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MOLECULAR METHODS TO DETERMINE THE PRESENCE OF HEPATITIS B VIRUS (HBV) GENOTYPES IN THE SERUM OF A HBV-INFECTED CHACMA BABOON.

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A research report submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Chemical Pathology.

Johannesburg, 2004

DECLARATION

I, **Tholsi Jocelyn Naicker**, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Chemical Pathology in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



17th day of March, 2004

To all the wonderful people I met in the Research Unit of Molecular Hepatology, University of Witwatersrand, who so graciously shared their knowledge with me.

ABSTRACT

In the course of a previous study (Kedda, et al., 2000), it became evident that the baboons had been inoculated with a mixture of HBV genotypes, namely, genotypes A and non-A. Therefore, a follow up study was undertaken to determine whether either A or non-A HBV genotypes predominated over the other during the time course of 52 weeks post-inoculation of HBV into baboons.

HBV DNA extracted from sera obtained from one of the four HBV positive baboons, at two time points during a 52 week post-HBV inoculation period, was amplified using primers specific for the HBV core region that is well conserved in all genotypes.

A specific 6 bp insertion present only in HBV genotype A and not in any of the non-A genotypes (B-H), made it possible to differentiate genotype A from non-A genotypes.

The amplicons were cloned into plasmid vectors to separate the genotypes and manually sequenced in both directions.

The manual molecular methods employed, determined that HBV genotypes A and non-A were present in all samples and that their relative proportions in the samples had altered over the total study time period.

At the initial time point, the proportions of both genotypes were approximately equal; then changed after four months to 75% of the sample comprising genotype non-A and after ten months the situation had reversed, this time 79% of the sample consisted of genotype A.

No definite conclusions concerning the dominance of one genotype over the other, the natural progression, or replication rates of the genotypes could be made because the changing proportions of genotypes may have been caused by sampling fluctuations at the time of HBV DNA extraction and cloning in the setting of low viral loads in the baboon sera.

ACKNOWLEGEMENTS

I wish to thank my mentor, Prof M.C. Kew of the Department of Medicine, Molecular Hepatology Research Unit, University of Witwatersrand, for giving me the opportunity to be part of his talented team, as well as my supervisors, Marina Baptista and Dr Anna Kramvis for providing me with the necessary samples, materials, training and support in molecular biology and Dr J.S. Galpin for statistical analysis of the results. The H.E. Griffin Cancer Trust funded this project.

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NOMENCLATURE

bp	base pairs
BQW	best quality water
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dH_2O	distilled water
dNTPs	deoxynucleotide triphosphate
dTTP	deoxythymine triphosphate
ddATP	di-deoxyadenine triphosphate
ddCTP	di-deoxycytosine triphosphate
ddNTPs	di-deoxynucleotide triphosphates
ddGTP	di-deoxyguanine triphosphate
ddTTP	di-deoxythymine triphosphate
EtBr	Ethidium Bromide
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B surface Antigen
kb	kilobases
nm	nanometer
nt	nucleotide
ORF	Open Reading Frame
PBS	phosphate buffered saline

rpm	revolutions per minute
v/v	volume for volume
cDNA	copy DNA

CHAPTER 1 INTRODUCTION

1.1 HEPATITIS B VIRUS (HBV)

1.1.1 HBV STRUCTURE

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family of DNA viruses that mainly infects primates and birds. Three particulate forms of HBV can be demonstrated by electron microscopy (Figure1.1, Fields, *et al.*, 1985).

The two most numerous forms are 22 nanometer (nm) spheres and tubules representing subviral particles that consist of excess viral envelope protein and host-derived lipid. These forms contain no viral DNA and are therefore non-infectious (Ganem, *et al.*, 1987). Despite this they are highly immunogenic and elicit a strong antibody response. The third, less numerous form, is the complete infectious virion (Figure 1.1). It is speculated that the subviral particles act as decoys to absorb neutralising host antibodies, thus facilitating proliferation of the infectious virions (Gocke, 1975).

The outer surface protein found in all 3 particulate forms is the hepatitis B surface antigen (HBsAg). The Dane particle consists of this outer envelope and an icosahedral viral nucleocapsid or core that is composed of a single polypeptide called the hepatitis B core antigen (HBcAg). This core contains the viral genome and consists of a partially double stranded 3.2 kilobase (kb) circular DNA molecule and a DNA polymerase (POL) that is involved in viral replication. (Ganem, *et al.*, 1987). HBeAg (hepatitis B e antigen), a soluble antigen found in

the serum, is not part of the nucleocapsid and is immunologically distinct from the HBcAg (Ganem, *et al*, 1987). HBeAg is usually considered to be a marker of increased infectivity.

1.1.2 HBV GENOME

The genome of HBV consists of between 3182 –3221 nucleotides (nts) depending on the genotype. It is circular and has a full-length (complete) strand, conventionally called the minus strand (Figure 1.2, Tiollais, *et al*., 1985) and a shorter (incomplete) strand called the plus strand. Whilst the 5' end of the incomplete strand is fixed, the 3' end is heterogeneous and varies in length (Gerber, *et al*., 1985).

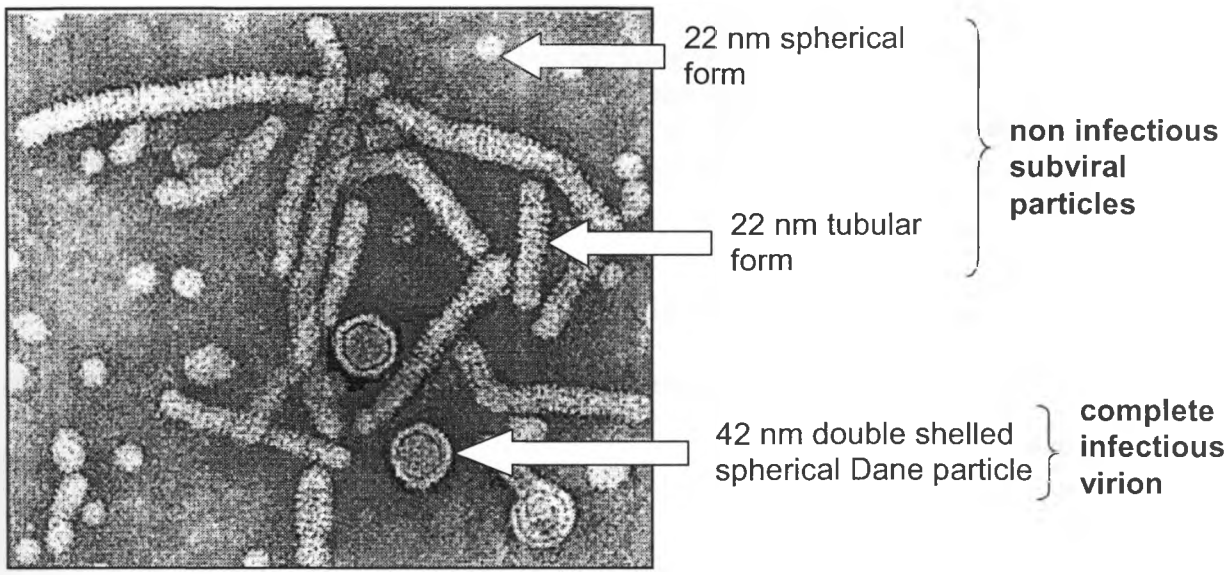

Figure1.1 Electron micrograph demonstrating the 3 particulate components of HBV (Fields, *et al*., 1985).

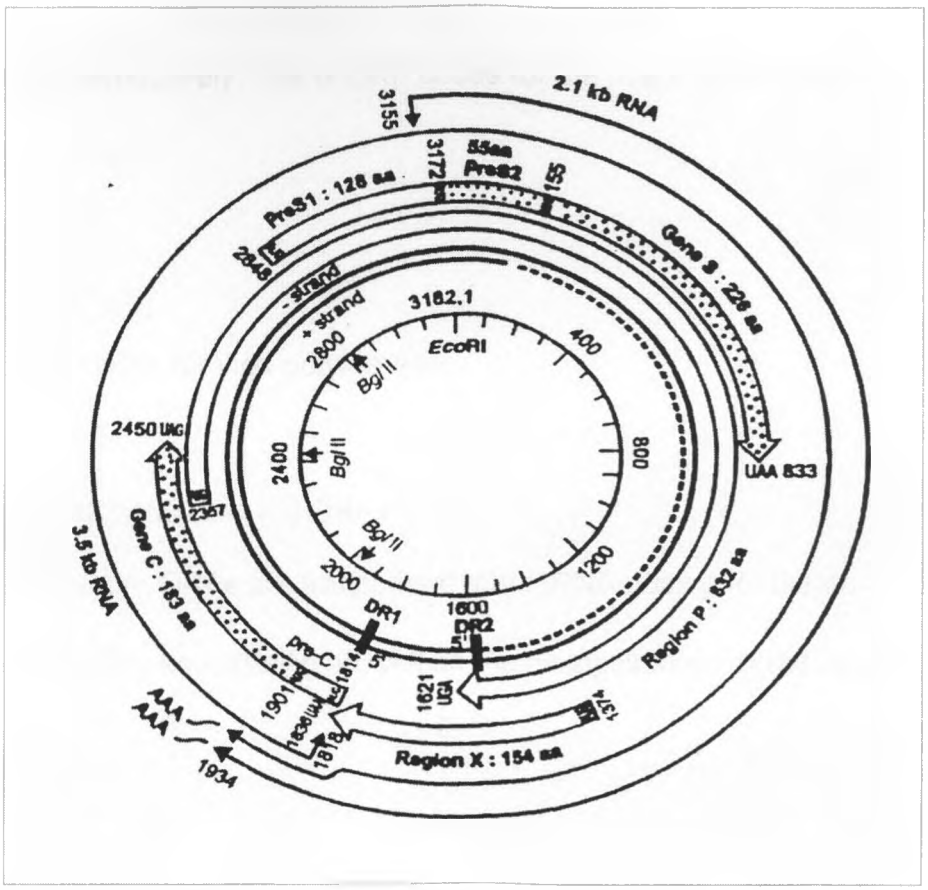

Figure 1.2 A map of the structure and genetic organisation of the HBV genome. The central circles represent the HBV genomic DNA, the complete circle being the minus (-) strand and the incomplete circle with the dotted line, the plus (+) strand. The broad surrounding arrows indicate the 4 HBV open reading frames, namely preC/C, P, preS/S and X. The outer arrows represent the 3.5 kb and 2.1 kb mRNAs respectively, which are the two major RNA templates (From Tiollais, *et al.*, 1985).

The HBV genome has 4 major open reading frames (ORFs), (Figure1.2, Gerber, *et al.*, 1985, Tiollais, *et al.*, 1988) all of which are encoded by the minus strand and are:

1. PreS and S ORFs

   The preS ORF comprises preS1 and preS2. S ORF, in combination with preS1 and preS2, codes for two HBsAg related proteins, the middle and large

S proteins, respectively. The S ORF codes for the major envelope protein, HBsAg.

2. P ORF

   The P ORF codes for viral polymerase.

3. Precore (preC) and core (C) ORFs

   The precore/core ORFs are transcribed into mRNA coding for the core proteins (HBcAg) and HBeAg as well as the encapsidation signal epsilon (ε).

4. X ORF

   X ORF codes for the X protein that can transactivate viral as well as host cellular gene expression during infection (Tiollais, *et al.*, 1985).

1.1.3. HBV REPLICATION

When the Dane particle fuses with the host hepatocyte cell membrane, the outer surface coat is shed and the viral nucleocapsid enters the cytoplasm where it is transported to the nucleus (Ganem, *et al.*, 1987). Despite being a DNA virus it has the replicative strategy typical of a retrovirus. Instead of the DNA replicating from a DNA template, HBV relies on the reverse transcription action of its endogenous POL on a RNA intermediate called pregenomic RNA (pgRNA) which is within subviral core particles in the cytoplasm of infected hepatocytes. The mechanism by which the virion enters hepatocytes has not yet been established.

1.1.3.1 Replicative steps

Step 1 – Production of Covalently Closed Circular DNA (cccDNA)

In the nucleus of infected hepatocytes, the incomplete double strand is repaired by the viral polymerase to form a completely double-stranded DNA called the covalently closed circular DNA (Summers, *et al.*, 1982).

Step 2 –Transcription of cccDNA

Once HBV DNA has been recircularised, subgenomic and genomic transcripts that are required for HBV protein synthesis are generated by the action of host RNA polymerase II (reviewed in Nassal, *et al.*, 1994).

The smaller subgenomic transcripts serve as mRNA for the expression of the HBsAg and X proteins and the larger genomic transcripts express HBcAg, POL and HBeAg proteins. The 3.5 kb pgRNAs are sequested from the cytoplasm together with POL and HBcAg to form subviral particles. The 5' end of pgRNA is folded over into a stem-loop structure called epsilon (ε) (reviewed in Kramvis, 1998), which provides the signal for pgRNA encapsidation.

Step 3 – Reverse transcription of the pregenomic RNA

The ε loop of pgRNA also activates reverse transcription by the polymerase in the hepatocyte nucleus, to generate minus DNA strands (reviewed in Kramvis, *et al.*, 1998).

Step 4 – Synthesis of the plus strands

At the end of minus strand synthesis, the RNAse activity of POL partially degrades the pgRNA template. The remaining 15–18 base pair (bp) oligonucleotides at the 5' end of the pg RNA act as primers for the synthesis of the plus DNA strands and are catalysed by POL. The 3' terminal end of the minus DNA strand is redundant and the plus strand is shorter, thus giving rise to a partially double stranded genome.

Then the subviral particles consisting of nucleocapsids with mature DNA genomes, acquire their outer envelopes. These completed virions are then exported out of the hepatocytes by the host's normal transport systems (Schaller, 1997), without causing cell lysis (Ganem, *et al.*, 1987). Viral nucleocapsids can also be recycled back into the nucleus.

1.2 EPIDEMIOLOGY

To date, established active infections with HBV have been found to only occur in humans and other primates. It is a global public health problem, and endemic in sub-Saharan Africa (Dusheiko, 1998). There are 8 known HBV genotypes named A to H and have different geographic distributions (reviewed in Dusheiko, 1998). In southern Africa, infections with HBV genotypes A and D predominate (Norder, *et al.*, 1994, Kew, *et al.*, 1997, Bowyer, *et al.*, 1997).

Mayerat and colleagues (1999) showed an association between HBV genotype A and the development of a chronic active hepatitis. It was postulated that this could possibly represent a lower antigenicity (than genotype D) and hence a lower capacity to induce immune clearance in patients infected with this genotype. To date no difference between the antigenicity of genotype A and D has been described in either humans or other primates.

HBV is contracted mainly via perinatal transmission from an acutely HBV-infected or chronic carrier mother to her newborn baby, horizontally through sexual contact or other undetermined means, parenterally to recipients of infected blood / blood products, needle-sharing drug addicts or needlestick injuries. Transmission occurs at a higher frequency if the acutely infected or chronic carrier is HBeAg positive (Jia-Horng Kao, *et al.*, 2002).

1.3 PATHOGENESIS

Infection with HBV is primarily a hepatic infection (hepatotropic) producing an acute hepatitis characterised by hepatocellular injury and inflammation. Clinical manifestations of disease include acute viral hepatitis, which is usually self-limiting, fulminant hepatitis resulting in death, subclinical hepatitis, an asymptomatic carrier state, and chronic persistent hepatitis progressing in later stages to cirrhosis and hepatocellular carcinoma (Jia-Horng Kao, *et al.*, 2002)

The acute infection is usually self-limiting and HBV is completely cleared from the blood with resulting lasting immunity from reinfection through the production of antibodies to HBsAg and HBcAg.

HBV does not appear to be cytotoxic to its host, and the hepatocellular injury and inflammation that is characteristically seen in acutely infected individuals has been attributed to the host immune response to infected cells (Sherlock, 1972). This explains the varying range of mild to severe clinical presentations between individuals.

HBV characteristically produces large amounts of viral antigens in the liver that can be detected in the blood with the exception of HBcAg, which is retained in the liver. The most useful of these serum HBV antigens is HBsAg. Although HBV replicates in the liver, it is also found in extrahepatic sites (Ganem, *et al.*, 1987).

In ≈ 5 – 10 % of people the infection does not resolve completely, but exists as a persistent chronic infection, varying in grade from mild to severe, with the possible subsequent development, many years later, of cirrhosis and hepatocellular carcinoma. In asymptomatic carriers HBsAg and / or HBeAg can persist in the blood in the absence of hepatitis and these individuals represent reservoirs of the virus.

1.4 STUDY BACKGROUND

There is a great demand for human donor organs for transplantation into patients with end-stage organ disease. The supply of available donor organs to meet these needs is insufficient and the annual mortality rate of patients on the waiting list is high. It was for this reason that animals were examined as alternative sources of donor organs.

Pigs were considered as sources of xenografts (grafts from animal to man) because of their rapid breeding and because no ethical issues would be raised by their use. A major drawback in the use of porcine organs was the serious problem of hyperacute rejection (HAR) (Doring, *et al.*, 1997). HAR can occur within minutes of transplantation, and is the hallmark pathological syndrome of graft rejection when immunologically distant species such as the pig and man are involved.

Primates like the baboons and chimpanzees are considered to be the most suitable donors because of their anatomical, physiological and immunological closeness to humans. The suggested use, however, of the slow breeding chimpanzees caused a huge ethical outcry because they are a highly intelligent and endangered species.

Baboons, on the other hand, are present in large numbers on the African continent and the judicious use of their organs would not endanger the survival of their species.

The transplanted allografts in recipients who had had HBV-induced end-stage liver disease, could become infected with HBV harboured in extra-hepatic tissues (Perillo, *et al.*, 1993). Long-term passive immunoprophylaxis (Samuel, *et al.*, 1991) can be used to delay graft reinfection. This treatment unfortunately, is expensive and duration of treatment not established (Dusheiko, 1994). The problem of graft reinfection would be solved if baboons were found to be resistant to HBV infection.

In earlier studies (between 1940 to 1970) when a primate model for HBV infection was being sought, investigators inoculated various primate species including *Papio sp* (baboons) with serum obtained from patients with clinical hepatitis (WHO, 1975; Deinhardt, 1976). None of the baboons developed either clinical or biochemical evidence of HBV infection.

At that time the technology for the detection of HBsAg was not available. With subsequent technological advancement, detection of HBV antigens and reactive antibodies became possible. Because of the apparent unsuccessful attempts to induce hepatitis with infectious material, and because of the absence of detectable HBsAg in a survey of 99 baboons (Prince, 1970), researchers concluded that baboons were resistant to infection with HBV and suggested that they would be useful sources of liver xenografts.

Subsequently, conflicting results were forthcoming. Serological surveys (Eichberg, *et al.*, 1980; Peterson, 1979) found that some baboons had anti-HBsAg and there was uncertainty whether baboons were in fact, resistant to HBV infection.

None of these earlier studies had used molecular techniques, as these were only possible after development of the polymerase chain reaction (PCR) technique by Mullis in 1984 (Mullis, 1986) that enabled the detection of minute quantities of viral DNA.

In 1993 two patients with end stage liver disease from chronic HBV infection received baboon xenografts (Starzl, *et al.*, 1993). Less than 3 months later, both recipients demised and samples of their livers were examined. The liver samples were negative for histological evidence of HBV infection as well as immuno-histochemical staining for HBsAg and HBcAg (Starzl, *et al.*, 1993). PCR studies on the liver biopsies obtained a positive result in only one of 5 samples (Lanford, *et al.*, 1995). Lymphocytes from the patient with this positive liver PCR result, were analysed separately and found to have HBV DNA (Lanford, *et al.*, 1995). The source of the positive PCR result was therefore proposed to be residual HBV DNA harboured in lymphocytes rather than from the liver itself.

Subsequently, Michaels, *et al.*, (1996) undertook to further examine the susceptibility of baboons to HBV infection using molecular techniques. When

they inoculated 4 baboons (*Papio Sp*) with HBV-containing serum, all the baboons remained clinically healthy. Biochemical and serological markers, HBV DNA, liver biopsies, and liver staining for HBsAg and HBcAg were all negative for infection after a 6-month period. During weeks 2 to 5, one of the baboon's serum samples yielded a weakly positive PCR result for HBV DNA. Subsequent testing of samples after week 6 on this same baboon was all negative.

As delayed clearance of a HBV inoculum had been reported previously in a study on macaques (Lazizi, *et al.*, 1993), Michaels, *et al.*, (1996) proposed this as a possible explanation for the confounding result. They concluded that despite their small sample size, the results of their study supported the contention that baboons were resistant to HBV infection.

In an attempt to confirm these observations using even more sensitive molecular methods, Kedda *et al* (2000) conducted a study on 10 chacma baboons (*Papio ursinus orientalis*). Pooled serum from 3 HBsAg and HBeAg positive patients with clinically overt acute viral hepatitis was inoculated into 6 baboons whilst the remaining 4 animals were controls.

Blood samples were taken at 4 weekly intervals in a 52 week period, after which time the baboons were euthanased and their livers removed for analysis. Seroconversion was demonstrated by the presence of anti-HBcAg in 4 of the 6 inoculated baboons 16 weeks post inoculation. Transmission electron

microscopic examination of the liver sample of one of these 4 baboons, showed Dane particles and spherical and tubular forms of HBV.

HBV-DNA was detected in low titre by nested–PCR in all the serum samples as well as in liver tissues of these same 4 baboons throughout the 52 week follow up period. The small serum viral quantities suggested low-grade HBV replication in the liver.

The specificity of the HBV-DNA was confirmed with Southern Hybridisation and by nucleotide sequencing. Nucleotide sequences in 4 regions of the HBV genome showed identical sequences between the HBV-DNA in the baboon sera and one of the inoculated HBV isolates.

The seroconversion as well as presence of HBV-DNA for as long as 52 weeks in the serum and liver samples was evidence for this being an infection rather than mere persistence of the inoculum. There was no biochemical evidence of either an acute or chronic hepatitis, although it was possible that a short-lived acute hepatitis could have been missed during the 4 weekly sampling intervals. The results indicated a chronic carrier state.

These findings demonstrated that baboons are in fact, susceptible to HBV infection, and that caution should be exercised when they are used as sources of liver xenografts in recipients who have chronic HBV infection-induced end-stage

liver failure. They further advised that recipients should be treated as if they had received an allograft and that passive immunoprophylaxis be used (Samuel, *et al.*, 1991).

Furthermore, if a difference in antigenicity between HBV genotype A and non-A exists, and patients with end-stage liver disease are infected with HBV genotype A (Mayerat, *et al.*, 1999), this might have relevance in predicting which transplant recipients are likely to develop HBV infection of the xenograft.

1.5 STUDY OBJECTIVES:

In the course of the Kedda, *et al* (2000) study, it became evident that the chacma baboons had been inoculated with a mixed population of HBV genotypes, namely, genotypes A and non-A. The aim of the follow up study was to determine whether either A or non-A HBV genotypes predominated over the other during the time course of the 52 weeks post-inoculation in the 4 susceptible baboons. One baboon and two time points were analysed in the study presented.

CHAPTER 2 MATERIALS AND METHODS

2.1 STUDY SAMPLES

The study was performed on serum samples of one of the baboons that had tested positive for HBV infection in the preceding Kedda *et al* (2000) study described above. This baboon was one of those 10 chacma baboons (*Papio sp*)

described that had been caught in the wild of the North-West Province of South Africa.

Permission to extend the original ethical clearance certificate (AESC no. 97/88/1) granted to the researchers (Kedda, *et al.*, 2000) was approved by the Animal Ethics Committee of the Medical University of Southern Africa (MEDUNSA). The procedures performed on the baboons as well as the care of the baboons had been in accordance with the guidelines of both this committee and those of the South African Medical Research Council.

Baboon serum samples for further analysis were only available from blood collections at two time points namely, 4 months (sample B2 531) and 10 months (sample B2 21/6) post-HBV inoculation.

2.2 DNA EXTRACTION

DNA was extracted using the QIAamp DNA blood mini kit (Qiagen Inc., Hilden, Germany). Included in the extraction process, was a serum sample determined positive for HBsAg, HBeAg and HBV DNA by slot blot hybridization (positive control) which was supplied as a standard by my supervisor Dr Kramvis (unpublished), and negative controls consisting of best quality water (BQW) (to measure contamination during the extraction procedure) and a sample of the baboon's serum before inoculation (time point 0).

2.2.1 METHOD

The extraction process was as follows:

Sample dilution

50 µL aliquots of each sample (including the positive and negative controls) were pipetted into 1.5 ml microfuge tubes and the volume made up to 200 µL with phosphate buffered saline (PBS).

Lysis

1) 25 µL of Qiagen proteinase K stock solution and 200 µL Buffer AL were added to each tube and vortexed immediately for 15 sec. The virions within the samples were thus lysed and the DNA released.
2) The samples were incubated for 10 mins at 70^0 C followed by a further incubation at 95^0 C for 15 mins in order to inactivate any potentially pathogenic agents present.

Purification of viral DNA

1) 210 µL 100 % ethanol was added to each sample and mixed by vortexing.
2) QIAamp spin columns were placed into the 2 ml collection tubes provided in the kit and the mixtures were carefully added to each column. Care was taken not to wet the rim of the columns thus preventing cross-contamination
3) The caps of the spin columns were closed and the collection tubes micro-centrifuged (Eppendorf centrifuge 5417C) at 8000 rpm for 1 min to allow the

DNA present to adsorb onto the QIAamp silica-gel membrane of the spin columns. The specific salt content and pH of the lysate ensured that proteins and other contaminants

within the samples that might inhibit downstream enzymatic reactions were not retained by the gel membranes.

4) The collection tubes containing filtrate were discarded and each spin column was placed into clean collection tubes.
5) The columns were each washed with 500 µL Buffer AW in order to remove any residual contaminants. This wash step did not interfere with the membrane DNA binding.
6) The collection tubes were again centrifuged at 8000 rpm for 1 min and the filtrate containing tubes discarded and replaced with clean collection tubes.
7) Column washes were repeated with 500 µL of Buffer AW and followed with centrifugation at 8000 rpm for 3 mins.
8) Once again the filtrate containing collection tubes were discarded and each spin column was placed into a clean microfuge tube.
9) 50 µL of Buffer AE (elution buffer that had equilibrated to room temperature) was added to each column and incubated for 5 mins at room temperature before centrifugation at 8000 rpm for 1min. This step allowed maximum elution of the viral DNA that had been retained by the membranes.
10) Microfuge tubes containing the purified DNA eluate were stored at -20^0 C.

2.3 POLYMERASE CHAIN REACTION (PCR)

2.3.1 PCR BACKGROUND

PCR is an *in vitro* method of amplification of specific DNA / RNA fragments of defined length and sequence. The principal indication for PCR is the amplification of normally sub-detectable quantities of DNA / RNA to achieve $>10^6$ fold quantities that are easily detectable.

A double-stranded DNA template and two oligonucleotide probes called primers that are complementary to the flanking ends of the target sequence of each DNA strand, are required for this technique. Each primer specifically anneals to opposite ends of each strand of target DNA forming an initiation site for DNA polymerase to catalyse the synthesis of a copy of the parent DNA strand (cDNA).

Extension of the primer 3' end is achieved by the addition of four deoxynucleotide triphosphates (dNTPs), which are, deoxyadenine triphosphate (dATP), deoxythymine triphosphate (dTTP), deoxyguanine triiphosphate (dGTP) and deoxycytosine triphosphate, respectively.

The primers used for amplification were chosen to bind to regions of the genome that were well conserved in all genotypes. A 6 bp insertion, 5'CGGGAC3', (between nucleotides 2354 –2359) that is present in HBV genotype A, is absent in the other genotypes, B to H. Thus, all HBV genotypes would be amplified without bias towards any particular genotype.

The oligonucleotide primers (Table 2.1) that were designed with the aid of the Oligo Analysis and Plotting Tool computer software program (http://oligos.qiagen.com /oligos/toolkit.php?), were within the terminal region of the HBV genome (Figure 1.2).

The PCR process involved several 3-step cycles (Figure 1.3):

2.3.1.1 Denaturation (Figure 2.1 a)

The two complementary strands of the DNA template were separated (denatured) by heating to 94° C.

2.3.1.2 Annealing (Figure 2.1 b)

The sense and antisense primers attached to each denatured strand.

2.3.1.3 Primer extension (Figure 2.1 c)

Addition of nucleotides (dNTPs) to the 3' ends of each primer initiation sequence was catalysed by *Taq polymerase.* At the end of the first cycle, target DNA within the samples, was doubled.

Taq polymerase is a DNA polymerase that was originally isolated from a naturally occurring thermophilic bacterium, *Thermus aquaticus,* which naturally inhabits hot springs. Its heat resistant property allows it to remain stable for extended periods at the high temperature that is required to denature DNA without itself

being denatured. Automation of repeated 3-step cycles in a thermocycler are thus possible without the addition of polymerase after each cycle.

After 40 of these 3-step cycles, the initial target DNA was amplified exponentially until more than a million copies were present.

Table 2.1 PCR primers

PCR I

Sense primer:	1687
Nucleotide position [x]:	1687 – 1706
Sequence:	5' – CGA CCG ACC TTG AGG CAT AC - 3'
Antisense primer:	2498
Nucleotide position [x]:	2477 – 2498
Sequence:	5' – AAG CCC AGT AAA GTT TCC CAC C - 3'
PCR I amplicon size =	**812 bp** (1687-2498)

PCR II

Sense primer:	2267
Nucleotide position [x]:	2267 – 2284
Sequence:	5' – GGA GTG TGG ATT CGC ACT - 3'
Antisense primer:	2436
Nucleotide position [x]:	2419 – 2436
Sequence:	5' – TGA GAT CTT CTG CGA CGC - 3'
PCR II amplicon size =	**170 bp** (2436 – 2267)

PCR I - First round polymerase chain reaction
PCR II - Second round polymerase chain reaction
[x] Denotes the nucleotide position of hepatitis B virus *adv* genome (GenBank accession # V00866) where *EcoRI* cleavage site is position 1.

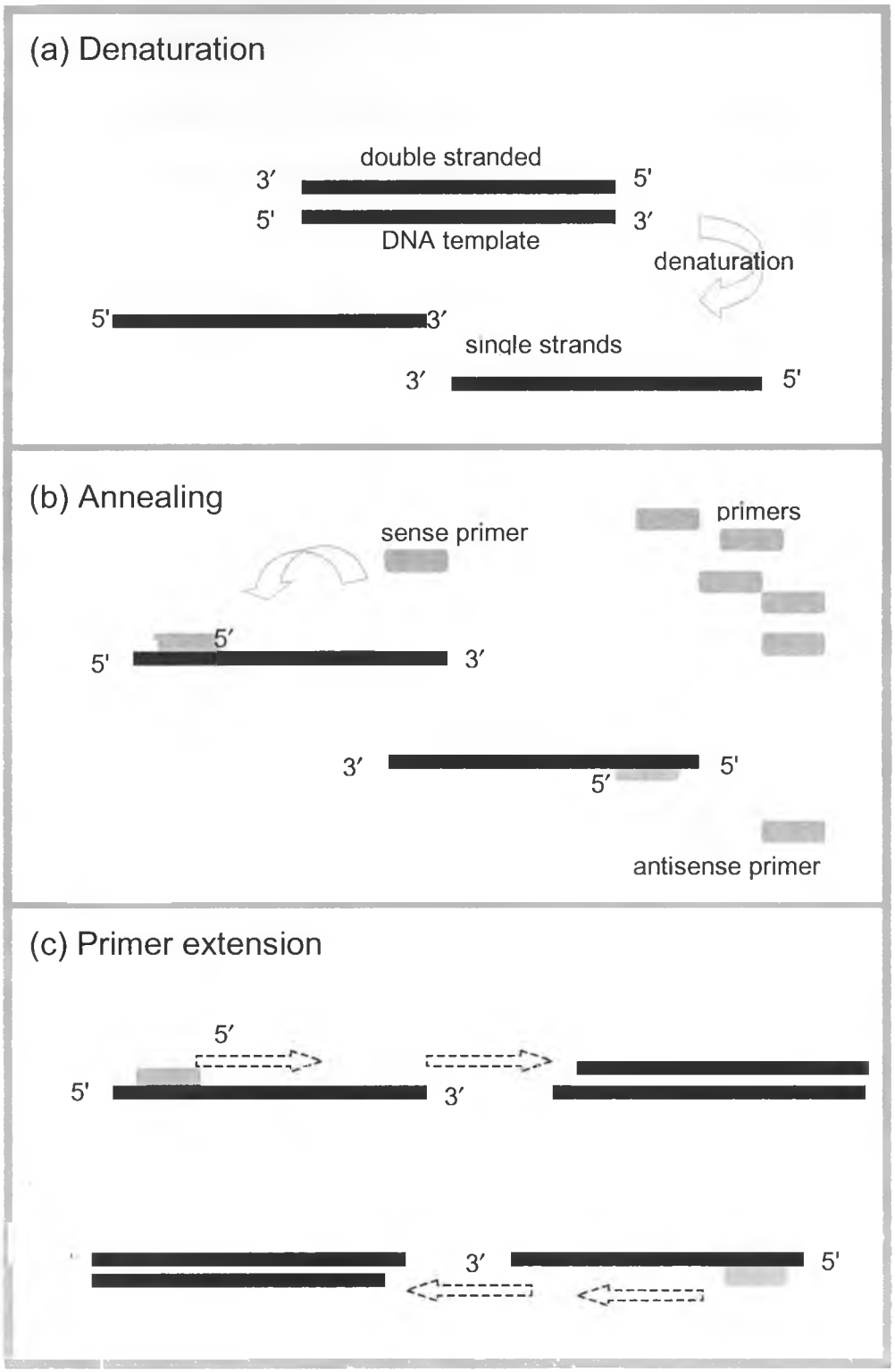

Figure 2.1 The 3 steps of the polymerase chain reaction (PCR)

2.3.1.4 PCR Precautions

The extreme sensitivity of PCR makes the procedure very susceptible to contamination by minute amounts of extraneous nucleic material. Careful laboratory precautions and techniques as suggested by Kwok *et al* (1989) had to be strictly adhered to in order to avoid cross–contamination and prevent false positive results. The following measures were taken:

1) Preparation of samples containing DNA was performed in a specifically demarcated area separate from the PCR laboratory. The sample tubes were given a short spin in order to settle fluid clinging onto the sides of the vessels. This prevented spillage of DNA prior to opening the caps.
2) Gloves were changed between, as well as at the end of each sample handling to prevent sample cross-contamination and transfer of DNA out of the area.
3) The special DNA-free PCR laboratory was dedicated to PCR reagent preparation alone.
4) Disposable gloves were removed and changed on entering in order to eliminate inadvertent transfer of DNA into this lab.
5) The laboratory contained a UV germicidal lamp, which degraded all DNA contaminants.
6) As a further precaution all working surfaces were wiped down first with 1N HCl and then 70 % ethanol before commencing work.

7) The PCR laboratory contained a complete set of pipettes, disposable pipette tips, pre-autoclaved micro centrifuge tubes, disposable gloves; all dedicated to PCR reagent preparation alone.

8) Buffer solutions, deionized water and all other reagents were of PCR standard, and hence DNA free.

9) Lyophilised primer stocks were made up and aliquoted there.

10) PCR reagents were also aliquoted in this DNA-free site. Aliquoting was necessary in order to minimize contamination of the stock reagent. Each aliquot was properly labeled for easy tracing back should contamination inadvertently occur.

11) Disposable gloves were changed frequently during reagent handling.

12) The master premixture of PCR reagents was prepared in this PCR location and directly pipetted into each micro-centrifuge tube before sample DNA was added in the specially demarcated area outside this room. This limited the number of sample transfers, thereby further reducing the chances of contamination.

13) Disposable filter-tips were used to prevent contamination of the pipette barrels.

14) After the addition of DNA, each tube was capped prior to loading the next sample.

2.3.2 PCR I

The buffer pH as well as the concentration of magnesium chloride ($MgCl_2$), dNTPs and Taq polymerase had to be optimised for each amplification. When the ideal parameters had been determined the following method was employed.

A reaction mixture was prepared with:

1 µmol/L of each outer primers described in Table 2.1

200 µmol/L of each of the dNTPs namely, dATP, dGTP, dTTP, dCTP

0.02 U/µl Dynazyme^{TM} *Taq* DNA polymerase (version 2.0 Finnzymes OY, Espoo, Finland)

4 mmol/L $MgCl_2$

10 mmol/L Tris HCl buffer (pH 8.8 at 25° C)

50 mmol/L KCl

0.1% Triton^{R}-X 100

2.5 µl of DNA extracted from each sample (10 % v/v) was added to micro-centrifuge tubes each containing 22.5 µl of the above reaction mixture, the final reaction volume being 25 µl. The positive control was a serum sample determined positive for HBsAg, HBeAg and HBV DNA by slot-blot hybridization; and the negative control was best quality water (BQW). The micro-centrifuge tubes were loaded onto a thermocycler (Perkin Elmer, Norwalk, CT) that was programmed according to the 3-step cycling protocol described in Table 2.2.

Table 2.2 PCR I cycling protocol

	TEMPERATURE	TIMING	
Denaturation	94° C	30 secs	per cycle
Annealing	62° C	50 secs	
Primer Extension	72° C	80 secs	
The above cycle was repeated 39 times			
Holding temperature	4° C		

2.3.3 DETECTION OF PCR I PRODUCTS

On completion of the 40 PCR cycles, 5 µl of the amplification product (amplicons) from each time point and controls was loaded into wells cut into a 2 % agarose gel (Appendix A). The DNA was visualised by incorporating 5 µl ethidium bromide (EtBr) into the gel during its preparation. EtBr binds to DNA molecules by intercalation with adjacent nucleotide base pairs.

After separation of the amplicons by electrophoresis, the gel was irradiated with UV light in a dark room. Visualization of the DNA was possible because the attached EtBr fluoresces.

The presence of a band for the positive control confirmed that the PCR had worked. No bands were seen for either test sample or the negative control.

A limitation to the use of EtBr is that there needs to be >25 ng DNA in the samples before fluorescence is detected. As it was suspected that the DNA concentration within the test samples was extremely low, a further PCR amplification was deemed necessary. This was achieved by subjecting aliquots of the PCR I amplification products to a further round of the above described PCR I amplification process, namely, PCR II.

This technique, referred to as nested–PCR, is a two-step process. In the first step, an outer primer set within the terminal core region was employed whilst the subsequent step used internally binding primers (Table 2.1).

2.3.4 PCR II

The same process as described above for PCR I was repeated using instead, 5 µl aliquots (10 % v/v) of the PCR I amplicons as the target DNA. The reaction mixture differed from PCR I in that it had 1.5 mmol/L $MgCl_2$, the primers were internally binding (Table 2.1) and the final reaction volume was 50 µL. All other components of the reaction mixture were in the same proportions as for PCR I. The cycling protocol used is described in Table 2.3.

Table 2.3 PCR II cycling protocol

	TEMPERATURE	TIMING	
Denaturation	94° C	30 secs	per cycle
Annealing	51° C	50 secs	
Primer Extension	72° C	50 secs	
The above cycle was repeated 39 times.			
Holding temperature	4° C		

2.3.5 DETECTION OF AMPLIFIED PCR II PRODUCTS

A 2 % agarose gel incorporating 5 µL of EtBr was prepared and 5 µL aliquots of each of the PCR II amplicon samples as well as that of a 100 bp DNA ladder were loaded in separate wells and electrophoresed. This time, irradiation with UV light showed DNA fluorescence in both samples (Figure 3.1). When compared with the molecular size marker (Sambrook, *et al.*, 1989), the sample bands were found to be within the size expected i.e. 170 bps (Table 2.1).

2.4 CLONING

2.4.1 BACKGROUND

Direct sequencing of the PCR II amplicons was not performed as this would deplete the original serum samples. Cloning further amplified the PCR II amplicons and was necessary to separate genotype A from genotypes non-A.

The PCR-Script™ Amp Cloning kit (Stratagene La Jolla, CA, USA) was used and involved the following steps:

1) Pre-treatment of target DNA, in this case, the blunt-ended PCR II amplicons, were prepared by purification and polishing.

2) Ligation of polished PCR II amplicon DNA with the pPCR-Script™ Amp SK (+) plasmid vector.

3) Introduction of the recombinant plasmid DNA thus formed into specially modified kit bacterial cells (Epicurian Coli XL10-Gold™ Kan) by a process called transformation.

2.4.2 PURIFICATION OF PCR II AMPLICONS

The blunt-ended PCR II amplicons were purified with the StrataPrep™ PCR purification kit. This pre-treatment of PCR products removes PCR primers,

unincorporated dNTPs, DNA polymerases and buffer components present in excess at the end of PCR. The following method was used:

1) Each sample of PCR II product was combined with an equal volume of DNA-binding solution.
2) They were transferred to microspin cups held within 2 ml receptacle collection tubes, the caps were closed and microcentrifuged at 14000 rpm for 30 secs.
3) The microspin cups were removed and retained because the PCR products were bound to the fibre matrix of the spin cups. The DNA-binding solution within the collection tubes was discarded.
4) The microspin cups were then each washed with 750 µl PCR wash buffer, the caps closed and the tubes centrifuged at 14000 rpm for 30 secs.
5) Again the wash buffer was discarded and the microspin cups retained.
6) In order to remove residual buffer the tubes were subjected to further centrifugation at maximun speed for 30 secs.
7) The microspin cups were transferred to 1.5 ml microfuge tubes.
8) Double-distilled water was added to each cup to make up to the original volume of each sample and the tubes incubated at room temperature for 5 mins.
9) Finally they were centrifuged at maximum speed for 30 secs, the microspin cups discarded and the purified DNA within the eluate retained.

2.4.3 3' EXONUCLEASE TREATMENT

To 0.5 ml microfuge tubes the following was added and mixed in order:

10 µl of the purified PCR products

1 µl 10 mM dNTP mix ie. (2.5 mM of each dNTP)

1.3 µl of 10x polishing buffer

1 µl cloned *Pfu* DNA polymerase (0.5 U)

20 µl mineral oil was added to the reaction mixture and the tubes were incubated for 30 mins at 72° C. Thereafter, these polished PCR products were stored at 4° C until the subsequent ligation process was commenced.

2.4.4 LIGATION

Ligation is the process whereby foreign DNA is incorporated into a vector to form recombinant DNA. The plasmid vectors supplied in the cloning kit were cut with the restriction enzyme *Srf I* at nucleotide position 728 (Figure 2.2) and the purified, polished PCR II DNA products were inserted at this site (Figure 2.3). The inserts were joined to the plasmid DNA by the enzyme DNA ligase to form recombinant plasmid DNA (recombinants).

The pPCR-Script Amp SK (+) plasmid, contained an ampicillin-resistance gene and a *LacZ'* gene with a *lac* promoter necessary for its expression (Figure 2.2). These genes enabled detection of the desired recombinant plasmid DNA molecules as opposed to other ligation products.

To 0.5 ml microfuge tubes the following was added and mixed in order:

1 µl of pPCR-Script Amp SK (+) cloning vector (10ng)

1 µl of pPCR-Script 10 x reaction buffer

0.5 µl of 10 mM rATP

4 µl of the samples of blunt-ended PCR products (50ng)

and controls (400ng)

1 µl of *Srf I* restriction enzyme (5 U/µl)

1 µl of T4 DNA ligase (4 U/µL)

1.5 µl distilled water to make up to a 10 µl volume

These reaction samples were mixed briefly and incubated at 16° C overnight for approximately 14 hrs.

The next day the ligation products were placed on a heating block at 65° C for 10 mins and then stored at 4° C until commencement of the subsequent transformation process.

At the end of the ligation procedure the mixture contained:

- The desired recombinant DNA plasmids, i.e. PCR II product inserts incorporated into the cloning vector DNA.
- Unligated or self-ligated plasmids that had recircularised without incorporating the PCR inserts.

2.4.5 TRANSFORMATION

2.4.5.1 Background

Transformation is the process whereby bacterial cells take up recombinant DNA and are called transformants. The ligation products were introduced into the Epicurian Coli XL10-Gold™ bacterial cells (*E. coli*) provided in the cloning kit. These *E. coli* cells had been specially treated by the manufacturer in order to enhance their ability to take up foreign DNA (ultracompetent).

2.4.5.2 Method

1) Within 1 hour of commencing, fresh SOC medium (Appendix A) was made up. This medium was used to enhance the transformation process.

2) A few minutes before proceeding, an aliquot of Epicurian Coli XL10-Gold™ Kan ultracompetent bacterial cells were removed from the –70° C freezer and left to thaw on ice. This is an important step as these cells quickly lose their competency if not handled carefully.

3) 20 µl of ultracompetent cells were pipetted into pre-chilled microfuge tubes and 5 µl of each sample of ligation products added. A control consisting of 1 µl of cloning vector alone was included to determine whether the transformation process had worked. The remaining ultracompetent cells were immediately returned to the –70° C freezer.

4) The mixtures were left on ice for 20 mins after which the temperature of the sample was raised briefly (90 secs) to 42° C in order to stimulate cellular uptake of plasmid DNA (Heat Shock Method) and then immediately immersed into ice for 4 mins.

5) 1 ml of the freshly prepared SOC medium was added to each sample and the tubes were then placed in a shaking incubator at 37° C for 60 mins.

6) Ampicillin-LB agar plates (Appendix A) which had been freshly prepared, were each first spread with 40µl of the enzyme inducer, isopropyl-thiogalactoside (IPTG) and left to stand for approximately 5 mins. They were then layered with 40 µl of the lactose analogue, 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal).

7) The samples were removed from the incubator and 500 µl of each (including the control sample) was plated out onto the prepared ampicilin-LB agar plates and left to incubate overnight at 37° C.

2.5 BLUE-WHITE COLONY SELECTION

2.5.1 BACKGROUND

The plasmid vector, pPCR-Script Amp SK (+) has an ampicillin resistance gene, which confers upon the transformants the ability to grow on ampicillin-agar. Each

transformed bacterium can multiply, forming an individual colony with ampicillin acting as the selectable marker.

The pPCR-Script Amp SK (+) vector also has a *LacZ* gene that codes for the enzyme β-galactosidase. This enzyme splits the lactose analogue, X-gal, in the presence of the enzyme-inducer, IPTG; both of which are incorporated into the culture medium. Splitting of X-gal results in the formation of a deep blue coloured product.

The *LacZ* gene, is however, inactivated by insertion of a PCR II amplicon into this site (Figure 2.2). Bacterial colonies of transformants with recombinant plasmid DNA would therefore be detected by the absence of the blue coloured product, and remain white.

2.5.2 SELECTION OF TRANSFORMANT COLONIES

After overnight incubation, the plates of the control and both samples showed growth of multiple bacterial colonies. The control plate, containing untransformed bacterial cells, had blue colonies only. After placing the sample culture plates in the fridge (4° C) to facilitate further development of colour, only the white colonies were selected for miniprep processing (Figure 2.3).

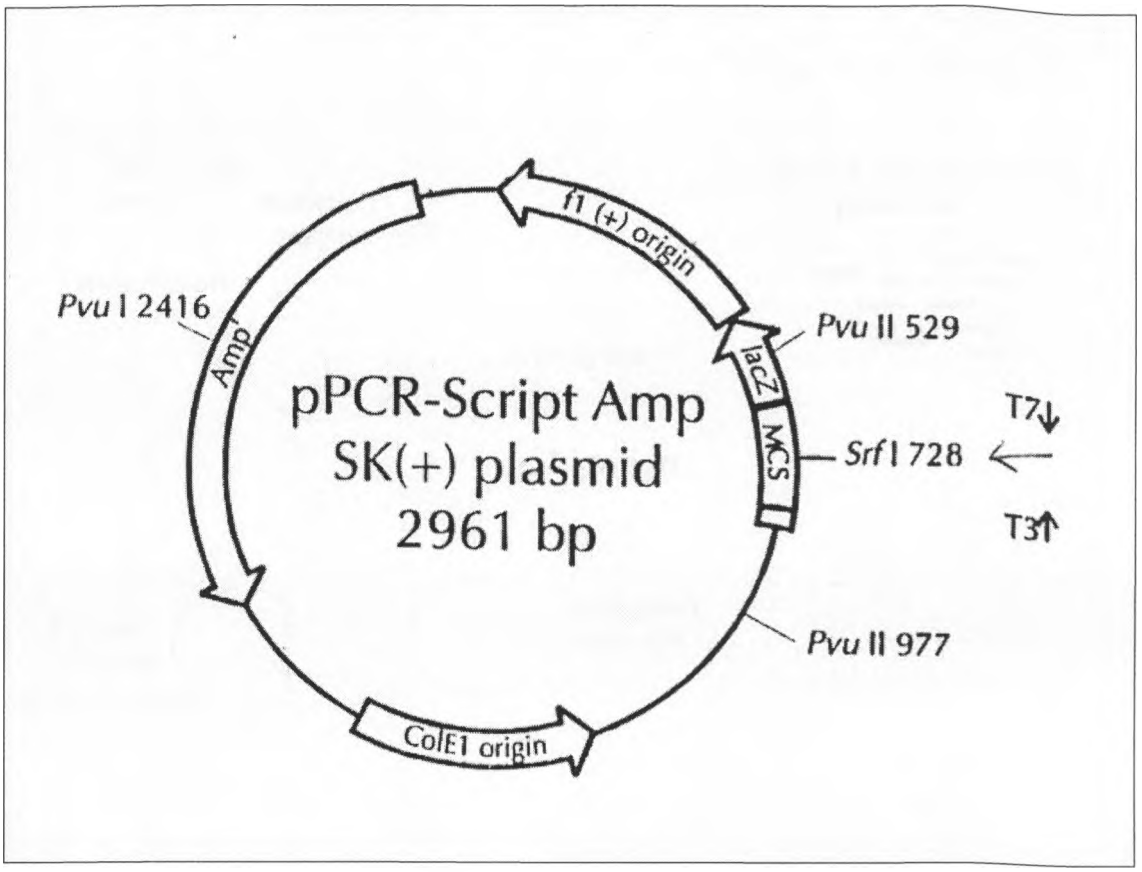

Figure 2.2 pPCR – SCRIPT AMP SK (+) 2961 bp plasmid (From PCR-Script™ Amp Cloning kit, Stratagene La Jolla,CA, USA. <http:/www.stratagene.com.manuals/ 211189.pdf> (22 Jan 2003)

Thirty white colonies were selected from both sample plates B2 531 and B2 21/6 and each colony plated out onto a freshly prepared ampicillin-LB agar plate. Throughout this study, fresh stocks of each colony were maintained by repeating this plating out procedure onto freshly prepared ampicillin-LB plates.

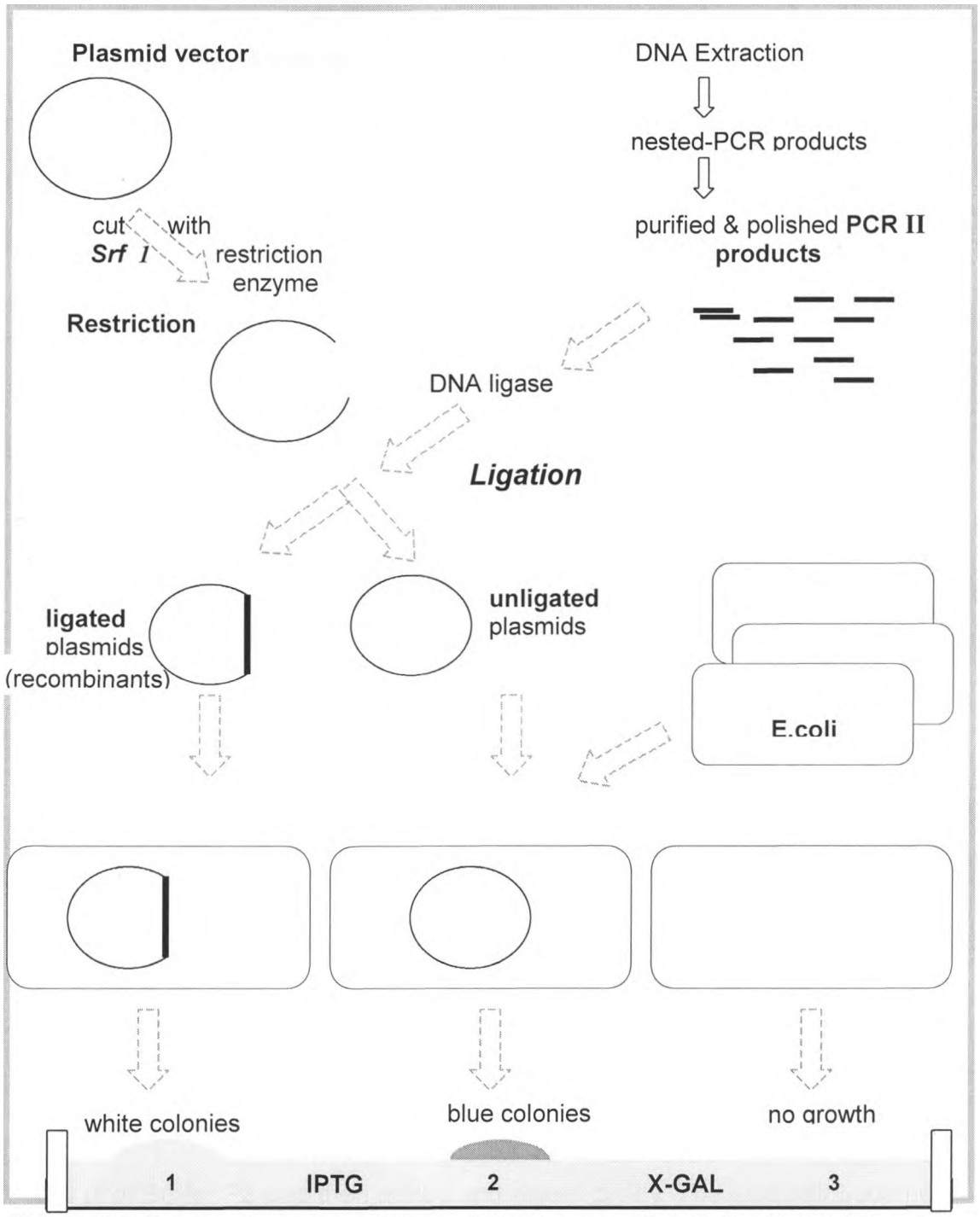

Figure 2.3 Schematic representation of the steps leading to the blue-white colony selection process using Amp LB agar plates containing IPTG & X-gal.

- Restriction – plasmid vectors cut with *Srf I* restriction enzyme
- Ligation – PCR II inserts incorporated into plasmid DNA producing recombinant plasmids.
- Transformation – recombinant plasmids being taken up by *E.coli* bacteria.
  (1) White colonies - transformed *E.coli* bacteria containing recombinant plasmid DNA
  (2) Blue colonies - transformed *E.coli* bacteria containing self-ligated/unligated plasmids that had recircularised without incorporating the PCR II inserts.
  (3) No growth from bacteria that were not transformed.

The colonies were allowed to grow at 37° C and then a sample of each colony was inoculated into a 50 ml Nunc tube containing 5 ml of a mixture of 1 ml ampicillin per litre Luria Broth (Appendix A). The tubes were placed in the shaking incubator and left at 37° C overnight. This facilitated active bacterial growth, which increased the number of copies of each transformant and hence recombinant DNA.

2.6 MINIPREPS OF PLASMIDS

2.6.1 BACKGROUND

In this extraction procedure, a number of the transformed colonies were simultaneously processed to extract plasmid DNA from the bacteria.

2.6.2 METHOD

This was performed with the QIA prep Spin Miniprep kit (Qiagen Inc., Hilden, Germany).

1) The Nunc tube cultures were removed from the incubator and centrifuged at 13000 rpm for 15 mins.
2) After discarding the supernatant, each pellet was resuspended in 250 µl of Buffer P1 containing RNase A.
3) 250 µl of Buffer P2 was then added and mixed gently. The mixtures became viscous and slightly clearer.
4) 350 µl of Buffer N3 was added and tubes inverted 4-6 times. The solutions then became slightly cloudy.

5) The samples were centrifuged at 13000 rpm for 10 mins.
6) The supernatant from each sample was added to QIAprep spin columns and centrifuged at 13000 rpm for 60 secs.
7) The flow-through was discarded, and each column was washed with 0.5 ml Buffer PB.
8) After centrifugation at 13000 rpm for 60 secs the flow-through was again discarded.
9) The columns were each washed with 0.75 ml of Buffer PE and centrifuged at 13000 rpm for 60 secs.
10) The flow-through was discarded and the samples centrifuged for an additional 60 secs.
11) The columns were then placed into clean 1.5 ml tubes and 70 µl Buffer EB was added to each in order to elute the plasmid DNA.
12) After standing for 60 secs, the tubes were centrifuged at 13000 rpm for a further 60 secs. The sample eluates comprising plasmid DNA were stored at 4° C until the subsequent restriction and sequencing processes were commenced.

2.7 RESTRICTION

2.7.1 BACKGROUND

In this process the PCR II inserts were restricted from a small volume of each eluted plasmid DNA sample by using the restriction enzyme *Pvu II*. This enzyme cuts the cloning vector specifically at two designated sites, namely, nucleotides

529 and 977 respectively (Figure 2.2), producing blunt-ended DNA inserts (as opposed to those with sticky overhangs). This process was necessary in order to confirm that each selected white clone did in fact, contain recombinant plasmid DNA.

2.7.2 METHOD

1) A mastermix of the following was made:

   0.5 µl *Pvu II*

   1 µl Buffer M

   2 µl distilled water

   The total volume made up was determined by multiplying this volume by the number of samples increased by one.

2) 5 µl of each sample of plasmid eluate was mixed with 5 ml of the mastermix, briefly centrifuged and incubated at 37° C for 1 hr.

3) These restricted samples were then electrophoresed to confirm that the white clones did contain recombinant DNA.

2.8 ELECTROPHORESIS OF RESTRICTION FRAGMENTS

2.8.1 METHOD

1) 5 µl of EtBr was incorporated into a 1 % agarose gel preparation after it had cooled down to less than 50° C.

2) 10 µl of each restricted sample was mixed with 5 µl Ficoll loading dye and loaded into a well in the gel. The Ficoll dye served to sink the DNA to the

bottom of the wells to prevent the buffer solution from floating out the DNA. Five µl samples of a 100 bp and 1 kb DNA size ladder were loaded before the test samples.

3) The gel was covered with TBE buffer (Appendix A) and the samples were run at 120 V for 45 mins.

2.9 VISUALISATION OF RESTRICTION FRAGMENTS

The gel was initially visualised under UV light and then photographed in a dark room with a Polaroid camera to make a permanent record (Figure 3.2). The high intensity of the bands gave a rough estimation that there was sufficient DNA present.

The molecular weight markers served as reference markers for determination of the molecular size of the DNA fragments in the lanes. The size of the insert was 170 bp and the restriction fragment without the insert was 449 bp. The samples with the inserts were therefore 619 bp (449 bp + 170 bp insert) (Figure 3.2).

The photograph showed that the correct restriction fragment size (619 bp) was present. This confirmed that the selected white colonies were positive for recombinants. Several gels were run until 20 positive clones for sample B2 531 and 19 positive clones for sample B2 21/6 were confirmed.

The unrestricted minipreps from the confirmed positive clones were sequenced to determine which genotype of HBV they contained.

2.10 NUCLEOTIDE SEQUENCING

2.10.1 BACKGROUND

The Sanger-Coulson chain termination sequencing method (Sanger, *et al.*, 1977) of determining the order of nucleotide bases in a DNA sample was used. In this method, DNA strands are synthesised from a single stranded template of the DNA in the sample. The method is based on interruption of new strand synthesis at varying sites by the incorporation of 2′ 3′-dideoxy-nucleotide-5′-triphosphates (ddNTPs) resulting in a mixture of strands of varying fragment lengths.

Like the PCR process, this method cycles target DNA through the three steps of denaturation, annealing of primers to each template and the elongation of the primer by DNA polymerase. In contrast to PCR however, a mixture of four ddNTPs are added during the sequencing reaction. They are, dideoxyadenine triphosphate (ddATP), dideoxycytosine triphosphate (ddCTP), dideoxyguanine triphosphate (ddGTP) and dideoxythymine triphosphate (ddTTP) respectively. These ddNTPs lack the 3′ hydroxyl group in their deoxyribose rings, therefore, further elongation is prevented when they are incorporated into the growing chain.

At the end of the sequencing reaction all strands will have terminated at random sites as determined by the ddNTP type inserted. This means that there will be a large population of strands of varying lengths, terminated at every possible nucleotide site after the primer sequence.

In this experiment, sequencing was performed with the Sequenase Version 2.0 DNA sequencing kit (Amesham Life Science Inc., Cleveland, OH, USA). The extracted positive clones from both baboon samples that had been identified by the blue white colony selection method and confirmed as having the correct restriction fragment size, were sequenced in both the forward and reverse directions using sense and antisense primers respectively.

2.10.2 METHOD

1. Alkaline denaturation of double-stranded sample DNA

- To 30 µl of each plasmid DNA sample, 0.1 volume (3 µl) of 2 M NaOH and 2 mM EDTA (pH 13) was added and the mixture was incubated for 30 mins at 37° C.
- The samples were neutralised with 0.1 volumes (3.3 µl) 3 M sodium acetate.
- The DNA was precipitated with 3 volumes (108.9 µl) of 70 % ethanol (-70° C).
- They were spun for 15 min at 4° C in a microfuge at top speed.

- The supernatant was discarded and the pelleted plasmid DNA washed with 70 % ethanol.
- They were spun in a microfuge at maximum speed at room temperature.
- The supernatant was discarded and the samples air-dried.
- The DNA was redissolved with 7 µl sterile water added to each pellet.

2. Annealing

- The annealing mixture was prepared by mixing the following:

DNA	7 µl
Reaction buffer	2 µl
Primers (sense/antisense)	1 µl

- Heating of the annealing mixture at 99° C for 2 mins allowed annealing of the DNA with the primers. The samples were allowed to cool slowly to below 35° C by standing at room temperature for between 5-30 mins.
- After a brief centrifugation the samples were chilled on ice for use in step four.

3. Preparation of microtitre plates

- Microtitre plate lanes were labeled as G, A, T and C. Termination mixes containing each ddNTP were added to the respective wells and covered to limit evaporation. They were left to stand at room temperature.

4. The labeling reaction

- The labeling mixture was prepared by adding:

DTT	1 µl
Diluted labeling mix (1:15)	2 µl
^{35}S-α dCTP (10µCi/µL)	0.5µl
Diluted Sequenase polymerase	2 µl
Total volume	**5.5 µl** per sample

- The actual volume of labeling mixture stock prepared was 5.5 µl multiplied by the number of samples. An extra 5.5 µl was added to accommodate for loss of volume through evaporation. Ice-cold annealed DNA samples from step 2 were each mixed with 5.5 µl of labeling mixture and incubated at room temperature for 2 mins.

5. Termination Reaction

- The microtitre plates were pre-warmed on a 42° C heating block for 1 min.
- Whilst remaining on the heating block, 3.5 µl of the combined labeling mixture and sample DNA was added to the each termination ddNTP already within the microtitre wells.
- After each addition the solutions were thoroughly mixed using a "pumping" action with the micropipette taking care to avoid the introduction of air bubbles.
- Disposable micropipette tips were discarded after each addition to prevent contamination of the labeling mixture stock.

- The additions were timed such that each sample was only allowed to incubate for 5 mins after which each reaction was terminated by the addition of 4 µl of stop solution.

All samples were sequenced in both forward (sense) and reverse (antisense) directions.

6. Storage

- The microtitre plates were stored at - 4° C. All these sequenced samples had to be electrophoresed and autoradiographed before the incorporated ^{35}S had lost its radioactivity.

2.10.3 SEQUENCE READING

Each G, A, T and C track on the autoradiograph contained fragments that had been terminated by one of the 4 types of ddNTPS during the sequencing strand synthesis. The sequence was read by identifying the track in which a band was present.

The smallest nucleotide fragments after the primer sequence, being the most mobile, migrated the fastest and were found at the bottom of the autoradiograph. Therefore, reading of each fragment length commenced from the bottom up. The next band further up represented a DNA molecule one nucleotide longer and was classified according to the ddNTP tract in which the band lay. The ddNTP sequence was thus progressively read, one nucleotide at a time.

The readings were entered on a sequence template (Table 2.4) for samples run in both the forward and reverse directions. Mutations were recorded only when they were identified in both directions.

2.11 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

2.11.1 POLYACRYLAMIDE GEL PREPARATION

Great care was taken in the preparation of the two sequencing gel glass plates. After being thoroughly scrubbed down with Sunlight liquid detergent and hot water, they were cleaned with ice cold 70 % ethanol and then isopropanol. They were placed together separated only by wedged plastic spacers positioned at each lateral edge. These spacers determined the thickness of the gel required. The plates were held together with tight metal clips at each corner and raised off the work surface with blocks, the top end being tilted upwards.

Five hundred mls of 7 % polyacrylamide gel solution (Appendix A) was prepared in the microwave oven. Prior to pouring the gel, both TEMED and ammonium persulphate (APS) (Appendix A) were added to initiate polymerisation and then the gel solution was immediately poured between the two glass plates from the top end with a large syringe, taking extreme care to not to create air bubbles. Any air bubbles trapped within the gel were eliminated by gently tapping on the plates.

Table 2.4 Sample of a sequence template of the terminal core region of HBV (nucleotide sequences 2351-2363 numbered from *EcoR I* site (forward direction) of sampled clones.

nt. 2267

Sample B2 531 [a] clones	67 G	68 G	69 A	70 G	71 T	72 G	73 T	74 G	75 G	76 A	77 T	78 T	79 C	80 G
1	•	•	•	•	•	•	•	•	•	•	•	•	•	•
↓	•	•	•	•	•	•	•	•	•	•	•	•	•	•
20	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Sample B2 21/6 [a] Clones														
1	•	•	•	•	•	•	•	•	•	•	•	•	•	•
↓	•	•	•	•	•	•	•	•	•	•	•	•	•	•
20	•	•	•	•	•	•	•	•	•	•	•	•	•	•

Distinguishing **6 bps** nt. 2464

Sample B2 531 [a]Clones	51 C	52 G	53 C	**54 C**	**55 G**	**56 G**	**57 G**	**58 A**	**59 C**	60 C	61 G	62 A	63 G	64 G
1	•	•	•							•	•	•	•	•
↓	•	•	•							•	•	•	•	•
20	•	•	•							•	•	•	•	•
Sample B2 21/6 [a] Clones														
1	•	•	•							•	•	•	•	•
↓	•	•	•							•	•	•	•	•
20	•	•	•							•	•	•	•	•

(Total 170 bps) nt. 2437

[a] Clones selected for sequencing

• Represents conservation of the sequences

6bps in the blocks without the dots represent nucleotides 2354 –2359 that are present in HBV genotype A but absent in genotype non-A

↓ Represents missing rows

⇨ Represents following nucleotide sequences not shown in this template

After pouring, the gel was placed horizontally and a well-forming plastic comb inserted into the top end of the gel. The gel was left to polymerise overnight and used for PAGE within 20 hrs of gel preparation.

2.11.2. ASSEMBLY OF ELECTROPHORESIS APPARATUS AND SAMPLE LOADING

After polymerisation, the metal clips and plastic comb were removed, the excess gel washed off the exterior of the plates with tap water and the plates dried. The comb was reinserted and clipped in place with the "teeth" facing downwards but not touching the gel.

The plates were clamped firmly to both the upper and lower buffer chambers of the vertical electrophoresis apparatus with the back plate facing anteriorly and the buffer outlets closed. Using a disposable pipette the top edge of the gel was sealed with 1 % agarose gel.

A dilution of glycerol tolerant buffer (GTB) (Appendix A) was used as the running buffer because this same buffer had been used previously in the gel preparation. A measuring cylinder was filled with 60 mls of GTB, made up to 1200 mls with distilled water and the apparatus buffer troughs filled.

The teeth of the comb were gently lowered into the gel and secured in place with small clips. Using a disposable pipette the same GTB buffer was used to flush out the urea in each well.

Ten μl of stop solution from the sequencing kit was pipetted into alternate wells. The circuit was closed and the electrophoresis apparatus run at 60 watts for a short while. The blue colour of the stop solution enabled the visualisation of leaks present in any of the wells. The power pack was switched off and the non-leaky wells were marked off on the glass plate and allocated to a sequenced specimen.

The sequenced specimens were then transferred from the freezer into a cooler bag containing ice. The plates were placed on an 83° C heating block for 2 mins to denature the DNA and then immediately placed back onto the ice.

The urea was once again flushed out of the wells with GTB buffer and 3.5 μl of each specimen loaded into the marked wells. Great care was taken to dispose micropipette tips between each specimen in order to avoid cross-contamination.

The apparatus was switched on again and the samples allowed to electrophorese for about three to three and a half hrs at 60 watts. The high voltage generated heat, and this was one of the factors that prevented the re-association of complementary strands of DNA.

2.11.3 GEL HANDLING AFTER ELECTROPHORESIS

At the determined run time, the machine was switched off and the buffer outlets opened to drain the buffer before unclamping the gel plates. The spacers and comb were removed and the gel plates carefully pried apart with the gel adherent to the back plate. A small nick was made in the left hand corner of the gel to identify where the sample loading had commenced.

The gel was placed into a trough containing fixing solution (Appendix A) and left for 20 mins. Excess fixative was blotted lightly with 3 MM Whatman paper. The gel was then allowed to adhere to a second dry sheet and then the gel-paper stripped off the backing glass plate. The excess paper around the gel was trimmed and cut at the nicked gel edge. It was covered with a layer of cling wrap and dried on a Hoefer dryer for two and a half hrs at 80° C. This whole process was repeated until all the sequenced specimens had been electrophoresed.

2.12 AUTORADIOGRAPHY

The dried gel on the Whatman paper was placed into an x -ray casette and exposed to the emulsion side of a Kodak XRP–1 radiograph film in the dark room. The films were exposed for a period of between two to five days.

2.13 STATISTICAL ANALYSIS

Fisher's exact and the Chi-squared tests were used to analyse the data where appropriate.

CHAPTER 3 RESULTS

3.1 PCR II PRODUCTS

The sample bands were compared with the molecular weight marker (Figure 3.1) and were found to be within the expected size i.e. 170 bps (Table 2.1).

3.2 TRANSFORMANT COLONIES

The control plate and both B2 531 and B2 21/6 sample plates showed growth of multiple bacterial colonies. The control plate, which contained untransformed bacterial cells, had blue colonies only. Thirty white colonies containing transformed bacterial cells, were chosen from each sample plate for mini-prepping.

3.3 RESTRICTION FRAGMENTS

Restriction fragments containing no inserts were 449 bp in size, whilst those that had inserts, were 619 bp (449 bp + 170 bp). Photographs that were taken (Figure 3.2) identified the colonies containing the inserts (positive clones). Twenty positive clones from sample B2 531 and 19 positive clones from sample B2 21/6 were identified for sequencing.

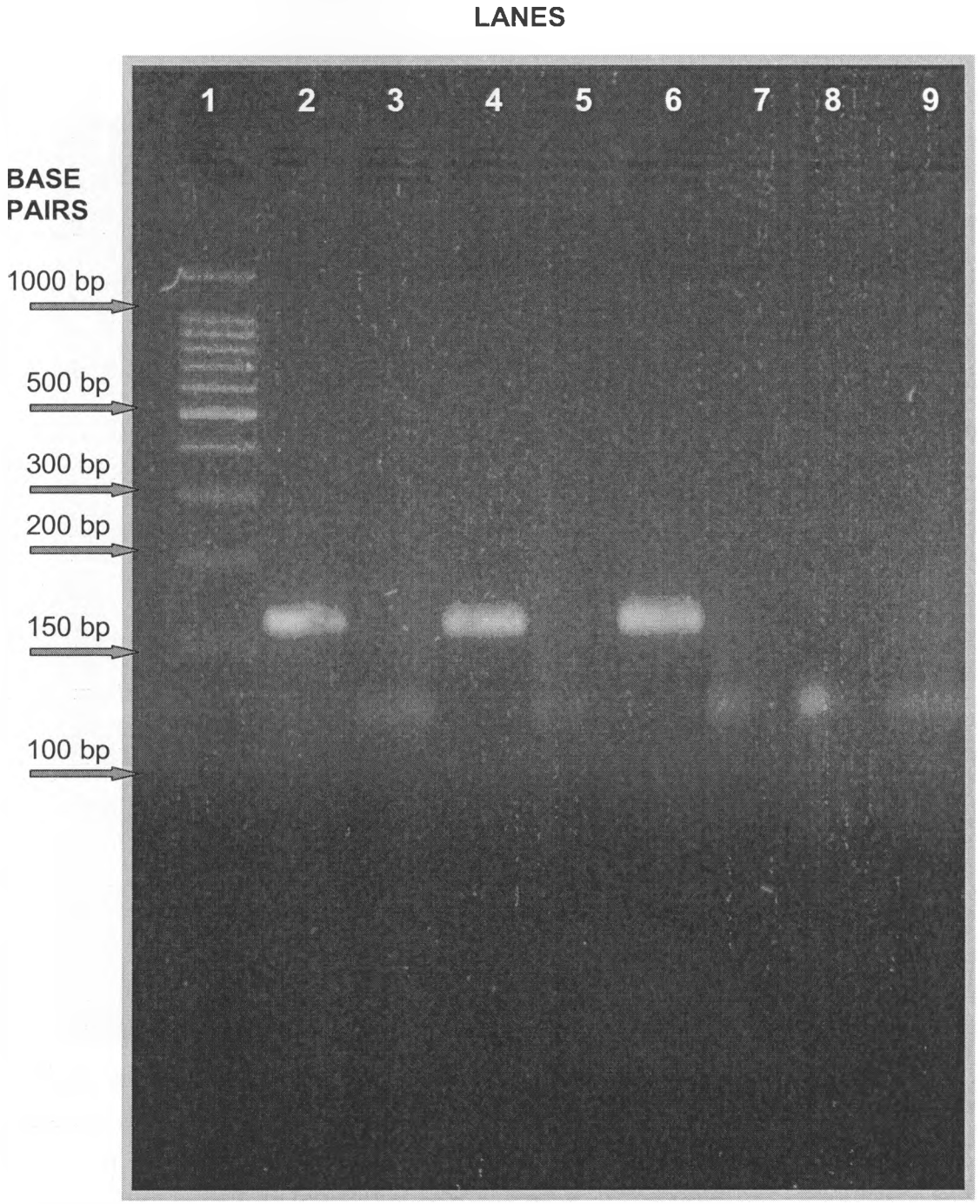

Figure 3.1 PCR II amplicons run on a 2 % agarose gel stained with ethidium bromide and visualised under UV light.

Lane **1** 100 bp molecular weight marker (Promega)
Lane **2** Positive control
Lane **3** PCR II negative control
Lane **4** Sample 1 (B2 531)
Lane **5** PCR II negative control
Lane **6** Sample 2 (B2 21/6)
Lane **7** PCR II negative control
Lane **8** PCR II negative control
Lane **9** PCR II negative control

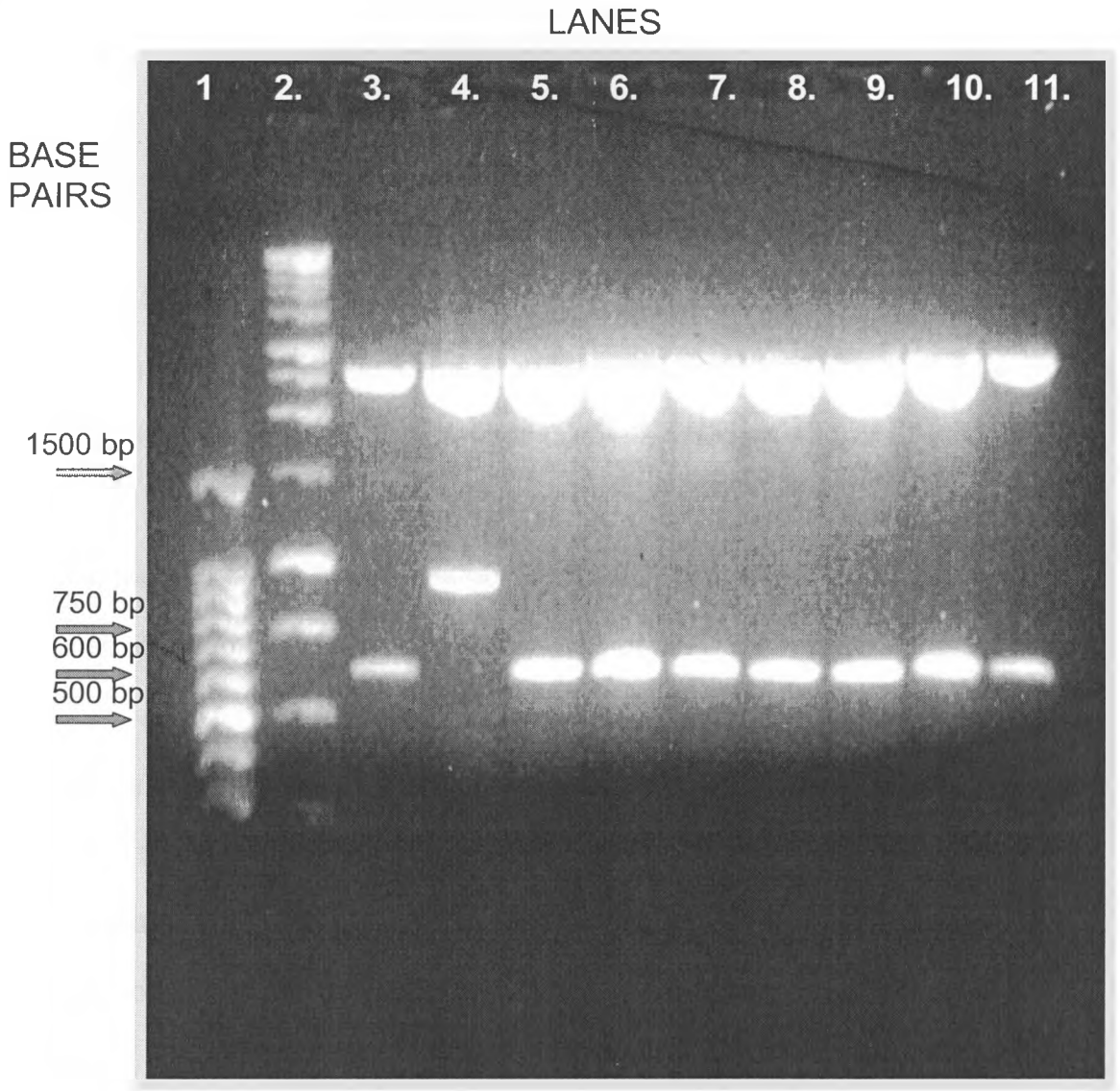

Figure 3.2 Restriction digest of different clones of sample B2 531 using *Pvu II* and run on a 2 % agarose gel.

Lane 1	100 bp molecular weight marker (Promega)
Lane 2	1 kb molecular weight marker (Promega)
Lanes 3 to 11	Different clones of sample B2 531
Lane 4	Clone containing a double insert
Lane 3, 5-11	Clones containing inserts (619 bp)

3.2 CLASSIFICATION OF GENOTYPES

Using the criterion of the 6 bp insertion (nucleotides 2354 –2359) (Figure 3.3) being present in HBV genotype A and absent in genotype non-A (Figure 3.4), the results were compared and graphed (Table 2.4, Figure 3.5).

The number of positive clones of A and non-A genotypes at each time point was used to determine the relative proportions of the two genotypes present.

During the study, it had been determined that the proportions of HBV genotypes within the inoculum were approximately 50 % of each genotype A and non-A (Baptista, *et al.*, 2003). In the sample 4 months post inoculation (sample B2 531), the proportions had changed, 75 % of the sample then consisting of genotype non-A. After 10 months (sample B2 21/6), the genotype proportions had reversed, this time 79 % of the sample population of HBV being represented by genotype A. The hypothesis of equal proportions of the genotypes over time was rejected ($P<0.002$).

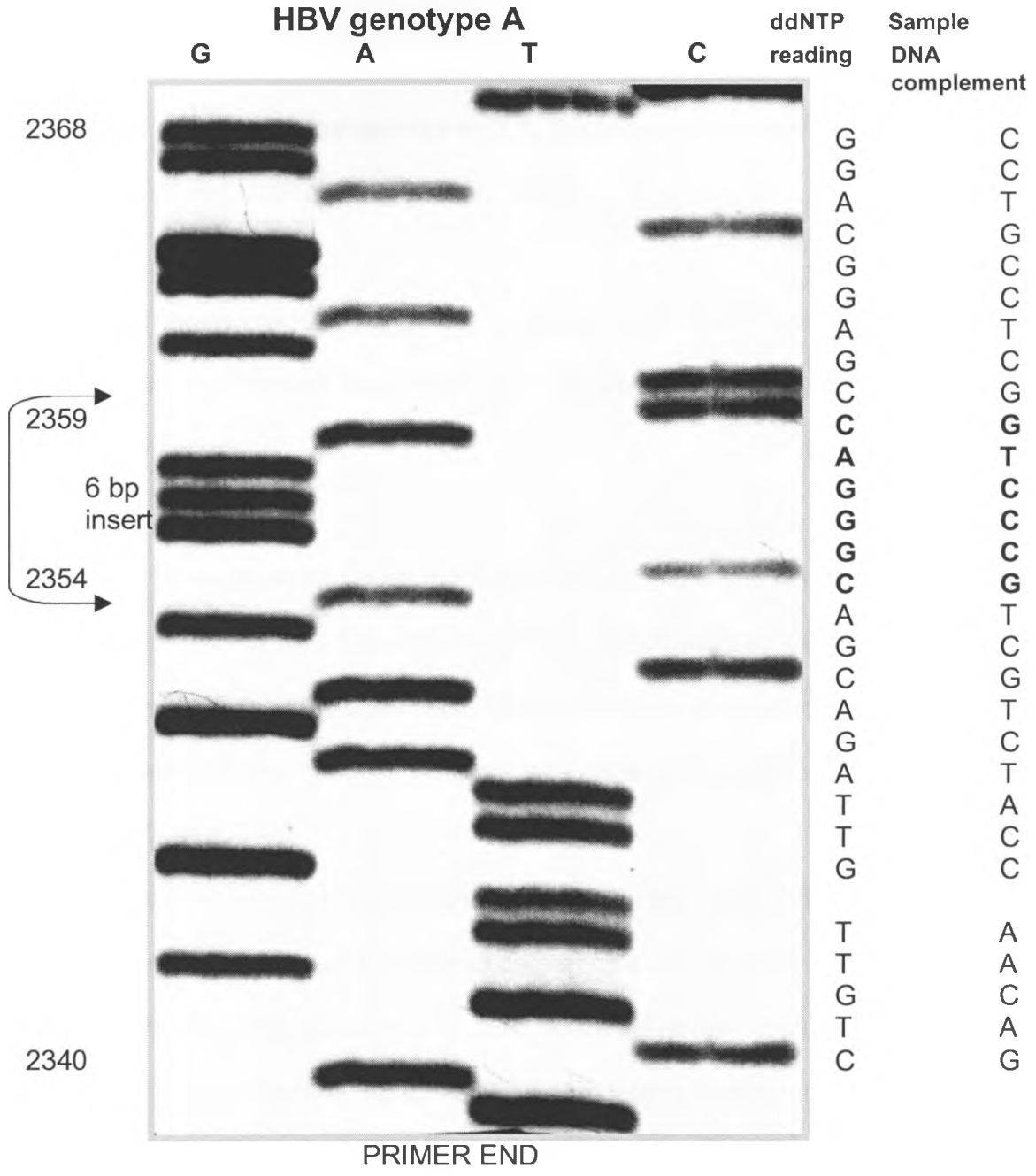

Figure 3.3 Enlargement of a section of an autoradiograph showing sequence results of the core region (nucleotides 2340-2368, numbered from the *EcoR I* site) obtained from a clone of HBV genotype A. The 6 bp insert, 5'CGGGAC3' is present and distinguishes it from HBV genotype non-A. The nucleotide tracks G, A, T and C are labeled.

viral forms were demonstrated as well as the presence of anti HBcAg at 16 weeks post inoculation (Kedda, *et al.*, 2000).

The viral titers were found to be low (Baptista, *et al.*, 2003), and a single round of PCR was insufficient to detect HBV DNA. Nested-PCR amplification was therefore necessary.

Because the baboon had been inoculated with a mixed population of HBV genotypes, namely, genotypes A and non-A, cloning was necessary to separate them. The DNA region cloned was well conserved in all genotypes. Thus, all HBV genotypes were amplified without bias towards any particular genotype.

A specific 6 bp insertion, 5'CGGGAC3', (between nts. 2354-2359) present only in HBV genotype A, made it possible to differentiate this genotype from non-A genotypes. All non-A genotypes (B to H) do not have this insertion. A further genotyping assay such as restriction fragment length polymorphism analysis would be necessary to distinguish between them.

The results reveal that HBV of both genotypes were persistently present throughout the study period (Figure 3.3), albeit in different proportions. No uniform pattern of replication between the genotypes in all the baboons throughout the 52-week study period could be discerned (Kedda, *et al.*, 2000,

Baptista, *et al.*, 2003) and an explanation for the changing proportions needed to be found.

Statistical analysis of the number of clones selected, excluded the possibility that the number of clones sampled was insufficient to accurately assess the relative proportions of genotypes in the serum per time point.

No definite conclusions concerning the dominance of one genotype over the other, the natural progression or replication rates of the genotypes could be made because the changing proportions of genotypes may have been caused by sampling fluctuations at the time of HBV DNA extraction and cloning in the setting of low viral loads in the baboon sera.

The persistence of HBV DNA at 10 months post inoculation (Figure 3.3) however, supports a conclusion that a low rate of viral replication had been occurring. This counters the possible argument that the HBV DNA present was a mere persistence of the original inoculum within the baboon bloodstream.

The manual sequencing employed in this study, showed that, when the viral DNA sequences of the genotypes are known, they are easily detected in a sample, although the processes are labour-intensive and time consuming. Automated sequencing would be a better alternative.

APPENDIX A: SOLUTIONS, REAGENTS AND GELS

40 % ACRYLAMIDE STOCK

- 38 g Acrylamide (Promega)
- 2 g Bis-acrylamide (Promega)
- 5 g Amberlite

Add 50 mls BQW

Cover with foil & stir at room temperature until completely dissolved

Make up to 100 mls with BQW

Filter sterilise & store at 4° C

AGAROSE GEL

Dissolve sealkem HGT agarose in TBE running buffer (350 ml per gel)

Boil in microwave

Add 3 μL EtBr (10 ng/ml stock) per 100 ml gel

10 % AMMONIUM PERSULPHATE (APS)

- 0.25 g APS (Biorad)
- 2.5 mls dH_2O

Cover with tin foil & store in fridge

FICOLL LOADING DYE

- 50 g sucrose (50 %) (Saarchem)
- 10 ml 50 mM EDTA (pH 7) (Boehringer Mannheim)
- 0.1 g Bromophenol blue (0.1 %) (Merck)
- 10 g Ficoll (10 %)

Make up to 100 ml with BQW

FIXING SOLUTION

- 150 mls 15 % Methanol (Saarchem)
- 50 mls 15 % Acetic acid (Labchem)

Make up to 1 L with dH_2O

GLYCEROL TOLERANT BUFFER (GTB)

- 108 g Tris base (Boehringer Mannheim)
- 36 g Taurine (USB)
- 2 g $Na_2EDTA.2H_2O$ (Boehringer Mannheim)

Make up to 500 ml

Filter sterilise or autoclave

8 % GT SEQUENCING GEL

- 42 g Urea (Promega)

- 20 ml 40 % Acrylamide stock
- 5 ml 20 x GTB
- 35 ml BQW

Dissolve

Make up to 100 ml WITH BQW

Add 600 μL10 % APS (Biorad), 25 μLTemed (Promega) and mix

Immediately pour onto sequencing plates

LURIA-BERTANI (LB) BROTH

- 10 g bacto-tryptone
- 5 g bacto-yeast extract
- 10 g NaCl

Make up to 1 L with dH_2O

Titrate the pH to 7.5 with NaOH

Aliquot into 100 ml bottles

Autoclave immediately

LB-AMPICILLIN AGAR PLATES

Add 1,4 g Sealkem HGT agarose to 100 mls LB broth

Autoclave

When cool enough add ampicillin 25 μg/ml ampicillin

Pour into plates

SOC MEDIUM

Mix the following:

- 2 g Tryptone
- 50 mg Yeast extract
- 50 mg NaCl

Make up to 100 mls using BQW

Autoclave

When cooled down add:

- 1 ml 1 M $MgCl_2$
- 1 ml 1 M $MgSO_4$

2 ml 20 % glucose (w/v) solution

Check pH (should be 7)

Filter sterilise and store at 4° C

Use within 1 hour of making up.

10 X TBE

- 108 g Tris base (Boehringer Mannheim)
- 55 g Boric acid (Sigma)
- 7.4 g Na_2EDTA (Boehringer Mannheim)

Make up to 1 L with dH_2O

Autoclave

1 M TRIS-HCl

Dissolve 121.1 g Tris (Boehringer Mannheim) in 800 mls in water

Titrate to pH 7.5 with HCl

Make up to 1 L dH_2O

Autoclave

APPENDIX B: PUBLICATIONS

Baptista, M., Kramvis, A., Jammeh, S., Naicker, J., Galpin J., Kew, M.C. 2003. Follow up of infection of chacma baboons with inoculum containing A and non-A genotypes of hepatitis B virus. *World Journal of Gastroenterology*, vol. 9, pp. 731-735.

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• VIRAL HEPATITIS •

Follow up of infection of chacma baboons with inoculum containing a and non-a genotypes of hepatitis B virus

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Abstract

AIM: To determine whether one genotype (A or non-A genotypes of HBV) predominated over the other during the course of HBV infection.

METHODS: Four baboons were inoculated with HBV. DNA was extracted from serum obtained at monthly intervals post-inoculation for 52 weeks and HBV DNA was amplified using primers specific for the core region containing an insert characteristic of genotype A (nt 2 354-2 359, numbering from the *EcoRI* site). The amplicons were cloned into PCR-Script™ and a minimum of 15 clones per time point were sequenced in both directions.

RESULTS: Both genotypes persisted for the entire follow-up period of 52 weeks. Genotype non-A predominated in two baboons and genotype A in one baboon. Neither genotype predominated in the fourth baboon, as shown at a 5 % level of testing.

CONCLUSION: No conclusions concerning the dominance of either genotype or the natural progression or replication rates of HBV could be drawn because the pattern of the genotypes found may have been caused by sampling fluctuations at the time of DNA extraction and cloning as a result of the very low viral loads in the baboon sera.

Baptista M, Kramvis A, Jammeh S, Naicker J, Galpin JS, Kew MC. Follow up of infection of chacma baboons with inoculum containing a and non-a genotypes of hepatitis B virus. *World J Gastroenterol* 2003; 9(4): 731-735
http://www.wjgnet.com/1007-9327/9/731.htm

INTRODUCTION

Xenografts from closely related non-human primates or pigs have been suggested as one way to alleviate the chronic shortage of donor organs for human liver transplantation. Baboons (*Papio* species) which are phylogenetically close to humans, have reasonably large livers, and are not endangered. They have already been the source of xenografts for two humans undergoing liver transplantation[1-3] (as well as for a few patients receiving kidney or heart transplants[2,4-6]). The use of liver xenografts would, however, be precluded if they were infected by zoonotic pathogens. In addition, because chronic hepatitis C and B virus infections are now the most frequent causes of end-stage liver disease requiring transplantation in humans[7], the donor livers should be resistant to infection with these viruses. Our study has previously shown that Chacma baboons (*Papio ursinus orientalis*) are resistant to infection of hepatitis C virus[8] but are susceptible to infection of hepatitis B virus (HBV)[9].

In the latter study[9], pooled serum from three patients with acute hepatitis B (the serum of all three was HBV surface (HBsAg)- and e antigen (HBeAg)-positive and had high titers of HBV DNA) had been injected into the baboons. Direct sequencing of HBV DNA amplified at various times post-inoculation indicated that the baboons had been inoculated with a mixed population of HBV. Cloning of the HBV DNA amplified from the inoculum revealed that it fortuitously contained approximately equal proportions of A and non-A genotypes of HBV. HBV has been classified into genotypes A-H, with an intergenotypic diversity of at least 8 %[10-13]. Genotype A accounts for 80 % and genotype D for 10 % of the genotypes found in southern Africa, with the other genotypes being either absent or present in very few isolates[14]. HBV genotypes have a characteristic geographical distribution[15], which help in tracing the route of HBV infection[16], and may influence the severity and outcome of infection with this virus[17]. However, little is known about the natural progression and severity of the infection when more than one genotype is present[18].

Co-infection with two or more genotypes may be the consequence of multiple exposures to infection at an early age when immune responses are immature or in older individuals with immune disorders[19], or the result from genotype changes during seroconversion from e antigen positivity to negativity[20]. Documented cases of co-infection with more than one genotype of HBV were rare[18,19,21,22], and the natural progression of HBV infection in this circumstance has not been thoroughly evaluated. In one patient studied recently, infection with genotypes D and A (with D predominant) was serologically "silent" (HBsAg-negative but HBV DNA-positive), although with pathological consequences because the patient was cirrhotic and died of liver failure[18]. This patient may be of particular interest in view of our failure to detect HBsAg in the serum of our HBV DNA-positive inoculated baboons[9]. Therefore, the opportunity was taken in the present study to monitor the changes over time in the relative proportions of genotypes A and non-A in the inoculated baboons and to ascertain if the two genotypes differed in their rate of replication or in their ability to persist in the inoculated baboons.

MATERIALS AND METHODS

Samples

For this study, serum samples which were collected at 4-weekly intervals from 4 baboons infected by inoculated HBV[9] were analyzed which was started at week 8,. Because the initial phases of the study included the housing and inoculation of the baboons, the collection of serum samples were carried out at the Medical University of Southern Africa, this study was

undertook with the permission given by the Animal Ethics Committee of that institution. The Committee approved the procedures, and the care of the baboons according to its guidelines and those of the South African Medical Research Council. Each of the baboons had received intravenous injection of 1 ml pooled serum obtained from 3 HBV surface antigen (HBsAg) and HBV e antigen (HBeAg)-positive patients with clinically-overt acute hepatitis B. The concentration of HBV DNA in the pooled sera was 2 133 pg/ml (which was 2 982 pg/ml in another laboratory) using the Digene Hybrid Capture System (Digene Diagnosis Inc., Beltsville, MD, USA); the values in the individual isolates were 2 870 pg/ml, 6 660 pg/ml and 692 pg/ml, respectively. Using methods of amplification and cloning (see below), the HBV DNA amplfied from the pooled inoculum was shown to contain equal proportions of genotype A and non-A of HBV. All of the baboon sera were tested for HBsAg, anti-HBc, and anti-HBs using commercially available assays (Abbott Laboratories, Chicago, IL, USA). All serum samples were stored at -20 ℃.

PCR assay of HBV

HBV DNA levels were assessed with a quantitative PCR assay (Amplicor™ HBV monitor test, Roche Diagnostics). Briefly, 50 μl of serum were prepared with pre-treatment with polyethylene glycol, alkaline lysis of the pelleted viral particles, and neutralization of the lysate. After adding a fixed amount of internal standard and a PCR mix, 30 cycles of PCR amplification were performed according to the manufacturer' s instructions. Biotinylated amplicons were captured on strepavidin-coated microwells and hybridized with specific dinitrophenyl-labelled oligonucleotide probes. It was incubated with alkaline phosphatase conjugated anti-DNP antibodies and a colorimetric substrate, then a kinetic of O.D. determination of HBV DNA levels was performed. The limit of detection of this PCR assay was 400 copies of viral genome per ml, and quantitation was linear up to 4×10^7 copies per ml[23,24].

DNA extraction

DNA was extracted from serum using the QIAamp blood kit (Qiagen Inc., Hilden Germany), according to the manufacturer' s instructions and as previously described[25]. Known positive and negative sera, as well as best quality water were used as controls for the extraction procedure.

PCR of HBV DNA

HBV DNA in the core region was amplified using primers designed to amplify all the HBV genotypes (Table 1A and 1B). PCR was performed in 25 μL and 50 μL final reaction volumes for the first and second rounds, respectively. The reaction for the first round of the PCR consisted of 0.02 U/μL Dynazyme™ Taq DNA polymerase (version 2.0, Finnzymes OY, Espoo, Finland), 200 μmol/L of each of the nucleotide triphosphates, 1 μmol/L of each of the primers, 4 mmol/L $MgCl_2$ and 10 mmol/L Tris-HCl (pH 8.8 at 25 ℃), 50 mmol/L KCl, and 0.1 % Triton® X-100. The reaction mixture for the second round of the PCR was the same as for the first round except that 1.5 mmol/L $MgCl_2$ was used. A third round of PCR was used on the serum from those time points that were negative after 2 rounds of PCR. The reaction mixture was the same as for the second round of PCR except that concentrations of $MgCl_2$ of 1.0 mmol/L, 1.5 mmol/L, and 1.5 mmol/L, were used for the first, second, and third rounds of PCR, respectively. All PCR assays were performed in a programmable thermal cycler (Perkin Elmer, Norwalk, CT, USA) with the 3-step cycling profile shown in Table 1B. Sera positive for HBsAg, HBeAg, and HBV DNA detected by slot-blot hybridization were used as positive controls and best quality water instead of DNA as negative controls. To avoid cross-contamination and false-positive results, the precautions and procedures recommended by Kwok and Higuchi[26] were strictly adhered to DNA extraction, the various stages of PCR amplification, and gel electrophoresis were performed in physically separate venues.

Table 1A Oligonucleotide primers

		Primer	Position[a]	Sequence
Double-round PCR	PCR1	1687(+)	1687-1706	5' CGA CCG ACC TTG AGG CAT AC 3'
		2498(-)	2498-2477	5' AAG CCC AGT AAA GTT TCC CAC C 3'
	PCR2	2267(+)	2267-2284	5' GGA GTG TGG ATT CGC ACT 3'
		2436(-)	2436-2419	5' TGA GAT CTT CTG CGA CGC 3'
Triple-round PCR	PCR1	1687(+)	1687-1706	5' CGA CCG ACC TTG AGG CAT AC 3'
		2498(-)	2498-2477	5' AAG CCC AGT AAA GTT TCC CAC C 3'
	PCR2	1795(+)	1795-1812	5' TGG TCT GTC GAC CAG CAC 3'
		2436(-)	2436-2419	5' TGA GAT CTT CTG CGA CGC 3'
	PCR3	2267(+)	2267-2284	5' GGA GTG TGG ATT CGC ACT 3'
		2400(-)	2400-2382	5' CTG CGA GGC GAG GGA GTT 3'

Table 1B Polymerase chain reaction cycling profiles

		Amplification conditions			Size[b]
		Denaturation	Annealing	Extension	
Double-round PCR	PCR1	94℃ 30 sec	62℃ 40 sec	72℃ 80 sec	812bp
	PCR2	94℃ 30 sec	57℃ 50 sec	72℃ 50 sec	170bp
Triple-round PCR	PCR1	94℃ 30 sec	62℃ 40 sec	72℃ 80 sec	812bp
	PCR2	94℃ 30 sec	48℃ 60 sec	72℃ 50 sec	642bp
	PCR3	94℃ 30 sec	50℃ 60 sec	72℃ 50 sec	134bp

Notes: PCR1: the first round polymerase chain reaction; PCR2: the second round polymerase chain reaction; PCR3: the triple round polymerase chain reaction. (+) sense; (-) anti-sense. [a]Denotes the nucleotide position of hepatitis B virus *adw* genome (GenBank accession #V00866) where the *EcoRI* cleavage site is position 1. [b]Size of the amplicons in base pairs.

Detection of amplified products

A 5 μl aliquot of the amplified PCR product was electrophoresed in a 2 % agarose gel. Bands of the appropriate size (Table 1A and 1B) were visualised under ultraviolet light after ethidium bromide staining.

Cloning and nucleotide sequencing

Following the double or triple round PCR, amplicons were cloned using PCR-Script (Stratagene, La Jolla, CA, USA). Plasmid DNA was extracted using the QIAprep spin miniprep kit (Qiagen Inc., Hilden, Germany) and restricted with *Pvu II* to confirm the presence of the correct insert (Table 1A and 1B). Sequencing of positive clones was performed using the T7 Sequenase Version 2.0 DNA sequencing kit (Amersham Life Science Inc., Cleveland, OH, USA). Sequences were analyzed in both forward and reverse directions with primers T3 and T7 on 8 % glycerol-tolerant acrylamide gels and autoradiographed. The number of clones of A and non-A genotypes obtained at each time point was used as a measure of the relative proportions of the two genotypes in the serum at that time.

Statistical analysis

Fisher' s Exact test and the Chi-squared tests were used for statistical analysis, where appropriate.

RESULTS

HBV DNA was detected in the serum using either double or triple round PCR in the four inoculated baboons at various time points during the 52-week follow up period. For baboons 1 and 13 HBV DNA concentration was determined at various time of post-inoculation using the Amplicor HBV Monitor™ Test (Table 2). When serum was available for further analysis and HBV DNA was successfully amplified, the amplicons were cloned and sequenced. Genotype A was distinguished from the other genotypes by the sequence 5' CGGGAC3' (nt 2 354 -nt 2 359, numbering from the Eco R1 site) that is specific to this genotype (Figure 1). Depletion of inoculum serum prevented us from amplifying the S region and carrying out restriction fragment length polymorphisms in order to assign the non-A genotype to one of genotypes B to H. Although we could not preclude the possibility that the non-A isolates were comprised of more than one genotype, the most likely genotype would be genotype D, the genotype besides A commonly found in South Africa[14].

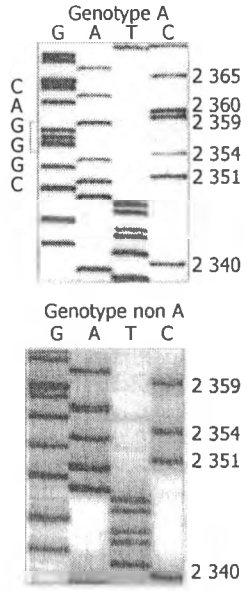

Figure 1 Sequence profiles of the core region (nucleotides 2 340-2 368 numbering from the *EcoRI* site) showing the insertion of 5' CGGGAC3' (nucleotide 2 354-2 359) found in genotype A and its absence in genotypes non-A. Tracks G, A, T and C are labelled.

Table 2 HBV DNA levels measured by the amplicor HBV monitor test

Time of post-inoculation (months)	HBV DNA Levels (genomes/ml)									
	2	3	4	5	6	7	8	9	10	10
Baboon 1	<400	<400	4 338	3 711	ns	ns	<400	ns	<400	<400
Baboon 13	<400	ns	ns	ns	ns	ns	2 6216	1 003	ns	ns

Notes: ns: no serum available.

Figure 2 showed the relative concentration of genotypes A and non-A at the various time points represented as a percentage of the total number of clones obtained and sequenced at that time point. The hypothesis of equal proportions of the genotypes over time was rejected for all four baboons ($P<0.002$ in each instance). For each specific time/baboon combination, the hypothesis that the proportions of genotype A and non-A were equal was rejected in all but 4 cases, namely, at 10 months for baboons 2 and 12, and at 11 months for baboons 1 and 13.

Genotype non-A predominated in two baboons (baboon 2 and 13) and genotype A in one baboon (baboon 1); neither genotype predominated in the fourth baboon (baboon 12), as shown at a 5 % level of testing. In the three baboons in which one or other genotype predominated, there was at least one time-point at which the non-dominant genotype was present in the highest concentration at a proportion significantly different from zero.

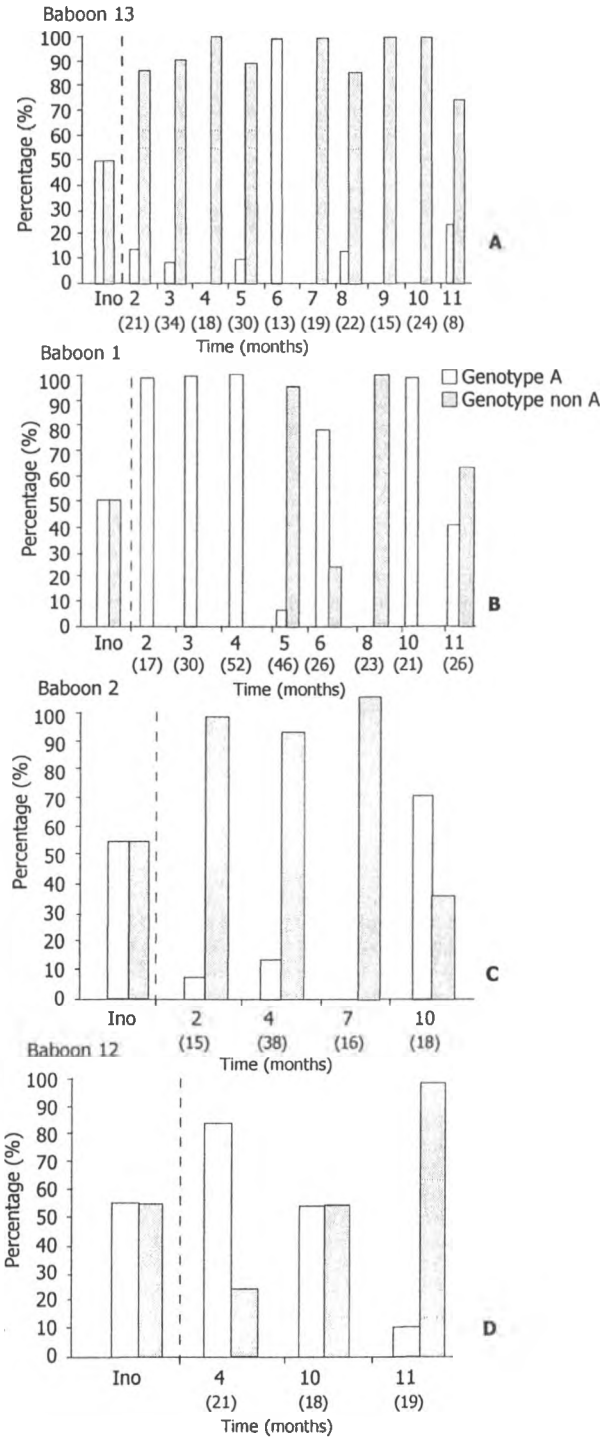

Figure 2 The change of genotype of hepatitis B virus at various time of post-infection which was represented as a percentage. The number of clones sequenced at each time point was showed in brackets. *ino: inoculum.

DISCUSSION

Neither HBsAg nor antibody to HBsAg (anti-HBs) was detected by conventional tests in the serum of the four inoculated baboons at any time during the 52 weeks they were monitored[9]. HBV DNA identical to either the A or non-A HBV genotypes inoculated was, however, detected in each baboon throughout the follow-up period, and the presence of anti-HBc and Dane particles, small spherical particles and tubular particles was demonstrated in the serum at 16 weeks[9]. The viral titers in the baboons were low (Table 2), as shown by the need to use nested PCR amplification to detect HBV DNA at all time points and three rounds of amplification at some time-points. No biochemical evidence of liver injury was evident at any stage and liver histology was normal at 52 weeks. These findings together suggested that the animals had become chronic asymptomatic carriers of the virus.

We had hoped that a clear pattern of different rates of replication of the two genotypes would be evident. However, no uniform pattern could be discerned in the relative concentration of genotypes A and non-A during the 52 weeks (Figure 2). Other explanations of a technical nature for the varying concentrations of the genotypes were needed therefore to be considered. One possibility was that the number of clones sampled at each time-point was too small to accurately assess the relative proportions of the genotypes in the serum at that time. This explanation was not supported by statistical analysis of the numbers of clones involved at each time point. A more likely explanation was that the low copy number of the genotypes resulted in sampling error at the time of HBV DNA extraction and cloning. This explanation was particularly apposite for the genotype pattern in baboon 13, in which genotype A alone was found in a single sample, whereas at all other time-points on either side of this sample, genotype non-A either predominated or was the sole genotype cloned (Figure 2A). It was also relevant that the conditions created in the study were artificial on two counts: the approximately equal concentrations of the two genotypes inoculated into the baboons resulted from the pooling of three serum samples from different patients, and we were assessing the effects of a human virus injected into a non-human primate.

Jeantet and co-workers were able to clone and sequence entire HBV genomes and found a number of mutations in the surface, precore and other regions affecting expression of the surface gene in both genotypes[18]. The A genotype was fully replication competent, although, surprisingly, this was not true of the predominant D genotype. Sequencing of the subgenomic amplicons of HBV from our infected baboons did not reveal any mutations in the core region of the HBV isolates. Full genome analysis was impossible in our study because the study was carried out retrospectively. Either the serum samples were depleted or when serum was available full genome amplification did not work, possibly because of the low viral load.

The very low concentrations of HBV in the serum of the infected baboons (Table 2) and the resulting likelihood of sampling error during viral DNA extraction and cloning prevented us from drawing firm conclusions about the natural progression over time of genotypes A and non-A replication in baboons. The study did however show that both genotypes persisted for the entire period of 52-week of follow-up.

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