Development and evaluation of an efficient cell-culture system for *Hepatitis E virus*

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Using a faecal suspension with high load of *Hepatitis E virus* (HEV) (2.0×10^7 copies ml^{-1}, genotype 3), we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5). HEV progeny released in the culture medium were passaged five times successively in PLC/PRF/5 cells. The initial day of appearance and load of HEV detectable in the culture supernatant after inoculation were dependent on the titre of seed virus in the inoculum. When 6.4×10^4 copies of HEV were inoculated on monolayers of PLC/PRF/5 cells in six-well microplates, HEV RNA was first detected in the culture medium on day 14 post-inoculation and increased to 9.1×10^5 copies ml^{-1} on day 60. When 8.6×10^5 copies of HEV were inoculated, HEV RNA was initially detected on day 12 and reached the highest titre of 8.6×10^7 copies ml^{-1} on day 60. HEV incubated at temperatures higher than 70 °C did not grow in PLC/PRF/5 cells, while HEV incubated at 56 °C for 30 min was infectious. Convalescent serum samples with IgM-class HEV antibodies obtained from patients infected with HEV of genotype 1, 3 or 4 neutralized the genotype 3 virus, indicating that HEV antibodies are broadly cross-reactive. Serum samples obtained from patients 8.7 or 24.0 years after the onset of HEV infection also prevented the propagation of HEV in PLC/PRF/5 cells, suggesting the presence of long-lasting HEV antibodies with neutralizing activity in individuals with past HEV infection.

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INTRODUCTION

Hepatitis E virus (HEV) was discovered in 1983 by immune electron microscopy (Balayan *et al.*, 1983) and was first cloned in 1990 (Reyes *et al.*, 1990). It is the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.*, 2004). Transmission of HEV occurs primarily by the faecal–oral route via contaminated water supplies in developing countries where sanitation is suboptimal (Purcell & Emerson, 2001; Smith, 2001). Accumulating lines of evidence have indicated that hepatitis E is a zoonosis (Harrison, 1999; Hsieh *et al.*, 1999; Meng, 2003; Meng *et al.*, 1997b, 1998, 2002; Okamoto *et al.*, 2001; Nishizawa *et al.*, 2003, 2005; Tei *et al.*, 2003; Yazaki *et al.*, 2003; Sonoda *et al.*, 2004). In addition, recent studies have indicated that zoonotic food-borne transmission of HEV from domestic pigs, wild boar or wild deer to humans may occur as autochthonous infection in Japan (Li *et al.*, 2005; Matsuda *et al.*, 2003; Tamada *et al.*, 2004; Tei *et al.*, 2003; Yazaki *et al.*, 2003).

The HEV virion is 27–34 nm in diameter and does not possess an envelope. The genome of HEV is a single stranded, positive-sense RNA of approximately 7.2 kb (Tam *et al.*, 1991). Based on the genomic variability noted among HEV isolates, HEV sequences have been classified into four genotypes: genotype 1 consists of epidemic strains in developing countries in Asia and Africa; genotype 2 has been described in Mexico and Africa; genotype 3 is widely distributed in the world and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in the USA, European countries and Japan; and genotype 4 contains strains from humans and/or domestic pigs in Asian countries including China, Taiwan and Japan (Schlauder & Mushahwar, 2001; Lu *et al.*, 2006; Inoue *et al.*, 2006).

Propagation and production of HEV *in vitro* have been attempted in various continuous cell lines (Huang *et al.*, 1992, 1995, 1999; Kazachkov *et al.*, 1992; Li *et al.*, 1996; Meng *et al.*, 1996, 1997a; Wei *et al.*, 2000) and in primary hepatocytes from non-human primates (Arankalle *et al.*, 1988; Kane *et al.*, 1984; Tam *et al.*, 1996a, b, 1997; Tsarev *et al.*, 1994). However, the lack of an efficient cell-culture system for HEV has greatly hampered detailed analysis of the virus replication cycle in infected cells to resolve many important questions. Recently, Emerson *et al.* (2005, 2006) reported a quantitative cell-culture assay for neutralization tests and thermal stability tests by detecting infected culture cells by immunofluorescence microscopy. However, high-titre HEV was not detected in the culture supernatant. Using

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a faecal suspension with high HEV load (2.0×10^7 copies ml^{-1}) as an inoculum, obtained from a Japanese patient who contracted domestic infection of genotype 3 HEV, we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5), which yielded the highest HEV load of up to 10^8 copies ml^{-1} in the culture supernatant and succeeded in propagating five generations of serial passages of culture supernatant. In addition, as preliminary applications of this culture system for HEV, we examined the thermal stability of HEV and the neutralizing activity of serum samples containing IgG-class and/or IgM-class HEV antibodies obtained from patients with clinical or subclinical HEV infection.

METHODS

Virus. We used an HEV strain (JE03-1760F) in faecal specimens obtained at the acute phase from a 67-year-old Japanese patient with chronic renal failure who contracted domestic infection of genotype 3 HEV in 2003, just before starting maintenance haemodialysis. The faecal suspension in Tris/HCl buffer (10 mM, pH 7.5) was centrifuged at 6200 *g* at 4 °C for 10 min and the resulting clear supernatant was aliquoted as virus stocks and stored at −80 °C. The HEV RNA titre of the virus stock was estimated to be 2.0×10^7 copies ml^{-1} by the method described below. Prior to inoculation of the faecal suspension, the virus stock was subjected to purification by passage through microfilters with a pore size of 0.45 and 0.22 μm (Millex-GV; Millipore).

Cell-culture. A total of 21 established cell lines including the PLC/PRF/5 (CRL-8024), A549 (CCL-185), HepG2 (HB-8065), HuH7 (RCB1366), IEC-6 (CRL-1592), NUGC-4 (JCRB0834), MDCK (CCL-34), MDBK (CCL-22), P19 (CRL-1825), LLC-MK2 (CCL-7), BC3H1 (CRL-1443), C2C12 (CRL-1772), HEK293 (CRL-1573), L929 (RCB1422), HT-1080 (JCRB9113), SK-N-MC (HTB-10), GOTO (JCRB0612), C6 (CCL-107), CV1 (CRL-10478), HeLa (CCL-13) and MCF (JCRB0314) cells, which were available from the ATCC or RIKEN Cell Bank (RIKEN BioResource Center), were used in the present study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS; PAA Lab GmbH), 100 U penicillin G ml^{-1}, 100 μg streptomycin $ml^{-1}$and 2.5 μg amphotericin B ml^{-1}, at 37 °C in a humidified 5 % CO_2 atmosphere. For virus infection, confluent cells were trypsinized and diluted 1 : 4 in medium and 2.0 ml was added to wells (diameter of 3.5 cm) of a six-well microplate (IWAKI) 1 or 2 days before virus infection.

Virus inoculation and passage. Monolayers of cultured cells in a six-well microplate were washed three times with 1 ml PBS(-), containing 0.2 % (w/v) BSA (Sigma-Aldrich), and 0.2 ml of the filtered virus stock that had been diluted with PBS(-) was inoculated on the cells. In serial passages, 0.2 ml culture supernatant that had been filtered through a microfilter with a 0.22 μm pore size was inoculated on a monolayer of PLC/PRF/5 cells. One hour after inoculation at room temperature, the solution was removed and 2 ml maintenance medium was added. The maintenance medium used for virus culturing consisted of 50 % DMEM and 50 % medium 199 (Invitrogen) containing 2 % (v/v) heat-inactivated FCS and 30 mM $MgCl_2$ at final concentration; other supplements were the same as in the growth medium. Culturing was performed at 35.5 or 37.0 °C in a humidified 5 % CO_2 atmosphere. On the day after inoculation, the cells were washed five times with 1 ml PBS(-) and 2 ml maintenance medium was added. Then, starting on day 2 after inoculation and then every other day, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium and the medium collected was stored at −80 °C until virus titrations were performed. In this study, duplicate, triplicate or quadruplicate sets of inoculum were inoculated in parallel on cells cultivated in a six-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells and representative data were adopted.

Detection and quantification of HEV RNA. For detection of HEV RNA in serum samples, faecal supernatants and culture media, nested RT-PCR with primers targeting the ORF2 region of HEV RNA was performed as described previously (Mizuo *et al.*, 2002). The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far (Mizuo *et al.*, 2002; Takahashi *et al.*, 2003; Yazaki *et al.*, 2003). Quantification of HEV RNA was performed by real-time detection RT-PCR according to the method described previously (Jothikumar *et al.*, 2006), with a slight modification. In brief, total RNA was extracted from 10–100 μl serum sample, faecal supernatant or culture medium with TRIZOL-LS reagent (Invitrogen) and subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR kit (Qiagen) using sense primer (5′-GGTGGTTTCTGGGGTGCA-3′), antisense primer (5′-AGGGGTTGGTTGGATGAA-3′), and a probe consisting of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3′) on an ABI Prism 7700 Sequence Detector (Applied Biosystems). Thermal cycler conditions were 50 °C for 30 min at stage 1; 95 °C for 15 min at stage 2; and 50 cycles of 94 °C for 15 s, 56 °C for 30 s and 76 °C for 30 s at stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate and the mean value was adopted.

Western blot analysis. For detection of HEV capsid proteins, 10 μl culture medium or diluted faecal supernatant as a control was mixed with an equal volume of 2 × gel-loading buffer with SDS and β-mercaptoethanol and a total of 15 μl solution was subjected to SDS-PAGE in a 5–15 % gradient gel (Bio-Rad), followed by transfer onto a nitrocellulose filter membrane (Hybond-ECL; Amersham Biosciences). The membrane was immersed in Tris-buffered saline (TBST) [10 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.1 % (v/v) Tween-20] containing 5 % ECL blocking agent (Amersham Biosciences) and, after washing with TBST, incubated at room temperature for 1 h with 10 μg anti-HEV ORF2 mouse monoclonal antibody ml^{-1} (IgG1 subclass: Hyb-H6210) that had been raised against the recombinant HEV ORF2 protein expressed in the pupae of silkworm (Mizuo *et al.*, 2002), as the primary antibody. After washing, the membrane was incubated with ECL anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody from sheep (1 : 2500; Amersham Biosciences) and examined using a chemical luminescence system (ECL Western blotting detection reagents and analysis system; Amersham Biosciences). ECL DualVue Western Blotting Markers (Amersham Biosciences) were used as protein markers.

Study of the thermal stability of HEV. One hundred microlitres of virus stock was dispensed to each of five MicroAmp reaction tubes (0.5 ml; Applied Biosystems). The tubes were incubated at 56 °C for 30 min, 70 °C for 10 min, 95 °C for 1 min or 95 °C for 10 min in a GeneAmp PCR System 2400 (Perkin Elmer). As control, a tube containing 100 μl virus stock was kept at room temperature (25 °C) for 30 min. The heat-treated virus and the control were diluted with PBS(-) to 6.0×10^4 copies in 0.2 ml solution and inoculated on a monolayer of PLC/PRF/5 cells in a six-well microplate. The protocol after infection and maintenance of cultured cells were as described in the virus inoculation and passage section.

Neutralization test. Serum samples (nos 1–3) that were positive for IgG, IgM and IgA classes of anti-HEV antibodies by in-house

Table 1. Serum samples used in the neutralization test

Serum sample no.	Days (years) after onset*	Relative titre of anti-HEV†			HEV RNA	Genotype of HEV recovered from patient
		IgG-class	IgM-class	IgA-class		
1	13–23‡	1:5200	1:2400	1:790	–	3
2	37–54‡	1:7100	1:610	1:590	–	1
3	24–36‡	1:98000	1:3500	1:2000	–	4
4	1113 (3.0)	1:7800	–§	–	–	4
5	3186 (8.7)	1:1500	–	–	–	3
6	8764 (24.0)	1:740	–	–	–	3
7	Unknown	1:600	–	–	–	Unknown

*Day on which the serum sample(s) was obtained after disease onset.
†Relative titre of anti−HEV was determined as described in Methods.
‡Pooled serum obtained on the indicated days after the disease onset from three patients with hepatitis E during the convalescent phase.
§−, Negative (<1:100) for the indicated class of anti−HEV.

ELISA (Takahashi *et al.*, 2005) were obtained from three patients with sporadic acute hepatitis E during the convalescent phase (Table 1). Three other serum samples (nos 4–6) containing only the IgG-class of anti-HEV antibody were obtained from patients with HEV infection 3.0, 8.7 or 24.0 years after disease onset (Kuno *et al.*, 2003; Tokita *et al.*, 2003; Mitsui *et al.*, 2004). One additional serum sample (no. 7) was obtained from an individual with detectable anti-HEV IgG. The relative titres of anti-HEV IgG, IgM or IgA antibodies were determined by end–point ELISA according to the methods described previously (Tokita *et al.*, 2003; Takahashi *et al.*, 2005); i.e. the serum dilution that gave the absorbance (measured at the wavelength of 450 nm) of each cut-off point was estimated by testing multiple dilutions of the serum. All serum samples used were negative for HEV RNA.

The serum samples were purified by passage through a microfilter with 0.45 μm pore size and then a microfilter with 0.22 μm pore size, prior to use in the neutralization test. A solution of 0.2 ml containing the same amount of virus stock (6.0×10^4 copies) and each of various serum samples diluted with PBS(-) at 1:5, 1:50, 1:500 or 1:5000, was incubated at room temperature for 1 h and then inoculated on monolayers of PLC/PRF/5 cells in a six-well microplate. After 1 h, the supernatant was removed and 2 ml maintenance medium was added. The protocol after infection and maintenance of cell-culture was as described above.

RESULTS

Inoculation of HEV on various cultured cells

Faecal supernatant containing an HEV strain (JE03-1760F) was inoculated on each of the 21 selected cell lines and cultured at 35.5 °C or 37.0 °C. HEV could propagate in only PLC/PRF/5 and A549 cells among the 21 cell lines (Fig. 1). HEV RNA was first detected in the culture medium on day 12 or 14 after inoculation and continued to be detectable up to the end of the observation period of 38 days in the culture media of both PLC/PRF/5 and A549 cells. When HEV was inoculated on PLC/PRF/5 cells and maintained at 35.5 °C, the HEV viral load was the highest throughout the observation period. Based on these results, culture of HEV was carried out using the PLC/PRF/5 cell line at a temperature of 35.5 °C thereafter.

Serial passages of HEV in PLC/PRF/5 cells

As the first step in serial passages of HEV (passage 0), a faecal supernatant containing an HEV strain (JE03-1760F) was inoculated on PLC/PRF/5 cells (Table 2). HEV RNA became detectable in the culture supernatant on day 10 after inoculation at 1.1×10^4 copies ml^{-1} and its load increased to 6.4×10^5 copies ml^{-1} on day 28 (Fig. 2). The first passage (passage 1) on a fresh monolayer of PLC/PRF/5 cells was carried out using 28 days culture medium that was filtered through a 0.22 μm microfilter. Contrary to our expectation, HEV RNA was not detectable in the harvested culture medium 2 weeks later, but was first detected on day 36 after inoculation and reached a maximum load of 8.6×10^6 copies ml^{-1} on day 56. For the second passage, the 56 days culture medium of passage 1 was inoculated on a fresh monolayer of PLC/PRF/5 cells. HEV RNA was first detected in the culture

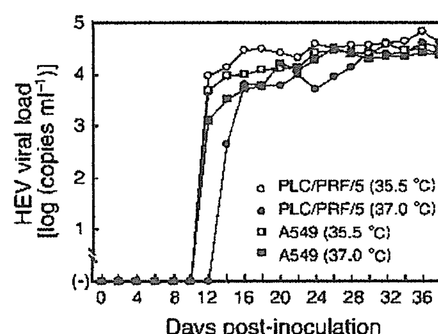

Fig. 1. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells maintained at 35.5 or 37.0 °C that were inoculated with a faecal supernatant containing an HEV (JE03-1760F) strain and cultured for the indicated number of days.

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

Passage	Inoculum source	HEV RNA titre (copies ml^{-1})	Viral load of HEV inoculated in each well (copies per well)*
0	Faecal supernatant (JE03-1760F)	2.0×10^7	6.0×10^4
1	Culture supernatant (day 28 after the first inoculation)	6.4×10^5	3.6×10^4
2	Culture supernatant (day 56 after the second inoculation)	8.6×10^6	9.8×10^4
3	Culture supernatant (day 88 after the third inoculation)	5.8×10^6	7.0×10^4
4	Culture supernatant (day 64 after the fourth inoculation)	1.1×10^6	1.2×10^5
5	Culture supernatant (day 54 after the fifth inoculation)	7.6×10^5	1.1×10^5

*Quantification of HEV RNA was performed after filtration of the faecal supernatant or culture supernatant through a 0.22 μm microfilter.

medium on day 16 and increased to 5.8×10^6 copies ml^{-1} on day 88. In the third, fourth and fifth passages, progeny were first detectable in the culture medium on day 12 or 14 after inoculation and grew in a similar manner as those in passage 0 and passage 2. The HEV RNA titre in the culture medium increased to 2.4×10^6 copies ml^{-1} on day 58 of the fifth passage. During these serial passages, no cytopathic effect (CPE) was observed in the PLC/PRF/5 cells, which continued to produce HEV progeny for at least 88 days as observed in passage 2.

Inoculation of HEV at different viral loads

The HEV RNA titre in the inoculum after filtration that was used for passage 1 was, at 3.6×10^4 copies per well, the lowest of the five passages (Table 2), suggesting that the day of the initial appearance of HEV in the culture medium may be affected by the HEV RNA titre in the inoculum. To elucidate the reason why HEV appeared 2 weeks later in the culture medium of passage 1 than in those of the other four passages, different amounts of HEV (2.4×10^4, 3.2×10^4, 6.4×10^4, 1.6×10^5 or 8.6×10^5 copies per well) were inoculated on fresh monolayers of PLC/PRF/5 cells and the HEV viral load was measured in the culture media until day 60 after inoculation (Fig. 3). When HEV was inoculated at 2.4×10^4 or 3.2×10^4 copies per well, HEV RNA was first detected in the collected culture media on day 22 or 24 after inoculation. On the contrary, when HEV of 6.4×10^4 or more copies per well was inoculated, HEV initially appeared in the culture medium on day 12 or 14 after inoculation. The greater the amount of HEV inoculated, the more rapidly HEV in the culture medium increased. On day 60 after inoculation of HEV of 8.6×10^5 copies per well, the amount of HEV RNA in the culture medium was the highest at 8.6×10^7 copies ml^{-1}. Based on these results, the viral load of HEV to be inoculated on PLC/PRF/5 cells for efficient propagation of HEV was considered to be 6.0×10^4 or higher copies per well in our culture system.

Western blot analysis with an ORF2 protein-specific mouse monoclonal antibody

Western blots of the day 0 and day 12 culture media did not show any bands, but those of the day 18, 36, 50 and 68 culture media with HEV viral loads of 3.8×10^4 to 1.5×10^8 copies ml^{-1} showed two bands of 65 kDa (major) and 74 kDa (minor) (lanes 3–6 in Fig. 4), the difference being most likely due to the absence/presence of glycosylation (Li *et al.*, 1997). Their migration on SDS-PAGE was indistinguishable from that of faecal supernatant used as an inoculum in the present study (lanes 7 and 8 in Fig. 4).

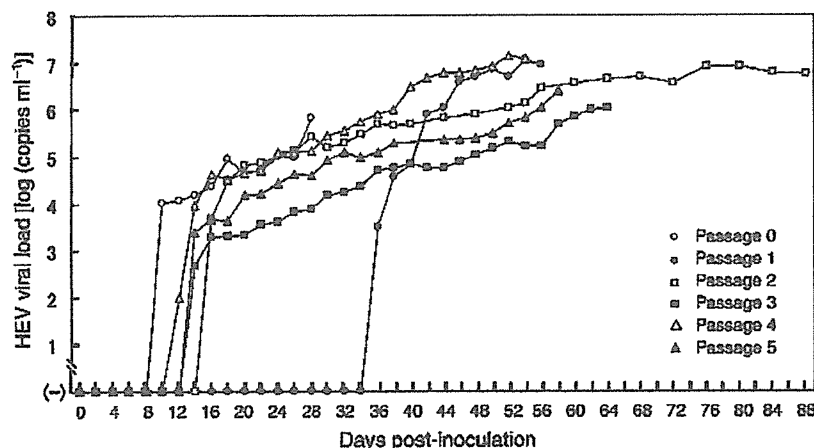

Fig. 2. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with faecal supernatant (passage 0) or culture supernatant of passage 0, 1, 2, 3 or 4 that was harvested on the final day of each passage (see Table 2). The culture supernatant of each passage was purified by passing through a microfilter of 0.22 μm pore size (see Methods) and inoculated on fresh PLC/PRF/5 cells.

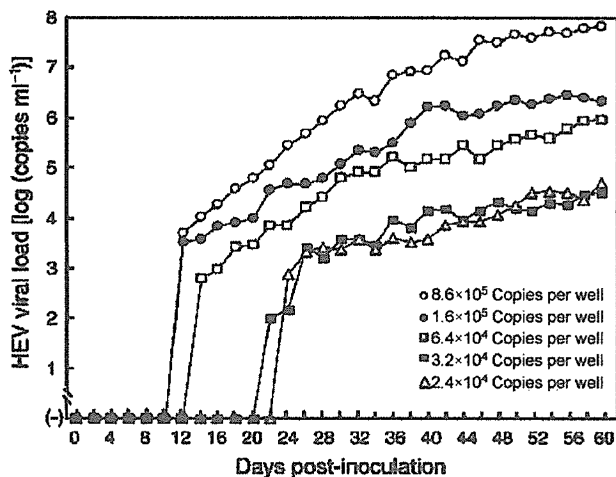

Fig. 3. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 cells after inoculation with diluted faecal supernatant containing various HEV loads.

Thermal stability of HEV

The same amounts of HEV inoculum were incubated at 95 °C for 10 min, 95 °C for 1 min, 70 °C for 10 min, 56 °C for 30 min or room temperature (25 °C) for 30 min. They were diluted to 6.0×10^4 copies per well and inoculated on PLC/PRF/5 cells. When the HEV inoculum was incubated at 95 °C for 10 min, 95 °C for 1 min or 70 °C for 10 min prior to inoculation on PLC/PRF/5 cells, HEV RNA was not detectable in the culture medium throughout the observation period of 50 days after inoculation (Table 3). However, when the HEV inoculum was incubated at 56 °C for 30 min or room temperature (25 °C) for 30 min prior to inoculation, HEV RNA was first detected in the culture medium on day 20 or day 16, respectively.

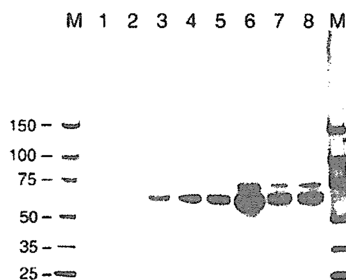

Fig. 4. Western blot analysis of HEV capsid proteins separated on a 5–15 % polyacrylamide gel using an anti-HEV ORF2 mouse monoclonal antibody. M denotes molecular masses of protein markers that are indicated in kDa. Culture medium was applied onto lanes 1–6: lane 1, day 0 (HEV RNA: undetectable); lane 2, day 12 (4.6×10^3 copies ml^{-1}); lane 3, day 18 (3.8×10^4 copies ml^{-1}); lane 4, day 36 (6.1×10^6 copies ml^{-1}); lane 5, day 50 (5.5×10^7 copies ml^{-1}); and lane 6, day 68 (1.5×10^8 copies ml^{-1}). Diluted faecal supernatants were applied onto lane 7 [2.0×10^4 copies ml^{-1} (1 : 1000 dilution)] and lane 8 [2.0×10^5 copies ml^{-1} (1 : 100 dilution)]. Arrowheads point to the 65 and 74 kDa bands.

Neutralization test

Three serum samples (nos 1–3) (Table 1) containing IgG, IgM and IgA classes of anti-HEV antibodies obtained from patients who had contracted infection of HEV of genotype 3, 1 or 4, respectively, were tested for their ability to neutralize an HEV strain of genotype 3 (JE03-1760F) in the present culture system. Each serum sample, diluted at 1 : 2.5 with PBS(-), was mixed with an equal volume of the diluted virus stock containing 6.0×10^4 copies of HEV, kept at room temperature for 60 min and inoculated on monolayers of PLC/PRF/5 cells in a six-well microplate. In each well containing the serum sample of no. 1, 2 or 3, HEV RNA was not detectable throughout the observation period of up to 50 days after inoculation. However, in wells with the control serum (anti-HEV-negative), HEV RNA was first detectable on day 14 post-inoculation and continued to be positive ($> 10^6$ copies ml^{-1}) up to the end of the observation period.

One hundred microlitres each of serum samples containing IgG-class anti-HEV only (nos 4–7 in Table 1) diluted at 1 : 2.5, 1 : 25, 1 : 250 or 1 : 2500, was mixed with an equal volume of the diluted inoculum (6.0×10^4 copies) and kept at room temperature for 60 min. When the serum samples obtained from patients (nos 4–6) 3.0 to 24.0 years after the onset of HEV infection were used, HEV RNA was not detectable in the culture supernatant up to 50 days after inoculation at the final dilutions of 1 : 5 and 1 : 50, but did become detectable in the culture supernatant on day 10 or 12 after inoculation at the final dilutions of 1 : 500 and 1 : 5000 (Table 4). In the serum sample (no. 7) with only anti-HEV IgG antibody that was obtained from an individual whose period of HEV infection is unknown, the culture supernatants were negative for HEV RNA throughout the observation period of 50 days when mixed at the final dilution of 1 : 5, but had detectable HEV RNA on day 12 and thereafter when mixed at the final dilutions of 1 : 50 and 1 : 500.

DISCUSSION

Establishment of a practical cell-culture system that facilitates the propagation of HEV *in vitro* is critical for virological characterization as well as for studies on prevention of HEV infection. Several *in vitro* culture systems for HEV replication, such as the human embryo lung diploid cell strain (2BS), A549, PLC/PRF/5, HepG2 and primary hepatocytes from non-human primates (chimpanzees, cynomolgus macaques, tamarins and African green monkeys), have been reported (Kane *et al.*, 1984; Arankalle *et al.*, 1988; Huang *et al.*, 1992, 1995, 1999; Kazachkov *et al.*, 1992; Tsarev *et al.*, 1994; Li *et al.*, 1996; Meng *et al.*, 1996, 1997a; Tam *et al.*, 1996a, b, 1997; Wei *et al.*, 2000). However, none of these culture systems can provide high-titre HEV in the

Table 3. Effect of exposure to different temperatures on the infectivity of an HEV strain (JE03-1760F)

Days after inoculation	HEV RNA titre (copies ml^{-1}) in culture medium				
	25 °C, 30 min*	56 °C, 30 min	70 °C, 10 min	95 °C, 1 min	95 °C, 10 min
10	0	0	0	0	0
12	0	0	0	0	0
14	0	0	0	0	0
16	6.8×10^2	0	0	0	0
18	1.2×10^3	0	0	0	0
20	6.6×10^3	3.1×10^3	0	0	0
30	4.7×10^4	1.7×10^4	0	0	0
40	7.1×10^5	1.1×10^5	0	0	0
50	1.2×10^6	2.1×10^5	0	0	0

*HEV inoculum was incubated at the indicated temperature for 1–30 min, diluted to 6.0×10^4 copies in 0.2 ml and inoculated on PLC/PRF/5 cells.

culture medium; therefore, they cannot be used for the biophysical and virological studies of HEV. In the present study, using a faecal suspension (the JE03-1760F strain/genotype 3) with high HEV load (2.0×10^7 copies ml^{-1}) as an inoculum, we tested 21 cell lines including A549, HepG2 and PLC/PRF/5 cells that had been reported to support *in vitro* replication of HEV (Huang *et al.*, 1995, 1999; Li *et al.*, 1996; Meng *et al.*, 1996, 1997a; Wei *et al.*, 2000), for the possible development of an efficient culture system for HEV. Of note, a high load of HEV was yielded from the culture supernatant of cultivated A549 and PLC/PRF/5 cells on day 12 to day 14 post-inoculation and thereafter, but HEV RNA was not detectable in the culture supernatant from the remaining 19 cell lines up to the end of the observation period of 30 days. Upon comparison of the load of HEV in culture supernatant obtained from A549 and PLC/PRF/5 cells that had been cultured at two distinct temperatures (35.5 or 37.0 °C), the highest yield of HEV was obtained in the culture supernatant of PLC/PRF/5 cells maintained at 35.5 °C throughout the observation period. Consequently, in the current study, we selected PLC/PRF/5 and 35.5 °C as the suitable cell line and culturing temperature, respectively. Although the PLC/PRF/5 cell line has been used for the same purpose as the present study since the late 1980s (Pillot *et al.*,

Table 4. Neutralization of an HEV strain (JE03-1760F) with serum samples containing IgG-class antibodies to HEV from pedigreed patients who contracted clinical or subclinical HEV infection 3 to 24 years ago or from a non-pedigreed individual

Days after inoculation	HEV RNA titre (copies ml^{-1}) in culture supernatant*												
	Control serum	Serum samples with IgG-class antibodies against HEV (dilution)											
		No. 4†			No. 5†			No. 6†			No. 7		
		1:50	1:500	1:5000	1:50	1:500	1:5000	1:50	1:500	1:5000	1:5	1:50	1:500
0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	3.2×10^2	0	0	0	0	0	4.8×10^2	0	0	0	0	0	0
12	4.8×10^3	0	2.8×10^2	8.0×10^2	0	6.0×10^2	3.2×10^3	0	4.0×10^3	9.4×10^3	0	1.2×10^3	4.4×10^3
14	7.0×10^3	0	1.0×10^3	4.4×10^3	0	4.0×10^2	1.9×10^4	0	8.0×10^3	1.1×10^4	0	2.1×10^3	7.6×10^3
18	5.0×10^4	0	1.0×10^3	9.6×10^3	0	1.1×10^3	2.9×10^4	0	7.8×10^3	4.2×10^4	0	6.9×10^3	7.1×10^4
20	6.8×10^4	0	4.4×10^3	2.0×10^4	0	7.4×10^3	3.0×10^4	0	1.1×10^4	5.6×10^4	0	6.2×10^3	8.5×10^4
30	2.2×10^5	0	NT‡	NT	0	NT	NT	0	NT	NT	0	NT	NT
40	4.0×10^5	0	NT	NT	0	NT	NT	0	NT	NT	0	NT	NT
50	9.0×10^5	0	NT	NT	0	NT	NT	0	NT	NT	0	NT	NT

*Each serum sample diluted at 1:2.5, 1:25, 1:250 or 1:2500 or control serum diluted at 1:2.5, was mixed with an equal volume of the diluted inoculum (6.0×10^4 copies), kept at room temperature for 60 min, inoculated on monolayers of PLC/PRF/5 cells and cultured for up to 50 days after inoculation. HEV RNA titre in the culture supernatant obtained on the indicated day after inoculation was measured.

†HEV RNA was negative in the culture supernatants obtained on day 0 to day 50 post-inoculation when mixed with an equal amount of 1:2.5 dilution of serum sample.

‡NT, Not tested.

1987; Meng *et al.*, 1996, 1997a), the precise reason why the high HEV load of up to 10^8 copies ml^{-1} was detected in the culture supernatant of cultivated PLC/PRF/5 cells in the current study is unknown. However, we speculate that the availability of a faecal suspension with high HEV load (2.0×10^7 copies ml^{-1}) was vital for establishment of an efficient culture system for HEV in the present study. In support of our speculation, when we used other faecal suspensions with low HEV load, HEV could not be propagated on PLC/PRF/5 cells in repeated experiments (data not shown). Since the genotype 3 HEV strain used in this study could present higher infective capabilities than other HEV strains, the possibility of differences in the multiplication efficiencies of diverse HEV strains in cell-culture should also be considered.

In the present study, five generations of serial passages (passages 1–5) of culture supernatant were successfully carried out (Fig. 2). Prior to inoculation on PLC/PRF/5 cells, each inoculum (culture supernatant) for passage 1, 2, 3, 4 or 5 was purified by passage through a microfilter of 0.22 μm pore size. It is reasonable to consider that the filtered solution of inoculum does not contain cells or cell debris derived from long-term cultivation of PLC/PRF/5 cells and it is likely that serial passage was achieved by infection of virions produced by the infected cells to fresh cells in the next generation. These results suggest that the infected cells in the serial passages continued to replicate infectious HEV virions. Of note, progeny were detected approximately 2 weeks after inoculation and the HEV load increased to 10^5–10^7 copies ml^{-1} within 1 or 2 months in passages 2–5. However, in passage 1, HEV RNA was first detected in the culture supernatant on day 36 after inoculation. In order to clarify the reason for the delayed appearance of HEV in passage 1, the HEV RNA titre of the inoculum for each passage after treatment with the microfilter was determined. To our surprise, the HEV RNA titre of the inoculum after filtration was lower than that before filtration, probably due to non-specific absorption of HEV to the filter membrane. The real HEV RNA titre in the inoculum used for passage 1 was, at 3.6×10^4 copies per well, the lowest of the five passages, suggesting that the day of the initial appearance of HEV in the culture medium may depend on the HEV RNA titre of the inoculum. To confirm this notion, diluted faecal supernatants with various HEV loads were inoculated on PLC/PRF/5 cells. In the present system, the day on which HEV RNA became detectable in the culture supernatant was dose-dependent: i.e. it became detectable on day 12 or day 14 post-inoculation when a faecal suspension containing 6.4×10^4 or more copies per well was inoculated, and on day 24 or day 26 when a faecal suspension containing 2.4×10^4 or 3.2×10^4 copies per well was inoculated. In addition, the greater the amount of HEV inoculated, the more rapidly the HEV load in the culture supernatant increased, reaching higher levels. Therefore, in our cell-culture system using faecal supernatant containing the JE03-1760F strain as the inoculum, inoculation of 6.0×10^4 copies of HEV per well is recommended for efficient replication of HEV. In some reports (Huang *et al.*, 1992, 1999; Li *et al.*, 1996), the occurrence of HEV propagation was proven by observation of CPE. However, in our experiments, CPE was not observed in any of the cultured cells supporting replication of HEV. In our culture system, HEV RNA continued to be detected up to 4 months post-inoculation (data not shown), as long as HEV-infected PLC/PRF/5 cells were alive and continued to produce HEV progeny.

Recent studies have indicated that zoonotic food-borne transmission of HEV from domestic pigs, wild boar or wild deer to humans may occur as domestic infection in Japan, where some people ingest uncooked or undercooked meat or viscera (such as raw liver and colon/intestines) (Matsuda *et al.*, 2003; Tei *et al.*, 2003; Yazaki *et al.*, 2003; Tamada *et al.*, 2004; Li *et al.*, 2005). Pig liver specimens from seven (1.9 %) of 363 packages sold in local grocery stores in Hokkaido had detectable HEV RNA. Of interest, one swine HEV isolate (swJL145) obtained from a packaged pig liver was 100 % identical to the virus recovered from an 86-year-old patient who had contracted sporadic hepatitis E after ingestion of undercooked pig liver, suggesting that consumption of undercooked pig liver/intestine is a potential risk factor for HEV infection (Yazaki *et al.*, 2003). Since little was known about the thermal stability of HEV, the newly developed culture system for HEV was used to examine the thermal stability of HEV. In the current study, HEV in the faecal suspension was inactivated by incubation at 70 °C for 10 min or at 95 °C for 1 min; however, upon incubation at 56 °C for 30 min, the HEV was still infectious, corroborating the previous report by Emerson *et al.* (2005). In the present study, the faecal suspension was heat-treated without the addition of proteins as stabilizing factors. Therefore, the temperature that would be required to inactivate virus embedded in an uncooked or undercooked meat or viscera from infected pigs, wild boars or deer is expected to be higher than that estimated in the present study.

In vitro neutralization tests for HEV were reported by two groups of investigators in 1997, but these tests have shortcomings. Tam *et al.* (1997) used primary hepatocytes isolated from cynomolgus monkeys for inoculation of HEV, but primary hepatocytes from this monkey are not available to most laboratories and the cells need to be cultured under fastidious conditions. Meng *et al.* (1997a) used PLC/PRF/5 cells for propagating HEV, as in our current study, but they detected only those antibodies that block binding of virus to cells; antibodies that neutralize at a step after binding would not be seen as neutralizing in their test. In our cell-culture system, only replicating viruses are detected, thus ensuring that a biologically relevant receptor is used and that neutralization post-attachment can be detected, similar to a recently reported neutralization assay for HEV that identifies the virus-infected cells (HepG2/C3A) by immunofluorescence microscopy (Emerson *et al.*, 2006). A previous study indicated that all convalescent serum samples from rhesus monkeys that had been experimentally infected with

HEV genotype 1, 2, 3 or 4 neutralized the genotype 1 virus (Emerson *et al.*, 2006). In the present study, serum samples with IgG, IgM and IgA classes of anti-HEV antibodies that were obtained from patients infected with HEV of genotype 1, 3 or 4 during the convalescent phase neutralized a genotype 3 virus, confirming that HEV antibodies are broadly cross-reactive. In addition, serum samples obtained from patients with IgG anti-HEV antibodies 8.7 or 24.0 years after the onset of HEV infection, that were detectable by ELISA using recombinant ORF2 protein expressed in the pupae of silkworm (Mizuo *et al.*, 2002; Takahashi *et al.*, 2005), also prevented propagation of HEV in PLC/PRF/5 cells, suggesting the presence of long-lasting HEV antibodies with neutralizing activity in individuals with past HEV infection.

In conclusion, using a faecal suspension with a high HEV load of 2.0×10^7 copies ml^{-1}, we developed an efficient cell-culture system for HEV in PLC/PRF/5 cells, with an HEV RNA titre of up to 10^8 copies ml^{-1} in the culture supernatant. HEV progeny released in the culture supernatant were passaged five times serially in PLC/PRF/5 cells. The cell-culture system developed for HEV would be useful for resolving many important questions regarding the biophysical and virological characteristics of HEV and for facilitating vaccine research.

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Analysis of the Full-Length Genome of Genotype 4 Hepatitis E Virus Isolates From Patients With Fulminant or Acute Self-Limited Hepatitis E

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It was suggested that hepatitis E virus (HEV) genotype 4 is associated more closely with the severity of hepatitis E than genotype 3, although the virological basis remains unknown. The aim of this study was to examine whether genomic differences among genotype 4 HEVs are responsible for the development of fulminant hepatitis. Full-length sequences of genotype 4 HEVs from three patients with fulminant hepatitis and six patients with acute self-limited hepatitis were determined. The sequences were analyzed with those of 13 genotype 4 HEV isolates whose entire nucleotide sequence is known. Analysis of 22 full-length sequences (fulminant hepatitis, 5; acute hepatitis, 17) revealed that C at nt 1816 and U at nt 3148 (U3148), both of which do not change the amino acid sequences, were significantly associated with fulminant hepatitis ($P = 0.0489$, respectively). When partial nucleotide sequences containing nt 1816 or nt 3148 were determined in 16 additional HEV isolates of genotype 4, a closer association between U3148 and fulminant hepatitis ($P = 0.0018$) was observed. The comparison of 86 HEV isolates of all four genotypes showed that U3148 had a stronger association with fulminant hepatitis than other nucleotides at nt 3148 ($P = 0.0006$). Patients infected with HEV with U3148 had a significantly lower value of the lowest prothrombin activity ($P = 0.0293$). Nt 3148 is located within the RNA helicase domain, and 22-nt sequence including nt 3148 was well conserved among all genotypes. A silent substitution of U3148 in HEV may be associated with the development of fulminant hepatitis. Further studies are needed to clarify the underlying mechanism. ***J. Med. Virol. 78:476–484, 2006.*** © 2006 Wiley-Liss, Inc.

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KEY WORDS: hepatitis E virus; fulminant hepatitis; full-length genome; genotype; silent mutation

INTRODUCTION

Hepatitis E virus (HEV) is a major cause of epidemic and sporadic hepatitis in many developing countries [Purcell and Emerson, 2001]. Recently, sporadic cases of HEV infections have been reported in industrialized countries [Harrison, 1999; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003], where zoonotic transmission of HEV has been suggested; animals such as swine serve as reservoirs for HEV [Meng, 2003; Tei et al., 2003; Yazaki et al., 2003]. HEV is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004a; http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_hepev.htm]. The genome of HEV is a single-stranded, positive-sense RNA of approximately 7.2 kb and consists of a short 5′-untranslated region, three partially overlapping open reading frames (ORF1, ORF2, and ORF3), and a short

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB220971–AB220979 (full-length sequences) and AB221706–AB221758 (partial ORF1 sequences).

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3′-untranslated region terminated by a poly(A) tract [Tam et al., 1991; Wang et al., 2000]. Based on sequence analysis, HEV sequences have been classified into four major genotypes (1–4). Genotype 1 is the main cause of hepatitis E in developing countries in Asia and Africa, and genotype 2 has been documented in Mexico and Nigeria. Genotype 3 or 4 has been described in the United States, European countries, China, Taiwan, and Japan [Schlauder and Mushahwar, 2001; Mizuo et al., 2002].

Infection of HEV induces self-resolving hepatitis or a subclinical state in most cases, but it can cause fulminant hepatitis. It was reported that HEV infection is a major cause of fulminant hepatitis in endemic areas for HEV [Nanda et al., 1994; Coursaget et al., 1998; Sheikh et al., 2002]. Patients with fulminant hepatitis have been reported in an industrialized country [Suzuki et al., 2002]. Recent observations suggest that the HEV genotype influences the severity of hepatitis E, and that genotype 4 is associated more strongly with the severe form of hepatitis E than genotype 3 [Mizuo et al., 2005]. To date, nine HEV-associated fulminant hepatitis cases have been reported in Japan, and seven of them were infected with genotype 4 HEV [Suzuki et al., 2002; Ohnishi et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003; Kato et al., 2004; Takahashi et al., 2005], although a larger number of patients are infected with genotype 3 than genotype 4 in Japan [Okamoto et al., 2003]. These observations suggest that there is a close relationship between infection with genotype 4 HEV and progression to fulminant hepatitis, which prompted us to investigate whether genomic differences among HEVs are responsible for the development of fulminant hepatitis. The full-length genome of genotype 4 HEV in patients with fulminant hepatitis and in those with acute self-resolving hepatitis was determined.

MATERIALS AND METHODS

Serum Samples

Sera collected between 1998 and 2004 in Japan from three patients with type E fulminant hepatitis and six patients with the mild form of type E acute hepatitis, who had a lowest prothrombin activity of ≥80%, were used for full-length sequencing of HEV. Clinical characteristics and laboratory data of the three patients with fulminant hepatitis (isolate names: HE-JF3, HE-JF4, and HE-JF5) [Suzuki et al., 2002; Yazaki et al., 2003] and six acute hepatitis patients (isolate names: HE-JA2, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41) [Mizuo et al., 2002, 2005; Yazaki et al., 2003] were reported previously.

Serum samples collected between 1993 and 2004 from 20 patients with genotype 3 HEV and 16 patients with genotype 4 HEV were used for partial sequencing of ORF1 of HEV. One each of the patients with genotype 3 or 4 was diagnosed with type E fulminant hepatitis whose isolate was designated as HE-JF2 and HE-JF1, respectively [Suzuki et al., 2002]. The remaining 34 patients were diagnosed as having type E acute hepatitis [Mizuo et al., 2002, 2005; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005].

Amplification of Full-Length HEV Genome

Total RNA was extracted from 120 to 300 μl of serum, and subjected to cDNA synthesis and nested polymerase chain reaction (PCR) for six overlapping regions excluding the extreme 5′- and 3′-terminal regions; the amplified regions were nt 37–1199 (1163 nt) (primer sequences excluded), nt 991–3148 (2158 nt), nt 3029–4603 (1575 nt), nt 4401–5325 (925 nt), nt 5240–5998 (759 nt), and nt 5985–7142 (1158 nt).

The 5′-end sequence (nt 1–60) 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 [Okamoto et al., 2001]. Amplification of the 3′-end sequence (nt 7071–7240: excluding poly [A] tail) was attempted by the RACE method as described previously [Okamoto et al., 2001].

Amplification of Partial Nucleotide Sequences of ORF1 Including Nt 1816 and 3148

Total RNA extracted from 50 μl of serum was reverse-transcribed and subjected to PCR with genotype-specific primers. To amplify the nt 1543–2086 sequence (primer sequences excluded) of genotype 4 HEV, first round PCR was performed with primers HE296 and HE269, and second round PCR was performed with primers HE297 and HE298 (Table I). To amplify the nt 2827–3286 sequence of genotype 4 HEV, HE299 and HE302 were used for the first round PCR and HE300 and HE301 for the second round PCR. Genotype 3-specific PCR was performed to amplify the nt 1546–2074 sequence with HE303 and HE306 in the first round and HE304 and HE282 in the second round. To amplify the nt 2995–3469 sequence of genotype 3 HEV, HE307 and HE310 were used in the first round and HE308 and HE309 in the second round.

Semi-Quantitation of HEV RNA

Semi-quantitation of HEV RNA was performed by the end-point dilution method, with primers targeting the ORF2 region as described previously [Mizuo et al., 2002]. The highest dilution (10^N) of extracted RNA that was found to be positive was estimated and it was converted to the relative titer per 1 ml of serum.

Sequence Analysis

The amplification products were sequenced on both strands either directly or after cloning into pT7Blue T-Vector (Novagen, Inc., Madison, WI), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequence analysis was performed using Genetyx-Mac (version 12.6.6; Genetyx Corp., Tokyo, Japan) and

TABLE I. Positions and Nucleotide Sequences of Primers Used for Polymerase Chain Reaction (PCR) Amplification of Partial Nucleotide Sequences of Hepatitis E Virus (HEV) ORF1

Primer	Polarity	Nucleotide position[a]	Specificity	Nucleotide sequence[b]
HE269	Antisense	2135–2154	Genotype 4	5′ ARSCCYGAMACCGACCAGGT 3′
HE282	Antisense	2075–2094	Genotype 3	5′ GACTCCCARVYRTGSCCRGG 3′
HE296	Sense	1509–1528	Genotype 4	5′ AGGGTTAYGAYAAYGAGGCA 3′
HE297	Sense	1523–1542	Genotype 4	5′ GAGGCATTTGARGGGTCGGA 3′
HE298	Antisense	2087–2106	Genotype 4	5′ AARGGRTTRGYWGACTCCCA 3′
HE299	Sense	2765–2784	Genotype 4	5′ GAYGCYTGGGARCGYAACCA 3′
HE300	Sense	2807–2826	Genotype 4	5′ CTKACYGAGCCRGCBATAGC 3′
HE301	Antisense	3287–3306	Genotype 4	5′ TTTGTWGGDACMAGCTCAGG 3′
HE302	Antisense	3303–3322	Genotype 4	5′ RGTAASRTGCCACCACTTTG 3′
HE303	Sense	1448–1467	Genotype 3	5′ CRGTGGYTVGGSCAGGAGTG 3′
HE304	Sense	1526–1545	Genotype 3	5′ GCYTAYGAGGRBTCYGAGGT 3′
HE306	Antisense	2087–2106	Genotype 3	5′ AADGGRTTVGCAGRCTCCCA 3′
HE307	Sense	2933–2952	Genotype 3	5′ CGTGCYTGYGCYGGYTGCAC 3′
HE308	Sense	2975–2994	Genotype 3	5′ TAYCAGTTYACYGCYGGGGT 3′
HE309	Antisense	3470–3489	Genotype 3	5′ TGRACHGTRATYGCACCAGG 3′
HE310	Antisense	3518–3537	Genotype 3	5′ GCYGTRGCTATRATTGTGGT 3′

[a]The nucleotide numbers are in accordance with HE-JA1 (AB097812).
[b]R = A or G; S = C or G; Y = C or T; M = A or C; V = A, C or G; W = A or T; K = G or T; B = C, G or T; D = A, G or T; H = A, C or T.

ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequences were aligned by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987]. Bootstrap values were determined with 1,000 resamplings of the data sets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

Statistical Analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and the Mann–Whitney U test for comparison of continuous variables between two groups. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Analysis of Full-Length Genome of Genotype 4 HEV Isolates

Seven of the nine HEV isolates, that is, HE-JF4, HE-JF5, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41, had the same genomic length of 7239 nt, excluding the poly(A) tract at the 3′-terminus. HE-JF3 and HE-JA2 had a genomic length of 7240 nt and 7243 nt, respectively; the differences in genomic length were attributed to an insertion of 1 nt or 4 nt in HE-JF3 and HE-JA2, respectively, in the 3′-untranslated region. When the full genome sequences were compared with those of reported HEV isolates, HE-JF3 was closely related to HE-JA1 [Nishizawa et al., 2003] with a nucleotide identity of 99.6%. HE-JF4 shared an identity of 99.7% with HE-JF5, and had the highest identity of 99.9% with both JYW-Sap02 and JTS-Sap02 [Takahashi et al., 2004]. HE-JA19 and HE-JA37, which shared an identity of 99.3%, were 99.2% and 98.9% similar to JKK-Sap00 [Takahashi et al., 2003], respectively. HE-JA28, HE-JA36, and HE-JA41 were 99.5–99.6% identical to each other and had an identity of 98.4–98.7% with JSM-Sap95 [Takahashi et al., 2004]. HE-JA2 was only 84.3–86.8% similar to the other genotype 4 isolates. A phylogenetic tree was constructed based on the full genome sequence of genotype 1–4 HEV (Fig. 1). HE-JF3 segregated into a cluster consisting of JSN-Sap-FH02 [Takahashi et al., 2003] and JSF-Tot03 [Takahashi et al., 2005], both of which had been obtained from fulminant hepatitis patients. HE-JF4, HE-JF5, HE-JA19, and HE-JA37 were grouped into another cluster consisting of JKK-Sap00, JYW-Sap02, and JTS-Sap02. HE-JA28, HE-JA36, and HE-JA41 formed a mini-cluster, separate from the latter cluster.

To investigate nucleotide differences that may be related to fulminant hepatitis, the nine full-length sequences of HEV determined in this study were examined along with 13 previously reported, entire or nearly entire HEV sequences [Wang et al., 2000; Kuno et al., 2003; Liu et al., 2003; Nishizawa et al., 2003; Takahashi et al., 2003, 2004, 2005]. No nucleotide substitutions within the 5′-and 3′-untranslated regions were specific for the HEV isolates from patients with fulminant hepatitis, although some of the reported isolates lacked the extreme 5′-terminal part of the 5′-untranslated region. A total of 7,145 nucleotides within the coding region of the 5 genomes (HE-JF3, HE-JF4, HE-JF5, JSN-Sap-FH02, and JSF-Tot03) recovered from patients with fulminant hepatitis were compared with those of the 17 genomes recovered from patients with acute hepatitis. At each nucleotide position, we examined whether a particular nucleotide was significantly more prevalent among the HEV genomes from patients with fulminant hepatitis than among those from patients with acute hepatitis. A P-value was determined for each nucleotide position.

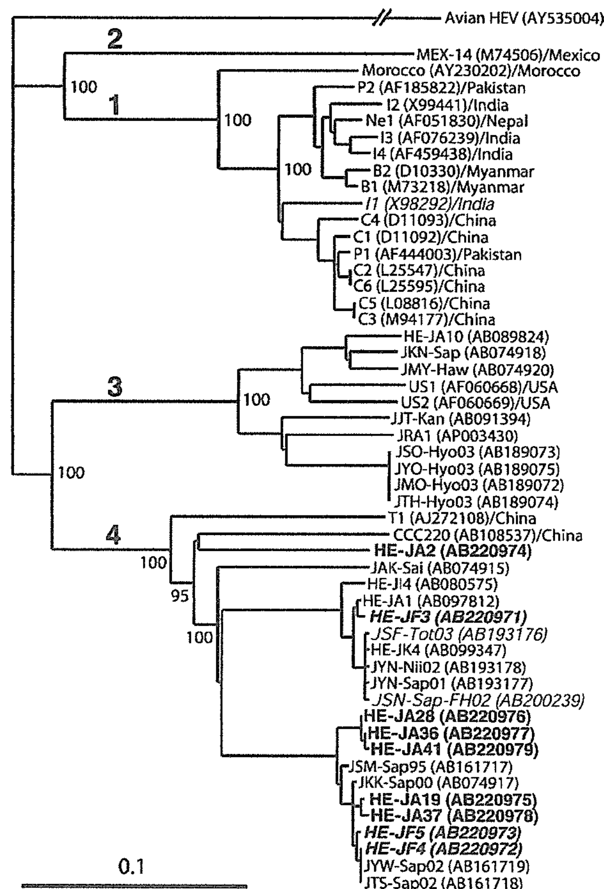

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 51 human hepatitis E virus (HEV) isolates, using a chicken HEV isolate (AY535004) as an outgroup. The nine HEV isolates whose full-length sequence was determined in the present study are indicated in bold type for visual clarity. Forty-two isolates whose entire or nearly entire sequence has been reported were included for comparison, with the accession number in parentheses. After the slash, the name of the country other than Japan where the HEV isolate was isolated is shown. The six isolates that were obtained from patients with fulminant hepatitis are indicated in italic type. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings.

All 21 nucleotide substitutions with $P < 0.2$ did not change the amino acids (Table II). Among them, C at nt 1816 (C1816) and U at nt 3148 (U3148) were seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis (100% vs. 47%, $P = 0.0489$; 100% vs. 47%, $P = 0.0489$).

Comparison of Nucleotides at Nt 1816 and 3148 in Genotype 4 HEV Isolates

To determine the nucleotides at nt 1816 and 3148 in an additional 16 HEV isolates from patients with genotype 4 HEV, including a patient with fulminant hepatitis (HE-JF1), the partial nucleotide sequences of two different regions within ORF1 (nt 1543–2086 and nt 2827–3286) were determined. The nucleotides at nt 1816 and 3148 in the obtained sequences and those in the 22 full-length genomes were compared between the fulminant and acute hepatitis patients (Table III). C at nt 1816 and U at nt 3148 remained significantly more prevalent among the patients with fulminant hepatitis than among those with acute hepatitis ($P = 0.0268$ and $P = 0.0018$, respectively). Of note, U3148 was found to have a closer relationship with fulminant hepatitis than C1816.

Comparison of the Nucleotides at Nt 3148 in HEV Isolates of All Four Genotypes

Figure 2A depicts the genomic organization of the four genotypes of HEV and the location of nt 3148. Nt 3148 is located within the RNA helicase domain of ORF1, and is the third base of a triplet codon encoding valine. The consensus sequence of each of the four genotypes of HEVs whose entire or nearly entire sequence was known was determined. When the consensus sequences of the 4 genotypes were aligned, a 22-nt sequence including nt 3148 was found to be well conserved (Fig. 2B). To compare the 22-nt sequence among additional isolates, the nucleotide sequence of nt 2995–3469 was determined for 20 additional isolates from patients infected with genotype 3 HEV, including that from a fulminant hepatitis patient (HE-JF2). Comparison of the 22-nt sequence among 86 isolates including the 20 genotype 3 and 16 genotype 4 isolates, whose partial nucleotide sequence was determined in the present study, disclosed that there are some minor substitutions in this conserved area (Fig. 2C). Notably, genotype 4 isolates had a much higher prevalence of U3148 than isolates of the other three genotypes (39.5% [15/38] vs. 4.2% [2/48], $P < 0.0001$), and U3148 was associated significantly more frequently with fulminant hepatitis than other nucleotides at nt 3148 (C or G) (35.3% vs. 2.9%, $P = 0.0006$). On the other hand, at nt 1816, no particular nucleotide was seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis when the 86 isolates of four distinct genotypes were compared (data not shown).

Comparison of Demographic Characteristics and Laboratory Parameters Among the Hepatitis E Patients According to the Presence of U3148

The demographic features and laboratory parameters were compared in relation to the presence or absence of U3148 among 48 patients with type E acute or fulminant hepatitis for whom such data were available [Mizuo et al., 2002, 2005; Suzuki et al., 2002; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005]. Twenty-seven patients were infected with genotype 4 HEV and the remaining 21 patients with genotype 3 HEV. A patient who was infected with both genotypes 3 and 4 HEV isolates [Takahashi et al., 2002]

TABLE II. Differences in the Nucleotide Sequence of Genotype 4 HEV Genomes Obtained From Patients With Fulminant or Acute Hepatitis E

Isolate name[a]	Diagnosis	Nucleotide no.[b]																				
		37	370	421	1345	1816	1963	2101	2128	2224	2725	3148	3185	3796	3856	4579	4888	5071	5907	5943	6126	6216
HE-JF3	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF4	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF5	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSN-Sap-FH02	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSF-Tot03	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JA2	AH	U	U	G	C	C	A	C	C	A	U	C	U	U	C	C	A	U	A	U	U	U
HE-JA19	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA28	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA36	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA37	AH	C	C	A	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA41	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
T1	AH	C	U	G	C	G	C	C	C	U	C	C	C	C	C	C	C	C	A	U	U	U
CCC220	AH	U	C	U	C	C	U	C	C	U	C	U	C	C	C	C	A	C	A	C	U	G
HE-JA1	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JK4	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JAK-Sai	AH	U	U	A	U	U	A	C	U	C	C	U	C	U	C	C	G	C	U	U	U	C
JSM-Sap95	AH	C	C	G	U	U	C	U	U	C	C	C	C	U	U	U	G	C	U	U	C	C
JKK-Sap00	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	U	C	C
JYN-Sap01	AH	U	C	G	U	C	C	U	U	C	U	U	U	C	U	U	G	U	C	C	C	C
JYW-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JTS-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JYN-Nii02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
		U/C	C/U	G/H	U/C	C/K	C/W	U/C	U/C	U/M	U/C	U/C	U/C	C/U	U/C	U/C	G/M	U/C	C/W	C/U	C/U	C/K
	FH	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
	AH	9/8	11/6	11/6	11/6	8/9	11/6	10/7	11/6	10/7	10/7	8/9	10/7	11/6	10/7	10/7	11/6	10/7	9/8	9/8	10/7	11/6
	P value[c]	0.076	0.166	0.166	0.166	**0.049**	0.166	0.114	0.166	0.114	0.114	**0.049**	0.114	0.166	0.114	0.114	0.166	0.114	0.076	0.076	0.114	0.166

FH, fulminant hepatitis; AH, acute hepatitis; H = A or C or U; K = G or U; W = A or U; M = A or C.

Nucleotide substitutions in which the prevalence of a particular nucleotide at a nt position was higher among the HEV genomes from FH patients than among those from AH patients with $P < 0.2$ are shown.

[a]The isolate names whose entire sequences have been determined in this study are indicated in bold face.

[b]Nucleotide numbers are in accordance with HE-JA1 (AB097812).

[c]*P* values (Fisher's exact probability test) that are significant are indicated in bold face.

TABLE III. Comparison of Nucleotides at Nt 1816 and 3148 Among 38 Isolates of Genotype 4 HEV Recovered From Patients With Fulminant or Acute Hepatitis E

	Nt 1816		Nt 3148	
Patients with	C	G or U	U	C
Fulminant hepatitis	5	1	6	0
Acute hepatitis	10	22	9	23
P value	0.0268		0.0018	

was excluded. The patients infected with HEV with U3148 tended to have a higher peak level of total bilirubin than those with C3148 or G3148 ($P = 0.0721$) (Table IV). Of note, the value of the lowest prothrombin activity was significantly lower in patients with U3148 than in those with C3148 or G3148 ($P = 0.0293$).

DISCUSSION

It is generally considered that the severity of hepatitis E depends on host factors of the infected patients such as

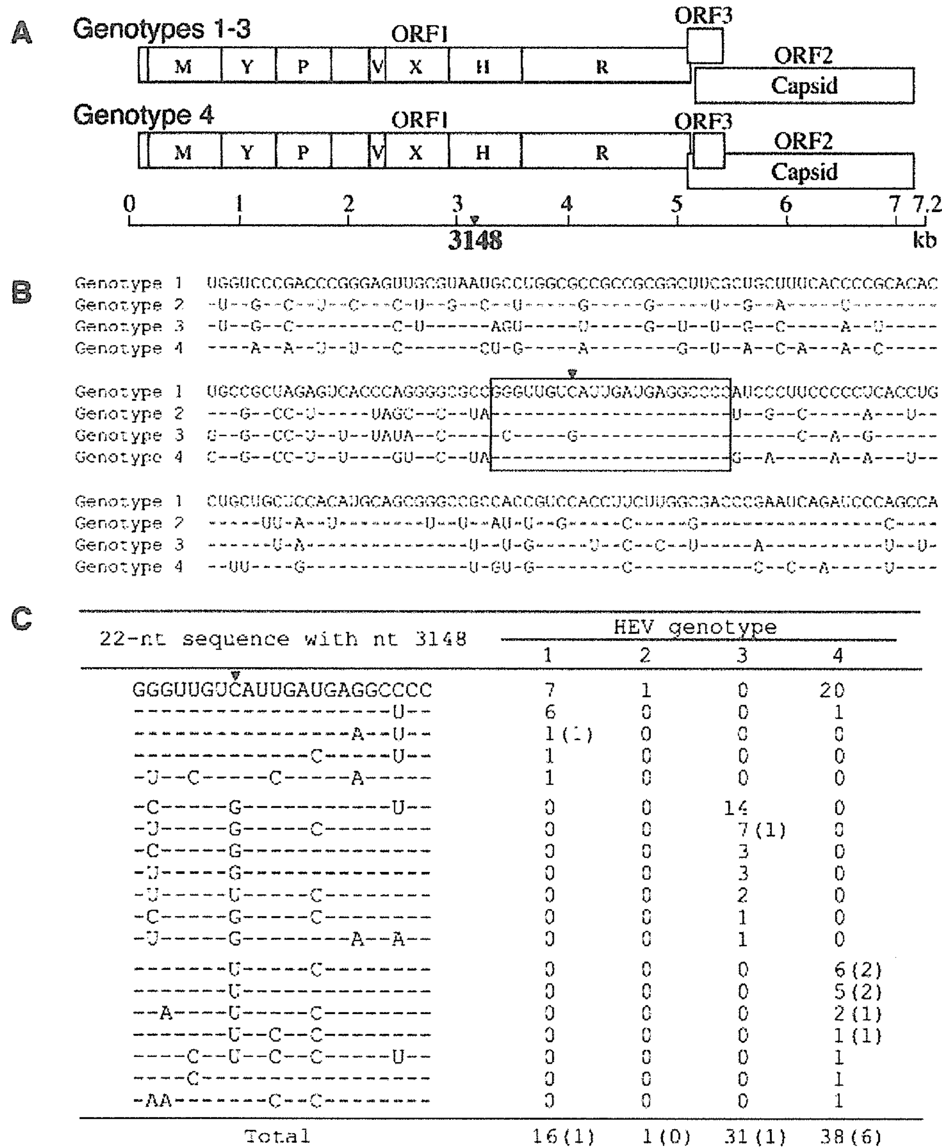

Fig. 2. Characteristics of nt 3148 in HEV and its surrounding sequence. A: Organization of the HEV genome of genotypes 1–3 and that of genotype 4 showing the location of nt 3148. M, methyltransferase; Y, Y domain; P, papain-like protease; V, proline-rich hinge domain; X, X domain; H, RNA helicase; R, RNA polymerase. B: Comparison of consensus sequences of the region including nt 3148 (201 nt) in each of the four distinct genotypes of HEV. The inverted triangle indicates the position of nt 3148, and the box indicates a conserved area including nt 3148. The consensus sequence of each genotype was deduced from nucleotides common to each of the four genotypes (genotype 1, 16 isolates; genotype 2, 1 isolate; genotype 3, 11 isolates; and genotype 4, 22 isolates). C: Comparison of a 22-nt sequence including nt 3148 among 86 HEV isolates of four distinct genotypes. The inverted triangle indicates nt 3148. The numbers on the right of the sequences indicate the number of isolates with the indicated sequences, and the number in parentheses indicate the number of isolates obtained from patients with fulminant hepatitis. The genotype 1 isolate from a patient with fulminant hepatitis (I1, X98292 [Donati et al., 1997]), and the genotype 3 isolate from a patient with fulminant hepatitis (HE-JF2 [Suzuki et al., 2002]) are shown.

TABLE IV. Comparison of Demographic Characteristics and Laboratory Parameters Between Patients Infected With HEV With U at Nt 3148 or Those With Another Nucleotide at Nt 3148

	Nt 3148		
	U (n = 8)	C or G (n = 40)	*P* value[a]
Age (years)	58.5 ± 8.9	57.7 ± 12.0	0.7085
Male [number (%)]	8 (100)	33 (82.5)	0.2532
Peak ALT (IU/l)	2,814 ± 1,009	2,459 ± 1,237	0.2685
Peak total bilirubin (mg/dl)	16.5 ± 10.3	9.6 ± 8.3	0.0721
Lowest prothrombin activity (%)	51.8 ± 29.1	76.8 ± 25.9	**0.0293**
High HEV load[b] [number (%)]	3 (37.5)	10 (25.0)	0.3704

The demographic characteristics and laboratory parameters of patients with type E acute or fulminant hepatitis were studied [Mizuo et al., 2002, 2005; Suzuki et al., 2002; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005]. Twenty-seven patients were infected with genotype 4 HEV and 21 patients were infected with genotype 3 HEV.

[a]*P* value that is statistically significant is indicated in bold face.

[b]With HEV RNA titer of $\geq 10^5$ copies/ml at the first examination.

pregnancy [Harrison, 1999; Purcell and Emerson, 2001; Smith, 2001] or aging [Harrison, 1999]. The mortality rate among pregnant women who acquired hepatitis E is as high as 20%. In addition, the presence of an underlying disease may influence the severity of hepatitis E [Mizuo et al., 2005]. However, similar to other known hepatitis viruses, viral factors may play a role in the pathogenesis of type E fulminant hepatitis. Hepatitis B virus variants with mutations in the precore region [Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Terazawa et al., 1991; Yotsumoto et al., 1992] and/or the core promoter [Sato et al., 1995] have been implicated in fulminant hepatitis. Possible associations were also suggested between the severity of hepatitis A and significant numbers of nucleotide substitutions in the 5′-untranslated region of the hepatitis A virus genome [Fujiwara et al., 2001, 2002]. Recently, it was suggested that the severity of hepatitis E is influenced by the genotype of HEV, based on the finding that patients infected with genotype 4 HEV tend to have more severe disease than those with genotype 3 HEV in Japan [Mizuo et al., 2005]. The mortality rate of hepatitis E in developing countries where genotype 1 HEV prevails is reported to be about 1% [Purcell and Emerson, 2001]. As for genotype 2 HEV, no fulminant cases have been reported thus far. In Japan where HEV isolates of genotypes 3 and 4 circulate, 4 (14.8%) of 27 patients with genotype 4 HEV and 1 (4.8%) of 21 patients with genotype 3 HEV died due to fulminant hepatitis, indicating that genotype 4 HEV may be more closely associated with the development of fulminant hepatitis than HEV of other genotypes. Genotype 4 HEV is unique in that there is an insertion of a single nucleotide (U) at nt 5159, which affects both ORF2 and ORF3 (Fig. 2A). The ORF2 of genotype 4 HEV overlaps ORF1 by one nt, whereas ORF2 in all reported isolates of genotypes 1–3 begins 41 nt downstream of ORF1. The first initiation codon of ORF3 in genotype 4 HEV isolates is 28 nt downstream of ORF1, in contrast with ORF3 in reported isolates of genotypes 1–3 which overlaps ORF1 by one nt. Consequently, genotype 4 HEV has an additional 14 codons in ORF2. The predicted size of ORF3 of genotype 4 HEV at the 5′-terminal portion is nine codons shorter than that of reported isolates of genotypes 1–3 [Wang et al., 2000; Takahashi et al., 2003]. The uniqueness of the genotype 4 HEV genome may explain, at least in part, its association with the severe form of hepatitis E.

Amino acid substitutions in viral proteins that are related with altered pathogenesis have been well documented [Brack et al., 1998; Raychaudhuri et al., 1998; Lum et al., 2003; Glenn and Novembre, 2004]. The viral RNA 5′- and 3′-untranslated region sequences can also affect the expression of disease symptom [Slobodskaya et al., 1996; Brack et al., 1998; Bryant et al., 2005]. In the present study, amino acid changes in the coding regions or nucleotide substitutions in the 5′- and 3′-untranslated regions, that may be associated with the development of fulminant hepatitis, were not observed among patients who were infected with genotype 4 HEV and diagnosed with fulminant hepatitis. However, a silent substitution of U at nt 3148, that is located within the RNA helicase domain of ORF1, was observed significantly more frequently among genotype 4 HEV isolates than among isolates of the other genotypes ($P < 0.0001$), and among HEV isolates obtained from patients with fulminant hepatitis than among those obtained from patients with acute hepatitis ($P = 0.0006$). The results suggest that U3148 in the HEV genome is associated with progression to fulminant hepatitis or the severe form of hepatitis E. The underlying reason for the association of a silent substitution at nt 3148 in the HEV genome with the progression to fulminant hepatitis remains unknown. Two possible explanations are as follows. One explanation is that the silent substitution at nt 3148 may influence the efficiency of replication of HEV. Nt 3148 is located at the RNA helicase domain [Koonin et al., 1992] and the particular nucleotide at nt 3148 may alter the secondary structure of the genome, thereby affecting the expression of RNA helicase. The secondary structure of the RNA genome with U3148 may be favorable for translation of the RNA helicase and RNA polymerase whose coding region is located downstream of the RNA

helicase domain, as discussed previously [Hirata et al., 2003]. Another explanation is that the nucleotide sequence containing nt 3148 may regulate the transcription of the subgenomic mRNA of the HEV genome. It was reported that in the liver of cynomolgus macaques infected with HEV, there was a subgenomic mRNA that was shorter than the entire genome and had a common 3′-end with genomic RNA [Tam et al., 1991; Yarbough et al., 1991]. The 22-nt sequence including nt 3148 was conserved among all four genotypes, supporting the latter explanation. As the 5′-end of the subgenomic mRNA has not been determined as yet, it is uncertain whether nt 3148 can affect the transcription of the mRNA. As to whether the sequence including nt 3148 plays a role at the genomic level, the 15-nt sequence including U3148 is homologous to the sequence 5′ UGCYAUUGAGCAGGC 3′ (nt 67–81) in the methyltransferase domain of ORF1, which is well conserved among the genotypes, and further investigation may be warranted.

Of interest, a plant virus, *Apple stem grooving virus* (ASGV), that contained a single silent substitution in the coding region, did not induce the symptoms in host plants that are characteristic of the wild-type virus [Hirata et al., 2003]. As the substitution did not affect the abundance of mRNA transcribed from the downstream, the mechanism of symptom attenuation is under discussion. Its genome consists of a single-stranded, positive-sense RNA of 6.5 kb that is 5′-capped and 3′-polyadenylated. Some features of the ASGV genome resemble those of the HEV genome, suggesting that a silent substitution in the HEV genome may also affect the symptoms in the host. HEV RNA replication occurred in primate cell cultures transfected with in vitro transcripts of an infectious cDNA clone of the HEV genome [Emerson et al., 2004b]. Studies using a mutagenized genotype 4 HEV with U3148 that is constructed in vitro, may elucidate the mechanism by which the silent substitution of U3148 leads to progression to fulminant hepatitis.

In conclusion, the results of this study suggest that a silent substitution of U at nt 3148 in genotype 4 HEV is associated closely with the occurrence of fulminant hepatitis. As the number of patients with type E fulminant hepatitis is limited, accumulation of patients with type E fulminant hepatitis not only in Japan but also in other countries and extensive clinical and virological analyses of a large number of such cases are needed in future studies to evaluate our proposal. Studies on the mechanism by which the silent substitution of U3148 leads to progression to fulminant hepatitis may elucidate a novel determinant of disease severity of HEV infection.

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Development and validation of an improved RT-PCR assay with nested universal primers for detection of hepatitis E virus strains with significant sequence divergence☆

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Abstract

Recent studies revealed that hepatitis E virus (HEV) genomes are more variable than previously thought and well-conserved regions suitable for designing universal primers are limited. In this study, based on alignment of 70 full-length HEV sequences of genotypes 1–4, a part of the ORF2/ORF3 overlapping region was found to be the best target region for PCR amplification of various HEV strains. Using the newly designed primers, an RT-PCR method (ORF2/3-137 PCR) that amplifies a 137-nucleotide (nt) sequence within the ORF2/ORF3 overlapping region and is capable of amplifying all known HEV sequences was developed. When compared with the previous RT-PCR method (ORF2-457 PCR) that amplifies a 457 nt ORF2 sequence, ORF2/3-137 PCR was two to three times more sensitive than ORF2-457 PCR upon testing serial dilutions of three HEV RNA-positive serum samples. The ORF2/3-137 PCR assay could detect viraemia in five patients with acute or fulminant hepatitis E 3–14 days longer than ORF2-457 PCR after disease onset. All 41 ORF2-457 PCR-positive serum samples of various genotypes tested positive for HEV RNA by the ORF2/3-137 PCR assay. Since the amplicons of ORF2/3-137 PCR contain variable sequences, a phylogenetic tree of the ORF2/3-137 products could clearly distinguish the different HEV genotypes.
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Keywords: Hepatitis E virus; RT-PCR; HEV RNA; Genotype; Phylogenetic tree

1. Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, has a single-stranded, positive-sense RNA genome within a non-enveloped capsid (Purcell and Emerson, 2001). The genome is approximately 7.2 kb in size and consists of a short 5′-untranslated region (UTR), three partially overlapping open reading frames (ORF1–3) and a short 3′-UTR terminated by a poly(A) tract (Tam et al., 1991). HEV is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson et al., 2004). In developing countries, HEV is an important public health concern because it causes water-borne outbreaks as well as sporadic hepatitis (Purcell and Emerson, 2001). Recently, besides imported cases of hepatitis E, locally acquired sporadic cases of hepatitis E have been reported in industrialized countries (Harrison, 1999; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003; Mansuy et al., 2004; Ijaz et al., 2005), where zoonotic transmission of HEV has been suggested (Meng et al., 1997, 1998, 2002; Meng, 2003; Nishizawa et al., 2003; Okamoto et al., 2001; Tei et al., 2003; Yazaki et al., 2003).

Sequence analyses of HEV isolates revealed extensive heterogeneity of the genome and four major genotypes (1–4) have been identified (Schlauder and Mushahwar, 2001). HEV is distributed worldwide and a difference in geographic distribution of the genotypes has been noted (Schlauder and Mushahwar, 2001; Lu et al., 2006). Genotype 1 was isolated from tropical and subtropical countries in Asia and Africa, and genotype 2 was described in Mexico and Africa. Genotype 3 is most widely

☆ The nucleotide sequence data reported in this study have been assigned GenBank/EMBL/DDBJ accession numbers AB259173 to AB259213.

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distributed in the world and has been isolated in North and South America, Europe, Oceania, and Asia. Genotype 4 has been found exclusively in Asia including China, Japan, Taiwan, and Vietnam. Of interest, there is also a difference in the hosts infected by each genotype: genotypes 1 and 2 have been isolated exclusively from humans thus far, whereas genotypes 3 and 4 have been recovered from not only humans but also several species of animals including pigs, wild boars, a wild deer, and a wild mongoose (Meng et al., 1997; Okamoto et al., 2001; Meng, 2003; Tei et al., 2003; Sonoda et al., 2004; Nishizawa et al., 2005; Nakamura et al., 2006). In some geographic areas where HEV of genotype 1 or 2 is prevalent in humans, HEV of genotype 3 or 4 has been identified in pigs (Arankalle et al., 2002; Cooper et al., 2005).

It was reported that the inter-genotype diversity over the entire genome of HEV is 23.6–27.7% (Zhai et al., 2006), and the intra-genotype diversities of genotypes 1, 3, and 4 are as high as 11.8, 19.3, and 17.0%, respectively (Inoue et al., 2006b). The presence of markedly heterogeneous sequences makes it difficult to design primers suitable for sensitive detection of all four genotypes of HEV by RT-PCR. At present, a total of 70 entire or nearly entire genomic sequences of HEV isolates are available from the GenBank/EMBL/DDBJ databases. When the sequences of previously reported universal primers for the detection of HEV RNA (Erker et al., 1999; Huang et al., 2002; Mizuo et al., 2002; Takahashi et al., 2003a; Jothikumar et al., 2006) were compared with the 70 HEV sequences, a set of primers and a probe designed within the ORF2/ORF3 overlapping region of the HEV genome for real-time RT-PCR (Jothikumar et al., 2006) was the best in view of being positioned in a highly conserved area. However, the amplified product of the real-time RT-PCR is too short for determining the genotype. In the present study, based on the analysis of 70 entire or nearly entire HEV sequences, nested universal primers were designed within the ORF2/ORF3 overlapping region for PCR that was capable of amplifying HEV sequences of all four genotypes and in which the amplified products could be used for HEV genotyping. The validity of this method for sensitively detecting HEV RNA was compared with that of the previous RT-PCR method that amplifies a 457 nt sequence within ORF2 (Mizuo et al., 2002).

2. Materials and methods

2.1. Serum samples

To assess the sensitivity of the newly developed RT-PCR assay by comparison with that of previously described RT-PCR assay (Mizuo et al., 2002), three serum samples were used: the first sample (04-1601) was obtained from a Japanese patient who contracted infection of a genotype 1 HEV while traveling in India; the second sample (03-1367) was obtained from a Japanese individual who acquired subclinical infection of a genotype 3 HEV; the third sample (06-0526) was obtained from a Japanese patient with sporadic acute hepatitis E who was infected with a genotype 4 HEV. Serial 10-fold dilutions with normal human serum were subjected to the two RT-PCR assays. In addition, the sensitivity of the two RT-PCR assays was compared using periodic serum samples that had been collected from five patients (Cases 1–5) with hepatitis E (Kuno et al., 2003; Yazaki et al., 2003; Mizuo et al., 2005). Case 2 was diagnosed with fulminant hepatitis E and the remaining four patients with acute hepatitis E.

To verify the validity of the new RT-PCR assay for detecting HEV strains with significant sequence variations, a total of 41 serum samples that were known to contain various strains of genotypes 1, 3, and 4 identified by the previous RT-PCR assay (Mizuo et al., 2002) were used. Of these, 10 serum samples containing a genotype 1 HEV were obtained from patients with acute hepatitis E in Nepal in 1997–2002 (Shrestha et al., 2004). Serum samples containing HEV genotype 3 were obtained from 10 patients with acute hepatitis E in Japan, whose clinical data were reported previously (Mizuo et al., 2002, 2005; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Mitsui et al., 2005) and nine pigs in different farms in Japan (Takahashi et al., 2003b, 2005). Serum samples containing genotype 4 HEV were obtained from 10 Japanese patients with acute hepatitis E (Yazaki et al., 2003; Hijioka et al., 2005) including a patient with imported hepatitis E who was presumed to have acquired HEV infection while traveling in Vietnam (Koizumi et al., 2004), and from two pigs in different farms in Japan (Takahashi et al., 2003b, 2005).

2.2. Extraction of RNA and RT-PCR

Total RNA was extracted from 100 μl of each serum sample with guanidinium thiocyanate and phenol–chloroform using TRIZOL LS Reagent (Invitrogen, Groningen, The Netherlands). The RNA precipitate was dissolved in 5.5 μl of water containing 20 U of RNaseOUT RNase inhibitor (Invitrogen) and reverse transcribed at 42 °C for 60 min with SuperScript II Reverse Transcriptase (Invitrogen) and antisense primer HE364 (5′-CTG GGM YTG GTC DCG CCA AG-3′ [M = A or C; Y = T or C; D = G, A or T]) in a reaction mixture of 10 μl. The cDNA was heat denatured at 95 °C for 15 min and subjected to nested PCR in the presence of *TaKaRa Ex Taq* (TaKaRa Bio Inc., Shiga, Japan). The first-round PCR was carried out with primers HE361 (sense: 5′-GCR GTG GTT TCT GGG GTG AC-3′ [R = A or G]) and HE364 for 35 cycles (94 °C for 2 min prior to cycling: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s [an additional 7 min in the last cycle]). The second-round PCR for 25 cycles was carried out with primers HE366 (sense: 5′-GYT GAT TCT CAG CCC TTC GC-3′) and HE363 (antisense: 5′-GMY TGG TCD CGC CAA GHG GA-3′ [H = A, T or C]) under the same conditions as the first-round PCR. The size of the amplification product of the first-round PCR was 164 base pairs (bp) (nt 5302–5465), and that of the second-round PCR was 137 bp (nt 5325–5461): nucleotide numbers are in accordance with the HE-JA10 isolate (AB089824). The second-round amplification products were electrophoresed on a 3% (w/v) NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME), stained with ethidium bromide, and photographed under UV light.

The second RT-PCR assay, which amplifies a 457 nt sequence in ORF2, was carried out according to the method described previously (Mizuo et al., 2002). For convenience, the previous