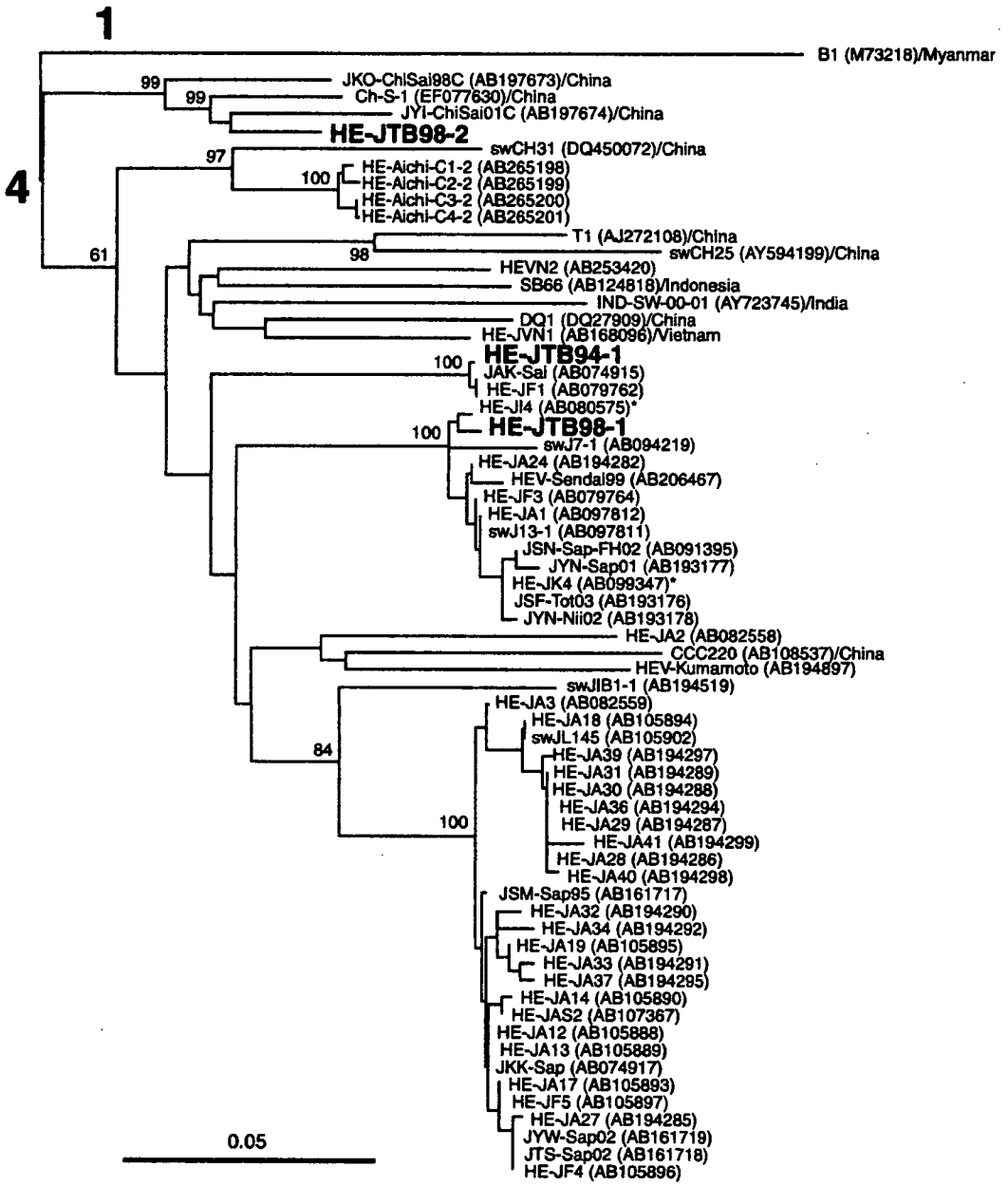

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 65 HEV isolates, using a genotype 1 HEV (M73218) as an outgroup. In addition to the HE-JTB94-1, HE-JTB98-1, and HE-JTB98-2 isolates found in the present study, which are indicated in bold type, 61 reported HEV isolates of genotype 4, 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 where it was isolated (non-Japanese origin only). An asterisk denotes human 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

mans, swine and wild boars, supporting the indigenous nature of these 8 blood donor isolates (Fig. 1). On the other hand, the remaining three HEV isolates obtained in the present study (HE-JTB94-1, HE-JTB98-1 and HE-JTB98-2) were close to the prototype genotype 4 isolate (T1) with nu-

cleotide identities of 84.7–87.6%, and were only 79.9–81.8, 77.7–78.4, and 79.9–81.3% similar to the B1 isolate of genotype 1, MEX-14 isolate of genotype 2, and JRA1 isolate of genotype 3, respectively, suggesting that the HE-JTB94-1, HE-JTB98-1 and HE-JTB98-2 isolates belong to genotype 4. The phylogenetic tree constructed based on the common 412-nt ORF2 sequence confirmed that the 3 HEV isolates obtained in the present study segregated to genotype 4 (Fig. 2). Of note, the HE-JTB94-1 and HE-JTB98-1 isolates segregated into two distinct clusters consisting of Japanese HEV strains, each with a bootstrap value of 100%, but HE-JTB98-2 segregated into a cluster comprising the Chinese HEV strains that had been recovered from a Chinese patient with autochthonous hepatitis E (accession no. EF077630) and Japanese patients with hepatitis E who had traveled to China (AB197673–AB197674), suggesting that the HE-JTB98-2 isolate may be of China origin.

Discussion

This study examined the prevalence of ongoing subclinical HEV infection among 4019 apparently healthy blood donors with an elevated ALT level of ≥61 IU/l who donated blood during the last 16 years at a Japanese Red Cross Blood Center located in the northern part of mainland Honshu of Japan. As for the geographical distribution of hepatitis E in Japan, it was reported that there was wide variation, with a higher prevalence in the northern part of Japan (Hokkaido Island and the northern part of mainland Honshu) [1, 30], suggesting that the results obtained in the present study cannot simply be generalized for the whole country. However, our study corroborated the previous study by Tanaka et al. [51], who reported that, based on the age-specific distribution of anti-HEV IgG in 1974, 1984, and 1994, exposure to HEV remained constant between 1974 and 1994 in Metropolitan Tokyo, Japan. The prevalence of clinical HEV infection among patients with acute hepatitis remained unchanged during the period from 1989 to 2005 in a city hospital in Aichi Prefecture, which is located in the central part of Honshu Island of Japan [29]. Therefore, our present study may represent the recent trends of HEV infection, at least in the northern and central parts of mainland Honshu of Japan, where hepatitis E is low-endemic [1].

The presence of anti-HEV IgG most likely reflects past subclinical HEV infection. The present study revealed that the prevalence of anti-HEV IgG among blood donors with ALT of ≥61 IU/l between 2004 and 2006 was similar to that in 1998 (5.3 vs. 5.2%), and that the prevalence of anti-HEV IgG among blood donors with ALT of ≥201 IU/l was comparable among the three year groups of 1991–1995, 1996–1999 and 2004–2006 (5.8, 4.3, and 6.6%, respectively) as well as that of 2002–2003 (4.1% or 23/560) [7]. Longitudinal seroepidemiological studies on transiently infected individuals suggested that anti-HEV IgG persisted much longer than expected, i.e., for more than 20 years [27, 28]. Even a low titer of anti-HEV IgG may reflect past subclinical HEV infection and has been detected in a significant proportion of healthy individuals not only in Japan but also in the United States and European countries [8, 16, 33, 36, 40, 51]. Therefore, in studies in which anti-HEV IgG is assayed at a single time point in each individual, it may be hard to specify when individuals with anti-HEV IgG contracted HEV infection and how prevalent de novo subclinical HEV infection was during a particular period.

In the present study, the genomic RNA of HEV was detected in a total of 11 donors with an ALT level of 63–2598 IU/l among the 4019 donors tested. When stratified by the year group of blood collection, the prevalence of HEV viremia among blood donors with ALT of ≥61 IU/l between 2004 and 2006 was comparable to that in 1998 (0.2 vs. 0.3%), and the prevalence of HEV viremia among blood donors with ALT of ≥201 IU/l was not statistically different among the three year groups of 1991–1995, 1996–1999, and 2004–2006 (1.3, 3.4, and 3.3%, respectively), or from that of 2002–2003 (4.4% or 1/23) [7], suggesting that de novo subclinical HEV infection occurred at an almost constant rate during the last 16 years in Tochigi Prefecture, Japan. In industrialized countries including Japan, maintenance of good hygiene of the water supply and sewage systems made the likelihood of waterborne infection of hepatitis A virus (HAV) extremely low [13]. However, our observations are consistent with the notion that transmission of HEV would not

be prevented by only improvement of sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [36].

Domestically acquired hepatitis E has been reported in industrialized countries including the United States and European countries since 1997 [2, 5, 10, 15, 21, 34, 35, 37, 55]. However, only a limited number of sporadic cases of acute hepatitis E have been reported in the United States and European countries, and the changing profiles of clinical and subclinical HEV infection have not been studied in these countries. In Japan, clinical hepatitis E is rare compared with clinical hepatitis A but is occurring more frequently than previously thought [30, 33], where the first case of autochthonous hepatitis E was reported in 2001 [43], and presumably indigenous HEV strains have been recovered from individuals who contracted HEV infection in the 1970s [27, 28]. It remains unknown, however, why the prevalence of domestic HEV infection has remained stable during the last few decades in Japan. It has recently been suggested that zoonotic foodborne transmission of HEV from domestic pigs and wild boars to humans plays an important role in the occurrence of cryptic hepatitis E in Japan, where Japanese people have distinctive habits of eating raw fish (sushi or sashimi) and, less frequently, uncooked or undercooked meat (including the liver and colon/intestine of animals) [17, 23, 31, 41, 57]. Of note, we found a high prevalence of swine anti-HEV antibodies and a high HEV viremia rate among Japanese pigs [46, 48]. The majority of patients with sporadic acute hepatitis E in Hokkaido had a history of consuming grilled or undercooked pig liver and/or intestine approximately 2–8 weeks prior to the onset of hepatitis E [31]. Pig liver specimens from 7 (1.9%) of 363 packages sold in local grocery stores in Hokkaido had detectable HEV RNA [57]. These results strongly suggest that consumption of undercooked pig liver/intestine is a potential risk factor for HEV infection. Transfusion-associated hepatitis E has also been reported in Japan [22, 27]. Recently, of interest, it was reported that HEV RNA was detected in bivalves called Yamato-Shijimi (*Corbicula japonica*) obtained from Japanese rivers, indicating that HEV contaminates river water in Japan [18]. However, the mode of HEV transmission in the 11 viremic donors in the present study was unclear. Further studies are needed to elucidate the mode(s) of clinical and subclinical HEV infection in the general population of Japan including Tochigi Prefecture.

As the 11 viremic donors identified in the present study had an elevated ALT level, the blood from the 11 donors was not used for transfusion, suggesting that ALT testing helps prevent transfusion-transmitted HEV infection. As one of the 11 infected donors had only a slightly elevated ALT level of 63 IU/l, it seems likely that even donors with a normal ALT level ($\leq$60 IU/l) may have detectable HEV RNA. The prevalence of HEV RNA decreased with the ALT level and was significantly less frequent among the 3000 donors with ALT of 61–100 IU/l than among the 93 donors with ALT of $\geq$201 IU/l (0.067 vs. 4.3%, $P<0.0001$) in 1998 and 2004–2006. Although the number of donors tested was limited, it is reasonable to speculate that the prevalence of ongoing HEV infection among donors with a normal ALT level may be less than 0.067% in Tochigi Prefecture. The proportion of such donors may be very small or negligible, particularly in the southern part of Japan, where only 1.7% (9/527) of blood donors with ALT of $\geq$61 IU/l had anti-HEV IgG [7].

Reflecting the polyphyletic nature of human and animal HEV isolates of Japanese origin [30, 33, 44], the HEV isolates recovered from the 11 viremic donors in the present study differed by 1.7–22.8% from each other and segregated into genotype 3 or 4. Ten human HEV strains of genotype 3 (HE-JI3 [AB080579], HE-JBD1 [AB112743], HE-JBD2 [AB154829], HE-JBD3 [AB154830], and 6 unpublished isolates) have been isolated in the same prefecture as that of the 11 viremic donors and shared identities ranging from 80.6 to 99.8% with the 8 genotype 3 HEV isolates obtained in the present study. As for human HEV strains of genotype 4, two strains (HE-JI4 [AB080575] and HE-JK4 [AB099347]) isolated in the same prefecture shared 87.9–99.3% identities with the 3 genotype 4 HEV isolates obtained in the present study. These results further support the marked heterogeneity of the HEV genome and its wide distribution in Japan, even within a certain prefecture in this country.

In conclusion, 11 blood donors with HEV viremia were identified among 4019 voluntary blood donors with an elevated ALT level at a blood center located in the northern part of mainland Honshu of Japan, where hepatitis E is low-endemic. In this study, 2.4% of individuals with ALT of ≥201 IU/l had ongoing subclinical infection of various HEV strains, and the prevalence of HEV viremia was distributed nearly evenly in the year groups of 1991–1995, 1996–1999, and 2004–2006, suggesting that the occurrence rate of subclinical infection with divergent HEV strains has essentially remained unchanged during 1991–2006 in Japan. Future studies are warranted to clarify the mode(s) of HEV transmission that may be responsible for the stable occurrence of clinical and, mostly, subclinical HEV infections over the past several decades in humans living in industrialized countries, where a significant proportion of the general population have HEV antibodies, but hepatitis E is believed to be non- or low-endemic.

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Prolonged Fecal Shedding of Hepatitis E Virus (HEV) during Sporadic Acute Hepatitis E: Evaluation of Infectivity of HEV in Fecal Specimens in a Cell Culture System[▽]

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To investigate the duration of fecal shedding and changing loads of hepatitis E virus (HEV) in feces and serum from patients with acute HEV infection, HEV RNA was quantitated in periodic serum and fecal specimens obtained from 11 patients with sporadic acute hepatitis E. All 11 patients had detectable HEV RNA in serum at admission, with the highest viral load being 1.9×10^3 to 1.7×10^7 copies/ml, and HEV viremia lasted until days 17 to 48 (mean, 28.3) after the onset of hepatitis. Even at the initial examination on days 10 to 29 (mean, 17.6), the HEV load in fecal supernatant was less than 5.7×10^4 copies/ml for 10 of the 11 patients, while for the remaining patient (patient 1) it was markedly high, 2.0×10^7 copies/ml on day 22. In addition, although HEV RNA in fecal supernatant continued to be positive until days 14 to 33 (mean, 22.4) for patients 2 to 11, that for patient 1 was detectable even on day 121. HEVs in fecal specimens obtained on days 22, 24, 26, 28, and 30, but not day 121, from patient 1 grew efficiently in PLC/PRF/5 cells, reaching the highest titer of up to 10^7 copies/ml in culture medium on day 50 postinoculation. The HEV genome recovered from patient 1 had 29 unique nucleotides that were not seen in any of the 25 reported HEV isolates of the same genotype over the entire genome, with six amino acid substitutions in the ORF1 protein.

Hepatitis E is an enterically transmitted viral disease caused by hepatitis E virus (HEV). The disease occurs in epidemic and sporadic forms in most developing countries of Asia, Africa, and Latin America (43). Sporadic cases of locally acquired hepatitis E also have been identified in industrialized countries, including the United States, European countries, and Japan (3, 7, 11, 19, 20, 24, 27, 32, 33, 39, 42, 62, 64, 69). A significant proportion of healthy individuals in industrialized countries are seropositive for antibodies to HEV (anti-HEV), and a high prevalence of anti-HEV of over 20% has been reported in some areas of the United States (57). Anti-HEV also has been detected in many animal species, and HEV has been isolated from domestic pigs and wild animals, including boars, a deer, and a mongoose (30, 34, 50, 51, 56). Accumulating lines of evidence indicate that hepatitis E is a zoonosis (19, 28–30, 37, 38, 47, 56, 68). HEV infection runs an acute course, normally resulting in resolution within a few weeks after onset. Although only a minority of HEV infections induce overt hepatitis, the contribution of HEV to the development of fulminant hepatitis is known not only in developing countries (35) but also in industrialized countries (42, 49). The presence of a chronic or persistent HEV infection, however, has not been described.

HEV is a nonenveloped RNA virus and is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (13). Its genome is a single-stranded, positive-sense RNA of approximately 7.2 kb. It contains a short 5′-untranslated region (5′UTR) followed by three open reading frames (ORFs; ORF1, ORF2, and ORF3) and then a short 3′UTR with a poly(A) tail (53, 63). Although only one serotype has been recognized, HEV sequences worldwide can be classified into four major genotypes, 1, 2, 3, and 4, which are represented by the Burmese isolates, the Mexican isolate, the U.S. isolates, and the new Chinese isolates, respectively. Genotype 1 is responsible for the majority of HEV infections in developing countries; genotype 2 consists of strains not only in Mexico but also in African countries including Chad, Namibia, and Nigeria; genotype 3 is widely distributed throughout the world except in Africa; and genotype 4 is distributed exclusively in Asian countries (26, 40, 46).

Although viremia and antibody response to HEV have been studied in many cases of hepatitis E (32, 52), fecal shedding of HEV has been studied for a limited number of patients, and changing profiles of load and infectivity of fecal HEV during acute HEV infection are poorly understood. In the present study, we detected HEV RNA quantitatively in periodic serum and fecal specimens obtained from 11 patients with sporadic acute hepatitis E and found a particular patient for whom virus fecal excretion lasted at least 121 days after the disease onset.

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TABLE 1. Characteristics and peak HEV RNA levels in serum and fecal samples from 11 patients with sporadic acute hepatitis E from whom periodic fecal specimens were available

Patient no.	Age (yr)/ sex [a]	Location of infection	HEV genotype	No. of samples tested		Peak level				
				Serum	Feces	ALT (IU/liter)	AST[c] (IU/liter)	Total bilirubin (mg/dl)	HEV RNA in serum[b] (copies/ml)	HEV RNA in feces[b] (copies/ml)
1	67/M	Japan	3	21	28	620	363	0.5	7.2×10^6 (5)	2.0×10^7 (22)
2	36/M	Bangladesh (imported)	1	16	7	3,863	1,887	68.6	3.8×10^6 (6)	5.7×10^4 (22)
3	56/M	Vietnam (imported)	4	13	9	997	704	11.9	1.9×10^3 (7)	2.7×10^3 (12)
4	58/M	Japan	4	69	14	2,635	1,410	18.8	7.2×10^5 (9)	<100 (+) (15)
5	56/M	Japan	4	11	5	2,046	2,566	15.8	1.3×10^5 (5)	2.7×10^3 (10)
6	86/M	Japan	4	34	41	1,305	1,619	26.0	9.8×10^4 (12)	2.4×10^3 (19)
7	67/M	Japan	4	35	14	3,866	3,321	31.5	5.7×10^4 (12)	<100 (+) (29)
8	30/M	Nepal (imported)	1	9	8	1,067	960	9.7	8.2×10^3 (13)	7.1×10^2 (14)
9	37/M	Japan	3	14	11	2,241	1,121	3.1	1.0×10^5 (2)	<100 (+) (18)
10	47/M	Japan	4	33	6	2,492	1,472	31.3	1.7×10^7 (7)	<100 (+) (22)
11	56/M	Japan	4	25	3	4,348	3,339	3.6	3.3×10^5 (3)	<100 (+) (11)

[a] M, male.
[b] The day after onset of the disease on which the initial sample was obtained, which was the day on which the peak HEV RNA level was observed, is shown in parentheses. (+), positive for HEV RNA by ORF2/3-137 PCR.
[c] AST, aspartate transaminase.

In addition, we evaluated the infectivity of HEV in fecal specimens obtained from the patient by using a recently developed cell culture system (55). Furthermore, the full-length genomic sequence was determined for the HEV isolate obtained from the patient with prolonged fecal shedding in an attempt to investigate whether there are HEV mutations that are responsible for the observed long-term fecal excretion and high level of replicative activity of HEV.

MATERIALS AND METHODS

Serum and fecal specimens. With informed consent, serum and fecal samples were collected periodically from 11 patients (patients 1 to 11) who contracted sporadic acute hepatitis E between 2002 and 2006 (Table 1). Three patients acquired HEV infection while traveling in Bangladesh, Vietnam, and Nepal, respectively, and the remaining eight patients contracted domestic HEV infection in Japan. Diagnosis of acute hepatitis E was based on the presence of immunoglobulin M (IgM) and IgA classes of antibodies to HEV (anti-HEV IgM and anti-HEV IgA) and HEV RNA in serum. The number of serum samples tested from each patient ranged from 9 to 69, and that of fecal samples ranged from 3 to 41. Fecal sampling was initiated soon after the diagnosis of acute hepatitis E. Fecal specimens (5 to 10 g) were suspended at 15% (wt/vol) in Tris-HCl buffer (0.01 M, pH 7.5) and were centrifuged in a refrigerated centrifuge (Hitachi High-Technologies Corp., Tokyo, Japan) at 1,600 × *g* at 4°C for 30 min, and the supernatant was recovered. It was spun down in a high-speed microrefrigerated centrifuge (Tomy Seiko, Tokyo, Japan) at 6,200 × *g* at 4°C for 10 min, and a clear supernatant was obtained. Aliquots were stored at −80°C.

Detection of antibodies to HEV. To detect anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA in serum, an enzyme-linked immunosorbent assay (ELISA) was performed using the purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm (32), as described previously (52). 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 ELISAs 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 cutoff values used in the anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA assays were 0.175, 0.440, and 0.642, respectively (52). Samples with ODs for anti-HEV IgG, IgM, or IgA greater than or equal to the respective cutoff 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, if the OD 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 and quantitation of HEV RNA. Total RNA was extracted from 100 μl of serum sample or fecal supernatant with TRIzol-LS reagent (Invitrogen, Carlsbad, CA). For detection of HEV RNA in the serum and fecal samples, nested reverse transcription-PCR (RT-PCR) (ORF2/3-137 PCR) with primers targeting the ORF2/ORF3 overlapping region of the HEV genome was performed as described previously (22). The size of the amplification product of the first-round PCR was 164 bp, and that of the second-round PCR was 137 bp. The ORF2/3-137 PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far (22). To confirm the presence of HEV RNA, a second RT-PCR (ORF2-457 PCR) assay that amplifies a 457-nucleotide (nt) sequence in ORF2 was carried out according to the method described previously (32).

Quantitation of HEV RNA was performed by real-time RT-PCR detection according to the previously described method (23) with a slight modification. In brief, total RNA was extracted from 2 to 100 μl of the serum sample, fecal supernatant, or culture medium with TRIzol-LS reagent and was subjected to real-time RT-PCR with the QuantiTect Probe RT-PCR kit (QIAGEN, Tokyo, Japan) using a sense primer (5′-GGT GGT TTC TGG GGT GAC-3′), an antisense primer (5′-AGG GGT TGG TTG GAT GAA-3′), and a probe consisting of an oligonucleotide with a 5′ reporter dye (6-carboxyfluorescein [FAM]) and a 3′ quencher dye (6-carboxytetrarhodamine [TAMRA]) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). The thermal cycler conditions were 50°C for 30 min, 95°C for 15 min, and 50 cycles of 94°C for 15 s, 56°C for 30 s, and 76°C for 30 s. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was determined.

Cell culture and virus inoculation. A hepatocarcinoma cell line (PLC/PRF/5; ATCC no. CRL-8024; Manassas, VA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; PAA Lab GmbH, Pasching, Austria), 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B at 37°C in a humidified 5% CO_2 atmosphere, as described previously (55). For virus infection, confluent cells were trypsinized and diluted 1:4 in medium, and 2.0 ml was added to each well (well diameter, 3.5 cm) of a 6-well microplate (IWAKI, Tsukuba, Japan) 1 or 2 days before virus infection. Monolayers of cultured cells in the 6-well microplate were washed three times with 1 ml of phosphate-buffered saline (pH 7.5) without Ca^{2+} and Mg^{2+} [PBS(−)], and 0.2 ml of the filtrated virus stock that had been diluted 1:5 in PBS(−) containing 0.2% (wt/vol) bovine serum albumin (BSA; Sigma Aldrich Inc., St. Louis, MO) was inoculated on the cells in each well. One hour after inoculation at room temperature, the solution was removed, and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing consisted of 50% DMEM and 50% medium 199 (Invitrogen) containing 2% (vol/vol) heat-inactivated FCS and 30 mM $MgCl_2$ at final concentration; other supplements were the same as those in the growth medium. The culture was done at 35.5°C in a humidified 5% CO_2 atmosphere. On the day following inoculation, the inoculated cells were washed five times with 1 ml of

PBS(−), and then 2 ml of maintenance medium was added. Every other day, half (1 ml) of the culture medium was replaced with fresh maintenance medium, and the collected media were stored at −80°C until virus titrations were performed. In this study, triplicate sets of inocula were inoculated in parallel on the cultivated cells in a 6-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, and representative data were determined.

Amplification of the full-length HEV genome. To determine the full-length sequence of an HEV isolate, total RNA was extracted from 200 μl of fecal suspension using TRIzol-LS, and the RNA preparation was reverse transcribed and subjected to nested PCR. Five overlapping regions excluding the extreme 5′ and 3′ termini were amplified; they were nt 25 to 1252 (1,228 nt) (primer sequences excluded), nt 1063 to 3109 (2,047 nt), nt 3088 to 4682 (1,595 nt), nt 4633 to 6362 (1,730 nt), and nt 6324 to 7181 (858 nt). The nucleotide numbers were in accordance with those of the sequence of strain JE03-1760F. The extreme 5′-end sequence (nt 1 to 31) was determined by a modified rapid amplification of cDNA ends (RACE) technique, called RNA ligase-mediated RACE (RLM-RACE), with the First Choice RLM-RACE kit (Ambion, Austin, TX) as described previously (38). Amplification of the 3′-end sequence [nt 7083 to 7226, excluding the poly(A) tail] was attempted by the RACE method described previously (38).

Cloning and sequence analysis of PCR products. The amplification products were sequenced on both strands either directly or after being cloned into the pT7Blue T vector (Novagen Inc., Madison, WI) using the BigDye Terminator v3.1 cycle sequencing kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems). 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 (National Institute of Genetics, Mishima, Japan) (21). Sequence alignments were generated by CLUSTAL W (version 1.8) (58). A phylogenetic tree was constructed by the neighbor-joining method (45). Bootstrap values were determined on 1,000 resamplings of the data sets (15). The final tree was obtained using the TreeView program (version 1.6.6) (41).

Nucleotide sequence accession numbers. The sequences determined in the present study have been deposited in the DNA Data Bank of Japan, GenBank, and EMBL databases under the following accession numbers: AB301710 (complete genome) and AB301698 to AB301709 (ORF2).

RESULTS

Detection and quantitation of HEV RNA in periodic serum and fecal specimens obtained from 11 patients with acute hepatitis E. All 11 patients studied had detectable HEV RNA in serum at admission, with the highest viral loads ranging from 1.9×10^3 copies/ml to 1.7×10^7 copies/ml (Table 1), and HEV viremia lasted until days 17 to 48 (mean, 28.3) after the onset of the hepatitis (Fig. 1). In contrast, even at the first examination on days 10 to 29 (mean, 17.6; $n = 11$), the peak HEV load in fecal supernatant was low, being <100 copies/ml in 5 of the 11 patients (patients 4, 7, and 9 to 11), although HEV RNA was reproducibly detectable by the two conventional RT-PCR methods (ORF2/3-137 PCR and ORF2-457 PCR), and only 7.1×10^2 to 5.7×10^4 copies/ml in patients 2, 3, 5, 6, and 8. Of interest, the peak HEV load in a fecal specimen from the remaining one patient (patient 1) was markedly high, at 2.0×10^7 copies/ml on day 22 after the disease onset, although the fecal specimen from patient 1 was obtained on a day comparable to the days on which fecal specimens with the highest HEV load were taken from the other 10 patients (days 10 to 29; mean, 17.2). In addition, although HEV RNA in fecal supernatant continued to be positive until days 14 to 33 (mean, 22.4) for patients 2 to 11, similar to the case with serum samples, fecal HEV from patient 1 was detectable even on day 121. The HEV in the fecal supernatant was typed as genotype 1 for patients 2 and 8, genotype 3 for patients 1 and 9, and genotype 4 for patients 3 to 7, 10, and 11 (sequences AB301698 to AB301708).

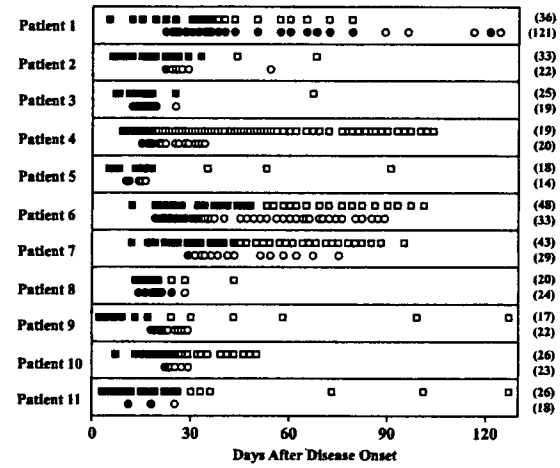

FIG. 1. Detection of HEV RNA in initial and follow-up serum and fecal samples from 11 patients (patients 1 to 11) with hepatitis E. For each patient, closed or open boxes in the top row represent positivity or negativity for HEV RNA in serum, respectively; closed and open circles in the bottom row represent positivity or negativity for HEV RNA in feces, respectively. The number in parentheses at the end of each row indicates the final day on which HEV RNA was detectable. Patient 4 contracted fulminant hepatitis E and died 105 days after onset of the illness.

Clinical characteristics, laboratory parameters, and HEV markers in patient 1. Patient 1, a 67-year-old Japanese man who had been noted to have renal dysfunction of unknown etiology and had received monthly follow-up examinations at a local hospital since 1993, was referred and admitted to the Department of Otorhinolaryngology at our university hospital in April 2003 because of otalgia and difficulty hearing. Although the patient was diagnosed with otitis media, he also had systemic edema and complained of general malaise and anorexia, and his renal function was found to have deteriorated, with elevated levels of blood urea nitrogen of 92 mg/dl (normal range, 8 to 20 mg/dl) and serum creatinine of 7.87 mg/dl (normal range, 0.38 to 0.90 mg/dl), accompanied by anemia with a markedly decreased hemoglobin level of 5.5 g/dl (normal range, 11.3 to 15.2 g/dl). On the day of admission, he was transferred to the Department of Nephrology in the same university hospital, because he required hemodialysis. Besides severe chronic renal failure, laboratory findings at admission revealed liver dysfunction with slightly elevated levels of alanine aminotransferase (ALT) of 118 IU/liter (normal range, 4 to 30 IU/liter) and aspartate aminotransferase of 164 IU/liter (normal range, 11 to 30 IU/liter). To clarify the etiology of liver dysfunction, his serum was tested for hepatitis virus markers. Serological and molecular markers of hepatitis A virus (HAV), hepatitis B virus, and hepatitis C virus were negative, except for anti-HAV (total) and antibodies to the hepatitis B virus core (anti-HBc). In addition, both cytomegalovirus and Epstein-Barr virus antibodies were IgG positive but IgM negative. However, he had high-titer IgG, IgM, and IgA classes of antibodies to HEV detectable by in-house ELISA as described below, and HEV RNA was detected in his serum. Consequently, the present patient was diagnosed as having sporadic acute hepatitis E, and periodic serum and fecal specimens

TABLE 2. Laboratory parameter, anti-HEV antibody levels, and HEV RNA in periodic serum and fecal samples obtained from patient 1

Day after onset	ALT (IU/liter)	Anti-HEV (absorbance at 450 nm) level for Ig class[b]:			HEV RNA (copies/ml) in[c]:	
		G	M	A	Serum	Feces
−5	26	NA[a]	NA	NA	NA	NA
5	118	>3.000 (+)	1.938 (+)	>3.000 (+)	7.2×10^6	NA
12	620	>3.000 (+)	2.712 (+)	>3.000 (+)	5.0×10^5	NA
15	285	>3.000 (+)	>3.000 (+)	>3.000 (+)	3.6×10^5	NA
19	49	>3.000 (+)	>3.000 (+)	>3.000 (+)	3.3×10^5	NA
22	15	>3.000 (+)	>3.000 (+)	>3.000 (+)	1.7×10^5	2.0×10^7
24	NA	NA	NA	NA	NA	1.1×10^7
25	19	>3.000 (+)	>3.000 (+)	>3.000 (+)	5.6×10^4	1.8×10^5
26	NA	NA	NA	NA	NA	4.7×10^6
28	NA	NA	NA	NA	NA	8.6×10^5
30	22	>3.000 (+)	>3.000 (+)	>3.000 (+)	7.7×10^2	1.4×10^5
32	15	>3.000 (+)	>3.000 (+)	>3.000 (+)	2.7×10^2	7.4×10^4
34	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	1.7×10^2	4.2×10^2
35	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	<100 (+)	2.8×10^3
36	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	<100 (+)	1.6×10^4
38	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	7.0×10^3
40	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	<100 (+)
43	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	3.4×10^4
50	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	4.9×10^4
57	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	9.8×10^3
60	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	3.9×10^4
65	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	4.9×10^2
68	NA	NA	NA	NA	NA	2.0×10^2
72	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	1.7×10^2
79	NA	>3.000 (+)	>3.000 (+)	>3.000 (+)	−	<100 (+)
89	NA	NA	NA	NA	NA	−
96	NA	NA	NA	NA	NA	−
116	NA	NA	NA	NA	NA	−
121	NA	NA	NA	NA	NA	1.3×10^5
124	NA	NA	NA	NA	NA	−
207	NA	NA	NA	NA	NA	−
212	NA	NA	NA	NA	NA	−
214	NA	NA	NA	NA	NA	−
261	NA	>3.000 (+)	0.671 (+)	1.589 (+)	−	NA

[a] NA, not available.
[b] (+), positive for anti-HEV of the indicated Ig class.
[c] (+), positive for HEV RNA by ORF2/3-137 PCR. −, negative for HEV RNA.

obtained from the patient were used for quantitation and characterization of HEV.

Although the exact day of onset of acute hepatitis was unclear in this case, the day of appearance of general malaise and anorexia, which also might have been due to the progression of chronic renal dysfunction, was tentatively regarded as the day of onset of hepatitis (5 days before hospitalization). The patient developed a mild form of self-limited acute hepatitis with a peak ALT level of 620 IU/liter (Table 2), and his serum ALT level normalized on day 22 after disease onset. IgG, IgM, and IgA classes of anti-HEV antibodies were detectable through the end of the observation period (day 261). The load of HEV RNA in the circulation was highest on admission (day 5), at 7.2×10^6 copies/ml, and viral RNA was detectable until day 36 despite normalization of liver enzymes on day 22. Fecal specimens were obtainable from the patient between day 22 and day 214. The highest HEV RNA titer was seen for the fecal specimen collected on day 22 (2.0×10^7 copies/ml), and fecal HEV RNA continued to be positive until day 79. HEV RNA in feces became undetectable on day 89 and remained negative on days 96 and 116. Surprisingly, however, fecal HEV RNA was reproducibly positive on day 121 at a titer of 1.3×10^5 copies/ml. On day 124 and thereafter, HEV RNA in feces was undetectable. All serum and fecal samples that were positive or negative for HEV RNA by real-time detection RT-PCR were subjected to ORF2/3-137 PCR, and the presence of HEV RNA was confirmed by ORF2-457 PCR. Furthermore, the specificity of the ORF2-457 PCR assay results was verified by sequence analysis of the amplicons. Of note, the 412-nt ORF2 sequence obtained from the serum sample on day 5 (AB301709) and those from fecal specimens on days 22, 50, and 121 were 100% identical to each other.

Infectivity of fecal HEV evaluated in a cell culture system. In addition to the fecal supernatant (JE03-1760F) collected on day 22 from patient 1 that had been demonstrated to grow in PLC/PRF/5 cells in our previous study (55), those collected on days 24, 26, 28, 30, and 121 from the same patient with lower HEV RNA titers (Table 2) were inoculated onto fresh monolayers of PLC/PRF/5 cells, and the HEV viral load was measured in the culture medium until 50 days postinoculation (dpi) (Fig. 2) to assess the infectivity of HEV in periodic fecal specimens obtained from the patient. When HEVs in fecal specimens that had been collected from the patient on days 22, 24, and 26 were inoculated, HEV RNA was first detected in the collected culture media at 12 or 14 dpi and increased to 6.4×10^5 to 1.7×10^7 copies/ml at 50 dpi. When HEVs in fecal

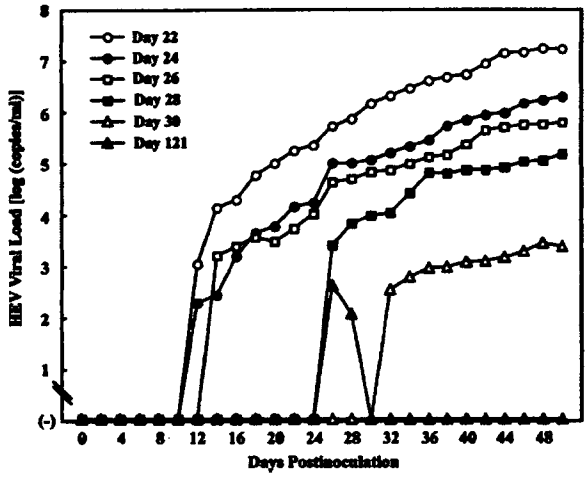

FIG. 2. Quantitation of HEV RNA in the culture supernatant of PLC/PRF/5 cells after inoculation with fecal supernatant that had been collected on the indicated day after disease onset from patient 1. The fecal supernatant that had been diluted 1:5 in PBS(−) containing 0.2% BSA and filtered through a 0.22-μm microfilter was inoculated into each well with the following viral loads: day 22 after onset, 6.5×10^5 copies per well; day 24, 4.1×10^5 copies per well; day 26, 1.9×10^5 copies per well; day 28, 3.3×10^4 copies per well; day 30, 3.4×10^3 copies per well; and day 121, 2.9×10^3 copies per well.

specimens (days 28 and 30) were inoculated, HEV initially appeared in the culture medium at 26 or 32 dpi. Consequently, fecal specimens on days 22 to 30 were found to be infectious in PLC/PRF/5 cells, and the HEV RNA level in the culture medium increased more rapidly as the amount of HEV inoculated increased. On the other hand, the HEV in fecal supernatant on day 121 did not grow efficiently in PLC/PRF/5 cells, and a transient and slight elevation of HEV in culture medium was observed at 26 and 28 dpi, as illustrated in Fig. 2. The reproducibility of HEV positivity was confirmed by the two conventional RT-PCR assays. The HEV in fecal suspension from patient 2 (day 22 after onset) was found to be noninfectious in the culture system.

Analysis of the full-length genomic sequence of HEV. The full-length genomic sequence of an HEV isolate obtained from the fecal specimen (JE03-1760F) of patient 1 that had been collected on day 22 after the onset of hepatitis was determined (AB301710). The JE03-1760F isolate had a genomic length of 7,226 nt, excluding the poly(A) tract at the 3′ terminus, and possessed three major ORFs, ORF1, ORF2, and ORF3, which had a coding capacity of 1,703 amino acids (aa) (nt 26 to 5134), 660 aa (nt 5172 to 7151), and 122 aa (nt 5134 to 5499), respectively. The 5′UTR and 3′UTR of JE03-1760F comprised 25 and 75 nt [excluding the poly(A) tail], respectively. Comparison of the JE03-1760F genome to 75 reported HEV genomes of genotypes 1 to 4, the entire or nearly entire nucleotide sequences of which were known, revealed that it was closest to wbJSG1 (a genotype 3 boar HEV isolate of Japanese origin; see Fig. 3 for the GenBank accession number) with an identity of 91.7%, but it was only 73.9 to 75.8% similar to the prototype HEV isolates of genotypes 1, 2, and 4 (Sar-55, MEX-14, and T1, respectively) in the nucleotide sequence of the full genome. A phylogenetic tree was constructed based on the overlapping almost-complete genomic sequence of 76 HEV isolates obtained from humans, swine, wild boars, a deer, and a mongoose, using an avian HEV as an outgroup. The tree confirmed that JE03-1760F belonged to genotype 3 and segregated into a cluster consisting of human, swine, boar, deer,

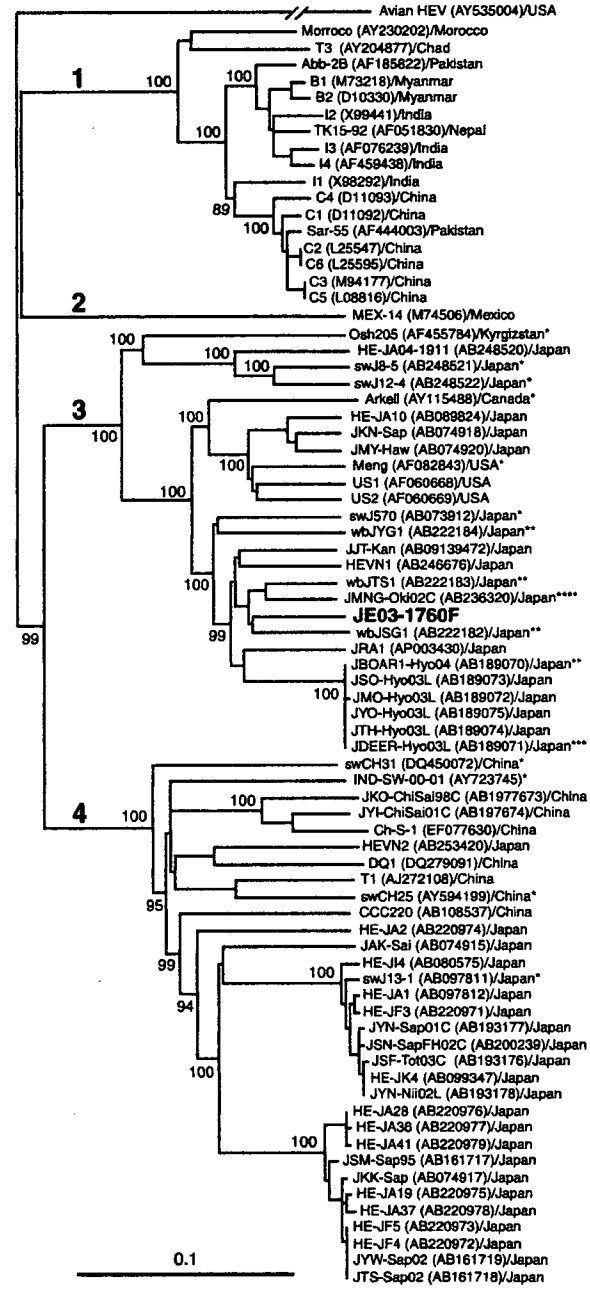

FIG. 3. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 76 HEV isolates, using an avian HEV isolate (AY535004) as an outgroup. The HEV isolate (JE03-1760F) for which the full-length sequence was determined in the present study is indicated in boldface for visual clarity. Seventy-five isolates for which the entire or nearly entire sequences have 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 (****). Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings. Bar, 0.1 nucleotide substitution per site.

TABLE 3. Nucleotides unique to the HEV isolate from patient 1 and comparison to sequences of the 25 reported HEV isolates of the same genotype (genotype 3) for which the entire or nearly entire genomic sequences are known

Nucleotide position	Nucleotide change	Genomic region	Amino acid change[a]
169	U to C	ORF1	
610	G to A	ORF1	
886	G to U	ORF1	
1132	C or U to A	ORF1	
1951	G to U	ORF1	
2310	A to G	ORF1	AAG (Lys), GAG (Glu), GAA (Glu), or GAC (Asp) to GGG (Gly)
2317	U to C	ORF1	
2368	A, C, or U to G	ORF1	
2372	A or C to U	ORF1	CCG (Pro), CCA (Pro), or AAG (Lys) to UCA (Ser)
2653	G to A	ORF1	
2808	C to U	ORF1	GCU (Ala) or GCC (Ala) to GUU (Val)
3280	G to A	ORF1	
3371	A to U	ORF1	ACA (Thr), ACG (Thr), or ACU (Thr) to UCG (Ser)
3631	G to A	ORF1	
3954	G to A	ORF1	AGC (Ser) or AGU (Ser) to AAC (Asn)
4228	C or U to A	ORF1	
4239	C to U	ORF1	GCC (Ala) to GUC (Val)
4252	G or U to C	ORF1	
4399	C, G, or U to A	ORF1	
5963	A or G to U	ORF2	
5990	C, G, or U to A	ORF2	
6128	U to C	ORF2	
6260	G to A	ORF2	
6449	C to U	ORF2	
6575	U to C	ORF2	
6590	C or U to A	ORF2	
6602	C or U to A	ORF2	
6650	U to C	ORF2	
6884	A or U to C	ORF2	

[a] Amino acid sequences are expressed as three-letter abbreviations (in parentheses) after the corresponding triplet codons.

and mongoose HEV isolates that are presumed to be indigenous to Japan (Fig. 3).

Upon comparison to the 25 reported HEV isolates of the same genotype (for accession numbers, see Fig. 3), the entire or nearly entire sequences of which are known, the JE03-1760F isolate had 29 nucleotides that were not seen in any of the 25 HEV isolates, resulting in six amino acid substitutions exclusively in the ORF1 protein (Table 3).

DISCUSSION

Given that the focus for viral detection in enterically transmitted non-A, non-B hepatitis historically had been fecal shedding, the first method available for detection of the virus (now known as HEV) responsible for this disease was immune electron microscopy (IEM) (4, 5, 48, 67), but the detection rate of HEV in fecal samples by IEM was low: the detection limit of HEV by IEM is estimated to be only 10^6 particles (59). Application of RT-PCR methods resulted in a dramatic increase in the detection of HEV RNA among patients with acute hepatitis E (76/166 [45.8%] or 47/67 [70.1%]) (9, 10). In the present study, although the number of patients studied was small, all 11 patients had detectable HEV RNA in their fecal specimens. The HEV load in fecal supernatant was low, at <100 copies/ml for 5 of the 11 patients, and was only 7.1×10^2 to 5.7×10^4 copies/ml for 5 other patients, indicating that a large proportion of patients were excreting the virus, albeit in small quantities, after the onset of clinical symptoms. To our surprise, high-titer HEV RNA of 2.0×10^7 copies/ml was detected in the stool sample of the remaining patient (patient 1), although the fecal sample was collected on day 22 after the presumed day of onset or 10 days after the day with the highest ALT value during the observation period. This finding encouraged us to investigate whether HEV in the fecal supernatant can replicate in established cell lines. Using the fecal suspension obtained on day 22 (JE03-1760F) from patient 1, we successfully developed an efficient cell culture system for HEV in PRC/PRF/5 and A549 cells in our previous study (55).

Except for experimental HEV infection in monkeys (9, 61), only a few studies had been conducted for detection of HEV RNA in serial fecal samples from patients with acute hepatitis E. In a study of HEV transmission in a single volunteer in which RT-PCR was employed for sequential detection of HEV RNA (8), HEV RNA was detected in fecal samples from the patient up to day 16 after the onset of symptoms; stools collected on days 4 and 7 transmitted disease to three monkeys. Since subsequent samples were not collected from the patient, it was unknown how long fecal shedding of HEV lasted. Based on data on serial fecal samples, Nanda et al. (36) reported that four patients with acute hepatitis E in India showed fecal virus shedding up to the 9th, 10th, 12th, and 52nd days of the illness, respectively. In the largest available data set on serial fecal samples, HEV RNA was detectable in 16 (80%) of 20 patients in an outbreak of hepatitis E in India, and the maximum duration after the onset of the first symptom at which a stool sample was positive for HEV RNA was 30 days (2). Most

previous studies on detection of HEV RNA in fecal samples from patients with epidemic or sporadic hepatitis E had been conducted cross-sectionally. In a study of single fecal samples from patients with acute hepatitis E, Clayson et al. (10) found HEV RNA in 53% (8/15), 77% (10/13), and 50% (1/2) of stool samples collected within the first 3 days of illness, within 8 to 11 days, and within 12 to 15 days, respectively; no stool samples were collected after 15 days. Several other studies of single stool samples from infected individuals indicated that fecal shedding occurs for approximately 2 weeks (1, 9, 59, 70). Therefore, to summarize the previous studies, prolonged fecal shedding of HEV in humans was shown in a small group of patients, and the longest duration of fecal excretion of HEV thus far reported is 52 days.

In the present study, we were able to obtain serial fecal samples from 11 sporadic cases of domestic or imported acute hepatitis E, and therefore it was possible to investigate the duration of HEV excretion and load of HEV shed into the feces. Nine of the 11 patients studied had short-term virus fecal excretion of 14 to 29 days' duration, corroborating the findings of the previous study in which loss of virus fecal shedding was observed within 30 days of illness for patients with acute HEV infection, except for one patient, who showed fecal shedding up to the 52nd day of illness (36). One of the most remarkable results of this study was that HEV RNA was detectable in the fecal specimen obtained from a patient (patient 1) even on day 121 after the presumed onset of hepatitis, or 109 days after the day on which the peak ALT level of 620 IU/liter was observed. Recently, prolonged fecal virus shedding (beyond 56 dpi) was observed in only 2 of 15 pigs experimentally infected with genotype 3 HEV (14) and only one of nine chickens experimentally infected with avian HEV (6). The observation of prolonged fecal virus shedding from a natural case of human hepatitis E, coupled with similar observations in HEV infections from a very small number of experimentally infected animals, indicates that, indeed, prolonged fecal shedding does occur during HEV infection. This has important implications for understanding HEV pathogenesis and transmission.

The precise reason for the markedly long duration of virus fecal excretion found for a particular patient remains unknown. Unlike the other 10 patients studied, patient 1 had an underlying disease of chronic renal failure and contracted de novo HEV infection just before the initiation of hemodialysis. As patients on maintenance hemodialysis have an impaired immune response to viral protein or to vaccination (12, 17, 18, 25, 44), they may be unable to raise an adequate immune response to viral protein and to efficiently eliminate an infecting virus. The impaired immune response in hemodialysis patients is thought to be related to low levels of T-cell proliferation upon mitogenic stimuli because of impaired costimulation by accessory cells (16). Prolonged viremia of HEV also was reported for a patient with T-cell lymphoma during chemotherapy (54). Therefore, one possible explanation is that protracted fecal shedding of HEV in patient 1 was attributable to impaired host immunity at the initiation of hemodialysis and during maintenance hemodialysis. However, high and persistent excretion of HAV by immunocompetent patients has been reported (60), and transient, short-term viremia of HEV was observed in three hemodialysis patients who acquired subclinical HEV infection (31), suggesting that it is important to consider an alternative explanation for the obtained results. HEV in the fecal suspension obtained from patient 1 on day 22 (JE03-1760F) could grow efficiently in our cell culture system, reaching a high titer of up to 10^8 copies/ml, and HEV progeny released in the culture medium were successfully passaged five times in culture cells (55). Therefore, it is likely that the JE03-1760F strain has a higher replicative capability than other HEV strains.

For patient 1, fecal shedding of HEV lasted approximately 100 days after normalization of the ALT level and 83 days after the cessation of viremia, suggesting extrahepatic replication of HEV. Of interest, it has been shown that both swine HEV and human HEV replicate in extrahepatic organs of experimentally infected pigs, including the colon and intestines (65), which may be responsible for the long duration of virus fecal excretion. Whether the JE03-1760F strain is likely to replicate efficiently and for a longer duration in the colon and/or intestines needs further investigation.

It remains unknown what mutations in the HEV genome are associated with the heightened multiplication ability of HEV. Therefore, in the present study, we determined the full-length genomic sequence of the JE03-1760F isolate obtained from the fecal suspension with the highest HEV load of 2.0×10^7 copies/ml of patient 1 and found 29 nucleotide substitutions over the entire genome that are unique to the JE03-1760F isolate and that are not seen in any of the 25 reported HEV isolates of the same genotype (genotype 3). Among the 29 nucleotide substitutions, six substitutions lead to amino acid changes in ORF1, which encodes nonstructural proteins: two substitutions were seen in the poly-proline hinge, one each in the X domain and helicase, and two in the RNA-dependent RNA polymerase. Therefore, it is tempting to speculate that these substitutions could be candidates for mutations associated with high levels of replication activity and long-term fecal shedding of HEV in infected hosts and the capability of efficient replication in a cell culture system; further studies are needed to clarify this important issue. Studies using a mutagenized, infectious cDNA clone of the HEV genome may elucidate the mechanism by which the observed nucleotide substitutions with or without amino acid changes lead to active replication and protracted fecal shedding of HEV.

Viral RNA became detectable again on day 121 but not on day 124 in patient 1. A better explanation as to why fecal virus shedding was positive again on day 121 even after 6 weeks of negative results may be required in future studies. A sequence comparison between the genome of day 121 virus and those of the earlier viruses (such as day 79 virus) may provide important information on the mechanism of prolonged fecal virus shedding. The precise reason why HEV in the fecal specimen on day 121 from patient 1 did not grow as efficiently as that on day 30 with a comparable HEV load remains unknown. One possible explanation is that antibodies against HEV secreted into the gastrointestinal tract have neutralized the virus. However, no significant signals of IgG, IgM, and IgA classes of HEV antibodies were detectable in fecal samples throughout the observation period in patient 1 or in the other 10 patients studied (data not shown). Fecal samples contain large amounts of phenolic and metabolic compounds and polysaccharides and have a very heterogeneous composition (66), suggesting that the performance of our cell culture system may vary between

samples, although we diluted fecal specimens 1:5 in PBS(−) containing 0.2% BSA prior to inoculation. It also is likely that replication-defective HEV genomes or those with lowered replicative activity have appeared at the end stage of long-term HEV infection. In this context, further studies by molecular approaches using the cell culture system for HEV and various clinical samples are needed.

In conclusion, the present study indicates that a delayed protracted virus shedding in feces occurs in a fraction of patients with acute sporadic HEV infection and that HEV shed into the feces at least on day 30 after the onset of hepatitis has the capability of efficiently replicating in PLC/PRF/5 cells, suggesting that careful attention to hygiene and sanitation is necessary even after patients with hepatitis E enter the recovery phase. Therefore, fecal-oral transmission of HEV also should be taken into consideration as a possible transmission route for individuals with no immunity against HEV, even in industrialized countries where the occurrence of domestic HEV infection as a zoonosis currently is emphasized.

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Review

Genetic variability and evolution of hepatitis E virus

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Abstract

Hepatitis E virus (HEV) is the sole member of the genus *Hepevirus* in the family *Hepeviridae*. HEV is transmitted primarily by the fecal–oral route, and water-borne epidemics are characteristic of hepatitis E in many developing countries in Asia, Africa and Latin America where sanitation conditions are suboptimal. Accumulating lines of evidence indicate that HEV-associated hepatitis also occurs domestically among individuals in industrialized countries, that there are animal reservoirs of HEV such as domestic pigs and wild boars, and that hepatitis E is a zoonosis. Based on the extensive genomic variability among HEV isolates, HEV sequences have been classified into four genotypes: genotype 1 consists of epidemic strains in developing countries in Asia and Africa; genotype 2 has been described in Mexico and several African countries; genotype 3 HEV is widely distributed and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in many countries in the world, except for countries in Africa; and genotype 4 contains strains isolated from humans and/or domestic pigs exclusively in Asian countries. This paper reviews current knowledge on the genomic variability, geographic distribution and zoonotic aspects of HEV as well as the clinical significance of genotype and evolution of HEV.

Keywords: Hepatitis E virus; Zoonosis; Genotype; Phylogenetic tree

Contents

1. Introduction

Hepatitis E virus (HEV; formerly known as enterically transmitted non-A, non-B hepatitis virus) is the causative agent of acute self-limited or fulminant hepatitis E, which is considered to be endemic in many developing countries in Asia, Africa and Latin America where sanitation conditions are suboptimal (Purcell and Emerson, 2001a). HEV is transmitted primarily by the fecal–oral route, and water-borne epidemics are characteristic of hepatitis E. Hepatitis E may occur in any of three forms: large epidemics, smaller outbreaks, or sporadic infections. Sporadic cases of HEV infection have also been reported in non-endemic, industrialized countries, where its occurrence is usually associated with travel to endemic countries (Dawson et al., 1992; Donati et al., 1997; Herrera, 1993; Hino et al., 1991; Ishikawa et al., 1995). Recently, however, accumulating lines of evidence indicate that HEV-associated hepatitis also occurs among individuals in industrialized countries who have no history of travel to areas where HEV is endemic (Amon et al., 2006; Harrison, 1999; Ijaz et al., 2005; Kwo et al., 1997; Mansuy et

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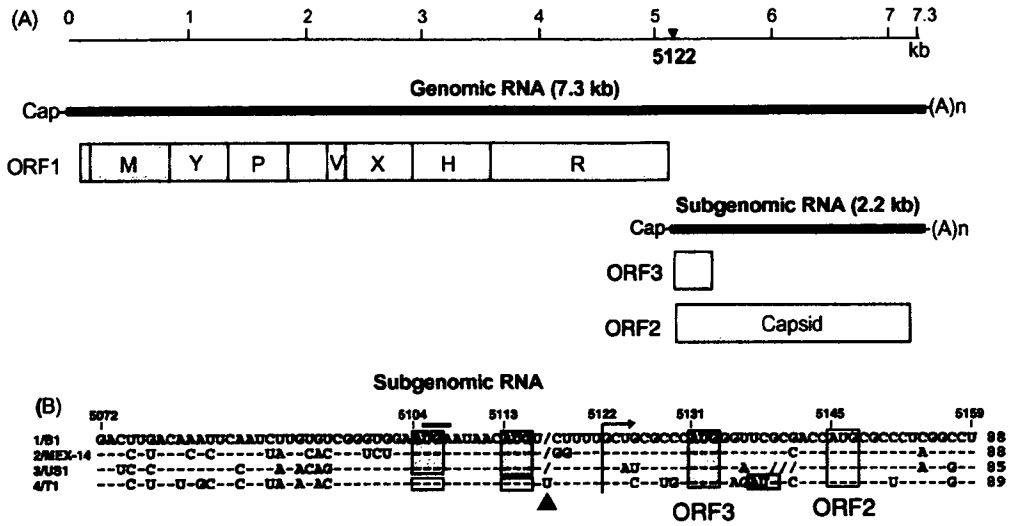

Fig. 1. Organization of the genomic and subgenomic RNAs of HEV. (A) Organization of the HEV genome showing the location of nt 5122, at which the subgenomic RNA of 2.2 kb initiates: nucleotide positions are in accordance with the Sar-55 isolate (GenBank/EMBL/DDBJ accession no. AF444003). M, methyltransferase; Y, Y domain; P, papain-like protease; V, proline-rich hinge domain; X, X domain; H, RNA helicase; R, RNA polymerase. (B) Comparison of the HEV sequences containing potential initiation codons for ORF2 and ORF3. Four representative HEV isolates of genotype 1, 2, 3 or 4 whose entire genomic sequence is known were compared. Potential initiation codons of ORF3 are indicated by shaded boxes, and those of ORF2 are shown by open boxes. The inserted U residue that is exclusively found in the genotype 4 isolates is marked with the closed triangle. The termination codon (UGA) of ORF1 is overlined. See Fig. 2 for accession no. of each isolate.

al., 2004; Mizuo et al., 2005; Okamoto et al., 2003; Purcell and Emerson, 2001a; Smith, 2001) and that hepatitis E is a zoonotic disease (Erker et al., 1999; Harrison, 1999; Meng, 2000, 2003; Meng et al., 1997, 1998; Nishizawa et al., 2003; Okamoto et al., 2001; Smith, 2001; Tei et al., 2003; Yazaki et al., 2003).

HEV was previously classified in the family *Calciviridae*, but it is now classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson et al., 2004). The virion is approximately 27–34 nm in diameter and does not possess an envelope. It is most likely icosahedral and is believed to be composed of a single capsid protein. Its genome consists of a single-stranded, positive-sense RNA of approximately 7.3 kilobases (kb) in length. It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and a short 3′ UTR that is terminated by a poly(A) tract (Tam et al., 1991; Wang et al., 2000). Although a single serotype has been proposed, extensive genomic diversity has been observed among HEV isolates. Several classification schemes have been proposed (Erker et al., 1999; Gouvea et al., 1998a; Schlauder and Mushahwar, 2001; Schlauder et al., 1999, 2000; Tsarev et al., 1999; van Cuyck-Gandre et al., 2000; Wang et al., 1999; Zhai et al., 2006). However, the most accepted scheme classifies HEV isolates into four major genotypes (1–4) (Emerson et al., 2004; Lu et al., 2006; Schlauder and Mushahwar, 2001; Zhai et al., 2006).

HEV was discovered by immune electron microscopy in 1983 (Balayan et al., 1983). Eight years after its discovery, the full genomic sequence of HEV was first determined for a strain from Myanmar (formerly Burma) (Tam et al., 1991; B1 in Fig. 1), which had greater than 88.2% nucleotide identity across the entire genome to isolates obtained from other countries in Asia including China, India, Nepal, and Pakistan (Aye et al., 1992; Bi et al., 1993; Donati et al., 1997; Gouvea et al., 1998b; Tsarev et al., 1992; van Cuyck-Gandre et al., 2000; Yin et al., 1994) and those in Africa including Chad and Morocco (Cheng and Meng, 2004; van Cuyck et al., 2003). In 1992, a Mexican strain that was implicated in an outbreak that occurred in Mexico in 1986, was reported (Huang et al., 1992). The Mexican strain is distinct from the Burmese variants, and constitutes a second genotype. In 1997, an HEV isolate from a patient with sporadic acute hepatitis E in the United States who had no history of travel abroad was reported (Kwo et al., 1997), and it constituted a third genotype, which was subsequently found to be widely distributed throughout the world (Banks et al., 2004; Inoue et al., 2006b; Mitsui et al., 2005; Mizuo et al., 2002; Pina et al., 2000; Schlauder et al., 1998, 1999, 2000; Takahashi et al., 2001; K. Takahashi et al., 2003; Wang et al., 2001; Worm et al., 2000; Zanetti et al., 1999). In 1999, HEV isolates recovered from Chinese patients with acute hepatitis that were distinct from the original Chinese isolates of genotype 1, were reported and they constitute a fourth group (Wang et al., 1999). HEV isolates classifiable into the fourth group have also been identified from sporadic cases of HEV infection not only in China but also in Taiwan and Japan (Hsieh et al., 1999; Inoue et al., 2006a; Kuno et al., 2003; Takahashi et al., 2002; K. Takahashi et al., 2003; Wang et al., 2000). Accordingly, HEV sequences have been classified into four major genotypes (genotypes 1–4) (Emerson et al., 2004; Lu et al., 2006; Schlauder and Mushahwar, 2001; Zhai et al., 2006).

2. Organization of the genomic and subgenomic RNAs of HEV

The HEV genome is a single-stranded, positive-sense RNA molecule of approximately 7.3 kb in size and is capped (Kabrane-Lazizi et al., 1999b) (Fig. 1A). The coding region is preceded by a short UTR of 25 nucleotides (nt) and is followed

by a UTR of 65–74 nt and a poly(A) tract. The coding region consists of three ORFs (ORF1, ORF2, and ORF3) (Tam et al., 1991; Wang et al., 2000). ORF1, which is approximately 5 kb in size, is located at the 5′ end and encodes non-structural proteins that are involved in RNA synthesis including guanylyl transferase, methyl transferase, papain-like cysteine protease, RNA helicase and RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992; Magden et al., 2001). ORF2, which is approximately 2 kb, occupies the 3′ end of the coding region and encodes the capsid protein. ORF3 is a small reading frame with a maximal coding capacity of 372 bases, and with a 3′ end that overlaps ORF2 by 331 nt; ORF3 encodes a small immunogenic phosphoprotein with a maximum length of 123 amino acids. The function(s) of the ORF3 protein has not been fully defined, but it is postulated to interact with the ORF2 protein (Tyagi et al., 2002) and with cellular proteins involved in cell signaling (Korkaya et al., 2001; Tyagi et al., 2002). Compared with genomes of genotypes 1, 2 and 3, genomes of genotype 4 contain a nucleotide insertion of U just after the second AUG codon of ORF3 which changes the downstream reading frames so that different AUG codons are believed to initiate translation in both ORF2 and ORF3 (Fig. 1B), and this frameshift is predicted to lengthen the ORF2 protein by 14 amino acids and shorten the ORF3 protein by 9 amino acids (Wang et al., 2000).

In an early study by Tam et al. (1991), genome-length RNA and two coterminal RNAs of 2 and 3.7 kb were detected in liver tissue from HEV-infected cynomolgus macaques by Northern blot analyses. Since the transfected recombinant full-length genome was infectious, it was thought that ORF1 of the genomic RNA is translated immediately upon entry into cells to produce the enzymes responsible for viral RNA synthesis (Emerson et al., 2004). In contrast, it was shown that production of ORF2 and ORF3 proteins following transfection of full-length genomes requires a functional viral polymerase, presumably for the synthesis of the subgenomic RNAs that encode ORF2 and ORF3 proteins (Emerson et al., 2004). The recent study by Graff et al. (2006) demonstrated that a single subgenomic RNA of 2.2 kb, which is capped, initiates at nt 5122 downstream of the first two AUG codons in ORF3 in genotypes 1–3 (downstream of the insertion site between nt 5115 and

Table 1
Geographic distribution of HEV genotypes

Genotype	Geographic region				
	Asia	Africa	America	Europe	Oceania
1	Bangladesh Cambodia China India Japan[a] Kyrgyzstan Myanmar Nepal Pakistan Uzbekistan Vietnam	Algeria CAR Chad Djibouti Egypt Morocco Namibia South Africa Sudan Tunisia	–	Russia UK[a]	–
2	–	CAR Chad DRC Egypt Namibia Nigeria	Mexico	–	–
3	Cambodia Japan Korea Kyrgyzstan Taiwan Thailand	–	Argentina Canada Mexico USA	Austria France Germany Greece Hungary Italy Netherlands Russia Spain UK	Australia New Zealand
4	China India Indonesia Japan Taiwan Vietnam	–	–	–	–

[a] Most likely imported from countries endemic for genotype 1 HEV.

5116 unique to genotype 4) (Fig. 1B) and is bicistronic, and that two closely spaced AUG codons at nt 5131 and 5145 in different reading frames are used for the initiation of ORF3 and ORF2 translation, respectively: nucleotide positions are in accordance with the Sar-55 isolate (AF444003). Their findings indicate that both the ORF2 and ORF3 proteins are produced from a single subgenomic RNA, and that these two proteins are similar in size (ORF2 [658–660 aa], ORF3 [112–114 aa]), respectively, among all four genotypes.

3. Geographic distribution of HEV

Genotype 1 is distributed in various countries including Bangladesh, Cambodia, China, India, Kyrgyzstan, Myanmar, Nepal, Pakistan, Uzbekistan, and Vietnam in Asia and Algeria, the Central African Republic (CAR), Chad, Djibouti, Morocco, Sudan, Tunisia, Namibia, Egypt, and South Africa in Africa (Table 1). HEVs that are commonly found in Asia and Africa have been classified as the Asian and African subgenotypes of genotype 1, respectively (Tsarev et al., 1999). Genotype 2 has been represented by the prototype sequence from an epidemic in Mexico (Huang et al., 1992) and new variants were recently identified from endemic cases in African countries including CAR, Chad, Democratic Republic of the Congo (DRC), Egypt, Namibia, and Nigeria (Buisson et al., 2000; Maila et al., 2004; Nicand et al., 2005). HEVs of genotypes 1 and 2 have caused epidemics and outbreaks of hepatitis E in tropical and some subtropical regions usually due to transmission by fecal contamination of water supplies (Purcell and Emerson, 2001a). In contrast, HEVs of genotypes 3 and 4 were found in sporadic acute hepatitis E cases in the United States, European countries, China, and Japan, and these cases were most likely zoonotic in origin (Harrison, 1999; Meng, 2000, 2003; Okamoto et al., 2003; Smith, 2001). Genotype 3 accounts for the largest number of isolates among all HEV sequences archived in the GenBank/EMBL/DDBJ databases, and many of them were identified in the United States or Japan (Table 2). However, genotype 3 HEV is widely distributed and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in a total of 22 countries including Argentina, Australia, Austria, Cambodia, Canada, France, Germany, Greece, Hungary, Italy, Japan, Korea, Kyrgyzstan, Mexico, the Netherlands, New Zealand, Russia, Spain, Taiwan, Thailand, the United Kingdom, and the United States: no genotype 3 HEV strains have thus far been identified in Africa. On the contrary, genotype 4 is restricted to Asian countries and contains strains from humans and/or domestic pigs in China, India, Indonesia, Japan, Taiwan, and Vietnam.

Among 38 countries where HEV strains have been isolated from infected patients, HEVs of a single genotype were isolated from infected patients in 29 countries (genotype 1 in 12 countries, genotype 2 in 3 countries, genotype 3 in 12 countries, and genotype 4 in 1 country) and HEVs of two distinct genotypes were isolated from infected patients in 8 countries (genotypes 1 and 2 in 4 countries, genotypes 1 and 3 in 2 countries, and genotypes 1 and 4 in 2 countries). Japan is unique in that three distinct genotypes (1, 3 and 4) of HEV strains have been identified in infected patients, although genotype 1 HEV is most likely imported (Table 2).

4. HEV infection as a zoonosis

Accumulating lines of evidence indicate that there are animal reservoirs of HEV and that hepatitis E is a zoonotic disease. The Meng isolate was the first reported strain of HEV isolated from an animal, namely, from an infected pig in the United States in 1997 (Meng et al., 1997). Since then, many swine HEV isolates have been identified in many countries worldwide (Table 2) (Arankalle et al., 2002; Cooper et al., 2005; Garkavenko et al., 2001; Hsieh et al., 1999; Lu et al., 2004; Okamoto et al., 2001; Huang et al., 2002; Meng, 2000, 2003; Nishizawa et al., 2003; Pina et al., 2000; Purcell and Emerson, 2001b; Y.C. Wang et al., 2002; Wu et al., 2002). In Japan, we found a high prevalence of swine anti-HEV antibodies among Japanese pigs of 3–6 months of age (71% or 2150/3009) and a high HEV viremia rate among pigs of 2–4 months of age (11% or 190/1798) (M. Takahashi et al., 2003; Takahashi et al., 2005). Thus, HEV is considered to be enzootic in pigs worldwide. The following results support the notion that animals such as pigs serve as reservoirs for HEV infection in humans: experimental cross-species infection of non-human primates by swine HEV and of pigs by human HEV was demonstrated (Balayan et al., 1990; Meng et al., 1998); there was a significantly higher prevalence of anti-HEV antibody among pig handlers than among age- and geography-matched control subjects (Drobeniuc et al., 2001; Hsieh et al., 1999; Meng et al., 2002); and numerous other animal species (chickens, monkeys, cats, dogs, cattle goats, sheep and rodents) are positive for anti-HEV antibodies (Arankalle et al., 1994; Hirano et al., 2003a,b; Kabrane-Lazizi et al., 1999a; Meng, 2000; Haqshenas et al., 2001; Huang et al., 2004; Kuno et al., 2003; Purcell and Emerson, 2001b). In addition, there have been reports of high genetic relatedness between HEV isolates obtained from humans and those obtained from swine in the same geographical regions including the United States, Taiwan, Spain, China, and Japan (Erker et al., 1999; Hsieh et al., 1999; Meng et al., 1997; Nishizawa et al., 2003; Pina et al., 2000; Y.C. Wang et al., 2002; Wu et al., 2002) (Table 3).

As reviewed by Smith (2001), it is likely that foods such as shellfish (Cacopardo et al., 1997; Koizumi et al., 2004; Mechnik et al., 2001) can act as vehicles for transmission of HEV. We recently reported that sporadic cases of acute hepatitis E in Hokkaido, where clinical HEV infection is most prevalent in Japan, may be food-borne with a zoonotic origin (Yazaki et al., 2003; Mizuo et al., 2005). At least 25 (78%) of the 32 patients with sporadic acute hepatitis E seen in two city hospitals in Hokkaido had a history of consuming grilled or undercooked pig liver and/or intestine approximately 2–8 weeks prior to the onset of hepatitis E (Mizuo et al., 2005). Pig liver specimens from 7 (1.9%) of 363 packages sold in local grocery stores in Hokkaido, had detectable HEV RNA, with a viral load of 10^2–10^7 copies/g, although it is not known if the virus in the packaged pig livers was still infectious. Importantly, the sequences of seven swine HEV isolates recovered from packaged pig livers were very closely related, or identical in a few cases, to the viruses recovered from

Table 2
Number of HEV isolates and distribution of HEV genotypes in different countries in the world

Region/country	Total no. of HEV isolates (full-length)	No. of HEV isolates with genotypes 1, 2, 3 and 4		
		Human	Swine	Others
America	130(5)	0-1-11-0[a]	0-0-117-0	0-0-1-0
Canada	2(1)	–	0-0-2-0	–
USA	80(3)	0-0-3-0	0-0-76-0	0-0-1[b]-0
Mexico	38(1)	0-1-0-0	0-0-37-0	–
Argentina	10(0)	0-0-8-0	0-0-2-0	–
Europe	164(1)	2-0-66-0	0-0-79-0	1-0-16-0
Russia	2(0)	1-0-1-0	–	–
Netherlands	41(0)	0-0-24-0	0-0-17-0	–
UK	17(1)	1-0-14-0	0-0-2-0	–
Spain	84(0)	0-0-9-0	0-0-59-0	1[b]-0-15[b]-0
Germany	2(0)	0-0-2-0	–	–
France	13(0)	0-0-11-0	0-0-1-0	0-0-1[b]-0
Austria	1(0)	0-0-1-0	–	–
Greece	2(0)	0-0-2-0	–	–
Italy	1(0)	0-0-1-0	–	–
Hungary	1(0)	0-0-1-0	–	–
Asia	1073(67)	294-0-93-265	1-0-245-118	18-0-24-15
Japan	461(43)	3-0-85-95	0-0-225-14	0-0-24[c,d,e]-15[c]
China	251(14)	27-0-0-146	0-0-0-78	–
Taiwan	22(0)	0-0-0-15	0-0-3-4	–
Korea	10(0)	0-0-7-0	0-0-3-0	–
Thailand	10(0)	–	0-0-10-0	–
Indonesia	1(0)	–	0-0-0-1	–
Myanmar	4(2)	4-0-0-0	–	–
Vietnam	10(0)	1-0-0-9	–	–
Cambodia	6(0)	1-0-1-0	1[f]-0-3-0	–
India	149(4)	110-0-0-0	0-0-0-21	18[b,g]-0-0-0
Nepal	134(1)	134-0-0-0	–	–
Bangladesh	3(0)	3-0-0-0	–	–
Pakistan	3(2)	3-0-0-0	–	–
Kyrgyzstan	7(1)	6-0-0-0	0-0-1-0	–
Uzbekistan	2(0)	2-0-0-0	–	–
Africa	68(2)	46-16-0-0	–	6-0-0-0
Egypt	8(0)	3-1-0-0	–	4[h,i]-0-0-0
Morocco	3(1)	3-0-0-0	–	–
Tunisia	1(0)	1-0-0-0	–	–
Sudan	13(0)	13-0-0-0	–	–
Chad	7(1)	5-2-0-0	–	–
Algeria	16(0)	16-0-0-0	–	–
Nigeria	6(0)	0-6-0-0	–	–
Namibia	5(0)	1-4-0-0	–	–
CAR	3(0)	1-2-0-0	–	–
DRC	1(0)	0-1-0-0	–	–
Djibouti	3(0)	3-0-0-0	–	–
South Africa	2(0)	–	–	2[a]-0-0-0
Oceania	4(0)	0-0-2-0	0-0-2-0	–
Australia	1(0)	–	0-0-1-0	–
New Zealand	3(0)	0-0-2-0	0-0-1-0	–
Total	1439(75)[j]	342-17-172-265	1-0-511-50	25-0-41-15

CAR = the Central African Republic; DRC = Democratic Republic of the Congo.
[a] X-X-X-X indicates number of HEV isolates of genotype 1, that of genotype 2, that of genotype 3, and that of genotype 4, respectively.
[b] Sewage.
[c] Wild boar.
[d] Deer.
[e] Mongoose.
[f] Accession no. DQ145799.
[g] Well water.
[h] Horse.
[i] Accession nos. AY963777–AY963780.
[j] Nucleotide sequence data of 1439 HEV isolates were retrievable from the DDBJ/EMBL/GenBank databases as of January 11, 2007.