Table 2
Sources and HEV RNA titer of inocula used for serial inoculations onto PLC/PRF/5 cells

Passage	Inoculum source	Viral load of HEV inoculated in each well (copies per well)[a]
Experiment A		
0[b]	Fecal supernatant (JE03-1760F)	6.0×10^4
1[b]	Culture supernatant (28th day after the first inoculation)	3.6×10^4
2[b]	Culture supernatant (56th day after the second inoculation)	9.8×10^4
3[b]	Culture supernatant (88th day after the third inoculation)	7.0×10^4
4[b]	Culture supernatant (64th day after the fourth inoculation)	1.2×10^5
5[b]	Culture supernatant (54th day after the fifth inoculation)	1.1×10^5
6	Culture supernatant (58th day after the sixth inoculation)	6.7×10^4
7	Culture supernatant (48th day after the seventh inoculation)	6.0×10^4
8	Culture supernatant (46th day after the eighth inoculation)	1.9×10^6
9	Culture supernatant (46th day after the ninth inoculation)	5.1×10^6
10	Culture supernatant (28th day after the tenth inoculation)	3.6×10^5
11	Culture supernatant (24th day after the eleventh inoculation)	3.0×10^3
12	Culture supernatant (48th day after the twelfth inoculation)	1.0×10^3
13	Culture supernatant (30th day after the thirteenth inoculation)	3.0×10^2
Experiment B		
0	Fecal supernatant (JE03-1760F)	5.0×10^5
1	Culture supernatant (56th day after the first inoculation)	1.1×10^6
2	Culture supernatant (52nd day after the second inoculation)	3.2×10^5
3	Culture supernatant (108th day after the third inoculation)	4.4×10^5
4	Culture supernatant (32nd day after the fourth inoculation)	4.4×10^4
5	Culture supernatant (30th day after the fifth inoculation)	1.3×10^5

[a] Quantification of HEV RNA was performed after filtration of the fecal supernatant or culture supernatant through a 0.22 μm microfilter.
[b] Retrieved from Tanaka et al. (2007).

56-dpi culture medium of Passage 1, HEV RNA emerged on 16 dpi and increased to 5.8×10^6 copies/ml on 88 dpi. In Passages 3–5, HEV RNA appeared on 12 or 14 dpi, and thereafter grew in a manner similar to those in Passages 0 and 2. The HEV RNA titer in the culture medium increased to 2.4×10^6 copies/ml on 58 dpi in Passage 5. In Passages 6–10 carried out in the present study, HEV RNA was first detectable in the culture medium on 8, 10, or 12 dpi, and reached a load of $>10^5$ copies/ml on 12–22 (mean, 16.0) dpi, contrasting with

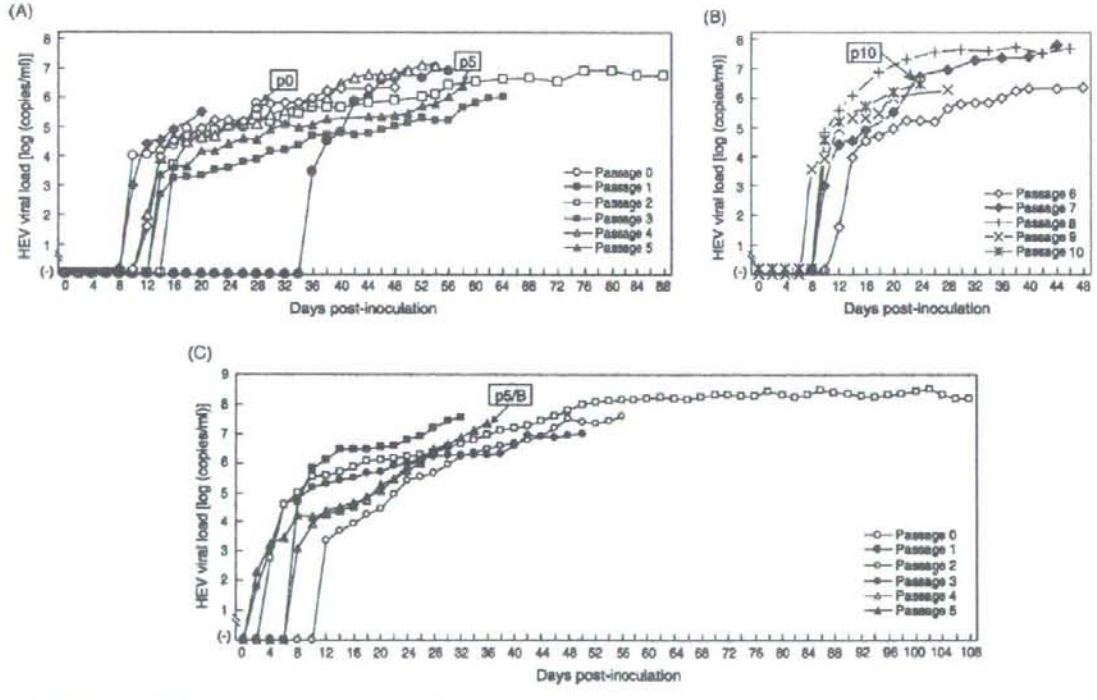

Fig. 2. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with fecal supernatant or culture medium of Passages 0, 1, 2, 3, or 4 in Experiment A (A), culture medium of Passages 5, 6, 7, 8 or 9 in Experiment A (B) or fecal supernatant or culture medium of Passages 0, 1, 2, 3, or 4 in Experiment B (C), that was harvested on the final day of each passage (see **Table 2**). The harvested culture supernatant of each passage was purified by passing through a microfilter with pore size of 0.22 μm (see Section 2) and then inoculated on fresh PLC/PRF/5 cells. The boxed letters of p0, p5, p10, and p5/B denote the days after inoculation when culture supernatants used for molecular cloning of the HEV genomes, were harvested.

Passages 1–5 where HEV load in the culture supernatant became >10^5 copies/ml on 24–48 (mean, 35.2) dpi (Fig. 2B).

The HEV load of the inoculum used was high in Passages 1–10, ranging from 3.6×10^4 copies per well to 5.1×10^6 copies per well (Table 2), and there was little or no well-to-well variability with regard to virus appearance in culture medium (dpi) and highest virus titer. However, when diluted culture medium containing a small amount of HEV (3.0×10^3, 1.0×10^3, or 3.0×10^2 copies per well) was inoculated in Passages 11, 12, and 13, respectively, marked well-to-well variability was observed (Fig. 3). In Passage 11, Well 11-2 showed virus appearance at 12 dpi, with the highest load of 6.5×10^7 copies/ml on 46 dpi, while Wells 11-3 to 11-5 showed virus appearance on 22–28 dpi, with the highest load of 4.6×10^4 to 3.2×10^5 copies/ml on 50 dpi (Fig. 3A). Well 11-1 showed only transient virus appearance, peaking at a titer of 5.0×10^3 copies/ml. In Passage 12 with the inoculation of HEV in Well 11-2 of Passage 11 that was harvested on 48 dpi, the earliest appearance on 14 dpi and the highest load of 1.5×10^7 copies/ml on 30 dpi were seen in Well 12-1 (Fig. 3B). In Passage 13 with the inoculation of HEV in Well 12-1 of Passage 12 that was collected on 30 dpi, HEV RNA first emerged on 10 dpi in Well 13-3, with the highest load of 4.7×10^6 copies/ml on 46 dpi (Fig. 3C). Of note, when larger amounts of HEV (1.0×10^4 or greater copies per well) were inoculated in Passages 11–13 for comparison, there was little or no discernible well-to-well difference regarding the date of virus appearance and the maximum titer of HEV RNA in culture medium (data not shown).

In an additional experiment (Experiment B in Table 2), after primary propagation of the JE03-1760F strain for 56 days, five generations of serial passages (Passages 1–5) were performed in PLC/PRF/5 cells. The highest HEV load in the harvested culture media was observed on 30–108 dpi and was in the range of 10^7 or 10^8 copies/ml (Fig. 2C).

During these serial passages, a CPE was not observed in the PLC/PRF/5 cells despite differences in the duration of cell culture and changing profile of HEV load in each passage.

3.3. Infectivity of wild-type HEV and its progenies in culture

In order to examine whether the JE03-1760F strain was able to adapt to growth in culture of PLC/PRF/5 cells during successive passages, the infectivity of wild-type JE03-1760F and its cell culture-produced variant (JE03-1760F.p13-3 in Experiment A: for simplicity, p13-3 hereafter) in the 46-dpi culture supernatant of Well 13-3 in Passage 13 as well as another variant (p5/B) in the 38-dpi culture supernatant of Passage 5 in Experiment B, were compared using the same viral loads of each inoculum (Table 3). When a small amount of HEV (1.0×10^3 copies per well) was inoculated, the yielded culture supernatants were negative for HEV

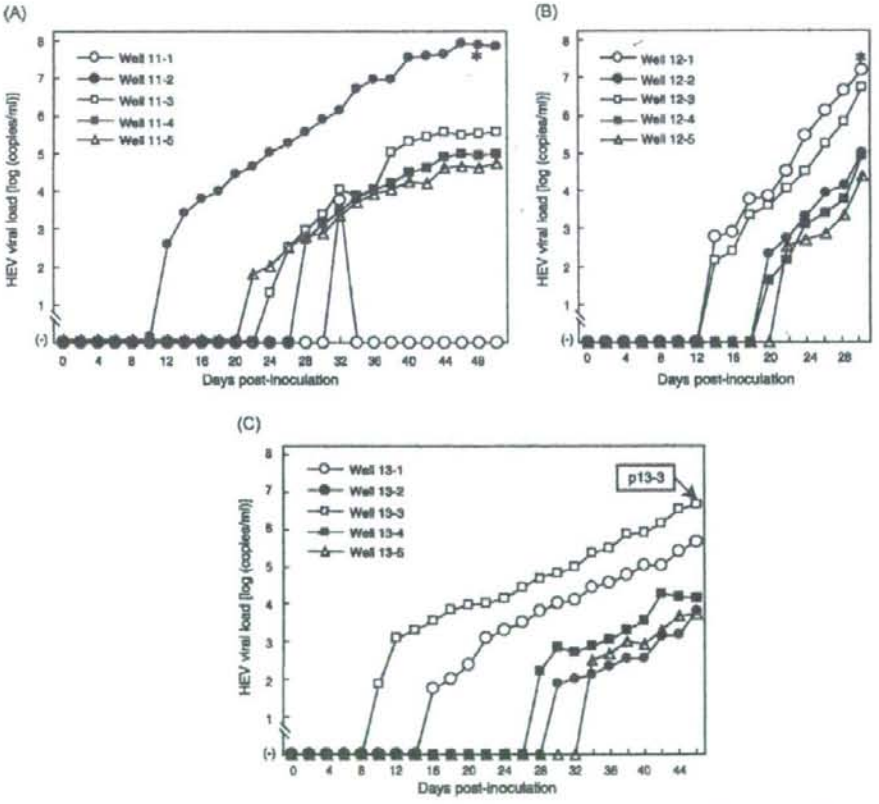

Fig. 3. Quantification of HEV RNA in culture supernatants in five wells of PLC/PRF/5 cells in (A) Passage 11 after inoculation with diluted 24-dpi culture supernatant of Passage 10, (B) Passage 12 after inoculation with diluted 48-dpi culture supernatant of Well 11-2 of Passage 11 (indicated with an asterisk in A), and (C) Passage 13 after inoculation with diluted 30-dpi culture supernatant of Well 12-1 of Passage 12 (indicated with an asterisk in B), which contain HEV at 3.0×10^3, 1.0×10^3, and 3.0×10^2 copies per well, respectively. The boxed letter of p13-3 denotes the day after inoculation when culture supernatant used for molecular cloning of the HEV genome, was harvested.

Table 3
Comparison of infectivity of wild-type and its cell culture-produced variants in PLC/PRF/5 cells

Inoculum	No. of wells tested	No. of wells with continuous HEV release after inoculation of the indicated amount of HEV (copies per well)			
		3.0×10^3	1.0×10^3	3.0×10^2	1.0×10^2
Fecal specimen (wild-type JE03-1760F)	5	4 (80%) [1 (20%)/32 dpi][a]	0 [0]	NT[b]	NT
The 46-dpi culture supernatant of Well 13-3 in Passage 13 in Experiment A[c]	5	5 (100%) [5 (100%)/18.0 dpi]	5(100%) [5 (100%)/20.0 dpi]	5 (100%) [5 (100%)/20.4 dpi]	1 (20%) [1 (20%)/22 dpi]
The 38-dpi culture supernatant in Passage 5 in Experiment B[d]	5	5 (100%) [5 (100%)/16.4 dpi]	5(100%) [4 (80%)/22.0 dpi]	1 (20%) [0]	NT

[a] The number (percentage) of wells with efficient multiplication of HEV with a load of $>10^4$ copies/ml in the culture supernatant/mean dpi when the HEV RNA titer in culture medium reached $>10^4$ copies/mL is shown in the brackets.
[b] Not tested.
[c] The p13-3 isolate was obtained from this inoculum and its entire genomic sequence was determined (see Fig. 3C and Table 4).
[d] The p5/B isolate was obtained from this inoculum and its entire genomic sequence was determined (see Table 4).

RNA in all five wells for wild-type virus throughout the observation period of 50 days. On the other hand, when HEV at 3.0×10^3 copies per well was inoculated, continuous growth was observed in four (80%) of the five wells for wild-type JE03-1760F. However, efficient multiplication of HEV with a load of $>10^4$ copies/ml in the culture supernatant was observed in only one well (20%) in the wild-type virus. In contrast, when p13-3 HEV was inoculated at 3.0×10^3, 1.0×10^3, or 3.0×10^2 copies per well, respectively, continuous growth was observed in all five wells (100%): it was seen in one (20%) of the five wells with the inoculation of HEV at 1.0×10^2 copies per well. As for p5/B, all five wells each with the inoculation of HEV at 3.0×10^3 or 1.0×10^3 copies per well showed continuous growth, and, upon inoculation of HEV at 3.0×10^2 copies per well, only one well (20%) showed persistent virus appearance. In addition, p13-3 and p5/B showed earlier appearance of progenies with HEV load of $>10^4$ copies/ml in the culture supernatant, compared with the wild-type (Table 3).

3.4. Mutational characteristics in consecutive passages of the JE03-1760F strain

Table 4 compares mutations over the entire genome and the predicted amino acid differences within the three ORFs between the wild-type HEV and its progenies. Four cell culture-produced variants in Experiment A including the p0 isolate obtained from the 28-dpi culture supernatant of the first inoculation of the wild-type HEV, the p5 isolate from the 58-dpi culture supernatant of Passage 5, the p10 isolate from the 24-dpi culture supernatant of Passage 10, and the p13-3 isolate from the 46-dpi culture supernatant of Well 13-3 of Passage 13 as well as one variant (p5/B) obtained from the 38-dpi culture supernatant of Passage 5 in Experiment B, were sequenced over the entire genome. They all had the same genomic length of 7226 nt as the wild-type HEV (the poly(A) tract at the 3′-terminus excluded). Of note, the p0 isolate had only one mutation at nt 22 in the 5′UTR that replaced U for A. The p5 isolate harbored eight additional mutations which were restricted to ORF1 or ORF2, including a mutation at nt 2938 with the mixed nucleotide population of U as well as the C that the wild-type possessed. A C-to-U mutation at nt 3453 in the p5 isolate changed amino acid 1143 in ORF1 from alanine to valine. Compared to the p5 isolate, nine additional mutations were present in ORF1, ORF2, or the ORF2/ORF3 overlapping region of the p10 isolate, which differed by 18 nt from the wild-type over the entire genome. In addition to the mutation at nt 3453 which first appeared in the p5 isolate, four mutations occurred in the p10 isolate at nt 591, 2246, 5378, and 5456 resulting in amino acid changes in ORF1 or ORF3 (Table 4). Upon comparison with the p10 isolate, two additional mutations at nt 3553 and 3620 in ORF1, unaccompanied by amino acid change, were seen in the p13-3 isolate that did not possess the U-to-A mutation at nt 22, probably due to a backward mutation. Of interest, the p5/B isolate in Experiment B differed by 21 nt from the wild-type over the entire genome, including the nucleotide substitutions at nt 2246, 5378, and 5456 that were commonly seen in the p13-3 isolate in another series of passages (Experiment A). In addition to these three nucleotide substitutions, there were five non-synonymous mutations at nt 2913, 2915, 3453, 5312, and 6652 that were not seen in any of the reported HEV isolates of the same genotype (genotype 3) whose entire or nearly entire genomic sequence is known (see Fig. 4 for isolate names and their accession nos.). An Ala to Val change at aa 189 of ORF1 protein and Asn to Asp alteration at aa 562 of ORF2 protein were seen in only two and one isolates of the reported genotype 3 HEV isolates, respectively, while Ala at aa 928 of ORF1 protein that was seen in the p5/B isolate was preserved in all reported isolates of the same genotype.

Table 4
Comparison of the sequences of wild-type JE03-1760F and its cell culture-produced variants (p0, p5, p10, p13-3, and p5/B) over the entire genome

Nucleotide position	Region	Nucleotide						Amino acid	
		wt	p0[b]	p5[b]	p10[b]	p13-3[b]	p5/B[c]	Position	Substitution
22	5'UTR	U	**A**	**A**	**A**	U	U	NA[d]	-
61	ORF1	U	U	U	U	U	**C**	12	-
370	ORF1	C	C	C	**U**	**U**	C	115	-
445	ORF1	U	U	U	U	U	**C**	140	-
591	ORF1	C	C	C	**U**	**U**	C	189	Ala to Val
829	ORF1	C	C	C	C	C	**U**	268	-
1378	ORF1	C	C	C	C	C	**U**	451	-
1549	ORF1	U	U	U	U	U	**C**	508	-
2191	ORF1	C	C	C	C	C	**U**	722	-
2236	ORF1	C	C	C	C	C	**U**	737	-
2246	ORF1	U	U	U	**C**[e,f]	**C**	**C**	741	Trp to Arg
2704	ORF1	U	U	**C**	**C**	**C**	U	893	-
2808	ORF1	U	U	U	U	U	**C**	928	Val to Ala
2913	ORF1	A	A	A	A	A	**G**	963	Glu to Gly
2915	ORF1	G	G	G	G	G	**U**	964	Val to Leu
2938	ORF1	C	C	**Y**[g]	**U**	**U**	C	971	-
3106	ORF1	A	A	**G**	**G**	**G**	A	1027	-
3223	ORF1	U	U	U	U	U	**C**	1066	-
3235	ORF1	C	C	C	**U**	**U**	C	1070	-
3453	ORF1	C	C	**U**	**U**	**U**	C	1143	Ala to Val
3496	ORF1	C	C	C	**U**	**U**	C	1157	-
3553	ORF1	C	C	C	C	**U**	C	1176	-
3620	ORF1	U	U	U	U	**C**	U	1199	-
4015	ORF1	C	C	**U**	**U**	**U**	C	1330	-
4309	ORF1	C	C	C	C	C	**U**	1428	-
4462	ORF1	C	C	**U**	**U**	**U**	C	1479	-
5312	ORF2	U	U	U	U	U	**C**	47	-
	ORF3							51	Ile to Thr
5378	ORF2	A	A	A	**G**	**G**	**G**	69	-
	ORF3							73	Asn to Ser
5456	ORF2	C	C	C	**U**	**U**	**U**	95	-
	ORF3							99	Pro to Leu
6047	ORF2	U	U	U	U	U	**C**	292	-
6470	ORF2	C	C	C	**U**	**U**	C	433	-
6578	ORF2	C	C	**U**	**U**	**U**	C	469	-
6611	ORF2	C	C	C	**U**	**U**	C	480	-
6626	ORF2	U	U	**C**	**C**	**C**	U	485	-
6652	ORF2	U	U	U	U	U	**C**	494	Val to Ala
6855	ORF2	A	A	A	A	A	**G**	562	Asn to Asp
6944	ORF2	U	U	U	U	U	**C**	591	-
7186	3'UTR	C	C	C	C	C	**U**	NA	-

[a] Nucleotide mutations are shaded for visual clarity.
[b] Progenies recovered from culture supernatants of primary propagation and Passages 5, 10, and 13 in Experiment A.
[c] A progeny recovered from culture supernatant of Passage 5 in Experiment B.
[d] NA, not applicable.
[e] Nucleotide mutations that are commonly seen in the two distinct series of passages in Experiments A and B, are shown in bold type.
[f] Nucleotide mutations that are not seen in any of the 27 reported genotype 3 HEV isolates whose entire or nearly entire sequence is known, are underlined.
[g] Y, mixture of U and C.

A phylogenetic tree was constructed based on the full-length nucleotide sequences of the wild-type JE03-1760F and its cell culture-produced variants in PLC/PRF/5 cells (118d/Alex, p0, p5, p10, p13-3, and p5/B) and A549 cells (114d/A549) as well as all reported genotype 3 HEV isolates and one representative isolate each of genotype 1, 2, or 4 whose entire sequence is known. The phylogenetic tree depicted that these eight wild-type and variant HEVs segregated into a cluster within genotype 3 with a bootstrap value of 100% and were closely related to the reported genotype 3 HEV isolates of Japan origin (Fig. 4), suggesting the indigenousness of the JE03-1760F strain among the genotype 3 isolates.

Based on the number of days after the initial inoculation and the mutations that were observed among HEV isolates over the entire genome, the mutation rate of the HEV strain of JE03-1760F during primary culture or consecutive passages of culture supernatant was estimated to be $1.3–3.4 \times 10^{-3}$ nucleotide substitutions per site per year in PLC/PRF/5 cells and 0.4×10^{-3} nucleotide substitutions per site per year in A549 cells (Table 5).

4. Discussion

The present study revealed that the HEV strain JE03-1760F can replicate efficiently in PLC/PRF/5 and A549 cells up to 118 and

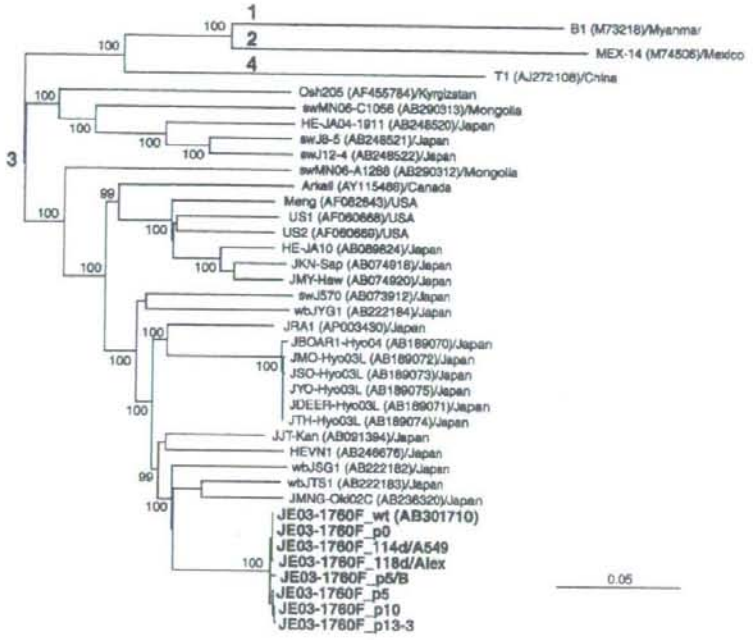

Fig. 4. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 38 HEV isolates. Twenty-seven genotype 3 HEV isolates as well as one each of genotype 1, 2 or 4 HEV isolates for which the entire or nearly entire sequences have been reported were included for comparison, with the accession number in parentheses followed by the name of the country where it was isolated. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. Bar, 0.05 nucleotide substitutions per site.

114 dpi, respectively. Furthermore, the JE03-1760F strain was successively propagated in PLC/PRF/5 cells at least up to thirteen generations of serial passages of culture supernatant with the highest HEV load of 10^8 copies/ml. These results furthered our previous study reporting an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells using the JE03-1760F strain (Tanaka et al., 2007).

Knowledge that has accumulated on cell culture of hepatitis A virus (HAV) (Frosner et al., 1979; Provost and Hilleman, 1979; Binn et al., 1984; Jansen et al., 1988; Day et al., 1992; Emerson et al., 1993; Graff et al., 1994; Brack et al., 1998) would be useful for evaluating and improving the recently established cell culture system for HEV (Tanaka et al., 2007). The JE03-1760F strain does not induce CPE in PLC/PRF/5 and A549 cells. Persistent infection is characteristic of this virus system, like most strains of HAV (Jansen et al., 1988; Graff et al., 1994; Hu et al., 2002). With successive passages, HAV becomes progressively adapted to growth in vitro (Frosner et al., 1979; Provost and Hilleman, 1979; Binn et al., 1984). Adaptation to growth in cell culture results in shortening of the interval between inoculation of cultures and maximum virus yield as well as increases in the yield of virus. To examine whether the JE03-1760F strain can adapt to growth in cell culture during consecutive passages, the infectivity of wild-type JE03-1760F and its cell culture-produced variants (p13-3 and p5/B) in PLC/PRF/5 cells were compared in the present study. When HEV at 3.0×10^3 copies per well (0.2 ml) was inoculated on PLC/PRF/5 cells (approximately 10^6 cells per well), continuous growth was observed in four of five wells for wild-type JE03-1760F and in all five wells for both p13-3 and p5/B. Upon inoculation of HEV at 1.0×10^3 copies per

Table 5
Comparison of cell culture-produced variants with the wild-type HEV JE03-1760F isolate

Feature	PLC/PRF/5 cells						A549 cells
	118d/Alex	p0	p5	p10	p13-3	p5/B	114d/A549
HEV RNA titer (copies/ml)	3.7×10^7	6.4×10^5	2.4×10^6	3.0×10^6	4.7×10^6	2.2×10^7	1.8×10^6
Days after the initial inoculation	118	28	348	540	664	316	114
Nucleotide substitution[a]							
Entire genome (7226 nt)	4	1	9	18	19	21	1
5′UTR (25 nt)	0	1	1	1	0	0	0
ORF1 (5109 nt) [1703 aa]	3 [0][b]	0	6 [1]	11 [3]	13 [3]	13 [4]	1 [0]
ORF2 (1980 nt) [660 aa]	1 [0]	0	2 [0]	6 [0]	6 [0]	7 [2]	0
ORF3 (339 nt) [113 aa]	0	0	0	2 [2]	2 [2]	3 [3]	0
3′UTR (75 nt)	0	0	0	0	0	1	0
Mutation rate ($\times 10^{-3}$ nucleotide substitutions per site per year)	1.71	1.80	1.31	1.68	1.45	3.36	0.44

[a] The cell culture-produced variants were compared with the wild-type HEV JE03-1760F isolate.
[b] Number of nucleotide mutations [number of amino acid substitutions] is shown.

well, the harvested culture supernatants were serially positive for HEV RNA in all five wells for both p13-3 and p5/B, but in none of the five wells tested for wild-type (Table 3). Based on continuous release of progenies into the culture medium, the 50% tissue culture infectivity dose ($TCID_{50}$) per well (0.2 ml) was estimated to be 2.0×10^3 copies for wild-type, but lower at 1.4×10^2 copies for p13-3 and 4.5×10^2 copies for p5/B. Furthermore, earlier appearance and greater increase in the yield of progenies in the culture supernatant were evident in both p13-3 and p5/B. Upon inoculation of HEV at 3.0×10^3 copies per well, efficient multiplication of HEV with a load of $>10^4$ copies/ml in the culture supernatant was observed in all five wells for p13-3 and p5/B, respectively, but in only one of five wells for the wild-type virus. The marked well-to-well variability in virus appearance in culture medium (dpi) and highest virus titers observed in Passages 11–13 with the inoculation of low-titer HEV (Fig. 3), suggests that the cell culture-produced variants have a mixed population forming quasispecies, of which some variants can lead to robust infection and replication, while others have only moderate effects. Repeated experiments confirmed the presence of variants with robust infection and replication in the p13-3 and p5/B populations, suggesting that the wild-type JE03-1760F HEV would have adapted to growth in cell culture during 13 or 5 consecutive passages in our cell culture system.

With regard to HAV, adaptive mutations that permit the virus to replicate efficiently in cell culture have been extensively characterized. Mutations within the internal ribosome entry site enhance cap-independent viral translation in a cell-type-specific fashion, whereas mutations in 2B promote viral RNA replication in all types of cultured cells (Day et al., 1992; Emerson et al., 1993; Yi and Lemon, 2002). In an attempt to define the molecular mechanisms underlying adaptation of HEV to growth in cell culture, we cloned the genomes of seven cell culture-generated variants (118d/Alex, 114d/A549, p0, p5, p10, p13-3, and p5/B) of JE03-1760F strain HEV, determined their complete nucleotide sequences, and compared the sequences of these variants to the sequence of their wild-type parent which was reported previously (Takahashi et al., 2007). Adaptation of HEV to growth in cell culture would require profound changes in the biologic characteristics of the virus. However, only a single nucleotide substitution of U to A at nt 22 in the 5′UTR was found in the p0 genome recovered from the 28-dpi culture supernatant of Passage 0 (a primary cell culture of the wild-type JE03-1760F strain). That the nucleotide change found in the 5′UTR of the genome of the cell culture-produced HEV variant has an effect on ribosome recognition or initiation of translation cannot be ruled out. However, the absence of the U to A mutation at nt 22 in the 118d/Alex genome that was recovered from the 118-dpi culture supernatant of another primary propagation of the wild-type strain in PLC/PRF/5 cells as well as the appearance of a revertant with regard to nt 22 (the p13-3 genome), suggests that this mutation may not play an essential role in adaptation of the JE03-1760 strain to growth in PLC/PRF5 cells. As for evolution of the JE-03-1760F strain in A549 cells, despite its continued growth for 114 days, the recovered viral genome (114d/A549) differed from the wild-type virus in only one nucleotide (nt 1402), the former having two distinct bases of U and a new nucleotide (C), suggesting the emergence of JE03-1760F with C at nt 1402 (C1402) in the presence of U1402. Whether such variants with a point mutation may have replication advantage, and adaptation by inference, would need to be verified by its eventual takeover during additional passages in culture. Similar emergence of a mutant and takeover by the mutant were seen during consecutive passages in the present study, as exemplified by the nucleotide at 2938 (C in p0, U and C in p5, and U in p10).

Although nucleotide substitutions accumulated in the HEV genome during serial passages in culture, even a variant recovered from Passage 13 (p13-3) possessed merely 19 nucleotide substitutions accounting for 0.3% of the entire genome of the parent virus. Hence, adaptation of HEV to growth in vitro was associated with a limited number of mutations. In agreement with this, HAV variants of p16 HM175 (16th in vitro passage level) exhibited 19 mutations (0.3% or 19/7478) in comparison with the wild-type genome (Jansen et al., 1988). Likewise, GBM/HFS displayed 24 mutations (0.3%) after 8 passages in human embryonic kidney cells followed by 23 passages in HFS cells (Graff et al., 1994), and H2K7 P12 displayed seven mutations (0.1%) during 12 passages (Hu et al., 2002). The p13-3 HEV genome had 10 synonymous and 3 non-synonymous substitutions in ORF1 and 6 synonymous substitutions in ORF2 accompanied by 2 non-synonymous substitutions in the overlapping ORF3. Of note, one of the three non-synonymous mutations within ORF1 mapped to the helicase region (aa 1143), and may be associated with heightened replication of p13-3 toward adaptation in culture. Two other non-synonymous mutations were found in ORF3 (aa 73 and 99) of p13-3, and these two mutations were also possessed by p5/B. Coding only for 113 aa, ORF3 underwent adaptive mutations most frequently, and on that basis, may be implicated in heightened infectivity. Of note, among the 11 non-synonymous mutations observed in p5, p10, p13-3 and/or p5/B, eight were not seen in any of the 27 reported HEV isolates of the same genotype, suggesting the uniqueness of the adaptive mutations.

The RNA secondary structure for the HEV genomic region from nt 130 to 250 is shown to be responsible for binding the ORF2 protein (Surjit et al., 2004). It is reported that two putative stem-loop structures comprising nt 7089–7163 and 7173–7194, respectively, at the 3′ end of the HEV genome are important in concert for binding to the RNA-dependent RNA polymerase (Agrawal et al., 2001). Mutations which affect the secondary structures in the 3′-terminal region can influence RNA replication (Graff et al., 2005a). Therefore, synonymous mutations observed in the cell culture-generated variants in the current study may also affect viral replication and protein expression. However, further studies using reporter assays and in vitro mutagenesis techniques are required to clarify whether the observed synonymous mutations have any drastic effects on RNA secondary structure and the formation of pseudoknots. The mutations we identified in p13-3 and p5/B, particularly those that were commonly seen in p13-3 and p5/B at nt 2246, 5378, and 5456, and those that will be found in extended consecutive passages in cell culture would provide further information for understanding the molecular basis of adaptation of HEV to growth in cell culture.

Since HEV infection in humans and animals is usually transient, it is not possible to estimate the mutation rate of HEV by analyzing serial samples collected from an individual host. Based on the assumption that HEV can persist in the community by transmission from host to host successively, the evolutionary rate of HEV has been estimated to be approximately $1.4–1.7 \times 10^{-3}$ nucleotide substitutions per site per year by analyzing the complete genomic sequence of four HEV isolates that formed a phylogenic cluster and that were isolated over a span of 7.5 years (Takahashi et al., 2004). However, this may not represent the true mutation rate of HEV since its serial transmissions through 7.5 years could not be verified. With the advent of persistent infection during long-term primary propagation or consecutive passages of culture supernatant, the mutation rate of HEV was estimated to be $1.3–3.4 \times 10^{-3}$ nucleotide substitutions per site per year in PLC/PRF/5 cells and lower at 0.4×10^{-3} nucleotide substitutions per site per year in A549 cells. The faster and more efficient growth of the HEV strain JE03-1760F during propagation on cell cultures seems to be associated with the appearance of mutations, similar to the HAV strain GBM (Graff et al., 1994).

In conclusion, mutations that occurred during primary propagation of the wild-type JE03-1760F and during serial passages

in culture in PLC/PRF/5 or A549 cells were examined to sort out the mechanism of adaptation. Similar to the adaptation of another enteric hepatitis virus (HAV) during culture, few genetic changes (0.3%) were found in HEV progenies thriving in cell culture. Viral adaptation in cell culture appears to be a multi-step process. Passage of virus in vitro may also be associated with attenuation of virulence as evidenced by a reduction in the ability of HAV to induce liver injury in both experimentally challenged primates and man (**Provost et al., 1982, 1986; Karron et al., 1988**). Extended in vitro passages of the JE03-1760F strain may result in attenuation of the virus, and this may be one approach to the development of an attenuated HEV vaccine. Infectious molecular constructs need to be engineered from the wild-type JE03-1760F and its cell culture-generated variants, for mapping viral functions that would be involved in adaptation and attenuation of HEV.

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Short Communication

Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells

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A full-length infectious cDNA clone (pJE03-1760F/wt) of a genotype 3 hepatitis E virus (HEV) (strain JE03-1760F) obtained from a faecal specimen was constructed in this study. Upon transfection of the capped *in vitro* transcripts of pJE03-1760F/wt into PLC/PRF/5 cells, the viral RNA levels in the culture supernatant started to increase on day 6 post-transfection (p.t.) and reached 10^7 copies ml^{-1} on day 28 p.t. Detection of increasing numbers of cells with ORF2 protein expression by immunofluorescence assay at 5, 7, 11 and 15 days p.t. indicated the spread of HEV infection in cell culture. When the cDNA-derived virus in culture supernatant was inoculated into PLC/PRF/5 or A549 cells, it grew as efficiently as the faeces-derived virus in both cells, reaching 10^6 copies ml^{-1} at 30 days post-inoculation. Our reverse genetics system for HEV that is usable in a robust cell-culture system will be useful for elucidation of the mechanism of HEV replication and functional roles of HEV proteins.

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Hepatitis E virus (HEV) causes acute and fulminant hepatitis E in humans. Genotype 1 and 2 HEVs have caused outbreaks of hepatitis E as water-borne epidemics, and genotype 3 and 4 HEVs were found in sporadic cases of hepatitis E, which were most likely zoonotic in origin (Okamoto, 2007). HEV is classified as the sole member of the genus *Hepevirus* (Emerson *et al.*, 2004a) in the family *Hepeviridae*. HEV is a non-enveloped virus and its genome is a single-stranded, positive-sense RNA, which is capped and polyadenylated (Kabrane-Lazizi *et al.*, 1999; Tam *et al.*, 1991). The genome is approximately 7.2 kb and contains three open reading frames (ORFs), ORF1, ORF2 and ORF3 (Tam *et al.*, 1991). The ORF1 encodes non-structural proteins including RNA helicase and RNA-dependent RNA polymerase (Agrawal *et al.*, 2001; Koonin *et al.*, 1992; Magden *et al.*, 2001). ORF2 and ORF3 proteins are translated from a single subgenomic RNA (Graff *et al.*, 2006; Huang *et al.*, 2007). The ORF2 protein is the viral capsid protein, consisting of 660 aa. The ORF3 protein is a small protein (113–114 aa) that is essential for viral infectivity in animals (Graff *et al.*, 2005; Huang *et al.*, 2007). However, the replication mechanism and protein functions of HEV are not fully understood.

Propagation of HEV *in vitro* has been attempted in various cell lines (Divizia *et al.*, 1999; Huang *et al.*, 1992; Kazachkov *et al.*, 1992; Meng *et al.*, 1997; Wei *et al.*, 2000), but an efficient cell culture system has not been developed. Several research groups have established infectious cDNA clones of HEV, which were observed to replicate in non-human primates or pigs (Emerson *et al.*, 2001, 2004b; Graff *et al.*, 2005; Huang *et al.*, 2005, 2007; Panda *et al.*, 2000). However, efficient HEV propagation in cultured cells has not yet been observed in any of these infectious cDNA clone systems, due to the inability of the virus progeny to spread to other cells in culture (Emerson *et al.*, 2004b). Recently, we developed an efficient cell-culture system for HEV in PLC/PRF/5 and A549 cells using a genotype 3 HEV (strain JE03-1760F) that had been obtained from a faecal specimen from a Japanese hepatitis E patient (Lorenzo *et al.*, 2008; Takahashi *et al.*, 2007; Tanaka *et al.*, 2007).

To develop a full-length infectious cDNA clone of HEV, RNA was extracted from the faecal specimen containing the JE03-1760F strain (Tanaka *et al.*, 2007) using TRIzol LS (Invitrogen) and cDNA was synthesized using SuperScriptII (Invitrogen) with primers ENDr and 4927r (Supplementary Table S1, available in JGV Online). Using the synthesized cDNA as template, three fragments covering the entire JE03-1760F genome were amplified by PCR

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The nucleotide sequences reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB437316 (pJE03-1760F/wt) and AB437319 (pJE03-1760F/ΔORF1).

A supplementary table of primer sequences is available with the online version of this paper.

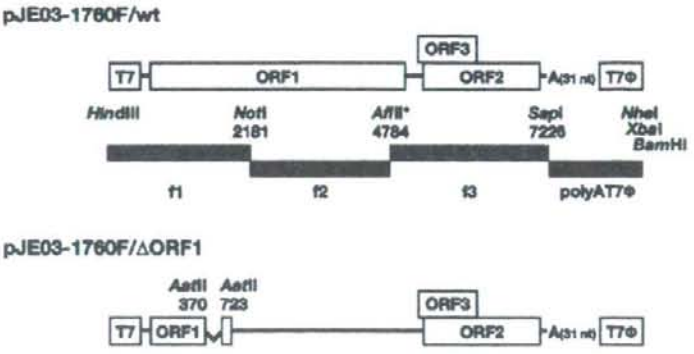

Fig. 1. Schematic diagram of the full-length cDNA clone of HEV strain JE03-1760F (pJE03-1760F/wt) and its ORF1-defective mutant (pJE03-1760F/ΔORF1). The pJE03-1760F/wt was constructed from four cDNA fragments (f1–f3 and polyAT7Φ). *, The *Afl*II genetic marker. The fragment polyAT7Φ contains T7 terminator (Φ) and 31 nt poly(A) sequences. pJE03-1760F/ΔORF1 was made from pJE03-1760F/wt by digestion with *Aat*II followed by blunting and self-ligation.

with KOD plus ver. 2 (Toyobo) (Fig. 1). Fragment 1 (f1), which contains the T7 RNA polymerase promoter sequence upstream of the extreme 5′-end of the JE03-1760F genome, was amplified with primers f1-f and f1-r (Supplementary Table S1). The f2 fragment was amplified with primers f2-f and f2-r, and the f3 fragment with primers f3-f and f3-r; an *Afl*II site was introduced as a genetic marker at the 3′ or 5′ end of the f2 and f3 fragments, respectively, by replacing T with C at nt 4784 and G with T at nt 4786, without amino acid substitution (Fig. 1). Fragment polyAT7Φ, consisting of 31 nt of adenine and a T7 terminator sequence, was amplified by PCR with primers polyAT7Φ-f and polyAT7Φ-r using pTnT vector (Promega) as a template. An A overhang was added to the amplified blunt-ended fragments using A-Addition kit (Qiagen) and these were cloned into pT7 Blue T vector (Novagen). After confirming the sequence of the cloned fragments, the four fragments were ligated stepwise at *Not*I, *Afl*II and *Sap*I sites, and inserted into the *Hin*dIII–*Bam*HI site of the pUC19ΔAatIISapI vector (kindly provided by Dr N. Ito, Gifu University, Japan). The full-length cDNA of the JE03-1760F strain that was constructed was designated pJE03-1760F/wt. In addition, a recombinant plasmid was constructed as a negative control. To achieve this, pJE03-1760F/wt plasmid DNA was digested with *Aat*II, followed by blunting with T4 DNA polymerase (TaKaRa Bio) and self-ligation, resulting in the formation of a frameshift mutant of ORF1, pJE03-1760F/ΔORF1 (Fig. 1).

First, we tried to use the nonlinearized genome plasmid as a template for *in vitro* transcription, but transcription was not completely terminated by the T7 terminator sequence. Therefore, using the *Nhe*I-linearized plasmid as a template, genomic RNA was transcribed with AmpliScribe T7-Flash Transcription kit (Epicentre Biotechnologies). The transcribed RNA was capped using ScriptCap m^7G Capping System (Epicentre Biotechnologies) and purified. An aliquot (3 μg) of the capped RNA was transfected into subconfluent (60–80 % confluent) PLC/PRF/5 cells (ATCC no. CRL-8024), which were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % (v/v) fetal calf serum (FCS), 100 U penicillin G ml^{-1}, 100 μg streptomycin ml^{-1} and 2.5 μg amphotericin B ml^{-1} (Tanaka *et al.*, 2007), in a well of six-well plates (Iwaki) in duplicate, using the TransIT-mRNA Transfection kit (Mirus Bio); the transfected cells were incubated at 37 °C. At 2 days post-transfection (p.t.), the culture medium was replaced with 2 ml growth medium and incubated at 35.5 °C. Then, every other day, half of the culture medium (1 ml) was replaced with fresh maintenance medium consisting of 50 % DMEM and 50 % medium 199 (Invitrogen) containing 2 % FCS, 30 mM $MgCl_2$, 100 U penicillin G ml^{-1}, 100 μg streptomycin ml^{-1} and 2.5 μg amphotericin B ml^{-1}. The collected medium was centrifuged at 800 *g* at 4 °C for 5 min and the supernatant was stored at −80 °C until use.

To monitor virus production in transfected cells (two wells each), HEV RNA levels in the culture supernatants of the transfected cells were serially quantified by real-time RT-PCR using the QuantiTect Probe RT-PCR kit (Qiagen) in a LightCycler apparatus (Roche Diagnostics) as described previously (Takahashi *et al.*, 2008a). The HEV RNA titre decreased to approximately 10^6 copies ml^{-1} in all samples tested at 4 days p.t., due to the medium change on day 2 p.t. (Fig. 2a). Thereafter, a gradual decrease in HEV load was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, whose titre probably reflects residual amounts of the introduced RNA transcripts in the culture medium. On the other hand, in the pJE03-1760F/wt RNA-transfected cells, the viral RNA levels started to increase at 6 days p.t. and reached greater than 10^7 copies ml^{-1} at 28 days p.t. The viral RNA load in the medium was maintained in the order of 10^7 copies ml^{-1} in the pJE03-1760F/wt RNA transfection through to 60 days p.t.

An immunofluorescence assay was performed as described previously (Takahashi *et al.*, 2008b). Briefly, on days 5, 7, 11 and 15 p.t., pJE03-1760F/wt RNA-transfected PLC/PRF/5 cells were fixed with 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100, incubated with a mouse monoclonal antibody (mAb) against the ORF2 protein (H6225) (Takahashi *et al.*, 2008a) and stained with Alexa

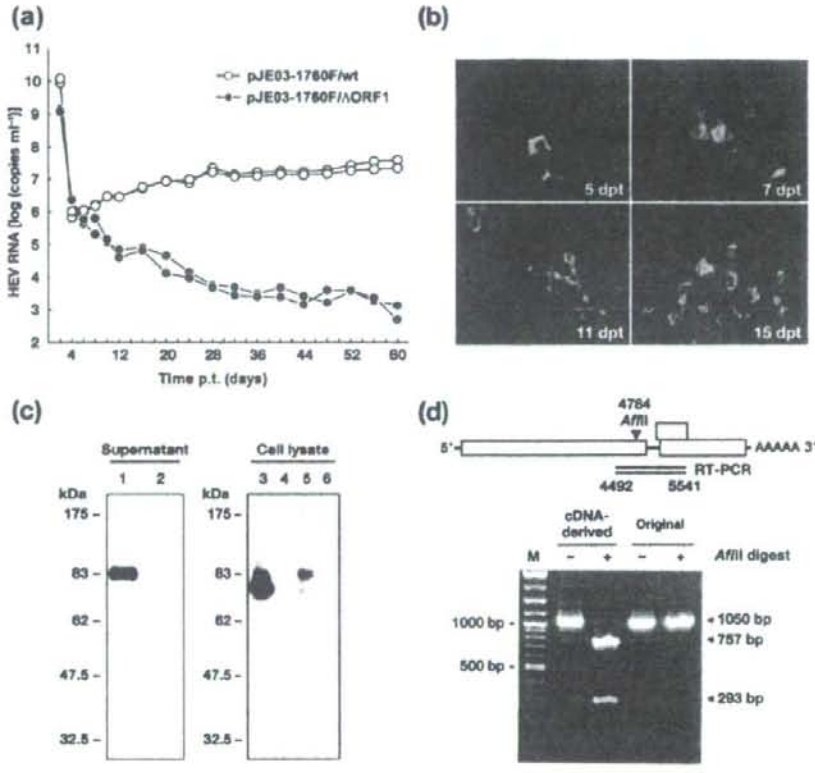

Fig. 2. Evaluation of a reverse genetics system for HEV using the JE03-1760F strain in cell culture. (a) HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells was quantified by measuring the RNA titre in culture supernatants by real-time RT-PCR (two wells for each sample). (b) Immunofluorescent staining of pJE03-1760F/wt RNA-transfected PLC/PRF/5 cells by an anti-ORF2 mAb at 5, 7, 11 and 15 days p.t. (dpt). The images of stained cells were obtained using the IN Cell Analyser 1000 (GE Healthcare). (c) On day 60 p.t., ORF2 protein in culture supernatants or cell lysates of the RNA-transfected PLC/PRF/5 cells was detected by Western blot with an anti-ORF2 mAb. The culture supernatant of pJE03-1760F/wt RNA (lane 1) and pJE03-1760F/ΔORF1 RNA (lane 2) that was directly mixed with an equal volume of 2× sample buffer was loaded at 20 µl per lane. The lysate of cells transfected with pJE03-1760F/wt (lane 5) or pJE03-1760F/ΔORF1 (lane 6) was loaded at 10 µl per lane. The lysates of cells transfected with pCI-HEVORF2 plasmid (lane 3) or mock-transfected (lane 4) were also loaded (10 µl per lane). (d) Amplification of a genomic sequence containing the genetic marker *Afl*II site by RT-PCR of the culture supernatant of the pJE03-1760F/wt RNA-transfected cells (cDNA-derived; 60 days p.t.) or faecal supernatant containing strain JE03-1760F (original). Amplification products were (+) or were not (−) digested with *Afl*II and then separated by electrophoresis. M, Marker.

Fluor 488-conjugated anti-mouse IgG (Invitrogen). ORF2 protein expression was detectable in the transfected cells at 5 days p.t., and increasing levels of ORF2 antigens on days 7, 11 and 15 p.t. suggested the spread of HEV infection in cell culture (Fig. 2b).

For detection of ORF2 protein in the culture supernatant, Western blot analysis was performed using the 60 days p.t. samples. The culture supernatant of HEV RNA-transfected cells was directly mixed with the same amount of 2× SDS-PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4 % SDS, 10 % (w/v) sucrose, 10 % (v/v) 2-mercaptoethanol and 0.01 % (w/v) bromophenol blue]. Proteins in the samples were separated by SDS-PAGE on an 8 % polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane (0.45 µm; Millipore). The membrane was incubated with anti-HEV ORF2 mAb (H6210) conjugated with horseradish peroxidase (HRP) (Takahashi *et al.*, 2008a) and visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo

Fisher Scientific). As shown in Fig. 2(c), a single 83 kDa band was detected in the culture medium of the pJE03-1760F/wt RNA transfection (lane 1) but not in that of the ΔORF1 mutant RNA transfection (lane 2). In addition, cell lysates prepared after DNA or RNA transfection were subjected to detection of ORF2 protein by Western blotting with H6210 mAb and enhanced chemiluminescence HRP-conjugated anti-mouse IgG from sheep (GE Healthcare). For preparation of cell lysates, DNA- or RNA-transfected cells on six-well plates were lysed in 100 µl lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % NP-40 and 150 mM NaCl] and the lysate was mixed with the same amount of 2 × SDS-PAGE sample buffer. The 83 kDa single band was also detected in the lysate of the pJE03-1760F/wt RNA-transfected cells (lane 5) but not in the lysate of ΔORF1 mutant RNA-transfected cells (lane 6) or mock-transfected cells (lane 4) (Fig. 2c). For comparison, an expression plasmid, pCI-HEVORF2, for the ORF2 protein of strain JE03-1760F was constructed in the following way. The full-length ORF2 sequence of the JE03-1760F genome was amplified with primers ORF2-f and ORF2-r (Supplementary Table S1). The amplified DNA was cloned into the *Nhe*I–*Xba*I site of pCI vector (Promega), and the resulting plasmid, pCI-HEVORF2, was transfected into subconfluent PLC/PRF/5 cells in wells of a six-well plate using *Trans*IT-LT1 Transfection reagent (Mirus Bio). The transfected cells were incubated at 37 °C for 3 days and then analysed by Western blotting. Interestingly, the molecular mass of the ORF2 protein expressed by the pCI expression plasmid (Fig. 2c, lane 3) was slightly less than that detected in the pJE03-1760F/wt RNA-transfected cells.

The genomic region containing the *Afl*II site (Fig. 1) was amplified by RT-PCR using primer 5811r for cDNA synthesis and primers 4511f and 5522r for PCR (Supplementary Table S1). In contrast with the original JE03-1760F strain in faeces, the amplified fragment from the culture supernatant of pJE03-1760F/wt RNA transfection was digestable with *Afl*II (Fig. 2d), confirming that the propagated cDNA-derived virus (pJE03-1760F/wt) possessed the introduced genetic marker.

The pJE03-1760F/wt and original JE03-1760F strains were inoculated into PLC/PRF/5 or A549 cells (ATCC no. CCL-185), which were grown in maintenance medium on six-well plates at 1.0×10^5 or 1.0×10^4 copies per well, respectively. Virus inoculation and maintenance of inoculated cells were carried out as described previously (Tanaka *et al.*, 2007). Fig. 3 indicates that the cDNA-derived pJE03-1760F/wt grew as efficiently as the original faeces-derived virus in both PLC/PRF/5 and A549 cells, reaching 10^6 or 10^5 copies ml^{-1} at 30 days post-inoculation, respectively.

In the present study, an infectious cDNA clone of HEV with efficient propagation capability was successfully established using strain JE03-1760F. We previously

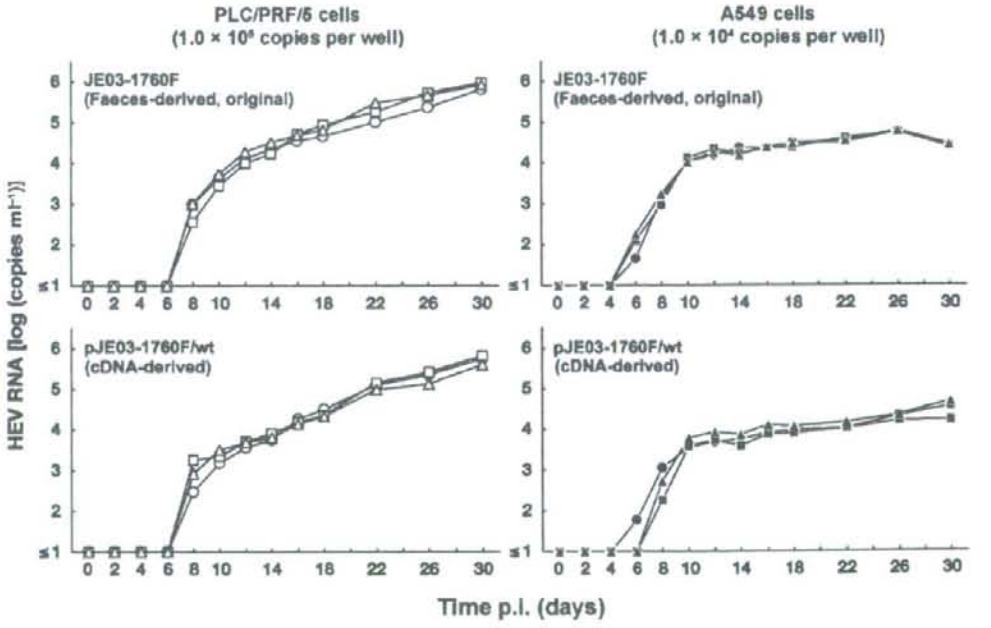

Fig. 3. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells that were inoculated with the indicated viruses (in triplicate) and cultured for up to 30 days.

reported that the HEV RNA titre in the faecal suspension of strain JE03-1760F was markedly high, at 2.0×10^7 copies ml^{-1}, compared with the specimens from 10 other patients studied (less than 5.7×10^4 copies ml^{-1}) (Takahashi *et al.*, 2007), suggesting that strain JE03-1760F has heightened replication activity. Therefore, the use of the JE03-1760F genome in the present study may have been the greatest factor contributing to the successful development of an infectious HEV cDNA clone with robust infection and propagation *in vitro*. The JE03-1760F genome has 29 unique nucleotide substitutions that were not seen in any of the 25 reported HEV isolates of the same genotype (Takahashi *et al.*, 2007). One or some of these substitutions may be responsible for the high replication capability of HEV.

Interestingly, the ORF2 protein was detectable as a single band of 83 kDa following Western blot analysis of the supernatant and lysate of the pJE03-1760F/wt RNA-transfected cells, and the molecular mass was slightly greater than that of the protein transcribed by the pCI-HEVORF2 plasmid vector (Fig. 2c). In the reported transient overexpression systems, both glycosylated and non-glycosylated forms of ORF2 proteins have been detected. Jameel *et al.* (1996) reported that the ORF2 protein was detected in three molecular forms, including a 74 kDa non-glycosylated form and 82 and 88 kDa glycosylated forms. It was reported that the glycosylated form of ORF2 protein gradually shifted to the non-glycosylated form in the cytoplasm through the retro-translocation pathway (Surjit *et al.*, 2007) and that only the non-glycosylated form was stable in the cytoplasm of mammalian cells (Torresi *et al.*, 1999). However, the 83 kDa ORF2 protein detected in the culture supernatants and lysates of pJE03-1760F/wt RNA-transfected cells (Fig. 2c) and cells infected with cell culture-generated HEV (data not shown) is likely to be glycosylated. In support of our speculation that the glycosylated form of ORF2 protein has a role in the HEV replication cycle, Graff *et al.* (2008) recently reported that the formation of infectious virus particles was prevented by mutations within the potential glycosylation sites in the ORF2 protein. In addition, it was shown that the recombinant replicase domain of HEV ORF1 protein is localized on the endoplasmic reticulum membrane (Rehman *et al.*, 2008). HEV particles in the culture supernatant banded at a low buoyant density of 1.15–1.16 g ml^{-1} in sucrose, suggesting that HEV virions released from infected cells associate with lipids (Takahashi *et al.*, 2008b). Therefore, the ORF2 capsid protein existing as a glycoprotein may be advantageous for virion assembly if cellular membranes play an important role in HEV particle maturation.

In conclusion, we established a reverse genetics system for HEV that is utilizable in a robust cell-culture system. This system will be useful for further elucidation of the mechanism of HEV replication and the functional roles of HEV proteins.

Acknowledgements

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Supplementary Table S1. Primer sequences used in this study

Primer name	Sequence (5′–3′)	Underlined sequence*
ENDr	CCAGGGAGCGCGAAAAGCAGAAAAAAG	
4927r	AAGAAAATCACAAACAGCAAGGCGC	
f1-f	AAGCTTAATACGACTCACTATAGCAGACCACGCATGT	*Hin*dIII and T7 promoter sequence
f1-r	ACTGACTGGTGGAGTGGGGTAGGAC	
f2-f	GTCCACATCTGGCTTTTCTAGTGAC	
f2-r	TTTCTAGAACCTTAAGTTTGAGCCCACAG	*Afl*II
f3-f	AAGCTTCAAACTTAAGGTTGATTACCGC	*Afl*II
f3-r	GCTCTTCTTTTTTCCAGGGAGCGCGAAA	*Sap*I
polyAT7Φ-f	GAGTCGACCCGGGGCTCTTCAAAAAAA	*Sap*I
polyAT7Φ-r	GGATCCTCTAGAGCTAGCCAAAAAACCCCTCAA	*Nhe*I, *Xba*I and *Bam*HI
5811r	AGTAGGGGTAGTGGTTGTCTG	
4511f	TATGGAAGAGTGCGGTATGC	
5522r	CAGGTACAGGGGCTGTGTCA	
ORF2-f	GCTAGCCACCATGCGCCCTAGGGCTGTTCTG	*Nhe*I
ORF2-r	ACTCTAGATTAAGACTCCCGGGTTTTACCT	*Xba*I

*The sequences underlined in the primers indicate the given restriction enzyme or T7 promoter sequence.

Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka , T. and Okamoto, H. (2009). Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells. *J Gen Virol* **90**, 457–462.

肝臓　50巻2号　60-64（2009）

＜症例報告＞

インドから帰国直後に重症E型肝炎を発症した日本人妊婦の1例

江林　明志[1]　井上　和明[1]*　高橋　和明[2]　渡邊　綱正[1]
山田　雅哉[1]　安田　宏[1]　三代　俊治[2]　与芝　真彰[1]

要旨：E型肝炎ウイルス（HEV）はアジア・アフリカにおける流行性肝炎の重要な原因で，一般には self-limited な経過を取るが，妊婦では重症化しやすく致死率が高く胎児の合併症も多いとされる．今回我々は本邦で初めて妊娠中の HEV 感染により急性肝炎重症型に陥った邦人妊婦例を経験した．本例はインドに長期滞在して帰国後2週間で発症し，入院時の血清より genotype 1 の HEV RNA（JHN-Kan07R，AB447389）が検出された．抗ウイルス治療開始後速やかに肝機能は回復し，早産であったものの周産期の合併症もなく，母児共にその後は順調な経過をとった．

索引用語：　E型肝炎ウイルス　　妊娠　　急性肝炎重症型　　早産

はじめに

E型肝炎はE型肝炎ウイルス（HEV）により引き起こされる急性肝炎である[1]．HEV は 7.2kb の一本鎖 RNA ウイルスであり，大別して四種の genotype の存在が知られている[2]．Genotype 1（1型）はアジアでの流行株，2型はメキシコでの集団発生株，3型は欧米での散発例から，4型は東アジアでの散発例から見つかってきている．日本に土着する HEV の genotype は3型と4型である．一般には予後良好な疾患であるが高齢者での罹患，genotype 4 と重症化の関係が指摘されている[3]．インドからは妊婦が妊娠第三期に感染すると重症化しやすく，20% が劇症肝炎で死亡するという報告があったが[4,5]，我が国では今まで妊婦の感染例は1例も報告されていない[3]．

今回我々はインドに長期滞在の後にタイ経由で帰国した31才の妊婦において，genotype 1 の HEV の感染による急性感染重症型の1例を経験し，劇症化を予知してメチルプレドニゾロンパルス療法とインターフェロン療法を行ったところ，肝炎が鎮静化して劇症化を回避することができた．その後は早産である点を除いて母児ともに順調な経過をとった．本邦初の妊婦例で且つ検出されたHEVの配列も既報のものとは若干異なっていたので報告する．

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＜受付日2008年7月28日＞＜採択日2008年12月5日＞

症　例

患者：31才　日本人女性．

主訴：全身倦怠感．

家族歴：夫がブータン人であることの外，特記事項なし．

既往歴：特記事項なし　薬物服用歴と飲酒歴はなし．

現病歴：2006年1月から2007年6月までインドに長期滞在して，ブータン人僧侶である夫の仏道修行に同行してインド国内旅行をした．インド国内では宿泊は現地人向けの宿に滞在し，現地人と同じような食事を取った．インドからタイ経由で2007年6月末に帰国した．帰国後7月中旬より軽度の倦怠感と体幹に皮疹が出現した為，初発症状から3日後に近くの病院の皮膚科医を受診したところ蕁麻疹と診断され，抗ヒスタミン剤の内服と強力ミノファーゲンCの注射が行われた．皮膚科受診の4日後に黄疸が出現した為に同病院の内科を受診した．血液検査上 AST 4315 U/L，ALT 1807 U/L，総ビリルビン 12.9 mg/dl と肝機能障害を認めた為に，急性肝炎と診断され近医へ入院となった．入院後は患者が妊婦であることも考慮して，安静と補液のみを行い自然治癒を期待して経過観察のみ行った．しかしその後トランスアミナーゼ，ビリルビンがともに上昇し，PT も 40% まで低下し本人の全身倦怠感も増悪し，Yoshiba らの予知式[6]では劇症化確率が83% であっ

Table 1 Laboratory data on admission.

〈Peripheral blood〉		〈Biochemistry〉		〈Viral marker〉	
WBC	13600 /μl	T-P	5.5 g/dl	IgM-HA Ab	0.0 (−)
RBC	376 × 10⁴ /μl	Alb	2.8 g/dl	HBs Ag	0.1 (−)
Hb	11.8 g/dl	BUN	2.7 mg/dl	HBs Ab	3.9 (−)
Hct	35.0 %	Cre	0.32 mg/dl	HBe Ag	0.4 (−)
Plt	196 × 10³ /μl	T-Bil	14.0 mg/dl	HBe Ab	1.7 (−)
		D-Bil	9.3 mg/dl	HBc Ab	15.9 (−)
〈Coagulation〉		(D/T ratio 0.66)		HBc Ab (×200)	< 5.0 (−)
PT	39.6 %	AST	5335 U/l	IgM-HBc Ab	< 0.1 (−)
APTT	57.5 %	ALT	2195 U/l	HCV Ab	(−)
Fib	277 mg/dl	LDH	440 U/l	HCV-RNA	(−)
HPT	35.1 %	Al-P	617 U/l	HEV-RNA	(+)
		ChE (135-413)	120 U/l	IgG-HEV Ab	(+)
		CRP	1.9 mg/dl	IgM-HEV Ab	(+)
		NH3	72.3 μg/dl		

Z value of predictive formula for Fulminant hepatitis + 1.65

た為に入院4日目に当院へ転院となった.

入院時現症：妊娠21週1日目　身長164 cm　体重60.5 kg　体温37.6℃　血圧100/60 mmHg　脈拍76回/分・整　意識レベル　肝性脳症Ⅰ度　Number connection test (NCT) は65秒 (回復後28秒) 及び4ケタ逆唱が全く出来なかった.

眼瞼結膜に貧血なく眼球結膜に黄染著明. 胸部　心雑音なし, 呼吸音清, 肺野にラ音なし. 腹部　肝臓1横指触知　圧痛なし, 脾臓　触知せず, 下肢　浮腫はなし.

入院時血液検査所見 (**Table 1**)：血液生化学検査でAST 5335 U/L, ALT 2195 U/Lとトランスアミナーゼの高値と総ビリルビン14 mg/dlの上昇と直接ビリルビン/総ビリルビン比の低下 (D/T比0.66) を認めた. 前医における原因検索ではHBs抗原陰性, HBs抗体陰性, HCV抗体陰性, IgM-HA抗体陰性, HIV抗体陰性, 抗核抗体40未満, 抗ミトコンドリア抗体陰性で, HEV RNAは検査中であった. 当院でも再確認を行いHAV, HBV, HCVなどの肝炎ウイルスマーカーと自己抗体は陰性であった.

妊婦であることを考慮してCT volumetryは施行せず, 腹部超音波検査のみ施行したところ, 肝の辺縁はやや鈍で肝の軽度腫大が認められたが脾臓の腫大は認められなかった. 腹部超音波検査による胎児推定重量は340 g.

入院後経過 (**Fig. 1**)：転院時に与芝の予知式による劇症化確率が83%であり, 劇症化の切迫が示唆されたため, 肝炎を早期に鎮静化させる必要があると考えた. 転院日に前医より, 前医に入院したときの血清からHEV RNAが検出されたとの報告があった. インドでは妊婦で高率に劇症化して死亡例も報告されていることより, 肝炎を早期に鎮静化させる為にメチルプレドニゾロンのパルスとその漸減療法とHEVがRNAウイルスであることよりインターフェロンに感受性があると想定してIFN-β静注を開始した. 治療開始後より血清トランスアミナーゼ及びビリルビンの値は急速に改善し, 第三病日にはNCTは50秒まで改善し, 4桁逆唱も可能となった. この時点でほぼ意識は清明になったと判断し, 食欲も出てきたので食事を開始し, アンモニアの値に注意しながら徐々に蛋白摂取量を上げていった. 肝機能が順調に回復したためにメチルプレドニゾロンを9日間で投与を終了し(総量5600 mg), またインターフェロンはインターフェロンβ3MUを14日間連日投与した(総量42MU). HEV RNAは第19病日に陰性となった. 肝機能は約一カ月で完全に正常化し第28病日に自宅安静を条件に退院とした. 分娩は妊娠第37週と早産であったが合併症もなく, その後も母児共に健康である.

HEV遺伝子解析：患者血清由来HEV株：ORF1のreplicaseをコードする821 ntの塩基配列(JHN-Kan 07R, DDBJ/EMBL/GenBank accession number AB 447389)を既報配列と比較したところ, 本例由来HEV株は, genotype 1型に属してはいたが, HEVの流行地域から過去に報告されたHEVの配列とは多少異なっていた. Phylogenetic tree解析の結果をFig. 2に示す.

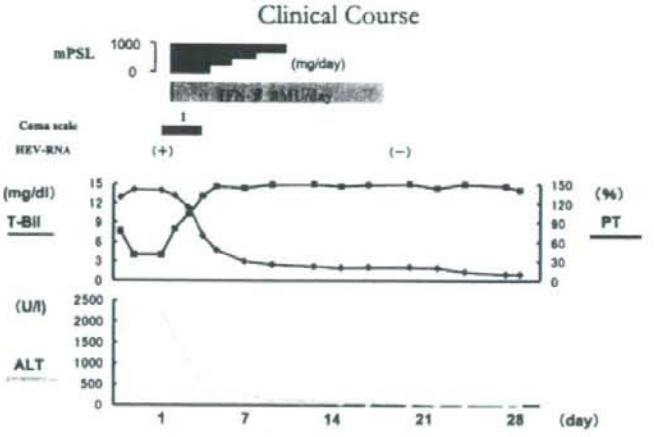

Fig. 1 Clinical course of the patient.

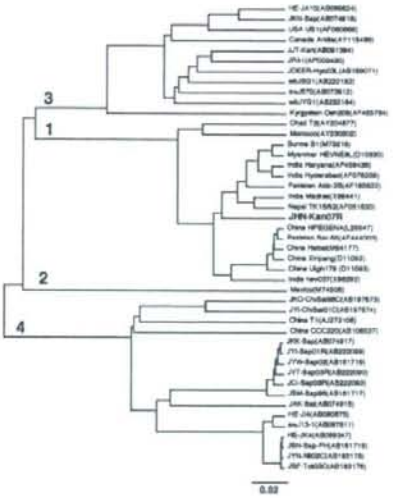

Fig. 2 Phylogenetic tree of HEV, based on 821 nt within ORF1. JHN-Kan07R, bold typed, is the isolate from the patient in this study.

考　　案

妊娠が急性肝炎の予後に影響を与えるか否かについての議論は未だに決着がついていない．先進国からの報告では栄養が十分であれば妊娠そのものは肝炎の経過に影響を及ぼさないとされているが[7]，一方発展途上国のうちでも肝炎の流行地域からの報告では妊娠中，とりわけ妊娠第三期の肝炎は重症化して死亡率が高いことが報告されている[8]．インドやバングラデシュからの報告では妊娠中の重症肝炎の原因として HEV が問題とされている[9][10]．HEV はこれらの流行地では一般に水系感染で伝播し，男性や非妊娠女性例では self-limited な経過をとり死亡率は 0.1% 以下である[11]．一方日本のようにsporadic caseが中心の地域では動物がリザーバーとして注目されている[12][13]．今回我々は HEV による劇症化が予知される急性肝炎重症型の妊婦を本邦で初めて経験した．

本症例は肝炎の発症前に約一年半インドに滞在し，帰国後2週目に肝炎を発症しており一般的な潜伏期が6〜8週であることから[14]，本症例は輸入感染症と考えられた．国内で発見された E 型肝炎や劇症化した症例が殆ど genotype 3，4 であるのに対し[3]，異なったウイルスの遺伝子型（genotype 1 HEV を検出）である事も本例が輸入感染症であることと支持し，genotype1 に感染した妊婦の重症化もこれまでの報告と一致する．また，本例は妊娠第二期の感染であり，これまでの報告からも高率の子宮内胎児死亡が報告されているが，本例では胎児の発育は順調であった．ただし第 37 週の分娩であり早産に分類される．この経過は HEV 感染妊婦の大半が早産であるというこれまでの報告に一致した[15]．

今回の患者は元々健康体で栄養状態も良好であり，

低栄養状態により重症化したとは考えにくい．実際，妊婦における重症化のメカニズムは，細胞での増殖系や小動物での実験系がないために未だに推察の域を出ないが，Th_1型サイトカインとTh_2サイトカインのimbalanceや[16]*in vitro*での発現系の実験からORF3が宿主側のシグナル伝達に影響を与えてウイルス増殖の促進に関与している可能性が示唆されている[17]．本例では肝細胞破壊を止めるためにメチルプレドニゾロンパルス療法を開始したところ，急速にトランスアミラーゼが改善した．本邦の他施設からもE型劇症肝炎にメチルプレドニゾロンパルス療法を行ったところトランスアミナーゼが急速に低下したとの報告があり[18)~20)]，E型肝炎ウイルスの劇症化のメカニズムはウイルスそのもののCytopathicな効果よりも，宿主の免疫応答による肝細胞破壊によると考えられる．

インターフェロンの使用に関しては今後とも議論が必要であろうが，厚労省の班会議の報告ではE型劇症肝炎の死亡例は3例全例亜急性型の経過をとり[21)]，ウイルス増殖の持続が肝細胞破壊の進行に関与している可能性が示唆される．本例は発症から脳症1度まで12日であることより，もし劇症化すれば亜急性型に分類され予後不良例と推定されるがメチルプレドニゾロンのパルス療法とインターフェロンを併用したところ，トランスアミナーゼの急速な改善とともにウイルス血症も急速に改善したので，本例では劇症化の予知と早期治療が有効であったと考えられる．インターフェロン投与は今後重症化例では治療上の一手段として検討すべきであろう．

副腎皮質ホルモンとインターフェロンの有害事象については，妊娠第二期以降の女性と胎児において重篤例の報告は無い[22)~24)]．

E型肝炎流行地域における妊婦のE型劇症肝炎の死亡率の高さは，日本のような人工肝補助療法がないことと，肝移植ができないことが原因と考えられる．

現在南アジアには日本人居住者が約10万人存在するとされる．このような地域から帰国した妊婦に原因不明の肝障害を認めた場合には，原因検索の一つにHEV抗体，HEV-RNAの検索を含めるべきであろう．本例では皮疹が病初期の特徴的身体所見の一つであったが，これまでの報告で特異性の高い身体所見はない[25)]．肝障害の原因がHEVと判明した場合には，周産期の合併症が多いので産婦人科医と協力して治療に当たることが望ましいと考えられる．

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A case of Japanese pregnant woman who developed severe acute hepatitis E right after back from India

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Hepatitis E virus (HEV) causes large-scale outbreaks in endemic areas and sporadic infections in developed countries: the latter includes "imported infection" by travelers back from endemic areas. In men and non-pregnant women, HEV infection is usually self-limited. It is reported, however, that acute hepatitis E in pregnant women is more severe, often resulting in fulminant hepatic failure. We experienced the first case of the pregnancy-associated acute hepatitis E in Japan: our patient was at 21st week of pregnancy and had stayed in India until 2 weeks before developing hepatitis. The HEV isolate recovered from her serum (JHN-Kan07R, AB447389) segregated to genotype 1, prevalent in India. Result of a predictive formula for development of fulminant hepatitis was positive on admission. Therefore we started daily administration of interferon and steroid pulse treatment, and the patient recovered rapidly. She was delivered of a baby without serious perinatal complications in spite of preterm delivery that is one of the reported obstetric complications of HEV infection.

Key words: hepatitis E virus　pregnancy　severe acute hepatitis　preterm delivery

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