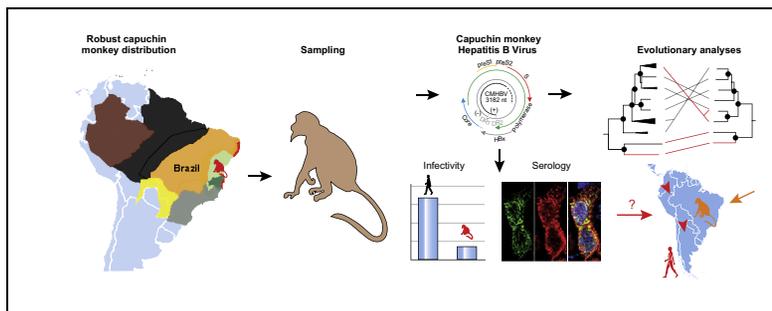


# A novel hepatitis B virus species discovered in capuchin monkeys sheds new light on the evolution of primate hepadnaviruses

## Graphical abstract



## Highlights

- A divergent HBV species termed CMHBV was discovered in Brazilian capuchin monkeys.
- CMHBV and the related WMHBV use the same receptor as HBV to infect human cells.
- CMHBV may cause chronic hepatitis B, potentially enabling new animal models.
- Primates may have been carrying HBV-related viruses for millions of years.
- New World HBV genotypes were likely introduced during the peopling of the Americas.

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## Lay summary

The origins of HBV are unclear. The new orthohepadnavirus species from Brazilian capuchin monkeys resembled HBV in elicited infection patterns and could infect human liver cells using the same receptor as HBV. Evolutionary analyses suggested that primate HBV-related viruses might have emerged in African ancestors of New World monkeys millions of years ago. HBV was associated with hominoid primates, including humans and apes, suggesting evolutionary origins of HBV before the formation of modern humans. HBV genotypes found in American natives were divergent from those found in American monkeys, and likely introduced along prehistoric human migration. Our results elucidate the evolutionary origins and dispersal of primate HBV, identify a new orthohepadnavirus reservoir, and enable new perspectives for animal models of hepatitis B.

# A novel hepatitis B virus species discovered in capuchin monkeys sheds new light on the evolution of primate hepadnaviruses

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**Background & Aims:** All known hepatitis B virus (HBV) genotypes occur in humans and hominoid Old World non-human primates (NHPs). The divergent woolly monkey HBV (WMHBV) forms another orthohepadnavirus species. The evolutionary origins of HBV are unclear.

**Methods:** We analysed sera from 124 Brazilian monkeys collected during 2012–2016 for hepadnaviruses using molecular and serological tools, and conducted evolutionary analyses.

**Results:** We identified a novel orthohepadnavirus species in capuchin monkeys (capuchin monkey hepatitis B virus [CMHBV]). We found CMHBV-specific antibodies in five animals and high CMHBV concentrations in one animal. Non-inflammatory, probably chronic infection was consistent with an intact preCore domain, low genetic variability, core deletions in deep sequencing, and no elevated liver enzymes. Cross-reactivity of antisera against surface antigens suggested antigenic relatedness of HBV, CMHBV, and WMHBV. Infection-determining CMHBV surface peptides bound to the human HBV receptor (human sodium taurocholate co-transporting polypeptide), but preferentially interacted with the capuchin monkey receptor homologue. CMHBV and WMHBV pseudotypes infected human hepatoma cells via the human sodium

taurocholate co-transporting polypeptide, and were poorly neutralised by HBV vaccine-derived antibodies, suggesting that cross-species infections may be possible. Ancestral state reconstructions and sequence distance comparisons associated HBV with humans, whereas primate hepadnaviruses as a whole were projected to NHP ancestors. Co-phylogenetic analyses yielded evidence for co-speciation of hepadnaviruses and New World NHP. Bayesian hypothesis testing yielded strong support for an association of the HBV stem lineage with hominoid ancestors. Neither CMHBV nor WMHBV was likely the ancestor of the divergent human HBV genotypes F/H found in American natives.

**Conclusions:** Our data suggest ancestral co-speciation of hepadnaviruses and NHP, and an Old World origin of the divergent HBV genotypes F/H. The identification of a novel primate hepadnavirus offers new perspectives for urgently needed animal models of chronic hepatitis B.

**Lay summary:** The origins of HBV are unclear. The new orthohepadnavirus species from Brazilian capuchin monkeys resembled HBV in elicited infection patterns and could infect human liver cells using the same receptor as HBV. Evolutionary analyses suggested that primate HBV-related viruses might have emerged in African ancestors of New World monkeys millions of years ago. HBV was associated with hominoid primates, including humans and apes, suggesting evolutionary origins of HBV before the formation of modern humans. HBV genotypes found in American natives were divergent from those found in American monkeys, and likely introduced along prehistoric human migration. Our results elucidate the evolutionary origins and dispersal of primate HBV, identify a new orthohepadnavirus reservoir, and enable new perspectives for animal models of hepatitis B.

Keywords: Hepatitis B virus; Viral evolution; New World; Primate; Human dispersal. Received 31 March 2017; received in revised form 19 January 2018; accepted 27 January 2018

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## Introduction

The hepatitis B virus (HBV) is one of the most important human pathogens, causing at least 680,000 deaths each year globally caused by chronic infection resulting in liver cirrhosis and hepatocellular carcinoma.<sup>1</sup> HBV is the prototype species of the genus *Orthohepadnavirus* in the family *Hepadnaviridae*. In humans, HBV comprises 10 genotypes named A–J.<sup>2</sup> Additional HBV genotypes infect Old World non-human primates (NHPs), including chimpanzees, gorillas, orangutans, and gibbons.<sup>3</sup> Infection of humans with HBV genotypes from NHPs has not been described yet. By contrast, NHPs can carry human HBV genotypes and HBV genotypes from other NHP species, illustrating the potential of primate HBV to cross the species barrier.<sup>3</sup> Different from other major blood-borne viruses, such as HIV, there is no evidence for an evolutionary origin of human HBV from viruses carried by Old World NHPs.<sup>4</sup> Similarly, the evolutionary origins of the divergent human HBV genotypes F and H associated with American natives inhabiting Alaska and Latin America are unknown.<sup>5</sup>

In the absence of known animal reservoirs for human HBV strains, the eradication of hepatitis B through universal vaccination and antiviral treatment might be possible.<sup>6</sup> We recently described a New World bat hepadnavirus that could infect human hepatocytes and was not neutralised by hepatitis B vaccine-induced antibodies.<sup>7</sup> Whether this bat virus may infect humans remains open, because direct contact between humans and bats (e.g. from hunting of bats as bushmeat) is rare in the New World.<sup>8</sup> By contrast, contact between indigenous American populations and NHP is more intense, including consumption of NHP, their keeping as pets, and the encroachment of NHP to human dwellings because of destruction of their natural habitats.<sup>9</sup> Additionally, the genetic relatedness of humans and NHP facilitates cross-species infections.<sup>10</sup>

The only other known primate hepadnavirus species beyond HBV, termed woolly monkey HBV (WMHBV), was described in 1998 from captive woolly monkeys (*Lagothrix lagotricha*).<sup>11</sup> Although the WMHBV forms a phylogenetic sister species to HBV, its description in a confined setting challenged definitive assertions on the role of New World NHPs for the evolutionary origins of HBV. Sampling of NHPs is difficult for ethical and technical reasons, and in consequence, South American NHPs are among the most understudied primate populations in terms of the infectious agents they may carry.<sup>12</sup> There are only four studies on HBV in New World NHPs that collectively analysed only about 100 animals.<sup>3</sup>

Here, we screened New World NHPs for hepadnaviruses and identified a novel HBV species in capuchin monkeys, which was highly divergent from WMHBV. Evolutionary and functional analyses enabled new hypotheses for primate HBV evolution, showed important similarities between the infection patterns of the novel hepadnavirus and HBV, and pointed at potential transmissibility of the novel HBV to humans.

## Materials and methods

Screening for hepadnaviruses, cloning, and infection assays were done, as described previously.<sup>7</sup> Evolutionary analyses

were done in a maximum likelihood and Bayesian framework using MEGA6 and BEAST.<sup>13,14</sup> Co-phylogenetic analyses were done using PACo, ParaFit, and CoRe-PA.<sup>15–17</sup> Ethical approval was obtained from Brazilian and German authorities.

For further details regarding the materials used, please refer to the [CTAT table](#) and [supplementary information](#).

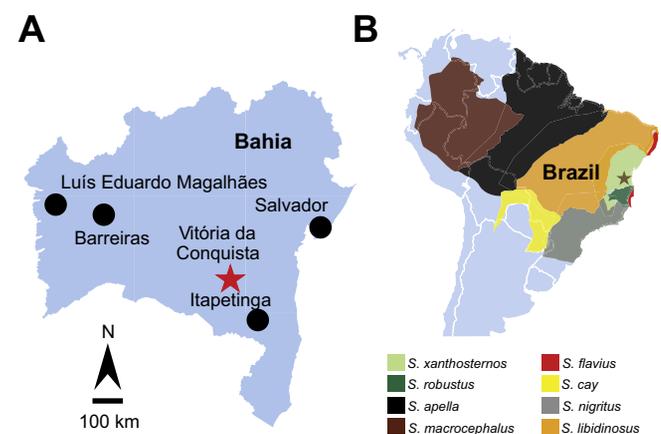
## Results

### Identification of a novel primate HBV

During 2012–2016, sera were sampled from 124 NHPs belonging to at least 10 species in three zoos and two shelters receiving confiscated animals from illegal trafficking in the state of Bahia, north-eastern Brazil (Fig. 1A). As shown in [Table S1](#), most sampled animals were robust capuchin monkeys (genus *Sapajus*, family Cebidae). Because of frequent hybridisation events between different *Sapajus* species (Fig. 1B) and because of animal trafficking over long distances, not all species could be unambiguously identified.<sup>18</sup> An adult female capuchin monkey kept in an animal shelter for about 1 year before sampling tested positive in a broadly reactive and highly sensitive *Hepadnaviridae* PCR assay,<sup>7</sup> whereas all other animals were PCR negative. The hepadnavirus-positive animal belonged to the species *Sapajus xanthosternos* (Table 1). This animal showed unspecific signs of disease, including thinness, lethargy, and mild dehydration, and died about 6 months after sampling. Unfortunately, no follow-up specimens were available. *Sapajus xanthosternos* is classified as “critically endangered” because of severe population decline and habitat loss,<sup>19</sup> and can currently only be found in small protected areas that overlap with our sampling sites (Fig. 1B).

### Genomic characterisation of the novel hepadnavirus

The full genome of the virus termed capuchin monkey HBV (CMHBV; GenBank accession number KY703886) was amplified by sets of overlapping PCRs directly from serum, as described previously,<sup>7</sup> and sequenced. The CMHBV genome spanned 3,182 nucleotides, and showed the typical length and organisation of an orthohepadnavirus genome, including four overlapping open reading frames encoding the predicted surface (S),



**Fig. 1. Sampling sites and *Sapajus* distribution.** (A) Bahia state with sampling sites. (B) Distribution of *Sapajus* species according to the Red List of Threatened Species from the International Union for Conservation of Nature. Red star, positive site.

Table 1. Virological and clinical findings in animals with signs of current or past hepadnavirus infection.\*

ID	Morphological species designation/mitochondrial DNA closest relative <sup>†</sup>	Weight (kg)/Temperature (°C)/Heart rate <sup>‡</sup>	PCR (DNA copies/ml)	Antibody status (end-point IFA titer)				Observations
				α HBV	α CMHBV	α HBV LHBs	α CMHBV LHBs	
M-05	<i>Sapajus xanthostermos</i> /S. xanthostermos	4.3/38.6/220	-	+	+	(1:40)	-	Adult; male; healthy; 1 year in shelter; sampled 2012
M-12	<i>S. xanthostermos</i> /S. xanthostermos	2.0/36.9/168	+ (9.2 × 10 <sup>8</sup> )	-	-	-	-	Adult; female; thinness, lethargy, mild dehydration; 1 year in shelter; sampled 2012
M-39	<i>Sapajus</i> sp./ <i>Sapajus robustus</i>	2.1/36.4/196	-	-	+	(1:80)	-	Adult; female; healthy; 1 year in shelter; sampled 2012
09	<i>Sapajus</i> sp./ <i>Sapajus apella</i>	1.8/37.9/230	-	-	+	(1:320)	-	Senile; female; very slim, multifocal alopecia in body; captured in 'Poço Escuro'; 2 years in shelter; sampled 2016
10	<i>Sapajus</i> sp./S. apella	1.8/37.8/234	-	-	+	(1:320)	-	Senile; female; very slim, multifocal alopecia in tail, enlarged submandibular lymph node; captured in 'Poço Escuro'; 2 years in shelter; sampled 2016
22	<i>Sapajus</i> sp./S. apella	2.5/37.7/258	-	+	+	(1:80)	(1:5,120)	Adult; female; lives in the wild in 'Poço Escuro'; sampled 2016

\* CMHBV, capuchin monkey hepatitis B virus; HBV, hepatitis B virus.

<sup>†</sup>Inherited only from the mother, thus not excluding hybridisation with different paternal species. COI reference entries were not available for all *Sapajus* species; cytochrome B reference entries from previous studies were conflicting, suggesting caution needs to be taken with definite species designations.<sup>‡</sup>Beats per minute.

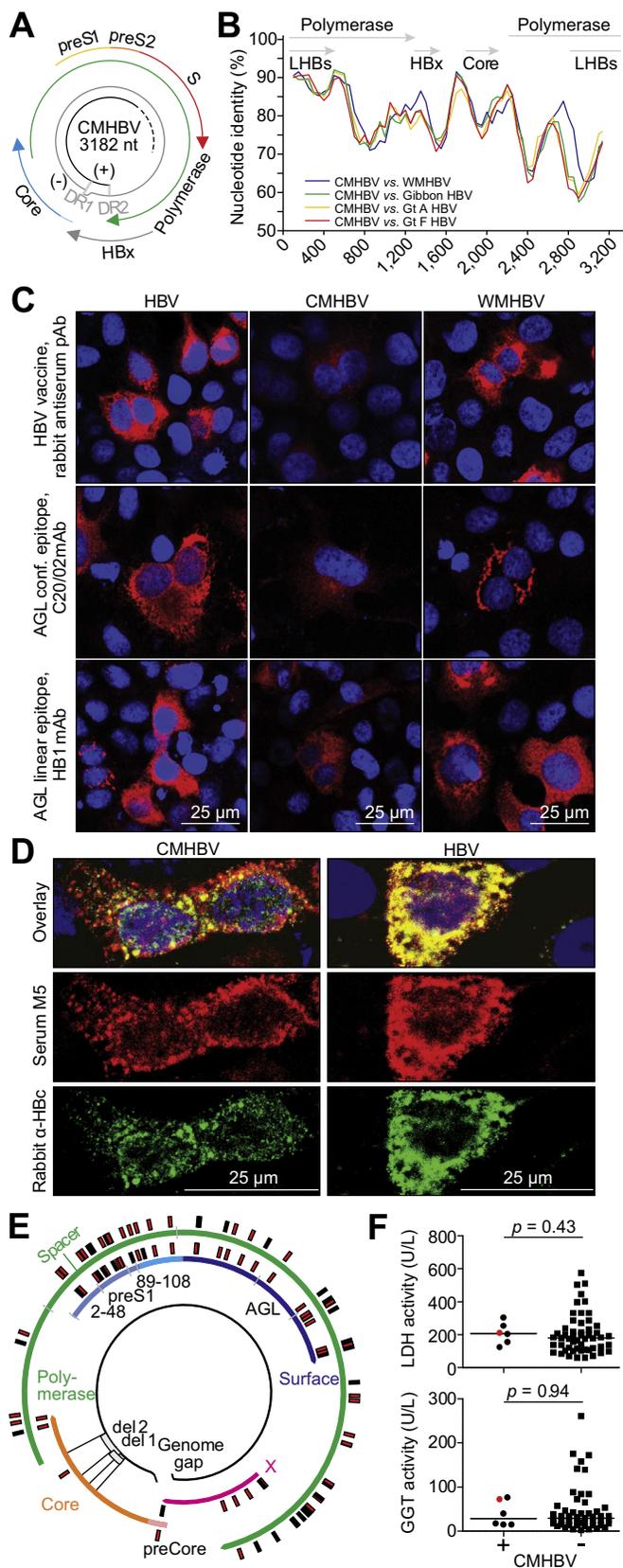
polymerase (Pol), core (C), and X proteins; conserved direct repeats; and the conserved RNA secondary structure epsilon involved in hepadnavirus replication (Fig. 2A and Fig. S1A). The CMHBV was equidistant from all other primate hepadnaviruses with a sequence distance averaged over the whole genome ranging between 20.4% (HBV genotype C) and 22.3% (HBV genotype F; Table 2). CMHBV was not significantly closer to WMHBV than to human and ape HBV (Fig. 2B). It significantly resembled WMHBV only in the preS1 and X domains. No evidence for genomic recombination involving the novel CMHBV was found using recombination detection tools. In sum, sequence distances exceeding the 20% threshold separating the primate hepadnavirus species WMHBV and HBV and the typical genome organisation confirmed the CMHBV as a new orthohepadnavirus species.

### Antigenic relatedness of HBV and New World monkey hepadnaviruses

The predicted antigenic loop of CMHBV showed conserved location and length compared to HBV and WMHBV, including eight cysteine residues that are essential for the stability of the conformational surface epitopes (Fig. S1B). To confirm the antigenic relatedness among CMHBV, WMHBV, and HBV, we comparatively analysed the reactivity of different antisera with the S proteins of these hepadnaviruses. As expected, a polyclonal rabbit antiserum (pAb) against the conventional HBV vaccine (HBVaxPRO) and two monoclonal (mAb) anti-HBs antibodies showed reactivity against the three hepadnavirus S proteins in an immunofluorescence assay (IFA) (Fig. 2C). Compared to the HBV S antigen (HBsAg), the WMHBsAg showed similar reactivity with the antisera, whereas a weaker signal was observed for the CMHBsAg. Consistent with the low reactivity against HBV antisera, a diagnostic quantitative HBsAg assay showed strongly reduced reactivity with secreted CMHBsAg (Fig. S1C).

### Resurrection of the CMHBV for serological investigation

The full CMHBV genome was cloned into an overlength expression vector, as described previously,<sup>7</sup> and used to transfect hepatoma cell lines expressing CMHBV proteins for serological analyses in an IFA. Whereas the CMHBV DNA-positive animal tested negative for CMHBV antibodies, five DNA-negative capuchin monkeys from the same sampling site tested positive for antibodies, showing IFA endpoint titres between 1:40 and 1:5,120 (Table 1). Comparative testing of all sera using cell cultures expressing overlength HBV constructs revealed only two HBV IFA-positive animals, and anti-HBV titres were at least two-fold and up to 64-fold lower than anti-CMHBV titres, consistent with cleared CMHBV infections in these animals. The cellular location of the IFA reactivity pattern was consistent with the typical anti-core reactivity pattern involving the nucleus and cytoplasm, and its specificity was confirmed by co-localisation using control antisera (exemplified in Fig. 2D). No animal tested positive in IFA using expression constructs for the CMHBV or HBV S antigen, which is comparable to the low production of anti-S antibodies frequently observed in HBV-infected humans after HBsAg clearance.<sup>20</sup> The occurrence of CMHBV antibody-positive animals during the whole observation period (2012–2016), including a free-ranging monkey, suggest maintenance of CMHBV in the location. According to morphological and genomic characteristics, CMHBV antibody-positive animals belonged to at least three different capuchin monkey species,



including *Sapajus robustus*, *S. apella*, and *S. xanthosternus* (Table 1).

### CMHBV infection patterns

All five CMHBV antibody-positive animals were apparently healthy. Whether the signs of disease in the DNA-positive animal were caused by infection with the CMHBV, and whether it had an acute or chronic CMHBV infection status remain unknown. In any case, the virus had an intact preCore domain, suggesting production of hepadnaviral e-antigen (eAg). In human HBV, the eAg-positive, non-inflammatory phase is characterised by a high viral load and low mutation rate in the intra-patient quasispecies correlating with limited immune pressure.<sup>21</sup> Comparable to HBV, the CMHBV load was high with  $9.2 \times 10^8$  genome copies per millilitre. A total of 57 genomic sites were identified with at least 1% variation in deep sequencing, which is comparable to humans chronically infected with HBV (Fig. 2E and detailed in Table S2).<sup>22</sup> Almost all non-synonymous substitutions ( $d_N$ ) in the Pol mapped to the predicted spacer region, which may be partially dispensable for hepadnavirus replication,<sup>23</sup> implying limited impact of these  $d_N$  on CMHBV replication efficiency. In agreement with limited antibody-mediated pressure, only two  $d_N$  occurred in the antigenic loop at CMHBV SHBs residues 111 (P/S) and 160 (A/V) at low frequencies of 1–2% (Fig. S1B). Several reads containing two large deletions of up to 78 and 150 nucleotides were observed in the C open reading frame (Fig. 2E). The occurrence, location, and size of these C deletions were consistent with the deletion patterns found in chronic hepadnavirus infections of humans and woodchucks.<sup>24,25</sup> In summary, genomic patterns were consistent with a non-recent chronic replicative infection. Lack of anti-C antibodies may appear in conflict with a chronic infection status, and instead suggest an acute infection preceding seroconversion. However, absence of a detectable anti-C antibody response has been described in vertically infected chronic HBV carriers caused by foetal exposure to the immunomodulatory eAg,<sup>26</sup> pointing at a potential vertical infection. Finally, biochemical markers of systemic infection (lactate dehydrogenase) and liver damage (gamma-glutamyltransferase) were compared between the five CMHBV antibody- and the single CMHBV DNA-positive, as well as 53 CMHBV-negative animals, for which sufficient sample volumes were available. Lactate dehydrogenase and gamma-glutamyltransferase levels were within the range described for capuchin monkeys,<sup>27</sup> and no significant differences were

**Fig. 2. CMHBV infection patterns.** (A) CMHBV genomic organisation. (B) Genomic identity between the CMHBV and reference hepadnaviruses. (C) Staining of primate hepadnavirus small surface p+roteins expressed in Huh7 cells by anti-HBs sera. (D) Co-localisation of signals of rabbit control serum and monkey serum (animal M5). (E) Polymorphisms in the CMHBV near-full genome; red, non-synonymous; black, synonymous. Del, deletion (del 1, C positions 219–296; del 2, C positions 244–393). (F) LDH and GGT activity in units (U)/L; n = 6 for CMHBV-positive and n = 53 for CMHBV-negative animals; red, CMHBV DNA-positive animal; line, median; p-values refer to Mann Whitney-U tests. AGL, antigenic loop; CMHBV, capuchin monkey hepatitis B virus; GGT, gamma-glutamyltransferase; HBV, hepatitis B virus; LDH, lactate dehydrogenase; WMHBV, woolly monkey hepatitis B virus.

Table 2. Percentage sequence distance between CMHBV, WMHBV, and other primate hepadnaviruses.\*

	CMHBV core (aa)	CMHBV LHBs (aa)	CMHBV Pol (aa)	CMHBV X (aa)	CMHBV complete genome (nt)	WMHBV complete genome (nt)
CMHBV						<b>20.5</b>
WMHBV	7.7–8.1	20.3	26.4–26.6	<b>28.7</b> –29.3	20.5	
ChHBV	8.1–12.9	21.1–23.1	26.9–28.4	30.7–34.0	21.1–21.8	21.5–22.5
GoHBV	8.6–9.5	21.1–21.3	27.4–27.9	30.1–31.6	21.2–21.7	21.6–22.0
OrHBV	<b>7.1</b> –8.6	21.1–22.6	27.0–28.1	29.4–31.4	20.6–20.9	21.2–21.9
GbHBV	<b>7.1</b> –12.4	<b>19.8</b> –26.0	<b>25.8</b> –28.4	30.1–35.9	20.5–21.8	21.3–23.1
HBV						
A	9.5–12.4	22.1–24.7	26.6–28.1	34.6– <b>37.9</b>	20.6–21.5	21.8–22.2
B	9.5–14.8	23.4–24.7	26.2–27.9	28.8–32.0	20.6–21.3	21.4–22.4
C	9.0–12.4	21.1–23.1	27.5–28.6	30.7–35.3	<b>20.4</b> –21.1	21.3–22.5
D	10.5–14.3	23.9–24.9	27.5– <b>29.0</b>	30.7–34.0	21.2–21.7	21.5–22.6
E	<b>16.2</b>	23.9	28.6	32.7	22.0	22.1–22.2
F	10.0–10.5	25.2– <b>26.5</b>	27.2–28.1	33.3–37.3	21.7– <b>22.3</b>	22.7–23.4
G	9.5	22.9	26.7	34.0	21.5	22.0–22.1
H	11.0	25.2	27.6	32.7	21.7	23.1–23.2
I	9.5–10.0	23.1–23.4	26.9–27.5	32.0–33.3	20.5–20.8	22.0–22.2
J	8.6	23.4	26.9	35.9	21.6	22.6–22.8
[A–J]	8.6–16.2	21.1–26.5	26.2–29.0	28.8–37.9	20.4–22.3	21.3– <b>23.4</b>
Overall	7.1–16.2	19.8–26.5	25.8–29.0	28.7–37.9	20.4–22.3	20.5–23.4
Mean (SD)	9.2 (1.8)	22.5 (1.3)	27.5 (0.6)	32.9 (1.9)	21.6 (0.4)	22.1 (0.4)

\* Highlighted in bold: lowest and highest distances (i.e. non-identity) within each column. Core open reading frame included the preCore domain. aa, amino acid; CMHBV, capuchin monkey hepatitis B virus; nt, nucleotide; WMHBV, woolly monkey hepatitis B virus.

observed between groups (Fig. 2F), which is compatible with a non-inflammatory CMHBV infection predicted by genomic comparisons.

### CMHBV receptor interactions

Attachment to the HBV receptor, sodium taurocholate (TC) co-transporting polypeptide (NTCP), and entry into hepatocytes are mediated by two subdomains of the hepadnaviral preS1 domain.<sup>28</sup> The NTCP-interacting preS1 domains of CMHBV, WMHBV, and HBV were highly conserved (Fig. 3A), suggesting similarities of cellular entry processes between these primate orthohepadnaviruses. Hepatitis delta virus (HDV) pseudotyped with the CMHBV and WMHBV S proteins (HDV<sub>CMHBV</sub> and HDV<sub>WMHBV</sub>) could infect human hepatocytes expressing NTCP from either human (hNTCP) or *Tupaia* origin (*Tupaia* is a surrogate animal model for HBV infection), albeit less efficient than HDV<sub>HBV</sub> (Fig. 3B). The addition of high amounts of HBV vaccine-induced anti-HBs IgG blocked the HDV<sub>HBV</sub> infection efficiently (>99.5%), whereas HDV<sub>CMHBV</sub> and HDV<sub>WMHBV</sub> infections were only reduced by about 40% (Fig. 3C). The fact that even very high amounts of anti-HBs could not efficiently neutralise HDV<sub>CMHBV</sub> and HDV<sub>WMHBV</sub> was consistent with the antigenic differences between the S proteins of CMHBV and WMHBV compared to the HBV vaccine strain (Fig. S1B) and with suboptimal vaccine-induced protection against heterologous serotypes in humans infected with HBV.<sup>29–31</sup>

The capuchin monkey NTCP homologue was unknown before this study. To assess the host associations of CMHBV comparatively, we characterised the NTCP homologue of a capuchin monkey (*Sapajus* Ntcp [sNtcp]). The sNtcp and the hNTCP differed by 29 amino acid residues (8.3%, Fig. S2). Since viral binding and entry compete with NTCP-mediated bile-acid transport,<sup>32</sup> we investigated the ability of sNtcp to mediate TC transportation. The sNtcp showed similar transport activity of TC compared to the hNTCP (Fig. 3D). Binding of HBV and WMHBV preS1 peptides to the hNTCP correlates with reduced TC transport in a quantitative manner.<sup>32,33</sup> Consistent with the host association of CMHBV, the CMHBV myr-preS1 peptide showed significantly stronger inhibition of TC transport by the

sNtcp compared to the hNTCP ( $p < 0.05$ ; Fig. 3E). Of note, the CMHBV myr-preS1 peptide-mediated reduction of TC transport by the hNTCP was still within the nanomolar range (Fig. 3E). This was consistent with the ability of CMHBV surface proteins to confer infection of human cells via the hNTCP (Fig. 3B).

### Phylogenetic relationships of the novel CMHBV

In a maximum likelihood (ML) phylogenetic reconstruction of the full genomes of primate hepadnaviruses, CMHBV clustered with WMHBV with high statistical support, forming a basal sister lineage to the HBV genotypes (Fig. 4A and Fig. S3). The same topology was observed upon inclusion of non-primate orthohepadnaviruses into the phylogenetic analysis (Fig. S4A). These phylogenetic relations corroborated the existence of an expanded primate hepadnavirus clade containing HBV and relatively more diversified viruses from New World NHPs.

### Co-segregation of hepadnaviruses and their hosts

Recent evidence suggests that orthohepadnaviruses may have co-evolved with their hosts.<sup>34</sup> Indeed, formal co-phylogenetic analyses comparing the overall agreement between virus and host trees<sup>15,16</sup> yielded statistically significant evidence ( $p < 0.005$ ) for co-segregation between primate hepadnaviruses and their hosts. However, of the 19 individual host–virus associations within the co-phylogenetic analysis, only the associations of CMHBV and WMHBV with their hosts were highly significant ( $p = 0.005$  and  $p = 0.014$ , respectively; red lines in Fig. 4B). For hominoid primates (apes and humans) and their hepadnaviruses, only a clade composed of HBV strains infecting *Nomascus* gibbons showed statistically significant co-segregation ( $p < 0.05$ ; Fig. 4B). In a confirmatory event-based co-phylogenetic reconstruction,<sup>17</sup> co-speciation was consistently observed for CMHBV and WMHBV and their hosts. By contrast, host-independent virus evolution was predominant among HBV. This included numerous duplication events (i.e. host-independent virus speciations) and sorting events (i.e. following host speciation, viruses remain on only one of the resulting species [Fig. 4C]). In sum, evidence for co-evolution was found chiefly for deep-branching hepadnaviruses from New

World NHPs, but less so for HBV strains infecting humans and hominoid primates. Therefore, co-evolution does not provide a likely explanation for the current phylogenetic relationships of HBV in hominoid primates.

### Reconstruction of ancestral host associations

Based on host associations in a Bayesian phylogeny, ancestral state reconstruction (ASR) at tree nodes was conducted. The most recent common ancestor (MRCA) of HBV was projected to a human host, rather than an NHP (posterior probability, 0.84) (Fig. 4D). This reconstruction was consistent with a higher patristic distance of HBV strains infecting humans (15.0%) than those infecting hominoid NHPs (10.9%). Only upon inclusion of CMHBV and WMHBV, hepadnavirus patristic distance was higher in NHPs than in humans with 23.1% (Fig. 4E). This was consistent with the projection of a NHP at the root of the primate hepadnavirus tree in ASR, albeit with lower statistical support (posterior probability, 0.72). Without the CMHBV, ASR yielded equal probability for a human or a NHP host at the root of the primate hepadnavirus tree (Fig. S4B), illustrating the relevance of CMHBV for the understanding of primate hepadnavirus origins. In sum, primate orthohepadnaviruses were projected to NHP ancestors, but less so to the HBV stem lineage.

### Hypothesis testing of HBV origins

To assess at what time HBV ancestors may have arisen within Old World primates (the Catarrhini), hypothesis testing was done in a time-calibrated Bayesian framework. In brief, the MRCA of HBV was calibrated by five different priors describing the origin of diverse primate ancestors. These priors included the origin of the Catarrhini (25.1 million years ago [mya];<sup>35</sup> Node 1 in Fig. 5A), of the Hominoidea (17.4 mya;<sup>35</sup> Node 2), of the genera *Homo* and *Pan* (5.54 mya;<sup>36</sup> Node 3), of the genus *Homo* (2.9 mya;<sup>37</sup> Node 4), and of anatomically modern humans (AMHs; 0.124 mya;<sup>38</sup> Node 5). Details of all calibrations are provided in the Supplementary methods.

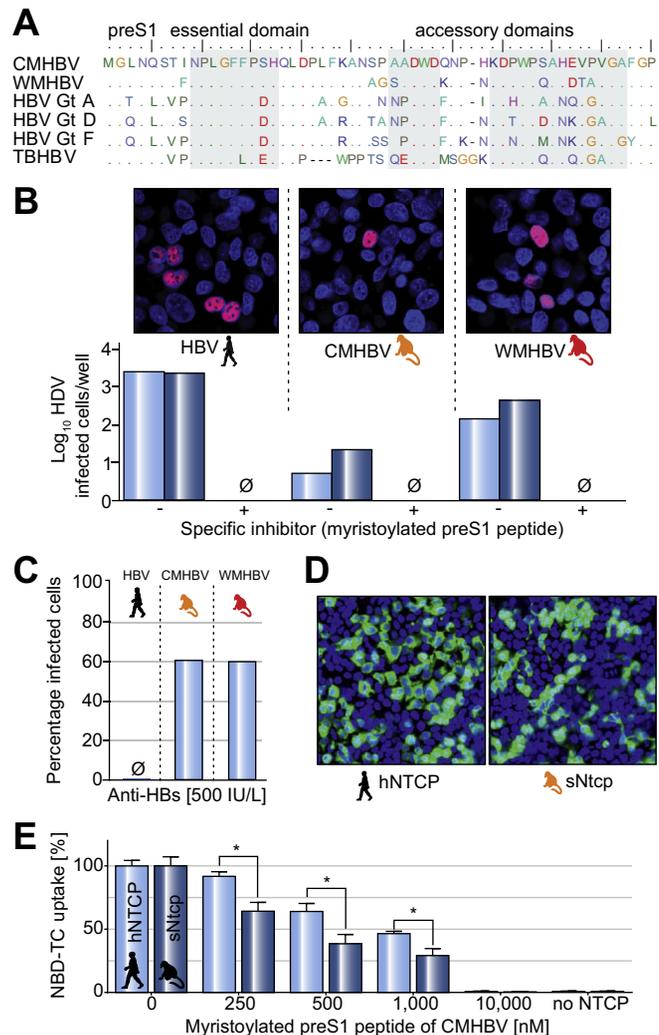
A relatively recent origin of HBV in AMH was not supported by these analyses, since all calibrations assigning MRCAs of primate ancestors were supported by significantly higher Bayes factors (BFs; commonly expressed as twice the natural logarithm [2 ln BF] in probabilistic theory;<sup>39</sup> Fig. 5A). The strongest support was found for an origin of HBV ancestors in Hominoidea (2 ln BF, 9.6–87.4 compared to all priors). Several confirmatory analyses using identical settings without the novel CMHBV also opposed an origin of HBV in AMH, but yielded considerably lower statistical support by up to five orders of magnitude for different calibrations (Fig. S4D), highlighting the importance of CMHBV for reconstructions of HBV origins.

In sum, evolutionary analyses supported an ancient and potentially co-evolutionary relationship between primate hepadnaviruses and NHP hosts, and strongly suggested an origin of HBV ancestors in Hominoid Old World primates, preceding the formation of the human stem lineage.

### Discussion

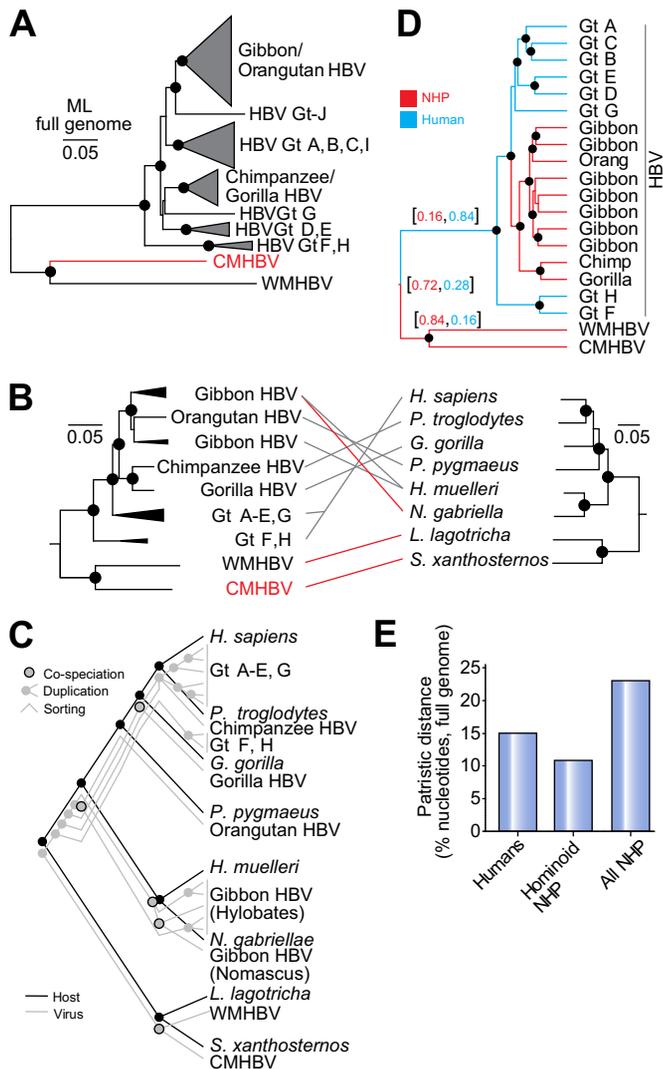
We discovered a new primate hepadnavirus species termed CMHBV from capuchin monkeys, and investigated its infection patterns and evolutionary history.

Our data suggest that catarrhine and platyrrhine primates have been carrying hepadnaviruses for millions of years, which disfavors a previous hypothesis assuming a split between



**Fig. 3. CMHBV–receptor interactions.** (A) PreS1 residues relevant for primate hepadnavirus entry. (B) Hepadnavirus LHB-mediated entry of HDV pseudotypes into human (light blue) or *Tupaia* (dark blue) NTCP-expressing hepatoma cells as detected by IFA for HDV antigen. (C) Neutralisation of pseudotypes by vaccine-induced antibodies (rabbit serum) relative to untreated inocula set as 100%. (D) NBD-TC (green) transport by human NTCP (hNTCP) and *Sapajus* NTCP (sNTcp) transiently expressed in HEK293 cells. Blue, nuclei. (E) Inhibition of hNTCP- and sNTcp-mediated transport of NBD-TC by myr-preS1-peptide of CMHBV relative to untreated controls (*t*-test,  $*p < 0.05$ ). CMHBV, capuchin monkey hepatitis B virus; HBV, hepatitis B virus; hNTCP, human sodium taurocholate co-transporting polypeptide; NBD-TC, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-taurocholate; sNTcp, *Sapajus* sodium taurocholate co-transporting polypeptide; WMHBV, woolly monkey hepatitis B virus.

hominoid HBV and hepadnaviruses from New World NHPs only several thousand years ago.<sup>40</sup> Similarly, an origin of all primate hepadnaviruses in New World monkeys is not supported by our results. A recent analysis consolidated an African origin of Platyrrhini and their arrival in South America at least 36 mya.<sup>41</sup> Our phylogenetic evidence is thus compatible with the existence of ancestral hepadnaviruses in African pre-platyrrhines introduced into South America during its transatlantic colonisation<sup>42</sup> (Fig. 5B). The strong evidence for an association of the HBV stem lineage with hominoid ancestors is compatible with the apparent absence of divergent HBV genotypes in cercopithecoïd Old World primates.<sup>43</sup> Whether other hepadnavirus species may exist in extant cercopithecoïd monkeys requires further



**Fig. 4. Evolutionary analyses including the novel hepadnavirus.** (A) Phylogenetic tree including NHP hepadnavirus complete genomes<sup>3</sup> and human HBV complete genomes.<sup>58</sup> (B) Co-phylogeny of primate hepadnaviruses and hosts; red lines, significant host-virus associations using ParaFit.<sup>16</sup> (C) Event-based co-phylogenetic reconstruction (CoRe-PA<sup>17</sup>). (D) Bayesian ancestral state reconstruction; filled circles in A, B, and E; bootstrap/posterior probability support >75%/>0.9; scale bar, substitutions per site. (E) Maximum sequence distances of complete hepadnavirus genomes from humans, hominoid NHPs, and all NHPs. CMHBV, capuchin monkey hepatitis B virus; Gt, genotype; HBV, hepatitis B virus; NHP, non-human primate; WMHBV, woolly monkey hepatitis B virus.

investigation, since the vast majority of primate species has not been investigated for hepadnaviruses.<sup>3</sup>

Whether indeed extant HBV genotypes infecting hominoid apes are the result of human-to-NHP transmission events,<sup>44</sup> as predicted in our analyses, or whether this apparent association with humans resulted from incomplete lineage sorting of HBV genotypes within hominoid NHPs remains to be determined in studies investigating whether divergent hepadnaviruses exist in hominoid NHPs.

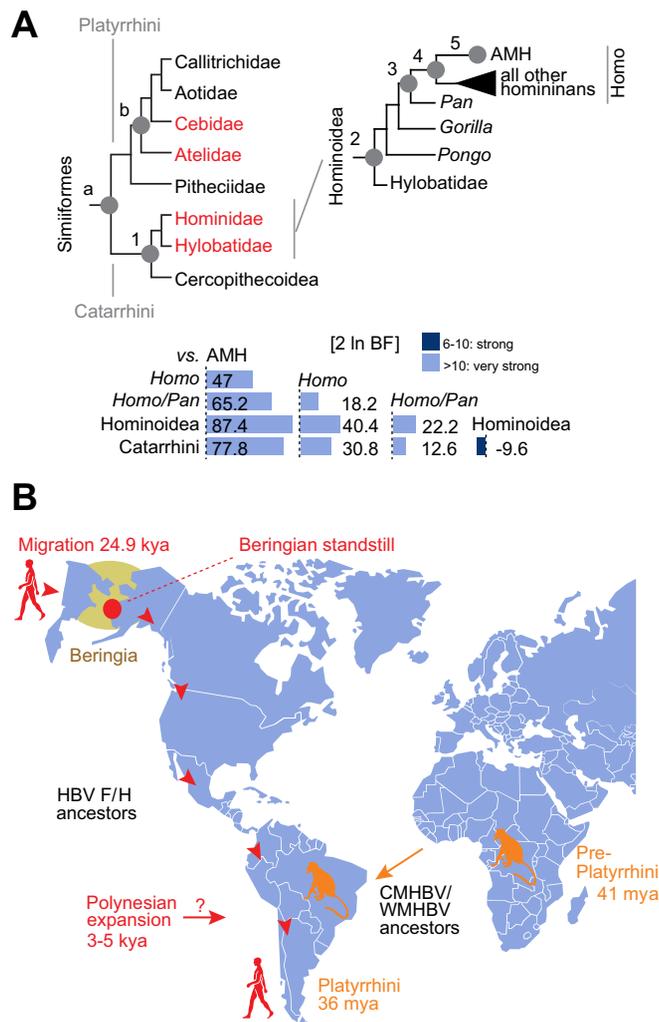
One of the major obstacles to reconcile the evolutionary history of humans and HBV has been the enigmatic origin of the

New World genotypes F and H.<sup>2</sup> Contrary to previous reconstructions,<sup>45</sup> neither the CMHBV nor the WMHBV appear to be direct evolutionary ancestors of HBV genotypes F and H. Our data thus suggest non-American origins of these divergent HBV genotypes. Recent evidence supports that human ancestors populating America were geographically isolated on the Beringian land bridge for about 9,000 years during the past glacial maximum.<sup>46</sup> It is conceivable that this relatively small group of people may have carried ancestors of HBV genotype F/H. The latter split into genotypes F and H<sup>47</sup> might have taken place during the rapid southward dispersal into the Americas<sup>46</sup> (Fig. 5B). HBV genotype F/H precursors in the Old World may have disappeared because of extinction events during the human dispersal history, as illustrated by the disappearance of HBV genotype C in Western Asia.<sup>2</sup>

Of note, recent evidence supports a potential pre-Columbian contact between Polynesians and South American natives about 3–5 thousand years ago,<sup>46</sup> which may be compatible with the occurrence of genotype F in Polynesia.<sup>48</sup> However, a hypothetical introduction of F/H ancestors into the Americas via the Polynesian route fails to explain the occurrence of genotype F in Alaska, and implies a relatively short time for the split into genotypes F and H and into four distinct F sub-genotypes. The slow long-term evolutionary rate of HBV challenging rapid speciation of genotypes F and H is best illustrated by the detection of a near-contemporary HBV genotype C sequence in a Korean mummy from the 16th century.<sup>2,49</sup> However, more than one introduction of genotype F/H ancestors into the Americas cannot be excluded at the current knowledge of the human dispersal history.

Our data on entry of viral pseudotypes into human hepatocytes and on interaction of CMHBV peptides with the human HBV receptor may imply a zoonotic potential of hepadnaviruses circulating in New World NHPs. However, infection experiments relying on full hepadnaviruses and primary human liver cells will be required to permit definite conclusions on the zoonotic potential of CMHBV. Of note, third-generation HBV vaccines containing preS1 epitopes<sup>50</sup> might be more potent to neutralise divergent hepadnaviruses, like CMHBV, efficiently, highlighting an additional value of newer vaccine generations.

Chronic hepatitis B infection remains a major human disease with no effective cure. Despite recent advances using transgenic mice,<sup>51</sup> research on hepatitis B cure is hampered by the lack of suitable animal models for persistent HBV infection.<sup>52</sup> Challenges to develop primate models for persistent hepatitis B infection include ethical constraints on the usage of chimpanzees, the endangered status of woolly monkeys, and non-persistent infection with WMHBV in spider monkeys.<sup>52</sup> Whether macaques may prove useful animal models for chronic hepatitis B remains to be confirmed.<sup>53</sup> Capuchin monkeys easily breed in captivity<sup>54</sup> and are among the most widely used New World primates in biomedical research.<sup>55</sup> Even though capuchin monkeys have not been widely used as animal models in recent infectious-disease research, pivotal studies on schistosomes and herpesviruses illustrate the technical usability of these animals for controlled infections.<sup>56,57</sup> The novel CMHBV showed preliminary evidence for the capacity to cause chronic infections and for a broad host range potentially facilitating infection studies in non-endangered capuchin monkey species. Our findings thus enable new perspectives to investigate HBV pathogenesis and cure.



**Fig. 5. Reconstructions of HBV origins.** (A) Cladogram of primates according to Springer *et al.*<sup>35</sup> Letters and numbers along primate cladograms represent nodes calibrated for hypothesis testing and results of Bayes factor tests (details provided in the Supplementary methods); strength of evidence from Bayes factor (BF) tests given as twice the natural logarithm (2 ln) according to Kass and Raftery.<sup>39</sup> (B) Biogeographic origins of American primates and potential hepadnavirus dispersal routes. Red, human origins and putative introduction of HBV genotypes F and H; orange, New World NHP origins and those of CMHBV and WMHBV ancestors. CMHBV, capuchin monkey hepatitis B virus; HBV, hepatitis B virus; kya, thousand years ago; mya, million years ago; NHP, non-human primate; WMHBV, woolly monkey hepatitis B virus.

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### Conflict of interest

The authors do not declare any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors' contributions

B.F.C.D.S., A.K., A.R.: acquisition, analysis, and interpretation of data. I.D.O.C.: design and execution of field work. V.M.C., N.S., P.L.R., N.G., R.K., S.F.M., C.V., A.M.S., A.J.S.D.S., M.S.G.G., A.S., M. N., C.R.F., J.R.R.P., M.D.C.P.S., J.G., P.L., C.D., E.M.N.: acquisition and analysis of data. D.G., J.F.D.: study concept, design, and supervision, and writing of the article.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jhep.2018.01.029>.

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Author names in bold designate shared co-first authorship

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