A novel marsupial hepatitis A virus corroborates complex evolutionary patterns shaping the genus *Hepatovirus*

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Abstract

The discovery of nonprimate hepatoviruses illuminated the evolutionary origins of hepatitis A virus (HAV) in small mammals. Marsupials are ancient mammals that diverged during the Jurassic from other Eutheria. Viruses from marsupials may thus provide important insight into virus evolution. To investigate Hepatovirus macroevolutionary patterns, we sampled 112 opossums in northeastern Brazil. A novel marsupial HAV (MHAV) was detected in a Brazilian Common Opossum (Didelphis aurita) by nested RT-PCR. MHAV concentration in liver was high at $2.5 \times 10^9$ RNA copies/gram and about 1000-fold higher than in other solid organs, suggesting hepatotropism. Hepatovirus seroprevalence in D. aurita was 26.6% using an ELISA. End-point titers in confirmatory immunofluorescence assays were high and marsupial antibodies co-localized with anti-HAV control sera, suggesting specificity of serological detection. MHAV showed all genomic hallmarks defining hepatoviruses, including late domain motifs likely involved in quasi-envelope acquisition, a predicted C-terminal pX extension of VP1, strong avoidance of CpG dinucleotides and a type 3 internal ribosomal entry site. Translated polyprotein gene sequence distances of at least 23.7% to other hepatoviruses suggested MHAV represents a novel Hepatovirus species. Conserved predicted cleavage sites suggested similarities in polyprotein processing between HAV and MHAV. MHAV was nested within rodent hepatoviruses in phylogenetic reconstructions, suggesting an ancestral hepatovirus host switch from rodents into marsupials. Co-phylogenetic reconciliations of host and hepatovirus phylogenies confirmed that host-independent macroevolutionary patterns shaped the phylogenetic relationships of extant hepatoviruses. Although Marsupials are synanthropic and consumed as wild game in Brazil, HAV community protective immunity may limit the zoonotic potential of MHAV.
Importance

The hepatitis A virus (HAV) is an ubiquitous cause of acute hepatitis in humans. Recent findings revealed the evolutionary origins of HAV and the genus *Hepatovirus* defined by HAV in small mammals. The factors shaping the genealogy of extant hepatoviruses are unclear. We sampled marsupials, one of the most ancient mammalian lineages and identified a novel marsupial HAV (MHAV). The novel MHAV shared specific features with HAV, including hepatotropism, genome structure and a common ancestor in phylogenetic reconstructions. Co-evolutionary analyses revealed that host-independent evolutionary patterns contributed most to the current phylogeny of hepatoviruses and that MHAV was the most drastic example of a cross-order host switch of any hepatovirus observed so far. The divergence of marsupials from other mammals offers unique opportunities to investigate HAV species barriers and whether mechanisms of HAV immune control are evolutionarily conserved.
Introduction

The hepatitis A virus (HAV) is an ubiquitous cause of acute viral hepatitis in humans, causing about 11,000 deaths worldwide per year (1). HAV belongs to the genus *Hepatovirus* within the family *Picornaviridae* (2). HAV stands out from other picornaviruses in its ability to occur as typical non-enveloped viruses in feces and as lipid-layered particles in blood (3). Additionally, the unique structural properties of HAV resembling those found in ancestral insect viruses suggest HAV is an ancient picornavirus (4).

HAV was long thought to be restricted to primates, with genotypes I-III found in humans and genotypes IV-VI, termed simian HAV (SHAV), found in nonhuman primates (2). Because HAV engenders long-lasting immunity following infection, how the virus may have survived in scattered pre-historic human populations has long been enigmatic (5). Only recently, nonprimate hepatoviruses were discovered, suggesting that HAV ancestors may have evolved in small mammals prior to their introduction into humans (5).

The expanded genus *Hepatovirus* now includes at least 16 putative virus species (6). The majority of novel hepatoviruses has been obtained from bats and rodents (5, 7). The corresponding host orders Chiroptera and Rodentia are the most speciose among mammals and both are major sources of novel viruses (8, 9). Other mammalian orders carrying hepatoviruses include Primates, Scandentia, Eulipotyphla and Carnivora (6, 10). These orders all belong to a clade of placental mammals termed Boreoeutheria (6) (Figure 1A).

Boreoeutherian orders diversified about 87 million years ago (mya) during the upper Cretaceous and their phylogenetic relationships are not easily reconciled (11). The rapid diversification that occurred at the root of known hepatovirus hosts challenges co-evolutionary assessments at ancestral nodes of the *Hepatovirus* phylogeny. Additionally, host genetic relatedness facilitates
cross-host infections of pathogens (12). Spill-over infections between genetically related hosts are thus not easily differentiated from co-evolutionary relationships.

The genealogy of hepatoviruses is likely complex. In preliminary work, we noted conflicting phylogenies of hosts and nonprimate hepatoviruses that suggested several non-recent hepatovirus host switches (5). In follow-up analyses, we showed that recombination events involving highly diverse hepatoviruses from different mammalian orders corroborated these host switches (6). On the other hand, we showed that surprisingly speciose monophyletic clades of bat hepatoviruses exist across different continents, which may hint at yet undefined long-term evolutionary relationships (6).

Comparative phylogenomic analyses of hepatoviruses from highly divergent hosts may provide important insights into the macroevolutionary patterns of this ancient viral genus. The order Marsupialia is one of the oldest mammalian orders that diverged from the ancestor of Boreoeutheria about 170 mya (11) (Figure 1A). Marsupials such as opossums likely originated from South American ancestors and nowadays are endemic to Australasia and the Americas only (13).

To further elucidate hepatovirus macroevolutionary patterns, we investigated opossums from Brazil for hepatoviruses using molecular, serological and bioinformatic tools.

Results

Fieldwork

During July-August 2015, we sampled opossums in remaining Atlantic rain forest patches in the northeastern Brazilian state of Bahia, including the capital Salvador and a municipality termed Mata de São João, located at 53 km distance (Figure 1B and 1C). Of 55 animals captured alive, only a serum specimen was taken. Another 57 animals were obtained dead from the wildlife
Hepatovirus detection and organ tropism

Serum and liver specimens were investigated for hepatovirus RNA by nested RT-PCR as described previously (5). Only a single liver specimen from an adult female Brazilian Common Opossum (Didelphis aurita) termed Br225 originating from Salvador tested positive. D. aurita was also the most abundantly sampled species in our study (Table 1). The 0.9% rate (1 of 112) of acute hepatovirus infection in opossums as evidenced by detection of hepatoviral RNA was not significantly different (Fisher exact, two-tailed p=0.56) from the 0.7% rate (117 of 15,987) found in a large investigation of different small mammals for hepatoviruses (5). This suggested similarities in the epidemiology of hepatovirus infection between marsupials and other mammals.

Following nucleotide sequencing of the screening RT-PCR amplicon, a real time RT-PCR assay relying on photometrically quantified cRNA standards was designed to determine virus concentrations in different organs of animal Br225 that tested positive for hepatovirus RNA. Hepatovirus RNA concentration in liver tissue was high at 2.5x10^9 genome copies per gram and between three to four orders of magnitude higher than in other solid organs (Figure 2A). This suggested hepatotropism of the marsupial HAV (MHAV). Whether the deceased animal Br225 suffered from acute hepatitis remained unknown, since the quality of the available tissue specimens was insufficient for histopathological analyses. During macroscopic examination, the...
animal showed signs of physical injury, including hemorrhages in liver and lungs. It is thus unlikely that MHAV infection was the direct cause of death in that animal.

**Hepatovirus seroprevalence**

HAV seroprevalence studies in small mammals are challenging due to lack of established methods. In pivotal investigations of African bats, HAV seroprevalence using an immunofluorescence assay (IFA) and confirmatory neutralization tests (NT) was 7.3% (5). Advantages of IFA include the ability to visualize and titrate antibody responses, but sensitivity may be limited. Advantages of NTs include high specificity, but the protocol is laborious and particularly time-consuming for HAV, thus not facilitating high-throughput analyses (14). In preliminary experiments, we determined that results of an HAV ELISA relying on competition of serum antibodies with a horseradish peroxidase-conjugated polyclonal human anti-HAV IgG were equivalent to those of NT for all bat sera tested previously for HAV (5). In 48 human sera used to assess ELISA performance, positive and negative results were clearly distinguishable (Figure 2B). Mean optical density (OD) in 24 positive sera from HAV vaccinees was 0.114 (standard deviation (SD), 0.122), compared to a mean OD of 1.916 (SD, 0.191) in 24 negative sera. In opossum sera, negative and positive samples were less clearly distinguished. Mean OD in 12 positive sera was 0.426 (SD, 0.205), compared to a mean OD of 0.807 (SD, 0.068) in 43 negative sera. Nonetheless, the difference in ODs between positive and negative sera was statistically significant in both humans and marsupials (Mann-Whitney test, p<0.001 for both hosts). High specificity of serological detection was also suggested by confirmation of positive ELISA results using an IFA relying on cells infected with human HAV in 10 out of 12 marsupial specimens and by co-localization of marsupial antibodies with a monoclonal control antiserum raised against human HAV (Figure 2C). Additionally, high specificity of serological detection
was suggested by relatively high end-point IFA titers (median, 1:900; range, 1:40-1:10,000, Table 2). In sum, we found common exposure of Brazilian Common Opossums to hepatoviruses at 26.6% (12 of 45 sera from *D. aurita*). None of the 10 sera available from other opossum species yielded positive ELISA results (Table 1). During clinical examination considering general condition, nutritional and hydration status, heart and respiratory rates, and body temperature, no abnormalities were observed in seropositive opossums. This may suggest that opossums generally survive hepatovirus infection. Seropositive animals originated from both sampling sites, suggesting relatively wide spread of MHAV, since the home range of Brazilian Common Opossums is only about 0.012 km² (15).

### Antigenic relatedness between HAV and MHAV

Marsupial sera reacted with HAV antigens in ELISA and IFA. This suggested either exposure of marsupials to human HAV or antigenic relatedness between HAV and MHAV. To further examine antigenic relatedness between HAV and MHAV, all epitopes associated with HAV neutralization were analyzed. As shown in Figure 2D, 17 out of 24 (77.3%) amino acid residues across VP2, VP3 and VP1 were either identical or homologous between HAV and MHAV.

Thermodynamic modelling of the MHAV VP3 revealed very high structural similarity between HAV and MHAV, including a conformational epitope constituting the major antigenic site of HAV (shown with an asterisk in Figure 2E) and all structural elements defining the structure of the HAV VP3 (Figure 2F). In sum, serological reactivity patterns including high seroprevalence and antibody titers together with genomic and structural similarities suggested exposure of animals to MHAV rather than HAV and considerable antigenic relatedness between HAV and MHAV.
Genomic characterization of the marsupial hepatovirus

In a Bayesian phylogeny of the partial VP2-VP3 region obtained upon sequencing of an extended amplicon yielded by the screening PCR assay as described previously (5), the MHAV clustered within a clade defined by rodent hepatoviruses from Chinese woodchucks (Marmota himalayana) and Mexican cotton rats (Sigmodon mascotensis) (5, 7) at high statistical support (Figure 3A). The near-complete MHAV genome was characterized by sets of nested PCR assays and a 5'/3'-RACE strategy as described previously (5) and submitted to GenBank under accession number MG181943. Similar in length to HAV, the MHAV polyprotein gene comprised 6,687 nucleotides and showed the typical properties defining hepatoviruses. This included the absence of a leader protein, and the presence of a truncated VP4 lacking an N-terminal myristoylation signal, of a tandem YPX3L late domain motif in VP2 likely involved in quasi-envelope acquisition (16), of a predicted transmembrane domain (TMD) in the 3A domain (17) and of a cis-acting replication element (cre) in the 3Dpol domain (18) (Figure 3B). The polyprotein gene was preceded by a 5'-untranslated region containing a predicted type 3 internal ribosomal entry site (IRES) (19), which consisted of five major domains and included typical pyrimidine-rich regions between domains II and III and before the start codon of the polyprotein gene (Figure 3C). Along the polyprotein gene, MHAV genomic identity was generally highest with a rodent hepatovirus sampled in Mexico in 2005 from a cotton rat (S. mascotensis) (5) (Figure 3D). Averaged over the translated full polyprotein gene, amino acid sequence distance between the MHAV and the Mexican rodent HAV was 23.7%. For comparison, amino acid sequence distance between the MHAV and human HAV genotypes ranged from 32.3-32.8% and the maximum sequence distance within HAV genotypes was 7.9%. This suggested MHAV is a new Hepatovirus species. As shown in Table 3, predicted polyprotein cleavage sites were highly conserved between HAV and MHAV. This suggested similarities in 3Cpro-mediated polyprotein processing between HAV and MHAV (20),
including a C-terminal extension of VP1 termed pX, potentially involved in capsid assembly and quasi-envelopment (16, 21). The close genetic relationship of the MHAV with Chinese and Mexican rodent hepatoviruses was confirmed along the major picornavirus domains P1, P2 and P3 in separate Bayesian phylogenies (Figure 3E). No evidence for recombination involving the MHAV was evident from these phylogenies and no recombination involving MHAV was detected in formal recombination analyses of the full MHAV genome.

**Codon usage of the novel hepatovirus and marsupial hosts**

One of the most distinctive features of HAV is a marked codon usage bias (CUB) reflected as a low effective number of codons (ENC) (22). In HAV, CUB has been associated with preferred usage of rare codons to modulate translation and evade host cell defenses (22). As shown in Figure 4A, codon usage was generally comparable between HAV and MHAV and between human and marsupial hosts. The CUB of HAV and MHAV was very similar as evidenced by comparable ENC counts and arginine indexes (Figure 4B). Rather than a preferred usage of codons that were underrepresented in their hosts, HAV and MHAV shared a pronounced avoidance of codons containing a CpG dinucleotide. In many RNA viruses, CUB may largely result from selective pressure against CpG dinucleotides (23), which were recently shown to be targeted by the zink-finger antiviral protein ZAP (24). In HAV and MHAV the CpG content was similarly low at 0.111 and 0.115 of the expected content based on a random dinucleotide composition and actual frequencies of genomic C and G nucleotides, much lower than the CpG content observed in other picornaviruses (25). Therefore, it is likely that HAV and MHAV are under similar selective pressure, hypothetically mediated by ZAP.

**Evolutionary relationships of the novel marsupial hepatovirus**
The level of congruence between host and virus phylogenies can provide information on the genealogy of extant viruses. Programs for co-evolutionary analyses can broadly be summarized into two groups (26). A first group of programs aims at testing the congruence between host and virus phylogenies by identifying symmetries in genetic distances. In the case of this study, we used a program termed ParaFit (27). A second group of programs aims at comparing the topology of host and virus phylogenies to infer the nature and frequency of different evolutionary events. In the case of this study, we used a program termed CoRe-PA (28). Both ParaFit and CoRe-PA were used for two different hepatovirus datasets. The first hepatovirus dataset consisted of the partial VP2-VP3 domains. This dataset was chosen because it contains the largest number of novel hepatovirus sequences from diverse hosts (5). The second hepatovirus dataset consisted of the full P1, P2 and P3 polyprotein domains. This dataset was chosen because evolutionary associations can differ between different domains of picornavirus polyprotein genes due to recombination events (29). The host phylogeny was reconstructed using a mitochondrial marker and taxonomic constraints detailed in (5) to increase phylogenetic resolution above family level. Using ParaFit, the overall agreement between virus and host phylogenies was highly significant for both the analyses of the partial Hepatovirus VP2-VP3 and the full polyprotein domains (p<0.01). Striking examples of co-segregating hepatoviruses and bat hosts from geographically distant sampling sites included closely related viruses from African and European Miniopterus and Rhinolophus bats (Figure 5A). In contrast, fewer nonprimate hepatoviruses and their hosts yielded statistical significance in analyses of the full polyprotein domains (Figure 5B). Consistent with the results of the partial VP2-VP3 dataset, most of the statistically significant individual associations were observed in the capsid-encoding P1 domain that includes the VP2-VP3 domains.
Despite the statistical significance of the distance-based analyses, the topology of the hepatovirus phylogenies did not match that of their hosts in numerous occasions. Additionally, near-identical SHAV genotype V genomic sequences were recovered from three distinct genera of nonhuman primates, namely *Papio anubis* (olive baboons), *Macaca mulatta* (rhesus macaques) and *Chlorocebus aethiops* (African green monkeys) (6, 30, 31). Similarly, near-identical nonprimate hepatoviruses were recovered from sympatric, yet genetically distinct, rodent and bat hosts (Figure 5A). The identification of the natural hosts for these hepatoviruses is thus challenging and recent spill-over infections facilitated by sympatry and host genetic relatedness (12) may bias the distance-based co-evolutionary reconstructions.

Event-based reconciliations of hepatovirus and host phylogeny using CoRe-PA revealed that indeed, between 17-21% of evolutionary events were projected to co-speciations for the partial VP2-VP3 domains (Figure 6A, left panel) and the P1, P2 and P3 domains (Figure 6B, left panel). The existence of co-evolutionary events was consistent with fewer co-speciation events in the majority of control runs relying on randomized host-virus associations compared to the original datasets (the red squares in Figure 6A and 6B). A similar quantity of co-speciation events in the VP2-VP3 dataset was also observed in control runs which excluded all reconstructions without at least one host switch (abbreviated as w/s in Figure 6A) or which facilitated reconstructions of co-speciations by low costs attributed to those events (abbreviated as co in Figure 6A). However, host-independent evolutionary events predominated the genealogy of extant hepatoviruses. As shown in Figure 6A and in Figure 6B, these events included predominantly sorting events, i.e., viruses were predicted to have remained on only one host progeny lineage after speciation. This may either result from failure of the virus to speciate along with the host, or from extinction of one virus progeny lineage. Hypothetically, sorting events may also represent unrecognized co-speciations due to lack of sampling of the respective hepatovirus
progeny counterparts. Additional host-independent events included duplications, i.e., host-independent viral speciation events, and host switches. As shown in Figure 6C, the phylogenetic relationship of the MHAV was reconstructed as the most deep-branching host switch among extant hepatoviruses, highlighting the relevance of including MHAV in hepatovirus evolutionary reconstructions.

Common challenges of co-evolutionary analyses include partially unresolved virus phylogenies and an inevitable sampling bias, which we tried to minimize by multiple levels of bioinformatic analysis. Distance-based and event-based co-evolutionary reconstructions yielded limited evidence for co-speciation of hepatoviruses and their hosts, whereas event-based reconstructions revealed that host-independent evolutionary events predominated the genealogy of extant hepatoviruses. Despite our thorough co-phylogenetic analyses, partially contradictory results suggest that the macroevolutionary history of hepatoviruses is too complex to be exhaustively described using the currently available tools. Future analyses would greatly benefit from event-based tools taking branch lengths into account and tools recognizing more than just four evolutionary events (32).

Discussion

We identified a new hepatovirus in marsupials and investigated its prevalence and organ distribution. Opossums are well-known reservoirs of human pathogenic parasites such as *Leishmania* and *Trypanosoma* (33, 34). In contrast, little is known about viruses in opossums despite the increased attention small mammals gained as evolutionary sources of major human viruses including HAV (5). Limited genomic data has been retrieved for hantaviruses, anelloviruses, orthopoxviruses, flaviviruses, and parvoviruses from opossums (35-39). The broad taxonomic range of viral findings may suggest an important role of opossums as sources of
human infections, because many American marsupial species are synanthropic (40). Additionally, opossums are commonly hunted and consumed as wild game by resource-limited Brazilian communities (41). Because the hepatovirus clade containing the MHAV shares a monophyletic ancestor with human HAV, the MHAV may hypothetically retain the ability to infect humans. However, the conserved antigenicity between HAV and MHAV may limit an introduction of MHAV or related viruses into humans due to community protective immunity to HAV. Notably, community protective immunity may both be elicited by frequent natural infection with HAV suggested by about 70% HAV seroprevalence in young adults in northeastern Brazil (42) and by vaccination programmes initiated in Brazil in 2014 (43). Our formal co-evolutionary analyses confirmed a complex genealogy of the genus *Hepatovirus*. We obtained statistically significant evidence for the existence of co-segregation of distinct hepatoviruses and their hosts separated by large geographic distances, as suggested before for some bat hepatoviruses (6). However, event-based reconstructions suggested that host-independent macroevolutionary patterns predominated the genealogy of extant hepatoviruses. Predominantly host-independent *Hepatovirus* evolution is consistent with lack of co-evolutionary relationships in other picornavirus genera (44, 45). Irrespective of the inherent uncertainties of co-evolutionary reconstructions, host switches such as that from putative rodent-borne MHAV ancestors into marsupials highlight that hepatoviruses can infect highly diverse hosts. The inferred cross-order hepatovirus host switch from rodents into marsupials may have been facilitated by sympatry of these hosts in many areas of Latin America, including competition for food and even marsupials feeding on rodents (46, 47). Our results strongly suggest that the species barriers towards hepatovirus infection seems penetrable, hinting at a broadly conserved cellular receptor across mammalian hosts, which may extend beyond the canonical HAV receptor HAVcr1/TIM-1 according to recent data (48).
Because for the hepatoviruses, cleavage of mitochondrial antiviral signaling protein (MAVS) is a major marker of viral immune evasion and host specificity (49), comparative investigation of MAVS cleavage by MHAV protease precursors may yield further insights into hepatovirus species barriers. On the other hand, major components of the adaptive immune system likely evolved before the divergence of marsupials from Eutherians (50). Ancestral hepatovirus immune evasion mechanisms may thus be efficient in divergent mammalian hosts. However, marsupial CD4 sequences share only about 40% amino acid identity with Eutherian mammals (51). Because immune control of HAV includes antiviral cytokines excreted by CD4+ T cells (52), MHAV may represent a unique opportunity to assess whether this part of the immune control of HAV is indeed evolutionarily conserved once virus isolates or reverse genetics system become available. Finally, opossums have been used previously in studies on viral pathogenesis and transmission, including rabies virus, vesicular stomatitis virus and La Crosse virus (53-55), highlighting the feasibility of hepatovirus experimental infections in these uniquely divergent animals.

Materials and methods

Ethical aspects

Sampling and export of specimens was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the Federal University of Bahia, permit number 25/2014, and by the System of Authorization and Information on Biodiversity (SISBio / ICMBio), permit numbers 43737-2/43737-4, and by the Ministry of Agriculture, Livestock and Food Supply (MAPA), permit numbers No. 005/2014 and 001/2017, and by CITES permits E-00306/17 (Germany) and 15BR018932/DF and 16BR022344/DF (Brazil).

Sampling
Animals were captured using tomahawk traps with fruit and meat as bait. After sedation by trained veterinarians, blood was collected from cephalic, lateral saphenous, jugular, femoral or caudal veins, depending on the species and size of the captured animal. Animals were subsequently released. Tissues from animals obtained dead were stored after necropsies in RNAlater solution (Qiagen, Hilden, Germany). Animals were typed according to morphological criteria by trained veterinarians.

**RNA purification**

Viral RNA was extracted of about 30 mg of tissue from solid organs or 10-50μL of sera. RNA was purified using the MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, Penzberg, Germany) for tissue specimens and the DNA and Viral NA Small Volume Kit (Roche) for sera.

**Hepatovirus detection and quantification**

Viral quantification was done by strain-specific real-time RT-PCR using in vitro transcribed cRNA control standards as described previously (56). Primers and probes for this assay were located in the VP2 domain and contained the following sequences: MHAV-rtF: AATCCTACACCCCTTTCAACAAGGA (polyprotein gene positions 409-432), MHAV-rtR: AGGGTATACAGGCAAAGAAGCAA (polyprotein gene positions 479-501) and MHAV-rtP: FAM-TTGCAGCAATGGTCCCAGCAGATC-TAMRA (polyprotein gene positions 440-463).

Thermocycling involved reverse transcription at 55 °C for 20 minutes followed by 94 °C for 3 minutes, and 45 cycles of 94 °C for 15 seconds, and 58 °C for 30 seconds. PCR chemistry was generally the OneStep SuperScript III RT-PCR kit and the Platinum Taq PCR kit (both Thermo Fisher, Darmstadt, Germany) for second round reactions as described previously (5).
Serology

An ELISA test system with increased sensitivity for the detection of antibodies to human HAV was used for antibody detection in marsupial sera, following the manufacturer’s specifications (Mediagnost, Reutlingen, Germany). The ELISA is based on the competition of serum antibodies with a high-avidity horseradish peroxidase-conjugated polyclonal human anti-HAV IgG for detection. Sera were tested at a 1:20 dilution. Due to limited serum volumes, only one replicate was tested in single determinations. Negative controls included ELISA dilution buffer and anti-HAV negative human sera. The cut-off was defined as 50% inhibition of the extinction of the negative controls.

An indirect IFA was done using FRhK-4 cells persistently infected with HAV as described previously (5). Opossum sera were diluted 1:40, 1:100, 1:200, 1:400, 1:800, 1:1,000, and 1:10,000. Reactions were detected with rabbit-anti-opossum IgG (Biomol, Hamburg, Germany) and a goat anti-rabbit cyanine 3-labeled IgG (Dianova, Hamburg, Germany). Infected cells were mixed with non-infected cells in a 1:1 ratio to allow internal negative controls. Additionally, mock-infected cell cultures were used as controls with opossum sera to exclude nonspecific reactivity. Both ELISA and IFA thus do not differentiate between IgM and IgG isotypes in analyzed sera.

Bioinformatics

Genome annotations and translation alignments were done using Geneious 6.1.8 (57). Nexus files for phylogenetic analyses were generated in MEGA7 (58). Bayesian phylogenies were generated using MrBayes V3.1 using a GTR+G+I substitution model (59). Trees were run for two million generations, sampled every 100 steps. After an exclusion of 5,000 of the total 20,000 trees as burn-in, final trees were annotated with TreeAnnotator and visualized with FigTree from the
The avian encephalomyelitis virus (genus Tremovirus) was used as an outgroup. Secondary structure predictions were done using Mfold. Sequence distances were plotted using SSE V1.2. Codon usage and relative CpG content (ratio of observed content to expected at actual C and G genomic frequencies) of viruses were determined using SSE V1.2. The arginine index (AI) was calculated as the ratio of genomic CGN/(CGN+AGR) codons.

Host codon usage was retrieved from the online database HIVe-CUT. Opossums were represented by Monodelphis domestica in HIVe-CUT. Host cytochrome B sequences were obtained from GenBank and used for reconstruction of phylogenetic relationships as described above. ParaFit was run in R, through the Rstudio environment, with the packages APE and Vegan including 100,000 random permutations of virus-host associations to test for statistical significance. Most parsimonious reconciliations between hepatovirus and host phylogenies were computed with the command-line version of CoRe-PA using three different event cost evaluation methods for distinct evolutionary events, namely co-speciation, duplication, sorting, and host switch. First, the adaptive cost method implemented in CoRe-PA was used with 1,000 random cost models. Second, the adaptive cost method was used as before, but only those reconciliations were considered that have at least one host switch (-s option in CoRe-PA). Third, reconciliations were maximized with respect to the number of co-speciation events (-x option in CoRe-PA). Host-virus associations were randomized yielding 100 replicates and the results were compared with the reconciliation obtained from the original data set. Thermodynamic modelling of the MHAV VP3 was done onto the HAV crystal structure using Chimera and ESPript 3.0.

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Figure legends

Figure 1. Phylogeny of hepatovirus hosts and sampling sites. (A) Mammalian phylogeny showing the time of divergence between marsupials and therians, including a monotreme outgroup, adapted from (11). Mya, million years ago. (B) South America, Brazil and sampling site in Bahia (red circle). Orange, geographic range of *Didelphis aurita*, the most frequently sampled marsupial in this study (66). Distribution was retrieved from the IUCN Red List of Threatened Species, [http://www.iucnredlist.org](http://www.iucnredlist.org), downloaded on January 2\textsuperscript{nd}, 2018 and mapped using QGIS ([www.qgis.org](http://www.qgis.org)) and open source data from Natural Earth ([http://www.naturalearthdata.com](http://www.naturalearthdata.com)). (C) Total of samples obtained per site. Image of *D. aurita* (copyright Pedro Lima given to author I.O.C.).

Figure 2. Infection patterns. (A) Marsupial HAV (MHAV) RNA concentrations in solid organs. (B) Adaptation of human HAV ELISA (triangles) for marsupials (squares). Abs., absorbance. (C) Co-localization of antibodies from marsupial serum and an anti-HAV monoclonal antibody (7E7) in FRhK-4 cells infected to 100% with human HAV, mixed with non-infected cells in a 1:1 ratio to generate internal negative controls. (D) Hepatovirus epitopes associated with neutralization as summarized in (4). Residue color indicates biochemical properties according to the Blosum62 matrix. (E) Thermodynamic modelling of the MHAV VP3 compared to HAV. *, central antigenic site. (F) Conservation of predicted structural elements within the VP3 domain between HAV and MHAV. Conserved characters are indicated with red boxes; α-helices and 310-helices (η) are represented by squiggles, β-strands are represented by arrows, strict β-turns are indicated with TT.
**Figure 3. Genomic characterization and evolutionary relationships.** (A) Partial VP2-VP3 hepatovirus phylogeny based on 864 nucleotides corresponding to genomic positions 1,124–1,988 in HAV genotype IA (GenBank accession no. AB020564). Gt, genotype. (B) Full genome organization of the marsupial HAV (MHAV). Sequencing of the 5′-terminus likely lacked 12 nucleotides compared to the most closely related rodent HAV strain from *Sigmodon mascotensis* (KT452685). TMD, transmembrane domain; cre, *cis*-acting replication element. (C) IRES prediction. Yellow, pyrimidine-rich regions. (D) Amino acid sequence identity between MHAV and rodent HAV from *Sigmodon* (red), *Marmota himalayana* (yellow; KT229612), and human HAV genotype IA (black; AB020564). Top, schematic representation of the HAV genome organization. All other hepatoviruses are given in gray for clarity of presentation. (E) Bayesian phylogenies of hepatovirus domains P1, P2 (only 2C) and P3 (only 3CD). Host genera and species are abbreviated for graphical reasons, full host details are provided in Figure 6C. Filled circles in A and E, Bayesian posterior probability support of grouping above 0.9. Scale bar indicates genetic distance; Virus names are colored according to host orders.

**Figure 4. Codon usage bias.** (A) Codon usage of HAV and MHAV compared to human and marsupial hosts. Stop codons are not shown for clarity of presentation; aa, amino acid residue. (B) AI, arginine index, corrected CpG dinucleotide content and effective number of codons (ENC) of HAV and MHAV. Error bars in HAV indicate the range of values among HAV genotypes.

**Figure 5. Distance-based co-evolutionary analyses.** (A) Partial VP2-VP3 domains. Dashed lines, significant (p<0.05) co-evolutionary associations between individual hosts and hepatoviruses. Filled circles, Bayesian posterior probability of grouping above 0.9. Scale bar
indicates genetic distance. (B) Hepatovirus domains P1, P2 and P3 represented as in panel A. The novel MHAV is highlighted in red and boldface.

**Figure 6. Event-based co-evolutionary analyses.** (A) Frequencies of evolutionary events (indicated above panels) in the partial VP2-VP3 dataset. Red squares, original dataset. Tukey boxplots, data from 100 randomizations of host-virus associations. Automated cost model, $a$; automated model excluding reconstructions without host switches, $w/s$; reconstructions facilitating co-speciations by low event costs, $co$. (B) Frequencies of evolutionary events as in A for P1, P2 and P3 domains. Confirmatory runs using cost models excluding reconstructions without host switches and those maximizing co-speciation yielded near-identical results and are not shown for clarity of presentation. (C) Representation of the most parsimonious reconciliation of host and hepatovirus phylogenies for the partial VP2-VP3 domains.
Table 1. Sample characteristics

| Species               | N  | Positive / total (%) | Blood PCR | Serology PCR | Liver PCR |
|-----------------------|----|----------------------|-----------|--------------|-----------|
| Didelphis aurita      | 96 | 0/45 (0)             | 12/45 (26.6) | 1/51 (1.96) |
| Didelphis albiventris | 2  | 0/1 (0)              | 0/1 (0)    | 0/1 (0)      |
| Didelphis sp.*        | 4  | nt                   | nt        | 0/4 (0)      |
| Marmosops incanus     | 1  | nt                   | nt        | 0/1 (0)      |
| Micoureus demerarae   | 7  | 0/7 (0)              | 0/7 (0)    | nt           |
| Metachirus nudicaudatus| 2 | 0/2 (0)              | 0/2 (0)    | nt           |
| **Total**             | 112| 0/55 (0)             | 12/55 (21.8)| 1/57 (1.8)  |

nt: not tested, *These species could not be unambiguously typed according to morphological criteria due to potential hybridization events between parental lineages.
Table 2. Individual test results

| Sample ID | ELISA optical density | IFA end-point titer | PCR Blood | PCR Live | Sampling site |
|-----------|-----------------------|--------------------|-----------|---------|---------------|
| 8         | 0.074                 | 1:10,000           | neg       | nt      | Mata de São João |
| 2         | 0.640                 | 1:1,000            | neg       | nt      | Salvador      |
| 67        | 0.349                 | 1:200              | neg       | nt      | Salvador      |
| 70        | 0.630                 |                    | neg       | nt      | Salvador      |
| 73        | 0.206                 | 1:800              | neg       | nt      | Salvador      |
| 80        | 0.544                 | 1:1,000            | neg       | nt      | Salvador      |
| 135       | 0.541                 |                    | neg       | nt      | Salvador      |
| 157       | 0.647                 | 1:400              | neg       | nt      | Salvador      |
| 158       | 0.408                 | 1:1,000            | neg       | nt      | Salvador      |
| 196       | 0.650                 | 1:100              | neg       | nt      | Salvador      |
| 209       | 0.535                 | 1:40               | neg       | nt      | Salvador      |
| 225       | nt                    | nt                 | nt        | pos     | Salvador      |
| 233       | 0.107                 | 1:10,000           | neg       | nt      | Salvador      |

All animals belonged to the species *D. aurita*; *Cut-off for positivity: ≤0.650; extinction at 620 nm; pos: positive; neg: negative; nt: not tested
### Table 3. Similarities between predicted polyprotein cleavage sites of MHAV and HAV

|              | VP4/VP2       | VP2/VP3       | VP3/VP1-PX     |
|--------------|---------------|---------------|---------------|
| **HAV**      | LSLA/DIEE     | LSTQ/MMRN     | VTTQ/VGDD     |
| **MHAV**     | LSLA/DVEE     | IMTQ/MMRN     | TVAQ/AGDD     |
| **VP1-px/2B**|               |               |               |
| **HAV**      | LFSQ/AKIS     | LRTQ/SFSN     |               |
| **MHAV**     | VCSQ/SGPI     | LHTQ/GFSD     |               |
| **2C/3A**    |               |               |               |
| **HAV**      | LWSQ/GISD     | IPA/EVYH      | VESQ/STLE     |
| **MHAV**     | LWSQ/SGDD     | IPAC/GVYH     | AESQ/STLE     |
| **3A/3B**    |               |               |               |
| **HAV**      |               |               | IESQ/RIMK     |
| **MHAV**     |               |               | IESQ/RIMK     |
| **3B/3C**    |               |               |               |
| **HAV**      |               |               |               |
| **MHAV**     |               |               |               |
| **3C/3D**    |               |               |               |

HAV = NC_001489; Green, conserved sites