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RAPID COMMUNICATION

Natural co-infection of divergent hepatitis B and C virus homologues in carnivores

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Abstract

In humans, co-infection of hepatitis B and C viruses (HBV, HCV) is common and aggravates disease outcome. Infection-mediated disease aggravation is poorly understood, partly due to lack of suitable animal models. Carnivores are understudied for hepatitis virus homologues. We investigated Mexican carnivores (ringtails, *Bassariscus astutus*) for HBV and HCV homologues. Three out of eight animals were infected with a divergent HBV termed ringtail HBV (RtHBV) at high viral loads of 5×10^9 –1.4×10^{10} copies/ml serum. Two of the RtHBV-infected animals were co-infected with a divergent hepacivirus termed ringtail hepacivirus (RtHV) at 4×10^6–7.5×10^7 copies/ml in strain-specific qRT-PCR assays. Immunofluorescence assays relying on HBV core and RtHV NS3/4a proteins indicated that none of the animals had detectable hepadnavirus core-specific antibodies, whereas one RtHV-infected animal had concomitant RtHV-specific antibodies at 1:800 end-point titre. RtHBV and RtHV complete genomes showed typical HBV and HCV structure and length. All RtHBV genomes were identical, whereas RtHV genomes showed four amino acid substitutions located predominantly in the E1/E2-encoding genomic regions. Both RtHBV (>28% genomic nucleotide sequence distance) and RtHV (>30% partial NS3/NS5B amino acid sequence distance) formed new species within their virus families. Evolutionary analyses showed that RtHBV

grouped with HBV homologues from different laurasiatherian hosts (carnivores, bats, and ungulates), whereas RtHV grouped predominantly with rodent-borne viruses. Ancestral state reconstructions showed that RtHV, but not RtHBV, likely emerged via a non-recent host switch involving rodent-borne hepacivirus ancestors. Conserved hepatitis virus infection patterns in naturally infected ringtails indicate that carnivores may be promising animal models to understand HBV/HCV co-infection.

KEYWORDS
carnivores, co-infection, evolution, HBV, HCV, hepatitis

1 | INTRODUCTION

Viral hepatitis accounts for over 1 million deaths globally, mainly due to infection with hepatitis B and C viruses (HBV, HCV) contributing to 96% of all deaths (WHO, 2017). HBV and HCV co-infections are common due to shared transmission routes and generally aggravate the disease outcome (Bellecave et al., 2009; Konstantinou & Deutsch, 2015). However, HCV superinfection in patients with chronic hepatitis B can also result in HBV seroconversion and vice versa, facilitating viral clearance (Konstantinou & Deutsch, 2015). Co-infection patterns are poorly understood due to lack of appropriate in vitro and in vivo systems (summarized in Rasche et al., 2021).

HBV and HCV homologues were discovered in diverse animals (Figure 1a), but naturally occurring co-infections have not been documented beyond humans (Rasche, Sander, et al., 2019). Donkeys harbour homologues of HBV and HCV (Rasche et al., 2021; Walter et al., 2017), and serologic evidence suggests exposure of individual donkeys to both viruses (Rasche et al., 2021). Carnivores may be important HBV/HCV hosts because they belong to the major HBV host clade laurasiatheria that includes bats and ungulates (Rasche, Lehmann, et al., 2019), and because they frequently predate on rodents, which are major sources of HCV homologues (Bletsa et al., 2021; Moreira-Soto et al., 2020). The only known carnivore-associated hepatitis virus is a divergent cat HBV (Aghazadeh et al., 2018; Lanave

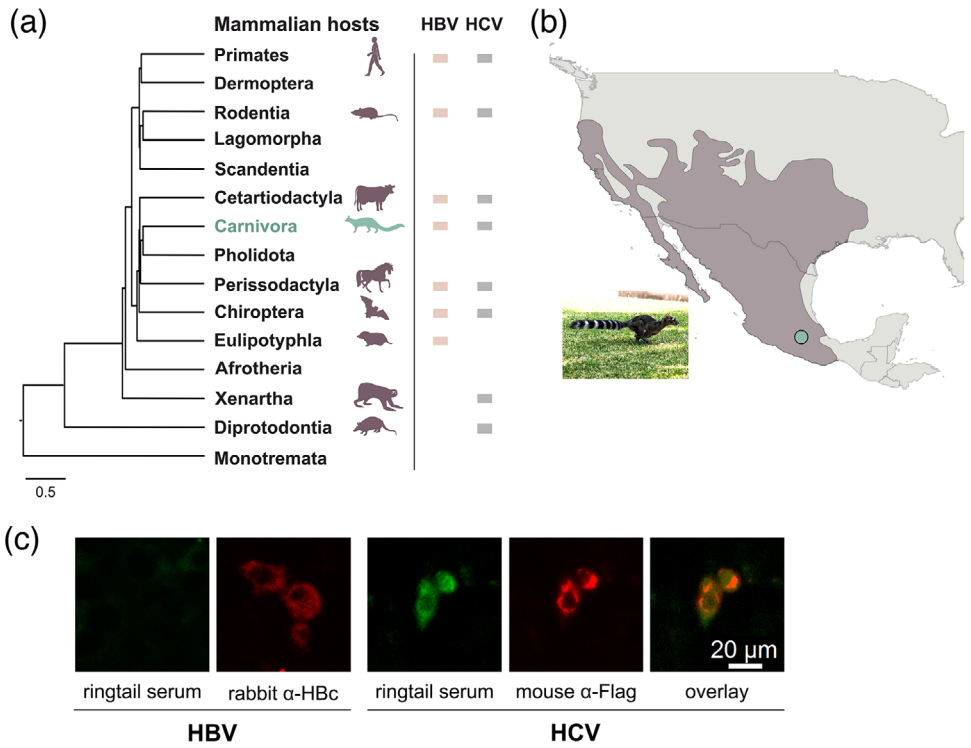

FIGURE 1 Host range of hepatitis B and C virus (HBV and HCV) homologues, sampling site and serology. (a) Mammalian phylogeny (Foley et al., 2016). Mammalian host orders of HBV (peach squares) and HCV (grey squares) are depicted. (b) Sampling site (Oaxtepec, Morelos, Mexico) of the captured ringtails depicted with a green dot using QGIS v3.10.13. Geographic distribution of ringtails in purple (data from IUCN; Reid et al., 2016). Image: Ringtail in Oaxtepec, Mexico taken by Álvaro Aguilar-Setién. (c) Immunofluorescence assay testing antibodies against HBV (left) and RtHV (right). Ringtail serum: CO-08/924

et al., 2019). Beyond domestic animals, wild carnivores have not been investigated for hepatitis viruses. Here, we identified divergent HBV and HCV species that naturally co-infect non-domesticated carnivores and reconstruct their infection patterns and genealogy.

2 | MATERIALS AND METHODS

2.1 | Animal sampling

Sampling of animals was carried out at the resort 'Centro Vacacional Oaxtepec' in Yautepec, Morelos, Mexico (18°54′23″N 98°58′13″W, 1368 masl). The site is located in a humid subtropical climate zone (Cwa, Köppen climate classification) with a temperature range of 15.3–29.0°C and annual rainfall of 945.7 mm^3 (SMN, National meteorological system acronym in Spanish). The capture of animals was carried out using Tomahawk live traps (Wisconsin, USA) baited with canned sardine and cookies with jam. Traps were placed in green areas and rocky hills from 19:00 in the evening until 6:00 the next day. Animals were anesthetized by trained veterinarians using 30 mg/kg of ketamine and 2 mg/kg of xylazine, and subsequently released at the site of capture after total recovery from anaesthesia (at least 6 h of recovery). Animals were released at sunset, when environmental temperature and light are lower compared to the rest of the day. All animals were monitored before, during, and after the anaesthesia. Blood samples were taken from the jugular vein with vacutainer system using tubes with clot activator and serum separating gel. The sera were transferred into new tubes and temporarily refrigerated until they were transferred to the laboratory and stored at −70°C.

2.2 | Nucleic acid purification and virus detection

Nucleic acids from sera were extracted using MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Molecular Systems, USA). Animal species were confirmed by molecular typing using an established protocol to characterize the *Cytochrome c oxidase* subunit I gene by PCR as previously described (de Carvalho Dominguez Souza et al., 2018). PCR tests targeting orthohepadnaviruses (Drexler, Geipel, et al., 2013) and hepaciviruses (Drexler, Corman, et al., 2013) were performed. Positive samples were sent for Sanger sequencing (Microsynth Seqlab, Germany). After obtaining the genetic sequence, a quantitative reverse transcription PCR (RT-qPCR) was designed specifically for the newly identified ringtail hepadna- and hepaciviruses. The following oligonucleotides (IDT Technology) were used for RtHBV: Fw 5′-TCATTTACCTCTTAGTCCTGGTGCTA-3′, Rv 5′-CAAACTGGGATATAACCCTTCCAA-3′, and probe 5′-CCTCACTTG/ZEN/CTTGTTGGG-3′; and for RtHV: Fw 5′-TCACAGGAGACAATGTGGTTGAG-3′, Rv 5′-GCGGGCGAGGAACATG-3′, and probe 5′-TGGAAGTCG/ZEN/CCTGACATA-3′. The RT-qPCR quantification relied on photometrically quantified plasmid controls.

2.3 | High throughput sequencing and genome retrieval

To obtain full length genomes, RNA libraries from positive samples (Table 1) were prepared according to KAPA HyperPrep manufacturer protocol (Roche) for sequencing either on a NovaSeq 6000 system (500 cycles paired-end) or NextSeq 550 system (150 cycles paired-end). Raw reads were quality trimmed with Trimmomatic V.0.4 (Bolger et al., 2014) removing all reads with quality <Q30. High quality reads were mapped to equine (EqHBV, GenBank accession no. MT134279) for ringtail hepadnavirus (RtHBV)-positive samples, or to Sloth HV (GenBank accession no. MH844501) for ringtail hepacivirus (RtHV)-positive samples, as reference genomes using the very-sensitive-local option in Bowtie2 (Langmead & Salzberg, 2012). These genomes were chosen as reference due to the high similarity between novel viruses and these genomes when comparing the PCR screening fragments. Once a consensus sequence was obtained based on the sequence reads, several iterations were performed assembling the total amount of reads against newest consensus sequence using the option sensitive-local with Bowtie2 until no more new reads were assembled. All sequences were confirmed by Sanger sequencing.

2.4 | Genome characterization

All open reading frames (ORFs) were predicted using Geneious. RtHV non-structural proteins were predicted by signal 5.0 (where applicable) and sequence homology to sloth HV (GenBank accession no. MH844501). RtHV secondary structures at genome ends were predicted using MFold (Zuker, 2003).

2.5 | Sequence data

Sequences used in this study were downloaded from GenBank via Geneious v11.1.5 (https://www.geneious.com). Complete genome sequences used for orthohepadnavirus evolutionary analyses include (GenBank accession no.|*species*/virus name|abbreviation): MZ393519|*Ringtail hepatitis B virus*|RtHBV, MT134279|*Equine hepatitis B virus*|EqHBV, MK620908|Tai Forest hepatitis B virus|TFoHBV, MH307930|*Domestic cat hepatitis B virus*|DCHBV, MG600410|*Pomona bat hepatitis B virus*|IRLBHBV, KY962705|*Pomona bat hepatitis B virus*|LHSBHBV, KF939649|*Pomona bat hepatitis B virus*|PBHBV, NC_024444|*Roundleaf bat hepatitis B virus*|HSBHBV, NC_024443|*Roundleaf bat hepatitis B virus*|RLBHBV, JX941466|*Long-fingered bat hepatitis B virus*|LFBHBV, AY334076|*Woodchuck hepatitis virus*|WHV, U29144|*Arctic ground squirrel hepatitis B virus*|AGSHV, K02715.1|*Ground squirrel hepatitis virus*|GSHV, MK345470|*Crowned shrew hepatitis B virus*|CSHBV, NC_024445|*Tent-making bat hepatitis B virus*|TMBHBV, AF046996|Woolly monkey hepatitis B virus|WMHBV, KY703886|*Capuchin monkey hepatitis B virus*|CMHBV, AP007263|*Hepatitis B virus*, genotype A|HBV-A, LC064368|*Hepatitis B virus*, genotype B|HBV-B, LC519799|*Hepatitis B virus*, genotype

TABLE 1 Sample characteristics

ID	Sex	Age	Material	Collection date	Locality	RtHBV			RtHV		
						RtHBV (copies/ml)	Genome coverage	IFA	RtHV (copies/ml)	Genome coverage	IFA
CO-03/918	Female	Adult	Serum	04.09.2018	CV, Oaxtepec	neg	na	neg	neg	na	neg
CO-04/919	Male	Adult	Serum	04.09.2018	CV, Oaxtepec	neg	na	neg	neg	na	neg
CO-06/921	Female	Adult	Serum	06.09.2018	CV, Oaxtepec	neg	na	neg	neg	na	neg
CO-07/922	Male	Adult	Serum	07.09.2018	CV, Oaxtepec	neg	na	neg	neg	na	neg
CO-08/923	Female	Adult	Serum	22.11.2018	CV, Oaxtepec	1.4×10^{10}	13781x[†]	neg	7.5×10^{7}	233335x[†]	neg
CO-09/924	Female	Adult	Serum	22.11.2018	CV, Oaxtepec	5.3×10^{9}	16033x[†]	1:800	4×10^{6}	16062x[†]	neg
CO-10/925	Male	Adult	Serum	23.11.2018	CV, Oaxtepec	neg	na	neg	neg	na	neg
CO-11/926	Male	Adult	Serum	23.11.2018	CV, Oaxtepec	5×10^{9}	13781x[§]	neg	neg	na	neg

Abbreviations: CV, Centro Vacacional; IFA, immunofluorescence assay; na, not applicable; neg, negative; RtHV; ringtail hepacivirus; RtHBV, ringtail hepadnavirus.

[†]Sequences were obtained by NovaSeq 6000 system.

[§]Sequence was obtained by NextSeq 550 system.

C|HBV-C, AB554024|*Hepatitis B virus*, genotype-D|HBV-D, LC513656|*Hepatitis B virus*, genotype E|HBV-E, AP007264|*Hepatitis B virus*, genotype-G|HBV-G, AB846650|*Hepatitis B virus*, genotype H|HBV-H, HE815465|*Hepatitis B virus*, genotype D|HBV-D, D00220|HBV, FJ798096|HBV, HQ603065|HBV, EU155826|HBV, EU155826, KR229754.1|*White sucker hepatitis B virus*|WSHBV. Complete genome sequences used for hepacivirus evolutionary analyses include (GenBank accession no.|*species*/virus name|abbreviation): MZ393518|Ringtail hepacivirus|RtHV, KC411784|*Hepacivirus F*|RHV-F; KY370094|Rodent hepacivirus|RHV; KC815310|*Hepacivirus E*|RHV-E; MG600412|Rodent hepacivirus|RHVa; KY370095|Rodent hepacivirus|RHVb; KJ950938|*Hepacivirus G*|NRHV1; MH844501|Sloth hepacivirus|Sloth HV; MG211815|Hepacivirus P|HV-P; KJ950939|*Hepacivirus H*|NRHV2; MH824541|Sifaka hepacivirus|Sifaka HV; MN635449|Possum hepacivirus|Possum HV; U22304|*Hepacivirus B*|GVB-B; KP641127|*Hepacivirus N*|BoHV; KC551801|*Hepacivirus D*|GHV; KC796077|*Hepacivirus L*|BHV-L; KC411806|*Hepacivirus I*|RHV-I; MH370348|Oligoryzomys hepacivirus|Oligoryzomys HV; KC796074|*Hepacivirus K*|BHV-K; KC796078|*Hepacivirus M*|BHV-M; KP325401|*Hepacivirus A*|NPHV; M62321|*Hepacivirus C*|HCV1a; KC411777|*Hepacivirus J*|RHV-J; KR902729|Wenling shark virus|WLSV-MHS-2.

The orthohepadnavirus and hepacivirus datasets were aligned with the MAFFT (Katoh & Standley, 2013) plugin with an iterative refinement algorithm G-INS-i implemented within Geneious. Translation alignments were performed when aligning ORFs. The orthohepadnavirus dataset used consisted of complete genome sequences, whereas the hepacivirus dataset used consisted of translated complete polyprotein sequences.

2.6 | Evolutionary analyses

Genetic divergence was assessed by calculating mean pairwise sequence distances in MEGA-X (Kumar et al., 2018). For orthohepadnaviruses, complete genomes were used, whereas for hepaciviruses we compared amino acid sequences at positions 1123–1566 of the NS3 and 2536–2959 of the NS5B according to HCV1a (GenBank accession no. M62321.1). Recombination analyses were performed for the complete polymerase gene of orthohepadnaviruses and the complete polyprotein gene of hepaciviruses with the methods RDP, GENECONV, Chimaera, MaxChi, BootScann, SiScan, and 3Seq using default parameters in package RDP4 (Martin et al., 2015). Regions with predicted recombination events that were detected with >2 methods and $p < .05$ were excluded for further analyses. Bayesian phylogenies were generated using MrBayes V3.2 (Huelsenbeck & Ronquist, 2001) using Hasegawa-Kishino-Yano (HKY) for the orthohepadanavirus dataset and Whelan And Goldman (WAG) for the hepacivirus dataset as substitution models as described previously (Moreira-Soto et al., 2020; Rasche, Lehmann, et al., 2019). Trees were run for two million generations with 25% burn-in. Ancestral state reconstructions (ASR) were generated using the BEAST package V1.10.4 as previously described (Moreira-Soto et al., 2020; Rasche, Lehmann, et al., 2019), using HKY+G as substitution model, uncorrelated clock with a lognormal distribution, and Yule speciation process as tree prior. The analyses were run for 10 million generations with 10% burn-in, sampling every 1000 steps. For the RtHBV ASR analysis, we excluded two potentially recombinant regions (263–920 and 1879–2269 nucleotides (nt) according to RtHBV) in the polymerase gene, whereas for RtHV, the complete polyprotein gene was used.

2.7 | Cells and plasmids

HuH-7 cells were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The RtHV-NS3/NS4A plasmid was constructed by subcloning a synthesized fragment containing the NS3/NS4A coding region of RtHV (IDT Technology) into the mammalian expression vector pREN2, which contained a C-terminal

FLAG-tag (Moreira-Soto et al., 2020). For RtHBV, a plasmid construct containing the core protein of HBV sub-genotype D3 in pcDNA3.1+ as vector was used (Rasche et al., 2021).

2.8 | Immunofluorescence assay

To detect the presence of antibodies against RtHBV or RtHV, an immunofluorescence assay (IFA) was performed. HuH-7 cells were seeded in a six-well plate at a confluency of 80%. Each well was transfected using 2.5 μg of individual plasmid using standard FuGene HD (Promega) protocol. At day 1 post-transfection, cells were trypsinized, resuspended in DMEM media with 10% FBS without antibiotics and re-seeded in multitest cover slides at a density of 2.5×10^5 cells/ml. Seeded cover slides were incubated at 37°C overnight in a humid chamber and subsequently fixed with ice-cold acetone/methanol (ratio 1:1). Cells were then permeabilized, followed by a blocking step with 10% goat serum. Detection of antibodies was performed by application of diluted ringtail sera at concentrations 1:40, 1:100, and 1:400. Once a positive sample was detected, a dilution series between 1:40 and 1:8000 was performed to determine the end-point titre. After application of ringtail sera, a goat anti-ferret antibody coupled with FITC (Abcam ab112769) was added at a concentration of 1:500. As no anti-ringtail antibody was available, we used an anti-ferret antibody due to genetic similarities between both species. Non-infected ferret serum was used as negative control. In parallel, positive control experiments for RtHV were set up using a mouse anti-Flag IgG1 (sigma F1804-50 μg) at 1:400 and as secondary antibody an anti-mouse Cy3 (Jackson ImmunoResearch) at 1:400. For RtHBV, rabbit anti-HBV core (Rasche et al., 2021) at 1:100 was used as primary antibody and anti-rabbit Cy3 (Jackson ImmunoResearch) as secondary antibody at 1:400. Immunofluorescence was visualized in a Leica DMi8 microscope.

3 | RESULTS AND DISCUSSION

3.1 | Identification of divergent HBV and HCV homologues causing infection in ringtails

We investigated wild ringtails (*Bassariscus astutus*) from Oaxtepec, Morelos, Mexico (Figure 1b). Ringtails are nocturnal solitary carnivores genetically related to raccoons and ferrets (Foley et al., 2016), that live in the south of the United States and Mexico (Reid et al., 2016). Serum was obtained from eight healthy adult animals sampled in 2018 (Table 1). Morphologic species classification was confirmed by characterization of *Cytochrome c oxidase* subunit I. Broadly reactive (RT-)PCR tests targeting orthohepadnaviruses (Drexler, Geipel, et al., 2013) and hepaciviruses (Drexler, Corman, et al., 2013) followed by strain-specific RT-qPCRs indicated the presence of these viruses in the samples investigated. The hepadnavirus detection rate was 37.5% (95% confidence interval (CI) 13.7–69.5) with viral loads between 5×10^9 and 1.4×10^{10} copies/ml and the hepacivirus detection rate was 25% (95% CI 7.2–59.1) with viral loads between 4×10^6 and 75×10^7 copies/ml (Table 1), suggesting intense infections and viral loads comparable to those observed in human HBV and HCV infections (Drexler et al., 2009; Rasche, Lehmann, et al., 2019). Both female animals infected with RtHV were also infected with RtHBV, whereas one male animal was infected only with RtHBV (Table 1). Serologic testing using IFA based on the conserved HBV core (Rasche et al., 2021) showed that none of the animals had detectable hepadnaviral anti-core antibodies, indicating acute infection or unusually low levels of cross-reactive anti-core antibodies. IFA based on RtHV NS3-NS4a (Moreira-Soto et al., 2020) showed that one RtHV PCR-positive animal had antibodies at 1:800 end-point titre (Figure 1c). It was not possible to investigate hepatitis virus-mediated disease as no pathological examination was performed due to the release of animals after blood collection. Of note, unclear link to disease has also been reported in other HBV and HCV homologues, as in the case of cattle hepacivirus (Rasche, Sander, et al., 2019). Nevertheless, high viral loads for both hepatitis viruses and adaptive immune responses against RtHV corroborated natural infection of ringtails, as opposed to potential contamination with viruses of environmental or dietary origin.

3.2 | Genomic characterization of new HBV and HCV homologues

Complete RtHBV and RtHV genomes were obtained by illumina-based high throughput sequencing. Genome lengths and organization showed typical properties of these viral families. The RtHBV had a genome size of 3158 nt, with overlapping ORFs encoding the predicted polymerase, pre-core/core, X, and surface proteins (preS1, preS2, and S) (Figure 2a left). Contrary to HBV, RtHBV showed no overlap of the pre-core domain with the carboxy-terminus of X. Despite high similarity of the essential pre-S1 domain required for interaction with the HBV cellular receptor Na^+-taurocholate cotransporting polypeptide (NTCP) between HBV and RtHBV, relatively higher similarity of RtHBV with EqHBV in other pre-S1 domains required for NTCP usage (Figure 2a right, upper insert) might suggest low zoonotic potential, because EqHBV could not infect primary human hepatocytes (Rasche et al., 2021). Conservation of a genomic pre-core domain suggested the production of an HBe antigen (HBeAg; Figure 2a right, lower insert), compatible with high viral loads in the immunotolerant phase of hepatitis B (Revill et al., 2020). No difference was observed between the three RtHBV genomes, suggesting either recent infection as also indicated by the lack of detectable antibodies or HBeAg-mediated evolutionary stasis (Rasche et al., 2021). The genome of RtHV had a size of 9418 nt with a polyprotein gene encompassing 8802 nt encoding the predicted structural proteins core, envelope (E) 1/2, p7 as well as non-structural proteins NS2-NS3-NS4A-NS4B-NS5A-NS5B (Figure 2b). The detection of a type IV internal ribosomal entry site (IRES) with two microRNA-122 binding sites (CACUCC) in the 5′-genome end was suggestive of liver tropism. Both the IRES and a poly-U/C rich region followed by four stem-loops in the 3′-genome end suggested similarities between HCV and RtHV replication (Figure 2b). The two sequenced RtHV differed at 42 positions along the genome, of which four were non-synonymous substitutions, all within E1–E2 (Figure 2b). This was

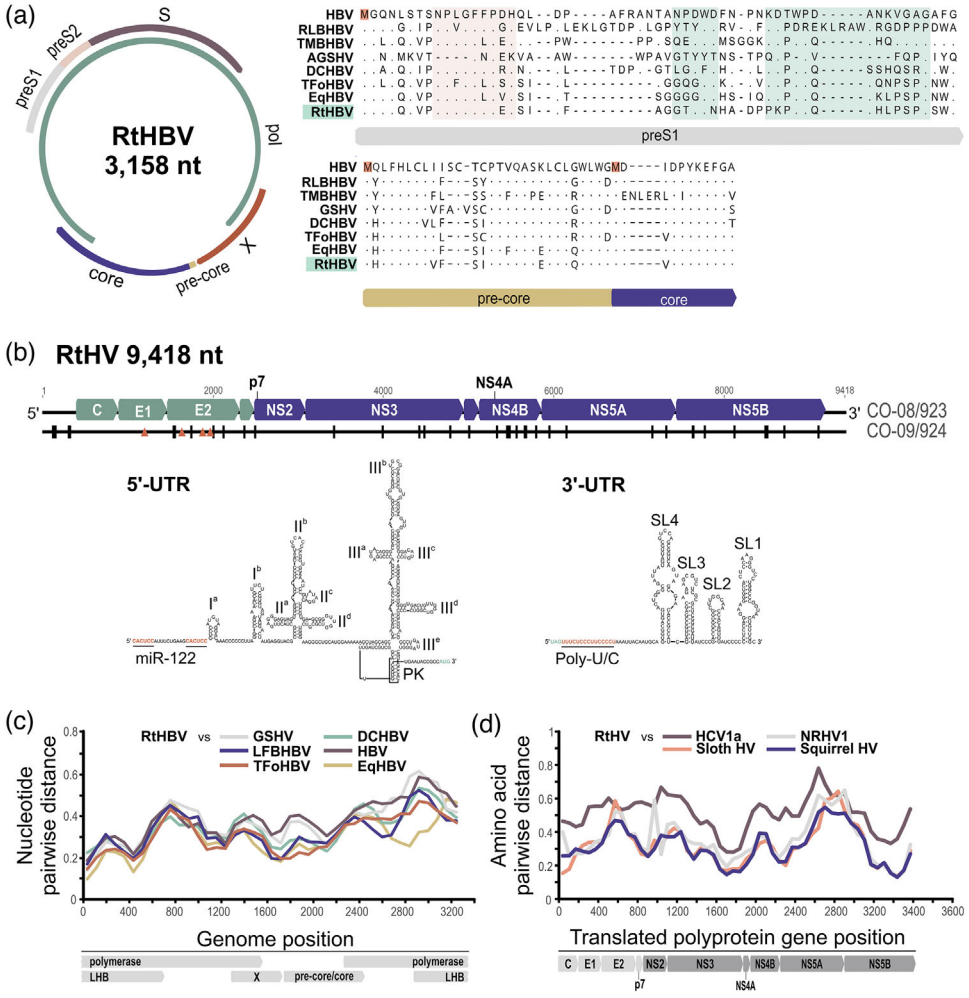

FIGURE 2 Genomic characterization of ringtail hepatitis viruses. (a) Left, genome organization of ringtail hepadnavirus (RtHBV). Right: upper insert, pre-S1 region. Essential NTCP-binding domains in human hepatitis B virus (HBV) are highlighted. Dots indicate identical amino acid residues. Right: lower insert, comparison of translated pre-core and N-terminal core domains. (b) Genome organization of ringtail hepacivirus (RtHV). Structural proteins of RtHV are coloured in green, whereas non-structural (NS) proteins are in blue. Differences between RtHV sample CO-08/923 compared to sample CO-09/924 are shown as black lines, and non-synonymous substitutions as orange triangles. Predicted structures of the RtHV internal ribosomal entry site (IRES) and 3′UTR. PK, pseudoknot. (c) Comparison of pairwise nucleotide sequence distance of RtHBV versus other orthohepadnaviruses. Sequence distances were calculated with SSE using a sliding window of 300 and step size of 80 nucleotides. LHB, large surface protein. (d) Comparison of pairwise amino acid sequence distance of RtHV versus other hepaciviruses. Sequence distances were calculated with SSE using a sliding window of 400 and step size of 200 amino acids. Virus abbreviation, name (GenBank accession number): RtHBV, ringtail hepadnavirus (MZ393519); GSHV, ground squirrel hepatitis virus (K02715.1); LFBHBV, long-fingered bat hepatitis B virus (JX941466); RLBHBV, roundleaf bat hepatitis B virus (NC_024443); TMBHBV, tent-making bat hepatitis B virus (NC_024445); AGSHV, arctic ground squirrel hepatitis virus (U29144); TFoHBV, tai forest hepatitis B virus (MK620908); DMHBV, domestic cat hepatitis B virus (MH307930); HBV, hepatitis B virus (AP007263); EqHBV, equine hepatitis B virus (MT134279); HCV, hepacivirus C (M62321); sloth HV (MH844501); NRHV1, hepacivirus G (KJ950938); squirrel HV, hepacivirus P (MG211815); RtHV, ringtail hepacivirus (MZ393518)

consistent with higher evolutionary rates within hepacivirus genomic regions encoding E compared to other proteins (Gray et al., 2011).

Based on genomic sequence distances, RtHBV forms a previously unknown viral species within the genus *Orthohepadnavirus* (0.28–0.32 pairwise nucleotide sequence distances, Figure 2c, Table S1) in the family *Hepadnaviridae* (ICTV, 2019b). Similarly, RtHV forms a previously unknown viral species within the genus *Hepacivirus* in the family *Flaviviridae* (ICTV, 2019a), showing pairwise amino acid sequence distances >0.3 in the NS3 and NS5B regions used for typing (Figure 2d, Table S2) (Smith et al., 2016).

3.3 | RtHBV and RtHV showed different evolutionary histories

In Bayesian phylogenetic reconstructions, RtHBV grouped within an HBV clade infecting three diverse laurasiatherian hosts orders, namely Carnivora, Perissodactyla and Artiodactyla (Figure 3a). In contrast, RtHV grouped within a clade composed predominantly of rodent-borne viruses (Figure 3b). Recombination analyses did not yield evidence for recombination events within the RtHV polyprotein genes, which was comparable to scarce recombination in human HCV

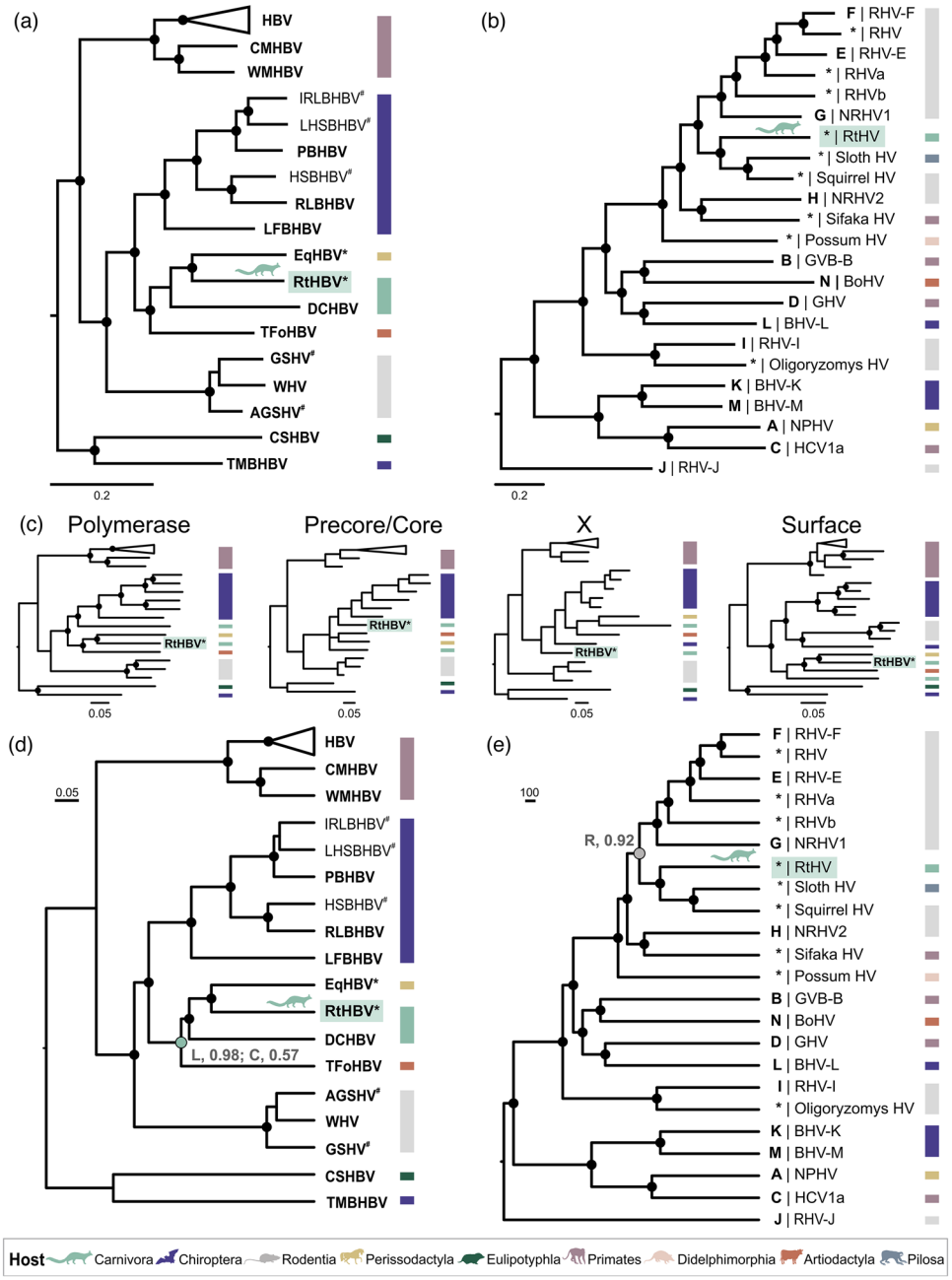

FIGURE 3 Evolution of ringtail hepatitis viruses. (a) Bayesian phylogeny of complete orthohepadnavirus genomes using the white sucker hepatitis B virus as an outgroup. (b) Bayesian phylogeny of complete hepacivirus translated polyproteins using Wenling shark hepacivirus as an outgroup. (c) Bayesian phylogeny of individual orthohepadnavirus open reading frames (ORFs). (d) Ancestral state reconstruction of orthohepadnaviruses. (e) Ancestral state reconstruction of hepaciviruses. Number at coloured node denote posterior probability support for a host order (C, carnivora; R, rodent) or superorder (L, laurasiatheria) from ancestral state reconstructions. Species names are in bold. Asterisk in tip label denotes novel species not yet evaluated by the ICTV. Posterior values ≥0.9 are shown as black circles at nodes. Scale bar indicates genetic distance. For hepatitis B virus (HBV), species demarcation criterion by the ICTV is >20% nucleotide sequence divergence averaged across complete genomes. Hashtag in tip labels highlights HBV homologues with <20% nucleotide sequence divergence compared to another hepadnavirus species classified as species for historic reasons. Information on the sequences used is given in Materials and Methods section

(Theze et al., 2015). In contrast, different tree topologies of individual RtHBV ORFs were compatible with recombination events (Figure 3c) and formal recombination analyses suggested non-recent recombination events in all genes (data not shown). After excluding recombinant regions within the polymerase, leaving c. 1300 nt for analyses, the topology resembled that of the complete genome with good support (data not shown). Hence, this region was used for ASR in a Bayesian framework. ASR revealed different evolutionary histories for both viruses. RtHBV had a laurasiatherian ancestor (posterior probability, 0.98), whereas its evolutionary origins could not be

reconstructed unambiguously due to the multiple host orders comprised in the RtHBV clade (Figure 3d). In contrast, ASR suggested rodents as ancestral hosts of RtHV (posterior probability, 0.92) indicating a non-recent host switching event during the RtHV genealogy (Figure 3e).

Ringtails are omnivorous animals that prey on rodents (Reid et al., 2016). It is thus plausible that RtHV was acquired by ringtails via its prey, which is consistent with cats showing antibodies against rodent-borne pathogens such as orthopoxviruses and hantaviruses (Nowotny, 1994), and ferret hepatitis E virus (HEV) clustering with rodent-associated HEV (Ryll et al., 2019). In humans, HCV is transmitted efficiently only via parenteral route, whereas HBV is transmitted efficiently via sexual, parenteral, and vertical routes (Rasche, Sander, et al., 2019). Co-detection of two blood-borne hepatitis viruses in two females and of a hepadnavirus in a male sampled during a short time span within one region is most compatible with aggressive territorial behaviour facilitating horizontal transmission (Reid et al., 2016). As the major limitation of our study was the small number of samples, future fieldwork should include ecologically validated sampling of prey and predator populations to assess hepatitis virus transmission routes between and to ringtails.

Finally, animal models for HBV/HCV co-infections are scarce (Rasche et al., 2021). Although ringtails would likely constitute a promising animal model, usage of ringtails may be inconvenient as they are not established laboratory animals. Hypothetically, ferrets may be susceptible to RtHBV/RtHV due to their close genetic relationship with ringtails. Ferret-specific reagents are readily available since ferrets are a well-established model to study viral pathogenesis for pathogens such as SARS-CoV-2 and influenza viruses (Kim et al., 2020). If these animals proved to be susceptible, co-infection studies relying on viraemic sera or resurrected virus (in the case of RtHBV) would be feasible.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Animal sampling was approved under permit R-2017-785-068 by the local Ethics and Research Committee from Comisión Nacional de Investigación Científica del IMSS. All efforts were made to leave animals unharmed or to minimize animal suffering.

DATA AVAILABILITY STATEMENT

New ringtail hepadnavirus and hepacivirus genomes are deposited in GenBank under accession numbers MZ393517-19, MZ397303-04.

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