzygnost HBsAg Monoclonal II	Immunofluorescence (pAb) % of Positive Cells per Field at ×10 Magnification
Gly Y*	3.59	21.94	12.89	9.02	4.78	8.83	4.73	30-40
1056Sp.	3.31	11.88	1.36	0.52 Neg†	1.16	6.17	0.35 low‡	40-50
BA2.4	3.24	7.07	0.69 low	0.37 Neg	0.76 low	6.66	0.33 low	40-50
BA3.4	2.20	4.08	0.029 Neg	5.67	0.27 Neg	2.20	2.47	40-50
SA7	2.79	5.20	9.59	4.27	7.59	8.04	5.59	40-50
M5	1.57	3.34	0.024 Neg	0.71 Neg	1.40	0.23 Neg	0.30 Neg	40-50
BA3.2	N/A§	N/A	N/A	N/A	N/A	N/A	N/A	20-30
Gly D*	3.50	33.40	13.22	18.78	7.01	8.70	4.15	50-60
Arg145	3.40	15.60	0.40 low	8.42	4.83	0.27 low	0.38 low	50-60
91-4696	3.66	29.88	12.97	14.50	5.76	8.35	5.11	40-50
HK188	3.64	22.40	12.94	32.97	11.52	8.51	5.01	40-50
AP3.1	3.31	17.10	11.60	15.46	5.18	6.93	6.71	40-50
SA4	1.89	2.91	3.46	6.06	4.58	2.31	1.38	40-50
SA6	2.70	1.43	8.21	9.52	5.02	4.45	3.77	20-30
pJI (plasmid)‖	0.52 Neg	0.11 Neg	0.024 Neg	0.32 Neg	0.196 Neg	0.16 Neg	0.27 Neg	Neg
COS7 cells‖	0.39 Neg	0.16 Neg	0.063 Neg	0.46 Neg	0.20 Neg	0.18 Neg	0.28 Neg	Neg
Working standard¶	1.0	1.0	1.0	1.0	1.0	1.0	1.0	Not tested
Monitor sample¶	0.68	0.31	0.30	0.73 Neg	0.40	0.39	0.53	Not tested
Assay cutoff**	0.53	0.23	0.14	0.95	0.28	0.24	0.31	

NOTE. Reactivity is expressed as an index = (OD) of sample/(OD) of working standard.

*Gly Y, Gly D are standard sequences of HBV subtype *ay* and *ad*, respectively.

†Neg = Negative, *i.e.*, reactivity below the kit cutoff level.

‡Low = reactivity below that obtained for the working standard.

§N/A = results not applicable. BA3.2 expressed antigen was later found to be retained within the cells.

‖Negative controls. *i.e.*, supernatants from COS7 cells alone and cells transfected with the plasmid pJI, which certains no insert of hepatitis B surface gene.

¶The working standard (0.5 IU/mL) and the monitor sample (0.125 IU/mL) are serum preparations from the National Institute of Biological Standards (London, UK) and distributed nationally to diagnostic laboratories as external controls.

**Assay cutoff = the value prescribed by the assay above which a serum sample would be deemed to be reactive.

for Biological Standards and Control, NIBSC UK), to give an index of reactivity (Table 2). The reactivities of the variants were also expressed as a percentage of that found for the standard HBV sequence, the activity of which was taken as 100% (Table 3). We recorded the number of variants detected by each assay at a level of ≥10% of the reactivity of the standard HBV sequence (Table 4). We considered this more appropriate than simply relying on the kit cutoff value, because in a diagnostic setting reactivities close to the cutoff value are not generally considered to be true positives without corroborative testing such as neutralization or detection of antibodies to hepatitis B core antigen. The assays were repeated to assess reproducibility of test results. To control for transfection/expression efficiency between experiments, supernatants from multiple transfections were tested using IMX HBsAg (V2) (Table 5). Also, we repeated 6 of the 7 assays using a separate batch of supernatants.

The assays used in the study were (1) bioELISA HBsAg colour, BIOKIT, Longfield, Kent, UK (standard procedure); (2) AUSRIA II-125, Abbott Laboratories Ltd., Maidenhead, UK (overnight room temperature procedure); (3) VIDAS HBsAg, bioMerieux SA, Marcy-l'Etoile, France (long protocol); (4) Enzymun-Test HBsAg ES300, semi-automated system, Boehringer Mannheim GmbH, Mannheim, Germany; (5) IMX HBsAg (V2) semi-automated system, Abbott Laboratories Ltd.; (6) Murex HBsAg GE14, Murex Biotechnology Ltd., Dartford, UK (2-hour procedure); and (7) Enzygnost HBsAg Monoclonal II, Behring Diagnostics GmbH, Marburg, Germany (manual procedure).

Immunofluorescence. COS7 cells on glass coverslips were methanol fixed, washed with PBS, and incubated with goat anti-HBsAg pAb (Dako, High Wycombe, UK) for 45 minutes at room temperature. After washing, the cells were incubated with the secondary antibody, anti-goat fluorescein isothiocyanate–labeled immunoglobulin, for 30 minutes at room temperature (Sigma-Aldrich Company). Cells were examined for fluorescence under a Nikon Microphot-SA microscope.

RESULTS

Sequencing

The mutations detected in each of the variant samples are detailed in Table 1. Also listed is the clinical background and country of origin of the serum samples. As sample T5N gave uninterpretable results, this is dealt with separately (see below).

Immunofluorescence

Immunofluorescence was performed to measure transfection efficiency (Table 2). Most samples (10/12) showed 40% to 50% of cells expressing HBsAg. BA3.2 and SA6 were lower at 20% to 30% but still within the lower range of the standard Gly Y and expected to produce detectable amounts of surface antigen.

Diagnostic Assay Reactivity

The reactivity of each variant is presented in two ways. First, it was calculated as an index (Table 2), *i.e.* reactivity of the variant divided by that of the NIBSC standard (0.5 IU/mL) for each assay. Secondly (Table 3), they are expressed as a percentage of the reactivity of the standard HBV sequence of the same subtype, *ay* or *ad*. These two analyses allow comparison both to a known amount of natural HBsAg and to an *in vitro* expressed standard sequence. Representing the

TABLE 3. Percentage Reactivity of HBsAg Variants in 7 Assays Compared With the Positive Control (100%)

	1	2	3	4	5	6	7
Sample	bioELISA HBsAg color	AUSRIA II −125	VIDAS HBsAg	Enzymun- Test HBsAg ES300	IMX HBsAg (V2)	HBsAg GE14	Enzygnost HBsAg Monoclonal II
Gly Y*	100	100	100	100	100	100	100
1056Sp.	92.2	54.2	10.6	5.8	24.3	69.9	7.4
BA2.4	90.3	32.2	5.35	4.1	15.9	75.4	6.98
BA3.4	61.3	18.6	0.2	62.9	5.7	24.9	52.2
BA3.2	N/A†	N/A	N/A	N/A	N/A	N/A	N/A
SA7	77.7	23.7	74.4	47.3	158.8	91.0	118.2
M5	44.9	10.0	0.2	3.8	20.0	2.6	7.2
Gly D*	100	100	100	100	100	100	100
Arg145	97.1	46.7	3.0	44.8	68.9	3.2	9.2
91-4696	104.6	89.5	96.1	77.2	82.2	96.3	123.1
HK188	104.0	67.1	97.9	175.6	164.3	97.8	120.7
AP3.1	94.6	51.2	87.7	82.3	73.9	79.7	161.7
SA4	54.0	8.71	26.2	32.3	65.3	26.6	33.3
SA6	77.1	4.28	62.1	50.7	71.6	51.1	90.8
pJI (plasmid)‡	14.5	0.51	0.2	3.6	4.1	1.8	5.8
COS7 cells‡	10.9	0.73	0.5	5.1	4.2	2.0	6.0
Working standard§	27.9	4.6	7.8	11.1	21.0	11.5	21.1
Monitor sample§	18.9	1.4	2.3	8.1	8.4	4.5	11.2
Capture antibody	Guinea pig pAb	Guinea pig pAb	Mouse mAb	Mouse mAb	Mouse mAb	Goat pAb	Sheep pAb
Detection antibody	Goat pAb	Human pAb	Mouse mAb	Mouse mAb	Goat pAb	Mouse mAb	Mouse mAb

NOTE. Reactivity = (OD) of sample/(OD) of standard sequence of same subtype ×100%.

*Gly Y, Gly D are standard sequences of HBV subtype *ay* and *ad*, respectively.

†N/A = results not applicable. BA3.2 expressed antigen was later found to be retained within the cells.

‡Negative controls. Supernatants from COS7 cells alone and cells transfected with the plasmid pJI, which certains no insert of hepatitis B surface gene.

§The working standard (0.5 IU/mL) and the monitor sample (0.125 IU/mL) are serum preparations from the National Institute of Biological Standards (London, UK), distributed nationally to diagnostic laboratories as external controls.

data in these ways allows both inter- and intra-assay comparisons to be made. All assays were performed within the sensitivity limits claimed by their manufacturers using the two preparations of standard sera.

Both standard sequences and 7 of the variants (Arg145, 91-4696, HK188, AP3.1, SA4, SA6, and SA7) were detected by all assays, although Arg145 was less reactive than the working standard in assays 3, 6, and 7 and was only marginally above cutoff level in 6 and 7. These contain variants in HBs regions 1, 3, 4, and 5 of the MHR.[1] Three samples (1056Sp, BA 3.4, and BA 2.4) were detected by most of the assays, although some displayed only low level reactivity: 1056Sp was low in assay 7 and negative in assay 4; BA3.4 was negative in assays 3 and 5; and BA2.4 was negative in assay 4 and low in assays 3, 5, and 7. This group contained variants in HBs regions 1, 2, and 4 of the MHR. Sample M5, which had mutations in HBs regions 1 to 4, was negative in assays 3, 4, 6, and 7. Sample BA 3.2, containing mutations in HBs region 2, could not be detected by any of the assays. To attribute nonreactivity of BA 3.2 to the mutations *per se*, we had to exclude intracellular retention of surface antigen particles. Loss of secretion of HBsAg from transfected cells has been noted previously.[25,26] Therefore, cell lysates of BA3.2 and appropriate controls (cell lysates of Gly Y and standard sera) were tested using assays 1, 5, and 6. Positive results for BA3.2 were detected in all three assays, with a reactivity of approximately 20% of that of the standard HBsAg sequence (data not shown). This indicated that the expressed BA3.2 antigen was not present in the culture supernatant and is separated accordingly from the other samples in Table 2. This suggests that the loss of cysteine at amino acid 124 has had a deleterious effect on secretion as is suggested by the 20% to 30% positivity found by immunofluorescence on the transfected cells (Table 2).

Table 4 compares assay performance of the variants that react at a level greater than or equal to 10% of the standard HBV sequence. The assays detected between 7 and 11 of the variant supernatants. In general, the use of pAb in the capture and/or detection phases was associated with higher detection rates: assays 1, 2, 5, and 6 detected 11, 9, 10, and 9 of the 11 variants, respectively. The exception was assay 7 (detected 7 variants), which used sheep pAb in the capture phase and mouse mAb for detection. Assays 3 and 4, which detected only 7 and 8 of the variants, respectively, used mAb in both capture and detection phases. Variants of HBV subtype *d* are

TABLE 4. Number of Variants Detected by Each Assay at a Level of ≥10% of the Standard HBsAg (GlyY/GlyD)

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7
ay Subtype variants	5/5	5/5	2/5	2/5	4/5	4/5	2/5
ad Subtype variants	6/6	4/6	5/6	6/6	6/6	5/6	5/6
Total variants detected	11/11	9/11	7/11	8/11	10/11	9/11	7/11
Capture antibody	Guinea pig pAb	Guinea pig	Mouse mAb	Mouse mAb	Mouse mAb	Goat pAb	Sheep pAb
Detection antibody	Goat pAb	pAb	Mouse mAb	Mouse mAb	Goat pAb	Mouse mAb	Mouse mAb

TABLE 5. Expressed Surface Antigen Reactivities From Multiple Transfection Experiments Using the IMX HBsAg (V2) Assay

Sample	Mean Index of Reactivity* ± SE (Number of Transfection Experiments)	Range of Reactivity	Multiple of Mean
Gly Y	4.80 ± 0.49 (10)	3.26-7.81	0.68-1.63
1056Sp.	1.43 ± 0.25 (6)	0.64-2.20	0.45-1.54
BA2.4	0.73 ± 0.21 (5)	0.24-1.43	0.33-1.96
BA3.4	0.23 ± 0.041 (4)	0.13-0.33	0.57-1.43
SA7	5.30 ± 0.37 (5)	4.38-6.47	0.83-1.22
M5	0.95 ± 0.22 (8)	0.43-2.07	0.45-2.18
Gly D	4.79 ± 0.36 (8)	3.50-6.32	0.73-1.32
Arg145	5.40 ± 1.10 (9)	1.59-10.38	0.3-1.92
91-4696	4.72 ± 0.84 (7)	1.96-8.04	0.42-1.70
HK188	8.58 ± 1.07 (10)	4.72-14.04	0.55-1.64
AP3.1	6.57 ± 1.33 (6)	1.93-10.72	0.30-1.63
SA4	4.88 ± 0.98 (4)	2.44-7.16	0.50-1.47
SA6	3.91 ± 0.64 (4)	2.51-5.24	0.64-1.34
PJI†	0.14 ± 0.014 (11)	0.08-0.27	0.57-1.93
COS7 cells†	0.15 ± 0.014 (11)	0.09-0.25	0.60-1.67

*Reactivity = (OD) of sample/(OD) of NIBSC standard serum.

†Negative controls are supernatants from COS7 cells alone and cells transfected with the plasmid pJI without cloned HBV surface gene.

also detected more readily than those of subtype *y* using this panel of assays (Tables 2, 3, and 4).

As a measure of transfection/expression efficiency, reproducibility of variant antigen reactivities was determined on supernatants from multiple transfection experiments using the IMX HBsAg (V2) assay (Table 5). The number of supernates tested for each antigen ranged from 4 to 11 and the range of reactivity was relatively consistent at a level of 0.5 to 1.6 times the mean value. Only sample M5 produced a wide range of values, being 0.45 to 2.2 times the mean value.

Transfection efficiency was also measured by repeating 6 of the 7 assays using a separate batch of supernatants from a different transfection experiment (data not shown). Only M5 in assay 1 (bioELISA) gave an obviously different reactivity on repeat testing; 44% of standard sequence activity compared with 13% in a previous experiment). Four other samples of low reactivity (1 in assay 3 and 3 in assay 7) became either borderline positive, having initially been negative, or became negative having initially been borderline positive. We do not consider this to be significant. In fact, the reactivity of these samples compared with that of the appropriate standard GlyY/D (set at 100%) ranged from 6% to 12.6%, a level at which repeat testing would be required in a clinical or diagnostic setting.

To assess intra-test variation, four of the assays (assays 1, 5, 6, and 7) were repeated using the original supernatants. The reactivities of the variants were comparable with the original results with only one borderline positive sample becoming negative on repeat and one negative sample becoming borderline positive in assay 7 (data not shown).

Finally, the variant T5N, containing a 2-aa insertion and mutations in HBs regions 1, 2, and 4, was not detected by any of the assays either in the supernatant or the cell lysate. This either implies gross antigenic diversity or a lack of production. The original serum of this variant was found to be negative by monoclonal antibody-based Auszyme assay, but positive by the polyclonal radioimmunoassay AUSRIA II (Abbott Laboratories Ltd).[4] Perhaps the AUSRIA II positivity was caused by higher viral load in serum or even a mixed population of viruses. However, we cannot exclude the possibility (albeit unlikely) of there being differences in secondary structure of expressed HBsAg compared with the native HBsAg, which would affect reactivity in diagnostic assays. On immunofluorescence, T5N showed only 5% to 10% of cells fluorescing, which indicates some binding by pAbs and therefore some degree of expression; however, it remains possible that the antigen level in the supernatant was insufficient for detection. Otherwise it is not clear why T5N variant was not detected by any of the 7 assays. It has been detected *in vitro* recently.[27] To confirm whether the sequence was adequately expressed, plasmid sequences upstream of the cloning site were found to be identical to those seen in the parent plasmid pJI, so cloned HBsAg expression was unlikely to have been affected by changes in the vector sequence.

DISCUSSION

Our main observation is that all assays are not equally able to detect expressed HBsAg variants, mainly because of the use of anti-HBs antibodies with variable specificities and sensitivities against different HBsAg epitopes. However different levels of HBsAg expression, whether *in vivo* or *in vitro*, could play a role.

Obviously, in any analysis of this nature, standardization of the number of HBsAg particles is required, but this presented difficulties. Electron microscopy was attempted (data not shown) but the particles were difficult to count because of clumping and an uneven distribution. Also, Bradford assay for total protein determination proved unhelpful because HBsAg was masked by large quantities of fetal calf serum. We have now developed an epitope tag system to quantify the numbers of particles in the supernatants (Basuni et al., manuscript submitted) independently of HBsAg antigenicity.

A mammalian expression system was used in the study because the availability of sera containing HBsAg variants is usually limited. We assume there is little or no alteration in the secondary structure of expressed HBsAg compared with the natural material, because all of the post-translational modifications should occur in COS7 cells. However, expressed HBsAg is not absolutely ideal for characterizing variation as measured reactivity depends both on antigenicity and on the total amount of protein. Because of the transient nature of expression and relatively low number of cells, the assay reactivities are lower than would be expected in serum.

In this cohort of samples we observed the following points. First, the samples that displayed similar reactivity to the standard sequence had variation in regions 1, 3, 4, and 5, whereas those with reduced reactivity all had variation within HBs region 2 (1056sp, BA 3.4 ,and BA 2.4 in regions 1, 2, and 4; M5 in regions 1 to 4; BA 3.2 in region 2; and T5N in regions 1, 2, and 4). This region, either solely or discontinuously with other regions clearly contributes to the loss of reactivity. There is also evidence that the 4 aas bounded by cysteines at aa 121 and 124, HBs region 2, form a distinct epitope on the tip of a loop.[28,29] Cysteines within the MHR are responsible for the formation of intramolecular and intermolecular disulfide bridges, which give the HBsAg its highly complex structure. Antigenicity is dependent on this structure, and substitution of many of the cysteine residues results in either reduced or complete loss of immunoreactivity.[25,26,30,31] Alternatively, it could be that there are secondary effects on other regions upstream or downstream from the mutations.[1,31]

Second, it was clear that there was no correlation between

the number of variants and altered antigenicity. It seems to be the site and not the number that is responsible for this reduced reactivity. For example, Arg145 and BA3.4 had single mutations and displayed less than 50% of standard reactivity in 5 and 4 assays, respectively. Samples SA4 and BA 2.4, each with 2 mutations, showed less than 50% of standard reactivity in 5 assays. However, AP3.1 with 1 mutation, HK188 and 91-4696 with 2 mutations, and SA6 with 3 mutations showed good levels of reactivity, with less than 50% in either one or no assays.

Third, some poor reactivity is caused by reduced secretion from cells. A comparison between samples BA3.4, which contains T123N, and BA3.2, which had the additional C124R, is instructive. The loss of cysteine at aa 124 completely abrogated reactivity in all 7 assays when the supernatant was used. When the cell lysate was tested in assays 1, 5, and 6, it displayed reactivities of 28%, 18%, and 15% of that of the GlyY, respectively. Because cell lysate BA3.2 is detected at lower levels compared with BA3.4, we can conclude that the addition of C124R and T123N has a dual effect on both immunoreactivity and secretion.

Fourth, *in vitro* results do not always confirm *in vivo* observations. Supernatants from samples HK188 and 91-4696 (Hino et al., unpublished data, 1997), which were initially HBsAg negative in serum, surprisingly displayed similar reactivity to the positive control samples. The failure to detect serum HBsAg may have been caused by the presence of a low level of HBsAg or, perhaps, because complexes between anti-HBs and HBsAg prevented the antigen from being detected.[32]

Fifth, it has previously been suggested that *ay* subtype samples react less well than those with an *ad* background.[33,34] This also appeared to be the case here; however, this needs confirmation using samples with the same mutations in both subtype backgrounds.

Finally, it is obvious that the ability of an assay to detect a variant depends critically on the choice of anti-HBs used. In general these samples were best detected by assays that used pAbs in the capture and/or detection phases (with the exception of assay 7). Assays that contained mAbs for both phases of the assay appeared to perform less efficiently in detecting this set of variants.

Although currently available HBsAg assays are an improvement on their predecessors, there is a need for further development with ongoing assessment of assay ability to detect the emerging HBsAg variants that are discovered in clinical settings or by other diagnostic methods, *e.g.*, PCR. Manufacturers could also investigate the possibility of using antibodies that are not affected by conformational change within the MHR perhaps by developing antibodies to linear epitopes outwith this region.

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SHORT REPORT

An efficient extraction method from blood clots for studies requiring both host and viral DNA

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SUMMARY. The clot from blood is usually discarded after the collection of serum. Yet, it contains nucleated white blood cells and substantial serum. Here, we have compared four methods to enable quick and efficient extraction of human genomic and viral DNA from clotted blood. Two of these methods, a phenol-based in-house method and Tripure isolation reagent, only achieved a low polymerase chain reaction (PCR) yield. In contrast, the QIAamp blood kit and the High Pure Viral Nucleic Acid kit were equally efficient, with similar sensitivity to serum for extraction of viral DNA.

Keywords: blood clots, DNA extraction, hepatitis B virus, human β-globin.

INTRODUCTION

Genomic markers, including human leucocyte antigen (HLA) typing, of individuals who have either cleared hepatitis B virus (HBV) infection, are chronic carriers or have no evidence of infection, allow the study of host immune factors that may influence the course of infection. Specific HLA class II alleles are associated with both hepatitis B and C viral clearance [1–3]. Many large-scale studies in this area are underway and this field is set to expand in the future. Thus, if testing for HLA and hepatitis viruses is required in addition to other serological markers, there is a need to minimize the blood volume used in laboratory testing, especially in infants. Direct polymerase chain reaction (PCR) amplification from whole blood without prior DNA isolation has been attempted [4], but sensitivity of viral DNA detection is low [5] and the DNA cannot be stored for further investigation [6]. Methods have been described in the literature for DNA extraction from whole liquid blood [7,8] as well as from clotted blood [6,9]. As clotted blood is usually discarded after collection of the serum, extraction of DNA from clots could be useful and efficient. Proteinase K, a powerful, broad-spectrum proteolytic enzyme, has been used in nucleic acid isolation for more than 25 years [10]. In this study we compared four methods, three of which are dependent on digestion with proteinase K, for extraction of both human (β-globin) and viral (HBV) DNA from clotted blood.

Abbreviations: HBV, hepatitis B virus; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

DNA extraction methods

In-house procedure. A pea-sized blood clot was transferred to a sterile Eppendorf tube and the following reagents added: 250 μl nucleic acid lysis mix (0.4 M NaCl, 10 mM Tris, 2 mM EDTA), 250 μl lysis buffer (Applied Biosystems Warrington, Cheshire, UK; cat. no. 400676) and 50 μl proteinase K at 10 mg ml^{-1}. Samples were incubated at 55 °C for 2–3 h or overnight at 37 °C followed by vortexing to dissolve the blood clot. Then, 500 μl phenol–chloroform was added and the DNA precipitated and washed with ethanol. The pellet was left to air-dry and then resuspended in 50 μl of 1× TE (10 mM Tris, 1 mM EDTA) buffer.

TriPureTM Isolation Reagent. One millilitre of TriPure isolation reagent (Roche Diagnostics, Lewes, UK) was added to a pea-sized blood clot in a sterile Eppendorf tube and the cells were lysed by repetitive pipetting. The samples were incubated for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added, the tube was capped securely and then shaken vigorously. Further incubation at room temperature for 2–15 min was carried out followed by centrifugation at 12 000 *g* for 15 min to separate the solution into three phases. After centrifugation, the upper, aqueous, colourless phase containing RNA was carefully removed.

DNA precipitation from the interphase and the red organic phase was performed using 96% ethanol. Samples were washed three times with 0.1 M sodium citrate in 10% ethanol and then once in 75% ethanol. The DNA pellet was air-dried and then resuspended in 50 μl 8 mM NaOH. Finally, the pH of the isolated DNA was adjusted to 8.4 using 0.1 M HEPES.

High Pure Viral Nucleic Acid kit and QIAamp blood kit. For the High Pure Viral Nucleic Acid kit (Roche Diagnostics), 200 μl of working solution [binding buffer supplemented with poly (A) carrier (RNA)], and 40 μl 20 mg ml^{-1} of proteinase K were added to a pea-sized blood clot in a sterile Eppendorf tube, mixed and incubated for 10 min at 72 °C. After incubation, 100 μl of isopropanol was added. The filters and collection tubes were combined and the samples pipetted into the upper reservoir followed by centrifugation for 1 min at 8000 *g* and the flowthrough discarded. The filter was washed twice with the wash buffer and the flowthrough discarded after each wash. Finally, centrifugation was performed for 10 s at full speed (12000 *g*) to remove all the residual wash buffer. Collection tubes were discarded and clean, nuclease-free 1.5-ml tubes were used to collect the eluted DNA in 50 μl of elution buffer.

Although the High Pure Viral Nucleic Acid kit (Roche Diagnostics) and the QIAamp blood kit (Qiagen Ltd, Crawley, UK) kits employ the same principle, the reagents are different. For the Qiagen kit, Buffer AL, Qiagen protease, ethanol and buffer AW were used, respectively, instead of the working solution, proteinase K, isopropanol, and wash buffer that were employed in the Roche kit.

β-*globin PCR*

Five microlitres of extracted DNA were amplified in 50 μl of master mix containing 1.25 U *Taq* polymerase (Gibco, Paisley, Strathclyde, UK), 2.5 U *Taq*Start™ antibody (Clontech Laboratories Inc., Palo Alto, CA), 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 10× PCR buffer (supplied with *Taq* polymerase) and 20 pmol of each primer, Pco3 (5′-ACACAACTGTGTTCACTAGC-3′) and Pco4 (5′-CAACTTCATCCACGTTCACC-3′). The amplification cycles were as follows: 5 min at 94 °C, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The product was 110 bp.

HBV DNA PCR

The same master mix used for β-globin PCR was used for HBV DNA PCR, except that the $MgCl_2$ concentration was 2.5 mM and HBV S-gene primers were used. The first-round primers were: 5′-CCTGCTGGTGGCTCCAGTTC-3′ and 5′-CCACAATTCKTTGACATACTTTCCA-3′, where K = G or T. Five cycles were performed at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 90 s, followed by 35 cycles with the denaturation temperature reduced to 90 °C. One microlitre of the first-round PCR product was reamplified in a second round with nested primers (5′-GCACACGGAATTCCGAGGACTGGGGACCCTG-3′ and 5′-GACACCAAGCTTGGTTAGGGTTTAAATGTATACC-3′) for five cycles at 95 °C for 1 min, 55 °C for 75 s and 72 °C for 90 s followed by 25 cycles with the denaturation temperature reduced to 90 °C. The product was 681 bp.

Materials: human β-globin and HBV DNA

In the initial extraction, we amplified β-globin from clots of HBV-negative blood. As a control, DNA was used that had been extracted, using a standard technique, from primary human embryonic lung cells (MRC5) infected with the human cytomegalovirus (HCMV).

The HBV DNA source was blood from two HBV carrier patients with a low level of viraemia. Subsequent precise analyses were performed on a dilution series of HBV-positive stock serum (our internal laboratory standard) diluted in normal whole blood that was negative for HBV. The dilution series ranged from 10^{-3} to 10^{-6}, equivalent to 4×10^4–4×10 genome equivalents per ml (gEq ml^{-1}). After leaving the blood to clot, sera and clots were separated by centrifugation and aliquoted. Our positive serum standard for HBV PCR at a titration of 10^{-6} (40 gEq ml^{-1}) was also extracted.

RESULTS AND DISCUSSION

The kits and reagents were assessed for extraction of human DNA (β-globin) on HBV-negative blood and then on HBV-carrier patients in order to measure the HBV DNA levels simultaneously. Equal volumes of the PCR products were run on 1% agarose gels stained with ethidium bromide. The PCR yield, reflecting the quantity and perhaps the purity of the isolated DNA, was used as an approximate estimate of the DNA isolation ability of each kit. The Qiagen and Roche High Pure kits had unmistakably brighter DNA bands, as shown in Table 1 and Fig. 1.

The Qiagen and Roche High Pure kits were then assessed for sensitivity using a dilution series of a positive serum sample containing HBV DNA of known concentration in

Table 1 Comparison of the polymerase chain reaction (PCR) yield using four different extraction methods

Method	Intensity of PCR bands*
QIAamp blood kit	+ + +
High Pure Viral Nucleic Acid kit	+ + +
In-house procedure	+ +
TriPure™ Isolation Reagent	–

*Intensity of PCR bands: + + +, high; + +, moderate, –, not detected.

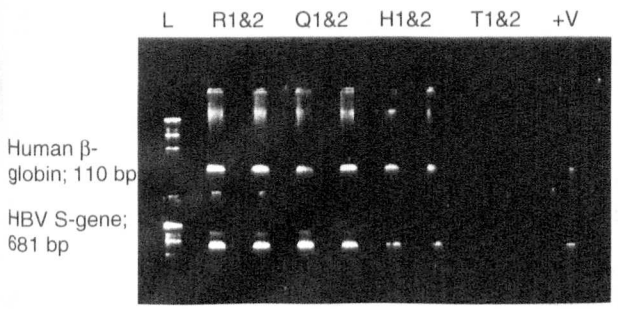

Fig. 1 Comparison of polymerase chain reaction (PCR) yield from two carrier patients with low viraemia, using four different extraction methods. R1&2, extraction using the Roche High Pure Viral kit; Q1&2, extraction using the Qiagen QIAamp blood kit; H1&2, extraction using an in-house procedure; T1&2, extraction using the TriPure Isolation Reagent; +V, positive control; L, 100-bp molecular weight ladder.

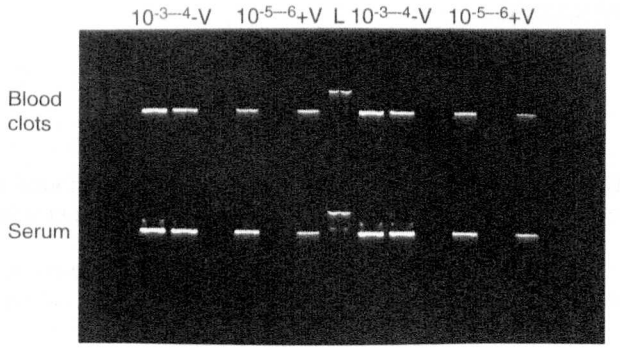

Fig. 2 Hepatitis B virus (HBV) DNA extraction sensitivity of Qiagen and Roche kits. Polymerase chain reaction (PCR) results are presented from 10^{-3} to 10^{-6} dilutions of the HBV-positive control extracted using the Qiagen and Roche kits. –V, negative control; +V, positive control; L, 100-bp molecular weight ladder.

negative blood. Both sera and blood clots of these dilutions were extracted and PCR was carried out in the same run. For both sera and blood clots, the PCR yield was consistent between the two kits: HBV DNA was detected up to a dilution of 10^{-5}, equivalent to 4×10^{2} gEq ml^{-1} (Table 2; Fig. 2). However, the positive control serum gave a positive result at 4×10 gEq ml^{-1}, perhaps because dilution of the stock serum was performed in negative serum and not whole blood.

Thus, both Qiagen and Roche High Pure kits were equally efficient and sensitive for extraction of DNA from clotted blood, as well as simple to use and widely available. Moreover, our experience with these methods revealed that:

1 Blood clots do not have to be completely dissolved because the required incubation time with proteinase K is only 10 min.
2 Extreme care must be taken on transferring the digested blood to avoid any debris that could obstruct the filter in the column.
3 A higher centrifugation speed (10 000 *g*) is preferred to that recommended (8000 *g*) because the partially digested blood is heavier than serum.

Table 2 Hepatitis B virus polymerase chain reaction (HBV PCR) results of serum and blood clots extracted by Qiagen QIAamp blood and Roche High Pure Viral Nucleic acid kits

	QIAamp blood kit		High Pure Viral Nucleic Acid kit	
Sample dilution	Serum	Blood clot	Serum	Blood clot
10^{-3}	+	+	+	+
10^{-4}	+	+	+	+
10^{-5}	+	+	+	+
10^{-6}	–	–	–	–

Control serum was also amplified by PCR; the titre was 10^{-6} (4×10 genome equivalents per ml).

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ERRATUM

Huang Y-H, Wu J-C, Chiang T-Y *et al.* Detection and viral nucleotide sequence analysis of transfusion-transmitted virus infection in acute fulminant and non-fulminant hepatitis. *J Viral Hepat* 2000; 7: 56–63

A copyediting mistake led to the affiliation and correspondence addresses being given as *Taiwan, China* rather than *Taiwan, Republic of China*. The corrected details are reprinted below.

Detection and viral nucleotide sequence analysis of transfusion-transmitted virus infection in acute fulminant and non-fulminant hepatitis

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