司，田中智之，野田 衛					
李 天成	動物由来Ｅ型肝炎ウイルス；Ｅ型肝炎ウイルスの多様性	病原微生物検出情報	35(1)	10-2	2014
石井孝司	Ｅ型肝炎の慢性化、肝外病変について	病原微生物検出情報	35(1)	13-4	2014

註：上記は 2013 年 2 月以降 2014 年 1 月末までに刊行された研究業績であり（一部印刷中を含む）、前年度報告書との重複は無い。研究代表者（班長）を二重下線、研究分担者（班員）を一重下線、研究協力者（班長付き及び班員付き）を一重破線で示した。

Ⅳ. 研究成果の刊行物・別刷

Virus Research 180 (2014) 59–69

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular characterization of a novel hepatitis E virus (HEV) strain obtained from a wild boar in Japan that is highly divergent from the previously recognized HEV strains☆

Masaharu Takahashi[a,1], Tsutomu Nishizawa[a,1], Shigeo Nagashima[a], Suljid Jirintai[a], Manri Kawakami[b], Yoshihide Sonoda[c], Tadahiro Suzuki[d], Shogo Yamamoto[e], Kazuhiro Shigemoto[f], Kozo Ashida[g], Yukihiro Sato[h], Hiroaki Okamoto[a,*]

[a] Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan
[b] Hepatology Research Center, Okayama Saiseikai General Hospital, Okayama-shi, Okayama 700-8511, Japan
[c] Nara Municipal Tahara Clinic, Nara-shi, Nara 630-2174, Japan
[d] Tessei-cho Clinic, Niimi-shi, Okayama 719-3701, Japan
[e] Miyazaki Prefectural Institute for Public Health and Environment, Miyazaki-shi, Miyazaki 889-2155, Japan
[f] Osaba Family Clinic, Yamaguchi-shi, Yamaguchi 753-0212, Japan
[g] Division of Internal Medicine, Okayama University Hospital Misasa Medical Center, Misasa, Tottori 682-0122, Japan
[h] Kamiichi General Hospital, Kamiichi, Toyama 930-0391, Japan

ARTICLE INFO

Article history:
Received 7 November 2013
Received in revised form 9 December 2013
Accepted 10 December 2013
Available online 24 December 2013

Keywords:
Hepatitis E virus
Wild boar
Phylogeny
Genotype

ABSTRACT

Although a consensus classification system for hepatitis E virus (HEV) genotypes is currently unavailable, HEV variants (JBOAR135-Shiz09 and wbJOY_06) from wild boars (*Sus scrofa leucomystax*) have provisionally been classified into two novel genotypes (5 and 6). While performing a survey of HEV infections among 566 wild boars that were captured in Japan between January 2010 and August 2013, we found 24 boars (4.2%) with ongoing HEV infections: 13 had genotype 3 HEV, 10 had genotype 4 HEV and the remaining boar possessed a novel HEV variant (designated wbJNN_13). The entire wbJNN_13 genome comprised 7247 nucleotides excluding the poly(A) tail, and was highly divergent from known genotype 1 to 4 HEV isolates derived from humans, swine, wild boars, deer, mongoose and rabbits by 22.4–28.2%, JBOAR135-Shiz09 and wbJOY_06 by 19.6–21.9% and rat, ferret, bat and avian HEV isolates by 40.9–46.1% over the entire genome. Phylogenetic trees confirmed that wbJNN_13 is distantly related to all known HEV isolates. A Simplot analysis revealed no significant recombination among the existing HEV strains. These results indicate the presence of at least three genetic lineages of presumably boar-indigenous HEV strains. Further studies to fully understand the extent of the genomic heterogeneity of HEV variants infecting wild boars are warranted.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans. Hepatitis E is an important public health problem, being primarily transmitted via the fecal-oral route through contaminated food or water in many developing countries in Asia, Africa and Latin America, where sanitation conditions are suboptimal (Emerson and Purcell, 2013). In contrast, sporadic and autochthonous cases of hepatitis E also occur in many industrialized countries, including the United States, European countries and Japan (Colson et al., 2010; Tei et al., 2003; Yazaki et al., 2003). Hepatitis E is now a recognized zoonotic disease in swine, and other animals likely serve as a reservoir for HEV infection in humans (Meng, 2013; Purcell and Emerson, 2008; Takahashi and Okamoto, 2013).

HEV is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Meng et al., 2012). The genome is a single-stranded, positive-sense RNA of approximately 7.2 kilobases (kb) in size, and contains three open reading frames that encode non-structural proteins involved in replication (ORF1), a capsid protein consisting of 660 amino acids (aa) (ORF2) and a small protein of only 113–114 aa (ORF3) that is essential for viral infectivity in

☆ The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB856243 for entire wild boar HEV genome and AB856220–AB856242 for 23 partial wild boar HEV sequences.

* Corresponding author. Tel.: +81 285 58 7404; fax: +81 285 44 1557.
E-mail address: hokamoto@jichi.ac.jp (H. Okamoto).

[1] These authors contributed equally to this work.

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.12.014

Table 1
Prevalence of anti-HEV IgG and HEV RNA among wild boars in Japan, stratified by year of capture.

Year	No. of boars tested	Serum samples			Liver samples		No. of HEV RNA-positive boars
		No.	Anti-HEV IgG-positive	HEV RNA-positive	No.	HEV RNA-positive	
2010	65	65	8 (12.3%)	1 (1.5%)	26	0	1 (1.5%)
2011	126	122	16 (13.1%)	7 (5.7%)	125	7 (5.6%)	7 (5.6%)
2012	240	214	18 (8.4%)	4 (1.9%)	238	4 (1.7%)	4 (1.7%)
2013	135	110	16 (14.5%)	11 (10.0%)	134	11 (8.2%)	12 (8.9%)
Total	566	511	58 (11.4%)	23 (4.5%)	523	22 (4.2%)	24 (4.2%)

animals (Graff et al., 2005; Huang et al., 2007) and virion egress (Emerson et al., 2010; Yamada et al., 2009). The ORF1 protein is translated directly from genomic RNA, while the ORF2 and ORF3 proteins are translated from a 2.2-kb bicistronic subgenomic RNA (Graff et al., 2006; Ichiyama et al., 2009). Four genotypes (1–4) of HEV that infect humans have been identified (Lu et al., 2006; Okamoto, 2007). Genotype 1 and 2 HEVs are restricted to humans, and are associated with outbreaks of hepatitis E as water-borne epidemics in developing countries, whereas genotype 3 and 4 HEVs are zoonotic, and are responsible for sporadic cases of hepatitis E worldwide. Genotype 3 and 4 HEVs have been isolated from various animal species, including pigs, wild boars, deer and mongooses (Meng, 2011; Nidaira et al., 2012; Sato et al., 2011; Takahashi et al., 2004), and as noted above, are capable of causing zoonotic disease (Colson et al., 2010; Izopet et al., 2012; Li et al., 2005; Tei et al., 2003; Yazaki et al., 2003). Japan-indigenous genotype 3 HEV strains have been provisionally classified into three subgenotypes: 3b (3jp), 3a (3us) and 3e (3sp), where "jp" stands for Japan-type, "us" for US-type and "sp" for Spanish (European) type (Lu et al., 2006; Okamoto et al., 2003; Takahashi et al., 2003).

Recently, a number of divergent HEV isolates have been identified in numerous other animal species, including chickens, rabbits, rats, ferrets, bats and fish (cutthroat trout) with an ever-expanding host range (Batts et al., 2011; Drexler et al., 2012; Johne et al., 2010; Kumar et al., 2013; Meng, 2013; Raj et al., 2012; Zhao et al., 2009). In addition, novel HEV sequences [JBOAR135-Shiz09 (AB573435) and wbJOY_06 (AB602441)] that may belong to new or unrecognized genotypes have been detected in wild boars in Japan (Takahashi et al., 2010, 2011), and are suggested to be divided into two additional genotypes (5 and 6) (Bouquet et al., 2012; Smith et al., 2013). Although there has been controversy about whether isolates from wild boars should be considered new genotypes or subtypes (subgenotypes), two wild boar strains (JBOAR135-Shiz09 and wbJOY_06) will be provisionally described in this study as genotype 5 and genotype 6, respectively, for simplicity.

In recent studies, 73 HEV strains had been isolated from 1885 wild boars in Japan (3.9% of the population) (Nakano et al., 2013; Takahashi and Okamoto, 2013). Although these boar HEV strains were classified into genotype 3 (69%), 4 (28%) or novel genotypes (5 and 6) represented by JBOAR135-Shiz09 and wbJOY_06, respectively, the spatial prevalence of HEV infections among the wild boars in Japan and the pattern and extent of genomic diversity among HEV strains infecting wild boars in Japan were not fully understood. Therefore, the present study was conducted to investigate the prevalence of the wbJOY_06 type HEV strain (genotype 6), focusing on Okayama Prefecture, where the particular strain had been isolated in our previous study (Sato et al., 2011), and other HEV strains, including those of unidentified genotype(s), if any, in a total of 12 prefectures (see Table 2). Unfortunately, the wbJOY_06-like strain was not found in any of the 566 wild boars studied. However, another divergent strain (wbJNN_13) that may be classifiable into a novel genetic lineage was identified in Nagano Prefecture, located in the central part of Honshu Island of Japan, and its genomic characteristics were analyzed, together with those of 23 other strains obtained in the current study.

2. Materials and methods

2.1. Serum and liver specimens obtained from wild boars

Paired serum and liver specimens, serum only or liver tissue samples only, were obtained from a total of 566 wild boars (*Sus scrofa leucomystax*) that were captured in 12 prefectures (located from north to south in Japan): Toyama (3 boars), Fukui (15), Nagano (62), Gifu (122), Hyogo (18), Nara (35), Tottori (11), Okayama (244), Hiroshima (2) and Yamaguchi (10) on mainland Honshu, the prefecture of Kochi (5) on Shikoku Island and the prefecture of Miyazaki (39) on Kyushu Island (Supplementary Fig. 1) between January 2010 and August 2013. A total of 511 serum samples and 523 liver tissue samples, including 468 paired serum and liver specimens, were available from the 566 boars: there were no overlaps between the 566 boars in the present study and the 578 boars evaluated in the previous study (Sato et al., 2011).

2.2. ELISA for detecting anti-HEV IgG

To detect anti-HEV IgG in the serum samples from wild boars, an enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein (genotype 4) that had been expressed in silkworm pupae (Mizuo et al., 2002) as described previously (Sato et al., 2011). An optical density (OD) value of 0.274 was used as the cut-off value for the swine anti-HEV IgG assay (Takahashi et al., 2005).

2.3. Qualitative and quantitative detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed to detect HEV RNA. Total RNA was extracted from 100 μl of each serum sample using the TRIzol LS reagent (Life Technologies, Carlsbad, CA) or 50 mg of each liver specimen using the TRIzol Reagent (Life Technologies) following the manufacturer's instructions. The extracted RNA was reverse-transcribed with SuperScript II RNase H$^-$ Reverse Transcriptase (Life Technologies), and subsequent nested PCR was performed with the ORF2 primers and *TaKaRa Ex Taq* (TaKaRa Bio, Shiga, Japan) as described previously (Mizuo et al., 2002). The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457bp. The sequences of primers used for the ORF2-457 PCR (Supplementary Table 1) were well-conserved across all four genotypes and two wild boar sequences (JBOAR135-Shiz09 and wbJOY_06) (Takahashi et al., 2011). The specificity of the RT-PCR assay was verified by a sequence analysis, as described below. The sensitivity of the RT-PCR assay was assessed as described previously (Mizuo et al., 2002; Takahashi et al., 2003).

Table 2
Prevalence of anti-HEV IgG and HEV RNA among wild boars in Japan, stratified by geographic region.

Region	No. of boars tested	Serum samples			Liver samples		No. of HEV RNA-positive boars
		No.	Anti-HEV IgG-positive	HEV RNA-positive	No.	HEV RNA-positive	
Toyama	3	3	0	0	3	0	0
Fukui	15	15	1 (6.7%)	1 (6.7%)	14	1 (7.1%)	1 (6.7%)
Nagano	62	62	4 (6.5%)	1 (1.6%)	60	0	1 (1.6%)
Gifu	122	120	16 (13.3%)	9 (7.5%)	122	9 (7.4%)	9 (7.4%)
Hyogo	18	18	0	0	18	0	0
Nara	35	35	9 (25.7%)	2 (5.7%)	35	3 (8.6%)	3 (8.6%)
Tottori	11	11	0	0	11	0	0
Okayama	244	191	18 (9.4%)	8 (4.2%)	243	8 (3.3%)	8 (3.3%)
Hiroshima	2	2	0	0	2	0	0
Yamaguchi	10	10	4 (40.0%)	1 (10.0%)	10	1 (10.0%)	1 (10.0%)
Kochi	5	5	1 (20.0%)	0	5	0	0
Miyazaki	39	39	5 (12.8%)	1 (2.6%)	0	0	1 (2.6%)
Total	566	511	58 (11.4%)	23 (4.5%)	523	22 (4.2%)	24 (4.2%)

To confirm the presence/absence of HEV RNA in serum and/or liver specimens from wild boars, another RT-PCR (ORF2/3-137 PCR) that amplifies a 137-nt sequence within the ORF2/ORF3 overlapping region and is capable of amplifying all four genotypes (Inoue et al., 2006b), was performed with primers that were slightly modified based on three wild boar sequences including wbJNN_13 (see below). The sequences of primers used for the modified ORF2/3-137 PCR assay are indicated in Supplementary Table 1.

HEV RNA was quantitated by real-time detection RT-PCR according to the previously described method (Takahashi et al., 2008), with slight modifications. In brief, total RNA extracted from 100 μl of a diluted serum sample, or 50 mg of liver tissue was subjected to real-time RT-PCR with the QuantiTect Probe RT-PCR Kit (Qiagen, Tokyo, Japan), using the sense primer HE311 (5′-GGT GGT TTC TGG GGT GAC-3′), antisense primer HE312 (5′-AGG GGT TGG TTG GAT GAA-3′) and a probe (HE313-P) consisting of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) on a LightCycler apparatus (Roche Diagnostics K.K., Tokyo, Japan). The thermal cycler conditions were 50 °C for 20 min, 95 °C for 15 min, 45 cycles of 95 °C for 1 s and 60 °C for 60 s. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

2.4. Amplification of the full-length HEV genome

To determine the full-length sequence of the wbJNN_13 genome that was markedly divergent from all HEV sequences of genotypes 1–4 and the two wild boar sequences (JBOAR135-Shiz09 and wbJOY_06) (see below), total RNA was extracted from a serum sample (1 ml) using the High Pure Viral RNA Kit (Roche Applied Science, Mannheim, Germany) and then the TRIzol-LS reagent (Life Technologies), and the RNA preparation thus obtained was reverse-transcribed with SuperScript III Reverse Transcriptase (Life Technologies) and subjected to nested or semi-nested PCR of six overlapping regions including the extreme 5′- and 3′-terminal regions, using enzymes [KOD FX Neo (Toyobo, Osaka, Japan), *TaKaRa LA Taq* with GC Buffer (TaKaRa Bio)] and primers whose sequences were derived from well-conserved areas across all HEV strains of genotypes 1–4 and two wild boar strains (JBOAR135-Shiz09 and wbJOY_06) whose entire genomic sequences are known, as well as those obtained during the amplification procedure (Supplementary Table 1). The amplified regions, excluding the primer sequences, were nucleotides (nt) 1–60 (60 nt), nt 41–2236 (2196 nt), nt 2143–4454 (2312 nt), nt 4330–5384 (1055 nt), nt 5349–6402 (1054 nt), and nt 6371–7263 (893 nt) (Supplementary Fig. 2). The extreme 5′-end sequence (nt 1–60) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE), using the First Choice RLM-RACE kit (Ambion, Austin, TX), as described previously (Okamoto et al., 2001). Amplification of the 3′-end sequence [nt 6371–7247 (877 nt): poly(A) tail excluded] was performed by the RACE method as described previously (Okamoto et al., 2001). To confirm the entire genomic sequence determined from the above amplified regions, three other overlapping regions, including nt 41–2832 (2792 nt), nt 2678–5170 (2493 nt) and nt 5063–7247 (2185 nt) (primer sequences at both ends were excluded), were amplified and sequenced (Supplementary Table 1 and supplementary Fig. 2).

2.5. Sequence analysis of PCR products

The amplification product was purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Ltd., Tokyo, Japan), after which both strands were sequenced directly or after cloning into the T-Vector, pMD20 (TaKaRa Bio), using an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The sequence analysis was performed using the Genetyx software program (version 11.1.2; Genetyx Corp., Tokyo, Japan), and multiple alignments were generated with the CLUSTAL Omega software program, version 1.2.0 (Goujon et al., 2010). Phylogenetic trees were constructed based on the 412-nt ORF2 sequence or full-length sequence, according to the neighbor-joining method (Saitou and Nei, 1987) with the Kimura two-parameter model (transition/transversion ratio was fixed and gamma correction was not used) and 1000 replicates of bootstrap resamplings, as implemented in the MEGA5 software program (version 5.2.1) (Tamura et al., 2011). Another phylogenetic tree was constructed by the PHYML method, version 3.0 (Guindon and Gascuel, 2003) implemented via the PALM web serve (http://palm.iis.sinica.edu.tw) (Chen et al., 2009), based on the full-length sequence. The maximum-likelihood phylogenetic tree was constructed using the best model (GTR + G) selected by the MODELTEST software program, version 3.7 (Posada and Crandall, 1998) under the corrected Akaike information criterion (AICc) and 500 replicates of bootstrap resamplings. The final trees were visualized by the FigTree software program, version 1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/).

For comparison, 220 complete genome sequences were downloaded from DDBJ/EMBL/GenBank databases on September 30, 2013. Sequences were removed from the dataset if there was evidence that they were recombinant (accession nos. D11093,

Table 3
Characteristics of the 24 wild boars with ongoing HEV infections.

ID no.	Date of sampling	Body weight (kg)	Region	Anti-HEV IgG (OD_{450}) in serum[a]	HEV RNA titer		HEV genotype (subgenotype)	HEV isolate name
					Serum (copies/ml)	Liver (copies/mg)		
WB0635	'10.01.01	20	Miyazaki	0.492 (+)	2.4×10^6	NA	3 (3b)	wbJMZ_10
WB0772	'11.01.10	20	Fukui	0.202 (−)	<200 (+)	<400 (+)	3 (3b)	wbJFI_11
WB0717	'11.01.15	20	Gifu	0.228 (−)	2.2×10^4	2.3×10^6	4	wbJGF_11
WB0736	'11.02.05	60	Nara	0.908 (+)	<200 (+)	<400 (+)	3 (3a)	wbJNR_11
WB0947	'11.12.31	Unknown	Okayama	0.472 (+)	8.8×10^5	1.4×10^7	3 (3e)	wbJOY_11-1
WB0948	'11.12.31	Unknown	Okayama	0.363 (+)	1.2×10^4	3.1×10^6	3 (3e)	wbJOY_11-2
WB0949	'11.12.31	Unknown	Okayama	0.148 (−)	2.2×10^4	3.3×10^7	3 (3e)	wbJOY_11-3
WB0950	'11.12.31	Unknown	Okayama	0.139 (−)	6.3×10^3	7.5×10^5	3 (3e)	wbJOY_11-4
WB0976	'12.07.14	25	Gifu	>3.000 (+)	2.9×10^4	5.0×10^3	4	wbJGF_12-1
WB0977	'12.07.28	40	Gifu	2.545 (+)	1.4×10^5	3.5×10^6	4	wbJGF_12-2
WB0985	'12.10.14	10	Gifu	0.154 (−)	<200 (+)	<400 (+)	4	wbJGF_12-3
WB1180	'12.12.31	20	Nara	2.802 (+)	5.0×10^5	2.4×10^7	3 (3b)	wbJNR_12
WB1068	'13.01.13	Unknown	Okayama	0.103 (−)	<200 (+)	<400 (+)	3 (3b)	wbJOY_13-1
WB1072	'13.01.19	Unknown	Okayama	0.086 (−)	<200 (+)	<400 (+)	3 (3b)	wbJOY_13-2
WB1182	'13.01.24	15	Nara	2.420 (+)	(−)	<400 (+)	3 (3b)	wbJNR_13
WB1122	'13.02.02	40	Okayama	2.807 (+)	<200 (+)	1.1×10^4	3 (3b)	wbJOY_13-3
WB1123	'13.02.02	40	Okayama	2.767 (+)	<200 (+)	2.3×10^5	3 (3b)	wbJOY_13-4
WB1116	'13.02.12	15	Gifu	2.833 (+)	9.4×10^7	1.7×10^7	4	wbJGF_13-1
WB1117	'13.02.12	15	Gifu	0.458 (+)	1.1×10^6	1.1×10^7	4	wbJGF_13-2
WB1118	'13.02.12	15	Gifu	0.040 (−)	2.6×10^3	<400 (+)	4	wbJGF_13-3
WB1119	'13.02.12	15	Gifu	1.221 (+)	1.2×10^5	7.3×10^6	4	wbJGF_13-4
WB1203	'13.02.24	50	Gifu	0.013 (−)	<200 (+)	<400 (+)	4	wbJGF_13-5
WB1178	'13.03.16	60	Nagano	0.041 (−)	3.0×10^2	NA	Unclassified	wbJNN_13
WB1206	'13.07.01	30	Yamaguchi	>3.000 (+)	3.6×10^2	1.5×10^5	4	wbJYG_13

NA, not available.
[a] The cut-off value for the anti-HEV IgG was set at 0.274.

AF051830 and DQ450072) (Fan, 2009; van Cuyck et al., 2005; Wang et al., 2010) and if they differed from any other sequence in the dataset by <2% of the nucleotide positions, which left a total of 110 sequences (see Fig. 2 for accession nos.).

3. Results

3.1. The prevalence of anti-HEV IgG and HEV RNA in wild boars

Serum samples obtained from 511 wild boars and liver tissues from 523 boars were tested for the presence of anti-HEV IgG antibodies and HEV RNA. Overall, 58 boars (11.4%) were positive for anti-HEV IgG, with the prevalence differing by year of capture, ranging from 8.4% in 2012 to 14.5% in 2013, and 24 boars (4.2%) had HEV RNA detectable by the ORF2-457 PCR assay in the serum and/or liver specimens, the prevalence being different by year of capture, ranging from 1.5% in 2010 to 8.9% in 2013 (Table 1). Another RT-PCR assay, ORF2/3-137 PCR, confirmed the presence/absence of HEV RNA in all 566 wild boars tested. The anti-HEV IgG was detectable in wild boars captured in eight (66.7%) of the 12 prefectures studied (Table 2). Among the eight prefectures with anti-HEV IgG-positive boars, the prevalence of boar anti-HEV IgG differed markedly by geographic region (prefecture), from 6.5% to 40% (median, 13.1%). Boars with ongoing HEV infections were found in seven prefectures (58.3%), with the prevalence ranging from 1.6% to 10% (median, 4.2%).

3.2. Characteristics of the 24 wild boars that were positive for HEV RNA in the serum and/or liver specimens

Serum samples were available from all 24 boars with ongoing HEV infections, and 14 boars (58.3%) tested positive for anti-HEV IgG, with an OD value ranging from 0.363 to >3.000 (Table 3). HEV RNA was detectable in the serum samples of 23 of the 24 boars tested, although the titer was variable, being 9.4×10^7 copies/ml at the highest. HEV RNA was detectable in all 22 liver specimens that were available, including five specimens with a high HEV RNA titer on the order of 10^7 copies/mg (Table 3).

3.3. Genetic heterogeneity of boar HEV isolates recovered from wild boars in Japan

The amplification products of ORF2 (412 nt) from 24 HEV-infected boars were sequenced and compared (Table 4). Among the 24 boar HEV isolates obtained from the infected boars, 13 isolates (54%) were close to genotype 3 HEV isolates, with nucleotide sequence identities of 81.7–96.8%, but were only 73.7–80.2% similar to known HEV isolates of other genotypes (1, 2 and 4). The 13 genotype 3 isolates shared identities ranging from 81.3% to 100% (86.9 ± 0.5%) within the 412-nt ORF2 sequence with each other, and were further classified into subgenotype 3a ($n = 1$), 3b ($n = 8$) or 3e ($n = 4$), sharing identities of 93.4%, 90.2–94.4% and 96.1–96.8%, respectively, with the representative 3a (AF060669), 3b (AP003430) and 3e (AB248520) isolates.

Ten isolates were 83.9–85.9% similar to the prototype genotype 4 HEV isolate (AJ272108), but were only 74.7–81.7% similar to known HEV isolates of the other three genotypes (1–3). These 10 isolates shared identities of 87.6–100 (97.1 ± 0.5)% within the 412-nt ORF2 sequence with each other. These results indicated that 13 and 10 of the Japanese boar HEV isolates obtained in the present study were classifiable into genotype 3 (3a, 3b or 3e) and genotype 4, respectively. Of interest, however, the remaining isolate (wbJNN_13) was only 77.0–81.5% identical to all genotype 1–4 isolates, 76.8% identical to a prototype rabbit HEV isolate (FJ906895) and 82.2% and 81.0% similar to two other wild boar HEV isolates (JBOAR135-Shiz09 and wbJOY_06, respectively), which are suggested to belong to additional genotypes (5 and 6, respectively) (Bouquet et al., 2012; Smith et al., 2013; Takahashi et al., 2010, 2011), within the 412-nt ORF2 sequence. The wbJNN_13 isolate shared only 57.7–64.1% nucleotide sequence identities with rat,

Table 4
Comparison of the identity (%) within the 412 nt ORF2 sequence of the 24 boar HEV isolates obtained in the present study with entire or near-entire sequences of previously reported HEV isolates.

HEV isolate	Accession no.	Genotype 3			Genotype 4 (n = 10)	Unclassified (n = 1)
		Subgenotype 3a (n = 1)	Subgenotype 3b (n = 8)	Subgenotype 3e (n = 4)		
Genotype 1	M73218	79.0	77.8–80.2 (79.3 ± 1.1)	77.6–78.3 (78.1 ± 0.4)	78.8–81.7 (81.4 ± 0.5)	77.6
Genotype 2	M74506	78.5	73.7–76.6 (76.0 ± 1.0)	76.1–76.3 (76.3 ± 0.1)	74.7–76.6 (76.2 ± 0.5)	78.6
Genotype 3						
Subgenotype 3a	AF060669	93.4	86.4–89.5 (88.4 ± 1.3)	83.2–83.4 (83.4 ± 0.1)	79.0–81.7 (81.1 ± 0.7)	77.6
Subgenotype 3b	AP003430	87.3	90.2–94.4 (91.3 ± 1.4)	84.3–84.6 (84.5 ± 0.4)	78.8–81.5 (81.2 ± 0.9)	77.1
Subgenotype 3e	AB248520	83.0	81.7–84.6 (83.0 ± 0.8)	96.1–96.8 (96.6 ± 0.4)	78.2–79.2 (78.3 ± 0.3)	77.0
Genotype 4	AJ272108	80.0	78.5–79.3 (79.0 ± 0.3)	78.0–78.1 (78.0 ± 0.1)	83.9–85.9 (85.7 ± 0.6)	81.5
Wild boar HEV (Genotype 5)	AB573435	80.4	78.3–79.5 (79.0 ± 1.0)	80.3–80.4 (80.4 ± 0.1)	78.1–79.6 (79.0 ± 0.4)	82.2
Wild boar HEV (Genotype 6)	AB602441	80.4	77.6–80.5 (78.7 ± 1.0)	77.3	78.6–79.8 (79.6 ± 0.4)	81.0
Rabbit HEV	FJ906895	80.0	80.2–83.6 (81.8 ± 1.2)	79.3–79.5 (79.5 ± 0.1)	78.5–79.3 (78.6 ± 0.3)	76.8
Rat HEV	GU345042	66.9	63.2–66.5 (65.7 ± 1.1)	65.6–66.3 (66.1 ± 0.4)	62.8–65.5 (64.9 ± 0.7)	62.3
Ferret HEV	JN948606	64.9	65.5–66.5 (66.0 ± 0.4)	64.4	64.1–66.3 (65.6 ± 0.6)	64.1
Bat HEV	JQ001749	59.2	58.6–60.7 (59.4 ± 0.8)	58.0	56.1–58.1 (57.4 ± 0.4)	57.7
Avian HEV	AY535004	55.5	55.6–59.0 (57.4 ± 1.5)	55.3–55.8 (55.7 ± 0.3)	55.8–56.8 (55.9 ± 0.3)	57.7

ferret, bat and avian HEV strains. These results suggest that the wbJNN_13 isolate can be classified into a previously unidentified genetic lineage.

The phylogenetic tree constructed by the neighbor-joining method based on the 412-nt ORF2 sequences (Fig. 1), confirmed that 23 of the 24 boar HEV isolates obtained in the present study belonged to genotype 3 or 4, and that the 13 genotype 3 isolates were classifiable into subgenotype 3a, 3b or 3e, with a bootstrap value of 98%, 72% and 99%, respectively. The tree also showed that the remaining isolate (wbJNN_13) was markedly different from all known HEV isolates of genotypes 1–4 and the two wild boar strains (JBOAR135-Shiz09 and wbJOY_06).

3.4. Analysis of the full-length genome of the wbJNN_13 strain

The wbJNN_13 isolate was recovered from a serum sample from a 60 kg female wild boar that had been caught in a forest in Ueda city, Nagano Prefecture (Supplementary Fig. 1), located in the central part of Honshu Island on March 16, 2013. The wbJNN_13 isolate had a genomic length of 7247 nt, excluding the poly(A) tract at the 3′ terminus, and possessed three major ORFs, similar to reported mammalian and avian HEV isolates (Meng et al., 2012). ORF1, ORF2, and ORF3 encoded 1709 aa (nt 26–5152), 660 aa (nt 5194–7173) and 112 aa (nt 5186–5521), respectively. The 5′ and 3′ untranslated regions of wbJNN_13 comprised 25 nt and 74 nt [excluding the poly(A) tail], respectively. Upon comparison with the HEV genomes of genotypes 1–4 and two wild boar HEV genomes whose entire nucleotide sequences are already known (see Fig. 2 for accession nos.), the wbJNN_13 genome shared nucleotide sequence identities of only 73.1–74.5% with human genotype 1 HEV (n = 11), 73.5% with human genotype 2 HEV (n = 1), 71.8–77.6% with genotype 3 HEV of human, swine, wild boar, deer, mongoose and rabbit origin (n = 64), 76.2–77.6% with genotype 4 HEV of human, swine, and wild boar origin (n = 32), and 78.1–80.4% with two wild boar HEV sequences. In addition, wbJNN_13 was only 53.9–59.1% identical to rat, ferret, bat and avian HEV strains over the entire genome (Table 5).

The nucleotide sequences of ORF1, ORF2 and ORF3 of wbJNN_13 were compared with those of other HEV genotypes, and the identities among them are shown in Table 5. Together, these results indicate that the wbJNN_13 isolate is distantly related to the known HEV isolates of genotypes 1–4, including seven boar isolates of genotypes 3 and 4 and two wild boar isolates (JBOAR135-Shiz09 and wbJOY_06), and is clearly distinct from the previously reported rat, ferret, bat and avian HEV isolates. The phylogenetic tree constructed by the neighbor-joining method with the Kimura-2-parameter model, based on overlapping the entire or nearly entire genomic sequence of the 111 HEV isolates, using a prototype rat HEV strain as an outgroup, confirmed that wbJNN_13 does not belong to any of the four known genotypes or two additional genotypes from wild boars, most likely being classifiable into an unrecognized genetic lineage (Fig. 2).

In an attempt to improve the phylogenetic analysis, a maximum-likelihood phylogenetic tree including the 111 full or near-full HEV genomes, was constructed with the PHYML method using the GTR+G substitution model selected by MODELTEST under the corrected Akaike information criterion (AICc). The tree further confirmed that wbJNN_13 is distantly related to all known HEV isolates of genotypes 1–4 and the two wild boar isolates of additional genotypes (Supplementary Fig. 3).

To investigate the possible presence of recombination in the wbJNN_13 genome, a window scanning analysis of aligned HEV genomes was performed using the Simplot software program. The wbJNN_13 isolate was slightly closer to a provisional genotype 6 from a wild boar (AB602441) than the remaining four genotypes (1–4) and a provisional genotype 5 from wild boar (AB573435), but no significant evidence of recombination between genotype 6 from the wild boar and the other five genotypes was revealed by this method (Supplementary Fig. 4).

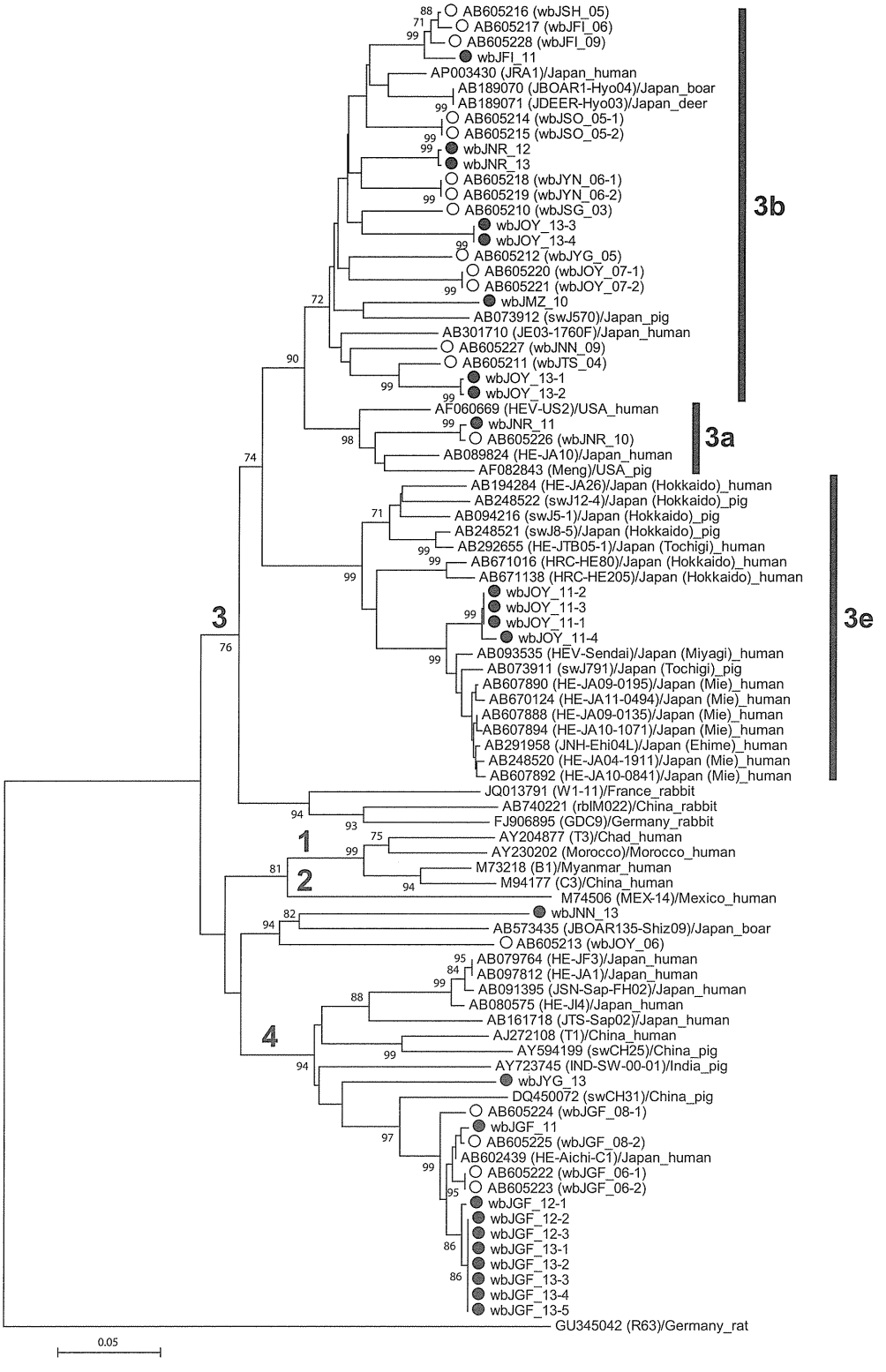


Fig. 1. The phylogenetic tree constructed by the neighbor-joining method based on the 412-nt ORF2 sequences of 83 HEV isolates of genotypes 1–4 and three HEV variants from wild boars, including the wbJNN_13 isolate obtained in the present study, using a rat HEV isolate (accession no. GU345042) as an outgroup. Nineteen wild boar isolates obtained in the previous study (Sato et al., 2011) are indicated by open circles, and 24 wild boar isolates obtained in the present study are shown by closed circles. Forty-two representative isolates from humans, pigs, wild boars, deer and rabbits were included for comparison, with the DDBJ/EMBL/GenBank accession number and isolate name given in parentheses. After the slash, the name of the country where the HEV strain was isolated and the name of animal species from which the HEV strain was isolated, are shown. For 3e strains, the name of prefecture where the HEV strain was isolated, is also indicated in parenthesis after Japan. Subgenotypes 3a, 3b and 3e within genotype 3 are highlighted by vertical bars. Bootstrap values (≥70%) are indicated as a percentage of the data obtained from 1000 resamplings. Bar, 0.05 substitutions per site.

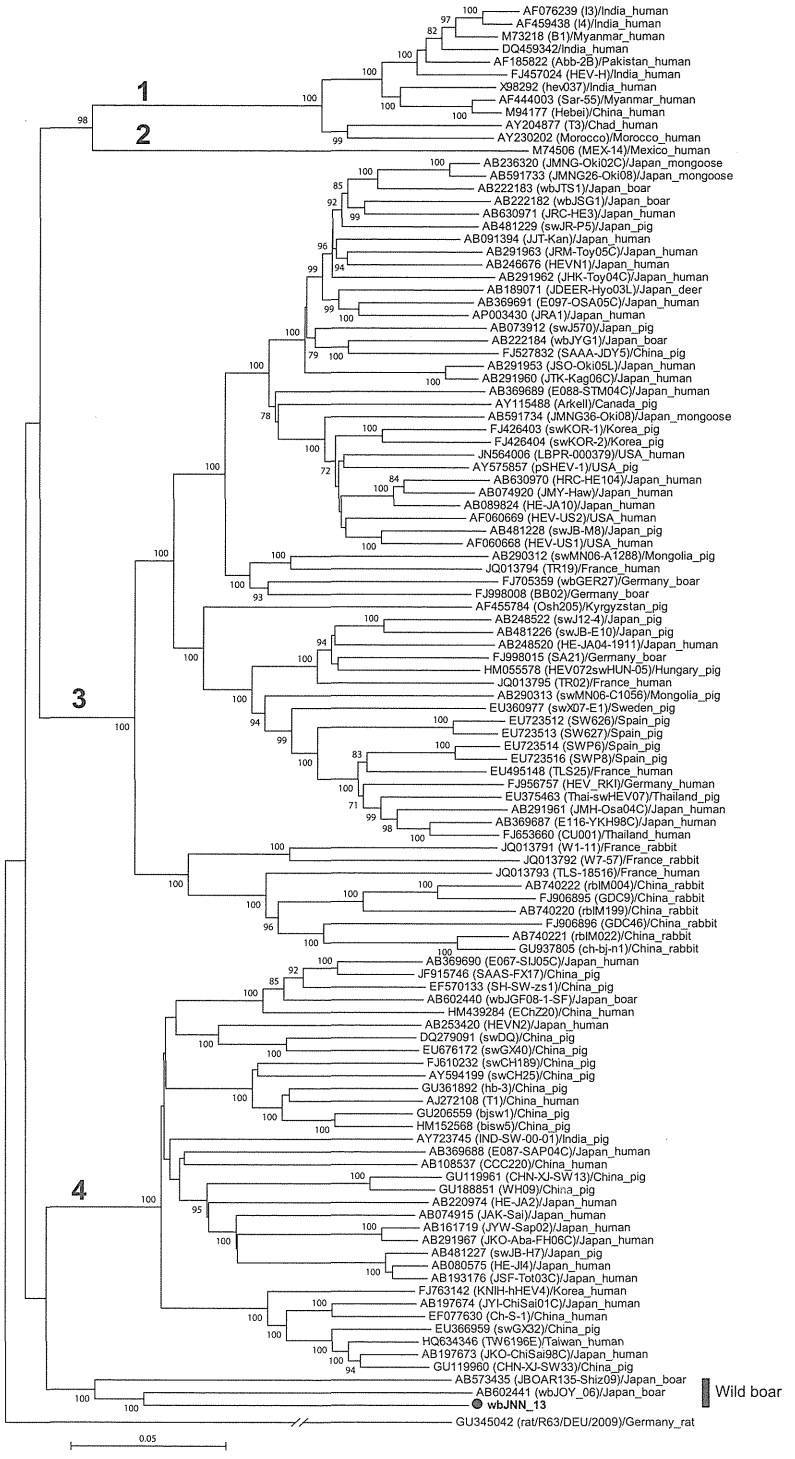


Fig. 2. The phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 108 HEV isolates of genotypes 1–4 and three HEV variants from wild boars (highlighted with a vertical bar), including the wbJNN_13 isolate obtained in the present study, which is indicated by a closed circle, using a rat HEV isolate (accession no. GU345042) as an outgroup. Fifty-four human isolates of genotypes 1–4, 35 swine isolates of genotypes 3 and 4, seven wild boar isolates of genotypes 3 and 4, one deer isolate and three mongoose isolates of genotype 3 and eight rabbits HEV isolates, as well as two wild boar sequences (JBOAR135-Shiz09 and wbJOY_06) whose entire or nearly entire sequence has been reported, and which differed from each other by ≥2% of the nucleotide positions over the entire genome, were included for comparison, with the DDBJ/EMBL/GenBank accession number and isolate name given in parentheses. After the slash, the name of the country where the HEV strain was isolated and the name of animal species from which the HEV strain was isolated, are shown. Bootstrap values (≥70%) are indicated as a percentage of the data obtained from 1000 resamplings. Bar, 0.05 substitutions per site.

The wbJNN_13 genome possessed a nucleotide insertion of C, just after a cis-reactive element with the sequence UGAAUAA-CAUGU [nt 5153–5164, corresponding to nt 5105–5116 of the genotype 1 HEV (AF444003, Sar-55)], which has been reported to be critical for the synthesis of 2.2-kb subgenomic RNA (Graff et al., 2005, 2006). This is similar to the genomes of genotype 4 (Inoue et al., 2006a; Wang et al., 2000) and the two wild boar strains (genotypes 5 and 6), although they harbored U in place of C at the same nucleotide position (Fig. 3). Of interest, the wbJNN_13 genome had A-to-G and G-to-U mutations at nt 5180 and nt 5182,

Table 5
Comparison of the identity (%) over the entire genome, and the ORF1, ORF2 or ORF3 sequences of the wbJNN_13 isolate obtained in the present study with the entire or near-entire sequences of previously reported HEV isolates.

HEV isolate	No. of isolates compared	Identity (%)			
		Entire genome	ORF1	ORF2	ORF3
Genotype 1[a]	11	73.1–74.5 (74.0 ± 0.4)	72.1–73.1 (72.6 ± 0.3)	77.3–78.1 (77.8 ± 0.3)	83.6–85.4 (84.6 ± 0.5)
Genotype 2[a]	1	73.5	71.9	77.5	82.4
Genotype 3[a]	64	71.8–77.6 (74.7 ± 0.6)	72.4–76.2 (72.1 ± 0.5)	77.2–81.3 (78.7 ± 0.7)	80.6–87.5 (84.3 ± 1.2)
Humans	26	74.1–77.6 (74.9 ± 0.7)	72.5–76.2 (73.2 ± 0.7)	77.2–81.3 (78.7 ± 0.8)	81.8–87.5 (84.7 ± 1.0)
Swine	20	74.1–75.3 (74.7 ± 0.3)	72.6–73.8 (73.1 ± 0.3)	77.4–80.0 (78.7 ± 0.7)	83.0–85.6 (84.3 ± 0.8)
Wild boars	6	74.2–74.9 (74.6 ± 0.2)	72.6–73.2 (72.9 ± 0.2)	78.1–79.1 (78.7 ± 0.4)	83.3–85.3 (84.6 ± 0.7)
Deer	1	74.3	72.9	78.7	85.1
Mongooses	3	74.5–75.0 (74.7 ± 0.3)	72.6–73.2 (72.9 ± 0.3)	78.3–79.3 (78.8 ± 0.5)	84.5–85.4 (85.0 ± 0.5)
Rabbits	8	71.8–74.6 (74.0 ± 0.9)	72.4–73.2 (72.8 ± 0.3)	77.3–78.7 (78.3 ± 0.5)	80.6–84.1 (82.4 ± 1.2)
Genotype 4[a]	32	76.2–77.6 (76.8 ± 0.3)	74.3–76.5 (75.4 ± 0.5)	79.6–81.6 (80.8 ± 0.5)	84.5–89.3 (87.6 ± 1.0)
Humans	16	76.4–77.6 (77.0 ± 0.3)	74.3–76.5 (75.6 ± 0.5)	79.6–81.6 (80.8 ± 0.6)	84.5–89.3 (87.4 ± 1.3)
Swine	15	76.2–77.1 (76.7 ± 0.2)	74.3–75.9 (75.3 ± 0.3)	80.4–81.3 (80.9 ± 0.3)	86.3–89.0 (87.8 ± 0.7)
Wild boars	1	76.8	75.1	80.5	88.1
Wild boar (Genotype 5)[a]	1	78.1	76.6	82.8	88.1
Wild boar (Genotype 6)[a]	1	80.4	79.1	83.2	89.9
Rat[b]	5	55.8–59.1 (57.4 ± 1.5)	57.7–58.6 (57.9 ± 0.4)	60.0–61.8 (60.8 ± 0.7)	54.4–55.7 (55.0 ± 0.6)
Ferret[c]	2	57.5–58.3 (57.9 ± 0.6)	56.8–58.0 (57.4 ± 0.8)	59.7–59.9 (59.8 ± 0.1)	47.8–48.9 (48.4 ± 0.8)
Bat[d]	1	53.9	53.2	56.0	52.3
Avian[e]	7	54.9–55.8 (55.2 ± 0.3)	54.6–56.0 (55.2 ± 0.5)	53.2–55.9 (54.6 ± 1.0)	46.6–51.7 (48.7 ± 1.8)

[a] See Fig. 2 for accession nos.
[b] The accession nos. for the five rat isolates compared are: GU345042, GU345043, JN167537, JN167538 and JX120573.
[c] The accession nos. for the two ferret isolates compared are: JN998606 and JN998607.
[d] The accession no. for the bat isolate compared is: JQ001749.
[e] The accession nos. for the seven avian isolates compared are: AM943646, AM943647, AY535004, EF206691, GU954430, JN597006 and JN997392.

respectively, with conversion from the methionine codon (AUG) to GUU, which was presumed to encode a shortened ORF3 protein of 112 aa (Fig. 3).

4. Discussion

The present study indicated that HEV infection is prevalent among wild boars in Japan, with the overall prevalence of anti-HEV IgG and HEV RNA being 11.4% and 4.2%, respectively, corroborating our previous study conducted from 2003 to 2010 reporting that anti-HEV IgG and HEV RNA were detected in 8.1% and 3.3% of the studied boars (Sato et al., 2011), respectively, and that polyphyletic HEV strains of genotypes 3 and 4, similar to those obtained from humans and domestic pigs in Japan, are also circulating among wild boars (Takahashi et al., 2003; Takahashi and Okamoto, 2013). In this study, we attempted to isolate a wbJOY_06-type (genotype 6) strain in Okayama Prefecture, where the wbJOY_06 strain had been identified (Sato et al., 2011; Takahashi et al., 2011), but this strain was not found in the serum or liver specimens from a total of 244 wild boars captured, although eight (3.3%) wild boars had detectable HEV RNA, suggesting that the wbJOY_06-like strain is rare even in Okayama Prefecture. Of interest, however, we identified a novel HEV variant (wbJNN_13) that may be classifiable into an additional genotype, next to those of two other wild boar HEV strains [JBOAR135-Shiz09 (AB573435) and wbJOY_06 (AB602441)] that are suggested to belong to genotype 5 and genotype 6, respectively (Bouquet et al., 2012; Smith et al., 2013; Takahashi et al., 2010, 2011) in another prefecture (Nagano). Nagano Prefecture is located approximately 500 km away from the place where the wbJOY_06 was isolated (Sato et al., 2011) and 200 km away from the location where the JBOAR135-Shiz09 strain was isolated (Takahashi et al., 2010), suggesting the independent distribution of these three unique wild boar HEV strains in three distinct areas in Japan.

Wild boars are indigenous to many countries worldwide, including Japan, posing concerns about ecological and infectious diseases. The consumption of boar meat and viscera provides an increased risk for the transmission of HEV from wild boars to humans (Li et al., 2005; Matsuda et al., 2003; Sonoda et al., 2004). The anti-HEV seropositivity in wild boars varied from 17% to 50.3%, with HEV RNA detected in 2.5–25% of samples in Germany, Spain, Italy,

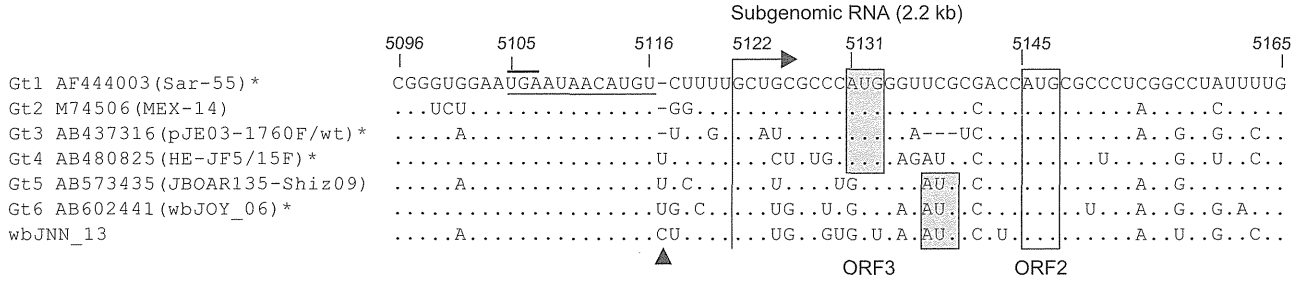


Fig. 3. The comparison of the HEV sequences containing putative initiation codons for ORF2 and ORF3, and the reported initiation site of the subgenomic RNA. Representative genotype 1–4 HEV isolates (Sar-55, MEX-14, pJE03-1760F/wt and HE-JF5/15F) and two boar HEV isolates (JBOAR135-Shiz09 and wbJOY_06) which are suggested to be classifiable into genotypes 5 and 6, respectively, were compared. The reported initiation site of the subgenomic RNA of Sar-55, pJE03-1760F/wt, HE-JF5/15F and wbJOY_06 (indicated by asterisks) is illustrated by a vertical bar with an arrow (Graff et al., 2006; Ichiyama et al., 2009; Takahashi et al., 2011). Putative initiation codons of ORF3 are indicated by shaded boxes, and those of ORF2 are shown by open boxes. The inserted U residue that was found in the genotype 4 isolate (HE-JF5/15F) and two reported wild boar isolates (JBOAR135-Shiz09 and wbJOY_06) and the inserted C that was found in the wbJNN_13 isolate, are marked with a closed triangle. The termination codon (UGA) of ORF1 has a line drawn above it, and a cis-reactive element of 12 nt (nt 5105–5116) (Graff et al., 2005, 2006) is underlined. The dots indicate nucleotides that are identical to the top sequence, and dashes denote a deletion of nucleotides. The nucleotide positions are in accordance with the Sar-55 genome.

Australia and Hungary (de Deus et al., 2008; Forgach et al., 2010; Kaba et al., 2010; Martelli et al., 2008; Reuter et al., 2009; Schielke et al., 2009), and most strains of HEV recovered from wild boars worldwide belonged to genotype 3. In Japan, the presence of anti-HEV antibodies in wild boars was also widely variable, ranging from 4.5% to 34.3% based on the geographic regions with HEV RNA detection rates ranged from 1.1% to 13.3%. Japan is unique in that not only genotype 3 HEV strains, but also genotype 4 strains, have been recovered from wild boars (Sato et al., 2011; Takahashi and Okamoto, 2013). These characteristics of HEV infection among wild boars in Japan were confirmed in the present study, and the genotype 4 HEV strains isolated in 2006 and 2008 in a previous study in Gifu Prefecture (Sato et al., 2011), as well as in 2011–2013 in the present study, formed a separate cluster supported by a high bootstrap value of 99%, indicating spread of a regional strain. The Gifu genotype 4 strains from wild boars segregated into a cluster together with a human HEV isolate of the same genotype (HE-Aichi-C1) that was 99.8% identical within the 412-nt ORF2 sequence to wild boar strains in Gifu (Fig. 1). The HE-Aichi-C1 strain was recovered from a male patient living in Aichi Prefecture, adjacent to Gifu Prefecture in the north, who had consumed undercooked wild boar meat approximately 30 days before the onset of acute hepatitis E (Shimizu et al., 2006). The long-term persistence of the same HEV strain among wild boars in the same area and the presence of hepatitis E patients who were infected with the same strain after consuming undercooked wild boar meat further the notion that the wild boar is an important reservoir of HEV in humans.

Although the presence of 10 subgenotypes (3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i and 3j) within genotype 3 has been proposed (Lu et al., 2006), only three subgenotypes; 3a, 3b and 3e, have been identified in hepatitis patients and domestic pigs in Japan (Okamoto et al., 2003; Takahashi et al., 2003). Of these, 3a (3us) and 3b (3jp) are frequently observed, while 3e (3sp) is rare in Japan (Nakano et al., 2012). The 3e strains have been isolated from HEV-infected humans and pigs in restricted areas of Japan, including Hokkaido, Miyagi, Tochigi, Mie and Ehime (see Supplementary Fig. 1) and those from HEV-infected wild boars in Mie (Nakano et al., 2013). Four 3e HEV strains identified from wild boars in Okayama Prefecture for the first time in the present study were 82.2–97.3 (91.9 ± 3.5)% identical to 37 3e Japanese strains, but only 85.1–91.7 (88.9 ± 1.8)% similar to European 3e strains (22 strains isolated in Italy, France, Germany, Hungary and the United Kingdom, retrievable from the DDBJ/GenBank/EMBL databases as of September 30, 2013) within the overlapping 304–412 nt sequence.

Subgenotype 3e strains are usually detected in European countries (Widen et al., 2011). A coalescent analysis indicated that the import of a breed of large-race pigs from Europe since the 1960s may be responsible for the introduction of subgenotype 3e isolates to Japan, and a phylogenetic analysis suggested that the direction of gene flow of HEV subgenotype 3e was from swine to wild boars (Nakano et al., 2013). The indigenization and spread of HEV in Japan are likely associated with the popularization of eating pork. Of note, a group of four wild boars hunted in the forest of Niimi city, Okayama on the same day (December 31, 2011) were infected with essentially the same 3e strain sharing nucleotide sequence identities of 99.2–100%, suggesting the occurrence of mass HEV infection in a group of wild boars, most likely via consumption of HEV-contaminated food and/or water in their wild life. The 3e isolates in Okayama were 95.6–97.3 (96.6 ± 0.4)% similar to those in Ehime, Mie, and Miyagi, but only 88.8–91.2 (90.0 ± 0.7)% identical to those in Hokkaido, suggesting region-dependent spread of 3e strains in Japan, although two distinct 3e strains were identified in Tochigi (Fig. 1). The HEV strains obtained from wild boars sampled in two other cities in Okayama in 2013 segregated into subgenotype 3b. Two strains sampled in the same city were nearly identical within the 412-nt ORF2 sequence (99.7%, respectively). However, the 3b strains obtained in the two different cities shared only 86.6–87.1% nucleotide sequence identities, suggesting sequestered distribution of various HEV strains among wild boars even in a prefecture in Japan.

Currently, the classification of HEV variants is devoid of a consensus definition for genotyping or for deeper taxonomic grouping into species and genera that could incorporate more recently identified viruses assigned to the *Hepeviridae* family that infect rats, ferrets, bats, chickens and fish (Batts et al., 2011; Drexler et al., 2012; Johne et al., 2010; Kumar et al., 2013; Meng, 2013; Raj et al., 2012; Zhao et al., 2009). Early classification schemes were based on partial genome sequences, with a suggestion that variants differing by >20% in the nucleotides in the ORF2 region should be classified into different genotypes (Worm et al., 2002). Since then, nucleotide sequence data from various HEV strains have accumulated, and more than 200 full-length or nearly full-length genomic sequences of HEV have been deposited in the DDBJ/EMBL/GenBank databases. The analyses of complete genomic sequences with or without a variety of subgenomic sequences led to the conclusion that HEV could be divided into four genotypes, as defined by the International Committee on the Taxonomy of Viruses (ICTV) (Meng et al., 2012), and 12 subtypes (Zhai et al., 2006), 24 subtypes (Lu et al., 2006) or at least seven subclusters or subgroups (Okamoto, 2007). However, the designations given in these studies still give rise to confusion due to a lack of agreed-upon criteria for the designation of subgenotypes (subtypes) (Bouquet et al., 2012).

Recently, Smith et al. (2013) reexamined published complete genome sequences, and proposed that human HEV strains and those from swine, wild boars, deer and mongooses, can be divided into six genotypes (genotypes 1–4 and two additional genotypes from wild boars). The presence of two additional genotypes represented by two isolates from wild boars (JBOAR135-Shiz09 and wbJOY_06) extends the conclusion of the previous analyses of wild boar isolates based on the complete genome sequences (Bouquet et al., 2012; Takahashi et al., 2011). The present phylogenetic analyses (Fig. 2 and supplementary Fig. 3) and pairwise comparisons (Table 5) between the complete genomic sequences indicated that the wbJNN_13 HEV strain identified from a wild boar in the present study is genetically highly divergent. However, it seems too early to conclude that wbJNN_13 can be classifiable into a novel genotype, considering the following conditions: (i) the classification of HEV variants into genotypes/subgenotypes lacks consensus criteria; (ii) there is controversy regarding the classification of variants isolated from rabbits, closely related to genotype 3, and some studies have concluded that these isolates represent an additional genotype (Geng et al., 2011; Izopet et al., 2012; Zhao et al., 2009), while others considered them to be a subtype of genotype 3 (Cossaboom et al., 2011; Meng et al., 2012; Takahashi et al., 2011), or to occupy an intermediate position (Smith et al., 2013); (iii) divergent HEV strains that are most closely related to those in humans are expected to be increasingly identified, as exemplified by those from wild boars in our previous (Takahashi et al., 2011) and present studies, and their species tropism remains unknown and (iv) as HEV variants have been identified from rats, ferrets, bats, chickens and fish (cutthroat trout) (Batts et al., 2011; Drexler et al., 2012; Johne et al., 2010; Kumar et al., 2013; Meng, 2013; Raj et al., 2012), the range of HEV-infected hosts is expected to continue expanding (Yugo and Meng, 2013). These conditions and the difference of 19.6% (less than 20%) between wbJNN_13 and wbJOY_06 over the entire genome may suggest that wbJNN_13 should be regarded as a markedly divergent variant of wbJOY_06 (genotype 6) or placed in an intermediate position of genotype and subgenotype. The findings obtained in the present study will aid in future re-classification of divergent HEVs infecting ever-expanding hosts.

In conclusion, the present study revealed that wild boars in Japan have an overall prevalence of HEV RNA of 4.2%, and harbor

various HEV strains of genotypes 3 and 4 and a highly divergent strain (wbJNN_13), and suggested the presence of multiple genetic lineages of HEV strains that may be indigenous to wild boars in Japan. Further efforts are therefore warranted to clarify whether the boar HEV strains that are markedly divergent from genotype 3 and 4 HEV strains are transmissible to humans and harbor the potential for zoonotic infection. More work is needed to search for new HEV strains that may be classified into novel genotype(s) not only in wild boars but also in humans and other animals, in order to deepen our understanding about the extent of the genomic heterogeneity of HEV strains, and to determine consistent criteria that could be used for the assignment of virus genotypes/subgenotypes (subtypes).

Acknowledgments

We thank all of the physicians and hunters who were involved in the collection of serum and/or liver specimens from wild boars. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.12.014.

References

Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (*Oncorhynchus clarkii*). Virus Res. 158 (1–2), 116–123.

Bouquet, J., Cherel, P., Pavio, N., 2012. Genetic characterization and codon usage bias of full-length Hepatitis E virus sequences shed new lights on genotypic distribution, host restriction and genome evolution. Infect. Genet. Evol. 12 (8), 1842–1853.

Chen, S.H., Su, S.Y., Lo, C.Z., Chen, K.H., Huang, T.J., Kuo, B.H., Lin, C.Y., 2009. PALM: a paralleled and integrated framework for phylogenetic inference with automatic likelihood model selectors. PLoS One 4 (12), e8116.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D., Gerolami, R., 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. J. Infect. Dis. 202 (6), 825–834.

Cossaboom, C.M., Cordoba, L., Dryman, B.A., Meng, X.J., 2011. Hepatitis E virus in rabbits, Virginia, USA. Emerg. Infect. Dis. 17 (11), 2047–2049.

de Deus, N., Peralta, B., Pina, S., Allepuz, A., Mateu, E., Vidal, D., Ruiz-Fons, F., Martin, M., Gortazar, C., Segales, J., 2008. Epidemiological study of hepatitis E virus infection in European wild boars (*Sus scrofa*) in Spain. Vet. Microbiol. 129 (1–2), 163–170.

Drexler, J.F., Seelen, A., Corman, V.M., Fumie Tateno, A., Cottontail, V., Melim Zerbinati, R., Gloza-Rausch, F., Klose, S.M., Adu-Sarkodie, Y., Oppong, S.K., Kalko, E.K., Osterman, A., Rasche, A., Adam, A., Muller, M.A., Ulrich, R.G., Leroy, E.M., Lukashev, A.N., Drosten, C., 2012. Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. J. Virol. 86 (17), 9134–9147.

Emerson, S.U., Nguyen, H.T., Torian, U., Burke, D., Engle, R., Purcell, R.H., 2010. Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. J. Virol. 84 (18), 9059–9069.

Emerson, S.U., Purcell, R.H., 2013. Hepatitis E virus. In: Knipe, D.M., Howley, P.M., Cohen, J.I., Griffin, D.E., Lamb, R.A., Martin, M.A., Racaniello, V.R., Roizman, B. (Eds.), Fields Virology, 2, sixth ed. Lippincott Williams & Wilkins, Philadelphia, pp. 2242–2258.

Fan, J., 2009. Open reading frame structure analysis as a novel genotyping tool for hepatitis E virus and the subsequent discovery of an inter-genotype recombinant. J. Gen. Virol. 90 (Pt 6), 1353–1358.

Forgach, P., Nowotny, N., Erdelyi, K., Boncz, A., Zentai, J., Szucs, G., Reuter, G., Bakonyi, T., 2010. Detection of hepatitis E virus in samples of animal origin collected in Hungary. Vet. Microbiol. 143 (2–4), 106–116.

Geng, Y., Zhao, C., Song, A., Wang, J., Zhang, X., Harrison, T.J., Zhou, Y., Wang, W., Wang, Y., 2011. The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China. Infect. Genet. Evol. 11 (2), 476–482.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R., 2010. A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. 38 (Web Server issue), W695–W699.

Graff, J., Nguyen, H., Yu, C., Elkins, W.R., St Claire, M., Purcell, R.H., Emerson, S.U., 2005. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. J. Virol. 79 (11), 6680–6689.

Graff, J., Torian, U., Nguyen, H., Emerson, S.U., 2006. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J. Virol. 80 (12), 5919–5926.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.

Huang, Y.W., Opriessnig, T., Halbur, P.G., Meng, X.J., 2007. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. J. Virol. 81 (6), 3018–3026.

Ichiyama, K., Yamada, K., Tanaka, T., Nagashima, S., Jirintai, S., Takahashi, M., Okamoto, H., 2009. Determination of the 5′-terminal sequence of subgenomic RNA of hepatitis E virus strains in cultured cells. Arch. Virol. 154 (12), 1945–1951.

Inoue, J., Nishizawa, T., Takahashi, M., Aikawa, T., Mizuo, H., Suzuki, K., Shimosegawa, T., Okamoto, H., 2006a. Analysis of the full-length genome of genotype 4 hepatitis E virus isolates from patients with fulminant or acute self-limited hepatitis E. J. Med. Virol. 78 (4), 476–484.

Inoue, J., Takahashi, M., Yazaki, Y., Tsuda, F., Okamoto, H., 2006b. Development and validation of an improved RT-PCR assay with nested universal primers for detection of hepatitis E virus strains with significant sequence divergence. J. Virol. Methods 137 (2), 325–333.

Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., Kamar, N., Abravanel, F., Guerin, J.L., 2012. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, france. Emerg. Infect. Dis. 18 (8), 1274–1281.

Johne, R., Plenge-Bonig, A., Hess, M., Ulrich, R.G., Reetz, J., Schielke, A., 2010. Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J. Gen. Virol. 91 (Pt 3), 750–758.

Kaba, M., Davoust, B., Marie, J.L., Colson, P., 2010. Detection of hepatitis E virus in wild boar (*Sus scrofa*) livers. Vet. J. 186 (2), 259–261.

Kumar, S., Subhadra, S., Singh, B., Panda, B.K., 2013. Hepatitis E virus: the current scenario. Int. J. Infect. Dis. 17 (4), e228–e233.

Li, T.C., Chijiwa, K., Sera, N., Ishibashi, T., Etoh, Y., Shinohara, Y., Kurata, Y., Ishida, M., Sakamoto, S., Takeda, N., Miyamura, T., 2005. Hepatitis E virus transmission from wild boar meat. Emerg. Infect. Dis. 11 (12), 1958–1960.

Lu, L., Li, C., Hagedorn, C.H., 2006. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev. Med. Virol. 16 (1), 5–36.

Martelli, F., Caprioli, A., Zengarini, M., Marata, A., Fiegna, C., Di Bartolo, I., Ruggeri, F.M., Delogu, M., Ostanello, F., 2008. Detection of hepatitis E virus (HEV) in a demographic managed wild boar (*Sus scrofa scrofa*) population in Italy. Vet. Microbiol. 126 (1–3), 74–81.

Matsuda, H., Okada, K., Takahashi, K., Mishiro, S., 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J. Infect. Dis. 188 (6), 944.

Meng, X.J., 2011. From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. Virus Res. 161 (1), 23–30.

Meng, X.J., 2013. Zoonotic and foodborne transmission of hepatitis E virus. Semin. Liver Dis. 33 (1), 41–49.

Meng, X.J., Anderson, D., Arankalle, V.A., Emerson, S.U., Harrison, T.J., Jameel, S., Okamoto, H., 2012. Hepeviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses ed. Elsevier/Academic Press, Oxford, pp. 1021–1028.

Mizuo, H., Suzuki, K., Takikawa, Y., Sugai, Y., Tokita, H., Akahane, Y., Itoh, K., Gotanda, Y., Takahashi, M., Nishizawa, T., Okamoto, H., 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J. Clin. Microbiol. 40 (9), 3209–3218.

Nakano, T., Takahashi, K., Arai, M., Okano, H., Kato, H., Ayada, M., Okamoto, H., Mishiro, S., 2013. Identification of European-type hepatitis E virus subtype 3e isolates in Japanese wild boars: molecular tracing of HEV from swine to wild boars. Infect. Genet. Evol. 18, 287–298.

Nakano, T., Takahashi, K., Pybus, O.G., Hashimoto, N., Kato, H., Okano, H., Kobayashi, M., Fujita, N., Shiraki, K., Takei, Y., Ayada, M., Arai, M., Okamoto, H., Mishiro, S., 2012. New findings regarding the epidemic history and population dynamics of Japan-indigenous genotype 3 hepatitis E virus inferred by molecular evolution. Liver Int. 32 (4), 675–688.

Nidaira, M., Takahashi, K., Ogura, G., Taira, K., Okano, S., Kudaka, J., Itokazu, K., Mishiro, S., Nakamura, M., 2012. Detection and phylogenetic analysis of hepatitis E viruses from mongooses in Okinawa, Japan. J. Vet. Med. Sci. 74 (12), 1665–1668.

Okamoto, H., 2007. Genetic variability and evolution of hepatitis E virus. Virus Res. 127 (2), 216–228.

Okamoto, H., Takahashi, M., Nishizawa, T., 2003. Features of hepatitis E virus infection in Japan. Intern. Med. 42 (11), 1065–1071.

Okamoto, H., Takahashi, M., Nishizawa, T., Fukai, K., Muramatsu, U., Yoshikawa, A., 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem. Biophys. Res. Commun. 289 (5), 929–936.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Purcell, R.H., Emerson, S.U., 2008. Hepatitis E: an emerging awareness of an old disease. J. Hepatol. 48 (3), 494–503.

Raj, V.S., Smits, S.L., Pas, S.D., Provacia, L.B., Moorman-Roest, H., Osterhaus, A.D., Haagmans, B.L., 2012. Novel hepatitis E virus in ferrets, the Netherlands. Emerg. Infect. Dis. 18 (8), 1369–1370.

Reuter, G., Fodor, D., Forgach, P., Katai, A., Szucs, G., 2009. Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. J. Clin. Virol. 44 (4), 277–281.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4 (4), 406–425.
Sato, Y., Sato, H., Naka, K., Furuya, S., Tsukiji, H., Kitagawa, K., Sonoda, Y., Usui, T., Sakamoto, H., Yoshino, S., Shimizu, Y., Takahashi, M., Nagashima, S., Jirintai, S., Nishizawa, T., Okamoto, H., 2011. A nationwide survey of hepatitis E virus (HEV) infection in wild boars in Japan: identification of boar HEV strains of genotypes 3 and 4 and unrecognized genotypes. Arch. Virol. 156 (8), 1345–1358.
Schielke, A., Sachs, K., Lierz, M., Appel, B., Jansen, A., Johne, R., 2009. Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain. Virol. J. 6, 58.
Shimizu, Y., Yamada, M., Tatematsu, H., Ishihara, M., Morita, K., Ishiguro, Y., Katano, Y., Goto, H., Takahashi, M., Okamoto, H., 2006. Four cases of hepatitis E after eating wild boar meats in Aichi, Japan. Kanzo 47 (10), 465–473.
Smith, D.B., Purdy, M.A., Simmonds, P., 2013. Genetic variability and the classification of hepatitis E virus. J. Virol. 87 (8), 4161–4169.
Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T., Okamoto, H., 2004. Prevalence of hepatitis E virus (HEV) infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. J. Clin. Microbiol. 42 (11), 5371–5374.
Takahashi, K., Kitajima, N., Abe, N., Mishiro, S., 2004. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology 330 (2), 501–505.
Takahashi, K., Terada, S., Kokuryu, H., Arai, M., Mishiro, S., 2010. A wild boar-derived hepatitis E virus isolate presumably representing so far unidentified "genotype 5". Kanzo 51 (9), 536–538.
Takahashi, M., Hoshino, Y., Tanaka, T., Takahashi, H., Nishizawa, T., Okamoto, H., 2008. Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. Arch. Virol. 153 (4), 657–666.
Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F., Okamoto, H., 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J. Gen. Virol. 84 (Pt 4), 851–862.
Takahashi, M., Nishizawa, T., Sato, H., Sato, Y., Jirintai, S., Nagashima, S., Okamoto, H., 2011. Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. J. Gen. Virol. 92 (Pt 4), 902–908.
Takahashi, M., Nishizawa, T., Tanaka, T., Tsatsralt-Od, B., Inoue, J., Okamoto, H., 2005. Correlation between positivity for immunoglobulin A antibodies and viraemia of swine hepatitis E virus observed among farm pigs in Japan. J. Gen. Virol. 86 (Pt 6), 1807–1813.
Takahashi, M., Okamoto, H., 2013. Features of hepatitis E virus infection in humans and animals in Japan. Hepatol. Res., http://dx.doi.org/10.1111/hepr.12175 [Epub ahead of print].
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28 (10), 2731–2739.
Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362 (9381), 371–373.
van Cuyck, H., Fan, J., Robertson, D.L., Roques, P., 2005. Evidence of recombination between divergent hepatitis E viruses. J. Virol. 79 (14), 9306–9314.
Wang, H., Zhang, W., Ni, B., Shen, H., Song, Y., Wang, X., Shao, S., Hua, X., Cui, L., 2010. Recombination analysis reveals a double recombination event in hepatitis E virus. Virol. J. 7, 129.
Wang, Y., Zhang, H., Ling, R., Li, H., Harrison, T.J., 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J. Gen. Virol. 81 (Pt 7), 1675–1686.
Widen, F., Sundqvist, L., Matyi-Toth, A., Metreveli, G., Belak, S., Hallgren, G., Norder, H., 2011. Molecular epidemiology of hepatitis E virus in humans, pigs and wild boars in Sweden. Epidemiol. Infect. 139 (3), 361–371.
Worm, H.C., van der Poel, W.H., Brandstatter, G., 2002. Hepatitis E: an overview. Microbes Infect. 4 (6), 657–666.
Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Nagashima, S., Tanaka, T., Okamoto, H., 2009. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. J. Gen. Virol. 90 (Pt 8), 1880–1891.
Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y., Okamoto, H., 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J. Gen. Virol. 84 (Pt 9), 2351–2357.
Yugo, D.M., Meng, X.J., 2013. Hepatitis E virus: foodborne, waterborne and zoonotic transmission. Int. J. Environ. Res. Public Health 10 (10), 4507–4533.
Zhai, L., Dai, X., Meng, J., 2006. Hepatitis E virus genotyping based on full-length genome and partial genomic regions. Virus Res. 120 (1–2), 57–69.
Zhao, C., Ma, Z., Harrison, T.J., Feng, R., Zhang, C., Qiao, Z., Fan, J., Ma, H., Li, M., Song, A., Wang, Y., 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. J. Med. Virol. 81 (8), 1371–1379.

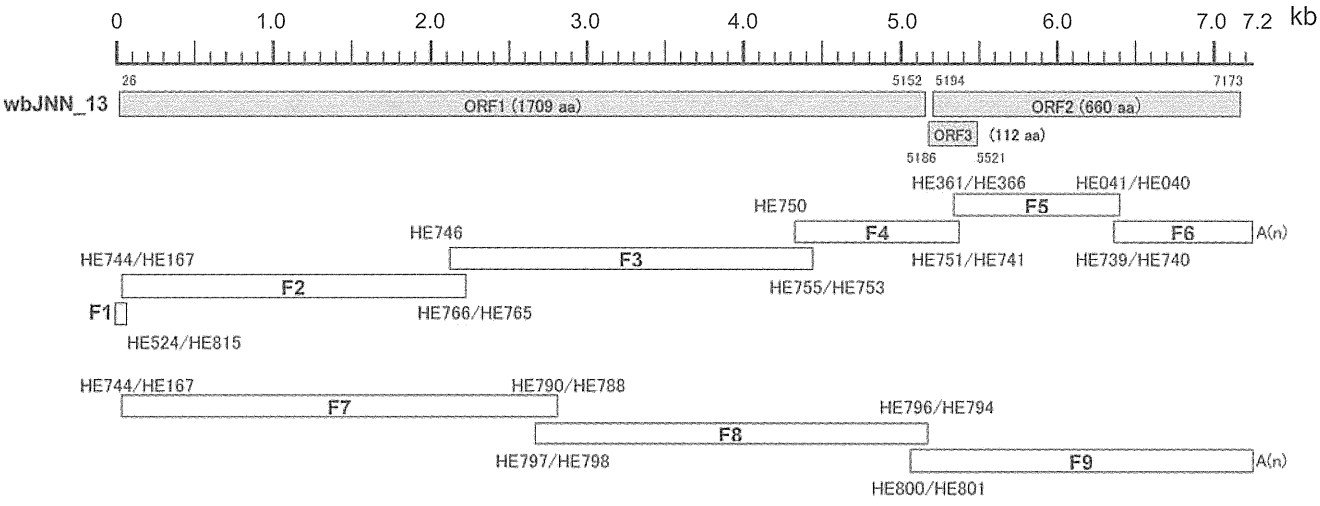
Supplementary Table 1
Primer sequences used in this study

Primer name	Polarity	Nucleotide position	Nucleotide sequence	Notes
HE044	Sense	595-5981	5'-CAA GGH TGG CGY TCK GTT GAG AC-3'	ORF2-457 PCR (1st)[a)]
HE040	Antisense	6442-6464	5'-CCC TTR TCC TGC TGA GCR TTC TC-3'	ORF2-457 PCR (1st)[a)]
HE110	Sense	5969-5990	5'-GYT CKG TTG AGA CCT CYG GGG T-3'	ORF2-457 PCR (2nd)[a)]
HE111	Sense	5969-5990	5'-GYT CKG TTG AGA CCA CGG GYG T-3'	ORF2-457 PCR (2nd)[a)]
HE112	Sense	5969-5990	5'-GYT CKG TTG AGA CCT CTG GTG T-3'	ORF2-457 PCR (2nd)[a)]
HE041	Antisense	6403-6425	5'-TTM ACW GTC RGC TCG CCA TTG GC-3'	ORF2-457 PCR (2nd)[a)]
HE361	Sense	5306-5325	5'-GCR GTG GTT TCT GGG GTG AC-3'	Modified ORF2/3-137 PCR (1st)[b)]
HE831	Antisense	5450-5469	5'-CTG GGM YTG GTC DCG CCA RG-3'	Modified ORF2/3-137 PCR (1st; Underlined R was changed from A in HE364)[b)]
HE830	Sense	5329-5348	5'-GYT GAY TCT CAG CCC TTC GC-3'	Modified ORF2/3-137 PCR (2nd; Underlined Y was changed from T in HE366)[b)]
HE832	Antisense	5446-5465	5'-GMY TGG TCD CGC CAR GHG GA-3'	Modified ORF2/3-137 PCR (2nd; Underlined R was changed from A in HE363)[b)]
HE815	Antisense	81-100	5'-ATT AGC AGC CGC CAG AGC AG-3'	Full genome amplification in the region F1, RLM-RACE (1st)
HE524	Antisense	61-81	5'-GCC TGC TCA ATA GCA GTA GTG-3'	Full genome amplification in the region F1, RLM-RACE (2nd)
HE744	Sense	9-28	5'-CGT WTG TGG YCG AYG CCA TG-3'	Full genome amplification in the region F2, nested RT-PCR (1st)
HE167	Sense	21-40	5'-AYG CCA TGG AGG CCC AYC AG-3'	Full genome amplification in the region F2, nested RT-PCR (2nd)
HE765	Antisense	2255-2274	5'-AGA GGC TGC TGG GAC ATT GG-3'	Full genome amplification in the region F2, nested RT-PCR (1st)
HE766	Antisense	2237-2256	5'-GGA AGG GCA GGG CAC AAA CC-3'	Full genome amplification in the region F2, nested RT-PCR (2nd)
HE746	Sense	2120-2142	5'-ACA CTY TAY ACY CGH ACY TGG TC-3'	Full genome amplification in the region F3, semi-nested RT-PCR (1st and 2nd)
HE753	Antisense	4531-4550	5'-AAA GCC GAA TCA ACC ACT GG-3'	Full genome amplification in the region F3, semi-nested RT-PCR (1st)
HE755	Antisense	4470-4493	5'-CTA GTG AGA AGT TAT TCT GGG TGC-3'	Full genome amplification in the region F3, semi-nested RT-PCR (2nd)
HE750	Sense	4307-4329	5'-GCB YTNT TYG GCC CNT GGT TCC G-3'	Full genome amplification in the region F4, semi-nested RT-PCR (1st and 2nd)
HE741	Antisense	5489-5510	5'-CCA GCT GGG GTA GAT CTA CGA C-3'	Full genome amplification in the region F4, semi-nested RT-PCR (1st)
HE751	Antisense	5385-5404	5'-CGG TGG TGG CGG TGA CAT CA-3'	Full genome amplification in the region F4, semi-nested RT-PCR (2nd)
HE361	Sense	5306-5325	5'-GCR GTG GTT TCT GGG GTG AC-3'	Full genome amplification in the region F5, nested RT-PCR (1st)
HE366	Sense	5329-55348	5'-GYT GAT TCT CAG CCC TTC GC-3'	Full genome amplification in the region F5, nested RT-PCR (2nd)
HE040	Antisense	6442-6464	5'-CCC TTR TCC TGC TGA GCR TTC TC-3'	Full genome amplification in the region F5, nested RT-PCR (1st)
HE041	Antisense	6403-6425	5'-TTM ACW GTC RGC TCG CCA TTG GC-3'	Full genome amplification in the region F5, nested RT-PCR (2nd)
HE739	Sense	6315-6334	5'-TCT TGC TGA CAC TCT CCT CG-3'	Full genome amplification in the region F6, 3'-RACE (1st)
HE740	Sense	6351-6370	5'-ATT GAT TTC GTC GGC TGG AG-3'	Full genome amplification in the region F6, 3'-RACE (2nd)
HE744	Sense	9-28	5'-CGT WTG TGG YCG AYG CCA TG-3'	Full genome amplification in the region F7, nested RT-PCR (1st)
HE167	Sense	21-40	5'-AYG CCA TGG AGG CCC AYC AG-3'	Full genome amplification in the region F7, nested RT-PCR (2nd)
HE788	Antisense	2899-2922	5'-GCA TCC AGC TCT AAA GCA AGA TTG-3'	Full genome amplification in the region F7, nested RT-PCR (1st)
HE790	Antisense	2833-2856	5'-GTC GGT TTG TTG GCC TCA AAC CAG-3'	Full genome amplification in the region F7, nested RT-PCR (2nd)
HE797	Sense	2623-2648	5'-AAT TCA TGC GGT TGC CCC TGA CTA TC-3'	Full genome amplification in the region F8, nested RT-PCR (1st)
HE798	Sense	2652-2677	5'-TCA AGC AAA ATC CCA AGA GGC TTG AG-3'	Full genome amplification in the region F8, nested RT-PCR (2nd)
HE794	Antisense	5224-5245	5'-GCA GCA TAG GCA AAA GCA TGA G-3'	Full genome amplification in the region F8, nested RT-PCR (1st)
HE796	Antisense	5171-5196	5'-CAT GAT GGC ATA TCA ACA CGC CAA GC-3'	Full genome amplification in the region F8, nested RT-PCR (2nd)
HE800	Sense	5006-5033	5'-GTT GTT TCG AGA GCT TAT GGG GTT AGT C-3'	Full genome amplification in the region F9, 3'-RACE (1st)
HE801	Sense	5037-5062	5'-GCC TGG TAC ATA ACC TTA TTG GCA TG-3'	Full genome amplification in the region F9, 3'-RACE (2nd)

For regions F1-F9, see Supplementary Fig. 1.
a) Mizuo et al. (2002)
b) Inoue et al. (2006b)

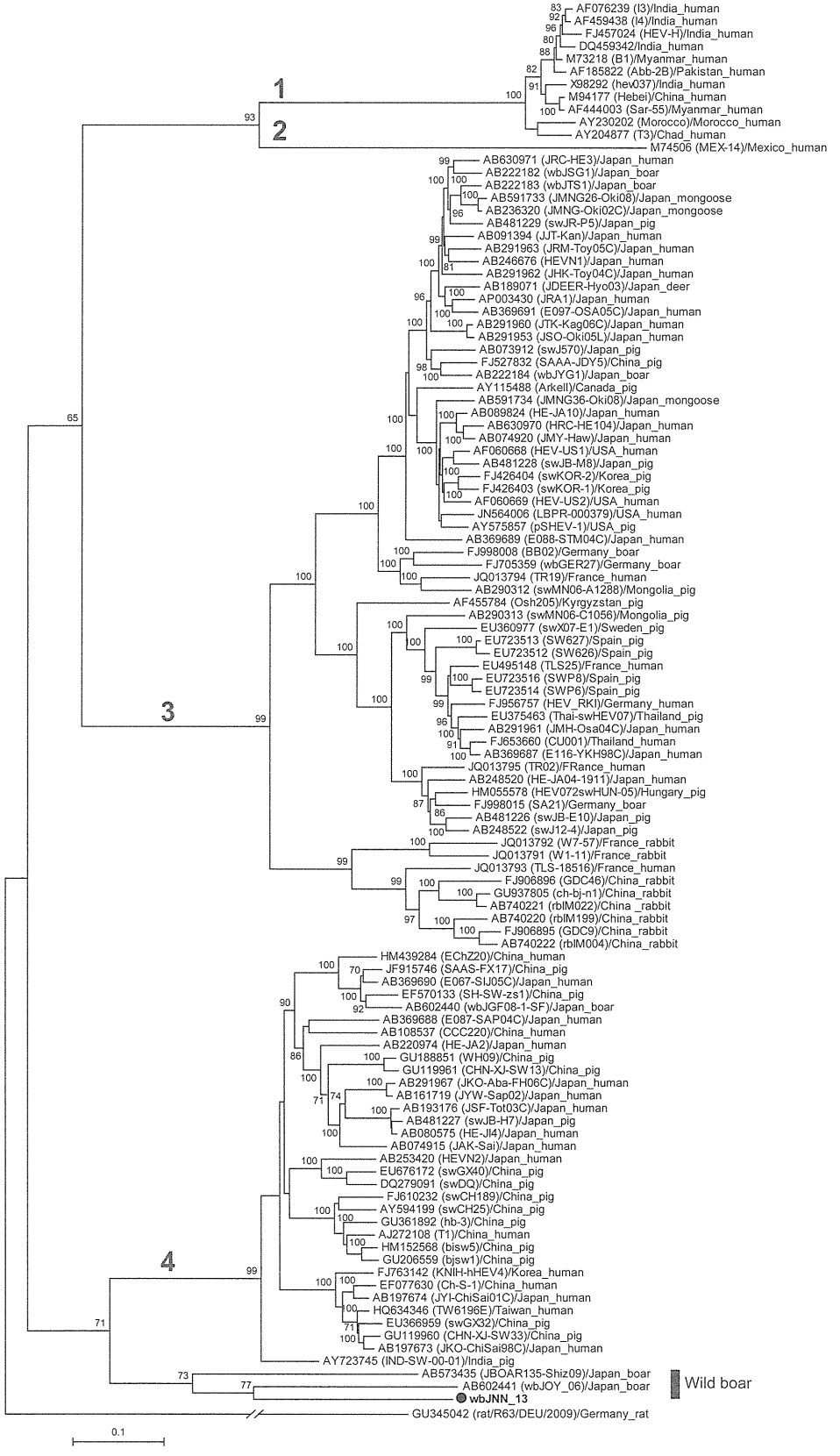
Supplementary Fig. 1

A map of Japan showing the locations of the 12 prefectures where the wild boars were captured, and Shizuoka Prefecture where the JBOAR135-Shiz09 (AB573435) strain was isolated (Takahashi et al., 2010). In addition, the locations of five prefectures (Hokkaido, Miyagi, Tochigi, Mie and Ehime) where subgenotype 3e HEV strains have been isolated, are indicated.



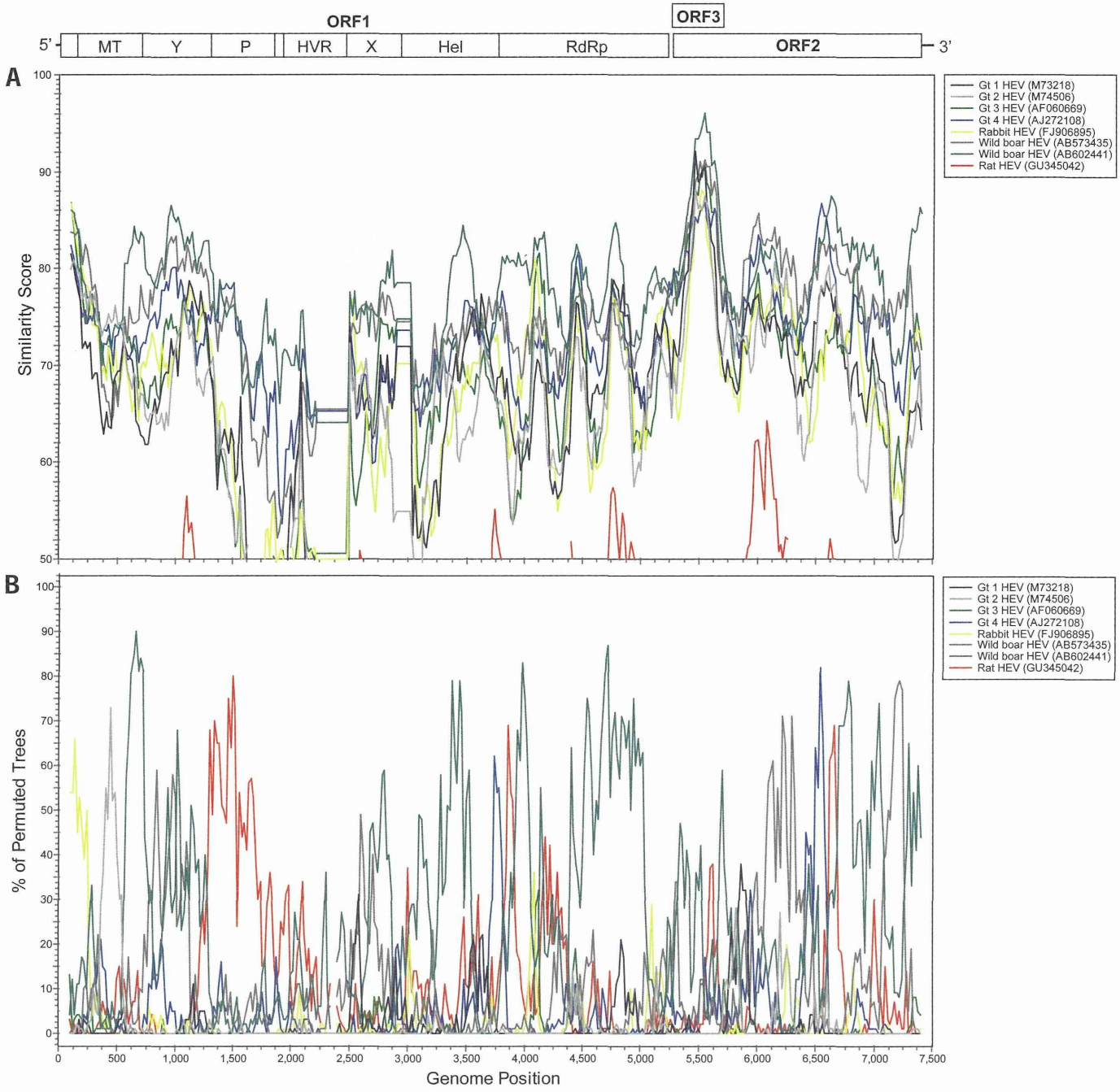
Supplementary Fig. 2

The strategy used to amplify the complete genomic sequence of the wbJNN_13 strain. The line at the top represents the nucleotide position of the HEV genome. The shaded bars indicate three open reading frames (ORF1, ORF2 and ORF3). The open boxes show regions amplified by RLM-RACE (F1), conventional RT-PCR (F2–F5, F7 and F8) and 3'-RACE (F6 and F9). The names of primers used for amplification (see Supplementary Table 1) are indicated at both ends of open boxes.



Supplementary Fig. 3

The maximum-likelihood relationships of the full-length genomic sequences of 111 reported human, swine, boar, deer, mongoose and rabbit HEV isolates and the wbJNN_13 isolate, using a rat HEV isolate (GU345042) as an outgroup. The tree was constructed using the PHYML program (model GTR + G), with optimized tree topology and branch lengths, and the numbers associated with tree branches are indicative of the percentages of 500 full maximum-likelihood bootstrap replicates that support the existence of the branches The wbJNN_13 isolate is indicated by a closed circle.



Supplementary Fig. 4

The results of the complete genome scanning carried out by the Simplot software program for wbJNN_13 versus four representative isolates of genotypes 1-4, two wild boar isolates (AB573435 and AB602441), a rabbit HEV isolate (FJ906895), and a rat HEV isolate (GU345042) as an outgroup. (A) The results from a Simplot analysis are shown. The y-axis shows the percentage of identity within a sliding window 200-bp wide centered on the position plotted, with a step size between plots of 20 bp. (B) The bootscanning of the HEV sequences. The y-axis shows the percentage of permutated trees using a 200-bp sliding window centered on the position plotted, with a step size between plots of 20 bp. The open reading frame map is schematically shown at the top of the figure. Abbreviations: MT, methyltransferase; Y, Y domain; P, papain-like protease; HVR, hypervariable region; X, X domain; Hel, helicase; and RdRp, RNA-dependent RNA polymerase.

Virus Research 179 (2014) 102–112

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Marked genomic heterogeneity of rat hepatitis E virus strains in Indonesia demonstrated on a full-length genome analysis☆

Mulyanto[a,b], Joseph Benedictus Suparyatmo[c], I Gusti Ayu Sri Andayani[b], Khalid[b], Masaharu Takahashi[d], Hiroshi Ohnishi[d], Suljid Jirintai[d], Shigeo Nagashima[d], Tsutomu Nishizawa[d], Hiroaki Okamoto[d,*]

[a] West Nusa Tenggara Hepatitis Laboratory, Mataram, Indonesia
[b] Immunobiology Laboratory, Faculty of Medicine, University of Mataram, Mataram, Indonesia
[c] Department of Clinical Pathology, Moewardi Hospital, University of Sebelas Maret, Solo, Indonesia
[d] Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke-Shi, Tochigi-Ken 329-0498, Japan

ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form 30 October 2013
Accepted 30 October 2013
Available online 11 November 2013

Keywords:
Hepatitis E virus
Rat
Indonesia
Phylogeny
Genotype

ABSTRACT

Rat hepatitis E virus (HEV) strains have recently been isolated in several areas of Germany, Vietnam, the United States, Indonesia and China. However, genetic information regarding these rat HEV strains is limited. A total of 369 wild rats (*Rattus rattus*) captured in Central Java (Solo) and on Lombok Island, Indonesia were tested for the presence of rat HEV-specific antibodies and RNA. Overall, 137 rats (37.1%) tested positive for rat anti-HEV antibodies, while 97 (26.3%) had rat HEV RNA detectable on reverse transcription-PCR with primers targeting the ORF1-ORF2 junctional region. The 97 HEV strains recovered from these viremic rats were 76.3–100% identical to each other in an 840-nucleotide sequence and 75.4–88.4% identical to the rat HEV strains reported in Germany and Vietnam. Five representative Indonesian strains, one from each of five phylogenetic clusters, whose entire genomic sequence was determined, were segregated into three genetic groups (a German type, Vietnamese type and novel type), which differed from each other by 19.5–23.5 (22.0 ± 1.7)% over the entire genome. These results suggest the presence of at least three genetic groups of rat HEV and indicate the circulation of polyphyletic strains of rat HEV belonging to three distinct genetic groups in Indonesia.

1. Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, is transmitted primarily via the fecal-oral route and often causes water-borne epidemics in developing countries in Asia and Africa, where sanitation conditions are suboptimal (Emerson and Purcell, 2013). Hepatitis E is now no longer confined to developing countries and has recently become a concern in many industrialized countries, including the United States, European countries and Japan, where transmission is primarily zoonotic (Colson et al., 2010; Dalton et al., 2008; Emerson and Purcell, 2013; Hoofnagle et al., 2012; Kamar et al., 2012; Meng, 2010; Mizuo et al., 2002; Purcell and Emerson, 2008; Takahashi and Okamoto, 2013). Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms. However, chronic HEV infection has recently been documented in immunocompromised patients, such as solid-organ transplant recipients and human immunodeficiency virus-infected patients (Kamar et al., 2013; Zhou et al., 2013).

HEV is classified as the sole member of the genus *Hepevirus* of the family *Hepeviridae* (Meng et al., 2012). The viral genome is a single-strand, positive-sense RNA that measures approximately 7.2 kilobases (kb) in length and contains a short 5′ untranslated region (5′-UTR), three open reading frames (ORFs: ORF1, ORF2 and ORF3) and a short 3′-UTR terminated by a poly (A) tract. ORF1 encodes non-structural proteins involved in viral replication and viral protein processing, with a hypervariable region on the central portion (Pudupakam et al., 2011). ORF2 encodes the viral capsid protein containing three potential glycosylation sites (Graff et al., 2008). ORF3 mostly overlaps with ORF2 and encodes a small protein of 113–114 amino acids (aa) required for virion egress from cells (Emerson et al., 2010; Yamada et al., 2009). The ORF1 protein is translated directly from genomic RNA, while the ORF2 and ORF3 proteins are translated from a 2.2-kb bicistronic subgenomic RNA (Graff et al., 2006).

☆ The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB847305–AB847309 for five entire rat HEV genomes and AB847310–AB847406 for 97 partial rat HEV sequences.

* Corresponding author. Tel.: +81 285 58 7404; fax: +81 285 44 1557.
E-mail address: hokamoto@jichi.ac.jp (H. Okamoto).

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.10.029

To date, at least four major genotypes (genotypes 1–4) of HEV have been identified in humans (Okamoto, 2007). Genotype 1 and 2 HEVs are restricted to humans and are often associated with large outbreaks and epidemics in developing countries in Asia and Africa. Genotype 3 and 4 HEVs have been isolated not only from humans, but also from other animal species, including pigs, wild boars, deer and mongooses (Meng, 2011; Nidaira et al., 2012; Sato et al., 2011; Takahashi et al., 2004), and are capable of causing zoonotic disease (Izopet et al., 2012; Li et al., 2005; Tei et al., 2003; Yazaki et al., 2003). Recently, novel HEV sequences belonging to new unrecognized HEV genotypes have been detected in wild boars (Takahashi et al., 2010, 2011), and a distant member of genotype 3 HEV has been identified in rabbits (Zhao et al., 2009). Avian HEV derived from chickens is phylogenetically distinct from mammalian hepeviruses and likely represents a new genus within the family *Hepeviridae* (Meng et al., 2012). A strain of HEV that may belong to a separate taxonomic unit of higher rank, e.g., a subfamily, has recently been identified in cutthroat trout (Batts et al., 2011).

In addition, divergent HEV strains have also been isolated from wild rats (Johne et al., 2010b), ferrets (Raj et al., 2012) and bats (Drexler et al., 2012). Rat HEV has been identified in wild rats in Germany (Johne et al., 2010b), the United States (Purcell et al., 2011), Vietnam (Li et al., 2013b), Indonesia (Mulyanto et al., 2013) and China (Li et al., 2013c). In Germany, rat HEV has been recovered from wild rats in at least four cities including Hamburg, Stuttgart, Esslingen and Berlin, and the geographical clustering of rat HEV has been suggested (Johne et al., 2012). Although entire genomic sequences have been determined for four German rat HEV strains (Johne et al., 2010a, 2012) and one Vietnamese strain (Li et al., 2013b), the extent of genomic heterogeneity and the global distribution of rat HEV strains are not fully understood. Therefore, a molecular epidemiological study was conducted to investigate the prevalence of HEV antibodies and viremia in wild rats in Central Java (Solo) and on Lombok Island, where frequent infection with rat HEV in wild rats was observed in our previous study (Mulyanto et al., 2013). In addition, the genomic characteristics of rat HEV in these two areas of Indonesia, where house rats are routinely trapped in residences, were analyzed in comparison with reported mammalian and avian HEV strains.

This report describes the frequent detection of polyphyletic rat HEV strains in both Central Java and Lombok in Indonesia and the genomic characteristics of Indonesian rat HEV strains segregated into three distinct genetic groups (a German type, Vietnamese type and novel type) that differ from each other by 19.5–23.5 (22.0 ± 1.7)% over the entire genome, suggesting a wide distribution of rat HEV with a markedly divergent genomic sequence.

2. Materials and methods

2.1. Serum samples obtained from wild rats

Wild black house rats (*Rattus rattus*) were trapped in Indonesia according to the previously described method (Mulyanto et al., 2013). Serum samples were obtained from 136 wild rats in Solo (also called Surakarta), a city in Central Java, between September 24 and October 5, 2012. Serum samples were also obtained from 233 wild rats in the capital city, Mataram, and one district (West Lombok) of Lombok Island between October 2 and 22, 2012 (Fig. 1). All serum samples were stored at −20 °C in Indonesia and at −80 °C after being sent to Japan and were preserved up until testing. Although two types of viremic rat sera (ratIDE079 and ratIDE113) collected in a previous study (Mulyanto et al., 2013) were used in the present study to determine the entire genomic sequence of rat HEV, there was no overlap in wild rats between the present study and the previous study (Mulyanto et al., 2013).

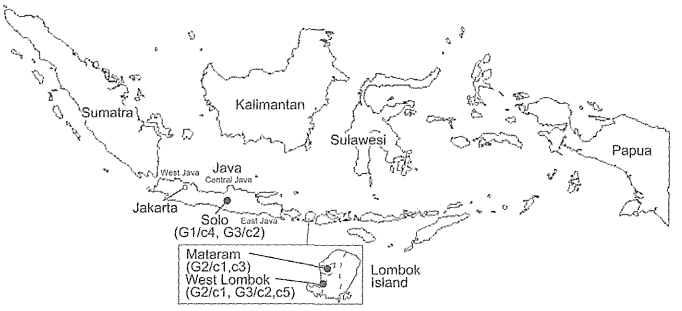


Fig. 1. A map of Indonesia showing the locations of Mataram and West Lombok on Lombok Island and Solo on Java Island (Central Java). The distribution of three genetic groups (G1–G3) and five phylogenetic clusters (1–5; designated c1–c5, respectively) are indicated in parentheses.

2.2. Production and purification of recombinant ORF2 proteins of rat HEV

A recombinant ORF2 protein of rat HEV with a truncated N terminus [amino acid (aa) residues 101–644 of ORF2] was expressed using a recombinant baculovirus according to the method described by Mizuo et al. (2002), with the following modifications. The putative capsid gene (ORF2) of a rat HEV isolate of ratIDE079, whose entire genomic sequence was determined in the present study, was amplified via reverse transcription (RT)-PCR with a sense primer (HE686: 5′-ATG GCG CAG GCC CCT GCG CCG A-3′) and antisense primer (HE687: 5′-GAC ACT GTC GGC TGC CGC GGC T-3′): the truncated ORF2 start codon (ATG) is underlined. The PCR product was cloned into a pT7BlueT vector (Novagen, Inc., Madison, WI), and digested with *Bam*HI and *Sac*I. The resulting 2-kb fragment was inserted into the *Bgl*II-*Sac*I site of a Transfer vector, pYMG (Sysmex Corp., Tokyo, Japan), and sequenced. The 5′-truncated putative capsid (ORF2) gene encoding 545 aa was cloned into a baculovirus expression vector and expressed in silkworm pupae.

The silkworm pupae were lysed in 20 mM PIPES [piperazine-*N*,*N*′-bis (2-hydroxypropane-3-sulfonic acid)] buffer (pH 6.6) containing 10% (v/v) glycerol, 0.1 M NaCl, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 10 mM benzamidine, and then homogenized in 10% (w/v) Triton X-100, followed by centrifugation at 100,000 × *g* at 4 °C for 15 min. The resulting supernatant was treated with polyethylene glycol at a final concentration of 4% (w/v) and centrifuged at 12,000 × *g* at 4 °C for 15 min. The precipitates were redissolved in 20 mM Tris–HCl (pH 8.0) and purified using anion-exchange chromatography. Following purification, the purified protein was shown to produce one predominant band of 60 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. The purified recombinant capsid protein was used in an enzyme-linked immunosorbent assay (ELISA).

2.3. ELISA for detecting rat anti-HEV IgG

To detect IgG class antibodies against rat HEV (rat anti-HEV IgG) in the serum samples obtained from the wild rats, ELISA was performed using purified recombinant ORF2 proteins isolted from the rat HEV strain (ratIDE079) expressed in silkworm pupae, as described above, with slight modifications of the described ELISA method used to detect swine anti-HEV IgG (Takahashi et al., 2005). Briefly, ELISA microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with the recombinant rat HEV ORF2 protein (100 ng/well). The samples were added to each well