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Detection of bat hepatitis E virus RNA in microbats in Japan

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Abstract

Several recent studies have reported that various bat species harbor bat hepatitis E viruses (BatHEV) belonging to the family *Hepeviridae*, which also contains human hepatitis E virus (HEV). The distribution and ecology of BatHEV are not well known. Here, we collected and screened 81 bat fecal samples from nine bat species in Japan to detect BatHEV RNA by RT-PCR using HEV-specific primers, and detected three positive samples. Sequence and phylogenetic analyses indicated that these three viruses were BatHEVs belonging to genus *Orthohepevirus D* like other BatHEV strains reported earlier in various countries. These data support the first detection of BatHEVs in Japanese microbats, indicating their wide geographical distribution among multiple bat species.

Keywords Bat · Hepevirus · Epidemiology

Bats are known to be natural reservoirs of various zoonotic viruses such as rabies virus, Nipah virus, and severe acute respiratory syndrome (SARS) coronavirus [1–3]. In addition, hepatitis E virus (HEV)-like viruses have been detected in bats in several countries [4, 5]. Bat HEV (BatHEV) belongs to family *Hepeviridae*, genus *Orthohepevirus* and is a non-enveloped, positive-sense, single-stranded RNA virus. *Orthohepevirus* is divided into four species, *Orthohepevirus A–D* [6, 7]. *Orthohepevirus A* contains HEV, which is a causative agent for human acute hepatitis. *Orthohepevirus B* includes avian-HEV associated with hepatitis-splenomegaly syndrome. *Orthohepevirus C* is divided into C1 (rodent-HEV) and C2 (carnivore-associated HEV) genotypes. *Orthohepevirus D* comprises BatHEV, which has 57.4–64.8% identity with HEV [8]. Recently, BatHEVs were detected from macrobats and microbats in a variety of countries [4, 5]. However, limited information about the distribution of BatHEVs in other regions and their ecology is available. Here, we surveyed several Japanese microbat species to detect BatHEVs.

To examine whether BatHEVs exist in Japanese bats, we collected 81 bat fecal samples from nine bat species captured in five different prefectures of Japan in 2015, with permission from the Ministry of the Environment, Japan and the respective local government (Fig. 1a). Bats were caught using a harp trap and kept in a pouch for an hour to check the signs of disease as well as to obtain fresh feces. All captured bats did not show any obvious symptoms. The feces were added in a medium containing antibiotics, and frozen in dry ice. We extracted RNA from the fecal samples and performed RT-PCR using a primer set (Table 1) that was designed specifically against the conserved region of the RNA-dependent RNA polymerase (RdRp) of HEV, to screen the HEV genomes. Two samples (BtHEV-Ej1 and BtHEV-Ej2) from the Japanese short-tailed bat (*Eptesicus japonensis*) and 1 sample (BtHEV-Ps1) from the Brown long-eared bat (*Plecotus sacrimontis*) were found to be positive. Although we attempted to sequence the entire genome of the BatHEVs, we failed to amplify the whole genome using RT-PCR. Therefore, we amplified partial ORF1, which

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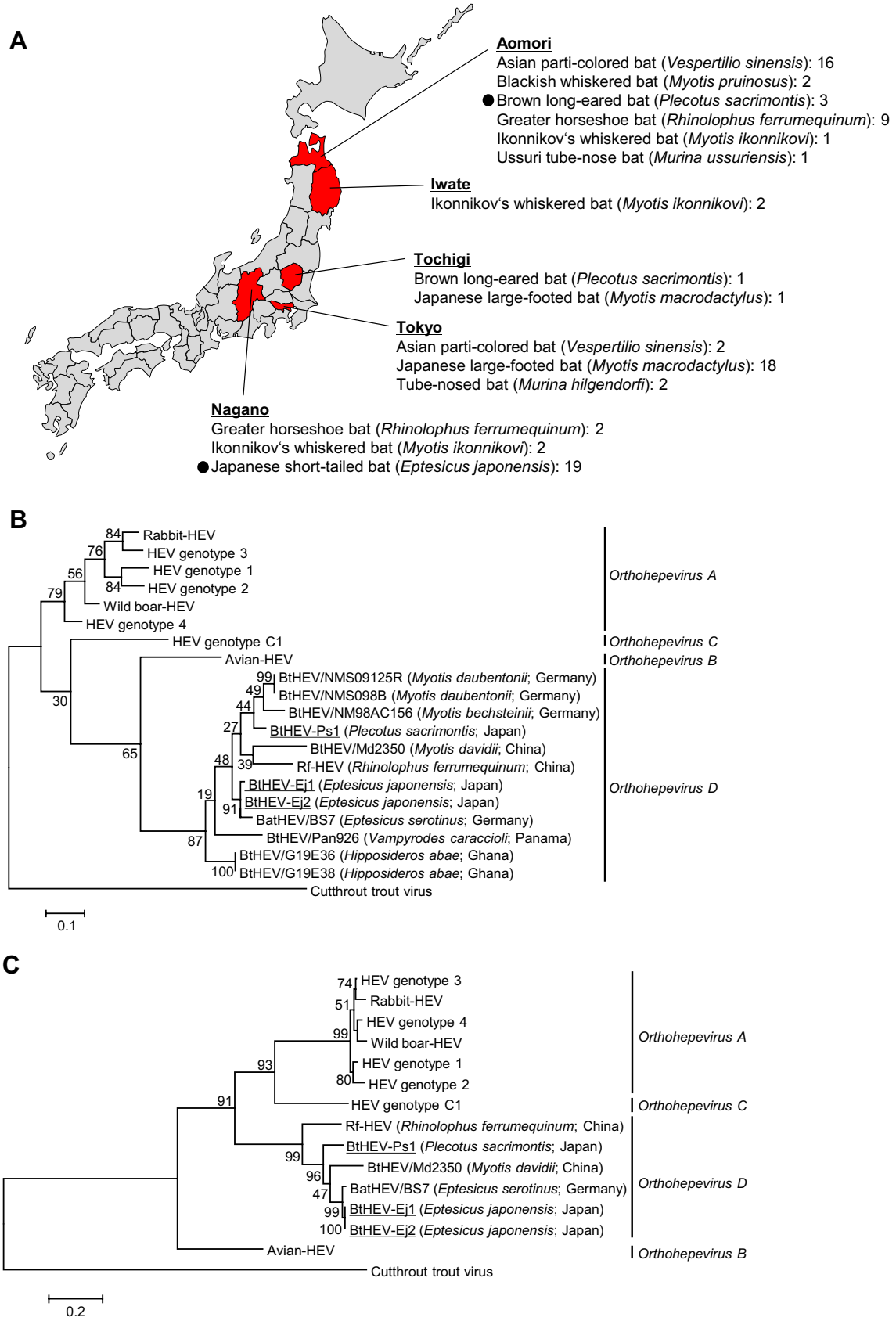
A
Aomori
Asian parti-colored bat (Vespertilio sinensis): 16
Blackish whiskered bat (Myotis pruinosus): 2
● Brown long-eared bat (Plecotus sacrimontis): 3
Greater horseshoe bat (Rhinolophus ferrumequinum): 9
Ikonnikov's whiskered bat (Myotis ikonnikovi): 1
Ussuri tube-nose bat (Murina ussuriensis): 1
Iwate
Ikonnikov's whiskered bat (Myotis ikonnikovi): 2
Tochigi
Brown long-eared bat (Plecotus sacrimontis): 1
Japanese large-footed bat (Myotis macrodactylus): 1
Tokyo
Asian parti-colored bat (Vespertilio sinensis): 2
Japanese large-footed bat (Myotis macrodactylus): 18
Tube-nosed bat (Murina hilgendorfi): 2
Nagano
Greater horseshoe bat (Rhinolophus ferrumequinum): 2
Ikonnikov's whiskered bat (Myotis ikonnikovi): 2
● Japanese short-tailed bat (Eptesicus japonensis): 19
B
Rabbit-HEV
HEV genotype 3
HEV genotype 1
HEV genotype 2
Wild boar-HEV
HEV genotype 4
HEV genotype C1
Avian-HEV
BtHEV/NMS09125R (Myotis daubentonii; Germany)
BtHEV/NMS098B (Myotis daubentonii; Germany)
BtHEV/NM98AC156 (Myotis bechsteinii; Germany)
BtHEV-Ps1 (Plecotus sacrimontis; Japan)
BtHEV/Md2350 (Myotis davidii; China)
Rf-HEV (Rhinolophus ferrumequinum; China)
BtHEV-Ej1 (Eptesicus japonensis; Japan)
BtHEV-Ej2 (Eptesicus japonensis; Japan)
BatHEV/BS7 (Eptesicus serotinus; Germany)
BtHEV/Pan926 (Vampyrodes caraccioli; Panama)
BtHEV/G19E36 (Hipposideros abae; Ghana)
BtHEV/G19E38 (Hipposideros abae; Ghana)
Cutthrout trout virus
Orthohepevirus A
Orthohepevirus C
Orthohepevirus B
Orthohepevirus D
0.1
C
HEV genotype 3
Rabbit-HEV
HEV genotype 4
Wild boar-HEV
HEV genotype 1
HEV genotype 2
HEV genotype C1
Rf-HEV (Rhinolophus ferrumequinum; China)
BtHEV-Ps1 (Plecotus sacrimontis; Japan)
BtHEV/Md2350 (Myotis davidii; China)
BatHEV/BS7 (Eptesicus serotinus; Germany)
BtHEV-Ej1 (Eptesicus japonensis; Japan)
BtHEV-Ej2 (Eptesicus japonensis; Japan)
Avian-HEV
Cutthrout trout virus
Orthohepevirus A
Orthohepevirus C
Orthohepevirus D
Orthohepevirus B
0.2

◂**Fig. 1** Bat capture sites and phylogenetic trees of the detected viruses and bats. **a** Prefectures where bats were captured are indicated in red. Bat species and the number of samples are noted in each prefecture. To obtain fresh feces, bats were kept in a pouch for an hour, and the feces were then collected by a sterilized cotton bud and transferred to 1 mL of Dulbecco's modified medium Eagle's minimum essential medium (DMEM) supplemented with 100 U/mL of penicillin, 1 mg/mL of streptomycin, 100 µg/mL of gentamycin, and 2 µg/mL of amphotericin. The feces were suspended well and then centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatants were used for RNA extraction with ISOGEN LS reagent (Nippon Gene). cDNA was synthesized using Prime Script RT reagent kit (Takara Bio) with a mixture of random hexamer and oligo dT primers. PCR amplifications were performed using the KOD FX Neo (Toyobo) with consensus HEV primer sets (PanHEV F and R), which were designed in this study to amplify a 191-bp fragment of the RNA-dependent RNA polymerase gene (corresponding to nt 4084–4274 of BatHEV/BS7) from the BatHEV genome. Black dots indicate Pan-HEV RT-PCR-positive bats. **b** and **c** Maximum likelihood phylogenetic trees of the partial RdRp and capsid genes (corresponding to nt 3970–4296 and nt 4777–6690 of BatHEV/BS7, respectively) of BtHEVs including the novel Japanese viruses (bold underline) at the amino acid level with Jones–Taylor–Thornton model. All phylogenetic trees were generated using ClustalW and MEGA software version 7.0 [9]. Bootstrap values are shown above and to the left of the major nodes. Scale bars indicate the number of substitutions per site. The accession numbers of sequences used in these trees are as follows: BtHEV-Ej1 (LC340968), BtHEV-Ps1 (LC340969), BtHEV-Ej2 (LC340970), HEV genotype 1 (D11092), HEV genotype 2 (M74506), HEV genotype 3 (EU723512), HEV genotype 4 (AB220974), Rabbit-HEV (FJ906895), Wild boar-HEV (AB573435), Avian-HEV (AM943647), HEV genotype C1 (GU345042), Rf-HEV (KJ562187), Bat-HEV/BS7 (JQ001749), BtHEV-Md2350 (KX513953), BtHEV/G19E36 (JQ001744), BtHEV/Pan926 (JQ001745), BtHEV/NMS098B (JQ001746), BtHEV/NMS09125R (JQ001747), BtHEV/NM98AC156 (JQ001748), BtHEV/G19E38 (JQ071861), and Cutthroat trout virus (NC015521)

encodes non-structural protein including RdRp, and entire capsid coding region of BtHEV-Ej1, -Ej2, and -Ps1 (corresponding to nucleotides (nt) 2171–6690, nt 3585–6690, and nt 3847–6690 of BatHEV/BS7, respectively) using specific primers (Table 1). The sequences of BtHEV-Ej1 and BtHEV-Ej2, which were collected in the same site on different days, were 99% identical (nt 2904 out of 2915). The identity between BtHEV-Ej1/-Ej2 and BtHEV-Ps1 using a part of the RdRp was about 75% at the nucleotide level, suggesting the presence of multiple BatHEVs in Japan. BLAST analysis indicated that BtHEV-Ej1/-Ej2 showed the highest sequence identities to BatHEV/BS7, a German strain detected from the Serotine bat (*Eptesicus serotinus*), among strains previously reported in other countries. On the other hand, BtHEV-Ps1 showed the highest sequence identity to BtHEV/NMS098B, a German strain detected from the Daubenton's bat (*Myotis daubentonii*). In particular, 82% identity observed between BtHEV-Ej1/-Ej2 and BatHEV/BS7 or 77% identity observed between BtHEV-Ps1 and BtHEV/NMS098B were greater than that observed between Japanese strains. These data suggest that similar viruses exist in geographically distant regions.

We then phylogenetically analyzed the sequences by maximum-likelihood analysis using ClustalW and MEGA version 7.0 [9]. A phylogenetic tree constructed using the partial amino acid sequences of RdRp indicated that the Japanese viruses were included in *Orthohepevirus D* (Fig. 1b), demonstrating that all BatHEVs are classified in this species. We also amplified the full-length capsid (ORF2) sequences by RT-PCR and analyzed them phylogenetically. The resulting tree confirmed that the novel Japanese viruses were included in *Orthohepevirus D* (Fig. 1c). The sequences of BtHEV-Ej1/-Ej2 were placed in a position neighboring the German BatHEV/BS7 strain, confirming the phylogenetic similarity between these strains.

All bats captured in this study were insectivores and hibernate in winter. Although there is no information about migration of *E. japonensis* and *P. sacrimontis*, bat species closely related to them were reported to migrate only a few kilometers from their colonies per night [10, 11]. They had different ecology in the terms of the habitat. BtHEV-Ej1 and -Ej2 were detected on different days from two *E. japonensis* bats, both of which used eaves of the same house as night roost in Nagano. Although the *E. japonensis* bats formed mixed colony at the roost with *Myotis ikkonikovi* and *Rhinolophus ferrumequinum* bats, BatHEVs were only detected in *E. japonensis*, implying BtHEV-Ej1/-Ej2 might have a narrow host range. *P. sacrimontis* bats usually form a small colony without other species of bats. Indeed, *P. sacrimontis* bats, from which BtHEV-Ps1 was detected in this study, were captured near such small colony in a ruin in Aomori. Thus, BtHEV-Ps1 is likely circulating in the *P. sacrimontis* since the bats have low opportunity to come in contact with other species of bats.

The closely related BatHEVs (BtHEV-Ej1/-Ej2 and Bat HEV/BS7) have been detected in different species of *Eptesicus* bats (*E. serotinus* and *E. japonensis*). Since the distribution areas of these bats are not overlapping, viruses ancestral to BtHEV-Ej1/-Ej2 and BatHEV/BS7 might have infected ancestral *Eptesicus* and might have branched into different species in the process of evolution.

For virus isolation, we inoculated the RT-PCR-positive fecal samples not only into several bat cells (BKT, FBKT, and DemKT1 cells) but also into other mammalian cell lines (Madin-Darby canine kidney (MDCK), African green monkey VeroE6, human A549, Madin-Darby bovine kidney (MDBK), and swine PK15 cells) since we suspected that the bat fecal samples may contain several pathogens other than BatHEVs. All inoculated cells were incubated for 12–15 days with media changes at 2–3 days interval. After the incubation, cells were blindly passaged three times. However, we could neither recover any infectious viruses nor detect BatHEV RNA in the inoculated cells by RT-PCR.

In conclusion, the present study showed the presence of several BatHEV strains, which were independently classified

Table 1 Primers used in this study

Primer	Sequence (5′-3′)
Ej1 1933F	CCTGGCGGTGGNATNTGCGG
Ej1 2790R	GACCGGACCACATCAGAAG
Ej1 2602F	CCTGTTGCTTCTGATCATGC
Ej1/2 3135 R	TCAGCTGGCTCAATAGGTGC
Pan HEV F	GTTTTTGAGAATGAYTTYTCTGA
Pan HEV R	TCACCGGAGTGYTTYTTCCA
Ej1/2 4058 F	CCTTTATTGGCTGCAGAG
Ej1/2 5096 R	CCACCGACAGACGCAGG
Ej1/2 5035 F	CACATCATGTCAACGGAGG
Ej1/2 Ps1 5834 R	GGGCGGTCNGNCTCATGGTTA
Ej1/2 5703 F	CTTTATGGGAAACCAGTGAC
Ej1/2 6123 R	GCCARAANGACAATTTGCCG
Ej1/2 6059 F	ACTGGACCAAGGCCACATTG
Ps1 2987 F	AGYCGTGCNCANGCCATTG
Ps1 4072 R	TGCATCCGGTGCAACAAGAG
Ps1 4008 F	GGCTAGAATGTGAGCTCCTG
Ps1 4658 R	GAAAAGGGAGCGGCATGTCC
Ps1 4647 F	ATGAATAACACCCCTTTCTG
Ps1 5277 R	TTAGCAGTCTCAACAGATCG
Ps1 5743 F	GACGGTCCAACTCTACGGTTC
Ps1 6331 R	TCGGGCATCGNGAAGGNCTC
Ps1 6194 F	CCCTTATCAGTATNACAACAATG
Ej1/2 Ps1 terminal R	TTTTTTTTAAGGGTATTACACTG

into *Orthohepevirus D*, in Japanese bats, suggesting wide geographical distribution of BatHEV among multiple bat species. Although these data suggest limited transmissibility of BatHEV to other animals, further studies are needed to determine its zoonotic potential.

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Author contributions TK, SM, and TH conceived and designed the experiments. TK, TY, KMi, MS, RS, and KMa performed the experiments. TK and SM analyzed the data. TK, SM, and TH wrote the paper and designed the figure. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest There is no conflict of interest associated with this article.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent Informed consent is not required because no human participants were involved in this article.

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