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The epidemiology and molecular characterstics of hepatitis B virus infection in children from a hyperendemic area of South Africa, including a field trial of the hepatitis B immunization programme and its impact on infection in this population

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THE EPIDEMIOLOGY AND MOLECULAR CHARACTERISTICS OF HEPATITIS B VIRUS INFECTION IN CHILDREN FROM A HYPERENDEMIC AREA OF SOUTH AFRICA, INCLUDING A FIELD TRIAL OF THE HEPATITIS B IMMUNIZATION PROGRAMME AND ITS IMPACT ON INFECTION IN THIS POPULATION

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A dissertation submitted to the Faculty of Medicine,
University of the Witwatersrand, in partial fulfilment of the requirements for the degree Masters of Medicine (Virology)

DECLARATION

I declare that this dissertation is my own, unaided work and has not been submitted for any degree or examination at any other university.



Eftyhia Vardas

7 January 1999

Ethics Clearance

Informed consent was obtained from the parents/guardians of all subjects. Clearance from the Committee for Research on Human Subjects and Ethics at the University of the Witwatersrand Johannesburg was obtained, protocol number *M951029* and human experimentation guidelines as specified by this committee were followed in the conduct of the clinical research.

Permission to conduct the study was granted from the Ethics Committee at Cecilia Makiwane Hospital where the field work was done and the Department of Information, Eastern Cape Province, South Africa.

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Abbreviations

anti-HBc	-	antibodies to hepatitis B virus core antigen
anti-HBe	-	antibodies to hepatitis B virus e antigen
anti-HBs	-	antibodies to hepatitis B virus surface antigen
bp	-	base pair
cccDNA	-	covalently closed circular DNA
CI	-	confidence interval
COOH-terminal	-	carboxyl terminal
DHBV	-	duck hepatitis B virus
DNA	-	deoxy-ribose nucleic acid
DR	-	direct repeat
ELISA	-	enzyme liked immunosorbant assay
EM	-	electron microscopy
ER	-	endoplasmic reticulum
EPI	-	expanded programme for immunisation
GSHV	-	ground squirrel hepatitis virus
HBcAg	-	hepatitis B virus core antigen
HBeAg	-	hepatitis B virus e antigen
HBIG	-	hepatitis B immune globulin
HBsAg	-	hepatitis B virus surface antigen
HBV	-	hepatitis B virus
HCC	-	hepatocellular carcinoma
HIV	-	human immune deficiency virus
kb	-	kilo-base
kD	-	kilo-Daltons
LDPD	-	low dose plasma derived
ml	-	millilitre
µg	-	micrograms
NIV	-	National Institute for Virology
nm	-	nanometre
nt	-	nucleotide
NH_2-terminal	-	amino terminal
ORF	-	open reading frame
PCR	-	polymerase chain reaction
PD	-	plasma derived
pgRNA	-	pregenomic RNA
RFLP	-	restriction fragment length polymorphism
RIA	-	radioimmunoassay
RNA	-	ribo-nucleic acid
RT	-	reverse transcriptase
SD	-	standard deviation
TP	-	terminal protein
WHO	-	World Health Organisation
WHV	-	woodchuck hepatitis virus

List of Tables

Table I *Hepadnavirus* family common biological characteristics.

Table II Summary of the historical progression of HBV classification systems starting with the early serotype based subtypes and progressing to the current genotypic sequence homology based classification system.

Table III Worldwide prevalence patterns of HBV infection.

Table IV World Health Organisation recommended HBV vaccination strategies based on hepatitis B prevalence data.

Table V Proportion of HBsAg positive tests per age group, Study 1.

Table VI Proportion of HBsAg positive tests, Study 1.

Table VII a. Characteristics of study population vaccine field trial, Study 2.
b. Response rates at each time point for each vaccine group.

Table VIII Mean ages and 95% CI's of vaccinations and timing of blood specimens taken for the LDPD and recombinant vaccine groups, Study 2.

Table IX Log mean anti-HBs levels (± SD) at each time point for the LDPD and recombinant HBV vaccine groups.

List of Figures

Figure 1 - The morphology and structure of Hepadnaviral virions and subviral particles.

Figure 2 - Structure and genetic organization of the HBV genome.

Figure 3 - Schematic representation of the HBV envelope proteins.

Figure 4 - Clinical events and measurable serological responses associated with acute HBV infection.

Figure 5 - Age and Sex Distribution of Sample Study 1.

Figure 6 - HBV Genotyping Algorithm used for Study 2, showing the restriction enzyme and the fragment used.

Figure 7 - Gel electrophoresis of amplified products of Pre-S1 fragment, (161 bp). Lanes 1, 4, 15 and 17 are sterile water controls. Lane 16 is a known positive control. Molecular weight marker V was loaded on either side of the gel. The specimens in lanes 2, 3,6,7,9 and 13 were taken as positive.

Figure 8 - Gel electrophoresis of amplified products of Pre-S2 fragment, (413 bp). Lanes 3, 7, 10 are sterile water controls. Lane 16 is a known positive control. Molecular weight marker XIV was loaded on the far side of the gel. The specimens in lanes 1,2, 4,5,6,8,9,12,14,15 were taken as positive.

Figure 9 - Summary of HBV RFLP genotyping results Study 2.

Figure 10 - The proportion (%) of children who had levels of anti-HBs ≥10 mIU/ml in each vaccine group at each time interval after vaccination.

Figure 11 - Mean anti-HBs levels in unimmunised controls demonstrating the natural decay of maternally derived anti-HBs with time.

Abstract

Three studies were done in children from the Eastern Cape Province of South Africa, an area highly endemic for hepatitis B virus (HBV) infection. The objective of the first study was to investigate the epidemiology and age of acquisition of HBV infection in a community based, age stratified sample of children from 0-6 years of age (n=2299), to provide a pre-immunisation baseline measure of this infection in the population targeted for HBV immunisation in South Africa. The results suggest that there is a significant burden of HBV infection in the population targeted for immunisation (overall, 10.4% HBsAg positivity and 15.7% positive 61-72 month age group) with a high rate of chronic carriers in the early age groups of 0-6 (8.1%) and 7-12 (8.9%) months.

In the second study the HBV genotypes in a randomly selected group of chronically infected individuals from the same population (n=57) was determined. The aim was to supply information regarding the naturally circulating HBV genotypes in children from this area similarly, to provide baseline information to enable the future detection of escape mutants after low dose HBV immunisation had been introduced. The predominant HBV genotype identified was A' (85.7%), genotype D was found in 11.4% and A in 2.9% of the amplified specimens. These findings suggest a unique circulation of HBV genotypes in the Eastern Cape compared to the other genotypes currently identified in South Africa.

In the final study, the effectiveness under field conditions of a low-dose plasma derived (LDPD) HBV vaccine chosen for the EPI programme in South Africa was assessed. Children presenting for routine immunisations at 6 weeks of age were randomly assigned to receive either LDPD HBV vaccine through the normal clinic route (n=119) or a recombinant paediatric HBV vaccine (n=108) given under controlled conditions. Both vaccines were administered at 6, 10 and 14 weeks of age. At one month after the last vaccination, LDPD vaccine induced levels of anti-HBs ≥ 10 mIU/ml in 42.2% (95% CI 27.99-57.77) of immunised infants whereas recombinant vaccine induced protective antibody levels in 88.6% (95% CI 74.66-95.74) of immunised infants. These results suggest a poor performance of the LDPD vaccine under field conditions in Eastern Cape infants.

Table of Contents

Abbreviations	i
List of Tables	ii
List of Figures	iii
Abstract	iv
Table of Contents	v-vii

1. INTRODUCTION

1.1. THE VIRUS

1.1.1. Overview	1
1.1.2. Classification Morphology and Structure	2-5
1.1.3. Genome Organisation and Viral Replication	5-7
1.1.4. HBV Gene Products	7-12
1.1.5. Serotypes and Genotypes	13-15
1.1.6. Clinical Aspects and Mechanisms of Persistence of HBV Infection	15-17
1.1.7. Laboratory Diagnosis of Hepatitis B virus infection	17
1.1.8. Epidemiology of HBV infection	17-19

1.2. CONTROL OF HBV INFECTION

1.2.1. HBV Vaccines	20-23
1.2.2. HBV Vaccination Programmes	23-25
1.2.3. Vaccine Escape Mutants	25-28

Table of Contents

Abbreviations	i
List of Tables	ii
List of Figures	iii
Abstract	iv
Table of Contents	v-vii

1. INTRODUCTION

1.1. THE VIRUS

1.1.1. Overview	1
1.1.2. Classification Morphology and Structure	2-5
1.1.3. Genome Organisation and Viral Replication	5-7
1.1.4. HBV Gene Products	7-12
1.1.5. Serotypes and Genotypes	13-15
1.1.6. Clinical Aspects and Mechanisms of Persistence of HBV Infection	15-17
1.1.7. Laboratory Diagnosis of Hepatitis B virus infection	17
1.1.8. Epidemiology of HBV infection	17-19

1.2. CONTROL OF HBV INFECTION

1.2.1. HBV Vaccines	20-23
1.2.2. HBV Vaccination Programmes	23-25
1.2.3. Vaccine Escape Mutants	25-28

2. AIMS AND ORGANISATION OF DISSERTATION 29

2.1. Study 1

Pre-immunisation epidemiology of hepatitis B virus infection in South African children from the Eastern Cape Province.

2.1.1. Abstract 30
2.1.2. Introduction 30-32
2.1.3. Materials and Methods 33-35
2.1.4. Results 35
2.1.5. Discussion 36-42

2.2. Study 2

Molecular Characteristics of the circulating genotypes of HBV in chronically infected children from the Eastern Cape Province.

2.2.1. Abstract 43
2.2.2. Introduction 43-45
2.2.3. Materials and Methods 46-48
2.2.4. Results 48-49
2.2.5. Discussion 49-55

2.3. Study 3

Immunogenicity of low dose plasma derived HBV vaccine under field conditions in South African infants from an area of highly endemic for HBV infection.

2.3.1. Abstract 56-57
2.3.2. Introduction 58-60
2.3.3. Materials and Methods 61-63
2.3.4. Results 63-66
2.3.5. Discussion 66-77

3.	CONCLUDING DISCUSSION	78-79
4.	REFERENCES	79-101
5.	APPENDIX I	102

1. Introduction

1.1. The Virus

1.1.1. Overview

Infection with hepatitis B virus (HBV) poses a significant public health problem in many parts of the world, particularly in sub-Saharan Africa [Kiire, 1993] despite the availability of safe and effective vaccines. Worldwide more than 300 million people are estimated to be chronic carriers of the virus and about 30% of these carriers who survive for 30 years or more may subsequently die of the chronic sequelae of HBV infection, cirrhosis and hepatocellular carcinoma (HCC) [Maynard, 1990].

In 1965 Blumberg [Blumberg, et al., 1965] identified a new antigen in leukemic sera of native Australians. This "Australian antigen" was later shown to be the surface antigen of HBV (HBsAg). In 1970, the complete HBV particle could be isolated from patients' serum and visualised by electron microscopy [Dane, et al., 1970]. Although, subsequently HBV was intensively studied, early investigations were limited to descriptive studies of the virus, it's associated antigens and characterisation of the serological responses identified with viral infection [Hoofnagle, 1981]. Since the 1970's significant progress has been made in understanding the molecular biology, replication, epidemiology, natural history, transmission and public health importance of HBV with over 1000 articles being published per year on this virus alone [Gerlich, 1995]. The discovery of natural animal models of HBV infection coupled with the advent of techniques for molecular cloning of the viral genome transformed the understanding of the life cycle of this virus and led to a significant turning point in the history of medicine; the development of the first successful recombinant vaccine for a human infectious disease.

1.1.2. Classification, Morphology and Structure

The *Hepadnaviridae* are a family of small (3.0-3.3 kb) hepatotropic DNA viruses that can infect a variety of extra-hepatic sites and may persistently infect liver cells, leading to chronic hepatitis and in some cases to hepatocellular carcinoma [Ganem, et al., 1994]. The prototype hepadnavirus is human HBV. The first non-human hepadnavirus to be discovered was the woodchuck hepatitis virus (WHV). This virus was first detected in a colony of captive woodchucks (*Marmota monax*) in which chronic hepatitis and hepatoma were frequently found at necropsy [Summers, et al., 1980]. Subsequently, a series of similar viruses were recovered from a variety of animal species, including the ground squirrel (ground squirrel hepatitis virus, GSHV), the Pekin duck (duck HBV or DHBV), wild herons, domestic geese, marsupials and other hosts [Ganem, 1996]. All these viruses share many similar biological properties (*Table I*), however there are important distinctions among the members of this family particularly between the avian and mammalian viruses. Compared to the mammalian hepadnaviruses the avian viruses generally have smaller viral genomes, share little primary nucleotide (nt) sequence homology, lack the X open reading frame (ORF) and code for two rather than three surface envelope proteins [Mandart, et al., 1984].

HBV is unusual among animal viruses because infected cells produce multiple types of virus-related particles. Electron microscopy (EM) of partially purified HBV preparations show three types of particles: a) 42-47 nm double-shelled particles (hepatitis B virus particles, also known as Dane particles), b) 20 nm spheres, usually vastly in excess of the Dane particles and c) smaller quantities of filaments of 20 nm diameter and variable length (*Figure 1*). Both the 20 nm spheres and filaments constitute excess HBsAg not incorporated into full virus particles. The 42 nm infectious virion consists of a 27 nm icosahedral nucleocapsid (the hepatitis B core antigen, HBcAg) and a 7 nm lipoprotein bilayer derived from the endoplasmic reticulum of the host cell [Yoffe and Noonan, 1993].

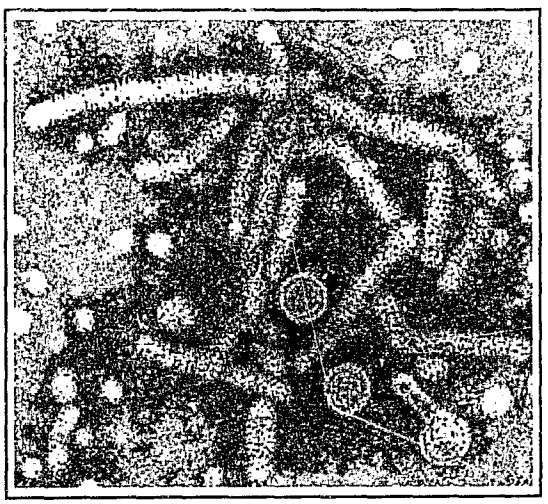
Table I *Hepadnavirus* family common biological characteristics. [from ref. Ganem, 1996]

	HBV	WHV	GSHV	DHBV
***Genome* (kb)**	3.2	3.3	3.3	3
ORF's	S, C, P, X	S, C, P, X	S, C, P, X	S, C, P
Hosts	humans chimpanzees	woodchucks	ground squirrels woodchucks chipmunks	ducks geese
Replication	liver kidney pancreas WBC	liver kidney pancreas WBC	liver	liver kidney pancreas spleen
Diseases	AC hepatitis cirrhosis HCC	AC hepatitis cirrhosis HCC	AC hepatitis HCC	AC hepatitis

HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; GSHV, ground squirrel hepatitis virus; DHBV, duck hepatitis B virus; kb, kilobase; ORF, open reading frame; WBC, white blood cells; AC, asymptomatic carrier; HCC, hepatocellular carcinoma.

Figure 1 Electron micrograph of HBV particles, including virions, 20 nm spheres and filaments.

(Reproduced from Linda Stannard's University of Cape Town, Department of Virology internet page: http://www.uct.ac.za/depts/mmi/stannard/linda.html).



HBV virion DNA is a relaxed circular, partially duplex species of 3.2 kb whose circularity is maintained by 5′ cohesive ends (*Figure 2*) [Ganem, et al., 1994; Ganem, 1996; Kramvis and Kew, 1998]. This molecule is unusual in that its two DNA strands are not perfectly symmetrical. The minus (-) strand is unit length and has protein covalently linked to its 5′ end [Gerlich and Robinson, 1980]. The plus (+) strand is less than unit length and has a capped oligoribonucleotide at its 5′ end [Lien, et al., 1986]. The positions of the 5′ ends of both strands map to the regions of short (11 nt) direct repeats (DR's) in viral DNA [Ganem, 1996]. The 5′ end of the minus strand DNA maps within the repeat called DR1, while the plus strand DNA starts within DR2 (*Figure 2*). These repeats are essential in priming the synthesis of their respective DNA strands.

1.1.3. Genome Organisation and Viral Replication

Molecular cloning of HBV DNA extracted from Dane particles reveals an extremely compact genomic coding organisation, every nt in the genome is found within a coding region and over 50% of the sequence is translated in more than one frame [Ganem, et al., 1994]. As shown in *Figure 2,* four open reading frames (ORFs) are present in the DNA. The viral polymerase, the central enzymatic activity in genomic replication as well as two additional proteins required for viral replication, the terminal protein (TP) and RNase H are encoded by the P gene. The C (core) region encodes the structural protein of the nucleocapsid (HBcAg) and the "e "antigen (HBeAg) [Pasek, et al., 1978]. ORF S/Pre-S encodes the viral surface glycoproteins [Valenzuela, et al., 1979]. The product of the fourth ORF, ORF X is a poorly understood regulatory protein which appears to enhance the expression of both homologous and heterologous genes *in vitro* and may play a role in hepatocellular carcinogenesis.

Important regulatory elements have been identified in hepadnaviral genomes (*Figure 2*); one designated ENH or enhancer I, is located immediately upstream of the X ORF within the P coding region and the other (designated enhancer II lies upstream of the

C promoter [Yoffe and Noonan, 1993]. A region of the genome which is required for a fivefold enhancement of gene expression following glucocorticoid treatment has been designated the glucocorticoid-responsive element (GRE) [Ganem, 1996]. This element functions independently of genome orientation, lacks species or cell type specificity and contains an 18 base pair (bp) sequence that is homologous to other known glucocorticoid response elements [Yoffe and Noonan, 1993]. Also a packaging signal called ε (epsilon) has been identified and mapped near the pre C/C ORF junction, a region which contains a highly conserved, 60-70 bp sequence with a high degree of homology to the U5 region of retroviral long terminal repeats (LTR). In retroviruses U5 plays a role in the packaging of the genome and may similarly influence the selective packaging of HBV genomic material [Hilleman, 1994; Kramvis and Kew, 1998].

These observations regarding the organisation of the HBV genome, derived from animal models and molecular cloning techniques, have permitted the formulation of a model of HBV replication [Summers, et al., 1982]. Following receptor binding, virions deliver their nucleocapsids to the cytoplasm. The nucleocapsids then translocate to the nucleus, where the relaxed circular viral DNA is converted to a covalently closed circular DNA (cccDNA) by a number of complex steps requiring completion of plus strand DNA synthesis, dissociation of the terminal proteins from both strands and removal of the terminal redundancy from the minus strand as well as the oligo-RNA primer from the plus strand. HBV replicates by reverse transcription of a 3.5 kb RNA intermediate, pregenomic RNA (pgRNA) which is transcribed from cccDNA in hepatocyte nuclei. PgRNA is bifunctional, serving as a template for both translation and reverse transcription. The transcription of cccDNA is by host RNA polymerase II enzyme. The resulting RNA's are translated to give rise to the P, C, pre-S/S and X gene products.

Both the C and P proteins are translated from full length pgRNA, whereas subgenomic

RNA's encode the S and X proteins. Before being reverse transcribed, pgRNA is sequestered from the cytoplasm by being packaged, together with the polymerase, into subviral particles composed of the core (C) protein. For pgRNA to be encapsidated, its 5′ end is folded into a stem loop structure known as the encapsidation signal or epsilon (ε) which is recognised by the viral polymerase [Kramvis and Kew, 1998]. The polyadenylation signal, TATAAA (*Figure 2*) located in the C ORF is required for the correct polyadenylation and processing of all of the HBV transcripts which occurs in the hepatocyte cytoplasm. Within the subviral particles, viral DNA synthesis is initiated following minus strand synthesis and concomitant degradation of the RNA template. Plus strand DNA synthesis then occurs. Once genomic DNA synthesis is completed, progeny cores bud into the intracellular membranes, primarily the endoplasmic reticulum (ER) and Golgi to acquire their glycoprotein envelope. Enveloped virions are then secreted via vesicular transport mechanisms.

1.1.4. HBV Gene Products

Surface Proteins

The S ORF contains three in-frame initiation codons and encodes the three structurally related envelope proteins of HBV, the large (L), middle (M) and small (S) HBV surface proteins. Historically, the sequence between the first and the second start site has been termed pre-S1 (108 or 119 amino acids, depending on subtype) and the sequence between the second and third start site pre-S2 (55 amino acids) [Prange et al, 1995]. The L (39 kD) protein covers the entire reading frame and is produced by initiation at the first initiation codon. Initiation at the second in frame initiation codon produces the M protein. The S protein, which is 226 amino acids (aa) long, is produced by initiation at the inner-most in frame initiation codon. The organisation of the three envelope proteins

Figure 2 Genetic organisation of the HBV genome. The broad arrows represent the four ORF's; P (blue), S (red), C (green) and X (yellow). The locations of DR1 and DR2, the major enhancer, GRE, the glucocorticoid response element, U5-like domain shared by some retroviruses are also shown.

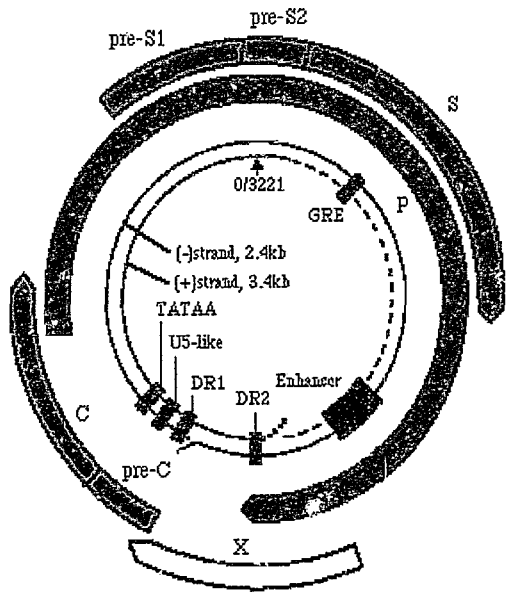
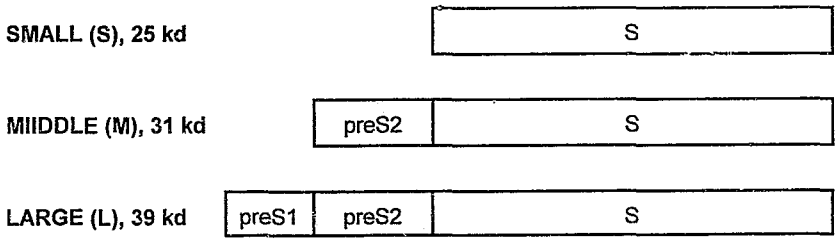


Figure 3 Schematic representation of the HBV envelope proteins showing the relative sizes in kilodaltons (kd) and coding regions included in each protein.



is schematically presented in *Figure 3*. Both the L and M proteins share a common carboxyl (COOH) terminal and differ mainly by the length and structure of their amino (NH_2) terminal or pre-S extensions. Classic hepatitis B surface antigen (HBsAg), which contains only the S domain is also referred to as the S protein. Each protein exists in two forms, differing only by the presence or absence of S-domain glycosylation.

Complete infectious virions (Dane particles) contain L, M and S in approximately equimolar amounts. The subviral 20 nm spheres are composed of mainly the S protein with variable amounts of the M protein. However, the filamentous 20 nm forms also carry some L protein. Both pre-S encoded polypeptides are quantitatively minor components of the circulating pool of S-related antigens, with M protein accounting for 5-15% of the total and L only 1-2% of the total. The L and M proteins appear to mediate binding of the virus to hepatocytes and L may also regulate the nuclear pool of cccDNA [Prange, et al., 1995]. The L, M and S proteins also appear to participate in the generation and secretion of enveloped virus [Ganem, 1996].

Surface protein biosynthesis and primary structure are discussed in further detail because of the importance of the S protein in the immunological response to HBV infection, the manufacture of recombinant vaccines and the serotyping and genotyping of the virus. All the surface proteins are synthesized in the endoplasmic reticulum (ER) as integral membrane proteins. In cell-free translation systems, nascent surface protein chains are found to be orientated in the lipid bilayer such that both the NH_2 and COOH terminals are in the vesicle lumen. Accordingly, the surface proteins can be divided into three hydrophobic and two hydrophilic regions. This transmembrane topology relative to the lipid bilayer is determined during synthesis by two signal sequences in the aa chain, signal I (sigI), residues 11-29, at the NH_2 terminal and signal II (sigII), residues 80-98, at the COOH terminal [Bruss, et al., 1996]. Between these two signal sequences, forming a cytoplasmic loop, is the sequence 120-160 aa which forms the

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HBV specific major antigenic determinant "a" which is common to all serological subtypes of HBV [Berting, et al., 1995]. All characterised HBV subtypes have mutations in the stretch between residues 120-160 [Ganem, 1996], however the most important parts of this conformational determinant are between residues 121-147, with residue 146 being N-glycosylated in 30-40% of the surface proteins [Ashton-Rickardt and Murray, 1989].

The S protein structure discussed above is common to all three of the HBV surface proteins. However, in addition to this structure the M protein carries a further NH_2-terminal 55 aa, the pre-S2 domain and the L protein has the entire pre-S region, pre-S1 and pre-S2, of 119 aa added to the NH_2-terminal [Bruss, et al., 1996].

Core and e Proteins

The C ORF contains two in-frame initiation codons which divide it into the pre-C and C domains. Translation initiation at the first initiation codon results in the synthesis of a precursor polypeptide (25 kD), the first 19 aa of which are a signal peptide derived from the pre-C area [Ou, et al., 1986]. The signal peptide targets the nascent protein for insertion into the ER where cleavage of the peptide occurs [Yoffe and Noonan, 1993]. The majority of the resulting protein (p23) is translocated into the ER lumen and proceeds through the secretory pathway where proteolytic cleavage of the COOH-terminal domain generates the final secreted product, the 16-18 kD hepatitis e antigen (HBeAg). [Ou, et al., 1986]. The function of HBeAg remains unclear, although high titres of the antigen are detected in the serum of viraemic individuals.

The hepatitis B core antigen (HBcAg) is a 22 kD phosphoprotein that is produced by translation initiation at the second initiation codon of the pre-C/C ORF. HBcAg is the major polypeptide of the nucleocapsid. HBcAg can self assemble into subviral particles in the absence of the other virion proteins.

Polymerase Proteins

The DNA polymerase or P protein is a virus associated enzyme coded for by the P ORF. The polymerase is multi-functional and has three independent domains which have been mapped along the protein from the NH_2-terminal to the COOH-terminal; the terminal protein TP, DNA and RNA dependant DNA polymerase and RNase H. The TP acts as the primer for reverse transcription and is found covalently linked to the 5 ′ end of minus strand DNA. The enzymatic activities associated with the P protein are responsible for the reverse transcription of pgRNA to minus strand DNA, concomitant degradation of the RNA template and synthesis of plus strand DNA [Ganem, 1996]. The P protein is also required as a structural component for the packaging of pgRNA into immature core particles.

The X Protein

The X ORF is conserved in all mammalian hepadnaviruses. The gene product, X, is a 16-18 kD protein which has been shown to have novel, intrinsic, protein kinase activity capable of auto-phosphorylation [Schek, et al., 1991]. In transient transfection assays, X protein can act as a transcriptional *trans*-activator, positively regulating a wide range of cellular and viral promoters, including ENH I and the S gene promoter [Yoffe and Noonan, 1993]. Details of the biosynthetic pathways involved in X protein synthesis are at present not clear, however further important biochemical properties of this protein have recently been elucidated. The X protein does not appear to have a sequence-specific DNA binding ability, it activates its own gene transcription and the tumour suppressor gene product p53 can repress X gene transcription [Koike, 1995]. It has, therefore, been postulated that one of the mechanisms of hepatocellular carcinogenesis is that the X protein inhibits hepatic serine protease activity, thus eliminating the suppressor effect of p53 on the basic transcription machinery in the hepatocyte nucleus.

1.1.5. Serotypes and Genotypes

The serologic heterogeneity of HBV has been established from early immunodiffusion experiments [Levene and Blumberg, 1969; Raunio, et al., 1970], although the fact that these forms represented genetically stable variants of the virus was not recognised until later. *Table II* summarises the historical progression in HBV classification systems, which were initially based on serological determinants and have evolved to the present day system of genotyping based on nucleotide sequence homology of the S gene.

Four serotypes called subtypes of the HBsAg were initially defined by two mutually exclusive determinant pairs *d/y* and *w/r* and a common "a" determinant [Le Bouvier, 1971; Bancroft, et al., 1972]. By subdivision of the four major subtypes in the mid-1970's, nine different subtypes were identified [Couroucé and Soulier, 1975; Magnius, et al., 1975]. (*Table II*). However, the value of HBV sequence data in tracing routes of infection and establishing phylogenetic relationships was soon realised and a genetic classification based on the comparison of complete genomes defined four groups of HBV, later called genotypes, A-D [Okamoto, et al., 1988]. The interrelation of the nine subtypes to these genotypes, the possible presence of more than four human genotypes as well as their global geographical prevalence remained to be determined. By sequencing the S-gene of HBV, the molecular basis for the serological variations of HBsAg was assessed [Norder, et al., 1993]. In this way, two new genotypes designated E and F were identified. F is the most divergent of HBV genomes sequenced as yet, and differs by 14 % from other HBV genomes. When the worldwide molecular epidemiology

Table II Summary of the historical progression of HBV classification systems starting with the early serotype based subtypes and progressing to the current genotypic sequence homology based classification system.

Study and Date	HBV Types	Classifying Characteristic
Le Bouvier, 1971 Bancroft, 1972	4 subtypes; *adw, ayw, adr, ayr.*	"a" determinant and two pairs of allelic variations; *d/y* and *w/r*
Couroucé, 1975	8 subtypes; *ayw1, ayw2, ayw3, ayw4, adw2, adw4, adr, ayr.*	four sub-determinants of "a", later redefined as w1-w4.
Magnius, 1975	9 subtypes; *ayw1, ayw2, ayw3, ayw4, adw2, adw4, adr, ayr,* ***adrq+, adrq-.***	acquisition of q determinant
Couroucé-Pauty, 1983	9 subtypes plus 2 compound types; *ayw1, ayw2, ayw3, ayw4, adw2, adw4, adr, ayr, adrq+, adrq-,* ***adyr, adwr.***	8 existing subtypes confirmed by monoclonal antibody studies and sequencing with the addition of two compound subtypes.
Okamoto, 1988	4 genotypes; A, B, C, D.	based on divergence of > 8% of entire genome sequence
Norder, 1993	6 genotypes; A, B, C, D, E, F	based on divergence of genomic sequence of S gene and inclusion of worldwide HBV samples

of HBV based on the variability of the S gene was defined, E and F seemed to originate in West Africa and South America respectively. The predominant genotype in Africa and North West Europe is A; D is commonly found in Mediterranean countries, the Middle East, Indonesia and Alaska; C and B are mainly found in the Far East. This distribution is not exclusive and a variety of genotypes may be found in certain populations, particularly with the current increased mobility of human populations with modern travel. The significance of potential biological differences between genotypes and their impact on the clinical and immunological responses of the host to HBV infection, remains to be described. Also, of particular importance in the molecular epidemiology of HBV is the potential for the appearance of vaccine HBsAg escape mutants with the increasing use of global HBV vaccination programmes.

1.1.6. Clinical Aspects and Mechanisms of Persistence of HBV Infection

Acute, primary infection with HBV in susceptible adults results from sexual contact with an infected individual or from parenteral or mucosal exposure to virus-containing blood or blood products [Ganem, 1982; Wright and Lau, 1993]. Primary infection is usually mild and the risk of chronicity is less than 5% in immunocompetent adults. However, this risk of chronicity is greatly increased in the newborn (up to 90%) and the immunocompromised, such as transplant recipients or those concomitantly infected with human immune deficiency virus (HIV) [Wright and Lau, 1993].

Accordingly, in the vast majority of adult acute hepatitis B infections and in approximately 10% of infections in infants, host immune responses to viral antigens results in the clearance of infected cells from the liver and the removal of free virions from the blood. The removal of circulating free virions is usually achieved by antibodies generated by the host humoral immune response mechanisms whereas the cellular immune response to envelope, nucleocapsid and polymerase antigens removes infected cells [Chisari, 1995]. Very rarely, acute infection may lead to fulminant liver

failure with a case fatality close to 80% [Hollinger, 1996].

Chronic HBV infection, in both adults and infants who do not resolve the infection and go on to chronic or persistent HBV infection, is characterised by active viral replication in hepatocytes with varying levels of viraemia. Clinically, chronic infection may range from being completely asymptomatic to severe chronic injury and inflammation of the liver. Asymptomatic carriers are of major epidemiological importance since they act as a reservoir for HBV infection [Ganem, 1996]. Additionally, chronic hepatitis B patients that survive for more than 30 years have a markedly increased risk of hepatocellular carcinoma [Beasley, et al., 1981; Beasley, 1988].

It has been suggested that both host and viral factors contribute to chronic HBV disease [Meuer and Moebius, 1994]. HBV itself is not directly cytopathogenic and clinical symptoms of infection result from the destruction of virally infected hepatocytes by host T lymphocytes [Chisari, 1995]. Both class-I and class-II restricted T cell responses to HBV are vigorous, polyclonal and multi-specific in acutely infected patients who clear the infection. However these responses are weak and narrowly focussed in patients who are chronically infected This narrow response has been postulated to be one of the main host mechanisms of persistence in chronic HBV infection [Chisari, 1995] and is illustrated by the tolerance to HBV infection after vertical transmission in the newborn. In this situation, HBV transmission occurs mainly at the time of delivery, when the newborn is exposed to large quantities of viraemic maternal blood during passage through the birth canal. The cellular immune response of the neonate is incompletely developed at birth, thus limiting its cellular response to HBV, resulting in almost 80-90% chronic infection [Ganem, 1996; Stevens, et al., 1975].

However, viral factors also may contribute to chronic HBV infection and it has been suggested that certain viral variants are specifically associated with chronicity [Meuer

and Moebius, 1994]. Other viral factors which influence HBV persistence have also been suggested and include viral evasion by epitope inactivation, T cell receptor antagonism, incomplete down regulation of viral gene expression and the infection of immunologically privileged sites [Chisari, 1995].

1.1.7. Laboratory Diagnosis of HBV Infection

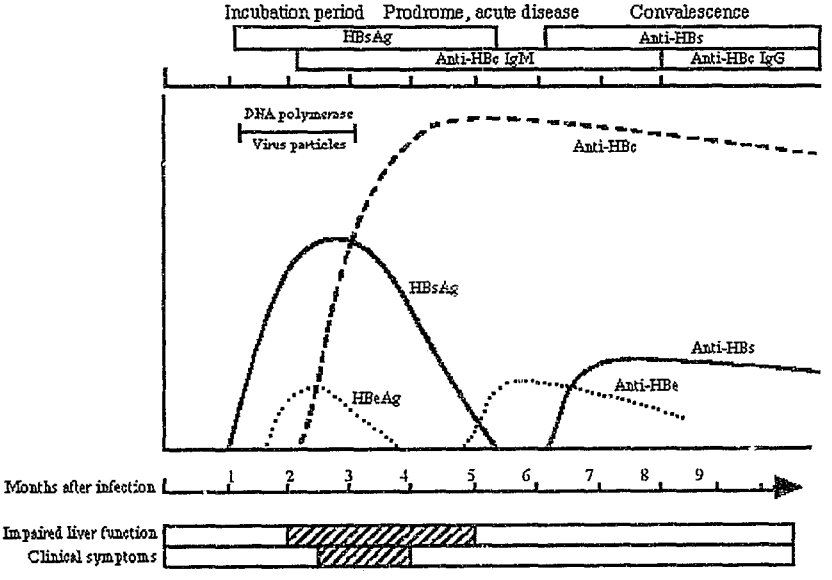
The laboratory diagnosis of HBV infection is dependant on the detection of various markers in the blood by radioimmunoassay (RIA), the gold standard for most HBV markers, or more recently by the much simplified technique of enzyme linked immunosorbant assay (ELISA).

Figure 4 illustrates the clinical course of acute HBV infection and the various markers that can be detected at each stage of infection incubation, prodromal period and convalescence. Assays for the following HBV markers currently exist HBsAg and anti-HBs antibody, HBeAg and anti-HBe, anti-HBc IgG and IgM. Persistently infected individuals can also be detected routinely by measuring the presence of HBsAg and HBeAg. Any individual who has HBsAg persisting for longer than 6 months is considered a carrier of HBV. Highly infectious carriers in addition to circulating HBsAg also have measurable HBeAg levels. In both types of carriers HBV DNA can be measured, but highly infectious carriers usually have higher levels of circulating viral DNA [Edmunds, et al., 1996 i].

1.1.8. Epidemiology of HBV Infection

HBV infection is the most important chronic infection of humans [Kiire, 1993; Maynard, 1990; Robson, et al., 1991]. The worldwide patterns of HBV prevalence and the status of HBV immunisation programmes are shown in *Table III*. The incubation period of HBV

Figure 4 Clinical events and measurable serological responses associated with acute HBV infection.



infection is 60-180 days. Three main routes of HBV transmission occur sexual, parenteral and vertical [Robson, et al., 1994]. HBsAg has been found in blood and blood products, saliva, seminal fluid, vaginal exudates, menstrual blood and serous exudates. Infection can, therefore occur from sexual contact, infusions of contaminated blood or blood products, intravenous drug use and occupational exposure in health care workers. In areas of low HBV endemicity, the predominant mode of HBV transmission appears to be sexual or parenteral with high risk individuals (health care workers, intravenous drug users, immunocompromised hosts, sexually active individuals) being targeted for immunisation [Edmunds, et al., 1996 i].

In areas of high and intermediate endemicity, the majority of HBV infections occur in the perinatal period, during infancy and childhood. Two main HBV transmission patterns have been described. Perinatal infection predominates in Asia, were most HBsAg positive mothers have circulating HBeAg and the risk of infant chronic infection is almost 90% [Stevens, et al., 1975]. Whereas in Africa and the Mediterranean basin where the proportion of HBeAg positive mothers is much lower, infection is transmitted mainly by chronically infected family members and playmates during infancy and childhood with a lower risk of chronicity of between 20-30% [Tabor, et al., 1985]. However, any infection acquired early in life carries the risks of chronic evolution, and thus the risks of expansion of the asymptomatic carrier pool and ultimately the risk of hepatocellular carcinoma [Bortolotti, 1994].

Other HBV transmission mechanisms have also been postulated in Africa, these include transmission via blood sucking insects (mosquitos and bedbugs) [Jupp, et al., 1983], tattooing, ritual scarification [Kew, et al., 1973], ritual circumcision, ear piercing and shared use of toothbrushes and razors [Kiire, 1993; Robson, 1991; Robson, 1994]. However these mechanisms of infection remain unclear, and have not been confirmed.

1.2. Control of HBV Infection

1.2.1. HBV Vaccines

HBV vaccines have two purposes: to prevent the morbidity and mortality associated with acute infection and to reduce the occurrence of chronic liver disease and hepatocellular carcinoma. First generation plasma derived (PD) HBV vaccines were introduced in the early 1980's [Szmuness, et al., 1980] and contain the small envelope protein (S) of HBV as 22 nm subviral particles. As discussed in sections *1.1.4.* and *1.1.5.*, the S protein contains the principal HBV surface antigen (HBsAg), which is made up of the common group determinant ("a") and several allelic subtype determinants [Berting, et al., 1995; Couroucé, et al., 1975; Magnius, et al., 1975]. Antibodies to HBsAg, anti-HBs, are neutralising and because of the shared "a" determinant are cross protective among different subtypes [Murphy, et al., 1974; Chisari,1995]. However, single substitutions in the S gene may alter expression of the "a" determinant resulting in reduced neutralisation by anti-HBs [Waters, et al., 1992]. This phenomenon is discussed in detail in the section *1.2.3.* which deals with vaccine escape mutants.

Two main types of HBV vaccine have been used widely: the first generation PD vaccines which are made from heat or chemically inactivated subviral particles derived from plasma collected from chronic carriers of HBsAg and second generation HBV recombinant vaccines in which HBsAg particles are expressed from recombinant DNA in the yeast, *Saccharomyces cerevisiae*. The HBsAg expressed in yeast is non glycosylated and is prepared for vaccine by physico-chemical purification, adsorption onto aluminium hydroxide as an adjuvant and preserved with thimerosal [Lemon and Thomas, 1997]. Recombinant products, first introduced in 1987, were found to be indistinguishable in immunogenicity, safety and efficacy from the first generation PD HBV vaccines in large clinical trials [Scolnick, et al., 1984]. Newer recombinant vaccines, called third generation HBV vaccines, which contain both pre-S1 and pre-S2

antigens expressed in yeast or stably transformed mammalian cell lines have recently also been produced [Katkov, et al., 1994; Moulia-Pelat, et al., 1994]. Third generation vaccines were developed in the hope that they would be more immunogenic, particularly in persons who do not respond to conventional vaccines [Katkov, et al., 1994]. Experiments in mice and early Phase II trials in humans have shown that pre-S1 and pre-S2 HBV vaccines produce strong humoral and cellular immune responses [Jones, et al., 1998].

Both immunisation factors and host factors may influence the immunogenicity of HBV vaccines (PD and recombinant). Host factors which are important are age, sex, background prevalence of HBV infection, genetic factors and immunological competence [Hollinger, 1996]. Immunisation factors include dosage of antigen administered, storage of the vaccine, administration of concurrent Expanded Programme for Immunisation (EPI) vaccines, site and route of inoculation and timing of inoculations [Katkov, et al., 1994; Lemon and Thomas, 1997; Maillard and Pillot, 1998]. HBV vaccine unresponsiveness is seen in the vast majority of immunocompromised persons, such as those on chronic renal dialysis, and renal transplant recipients [Stevens, et al, 1984; Jacobson, et al, 1985]. However, 5% of immunocompetent adults immunised with HBV vaccine do not acquire anti-HBs and, therefore do not respond to immunisation [Szmuness, et al., 1980; Dienstag, et al., 1984]. Vaccine unresponsiveness in immunocompetent persons may be linked to genetic factors since some studies have suggested that there is a specific gene which controls responsiveness to HBsAg [Craven, et al., 1986; Alper, et al., 1989]. A similar immune response gene appears to account for humoral HBsAg responses in mice which controls the response to the S protein of HBV [Milich, et al., 1985]. Response to the pre-S regions in mice are controlled by a different gene so that addition of these regions on the vaccine antigen may increase the overall humoral response [Katkov, et al., 1994].

Protective serum titres of anti-HBs (≥10 mIU/ml) develop in 95-90% of healthy infants, children and young adults who receive a series of three intramuscular injections [Andre and Zuckerman, 1994; Lemon and Thomas, 1997; Hollinger, 1996; Resti, et al., 1997] . However, this response is reduced in persons greater than forty years of age, the obese, immunocompromised individuals or those who smoke [Hollinger, 1996]. The recommended dosages and immunisation schedules for HBV vaccines vary according to age and immune status; patients on haemodialysis or those that are immunocompromised in any way should be given higher doses [Lemon and Thomas, 1997]. Various immunisation schedules have been tested with HBV vaccines [Jilg, 1989] and in general include a booster dose four to six months after primary immunisation in order to achieve higher antibody titres. Administering three doses on an accelerated schedule, 0, 1 and 2 months, should result in more rapid antibody production but may reduce the peak titres achieved [Hadler, et al., 1989; Jilg, et al., 1989]. Approximately 50% of individuals who do not develop anti-HBs after three doses of HBV vaccine will do so after one or two additional doses [Hadler, et al., 1986; Hadler, et al., 1992]. The immunogenicity of HBV vaccines is not reduced when administered simultaneously (at different sites) with hepatitis B immune globulin (HBIG) and similarly when administered with other vaccines [Lemon and Thomas, 1997].

Well designed clinical trials have demonstrated the efficacy of both PL and recombinant second generation HBV vaccines. Immunisation reduced the incidence of HBV infection in homosexual men by 90-95% [Szmuness, et al., 1980; Szmuness, et al., 1981; Francis, et al., 1982] and in healthcare workers frequently exposed to infected blood [Szmuness, et al., 1982]. Immunisation has been similarly shown to reduce rates of HBV infection in infants born to HBsAg positive mothers, particularly if administered in conjunction with HBIG at birth [Stevens, et al., 1987; Andre and Zuckerman, 1994; Chen, et al., 1996]. Furthermore HBV immunisation programmes have also been shown to decrease the incidence of hepatocellular carcinoma in children from Taiwan [Chang, et al., 1997] and

early immunisation lowers the prevalence of HBsAg carriers among children from areas highly endemic for this infection [Whittle, et al., 1995].

HBV vaccine protection is evident within weeks after the first two doses in adults, and in large prospective studies is correlated with anti-HBs titres greater or equal to 10 mIU/ml [Szmuness, et al., 1980; Szmuness, et al., 1981; Francis, et al., 1982; Hollinger, 1996; Gesemann and Scheiermann, et al., 1995]. Symptomatic HBV infection is very rare in immunised persons with anti-HBs 10 mIU/ml or higher, although there is eventual loss of detectable antibody in over 50% of these persons within 5 to 10 years after immunisation [Goldfarb, et al., 1994]. The duration of anti-HBs antibodies is directly proportional to the initial titre obtained after the primary immunisation schedule of three doses of vaccine [Gesemann and Scheiermann, 1995]. However, even though anti-HBs levels may eventually decline, there may be a strong anamnestic response in children for almost 10 years after vaccination [West, et al.,1994]. Furthermore, although there is currently no direct experimental proof, cellular immune memory may also exist depending on the quality of the original antigen used in the primary vaccination schedule [Katkov, et al., 1994; Jones, et al., 1998].

HBV vaccines are among the safest vaccines available [Hadler, et al 1992], with few side effects ever having being reported apart from injection-site tenderness which occurs in approximately 22% of vaccine recipients. Anaphylaxis is rare, but has been reported [Stratton, et al., 1994] and Guillain-Barré syndrome has also occurred in vaccine recipients, however the precise cause of this side-effect is unknown at present. The only contraindication to HBV vaccine administration is hypersensitivity to yeast or to a component of the vaccine.

1.2.2. HBV Vaccination Programmes

Systematic immunisation on a worldwide scale was not recognised as a practical

possibility until 1974, when WHO launched its EPI initiative. Six vaccine preventable infections in infants and children were initially targeted; diphtheria, tetanus, pertussis, polio, measles and tuberculosis [World Health Organisation, 1996]. The EPI underwent revision in 1993 with the addition of HBV and yellow fever vaccines as well as supplements of vitamin A and iodine. These additions were made based on cost-benefit analyses from the *World Bank* which showed that this "package" of vaccines and supplements had the highest cost-effectiveness in children of any health measure currently available [World Health Organisation, 1996]. Currently, 80% of the world's children receive at least the original six EPI vaccines [Bland and Clements, 1998]. However because of the high cost of HBV and yellow fever vaccines, their integration into the EPI programme remains poor, particularly in developing countries [Kane, 1996].

The purpose of HBV vaccination is to interrupt transmission and ultimately eliminate HBV from populations in the world. The vaccine coverage level and herd immunity required in a population to arrest HBV transmission are not know [Anderson and May, 1985; Anderson and May, 1990]. However, based on a model developed in The Gambia, it has been suggested that HBV vaccine coverage of 70% may be adequate to interrupt transmission of this virus in a population [Edmunds, et al., 1996 ii].

There are a number of vaccination strategies which may be implemented once the decision to introduce HBV immunisation as a national programme has been made. The choice of strategy is largely dependant on the HBV epidemiology in the country, which is dependant on socioeconomic factors, the proportion of individuals with high risk life styles, the pre-existing prevalence of HBV and the availability of vaccination programme resources [Grob, 1995]. WHO recommendations for HBV vaccination strategies based on HBV prevalence data are shown in *Table IV* . In countries with an HBV carrier prevalence $\geq$ 2%, the use of four doses of vaccine, with the first at birth preferably with the concurrent use of HBIG has been found to be the most effective HBV immunisation

strategy. However, financial constraints in some African countries with ≥ 2% HBsAg carrier rates have not allowed this option to be considered. The HBV vaccination programme adopted by South Africa in April 1995 [Department of Health, 1995] is an accelerated programme based on the existing EPI schedule of 6,10 and 14 weeks of age. HBV vaccine is administered with oral polio, and diphtheria-pertussis-tetanus (DPT) vaccine. No birth dose of HBV vaccine and no HBIG is included. Furthermore, no catch-up HBV vaccination programme in older children 1- 5 years of age was included, which considering the high transmission in this group, may have been the best option for HBV control in South Africa [Abdool Karim, 1998].

1.2.3. Vaccine Escape Mutants

The first indications of a significant change in the antigenic structure of HBV were discovered in children given both HBV vaccine and HBIG [Harrison, 1992; Okamoto, et al., 1992]. This change was demonstrated by a substitution of the aa arginine (R) for glycine (G) at position 145 in the S protein. Changes at other aa residues have subsequently been found, however the change at position 145 (G to R) appears to have the greatest effect on antigenicity because of alterations which occur in the "a" determinant (positions 139-147) [Howard, 1995]. Mutant viruses have been associated with severe fulminating liver disease in individuals that had circulating anti-HBs and they have also proven to be difficult to detect by conventional antibody assays [Carman, et al., 1995].

Breakthrough HBV infections in vaccinated children have been described in many countries including The Gambia [Whittle, et al., 1990], Italy [Carman, et al., 1990], Singapore [Oon, et al., 1995], Japan [Okamoto, et al., 1992], the USA [Nainan, et al., 1997] and the United Kingdom [Ngui, et al., 1997]. In The Gambia over half of the vaccinated children who became infected showed a transient anti-HBc response [Whittle, et al., 1990]. The significance of these vaccine induced escape variants of HBV

has not been fully established, however it has recently been estimated with the use of mathematical models [Wilson, et al., 1998] that although the number of vaccine escape mutants currently being detected in countries with universal childhood HBV immunisation programmes is low, the potential for the generation of highly infectious HBV variants exists. The cross protection afforded by present HBV vaccines would be lost, leading to vaccine failure and thus the explosive transmission of escape mutants which may cause fulminant liver disease would occur. It has been suggested that vaccine escape mutants take approximately ten years to develop, so that steps must be taken now to ensure that vaccine formulations may be adequately adjusted to make provision for these changes in the variant viruses [Wilson, et al., 1998].

Table III Worldwide prevalence patterns of HBV infection. [modified from Kiire, 1993]

	Low	Intermediate	High
Region and Presence of National Vaccination Programme (yes/no)*	Western Europe (yes) Australia (yes) North America (yes) South America (no)	Mediterranean (yes) Eastern Europe (no) Russia (no) North Africa (yes) Middle East (yes) Central America (no) South America (no) India (no)	China (yes) Southern Asia (yes) Sub-Saharan Africa (no) South Africa (yes) Pacific Islands (yes) Amazon region (yes)
Prevalence of HBV markers (%)	HBsAg: 0.2-0.5 HBsAb: 4-6	HBsAg: 3-7 HBsAb: 20-55	HBsAg: 8-20 HBsAb: 70-95
Extent of childhood/ neonatal infection	Rare	Frequent	Very frequent

* based on Kane, 1996.

Table IV World Health Organisation recommended HBV Vaccination Strategies based on hepatitis B prevalence data.

HBV Prevalence	Recommended Schedule	Countries
< 2%	Routine infant immunisation and adolescent programmes	Europe North America
≥ 2%	Routine infant immunisation*	Sub-Saharan Africa Far East

* three doses HBV vaccine, +/- HBIG

2. AIMS AND ORGANISATION OF DISSERTATION

This work was initiated prior to the introduction of universal childhood hepatitis B virus (HBV) immunisation in South Africa and continued until the end of 1996 after HBV immunisation had been formally introduced as part of the Expanded Programme for Immunisation (EPI). All the field work was done in the Eastern Cape Province of South Africa, an area highly endemic for HBV infection.

The objectives of this work were:

- To determine the prevalence and age of acquisition of HBV infection in children 0-6 years of age from the Eastern Cape Province.
- To define the circulating HBV genotypes in chronically infected children 0-6 years of age from the same area.
- To assess the effectiveness of LDPD HBV vaccine in Eastern Cape infants under field conditions following the revised EPI vaccination programme.

This research report is divided into the following three sections:

Study 1. Pre-immunisation epidemiology of hepatitis B virus infection in South African children from the Eastern Cape Province.

Study 2. Molecular characteristics of the circulating genotypes of HBV in chronically infected children from the Eastern Cape Province.

Study 3. Immunogenicity of low dose plasma derived HBV vaccine under field conditions in South African infants from an area highly endemic for HBV infection.

2.1. Study 1

Pre-immunisation epidemiology of hepatitis B virus infection in South African children from the Eastern Cape Province.

2.1.1. Abstract

The prevalence of hepatitis B surface antigen (HBsAg) was determined in a community-based, cross-sectional, age-stratified sample of children from 0 to 6 years of age (n=2299) from the Eastern Cape Province of South Africa. The purpose of the study was to investigate the epidemiology and the age of acquisition of hepatitis B virus (HBV) infection in children, thus providing a pre-immunisation baseline measure of this infection in the population targeted for HBV immunisation in South Africa. Overall, 10.4% (95% CI 9.2-11.7) of the children tested were HBsAg positive. There was a high rate of positivity in the 0-6 and 7-12 month age groups at 8.1% (95% CI 5.5-11.7) and 8.9% (95% CI 6.1-12.7) respectively, suggesting a higher rate of early acquisition of this infection than previously reported in South Africa. The proportion of HBsAg positive children increased significantly with increasing age ($\chi^2_{trend} = 5.9$, df=1, p=0.02), reaching 15.7% in the 61-72 month age group. This is the highest rate of HBV infection reported in community-based children from South Africa indicating a significant burden of this infection. The difference in HBsAg prevalence between urban and rural children was not statistically significant ($\chi^2 = 0.32$, df = 1, p = 0.57). There was also no difference in positivity between males (10.5%; 95% CI 8.7-12.5) and females (9.8%; 95% CI 8.1-11.7), ($\chi^2 = 0.006$, df = 1, p = 0.94). This study provides the most recent pre-immunisation, community-based baseline investigation of the epidemiology of HBV infection in children targeted for universal immunisation in South Africa.

2.1.2. Introduction

Infection with hepatitis B virus (HBV) poses a significant public health problem in many parts of the world, particularly in sub-Saharan Africa [Kiire, 1993]. Worldwide more than

300 million people are estimated to be chronic carriers of the virus and about 30% of these carriers who survive for 30 years or more may subsequently die of the chronic sequelae of HBV infection, cirrhosis and hepatocellular carcinoma [Maynard, 1990]. Almost 90% of infants infected with HBV at a young age will become chronic carriers, whereas 10% or less of adults develop the carrier state [Hyams, 1995]. Therefore, immunisation in infancy and childhood remains the most effective intervention to prevent the transmission of HBV and the sequelae associated with early acquisition of infection and the carrier state [World Health Organisation, 1992; Kane, 1995].

The epidemiology of HBV varies greatly in different parts of the world. Nevertheless, it is possible to broadly classify geographic regions according to the prevalence of HBV infection. In areas of high endemicity, defined as regions in which more than 75% of adults have serological evidence of past or current infection with HBV [Edmunds et al., 1996], infection primarily occurs during childhood. Within South Africa, which is identified as highly endemic for HBV infection [Kew, 1992; Dusheiko, et al., 1989 i; Dusheiko, et al.,1989 ii], most children acquire the infection, by as yet largely undescribed horizontal routes, between the ages of 1 to 5 years. Vertical transmission appears to be rare, with estimates of highly infectious hepatitis B e antigen (HBeAg) positive pregnant women ranging from 4.6%-10.3% [Guidozzi, et al.,1992; Prozesky, et al.,1983] compared to South East Asia where greater than 40% of pregnant women are HBeAg positive [Edmunds, et al., 1996; Stevens, et al.,1975]. Presumably, perinatal and neonatal infection still occur at a low rate in South Africa [Kew, 1992].

Details regarding the epidemiology of hepatitis B virus infection in children from Southern Africa has been reported in a number of published studies [Prozesky, et al.,1983; Dusheiko, et al.,1989 i; Dusheiko, et al.,1989 ii; Joubert, et al.,1991; Di Bisceglie, et al.,1986;Botha, et al.,1984; Abdool Karim, et al., 1988; Abdool Karim, et al., 1989, Schoub, et al.,1991; Schoub, et al., 1992 ii; Schoub, et al., 1993]. Previous

exposure to HBV as measured by HBsAg positivity ranges from 0.97% in urban black children [Dibisceglie, et al.,1986] to 25.1% in institutionalised children from Kwazulu-Natal [Abdool Karim et al.,1988]. The results from these studies form the basis upon which South African health policy decisions have been made considering the inclusion of hepatitis B virus immunisation for children. However, each of these studies has limitations, which restricts its use as a reliable, recent, community-based pre-immunisation baseline estimate of HBV infection in infants and children from South Africa. The most important constraints are; very small sample sizes were studied [Prozesky, et al.,1983; Botha, et al.,1984; Schoub, et al.,1991] samples were taken from diversely different groups (urban, rural, institutions and undefined) [Abdool Karim, et al.,1988; Schoub, et al., 1993, Botha, et al.,1984] and various age groups were examined which included mainly older children and adults and not children less than 6 months of age [Dibisceglie, et al.,1986; Dusheiko, et al.,1989 i; Dusheiko, et al.,1989 ii; Joubert, et al.,1991; Abdool Karim, et al.,1989]. In addition all these studies were done in the late 1980's and early 1990's. Since then there have been considerable socio-political changes in Southern Africa with significant changes in economic and cultural activities, increased population mobility and blurring of strict urban/rural boundaries which may impact on the epidemiology of infectious diseases particularly HBV infection.

The introduction of universal infant HBV immunisation in South Africa [Department of Health South Africa,1995; Kane, 1995], which would undoubtedly alter the characteristics of natural HBV infection and the inadequate existing baseline data on the epidemiology of HBV in the group targeted for universal HBV immunisation prompted the present study. The aim was to provide a recent pre-immunisation quantitative assessment of the burden of HBV infection in children between the ages of 0 to 6 years and to approximate the age of acquisition of this infection, thus providing a community-based baseline estimate of HBV infection against which to measure the impact of the South African HBV infant immunisation strategy in the future.

2.1.3. Materials and Methods

Study Population

The study was done in the Mdantsane district of the Eastern Cape Province which includes both urban and rural communities. A large, densely populated urban township is located in the centre of the district. Most inhabitants of the township live in formal brick houses or backyard shacks and have access to basic amenities such as electricity, water and sanitation [Orkin, et al., 1998]. The township is surrounded by a sparsely populated rural area made up of isolated traditional settlements. Few rural households have electricity, water is obtained from local rivers or springs, and pit latrines are the predominant form of sanitation [Orkin, et al., 1998]. In 1996, it was estimated that a total of 359,978 children under 5 years of age live in Mdantsane district, approximately 67% of whom are from rural communities [Department of Health, 1996].

Health services in the district are provided by Cecilia Makiwane Hospital (CMH) with a capacity of 1000 beds and 18 primary health care clinics associated with the hospital. Eleven of these clinics are located within the township of Mdantsane and seven are in the surrounding rural areas. Four urban clinics and three rural clinics were selected for the study. The clinics were selected for logistic reasons in that their immunisation days were staggered throughout the five day working week. Nevertheless, the clinics were thought to be representative of others in the district.

The study population consisted of children visiting the primary health clinics for routine primary care, between the ages of 0 to 72 months from July 1995 to April 1996. Children were included if there was no history of hepatitis B immunisation. This was confirmed with the parent or guardian and by examining the *Road to Health* card, a patient held record of immunisations and birth data. Children older than 72 months or those that had been already immunised against hepatitis B were excluded from the study. The purpose of the study was explained to the parent or guardian of each eligible child. Children were

then included if the mother or guardian gave written consent for their participation. The study aimed to obtain a cross-sectional age stratified sample of 2000 children equally stratified into the following age groups; 0-6 months, 7-12 months, 13-24 months, 25-36 months, 37-48 months, 49-60 months and 61 to 72 months. Two registered nurses from the area (field workers) recruited the subjects, documented the relevant demographic information and took the blood samples.

Sample Collection

Heel or finger prick blood specimens were obtained from children less than 24 months of age, using *Microtainer®* tubes. The maximum volume of blood that these tubes contain is 700 µl and this was the maximum limit set for children below 24 months of age. Children over the age of 24 months had either a heel/finger prick specimen taken or formal venepuncture from the antecubital fossa with a maximum volume of 5 ml allowed. Strictly hygienic and safe conditions were maintained at all times during blood sample collection. Name, age, gender, district and immunisation histories were recorded on standardised patient information forms (Appendix 1). Blood specimens were kept in a cooler box on wet ice blocks at each clinic and transported back to Cecilia Makiwane Hospital to be stored at 4°C. At the end of each week the specimens were sent to the National Institute for Virology (NIV) in Johannesburg by overnight courier for analysis. On arrival at the NIV, the serum was separated, the specimens were labelled and stored at -20°C until testing.

Serological Testing

Serum samples were batched and tested for HBsAg using a standard enzyme immunoassay (ELISA) kit, (*Wellcozyme HBsAg,* Murex Laboratories®, UK). The manufacturers conditions for the tests were strictly adhered to at all times and positives were defined according to the kit instructions.

Statistical Analysis

SAS statistical software was used for all the analyses. The 95% confidence intervals for the proportions positive per age group stratum and for the whole group were calculated

[Fleiss, 1981]. Differences in prevalence between gender and rural/urban groups were evaluated for significance using the χ^2 test. The χ^2 test for trend with one degree of freedom was used to evaluate linear trends for the age stratified results.

2.1.4. Results

A total of two thousand two hundred and twenty nine (2299) serum samples were tested. The age and sex distribution of the children tested are shown (*Figure 5*). In total 47.1% of the sample were boys, 48.0% were girls and information on gender was missing for 114 children (4.9%). The proportion of girls and boys were similar in each stratum. Children in the 13-24 month age group were slightly over-represented whereas there were fewer children in the 61-72 month group.

The proportion of HBsAg positive children in each age group is shown in *Table V*. The differences between age groups is statistically significant (χ^2=13.0, df=6, p=0.04). Overall, 10.4% (95% CI 9.2-11.7) of the children were positive for HBsAg. The prevalence of HBsAg was surprisingly high in the two lower age groups 0-6 and 7-12 months at 8.1% (95% CI 5.5-11.7) and 8.9% (95% CI 6.1-12.7) respectively. The proportion of HBsAg positive children increased gradually from 8.1%(95% CI 5.5-11.7) in the 0-6 month age group to 15.7% (95% CI 10.7-22.3) in the children aged 61-72 months. This increase is statistically significant (χ^2_{trend} =5.9, df=1, p=0.02).

HBsAg positivity for the 2175 children with information on gender and place of residence is shown in *Table VI*. Of the girls, 9.8% (95% CI 8.1-11.7) were HBsAg positive compared to 10.5% (95% CI 8.7-12.5) of the boys. However, this difference is not statistically significant (χ^2=0,32, df=1, p=0.57). 2298 children of the 2299 had information on the area of residence. Fifty six percent (1281/2298) were from urban areas and 44% (1017/2298) were from areas classified as rural. *Table VI* indicates that the proportion of HBsAg positive children in these two groups was very similar and not statistically

different (χ^2=0.006, df=1, p=0.94).

2.1.5. Discussion

This study quantitatively assesses the burden of HBV infection in children between the ages of 0 to 6 years and provides a recent, community-based pre-immunisation baseline estimate against which to measure the impact of the immunisation strategy recently introduced in South Africa.

The average rate of HBsAg positivity of 10.4% in children from the Eastern Cape, as a single indicator of infection with HBV, is similar to previously published data in adult males from this area (9%) [Dusheiko, et al.,1989 i; Dusheiko, et al.,1989 ii]. This work in adults is the only other study done estimating the burden of HBV infection in people from the Eastern Cape and was conducted on adult black men employed as gold miners in the Witwatersrand in the 1980's. The older age groups of children in the present study (49-60 and 61-72 months) show an increase in HBsAg positivity consistent with later acquisition of infection, probably by horizontal routes. However, the peak of HBsAg positive children in the 61-72 month age group (15.7%) suggests a much higher rate of HBV carriers in adolescents and adults than demonstrated in the earlier published study from the Eastern Cape [Dusheiko, et al.,1989 i; Dusheiko, et al.,1989 ii], therefore there is a significant burden of HBV infection in this population which may have been underestimated in the past due to the limited study designs.

We also found that there were no differences in infection with HBV between Eastern Cape children from rural and urban areas, although differences in HBsAg prevalence in South African children from rural and urban environments have previously been demonstrated [Prozesky, et al.,1983; Abdool Karim, et al., 1988; Di Bisceglie, et al.,1986] The range of HBsAg positivity in these other studies is quite wide, within the rural areas 4.5% [Prozesky, et al.,1983] to 18.5% [Abdool Karim, et al.,1988] and the

urban areas 0.97% [Dibesceglie, et al.,1986] to 10% [Abdool Karim, et al.,1988]. The samples for these studies were taken from diversely different parts of the country thus making it difficult to generalise their results to the whole of South Africa. Moreover, one study clearly showed that socio-economic disparities *per se* did not appear to contribute to the differences seen in HBsAg rates, since no obvious differences in HBV infection rates was found between the highest and lowest socio-economic groups in one area [Dibesceglie, et al.,1986]. However, all these investigations were conducted almost ten years ago, when the rural/urban divide in South Africa was more obvious. In the past, population movements in South Africa were enforced by racially motivated apartheid laws which began to collapse in the early 1990's. In the Eastern Cape Province the rural/urban boundaries have certainly been blurred in recent years due to the mobility and peri-urban settlement patterns of the population [Orkin, et al., 1998]. This may account for the loss of the strict division of groups as rural versus urban and explain the similarity in the prevalences of HBV infection found in our study for rural and urban children.

Throughout Africa, lifetime exposure rates to HBV are the same in the two sexes [Kew,1992]. However, the carrier rate is usually higher in men than in women, with ratios ranging from 1.1:1 and 3.2:1 [Kew,1992; Abdool Karim, et al.,1989; Schoub, et al., 1992]. In South African children no differences in chronic HBV infection have been shown between children of different sexes [Abdool Karim, et al.,1988]. Our findings support this and we did not demonstrate any difference in HBsAg positivity rates between boys and girls up to the age of 72 months .

The role of vertical transmission, from mother to infant, has been thought to be less important in Southern Africa with the predominant mode of transmission being by undefined horizontal routes in older children (24 to 60 months of age), probably occurring during play activities or ritual scarification [Kiire,1993; Edmunds, et al.,1996;

Kew,1992; Abdool Karim, et al.,1988; Martinson, et al.,1996; Chiaramonte, et al.,1991]. Also, since the proportion of Southern African women of child-bearing age that are considered highly infectious carriers (HBsAg and HBeAg positive) is relatively low (ranging from 4.6% in urban areas [Guidozzi, et al.,1992] to 10.3% in rural areas [Prozesky, et al.,1983]) it is thought that vertical transmission is not a major mechanism of HBV infection in South Africa. The high levels of HBsAg in the young age groups 0-6 and 7-12 months of 8.1% and 8.9% respectively shown here were, therefore, not expected. These high levels imply that early HBV transmission is occurring. This transmission may be from mother to infant during the perinatal/neonatal period or via other modes of HBV transmission in this young age group such as ritual scarification, ear piercing and exposure to HBV infected siblings and family members. Since none of these mechanisms of early infection with HBV were directly measured and because the first age stratification in the our study from 0-6 months is relatively crude, we are, therefore, only able to observe that early transmission is probably occurring and speculate about the likely mechanisms in South Africa infants. Further epidemiological studies in the age group below 6 months are needed to clarify this issue further.

Hepatitis B infection is a major problem in South African children and may have been underestimated in the past. The recent introduction of universal childhood immunisation against HBV in South Africa should be an important step in reducing the long term sequelae of this disease. The current HBV immunisation schedule in South Africa (at 6,10 and 14 weeks of age) was based on data that showed a predominance of horizontal transmission [Eggers, 1995; Robson, et al.,1991; Robson, 1992]. This assumption may need to be re-evaluated in the light of the high levels of early HBV transmission demonstrated in this study. Preventing perinatal or early neonatal transmission would require changes to the current immunisation schedule. It would probably be too expensive for South Africa to provide hepatitis B immune globulin (HBIG) at birth but it might be feasible to include an additional dose of HBV vaccine at

Kew,1992; Abdool Karim, et al.,1988; Martinson, et al.,1996; Chiaramonte, et al.,1991]. Also, since the proportion of Southern African women of child-bearing age that are considered highly infectious carriers (HBsAg and HBeAg positive) is relatively low (ranging from 4.6% in urban areas [Guidozzi, et al.,1992] to 10.3% in rural areas [Prozesky, et al.,1983]) it is thought that vertical transmission is not a major mechanism of HBV infection in South Africa. The high levels of HBsAg in the young age groups 0-6 and 7-12 months of 8.1% and 8.9% respectively shown here were, therefore, not expected. These high levels imply that early HBV transmission is occurring. This transmission may be from mother to infant during the perinatal/neonatal period or via other modes of HBV transmission in this young age group such as ritual scarification, ear piercing and exposure to HBV infected siblings and family members. Since none of these mechanisms of early infection with HBV were directly measured and because the first age stratification in the our study from 0-6 months is relatively crude, we are, therefore, only able to observe that early transmission is probably occurring and speculate about the likely mechanisms in South Africa infants. Further epidemiological studies in the age group below 6 months are needed to clarify this issue further.

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birth. This would be much cheaper than using HBIG and could be included with the oral polio and BCG vaccines currently given at birth. A study to evaluate whether an additional dose of HBV vaccine at birth would reduce the early transmission of HBV in South Africa is an urgent priority.

We have described the pre-immunisation prevalence of HBV infection in a cohort of children from a highly endemic area of South Africa using a single measure of infection, HBsAg. Similar baseline studies in areas of high endemicity for HBV infection, have proven extremely valuable to evaluate the impact of HBV immunisation strategies [Ruff, et al.,1995; Chen and Orenstein, 1996]. Although these studies in general are costly in terms of time and resources, they remain the only way to quantitate the impact of HBV immunisation programmes, detect vaccination failures, monitor the level of vaccine coverage in the target age groups and observe the development of vaccine escape mutants of HBV. This baseline study used in conjunction with follow up studies of vaccinated children from the Eastern Cape will allow an assessment of the impact of the HBV immunisation strategy in South Africa.

Figure 5 Age and Sex Distribution of Sample Study 1.

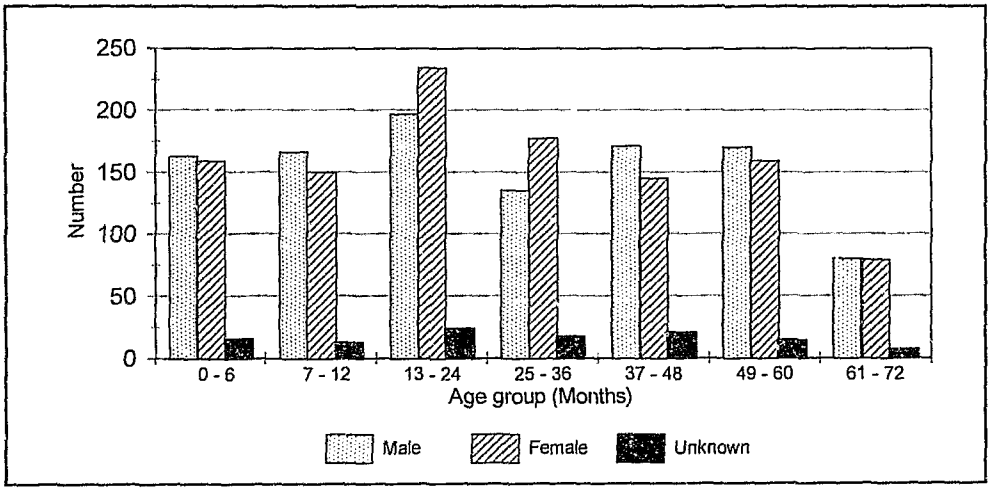


Table V Proportion of HBsAg positive tests per age group.

Age (Months)	Total	HBsAg Positive		
		No	%	[95% CI]
0 - 6	333	27	8.1%	[5.5-11.7]
7 - 12	326	29	8.9%	[6.1-12.7]
13 - 24	453	45	9.9%	[7.4-13.2]
25 - 36	329	42	12.8%	[9.5-17.0]
37 - 48	337	27	8.0%	[5.4-11.6]
49 - 60	344	42	12.2%	[9.0-16.3]
61 - 72	166	26	15.7%	[10.7-22.3]
Total	2288	238	10.4%	[9.2-11.7]

95%CI : 95% Confidence Interval

Table VI Proportion of HBsAg positive tests.

	Total	HBsAg Positive		
		No	%	[95% CI]
Male	1078	113	10.5%	[8.7-12.5]
Female	1097	107	9.8%	[8.1-11.7]
Rural	1281	132	10.4%	[8.8-12.2]
Urban	1017	106	10.5%	[8.7-12.6]

95%CI : 95% Confidence Interval

2.2. Study 2

Molecular characteristics of the circulating genotypes of HBV in chronically infected children from the Eastern Cape Province.

2.2.1. Abstract

The naturally occurring HBV viral genotypes in a community based sample of chronically infected children between the ages of 0-72 months, before the introduction of universal childhood HBV immunisation, were defined by a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technique. The children were all from an area considered to be highly endemic for HBV infection, with an overall HBsAg prevalence in the 0-72 month age group of 10.4%. From the 238 children found to be hepatitis B surface antigen (HBsAg) positive in Study 1 (Section 2.1.), a randomly selected sample of children (n=57) were used for the present study. The pre-S1 and pre-S2 regions of HBsAg were successfully amplified by the polymerase chain reaction (PCR) in 64.9% (37/57) of these specimens. Genotype identification was then done using appropriate restriction enzyme digests of the amplified segments. Two specimens could not be classified after the RFLP analysis. In the remaining 35 specimens, the predominant genotype identified in 85.7% (30/35) of the specimens was A'. Genotype D was found in 11.4% (4/35) and A in 2.9% (1/35) of the amplified specimens. This is the first assessment of HBV genotypes in a sample of community infected children from the Eastern Cape Province, and the results show a unique clustering of mainly genotype A' in this area.

2.2.2. Introduction

The serologic heterogeneity of HBV, into nine different subtypes was identified in the 1970's [Couroucé and Soulier, 1975; Couroucé-Pauty, et al., 1978]. The geographic distribution of these subtypes was later established [Couroucé-Pauty, et al., 1983]. Early sequence analysis of HBV viral DNA then led to the definition of six genotypes,

based on the relatedness of whole genomes [Okamoto, et al., 1988]. To associate the extended subtype nomenclature to the genetic classification of HBV, studies were done in which the entire S-gene of all the representative subtypes were sequenced [Norder, et al., 1992]. The smallest difference between genotypes at the level of the S gene was found to be 4.1%, therefore a difference of 4% is used to delimit a genotype at the level of the S gene [Norder, et al., 1992]. Based on this methodology, six genotypes of HBV have been characterised, A-F. The HBV strains within each genotype show a characteristic geographic distribution. It has been proposed that the geographical distribution of humans with various genetic backgrounds may have influenced the divergence of HBV into these various strains by a process of evolutionary selection [Telenta, et al., 1997]. However, phylogenetic studies of HBV are incomplete, because the number of HBV isolates analysed in some parts of the world, particularly in southern Africa, is very small.

Genetic diversity of HBV and amino acid substitutions may give rise to variations in viral epitopes that are important for viral neutralisation [Magnius and Norder, 1995]. During studies of the immunogenicity and efficacy of hepatitis B vaccines in Italy, a number of individuals who had apparently mounted a successful immune response, as shown by the production of anti-HBs, later became infected with hepatitis B virus [Carman and Thomas, 1991; Carman, et al., 1995]. Sequence analysis of the virus from one of these cases revealed a mutation in the nucleotide sequence encoding the "a" determinant, the consequence of which was a substitution of arginine for glycine at amino acid position 145 [Carman, et al., 1990]. Similar mutations have found in HBV from a variety of other countries, including Japan, USA, UK and Singapore [Oon, et al., 1995]. The emergence of these escape mutants is a cause for concern for the laboratory detection of virus and possibly for future vaccination programmes. The "a" determinant in which the escape mutations occur is an important virus determinant to which vaccine-induced neutralising antibody binds. Neutralising antibodies do not recognise the mutants. Therefore, the

threat of a fully replication competent virus that is unaffected by the presence of vaccine induced neutralising antibodies and may be easily transmitted must be monitored. Worldwide surveys of HBV molecular epidemiology in order to monitor the circulating pool of wild-type and immune-selected mutant viruses, particularly as universal HBV immunisation is introduced into more countries, must be done.

Little is known about the circulating HBV genotypes in South Africa. Early serological subtype analysis on specimens from this country showed that the major HBV subtype in South Africa was *adw2* (97.7%) with the remaining 2.3% being *ayw*. To date the identified genotypes in this country are based on the data from the initial characterisation of subtypes *adw2* and *ayw* belonging to genotypes A and D respectively [Couroucé-Pauty, et al., 1983] and on work done on a hepatocellular carcinoma cell line 9PLC/PRF/5 [Rivkina, et al., 1988]. The most recent and extensive study to date, in which HBV from adult chronic carriers from the Gauteng province was sequenced, identified genotypes A, B, C and D and also found a distinct clustering of the genotype A viruses from Gauteng into a subgroup called A' [Bowyer, et al., 1997].

HBV infection is endemic in South Africa, with some areas of the country including the Eastern Cape Province and Kwazulu-Natal having higher prevalence rates of carriers compared with other parts of the country [Abdool Karim, et al., 1988; Dusheiko, et al., 1989 i; Dusheiko, et al., 1989 ii]. The recent introduction of a LDPD HBV vaccine as part of the infant immunisation programme in this country [Department of Health, 1995], and the potential for the generation of virus escape mutants prompted the present study to describe the naturally occurring HBV variants in a pre-immunisation community-based group of children identified as chronic carriers from the Eastern Cape Province.

2.2.3. Materials and Methods

Subjects

238 HBsAg positive, community infected children between the ages of 0-72 months were identified as part of a large cohort from the Eastern Cape Province which was used to describe the epidemiology of HBV infection in this region. The selection of this sample is described in detail in Section *2.1.3.* above. Fifty seven (57) randomly selected HBsAg positive samples were chosen for the PCR and RFLP analysis of HBV genotypes.

HBV DNA Extraction

HBV DNA was extracted from 210 µl of serum using QIAamp® Blood Kit (Qiagen, Hilden, Germany) rapid purification columns according to the manufacturer's instructions. Briefly, the serum, buffer and proteinase K are all mixed together and incubated at 70 °C for 10 minutes. Isopropanol was then added to the treated sample, which was then centrifuged at 6000 x *g* (8000 rpm) to extract the DNA. The DNA was eluted from the column using supplied buffers at pH > 9 to maximise the DNA yield.

DNA Amplification

Two polymerase chain reaction (PCR) fragments were used to genotype the HBV specimens by RFLP and size analysis [Bowyer, unpublished results]. The first of these, referred to as fragment PS1 since it is part of the pre-S1 region of the large (L) envelope surface protein of HBV has previously been described (Sense 2839: 5′ CTTGGGAACAAGAGCTACAGCAT 3' and antisense 2979: 5′ GGGTTGAAGTCCCAATCTGGA 3′) and has a length of 128 or 161 bp depending on HBV type [Li, et al1993]. The second fragment, referred to as PS2, includes the entire pre-S2 region together with portions of the pre-S1 and HBsAg (small, S protein) at the carboxyl and amino termini respectively. This amplicon was obtained using sense primer 3094, 5′ CCTCAGGCTCAGGGCATA 3′, and anti-sense primer 253, 5′ AGAAAATTGAGAGAAGTCCACCACG 3′, to yield a 413 bp fragment.

When sub-optimal amounts of serum were available, or no product was evident on first round PCR, a nested PCR was done using sense primer 2839, 5′ CTTGGGAACAAGAGCTACAGCAT 3′, and antisense primer 690, 5′ AGCCCTACGAACCACTGAACAAAT 3′. This gave a 1067 or 1100 bp amplicon depending on genotype. All primer numbers refer to the distance from the *Eco R1* site.

All PCR reactions were performed using 10 µl of extracted DNA, 200 µM of each dNTP, 1 µM of each primer (forward and reverse), 5 U *Taq* polymerase (Boehringer Mannheim, Germany) and 1x PCR buffer supplied with this product in a final volume of 100 µl. Thin walled PCR tubes were used for all reactions. After an initial denaturation for 2 minutes at 94°C, DNA was amplified in 36 cycles (94°C for 1 minute, 48°C for 1 minute, 72 °C for 2 minutes) followed by a final elongation for 7 minutes at 72 °C. Then, 10 µl of the PCR reaction mix was loaded on a 2.5% or 3% agarose gel, depending on fragment size, containing ethidium bromide (5 µg/ml) and electrophoresed. DNA was visualised under UV light. The nested PCR for the Pre-S1 and Pre-S2 portions of the genome were done under identical conditions in the same PCR reaction mix. The expected sizes of the PCR products were 128/161, 413 and 1067/1100 bp for the pre-S1, pre-S2 and nested PCR's respectively, and the size markers used were Boehringer Mannheim DNA Markers V, XIV and VI (Boehringer Mannheim, Germany) for the respective reactions.

To avoid contamination, pre-PCR preparation, DNA extraction, DNA amplification and gel electrophoresis of PCR products were performed in separate rooms. In each series of experiments, six samples, four negative and two positive controls were subjected to PCR. Negative controls were sterile water and positive controls were sera from highly infectious carriers (HBsAg and HBeAg positive) identified by serology.

Restriction Digests

A summary of the HBV genotyping algorithm [Bowyer, unpublished results] specifying

the restriction enzyme and fragment used for each specimen is shown in *Figure 6*. For each reaction, a volume of 10 µl of the appropriate PCR product was mixed with 2.5 µl of x10 buffer, 10.5 µl water, 1 µl casein (final concentration 40 µg/ml) and 1 µl of the respective enzyme. Casein was added to enhance restriction enzyme activity. For Pre-S1 fragments, the enzymes used were *Bam HI* and *Taq I* [Shih, et al., 1991] whereas for the pre-S2 fragment the enzymes used were *Hpa II* and *Bam HI*. All restriction enzymes were used with their designated buffers and conditions recommended by the manufacturer (Boehringer Mannheim, Germany) in a final reaction volume of 25 µl. After incubating for 2-3 hours at the temperature appropriate for the particular restriction enzyme, the entire volume of the sample was loaded into the slots of an agarose gel of appropriate grade and percentage containing ethidium bromide (5 µg/ml) for visualisation under UV light. Fragments greater than 150 bp were visualised using agarose D1 LE, molecular grade (supplied by Whitehead Scientific) whereas smaller fragments were run on 3% Metaphor agarose gels (supplied by FMC Bioproducts). The gels were hydrated and cast according to the manufacturers instructions. For each enzyme treated reaction, 10 µl of the PCR product was mixed with 2.5 µl of x10 buffer, 10.5 µl water, 1 µl casein and no restriction enzyme was run in parallel.

2.2.4. Results

Of the 57 samples subjected to PS1, PS2 and nested PCR's, 64.9% (37/57) yielded suitable material for RFLP analysis. All "low" positive or equivocal specimens, with faint bands on the initial PCR (either PS1 or PS2) were confirmed on the nested reaction. The remaining 20 specimens (35.1%) either did not amplify on any of the PCR's (13 specimens) or had discordant results on the PS1 and PS2 PCR's with only one of the two segments amplifying (7 specimens). No amplification product was obtained for these latter 7 specimens on the nested PCR. Therefore, these 20 specimens were not included in the RFLP analysis and were discarded from further study at this stage.

Figure 7, shows an example of a gel electrophoresis of amplified products of Pre-S1 fragment (161 bp), with negative sterile water controls and a known highly infectious carrier as a positive control. Similarly, *Figure 8* shows an example of the gel electrophoresis of amplified products of Pre-S2 fragment, (413 bp), once again negative and positive controls were included.

A summary of the RFLP results for the 35 specimens with suitable fragments for restriction enzyme digestion is shown in *Figure 9*. The majority of the specimens (85.7%) genotyped as A′, 11.4% as genotype D and only one specimen (2.9%) as genotype A.

2.2.5. Discussion

This report describes a rapid RFLP method for genotyping HBV based on the analysis of the pre-S region, which has been shown to be the most variable part of the genome [Norder, et al., 1993]. This is the first study to describe the circulating HBV genotypes in community infected children from an area highly endemic for HBV infection in South Africa. A unique circulation of primarily genotype A′ was found with only minor representation of genotypes D (11.4%) and A (2.9%) which where previously thought to be the main genotypes in this country.

In this study, HBV DNA was obtained from a relatively low proportion of the samples (64.9%) subjected to extraction and PCR amplification. This may have been due to a number of reasons including the poor quality of some of the serum specimens due to haemolysis of red blood cells before separation of the serum and thus the introduction of polymerase inhibitors. Secondly, the relatively low sensitivity of the DNA extraction process in commercial columns compared to formal phenol-chloroform extraction [Kramvis, et al., 1996] may have affected the DNA yield. Finally, the PCR conditions may also have been sub-optimal allowing for incomplete denaturation of DNA. The latter reason seems unlikely however, due to the good DNA yield obtained in numerous other

reactions using many other specimens which were collected and stored differently [Bowyer , unpublished data].

Methods based on RFLP [Shih, et al 1991] and the use of type specific primers [Norder, et al., 1992] have previously been described for HBV genotyping. A recent large scale study, in which 99 serum samples from highly infectious HBeAg carriers were subjected to RFLP analysis and then confirmed by sequencing has shown the reliability of this technique [Lindh, et al., 1998]. Therefore, large scale rapid epidemiological genotypic characterisation of HBV positive specimens can be done by RFLP without having to resort to sequencing. The molecular epidemiology of HBV based on the described relatively rapid PCR and RFLP techniques on S gene sequences has four major functions; to describe the phylogenetic relationships of naturally circulating HBV in specific geographic regions [Magnius and Norder, 1995], to confirm the transmission of HBV within defined epidemiological circumstances thus tracing the source of infection in outbreaks of hepatitis B [Uy, et al., 1992], to monitor the appearance of vaccine escape mutants of the virus in naturally circulating genotypes or in response to vaccine induced immune pressure [Carman and Thomas, 1991] and to determine the differences in pathogenicity and response to treatment between genotypes [Lindh, et al., 1998].

The endemic occurrence of HBV in South Africa, with some areas of the country having markedly higher prevalences of chronic carriers than other areas offers the opportunity to study the natural transmission patterns and phylogenetic relationships of this virus within regions of the country where the epidemiology of the virus may differ. Some description of circulating HBV genotypes has been done in South African adult carriers from the Gauteng province [Bowyer, et al., 1997]. The overall HBV carrier rates in the Gauteng population is low, adults (5.8%) [Dusheiko, et al., 1983 i; Dusheiko, et al., 1989 ii] and children (0.97%) [Prosesky, et al., 1983]. In the study by Bowyer et al., 1997, the pre-S2/S genes from 29 acutely or chronically infected individuals were sequenced.

Genotype A was identified in 24 specimens, 3 with Group D infection and one each with genotypes B and C. Furthermore, it was also shown that 59.1% of the sequences clustered to form a distinct segment called genotype A′. These genotype A′ specimens had a series of amino acid differences in the pre-S2 region as well as in the major surface antigen.

In the Eastern Cape population the overall HBV carrier rate in adults is 9% [Dusheiko, et al., 1989 i; Dusheiko, et al., 1989 ii] and 10.4% in children (10.4%) [Vardas, et al., 1999]. The predominant genotype we identified in community infected children is A′ . Whether variations in the prevailing genotypes in various regions of South Africa may explain the regional differences in prevalence and natural course of HBV infection needs to be shown by doing further studies in other regions of this country. Differences in circulating genotypes may also reflect biological differences in the virus which may influence the vertical transmission of HBV infections [Magnius and Norder, 1995] and also the long-term replicative potential of different strains.

Importantly though, responses to HBV vaccination may also be different in areas where the circulating naturally infecting genotypes are different. With the introduction of universal infant HBV immunisation in South Africa and the use of an extremely low dose plasma derived product, the poor response shown to this vaccine in Eastern Cape infants compared with those in Gauteng [Aspinall and Kocks, 1998] may partly be explained by genotypic variation of the virus in these two regions of the country. The long term effects of the immune pressure proffered by a sub-optimal LDPD HBV vaccine on the circulating genotypes in the Eastern Cape, particularly since genotype A′ shows variations in the HBsAg portion of the HBV S protein need to be monitored. Sequencing of the genotypes in this area at intervals after the introduction of LDPD HBV vaccine will enable the early detection of vaccine escape mutants, if they occur at all under these circumstances.

Finally, the mapping of the naturally circulating HBV genotypes in South Africa should be useful for studying differences in the pathogenicity and response to treatment between genotypes, both within the country and in comparison to other countries. Although these differences in pathogenicity and treatment response have not been studied systematically, due to the paucity of genotypic data, it has been suggested that genotypes B and C are associated with more severe liver damage [Lindh, et al., 1998]. In therapeutic studies using interferon it has been shown that genotype A strains are more susceptible to respond to treatment than other strains [Zhang, et al., 1996]. These findings encourage further study of the clinical importance of genotypes and emphasize the need for a simple genotyping method using PCR and RFLP techniques.

Figure 6 HBV Genotyping Algorithm used for Study 2, showing the restriction enzyme and the fragment used.

<table>
<tr><td colspan="4">STEP 1

Pre-S1 fragment (128/161 bp), digest with Bam HI</td></tr>
<tr><td>Digest
D=79/50 bp
Confirms D</td><td></td><td>Digest
E=109/50
F=112/50

Go to Step 2 or 4</td><td>No Digest
A and A'/B/C

Go to step 3</td></tr>
<tr><td colspan="4">STEP 2

Pre-S1 fragment (128/161bp), digest with Taq I</td></tr>
<tr><td colspan="2">Digest
B=45 bp and C=116 bp
E=111/47</td><td colspan="2">No Digest
A and A'
D/F</td></tr>
<tr><td colspan="4">STEP 3

Pre-S2 fragment 413 bp, digest Bam HI</td></tr>
<tr><td colspan="2">Digest
A/E/F
251 and 162 bp</td><td colspan="2">No Digest
A'/B/C/D
Go to Step 2 for A' or B/C
Go to Step 4 B/C</td></tr>
<tr><td colspan="4">STEP 4
Pre-S2 fragment (413 bp), digest with Hpa II</td></tr>
<tr><td colspan="2">Digest
C 203 bp and E 210 bp confirmed</td><td colspan="2">No Digest
B, A, A', D/F confirmed</td></tr>
</table>

Figure 7 Gel electrophoresis of amplified products of Pre-S1 fragment, (161 bp). Lanes 1, 4, 15 and 17 are sterile water controls. Lane 16 is a known positive control. Molecular weight marker V was loaded on either side of the gel. The specimens in lanes 2, 3, 6, 7, 9, 13 were taken as positive.

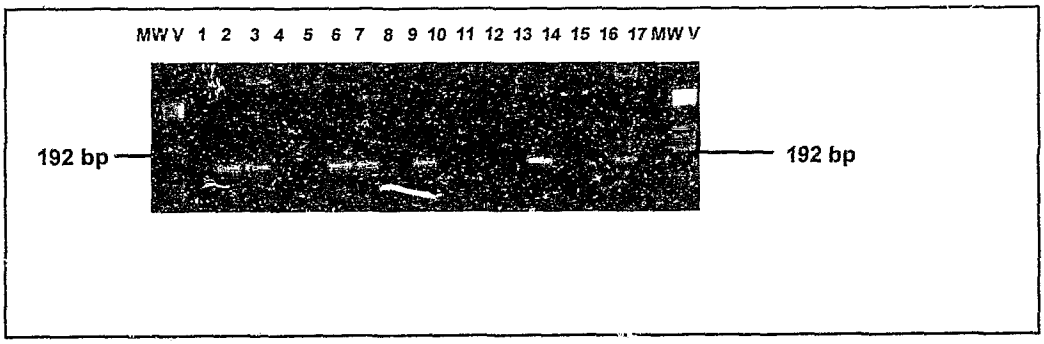


Figure 8 Gel electrophoresis of amplified products of Pre-S2 fragment, (413 bp). Lanes 3, 7, 10 are sterile water controls. Lane 16 is a known positive control. Molecular weight marker XIV was loaded on the far side of the gel. The specimens in lanes 1, 2, 4, 5, 6, 8, 9, 12, 14, 15 were taken as positive.

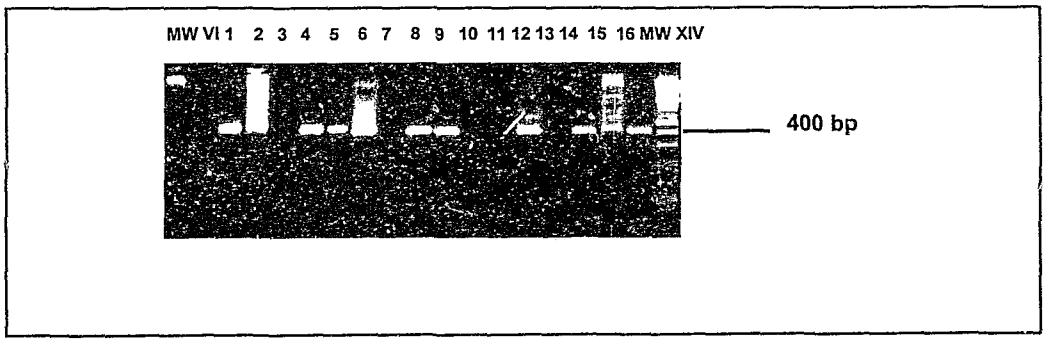


Figure 9 Summary of RFLP results Study 2.

Genotype Result	Number of Specimens (%)
A'	30 (85.7)
D	4 (11.4)
A	1 (2.9)
Total	35

2.3. Study 3

Immunogenicity of low dose plasma derived HBV vaccine under field conditions in South African infants from an area highly endemic for HBV infection.

2.3.1. Abstract

The effectiveness, under field conditions, of a low dose plasma derived (LDPD) HBV vaccine containing 1.5 µg of HBsAg per paediatric dose was assessed. Infants presenting for routine immunisations at 6 weeks of age, with no previous history of HBV vaccination were randomly assigned to receive either LDPD HBV vaccine through the normal clinic route (n=119) or a recombinant paediatric HBV vaccine (n=108) given under controlled conditions. Infants in both groups received vaccine at 6, 10 and 14 weeks of age according to the revised accelerated South African EPI schedule. Antibodies to hepatitis B surface antigen (anti-HBs) were measured at baseline, before vaccination at 6 weeks of age, and then every four weeks after one, two and three doses of vaccine. Serological protection was taken to be any anti-HBs level ≥ 10 mIU/ml. A control group (n=80) of age-matched, HBsAg negative infants with no prior history of HBV immunisation were also included in the analysis to demonstrate the natural levels and decay of maternal anti-HBs antibody in unimmunised children with time up to and including 18 weeks of age.

Follow up rates for both vaccine groups at each of the early time intervals (10 and 14 weeks) was good (81%-62%) except for the last visit at 18 weeks of age where the follow up rates dropped to less than 45% for both groups. Baseline anti-HBs levels between the two vaccine groups and between the vaccine groups and the unimmunised controls were not significantly different (ANOVA, F=2.83, p=0.06). However, at the 14 and 18 week time points, one month after two and three HBV vaccines, the levels of anti-HBs were significantly higher in the recombinant vaccine group compared to the LDPD group, (χ^2=8.865, p=0.003) and (χ^2=21.114, p=0.000) respectively. This

significant difference in anti-HBs levels remained even after log transformation of the skewed anti-HBs data, at 14 weeks (t=2.9 [illegible] 004) and 18 weeks (t=5.14, p=0.000). At one month after the last vaccination, L[illegible] vaccine induced levels of anti-HBs ≥ 10mIU/ml in 42.2% (95% CI 27.99-57.77) of immunised infants whereas recombinant vaccine induced protective antibody levels in 88.6% (95% CI 74.66-95.74) of immunised infants. The unimmunised age-matched control group showed a typical pattern of initially high maternally derived anti-HBs antibodies which progressively declined with time. In a logistic regression model, the only factor that was found to influence anti-HBs levels was vaccine group (p=0.0002). These results suggest an inadequate performance of the LDPD vaccine under field conditions in Eastern Cape infants.

2.3.2. Introduction

Hepatitis B virus (HBV) infection is an important health problem in many parts of the world, particularly in developing countries [Maynard, 1990]. In 1991, the Expanded Programme on Immunisation (EPI) Global Advisory Group of the World Health Organisation (WHO) recommended that hepatitis B vaccine should be included in national immunisation programmes in all countries with a hepatitis B carrier rate of 8% or more by 1995 and in every country by 1997 [World Health Organisation, 1996]. The objective of the WHO recommendations is to reduce the incidence of new carriers among children by 80% by 2001 and to eventually eliminate the approximately one million deaths per year that occur from HBV associated cirrhosis and primary liver cancer [Kane, 1996].

South Africa is an area of high endemicity for HBV infection with more than 75% of adults having evidence of past infection with this virus [Dusheiko, et al., 1989 i; Edmunds, et al., 1996 i]. The main mode of transmission appears to be by horizontal routes in children [Prozesky, et al., 1983; Abdool Karim, et al., 1989; Kew, 1992] although a recent study has demonstrated some earlier transmission in the 0-6 month

age group [Vardas, et al., 1999] HBV carrier rates in South African children, measured as HBsAg positivity, are variable ranging from 0.97% in urban areas like Soweto [Prozesky, et al., 1983] to 18.5% in rural areas of Kwazulu-Natal [Abdool Karim, et al., 1988].

Despite these regional variations in HBV prevalence, the overall burden of HBV infection in South Africa is significant [Schoub, et al., 1992]. Therefore, in April 1995 , following WHO recommendations, a revised EPI programme was introduced for this country which included HBV immunisation, to be administered with oral polio and DPT at 6, 10 and 14 weeks of age [Eggers, 1995; Voigt and Kirsch, 1995]. However, the high prices of HBV vaccines, which impedes their wide spread use in developing countries [Lee, et al., 1989; Ndumbe, 1996; Demicheli and Jefferson, 1996] limited the choice of vaccine to be used in South Africa. Therefore a relatively cheaper LDPD HBV vaccine containing 1.5 µg of HBsAg per paediatric dose which is endorsed by WHO [World Health Organisation, 1996] and has been shown to induce protective antibody responses in trials in infants from the Far East [Kim, et al., 1985; Chung, et al., 1988; Lee and Kim, 1989] was chosen for use as part of the South African infant immunisation programme [Eggers, 1995].

The safety record and efficacy of PD HBV vaccines is not in question, since over 30 million doses have been given safely and successfully over a number of years in many countries [Szmuness, et al., 1980; Szmuness, et al.,1981; World Health Organisation, 1989; Hollinger, 1996; Hall, 1996; Lemon and Thomas, 1997]. Studies of PD hepatitis B vaccine in South African paediatric populations confirm the efficacy of this type of vaccine in local populations, with high serological protection rates of ≥10 mIU/ml in 93% [Prozesky, et al., 1983] and 96% [Schoub, et al., 1991] of immunised children. However, HBV vaccine containing 10 µg of HBsAg per dose was used in both these studies and more prolonged vaccination schedules (birth, 1 and 6 months of age) were followed.

The current EPI HBV vaccine schedule uses an accelerated programme of immunisation at 6, 10 and 14 weeks of age which is known to decrease the peak titres of anti-HBs achieved [Hadler, et al., 1989; Jilg, et al., 1989]. Furthermore, the use of low dose recombinant HBV vaccine has previously been shown to provide poor serological protection in South African children with only 63.8% of children between the ages of 5-17 years demonstrating anti-HBs levels ≥10mIU/ml [Manyike, et al., 1992]. An accelerated schedule of immunisation similar to the current HBV schedule of 0, 1 and 2 months was used in this latter study. Therefore, although the introduction of HBV vaccine for South Africa infants was seen as a major positive public health intervention, the use of a low dose vaccine on an accelerated schedule has been questioned because of the increased likelihood of lower vaccine effectiveness. Additionally, because of the low HBsAg concentration per dose in this particular LDPD vaccine formulation, 1.5 µg/0.5 ml paediatric dose, the durability under less than perfect field, cold chain and storage conditions in South Africa [Battersby, 1995] has also been questioned, as there is no margin of safety for decline in antigen concentration.

The first published South African trial with this LDPD HBV vaccine showed a good response in children receiving three doses under controlled conditions with 93% of the children attaining anti-HBs levels ≥10mIU/ml [Aspinall and Kocks, 1998]. However, this trial was done in an urban area with an undefined background prevalence of HBsAg carriers and vaccine handling which was strictly controlled by the researchers and thus did not test the performance of the vaccine under field conditions. Therefore, the aim of the present study was to evaluate the immunogenicity of the South African EPI LDPD HBV vaccine under field conditions, in the Eastern Cape Province which is highly endemic for HBV infection, with HBsAg carrier rates in the 0-6 month age group being 8.1%.

2.3.3. Materials and Methods

Study Population and Vaccine Handling

The study was conducted in the Mdantsane district of the Eastern Cape Province which consists of a large urban township surrounded by a number of rural settlements. Details of this area and the population living here are described in Section *2.1.3.* Health services in the district are provided by Cecilia Makiwane Hospital (CMH) with a capacity of 1000 beds and 18 primary health care clinics associated with the hospital. Eleven clinics are located within the township of Mdantsane and 7 are in the surrounding rural areas. Four urban clinics and three rural clinics were selected for the study. The clinics were selected for logistic reasons in that their immunisation days were staggered throughout the five day working week but the clinics used were thought to be representative of others in the district. Sample collection started in July 1995 and ended in April 1996.

Two qualified nurses were hired as field workers to recruit the subjects, to administer both LDPD (Hepaccine®, Chiel Sugar and Foods, Korea) and recombinant HBV (Engerix B®, Smith Kline and Beecham, Belgium) vaccines, and to take the blood samples as required. The recombinant HBV vaccine delivery and storage was handled by the field researchers, whereas single dose vials of the LDPD vaccine used was delivered to the clinics by the normal route of vaccine distribution from the central Department of Health stores in the Eastern Cape Province. On each vaccination day, the necessary number of vials of recombinant vaccine were transported with cold packs to the clinics. LDPD vaccine was taken from the supply of the clinic being visited on that particular day, no attempt was made to assess the conditions of storage and transport of the LDPD vaccine. All vaccine doses in both the groups was administered in the same way by the research workers, intramuscularly into the antero-lateral thigh of the infant.

All children at the appropriate age (6 weeks) arriving at the clinics for their scheduled

vaccinations were eligible to participate in the study. Children were included if their mother or guardian gave written informed consent for participation in the study. Infants were excluded if they had already received any HBV immunisation or if their parents refused to give consent for participation. A total of 119 infants were enrolled for the LDPD group and 108 infants for the recombinant vaccine group. Blood samples from 80 age-matched, HBsAg negative, HBV unimmunised children (n= 20 for each age group of 6, 10, 14 and 18 weeks), obtained from the cohort in *Study 1* (Section *2.1.* above) were included in the analysis. These samples were analysed to provide a baseline measure of anti-HBs levels at each EPI time interval, so that natural decay of maternal anti-HBs levels could be assessed.

Sample Collection and transport

Heel or finger prick blood specimens were obtained from the enrolled children at baseline before vaccination at 6 weeks of age (Blood 1), and then every four weeks after one, two and three doses of vaccine. These specimens are referred to as Blood 2 ,3 and 4 respectively. Specimens from the unimmunised controls was collected in an identical fashion as for the vaccine group infants, but only once at the appropriate times of 6, 10, 14 and 18 weeks of age. There was no follow up of these children.

Name, age, gender, district and immunisation histories for all the EPI vaccines were recorded on standardised patient information forms for all infants enrolled (Appendix 1). After each session blood specimens were kept in a cooler box on ice blocks and transported back to Cecilia Makiwane Hospital (CMH) in this way to be stored at 4°C. At the end of each week the specimens were sent to the National Institute for Virology (NIV) in Johannesburg by overnight courier for analysis. On arrival at the NIV, the serum was separated, the specimens were labelled and stored at - 20°C until testing.

Serological Testing and Statistical Analysis

Serum samples were batched and tested for anti-HBs using a standard enzyme linked immunosorbant assay (ELISA) kit, (*Wellcozyme anti-HBs,* Murex Laboratories®, UK). The manufacturers conditions for the tests were strictly adhered to and positives were defined according to the kit instructions. Kinetic readings were done for quantification of the level of anti-HBs in milli-international units per millilitre (mIU/ml). The detection of HBsAg was carried out using a standard ELISA kit (*Wellcozyme HBsAg,* Murex Laboratories®) was done only for those samples in which there was sufficient volume.

Descriptive statistics, 95% confidence intervals, *univariate analysis using* χ^2 tests of association and trend, Student's t tests for comparison of means, log transformation of the data as well as multivariate testing with a logistic regression model were done. All the statistical testing was done using *SAS and SPSS statistical software.*

2.3.4. Results

A total of 227 infants were enrolled in the two vaccine groups, 119 in the LDPD and 108 in the recombinant groups respectively. The mean age at enrollment was 7.0 (SD ± 1.59) and 7.1 (SD ± 1.69) weeks for the LDPD and recombinant vaccine groups respectively. There were no statistically significant differences between the two vaccine groups regarding mean age at enrollment in weeks (p=0.66) and sex distribution of the infants (p=0.49). The characteristics of the vaccine sample are shown in *Table VIIa.*

After enrollment 18 infants did not have blood specimens taken and withdrew from the study. Baseline blood specimens (blood 1) were taken from 108 infants in the LDPD group and 101 infants in the recombinant vaccine group before vaccination. All children then received their first vaccination during the same visit. The response rates at each follow up time point in each of the vaccine groups is shown in *Table VIIb.* Follow-up of the infants was done at the 10 week immunisation time and a second blood specimen

(blood 2) was taken before the second dose of vaccine was administered at that time. Seventy nine of 108 (73.1%) of the LDPD group and 82/101 (81.2%) of the recombinant group received a second dose of their respective vaccine and had blood 2 taken. Overall, a total of 130 infants had blood specimens taken at the 14 week visit (blood 3) before administration of the final dose of vaccine to complete the three dose schedule, the response rate in the LDPD group was 57.4% and 67.3% in the recombinant group. However, the follow up at 18 weeks, one month after the last dose of vaccine, was poor for both vaccine groups, and blood 4 specimens were collected from 43.6 % (44/101) of the recombinant group and 41.7% (45/108) of the infants in the LDPD group. At each time interval in a logistic regression model, there were no significant differences in the drop out rate by vaccine group (p=0.444). Attempts were made to follow up the infants who did not present at each of the time intervals (non-compliers), however only a few of these infants were ever traced again, so limited information was available for these infants. However, the main reason for not completing the immunisation schedule and the study for non-compliers in both groups was migration from the study area (55%), in 12% of cases the guardians refused consent for subsequent blood samples, even though the infant received vaccination. In 33% of infants lost to follow up, no reason was ever identified.

For the infants who remained in the study and were followed up, vaccination schedules varied, with few children arriving for vaccination on the correct day, mean ages and 95% confidence intervals at which vaccine was administered and for the follow up time at the scheduled 18 weeks visit for the two vaccine groups are shown in *Table VIII*. There were no statistically significant differences between vaccine groups regarding mean ages at vaccination and mean age at follow up at 6, 10 and 14 weeks (t=219.6, p=0.66), (t=167.2, p=0.73), (t=136.0, p=0.33) respectively or the timing of the last specimen at 18 weeks (t=89.1, p=0.69).

Anti-HBs levels at baseline and at blood 2 (10 weeks), were similar in both groups and were not statistically significantly different (χ^2 1.46, p=0.482) and (χ^2 1.96, p=0.376) . However at both blood 3 (14 weeks) and at blood 4 (18 weeks) the differences in anti-HBs levels were statistically significantly different (χ^2 9.43, p=0.009) and (χ^2 23.97, p=0.000) respectively, with higher anti-HBs levels seen in the recombinant vaccine group.

Due to the skewed distribution of the anti-HBs data with the majority of the measurements being low, a log transformation was applied to compare the levels of anti-HBs at each time interval for each vaccine groups to determine if the statistical differences at 14 and 18 weeks between the two vaccine groups show by the univariate (χ^2) analysis remained. *Table IX* shows the log mean anti-HBs levels (mIU/ml) for the two vaccine groups (± SD) and results of independent t tests applied to the log transformed anti-HBs levels for each of the vaccine groups at baseline (blood 1) and for blood 2, 3 and 4 at 10, 14 and 18 weeks respectively. There were no differences between the two vaccine groups at baseline (t=1.63, p=0.104) and at the 10 week visit (t=1.43, p=0.154), however, the statistically significant differences between LDPD and recombinant vaccine at 14 (t=2.92, p=0.004) and 18 weeks (t=5.14, p=0.000) persisted, with higher log levels of anti-HBs in the recombinant vaccine group. The mean baseline anti-HBs measurements for the unimmunised controls are shown for the various time intervals in *Figure 11*. The levels of anti-HBs start high at baseline and decrease with time reaching the lowest mean level at 18 weeks of follow up. Baseline anti-HBs levels between the two vaccine groups and between the vaccine groups and the unimmunised controls were not significantly different (ANOVA, F=2.83, p=0.06).

Figure 10 shows the percentage of children who had anti-HBs levels ≥10 mIU/ml in each vaccine group at each time interval after vaccination. The proportion of children with protective anti-HBs levels (≥10 mIU/ml) at baseline and at the 10 week visit were not

statistically significantly different between the LDPD and recombinant groups (χ^2=0.975, p=0.323) and (χ^2=0.600, p=0.439) respectively. However, at 14 and 18 weeks, the proportion of protected children in the LDPD group was lower than in the recombinant vaccine group. These differences were statistically significant at both 14 weeks (χ^2=8.865, p=0.003) where 63.2% of infants in the recombinant group and 37.1% of those in the LDPD group were protected and at 18 weeks where 88.6% of infants in the recombinant group and 42.2% in the LDPD group had anti-HBs levels ≥10 mIU/ml, (χ^2=21.114, p=0.000).

After doing all the HBsAb testing, sufficient volumes of serum for HBsAg testing was available in 110/227 (48.5%) of the enrolled infants. Of these infants, 95.5 % were HBsAg negative and 4.5 % were positive for HBsAg. All the positive infants (n=5) were in the LDPD group. However, in a logistic regression model applied to determine which factors (vaccine type, sex or HBsAg) were responsible for the differences in anti-HBs responses in the two vaccine groups, it was shown that the only factor that had any significant impact on the anti-HBs levels and therefore the number of children with protective anti-HBs ≥10 mIU/ml was vaccine type (p=0.0002).

2.3.5. Discussion

This study is the first to determine the immunogenicity of a low dose plasma derived vaccine under field conditions in South Africa. The results indicate that LDPD HBV vaccine administered in these infants in the field is less immunogenic than a recombinant vaccine administered under controlled conditions. The LDPD vaccine achieved both lower mean anti-HBs levels after one, two and three doses as well as lower final titres at one month after the last vaccination of a three dose schedule. Eventually, only 42.4% of vaccinated infants with LDPD vaccine had anti-HBs levels ≥10 mIU/ml compared to 88.6% of infants in the recombinant vaccine group.

Factors which may have influenced the immunogenicity of this vaccine can be separated into two broad categories, immunisation factors and host factors. The former category includes dosage, storage of the vaccine, administration of concurrent EPI vaccines, site and route of inoculation and timing of inoculations. Host factors which should be considered are age, sex, background prevalence of HBV infection, genetic factors and immunological competence.

HBV vaccines are not generic products and the optimal dose for each product is usually different and determined using dose titration curves in studies that vary the dose, age, schedule and number of doses [Hollinger, 1996]. Several studies have shown unequivocally that the dose of vaccine administered to an individual is of considerable importance in determining the peak immune response achieved by that person [Zahradnik, et al., 1985; Hollinger, et al., 1986; Yeoh, et al., 1986; Zahradnik, et al., 1987; Phanuphak, et al., 1989; Lee, et al., 1995]. The magnitude of the anti-HBs response induced by a primary HBV vaccination schedule appears to be predictive of long term antibody persistence and a logarithmic decline of antibodies usually occurs over time [Gesemann and Scheiermann, 1995]. The recombinant vaccine used in this study contains 10 µg of HBsAg per paediatric dose compared to 1.5 µg per dose in the LDPD vaccine. Therefore, one factor which may have contributed to the poor responses in the infants in the LDPD group may have been the almost ten times lower HBsAg concentration in the LDPD product. However, the manufacturer of the LDPD vaccine claims that a unique flash heating process of pooled donor plasma in the production of this vaccine preserves the pre-S portions of HBV surface protein, thus making it more immunogenic and justifying the lower concentration of antigen in each dose [Chiel Foods & Chemicals, 1995]. This increased immunogenicity of the LDPD product was not demonstrated in our study and the lower dose of HBsAg was probably the most likely reason for the poor vaccine response. Low dose recombinant vaccine has also been shown to induce poor anti-HBs responses in South African children, with only 63.8% of

children 7-17 years of age showing an anti-HBs response ≥ 10 mIU/ml after three doses of vaccine containing 2 µg HBsAg per dose [Manyike, et al., 1992].

Other important factors which may have influenced the immunogenicity of the LDPD product in this study include transport and storage of the vaccine. The recombinant vaccine was transported and stored by the field researchers, whereas the LDPD product went through the normal delivery systems in the Department of Health. Whether these conditions were less than ideal is not known as cold chain, storage and vaccine potency at the end point of delivery, for both LDPD and recombinant vaccines were not monitored directly. However, the possibility that the LDPD vaccine was damaged in some way en route to the clinics in the Eastern Cape exists. Some manufacturers try to build in a "safety margin" into HBV vaccine antigen dose because the vaccine will be administered under various conditions of storage and delivery, as well as on individuals of different ages and immunocompetence [Kane, 1995]. The LDPD vaccine used in the South African EPI has such a low concentration of HBsAg per single vial dose, that even small cold chain breeches or poor storage conditions may have considerably decreased the potency of the final product in the field. Thus this product does not appear to be robust enough to survive within the less than ideal cold chain and vaccine storage conditions currently in place in South Africa [Battersby, 1995].

Other immunisation factors such as the site, route and timing of the inoculations were identical in both vaccine groups and could not have influenced the poor immunogenicity of the LDPD vaccine. Both LDPD vaccine and the recombinant HBV vaccine were given on an accelerated schedule at 6, 10 and 14 weeks of age which may have contributed to the overall low levels of anti-HBs seen in both groups [Jilg, 1989; Lemon and Thomas, 1997]. Studies in Thai infants using this particular LDPD product have demonstrated that the greater the interval between the second and third vaccines, the better the overall immunogenicity of the vaccine [Phanuphak, et al 1989]. The present

study clearly demonstrates that the last dose of vaccine (at 14 weeks) plays a vital role in inducing protective immunity in vaccinated infants (*Figure 10*). This has important implications for the long term durability of the anti-HBs levels in infants since the lower the one month antibody levels are, the less likely it is that vaccine protection will last [Gonzalez, et al., 1993; Tabor, et al., 1993; Gesemann and Scheiermann, 1995]. Accordingly, it would be essential for an individual to complete the HBV vaccination schedule with LDPD HBV vaccine to ensure serological protection. Although the difficulties of maintaining good vaccine coverage with HBV vaccine in South Africa have previously been demonstrated [Schoub. et al., 1991] with only 39 % of infants completing their scheduled HBV immunisations in this study.

The concurrent administration of other routine EPI vaccinations at 6, 10 and 14 weeks were identical for both vaccine groups and there is no published evidence to suggest that there is cross interference with other EPI vaccines [Coursaget, et al., 1992; Da Villa, et al., 1995; Papaevangelou, et al., 1995; Lemon and Thomas, 1997]. Therefore the other EPI vaccines are unlikely to have influenced the poor immunogenicity seen with the LDPD HBV vaccine.

Host factors like age and sex distributions for the two vaccine groups in this field trial were not different, and so probably did not influence the different immunogenicities of the two HBV vaccines. The background prevalence of HBsAg carriers in this population in the Eastern Cape, has been estimated to be high, close to 8.1% in the 0-6 month age group [Vardas, et al., 1999]. However, since infants were taken from the same population and randomly assigned to one or the other vaccine group, it is unlikely that there was any difference in background HBV infection rates between the two vaccine groups. Another important host factor which was unlikely to be different in the two vaccine groups was genetic background, however, no genetic markers were actually measured in this study. Population-specific characteristics have been shown to

adversely affect the immunogenicity of this LDPD vaccine in Melanesian infants from the Solomon Islands [Milne, et al., 1992; Milne, et al., 1995] and although there have been some published studies from the Far East [Kim, et al., 1985; Chung, et al., 1988; Lee and Kim, 1989] and one South African study [Aspinall and Kocks, 1998] suggesting that the immunogenicity of this vaccine is good in these regions, the genetic characteristics of the population in which the vaccine is used may ultimately influence the general applicability of the vaccine on a worldwide basis.

Interference with the vaccine response by high levels of maternal anti-HBs transplacentally passed on to infants is another possible reason for the poor performance of the LDPD vaccine. However, there was no difference in the baseline anti-HBs levels for the two vaccine groups and the unimmunised control group. The natural decay of maternal anti-HBs was shown in the unimmunised group. In the two vaccine cohorts, the anti-HBs levels initially declined, probably signifying loss of maternal antibody but then increased after the second or third dose of vaccine took effect. It is generally accepted that maternal antibody interference with the response to HBV vaccine is unlikely [Kane, 1995].

The final host factor that may have influenced the immunogenicity of the LDPD vaccine in this study is the immunological competence of the population studied [Hollinger, 1996; Lemon and Thomas, 1997]. Once again, this was not measured directly, but only healthy children presenting for their routine immunisations were eligible for enrollment in the study. This would have excluded severely ill infants, but may not have excluded infants perinatally infected with HIV. Whether the response to LDPD HBV vaccine is different in HIV infected children has not been clearly demonstrated as yet, although some studies suggest that even healthy HIV positive children respond poorly to HBV vaccinations [Zuin, et al., 1992; Zucotti, et al., 1994; Rutstein, et al., 1994]. Once again, because of randomising to the vaccine groups, the numbers of HIV infected children

would likely be the same in the recombinant and LDPD vaccine groups. However, because of the possibility of a poorer response to vaccine antigens in HIV infected children, the EPI policy in South Africa should probably take into account the substantial burden of HIV infection in South African women of child bearing age [Department of Health, 1994] and ensure that highly immunogenic vaccines are used as part of the EPI programme.

The poor performance of the LDPD HBV vaccine in this study in South Africa is in contrast to studies in infants from the Far East [Kim, et al., 1985; Chung, et al., 1988; Lee and Kim, 1989] and the single South Africa study done in an area of undefined HBV prevalence and under controlled conditions [Aspinall and Kocks, 1998]. However, these trials use variable study designs; different immunisation schedules and initial ages of vaccination, thus making it difficult to generalise their results. In a recent study in which LDPD was introduced as an EPI vaccine for Thai infants, serological protective rates of > 10 mIU/ml, were found in 88% of infants one month after a three dose schedule of vaccine [Chunsuttiwat, et al., 1997]. However, a schedule of 3 doses birth, 2 and 6 month was used in this study and double the recommended paediatric dose of vaccine (3 µg/dose) was administered, suggesting a poor overall response to LDPD vaccine in Thai infants. This strategy of doubling the dose, remains an option for the South African EPI and should be tested in the field as a matter of urgency. The impact and cost effectiveness of doubling the dose of LDPD HBV vaccine must also be assessed, as it may be cheaper to use another type of vaccine with a higher concentration of HBsAg.

The main limitation of this study in the Eastern Cape, was the considerable drop out rate for the 18 week visit, which limited the samples sizes at that time. However, due to the dramatically different serologically protective antibodies in the two vaccine groups at this time, the statistical power of the estimate remained highly significant. The main reason for the poor response rate at 18 weeks can only be speculated on, but appears to be

related to the fact that there is no real incentive for mothers to return to have their children bled for a study and that this is an unscheduled EPI time. Every attempt was made to search for patients not returning for any of the follow up times but difficulties were encountered mainly related to the migration of people from this area for economic reasons [Orkin, et al., 1998]. Finally, the conventional response to HBV vaccination, as measured by serological means [Hollinger, 1996; Lemon and Thomas, 1997] was used as the only indicator of vaccine immunogenicity in this study. We did not examine any differences, if any, in the cellular responses to HBV vaccine between recombinant and LDPD vaccine.

This study provides valuable information regarding the immunogenicity of LDPD HBV vaccine in South African and emphasises the need for pilot studies with new vaccines in the targeted communities. Furthermore the need for continuous surveillance of vaccine responses, coverage , cold-chain maintenance and vaccine storage are also highlighted [Aylward, et al., 1994; Chen and Orenstein, 1996, Chen, et al., 1996]. The long term risks of sub-optimal immunity providing immune pressure on natural HBV infection to create viral escape mutants that eventually will not be protected against by the immunity induced by conventional HBV vaccination needs to be monitored [Carman and Thomas, 1991]. Adjustments may need to be made to this essential new addition to the South African EPI for it to remain cost effective. Most importantly the dose of antigen per vaccine must be optimised to ensure a sufficient serological protection of children under field conditions in South Africa which may be done by doubling the normal paediatric dose, as was done in Thailand [Chunsuttiwat, et al., 1997], however this strategy will increase the cost of the HBV vaccine initiative in South Africa. Booster HBV vaccine doses may need to be administered at a later date (five years of age) to ensure sufficient serological protection against HBV infection of the targeted population.

Table VII

a. Characteristics of study population HBV vaccine field trial

	Total	Vaccine group		
		LDPD	Recombinant	p Value
N	227	119	108	
Age (Weeks) *	7.0 ± 1.64	7.0 ± 1.59	7.1 ± 1.69	p=0.66 †
Gender				
Males	106 (46.7%)	53 (44.5%)	53 (49.1%)	p=0.49 ‡
Females	121 (53.3%)	66 (55.5%)	55 (50.9%)	

* Mean ± SD
† Students t-test
‡ χ^2 Test

b. Response rates at each time point for each vaccine group.

	Total		LDPD		Recombinant	
	No	% Completed	No	% Completed	No	% Completed
Dose 1	209	100.0%	108	100.0%	101	100.0%
Dose 2	161	77.0%	79	73.1%	82	81.2%
Dose 3	130	62.2%	62	57.4%	68	67.3%
18 weeks	89	42.6%	45	41.7%	44	43.6%

Table VIII Mean ages at each vaccination data and at one month after the last vaccine demonstrating the timing of vaccine doses and specimens.

	Weeks *			
		Vaccine group		
	Total	LDPD	Recombinant	p Value†
Dose 1	7.0 [6.8; 7.3]	7.0 [6.7; 7.3]	7.1 [6.8; 7.4]	
Dose 2	13.0 [12.4; 13.6]	12.9 [11.9; 13.9]	13.1 [12.3; 13.9]	p=0.73
Dose 3	18.4 [17.6; 19.2]	18.0 [16.8; 19.2]	18.8 [17.7; 19.9]	p=0.33
18 weeks	22.3 [21.5; 23.1]	22.1 [21.0; 23.2]	22.4 [21.2; 23.7]	p=0.69

* Mean [Confidence limits]

† Student's t-test

Table IX Log mean anti-HBs levels (± SD) at each time point for the LDPD and recombinant HBV vaccine groups.

	Log Anti-HBs Levels		
	LDPD	**Recombinant**	**p value**
Dose 1	1.0 [1.14]	1.2 [1.04]	0.104
Dose 2	0.8 [1.07]	1.0 [0.92]	0.154
Dose 3	0.7 [0.79]	1.1 [0.72]	0.004
18 weeks	0.9 [0.79]	1.7 [0.65]	0.000

* Mean [± SD]
† Student's t-test

Figure 10 The proportion (%) of children who had levels of anti-HBs ≥10 mIU/ml in each vaccine group at each time interval after vaccination.

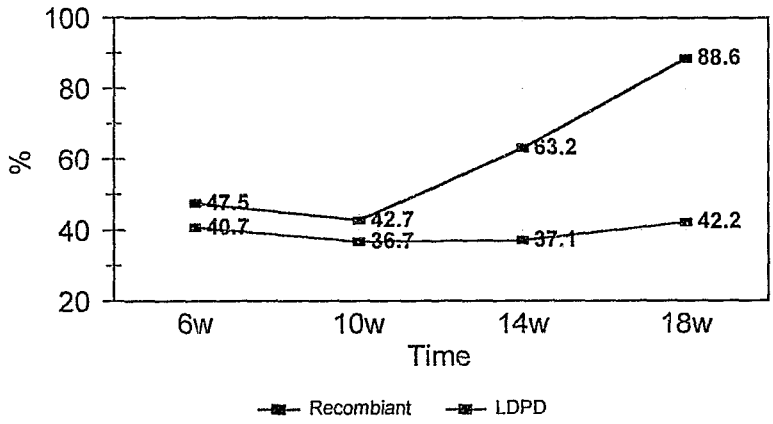
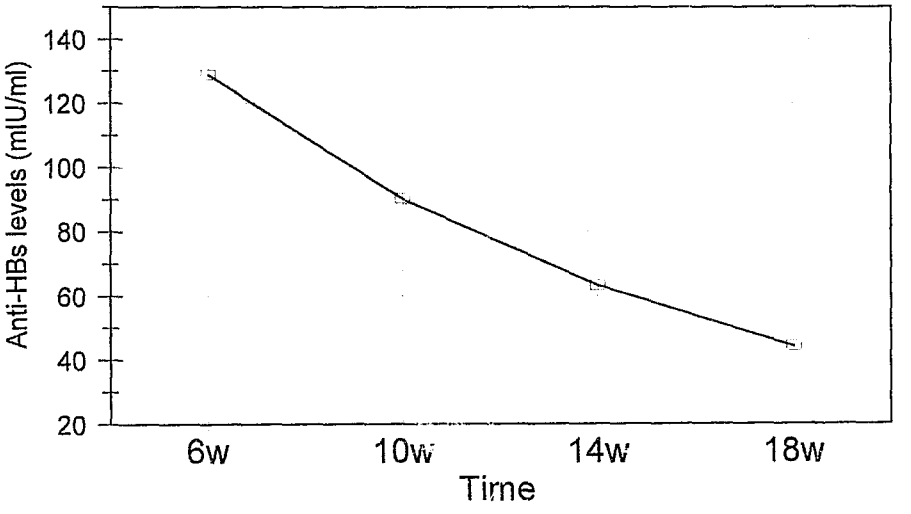


Figure 11 Mean anti-HBs levels in unimmunised controls demonstrating the natural decay of maternally derived anti-HBs with time.



CONCLUDING DISCUSSION

These three studies attempt to answer important issues regarding HBV and its control in South Africa. The first study provides the most recent pre-immunisation, community-based baseline investigation of the epidemiology of HBV infection in children targeted for universal immunisation in South Africa. The results from this study showed that there may be a higher rate of earlier transmission of this infection in the 0-6 month age group than previously expected. Based on this finding the current HBV EPI schedule (vaccine administered at 6, 10, 14 weeks) may have to be amended to include a birth dose of vaccine. Furthermore, the overall rate of chronic HBV carriers in the 61-72 month group (15.7%) is the highest rate shown for South African, non institutionalised children of this age. This result emphasises the importance of a universal infant HBV programme in South Africa. The second study provides the first assessment of HBV genotypes in a sample of community infected children from the Eastern Cape Province, and the results show a unique clustering of mainly genotype A' in this area. Measuring the chronic carrier rates and circulating HBV genotypes in the Eastern Cape periodically after the introduction of universal infant HBV immunisation will enable an assessment of the EPI intervention based on the baseline information generated by Study 1 and 2.

Study 3 assessed the effectiveness, under field conditions, of a low dose plasma derived (LDPD) HBV vaccine containing 1.5 µg of HBsAg per paediatric dose. This vaccine was introduced as part of the revised EPI schedule in South Africa in April 1995 and was administered on a rapid schedule at 6, 10 and 14 weeks of age. The results from Study 3 suggest an inadequate performance of the LDPD vaccine under field conditions in Eastern Cape infants. The reasons for this were difficult to assess because the design of the study did not take into account vaccine potency and cold-chain management. However, the poor performance of the LDPD HBV vaccine in the field, regardless of the

reasons, suggests that the HBV EPI programme in South Africa should be reassessed regarding the choice of HBV vaccine.

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5. APPENDIX 1

An example of the patient data form used to collect demographic and vaccine related data from all study participants (Study 1, 2 and 3). Forms were colour coded for the various studies and study numbers were pre-printed.

No. 2998 NATIONAL INSTITUTE FOR VIROLOGY: HEPATITIS PROJECT (011 8829910)

CLINIC DATE

CLINIC NUMBER

NAME

RESIDENTIAL AREA

DATE OF BIRTH

SEX *M* *F* ***PLEASE ✓***

VACCINATION HISTORY			
	DATE		DATE
1st BIRTH POLIO		1ST HBV	
2ND POLIO		2ND HBV	
3RD POLIO		3RD HBV	
4TH POLIO			
5TH POLIO			
1ST MEASLES			
2ND MEASLES			

PLEASE SUPPLY DATES WHERE AVAILABLE

(Reverse Side)

CONSENT FORM

I, the undersigned, understand the aim of the hepatitis project as fully explained to me by the study research workers.

I hereby give consent for my child to take part in this project. I understand that I do not have to allow a blood sample to be taken from my child and that I can remove my self and my child from the study at any time.

....

Author Vardas E
Name of thesis The Epidemiology And Molecular Characteristics Of Hepatitis B Virus Infection In Children From A Hyperendemic Area Of South Africa, Including A Field Trial Of The Hepatitis B Immunization Programme And Its Impact On Infection In This Population Vardas E 1999

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