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ORIGINAL ARTICLE

# Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon

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**Keywords**
ELISA – gibbon – hepatitis B virus – infection – non-human primate – orangutan – phylogenetic tree – real-time PCR – serological markers – transmission

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## Abstract

*Background* Hepatitis B virus (HBV) is a public health problem worldwide and apart from infecting humans, HBV has been found in non-human primates.
*Methods* We subjected 93 non-human primates comprising 12 species to ELISA screening for the serological markers HBsAg, antiHBs and anti-HBc. Subsequently, we detected HBV DNA, sequenced the whole HBV genome and performed phylogenetic analysis.
*Results* HBV infection was detected in gibbon (4/15) and orangutan (7/53). HBV DNA isolates from two gibbons and seven orangutans were chosen for complete genome amplification. We aligned the *Pre-S*/*S*, *Pre-C*/*C* and entire genomes with HBV sequences and performed phylogenetic analysis. The gibbon and orangutan viruses clustered within their respective groups.
*Conclusions* Both geographic location and host species influence which HBV variants are found in gibbons and orangutans. Hence, HBV transmission between humans and non-human primates might be a distinct possibility and additional studies will be required to further investigate this potential risk.

## Introduction

Human hepatitis B virus (HBV) is the prototype member of the family *Hepadnaviridae*. It is a spherical enveloped particle containing partially double stranded DNA and RNA dependent DNA polymerase. The majority of infections by this diminutive viral genome affect humans. Hence, various research projects have been aimed at accumulating information on human hepatitis B. In nature, HBV has been found in non-human primate species such as chimpanzees (*Pan troglodytes*) (ChHBV) [1], orangutans (*Pongo pygmaeus*) (OuHBV) [2], wild and captive gibbons (*Hylobates* sp. and *Nomascus* sp.) (GiHBV) [3], gorillas (*Gorilla gorilla*) (GoHBV) [4] and woolly monkeys (*Lagothrix lagothricha*) (WMHBV) [5]. However, information on epidemiology, genome and pathogenicity of non-human primate hepatitis B virus has remained rather limited and mainly been gleaned from captive animals. According to Deinhardt's

survey (1976), hepatitis B surface antigen (HBsAg) has been found in chimpanzees, gibbons and orangutans, whereas marmosets (*Callithrix jacchus*), squirrel monkeys (*Saimiri* sp.), baboons (*Papio* sp.), rhesus macaques (*Macaca mulatta*) and vervet monkeys (*Cercopithecus aethiops*) apparently are devoid of both HBsAg and antiHBs [6]. Up to now, there have been several studies on serological markers of HBV infection in *Cercopithecidae* monkeys [7, 8]. However, all studies showed negative results for serological HBV markers and no attempt at HBV amplification has been successful in this family [8]. Southeast Asia is an area endemic for HBV infection. Several studies have undertaken serological surveys on the families *Pongidae* and *Hylobatidae* to determine epidemiology, phylogenetic relationships and route of cross-species transmission. For example, Warren et al. have examined 195 orangutans from Borneo and Sumatra [2], Grethe et al. have investigated 12 gibbons from different parts of Thailand and one gibbon from Vietnam [4]. Noppornpanth et al. have performed studies on 101 captive gibbons from central Thailand [3]. Sall et al. have investigated the population of pileated gibbon and yellow-cheeked gibbon in the northern and south-western regions of Cambodia and east of the Mekong river [9]. From all these studies, a high prevalence (40–46%) of HBV infection in gibbons and orangutans in this region has become evident.

Upon characterization of the respective nucleotide sequences human hepatitis B virus was divided into distinct genetic groups. Accordingly, Okamoto et al. differentiated HBV into four genetic groups or genotypes (A, B, C, and D) based on nucleotide differences between sequences of 8% or above [10]. Subsequently, four additional genotypes of HBV (E, F, G and H) have been identified [11–16]. Genetic characterization is advantageous in that it reveals the relationship among these sequences as well as to the known primate HBV sequences. Many experiments have shown that the sequences of non-human primates are on a genetic branch separate from other known human HBV sequences [12, 13, 17–19]. Moreover, the phylogenetic clusters of this virus are directly related to geographic host distribution [4, 8, 9]. Cross–species transmission has been documented. GiHBV and OuHBV were grouped together because both species share the same habitat in Southeast Asia [20]. Interestingly, the primate viruses are closely related to the human HBV [21]. In addition, the structure of HBV virus found in humans and non-human primates is very similar. HBV transmission from humans to non-human primates or vice versa might be possible once their habitats overlap.

The study reported here has been aimed at elucidating epidemiology, pathogenicity and potential reservoirs of HBV infection in non-human primates. In addition, our group has sequenced the entire genomes of HBV isolated from the carriers. Furthermore, we have performed phylogenetic analysis on both virus isolates to investigate their genetic relatedness to the previously identified human and non-human primate strains of HBV.

## Materials and methods

### Study population

To investigate the potential reservoirs of HBV infection among captive non-human primates, 17 macaques (10 long-tailed macaques, *Macaca fascicularis*, four southern pig-tailed macaques, *Macaca nemestrina*, two stump-tailed macaques, *Macaca arctoides* and one rhesus macaque, *Macaca mulatta*), 15 gibbons (six white-cheeked gibbons, *Nomascus leucogenys*, one yellow-cheeked Gibbon, *Nomascus gabriellae,* six pileated gibbons, *Hylobates pileatus* and two white-handed gibbons, *Hylobates lar*), eight langurs (four silvered langurs, *Semnopithecus cristatus*, one Phayre's langur, *Semnopithecus phayrei*, and three dusky langurs, *Semnopithecus obscurus*) kept at Dusit zoo, Bangkok and 53 orangutans (*Pongo pygmaeus*) kept at Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand were subjected to this research project, which had been approved by the Faculty of Veterinary Science, Animal Care and Use Committee (FVS – ACUC), Mahidol University.

### Sample collection

During the routine health check, all primates were anaesthetized. From each animal blood samples were collected by venipuncture and transferred to EDTA anticoagulant coated test tubes. Plasma was separated by centrifugation at 3000 rpm for 10 minutes and kept at −70°C until tested. The demographic data of the primates have been obtained from the records of the zoo and wildlife breeding center.

### Serological method

Plasma samples were subjected to a biochemical analyzer (Hitachi 912, Roche Diagnostics, Mannheim, Germany) for biochemical analysis of alanine aminotransferase (ALT) and aspartate transaminase (AST). Plasma was assayed for HBsAg, antibodies to HBsAg (antiHBs), and antibodies to the HBV core antigen


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(antiHBc) by enzyme linked immunosorbent assay (ELISA) using the Murex HBsAg Version 3, Murex antiHBs and Murex antiHBc kit, respectively (Murex, Biotech Limited, Dartford, Kent, England).

### HBV DNA extraction and detection

HBV DNA was extracted from 100-$\mu$l plasma samples using proteinase K in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation [22]. The DNA pellets were dissolved in 30 $\mu$l sterile distilled water. Subsequently, our team performed PCR and real-time PCR assays to determine the quantitative HBV DNA levels as previously described [23].

### Whole genome amplification and sequencing

Two HBsAg positive samples of gibbons (*Nomascus leucogenys*) with high viral load and all seven samples of orangutans (*Pongo pygmaeus*) were subjected to complete HBV genome amplification by PCR using four primer sets selected from conserved regions so that the resulting amplicons overlapped contiguous fragments. The primer sequences of set one were PreS1F+ (5′-GGGTCACCATATTCTTGGGAAC-3′: position 2814 to 2835) and R5 (5′– AGCCCAAAAG-ACCCACAATTC–3′: position 1015 to 995); of set 2, F6 (5′– ATATGGATGATGTGGTATTGGG–3′: position 737 to 758) and X102 (5′– ACCTTTAACC-TAATCTCC-3′: position 1764 to 1748); of set 3, X101 (5′-TCTGTGCCTTCTCATCTG-3′: position 1552 to 1569) and CORE2 (5′-CCCACCTTATGAG-TCCAAGG-3′: position 2476 to 2457) and of set 4, CORE1 (5′-GAGTGTGGATTCGCACTCCTCC-3′: position 2268 to 2289) and R1 (5′-TGTAACACGAG-CAGGGGTCCTA-3′: position 201 to 180). The total 25-$\mu$l reaction mixture comprised 2 $\mu$l of a resuspended HBV viral DNA solution, 10 $\mu$l of 2.5X Eppendorf® MasterMix (Eppendorf, Hamburg, Germany), 0.5 $\mu$l of 25 $\mu$M primer, and sterile water. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 3 minutes followed by 35 cycles at 94°C for 30 s (denaturation), 55°C for 30 s (primer annealing), 72°C for 1.30 minutes (extension) and a final extension step at 72°C for 7 minutes. PCR-amplified products were examined by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Subsequently, the bands of interest were purified applying the Perfectprep®Gel Cleanup kit (Eppendorf, Hamburg, Germany). Cycle sequencing was performed using the AmpliTaq™ DNA Polymerase FS dye terminator cycle sequencing chemistry of the ABI PRISM™ BigDye™ Terminator CyCle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA). The reaction was performed according to the manufacturer's specifications. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and submitted to the GenBank database.

### Phylogenetic analyses

The two sequences of captive gibbon HBV (G25 and G26) determined in the course of our previous study [3] in the Krabok Koo Wildlife Breeding Center, and the nine HBV sequences (two GiHBV and seven OuHBV sequences) obtained in the course of this study were aligned with each human genotype. Sequences were also compared with available complete genome sequences from chimpanzee, gibbon, orangutan, woolly monkey and gorilla. Our team performed phylogenetic analyses and genetic comparisons of HBV isolates applying the Clustal X (1.83) multiple alignment program. Subsequent analysis was performed by Molecular Evolutionary Genetics Analysis (MEGA) software version 3.1. Amino acid translations were accomplished using the ExPASy translation tool (available on http://www.expasy.ch/tools/dna.html).

## Results

### Seroprevalence of HBV in non-human primates

Ninety-three plasma samples of various non-human primates were tested for the presence of HBsAg and antiHBs and antiHBc antibodies. Sera positive for at least one marker of HBV infection were found only in gibbon (9/15; 60%) and orangutan (40/53; 75.47%). The results are shown in Table 1. Moreover, four gibbons and seven orangutans were identified as chronic carriers. To determine the liver pathology associated with this infection, ALT and AST levels were determined in the plasma of four HBsAg positive gibbons and 36 HBsAg negative animals. With one exception (GD14), the ALT and AST levels of infected individuals were within normal limits (0–40 U/l) upon comparison with HBsAg negative animals (Table 2). The mean ± SD of ALT and AST values in HBsAg negative animals were 33.64 ± 17.56 and 34.82 ± 15.15, respectively. Approximately 55.56% (5/9) of gibbons and 82.5% (33/40) of orangutans were non-carrier animals, as indicated by the presence of antiHBs and antiHBc antibodies.

**Table 1** Seroprevalence of HBV and HBV DNA among captive non-human primates

| Primate (n) | n | Positive HBV marker | | | |
|---|---|---|---|---|---|
| | | HBsAg and antiHBc n (%) | antiHBs and antiHBc n (%) | antiHBc only n (%) | HBV DNA n (%) |
| 1. Macaque (17) | | | | | |
| *Macaca fascicularis* | 10 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Macaca nemestrina* | 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Macaca arctoides* | 2 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Macaca mulatta* | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 2. Langur (8) | | | | | |
| *Semnopithecus cristatus* | 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Semnopithecus phayrei* | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Semnopithecus obscurus* | 3 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 3. Gibbon (15) | | 4 (26.67) | 5 (33.33) | 0 (0) | 4 (26.67) |
| *Nomascus leucogenys* | 6 | 2 (33.33) | 3 (50) | 0 (0) | 2 (33.33) |
| *Nomascus gabriellae* | 1 | 1 (100) | 0 (0) | 0 (0) | 1 (100) |
| *Hylobates pileatus* | 6 | 1 (16.7) | 1 (16.7) | 0 (0) | 1 (16.7) |
| *Hylobates lar* | 2 | 0 (0) | 1 (50) | 0 (0) | 0 (0) |
| 4. Orangutan (53) | | | | | |
| *Pongo pygmaeus* | 53 | 7 (13.21) | 25 (47.17) | 8 (15.09) | 7 (13.21) |
| Total | 93 | | | | |

**Table 2** Demographic data, ALT, AST, HBV serological markers and HBV viral load of captive non-human primates

| No | Species | Sex | Age | Cage | Code | GenBank accession no. | ALT (U/l) | AST (U/l) | HBsAg | antiHBs | antiHBc | HBV DNA | HBV DNA (copies/$\mu$l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *N. gabriellae* | M | 8 | – | GD13 | – | 17 | 14 | + | – | + | + | $2.830 \times 10^6$ |
| 2 | *N. leucogenys* | F | 21 | – | GD14 | EU155828 | 79 | 75 | + | – | + | + | $2.002 \times 10^7$ |
| 3 | *N. leucogenys* | M | 11 | – | GD21 | EU155829 | 18 | 14 | + | – | + | + | $3.085 \times 10^7$ |
| 4 | *H. pileatus* | F | ND | – | GD22 | – | 16 | 9 | + | – | + | + | $5.26 \times 10^6$ |
| 5 | *P. pygmaeus* | M | 6–8 | 3/10 | OS6 | EU155821 | ND | ND | + | – | + | + | $3.660 \times 10^6$ |
| 6 | *P. pygmaeus* | F | 3–4 | 3/10 | OS9 | EU155822 | ND | ND | + | – | + | + | $1.875 \times 10^6$ |
| 7 | *P. pygmaeus* | M | 5 | 3/6 | OS23 | EU155823 | ND | ND | + | – | + | + | $4.680 \times 10^5$ |
| 8 | *P. pygmaeus* | M | 5 | 3/5 | OS25 | EU155824 | ND | ND | + | – | + | + | $7.070 \times 10^6$ |
| 9 | *P. pygmaeus* | M | 6–8 | 3/5 | OS27 | EU155825 | ND | ND | + | – | + | + | $1.210 \times 10^7$ |
| 10 | *P. pygmaeus* | F | 6–8 | 3/5 | OS28 | EU155826 | ND | ND | + | – | + | + | $5.440 \times 10^6$ |
| 11 | *P. pygmaeus* | M | 5 | 3/1 | OS39 | EU155827 | ND | ND | + | – | + | + | $8.250 \times 10^5$ |

ALT and AST normal range: 0–40 U/l. ALT and AST (Mean ± SD) values in animals with HBsAg negative were 33.64 ± 17.56 and 34.82 ± 15.15.
ND, no data.

### Detection of HBV DNA in non-human primates

To quantify HBV DNA in non-human primates, we screened available plasma samples collected from various species by real-time PCR according to the previously described method [23]. The limit of this method is 100 copies per $\mu$l. The results from real-time PCR perfectly correlated with the results obtained by HBsAg screening. The levels of HBV DNA are shown in Table 2.

### Gibbon and orangutan HBV nucleotide sequences

The complete HBV genomes from nine non-human primates, two from gibbon (*Nomascus leucogenys*) and seven from orangutan (*Pongo pygmaeus*), comprised 3182 nucleotides and showed genetic organization compatible with the human virus. The nucleotide sequences determined in this research study have been submitted to the GenBank database and assigned accession numbers EU155821–EU155827 (Orangutan), EU155828–EU155829 (Gibbon). All gibbon and orangutan HBV sequences were compared with the representative sequences in GenBank including the eight human HBV genotypes, orangutan, gibbon, chimpanzee, gorilla and woolly monkey sequences.

#### *Pre-S/S gene*

Our group aligned the nucleotides of the *Pre-S/S* region in order to establish nucleotide and amino acid


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differences between HBV isolates from gibbons, orangutans and human HBV genotypes B and C. Moreover, the GiHBV and OuHBV sequences were compared with other HBV strains. Based on the percentage of similarity, the highest percentage of similarity within each respective group, GiHBV and OuHBV displayed a higher percentage of similarity than to the human HBV (data not shown). The nucleotide sequences of two gibbons of this study, two gibbons from the previous study [3], seven orangutans and human HBV genotypes B and C were translated into amino acid sequences (Fig. 1A). Comparison of gibbon and orangutan *Pre-S/S* gene sequences with human HBV genotypes B and C, the genotypes endemic in Southeast Asia, revealed that HBV isolated from gibbons and orangutans had a deletion of 33 nucleotides representing 11 codons at the 5′end of the Pre-S1 region as had already been established (Fig. 1A). For G25, we discovered an insertion of Gln (Q) between $Gly^{83}$ (G) and $Ile^{84}$ (I). In addition, several variability were found in the Pre-S2 and S regions, but neither deletions nor insertions could be detected in all isolates.

On closer investigation, the only non-human primate (orangutan and gibbon) amino acids we found in the Pre-S1 region were $Gln^{14}$ (Q), $Glu^{27}$ (E), $Leu^{33}$ (L), $Thr^{56}$ (T), $Val^{92}$ (V), in the Pre-S2 region, $Val^{7}$ (V), and in the S region, $Ala^{190}$ (A), $Leu^{193}$ (L) and $Ile^{213}$(I).

*Pre-C/C gene*

The *Pre-C/C* gene sequences were less divergent than the *Pre-S/S* gene. Our team could not discern any mutations in either the core promoter region (nucleotide positions T1753C/A, A1762T and G1764A) or the *Pre-C* region (nucleotide positions T1858C, G1896A and G1899A). Yet, all our gibbon and orangutan sequences showed a G to T mutation at position 1896. This mutation induced an amino acid change from Try (W) to Leu (L) at amino acid residue 28 of the Pre-C region. The nucleotides at positions 2174 to 2413 of the core region were highly conserved; the resulting amino acid sequences were similar for all isolates. Alignment of the core protein's amino acids showed that the amino acids in this region are highly conserved among OuHBV, GiHBV and human HBV. The only non-human primate (orangutan and gibbon) amino acids are $Leu^{28}$ in the Pre-C region and $Val^{27}$ (V), $Asn^{51}$ (N), $Val^{59}$ (V), $Thr^{67}$ (T), $Ser^{70}$ (S), $Asn^{74}$ (N), $Pro^{179}$ (P) and $Ala^{180}$ (A) in the C region (Fig. 1B).

*Complete HBV genome*

All sequences were analyzed by comparison with each of the human HBV genotypes A-H. The results showed that all isolates were 98–99% identical within the orangutan group and 93–98% within the gibbon group. Comparison between gibbon and orangutan sequences showed 90–91% identity (data not shown).

## Phylogenetic analyses of gibbon and orangutan HBV

To determine the phylogenetic relationships, phylogenetic trees of the *Pre-S/S*, the *Pre-C/C* region and the complete nucleotide sequence were constructed.

*Phylogenetic tree of Pre-S/S* gene

This phylogenetic tree comprises the *Pre-S/S* nucleotide sequences of OuHBV and GiHBV isolates from the present project, representative of non-human primates and of each human HBV genotype from GenBank. All *Pre-S/S* sequences including gibbon sequences from our previous study were examined by neighbor joining analysis. The results are shown in Fig. 2A. Furthermore, the HBV isolates from the gibbons (GD14, GD21, G25 and G26) were found distantly (91–93%) related to the OuHBV sequences (OS6, OS9, OS23, OS25, OS27, OS28, and OS39). OuHBV in this study clustered with orangutan from Indonesia (Y17559) by 100% bootstrap value.

*Phylogenetic tree of the Pre-C/C* gene

The results of phylogenetic analysis of the *Pre-C/C* gene were similar to the Pre-S/S region in that the gibbon and orangutan. *Pre-C/C* gene was on separate branches from each human genotype (Fig. 2B). The GiHBV sequence determined from our earlier research branched most closely with AJ131574 for G25 and clustered with AY330914 and AJ131568 for G26. The bootstrap values were 49 and 98%, respectively. Whereas GD14 and GD21 were different from the GiHBV of the preceding study because they clustered with AJ131573 with a bootstrap value of 100%. Both AJ131573 and AJ131574 had been isolated from *H. concolor* kept in Dusit zoo, Thailand. All orangutan sequences related with AF193863, an orangutan virus from Kalimantan, Indonesia.

*Phylogenetic tree of the complete HBV genome*

The complete HBV sequences of non-human primates our team had arrived at were compared with sequences representative of each group of human HBV genotype, orangutan, gibbon, gorilla, chimpanzee, and woolly monkey HBV in the GenBank database (Fig. 2C). The woolly monkey sequences were used as an out group. The data support that each of the human genotypes clusters separately from non-human primates whereas all sequences obtained from gibbons and orangutans

## (A) Pre-S/S protein

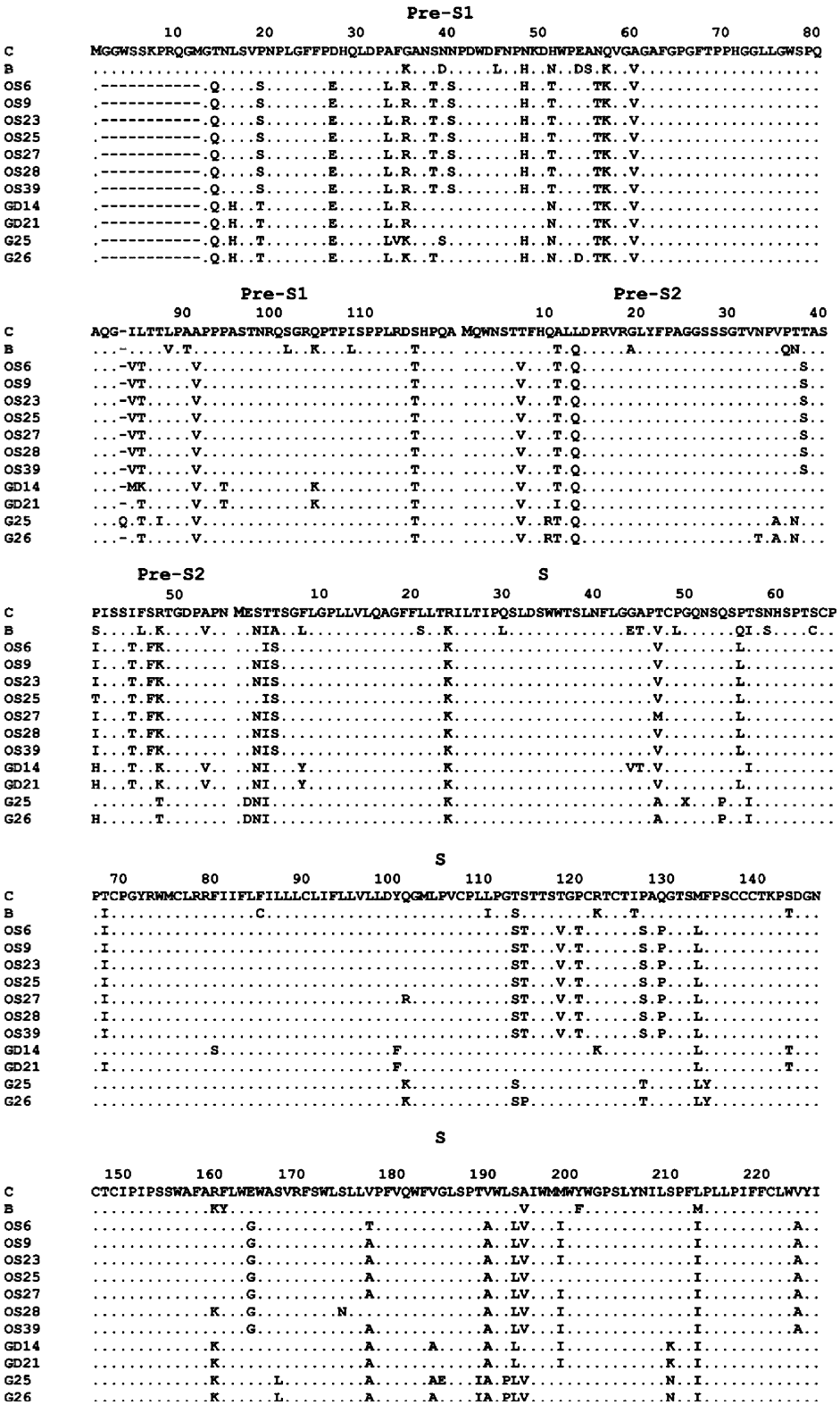




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**Fig. 1** Alignment of amino acid sequences of the complete *S* gene (Pre-S1, Pre-S2 and S domain) (A) and complete *C* gene (Pre-C and Core domain) (B) from four gibbons (GD14, GD21, G25 and G26) and seven orangutans (OS6, OS9, OS23, OS25, OS27, OS28 and OS39) with human HBV genotypes B (accession no. D00331) and C (accession no. X04615). Dots indicated conserved amino acids. Dashes indicated deletion amino acids. Changing amino acids were indicated in letters. A master sequence based on the comparison was shown in the upper line. The percentages of similarity of the complete *S* genes were 99% within the orangutan group, 94% within the gibbon group, 89–91% between the orangutan and human HBV, 89–92% between the gibbon and human HBV and 91–93% between the orangutan and gibbon. While, *Pre-C/C* gene were 98–99% within the orangutan group, 94–98% between gibbon group, 90–92% between the orangutan and human HBV, 90–93% between the gibbon and human HBV and 92–94% similarity between the orangutan and gibbon.

**(B) Pre-C/C protein**

```
                Pre-C                                   C
                10        20                  10        20        30        40        50
C       MQLFHLCLIISCSCPTVQASKLCLGWLWG MDIDPYKEFGASVELLSFLPSDFFPSIRDLLDTASALYREALESPEHCSP
B       ............................. ..................................................
OS6     .........................L. ..........T.............V.....................
OS9     .........................L. ..........T.............V.....................
OS23    .........................L. ..........T.............V..V..................
OS25    .........................L. ......Y...............V.....................
OS27    .........................L. .........GT....F....E....V.....................
OS28    .........................L. ..........T.............V..R..................
OS39    .........................L. ..........T.............V.....................
GD14    .........................L. ..........TA.......P.....V.....................
GD21    .........................L. ..........T.............V.....................
G25     ................F........L. ..........T.............V.....................
G26     ................I........L. ..........T.............V.....................

                                        C
                60        70        80        90       100       110       120       130
C       HHTALRQAILCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPP
B       ..............................................................................
OS6     N.......V.......T..S...N............N...N.....................................
OS9     N.......V.......T..S...N............N...N.....................................
OS23    N...F...V.......T..S...N............N...N.....................................
OS25    N.......V.......T..S...N............N...N.....................................
OS27    N.......V.......T..S...N............N...N.....................................
OS28    N......GV.......T..S...N............N...N.....................................
OS39    N.......V.......T..S...N............N...N.....................................
GD14    N.......V.......T..S...N............N...H.....................................
GD21    N.......V.......T..S...N............K.....................................
G25     N.......V.......T..S...N.............N.....................................
G26     N.......V.......T..S...N............N...N.....................................

                                C

                140       150       160       170       180
C       AYRPPNAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPGRRRSQSRESQC
B       ..........................................R...........
OS6     ..........................................R......PA...
OS9     ..........................................R......PA...
OS23    ..........................................R......PA...
OS25    ..........................................R......PA...
OS27    ..........................................R......PA...
OS28    ..........................................R......PA...
OS39    ..........................................R......PA...
GD14    ..........................................R......PA...
GD21    ..........................................R......PA...
G25     ..........................................R......PA...
G26     ..........................................R......PA...
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**Fig. 1** (Continued)

in this study can be grouped with previously published GiHBV and OuHBV sequences. The novel gibbon sequences clustered as a subgroup with the gibbon sequences previously obtained.

## Discussion

Upon screening various non-human primate species for HBV infection, our group established that approximately 60% of gibbons and 75% of orangutans showed at least one marker of HBV. This rate in gibbons is higher than that found in our previous study (approximately 40%) [3]. In addition, the rates of active infection defined by detectable HBV DNA in gibbons (26.7%) and orangutans (13.2%) are similar to those of non-human primates in Central Africa and Southeast Asia [8]. However, the results in this study were obtained from wild-born primates kept in captivity, and the prevalence of HBV infection in wild gibbons and orangutans is unknown. In contrast, we

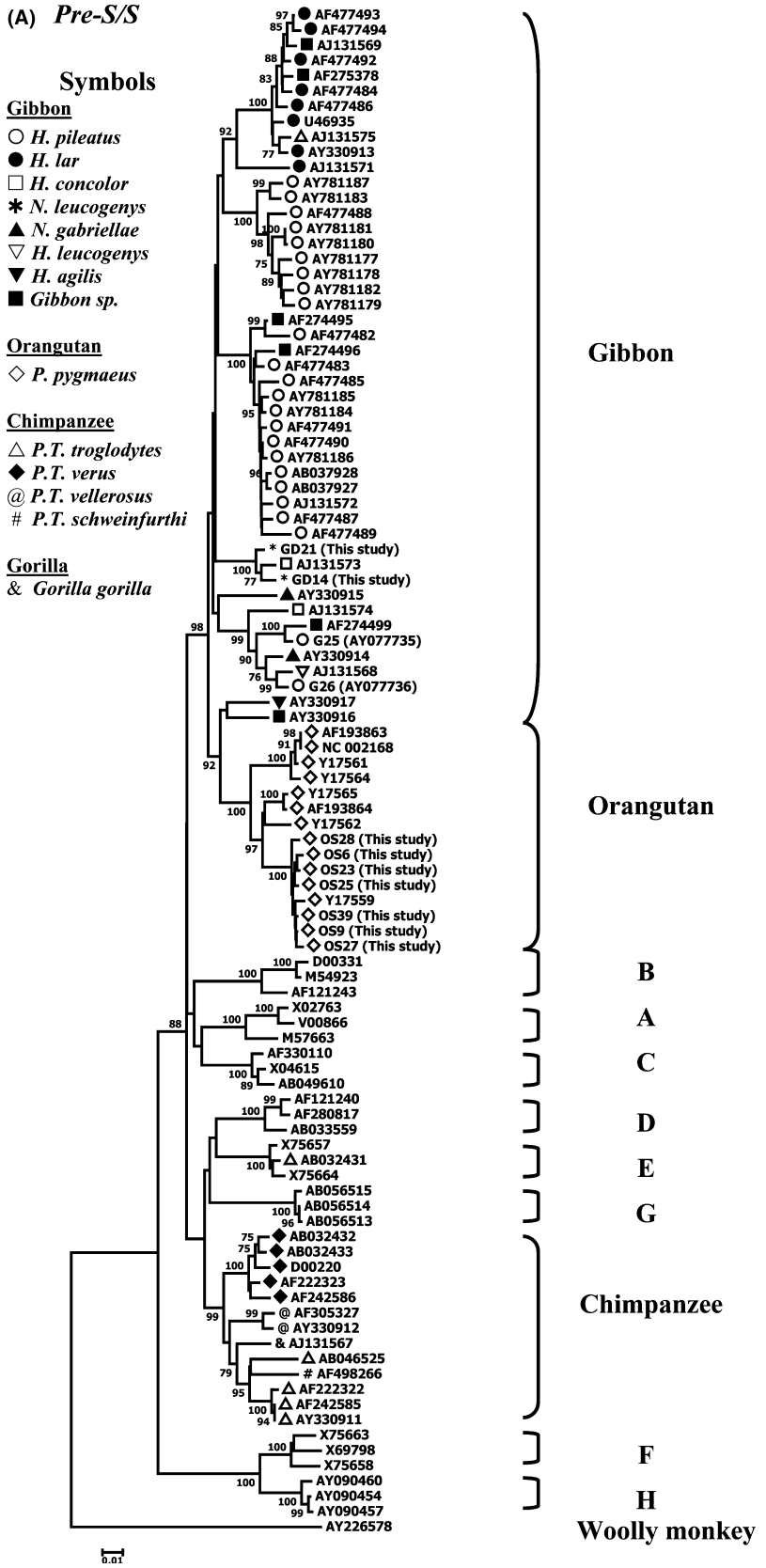


**Fig. 2** Phylogram depicting the phylogenetic relationship of the sequence obtained from the present study and representative sequences of non-human HBV strains from GenBank. Regions included in the comparison were: (A) the large *S* gene including *Pre-S1*, *Pre-S2* and HBsAg gene; (B) the *C* gene, including Pre-C and Core region; (C) entire genome. Percentage bootstrap values (>75%) were shown at the respective nodes. The scale bar at the bottom indicated the genetic distance. The species origin of sequences obtained in this study and in previous studies was indicated by the symbol. [GenBank accession numbers: Gi-HBV – GD14 (EU155828); GD21 (EU155829), OuHBV – OS6 (EU155821); OS9 (EU155822); OS23 (EU155823); OS25 (EU155824); OS27 (EU155825); OS28 (EU155826); OS29 (EU155827)].



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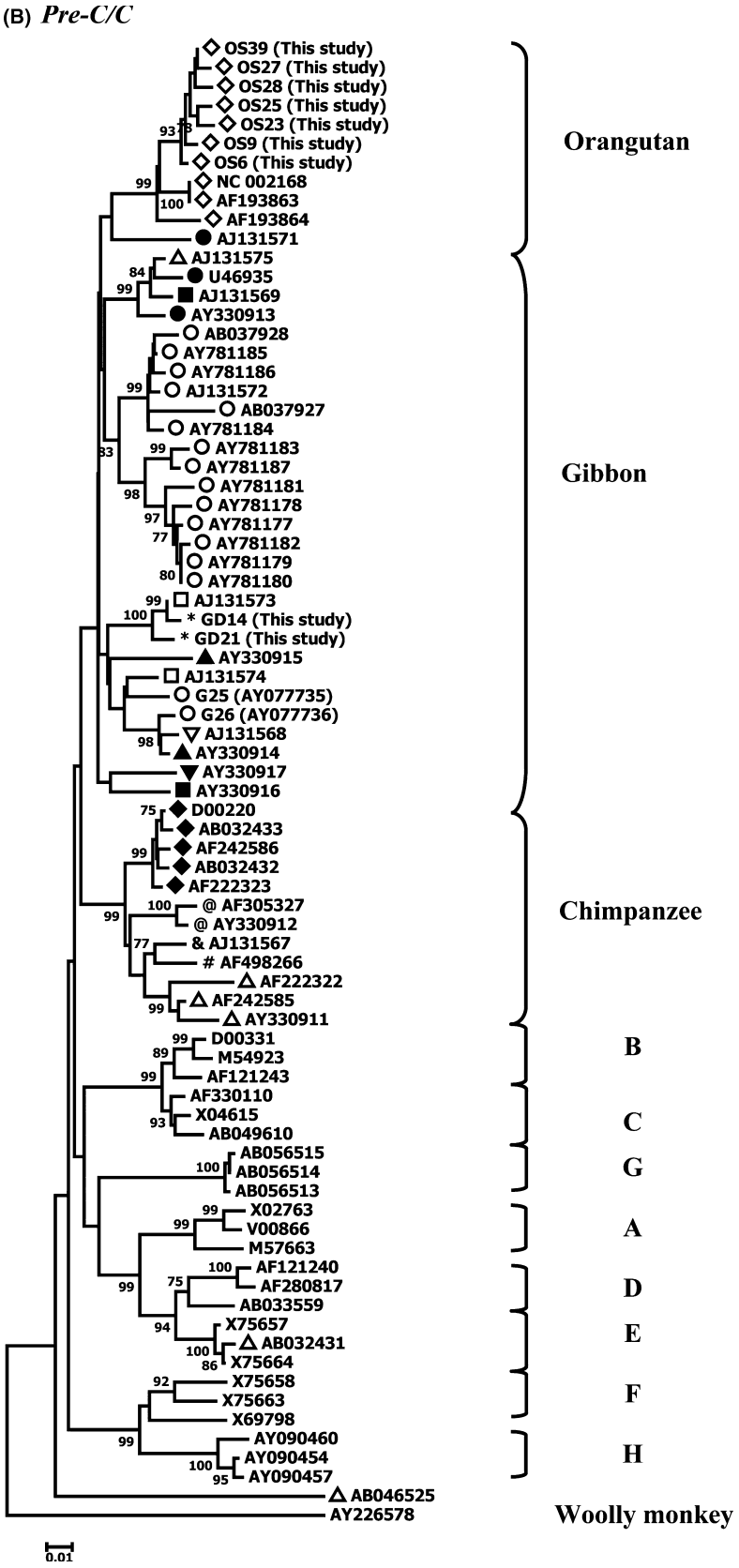


**Fig. 2** (Continued)

(C) **Entire genome**

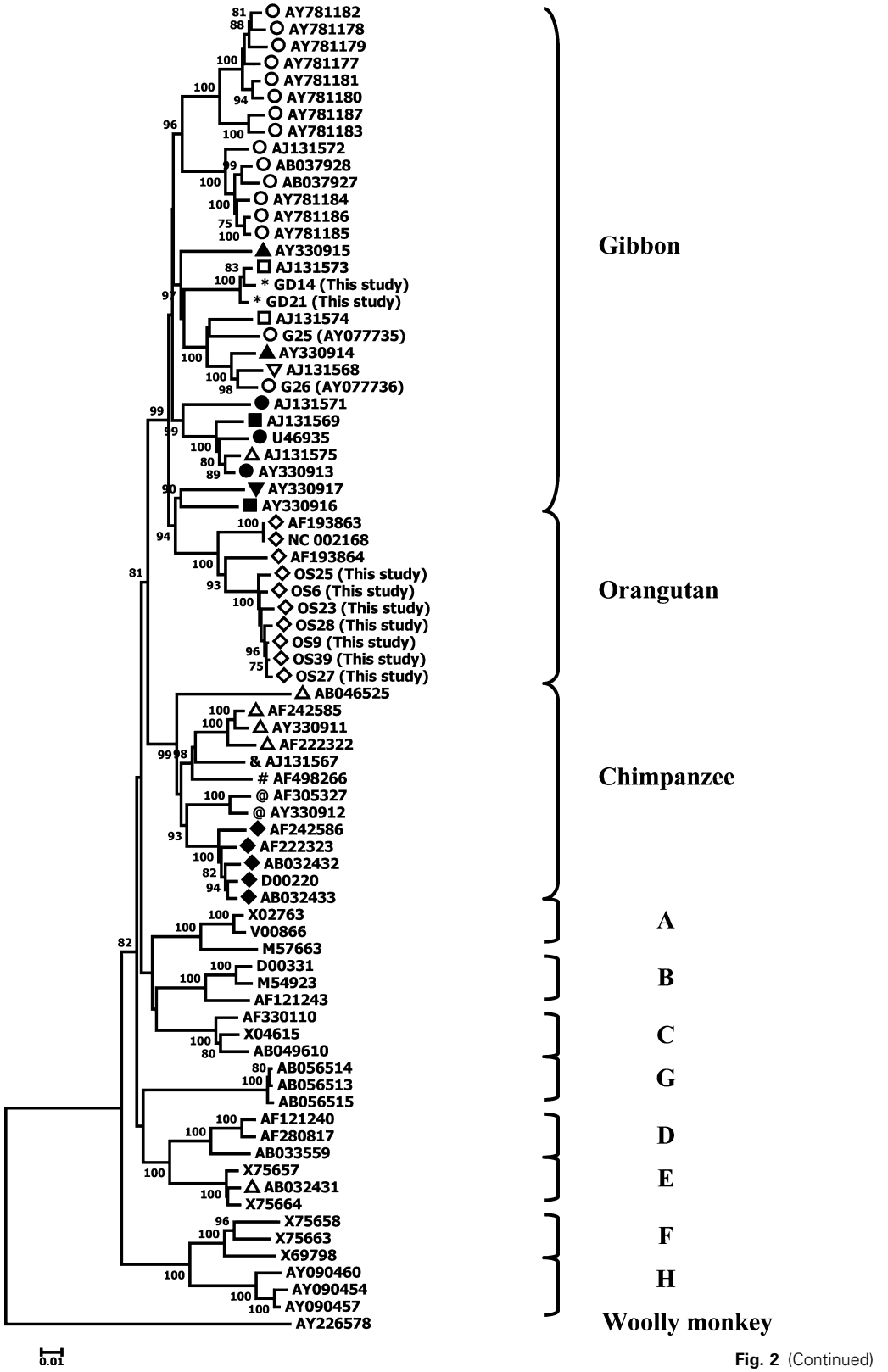


**Fig. 2** (Continued)



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could neither detect any HBV marker nor HBV DNA by real-time PCR in either macaques or langurs. The finding that HBV can and does infect only the members of the families *Pongidae* and *Hylobatidae* strongly supports previous evidence for their rather narrow host range [6–8].

Our results regarding HBV serological markers showed that the majority of orangutans and gibbons had been infected with the virus but managed to resolve the infection, which became apparent by positive results for antiHBs, and negative results for HBsAg and HBV DNA. In contrast, some orangutans and gibbons became chronic carriers and displayed positive results for serum HBsAg and HBV DNA. Interestingly, approximately 15% of orangutans displayed only antiHBc without HBsAg or antiHBs usually accompanying this marker. Such atypical serology is likely attributable to resolved HBV infection since HBV DNA could not be detected in any of the orangutans' plasma. In humans, antiHBc-positive individuals lacking HBsAg are usually considered to have been previously exposed to HBV infection, but a proportion of these patients may have subclinical or occult HBV infection as HBV DNA can be detected in the liver. Occult HBV status is in some cases associated with mutant virus undetectable by commercial HBsAg assays, but more frequently results from a strong suppression of virus replication and gene expression [24]. Although the significance of isolated antiHBc in non-human primates is unclear, the serological markers of HBV infection in gibbons and orangutans may be similar to those described in humans.

In humans, several researchers have reported that patients with chronic HBV infection often present mutations in the basic core promoter region [24]. Accordingly, we aligned the Core regions to analyze nucleotide positions 1753, 1762 and 1764 including the *Pre-C* variant's nucleotide positions 1858, 1896 and 1899. We could not detect any mutations at those positions except for a G to T substitution at nucleotide position 1896. This substitution had occurred in all gibbon and orangutan HBV sequences described here, and was commonly found in non-human primate sequences. It has been proposed that the difference of RNA secondary structure between infected humans and non-human primates may be responsible for this discrepancy [25, 26].

Upon phylogenetic analysis, all seven complete genome sequences of HBV-infected orangutans obtained in this study grouped with those derived from previously published sequences. Indeed, they showed genetic relatedness to the HBV isolates from gibbons, particularly the *Hylobates* species that share geographical habitat ranges. The branches occupied by all orangutans analyzed clustered together, and displayed very close phylogenetic relatedness to an HBV isolate obtained from Indonesia (Y17559) [2]. This isolate showed a very high percentage of sequence similarity (approximately 98–99% identity of the *Pre-S* gene) to all isolates described here, suggesting that they had originated from a common source. Although the geographic origin of the orangutans described in this study was unknown, prior to their capture they probably inhabited Borneo and Sumatra, as wild living orangutans are generally restricted to these islands. Thus, the primary source of this HBV strain found in captive orangutans may have originated from the wild.

HBV isolates from gibbons in this study (*N. leucogenys*) were phylogenetically separated from gibbons (*H. pileatus and H.lar*) described in our preceding research, but were almost identical to a gibbon isolate (*H. concolor*) that was reported to have originated from a Thai zoo [4], suggesting several strains of HBV circulate in gibbons in Thailand. Previous data have shown that the HBV isolates from gibbons in different regions of Thailand and Vietnam could be classified into four phylogenetically distinct genomic groups [4]. Likewise, there appears to be a substantial difference in HBV strain distribution between gibbons from Thailand and those from Cambodia [9]. These observations can be explained by the different geographical location as well as different species (and sub-species) of non-human primates, which in turn may have determined the particular HBV strains infecting those animals in this geographic region.

In humans, a high percentage of individuals who become infected by horizontal transmission during adolescence or adulthood have a short duration of infectivity and clear the virus. In contrast, mother-to-child perinatal transmission generally leads to lifelong chronic HBV infection due to a prolonged stage of immunological tolerance and it is considered to be an essential mechanism for the persistence of HBV infection in human populations. Similar to HBV infection in humans, our previous study has documented that HBV in captive gibbons can be transmitted by vertical and horizontal routes [3]. Frequent vertical transmission in captive gibbons supports the assumption that this may be a main mechanism for the continuation of HBV infection in gibbons and potentially other ape species in the wild [8]. In addition, horizontal transmission also represents an important route for HBV distribution in the wild as well as in captivity. For instance, orangutans in the

wild are solitary apes with restricted contact with other individuals and thus, the possibility of horizontally acquired infection is limited. When captured and housed together, the probability of orangutans to be exposed to HBV appears to be increased. Indeed, the pronounced sequence similarity of HBV among infected orangutans described here strongly suggests that the transmission of HBV among these apes might have been relatively recent, possibly due to horizontal spread from an animal infected in the wild prior to its capture.

Despite the previous hypothesis of species-specific HBV infection, a geographical basis rather than species association accounting for the distribution of HBV variants has been increasingly recognized. For instance, HBV in orangutans consistently grouped within the gibbon clade in South-East Asia and similarly, a gorilla sequence (AJ131567) clustered with chimpanzee sequences in Central Africa. Lack of strict species-specificity of HBV variants was also reported for a chimpanzee sequence (AJ13575) grouped with a gibbon cluster, and another chimpanzee sequence (AB032431) grouped with human HBV genotype E. These observations support probable interspecies transmission which could be explained by sharing a common habitat in geographic regions with high prevalence of HBV infection, such as South East Asia and Central Africa. Thus, the more the regions both species inhabit overlap, the higher the probability of cross species transmission [8, 9]. This probability has currently been investigated by several researchers in order to elucidate the ultimate origin and evolution of HBV in humans and non-human primates.

Experimental transmission of human HBV to non-human primates by exposure to human saliva containing HBV has been reported [27, 28]. Hu et al. constructed a phylogenetic tree and found that the S gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from humans to chimpanzees [17]. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV [3]. Yet, non-human primate virus could probably be transmitted to humans as the respective HBV genomes are largely similar. Due to this similarity, HBV vaccine can be used to prevent cross-transmission between species. In fact, HBV isolated from our gibbons and orangutans contain glycine at position 145 of the '*a*' determinant indicating that HBV vaccines should be effective. However, the route of HBV transmission from non-human primates to humans ought to be elucidated.

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## References

1 Voudin M, Wolstenholme AJ, Tsiquaye KN, Zuckerman AJ, Harrison TJ: The complete nucleotide sequence of the genome of a hepatitis B virus isolated from a naturally infected chimpanzee. *J Gen Virol* 1988; **69**:1383–9.

2 Warren KS, Heeney JL, Swan RA, Verschoor HJ, Verschoor EJ: A new group of hepadnaviruses naturally infecting orangutans (*Pongo pygmaeus*). *J Virol* 1999; **73**:7860–5.

3 Noppornpanth S, Haagmans BL, Bhattarakosol P, Ratanakorn P, Niester HG, Osterhaus AD, Poovorawan Y: Molecular epidemiology of gibbon hepatitis B virus transmission. *J Gen Virol* 2003; **84**:147–55.

4 Grethe S, Heckel J, Rietschel W, Hufert F: Molecular epidemiology of hepatitis B virus variants in nonhuman primates. *J Virol* 2000; **74**:5377–81.

5 Lanford RE, Chavez D, Brasky KM, Burns RBI, Rico-Hesse R: Isolation of a hepadnavirus from the woolly monkey, a new world primate. *PNAS* 1998; **95**:5757–61.

6 Deinhardt F: Hepatitis in primates. *Adv Virus Res* 1976; **20**:113–57.

7 Makuwa M, Souquiere S, Telfer P, Bourry O, Rouquet P, Kazanji M, Roques P, Simon F: Hepatitis viruses in non-human primates. *J Med Primatol* 2006; **35**:384–7.

8 Starkman SE, MacDonald DM, Lewis JCM, Holmes EC, Simmonds P: Geographic and species association of hepatitis B virus genotypes in non-human primates. *Virology* 2003; **314**:381–93.

9 Sall AA, Starkman S, Reynes JM, Lay S, Nhim T, Hunt M, Marx N, Simmonds P: Frequent infection of *Hylobates pileatus* (pileated gibbon) with species-associated variants of hepatitis B virus in Combodia. *J Gen Virol* 2005; **86**:333–7.

10 Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M: Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69**:2575–83.

11 Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO: Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; **73**:1201–8.
12 Norder H, Hammas B, Lee SD, Bile K, Courouce AM, Mushawar IK, Magnius LO: Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol* 1993; **74**:1341–8.
13 Norder H, Courouce AM, Magnius LO: Complete genomes, phylogenetic relatedness, and structural protein of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; **198**:489–503.
14 Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R, Gerlich WH: Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. *J Gen Virol* 1993; **74**:1627–32.
15 Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**:67–74.
16 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO: Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**:2059–73.
17 Hu X, Margolis HS, Purcell RH, Ebert J, Robertson BH: Identification of hepatitis B virus indigenous to chimpanzees. *PNAS* 2000; **97**:1661–4.
18 Lanford RE, Chavez D, Rico-Hesse R, Mootnick A: Hepadnavirus infection in captive gibbons. *J Virol* 2000; **74**:2955–9.
19 MacDonald DM, Holmes EC, Lewis JC, Simmonds P: Detection of hepatitis B virus infection in wild – born chimpanzees (*Pan troglodytes* verus): phyogenetic relationships with human and other primate genotypes. *J Virol* 2000; **74**:4253–7.
20 Fares MA, Holmes E: A revised evolutionary history of hepatitis B virus (HBV). *J Mol Evol* 2002; **54**:807–14.
21 Verschoor EJ, Warren KS, Langenhuijzen S, Heriyanto, Swan RA, Heeney JL: Analysis of two genomic variants of orang-utan hepadnavirus and their relationship to other primate hepatitis B - like virus. *J Gen Virol* 2001; **82**:893–7.
22 Sambrook J, Russell DW: Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 2001.
23 Payungporn S, Tangkijvanich P, Jantaradsamee P, Theamboonlers A, Poovorawan Y: Simultaneous quantitation and genotyping of hepatitis B by real – time PCR and melting curve analysis. *J Virol Methods* 2004; **120**:131–40.
24 Raimondo G, Pollicino T, Cacciola I, Squadrito G: Occult hepatitis B virus infection. *J Hepatol* 2007; **46**:160–70.
25 Jardi R, Rodriguez F, Buti M, Costa X, Valdes A, Allende H, Schaper M, Galimany R, Esteban R, Guardia J: Mutations in the basic core promoter region of hepatitis B virus. Relationship with precore variants and HBV genotypes in a Spanish population of HBV carriers. *J Hepatol* 2004; **40**:507–14.
26 Ingman M, Lindqvist B, Kidd-Ljunggren K: Novel mutation in hepatitis B virus preventing HBeAg production and resembling primate strains. *J Gen Virol* 2006; **87**:307–10.
27 Alter HJ, Purcell RH, Gerin JL, London WT, Kaplan PM, McAuliffe VJ, Wagner J, Holland PV: Transmission of hepatitis B to chimpanzees by hepatitis B surface antigen-positive saliva and semen. *Infect Immun* 1977; **16**:928–33.
28 Bancroft WH, Snitbhan R, Scott RM, Tingpalapong M, Watson WT, Tanticharoenyos P, Karwacki JJ, Srimarut S: Transmission of hepatitis B virus to gibbons by exposure to human saliva containing hepatitis B surface antigen. *J Infect Dis* 1977; **135**:79–85.