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Bats Worldwide Carry Hepatitis E Virus-Related Viruses That Form a Putative Novel Genus within the Family *Hepeviridae*

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Hepatitis E virus (HEV) is one of the most common causes of acute hepatitis in tropical and temperate climates. Tropical genotypes 1 and 2 are associated with food-borne and waterborne transmission. Zoonotic reservoirs (mainly pigs, wild boar, and deer) are considered for genotypes 3 and 4, which exist in temperate climates. In view of the association of several zoonotic viruses with bats, we analyzed 3,869 bat specimens from 85 different species and from five continents for hepevirus RNA. HEVs were detected in African, Central American, and European bats, forming a novel phylogenetic clade in the family *Hepeviridae*. Bat hepeviruses were highly diversified and comparable to human HEV in sequence variation. No evidence for the transmission of bat hepeviruses to humans was found in over 90,000 human blood donations and individual patient sera. Full-genome analysis of one representative virus confirmed formal classification within the family *Hepeviridae*. Sequence- and distance-based taxonomic evaluations suggested that bat hepeviruses constitute a distinct genus within the family *Hepeviridae* and that at least three other genera comprising human, rodent, and avian hepeviruses can be designated. This may imply that hepeviruses invaded mammalian hosts nonrecently and underwent speciation according to their host restrictions. Human HEV-related viruses in farmed and peridomestic animals might represent secondary acquisitions of human viruses, rather than animal precursors causally involved in the evolution of human HEV.

A third of the world's human population may have been infected with hepatitis E virus (HEV), the prototype of the family *Hepeviridae* (59). HEVs are small, nonenveloped viruses with an approximately 7,200-nucleotide (nt) positive-sense, single-stranded RNA genome. Human HEV is classified into four genotypes (41). Following food-borne and waterborne fecal-oral infection, clinical symptoms range from asymptomatic to severe hepatitis (57). Hepatitis B virus and HEV constitute the most common causes of acute hepatitis in developing countries (57). In industrialized countries, hepatitis E has long been considered rare, but there is now a growing body of evidence suggesting that particularly HEV genotypes 3 and 4 constitute major etiologies of acute viral hepatitis (13). Moreover, HEV was found to be present in blood donors at rates between 0.07% in Chinese blood donors and 19.5% in Japanese donors with elevated liver enzyme levels (27, 63) as well as in plasma fractionation pools at rates of up to 10% (5). HEV infection can become chronic in immunocompromised patients and has been associated with high rates of mortality in pregnant women (6, 14, 36). Along with a growing awareness of the clinical relevance of HEV, candidate vaccines have been developed and tested in phase II and III clinical trials (64, 72, 75).

HEV differs from all other human hepatitis viruses in that it has been linked to animal reservoirs, beginning with its isolation from swine in 1997 (43). In particular, HEV genotypes 3 and 4 are associated foremost with food-borne zoonotic transmission from deer, domestic pigs, and wild boar (55, 58, 68). Antibodies against human HEV have been detected in several other animal species, including cattle, sheep, and horses (55). Zoonotic infection seems to contribute to a high seroprevalence in swine handlers and veterinarians (7, 22, 42, 44). Critically, those viruses detected in livestock animals such as swine, deer, farmed rabbits, and mongooses all cluster closely with human viruses (12, 23, 50, 73). Beyond this monophyletic clade, more divergent animal hepeviruses have been described recently. Rat sera were long known to cross-react with human HEV antigens serologically (16, 55), and a distinct hepevirus lineage associated with mild hepatitis in infected animals was detected in wild Norway rats (31, 38, 60). Avian hepeviruses have been identified in farmed chickens but in no other birds so far (28). These viruses are globally widespread in poultry farms, causing a range of symptoms from asymptomatic infection to severe hepatitis and splenomegaly (40, 74). The genetically most distant animal hepevirus was already isolated from apparently

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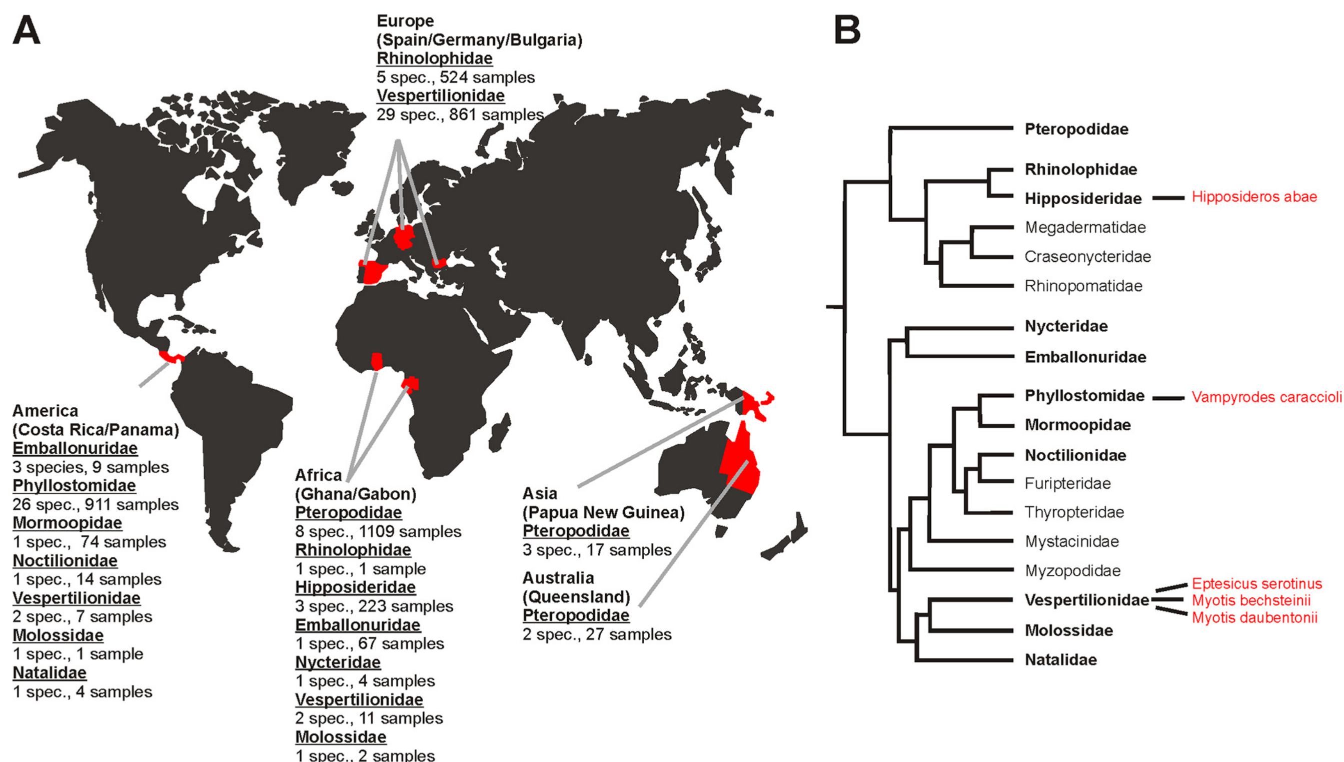

FIG 1 Sampling sites and covered bat evolutionary lineages. (A) Sampling sites and numbers of sampled species and specimens per family. (B) Bat evolutionary lineages according to data reported previously (67). Bat families for which samples were tested in this study are shown in boldface type. The names of bat species which tested positive for hepeviruses are shown in red type next to their family designations.

healthy trout in 1988 but was identified as a member of the family *Hepeviridae* only in 2011 (3).

Because of the zoonotic nature of HEV infection, it is highly relevant to learn more about its genetic diversity and association with mammalian hosts. Current data suggest the existence of specific virus clades in specific mammalian hosts, but the restriction of previous studies to farmed and peridomestic animals provided little opportunity to investigate the origin of human viruses. The phylogenetic placement of viruses of several unrelated animal taxa with human viruses suggests an acquisition from humans, but data are insufficient to reject alternative hypotheses. Critically, the very recent detection of rodent viruses in a sister relationship with human HEV suggests the existence of a wider, undiscovered diversity of *Hepeviridae* in mammals. In several studies by us and other groups, bats have proven to be highly efficient indicators of mammalian virus diversity, which may be due to their exceptionally large social group sizes, which promote the acquisition and maintenance of viruses (8, 15, 19, 20, 69, 70). Bats have been linked to a growing number of emerging viruses, including lyssaviruses, coronaviruses, henipaviruses, and filoviruses (8). For all of these viruses, bats carry larger viral diversities than other mammals, supporting the notion that bats might act as viral reservoirs. To examine if bats may also play a specific role in the ecology and evolution of mammalian *Hepeviridae*, we investigated a globally representative biological sample from 85 different bat species, including over 3,000 specimens. Our results suggest the existence of a genetic clade of viruses whose patristic distance resembles that encountered in rodents and humans (including the associated livestock viruses), altogether yielding genetic criteria to define putative genera within the family *Hepeviridae*. We conclude that three putative genera of mammalian hepeviruses may exist.

MATERIALS AND METHODS

Bat sampling and specimen preparation. Bat fecal and blood specimens were collected in Germany, Bulgaria, Spain, Ghana, Gabon, Papua New Guinea, Australia, Costa Rica, and Panama throughout 2002 to 2011 (Fig. 1) (sampling permits are provided in Acknowledgments). Bats were caught by using mist nets and kept in individual cloth bags until examination by trained ecologists. Fecal samples were collected directly from individual bags or from plastic sheets placed onto the ground below bat roost sites and stored after suspension in RNAlater solution (Qiagen, Hilden, Germany) at 8°C until further processing. Blood was taken by puncturing the wing or tail veins. Bats were released unharmed at the capture site the same night. Bat organs were available from 35 animals found dead in Germany and delivered to centers for bat protection and from 37 *Eidolon helvum* bats from a study site in Ghana (18).

Viral RNA was purified from bat fecal and blood specimens by using the viral RNA minikit and from solid-organ specimens by using the RNeasy minikit (both from Qiagen).

Human specimens. Sera were collected in 1998 from 453 otherwise healthy HIV-infected patients in Cameroon for studies of HIV and hepatitis C virus (51). The anonymized samples were extracted in pools of 10 to 40 by using the viral RNA minikit (Qiagen). Anonymized blood donor plasma samples collected from 2009 to 2010 in Germany were pooled in up to 96-member pools as described previously (61). Briefly, 96-member pools containing 100 μl of individual plasma donations were concentrated by ultracentrifugation, followed by RNA purification and elution in 65 μl. Due to the scarcity of available material, 20 RNA samples from these pools were merged before testing. The total number of analyzed individual donations was 93,146.

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TABLE 1 Oligonucleotides used for RT-PCR screening, complete genome sequencing, and virus quantification

Virus targeted	Oligonucleotide[a]	Sequence (5′–3′)[b]	Polarity	Use
Hepeviridae	HEV-F4228	ACYTTYTGTGCYYTITTTGGTCCITGGTT	+	Heminested screening RT-PCR
	HEV-R4598	GCCATGTTCCAGAYGGTGTTCCA	−	
	HEV-R4565	CCGGGTTCRCCIGAGTGTTTCTTCCA	−	
	HEV-BS7like-R4602	ACGACCATRTTCCAIACIGT	−	5′ completion of 1st-round screening amplicons
	HEV-F14a	GTGGTCGATGCCATGGAGGCCCATCAGT	+	Heminested RT-PCR assay 1 for amplification of small sequence fragments along *Hepeviridae* genomes to permit full-genome sequencing by lineage-specific bridging primers
	HEV-F14b	GCCAGGGTAAGAATGGACGTCTCGCAGT	+	
	HEV-F14c	GCAACCCCCGATGGAGACCCATCAGT	+	
	HEV-F79a	GGCWGCTYTGGCWGCGGC	+	
	HEV-F79b	GGCTACTGCGGCGGCGGC	+	
	HEV-F102	CYGYCTTGGCGAATGCTGTGGTGGT	+	
	HEV-R106	GGCGGTGTTCGCTGCAGCTAGAGYWGC	−	
	HEV-R390a	GGGGCAGAATACCAGCGCTG	−	
	HEV-R390b	GGGGCTGAGTACCAGCGCTG	−	
	HEV-R390c	GGGGCGGTGTACCACCGCTG	−	
	HEV-R390d	GGGCAATCTCGCCAGCGCTG	−	
	HEV-F400	GGIMGIGAYGTICAGCGITGG	+	Heminested RT-PCR assay 2 for full-genome sequencing
	HEV-F795	GGGCIRTIGGITGYCAYTTYGT	+	
	HEV-F848	CCIATGCCITAYGTICCITACCC	+	
	HEV-R1045	GTCAKIAGICKIGARCARCARARIGC	−	
	HEV-R1065	ATVCCICGIAGRTAIGTCATDAG	−	
	HEV-R1075	GTIACYTTGTAYSWRATICCICGIAGRTAIGTCA	−	
	HEV-F5760	CTGACGTTTTCGACCTGTCGT	+	Heminested RT-PCR 3 for full-genome sequencing
	HEV-F5770	GCGTCTGTCGGTGGGTTTTC	+	
	HEV-F5790	GGCCACAGTCCAACAATGTTC	+	
	HEV-R5780	CTTATAGAAAACCCACCGACAGAC	−	
	HEV-R5810	ATGTTGGAACATTGTTGGACTGTG	−	
	HEV-R5960	GTYTCGACAGAGCGCCAICC	−	
	HEV-R6473	CCIAGGTCTATRTCGTGIGG	−	
	HEV-R6493a	TCCTGCTCRTGCTGRTTATCATARTCCTG	−	
	HEV-R6493b	TCCTGGAGRTGCTGRTTATCAAARTCCTG	−	
	HEV-F6376a	GTCTCGGCCAATGGCGAGCC	+	Heminested RT-PCR 4 for full-genome sequencing
	HEV-F6376b	GTGTCTGAGAACGGTGAGCC	+	
	HEV-F6493a	CAGGAYTATGATAAYCAGCAYGAGCAGGA	+	
	HEV-F6493b	CAGGAYTTTGATAAYCAGCAYCTCCAGGA	+	
	HEV-R6865	CRGTRGTRTTRTAATTRTARGGRTARCCRGC	−	
M. bechsteinii bat hepevirus	HEV-NM8AC-rtF	TGGGTGGTTTTATGGTGATCTCT	+	Virus-specific quantitative real-time RT-PCR assays
	HEV-NM8AC-rtP	FAM-AGGCCGACTTGCACGCGCA-BBQ1	+ (probe)	
	HEV-NM8AC-rtR	CGTCAGGCACAGCCATAGC	−	
M. daubenonii bat hepevirus	HEV-NMS09-B-R-rtF	GCCCTGGAAAAGCGTATTGTT	+	
	HEV-NMS09-B-R-rtP	FAM-TCAGCTTCCCCCTGGCTGGTTTTATG-BHQ1	+ (probe)	
	HEV-NMS09-B-R-rtR	TGAAGGTCAGCCTCAGTATAAAGRT	−	
H. abae bat hepevirus	HEV-G19E-rtF	CCTGGTTGGTTCTATGGTGATCT	+	
	HEV-G19E-rtP	FAM-AATCAGACCTGCATGCTCACACTATGGCT-BHQ1	+ (probe)	
	HEV-G19E-rtR	TCTCAAAAACCTTACAGCCATCAG	−	
E. serotinus bat hepevirus	HEV-BS7-rtF	GCTGGTTTTACGGCGACTTG	+	
	HEV-BS7-rtP	FAM-ATACCGAGGCTGATCTG-BHQ1	+ (probe)	
	HEV-BS7-rtR	AGGAACTGCCATTGCATGTG	−	
V. caraccioli bat hepevirus	HEV-Pan-rtF	CCGGGCGATAGAAAAGCAT	+	
	HEV-Pan-rtP	FAM-TTGTTGCACAGCTGCCACCTGGAT-BHQ1	+ (probe)	
	HEV-Pan-rtR	GGTGTAAGCGTGTATGTCAGACTCA	−	

[a] Named after the position in the reference sequence under GenBank accession number NC_001434.
[b] R is G/A, Y is C/T, S is G/C, W is A/T, M is A/C, K is G/T, H is A/C/T, B is C/G/T, N is A/T/C/G, and I is inosine. FAM, 6-carboxyfluorescein; BBQ/BHQ1, blackberry quencher/black hole quencher 1.

Screening for hepeviruses. Screening for novel hepeviruses was done by heminested reverse transcription (RT)-PCR using broadly reactive oligonucleotides targeting viral RNA-dependent RNA polymerase (RdRp). The assay was designed to amplify all members of the family *Hepeviridae* available in GenBank. The assay sensitivity was determined to be on the order of 10 copies per reaction by using a quantified *in vitro* transcript (HEV genotype 3). For the generation of transcript controls, the screening RT-PCR amplicons were TA cloned (Invitrogen, Karlsruhe, Germany), reamplified with vector-specific primers, and *in vitro* transcribed by using T7 RNA polymerase (Asuragen, Austin, TX). Further broadly reactive nested RT-PCR assays were designed for additional genomic regions to enable a full-genome characterization (Table 1). Additional primers used for full-genome characterization are available upon request.

First-round RT-PCRs were carried out by using a touchdown protocol with reverse transcription at 50°C for 20 min and subsequent PCR at 95°C for 3 min, 10 cycles with 95°C for 15 s, a 1°C touchdown decrease of the

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TABLE 2 Sample characteristics

Chiroptera family	Species	No. of samples			No. of RT-PCR-positive samples (%)	Virus concn[a] (specimen type)	Sampling site(s) (yr)
		Feces	Blood	Liver			
Pteropodidae	*Dobsonia praedatrix*		9				Papua New Guinea (2002)
	Eidolon helvum	438	100	37			Ghana (2008/2009/2010)
	Epomophorus sp.	3					Ghana (2009)
	Epomops franqueti		100				Gabon (2009)
	Hypsignathus monstrosus		100				Gabon (2009)
	Melonycteris melanops		7				Papua New Guinea (2002)
	Micropteropus sp.	10					Ghana (2009)
	Micropteropus pusillus		100				Gabon (2009)
	Myonycteris torquata		100				Gabon (2009)
	Nanonycteris sp.	7					Ghana (2009)
	Pteropus alecto		3				Australia (2006)
	Pteropus poliocephalus		24				Australia (2006)
	Rousettus aegyptiacus	14	100				Gabon (2009); Ghana (2008)
	Rousettus amplexicaudatus		1				Papua New Guinea (2002)
Rhinolophidae	*Rhinolophus blasii*	82					Bulgaria (2008)
	Rhinolophus euryale	243					Bulgaria (2008)
	Rhinolophus ferrumequinum	40					Bulgaria (2008)
	Rhinolophus hipposideros	146					Bulgaria (2008), Spain (2010)
	Rhinolophus landeri	1					Ghana (2009)
	Rhinolphus mehelyi	13					Bulgaria (2008)
Hipposideridae	*Hipposideros abae*	57			2 (3.51)	8.50×10^8 (feces), 6.05×10^{10} (feces)	Ghana (2008/2009[b])
	Hipposideros cf. *caffer-ruber*	166					Ghana (2008/2009)
Nycteridae	*Nycteris* sp.	4					Ghana (2008/2009)
Noctilionidae	*Noctilio leporinus*	3	11				Panama (2010)
Emballonuridae	*Coleura afra*	67					Ghana (2008/2009)
	Peropteryx kappleri	5					Costa Rica (2010)
	Saccopteryx bilineata	1	1				Panama (2010/2011)
	Saccopteryx leptura		2				Panama (2011)
Phyllostomidae	*Anoura geoffroyi*	99					Costa Rica (2010)
	Artibeus jamaicensis	48	298				Panama (2010/2011)
	Artibeus lituratus	4	22				Panama (2010/2011)
	Artibeus phaeotis		3				Panama (2011)
	Artibeus watsoni		6				Panama (2011)
	Carollia castanea	11	18				Costa Rica (2010), Panama (2010/2011)
	Carollia perspicillata	207	13				Costa Rica (2010), Panama (2010/2011)
	Desmodus rotundus		1				Panama (2011)
	Enchisthenes hartii	3					Costa Rica (2010)
	Glossophaga commissarisi	3					Costa Rica (2010)
	Glossophaga soricina	28	11				Costa Rica (2010), Panama (2010/2011)
	Lampronycteris brachyotis		2				Panama (2011)
	Lophostoma silviculum	3	10				Panama (2010/2011)
	Micronycteris hirsuta	1					Panama (2010)
	Micronycteris microtis	4	7				Panama (2010)
	Micronycteris minuta		1				Panama (2011)
	Mimon crenulatum	1	2				Panama (2010/2011)
	Phylloderma stenops	2	5				Panama (2010/2011)
	Phyllostomus discolor		11				Panama (2011)
	Phyllostomus hastatus	4	11				Panama (2010/2011)
	Platyrrhinus helleri	1	1				Panama (2010)
	Tonatia saurophila	10	9				Panama (2010/2011)
	Trachops cirrhosus	4	8				Panama (2010/2011)
	Uroderma bilobatum	3	23				Panama (2010/2011)
	Vampyressa pusilla		3				Panama (2011)
	Vampyrodes caraccioli		10		1 (10.0)	1.75×10^5 (blood)	Panama (2011[b])
Mormoopidae	*Pteronotus parnellii*	36	38				Costa Rica (2010), Panama (2010/2011)
Vespertilionidae	*Barbastella barbastellus*	8					Bulgaria (2008)
	Eptesicus serotinus	2		3	1 (20.0)	5.38×10^9 (liver)	Germany (2008[b]/2009)
	Glauconycteris beatrix	1					Ghana (2008)

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TABLE 2 (Continued)

Chiroptera family	Species	No. of samples			No. of RT-PCR-positive samples (%)	Virus concn[a] (specimen type)	Sampling site(s) (yr)
		Feces	Blood	Liver			
	Myotis brandtii	18					Germany (2008)
	Myotis alcathoe	2					Bulgaria (2008)
	Myotis bechsteinii	69		1	1 (1.43)	2.51×10^8 (feces)	Bulgaria (2008), Germany (2008[b]/2009)
	Myotis capaccini	1					Bulgaria (2008)
	Myotis dasycneme	79		2			Germany (2006/2007/2008/2009)
	Myotis daubentonii	101		3	2 (1.92)	4.41×10^8 (feces), 3.73 × 109 (feces)	Bulgaria (2008), Germany (2007/2008[b]/2009/2010)
	Myotis emarginatus	5					Bulgaria (2008)
	Myotis myotis	243		2			Bulgaria (2008), Germany (2008/2009)
	Myotis mystacinus	56					Bulgaria (2008), Germany (2008)
	Myotis nattereri	70		2			Bulgaria (2008), Germany (2008/2009)
	Myotis nigricans	4	2				Panama (2010)
	Myotis oxygnathus	1					Bulgaria (2008)
	Myotis schreibersii	38					Bulgaria (2008)
	Nyctalus leisleri	7					Bulgaria (2008), Germany (2008)
	Nyctalus noctula	3		3			Germany (2007/2008/2009/2011)
	Pipistrellus cf. *nanus*	3					Ghana (2008/2009)
	Pipistrellus nathusii	17		5			Germany (2006/2007/2009)
	Pipistrellus pipistrellus	44		7			Germany (2006/2008/2009/2010)
	Pipistrellus pygmaeus	54		1			Bulgaria (2008), Germany (2007/2008/2009)
	Pipistrellus sp.	7		3			Ghana (2009), Germany (2009)
	Plecotus auritus	8		3			Bulgaria (2008), Germany (2006/2008/2010)
	Plecotus austriacus	3					Germany (2008)
	Rhogeessa tumida	1					Panama (2010)
Molossidae	*Molossus molossus*	1					Panama (2010)
	Tadarida major	1					Ghana (2008)
	Tadarida sp.	1					Ghana (2009)
Natalidae	*Natalus lanatus*	4					Costa Rica (2010)
Total	85 species	2,624	1,173	72	7 (0.18)		

[a] Concentration per gram of feces or tissue or per milliliter of serum.
[b] Positive sampling year per site.

annealing temperature down to 50°C, and extension at 68°C for 30 s, followed by another 40 cycles at an annealing temperature of 52°C. Second-round reactions used the same cycling protocol but without the RT step. Amplicons of the expected size (approximately 371 and 338 bp in the first and second rounds, respectively) were visualized on 2.0% agarose gels with ethidium bromide staining. First-round RT-PCR was performed by using the SuperScript III (SSIII) one-step RT-PCR kit (Invitrogen) with 5 μl of RNA, 400 nM (each) first-round primers, 1 μg bovine serum albumin, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 2.4 mM $MgSO_4$. Second-round 50-μl Platinum *Taq* reactions were carried out as recommended by the manufacturer (Invitrogen), using 1 μl of the first-round PCR product, 2.5 mM $MgCl_2$, and 400 nM (each) second-round primers. All PCR products were extended up to the size of the first-round fragment for phylogenetic analyses using heminested RT-PCR oligonucleotides and an additional bat hepevirus-specific reverse primer (Table 1).

Full-genome characterization. Following cDNA synthesis using the SuperScript III reverse transcription kit (Invitrogen), amplicons from generic PCR assays were bridged by long-range PCR using gene-specific primers and the Expand high-fidelity (Roche, Mannheim, Germany) and Phusion PCR (New England BioLabs, Frankfurt, Germany) kits. Additionally, Phi29-driven whole-transcriptome amplification (WTA) was done for the enrichment of viral sequences against the DNA/RNA background in specimens. Gene-specific reverse primers in the screening RT-PCR amplicon were designed with 5′-phosphate moieties, reverse transcribed with the SSIII kit (Invitrogen), digested with RNase H (Invitrogen), and finally ligated and amplified by using the Qiagen WTA kit (Qiagen). Genome ends were amplified by using the 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche).

Quantification of novel hepeviruses. Viral RNA quantification was done by using photometrically quantified *in vitro* RNA transcripts as described above and specific real-time RT-PCR assays (see Table 1 for oligonucleotides). Quantification was done by using 5 μl of RNA extract, 300 nM each primer, and 180 nM probe, using the SSIII one-step kit as described above. Cycling in a Roche LightCycler480 instrument involved the following steps: 55°C for 15 min, 95°C for 3 min, and 45 cycles of 95°C for 15 s and 58°C for 30 s with measurement of fluorescence.

Serological analysis. An indirect immunofluorescence assay was done by using human embryonic kidney 293T (HEK293T) cells transiently expressing the full-length ORF2-encoded capsid protein from a human HEV genotype 1 isolate (A. Osterman, unpublished data). Sera ($n = 49$) from eight different bat species were diluted 1:40, and detection was done by using a goat anti-bat immunoglobulin G polyclonal serum (Bethyl Laboratories, Montgomery, TX), followed by a cyanine 2-labeled donkey anti-goat serum (Dianova, Hamburg, Germany), as described previously (48). Human positive-control serum was used in dilutions of 1:40 and 1:80. Cyanine 2-labeled goat anti-human serum (Dianova) was applied as a secondary antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). All pictures were taken at the corresponding microscopic settings with a Motic Axiovision microscope (Zeiss).

Nucleotide sequence accession numbers. Nucleotide sequences from all novel bat hepeviruses described in this study are available in GenBank under accession numbers JQ001744 to JQ001749 and JQ071861, with JQ001749 representing a full bat hepevirus genome. Nucleotide sequences from human hepeviruses determined in this study are available under GenBank accesssion numbers JQ034512 to JQ034522.

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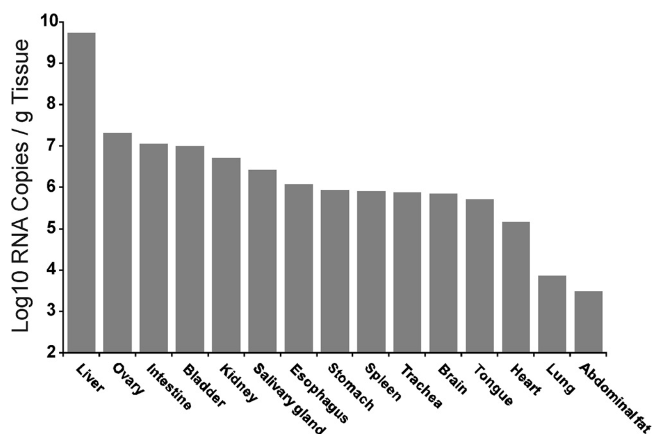

FIG 2 Solid-organ distribution of bat hepevirus BS7 in an *Eptesicus serotinus* bat. Virus concentrations assessed by strain-specific real-time RT-PCR using quantified *in vitro*-transcribed RNA controls are given for individual tissue specimens. Since this animal was brought dead to a bat shelter, no blood specimen could be taken due to coagulation.

RESULTS

Heminested RT-PCR was used to screen a total of 3,869 fecal, liver, and blood specimens from 85 different bat species from five continents (Table 2). Hepevirus RNA was found in seven (0.18%) specimens, including five fecal samples, one blood sample, and one liver specimen. RNA-positive samples stemmed from Europe, Central America, and Western Africa (Fig. 1A). Detected sequences originated from five bat species of three different families (Hipposideridae, Vespertilionidae, and Phyllostomidae), representing all three major stem lineages in the phylogenetic tree of bats (Fig. 1B). The detection rate in feces (5 of 2,624) was not significantly higher than those in blood (1 of 1,173) and liver (1 of 72) ($P = 0.40$ and 0.15, respectively, by two-tailed Fisher's exact test). Very high virus RNA concentrations were found in all fecal samples (median, 8.5×10^8 RNA copies per gram of feces; range, 2.5×10^8 to 6.1×10^{10} RNA copies per gram of feces). These concentrations were several orders of magnitude higher than that in the single positive blood specimen (1.8×10^5 RNA copies per ml). Hepevirus RNA was also detected in a liver sample at a high concentration comparable to those detected in feces (5.4×10^9 RNA copies per gram of tissue). Virus concentrations were analyzed in all solid organs of the animal whose liver tested positive. As shown in Fig. 2, RNA concentrations in liver tissue exceeded those in any other organ by at least 1,000-fold. The comparable virus concentrations in solid organs other than liver were likely the result of a high level of viremia. Unfortunately, no blood sample was available from this animal.

For phylogenetic analysis, a putative RNA-dependent RNA polymerase (RdRp) gene fragment corresponding to 108 amino acids (aa) was amplified from all seven positive specimens. Distance-based and probabilistic phylogenetic analyses provided an acceptable robustness of major nodes, placing all chiropteran viruses in a separate monophyletic clade within the family *Hepeviridae* (Fig. 3). Long phylogenetic branches linking the bat viruses to their common ancestor suggested the existence of a high level of diversity of related viruses. The clade of bat viruses was about as diversified as all human HEVs, including related HEVs found in nonhuman hosts such as rabbits, deer, and wild boar (Table 3; see also Table S1 in the supplemental material). The rodent-specific hepeviruses described more recently in wild Norway rats (*Rattus norvegicus*) (31, 32, 38) had a common ancestor with human HEV and related viruses but not with bat or avian HEV. The patristic distance within the clade of bat viruses exceeded that in the rat-associated clade more than 2-fold (21.1% versus 9.3% amino acid divergence) (Table 3). The bat viruses were also considerably more diversified than all known avian viruses.

Several viruses from livestock and peridomestic animals are placed phylogenetically within and between the four human HEV genotypes. To look for signs of human HEV-related viruses in bats, a subset of 49 bat sera representing major bat lineages, including 20 *Myotis dasycneme*, 5 *Hipposideros gigas*, 5 *Hipposideros* cf. *caffer-ruber* (*Hipposideros* sp. that looks like *Hipposideros caffer* or *H. ruber*), 4 *Rhinolophus alcyone*, 5 *Rousettus aegyptiacus*, 5 *Miniopterus inflatus*, 5 *Coleura afra*, and 5 *Vampyrodes caraccioli* sera, was analyzed. These sera were tested for antibodies to human HEV in an immunofluorescence assay using HEK293T cells transiently expressing the HEV genotype 1 capsid gene. No reactivity was observed for any sample (representative reactions are shown in Fig. 4).

To determine, on the contrary, if descendants of bat viruses are encountered in humans, pooled plasma samples from 93,146 blood donors were tested. As a control, all samples were first screened for human HEV. HEV genotype 3 viruses were detected in 11 pools. This was in agreement with previously determined prevalences of HEV RNA in blood products (2, 27, 63), confirming the suitability of the pooled samples for HEV detection in principle. Notably, the method of blood donor pooling involved the concentration of viral particles by high-speed centrifugation, allowing a nearly quantitative recovery of viruses from pools. The sensitivity limit of the assay for individual blood donations contained in the pools could therefore be projected to be approximately 4.4 $\log_{10}$ copies/ml. This was compatible with previously reported viral loads in individual plasma donations ranging between 3.2 and 5.7 $\log_{10}$ IU/ml (4). None of the blood donor pools yielded positive results in single and heminested RT-PCR assays capable of detecting all bat-associated HEVs, ruling out the existence of human-specific viruses related to the bat-specific clade in these samples. To investigate humans with potentially closer exposures to bats and a propensity for persistent infection (11, 14, 45), an additional test of 453 anonymized sera from Cameroonian HIV-positive patients was done. No evidence of bat or human hepevirus RNA was obtained, whereas the suitability of these samples for the detection of viral RNA was proven previously (51). This may indicate that the level of immunosuppression might not have been as severe in these patients as in other cohorts of HIV-infected individuals tested for HEV (11, 14).

To compare the genome properties of the novel bat viruses with those of other members of the family *Hepeviridae*, the full genome of one virus, termed BS7, from a German *Eptesicus serotinus* bat was sequenced. As shown in Fig. 5A, the bat virus represented an independent branch among the full-length hepevirus genomes. The complete genome comprised 6,767 nucleotides (nt), excluding the poly(A) tail at the 3′ end, constituting the shortest mammalian hepevirus genome, with a size comparable to that of the avian viruses (6,654 nt in the U.S. prototype strain [GenBank accession number AY535004]). The coding regions were flanked by a 33-nt 5′-untranslated region (UTR) and a 77-nt 3′-UTR. In the coding region, at least three open reading frames

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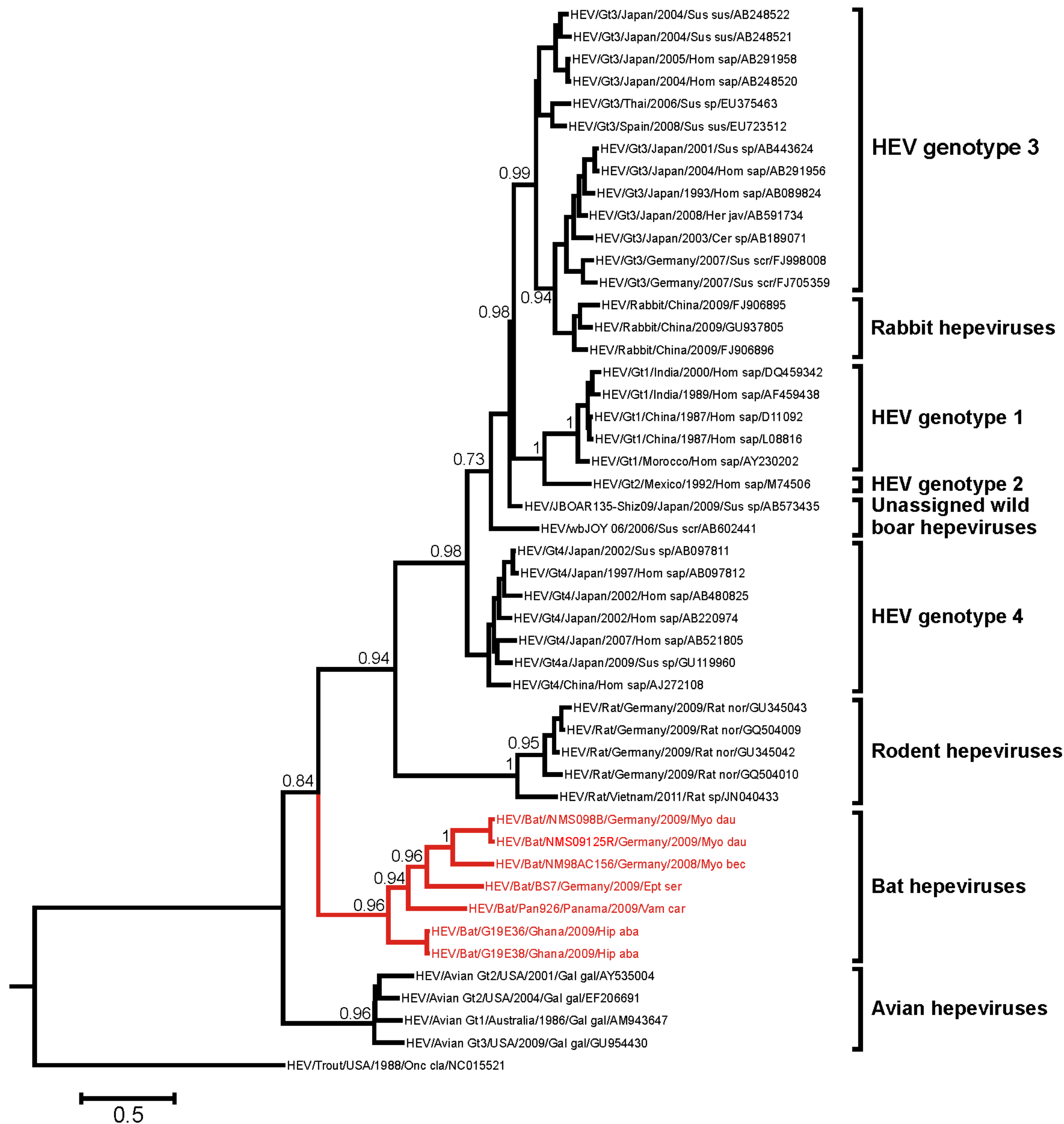

FIG 3 Partial RdRp gene phylogeny of the family *Hepeviridae*, including novel bat viruses. The Bayesian phylogeny was generated with MrBayes V3.1 (30), using a general time-reversible (GTR) model with a gamma distribution (G) across sites and a proportion of invariant sites (I) (GTR+G+I) as the substitution model; otherwise default settings were used, and 4,000,000 generations were sampled every 100 steps. In agreement with tree topologies from the full-length ORF1 genes (Fig. 5C), a monophyly prior was set on the root of all mammalian hepeviruses in order to stabilize the phylogenetic reconstruction over this shorter sequence fragment. After an exclusion of 15,000 of the total 40,000 trees, the final tree was annotated and visualized with TreeAnnotator and FigTree from the BEAST package. Values at the nodes indicate the fraction of times that each node was represented within the 95% highest posterior density interval of the trees. Values below 0.7 and those overlapping with taxon names are hidden for clarity of presentation. Branches leading to novel bat viruses and the corresponding taxon names are shown in red. The scale bar indicates genetic distance. The partial RNA-dependent RNA polymerase (RdRp) alignment comprised 324 nucleotides corresponding to positions 4,282 to 4,605 in an HEV genotype 1 prototype strain (GenBank accession number AF459438).

(ORFs) typical of all hepeviruses were identified (Table 4). An internal putative 660-nt ORF overlapping ORF1 in the −2 reading frame was identified at positions 326 to 985 and provisionally termed ORF NX (for N-terminal unknown). Whether ORF NX corresponded to the putative ORF4 or ORF5 described for rodent hepeviruses remains unclear, since the sequence identity with these ORFs was very low (33.3% and 14.4% nucleotide identities and 30.5% and 17.5% amino acid identities with ORF4 and ORF5, respectively), and no significant similarity of the putative gene product of the internal reading frame to any described protein domain could be detected by BLAST. Similar to bat hepevirus ORF NX, rat hepevirus ORF4 is located at the N terminus of ORF1, while ORF5 is located approximately 2,000 nucleotides downstream of ORF1 (31).

In the first ORF of BS7 (ORF1) (nt 34 to 4776), several domains could be predicted (Table 5). A putative methyltransferase domain was found to contain all four conserved amino acid residues identified previously in plus-stranded RNA viruses belonging to

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TABLE 3 Percent nucleotide (below the diagonal) and amino acid (above the diagonal) sequence identities between hepeviruses[a]

Hepevirus lineage (no. of strains compared)	% identity									
	Bat	Avian	Rodent	HEV genotype 1	HEV genotype 2	HEV genotype 3	HEV genotype 4	Rabbit	Unassigned boar	Trout
Bat (7)	78.9–100 **70.3–100**	67–72.5	58.3–63.9	57.4–62	57.4–62	58.3–63.9	63–64.8	58.3–62	59.3–68.5	44–46.8
Avian (4)	**60.6–65.4**	94.5–100 **78.3–89.3**	59.3–62	60.2–62	59.3–60.2	60.2–63	62–63.9	61.1–63	62.3–65.7	45.0
Rodent (5)	**56.5–60.8**	**53.7–61.4**	90.7–96.3 **77.2–95.4**	68.5–75.0	68.5–72.2	65.7–73.1	71.3–73.1	66.7–72.2	67–71.3	39.8–43.5
HEV genotype 1 (4)	**58–63.6**	**59.6–62.7**	**63.3–66.7**	96.3–99.1 **93.8–98.8**	87–88.9	80.6–86.1	77.8–83.3	83.3–87	82.4–85.8	41.7–43.5
HEV genotype 2 (1)	**54.6–60.5**	**57.7–61.7**	**62.0–63.9**	**76.5–77.5**	100 **100**	83.3–85.2	81.5	86.1–87	83–84.3	42.6
HEV genotype 3 (13)	**57.4–63.9**	**57.4–64.8**	**58.6–66**	**71.9–76.9**	**72.2–76.5**	91.7–100 **78.1–99.4**	82.4–86.1	90.7–95.4	83–88	45.4–48.1
HEV genotype 4 (6)	**56.8–64.2**	**59.6–65.1**	**61.4–65.7**	**70.1–74.4**	**69.4–72.5**	**70.7–78.4**	94.4–100 **82.7–97.8**	82.4–85.2	84–88.9	44.4–45.4
Rabbit (3)	**56.8–63**	**57.4–63.9**	**60.2–63.6**	**70.7–76.2**	**71.6–75.3**	**77.5–83.3**	**70.4–75.9**	96.3–97.2 **86.4–88.3**	81.1–85.2	43.5–44.4
Unassigned boar (2)	**55.9–63.9**	**60.8–64.8**	**59.6–64.2**	**72.7–76.2**	**73–74.4**	**71.1–78.1**	**74.5–78.7**	**71.3–72.2**	88.7 **76.5**	44.4–45.3
Trout (1)	**47.4–50.5**	**46.5–50.8**	**45.4–49.1**	**44.4–46**	**50.3**	**48.5–51.5**	**48.8–50.6**	**48.5–51.2**	**47.5–51.2**	100 **100**

[a] Evolutionary analyses were conducted with MEGA5 (66). GenBank accession numbers are as follows: JQ001744 to JQ001749 and JQ071861 for bat HEV; AM943647, EF206691, AY535004, and GU954430 for avian hepevirus; GU345042, GU345043, GQ504009, GQ504010, and JN040433 for rodent HEV; AF459438, DQ459342, D11092, and L08816 for HEV genotype 1; M74506 for HEV genotype 2; EU723512, EU375463, AB291958, AB248520, AB248521, FJ998008, FJ705359, AB089824, AB443624, AB291956, AB591734, AB189071, and AB301710 for HEV genotype 3; AB097812, AB097811, AB480825, AB220974, AB521805, and GU119960 (genotype 4a) for HEV genotype 4; FJ906895, FJ906896, and GU937805 for rabbit HEV; AB602441 and AB573435 for unassigned boar HEV; and NC_015521 for trout HEV. Boldface type indicates percent nucleotide identity, and lightface type indicates percent amino acid identity.

the alphavirus-like supergroup (62) at ORF1 amino acid positions 65 (H), 115 (D), 118 (R), and 229 (Y). The beginning of a putative Y domain, as typical for hepeviruses, was found at around aa 216 (VVTY). The level of conservation decreased downstream, and the end of the domain could not be identified. The genome region encoding the papain-like protease in HEV was highly divergent in hepeviruses from different hosts, including bats, and the amino acid residues around a putative catalytic cysteine (TCFL) defined previously by Koonin et al. (35) were found in neither the bat hepevirus nor the previously described rat, bird, or fish hepeviruses.

The putative proline-rich hinge region of the highly variable sequence had a maximum proline density of 12 proline residues within 68 amino acid positions (P/68 aa). The X domain of unknown function in human HEV (35) could not be unambiguously identified in BS7, since only the start and not the end of this putative region could be mapped in comparison to human HEV (Table 5). The putative helicase contained the nucleoside triphosphate (NTP)-binding site GVPGSGKS (aa 874 to 881) and DEAP motif (aa 927 to 930) described previously (65), with serine being replaced by alanine at aa 878. The putative RdRp contained the conserved tripeptide GDD. Out of the eight RdRp motifs described previously, two (motifs I [KDCNKFTT] and IV [NDFSE FDSTQNN]) were completely conserved in BS7. The other motifs could be identified but were less conserved (Table 5) (35).

The second ORF, coding for the putative 638-aa capsid protein, was found at nt 4777 to 6690 (Tables 4 and 5). The conserved sequence TGAATAACA within a *cis*-active element overlapping the ORF2 start codon in human and avian HEVs (26) was present in the bat virus genome at the homologous position. The first half of the polypeptide had a basic charge, with a predicted isoelectric point (pI) of 10.97, suggesting a potential involvement in the encapsidation process (54). In contrast to rodent hepeviruses (32), the ORF2 domains of BS7 seemed to be less conserved (Table 5) (71). Several hydrophobic residues occurred after the ORF2 start codon (FAYLLLLFL [ORF2 aa residues 16 to 24]), which characterize the N-terminal signal sequence in other mammalian hepeviruses. ORF2 was most conserved in the shell (S) domain, including tyrosine at position 288, which was described previously to be crucial for capsid formation in HEV genotype 3 (71). The middle (M) and protruding (P) domains were less conserved.

A putative ORF3 was detected in an alternative reading frame overlapping the capsid-encoding ORF2 (Table 4). In HEV, this protein encodes a phosphoprotein that is not essential for replica-

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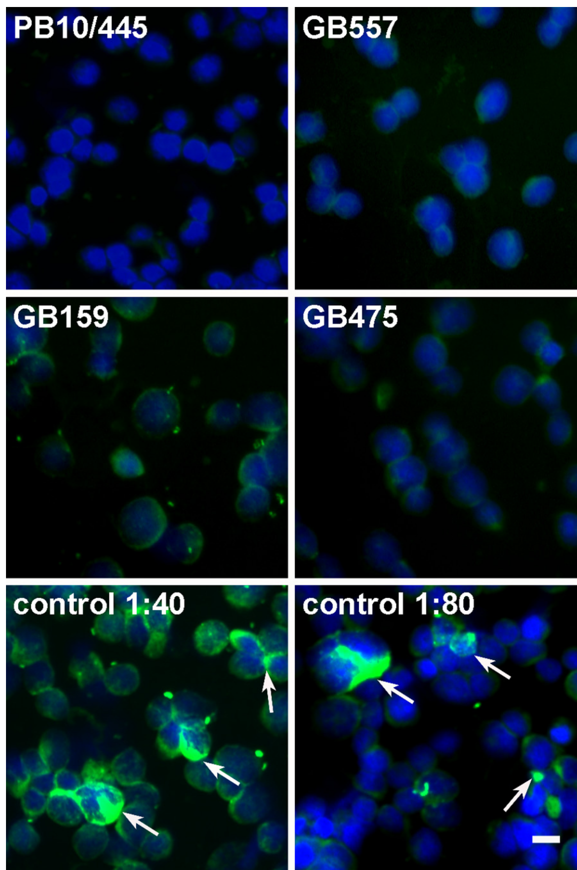

FIG 4 Serologic testing of bat sera for antibodies to human HEV with an HEV-specific indirect immunofluorescence assay. Slides carrying human embryonic kidney 293T cells transiently expressing the full-length ORF2 protein from a human HEV genotype 1 strain were incubated with bat sera (diluted 1:40) from eight different species. To allow the evaluation of the reaction specificity, the transfection efficiency was optimized to yield only 5 to 10% of cells expressing HEV antigen. One HEV RT-PCR-positive species (*Vampyrodes caraccioli* [PB10/445]) and three different RT-PCR-negative species (*Hipposideros gigas* [GB557], *Rousettus aegyptiacus* [GB159], and *Miniopterus inflatus* [GB475]) are shown. Notably, *R. aegyptiacus* specimen GB159 was chosen because we realized that it reacted nonspecifically with all cells, including those not expressing HEV antigen, and we wanted to demonstrate its clear discrimination from seropositive human sera. Detection was done by incubation with goat anti-bat immunoglobulin (Ig), followed by donkey anti-goat Ig labeled with cyanine 2. As a control, an anonymous human serum sample from a patient infected with HEV was applied in dilutions of 1:40 and 1:80 (to reduce the background signal). White arrows indicate specific serologic reactivity with HEV ORF2-expressing cells. The bar represents 25 μm. All pictures were taken with identical microscope and camera settings.

tion in hepatoma cells (21) and that might be involved in virion release, transcription, or interactions with cellular factors (34, 47, 49). The putative ORF3 product in the bat hepevirus showed little similarity to proteins encoded by ORF3 in other hepeviruses (Table 4) and did not yield significantly similar sequences in a BLAST search. It did not overlap the 3′ terminus of ORF1, as in HEV genotypes 1 to 3 (31), and its size was between that of trout hepevirus (678 nt) and that of the human or rodent hepeviruses (372 and 309 nt, respectively) (Table 5) (3, 31). The low level of similarity of ORF3 between viruses from different hosts such as bats, rodents, birds, and humans is in contrast to its marked conservation in viruses from identical hosts (Table 6) and may represent viral adaptation to particular hosts.

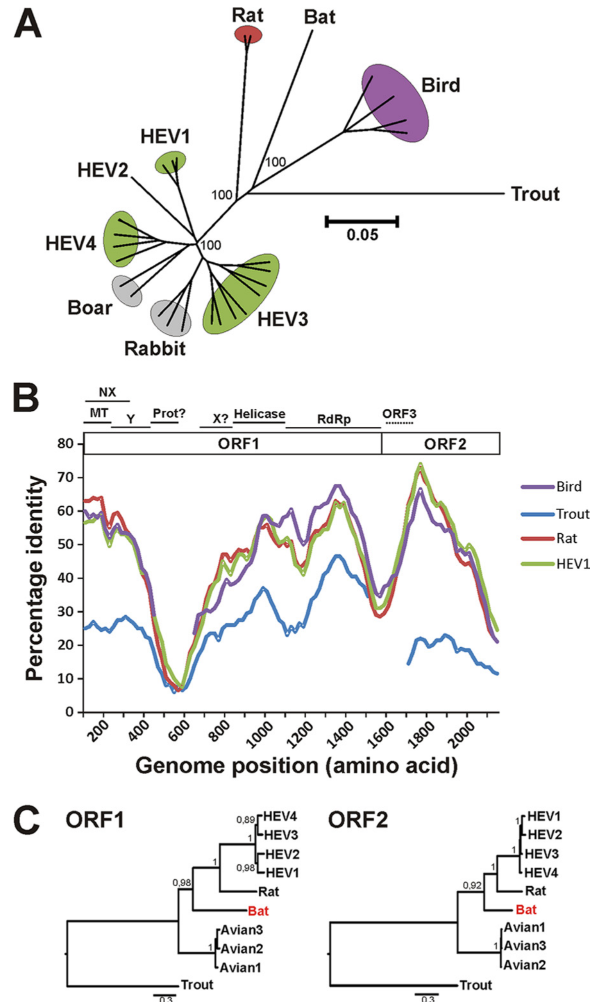

FIG 5 Complete genome nucleotide phylogeny, amino acid sequence identity, and ORF1/ORF2 amino acid phylogeny of bat hepevirus BS7 and prototype hepeviruses. (A) Neighbor-joining phylogeny of the complete genomes of members of the *Hepeviridae* using the nucleotide percentage distance substitution matrix and complete deletion option in MEGA5. Values at deep node points indicate support from 1,000 bootstrap reiterations; those at apical nodes are hidden for clarity of presentation. (B) Amino acid identity plot. The complete ORF1 and ORF2 were translated, concatenated, and compared to avian, rodent, human, and trout prototype hepeviruses. Positions containing gaps in the bat hepevirus were stripped from the alignment. The uncorrected amino acid identity was plotted with a sliding window size of 200 and a step size of 20 amino acids. For orientation, a schematic representation ORF1 and ORF2 is shown with putative nonstructural functional domains as approximated by BLAST comparisons with GenBank reference sequences depicted at the top (MT, methyltransferase; NX, putative ORF NX; Y, Y-like domain; Prot, papain-like cysteine protease; X, X domain/ADP-ribose-binding module; RdRp, RNA-dependent RNA polymerase). The protease and X domains could not be unambiguously identified and are therefore given with question marks. ORF3 is shown with a dotted line, since it is translated in a different reading frame than ORF2 and is shown only for an indication of its genomic position. (C) Bayesian phylogeny of the complete ORF1 and ORF2. Inference of Bayesian phylogenies was done by using MrBayes V3.1 with a WAG amino acid model and 4,000,000 generations sampled every 100 steps. After the exclusion of 10,000 trees as a burn-in, 15,000 final trees were annotated and visualized with TreeAnnotator and FigTree from the BEAST package. Values at the node points indicate posterior probability support (scale bar, genetic distance). GenBank accession numbers for taxa are AF459438 (HEV genotype 1), M74506 (HEV genotype 2), AB301710 (HEV genotype 3), AB220974 (HEV genotype 4), GU345042 (rat hepevirus), AM943647 (avian hepevirus genotype 1), EF206691 (avian hepevirus genotype 2), GU954430 (avian hepevirus genotype 3), and NC_015521 (trout HEV).

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TABLE 4 Identities of bat HEV (BS7) ORFs with HEV prototype strains

Hepevirus[a]	% identity					
	ORF1 (genome positions 34–4776)		ORF2 (genome positions 4777–6690)		ORF3 (genome positions 4859–5272)	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
HEV genotype 1	49.1–49.5	43.5–44.1	52.5–52.7	49.8–50.7	31.4–31.9	9.3–10.1
HEV genotype 2	48.5	44.1	51.2	49.1	33.6	10.9
HEV genotype 3	48.8–49.4	43.5–44.0	52.0–53.0	50.3–50.7	29.9–31.2	10.2–12.8
HEV genotype 4	48.1–49.3	44.1–44.3	51.6–52.7	50.3–50.8	28.2–29.1	10–10.9
Rabbit	48.5–49.1	43.5–44.1	51–52.1	47–49.8	27.4–28.0	10.2–10.9
Wild boar	49–49.2	43.8–43.9	51.6–52.8	49.7–51.2	29.9–30.6	11.1–12.0
Rodent	48.5–48.7	45.1–45.3	51.9–52	48.9–49.4	28.9–29.6	7.1
Avian	51.6–52.3	47.5–47.6	47–48.8	42.9–43.1	22.7–23.5	6.0
Trout	38.3	26.5	33.8	17.3	28.2	13.2

[a] GenBank accession numbers of prototype strains are as follows: D11092, L08816, AF459438, and DQ459342 for HEV genotype 1; M74506 for HEV genotype 2; EU723512, FJ705359, FJ998008, AB089824, AB248521, AB301710, AB248520, AB591734, and EU375463 for HEV genotype 3; FJ906895, FJ906896, and GU937805 for rabbit hepevirus; AB220974, GU119960, AB480825, AB097811, and AB097812 for HEV genotype 4; AB573435 and AB602441 for unassigned wild boar hepevirus; GU345042 and GU345043 for rodent hepevirus; AM943647, EF206691, AC535004, and GU954430 for avian hepevirus; and NC_015521 for trout hepevirus.

The amino acid identity plot (Fig. 5B) indicated that the bat hepevirus was closely related to avian hepeviruses in some parts of ORF1 and to primate hepeviruses in ORF2 (Fig. 5B). However, multiple-change-point analysis with Dual Brothers (46) and bootscan analysis with Simplot V3.5 (39) yielded no evidence of recombination, and the complete ORF1 and ORF2 sequences clustered reliably with other mammalian hepeviruses (Fig. 5C).

According to the International Committee on Taxonomy of Viruses (ICTV), the mammalian hepeviruses known at the time of assessment constituted a single genus (41). The avian viruses were suggested previously to form another independent genus (40). To test how the bat hepeviruses fit these proposals and to develop a working criterion for the tentative classification of partially sequenced viruses, we evaluated distance-based classification criteria. First, an amino-acid-based criterion for members of the *Hepeviridae* was calculated by using the comparably small 108-amino-acid RdRp fragment used for this study. The fragment overlaps largely with the amplicon of another widely used RT-PCR assay (32, 60) and might therefore enable the expansion of our taxonomic attempts to previous and future studies. The distribution of pairwise distances indicated several potential taxonomic ranks (Fig. 6A). Amino acid distance values of up to 9.3% but less than 11.1% were seen within the established HEV genotypes. Distances between 11.1% and 22.2% separated established HEV genotypes 1 to 4. The rabbit viruses would thus belong to HEV genotype 3, while the unclassified wild boar viruses would correspond to a distinct genotype. The latter might therefore constitute a fifth HEV genotype. The recently described rat hepeviruses were even more divergent, with up to 34.3% substitutions relative to other mammalian HEV isolates (Table 3).

The range of pairwise distances between all bat hepeviruses (up to 19.4%) suggested that they formed a taxonomic entity of the same rank as human HEV or rodent or avian hepeviruses.

Sequence distances between the closely related *Myotis* bat viruses from Germany (strains NMS098B and NMS09125R from *M. daubentonii* and NM98AC156 from *M. bechsteinii*) corresponded to distances observed within genotypes (Fig. 6A). Distances between PAN926 from Panama (*V. caraccioli*), G19E36 from Ghana (*Hipposideros abae*), and all other bat hepeviruses indicated that each of these viruses could be classified as a distinct genotype if the above-described criteria are applied. The classification of bat hepevirus BS7 from *E. serotinus* remained questionable because its sequence distances to the *Myotis* bat viruses fell on the border of inter- and intragenotypic distances characteristic for HEV genotypes 1 to 4.

The distance criterion within our partial RdRp gene fragment failed to discriminate bat and avian hepeviruses into two different tentative genera, which did not match the closer phylogenetic relatedness of mammalian viruses in larger parts of the ORF1-encoded polyprotein and the ORF2-encoded capsid protein (Fig. 5B and C). For better resolution, the distribution of pairwise amino acid distances was also plotted over those complete ORF1 and ORF2 sequences represented in Fig. 3. There was a clear separation between genetic distances within and between the four major clades: human HEV-like, rodent, avian, and bat hepeviruses. The distances within and between these groups were <22% and >46% in ORF1 and <18% and >42% in ORF2 (Fig. 6B and C), supporting the existence of four putative genera in the family *Hepeviridae*. Notably, these four putative genera were also well supported in the partial RdRp gene phylogenetic tree (Fig. 3).

DISCUSSION

In this study, we have described novel hepeviruses from a globally representative sample of bat specimens. The genomic characterization of a bat hepevirus clearly supported their classification as members of the recently established family *Hepeviridae* and indicated that they may be the most divergent mammalian hepeviruses described so far.

While a full genomic characterization is generally required for exact conclusions on taxonomy, the RdRp-based criterion established here may represent a useful tool for the typing of partially sequenced field specimens, similar to, e.g., the VP1 gene in enteroviruses and the RdRp gene in coronaviruses (20, 53). Our approach might help to resolve the classification of rodent hepeviruses, for which a fifth genotype has been proposed (38), as well as that of the rabbit hepeviruses, which seem to belong to HEV genotype 3 (23, 24). On the other hand, classification based on the RdRp gene may not always be sufficient to assign novel genera, and criteria based on full-ORF data may be required. The sequence of ORF3, which was found to be highly distinct between

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TABLE 5 Genomic features of bat hepevirus BS7[a]

Bat hepevirus genomic region and domain	Start site	End site	Length	Description
5′-UTR				
Full region	nt 1	nt 33	33 nt	
ORF1				
Full ORF	nt 34	nt 4776	4,743 nt	$pI^2 = 7.5$
Methyltransferase				Start and end could not be defined
4 conserved motifs	aa 65 (H), 115 (D), 118 (R), and 229 (Y)			All four conserved amino acid residues exist in BS7
Y domain	aa 216 (VVTY)	NI		Similarities in BLAST to corresponding regions in human HEV but unidentifiable end
Papain-like protease				Not conserved
Proline-rich hinge region				Not conserved
X domain	aa 684 (PDGSK)			Similarities in BLAST to corresponding regions in human HEV but unidentifiable end
Helicase				Start and end could not be defined
NTP-binding site in helicase (GVPGSGKS)	aa 874	aa 881	8 aa	Conserved with serine replaced by alanine
DEAP motif	aa 927	aa 930	4 aa	100% identity
RdRp	NI	NI		Start and end could not be defined
RdRp motifs				
I (KDCNKFTT)	aa 1277	aa 1284	8 aa	100% identity (KDCNKFTT)
II (ISAWSKTFCALFGPWFRA)	aa 1297	aa 1314	18 aa	83% identity (ISAWPKTLCALFGPWFRA)
III (FYGDAFDDTVF)	aa 1328	aa 1338	11 aa	36% identity (FYGDLYTEADL)
IV (NDFSEFDSTQNN)	aa 1354	aa 1365	12 aa	100% identity (NDFSEFDSTQNN)
V (KHSGEPGTLLWNTVW)	aa 1410	aa 1424	15 aa	93% identity (KHSGEPGTLLFNTVW)
VI (AAFKGDDSIVL)	aa 1441	aa 1451	11 aa	64% identity (HVYKGDDSVVL)
VII (LYAGVVVAPG)	aa 1481	aa 1490	10 aa	40% identity (VFSHYIVAPG)
VIII (ALPDVVRFAGRLT)	aa 1493	aa 1505	13 aa	39% identity (VVKDLLRTWGRMT)
Conserved tripeptide GDD	aa 1445	aa 1447	3 aa	100% identity
ORF2				
Full ORF	nt 4777	nt 6690	1,914 nt	$pI = 8.0$
cis-reactive element overlapping ORF2 start codon (TGAATAACA)	nt 4778	nt 4786	9 nt	Conserved nucleotides in avian and human strains were also conserved in BS7
Signal sequence				Not conserved at the N terminus but followed by a hydrophobic domain at aa 16-24 (FAYLLLLFL)
Arginine-rich domain	NI	aa 69		Conserved C terminus (PLT) but deletions and fewer R residues (7 compared to 12) than human hepeviruses
S domain	aa 87	aa 277	191 aa	Conserved
M domain	aa 278	aa 412	135 aa	Divergent N terminus, conserved C terminus
P domain	aa 413	aa 561	149 aa	Partially conserved
C-terminal domain				Not conserved
Termination codon ORF2	nt 6688	nt 6690	3 nt	TAA typical of genotype 3
ORF3				
Full ORF	nt 4859	nt 5272	414 nt	$pI = 8.1$
3′-UTR				
Full region	nt 6691	nt 6767	77 nt	

[a] nt, nucleotide; pI, isoelectric point (determined online at www.isoelectric.ovh.org/); aa, amino acids; NI, not identifiable.

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TABLE 6 Percent nucleotide (below the diagonal) and amino acid (above the diagonal) sequence identities between hepevirus ORF3s[a]

Hepevirus host or lineage (no. of compared strains)	% identity: Bat	Avian	Rodent	HEV genotypes 1-4
Bat (1)		9.3–12.8	9.8	12.3–16.7
Avian (4)	**26.4–28.0**	88.4–96.5 / **93.9–96.9**	24.3	25.6–31.2
Rodent (2)	**25.2–25.5**	**36.5–38.3**	94.1 / **97.4**	20.8–32.4
HEV genotypes 1–4 (22)	**32.5–35.1**	**33.3–38.6**	**37–42.5**	72.1–100 / **80.4–99.2**

[a] Evolutionary analyses were conducted with MEGA5 (66). GenBank accession numbers of strains are as follows: D11092, L08816, AF459438, DQ459342, M74506, EU723512, FJ705359, FJ998008, AB089824, AB248521, AB301710, AB248520, AB591734, EU375463, FJ906895, FJ906896, GU937805, AB220974, GU119960, AB480825, AB097811, AB097812, AB573435, and AB602441 for HEV genotypes 1 to 4; GU345042 and GU345043 for rodent hepevirus; and AM943647, EF206691, AC535004, and GU954430 for avian hepevirus. Boldface type indicates percent nucleotide identity, and lightface type indicates percent amino acid identity.

hepeviruses from different hosts, might be useful as an additional marker for classification. Our cumulative classification efforts suggested the existence of at least four putative genera in the family *Hepeviridae*. One genus would comprise human HEV genotypes and closely related animal viruses, while the other three would include viruses from chiropteran, rodent (rat), and avian (chicken) hosts. The trout hepevirus might correspond to a separate taxonomic unit of a higher rank, e.g., a subfamily (3).

The rate of hepevirus detection in bats was similarly low compared to that in other mammals, including humans (1, 9, 16, 24, 38, 58, 60). The highest anti-HEV seroprevalence and RNA detection rates described so far were for farmed animals, e.g., rabbits, chickens, and piglets (23, 33, 40). The accumulation of very large groups of susceptible individuals is uncommon in wild animals, with bats probably constituting the only mammals beside humans that form social groups exceeding several hundred thousand individuals in one place (52). Our study was performed on samples from very large social groups, including a breeding roost of *Eidolon helvum* bats in Ghana with more than 300,000 individuals (19) as well as vespertilionid bats hibernating in different roosts in Germany and Bulgaria, all exceeding 10,000 members (20, 25). While we have not observed any particularly high prevalence of viruses in these samples, the diversity of bat viruses found in our whole sample was comparable to that of the extensively studied human hepeviruses and much higher than that of the known avian or rodent viruses. We can thus be confident that our study provides a fair representation of the wider bat hepevirus diversity. Despite this, all chiropteran hepeviruses were monophyletic. In contrast to other viruses for which bats are assumed to constitute relevant mammalian reservoirs, such as mammalian coronaviruses and paramyxoviruses (19, 56), bat hepeviruses were not interspersed with human or other mammalian viruses. Bats therefore do not seem to constitute reservoirs of mammalian hepeviruses in general. The clear distinction of the four proposed hepevirus genera implicated rather that hepevirus evolution might have involved a nonrecent invasion of ancestral mammalian hosts. The marked exception to this idea is human hepeviruses, which can be found in both humans and peridomestic animals. It may be plausible that the intensification of farming activities during human history led to transmissions of some but not all human viruses to farmed animals, which would then have

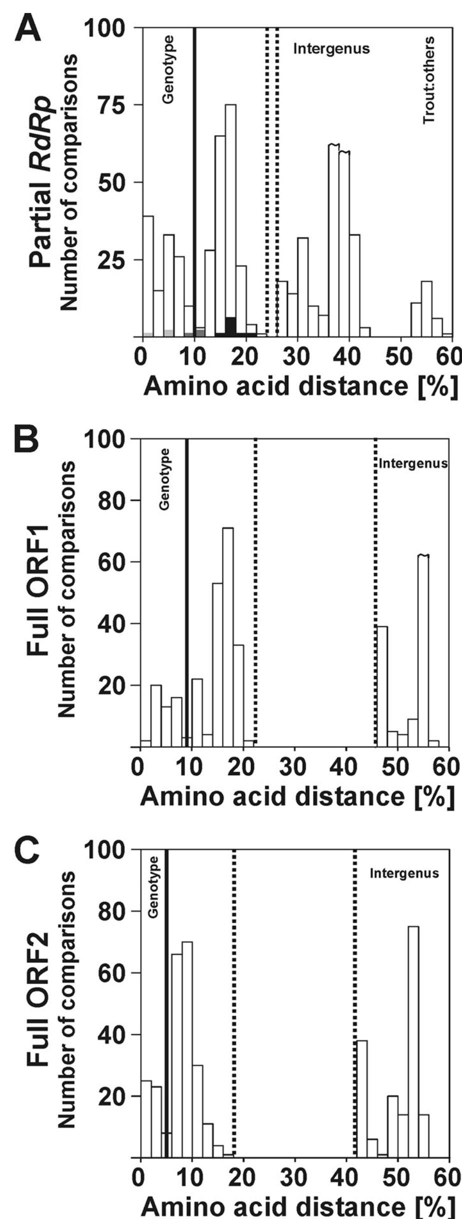

FIG 6 Distribution of *Hepeviridae* partial RdRp and full ORF1 and ORF2 pairwise amino acid distances. Uncorrected pairwise amino acid distances were calculated between members of the family *Hepeviridae* in the same 108-amino-acid RNA-dependent RNA polymerase (RdRp) alignment as that used for Fig. 3 (A) and in the complete ORF1 (B) and ORF2 (C). The *y* axis indicates the number of pairwise identity scores within each range represented on the *x* axis. The bold line indicates a distance cutoff that separates intratypic and intertypic distances within HEV genotypes 1 to 4. The dotted lines indicate a range of possible sequence cutoffs between sequence distances within and between the four suggested hepevirus genera. Distances within NM bat hepeviruses are indicated in light gray. Distances between BS7 bat virus and NM viruses are shown in gray. Distances between PAN926, G19E36, and other bat viruses are shown in black.

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maintained these viruses and formed a potential source of zoonotic reinfection. This hypothesis is coherent with the observation that HEV genotypes 1 and 2 have so far been detected only in humans and not in any other animals.

Based on our data, it would now be possible to search for hepeviruses in more distant relatives of farmed or peridomestic animals. This search should include lagomorphs other than rabbits, wild (nonlivestock) ungulates, as well as carnivores other than mongooses. If, indeed, hepeviruses very distinct from human HEV could be found in these taxa, the idea of an acquisition of human hepeviruses by farmed animals would be strongly supported. HEV in wild primates should also be studied to confirm a possibly more general association of human-related viruses with primates.

As with other newly described viruses in animals, we have to address potential zoonotic risks. Human infection has been associated with the consumption of pig, wild boar, and deer meat in industrialized countries (10, 37, 58, 68). Even though bats are consumed by humans in parts of the world (45), we consider it unlikely that bat hepeviruses would easily transmit to humans. As discussed above, the viruses carried by pig, boar, and deer are closely related to human viruses. Neither avian nor rodent hepeviruses were transmissible to primates experimentally (29, 60), and the latter are even more closely related to human viruses than the bat viruses. Despite reports on the serologic reactivity of human and rodent sera with hepevirus antigens from the opposed species (16, 17), proof of zoonotic transmission would require direct virus detection. In our study, we did not find any bat hepevirus RNA in a very broad range of human specimens.

Our data underline the importance of investigating targeted and balanced samples when studying viral host associations. While the surveillance of pathogens in livestock and peridomestic animals is important for epidemiology, ecologically valid conclusions regarding host associations can be very hard to reach using such samples. It is essential to cover large geographic and phylogenetic samples from the spectrum of potential viral hosts (19).

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