hematology and biochemical tests 30 days before admission. He developed fatigue and anorexia 1 week before admission and subsequently noticed dark urine approximately 5 days before admission. He came for a periodic checkup 1 day before his admission, and showed jaundice and a marked elevation of his serum liver enzyme levels. He had no history of drinking unsterilized spring water, or consuming raw fish or shell fish, or raw or undercooked meat/offal from livestock or wild animals such as deer and boar.

Table 1 shows the laboratory findings at the time of admission. There was a substantial elevation of the liver enzymes and total bilirubin levels, although the prothrombin time was not prolonged. Routine serological markers for hepatitis viruses suggested no active infection with these viruses. He was diagnosed with acute hepatitis of unknown etiology with no sign of liver failure. He underwent supportive therapy with parenteral fluid resuscitation in order to address such symptoms as nausea and anorexia.

The symptoms and the liver enzymes immediately recovered after admission, even though the patient kept taking valsartan and cibenzoline and a drug-induced lymphocyte stimulation test (DLST) showed a positive result against the *da jian zhong tang* and cibenzoline. The etiology of the liver injury was thought to be drug-induced liver injury (DILI) due to *da jian zhong tang*, based on the positive result (9 points) of the criteria for DILI score (Digestive Disease Week, Japan 2004) and the rapid decrease in his serum alanine aminotransferase (ALT) levels since the discontinuation of *da jian zhong tang*. In contrast to the liver enzymes, the total bilirubin continuously increased and peaked at 12.8 mg/dL at the 14th hospital day (Fig. 1). Prednisolone (initially 30 mg/day and then tapered) was administrated to treat his cholestasis for 30 days and thereafter the serum bilirubin level rapidly returned to normal level (Fig. 1).

Hepatitis E virus serology and HEV RNA were thereafter examined using the stocked serum samples, which

Table 1 Laboratory findings on admission

Hematology		Blood chemistry		Virus markers	
WBC	5970/μL	T.Bil.	2.5 mg/dL	IgM HAVAb	(−)
RBC	421×10^4/μL	D.Bil.	1.5 mg/dL	IgMHBcAb	(−)
Hb	12.4 g/dL	AST	719 IU/L	HBsAg	(−)
Ht	38.0%	ALT	1120 IU/L	HBsAb	(−)
Platelet	19.3×10^4/μL	LDH	451 IU/L	HBcAb	(+)
Neutrophil	67.5%	γ-GTP	86 IU/L	HCVAb	(−)
Lymphocyte	17.3%	Al-P	618 IU/L	HCV RNA	(−)
Monocyte	11.3%	LAP	82 IU/L	EBVCA IgG	(−)
Eosinophil	0.4%	T.P.	6.6 g/dL	EBVCA IgM	(−)
Basophil	0.2%	Albumin	3.9 g/dL	EBNA Ab	(−)
		IgG	1405 mg/dL	CMV IgG	(+)
Electrolytes and Renal function		IgA	221 mg/dL	CMV IgM	(−)
Na	140 mEq/L	IgM	79 mg/dL		
K	4.6 mEq/L	CRP	<0.1 mg/dL	Autoantibodies	
Cl	104 mEq/L			ANA	(−)
BUN	17.8 mg/dL	Blood coagulation			
CRNN	0.77 mg/dL	PT	95%	Tumor marker	
		HPT	58.4%	AFP	2.1 ng/mL
Urinalysis		Fibrinogen	319 mg/dL		
pH	6.0	Antithrombin	105%		
Sp.G.	1.022	FDP D-dimer	0.8 μg/mL		
Protein	(−)				
Sugar	(+)				

WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; BUN, blood urea nitrogen; CRNN, creatinine; Sp.G, specific gravity; T.Bil., total bilirubin; D.Bil., direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; LAP, leucine aminopeptidase; TP, total protein; Ig, immunoglobulin; CRP, C-reactive protein; PT, prothrombin time; HPT, hepaplastin test; FDP, fibrin degradation products; HAV, hepatitis A virus; Ab, antibody; Ag, antigen; HB, hepatitis B virus; HCV, hepatitis C virus; EB, Epstein–Barr virus; CMV, cytomegalovirus; ANA, antinuclear antibody; AFP, α-fetoprotein.

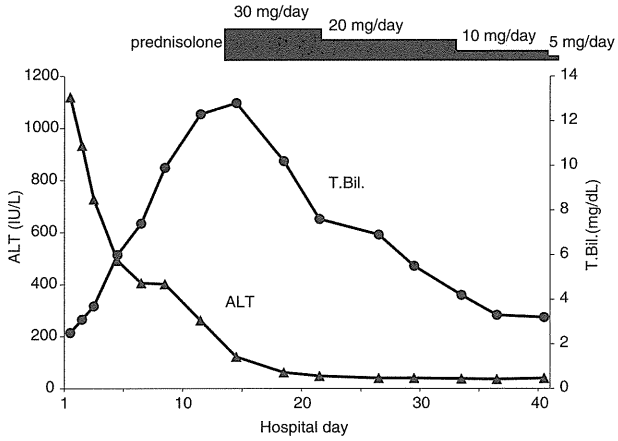

Figure 1 Clinical course of the patient. Hospital day represents the days after admission. ALT, alanine aminotransferase; T.Bil, total bilirubin.

were determined as previously described (Table 2).[3,4] The genomic RNA of genotype 3 HEV was detectable in serum obtained 3 weeks before the onset of the symptoms of acute hepatitis and it then disappeared 3 months after onset. Both IgG and IgA class anti-HEV Ab turned positive at the time of disease onset, although they were negative at 3 weeks prior to the onset, and remained positive for at least 3 months. The titer (optical density) of IgG class Ab exceeded 3.0 throughout the disease course, whereas the titer of the IgA class Ab increased and peaked on the 19th hospital day and thereafter gradually decreased. In contrast, IgM class Ab was negative throughout the course. Although the patient's history of food intake in the 3 months before the onset of acute hepatitis was carefully rechecked after the etiology was determined, the patient had had no opportunity to consume any food considered to be a possible source of HEV infection, because he had been paying attention to his food intake since he had undergone surgery for gastric cancer.

Table 2 Serial changes in HEV RNA and the titer of anti-HEV antibody

Hospital day (days from admission date)	HEV RNA	Anti-HEV antibody (OD_{450})		
		IgG	IgM	IgA
−27	+	0.031 (−)	0.060 (−)	0.036 (−)
3	+	>3.0 (+)	0.153 (−)	1.488 (+)
19	+	>3.0 (+)	0.170 (−)	2.097 (+)
27	+	>3.0 (+)	0.127 (−)	1.923 (+)
34	+	>3.0 (+)	0.096 (−)	1.672 (+)
41	+	>3.0 (+)	0.079 (−)	1.517 (+)
97	−	>3.0 (+)	0.036 (−)	1.117 (+)

HEV, hepatitis E virus; Ig, immunoglobulin; OD, optical density.

DISCUSSION

THERE ARE AT least four HEV genotypes capable of infecting humans, and genotypes 3 and 4 are thought to be autochthonous in Japan.[5] Although HEV infection is reported to account for a small percentage of acute hepatitis from major liver centers in Japan,[2] substantial HEV infection is thought to be overlooked in general hospitals, because the screening test for a diagnosis of the HEV infection has not been provided to general clinical practice. Indeed, this case could have been misdiagnosed as DILI due to the presence of *da jian zhong tang* if HEV tests had not been performed. On the other hand, there are several reports describing HEV infection to be associated with drug hypersensitivity[6–8] or an autoimmune hepatitis-like response.[9,10] Moreover, DLST for herbal medicine is known to be prone to being false-positive.[11,12] These facts indicate that the establishment of an accurate diagnostic method for HEV infection is therefore urgently needed.

Hepatitis E virus infection is thought to be more frequently asymptomatic in young people than in the elderly and thus it results in a high prevalence of IgG class anti-HEV Ab in the general population in comparison to the low incidence of overt acute hepatitis E.[13] IgM class anti-HEV Ab is occasionally negative in patients with an asymptomatic infection.[14] In contrast, the present report is the first case that showed a negative result for the IgM class anti-HEV Ab throughout the clinical course of overt and icteric acute hepatitis. Furthermore, the patient showed high levels of serum ALT and total bilirubin (1120 IU/L and 12.8 mg/dL, respectively), suggesting that the patient was immunologically responsive to HEV. The patient had not received any immunosuppressive therapy including anticancer drugs before the onset of the hepatitis, although he had undergone subtotal gastrectomy for early gastric cancer. Both IgG and IgA class Ab had already and fully responded when the patient received prednisolone. Therefore, prednisolone administration is not thought to have affected the poor response of the patient with IgM class Ab.

The reason for the lack of IgM class Ab response in this case was unclear. The ELISA system used in this report for detecting anti-HEV antibodies is well established[4] and had been used in several previous studies as a reliable system with high sensitivity and specificity for at

least genotypes 1, 3 and 4 HEV infection.[3,14] In addition, this ELISA system uses the same recombinant HEV protein (550 amino acid residues 111–660 of open reading frame 2 protein of genotype 4 [HE-J1 strain, accession no. AB082545], which was precisely described in previous reports)[4,15] for detecting all classes of HEV antibodies, and the classes of captured antibodies are distinguished using different enzyme-labeled secondary antibodies corresponding to each Ab class.[3] In this patient, IgG and IgA class HEV Ab was detectable at the disease onset, and continued to be positive for more than 3 months. IgM class anti-HEV Ab was undetectable even when recombinant open reading frame 2 proteins of various genotypes (1, 3 and 4) were used as antigen probes.[15] Therefore, it is likely that the negativity of IgM class anti-HEV Ab in this case was attributable to poor response of IgM class antibody, not to infection of a specific viral strain.

In contrast to IgM, the IgA class Ab apparently responded to the HEV infection and the titer of the Ab was clearly associated with the hepatitis symptoms such as the jaundice. Supportively, a diagnostic system using IgA class Ab to HEV is now being developed for clinical use in Japan, based on the report that IgA class anti-HEV Ab is more sensitive and specific than IgM class anti-HEV Ab for the diagnosis of acute hepatitis E.[16,17]

This case suggests and supports the notion that screening for HEV infection only with IgM class anti-HEV Ab is insufficient to diagnose hepatitis E and that the detection of IgA class Ab may therefore be useful for making an accurate diagnosis.

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ORIGINAL ARTICLE

Frequent detection and characterization of hepatitis E virus variants in wild rats (*Rattus rattus*) in Indonesia

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Abstract One hundred sixteen rats (*Rattus rattus*) captured in Indonesia from 2011 to 2012 were investigated for the prevalence of hepatitis E virus (HEV)-specific antibodies and HEV RNA. Using an ELISA based on HEV genotype 4 with an ad hoc cutoff value of 0.500, 18.1 % of the rats tested positive for anti-HEV IgG. By nested RT-PCR, 14.7 % of the rats had rat HEV RNA, and none were positive for HEV genotype 1-4. A high HEV prevalence among rats was associated with lower sanitary conditions in areas with a high population density. Sixteen of the 17 HEV isolates obtained from infected rats showed >93.0 % nucleotide sequence identity within the 840-nucleotide ORF1-ORF2 sequence and were most closely related to a Vietnamese strain (85.9-87.9 % identity), while the remaining isolate differed from known rat HEV strains by 18.8-23.3 % and may belong to a novel lineage of rat HEV. These results suggest a wide distribution of rat HEV with divergent genomes.

The nucleotide sequences of rat HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB725884-AB725900 (840 nt).

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Introduction

Acute or fulminant hepatitis E, caused by hepatitis E virus (HEV), is a worldwide human disease that is a major public health concern in many developing countries with poor sanitation conditions because it is primarily transmitted by the fecal-oral route [5]. In contrast, sporadic cases of hepatitis E that are not related to travel to endemic areas, most likely of zoonotic origin, have been increasingly described in many industrialized countries, including the United States, European countries, and Japan [21, 24, 26, 28].

HEV is a positive-sense, single-stranded RNA virus that belongs to the genus *Hepevirus* of the family *Hepeviridae* [23]. The genome of HEV is approximately 7.2 kb in size and contains a short 5' untranslated region (5'UTR), followed by three open reading frames (ORFs; ORF1, ORF2 and ORF3) and a short 3' UTR terminated by a poly (A) tract [35]. ORF1 at the 5' end of the genome encodes several non-structural proteins involved in replication, while ORF2 codes for a capsid protein of 660 amino acids (aa). ORF3, which partially overlaps with ORF2, encodes a small protein of only 113-114 aa that is required for virion egress [39]. Four genotypes (1-4) of HEV have been identified in mammalian species [27]. Genotype 1 and 2 HEVs are restricted to humans and are often associated with epidemic waterborne and sporadic hepatitis in developing countries. Genotype 3 and 4 HEVs are believed to undergo zoonotic transmission, using pigs and, possibly, a range of other mammals as reservoirs, and they are responsible for sporadic cases of hepatitis E worldwide. Infection of humans caused by eating raw or undercooked meat/viscera from domestic pigs, wild boars, and deer has also been documented [16, 22, 37, 40].

In addition to the strains of HEV representing genotypes 3 and 4 recovered from domestic pigs, wild boars, deer, and mongoose, novel HEV sequences belonging to new unrecognized HEV genotypes have been detected in wild boars [31, 34], and a distant member of genotype 3 HEV has been identified in rabbits [41]. Avian HEV from chickens likely represents a new genus within the family *Hepeviriade* [3, 21]. 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 [2].

Other mammalian species, including rats, have also been found to have antibodies reactive with the ORF2 protein of human HEV strains. However, an HEV strain recently identified from wild rats in Germany is unique in that it is distantly related to known human HEV strains, with only 55 % sequence identity over the entire genome [9, 10]. Rat HEV was also identified from wild rats in the United States [29] and Vietnam [17], and their partial sequences have been determined. In Germany, rat HEV was recovered from wild rats in at least four cities, including Hamburg, Stuttgart, Esslingen, and Berlin, and a geographical clustering of rat HEV has been suggested [11]. However, 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 antibody and viremia in wild rats and the genomic characteristics of rat HEV on Lombok Island, Indonesia, where house rats are routinely trapped in every residence. Lombok is an island in West Nusa Tenggara Province, Indonesia. It forms part of the chain of the Lesser Sunda Islands, with the Lombok Strait separating it from Bali to the west and the Alas Strait between it and Sumbawa to the east. The island is divided into four districts (North Lombok, West Lombok, Central Lombok, and East Lombok) and one kotamadya (Mataram City), which is the provincial capital and largest city on the island (see Fig. 1).

This report describes the frequent detection of HEV viremia and genomic characteristics of Indonesian strains of rat HEV, which are distantly related to the German and U.S. strains, thereby suggesting a wide distribution of rat HEV with divergent genomic sequences.

Materials and methods

Serum samples from wild rats

Wild black house rats (*Rattus rattus*) were trapped and thus caught alive in the capital city, Mataram, and two districts (Central Lombok and West Lombok) of Lombok Island, Indonesia (Fig. 1) between August 2011 and February 2012. The rats were caught using a rat trap, as is commonly done by residents to capture rats in their houses. Traps were placed in the evening around the strategic places in the house of every resident who was involved in this study. The trap was observed in the morning of the following day, and trapped rats were immediately taken to the laboratory to have their blood drawn. Data and information regarding the rats and the capture location were recorded. The rats were anesthetized, and blood was obtained by cardiac puncture. Subsequently, serum was separated and stored frozen at -20 °C in Indonesia and at -80 °C after being sent to Japan and was thus preserved up until testing.

This study also recorded the number of houses and the number of occupants per house, as well as sewage and garbage disposal in a radius of about 100 m from the house where the traps to catch rats were situated (total area observed = around 0.04 km^2 per village) in order to get an overview and to estimate the sanitation conditions in the villages where rats were trapped.

ELISA for detecting anti-HEV antibodies

Serum samples were tested for IgG-class antibodies against HEV (anti-HEV IgG) by using an enzyme-linked immunosorbent assay (ELISA) that was a slight modification of an ELISA for the detection of swine HEV antibodies [33]. The antigen used was purified recombinant ORF2 protein from the human genotype 4 HEV strain (HE-J1) that had been expressed in silkworm pupae [25]. The samples were added to each well of an ELISA microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) coated with the recombinant ORF2 protein (250 ng/well) at a 100-fold dilution in 10 mM Tris-buffered saline containing 40 % Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), 0.18 % Tween 20, and a mock protein preparation (optical density [OD] at 280 nm = 0.1) that had been obtained from the pupae of silkworms infected with non-recombinant baculovirus. The peroxidase-conjugated goat IgG fraction to rat IgG (whole molecule: MP Biomedicals, LLC., Solon, OH) was used for the rat anti-HEV IgG assay. The OD value of each sample was read at 450 nm. Although 75 of the samples tested (64.7 %) had an OD value of < 0.200 (see Fig. 2), the cutoff value was set at 0.500 as an ad hoc value in the present study. Test samples with OD values equal to or greater than the cutoff value were considered to be positive for anti-HEV IgG. The specificity of the anti-HEV assay was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, if the OD value of the tested sample was less than 30 % of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV IgG.

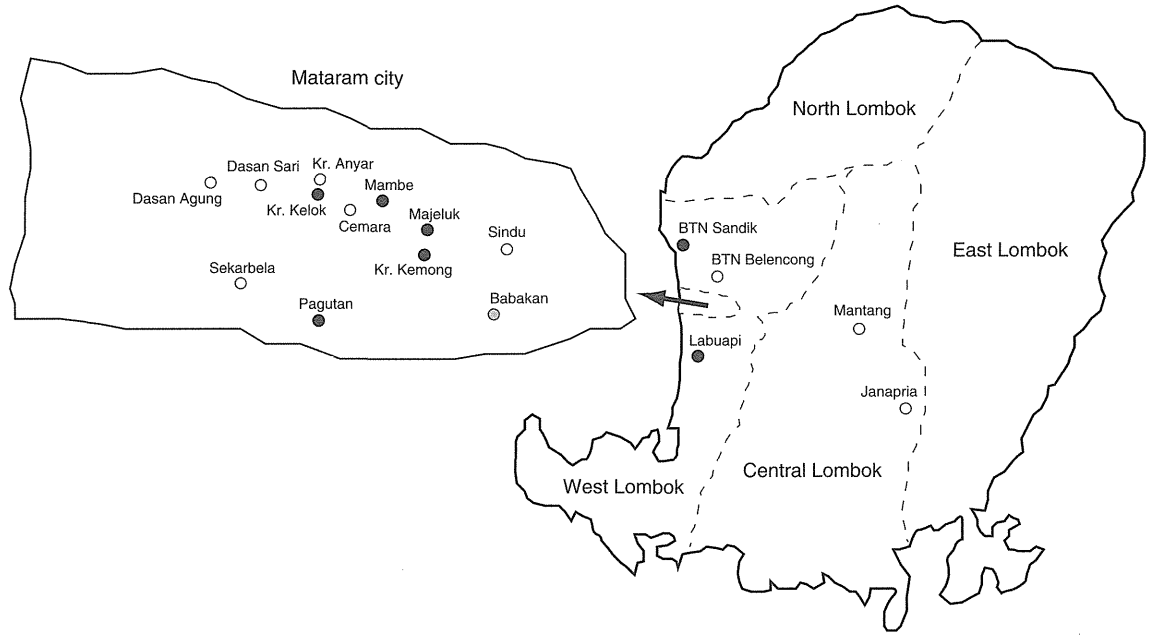

Fig. 1 Map of Lombok, Indonesia, showing the locations of the 17 villages where wild rats were captured. A closed circle indicates the village where HEV-infected rats were captured, a shaded circle represents the village where an HEV-antibody-positive rat without viremia was captured, and open circles indicate the villages with no infected rats

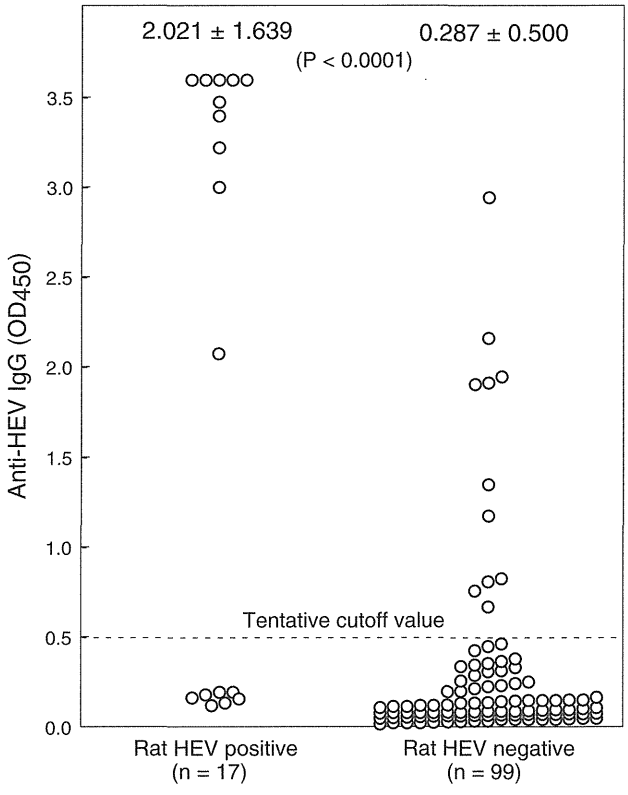

Fig. 2 Comparison of the distribution of the OD value in the anti-HEV IgG ELISA between rat-HEV-positive and -negative rats. The number at the top represents the mean ± standard deviation of the OD values

Detection of HEV RNA

Total RNA was extracted from 100 μl of each serum sample (or serum sample diluted at 1:100 or 10,000 with phosphate-buffered saline containing 2 % bovine serum albumin) using TRIzol LS Reagent (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. Two protocols for nested reverse transcription (RT) polymerase chain reaction (PCR) were used for detection of HEV RNA. The extracted RNA was reverse-transcribed with SuperScript II (Invitrogen), and subsequent nested PCR was performed with TaKaRa *Ex Taq* (TaKaRa Bio, Shiga, Japan) and primers (Table 1) derived from the areas of the ORF1-ORF2 region that are well-conserved across all five reported rat HEV sequences (GQ504009, GQ504010, GU345042, GU345043, and JN040433) to detect the genomic RNA of rat HEV. The PCR amplification was carried out for 35 cycles in the first round (94 °C for 30 s [an additional 2 min was used in the first cycle], 55 °C for 30 s, and 72 °C for 90 s [an additional 7 min was used in the last cycle]) and for 25 cycles in the second round under the same conditions used for the first round. The size of the amplification product of the first-round PCR was 899 base pairs (bp), and that of the amplification product of the second-round PCR was 880 bp. The RT-PCR assay was performed in duplicate, and reproducibility was confirmed. To avoid contamination during PCR procedures, the guidelines of Kwok and Higuchi [15] were strictly

Table 1 Primers used for nested RT-PCR and nucleotide sequencing

Primer name	Sequence (5'-3')[a]	Notes[b]
HE607	CTTGGTTYAGGGCCATAGAG	nt 4098-4117 (for 1st round PCR, sense)
HE604	CAGCAGCGGCACGAACAGCA	nt 4977-4996 (for 1st round PCR, antisense)
HE608	TTYAGGGCCATAGAGAAGGC	nt 4103-4122 (for 2nd round PCR and sequencing, sense)
HE606	ACAGCAAAAGCACGAGCACG	nt 4963-4982 (for 2nd round PCR and sequencing, antisense)
HE609	GGAATACAGTCTGGAAYATGGC	nt 4425-4446 (for sequencing, sense)
HE610	YTTCTTCCAYCGRCCCCGCA	nt 4377-4396 (for sequencing, antisense)

[a] Y denotes T or C, and R represents G or A

[b] Nucleotide numbers were in accordance with a prototype rat HEV strain (R63, GU345042)

followed. The specificity of the RT-PCR assay was verified by sequence analysis as described below.

A second RT-PCR assay, which had the capability of amplifying all four known genotypes of human HEV strains, was carried out according to a method described previously [25].

Sequence analysis of PCR products

The amplification product was sequenced directly on both strands using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using the Genetyx ver.11.0.4 software program (Genetyx Corp., Tokyo, Japan), and multiple alignments were generated using the CLUSTAL W software program version 2.1 [38]. A phylogenetic tree was constructed by the neighbor-joining method [30] with the Kimura two-parameter correction model and 1,000 replicates of bootstrap re-sampling as implemented in MEGA 5 (version 5.0.5) [36].

Results

The prevalence of anti-HEV IgG and HEV RNA among wild rats

A total of 116 house rats weighing 26-215 g (53 males and 63 females) were trapped in the 12 villages of Mataram City, three villages of the West Lombok district, and two villages of the Central Lombok district on Lombok Island, Indonesia, between August 2011 and February 2012 (Fig. 1), and their serum samples were tested for the presence of anti-HEV antibodies. The OD values in anti-HEV IgG ELISA ranged from 0.020 to >3.500. Although 75 samples (64.7 %) had an OD value of <0.200, the cutoff value was provisionally set at 0.500 in the present study. Twenty-one samples (18.1 %) had OD values that were equal to or greater than the cutoff value and were considered to be positive for anti-HEV IgG (Table 2). All 116 rat serum samples were also tested for the presence of HEV RNA by RT-PCR with nested primers targeting the ORF1-ORF2 junction region of the rat HEV genome, and 17 samples (14.7 %) tested positive for rat HEV RNA (Table 2). No samples had HEV RNA detectable by RT-PCR with nested primers targeting the ORF2 region of the human HEV genomes of genotypes 1-4.

Stratifying the prevalence of HEV infection in wild rats by geographic region revealed both anti-HEV IgG and HEV RNA to be most frequently detectable in Mataram City (20.5 % and 17.9 %, respectively), followed by the West Lombok district, where the prevalence of anti-HEV IgG was 17.2 % and that of HEV RNA was 10.3 %. In contrast, neither anti-HEV IgG nor HEV RNA was detectable in the wild rats from the Central Lombok district, although the sample size was small (n = 9; Table 2). When the prevalence of HEV infection in the rats studied was also stratified by weight as a measure of age, the rats showed an increasing tendency in the prevalence of anti-HEV IgG with weight (Table 3). The prevalence of HEV viremia was highest (27.3 %) in the 151-200 g weight group, followed by the 101-150 g weight group (18.4 %).

The prevalence of HEV infection in wild rats differed by village as well, even within Mataram City, with a high viremia rate of rat HEV in some villages, including Kr. Kelok (32.0 % or 8/25), BTN Sandik (22.2 % or 2/9), Kr. Kemong (17.6 % or 3/17; Table 2). Although only two or four rats were trapped in the Majeluk, Mambe, and Pagutan villages in Mataram City, viremic rats were successfully identified.

Characteristics of the 17 wild rats with ongoing infection of rat HEV

The 17 HEV-RNA-positive rats had body weights ranging from 36 to 173 g, and 59 % of them were male (Table 4). Fourteen rats with HEV viremia were from Mataram City, and the remaining three rats were from the West Lombok district. Ten of the 17 viremic rats had detectable anti-HEV

Table 2 Prevalence of anti-HEV IgG and HEV RNA in house rats from Lombok, Indonesia

Geographic region (City or district /village)	n	Body weight (mean ± SD g)	Anti-HEV IgG-positive	HEV RNA-positive
Mataram	78	110.9 ± 37.3	16 (20.5 %)	14 (17.9 %)
Babakan	8	125.8 ± 32.3	1 (12.5 %)	0
Cemara	9	90.5 ± 16.8	0	0
Dasan Agung	2	112.0 ± 16.9	0	0
Dasan Sari	3	101.0 ± 32.5	0	0
Kr. Anyar	4	92.9 ± 38.9	0	0
Kr. Kelok	25	113.9 ± 33.2	9 (36.0 %)	8 (32.0 %)
Kr. Kemong	17	109.7 ± 42.7	3 (17.6 %)	3 (17.6 %)
Majeluk	2	111.6 ± 11.3	1 (50.0 %)	1 (50.0 %)
Mambe	2	97.0 ± 16.8	2 (100 %)	1 (50.0 %)
Pagutan	4	131.5 ± 73.3	0	1 (25.0 %)
Sekarbela	1	199.3	0	0
Sindu	1	74.0	0	0
West Lombok	29	80.4 ± 42.1	5 (17.2 %)	3 (10.3 %)
BTN Blencong	2	139.4 ± 51.5	0	0
BTN Sandik	9	91.8 ± 40.0	3 (33.3 %)	2 (22.2 %)
Labuapi	18	69.3 ± 37.5	2 (11.1 %)	1 (5.6 %)
Central Lombok	9	76.9 ± 24.4	0	0
Janapria	6	92.1 ± 10.8	0	0
Mantang	3	46.7 ± 6.0	0	0
Total	116	101.0 ± 40.2	21 (18.1 %)	17 (14.7 %)

Table 3 Prevalence of anti-HEV IgG and HEV RNA in wild rats stratified by weight as a measure of age

Weight (g)[a]	n	Anti-HEV IgG-positive	HEV RNA-positive
≤50	14	0	1 (7.1 %)
51-100	50	9 (18.0 %)	6 (12.0 %)
101-150	38	6 (15.8 %)	7 (18.4 %)
151-200	11	5 (45.5 %)	3 (27.3 %)
≥201	1	1 (100 %)	0
Total	114	21 (18.4 %)	17 (14.9 %)

[a] The weight was not known for two rats that had no detectable HEV antibody and HEV RNA

IgG, representing an ongoing HEV infection accompanied by an early antibody response, and the remaining seven viremic rats were negative for anti-HEV IgG, most likely representing viremia before the development of an immune response in the infected rats (Table 4). The finding that 11 anti-HEV-IgG-positive rats were negative for HEV RNA may therefore represent a past HEV infection in the studied rats. The OD value of anti-HEV IgG was significantly higher in the viremic rats than in the non-viremic rats ($P < 0.0001$, Mann-Whitney's U test; Fig. 2). Genomic RNA of rat HEV was detectable in serum even at a dilution of 1:100 in 15 samples and of 1:10,000 in four samples, thus suggesting that the HEV load in wild rats differs markedly for each individual rat.

Evaluation of the sanitation conditions in the villages where rats were trapped

The number of individuals per house and population density in the areas where HEV-infected rats were found in the area of Mataram was about seven per house and 6,000/km^2, respectively, in comparison to about five per house and 4,600/km^2, respectively, in the areas where no infected rats were observed. The number of individuals per house and the population density were about five per house and 2,500/km^2, respectively, in two villages with HEV-infected rats in the West Lombok district, while in the remaining village without infected rats, the number of individuals per house was about 3, and the population density was about 1,800/km^2. The number of individuals per house was about 5 in Central Lombok, where no infected rats were identified, and the population density was lower, at about 1,500/km^2. The accumulation of biological waste, including food scraps, was higher in the locations with HEV-infected rats in comparison to the area where there were no HEV-infected rats were found.

Comparison of rat HEV isolates obtained from wild rats in the present study with reported HEV strains

The amplification products of the ORF1-ORF2 junction region (840 nt; primer sequences at both ends excluded)

Table 4 Characteristics of the 17 wild rats with an ongoing infection of rat HEV

Date of sampling	Body weight (g)	Sex	City or district/village	Anti-HEV IgG (OD_{450})	Relative HEV RNA titer[a]	HEV group[b]	HEV isolate name
'11.08.12	137	F	Mataram/Kr. Kelok	0.165 (−)	1+[c]	1	ratIDE003
'11.08.13	105	F	Mataram/Kr. Kelok	0.138 (−)	2+	1	ratIDE004
'11.09.28	75	M	Mataram/Kr. Kelok	>3.500 (+)	1+[c]	1	ratIDE022
'11.10.08	109	F	Mataram/Mambe	3.224 (+)	4+	1	ratIDE026
'11.10.08	120	F	Mataram/Majeluk	3.401 (+)	2+	1	ratIDE027
'11.10.18	66	M	Mataram/Kr. Kemong	0.184 (−)	3+	1	ratIDE045
'11.10.18	65	M	Mataram/Kr. Kemong	0.198 (−)	2+	1	ratIDE047
'11.10.21	154	F	Mataram/Kr. Kelok	>3.500 (+)	2+	1	ratIDE054
'11.10.25	173	F	Mataram/Kr. Kemong	>3.500 (+)	2+	1	ratIDE056
'11.11.01	94	M	Mataram/Kr. Kelok	2.077 (+)	3+	1	ratIDE060
'11.11.22	98	M	Mataram/Kr. Kelok	3.004 (+)	2+	1	ratIDE075
'11.12.06	36	F	West Lombok/BTN Sandik	0.161 (−)	2+	1	ratIDE077
'11.12.06	71	M	West Lombok/BTN Sandik	0.124 (−)	2+	1	ratIDE078
'11.12.08	138	M	Mataram/Kr. Kelok	>3.500 (+)	4+	1	ratIDE079
'11.12.15	144	M	Mataram/Kr. Kelok	3.478 (+)	4+	1	ratIDE080
'12.01.11	146	M	Mataram/Pagutan	0.198 (−)	2+	1	ratIDE094
'12.02.13	165	M	West Lombok/Labuapi	>3.500 (+)	4+	2	ratIDE113

[a] Relative HEV RNA titer was expressed as 1+, 2+, 3+, and 4+, based on the detectability of HEV RNA in original or diluted serum corresponding to 1, 0.1, or 0.01 μl of serum, respectively

[b] See Figure 3

[c] HEV RNA was also detectable at the dilution of 1:4, corresponding to 25 μl of serum

from 17 HEV-infected rats were sequenced, and the 840-nt sequence corresponding to nt 4123-4962 of the rat HEV genome (GU345042) comprising the C-terminal ORF1 (796 nt), the junction region (30 nt), and the N-terminal ORF2 (14 nt) were compared with the corresponding sequences of other HEV strains (Supplementary Table 1). The 17 HEV isolates obtained in the present study shared identities of 76.7-87.9 % within the 840-nt ORF1-ORF2 sequence with five rat HEV isolates reported from Germany (GQ504009, GQ504010, GU345042, and GU345043) and Vietnam (JN040433), while they were only 55.5-58.9 % identical to the prototype human HEV isolates of genotypes 1-4, rabbit HEV, and two novel wild boar HEV strains, and they were 49.0-52.1 % similar to avian HEV strains. The 17 rat HEV strains in Indonesia were only 77.5-79.3 % similar to the US strain (JF516246) within the overlapping 292-nt ORF1 sequence. Sixteen of the 17 HEV isolates obtained from the infected rats (94 %), including the ratIDE003 isolate, were closely related to each other, with nucleotide sequence identities of 93.0-100 %, but were only 79.9-82.0 % similar to the remaining isolate (ratIDE113). These results indicate that 17 Indonesian rat HEV isolates obtained in the present study are most closely related to the HEV strains from wild rats among all known mammalian and avian HEV strains, but they were different from reported rat HEV strains, thus suggesting that the Indonesian rat HEV strains obtained in the present study are classifiable into two groups, and ratIDE113 may therefore belong to a novel lineage of rat HEV.

A phylogenetic tree constructed by the neighbor-joining method, based on the 840-nt ORF1-ORF2 sequence, indicated that all 17 HEV isolates obtained in the present study form a cluster with all five known rat HEV strains whose overlapping 840-nt sequence is avaialble, supported by a bootstrap value of 100 %, that 16 of the 17 Indonesian rat HEV strains are closely related to a Vietnamese rat HEV strain, and that the remaining rat HEV (ratIDE113) is remotely related to other rat HEV strains (Fig. 3).

Discussion

Johne et al. [10] first reported the identification of rat HEV in feces of a wild Norway rat (*R. norvegicus*) in the city of Hamburg, Germany. The presence of rat HEV in wild rats has been reported from only two additional countries, including the United States and Vietnam [17, 29]. The present study revealed that rat HEV also circulates in Indonesia. Seventeen (14.7 %) of the 116 house rats trapped for the current study were viremic for rat HEV, and the genomic sequences of the 17 HEV strains obtained from

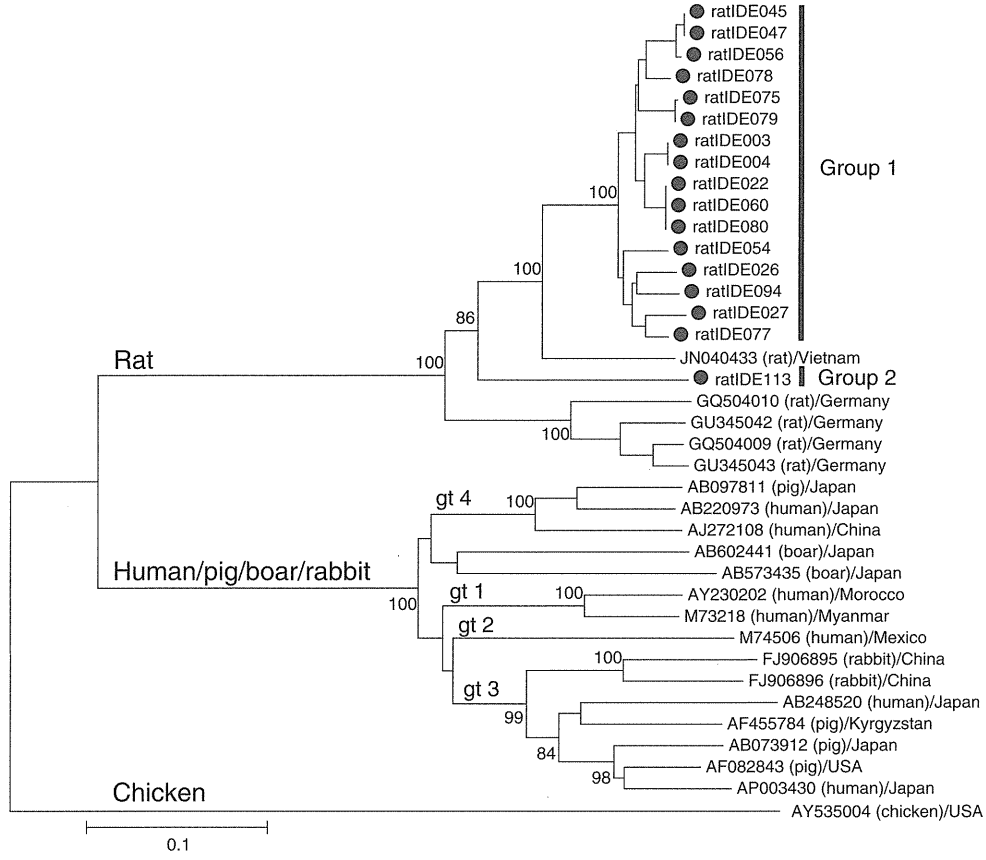

Fig. 3 Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF1-ORF2 regions (840 nt) of 37 HEV isolates, using an avian HEV strain (AY535004) as an outgroup. The 17 rat HEV isolates found in the present study are indicated by closed circles, and the five reported rat HEV isolates of German and Vietnamese origin, 15 representative HEV isolates derived from humans, pigs, wild boars, or rabbits whose common 840-nt sequence is known are included for comparison, with their host species shown in parentheses. The name of the country of isolation is indicated after the slash. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data. Bar: 0.1 nucleotide substitutions per site

the viremic rats were also remotely related to the reported German and U.S. strains, with nucleotide sequence differences of 21.7-23.3 %. Although 16 of the 17 Indonesian strains were suggested to share a common ancestor with a Vietnamese strain, with a nucleotide sequence difference of 12.1-14.1 %, the remaining Indonesian strain (ratIDE113) differed from the Vietnamese strain by 18.8 %, thus suggesting the presence of a rat HEV strain in Indonesia that may belong to a novel genotype. However, the designation "genotype" has a specific meaning in HEV terminology (e.g., human HEV genotypes 1-4). For rat HEV, no threshold has been defined so far for distinction of genotypes. Until a distinct definition of a rat HEV genotype is available, another designation "lineage" or "group" should be used for the novel strain.

The present study showed that the prevalence of anti-HEV IgG among rats in Indonesia was 21/116 (18.1 %) based on the ad hoc cutoff value of 0.500 in the in-house ELISA. Since a sufficient number of serum samples from rats that are known to be uninfected was not available during the current study, the cutoff value was tentatively set at 0.500, although approximately 65 % of the samples tested had an OD value of <0.200. It is therefore possible that the prevalence of anti-HEV IgG, at 18.1 % as determined in the present study, was underestimated because the OD value of some of the samples with OD values between 0.200 and 0.500 was less than 30 % of the original value after absorption with the recombinant ORF2 protein. Many previous studies have shown that various species of rats, including Norway (*R. norvegicus*), black (*R. rattus*), and cutton (*Sigmodon hispidus*) rats, have antibodies detectable by ELISA with recombinant ORF2 protein from human HEV strains [1, 6, 7, 12]. Two recently published studies show that cross-reactions between human HEV and rat HEV are present but that homologous antigens react more effectively with their respective antibodies [11, 17]. Therefore, recombinant ORF2 protein of rat HEV should be used as an antigen probe in future studies in order to determine the accurate prevalence of anti-HEV antibodies in rats.

Usually, HEV RNA is correlated with anti-HEV IgM but not with anti-HEV IgG in humans [14]. Titers of anti-HEV IgM antibodies may be useful in determining which rats have acute infections and which rats have past infections. However, anti-HEV IgM was detected in a very small percentage of the rats, and the result was not considered to be useful (data not shown), as was experienced in the assay of anti-HEV IgM in pigs [20, 33]. The correlation between HEV RNA and the titer of anti-HEV IgG found in the current study (Fig. 2) was somewhat surprising, and not in line with studies of human HEV. When a total of 1,425 one- to six-month-old pigs were studied, the OD value of anti-HEV IgG tended to be higher in the 55 viremic pigs than in the 1,370 non-viremic pigs (2.222 ± 0.824 vs. 1.549 ± 0.872), but not statistically significant ($p = 0.0785$) [33]. Therefore, to draw a plausible conclusion about the relationship between the positivity of HEV RNA and the titer of anti-HEV IgG in rats, further studies on a larger number of rats are needed.

In previous studies of rat HEV, only one or two strains were isolated from the studied populations. First, rat HEV was recovered from two of 30 fecal samples [10] or two of six liver specimens [9] of wild rats in Germany. Subsequently, a Vietnamese rat HEV strain was isolated from one of the five IgM antibody-positive serum samples that were collected from 139 wild rats (*R. tanezumi* or *R. norvegicus*) in Vietnam with an IgG antibody positivity rate of 20.9 % [17], and two strains of rat HEV were isolated from serum samples from 134 wild rats (*R. norvegicus*) trapped in urban Los Angeles that had a seroprevalence of antibodies against HEV of 78.4 % [29]. In contrast, the present study identified a total of 17 viremic rats (*R. rattus*) that had detectable genomic RNA of rat HEV, accounting for 14.7 % of the rats studied. The precise reason for the high viremia rate of rat HEV in wild rats in the current study is unknown. However, the selection of PCR primers and probes may crucially influence the detection rate of rat HEV with a divergent genome. It is likely that the current RT-PCR method to detect rat HEV used primers derived from well-conserved areas of the rat HEV genome, based on the five rat HEV sequences of Germany and Vietnam origin (GQ504009, GQ504010, GU345042, GU345043, and JN040433), which may have thus contributed to the higher detection rate of rat HEV viremia. The reported primers and a probe for quantitative detection of rat HEV [29] had two to five mismatches in their 21-to-29-nt sequences, including those at the third nucleotide from the 3' terminus, against the corresponding sequences of the Indonesian strains obtained in the present study. Therefore, the extent of genomic heterogeneity of HEV complicates the detection of novel strains. Another possibility is that, although the seroprevalence rate of HEV infection was not very high in the present study in comparison to previous studies [12, 29], the prevalence rate of HEV viremia was very high among the wild rats captured in Lombok, Indonesia. The rats reach sexual maturity at a weigh of approximately 150-200 g. All but one of the 116 rats trapped for the current study had a weight of < 200 g, thus suggesting that the majority of the rats studied were at the age of susceptibility to HEV infection, comparable to domestic pigs aged 2-3 months with a high viremia rate [32].

In general, the number of houses and population density per village tended to be higher in the locations with HEV-infected rats in comparison to those without. The sanitary conditions in locations with HEV-infected rats were poorer than those in locations with no infected rats. The differences were especially apparent based on the criteria for waste disposal and household sewage. The accumulation of biological waste was higher at the locations with HEV-infected rats in comparison to the areas where no HEV-infected rats were found. Therefore, these observations suggest that a high prevalence of HEV infection among wild rats is associated with lower sanitary conditions in areas with a high population density.

The role of rats in human HEV infections remains controversial. Rats are interesting as a potential source of human infections because, although they are not a human food, they are ubiquitous and in close contact with humans everywhere. Kanai et al. [13] reported that 10 of the 56 (17.9 %) rats captured around a pig farm were positive for HEV genotype 3 and suggested that there is a contamination risk of human HEV via rats. Successful experimental infections of genotype 1 HEV to Wistar rats [19] and genotype 4 HEV to Mongolian gerbils [18] or nude mice [8] have been reported. However, none of the laboratory rats inoculated with various HEV strains of genotypes 1, 2, and 3 had evidence of infection at an infectious titer of up to 10^5 [29], thus suggesting that rats are not readily susceptible to infection with other mammalian HEVs. This is supported by the observation that none of the 116 rats in the present study had any detectable human HEV genomic RNA. Of interest, Dremsek et al. [4] reported the presence of rat HEV-specific antibodies in forestry workers. Future studies should further investigate the epidemiological role of rat HEV in human HEV infections.

In conclusion, the present study revealed that wild rats in Lombok, Indonesia, have an overall prevalence of anti-HEV IgG of 18.1 % and HEV RNA of 14.7 %, and they harbor heterogeneous rat HEV strains that differ from the prototypic German strains by 20.3-23.3 % and a Vietnamese strain by 12.1-18.8 %. This study suggests a wide distribution of rat HEV strains with divergent genomes in wild rats, and thus further efforts to search for new rat HEV strains that may be classified into novel genotype(s) in wild rats in various geographic regions in the world are warranted.

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Supplementary Table 1

Nucleotide and deduced amino acid sequence identities between reported rat, human, rabbit, wild boar, avian HEV strains and rat HEV strains isolated from 17 wild rats in Lombok, Indonesia in the present study

Strain/accession no.	Rat HEV strains from Lombok, Indonesia obtained in the present study			
	ratIDE003 and 15 other isolates in Group 1[a]		ratIDE113 in Group 2[a]	
	nt	aa	nt	aa
Rat HEV_Germany/GQ504009	78.0–79.3 (78.5 ± 0.7)	91.9–93.0 (92.6 ± 0.3)	78.3	92.3
Rat HEV_Germany/GQ504010	77.6–79.4 (78.2 ± 0.5)	91.5–92.6 (92.3 ± 0.3)	77.1	92.3
Rat HEV_Germany/GU345042	77.7–79.7 (78.5 ± 0.5)	92.3–93.4 (93.0 ± 0.3)	76.7	92.6
Rat HEV_Germany/GU345043	77.7–79.3 (78.4 ± 0.5)	91.2–92.3 (91.9 ± 0.3)	77.6	91.5
Rat HEV_USA/JF516246	77.5–78.1 (78.1 ± 0.5)	95.1	79.3	98.3
Rat HEV_Vietnam/JN040433	85.9–87.9 (87.1 ± 0.5)	97.8–99.3 (98.8 ± 0.4)	81.2	96.0
ratIDE003 and 15 other isolates (Group 1)	93.0–100 (95.0 ± 1.7)	98.5–100 (99.4 ± 0.4)	79.9–82.2 (80.8 ± 0.5)	95.2–96.3 (96.0 ± 0.3)
ratIDE113 (Group 2)	79.9–82.2 (80.8 ± 0.5)	95.2–96.3 (96.0 ± 0.3)	-	-
Genotype 1 human HEV_Myanmar /M73218	57.4–58.3 (57.6 ± 0.5)	61.4–61.8 (61.7 ± 0.1)	57.4	61.0
Genotype 2 human HEV_Mexico/M74506	55.5–56.5 (56.1 ± 0.4)	59.2–59.6 (59.5 ± 0.1)	56.1	59.2
Genotype 3 human HEV_USA/AF060668	56.7–57.8 (57.0 ± 0.4)	60.7–61.0 (61.0 ± 0.1)	57.5	60.3
Genotype 4 human HEV_China/AJ272108	57.2–58.9 (57.8 ± 0.5)	61.2–61.4 (61.3 ± 0.1)	57.8	61.4
Rabbit HEV_China/FJ906895	55.7–56.8 (56.4 ± 0.3)	59.9–60.3 (60.2 ± 0.1)	56.1	59.6
Wild boar HEV_Japan/AB573435	55.8–57.5 (56.6 ± 0.8)	59.9–60.3 (60.2 ± 0.1)	56.9	59.6
Wild boar HEV_Japan/AB602441	56.5–58.3 (57.2 ± 0.5)	60.3–60.7 (60.6 ± 0.1)	58.5	60.3
Genotype 1 avian HEV_Australia/AM943647	50.8–51.5 (51.1 ± 0.3)	50.0	50.3	48.5
Genotype 2 avian HEV_USA/AY535004	49.0–49.7 (49.3 ± 0.2)	50.4	50.5	48.5
Genotype 3 avian HEV_Hungary/AM943646	50.9–52.1 (51.5 ± 0.3)	50.0–50.4 (50.3 ± 0.1)	50.3	48.9

[a] See Figure 3.

[b] The nucleotide and amino acid sequence identities were compared within the 840-nt ORF1-ORF2 sequence and the 265-aa ORF1 sequence, respectively, but for the US rat HEV strain (JF516246) which was compared within the overlapping 292-nt and 97-aa ORF1 sequences.

Hepatitis E Virus Outbreak in Monkey Facility, Japan

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An outbreak of hepatitis E virus occurred in an outdoor monkey breeding facility in Japan during 2004–2006. Phylogenetic analysis indicated that this virus was genotype 3. This virus was experimentally transmitted to a cynomolgus monkey. Precautions should be taken by facility personnel who work with monkeys to prevent infection.

Wild or reared monkeys have been used as disease models in animal facilities worldwide. Because disease caused by hepatitis E virus (HEV) is a zoonosis (*1–4*), monkeys might be infected. We examined the prevalence of antibodies against HEV in serum and fecal samples collected from monkeys in animal facilities at the Primate Research Institute of Kyoto University in Japan for 6 years (2004–2009). We found that spontaneous infection and transmission of HEV occurred in a monkey facility.

The Study

There are 9 monkey colonies (A–I) at the Primate Research Institute of Kyoto University. Colonies A–G contained Japanese monkeys (*Macaca fuscata*), and colonies H and I contained rhesus monkeys (*Macaca mulatta*). Each colony was bred in a separate outdoor breeding facility. A total of 588 monkey serum samples were collected during September–November 2004–2009 and tested for IgG and IgM against HEV and for HEV RNA by ELISA or reverse transcription PCR (RT-PCR) as described (*5–7*). Samples from colonies G and F were collected during 2004–2006, whereas in 2009 samples were collected from colonies A, C, D, and I.

The prevalence of IgG against HEV was 0% in 2004, 20.0% in 2005, and 78.5% in 2006, followed by a gradual decrease to 35.9% in 2009 (Table 1). The prevalence of IgM against HEV increased from 0% in 2004 to 2.5% in 2005 and to 6.6% in 2006, and then decreased to 1.1% in 2007 and 0% in 2008 and 2009.

Table 1. Prevalence of IgG and IgM against hepatitis E virus in monkeys at monkey facility, Japan, 2004–2009

Year	No. positive/no. tested (%) IgG	IgM
2004	0/110	0/110
2005	24/120 (20.0)	3/120 (2.5)
2006	96/121 (78.5)	8/121 (6.6)
2007	73/96 (76.0)	1/96 (1.1)
2008	47/90 (52.2)	0/90
2009	18/51 (35.3)	0/51

IgG against HEV was not detected in any of the 9 colonies in 2004, indicating that HEV infection did not occur before October 2004. However, in 2005, the prevalence of IgG reached 100% in colony D and 20% in colony G (Figure 1). ELISA titers were high, ranging from 0.293 to 1.641 in colony D and from 0.230 to 0.845 in colony G. These results suggested that HEV infection occurred after October 2004 in the monkey facility. The prevalence of IgG was higher in colony D than in colony G, and IgM was not detected in colony D, suggesting that HEV infection occurred earlier in colony D than in colony G. These colonies adjoined each other, indicating that the first HEV infection occurred in colony D and was then transmitted to colony G. Colonies A, C, D, E, and H each had an IgG prevalence of 90%–100%, and colonies B and G had an IgG prevalence >80% in 2006 (Figure 1). These results demonstrated that infection spread over a large area, except for colony F, during 2005 and 2006.

To compare the kinetics of IgG formation during 2004–2009, serum samples from 25 monkeys awhose peak ELISA optical density (OD) values for IgG against HEV were each higher than 1.0 were selected. In most monkeys, OD values for IgG increased rapidly and then decreased gradually year by year. The kinetic pattern of monkey M1543 was different from those of other monkeys that had high OD values (2.568–2.738). IgM was detected exclusively in this monkey in 2006 (OD value 0.620).

Serum samples from the 25 monkeys were used to detect HEV RNA by RT-PCR. Four serum samples were positive for HEV RNA; all were from the same monkey (M1543) from which samples were collected in 2006, 2007, 2008, and 2009. Nucleotide sequences of 348 bp coding the partial open reading frame 2 showed 100% identity. This result indicated that monkey M1543 was infected persistently with HEV and produced virus continuously.

To examine whether HEV was present in feces, 2 fecal samples were collected from monkey M1543 in September and November 2009 for detection of HEV RNA. Both samples were positive for HEV RNA. Nucleotide sequences of these samples were identical to those detected from serum samples.

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Primers were designed on the basis of sequences of swine HEV (GenBank accession no. AB248522), and RT-PCR was performed to amplify the viral genome except for the N terminus noncoding region. This strain was designated the monkey HEV Inuyama strain (JQ026407). Phylogenetic analysis of its genome indicated that this strain belongs to HEV genotype 3 (Figure 2). Infectivity of the monkey HEV strain was examined ex vivo with a human hepatocarcinoma cell line (PLC/PRF/5), and in vivo with

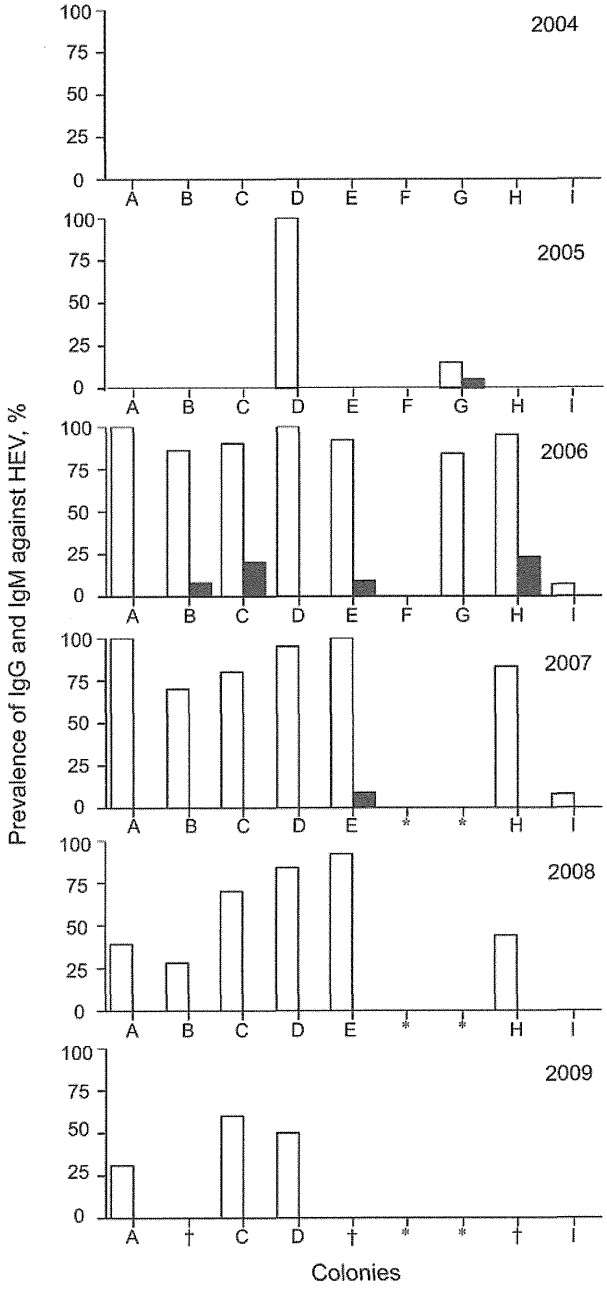

Figure 1. Prevalence of IgG (white bars) and IgM (black bars) against hepatitis E virus (HEV) in monkey facility, Japan, 2004–2009. *Monkeys were moved to another animal facility; †specimen not available.

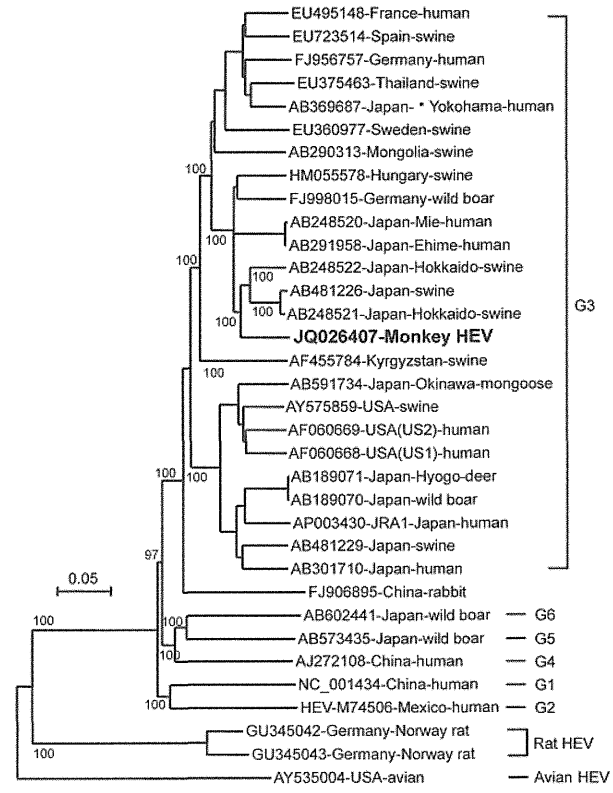

Figure 2. Phylogenetic analysis of monkey hepatitis E virus (HEV) Inuyama strain on the basis of nucleotide sequence of the HEV genome except for a 5′ noncoding region (7,206 nt) by using avian HEV as an outgroup. Values along the branches are bootstrap values determined on the basis of 1,000 resamplings of datasets. **Boldface** indicates strain isolated in this study. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

2 HEV-negative cynomolgus monkeys. Both experiments showed that the virus was infectious (online Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/pdfs/12-0884-Techapp.pdf).

A total of 94 human serum samples were collected from staff of the Primate Research Institute and subjected to ELISA for detection of IgG and IgM against HEV. All serum samples were negative for IgM against HEV, but the prevalence of IgG was 6.9% in 2007, 9.7% in 2008, and 11.8% in 2009, although differences among these years were not significant ($p>0.05$) (Table 2). No HEV RNA was detected in serum samples, and none of the staff had symptomatic hepatitis E during the 6-year study.

Conclusions

We conducted long-term monitoring of HEV infection in monkeys and report natural infection and transmission of HEV in a monkey facility. We sought to determine the source of the HEV outbreak and where HEV was intro-

Table 2. Prevalence of IgM against hepatitis E virus in serum samples from animal handlers at monkey facility, Japan, 2007–2009*

Year	No. positive/no. tested (%)
2007	2/29 (6.9)
2008	3/31 (9.7)
2009	4/34 (11.8)

*All samples were negative for IgG against hepatitis E virus and for virus RNA.

duced to colony D. At our research institute, each monkey colony is bred in a separate outdoor breeding facility built on a mountain, and the monkeys live in an environment similar to their natural habitat. Because each outdoor feeding facility is isolated by a double fence, natural reservoirs of HEV (wild boars and deer) cannot enter it. Phylogenetic analysis of monkey HEV strains indicated that this virus was genotype 3, and BLAST analysis showed that the monkey isolate is closest to HEV strains isolated from pigs in Japan. Nucleotide identities were 92%–93% (AB248521, AB248522, and AB481226). However, no evidence indicates that HEV is transmitted from pigs or wild boars to monkeys.

A notable finding in this study was the persistence of HEV infection. Generally, HEV infection is self-limiting and symptoms are transient. Persistent HEV infection occurs in solid-organ transplant recipients who have received immunosuppressive drugs (*8*) or in patients with other conditions associated with immunosuppression, such as HIV infection (*9*) and hematologic malignancies (*10*,*11*). However, there is no evidence of immunosuppression in monkey M1543, and the cause of the persistent HEV infection in this monkey is unknown.

The fact that the infectious HEV strain was detected in a monkey facility and caused an HEV outbreak cast doubt and apprehension on the safety of handling monkeys. Although no staff member showed development of symptomatic hepatitis E, precautions should be taken by facility workers who work with monkeys to prevent infection with HEV.

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Characterization of Full Genome of Rat Hepatitis E Virus Strain from Vietnam

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We amplified the complete genome of the rat hepatitis E virus (HEV) Vietnam strain (V-105) and analyzed the nucleotide and amino acid sequences. The entire genome of V-105 shared only 76.8%–76.9% nucleotide sequence identities with rat HEV strains from Germany, which suggests that V-105 is a new genotype of rat HEV.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (*1*), classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (*2*). Hepatitis E, caused by HEV infection, is a serious public health concern in developing countries and is recognized as sporadic and endemic acute hepatitis (*3*). To date, at least 4 genotypes of HEV have been isolated from humans (*4*). In addition, HEV has been isolated from other mammals, including pigs, wild boars, wild deer, rabbits, ferrets, bats, chickens, and wild rats (*5–9*). Much direct evidence indicates that HEV is transmitted from pigs or wild boars to humans, and therefore hepatitis E caused by genotypes 3 and 4 is recognized as a zoonotic disease (*6,8,10*).

Rat HEV was first isolated from Norway rats in Germany (*7,11*). Since then, rat HEV strains have been isolated from wild rats in other areas of Germany and detected in wild rats in the United States and Vietnam (*12–14*). Those results suggest that rat HEV infection is not restricted to Germany but is broadly distributed in wild rats throughout the world. The nucleotide sequences of the rat HEV isolated in Germany and the United States are similar; however, the partial sequences of the Vietnam rat HEV strain (V-105, JN040433) have been found to have 78.18%–79.43% identities with isolates from Germany, R63 and R68 (*14*). To confirm whether new genotypes of rat HEV exist, we amplified the entire genome of the rat HEV V-105 strain and analyzed the sequences. We confirmed that the rat HEV strain isolated in Vietnam belongs to a new genotype of rat HEV.

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The Study

The rat HEV used in this study was isolated from a 10% lung homogenate of a wild rat from Vietnam, which was positive for rat HEV RNA by reverse transcription PCR (RT-PCR) (*14*). Because of the limited availability of rat specimens that are positive for HEV RNA, we first transmitted the rat HEV to a laboratory rat (Wistar) to produce a large amount of virus for RNA extraction and genome amplification. After intravenous inoculation of the rat, fecal specimens positive for HEV RNA were collected, and a 10% suspension was prepared as described (*15*). RT-PCR was performed by using Superscript II RNase H^- (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN (*14*). The full-length genome of the V-105 strain was amplified by RT-PCR with primers based on the nucleotide sequences of GU345042 and JN040433 (Table 1). All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequencing was carried out by using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA).

Because 901 nt of V-105, corresponding to nt 4108–5008 of the R63 genome, were already known (*14*), primers F13 and open reading frame (ORF) 1–R12 were designed. An ≈2,100-nt fragment of the C-terminus of the rat HEV V-105, nt 4923-poly (A) tail, was amplified with a pair of primers, F13 and TX30SXN, by the first RT-PCR. The ORF1 region was amplified with primers ORF1-F1 and ORF1-R12. Two fragments, 440 nt (nt 11–450) and 1,182 nt (nt 2990–4171), were amplified by nested PCR with 2 sets of primers, ORF1-F2/ORF1-R1 and ORF1-F7/ORF1-R12, respectively. On the basis of the nucleotide sequences of those amplified fragments, ORF1-F9, ORF1-F16, ORF1-R16, ORF1-F18, and ORF2-R21 were designed, and 3 fragments, 1,830 nt (nt 388–2217), 996 nt (nt 2080–3075), and 1,110 nt (nt 3991–5100), were amplified with 3 sets of primers, ORF1-F9/ORF1-R10, ORF1-F16/ORF1-R16, and ORF1-F18/ORF2-R21, respectively.

To amplify the N-terminus nonstructural region of V-105, we synthesized cDNA with primer ORF1-R14, and a DNA anchor (P-CACGAATTCACTATCGATTCTGG AACCTTCAGAGG-NH_3) was linked to the N-terminus of the cDNA by T4 RNA Ligase I (BioLabs, Tokyo, Japan). By using this anchor-cDNA as the template, the first and the nested PCRs were carried out with 2 sets of primers, anchor-1/ORF1-R14 and anchor-2/ORF1-R13, respectively.

Table 1. Oligonucleotides used in amplifying the complete genome of the rat HEV Vietnam strain, V-105*

Primers	Product length, b
Forward ORF1-F1 (1-21)† 5'-GCAACCCCCGATGGAGACCCA-3'‡ Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	§
Forward ORF1-F2 (11-30) 5'-ATGGAGACCCATCAGTATGT-3'† Reverse ORF1-R1 (431-450) 5'-GTGCAAAAGGAAAGATCAGT-3'	440
Forward ORF1-F9 (388-408) 5'-AGCTAACAACATCCGCCGTTG-3' Reverse ORF1-R10 (2197-2217) 5'-TGGGTTCGGTCGAAGGCCTCT-3'†	1,830
Forward ORF1-F16 (2080-2100) 5'-TGCAGCCGTTTATGAGGGAGA-3' Reverse ORF1-R16 (3055-3075) 5'-CGCCATTCTGTGGGTTCTAGA-3'	996
Forward ORF1-F7 (2990-3009) 5'-GACCCAAGGCAGATCCCTGC-3'† Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	1,182
Forward ORF1-F18 (3991-4011) 5'-ATTCACCACAGACGAGCCAGT-3' Reverse ORF2-R21 (5079-5100) 5'-GGTGATAGCCAATTGGTAAGCT-3'	1,110
Forward F13 (4896-4915) 5'-AATAACACTCTGGGCTGTAG-3' ReverseTX30SXN 5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	2,092
Forward primer Anchor-1: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3' Reverse primer ORF1-R14 (276-296) 5'-TAGACCTAGGGTGCGCACCGA-3'	§
Forward primer Anchor-2: 5'-GAATCGATAGTGAATTCGTG-3' Reverse primer ORF1-R13 (200-220) 5'-AACACGCTGTACCGGATGCGA-3'	240

*HEV, hepatitis E virus; ORF, open reading frame.
†Numbers in a parentheses show the positions of primers corresponding to the entire genome of rat HEV V-105.
‡Primer designed based on rat HEV (GU345042).
§The PCR product was not detected.

The V-105 genome consisted of 6,927 nt plus a poly (A) tail of a still-undetermined length (GenBank accession no. JX120573). The genomic structure of V-105 was, from the N-terminus toward the C-terminus, the N 5′–untranslated region (UTR) at nt 1–10, ORF1at nt 11–4900, ORF3 at nt 4917–5225, ORF2 at nt 4928–6862, the 3′-UTR at nt 6863–6927, and the poly (A) tail starting at nt 6928. ORF2 and ORF3 encode 644 aa and 102 aa, respectively, as do R63 and R68. However, ORF1 of V-105 encodes 1,629 aa, which is 7 aa shorter than either R63 or R68. The V-105 genome possessed 2 aa insertions (Ser-Pro) between the aa residues 591 and 592 and 9 aa deletions (Ser-Pro-Pro-Gly-Pro-Pro-Pro-Ala-Gly) between aa residues 852 and 853, corresponding to those of R63. The 3′-UTR was 65 nt as were R63 and R68. Unlike R63 and R68, only 1 additional putative ORF, corresponding to ORF4 (nt residues 27–578), was found in V-105, suggesting that other putative ORFs, ORF5 and ORF6 found in R63 and R68, are not common in rat HEV.

When the V-105 genome was compared with reported HEV genomes, the V-105 genome shared identities of only 50.5% with avian HEV, 53.6% with rabbit HEV, 53.7%–54.0% with wild boar HEV, and 53.1%–53.5% with HEV genotypes 1–4. In contrast, V-105 shared relatively high nucleotide sequence identities (76.8%–76.9%) with rat HEV strains (R63 and R68) (Table 2). The nucleotide and amino acid sequences of ORF1, ORF2, and ORF3 of V-105 were compared with those of other HEV genotypes, and the identities among them are shown in Table 2. Together, these results suggest that V-105 is more similar to rat HEV than to other HEV genotypes.

Phylogenetic trees were generated on the basis of the nucleotide sequences derived from the entire genome and ORF3 of the genotypes 1–4, wild boar, rabbit, chicken, and rat HEV isolates. These trees demonstrated that V-105 does not belong to any known genotype and should probably be classified into a new genotype (Figure).

Table 2. Nucleotide and deduced amino acid sequence identities between human, wild boar, rabbit, rat, and avian HEV strains, compared with Vietnam rat HEV V-105 strain*

HEV strain (GenBank accession no.)	Vietnam rat HEV strain						
	Entire genome	Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC_001434)	53.5	50.7	60.8	51.0	54.1	55.5	33.3
Genotype 2 (M74506)	53.3	51.2	59.1	51.4	53.1	54.6	30.6
Genotype 3 (AF060668)	53.3	50.8	59.2	53.8	51.6	57.1	26.5
Genotype 4 (AJ272108)	53.1	50.9	58.9	52.6	50.7	55.4	24.5
Wild boar HEV (AB573435)	53.7	51.5	59.6	53.4	50.3	56.3	28.2
Wild boar HEV (AB602441)	54.0	51.5	59.6	53.4	50.3	56.3	28.2
Rabbit HEV (FJ906895)	53.6	51.3	59.3	51.5	52.1	56.2	26.2
Rat HEV (GU345042)/R63	76.9	75.7	79.6	80.6	87.0	91.6	66.7
Rat HEV (GU345043)/R68	76.8	75.5	79.8	80.9	86.4	92.1	66.7
Avian (chicken) HEV (AY535004)	50.5	49.7	54.2	47.0	44.7	47.4	33.9

*HEV, hepatitis E virus; ORF, open reading frame.

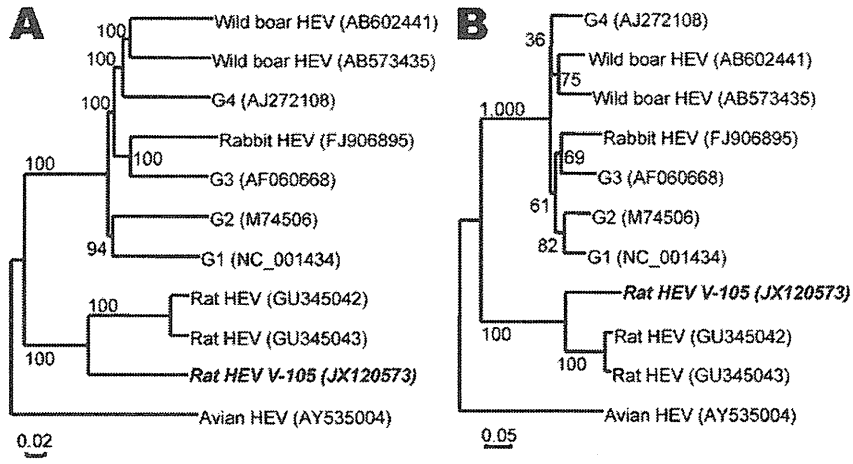

Figure. Phylogenetic relationships among genotypes 1–4, wild boar, rabbit, avian (chicken), and rat hepatitis E virus (HEV) isolates. The nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by the Kimura 2-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method, based on the entire genome (A) and open reading frame 3 (B) of the genotypes 1–4, wild boar, rabbit, avian (chicken), and rat HEV isolates. Scale bar indicates nucleotide substitutions per site. **Boldface** indicates isolate used in this study.

Conclusions

In this study we successfully amplified the entire genome of an HEV strain isolated from a wild rat in Vietnam. Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4, wild boar, rabbit, and chicken HEV and close to those of the rat HEV strains. Although the entire genome of V-105 shared nucleotide sequence identities of only 76.8%–76.9% with the isolates from Germany (R63 and R68), the ORF1 and ORF3 amino acid identities between V-105 and these isolates were 86.4%–87.0% and 66.7%, respectively, which suggests that V-105 can be classified into a new genotype of rat HEV. However, ORF2 has relatively high amino acid identities with R63 and R68 (91.6%–92.1%), indicating that the V-105 and rat HEV isolates from Germany share similar antigenicity. In fact, rat HEV–like particles derived from R63 are cross-reactive to serum from V-105–infected wild rats (*14*).

In conclusion, we isolated and identified rat HEV strain V-105 from a wild rat in Vietnam, and this strain was highly divergent from known rat HEV isolates. We propose that the strain from Vietnam, V-105, is a new member of the rat HEV genotype.

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