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ORIGINAL ARTICLE

Genomic and spatial variability of a European common vole hepevirus

René Ryll[1] · Gerald Heckel[2,3] · Victor M. Corman[4,5] · Jan Felix Drexler[4,5] · Rainer G. Ulrich[1,6]

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Abstract

Rodents host different orthohepeviruses, namely orthohepevirus C genotype HEV-C1 (rat hepatitis E virus, HEV) and the additional putative genotypes HEV-C3 and HEV-C4. Here, we screened 2,961 rodents from Central Europe by reverse transcription polymerase chain reaction (RT-PCR) and identified HEV RNA in 13 common voles (*Microtus arvalis*) and one bank vole (*Myodes glareolus*) with detection rates of 2% (95% confidence interval [CI]: 1-3.4) and 0.08% (95% CI: 0.002-0.46), respectively. Sequencing of a 279-nucleotide RT-PCR amplicon corresponding to a region within open reading frame (ORF) 1 showed a high degree of similarity to recently described common vole-associated HEV (cvHEV) sequences from Hungary. Five novel complete cvHEV genome sequences from Central Europe showed the typical HEV genome organization with ORF1, ORF2 and ORF3 and RNA secondary structure. Uncommon features included a noncanonical start codon in ORF3, multiple insertions and deletions within ORF1 and ORF2/ORF3, and the absence of a putative ORF4. Phylogenetic analysis showed all of the novel cvHEV sequences to be monophyletic, clustering most closely with an unassigned bird-derived sequence and other sequences of the species *Orthohepevirus C*. The nucleotide and amino acid sequence divergence of the common vole-derived sequences was significantly correlated with the spatial distance between the trapping sites, indicating mostly local evolutionary processes. Detection of closely related HEV sequences in common voles in multiple localities over a distance of 800 kilometers suggested that common voles are infected by cvHEV across broad geographic distances. The common vole-associated HEV strain is clearly divergent from HEV sequences recently found in narrow-headed voles (*Microtus gregalis*) and other cricetid rodents.

Introduction

The family *Hepeviridae* comprises two genera, genus *Piscihepevirus*, with only one fish-associated virus [2], and genus *Orthohepevirus*, with four species. Species *Orthohepevirus A* comprises eight genotypes, of which HEV-1 to HEV-4 and HEV-7 have been found in humans. Genotypes HEV-1 and HEV-2 seem to be restricted to humans. The

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✉ Jan Felix Drexler
felix.drexler@charite.de

✉ Rainer G. Ulrich
rainer.ulrich@fli.de

[1] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Novel and Emerging Infectious Diseases, Südufer 10, 17493 Greifswald-Insel Riems, Germany

[2] University of Bern, Institute of Ecology and Evolution, Bern, Switzerland

[3] Swiss Institute of Bioinformatics, Quartier Sorge, Batiment Genopode, Lausanne, Switzerland

[4] Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Virology, Campus Charité Mitte, Charitéplatz 1, 10098 Berlin, Germany

[5] German Centre for Infection Research (DZIF), Associated Partner Site Berlin, Berlin, Germany

[6] German Centre for Infection Research (DZIF), Partner Site Hamburg-Lübeck-Borstel-Insel Riems, Greifswald-Insel Riems, Germany

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other genotypes occur in various animals, including pigs and wild boar (HEV-3 to HEV-6), rabbits (HEV-3), and camelids (HEV-7 and HEV-8) [44]. The species *Orthohepevirus B* and *Orthohepevirus D* include only non-zoonotic avian HEV and bat HEV strains, respectively. Members of the species *Orthohepevirus C* were first detected in Norway rats (*Rattus norvegicus*; genotype HEV-C1) and in different carnivore species (genotype HEV-C2) [20, 37].

Hepeviruses have a single-stranded RNA genome of positive polarity with a size of about 6.9–7.2 kilobases (kb), a short 5' untranslated region (UTR), three major open reading frames, and a 3'-UTR with a poly-A tail [23, 44]. Open reading frame (ORF) 1 is 4.6 to 5.2 kb in length and encodes a polyprotein including different non-structural proteins (Mt, methyltransferase; Y, Y-like domain; PCP, papain-like cysteine protease; X, X domain/ADP-ribose-binding module; Hel, helicase; RdRp, RNA-dependent RNA polymerase) [23]. ORF2 is about 2.0 kb in length and encodes the capsid protein [55]. ORF3 is approximately 342 nucleotides (nt) long and encodes a multifunctional protein that seems to function as an ion channel (viroporin) and is needed for the release of viral particles [9, 54]. In addition to these three ORFs, members of the species *Orthohepevirus C* share an additional putative fourth ORF [20].

Rodents represent the largest mammalian order, in terms of both the number of species and the number of individuals, and have various life history traits [50]. They are well-known carriers of a large number of zoonotic viral, bacterial and parasite agents [31], but although the number of pathogens identified in rodent species is steadily increasing, our current knowledge of rodent-borne agents is still very limited [34, 53].

Norway rat-associated HEV (rat HEV) was detected first in Germany [18, 19], but was found later in Norway rats and Black rats (*Rattus rattus*) from various other European countries, Asia and the USA [26, 27, 32, 33, 35, 36, 39, 51]. This virus has also been detected in Asian house shrews (*Suncus murinus*), which might have been due to spillover infections [13]. Similarly, rat HEV RNA was detected in a Syrian brown bear (*Ursus arctos syriacus*) in a zoo in Germany, probably due to a spillover infection from free-living rats in the same zoo [46]. Serological and experimental infection studies suggest that rat HEV could have zoonotic potential although this is controversial [7, 10, 20, 36, 42]. Recently, HEV-C1-related RNA was detected in an immunocompromised patient from Hong Kong, China, and a healthy, immunocompetent patient from Halifax, Canada, underlining the need for a critical assessment of the zoonotic potential of members of the genotype HEV-C1 [1, 47]. Additionally, HEV-3 strains of the species *Orthohepevirus A* were detected in rats, probably transmitted by spillover infection [22, 25, 39].

Recently, two novel rodent-associated hepeviruses were identified in a Chevrier's field mouse (*Apodemus chevrieri*) and a Père David's vole (*Eothenomys melanogaster*) from China, and proposed as genotypes HEV-C3 and HEV-C4 [49] within the species *Orthohepevirus C*. Additional novel, unassigned rodent-associated HEV-strains were detected in cricetid and murine rodents in Brazil and China, namely in a hairy-tailed bolo mouse (*Necromys lasiurus*), a delicate vesper mouse (*Calomys tener*), a striped field mouse (*Apodemus agrarius)*, a Chinese striped hamster (*Cricetulus barabensis)*, a grey-sided vole (*Myodes rufocanus)*, a narrow-headed vole (*Microtus gregalis)*, a grey dwarf hamster (*Cricetulus migratorius)*, and a kolan vole (*Eothenomys inez*) [8, 53]. Furthermore, a HEV-strain originating from a kestrel (*Falco tinnunculus*) showed higher sequence similarity to members of the species *Orthohepevirus C* than to the avian-associated HEV-strains [38]. Investigation of more than 330 rodents from five different species in Hungary resulted in the detection of HEV RNA exclusively in eleven common voles (*Microtus arvalis*). Phylogenetic reconstruction of the short screening fragment within the coding region of the RdRp of HEV revealed a close relationship to the previously described kestrel-derived HEV-strain. The complete genome sequences of these novel HEV strains were not determined [24].

Here, we describe the screening of nearly 3,000 rodents originating from Central Europe by broad-spectrum, nested reverse transcription polymerase chain reaction (RT-PCR) for the presence of HEV RNA. We determined and analyzed the complete genome sequences of novel hepevirus strains associated with common voles.

Materials and methods

Animal collection

A total of 2,961 rodents were used for this study (for detailed information see Table 1 and Figure 1). This includes 2,662 animals collected previously and screened for novel rodent hepaciviruses [12] and 299 animals trapped along a transect at the border of Germany and the Czech Republic [3, 41].

RNA extraction

Viral RNA was extracted from serum pools or individual liver samples. Viral RNA was extracted from about 30 mg of liver tissue or 10-50 µL of serum. RNA was purified using a MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, Penzberg, Germany) for tissue specimens and a DNA and Viral NA Small Volume Kit (Roche) for serum.

Molecular detection of HEV RNA

For the detection of HEV RNA, a nested RT-PCR targeting the RdRp-encoding domain of the ORF1 of HEV was used

Table 1 Results of RT-PCR screening of rodents collected in Germany and the Czech Republic

Country	Federal state/region	Trapping site	Number of RT-PCR positive/total number of animals tested						
			Microtus arvalis	*Microtus agrestis*	*Apodemus flavicollis*	*Apodemus agrarius*	*Apodemus sylvaticus*	*Myodes glareolus*	Subtotal
Germany	Berlin	Berlin	-	-	0/10	0/40	-	-	0/50
	Brandenburg	Muckrow (Muc)[b]	1/16	-	-	-	0/2	-	1/18
		four other sites	0/14	-	0/3	0/3	0/14	0/1	0/35
	Baden-Wuert-temberg	Weissach (Wei)[b]	1/24	0/4	0/34	-	0/2	0/150	1/214
		Ditzingen (Dit)[a]	-	-	0/20	-	-	1/28	1/48
		ten other sites	0/25	0/2	0/118	-	0/7	0/213	0/365
	Bavaria	Falkenstein (Fal)[b]	2/32	-	-	-	-	-	2/32
		eleven other sites	0/73	-	0/1	-	0/4	0/1	0/79
	Hesse	seven sites	0/15	0/13	0/17	-	0/27	0/88	0/160
	Mecklenburg-Western Pomerania	nine sites	0/20	0/18	0/76	0/20	-	0/73	0/207
	North Rhine-Westphalia	twelve sites	0/11	-	0/80	-	0/35	0/177	0/303
	Lower Saxony	twelve sites	0/3	-	0/192	-	0/55	0/187	0/437
	Thuringia	Creuzburg (Cre)[a]	1/14	0/1	0/1	0/4	0/16	-	1/36
		eight other sites	0/211	0/113	0/145	0/19	0/13	0/288	0/789
Subtotal	**9**	**79**	**5/458**	**0/151**	**0/697**	**0/86**	**0/175**	**1/1206**	**6/2,773**
Czech Republic	Plzen Region	Hayek (Hay)[b]	5/39	-	-	-	-	-	5/39
		Zalesi (Zal)[b]	3/17	-	-	-	-	-	3/17
		four other sites	0/132	-	-	-	-	-	0/132
Subtotal	**1**	**6**	**8/188**	-	-	-	-	-	**8/188**
Total	**10**	**85**	**13/646**	**0/151**	**0/697**	**0/86**	**0/175**	**1/1206**	**14/2,961**

[a] Trapping site with at least one hepatitis E virus (HEV)-RNA-positive animal (see Fig. 1, filled squares or circles); [b] Trapping sites where additionally at least one complete genome sequence of the novel common-vole-associated HEV was obtained (see Fig. 1, trapping sites indicated by asterisks)

as described before [11]. The PCR product has an expected size of 279 nt, without the primers. PCR products were separated by agarose gel electrophoresis and visualized by staining with Midori Green Advance (Biozym, Hessisch Oldendorf, Germany).

Complete genome sequence determination

For complete genome sequence determination of five representative HEV strains of the common vole-associated HEV, a primer-walking approach was used, including a 5′ and 3′ rapid amplification of cDNA ends (5′/3′ RACE System, Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out under the following conditions: 50 °C for 15 min, 94 °C for 2 min, 40 PCR cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 1-2 min, and a final extension at 72 °C for 10 min. The primers used for determination of the complete genome sequences are listed in Supplementary Table 1. RT-PCR products were sequenced directly using a BigDye Terminator 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

Sequence and phylogenetic analysis

Sequence alignments were produced using BioEdit [14], and phylogenetic analysis was done using MEGA 6 [48]. The amino acid (aa)-sequence-based phylogenies were reconstructed by a maximum-likelihood analysis with the Jones-Taylor-Thornton (JTT) substitution model with invariant sites, a gamma distribution shape parameter of five, and 1,000 bootstrap replicates. The phylogenetic analysis

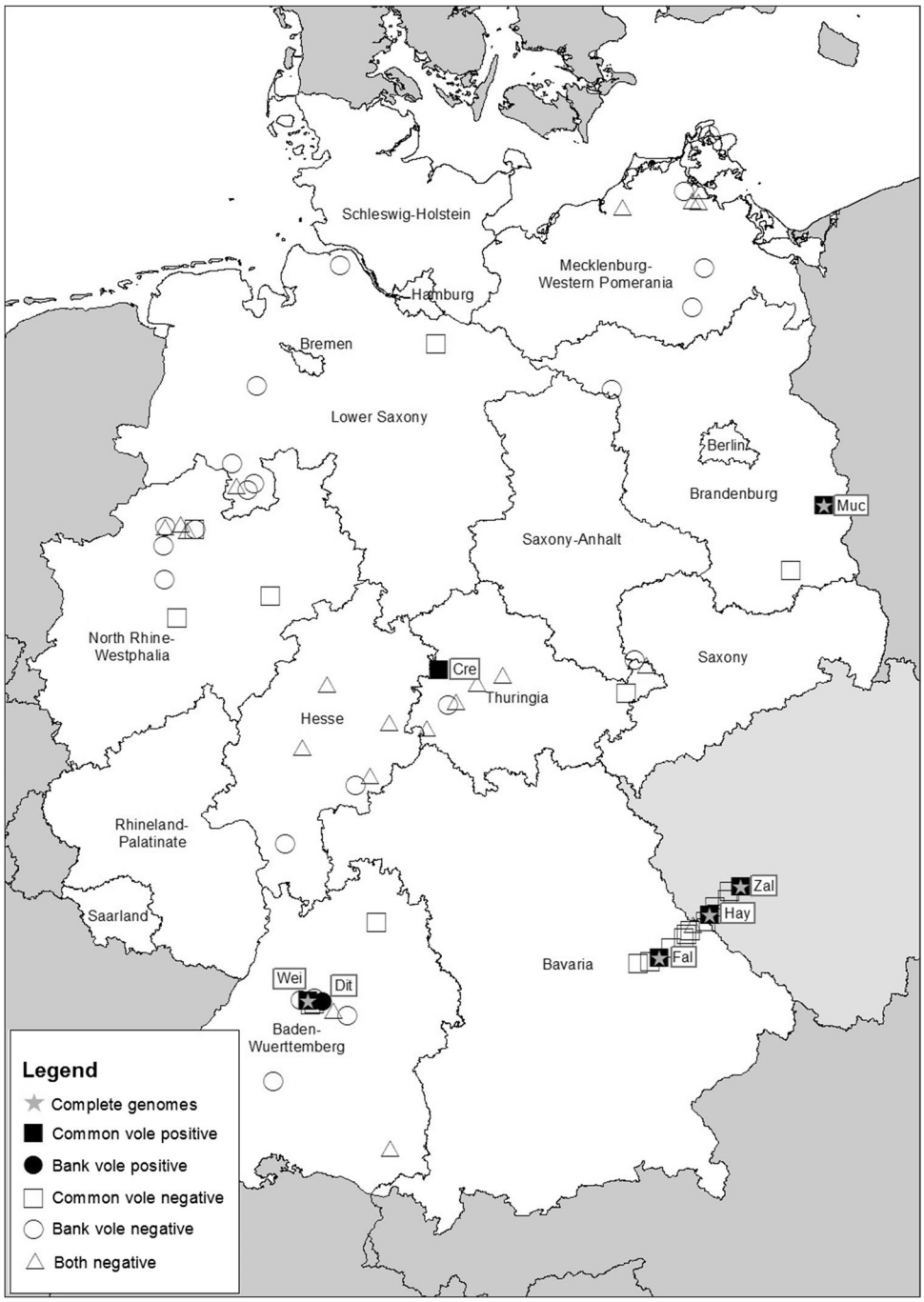


Fig. 1 Map of the trapping sites of common voles (*Microtus arvalis*) and bank voles (*Myodes glareolus*) in Germany and the Czech Republic. Trapping sites of hepatitis E virus (HEV)-RNA-positive common voles and bank voles are indicated by filled squares and circles, respectively. Asterisks indicate the origins of complete common vole-derived HEV sequences

used the proposed reference sequences of HEV [44, 45], sequences for the proposed genotypes HEV-8, HEV-C3, and HEV-C4 [49, 52], two unassigned sequences [8, 38], five rodent-associated HEV-strains from China [53] and recently described HEV sequences from common voles [24]. If available, complete genome sequences were used. Otherwise, a fragment of the RdRp-encoding region was used for phylogenetic analysis. Pairwise distances were calculated at the nt and aa level with MEGA 6 [48]. Tests for potential recombination events were done using Bootscan [29], implemented within RDP4 software [30], using the reference sequences listed above. For Simplot analysis with a window size of 100 nt and a step size of 25 nt, scripts were written in R [6]. The identification of conserved protein coding domains was done using CDD/SPARCLE, using the default settings [28]. For the prediction of RNA secondary structures within the intergenic region between ORF1 and ORF2, the program mfold [57] was used with default settings. We tested for largely local transmission of cvHEV resulting in "isolation-by-distance" patterns by computing

pairwise genetic and geographic distances between sampling locations [40]. Pairwise genetic distances were calculated using MEGA 6 [48] for all common vole-associated HEV sequences, including the kestrel-derived HEV sequence (see Fig. 2A, clades I-III), and geographic distances were calculated using the "dist"-function, implemented in R [6], by using the coordinates of the individual trapping sites. We tested for statistical significance between half matrices at the nt and aa level using Mantel tests, implemented in the package "ade4" of R. The 95% confidence interval (CI) was calculated in R by using the binom.test() function [5, 6].

Results

Screening of rodents by nested RT-PCR

RT-PCR screening of 432 serum pools with 2,961 individual samples (Table 1) resulted in the detection of 14 positive pools with a band of the expected size in agarose gel electrophoresis. Subsequent analysis of individual samples from the positive pools showed 14 animals, including 13 common voles and one bank vole (*Myodes glareolus*), to be RT-PCR positive. HEV-RNA-positive animals originated from seven trapping sites (Fig. 1). The detection rate of HEV RNA was 2% (13/646; 95% CI: 1-3.4) for all common voles tested and 0.08% (1/1206; 95% CI: 0.002-0.46) for all bank voles tested using broad-spectrum nested RT-PCR (Table 1). For single trapping sites, the detection rate in common voles reached 4.2% (1/24, site Wei; 95% CI: 0.1-21.1), 7.1% (1/14, site Cre; 95% CI: 0.18-33.86), 6.2% (2/32, site Fal; 95% CI: 0.76-20.8 and 1/16, site Muc; 95% CI: 0.1-30.2), 12.8% (5/39, site Hay; 95% CI: 4.2-27.4), 17.6% (3/17, site Zal; 95% CI: 3.7-43.4) and in bank voles 3.5% (1/28, site Dit; 95% CI: 0.01-18.34). For the locations of the trapping sites, see Fig. 1.

Phylogenetic analysis and spatial relationships

Phylogenetic analysis based on predicted aa sequences of a portion of ORF 1 (GenBank accession numbers MK192405 to MK192409 and MK192412 to MK192420) revealed a monophyletic group of three major clades, I–III, including the previously reported common vole-associated HEV sequences and a kestrel-derived HEV strain from Hungary (Fig. 2A, indicated by an arrow) within the species *Orthohepevirus C*. Clade I includes sequences from common voles trapped at different sites at the border between Germany (GER) and the Czech Republic (CZE; Fig. 1). The second clade, clade II, includes only sequences derived from four common voles trapped at Hayek (Hay), CZE. Clade III includes sequences from voles from four trapping sites in GER, including three sequences from common voles and one sequence from a bank vole, and several common vole derived HEV sequences and a kestrel-derived sequence from Hungary [24].

Pairwise sequence identity values for the sequences within clade I ranged from 91.7 to 100% at the nt level and were 100% at the aa level. The identity values for nt and aa sequences within clades II and III were similar (clade II: 99.6 to 100% and 100%, respectively; clade III: 86.6 to 99.6% and 98.9 to 100%, respectively). Comparing the sequences from clades I, II and III with the kestrel-derived sequence, the sequence identity ranged from 83.7 to 92.4% and 94.6 to 98.9% at the nt and aa level, respectively (Supplementary Table 2).

The nt and aa sequence divergence of all common vole-derived HEV sequences and the spatial distances of the corresponding trapping sites showed significant correlations ($r = 0.7$; $p < 0.00001$ and $r = 0.6$; $p < 0.00001$, respectively; see Fig. 3).

The phylogenetic tree revealed that the common vole (and kestrel-) associated HEV sequences clustered together with a narrow headed vole-derived sequence from China (HEV-RtMg/XJ2016, indicated by a dotted arrow in Fig. 2A). Members of the species *Orthohepevirus C* not associated with cricetid rodents were only found in a separate clade (rat-associated HEV-C1, carnivore-associated HEV-C2, and *Apodemus chevrieri*-associated putative genotype HEV-C3).

Generation of complete genome sequences from novel common vole-associated hepatitis E virus

Complete genome sequences of this novel common vole-associated HEV (cvHEV) were obtained by a primer-walking approach for five common vole-derived strains (GenBank accession numbers MK192405 to MK192409; Fig. 1). The genomes of these novel strains all have the same organization but with slight variation in length, ranging from 7020 to 7077 nt (Fig. 4A and B). The 5' UTR has a length of 33 nt, and ORF1 has a length of 4914-4971 nt and encodes a polyprotein of 1638-1657 aa. ORF2 had a length of 1992 nt and encodes a protein of 664 aa residues. The 3′ UTR has a length of 55 nt, excluding the poly-A tail. The different lengths of ORF1 are caused by short in-frame insertions/deletions (indels) of different lengths within the Y-domain and PCP- and RdRp-encoding regions of ORF1 (Fig. 4C).

ORF3 has a length of 363 nt and encodes a protein of 121 aa, overlapping with ORF2 and starting within the non-coding region between ORF1 and ORF2 (Fig. 4A-C). It contains an alternative start codon (UUG encoding leucine) at the 5′ end, as it was described previously for the kestrel-derived HEV genome [16, 38]. In addition, all five novel genome sequences contain an in-frame indel within the ORF2/ORF3 overlapping region in comparison to the

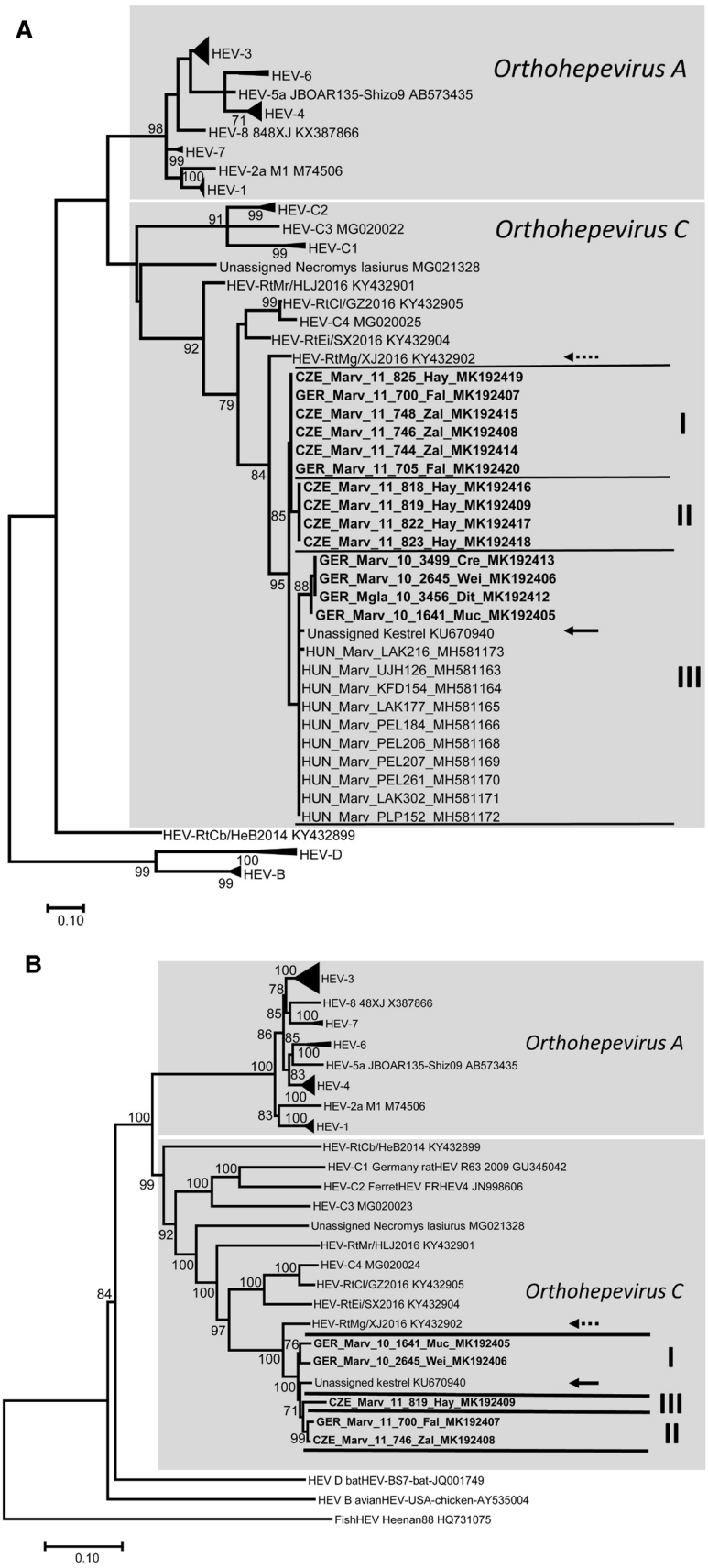


Fig. 2 Phylogenetic reconstruction of amino-acid (aa)-sequence-based trees for novel rodent-associated hepatitis E virus (HEV) sequences. The phylogenetic relationships of the novel common vole-associated HEV were calculated by maximum-likelihood analysis using the Jones-Taylor-Thornton (JTT) substitution model with invariant sites, a gamma-distributed shape parameter, and 1,000 bootstrap replicates. Support values below 70 are not shown. The phylogenetic analysis of HEV was based on proposed reference sequences [44, 45], sequences for the proposed genotypes HEV-8, HEV-C3 and HEV-C4 [49, 52], two unassigned sequences [8, 38], five rodent-associated HEV-strains from China [53], and a recently discovered HEV from common voles in Hungary [24]. Next to the individual identification code for each positive animal, the abbreviation of the species (Mgla, *Myodes glareolus*; Marv, *Microtus arvalis:* Cb, *Cricetulus barabensis*; Mr, *Myodes rufocanus*; Cl, *Eothenomys melanogaster*; Ei, *Eothenomys inez*; Mg, *Microtus gregalis*), the trapping site, and country are shown. An unassigned kestrel-derived HEV sequence is indicated by an arrow. (A) Relationships based on the protein encoded by the 92-aa screening fragment encoded within ORF1. (B) Relationships based on the concatenated ORF1/ORF2 encoded sequence, with a length of 2139-2384 aa

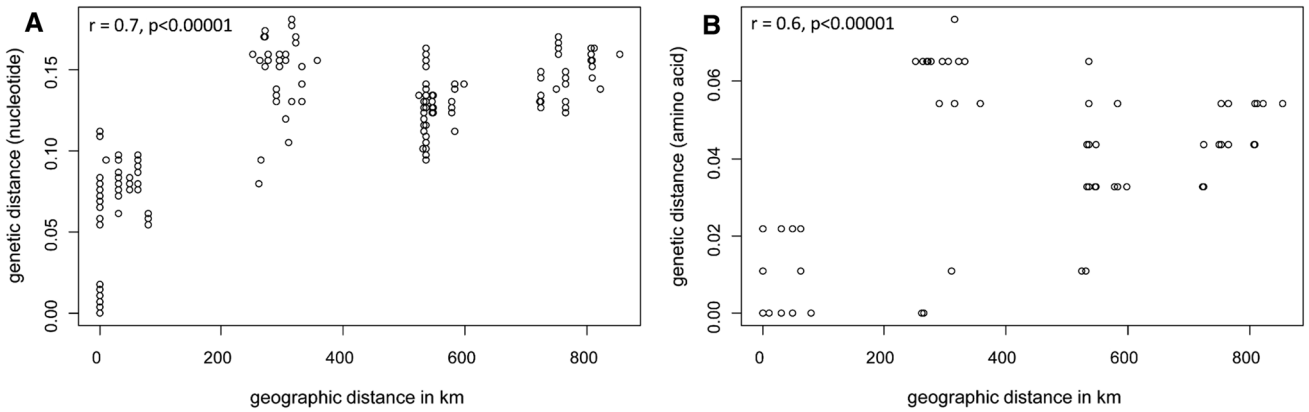


Fig. 3 Relationships between geographical distance and genetic divergence based on a portion of the RdRp coding region of rodent-associated hepatitis E virus (HEV) sequences at the nt level (A) and the corresponding amino acid sequence level (B). Sequences of all common vole-associated HEV strains (see Fig. 2A) were included. Pairwise geographic distances were calculated by using the coordinates (longitude and latitude) of each trapping site, the "dist" function implemented in R [6], and the genetic distance (p-distance), which was calculated using MEGA 6 [48]. Pairwise distance plots were generated using the "plot" function implemented in R [6]

kestrel-derived sequence (Fig. 4B and C). In contrast to other members of the species *Orthohepevirus C*, an additional putative ORF4 overlapping with the ORF1 was not found at the 5' end (Fig. 4A) [20].

An RNA secondary structure prediction revealed a similar stem-loop structure in all five novel common vole-derived genomes and the kestrel-derived sequence at the same position within the junction region between ORF1 and ORF2 (Fig. 4A and Supplementary Fig. 1) as described for members of the species *Orthohepevirus A* [4].

Comparison of the complete common vole-associated HEV genome sequence with those of other members of the family *Hepeviridae*

Phylogenetic analysis and pairwise sequence comparison of the concatenated ORF1 and ORF2 nt and aa sequences showed almost the same patterns as the analysis of the screening fragment (Fig. 2B and Supplementary Tables 2 and 3). The cvHEV sequences again had the highest similarity to the narrow headed vole-derived sequence from China (HEV-RtMg/XJ2016). Together with other cricetid-rodent-associated HEV strains, they formed a sister clade to murid-rodent- and carnivore-associated HEV strains. The Chinese striped hamster-associated sequence formed a separate clade (Fig. 2B).

In a comparison to the kestrel-derived HEV sequence, the sequence identity values ranged from 83.7 to 86.2% and 94.6 to 96.7% for the clades I, II and III at the nt and aa level, respectively. The similarity of the concatenated ORF1 and ORF2 sequence to other members of the species *Orthohepevirus C* ranged from 59.6 to 67.8% and 61.1 to 76.6% at the nt and aa level, respectively (Supplementary Table 3).

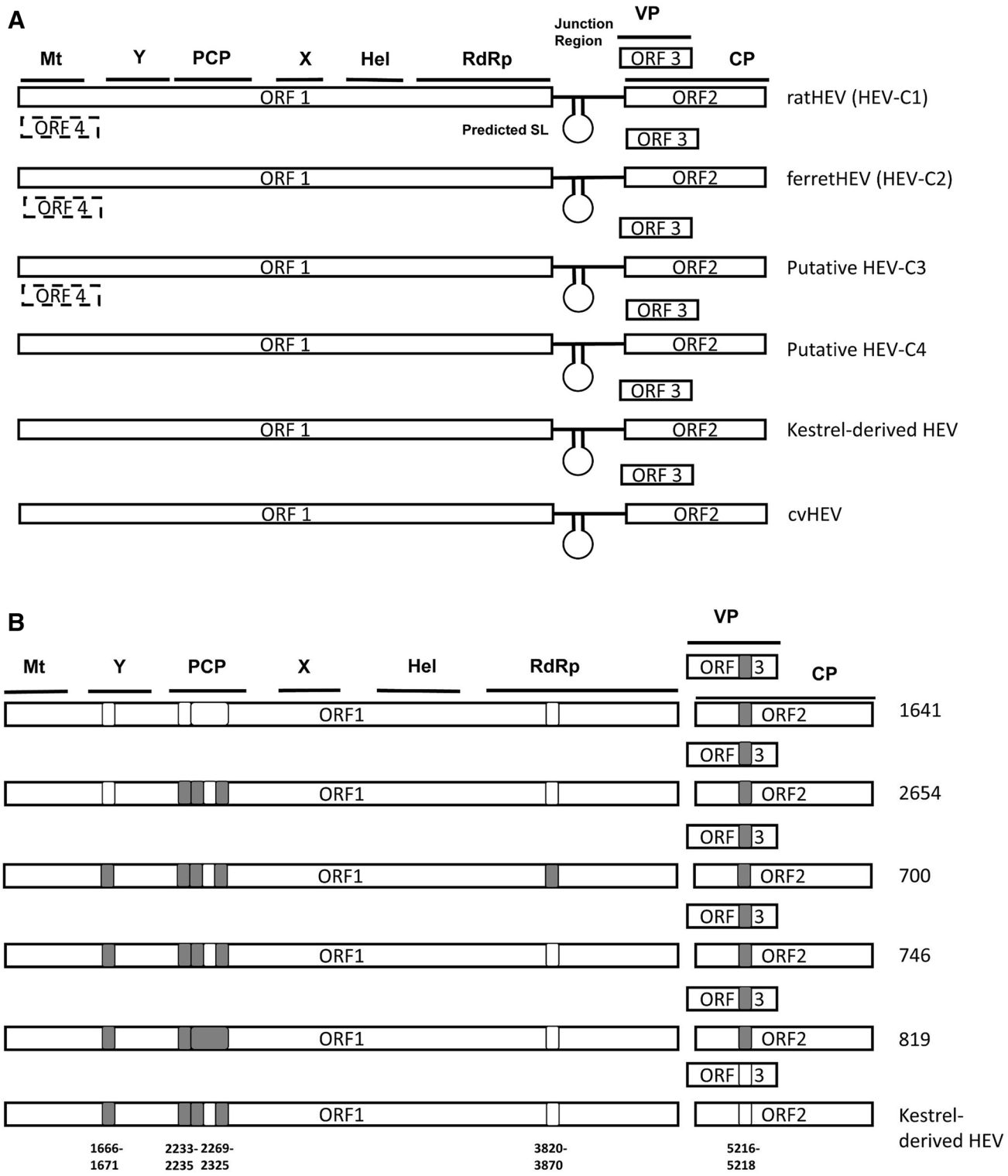
Simplot analysis of the novel cvHEV revealed no obvious differences compared to other members of the species *Orthohepevirus C*. Additionally, no recombination events were detected by Bootscan analysis (data not shown).

Five conserved protein coding domains were identified in all five complete genome sequences of the novel cvHEV, the reference sequence originating from a kestrel (GenBank accession number KU670940), and the narrow-headed vole-derived sequence (GenBank accession number KY432902): viral methyltransferase, Appr-1"-p processing enzyme, RNA helicase, RNA-dependent RNA polymerase, structural protein 2 (Supplementary Table 4).

Discussion

By screening nearly 3,000 rodents from Central Europe, we identified a novel HEV strain in 14 samples and determined the complete genome sequences of five isolates of this strain from common voles collected at different sites. The multiple detection of this strain in common voles from different trapping sites (this study and [24]) and its complete absence in other rodent species from the same region suggests that this novel virus is specific for common voles. The detection of cvHEV in one bank vole might be explained by a spillover infection. These findings again underline the necessity of multiple detections of a pathogen in a single species from different geographical regions in order to make a reliable conclusion about a reservoir-virus association, as discussed previously for hantaviruses [15].

The average infection rate observed here (2%; 95% CI: 1-3.4) was low; however, when calculating the detection rate for single trapping sites it ranged between 4.2% (95% CI: 1-21.1) and 17.6% (95% CI: 3.7-43.4). A previous study in


A
Junction Region
VP
Mt
Y
PCP
X
Hel
RdRp
ORF 3
CP
ORF 1
ORF2
ratHEV (HEV-C1)
ORF 4
Predicted SL
ORF 3
ferretHEV (HEV-C2)
Putative HEV-C3
Putative HEV-C4
Kestrel-derived HEV
cvHEV
B
VP
Mt
Y
PCP
X
Hel
RdRp
ORF 3
CP
ORF1
ORF2
1641
2654
700
746
819
Kestrel-derived HEV
1666-1671
2233-2235
2269-2325
3820-3870
5216-5218

◀**Fig. 4** Comparison of the genomic organization of members of the species *Orthohepevirus C* (genotypes HEV-C1 and HEV-C2), including the putative genotypes HEV-C3/HEV-C4, the kestrel-derived HEV and the novel common vole-associated HEV. (A) A schematic representation showing the length and position of the open reading frames (ORFs) 1, 2 and 3, including the putative ORF4 (dotted lines). The functional domains encoded by ORF1 (Mt, methyltransferase; Y, Y-like domain; PCP, papain-like cysteine protease; X, X domain/ADP-ribose-binding module; Hel, helicase; RdRp, RNA-dependent RNA polymerase [23]), the ORF2-encoded capsid protein (CP), and the ORF3-encoded viroporin (VP) are located at the corresponding positions, and the junction region with a stem-loop (SL) secondary structure between the ORF1 and the overlapping ORF2/ORF3 of rat HEV (HEV-C1, GU345042), ferret HEV (HEV-C2, JN998606), putative HEV-C3 (MG020023), putative HEV-C4 (MG020024), kestrel-derived HEV (KU670940), and common-vole associated HEV (cvHEV, strain MK192496) are shown. (B) Insertions or deletions (indels) of the five cvHEV strains 1641, 2645, 700, 746 and 819 (GenBank accession numbers MK192405-MK192409) compared to the kestrel-derived HEV-strain (KU670940) are indicated by empty or filled squares. Indels are located at the nucleotide positions 1666-1671, 2233-2235, 2269-2325, 3820-3870 and 5216-5218. Nucleotide sequences of indels are highlighted by a grey colored box. (C) Nucleotide sequences of the indels within ORF1 and ORF2/ORF3

Hungary indicated a detection rate of 10.2% at the single site investigated [24]. Future investigations would have to apply a cvHEV-specific RT-PCR assay for potentially improved sensitivity.

The detection of this virus in common voles from Germany, the Czech Republic (this study) and Hungary [24] suggests a broad geographical distribution and individual strains of the novel cvHEV in Europe. This conclusion was confirmed by an "isolation-by-distance" pattern that showed a strong, positive correlation between the geographic and genetic distances of the novel cvHEV strains. This suggests that the spread and evolution of cvHEV occur mostly at a local or regional spatial scale, probably tightly associated with common voles [40]. Further investigations are needed to evaluate if the presence of various cvHEV strains can be explained by their association with different lineages of common voles in Europe [41].

The high sequence similarity and phylogenetic relationship of the cvHEV sequences, and in particular those from Hungary, may indicate a dietary origin of the kestrel-derived HEV strain [38]. Supporting this assumption, the kestrel-derived HEV strain and the cvHEV strains share attributes that are unique among the members of the family *Hepeviridae*: an alternative noncanonical start codon for ORF3 and the absence of a putative ORF4, previously found in members of genotypes HEV-C1 and HEV-C2 and the putative genotype HEV-C3 ([16], Fig. 4A).

The cvHEV strains show a high degree of sequence similarity to other members of the species *Orthohepevirus C* and form a large cluster with HEV strains from different cricetid rodents, including the putative HEV-C4 genotype [49]. A coevolution scenario might explain the phylogenetic relationships of the rodent-associated hepeviruses, at least in part. The common vole-associated HEV is closely related to the narrow-headed vole-associated HEV strain from China, and both reservoir species belong to the genus *Microtus*. The next most closely related HEV strains were identified

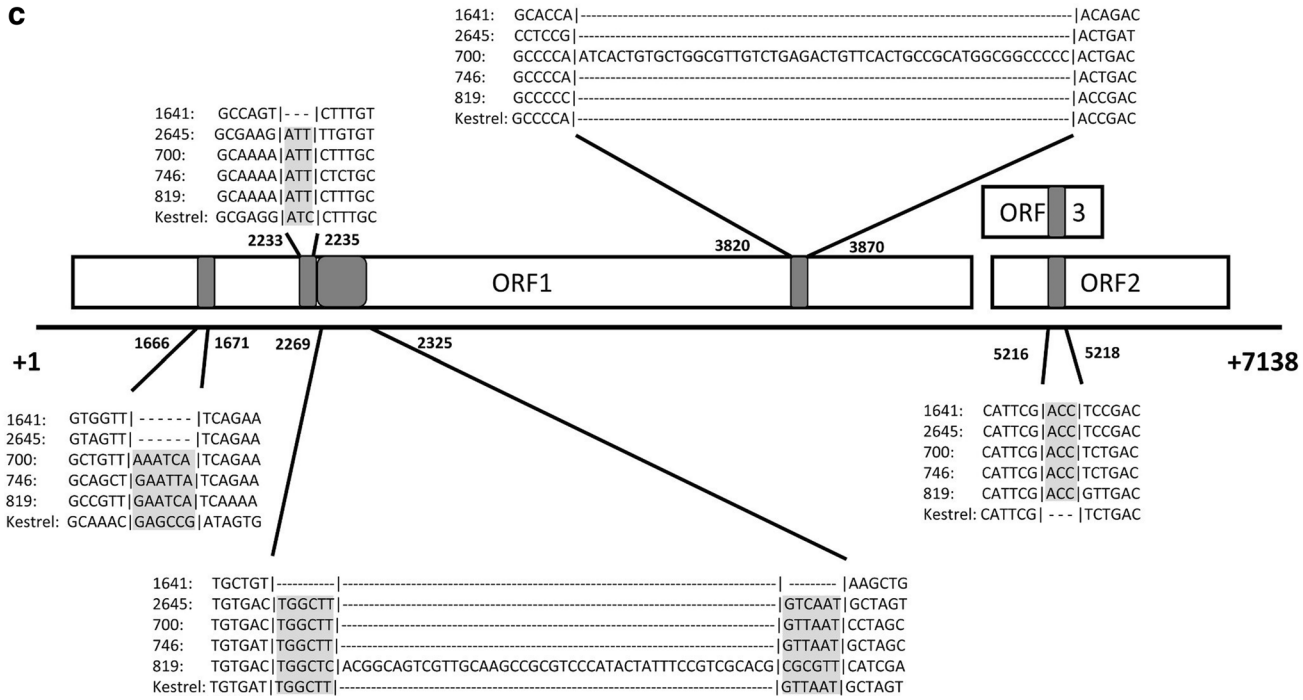


Fig. 4 (continued)

in other arvicoline rodents, followed by a strain from a sigmodontine rodent from South America. Members of the previously proposed genotype HEV-C3 are most closely related to rat HEV (genotype HEV-C1) and have also been discovered in the murine species *Apodemus chevrieri*, which belongs to the same subfamily as rats, which are reservoirs of rat HEV. The most divergent HEV strain within the species *Orthohepevirus C* was found in Chinese striped hamster (HEV-RtCb/HeB2014 KY432899).

Interestingly, multiple insertions/deletions were identified in the genomes of common vole-associated HEV strains; all indels were in-frame, and none of them interrupted the ORF1 coding sequence. The positions of these indels within ORF1 of common vole-associated HEV differed from those of indels previously detected in HEV-3 strains from chronically infected patients or in rabbit HEV strains [17, 21, 43, 56]. The occurrence of these indels suggests genomic plasticity in the ORF1 region, and this might be used in the future as a marker for molecular epidemiological studies.

In conclusion, this study confirms that members of the species *Orthohepevirus C* are associated with rodents and refutes an evolutionary origin of these viruses in avian hosts. The overrepresentation of cricetid-rodent-associated hepeviruses needs to be confirmed by large-scale studies of other rodent families.

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animal experiments performed by any of the authors.

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