OCCULT HEPATITS B VIRUS (HBV) INFECTION IN THE CHACMA BABOON (*PAPIO URSINUS ORIENTALIS*)

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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

Signed: .....

..... day of ....., 20.....

For my grandmother

Mary Clark Milner Dickens-Smith

‘Hippie’

29/06/1918 – 5/12/2002

Presentations

International Conference:

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Presentation: Molecular characterisation of the baboon hepadnavirus.

Abstract

Members of the family *Hepadnaviridae* have been detected in both avian and mammalian species. They have a very limited host range, and among the non-human primates, have been found to occur naturally in chimpanzees, gorillas, gibbons, orang-utans and woolly monkeys. The human hepatitis B virus (HBV) has been shown to infect chimpanzees, Barbary macaques and tree shrews. During the course of a previous study, to determine the susceptibility of baboons (*Papio ursinus orientalis*) to HBV infection, HBV DNA was detected in the serum of 2 baboons prior to their inoculation with HBV-positive human serum, raising the possibility that baboons are naturally infected with a hepadnavirus. Therefore the aim of this study was to determine the prevalence of HBV in wild-caught baboons and to molecularly and functionally characterise the virus isolated from these baboons.

DNA was extracted from the sera of wild-caught baboons and four separate regions of the HBV genome amplified by nested polymerase chain reaction (PCR). Samples were only considered to be positive for HBV if at least three of these regions amplified. DNA was extracted from the liver tissue of one of the HBV DNA-positive baboons using a proteinase K digestion followed by a phenol-chloroform extraction and ethanol precipitation. From this extract, the complete HBV genome was amplified by nested PCR of eight overlapping subgenomic fragments, and sequenced. This sequence was analysed phylogenetically using both the PHYLIP and Simmonic software packages. A selective real time PCR using SYBR®-green detection was used to detect covalently closed circular (ccc) DNA. RNA was extracted from the baboon liver tissue using a guanidinium-acid-phenol extraction method, reverse transcribed and portions of the HBV genome amplified by nested PCR. Transmissibility of the virus was tested by injecting four experimentally naïve baboons individually with serum from four HBV DNA-positive baboons and followed for 26 weeks.

HBV was detected in the serum of 5/69 (7,2%) wild-caught baboons by Southern hybridization and in 11/49 (22,4%) adult and 4/20 (20,0%) juvenile wild caught

baboons. This gave an overall prevalence of 21,7% in the baboon population surveyed. Serologically, the baboon sera were negative for all markers of HBV infection and alanine aminotransferse (ALT) levels were normal. In the one baboon liver tissue available, HBcAg was detected by immunohistochemical staining in some of the hepatocyte nuclei, but HBsAg was not detected.

Phylogenetic analysis of the complete genome of the HBV isolate found it to cluster with subgenotype A2, a surprising result considering that subgenotype A1 predominates in South Africa. However, unlike other subgenotype A2 isolates, the basic core promoter had the G1809T / C1812T double mutation characteristic of subgenotypes A1 and A3 and the precore region had the G1888A mutation unique to subgenotype A1. These mutations in the basic core promoter and pre-core regions have previously been shown to reduce the expression of the precore and core proteins. Four additional mutations in the polymerase, surface, X and core open reading frames (ORFs) further differentiated the baboon HBV strain form the majority of previously sequenced subgenotype A2 isolates.

cccDNA was detected at low levels in the baboon liver tissue. Regions of the precore/core and surface ORFs were amplified off reverse transcribed cDNA. These results demonstrate HBV replication in the baboon liver. Transmission of the virus was shown by the detection of HBV DNA in the sera of the four inoculated baboons at various times throughout the 26 week follow-up period. These baboons also showed transient seroconversion for HBsAg and HBeAg during this period with intermittent fluctuations in ALT levels. Moreover, using DNA extracted from liver tissue obtained at necropsy from one of the injected baboons, the sequence of the HBV surface gene amplified was found to be identical to the sequence of the isolate from inoculum.

The finding of subgenotype A2 in the baboon is paradoxical because subgenotypes A1 and A3 as well as genotypes D and E predominate in Africa. The possibility exists that subgenotype A2 is an older strain that has been overtaken by these other strains. There is however a scarcity of subgenotype A2 sequencing data from Africa and a higher circulation of this subgenotype could be uncovered with more extensive molecular epidemiological studies in more remote

areas. Alternatively, a recent discovery of alternative compartmentalization of subgenotype A2 infections in the peripheral blood lymphocyte population of individuals from India, where subgenotype A1 also predominates, could explain the lack of detection of this subgenotype in Africa.

Occult hepatitis B infection is defined as the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for HBsAg by currently available assays. The detection of HBV DNA in the baboon liver and serum in the absence of serological markers therefore classifies this infection as occult. To our knowledge, this is the first study to demonstrate a naturally occurring occult HBV infection in non-human primates.

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List of Abbreviations

ALT		-	Alanine aminotransferase levels
Amino acids:	A	-	Alanine
	C	-	Cysteine
	E	-	Glutamic acid
	G	-	Glycine
	P	-	Proline
	R	-	Arginine
	S	-	Serine
	V	-	Valine
Anti-HBc		-	Antibodies to the HBV core antigen
Anti-HBe		-	Antibodies to the HBV e antigen
Anti-HBs		-	Antibodies to the HBV surface antigen
Anti-WHc		-	Antibodies to the WHV core antigen
Anti-WHs		-	Antibodies to the WHV surface antigen
AP-1		-	Activating protein-1
ASHV		-	Arctic squirrel hepatitis virus
BCP		-	Basic core promotor
bp		-	Base pair
C&G		-	Church and Gilbert buffer
C/EBP		-	CCAAT/enhancer-binding protein
cccDNA		-	Covalently closed circular DNA
cDNA		-	Complimentary DNA
ChHBV		-	Chimpanzee hepatitis B virus
cm		-	Centimeter
°C		-	Degrees celsius
DEPC		-	Diethyl pyrocarbonate
DHBV		-	Duck hepatitis B virus
DNA		-	Deoxribonucleic acid
DR1		-	Direct repeat 1
DR2		-	Direct repeat 2
ε		-	Encapsidation signal

EDTA	-	Ethylene diamine tetra-acetic acid
ELISA	-	Enzyme-linked immunosorbent assay
Enh1	-	Enhancer 1
Enh2	-	Enhancer 2
EtBr	-	Ethidium bromide
Extn	-	Extraction
GAPDH	-	Glyceraldehyde – 3 – phosphate dehydrogenase
gGT	-	Gamma-glutamyltranspeptidase
GiHBV	-	Gibbon hepatitis B virus
GoHBV	-	Gorilla hepatitis B virus
GSHV	-	Ground squirrel hepatitis virus
HAV	-	Hepatitis A virus
HBcAg	-	Hepatitis B virus core antigen
HBeAg	-	Hepatitis B virus e antigen
HBsAg	-	Hepatitis B virus Surface antigen
HBV	-	Hepatitis B virus
HBx	-	Hepatitis B virus x protein
HCC	-	Hepatocellular carcinoma
HCV	-	Hepatitis C virus
HDV	-	Hepatitis delta virus
HEV	-	Hepatitis E virus
HHBV	-	Heron hepatitis B virus
HIV	-	Human immunodeficiency virus
HNF3	-	Hepatocyte nuclear factor 3
HNF4	-	Hepatocyte nuclear factor 4
HRP	-	Horseradish peroxidase
ICD	-	Isocitrate dehydrogenase
Ig	-	Immunoglobulin
kb	-	kilobase
LB	-	Luria-Bertani
mAU	-	One mAU is the activity that releases folin-positive amino acids and peptides corresponding to 1 µmol tyrosine per minute
µg	-	Microgram

mg		-	Milligram
min		-	Minute
µl		-	Microliter
ml		-	Milliliter
µm		-	Micron
µM		-	Micromolar
MOPS		-	3-[N-morpholino]-2-hydroxypropanesulphonic acid
mRNA		-	Messenger RNA
NAT		-	Nucleic acid testing
NF-1		-	Nuclear factor 1
NF-κB		-	Nuclear factor-kappa B
ng		-	Nanogram
NIH		-	National Institute of Health
nm		-	Nanometer
nt		-	Nucleotide
nucleotides:	A	-	Adenine
	C	-	Cytosine
	G	-	Guanine
	T	-	Thymine
OBI		-	Occult hepatitis B virus infection
OD		-	Optical density
OHB		-	Occult hepatitis B
ORF		-	Open reading frame
OuHV		-	Orang-utan hepadnavirus
PBMC		-	Peripheral blood mononuclear cell
PBS		-	Phosphate buffered saline
PCR		-	Polymerase chain reaction
pgRNA		-	Pregenomic RNA
POI		-	Primary occult infection
RFLP		-	Restriction fragment length polymorphism
RNA		-	Ribonucleic acid
rRNA		-	Ribosomal RNA
rpm		-	Revolutions per minute

s	-	Second
SDS	-	Sodium dodecyl sulphate
SOI	-	Secondary occult infection
SSC	-	Saline sodium citrate
U	-	Unit
v/v	-	Volume per volume
vge	-	Viral genome equivalents
WHO	-	World Health Organisation
WHsAg	-	Woodchuck hepatitis virus surface antigen
WHV	-	Woodchuck hepatitis virus
WMHBV	-	Woolly monkey hepatitis B virus
w/v	-	Weight per volume

1. Introduction

Hepatitis viruses infect the liver, causing both acute and chronic diseases. According to the World Health Organisation (WHO), approximately 2 billion people worldwide have been infected with the hepatitis B virus (HBV), 350 million people are chronic carriers of the virus and 600 000 die each year as a result of either acute or chronic infections with the virus (WHO, 2008). Approximately 10% of adults and 90% of children infected with HBV become chronically infected (Sugiyama *et al.*, 2007).

1.1 The hepatitis B virus

One of the first records of hepatitis or yellow jaundice epidemics was by Pope Saint Zacharias in the 8th century, and descriptions of the disease can be found as far back as the writings of Hippocrates (Deinhardt, 1976). Hippocrates described hepatitis as a disease "produced by black bile when it flows into the liver" and correctly listed the symptoms as anorexia, vomiting, fever, livid or pale-yellow complexion, and liver pain (Lai and Locarnini, 2002).

The hepatitis viruses comprise a group of five unrelated, often unusual pathogens, designated hepatitis A through E, grouped according to the disease they cause rather than their virological properties (Zuckerman, 1997; Howard, 2002).

HBV is a partially double-stranded DNA virus that replicates by reverse transcription. It is classified in the family *Hepadnaviridae* (for HEPAtropic DNA VIRUSES). The *hepadnaviridae* are further separated into two genera – the orthohepadnaviruses found in mammals and the avihepadnaviruses found in birds (Ganem, 1996; Zuckerman, 1997; Howard, 2002). Although these two genera differ in genetic organisation, structure and biological properties – for example the avihepadnaviridae lack a separate gene encoding the HBV-X protein - all the hepadnaviruses are able to induce persistent infections in their natural hosts (Howard, 1994; Scaglioni *et al.*, 1996).

All hepadnaviruses share a number of common properties. They: are enveloped viruses containing a 3 - 3,3 kb relaxed-circular, partially double-stranded genome; have a viral polymerase able to repair the gap in the DNA template; produce an excess of subviral lipoprotein particles composed of envelope proteins; have a narrow host range infecting only species closely related to their natural host and produce persistent infections that are largely (but not completely) hepatotropic (Ganem, 1996).

HBV infections are often associated with hepatocellular carcinoma (HCC) in humans and the integration of viral DNA into the host's genome has been found in many of these tumours. Approximately one-third of all cases of cirrhosis and one-half of all HCC cases can be attributed to chronic HBV infections. Because HCC is the 4th most common cause of cancer- related deaths globally, chronic HBV infections are considered to be, after tobacco, the most common carcinogen to which humans are exposed (de Franchis *et al.*, 2003; Shepard *et al.*, 2006; WHO, 2009). HBV is transmitted via sexual contact, blood and blood products and, according to the WHO, is 50 to 100 times more infectious than the human immunodeficiency virus (HIV) (Zuckerman, 1997). It can remain stable and infectious on an environmental surface for more than 7 days (Shepard *et al.*, 2006).

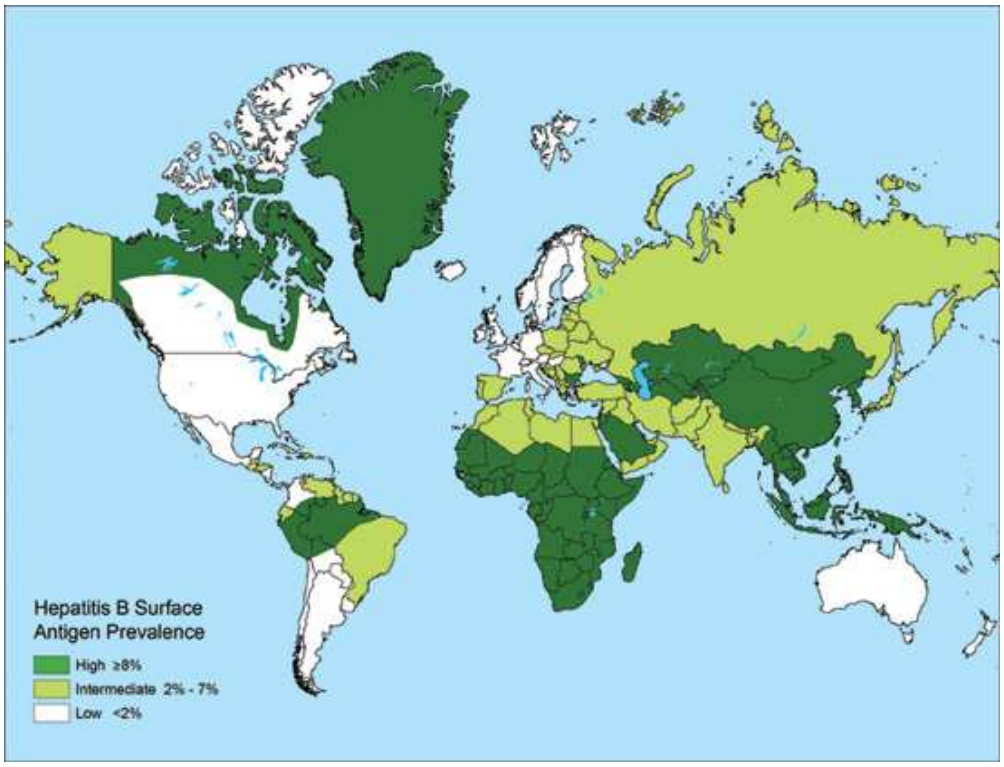


Figure 1: Geographical prevalence of HBV. The prevalence of the hepatitis B surface antigen, a marker of chronic HBV infections, can be used to show the worldwide endemicity of HBV. Source: http://www.library.northwestern.edu/govinfo/news/090519.jpg (accessed 9 December 2009)

1.2 Clinical manifestations of HBV infection

The prevalence of HBV differs greatly in different parts of the world (figure 1). Areas with low HBV endemicity (less than 1% chronic infections) include North-western Europe, North America and Australia (de Franchis *et al.*, 2003). Transmission of the virus in these countries occurs mainly via high risk sexual activity and injection drug use. Areas with intermediate HBV endemicity (between 1% and 8% chronic infections) include the Mediterranean, Middle East and the Indian subcontinent. Sub-Saharan Africa, South East Asia, the Pacific Islands and parts of the Arctic are classified as regions highly endemic for HBV with a chronic infection prevalence of greater than 8% (Brechot, 1996; de Franchis *et al.*, 2003; Fattovich, 2003). In South East Asia, the source of infection in highly endemic regions is mainly perinatal (with a small contribution from

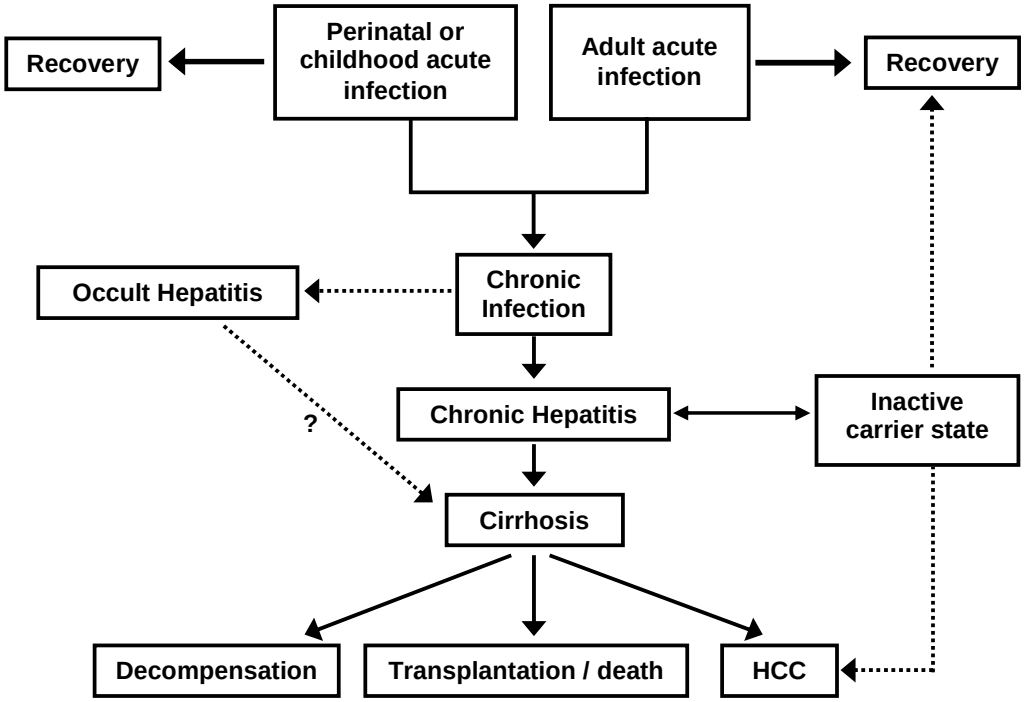


Figure 2: Outcomes of HBV infection. This schematic representation provides a summary of the natural history of HBV infections.

transplacental transmission) whereas in sub-Saharan Africa, infections are mainly transmitted through horizontal infection during infancy and early childhood. Clinical observations have shown that HBV infection can evolve from acute hepatitis to chronic hepatitis, cirrhosis and finally HCC (figure 2) (Brechot, 1996). The outcome of an infection is determined largely by the host's immune response, with the severity of the hepatic injury correlating directly with the strength of this immune response to one or more of the viral proteins (Rehermann *et al.*, 1995; Villeneuve, 2005). Persistent infections can be separated into three distinct clinical states based on their serological profiles: chronic HBV, inactive or asymptomatic carriers, and occult HBV (table 1) (Torbenson and Thomas, 2002).

Table 1: Serological profiles of patients with HBV infection (Torbenson and Thomas, 2002).

Serological Tests	HBV Immunisation	Acute HBV	HBV Recovery	Chronic HBV	Inactive or asymptomatic carrier	Occult HBV
Anti-HBs	+	-	+	-	-	- / +
Anti-HBc	-	+	+	+	+	- / +
Anti-HBe	-	-	+	-	+	- / +
HBsAg	-	+	-	+	+	-
HBeAg	-	+	-	+	-	- / +
HBV DNA	-	+	-	+, $>10^5$ copies	+, $<10^5$ copies	+, $<10^3$ copies

1.2.1 Acute infections

Acute HBV infections have an average incubation period of 90 days (range: 60 – 150 days) and can be defined as an abrupt clinical, biochemical, and/or histopathological manifestation of hepatic injury that occurs within six months of HBV exposure and that resolves spontaneously, in more than 90% of cases, within six months of the onset of symptoms (de Franchis *et al.*, 2003; Raimondo *et al.*, 2003; Shepard *et al.*, 2006). Clinical symptoms include nausea, vomiting, abdominal pain, fever, jaundice, dark urine, changes in stool colour, and hepatomegaly (Shepard *et al.*, 2006). HBV DNA can normally be detected one to two weeks after exposure, during which time patients generally have no symptoms and only very slightly increased serum alanine aminotransferase (ALT) levels (Fattovich, 2003; Huang *et al.*, 2006). The first serological HBV markers, namely HBsAg and hepatitis B e antigen (HBeAg) may be detected (Fattovich, 2003; Shepard *et al.*, 2006).

Two to six weeks after exposure patients enter an immunoactive phase that is characterised by a decrease in serum HBV DNA and an increase in ALT levels and histological activity, indicating lysis of infected hepatocytes by the host's immune system. HBV antibodies, including antibodies to the hepatitis B core antigen (Anti-HBc), can be detected between four and eight weeks post exposure (Huang *et al.*, 2006; Shepard *et al.*, 2006). In the third phase of an acute HBV infection, there is a reduction of serum HBV DNA to below 10^5 copies per ml, normalisation of ALT levels, resolution of any necroinflammation and seroconversion from HBeAg to anti-HBe (Fattovich, 2003).

Patients are considered to have resolved the HBV infection when they become HBsAg negative and develop antibodies to HBsAg (anti-HBs), indicating clearance of the virus (Yim and Lok, 2006).

A severe form of acute HBV infection is fulminant hepatitis in which the course of the infection is complicated by encephalopathy, with HBsAg often being undetectable at the time of diagnosis (de Franchis *et al.*, 2003). The severity of

the liver injury in fulminant hepatitis indicates a vigorous immune response from the host and is associated with rapid viral clearance (Villeneuve, 2005).

1.2.2 Chronic infections

Chronic HBV infections can be defined as the presence of HBsAg in the serum of an infected individual for at least six months or as the presence of HBsAg in a patient negative for immunoglobulin (Ig) M antibodies to the hepatitis B core antigen (HBcAg) (Shepard *et al.*, 2006). Approximately 90% of babies perinatally infected with HBV and 30% of children horizontally infected develop chronic infections, but only 5 - 10% of adult infections become chronic (de Franchis *et al.*, 2003; Huang *et al.*, 2006; Yim and Lok, 2006). Most chronically infected individuals present with HBeAg-positive chronic hepatitis B ten to thirty years after their initial infection (Fattovich, 2003). Chronic HBV infections can be divided into four phases: immune tolerance; immune clearance (HBeAg positive); inactive carrier (HBeAg negative) and reactivation, although not all individuals pass through every phase (Yim and Lok, 2006).

In the initial immune tolerance phase, analogous to the incubation period of an acute infection, infected individuals have high HBV viral loads and are positive for HBeAg (Villeneuve, 2005). Their ALT levels are normal and there is minimal or no liver inflammation. Most of those infected at birth or in early childhood pass through this phase, which can last between one and four decades (de Franchis *et al.*, 2003; Yim and Lok, 2006). In individuals infected in later childhood or as adults this phase is either very short or completely absent.

The second phase, the immune clearance phase, can be compared to symptomatic hepatitis in acutely infected individuals. HBeAg persists along with high HBV viral loads and abnormal ALT levels (de Franchis *et al.*, 2003; Villeneuve, 2005; Yim and Lok, 2006). The immunological response of the host results in active inflammation of the liver tissue, and hence progressive liver damage. There is an increased T cell response to HBcAg and HBeAg resulting in the lysis of infected hepatocytes and flares of aminotransferase levels. The frequency and the severity of these flares increase the risk of cirrhosis and HCC developing. The immune

clearance phase culminates in seroconversion from HBeAg to anti-HBe. This seroconversion occurs spontaneously at a rate of 5 – 10% per year, but may also result from treatment with interferon or other nucleoside analogs.

The third phase of chronic HBV infection, the inactive carrier state, is characterised by the absence of HBeAg, the presence of HBsAg and anti-HBe, low or undetectable serum HBV DNA levels ($<10^5$ copies/ml), minimal to no liver inflammation and normal ALT levels. In some individuals, this state may persist indefinitely resulting in a sustained remission and a generally good prognosis.

Approximately 1 - 5% of chronically infected individuals progress from the inactive carrier state to the fourth phase of infection, where there is a reactivation of HBV replication leading to HBeAg-negative chronic hepatitis B. HBsAg, anti-HBe and HBV DNA are all detectable in the serum but HBeAg is not, ALT levels are elevated and there is necro-inflammation of the liver tissue.

Annually, approximately 8 - 10% of patients with HBeAg-negative chronic HBV infections develop cirrhosis compared to 2 - 5% in HBeAg-positive patients (Villeneuve, 2005; Yim and Lok, 2006). Patients with chronic HBV infections and cirrhosis have a five year cumulative risk of 15 - 20% of developing HCC. Additional risk factors for the progression to cirrhosis and HCC include being male (suggesting a tumourogenic effect of androgens especially high serum testosterone levels); older age; alcohol abuse (a six fold higher risk of progression to cirrhosis); co-infection with HCV, HDV or HIV; aflatoxin exposure; cigarette smoking; increased levels of HBV replication and the genotype of the HBV infection (Fattovich, 2003; Villeneuve, 2005; Chan and Sung, 2006; Yim and Lok, 2006).

1.2.3 Occult Infections

Occult HBV infections (OBIs) are defined as

"the existence of HBV DNA in serum, lymphoid cells (PBMC) and / or the liver and virus genome replicative intermediates (i.e. covalently closed circular DNA (cccDNA) and / or mRNA) in lymphoid and / or hepatic tissue in the absence of serum HBV surface antigen, symptoms and biochemical evidence of liver injury" (Michalak *et al.*, 2007).

OBIs can therefore only be definitively diagnosed by the detection of HBV DNA in the liver tissue. Screening for HBV DNA in the serum alone underestimates up to 40% of the OBI cases (Gibney *et al.*, 2008).

The existence of OBI was first suspected early in the 1980s with the first cases being reported from the mid 1980s (Raimondo *et al.*, 2007). In these cases, a number of both alcoholic and non-alcoholic patients with varying degrees of liver damage from normal liver function to alcoholic cirrhosis were reported to have HBV DNA present in their serum in the absence of HBsAg (Nalpas *et al.*, 1985). The transmissibility of these infections was demonstrated by injecting serum from two individuals (negative for all conventional HBV markers, but positive for HBV DNA) into two chimpanzees, which subsequently developed acute hepatitis (Thiers *et al.*, 1988). Over the years, OBIs have been variably called "serologically silent hepatitis B", "silent hepatitis B", "surface antigen negative carriers", "inapparent HBV", "unrecognised HBV" and "cryptic" infections (Torbenson and Thomas, 2002; Pollicino *et al.*, 2004). Approximately 20% of all OBIs are negative for all serological HBV markers (termed seronegative OBI), with the remainder being positive for anti-HBc and/or anti-HBs in the serum (seropositive OBI) (Raimondo *et al.*, 2008a; Hollinger and Sood, 2010).

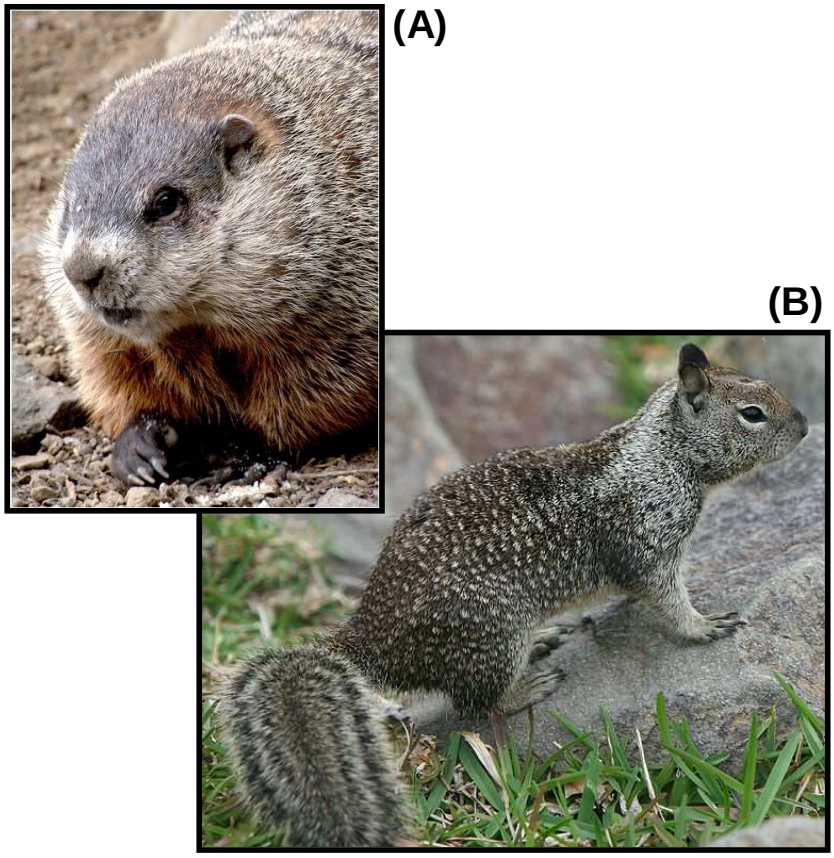


Figure 3: Animal models of occult infections. Occult HBV infections have been detected in (A) woodchucks (*Marmota monax*) and (B) ground squirrels (*Spermophilus beecheyi*). Photograph (A) by Eiffelle and photograph (B) by Thomas O'Brien.

1.2.3.1 Occult woodchuck hepatitis virus infections

Occult infections have been identified in both ground squirrels and woodchucks (figure 3) infected with the ground squirrel hepatitis virus (GSHV) and woodchuck hepatitis virus (WHV) respectively (Raimondo *et al.*, 2007). WHV is closely related to HBV especially in terms of its molecular and pathobiological properties (Coffin *et al.*, 2004). Woodchucks that have recovered both serologically and histologically from acute hepatitis, maintain a lifelong persistence of small amounts of viral DNA in both their liver and lymphoid tissues, with the liver being the main site of viral replication (Michalak *et al.*, 1999). The occult infections frequently result in a low grade inflammation of the liver tissue and HCC develops in approximately 20% of woodchucks within three to five years of apparent resolution of the acute infection (Michalak *et al.*, 1999).

Antibodies against WHV core antigen (anti-WHc) alone can be detected continuously in seropositive OBI as opposed to seronegative OBI, where serological markers are absent (Michalak *et al.*, 1999; Coffin *et al.*, 2004). The maintenance of anti-WHc indicates a sustained stimulation of the woodchuck immune system by the nucleocapsid proteins and the presence of small amounts of WHV covalently closed circular (ccc) DNA indicate low levels of ongoing viral RNA transcription and protein translation (Menne *et al.*, 2007). A subsequent study has shown that female woodchucks with occult infections can transmit WHV to their offspring as an asymptomatic, serologically-negative infection that progresses for years post-natally and, in some cases, results in HCC. This virus was shown to be biologically competent because WHV could be transmitted from the offspring to healthy animals (Coffin and Michalak, 1999).

In the woodchuck, OBIs have been divided into two categories:

- ***primary occult infections*** (POIs), which are confined to the lymphatic system and have very low levels of replication (Michalak *et al.*, 2004; Michalak *et al.*, 2007). These infections are found in offspring born to dams recovered from acute infections and experimentally in adult woodchucks inoculated with either small doses of WHV ($<10^3$ viral genome equivalents (vge)) or with lymphoid cells from animals with long resolved acute infections. The POIs found in the woodchuck are negative for all serological markers and are thus analogous to seronegative OBIs in humans and do not confer protection against subsequent infection.

- ***secondary occult infections*** (SOIs) occur in both the liver and lymphatic tissues of woodchucks following recovery from an acute infection (Michalak *et al.*, 2004; Michalak *et al.*, 2007). SOIs are negative for WHV surface antigen (WHsAg) but positive for anti-WHc and sometimes antibodies to WHsAg (anti-WHs), making them analogous to seropositive human OBIs. They provide protection against re-exposure to pathogenic doses of WHV (Mulrooney-Cousins and Michalak, 2007).

1.2.3.2 Methods of OBI detection

Occult HBV is characterised by low copy numbers of viral genomes per cell in liver tissue and very low levels of circulating viral DNA - serum levels below 10^3 vge/ml and averaging 10^2 vge/ml (Brechot *et al.*, 2001). These levels are much lower than those found in HBsAg positive, HBeAg negative sera, which average 10^4 vge/ml and HBsAg positive, HBeAg positive sera which have more than 10^8 vge/ml (Brechot *et al.*, 2001; Chemin and Trepo, 2005). The reasons for these very low levels are not known but it is thought that both host and viral factors could contribute (Carreno *et al.*, 2008). The host immune response is hypothesised to play a role in keeping viral replication levels low, a theory supported by the fact that viral replication is reactivated in immunosuppressed patients with OBI.

Although OBIs can be diagnosed by the testing of serum or peripheral blood mononuclear cells (PBMCs) for HBV DNA using a highly sensitive PCR, the most accurate way of identifying these infections is by the detection of HBV DNA in the liver (Mulrooney and Michalak, 2003; Carreno *et al.*, 2008; Raimondo *et al.*, 2008a). Total DNA extractions using Proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation, result in maximum amounts of high quality nucleic acid and so are preferred to ready-to-use extraction kits (Michalak *et al.*, 2007).

The detection of HBV DNA in the absence of HBsAg by the nested PCR amplification of at least three non-overlapping, highly conserved regions of the HBV genome, with viral DNA being detected in at least two of these regions, is the gold standard for detection of OBI (Brechot *et al.*, 2001; Raimondo *et al.*, 2007; Raimondo *et al.*, 2008a). The sensitivity and specificity of detection can be increased by Southern hybridisation of the nested PCR amplicons using an HBV specific probe (Mulrooney-Cousins and Michalak, 2007).

1.2.3.3 Molecular characterisation of HBV isolated from OBIs

It is very difficult to clone full-length genomes from low viraemic carriers and therefore only a limited number of such genomes have been isolated and characterized from OBIs (Hass *et al.*, 2005). Irrespective of genotype, amino acid diversity within the pre-S / S region was shown to be significantly higher in strains from OBI sera compared to strains from chronic carriers, (Candotti *et al.*, 2008). There was also a significant increase in nucleotide diversity in strains isolated from anti-HBs-positive individuals compared to samples without this marker. Substitutions appeared to be concentrated in the Major Hydrophilic Region of the S antigen as well as the CD8+ cytotoxic T-lymphocyte epitopes (Candotti *et al.*, 2008).

1.2.3.4 Clinical implications of occult infections

There are three main clinical implications of OBIs:

- **Transmission**

  The presence of anti-HBs was previously considered to signal resolution of HBV infection, but the identification of OBIs means that blood positive for anti-HBs alone cannot be safely used for transfusion. Transmission of HBV to two immunocompetent transfusion recipients from an anti-HBs positive occult HBV carrier was recently demonstrated (Levicnik-Stezinar *et al.*, 2008). Thus, serological testing of blood alone is not sufficient to guarantee the safety of the blood. Nucleic acid testing (NAT) of blood donations for the presence of HBV DNA is therefore routinely used and in South Africa and Brazil, this method of testing has detected a number of occult infections (Almeida and Cardoso, 2006; Allain *et al.*, 2009). Furthermore, there is a risk of transmission of HBV to orthotopic liver transplant recipients from OBI donors. Therefore NAT of donors is recommended (Ciesek *et al.*, 2008; Raimondo *et al.*, 2008b).

- **Reactivation**

  Reactivation of OBI occurs frequently in individuals undergoing immunosuppressive therapy, for example in patients who have received organ transplants - particularly liver, kidney and bone marrow – and in those receiving systematic chemo-, radio- or immuno-therapy (Besisik *et*

al., 2003; Knoll *et al.*, 2006; Raimondo *et al.*, 2008a). OBIs are also often reactivated in HIV-positive individuals (Mulrooney-Cousins and Michalak, 2007). The prevalence of occult HBV and HIV co-infections remains controversial with reports varying from 0% to 89% (Cohen Stuart *et al.*, 2009). In South Africa, between 7,5% and 22% of HIV-positive and 2,4% of HIV-negative individuals have been reported to be positive for OBIs (Mphahlele *et al.*, 2006; Firnhaber *et al.*, 2009).

- **Liver disease**

  Individuals with OBI and HCV co-infections have an increased risk of developing severe liver disease (Cacciola *et al.*, 1999). OBIs appear to interfere with the clinical outcomes of chronic HCV infections and favour or accelerate the progression to cirrhosis. Individuals with OBI alone also have an increased risk of developing chronic liver disease and HCC (Pollicino *et al.*, 2004; Ikeda *et al.*, 2009). The precise role of OBIs in malignant transformation is however unclear (Ikeda *et al.*, 2009).

In a recent paper, Hollinger and Sood (2010) cautioned that not all cases of occult HBV are clinically relevant because detection of HBV DNA only in the serum does not always translate into infectivity of the virus and they therefore differentiate between occult hepatitis B (OHB) and OBI.

"False" cases of OBI can be identified by serum HBV DNA levels that are comparable to those detected at the various stages of serologically evident infections (Raimondo *et al.*, 2008a; Raimondo *et al.*, 2008b). These "false" cases are usually the result of infections with surface gene mutant HBV strains that produce a modified HBsAg that is not recognised by the detection assay leading to the HBsAg-negative phenotype.

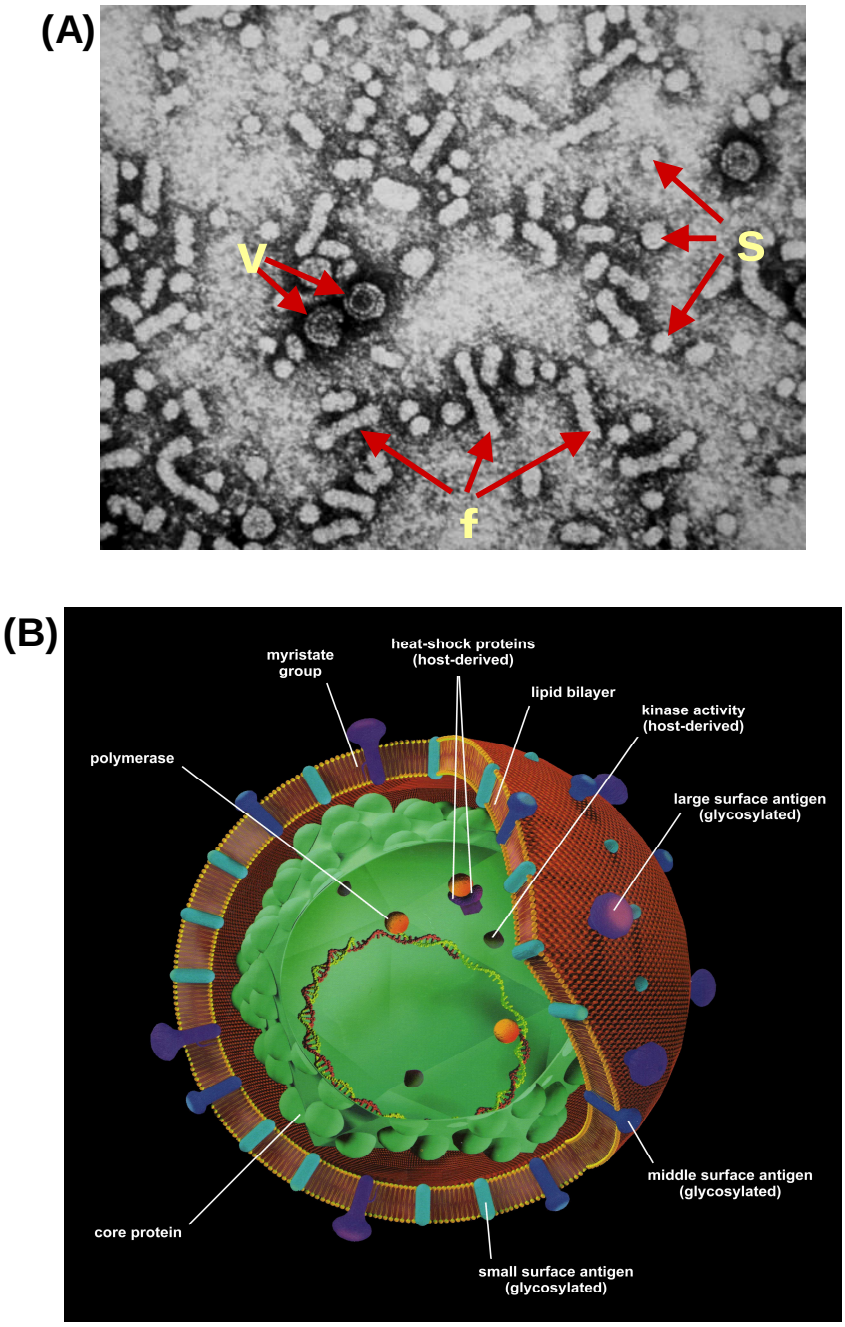


Figure 4: Structures of HBV
Panel A: Electron micrograph of HBV virions. This electron micrograph shows complete virions (v) as well as spherical (s) and filamentous (f) subviral particles which can be detected in the blood of chronic carriers.
Panel B: The architecture of a Dane particle. This schematic shows the structural and enzymatic proteins found within the Dane particle (modified from Lai and Locarnini, 2002).

1.3 HBV structure

The viral agent responsible for hepatitis B was first isolated by Blumberg *et al.* in 1965. Five years later, in 1970, Dane *et al.* described the 42 nm complete HBV virion particle, which they called the Dane particle (Kann, 2002). Electron microscopy of the serum of infected individuals has shown two types of particles – the 42 nm to 47 nm Dane particles at a titre of up to 10^9 particles per ml, as well as an excess of 20 nm spherical subviral particles at a concentration of up to 10^{14} spheres per ml (Kann, 2002). These subviral particles (figure 4, panel A) consist of viral surface proteins and host-derived lipid components and are not infectious as they lack nucleic acids (Ganem, 1996). The Dane particle (figure 4, panel B) is double shelled with the outer shell or envelope consisting of viral surface particles embedded in host derived lipid and the inner shell consisting of an icosahedral nucleocapsid composed of viral core protein (Kann, 2002; Huang *et al.*, 2006).

A 3,2 kb relaxed circular partially double stranded DNA molecule, together with a covalently bound viral polymerase protein on the 5' end of the negative strand, is contained within the nucleocapsid. The negative or minus strand of the DNA molecule has a defined nick and a terminal redundancy of seven to nine nucleotides (Kann, 2002; Huang *et al.*, 2006). The positive or plus strand is of variable length and shorter than the negative strand (Kann, 2002; Yokosuka and Arai, 2006). The binding of the viral polymerase protein to the 5'-end of the negative strand, means that the negative strand is not covalently closed. The positive strand bridges this gap and keeps the genome circular by base pairing on either side of the gap to the complementary negative strand.

1.3.1 HBV gene products

The HBV genome has four overlapping open reading frames (ORFs) encoding the polymerase, envelope, core and X gene products (figure 5).

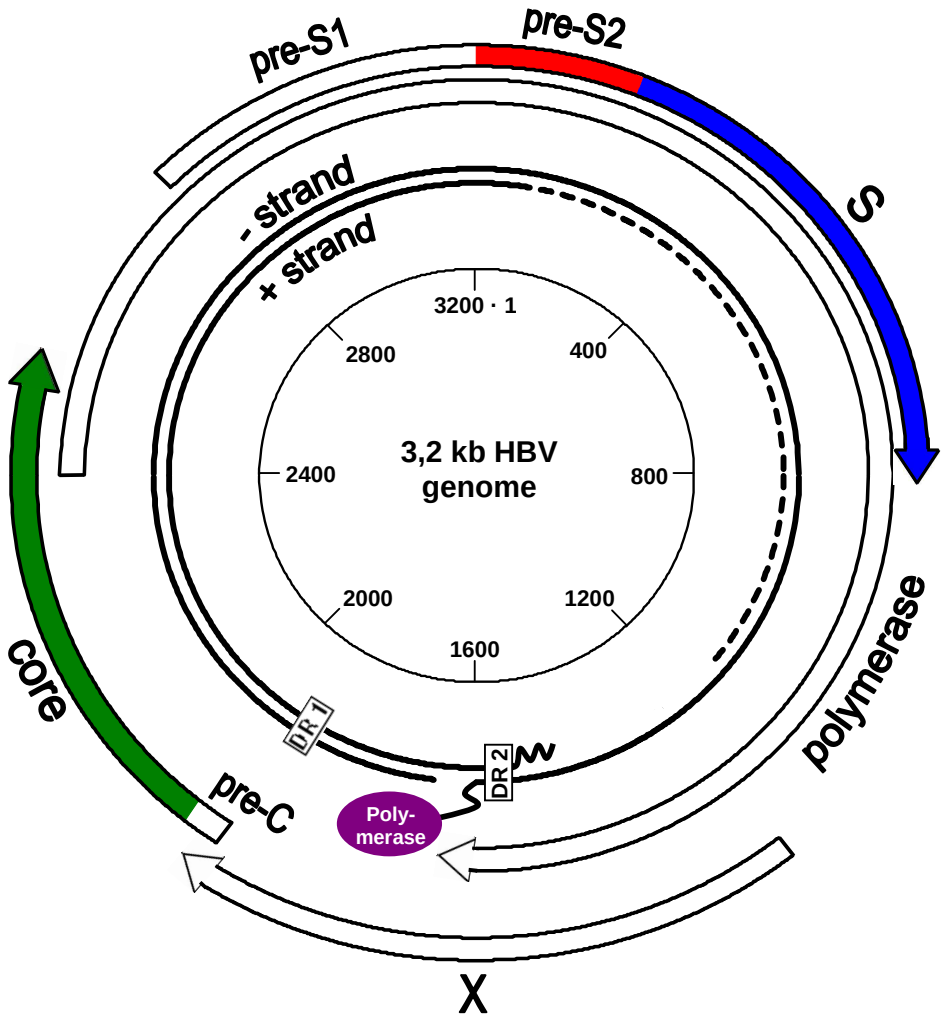


Figure 5: The organisation of the HBV genome. The 3,2 kb HBV genome has four overlapping open reading frames (drawn as arrows) encoding seven different transcripts. The negative strand has a 7 to 9 nucleotide terminal redundancy and the viral polymerase (purple oval) is linked to its 5' end. The variable length of the positive strand is represented by the dotted line. The 5' end of the positive strand is capped by an RNA primer (∿). The positions of the two 11 nucleotide direct repeat sequences (DR1 and DR2) are shown.

1.3.1.1 Polymerase gene

The polymerase protein is translated from the pregenomic RNA and consists of between 834 and 845 amino acids. It has at least four domains – the N-terminal; spacer; polymerase and C-terminal domains (Kann, 2002; Yokosuka and Arai, 2006). The N-terminal domain contains a tyrosine residue, whose hydroxyl group is necessary for the binding of the polymerase protein to the first nucleotide of the negative DNA strand, and hence it primes negative strand synthesis. The C-terminal domain encodes an RNaseH that removes the template RNA during

replication, allowing for the synthesis of the positive DNA strand. The HBV polymerase protein is both structurally and functionally similar to the HIV reverse transcriptase, both of which contain a classical tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif in their catalytic centres. Mutations within this motif are often associated with resistance to nucleoside analogs, such as lamivudine (Yokosuka and Arai, 2006).

1.3.1.2 Surface gene

The surface gene has three in-frame initiator codons encoding three different proteins, all of which terminate at a single stop codon (Chouteau *et al.*, 2001). These three proteins, termed the small (S), middle (M) and large (L) proteins, all share an identical 226 amino acid C-terminal region called the surface domain (Barrera *et al.*, 2005). The M protein contains an additional 55 amino acid N-terminal domain called the pre-S2 domain, and the L protein is further extended in the N-terminal direction with the addition of the 108 amino acid pre-S1 domain. Posttranslational modifications of all three proteins into glycoproteins contribute to their biological activities (Chouteau *et al.*, 2001).

The three surface glycoproteins all contain the "a" determinant epitope, located at codon positions 124 to 147 of the S gene. The "a" determinant is one of the main initial immune response targets of anti-HBs antibodies during acute hepatitis B and amino acid substitutions within this determinant can lead to conformational changes affecting the binding of neutralising antibodies (Chongsrisawat *et al.*, 2006; Lada *et al.*, 2006). The serological subtype of the virus is determined by two additional determinants: a lysine residue at codon 122 denotes a subtype *d* whereas an arginine at this position denotes a subtype *y* ; a lysine at codon position 160 denotes subtype *w* whereas an arginine denotes subtype *r* (Kann, 2002). The amino acid residue at codon 127 further differentiates the *w* serological subtypes into w1 to w4. The *adr* subtypes are further divided into q^+ and q^- (Courouce-Pauty *et al.*, 1978). By combining these different determinants, a total of nine different serological subtypes have been identified (Yokosuka and Arai, 2006). Initially, HBV isolates were grouped according to their serological subtype or serotype (Magnius and Norder, 1995). These serotypes however did not correspond with the geographical distribution of HBV and so this

classification was superseded by the classification of HBV into genotypes (section 1.5), which display a geographical distribution.

The surface or envelope proteins play a vital role in the host specificity of the virus. During the viral life cycle, the virus binds to the surface of the hepatocyte and fuses with it (Chouteau *et al.*, 2001). The nucleocapsid is then released into the cytosol where it migrates to the hepatocyte nucleus. *In vitro* studies have shown that if the normal pathway of viral entry is artificially bypassed, HBV is able to replicate in the hepatocytes of a different species, indicating that it is the binding of the virus to the hepatocyte's surface that confers host specificity (Chouteau *et al.*, 2001). This binding depends on the interaction of the viral envelope glycoproteins with cellular receptors on the surface of the hepatocyte (Barrera *et al.*, 2005). Differences in the structural features of the receptors between species would explain the specificity of hepadnaviral infections. *In vitro* experiments have shown that if the N-terminal portion of the L protein (specifically amino acids 1 to 10 and 21 to 30) from the woolly monkey hepatitis B virus, which normally cannot infect human hepatocytes, is replaced with its HBV counterpart, infection can be achieved (Chouteau *et al.*, 2001). Studies using the duck hepatitis B virus (DHBV) have identified carboxypeptidase D as the cellular receptor to which the DHBV surface proteins bind on duck hepatocytes, but the cellular receptor for HBV in humans has not yet been identified (Chisari, 2000; Chouteau *et al.*, 2001). A recent study using primary *Tupaia* hepatocyte cultures has suggested that cell surface heparan sulphate proteoglycans are low affinity receptors for HBV (Leistner *et al.*, 2008).

1.3.1.3 Core gene

The core gene encodes two proteins that are translated from two different transcripts. The pregenomic RNA (pgRNA) is translated into the core protein and the polymerase, whereas the precore messenger RNA (mRNA) is translated into the precursor of HBeAg (Yokosuka and Arai, 2006; Sugiyama *et al.*, 2007). The core and precore proteins are post-translationally processed, differing only in their N-terminus amino acids (Huang *et al.*, 2006; Sugiyama *et al.*, 2007).

The core protein, a structural protein, is 183 – 185 amino acids in length depending on the genotype of the virus and, compared to the envelope gene, is relatively well conserved (Jazayeri *et al.*, 2004; Yokosuka and Arai, 2006). This protein dimerises in the cytoplasm of the hepatocyte and, after reaching a concentration threshold of 0,8 μM, spontaneously assembles into the icosahedral capsid (Kann, 2002; Yokosuka and Arai, 2006). World-wide, the distribution of HBV tends to follow a geographical pattern, but the ethnic background of an infected individual also seems to play a role. Infected individuals with related ethnicity often have common amino acid substitutions in specific regions of the core gene, despite the individual's current geographical location. It is hypothesised that these substitutions may be driven by host T-cell selection (Jazayeri *et al.*, 2004).

The HBeAg is a soluble, secreted, non-particulate form of the viral nucleocapsid that is not necessary for either viral replication or infection (Cabrerizo *et al.*, 2000; Visvanathan *et al.*, 2007). It differs from the core protein in that it contains an extra 29 amino acids at the amino end (Kann, 2002). The role of HBeAg in the viral life cycle is not well understood although production of this antigen is thought to be required for the establishment of persistent infections in acutely infected individuals (Jazayeri *et al.*, 2004). *In vitro,* HBeAg has been shown to slow the replication of HBV DNA by reducing dimerisation of the core antigen and hence decreasing the encapsidation of pgRNA, which in turn induces immunological tolerance (Yokosuka and Arai, 2006). HBeAg negative chronic HBV is often associated with more aggressive liver disease (Visvanathan *et al.*, 2007).

1.3.1.4 X gene

The HBV X-protein (HBx) is another non-structural protein, 154 amino acids in length (Hoare *et al.*, 2001). Found only in the orthohepadnaviridae, the precise function of HBx is not well understood although it appears to be essential for the establishment of viral infections in mammalian animal models (Scaglioni *et al.*, 1996; Kann, 2002; Yokosuka and Arai, 2006). *In vitro,* HBx has been shown to activate the AP-1 and NF-κB signalling pathways and to bind to p53 (Yokosuka

and Arai, 2006). HBx also appears to have an enhancing effect on the metabolism of intrahepatic purines and pyrimidines needed for the efficient replication of HBV. Carboxyl terminally truncated forms of HBx have been known to integrate into the host genome of chronically infected individuals and are believed to play a role in the development of HCC (Poussin *et al.*, 1999; Yokosuka and Arai, 2006).

1.3.2 *cis*-acting elements

The HBV genome contains a number of both ubiquitous and hepatocyte specific transcription factor binding sites, parts of which cluster in the enhancer 1 (Enh1) and 2 (Enh2) regions. Enh1 spans approximately 200 nucleotides between the S and X genes and is the major enhancer, stimulating transcription of all the viral RNAs (Kann, 2002). Both ubiquitous (e.g. NF-1, AP-1 and NF-κB) and liver cell specific (e.g. C/EBP, HNF4 and HNF3) transcription factors bind to the Enh1 region. Enh2 is located immediately upstream of the core ORF and confers hepatocyte specificity on the virus as it binds only liver specific transcription factors such as C/EBP and HNF-4 (Kann, 2002; Chang *et al.*, 2004). Two 11 nucleotide direct repeat sequences (DR1 and DR2) are located one on either side of the gap in the negative DNA strand. DR1 is located at nucleotides 1826 – 1836 and DR2 at nucleotides 1592 – 1602 (Jilbert *et al.*, 2002). Both these direct repeat sequences are necessary for the replication of HBV.

1.4 HBV replication

The HBV life cycle (figure 6) begins with the binding of the enveloped virion, most likely via the pre-S proteins, to a specific but as yet unidentified receptor on the surface of the hepatocyte (Ganem, 1996; Pawlotsky, 2005). The viral envelope fuses with the membrane of the host cell and delivers the nucleocapsid into the cytoplasm. The viral genomic DNA is transferred to the nucleus where it is converted into cccDNA using the host cell's enzymes (He *et al.*, 2002). During this process, the viral polymerase protein bound to the 5'-end of the negative strand, as well as the 7 - 9 nucleotide terminal redundancy, is removed and the two ends of the DNA strand are ligated (Caselmann, 1994). An endogenous DNA

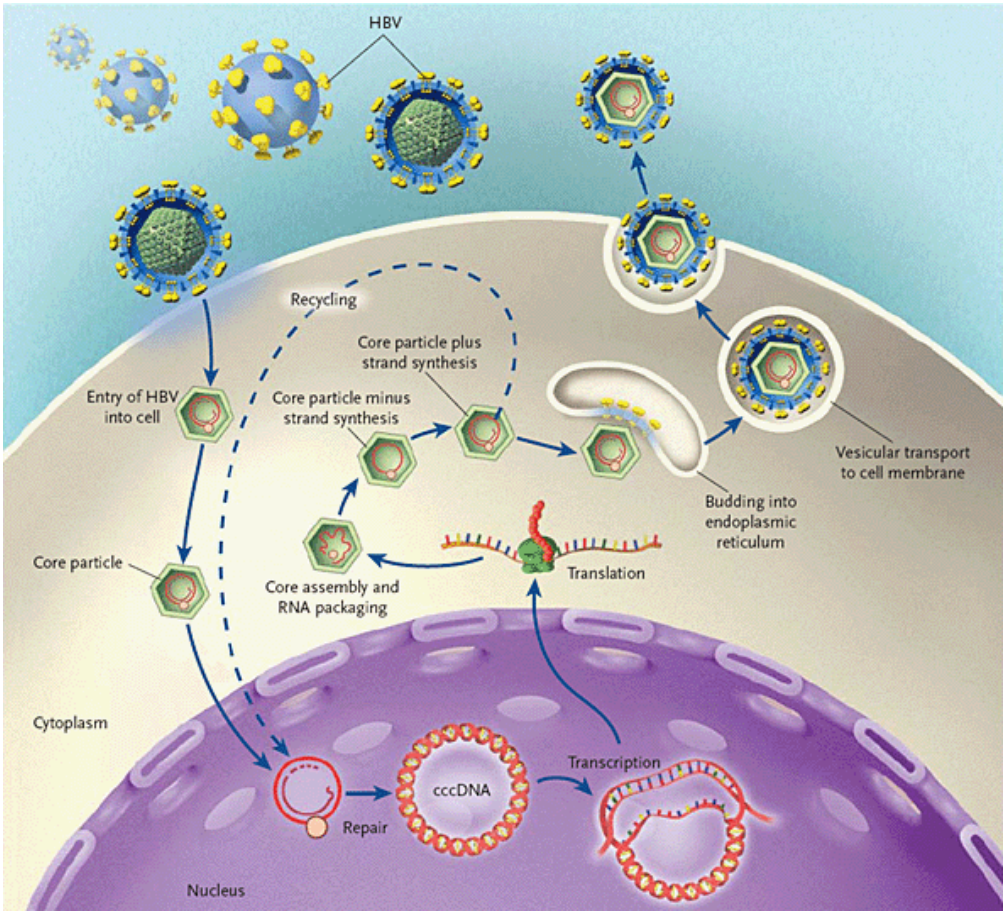


Figure 6: **The life cycle of hepadnaviruses** (Ganem and Prince, 2004). HBV virions bind to an as yet unidentified receptor on the hepatocyte surface and are internalised. The nucleocapsid is released into the cytoplasm and migrates to the nucleus where the partially double stranded DNA genome is converted into cccDNA. This cccDNA serves as template for the transcription of the pgRNA and subgenomic mRNAs that are translated in the cytoplasm into the viral proteins. Progeny viral capsids are assembled using the pgRNA, core and polymerase proteins. The pgRNA is reverse transcribed into viral DNA within the capsid and the mature nucleocapsids either bud into the preGolgi compartment where they are enveloped and exported from the cell via the endoplasmic reticulum or else the genome is recycled back into the nucleus to be converted into cccDNA.

polymerase then completes the positive strand forming the cccDNA (Laras *et al.*, 2006). As chronic HBV infections progress, there is a decrease in viral replication and cccDNA becomes the predominant form of HBV DNA with a stable pool of between 5 and 50 cccDNA molecules found inside an infected hepatocyte's nucleus (He *et al.*, 2002). In patients with HCC, the levels of cccDNA in tumourous compared to non-tumourous liver tissue are significantly higher (Wong *et al.*, 2006). The cccDNA molecules serve as template for the transcription of five different unspliced mRNAs of 3,5 kb (pgRNA and envelope

mRNAs); 2,4 kb (LHBs mRNA); 2,1 kb (MHBs and SHBs mRNA) and 0,9 kb (X mRNA) which are translated into seven different proteins – the pre-S1, pre-S2, S, polymerase, X, core and precore (Kann, 2002). Once the pgRNA is synthesised, packaged into nucleocapsids and translocated to the cytoplasm, genomic replication begins.

Replication of the HBV genome begins with the 5' capping and 3' polyadenylation of the 3,5 kb pgRNA transcript. This transcript has identical terminal redundancies of roughly 200 bp (including the 11 nucleotide DR1 sequence) at both the 3' and 5' ends (Ganem, 1996). DR2 is found on the 3' end of the pgRNA only (Jilbert *et al.*, 2002). Approximately 100 bp of this terminal redundancy encodes for a RNA stem-loop structure called the encapsidation signal, ε, which is phylogenetically conserved in all hepadnaviruses although there is significant sequence variation between the avi- and orthohepadnaviruses. The HBV polymerase binds to the bulge of the 5' ε structure initiating encapsidation of the pgRNA (Jilbert *et al.*, 2002). In the cytoplasm, newly synthesised core protein dimerises, and then multimerises, to form a single capsid of 180 – 240 core particles which encapsidate the HBV pgRNA and polymerase (Zhou and Standring 1992; Seifer *et al.* 1993; Lingappa *et al.* 2005).

Transcription is initiated by the binding of the HBV polymerase to the 5' ε structure and the synthesis of the first three nucleotides (5' – GAA – 3') of the negative strand. The polymerase, together with this covalently attached 3 nucleotide oligonucleotide, transfers to the 3' DR1 region which contains a complementary acceptor site for this oligonucleotide primer, and synthesis of the negative DNA strand proceeds. As the negative strand extends, the RNaseH activity of the HBV polymerase simultaneously degrades the pgRNA template, stopping at the 5' capped terminal 18 nucleotides which include the DR1 sequence. This 18 nucleotide RNA primer is then transferred to the 5' end of the newly synthesised negative strand where it anneals to DR2, priming synthesis of the positive strand. The positive strand contains the 18 nucleotide terminal redundancy on its 3' end, enabling it to "jump" to the 3' end of the negative strand, circularising the genome and allowing synthesis of the positive strand to

continue for a variable length. This process results in a partially double stranded, relaxed circular DNA genome with the polymerase protein covalently attached to the 5' end of the negative strand, characteristic of HBV. The DNA containing nucleocapsids are enveloped by HBV surface proteins in the pre-Golgi compartment of the endoplasmic reticulum and are secreted, via the vesicular transport pathways, into the blood as complete Dane particles (Caselmann, 1994; Ganem, 1996; Schormann *et al.*, 2006).

The HBV reverse transcriptase lacks 3' – 5' exonuclease proof-reading activity resulting in an increased number of nucleotide substitutions during replication and hence the formation of viral 'quasispecies' within an infected individual (Pawlotsky, 2005). The frequency of mutation in HBV ranges from 10^{-5} to 10^{-4} substitutions per site per year, which is 10^{4} times higher than that of DNA viruses, but approximately the same as that of RNA retroviruses $\left(10^{-5}\right)$ (Holland *et al.*, 1982; Kramvis *et al.*, 2005a; Jazayeri *et al.*, 2009). Furthermore, the four overlapping ORFs of HBV places certain conservatory constraints on its variability (Miyakawa and Mizokami, 2003). Over the course of an infection, the immune response of the individual, as well as any drug therapies, can result in different variants or mutations being selected for (Tong, 2005).

1.5 HBV Genotypes

An organism's genotype can be defined as its genetic constitution (Hale and Margham, 1988). In viruses, the term genotype refers to the replication competent sequence into which the genome has stabilised over a prolonged period of time (Francois *et al.*, 2001). The first four HBV genotypes (A – D) were identified by comparing 18 complete genomes and were shown to have an inter-group divergence of between 8,5% and 10% and an intragenotype divergence of less than 5,6% (Okamoto *et al.*, 1988). In 1992, work by Norder *et al.* extended this to six genotypes and currently eight different genotypes (A – H) of HBV are recognised with two additional genotypes, I and J, having been proposed (Cao, 2009; Kurbanov *et al.*, 2009). The different HBV genotypes are believed to influence HBeAg seroconversion rates, mutational patterns in the precore and core

promoter regions and the clinical manifestations and severity of HBV-related liver disease (Buti *et al.*, 2005).

When the first four genotypes of HBV were identified, none of the nucleotide divergences fell in the 6 - 8% range and so a sequence divergence of greater than 8% in the entire HBV genome was used to separate isolates into genotypes (Kramvis *et al.*, 2008). As more sequences of complete HBV genomes have become available, an overlap between the inter- and intra-genotype divergences occurring between 6% and 8% has become evident. A nucleotide divergence of greater than 7,5% across the complete viral genome has therefore been proposed to separate the different strains of HBV into genotypes (Kramvis *et al.*, 2008). Additionally, a sequence divergence of greater than 4% at the level of the S-gene has been shown to be sufficient to differentiate isolates into separate genotypes (Norder *et al.*, 1993; Magnius and Norder, 1995). The core and basic core promoter (BCP) regions of the HBV genome have also been used as an indication of genotype (Myers *et al.*, 2006). These two regions however contain greater variation than the surface gene and so the changes are not always genotype specific. The classification of genotypes using these regions is therefore not as accurate as when the S ORF is used.

Subgenotypes are separated on the basis of a complete nucleotide sequence divergence of between 4% and 7,5% and good bootstrap support (Kramvis *et al.*, 2008). Five of the HBV genotypes (A, B, C, D and F) have been split into subgenotypes which, in certain instances, are separated either geographically or ethnically (table 2).

Table 2: **Relationship between HBV genotypes, subgenotypes, serotypes and their geographical distribution**

Genotype	Sub-genotype	Predominant Serotype	Geographical Distribution	References
A	A1	*adw2*	Sub-Saharan Africa, Southern Asia, Brazil, the Philippines	2, 6, 11, 12
	A2	*adw2*	Northern Europe, United States, the Arctic	10, 23, 36
	A3	*ayw1*	Western and Central Africa, Cameroon, Gabon	9, 14, 17
	A4	*ayw1*	Mali, the Gambia	11, 19
	A5	*ayw1*	Western Africa, Haiti	19, 31, 42
	A6	*ayw1, adw2*	Democratic Republic of Congo, Rwanda	43
	A7	*ayw1, adw2*	Cameroon, Rwanda	42
B	B1	*adw2*	Japan	10, 21
	B2	*adw2*	Most of Asia except Korea	10, 21, 32
	B3	*adw2, ayw1*	Indonesia	10, 21
	B4	*ayw1*	Indonesia, Vietnam	10, 21, 32
	B5	*ayw1*	Indonesia, Philippines	18, 21, 24, 32
	B6	*adw2*	Alaska, Northern Canada, Greenland	22, 36
	B7	*ayw*	Eastern Indonesia	26
	B8	*ayw*	Indonesia	38
C	C1	*adr, ayr*	Vietnam, Thailand, Myanmar	7
	C2	*adr*	China, Korea, Japan, Rwanda	7, 35
	C3	*adr*	Pacific Islands (Micronesia, Melanesia and Polynesia), Indonesia	24, 36
	C4	*ayw3*	Australia	4, 10
	C5	*adw2*	Indonesia, Philippines, Vietnam	24
	C6	*adr*	Papua Indonesia	25
	C7	*adr*	Indonesia	38
	C8		Philippines	33
D	D1	*ayw2*	Mediterranean Europe, Middle East, Egypt, India, Asia	20, 23, 32
	D2	*ayw3*	Russia, the Baltic region, India, Japan	28, 32
	D3	*ayw2, ayw3*	Europe, Asia, South Africa, United States	20, 36

	D4	*ayw2*	Australia, Japan, Papua New Guinea, Rwanda	24, 35
	D5	*awy3*	Eastern India	15, 34
	D6	*ayw2*	Papua Indonesia	25
	D7	*ayw2*	Tunisia, Algeria, Morocco	37
	D8	*ayw2*	Niger	41
E		*ayw4*	West and Central Africa	9, 13, 30
F	F1	*adw4*	Alaska, Argentina, Bolivia, Chile	1, 32
	F2	*adw4*	Venezuela, Brazil, Nicaragua	1, 32
	F3	*adw4*	Venezuela, Columbia, Panama	1, 32
	F4	*adw4*	Argentina, Bolivia, France	32
G		*adw2*	France, United States, Vietnam, Mexico, Canada	3, 16, 36
H		*adw4*	Mexico, Nicaragua, California	5, 8, 36
I		*adw, ayw*	Vietnam, Laos, Northeast India, Northwest China	27, 29, 40, 44
J		*ayw*	Japan (might be from Borneo)	39

[1](Arauz-Ruiz *et al.* 1997)
[2](Bowyer *et al.* 1997)
[3](Stuyver *et al.* 2000)
[4](Sugauchi *et al.* 2001)
[5](Arauz-Ruiz *et al.* 2002)
[6](Kramvis *et al.* 2002)
[7](Huy *et al.* 2004)
[8](Kirschberg *et al.* 2004)
[9](Mulders *et al.* 2004)
[10](Norder *et al.* 2004)
[11](Hannoun *et al.* 2005)
[12](Kramvis *et al.* 2005a)
[13](Kramvis *et al.* 2005b)
[14](Kurbanov *et al.* 2005)
[15](Banerjee *et al.* 2006)
[16](Chudy *et al.* 2006)
[17](Makuwa *et al.* 2006)
[18](Nagasaki *et al.* 2006)
[19](Olinger *et al.* 2006)
[20](Kramvis and Kew 2007)
[21](Liu *et al.* 2007)
[22](Sakamoto *et al.* 2007)
[23](Schaefer 2007)
[24](Schaefer 2007b)
[25](Lusida *et al.* 2008)
[26](Nurainy *et al.* 2008)
[27](Olinger *et al.* 2008)
[28](Tallo *et al.* 2008)
[29](Tran *et al.* 2008)
[30](Andernach *et al.* 2009a)
[31](Andernach *et al.* 2009b)
[32](Cao 2009)
[33](Cavinta *et al.* 2009)
[34](Chandra *et al.* 2009)
[35](Hubschen *et al.* 2009)
[36](McMahon 2009)
[37](Meldal *et al.* 2009)
[38](Mulyanto *et al.* 2009)
[39](Tatematsu *et al.* 2009)
[40](Arankalle *et al.* 2010)
[41](Abdou Chekaraou *et al.* 2010)
[42](Hubschen *et al.* 2010)
[43](Pourkarim *et al.* 2010)
[44](Yu *et al.* 2010)

1.5.1 Genotypes and subgenotypes found in Africa

1.5.1.1 Genotype A and its subgenotypes

Genotype A has been found in Africa, Asia, Northern Europe, the United States and the Arctic (Hannoun *et al.*, 2005; Kramvis *et al.*, 2005b; McMahon, 2009). It is characterised by a six nucleotide insertion at the carboxyl terminus of the core ORF resulting in an overall genomic length of 3221 nucleotides (Kramvis *et al.*, 2005a). Genotype A isolates have a cytosine (C) residue at position 1858 that prohibits a guanidine (G) to adenine (A) point mutation at position 1896 and stabilises the encapsidation (ε) signal's stem-loop structure (Sugauchi *et al.*, 2004; Schaefer, 2007).

The splitting of this genotype into subgenotypes is strongly supported phylogenetically, with 100% bootstrap values when complete genome sequences are analysed (Makuwa *et al.*, 2006). Subgenotype A1 is predominant in sub-Saharan Africa including South Africa, Zimbabwe, Mozambique, Somalia and Malawi, and in India and Nepal and has also been found in Brazil, the Philippines and Yemen (Kramvis *et al.*, 2005a). Subgenotype A2 is predominant in Western Europe and the United States, as well as Alaska and Greenland. Subgenotype A2 has also been found in Argentina, Mexico, Venezuela, Nicaragua, Zanzibar and Kenya (Mwangi *et al.*, 2008; Mbayed *et al.*, 2009). Subgenotype A3 can be found in Western African countries such as Cameroon and Gabon (Kurbanov *et al.* 2005; Makuwa *et al.* 2006; Olinger *et al.* 2006). Newer subgenotypes of genotype A have been tentatively designated subgenotypes A4 (found in Mali and the Gambia), A5 (found in Nigeria, Cameroon and Haiti) and A6 (found in Rwanda and the Democratic Republic of Congo) (Hannoun *et al.* 2005; Olinger *et al.* 2006; Andernach *et al.* 2009b; Hubschen *et al.* 2010; Pourkarim *et al.* 2010).

1.5.1.1.1 Subgenotypes A1 and A2

Subgenotypes A1 and A2 can be separated on the basis of their distinct epidemiological distributions, but they also have marked virological differences. An example is an asparagine at position 207 and a leucine at position 209 in

subgenotype A1 isolates whereas subgenotype A2 isolates have a serine at position 207 and a valine at position 209 (Kimbi *et al.*, 2004). The genetic distance between subgenotypes A1 and A2 suggests that they diverged a relatively long time ago (Hannoun *et al.*, 2005).

Individuals infected with subgenotype A1 have lower levels of HBV DNA in their serum compared to those infected with A2, as well as a lower prevalence of HBeAg and a higher incidence (4,5 fold increased risk) of HBV-related HCC (Hasegawa *et al.*, 2004; Kimbi *et al.*, 2004; Kusakabe *et al.*, 2007; McMahon, 2009). Individuals infected with subgenotype A2 have a lower rate of liver disease related deaths compared to individuals infected with the other genotypes, as well as a higher rate of sustained remission after seroconversion (Hasegawa *et al.*, 2004).

Subgenotype A1 isolates have unique double or triple point mutations (G1809T, A1811T and C1812T/G) in the precore Kozak sequence (Kimbi *et al.*, 2004). Mutations in this region have been shown to reduce the translation of HBeAg by a ribosomal leaky scanning mechanism and could be responsible for the early loss of HBeAg seen in Southern African Blacks (Ahn *et al.*, 2003). Subgenotype A2 isolates that have either decreased levels or a lack of HBeAg have a greater prevalence of the 1762T and 1764A double mutation in the precore promoter region (Schaefer, 2007).

Within the pregenome ε signal there are a number of nucleotides that differ between the A1 and A2 subgenotypes (figure 7). The third nucleotide in the six nucleotide ε bulge, nucleotide 1862, is a G in the majority of subgenotype A2 isolates, as well as in the wild-type HBV, but most A1 isolates have a thymine (T) in this position (Kramvis *et al.*, 1998). At nucleotide 1888 in the upper stem, the A2s have a G while most A1s have an A, introducing an additional start codon in the precore region (Kimbi *et al.*, 2004). This extra start codon can affect the expression of the core protein leading to a decrease in levels of the virus (Kimbi, 2005). These nucleotide substitutions could account for the clinical differences observed between these two subgenotypes.

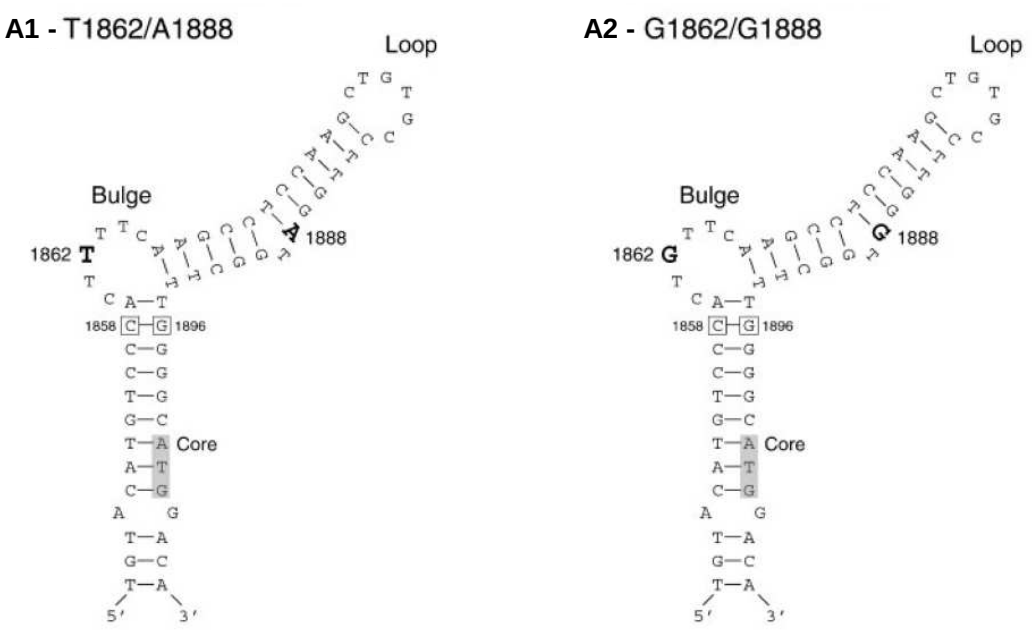


Figure 7: Conformation of the pregenome (ε) signal for subgenotypes A1 and A2 (Sugauchi *et al.*, 2004). A characteristic of genotype A isolates is the Watson-Crick pair between the C at nucleotide position 1858 and the G at nucleotide 1896. Subgenotype A2 isolates have a G at both nucleotide positions 1862 and 1888. Approximately 80% of subgenotype A1 isolates have an 1862T and approximately 75% have an 1888A. The initiation codon of the core gene is shaded in grey.

1.5.1.1.2 Subgenotypes A3, A4, A5 and A6

Subgenotype A3 has been found in Western and Central Africa (Kurbanov *et al.*, 2005; Makuwa *et al.*, 2006). Similar to subgenotype A1, subgenotype A3 isolates have a high intra-subgenotype nucleotide divergence (mean of 3,9% ± standard deviation of 1,1% (range 1,8% - 4,8%)) suggesting a long natural history in its native population (Kurbanov *et al.*, 2005). Three amino acid substitutions in the pre-S2 domain - N152S, P155Q and N174T – are specific for this subgenotype (Makuwa *et al.*, 2006).

Subgenotype A4, found in Mali and the Gambia, has also been proposed with a bootstrap value of 97% based on the complete genome (Olinger *et al.*, 2006). The mean nucleotide divergence between this subgenotype and subgenotype A3, however, is only 3,8%, so it has been suggested that subgenotype A4 be considered a clade of subgenotype A3 as opposed to a separate subgenotype (Kurbanov *et al.*, 2009).

A fifth subgenotype from Nigeria, subgenotype A5, has also been proposed (Olinger *et al.*, 2006). Additional complete genome sequences from Haiti and Cameroon, that cluster with the subgenotype A5s from Nigeria, have also been published (Andernach *et al.* 2009b; Hubschen *et al.* 2010).

The proposed subgenotype A6 was isolated from three African-Belgian patients two of whom were originally from the Democratic Republic of Congo and one of whom was from Rwanda (Pourkarim *et al.* 2010). The A6 isolates diverge at the main node from all other subgenotypes of A with high bootstrap support (94%) and have an inter-subgenotypic nucleotide divergence over the complete genome of greater than or equal to 4% when compared to the other subgenotypes of A.

A seventh subgenotype of A was recently described in isolates from Rwanda and Cameroon (Hubschen *et al.* 2010). Designation of these strains as a new subgenotype is based on good bootstrap support and a mean inter-subgenotype nucleotide divergence of at least 3,81 ± 0,24%.

1.5.1.2 Genotype D and its subgenotypes

Worldwide genotype D, with eight subgenotypes, is the most widely spread of all the genotypes but is found predominantly in the Mediterranean area, Eastern Europe, Russia and the Near and Middle East extending into India (Lazarevic *et al.*, 2007; Abdou Chekaraou *et al.* 2010). In Africa, isolates from subgenotype D1, D3, D4, D7 and D8 have been found in Egypt, South Africa, Rwanda, Tunisia and Niger respectively (Kramvis and Kew 2007; Hubschen *et al.* 2009; Meldal *et al.* 2009; Abdou Chekaraou *et al.* 2010). Genotype D is characterised by a 33 nucleotide deletion at the N terminus of the preS1 region resulting in it having the shortest genome of the eight HBV genotypes (3182 nucleotides) (Kramvis *et al.* 2005a; Schaefer 2007). Individuals infected with this genotype are found to be anti-HBe positive significantly more often than individuals infected with the other genotypes and to have significantly higher ALT levels indicating a more active form of the disease (Kidd-Ljunggren *et al.*, 2004).

1.5.1.3 Genotype E

Genotype E strains are characterised by a very low genetic diversity spanning a large geographic area and could be a recent introduction into the human population (Mulders *et al.*, 2004; Hubschen *et al.*, 2008). They have been found in Western and Central Africa including Angola, Liberia, Senegal, Ivory Coast, the Gambia, Ghana, Nigeria, Mali, Burkina Faso, Togo, Benin, Cameroon, Namibia and the Democratic Republic of Congo (Kramvis *et al.*, 2005b). The absence of this genotype in the African-American population, despite the slave trade in the 19th century, supports a recent introduction of this genotype, possibly within the last 200 years (Andernach *et al.*, 2009a).

1.5.2 Putative genotypes

A new genotype, genotype I, has been proposed to describe an aberrant strain originating from Hanoi in the northern part of Vietnam and has also been found in voluntary blood donors from Laos (Olinger *et al.*, 2008; Tran *et al.*, 2008). Recently, additional genotype I isolates have been reported in a primitive tribe from northeast India as well as in the northwest of China (Arankalle *et al.* 2010; Yu *et al.* 2010). This variant seems to have evolved from a series of complex recombination events combining genotypes C, A and G – a surprising result as the prevailing genotypes in this region are B and C with genotype A being identified very rarely and genotype G so far undetected in Vietnam.

A novel HBV strain has been isolated from a Japanese patient with HCC who served in Borneo during World War II (Tatematsu *et al.*, 2009). Tentatively designated genotype J, this isolate occupies a unique phylogenetic position, clustering amongst the non-human HBV isolates closest to isolates from the gibbon and orang-utan.

1.5.3 Recombination of genotypes

When more than one genotype co-circulate in a region, there can be recombination of these genotypes, resulting in viral variants in both individuals

and the population in general (Kramvis *et al.*, 2005a; Cao, 2009). Recombination events between genotypes A and D in South Africa, India and Italy, A and G in the USA, B and C in Asia, C and D in Tibet and C and G in Thailand have been reported (Owiredu *et al.* 2001; Chauhan *et al.* 2008; Tran *et al.* 2008). Recombination events can contribute to the genetic diversity of HBV and lead to the formation of new genotypes. An example is subgenotype B2, which predominates in mainland Asia, is composed of genotype B recombined in the precore/core region with genotype C (Sugauchi *et al.*, 2002; Sugauchi *et al.*, 2003). Recombination may also help the virus evade the host's immune response by generating unfamiliar antigenetic configurations (Owiredu *et al.*, 2001).

The study of HBV is limited by the absence of suitable tissue culture systems and therefore, as an alternative, animal models have been used.

1.6 Non-human hepadnaviruses

Hepadnaviruses infect both avian and mammalian hosts.

1.6.1 *Avihepadnaviridae*

DHBV was first detected in the serum of Pekin ducks (*Anas domesticus*) from both China and the United States in the early 1980s (Mason *et al.*, 1980). Its 3,0 kb genome is the smallest of the hepadnaviruses and contains just three overlapping ORFs: the precore/core, pre-S/S and polymerase (Sprengel *et al.*, 1988). DHBV, along with the other avian hepatitis viruses, lacks the X ORF and infected ducks do not develop HCC (Feitelson and Larkin, 2001). The duck model system is extremely well characterised and has been used to analyse the early steps of hepadnaviral infections, to discover how the hepatitis viruses replicate, and for the testing of drugs that inhibit viral replication (Feitelson and Larkin, 2001; Stoeckl *et al.*, 2006). A closely related avihepadnavirus, designated the heron hepatitis B virus (HHBV), has been found in the grey heron (*Ardea cinerea*) (Sprengel *et al.*, 1988).

1.6.2 *Orthohepadnaviridae*

The orthohepadnaviruses all have similar genomic organisation and infect both rodents and primates. Animal models have been used to study hepadnaviral infections, especially the pathogenesis of chronic liver disease and the development of HCC (Feitelson and Larkin, 2001).

1.6.2.1 Rodent hepadnaviruses

In 1978 WHV was discovered at the Philadelphia Zoo in a colony of captive woodchucks (*Marmota monax*) with chronic hepatitis and HCC (Summers *et al.*, 1978). Woodchucks infected with this virus have a disease course that is very similar to that seen in HBV-infected humans, making them a good model for investigating the viral life cycle and the oncogenic potential of hepadnaviruses (Hodgson and Michalak, 2001). Following acute WHV infection, woodchucks can develop an occult form of the infection that can be transmitted to virus-naïve animals.

GSHV was first isolated from northern Californian Beechey ground squirrels (*Spermophilus beecheyi*) (Marion *et al.*, 1980). Compared to HBV, GSHV has a slightly larger Dane particle (47 nm compared to 42 nm) and it has slightly longer and more abundant filamentous forms in the serum.

The arctic squirrel hepatitis virus (ASHV) has been found in wild Alaskan arctic ground squirrels (*Spermophylus parryi kennicotti*) with liver disease and large hepatic nodules (Testut *et al.*, 1996). This virus is more closely related to WHV and GSHV having a similar genome size and virtually identical genetic organisation.

1.6.2.2 Non-human primate hepadnaviruses

Phylogenetically, non-human primates are very closely related to humans, making them excellent model systems (Sibal and Samson, 2001). Of the more than 200 species of non-human primates, only about 30 are used in research.

In the late 1960s and early 1970s, a number of studies looked for hepadnaviral infections in non-human primate serum samples by screening for the HBsAg (Robertson and Margolis, 2002). Evidence of these infections was found most consistently in chimpanzees but also in gibbons and orang-utans (Hirschman *et al.*, 1969; Blumberg *et al.*, 1972). Subsequently, using more sensitive molecular techniques, naturally occurring strains of HBV have been found in a number of Old and New World primates, namely the chimpanzee, gibbon, woolly monkey, orang-utan and gorilla (figure 8) (Robertson and Margolis, 2002). The rate of infection in the non-human primates is approximately 16,7%, a rate similar to that observed in humans in regions of endemic infection such as Central Africa and South East Asia (Starkman *et al.*, 2003). The non-human primate hepadnaviral sequences have a number of amino acid residues differentiating them from the consensus sequences of each of the human HBV genotypes (Robertson and Margolis, 2002). These include a glutamic acid at position 16 of the pre-S region, a leucine at position 133 of the S region and a leucine at position 28 of the precore region (Revill *et al.*, 2010). They also have an 11 amino acid deletion at the amino terminal end of the preS region, found in genotype D (Aiba *et al.*, 2003).

1.6.2.2.1 Chimpanzee Hepatitis B Virus

In 1978 it was reported that a colony of chimpanzees (*Pan troglodytes*) at London Zoo had a high carrier rate of HBV infection (Zuckerman *et al.*, 1978). At that time, the source of this infection was believed to be the practice of inoculating newly captured chimpanzees with pooled human HBV-positive blood to passively immunize the animals (Zuckerman *et al.*, 1978). Two of the infected chimpanzees, a male and a female, had been captured in the wild and three of their offspring were also HBV carriers, indicating perinatal transmission. Subsequent publication of the complete sequence of the HBV isolated from one these animals showed it to be approximately 10% divergent from human HBV sequences (Vaudin *et al.*, 1988). Genomic and serological analyses of HBV isolated from additional infected chimpanzees confirmed that the virus was genetically distinct from the known human HBV genotypes and therefore indigenous to chimpanzees (Hu *et al.*, 2000; MacDonald *et al.*, 2000; Takahashi *et al.*, 2000). This led to the designation of this virus as the chimpanzee HBV (ChHBV) (Takahashi *et al.*, 2000).

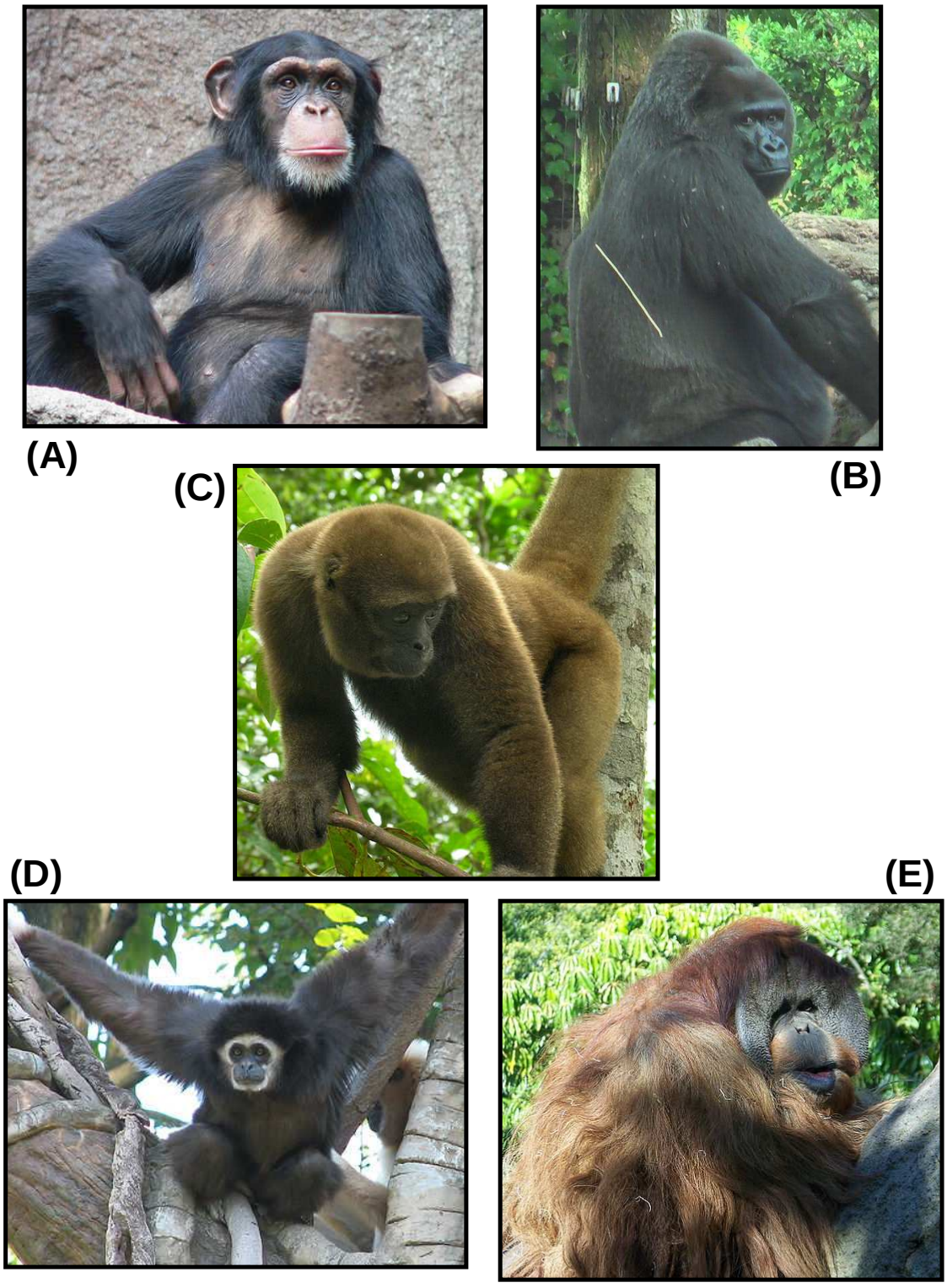


Figure 8: Hepadnaviral animal models. *Orthohepadnaviridae* occur naturally in both Old and New World non-human primates namely: (A) chimpanzees; (B) gorillas, (C) woolly monkeys, (D) gibbons and (E) orang-utans.
Photographs by: (A) Thomas Lersch; (B) Nesnad; (C) Evgenia Kononova; (D) Trisha Shears and (E) Nehrams2020.

The various subspecies of chimpanzee are found in geographically distinct regions of Africa – *Pan troglodytes troglodytes* and *P.t. vellerosus* in Central Africa, *P.t. versus* in West Africa and *P.t. schweinfurthi* in East Africa (Takahashi *et al.*, 2001; Vartanian *et al.*, 2002; Makuwa *et al.*, 2005). Phylogenetically, when compared to the human and other non-human primate HBV sequences (with the exception of the gorilla) the ChHBV sequences cluster together. When compared to each other however, the ChHBV strains from different subspecies appear to cluster according to their geographical origins, similar to the clustering seen in the human subgenotypes (Hu *et al.*, 2001; Vartanian *et al.*, 2002).

1.6.2.2.2 Gorilla Hepatitis B Virus

Only one complete HBV sequence from a Western lowland gorilla (*Gorilla gorilla gorilla*) has been reported (Grethe *et al.*, 2000). Phylogenetically gorilla HBV (GoHBV) clusters with the chimpanzee strains. HBV-DNA has also been reported in gorillas from Gabon (Makuwa *et al.*, 2003).

1.6.2.2.3 Gibbon Hepatitis B Virus

Gibbons are found throughout the tropical rainforests of South and Southeast Asia including Thailand, Laos, Cambodia, Indonesia and Malaysia (Noppornpanth *et al.*, 2003). HBV has been found in a number of gibbon subspecies including *Hylobates agilis, H. lar H. moloch* and *H. pileatus* (Lanford *et al.*, 1994; Norder *et al.*, 1996; Aiba *et al.*, 2003) and NAT has established the carrier rate to be 26.7% (Sa-nguanmoo *et al.*, 2008). Inoculation of HBsAg-positive gibbon serum into chimpanzees was shown to transmit the infection (Mimms *et al.*, 1993). The gibbon hepatitis B virus (GiHBV) genome is more closely related to the ChHBV genome than to the human HBV genome (Norder *et al.*, 1996). The pre-S region is the most divergent, differing from the ChHBV genome by 15,7% at the nucleotide level and 12,9% at the amino acid level, and has a number of changes including a glutamic acid at position 16 (also found in the ChHBV), a leucine at position 22 and a valine at position 115. Gibbons chronically infected with GiHBV show no overt signs of liver disease or mortality as a result of liver disease (Lanford *et al.*, 2000).

1.6.2.2.4 Orang-utan Hepadnavirus

Orang-utans (*Pongo pymarus*), restricted to the islands of Borneo and Sumatra, are the only great apes found outside Africa (Warren *et al.*, 1999). NAT has shown 13,2% of orang-utans to be infected with orang-utan hepadnavirus (OuHV), which is most closely related to the gibbon HBV (Verschoor *et al.*, 2001; Noppornpanth *et al.*, 2003; Sa-nguanmoo *et al.*, 2008). Orang-utans infected with OuHV do not develop hepatitis and therefore, this virus has been classed as a hepadnavirus as opposed to a hepatitis B virus (Takahashi *et al.*, 2000). The human genotype C strains, which also originate from Southeast Asia, are the human strains most closely related to OuHV (Warren *et al.*, 1999).

1.6.2.2.5 Woolly Monkey Hepatitis B Virus

The woolly monkey (*Lagothrix lagotricha*), a New World primate, is found in Colombia, Ecuador, Peru and Brazil. The woolly monkey hepatitis B virus (WMHBV) was first identified in an animal with fulminant hepatitis, and has subsequently been found in approximately 80% of the offspring of infected woolly monkey females, suggesting a vertical mode of transmission (Lanford *et al.*, 1998). WMHBV has also been shown to infect the black-handed spider monkey (*Ateles geoffroyi*), a close relative of the woolly monkey (Lanford *et al.*, 2003). The 3197 nucleotide genome has the same genetic organisation as human HBV and is the most divergent of the primate strains, showing a 35% divergence from human HBV (Lott *et al.*, 2003). Phylogenetically, WMHBV is closest to the human genotype F isolates found in South and Central America (Lanford *et al.*, 1998).

1.7 Animal models used to study HBV

The limited host range of the hepadnaviruses means that these infections are optimal in their natural hosts. Human HBV can infect other species although not very efficiently (figure 9).

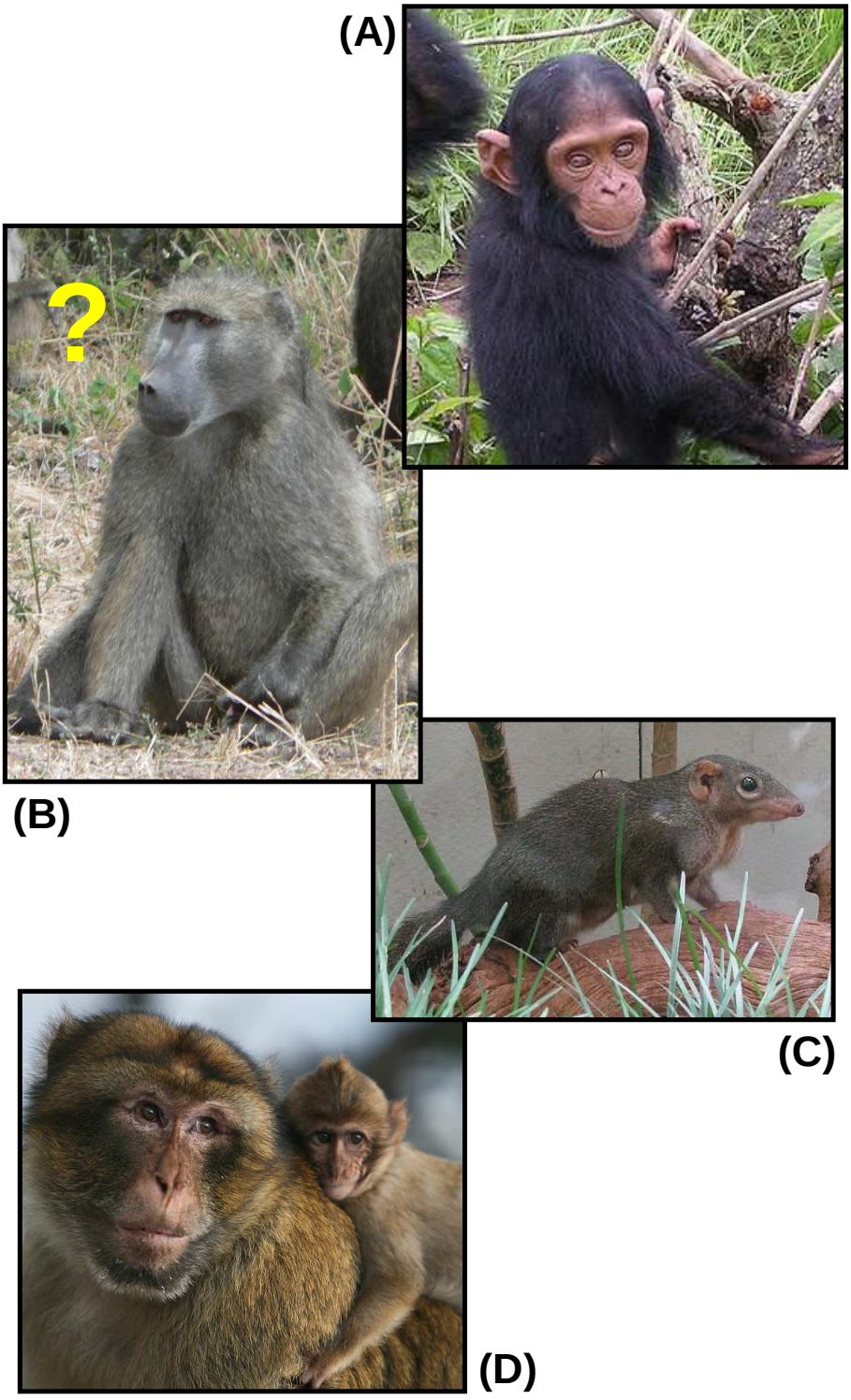


Figure 9: Animal models used to study HBV. The chimpanzee (A), tree shrew (C) and Barbary macaque (D) can all be experimentally infected with HBV. Baboons (B) have also been proposed as a model for HBV infection.
Photographs by: (A) Delphine Bruyere; (B) David Dickens; (C) Cymothoa exigua and (D) Karyn Sig.

1.7.1 Chimpanzee model

The chimpanzee is one of the only non-human primates that can be persistently infected with human HBV (Ruiz-Opazo *et al.*, 1982b). Infected chimpanzees have elevated levels of HBsAg in their serum as well as anti-HBc, HBeAg and 42 nm Dane particles (Ruiz-Opazo *et al.*, 1982b). In the liver tissue, HBV has been shown to exist in a covalently closed, supercoiled circular configuration (Ruiz-Opazo *et al.*, 1982a). Although experimentally infected chimpanzees generally display a mild form of liver disease, they have been invaluable for the study of the transmission, natural history and pathogenesis of HBV infections (Feitelson and Larkin, 2001). However, the availability of wild-caught chimpanzees for research is restricted as by nature they are highly intelligent animals. Furthermore, the expansion of logging, large-scale hunting and agriculture into their forest habitats has led to them being classed as an endangered species (Feitelson and Larkin, 2001; Prince and Brotman, 2001).

1.7.2 Macaque model

Barbary macaques (*Macaca sylvanus*) can be found in the Atlas Mountains of Algeria and Morocco as well as in Gibraltar. Macaques transfected with cloned HBV DNA develop markers of an acute HBV infection and HBsAg can be detected in the serum of infected macaques for between 4 and 7 weeks (Gheit *et al.*, 2002). HBV DNA can be detected by PCR and histological analysis of liver tissue shows evidence of pathological changes indicating an acute HBV infection (Gheit *et al.*, 2002).

1.7.3 Tree shrew model

The tree shrew (*Tupaia belangeri*) is a non-rodent, primate-like animal found in tropical forests and plantation areas of Southeast Asia. *In vitro*, primary *Tupaia* hepatocytes can be infected with HBV resulting in the synthesis of viral DNA and RNA and the secretion of HBsAg and HBeAg (Cao *et al.*, 2003). *In vivo* 55,2% of infections result in acute infections in which serum HBsAg levels decline rapidly followed by seroconversion to anti-HBs and anti-HBe. Replication of

viral DNA, viral gene expression and cccDNA are evident in the liver. The tree shrew model mirrors the epidemiology of liver cancer in humans and is thus useful for studying the mechanisms involved in the pathogenesis of liver disease.

1.7.4 Baboon as a possible model of HBV infection?

Baboons (*Papio sp.*) are one of the most intensively studied non-human primate species because their growth and aging parallels that of humans in many biological respects (Mukai *et al.*, 1980). Phylogenetically, baboons are very close to humans showing an approximately 96% homology at the DNA level (Murthy *et al.*, 2006). They also have an immune system very similar to that of humans (Murthy *et al.*, 2006).

Early studies involving the inoculation of baboons with HBV-positive sera failed to detect any clinical or biochemical signs of infection in these primates and initial serological surveys failed to detect HBsAg in the serum, leading to the conclusion that baboons were not susceptible to HBV infections (Deinhardt, 1976). A subsequent serological survey, however, found anti-HBsAg in 36,2% of baboons tested (Eichberg and Kalter, 1980). A more recent serological screening of 31 adult male baboons (*Papio cynocephalus*) found one baboon to be positive using a human enzyme linked immunoabsorbent assay (ELISA) but this same animal was found to be negative when tested in a different laboratory using a different human ELISA test (Michaels *et al.*, 1994). In 1996, a pilot study involving four baboons inoculated with HBV positive serum was unable to detect any signs of HBV infection by PCR, liver biopsy evaluation or serology, although one of the baboons showed low levels of HBV DNA in weeks two through five (Michaels *et al.*, 1996). When these tests were repeated, HBV DNA was not detected.

1.8 Interactions between humans and baboons

Interactions between man and baboon have a long history making the transmission of viruses between species quite plausible. Baboons are the most widely distributed of the African monkeys, found in virtually all parts of sub-

Saharan Africa (Barrett, 2000). They often appear in ancient Egyptian mythology and art being depicted as captives brought from the south, pets on leashes, dancers and jesters (Cheney and Seyfarth, 2007). They have been shown climbing trees to collect dates and figs for their owners and are even depicted as police assistants attacking thieves in the marketplace (figure 10).

Figure 10: Baboons acting as police assistants (Cheney and Seyfarth, 2007). This illustration from the Old Kingdom mastaba of Tepemankh at Saqarra, an Egyptian tomb, depicts two baboons, on leashes, attacking a thief in the marketplace. The accompanying hieroglyphic reads, "Fear for this baboon".

In Southern Africa, interactions between humans and baboons are well documented. Baboons have been kept as pets and even trained to work as oxcart drivers, railway labourers and goatherds on farms (Cheney and Seyfarth, 2007). One such story is of a baboon called Jack the Signalman. In the latter part of the 1800s, in a town called Uitenhage in the Eastern Cape, a railway guard fell under a moving train and had to have both legs amputated at the knees. He was hired as a signalman but struggled with the work until he acquired a young baboon called Jack who quickly learned how to perform a signalman's duties and operate the switches. Jack died of tuberculosis in 1890.

There have also been reports of children and young baboons playing together. Marais (1971) recounts one such incident on the bank of a stream where some young native children were digging clay from a hole in the river bank and fashioning it into small clay animals. Joining in the digging was a group of young baboons. The play got quite raucous as both children and baboons tried to access the hole containing the clay with plenty of pinching, kicking and dragging by both sides albeit all in jest.

More common, however, are reports of conflict between humans and baboons. In the rural areas, baboons raid orchards, destroy irrigation pipes and kill sheep and goats. Baboons can become aggressive when challenged and so are often killed by farmers for being pests. In Africa, primates are often slaughtered for bushmeat, providing another source of exposure to viruses (Robertson, 2001).

1.9 Rationale and objectives of study

Patients with end stage liver disease require liver transplants, but the increasing need for donor organs means that a substantial number of these patients die on waiting lists before a liver becomes available (Williams, 2006). HBV infected individuals are not good candidates for liver transplants as residual HBV in these patients can infect the donated organ resulting in rapid liver destruction (Lanford *et al.*, 1995). The use of xenotransplants from pigs and non-human primates to humans was considered in order to overcome the donor shortage and/or "bridge" patients with terminal hepatic failure until a human donor organ became available (Chapman *et al.*, 1995; Luo *et al.*, 1996). The supposed lack of susceptibility of baboons to infection with HBV meant that they were good candidates for liver xenotransplants. Moreover, baboons are phylogenetically close to humans, can be matched to humans for tissue and blood group using standard immunological tests and, unlike chimpanzees, they are not an endangered species (Gridelli *et al.*, 1993; Gridelli *et al.*, 1994).

Baboon to human liver xenotransplants were attempted in two individuals: a 35-year-old HBV and HIV co-infected male who survived 70 days post transplant (Starzl *et al.*, 1993) and a 62 year old male with chronic active HBV who survived 27 days post transplant (Lanford *et al.*, 1995). Replication of HBV was not detected in either of the baboon donor livers at autopsy, but that could have been a consequence of the limited life-span of the transplants or the sensitivity of the detection methods.

Initial studies had shown baboons to be resistant to HBV infection (see section 1.7.4) but these early studies used mainly biochemical and histological findings as well as serological markers to detect HBV infection. In an attempt to confirm the previous findings, six wild caught Chacma baboons (*Papio ursinus orientalis*) were inoculated with pooled HBV positive serum and followed for 52 weeks using sensitive molecular techniques to detect evidence of transmission (Kedda *et al.*, 2000). HBV DNA was detected by nested PCR in both the serum and liver tissue of four of the baboons up to 52 weeks post inoculation. Liver function and histology were normal and HBsAg was not detected in the serum. In two of the six baboons, HBV DNA was detected in the serum using nested PCR, at baseline prior to inoculation with HBV. This was initially thought to be the result of experimental error but retesting in independent laboratories confirmed the presence of HBV DNA. This raised the possibility that baboons are naturally infected with a hepadnavirus.

The objective of the present study was to determine the prevalence of 'HBV' in baboons in the wild and to molecularly characterize the virus isolated from these baboons. Thus the aims included:

- Detection of HBV DNA in sera from 69 wild-caught baboons
- Amplification of the complete genome of the virus isolated from baboon liver and/or serum samples
- Sequencing of the complete genome of the isolate and determination of its phylogenetic relationship to other hepadnaviruses
- Demonstration of replication in baboon liver
- Transmission of the virus to experimentally naïve baboons.

2. Materials and Methods

Permission for this study was obtained from the Animal Ethics Committee of the University of the Witwatersrand (Ethics Clearance Number: 97/88/1, Appendix I). All procedures were approved by the committee and the baboons were cared for according to the guidelines of the South African Medical Research Council.

2.1 Baboons

Ten large, adult Chacma baboons (*Papio ursinus orientalis*) were wild-caught in the Eastern Cape province of South Africa. The baboons were quarantined for six weeks in the Eastern Cape, but were not involved in any form of medical or veterinary research during this time. The only injection these baboons received was an intradermal tuberculin test performed with a disposable needle and syringe. Each baboon was housed in a separate cage during both the quarantine period and after their arrival at the Animal Unit of the University of the Witwatersrand Medical School in Johannesburg. Ten millilitres of whole blood was obtained from each of the ten baboons, allowed to clot and separated by centrifugation at ~5000 revolutions per minute (rpm) for 15 minutes (min) at room temperature. The serum was removed using a disposable pipette and stored at -70 °C.

Additional blood samples were obtained from both 39 adult and 20 juvenile Chacma baboons wild-caught in the Western Cape and Limpopo provinces of South Africa. Serum was isolated as described above and stored at -70 °C.

One of the ten baboons, baboon 9732, arrived in Johannesburg with a fracture of the femur and the Animal Ethics Committee decided that it should be euthanized. During necropsy, liver tissue and serum were obtained from this baboon. A section of the liver tissue was placed in formalin for histological examination while the remainder was snap frozen in liquid nitrogen and stored at -70 °C.

2.2 Immunocytochemical Examination of Samples

Baboon serum samples were tested at the National Health Laboratory Services (NHLS, Johannesburg, South Africa) for HBsAg and for anti-HBc using the Abbott AxSYM Microparticle Enzyme Immunoassay system (Abbott Laboratories, Chicago, Ill. USA). The alanine and aspartate aminotransferase levels were also measured using a Hitachi 747 Automatic Analyser.

Histological and immunohistochemical analysis of baboon liver tissue was kindly performed by Prof. A Paterson from the Department of Anatomical Pathology, University of the Witwatersrand. Formalin (10% v/v) fixed liver tissue from baboon 9732 was used for both histological and immunohistological preparations. The liver tissue was embedded in paraffin wax and 5 micron (μm) sections cut.

2.2.1 Haematoxylin and Eosin Staining

Sections were stained with haematoxylin and eosin for histological examination (Gamble and Wilson, 2002). Briefly, sections were dewaxed in xylene and rehydrated by passing them through decreasing concentrations of alcohol. After immersion in haematoxylin for five minutes, the sections were blued by placing them under running tap water and the nuclei differentiated by dipping the sections in acid alcohol (1% hydrochloric acid in 70% ethanol) once or twice. Sections were rinsed in tap water, stained in 0,2% eosin for one minute and rinsed again in tap water. The sections were dehydrated through increasing concentrations of alcohol, cleared in two changes of xylene, mounted in Entellan (Merck, Darmstadt, Germany) and viewed using a light microscope.

2.2.2 Immunohistochemistry against HBsAg and HBcAg

To detect HBsAg and HBcAg in the hepatocytes, 5 μm sections were dewaxed in xylene, rehydrated by passing through decreasing concentrations of alcohol, rinsed in distilled water and washed twice in PBS, pH 7,4 (Appendix II). Endogenous peroxidase activity was quenched by incubating the sections in

1% hydrogen peroxide (Merck) for 30 min and washed twice in PBS. A serum block was performed by incubating the sections in 1% goat serum (made up in PBS) (Dako, Glostrup, Denmark) for 20 min. The serum block was aspirated and primary antibody (monoclonal mouse anti-HBsAg (M3506, Dako) or polyclonal rabbit anti-HBcAg (B0586, Dako) added. Sections were incubated overnight in primary antibody at 4 °C in a humidified chamber. The following day, the sections were washed twice in PBS and incubated in secondary antibody (goat-anti-mouse IgG / horseradish peroxidise (HRP) (P0447, Dako) or goat-anti-rabbit IgG/HRP (P0448, Dako)) for one hour at room temperature. After washing three times in PBS, the sections were incubated for five minutes in diaminobenzidine (DAB) (Roche Diagnostics Ltd., Mannheim, Germany), rinsed in distilled water, dehydrated through increasing concentrations of alcohol, cleared in xylene and mounted in Entellan. Sections were viewed using a light microscope.

2.3 Extraction of DNA

DNA was extracted from both the serum and liver tissue of the baboons using a variety of different methods.

2.3.1 QIAGEN QIAamp® DNA Blood Mini Kit

DNA was extracted from 200 µl of baboon serum using QIAamp® DNA Blood Mini Kit (QIAGEN, Inc., Hilden, Germany) according to the manufacturer's instructions. Briefly, the 200 µl aliquot of serum was incubated with QIAGEN protease and a lysis buffer at 56 °C from 10 min. The lysate was applied to a QIAamp® spin column, washed twice and eluted in 50 µl best quality water (Sabax, Adcock Ingram Critical Care Ltd., Johannesburg, South Africa).

2.3.2 GeneReleaser®

GeneReleaser® (Bioventures Inc., Murfyboro, Tenn.) is a proprietory agent which quickly facilitates the release of DNA from whole blood and cells. DNA was extracted from 5 µl of baboon serum using GeneReleaser® according to the manufacturer's instructions. A 20 µl volume of GeneReleaser® was added to the serum sample and treated using the following thermocycle lysis program: 65 °C held for 30 s, 8 °C held for 30 s, 65 °C held for 180 s, 97 °C held for 60 s and 65 °C held for 60 s. Treated samples were held at 80 °C until the PCR mixture was added. The full 25 µl of treated sample was used as template for a 100 µl first round PCR reaction (section 2.5.3).

2.3.3 QIAGEN QIAamp® DNA Mini Kit

DNA was extracted for cccDNA analysis from liver tissue using the QIAamp® DNA Mini kit tissue protocol. Although this kit does not require any mechanical disruption of the tissue sample, 10-25 mg of liver tissue was ground in liquid nitrogen with a pestle and mortar to facilitate lysis of the cells. The ground tissue was transferred to a 1,5 ml micro-centrifuge tube and DNA extracted according to the manufacturer's instructions. The samples were incubated with QIAGEN Proteinase K (>600 mAU/ml) and a lysis buffer overnight at 56 °C on a rocking platform to ensure complete lysis. The following day, the lysed sample was transferred to a QIAamp® spin column, washed twice and eluted in 50 µl best quality water. Eluting the DNA in 50 µl as opposed to 200 µl decreases the overall yield of DNA but increases the concentration significantly. The samples were stored at -20 °C.

To maximise the quantity of DNA in each sample extracted from the baboon liver tissue, the extractions were repeated, using a number of variations: (1) the starting amount of liver tissue was increased to 50 mg; (2) multiple extracts were pooled and re-extracted using a salt-ethanol precipitation; (3) DNA was extracted from both 25 mg and 50 mg of baboon liver tissue as before but in the final step of the extraction procedure, DNA was eluted from the extraction column three times,

using the maximum volume (200 µl) of elution buffer permitted each time, resulting in a final elution volume of 600 µl. These extracts were reprecipitated using a salt-ethanol precipitation method and resuspended in a smaller volume of best quality water.

2.3.4 Phenol-Chloroform Extractions

This protocol is a modification of that described by Sykes in 1983 (Kramvis *et al.*, 1996; Sykes, 1983). Approximately 500 mg of liver tissue was cut from frozen baboon liver. The liver tissue had been stored for a number of years at -70 °C so the section was obtained from approximately 1 cm below the surface of the frozen tissue to prevent contamination and loss of DNA quality as a result of degradation by freezer burn. The tissue was finely minced using a sterile scalpel blade and homogenised with a sterile 2 ml manual glass homogeniser in 1 ml phenol-chloroform lysing buffer (Appendix II). The homogenate was transferred to a sterile 50 ml Nunc™ tube (Nunc A/S, Roskilde, Denmark) and an additional 9 ml lysing buffer added. The homogenate and extra buffer were blended using a blunt, sterile glass rod. Two ml of 10% SDS (Appendix II) was added, the tube tightly capped and placed on a gentle shaker at 37 °C for 20 min. Following this incubation, 10 ml tris-saturated phenol (pH 7) (SIGMA, Sigma-Aldrich Inc., St Louis, Mo.) and 5 ml chloroform: iso-amyl alcohol (24:1) (Appendix II) was added to the tube, which was tightly capped and shaken vigorously. The sample was centrifuged at 2000 x *g* in a Sorvall® RT6000 tabletop refrigerated centrifuge (Global Medical Instrumentation Inc., Ramsey, Minn.) for 15 min at 4 °C to separate the phases. The aqueous (upper) phase was removed using a sterile 10 ml pipette, carefully avoiding the interphase, and transferred to a clean, sterile 50 ml Nunc Tube. This extraction with phenol and chloroform was repeated with the aqueous phase being transferred once more to a clean, sterile 50 ml Nunc tube. The sample was then extracted once with chloroform alone by adding 10 ml chloroform: iso-amyl alcohol (24:1) (Appendix II) to the tube, tightening the cap and shaking vigorously prior to centrifuging it at 2000 x *g* for 10 min at 4 °C to separate the phases. After transferring the aqueous (upper) phase to a clean, sterile 50 ml Nunc tube, the DNA was precipitated by the addition of 2,5 volumes

(approximately 25 ml) ice-cold ethanol (Saarchem, Merck Ltd. Modderfontein, South Africa) and left overnight at -20 °C. The following day, the DNA was pelletted by centrifuging at 200 x *g* in a Sorvall® RT6000 centrifuge for 30 min at 4 °C, the supernatant was carefully decanted and discarded and the pellet resuspended overnight on a gentle shaker in 10 ml TE Buffer (pH 8) (Appendix II). Two mg Proteinase K (Roche, F. Hoffmann – La Roche Ltd., Basel, Switzerland) was added to the sample and digested at 60 °C for 2 hours with occasional mixing after which an additional 200 µg Proteinase K was added and digestion continued at 60 °C for a further 2 hours. The sample was extracted twice with phenol-chloroform and once with chloroform alone as described previously, the DNA re-precipitated by the addition of 2,5 volumes (approximately 25 ml) ice-cold ethanol and the sample left overnight at -20 °C. The DNA was pelletted by centrifuging at 200 x *g* for 30 min at 4 °C in a centrifuge and the pellet allowed to air-dry almost completely before being resuspended overnight on a gentle shaker in 1 ml sterile water (Sabax). Once the sample was completely resuspended, it was stored in 100 µl aliquots at -70 °C.

2.3.5 Quantification of DNA

The DNA extracted from the samples was quantified using spectrophotometric analysis on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, Del.). The phenol-chloroform extracted DNA samples were highly concentrated and were thus diluted ten fold prior to analysis. The concentration of the DNA was calculated by using the standard that 1 OD_{260} = 50 µg DNA/ml. The quality of the samples was assessed by calculating the OD_{260}/OD_{280} ratio.

2.4 Genotyping of HBV

HBV DNA extracted from liver tissue using the phenol-chloroform extraction method was genotyped using a modification of the method first described by Lindh *et al.* in 1997.

2.4.1 PCR amplification

A region of the surface gene of HBV (nt 256 – 796) was amplified by nested PCR using the primers in table 3. In the first round of amplification, a 22,5 µl mixture comprising 0,5 units (U) of BIOTAQ™ DNA polymerase (Bioline Ltd, Luckenwalde, Germany), 200 µM of each deoxynucleoside triphosphate (dNTP), 1 µM of each primer (230F and 800R), 3 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8,8) and 0,01% Tween-20, was added to 2,5 µl sample DNA. Amplification was performed in a Techgene programmable thermocycler (Techne Inc., Princeton, NJ.) as follows: denaturation at 94 °C held for 60 s, annealing at 53 held for 300 s and extension at 72 °C held for 180 s for a total of 40 cycles with a final extension at 72 °C held for 7 min. The second round of amplification added 5 µl of first round product to 45 µl of a mixture containing 1 U of BIOTAQ™ DNA polymerase (Bioline), 200 µM of each dNTP, 1 µM of each primer (P7 and P8), 1,5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8,8) and 0,01% Tween-20. Amplification was performed in a programmable thermocycler (Techne) as follows: denaturation at 94 °C held for 45 s, annealing at 53 °C held for 60 s and elongation at 72 °C held for 90 s for a total of 40 cycles with a final extension at 72 °C held for 10 min.

Table 3: First and second round primers for genotyping of HBV

Name	Position[a]	Nucleotide Sequence	Orientation
230F	231 - 249	5' – TCA CAA TAC CGC AGA GTC T – 3'	Sense
800R	801 - 782	5' – AAC AGC GGT ATA AAG GGA CT – 3'	Antisense
P7	256 - 278	5' – GTG GTG GAC TTC TCT CAA TTT TC – 3'	Sense
P8	796 - 776	5' – CGG TAW AAA GGG ACT CAM GAT – 3'	Antisense

[a] Denotes position on HBV subgenotype A2 genome (GenBank accession #X70185) with the *EcoR*I cleavage site at position 1.
Abbreviations: W – A and T; M – C and A

A 10 µl aliquot of the second round PCR product was resolved on a 1% agarose gel (section 2.6) to confirm the successful amplification of the required product prior to restriction fragment length polymorphism (RFLP) analysis.

2.4.2 Restriction Fragment Length Polymorphism (RFLP) analysis

RFLP analysis was performed on the second round PCR product to determine the HBV genotype (A – F) (Lindh *et al.*, 1997). Fifteen µl of the second round PCR product was dispensed into two separate 0,5 ml thin-walled PCR tubes. To one of these tubes, 5 U *Hinf*I (New England Biolabs, Inc, Ipswich, Mass.) and 1x NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM Dithiothreitol; pH 7,9) was added, while to the other, 5 U *Tsp*509I (New England Biolabs) and 1x NEBuffer 1 (10mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$ and 1 mM Dithiothreitol; pH 7,0) was added. Each reaction was overlaid with a thin layer of mineral oil before being incubated for 3 hours at 37 °C (for the *Hinf*I restriction) or 65 °C (for the *Tsp*509I restriction). The reaction was terminated by adding 4 µl gel loading dye (Promega Corp, Madison, Wis.).

The entire restriction reaction was resolved on a 3% agarose gel (Appendix II) containing 10 µg/ml ethidium bromide (Bio Basics Inc., Markham, Ontario) at ~3,5 volts/cm along with 10 µl 100 bp DNA ladder (Promega) and 10 µl 50 bp DNA ladder (Promega) until the bromophenol blue tracking dye had migrated approximately 80% of the length of the gel. An image of the gel was recorded using the Bio-Rad Gel Doc XR Imaging System (Bio-Rad Laboratories Inc., Hercules, Calif.) and the sizes of the restricted fragments compared to those in table 3 of Lindh *et al.* (1997, p 1290) to determine the genotype of the samples.

2.5 PCR amplification

2.5.1 Full length PCR

Amplification of the full HBV genome was performed using a modification of the protocol described by Günther *et al.* in 1995 (primers listed in table 4) and the Expand High Fidelity PCR System (Roche Diagnostics Ltd., Mannheim, Germany).

Table 4: Primers for amplification of full length HBV genome (Gunther *et al.*, 1995)

Name	Position[a]	Nucleotide Sequence	Orientation
P1	1820 – 1841	5' – CTT TTT CAC CTC TGC CTA ATC A – 3'	Sense
P2	1825 - 1806	5' – AAA AAG TTG CAT GCT GAG GG – 3'	Antisense

[a] Denotes position on HBV subgenotype A2 genome (GenBank accession #X70185) with the *EcoR*I cleavage site at position 1.

The full length PCR amplification used a manual "hot start" in which the polymerase was omitted from the initial reaction mixture, and only added once the samples reached annealing temperature for the first time. The initial reaction therefore contained a 20 µl mixture comprising 1x Expand High Fidelity buffer with 1,5 mM $MgCl_2$, 50 µM of each dNTP, 1 µM of each primer (P1 and P2) and 5 µl sample DNA. Amplification was preformed using a RoboCycler® Gradient 40 programmable thermocycler (Stratagene, La Jolla, Calif.) as follows: denaturation at 94 °C held for 40 s, annealing using a gradient of 57 °C – 60 °C held for 90 s and elongation at 68 °C for 180 s with an increase of 120 s after each 10 cycles for a total of 40 cycles. Five µl of an enzyme mixture comprising 1x Expand High Fidelity buffer and 3,75 U Expand High Fidelity enzyme mix was added to each reaction 30 s into the first annealing step. Following full length PCR amplification, a 5 µl aliquot of each sample was resolved on a 1% agarose gel (section 2.6), together with a 1 kb DNA ladder (Promega) to confirm successful amplification of the sample.

2.5.2 Amplification of the full HBV genome using two overlapping PCR products

This amplification was performed using the primers described by Takahashi *et al.* in 1998 (table 5) and the Expand High Fidelity PCR system.

Table 5: Primers for amplification of two overlapping PCR products (Takahashi *et al.*, 1998)

Name	Position[a]	Nucleotide Sequence	Orientation
T715	1553 - 1572	5' – CTG TGC CTT CTC ATC TGC CG - 3'	Sense
#S1-2	704 - 685	5' – CGA ACC ACT GAA CAA ATG GC - 3'	Antisense
#ep1-1	1606 - 1625	5' – GCA TGG AGA CCA CCG TGA AC - 3'	Sense
#S2-2	687 - 668	5' – GGC ACT AGT AAA CTG AGC CA - 3'	Antisense
#S1-1	192 - 211	5' – TCG TGT TAC AGG CGG GGT TT - 3'	Sense
T734	3161 - 3143	5' – CTT CCT GAC TGS CGA TTG G - 3'	Antisense
#S2-1	455 - 474	5' – CAA GGT ATG TTG CCC GTT TG - 3'	Sense
T733	3100 - 3081	5' – CCT GAG CCT GAG GGC TCC AC - 3'	Antisense

[a] Denotes position on HBV subgenotype A2 genome (GenBank accession #X70185) with the *Eco*RI cleavage site at position 1.
Abbreviations: S – G or C

The amplification protocol used was similar to that described in section 2.5.1 for the full length amplification of the HBV genome except that a nested PCR was used. The first round of amplification was identical to that of the full length amplifications except that the gradient for the annealing temperatures was 55 °C – 57 °C. The second round of the nested PCR used 5 µl of first round PCR product as template and a gradient of annealing temperatures of 53 °C – 55 °C but was identical to the full length PCR in all other respects. The success of the amplifications was confirmed by resolving a 5 µl aliquot of each sample on a 1% agarose gel (section 2.6), together with a 1 kb DNA ladder (Promega).

2.5.3 Subgenomic PCR

Subgenomic PCR amplifications using HBV-specific primers were used both to confirm the presence of HBV DNA in the extracts and to amplify the complete viral genome by the amplification of eight overlapping subgenomic fragments.

The primers used for these PCR amplifications were obtained either from Hu *et al*. (2000) or else were primer combinations used routinely in the Molecular Hepatology Research Unit laboratory. The subgenomic PCR amplifications were nested PCRs and the primers used, as well as the thermocycling conditions, are shown in table 6. For the first round of the nested PCR amplification, a 22,5 µl mixture comprising 0,5 U of BIOTAQ™ DNA polymerase (Bioline), 200 µM of each dNTP, 1 µM of each primer, 3 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8) and 0,01% Tween-20, was added to 2,5 µl sample DNA and amplified in a Techgene programmable thermocycler (Techne). All amplifications contained a final elongation step at 72 °C held for 7 min. For the second round of amplification, 5 µl of first round product was added to a 45 µl mixture containing 1 U of BIOTAQ™ DNA polymerase (Bioline), 200 µM of each dNTP, 1 µM of each primer, 1,5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8) and 0,01% Tween-20. Amplification was once again performed in a Techgene programmable thermocycler (Techne) and included final elongation step at 72 °C held for 10 min.

Table 6: **Primers combinations and thermocycling profiles for amplification of subgenomic regions of the viral genome.**

Name	Position[a]	Nucleotide Sequence	Orientation	Thermocycling Conditions			
				Denaturation	Annealing	Elongation	No. of cycles
58F[d]	56 – 75	5- CCT GCT GGT GGC TCC AGT TC - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
1450R[d]	1447 – 1426	5- GAT TCA GCG CCG ACG GGA CGT A - 3’	Antisense				
409F[d]	409 – 431	5- CAT CCT GCT GCT ATG CCT CAT CT - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
1101R	1120 – 1098	5- GAA AGG CCT TGT AAG TTG GCG AG - 3’	Antisense				
255F	253 – 275	5- CTC GTG GTG GAC TTC TCT CAA TT - 3’	Sense	94 °C for 30 s	56 °C for 50 s	72 °C for 60 s	40
759R	759 – 739	5- CCC CAA TAC CAC ATC ATC CAT - 3’	Antisense				
459F	460 – 480	5- TAT GTT GCC CGT TTG TCC TCT - 3’	Sense	94 °C for 30 s	56 °C for 50 s	72 °C for 50 s	40
710R	711 – 691	5- AGC CCT ACG AAC CAC TGA ACA - 3’	Antisense				
409F[d]	409 – 431	5- CAT CCT GCT GCT ATG CCT CAT CT - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
1800R	1800 – 1774	5- AGA CCA ATT TAT GCC TAC AGC CTC CTA - 3’	Antisense				
730F[d]	682 – 704	5- AGT GCC ATT TGT TCA GTG GTT CG - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
1575R	1573 – 1549	5- CCG GCA GAT GAG AAG GCA CAG ACG G - 3’	Antisense				
1528F	1528 – 1547	5- ACC TCT CTT TAC GCG GTC TC - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
1921R	1921 – 1904	5- TTT ATA CGG GTC AAT GTC - 3’	Antisense				
1552F	1552 – 1571	5- TCT GTG CCT TCT CAT CTG CC - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
1803R	1803 – 1783	5- CGC AGA CCA ATT TAT GCC TAC – 3’	Antisense				
1732F	1730 – 1747	5- CTG GGA GGA GTT GGG GGA - 3’	Sense	94 °C for 30 s	62 °C for 50 s	72 °C for 50 s	40
2045R	2043 – 2022	5- CAA TGC TCA GGA GAC TCT AAC GG - 3’	Antisense				
1765F	1763 – 1783	5- GGT CTT TGT ACT AGG AGG CTG - 3’	Sense	94 °C for 30 s	58 °C for 50 s	72 °C for 50 s	40
1968R	1966 – 1946	5- GTC AGA AGG CAA AAA CGA GAG - 3’	Antisense				
1860F[d]	1859 – 1879	5- ACT GTT CAA GCC TCC AAG CTG - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
58R[d]	75 – 56	5- GAA CTG GAG CCA CCA GCA GG - 3’	Antisense				
1898F	1898 – 1917	5- GGC ATG GAC ATT GAC CCG TA - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
2898R	2917 – 2896	5- GAG GAT TGG GAA CAG AAA GAT T - 3’	Antisense				
1860F[d]	1859 – 1879	5- ACT GTT CAA GCC TCC AAG CTG - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
58R[d]	75 – 56	5- GAA CTG GAG CCA CCA GCA GG - 3’	Antisense				

2440F[d]	2416 – 2437	5- GCC GCG TCG CAG AAG ATC TCA A - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
2853R[d]	2842 – 2823	5- TGT TCC CAA GAA TAT GGT GA - 3’	Antisense				
1860F[d]	1859 – 1879	5- ACT GTT CAA GCC TCC AAG CTG - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
409R[d]	431 – 409	5- AGA TGA GGC ATA GCA GCA GGA TG - 3’	Antisense				
2440F[d]	2416 – 2437	5- GCC GCG TCG CAG AAG ATC TCA A - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
58R[d]	75 – 56	5- GAA CTG GAG CCA CCA GCA GG - 3’	Antisense				
1687F	1687 – 1706	5- CGA CCG ACC TTG AGG CAT AC - 3’	Sense	94 °C for 30 s	62 °C for 40 s	72 °C for 80 s	40
2498R	2498 – 2477	5- AAG CCC AGT AAA GTT TCC CAC C - 3’	Antisense				
2267F	2267 – 2284	5- GGA GTG TGG ATT CGC ACT - 3’	Sense	94 °C for 30 s	51 °C for 50 s	72 °C for 50 s	40
2436R	2436 – 2419	5- TGA GAT CTT CTG CGA CGC - 3’	Antisense				
2540F	2540 – 2561	5- TCC CTC CTT TCC TAA CAT TCA T - 3’	Sense	94 °C for 30 s	50 °C for 50 s	72 °C for 50 s	40
2896R	2896 – 2917	5- GAG GAT TGG GAA CAG AAA GAT T - 3’	Antisense				
2566F	2565 – 2586	5- CAG GAG GAC ATT ATT AAT AGA T - 3’	Sense	94 °C for 30 s	48 °C for 50 s	72 °C for 50 s	40
2858R	2857 – 2838	5- CCA TGC TGT AGC TCT TGT TC - 3’	Antisense				
2800F	2800 – 2834	5- CAG GTA GCG CCT CAT TTT GTG GGT CAC CAT ATT CT - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
1101R	1120 – 1098	5- GAA AGG CCT TGT AAG TTG GCG AG - 3’	Antisense				
2853F[d]	2823 – 2842	5- TCA CCA TAT TCT TGG GAA CA - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
409R[d]	431 – 409	5- AGA TGA GGC ATA GCA GCA GGA TG - 3’	Antisense				
2853F[d]	2823 – 2842	5- TCA CCA TAT TCT TGG GAA CA - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	45
1101R	1120 – 1098	5- GAA AGG CCT TGT AAG TTG GCG AG - 3’	Antisense				
58F[d]	56 – 75	5- CCT GCT GGT GGC TCC AGT TC - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35
730R[d]	682 – 704	5- CGA ACC ACT GAA CAA ATG GCA CT - 3’	Antisense				
2440F[d]	2416 – 2437	5- GCC GCG TCG CAG AAG ATC TCA A - 3’	Sense	94 °C for 45 s	55 °C for 45 s	72 °C for 180 s [b]	35 [c]
58R[d]	75 - 56	5- GAA CTG GAG CCA CCA GCA GG - 3’	Antisense				

[a] Denotes position on HBV subgenotype A2 genome (GenBank accession #X70185) with the *Eco*RI cleavage site at position 1.

[b] Ramping from annealing temperature to elongation temperature was performed at 0,4 °C per second.

[c] This was a single round PCR amplification using a clone containing a portion of the HBV genome as template. The conditions used for this amplification were identical to those used for the second round of nested PCR amplifications.

[d] Hu *et al.* 2000

2.5.4 Real Time PCR

Real time PCR was used to detect cccDNA in the liver tissue. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Amplified product was detected with SYBR® Green.

2.5.4.1 Plasmid-Safe™ DNase treatment

The DNA extracts were treated with Plasmid-Safe™ ATP-Dependent DNase (Epicentre® Biotechnologies, Madison, Wis.), which selectively hydrolyses linear double-stranded DNA and, at a lower efficiency, linear and closed-circular single-stranded DNAs. This meant that the chromosomal DNA would be digested while the HBV cccDNA would remain intact. Ten µl of total DNA extract was digested with 2 mM ATP solution and 20 U Plasmid-Safe DNase in 1 x reaction buffer (33 mM Tris-acetate (pH 7,8), 66 mM potassium acetate, 10 mM magnesium acetate and 0,5 mM DTT) for 45 min at 37 °C. The reaction was terminated by incubation at 70 °C for 30 mins. The restricted DNA was used directly as template for real time PCR analysis.

2.5.4.2 PCR amplification using SYBR® green detection

The *Power* SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, Calif.) was used for this PCR. To obtain optimal sensitivity, three primers were used for this amplification, two sense primers and one antisense primer (table 7).

Table 7: Primers for real time PCR using SYBR® Green detection (Bowden *et al.*, 2004)

Name	Position [a]	Nucleotide Sequence	Orientation
CCC1	1540 – 1559	5' – GCG GWC TCC CCG TCT GTG CC – 3'	Sense
DRF1	1551 – 1570	5' – GTC TGT GCC TTC TCA TCT GC – 3'	Sense
CCC2	1906 – 1887	5' – GTC CAT GCC CCA AAG CCA TC – 3'	Antisense

[a] Denotes position on HBV subgenotype A2 genome (GenBank accession #X70185) with the *Eco*RI cleavage site at position 1.
Abbreviations: W – A or T

For each sample, a 50 µl reaction containing 25 µl *Power* SYBR® Green PCR Master Mix, 0,5 mM of each of the three primers and 5 µl of sample DNA was prepared. The amplification was performed using a 7500 Real-Time PCR system (Applied Biosystems) as follows: initial denaturation at 95 °C held for 10 min and a total of 45 cycles of denaturation at 95 °C held for 15 s and annealing and elongation at 65 °C held for 60 s. Data was collected during the 65 °C incubation. A dissociation curve was run at the end of the amplification using the following protocol: 95 °C held for 15 s, 64 °C held for 60 s and 99 °C held for 15 s.

2.6 Agarose Gel Electrophoresis

Products were resolved on agarose gels of the appropriate volume and concentration, prepared as described in appendix II, in order to visualise the DNA. Once completely solidified, the agarose gel was immersed fully in an electrophoresis tank containing 1x TBE Buffer (Appendix II). The samples were prepared by adding 0,4 volumes 6x Blue/Orange Loading Dye (15% Ficoll® 400, 0,03% bromophenol blue, 0,03% xylene cyanol FF, 0,4% orange G, 10mM Tris-HCl (pH 7,5) and 50 mM EDTA) (Promega) and mixed thoroughly. The entire sample volume was loaded into the agarose gel and the gel electrophoresed at ~4 volts / cm until the bromophenol blue tracking dye front had migrated ~75% of the length of the gel. An image of the gel was recorded using the Gel Doc XR Imaging System (Bio-Rad).

2.7 Cloning

Two different methods were used to clone fragments amplified by PCR. The TOPO® XL PCR Cloning Kit (Invitrogen Corp., Carlsbad, Calif.) was used to clone fragments greater than 2 kb in length and the InsTAclone™ PCR Cloning Kit (Fermentas Life Sciences, Burlington, Ontario) was used to clone fragments smaller than 2 kb. Both kits were used according to the manufacturer's instructions.

PCR amplicons cloned with the TOPO® XL PCR Cloning Kit were isolated by resolving them on a 0,8% Crystal Violet agarose gel (Appendix II), and the fragments of interest excised and extracted using a S.N.A.P.™ purification column (Invitrogen). The extracted DNA was eluted in 40 µl TE Buffer (Invitrogen) and 4 µl of this gel-purified product was used for the cloning reaction. The TOPO® cloning reaction was incubated for 5 min at room temperature, after which 1 µl 6x TOPO® Cloning stop solution was added. A 2 µl aliquot of the TOPO® cloning reaction was used immediately for the transformation of One Shot® TOP10 chemically competent cells (Invitrogen). Three different volumes of the transformation reaction (50 µl, 100 µl and 150 µl) were spread onto prewarmed Luria-Bertani (LB) agar plates containing 50 µg/ml kanamycin (Bio Basic Inc.) and incubated overnight at 37 °C. Individual colonies were selected and re-plated onto fresh LB agar-kanamycin plates, incubated at 37 °C overnight and stored at 4 °C until required.

For PCR amplicons cloned with the InsTAclone™ PCR Cloning Kit, the final elongation step of the second round of the nested PCR amplification was increased from 10 min to 30 min. This was to increase the number of 3'-A overhangs on the ends of the amplicons. The amplicons were gel purified using the QIAGEN QIAquick Gel Extraction Kit according to the manufacturer's instructions and eluted in 50 µl best quality water. A 10 µl aliquot of this elute was resolved on a 1% agarose gel (section 2.6) together with a O'GeneRuler 1 kb DNA ladder (Fermentas). This molecular weight marker is composed of fragments of known concentration and the concentration of the eluted DNA can be determined by comparing the intensity of the resolved fragment to the intensities of the fragments in the molecular weight marker. This concentration was used to determine the volume of extracted DNA equivalent to the 0,54 pmol ends required for the cloning reaction which was set up according to the manufacturer's instructions and incubated overnight at 22 °C. A 2,5 µl aliquot of the cloning reaction was transformed into One Shot® TOP10 chemically competent cells (Invitrogen). As with the TOPO® XL PCR Cloning Kit, three different volumes of the transformation reaction (50 µl, 100 µl and 150 µl) were spread onto prewarmed LB agar plates containing 50 µg/ml ampicillin (Roche) and incubated overnight at 37 °C. The next day, individual colonies were selected

and re-plated onto fresh LB-ampicillin plates, incubated at 37 ℃ overnight and stored at 4 ℃ until required.

Plasmid DNA was isolated from individual colonies using the GenElute™ Plasmid Miniprep Kit (SIGMA) according to the manufacturer's instructions. The cells were cultured overnight in 5 ml LB medium containing either kanamycin or ampicillin as required. The cells were harvested, lysed and the DNA purified using a GenElute™ Miniprep Binding Column. The DNA was eluted in 50 µl best quality water.

A 5 µl aliquot of extracted plasmid DNA was restricted at 37 ℃ for 1 hour with 20 U *EcoR*I (New England BioLabs) in 1x NEBuffer *EcoR*I, comprising 50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$ and 0,025% Triton X-100 at a pH of 7,5. The restricted products were resolved overnight on a 1% agarose gel (section 2.6) together with a 1 kb DNA ladder (Promega) to determine whether or not the plasmids contained the fragment of interest.

2.8 Sequencing of amplified products

Products amplified by PCR, as well as cloned PCR products, were sequenced by Inqaba Biotechnical Industries Ltd (Pretoria, South Africa) using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA), and the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). The primers used for sequencing the PCR amplicons were the same as those used for the second round PCR or, in the case of the cloned products, the vector specific primers recommended by the manufacturers. Chromatograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Sequences were subjected to a standard nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the amplicons belonged to the family *Hepadnaviridae* rather than being the result of the non-specific amplification of genomic DNA

2.9 Phylogenetic Analysis

Sequences were aligned manually with complete HBV genome sequences from GenBank® (http://www.ncbi.nlm.nih.gov/Genbank/index.html) using the GeneDoc program (Nicholas and Nicholas, 1997). Overlapping sequences were used to generate a complete sequence which was fed into PHYLIP (PHYLogeny Inference Package) version 3.6 (Felsestein, 1995). Neighbour joining phylogenetic trees were generated by calculating the evolutionary distances using the DNADIST program and the Kimura-2-parameter model and a dendogram was generated with the NEIGHBOR program. Bootstrap analysis using 1 000 bootstrap replicates was performed using the SEQBOOT, DNADIST and NEIGHBOR programs. Bootstrap values below 75% indicated that a monophyletic group was not well supported, while values greater that 95% indicated very solid nodes. A consensus tree was generated using the CONSENSE program and the trees visualised using the TreeView Win 32 program (Page, 1996). Trees were rooted using either a WMHBV isolate (GenBank accession no. AY226578) or an HBV genotype F isolate (GenBank accession no. X69798) and the scale bar at the bottom of the dendogram showed the scaled evolutionary distance.

2.10 Recombination Analysis

Evidence of possible recombination with the complete HBV genome from baboons was investigated using the Simmonic 2005 version 1.6 software package implementing PHYLIP (Simmonds and Midgley, 2005). The complete genome sequence was compared to a reference set of 228 non-recombinant HBV isolates and a GroupScanning plot generated (Tatematsu *et al.*, 2009). Consecutive fragments of 500 bp in length, with 15 bp incremental steps between fragments, were analysed.

2.11 Southern Blotting DNA samples

This protocol was obtained from Martins-Furness (2009)

2.11.1 Blotting of samples onto a nylon membrane

Samples were blotted onto nylon membrane either by using a slot blot manifold or by first resolving the samples on an agarose gel and then transferring the DNA to the membrane using the capillary transfer method.

2.11.1.1 Slot Blotting

Serum samples from a number of different baboons and vervet monkeys were blotted onto a nylon membrane using a modification of the protocol described by Zaaiger *et al.* (1994). A 50 µl aliquot of serum was incubated with 50 µl slot blot denaturing solution (Appendix II) for two hours at 56 °C followed by a 95 °C incubation for ten min. The samples were quick chilled on ice to allow them to thicken and 200 µl 20 x SSC (Appendix II) added. After mixing thoroughly by vortexing, the samples were clarified by centrifuging at 13 000 rpm in a microfuge (Eppendorf GmBH., Hamburg, Germany) for 15 min. Meanwhile, the Bio-Dot® SF microfiltration apparatus (BioLab Laboratories, South Richmond, Calif.) was prepared by cutting a piece of Hybond N nylon membrane (Amersham Biosciences UK Ltd., Buckinghamshire, UK), to the size of the slot blot manifold. This membrane, together with a piece of Bio-Dot® SF filter paper (BioLab) the same size as the membrane, was pre-wet in 20 x SSC (Appendix II) and placed on the manifold on top of the filter paper before being clamped tightly in place. Two hundred µl of supernatant from each of the centrifuged samples was loaded, one sample per well, and allowed to blot by applying a vacuum to the manifold. Once the entire sample had passed through the membrane, the vacuum was released, the apparatus disassembled and the bound DNA denatured by placing the membrane (DNA side up) on a pad of filter paper soaked in 0,4 M NaOH (Appendix II) for 5 min. The membrane was transferred to a neutralising solution comprising 2 x SSC for one minute and placed, DNA side up, on a clean piece of filter paper to air-dry. The DNA was immobilised by baking the blot at 80 °C for two hours

and the blot was then stored, between two pieces of filter paper, at room temperature until required.

2.11.1.2 Transfer of DNA from the agarose gel

A 10 µl aliquot of each of the PCR amplified products to be blotted was resolved on a 1% agarose gel (section 2.6). Following electrophoresis, the DNA was prepared for transfer to the nylon membrane by trimming any excess agarose from the sides of the gel and placing it in a trough and covered with depurination solution (Appendix II) on a gentle horizontal shaking platform for 20 min at room temperature. Soaking the gel in depurination solution for 10 – 20 min increases the efficiency of the transfer of large DNA fragments (>15 kb) to the nylon membrane by nicking the DNA (Sambrook *et al.*, 1989). The depurination solution was poured off, the gel rinsed twice in distilled water and then covered in denaturation solution (Appendix II) to ensure that the DNA is single stranded (Sambrook *et al.* 1989). After gently shaking for 45 min at room temperature, the denaturation solution was poured off and the gel rinsed twice in distilled water. Following denaturation, the gel was covered in neutralisation solution (Appendix II) and placed on the shaking platform for 30 min at room temperature after which the neutralisation solution was poured off and the gel rinsed twice in distilled water. The DNA was transferred to the nylon membrane using the capillary transfer method.

Approximately one litre of 20 x SSC (Appendix II) was poured into a trough and a glass plate, slightly wider than the length of the agarose gel, was placed across the width of the trough (figure 11). A wick, composed of a long piece of 3MM Whatman paper (Whatman Plc., Maidstone, Kent, UK), was wet thoroughly with 20 x SSC and placed across the glass plate with both ends sitting in the buffer. A glass rod was carefully rolled across the wick to remove any air bubbles. The agarose gel was positioned upside down on the wick, the glass rod rolled carefully over the gel to remove any air bubbles and the areas around the gel covered with plastic cling wrap.

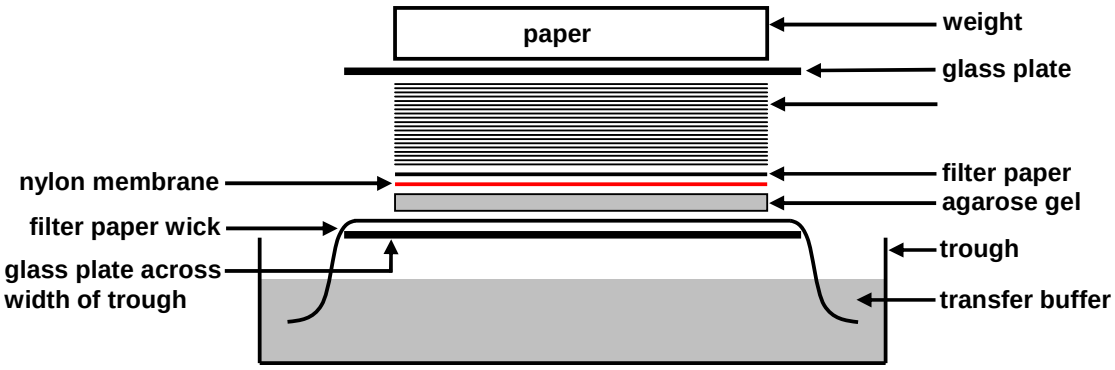


Figure 11: Capillary transfer of DNA from an agarose gel. This schematic illustrates the set-up required to transfer DNA from an agarose gel to a nylon membrane. Buffer is drawn up from the trough via the wick passing through the gel and into the stack of paper towels. As the buffer passes through the agarose gel, it elutes the DNA from the gel and deposits it on the nylon membrane. A weight is applied to the top of the paper towels to ensure that there is a tight connection between the various layers of the transfer set-up (Sambrook *et al.* 1989).

A piece of Hybond-N nylon membrane (Amersham Biosciences) was cut to the exact size of the gel and pre-wet in distilled water and followed by 20 x SSC (Appendix II). The membrane was placed on top of the agarose gel and the glass rod rolled carefully over the membrane to remove air bubbles. One corner of the membrane was carefully marked at this time to allow for the correct orientation of the membrane after transfer. Two sheets of 3MM Whatman filter paper cut to the same size as the gel were pre-wet in 20 x SSC (Appendix II) and placed over the membrane, removing any air bubbles by rolling the glass rod over the filter paper. An additional four sheets of dry 3MM Whatman filter paper cut to the same size as the gel were placed on top of the wet sheets and any air bubbles removed as before. The filter paper was covered with a stack of paper towels ~15 cm high, trimmed to the same size as the agarose gel, ensuring that there was no contact between the gel and the paper towels to prevent short-circuiting the transfer of the DNA. A second glass plate was placed on top of this stack and covered with a weight of ~800g evenly distributed across the entire gel. Transfer was allowed to take place for between 12 and 48 hours, replenishing the buffer in the bottom tank if and when needed, being careful not to disturb the transfer set-up in the process. Once transfer was complete, the set-up was dismantled, the membrane washed in 2 x SSC (Appendix II) and placed DNA side up on a clean piece of filter paper to

air-dry. The membrane was baked at 80 °C for two hours to immobilise the DNA and stored between two pieces of filter paper at room temperature until required.

2.11.2 Preparation of probe for Southern hybridisation

DNA was extracted from the serum of a patient known to be positive for a HBV infection using the QIAGEN QIAamp® DNA Blood Mini Kit and used as template for a full length PCR amplification (section 2.5.1) of the complete viral genome. The full length amplicon was cloned using the TOPO® XL PCR Cloning Kit (section 2.7) and the plasmid DNA extracted using the GenElute™ Plasmid Miniprep Kit (SIGMA). The plasmid DNA extract, diluted one in ten, was used as template for amplification of the complete genome by full length PCR amplification (section 2.5.1). The full length amplicon was resolved on a 1% agarose gel (section 2.6), the 3,2 kb fragment excised with a clean scalpel blade and the DNA purified using the QIAGEN QIAquick Gel Extraction Kit according to the manufacturer's instructions. The DNA was eluted in 10 µl elution buffer (10mM Tris-HCl, pH 8.5) and stored at -20 °C until needed.

The gel purified full length HBV genome amplicon was quantified by resolving a dilution series of the DNA on a 1% agarose gel (section 2.6). The DNA was diluted in sterile water and 10 µl of each dilution resolved on the agarose gel. The concentration of the probe was approximated from this gel using the standard that the fragment just visible contains 10 ng of DNA.

2.11.3 Radioactive labelling of probe DNA

The probe was labelled using the Megaprime™ DNA Labelling System (Amersham Biosciences). A 200 ng aliquot of the gel purified 3.2 kb full length HBV amplicon (section 2.11.2) was placed in a 1,5 ml microcentrifuge tube together with 5 µl primer solution (random nonamer primers in an aqueous solution) and the volume adjusted to 33 µl with TE Buffer (Appendix II). The probe was denatured at 99 °C for five min, briefly centrifuged and placed on ice. Five µl of labelling buffer (dATP, dTTP and dGTP in Tris-HCl (pH 7,5),

2-mercaptoethanol and $MgCl_2$), 2 µl enzyme solution (1 U/µl DNA polymerase I Klenow fragment (cloned) in 100 mM potassium phosphate (pH 6,5), 10 mM 2-mercaptoethanol and 50% glycerol) and 5 µl Amersham Redivue™ α-^{32}P-dCTP 250 µCi (Amersham Biosciences) was added and the reaction incubated at 37 °C for 20 min after which it was briefly centrifuged, placed on ice and 50 µl of TE Buffer (Appendix II) added to adjust it to a total volume of 100 µl.

The radioactively labelled probe was purified by passing it through a Sephadex column (Appendix II) to remove any unincorporated nucleotides. A labelled 1,5 ml microcentrifuge tube was placed at the bottom of an equilibrated sephadex column to collect the labelled probe. The entire 100 µl of probe was added to the Sephadex column and centrifuged at ~3 500 x *g* for 5 min. An equal volume (100 µl) of TE buffer (Appendix II) was added to the column and the columns left to stand at room temperature for 5 min before centrifuging once again for 5 min at ~3 500 x *g*. The collection tube containing the purified probe was removed from the bottom of the column, the volume of the probe adjusted to 1 ml with TE buffer (Appendix II) and placed on ice.

The amount of radioactivity incorporated into the probe was established using a scintillation counter. The sample was prepared by adding 10 µl of labelled probe to 5 ml INSTA-GEL scintillation gel (United Technologies, Packard Instrument Company, Dowers Grove, Ill.) in a glass scintillation vial (Packard Instrument Company). After mixing thoroughly by vigorous shaking, the sample was counted for one minute on the scintillation counter and the amount of probe required for the Southern hybridisations calculated. For each blot, between 25 x 10^6 and 100 x 10^6 incorporated counts per minute (cpm) of probe was necessary.

2.11.4 Hybridisation of Southern Blots with Radioactive probes

A piece of nylon mesh was cut slightly larger (~1 cm) than the dimensions of the nylon membrane to be hybridised and pre-wet in distilled water. The nylon membrane onto which the DNA had been blotted (section 2.11.1) was placed in a

suitable trough and pre-wet in distilled water before being rolled, DNA side up, inside the pre-wet piece of nylon mesh allowing any overlapping areas of the blot to remain separated during hybridisation. The rolled blot was placed in a hybridisation tube (Hybaid Ltd., Middlesex, UK) in such a way as to ensure that, when placed in the hybridisation oven, the movement of the rotor would keep the blot unrolled. If more than one membrane was to be blotted simultaneously, each blot was placed in a separate hybridisation tube. After unrolling the blot in the hybridisation tube, it was rinsed in a small volume of Church and Gilbert (C&G) hybridisation buffer (Appendix II), which was discarded, and any excess liquid in the tube drained off. Fifty ml C&G Hybridisation Buffer was added to each tube and the blots pre-hybridised at 65°C for 30 min in a Hybaid hybridisation oven. Meanwhile, an appropriate volume of radioactively labelled probe (8×10^7 cpm) was placed in a 2 ml microcentrifuge tube together with 100 µl salmon sperm DNA (Appendix II) and mixed thoroughly. A separate tube of probe was prepared for each blot to be hybridised. The probe was incubated at 99 °C for 5 min, snap cooled on ice and briefly centrifuged. One ml of pre-heated C&G Hybridisation buffer was added to the probe and mixed thoroughly just prior to the addition of the entire mixture to the pre-hybridised blot in the hybridisation tube. Care was taken to avoid placing the probe directly on the membrane; instead it was added to the hybridisation buffer already in the tube. The blot was hybridised overnight at 65 °C.

2.11.5 Washing of the Southern Blot after hybridisation

The following day the blots were washed to remove any excess probe and any non-specifically bound probe. The hybridisation tube was removed from the oven and the C&G hybridisation buffer poured off and discarded. Approximately 200 ml of the first wash buffer (Appendix II) was added to the hybridisation tube and the blot washed at room temperature for 8 min in the hybridisation oven. This first wash buffer was poured off and discarded and the wash repeated. After pouring the wash buffer off once again, ~200 ml of the second wash buffer (Appendix II) was added to the tube. The tube was returned to the hybridisation oven and the blot washed at 65 °C for 10 min. The second wash buffer was

poured off and discarded and the blot rinsed twice at room temperature with the third wash buffer (Appendix II). The blotted nylon membrane, together with the nylon mesh, was removed from the hybridisation tube and unrolled. The membrane was placed between two sheets of plastic and sealed on all sides with a plastic sealer being careful to remove any air-bubbles. The nylon mesh was placed in a 10% SDS solution so that it could be re-used, after extensive rinsing in distilled water, if ever the membrane was re-probed with the same probe. The membrane, sealed in plastic, was placed in a light-tight X-ray cassette with an intensifying screen and exposed to α-^{32}P-sensitive KODAK BioMax MS Film (Carestream Health, Rochester, NY.) for ~2 weeks at -70 °C before developing.

2.12 Analysis of RNA

2.12.1 Extraction of RNA from liver tissue

RNA was extracted using the guanidinium-acid-phenol method first described in 1987 by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987; Farrell, 1993). Liver tissue was obtained from ~1cm below the surface of the frozen baboon 9732 liver to prevent contamination and the degradation of nucleic acids by freezer burn. A section of the liver tissue weighing approximately 100 mg was ground in liquid nitrogen using an RNase-free mortar and pestle and the ground liver tissue transferred to a 2 ml glass homogeniser. The tissue was homogenised in 1ml Solution D (Appendix II) and the homogenate transferred to a 15 ml disposable, RNase free polypropylene tube after which the following were added in order: 100 µl 2M sodium acetate, pH 4,0 (Appendix II), 1 ml water saturated phenol (Appendix II) and 200 µl 49:1 chloroform: iso-amyl alcohol (Appendix II). The tube was capped and mixed thoroughly by inversion following the addition of each reagent and, after all the reagents had been added, shaken vigorously for ten seconds. The sample was cooled on ice for 15 min and then centrifuged at 2000 x *g* at 4 °C to separate the phases. The aqueous (upper) phase, which contained the RNA, was transferred to a fresh 15 ml tube and mixed with an equal volume of isopropanol (approximately 1ml, Associated Chemical Enterprises cc., Johannesburg, South Africa) and stored at –20 °C overnight to precipitate the

RNA. The following day, the precipitate was collected by centrifugation at maximum speed (>2000 x *g*) for 20 min at 4 °C, the supernatant was carefully decanted and discarded and the RNA pellet completely resuspended in 300 µl of Solution D (Appendix II). This resuspension was transferred to an RNase free 1,5 ml microcentrifuge tube (Eppendorf) and the RNA precipitated by adding an equal volume of ice cold isopropanol and storing at –20 °C for one hour. The RNA pellet was collected at top speed in a microcentrifuge for ten minutes at 4 °C and the supernatant carefully decanted and discarded. The pellet was washed twice in 500 µl 75% ethanol (Appendix II), recentrifuged and incubated to near dryness. Finally, the pellet was redissolved in ~100 µl DEPC-treated water (Invitrogen) and stored at –70 °C in suitable aliquots. Repeated freezing and thawing of the RNA was avoided as this has been known to increase the risks of RNA degradation.

2.12.2 Quantification of RNA

The concentration of each RNA sample was determined by spectrophotometry using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The concentration of the RNA was calculated by using the standard that 1 OD_{260} = 40 µg RNA/ml. The quality of the samples was assessed by calculating the OD_{260}/OD_{280} ratio.

2.12.3 DNase treatment of extracted RNA

An appropriate volume of guanidinium-acid-phenol extracted RNA was digested with RNase-free Deoxyribonuclease I (DNase I, Fermentas) to remove any contaminating DNA from the sample. Digestions were set up in an RNase-free 0,2 ml thin-walled PCR tube (Eppendorf). For each µg of RNA, one U of DNase I (1 U/µl) and one tenth the final volume of 10 x Reaction Buffer (100 mM Tris-HCl (pH 7,5), 25 mM $MgCl_2$, 1 mM $CaCl_2$) were added and the sample made up to the correct volume using DEPC-treated water (Invitrogen). The digestions were incubated at 37 °C for 30 min in a Techgene programmable thermocycler (Techne) and, after the addition of a volume of 25mM EDTA (Fermentas) equal

to one tenth of the volume of the restriction, the digestions were inactivated by incubating at 65 ºC for 10 min in the Techgene thermocycler. The EDTA was added at this stage as RNA is known to hydrolyse during heating in the absence of a chelating agent (Wiame *et al.*, 2000).

Following digestion of the RNA sample with DNase I, the RNA was reprecipitated by adding one tenth the volume of RNase-free 2M Sodium Acetate (Appendix II) and an equal volume of ice-cold isopropanol and placing the tube at -20 ºC for one hour. The RNA pellet was collected at top speed in a microcentrifuge (Eppendorf) for 10 min at 4 ºC and the supernatant carefully decanted and discarded. The pellet was washed twice in 500 µl 75% ethanol (Appendix II), recentrifuged and incubated to near dryness. The RNA pellet was dissolved in an appropriate volume (~1 µl for each µg of RNA digested) of DEPC-treated water. The concentration of RNA in each sample was determined by spectrophotometric analysis using a NanoDrop spectrophotometer as described in section 2.12.2.

2.12.4 Agarose gel electrophoresis of RNA

The DNase treated RNA was resolved on a 1% agarose gel containing formaldehyde and 1 x MOPS buffer to assess the quality of the extraction. A 50 ml gel was prepared by completely dissolving 0,5 g agarose (Bioline) in 37,5 ml distilled water in a microwave oven (Farrell, 1993). The gel was cooled to between 55 ºC and 60 ºC and 10 ml pre-warmed 5 x MOPS buffer (Appendix II) and 2,75 ml pre-warmed formaldehyde (Saarchem) were added. This produced a 1% agarose solution in 1 x MOPS buffer and 0,66 M formaldehyde. The gel was cast under a fume hood to minimise formaldehyde fumes in the room and allowed to set. The RNA samples were prepared by the addition of 5 µg RNA, 2 µl 5 x MOPS buffer, 3,3 µl formaldehyde, 10 µl formamide (Saarchem) and DEPC-treated water (Invitrogen) to a final volume of 20 µl, in a sterile, RNase free 0,5 ml thin-walled PCR tube. The samples were mixed thoroughly and centrifuged briefly in a microcentrifuge to collect the components at the bottom of the tube. They were then denatured at 55 ºC for fifteen minutes after which 2 µl

of 10 x RNA gel loading buffer (Appendix II) was added to each tube. During the incubation step, the now solidified gel was immersed in an electrophoresis tank containing sufficient 1 x MOPS running buffer to completely cover the gel. The comb was only removed after immersion of the gel as this reduced the vacuum effect created when the comb was pulled out and thus reduced the chance of damaging the bottom of the wells. The samples were loaded immediately after the addition of the loading buffer and electrophoresed at ~ 5 V per centimetre of gel length until the bromophenol blue dye front had migrated approximately 80% of the length of the gel. The gel was removed from the tank and stained in a 0,5 µg/ml solution of ethidium bromide in DEPC-treated water for one hour. The gel was destained overnight in DEPC-treated water to remove the excess formaldehyde from the gel. The following morning, the gel was examined under UV light and an image recorded using the Gel Doc XR Imaging System (Bio-Rad).

2.12.5 Reverse transcription of RNA

DNase-treated RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. A 10 µl reaction volume containing 1 µg of total RNA together with 250 ng oligo$(dT)_{18}$ primer and 100 U SuperScript™ III Reverse Transcriptase enzyme was used resulting in the synthesis of ~100 ng/µl first-strand cDNA. Non-reverse transcribed negative controls were prepared in an identical manner except that DEPC-treated water (Invitrogen) was added to each reaction instead of the reverse transcriptase enzyme.

The success of the reverse transcription reaction was confirmed by the PCR amplification of a portion of the glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) gene. Two hundred ng (2 µl) of cDNA was added to a 25 µl mixture containing 0,5 U of BIOTAQ™ DNA polymerase (Bioline), 200 µM of each dNTP, 500 nM of each primer (GAPDHF: 5' – CCC TTC ATT GAC CTC AAC TAC ATG -3'; GAPDHR: 5' – CAT GCC AGT GAG CTT CCC GTT CAG -3'; Weinberg *et al.*, 2000), 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1,5 mM $MgCl_2$

and 0,1% Triton X-100 15 mM. Amplification was performed in a MyCycler™ programmable thermocycler (Bio-Rad) as follows: denaturation at 94 °C held for 60 s, annealing at 52 °C held for 30 s and elongation at 72 °C held for 90 s for a total of 30 cycles followed by a final extension at 72 °C held for 10 min. Both reverse transcribed and non-reverse transcribed products were subjected to PCR analysis. Amplified products were resolved on a 1% agarose gel (section 2.6).

2.12.6 Amplification of cDNA

Amplification of subgenomic regions of the HBV genome was performed as described in section 2.5.3, except that cDNA generated from the reverse transcription reaction was used as template instead of extracted DNA.

2.13 Infection of Experimentally Naïve Baboons

The transmission of HBV to experimentally naïve baboons was performed by Professor R. Purcell at the National Institute of Health (NIH), Bethesda, USA. Permission for this portion of the study was obtained from the Animal Ethics Committee of the NIH (Ethics Clearance Number: 98-B-428 Appendix I). In order to show that HBV could be transmitted from an infected baboon to an experimentally naïve, domestically raised baboon, serum from 4 of the original 10 baboons, namely baboon 9712, baboon 9732, baboon 9733 and baboon 9734, was used to inoculate 4 baboons at the NIH (figure 12). The naïve baboons were obtained from a domestic breeder, the Mannheimer Foundation, and were free of all markers of HBV replication prior to their inclusion in this study. The baboons were each housed separately and were each inoculated intravenously with 500 µl of serum from one of the original 4 infected baboons. Following inoculation, a liver biopsy as well as serum was obtained from each of the 4 baboons weekly. The serum was used to measure ALT, isocitrate dehydrogenase (ICD) and gamma-glutamyltranspeptidase (gGT) levels as well for the detection of HBsAg, HBeAg, anti-HBe, anti-HBs and anti-HBc.

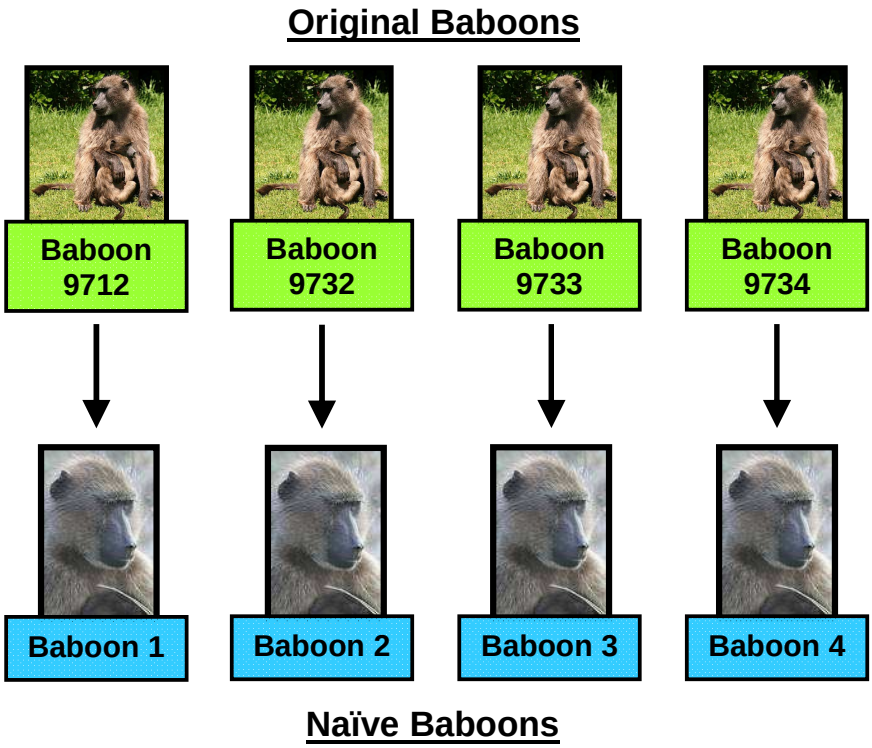


Figure 12: Infection of experimentally naïve baboons. Serum from 4 of the original 10 baboons wild-caught in the Eastern Cape Province was used to infect four experimentally naïve baboons. This diagram indicates from which of the original baboons serum was obtained to infect the naïve baboons

Twenty five weeks post inoculation, the study was terminated and the baboons euthanized. At necropsy, liver tissue, snap frozen in liquid nitrogen, as well as serum was obtained from each baboon and these samples were shipped on dry-ice to Professor Kew at the Molecular Hepatology Research Unit in Johannesburg for further testing. DNA was extracted from the serum using the QIAGEN QIAamp® DNA Blood Mini Kit (section 2.3.1) and from the tissue using the phenol-chloroform extraction method (section 2.3.4). Subgenomic regions of the HBV genome were amplified by nested PCR (section 2.5.3). The amplified products were either subjected to Southern blotting analysis (section 2.11) in the case of serum extracts or, in the case of the tissue extracts, sequenced (section 2.8). The sequences were aligned manually using the GeneDoc program and a phylogenetic tree generated (section 2.9) to confirm that the virus extracted from the experimentally naïve baboons was the same as that found in the original baboons.

3. Results

HBV DNA was detected in the sera of Chacma baboons by Southern hybridization. The prevalence of HBV in the South African baboon (*Papio ursinus orientalis*) population was determined by extracting DNA from the serum of 69 wild-caught baboons and amplifying four different regions – the precore/core, core, polymerase and surface regions - of the viral genome. If a sample amplified for three or more of these regions, the baboon was considered to be HBV-DNA positive. The complete viral genome was sequenced using overlapping subgenomic fragments amplified from HBV DNA isolated from the liver tissue of baboon 9732. Viral antigens were detected in the liver tissue using immunohistochemistry, and liver function tests, including alanine and aspartate aminotransferase levels, were performed using serum from this baboon. Replication of the virus was demonstrated by extracting RNA from the liver tissue, reverse transcribing it and amplifying subgenomic regions of the viral genome off this cDNA. Replication was further confirmed by the detection of cccDNA in the baboon liver tissue using real-time PCR analysis. HBV-positive sera from four baboons was used to infect four experimentally naïve baboons to demonstrate transmission.

3.1 Prevalence of HBV in South African baboons

3.1.1 Detection of HBV by nested PCR amplification

Initially DNA was extracted from the sera of ten wild-caught baboons from the Eastern Cape Province (figure 14). HBV DNA could not be amplified using a single round of PCR, indicating that HBV DNA was present at low levels in baboon serum. Using nested PCR of four different regions of the HBV genome, HBV DNA was amplified successfully (figure 13). The precore/core gene region (panel A) amplified for most samples and the surface gene region (panel D) for the least samples.

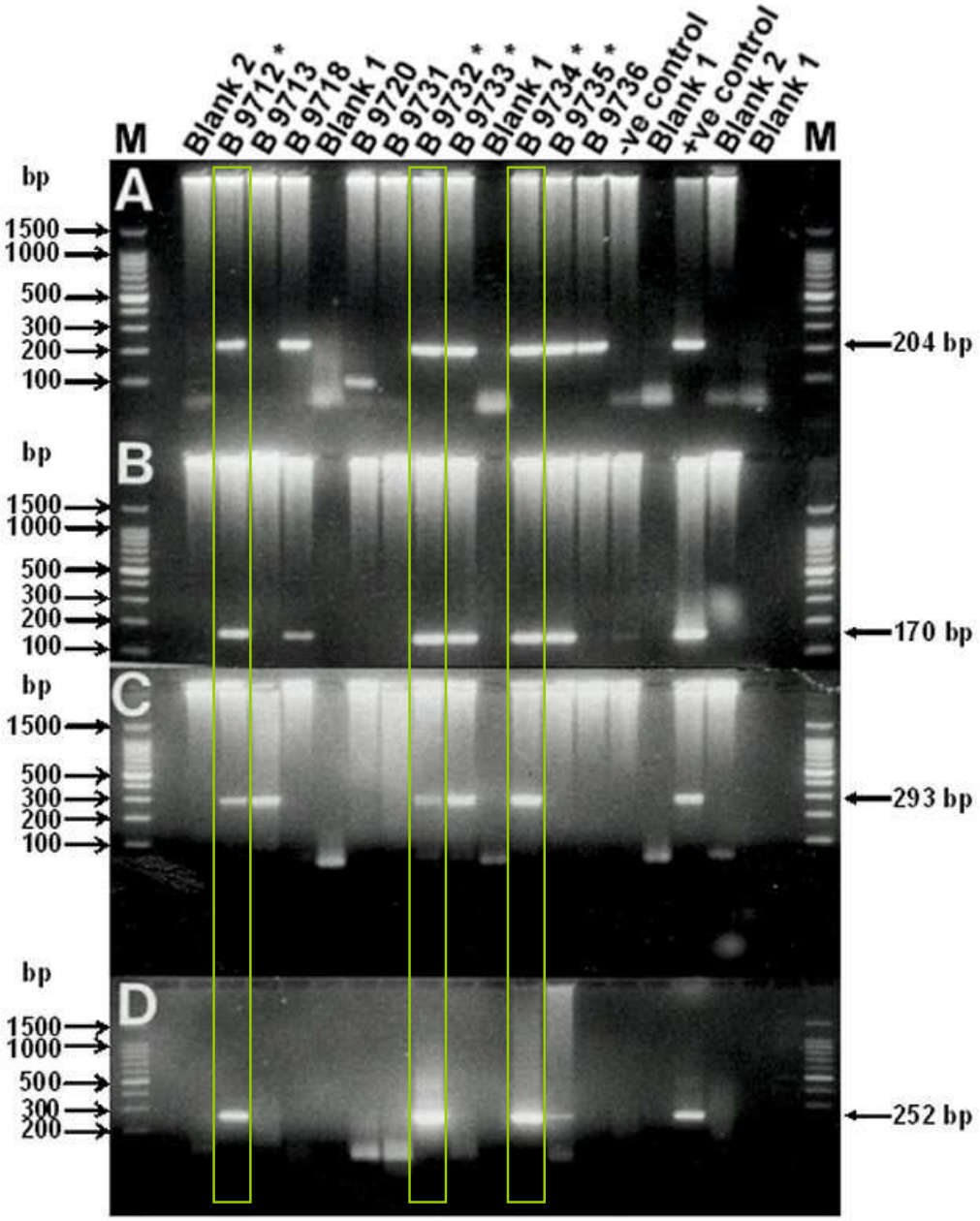


Figure 13: Nested PCR amplification of four different regions of the HBV genome. DNA extracted from the serum of ten wild-caught baboons was used as template for a nested PCR of four different regions of the HBV genome. These amplicons were resolved on a 1% EtBr agarose gel alongside a 100 bp DNA ladder.
Panel A shows the amplification of a 204 bp region of the precore/core gene (1765F – 1968R).
Panel B shows the amplification of a 170 bp region of the core gene (2267F – 2436R).
Panel C shows the amplification of a 293 bp region of the polymerase gene (2566F – 2858R).
Panel D shows the amplification of a 252 bp region of the surface gene (459F – 710R).
Abbreviations: M – 100 bp DNA ladder; Blank 1 and Blank 2 – single and double round PCR amplifications respectively, containing best quality water instead of DNA; -ve control - negative extraction control which used DNA extracted from HBV-negative human serum as template; +ve control - positive control using DNA extracted from HBV-positive human serum as template.
Samples positive for HBV are marked with an asterisk (*) and those positive for all four regions are boxed.

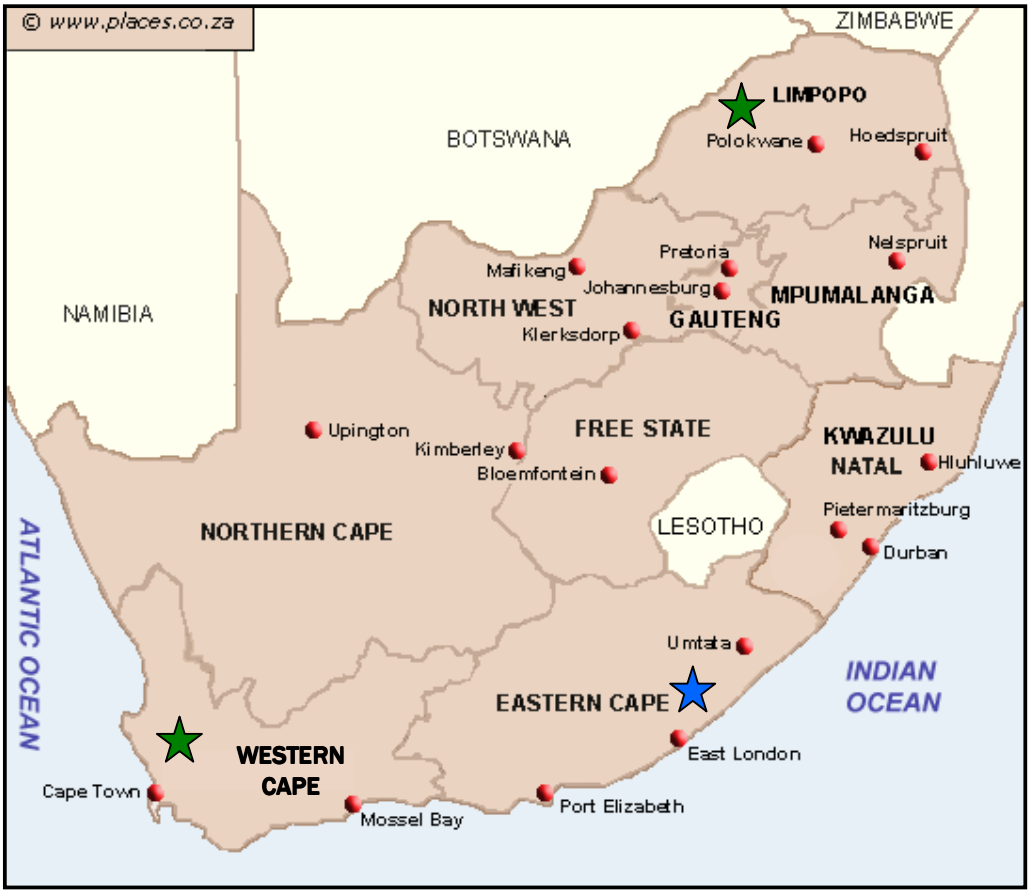


Figure 14: **Map of South Africa.** This map indicates the nine South African provinces and the regions from which wild baboons were captured.
★ - The region from which the original ten baboons were captured.
★ - The regions from which baboons were subsequently captured.

The samples from baboons 9712, 9732 and 9734 amplified successfully for all four regions tested and the samples from baboons 9733 and 9735 amplified successfully for three of the four regions (figure 13). The samples from baboons 9713, 9718, and 9736 amplified for only one or two of the regions tested, whereas samples from baboons 9720 and 9731 failed to amplify for any of the four regions. Repetition of the amplification yielded the same results.

HBV DNA-positive sera from baboons 9712, 9732, 9733 and 9734 were negative for both HBsAg and anti-HBc. Alanine and aspartate aminotransferase levels were normal and, following high speed centrifugation and treatment with anti-HBs, no viral particles were observed in the serum using electron microscopy.

Figure 15: Dendogram of precore/core region amplicons (nt 1800 – 1961) from the sera of six different baboons. Representative samples of all eight HBV genotypes as well as the non-human primate hepadnaviruses are included. Samples are numbered according to their GenBank accession numbers followed by their country of origin and a letter indicating which genotype they cluster with. The samples from the baboons are highlighted in yellow.

Fifty nine additional serum samples were subsequently obtained from both adult and juvenile baboons in the Limpopo and Western Cape provinces (figure 14) and tested for HBV DNA by nested PCR. Using the criterion of at least three of the four regions being PCR-positive, in total 11/49 (22,4%) adult and 4/20 (20%) juvenile wild-caught baboons were positive for HBV. Taken together the overall prevalence of hepadnaviral DNA in baboons was 21,7% (15/69).

3.1.2 Phylogenetic analysis of HBV precore region

In order to confirm the specificity of the amplification, representative samples of the HBV precore region (nt 1800 – 1961) amplified off DNA extracted from the sera of six different baboons were sequenced and analysed phylogenetically. The baboon isolates clustered with genotype A (figure 15).

A)

J 9755	J 9757	A H	J 9754	B 9718	B 9713
J 9745	J 9751	J 9749	M 9656	B 9753	J 9756
J 9750	B 9702	B 9731	B 9732	J 9746	V 42
J 9740	J 9758	V 34	Probe	B 1845	B 1894
J 9761	M 9651	B 9726	J 9743	B 9734	J 9747
B 1897	J 9738	B 9733	J 9742	J 9747	B 1896
V 28	B 9712	B 1899	J 4572	V 41	B 9720
V 35	B 9717	J 9760	B 1869	B 1858	B 9735

B)

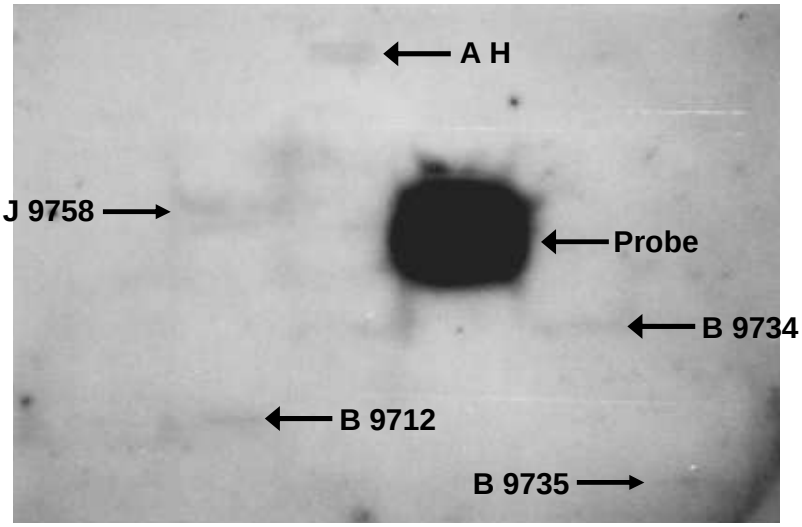


Figure 16: Southern slot blot of baboon serum samples. Baboon sera were blotted onto a nylon membrane and hybridized with an HBV-specific probe.
Panel A shows the slot blot template. Abbreviations: AH – serum sample from a patient suffering from acute hepatitis (positive control); B – adult baboon serum sample; J – juvenile baboon serum sample; V/M – serum sample from vervet monkey (negative control); Probe – HBV specific probe diluted in HBV-negative serum (positive control). The samples that hybridized to the HBV-specific probe are highlighted in yellow.
Panel B shows the autoradiograph of the slot blot after a seven day exposure. HBV DNA positive sera, as well as the positive controls, are indicated.

3.1.3 Detection of HBV DNA by Southern hybridization

Using Southern blot analysis, serum samples were tested for HBV DNA. Of the 39 samples shown in figure 16, four were positive. Longer exposures to autoradiography film did not improve the detection of HBV DNA. Sera from all sixty-nine baboons were screened for HBV DNA using Southern blot analysis, but only five were found to be positive.

The low viral loads meant that the complete HBV genome could only be amplified subgenomically. Because of the small volumes of sera available, liver tissue obtained from a single baboon (9732), which was euthanized for medico-ethical reasons, was used to further characterise the HBV present in baboons.

3.1.4 Summary of HBV DNA prevalence study

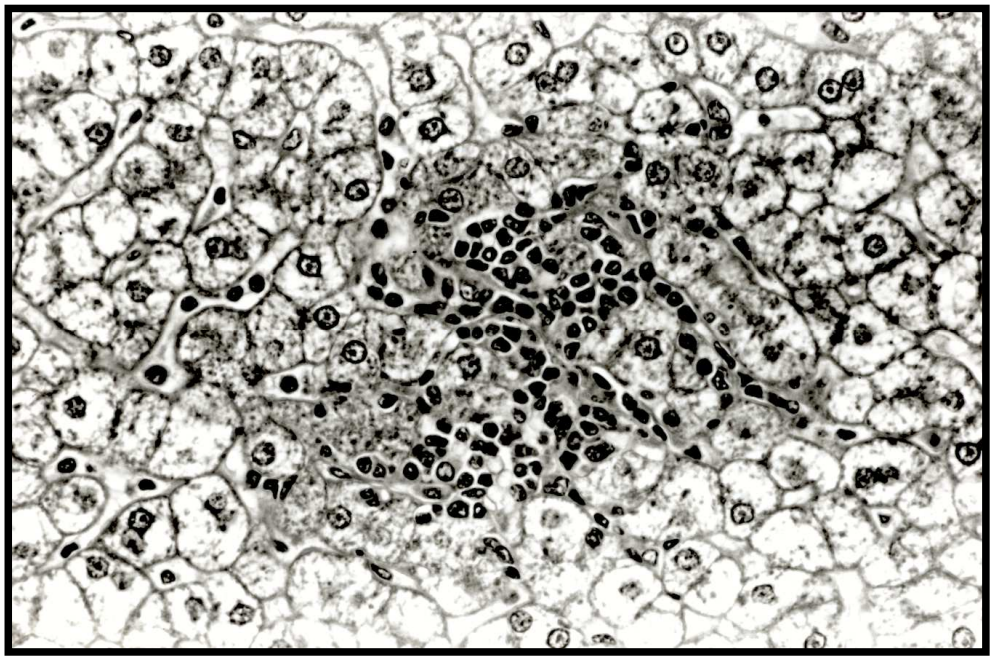
A summary of the results of the initial prevalence studies for the 11 adult and 4 juvenile HBV DNA positive baboons is shown in table 8. The remaining 38 adult and 14 juvenile baboons tested did not meet the criteria to be considered positive for HBV infection.

Table 8: Summary of results for HBV DNA positive baboons

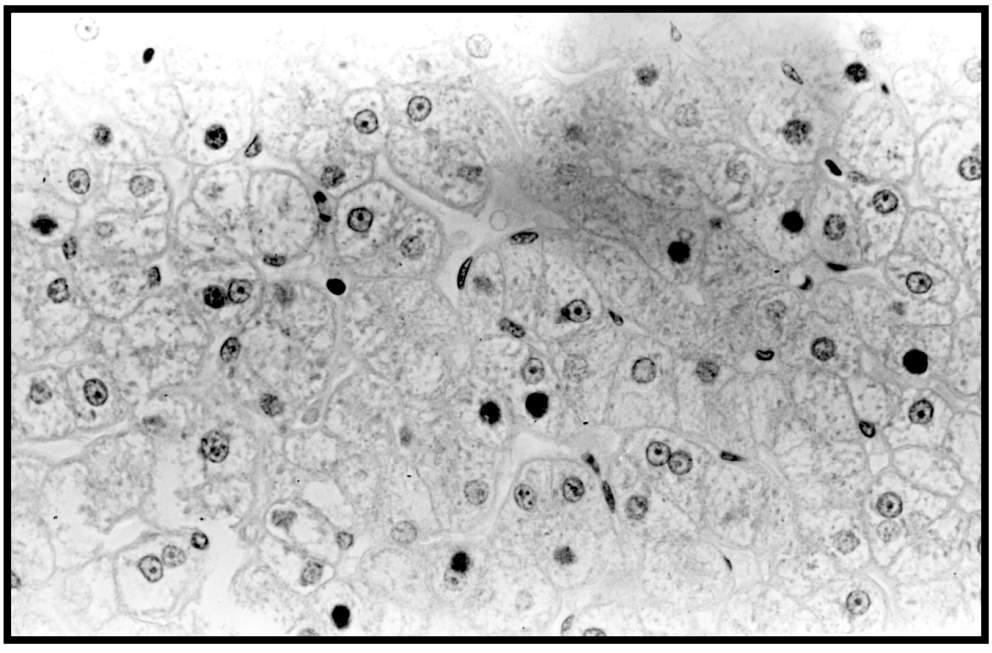
Baboon[a]	Origin[b]	SH[c]	Nested PCR			
			Polymerase	Core	Surface	Pre-core/ core
B9712	E. Cape	+	+	+	+	+
B9732	E. Cape	+	+	+	+	+
B9733	E. Cape	-	+	+	-	+
B9734	E. Cape	+	+	+	+	+
B9735	E. Cape	+	-	+	+	+
B9768	W. Cape	-	+	+	+	+
B9770	W. Cape	-	-	+	+	+
B9775	W. Cape	-	+	+	+	+
B97116	Limpopo	-	-	+	+	+
B97118	Limpopo	-	-	+	+	+
B97122	Limpopo	-	+	+	+	+
J9747	W. Cape	-	-	+	+	+
J9752	W. Cape	-	+	-	+	+
J9758	W. Cape	+	+	+	+	+
J9761	W. Cape	-	+	-	+	+

[a] Prefix of B indicates an adult baboon and prefix of J indicates a juvenile baboon
[b] E. Cape – Eastern Cape province ; W. Cape – Western Cape province
[c] SH – Southern Hybridisation

A)



B)



<u>Figure 17:</u> Micrograph of liver tissue from baboon 9732 showing lobular hepatitis. Liver tissue obtained from baboon 9732 at necropsy was fixed in formalin, embedded in paraffin and sectioned.

Panel A was stained with hematoxylin and eosin. It shows a focus of mild lobular hepatitis but there is no evidence of interface hepatitis or bridging necrosis. The portal tracts are normal. Magnification: 400x

Panel B: the section is stained with immunoperoxidase using a polyclonal antibody to the HBV core antigen. HBcAg can be detected in the occasional hepatocyte nucleus. Magnification: 400x

3.2 Immunohistology of HBV DNA positive baboon liver tissue

Histological examination of liver tissue from baboon 9732 showed the presence of mild focal lobular hepatitis, but no evidence of interface hepatitis, bridging necrosis, dysplasia of hepatocytes, cirrhosis, or hepatocellular carcinoma (figure 17, panel A). Immunohistochemical staining of the liver tissue detected HBcAg in the nuclei of some of the hepatocytes, with a patchy distribution (figure 17, panel B). HBsAg and 42nm enveloped (Dane) particles were not detected in the cytoplasm.

3.3 Extraction of DNA from liver tissue

3.3.1 Quality control and quantification of the DNA extracts

Using phenol chloroform, DNA was extracted from two liver tissue samples from baboon 9732 and from an extraction blank. The OD 260/280 ratio for both samples was 1,82 indicating that the DNA was of a very high quality. The quality and quantity of the DNA extracts were confirmed by resolving 20 µg of each extract on a 0,8% agarose gel overnight (figure 18).

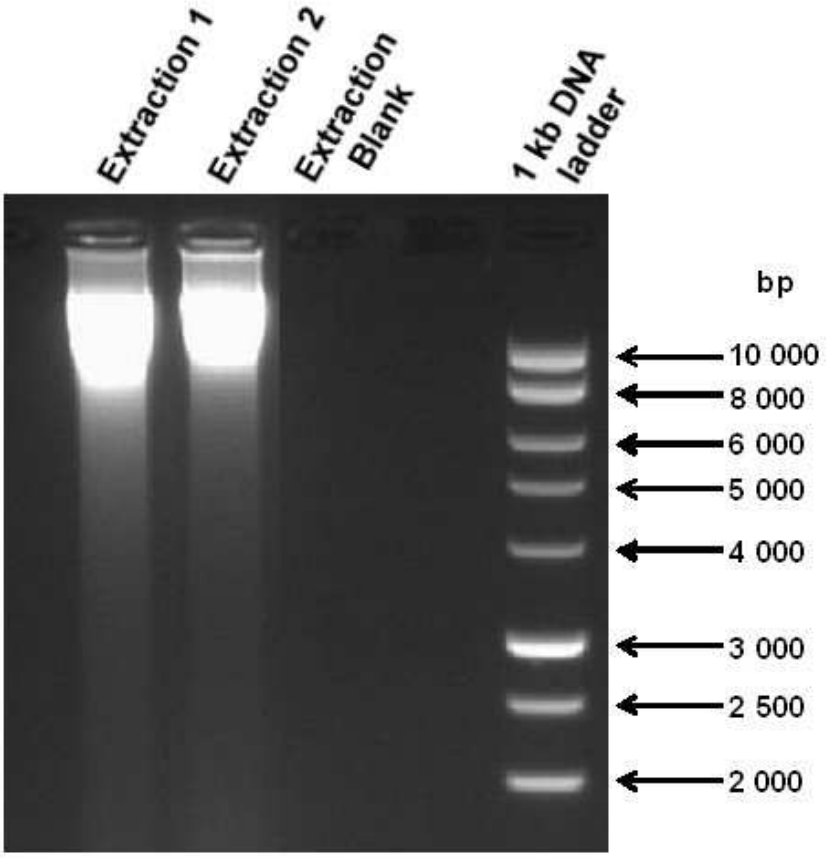


Figure 18: Confirmation of the quality of the phenol-chloroform DNA extractions. This composite figure shows the phenol-chloroform DNA extracts from the liver tissue of baboon 9732 that were resolved overnight on a 0,8% EtBr agarose gel. For each extract 20 µg of DNA was resolved. Both extractions are of high quality and quantity and the extraction blank shows no evidence of contamination.

3.3.2 Identification of HBV DNA in the liver tissue extracts

The presence of hepadnaviral DNA in the extracts was confirmed by nested PCR of two viral genomic regions, namely a portion of the precore/core gene (nt 1763-1966) and a portion of the surface gene (nt 460-711) (figure 19). The PCR worked well for both regions, without any signs of amplification in the negative controls.

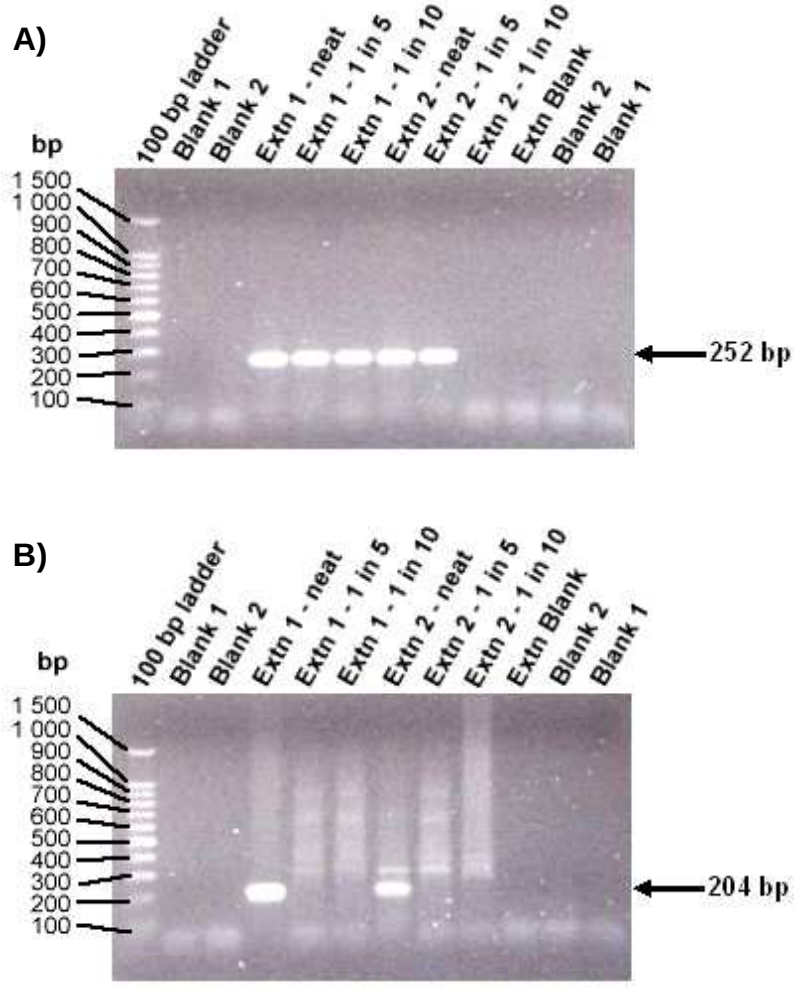


Figure 19: Amplification of HBV DNA from baboon 9732 liver extracts. Amplicons of two different regions of the HBV genome were resolved on a 1% agarose gel.
Panel A shows the amplification of a 252 bp portion of the surface gene (459F – 710R).
Panel B shows the amplification of a 204 bp region of the precore/core gene (1765F – 1968R).
Phenol chloroform extracted DNA from the liver tissue of baboon 9732 was used as template either neat, diluted 1 in 5, or diluted 1 in 10.
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn - extraction.

3.4 Genotyping of the HBV

The HBV extracted from the baboon liver tissue was shown to belong to genotype A using the method described by Lindh *et al.* in 1997 (figure 20).

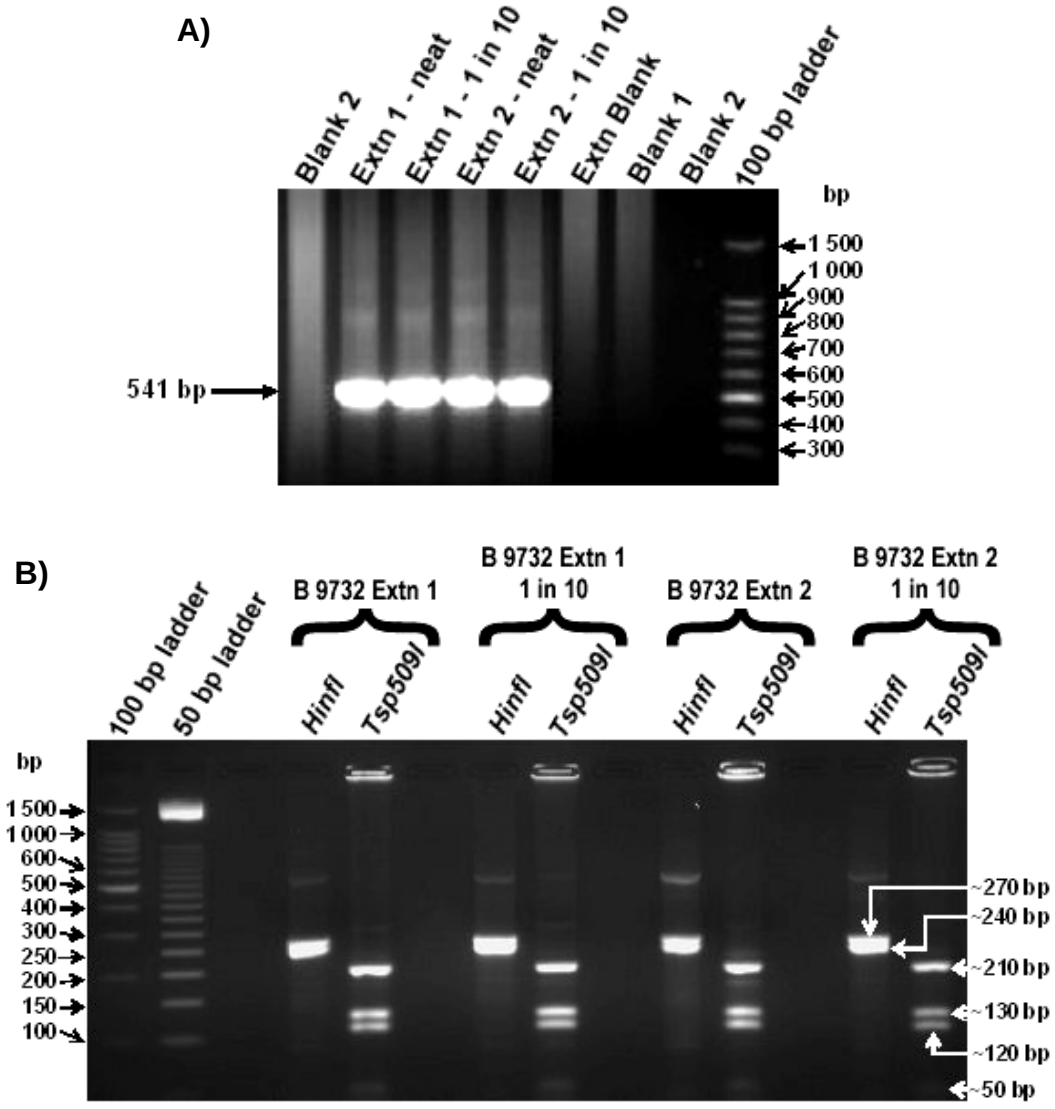


Figure 20: Lindh Genotyping of B 9732 liver extracts.
Panel A – The 541 bp nested PCR amplicons (nt 256 – 796) were resolved on a 1% EtBr agarose gel to confirm successful amplification. DNA extracted from the liver tissue of baboon 9732 was used as template either neat or diluted 1 in 10.
Panel B – The restricted amplicons were resolved on a 3% EtBr agarose gel. When compared to the patterns described by Lindh *et al*. (1997), the HBV from baboon 9732 was genotype A.
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn - extraction.

3.5 Amplification and sequencing of the HBV genome from the baboon

3.5.1 Amplification of the HBV genome

Attempts to sequence the entire genome of baboon HBV, using full genome amplification (Günther *et al*., 1995), or amplification of two overlapping subgenomic fragments (Takahashi *et al*, 1998), proved unsuccessful. The amplicons generated by either of these methods failed to hybridize to a HBV DNA probe following Southern hybridization. Furthermore, when these amplicons were gel extracted and cloned into the pCR®-XL-TOPO® vector using the TOPO® XL PCR Cloning Kit (Invitrogen), HBV DNA could not be amplified off the resulting clones.

3.5.2 Amplification of the viral genome by nested PCR amplification of numerous overlapping subgenomic fragments.

In order to obtain the sequence of the complete HBV genome, a number of different nested PCR amplifications of overlapping subgenomic fragments were attempted (table 6, section 2.5.3). The regions that amplified successfully and were sequenced to generate the complete sequence of the virus are summarised in figure 21 with the amplicons shown in figures 22-24.

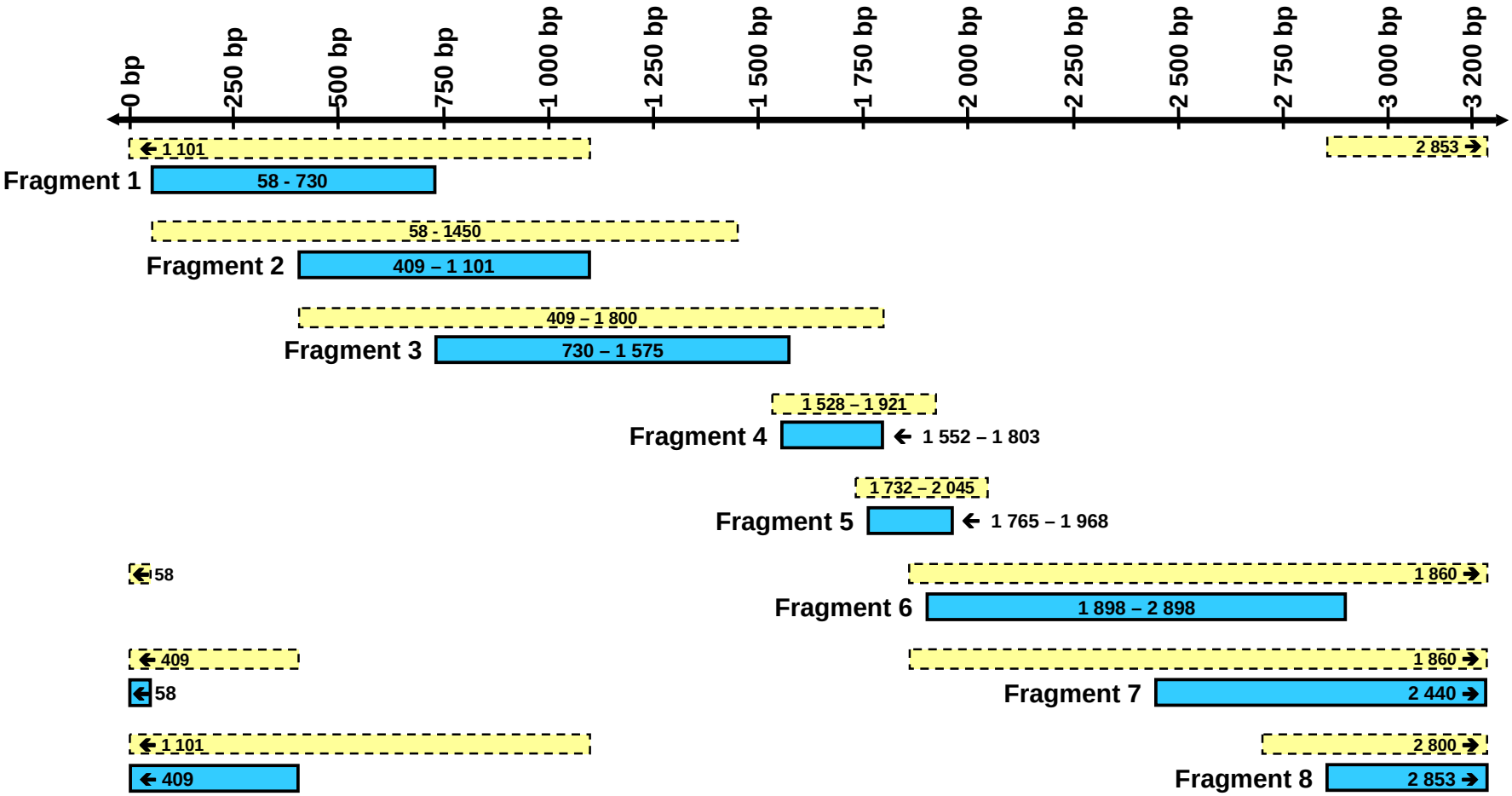


Figure 21: Amplification of the HBV genome using overlapping subgenomic fragments. This schematic shows the eight overlapping subgenomic fragments, amplified by nested PCR, which were used to generate the complete HBV sequence isolated from the liver tissue of baboon 9732. The first round of the nested PCR is shown in yellow and the second round in blue.

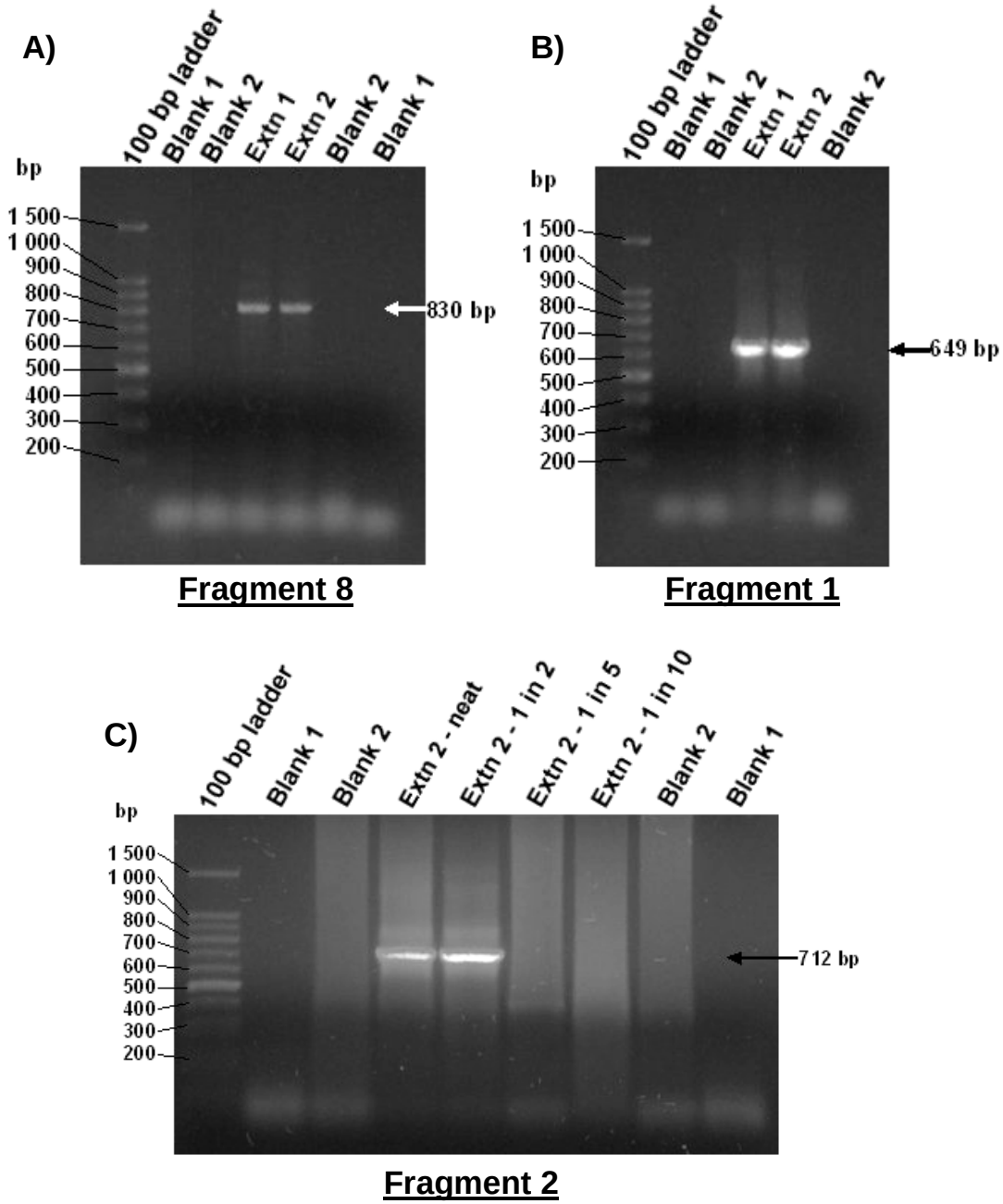


Figure 22: Nested PCR of overlapping regions of the HBV preS1/preS2/S open reading frame. Subgenomic fragments amplified by nested PCR, using DNA extracted from the liver tissue of baboon 9732, were resolved on a 1% EtBr agarose gel. Both the primers and thermocycling conditions described by Hu *et al.* (2000) were used.
Panel A – PCR 1: 2800F – 1101R; PCR 2: 2853F – 409R
Panel B – PCR 1: 2853F – 1101R; PCR 2: 58F – 730R
Panel C – PCR 1: 58F – 1450R; PCR 2 409F – 1101R
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn - extraction.

The nested PCR of fragment 7 gave two distinct amplicons of equal intensity – one at the expected size of 880 bp and the other a smaller fragment of ~520 bp (figure 23). The fragments were cloned into the pTZ57R/T plasmid vector using the Fermentas cloning kit. Clones containing inserts were screened for HBV DNA by PCR. Only the clones containing the larger of the two fragments amplified successfully indicating that only the larger fragment was hepadnaviral, whereas the smaller fragment was of chromosomal origin, as shown by sequencing.

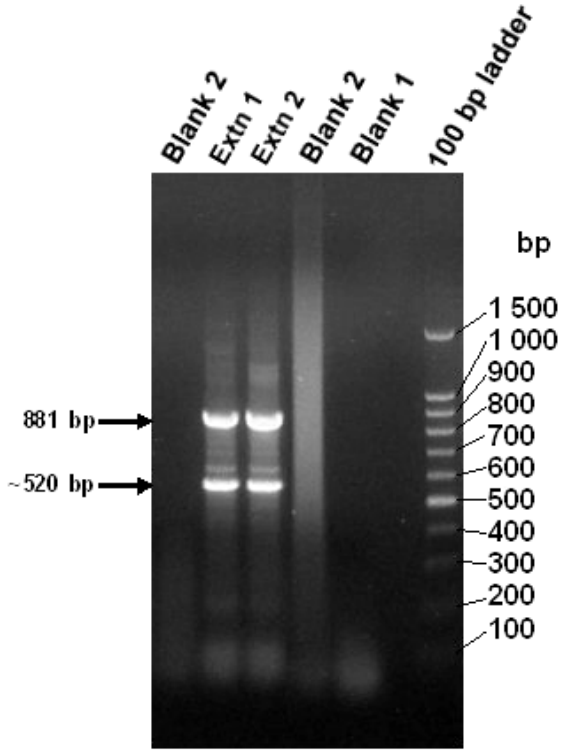


Figure 23: Nested PCR amplification of the 2440F – 58R region of the HBV genome. This nested PCR (PCR 1: 1860F – 409R; PCR 2: 2440F – 58R) which includes a portion of the surface gene of the HBV genome, was expected to yield a 881 bp fragment. When the products were resolved on a 1% EtBr agarose gel however, two fragments – one the expected size of 881 bp and the other a smaller fragment of ~520 bp - were observed. The fragments were excised individually, cloned into a suitable vector and subjected to an additional round of subgenomic PCR using primers internal to the 2440F – 58R region. Only the larger of the two fragments amplified. Sequencing of the clones confirmed that only the 881 bp fragment was HBV.
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn - extraction.

The remaining regions of the hepatitis B viral genome were amplified by applying the thermocycling conditions described by Hu *et al*. (2000) to combinations of primers used routinely in the laboratory (figure 24). To avoid the interference of non-specific amplicons with downstream automated sequencing, the correct sized fragments were gel purified.

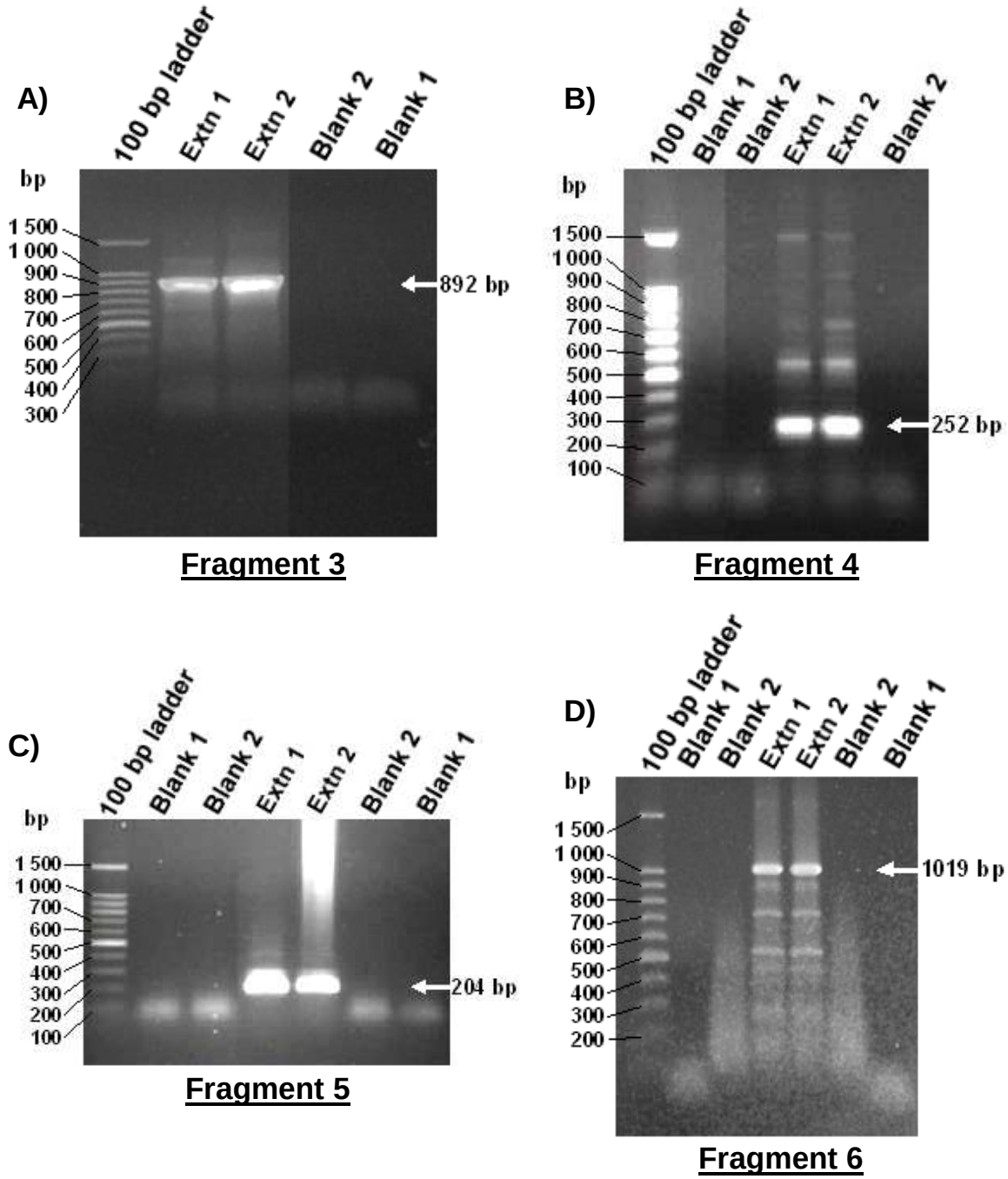


Figure 24: Agarose gel electrophoresis of nested PCR amplicons. Subgenomic fragments amplified off DNA extracted from baboon 9732 by nested PCR, using the thermocycling conditions described by Hu *et al.* (2000), were resolved on 1% EtBr agarose gels.
Panel A – PCR 1: 409F – 1800R; PCR 2: 730F – 1575R
Panel B – PCR 1: 1528F – 1921R; PCR 2: 1552F – 1803R
Panel C – PCR 1: 1732F – 2045R; PCR 2: 1765F – 1968R. The DNA extracts were both diluted 1 in 10 before being used as template for the first round of amplification.
Panel D – PCR 1: 1860F – 58R; PCR 2: 1898F – 2898R
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn - extraction.

3.5.3 Phylogenetic analysis of the HBV from baboons

The overlapping subgenomic amplicons covering the entire HBV genome were aligned and a sequence for the complete HBV genome, isolated from the liver of baboon 9732, determined. Translation of the 4 ORF'S showed them to be well conserved relative to the consensus sequence of subgenotype A2 with the following exceptions:

- T380C $\Rightarrow$ rtV84A in conserved region A of the polymerase
  $\Rightarrow$ C76R in HBsAg
- A2019G $\Rightarrow$ E40G core protein
- C1470T $\Rightarrow$ P33G in the X protein
- T1765C $\Rightarrow$ P145S in the X protein

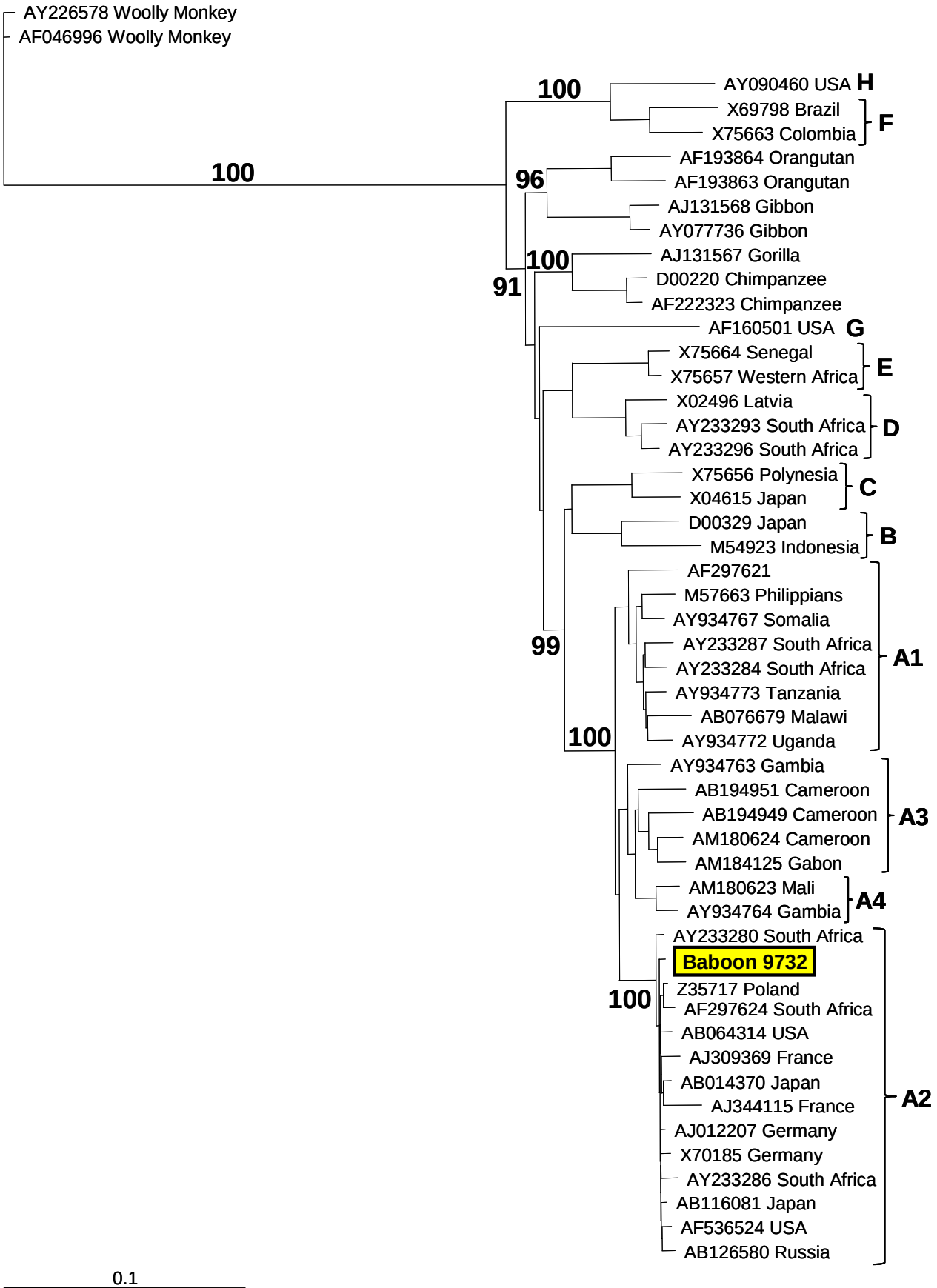
Mean nucleotide divergence calculations (table 9) showed that the HBV from the baboon was closely related to subgenotype A2 and this was confirmed by phylogenetic analyses of the complete genome, as well as each of the four individual open reading frames, (figures 25 – 27).

Table 9: Mean nucleotide divergence.

	Baboon vs A1	Baboon vs A2
Complete Genome	4,52 ± 0,42 (3,85 – 6,04)	1,00 ± 0,55 (0,50 – 3,59)
Polymerase	4,60 ± 0,31 (4,09 – 5,52)	0,87 ± 0,57 (0,28 – 4,08)
Pre-S1/S2/S	3,61 ± 0,42 (2,78 – 4,81)	0,84 ± 0,46 (0,17 – 2,52)
Precore/core	4,04 ± 0,95 (2,96 – 7,64)	0,98 ± 0,69 (0,31 – 3,41)
X	3,34 ± 0,74 (2,16 – 5,62)	1,49 ± 0,66 (0,43 – 4,30)

The mean nucleotide divergences (expressed as a percentage) for the complete and the individual open reading frames were calculated using DAMBE (http://dambe.bio.uottawa.ca/dambe.asp). The sequences compared are those shown in Appendix III (51 subgenotype A1 and 51 subgenotype A2 sequences). The mean nucleotide divergence is followed by the standard deviation with the range given in parentheses.

Figure 25: Dendogram of the complete HBV genome isolated from baboon 9732.
Samples representative of all eight HBV genotypes, as well as the primate hepadnaviruses, have been included. Samples are numbered according to their GenBank accession numbers, followed by their country of origin. The sample from baboon 9732, highlighted in yellow, clusters strongly with the genotype A2 isolates (bootstrap value of 100).


AY226578 Woolly Monkey
AF046996 Woolly Monkey
100
100
AY090460 USA
H
X69798 Brazil
X75663 Colombia
F
96
AF193864 Orangutan
AF193863 Orangutan
AJ131568 Gibbon
AY077736 Gibbon
100
91
AJ131567 Gorilla
D00220 Chimpanzee
AF222323 Chimpanzee
AF160501 USA
G
X75664 Senegal
X75657 Western Africa
E
X02496 Latvia
AY233293 South Africa
AY233296 South Africa
D
X75656 Polynesia
X04615 Japan
C
D00329 Japan
M54923 Indonesia
B
99
AF297621
M57663 Philippians
AY934767 Somalia
AY233287 South Africa
AY233284 South Africa
AY934773 Tanzania
AB076679 Malawi
AY934772 Uganda
A1
100
AY934763 Gambia
AB194951 Cameroon
AB194949 Cameroon
AM180624 Cameroon
AM184125 Gabon
A3
AM180623 Mali
AY934764 Gambia
A4
AY233280 South Africa
Baboon 9732
100
Z35717 Poland
AF297624 South Africa
AB064314 USA
AJ309369 France
AB014370 Japan
AJ344115 France
AJ012207 Germany
X70185 Germany
AY233286 South Africa
AB116081 Japan
AF536524 USA
AB126580 Russia
A2
0.1

<u>Figure 26:</u> Unrooted dendogram showing the complete HBV genome isolated from baboon 9732 relative to other subgenotype A2 strains.
Fifty one subgenotype A2 samples, isolated from five different continents, have been included. The GenBank accession numbers, as well as country of origin for each of these samples, are shown in the table below.

	Genbank Accession Number	Country of Origin
1	V00866	
2	S50225	
3	X02763	USA
4	X70185	Germany
5	Z35717	Poland
6	AY233286	South Africa
7	AY233280	South Africa
8	AF297624	South Africa
9	AF090838	Belgium
10	AF090841	Belgium
11	X51970	Germany
12	AF090839	Belgium
13	AF090840	Belgium
14	Z72478	Germany
15	AB064314	USA
16	AF537371	USA
17	AF537372	USA
18	AB014370	Japan
19	AJ344115	France
20	AY034878	USA
21	AJ309370	France
22	AJ309369	France
23	AJ309371	France
24	AY128092	Canada
25	AF536524	USA
26	AJ012207	Germany

	Genbank Accession Number	Country of Origin
27	L13994	
28	AB205118	Japan
29	AY902775	USA
30	AB116080	Japan
31	AB116079	Japan
32	AB116078	USA
33	AB116077	USA
34	AB116076	USA
35	AB116081	Japan
36	AB126580	Russia
37	AY152726	USA
38	AY738142	Germany
39	AY738141	Germany
40	AY738140	Germany
41	AY862868	China
42	AY862867	China
43	AB222708	Uzbekistan
44	AM282986	New Zealand
45	EF208113	Germany
46	DQ788725	Germany
47	AB222707	Uzbekistan
48	AM295797	France
49	AB246338	Japan
50	AB246337	USA
51	AY738139	Germany
★	Baboon 9732	South Africa

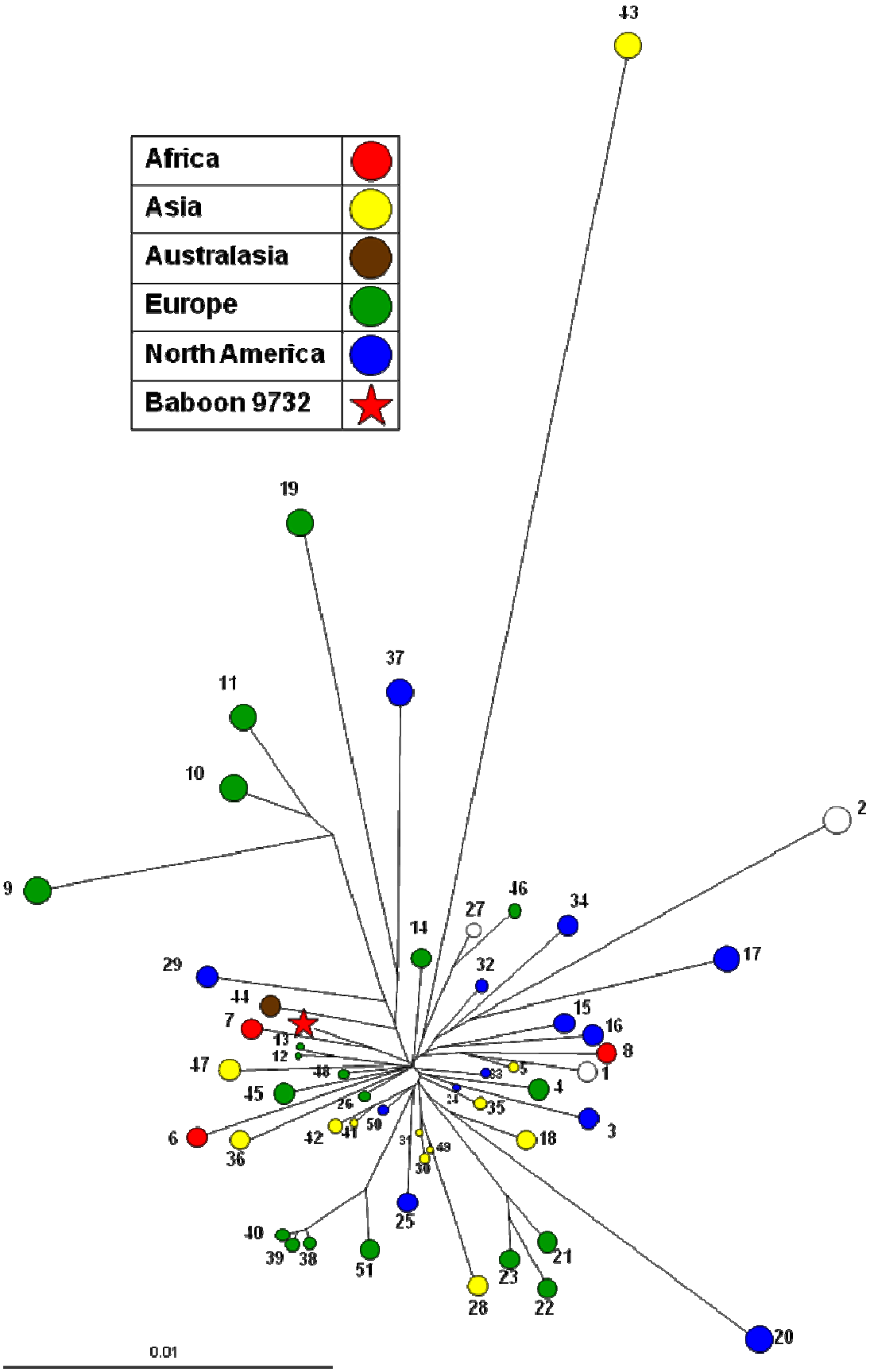
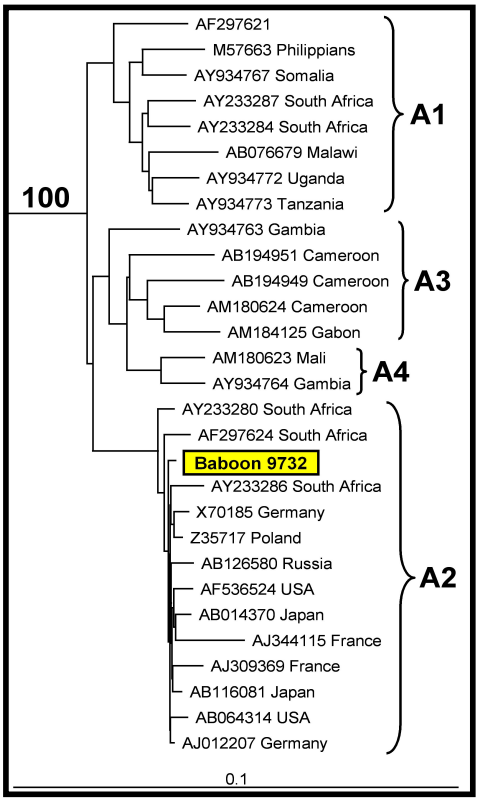
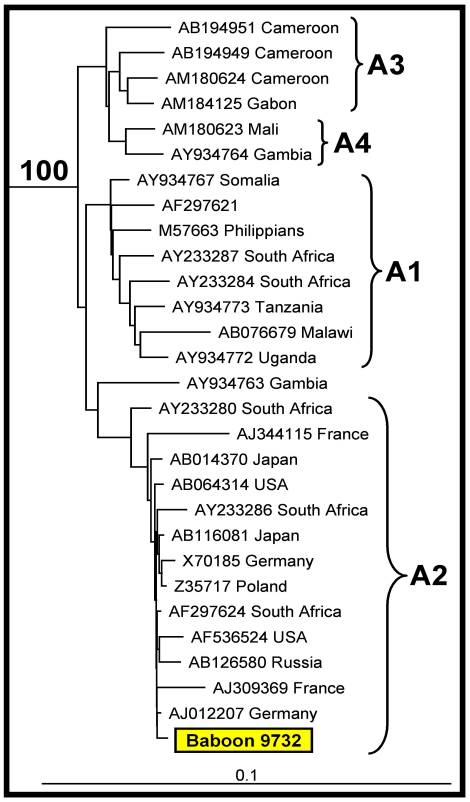

Africa
Asia
Australasia
Europe
North America
Baboon 9732
0.01

Figure 27: Dendograms of the four individual HBV open reading frames
A portion of each dendogram, showing the genotype A isolates, is shown. Samples are numbered according to their GenBank accession numbers, followed by their country of origin. The sample from baboon 9732, highlighted in yellow, clusters within genotype A for all four ORFs.
Panel A shows the polymerase ORF (nt 2307 – 1623)
Panel B shows the preS1/preS2/surface ORF (nt 2854 – 835)
Panel C shows the precore/core ORF (nt 1814 – 2458)
Panel D shows the X ORF (nt 1374 – 1838)

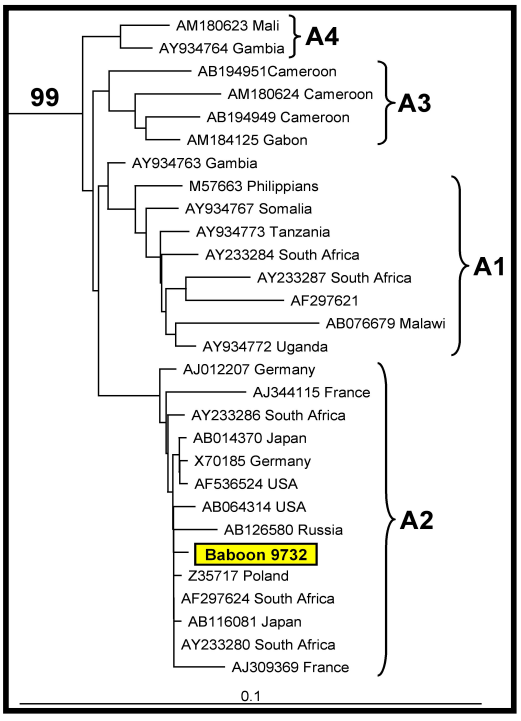
A) Polymerase ORF



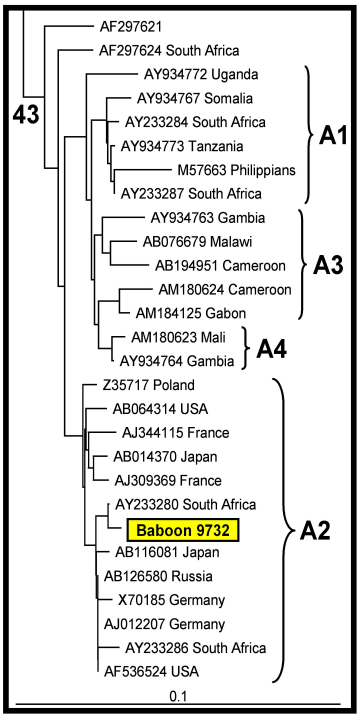
B) PreS1/preS2/surface ORF



C) Precore/core ORF



D) X ORF



3.5.4 Screening for recombination in HBV from the baboon

Possible recombination with other human and/or primate genotypes was investigated by aligning the complete HBV genome isolated from the liver of baboon 9732 with HBV genomes from 228 non-recombinant HBV isolates and running a GroupScan analysis using Simmonic. There was no evidence of recombination in the strain isolated from baboon 9732, with the HBV sequences deeply embedded in genotype A (figure 28).

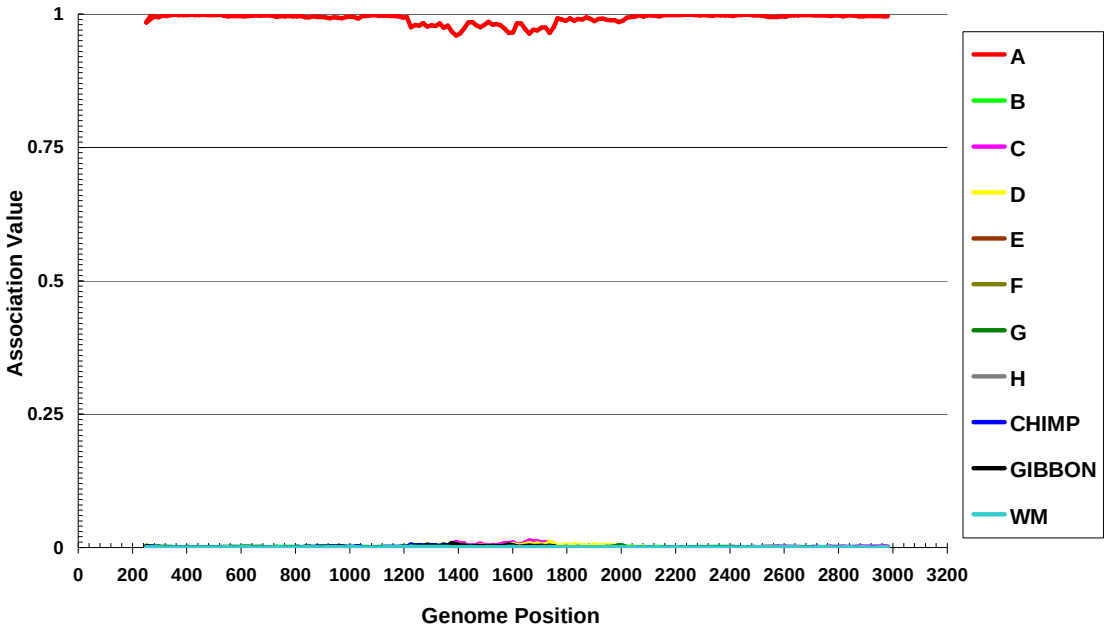


Figure 28: Recombination analysis of complete HBV genome from baboon 9732. A grouping scan using the Simmonic software package for the HBV strain from baboon 9732 versus 228 selected non-recombinant HBV genotype reference strains grouped by genotype, was performed. The X-axis shows the genomic position and the Y-axis indicates the mean distances between the baboon HBV and the reference groups. Values close to 1 on the Y-axis indicate that a sample is deeply rooted in that genotype.
Abbreviations: Chimp – chimpanzee; WM – woolly monkey.

3.6 Expression of HBV RNA in baboon liver tissue

3.6.1 Extraction of RNA from liver tissue

RNA was extracted from the liver tissue from baboon 9732 and the quantity and quality assessed. The A_{260}/A_{280} ratios for all the extracts were in the expected $2 \pm 0{,}05$ range. The quality of the RNA was further assessed by resolving a 2 µg aliquot on a 1% agarose gel (figure 29).

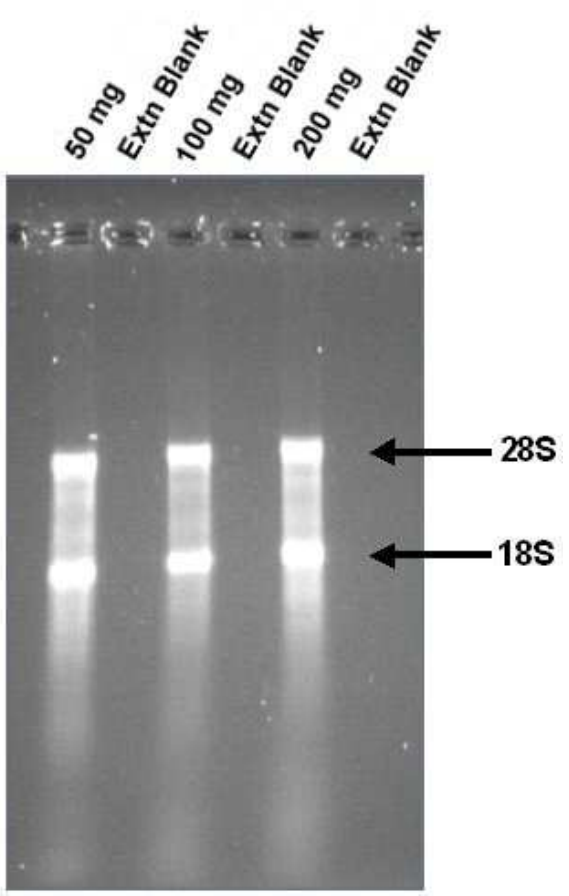


Figure 29: Assessment of the quality of the DNaseI treated RNA extracts. RNA extracted from different starting amounts (50 mg, 100 mg and 200 mg) of liver tissue, from baboon 9732, was treated with DNaseI and resolved on a 1% denaturing agarose gel. For each extract, 2 µg of sample was resolved. For all three extracts, the RNA appears visually to be of very good quality as no degradation of either the 28S and 18S rRNA subunits can be seen.

3.6.2 Assessment of the messenger RNA quality

One microgram of each of the three DNase treated RNA extracts was reverse transcribed using the Superscript III Reverse Transcription System from Invitrogen. The resulting cDNA was amplified by nested PCR using HBV specific primers, to confirm the presence of hepadnaviral RNA. Prior to this amplification however, the quality of the messenger RNA within the extracts was assessed by amplifying a portion of the glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) gene using the cDNA as a template (figure 30). All three of the RNA extracts amplified successfully, and no amplification of the non-reverse transcribed RNA extracts or any of the other negative controls was obtained.

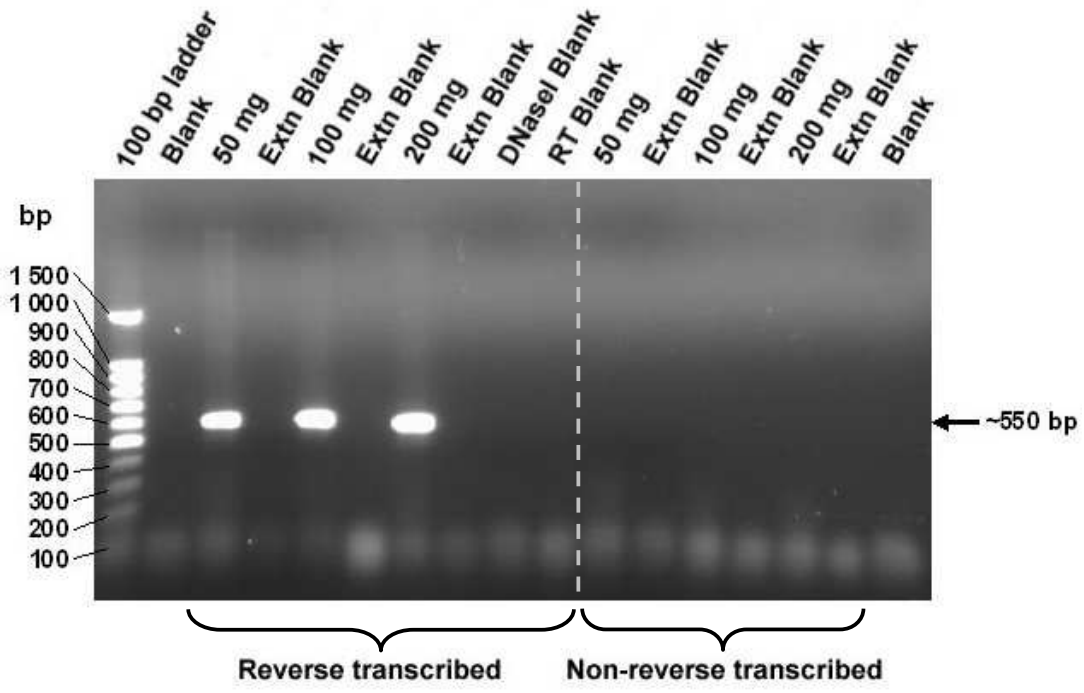


Figure 30: Amplification of GAPDH gene to assess mRNA quality. A ~550 bp region of the glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) gene was amplified using the DNaseI treated reverse transcribed RNA extracts as template and resolved on a 1% EtBr agarose gel. Non-reverse transcribed extracts (in which DEPC-treated water was added instead of enzyme during the reverse transcription reaction) were amplified as negative controls. The amplification worked perfectly for all three samples with no amplification in any of the negative controls indicating that the mRNA was of good quality and free of contamination.
Abbreviations: Blank - PCR negative control containing best quality water instead of cDNA; DNaseI Blank - DNaseI treatment negative control where DEPC-treated water was added instead of RNA; RT Blank - reverse transcription negative control where DEPC-treated water was added instead of cDNA; Extn - extraction.

3.6.3 Amplification of subgenomic regions of HBV using reverse transcribed cDNA

Following successful amplification of the GAPDH gene from all three samples, two different regions of the viral genome – parts of the precore/core (1765 - 1968) and surface (459 – 710) open reading frames – were amplified by nested PCR using the cDNA as template (figure 31). Amplification of the precore/core region was only successful when the cDNA template was diluted 1 in 5 (figure 31, panel B). Identical results were obtained when the amplification was repeated on reverse transcribed and non-reverse transcribed RNA diluted 1 in 5. Sequencing of these amplicons found them to be identical to the sequences of the DNA isolated from the liver of baboon 9732.

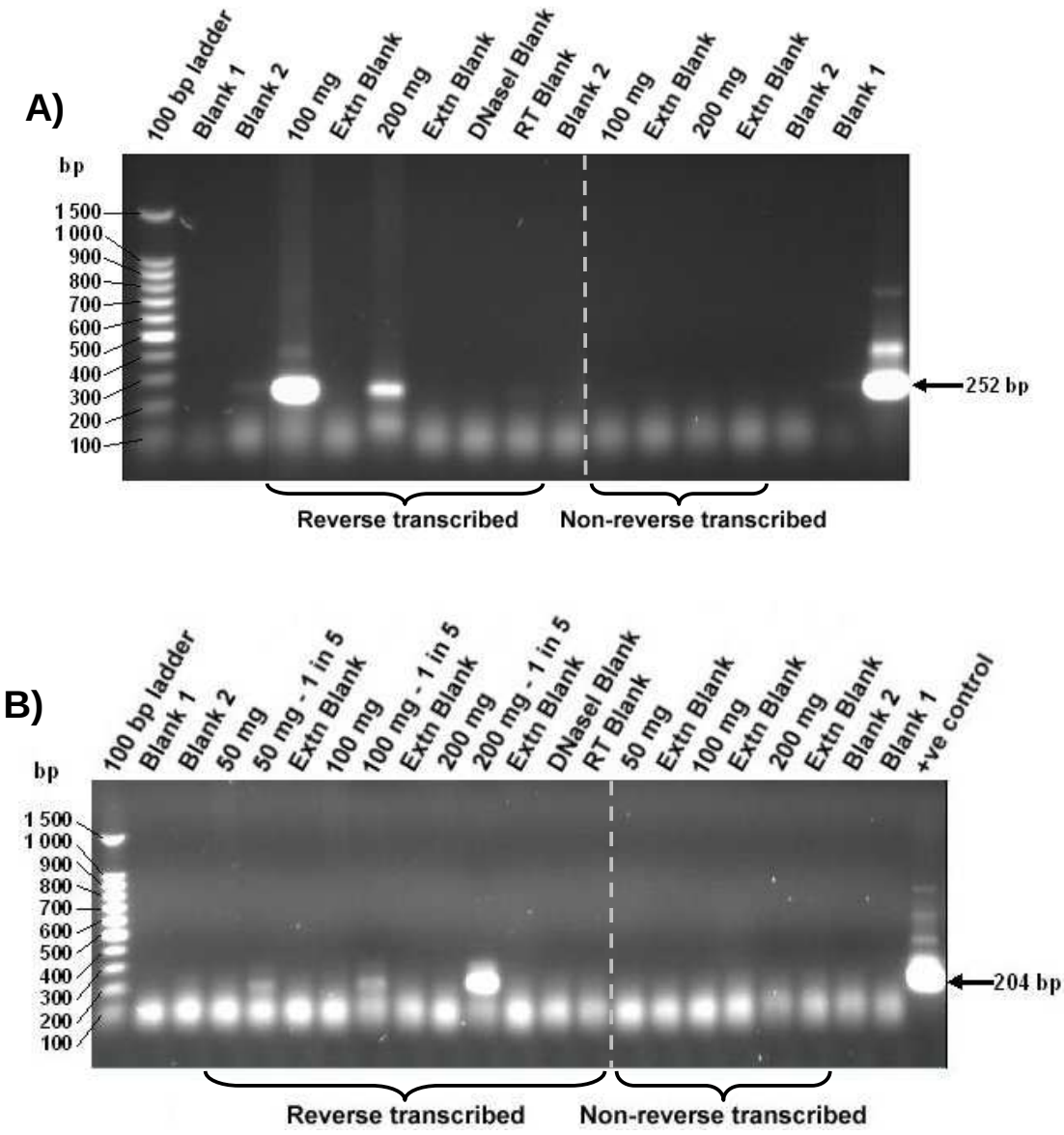


Figure 31: Nested PCR amplification of subgenomic regions from cDNA. The reverse transcribed, DNaseI treated cDNA products were amplified by nested PCR and the amplicons resolved on a 1% EtBr agarose gel. Non-reverse transcribed samples (in which DEPC-treated water was added instead of enzyme during the reverse transcription reaction) were included as negative controls.

Panel A – PCR 1: 255F – 759R; PCR 2: 459F – 710R

Panel B – PCR 1: 1723F – 2045R; PCR 2: 1765F – 1968R. This amplification was only successful when the cDNA was diluted 1 in 5.

Abbreviations: Blank 1 - single round PCR containing best quality water instead of cDNA; Blank 2 – double round nested PCR containing best quality water instead of cDNA; DNaseI Blank - DNaseI treatment negative control where DEPC-treated water was added instead of RNA; RT Blank - reverse transcription negative control where DEPC-treated water was added instead of cDNA; +ve control - PCR positive control using DNA extracted from the liver tissue of baboon 9732 as template; Extn - extraction

Small amounts of liver tissue were obtained from five additional wild-caught baboons. RNA was extracted from ~1,2 g of this tissue using the Promega SV total RNA isolation kit. The RNA extracts were reverse transcribed using the Qiagen Sensiscript reverse transcriptase system and a region of the core open reading frame (2267 - 2436) amplified by nested PCR. The products were resolved on an agarose gel (figure 32, panel A) and a product was visible only for the PLC/PRF5 positive control. In order to increase the sensitivity of detection, Southern hybridisation was performed on the nested PCR amplicons and expression of HBV RNA was demonstrated in baboon 1858 (figure 32, panel B).

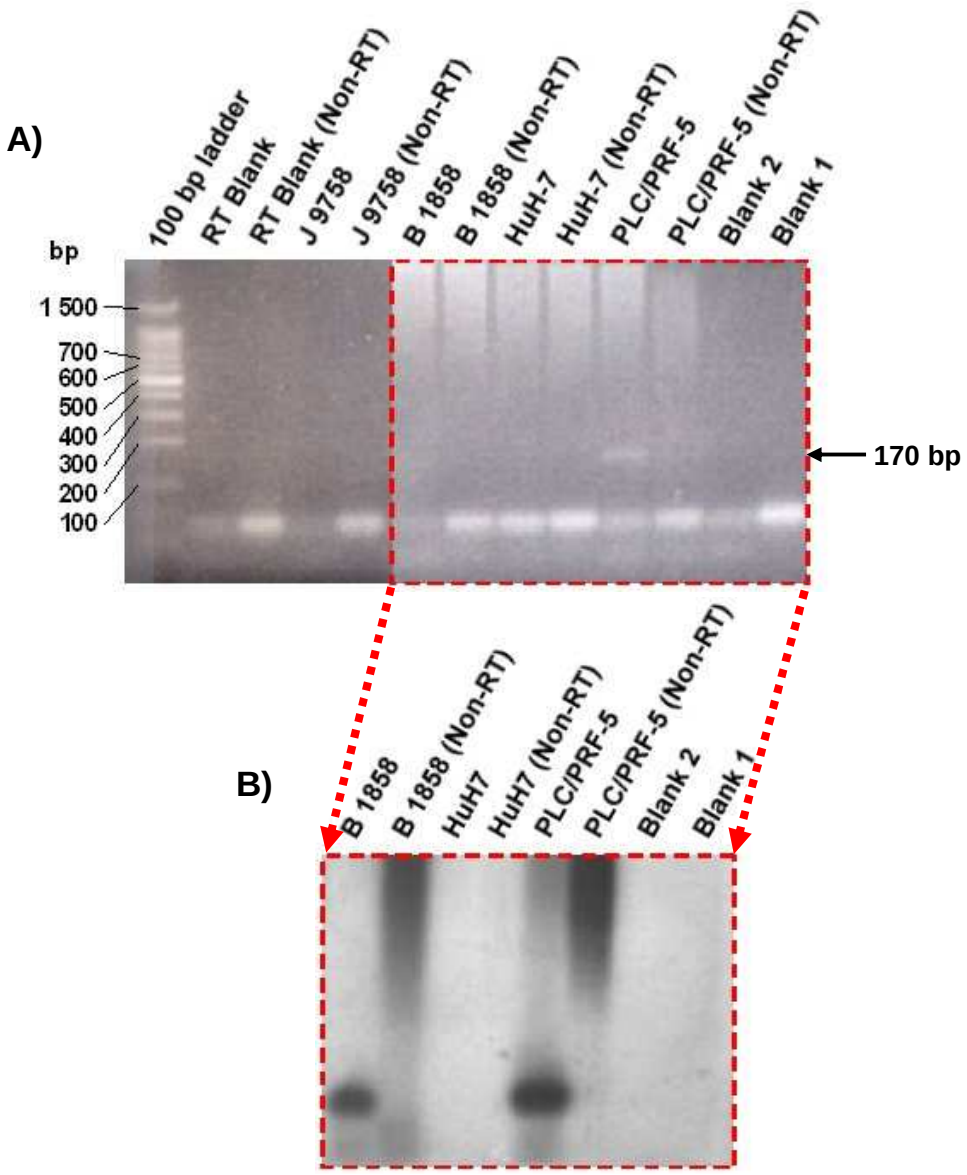


Figure 32: Nested PCR of HBV core region from RNA.
Panel A is a composite figure of a 2% EtBr agarose gel on which nested PCR amplicons (1689 – 2509 in the first round and 2267 – 2436 in the second round), using RNA extracted from the liver tissue of wild-caught baboons as template, were resolved. The only amplicon visible was from the positive RNA extraction control from PLC/PRF-5 cells. The amplicons were therefore subjected to Southern hybridization to detect low levels of amplification.
Panel B shows a portion of the autoradiograph of the Southern hybridization for the samples resolved in panel A. The RNA extraction positive control from PLC/PRF-5 cells hybridized as expected and there was no hybridization to any of the negative controls. The sample extracted from the liver tissue of B 1858 also hybridised, indicating that there was amplification of the core open reading frame of HBV in this baboon.
Abbreviations: Blank 1 - single round PCR containing best quality water instead of cDNA; Blank 2 – double round nested PCR containing best quality water instead of cDNA; Non-RT - Non-reverse transcribed negative controls in which DEPC-treated water was added instead of enzyme during the reverse transcription reaction; RT Blank - reverse transcription negative control where DEPC-treated water was added instead of cDNA.

3.7 Identification of covalently closed circular DNA in the liver tissue of the baboon

3.7.1 Extraction of DNA for cccDNA analysis

As a positive control for the cccDNA PCR, DNA was extracted from HBV DNA positive tumourous and non-tumourous human liver. DNA was extracted from rat liver tissue to be used as a negative control. The efficiency of the extractions was assessed by resolving an aliquot of each extraction overnight on a 1% agarose gel (figure 33). Relatively lower levels of DNA were extracted from the baboon liver tissue compared to the controls. The non-tumourous human liver tissues showed the highest levels of DNA per extract followed by the rat liver.

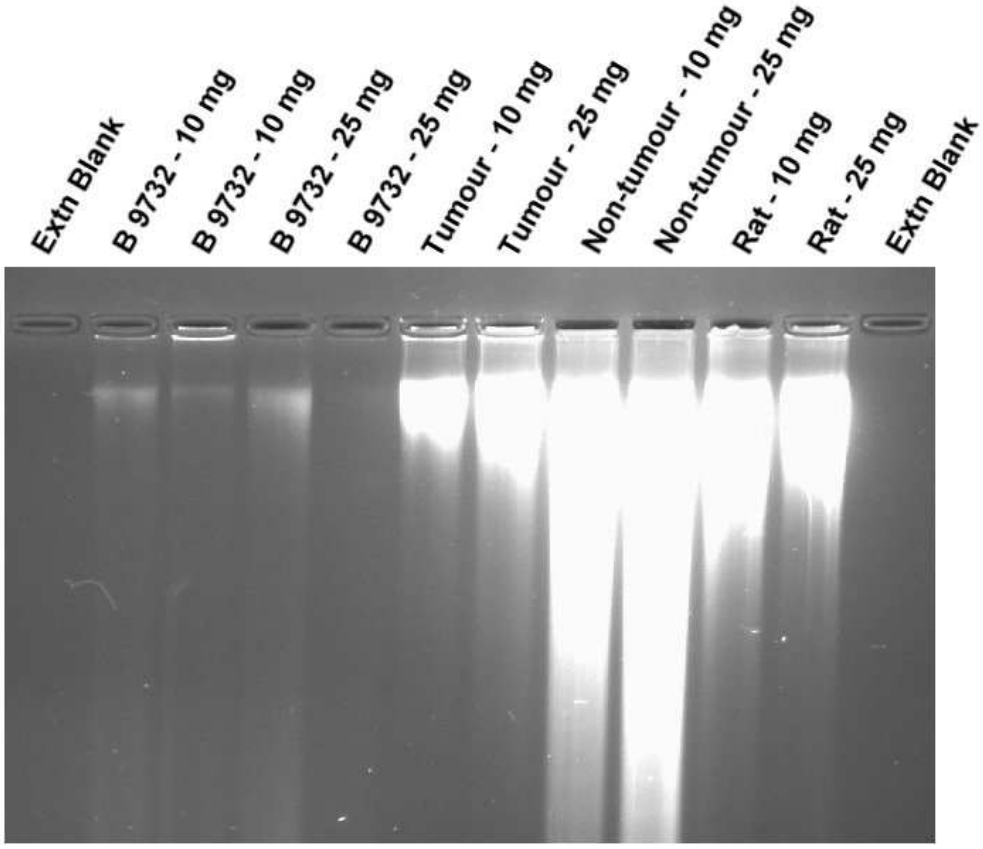


Figure 33: Assessment of DNA extracted using the QIAmp DNA Mini Kit. DNA extracted from the liver tissue of baboon 9732, tumourous and non-tumourous human liver tissue and rat liver tissue was resolved on a 0,8% EtBr agarose gel. Five µl of each extract was resolved. All the extractions had DNA of good quality as shown by the abundance of intact high molecular weight DNA. Extractions from the baboon liver tissue yielded much lower concentrations of DNA than the control tissues.

3.7.2 Real Time PCR analysis using SYBR®-green

cccDNA was detected in the liver tissue of baboon 9732 using real time PCR with SYBR®-green dye (table 10). The melting temperature of each sample (Tm) was determined by performing a dissociation curve at the end of the PCR. The HBV cccDNA positive controls (human tumourous and non-tumourous HBV DNA positive liver tissue and plasmid DNA containing a greater than full length HBV genome) had melting temperatures of between 82,7 °C and 84 °C while the negative controls (HBV DNA negative rat liver tissue and extraction and reagent blanks) had melting temperatures below 80,0 °C. The samples extracted from the baboon liver tissue showed similar melting temperatures to the positive controls indicating that HBV cccDNA was present. The amplification plots showed that the baboon extracts reached log phase after ~36 cycles compared to the positive control liver samples, which reached log phase after ~30 cycles and the plasmid positive controls, which reached log phase after ~5 cycles. This low cccDNA concentration in the baboon liver was also apparent in the dissociation curves (figure 34).

Table 10: **Real Time cccDNA SYBR® green detection results**

A)

Sample Name	Detector	Tm
B 9732 Extraction 1	SYBR	83.1
B 9732 Liver 10 mg - Extn1	SYBR	83.1
B 9732 Liver 10 mg - Extn2	SYBR	83.9
B 9732 Liver 25 mg - Extn1	SYBR	83.1
B 9732 Liver 25 mg	SYBR	82.7
Tumour 10 mg	SYBR	82.7
Tumour 25 mg	SYBR	83.9
Non-Tumour 10 mg	SYBR	83.1
Non-Tumour 25 mg	SYBR	82.7
Rat 10 mg	SYBR	79.5
Rat 25 mg	SYBR	79.1
Reagent Blank	SYBR	71.1

B)

Sample Name	Detector	Tm
B 9732 Liver Pooled - 1	SYBR	82.7
B 9732 Liver Pooled - 2	SYBR	82.3
B 9732 Liver Pooled - Blank	SYBR	78.1
B 9732 Liver Re-eluted – 25 mg	SYBR	82.7
B 9732 Liver Re-eluted – 50 mg	SYBR	83.1
B 9732 Liver Re-eluted - Blank	SYBR	78.1
Tumour – 10 mg	SYBR	83.1
Tumour – 25 mg	SYBR	83.1
Non-Tumour – 1 0mg	SYBR	83.1
Non-Tumour – 25 mg	SYBR	83.1
Extraction Blank	SYBR	78.1
Rat Liver – 25 mg	SYBR	79.4
Rat Liver – 50 mg	SYBR	79.8
Rat Liver - Blank	SYBR	76.0
Plasmid diluted 1 in 10	SYBR	84.0
Plasmid diluted 1 in 25	SYBR	84.0
Water Blank	SYBR	78.6

Abbreviations: Tm – melting temperature; Tumour – DNA extracted from human tumour liver tissue was used as template for the real-time PCR; Non-Tumour – DNA extracted from non-tumourous human liver tissue; Rat – DNA extracted from rat liver tissue; Plasmid – DNA extracted from a plasmid containing a greater than full length HBV genome was used as template.

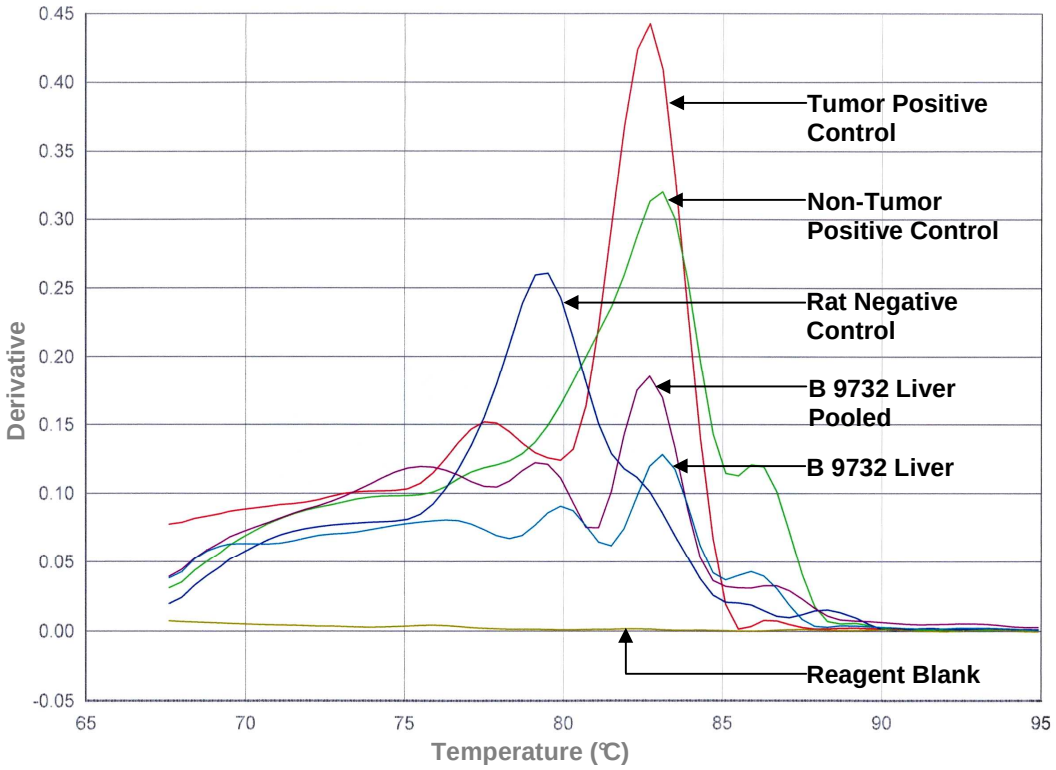


Figure 34: Dissociation curve of Real Time PCR amplicons. The dissociation curve was performed after a real time selective PCR for covalently closed circular (ccc) DNA using SYBR®-green for detection. The samples shown in this figure include two positive controls extracted from tumourous and non-tumourous HBV positive liver tissue respectively and two negative controls - a Rat Negative Control and a Reagent Blank using best quality water instead of DNA. The B 9732 liver sample was extracted using the phenol-chloroform extraction method and the B 9732 Liver pooled sample was from ten identical extractions performed using the QIAmp DNA Mini Kit, which were pooled and re-extracted using a salt-ethanol precipitation. The samples from the baboon liver tissue show a similar melting temperature to the positive controls, although the heights of the peaks indicate that they are at a much lower concentration.

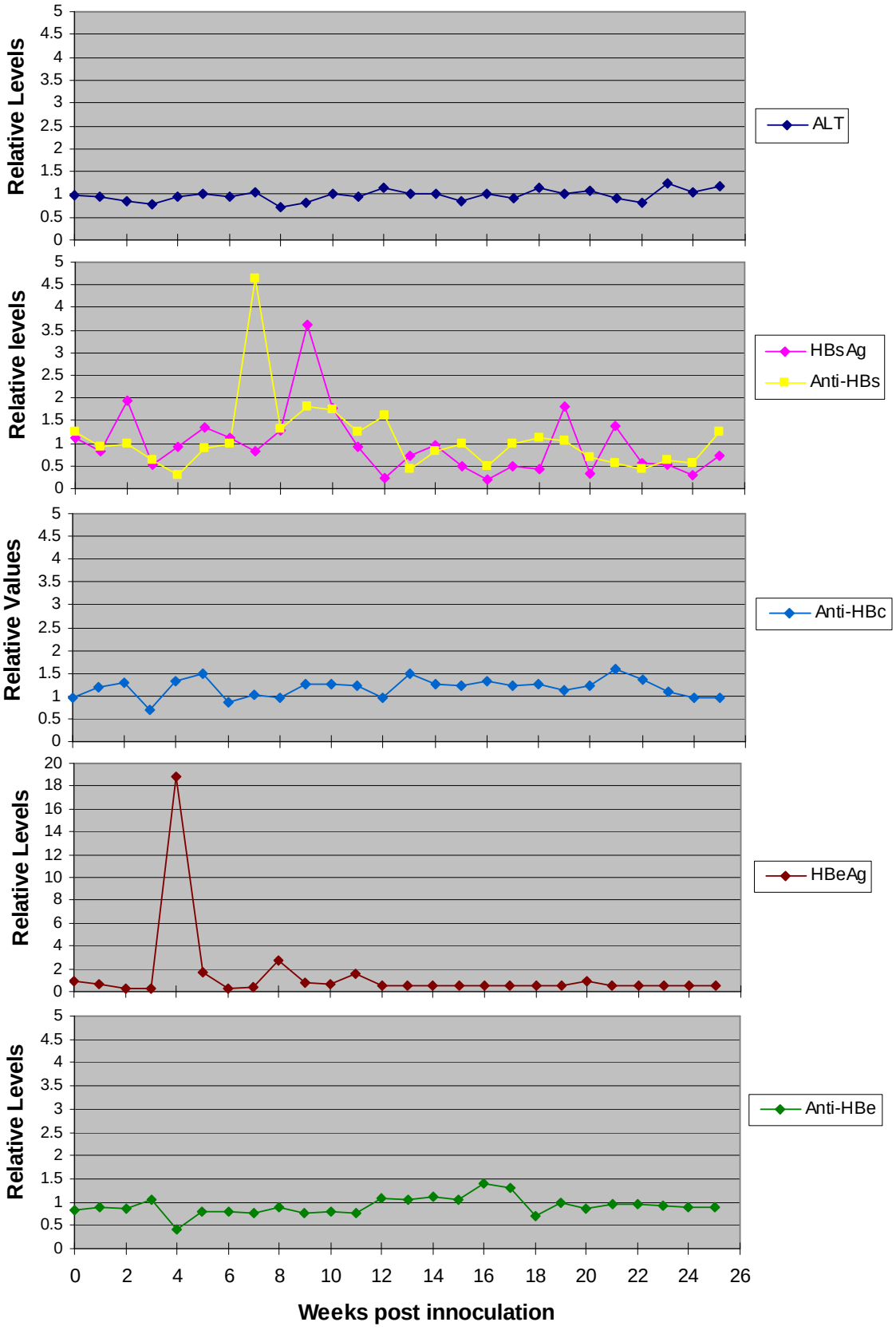
3.8 Transmission of HBV to experimentally-naïve baboons

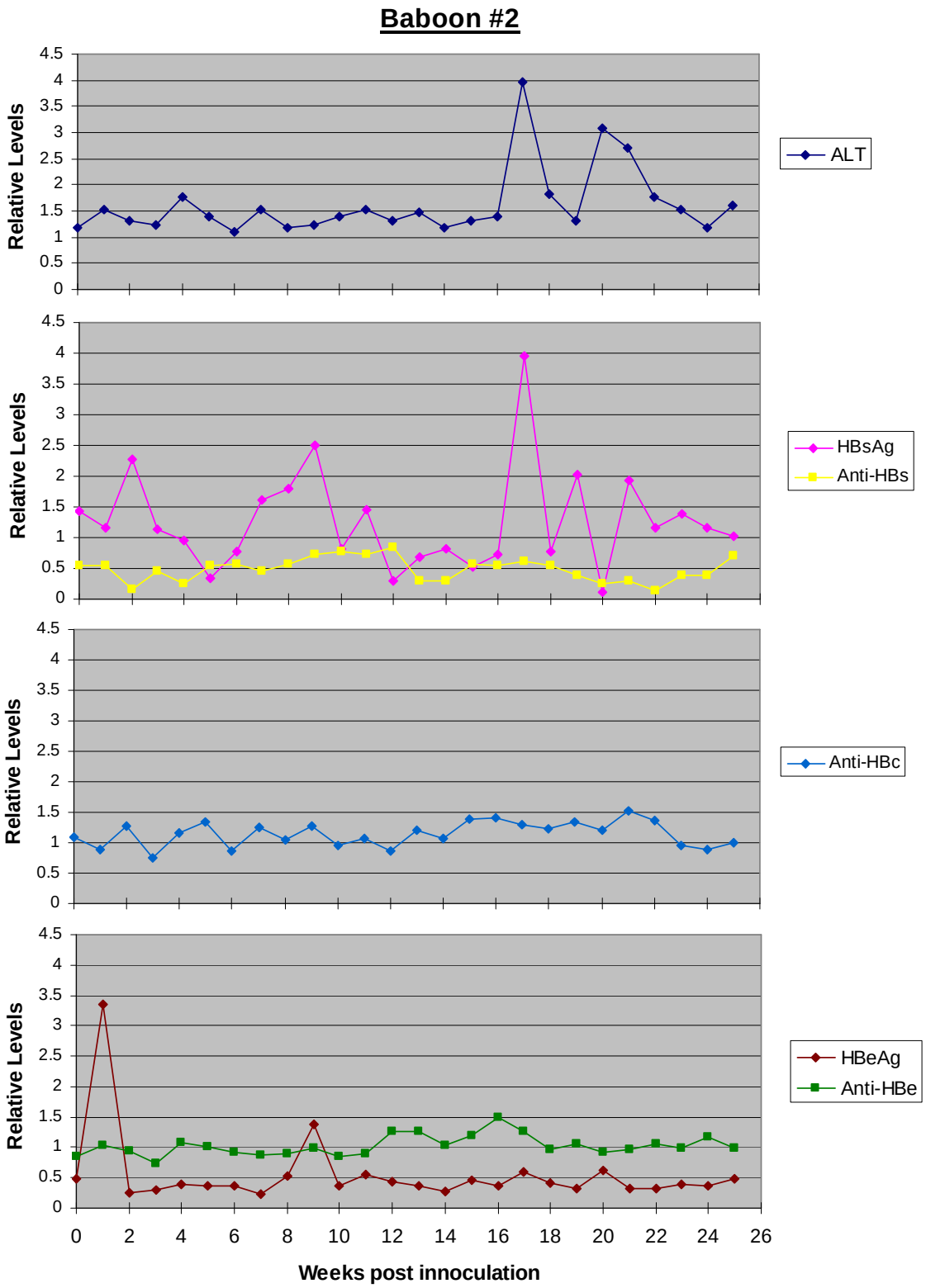
Transmission of the HBV found in baboons to experimentally naïve baboons was performed by Professor R. Purcell at NIH in America. Four experimentally naïve, domestically raised baboons were each injected with serum from one of four of the original HBV DNA positive baboons. Prior to inoculation, blood was drawn from the baboons to determine their baseline AST, ALT and IGG levels and for PCR analysis to show that they were not HBV DNA positive at baseline. Post inoculation, serum was drawn and a liver biopsy performed on each baboon on a weekly basis to determine the ALT, ICD and gGT levels as well as serological markers HBsAg, HBeAg, anti-HBc, anti-HBe and anti-HBs levels. The baboons were transiently positive for a number of these markers, as shown in figure 35. Baboon #1, inoculated with serum from baboon 9712, had an increase in the level of HBsAg 9 weeks post inoculation with smaller increases 2, 19 and 21 weeks post inoculation. This baboon also had increases in the levels of anti-HBs and HBeAg 7 and 4 weeks post inoculation respectively. Baboon #2, inoculated with serum from baboon 9732, showed an increase in HBsAg and HBeAg levels 9 weeks post inoculation. Moreover, this baboon showed a coincident sharp increase in both ALT and HBsAg levels 17 weeks post inoculation, with the levels of both these markers being intermittently elevated for the following four weeks. Baboon #3, inoculated with serum from baboon 9733, showed an increase in HBsAg levels 2, 9, 10 and 17 weeks post inoculation, an increase in anti-HBs 24 weeks post inoculation and an increase in HBeAg levels 0 weeks post inoculation as well as 8 and 20 weeks post inoculation. Baboon #4, inoculated with serum from baboon 9734, had increased levels of HBsAg 9 weeks post inoculation. Anti-HBs levels in this baboon increased dramatically 15 weeks post inoculation and remained elevated for the next 6 weeks. After six months (26 weeks), the four baboons were sacrificed and serum and liver tissue obtained from each baboon.

Figure 35: Relative levels of ALT and HBV serological markers in experimentally-naive baboons.

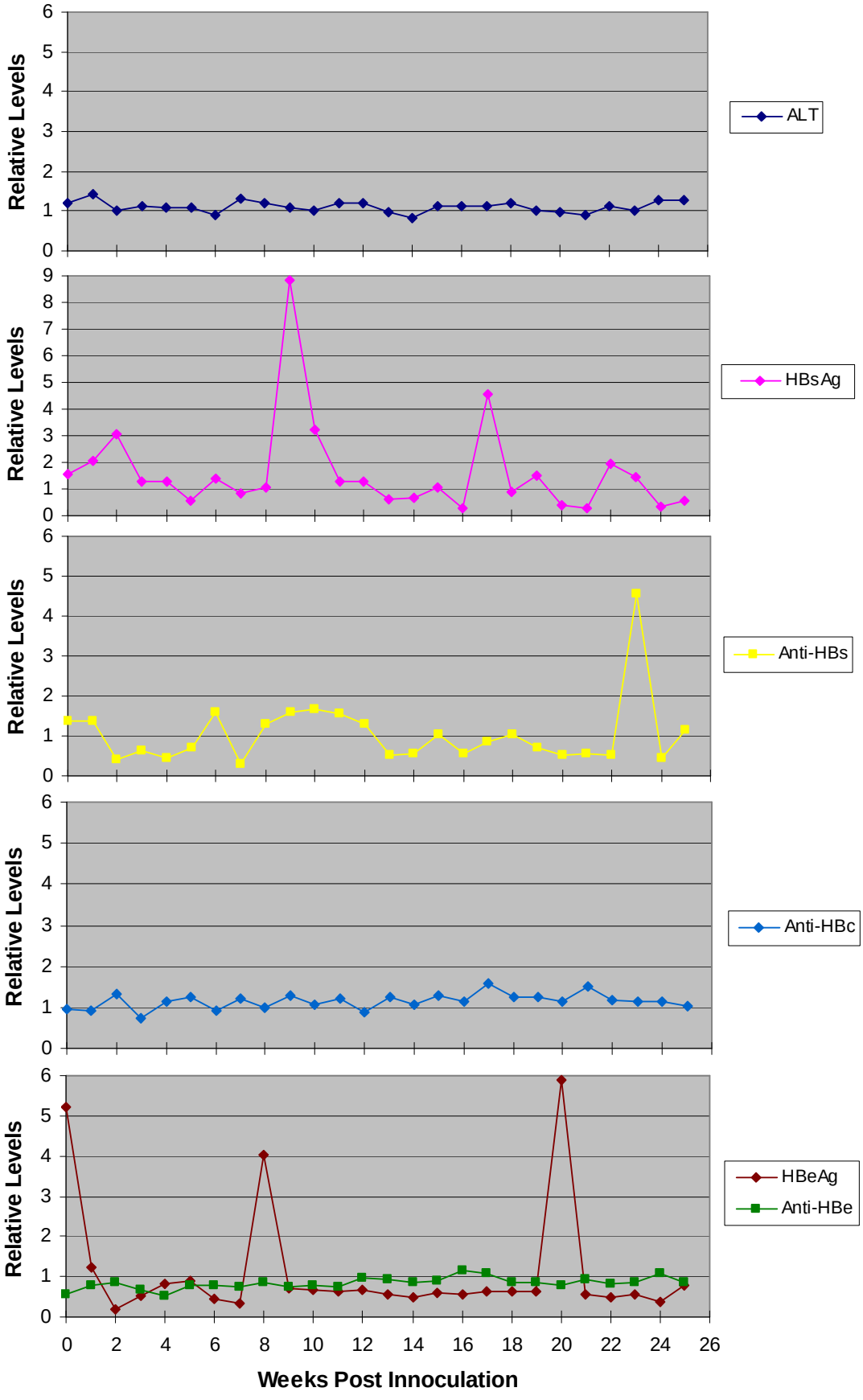
Using serum obtained from experimentally-naive baboons, the ALT and HBV serological marker levels were measured at weekly intervals post inoculation and computed relative to baseline level. The baseline level was taken to be the average of the values obtained at weekly intervals for the month prior to inoculation. Serum for 'Week 0' levels was drawn just after inoculation.

Baboon #1

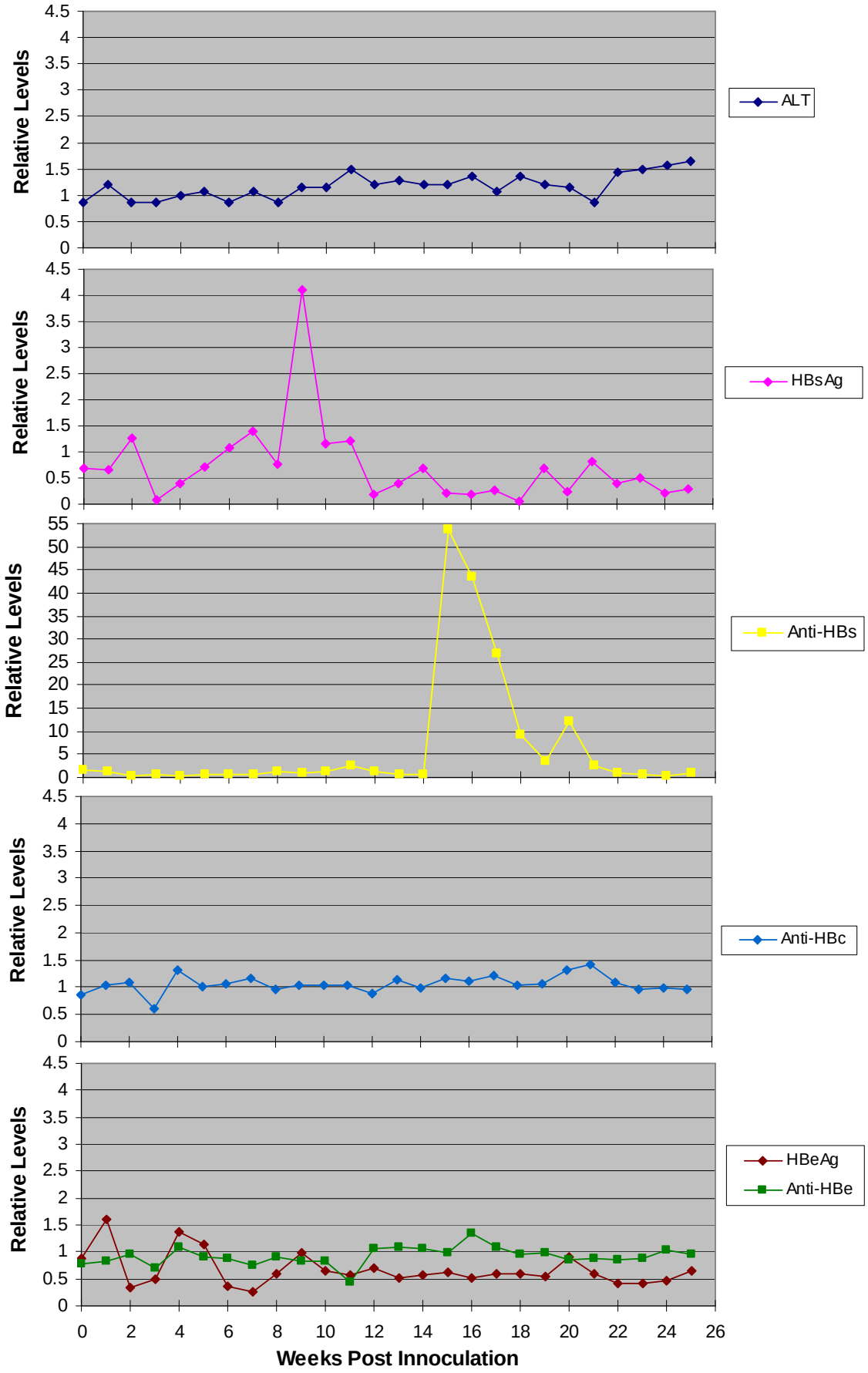

Relative Levels
ALT
Relative levels
HBsAg
Anti-HBs
Relative Values
Anti-HBc
Relative Levels
HBeAg
Relative Levels
Anti-HBe
Weeks post innoculation


Baboon #2
Relative Levels
ALT
HBsAg
Anti-HBs
Anti-HBc
HBeAg
Anti-HBe
Weeks post innoculation

Baboon #3


Relative Levels
ALT
HBsAg
Anti-HBs
Anti-HBc
HBeAg
Anti-HBe
Weeks Post Innoculation

Baboon #4


Relative Levels
ALT
HBsAg
Anti-HBs
Anti-HBc
HBeAg
Anti-HBe
Weeks Post Innoculation

3.8.1 Identification of HBV in the serum of infected baboons

DNA was extracted from the serum collected at each time point using the QIAamp DNA extraction kit (Qiagen) and nested PCR analysis for the surface gene (459 – 710) was performed. The PCR amplicons were visualised by agarose gel electrophoresis and subjected to Southern hybridization analysis with an HBV specific probe both to confirm that the amplicons were HBV and to detect any low levels of amplification, too faint to be visualised on the agarose gel. The results for all four baboons are shown in table 11. There was no correlation between the serology results shown in figure 35 and the time points at which HBV DNA was detected.

Table 11: Amplification of surface gene (nt 459 – 710) from infected baboons

Week	Baboon #1	Baboon #2	Baboon #3	Baboon #4
Baseline	-	-	-	-
1	-	-	+	-
2	-	+	-	-
3	-	-	+	+
4	-	-	-	+
5	+	-	+	-
6	-	+	-	+
7	-	-	-	-
8	-	-	-	+
9	+	-	+	-
10	+	-	-	-
11	-	+	+	-
12	-	+	-	-
13	-	-	-	-
14	+	+	+	-
15	-	-	-	+
16	-	-	-	-
17	-	-	+	-
18	-	+	+	+
19	-	-	+	-
20	-	+	-	-
21	+	-	+	-
22	-	-	-	+
23	-	-	-	-
24	-	+	-	-
25	-	-	+	-
26	-	-	+	-
Necropsy	-	+	-	+

DNA was extracted from serum drawn from the infected baboons each week and a nested PCR amplification (255-761 in the first round and 459-710 in the second round) for a 252 bp region of the surface gene performed. Baboon #1 was inoculated with serum from baboon 9712, baboon #2 with serum from baboon 9732, baboon #3 with serum from baboon 9733 and baboon #4 was inoculated with serum from baboon 9734. The (+) in the table indicates that the sample amplified successfully at this time point and the (-) indicates that the PCR failed.

3.8.2 Identification of HBV in the liver tissue of experimentally infected baboon #2

DNA was extracted from the liver tissue of baboon #2 (inoculated with serum from baboon 9732), using the phenol-chloroform extraction method and a portion of the surface gene (409 – 1101) was amplified by nested PCR (figure 36). The amplicon was sequenced using automated sequencing and the sequence aligned with that obtained from baboon 9732. The sequence of the virus extracted from the liver tissue of baboon #2, six months after inoculation, was identical to the HBV sequence of this region found in the original baboon 9732.

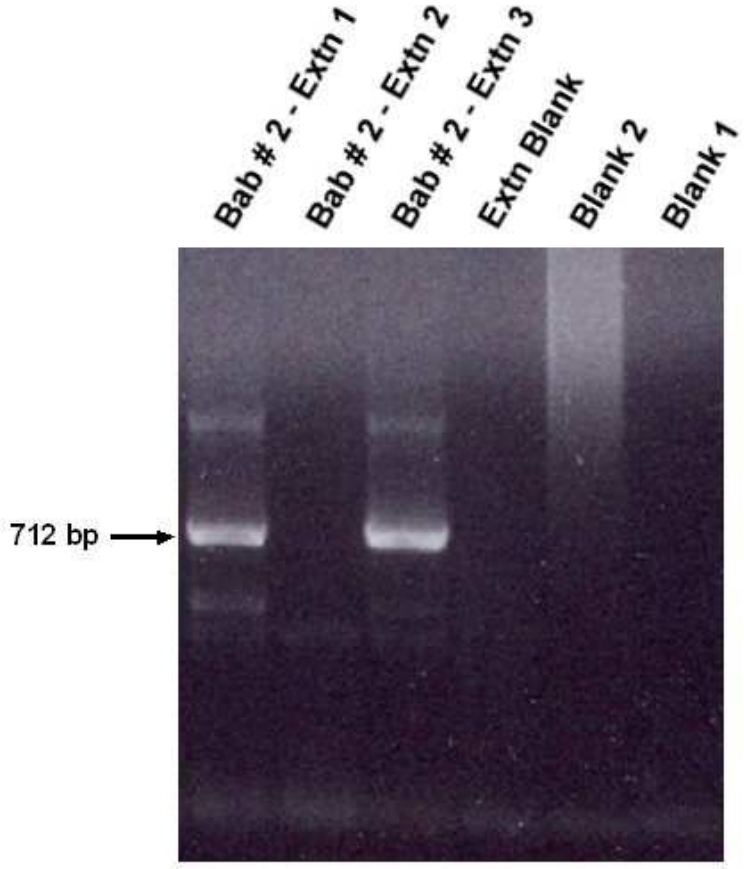


Figure 36: Amplification of a portion of the HBV surface gene from infected baboon #2. A nested PCR amplification (58 – 1450 in the first round of amplification and 409 – 1101 in the second round) of a 712 bp region of the surface gene was performed using DNA extracted from baboon #2 (inoculated with serum from baboon 9732). Although three identical DNA extractions were performed, only two of them (extractions 1 and 3) amplified successfully.
Abbreviations: Blank 1 - single round PCR containing best quality water instead of DNA; Blank 2 – double round nested PCR containing best quality water instead of DNA; Extn – extraction.

4. Discussion

The results of previous studies carried out in our laboratory intimated to the possibility that baboons may be infected with an indigenous strain of HBV. In the first study, four Chacma baboons, which were HBV DNA negative at baseline, were inoculated with human serum and shown to be susceptible to HBV infection, with no biochemical evidence of liver injury at any stage and with normal liver histology at necropsy, 52 weeks post-inoculation (Kedda *et al.*, 2000). The inoculum was pooled serum from three patients with acute hepatitis B, and contained both genotype A and non-A (most likely D) strains of HBV (Baptista *et al.*, 2003). The baboon sera were negative for HBsAg and anti-HBs using conventional assays but positive for anti-HBc at 16 weeks post inoculation. In a follow up study, very low concentrations of HBV DNA were found in the serum of the infected baboons and genotypes A and non-A were detected in varying concentrations in four baboons for the entire 52-week follow up period (Baptista *et al.*, 2003). Two baboons were excluded from these studies because they were HBV DNA-positive at baseline, prior to inoculation, and we therefore proceeded to determine the prevalence of HBV in wild-caught baboons.

4.1 Prevalence of HBV DNA in Chacma baboons

The Chacma baboon (*Papio ursinus orientalis*) is widely distributed in South Africa, ranging from the Cape Peninsula to just north of the Limpopo River (Marais, 1971). Sixty nine wild caught Chacma baboons - forty nine adults and twenty juveniles - from three distinct geographical regions of South Africa were screened to determine the prevalence of HBV in these primates. Serum samples from seven vervet monkeys (*Cercopithecus pygerythrus*) were initially also screened for HBV but, were found to be negative for all four regions and, thereafter used as negative controls.

Two different extraction methods, the QIAGEN QIAamp® DNA Blood Mini Kit and GeneReleaser® were used to extract DNA from the primate serum samples and this DNA used as the template for the nested-PCR amplification of four non-

overlapping regions of the HBV genome. Although the GeneReleaser® system is more economical, time effective and requires a smaller amount of serum, it is more prone to false-negative results when HBV DNA concentrations are low (Kramvis *et al.*, 1996). It is however slightly more sensitive than the QIAamp® kit for detecting HBV DNA at low-titres. In the present study, the efficiency of DNA extraction from the baboon sera did not differ between the two methods.

Although nested PCR increases the sensitivity of detection in samples with low titres of HBV, there is an increased risk of false-positivity because of inadvertent contamination. All the necessary controls and precautions recommended by Kwok and Higuchi (1989) were therefore strictly adhered to. Moreover, the amplifications were repeated at different times, by different researchers, in independent laboratories. The variation shown phylogenetically between the precore/core sequences of a representative number of samples (figure 15) furthermore confirmed that there were differences in the HBV strains from individual baboons, ruling out the possibility of a contaminant.

HBV DNA was detected in five baboon sera using Southern hybridization (figure 16). This detection of HBV in baboon serum samples by Southern hybridization is an important control because it eliminates the possibility that the sera were contaminated in the laboratory during subsequent analyses.

Samples were only considered to be positive for HBV DNA if they amplified for at least three of the four regions of the HBV genome. Using this criterion, 15 out of 69 baboon sera were HBV DNA positive giving an overall prevalence of 21,7%. Considering that less stringent criteria (i.e. nested-PCR amplification of at least two out of three different regions (Cacciola *et al.*, 1999; Raimondo *et al.*, 2007)) are used to detect HBV in serologically negative individuals and that we did not determine the prevalence of HBV in the liver, this may be an underestimation of the prevalence of HBV in baboons (Gibney *et al.*, 2008).

Using commercially available ELISA assays, the sera of four HBV DNA positive baboons were tested and found to be serologically negative for HBsAg and anti-HBc. On the other hand, immunohistochemical staining of liver tissue from one

of these baboons did detect HBcAg in several hepatocyte nuclei (figure 17, panel B) but could not detect HBsAg in the cytoplasm. This detection of HBcAg and the detection by others of HBsAg and/or HBcAg in a number of naturally infected non-human animals including chimpanzees, gibbons, orang-utans, woolly monkeys, Beechy ground squirrels and tree squirrels (Marion *et al.*, 1980; Feitelson *et al.*, 1986; Lanford *et al.*, 1998; Warren *et al.*, 1999; Hu *et al.*, 2000; Lanford *et al.*, 2000) excludes the possibility that the lack of detection of HBsAg and anti-HBc was a result of the lack of cross-reactivity between the human and baboon antigens. The baboon immune system is similar to that of humans (Murthy *et al.*, 2006) and antigenic cross-reactivity to a number of other viruses including HIV and simian immunodeficiency virus (SIV) has been demonstrated in baboons (Langat *et al.*, 1999). Moreover, interspecies cross-reactivity to human immunoglobulins and to three distinct epitopes on human plasminogen has been demonstrated in baboons (Cummings *et al.*, 1984; Shearer *et al.*, 1995).

In the present study, we could not detect HBV infection using serological tests alone, concurring with the findings of previous studies in baboons (Blumberg *et al.*, 1972; Deinhardt, 1976; Michaels *et al.*, 1994). In contrast HBsAg positivity has been reported in 6,7% of chimpanzees, 4,6% of gorillas, 20,7% of gibbons, 21,3% of orang-utans and 53,8% of woolly monkeys tested (Sa-Nguanmoo *et al.* 2009 and references therein). On the other hand, using nucleic acid testing, hepadnaviral infections were detected in 19,5% of chimpanzees, 15,1% of gorillas, 37,5% of gibbons, 27,3% of orang-utans and 60% of woolly monkeys (Sa-Nguanmoo *et al.* 2009 and references therein). Michaels *et al.* (1996) failed to detect HBV DNA in four experimentally infected baboons but, since no details are given on the regions amplified or whether a nested PCR amplification was used, it is difficult to gauge whether the amplification used was sensitive enough to detect low levels of viral DNA. Kedda *et al.* (2000) detected HBV DNA in experimentally infected baboons, 52 weeks post-infection using nested PCR. In the present study, the first large scale cross sectional study of the prevalence of HBV DNA in wild-caught baboons, we found 21,7% of wild-caught Chacma baboons to be positive for HBV DNA, a prevalence similar to that found in other non-human primates (Sa-Nguanmoo *et al.*, 2009).

Histological examination of the baboon liver tissue from a single baboon showed mild focal lobular hepatitis but no interface hepatitis, bridging necrosis or other signs of liver injury (figure 17, panel A). This is similar to what has been observed in chimpanzees and Barbary macaques experimentally infected with human HBV that develop characteristically mild symptoms of liver disease (Feitelson and Larkin, 2001; Gheit *et al.*, 2002).

4.2 Amplification of the complete HBV genome

Both Kedda *et al.* (2000) and Baptista *et al.* (2003) showed that HBV DNA levels in experimentally infected baboons are very low. Similarly, in the present study, the fact that HBV DNA could only be amplified using nested PCR indicated that low viral levels were found in the sera of naturally infected baboons. These low levels made amplification of the complete genome challenging. It has previously been shown that the best source of hepadnaviral DNA in carriers with low titres of virus is liver tissue and that the maximum amounts of high quality nucleic acids can be recovered using proteinase K digestion followed by phenol chloroform extraction and precipitation with ethanol (Michalak *et al.*, 2007; Raimondo *et al.*, 2008a). Therefore in order to ensure optimal levels of HBV DNA to allow for downstream sequencing, DNA was extracted from the liver tissue of baboon 9732 using this method.

In 2000, Hu *et al.* successfully amplified the complete hepatitis B viral genome from the sera of two of thirteen chimpanzees using overlapping sub-genomic fragments but only a portion of the surface gene from the remaining eleven chimpanzees. The primers used in that study all annealed to very conserved regions of the viral genome and were synthesised in both the forward and reverse orientations so that they could be used as either forward or reverse primers. Using these primers together with primers designed and used routinely in our laboratory (table 6), the complete genome of HBV isolated from baboon 9732 liver was successfully amplified by the nested PCR of eight overlapping fragments.

4.3 Sequencing and phylogenetic analysis of baboon HBV

Phylogenetic analysis of the complete genome (figures 25), as well as each of the four open reading frames (figure 27), of the HBV isolated from the liver of baboon 9732, showed it to belong to genotype A and to cluster with subgenotype A2. The relatedness of the HBV isolate from the baboon to subgenotype A2 isolates is reinforced by a mean nucleotide divergence of 1,0% across the complete genome compared to 4,5% when the baboon isolate is compared to subgenotype A1. Phylogenetic comparison of the baboon HBV sequence with 51 other subgenotype A2 complete HBV genomes showed that this subgenotype does not cluster according to geographical location (figure 26). The baboon isolate branched with one of three subgenotype A2 isolates from South Africa for which complete sequences are available, but separately from the remaining two (figure 26). Subgenotype A2 is rarely detected in South Africa, where subgenotype A1 predominates (Kimbi *et al.*, 2004).

GroupScanning analysis showed no evidence of recombination in the baboon HBV isolate. The Simmonic software used tests to see how deeply a query sequence lies within each clade formed by a particular genotype with values ranging from close to 0 (showing no grouping) to 1 (the query sequence is buried deep within a specific genotype) (Simmonds and Midgley, 2005). The values for the baboon HBV isolate never fell below 0,96 indicating a strong association with genotype A.

Analysis of the nucleotide sequence of the complete HBV genome isolated from the baboon liver showed that it contained the six nucleotide insertion in the core ORF as well as a C at nucleotide position 1858, both characteristics of genotype A. The 1858C variation is important as it stabilises the ε stem-loop structure by preventing a G to A mutation at position 1896 (Lok *et al.*, 1994).

The baboon HBV sequence included variations that differentiate subgenotype A2 from A1 at both the nucleotide and amino acid levels. These variations are concentrated in the pre-S1 region overlapping the polymerase spacer domain and

in the pregenome ε signal (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Datta *et al.*, 2009). The pre-S region, particularly peptides 21 to 47 are involved in the binding of the virus to the hepatocytes (Neurath *et al.*, 1986) and the ε signal is essential for the replication and biological functions of HBV (Kramvis and Kew, 1998b). The "a" determinant of the HBsAg did not contain any mutations that could lead to conformational changes affecting antibody binding and hence cannot be implicated in the failure to detect HBsAg in both the serum and liver tissue of the baboon (Pawlotsky, 2005; Lada *et al.*, 2006).

In contrast to other subgenotype A2 sequences, the basic core promoter and precore regions of the baboon HBV isolates contained the G1809T / C1812T double point mutation as well as the G1888A mutation. The former is found in subgenotypes A1 and A3 and latter only in subgenotype A1 (Kimbi *et al.*, 2004; Kramvis *et al.*, 2008). The 1809 / 1812 double nucleotide substitution is upstream of the precore initiation codon at position 1814. Nucleotides 1808 – 1817 (5' – AGCACCATGC – 3') are well conserved in genotypes A – H and conform to the Kozak sequence (5' - GC$^{A}/_{G}$CCATGG – 3'), which is optimal for the initiation of translation (Ahn *et al.*, 2003). The 1809 / 1812 variation changes this sequence to: 5' – A***T***CA***T***CATGC – 3' (initiation codon underlined; substitutions in bolded italics) and results in a more than 20% reduction of HBeAg expression through a ribosomal leaky scanning mechanism (Ahn *et al.*, 2003). This reduction in HBeAg expression is thought to facilitate early seroconversion to anti-HBe and is accompanied by a striking reduction in the replication of HBV. Nucleotide 1888 is found on the upper stem of the encapsidation signal and the G to A mutation creates a Watson-Crick pair with T1871 stabilising this structure (figure 7) (Kimbi, 2005). This mutation also affects translation of the core protein as it introduces an out-of-frame start codon that plays a role in modulating translation of the core protein by a leaky scanning mechanism (Kimbi, 2005). This decrease in core protein is thought to result in a less severe immune response and hence to less liver damage and possible persistence of the virus.

Four other mutations differentiated the baboon strain of HBV from the majority of previously sequenced human subgenotype A2 isolates. These included mutations:

T380C (rtV84A in conserved region A of the HBV polymerase and C76R in HBsAg), C1470T (P33G in the X protein), T1765C (P145S in the X protein) and A2019G (E40G in the core protein). The mutations in the polymerase and X proteins are not predicted to cause any significant conformational changes. The cysteine (C) to arginine (R) mutation at position 76 of HBsAg could affect this protein as the C residues are known to form disulfide bond cross linkages important in the tertiary structure of proteins. sC76R on the other hand, located in the first hydrophilic loop, would not be expected to have a significant effect because the antigenicity and infectivity of the virus are mainly affected by residues 101 to 172 of HBsAg (Salisse and Sureau, 2009). Furthermore, C76 is not essential for assembly and secretion of the S protein (Mangold *et al.*, 1995). The A2019G mutation causes an E40G substitution in the core protein. This could lead to conformational changes in the core protein as this mutation results in the replacement of a negatively charged polar side chain with a non-polar one. Further functional studies would be necessary to determine the effect of these changes found in the baboon HBV isolate.

4.4 Replication of HBV in the baboon liver

Hepadnaviruses replicate by the reverse transcription of an RNA intermediate from cccDNA. The presence of both cccDNA and viral RNA was therefore used to demonstrate viral replication in the baboon liver because the detection of HBV DNA alone does not necessarily correspond to infection (Hollinger and Sood, 2010). Moreover, HBV DNA positive baboons did not show significant biochemical or histological evidence of liver damage, and thus identification of viral replication was important to show infectivity.

Using real-time PCR, HBV cccDNA was detected in the baboon liver. This selective cccDNA PCR, originally devised by Kock *et al.* (1996), differentiates between intracellular HBV cccDNA, virion open circular DNA and other HBV replicative intermediates by using the fact that the HBV genome is partially double-stranded, while the cccDNA molecule has both strands complete. The primers are designed to selectively amplify a region corresponding to the gap and incomplete regions of the viral genome, and thus amplification is unhindered in

cccDNA but, because of the gap found in this region in the other forms of HBV, it is inefficient in these forms.

Extracted samples were treated with Plasmid-Safe™ ATP-Dependent DNase, which hydrolyses linear double stranded DNA into deoxynucleotides without affecting either closed circular, supercoiled or nicked circular double stranded DNAs (Epicentre biotechnologies). Bowden *et al.* (2004) noted that extracts treated with Plasmid-Safe™ ATP-Dependent DNase had a log reduction in background amplification, so a 1000-fold excess of non-ccc HBV DNA would be needed in order to make a significant contribution to the HBV cccDNA assay.

HBV DNA levels in the baboon are very low and similarly, low levels of cccDNA were detected in the liver tissue with the samples only reaching log phase after more than 35 cycles. The real-time PCR made use of SYBR®-green for detection and so a dissociation curve was used to confirm that both the samples extracted from the baboon liver and the positive controls had the same melting temperature (figure 34).

Detection of transcripts from both the core and surface ORFs showed that the virus is transcriptionally active in baboon hepatocytes (figure 31). Maximum amounts of high quality RNA were obtained from the baboon liver using the guanidinium–acid-phenol extraction method. This was found to be more efficient that the Promega SV total RNA isolation kit, which could only detect amplified products by Southern hybridisation (figure 32). Extracts were treated with DNaseI to prevent any HBV DNA carryover and to ensure that only HBV transcripts were detected (Mason *et al.*, 1998).

4.5 Transmission of the virus to experimentally naïve baboons

Transmission of the HBV found in baboons was demonstrated by successfully infecting four domestically raised, experimentally naïve baboons with sera from infected baboons. Serum samples were collected and analysed on a weekly basis for 26 weeks post-inoculation. At 26 weeks post-inoculation, the baboons were sacrificed and liver tissue obtained at necropsy. Transient HBsAg and HBeAg

seroconversion provided serological evidence of HBV infection in all four baboons as illustrated in figure 35. HBsAg and HBeAg derived from the inoculum have only been reported to persist in the serum of inoculated rhesus monkeys (*Macacca mulata*) for up to three weeks post inoculation (Lazizi and Pillot, 1993). Therefore, we did not consider elevations in the levels of the HBV serological markers within the first four weeks post inoculation as an indication of infection. There was however an increase in HBsAg levels in all four of the inoculated baboons nine weeks post inoculation indicating successful transmission of the virus to the experimentally naïve baboons by this stage.

HBV DNA was detected intermittently in the serum of all four baboons for the entire six month period of follow-up (table 11). The length of time following inoculation during which detectable HBV DNA from the inocula can be detected in the recipient serum, is contentious. On the one hand, Lanford *et al.* (2003) used the detection of WMHBV DNA in the serum of a spider monkey six weeks post inoculation as evidence of replication whereas Lazizi *et al.* (1993) amplified HBV DNA in rhesus monkeys, which are resistant to HBV infection, for a maximum of three months post-inoculation but not thereafter. In our case the time of follow up was longer than three months and since HBV DNA could be detected six months post inoculation, this did not represent persistence of the inoculum.

The sequence of the HBV isolated from the liver of baboon #2 at necropsy was identical to the sequence of the inoculum, demonstrating successful transmission of the virus to experimentally naïve baboons.

Table 12: **A comparison of hepadnavirus infection in the woodchuck and baboon (modified from Michalak *et al.*, 2007).**

Characteristic	Woodchuck Model		Baboon
	Primary Occult Infection	Secondary Occult Infection	
Serology:			
• WHVsAg	Negative	Negative	HBsAg Negative
• Anti-WHVc	Negative	Positive	HBcAg Positive
• Anti-WHVs	Negative	Positive or Negative	Anti-HBs Negative
WHV-DNA load			
• Serum	$\leq 10^2$ vge / ml	$\leq 10^2$ vge / ml	Low levels
• PBMC	$\leq 10^3$ vge / µg	$\leq 10^3$ vge / µg	nd*
• Liver	Not detectable	$\leq 10^3$ vge / µg	Low levels
Longevity of Persistence	Unknown	Life-Long	Life-Long
Infectivity	Yes	Yes	Yes
Spectrum of Organs Involved:			
• Lymphatic System	Yes	Yes	nd*
• Liver	No	Yes	Yes
Liver Histology	Normal	Intermittent minimal to moderate inflammatory changes with periods without alterations	Mild focal lobular hepatitis
Hepatocellular Carcinoma Development	Unknown	~ 20%	?

* nd – not determined

4.6 Characterization of the baboon HBV infection

Michalak *et al.* (2007) defined as occult HBV infection as:

"the existence of HBV DNA in serum, lymphoid cells (PBMC) and / or the liver and virus genome replicative intermediates (i.e. covalently closed circular DNA (cccDNA) and / or mRNA) in lymphoid and / or hepatic tissue in the absence of serum HBV surface antigen, symptoms and biochemical evidence of liver injury".

Using this definition, it is evident that the HBV infection found in wild-caught baboons meets the criteria of an occult infection. Because OBIs in humans occur in asymptomatic carriers of the virus, who are not frequently screened, the woodchuck model, which has been extensively studied, provides a good alternative model system. We therefore compared the characteristics of the infection in the baboon with this animal model (table 12). The baboon infection had the same characteristics of infection defined as secondary occult in the woodchuck model. The baboon had low viral loads in the serum and the liver, was serologically negative and exhibited mild focal lobular hepatitis. Furthermore, HBV DNA was detected in both juvenile and adult baboons, demonstrating the lifelong persistence of the virus. The infection was successfully transmitted to experimentally naive baboons using HBV DNA-positive serum.

This study is the first to report an OBI in a non-human primate, which is similar to the seronegative OBI seen in humans. These OBIs are characterised by a lack of all serological markers and very low or even undetectable levels of circulating HBV DNA (Raimondo *et al.*, 2010), with HBV DNA detected more frequently intrahepatically rather than in the serum (Hu, 2002). The detection of HBV DNA in liver biopsies is therefore considered to be the only reliable marker of this type of infection (Raimondo *et al.*, 2008b; Raimondo *et al.*, 2010). OBIs in humans without liver injury have not been extensively studied and therefore our current knowledge of OBIs comes mainly from the woodchuck model. The detection of an OBI in the baboon may provide a good alternative model system in an animal closely related to humans and not endangered.

4.7 Origins of HBV infection

The HBV genome has a very complex organisation with more than 67% of the genome coding for multiple proteins and regulatory functions within overlapping open reading frames (Mizokami *et al.*, 1997). A synonymous change in one ORF therefore could result in a non-synonymous change in the overlapping ORF and so, in order to maintain its functionality, evolution of the HBV genome is constrained. The HBV polymerase enzyme lacks proof-reading activity and has mutation rates similar to those of the related retroviruses or other RNA viruses (Simmonds, 2001). If evolution of the genome, in the absence of immune pressure, is taken to be $2{,}1\times10^{-5}$ substitutions per site per year, it can be extrapolated that the HBV genotypes evolved from a common ancestor between 2300 and 3100 years ago. The identification of HBV strains in wild caught chimpanzees, gibbons and orang-utans, equidistant both from each other and from the human genotypes, would mean that if the same mutation rate is adhered to, the primate associated HBV strains would also have originated in the last 2000 – 3000 years (Simmonds, 2001). Humans are the only species known to have travelled between continents during this period and so they would have had to be the vectors that spread the disease to other species.

An alternate hypothesis is that HBV co-evolved with modern humans when they migrated from Africa approximately 100 000 years ago (Norder *et al.*, 1994; Magnius and Norder, 1995). The difficulty with this theory is that the distribution of the HBV genotypes does not correspond between human population groups (e.g. genotype F is found in the Native American population whereas genotypes B and C are found in their nearest genetic relatives, the Mongoloid northeast Asians) (Simmonds, 2001). The other problem with this theory is the close relationship between the human and non-human primate strains. In this scenario, the primate viruses should be far more divergent from the human variants and from each other given the much longer period of primate co-speciation (Simmonds, 2001).

A third hypothesis for the origin of HBV speculates that the non-human primate HBV variants coevolved with their primate hosts over a period of 10 - 35 million

years (MacDonald *et al.*, 2000). The numerous genotypes found in humans would have resulted from multiple zoonotic transmissions from several non-human primate species infected with different species-specific strains. This hypothesis is supported by the fact that in areas of high HBV endemicity, there is close contact between humans and primates increasing the chances of cross species transmission (Simmonds, 2001). Transmission by multiple zoonotic events is not unprecedented. HIV type 1 has been shown to have originated from three separate cross species transmissions from chimpanzees and HIV type 2 originated through multiple contacts with sooty mangabeys (Gao *et al.*, 1992; Gao *et al.*, 1999). Supporting this theory is the fact that the most divergent of the human HBV genotypes, genotype F, and the most divergent of the non-human primate HBV strains, the woolly monkey HBV, are both found in South America. Furthermore, hepadnaviral isolates from the gibbon and orang-utan resident in east Asia grouped closest to isolates from genotype B, whereas isolates from chimpanzees in Africa grouped closest to genotype A, when ~60% of the polymerase ORF and the overlapping S gene was compared (Simmonds and Midgley, 2005). In both cases, these are the predominant human HBV genotypes found in humans sharing the natural habitats of these primates. The recently described "genotype J" isolate was found to cluster phylogenetically with isolates from the gibbon and the orang-utan, both of which occur in Borneo, where the patient is presumed to have been exposed (Tatematsu *et al.*, 2009).

A shortcoming of the "non-human primate origin" hypothesis has been that, apart from one HBV genotype E strain in a wild-caught chimpanzee (Takahashi *et al.*, 2000), there have been no reports of an HBV genotype being shared by both humans and primates and thus the actual species involved in transmission of HBV to humans has so far remained unidentified (Simmonds, 2001). The identification in the present study of the occult HBV infection in wild-caught baboons with subgenotype A2 share by humans strengthens the argument for a non-human primate origin of HBV infection and may provide a key to the puzzle.

The nucleotide divergence of genotype A is highest in Africa (4%) than elsewhere (2,96%) leading to the hypothesis that genotype A originated in Africa and then spread to the rest of the world (Kramvis and Kew, 2007; Andernach *et al.*, 2009a).

The relatively large nucleotide divergence between subgenotypes A1 and A2 suggests that they diverged more than 2000 years ago. The low variability seen within the circulating strains of subgenotype A2, which is characterized by a relatively slow evolutionary rate ($\sim 0{,}9\times10^{-4}$) (Zehender *et al.*, 2008), suggests that this subgenotype arrived in Europe from Africa in the last 500 years, most likely by the Portuguese sailors who first came to Southern Africa in the late 1400s (Hannoun *et al.*, 2005). Subgenotype A2 has been shown to preferentially be transmitted sexually especially in men-having-sex-with-men (De Maddalena *et al.*, 2007) The greater intragroup genetic diversity of African subgenotype A2 strains (1,97%) compared to A2 strains from the rest of the world (1,17%) (Andernach *et al.*, 2009a) supports this hypothesis.

The finding of subgenotype A2 in the HBV isolate from the liver of baboon 9732 may seem paradoxical as subgenotype A1 is the predominant strain presently circulating in South Africa. One possible explanation for this is that subgenotype A1 has over time become more predominant than subgenotype A2. An analogous trend may have occurred in the Mediterranean region where genotype D now predominates over genotype A (Norder *et al.*, 1993). Similarly a change in the prevalent HBV genotype in central and western Africa has been postulated to have occurred over the past 200 years, with the genotype E expanding in the human population (Andernach *et al.*, 2009a; Forbi *et al.*, 2010). Genotype E, which was originally restricted to the west coast of Africa, has now spread over a large crescent stretching from The Gambia, through Nigeria and the Democratic Republic of Congo into Namibia and Mozambique (Andernach *et al.*, 2009a). This recent spread is supported by the low sequence divergence (1,75%) of this genotype (Mulders *et al.*, 2004; Kramvis *et al.*, 2005b; Forbi *et al.*, 2010) and by the very sporadic detection of genotype E outside of Africa (Singh *et al.*, 2009; Alvarado Mora *et al.*, 2010), suggesting that this genotype was rare in Africa at the time of the forced migration of African slaves to the New World between the 16th and 19th centuries (Andernach *et al.*, 2009a; Forbi *et al.*, 2010). Instead, the subgenotypes found in descendants of Africans in South America are subgenotype A1 in Brazil (Araujo *et al.*, 2004) and subgenotype A2 in Venezuela (Quintero *et al.*, 2002).The higher nucleotide divergence of African subgenotype A2 strains

relative to non-African strains intimates that subgenotype A2 may be of African origin (Hannoun *et al.*, 2005). However, since only three complete subgenotype A2 HBV genomes from Africa have been published with a few partial genomes from Kenya (Mwangi *et al.*, 2008), no firm conclusions can be drawn.

4.8 Cross species transmission of HBV between humans and baboons

HBV is highly contagious virus and is approximately ten times more contagious than HCV and a hundred more times contagious than HIV (WHO, 2008). HBV is transmitted through the exchange of blood and bodily fluids including via bites and saliva (Scott *et al.*, 1980; Stornello, 1991). Documented close links between humans and baboons mean that cross species transmission of the virus is extremely plausible.

In Africa, primates are often slaughtered for bushmeat, so this could be another source of exposure to the virus (Robertson, 2001). Simian foamy virus, for example, regularly infects hunters of bushmeat as do avian influenza and Hendra viruses (Wolfe *et al.*, 2005). These viruses show little to no evidence of human-to-human transmission but it is hypothesised that through repeated transmissions, they will gradually adapt and ultimately evolve into viral strains capable of being transmitted by humans. This is what is believed to have happened with SIV and HIV. SIV naturally infects the red-capped mangabey (*Cercocebus torquatus*) and the greater spot-nosed monkey (*Cercopithecus nictitans*) (Lovgren, 2003). Chimpanzees (*Pan troglodytes*) acquired SIV by eating these monkeys giving rise to a hybrid form of the virus. This hybrid virus was transmitted to humans as HIV-1, probably through the hunting and butchering of chimpanzees for bushmeat (Lovgren, 2003).

Zoonotic transmissions of viruses are common. Avian influenza A virus (subtype H5N1) can be spread to domestic cats, tigers, leopards and of course humans (Keawcharoen *et al.*, 2004; Kuiken *et al.*, 2006). Human influenza A viruses can be transmitted to ferrets, guinea pigs and chimpanzees (Ferber, 2000; Lowen *et al.*, 2006) and the influenza B virus, supposedly restricted to humans, has been

found in harbour seals (Osterhaus *et al.*, 2000). The herpes simplex virus can be passed from humans to various species of ape including gorillas, chimpanzees and apes (Eberle and Hilliard, 1989) and an outbreak of the human measles virus in gorillas in Rwanda killed six of these primates (Ferber, 2000). Other human viruses have simian origins, for example the human T-cell leukaemia virus (HTLV) is the counterpart to the simian T-cell leukaemia virus (STLV) (Courgnaud *et al.*, 2004). Among the hepatitis viruses, hepatitis E has zoonotic tendencies and is found in humans, domestic pigs, rodents, deer and macaques (Hirano *et al.*, 2003; Christensen *et al.*, 2008). Baboons are susceptible to STLV (Takemura *et al.*, 2002) and are one of only a few non-human primates that when infected with HIV, develop an AIDS-like disease (Locher *et al.*, 2003). This latter fact, together with the fact that the baboon immune system, like its human counterpart, has all four IgG subclasses makes the baboon an ideal animal model system for human infections and vaccine development (Locher *et al.*, 2003).

In Africa, the majority of HBV carriers are HBeAg negative, and in these individuals, the spread of HBV is mainly horizontal (Kramvis and Kew, 2007). In South Africa, HBsAg positivity peaks between five and six years of age (Kew, 1996; Vardas *et al.*, 1999). In baboons, their natural habits mean that horizontal baboon to baboon transmission of HBV is highly likely. Baboons are very sociable animals living in troops of between 20 and 150 animals (Barrett, 2000). Bonds are strengthened by daily grooming with several related and unrelated partners including offspring (Cheney and Seyfarth, 2007). Infant baboons hold immense attraction for both adult and juvenile females who gather round to touch, hug and examine the infant. Young baboons, like their human counterparts, spend much of the day playing together. The games can become quite physical often leading to mock fights. This behaviour is generally tolerated by the other members of the troop although the occasional old male has been observed to heavily punish youngsters who disturb him (Marais, 1971). There is a strict hierarchy within troops of baboons, which is actively enforced. Although physical fights amongst both males and females are rare, they are not unknown. Alpha males especially tend to be very aggressive and displays of dominance and chases are a daily occurrence. An alpha male seldom lasts in that position for

more than a year and the new alpha male will often kill infants fathered by the male they have just usurped (Cheney and Seyfarth, 2007).

Subgenotype A2, which was found in the baboon has the unusual characteristic of being confined to the peripheral blood leukocytes (PBLs) of Indians, a population where subgenotype A1 and genotype D predominate (Datta *et al.*, 2009). The active replication of the virus in the PBLs may be the result of differential immune pressures, which allow different viral strains to evolve independently (Datta *et al.*, 2009). Although the results of the study by Datta *et al.* were challenged as being the result of contamination (Thio *et al.*, 2010) , the authors defended their work by explaining the necessary precautions and controls taken to prevent contamination. They also pointed out that HBV has a much slower mutation rate than RNA based viruses such as HIV, and so the publication of HBV sequences from unrelated isolates that are identical or differ in only one or two nucleotides is quite common. This unique compartmentalization of subgenotype A2 in the PBLs may explain the spread and transmission of HBV in baboons but further studies are necessary.

From the results of the present study, it is impossible to determine whether the HBV found in baboons initially came from humans, or if humans were infected by baboons. However, as noted by Michael Lai, a virus expert at the University of Southern California, Los Angeles, “When we expose ourselves to exotic animals, there is always a risk of being exposed to something unknown…When we perturb the existing peace between human beings and nature, we are opening a Pandora box, which may contain surprises.” (Lovgren, 2003).

5. Conclusion

The detection of HBV DNA in the sera of two Chacma baboons prior to inoculation with human HBV intimated that baboons are chronically infected with HBV. The objective of the present study was to determine the prevalence of HBV in wild caught Chacma baboons and to molecularly characterise the virus isolated from these animals.

Using nested PCR of four separate genomic regions, and taking a sample to be positive only if it amplified for three or more of these regions, HBV DNA was detected in the serum of 11/49 adult and 4/20 juvenile baboons resulting in an overall prevalence of 21,7%. This prevalence is similar to the HBV prevalence in humans and other non-human primates in highly endemic areas, including sub-Saharan Africa. Serologically, the baboons were negative for all markers of HBV infection, although HBcAg was detected in liver tissue by immunohistochemical staining. The detection of low levels of HBV DNA in the baboon liver tissue, as well as the serum, in the absence of HBsAg classifies this infection as occult (Raimondo *et al.* 2008a). The lack of any serological markers of HBV infection further distinguishes this as a seronegative OBI. OBIs are a common consequence of the resolution of acute hepatitis infections but may also occur after asymptomatic exposures to HBV (Michalak *et al.* 2007).

The complete HBV genome isolated from the liver of an adult male Chacma baboon, baboons 9732, was amplified by the nested PCR of eight overlapping subgenomic fragments. A 160 bp portion of the precore/core region of the viral genome was also successfully amplified and sequenced using DNA isolated from the serum of baboon 9732 as well as five other baboons. Phylogenetic and GroupScanning analysis of the baboon HBV isolate showed it to belong to genotype A, clustering with subgenotype A2, an unexpected result considering that subgenotype A1 predominates in South Africa. The baboon HBV had mutations in both the basic core promoter and precore regions not found in subgenotype A2. These mutations included the G1809T / C1812T double mutation in the Kozak sequence preceding the precore protein start codon

characteristic of subgenotypes A1 and A3, and G1888A in the precore region unique to subgenotype A1. The G1809T / C1812T double mutation and the G1888A mutation affect the translation of the precore and core proteins, respectively, by a leaky scanning mechanism, resulting in reduced expression of both proteins (Ahn *et al.* 2003; Kimbi 2005). Four additional mutations in the polymerase, surface, X and core regions of the baboon HBV strain differentiated the baboon isolate from the majority of previously sequenced subgenotype A2 isolates.

Low levels of cccDNA were detected in the liver tissue of baboon 9732 using a selective real-time PCR and SYBR®-green detection. The low levels were clearly demonstrated by length of time taken for the baboon HBV isolate to reach log phase (~35 cycles) when compared to isolates from both the tumorous and non-tumorous controls (~30 cycles). RNA extracted from the liver tissue of baboon 9732 was reverse transcribed and regions of the HBV precore/core and surface ORFs amplified by nested PCR. This detection of cccDNA and viral RNA in the liver tissue of baboon 9732 shows that the HBV is replicating at low levels in the baboon liver, , a characteristic of occult infections

The HBV found in baboons was successfully transmitted to four experimentally naïve baboons by injecting them individually with serum from four HBV DNA positive baboons. The inoculated baboons were transiently seropositive for both HBsAg and HBeAg and had intermittent increases in ALT levels. HBV DNA was detected at various time points post inoculation throughout the 26 week period and amplification of a portion of the HBV genome isolated from the liver tissue of one of the baboons post inoculation showed it to be identical in sequence to the inoculum. This demonstration of transmissibility of the baboon HBV is important as it shows that the virus is not only present in baboons but, taken together with the evidence of viral replication, establishes infectivity of the virus. The HBV found in baboons can therefore be classified as an occult HBV **infection** (Hollinger and Sood, 2010).

The well documented interactions between humans and baboons make cross species transmission of this virus a possible event. The identification of

subgenotype A2 in the baboon when subgenotypes A1 and A3 predominate in Africa, may indicate that subgenotype A2 is an older strain that previously circulated on the African continent. There is a paucity of sequencing data for subgenotype A2 from Africa, with only four complete subgenotype A2 genomes from South Africa deposited in GenBank. Shorter subgenomic sequences of subgenotype A2 from South Africa (Bowyer and Sim 2000), Tunisia (Meldal *et al.* 2009) and Kenya (Mwangi *et al.* 2008) have also been deposited. It is possible that more extensive molecular epidemiological studies in Africa may uncover a higher circulation of subgenotype A2 in more remote regions. Furthemore, in a recently published study it was shown that, in India, subgenotype A2 preferentially infects the peripheral blood lymphocytes as opposed to the liver (Datta *et al.* 2009). Subgeneotype A1 is the major strain of genotype A circulating in India. Therefore the possibility exists that this alternative compartmentalization of the subgenotype A2 infection has resulted in the lack of detection of this subgenotype in Africa. This possibility, which was not explored in the present study because of ethical and time constraints, should be investigated by extracting DNA from the PBL compartment of both baboons and Africans.

The detection of HBV in both adult and juvenile baboon sera, albeit at low levels is an indication of the lifelong persistence of this virus in these animals. HBV DNA was detected in the absence of serological markers, an indication of secondary occult infection (Michalak *et al.* 2007). The detection of cccDNA and HBV RNA in the liver tissue of baboons provided evidence of replication of the virus and the infection was transmitted to experimentally naive baboons. Although secondary occult infection has been demonstrated in the woodchuck animal model, to our knowledge, this is the first study to demonstrate, a naturally occurring occult HBV infection in a non-human primates.

Appendix I

Animal ethics clearance certificate from the University of the Witwatersrand:

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

97	88	1

APPLICANT: Professor M C Kew

DEPARTMENT: Medicine

PROJECT TITLE: Are baboons naturally infected with a hepadnavirus?

Species	Number	Expiry Date
10 ml baboon blood	10	5 December 1999

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available.

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

nil.

SIGNED _____ DATE: 5 December 1997

(Chairman: Animal Ethics Screening Committee)

Animal ethics clearance certificate from the National Institute of Health, Bethesda, USA:

ORIGINAL

NATIONAL INSTITUTES OF HEALTH

ANIMAL STUDY PROPOSAL

Leave Blank

Proposal # **LID 65**
Approval Date **5/99**
Expiration Date **5/02**

Annual Review Due:
1st Year **5/00**
2nd Year **5/01**

Please Type

A. ADMINISTRATIVE DATA:

Institute, Center, or Division	**NIAID**
Principal Investigator	**Robert H. Purcell, M.D.**
Building/Room	**7/202**
Telephone	**301-496-6227**
Fax/Email	**301-402-0524 /** rpurcell@niaid.nih.gov
Division, Laboratory, or Branch	**Hepatitis Viruses Section, LID**
Project Title	**Studies of human hepatitis viruses and other viruses related to studies of pathogenesis and vaccine development in Old World primates.**

Initial Submission [ ] Renewal [**X**] or Modification [ ] of Proposal Number **LID 65** .

List the names of all individuals authorized to obtain animals under this proposal and identify personnel (*) anticipated to have substantial animal contact:

NIAID: ~~**Randy Elkins**~~ **(removed 5/02), DVM*** **Sue Emerson* (added 12/01)**
Bioqual*: Richard Bradbury, DVM, MS **Floyd Cesler** **Marisa St. Claire, DVM, MS**
Tammy Tobery **Boris Skopetts, DVM, Ph.D.** **Betty Libby** **Max Shapiro**

B. ANIMAL REQUIREMENTS:

Species	**Old World monkeys, including *Macaca, Cercopithicus, Cercocebus, Papio spp.***
Stock/Strain	**Macaque, African Green, Mangabey, Baboon**
Age/Weight/Size	**Juveniles and Adults**
Sex	**Male/Female**
Source(s)	**Morgan Island, Three Springs Scientific, Mannheimer Foundation, and other NIH approved sources**
Animal Holding Location(s)	**Bioqual , Inc., 2501 Research Blvd., Rockville, MD 20850**
Animal Procedure Location(s)	**Bioqual , Inc., 2501 Research Blvd., Rockville, MD 20850**

Number of Animals To Be Used

Species	Year 1	Year 2	Year 3	Total
Old World species	**0/80**	**80**	**80**	**240**

1

NIH-0000-0 LID 65 1999

PROPRIETARY
DO NOT DUPLICATE
DO NOT DISTRIBUTE

December 1, 1998 98-B-428

Protocol 98-B-428: Attempt to transmit hepatitis B virus to baboons.

Purpose: Despite numerous attempts to transmit HBV to a variety of primate species, the chimpanzee remains the only useful animal model for this purpose. The list of primate species for which attempts to transmit HBV were unsuccessful includes baboons. However, recently Dr. Michael Kew discovered evidence of natural infection of baboons with a hepatitis B-like agent. He has partially characterized this agent but, because baboons in South Africa may have been infected and because the level of replication is low and difficult to document in some cases, he has requested that we collaborate with him on attempts to transmit the HBV he has recovered from South African baboons to domestically raised baboons. We have obtained four baboons from a domestic breeder. These all appear to be negative for markers of HBV replication. We will attempt to transmit a hepatitis B-like agent from the serum of South African baboons to the domestic baboons. They will be monitored with tests for HBV replication and samples will be sent to Dr. Kew for comparison with results of South African experiments.

Procedure:

Baboons	Inoculum	Dose/Route
1	Bab. 9712	0.5ml/IV
2	Bab. 9732	0.5ml/IV
3	Bab. 9733	0.5ml/IV
4	Bab. 9734	0.5ml/IV

Pre- and Post-Inoculation: See attached sheet.

Duration: Six months or until terminated by Dr. Purcell

Assurances:

1. There is at present no animal model except the chimpanzee for the study of infection with hepatitis B virus. Specifically, cell culture systems, computer models, etc., are inappropriate and incapable of yielding the necessary information to be derived from this protocol. Recently, Dr. Michael Kew, of South Africa, has obtained evidence for the natural infection of baboons with a hepatitis B-like agent. Preliminary sequence analysis suggests that it is very similar, if not identical, to certain human strains of HBV. Since the purpose of this protocol is to attempt to confirm Dr. Kew's findings and to characterize the baboon as a potential animal model for HBV studies, the species of animal assigned to this protocol has been considered and deemed to be the most appropriate for these studies. The number of animals on this protocol (4) has been considered and deemed to be appropriate in terms of interpretability of the anticipated data and conservation of animals. Additional animals will be added at a later date if the data warrants such an addition. The procedures used in this protocol have been approved in previous blanket protocols and are described in the Standard Operating Procedures of the facility housing animals.

2. A search of the literature (MEDLINE) and consultation with other individuals having expertise in the field have failed to yield alternatives to the procedures described herein or evidence that the research is unnecessarily duplicative of previously reported research.

3. Justification for single housing: Animals are housed socially in pairs when possible. Exceptions to pair housing may include the following and appropriate reasons in this experiment are checked: (1) Incompatibility (aggressiveness, size differential, etc.) [ ]; (2) The animals are receiving different infectious inocula [X]; (3) Inadvertent transmission to a cage mate would make the results uninterpretable [ ]; (4) Titration of virus stocks (pair housing would require twice as many animals as single housing) [ ]; (5) Collection of excreta (feces or urine from individual animals must be collected separately) [ ]; (6) Only one animal is being inoculated with an infectious agent: pair housing would expose a cage mate to inadvertent infection [ ]. Animals may be pair housed before and after the experiment.

Distribution: Drs. Purcell, Emerson, Gerin, Elkins (2 copies, one for Sec., NIAID ACUC) Bradbury, St. Claire, Skopets, Sibal (chimps only); Mr. Shapiro, Engle; Mrs. Wong
Others: Dr. Kew

Draft: _____
Final: _____

Revisions: _____
Distributed _____

Appendix II

0,7% or 1% or 3% Agarose gel

0,7 g (0,7%) or 1 g (1%) or 3 g (3%) Agarose

100 ml 1 x TBE buffer

Dissolve agarose in 1 x TBE buffer by heating in a microwave oven for ~4 minutes, swirling occasionally, until all the agarose particles are completely dissolved. Care must be taken that especially the high percentage gels do not boil over.

Cool to ~60 °C.

Add 10µl mg / ml Ethidium Bromide and swirl to mix.

Pour into a prepared casting tray and allow to solidify completely at room temperature.

0,8% Agarose gel with Crystal Violet

0,8g Agarose

100 ml 1 x TAE buffer

Dissolve agarose in 1 x TAE buffer by heating in a microwave oven for ~4 minutes, swirling occasionally, until all the agarose particles are completely dissolved.

Cool to ~60 °C.

Add 30µl 20 mg/ml Crystal Violet solution and swirl to mix.

Pour into a prepared casting tray and allow to solidify completely at room temperature.

24:1 Chloroform: isoamyl alcohol

To a sterile bottle, add:

48 ml Chloroform

2 ml Isoamyl alcohol

Mix thoroughly before using and store at room temperature.

0,5 M Ethylene diamine tetra-acetic acid (EDTA) (pH 8.0)

18,61 g Ethylene diamine tetra-acetic acid

80 ml Distilled water

Stir vigorously on a magnetic stirrer.

Adjust pH to 8.0 with 10 M Sodium hydroxide.

Adjust volume to 100ml with distilled water.

Sterilize by autoclaving and store at room temperature.

Luria-Bertani (LB) medium

10 g Tryptone

5 g Yeast

10 g Sodium chloride

Adjust volume to 1000ml with distilled water.

Sterilize by autoclaving and store at 4 °C.

LB agar plates

10 g Tryptone

5 g Yeast

10 g Sodium chloride

15 g Bacteriological agar

Adjust volume to 1000ml with distilled water.

Sterilize by autoclaving.

Cool to approximately 50 °C before adding 1 ml of either 50 mg/ml ampicillin or 50 mg/ml kanamycin as required.

After pouring, allow plates to solidify completely at room temperature before storing at 4 °C.

5 x MOPS buffer

41,86 g 3-[N-morpholino]-2-hydroxypropanesulphonic acid (MOPS)

4,12 g Sodium acetate

10 ml 0,5 M EDTA (pH 8.0)

800 ml Distilled water

Adjust pH to 7.0 with 10 M Sodium hydroxide.

Adjust volume to 1000 ml with distilled water.

Add 1 ml DEPC and stir at room temperature overnight.

Sterilise by autoclaving and store at room temperature.

Phosphate Buffered Saline (PBS) (pH 7.4)

8 g Sodium chloride

0,2 g Potassium chloride

1,44 g Na_2HPO_4 (Di-sodium hydrogen orthophosphate)

0,24 g KH_2PO_4 (Potassium di-hydrogen orthophosphate)

800 ml Distilled water

Adjust pH to 7.4 with hydrochloric acid.

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

Phenol chloroform extraction lysing buffer

7 M Urea

0,3 M Sodium chloride

10 mM EDTA

10 mM Tris base

Sterilise by autoclaving and store at room temperature.

RNA extraction Solutions

49:1 Chloroform: isoamyl alcohol

To a RNase free, sterile bottle, add:

49 ml Chloroform

1 ml Isoamyl alcohol

Mix thoroughly before using and store at room temperature**.**

Diethyl pyrocarbonate (DEPC) treated water

Prepare a 0,1% solution of DEPC in distilled water.

Stir overnight at room temperature.

Sterilize by autoclaving and store at room temperature.

75% Ethanol

To a RNase free, sterile bottle, add:

75 ml Absolute ethanol

25 ml DEPC treated water

Store at -20 °C.

2M Sodium acetate

16,42 g Sodium acetate

35 ml Glacial acetic acid

Adjust volume to 100 ml with distilled water.

Add 100 µl DEPC and stir overnight at room temperature.

Sterilize by autoclaving and store at room temperature.

0,75 M Sodium citrate

22 g Sodium citrate

Adjust volume to 100 ml with distilled water.

Sterilize by autoclaving and store at room temperature.

Solution D

47,28 g Guanidinium thiocyanate

3,33 ml 0,75 M sodium citrate (pH 7.0)

5 ml sarcosyl

700 µl β-mercaptoethanol

Adjust volume to100 ml with distilled water.

Add 100 µl DEPC and stir overnight at room temperature.

Sterilize by autoclaving and store at room temperature.

Water saturated phenol

10 ml UltraPure™ Phenol: Water (3,75 : 1 v/v) (Invitrogen)

1,3 ml RNase free water (Invitrogen)

Mix thoroughly.

RNA gel loading buffer

5 ml Glycerol

20 µl 0,5 M EDTA (pH 8.0)

0,04 g Bromophenol Blue

0,04 g Xylene Cyanol

Adjust volume to 10 ml with DEPC treated water.

Store in 1 ml aliquots at room temperature.

10 mg/ml Salmon sperm DNA

100 mg Salmon sperm DNA

Adjust volume to 10 ml with TE buffer (pH 8.0).

Dissolve overnight.

Sonicate 10 times (2 minutes on, 30 seconds off) and denature by boiling for 10 minutes.

Store frozen at -20 °C in 1 ml aliquots.

5% Sephadex

5 g Sephadex G50

100 ml TE buffer (pH 8.0)

Allow to stand overnight to equilibrate.

Sephadex spin columns

Plug the bottom of a 1 ml syringe with fish wool and place the syringe in a 10 ml collection tube.

Pipette 5% Sephadex into the syringe until full and centrifuge at 35 000 x g for 5 min, discard the filtrate and return the syringe to the 10 ml collection tube.

Repeat until the swollen sephadex is compacted to ~0,9 ml in the syringe.

Equilibrate the column by adding 100 µl TE buffer to the column and centrifuge for 5 min. Discard the filtrate, return the syringe to the 10 ml collection tube and repeat.

Place the syringe in a clean 10 ml collection tube, add 100 µl TE buffer to the column and centrifuge for 5 min.

Check that the volume of filtrate in the collection tube is 100 µl. If not, repeat the final step until 100 µl is eluted.

Slot Blot Denaturing Solution

5 mM	Tris-HCl (pH 8,0)
2,5 mM	EDTA
0,125% (w/v)	SDS
250 µg / ml	Proteinase K

Filter sterilise and use immediately.

10% Sodium dodecyl sulphate (SDS)

10 g	SDS

Adjust volume to 100 ml with distilled water.

Filter sterilise and store at room temperature.

0,4 M Sodium Hydroxide (NaOH)

16 g	Sodium hydroxide

Adjust volume to 100 ml with distilled water.

10 M Sodium Hydroxide

40 g	Sodium hydroxide

Adjust volume to 100 ml with distilled water.

Southern blot Church and Gilbert (C&G) hybridisation buffer (Church and Gilbert, 1984)

12,78 g Na_2HPO_4 Di-sodium hydrogen orthophosphate
4,20 g NaH_2PO_4 Sodium di-hydrogen orthophosphate
17,5 g SDS
5 ml 0,5 M EDTA (pH 8.0)
200 ml Distilled water
Adjust pH to 7.2
Adjust volume to 250 ml with distilled water.
Filter sterilise and store at room temperature.

Southern blot denaturation solution

87,66 g Sodium Chloride
20 g Sodium hydroxide
Adjust volume to 1000 ml with distilled water.
Sterilise by autoclaving and store at room temperature.

Southern blot depurination solution

22 ml Hydrochloric acid
978 ml Distilled water
Store at room temperature.

Southern blot neutralisation solution

175,2 g Sodium Chloride
120 g Tris base
800 ml Distilled water
Adjust pH to 7.5 with concentrated HCl.
Adjust volume to 1000 ml with distilled water.
Sterilise by autoclaving and store at room temperature.

Southern blot first wash buffer

50 ml 20 x SSC

5 ml 10% SDS

Adjust volume to 500 ml with distilled water and use immediately.

Southern blot second wash buffer

25 ml 20 x SSC

5 ml 10% SDS

Adjust volume to 500 ml with distilled water and use immediately.

Southern blot third wash buffer

2,5 ml 20 x SSC

Adjust volume to 500 ml with distilled water and use immediately.

2 x Saline sodium citrate (SSC)

100 ml 20 x SSC

800 ml Distilled water

Mix thoroughly.

20 x SSC

88,23 g Tri-sodium citrate

175,32 g Sodium Chloride

800 ml Distilled water

Adjust pH to between 7.0 and 8.0 with concentrated HCl.

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

1 x TAE buffer

100 ml 10 x TAE Buffer

900 ml Distilled water

Mix thoroughly.

10 x TAE buffer

48,4 g Tris base

11,4 ml Glacial acetic acid

3,7 g EDTA

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

1 x TBE buffer

100 ml 10 x TBE Buffer

900 ml Distilled water

Mix thoroughly.

10 x TBE buffer

108 g Tris base

55 g Boric acid

9,3 g EDTA

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

TE Buffer (pH 8)

2 ml 0,5 M EDTA

10 ml 1 M Tris-HCl (pH 8.0)

800 ml Distilled water

Adjust pH to 8.0 with concentrated HCl.

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

1 M Tris-HCl (pH 8.0)

121,1 g Tris base

800 ml Distilled water

Adjust pH to 8.0 with concentrated HCl.

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

Appendix III

GenBank accession numbers and countries of origin for the subgenotype A1 and A2 isolates used to calculate the mean nucleotide divergences shown in table 8.

Subgenotype A1

AB076678 - Malawi
AB076679 - Malawi
AB116082 - Bangladesh
AB116083 - Bangladesh
AB116084 - Bangladesh
AB116085 - Bangladesh
AB116086 - India
AB116087 - India
AB116088 - Nepal
AB116089 - Nepal
AB116091 - Philippines
AB116092 - Philippines
AB116093 - Philippines
AB116094 - Philippines
AB241114 - Philippines
AB241115 - Philippines
AB246335 - India
AB246336 - South Africa
AF297621 - South Africa
AF297625 - South Africa
AY161140 - India
AY233274 - South Africa
AY233275 - South Africa
AY233276 - South Africa
AY233277 - South Africa
AY233278 - South Africa
AY233279 - South Africa
AY233281 - South Africa
AY233282 - South Africa
AY233283 - South Africa
AY233284 - South Africa
AY233285 - South Africa
AY233287 - South Africa
AY233288 - South Africa
AY233289 - South Africa
AY233290 - South Africa
AY373432 - India
AY903452 - South Africa
AY934765 - Somalia
AY934766 - Somalia
AY934767 - Somalia
AY934768 - Somalia
AY934769 - Somalia
AY934770 - Somalia
AY934771 - Somalia
AY934772 - Uganda
AY934773 - Tanzania
AY934774 - Philippines
DQ020002 - Congo
DQ020003 - United Arab Emirates
M57663AP - Philippines

Subgenotype A2

AB014370 - Japan
AB064314 - USA
AB116076 - USA
AB116077 - USA
AB116078 - USA
AB116079 - Japan
AB116080 - Japan
AB116081 - Japan
AB126580 - Russia
AB205118 - Japan
AB222707 - Uzbekistan
AB222708 - Uzbekistan
AB246337 - USA
AB246338 - Japan
AF090838 - Belgium
AF090839 - Belgium
AF090840 - Belgium
AF090841 - Belgium
AF297624 - South Africa
AF536524 - USA
AF537371 - USA
AF537372 - USA
AJ012207 - Germany
AJ309369 - France
AJ309370 - France
AJ309371 - France
AJ344115 - France
AM282986 - New Zealand
AM295797 - France
AY034878 - USA
AY128092 - Canada
AY152726 - USA
AY233280 - South Africa
AY233286 - South Africa
AY738139 - Germany
AY738140 - Germany
AY738141 - Germany
AY738142 - Germany
AY862867 - China
AY862868 - China
AY902775 - USA
DQ788725 - Germany
EF208113 - Germany
L13994 -
S50225A -
V00866A -
X02763 - USA
X51970 - Germany
X70185A - Germany
Z35717 - Poland
Z72478 - Germany

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