A novel hepatovirus identified in wild woodchuck *Marmota himalayana*

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Hepatitis A virus (HAV), a causative agent of water- and food-borne hepatitis, is the only reported member of the genus *Hepatovirus* within the family *Picornaviridae*. HAV is widely distributed in humans and primates and has caused hepatitis A epidemics and outbreaks worldwide, particularly in developing countries. HAV has a single-stranded, positive-sense RNA genome of 7,500 base pairs (nt) with a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The polyprotein of HAV is cleaved co- and post-translationally into 11 proteins: 4 structural proteins, VP1–VP4 (P1 region) that comprise the viral capsid, and 7 non-structural proteins, 2A–2C (P2 region) and 3A–3D (P3 region). The first comparative study of HAV strains in primates demonstrated significant inter-strain heterogeneity. In the mid-1980s, various HAVs were isolated from hepatitis outbreaks of diverse origin. Based on analysis of the 900 nt of the complete VP1 protein, HAV is grouped into six genotypes (I-VI), among which, genotypes I, II and III are of human origin, while IV, V and VI are of simian origin. Despite its considerable genetic variability, HAV exhibits low antigenic variability. Only a single serotype has been described, and the only naturally antigenic variants are HAV strains collected from Old World monkeys. Furthermore, studies using monoclonal antibodies have suggested the presence of a limited number of antigenic epitopes of VP3 and VP1 clustered at the surface of the virus. A recent study using a structure-based predictive method suggested that additional epitopes might exist in VP2 and VP3.

HAV differs greatly from other picornaviruses in many respects, and has specific molecular mechanisms for viral adaptation, including deoptimization of codon usage, Treg shut-off and ‘membrane hijacking’. Furthermore, the clinical spectrum of disease caused by HAV varies considerably, from asymptomatic cases to mild and transient or severe hepatitis. The tropism of HAV for the liver also remains unresolved. Animal models used for HAV most commonly are primates. No closely related homolog virus has been identified in animals other than primates (human and non-human primates) except for a hepatovirus (*phopivirus*) was very recently discovered in seals. The restricted host range and lack of homologous viruses in other species has

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hampered the understanding of HAV pathogenesis and immune response. A renewed effort is needed to better characterize animal HAV-related viruses and to develop new animal models for hepatitis A virus infection24.

Here, we report on the discovery of a novel Hepatovirus from wild woodchuck Marmota himalayana, provisionally named Marmota himalayana hepatovirus (MHHAV) which may represent a new species and serotype in the genus Hepatovirus within the family Picornaviridae.

Results

**MiSeq high-throughput sequencing.** Two unique reads with lengths of 2,738 nt (contig1) and 3,324 nt (contig2) were assembled from the initial sequencing data. Blasts showed that contig1 had the best hit to simian HAV, with 75% nt identity, while contig2 had the best hit to human HAV, with 70% nt identity. Contigs1 and 2 were confirmed using specific primers.

**Genomic characterization.** Based on the sequences of contigs1 and 2, specific primers were designed to generate overlapping polymerase chain reaction (PCR) products. Two full-length genome sequences showing 99% nt identity from two different samples (ID 2 and 3) were acquired and deposited into GenBank under accession numbers KT229611 and KT229612. The MHHAV genome comprises 7,566 bp (excluding the polyadenylated tail), with a 713 nt 5′ UTR, an ORF of 6,756 nt (encoding a potential polyprotein precursor of 2,252 amino acids [aa]), followed by a 100 nt 3′ UTR and a poly (A) tail. The base content of the MHHAV genome is 31.39% A, 13.30% C, 20.41% G, and 34.89% U, with a pyrimidine content of 48.19%, which is similar to the known HAVs (simian HAV prototype, 49.21%; human HAV prototype, 48.95%; phosphivirus prototype, 46.83%). Sequence analysis revealed that the polyprotein of MHHAV showed 67% aa identity to that of HAV and 58% aa identity to that of phosphivirus. Furthermore, MHHAV displays a strong codon bias that is complementary to that of the woodchuck (Table 1). The rare codons of MHHAV are very abundant in woodchuck codons (Table 2).

A hypothetical cleavage map of the MHHAV polyprotein was derived by alignment with other HAVs (Fig. 1a). Only 3 cleavage sites are conserved in MHHAV, primate HAV and phosphivirus (VP2–VP3, 3B–3C, 3C–3D) (Fig. 1b). The P1 region of MHHAV is 2,400 nt in length, 27, 15 and 90 nt longer than those of the prototypic human HAV, simian HAV and phosphivirus, respectively. Interestingly, compared to previously known HAVs an additional 18 nt sequence was detected in the VP1 region (positions 3,009–3,026 nt, sequence: TCTCCTCTAGGAGAACA), coding for six aa “SSSRRRT”; whereas only 15 nt was found in the recently reported phosphivirus (TCCCTTAGGAGAACA). An RGD motif was found in the middle region of VP3 in MHHAV.

The P2 regions of the prototypic human and simian HAVs are 1,893 nt in length, but it is 1,896 nt in MHHAV and 1,932 nt in phosphivirus; highly conserved aa motifs, GXGXGKT (G 1250KRGGGKS) and D 1301DIGQ, in the nt-binding domain of the putative picornavirus NTPase and helicase, were also found in the 2C protein of MHHAV. The P3 region of MHHAV is 2,460 nt in length, which is 48 and 51 nt longer than those of the prototypic human and simian HAV, respectively, but 15 nt shorter than that of the phosphivirus. The 3D region includes the KDELR, YGDD and FLKR motifs of the RNA-dependent RNA polymerase.

**Antigenic site analysis.** HAV has a conformation-dependent immunodominant neutralization site. Residues S102, V171, A176, and K221 of VP1 as well as Q70, S71, E74 and 102–121 of VP3 have been implicated in neutralizing epitopes; residues 71 and 198 of VP2 as well as residues 89–96 of VP3 may harbor other epitopes14. Sequence alignment showed that all the aa in the antigenic sites were different between MHHAV and HAV prototype Fig. S1a) with the exception of S102 of VP1 and T71 of VP2. In addition, only two aa (A70 of VP3 and T71 of VP2) were identical between MHHAV and simian prototype. Furthermore, three aa are the same between MHHAV and the recently reported phosphivirus, T74 of VP3 as well as T71 and P198 of VP2. The detailed alignment of the MHHAV capsid protein with counterparts was shown in Fig. S2. The antigenic sites in the MHHAV model are presented in both cartoon (Fig. S1a) and surface (Fig. S1c) forms.

**Secondary RNA structure of the 5′ UTR.** The 5′ UTR of MHHAV is 20 nt shorter than that of human HAV (734 nt), 45 nt and 73 nt longer than those of simian HAV (669 nt) and phosphivirus (648 nt), respectively. It shares 56.72%, 54.48% and 41.73% nucleotide identity with human HAV, simian HAV and phosphivirus, respectively. The predicted secondary structure of MHHAV (Fig. 2a) shows that MHHAV 5′ UTR contains five major structural domains (including six stem-loops) labeled from 1 to V beginning at the 5′ terminus of the 5′ UTR, and lacks the first domain found in human HAV. The first domain (stem-loop Ia and Ib) of human HAV correlates to domain II (stem-loop) of human HAV. The predicted secondary structure of simian HAV 5′ UTR (Fig. S3) is similar to that of human HAV but lacks the first domain and stem-loop Ia. The stem-loop Ia and Ib of domain I, present in human HAV domain II, may form a pseudoknot; however, the pseudoknot in domain V of human and simian HAVs was not found in MHHAV.

The segment from 79 to 120 nt of the MHHAV 5′ UTR represents a polypyrimidine tract that has been suggested not to be involved in the formation of conserved helical structures25. In the 5′ UTR secondary structure of MHHAV, stem-loop III is a long multi-loop cloverleaf structure that corresponds in position and shape to the previously designated stem-loop IV (in primate HAVs and phosphivirus), is an internal ribosome entry site (IRES). The IRESes in MHHAV, primate HAV and phosphivirus have highly conserved base paired regions governing internal translation initiation and belong to IRESes of type III.

Two important motifs near the 3′ border of the picornavirus IRES were also found in the MHHAV 5′ UTR. The first motif is an UUUC sequence (box A) within the second pyrimidine-rich tract; the second motif is an AUG triplet (box B) that functions as an initiation codon in HAV and phosphivirus26. Similar to human and simian HAVs a putative cis-acting RNA replication element (cre) is located near the 5′ end of the 3D Pol-coding sequence of MHHAV. It contains a top loop of 18 nt with a stem segment of 34 nt, interrupted by four internal loops and
| Amino acid (symbols) | Codon (s) | Occurrence (%) of codon in virus/host |
|----------------------|-----------|----------------------------------------|
|                      |           | MHHAV | Woodchuck | Human | HAV |
| Phenylalanine (Phe, F) | UUU       | 100.00 | 48.83 | 86.70 | 100.00 |
|                      | UUC       | 13.33  | 100.00 | 100.00 | 28.20 |
| Leucine (Leu, L)     | UUA       | 91.57  | 9.57  | 19.40 | 49.32 |
|                      | UUG       | 100.00 | 25.18 | 32.60 | 100.00 |
|                      | CUU       | 20.48  | 20.29 | 33.30 | 44.66 |
|                      | CUC       | 1.20   | 53.90 | 49.50 | 8.22  |
|                      | CUA       | 4.82   | 14.46 | 18.20 | 9.32  |
|                      | CUG       | 10.84  | 100.00| 100.00| 26.03 |
| Isoleucine (Ile, I)  | AUU       | 100.00 | 56.46 | 76.90 | 100.00 |
|                      | AUC       | 5.45   | 100.00| 100.00| 16.63 |
|                      | AUA       | 46.36  | 30.03 | 36.10 | 31.62 |
| Valine (Val, V)      | GUU       | 100.00 | 24.96 | 39.10 | 100.00 |
|                      | GUC       | 12.36  | 58.23 | 51.60 | 14.81 |
|                      | GUA       | 24.72  | 14.43 | 25.30 | 16.99 |
|                      | GUG       | 35.96  | 100.00| 100.00| 45.15 |
| Serine (Ser, S)      | UCU       | 100.00 | 86.18 | 77.90 | 100.00 |
|                      | UCC       | 16.67  | 90.24 | 90.80 | 29.87 |
|                      | UCA       | 79.17  | 42.55 | 62.60 | 88.93 |
|                      | AGU       | 40.28  | 45.53 | 62.10 | 33.56 |
|                      | AGC       | 5.56   | 100.00| 100.00| 6.71  |
|                      | UCG       | 1.39   | 18.16 | 22.60 | 4.70  |
| Proline (Pro, P)     | CCU       | 100.00 | 91.58 | 84.40 | 100.00 |
|                      | CCC       | 9.62   | 100.00| 100.00| 21.74 |
|                      | CCA       | 71.15  | 53.16 | 85.40 | 81.64 |
|                      | CCG       | 3.85   | 23.95 | 34.80 | 2.42  |
| Threonine (Thr, T)   | ACU       | 100.00 | 55.51 | 69.30 | 100.00 |
|                      | ACC       | 9.21   | 100.00| 100.00| 20.74 |
|                      | ACA       | 65.79  | 38.67 | 79.90 | 92.59 |
|                      | AGC       | 1.32   | 17.88 | 32.30 | 4.44  |
| Alanine (Ala, A)     | GCC       | 100.00 | 85.96 | 66.40 | 100.00 |
|                      | GCA       | 6.68   | 100.00| 100.00| 28.37 |
|                      | GCG       | 50.70  | 40.99 | 57.00 | 58.51 |
|                      | GAG       | 1.41   | 26.76 | 26.70 | 1.42  |
| Tyrosine (Tyr, Y)    | UAU       | 100.00 | 55.35 | 79.70 | 100.00 |
|                      | UAC       | 12.99  | 100.00| 100.00| 28.41 |
| Histidine (His, H)   | CAU       | 100.00 | 61.04 | 72.20 | 100.00 |
|                      | CAC       | 20.59  | 100.00| 100.00| 22.11 |
| Glutamine (Gln, Q)   | CAA       | 100.00 | 26.62 | 36.00 | 92.86 |
|                      | CAG       | 53.57  | 100.00| 100.00| 100.00 |
| Asparagine (Asn, N)  | AAU       | 100.00 | 75.00 | 89.00 | 100.00 |
|                      | AAC       | 9.09   | 100.00| 100.00| 21.75 |
| Lysine (Lys, K)      | AAA       | 100.00 | 74.48 | 76.50 | 100.00 |
|                      | AAG       | 43.16  | 100.00| 100.00| 58.85 |
| Aspartic acid (Asp, D)| GAU      | 100.00 | 58.87 | 86.90 | 100.00 |
|                      | GAC       | 12.40  | 100.00| 100.00| 21.04 |
| Glutamic acid (Glu, E)| GAA     | 100.00 | 56.37 | 73.20 | 100.00 |
|                      | GAG       | 38.10  | 100.00| 100.00| 76.51 |
| Cysteine (Cys, C)    | UGU       | 100.00 | 61.04 | 84.10 | 100.00 |
|                      | UGC       | 13.51  | 100.00| 100.00| 26.58 |
| Tryptophan (Trp, W)  | UGG       | 100.00 | 100.00| 100.00| 100.00 |
| Arginine (Arg, R)    | CGU       | 1.32   | 27.60 | 36.90 | 4.21  |
|                      | CGC       | 0.00   | 44.80 | 85.20 | 2.11  |
|                      | CGA       | 2.63   | 28.32 | 50.80 | 3.51  |
|                      | CGG       | 2.63   | 45.88 | 93.40 | 2.11  |
|                      | AGA       | 100.00 | 91.04 | 100.00| 100.00 |
|                      | AGG       | 21.05  | 100.00| 98.40 | 31.58 |

Continued
two 1 nt bulges (Fig. 2b). However, in spite of the 68% nt identity between the cres of MHHAV and phopivirus, the similar region in phopivirus includes three loops and a stem segment (Fig. S4).

Detection of MHHAV in wild woodchucks. Sixteen (16.16%) (ID1-16) of 99 enteric lysates from wild woodchucks were positive for MHHAV RNA by RT-PCR, among which seven animals (ID1-7) were chosen for the collection of blood, liver, spleen, lung, and trachea samples. All sample types were MHHAV RNA-positive, with the highest virus load in the liver and the lowest in the trachea. T-test showed the viral loads in liver and other tissues were statistically significant (blood, p = 0.000; spleen, p = 0.022; lung, p = 0.000; trachea, p = 0.000). Two woodchucks (ID 17 and 18) had MHHAV RNA in their tissues and blood, but not in their feces (Fig. 3a). The sequences of the complete VP1 region amplified from these positive samples were determined. The 34 VP1 sequences showed 99% nt identity. Sequences within an individual woodchuck showed 100% nucleotide identity. All sequences were deposited into GenBank under accession numbers KT229577-KT229610. Furthermore, negative-stranded MHHAV RNA was detected only in the seven MHHAV-positive livers, while it is not detected in the spleen, lung, trachea, blood, and feces samples (Fig. 3b).

Phylogenetic and evolutionary analysis. Recombination analysis showed no evidence of inter-genotype recombination among primate HAVs, phopivirus and MHHAV (Fig. S5). Phylogenetic analysis suggested that MHHAV forms a distinct lineage compared to previous reported HAVs by the neighbor-joining method and 1,000 bootstrap replications. In contrast, the distance between phopivirus and previously reported HAVs is much closer (Fig. 4).

Table 1. Relative occurrence of synonymous codons for each amino acid. Note: The codon of highest frequency for each amino acid was assigned to 100%, the occurrence of other codons for each amino acid was relative to the occurrence of the most abundant frequency.

| Amino acid (symbols) | Codon (s) | Occurrence (%) of codon in virus/host |
|----------------------|----------|--------------------------------------|
| Glycine (Gly, G)    | GGU      | 100.00                               |
|                     | GCC      | 10.17                                |
|                     | GGA      | 100.00                               |
|                     | GGG      | 27.12                                |

Table 2. Rare codon usage in hosts and viruses.
Under the best-fit model, the mean substitution rate was $8.62 \times 10^{-4}$ substitutions per site per year (ssy), with a 95% HPD of $6.96 \times 10^{-3}$–$7.03 \times 10^{-3}$. The time of the most recent common ancestor of MHHAV and the primate HAV isolates was estimated to be around 1,000 years ago. In contrast, the common ancestor of MHHAV and phopivirus was estimated to be 1800 years ago (Fig. 5).

**Electron microscopy.** MHHAV was visualized by negative-staining electron microscopy, which revealed the presence of spherical, non-enveloped virus particles of ~27 nm in diameter, morphologically similar to HAV. Chloroform-purified virions were only rarely detected in feces, but were readily apparent following incubation with a polyclonal antibody (Fig. 6).

**Discussion**

HAV continues to be a source of morbidity and mortality despite the availability of an effective vaccine. Recently a new HAV-related hepatovirus known as phopivirus was reported in seals. Here, we report on the identification of a novel hepatovirus, tentatively named MHHAV, from wild woodchuck Marmota Himalayana. The purified virus particles from the fecal sample are morphologically similar to HAV virions by negative-staining electron microscopy. The complete genome and VP1 capsid protein of MHHAV was best hit to simian HAV, with 67% nt and 75% aa identities, respectively. Based on the classification of enteroviruses, in which a VP1 sequence identity of 70 to 85% is defined as a heterologous serotype, it is suggested that MHHAV may be a new serotype of HAV. Phylogenetic analyses of the polyprotein and VP1 protein indicate that MHHAV forms a separate branch from previous HAVs and may represent a new species in the genus Hepatovirus within the family Picornaviridae.

Although MHHAV has a genome organization identical to that of previously reported HAVs, the length of MHHAV polyprotein is different to those of the prototypic human HAV, simian HAV and phopivirus, respectively. Furthermore, the aa sequences of the predicted cleavage sites of MHHAV differ substantially from these viruses with only three cleavage sites (VP2/VP3, 3B/3C, and 3C/3D) conserved. The common motifs in the non-structural proteins of picornaviruses, such as the NTPase and helicase motifs, were also found in MHHAV. In most picornaviruses, RGD motif is located near the C terminus of VP1, however, this motif was found in the middle of the VP3 region in MHHAV, primate HAVs and phopivirus. The RGD motif is conserved in

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**Figure 2.** Predicted partial secondary structure of MHHAV. (a) Secondary structure of the MHHAV 5′ UTR; domains are labeled I to V. The putative initiator codon (AUG) and UUUCC sequence are indicated in red. (b) Cis-acting secondary structures of MHHAV in the 3D pol-coding region.
picornaviruses and functions in recognizing and attaching to host cells or enabling cell-to-cell and cell-to-matrix interactions. It is inferred from differences in the positions of RGD motifs that HAV interactions with host cell-surface integrins differ from those of other picornaviruses. Furthermore, hepatovirus differ from other picornaviruses in that they rarely use codons most often preferred by their hosts. MHHAV appears to follow HAV and phopivirus in codon usage. We speculate that this strategy may minimize direct competition of hepatovirus with host cell systems and enable persistence.

The antigenic sites of HAVs have been characterized by several research teams. Sequence alignment showed that these antigenic sites differed between MHHAV, primate HAVs, and phopivirus. Further sequence alignment of the whole genome of MHHAV, primate HAVs, and phopivirus revealed an 18 nt and 15 nt insertion encoding six aa ([S]SSRRT) at the C terminus of VP1, with three or two potential O-glycosylation sites (glycosylation site prediction website: http://www.cbs.dtu.dk/services/NetNGlyc/). As glycosylation is essential for antigen processing and presentation and VP1 is a major antigenic protein, we speculate that these sequence differences may indicate antigenic differences between MHHAV and other HAVs. However, the real antigenic characteristics should be determined by a neutralization assay when an in vitro cell culture system for these viruses has been developed.

The predicted secondary structure of the 5′ UTRs of MHHAV, human HAV, simian HAV, and phopivirus is very similar. The most important structure in the 5′ UTR is the IRES, which directs internal initiation of translation. The IRESes of primate HAVs, MHHAV and phopivirus exhibit evolutionarily conserved secondary structure including a long multi-loop cloverleaf structure, which has been grouped into type III. This is different from IRESes of poliovirus and human rhinovirus (type I), encephalomyocarditis virus and foot-and-mouth disease virus (type II) and HCV like IRES (type IV). A conserved RNA structure, the 110 nt HAV cre element located near the 5′ end of the 3Dpol region, is present in both human and simian HAVs as well as in the distantly related avian encephalomyelitis viruses, but the similar cre element was not present in the corresponding region in phopivirus. However, this element was also found in MHHAV, as was the AAACA/G motif that serves as the template for uridylylation of VPg by a slide-back mechanism. These findings suggest that MHHAV might have replication mechanisms and tissue tropism similar to those of other known HAVs, but the shape and position of cre element in phopivirus were still unknown. Further studies are needed to address the subtle difference in the secondary structure of the 5′ UTR among MHHAV, HAV, and phopivirus.

HAV is highly transmissible and HAV infection is acquired primarily by the fecal-oral route. The poor sanitary condition can cause HAV outbreak locally. In the present study, 16 out of 99 (16.16%) wild woodchucks were detected to carry the MHHAV. Furthermore 34 MHHAV VP1 sequences from different animals in this study shared 99% nt identity and sequences in the same woodchuck showed 100% nt identity. All these indicated that an MHHAV outbreak might happen in wild woodchucks at the time we collected the samples. As anticipated for an HAV replication in the liver, negative-sense RNA complementary to the positive-sense genomic MHHAV RNA was only detected in the liver, however, though with the highest RNA viral load in the livers, MHHAV distributed widely in different organs in the wild woodchucks, which was in accordance with the previous studies that HAV (phopivirus included) can also be detected in extrahepatic organs in the hosts. This phenomenon may be
partly explained by the fact that the HAV cellular receptor 1 was expressed wildly in different organ, such as liver, spleen, kidney and testis, so these organs can capture HAV virons. However, they cannot support the virus replication by some specific reasons. These findings suggest that the liver is the target organ of MHHAV infection and replication in wild woodchucks. Although its hepatotropism and ability to cause disease remain to be determined, the presence of MHHAV in the liver of wild woodchuck *Marmota Himalayana* is reminiscent of human HAV infection.

The phopivirus reported in seals in USA has similar genomic organization, codon usage bias and hepatic tropism to HAV and MHHAV. Phylogenetic analyses in this study indicated that the phopivirus is more distant to the previously reported HAVs than MHHAV. Evolutionary analysis suggested that phopivirus has a common evolutionary ancestry with HAVs and MHHAV. The estimated substitution rate of these viruses was $8.62 \times 10^{-4}$ ssy, similar to a French study based on VP1 sequences from primate genotype IA HAVs ($9.76 \times 10^{-4}$ ssy). This substitution rate is lower than those found in other picornaviruses. However, the common ancestor of MHHAV, primate HAVs and phopivirus was older than that of MHHAV and primate HAVs, indicating that the diversity and evolutionary pathway of HAV are far more complex than previously thought.

Human hepatotropic viruses or related viruses that infect wild woodchuck include woodchuck hepatitis virus (WHV) and hepatitis delta virus (HDV). Natural infection with WHV results in liver disease similar to that induced by HBV in humans. Woodchuck *Marmota monax* is commonly used as an animal model for hepatitis B virus (HBV) infection. There are currently no non-primate models of HAV infection. Guinea pigs can be infected by HAV but do not develop signs of disease or seroconvert. The discovery of MHHAV in woodchuck may facilitate the development of a new tractable animal model of human HAV infection and thus provide further insights into the evolution and pathogenesis of HAVs.

**Materials and Methods**

**Specimens and high-throughput sequencing.** Ninety-nine wild woodchucks were caught from Haixi, Qinghai Province in 2013. Enteric lysates of all the animals and the liver, spleen, lung, and trachea specimens of some animals were collected after exsanguination. The samples were transported on dry ice and stored at $-80^\circ$C at the China Center for Disease Control (CDC). After dilution (1:5 ratio, wt/vol) and filtration (0.45 μm and 0.22 μm membranes), total nucleic acid was extracted from 99 enteric lysates, followed by cDNA synthesis. Random PCR amplification was performed on each sample using primers with different barcodes. The PCR products were pooled for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA). Metagenomic
Figure 5. The Bayesian Markov Chain Monte Carlo (MCMC) tree of the VP1 regions of MHHAV and the known primate HAVs and phopivirus. Horizontal branches are drawn to scale of estimated year of divergence, with tip times reflecting sampling data (year). The estimated time for the most recent common ancestors of the major nodes of the lineages are shown. IA, IB, IIIB: subgenotypes of HAV genotypes I and III.

Figure 6. Spherical, non-enveloped virus particles of ~27 nm in diameter visualized by negative-staining electron microscopy: (a) unclumped MHHAV; (b) immune-complexed MHHAV. The black arrow indicates an intact particle; the white arrow indicates a potential empty particle.
profiling of the shotgun datasets was carried out using the customized informatics pipeline VirusSeeker to computationally identify viral sequences[48]. The study protocol was approved by the Ethics Committee of the China CDC, and was performed according to Chinese ethics laws and regulations. Furthermore, the methods were carried out in accordance with the approved guidelines.

**Full-length genomic amplification.** To determine the full-length genomic sequence of MHHAV, primers were initially designed based on contigs obtained by miseq high-throughput sequencing. Further synthesis was based on newly amplified MHHAV sequences. Long fragments (1500–3000 bp in length) were amplified for final confirmation. All PCR amplifications were performed using Ex Taq DNA polymerase. The extreme 5’ and 3’ ends of the genome were determined using a SMART RACE cDNA Amplification Kit (Clontech, US) and Genome Walking Kit (Takara, Japan). Sequences were assembled and manually edited to produce the final sequence of the viral genome. Codon usage was assessed both for the MHHAV and woodchuck. For woodchuck, codon usage tables were obtained from a database based on genomes in NCBI GenBank (http://www.kazusa.or.jp). For MHHAV, codon usage frequencies were determined by the Cusp program (http://emboss.sourceforge.net/apps/cvs/emboss/apps/cusp.html).

**Detection of MHHAV and amplification of complete VP1 region.** The presence of the MHHAV in wild woodchucks was confirmed by using the primers (forward: 5’-GATCCCAATATCCAGTTGGG-3’, reverse: 5’-CATGTTGCTACATTACTAGG-3’) targeting 658nt region spanning the VP2/VP3-coding junction of the virus using RT-PCR. Complete VP1 genome sequences were amplified with the primers listed below: forward: 5’-CTTTGAGGAGCCACTACTGGACC-3', reverse: 5’-AAGAGATAGGTTCCCTGTGTGTG-3’. The reaction mixture included 20 pmol of each primer and 2.5 U of Ex Taq DNA polymerase (Takara Bio). After 5 min at 94°C, 35 cycles of amplification (94°C for 30 s, 59°C for 30 s, and 72°C for 1 min) were performed, followed by a 7-min extension at 72°C. Products were resolved on a 1.5% agarose gel and purified (QIAquick PCR purification kit, QIAGEN). Nucleotide sequences were determined using the Big-Dye terminator cycle sequencing kit and the ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequences were determined and analyzed using the software package DNAStar.

**Quantitative PCR.** Viral RNA copies of MHHAV were quantified by real-time PCR. A forward primer (MHHAVF: 5’-GTCTCTTTTAAGGCACTCAT -3’), a reverse primer (MHHAVR: 5’-TGGGT CAGTCCCATCTGGCAAG-3’), and a probe (MHHAV Probe: 5’-FAM- CATCTTCATTCTCCCTCCTTACC-MGB -3’) were designed from sequences in the 5’ UTR region of the virus. The reaction condition involved 50°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 30 s.

**Negative-strand MHHAV RNA testing.** A tagged primer system was used to detect the negative-strand RNA. Negative-strand cDNA was generated with Tag MHHAV primer (5’-GATCCCAATATCCAGTTGGG-3’), targeting 5’ UTR of MHHAV), performed at 65°C for 5 min, 25°C 10 min, 50°C 50 min and 72°C 15 min. Then quantitative PCR was established to quantify the negative-strand RNA, a forward primer (TagF: 5’-CTTCCGACATCTGGTGGTG-3’), a reverse primer (UTRR: 5’-TGGGTCAGTCCATCTGGCAAG-3’), and a probe (NProbe: 5’-FAM-CATCTTCATTCTCCCTCCTAC-MGB -3’) were used. The reaction condition involved 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 30 s.

**Recombination and phylogenetic analysis.** Complete genomes of the known HAVs were downloaded. To detect potential combination, aligned sequences were analyzed by using the Bootscanning method and the neighbor-joining algorithm was run with 100 pseudo replicates implemented in Simplot software. Phylogenetic tree were performed using nucleic acid sequences of VP1 and polyprotein by the Neighbor-joining method and subsequently subjected to bootstrap analysis with 1000 replicates. Tree figures were produced using MEGA software (version 5).

**Evolutionary analysis.** To precisely estimate MHHAV, phopivirus and HAV substitution, a Bayesian Markov chain Monte Carlo (MCMC) approach was implemented in the BEAST package (v 1.8.2, available from http://beast.bio.ed.ac.uk/downloads). The jModelTest software 2.1.7 was used to identify the optimal evolutionary model. Different population dynamic models were used (constant size, exponential growth, logistic growth, expansion growth and Bayesian skyline). Different models were evaluated (GTR, Y94, K80, TN and Jukes) using the log-likelihood ratio test. The GTR model best fitted the sequences in this study. The effective sample sizes for the estimated parameters were calculated using Tracer 1.6. The effective sample size values for the estimated parameters in the MCMC analysis were greater than 200. Statistical uncertainty in the data was reflected in the 95% highest probability density values (HPD).

**RNA structure prediction of the 5’ UTR.** The secondary structure of the 5’ UTR RNA of MHHAV was predicted using consecutive fragments of the complete nucleotide sequence of the 5’ UTR of MHHAV and a thermodynamic folding energy minimization algorithm with RNA structure software (version 5.3); the graph was integrated using RnaViz (version 2.0.3).

**Structure prediction of MHHAV.** Based on a previously solved HAV particle structure (PDB: 4QPG)4, the structure of MHHAV was modeled using Phyre49 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id = index).
**Virus purification.** Stool samples were diluted to 20% suspensions in phosphate-buffered saline (PBS). Beads and chloroform were added to the suspensions, followed by 20 min centrifugation at 1,500 × g. Supernatant was collected and then subjected to a single ultracentrifugation step through a discontinuous sucrose/glycerol density gradient for HAV purification, as described previously.

**Electron microscopy.** Fifty-microliter volumes of chloroform-purified MHHAV (1 × 10^9 copies/ml) in PBS were examined directly by negative staining with 1% phosphotungstic acid (pH 6.8). Chloroform-purified MHHAV (450 µl) was incubated with a 1:10 dilution of 50 µl MHHAV polyclonal antibody (BALB/c mice immunized subcutaneously with purified MHHAV) at 37 °C for 1 h. Following centrifugation at 23,000 rpm for 1 h, the sediment was resuspended in 50 µl PBS and the suspension subjected to negative staining. Grids were examined using a transmission electron microscope (TECNAI 12, FEI, Blackwood, NJ).

**Statistical Analysis.** The statistical significance of viral load means between the liver and other tissues was assessed using the Student’s t test, and the statistical analyses were performed using SPSS 16.0.

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Author Contributions

Z.J.D., J.G.X. and J.M.Y. designed research; L.L.L., J.M.Y., C.Y.Z., Y.Y.A., H.C.G., Z.P.X., G.C.X. and L.L.P. performed research; J.M.Y., L.L.L., X.M.S., Z.J.D. and W.I.L. analyzed data; S.L. collected the sample and contributed new reagents; J.M.Y., Z.J.D. and W.I.L. wrote the paper. All authors reviewed the manuscript.

Additional Information

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