Frequent infection of *Hylobates pileatus* (pileated gibbon) with species-associated variants of hepatitis B virus in Cambodia

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As well as being distributed widely in human populations, hepatitis B virus (HBV) infections occur frequently in chimpanzee, gibbon and other ape populations in sub-Saharan Africa and South-East Asia. To investigate the frequency and genetic relationships of HBV infecting gibbons in Cambodia, pileated gibbons (*Hylobates pileatus*) that were originally wild-caught were screened for surface antigen. Twelve of 26 (46%) were positive, of which 11 were positive for HBV DNA. Phylogenetic analysis of complete genome sequences revealed two distinct genetic groups in the gibbon/orangutan clade. Three were similar to previously described variants infecting *H. pileatus* in Thailand and eight formed a distinct clade, potentially representing distinct strains of HBV circulating in geographically separated populations in South-East Asia. Because of the ability of HBV to cross species barriers, large reservoirs of infection in gibbons may hamper ongoing attempts at permanent eradication of HBV infection from human populations in South-East Asia through immunization.

Infection with hepatitis B virus (HBV) represents a major global health problem, affecting approximately one-third of the world’s human population and accounting for approximately one million deaths from chronic liver disease and hepatocellular carcinoma each year (Thomas & Jacyna, 1993). Human populations in South-East Asia, sub-Saharan Africa and Central and South America show particularly high frequencies of HBV infection that is often maintained through mother-to-child perinatal transmission; in Asia, the establishment of a highly infectious carrier state frequently perpetuates transmission to the next generation (André, 2000). Internationally coordinated attempts by the World Health Organization and the Global Alliance for Vaccines and Immunization to introduce universal immunization are aimed at reducing HBV carriage and, ultimately, eradication of human HBV infection (Kao & Chen, 2002).

Human HBV variants from different geographical regions differ in nucleotide sequence by up to 10–13% from each other, and are currently classified into a total of eight genotypes. Genotypes A, D and possibly G have global distributions, genotypes B and C are found predominantly in East and South-East Asia, genotype E is found in West Africa and genotypes F and H predominate amongst various population groups, including indigenous peoples, in Central and South America (Norder et al., 1994; Arauz-Ruiz et al., 1997, 2002). Active and resolved HBV infections are also found in chimpanzees (Hu et al., 2000; MacDonald et al., 2000; Takahashi et al., 2000; Vartanian et al., 2002) and in gibbons and orangutans in South-East Asia (Warren et al., 1999; Grethe et al., 2000; Verschoor et al., 2001; Noppornpanth et al., 2003) at frequencies approaching those found in human populations in endemic areas (reviewed by Starkman et al., 2003). HBV variants infecting chimpanzees and gibbons are genetically distinct from each other and from each of the human genotypes, providing evidence for the existence of separate, indigenous virus populations. Although there is currently no evidence that human populations have been or are infected with chimpanzee- or gibbon-associated HBV variants, the existence of this non-human primate reservoir of HBV will clearly hamper attempts at eradication of human HBV infection.

To investigate the extent of this potential reservoir of HBV infection, we surveyed populations of the pileated gibbon...
(Hylobates pileatus) and yellow-cheeked gibbon (Nomascus gabriellae) indigenous to the northern and south-western regions of Cambodia (H. pileatus) and east of the Mekong River (N. gabriellae) for HBV infection. Although threatened by hunting and habitat destruction, these species are the most abundant non-human apes in this part of South-East Asia and represent a possible source of human HBV reinfection in the future. To estimate the size of this potential reservoir, blood samples were collected for HBV testing from 26 originally wild-caught H. pileatus and two N. gabriellae gibbons on entry or after entry into the Phnom Tamao Wildlife Rescue Centre, just outside Phnom Penh, Cambodia. Although 19 of the 28 gibbons had been housed with conspecifics for short, variable periods before HBV testing, a similar frequency of HBV infection was found in wild-caught gibbons that were tested whilst in initial quarantine [four out of nine (MGI, GI_19, BG21 and SGIII), compared with seven out of 19]. None of the animals had been housed with other gibbon or primate species.

Gibbons were anaesthetized with ketamine and heparin-anticoagulated samples were collected by femoral venepuncture from each animal. Plasma from the separated sample was assayed for HBV surface antigen (HBsAg) by using a commercially available automated assay (AxSYM; Abbott Laboratories). Samples from 12/26 (46 %) plicated gibbons were found to be positive, whereas those from the two yellow-cheeked gibbons were negative. Among HBsAg-positive samples, 11/12 (91-7 %) were positive for HBV DNA by PCR using DNA extracted from 100 µl sample volumes of plasma [QIAamp DNA blood mini kit (Qiagen), following the manufacturer’s instructions], as described by Nakai et al. (2001). The observed high frequency of active infection in H. pileatus provides further evidence for a widespread and endemic pattern of HBV infection in gibbons in South-East Asia, and is comparable to that found in this species in Thailand (Noppornpanth et al., 2003).

To characterize the HBV variants infecting the 11 HBsAg-positive plicated gibbons, HBV sequences were amplified by PCR using previously described sets of nested primers that amplify overlapping segments of the genome, spanning the complete genome (MacDonald et al., 2000). These comprised the following primer combinations (first round, followed by second round): fragment A, S1/24, S3/23; fragment B, 41/16, 42/15; fragment C, 2/40, 4/38; fragment D, 28/3, 29/34; fragment E, 11/PS44 (new primer, sequence 5'-GGCCGAGACCKGCTGCGAGCRAA-3'), 25/PS45 (new primer, sequence 5'-AGGAGTTCGCCNGTATGGA-TCGG-3'). All amplification reactions were carried out by using the following thermal cycles for first and second rounds of amplification: 94 °C for 18 s, 50 °C for 24 s, 72 °C for 90 s (30 cycles) and a 7 min final extension step at 72 °C. Nucleotide sequencing was carried out directly on second-round amplification products by using an ABI BigDye kit (Applied Biosystems), followed by capillary electrophoresis on an ABI 3730 sequencer. Complete genome sequences were aligned with previously published HBV sequences by using CLUSTAL W with default settings and the SIMMONIC sequence editor (Simmonds & Smith, 1999). Sequences comprised three representative HBV sequences of human genotypes A–H (Fig. 1) and non-human primate HBV sequences from chimpanzees (n = 12), gorilla, gibbon species (n = 15), orangutan (n = 2) and the woolly monkey (GenBank accession numbers are shown in Fig. 1). Sequence comparisons in the S gene included additional further partial sequences from chimpanzees, gibbons and orangutans (listed in Fig. 2).

Each HBV sequence was 3182 bp in length and co-linear with the previously published gibbon HBV sequences. Each sequence similarly contained intact reading frames for the pol, core, X and pre-S1, pre-S2 and HBsAg genes that were equivalent in location to those in other gibbon HBV sequences. In the pre-core region, the HBV variant from GI_8 contained a pre-core stop mutation at position 1896 (codon 29), classified as M2 (Lok et al., 1994). GII_16 showed a wild-type G at position 1896 (tryptophan), whereas the remainder of the gibbon sequences contained T (leucine). The sequence from GIII_16 showed a further G to A (M4) change at position 1899. All gibbon sequences contained a T residue at position 1858 (M1). The pre-core encapsidation sequence was therefore destabilized in nine of the 11 gibbon sequences.

HBsAg gene sequences from the gibbon samples showed variability in the major hydrophilic region containing the a, d,y and r/w antigenic determinants. By using the previously established association between amino acid residues at positions 122 and 160 in the HBsAg gene (Okamoto et al., 1986), HBV variants from GII-8, GI-19 and MGI could be predicted to have an adr serotype, whereas the remainder would be ayrw. The region between residues 100 and 160 was otherwise characterized by the presence of mostly conservative and frequently polymorphic amino acid substitutions (110L, S113T, T114S, T126I, N131T, F134Y and T1435) that were generally shared with previously published gibbon- and other primate-derived HBV sequences.

By phylogenetic analysis, all 11 sequences obtained in this study grouped with those derived previously from gibbons and orangutans, together producing a clade with 100 % bootstrap support. Inclusion of the new gibbon sequences did not alter the interspersed position of the orangutan sequences in this phylogenetic group. The topology of the phylogenetic tree was constructed by the neighbour-joining method was congruent with those constructed by the maximum-likelihood and parsimony tree-building methods (data not shown).

A further comparison was made with a larger number of gibbon sequences derived from the HBsAg gene (Fig. 2). In this analysis, gibbon sequences were identified by species and, for H. pileatus gibbons, also by geographical origin (if known). By this analysis, gibbon sequences were divided into several distinct, bootstrap-supported
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phylogenetic groups, some corresponding to the previously described, species-associated genetic groups I–V (Grethe et al., 2000). Some species of gibbons were infected with variants from a single genetic group, such as those from *Hylobates lar* [derived from four independent studies; Grethe et al., 2000 (genetic group I); Lanford et al., 2000; Noppornpanth et al., 2003; Starkman et al., 2003]. Similarly, all but two of the sequences from the various *Nomascus* species grouped together (group N; genetic group V). These groupings were generally comparable with those observed for HBV sequences derived from orangutans (group PP).

![Fig. 1. Phylogenetic analysis of complete genome sequences of HBV from gibbons and comparison with previously published sequences of HBV from other apes and representative sequences of human genotypes A–H. Phylogenetic trees for these datasets were estimated by using the neighbour-joining tree-building method with Jukes–Cantor-corrected pairwise distances (scale indicated under key). The robustness of each phylogenetic grouping was demonstrated by bootstrap resampling using 100 sets of permuted data. The tree was rooted with the woolly monkey HBV sequence, GenBank accession no. AF046996. Bootstrap values of 70% or greater are shown on branches. The species origin of sequences obtained in this study and in previous studies is indicated by the key.](http://vir.sgmjournals.org)

![Fig. 2. Phylogenetic analysis of HBsAg gene sequences from different species of gibbons and orangutans, as identified in the key, using neighbour-joining with Jukes–Cantor-corrected pairwise distances (scale indicated under key). The tree was rooted with a human HBV genotype F sequence, HHVBFFOU. Bootstrap values of 70% or greater are shown on branches. The geographical origin of the *H. pileatus* sequence HBV131572 is unknown.](http://vir.sgmjournals.org)
In contrast to these species/genetic group associations, HBV variants infecting *H. pileatus* fell into two distinct lineages (HP1 and HP2; Fig. 2). HP1 includes sequences from three of the gibbons analysed in this study (MGI, GI_19 and GIH_8), as well as several sequences derived from HBV-infected pileated gibbons that were previously obtained in four independent studies [Grethe et al., 2000 (genetic group III); Aiba et al., 2003; Noppornpanth et al., 2003; Starkman et al., 2003]. In cases where the geographical location of the infected animals was stated, all were reported to have originated from Thailand. However, the majority of *H. pileatus* sequences obtained in this study (eight from 11) fell into a separate lineage (HP2) and were genetically distinct from all but one of the previously published *H. pileatus* sequences (GenBank accession no. AF477488; Noppornpanth et al., 2003). Despite the frequent lack of precision in locating the original sources of infection in the gibbons analysed in the current and previous studies, there appears to be a substantial difference in HBV genotype distribution between apes from Thailand and those from Cambodia.

Although there remains the possibility that some of the HBV infections that were reported in *H. pileatus* primates in previous studies were acquired in captivity from other gibbon or primate species, all variants in these and the current study fell into genetic groups HP1 and HP2. Indeed, the consistent grouping of *H. pileatus* sequences into the two groups suggests that at least two distinct strains of HBV circulate in this species in South-East Asia, and that the primary source of infection in captive animals is from the wild. In contrast to earlier suggestions (Vaudin et al., 1988; Lanford et al., 2000), the consistent finding of species-associated variants of HBV in gibbons adds further weight to the view that HBV infection in captive apes is not generally the result of human-to-ape transmission.

The ultimate origin of HBV in the various ape species (including man) remains unclear. We previously obtained evidence that the distribution of non-human ape-derived variants of HBV was most congruent with geographical distribution in South Asia, rather than with host species (Starkman et al., 2003). Thus, the northern-dwelling *Nomascus* species in Vietnam, north and eastern Laos and southern China carried HBV variants that were distinct from those in species in central regions (such as Cambodia and Thailand) and again distinct from those in Sumatra and Borneo, representing the southern end of the distribution of gibbons. The finding that HBV variants from orangutans were related most closely to, and frequently interspersed with, those from gibbons from these southern regions provides strong evidence for the proposed geographical, rather than strict species, association of HBV.

Although the subjects in the current study were obtained from a single geographical region, the observation that the infected pileated gibbons carried two distinct lineages of HBV provides further evidence against the previously proposed species association of HBV (Grethe et al., 2000).

Furthermore, the observation of differences in the frequencies of infection with HBV groups HP1 and HP2 between gibbons from Thailand and Cambodia strengthens the hypothesis for the existence of geographically differentiated circulating populations of HBV in the wild. Future investigation of HBV variants infecting *N. gabriellae*, which is isolated geographically from *H. pileatus* by the Mekong river, would allow the link between geographical distribution and HBV sequence differentiation to be further explored.

In summary, the data provide further evidence for a scenario in which reservoirs of HBV infection are maintained amongst South-East Asian ape species in the wild. From observations of HBV infections in orangutans and gorillas (Starkman et al., 2003), it is clear that there is potential for cross-species transmission between animals in adjacent or overlapping ranges and that this accounts for the geographical rather than strict species association of HBV genotypes that follows from previously proposed co-speciation theories (Norder et al., 1994; Magnus & Norder, 1995; MacDonald et al., 2000). The existence of these reservoirs and the potential for ape-to-human transmission may lead to problems with HBV eradication from human populations in the future.

References


