A hepatitis B virus causes chronic infections in equids worldwide

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Preclinical testing of novel therapeutics for chronic hepatitis B (CHB) requires suitable animal models. Equids host homologs of hepatitis C virus (HCV). Because coinfections of hepatitis B virus (HBV) and HCV occur in humans, we screened 2,917 specimens from equids from five continents for HBV. We discovered a distinct HBV species (Equid HBV, EqHBV) in 3.2% of donkeys and zebras by PCR and antibodies against EqHBV in 5.4% of donkeys and zebras. Molecular, histopathological, and biochemical analyses revealed that infection patterns of EqHBV resembled those of HBV in humans, including hepatotropism, moderate liver damage, evolutionary stasis, and potential horizontal virus transmission. Naturally infected donkeys showed chronic infections resembling CHB with high viral loads of up to 2.6×10^9 mean copies per milliliter serum for >6 mo and weak antibody responses. Antibodies against Equid HCV were codetected in 26.5% of donkeys seropositive for EqHBV, corroborating susceptibility to both hepatitis viruses. Deltavirus pseudotypes carrying EqHBV surface proteins were unable to infect human cells via the HBV receptor NTCP (Na^+/taurocholate cotransporting polypeptide), suggesting alternative viral entry mechanisms. Both HBV and EqHBV deltavirus pseudotypes infected primary horse hepatocytes in vitro, supporting a broad host range for EqHBV among equids and suggesting that horses might be suitable for EqHBV and HBV infections in vivo. Evolutionary analyses suggested that EqHBV originated in Africa several thousand years ago, commensurate with the domestication of donkeys. In sum, EqHBV naturally infects diverse equids and mimics HBV infection patterns. Equids provide a unique opportunity for preclinical testing of novel therapeutics for CHB and to investigate HBV/HCV interplay upon coinfection.

HBV | equids | animal model | coinfection | evolution

Chronic hepatitis B (CHB) is a major burden to human health. CHB is caused by infection with hepatitis B virus (HBV), the prototype species of the viral family *Hepadnaviridae*. Despite the availability of an effective vaccine, CHB causes about 900,000 deaths worldwide annually (1) due to liver cirrhosis and hepatocellular carcinoma (2). CHB treatment is currently limited to administration of pegylated-interferon-α (PegIFN-α) and nucleos(t)ide analogs, which are costly and can have side effects. HBV replication can be suppressed effectively by long-term treatments, but there is no cure for CHB (3). Promising novel drug candidates for CHB interfere with HBV entry, covalently closed circular DNA formation, transcription, capsid assembly, and egress or act as immune modulators (reviewed in ref. 4).

Progress of drug development for CHB is hampered by the lack of suitable preclinical animal models (5). Traditional animal models comprise chimpanzees, tree shrews, and woodchucks. However, experimental HBV infection of chimpanzees is restricted due to ethical concerns. HBV infection efficacy in tree shrews is low and autochthonous tree shrew hepadnaviruses are unknown. Woodchuck hepatitis B virus can cause chronic infection, but the hibernation of woodchucks influences the course of infection and complicates experimental set-ups (6). Chimeric humanized mice can be infected with HBV, but are immunodeficient and thus not generally suitable for the study of new drug candidates (7).

Newly discovered animal homologs of human viruses may afford novel in vivo models (6), exemplified by new hepatitis C infection

Significance

The development of urgently needed hepatitis B virus (HBV) treatments is challenged by the lack of suitable animal models. Here, we describe an HBV-related virus, which occurs globally in donkeys and zebras (Equid HBV, EqHBV). EqHBV presumably originated in Africa, approximately at the time when donkey domestication started. EqHBV causes a hepatotropic disease comparable to human HBV infection and prolonged infections, resembling chronic hepatitis B in humans. In vitro infection studies suggested that horses are also susceptible to EqHBV. Both donkeys and horses also host a hepatitis C virus (HCV)-like virus. EqHBV offers unique opportunities for in vivo studies of both chronic hepatitis B and HBV/HCV coinfections in terms of pathogenesis and therapy optimization.

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models based on hepatitis C virus (HCV)-related viruses from rats and horses (8–10). A variety of distantly HBV-related hepadnaviruses were recently identified in diverse animals such as bats and shrews (summarized in ref. 6). However, their potential usability as animal models for CHB is unclear due to challenges of housing these animals, short life spans, and unclear infection patterns.

Because horses and donkeys host the closest known homolog of HCV (6), and because human HBV/HCV infections frequently cooccur due to similar transmission routes (11), we investigated equids sampled globally for HBV homologs. We characterized a ubiquitous new HBV species infecting donkeys and zebras that causes chronic infections resembling human CHB.

Results

A Divergent Hepadnavirus Species in Donkeys and Zebras. To assess whether hepadnaviruses occur in equids, we investigated 2,917 specimens from donkeys, mules, horses, and zebras sampled in 13 countries on five continents (Table 1). Using a broadly reactive and highly sensitive nested PCR assay (12), we detected hepadnavirus DNA in 24 domestic donkeys (*Equus asinus*) from Bulgaria, Italy, Germany, Israel, Kenya, and Ghana and in five wild plain zebras (*Equus quagga*) from Tanzania (3.2% of donkeys and zebras; 95% CI, 2.2 to 4.6). In contrast, 1,958 horses were negative in the same assay (Fisher's exact test, $P < 0.001$) (Table 1). Complete viral genomes were characterized using a PCR-based approach (*SI Appendix*) from 21 strains (GenBank accession nos. MT134279, MT134282–MT134284, MT134294, MT134306–MT134321), while limited quantities of viral DNA hampered full genome characterization of the remaining PCR^+ samples. In all cases, the genome composition resembled that of HBV in size and its organization characterized by four overlapping open reading frames (ORFs) encoding polymerase, surface, X and core proteins, including likely production of an e antigen (HBeAg) as deduced from the presence of a preC domain. Uncommon genomic patterns included lack of an MHBs-encoding region within the surface ORF and lack of an overlap between the preC-encoding region and the X ORF (Fig. 1*A*). The maximum nucleotide sequence diversity among equid hepadnavirus genomes was low at 4.6% (4.1% among donkey-derived sequences and 0.6% among zebra-derived sequences). This was below the 8% sequence distance threshold separating genotypes in human HBV, suggesting that all equid-associated HBV strains correspond to one genotype. Based on nucleotide sequence distances to other mammalian hepadnaviruses exceeding 30.4 to 42.7% (*SI Appendix*, Table S1), the equid-associated hepadnavirus formed a distinct species termed Equid HBV (EqHBV). In a maximum-likelihood phylogeny based on the complete viral genome, EqHBV clustered distantly with recently described duiker and cat HBVs (Fig. 1*B*) and was approximately equidistant to hepadnavirus reference species along the complete viral genome (*SI Appendix*, Fig. S1). Deep sequencing of PCR amplicons showed no evidence for presence of another hepadnavirus in PCR^+ specimens, suggesting EqHBV is the major hepadnavirus infecting equids.

Zebras and Donkeys Have Antibodies against Hepadnaviruses. To elucidate the geographic distribution of EqHBV and to test whether EqHBV elicits an adaptive immune response, we performed an immunofluorescence assay (IFA) relying on the antigenically conserved HBV core protein (HBc) for antibody detection (12). The serological reactivity pattern of donkey sera was dispersed across both cytoplasm and nucleus, resembling human anti-HBc antibody reactivity (Fig. 1*C*). Anti-HBV antibodies were detected in 5.4% (95% CI, 4.0 to 7.2) of donkey and zebra sera originating from almost all sampled countries (Fig. 1*D* and Table 1), suggesting a worldwide distribution of EqHBV. Consistent with the absence of positive results during PCR testing, 150 horses sampled in Germany, Spain, and Brazil showed no evidence for HBV antibodies in the same IFA (Fisher's exact test, $P = 0.002$). The overall EqHBV seroprevalence in donkeys and zebras was low compared to about 20% HBV antibody prevalence in nonvaccinated human populations (13) or in nonhuman primates (20 to 40%) (14, 15). However, similarly low seroprevalence rates occur in other nonprimate hepadnavirus hosts, such as bats and shrews with 1.2 to 3.4% (16, 17), suggesting robustness of serological testing and commonalities of HBV spread in nonprimate hosts. Notably, the total EqHBV seroprevalence closely

Table 1. Sample characteristics

Equus species	Country	Year	No. of animals	PCR^+ (%)	$Antibody^+$ (%)
E. asinus	Germany	2007, 2008, 2015	56	6 (10.7)	7 (12.5)
	Spain	2011	82	0	2 (2.4)
	Italy	2013	356	2 (0.6)	5 (1.4)
	Bulgaria	2015	148	1 (0.7)	12 (8.1)
	Israel	2014	44	4 (9.1)	2 (4.5)
	Kenya	2015	34	1 (2.9)	2 (5.9)
	Mexico	2016	58	0	5 (8.6)
	Costa Rica	2016	15	0	3 (20.0)
	Ghana	2015	84*	10 (11.9)	5 (19.2)
Total (donkey)			877	24 (2.7)	43 (5.3)
E. mulus	Bulgaria	2015	53	0	0
Total (mule)			53	0 (0.0)	0 (0.0)
E. quagga/E. zebra	Namibia	2015	15	0	0
E. quagga	Tanzania	2016	14	5 (35.7)	3 (21.4)
Total (zebra)			29	5 (17.2)	3 (10.3)
Total (donkey/zebra)			906	29 (3.2)	46 (5.4)
E. ferus caballus	Spain	N.D.	607	0	0/50†
	Brazil	2013–2018	1,170	0	0/50†
	Germany	2017–2018	181	0	0/50†
Total (horse)			1,958	0	0
Total (study)			2,917	29 (1.0)	46 (1.6)

N.D., no data.
*Liver samples of all animals and sera samples of a subset of 26 animals.
†A subset of 50 samples was tested for antibodies.

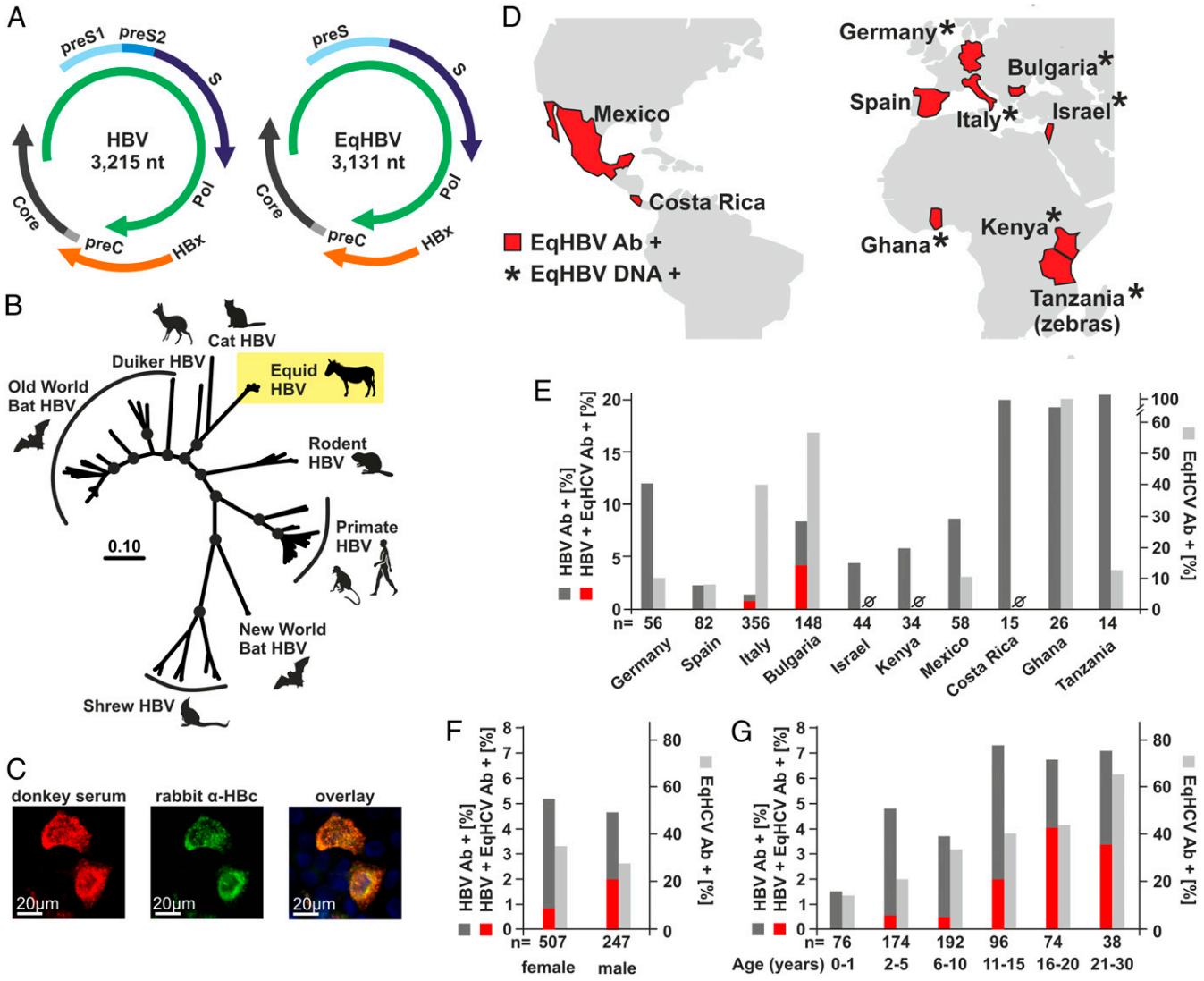

Fig. 1. Phylogeny and epidemiology of EqHBV. (*A*) Organization of HBV and EqHBV genomes. (*B*) Complete genome-based maximum-likelihood phylogeny. (*C*) Immunofluorescence costaining using an anti-EqHBV antibody$^+$ donkey serum (Do-15, Germany) and a rabbit anti-HBc serum. (*D*) EqHBV DNA and antibody detection in donkeys and zebras. (*E–G*) Seroprevalence per country (*E*), sex (*F*), and age group (*G*). Ab, antibodies; EqHBV, Equid HBV; EqHCV, Equid HCV; S, surface protein; Pol, polymerase; HBx, hepatitis B virus X protein.

corresponded to the EqHBV DNA detection rate by PCR, suggesting uncommon abortive infection or spontaneous virus clearance.

EqHBV and EqHCV Cocirculate in Donkeys. In humans, coinfections with HBV and HCV occur frequently due to shared transmission routes (11). We previously detected an HCV homolog in donkeys termed Equid HCV (EqHCV) (18). Of 34 EqHBV-seropositive donkeys, nine animals (26.5%; 95% CI, 14.4 to 43.3) originating from Italy and Bulgaria were seropositive for EqHCV using an established luciferase immunoprecipitation system assay (18), which corroborated susceptibility of donkeys for both hepatitis viruses (Fig. 1*E*). No statistically significant differences in EqHBV seroprevalence were detected between sexes (Fisher's exact test, $P = 1.000$) (Fig. 1*F*) or age groups (χ^2 test for trend, $P = 0.067$) (Fig. 1*G* and *SI Appendix*, Table S2). For EqHBV/EqHCV antibody codetections, no significant difference was observed between sexes (Fisher's exact test, $P = 0.108$) (Fig. 1*F*, red bar). However, a statistically significant increase of codetections with age was detected (χ^2 test for trend, $P = 0.026$) (Fig. 1*G*, red bar), which may be explained by elevated EqHCV seroprevalence in older animals (Fig. 1*G*, light gray bar). In sum, donkeys of different age groups, sexes, and geographic origins are exposed to equid HBV and HCV homologs at low rates.

EqHBV Is Hepatotropic. To investigate whether organ tropism and pathogenicity of EqHBV are similar to HBV, we tested organ and blood samples of 84 donkeys from an abattoir in northern Ghana. Mean virus concentrations in liver were significantly higher compared to those of other solid organs and blood (*t* test, $P = 0.001$) (Fig. 2*A*). In analogy to human HBV infections, virus concentrations varied among individual donkeys, reaching high levels of up to 10^{11} copies per gram of liver tissue and of up to 10^9 copies per milliliter of blood, likely due to viremic spread from infected hepatocytes. Next, we investigated pathological changes in infected donkeys. In humans, chronic hepatitis commonly leads to portal and periportal inflammation and successively causes progressive fibrosis (19). In EqHBV antibody$^+$ or DNA$^+$ samples, we observed significantly higher inflammatory activity (Ludwig-Batts scores I and II, exemplified in Fig. 2*B*) (Fisher's exact test, $P = 0.002$), and

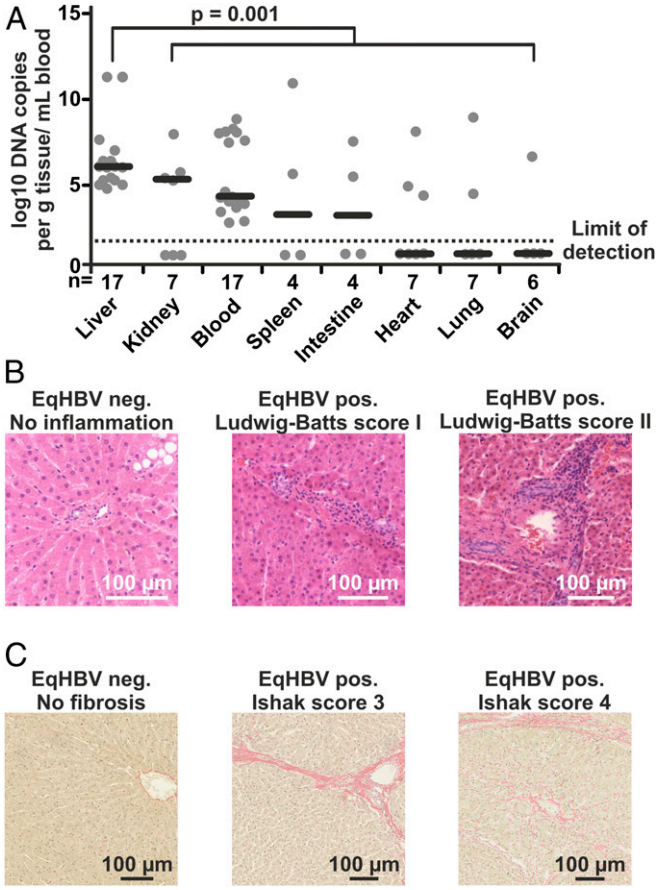

Fig. 2. Hepatotropism and pathogenicity of EqHBV. (*A*) EqHBV concentrations in Ghanaian donkey samples. Black bars, medians. (*B* and *C*) Pathological analyses. H&E-stained (*B*) and Sirius red-stained (*C*) liver tissue from donkeys. Three representatives are shown, sample IDs (*B*, left to right) Li-40, Li-34, Li-35, and (*C*, left to right) Li-09, Li-10, Li-41. EqHBV, Equid HBV; neg., negative; pos., positive.

portal to periportal fibrosis (Ishak scores 3 and 4, exemplified in Fig. 2*C*) (Fisher's exact test, $P = 0.038$) compared to EqHBV PCR$^-$ and antibody$^-$ animals (*SI Appendix*, Table S3). Acute EqHCV infection was excluded using a strain-specific real-time RT-PCR (18), but all animals were EqHCV-seropositive. In sum, EqHBV shows liver tropism and may lead to moderate liver damage, resembling the initial stages of CHB in humans.

EqHBV and HBV Differ in Receptor Usage. Human HBV enters hepatocytes via the Na^+/taurocholate cotransporting polypeptide (NTCP) (20). Despite the large genetic divergence of EqHBV and human HBV, EqHBV and HBV showed similarities in the HBV receptor-binding domain, including the essential NPLGF amino acid motif at the N-terminal domain of the large HBs (Fig. 3*A*). This motif is present in all hepadnaviruses known to use the NTCP for viral entry, including all primate HBVs and the tent-making bat HBV (TBHBV) (12). However, it is absent in shrew and woodchuck hepadnaviruses, which likely enter cells via an unknown receptor (16). To investigate the ability of the EqHBV surface protein to enter cells via the equid NTCP ortholog, we generated hepatitis D viruses (HDV) pseudotyped with surface proteins of HBV (HDV_{HBV}) and two EqHBV strains from a donkey ($HDV_{Do\text{-}EqHBV}$) and a zebra ($HDV_{Ze\text{-}EqHBV}$). $HDV_{Do\text{-}EqHBV}$ and $HDV_{Ze\text{-}EqHBV}$ could infect neither hepatoma cells expressing the human NTCP, nor the donkey NTCP ortholog (Fig. 3*B*). Consistently, neither $HDV_{Do\text{-}EqHBV}$ nor $HDV_{Ze\text{-}EqHBV}$ could infect primary human hepatocytes (PHHs) (Fig. 3*C*). To assess postentry replicative capacity of EqHBV, HepG2 cells were transfected with EqHBV-encoding 1.5mer overlength constructs. Detection of intracellular HBc and secreted HBeAg of two donkey- and zebra-associated EqHBV strains indicated ability of human hepatoma cells to express EqHBV proteins under viral endogenous promoters (*SI Appendix*, Fig. S2). Those data suggested that EqHBV enters cells via a different receptor or depends on unknown coreceptors, and that entry is a major barrier limiting the zoonotic potential of EqHBV.

EqHBV and HBV Infect Horse-Derived Hepatocytes In Vitro. Because cross-species transmission (CST) can occur in hepadnaviruses and because host genetic relatedness may facilitate CST (12, 21), we investigated whether horse-derived primary equine hepatocytes (PEHs) are susceptible to EqHBV. Expression and physiological functionality of the NTCP ortholog in PEHs was confirmed by IFA using a cross-reactive NTCP-antibody and a taurocholate transport assay (16) (*SI Appendix*, Fig. S3). PEHs were susceptible to both HDV_{HBV} and $HDV_{Ze\text{-}EqHBV}$, but not to $HDV_{Do\text{-}EqHBV}$ (Fig. 3*D*), potentially associated with 10 amino acid residues differing between donkey- and the zebra-associated EqHBV strains within the ORF encoding the viral surface proteins likely mediating entry (*SI Appendix*, Fig. S4). To further analyze the ability of human HBV to replicate in PEHs, we used cell culture-derived infectious HBV particles. Successful infection was demonstrated by de novo HBeAg production characteristic for HBV infection (22) (*SI Appendix*, Fig. S5). Productive HBV infection could be inhibited by vaccine-derived anti-HBs antibodies (500 IU/mL), but not by a high-titered EqHBV antibody$^+$ donkey serum (end-point IFA titer 1:4,000), compatible with antigenic divergence of HBV and EqHBV surface proteins. Infection of PEHs with EqHBV$^+$ sera from donkeys or zebras was not observed, potentially due to degradation after long-term storage and sampling in tropical settings and due to limited sample volumes preventing generation of high-titered inocula (Fig. 3*E*).

EqHBV Causes CHB in Donkeys. In humans, CHB is defined as prolonged HBV infection exceeding 6 mo (23). Hallmarks of CHB include high viral load and limited inflammation, associated with presence of the immunoregulatory viral protein HBeAg (3). To investigate whether EqHBV infection leads to CHB, we evaluated biochemical markers and individual EqHBV infection histories of seven female donkeys (jennies) from Germany that were naturally infected with EqHBV and sampled longitudinally during pregnancy up to 12 mo (24). All jennies were negative for EqHCV RNA and antibodies. Of the seven jennies, one was newly infected during the study period, indicating that EqHBV can be acquired by adult animals (Fig. 4*A*). Another two jennies showed serologic evidence for past EqHBV infection (Fig. 4 *B* and *C*), one of them potentially with a viremic flare during the study period (Fig. 4*C*). The four remaining jennies showed evidence for chronic infection with EqHBV, indicated by prolonged viremia of EqHBV for >6 mo with dynamic viral loads of up to 2.6×10^9 mean copies per milliliter serum and presence of relatively constant levels of anti-HBc antibodies (Fig. 4 *D*–*G*). Biochemical parameters of liver function were in a normal range in all EqHBV-infected jennies compared to those of noninfected animals (Fig. 4 and *SI Appendix*, Fig. S6). High-throughput sequencing (HTS) of the preC/Core region of samples from all time-points confirmed a functional HBeAg-coding region during the complete course of infection, including absence of single nucleotide variants hypothetically preventing HBeAg translation (represented by + symbols on top of panels in Fig. 4).

Next, we analyzed intrahost genomic variation in animals with CHB by HTS. Intrahost consensus nucleotide sequences of EqHBV strains did not change during a time span of up to 15 mo. Single nucleotide polymorphisms (SNPs) (defined by occurrence

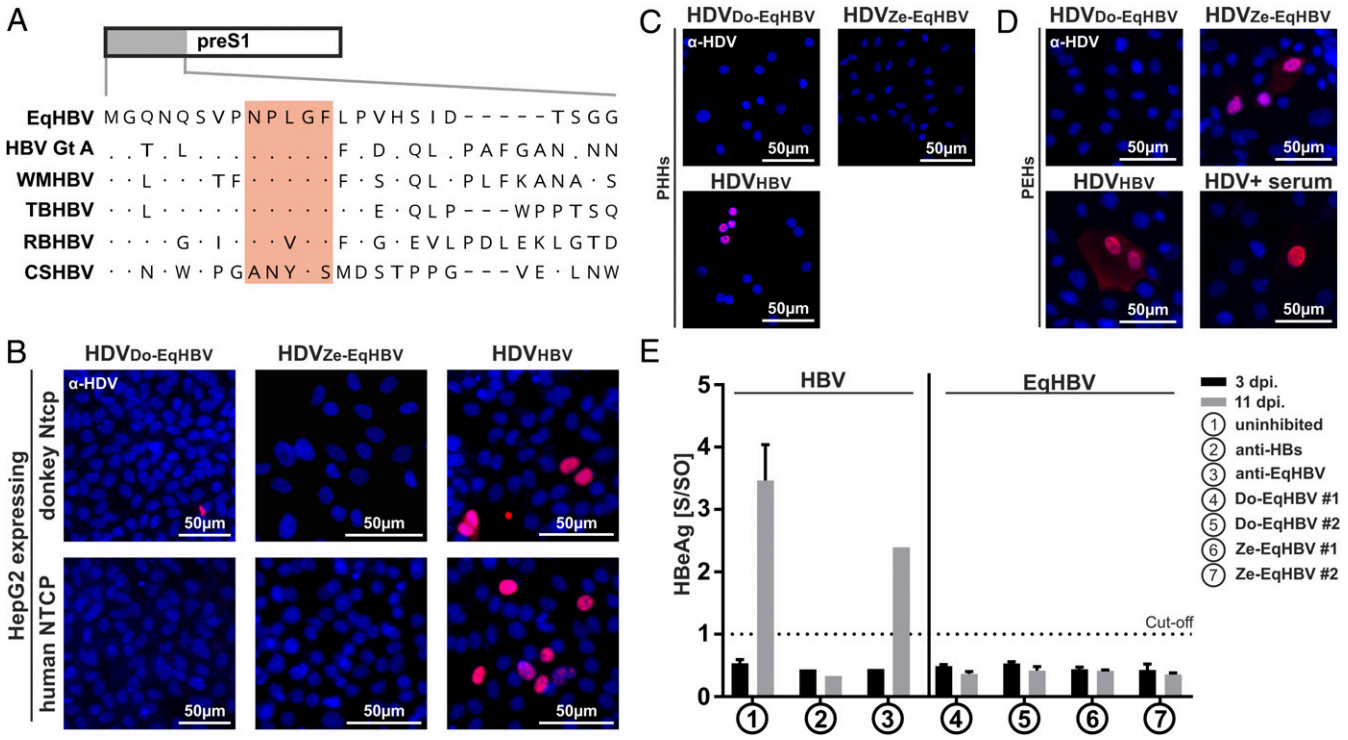

Fig. 3. Cell entry studies of EqHBV and HBV. (*A*) preS1-encoding region. Highlighted, essential NTCP-binding domain in human HBV. Dots, identical amino acids. The translated EqHBV sequence was identical in all strains. Additional sequence elements in HBV genotype A are not shown for clarity of presentation. (*B*) Infection of HepG2 cells expressing the human or the donkey NTCP ortholog. (*C*) Infection of PHHs and (*D*) Infection of PEHs with HDV pseudotyped with EqHBV-derived ($HDV_{Do\text{-}EqHBV}$/$HDV_{Ze\text{-}EqHBV}$) or HBV-derived HBs (HDV_{HBV}). Red, infected cells detected via Delta antigen staining using a patient-derived polyclonal HDV-antiserum. Blue, nuclei. (*E*) HBeAg production in PEHs (Architect; Abbott). S/CO, signal to cutoff ratio. anti-EqHBV, EqHBV-antibody$^+$ donkey serum; anti-HBs, vaccine-derived antiserum; CSHBV, crowned shrew HBV; WHV, woodchuck hepatitis B virus; WMHBV, woolly monkey HBV.

in >2.5% of at least 200 reads) occurred with a mean rate of 0.5 per 100 nucleotides. This was low compared to a mean rate of 1.8 SNPs per 100 nucleotides detected in HTS analyses of a capuchin monkey HBV (25) and resembled HBV during the $HBeAg^+$ phase of CHB (26). In sum, EqHBV can cause chronic infections resembling $HBeAg^+$ human CHB with respect to virus infection markers and genome conservation.

EqHBV Likely Originated Nonrecently in African Donkeys. Hepadnaviruses are ancient viruses that have likely existed in vertebrates for >200 million y (27) and have evolved within the mammalian lineage for about 80 million y (16). Our detection of EqHBV in the order Perissodactyla (odd-toed ungulates) extends the number of mammalian orders hosting hepadnaviruses to seven (Fig. 5*A*). The near-global distribution of EqHBV and the detection of EqHBV in both donkeys and zebras raise questions on the timespan of hepadnavirus existence in equids. Complex recombination patterns within EqHBV genomes and with nonequid hepadnaviruses were suggested by different topologies of phylogenies based on individual ORFs (*SI Appendix*, Fig. S7*A*) and formal recombination analyses (Fig. 5*B* and *SI Appendix*, Fig. S7*B*). According to our data, acute EqHBV infection occurs at low rates. Complex recombination patterns by coinfection of highly divergent viruses thus likely arose during a long timespan, hinting at a nonrecent origin of EqHBV. To probe the time and place of EqHBV origins, we reconstructed the geographic origin of EqHBV using an ancestral state reconstruction (ASR) in a Bayesian framework (*SI Appendix*). The geographic origin of EqHBV was reconstructed to Africa with substantial evidence (Bayes factor [BF], 3.1) (28) (Fig. 5*C*). Because the domestication of donkeys presumably started in Africa (29, 30), the ASR would be compatible with a long evolutionary history of EqHBV in equids. Of note, zebra- and donkey-associated EqHBV did not form separate clades (Fig. 5*C*), contrasting the phylogeny of their hosts (Fig. 5*D*). An ASR of the presumable host of the EqHBV ancestor yielded decisive evidence for donkeys compared to zebras (BF, 141.9) (28). This suggested a CST among genetically related and frequently sympatric donkey and zebra species, which is consistent with our experimental data showing the susceptibility of horse hepatocytes to noncabbaline, zebra-associated EqHBV. Notably, the likely origin of zebra-associated EqHBV in ancestors carried by donkeys may hint at a larger genetic diversity of EqHBV existing in donkeys.

EqHBV Origins Coincide with the Domestication of Donkeys. Next, we estimated the time to the most recent common ancestor (TMRCA) in a time-calibrated Bayesian framework, assuming a mean evolutionary rate of 1×10^{-5} according to ancient DNA data of human HBV (31). This analysis suggested that the common ancestor of EqHBV dated back ~2,400 y (Fig. 5*C*). To probe whether this calculation was appropriate, we used a second approach, based on the likelihood of different priors for the TMRCA of EqHBV. For this analysis, we constructed a dataset including all hepadnavirus species infecting members of the clade of mammals that includes ungulates, termed Laurasiatheria (Fig. 5*A*) and calibrated the root of the resulting phylogeny by the origin of Laurasiatherian mammals (16) (*SI Appendix*, Fig. S7*C*). Next, the TMRCA of EqHBV was calibrated by different priors describing the evolution and dispersal of its hosts, including the rise of the noncaballine clade, the time of donkey domestication, and the time of global donkey dispersal, calibrated by the arrival of Christopher Columbus in the Americas in 1492 (*SI Appendix*, Fig. S5*D*). The prior describing the time of donkey domestication ~5,000 to 6,000 y ago (32) was favored with strong to decisive statistical support over all other

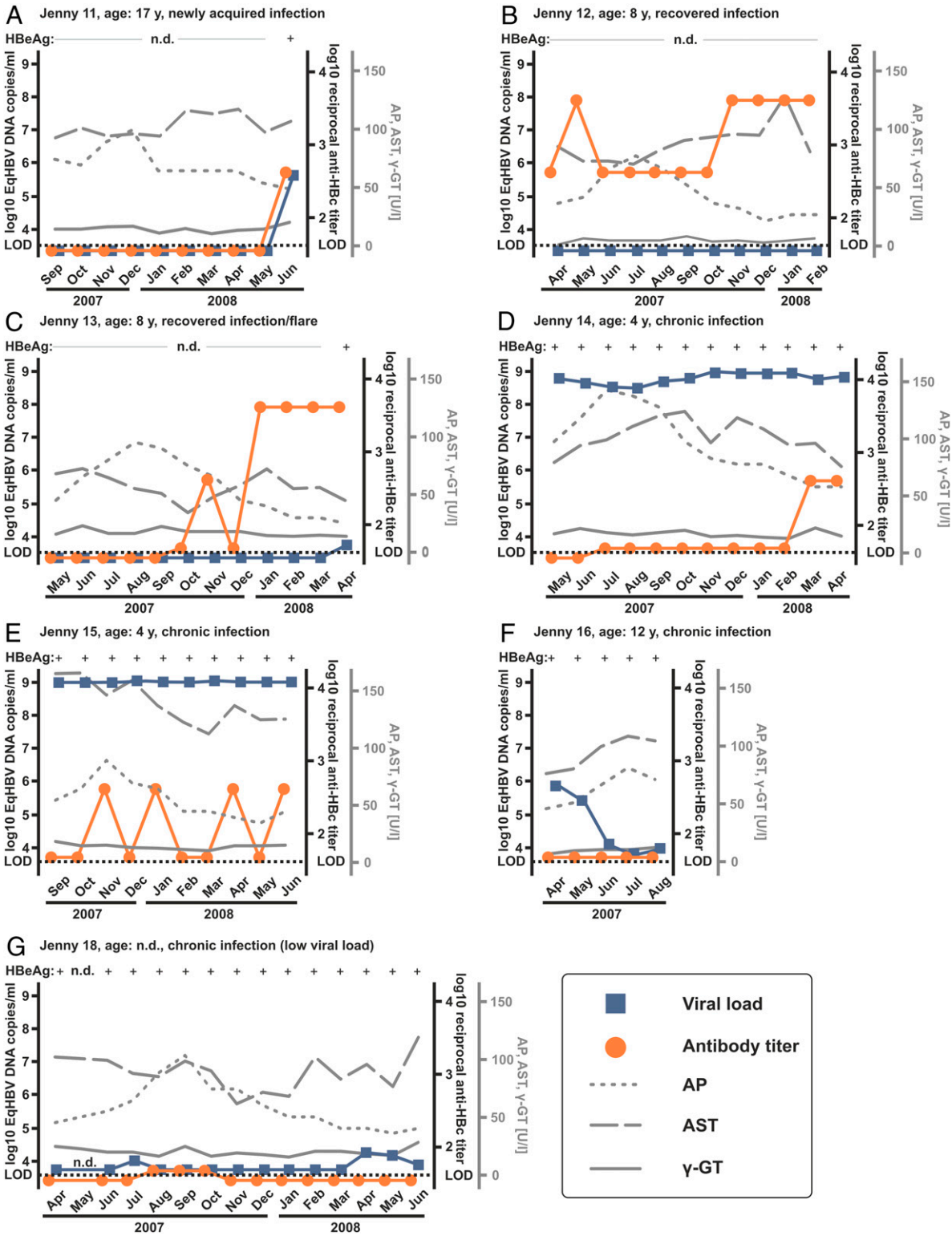

Fig. 4. (*A–G*) EqHBV infection markers in longitudinally sampled donkeys. Alkaline phosphatase (AP), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT) units per liter (U/L). Upper limit in five EqHBV^{-} animals (*SI Appendix*, Fig. S6): AP, 140 U/L; AST, 180 U/L; γ-GT, 35 U/L. n.d., no data. LOD, limit of detection.

priors (Fig. 5*D*). Robustness of these evolutionary reconstructions despite nonrecent recombination in the dataset was suggested by similar analyses done previously (16). Both time-calibrated analyses thus favored a scenario in which EqHBV emerged several thousand years ago. Human activities, such as rearing and handling of animals or veterinary practices, presumably dispersed EqHBV among donkey populations globally, similar as suggested before for EqHCV (18).

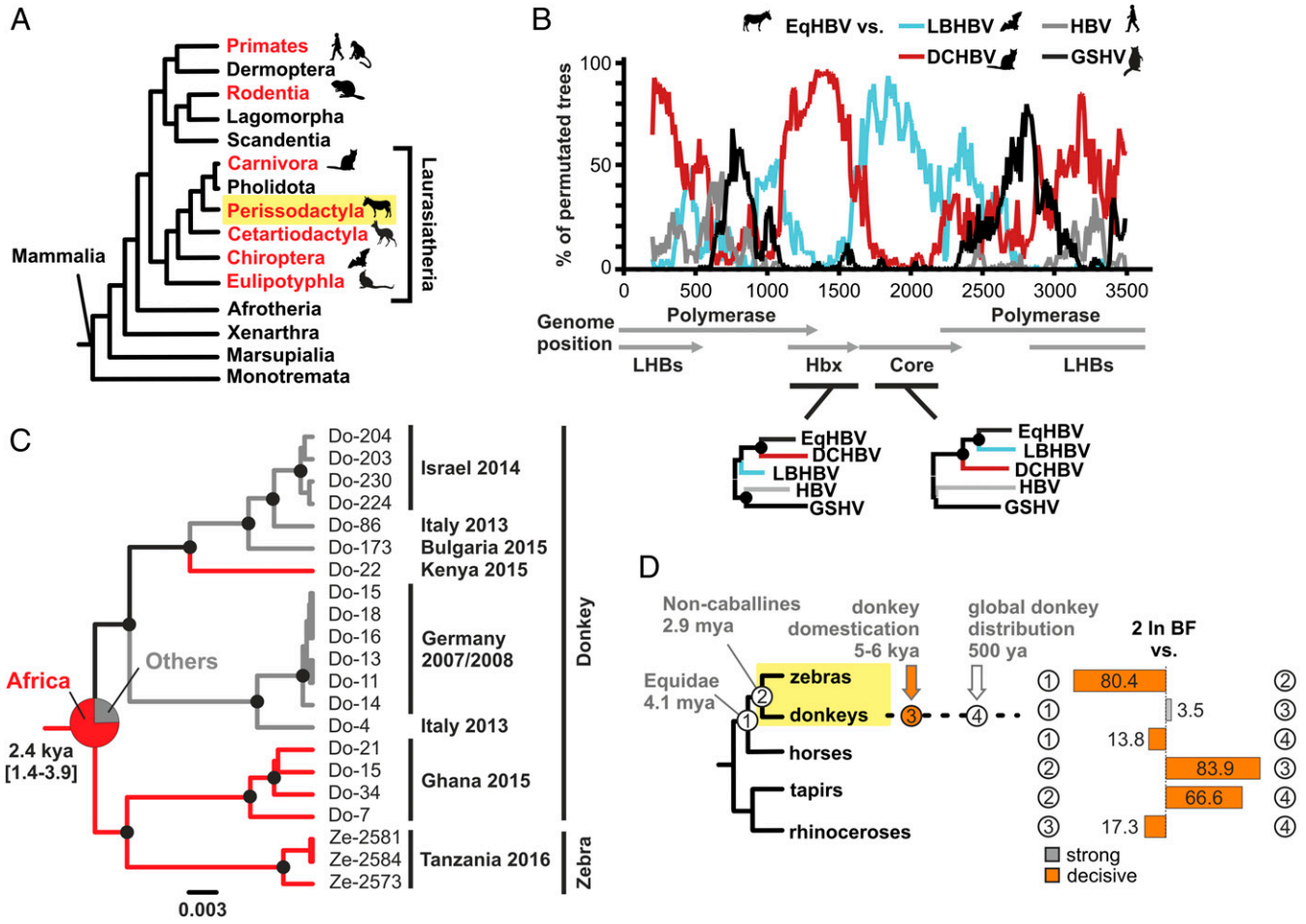

Fig. 5. Evolutionary origin of EqHBV. (*A*) Simplified cladogram of mammals (47). Red, taxa positive for hepadnaviruses. (*B*) Bootscan graph of EqHBV and reference sequences. Below, phylogenetic trees of genome regions as indicated by black bars. (*C*) Ancestral state and TMRCA reconstruction using EqHBV complete genome sequences. Filled dots, posterior probability of grouping >0.90. (*D*) Hypothesis testing. (*Left*) Cladogram of Perissodactyla (48). Priors used for hypothesis testing are labeled with numbers. (*Right*) Bayes factors (BF) supporting the comparison of two priors. Details provided in the *SI Appendix*. kya, thousand years ago; mya, million years ago; ya, years ago. 2 ln BF, twice the natural logarithm of the BF.

Discussion

Here, we elucidated the infection patterns and origins of a distinct hepadnavirus species infecting equids worldwide, termed EqHBV.

It is difficult to estimate the health cost of newly described pathogens. Donkeys are reared and traded as livestock and for transport worldwide (33). In many arid and semiarid countries in Africa and Asia, donkeys are the most important domestic animals, mainly used for work, milk, and meat production (34). Based on our data, EqHBV seems to cause moderate health problems in infected animals. Since donkeys are long-lived animals with a life expectancy of 25 to 30 y in the wild, and of up to 50 y in captivity (35), the medium- and long-scale impact of HBV infection on these important livestock animals should be investigated, ideally by combining case investigations and longitudinal studies. Importantly, the potential impact of EqHBV infection on genetically related perissodactylan hosts threatened by extinction, such as rhinoceros, deserves investigation.

The current coronavirus pandemic illustrates the impact of zoonotic pathogens on human health (36). HBV was long thought to be highly host-specific (37), a view that is challenged by recent data on new animal hepadnaviruses, including EqHBV, for which a CST from donkeys to zebras is likely according to our data. We showed previously that a New World bat HBV can enter human hepatocytes via the HBV receptor NTCP, suggesting that zoonotic transmission of reservoir-bound HBV to humans cannot be excluded (12). However, direct blood contact between hosts would presumably be needed for a zoonotic transmission event involving hepadnaviruses and direct bat-to-human contact is very rare (38). In contrast, slaughtering practices of donkeys may increase the potential of blood-borne pathogen transmission, best exemplified by the likely CST of HIV ancestors from nonhuman primates to humans (39). Beyond slaughtering donkeys for meat, there is a growing demand for donkey skin-derived gelatin, termed *ejiao*, used in traditional Chinese medicine, which led to a great decline in donkey populations in African countries (40). While the potential risk of zoonotic EqHBV infection during handling and slaughtering of donkeys seems limited due to the inability of EqHBs-pseudotyped HDV to establish infection in PHHs, post-entry replication competence was observed in human hepatoma cells. Definite assessments of the zoonotic potential of EqHBV will require identification of the EqHBV cellular receptors, ultimately enabling establishment of primate surrogate models (41).

Describing the infection patterns of newly discovered viruses is of utmost importance to understand virus transmission and pathogenesis and to envisage new animal models. Although human HBV and EqHBV are not genetically closely related, we found striking similarities between the infection patterns of these two HBV species, including liver tropism, moderate liver damage, high

viremia and, most strikingly, the development of chronic infection with characteristics resembling human CHB. To confirm these field-derived data and to investigate the susceptibility of equids to human HBV, in vivo infection experiments of equids with both EqHBV and HBV are required. The suitability of using equids for controlled hepatitis virus infections is best demonstrated by usage of horses as hepacivirus infection models in recent years (9).

Finally, understanding the mechanisms of HBV/HCV interference is of great clinical importance. Increased HBV replication and progression to severe liver disease can occur as a consequence of HCV treatment and coinfections with HBV and HCV are associated with a higher risk of hepatocellular carcinoma compared to HBV monoinfections (reviewed in ref. 42). HBV/HCV interference cannot be modeled using in vitro systems (43, 44). Tree shrews have been used in the past for experimental coinfection with both HBV and HCV. However, low infection efficacies, limited availability of host-specific tools, and variation between animal strains has severely limited the use of tree shrews for infection experiments (5). Our data suggest that equids represent a unique nonprimate animal host that is naturally infected by both HBV and HCV homologs (6). High viral loads of EqHBV and EqHCV (18) in infected equids suggest that both viruses can be efficiently used for in vivo infection studies.

Our study has three major limitations. First, although our collection comprises almost 3,000 samples and roughly comparable numbers from different continents, a sampling bias cannot be excluded. This may mainly affect the apparent absence of EqHBV in horses despite the susceptibility of horse-derived primary hepatocytes to EqHBV and although donkeys and horses are often kept in proximity and comingle (45), potentially facilitating CST. Alternatively, horse domestication and breeding practices may have led to extinction of EqHBV in horses, in analogy to the differential spread of equid herpesviruses among donkeys, zebras, and horses (46). Similarly, the small number of young animals in our study impedes definite conclusions regarding vertical virus transmission, which is a hallmark of human HBV infection. In addition, the number of sampled zebras in our study was small and comprised only two populations. The geographic distribution of EqHBV in zebras is thus unclear. Follow-up epidemiologic studies investigating natural infection of horses and other perissodactylan hosts with EqHBV can readily be conducted using the tools developed in our study. Second, the number of $EqHBV^+$ donkeys used for pathological investigation was low, due to the challenging access to donkey abattoirs in Africa. Additionally, correlating histopathological changes with a specific pathogen in the wild is challenging as infection with diverse other pathogens potentially eliciting liver damage cannot be excluded. Controlled infection studies will be needed to yield definite conclusions. Third, our in vitro infection studies are based on HDV pseudotypes with EqHBV surface proteins, thus only addressing the entry of EqHBV. Whether EqHBV can successfully complete its replication cycle in horse-derived hepatocytes needs confirmation using full EqHBV particles, requiring highly efficient cell culture-based EqHBV resurrection.

Despite these limitations, our groundbreaking study gives new perspectives on the evolution of animal homologs of human hepatitis viruses and provides unique opportunities to develop new animal models to investigate potential treatment for CHB and HBV/HCV coinfections.

Materials and Methods

Sampling and shipment were approved by local authorities (*SI Appendix*). Hepadnavirus screening and quantification, phylogenetic analyses, antibody detection, generation of pseudotypes, in vitro infection studies, and pathological studies were conducted as described previously and detailed in *SI Appendix* (12, 16).

Data Availability. All study data are included in the article and *SI Appendix*.

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