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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 $1,600 \times g$ 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 $6,200 \times g$ 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).

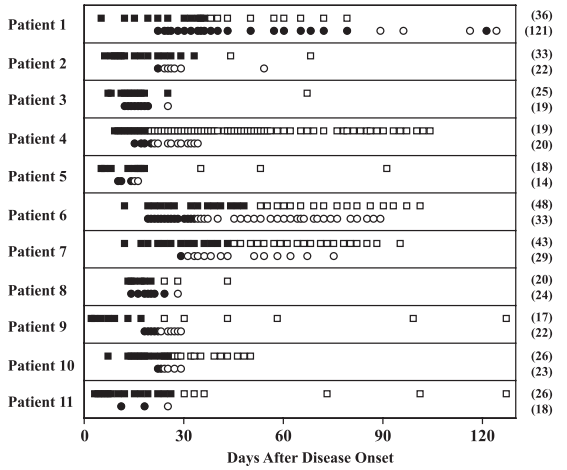

FIG. 1. Detection of HEV RNA in initial and follow-up serum and fecal samples from 11 patients (patients 1 to 11) with hepatitis E. For each patient, closed or open boxes in the top row represent positivity or negativity for HEV RNA in serum, respectively; closed and open circles in the bottom row represent 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

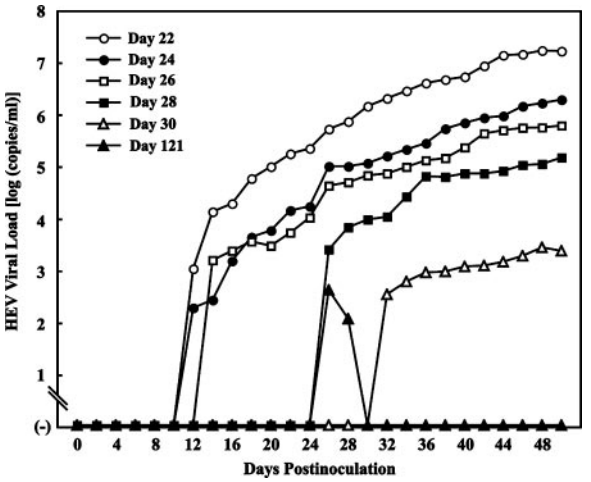

FIG. 2. Quantitation of HEV RNA in the culture supernatant of PLC/PRF/5 cells after inoculation with fecal supernatant that had been collected on the indicated day after disease onset from patient 1. The fecal supernatant that had been diluted 1:5 in PBS(−) containing 0.2% BSA and filtered through a 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,

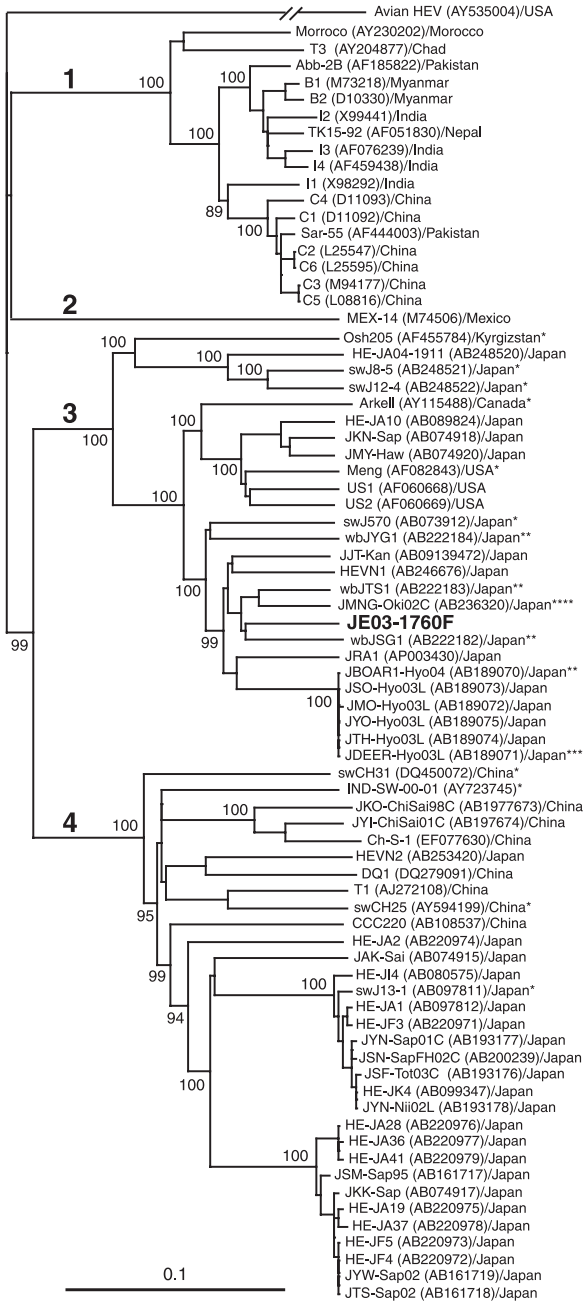

FIG. 3. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 76 HEV isolates, using an avian HEV isolate (AY535004) as an outgroup. The HEV isolate (JE03-1760F) for which the full-length sequence was determined in the present study is indicated 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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