Hepatitis B Virus in Silvery Gibbons (Hylobates moloch)

by

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BVSC (Hons)

A dissertation submitted to Murdoch University in fulfilment of the requirements for the degree of Masters in Philosophy in Zoo and Wildlife Medicine

Division of Veterinary and Biomedical Sciences

July 2004
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

....................................

Karen Louise Payne

30th July, 2004
Abstract

This research investigated a number of issues regarding hepatitis B virus (HBV) in the silvery gibbon (Hylobates moloch). Due to the relatively recent discovery of the virus in nonhuman primate populations, specific knowledge of the biological behaviour of the virus is presently lacking, with current information largely extrapolated from the behaviour of HBV in human infections. In order to manage the captive and wild populations of this critically endangered species, information regarding the behaviour of the virus in gibbons and the likely impact of the viral infection is essential.

The research was performed at Perth Zoo, with the study population consisting of the current and historical members of the zoo’s silvery gibbon colony. Because this gibbon species is critically endangered, the study was conducted with minimal intervention to the population with samples collected largely on an opportunistic basis from a small study population.

Review of the history of the virus within the Perth Zoo colony provided epidemiological evidence to indicate vertical transmission in three gibbons (Hecla, Uban and Jury). It would appear that vertical transmission is the primary mode of transmission leading to dispersal of the virus through the captive population of silvery gibbons.

Elevated concentrations of the liver enzymes alanine aminotransferase and aspartate aminotransferase were found in three gibbons (Perth 2, Uban and Jury), and may suggest a pathogenic role of the virus in this species. Histological examination of the livers of Uban and Perth 2 failed to demonstrate definitive evidence of cirrhosis, however mild fibrosis was seen in both cases and may represent an early stage of liver pathology associated with chronic hepatitis B infection.
The vaccination protocol developed at Perth Zoo was successful in preventing neonatal transmission of the virus from a high infectivity carrier mother in at least two individuals, and was also successful in producing a protective level of immunity against the virus in all three of the individuals tested.

Sequencing of the complete hepatitis B genome from one gibbon (Hecla) revealed that she was infected with GiHV (Gibbon hepatitis B virus), an indigenous strain of HBV previously identified in a number of gibbon species, but not previously confirmed in the silvery gibbon. Hecla’s strain of HBV was shown to be more closely related to other nonhuman primate strains of HBV than to any of the human strains of HBV. 100% nucleotide similarity to two of Hecla’s siblings indicates that infection in all three animals was the result of vertical transmission from their mother. Partial sequencing of the virus from a second gibbon (Uban) identified another strain of GiHBV which supports the results of the epidemiological study. Neither gibbon showed a high sequence similarity to the virus sequenced from Ivan, the father of the third carrier gibbon (Jury), although only limited sequence data was available from Ivan. Consequently it is likely that at least three different strains of GiHBV are present within the silvery gibbon population.

The information contained in this thesis will assist in the understanding and management of hepatitis B infection in silvery gibbons, as well as the numerous other species of nonhuman primates now shown to be susceptible to this virus.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>anti-HBc</td>
<td>antibody to hepatitis B core antigen (HBCAg)</td>
</tr>
<tr>
<td>anti-HBe</td>
<td>antibody to hepatitis B e antigen (HBeAg)</td>
</tr>
<tr>
<td>anti-HBs</td>
<td>antibody to hepatitis B surface antigen (HBsAg)</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>ChHBV</td>
<td>Chimpanzee hepatitis B virus</td>
</tr>
<tr>
<td>CITES</td>
<td>Convention on International Trade in Endangered Species of Wild Fauna and Flora</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>Oligodeoxynucleotide containing CpG immunostimulatory motifs</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>DEPCH$_2$O</td>
<td>Diethyl Pyrocarbonated water</td>
</tr>
<tr>
<td>DHBV</td>
<td>Duck hepatitis B virus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>GCC</td>
<td>Gibbon Conservation Centre</td>
</tr>
<tr>
<td>GiHBV</td>
<td>Gibbon hepatitis B virus</td>
</tr>
<tr>
<td>GSHV</td>
<td>Ground Squirrel Hepatitis virus</td>
</tr>
<tr>
<td>HBIG</td>
<td>hepatitis B immune globulin</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBCAg</td>
<td>hepatitis B core antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HBeAg</td>
<td>hepatitis B e antigen</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>ISIS</td>
<td>International species information systems</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>MedA.R.K.S.</td>
<td>Medical animal records keeping system</td>
</tr>
<tr>
<td>MEIA</td>
<td>Microparticle enzyme immunoassay</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mIU/ml</td>
<td>Milli-international units per millilitre</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mths</td>
<td>Months</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>OuHV</td>
<td>Orangutan hepadnavirus</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDV</td>
<td>Plasma derived vaccine</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SGOT</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>SGPT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>T°C</td>
<td>Temperature</td>
</tr>
<tr>
<td>U/L</td>
<td>Units per litre</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
</tbody>
</table>
µmol/L  micromoles per litre
USA  United States of America
WHO  World Health Organisation
WHV  Woodchuck hepatitis virus
WMHBV  Woolly monkey hepatitis B virus
YDV  Yeast derived vaccine
yrs  years
+ve  positive
-ve  negative
Acknowledgements

This thesis is dedicated to the memory of Uban, whose cheeky antics and soulful eyes captured my heart, and the hearts of many others. Through his death he has helped to shed light on the pathogenicity of hepatitis B in nonhuman primates, and silvery gibbons in particular. It is hoped that the knowledge gained from this thesis will aid in the conservation of other wild and captive nonhuman primates, including the critically endangered silvery gibbon.

This thesis is the result of a new program jointly run by Murdoch University and the Perth Zoo, a three year Master of Philosophy program which combines both a research thesis and a full time clinical residency in the Perth Zoo Veterinary Department. It has always been my dream to work as a veterinarian in a zoo or wildlife setting. This program has allowed me to achieve this dream whilst at the same time being able to contribute, through this research, to the conservation of the critically endangered silvery gibbon and to the pool of knowledge surrounding this emerging viral disease. Whilst I am proud to call this thesis my own, I could not have achieved this work without the support and contributions of the people acknowledged below.

Initially I would like to thank Perth Zoo and the Murdoch University Veterinary Trust for creating this position and providing the necessary funding and financial support, without which this thesis would never have been able to get off the ground. In particular, I would like to thank Associate Professor Dr John Bolton and Dr Cree Monaghan for their work in the inception and development of this program, and for their constant enthusiasm and support for this position.

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endeared themselves to me.

A major component of this thesis is the sequencing work, and I would like to express
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with the project and for providing all of the hepatitis B testing of the Perth Zoo gibbons.
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I would also like to thank Dr Kris Warren for her input into the project and her feedback. I admire her obvious enthusiasm for the work, the animals, and wildlife in general. I am
extremely grateful to have had the opportunity to work in such proximity with what I consider to be a true expert in this field. Her advice has been invaluable.

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Chapter 1: Literature Review

1.1 Introduction

A parenteral form of hepatitis in humans was first documented in 1885, but it was not until the late 1930's that the existence of the disease now known as hepatitis B virus (HBV) was firmly established (Hollinger and Liang, 2001). Since that time numerous studies have been performed investigating the virus. As a result, there is a relative wealth of information currently available regarding human infection with HBV. In contrast, hepatitis B viruses in nonhuman primates have only been discovered in the last two decades. Initially it was thought that these viruses were human strains, potentially contracted by the process of inoculating wild caught primates with human blood in order to protect them from human diseases (Zuckerman et al. 1978). Further research has since identified a number of separate nonhuman primate HBV viruses infecting great and lesser apes, and a single strain infecting a new world primate species, the woolly monkey (Lagothrix lagotricha). In recent years a number of researchers have studied these viruses and provided valuable information about the genetic relatedness of these viruses to each other and to human HBV. There appears to be a general consensus that the viruses detected in these nonhuman primate species are indigenous to their primate hosts, and are closely related to but distinct from human HBV. However debate continues as to the origin of the viruses and at what stage the various nonhuman primate and human strains diverged from each other.

There is currently very little information available on the epidemiology and pathogenicity of the nonhuman primate hepatitis B viruses in their natural hosts, with most of this information extrapolated from the human virus. As a result, little is also known about the prevention and treatment of the virus in the various primate hosts. Since 1992, four silvery gibbons (Hylobates moloch) at Perth Zoo have been identified as chronic carriers of hepatitis B. Two of these animals died of liver-related disease,
while the other two animals remain at Perth Zoo and are part of a successful breeding program of this critically endangered species.

In 1993, a single hoolock gibbon (Bunopithecus hoolock) was also found to be a chronic carrier of hepatitis B. This may represent the first reported case of hepatitis B infection in this species.

### 1.1.1 The Hepatitis B Virus


The viruses in the Hepadnaviridae family share certain features including virion size and ultrastructure. All hepadnaviruses contain partially double stranded DNA and replicate via reverse transcription using endogenous DNA polymerase, and all have 3 distinct morphological forms (intact virions, and spherical and filamentous forms of the viral envelope protein which circulate in excess) (Dienstag and Isselbacher, 2001a). These viruses replicate in the liver but can exist in extrahepatic sites, and are associated to varying degrees with acute and chronic hepatitis and hepatocellular carcinoma in their natural hosts.
Hepatitis B virus is a remarkably compact virus of 3200 base pairs and has four open reading frames: the pre-S region and S gene which encode the protein of the envelope (surface antigen proteins); the pre-C region and C gene which encode the core antigen and the e antigen; the X gene which encodes a protein involved with transcriptional and transactivating properties, and the P gene which encodes the DNA polymerase and overlaps the pre-S region and S gene completely and the X and core genes partially (Tiollais et al. 1985, Norder et al. 1996) (Figure 1.1). The surface and e antigen proteins serve as serological markers of infection, while the core protein can serve as a histological marker of infection. The presence or absence of these antigens, in combination with the respective antibodies, are used to determine whether a patient has been exposed to hepatitis B virus, and the type of infection present (acute vs chronic, high infectivity vs low infectivity). A summary of the significance of each of the serological markers is contained in Table 1.1, with further discussion of the significance of these markers in section 1.1.4 of this literature review.

Human hepatitis B isolates fall into one of at least eight genotypes (Terrault and Wright, 1998). These genotypes are defined by a difference in complete genome sequence of at least 8% (Okamoto et al. 1988) and display specific geographical distributions. Genotype A is commonly detected in Northwest Europe and sub-Saharan Africa, genotypes B and C in indigenous populations in East and Southeast Asia, genotype D
throughout the Mediterranean, India and aboriginal populations in Southeast Asia and Alaska, genotype E in West Africa only and genotype F in indigenous populations in the Americas (Norder et al. 1993, Magnus & Norder 1995). Recently a seventh and eighth human genotype (G and H respectively) have been described (Stuvyer et al. 2000, Kato et al. 2002, Arauz-Ruiz et al. 2002). There are currently only a small number of isolates that have been attributed to these new genotypes so the geographic distribution of these genotypes has not yet been established. The cases to date involving genotype G have originated in France and the USA, while those from genotype H originated from Central America.

Table 1.1: Serological markers of HBV infection (adapted from Deinstag and Isselbacher 2001a)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>Indicates presence of HBV surface antigen in serum and <strong>current HBV infection.</strong></td>
</tr>
<tr>
<td>anti-HBs</td>
<td>Indicates presence of antibody to HbsAg in serum. Considered to be the protective antibody. Usually appears after the disappearance of HBsAg from the serum and can persist indefinitely. <strong>May be the result of vaccination or actual infection.</strong></td>
</tr>
<tr>
<td>HBcAg</td>
<td>HBV core antigen. Remains within the hepatocyte and does not circulate in the serum.</td>
</tr>
<tr>
<td>anti-HBc</td>
<td>Indicates presence of antibody to HBcAg in serum. Appears in the serum shortly after HbsAg and before the appearance of anti-HBs. Can persist indefinitely in the serum. <strong>Indicative of actual infection rather than vaccination.</strong></td>
</tr>
<tr>
<td>HBeAg</td>
<td>Indicates the presence of HBV “e” antigen in serum, a qualitative marker of HBV replication and relative infectivity. High concentrations of HBeAg in the absence of anti-Hbe indicate <strong>high infectivity</strong> and the presence of circulating HBV DNA. Can persist indefinitely in chronic infections.</td>
</tr>
<tr>
<td>anti-HBe</td>
<td>Indicates the presence of antibody to HBeAg and conversion to <strong>lower infectivity</strong>. In acute infections is indicative of resolution of infection (combined with anti-HBs). Can persist indefinitely in chronic infections.</td>
</tr>
</tbody>
</table>
1.1.2 Epidemiology of Hepatitis B Virus in Humans

Hepatitis B is a ubiquitous organism that is globally distributed, with an estimated global prevalence of more than 300 million carriers, or approximately 5% of the world’s population (Terrault and Wright, 1998). Patterns of infection and average age at infection vary with different geographical regions, largely due to socioeconomic factors such as level of healthcare services, rather than physical factors.

Blood is by far the most infectious source of the virus, however virus particles have been found in almost every type of body fluid from infected persons, including saliva, semen, breast milk and menstrual fluid. It should be assumed that all bodily fluids from HBV-infected persons may be infectious and capable of transmitting HBV (Hollinger and Liang, 2001), however for transmission to occur from these less common sources, high concentrations of virus and percutaneous or mucous membrane exposure are likely to be required. Such high viral concentrations could easily occur in high infectivity chronic carriers (HBV e Antigen (HBeAg) positive). The hepatitis B virus is quite stable in the environment and remains infectious for up to seven days or longer, increasing the risk of possible exposure from surfaces contaminated by infectious body fluids (Terrault and Wright, 1998).

Transmission of the virus occurs by vertical or horizontal means. Vertical transmission occurs at a higher frequency in endemic regions and is a result of transmission from mother to offspring in the perinatal period. The majority of these infections are thought to occur at the time of delivery and are unrelated to breastfeeding (Terrault and Wright, 1998, Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001). It is thought that up to 10% of these cases may be due to intrauterine infection (Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001). The incidence of perinatal infection is greatly increased in those offspring born to high infectivity carrier (HBeAg positive) mothers, with approximately 90% of infants born to these mothers becoming infected, compared with 10-15% in low infectivity carrier (anti-HBe positive) mothers. Infants infected with
HBV at birth will usually undergo a clinically silent acute infection, but there is an 80-90% chance of the infant progressing to become a chronic carrier (Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001) (Figure 1.2). In contrast, the risk of chronicity for an immunologically competent person exposed to the virus in young adulthood is only approximately 1% (Dienstag and Isselbacher, 2001b). Further mother to offspring transmission can occur during early childhood, with 60% of infants born to high infectivity carrier mothers and not infected at birth becoming infected by the age of five (Terrault and Wright, 1998).

Figure 1.2: Relationship between age and outcome after acute HBV infection (Hollinger and Liang, 2001).

In non-endemic regions, transmission of HBV occurs primarily through horizontal means such as sexual contact and percutaneous inoculation. Recent modifications in sexual behaviour in response to the AIDS epidemic have led to a significant decrease in the percentage of new cases now arising through sexual contact, which previously accounted for 20% of all cases of HBV infection in the United States. Similarly, the number of acute hepatitis B cases in injectable drug users has fallen by 90% since
1988. Other risk factors for infection include: working in a health care environment, transfusion and dialysis, acupuncture, tattooing and living in the same household as a chronic carrier (Terrault and Wright, 1998, Hollinger and Liang, 2001). In these situations, infection usually arises from inoculation with contaminated blood products or via mucosal contact with infectious material (Hollinger and Liang, 2001).

In endemic areas such as the Far East (e.g. Southeast Asia, China, the Philippines, and Indonesia), the Middle East, Africa, and parts of South America, the prevalence of HBV infection is high, with hepatitis B surface antigen (HBsAg) positivity (indicating current infection) ranging from 8% to 15% (Terrault and Wright, 1998) (Figure 1.3). Serologic evidence of previous infection is almost universal in those without active infection. In these endemic regions, transmission tends to be primarily vertical with a high rate of chronic infection. Development of chronic infection and carrier status occurs more frequently in infants than in adults, perpetuating the transmission of the virus. Other forms of transmission such as sexual and via injectable drug use are also possible in endemic areas, however they represent a much smaller proportion of infections.

Figure 1.3: Global distribution of chronic hepatitis B infection (Robert G. 2002)
In contrast, areas of low prevalence such as the USA and Canada, northern Europe, Australia and the southern part of South America have <2% of the population with HBsAg positivity (Figure 1.3). In these areas, transmission tends to be primarily horizontal with adults and adolescents at greatest risk of acquiring HBV. The prevalence of chronic carriers is lower than in areas where HBV infection is endemic. Within the regions of low prevalence there are variations in the level of infection - some ethnic groups show greater representation, and infection rates are also higher in those who fall into the high risk categories mentioned previously.

1.1.3 Pathogenicity of Hepatitis B Virus in Humans

Infection with HBV causes a spectrum of liver disease ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The majority of patients recover from their infection, with 95-99% of previously healthy adults with acute hepatitis B having a favourable course and complete recovery (Dienstag and Isselbacher, 2001a). A large proportion of infections are subclinical infections, which are detectable only by the presence of serological markers and/or mild elevations in the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Dienstag and Isselbacher, 2001b). Chronic hepatitis will develop following HBV infection in 2-10% of immunocompetent adults, however 90% of infants infected in the perinatal period by HBV-carrier mothers will become chronic carriers. Of those who become persistently infected, many have mild liver disease with little or no associated long term morbidity or mortality (Hollinger and Liang, 2001).

The hepatitis B virus is not considered a cytopathic virus. Host immune reactions, particularly non-specific cell mediated immune mechanisms, are felt to be the main mediator of pathologic changes associated with HBV infection (Terrault and Wright, 1998, Hollinger and Liang, 2001, Dienstag and Isselbacher, 2001a). Some viral factors also influence the severity of disease, with certain genomic mutations known to be
associated with more severe forms of disease. Development of chronic versus acute infection is also felt to be related to the host’s immune response, in particular the ability to develop anti-envelope antibodies. Chronic infection occurs in patients with a poor immune response, whilst those with a good antibody response are likely to undergo acute infection and subsequent recovery, however the specific mechanisms by which this occurs are not yet fully understood (Hollinger and Liang, 2001). The role of the immune response in developing chronic infection is further supported by infants who become infected at birth. These infants, who have immature immune systems, typically have a very high rate of chronic infection but usually have only mild or asymptomatic clinical disease (Terrault and Wright, 1998).

The incubation period of HBV ranges from 45-120d (Hollinger and Liang, 2001). Variations in the length of incubation may be attributed to a number of factors including the size of the inoculum and route of infection. Once inside the host, HBV is transported via the bloodstream to the liver, which is the primary site of infection. The virus spreads rapidly through the liver, potentially infecting all hepatocytes. Viraemia follows approximately 6 weeks after inoculation. At this time, large numbers of non-infectious excess surface antigen (HBsAg) and infectious viral particles begin to circulate in the blood. Clinical signs are absent at this stage. One to three weeks thereafter markers of cell-mediated immunity become evident, coinciding with a rise in serum markers of liver injury (AST, ALT and bilirubin). Clinical signs become apparent at this stage and can include fatigue, malaise, ‘flu-like’ syndrome, abdominal pain, fever, anorexia, headache and clinical jaundice (Hollinger and Liang, 2001).

In most situations, the activated immune response can rapidly and effectively control the infection despite extensive infection of the hepatocytes. This is the case with typical subclinical and clinical cases of acute hepatitis. In approximately 1% of cases of acute hepatitis B infection, patients can develop acute fulminant hepatitis which is usually fatal (Terrault and Wright, 1998, Hollinger and Liang, 2001). It is postulated that these
patients may suffer massive hepatic necrosis as a result of a markedly augmented non-specific inflammatory response, although the mechanism for this is unknown (Hollinger and Liang, 2001).

In cases where there is ineffective viral clearance, infection progresses to chronicity. Chronic infection can involve asymptomatic carriers with little or no immune-mediated damage in the infected liver, and patients with varying degrees of associated liver damage (e.g. cirrhosis, hepatocellular carcinoma). The majority of carriers are anti-HBe positive (low infectivity) and show little or no histological or liver enzyme abnormalities (Hollinger and Liang, 2001). The presence of HBeAg in carriers is often associated with more severe chronic hepatitis, with the exception of some viral mutations (pre-core mutants). Development of chronic hepatitis and progression of the disease usually occurs a considerable period of time after infection. Hepatocellular carcinoma (HCC) is usually associated with an interval of at least 30 years between inoculation with HBV and development of disease (Ganem and Schneider, 2001). Carriers of HBV who become infected in infancy or early childhood have an enhanced risk of developing HCC (Dienstag and Isselbacher, 2001a).

A number of extrahepatic manifestations of hepatitis B infection are seen in 10-20% of patients, all of which are associated with the deposition of circulating antigen-antibody complexes. These can occur in both the acute and chronic stages of the disease and include arthralgias, arthritis, rashes, polyarteritis nodosa and glomerulonephritis.

1.1.4 Diagnosis of Hepatitis B Virus in Humans

Diagnosis of human HBV infection is based on the detection of serological markers in the blood in combination with the presence of clinical signs. In some cases of asymptomatic infection, diagnosis is based purely on serological markers. A description of the serological markers used along with a summary of their significance is contained in the following paragraphs. In Figure 1.4 the temporal relationship of the serologic and
clinical patterns observed during acute HBV infection is outlined. In Table 1.2, the serological patterns seen with acute and chronic infection are depicted.

HBV surface antigen (HBsAg) refers to the complex of antigenic determinants expressed on the surface of the HBV virion and the circulating forms described in 1.1. The presence of HBsAg in the blood is indicative of current HBV infection. In a typical case of acute infection with HBV, HBsAg will be detectable in the blood at 7-9 weeks post exposure, preceding elevations in serum aminotransferase activity (ALT, AST) and the development of clinical symptoms and/or jaundice. Serum concentrations reach a peak during the acute stage of illness, and then slowly decline to undetectable levels within 4 to 6 months. The persistence of HBsAg for greater than 6 months constitutes the presence of chronic infection (Terrault and Wright, 1998, Hollinger and Liang, 2001, 295).

Antibody to HBsAg (anti-HBs) is considered the protective antibody and its presence is associated with immunity to HBV. After natural infection, anti-HBs usually becomes detectable in the serum as concentrations of HBsAg fall. Anti-HBs can persist for a lifetime in over 80% of patients (Hollinger and Liang, 2001). In cases of chronic infection, anti-HBs is either undetectable or detectable only at low serum concentrations (less than 10% of carriers) (Terrault and Wright, 1998, Dienstag and Isselbacher, 2001a). Due to the protective nature of anti-HBs, modern vaccines employ the HBsAg subunit and therefore will also produce anti-HBs.

HBV core antigen (HBCAg) is the antigen expressed on the surface of the nucleocapsid core. These antigen particles remain within the hepatocyte and do not circulate unless encapsulated by an HBsAg coat, therefore naked HBCAg will not be detected in the serum. HBCAg particles can be detected readily in liver tissue with immunochemical staining.
Antibody to HBcAg (anti-HBc) appears in the serum 1-2 weeks after the appearance of HBsAg, preceding the appearance of anti-HBs by weeks to months and lasting indefinitely in most cases. Occasionally, due to the variable length of time between the disappearance of HBsAg and the appearance of anti-HBs in acute infections, there may be a “window period” where anti-HBc is the only detectable marker of infection. These cases can be differentiated from previous HBV infections by the determination of the immunoglobulin class of anti-HBc. IgM anti-HBc will be present in current and recent infections, and IgG anti-HBc in remote HBV infections (Dienstag and Isselbacher, 2001a). As HBCAg is not present in commercial vaccines, the presence of anti-HBc in serum is indicative of actual infection rather than vaccination-induced immunity.

Figure 1.4: Serological pattern after acute hepatitis B infection (Robert G. 2002)

Hepatitis B e antigen (HBeAg) is closely associated with the nucleocapsid of HBV and circulates in the serum during infection. HBeAg is a readily detected qualitative marker of HBV replication and relative infectivity (Hollinger and Liang, 2001, Dienstag and Isselbacher, 2001a). In acute infections, HBeAg appears with or shortly after HBsAg,
coinciding temporarily with high rates of virus replication, then becomes undetectable before the disappearance of HBsAg. Shortly after the disappearance of HBeAg in the serum, antibody to HBeAg (anti-HBe) appears in the serum (Terrault and Wright, 1998, Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001). In cases of chronic infection, HBeAg persists for greater than six months and may persist indefinitely. The presence of HBeAg in the serum of carriers constitutes the replicative phase of infection and is indicative of a high relative infectivity, coinciding with high circulating concentrations of HBV DNA. Carriers with evidence of active viral replication account for approximately 50% of all chronic carriers and often develop more severe chronic hepatitis, with 15% to 20% developing cirrhosis within 5 years (Terrault and Wright, 1998).

Seroconversion of carriers from HBeAg-positive to anti-HBe-positive is associated with conversion to the non-replicative phase and a low relative infectivity. Those in the non-replicative phase tend to have minimal or mild chronic hepatitis or are asymptomatic carriers. Conversion can occur spontaneously among carriers at a rate of 10-15% per year (Dienstag and Isselbacher, 2001b) and is usually associated with a transient increase in liver enzymes. Occasionally, low infectivity carriers can convert to high infectivity carriers. Because HBeAg is invariably present during early acute hepatitis B, HBeAg testing is indicated primarily during follow-up of chronic infection.

Table 1.2: Typical serological patterns of acute and chronic HBV infection (adapted from Dienstag and Isselbacher, 2001 and Hollinger and Liang, 2001)

<table>
<thead>
<tr>
<th>Classification</th>
<th>HBsAg</th>
<th>anti-HBs</th>
<th>anti-HBc</th>
<th>HBeAg</th>
<th>anti-HBe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never exposed</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>High infectivity chronic carrier</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Low infectivity chronic carrier</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Current acute infection</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Vaccine immunity</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Past exposure (exposure immunity)</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+/-</td>
</tr>
</tbody>
</table>

*HBsAb > 10 mIU/mL considered protective in humans
Hepatitis B virus DNA is considered a quantitative marker of HBV infections, with high circulating concentrations of HBV DNA coinciding with high infectivity and often with more severe liver damage. During acute infection HBV DNA can be detected by polymerase chain reaction (PCR) 3-5 weeks after infection, although more sensitive tests are reducing the period between infection and detection of HBV DNA. HBV DNA concentrations rise slowly prior to the emergence of HBsAg, then peak during the time of acute illness, before tapering off as the infection is cleared. In chronic cases, high levels of circulating HBV DNA are found in the serum during the replicative phase of infection (HBeAg positive), and DNA can be detected in the liver in extrachromosomal form. During the non-replicative phase (anti-HBe positive), little or no DNA can found in serum, although HBV DNA can be found in the liver integrated into the host genome.

1.1.5 Treatment of Hepatitis B Virus in Humans

There is no specific treatment for benign acute hepatitis B infection thus therapy is supportive and aimed at making the patient comfortable and maintaining adequate nutritional balance. Recovery from clinically apparent acute hepatitis B occurs in nearly 99% of previously healthy adults therefore antiviral therapy is not likely to improve the rate of recovery and is not required (Dienstag and Isselbacher, 2001b).

In fulminant hepatitis, the goal of therapy is to provide supportive therapy through the control of bleeding, correction of hypoglycaemia and treatment of other complications of the comatose state. Liver transplantation may also be beneficial in patients with fulminant hepatitis.

Specific antiviral therapy is reserved for cases of chronic HBV infection where the goal of therapy is to suppress HBV replication in order to reduce symptoms, to minimize chronic inflammation, and to prevent progression to cirrhosis and HCC. Because complete eradication of HBV infection often is not possible, the treatment is aimed at achieving a quiescent state of viral replication and remission of liver disease (Hollinger and Liang,
The most common indication for treatment is chronic ‘replicative’ hepatitis B infection, with detectable HBeAg and HBV DNA, elevated ALT activity, and histologic evidence of chronic hepatitis on liver biopsy in an immunocompetent adult.

A number of treatment options have been investigated for chronic HBV infection, but only two are currently used for this purpose: the immunomodulatory agent alpha interferon and the nucleoside analogue lamivudine. Both drugs are comparable in efficacy as initial treatment for chronic hepatitis B and achieve approximately 35% seroconversion rate from the ‘replicative’ to the ‘non-replicative’ phase, with concomitant improvement of liver histology (Dienstag and Isselbacher, 2001b). Alpha interferon has the benefit of short term therapy without the development of drug resistance, however it requires subcutaneous injection and is frequently associated with side effects. Lamivudine is a well tolerated, oral medication that can be used in patients who fail to respond to interferon. It requires longer term therapy and can produce drug resistant strains. Although some physicians and patients prefer to start with interferon, most prefer lamivudine as first-line therapy (Dienstag and Isselbacher 2001b).

With the ongoing development of a number of drugs, more treatment agents may be available in the future. Adefovir dipivoxil is a new nucleotide analogue that has recently become available. It has shown promise in early trials in both HBeAg positive and HBeAg negative chronic HBV carriers (Hadziyannis et al. 2003, Marcellin et al. 2003). Adefovir is an oral medication with minimal reported side effects, and there has been no reported development of drug resistance. Further trials are required however this drug appears promising as an alternative treatment for chronic HBV.

Liver transplantation is the only potential lifesaving intervention in many patients with end stage chronic hepatitis B, however reinfection of the new liver is common, accompanied by a variable degree of associated liver injury (Dienstag and Isselbacher,
Concurrent treatment with lamivudine and hepatitis B immunoglobulin can reduce the rate of reinfections.

1.1.6 Prevention and Control of Hepatitis B Virus in Humans

There are three main methods in which prevention and control of hepatitis B infection is effected – environmental control, passive immunoprophylaxis and active immunisation.

**Environmental control** includes changes in sexual practices, needle exchange programs for drug users, improved screening measures of blood products to reduce the risk of transfusion-associated hepatitis, and the use of standard blood and body fluid precautions including gloves, masks and eye protection, use of sterile instruments and single use needles and syringes, proper handling and disposal of sharps to prevent self injury (e.g. accidental needle-stick injury) and the use of appropriate disinfectants for instruments and work surfaces.

**Passive immunoprophylaxis** involves the use of human immunoglobulin preparations containing anti-HBs Ig (HBIG). The HBIG provides an immediate source of preformed antibodies to HBsAg, helping to prevent the development of clinical illness in cases where exposure to the virus has likely already occurred. Situations where HBIG would be used include neonates born to HBsAg-positive mothers, after a needle stick injury, after sexual exposure, and after liver transplantation in patients who were HBsAg positive before transplantation. Administration of HBIG should be performed as close as possible to the time of exposure since effectiveness appears to diminish rapidly if administration is delayed for more than three days (Hollinger and Liang, 2001). Immunoprophylaxis of neonates born to HBsAg-positive mothers is recommended immediately after birth or within 12 hrs, and in cases of needle stick injury or sexual exposure within 48 hrs if possible, but no later than seven days after exposure. In most cases, active immunisation by vaccination is recommended at the same time as HBIG administration to produce a more durable immunity.
Active immunisation employs the use of vaccination to prevent the development of infection and clinical illness associated with HBV. Patients are exposed to a non-infectious form of HBsAg against which the patient’s body mounts an antibody response. Immunisation is predominantly performed with the aim of developing an immune response prior to exposure to the virus, however it is also used in combination with HBIG to prevent development of infection in cases where exposure has already occurred (e.g. neonates born to HBV-positive mothers). Early vaccines were developed using inactivated HBsAg from the plasma of asymptomatic carriers, however these were expensive and time-consuming to produce. They have been replaced by recombinant vaccines that incorporate the surface gene of HBV into different expression vectors (yeast, Escherichia coli, or mammalian cell lines). These newer vaccines are more efficiently produced and cost-effective, the yeast derived vaccines being the most widely available. Further work is underway looking into the use of DNA-based vaccination against hepatitis B for use as both prophylaxis and treatment for chronic hepatitis. The proposed benefit of these vaccines would be the ability to produce cell-mediated immunity in addition to the humoral immunity produced by current vaccines (Davis et al. 1997).

A number of vaccination schedules have been tested. The most commonly recommended protocol involves three intramuscular injections at 0, 1 and 6 months with a standard dose (volumes vary with vaccine brand) (Terrault and Wright, 1998, Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001). More than 95% of immunocompetent vaccinates develop antibodies against surface antigen, with 80-90% of these maintaining protective levels (>10 mIU/mL) for five years, and 60-80% for ten years. Protective antibody concentrations appear to diminish after this time but protection appears to persist in many cases despite these low concentrations. Booster vaccinations after the initial course remain controversial. Hollinger and Liang (2001) recommend the use of at least one booster dose 5-10 years after completing the initial immunisation course. The World Health Organisation (WHO) no longer recommends the
use of boosters in healthy adults. More regular boosters may be required in immunocompromised people and those in high risk situations (e.g. children of HBV-infected mothers) whose anti-HBs levels have declined to less than 10 mIU/mL.

Widespread vaccination programs are recommended as the most effective means to control HBV in endemic regions, and to date more than 60% of the countries of the world have a national program. In neonates born to HBeAg positive mothers, the combination of HBIG and the standard vaccination schedule using a yeast-derived vaccine is associated with a 95% protective efficacy rate (Figure 1.5). Carrier prevalence has been reduced to < 1% in many countries where perinatal transmission is high and a national immunisation program is in place (Hollinger and Liang, 2001).

Figure 1.5: Protective efficacy rates in neonates born to HBeAg-positive mothers after immunisation with hepatitis B immune globulin alone, HBsAg vaccine alone, and a combination (Hollinger and Liang, 2001).

```
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protective Efficacy Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>HBIG x 3</td>
<td>72</td>
</tr>
<tr>
<td>PDV</td>
<td>75</td>
</tr>
<tr>
<td>YDV</td>
<td>95</td>
</tr>
<tr>
<td>PDV+HBIG</td>
<td>91</td>
</tr>
<tr>
<td>YDV+HBIG</td>
<td>95</td>
</tr>
</tbody>
</table>

HBIG: hepatitis B immune globulin          PDV: plasma-derived vaccine          YDV: yeast-derived vaccine
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1.2 Hepatitis B Virus in Nonhuman Primates

1.2.1 HBV in Nonhuman Primates

Susceptible Species


Surveys of large numbers of non-ape primate species have been performed by numerous authors (Eichberg et al. 1980, Heckel et al. 2001, Makuwa et al. 2003, Starkman et al. 2003). In his review of hepatitis in nonhuman primates, Deinhardt (1976) reported that HBsAg had been demonstrated in a number of chimpanzees as well as a few gibbons and orang-utans, however baboons (Papio sp), vervet monkeys (Cercopithecus aethiops), rhesus macaques (Macaca mulatta), squirrel monkeys (Saimiri sp.) and marmosets (Callithrix jacchus) seemed to be free of HBsAg and anti-HBs. In 1980, Eichberg et al. surveyed a number of nonhuman primates held in primate research centres in the USA for hepatitis A and B. Only two of 82 chimpanzees were positive for HBsAg, however 29.9% of the chimpanzees were positive for anti-HBs indicating previous exposure. Of 65 baboons, 19 vervet monkeys, 10 cebus monkeys (Cebus sp.), 20 squirrel monkeys and 18 marmosets tested, no evidence of current infection with HBV was found (HBsAg). However, 36.2% of the baboons and 5% of the squirrel monkeys were anti-HBs positive, indicating previous exposure to the virus.
Heckel et al. (2001) detected HBV core antibody (anti-HBc) in one vervet monkey from 454 monkeys tested from a range of families including Lemuridae, Cercopithecidae, Cebidae and Callimiconidae. This finding was indicative of previous exposure rather than current infection. Starkman et al. (2003) and Makuwa et al. (2003) performed HBV serology on 93 and 185 animals respectively from the Old World primate family Cercopithecidae spp. and failed to detect any positive animals. To account for the possibility that these non-ape primate species may have a more divergent virus that is not detectable by the human serological tests, Starkman retested the samples using PCR and a more divergent set of primers that also detects the highly divergent rodent HBVs. However the author found no evidence of infection in the non-ape primates tested.

Studies by Kedda et al. (2000) and Baptista et al. (2003) have suggested that baboons (Papio ursinus orientalis) may be susceptible to HBV; however cases to date are the result of experimental inoculation with human HBV, and the scientists failed to detect either of the conventional serological markers of infection, HBsAg or anti-HBs. HBV DNA was detected in baboon serum in very low levels but only by very sensitive methods (second and third round nested PCR). No evidence of clinical illness was demonstrated. There have been no reported cases of infection of baboons with HBV outside of the laboratory, and thus baboons are not currently considered a natural host of HBV infection. Michaels et al. (1996) failed to demonstrate HBV infection in four baboons challenged with the virus, a result supporting the theory that baboons are not natural hosts.

A similar study by Gheit et al. (2002) demonstrated acute hepatitis and viral replication in Macaca sylvanus, an Old World monkey found in Morocco. To achieve these results the researchers inoculated the liver directly with human HBV. HBsAg could be detected for a period of time and an elevation in ALT occurred, however there was no evidence of seroconversion as demonstrated by a lack of anti-HBs and anti-HBc in the serum. The
method of inoculation used in this experiment ensured a much higher chance of viral replication by bypassing the normal host immune mechanisms. Given the limited success despite this technique, it is unlikely that natural infection would occur in this species.

Lanford et al. 2003 were able to successfully infect a spider monkey (Ateles geoffroyi) with HBV from an infectious clone of Woolly monkey HBV (WMHBV). Previous studies had failed to produce infection in tamarins, macaques or baboons. Serological evidence of infection in the spider monkey was short lived (six weeks) and the infection did not result in clinical illness or elevated liver enzymes. This study indicates that the spider monkey may serve as a suitable host for the New World WMHBV, however it is not considered a natural reservoir.

The earlier serological survey by Eichberg et al. (1980) involved animals only from research centres. It is therefore difficult to know what role previous research may have played in the HBV status of these animals. It is possible that some may have been previously inoculated with the virus, intentionally or otherwise, as part of other studies. Testing used for detection of the virus has also advanced significantly since this time and it is possible that retesting of the same animals using the more sensitive methods available today may provide different results.

The studies on baboons (Michaels et al. 1996, Kedda et al. 2000, Baptista et al. 2003) and spider monkeys (Lanford et al. 2003) involved experimental inoculation of the primates with HBV. None of these studies was able to demonstrate clinical illness or active viral replication at levels which would be considered infective in the animals inoculated. Therefore whilst they may be capable of ‘infection’ based on certain laboratory criteria, there have been no reported cases of natural infection in these species and they are not considered natural hosts of the virus. In contrast, the studies by Heckel et al. (2001), Starkman et al. (2003), and Makuwa et al. (2003) involved
surveys of large numbers of wild caught and captive nonhuman primates from zoological parks and rehabilitation centres. Such animals are unlikely to have been exposed to human or primate virus experimentally, and thus would have required exposure to infected nonhuman primates or infected human handlers, to have acquired HBV infection. Viral testing in these later studies involved more sensitive testing methods than those available in the 1970's and 1980's. Given both of these factors, it is likely that these studies provide a more true representation of the susceptibility of various nonhuman primate species to HBV. Thus it is generally accepted that all ape species (gibbons, gorillas, chimpanzees and orang-utans) are susceptible to experimental HBV infection or have indigenous viral infections, and that HBV infection in monkeys is a rare event.

**History of Hepatitis B Virus in Apes and the Woolly Monkey**

Sequencing of the complete viral genome in combination with sequencing of the pre-S, surface and core regions, has demonstrated that viruses found in the ape species and the woolly monkey are closely related but genetically distinct from the human HBV virus (Vaudin et al. 1988, Mimms et al. 1993, Norder et al. 1996, Warren et al. 1999, Lanford et al. 1998, 2000, Grethe et al. 2000, Hu et al. 2000, 2001, MacDonald et al. 2000, Takahashi et al. 2000, 2001, Verschoor et al. 2001, Vartanian et al. 2002, Aiba et al. 2003, Noppornpanth et al. 2003, Starkman et al. 2003). Initially it was thought that these infections were the result of virus transmission from humans, possibly through the practice of inoculating newly caught chimpanzees with pooled human blood for ‘protection against human disease’ (Zuckerman et al. 1978). However, with the discovery of HBV in a number of primate species, and subsequent sequencing of these viruses, it is now accepted that they are indigenous to their nonhuman primate hosts.

**Chimpanzees** - Zuckerman et al. (1978) identified hepatitis B in a chimpanzee at London Zoo. Subsequent surveys at the zoo revealed that four of the eight other chimpanzees in the colony were HBsAg positive (indicating current infection), while the
remaining four were anti-HBs positive (indicating previous exposure). A gorilla and two
orang-utans were also found to be positive for anti-HBs. The virus from the original
chimpanzee at London Zoo was subsequently sequenced by Vaudin et al. (1988) and
found to show >10% sequence divergence from all other hepatitis B viruses previously
published. Based on the definition by Okamoto et al. (1988), this constituted a new
genotype of HBV, designated ChHBV.

Since this study, various authors have demonstrated the presence of nonhuman
primate-specific HBV in both wild and captive born chimpanzees (Vaudin et al. 1988, Hu
(Pan paniscus) (Heckel et al. 2001). Infection with HBV has been found to be
widespread among chimpanzees, with virus being detected in a number of subspecies
with varied geographical distributions (Pan troglodytes troglodytes, P. t. verus, P. t.
schweinfurthi, P. t. vellerosus) (Vaudin et al. 1988, Hu et al. 2000, 2001, MacDonald et

**Gorillas** - In 1978, Zuckerman et al. reported the detection of anti-HBs in a gorilla held
at London Zoo. Hepatitis B virus was subsequently reported in a colony of gorillas at the
Cincinnati Zoo, USA by Linnemann et al. (1984). Grethe et al. (2000) sequenced the
HBV genome from a gorilla held at a German zoo, and this remains the only gorilla HBV
sequence to date, designated GoHBV. On phylogenetic analyses the sequence from this
animal consistently clusters with sequences from chimpanzees, with which the gorilla
shares a common habitat range. Work by Thornton et al. (2001) and the recent surveys
by Heckel et al. (2001) and Makuwa et al. (2003) have continued to demonstrate cases
of hepatitis B infection in wild and captive gorillas.

**Gibbons** - In 1993, Mimms et al. sequenced the PreS gene from a white handed gibbon
(Hylobates lar) after passage of the virus through a chimpanzee. Norder et al. (1996)
subsequently sequenced the entire genome from this chimpanzee. Analysis of the virus demonstrated that it was most closely related to the previously published chimpanzee sequence (Vaudin et al. 1988), differing from it by 10% and from previously published human sequences by >10%. Again, this constituted a new genotype (GiHBV). Further work on the virus in gibbons by Grethe et al. (2000), Lanford et al. (2000), and Heckel et al. (2001), and more recently by Aiba et al. (2003), Noppompanth et al. (2003) and Starkman et al. (2003) have demonstrated the presence of HBV in a wide range of gibbon species within the Hylobates and Nomascus genera (H. lar, H. pileatus, H. agilis, H. moloch, N. concolor, N. leucogenys, N. gabriellae). There have been no reported cases in either the Bunopithecus or Symphalangus genera, each of which are represented by a single species (B. hoolock and S. syndactylus respectively).

Grethe et al. (2000) demonstrated the existence of genomic groups within the gibbon genotype, identifying five genomic groups among the 19 gibbons sampled, with intergroup differences of 6.3 to 7.9%, just below the threshold defined by Okamoto et al. (1988) for intergenotype distances. These genomic groups appeared to associate with the different geographical regions from which the gibbons originated in a similar manner to different geographical distributions of human HBV genotypes described by Norder et al. (1993).

**Orang-utans** - Warren et al. (1999) first described HBV in captive and wild caught Bornean orang-utans at the Wanariset rehabilitation centre in East Kalimantan, Indonesia. The entire viral genome from two of these orang-utans was subsequently sequenced by Verschoor et al. (2001) and differed from published human genotypes by >8%, thus comprising a new genotype, designated OuHV. Two distinct genetic variants of OuHV relating to the geographical origins of the affected orang-utans were described by Verschoor, a finding similar to that of Grethe et al. (2000) in gibbons. Heckel et al. (2001) also documented evidence of previous and current infection in a number of orang-utans held in European nonhuman primate collections. Sequencing has not been
performed on these individuals and subspecies of orang-utans involved have not been identified.

**Woolly Monkeys** - In 1998, Lanford et al. first described woolly monkey hepatitis B virus (WMHBV) after the discovery of the virus in a woolly monkey who died of fulminant hepatitis at the Louisville Zoo, USA. The authors sequenced the virus isolated from this animal and found it to be the most diverse of the nonhuman primate HBV strains to date, differing from human and nonhuman primate viruses by >14%. Phylogenetic analysis of the virus revealed that of all the published human and nonhuman primate genotypes, WMHBV was more closely related to human genotype F, the most divergent of all the human genotypes.

**Origin of Hepatitis B Virus in Nonhuman Primates**

The origin of the various nonhuman primate HBV genotypes has yet to be elucidated. The similarities in the genetic makeup of the various human and nonhuman primate genotypes is suggestive of a common origin, however the time frame of the divergence of the viral genotypes remains controversial. Numerous theories exist as to whether the virus originated in humans and spread to nonhuman primates or vice versa, or whether HBV evolution was host-independent (Robertson, 2001). Time frames for the evolution of the viruses range from the last 400 years, with the virus originating in the Americas and spreading rapidly to Europe, Africa and Asia; 100 000 to 150 000 years ago with the migration of humans from Africa; or over 20-35 million years ago, in line with divergence of the individual primate hosts (Robertson and Margolis, 2002). The existence of different genomic subgroups within the various nonhuman primate HBV genotypes described by Grethe et al. (2000), Hu et al. (2001) and Verschoor et al. (2001), lends itself to the theory of independent evolution within the host species and implies a much older infection event rather than a recent zoonotic event. In contrast to this, recent work by Fares et al. (2002) on the evolutionary history of HBV suggests that
the divergence of HBV in humans and apes has only occurred in the last 6,000 years, based on the use of an alternative analytical method.

There appears to be a close geographical association between the human and nonhuman primate genotypes, with WMHBV and human type F sharing a natural geographic range in the Americas, suggesting a common origin in the New World. This close relationship is also demonstrated on phylogenetic analysis, where both genotypes cluster closely together, yet separate from other human and nonhuman primate genotypes. Similarly, the Old World human and nonhuman primate viruses cluster closely, with the Asian OuHV and GiHBV clustering closest to human genotype C which is found in Asia, and the African ChHBV and GoHBV clustering closest to human genotype E, also found in Africa (Figure 1.6). Despite this, the various nonhuman primate genotypes, with the exception of the WMHBV, consistently cluster from the same branch, suggesting that they are more closely related to each other than any of the human genotypes.

Starkman et al. (2003), hypothesise further that within the geographical regions of the apes, relatedness of the nonhuman primate HBV viruses is affected more by the geographical location than by the host species. This is evidenced by the fact viruses from the more southerly distributed gibbon species (H. agilis and H. moloch) cluster more closely with those from the Bornean orang-utan (with which they share overlapping or adjacent habitat ranges) than with viruses from the gibbons from mainland Asia (H. lar, N. concolor, N. leucogenys, N. gabriellae) (Figure 1.7B). Geissmann (1991) describes the sympatry between white-handed gibbons (H. lar) and pileated gibbons (H. pileatus) in an area of southeastern Thailand, where the two species share an overlapping habitat range and where interaction between the two species has been observed. Another contact zone between these two species is known to occur northeast of Bangkok, and in this area limited hybridization between the two species has been demonstrated (Brockelman and Gittins, 1984).
Whilst the sharing of adjacent habitat by different species may allow for viral transmission between the gibbon species, spread of the HBV virus between gibbons and orang-utans is more difficult to explain given that effective viral transmission requires parenteral or sexual exposure, and that orang-utans in particular are solitary animals with limited contact with other animals in the wild (Warren et al. 1999). Such cross species transmissions may be more likely to occur in captivity, where a number of different species of primates may be housed in the same or adjacent enclosures. Although HBV infection has been detected in wild populations of nonhuman primates, as demonstrated by the presence of serological markers in animals caught directly from the
wild, all of the current nonhuman primate HBV sequences have been obtained from animals that have spent all or part of their lives in captivity. It is therefore difficult to determine the true likelihood of such interspecies transmissions in the wild situation and further sequencing from wild individuals is required.

**Figure 1.7:** Distribution of (A) chimpanzee subspecies and lowland gorillas in Africa, and (B) species of gibbons and orang-utans in South East Asia (Starkman et al. 2003)

### 1.2.2 Epidemiology of Hepatitis B Virus in Nonhuman Primates

Epidemiological studies into the transmission of the hepatitis B virus in nonhuman primates have relied upon tracing the history of known cases of infection within nonhuman primate collections (Kessler et al. 1982, Linnemann et al. 1984, Lanford et al. 1998, Aiba et al. 2003, Noppompanth et al. 2003). Large scale specific studies to determine the methods and rates of transmission of the indigenous HBVs isolated from
nonhuman primates have not been performed in the host animals as they have been with human HBV. Due to the high intelligence of these animals, as well as the highly endangered nature of many of the nonhuman primate species in which HBV infection has been identified, it is unlikely that such specific studies will be performed in the future. As a result, rates and modes of transmission of the virus in nonhuman primates are largely extrapolated from human HBV.

Recent studies have demonstrated patterns of HBV infection in captive primates consistent with both vertical and horizontal transmission as seen in humans, although frequencies at which transmission occurs via these modes has not yet been determined (Kessler et al. 1982, Linnemann et al. 1984, Lanford et al. 1998, 2000, Grethe et al. 2000, Aiba et al. 2003, Noppornpanth et al. 2003). Based on current epidemiological information it would appear that nonhuman primate HBV transmission is mainly vertical, with limited horizontal transmission, depending upon the geographic range and the population size of the host species (Robertson and Margolis, 2002).

chimpanzees and gibbons, consistent with that seen in humans. As with human HBV infection, it is thought that dose of the inoculum and route of infection play a large role in determining the length of incubation of the virus in nonhuman primates.

High and low infectivity carriers have also been identified in the various nonhuman primate species based on the presence of HBeAg and anti-HBe respectively. These carrier states have been further supported by patterns of transmission which are consistent with the serologic findings (Lanford et al. 1998, 2000, Aiba et al. 2003, Noppornpanth et al. 2003).

In addition to the presence of HBV DNA in the serum of infected individuals, HBV DNA has been detected in the saliva of nonhuman primates (Zuckerman et al. 1978, Noppornpanth et al. 2003), demonstrating the potential for infection through contact with either of these body fluids.

**Chimpanzees** - Zuckerman et al. (1978), reported on the discovery of chronic hepatitis B infection in five out of nine chimpanzees housed at London Zoo, with the remaining animals demonstrating evidence of previous infection (anti-HBs positive). Anti-HBs was also detected in one gorilla and two orang-utans held at London Zoo at the same time. Analysis of frozen serum from two other chimpanzees that had died in 1966 and 1972 revealed that these animals were also positive for HBsAg, and it was proposed that these may have been the source of infection for the animals diagnosed in 1978. Three of the carrier chimpanzees that were born at the zoo were the offspring of a carrier mother or a carrier father, suggesting a vertical mode of transmission or horizontal transmission in the perinatal period in the case of the mother, and probable perinatal infection in the case of the father. In both cases infection led to the persistent carrier state in the offspring. This is consistent with human HBV infection where development of the chronic carrier state occurs in 90% of children infected in the perinatal period (Hollinger and Liang, 2001). Both of the carrier parent chimpanzees at London Zoo had
been wild caught, raising the possibility that the infection was acquired naturally in the wild.

Further information on the transmission of ChHBV is limited. A number of researchers have sequenced viral DNA from chimpanzees, although most studies have involved individual, unrelated animals and therefore do not provide information regarding vertical or horizontal transmission (Hu et al. 2000, 2001, MacDonald et al. 2000, Takahashi et al. 2000, 2001, Vartanian et al. 2002). Infection in the wild or through human contact at the time of capture has been proposed as the source of infection in most cases.

Prevalence of HBV among populations of wild caught and captive chimpanzees has been investigated by a number of authors. Deinhardt (1976) reported that up to 25% of chimpanzees caught in the wild were found to be anti-HBs positive within weeks or months of arrival in the USA according to reports from the World Health organization (WHO) in 1975. A survey of captive chimpanzees across Europe and Asia by Heckel et al. (2001) found 12.2% of the chimpanzees (Pan troglodytes) and 18% of the bonobos (Pan paniscus) studied to have evidence of past or current infection with HBV. Of these seropositive animals, HBsAg (indicating current infection) could be found in only 1.7% of chimpanzees and 3.7% of bonobos. This is the first study to report HBV infection in bonobos. A similar survey of wild caught and captive chimpanzees in Gabon, Africa by Makuwa et al. (2003) found approximately 30% of chimpanzees tested had evidence of past or previous infection with HBV. Current infection was found to be present in 4.1% of these chimpanzees. MacDonald et al. (2003) found 25% (two of eight) chimpanzees were seropositive for HBV DNA by PCR, indicating current infection. Based on these three studies, the average level of current HBV infection would be estimated at approximately 10%, suggesting that the level of endemic HBV infection in chimpanzees is high, according to the human definitions used in section 1.1.2 of this literature review. Further studies would be required to make solid conclusions in this regard.
Gorillas - Linnemann et al. (1984) reported on the familial clustering of hepatitis B infections in two groups of gorillas at the Cincinnati Zoo. In both families, the patriarch was found to be a high infectivity chronic carrier, based on the presence of HBsAg and HBeAg, and the absence of anti-HBs. A serosurvey of the in-contact animals found that eight of 11 tested demonstrated evidence of previous infection (anti-HBs and anti-HBc). These 11 animals constituted the matriarchs and the first generation offspring in the colony at the time of testing. A further four second generation offspring were tested and all four were negative, however all were a year or less at the time of testing. One of the first generation offspring demonstrated seroconversion during the time of the study indicating that infection had occurred between the ages of seven and ten years. A breeding female on loan from another zoo also demonstrated seroconversion after her transfer, indicating probable horizontal transmission via sexual contact. This study demonstrates horizontal transmission of the virus from the carrier males, resulting in transient infection and recovery. It is likely that these exposures occurred in late childhood/early adulthood as indicated by the lack of infection in younger animals and the absence of carriers. The female partners were likely exposed via sexual contact. One of the first generation offspring received a blood transfusion from a carrier male, which may have been a source of infection for this animal.

In the studies mentioned previously by Heckel et al. (2001) and Makuwa et al. (2003), evidence of past infection with HBV was found in 11% and 30% of gorillas tested respectively, however none demonstrated current infection. The level of endemic infection of HBV among gorillas would therefore appear to be low in both captive and wild populations. This data may also suggest that gorillas are less prone to the development of chronic infection than other nonhuman primate species.

Gibbons - Grethe et al. (2000) sequenced partial and complete HBV genomes from 19 gibbons held in institutions across Europe and Asia. In this work, the authors were able to identify two probable transmission events among gibbons housed together, based on
nearly identical sequences found in each of the animals involved in the transmission events. The HBV sequence from a chimpanzee in this study was found to cluster more closely with gibbon sequences than with other chimpanzee sequences. The authors have postulated that this may represent the first ‘quasinatural’ interspecies transmission of HBV, proposing that it may have occurred at the previous institution where this chimpanzee had been co-housed with a group of gibbons. To date, no other such cases of interspecies HBV transmission among nonhuman primates have been reported to support this theory.

Also in 2000, Lanford et al. investigated hepatitis B infection in gibbons, testing 30 different animals from six species of gibbon housed at the Gibbon Conservation Centre in California (GCS). Of the 30 animals tested, 14 were positive for at least one marker of HBV infection (46.7%), including animals from three of the six species tested. Approximately 50% of the infected animals (23.3% of the tested animals) were classified as chronic carriers based on the presence of HBsAg without anti-HBs, with chronic carriers identified among the Hylobates agilis (Agile gibbon) and H. moloch (Silvery gibbon) species. Examination of the family tree of both species was suggestive of a pattern of vertical transmission resulting in chronic infection, and horizontal transmission resulting in viral clearance. In the H. agilis group, three siblings from a negative sire and an untested dam were all chronic carriers, suggesting that the dam was most likely a chronic carrier. Mating between two of these offspring also produced a chronic carrier. Mating with one of the carrier males and an anti-HBs, anti-HBc positive dam produced serologically negative offspring, suggesting that, as in humans, the carrier state of the mother is the major contributing factor in perinatal transmission of infection. Similarly in the H. moloch group, two of the chronic carriers (‘Chloe’ and ‘Chilibi’) were siblings and were likely to have been exposed to infection by their mother (not involved in the study). Two other animals (‘Shelby’ and ‘Ushko’) showed evidence of previous infection but apparent recovery (anti-HBs, anti-HBc positive) and are thought to have become horizontally exposed after having been housed with one or
more chronic carriers in the time prior to testing. Four of the animals in this study (Chloe, Chilibi, Ivan and Shelby) are relatives of animals at Perth Zoo and their status will be discussed in Chapter 2.

A study of 101 captive gibbons in a wildlife breeding centre in Thailand by Noppompanth et al. (2003) looked at the molecular epidemiology of gibbon hepatitis B transmission. This study found that approximately 40% of the animals tested, comprising four species of gibbons, were positive for at least one marker of HBV infection. Based on the presence of HBV DNA and HBsAg, in the absence of anti-HBs, 19 of the 101 (18.8%) were classified as chronic carriers. The remaining 20 animals with markers of infection demonstrated apparent recovery from the infection as judged by the presence of anti-HBs and anti-HBc. The study demonstrated that all partners of carriers showed evidence of previous or current HBV infection and a high sequence similarity in those currently infected, indicating a high likelihood of horizontal transmission. Two carrier families had offspring born at the centre. The mothers in these families were both found to be HBeAg positive and were therefore classified as high infectivity carriers. Both offspring were found to also be chronic carriers, with a 99.5% similarity of the S gene sequences from mother and offspring. These findings are strongly supportive of vertical HBV transmission. Saliva from 14 carrier gibbons was tested and found to be positive for HBsAg, six of these also being positive for HBV DNA. In contrast, neither HBV DNA nor HBsAg could be detected in the saliva of seronegative and HBV recovered animals. The presence of HBV DNA in the saliva of chronic carriers is similar to the findings in human carriers and confirms saliva as a possible source of horizontal transmission of HBV in gibbons.

Aiba et al. (2003) described the vertical transmission of HBV from a carrier female pileated gibbon at the Yokohoma Zoological Gardens which resulted in the offspring being HBsAg seropositive after birth and becoming a chronic carrier.
Heckel et al. (2001) demonstrated evidence of HBV exposure in 38% of 104 gibbons tested among European and Asian institutions holding nonhuman primates, as defined by the presence of one or more serological markers of infection. Of the 104 gibbons tested, 19.2% had a current infection as demonstrated by the presence of HBsAg in the serum. Sall et al. (unpublished) recently examined 26 pileated gibbons in Cambodia and found 12 of the 26 (46%) originally wild caught animals to be positive for HBsAg. These figures are similar to the 23.3% and 18.8% of gibbons tested by Lanford et al. (2000) and Noppompanth et al. (2003), and suggest that endemic HBV infection in gibbons is common among captive and wild caught populations.

**Orang-utans** - Hepatitis B virus in orang-utans was investigated by Warren et al. (1999) at the Wanariset Orang-utan Rehabilitation Centre in East Kalimantan, Indonesia. This study found 42.6% of the 195 orang-utans tested were positive for at least one marker of HBV infection, with 7.6% of these classified as chronic carriers based on the presence of HBsAg in the serum for greater than one year. Conversion to anti-HBs positive during the time of the study occurred in 40 animals that had demonstrated active infection (HBsAg positive at one or more sampling dates). Whilst most of the animals in the study had had direct or indirect contact with humans prior to sampling, in an area where HBV infection is endemic in the human population, three animals were caught directly from the wild. Two of the three wild orang-utans were found to be anti-HBs positive, suggesting an indigenous virus. Verschoor et al. (2001) sequenced the entire genome from two of the orang-utans in the Warren et al. study. These sequences clearly clustered separately to human genotypes, further supporting the indigenous nature of the hepatitis B virus found in the orang-utans (OuHV).

**Woolly Monkeys** - Lanford et al.’s (1998) study of the woolly monkey hepatitis B virus (WMHBV) demonstrated a high percentage of HBsAg positive animals in the Louisville Zoo colony where the virus was first discovered. 54% of the animals tested were positive for HBsAg, and 60% were positive for HBV detected by PCR. A further three
animals demonstrated previous infection. An overall 81% of animals in the colony were positive for one or more markers of WMHBV infection. Retrospective analysis of samples demonstrated that a number of these infections were chronic and were present in the colony at least nine years prior to first detection. Analysis of the family tree of this colony demonstrated a pattern of infection consistent with vertical transmission, with three female offspring of the founding dam being chronic carriers, and 80% of the offspring from these females also being positive for HBsAg. With 54% of the population seropositive for HBsAg, WMHBV would be considered highly endemic in this study population.

The zoonotic potential of the various nonhuman primate hepatitis B viruses is unknown, although it is likely that the close relatedness of the primate viruses to human HBV could enable transmission to humans. Experimental transmission of human HBV to nonhuman primates (chimpanzees, gibbons) has been documented (Deinhardt et al. 1976, Bancroft et al. 1977, Will et al. 1982, Sureau et al. 1988). The infectivity of human HBV to chimpanzees has been further demonstrated with the regular utilization of this species as a model to study the behaviour of human HBV in vivo (Prince and Brotman 2001), and to test the efficacy of HBV vaccines (Davis et al. 1996, Davis 1997, Prince and Brotman 2001).

The HBV sequence from one of three chimpanzees sequenced by Takahashi et al. (2002) appears to cluster more closely to human genotype E than to published chimpanzee or other nonhuman primate sequences. Similarly, sequences from the S gene from two chimpanzees in the study by Hu et al. (2000) appear to cluster with human genotypes A and C. However, as the sequences in the second study did not involve the entire genome, it is possible that the virus in these animals would have clustered elsewhere if based on the entire viral genome. These findings could suggest possible zoonotic (zooanthroponotic) transmission of the virus from humans to chimpanzees. The practice of inoculating wild caught chimpanzees with pooled human
serum to ‘protect from human diseases’ has been postulated to be the source of infection in these animals. If this were so, these cases would not represent natural interspecies transmission of the virus. To date there are no reported cases of natural infection of humans with nonhuman primate HBV viruses (Noppompanth et al. 2003). Until the origin of the viruses in the previous cases can be clarified, the situation remains that there are also no documented cases of natural infection of nonhuman primates with human HBV. However, the potential for both cases theoretically exists.

1.2.3 Pathogenicity of Hepatitis B Virus in Nonhuman Primates

The pathogenicity of hepatitis B virus in nonhuman primates is largely unknown due to a number of factors including the limited number of animals that have been studied. The viruses have only been discovered in the last few decades, and studies into the long term effects of the viruses, particularly the course of chronic infection, have not been performed. Hopefully more information will be revealed with time and further study into these diseases.

**Chimpanzees** - Deinhardt (1976) describes elevation of serum concentrations of liver enzymes (SGPT, SGOT) 1-13 weeks after the appearance of HBsAg in HBV infected chimpanzees. These levels persisted from a few days to 47 weeks. Histological liver changes were observed to coincide with enzyme elevations and persisted for a similar amount of time. Progression to cirrhosis was not observed in this study, although the length of the study period is not mentioned. Similar findings are reported by Will et al. (1982) and Sureau et al. (1988), who also documented a rise in liver enzymes with concurrent histologic liver changes consistent with acute viral hepatitis. Both of these changes resolved with the development of anti-HBs. These studies document in chimpanzees the typical pathological changes that occur in human cases of acute HBV. It should be noted though that these studies involved infection with human HBV rather than indigenous ChHBV.
In 1978, Zuckerman et al. demonstrated histological changes in liver biopsies from two chimpanzees at the London Zoo with chronic hepatitis B infection. These changes were consistent with a diagnosis of chronic persistent hepatitis in humans. Two other chronically infected chimpanzees demonstrated mild mesenchymal reaction and occasional inflammatory changes. No elevations were detected in serum aminotransferase concentrations from any of the chronically infected chimps in the study. Subsequent sequencing of the virus from one of the infected chimpanzees in this study by Vaudin et al. (1988) revealed that the virus infecting these animals clustered separately from human HBV genotypes, and continues to cluster with newly published chimpanzee strains. This therefore represents the first documented case of HBV associated pathology resulting from infection with indigenous ChHBV rather than human HBV.

Mimms et al. (1993) induced hepatitis B infection in a chimpanzee through inoculation with serum from a chronically infected gibbon (H. lar). The chimpanzee subsequently developed a pattern of serological markers consistent with acute HBV infection, however a marginal ALT elevation was detected on only one occasion. The HBV sequence obtained from this chimpanzee has been demonstrated to cluster with other gibbon sequences, suggesting that indigenous GiHBV was the infectious agent rather than HBV. Interpretation of this finding, with respect to pathology of the virus, is difficult given that we are not dealing with infection of the natural host.

**Gorillas** - Currently there are no documented studies investigating the pathogenicity of HBV infection in gorillas. Evidence of pathology associated with HBV infection in this species is also limited. A review of the medical records of two male carriers discussed in the paper by Linnemann et al. (1984) has revealed mild elevations in serum concentrations of AST in both animals on a couple of occasions, accompanied by a mild elevation in ALT in one male on one occasion (personal communication G. Levens, May 2004). Whilst it is not possible to determine the exact cause of the enzyme elevations in
these two gorillas, the findings indicate a possible pathogenic role for chronic hepatitis B infection in gorillas. Necropsy examination of the former animal demonstrated no overt signs of cirrhosis or other viral related pathology, although no specific tests were run (personal communication G. Levens, June 2004).

**Gibbons** - Lanford et al. (2000) reported no overt signs of clinical illness or mortality in six chronically infected gibbons, however liver enzyme levels were not measured and liver biopsies were not collected. Noppornpanth et al. (2003) demonstrated elevated serum concentrations of the liver enzyme alanine aminotransferase (ALT) in HBsAg positive gibbons (68.8 +/- 48.1 IU) compared with control animals (33.0 +/- 15.9 IU, P<0.05). Other measures of pathogenicity (liver biopsies, clinical signs) were not investigated in this study. Elevated ALT concentrations are a common finding in both acute and chronic HBV infection in humans (Hollinger and Liang, 2001).

**Orang-utans** - Serum alanine aminotransferase (ALT) concentrations were measured in 18 orang-utans at the Wanariset Orang-utan Reintroduction Centre in East Kalimantan, Indonesia (Warren et al. 1999). Of these 18 animals, eight were chronic HBV carriers, four were antibody positive individuals (indicating previous exposure to virus and subsequent recovery) and six individuals were seronegative for HBV (indicating no previous exposure to the virus). Chronic carriers were sampled between two and nine times. Serum ALT concentrations from all infected animals were within normal limits in comparison with unexposed animals (10 to 44 U/L), with one exception (83 U/L). This individual was HBV PCR positive and had a record of being anti-HBs negative followed by seroconversion four months later. Elevation of ALT at this time may have been associated with seroconversion as occurs in humans. Histology of a liver sample from a chronic carrier that died of unrelated causes found no evidence of overt hepatitis infection. This study therefore failed to find conclusive evidence of pathology associated with HBV infection in orang-utans; however it demonstrated a pattern of serological conversion similar to that seen in human HBV infection. The small sample
size, the limited number of biopsy samples, and the limited length of the study, precludes us from making further interpretation regarding the pathogenicity of OuHV.

**Woolly Monkeys** - Lanford et al. (1998) described a case of fatal fulminant hepatitis in a woolly monkey positive for WMHBV at the Louisville zoo, Kentucky USA. No details are given of the clinical or histological findings in this animal. A retrospective study of autopsy reports from 74 woolly monkeys that died in US zoos between 1974 and 1988 found evidence of liver disease in 28 cases, with hepatitis and liver necrosis being the most common findings. No evidence of cirrhosis or hepatocellular carcinoma (HCC) was found. Testing of these animals for WMHBV was not possible, and therefore the pathology could not be definitively linked to viral infection.

Woodchuck Hepatitis virus (WHV) is a much more potent carcinogen than HBV, with nearly 100% of woodchucks infected from birth with WHV developing HCC from around 18 to 24 months of age. Many animals develop multiple independent hepatic tumours, and animals that experience only transient WHV infection display an increased incidence of HCC. Ground squirrels infected with Ground Squirrel Hepatitis virus (GSHV) also display an increased prevalence of HCC, although the tumours are less prevalent and occur later in life than those in the woodchucks (Ganem and Schneider, 2001). Ducks infected with Peking duck hepatitis B virus (DHBV) display a low prevalence of HCC. These studies demonstrate the range of carcinogen capacity of the various hepadnaviruses.

Given the close genetic relationship of the primate hepadnaviruses to HBV, we might expect to see a similar a pattern of pathogenicity in nonhuman primates as that seen in humans infected with hepatitis B virus. Observations from the authors in the articles described previously suggest that this is the case in some HBV infected animals, with various animals displaying signs of acute fulminant hepatitis, elevated serum concentrations of aminotransferase, and histologic changes of acute and chronic
hepatitis in affected animals. Whilst all of these changes are consistent with pathology seen in humans as a result of acute and chronic infection with HBV, the full range of clinical changes has not been described in any one species. Further work is required to detail the full effect of these viruses in the individual species involved. Longer term studies are required to determine if the nonhuman primate hosts are susceptible to the range of pathologies seen in humans with chronic HBV, as such abnormalities develop over 30-40 years of chronic carriage (Tiollais et al. 1985).

1.2.4 Diagnosis of Hepatitis B Virus in Nonhuman Primates

As no tests are widely available for the specific detection of hepatitis B virus in nonhuman primates, detection of the virus in these species relies on the use of serologic assays designed to detect the markers of human HBV infection. Thus researchers and zoos have routinely screened nonhuman primates for the presence of HBsAg, anti-HBs and anti-HBc using commercially available ELISA and EIA methods designed for human HBV (Warren et al. 1999, Hu et al. 2000, 2001, Lanford et al. 2000, Noppornpanth et al. 2003). Additionally HBeAg and anti-HBe have been used to determine the infectivity status of chronically infected gibbons and chimpanzees (Vaudin et al. 1988, Noppornpanth et al. 2003, personal communication A. Mootnick, December 2003, personal communication G. Glover, March 2004).

In addition, HBV DNA has been detected using PCR methods, and sequencing of the pre-S, surface and core regions of the HBV genome, as well as the entire genome, has subsequently been performed by numerous researchers on isolates from chimpanzees, gorillas, orang-utans, gibbons and woolly monkeys (Vaudin et al. 1988, Mimms et al. 1993, Norder et al. 1996, Lanford et al. 1998, 2000, 2003, Warren et al. 1999, Grethe et al. 2000, Hu et al. 2000, 2001, Takahashi et al. 2000, 2001, MacDonald et al. 2000, Verschoor et al. 2001, Vartanian et al. 2002, Aiba et al. 2003, Noppornpanth et al. 2003). Phylogenetic analysis has also been performed on the sequences to determine the genetic relatedness of the viruses found in these animals to known human and
other nonhuman primate hepatitis B viruses. While sequencing of the pre-S, surface and core regions is useful as a means of comparison, conclusions concerning possible new genotypes can only be drawn on the basis of complete genome sequences as defined by Okamoto et al. (1988).

Verschoor et al. (2001) and Noppompanth et al. (2003) have developed combined polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) assays to discriminate between human and nonhuman primate (OuHV and GiHBV respectively) hepatitis B infections based on sequence variations between the human and nonhuman primate viruses. Aiba et al. (2003) developed a gibbon-specific PCR technique which similarly discriminated between human and gibbon infection based on sequences specific to the gibbon virus. In all three studies, all animal isolates examined tested positive with the nonhuman primate-specific tests whilst being negative for human HBV. These tests are extremely useful for confirming the origin of the virus in infected nonhuman primates as well as in infected people in contact with these animals, however they are not widely available.

It has not yet been determined that zoonotic transmission of HBV from humans to primates cannot occur. Therefore, while it has been demonstrated by both sequencing and PCR-RFLP that the nonhuman primate hepatitis B viruses identified to date crossreact with the human serological tests (and can therefore be detected using these tests), the presence of HBV serological markers in a nonhuman primate does not necessarily mean that the primate has been exposed to a nonhuman primate specific HBV. Current findings would suggest that this is the most likely scenario, however further testing in the form of DNA sequencing or PCR-RFLP tests is required to confirm virus identity on a case by case basis.
1.2.5 Treatment of Hepatitis B Virus in Nonhuman Primates

In 1978, Zuckerman et al. attempted treatment of chronically HBV infected chimpanzees at London Zoo with interferon, and two early nucleoside analogues - Ribavarin and adenine arabinoside. Little or no response to therapy was seen in the four chimpanzees treated, barring a temporary improvement in DNA polymerase activity during treatment with adenosine arabinoside, which returned to pre-treatment levels on cessation of treatment. To date, this remains the only investigation specifically directed at the treatment of nonhuman primates with indigenous HBV infection. This is probably largely due to the fact that pathogenicity of HBV in the various nonhuman primates has not been well documented and a need for treatment of these infections has not been clearly identified. The treatments used for management of human cases are aimed at suppressing HBV replication in order to reduce symptoms, minimize chronic inflammation, and prevent progression to cirrhosis and HCC. None of these findings has been consistently present in cases of nonhuman primate HBV infection documented to date, and therefore treatment of infected animals has not been warranted. Further work specifically investigating the pathogenicity of the nonhuman primate HBVs is necessary in order to determine whether such pathological changes do in fact occur in the indigenous hosts during the course of chronic infection.

Long term lamivudine therapy has been tested in woodchucks but with little success. A study by Mason et al. (1998) involved nine chronically infected woodchucks that were treated with lamivudine for greater than 12 months. Whilst there was a significant decrease observed in the WHV viral titres, this occurred at a much slower rate than seen in humans on lamivudine therapy. There was little to no change in the number of infected hepatocytes during the period of treatment and the development of hepatic neoplasms in the chronically infected woodchucks remained unchanged. In addition, there was rapid development of drug resistant viral mutant strains. The lack of success of lamivudine treatment in woodchucks demonstrates the variation in response between humans and woodchucks to the same therapy.
Given the close association between humans and nonhuman primates, in terms of both biological makeup of the hosts and genetic makeup of their respective HBVs, it would seem likely that those treatments that are useful in the management of chronic HBV would potentially be useful in the management of chronic HBV in nonhuman primates if the need arose.

### 1.2.6 Prevention and Control of Hepatitis B Virus in Nonhuman Primates

Chimpanzees have been used in a large number of studies to evaluate the effectiveness of various human hepatitis B vaccines (Prince and Brotman, 2001). In one study by Davis et al. (1996), 23 chimpanzees that had been vaccinated by various means were challenged with live human hepatitis B virus to determine the protective anti-HBs titre in chimpanzees. The study showed that chimpanzees with titres <1 mIU/ml became viraemic and subsequently developed anti-HBc. Each of these animals developed hepatitis, as demonstrated by elevated serum ALT concentrations (>40 IU/ml). Of the 14 animals with a titre of 1-10 mIU/ml, seven showed evidence of infection and hepatitis, six were infected but didn't develop hepatitis, and one remained uninfected. Of the eight chimpanzees with anti-HBs titres >10 mIU/ml, all were protected from infection except for one, that had the lowest titre at 12 mIU/ml. These results are in close alliance with the critical protective level of 10 mIU/ml anti-HBs determined for humans by the United States Centers for Disease Control (CDC). As the trial was based on the use of human strains of HBV, it is unknown if these anti-HBs titres would correlate similarly to challenge with ChHBV. However given the highly conserved nature of the protective S region amongst human and nonhuman primate strains of HBV, it would be expected that cross protection would occur.

In a study by Prince et al. (1997), two newborn chimpanzees were immunised using a DNA based hepatitis B vaccine. The vaccination regimen involved primary immunisation on the day of birth, with boosters at 6 and 24 weeks. Challenge with human HBV at 33
weeks elicited an antibody response in both animals, with no evidence of any of the conventional markers of infection with HBV (HBsAg, anti-HBc). Whilst this regimen demonstrated success in terms of protection against challenge at this time, the study was designed to confer protection against vertical transmission, where viral exposure occurred at the time of birth. A subsequent study revealed that protection did not occur with this regimen when viral challenge occurred on the day of birth (Prince and Brotman, 2001).

Davis et al. (2000) undertook a vaccination trial on orang-utans at the Wanariset Orangutan Rehabilitation Centre in East Kalimantan, Indonesia. Previous prophylactic vaccination of orang-utans at the centre with a paediatric dose of the commercial vaccine Energix-B® had resulted in no detectable antibodies to HBsAg four weeks after the second dose in the two animals vaccinated. Due to this unexpected hyporesponsiveness, the authors added CpG ODN, a potent adjuvant that has stimulatory effects on the immune system, to the vaccine in the hope of producing a more suitable vaccination response. This second trial involved 51 animals and confirmed the previous hyporesponsiveness seen in the original two animals. Of the 13 animals in this trial that received two doses of the commercial vaccine, only five animals attained anti-HBs levels that were considered protective (>10 mIU/mL) by 12-16 weeks after the initial vaccination. In contrast, 98% of the remaining animals in the study achieved protective levels by this time when CpG ODN was included in the vaccine, with 93% protected by eight weeks. The authors concluded that the addition of CpG ODN in future vaccination regimens could produce protective titres after two doses. Whether higher doses of the commercial vaccine would have increased immunogenicity may warrant further trials since the animals were administered human paediatric doses.

Thornton et al. (2001) commented on the management of hepatitis B infections in two gibbons and a western lowland gorilla at London Zoo. A newly arrived male white-cheeked gibbon (Hylobates leucogenys siki) was found to be positive for HBsAg, HBeAg,
HBV DNA, and anti-HBs, and was considered to be infected with HBV. The mate for this animal was found to be negative for all markers of infection and therefore was immunised with an accelerated course of vaccination with Energix-B®. Four doses were administered intramuscularly at zero, one, two and 12 months. The animal’s anti-HBs titre was measured one month after the second inoculation and was found to be >100 miu/litre and the animal was considered by the authors to be protected. Similarly, a second pair of white-cheeked gibbons (H. leucogenys leucogenys) was found to consist of a male carrier and an unexposed female. The female was subsequently immunised against HBV. The pair conceived an offspring that was immunised at 12 weeks of age and at this time was found to be negative for markers of HBV infection. Death of the offspring four weeks later of an unrelated cause meant that further follow-up was not possible. The plan had been to immunise with the rapid schedule, starting at 12 weeks when maternal protection was beginning to wane. A third animal, a western lowland gorilla (Gorilla gorilla gorilla) newly arrived from another zoo in the UK, was found to be a carrier of HBV during its quarantine period. The females with which he was to be housed were immunised immediately, one and two months later. A third booster was required at three months in these animals, before they were considered protected (anti-HBs >100 miu/litre). In addition, keeping and veterinary staff were immunised to protect against potential zoonotic spread of the virus.

Doses of vaccine were not mentioned in the Thornton et al. (2001) paper, making it difficult to determine the viral dose required to provide protective immunity in the animals studied. In addition, the authors consistently refer to miu/litre as the units for measuring protective anti-HBs titres. Studies by other authors (Davis et al. 1996, 2000, Prince et al. 1997) and the CDC measure anti-HBs titres in units of mIU/ml, considering >10 mIU/ml to be the critical protective level for infection with HBV in both humans and chimpanzees. An anti-HBs titre of 100 miu/litre, used by Thornton et al. (2001) as the protective level, would equate to 0.1 mIU/ml and therefore would be well below the critical protective level for humans and chimpanzees of >10 mIU/ml. This may represent
a typographical error. Attempts to contact the authors regarding this issue have been unsuccessful.

Vaccination of the gorilla colony at the Cincinnati Zoo was employed during the late 1980’s through to the mid-1990’s, due to the presence of hepatitis B positive gorillas in the collection (personal communication G. Levens, March 2004). This practice was ceased when HBsAg animals were no longer in the collection. Adult gorillas were vaccinated intramuscularly with 1 ml of an unspecified hepatitis B recombinant vaccine. A single 9 month old male was vaccinated with 0.5 ml of the same vaccine. Serum anti-HBs titres were not measured to determine the effectiveness of vaccination.

Agile gibbons (Hylobates agilis) at the Gibbon Conservation Centre (GCC) in California, USA have been successfully vaccinated with a three vaccination regimen (0, 1 month, 5 months) using 0.5ml of Energix-B (personal communication A. Mootnick, March 04). Anti-HBs titres measured one year after the second booster demonstrated protective levels (>10 mIU/ml). This regimen was used in gibbons of 1, 2 and 4 years of age, to provide immunity to HBV prior to introduction to chronic carrier animals.

The woolly monkey colony at Louisville Zoo has also employed a vaccination regimen to protect against WMHBV, with boosters given at 1 and 6 months after the original vaccination, in accordance with human vaccination recommendations (personal communication R. Burns, June 2004). Anti-HBs titres were measured at 7 months after the initial vaccination and demonstrable antibody production was observed. Due to the divergent nature of the virus in woolly monkeys, it is not known whether this reflects true “protective” immunity, as the vaccines are based on the S gene of human HBV.

Whilst it is possible that a number of other zoos and rehabilitation centres do vaccinate nonhuman primates against hepatitis B, published data on the various regimens used is not widely available. Although it would appear that hepatitis B virus has been present in
a number of nonhuman primate species for a significant period of time, the recognition of the disease in nonhuman primates has only occurred in recent years. As a result there is a lack of information regarding the pathogenicity and epidemiology of the virus, which may account for the lack of awareness of the need to vaccinate.

1.3 Aims and Objectives

The following chapters in this thesis look at the epidemiology and pathogenicity of hepatitis B virus infection in the silvery gibbon through investigation of the history of the virus at Perth Zoo. The effectiveness of vaccination as a strategy to prevent the transmission of the virus is also evaluated. In addition, HBV DNA obtained from one of the current chronic carriers at Perth Zoo has been sequenced along the entire genome, and a partial genome from a second gibbon has also been sequenced. Phylogenetic analysis has been performed on both viral isolates to investigate the genetic relatedness of this virus to those previously identified human and nonhuman primate strains of HBV. To date, only partial sequencing has been performed on HBV from members of the H. moloch species. As the genetic relationship of the different genotypes of HBV can only be fully assessed using the entire genome (Okamoto et al. 1988), this will be the first time that such information has been obtained regarding HBV in silvery gibbons.

It is hoped that this thesis will contribute significantly to the current knowledge of hepatitis B virus in silvery gibbons, and that these findings will also contribute to the understanding of nonhuman primate HBV in general. Such information will have implications on the management of captive and wild populations of this and other critically endangered nonhuman primate species.
Chapter 2: History of Hepatitis B in Silvery Gibbons at the Perth Zoo

2.1 Introduction

Perth Zoo currently houses a family group of four silvery gibbons, comprising an adult male, an adult female, and two of their offspring, who range in age from sixteen months to three years. The first offspring born to this same pair, Khusus, was transferred to an institution in California for breeding purposes in 2000. Regina, the second surviving offspring, was transferred to a zoo in Sydney in 2004.

A solitary hand-raised adult male gibbon, Uban, was also part of the collection but died in 2001 after a period of liver-related illness. Uban was the offspring of a female gibbon, Perth 2, who was part of the Perth Zoo collection from 1975 until her death in 1994.

Both adults in the current group are considered to be chronic carriers of hepatitis B virus, based on serological testing that has demonstrated cross-reactivity with human HBV. The female, Hecla, is classified as a high infectivity carrier due to the presence of HBeAg. Jury, the adult male, is considered a low infectivity chronic carrier due to the lack of HBeAg in his serum and the presence of anti-HBe. Uban was considered to be a chronic carrier of hepatitis B virus.

Vaccination programs developed at Perth Zoo appear to have been successful in preventing transmission of the virus to two of the three offspring tested. The first offspring in the program, Khusus, demonstrated evidence of immunity to HBV however also showed evidence of natural infection indicating exposure to the virus. Vaccination was not regarded as having been successful in preventing viral transmission in this case, however Khusus was prevented from becoming a chronic carrier and therefore does not pose a transmission risk to other animals or people. Due to the young age of the fourth
offspring (Sinta) at the time of writing, we are unable to assess the success of vaccination in this individual at this time. The details of the vaccination programs are discussed in Chapter 3 of this thesis.

Historical records also indicate hepatitis B infection in a hoolock gibbon (*Bunopithecus hoolock*), Horace, housed at Perth Zoo from 1980 until 1993. This may represent the first recorded case of HBV infection in this species of gibbon.

Through the examination and review of the history of the Silvery gibbon colony at Perth Zoo, the epidemiology and pathogenicity of hepatitis B virus in this species are discussed in this chapter. Hepatitis B infection in a single hoolock gibbon is also discussed.

### 2.2 Materials and Methods

The information contained in this chapter has been sourced from the medical records of Perth Zoo collection animals using the Med.A.R.K.S. computer records system (version 5.31j, International Species Information Systems, Apple Valley MN, USA 2001) and from hard copy medical files. Further information has been obtained from staff at the Gibbon Conservation Centre (GCC) in California, USA, the Assiniboine Park Zoo in Manitoba, Canada, and Howlett’s Wild Animal Park in Kent, UK. Family trees were constructed using information contained in the 2003 silvery gibbon studbook (Cocks, 2003) and confirmed by medical records from Perth Zoo, GCC, Assiniboine Park Zoo and Howlett’s Wild Animal Park.

Blood was collected from animals under general anaesthesia during routine physical examinations performed on different occasions over the past 12 years. Blood samples were subject to biochemical and haematological analysis. Additional blood was centrifuged using a Hettich Universal 1200 centrifuge at 5000 rpm for 15 minutes.
Serum was separated and submitted for hepatitis B testing at the Division of Microbiology and Infectious Diseases, in the Western Australian Centre for Pathology and Medical Research (PathCentre), Perth. Any remaining serum was held at -80°C for serum banking purposes.

Hepatitis B serology was performed using the Abbott AxSYM microparticle enzyme immunoassay (MEIA) system. Hepatitis B surface antigen (HBsAg) was detected by Abbott AxSYM HBsAG (V2) MEIA. Positive samples were confirmed using the Abbott AxSYM HBsAG (Confirmatory) MEIA neutralisation test and/or the Abbott Determine immunochromatographic card test. Anti-HBs, anti-HBc, HBeAg and anti-HBe were detected by Abbott AxSYM AUSAB MEIA, Abbott AxSYM CORE MEIA, Abbott AxSYM HBe 2.0 MEIA and Abbott AxSYM Anti-HBe 2.0 MEIA respectively.

An in-house PCR assay was used to detect HBV DNA. Viral DNA was extracted from the serum samples using the QIAamp® Viral RNA Mini Kit (QIAGEN, Doncaster, Vic) according to the manufacturer’s instructions. Extracted HBV DNA was then amplified by PCR using a standard TaqGold DNA Polymerase (Applied Biosystems Division, Foster City, CA). 8 µL of DNA was added to 12 µL of a mix of 2µL HB1763, 2µL HB2032R (Table 2.1), 200 µM deoxynucleoside triphosphate (dNTP) mix, PE Buffer II 1x, 1.25mM MgCl₂, and 0.5 units of Taq DNA polymerase. Samples were preheated for 10 minutes at 94°C to activate the enzyme and then cycled for 30 seconds at 94°C (denaturation), annealing for 30 seconds at 60°C, and extension for 45 seconds at 72°C for 45 rounds of amplification, followed by a final seven minute cycle at 72°C. After cycling, 0.4µL of each product was inoculated into 20µL of nested hepatitis B PCR mix (2µL HB1778E, 2µL HB2017RB (Table 2.1), 200 µM dNTP mix, PE Buffer II 1x, 2.25mM MgCl₂, 0.5 units of Taq DNA polymerase) and cycling repeated under the same conditions. Products were then run on a 1.8% agarose gel and stained with ethidium bromide to detect fragments.

**Table 2.1: Primers used in hepatitis B virus detection**

```markdown
<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>HB1763</td>
<td>Forward primer</td>
</tr>
<tr>
<td>HB2032R</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>HB1778E</td>
<td>Nested forward primer</td>
</tr>
<tr>
<td>HB2017RB</td>
<td>Nested reverse primer</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB1763 (forward)</td>
<td>GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA</td>
</tr>
<tr>
<td>HB2023R (reverse)</td>
<td>CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT</td>
</tr>
<tr>
<td>HB 1778E (forward)</td>
<td>GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT</td>
</tr>
<tr>
<td>HB 2017RB (reverse)</td>
<td>ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA</td>
</tr>
</tbody>
</table>

Full blood biochemistry and haematology profiles on blood collected prior to 1999 were performed and analysed at the Clinical Pathology Laboratory of Murdoch University Veterinary Hospital, Perth. Liver enzymes (ALT, AST, ALKP and bilirubin) were measured using a Cobas Mira Automated Chemistry Analyzer (Roche) using Roche and Boehringer reagents. Blood collected subsequently was analysed at Vetpath laboratories in Perth. Between 1999 and November 2002, liver enzymes (ALT, AST, ALKP and bilirubin) were measured using a Hitachi® 717 Chemistry Analyzer (Roche) and Roche reagents. Since November 2002, liver enzymes have been measured using an Olympus AU400 Chemistry Analyzer (Olympus) and Olympus reagents.

Specific reference ranges for biochemistry and haematology of silvery gibbons were not available so results were compared with the mean results of a number of other gibbon species as published in International Species Information System (ISIS) Reference Ranges for Physiological Values in Captive Wildlife (2002). Values were also compared with previous blood profiles on silvery gibbons in the Perth Zoo collection to account for possible species variation.

Gross post mortem examinations of Perth 2, Uban and Horace were performed at the time of death by Perth Zoo veterinarians. Tissue samples were fixed in 10% buffered formalin solution and examined histologically by veterinary pathologists at the Western Australian Department of Agriculture (Perth 2 and Horace) and Vetpath laboratories (Uban). Samples of fixed liver from Perth 2, Uban and Horace were examined in 2004 by veterinary pathologists at Vetpath laboratories and a pathologist in human medicine from PathCentre.
2.3 Individual Animal Profiles

2.3.1 Perth 2

Sex: Female
Born: Wild caught in Java Jan 1971
Birth date unknown
Arrived Perth Zoo: October 1975
Death: 31st October, 1994 (>23 yrs)

Hepatitis B status: Positive (low infectivity carrier)
Perth 2 was first tested for HBV in 1993 and at that time was classified as a low infectivity carrier (HBsAg +ve, anti-HBs -ve, HBeAg -ve, anti-HBe +ve). She died approximately one year later, with no further hepatitis B testing performed.

Epidemiology
Perth 2 had been in the Perth Zoo collection for 18 years prior to the time of first testing. It is therefore difficult to say at what stage she became infected with HBV. After being caught from the wild in 1971, Perth 2 had been held for 4 years in a private collection in Jakarta, an area with a high level of endemic HBV infection within the human population. She was transferred to Perth Zoo in 1975. It is possible that Perth 2 was infected in the wild, although evidence for this is lacking.

Pathogenicity
Perth 2 died in 1994 of liver disease. Blood testing in 1993 demonstrated mildly elevated serum ALT (131 U/L), with AST, ALKP and bilirubin in the normal ranges (33 U/L, 99 U/L, 8 µmol/L – ISIS mean for bilirubin 3 – 9 µmol/L). Repeat testing on the day before her death in 1994 showed significant increases in three of the tested liver parameters (ALT 364 U/L, AST 287 U/L, bilirubin 47 µmol/L), however ALKP remained within the normal range at 62 U/L.
The post mortem report described infiltration of the liver and spleen with numerous microabscesses, attributed to infection with Yersinia enterocolitica. On histopathological examination of the liver the primary pathologist described mild portal inflammation and mild periportal fibrosis. These changes are consistent with chronic hepatitis B viral infection, however the changes were mild and could have been clinically silent. Re-examination of these slides by a pathologist in human medicine reported mild fibrosis with septa formation (Figure 2.1). No definitive evidence of pathology associated with chronic hepatitis B infection was noted on this examination. Orcien stain and immunohistochemistry for HBsAb and anti-HBc were negative, however the liver was positive for HBV DNA using PCR. The widespread bacterial embolic disease caused by Y. enterocolitica infection appears to have been the cause of death in this animal.

Extensive iron accumulation within the liver parenchyma was observed by both pathologists. Liver tissues demonstrated strong positive staining to Perl’s stain (Figure 2.2), a histochemical stain specific for iron. Heavy iron deposition was seen throughout the liver parenchyma. Such heavy iron deposition can be associated with mild chronic inflammation. The cause of the iron overload in this case is unknown, however could be the result of a chronic disease process, a dietary overload of iron, or a genetic condition.
Figure 2.1: Masson trichrome staining of liver portal tract from Perth 2 demonstrating fibrous expansion with early septa (arrows). Original magnification 100x.

Figure 2.2: Haematoxylin and eosin staining of liver from Perth 2 demonstrating abscess (big arrows) and adjacent portal tract with iron deposition (small arrows). Original magnification 40x.
2.3.2 Uban

Sex: Male

Born: 6th November 1980, Perth Zoo

Sire: Perth 1

Dam: Perth 2

Death: 26th October, 2001 (21 yrs)

Hepatitis B status: Positive (low infectivity carrier)

Uban was born at Perth Zoo in 1980 from wild-caught parents, who both arrived at the zoo in 1975 from Jakarta. Uban was first diagnosed with hepatitis B in 1993, when screening of all the primates in the zoo for the virus was first performed. He was found at this time to be a high infectivity chronic carrier due to the presence of both HBsAg and HBeAg. Repeat testing on a number of occasions confirmed that he remained a chronic carrier until his death in 2001. However, seroconversion from high infectivity to low infectivity was determined to have occurred between 1993 and 1996, at which point anti-HBe became detectable and HBeAg could no longer be detected in serum.

Epidemiology

Due to the lack of testing prior to 1993, it is impossible to be sure at what stage Uban became HBV positive and how he was exposed to the virus. Uban was separated from his parents at three days of age due to mis-mothering, and was subsequently hand-raised by a member of staff. The staff member who raised Uban tested negative for HBV. Uban was never re-introduced to his parents or other gibbons.

Uban’s mother, Perth 2, was first tested for HBV in 1993 and was classified as a low infectivity carrier. The sire, Perth 1, died in 1984 and had not been tested for HBV.
Given the chronic nature of Uban's infection and the lack of other likely sources of viral exposure, it is probable that Uban was exposed to the virus in the perinatal period via his chronic HBV carrier mother, Perth 2.

Shelby, a sibling of Uban's, was born in 1983 and was transferred to an institution in California in 1993. Testing at Perth Zoo in 1993 showed that Shelby had evidence of past exposure to HBV but had developed immunity and had not become a chronic carrier. Shelby had been housed with his mother until the time of testing in 1993, and with his father (Perth 1) until Perth 1’s death in 1984. Shelby had not been exposed to other animals prior to testing.

Figure 2.3:  Uban’s family tree
Pathogenicity

Uban had blood collected for biochemical profiles on a number of occasions between 1994 and 2001, both as part of routine physical examinations and during investigation of his illness. In all results on record, ALT concentrations were moderately to markedly elevated (210 – 613 U/L) when compared with other mean gibbon ALT reference concentrations of 25 - 61 U/L (ISIS 2002). On all but one of these occasions, AST concentrations were also elevated, ranging from 150 - 262 U/L (ISIS mean 22 – 37 U/L). Alkaline Phosphatase (ALKP) concentrations were within the normal ranges on all occasions (111 – 535 U/L, ISIS 2002).

In 2001, Uban died after a period of liver-related illness, during which time he displayed a range of clinical signs including depression, inappetence and in the later stages of the disease, jaundice. A liver biopsy performed on the day before Uban died was consistent with the post mortem findings of acute, multifocal, necrotising abscesses involving the liver, with underlying cirrhosis consistent with chronic viral hepatitis. Multifocal abscessation also involved the kidney and spleen. Systemic staphylococcal abscessation involving the liver and other organs was the cause of death in this animal, a condition not routinely associated with chronic hepatitis B infection in humans. The histological diagnosis of cirrhosis however, was consistent with chronic viral hepatitis.

Recent re-examination of the post mortem samples by a pathologist in human medicine has disputed the finding of cirrhosis in this case, reporting only mild fibrosis present rather than the severe fibrosis seen in cases of cirrhosis (Figure 2.4). Widespread coagulative necrosis and abscess formation was described, and was attributed to the systemic bacterial infection as described above. Extensive iron accumulation is also noted, in a similar pattern to that observed in Perth 2 (Figure 2.5). Orcien stain and immunohistochemistry for HBsAb and anti-HBc were negative.
Figure 2.4: Masson trichrome stain of liver from Uban demonstrating minor fibrosis (arrows). Original magnification 100x.

Figure 2.5: Haematoxylin and eosin stain of liver from Uban demonstrating necrosis (small arrows) with adjacent viable parenchyma and portal tract containing iron deposition (large arrows). Original magnification 100x.
2.3.3 Hecla

**Sex:** Female

**Born:** 21st July 1983, Winnipeg Zoo, Canada

**Sire:** Studbook #2 - wild caught, Java

**Dam:** Studbook #1 – wild caught, Java

**Arrival Perth Zoo:** 11th August 1992 (6 yrs)

**Current age:** 20 yrs

Partnered with Jury. Gave birth to:

- Khusus born 13th January, 1995
- 970711 born 20th August, 1997 – died same day.
- Regina born 20th April, 1998
- Arjuna born 24th November, 2000
- Sinta born 24th March, 2003

**Hepatitis B status: Positive (high infectivity carrier)**

Hecla came to the zoo in 1992 from a zoo in Canada where she was born. She was first tested for hepatitis B in December 1992, approximately two months after her arrival, at which time she was suspected of being a chronic carrier, based on the presence of HBsAg and lack of anti-HBs. There was insufficient serum at this time to identify her level of infectivity, however, testing one year later revealed her to be a high infectivity chronic carrier, due to the presence of HBeAg. There is no record of testing for HBV prior to her arrival at the zoo, nevertheless the incubation period of the virus, in conjunction with her chronic carrier status, are strongly suggestive of her being positive for HBV on arrival. This is further supported by the fact that three full siblings of hers have since been identified as chronic carriers (Lanford et al. 2000, personal communication G. Glover, March 2004), suggesting a vertical mode of transmission.
Repeat testing over the last 10 years, most recently in April 2003, has confirmed that she remains a high infectivity chronic carrier.

**Epidemiology**

In her previous institution, Hecla was housed in a family group with her parents and two younger siblings. One year prior to her departure she was forced out of the group by her mother and was housed separately until her transfer to Perth Zoo. After a quarantine period at Perth Zoo, she was housed on her own until being introduced to an adult male (Jury), with whom she has been housed ever since. She is currently housed with Jury and two of their offspring, ranging in age from one to three years.

It is probable that Hecla became infected with HBV at her previous institution. Her chronic carrier status is most consistent with vertical transmission, suggesting exposure in the perinatal period. Currently the HBV status of her mother Bobby Jean remains unknown although there are plans to test her the next time general anaesthesia is performed. Due to Bobby Jean’s advanced age (greater than 44 yrs) general anaesthesia has been postponed until clinically necessary. Hecla’s father, Billy J, died in 1995 and was never tested for HBV. His infectivity status therefore remains unknown.

Hecla’s parents gave birth to 11 offspring since 1970. Four of these animals died prior to 1988, and two other offspring died on the day they were born. Hepatitis B testing was not performed on any of these animals (personal communication G. Glover, August 2003). Assini, born three years before Hecla, is now housed at Howlett’s Wild Animal Park. Testing of Assini for HBV has not been performed to date, however one of her five offspring has been found to be a high infectivity carrier, suggesting vertical transmission from Assini (personal communication C. Furley, February 2004).

A further two siblings of Hecla, Chilibi and Chloe (born three and six years after Hecla), have been shown by Lanford et al. (2000) to also be chronic carriers of HBV. Based on
these findings, the authors proposed that both these animals were infected via vertical transmission from their mother, a theory consistent with our findings. The infectivity status of these two animals has not been confirmed.

A fourth sibling, Willow, has recently been tested for HBV and also found to be a high infectivity carrier (personal communication G. Glover, March 2004).

Therefore, four of the five remaining offspring of Bobby Jean and Billy J are confirmed chronic carriers of HBV, with two of these confirmed as high infectivity carriers (HBeAg positive). The fifth offspring, Assini, is suspected of also being a chronic carrier, and likely to have been the source of viral transmission to at least one of her offspring. The infectivity status of Chloe and Chilibi is still to be determined, however it is likely that they would also be high infectivity carriers. The pattern of transmission seen in Bobby Jean and Billy J’s offspring is consistent with the pattern of vertical transmission seen in humans. Bobby Jean is therefore likely to have been the source of infection for Hecla and her siblings. The high transmission rate suggests that she is likely to be a high infectivity carrier. Serology from Bobby Jean will ultimately confirm this hypothesis.

**Pathogenicity**

Blood testing of Hecla on 11 separate occasions over the last 10 years has shown no elevation in any of the liver enzymes routinely tested (ALT, AST, ALKP, total bilirubin). On one occasion she was shown to have decreased serum protein concentrations which can be associated with hepatopathies, however no other changes were present at that time. Hecla has never demonstrated any clinical signs of liver-related illness. No liver biopsies or ultrasound examinations have been performed.
Figure 2.6 Hecla’s family tree

Sire: ‘Billy J’
Wild caught 1959
Hep B status unknown

Dam: ‘Bobbie Jean’
Wild caught 1959
Hep B status unknown
Probable high infectivity carrier

X
Born: 1970
Died: 1979
No data

‘Bebé’
Born: 1973
Died: 1988
No data

‘Togo’
Born: 1976
Died: 1982
No data

X
Born: 1979
Died: 1979
No data

‘Assini’
Born: 1980
Hep B status unknown
? High infectivity carrier

‘Hecla’
Born: 1983
High infectivity chronic carrier
(HBsAg +ve, anti-HBs -ve, anti-HBe -ve)

‘Chilibi’
Born: 1986
Chronic carrier
(HBsAg +ve, anti-HBs -ve)

‘Chloé’
Born: 1990
Chronic carrier
(HBsAg +ve, anti-HBs -ve)

X
Born: 1992
Died: 1992
No data

X
Born: 1993
Died: 1993
No data

‘Willow’
Born: 1994
High infectivity carrier
(HBsAg +ve, anti-HBs -ve, anti-HBe -ve)
2.3.4 Jury

**Sex:** Male

**Born:** 17th July 1986, Berlin Zoo, Germany

**Sire:** Studbook #16 - wild caught, Java

**Dam:** Studbook #5 – wild caught, Java

**Arrival Perth Zoo:** 23rd August 1992 (6 yrs)

**Current age:** 17 yrs

Partnered with Hecla. Sired:

- Khusus born 13th January, 1995
- 970711 born 20th August, 1997 - died same day.
- Regina born 20th April, 1998
- Arjuna born 24th November, 1998
- Sinta born 24th March, 2003

**Hepatitis B status: Positive (low infectivity carrier)**

Jury was born in a zoo in Germany and came to Perth zoo in 1992. He was first tested for hepatitis B in September 1992, within two weeks of his arrival. Based on the presence of HBsAg and lack of anti-HBs he was suspected of being a chronic carrier. A lack of sufficient serum at that time meant that his level of infectivity could not be determined, however testing one year later revealed him to be a low infectivity chronic carrier, due to the absence of HBeAg and the presence of anti-HBe. There is no record of Jury being tested for HBV prior to his arrival at Perth Zoo, however the incubation period of the virus suggests that he was infected with HBV upon arrival.

Results of repeat testing reported in 1996 and 1997 are currently unavailable, and no further testing has been performed. Due to the chronic nature of his infection he is considered to still remain a low infectivity carrier.
Epidemiology

As explained previously, testing for HBV on arrival at Perth Zoo indicated that Jury was already infected with HBV at the time he entered the collection, and that he would therefore have been exposed to the disease during his time at Berlin Zoo. His chronic carrier status is most consistent with vertical transmission from a carrier mother, especially considering his subadult age at the time of his arrival at Perth Zoo. Reports from the original institution indicate that Jury had been housed with his parents until the time he was sent to Perth Zoo. However, it is unclear whether other animals were also present in the enclosure during this time. Given the social behaviour of this species and the formation of monogamous family groups, it is likely that any other animals that may have been housed with Jury and his parents would have been siblings, as non-family members are not usually accepted by gibbon family groups (Rowe 1996). Studbook records for 2003 indicate that Jury is the sole offspring from his parents.

Grethe et al. (2000) included serologic testing of Jury's father Ivan in their study of HBV in gibbons, using a sample collected during his time at Berlin Zoo (prior to 1996). This sample tested HBsAg -ve and positive for both anti-HBs and anti-HBc, a serological pattern consistent with exposure to the virus and the subsequent development of immunity and typical of horizontal transmission. These authors were also able to partially sequence the HBV genome using the very sensitive method of nested PCR. The authors of this paper suggest that Ivan was exposed to HBV either at Berlin Zoo or in the wild. Lanford et al. (2000) also found Ivan to be HBsAg –ve, and positive for both anti-HBs and anti-HBc when tested in 1996, at the time of his arrival at his current institution in California. This supports the findings by Grethe et al. (2000) and confirms that Ivan was infected at the Berlin Zoo, where Jury was born, or in the wild.

The HBV status of Jury's mother, Paula, remains unknown. She died in 1993 from a systemic Yersinia infection. No evidence of chronic HBV infection was apparent based on liver pathology. HBV testing was not performed prior to her death. The patterns of
infection of Jury and Ivan suggest that she may have been a chronic carrier, potentially a high infectivity carrier, and could have been the source of infection for both Jury and Ivan.

Figure 2.7: Jury's family tree

Pathogenicity

Jury has had a number of blood samples taken for routine analysis during the past 11 years. Liver enzymes have remained within normal limits on three occasions, however there has been a mild elevation in his ALT concentrations on four occasions, including the three most recent samples (102 U/L September 1992, 130 U/L September 1999, 170 U/L March 2000, 105 U/L April 2004). AST has also been above the normal range on the three most recent samples (70 U/L September 1999, 61 U/L March 2000, 60 U/L April 2004). Values for ALKP and bilirubin have remained in the normal ranges.

In March 2000 Jury was anaesthetised for a physical examination to investigate progressive abdominomegaly and apparent abdominal discomfort. Blood was collected at this time (ALT 170 U/L, AST 61 U/L) and an ultrasound examination was performed.
Mild hepatomegaly was noted with dilation of the cystic and common bile ducts. The hepatic parenchyma appeared to be within normal limits. No biopsies were taken. Clinical signs resolved and no further illness has been reported, however the abdominomegaly has persisted.

In April 2004, a repeat GA was performed to determine if there had been any advancement in his condition. Ultrasound examination at this time demonstrated improvement, with liver parameters now within normal ranges. Mild elevations were again demonstrated in ALT and AST values (ALT 105 U/L, AST 60 U/L), however these were marginally reduced from the concentrations seen in 2000.

### 2.3.5 Khusus

**Sex:** Female

**Born:** 13th January 1995, Perth Zoo

**Sire:** Jury – see previous

**Dam:** Hecla – see previous

**Transferred from Perth Zoo:** to the Gibbon Conservation Centre (GCC), California, USA. 9th October 2000.

**Current age:** 9 yrs

**Hepatitis B status:** Exposure induced immunity

Khusus was born in 1995, the first offspring of Hecla and Jury, both chronic HBV carriers. Due to the high risk of infection with the virus via vertical transmission in the perinatal period, a vaccination program was developed for Khusus with the aim of inducing immunity to HBV. Details of the vaccination program used in Khusus and the subsequent offspring born at Perth Zoo are contained in Chapter 3 of this thesis.
Blood testing at 15 months of age revealed that Khusus had developed a serological pattern consistent with immunity (HBsAg -ve and anti-HBs +ve), however it also demonstrated evidence of viral exposure (anti-HBc +ve). It was concluded from these results that the vaccinations had not been successful in preventing viral infection, but they appeared to have been successful in preventing her from becoming a chronic carrier of the virus. Repeat testing at four and five years of age demonstrated that this immunity has been maintained. Testing at her current institution has confirmed that she is still immune (personal communication A. Mootnick, December 2003).

**Epidemiology**

Khusus was born at Perth Zoo and at the time of her first hepatitis B testing at 15 months of age had only been housed with her parents, Hecla and Jury. At this time she was found to have anti-HBs and anti-HBc in her serum, with no detectable HBsAg, indicating previous infection that had resolved. The exact time of infection is unknown. Both Hecla and Jury could have been sources of infection for Khusus. The most likely scenario would have been infection in the perinatal period via vertical transmission from Hecla, although horizontal transmission from Hecla or Jury in the period between birth and testing (i.e. first 15 months) cannot be ruled out.

*Figure 2.8: Family tree of Khusus, Regina, Arjuna and Sinta*
Pathogenicity

Khusus had blood collected on four occasions during the five years she was at Perth Zoo. On one occasion (July 2000) she demonstrated an elevated serum concentrations of ALT (162 U/L), however she had no other evidence of clinical illness at that time. No clinical illness has been demonstrated at any stage and all other liver parameters have remained within normal ranges.

2.3.6 Regina

Sex: Female

Born: 20th April 1998, Perth Zoo

Sire: Jury – see previous

Dam: Hecla – see previous

Transferred from Perth Zoo: to Taronga Zoo, Sydney, Australia. 12th February 2004.

Current age: 6 yrs

Hepatitis B status: Vaccine-induced immunity

Regina was the third offspring born to Hecla and Jury. A second offspring had been born in 1997 but died the same day – no HBV testing was performed on this second offspring.

Due to the chronic carrier status of the parents, a vaccination program was again adopted in the hope of preventing transmission of the virus to Regina. Minor adjustments to the program used for Khusus were made due to the evidence of exposure to the virus in Khusus’s results (see Chapter 3). Blood testing at 13 months of age demonstrated a serological response consistent with vaccine-induced immunity (HBsAg -ve, anti-HBs +ve, anti-HBc -ve). Repeat testing 4.5 years after the initial vaccination indicated that this immunity has been maintained.
Pathogenicity

Biochemical profiles on Regina have been conducted on two occasions, neither of which has demonstrated any elevations in liver or other enzymes. No clinical illness has been demonstrated by Regina at any stage.

2.3.7 Arjuna

Sex: Male

Born: 24th November 2000, Perth Zoo

Sire: Jury – see previous

Dam: Hecla – see previous

Current age: 3 yrs

Hepatitis B status: Vaccine-induced immunity

Given the apparent success of the vaccination program used with Regina, it was decided to also vaccinate Arjuna. Due to practical difficulties with the program used on Regina, slight modifications were made for Arjuna. Testing of Arjuna at two years of age displayed a serological pattern consistent with vaccine-induced immunity (HBsAg –ve, anti-HBs +ve, anti-HBc –ve).

Due to his young age, repeat testing has not yet been performed to determine whether this immunity will be maintained. It would seem reasonable to expect, based on the results from Khusus and Regina, that his immunity will also be maintained for approximately four to five years post-vaccination.

Pathogenicity

Arjuna has had a biochemical profile run on one occasion with no abnormalities detected. No clinical illness has been demonstrated.
2.3.8 Sinta

**Sex:** Female

**Born:** 24th March 2003, Perth Zoo

**Sire:** Jury – see previous

**Dam:** Hecla – see previous

**Current age:** 1 yr

**Hepatitis B status: unknown**

Sinta was vaccinated following the same protocol used for Arjuna. Due to her young age, blood will not be collected to determine the success of vaccination for a further 12 months or more, unless anaesthesia is required for other purposes in the interim. It is hoped that the regimen used will achieve the same success as that seen in Regina and Arjuna, in providing vaccine-induced immunity and preventing exposure to the disease.

**Pathogenicity**

At the time of writing, no biochemical profiles have been run on this individual. No clinical illness has been demonstrated.
Figure 2.9: Combined Hecla and Jury family tree

- **Sire: 'Billy F'**
  - Wild caught 1959
  - Hep B status unknown

- **Dam: 'Bobbie Jean'**
  - Wild caught 1959
  - Hep B status unknown
  - Probable High infectivity carrier

- **'Bebe'**
  - Born: 1970
  - Died: 1979
  - No data

- **'Togo'**
  - Born: 1976
  - Died: 1982
  - No data

- **'Assini'**
  - Born: 1980
  - Hep B status unknown
  - High infectivity carrier
  - (HBsAg +ve, anti-HBs -ve, anti-HBe -ve)

- **'Hecla'**
  - Born: 1983
  - High infectivity chronic carrier
  - (HBsAg +ve, anti-HBs +ve)

- **'Chilhi'**
  - Born: 1986
  - Chronic carrier
  - (HBsAg +ve, anti-HBs -ve)

- **'Chloe'**
  - Born: 1990
  - Chronic carrier
  - (HBsAg +ve, anti-HBs -ve)

- **'Willow'**
  - Born: 1994
  - High infectivity carrier
  - (HBsAg +ve, anti-HBs +ve, anti-HBe -ve)

- **Site: 'Ivan'**
  - Wild caught 1974
  - Exposed, immune
  - (HBsAg +ve, anti-HBs +ve, anti-HBe +ve)

- **Dam: 'Paula'**
  - Wild caught 1964
  - Hep B status unknown
  - Suspected high infectivity carrier

- **'Jury'**
  - Born: 1986
  - Low infectivity carrier
  - (HBsAg +ve, anti-HBs -ve, anti-HBe +ve)

- **'Khusus'**
  - Born: 1995
  - Vaccinated
  - Exposed, immune
  - (HBsAg +ve, anti-HBs +ve, anti-HBe +ve)

- **'Regina'**
  - Born: 1998
  - Vaccine induced immunity
  - (HBsAg +ve, anti-HBs +ve, anti-HBe +ve)

- **'Arjuna'**
  - Born: 2000
  - Vaccine induced immunity
  - (HBsAg +ve, anti-HBs +ve, anti-HBe +ve)

- **'Sinta'**
  - Born: 2003
  - Vaccinations commenced
  - No data
2.3.9 Horace (hoolock gibbon)

**Sex:** Male

**Born:** unknown (possibly wild caught)

**Arrival Perth Zoo:** 17th January, 1980

**Sire:** unknown

**Dam:** unknown

**Death:** 27th July, 1993 (>13 yrs)

**Hepatitis B status: Positive (high infectivity carrier)**

Horace was housed at the Perth Zoo from 1980 until 1993. In June 1993 Horace was found to be HBsAg +ve. Further testing detected the presence of HBeAg and anti-HBe. This pattern is consistent with a high infectivity carrier, although may also been seen at the time of serocnversion from high infectivity to low infectivity state.

**Epidemiology**

Horace had been transferred to Perth Zoo from a zoo in Asia, along with a female, Flossie. Both animals were assumed to have been caught from the wild. Records from this time are scarce, however it would seem that Horace and Flossie had been housed together at Perth Zoo from the time of their arrival until her death in 1992, and had not been housed with other gibbons during this time. Flossie had never been tested for hepatitis B infection.

It is difficult to say where Horace may have acquired his infection. The fact that he was a high infectivity carrier is suggestive of perinatal infection, however this cannot be confirmed given that his parentage is unknown.

**Pathogenicity**

Horace was euthanased due to public health concerns at the time. No haematology or serum biochemistry results were available for this animal. Post mortem examination did
not reveal any pathology in the liver associated with the hepatitis B infection. Re-
examination of the liver slides by a pathologist in human medicine has revealed the
presence of a low grade chronic hepatitis, typical of the chronic persistent hepatitis seen
in human chronic hepatitis B patients. Orcien staining to detect HBsAg within the
cytoplasm of infected hepatocytes was strongly positive throughout the hepatic
parenchyma, a pattern typical for HBV infection in humans (Figure 2.10). Hepatocytes
also demonstrated the typical "ground glass" appearance of cytoplasm seen as a result
of HBsAg within the dilated endoplasmic reticulum (Figure 2.11). Similarly, strong anti-
HBc positivity was also demonstrated within the nuclei of infected hepatocytes
throughout the liver (Figure 2.12). Immunohistochemistry for HBsAg however was
negative. HBV DNA was detected in paraffin embedded formalin fixed liver samples.

Figure 2.10: Orcien stain of liver from Horace demonstrating positive cytoplasmic staining of
hepatocytes indicative of hepatitis B surface antigen within dilated endoplasmic reticulum
(arrows). This corresponds to the "ground glass" appearance of the hepatocyte cytoplasm seen
in figure 2.6. Original magnification 400x.
Figure 2.11: Haematoxylin and eosin staining of the liver from Horace demonstrating the "ground glass" appearance of the cytoplasm indicative of hepatitis B surface antigen within dilated endoplasmic reticulum (arrows). Original magnification 400x.

Figure 2.12: Liver from Horace demonstrating positive nuclear staining for hepatitis B core antigen using immunohistochemistry (arrows). Original magnification 400x.
2.4 Discussion

2.4.1 Epidemiology

Uban’s high infectivity carrier status is consistent with the human pattern of vertical transmission of HBV. The known carrier status of his mother Perth 2, combined with the lack of contact with other known sources of infection, further support the theory that he was infected in the perinatal period prior to separation from Perth 2 at 3 days of age for hand-raising. The lack of any known period of liver related illness in his neonatal period is also consistent with the typically silent infections seen with human neonatal infection with HBV.

The evidence of previous infection with HBV in Uban’s sibling Shelby, who had only been housed with Perth 1 for one year and Perth 2 up until the time of testing, further demonstrates the infectious potential of Perth 2 and supports the theory that Perth 2 was the source of infection for both animals.

The fact that Uban was a chronic carrier and Shelby was not, could be explained by a number of different scenarios:

- Perth 2 may have been a high infectivity carrier at the time of Uban’s birth but subsequently seroconverted to become a low infectivity carrier between the birth of Uban and Shelby (such seroconversion occurs at a rate of 10-15% per year in human cases (Dienstag and Isselbacher, 2001b).

- Uban may have been in the 10-15% of cases that become infected in the perinatal period by low infectivity carrier mothers, whilst Shelby could have been in the 85-90% that don’t. Shelby might then have been exposed by horizontal transmission as a juvenile by intimate contact with Perth 2 and subsequently developed immunity.
• Shelby may have been exposed in the perinatal period but was in the 10% of cases that do not become chronic carriers at this age.

• Being the first offspring born to Perth 2, Uban’s birth and early perinatal period may have been more traumatic than Shelby’s. The mis-mothering that occurred with Uban’s birth could have increased his chances of becoming infected during the perinatal period.

Unfortunately we cannot be sure which, if any, of these scenarios actually occurred. Given Uban’s chronic carrier status and the small window of time when he had known exposure to the virus, it is likely that he became HBV positive as a result of vertical transmission. However, it is difficult to say if Shelby’s exposure occurred as a result of vertical or horizontal transmission.

Both Hecla and Jury appear to have come to Perth Zoo as chronic carriers. Transmission data from these animals is speculative, but it would seem that Hecla was most likely exposed via vertical transmission from her mother Bobby Jean, as evidenced by the fact that three of her siblings (Chloe, Chilibi and Willow) are also carriers. A fourth sibling, Assini, is likely to also be a carrier, as evidenced by the fact that one of her offspring is a high infectivity carrier, although testing of Assini has not yet been performed to confirm this. The high percentage of carrier offspring in this family (100% of those tested) is typical of the vertical transmission pattern seen with high infectivity (HBeAg +ve) carrier mothers in cases of human hepatitis B infection. Serology from Bobby Jean will be required to ultimately confirm Bobby Jean as the source of infection to her offspring. As four of the five carriers above are females involved in successful breeding programs, continued vertical transmission and perpetuation of infection is likely without preventive measures such as vaccination.

Jury’s father Ivan demonstrated evidence of previous exposure and subsequent recovery, but no HBV results are available for his mother Paula, who died in 1993. Given
that Jury has no other siblings, he was probably housed only with his parents until the
time of his arrival at Perth Zoo. Therefore his mother would appear to be the most likely
source of his infection. Alternatively, Jury may have been exposed to infection from his
father Ivan at the time of his acute infection, provided that Ivan’s infection occurred
prior to Jury’s.

In Ivan, HBV DNA could be detected and sequenced from the serum despite a lack of
detectable HBsAg and a serological pattern consistent with recovery from infection (anti-
HBC and anti-HBs positive). This demonstrates that low levels of HBV DNA can persist in
gibbons in cases of apparent recovery and in the presence of neutralising antibodies.
Heckel et al. (2001) has also demonstrated this finding in 11 of 23 nonhuman primate
samples (comprising two chimpanzees, three gorillas, two orang-utans and 16 gibbons)
which had the same serologic pattern as Ivan. These animals all had detectable levels of
DNA by nested PCR despite a lack of detectable HBsAg. Such cases also exist in human
HBV infections (Hollinger and Liang, 2001) although the significance of these cases is
yet to be determined. In human infections, researchers have been able to demonstrate
transmission and viral replication from such seronegative infections (Thiers et al. 1988,
Liang et al. 1990). Reactivation of HBV infection has been reported in patients with
serological evidence of recovery who have developed immunosuppression or undergone
chemotherapy (Thiers et al. 1988, Lok et al. 1991). It is thought that although
transmission has been demonstrated in the above scenarios, these occult HBV infections
are not likely to be clinically important except in the case of immunosuppression
(Hollinger and Liang, 2001). The levels of HBV DNA detected in the nonhuman primate
cases have been low and only detectable by very sensitive nested PCR assay, thus it is
probable that these individuals are unlikely to be infectious under normal circumstances.
It is not known if the virus in these animals would replicate to infective levels or
undergo reactivation if these animals also became immunosuppressed, however this
should be regarded as a possibility.
The presence of anti-HBc in Khusus is evidence that she was exposed to natural infection, however it is difficult to say whether infection occurred in the perinatal period as a result of vertical transmission, or via horizontal transmission from either Hecla or Jury in the following year. It would seem most likely that transmission was vertical, given Hecla’s high infectivity status; that stress associated with the birthing process may have enhanced shedding; and that trauma associated with the birthing process is conducive to the spread of the virus. Development of the chronic carrier status is a common feature in cases of neonatal infection, particularly vertical transmission. The lack of this response in Khusus is thought to be a result of protection attained by the vaccination process. Regardless of the origin of the infection, these results demonstrate that transmission of the virus is possible in the captive situation.

The lack of solid data about Horace makes implications on the epidemiology of his infection difficult. His chronic carrier status is suggestive of vertical transmission, therefore if he was truly wild caught, this would imply that the virus exists in the wild hoolock gibbon population and has done so prior to 1980. This is purely speculation, since Horace could also have become infected in his previous institution in Asia, or even during his time at Perth Zoo. As there are no currently held serum samples from Horace we cannot confirm that his hepatitis B infection is truly of gibbon origin rather than a human strain. However, given the current knowledge of this virus in nonhuman primates, and the lack of any confirmed zoonotic infections, it would seem likely that this infection represents a gibbon virus. Attempts have been made at obtaining HBV DNA from Horace’s fixed tissues but no success has yet been achieved. There are no published cases of hepatitis B infection in a hoolock gibbon. This would represent the first such case and demonstrates that animals within all four gibbon genera are susceptible to hepatitis B infection.
2.4.2 Pathogenicity

Chronic HBV carriers Uban and Perth 2 both died from systemic disease with liver involvement. Severe pathologic changes were seen in the livers of both animals, although the role of hepatitis B infection in these cases is disputed. One pathologist described histologic changes of cirrhosis in Uban, a finding consistent with chronic HBV infection in humans (Dienstag and Isselbacher, 2001b). However, examination of liver histopathology by a second pathologist working with cases of human hepatitis B cases, found no convincing evidence of pathology associated with chronic HBV infection. Similarly, histopathological examination of the liver of Perth 2 revealed changes that were consistent with chronic hepatitis B viral infection, however these changes were mild and could have been clinically silent. Review of these slides by a second pathologist failed to find evidence of hepatitis B-associated pathology. Specific stains applied to the fixed liver tissue of both animals failed to demonstrate the presence of HBsAg and anti-HBc.

The lack of definitive pathology associated with the chronic hepatitis B infections in Uban and Perth 2 does not exclude the possibility that the virus is pathogenic in silvery gibbons. A number of reasons may exist to explain the lack of definitive evidence of HBV infection on histologic examination of the tissues of Uban and Perth 2:

- Many cases of chronic HBV infection in humans occur without histologic changes in mild infections and in the early stages of more severe infection.
- In humans who are chronic HBV carriers, liver pathology associated with chronic hepatitis (e.g. cirrhosis and hepatocellular carcinoma) often does not occur until later in life, often 30 to 50 years post infection in cases of HCC (Dienstag and Isselbacher, 2001b). Gibbons can live to greater than 35 years (Rowe 1996), and Hecla’s mother is currently more than 44 years of age. It may have been too early in the course of the disease to see clinically evident changes in the livers of either of these animals.
In humans, certain genotypes and strains of HBV are known to be more pathogenic than others (Dienstag and Isselbacher, 2001b, Locarnini 2002). This may also be the case in silvery gibbons.

It is possible that the overwhelming histopathological changes associated with the systemic infections in both animals prevented mild changes resulting from viral infection from being detected.

The lack of reactivity to HBsAg and anti-HBc may be a result of variations in the genetic structure of the human and silvery gibbon strains of hepatitis B, rather than an absence of these substances within the gibbon hepatocytes. Lack of reactivity to these specific tests can also be seen in human cases of chronic HBV infection.

Whilst there is no overt evidence of liver pathology associated with chronic hepatitis B infection in either of these animals, it is possible that there is an indirect relationship between the viral infection and the death of these two animals, with the liver changes in both animals leading to a decreased immune response that may have made them more susceptible to systemic bacterial infections, such as the ones which caused their deaths.

In humans with chronic HBV infection there is a well known association between chronic hepatic disease and increased susceptibility to a variety of infectious diseases (personal communication D. Smith, May 2004).

Uban demonstrated moderate to marked elevations in liver enzymes (ALT and AST) on a number of occasions prior to his death, including a number of samples unrelated to his period of illness. Only a limited number of biochemistry results are available for Perth 2 but these also demonstrated mildly elevated serum ALT concentrations a year prior to death, as well as elevated AST, ALT and bilirubin concentrations on the day before her death. In both animals, ALKP concentrations remained in the normal range. Such elevations in serum aminotransferase concentrations, in conjunction with normal ALKP values, are consistent with chronic HBV infection in humans (Dienstag and Isselbacher,
2001b). It is difficult to say if these results were related to the hepatitis B infection, or purely a result of the systemic illness. However, given the acute nature of the illnesses in both animals, combined with the presence of elevated liver enzymes a significant period prior to their illness, it is possible that these enzyme elevations could be a result of chronic hepatitis B infection.

Hecla has not demonstrated any liver enzyme abnormalities despite numerous biochemical profiles being generated over the last 11 years. She has also not demonstrated any evidence of clinical illness. Hollinger and Liang (2001) report that human patients who acquire HBV infection in the perinatal period generally have low aminotransferase concentrations. This may explain the lack of enzyme elevation in Hecla, who was most likely infected at birth. Hecla’s age may also be a factor in the lack of liver enzyme changes. As noted previously, severe changes associated with chronic HBV infection in human often take 30-50 years to develop (Dienstag and Isselbacher, 2001b). The presence of strains with differing pathogenicity may also explain the lack of liver enzyme elevations.

In Jury, the presence of mild hepatomegaly, in combination with mildly elevated ALT concentrations on the three most recent blood tests, may indicate a low grade hepatopathy that could be a result of his chronic HBV carrier state. Chronic, persistent hepatitis in human patients with chronic HBV can present with little or no clinical signs, or mild hepatomegaly and modest elevations in aminotransferase activity (ALT, AST). Progressive disease often occurs in such cases however, and patients can go on to develop cirrhosis and hepatocellular carcinoma (Dienstag and Isselbacher, 2001b; Hollinger and Liang, 2001). Whilst there is currently no ultrasonographic evidence of liver pathology, it would be reasonable to expect progression of Jurys’ condition with time if his liver enzyme elevations were a result of the HBV infection. Liver biopsy would help to determine if there is any histological evidence of infection, however this invasive procedure has not been deemed clinically necessary in Jury at this stage.
Khusus demonstrated no clinical signs associated with her period of acute infection and has shown elevated serum concentrations of liver enzymes on only one occasion, at which time there was no other evidence of clinical illness. It is probable that a transient elevation in serum aminotransferase concentrations would have occurred during her acute infection, however this would likely have gone undetected due to a lack of sampling during the perinatal period.

No clinical illness or biochemical abnormalities have been detected in the three uninfected animals (Regina, Arjuna, Sinta).

Histological examination of the liver of the hoolock gibbon, Horace, revealed changes consistent with chronic persistent hepatitis, a pattern commonly seen in cases of chronic HBV infection in humans (Dienstag and Isselbacher, 2001a). This finding suggests that hepatitis B infection in hoolock gibbons can be associated with liver pathology similar to that seen in human infections. The reactivity of HBsAg and anti-HBc within the hepatocytes confirm his infection and demonstrate a similar pattern of liver infiltration with the virus to that seen in human cases of HBV infection. Given that pathology seen in human HBV is thought to be largely related to the immune response to the presence of HBV within the hepatocytes, it might also be expected that HBV infection in gibbons would play a similar pathologic role. The lack of immunohistochemical positivity to HBsAg, despite the positive reaction to Orcien stain (which also detects HBsAg), may reflect an antigenic difference between the human and gibbon strains. The fact that Horace displayed reactivity to both HBsAg and anti-HBc within the hepatocytes, whilst Uban and Perth 2 were both negative, may suggest that the virus in hoolock gibbons is genetically distinct to that in the silvery gibbons, and may be more closely related to human HBV. Sequencing of the virus in Horace would help to clarify this.
2.4.3 Prevention

It appears that the vaccination regimen used in Khusus was not successful in preventing infection with HBV, however it is difficult to say whether the presence of anti-HBs in her serum was the result of infection only, or a combination of infection and vaccination. Whilst there was serological evidence of infection, no clinical illness was demonstrated and the development of the carrier state was also prevented, implying that she does not pose a threat of viral transmission to other animals or humans.

Modified vaccination protocols used subsequently at Perth Zoo appear to have been successful in inducing immunity in Regina and Arjuna, with both animals demonstrating a serological pattern typical of vaccine-induced immunity (HBsAg –ve, anti-HBs +ve, anti-HBc –ve). It is anticipated that Sinta will also become immune, however confirmation of this will require further testing at a later date.

A detailed discussion of the vaccination protocols used in the above cases is contained in the following chapter.
Chapter 3: Vaccination of Silvery Gibbons (Hylobates moloch) Against Hepatitis B Virus at Perth Zoo

3.1 Introduction

Perth Zoo currently houses one of the few successful breeding colonies of silvery gibbons in the world and the only one in the Australasian region. As discussed in Chapter 2, both of the breeding adults at Perth Zoo are considered chronic carriers of HBV. The female Hecla is further classified as a high infectivity chronic carrier, due to the presence of HBeAg, and the male Jury is considered a low infectivity carrier (HBeAg –ve, anti-HBe +ve). Whilst transmission studies of the disease in nonhuman primates have been limited, previous studies have demonstrated a similar perinatal transmission pattern to that of HBV in humans (Zuckerman et al. 1978, Lanford et al. 1998, Noppornpanth et al. 2003). Vertical transmission in the perinatal period in humans is associated with a 90% transmission rate of the virus to the offspring, as well as a 90% chance of developing chronic infection in those infected in the perinatal period (Dienstag and Isselbacher, 2001b, Hollinger and Liang, 2001).

Based on this information, Perth Zoo was concerned that there would be a high probability that offspring born to this silvery gibbon breeding pair might become infected with the virus, develop chronic infection and become carriers, and potentially develop liver pathology associated with the infection in later life. Given the critically endangered status of the species however, and the obvious compatibility of the pair, it was decided to continue breeding these animals. A vaccination program to immunise newborn silvery gibbons against HBV was developed with the help of human virologists, to reduce the risk of transmission of the disease to future offspring.
In this chapter the development of a hepatitis B vaccination program for silvery gibbons at Perth Zoo over the last nine years is discussed and the efficacy of this program in providing protection against HBV infection is assessed.

### 3.2 Materials and Methods

Based on discussions with human virologists, it was initially recommended that newborn gibbons should be vaccinated within 24-72 hrs of birth with a combination of 0.5 ml of recombinant hepatitis B vaccine (Energix-B® SmithKline Beecham Biologicals, B-1330 Rixensart, Belgium) and 0.3 ml HBV Immunoglobulin (HBIG) (100 IU/ml). Both vaccine and HBIG were to be administered by intramuscular injection at separate sites. Booster vaccinations of 0.5 ml Energix-B® vaccine administered intramuscularly were to be given at four and twelve months of age. After the initial course of vaccination, opportunistic boosters of 0.5 ml Energix-B® vaccine intramuscularly were to be given approximately every five years or when anti-HBs titres dropped to <10 mIU/ml. A summary of the vaccination regimen used is contained in Table 3.1.

**Khusus** - The first offspring, Khusus, was vaccinated in accordance with the above protocol, receiving the recommended vaccination of Energix-B® and HBIG 24 hrs after birth, and a booster of Energix-B® at four months of age. The second booster was delayed from the recommended 12 months of age and given at 15 months due to management reasons.

**Regina** - In view of the lack of an ideal response in the results obtained from Khusus, a revised vaccination schedule of initial vaccination with 0.5 ml Energix-B® and 0.1 ml HBIG at 72 hrs after birth, followed by boosters of 0.5 ml Energix-B® one and six months later was employed for the second offspring in the program, Regina. Due to management and animal factors, the first booster was delayed to six weeks of age and
the second booster was given at five months of age. An opportunistic third booster was given at four and a half years of age.

**Arjuna** - The vaccination regimen was modified further for the third offspring in the program Arjuna, who was vaccinated with 0.5 ml Energix B® vaccine and 0.1 ml HBIG at 72 hrs after birth, and with a single booster of 0.5 ml Energix B® vaccine given at eight months of age. An opportunistic booster of 0.5 ml Energix B® was given two years after the initial vaccination.

**Sinta** - The above vaccination regimen used for Arjuna was repeated for Sinta, the fourth offspring in the program (0.5 ml Energix B® vaccine and 0.1 ml HBIG at 72 hrs after birth, and a single booster of 0.5 ml Energix B® vaccine given at eight months of age).

Anaesthesia of the dam Hecla, was performed at the time of initial vaccination of Regina, Arjuna and Sinta to reduce the risk of maternal rejection of the offspring. Anaesthesia of the dam was also required for the first booster of Khusus and the first and second boosters of Regina. Administration of the second booster to Khusus was performed while she was under anaesthesia to investigate signs of lameness. Physical separation of the offspring and Hecla for the booster vaccinations of Arjuna and Sinta allowed vaccination to be performed without the need for anaesthesia.

Serum was collected from each individual under general anaesthesia at opportunistic occasions after the initial course of vaccination in order to assess the success of the vaccination protocol. The serological markers HBsAg and anti-HBc were used to detect evidence of natural infection and anti-HBs titres were measured to determine the level of protective immunity produced by vaccination (see Materials and Methods 2.2)
Veterinary and keeping staff involved in husbandry and handling of the animals were required to have adequate immunity to hepatitis B, as demonstrated by a protective anti-HBs titre (>10 mIU/ml) on serological testing. Staff with negative anti-HBs titres, or titres below the protective level, were required to undergo an immunisation program and were prevented from working with the silvery gibbons until a protective titre had been achieved. Face masks and gloves were required to be worn during all procedures involving physical contact with the animals.

A serological survey of staff was also undertaken to determine if any staff members had been exposed to hepatitis B or were carriers of the virus. The survey involved 36 people and included staff working with the silvery gibbon colony and other nonhuman primates at Perth Zoo, as well as laboratory staff involved in the processing of blood and tissue samples from nonhuman primates at Perth Zoo.

<table>
<thead>
<tr>
<th>Offspring</th>
<th>1st vacc (HBIG, Energix-B®)</th>
<th>2nd vacc (Energix-B®)</th>
<th>3rd vacc (Energix-B®)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khusus</td>
<td>24 hr</td>
<td>4 mths</td>
<td>15 mths</td>
</tr>
<tr>
<td>Regina</td>
<td>72 hr</td>
<td>6 wks</td>
<td>5 mths</td>
</tr>
<tr>
<td>Arjuna</td>
<td>72 hr</td>
<td>8 mths</td>
<td>-</td>
</tr>
<tr>
<td>Sinta</td>
<td>72 hr</td>
<td>8 mths</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1: Hepatitis B vaccination regimen at Perth Zoo
3.3 Results

**Khusus** - Blood testing of Khusus at the time of the second booster demonstrated the presence of anti-HBs and anti-HBc, however she was negative for HBsAg (Table 3.2). This serological response is consistent with immunity to HBV, however the presence of anti-HBc indicates that natural infection with HBV had occurred. The lack of HBsAg and the presence of anti-HBs indicates that Khusus is not a carrier. An anti-HBs titre of >150 mIU/ml at this time is consistent with human and chimpanzee standards of protective immunity (>10 mIU/ml) (Davis et al. 1996, Terrault and Wright, 1998, Hollinger and Liang, 2001). Repeat blood testing five years after the initial vaccination demonstrated an anti-HBs titre >1000 mIU/ml, well in excess of protective levels. Transfer of this animal to another institution shortly after this time meant that further testing could not be performed.

**Regina** - Blood testing of Regina one year after the second booster demonstrated the presence of anti-HBs and the absence of anti-HBc and HBsAg (Table 3.2). This serological response is consistent with vaccine-induced immunity. A protective anti-HBs titre of >150 mIU/ml was found at this time. Serum from Regina was collected at the time of the third booster, 4.5 years after the initial vaccination and the immunity had been maintained (anti-HBs 270 mIU/ml). Serum collected 14 months after this third booster showed a further increase in protective titre to >1000 mIU/ml.

**Arjuna** - The presence of anti-HBs and the absence of anti-HBc and HBsAg on serology performed at two years after the initial vaccination demonstrated that Arjuna had also developed a serological response consistent with vaccine-induced immunity and had no evidence of exposure to the virus (Table 3.2). His anti-HBs titre of 218 mIU/ml was considered protective based on human and chimpanzee standards.
Table 3.2  Summary of vaccination regimen at Perth Zoo

<table>
<thead>
<tr>
<th>Offspring</th>
<th>1st vacc (HBIG, Energix-B®)</th>
<th>2nd vacc (Energix-B®)</th>
<th>3rd vacc (Energix-B®)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khusus</td>
<td>24 hr</td>
<td>4 mths</td>
<td>15 mths</td>
<td>HBsAg -ve, anti-HBc +ve, anti-HBs +ve (&gt;10 mIU/ml). Therefore immune but evidence of natural exposure (exposure immunity). Immunity maintained at 15 months (&gt;150 mIU/ml) and 5 years after initial vaccination (&gt;1000 mIU/ml). Opportunistic booster at 5 years.</td>
</tr>
<tr>
<td>Regina</td>
<td>72 hr</td>
<td>6 wks</td>
<td>5 mths</td>
<td>HBsAg -ve, anti-HBc -ve, anti-HBs +ve (&gt;10 mIU/ml). Therefore apparent vaccine-induced immunity and no evidence of infection. Immunity maintained at 4.5 years after initial vaccination (270 mIU/ml). Opportunistic booster given at 4.5 years and anti-HBs increased to &gt;1000 mIU/ml 1.5 years after booster.</td>
</tr>
<tr>
<td>Arjuna</td>
<td>72 hr</td>
<td>8 mths</td>
<td>-</td>
<td>HBsAg -ve, anti-HBc -ve, anti-HBs +ve (&gt;10 mIU/ml). Therefore apparent vaccine-induced immunity and no evidence of infection. Immunity maintained at least 2 years after initial vaccination (218 mIU/ml). Opportunistic booster at 2 years.</td>
</tr>
<tr>
<td>Sinta</td>
<td>72 hr</td>
<td>8 mths</td>
<td>-</td>
<td>No results available.</td>
</tr>
</tbody>
</table>

**Sinta** - Serological testing has not been performed for the fourth offspring Sinta at this stage.

The serological survey of staff revealed that of the 36 people tested, two had serological evidence of previous exposure to HBV. Both of these people were laboratory staff, neither of whom had had direct contact with infected gibbons, nor with their blood products. These results suggest that the gibbon HBV is not highly transmissible to humans through routine husbandry and handling of infected gibbons.
3.4 Discussion

The recommended regimen for human infants born to HBeAg +ve mothers is to vaccinate with HBIG and Energix-B® at birth, followed by booster vaccinations of Energix-B® at one and two months of age. The purpose of the immunoglobulin administered with the initial vaccination is to provide passive transfer of immunity and protection against potential viral infection and disease resulting from exposure during the birthing process. Delayed administration of HBIG beyond 72 hrs after exposure is associated with significantly decreased efficacy in humans (Hollinger and Liang, 2001). Previous trials in humans have shown that variations from the ideal schedule of vaccination at 0, 1 and 2 months for newborns and 0, 1 and 6 months for adults can still provide adequate levels of protective immunity (Hollinger and Liang, 2001).

The vaccination schedule developed for the silvery gibbon newborns was designed to provide maximum protection with minimal intervention during the critical bonding period between mother and newborn. Timing of the initial vaccination and HBIG administration was deemed to be critical to the success of vaccination, and in all of the protocols used both drugs were to be administered within 72 hrs of birth. Timing of booster doses was designed to allow a degree of maternal bonding to occur before intervention for the vaccination. Recommended dose rates were extrapolated from human dose rates.

The serological results from Khusus (anti-HBc +ve, anti-HBs +ve and HBsAg -ve) were consistent with immunity and previous natural infection with HBV. As the vaccine contains surface antigen only, the presence of antibodies to the core antigen indicates that the animal had been exposed to actual virus. It is not possible from these results to determine if the anti-HBs resulted from exposure to vaccine, virus or a combination of both. Nevertheless, the presence of an anti-HBs titre greater than 10 mIU/ml (>150 mIU/ml at 15 months after initial vaccination, and >1000 mIU/ml at five years) is indicative of protective immunity.
It is unclear why vaccination of Khusus did not prevent infection, given that she received a higher dose of HBIG and was given her first vaccination within 24 hrs of birth. Some possible explanations include:

- Khusus may have fallen in the 5% of newborns vaccinated with this regimen that do not develop immunity (Hollinger and Liang, 2001).
- Khusus may have acquired the infection in utero, as is thought to occur in 10% of human neonates born to HBeAg +ve mothers (Dienstag and Isselbacher, 2001a, Hollinger and Liang, 2001). Therefore she would have already been infected at the time she received the HBIG, making the HBIG ineffective.
- As Khusus was the first offspring born to this pair, there may have been more trauma associated with the birthing process and thus a greater chance of viral exposure during this period.
- Khusus may have acquired the infection by horizontal transmission shortly after birth, in the small window of time when passive immunity from HBIG had diminished and active immunity from vaccination had not yet developed.
- There may have been quality issues with either the vaccine or HBIG making them less efficacious.

Despite the fact that Khusus appeared to have been naturally infected, the lack of HBsAg in combination with the presence of anti-HBs implies that she is not a carrier of the virus and therefore does not pose a transmission risk to other nonhuman primates or humans. As the aims of the vaccination protocol were to provide immunity and prevent the development of the carrier state, vaccination of Khusus appears to have been successful in this respect, although the precise role of vaccination in achieving this outcome is undetermined.

Whilst Khusus did not develop clinical signs associated with her hepatitis B infection, it was still deemed undesirable to allow natural infection to occur and therefore the
vaccination schedule for Regina was modified from the original schedule to prevent this. A revised schedule of initial vaccination with 0.5 ml Energix-B® and 0.1 ml HBIG at 24-72 hrs after birth, followed by boosters of 0.5 ml Energix-B® at one and six months of age was advised, in line with the schedule used in adult humans (Terrault and Wright, 1998; Hollinger and Liang, 2001). This modified regimen was successful in producing immunity with no evidence of natural exposure (anti-HBs +ve, anti-HBc -ve, HBsAg -ve) and with a protective anti-HBs titre of >150 mIU/ml.

The regimen used on Regina was successful in terms of serological response, however there were difficulties encountered in the darting process required to administer both boosters. Following discussions with human virologists, it was decided to delay the initial booster for Arjuna (given to Regina at 6 weeks) until six months of age for management reasons. It was recognised that administration of the first vaccination and the HBIG within 72 hrs of birth was of vital importance to the success of the regimen and thus this was maintained in the vaccination program, despite the inherent risks of maternal rejection. Delaying the first booster meant that anaesthesia at this time may not have been required. The second booster was to be given at any opportunistic time greater than two months after the first booster. The first booster was delayed a further two months and was given at eight months of age, however serological testing two years after the initial vaccination indicated that Arjuna had a serological response typical of vaccine-induced immunity, with a protective anti-HBs titre of 218 mIU/ml.

Despite the fact that this vaccine regimen deviated from the recommended schedule, it was still successful in all regards, and the timing of the booster meant that anaesthesia of mother or offspring was not required. All vaccinations given prior to this age had required general anaesthesia of the dam to allow access to the offspring and to ensure good acceptance of the offspring when returned to the dam. Given this ideal result, the same schedule was repeated on Sinta, however serological testing has not yet been performed to determine if the results have been repeated.
The levels of anti-HBs achieved by vaccination in Khusus, Regina and Arjuna were well above the recommended levels for protection in both humans and chimpanzees. It is not known at this stage how these levels correlate to protection against viral challenge in gibbons, however it would appear that those achieved in Regina and Arjuna are above the protective level as evidenced by their lack of infection in a high risk environment. The minimum level in these animals was 150 mIU/ml, and thus levels above this should be considered protective in gibbons. Nevertheless, levels above 10 mIU/ml may well be protective in gibbons as they are in humans and chimpanzees (Davis et al. 1996, Terrault and Wright, 1998, Hollinger and Liang, 2001).

Although the number of animals in this study is low, it appears that the modified regimen, involving vaccination at 24-72 hrs with 0.5 ml Energix-B® vaccine intramuscularly and 0.1 ml HBV Immunoglobulin (100IU/ml) intramuscularly (at separate sites), followed by a booster vaccination of 0.5 ml Energix-B® vaccine intramuscularly at eight months of age, is successful in providing a serological response consistent with vaccine-induced immunity. It is recommended that this vaccination regimen be used for any future offspring born to this pair of silvery gibbons at Perth Zoo.

The need for further boosters beyond the initial regimen is undetermined. Booster vaccinations in humans were previously recommended at five and ten years after the initial course. More recently this booster dose is no longer recommended except in immune compromised people (World Health Organisation, 2003). The length of protection provided by the current vaccination regimen in the gibbons is undetermined. Virologists have therefore recommended that anti-HBs titres are measured every five years, on an opportunistic basis, in order to determine the duration of immunity. Repeat boosters of Energix-B® vaccine can be administered if levels of anti-HBs have been decreased below 10 mIU/ml.
Whilst the programme has been successful, there are inherent risks to the dam, offspring and staff associated with the vaccination procedures, especially when darting and general anaesthesia are involved. Among these risks is the very real risk of maternal rejection at the time of the first vaccination. At Perth Zoo we have found that the best method for administering the initial vaccination and HBIG is to perform the procedure as close to 72 hrs after birth as possible, anaesthetise the dam, administer the vaccine and HBIG to the newborn, then allow the newborn to suckle from the dam during her recovery under minimal staff supervision. This method allows for a full physical examination of both mother and offspring. The lack of overt supervision during the recovery process has been associated with good maternal acceptance of the offspring.

A number of unsuccessful attempts have been made at blood collection from the offspring for serology at the time of this vaccination. In view of the small size of the offspring (approximately 350-400g at birth), and the relatively large volume of blood required for testing (200µL of serum per parameter tested), blood collection for detection of HBV infection associated with in utero transmission is no longer performed.

Administering the initial booster at the age of eight months has allowed the procedure to be performed using only minimal physical restraint of the offspring after separation from the mother, rather than under general anaesthesia. The benefit of this is that the procedure is much quicker and the maternal bond much greater by this age such that rejection is unlikely to occur. However, the degree of restraint involved precludes blood collection at this time and therefore serological response to the primary vaccination cannot be assessed at this point. As HBIG administration is not required at this time, the timing of this first booster is less critical and therefore the procedure can be easily aborted and attempted again at a later date if separation is not easily managed.
Despite the risks involved, the benefits of the vaccination procedure in successfully establishing vaccine-induced immunity in silvery gibbon offspring at Perth Zoo was deemed to outweigh the risks involved. The development of behavioural training programs to enable hand administration of anaesthetics, and potentially to allow handling of the newborn offspring without the need for anaesthesia of the dam, would certainly help further reduce the risks involved with these procedures.

The effectiveness of vaccination of exotic species has often been questioned, as it is difficult to ascertain the extent of true protection afforded by vaccines that have been designed for other species (in this case, humans). This concern is certainly applicable to this situation, and whether these animals are truly “immune” to infection is debatable. In a critically endangered species such as the silvery gibbon however, we do not have the luxury of being able to run challenge tests to determine whether or not the immunity is truly protective. Nevertheless, based on work in humans and chimpanzees (Davis et al. 1996, Terrault and Wright, 1998, Hollinger and Liang, 2001), an anti-HBs titre of >10 mIU/ml is considered protective for challenge with HBV. In all cases in the Perth Zoo study, post vaccination anti-HBs titres were well in excess of 10 mIU/ml and have been maintained for a number of years. The fact that Khusus showed evidence of exposure-induced immunity demonstrates that the virus is transmissible in the captive situation, and as such there is a real risk of infection to all future offspring. Neither Regina or Arjuna have shown evidence of exposure despite living in very close contact with two chronic HBV carriers, suggesting that vaccination has provided true protective immunity in both of these animals. Serum samples collected from Hecla at the time of vaccinating Sinta have confirmed that Hecla is still a high infectivity carrier and would therefore be expected to pose a high risk of vertical transmission all offspring.

Given all of the above, it would appear that the vaccination program at Perth Zoo has been successful in producing immunity to HBV and in preventing the development of chronic infection in all four offspring. In addition, vaccination has been successful in
preventing infection in Regina and Arjuna in the presence of a high risk of exposure both vertically in the perinatal period, and horizontally during infancy from both Hecla and Jury. It is hoped that results from Sinta will be available in late 2004.

Hepatitis B vaccination regimens used at other institutions, as discussed in the literature review, have involved vaccination of subadult and adult animals. Thornton et al. (2001) and Mootnick (personal communication, March 2004) appear to have been successful in vaccinating gibbons (H. leucogenys and H. agilis respectively) using Energix-B®. Thornton et al. (2001) vaccinated adult animals at 0, 1, 2 and 12 months and achieved anti-HBs titres of > 100 mIU/litre, which they referred to as protective. Vaccination of a juvenile (12 weeks) was also attempted, however the animal died before booster vaccinations could be administered. The authors do not mention doses used and the units of anti-HBs are misleading, making comparison to the Perth Zoo regimen difficult.

Mootnick (personal communication, March 2004) vaccinated Agile Gibbons (H. agilis) of one, two and four years of age at the Gibbon Conservation Centre (GCC) in California, USA. Gibbons were vaccinated with 0.5ml of Energix-B® using a three vaccination regimen (0, 1 and 5 months). Anti-HBs titres measured one year after the second booster demonstrated protective titres (>10 mIU/ml). This regimen was used to provide immunity to HBV prior to introduction to chronic carrier animals, and therefore to protect against horizontal transmission. To date, only a small number of animals have been vaccinated, however protective titres (based on the chimpanzee and human standards) have been achieved on each occasion.

The doses used by Mootnick are the same as those used at Perth Zoo, and would suggest that 0.5ml of Energix-B® (the human paediatric dose) produces good immunity in gibbons when used in a multi-dose regimen. It should however be noted that all animals involved in these vaccinations have been subadults of less than five years of
age. Further work is required to determine the appropriate dose and regimen to vaccinate adult gibbons against HBV.

Orang-utan and gorilla HBV vaccination studies (Davis et al. 2000, Thornton et al. 2001) suggest that these species may be less responsive to vaccination with Energix-B®. Higher doses, additional boosters, and possibly the use of adjuvants, may be required to achieve protective anti-HBs titres in these species.

The regimen used at Perth Zoo appears to provide protection against potential vertical and horizontal transmission of HBV in a situation where the risk of transmission is high. The suitability of this vaccination regimen for other institutions would depend on the carrier status and number of the animals, and possibly the species involved, however it is hoped that the results of this trial will be of use to other institutions housing HBV-positive primates, including rehabilitation centres. The pertinent use of vaccination strategies such as the one described could aid in the release of endangered primates into the wild, which may not have otherwise been possible.
Chapter 4: Sequencing of the Hepatitis B Virus From Silvery Gibbons

4.1 Introduction

Hepatitis B virus has been identified in a number of gibbon species (Mimms et al. 1993, Norder et al. 1996, Grethe et al. 2000, Lanford et al. 2000, Heckel et al. 2001, Aiba et al. 2003, Noppompanth et al. 2003, Starkman et al. 2003). DNA sequence data obtained to date has confirmed the presence of gibbon-specific HBV strains, designated GiHBV.

There are currently no published sequences of the entire HBV genome from a silvery gibbon. Analysis of partial viral sequences obtained from silvery gibbons suggests that they carry a gibbon-specific variant, most likely GiHBV. According to the definition set by Okamoto et al. (1988), sequence analysis involving the entire genome is required to determine the HBV genotype, and therefore is necessary to confirm that the virus found in silvery gibbons is a nonhuman primate-specific strain and not of human origin.

This chapter describes the successful sequencing of the entire HBV genome obtained from Perth Zoo gibbon Hecla. This work represents the first complete genomic sequence of hepatitis B from a silvery gibbon to be entered into GenBank, the international genomic database. Partial sequencing of the viral genome from Uban is also described, and the results of phylogenetic analysis of both of these sequences are discussed.

Previously, evidence of HBV infection has been demonstrated in five animals from the silvery gibbon species (H. moloch), with three of these animals identified as chronic HBV carriers (Grethe et al. 2000, Lanford et al. 2000). In their study, Lanford et al. (2000) performed partial sequencing on the surface and core regions of the HBV genome from silvery gibbons Chloe and Chilibi, both siblings of Hecla, and a third gibbon Ling. Grethe et al. (2000) sequenced a further two HBV genomic regions from Ivan, the father of
Perth Zoo silvery gibbon, Jury. In 2003, Starkman et al. obtained the entire HBV genomic sequence from TB Black, a gibbon identified as belonging to the Hylobates genus, most likely belonging to the species agilis or moloch. Speciation of TB Black was attempted using sequencing of the mitochondrial 12S region, however the species of gibbon could not be confirmed.

Serum was available from three silvery gibbons in the Perth Zoo collection that were all identified as chronic carriers of HBV (Uban, Hecla and Jury). Based on a review of the histories of these animals (Chapter 2), it seemed likely that all three were infected from different sources and therefore might carry three different viral strains, possibly related to the partial silvery gibbon sequences obtained previously. Comparison of sequences from these related animals may help to confirm suspected sources of infection and therefore provide information on the epidemiology of HBV in silvery gibbons.

4.2 Materials and Methods

Blood from Uban, Hecla and Jury was collected under general anaesthesia during routine physical examinations performed on different occasions over the past 12 years. After centrifugation using a Hettich Universal 1200 centrifuge at 5000 rpm for 15 minutes, serum was separated and sent to the Division of Microbiology and Infectious Diseases, PathCentre for hepatitis B testing. Any remaining serum was held at -80°C for serum banking purposes.

To confirm the presence of HBV infection and the level of infectivity, hepatitis B serology was performed using the Abbott AxSYM microparticle enzyme immunoassay (MEIA) system as described in section 2.2 of this thesis. An in-house PCR assay was used to detect HBV DNA using the banked serum samples.
Sequencing of the virus recovered from all 3 gibbons by PCR was attempted at two institutions: the Western Australian Centre for Pathology & Medical Research (PathCentre) in Perth, and the Fiji School of Medicine in Fiji. Full genome sequences from Hecla were obtained at both institutions, whilst sequencing of the virus from Uban was only successful in Perth. Neither institution was able to successfully sequence viral DNA from Jury.

Sequencing at the Western Australian Centre for Pathology & Medical Research (PathCentre) - Hecla and Uban

Viral DNA was extracted from the serum samples using the QIAamp® Viral RNA Mini Kit (QIAGEN, Doncaster, Vic) according to the manufacturer’s instructions. The kit is suitable for extracting both DNA and RNA. Extracted HBV DNA was then amplified by PCR using a hot start TaqGold DNA Polymerase (Applied Biosystems Division, Foster City, CA) and a combination of primers from the table below (8 µL of DNA were added to 12 µL of a mix of forward and reverse primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM MgCl2, 200 µM deoxynucleoside triphosphate (dNTP) mix and 0.75 units of Taq DNA polymerase). Samples were preheated for 10 minutes at 95°C to activate the enzyme and then cycled for 15 seconds at 95°C (denaturation), annealing for 30 seconds at 50-60°C (depending on the primer combination), and extension for two minutes at 72°C for 30 rounds of amplification. Samples were run in duplicate, with one set of PCR products run on a 1.8% agarose gel and stained with ethidium bromide to detect fragments.

Purified PCR products were subjected to direct sequencing in both directions using the Applied Biosystems PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems Division, Foster City, CA) using primers from Table 4.1. 2.5 µL of the sequencing PCR template was added to 7.5 µL of the sequencing mix (1.0 µL reaction mix (v3.1), 1.5 µL buffer x5 (ABI), 3.2 µL primer (1 µM), 1.8 µL H2O). Samples were then thermocycled at 95°C for 10 seconds and 60°C for 30 seconds for a
total of 45 cycles. After cycling, 10 µL of sequence reaction product was added to 490 µL diethyl pyrocarbonated water (DEPCH₂O) through a Microcon-PCR (Millipore) filter and centrifuged for 15 minutes at 2900 rpm. Filters were then inverted and placed in a new collection tube to which 10 µL of DEPCH₂O was added. Samples were then centrifuged for a further two minutes at 2900 rpm. 20 µL of Formamide was added to the purified sequencing product, vortexed, and heated at 95°C for four minutes, then briefly centrifuged. 20 µL of the product was transferred to an ABI optical well and loaded into the ABI PRISM Genetic Analyzer. Sequences of amplified DNA were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) in the case of Hecla, and an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) in the case of Uban.

**Sequencing at Fiji School of Medicine - Hecla**

Viral DNA was extracted using the High Pure PCR Template Purification Preparation Kit (Roche Applied Science, Indianapolis, IN) as per the manufacturer’s instructions with the addition of Poly A (catalogue number 108 626 Roche Diagnostics) to enhance HBV extraction. 40 µL of Poly A (5 mg/ml) was added to 2.5 ml of binding buffer to create a working extraction solution. 200 µL of this working extraction solution was then added to 200 µL of serum and 40 µL of Proteinase K. Amplification of the entire genome was performed using the Taq Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN), with subsequent internal amplifications performed using standard Taq DNA Polymerase (Roche Applied Science, Indianapolis, IN) conditions according to the manufacturer’s protocols. Primers were selected from Table 4.1. The entire genome amplification was performed using primers 1877F and 1849R. Initial denaturation was performed at 94°C for two minutes. Samples were then cycled for 30 seconds at 94°C, annealing for 30 seconds at 65°C (decreasing by 1°C/cycle until 55°C), and extension at 68°C for three minutes for the first nine cycles. Extension times were then increased by five seconds per cycle for to a maximum two minutes over a further 20-30 cycles, as per the manufacturer’s protocol. Final extension was conducted at 68°C for five minutes.
Table 4.1: Primers used in sequencing for silvery gibbon hepatitis B virus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Cycle °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB0050R*</td>
<td>TGGAGCCACC AGCAGGRAAR TA</td>
<td>66</td>
</tr>
<tr>
<td>HB0400F*</td>
<td>TCTCTTCGCA TTCTCCGTCT ATG</td>
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</tr>
<tr>
<td>GH0547R*</td>
<td>CAGCAACATT AGGGATACAA AGAG</td>
<td>60</td>
</tr>
<tr>
<td>HB0680R*</td>
<td>CACCTGAGCA AATGCRCCTA GTAAACTGA</td>
<td>76</td>
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<tr>
<td>GH0780F*</td>
<td>GAGATCCCTT TATACCCGCTG TT</td>
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<td>GH0997R*</td>
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<tr>
<td>HB1065F*</td>
<td>CCCCCCATCR CAATGTGGNT ATC</td>
<td>66</td>
</tr>
<tr>
<td>HB1285R</td>
<td>GAGATCCGCA AGTATGGATC G</td>
<td>66</td>
</tr>
<tr>
<td>HB1324F*</td>
<td>CGATCCATA CCGGAAACTC</td>
<td>62</td>
</tr>
<tr>
<td>HB1609g</td>
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* Primers used at Sir Charles Gardiner Hospital
f Primers used at Fiji School of Medicine

Internal amplifications were performed as per the manufacturer's protocol as described for the Taq DNA polymerase (Roche Applied Science, Indianapolis, IN) in the previous section. Annealing temperatures ranged from 50-70°C (depending on the primer combination) for 30 seconds. Extension times of 45 seconds were used for fragments up to 1 Kb and one minute for fragments up to 1.5 Kb. Purified PCR products were subjected to direct sequencing in both directions using the ABI PRISM™ BigDye™
Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems Division, Foster City, CA) as described previously. Sequences of amplified DNA were determined using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The sequence of the complete viral genome was obtained after alignment of the smaller fragments using BioEdit Sequence Alignment Editor Version 5.0.9 (Hall, 1999) software. Multiple overlapping fragments of the genome were used such that each area of the genome was covered by a minimum of three sequences and a consensus sequence obtained.

Phylogenetic analysis was performed on multiple sequences aligned using the Clustal X program (Thompson et al. 1997). Subsequent analysis was performed using programs within the PAUP* package version 4 (Swofford D. L. 2003). Bootstrap analysis was performed using 1000 data sets. Phylogenetic trees were constructed using the Neighbour joining method within PAUP*.

Sequences used for phylogenetic analysis were obtained from the GenBank database. The isolate accession numbers and names (in parentheses) are listed below:

**Genotype A:** X70185 (HBVXCPS), S50225, X51970 (HVHEPB)  
**Genotype B:** AF121249, AF282918, AY033073  
**Genotype C:** AB112472, AF233236, X75665 (HHVBC)  
**Genotype D:** AF121240, AF280817, AJ344116 (HEBVBC)  
**Genotype E:** AB091255, AB106564, X75657 (HHVBBAS)  
**Genotype F:** AB036905, AF223962, X75658 (HHVBFFOU)  
**Genotype G:** AB056514, AB064310, AF160501  
**Genotype H:** AY090454, AY090457, AY090460  
**Gibbon complete genomes:** AY330913 (Tamang), AY330914 (Wendy), AY330915 (Happy), AY330916 (TB Black), AY330917 (Crazy Woman), AB037927 (Makiko), AB037928 (Yohko), U46935 (Hope), HBV131568 (gib824), HBV131569 (gib759), HBV131571 (gib645), HBV131572 (gib160), HBV131573 (gib153), AY077735 (G25), AY077736 (G26), BG21*, G12*, G11_14*, G11_15*, G11_16*, G11_17*, G11_11*, G11_8*, G19*, MGI*, SG_III*  
**Gibbon Surface genes:** AF213005 (Ling),
AF213006 (Chilibi), AF213007 (Pepino), AF274495 (G3), AF274496 (G9), AF274499 (G28), AF2745378 (G32), AF477482 (ChmiC14), AF477483 (GomezC14), AF477484 (JackoR4), AF477485 (JockC20), AF477486 (MidnightR27), AF477487 (NongchaiC16), AF477488 (SaanC13), AF477489 (SabooC15), AF477490 (NiC15), AF477491 (TaoC15), AF477492 (PokC2), AF477493 (JiebR6), AF477494 (BabyR6) Gibbon core genes:
AF213008 (Chilibi), AF213009 (Ling), AF213010 (Pepino) Chimpanzee complete genomes: AY330911 (Louisa), AY330912 (Osang), AF498266 (Chimp FG), AF305327 (CB0376), AF242585 (Chimp_2), AF242586 (Chimp_4), AF222322 (Ch109), AF222323 (Ch926), AB046525 (Bassi), AB032431 (Ch195), AB032432 (Ch256), AB032433 (Ch258), HBV131575 (chimp82), D00220 (HPBVCG) Chimpanzee Surface genes:
AF222311 (NIH28), AF222312 (NIH29), AF222313 (NIH39), AF222314 (NIH40), AF222315 (NIH44), AF222316 (NIH56), AF222317 (NIH57), AF222318 (NIH800), AF222319 (NIH814), AF222320 (NIH821), AF222321 (NIH904), AF305326 (CB0031), AF305328 (CH116), AF305329 (CH1435), AF305330 (CH1436) Orang-utan complete genomes: AF193863 (Somad), AF193864 (Papa) Orang-utan Surface genes: HBVY17559 (Doel), HBVY17560 (Somad), HBVY17561 (Romeo), HBVY17562 (O’on), HBVY17563 (Papa), HBVY17564 (Lisa), HBVY17565 (Mojo) Gorilla complete genome: HBV131567 (gor97) Woolly Monkey complete genomes: AF046996 (WMHBV), AY226578 (WMHBV-2).

* Not submitted to GenBank

### 4.3 Results

The results of sequencing of the entire genome from Hecla reveal that the virus she carries is most closely related to virus from AY330916 (TB Black) (data not shown), an animal identified as belonging to either the H. moloch or H. agilis species (Starkman et al. 2003), with a nucleotide divergence of 6.2%. Table 4.2 demonstrates that Hecla’s HBV sequence showed greatest similarity (i.e. lowest nucleotide divergence) to virus found in gibbons as a group (6.9%). Within the gibbon group her virus showed highest similarity to those seen in other species of gibbons from the Hylobates genus (H. agilis
Table 4.2: Unweighted nucleotide distances between representatives of all known human HBV genotypes, and all currently available full length HBV genomes from nonhuman primates.

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<th>H.lar (n=3)</th>
<th>N. leucogenys (n=1)</th>
<th>N. concolor (n=2)</th>
<th>N. gabriellae (n=2)</th>
<th>GiHBV (n=15)</th>
<th>OuHV (n=2)</th>
<th>GoHBV (n=1)</th>
<th>ChHBV (n=14)</th>
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6.8%, H. lar 6.7%, H. pileatus 7.2%). Hecla’s strain of HBV is also closely related to the virus found in gibbons within the Nomascus genus (N. concolor 7.1%, N. gabriellae 7.4%, N. leucogenys 7.4%) and HBV from the Bornean orang-utan (P. pygmaeus pygmaeus 7.3%). Hecla’s virus demonstrates >8% nucleotide similarity to published chimpanzee (9.3%), gorilla (9.1%) and woolly monkey (21.6%) HBV sequences, and to all published human HBV genotypes (A 9.7%, B 9.9%, C 9.4%, D 11%, E 10.2%, F 12.5%, G 11.6%, H 12.6%).

Phylogenetic analysis was performed on the entire genomic sequence obtained from Hecla. The sequence was compared to all other published entire HBV genomes from nonhuman primates in GenBank (26 gibbon, 14 chimpanzee, two orang-utan, one gorilla and two woolly monkey sequences), and three representative genomes from each of the eight currently recognised human HBV genotypes (A-H) (Figure 4.1). The two published woolly monkey sequences were used as an outgroup, due to their demonstrated dissimilarity from other human and nonhuman primate HBV sequences.

Hecla’s viral sequence consistently clustered among the gibbon viral sequences, grouping most closely to sequences from gibbons within the Hylobates genus (H. moloch and H. agilis in particular) and the Bornean orang-utan sequences. There is 100% bootstrap support for the viruses from all of the gibbon species branching from a single point, with orang-utans falling within that clade. There is also high bootstrap support for the branching of this gibbon clade into the separate Hylobates and Nomascus genera, and further branching into the gibbon species within these genera, particularly H. lar, and H. pileatus. Similarly, there is 100% bootstrap support for all of the chimpanzee viral sequences branching together with two exceptions, AB032431 (Bassi) and HBV131575 (Chimp 82), that group with human genotype E and H. lar respectively. Within the chimpanzee clade, there is strong support for separation into the chimpanzee subspecies P. troglodytes verus, P. troglodytes vellerosus, P. troglodytes troglodytes and P. troglodytes schweinfurthi. There was 100% bootstrap
Figure 4.1: Phylogenetic tree created from the analysis of full length genomes of all available nonhuman primate and human HBV sequences. Analysis was performed using the Neighbor joining method with bootstrap re-samplings using 1000 sets of permuted data. Bootstrap values >75% are shown on branches.
support for each of the human genotypes branching separately from each other and from the nonhuman primate sequences, with the exception of Bassi and Chimp 82 (mentioned previously).

A larger database exists of DNA sequences encompassing the S gene region of the HBV genome in nonhuman primates. Although less accurate than analysis of the entire genome at determining genotype of origin, analysis of the surface gene of the virus isolated from Hecla allowed comparison with a greater number of nonhuman primate sequences. Comparison was made with sequences encompassing the S gene region from a further 20 gibbon, 15 chimpanzee and seven orang-utan, in addition to the S gene region from the genome of the nonhuman primate sequences included in the previous analysis. A total of 88 sequences were examined by neighbour joining analysis with the resultant phylogenetic tree depicted in Figure 4.2.

Hecla’s viral sequence demonstrated 100% bootstrap support for branching with the virus obtained from her brother Chilibi, and also clustered closely to virus from Ling (H. moloch). Hecla’s virus also demonstrated 100% sequence similarity to that obtained from Chilibi over the S gene region (Figure 4.3).

Similarly to the tree based on the entire genome, the phylogenetic tree in the S gene analysis demonstrates strong grouping into gibbon, orang-utan, chimpanzee and woolly monkey HBV sequences, with high bootstrap support for the separate branches. There continues to be strong support for grouping into the gibbon genera and chimpanzee subspecies, although a number of individual sequences form branches separate to the major groupings. Speciation of the chimpanzees from which the S gene sequences were obtained is not provided and therefore it is difficult to know if these sequences represent subgroups of the previously defined subspecies, or a new grouping.
Figure 4.2: Phylogenetic tree created from the analysis of the S gene region of all available nonhuman primate HBV sequences. Analysis was performed using the Neighbour joining method with bootstrap re-samplings using 1000 sets of permuted data. Bootstrap values >75% are shown on branches.
Figure 4.3: Nucleotide alignment of the surface gene region of Hecla*. Nucleotide identities to the Hecla’s sequence are represented by a dot.

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* H. moloch
†H. agilis
‡H. moloch/H. agilis
* closest gibbon species used for comparison
The sequence of the core region obtained from Uban was compared to published sequences from the core region of the viruses isolated from Chilibi and Ling (H. moloch) and Pepino (H. agilis), in addition to the core region of sequences obtained from the genome of the nonhuman primates used in the initial analysis. A total of 50 sequences were examined by neighbour joining analysis and the resultant phylogenetic tree is depicted in Figure 4.4.

Uban's viral sequence was most closely related to HBV131575 (Chimp 82) based on a nucleotide similarity of 94.9%, however it also showed 94.75% similarity to the core regions obtained from both Hecla and Chilibi. As discussed previously, it is thought that Chimp 82 carries a strain of HBV related to that carried by H. lar gibbons rather than ChHBV.

In the phylogenetic tree Uban's HBV sequence branched most closely to the viral sequences of Ling (H. moloch) and TB Black (AY330916 – H. moloch/H. agilis), and among the other H. moloch and H. agilis gibbons, although showed no strong support for branching with any particular group of gibbons. Hecla's viral sequence again demonstrated 100% bootstrap support for branching with that of sibling Chilibi, with 100% nucleotide similarity also occurring across this region (Figure 4.5).

Distinct branches were again demonstrated for the chimpanzee, gibbon and woolly monkey sequences, with orang-utans falling within the gibbon clade as seen in the full genome analysis. Separate branches within these groups were again seen for the various chimpanzee subspecies as well as the gibbon genera, although a number of outlying sequences were seen within the gibbon branch. Two chimpanzee sequences also fell outside of the main cluster, AB032431 (Takahashi et al. 2000) and AB046525 (Takahashi et al. 2001).
Figure 4.4: Phylogenetic tree created from the analysis of the core gene region of all available nonhuman primate HBV sequences. Analysis was performed using the Neighbour joining method with bootstrap re-samplings using 1000 sets of permuted data. Bootstrap values >75% are shown on branches.
Figure 4.5: Nucleotide alignment of the core region of Hecla and Uban*. Nucleotide identities to 

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* H. moloch
* H. moloch/H. agilis
* H. agilis
* H. lar
* closest gibbon sequences used for comparison
Viral sequence data available from Jury’s father, Ivan (Grethe et al. 2000) comprised two small regions of the genome (A: 366 bp, B: 439 bp), not correlating to either the surface or core regions. Both of these regions were analysed against the corresponding regions from the full genomes used in the previous analyses. As the two fragments combined account for only one quarter of the length of the entire genome, it is difficult to make assumptions on the origins of the virus based on either of these fragments. However, in both analyses the virus from Ivan showed poor nucleotide similarity to all of the human genotypes, as well as the woolly monkey HBV. A closer relationship was seen with the various nonhuman primate sequences, and particularly with the various gibbon sequences. Fragment A showed 99% similarity to HBV131573 (species not confirmed, but clustering with the Nomascus gibbons in Figure 4.1), with the next closest sequences belonging to AY077735 (N. concolor) and Hecla (H. moloch), both demonstrating 93.3% similarity. Fragment B showed 98% similarity to HBV131572 (H. pileatus) and 97.7% similarity to AY077735 (N. concolor). A 95.9% similarity was seen to HBV131573 and 94% similarity to HBV from Hecla. No strong relationship was seen with any particular sequence over both fragments, and therefore origins of this strain are unclear, however it is certain that this represents a separate strain to that isolated from Hecla. As neither fragment encompassed the core region, no comparison could be made against Uban’s virus, however it is possible that this represents a third strain of HBV circulating within the H. moloch species.

4.4 Discussion

It is not known why DNA extraction was unsuccessful for Jury, given that he was confirmed as a low infectivity carrier in May 2004, based on a serological pattern of HBsAg +ve, anti-HBe +ve, anti-HBs –ve. Grethe et al. (2000) successfully sequenced DNA from silvery gibbon Ivan, Jury’s father, despite the fact that he was not a carrier but had demonstrated evidence of a resolved infection. Only partial sequencing was
performed on the virus from Ivan, with the areas sequenced not containing the surface or core regions, therefore his sequence was not able to be included in the analysis of sequences from Uban or Hecla. The complete genome of HBV from other gibbons was successfully sequenced by the authors using the same method and some additional primers. Very low levels of DNA would have been expected in Ivan, and sensitive nested PCR was employed to detect these low levels. One would expect much higher circulating levels of HBV DNA to have been present in Jury, given his infectivity and chronic carrier status. However, a significant minority of chronic HBV carriers are PCR negative, particularly if their liver function tests are normal.

Repeated attempts at DNA recovery were made in the Perth and Fiji laboratories, including nested PCR, however no success was obtained. In this study the relationship between Jury and Ivan (gib156 in the Grethe study) was not clarified until after successful sequencing of virus from Hecla had been achieved, at which point it was decided to continue with the current primers. Time constraints prevented further sequencing attempts on virus from Jury and Uban. Designing more specific primers based on the viral sequences obtained from Hecla or Ivan may allow successful sequencing in the future. Use of the primers and sequencing conditions described by Grethe et al. (2000) might also provide greater success in obtaining viral DNA from Jury.

Analysis of the full length HBV genomic sequence obtained from Hecla demonstrated 100% similarity to the previously published sequence from her brother Chilibi across both the S gene and core regions of the genome (Lanford et al. 2000). In their study, Lanford et al. (2000) found both the S and core regions of virus isolated from Chilibi to be identical to that from another sibling, Chloe. The sequence obtained from Hecla is therefore identical to those of both siblings across both of these regions, suggesting a common source of infection and a likely infection chain. This strongly supports the hypothesis from Chapter 2 that Hecla’s mother Bobbie Jean was the source of infection for Hecla and her siblings, and is consistent with a pattern of vertical transmission from
a high infectivity carrier mother. Sequence data of the complete viral genome from Chilibi and Bobbie Jean would confirm this beyond all doubt.

Among the full length viral genomes sequenced, Hecla’s virus showed highest nucleotide similarity to AY330916 (TB Black), a gibbon classified by Starkman et al. (2003) as belonging to either the H. moloch or H. agilis species. Among the gibbon sequences, Hecla’s virus showed greatest nucleotide similarity to those obtained from gibbons within the Hylobates genus (H. agilis, H. lar and H. pileatus) with which the silvery gibbon shares adjacent habitat (Table 4.2). A close relationship was also demonstrated with virus from the Nomascus genus of gibbons (N. concolor, N. gabiellae, N. leucogenys) and from the Bornean orang-utan. A nucleotide divergence of <8% exists between the virus from Hecla and from each of the gibbon species, and from the Bornean orang-utan. As defined by Okamato et al. (1988), HBV strains demonstrating greater than 8% nucleotide divergence constitute new genotypes. Based on this definition, Hecla’s virus falls within the gibbon genotype to which all of the published gibbon HBV genomes belong, and can be classified as GiHBV.

The Bornean orang-utan viral sequences show <8% divergence from the genomes of the viruses from gibbon species H. lar and H. agilis, and from the sequence obtained from Hecla. These orang-utan sequences however show >8% nucleotide divergence from the viruses from the remaining gibbon species and the gibbon group as a whole. Distribution maps of the Hylobates species and the Bornean orang-utan (Figure 4.6) demonstrate the close geographical proximity of the Bornean orang-utan and the Hylobates species lar, agilis and moloch, which may explain why the orang-utan virus (OuHV) is so closely related to these particular gibbon species. The grouping of the viruses from the various gibbon species in the phylogenetic tree based on the full length genomes also demonstrates a geographical association (Figure 4.1). All of the Hylobates species and the Bornean orang-utan viral sequences arise from a single branch to form a distinct clade. Within this clade, there is high bootstrap support for H. lar, H. pileatus
and P. pygmaeus pygmaeus subclades. Hecla’s virus formed a separate branch but clustered with the virus from a H. agilis gibbon (Crazy woman) and the H. moloch/H. agilis gibbon (TB Black), which both clustered closely with the Bornean orang-utans (Papa and Somad - P. pygmaeus pygmaeus). The gibbon species H. moloch and H. agilis are the two gibbon species that are most closely related geographically to the Bornean orang-utan (Figure 4.6). The viruses from the group of pileated gibbons shows 100% bootstrap support for division into two distinct subgroups. Sall et al. (unpublished) argue that these subgroups represent two different strains of HBV within the H. pileatus species, originating from two distinct geographical areas (Thailand and Cambodia) within the natural range of this species.

**Figure 4.6:** Geographic distribution of orang-utan and gibbon spp. (adapted from Starkman et al. 2003)
The viruses from the gibbons belonging to the Nomascus genus form a separate branch to those from the Hylobates species and from the orang-utan. The distribution map of the four gibbon genera (Figure 4.7) demonstrates that the Hylobates genus shares adjacent and overlapping territory with the Bornean orang-utan, whereas the Nomascus species are geographically isolated from the orang-utans. Similar clustering of the gibbon species is demonstrated in the analysis of the S gene and core regions.

Figure 4.7: Geographic distribution of the four gibbon genera and the Bornean orang-utan (adapted from Gleissman 1995).
Similarly, the single gorilla HBV sequence fell within the same genotype as the chimpanzee viruses, a finding reported previously by numerous authors (Grethe et al. 2000, Verschoor et al. 2001, Starkman et al. 2003). The distribution map of the western lowland gorilla (G. gorilla) shows that this species shares an overlapping geographical distribution with the troglodytes subspecies of chimpanzee, with whose virus the gorilla virus clusters most closely in the phylogenetic analysis (Figure 4.8). Again, this is suggestive of a geographical rather than species associated relationship, consistent with the theory of Starkman et al. (2003) that geographical distribution may play a stronger role in the origin of HBV subtypes among nonhuman primates than species of origin.

Figure 4.8: Geographic distribution of chimpanzees and gorillas (adapted from Starkman et al. 2003)

Gibbons HBV131569 and HBV131573 from the study by Grethe et al. (2000), were not identified by species. Comparison of the nucleotide identities of the viruses from these gibbons shows that HBV131569 is most closely related to the H. lar viruses (96.3% similarity) and more closely related to those from each of the Hylobates species than to any of the Nomascus species. The virus from HBV131575 is most closely related to the
N. gabriellae viruses (94.5% similarity) and more closely related to virus from the remaining Nomascus species than the Hylobates species. Both of these relationships are demonstrated in the three phylogenetic trees, where HBV131569 and HBV131573 consistently branch within the H. lar and Nomascus groups.

Hecla’s virus shows >8% nucleotide divergence from the sequences obtained from the other nonhuman primate species, as do all of the published gibbon sequences. Gibbons therefore carry viruses from a separate HBV genotype to those previously identified in orang-utans (OuHV), chimpanzees (ChHBV), gorillas (GoHBV) and woolly monkeys (WMHBV). This is consistent with findings by others (Hu et al. 2000, Takahashi et al. 2000, Vartanian et al. 2002, Aiba et al. 2003, Starkman et al. 2003). Among the human genotypes, Hecla’s virus was most closely related to genotype C, a genotype found to be widespread among the human populations of East and South East Asia, where the silvery gibbon is found (Figure 4.9). Genotype C is also the human genotype most closely related to all of the gibbon viruses and to the viruses obtained from the Bornean orang-utan, a species also distributed in East and South East Asia. The chimpanzee and gorilla sequences show greatest similarity to the human genotypes A and E, which are the predominant human genotypes in Africa. The three distinct nonhuman primate clusters (gibbon, chimpanzee and woolly monkey) showed >8% divergence from each of the eight human genotypes, therefore constituting distinct nonhuman primate genotypes. Whilst the single gorilla sequence and the two Bornean orang-utan sequences fell within the chimpanzee and gibbon genotypes respectively, they were >8% divergent from each of the human genotypes and therefore also represent nonhuman primate strains rather than human strains of HBV. Infection of nonhuman primates with HBV would therefore appear to be largely the result of indigenous infection with nonhuman primate-specific strains of HBV, rather than zoonotic transmission.
The full length genomes of the viruses obtained from the chimpanzees in this study formed a distinct clade in the phylogenetic tree, with 100% bootstrap support. Within this clade, as with the gibbons, a number of subclades could be demonstrated, correlating to the verus, vellerosus, troglodytes and schweinfurthi subspecies of P. troglodytes. Chimpanzee AB046525 (Bassi), failed to group within its subspecies group, and appears as an outlying branch within the chimpanzee clade. This animal has been identified as belonging to the troglodytes subspecies, however it has been demonstrated to have an unusual core gene sequence that is thought to have originated through recombination with a highly divergent (and hitherto undiscovered) genotype of HBV (Starkman et al. 2003). This divergent region explains the lack of subspecies clustering, and is evident in the phylogenetic analysis of the core region (Figure 4.4) where it appears as an outlying sequence.
The chimpanzee AB032431 (ch195) shows 1.4% nucleotide divergence from the three representatives of human genotype E (data not shown), but 9.3% divergence from the other chimpanzee sequences. This sequence therefore does not fall within the chimpanzee genotype (ChHBV), but does fall within the human genotype E, based on the definition set by Okamoto et al. (1988). Phylogenetic analysis further supports this relationship, with 99% bootstrap support that this individual branches within the human genotype E clade. The animal involved was HBsAg +ve on arrival at the study institution, however no mention is made of the origin of the animal nor its time in captivity prior to arrival at the study institution (Takahashi et al. 2000). This is in contrast to the other two animals in the study which were found to carry chimpanzee strains of HBV, where a detailed history is provided. The authors raise the possibility that this sequence may represent a case of anthropozoonotic transmission of the virus from humans, given the high prevalence of genotype E in Africa. However they favour the theory of transmission from another chimpanzee carrying the genotype E strain. The authors suggest that genotype E may have originated in nonhuman primates, and that nonhuman primates may have been the reservoir from which human HBV strains arose. Regardless of the origin, the high nucleotide similarity between ch195 and human genotype E demonstrated in the current study, in comparison to the relatively low nucleotide similarity between ch195 and other chimpanzee sequences, suggests that this individual is infected with a human strain of HBV and not with ChHBV.

Similarly, chimpanzee HBV131575 (chimp 82) demonstrates 3.1% nucleotide divergence from the H. lar group, and 6.4% divergence from the gibbon group of viruses as a whole, however shows 9.2% divergence from the chimpanzee group (ChHBV). Phylogenetic analysis also demonstrates high bootstrap support for the branching of chimp 82 within the viruses from the lar species of gibbon. It would seem that this individual therefore carries a strain of HBV most closely related to HBV found in gibbons, in particular H. lar, rather than a chimpanzee-specific strain. The authors proposed that the chimpanzee may have acquired the infection at a previous institution where it had
been housed with gibbons, and that this may be the first “quasinatural” interspecies transmission of HBV. The nucleotide identities obtained in the current study support the theories proposed by Grethe et al. (2000) that chimp 82 carries a strain of GiHBV. The different geographical ranges of chimpanzees and gibbons (Africa and Asia) further support transmission in the captive situation rather than in the wild. It is therefore likely that interspecies transmission of HBV can occur in the captive situation, and thus interspecies transmission may also occur in the wild where different species share adjacent habitat, however this remains to be demonstrated.

The HBV core sequence obtained from Uban showed highest nucleotide similarity to that from HBV131575 (Chimp 82), the chimpanzee reported by Grethe et al. (2000) to be infected with a gibbon strain of HBV (H. lar), rather than to the virus demonstrated in Hecla (H. moloch) (data not shown). However, there was not strong bootstrap support for a relationship with virus from any of the gibbon species in the phylogenetic analysis. This finding confirms that Uban was infected with a different strain of HBV to Hecla, and may possibly be infected with a strain with origins in the H. lar species, although full sequencing will be necessary to confirm the origin of the virus Uban carried. If such sequencing were to confirm the close relationship between the virus from Uban and that from H. lar gibbons, this may represent another incidence of interspecies transmission of HBV. Given that Uban was almost certainly infected by vertical transmission, such interspecies transmission would have to have occurred at the level of Perth 2 or above (i.e. prior to 1980). Anecdotal evidence suggests that Perth 2 was not housed with any other gibbon species during her time at Perth Zoo, although records from this time are lacking. Such an incidence of interspecies transmission would therefore most likely have occurred in the period of captivity prior to arrival at Perth Zoo, or potentially in the wild.
Whilst we cannot make phylogenetic inferences about the origin of the virus based on partial sequencing, the high similarity of Uban’s HBV sequence to virus from other gibbons over and above human HBV genotypes (data not shown) suggests that he is also carrying a strain of GiHBV. Complete sequencing of the genome will be required to confirm this, and to elucidate the origin of his virus.

The limited viral sequences available from Jury’s father Ivan suggest that his virus belongs to GiHBV and represents a different strain of HBV to that demonstrated in Hecla. Comparison to the virus sequenced from Uban was not possible, due to the lack of overlapping segments, however the relationships to other gibbon sequences demonstrated by virus from Uban are different to those demonstrated by virus from Ivan, suggesting that they represent two different strains of HBV, both also different to that seen in Hecla. It therefore seems likely that three separate strains of HBV are present within the captive population of silvery gibbons, as suggested in Chapter 2. Complete sequencing of the viral genomes from Uban and Ivan, as well as Jury and Perth 2, would help to clarify the origins of the strains of hepatitis B within the silvery gibbon population.
Chapter 5: General Discussion

This thesis has investigated a number of factors relating to hepatitis B infection in silvery gibbons, and has helped to provide new information on the biological behaviour of this poorly understood virus, as well as its phylogenetic origins in this species.

Based on the findings of this research, vertical transmission appears to be a prominent feature of the infection pattern in silvery gibbons. This is similar to the findings in other gibbons by Noppornpanth et al. (2003). Vertical transmission also appears to be a feature of HBV infection in gorillas and woolly monkeys (Linnemann et al. 1984, Lanford et al. 1998) and may be the primary mode of infection in nonhuman primates (Robertson and Margolis, 2002), with some limited horizontal transmission occurring.

Vertical transmission is highly likely in the case of Uban, who was born to a high infectivity carrier mother and was removed from her within three days of birth for hand raising. The lack of cohabitation with other gibbons, and the likely nonhuman primate-specific nature of his infection, suggest that he was exposed during the first three days of life. Uban also demonstrated seroconversion from a high infectivity carrier (HBeAg +ve, anti-HBe -ve) to a low infectivity carrier (HBeAg -ve, anti-HBe +ve) during the period of study. Seroconversion such as this is reported to occur in humans at a rate of 10-15% per year (Dienstag and Isselbacher, 2001b), but has not previously been reported in nonhuman primates.

Vertical transmission also appears likely in the case of Hecla and her siblings, with 100% of the siblings tested to date demonstrating evidence of chronic infection. This is further supported by the 100% sequence similarity of the S and core genes from three of these siblings (Hecla, Chilibi and Chloe). This high rate of transmission, combined with the
high level of chronic infection, is consistent with the pattern of transmission seen in humans from high infectivity carrier mothers. In these cases, 90% of infants born to high infectivity carrier mothers become infected at birth, and 80-90% of infants infected at birth develop chronic infection (Hollinger and Liang, 2001). Serological testing of Hecla’s mother Bobby Jean would ultimately confirm this hypothesis.

Bobby Jean is one of the founding animals of the captive silvery gibbon population, with her first and second generation progeny representing close to one quarter of the captive population. If vertical transmission, and subsequent development of chronic infection, occurs at similar rates in silvery gibbons to those demonstrated in humans (as this research suggests), we might expect that close to one quarter of the current captive population of this critically endangered species may have become chronically infected with HBV from this single source. The existence of other successful breeding females with high infectivity chronic infection is also likely, as seen with the likely vertical transmission to both Uban and Jury. It therefore seems likely that the rate of chronic HBV infection in the silvery gibbon population may be significantly higher than one quarter.

Analysis of the family trees of Uban, Hecla and Jury suggests that all three gibbons were infected from different sources, which can potentially be traced back to individuals caught from the wild. Sequencing of the virus from Hecla and Uban confirms that these animals were infected with different viral strains.

Whilst there is significant data now to suggest that the hepatitis B viruses found in nonhuman primates is not of human origin, but rather are indigenous strains that are host specific, information on the pathogenicity of these viruses in their indigenous hosts is limited.
This study demonstrated significant elevations in liver enzymes in three chronic HBV carriers over an extended period of time. Noppompanth et al. (2003) also demonstrated elevated liver enzyme concentrations in chronically infected gibbons, and similar elevations are typical of chronic HBV infection in humans (Hollinger and Liang, 2001) and chimpanzees (Deinhardt 1976, Will et al. 1982 and Sureau et al. 1988). These findings suggest a degree of hepatocellular damage associated with chronic HBV infection in gibbons.

Although no definitive evidence of liver pathology was found on post mortem of Uban and Perth 2, the findings of mild fibrosis may indicate an early stage in the development of cirrhosis. Previous studies in a number of nonhuman primates infected with HBV have also failed to demonstrate the presence of cirrhosis, a common finding in cases of chronic HBV infection in humans, which often progresses to hepatocellular carcinoma (HCC), however specific pathologic studies of HBV carrier nonhuman primates have been lacking. Given that the common pathologic conditions associated with chronic HBV infection in humans (cirrhosis, HCC) can take 30-40 years to develop, detection of similar changes in nonhuman primates would necessitate long term pathologic studies involving chronic carriers.

The presence of elevated liver enzymes in chronically infected silvery gibbons may indicate that HBV infection in this species can be associated with pathologic changes in the liver, as is commonly seen in human HBV infection. This finding has implications for the release of HBV infected animals into the wild from rehabilitation centres. Whilst natural infection may already be present in the wild populations, releasing rehabilitated animals which carry the virus may serve to increase the infection level to that which could compromise the already vulnerable population. This is particularly so in the case of female carriers, where there is a high risk of vertical transmission and therefore a greater potential to spread the virus. The chronic nature of HBV infection means that effects of the virus in the population may not be immediately obvious, however the
presence of a reservoir of infection and potential premature deaths could have a significant impact on the vulnerable population.

Hepatitis B infection in a hoolock gibbon (B. hoolock) has not previously been reported. The finding of hepatitis B infection in Horace therefore represents the first case of chronic HBV infection in this species and in the Bunopithecus genus. Histopathological examination of the liver from this animal revealed the presence of changes consistent with ‘chronic active hepatitis’, a syndrome associated with chronic HBV infection in humans (Dienstag and Isselbacher, 2001a). This finding suggests that infection with HBV in hoolock gibbons can be associated with liver pathology, and further supports the theory that the nonhuman primate strains of HBV can be pathogenic to their hosts. In addition, the presence of HBsAg and anti-HBc in the hepatocytes demonstrates that the virus behaves similarly in humans and nonhuman primates, and may therefore be expected to play a similar pathologic role.

The vaccination protocol developed for silvery gibbons at Perth Zoo has proven to be successful in preventing vertical transmission in two of the gibbons tested to date. Furthermore, in all three of the tested gibbons it has been successful in providing immunity and preventing the development of chronic infection. The anti-HBs titres achieved by vaccination appear to be protective when compared with established human and chimpanzee standards, and in view of the high risk of transmission in the study environment. Given the potentially high level of endemicity of HBV infection in the captive silvery gibbon population, and the potential pathogenicity of the virus in this species, it would seem prudent to employ a similar vaccination protocol in other breeding situations to prevent further spread of the virus, particularly in those collections that house high infectivity carrier females. The basic principles of the vaccination program would also be applicable in the lower risk situation of horizontal transmission. This vaccination protocol could be applied in rehabilitation centres,
enabling protection against infection for naïve animals during the period of rehabilitation and after release to the wild.

Sequence analysis of the entire HBV genome from Hecla demonstrates that she is infected with a strain of virus identical to those from her two siblings, that falls within the GiHBV genotype. It therefore appears to be a strain of HBV indigenous to gibbons. It would appear to be a silvery gibbon specific strain, based on the similarity to the isolate from TB Black, however other full HBV genomes from H. moloch are lacking.

The full genomic analysis of the virus obtained from Hecla demonstrated the distinct clustering of the nonhuman primate viruses within species- and subspecies-specific groups, as well as the geographical relationship between the viruses from the various Asian and African species (human and nonhuman primate). In addition, a single case of possible zoonotic transmission (human to chimpanzee or vice versa) and a single case of probable interspecies transmission (gibbon to chimpanzee) are demonstrated and confirm previous findings by other authors (Grethe et al. 2000, Takahashi et al. 2000). The conditions surrounding these individual cases need clarification to determine the true likelihood of interspecies transmission of the virus in the captive and wild situations.

The partial sequence of the virus obtained from Uban indicates that he is likely to be infected with a separate strain of HBV than Hecla, as suggested by the epidemiologic information in Chapter 2 of this thesis. The viral sequence appears to belong to GiHBV, and may be most closely related to those recovered from H. lar. Full genomic sequence analysis would be required to make further assumptions on the origin of the virus found in Uban.
5.1 Further Work

Whilst this research has helped to provide some new information on hepatitis B virus infection in silvery gibbons, there is still a lot more that needs to be studied if we hope to fully understand how this virus affects the species, as well as the numerous other nonhuman primate hosts that have been identified to date.

The publication of more sequences encompassing the entire HBV genome will help to gain a clearer picture of the origin of the viruses involved, and their relationship to each other. To better understand the virus in silvery gibbons, sequencing of viruses from both related and unrelated animals will help to more clearly establish the role of vertical and horizontal transmission in the spread of the disease. In addition, further hepatitis B testing of the current captive population of silvery gibbons would help to identify the existing level of infection, and enable the rational use of vaccination protocols such as the one described in this thesis to prevent further spread of the virus. In doing so, it may be possible to create a population of animals that are immune to the virus and could be successfully released into the wild without the risk of infecting wild populations.

In addition, an epidemiological survey of free living silvery gibbons would enable us to determine the level of endemic infection in the wild population. It is possible that captive breeding programs that have unknowingly involved carrier animals, and in particular high infectivity carrier females, have artificially raised the level of infection in the captive population above that which is present in the wild population. Sequencing of the virus from wild-caught silvery gibbons would help to determine if the viruses found to date in the captive population were originally derived from the wild.

Sequencing of the virus found in the hoolock gibbon Horace is required to confirm the origin of the virus found in this animal. If, as suspected, a gibbon-specific strain is
identified, confirmed cases of HBV will have been documented in three of the four
gibbon genera. The genus Syndactylus, containing the single species S. siamang, would
therefore be the only remaining gibbon genus not to have had HBV identified. HBV
testing of this species is also strongly recommended, as the large number of HBV cases
already identified in nonhuman primates, particularly gibbons and chimpanzees,
suggests that the virus is widespread among apes.

Further work is required to determine the extent of pathological changes associated
with HBV infection in nonhuman primates. Long term studies of carrier animals would
be most useful due to the chronic nature of the disease. To determine the effect of
chronic infection in nonhuman primates, progressive studies of both histological changes
in the liver and liver enzyme concentrations in animals known to have been infected
with HBV for greater than 20 years would be necessary. Post mortem liver samples from
any captive silvery gibbon that dies should preferably have histological examination
performed. This would facilitate accumulation of data from infected and uninfected
animals, and enable more thorough evaluation of HBV associated pathology.

It is hoped that serial liver enzyme concentrations can be measured in both Jury and
Hecla over the next decade to help provide further information in this regard. Further
evaluation of Bobbie Jean, Hecla’s mother, may provide invaluable information on the
effect of chronic infection if as suspected she does prove to be a high infectivity carrier.

To gain a fuller understanding of the relationship between the hepatitis B viruses
affecting the various nonhuman primate species, sequencing of the complete genome of
HBV from more animals is required. Full HBV genome sequences are particularly lacking
in orang-utans and gorillas, with a total of only three sequences from these species
published to date, and sequencing has been confined to the Bornean orang-utan (P.
pygmaeus pygmaeus) and the western lowland gorilla (G. gorilla gorilla). HBV has been
reported in the Bonobo chimpanzee (Pan paniscus), however sequencing has not yet
been performed on this species to determine the relationship of the virus in this species to those identified in other chimpanzees. Further sequences will help to determine if there truly is a geographical relationship between the various nonhuman primate strains. The inclusion of full length sequences from wild-caught animals, that have not had a period of time in captivity prior to testing, would enable more accurate phylogenetic analysis of HBV. The comparison of such sequences to currently published sequences may help to confirm the origin of some of the published sequences. Whilst current information suggests that HBV infection is confined to the ape species, the existence of WMHBV in woolly monkeys at the Louisville Zoo suggests that other primates may potentially be hosts for the virus. Continued testing of non-ape species, particularly New World species, for evidence of HBV infection is recommended to confirm the species that are susceptible to HBV infection.

The vaccination protocol described in this thesis appeared to be successful in preventing vertical transmission of HBV in the silvery gibbons. It is likely that such a regimen would give similar protection in other gibbon species, although this has yet to be established. The transferability of the regimen to other gibbon species and to other nonhuman primate species is not known. Protocols for vaccination in lower risk transmission situations (eg horizontal transmission, adult animals etc) need to be established to provide protection against further viral spread in both captive and wild populations.

Finally, development of a commercially available test to readily differentiate between human HBV and the various nonhuman primate strains would help to clarify the existence (or lack thereof) of zoonotic or anthropozoonotic infection.
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