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Paired chimpanzee hepatitis B virus (ChHBV) and mtDNA sequences suggest different ChHBV genetic variants are found in geographically distinct chimpanzee subspecies

Xiao-lei Hu [a], Ali Javadian [b], Pascal Gagneux [c], Betty H. Robertson [a,*]

[a] *Hepatitis Branch A33, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Atlanta, GA 30333, USA*

[b] *Experimental and Applied Research, Coulston Foundation, Alamogordo, NM, USA*

[c] *Glycobiology Research and Training Center, University of California at San Diego, La Jolla, CA, USA*

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Abstract

The surface antigen gene region from five chronic hepatitis B virus (HBV) infected chimpanzees was amplified by PCR and the sequence determined. Sequence comparison confirmed that all of the sequences were chimpanzee hepatitis B virus (chHBV) and they appeared to represent three distinct clusters or branches. To address the question of whether the three branches represented recently identified subspecies of chimpanzees, we determined the sequence of the mitochondrial DNA hypervariable D loop from hair samples obtained from these five chimpanzees. The results indicated that the three chHBV branches reflected three distinct subspecies of chimpanzees that are from different geographic regions in West Africa. The complete HBV sequence from members of the *Pan troglodytes troglodytes* cluster and the *Pan troglodytes verus* cluster are in the published literature; we determined the complete genome sequence for the third branch of HBV present in *Pan troglodytes vellerosus*. © 2001 Elsevier Science B.V. All rights reserved.

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Chimpanzee hepatitis B virus (ChHBV) sequences are genetically distinct from the viruses that cause human hepatitis B virus (HBV) infections (Hu et al., 2000; MacDonald et al., 2000; Takahashi et al., 2000; Vaudin et al., 1988). These sequences have been obtained from chronic ChHBV infected chimpanzees who were wild-born, captured prior to the international ban on chimpanzee capture in the late 1970s, or who were born in captivity to parents who were wild-born. In addition, HBV sequences have now been identified in all the Old World apes – gorilla (Grethe et al., 2000), gibbons (Norder et al., 1996; Grethe et al., 2000; Lanford et al., 2000), and orangutans (Warren et al., 1999) and in a New World primate, the woolly monkey (Lanford et al., 1998);

* Corresponding author. Tel.: +1-404-639-2369; fax: +1-404-639-1563.

E-mail address: bjr1@cdc.gov (B.H. Robertson).

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each primate group appears to have a unique HBV sequence distinct from each other and human HBVs.

We recently evaluated the HBV sequence from archived sera of five additional chronic HBV infected chimpanzees (Table 1) that are currently residents of the Coulston Foundation. The birth dates for CH116 and CB0031 indicate that they were wild born, seized prior to the international ban on chimpanzee capture in the late 1970s. In fact, CH116 was one of the first chimpanzees to be identified as an HBV carrier while she was at Holloman Air Force Base (Lichter, 1969). CH1435 and CH1436 were captive born in Busch Gardens, Tampa, Florida, and were donated to Coulston Foundation. Chimp CB0367 was illegally captured and sold to a seaman who brought her to this country in 1982, when she was about 6-months-old. She was confiscated by US Fish and Wildlife and spent time at the Primate Foundation in Arizona and the Laboratory for Experimental Medicine and Surgery in Primates, NYU prior to being donated to the Coulston Foundation in 1996. Archived sera from these five chronic carrier chimpanzees were used for amplification and sequencing of the surface antigen (S) region.

The complete S-region sequence from these five chronic HBV infected chimpanzees was compared to other chimpanzee S-region sequences, the gorilla and a gibbon S-region sequence and is illustrated graphically in Fig. 1. This phylogenetic tree clearly indicates that all of these HBV infections were the result of chHBV (asterisks, Fig. 1). Fig. 1 includes all the non-human chHBV sequences that have been reported (Grethe et al., 2000; Hu et al., 2000; Takahashi et al., 2000; MacDonald et al., 2000; CHBassi, Genbank Accession number AB046525) in addition to the five from this study. In our previous study (Hu et al., 2000), we observed what appeared to be two distinct branches within the group of chHBV strains that we investigated, while this analysis suggests that three to four branches can be observed. It is clear from this figure that CH1435, CH1436, and CB0031 were all infected with a strain of HBV most closely related to the original London Zoo chimpanzee sequence (D00220; Vaudin et al., 1988). The HBV in CH116 was most closely related to the HBV strain we identified in CH109 (AF222322; Hu et al., 2000). On the other hand, CB0376 was infected with a strain of HBV that was clearly on a separate branch.

Chronic HBV infections are usually the result of perinatal infection, most commonly mother–infant transmission. The presence of chHBV sequences in these chronically infected captive wild-born chimpanzees suggests that natural perinatal transmission is responsible for their infections. We reasoned that if these infections are the result of perinatal mother–infant transmission, identification of geographically distinct subspecies of chimpanzees based upon mitochondrial DNA sequencing (Morin et al., 1994; Gonder et al., 1997; Gagneux et al., 1999) should correspond with these three branches. Sequencing of the mtDNA extracted from hair samples of these chimpanzees revealed that the HBV in these three branches was derived from three different subspecies (Fig. 1, italics and Fig. 2). The three chimpanzees (CH1435, CH1436, and CB0031) who were infected with the most frequently found chHBV had mtDNA sequence indicating they were *Pan troglodytes verus*. CH116 belonged to the *Pan troglodytes troglodytes* group and the

Table 1
Background information of chimpanzees

Chimpanzee	Date of birth (Est)	Birth information	Serum collected	Gender
CH116 (Shirley)	(1958)	Wild-born captive	1998	F
CH1435 (Opal)	9/16/1980	Captive born[a]	1998	F
CH1436 (Angel)	8/12/1979	Captive born[a]	1998	F
CB0031 (Leppy)	(1967)	Wild-born captive	1998	M
CB0376 (Muna)	(1982)	Wild-born captive	1998	F

[a] These chimpanzees are siblings born to the same set of parents who were wild-born captives.

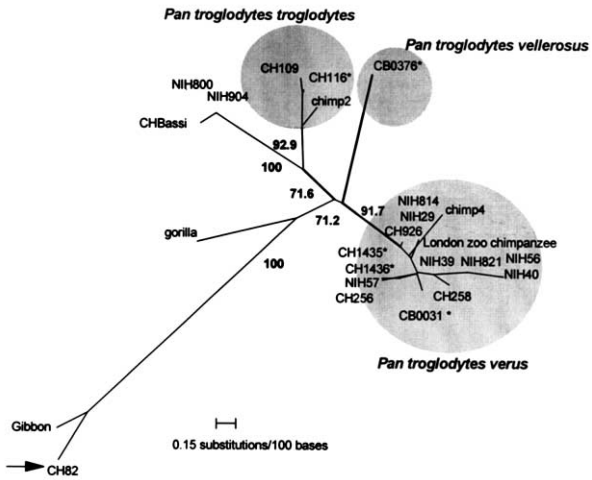

Fig. 1. Neighbor joining tree illustrating genetic relatedness of chimpanzee, gorilla, and gibbon HBV S-region sequences and identification of chimpanzee subspecies based upon mtDNA sequence analysis. HBV DNA was extracted and PCR amplification was performed as described previously (Hu et al., 2000). Fragment 2853-1101 (Table 2) was used as the template for S-region sequencing. Sequencing of the S-region (678 base pairs) was performed using dRhodamine terminators and analyzed on an ABI377 (Applied Biosystems). Sequences were aligned using the Pileup program within the GCG software package (Genetics Computer Group, Madison, WI) followed by phylogenetic analysis with DNADIST using the Kimura 2 parameter option, NEIGHBOR, and DRAWTREE within Phylip (Felsenstein, 1993). Bootstrap analysis was performed using SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in the Phylip package. Asterisks indicate the samples for which both HBV and mtDNA sequences were obtained for this study; the arrow highlights a sequence that probably resulted from transmission in captivity. The shaded circles indicate HBV branches that correlate with different chimpanzee subspecies based upon mtDNA sequences. Genbank accession numbers for the sequences identified by common names – gorilla: AJ131567; NIH800:AF222318; NIH904: AF222321; CH109:AF222322; CH116:AF305328; chimp2: AF242585; CB0376:AF305327; NIH814:AF222319; NIH29: AF222312; CH926:AF222323;chimp4: AF242586; NIH39: AF222313; NIH821:AF222320; NIH56:AF222316; NIH40: AF222314; CB0031:AF305326; NIH57:AF222317; CH1435: AF305329; CH1436:AF305330; CHBassi:AB046525; CH256: AB032432; CH258:AB032433; CH82:AJ131575; London zoo chimpanzee: D00220.

HBV sequence in this chimpanzee was present on the same branch with sequence from CH109 that we had previously reported (AF222322; Hu et al., 2000). The chimpanzee (CB0376) represented in the third HBV branch was a third subspecies, *Pan troglodytes vellerosus*. There has been a fourth chimpanzee subspecies cluster proposed, *Pan troglodytes schweinfurthii* (Morin et al., 1994), although it is not clear that this group can be distinguished from *P. t. troglodytes*, based upon mtDNA sequence analysis or geographic range (Gagneux et al., 1999; Deinard and Kidd, 2000; Gonder, 2000). It is possible that the HBV branches in Fig. 1 represented by NIH800 and NIH904 (AF222318 and AF222321) might reflect this subspecies distinction.

Previous studies have demonstrated that *P. t. troglodytes* is present within Central Africa and covers the region south of the Sanaga River in Cameroon and west of the Oubangui River in Congo, Zaire (Fig. 3, dark gray shading); one of the chimpanzees (CH116) we evaluated belonged to this group. Three of the chimpanzees (CH1435, CH1436 and CB0031) are clearly members of the Upper Guinean cluster, *P. t. verus*, which is geographically west of the Niger River (Fig. 3). In contrast, chimpanzee CB0376 groups unambiguously with Nigerian samples that represent the *P. t. vellerosus* cluster of chimpanzees whose geographic range is east of the Niger and north of the Sanaga River in Nigeria and Cameroon (Fig. 3).

Since the CB0376 sequence appeared to represent a strain of HBV indigenous to *P. t. vellerosus*

Table 2
Primer pairs used for PCR-amplification

Fragment location	Sense	Sequence
Fragment 2853-1101	Forward	5′ TCACCATATTCTT-GGGAACAAGA
	Reverse	5′ GAAAGGCCTTGT-AAGTTGGCGAG
Fragment 602-1830	Forward	5′ TGTATTCCCATCC-CATC
	Reverse	5′ AAAGTTGCATGG-TGCTGG
Fragment 1450-2275	Forward	5′ TACGTCCCGTCGG-CGCTGAATC
	Reverse	5′ GGAGTGCGAATC-CACACTCC
Fragment 1830-58	Forward	5′ TTTCACCTCTGCC-TAATCATCT
	Reverse	5′ GAACTGGAGCCA-CCAGCAGG

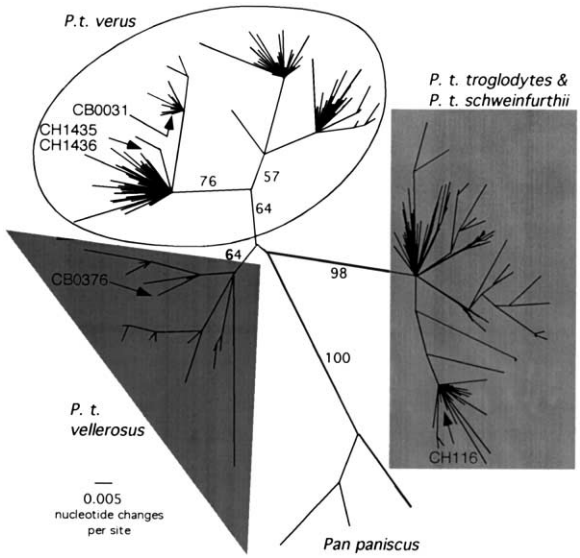

Fig. 2. Neighbor joining tree illustrating the chimpanzee subspecies determined by mtDNA sequencing. mtDNA was extracted from a plucked hair and a 400 bp segment of the hypervariable region 1 (HV1) of the mitochondrial control region (D Loop) was amplified and sequenced as previously described (Gagneux et al., 1999). For each animal sequences were obtained in duplicate from two different hairs. The sequences were aligned with 340 different haplotypes from chimpanzees of known geographic origin, genetic distances calculated using the Kimura 2 parameter option within PAUP and phylogenetic trees were generated to determine the taxonomic affiliation of our chimpanzees.

chimpanzees, and a representative isolate from this branch has not been previously characterized, we determined the complete genome sequence of this strain of HBV. A comparison of the complete genome sequence of CB0376 to other representative complete genome sequences confirmed that this sequence represented a separate branch (data not shown). A comparison to previously characterized chHBV amino acid sequences revealed that the conserved amino acid sequence patterns identified in our previous study (Hu et al., 2000) which appear to be non-human primate HBV-specific were conserved in these samples. These included E_{16} in the pre S-region, L_{133} and A_{177} in the S gene, L_{28} at the pre-core stop codon position and Q_{113} within core, and the 11 amino acid deletion in the polymerase gene which is common to all the primate HBV sequences and human genotype D HBV. These observations indicate that all three genetic branches of ChHBV have characteristic ChHBV amino acid sequences, which further suggests that these structural characteristic features may be used to distinguish non-human primate HBV isolates from human HBV isolates.

The correlation between HBV sequences and different subspecies of chimpanzees may suggest that they have co-evolved. Co-evolution of virus and host has been observed within other primate associated viruses. Within simian immunodeficiency virus (SIV) infections in African green monkeys, four distinct lineages have been identified which correspond to the four defined species, commonly referred to as vervet (*Chlorocebus pygerythrus*), grivet (*Chlorocebus aethiops*), sabaeus (*Chlorocebus sabaeus)* tantalus (*Chlorocebus tantalum*) that indicate ancient infection followed by divergence (Hirsch et al., 1993; Muller et al., 1993; Jin et al. 1994; Sharp et al., 1995). In addition, it has been proposed that chimpanzee

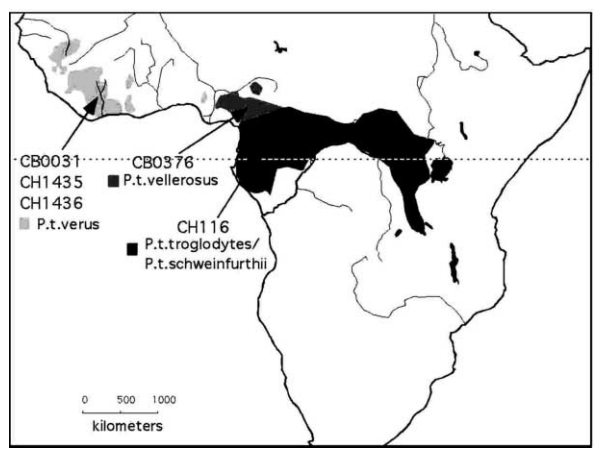

Fig. 3. Geographic regions within Africa where different subspecies represented in these five chimpanzees are located. The different chimpanzee ranges are indicated by the gray shaded areas. The northernmost genetic and geographically separated group, represented by CB0031, CH1435 and CH1436, are *P. t. verus*; to the west of this group are *P. t. vellerosus*, represented by CB0376. CH116 represents *P. t. troglodytes*. These ranges are based upon currently available information complied from the following sources: Morin et al. (1994), Gonder et al. (1997), Gagneux et al. (1999), Deinard and Kidd (2000) and Gonder (2000).

SIV co-evolved with different subspecies of chimpanzees followed by subsequent cross-species transmission to humans (Gao et al., 1999; Hahn et al., 2000).

Given that HBV and HIV are transmitted by similar routes and practices (i.e. blood and body fluid exposure), there exists the theoretical possibility of chimpanzee to human transmission of HBV. Chimpanzees are susceptible to human HBV infection and develop biochemical evidence of disease with liver enzyme elevations and have therefore been used as a laboratory model for human disease. However, there is no evidence to date that chimpanzee, orangutan, or gibbon HBV is responsible for human disease in the geographic regions where they reside. Knowledge of the genetic features of these non-human primate strains of HBV provides us with the background information needed to determine this in case such a transmission does occur.

One of the limitations of our study is the use of captive chimpanzees who were wild born or born in captivity. Chronic HBV infections generally result from mother to infant perinatal or horizontal early childhood infection. Our conclusions from this study could be influenced by potential horizontal (chimpanzee to chimpanzee) transmission in captivity between different subspecies of captured infants. Transmission of hepatitis B between different primates is clearly indicated by the presence of a gibbon sequence (AJ131575; Fig. 1, arrow) in a captive chimpanzee from a zoo and the presence of human genotypes A, C, and E in three chronic carrier chimpanzees (Hu et al., 2000; Takahashi et al., 2000) which probably resulted from the past practice of inoculating captured infants with human sera to protect against human disease. In addition, the sequences labeled chimp2 and chimp4 in Fig. 1 (AF242585, AF242586; MacDonald et al., 2000) were both published as being isolated from *P. t. verus* chimpanzees, while only chimp4 clusters with the branch that we have assigned to this subspecies. The CHBassi sequence (AB046525) was obtained from a captive chimpanzee in Gabon, which is within the *P. t. troglodytes* geographic range, while the sequence indicates that it is on a distinct branch that appears to represent the a separate HBV cluster. If non-invasive sampling approaches can be developed, sequence information of HBV from wild chimpanzees who have not lived in captivity and the corresponding mtDNA sequences are needed to definitively determine the relationship between different chimpanzee subspecies and chHBV sequences.

The HBV S-region sequences have been submitted to GenBank/EMBL/DDBJ and have accession numbers AF305326, and AF307328–AF305330; the accession number for the full genome sequence of HBV from CB0376 is AF305327. Accession numbers for the mitochondrial DNA hypervariable loop sequences are AF315497–AF31550.

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