

IV. 研究成果の刊行物・別刷

Hepatitis E Virus (HEV) Strains in Serum Samples Can Replicate Efficiently in Cultured Cells Despite the Coexistence of HEV Antibodies: Characterization of HEV Virions in Blood Circulation[▽]

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We recently developed a cell culture system for hepatitis E virus (HEV) in PLC/PRF/5 and A549 cells, using fecal specimens from HEV-infected patients. Since transfusion-associated hepatitis E has been reported, we examined PLC/PRF/5 and A549 cells for the ability to support replication of HEV in various serum samples obtained from 23 patients with genotype 1, 3, or 4 HEV. HEV progenies emerged in culture media of PLC/PRF/5 cells, regardless of the coexistence of HEV antibodies in serum but dependent on the load of HEV inoculated (31% at 2.0×10^4 copies per well and 100% at $\geq 3.5 \times 10^4$ copies per well), and were successfully passaged in A549 cells. HEV particles in serum, with or without HEV antibodies, banded at a sucrose density of 1.15 to 1.16 g/ml, which was markedly lower than that for HEV particles in feces, at 1.27 to 1.28 g/ml, and were nonneutralizable by immune sera in this cell culture system. An immuno-capture PCR assay of HEV virions treated with or without detergent indicated that HEV particles in serum are associated with lipids and HEV ORF3 protein, similar to those in culture supernatant. By immunoprecipitation, it was found that >90% of HEV particles in the circulation exist as free virions not complexed with immunoglobulins, despite the coexistence of HEV antibodies. These results suggest that our *in vitro* cell culture system can be used for propagation of a wide variety of HEV strains in sera from various infected patients, allowing extended studies on viral replication specific to different HEV strains.

Hepatitis E, an acute viral hepatitis caused by infection with hepatitis E virus (HEV), is a globally distributed human disease. In developing countries of Asia, Africa, and Latin America, where sanitation conditions are not well maintained, HEV infection is transmitted via the fecal-oral route through virus-contaminated water or food, with substantial mortality in pregnant women (7, 33). In industrialized countries, autochthonous hepatitis E is far more common than previously recognized and has a predilection for older men, in whom it causes substantial morbidity and mortality (5, 13, 31, 36, 44). HEV is the sole member of the genus *Hepevirus* within the family *Hepeviridae* (6). It is a single-stranded, positive-sense, polyadenylated RNA molecule of approximately 7.2 kb in size, with short 5'- and 3'-untranslated regions (53). The genomic RNA contains three open reading frames (ORFs). ORF1 encodes nonstructural proteins involved in virus replication and virus protein processing. ORF2 and ORF3 overlap, and the ORF2 and ORF3

proteins are translated from a single bicistronic subgenomic RNA (11, 16). ORF2 encodes a 660-amino-acid (aa) capsid protein. ORF3 encodes a small phosphorylated protein (113 or 114 aa) that is essential for viral infectivity *in vivo* and for virion release (10, 16, 60, 63). The viral capsid protein induces neutralizing antibodies after immunization (8, 14, 26, 51) or during the course of infection (40, 41).

Four major genotypes (genotypes 1 to 4) of HEV have been identified in mammalian species. The viruses in genotypes 1 and 2 are maintained among humans only and are responsible for waterborne epidemics of HEV infection in developing countries. Genotype 3 HEV has been found worldwide, and genotype 4 HEV was isolated in Asia (23, 32, 39). Recent comprehensive molecular and serological studies have led to the consensus that hepatitis E is a zoonosis with a reservoir in pigs and, possibly, a range of other mammals (27, 43, 47, 48, 57, 65). Genotype 3 and 4 HEVs are considered to undergo zoonotic transmission (23, 32). Recently, HEV was found in farmed rabbits in China, possibly representing a novel genotype (66). These rabbits are reared for their fur, and there is, as yet, no evidence of transmission to humans.

Recently, using inocula comprised of fecal suspensions with high HEV loads, originally obtained from Japanese patients

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TABLE 1. Serum samples obtained from hepatitis E patients and inoculated onto cultured cells

Patient no.	Age (yr) and sex ^a	HEV genotype	Sample no.	Days after onset	ALT level (IU/liter)	Relative titer of anti-HEV ORF2/relative titer of anti-HEV ORF3 ^a			HEV RNA titer (copies/ml)
						IgG class	IgM class	IgA class	
1	60 M	4	S1	2	1,036	—/—	—/—	—/—	2.3 × 10 ⁷
2	47 M	3	S2	Unknown	178	—/—	—/—	—/—	1.1 × 10 ⁶
3	35 M	4	S3		829	—/—	—/—	—/—	1.8 × 10 ⁶
4	67 M	3	S4a	15	285	1:9,500/1:200	1:3,300/1:1,000	1:3,100/1:190	3.6 × 10 ⁵
	67 M	3	S4b	22	15	1:13,000/1:340	1:3,200/1:300	1:1,600/1:130	1.7 × 10 ⁵
5	23 M	1	S5	2	4,538	1:4,200/1:110	1:1,600/1:650	1:1,700/1:480	2.8 × 10 ⁶
6	78 F	4	S6	10	1,763	1:340,000/1:210,000	1:7,000/1:260	1:11,000/1:8,900	7.6 × 10 ⁶
7	42 M	3	S7	Unknown	1,364	1:1,500/1:120	1:820/1:170	1:2,000/1:870	9.6 × 10 ⁶
8	44 M	3	S8		1,082	1:7,100/1:3,400	1:30,000/1:1,900	1:5,000/1:2,800	1.1 × 10 ⁷
9	46 M	4	S9	7	4,100	1:88,000/1:6,300	1:550/1:7,900	1:280/1:9,800	5.5 × 10 ⁵
10	47 M	4	S10	7	2,492	1:6,300/1:4,400	1:5,500/1:3,700	1:5,000/1:910	1.7 × 10 ⁷
11	61 M	3	S11	7	1,287	1:37,000/1:7,900	1:13,000/1:110	1:40,000/1:5,000	8.8 × 10 ⁶
12	59 M	4	S12	3	3,660	1:10,000/1:990	1:2,000/1:7,400	1:32,000/1:9,500	3.7 × 10 ⁶
13	52 M	3	S13	2	2,965	—/1:6,300	1:20,000/1:5,600	1:170/1:63,000	1.2 × 10 ⁷
14	61 M	4	S14	0	4,471	1:130/1:1,900	1:200/1:5,000	1:760/1:3,500	3.4 × 10 ⁶
15	63 M	4	S15	7	1,140	1:230,000/1:64,000	1:430/1:5,000	1:3,700/1:7,900	8.1 × 10 ⁵
16	60 M	4	S16	7	1,412	1:22,000/1:4,000	1:28,000/1:10,000	1:7,900/1:5,600	7.0 × 10 ⁵
17	66 M	3	S17	7	1,813	1:14,000/1:1,000	1:3,400/1:1,100	1:7,100/1:1,100	2.2 × 10 ⁶
18	42 M	3	S18	5	1,434	1:400/1:6,600	1:12,000/1:10,000	1:530/1:3,400	7.8 × 10 ⁶
19	46 M	3	S19	7	3,047	1:6,900/1:140	1:3,800/1:610	1:5,000/1:790	1.8 × 10 ⁷
20	56 F	3	S20	4	427	1:6,000/1:330	1:4,000/1:750	1:2,300/1:180	2.6 × 10 ⁶
21	46 F	4	S21	7	1,548	1:8,800/1:3,500	1:6,300/1:20,000	1:40,000/1:2,100	4.1 × 10 ⁷
22	54 M	4	S22	6	3,035	1:3,800/1:120	1:4,500/1:1,100	1:20,000/1:1,000	4.8 × 10 ⁶
23	48 M	4	S23	8	6,102	1:15,000/1:14,000	1:13,000/1:4,300	1:7,900/1:11,000	3.2 × 10 ⁶

^a Relative titers of anti-HEV ORF2 and anti-HEV ORF3 were determined by the methods described in Materials and Methods. —, negative (<1:100) for the indicated class of antibody.
^b F, female; M, male.

who contracted domestic infection of genotype 3 HEV (the JE03-1760F strain) or genotype 4 HEV (the HE-JF5/15F strain), we developed an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells, which yielded the highest HEV load of 10⁸ copies/ml in the culture supernatant, and we successfully propagated six or more generations in serial passages of culture supernatant (22, 55, 56). Infection of humans with HEV via blood transfusion has been reported not only in developing countries (genotype 1) (1, 18) but also in industrialized countries, including Japan (genotypes 3 and 4) (2, 24, 25, 28, 54), suggesting that HEV in serum samples can also be propagated in cultured cells. Therefore, in the present study, we examined whether HEV strains in serum samples obtained from various patients with sporadic acute hepatitis E can replicate in PLC/PRF/5 and A549 cells and produce infectious progenies in culture media, in relation to HEV load, genotype, and coexistence of HEV antibodies. In addition, in an attempt to clarify why HEV strains in sera are infectious in cultured cells despite the presence of HEV antibodies, HEV particles in serum samples were characterized and compared with those in culture supernatant and feces.

MATERIALS AND METHODS

HEV. With informed consent from patients, 24 serum samples (S1 to S3, S4a, S4b, and S5 to S23) with IIEV loads of 1.7 × 10⁵ to 4.1 × 10⁷ copies/ml were collected at the acute phase from 23 patients who contracted domestic or imported infection with a genotype 1, 3, or 4 HEV strain and developed acute hepatitis E during 2001 to 2008 in Japan, and these were used as inocula (Table 1). A fecal suspension containing a high load of wild-type HEV (JE03-1760F strain; 2.0 × 10⁷ copies/ml) (55) and a culture supernatant containing a high load of cell culture-generated JE03-1760F variant (1.2 × 10⁸ copies/ml) (22) were used as the references in this study.

Detection of antibodies to HEV. To detect anti-HEV ORF2 IgG, anti-HEV ORF2 IgM, and anti-HEV ORF2 IgA in serum samples, enzyme-linked immunosorbent assays (ELISAs) were performed using purified recombinant ORF2 protein that had been expressed in silkworm pupae (29), as described previously (49). The optical density (OD) of each sample was read at 450 nm. The cutoff values used for the anti-HEV ORF2 IgG, anti-HEV ORF2 IgM, and anti-HEV ORF2 IgA assays were 0.175, 0.440, and 0.642, respectively (49). Samples with OD values for anti-HEV ORF2 IgG, IgM, or IgA equal to or greater than the respective cutoff value were considered to be positive for anti-HEV ORF2 IgG, IgM, or IgA, respectively. The specificity of the anti-HEV ORF2 assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe, as described previously (49). The relative titers of anti-HEV ORF2 IgG, IgM, and IgA antibodies were determined by end-point ELISA, i.e., the serum dilution that gave the OD value of each cutoff point was estimated by testing multiple dilutions of the serum.

In addition, anti-HEV ORF3 IgG, IgM, and IgA antibodies were detected by an ELISA using the synthetic peptide spORF3-C24, with the sequence CIG ATS PSA PPL PPV VDI PQL GLR R, covering aa 90 to 113 at the C terminus of the ORF3 protein of the JE03-1760F strain (52), and the cutoff value was provisionally set at 0.2 for each class of ORF3 antibodies. The relative titers of anti-HEV ORF3 IgG, IgM, and IgA antibodies were also determined by end-point ELISA, i.e., the serum dilution that gave an OD value of 0.2 was estimated by testing multiple dilutions of the serum.

Detection and sequence analysis of HEV RNA. For detection of HEV RNA, total RNA was extracted from 10 to 100 µl of serum sample or culture supernatant by use of TRIzol-LS reagent (Invitrogen, Carlsbad, CA) and subjected to a nested reverse transcription-PCR (RT-PCR) that amplifies a 457-nucleotide (nt) sequence in ORF2, as described previously (29). The amplification product was sequenced directly on both strands, using a BigDye Terminator v3.1 cycle sequencing kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN, version 1.1.1, from the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan) (17). A phylogenetic tree was constructed by the neighbor-joining method (38), based on the 412-nt ORF2 sequence. Bootstrap values were determined for 1,000 resamplings of the data sets (9).

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TABLE 2. Serum samples used in neutralization test^a

Serum sample	Days (yr) after disease onset ^b	Relative titer of anti-HEV ORF2/relative titer of anti-HEV ORF3 ^c			Genotype of HIEV recovered from patient
		IgG class	IgM class	IgA class	
Pool 1	13–23	1:5,200/1:620	1:2,400/1:140	1:790/1:250	3
Pool 2	37–54	1:7,100/–	1:610/1:110	1:590/–	1
Pool 3	24–36	1:98,000/1:4,300	1:3,500/1:4,600	1:2,000/1:1,900	4
03-2150	40	1:32,000/1:4,000	1:4,200/1:1,200	1:2,400/1:630	3
08-1340	2,088 (5.7)	1:3,400/–	–/–	–/–	3

^a Relative titers of anti-HEV ORF2 and anti-HEV ORF3 were determined by the methods described in Materials and Methods. –, negative (<1:100) for the indicated class of antibody.

^b Pooled sera were obtained during the convalescent phase, on the indicated days after disease onset, from three patients with hepatitis E.

^c All samples were negative for HEV RNA.

Quantitation of HEV RNA. HEV RNA was quantitated by real-time RT-PCR according to a previously described method (55), with slight modifications. In brief, total RNA extracted from 100 μ l of diluted serum sample, fecal supernatant, or culture medium was subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR kit (Qiagen, Tokyo, Japan), a sense primer (5'-GGT GGT TTC TGG GGT GAC-3'), an antisense primer (5'-AGG GGT TGG TGG GAT GAA-3'), and a probe consisting of an oligonucleotide with a 5' reporter dye (6-carboxyfluorescein [FAM]) and a 3' quencher dye (6-carboxytetramethylrhodamine [TAMRA]) (5'-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3') on an Applied Biosystems 7900HT Fast real-time PCR system (Applied Biosystems). The thermal cycler conditions were 50°C for 30 min, 95°C for 15 min, and 50 cycles of 94°C for 15 s, 56°C for 30 s, and 76°C for 30 s.

Cell culture and virus inoculation. PLC/PRF/5 (ATCC CRL-8024) and A549 (ATCC CCL-185) cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/ml of penicillin G, 100 μ g/ml of streptomycin, and 2.5 μ g/ml of amphotericin B (growth medium) at 37°C in a humidified 5% CO₂ atmosphere as described previously (55). For virus infection, cells (5×10^5 cells) in 2 ml of medium were added to wells (diameter of 3.5 cm) of a six-well plate (Iwaki, Tsukuba, Japan) 2 days before virus infection. Monolayers of confluent cultured cells in a six-well plate were washed three times with 1 ml of phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ [PBS(–)] and inoculated with 0.2 ml of serum sample, culture supernatant, or fecal suspension that had been diluted with PBS(–) containing 0.2% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) and filtered through a microfilter with a pore size of 0.22 μ m (Millex-GV; Millipore Corp., Bedford, MA). In passage, 0.2 ml of culture supernatant that had been filtered through a 0.22- μ m microfilter was inoculated on a monolayer of PLC/PRF/5 or A549 cells. After being inoculated at room temperature for 1 h, the solution was removed and 2 ml of maintenance medium was added. The maintenance medium used for virus culture was a 1:1 mixture of DMEM and medium 199 (Invitrogen) containing 2% (vol/vol) heat-inactivated FBS and 30 mM MgCl₂ (final concentration); other supplements were the same as those in the growth medium. Culture was performed at 35.5°C in a humidified 5% CO₂ atmosphere. On the day following inoculation, the inoculated cells were washed five times with 1 ml of PBS(–), and thereafter, 2 ml of maintenance medium was added. Then, every other day from day 2 after inoculation, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium, and harvested media were stored at –80°C until virus titration. The infected tissue culture microplates were examined daily under an inverted microscope for specific cytopathic effect (CPE).

Neutralization test. Serum samples that were positive for the IgG, IgM, and IgA classes of anti-HEV antibodies (pool 1 to pool 3 and sample 03-2150) were obtained during the convalescent phase from four patients with sporadic acute hepatitis E (Table 2). One additional serum sample (08-1340), containing only the IgG class of anti-HEV antibody, was obtained 5.7 years after disease onset from a patient with HEV infection, from whom the serum sample 03-2150 and fecal specimen JE03-1760F had been obtained (50). All serum samples used for the neutralization test were negative for HEV RNA.

A solution of 0.2 ml containing the same amount of HEV RNA (serum sample, fecal suspension, or culture supernatant; 2.0×10^4 or 5.0×10^4 copies) and each of various antibody-positive serum samples diluted at 1:2.5, 1:25, or 1:250 with PBS(–) containing 0.2% BSA as well as anti-ORF2 monoclonal antibody (MAb) (H6225) (51), anti-ORF3 MAb (TA0536) (52), or negative-control MAb (905) (46) at a concentration of 2 mg/ml, was incubated at room temperature for 1 h and then inoculated onto monolayers of A549 cells in a

six-well microplate. In addition, a solution of 0.2 ml containing the same amount of culture supernatant treated with lipid solvent and/or protease, as well as anti-ORF2 MAb (H6225; 0.2 or 2 mg/ml) (51), anti-ORF3 MAb (TA0536; 0.2 or 2 mg/ml) (52), and antibody-positive serum sample pool 1 diluted 1:25 or 1:250, was incubated at room temperature for 1 h and then inoculated onto A549 cells. After 1 h, the supernatant was removed and 2 ml of maintenance medium was added. The protocol after infection and the maintenance of cell culture were performed as described above.

Equilibrium centrifugation in sucrose density gradient. A density gradient was prepared in an SW60Ti tube (Beckman Coulter Inc., Fullerton, CA) (0.8 ml of 60% [wt/wt] sucrose, 0.6 ml each of 50%, 40%, 30%, and 20% [wt/wt] sucrose, and 0.3 ml of 10% [wt/wt] sucrose in Tris-HCl buffer [0.01 M, pH 7.5] supplemented with 1 mM EDTA and 150 mM NaCl [TEN]). One hundred microliters each of serum sample, a fecal suspension containing the JE03-1760F strain, and a culture supernatant containing a cell culture-generated JE03-1760F variant, as well as culture supernatants treated with 5% (vol/vol) Tween 20, 10% (vol/vol) chloroform, or a solution containing 0.1% (vol/vol) NP-40, 0.1% (vol/vol) 2-mercaptoethanol (2-ME), and/or 0.1% (wt/vol) pronase E, was layered onto the surface of the gradient and overlaid with 0.4 ml of TEN. The tube was centrifuged at $179,200 \times g$ at 10°C for 48 h, and 150- μ l fractions were then recovered from the surface. The density of each fraction was measured by refractometry.

Immuno-capture RT-PCR assay. The immuno-capture RT-PCR assay was performed by a previously described method (51). Briefly, anti-ORF2 MAb (H6225), anti-ORF3 MAb (TA0536), anti-human IgG MAb (G19) (Institute of Immunology Co. Ltd., Tokyo, Japan), anti-human IgM MAb (M49) (58), anti-human IgA MAb (A13) (30), and MAb 905, raised against hepatitis B virus (HBV) e antigen (46) and used as a negative control, were used in this assay. These MAbs were biotinylated and purified by a previously described method (51). The wells of a Reacti-Bind high-binding-capacity streptavidin-coated plate (Thermo Fisher Scientific Inc., Pittsburgh, PA) were washed with saline, and 100 μ l of 1 μ g/ml biotinylated MAb in PBS containing 0.1% BSA was added to each well. The wells were incubated with gentle shaking at room temperature for 1 h and were then washed with saline. One hundred microliters of each virus sample was added to each well, incubated with gentle shaking at room temperature for 2 h, and then left standing at 4°C overnight. The solution in each well was removed, and the wells were washed with saline. The RNA in each well was then extracted with TRIzol-LS reagent and subjected to quantitative detection of HEV RNA. In addition, to examine whether prior treatment with detergents and/or protease affects the binding efficiency of MAbs and HIEV particles, 5 μ l of serum sample diluted 1:50 with TEN, of culture supernatant, or of fecal suspension was mixed with 50 μ l of Tween 20 (0.22% or 5.5%) or a solution containing 0.11% NP-40, 0.11% 2-ME, and/or 0.11% pronase E, incubated at 37°C for 2 h, diluted 1:100 with PBS containing 0.1% BSA, and then subjected to the immuno-capture RT-PCR assay as described above.

Immunoprecipitation of HEV particles. HEV particles complexed with immunoglobulins were detected as follows. A 50- μ l aliquot of test serum diluted 1:200 with saline was added to 50 μ l of goat antiserum to human IgG/IgM/IgA (whole molecule) (ICN Pharmaceuticals Inc., Aurora, OH) or to normal goat serum (Chemicon International Inc., Temecula, CA). As a control, fecal supernatant mixed with an immune serum (03-2150) was added to goat antiserum to human IgG/IgM/IgA or to normal goat serum. Each mixture was incubated at 37°C for 1 h and at 4°C overnight and then separated into precipitate and supernatant fractions by centrifugation (at $1,600 \times g$ at 4°C for 5 min). HEV RNA in the precipitate fraction was quantitated.

TABLE 3. Detection of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with serum samples at the indicated HEV RNA titers

Sample no.	HEV RNA titer in inoculum (copies per well)	Detectability of HEV RNA ^a	HEV RNA titer at 30 dpi (copies/ml) ^b	HEV isolate in culture medium
S1	2.2 × 10 ⁶	+	3.7 × 10 ⁶	S1_p0
S2	7.2 × 10 ⁴	+	1.8 × 10 ⁵	S2_p0
S3	1.5 × 10 ⁵	+	8.9 × 10 ⁵	S3_p0
S4a	5.8 × 10 ⁴	+	8.3 × 10 ⁴	S4a-1_p0
	2.0 × 10 ⁴	+	3.4 × 10 ⁴	S4a-2_p0
S4b	2.0 × 10 ⁴	+	5.0 × 10 ³	S4b_p0
S5	1.5 × 10 ⁶	+	2.9 × 10 ⁵	S5_p0
S6	3.0 × 10 ⁶	+	5.8 × 10 ⁵	S6_p0
S7	2.6 × 10 ⁶	+	7.2 × 10 ⁶	S7-1_p0
	5.8 × 10 ⁵	+	2.6 × 10 ⁶	S7-2_p0
	3.5 × 10 ⁴	+	2.0 × 10 ⁵	S7-3_p0
S8	3.8 × 10 ⁵	+	1.7 × 10 ⁶	S8_p0
	2.0 × 10 ⁴	—	NA	
S9	2.0 × 10 ⁴	—	NA	
S10	2.0 × 10 ⁴	—	NA	
S11	1.9 × 10 ⁵	+	6.0 × 10 ⁶	S11_p0
	2.0 × 10 ⁴	—	NA	
S12	2.0 × 10 ⁴	—	NA	
S13	2.0 × 10 ⁴	+	4.3 × 10 ⁴	S13_p0
S14	4.4 × 10 ⁵	+	6.7 × 10 ⁴	S14_p0
	2.0 × 10 ⁴	—	NA	
S15	2.0 × 10 ⁴	—	NA	
S16	2.0 × 10 ⁴	—	NA	
S17	2.0 × 10 ⁴	+	4.7 × 10 ³	S17_p0
S18	2.0 × 10 ⁴	+	1.7 × 10 ⁴	S18_p0
S19	3.8 × 10 ⁵	+	7.4 × 10 ⁴	S19_p0
	2.0 × 10 ⁴	—	NA	
S20	1.5 × 10 ⁵	+	1.9 × 10 ⁵	S20_p0
	2.0 × 10 ⁴	—	NA	
S21	2.0 × 10 ⁴	—	NA	
S22	4.4 × 10 ⁵	+	6.7 × 10 ⁴	S22_p0
S23	1.6 × 10 ⁵	+	5.3 × 10 ³	S23_p0

^a —, negative for HEV RNA during the observation period (0 to 30 dpi).
^b NA, not applicable.

Nucleotide sequence accession numbers. The nucleotide sequences of HEV isolates determined in the present study have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: AB525039 to AB525077.

RESULTS

Inoculation of serum HEV onto PLC/PRF/5 or A549 cells. Three serum samples (S1 to S3) containing an HEV strain of genotype 3 or 4, but without HEV antibodies, were inoculated onto fresh monolayers of PLC/PRF/5 cells in a six-well microplate at a viral load of 7.2 × 10⁴ to 2.2 × 10⁶ copies per well (Table 3). In the primary propagation (passage 0), HEV RNA was first detected in the culture medium of PLC/PRF/5 cells on the 4th to 6th day postinoculation (dpi), with a viral load of 1.8 × 10² to 7.2 × 10³ copies/ml, and continued to increase thereafter, with a maximum load of 1.8 × 10⁵ to 3.7 × 10⁶ copies/ml on 30 dpi (Fig. 1a). When 21 other HEV RNA-positive serum samples (S4a, S4b, and S5 to S23) that tested simultaneously positive for IgG, IgM, and IgA classes of HEV ORF2 and ORF3 antibodies were inoculated onto PLC/PRF/5 cells (7 samples were inoculated at two or three distinct viral loads), HEV progenies were released into the culture medium in all 13 samples upon inoculation at an HEV load of 3.5 × 10⁴

to 3.0 × 10⁶ copies per well and in 5 (31%) of 16 samples upon inoculation at an HEV load of 2.0 × 10⁴ copies per well. At 30 dpi, the HEV load in the culture supernatant was high, at 2.9 × 10⁵ to 7.2 × 10⁶ (median, 5.8 × 10⁵) copies/ml, upon inoculation of HEV on the order of 10⁶ copies per well (Fig. 1b), while it was 5.3 × 10³ to 6.0 × 10⁵ (median, 1.3 × 10⁵) copies/ml upon inoculation of HEV on the order of 10⁵ copies per well (Fig. 1c). When inoculated at 2.0 × 10⁴ to 5.8 × 10⁴ copies per well, the viral load in the culture supernatant reached only 4.7 × 10³ to 2.0 × 10⁵ (median, 3.4 × 10⁴) copies/ml on 30 dpi (Fig. 1d).

The serum-derived wild-type HEV and its cell culture-generated variant were 100% identical in nucleotide sequence for 412 nt within the ORF2 gene for all 21 pairs (Fig. 1a to d). The phylogenetic tree constructed based on the 412-nt ORF2 sequence depicted that each pair of the wild type and its cell culture-produced variant segregated into a cluster within genotype 1, 3, or 4, with a bootstrap value of 100% (Fig. 2), confirming that various HEV strains of serum origin were successfully propagated in cultured cells.

When representative serum samples, including 3 antibody-negative (S1 to S3) and 6 antibody-positive (S4a, S7, S13, S17, S18, and S20) samples, were inoculated onto A549 cells at an HEV load ranging from 1.0 × 10⁴ copies per well to 1.9 × 10⁶ copies per well, HEV progenies emerged in the culture medium in all samples tested, regardless of the presence or absence of HEV antibodies in the serum samples (Fig. 1e). It was notable that during primary propagation, CPE was not observed in the PLC/PRF/5 and A549 cells, despite differences in the titer of the inoculum and HEV load in each inoculation.

Supernatant passage of HEV in A549 cells. When HEV progenies in randomly selected culture supernatants of primary propagation (passage 0) were inoculated onto PLC/PRF/5 and A549 cells at a load of 1.0 × 10⁴ copies per well, A549 cells supported more efficient multiplication of HEV than did PLC/PRF/5 cells. Therefore, in passage, A549 cells were used as host cells for virus culture in place of PLC/PRF/5 cells. All HEV progenies (S1_p0 to S3_p0, S4a_p0, S4b_p0, and S5_p0 to S23_p0) released into the culture medium of PLC/PRF/5 cells that had been inoculated with serum samples (S1 to S3, S4a, S4b, and S5 to S23, respectively) (Table 3) were successfully passaged in A549 cells, as depicted in Fig. 1f for representative progenies. Although inoculated at various HEV loads, ranging from 4.6 × 10³ copies per well to 6.4 × 10⁶ copies per well, HEV RNA in the passage appeared at 2 to 6 dpi and reached a maximum load of 5.3 × 10⁴ to 6.6 × 10⁶ copies/ml at 30 dpi in the culture medium of A549 cells (Fig. 1f). During the passage, CPE was not observed in the A549 cells, despite differences in the changing profile of HEV load in each passage.

Neutralization of serum HEV by anti-HEV immune sera or MAbs. Three serum samples (pool 1 to pool 3) (Table 2), containing IgG, IgM, and IgA classes of anti-HEV antibodies obtained from patients who had contracted infection with HEV genotypes 3, 1, and 4, respectively, were tested for the ability to neutralize a serum-derived HEV strain of genotype 3 without concurrent HEV antibodies (S1) in the present cell culture system. One hundred microliters of each serum pool (pool 1 to pool 3), diluted 1:2.5 with PBS(–), was mixed with an equal volume of the diluted serum (S1) containing 5.0 × 10⁴ copies of HEV, kept

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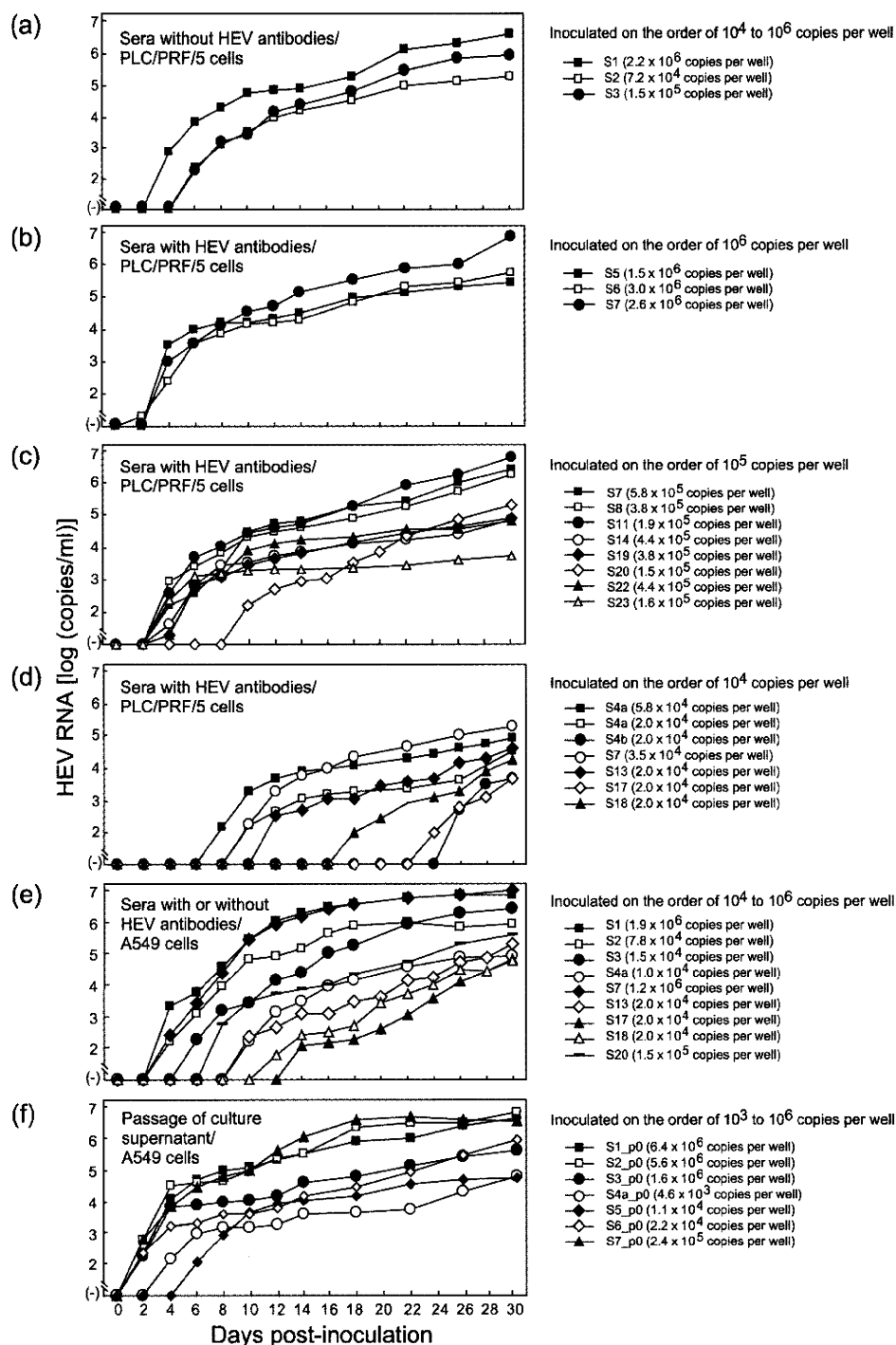


FIG. 1. Quantitation of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells inoculated with serum samples, with or without HEV antibodies (passage 0) and at various HEV loads (10^4 to 10^6 copies per well) (a to e), or in culture medium of passage 0 (f). (f) The harvested culture supernatant of passage 0 was purified by passage through a microfilter with a pore size of $0.22 \mu\text{m}$ (see Materials and Methods) and then inoculated onto fresh A549 cells.

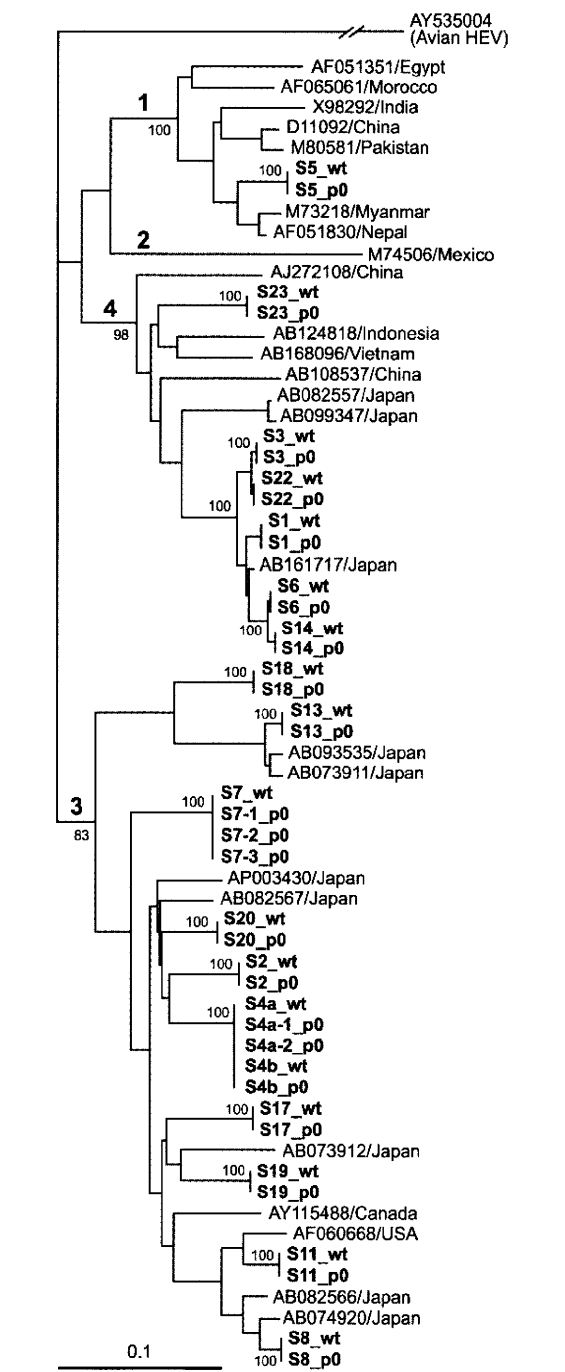


FIG. 2. Phylogenetic tree constructed by the neighbor-joining method, based on the partial nucleotide sequence of the ORF2 region (412 nt) of 64 HEV isolates, using an avian HEV (GenBank accession no. AY535004) as an outgroup. In addition to 39 HEV isolates obtained in the present study, which are indicated in bold, 24 representative HEV isolates of genotypes 1 to 4 whose common 412-nt sequence is known were included for comparison, and their accession numbers, followed by the names of the countries where they were isolated, are indicated. "wt" denotes an HEV isolate recovered from a

at room temperature for 1 h, and then inoculated onto monolayers of A549 cells in a six-well microplate. In each well containing a sample from pool 1, pool 2, or pool 3, HEV RNA was first detectable at 12 dpi and continued to increase, reaching 10^6 copies/ml at 50 dpi, as in wells with the control serum (anti-HEV-negative serum) (Fig. 3a). Of note, HEV particles in feces were neutralized by each serum pool (pool 1 to pool 3), even diluted 1:250, indicating that HEV particles in serum are nonneutralizable by immune sera with a 100-fold-higher concentration of anti-HEV antibodies in this cell culture system. In addition, HEV particles in the serum samples (S1) were not neutralized by anti-ORF2 MAb (H6225) or anti-ORF3 MAb (TA0536), even at a final concentration of 1 mg/ml (Fig. 3b).

To confirm this observation, 100 μ l each of serum samples containing IgG, IgM, and IgA classes of anti-HEV antibodies (03-2150 in Table 2) or IgG-class anti-HEV only (08-1340), diluted 1:2.5, both of which were obtained from the JE03-1760F patient (patient 4), 40 days and 5.7 years, respectively, after disease onset, was mixed with an equal volume of diluted inoculum of JE03-1760F origin (2.0×10^4 copies per well) and kept at room temperature for 1 h. When a fecal suspension containing the JE03-1760F strain was used as an inoculum and mixed with serum 03-2150 or 08-1340, the harvested culture supernatants were negative for HEV RNA throughout the observation period of 50 days, although HEV RNA appeared at 12 dpi and increased thereafter (1.8×10^6 copies/ml at 50 dpi) when mixed with the control serum (anti-HEV-negative serum), indicating that sera 03-2150 and 08-1340 have the ability to neutralize fecal HEV in this cell culture system (Fig. 3c). However, when serum S4a, obtained from patient 4 15 days after disease onset, was used as an inoculum, HEV RNA became detectable in the culture supernatant on the 10th day and thereafter, even with prior incubation with serum 03-2150 or 08-1340. Similarly, when the cell culture-generated JE03-1760F variant was used as an inoculum, the harvested culture supernatants were positive for HEV RNA on the 6th day after inoculation (Fig. 3c). These results indicate that immune sera have little or no ability to neutralize even HEV particles of the same strain in serum and culture supernatant.

Buoyant density of serum HEV. In an attempt to clarify why HEV in serum was nonneutralizable, HEV RNA-positive serum samples, a fecal suspension containing the JE03-1760F strain, and a culture supernatant containing the cell culture-produced JE03-1760F variant were subjected to ultracentrifugation in a sucrose density gradient. Like the case for the HEV progenies in culture medium, HEV particles in serum samples, with or without HEV antibodies, banded at a density of 1.15 to 1.16 g/ml, which was markedly lower than that for HEV particles in feces, at 1.27 to 1.28 g/ml (Fig. 4), suggesting that HEV particles in serum and culture supernatant are associated with a very-low-density material, possibly lipids.

When HEV particles in the peak fraction from the ultracen-

serum sample that was used to inoculate cells, and "p0" represents an HEV isolate obtained from culture supernatant harvested 30 days after inoculation of the corresponding serum sample. Bootstrap values are indicated for the major nodes as percentages of the data obtained from 1,000 resamplings. Bar, 0.1 nucleotide substitution per site.

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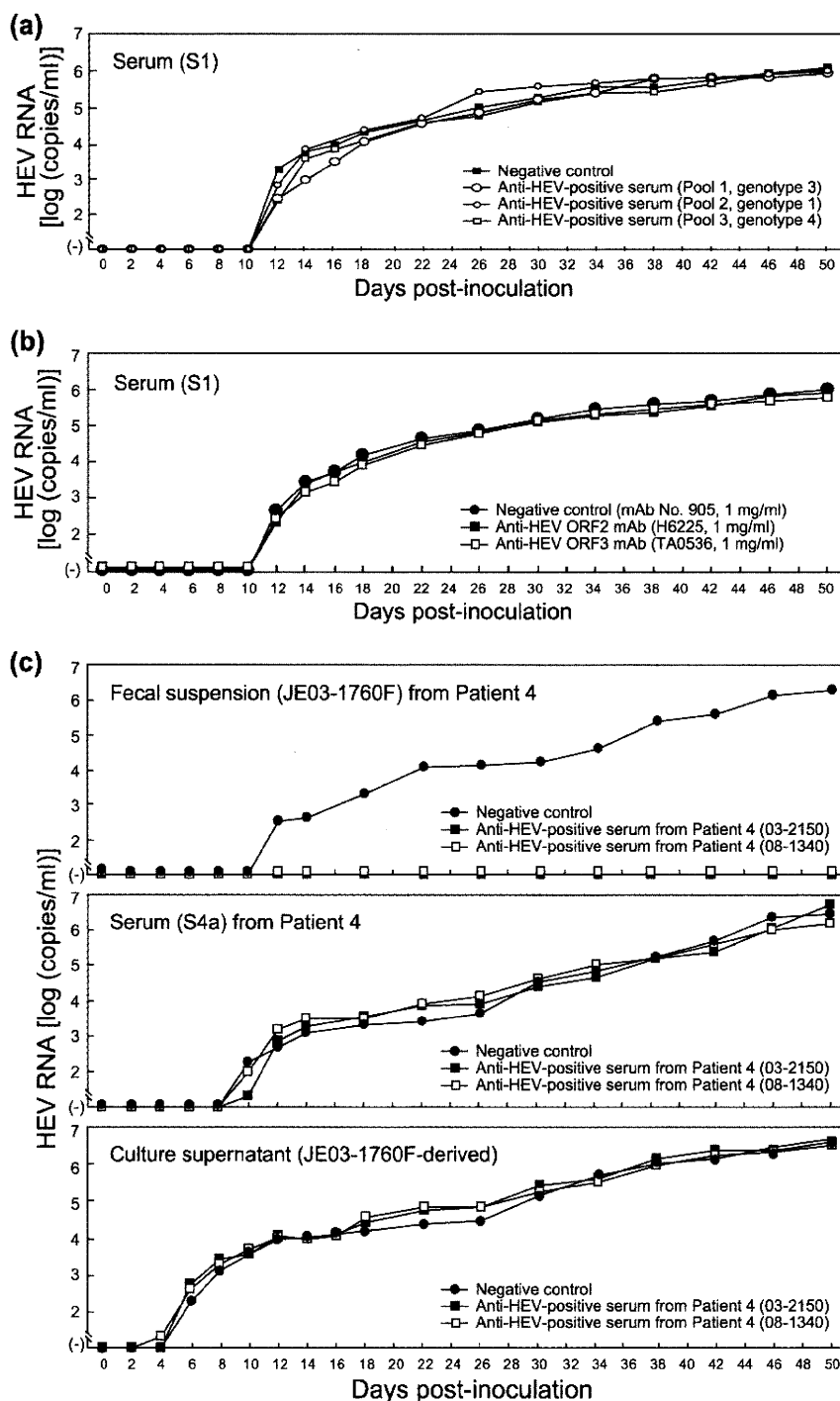


FIG. 3. (a) Quantitation of HEV RNA in culture supernatants of A549 cells inoculated with a serum sample (S1) with a viral load of 5.0×10^5 copies/ml that had been mixed with a pooled serum sample obtained during the early convalescent phase (pool 1, 2, or 3) and positive for IgM-, IgA-, and IgG-class anti-HEV antibodies or with an antibody-negative serum and cultured for 50 days. (b) Quantitation of HEV RNA in culture supernatants of A549 cells inoculated with a serum sample (S1) with a viral load of 5.0×10^5 copies/ml that had been mixed with an anti-ORF2 MAb (H6225) (S1), anti-ORF3 MAb (TA0536) (S2), or negative-control MAb (905) (46) at a final concentration of 1 mg/ml and cultured for 50 days. (c) Quantitation of HEV RNA in culture supernatants of A549 cells inoculated with a fecal suspension (JE03-1760F), a serum sample (S4a), or a culture supernatant of JE03-1760F (patient 4) origin with an HEV load of 2.0×10^5 copies/ml that had been mixed with a serum sample

trifugation procedure that were derived from a serum sample (S7), a culture supernatant, and a fecal suspension (JE03-1760F) were inoculated onto A549 cells at HEV loads of 1.0×10^4 , 1.0×10^5 , and 1.2×10^4 copies per well, respectively, the appearance and increase of the HEV progenies in the culture supernatant were nearly identical to those of the respective original samples (Fig. 5), indicating that HEV particles in serum and culture supernatant banding at a density of 1.15 to 1.16 g/ml are infectious in cell culture and that the peak fraction from ultracentrifugation contains infectious particles corresponding to those in the original, unfractionated sample, like the case for those in feces, banding at 1.27 to 1.28 g/ml.

Association of serum HEV with human immunoglobulins. To characterize the association of HEV particles with human immunoglobulins in the sera of HEV-infected patients, the HEV virions in the serum samples (S1 to S3, S6, S7, S19, and S21), with or without circulating HEV antibodies, were subjected to immunoprecipitation with goat anti-human IgG/IgM/IgA, and HEV RNA was quantitated in the precipitate fraction. Only $\leq 8.1\%$ of the PCR signals were recovered from the precipitate fraction (Table 4), suggesting that HEV exists in the circulation predominantly as free virions, even in the presence of high-titer HEV antibodies. To confirm this finding, immunocapture RT-PCR was performed to detect HEV virions in the peak fraction from sucrose density gradient ultracentrifugation, using anti-human IgG/IgM/IgA MAbs. Only $\leq 8.5\%$ of viral particles in serum were captured by anti-human IgG/IgM/IgA MAbs, despite the presence of HEV antibodies (Table 5), indicating that the great majority of HEV particles in the circulation exist as free virions not complexed with IgG, IgM, or IgA.

Association of serum HEV with lipids and ORF3 protein. To examine whether HEV particles in serum are associated with lipids and HEV ORF3 protein, similar to those in culture supernatant (63), the immuno-capture RT-PCR assay was performed using anti-ORF2 MAb (H6225) and anti-ORF3 MAb (TA0536) (Table 6). Like the case for fecal HEV, although 92.2 to 93.1% of viral particles were captured by anti-ORF2 MAb, no virus was captured by anti-ORF3 MAb, regardless of the presence or absence of 0.2% Tween 20. On the other hand, when viral particles in a viremic serum (S1) and a culture supernatant of HEV-infected PLC/PRF/5 cells were tested, they were both captured by anti-ORF2 and anti-ORF3 MAbs, although only partially (16.5 to 38.5%) in the presence of 0.2% Tween 20. Of note, upon treatment with 5% Tween 20, the efficiency of binding of HEV particles in serum and culture supernatant by anti-ORF2 MAb increased to 55.7% and 69.0%, respectively, and that by anti-ORF3 MAb increased to 90.4% and 76.8%, respectively. However, the buoyant density of HEV particles treated with 5% Tween 20, even after treatment with 10% chloroform, did not shift to 1.27 to 1.28 g/ml, corresponding to that of fecal HEV: the Tween 20-treated HEV particles were distributed at 1.17 to 1.29 g/ml, peaking at

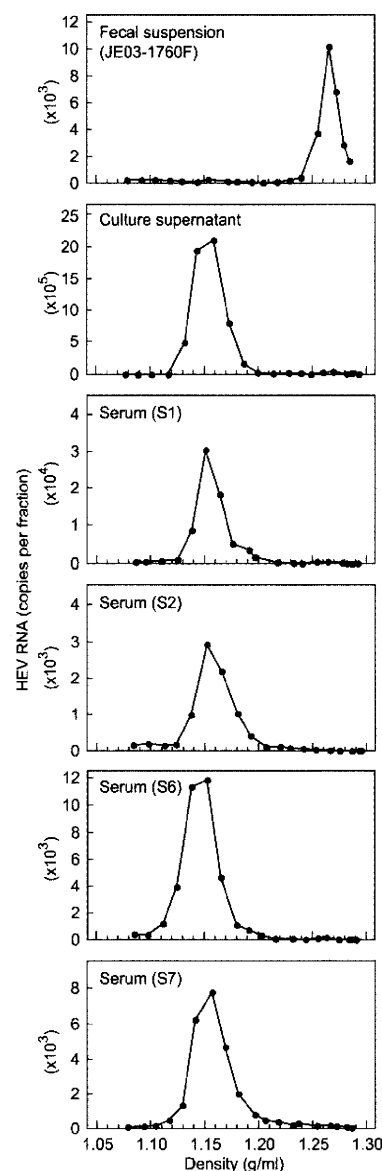


FIG. 4. Sucrose density gradient fractionation of HEV in a fecal specimen (JE03-1760F) and in a culture supernatant containing cell culture-produced HEV, as controls, and in serum samples with (S6 and S7) and without (S1 and S2) HEV antibodies.

1.23 g/ml, and the chloroform-treated HEV particles were found at 1.19 to 1.27 g/ml, with a peak density of 1.23 g/ml (Fig. 6). Next, 5 μ l of the HEV RNA-positive serum sample (S1), diluted 1:50 with TEN, was mixed with 50 μ l of TEN buffer

(03-2150) positive for IgM-, IgA-, and IgG-class anti-HEV antibodies but negative for HEV RNA, another serum sample (08-1340) positive only for IgG anti-HEV, or an antibody-negative serum and cultured for the indicated number of days. The fecal suspension, serum sample (S4a), and culture supernatant contained HEV of the same strain (JE03-1760F), and serum samples (S4a, 03-2150, and 08-1340) were obtained from the JE03-1760F patient (patient 4 in this study) 15, 40, and 2,088 days, respectively, after disease onset.

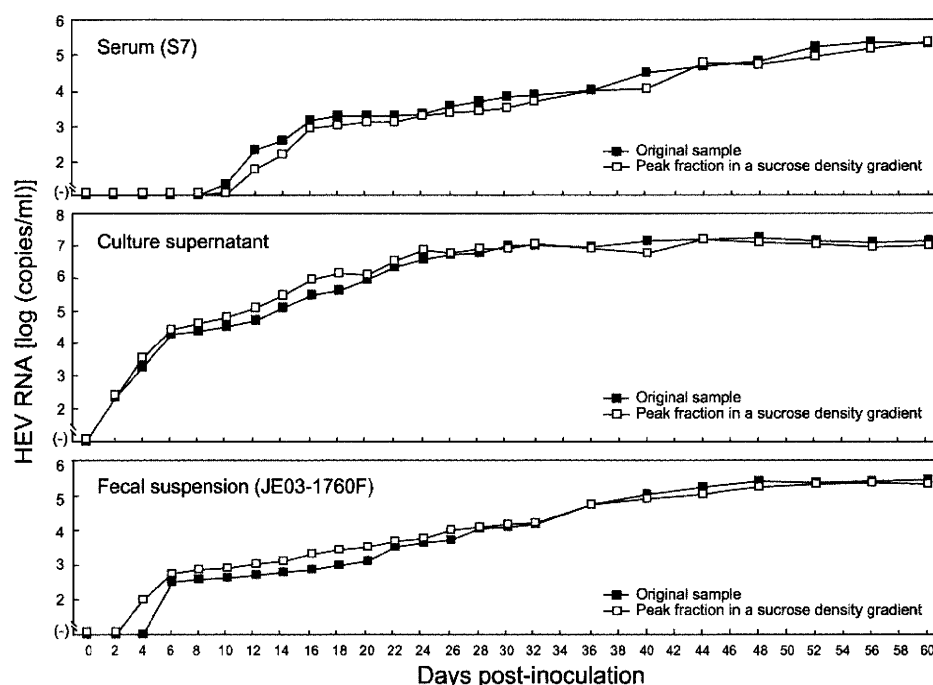


FIG. 5. Comparison of HEV RNA titers in culture supernatants of cultured cells obtained during the observation period of 60 days after inoculation between an original serum sample (S7) and its peak fraction in a sucrose density gradient, between an original culture supernatant and its peak fraction in a density gradient, and between an original fecal suspension (JE03-1760F) and its peak fraction in a density gradient (Fig. 4). The loads of HEV inoculated were 1.0×10^4 copies per well for the serum sample and its peak fraction, 1.0×10^5 copies per well for the culture supernatant and its peak fraction, and 1.2×10^5 copies per well for the fecal specimen and its peak fraction.

containing 0.1% NP-40, 0.1% 2-ME, and 0.1% pronase E and incubated at 37°C for 2 h, a method reported to liberate core particles from HBV virions (45). The efficiency of binding of HEV particles in serum by anti-ORF2 MAb increased substan-

TABLE 4. Immunoprecipitation of HEV particles in serum with goat serum against human IgG/IgM/IgA

Sample with HEV RNA (copies/test)	Immunoprecipitation (%) with:	
	Goat anti-human IgG/IgM/IgA	Normal goat serum
HEV RNA-positive sera ^a		
Anti-HEV-negative sera		
S1 (5.8×10^3)	3.5	1.7
S2 (2.8×10^2)	6.0	0.0
S3 (4.5×10^2)	0.9	0.0
Anti-HEV-positive sera		
S6 (1.9×10^3)	5.4	1.8
S7 (2.4×10^3)	5.4	0.4
S19 (4.5×10^3)	8.1	0.4
S21 (1.0×10^4)	2.4	1.0
Fecal suspension (7.0×10^4) mixed with serum sample 03-2150 ^b	98.2	0.0

^a See Table 1.

^b A fecal suspension containing HEV was mixed with a serum sample containing IgG, IgM, and IgA classes of HEV antibodies (see Table 2) prior to immunoprecipitation.

tially, to 86.3%, and that by anti-ORF3 MAb decreased to 0%. The buoyant density of the treated HEV particles in serum shifted to 1.27 g/ml, similar to that of fecal HEV particles as well as cell culture-generated HEV particles that had been treated with NP-40, 2-ME, and pronase E. Upon treatment with NP-40 and pronase E (without the addition of 2-ME), a similar finding was obtained (Fig. 6). These results suggest that serum-derived HEV particles are associated with lipids and ORF3 protein, similar to those in culture supernatant.

Neutralization of HEV particles in cell culture treated with lipid solvents and/or protease by an anti-HEV immune serum or MAb (anti-ORF2 or anti-ORF3). To examine whether the HEV particles in a cell culture treated with lipid solvent can be neutralized by an anti-HEV immune serum or MAb, 100 μ l of a serum pool (pool 1; diluted 1:25 or 1:250), anti-ORF2 MAb (H6225; 0.2 or 2 mg/ml), or anti-ORF3 MAb (TA0536; 0.2 or 2 mg/ml) was mixed with an equal volume of the diluted culture supernatant (treated with 5% Tween 20 and containing 2.4×10^4 copies of HEV), kept at room temperature for 1 h, and then inoculated onto A549 cells in a six-well microplate. The treated HEV particles replicated as efficiently as those without detergent treatment and were neutralized partially by anti-HEV immune serum, anti-ORF2 MAb, and anti-ORF3 MAb, in an antibody dose-dependent manner (Table 7).

To further explore whether cell culture-produced HEV particles treated with detergent and protease, with a buoyant density of 1.27 to 1.28 g/ml in sucrose, can be neutralized by an

TABLE 5. Bound HEV after immuno-capture by anti-human IgG, IgM, and IgA MAbs			
Serum or fecal sample	Total copy no. of HEV applied to each well	% of captured HEV in total HEV per well	
		Anti-human IgG/IgM/IgA MAbs (G19/M49/A13)	Negative control (MAb 905)
HEV RNA-positive sera ^a			
Anti-HEV-negative sera			
S1	2.5×10^3	1.7	0.0
S2	2.7×10^2	2.1	0.0
S3	3.5×10^2	1.8	0.0
Anti-HEV-positive sera			
S4a	2.9×10^2	0.9	0.0
S6	6.5×10^3	8.5	0.2
S7	3.7×10^3	6.6	0.5
Fecal suspension mixed with serum sample 03-2150 ^b	7.8×10^3	84.4	0.1

^a See Table 1.
^b A fecal suspension containing HEV was mixed with a serum sample (03-2150) containing IgG, IgM, and IgA classes of HEV antibodies (see Table 2) prior to immuno-capture PCR assay.

anti-HEV immune serum, 100 µl of immune serum (pool 1; diluted 1:25) was mixed with an equal volume of the culture supernatant (containing 1.0 × 10⁴ copies of HEV that had been treated with 0.1% NP-40 and 0.1% pronase E and diluted 1:100), kept at room temperature for 1 h, and then inoculated onto A549 cells. When cells were inoculated with the treated HEV particles, HEV RNA was first detected in the culture medium at 4 dpi and continued to be detectable up to the end of the observation period of 30 days, reaching a maximum load of 6.3 × 10⁶ copies/ml, similar to the case for particles without treatment (Fig. 7). In contrast, in wells inoculated with the treated HEV particles that had been incubated with an im-

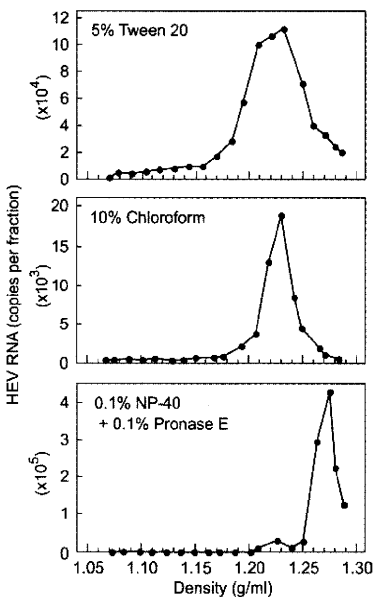


FIG. 6. Sucrose density gradient fractionation of HEV in culture supernatants treated with 5% Tween 20, 10% chloroform, or a solution containing 0.1% NP-40 and 0.1% pronase E.

mune serum, HEV RNA was not detectable until 30 days, indicating that virions treated with lipid solvent and protease, possessing the same character as virions from feces, are neutralizable by anti-HEV immune sera.

DISCUSSION

Only a limited number of HEV strains of genotype 3 (JE03-1760F) and genotype 4 (HE-JF5/15F) in fecal specimens have been propagated efficiently in PLC/PRF/5 and A549 cells (55,

Treatment	% of captured HEV in total HEV per well					
	Serum		Culture supernatant		Feces	
	Anti-ORF2 MAb (H6225)	Anti-ORF3 MAb (TA0536)	Anti-ORF2 MAb (H6225)	Anti-ORF3 MAb (TA0536)	Anti-ORF2 MAb (H6225)	Anti-ORF3 MAb (TA0536)
Tween 20						
0.2%	38.5	37.6	22.1	16.5	92.2	0.0
5%	55.7	90.4	69.0	76.8	95.7	0.4
10% Chloroform						
25°C, 10 min	28.4	64.7	87.3	54.4	92.0	0.1
37°C, 30 min	35.7	21.5	62.9	9.5	99.2	0.0
NP-40 + 2-ME + pronase E ^a						
NP-40 + 2-ME ^a	86.3	0.0	98.7	0.2	99.9	1.3
NP-40 + pronase E ^a	78.3	84.2	97.2	77.9	99.8	0.4
NP-40 + pronase E ^a	90.4	0.6	99.0	0.2	99.4	0.0
None	1.0	1.5	6.3	5.7	93.1	0.0

^a Treated with 0.1% NP-40, 0.1% 2-ME, and/or 0.1% pronase E.

TABLE 7. Neutralization of cell culture-generated HEV particles treated with 5% Tween 20 by an anti-HEV immune serum or MAb (anti-ORF2 or anti-ORF3)

Days after inoculation	HEV RNA titer (copies/ml) in culture supernatant ^a							
	Nontreated HEV (without antibody) ^b	5% Tween 20-treated HEV						
		Without antibody ^b	Immune serum (pool 1)		Anti-ORF2 MAb (II6225)		Anti-ORF3 MAb (TA0536)	
			1:50	1:500	1 mg/ml ^c	0.1 mg/ml ^c	1 mg/ml ^c	0.1 mg/ml ^c
0	—	—	—	—	—	—	—	—
2	20	40	—	—	—	—	—	—
4	9.9 × 10 ³	7.5 × 10 ³	—	—	—	—	—	160
6	5.7 × 10 ⁴	3.1 × 10 ⁴	—	60	—	160	120	3.3 × 10 ⁴
8	2.5 × 10 ⁵	6.1 × 10 ⁴	40	160	—	760	3.6 × 10 ³	8.8 × 10 ⁴
10	9.9 × 10 ⁵	7.5 × 10 ⁵	180	720	—	1.0 × 10 ³	1.7 × 10 ⁴	3.4 × 10 ⁵
12	2.8 × 10 ⁶	2.2 × 10 ⁶	660	3.4 × 10 ³	140	1.1 × 10 ⁴	2.0 × 10 ⁴	9.4 × 10 ⁵
14	5.9 × 10 ⁶	5.5 × 10 ⁶	1.1 × 10 ³	2.1 × 10 ⁴	340	7.2 × 10 ⁴	4.5 × 10 ⁴	4.0 × 10 ⁶

^a —, negative for HEV RNA.
^b No prior incubation with anti-HEV antibody.
^c Final concentration of anti-HEV antibody in each inoculum.

56). However, the present study revealed that various HEV strains of genotype 1, 3, or 4 in serum samples obtained from patients with domestic or imported hepatitis E can also infect and replicate efficiently in PLC/PRF/5 and A549 cells. Of note, when inoculated at an HEV load of $\geq 3.5 \times 10^4$ copies per well in a six-well microplate, HEV strains in all serum samples tested were successfully propagated in cultured cells, while upon inoculation at 2.0×10^4 copies per well, HEV strains in only 30% of serum samples grew in cultured cells. In other words, if the HEV load in serum is 3.5×10^5 copies/ml or higher, PLC/PRF/5 and A549 cells can support multiplication of HEV strains in all serum samples, with or without concurrent HEV antibodies, irrespective of HEV genotype. Progenies of serum-derived HEV strains in culture supernatant were successfully passaged in A549 cells, indicating that HEV progenies of serum origin released from cultured cells are infectious, similar to those of feces origin.

It has been reported that fecal samples contain various amounts of metabolic and phenolic compounds and polysaccharides and have a very heterogeneous composition (62). Like the case for the HE-JF5/15F strain which was used for the establishment of a genotype 4 HEV cell culture system (56), when the fecal specimen was inoculated onto monolayers of PLC/PRF/5 cells at a dilution of 1:5, as was done for the

JE03-1760F specimen (55), nearly all cells detached from the bottoms of all six wells inoculated, probably due to cell toxicity of the fecal sample. Approximately 30% of cells detached from the bottoms of the wells just after inoculation of the 10-fold-diluted fecal suspension, while such cell damage was not observed in any of the six wells tested when the 25-fold-diluted fecal specimen was used for inoculation of HEV onto PLC/PRF/5 or A549 cells, indicating that at least 5- to 25-fold dilution may be required when a fecal specimen is used as the inoculum in our cell culture system for HEV. In contrast, serum samples were successfully inoculated at a dilution of only 1:2, without apparent cell detachment, in the present study. In general, serum samples are routinely collected at the first examination from patients with a clinical diagnosis of acute hepatitis and are used for serological and molecular tests of hepatitis virus infection, while fecal specimens are rarely collected from patients for such purposes, although they may be collected after the diagnosis of hepatitis A or E is established. Therefore, serum samples are available earlier after disease onset than fecal specimens for all patients diagnosed as having hepatitis E and most likely have higher HEV loads (50), which will greatly increase the ease with which inoculation of clinical samples with HEV onto cultured cells can be carried out.

Since transmission of HEV and subsequent development of

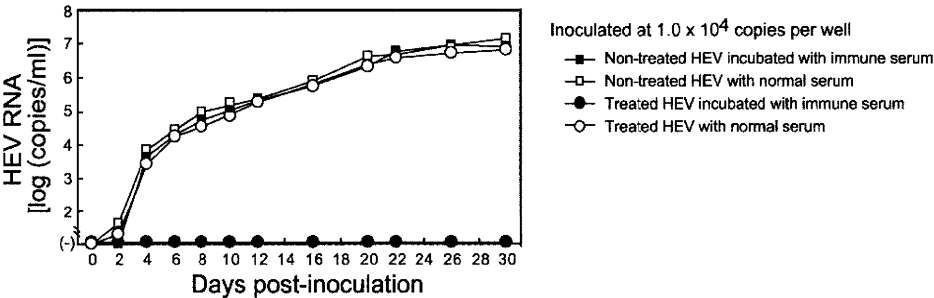


FIG. 7. Quantitation of HEV RNA in culture supernatants of A549 cells inoculated with a culture supernatant with a viral load of 1.0×10^5 copies/ml that had been treated with or without 0.1% NP-40 and 0.1% pronase E, mixed with a normal serum or pooled serum sample obtained at the early convalescent phase (pool 1) and positive for IgM-, IgA-, and IgG-class anti-HEV ORF2 and ORF3 antibodies, and cultured for 30 days.

hepatitis E via transfusion of viremic blood have been reported (1, 2, 18, 24, 25, 28, 54), our present observation that various HEV strains in serum samples were able to replicate in cultured cells and produce infectious progenies in the culture supernatant may be conceivable. Unexpectedly, however, HEV particles in serum samples grew in cultured cells despite the presence of HEV antibodies. The present studies indicated that over 90% of HEV particles in the circulation are free of immunoglobulins, even in the presence of IgM HEV antibodies. Similar to cell culture-generated HEV particles, HEV particles in serum were nonneutralizable by immune sera that can definitely neutralize the infection of HEV in feces in the cell culture system, and they banded at a sucrose density of 1.15 to 1.16 g/ml, which was identical to that in culture supernatant but markedly lower than that in feces (1.27 to 1.28 g/ml). Without prior treatment with detergent, few or no virus particles in both serum and cell culture were captured by anti-ORF2 MAb and anti-ORF3 MAb. However, after treatment with 5% Tween 20, the efficiencies of binding of HEV particles in serum by anti-ORF2 MAb and anti-ORF3 MAb increased markedly, to 55.7% and 90.4%, respectively, showing a similar pattern to that for cell culture-produced HEV particles (Table 6). These results suggest that HEV virions in serum possess the ORF3 protein on their surfaces, in association with lipids, similar to those in the supernatant of cultured cells (63), and would reconcile the apparently contradictory observation that IgM and IgA antibodies, which are capable of viral neutralization, and infectious virus coexist in acute-phase serum specimens, as reported for hepatitis A virus (HAV) (19, 35).

Provost et al. (34) reported that the infectious HAV present in acute-phase marmoset sera banded at two densities in CsCl (1.34 g/ml and 1.15 g/ml), suggesting that the less-dense particles represent lipid-associated virions. A similar banding pattern was observed by Lemon and Binn (20), who reported that the HAV released from infected BS-C-1 cells was incompletely neutralized when it was incubated with a variety of convalescent-phase sera (nonneutralizable fraction of 17 to 32%) and that chloroform extraction of virus resulted in a substantial reduction of the nonneutralizable fraction (to <1%). Nonneutralizable HAV recovered from untreated cell culture supernatant fluids sedimented heterogeneously and less rapidly than normal virus in rate-zonal sucrose gradients and also banded at a lower density in CsCl (1.14 to 1.18 g/ml) than did normal, neutralizable virus (1.32 g/ml). It seems that HEV particles in serum and culture supernatant are similar to the nonneutralizable fraction of HAV in cell culture in that they are resistant to antibody-mediated neutralization and are likely to be associated with lipids. However, in contrast to HAV, HEV particles in serum samples and culture supernatant showed a monophasic pattern in sucrose gradients, and essentially all HEV particles in these samples appeared to be nonneutralizable. In addition, chloroform treatment did not result in a shift of HEV particles in serum and culture medium to those with a density of 1.27 to 1.28 g/ml, corresponding to that of fecal HEV. Treatment with TEN buffer containing 0.1% NP-40 and 0.1% pronase E caused a shift of HEV particles in serum and cell culture to peak at a sucrose density of 1.27 g/ml. Of practical importance, these observations suggest that HEV exists as an "enveloped" virus in the circulation, similar to cell culture-produced HEV (63).

The inability of serum anti-HEV antibodies to neutralize infection of serum-derived virions appears to be at odds with the epidemiology of HEV infection (7, 36) and with the results of preclinical and clinical trials of a recombinant ORF2-based vaccine (42). The titers of serum antibodies (at the dilution used) affect the efficiency of virus neutralization. Therefore, we cannot rule out the possibility that the titer of anti-HEV antibodies that can neutralize feces-derived virions was too low to neutralize serum-derived HEV given the different membrane compositions. However, HEV in serum was not neutralized by each immune serum (pool 1 to pool 3 or 03-2150) diluted 1:5 (final concentration), although HEV in feces was neutralized by the immune sera diluted 1:500, suggesting that HEV in serum is nonneutralizable even by immune sera with a 100-fold-higher titer of anti-HEV antibodies in our cell culture system for HEV. In support of this finding, serum-derived HEV was not neutralized by anti-ORF2 MAb (H6225) or anti-ORF3 MAb (TA0536), even at a final concentration of 1 mg/ml, although the anti-ORF2 MAb could protect efficiently against infection of fecal HEV at an antibody concentration of 10 μ g/ml in our previous study (51). Natural infection with HEV usually follows ingestion of virus from water or food contaminated with HEV. Although the sequence of events that begins with entry via the gastrointestinal tract and ultimately results in hepatitis has not been resolved completely (7), and although extrahepatic sites of HEV, such as lymph node, small intestine, and colon tissues, have been identified in pigs infected with HEV (61), it is tempting to speculate that the primary site of replication for HEV is the hepatocyte, similar to that for HAV (15), and that HEV particles entering through the gastrointestinal tract, unaccompanied by lipids, can be neutralized by immune sera before reaching the liver via the portal vein. This speculation may help to explain how humans who were immunized with a recombinant ORF2-based vaccine can be protected against infection with HEV via the fecal-oral route.

It was previously reported that the ORF3 protein binds to the ORF2 capsid protein (59) and localizes to endocytic membranes (3). The apparent association of HEV virions with lipids suggests that cellular membranes play an important role in the assembly and release of HEV from noncytopathically infected cells. The immuno-capture RT-PCR assay in the present study indicated the presence of the ORF3 protein on the surfaces of not only HEV particles in cell culture but also those in serum samples, despite the absence of ORF3 protein on fecal HEV. In contrast to the HEV particles in cell culture treated with detergent and protease, those treated with detergent only (5% Tween 20) were neutralized only partially by immune serum containing anti-HEV ORF2 and ORF3 antibodies, an anti-ORF2 MAb (H6225) that can neutralize fecal HEV (51), or an anti-ORF3 MAb (TA0536) directed against a C-terminal immunodominant epitope (52) (Table 7 and Fig. 7). The ORF3 protein was accessible to anti-ORF3 MAb (TA0536) following detergent or chloroform treatment. These findings suggest that the C-terminal portion of the ORF3 protein is not exposed on the surfaces of HEV particles in serum and culture supernatant and may be covered with lipid membranes. The partial neutralization of the detergent-treated HEV virions in cell culture by anti-HEV antibodies may be ascribable to incomplete exposure of ORF2 and ORF3 pro-

teins after treatment with detergent only. In support of this speculation, 10 to 44% of the 5% Tween 20-treated HEV particles in cell culture and serum were not captured by anti-ORF2 and anti-ORF3 MAbs in the immuno-capture studies (Table 6). The ORF3 protein is likely to act as an adapter to link various intracellular transduction pathways, creating a host cell environment favorable for HEV replication and assembly (4). Our previous study using a wild-type JE03-1760F cDNA clone and its ORF3-null mutant demonstrated that the ORF3 protein is required for virion release from cultured cells (63). Taken together, the data indicate that it is very likely that HEV particles are released from both infected cultured cells (*in vitro*) and infected hepatocytes (*in vivo*) as lipid-associated virions accompanied by ORF3 protein and that the ORF3 protein and lipids are dissociated from the virion after it is shed into the bile duct and then the duodenum, which contain detergent (deoxycholic acid) and protease (trypsin), respectively.

The crystal structure of HEV-like particles was recently elucidated (12, 64). The outer surface of the particle, which is a target for antibodies, is mainly formed by the middle and protruding domains. It was also recently reported that the dimerization domain of the recombinant capsid protein (ORF2 aa 455 to 602), most probably the surface protrusion, is the neutralizing antibody recognition site of HEV and is essential for HEV-host interactions (21). However, a putative receptor for HEV has not yet been isolated, and virtually nothing is known about the mechanism by which HEV enters susceptible cells. Our previous study on virus attachment determined that cloned pJE03-1760F/wt virus in culture medium adsorbed to A549 cells with lower efficiency than that in cell lysate (approximately 3% and 14%, respectively, of the input virus within 1 h), peaking at a sucrose density of 1.27 to 1.28 g/ml (63), suggesting that nonneutralizable HEV particles in serum samples and culture supernatant, which are associated with lipids and band at 1.15 to 1.16 g/ml in sucrose gradients, can bind (though inefficiently) to cultured cells. Our present study indicated that even nonneutralizable HEV particles in serum samples and culture supernatant can be propagated in PLC/PRF/5 and A549 cells. It is interesting that a lipid solvent did not abolish or increase the infectivity of the newly released virions in the infected cell culture, and both cell culture-generated HEV particles and those that were treated with detergent and/or protease were propagated in cultured cells, with nearly identical efficiencies (Table 7 and Fig. 7), regardless of the presence or absence of the ORF3 protein and lipids on the surface. Taken altogether, it seems unlikely that only a fraction of HEV particles in serum samples and culture supernatant, partially unaccompanied by lipids, can bind to the cell surface receptor and enter susceptible cells. In an attempt to provide a plausible explanation(s) for this unexpected and intriguing observed phenomenon, it should be clarified in future studies whether and how nonneutralizable HEV particles in serum and culture supernatant can enter susceptible cells.

In conclusion, the present study indicated that PLC/PRF/5 and A549 cells can support efficient multiplication and passage of a wide variety of HEV strains (genotype 1, 3, or 4) in serum samples obtained from patients with imported or domestic hepatitis E and that HEV particles in serum samples are associated with lipids and ORF3 protein, similar to those in culture supernatant, most likely contributing to the assembly

and release of HEV from noncytopathically infected cells *in vivo* and *in vitro*. Although lipid-associated virions of other nonenveloped viruses, such as HAV, have been reported (20, 34, 37), the underlying mechanism by which HEV particles in the circulation can be propagated in cultured cells, similar to those in culture supernatant, despite the fact that they are seemingly "enveloped" and nonneutralizable by immune sera, needs to be clarified in future studies.

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Molecular and serological survey of hepatitis E virus infection among domestic pigs in Inner Mongolia, China

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Abstract To evaluate the prevalence and characteristics of swine hepatitis E virus (HEV) infection in Inner Mongolia, China, serum samples obtained from 356 2- to 4-month-old pigs on 14 farms in Inner Mongolia were tested for the presence of anti-HEV antibodies and HEV RNA. Overall, 186 pigs (52%) tested positive for anti-HEV antibodies, while 30 pigs (8%) had detectable HEV RNA levels. The 30 HEV isolates recovered from the viremic pigs were phylogenetically classified into genotype 4 and differed from each other by up to 15.3% in a 412 nt sequence within ORF2. The Inner Mongolian swine HEV strains were most similar to human or swine HEV strains isolated in the other provinces of China but differed by 15.9–18.9% from those in Mongolia (formerly known as Outer Mongolia). These results indicate that farm pigs in Inner Mongolia are frequently infected with markedly divergent genotype 4 HEV strains that may be indigenous to China.

Introduction

Hepatitis E virus (HEV) is a major cause of epidemic and acute sporadic hepatitis in many developing countries in Asia, Africa and Latin America. HEV infection is also endemic in many industrialized countries, including the United States, European countries and Japan [4, 6, 26, 33, 44]. HEV has been characterized as an icosahedral, non-enveloped, spherical particle with a diameter measuring approximately 30–32 nm in size, and it is classified in the genus *Hepevirus* of the family *Hepeviridae* [3]. Its genome is a single-strand, positive-sense RNA of approximately 7.2 kb in size and contains a short 5' untranslated region (5' UTR), three open reading frames (ORFs: ORF1, ORF2 and ORF3) and a short 3' UTR terminated by a poly (A) tract [28]. Four major genotypes (genotypes 1–4) of HEV have been identified in mammalian species [30]. Genotype 1 and 2 HEVs are restricted to humans and are often associated with large outbreaks and epidemics in developing countries with poor sanitation. Genotype 3 and 4 HEVs are believed to undergo zoonotic transmission, with a reservoir in pigs and, possibly, a range of other mammals, and are responsible for sporadic cases of hepatitis E in both developing and industrialized countries [2, 7, 19, 20, 24, 26, 27, 38, 40, 43, 47]. Genotype 3 is prevalent in North America, Europe, New Zealand and some Asian countries including China, Japan, Korea, Taiwan and Thailand. Genotype 4 is distributed mainly in Asian countries including China, India, Indonesia, Japan, and Vietnam [1, 18, 25].

In China, HEV has been attracting attention since approximately 120,000 individuals were infected with HEV and 707 persons died in a large outbreak of HEV in Xinjiang between July 1986 and April 1988 [54]. Molecular and seroepidemiological investigations have been performed in many of the provinces of China, and two

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major genotypes (1 and 4) were found to be the most prevalent [12, 17, 53]. Recent reports indicated that zoonotic transmission of genotype 4 HEV from pigs to humans has become the dominant cause of hepatitis E in eastern and southern China [13, 52]. In addition, genotype 3 HEV has also been identified in pigs in some areas of China [23].

Inner Mongolia, an autonomous region of China, borders Russia and Mongolia (formerly known as Outer Mongolia) in the north and neighbors eight other provinces and regions of China on its other three sides (see Fig. 1). Although serological surveys conducted during the 1990s indicated the presence of HEV infection in individuals living in Inner Mongolia [45, 54], no molecular or serological data on HEV infection in pigs have been reported. The aim of the present study was to investigate the prevalence of HEV infection among pigs in Inner Mongolia and to determine the extent of genetic variation among swine HEV strains in Inner Mongolia. We also attempted to

define the genetic relatedness of swine HEV strains in Inner Mongolia with those in the other areas of China and Mongolia.

Materials and methods

Serum samples

A total of 356 serum samples were obtained from pigs (mean age: 3.1 ± 0.8 months; age range 2–4 months) raised on 14 farms in 4 regions including Hulunbuir, Ulanhad, Hohhot and Alxa, which represent an area at the far east of the region, two central areas, and an area at the far western edge of the region, respectively, of Inner Mongolia (Fig. 1). Sample collection was performed between May and September in 2009, and the number of pigs and the place(s) where pigs were purchased were

Fig. 1 A map of China, showing provinces and regions including Inner Mongolia. The map of Inner Mongolia is expanded at the top, with the four shaded areas representing the four regions (Hulunbuir, Ulanhad, Hohhot and Alxa) where serum samples were obtained from farm pigs



recorded for each farm. Serum samples were stored at -40°C or below until testing for serological and molecular markers of HEV infection.

ELISA for detecting anti-HEV antibodies

To detect swine anti-HEV IgG, an enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein from the HE-J1 strain (genotype 4) that had been expressed in silkworm pupae [21], as described previously [35]. Briefly, ELISA microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with the recombinant ORF2 protein (250 ng/well). The samples were added to each well of the microplates at a dilution of 1:100 in 10 mM Tris-buffered saline containing 40% Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), 0.18% Tween 20, and a mock protein [optical density (OD) at 280 nm = 0.1] that had been obtained from the pupae of silkworms infected with non-recombinant baculovirus. As enzyme-labeled antibodies, peroxidase-conjugated rabbit IgG fraction to swine IgG (whole molecule) (MP Biomedicals, LLC., Solon, OH, USA) was used for the swine anti-HEV IgG assay. The OD of each sample was read at 450 nm. To determine the cut-off values in the swine anti-HEV IgG assay, 118 control swine serum samples that were exclusively negative for HEV RNA [34] were used as a panel. The OD value of 0.274 (mean + 6 standard deviations) was used as the cut-off value for the swine anti-HEV IgG assay [35]. Test samples with OD values greater than or equal to the cut-off value were considered to be positive for anti-HEV IgG.

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA. Total RNA was extracted from 100 μl of each sample using TRIzol LS Reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed with SuperScript II (Invitrogen), and subsequent nested PCR (ORF2-457 PCR) was performed with primers derived from the areas of the ORF2 region that are well conserved across all four genotypes, using the method described previously [21]. The size of the amplification product of the first-round PCR was 506 bp, and that of the amplification product of the second-round PCR was 457 bp. The RT-PCR assay was performed in duplicate, and reproducibility was confirmed. To avoid contamination during PCR procedures, the guidelines of Kwok and Higuchi [11] were strictly followed. Four negative controls and two positive controls were included for every 24 test samples. The results were recorded only when false-positive results were not obtained for the negative controls

and HEV RNA was detected in the positive controls. The negative control was water, which was processed in the same manner as for the serum samples. The positive control was serum from a Nepali patient with sporadic acute hepatitis E infected with HEV of genotype 1 [32], at a dilution of 1:1,000 in anti-HEV-negative human sera obtained from healthy individuals. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [21, 34].

To confirm the presence or absence of HEV RNA, a second RT-PCR (ORF2/3-137 PCR) assay using nested primers targeting the ORF2/ORF3 overlapping region was carried out according to the methods described previously [9]. The size of the amplification product of the first-round PCR was 164 bp, and that of the second-round PCR was 137 bp. The ORF2/3-137 PCR assay has the capability of amplifying all four known genotypes of HEV strains reported thus far, and the sensitivity of the RT-PCR assay was then assessed as described previously [9].

Sequence analysis of PCR products

The amplicons of the second-round PCR were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and then sequenced directly on both strands employing an ABI 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was performed using Genetyx-Win version 8.1.0 (Genetyx, Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan) [8]. Sequence alignments were generated by CLUSTAL W (version 1.8) [39]. A phylogenetic tree was constructed by the neighbor-joining method [29] with Kimura's two-parameter procedure [10] and 1,000 replicates of bootstrap re-sampling as implemented in MEGA 4.1 [36]. Genotypes/subgenotypes of HEV were assigned as described previously [18, 25, 30, 48, 50].

Results

Prevalence of anti-HEV antibodies among pigs in Inner Mongolia

Serum samples obtained from 356 2- to 4-month-old pigs at 14 swine farms in four areas of Inner Mongolia were tested for the presences of anti-HEV antibodies. Overall, 186 pigs (52%) were positive for anti-HEV IgG, with the prevalence differing by age, being 42% (42/101) among the 2-month-old pigs, 61% (80/132) among the 3-month-old

Table 1 Age-dependent prevalence of anti-HEV IgG in the sera of farm pigs in 14 herds in Inner Mongolia, China

Farm	Location	Total no. of pigs at sampling	Prevalence of anti-HEV IgG among pigs of age							
			2 months		3 months		4 months		Total	
			No. ^a	Positive ^b	No.	Positive	No.	Positive	No.	Positive
1	Hulunbuir	1,100	6	5 (83%)	21	16 (76%)	5	4 (80%)	32	25 (78%)
2	Hulunbuir	120	0		12	2 (17%)	4	0	16	2 (13%)
3	Hulunbuir	1,500	7	7 (100%)	6	6 (100%)	8	8 (100%)	21	21 (100%)
4	Ulanhad	154	4	2 (50%)	6	6 (100%)	1	1 (100%)	11	9 (82%)
5	Ulanhad	401	8	4 (50%)	11	4 (36%)	21	11 (52%)	40	19 (48%)
6	Ulanhad	350	10	4 (40%)	19	14 (74%)	15	7 (47%)	44	25 (57%)
7	Ulanhad	360	8	5 (63%)	15	11 (73%)	18	14 (78%)	41	30 (73%)
8	Hohhot	200	5	2 (40%)	4	2 (50%)	11	9 (82%)	20	13 (65%)
9	Hohhot	260	4	2 (50%)	6	2 (33%)	0		10	4 (40%)
10	Hohhot	220	14	3 (21%)	7	3 (43%)	0		21	6 (29%)
11	Alxa	550	23	4 (17%)	5	0	8	0	36	4 (11%)
12	Alxa	610	0		14	14 (100%)	15	10 (67%)	29	24 (83%)
13	Alxa	52	6	4 (67%)	0		8	0	14	4 (29%)
14	Alxa	101	6	0	6	0	9	0	21	0
Total		5,978	101	42 (42%)	132	80 (61%)	123	64 (52%)	356	186 (52%)

^a No. of pigs examined
^b No. of pigs positive for anti-HEV IgG

pigs, and 52% (64/123) among the 4-month-old pigs (Table 1). The prevalence of swine anti-HEV differed markedly by farm, being 0% in one herd (Farm 14), 11–50% in six herds, and 57–100% in seven herds. Of note, the prevalence of swine anti-HEV was only 0–29% in three farms with a small number of pigs (52 in Farm 13, 101 in Farm 14, and 120 in Farm 2). In contrast, its prevalence ranged from 78 to 100% in farms where 610–1,500 pigs were being raised (Farms 1, 3 and 12).

Figure 2 depicts the distribution of the OD value of swine anti-HEV according to age (months after birth) of the pigs tested. Swine anti-HEV was detected at the lowest OD value (0.567 ± 0.755) in the 2-month-old pigs, and then the titer increased gradually and was detectable at the mean OD value of 0.808 in the 3-month-old pigs and at 0.834 in the 4-month-old pigs.

Prevalence of HEV RNA in pigs in Inner Mongolia

The serum samples obtained from all 356 pigs were tested for the presence of HEV RNA by RT-PCR with nested primers targeting the ORF2 region (ORF2-457 PCR) (Table 2). HEV RNA was detected in the serum of 7 (7%) of the 2-month-old pigs, 12 (9%) of 3-month-old pigs and 11 (9%) of 4-month-old pigs. In total, 30 viremic pigs (8%) were identified in the current study. All 30 of the viremic

pigs were confirmed to be reproducibly positive for HEV RNA, and the remaining 326 pigs, including 166 anti-HEV-IgG-positive pigs, were exclusively negative for HEV RNA by a second RT-PCR (ORF2/3-137 PCR) assay with nested primers targeting the ORF2/ORF3 overlapping region. Among the 30 viremic pigs, 20 had detectable anti-HEV IgG, representing an ongoing HEV infection accompanied by an early antibody response, and the remaining 10 viremic pigs were negative for anti-HEV IgG, most likely representing viremia before the development of an immune response in infected pigs. The observation that 166 pigs with anti-HEV IgG were non-viremic may therefore indicate a past HEV infection in these pigs.

Genetic analysis of swine HEV recovered from viremic pigs

The 412-nt amplification products of ORF2-457 PCR (the primer sequence at each end was excluded) from 30 viremic pigs were sequenced and deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB550613–AB550642). The 30 swine HEV isolates were 84.7–100% identical to each other and were most closely related to the prototype human HEV isolate of genotype 4 [T1 (AJ272108)] [41], with identities of 83.0–92.7% at the nucleotide level. However, the isolates differed by

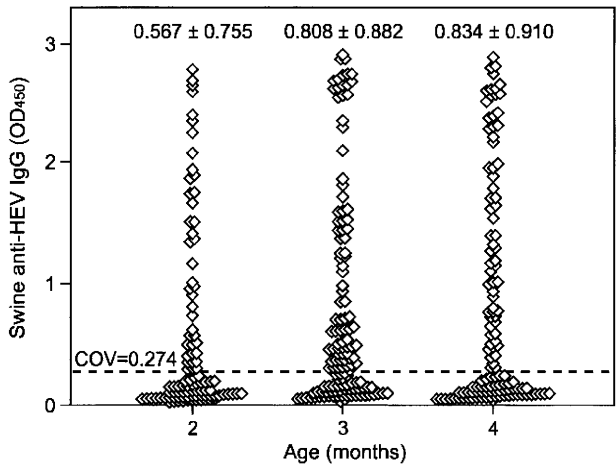


Fig. 2 Age-dependent distribution of the OD values obtained with swine anti-HEV IgG. Mean \pm SD OD values of swine anti-HEV IgG are indicated at the top

17.7–23.3% from a prototype HEV isolate of genotype 1 [B1 (M73218)], 20.9–25.7% from genotype 2 [MEX-14 (M74506)], and 19.2–23.1% from genotype 3 [US1 (AF060668)]. These results suggest that all 30 Inner Mongolian swine HEV isolates obtained in the present study are classifiable as being genotype 4.

The nucleotide sequence identity among swine HEV isolates from the same herd ranged from 99.5 to 100% at Farms 4 and 6, while that at Farms 8 and 12 was 85.9–100%, suggesting the co-existence of divergent isolates belonging to multiple clusters within the same herd (Table 3). One swine HEV isolate (swIM1-21) from Farm 1 was only 84.7–87.1% identical to those in the remaining four farms where viremic pigs were identified in the present study.

In order to further characterize the 30 swine HEV isolates obtained in the present study, a phylogenetic tree was constructed by the neighbor-joining method based on the 412 nt ORF2 sequence (Fig. 3). The tree confirmed that the 30 swine HEV isolates in Inner Mongolia belonged to genotype 4, and that they segregated into six clusters provisionally designated as 4a-1, 4a-2, 4a-3, 4b, 4d, and 4uc in the present study. The 4a-1, 4a-2, and 4a-3 clusters represent three distinct clusters within subgenotype 4a proposed by Lu et al. [18]; 4b represents a cluster of subgenotype 4b [18]; 4d represents a cluster of subgenotype 4d [18]; and 4uc stands for an as yet unclassified subgenotype within genotype 4. One swine HEV isolate from Farm 1 was grouped into Cluster 4uc, all seven isolates from Farm 4 were grouped into Cluster 4a-1, and all four isolates from Farm 6 were grouped into Cluster 4a-2. Of note, the nine isolates from Farm 8 were divided into two distinct clusters, 4a-2 ($n = 7$, swIM8-4/8/11/12/14/15/17) and 4d ($n = 2$, swIM8-1/6), and the nine isolates from Farm 12

were grouped into three different clusters, 4a-2 ($n = 6$, swIM12-2/3/5–8), 4a-3 ($n = 2$, swIM12-19/21), and 4b ($n = 1$, swIM12-9).

A BLAST screening was performed on DDBJ/EMBL/GenBank databases to search for overlap with HEV strains whose sequence (of >300 nt) has been determined, with nucleotide identities of >95% or with the highest identity to those obtained in the present study (Table 4). The swIM1-21 of Cluster 4uc was similar to the swine HEV strain (DQ294630) isolated in Heilongjiang, China, with the highest identity of 94.2% among the reported HEV strains. All seven isolates of Cluster 4a-1 obtained from Farm 4 had the highest similarity (97.7%) to the human HEV strain (AF134916) recovered from a hepatitis patient in Anhui, China. Four isolates from Farm 6, seven isolates from Farm 8, and six isolates from Farm 12 that were classified into Cluster 4a-2 shared 92.4–97.7% identity, and showed the highest similarity to the human or swine HEV strains of Chinese origin [DQ445493 (97.7%), FJ642016 (96.1%), and AF134812 (95.7%), respectively] (Table 4). Two HEV isolates of Cluster 4a-3 from Farm 12 had the highest nucleotide sequence identity (96.3%) to the swine HEV strain in China (AJ428852). Two 4d isolates from Farm 8 had the highest similarity (98.0%) to the HEV strain recovered from a Chinese patient with hepatitis E (FJ461765). These results indicate that 29 of the 30 swine HEV isolates obtained in the present study were most closely related to Chinese human or swine HEV strains among the known genotype 4 HEV strains. Of interest, one HEV isolate of Cluster 4b from Farm 12 was closest to the HEV strains of Vietnamese (AB168096) and Japanese (Okinawa) (AB194831) origin.

Discussion

The present serological and molecular survey revealed that HEV infection is highly prevalent among pigs in Inner Mongolia, with the overall prevalence of anti-HEV IgG at 52% (186/356) and HEV RNA at 8% (30/356), and that all HEV isolates recovered from the viremic pigs were classifiable into genotype 4. Swine anti-HEV antibodies have been detected in pigs from more than ten provinces, with nearly equal seropositivity rates (73.5–78.8%) in the northern and southern parts of China [42, 48]. This rate is higher than that in Inner Mongolia in the current study. The reason for the lower prevalence of swine anti-HEV in the present study remains unknown. However, the inclusion of swine farms with a small number of pigs being reared may have affected the seroprevalence rate, as discussed above. Furthermore, HEV RNA has been detected from serum, bile or stool of pigs in over 16 provinces and regions of China [5, 14, 31, 49–51, 53], with a prevalence of