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Short communication

The "original" Hepatitis B virus of Eastern chimpanzees (*Pan trogrodytes schweinfurthii*)

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ABSTRACT

Little is known about Hepatitis B Virus (HBV) infections in chimpanzees. Therefore, we investigated the prevalence of chimpanzee HBV (chHBV) infections in captive, wild born chimpanzees in the sanctuary on Ngamba Island, Uganda and one sample from a wild free ranging chimpanzee. In one third of the plasma samples (32.4%; 12/37) we detected antibodies to Hepatitis B (core) antigen. Amongst those individuals HBV DNA was detected in one captive wild born and the wild chimpanzee. In contrast to the only available earlier described HBV sequence from the subspecies *Pan troglodytes schweinfurthii*, there was no evidence of recombination with human HBV. Our sequences therefore are likely to present the "original" chHBV from *P. t. schweinfurthii*.

1. Main text

HBV infections have been identified in all subspecies of chimpanzees and seem to be asymptomatic in their natural hosts (MacDonald et al., 2000; Hu et al., 2000; Takahashi et al., 2000; Vartanian et al., 2002). For the chimpanzee subspecies *Pan troglodytes schweinfurthii* from East Africa, only one chHBV sequence is available (Vartanian et al., 2002) but subsequently determined to be a recombinant with human HBV type C (Magiorkinis et al., 2005). The events that led to recombination of chHBV with human HBV genotype C, not known to circulate in Africa, are puzzling since the samples were derived from a wild chimpanzee. However, only recently, evidence of recombination between HBV *Pan t. troglodytes* and *Pan t. ellioti* (formally known as *vellerosus*) was reported in two of the Cameroonian chHBV strains (Njouom et al., 2010). In captivity several cross-species transmissions have been reported between different primate species, examples are transmissions between gibbons and chimpanzees, chimpanzees and gorillas and between chimpanzees and humans (Norder et al., 1996; Grethe et al., 2000; Takahashi et al., 2000; Hu et al., 2000).

In order to investigate if the recombined chHBV from *P. t. schweinfurtii* is a general phenomenon and to test what percentage of captive (but wild born) chimpanzee are chronically infected with HBV, blood was collected in EDTA tubes from 37 chimpanzees living on Ngamba Island Chimpanzee Sanctuary, Uganda, between 2001 and 2008 during the annual routine health checks under anaesthesia. Plasma was separated by centrifugation at 3000 rpm for 10 min at room temperature. In addition fecal samples were collected from the same individuals. Two millilitre aliquots of the plasma and fecal samples were initially stored under liquid Nitrogen and later transferred to −80 °C freezer until transported on dry ice to the Robert Koch-Institut for analysis. In addition a serum sample obtained from a wild chimpanzee habituated to human presence (named Kiiza) in Kibale National Park in Uganda in 2006 during snare wire removal was included in the analyses.

For initial testing, plasma collected in 2008 was analyzed for antibodies to Hepatitis B (core)-antigen by Enzygnost Anti-HBc monoclonal enzyme immunoassay kit (Dade Behring Marburg GmbH, Germany) which indicate any HBV infection in the past except the early beginning of acute infection. In this test, 32.4% (12/37) of the chimpanzees were positive, including the sample

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Table 1
Ngamba chimp age/sex distribution 2007 and HBV ELISA results.

	Age category	Females tested/positive	Male tested/positive	Total
Infants	1–5 years	1/0	3/0	3
Juveniles	6–8 years	2/0	4/2	6
Sub adults	9–11 years	4/0	6/1	10
Adults	>12 years	12/7	6/2	18
Total		19/7	18/5	37

of the wild chimpanzee (Kiiza). Of the positive chimpanzees, 5 were males and 7 females. There were significantly more adults sero-positive than infants, juveniles and subadults (Sided Fishers exact analysis ($P = 0.004$)) (Table 1). The high seroprevalence of HBV infection reported in this paper corresponds with previous findings of 29.2% ($n = 156$ chimpanzees and 14 gorilla) from Gabon and Congo (Makuwa et al., 2006); 38.6% seropositivity ($n = 101$) was also observed in captive gibbons from Thailand positive for at least one marker of HBV infection (Noppornpanth et al., 2003). Even higher seropositivity for at least one marker of HBV infections in non-/human primates in captivity has been reported in gibbons (5/15: 33.3%) and orangutan (40/43: 93%) (Sa-nguanmoo et al., 2008).

To get information on the possible time point of infection, plasma samples collected from the same animals earlier (between 2001 and 2007) were investigated. All individuals positive for anti-HBV antibodies in the 2008 panel were also positive in the samples collected between 2001 and 2007.

To test for the presence of chHBV, DNA was extracted from plasma of all chimpanzees and serum of the wild chimpanzee using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Using a real time qPCR targeting all known HBV (Pas et al., 2000) each sample was analyzed in duplicate. Of the 37 chimpanzee samples tested initially, only one captive individual (Mika) and the wild chimpanzee were positive for chHBV.

To follow the course of infection plasma samples of Mika, the alpha male of the group, collected in previous health checks in the years 2001, 2004, 2005 and 2007 were analyzed by qPCR for HBV. All samples were positive for HBV DNA (Table 2), viral loads are comparable to the HBV viral load found in wild-born chimpanzees in Central and West Africa (Makuwa et al., 2003, 2007). The observed peak of viral load in 2005 corresponds to the hierarchy taking over demands as Mika established himself as an alpha male between 2004 and 2005 which was a very stressful period resulting in less immuno-reactivity than usual. Results from annual haematologic and serum biochemistry analyses from Mika revealed high level in 2007 and moderate increase in 2009 in enzyme levels of γ-glutamyl transferase (GT/GGT—123 u/l; 91 u/l) and Alanine aminotransferase (GPT/ALT—76 u/l; 71 u/l) respectively and a decrease/increase in alkaline phosphatase (ALP—76 u/l; 114 u/l) in 2007 and 2009 respectively, compared to non infected chimpanzees (data not shown), an indicator of reduced liver function in reference to reported values from healthy chimpanzees (Stone et al., 2000). However, the chimpanzee Mika showed no obvious symptoms of an HBV infection or reduced activity levels and changes in serum clinical chemistry should be interpreted with caution as they can be affected by other factors like age and sex (Videan et al., 2008).

Table 2
Quantitative real-time PCR for HBV for chimpanzees Mika and Kiiza.

Chimp	Sample year	Ct-.value	Copies/μl DNA	Copies/ml plasma
Mika	2001	19.2	1.8×10^5	1.0×10^8
	2004	22.1	2.7×10^4	1.5×10^7
	2005	19.8	1.2×10^5	7.1×10^7
	2007	27.6	6.8×10^2	3.9×10^5
	2008	31.6	1.5×10^2	2.2×10^4
Kiiza	2006	22.7	2.5×10^4	1.4×10^7

Transmission of HBV in apes has not been described in detail but presumably occurs through the same routes as described for humans (sexual, blood–blood contact, infectious fluids). The role of an infected alpha male in transmission of HBV especially to females and other individuals requires further investigation and may be useful in understanding the epidemiology of HBV infections in wild great apes. Unfortunately, no other chimpanzee of the group was PCR positive to allow molecular epidemiological tracing of these infections.

To test shedding of the virus we also extracted DNA from faeces samples available from Mika using the EURx GeneMATRIX Stool DNA Purification Kit (Roboklon). All these samples were positive in PCR confirming the applicability of the non-invasively collected samples for PCR analysis as described before (Makuwa et al., 2006).

In order to test if the two PCR positive chimpanzees are infected with a recombinant variant of HBV as described for this subspecies of chimpanzees, the near full-length genome of HBV was amplified and sequenced from DNA isolated from blood (primers are listed in supplementary Table S1). The following primer combinations were used HBV1802F/HBV803R, HBV PreS F/HBV1626R and HBV1446F/HBV1752R to amplify a 2.2 kb, 1,4 kb and 0,3 kb fragment, respectively. PCR products were separated by 1.5% agarose gel electrophoresis, purified with QIAGEN QIA Quick Gel extraction Kit (Qiagen) and sequenced with the ABI PRISM Big Dye Terminator cycle sequencing kit, according to the manufacturer's protocol. Sequences were determined using the ABI 310 automated DNA sequencer and analyzed with the ABI PRISM DNA Sequencing Analysis Software (Version 3.7, Applied Biosystems) and MEGA4 software program (http://www.megasoftware.net).

The nearly complete HBV genomes (positions 1–1730 and 1850–3263 compared to the reference sequence V00866) identified from Mika and Kiiza (sequence accession number HQ018763 and HQ018764 respectively) were aligned to a selection of published complete HBV genomes using MUSCLE (Edgar, 2004) as implemented in SeaView (Gouy et al., 2010). Following visual inspection, the alignment was then manually edited, being only slightly modified. The resulting dataset comprised 50 sequences and 3181 nucleotide positions.

Because the only *P. t. schweinfurthii* HBV sequence published has been identified as a recombinant comprising a sequence closely related to human HBV subtype C (Magiorkinis et al., 2005), it was particularly tempting to examine Mika and Kiiza HBV for evidence of recombination. Therefore both genomes were analyzed by bootscanning using SimPlot (Lole et al., 1999), which failed to identify any sign of recombination with known human subtypes or strains from other species (data not shown).

In a similar way, we also produced maximum likelihood (ML) phylogenies for the three fragments identified as putatively not recombinant and recombinant respectively in the FG genome (Magiorkinis et al., 2005). For this, likelihoods of models of evolution (JC, HKY and GTR; +F; +I, +G, +I + G) were first estimated and then compared according to the Akaike information criterion using jModelTest (Posada, 2008). For all three fragments the model of evolution to which the dataset was a better fit was GTR + I + G. ML phylogenetic trees were then estimated under this model using PhyML (Guindon and Gascuel, 2003) as implemented on a dedicated webserver http://www.atgc-montpellier.fr/phyml/. Equilibrium frequencies, topology and branch lengths were optimized and the tree search was realized using a combination of hill-climbing algorithms (NNI & SPR). Branch robustness was assessed by non-parametric bootstrapping (500 pseudo-replicates).

All three analyses supported the inclusion of Kiiza and Mika HBVs in the *P. troglodytes* clade (data not shown). For two of the

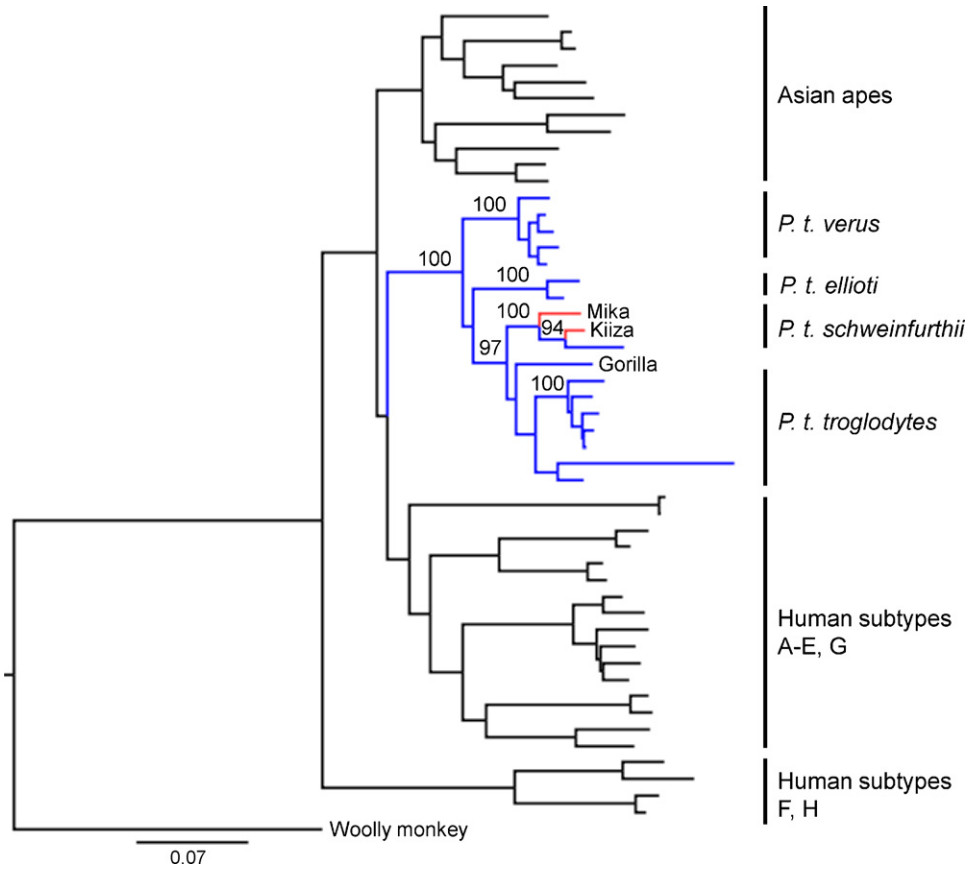

Fig. 1. Maximum likelihood tree of HBV complete genomes. Colored external branches stand for genomes identified from African apes, Mika and Kiiza HBV branches being highlighted in red. Bootstrap values above 90 are shown for the African ape clade only. The tree was rooted using one HBV sequence isolated from a woolly monkey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

analyses (corresponding to those performed on fragments 0–550 and 1051–3181 in Magiorkinis et al., 2005 analyses), the FG strain was found in close association with Kiiza and Mika strains, while in the analysis of fragments 551–1050 the same strain was found to be a part of the HBV type C clade (data not shown). ML analysis of entire genomes confirmed this pattern. The phylogenetic tree derived (Fig. 1) indeed shows that the complete HBV sequences from Kiiza and Mika cluster closely with the HBV isolate FG published previously for *P. t. schweinfurthii* (which likely reflects the small size of the recombinant HBV type C region (Vartanian et al., 2002)).

It is especially worthwhile noticing that the HBV sequence obtained from Kiiza is more closely related to FG's corresponding to the fact that Kiiza originates from the very same habituated chimpanzee group. The co-existence of a potential human-chimpanzee recombinant virus and a non-recombinant chimpanzee specific HBV strain in one and the same community is surprising and further investigations are needed to clarify the "natural" existence of the recombinant chHBV FG. However, the two new sequences described in this investigation may represent the "original" *P. t. schweinfurthii* sequence, which was at the origin of the recombination event leading to the published isolate FG (Vartanian et al., 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.10.017.

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