TABLE II. (*Continued*)

Isolate name	Host	Accession number	Country	Sequence length compared (nt)	Region	Identity (%)[a]	
						swMN06-A1288[b]	swMN06-C1056[b]
swNZ	Swine	AF215661	New Zealand	570	ORF1+ORF2	87.7	84.0
UIAS268	Human	DQ061078	Russia	683	ORF2	82.7	88.9
VH1	Human	AF195064	Spain	823	ORF1+ORF2	83.4	87.1
VH2	Human	AF195065	Spain	823	ORF1+ORF2	82.0	85.7
E11	Swine	AF195063	Spain	304	ORF2	82.2	86.8
SpswfCV3	Swine	DQ093564	Spain	307	ORF2	82.6	87.5
65103 and 25 others	Swine	DQ315745–DQ315770	Spain	168	ORF2	77.4–82.1	82.1–87.5
UAB1 and 11 others	Swine	DQ383734–DQ383745	Spain	196–216	ORF2	82.5–85.2	84.9–86.8
TW3SW	Swine	AF296167	Taiwan	304	ORF2	83.9	79.9
T2-4s	Swine	AY858893	Thailand	304	ORF2	84.5	83.6
UK	Human	AJ315768	UK	287	ORF1	81.9	82.2
NT1 and 8 others	Human	AJ879566–AJ879574	UK	280	ORF2	78.2–83.2	83.6–86.7
UK7518	Human	AY582797	UK	304	ORF2	80.3	87.5
UK8734	Human	AY362357	UK	304	ORF2	79.6	87.8
P354/1/02	Swine	AF503511	UK	473	ORF2	83.3	87.1
P143/11/02	Swine	AF503512	UK	473	ORF2	81.0	86.4
sw01-21160-1 and 26 others	Swine	AF466659–AF466685	USA	300–301	ORF2	84.6–87.3	80.6–83.9
2A and 17 others	Swine	EF088382–EF088399	USA	191–222	ORF2	82.2–86.9	80.6–84.3

[a]Identities of over 90.0% are indicated in boldface.
[b]HEV isolates whose entire genomic sequence was determined in the present study.
[c]NA, not applicable.

needed to elucidate whether people in Mongolia are infected with genotype 1 HEV strains, similar to other developing countries in Asia and Africa, or with genotype 3 HEV strains, as a zoonotic disease, that are homologous to HEV strains circulating among pigs in Mongolia.

The shedding peak of HEV in infected pigs was observed mainly at 2–3 months of age, which is considered to be the age range at which infected pigs are at highest risk of spreading the infection [Takahashi et al., 2003]. In the present study, in an attempt to isolate Mongolia-indigenous swine HEV in farm pigs, serum samples were collected from 2- or 3-month-old pigs in all four swine farms surrounding Ulaanbaatar. The rate of swine HEV seropositivity varied by farm, ranging from 6.3% (Farm D) to 99.0% (Farm A) with a nearly equal distribution between 2-month-old and 3-month-old pigs in Farms A and B. Almost all of the samples from the 2-month-old pigs at Farms A, B, and C were positive for HEV antibodies, suggesting that the pigs must have been exposed to HEV and seroconverted at an earlier age. The conditions of the farms such as their structure, location, water source, vermin control, handling, and entry and exit decontamination from other piggeries may have differed and contributed to the different seropositive rate in the different farms. However, the precise reason for this difference remains unknown. The four private swine farms where serum samples were collected from pigs in the present study had been founded in the 1990s, and are located 50–150 km away from each other around Ulaanbaatar. The present results that swine HEV strains from Farms A and B and those from Farms C and D segregated into two distinct phylogenetic clusters, respectively (Fig. 1), prompted us to ask the farmers about the origin of their pigs raised in the four farms. They informed us that pigs in Farm B were purchased from Farm A and that pigs in Farm D were purchased from Farm C, suggesting that HEV strains in Farms A and B had the same origin, as did those in Farms C and D. In response to our further inquiries, the farmers reported that at the time of establishment of the pig farms, the pigs in Farm A had been imported from Novosibirsk, Russia and those in Farm C had been imported from Inner Mongolia, an autonomous region in China that is bordered to the north by Mongolia. Of note, the majority of the pigs in Farm C died of an unconfirmed cause shortly thereafter, and new pigs from the United Kingdom were introduced. However, as indicated in Table II, the low nucleotide similarity of 82.2–87.8% between swMN06-C1056 from Farm C and human and swine HEV isolates reported thus far from the United Kingdom do not support the UK-origin of swine HEV isolates in Farm C.

The swMN06-A1288 isolate from Farm A and swMN06-C1056 isolate from Farm C, as representatives of two distinct clusters of Mongolian swine HEV strains, differed by 18.4% from each other over the entire genome, and were only up to 84.9 or 85.9% identical, respectively, to the reported genotype 3 HEV isolates whose entire nucleotide sequence is known. Recently, Lu et al. [2006] proposed provisional criteria to classify HEV isolates of genotype 3 or 4 at three levels: genotype, subgenotype, and isolate. When the entire genomic sequence was compared among the HEV isolates whose entire sequence has been determined, the nucleotide

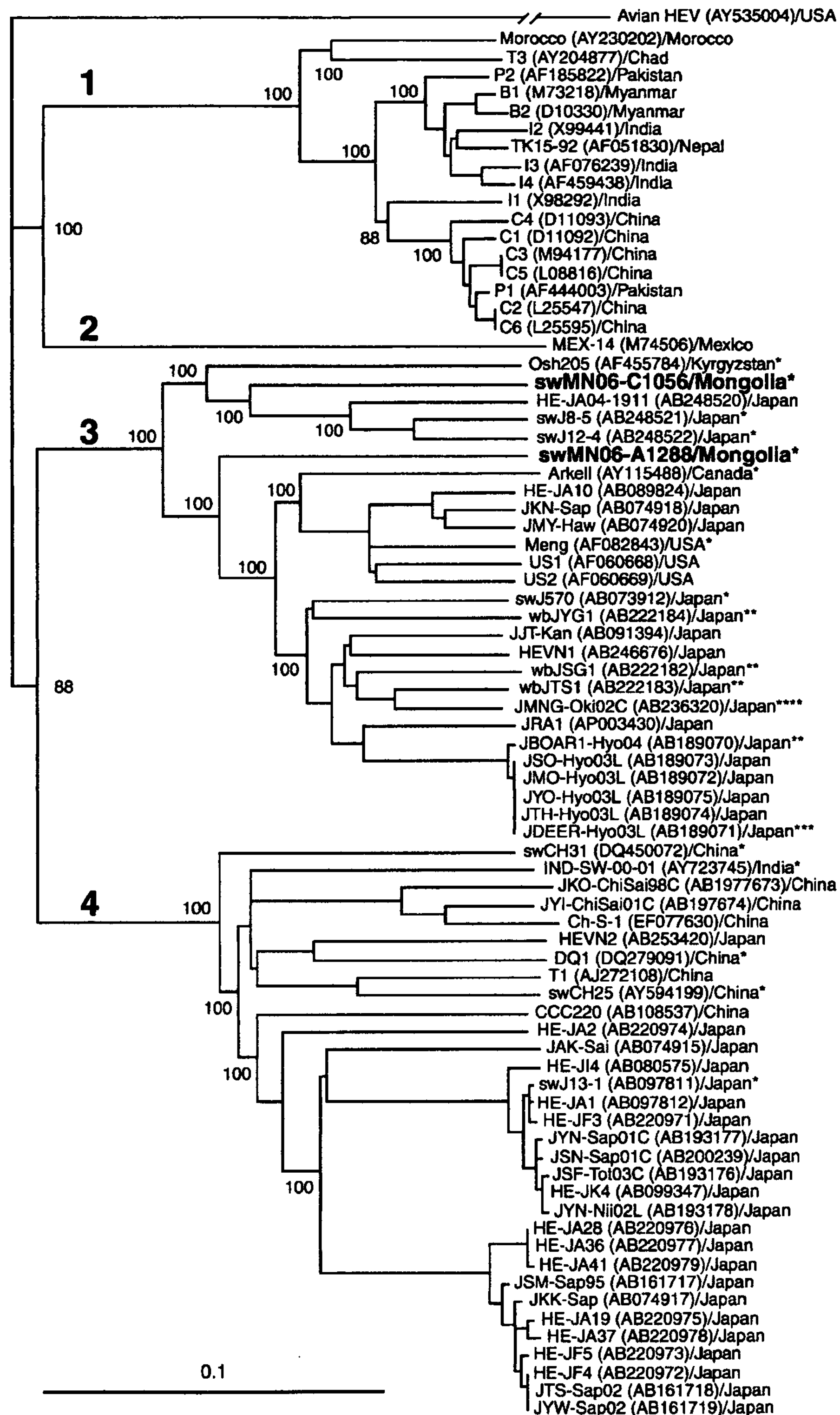

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 77 human HEV isolates, using an avian HEV isolate (AY535004) as an outgroup. The two swine HEV isolates (swMN06-A1288 and swMN06-C1056) whose full-length sequence was determined in the present study are indicated in bold type for visual clarity. Seventy-five isolates whose entire or nearly entire sequence has been reported were included for comparison, with the accession number in parentheses followed by the name of the country where it was isolated. Asterisks indicate isolates obtained from pigs (*), wild boars (**), a deer (***), and a mongoose (****), respectively. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings. Bar, 0.1 nucleotide substitutions per site.

identities were 74.4–75.6% at the genotype level, 82.0–87.9% at the subgenotype level, and 89.8–94.2% at the isolate level. The low nucleotide identities of 80.8–84.9% between the swMN06-A1288 isolate and the reported HEV isolates of genotype 3 and those of 80.9–85.9% between the swMN06-C1056 isolate and the reported genotype 3 isolates suggest that the two HEV isolates whose entire nucleotide sequence was determined in the present study can be grouped into two novel subgenotypes under genotype 3.

Although a sole genotype 3 HEV strain (UIAS268 [DQ061078]) from West Siberia of Russia has been deposited in the GenBank/EMBL/DDBJ databases, it was only 82.7% identical to the swMN06-A1288 isolate and 88.9% identical to the swMN06-C1056 isolate within the 683-nt ORF2 sequence. No genotype 3 HEV isolates had been reported from China including Inner Mongolia. A high similarity of 94.6% between the swMN06-C1056 isolate and the HU/NL2005-0825 [DQ200292] isolate from the Netherlands suggests that some of the Mongolian swine HEV strains might have originated from the Netherlands, although the nucleotide length for comparison was quite short at 148 nt. Of note is that the Mongolian government established swine farms in the early 1970s and maintained their pigs until 1989. However, it is unknown whether the pigs were infected with swMN06-A1288-like and/or swMN06-C1056-like strains. Therefore, we cannot rule out the possibility that the pigs in the four swine farms studied are infected with indigenous HEV strains that might have been prevalent in pigs raised in the 1970s in Mongolia. Further epidemiological studies on HEV strains circulating worldwide are warranted to better understand the origin and geographical distribution of extremely divergent strains of HEV.

There are accumulating lines of evidence that a number of animal species other than pigs such as rats, mice, dogs, cows, sheep, and goats may also act as natural reservoirs of HEV [Tien et al., 1997; Favorov et al., 1998, 2000; Kabrane-Lazizi et al., 1999; Purcell and Emerson, 2001b]. Besides farm pigs, approximately 30 million domestic animals including sheep, goats, cattle, horses, and camels are raised in Mongolia [http://www.discovermongolia.mn/country/Nomad_lifestyle.html]. Although the prevalence of HEV infection has not yet been studied in these domestic animals in Mongolia, numerous animals have serological evidence of prior infection of HEV [Purcell and Emerson, 2001b]. Therefore, it is tempting to speculate that cross-species transmission occur from pigs to other animals and that these animals may act as vehicles to transfer HEV from pigs to humans.

In conclusion, the present study revealed for the first time that farm pigs in Mongolia are frequently infected with genotype 3 HEV. Two novel clusters of swine HEV strains, represented by the swMN06-A1288 and swMN06-C1056 isolates, comprised distinct subgenotypes under genotype 3. Mongolia is one of the developing countries in Asia, and the livelihood of its people mostly depends on livestock herding including sheep, goats, camel, horses, cattle, and pigs: mutton, beef, and horse meat are the major meat products for human consumption. Therefore, further studies are warranted to clarify whether other animal species in addition to pigs that are raised in Mongolia are also infected with HEV and whether hepatitis E occurs as a zoonosis in this country.

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Identification of Genotype 4 Hepatitis E Virus Strains From a Patient With Acute Hepatitis E and Farm Pigs in Bali, Indonesia

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A previous study revealed that antibodies to hepatitis E virus (HEV) (anti-HEV) are highly prevalent among healthy individuals and farm pigs in Bali, Indonesia, and suggested that HEV infection may occur via zoonosis among Balinese people. However, there were no reports of acute hepatitis E in Bali. To elucidate whether Balinese HEV strains recovered from infected humans and pigs have significant sequence similarity, serum samples obtained from 57 patients (age, mean ± standard deviation, 31.1 ± 11.9 years) with sporadic acute hepatitis and from one hundred and one 2- or 3-month-old farm pigs in Bali were tested for anti-HEV and HEV RNA. Among the 57 patients, 2 (3.5%) had high-titer IgM/IgA class anti-HEV antibodies and one of them had detectable HEV RNA (BaliE03-46). Overall, 58 pigs (57.4%) tested positive for anti-HEV, while 5 pigs (5.0%) had detectable HEV RNA. Based on the 412-nucleotide sequence within open reading frame 2, the BaliE03-46 isolate and the 5 swine HEV isolates recovered from the viremic pigs were phylogenetically classified in genotype 4, but were only 77.3–90.8% identical to the genotype 4 HEV isolates reported thus far in China, India, Japan, Taiwan, and Vietnam. The BaliE03-46 isolate of human origin shared high identities of 97.3–98.3% with 4 of the 5 Balinese swine isolates, but differed by 16.1% from the remaining swine isolate. These results suggest that indigenous HEV strains of genotype 4 with marked heterogeneity are circulating in Bali, Indonesia, and that pigs are reservoirs of HEV for Balinese people who have a habit of ingesting uncooked pigs. ***J. Med. Virol. 79:1138–1146, 2007.*** © 2007 Wiley-Liss, Inc.

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KEY WORDS: hepatitis E virus; acute hepatitis; swine; zoonosis; Indonesia

INTRODUCTION

Hepatitis E, which is caused by hepatitis E virus (HEV), is a major public health problem in many developing countries where the sanitation condition is suboptimal. HEV is endemic in many areas of Asia and Africa and one epidemic in Mexico was documented [Purcell and Emerson, 2001]. Transmission of HEV occurs primarily by the fecal-oral route through contaminated water supplies in developing countries. Recent studies indicate that domestically infected hepatitis E also occurs among individuals in industrialized countries where hepatitis E had been believed to

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB298157–AB298166 (10 HAV isolates), AB298167–AB298177 (11 HBV isolates), and AB298178–AB298183 (6 HEV isolates).

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be non-endemic [Kwo et al., 1997; Harrison, 1999; Purcell and Emerson, 2001; Mizuo et al., 2002; Okamoto et al., 2003; Mansuy et al., 2004; Ijaz et al., 2005; Amon et al., 2006], and that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Harrison, 1999; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Meng, 2005]. Hepatitis E is usually self-limited and typically occurs in locations where laboratory diagnosis is unavailable. Consequently, the true burden of HEV infection remains unknown especially in developing countries.

HEV is a small nonenveloped virus and is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004]. Its genome is a single-stranded, positive-sense RNA of approximately 7.2 kilobases. It consists of a short 5′ untranslated region (UTR) followed by three partially overlapping open reading frames (ORFs: ORF1, ORF2 and ORF3), and then a short 3′UTR terminated by a poly(A) tract [Tam et al., 1991]. Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates, and mammalian HEV sequences have been classified into four genotypes (genotypes 1–4): the majority of HEV infections in developing countries are caused by genotype 1; genotype 2 consists of strains in Mexico and African countries including Chad, Namibia and Nigeria; genotype 3 is widely distributed throughout the world and has been isolated from sporadic cases of hepatitis E and domestic pigs in the United States, European countries and Japan; and genotype 4 is distributed exclusively in Asian countries including China, Taiwan, and Japan [Schlauder and Mushahwar, 2001; Lu et al., 2006].

In Indonesia where HEV is believed to be highly endemic, to date, only three outbreaks of HEV transmission have been documented in restricted areas (West Kalimantan and East Java) [Corwin et al., 1997, 1999; Sedyaningsih-Mamahit et al., 2002], and no epidemic or sporadic HEV transmission has ever been reported in other parts of Indonesia. In a previous study, antibodies to HEV (anti-HEV) were detected in 20% of the tested population in Bali where most of the people are Hindu and have a habit of consuming pork, while anti-HEV were detected in only 4% of the tested population in Lombok and 0.5% in Surabaya, where the majority of the populations are Muslim [Wibawa et al., 2004]. In addition, a high prevalence of anti-HEV was noted among farm pigs in Bali and a genotype 4 HEV strain (SB66-Bali) was identified [Wibawa et al., 2004], suggesting that HEV infection in Bali may occur via zoonosis. However, no cases of acute hepatitis E have been reported thus far in Bali. Therefore, the present study was conducted to search for acute hepatitis E cases among patients with sporadic acute hepatitis and for infected pigs raised in swine farms in Bali, and to characterize both human and swine HEV strains circulating there, in an attempt to gain further insight into the possible zoonotic transmission of HEV in Indonesia.

MATERIALS AND METHODS

Serum Samples

A total of 57 patients (42 males and 15 females; age, mean ± standard deviation [SD], 31.1 ± 11.9 years; range, 12–62 years) who were clinically diagnosed as having acute hepatitis during the period from August 2003 to October 2006, at Sanglah Hospital of Udayana University, Denpasar on Bali Island of Indonesia, were studied. This study included patients with an acute illness, presenting with clinical signs or symptoms of acute hepatitis such as jaundice, dark urine, general fatigue, anorexia, nausea, vomiting and fever, and who had a serum alanine aminotransferase (ALT) level that was at least two-and-one-half times the upper limit of normal. Excluded from this study were patients with alcoholic liver disease and those with a history of exposure to hepatotoxic drugs or chemicals. Autoimmune liver disease was not excluded by the routine test. Serum samples were obtained after informed consent at the first visit and were stored at −20°C or below until testing for serological and molecular markers of hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis delta virus (HDV) and HEV infections.

Serum samples were also collected from 101 pigs raised in swine farms in three distinct geographic regions (Kapal, Kerambitan and Dalung) in Bali Island in 2004.

Serological Testing

Sera from patients with acute hepatitis were tested for IgM antibody against HAV (anti-HAV IgM) by enzyme-linked immunosorbent assay (ELISA) (AxSYM HA-M Dainapack v.2.0, Abbott Japan, Tokyo, Japan), and for hepatitis B surface antigen (HBsAg) and antibodies to HBV core (anti-HBc [total]) by passive hemagglutination with commercial assay kits (Mycell II HBsAg and Mycell anti-rHBc, respectively; Institute of Immunology Co. Ltd., Tokyo, Japan). The IgM-class antibodies to HBV core (anti-HBc IgM) were detected by in-house ELISA according to the previously described method [Shimizu et al., 1983] with slight modifications, using purified recombinant HBV core particles that had been expressed in *Bacillus brevis* (Institute of Immunology Co. Ltd.) and peroxidase-conjugated mouse monoclonal anti-HBc (No. 3105) [Takahashi et al., 1983]. Antibodies to HCV (anti-HCV) were assayed by the hemagglutination method (Abbott HCV-PHA; Abbott Japan). The presence of antibodies to HDV was determined by in-house ELISA, using purified recombinant S-HDAg protein that had been expressed in the pupae of silkworm, as described previously [Inoue et al., 2005]. The IgG, IgM and IgA classes of antibodies to HEV (anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, respectively) as well as HEV RNA were detected as described below.

Detection of HAV, HBV, HCV and HDV Genomes and Determination of Their Genotypes

Sera from individuals with anti-HAV IgM were assayed for HAV RNA by reverse transcription (RT)-polymerase chain reaction (PCR) with nested primers derived from the VP1-2B region of the HAV genome as described previously [Mitsui et al., 2006]. Briefly, the amplification product of the first-round PCR was 548 base pairs (bp) (nucleotides [nt] 2,885–3,432), and that of the second-round PCR was 522 bp (nt 2,900–3,421): nucleotide numbers are in accordance with the prototype genotype IA HAV isolate (accession no. K02990). The HAV genotype was determined by phylogenetic analysis of the VP1-2B region sequence of the HAV isolates.

For serum samples with anti-HBc IgM, the presence of HBV DNA was determined by nested PCR targeting the S gene region as described previously [Takahashi et al., 2004]. The amplification product of the first-round PCR was 461 bp (nt 244–704), and that of the second-round PCR was 437 bp (nt 251–687): nucleotide numbers are in accordance with a genotype C HBV isolate of 3,215 nt (AB033550). The HBV genotype was determined by phylogenetic analysis of the S gene of the HBV isolates.

Sera from individuals with anti-HCV were assayed for HCV RNA by RT-PCR using nested primers derived from the 5′ UTR of the HCV genome as previously described [Okamoto et al., 1994]. HCV genotypes 1a, 1b, 2a, 2b and 3a were determined by the previously described method with a slight modification [Okamoto et al., 1993]. In brief, the original genotype 1b-specific antisense primer (No. 133) was replaced by another primer, No. 492 [Holland et al., 1996].

No patients were positive for anti-HDV and therefore no samples were subjected to detection of HDV RNA.

Detection of Human and Swine Antibodies to HEV

To detect anti-HEV IgG, anti-HEV IgM and anti-HEV IgA in human serum, ELISA was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm [Mizuo et al., 2002], as described previously [Takahashi et al., 2005]. Peroxidase-conjugated mouse monoclonal anti-human IgG antibody, peroxidase-conjugated mouse monoclonal anti-human IgM antibody, or peroxidase-conjugated mouse monoclonal anti-human IgA antibody was used in the ELISA assays for anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, respectively. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA assays was 0.175, 0.440, and 0.642, respectively [Takahashi et al., 2005]. Samples with OD values for anti-HEV IgG, IgM, or IgA greater than or equal to the respective cut-off value were considered to be positive for anti-HEV IgG, IgM, or IgA, respectively.

Swine anti-HEV IgG was detected by ELISA as described previously [Takahashi et al., 2003] with a slight modification. In brief, sample diluent was mixed with a mock protein obtained from the pupae of silkworm infected with nonrecombinant baculovirus, in an attempt to minimize non-specific reactions. The cut-off value used for the swine anti-HEV IgG assay was 0.274. Test samples with OD values for swine anti-HEV IgG greater than or equal to the cut-off value were considered to be positive for anti-HEV IgG.

The specificity of the human and swine anti-HEV assays 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% (anti-HEV IgG/IgA) or 50% (anti-HEV IgM) of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

Sera from patients with anti-HEV IgM/IgA and from farm pigs, were tested for HEV RNA by RT-PCR with the ORF2 primers as described previously [Mizuo et al., 2002]. The size of the amplification product of the first-round PCR was 506 bp (nt 5,955–6,460), and that of the second-round PCR was 457 bp (nt 5,965–6,421): nucleotide numbers are in accordance with the HE-JA10 isolate (AB089824). The nested RT-PCR assay was performed in duplicate, and reproducibility was confirmed. The specificity of the RT-PCR assay was verified by 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].

Sequence Analysis of PCR Products

The amplification product was sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from DDBJ (National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequence alignments were generated by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987] based on the partial nucleotide sequence of the ORF2 region (412 nucleotides [nt]). Bootstrap values were determined on 1,000 resamplings of the data sets [Felsenstein, 1985].

RESULTS

Etiology of Acute Hepatitis

The prevalence of hepatitis virus infections among 57 patients with acute hepatitis in Bali, Indonesia, was surveyed by testing serum samples for the IgM class of antibodies against HAV, HBV and HEV, HBsAg, and antibodies against HCV and HDV. As shown in Table I, hepatitis A (anti-HAV IgM-positive) was diagnosed in 16 patients (28.1%), two of whom had HBsAg

TABLE I. Demographic Features and Laboratory Findings in 57 Patients With Acute Hepatitis in Bali, Indonesia

Feature	Acute hepatitis A (n = 16)	Acute hepatitis B (n = 11)	Acute exacerbation of chronic HBV infection (n = 9)	Acute hepatitis C (n = 3)[a]	Acute hepatitis E (n = 2)	Non-A–E acute hepatitis (n = 16)
Mean age ± SD (years)	28.9 ± 11.5	27.1 ± 11.2	30.1 ± 12.8	35.3 ± 21.4	24.5 ± 4.9	36.7 ± 10.4
Male (%)	12 (75.0)	11 (100)	8 (88.9)	3 (100)	1 (50.0)	7 (43.8)
Laboratory data						
Total bilirubin (mg/dl)	8.9 ± 7.2	11.3 ± 9.5	7.4 ± 6.9	6.1 ± 5.6	3.7 ± 2.1	3.6 ± 2.8
ALT (IU/L)	688.4 ± 756.6	1007.8 ± 746.4	550.8 ± 539.9	874.3 ± 572.1	299.5 ± 30.4	733.6 ± 486.1
AST (IU/L)	298.3 ± 320.0	502.3 ± 386.3	283.2 ± 494.6	689.7 ± 516.6	369.5 ± 201.5	667.1 ± 710.8

[a]There is a possibility that they were chronic carriers of HCV.

unaccompanied by anti-HBc IgM, suggesting that these two patients acquired de novo HAV infection on the background of chronic HBV infection. Ten of the 16 patients with anti-HAV IgM were found to have detectable HAV RNA of genotype IA (accession nos. AB298157–AB298166). Type B acute hepatitis (anti-HBc IgM-positive) was diagnosed in 11 patients (19.3%) including one patient who was negative for HBsAg. All 11 patients with anti-HBc IgM were positive for HBV DNA and the HBV isolates were recovered. Phylogenetic analysis based on the S gene sequence revealed that 9 of the 11 HBV isolates were classifiable into genotype B and the remaining 2 isolates into genotype C (accession nos. AB298167–AB298177). Among the patients with acute hepatitis B, no patient had anti-HDV IgG, indicating that none of the patients studied contracted coinfection of HBV and HDV. Nine patients had HBsAg unaccompanied by anti-HBc IgM or any other hepatitis virus markers. These nine patients were suspected of having acute exacerbation of the chronic carrier state. Including the 23 patients with HBsAg, 45 patients (78.9%) had anti-HBc (total), suggesting a high prevalence of present or past HBV infection in Bali. Three patients (5.3%) were positive for both anti-HCV and HCV RNA and negative for anti-HAV IgM, anti-HBc IgM and anti-HEV IgM, suggesting that they contracted type C acute hepatitis, although there is a possibility that they were persistent HCV carriers. The genotype of HCV in these three patients was exclusively 1b. There was a high prevalence of anti-HEV IgG (40.4% or 23/57) most likely representing past infection of HEV, among the studied population. Two patients (3.5%) had IgG, IgM, and IgA classes of anti-HEV antibodies with high OD values of >2.0, respectively, and were diagnosed with sporadic acute hepatitis E: one of them had detectable HEV RNA (Table II). There were no appreciable differences in the demographic features and laboratory data between the patients with acute hepatitis E and those with other types of hepatitis (Table I), probably due to the small number of acute hepatitis cases in the present study. Sixteen patients (28.1%) were serologically negative for all known markers of hepatitis viruses, and were tentatively categorized as "non-A to E" cases in the present study.

Prevalence of Anti-HEV and HEV RNA Among Farm Pigs in Bali

Serum samples obtained from one hundred and one 2- or 3-month-old farm pigs in Bali were tested for anti-HEV IgG. The sera from 58 pigs (57.4%) were positive for anti-HEV, with comparable rates among the three farms studied (Table III). HEV RNA was detectable in three (15.0%) of the twenty 3-month-old pigs in Kapal and two (3.6%) of the fifty-five 2- or 3-month-old pigs in Kerambitan. One of the three viremic pigs in Kapal was positive for anti-HEV, while both viremic pigs in Kerambitan were 2 months old and seropositive for anti-HEV.

Genetic Analysis of Human and Swine HEV Isolate Circulating in Bali

The human HEV isolate recovered from the viremic patient was named BaliE03-46 and the five swine HEV isolates obtained from viremic pigs were named swBaliE04-08, swBaliE04-10, swBaliE04-18, swBaliE04-84,

TABLE II. Characteristics of Two Patients Who Were Diagnosed as Having Sporadic Acute Hepatitis E in Bali, Indonesia

Case no.	Age (years)/sex	Date of sampling	Total bilirubin (mg/dl)	ALT (IU/L)	AST (IU/L)	Anti-HEV (OD value) IgG-class	IgM-class	IgA-class	HEV RNA
03-39	21/F	30 August 2003	2.2	321	512	2.100 (+)	2.850 (+)	2.329 (+)	−
03-46	28/M	15 October 2003	5.1	278	227	2.169 (+)	2.885 (+)	2.293 (+)	+

TABLE III. Prevalence of Anti-HEV and HEV RNA Among Pigs From Three Areas in Bali, Indonesia

Region	No. of pigs	Age (months)	Anti-HEV IgG No. (%)	Anti-HEV IgG Optical density (OD) (mean ± SD)	No. (%) of pigs with HEV RNA
Kapal	20	3	11 (55.0)	1.683 ± 0.985	3 (15.0)
Kerambitan	55	2–3	33 (60.0)	1.368 ± 0.981	2 (3.6)
Dalung	26	2–3	14 (53.8)	1.270 ± 0.785	0
Total	101	2–3	58 (57.4)	1.404 ± 0.933	5 (5.0)

and swBaliE04-88, respectively. The 412-nt sequence of ORF2 of the human and swine HEV isolates obtained in the present study was determined and compared with each other. When compared with the 5 swine HEV isolates, the BaliE03-46 isolate was most closely related with the swBali04-18, with an identity of 98.3%. The 5 isolates of BaliE03-46, swBaliE04-08, swBaliE04-10, swBaliE04-18, and swBaliE04-88 showed high nucleotide identities of 97.3–99.8% with each other, but were only 83.9–85.4% similar to swBaliE04-84. Upon comparison with the prototype HEV isolates of genotypes 1–4, the 6 Balinese HEV isolates were closest to the prototype genotype 4 isolate of T1 (accession no. AJ272108) with nucleotide sequence identities of 85.2–87.1%, and were only 77.4–81.6%, 75.2–77.7%, and 77.2–78.6% similar to the B1 isolate (M73218) of genotype 1, MEX-14 isolate (M74506) of genotype 2, and US1 isolate (AF060668) of genotype 3, respectively. The phylogenetic tree constructed based on the common 412-nt sequence within ORF2 confirmed that the 6 Balinese human and swine HEV isolates obtained in the present study belonged to genotype 4, but that they segregated into two groups (Fig. 1): one group consisted of the BaliE03-46, swBaliE04-08, swBaliE04-10, swBaliE04-18, swBaliE04-88 and the reported Balinese swine HEV isolate (SB66-Bali) [Wibawa et al., 2004] and the second group comprised a single isolate, swBaliE04-84. Upon comparison with the reported HEV strains in the common 239- to 412-nt ORF2 sequence, the BaliE03-46, swBaliE04-08, swBaliE04-10, swBaliE04-18, and swBaliE04-88 isolates were most closely related with the SB66-Bali isolate at 97.1–98.3% identities, but were only 77.3–90.8% similar to the reported Chinese, Indian, Japanese, Taiwanese and Vietnamese HEV strains of the same genotype that had been recovered from humans or swine (Table IV). In addition, the swBaliE04-84 isolate had merely 84.7% identity with the SB66-Bali isolate and was only 80.0–87.9% identical to human or swine HEV strains that circulate in China, India, Japan, Taiwan and Vietnam.

DISCUSSION

A previous study indicated that the prevalence of HEV infection differs according to the religion and custom of the resident human population in different regions in Indonesia and that HEV antibodies are highly prevalent at 20% of the tested population in Bali, although epidemic HEV infection has not been documented thus far in regions other than West Kalimantan and East Java in this country [Wibawa et al., 2004]. Based on Balinese people's dietary habits, the finding of a high prevalence of swine anti-HEV IgG among Balinese pigs of 2–6 months of age and identification of a pig infected with a genotype 4 HEV that may be indigenous to Indonesia, it was suggested in the previous study that HEV infection may occur via zoonosis in Bali [Wibawa et al., 2004]. However, it remained unknown how prevalent hepatitis E is among Balinese patients with acute hepatitis and whether HEV strains recovered from patients with clinical HEV infection are closely related to swine HEV strains in Bali. Therefore, the present study was conducted in an attempt to answer these questions. Although the incidence rate of acute hepatitis E was lower than those of acute hepatitis A (28.1%) and acute hepatitis B (19.3%), two (3.5%) of the 57 patients studied were diagnosed as having hepatitis E based on the presence of high-titer anti-HEV IgM/IgA, and a genotype 4 HEV (BaliE03-46) was recovered from one of the two patients with hepatitis E.

Accumulated evidence indicates that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Harrison, 1999; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Meng, 2005] and that pigs are animal reservoirs of HEV [Balayan, 1997; Okamoto et al., 2001; Takahashi et al., 2003; Meng, 2005]. Anti-HEV IgG has been detected among pigs in high HEV-endemic countries such as China, Nepal, and Thailand as well as among pigs in low- or non-endemic countries such as Australia, Canada, Germany, Japan, New Zealand, Taiwan, the United Kingdom, and the United States [Clayson et al., 1995; Chandler et al., 1999; Hsieh et al., 1999; Meng et al., 1999; Garkavenko et al., 2001; Yoo et al., 2001; Takahashi et al., 2003; Meng, 2005]. In the current study, a high prevalence of swine anti-HEV IgG was also noted among 2- and 3-month-old pigs in Bali (57.4% or 58/101), being consistent with the high prevalence noted among Balinese pigs of 2–6 months of age (71.7% or 71/99) in the previous study [Wibawa et al., 2004]. These results support that HEV is endemic in pigs whether or not hepatitis E is common in the resident human population.

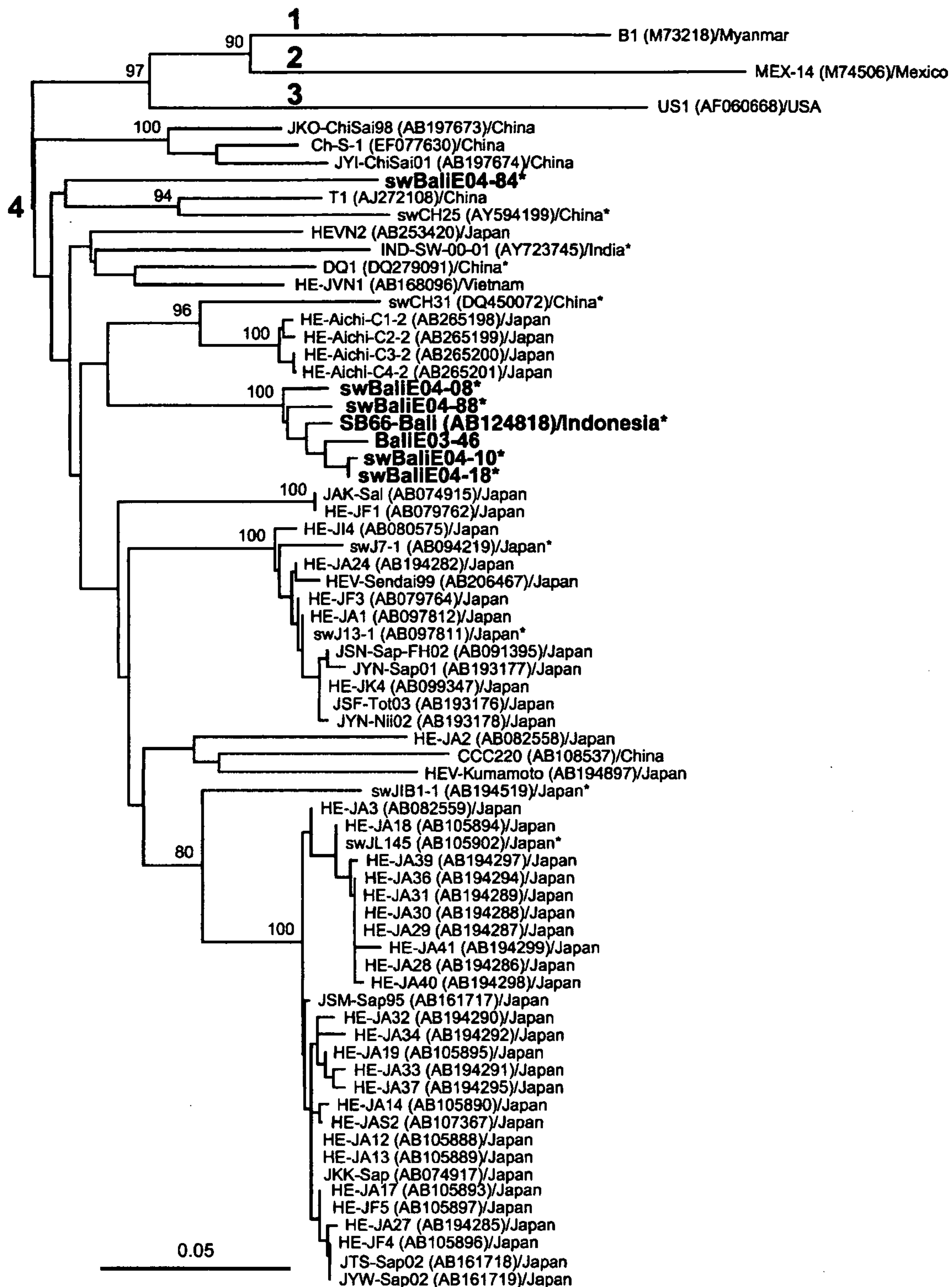

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 70 HEV isolates. In addition to the six human and swine HEV isolates obtained in the present study and the SB66-Bali Indonesian isolate found in our previous study which are indicated in bold type for visual clarity, one each of genotype 1–3 isolates and 60 reported genotype 4 HEV isolates whose common 412-nt sequence is known are included for comparison and their accession nos. are shown in parentheses followed by the name of the country where it was isolated. Asterisks denote swine HEV strains. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data.

TABLE IV. Comparison of Human and Swine HEV Isolates Obtained in the Present Study With the Reported HEV Isolates of Genotype 4 Whose Common 239–412 Nucleotide Sequence is Known

Country/host	No. of isolates compared[a]	Nucleotide length compared (nt)	Identity (%)	
			BaliE03-46 and 4 swine isolates[b]	swBaliE04-84
China				
Swine	39	300–412	84.5–89.1	82.2–87.9
Human	37	301–412	84.2–89.4	84.2–87.8
India				
Swine	16	239–412	83.0–86.9	82.4–85.6
Indonesia				
Swine	1	412	**97.1–98.3**	84.7
Japan				
Swine	15	412	77.3–88.8	80.0–87.6
Human	59	412	85.4–90.5	83.9–87.5
Taiwan				
Swine	3	304–346	84.2–89.3	84.4–85.5
Human	12	304–346	83.6–90.2	84.1–85.5
Vietnam				
Human	1	412	89.6–90.8	87.6

[a]Nucleotide sequence data were retrieved from the DDBJ/EMBL/GenBank databases on March 9, 2007.
[b]Include the swBaliE04-08, swBaliE04-10, swBaliE04-18, and swBaliE04-88 isolates.

In Thailand and Mexico, genotype 3 HEV was identified in serum and fecal samples from pigs, while genotype 1 or 2 HEV strains are prevalent in the respective human populations [Cooper et al., 2005]. In India, genotype 1 HEV is epidemic in humans, whereas genotype 4 HEV strains have been recovered from pigs [Arankalle et al., 2002, 2003]. On the other hand, there have been reports of high genetic relatedness between HEV isolates obtained from humans and those obtained from swine in the same geographical region in the United States (genotype 3) [Meng et al., 1997; Erker et al., 1999], Spain (genotype 3) [Pina et al., 2000], China (genotype 4) [Wang et al., 2002; Zheng et al., 2006], Taiwan (genotype 4) [Hsieh et al., 1999; Wu et al., 2002], and Japan (both genotypes 3 and 4) [Mizuo et al., 2002; Nishizawa et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003]. Similar to the previous observations in China, Taiwan, and Japan, the Balinese human HEV isolate of BaliE03-46 was found to be closely related to a Balinese swine HEV isolate (SB66-Bali) obtained in the previous study [Wibawa et al., 2004] and to 4 (swBaliE04-08, swBaliE04-10, swBaliE04-18, and swBaliE04-88) of the 5 swine HEV isolates obtained from farm pigs in Bali in the present study, with nucleotide identities of 97.3–98.3%, suggesting that HEV-associated hepatitis occurs via zoonotic transmission from pigs to humans among Balinese people.

In Japan, cluster cases of acute hepatitis E were linked epidemiologically and genetically to the consumption of uncooked or undercooked pig livers, deer meat and meat from wild boars [Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004; Mizuo et al., 2005]. HEV contamination was found in raw pig livers available in grocery stores in Hokkaido, which is located in the northern part of Japan and where hepatitis E is most prevalent in Japan [Yazaki et al., 2003]. Recently, Feagins et al. [2007] demonstrated that commercial pig livers sold in grocery stores in the United States are also contaminated by HEV and that pigs inoculated with two of the three PCR-positive pig-liver homogenates became infected, thus raising a public health concern about food-borne HEV infection. The custom in Bali is quite different from that in other places in Indonesia. The majority of Balinese people are Hindus, and have a custom of raising domestic animals such as pigs within their housing sites and of ingesting "lawar matah" with uncooked pig meat and viscera in the Balinese Hinduism ceremony, which is held frequently year-round. Some Balinese people ingest certain vegetables mixed with fresh blood from pigs. As reported in Japan [Tei et al., 2003; Yazaki et al., 2003], it is very likely that foods can act as vehicles for transmission of HEV. In fact, the two patients who were diagnosed with hepatitis E in the present study, are Hindus and had no history of travel outside Bali Island within the last 1 year, and reported that they ate raw pig meat around 1 month before the onset of acute hepatitis. Muslims are prohibited from eating pork. In support of possible zoonotic food-borne transmission of HEV in Bali, Surya et al. [2005] reported that anti-HEV was detected in 19.6% (20/102) of Hindus, compared with only 2.0% (2/101) of Muslims in Bali ($P < 0.0001$).

Viremic blood donors are potentially able to cause transfusion-associated hepatitis E not only in areas of high endemicity [Arankalle and Chobe, 2000], but also in low- or non-endemic countries. Several cases of transfusion-transmitted HEV infection with identical HEV sequences carried by the donor and recipient, have

been reported in Japan [Matsubayashi et al., 2004; Mitsui et al., 2004; Tamura et al., 2007] and the United Kingdom [Boxall et al., 2006]. It is probable that blood-borne transmission of HEV occurs in Bali. However, the two patients with hepatitis E in the present study had no history of blood transfusion.

The six Balinese human and swine HEV isolates obtained in the present study and a Balinese swine isolate (SB66-Bali) obtained in the previous study [Wibawa et al., 2004] belonged exclusively to genotype 4, but differed by 9.2–22.7% from the reported Chinese, Indian, Japanese, Taiwanese and Vietnamese HEV strains of the same genotype that had been recovered from humans or swine. In addition, the Balinese isolates segregated into two groups: the BaliE03-46, swBaliE04-08, swBaliE04-10, swBaliE04-18, swBaliE04-88 and SB66-Bali isolates constituted one group and differed by 14.6–16.1% from the remaining Balinese swine isolate, swBaliE04-84. Recently, Lu et al. [2006] proposed provisional criteria to classify HEV isolates of genotype 3 or 4 at three levels: genotype, subtype, and isolate. Based on the 300-nt sequence at the 5′ end of ORF2 that is included within the 412-nt ORF2 sequence whose sequence was determined in the present study, the nucleotide differences were 22.1–26.7% at the genotype level, 12.6–19.8% at the subtype level and 2.0–10.1% at the isolate level. The criteria suggest that the two phylogenetic groups of Balinese human and swine HEV isolates constitute two novel subtypes under genotype 4. A possible explanation for the observed marked divergence of the HEV genome would be the long-term circulation of HEV in different geographic regions and the independent evolution of virus in specific animal species including pigs.

In conclusion, the present study indicated for the first time the presence of clinical HEV infection in Bali, Indonesia, although the incidence rate of hepatitis E was low at 3.5% (2/57) and only one patient was positive for HEV RNA, and that indigenous genotype 4 HEV strains with marked heterogeneity are circulating in Bali, and suggested that pigs are reservoirs of HEV infection that may be food-borne in Balinese people who have a habit of consuming raw pig meat or viscera. To draw a definitive conclusion on the possible zoonotic food-borne transmission of HEV in Bali, further studies with larger numbers of patients with hepatitis E are needed. Thus far, the distribution of genotype 4 HEV is restricted to Asian countries, but the genomic variability of genotype 4 is as significantly marked as that of genotype 3, which is distributed widely throughout the world except in Africa. Therefore, studies on the extent of genomic variability of HEV strains that are associated with zoonotic HEV infection are warranted.

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Unchanged high prevalence of antibodies to hepatitis E virus (HEV) and HEV RNA among blood donors with an elevated alanine aminotransferase level in Japan during 1991–2006*

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Summary

Hepatitis E is rare in Japan but is occurring more frequently than previously thought. To investigate whether de novo subclinical infection of hepatitis E virus (HEV) has recently increased in Japan, HEV RNA was assayed in serum samples obtained from 4019 Japanese voluntary blood donors with alanine aminotransferase (ALT) of ≥61 IU/l, who are likely to have ongoing HEV infection, during 1991–2006. The overall rates of IgG-class antibody to HEV (anti-HEV IgG), anti-HEV IgM/IgA and HEV RNA among 3185 donors in 2004–2006 were comparable with those among 594 donors in 1998 (5.3 vs. 5.2%, 0.2 vs. 0.5%, and 0.2 vs. 0.3%, respectively). Among blood donors with ALT ≥ 201 IU/l in three groups according to the year of blood collection (1991–1995 [$n = 156$], 1996–1999 [$n = 116$] and 2004–2006 [$n = 61$]), there were no appreciable differences in the prevalence of anti-HEV IgG (5.8, 4.3, and 6.6%, respectively), anti-HEV IgM/IgA (1.9, 3.4, and 3.3%, respectively) and HEV RNA (1.3, 3.4, and 3.3%, respectively). The eleven HEV isolates obtained in the present study differed from each other by 1.7–22.8% in the ORF2 sequence and segregated into genotype 3 or 4. The occurrence rate of subclinical infection with divergent HEV strains has essentially remained unchanged during 1991–2006 in Japan.

Introduction

Hepatitis E, which is caused by hepatitis E virus (HEV), is found in many parts of the world. The disease is transmitted via the fecal-oral route through virus-contaminated water or food in developing countries where sanitation is suboptimal [36]. HEV infection is also endemic in industrialized countries, and IgG-class antibodies against HEV (anti-HEV IgG), most likely due to past subclinical HEV

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infection, have been detected in a significant proportion of healthy individuals in the United States, European countries and Japan [8, 16, 33, 36, 40, 51]; however, only a limited number of sporadic cases of acute hepatitis E have been reported in industrialized countries. Increasing lines of evidence indicate that hepatitis E is a zoonosis and that there exist animal reservoirs of HEV [9, 24, 25, 31–33, 40, 48, 52, 57].

HEV is a single-stranded, positive-sense RNA virus without an envelope and is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [4]. Its genome is approximately 7.2 kilobases (kb) in length and contains three open reading frames (ORFs: ORF1, ORF2, and ORF3) flanked by short untranslated regions [49]. ORF1 encodes non-structural proteins that are involved in virus replication and viral protein processing. ORF2 encodes the capsid protein and ORF3 encodes a small phosphorylated protein [14, 58]. Due to the extensive genomic diversity noted among HEV isolates, HEV sequences have been classified into four genotypes (genotypes 1–4) [39, 56]. Genotype 1 HEV was responsible for a number of waterborne epidemics of hepatitis E in Asia and Africa. Although HEV of genotype 2 has been detected less frequently, it was responsible for outbreaks in Mexico in 1986–1987 [54] and has been implicated in sporadic infections in Africa [3, 20]. On the other hand, genotypes 3 and 4 HEV cause sporadic cases of acute hepatitis but have not been found to be responsible for epidemics in humans; these infections seem to be zoonotic and both genotypes have been detected in pigs (genotype 3 worldwide, and genotype 4 in East Asia), which may constitute the major reservoir of HEV genotypes 3 and 4 [8, 19, 24, 25].

In Japan, multiple HEV strains of genotypes 3 and 4 have been recovered from patients with domestically acquired hepatitis E [12, 30, 42–45], and HEV has been recognized as an important causative agent of sporadic acute hepatitis of non-A, non-B, non-C etiology [30, 33]. A high prevalence of anti-HEV IgG has been reported [16, 27, 28, 50, 51], and HEV-viremic subjects have been identified among symptom-free blood donors with an elevated alanine aminotransferase (ALT) level [7]. However, it remains unknown whether or not subclinical HEV infection is increasing recently in Japan. Therefore, in an attempt to investigate the changing prevalence of de novo subclinical HEV infection in Japan, HEV RNA was assayed in serum samples obtained from Japanese voluntary blood donors with an elevated ALT level of ≥61 IU/l, who are likely to have ongoing HEV infection, during 1991–2006.

Materials and methods

Serum samples

Serum samples were collected from a total of 3185 voluntary blood donors (age: 32.5 ± 10.9 [mean ± standard deviation, SD] years; 2863 men and 322 women) with an elevated ALT level of 61–967 (range: 87.9 ± 41.8, mean ± SD) IU/l at the Japanese Red Cross Tochigi Blood Center, Japan, between April 2004 and December 2006. The Blood Center is located in Tochigi Prefecture, a prefecture in the northern part of mainland Honshu of Japan. Serum samples collected from 594 blood donors with an elevated ALT level of 61–2178 (100.4 ± 106.9) IU/l between February and November 1998 at the same blood center were also used in the present study. In addition, serum samples obtained from 240 blood donors with an elevated ALT level of ≥201 IU/l at the same blood center, from 1991–1997 and 1999, were used. Serum samples obtained from repeat donors during the study period were excluded; that is, each sample was obtained from a unique individual.

All 4019 serum samples were negative for hepatitis B surface antigen, and antibodies to hepatitis C virus (HCV) and human immunodeficiency virus (HIV) type 1. The 3198 samples obtained since 1999 were additionally negative for hepatitis B virus DNA, HCV RNA and HIV type 1 RNA by the nucleic acid amplification test using Roche's Multiplex reagent [26].

Detection of antibodies to HEV

To detect anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm [30], as described previously [47]. In the ELISA assays for anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, peroxidase-conjugated mouse monoclonal anti-human IgG antibody, peroxidase-conjugated mouse monoclonal anti-human IgM, or peroxidase-labeled mouse monoclonal anti-human IgA, respectively, was used. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA assays was 0.175, 0.440, and 0.642, respectively [47]. Samples with OD values for anti-HEV IgG, IgM, or IgA equal to or greater

than the respective cut-off value were considered to be positive for anti-HEV IgG, IgM, or IgA, respectively. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, when the OD value of the tested sample was less than 30% (anti-HEV IgG/IgA) or 50% (anti-HEV IgM) of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA in serum samples with anti-HEV IgM and/or anti-HEV IgA, using nested primers targeting the ORF2 region, as described previously [30]. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far [30, 46, 57]. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [30]. For serum samples that were negative for HEV RNA when 100 μl of serum sample was used, total RNA was extracted from 500 μl of serum, reverse transcribed, and then subjected to nested PCR as described above. To extract RNA from 500 μl of serum, test serum diluted 2-fold in saline was centrifuged at 287,582 × *g* at 4 °C for 2 h in a TLA-100.2 rotor (Beckman Coulter K. K., Tokyo, Japan), and the resulting pellet was suspended in 100 μl of saline and subjected to the RT-PCR assay. To confirm the reproducibility, this assay was performed in duplicate.

For serum samples without anti-HEV IgM and anti-HEV IgA, 10 μl each from 50 serum samples were pooled, and each pool was tested for HEV RNA by the above-mentioned RT-PCR. If a pool was positive for HEV RNA, the 50 serum samples of that pool were individually tested for the presence of HEV RNA.

Sequence analysis of PCR products

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [11]. Sequence alignments were generated by CLUSTAL W (version 1.8) [53]. Phylogenetic trees were constructed by the neighbor-joining method [38] based on the partial nucleotide sequence of the ORF2 region (412 nucleotides [nt]). Bootstrap values were determined on 1000 resamplings of the data sets [6].

Statistical analysis

Statistical analyses were performed using the Chi-Square-test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

Results

Age- and sex-specific prevalence of anti-HEV antibodies and HEV RNA during 2004–2006

A total of 3185 serum samples obtained from apparently healthy blood donors with an elevated ALT level between April 2004 and December 2006 were tested for the presence of anti-HEV IgG. Anti-HEV IgG was detected in 5.3% (168/3185) of the tested population including 5.1% of the 2863 male donors and 6.5% of the 322 female donors, the difference not being significant (Table 1). The prevalence of anti-HEV IgG increased with age among both the male and female donors, and was significantly higher among donors aged ≥40 years than among those aged <40 years in total (10.9 vs. 3.4%, $P < 0.0001$) and in the males (11.0 vs. 3.2%, $P < 0.0001$). All 168 serum samples with anti-HEV IgG were tested for anti-HEV IgM and anti-HEV IgA. Among them, anti-HEV IgM and anti-HEV IgA were simultaneously detected in six samples (3.6%), of which four samples tested positive for HEV RNA in a sample volume of both 10 and 100 μl, and one sample in 500 μl. As for the 3179 serum samples without anti-HEV IgM and anti-HEV IgA, although sixty-three 50-sample pools and one 29-sample pool were tested for the presence of HEV RNA, none of them had detectable HEV RNA. Consequently, 5 (0.2%) of the 3185 samples were found to be viremic for HEV. As for the prevalence of HEV viremia, there were no appreciable differences between males and females (0.1 vs. 0.3%, $P = 0.4628$), and between donors aged ≥40 years and those aged <40 years (0.4 vs. 0.1%, $P = 0.0700$).

Prevalence of anti-HEV antibodies and HEV RNA during 2004–2006, stratified by ALT level

In the present study, 168 donors with anti-HEV IgG were found during 2004–2006, including 143 (5.6%) with an ALT level of 61–100 IU/l, 21 (3.6%)

Table 1. Age- and sex-dependent prevalence of anti-HEV antibodies and HEV RNA among voluntary blood donors with an elevated ALT level between April 2004 and December 2006

Age (years)	No. of total donors (%) with				No. of male donors (%) with				No. of female donors (%) with			
	No. of donors	Anti-HEV		HEV RNA	No. of donors	Anti-HEV		HEV RNA	No. of donors	Anti-HEV		HEV RNA
		IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class	
16–19	538	9 (1.7)	1 (0.2)	1 (0.2)	462	7 (1.5)	1 (0.2)	1 (0.2)	76	2 (2.6)	0	0
20–29	702	19 (2.7)	1 (0.1)	1 (0.1)	640	14 (2.2)	1 (0.2)	1 (0.2)	62	5 (8.1)	0	0
30–39	1150	53 (4.6)	0	0	1054	48 (4.6)	0	0	96	5 (5.2)	0	0
40–49	561	55 (9.8)	2 (0.4)	1 (0.2)	518	54 (10.4)	2 (0.4)	1 (0.2)	43	1 (2.3)	0	0
50–59	200	24 (12.0)	0	0	169	19 (11.2)	0	0	31	5 (16.1)	0	0
60–68	34	8 (23.5)	2 (5.9)	2 (5.9)	20	5 (25.0)	1 (5.0)	1 (5.0)	14	3 (21.4)	1 (7.1)	1 (7.1)
Total	3185	168 (5.3)	6 (0.2)	5 (0.2)	2863	147 (5.1)	5 (0.2)	4 (0.1)	322	21 (6.5)	1 (0.3)	1 (0.3)

with an ALT level of 101–200 IU/l, and 4 (6.6%) with an ALT level of ≥201 IU/l (Table 2). The prevalence of anti-HEV IgG was comparable between donors with an ALT level of ≥201 IU/l and those with an ALT level of 61–200 IU/l (6.6 vs. 5.2%). As for the prevalence of HEV RNA, however, there was a significant difference between donors with an ALT level of ≥201 IU/l and those with an ALT level of 61–200 IU/l in total (3.3 vs. 0.1%, $P<0.0001$), in males (3.7 vs. 0.1%, $P<0.0001$), but not in females, probably due to the small number of female donors tested (0 vs. 0.3%, $P=0.8813$).

Comparison of the prevalence of anti-HEV antibodies and HEV RNA between donors with an elevated ALT level in 1998 and those in 2004–2006

The overall rates of anti-HEV IgG, anti-HEV IgM/IgA and HEV RNA among donors in 1998 were comparable with those among donors in 2004–2006 (5.2 vs. 5.3%, 0.5 vs. 0.2%, and 0.3 vs. 0.2%, respectively) (Table 3). The prevalence of anti-HEV IgG increased with age in the two year groups, although none of the two donors in the age group of 60–68 years in 1998 had anti-HEV IgG. As in the year group of 2004–2006, the prevalence of HEV RNA was significantly higher among donors with an ALT level of ≥201 IU/l than among those with an ALT level of 61–200 IU/l in 1998 (6.3 vs. 0%, $P<0.0001$).

Prevalence of anti-HEV antibodies and HEV RNA among donors with an elevated ALT level of ≥201 IU/l, stratified by the year group of blood collection

Table 4 compares various features of the blood donors with an elevated ALT level of ≥201 IU/l, who are likely to have ongoing HEV infection, in the three year groups (1991–1995, 1996–1999 and 2004–2006) according to the year of blood collection. There were no appreciable differences in the age distribution, gender ratio, ALT level and prevalence of anti-HEV IgG among the three year groups of 1991–1995, 1996–1999 and 2004–2006. The prevalence of anti-HEV IgM/IgA and HEV RNA, indicative of present HEV infection, was low at 1.9

Table 2. Prevalence of anti-HEV IgG and HEV RNA among voluntary blood donors with an elevated ALT level during 2004–2006, stratified by ALT level

ALT (IU/l)	Total			Male			Female		
	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA
61–100	2546	143 (5.6%)	2 (0.1%)	2296	128 (5.6%)	1 (0.04%)	250	15 (6.0%)	1 (0.4%)
101–200	578	21 (3.6%)	1 (0.2%)	513	15 (2.9%)	1 (0.2%)	65	6 (9.2%)	0
201–967	61	4 (6.6%)	2 (3.3%)	54	4 (7.4%)	2 (3.7%)	7	0	0
Total	3185	168 (5.3%)	5 (0.2%)	2863	147 (5.1%)	4 (0.1%)	322	21 (6.5%)	1 (0.3%)

Table 3. Comparison of the prevalence of anti-HEV antibodies and HEV RNA among blood donors with an elevated ALT level between 1998 and 2004–2006

Feature	1998				2004–2006			
	N	Anti-HEV-positive (%)		HEV RNA-positive (%)	N	Anti-HEV-positive (%)		HEV RNA-positive (%)
		IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class	
Age (years)								
16–19	43	0	0	0	538	9 (1.7)	1 (0.2)	1 (0.2)
20–29	216	6 (2.8)	1 (0.5)	1 (0.5)	702	19 (2.7)	1 (0.1)	1 (0.1)
30–39	200	11 (5.5)	0	0	1150	53 (4.6)	0	0
40–49	108	10 (9.3)	1 (0.9)	0	561	55 (9.8)	2 (0.4)	1 (0.2)
50–59	25	4 (16.0)	1 (4.0)	1 (4.0)	200	24 (12.0)	0	0
60–68	2	0	0	0	34	8 (23.5)	2 (5.9)	2 (5.9)
ALT (IU/l)								
61–100	454	23 (5.1)	1 (0.2)	0	2546	143 (5.6)	3 (0.1)	2 (0.1)
101–200	108	7 (6.5)	0	0	578	21 (3.6)	1 (0.2)	1 (0.2)
201–2178	32	1 (3.1)	2 (6.3)	2 (6.3)	61	4 (6.6)	2 (3.3)	2 (3.3)
Total	594	31 (5.2)	3 (0.5)	2 (0.3)	3185	168 (5.3)	6 (0.2)	5 (0.2)

Table 4. Prevalence of anti-HEV antibodies and HEV RNA among blood donors with an elevated ALT level of ≥201 IU/l, stratified by the year group of blood collection

Feature	Year of blood collection		
	1991–1995 ($n=156$)	1996–1999 ($n=116$)	2004–2006 ($n=61$)
Age (mean ± SD, years)	26.0 ± 9.9	26.4 ± 9.9	27.6 ± 12.5
Male	145 (92.9%)	105 (90.5%)	54 (88.5%)
ALT (mean ± SD, IU/l)	294.6 ± 216.4	299.8 ± 211.6	289.4 ± 135.8
Anti-HEV IgG	9 (5.8%)	5 (4.3%)	4 (6.6%)
Anti-HEV IgM/IgA	3 (1.9%)	4 (3.4%)	2 (3.3%)
HEV RNA	2 (1.3%)[a,b]	4 (3.4%)[a,c]	2 (3.3%)[b,c]
HEV genotype			
Genotype 3	1 (50.0%)	2 (50.0%)	2 (100%)
Genotype 4	1 (50.0%)	2 (50.0%)	0

[a] $P=0.2290$.
[b] $P=0.3256$.
[c] $P=0.9528$.

and 1.3%, respectively, in the year group of 1991–1995, but the difference among the three year groups was not statistically significant.

Genetic analysis of HEV isolates recovered from 11 viremic donors

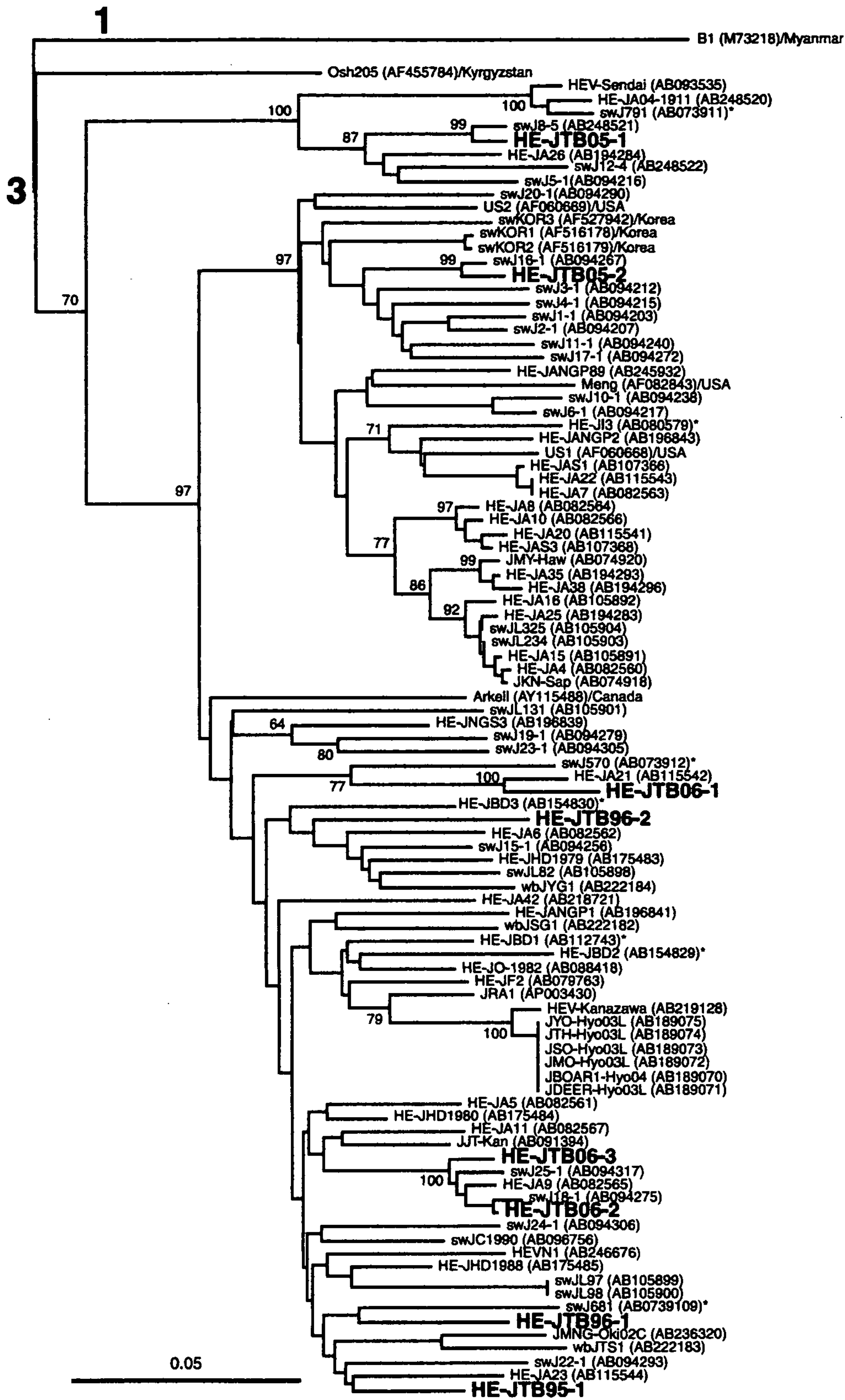
The 11 HEV isolates recovered from the transiently viremic donors were named with the prefix of HE-JTB followed by the year of isolation and the sequential number of the viremic samples obtained that year (Table 5). The 412-nt sequence of ORF2 of these HEV isolates was determined and compared with each other and with that of known HEV isolates of genotypes 1–4. These 11 HEV isolates were markedly variable, sharing nucleotide identities ranging from 77.2 to 98.3%, and were classifiable into two groups differing by 18.9–22.8%. Eight HEV isolates (HE-JTB95-1, HE-JTB96-1, HE-JTB96-2, HE-JTB05-1, HE-JTB05-2, HE-JTB06-1, HE-JTB06-2, and HE-JTB06-3) comprised one group and were close to the prototype Japanese isolate of genotype 3 (JRA1 [accession no. AP003430]) with nucleotide identities of 85.9–93.0%, and were only 77.9–80.3, 74.8–77.4, and 77.9–80.1% similar to the B1 isolate (M73218) of genotype 1, MEX-14 isolate (M74506) of genotype 2, and T1 isolate (AJ272108) of genotype 4, respectively. This finding suggests that these 8 HEV isolates are classifiable into genotype 3. The phylogenetic tree constructed based on the common 412-nt sequence within the ORF2 sequence confirmed that these 8 HEV isolates obtained in the present study belonged to genotype 3 and showed that they segregated into clusters consisting of Japanese HEV strains of the same genotype that had been recovered from hu-

Table 5. Characteristics of blood donors with an elevated ALT level who had detectable HEV RNA

Year of isolation	Age (years)/sex	ALT (IU/l)	Anti-HEV (OD_{450} value)			HEV RNA (μl)[a]			HEV genotype	Isolate name
			IgG-class	IgM-class	IgA-class	10	100	500		
1994	44/M	457	>3.000 (+)	2.325 (+)	2.137 (+)	+[b]	+	NT[c]	4	HE-JTB94-1
1995	58/M	2598	0.415 (+)	0.611 (+)	1.171 (+)	+	+	NT	3	HE-JTB95-1
1996	47/F	215	1.629 (+)	1.900 (+)	>3.000 (+)	+	+	NT	3	HE-JTB96-1
1996	49/M	262	1.624 (+)	0.981 (+)	1.983 (+)	+	+	NT	3	HE-JTB96-2
1998	29/M	628	0.127 (−)	1.272 (+)	0.146 (−)	+	+	NT	4	HE-JTB98-1
1998	54/M	2178	0.439 (+)	1.030 (+)	1.265 (+)	+	+	NT	4	HE-JTB98-2
2005	61/M	967	1.762 (+)	1.967 (+)	2.825 (+)	+	+	NT	3	HE-JTB05-1
2005	25/M	85	>3.000 (+)	2.792 (+)	2.683 (+)	+	+	NT	3	HE-JTB05-2
2006	60/F	63	1.170 (+)	0.665 (+)	2.928 (+)	+	+	NT	3	HE-JTB06-1
2006	17/M	138	2.278 (+)	1.804 (+)	2.939 (+)	−	−	+	3	HE-JTB06-2
2006	49/M	758	2.866 (+)	>3.000 (+)	2.313 (+)	+	+	NT	3	HE-JTB06-3

[a] HEV RNA was assayed using the indicated volume of serum samples.
[b] +, positive for HEV RNA; −, negative for HEV RNA.
[c] NT, not tested.

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 99 HEV isolates, using a genotype 1 HEV (M73218) as an outgroup. In addition to the HE-JTB95-1, HE-JTB96-1, HE-JTB96-2, HE-JTB05-1, HE-JTB05-2, HE-JTB06-1, HE-JTB06-2, and HE-JTB06-3 isolates found in the present study, which are indicated in bold type for visual clarity, 90 reported HEV isolates of genotype 3, whose common 412-nt sequence is known, are included for comparison. The reported isolates are indicated with the accession no. followed by the name of the country of isolation (non-Japanese origin only). An asterisk denotes human or swine HEV strains that were isolated in the same prefecture as those obtained in the present study. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data


1
B1 (M73218)/Myanmar
3
Osh205 (AF455784)/Kyrgyzstan
HEV-Sendai (AB093535)
HE-JA04-1911 (AB248520)
swJ791 (AB073911)*
swJ8-5 (AB248521)
HE-JTB05-1
HE-JA26 (AB194284)
swJ12-4 (AB248522)
swJ5-1(AB094216)
swJ20-1(AB094290)
US2 (AF060669)/USA
swKOR3 (AF527942)/Korea
swKOR1 (AF516178)/Korea
swKOR2 (AF516179)/Korea
swJ16-1 (AB094267)
HE-JTB05-2
swJ3-1 (AB094212)
swJ4-1 (AB094215)
swJ1-1 (AB094203)
swJ2-1 (AB094207)
swJ11-1 (AB094240)
swJ17-1 (AB094272)
HE-JANGP89 (AB245932)
Meng (AF082843)/USA
swJ10-1 (AB094238)
swJ6-1 (AB094217)
HE-JI3 (AB080579)*
HE-JANGP2 (AB196843)
US1 (AF060668)/USA
HE-JAS1 (AB107366)
HE-JA22 (AB115543)
HE-JA7 (AB082563)
HE-JA8 (AB082564)
HE-JA10 (AB082566)
HE-JA20 (AB115541)
HE-JAS3 (AB107368)
JMY-Haw (AB074920)
HE-JA35 (AB194293)
HE-JA38 (AB194296)
HE-JA16 (AB105892)
HE-JA25 (AB194283)
swJL325 (AB105904)
swJL234 (AB105903)
HE-JA15 (AB105891)
HE-JA4 (AB082560)
JKN-Sap (AB074918)
Arkell (AY115488)/Canada
swJL131 (AB105901)
HE-JNGS3 (AB196839)
swJ19-1 (AB094279)
swJ23-1 (AB094305)
swJ570 (AB073912)*
HE-JA21 (AB115542)
HE-JTB06-1
HE-JBD3 (AB154830)*
HE-JTB96-2
HE-JA6 (AB082562)
swJ15-1 (AB094256)
HE-JHD1979 (AB175483)
swJL82 (AB105898)
wbJYG1 (AB222184)
HE-JA42 (AB218721)
HE-JANGP1 (AB196841)
wbJSG1 (AB222182)
HE-JBD1 (AB112743)*
HE-JBD2 (AB154829)*
HE-JO-1982 (AB088418)
HE-JF2 (AB079763)
JRA1 (AP003430)
HEV-Kanazawa (AB219128)
JYO-Hyo03L (AB189075)
JTH-Hyo03L (AB189074)
JSO-Hyo03L (AB189073)
JMO-Hyo03L (AB189072)
JBOAR1-Hyo04 (AB189070)
JDEER-Hyo03L (AB189071)
HE-JA5 (AB082561)
HE-JHD1980 (AB175484)
HE-JA11 (AB082567)
JJT-Kan (AB091394)
HE-JTB06-3
swJ25-1 (AB094317)
HE-JA9 (AB082565)
swJ18-1 (AB094275)
HE-JTB06-2
swJ24-1 (AB094306)
swJC1990 (AB096756)
HEVN1 (AB246676)
HE-JHD1988 (AB175485)
swJL97 (AB105899)
swJL98 (AB105900)
swJ681 (AB073909)*
HE-JTB96-1
JMNG-Oki02C (AB236320)
wbJTS1 (AB222183)
swJ22-1 (AB094293)
HE-JA23 (AB115544)
HE-JTB95-1
100
100
99
87
97
99
70
71
97
97
77
99
86
92
64
80
100
77
79
100
100
0.05