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Molecular epidemiology of gibbon hepatitis B virus transmission

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Although transmission of human hepatitis B virus (HBV) variants to nonhuman primates is well documented, it remains to be elucidated whether nonhuman primate HBV is transmissible to humans. The prevalence and transmission routes of gibbon HBV were analysed in 101 captive gibbons in Thailand. Approximately 40 % of these animals showed at least one marker of HBV infection; 19 animals were chronic HBV carriers, characterized by elevated levels of alanine amino transferase and the presence of HBV DNA. Some of the chronic animals were found to be anti-HBc (HBV core antigen) negative (4 of 19), while precore promoter point mutations (nt 1762 or 1764) were determined in four animals by RFLP analysis. Phylogenetic tree analysis of the complete surface gene sequences revealed that gibbon viruses clustered separately from hepadnaviruses of other hosts. Evidence for horizontal and vertical transmission in captive gibbons was obtained. HBV DNA was also detected in the saliva of HBV carrier gibbons. Although some of the animal caretakers at the Krabok Koo Wildlife Breeding Centre were found to be chronic HBV carriers, genotype and sequence analysis did not reveal any evidence for zoonotic disease transmission.

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INTRODUCTION

Hepatitis B virus (HBV), a small double-shelled virus that contains a partially double-stranded DNA genome of approximately 3200 bases (White & Fenner, 1994), is found in several species, including woodchuck, ground squirrel, a range of bird species, such as duck, goose and grey heron (Mason *et al.*, 1980; Marion *et al.*, 1980; Summers *et al.*, 1978), and nonhuman primates like chimpanzee (*Pan troglodytes*), woolly monkey (*Lagothrix lagothrica*), orang-utan (*Pongo pygmaeus*) and gorilla (*Gorilla gorilla*) (Grethe *et al.*, 2000; Lanford *et al.*, 1998; Vaudin *et al.*, 1988; Warren *et al.*, 1999). HBV was also isolated from a gibbon, infected in the wild and housed at the CDC for 2 years (Mimms *et al.*, 1993). Phylogenetic analysis of the complete genome revealed that gibbon HBV represents a unique group when compared to HBV genotypes described previously. Remarkably, a 33 bp deletion after the start codon of preS1, the most divergent part of the genome, was observed (Norder *et al.*, 1996). Nonrecognition of an anti-preS1 monoclonal antibody at aa 27–35 to gibbon virus particles confirmed that the gibbon HBV surface protein conformation is different from that of human HBV (Mimms *et al.*, 1993).

Phylogenetically, HBV isolated from gibbons and chimpanzees share an early lineage, indicating that these viruses were indigenous to their respective hosts (Norder *et al.*, 1996). On the other hand, infection of chimpanzees with human and gibbon HBV can be accomplished (Gallagher *et al.*, 1991). Experimental transmission of human HBV to gibbons by exposure to human saliva containing HBV has been reported also (Bancroft *et al.*, 1977; Scott *et al.*, 1980). Replication of human HBV in the respective animals supported the close relation of these hosts and may indicate natural HBV cross-transmission. On the other hand, no evidence has been obtained thus far for HBV transmission from gibbon or chimpanzee to human. HBV is present at levels as high as 1×10^{13} virions ml^{-1} in the blood of HBV e antigen (HBeAg)-positive patients but virus particles have also been found in other body fluids, including saliva/nasopharyngeal fluids, semen, cervical secretions and

The GenBank accession numbers of the sequences reported in this paper are AF274495–96, AF274499, AF275378, AF477482–94, AY077735–36 and AF529308–09.

leukocytes (Alter *et al.*, 1977; Davison *et al.*, 1987). The possibility of human HBV transmission through contact with saliva from HBV chronic carriers has been obtained both in humans as well as in gibbons (Bancroft *et al.*, 1977; MacQuarrie *et al.*, 1974; Scott *et al.*, 1980; Stornello, 1991).

In order to analyse possible routes of gibbon HBV transmission, we determined the presence of HBV in captive gibbons in Thailand by serological testing and HBV DNA detection in chronic carriers. Sequencing and RFLP analysis of the viruses permits molecular characterization of gibbon HBV and possible routes of transmission between gibbons, including vertical transmission. To investigate horizontal transmission to humans, animal caretakers, some of whom are HBV carriers, were analysed for the presence of gibbon HBV. The unique deletion at the preS1 gene present in gibbon HBV permits the accurate identification of a zoonotic event.

METHODS

Study population. A total of 101 captive gibbons kept at the Krabok Koo Wildlife Breeding Centre, Royal Forest Department in Cha-Cheng-Sao, Thailand were included in the study. The gibbons are housed in small monogamous families. Most of them were born in the wild and all were examined in good health. The population consists of 52 males and 49 females ranging from 1 to 21 years old and of different species: *Hylobates lar* (white-handed gibbons, $n=72$), *H. pileatus* (pileated gibbons, $n=20$), *H. agilis* (black-handed gibbon, $n=2$) and *Nomascus concolar* (Tonkin black-crested gibbon, originally found in North Vietnam and central China, $n=7$). Two of the HBeAg-positive gibbons had their offspring aged 1 and 2 years.

Animal caretakers at the Krabok Koo Centre ($n=34$) were screened for the HBV surface antigen (HBsAg) during the prevaccination screening programme.

Sample collection. Gibbon blood was obtained by venepuncture during a brief period of anaesthesia by ketamine, part of the routine health-care programme. Sera were separated from clotted blood within 12 h by centrifuging at 1500 r.p.m. for 10 min and kept at −70 °C. Human blood was separated using the same procedure. Saliva samples were collected from 30 gibbons using the OraSure collection system (OraSure Technologie) following the manufacturer's protocol. Samples were kept at −70 °C until further testing.

HBV detection and liver function test analysis. All sera were analysed for HBsAg, antibodies to HBsAg (anti-HBs) and anti-HBc (antibodies to the HBV core antigen HBcAg) by enzyme immunoassay methodology (EIA) using a commercially available kit (DiaSorin), according to the manufacturer's instructions. HBeAg, anti-HBc and anti-HBs antibodies of some samples were reconfirmed by EIA kits obtained from Abbott Laboratories.

Gibbon HBV DNA was detected as described previously (Theamboonlers *et al.*, 1999). Briefly, DNA was extracted from 200 µl of serum with proteinase K in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation. The S gene was amplified by nested PCR using two sets of primers: primer F1 (5′-GGAGCGGGAGCATTCGGGCCA-3′, nt 3022–3042) and R6 (5′-GGCGAGAAAGTGAAAGCCTG-3′, nt 1103–1084) were used for first-round amplification and primers F2 (5′-CATCCTCAGGCCATGCAGTGGA-3′, nt 3192–3214) and R5 (5′-AGCCCAAAAGACCCAGAAATTC-3′, nt 1015–995) were used for second-round amplification. The amplification reaction required 30 cycles comprising initiation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, concluded by a final extension step at 72 °C for 10 min. The 1038 bp PCR product was detected by electrophoresis on a 1.5 % agarose gel with ethidium bromide and visualized under UV light.

The preS1/preS2 gene was detected using PCR primers P1 (5′-TCACCATATTCTTGGGAACAAGA-3′, nt 2817–2839) and P2 (5′-TTCCTGAACTGGAGCCACCA-3′, nt 80–61). The 478 bp PCR product was used as template for RFLP analysis and DNA sequencing. The X gene was amplified using primers Xo1 (5′-CTCTGCCGATCCATACTGC-3′, nt 1256–1274) and PC1 (5′-GGAAAGAAGTCAGAAGGC-3′, nt 1974–1957). Amplified products were used for precore mutant detection of gibbon HBV.

Sera of gibbon HBV carriers and HBsAg-negative animals were analysed for alanine aminotransferase (ALT) at the Central Laboratory of the Chulalongkorn University and Hospital (Bangkok, Thailand) using an automated analyser (Hitachi 912). Data were expressed as mean ± SD. The normal range of ALT is 0–38 U l^{-1}. Student's *t*-test was used to test statistical differences between groups.

Identification of gibbon HBV genotypes by RFLP. Two restriction enzymes, *Ava*II and *Dpn*II (New England Biolabs), were used for digestion of the preS1/preS2 PCR products. The RFLP patterns obtained were compared with the restriction endonuclease analysis reference profiles for human HBV genotype classification as described previously (Lindh *et al.*, 1998). Samples with different RFLP patterns were classified by direct sequencing.

The 720 bp PCR product of the X gene was digested with *Sau*3A I (New England Biolabs) for precore promoter mutant gene detection (nt 1762 and 1764), as reported previously (Takahashi *et al.*, 1995).

Gibbon HBV sequencing and phylogenetic analysis. HBV genes amplified by PCR were sequenced using the primer pairs P1 and P2, F2 and R5, and Xo1 and PC1 for the preS1/preS2, S and core region, respectively. Sequencing reactions were performed using the commercially available PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems). The sequence products were electrophoresed on a Perkin Elmer 310 sequencer (PE Biosystems). Each sample was sequenced in both directions and nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and submitted to GenBank.

PreS1, S and core genes of two HBV carrier families were aligned using BIOEDIT, the sequence alignment editor (version 5.0.9), while other gibbon HBV strains were used as a control. For phylogenetic analysis, S nucleotide sequences of 19 HBV carrier gibbons and two animal caretakers were multiply aligned using the CLUSTAL X program. Genetic distances were calculated using the Kimura two-parameter method within DNADIST of PHYLIP, version 3.6 (J. Felsenstein, Department of Genetics, University of Washington, USA) and the result was illustrated graphically as a neighbour-joining tree. Bootstrap values representing 1000 replicates (100 multiple data sets) were determined using the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs from the PHYLIP package. The TREEVIEW program (version 1.5) was run for unrooted phylogenetic construction.

To analyse the relationship between HBV isolates from animals in cage C, the best phylogenetic tree of the complete S gene was generated with the maximum-likelihood algorithms within the DNAML program using 1000 replicates of bootstrap values. The transition/transversion ratio was 2·0.

Table 1. Seroprevalence of gibbon HBV at the Krabok Koo Centre

Stage of infection	HBsAg	HBV DNA	Anti-HBs	Anti-HBc	No.
No infection	–	–	–	–	62
Recovered	–	–	+	+	20
HBV carrier	+	+	–	+	15
HBV carrier	+	+	–	–	4

RESULTS

Seroprevalence of HBV in gibbons

To analyse the prevalence of HBV infection in Thai gibbons, 101 serum samples were tested for the presence of HBsAg and antibodies to HBsAg and HBcAg. Serological testing indicated that 38·6 % of the animals were positive for at least one marker of HBV infection (Table 1). A total of 19 gibbons were chronic carriers, as defined by the presence of HBV DNA and HBsAg, in the absence of antibodies to the S protein. Interestingly, four of these animals were negative for anti-HBc even when tested repeatedly using a different EIA test. To test whether HBV infection was associated with liver damage, ALT enzyme levels were determined in the sera of 12 HBsAg-positive animals (Table 2) and 28 HBsAg-negative animals (Table 2 and data not shown). Elevated ALT levels were detected in HBV-infected gibbons ($68{\cdot}8 \pm 48{\cdot}1$ U l^{-1}) as compared to control animals ($33{\cdot}0 \pm 15{\cdot}9$ U l^{-1}, $P<0{\cdot}05$). Approximately 20 % (20 of 101) of the animals recovered from the infection, as evidenced by the presence of antibodies to S and core.

Serological markers of HBV infection in the chronic gibbons are shown in Table 2, including the stage of HBV of their families. Most of the HBV-infected gibbons were of the species *H. pileatus*, kept in the cage C area (10 of 19). All carriers' partners were HBsAg-positive or had been infected with HBV some time earlier. Two carrier families had babies born in the Krabok Koo Centre (cages C15 and R6). Tao C15, offspring of Ni and Saboo, was born in 1999 and

Table 2. HBV in serum and saliva of HBV carriers of gibbons and their couples

					Serum				Saliva	
Cage	Name	Species	Sex	ALT (U l^{-1})	HBV DNA	HBsAg	Anti-S	Anti-core	HBV DNA	HBsAg
C22	Daew	*H. pileatus*	F	48	+	+	–	+	+	+
C21	Candy	*H. pileatus*	F	69	+	+	–	–	–	+
C20	Jock	*H. pileatus*	M	46	+	+	–	+	–	+
	Koo	*H. pileatus*	F	36	–	–	+	+	–	–
C16	NongChai	*H. pileatus*	M	50	+	+	–	+	–	+
C15	Saboo	*H. pileatus*	M	207	+	+	–	+	–	+
	Ni	*H. pileatus*	F	70	+	+	–	–	–	+
	Tao*	*H. pileatus*	M	ND	+	+	–	–	ND	ND
C14	Gomez	*H. pileatus*	M	93	+	+	–	+	–	+
	Chmi	*H. pileatus*	F	87	+	+	–	+	+	+
C13	Saan	*H. pileatus*	M	46	+	+	–	+	+	+
	Kristine	*H. pileatus*	F	42	–	–	+	+	–	–
C2	Pok	*H. lar*	M	39	+	+	–	+	+	+
	Mek	*H. lar*	F	21	–	–	+	+	–	–
R4	Jacko	*H. lar*	M	50	+	+	–	+	–	+
	Ivana	*H. lar*	F	11	–	–	+	+	ND	ND
R6	Jieb	*H. lar*	F	20	+	+	–	+	+	+
	Baby R6*	*H. lar*	M	ND	+	+	–	–	ND	ND
	Kingkong	*H. lar*	M	ND	–	–	+	+	ND	ND
R27	Midnight	*H. lar*	M	ND	+	+	–	+	–	+
L14	Nin	*H. lar*	M	ND	+	+	–	+	+	+
	Sang	*H. lar*	F	ND	–	–	+	+	ND	ND
	Baloo	*H. lar*	M	ND	–	–	+	+	ND	ND
L10	Belle	*H. concolar*	F	ND	+	+	–	+	ND	ND
	Caesar	*H. concolar*	M	ND	+	+	–	–	ND	ND
L9	Charlie	*H. concolar*	M	ND	+	+	–	+	ND	ND
	Ozzy	*H. concolar*	M	ND	–	–	+	+	ND	ND

*Gibbons born recently at the Centre.

ND, Not determined.

all family members were found to be positive for S antigen and HBV DNA. Baby R6 was born in 2000 and his mother, Jieb, is a HBV chronic carrier, while his father, Kingkong, had natural immunity against HBV. Both mother gibbons were found by EIA methodology to be HBeAg positive.

Molecular characterization of gibbon HBV

HBV DNA isolated from 19 positive animals was used as target for DNA sequencing and RFLP analysis. The absence of the 33 bp after the preS1 start codon of gibbon HBV was confirmed. Grouping of gibbon HBV was performed by phylogenetic tree analysis; S phylogram analysis, including human genotype A–G, orang-utan, chimpanzee and woolly monkey, revealed the separate clustering of these gibbon HBVs (Fig. 1). Most animals from the three different cages cluster into the three separate groups. Animals in the closed cage, such as area C, were infected with a closely related strain of HBV since these viruses shared the root of the phylogenetic tree. HBV from two chronically infected mothers (Jieb and Ni) was more closely related to their infants. The R6 family showed a higher internal edge value at the node of the phylogram than the C15 family.

In order to analyse gibbon HBV transmission further, comparison of HBV sequences from two pairs of mother and baby gibbons was performed (GenBank accession nos AF477490–91 and AF477493–94). Comparison of preS1 sequences, the most divergent part of the HBV genome, indicated the close relation of these virus strains; Tao and Baby R6 showed a difference of only 4 and 2 bases from their mother, respectively, while 33–42 bases of the HBV sequences from both couples differed as compared to a nonrelated gibbon isolate (Fig. 2). Moreover, the preS1 gene sequence isolated from Saboo (the C15 father gibbon), showed a difference of 2 and 4 bases from dam and offspring sequences, respectively. The HBV S gene from animals in cage C showed a high percentage of sequence similarity, indicating the close relation between these viruses; mother and baby strains showed 99·5 % similarity (Table 3). Nongchai (from the neighbouring cage C16) also showed a high percentage of sequence similarity to Ni and Tao (C15 cage). However, maximum-likelihood phylogenetic analysis indicated the close relationship between Ni and Tao strains (Fig. 3). Additionally, the partial core region of both families showed 100 % identity among mothers and babies (data not shown). Thus, taking into account the relationships of these animals and the sequencing results, vertical transmission may seem quite likely to have occurred.

Precore promoter mutations at nt 1762 and 1764 were detected by the *Sau*3A I restriction pattern (Fig. 4a). Four carrier gibbons showed point mutations (G1-like pattern) in this region, while one sample could not be typed (G4). All mutated samples were positive for HBeAg testing.

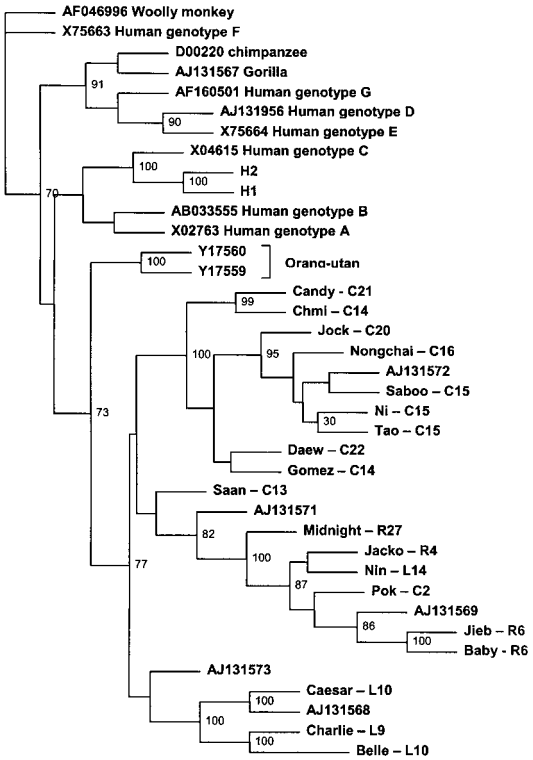

Fig. 1. Phylogram based on nucleotide sequences of the complete S gene using the Kimura two-parameter matrix and neighbour-joining method. Bootstrap values (1000 total) of ⩾70 % are indicated on the branches. Human and nonhuman primate HBV were compared to gibbon and human HBV isolates identified in this study. The GenBank accession numbers of reported sequences are indicated in the phylogram. S gene sequences from gibbon HBV presented in this tree are indicated by the name and cage of animals, as shown in Table 2. All sequences were submitted to GenBank: accession nos AF477482–94 and AF274495 (Candy C21), AF274496 (Daew C22), AY077735 (Belle L10), AY077736 (Caesar L10), AF274499 (Charlie L9), AF275378 (Nin L14), AF529308 (H1) and AF529309 (H2).

Gibbon HBV transmission to humans

As shown in Table 2, HBsAg was found in all saliva samples kept from carrier gibbons (n=14), while HBV DNA was detected in only six samples. Neither HBV DNA nor HBsAg was detected in HBV seronegative (n=7) and HBV recovered gibbons (n=9, data not shown).

A total of 34 animal caretakers of the Krabok Koo Centre were screened during part of a prevaccination programme and we found that 5·9 % (2 of 34) were positive for HBsAg and HBV DNA. Subsequently, gibbon and human HBV were identified by RFLP using *Ava*II/*Dpn*II digestion of preS1/preS2 PCR products, as indicated in Fig. 4(b, c), respectively. Shown are representative digestion patterns observed in samples G1–G4 and G6, gibbon virus strains spreading in the Krabok Koo Centre; the G5 strain is similar to G3. Two workers, H1 and H2, found to be HBs DNA

positive might have been exposed to gibbon viruses. Patterns of restriction enzyme digestion of gibbon HBV (G1–G6), however, showed different profiles as compared to human HBV (H1–H2). The 33 bp deletion in the preS1 region caused the different RFLP patterns, which cannot be grouped to any human virus genotype, including genotype D viruses, which are reported to have a preS1 deletion in the same region. These results were confirmed by sequencing and phylogenetic analysis of the S gene (Fig. 1).

DISCUSSION

Wild gibbons (*Hylobates* species) can be found throughout the tropical rainforests of South and Southeast Asia,

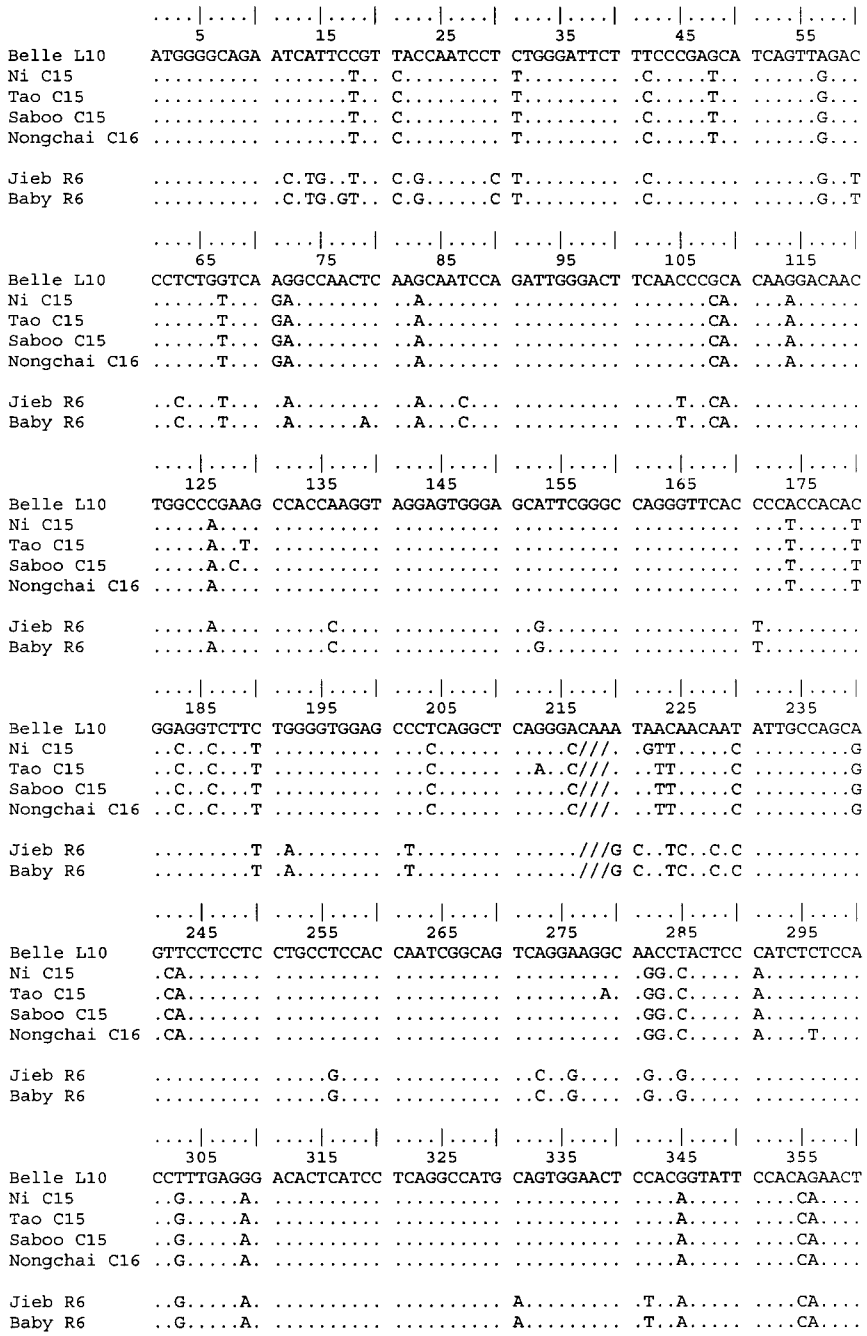

Fig. 2. Alignment of preS1 sequences from gibbon HBV. Sequences from two chronic carrier families and Nongchai (C16) were aligned using the BIOEDIT program to analyse vertical transmission in gibbons. All sequences were compared to Belle (L10) strain, as a control. Dots indicate identical nucleotide sequences, while slashes indicate insertion/deletion sequences. GenBank accession numbers are given in the legend to Fig. 1.

Table 3. Percentage sequence similarity of gibbon HBs gene isolated from family R6 and cage C animals

	Candy	Chmi	Daew	Gomez	Jock	Nongchai	Saboo	Tao	Ni	Jieb	Baby R6
Candy	100	98.7	98.1	98.6	97.8	98.2	97.2	98.3	98.4	93.7	93.3
Chmi	–	100	96.8	97.6	96.9	97.3	96.3	97.4	97.5	92.8	92.3
Daew	–	–	100	98.4	97.6	98.1	97.0	98.2	98.2	94.1	93.6
Gomez	–	–	–	100	98.3	98.6	97.6	98.8	98.9	93.7	93.3
Jock	–	–	–	–	100	98.5	97.6	98.8	98.8	93.3	92.9
Nongchai	–	–	–	–	–	100	98.1	**99.2**	**99.3**	93.7	93.3
Saboo	–	–	–	–	–	–	100	98.2	98.3	92.5	92.5
Tao	–	–	–	–	–	–	–	100	**99.5**	93.8	93.4
Ni	–	–	–	–	–	–	–	–	100	94.1	93.6
Jieb	–	–	–	–	–	–	–	–	–	100	**99.5**
Baby R6	–	–	–	–	–	–	–	–	–	–	100

including Thailand, Laos, Cambodia, Indonesia and Malaysia. The genus *Hylobates* is represented in these areas by three species, the white-handed gibbons (*H. lar*), the pileated gibbons (*H. pileatus*) and the black-handed gibbons (*H. agilis*). They are at high risk of extinction due to habitat loss and increased illegal pet trade. After categorization as a conserved wild animal in Thailand, hundreds of appropriated and abandoned gibbons have been handed over to the authorities of the Thai government. An infectious disease screening process was done to prevent possible disease spreading that may interfere with the health and reproduction of the wild gibbon population after reintroduction to the forest. Transmission of these viruses to humans, which may constitute a public health risk, was also evaluated.

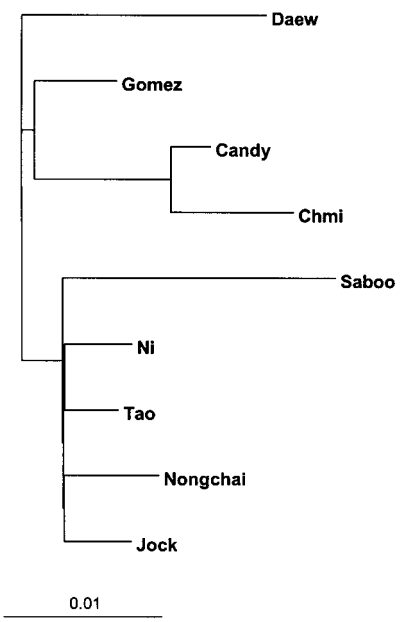

Fig. 3. Maximum-likelihood phylogenetic analysis of S gene sequences of gibbon HBV isolated from animals in cage C. The best tree was constructed using 1000 bootstrap values. Accession numbers of all sequences are described in the legend to Fig. 1.

Serological analysis of HBV infection in the gibbons kept at the Krabok Koo Centre showed that approximately 40 % of the animals were infected with HBV; 19 animals were HBV DNA-positive carriers and could be a source of virus spread in the gibbon population. Similar figures have been reported recently in captive gibbons housed in the Centre for Gibbon Studies in California, USA, by Lanford *et al.* (2000). On the other hand, Grethe *et al.* (2000) reported even higher frequencies in animals housed at different zoos. Similar to the first report on gibbon HBV (Mimms *et al.*, 1993), four of the chronically infected gibbons were negative for anti-HBc, even when tested repeatedly with different EIA tests. The specific immune response against core may be undetectable due to the lack of specific anti-gibbon IgG reagents.

Precore promoter mutation AGG→TGA at nt 1762 and 1764, linked previously to a relatively moderate down-regulation of the synthesis of HBeAg (Buckwold *et al.*, 1996; Moriyama *et al.*, 1996) but also observed in the HBeAg-positive stage (Takahashi *et al.*, 1995), has been associated with more severe liver damage (Lindh *et al.*, 1999) and increased responses to interferon (Kanai *et al.*, 1996). In gibbon HBV carriers, this mutation was detected in four HBeAg-positive animals, including Pok C2, Midnight R27, Nin L14 (all anti-core positive) and Caesar L10 (anti-core negative). Unfortunately, core gene sequencing and ALT status in these gibbons could not be analysed in this study due to the limitation of samples.

Molecular characterization by RFLP and phylogenetic analyses confirmed the separate clustering of gibbon HBV compared to human and nonhuman primate HBV. The three different clusters within the gibbon viruses observed could relate to genomic variants as described by Grethe *et al.* (2000). Interestingly, animals in the C and L cage areas shared the same branches of each group, suggesting the possibility of brother-and-sister relationships of these animals or horizontal transmission by infectious viruses in saliva during fights or feeding at an early age.

The recent finding that chimpanzees as well as orang-utans can be infected with HBV in the wild makes any proposed spread of HBV from the New World extremely unlikely

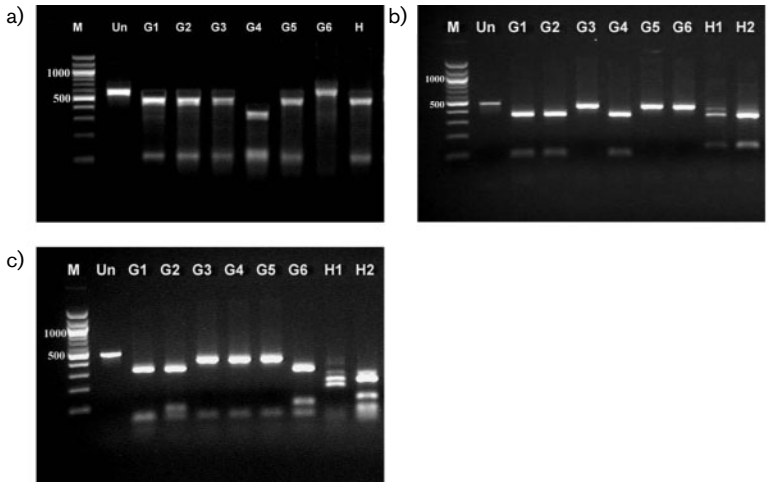

Fig. 4. HBV genotype characterization by RFLP analysis of gibbon HBV. DNA patterns after *Sau*3A I digestion are presented to detect precore promoter sequences, including a positive control (H) human HBV sample (a). Gel electrophoresis of preS1 DNA isolated from gibbons and humans, digested with *Ava*II (b) and *Dpn*II (c). Six samples isolated from gibbons (G1–G6) and two samples from humans (H1–H2) were analysed. Indicated are the 1000 and 500 bp DNA marker (M) and the uncut sample (Un).

(Hu *et al.*, 2000; MacDonald *et al.*, 2000; Takahashi *et al.*, 2000; Warren *et al.*, 1999). In contrast, all data support the idea that these viruses are indigenous to the different nonhuman primate populations. The geographical distribution of human genotype C viruses, the orang-utan HBV and gibbon HBV, all found in Southeast Asia, could indicate that spread through cross-species transmission in Southeast Asia may have occurred (Simmonds, 2001). However, gibbon and orang-utan HBV are more closely related to each other than to the human genotypes. Thus, more sequence data on different isolates are needed as well as possible evidence for transmission of nonhuman primate HBV to humans.

HBV is transmitted by sexual contact and parental exposure, although it is thought that mother-to-child prenatal transmission is responsible for high rates endemically in several regions of the world; 25–50 % of the HBV chronic carriers result through vertical transmission or horizontal transmission by nosocomial exposure in early childhood (Mast *et al.*, 1999; Mahoney, 1999; Wang & Zhu, 2000). In gibbons, vertical transmission was supported by 99.5 % identity of the S gene of HBV isolated from mother and baby gibbons. Hence, base sequence changing may have occurred through mutations in time; for example, Baby R6, younger than Tao at the date of blood sampling, showed higher sequence similarity of the preS1 region (Fig. 2). Unexpectedly, HBV isolated from Nongchai (C16 cage) showed a high percentage of sequence similarity to Ni and Tao (cage C15), possibly due to horizontal transmission. However, HBsAg isolated from Ni and Tao were more closely related, as determined by maximum-likelihood phylogram analysis. Noticeably, all animals that share cages with gibbon carriers were infected with HBV and anti-core antibodies were detected (Table 2). These data suggest that sexual contact or horizontal HBV transmission of family members could be a source of virus spread in this gibbon population.

Similar to observations made in human HBV (Noppornpanth *et al.*, 2000), HBsAg could be detected in the saliva of all HBV carriers. Until now, few cases have been reported on HBV infection from saliva by a human bite (MacQuarrie *et al.*, 1974; Stornello, 1991) but the high prevalence of HBV among dentist personnel and family members suggests that HBV might spread by saliva (Heathcote *et al.*, 1974). However, no report of HBV transmission from captive animals to humans currently exists. In the present study, HBV DNA was detected in the saliva of six gibbons, representing circulating HBV infectious particles in body fluids (Ljunggren *et al.*, 1993). HBV-positive human saliva administered intradermally, but not orally, to gibbons caused HBV transmission (Scott *et al.*, 1980). Thus, HBV DNA detected in gibbon saliva may indicate a potential risk for horizontal transmission, especially if the animals are injured after fighting or through accidental injury of animal caretakers. Two workers of the Kabok Koo Centre were found to be positive for HBsAg and HBV DNA, which corresponds to seroprevalence of HBV in the general adult Thai population (Tandon & Tandon, 1997). RFLP and phylogenetic analyses of the S gene from both isolates indicated that they were infected by human HBV.

The high prevalence of HBV infection in captive gibbons of the Krabok Koo Centre shows that HBV is an important infectious agent in captive gibbons. Since chronic carrier gibbons are the main source of HBV in the Centre, separating them and vaccination of newborn babies should be done to reduce the number of infected animals. Although transmission of HBV between nonhuman primates and humans, similar to zoonotic human immunodeficiency virus transmission (Hahn *et al.*, 2000), could not be confirmed, these studies could provide more insight in the molecular evolution and transmission routes of HBV and facilitate our understanding of the origin of HBV and its pathogenesis.

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