RT-PCR method was designated as "ORF2-457 PCR", and the newly developed RT-PCR method as "ORF2/3-137 PCR" in this paper.

2.3. Sequence analysis

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac (Version 12.2.6; Genetyx Corp., Tokyo, Japan) and ODEN (Version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) (Ina, 1994). Sequence alignments were generated by CLUSTAL W (Version 1.8) (Thompson et al., 1994). The percentages of nucleotides that were identical to all 70 HEV isolates within every 40 nt sequence were calculated over the entire genome and plotted with increments of 1 nt, using the Microsoft Excel 2003. Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), using an avian HEV strain (Huang et al., 2004) as an outgroup. Bootstrap values were determined with 1000 resamplings of the data sets (Felsenstein, 1985). The final tree was obtained with the TreeView program (Version 1.6.6) (Page, 1996).

3. Results

3.1. Designing primers

The sequences of 70 HEV isolates of genotypes 1–4 whose entire or nearly entire sequences are known, were obtained from the GenBank/EMBL/DDBJ databases and aligned so as to obtain maximal match. The percentages of nucleotides that were identical to all 70 isolates within every 40 nt sequence were calculated over the entire genome and plotted with increments of 1 nt (Fig. 1). The percentage of nucleotides that were identical to all 70 isolates within the first 40 nt sequence (nt 1–40) was plotted at position 1, and that within the second 40 nt sequence (nt 2–41) at position 2, and so on. This analysis revealed that there are three highly conserved regions among the 70 HEV genomes, with percentages of conserved nucleotides above 75%: (1) in the 5′-UTR and 5′-terminal part of ORF1; (2) within the ORF2/ORF3 overlapping region where three peaks were found (Fig. 1); (3) the central portion of ORF2. Among these, the ORF2/ORF3 overlapping region was selected as the target region for nested RT-PCR in the present study, and the sequences of two of the three peaks within the ORF2/ORF3 overlapping region were selected to design primers (the 137 nt sequence that was amplified by this PCR is indicated by the horizontal bar on the left in Fig. 1). Based on the sequence alignment of 70 isolates in this particular region, two sets of sense and antisense primers for ORF2/3-137 PCR were designed (Fig. 2).

3.2. Sensitivity of ORF2/3-137 PCR

To compare the sensitivity of ORF2/3-137 PCR and ORF2-457 PCR, serial 10-fold dilutions of three HEV-viraemic serum

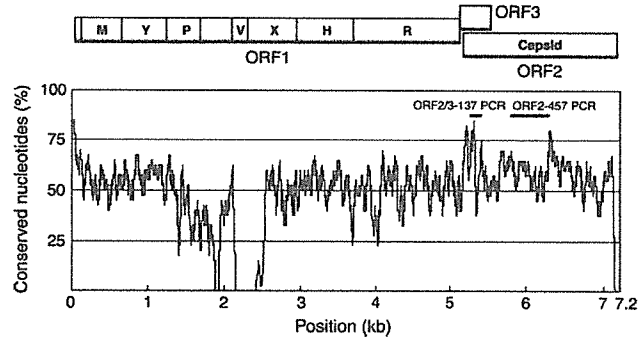

Fig. 1. Distribution of conserved nucleotides among 70 HEV isolates over the entire genome. Nucleotides that were identical to all 70 HEV isolates whose entire or nearly entire sequences are known were calculated within every 40 nt sequence over the entire genome and plotted with increments of 1 nt. The extreme 5′-end sequence has not been determined in 18 of the 70 HEV isolates, and the percentage of conserved nucleotides was calculated only among HEV isolates whose nucleotide sequences were available. Internal deletions/insertions were regarded as divergent nucleotides. The horizontal bars indicate the genomic region within the ORF2/ORF3 overlapping region that is amplifiable by the new RT-PCR assay (ORF2/3-137 PCR) developed in the present study, and the genomic region in the central portion of ORF2 that is amplifiable by the previous RT-PCR assay (ORF2-457 PCR) (Mizuo et al., 2002). At the top, the organization of the HEV genome is shown. M, methyltransferase; Y, Y domain; P, papain-like protease; V, proline-rich hinge domain; X, X domain; H, RNA helicase; R, RNA-dependent RNA polymerase.

samples were subjected to the two RT-PCR assays (Table 1). In 10^{-5} dilutions of strain 04-1601 with HEV of genotype 1, HEV RNA was detectable in nine (90%) of 10 samples by ORF2/3-137 PCR, while it was detectable in 6 (60%) of 10 samples by ORF2-457 PCR. In 10^{-6} dilutions of strain 03-1367 with

Table 1
Sensitivities of two RT-PCR assays for detection of HEV RNA

Virus strain	Dilution	ORF2-457 PCR[a]	ORF2/3-137 PCR[b]
		No. of positive samples/no. of tested samples (%)	No. of positive samples/no. of tested samples (%)
04-1601 (genotype 1)	10^{-2}	5/5 (100)	5/5 (100)
	10^{-3}	5/5 (100)	5/5 (100)
	10^{-4}	10/10 (100)	10/10 (100)
	10^{-5}	6/10 (60)	9/10 (90)
	10^{-6}	0/10	2/10 (20)
03-1367 (genotype 3)	10^{-2}	5/5 (100)	5/5 (100)
	10^{-3}	5/5 (100)	5/5 (100)
	10^{-4}	5/5 (100)	5/5 (100)
	10^{-5}	10/10 (100)	10/10 (100)
	10^{-6}	2/10 (20)	5/10 (50)
	10^{-7}	0/10	0/10
06-0526 (genotype 4)	10^{-2}	5/5 (100)	5/5 (100)
	10^{-3}	5/5 (100)	5/5 (100)
	10^{-4}	5/5 (100)	5/5 (100)
	10^{-5}	10/10 (100)	10/10 (100)
	10^{-6}	3/10 (30)	5/10 (50)
	10^{-7}	0/10	0/10

[a] Previously described RT-PCR method for amplification of a 457 nt sequence within ORF2 of HEV RNA (Mizuo et al., 2002).
[b] New RT-PCR method developed in the present study for amplification of a 137 nt sequence within the ORF2 and ORF3 overlapping region of HEV RNA.

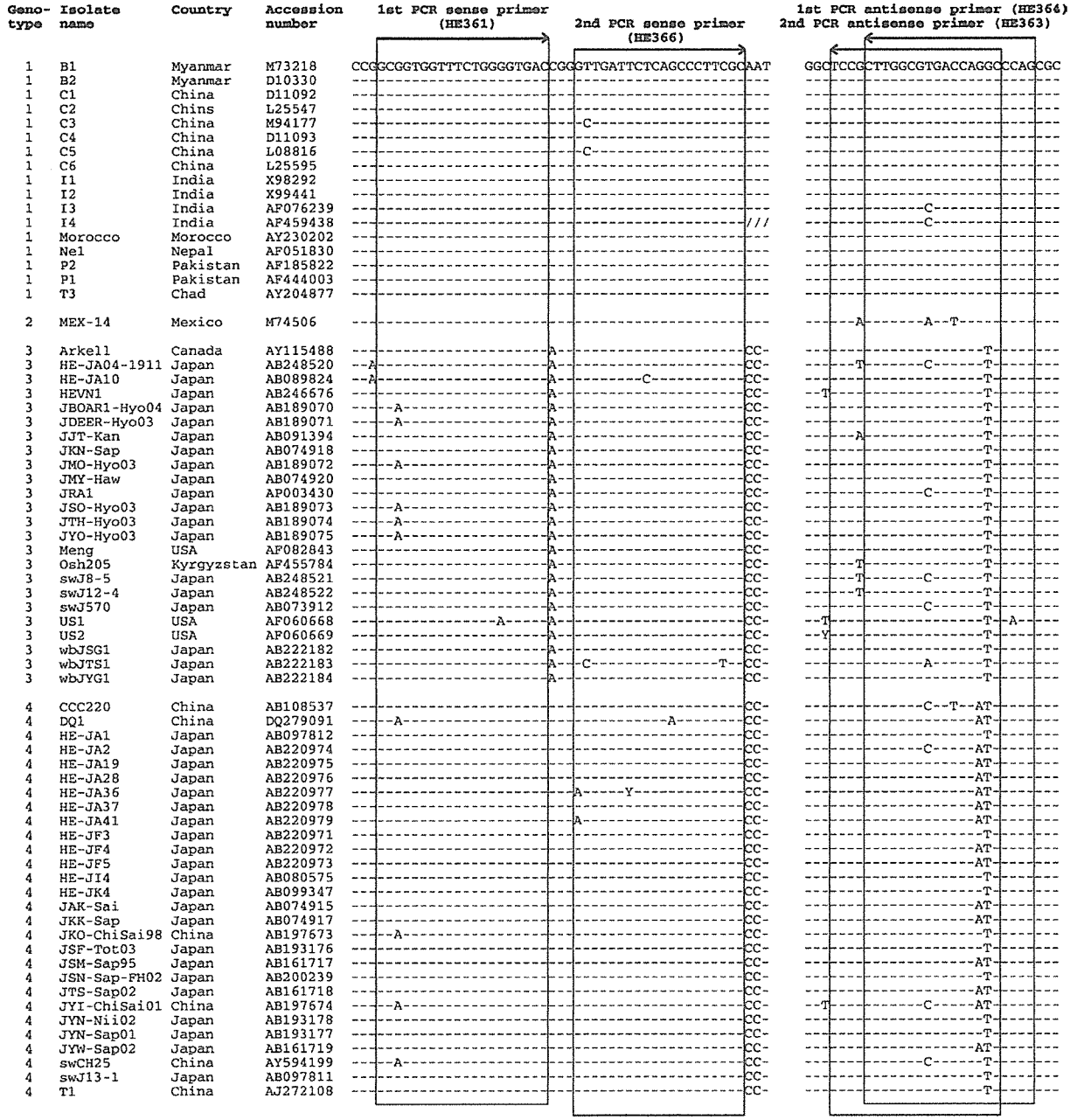

Fig. 2. Alignment of two partial sequences of the ORF2/ORF3 overlapping region of 70 isolates whose entire or nearly entire sequences are known. Boxes indicate the positions of the primers and arrows denote the polarity (sense and antisense) of the primers designed in the present study.

HEV of genotype 3, 5 (50%) of 10 samples were positive for HEV RNA by ORF2/3-137 PCR, while only 2 (20%) of 10 samples were positive for HEV RNA by ORF2-457 PCR. When the strain 06-0526 with HEV of genotype 4 was used to compare the sensitivity of the two assays, ORF2/3-137 PCR could also detect HEV RNA in 10^{-6} dilution samples more frequently than ORF2-457 PCR (50% [5/10] versus 30% [3/10]).

When stored periodic serum samples obtained from five patients with hepatitis E were tested, HEV RNA was detectable by ORF2/3-137 PCR 3–14 days longer after the disease onset than by ORF2-457 PCR (Table 2). Notably, in Case 4, HEV RNA was detectable by ORF2/3-137 PCR up to 43 days after the onset of acute hepatitis E, being detectable 14 days longer than by ORF2-457 PCR.

3.3. Applicability of ORF2/3-137 PCR

Serum samples obtained from 30 patients with acute hepatitis E and 11 domestic pigs, which had tested positive for HEV RNA by ORF2-457 PCR, were used to evaluate whether ORF2/3-137 PCR can amplify genomic sequences of various HEV strains. As expected, all 41 samples containing HEV of genotypes 1,

Table 2
Detection of HEV RNA in sequential serum samples obtained from five patients with sporadic acute or fulminant hepatitis E by two RT-PCR methods

Case no.	Days after onset	ALT (IU/l)	Total bilirubin (mg/dl)	Detectability of HEV RNA by	
				ORF2-457 PCR[a]	ORF2/3-137 PCR[b]
1	17	74	7.9	+	+
	19	61	7.2	–	+
	21	57	6.9	–	+
	34	31	3.0	–	–
2	16	409	9.5	+	+
	17	472	7.8	–	+
	18	257	6.5	–	+
	19	167	6.4	–	–
	20	107	6.1	–	–
	21	113	7.3	–	–
	22	93	7.5	+	+
	23	79	7.6	–	–
	24	72	7.6	+	+
	25	81	9.3	–	+
	26	72	9.2	–	+
	27	68	10.4	–	+
	28	49	12.2	–	–
3	27	125	17.4	+	+
	28	98	16.2	–	+
	30	63	16.5	–	+
	31	53	16.5	–	–
4	29	80	28.9	+	+
	31	68	28.0	–	+
	36	65	23.2	–	+
	40	59	17.6	–	+
	43	50	14.9	–	+
	45	52	14.6	–	–
5	26	38	27.1	+	+
	27	36	30.4	–	+
	28	30	31.3	–	+
	29	23	25.0	–	+
	32	18	26.2	–	+
	35	17	24.3	–	+
	39	15	25.5	–	–

[a] Previously described RT-PCR method for amplification of a 457 nt sequence within ORF2 of HEV RNA (Mizuo et al., 2002).
[b] New RT-PCR method developed in the present study for amplification of a 137 nt sequence within the ORF2 and ORF3 overlapping region of HEV RNA.

3, or 4 also tested positive for HEV RNA by ORF2/3-137 PCR. The amplified products were sequenced to confirm the specificity of the ORF2/3-137 PCR assay, and deposited in the GenBank/EMBL/DDBJ databases (AB259173 to AB259213). A phylogenetic tree was constructed based on the 97 nt sequence of the ORF2/ORF3 overlapping region of the 41 HEV isolates, along with the corresponding sequences in 70 HEV isolates whose entire or nearly entire sequences are known (Fig. 3). The tree could clearly distinguish the different HEV genotypes, and the genotypes of the 41 isolates determined based on the phylogenetic analysis of the 97 nt ORF2/ORF3 sequence were concordant with those determined based on phylogenetic analysis of the 412 nt ORF2 sequence.

4. Discussion

In general, when the genome of a virus exhibits marked heterogeneity, it is difficult to detect the viral nucleic acid sensitively and specifically. Particularly, many RNA viruses have remarkably heterogeneous genomes, and conserved regions that are suitable for designing primers and probes are limited. Since the 5′-UTR of a positive-strand RNA virus is generally conserved among isolates, this genomic region is often selected to design universal primers for detecting viral RNA by nucleic amplification tests such as RT-PCR and real-time detection PCR, as exemplified by hepatitis C virus (Okamoto et al., 1990; Desombere et al., 2005) and hepatitis A virus (Jothikumar et al., 2005; Sanchez et al., 2006). As for HEV, the 5′-UTR is also well conserved among all four genotypes, but it is too short (25–35 nt) to design a set of sense and antisense primers. Several sets of nested universal primers targeting the coding regions to sensitively detect HEV RNA, have previously been reported by various researchers (Erker et al., 1999; Huang et al., 2002; Mizuo et al., 2002; Takahashi et al., 2003a; Zhai et al., 2006). However, recent analyses of the full-length genomes of various human and animal HEV strains revealed that the HEV

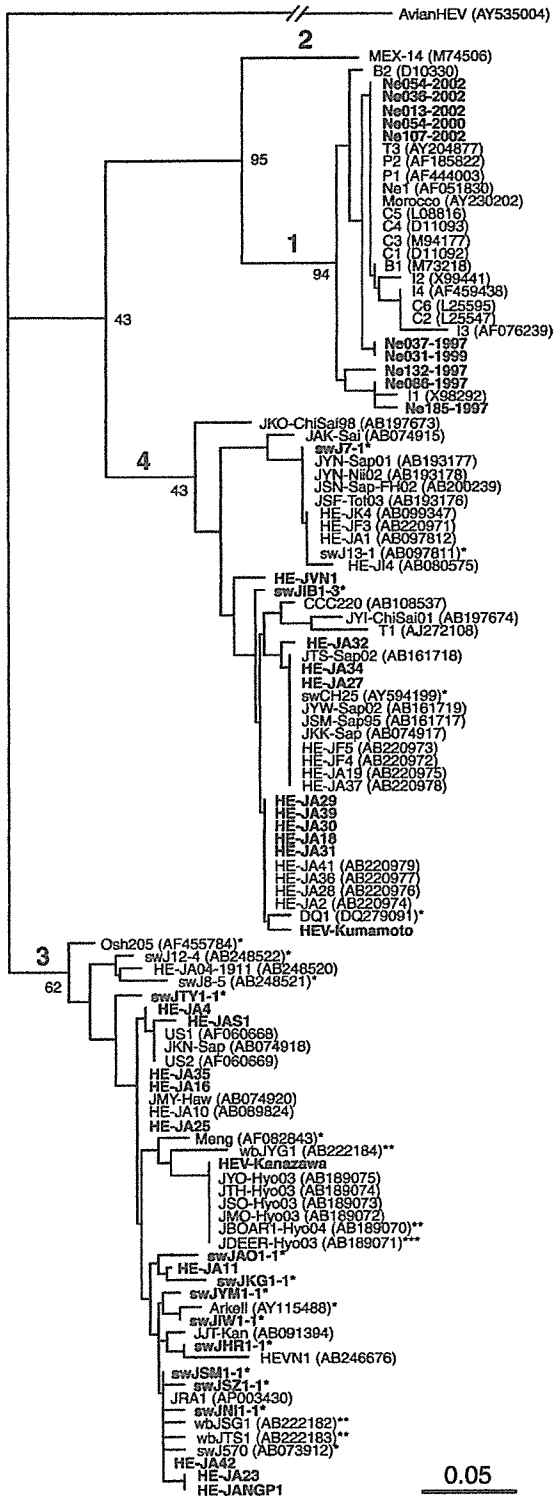

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2/ORF3 overlapping region (97 nt) of 111 human and swine isolates, using an avian HEV isolate (Huang et al., 2004) as an outgroup. The 30 human HEV isolates and 11 swine isolates whose partial sequence was determined in the present study are indicated in bold for visual clarity. Seventy isolates whose entire or nearly entire sequences have been reported were included for comparison, with the accession number in parentheses. Asterisks indicate isolates obtained from pigs*, wild boars** and deer***, respectively.

genome has more marked variability than previously thought (Nishizawa et al., 2005; Takahashi et al., 2004; Inoue et al., 2006a,b), and there are an increasing number of HEV isolates that have mismatches with one or more of the nested sense and antisense primers described thus far. It is likely that PCR using primers that have several mismatches with HEV variant strains, including a mismatch at the first and/or second nucleotide from the 3′-terminus, would lead to false negative results or have decreased sensitivity. In the present study, based on alignment of the full-length genomic sequences of 70 isolates so as to obtain maximal homology, three conserved genomic areas with percentages of conserved nucleotides above 75% were identified: they are the 5′-UTR and 5′-terminal part of ORF1; the ORF2/ORF3 overlapping region; the central portion of ORF2. Of these, the ORF2/ORF3 overlapping region contained three areas with highly conserved nucleotides, and was found to be the most suitable for designing primers for broadly reactive PCR; the ORF2/ORF3 overlapping region was also previously used as the target region for sensitive real-time detection PCR by Jothikumar et al. (2006). Among the three areas with highly conserved nucleotides within the ORF2/ORF3 overlapping region, 5′-terminal area (nt 5233–5275) had a high-GC content of 72.1–79.1% in the 70 HEV isolates whose entire or nearly entire genomic sequences are known; therefore, the remaining two areas within the ORF2/ORF3 overlapping region were used to design primers for a new RT-PCR (ORF2/3-137 PCR) assay in the present study (the area of the amplified products is shown by the horizontal bar on the left in Fig. 1). In the previous RT-PCR (ORF2-457 PCR) assay, the antisense primers used for cDNA synthesis, first-round PCR and second-round PCR were derived from the well-conserved area in the central portion of ORF2 (Fig. 1). However, in the genomic region used for the sense primers of ORF2-457 PCR, approximately 70% of the nucleotides are conserved: therefore, a mixture of three degenerate primers has been used for the second-round ORF2-457 PCR (Mizuo et al., 2002). This may have a large effect on the sensitivity of the ORF2-457 PCR assay.

In fact, the newly developed ORF2/3-137 PCR assay was two to three times more sensitive than the previous RT-PCR assay (ORF2-457 PCR) (Table 1), although the comparison was made in only three serum samples with HEV of genotypes 1, 3 or 4. Using the ORF2/3-137 PCR assay, the duration of viraemia in patients with acute or fulminant hepatitis E was revealed to be longer than that tested by the ORF2-457 PCR assay, with a difference of 3–14 days (Table 2). Even if serum samples are obtained from patients with hepatitis E at the late period of the acute phase or the early convalescent phase of hepatitis, the ORF2/3-137 PCR assay with increased sensitivity might be useful for molecular diagnosis of hepatitis E. Therefore, this improved method could be applied to testing for HEV RNA in patients with hepatitis E who have been underdiagnosed, and would help clarify the molecular aspects of HEV infection by providing the exact duration of viraemia and excretion of virion into feces in patients with clinical and subclinical HEV infection.

Phylogenetically, HEV is classified into four major genotypes (1–4). Currently, genomic regions that are most commonly

used to construct phylogenetic trees and define HEV genotypes include the 5′-end of ORF1 region (common 287 nt sequence), the 3′-end of the ORF1 region (common 307 nt sequence), the central portion of the ORF2 region (common 301 nt sequence), and the 3′-end of the ORF2 region (common 318 nt sequence): among them, the central portion of the ORF2 region with a span of approximately 300–450 nt accounts for the majority of HEV sequences reported thus far (Lu et al., 2006). In the present study, although the sequence of the amplicon of the ORF2/3-137 PCR assay was only 97 nt (primer sequences at both ends excluded), it contained genotype-dependent, variable areas. Therefore, a phylogenetic tree was constructed based on the 97 nt sequence of 70 HEV isolates whose entire or nearly entire sequences are known: genotype assignment was concordant with that based on the entire HEV genome. Furthermore, an additional 41 isolates whose genotype had been determined by phylogenetic analysis based on the 457 nt ORF2 sequence in the previous studies were subjected to the ORF2/3-137 PCR assay, and the resulting PCR products were used for analyzing the nucleotide sequences to confirm the specificity of the PCR assay and for constructing a phylogenetic tree, along with the corresponding sequence in the 70 HEV isolates. The present study revealed that phylogenetic analysis based on the 97 nt ORF2/ORF3 sequence could clearly distinguish the different HEV genotypes: mostly HEVs of genotypes 1, 3 and 4 were assessed, since the number of genotype 2 sequences is limited. Geographically, genotype 2 has been isolated from Mexico and African countries including Chad, the Central African Republic, Democratic Republic of the Congo, Egypt, Namibia, and Nigeria. The nucleotide sequences of 17 genotype 2 HEV isolates are retrievable from the GenBank/EMBL/DDBJ databases as of May 2006, and they include only one isolate whose entire sequence is known and 16 isolates whose partial sequences (the 3′-end of ORF2: 359–451 nt) are known (Buisson et al., 2000; Maila et al., 2004; Nicand et al., 2005): the ORF2/ORF3 overlapping region sequence is available for only a Mexican isolate. Regrettably, it was not examined whether the ORF2/3-137 PCR assay can sensitively and specifically detect genotype 2 HEV strains circulating in Africa in the present study.

In conclusion, it was found that a newly established RT-PCR method that amplifies a 137 nt sequence within the ORF2/ORF3 overlapping region, using primers designed based on highly conserved areas among 70 HEV isolates whose entire or nearly entire sequences are known, was considerably more sensitive than the previous method (Mizuo et al., 2002), and that the amplified products of the new method could be used for genotypic analysis of various HEV strains. The extent of genomic variability of HEV needs to be investigated more extensively in future studies and the presence of as yet unidentified HEV genotype(s) cannot be ruled out. However, in the interim, this improved RT-PCR method would be useful for detection of HEV strains with significant sequence variations, molecular diagnosis of acute or fulminant hepatitis E in patients with low viral load, and molecular epidemiological studies of HEV infection, not only in humans but also in animals including those previously unexamined.

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Case Report

A case of acute hepatitis E associated with multidrug hypersensitivity and cytomegalovirus reactivation

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A 65-year-old Japanese man was hospitalized because of acute hepatitis and severe cholestasis due to hepatitis E virus (HEV) infection combined with a drug reaction to a cold preparation. He died of disseminated intravascular coagulation and severe intestinal bleeding due to systemic cytomegalovirus reactivation following the development of severe eruptions with marked eosinophilia due to drug hypersensitivity to taurine and ursodeoxycholate preparations. The close interaction between viral infection or reactivation and drug hypersensitivity was considered as a pathophysiology in this case, which emphasizes the need for further study of the immunological mechanism of the interaction.

Key words: cholestasis, drug hypersensitivity, eosinophilia, eruption, hepatitis E, hypersensitivity syndrome

INTRODUCTION

SOME VIRAL SPECIES, such as the Epstein–Barr (EB) virus,[1–3] induce drug hypersensitivity associated with eruptions. Cases of severe eruptions caused by viral reactivation, usually by human herpesvirus-6 (HHV-6), induced by primarily occurring drug hypersensitivity, have recently been reported and designated as hypersensitivity syndrome (HS).[4] The association between viral infection or reactivation and drug allergy is therefore a major area of concern in studying the immunological mechanism of hypersensitivity. Here, we report a case demonstrating multidrug hypersensitivity and cytomegalovirus reactivation following acute hepatitis E virus (HEV) infection.

CASE REPORT

THE PATIENT WAS a 65-year-old Japanese man. He took a commercially available medicine for a common cold, Jikinin, because of his rhinorrhea and coughing in the middle of February, 2004. He noted dark urine and pruritus of the whole body on 1 March, and visited Iwate Prefectural Ohfunato Hospital on 9 March. He was hospitalized on the day of his visit with a diagnosis of acute hepatitis from the clinical findings of overt jaundice and elevated levels of liver enzymes (Table 1). The laboratory data obtained at this stage demonstrated acute hepatic injury with cholestasis, but without any sign of hepatic failure. He was then transferred to the Iwate Medical University Hospital on 12 March because of further elevation in the levels of serum bilirubin and liver enzymes.

On admission, he showed marked jaundice on the bulbar conjunctiva and skin, but no abnormality in consciousness and vital signs. Laboratory findings showed a marked increase in serum bilirubin level and a moderate increase in the levels of liver enzymes, but no abnormality in total protein concentration, albumin level or blood coagulation test results (Tables 1 and 2). Leukocyte bands showed no abnormal classification, and no eosinophilia was found at this stage. Serological screening tests for viral hepatitis revealed that he had acute hepatitis E virus (HEV) infection, and the genotype III HEV RNA was detected in the serum sample. This isolate designated HE-JA42 and the sequence of the open reading frame 2 region (412 nucleotides) was registered as the accession number of AB218721 for the DNA databank of Japan, and showed a relatively close identity of approximately 92% with isolates from

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Table 1 Laboratory findings on admission in former hospital

Hematology		Blood chemistry	
Neutrophil	56.6%	D.Bil.	15.7 mg/dL
Lymphocyte	27.6%	AST	1548 IU/L
Monocyte	14.0%	ALT	1483 IU/L
Eosinophil	0.6%	LDH	744 IU/L
Basophil	1.2%	γ-GTP	224 IU/L
White blood cell	4900/μL	T.Bil.	22.2 mg/dL
Red blood cell	436×10^4/μL	Al-P	1644 IU/L
Haemoglobin	13.7 g/dL	TBA	197.6 μM/L
Hematocrit	39.8%	T.P.	6.3 g/dL
Platelet	26.2×10^4/μL	IgG	1430 mg/dL
		IgA	494 mg/dL
Electrolytes and renal function		IgM	236 mg/dL
Na	141 mEq/L	CRP	1.0 mg/dL
K	4.4 mEq/L		
Cl	107 mEq/L	Blood coagulation	
Urea nitrogen	16.1 mg/dL	PT	145%
Creatinine	0.6 mg/dL	HPT	110%
		Fibrinogen	338 mg/dL
		Antithrombin	156%
		FDP D-dimer	1.1 μg/mL

T.Bil., total bilirubin; D.Bil., direct bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; γ-GTP, γ-glutamyltranspeptidase; Al-P, alkaline phosphatase; TBA, total bile acid; T.P., total protein; Ig, Immunoglobulin; CRP, C-reactive protein; PT, prothrombin time; HPT, hepaplastin test (normotest); FDP, fibrin and fibrinogen degradation products.

Table 2 Laboratory findings on admission in Iwate Medical University

Hematology		Blood chemistry		Virus markers	
Neutrophil	46.0%	D.Bil.	26.3 mg/dL	HBsAb	(-)
Lymphocyte	22.0%	AST	427 IU/L	HCVAb	(-)
Monocyte	23.0%	ALT	765 IU/L	EBVCA IgG	(+)
Eosinophil	4.0%	LDH	295 IU/L	EBVCA IgM	(-)
Basophil	1.0%	γ-GTP	295 IU/L	EBNA Ab	(+)
White blood cell	4530/μL	T.Bil.	29.0 mg/dL	HBsAg	(-)
Red blood cell	493×10^4/μL	Al-P	1716 IU/L	CMV IgG	(+)
Haemoglobin	15.1 g/dL	T.P.	7.0 g/dL	CMV IgM	(-)
Hematocrit	44.2%	Albumin	3.7 g/dL	HEV IgG	(+)
Platelet	24.6×10^4/μL	IgG	1780 mg/dL	HEV IgM	(+)
		IgA	563 mg/dL	HEV RNA	(+)
Electrolytes and renal function		IgM	240 mg/dL	Genotype III	
Na	136 mEq/L	CRP	0.7 mg/dL		
K	4.6 mEq/L			Autoantibodies	
Cl	102 mEq/L	Blood coagulation		ANA	(-)
Urea nitrogen	15.3 mg/dL	PT	114%		
Creatinine	0.8 mg/dL	PT-INR	0.85	Others	
		HPT	115.4%	AFP	2.7 ng/mL
Urinalysis		Fibrinogen	245.8 mg/dL	HGF	0.44 ng/mL
pH	6.0	Antithrombin	112%	4/8 CD	5.25
Sp.G.	1.015	FDP D-dimer	0.5 μg/mL		
Protein	(-)				
Sugar	(-)				

PT-INR, prothrombin time-international normalizaion ratio; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; EBVCA, Epstein-Barr virus capsid antigen; EBNA, Epstein-Barr virus nuclear antigen; HEV, hepatitis E; ANA, antinuclear antibody; AFP, alpha fetoprotein; HGF, hepatocyte growth factor; 4/8 CD, ratio of clusters of differentiation 4 to 8.

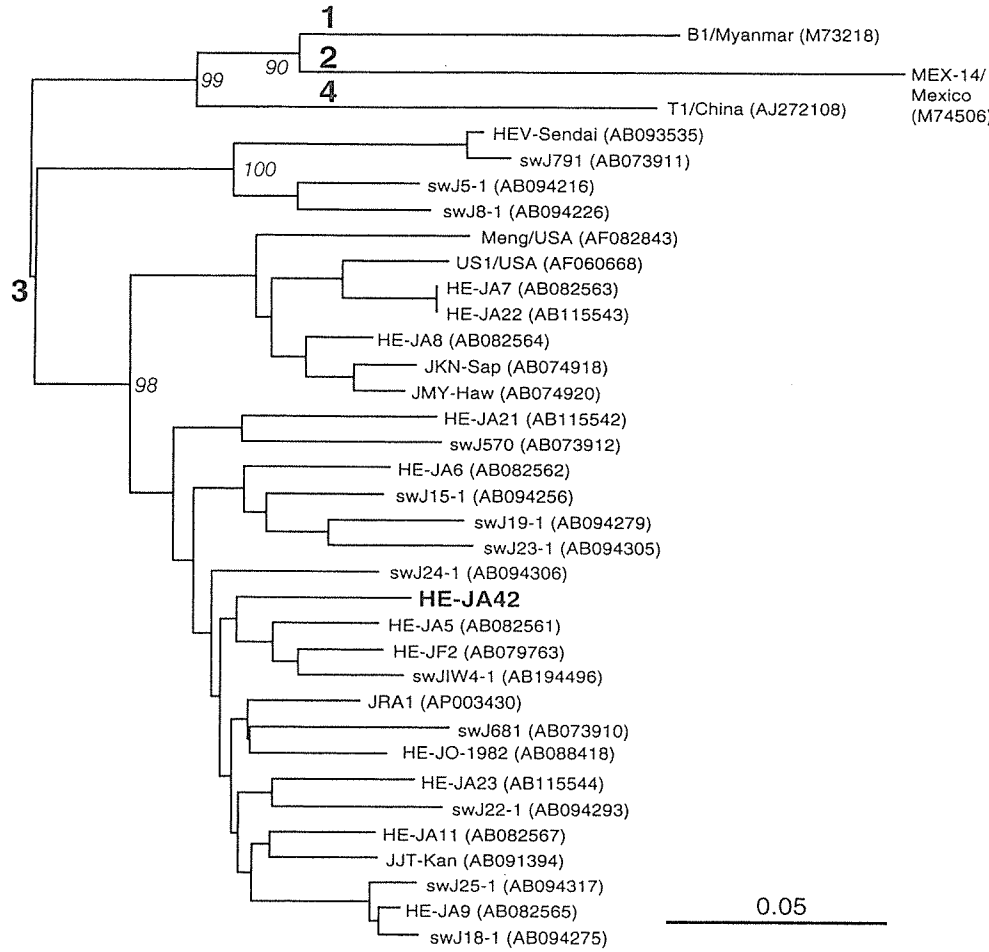

Figure 1 Phylogenetic tree constructed by neighbor-joining method on the basis of partial nucleotide sequence of open reading frame 2 region (301 nucleotides; nt 6037–6337 of the HE-JA10 genome [AB089824]) of reported human and swine genotype III HEV isolates. The HEV isolated from this patient is in bold face (HE-JA 42).

humans (HE-JA5 and HE-JF2) and swine (swJIW4-1) in Iwate prefecture (Fig. 1). Furthermore, a drug-induced lymphocyte stimulation test (DLST) showed a positive result for the drug, Jikinin, which he took for a common cold four weeks before the test. The DLST was carried out as follows: 1×10^6 peripheral blood lymphocytes of the patient per reaction were prepared using Ficoll–Paque, cultured and stimulated by medium with and without the drug solution. Lymphocyte proliferation measured by ^{3}H-incorporation to the DNA was 2.04-fold higher in drug-stimulated lymphocytes than in control. The drug Jikinin is a popular over-the-counter medicine for the common cold, containing some antiphlogistic and analgesic agents as shown in Table 3. Therefore, it was not clear which of these agents was responsible for the hypersensitive response.

Table 3 Active agents and additives included in the drug, Jikinin

Active agents
Dihydrocodeine phosphate
dL-Methylephedrine
Acetaminophen
Chloropheniramine maleate
Anhydrous caffeine
Liquorice extract
Additives
Talc
hydroxypropylcellulose
D-mannitol
Magnesium stearate
Cellulose
Sucrose

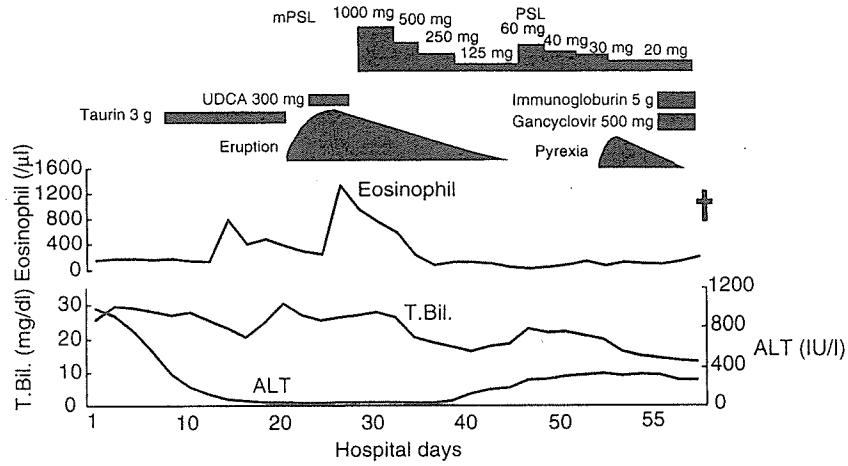

Figure 2 Clinical course of patient. ALT, alanine aminotransferase; mPLS, methylprednisolone; PLS, prednisolone; T. Bil., total bilirubin.

Abdominal computed tomography (CT) scan showed no signs of hepatic failure, such as liver atrophy, density irregularities or ascites, but showed the collapse and thickening of the wall of the gall bladder. Galactose receptor scintigraphy showed no decrease in functional liver mass. From these laboratory and imaging results, he was diagnosed to have acute hepatitis with intrahepatic cholestasis due to acute HEV infection and drug reaction to Jikinin.

Because the serum bilirubin level was maintained at approximately 30 mg/dL despite the smooth decrease in aminotransferase level after admission, 3 g/day taurin was administered on the tenth hospital day (HD) to induce choleresis (Fig. 2). Although the bilirubin level transiently decreased to 20 mg/dL after taurin administration, the level increased to 30.4 mg/dL on the 27th HD following an increase in eosinophil count up to 784/mL on the 13th HD, with numerous pruritic eruptions and erythema exsudativum multiforme appearing on his whole body on the 23rd HD (Fig. 3). Histopathological examination of his thigh lesion showed the presence of slight spongiosis and cell degradation in the epidermis, and marked eosinophilic infiltration around vessels and hair follicles in the upper dermis (Fig. 4). Taurin administration was stopped on the 23rd HD with the assessment of a possible allergic reaction to taurin. Instead, Ursodeoxycholic acid (UDCA) preparation was given on the 31st HD. However, the eosinophil count increased up to 20% on the 33rd HD, two days after the start of UDCA administration, followed by pyrexia (38.5°C), severe eruptions on the whole body with pruritus and facial edema. With a diagnosis of multidrug hypersensitivity despite the negative result of the DLST for taurin and UDCA, 1000 mg/day methylprednisolone was administered for three days, from the 35th to the 37th HD, and tapered by switching to oral prednisolone administration. Since the start of methylprednisolone treatment, symptoms of pyrexia, eruptions and facial edema improved, but urea nitrogen and creatinine levels gradually elevated. When the dose of prednisolone was tapered to 30 mg/day on the 54th HD, a high fever (39.5°C) abruptly developed. Although the administration of ganciclovir and immunoglobulin preparation was started on the 58th HD with a positive result for blood cytomegalovirus (CMV) antigen, the pyrexia did not subside and was followed by hemorrhagic shock originating from multiple hemorrhagic duodenal ulcers. Although an emergency hemostatic treatment was performed through gastrointestinal endoscopy, hemorrhage did not subside and the patient died from multiple organ failure associated with disseminated intravascular coagulation (DIC) on the 68th HD.

Autopsy and subsequent histopathology showed a number of findings indicating DIC and CMV infection: (i) gangrenous necrosis of the whole intestine with multiple fibrin thrombi in small vessels and microscopic infarction in the spleen with fibrin thrombi (Fig. 5a); (ii) multiple fresh infarcts in the liver, skin in the thumb tip and spleen (Fig. 5b); (iii) multiorgan CMV infection including the duodenum, small and large intestines and bilateral lungs (Fig. 6); and (iv) diffuse alveolar damage of both lungs.

DISCUSSION

THE PATHOPHYSIOLOGICAL FEATURES of the patient precipitating into death was not due to liver failure but to drug hypersensitivity and severe CMV reactivation, although the initial symptoms were those of

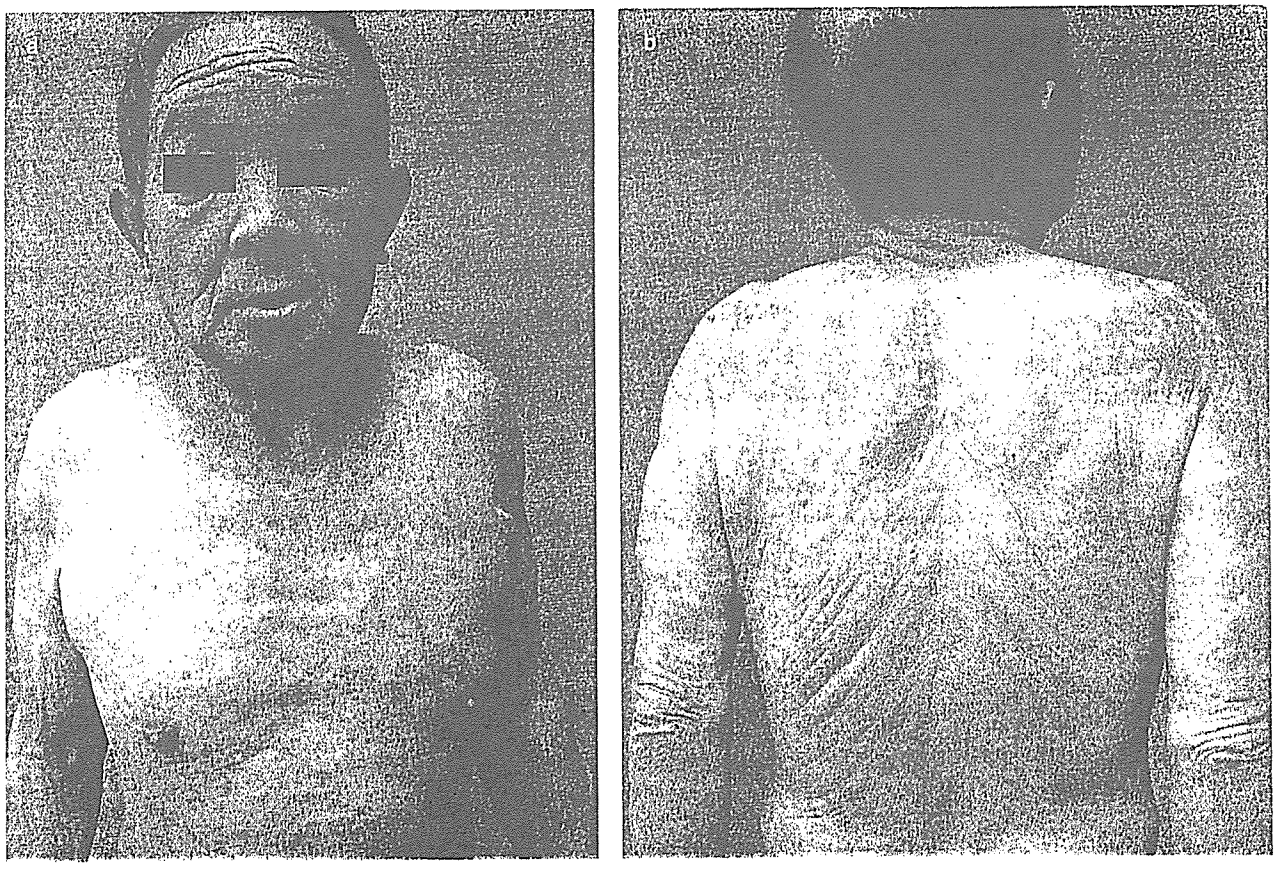

Figure 3 Clinical features of skin. Erythropapular eruptions and erythema exudativum multiforme were observed with icterus

acute hepatitis due to HEV infection, which is recently regarded to be endemic in Japan[7] and occasionally causes fatal hepatic failure.[6] The eruptions were multiform exudative erythema-type, and histopathologic finding of these eruptions showed marked eosinophilic infiltration. These findings indicated typical allergic dermatitis. The acute onset of rashes associated with pyrexia and eosinophilia following the start of taurin or UDCA administration suggests that allergic reactions to these drugs are responsible for the eruptions. However, neither taurin nor UDCA is listed as a high-risk compound for drug allergy.[1] Therefore, the condition of this patient at the onset of eruptions was considered to be highly susceptible to drug hypersensitivity.

A number of reports have shown a strong relationship between drug hypersensitivity and viral infection.[1–4] In both interactions, that is viral infection-induced drug hypersensitivity and drug hypersensitivity-induced viral reactivation, lymphocyte activation is considered to play an important role in the pathophysiology, although the precise mechanism of this role has not yet been elucidated. The EB virus, which infects B-lymphocytes and induces the development of infectious mononucleosis,[5] induces allergic reaction to ampicillin.[1–3] A reactivation of HHV-6, which infects T-lymphocytes and induces the development of exanthem subitum during the initial infection,[8] is hypothesized to induce the pathogenesis of hypersensitivity syndrome following the intake of specific drugs such as anticonvulsants and alloprinol.[4] In our patient, neither such a virus nor drugs were accounted for, at least during the initial phase of the disease. The CMV virus, which persistently infects white blood cells, endothelial cells and other cells, causing a symptomatic disease in an immunocompromised host, was remarkably activated only in the late phase of the disease, after the glucocorticoid therapy.

Therefore, hypersensitivity to multidrugs such as taurin and UDCA in this case cannot be categorized in any known disease entity of drug allergy. One of the issues in this case was whether HEV infection alone was sufficient to cause drug hypersensitivity, or whether an unfortunate coincidental HEV infection and independ-

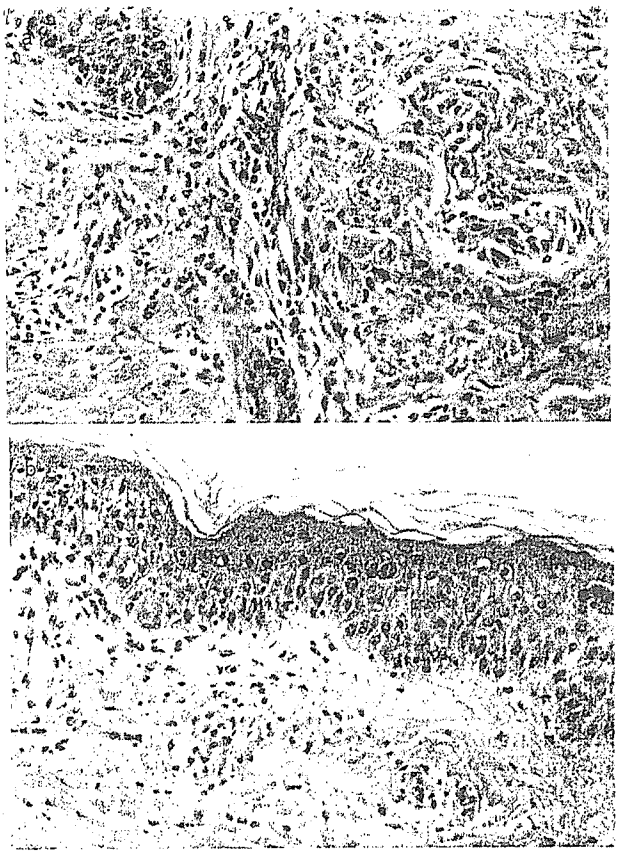

Figure 4 Marked eosinophilic infiltration around vessels and hair follicles were observed in upper dermis (hematoxylin-eosin staining, 200×).

ent reaction to Jikinin led to an accidental hypersensitive reaction. The other issue was whether the glucocorticoid therapy alone led to the severe CMV reactivation in the late phase.

The feature of hepatic injury in this case was marked cholestasis, which is unusual in HEV hepatitis[9] but common in drug-induced hepatitis.[10] Drug-induced hepatitis accounts for approximately 10% of all cases of fulminant hepatitis in Japan.[11] The use of some drugs during the early stages of acute hepatic injury may be implicated in the progression of such an injury to acute liver failure.[12,13] These findings suggest that drugs act not only as the primary cause, but also as the aggravating cofactor of acute liver injury. Although the precise mechanism by which drugs induce hepatic injury remains to be elucidated, two major types of hepatic injury are known: toxic hepatic injury and immunoallergic hepatitis.[14] In this case, the primary cause of liver injury might have been acute HEV infection, as shown by the positivity for the IgM anti-HEV antibody and HEV RNA; and the initial symptoms such as a cold might have been the onset symptom of acute hepatitis. The marked cholestasis associated with acute hepatitis might have been a result of drug reaction to Jikinin superimposed to acute HEV hepatitis, although it is not clear whether the HEV infection accelerated drug hypersensitivity or whether the HEV infection was just coincidental. Nagasaki *et al.* have recently reported that two patients with acute HEV hepatitis demonstrated acute onset autoimmune hepatitis-like features such as positivity for the antinuclear antibody and an elevated serum immunoglobulin G level.[15,16] This indicates the possibility that HEV infection induces an excessive immune response or aggravates asymptomatic autoimmune dis-

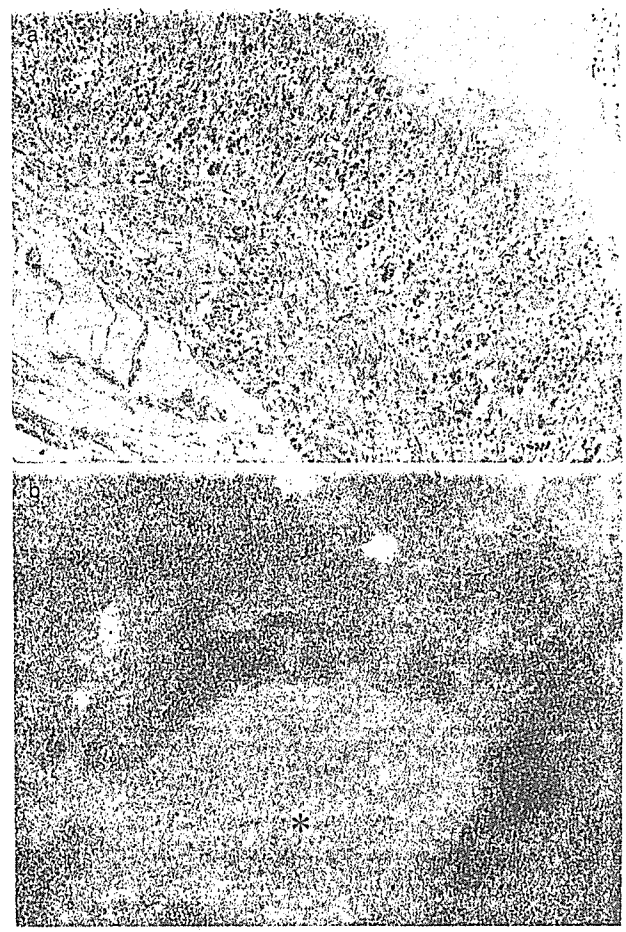

Figure 5 Histopathology of small intestine (a) and spleen (b). Multiple fibrin thrombi were observed in small vessels of the small intestine and spleen. Small infarcts (*) were observed in the spleen.

Figure 6 Histopathology of lung (a) and duodenum (b). Multiple inclusion bodies of cytomegalovirus were observed in epithelial cells.

eases, which supports the possible mechanism of HEV-induced drug hypersensitivity in this case. Indeed, a case of hepatitis E-associated hypersensitivity to dapsone, an antileprosy drug, was reported.[17] Besides HEV infection, many extrahepatic symptoms associated with acute and chronic viral hepatitis have been reported in relation to immunoallergic mechanisms such as Guillain–Barré syndrome[18] and Schönlein–Henoch purpura.[19] However, there is no report demonstrating the association between drug hypersensitivity and the hepatitis virus, although a close relationship between drug hypersensitivity and acute infection or reactivation of herpesviruses, HHV-6,[4] EBV[20] and CMV[21] has been reported.

The pathophysiologies leading to the death of the patient were DIC and a massive hemorrhage from duodenal ulcer, both of which were induced by CMV reactivation in multiple organs. CMV infects many types of cell including lymphocytes,[5] remains latent within the host and reactivates when the host's immune system is compromised.[22] On the other hand, CMV is considered as one of the causative viruses of hypersensitivity syndrome, as with other herpesviruses. Indeed, Aihara *et al.*[21] reported a case of hypersensitivity syndrome associated with CMV reactivation, which developed jaundice, renal failure and DIC, similarly to our present case. Therefore, CMV reactivation in this case may be induced by not only glucocorticoid therapy but also pathogenic mechanism that is the same as that underlining HHV-6 reactivation in hypersensitivity syndrome.

In summary, this case suggests the possibility that HEV infection is a cause of multidrug hypersensitivity, and that drug hypersensitivity induces CMV reactivation instead of HHV-6. These findings emphasize the need for further study of the immunological mechanism of the interaction between drug hypersensitivity and viral infection.

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Short Communication

Analysis of human and swine hepatitis E virus (HEV) isolates of genotype 3 in Japan that are only 81–83 % similar to reported HEV isolates of the same genotype over the entire genome

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Full-length sequences were determined for a human hepatitis E virus (HEV) isolate (HE-JA04-1911) and two swine HEV isolates (swJ8-5 and swJ12-4) that belong to one of three clusters within genotype 3 in Japan and are close to Spanish isolates according to their partial sequences. The three HEV isolates were 89·7–92·9 % identical to each other, but only 80·7–83·0 % similar to 21 HEV strains of the same genotype isolated in Canada, Kyrgyzstan, the USA and Japan over their entire genome. On comparison with HEV isolates whose partial sequence is known, the HE-JA04-1911, swJ8-5 and swJ12-4 isolates segregated into a phylogenetic cluster consisting of human and swine HEV isolates in Japan and the UK, with identities of 89·8–100 % and 87·9–92·4 %, respectively. Genotype 3 HEV isolates were found to be markedly heterogeneous. The UK-isolate-like HEV strains in Japan may have originated from the UK via the importation of pigs since 1900.

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Hepatitis E virus (HEV), the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.*, 2004), is the major cause of enterically transmitted non-A, non-B hepatitis. 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; Meng, 2003; Meng *et al.*, 1997, 1998, 2002; Nishizawa *et al.*, 2003; Okamoto *et al.*, 2001; Tei *et al.*, 2003; Yazaki *et al.*, 2003). 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, where some people ingest uncooked or undercooked meat or viscera (such as raw liver and colon/intestines) from pigs, wild boar and deer (Li *et al.*, 2005; Matsuda *et al.*, 2003; Tamada *et al.*, 2004; Tei *et al.*, 2003; Yazaki *et al.*, 2003). The genome of HEV is a single-stranded, positive-sense RNA of approximately 7·2 kb and consists of a short 5′ untranslated region (UTR), three open reading frames (ORF1–3) and a short 3′ UTR terminated by a poly(A) tract (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 and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in a total of 22 countries (Argentina, Australia, Austria, Cambodia, Canada, France, Germany, Greece, Italy, Japan, Korea, Kyrgyzstan, Mexico, The Netherlands, New Zealand, Russia, South Africa, Spain, Taiwan, Thailand, the UK and the USA); and genotype 4 contains strains from humans and/or domestic pigs in China, India, Indonesia, Japan, South Africa, Taiwan and Vietnam (Schlauder & Mushahwar, 2001; see Table 1).

In Japan, polyphyletic HEV strains of genotypes 3 and 4 that are presumably indigenous to Japan have been isolated not only from humans and domestic pigs but also from boar, a deer and a mongoose captured in the wild (Nakamura *et al.*, 2006; Okamoto *et al.*, 2001; Sonoda *et al.*, 2004; Takahashi *et al.*, 2001; Tei *et al.*, 2003). Genotype 3 HEV isolates are

The GenBank/EMBL/DDBJ accession numbers for the sequences of the HE-JA04-1911, swJ8-5 and swJ12-4 isolates determined in this work are AB248520–AB248522, respectively.

Table 1. Comparison of the three HEV isolates of genotype 3 whose entire sequences were determined in this study with reported HEV isolates of the same genotype

NA, Not applicable.

Isolate name	Host	GenBank accession no.	Country of origin	Sequence length compared (nt)	Region of genome	Identity (%)*		
						HE-JA04-1911†	swJ8-5†	swJ12-4†
HE-JA04-1911†	Human	AB248520	Japan	7280	Full-length	NA	89·7	89·7
swJ8-5†	Swine	AB248521	Japan	7241	Full-length	89·7	NA	**92·9**
swJ12-4†	Swine	AB248522	Japan	7241	Full-length	89·7	**92·9**	NA
Osh205	Swine	AF455784	Kyrgyzstan	7239	Full-length	83·0	82·8	82·9
Arkell	Swine	AY115488	Canada	7255	Full-length	81·4	81·1	81·5
wbJTS1	Wild boar	AB222183	Japan	7241	Full-length	81·4	81·8	81·6
US2	Human	AF060669	USA	7277	Full-length	81·3	80·8	81·1
Meng	Swine	AF082843	USA	7242	Full-length	81·3	81·2	81·3
swJ570	Swine	AB073912	Japan	7257	Full-length	81·2	80·9	81·4
JBOAR1-Hyo04	Wild boar	AB189070	Japan	7247	Full-length	81·2	81·4	81·3
JDEER-Hyo03	Wild deer	AB189071	Japan	7230	Full-length	81·2	81·5	81·2
JRA1	Human	AP003430	Japan	7230	Full-length	81·1	81·2	81·4
JJT-Kan	Human	AB091394	Japan	7218	Full-length	81·1	81·2	80·9
JMY-Haw	Human	AB074920	Japan	7233	Full-length	81·1	81·4	81·1
JSO-Hyo03	Human	AB189073	Japan	7180	Full-length	81·1	81·4	81·2
JYO-Hyo03	Human	AB189075	Japan	7180	Full-length	81·1	81·4	81·1
wbJYG1	Wild boar	AB222184	Japan	7240	Full-length	81·1	81·1	81·1
HE-JA10	Human	AB089824	Japan	7262	Full-length	81·0	81·3	81·2
JMO-Hyo03	Human	AB189072	Japan	7180	Full-length	81·0	81·4	81·1
JTH-Hyo03	Human	AB189074	Japan	7180	Full-length	81·0	81·4	81·1
US1	Human	AF060668	USA	7202	Full-length	81·0	80·9	81·4
wbJSG1	Wild boar	AB222182	Japan	7240	Full-length	81·0	81·6	81·4
JKN-Sap	Human	AB074918	Japan	7256	Full-length	80·9	81·3	81·2
HEVN1	Human	AB246676	Japan	7231	Full-length	80·7	81·1	81·2
JNH-Ehi04	Human	AB200240	Japan	740	ORF1	**100·0**	**90·7**	**90·3**
swJ791	Swine	AB073911	Japan	412	ORF2	**98·1**	89·8	**90·3**
HEV-Sendai	Human	AB093535	Japan	412	ORF2	**97·8**	**90·0**	**90·5**
P354/1/02	Swine	AF503511	UK	473	ORF2	**92·4**	**90·1**	**90·3**
Gr2	Human	AF110389	Greece	519	ORF1+ORF2	**90·5**	**92·1**	**90·9**
NT3	Human	AJ879569	UK	280	ORF2	**90·3**	**91·0**	**91·8**
NT6	Human	AJ879572	UK	280	ORF2	**90·0**	**90·0**	**91·1**
swJ5-1	Swine	AB094216	Japan	412	ORF2	**90·0**	**94·7**	**93·7**
HE-JA26	Human	AB194284	Japan	412	ORF2	89·8	**94·2**	**93·4**
UK	Human	AJ315768	UK	287	ORF1	88·9	88·5	88·2
P143/11/02	Swine	AF503512	UK	473	ORF2	88·8	**92·0**	**90·9**
MP14	Human	AY626042	France	189	ORF2	88·4	89·9	89·4
swRSA-1	Swine	AY621664	South Africa	197	ORF2	88·3	86·8	86·8
UK7518	Human	AY582797	UK	304	ORF2	88·2	**91·8**	**91·8**
MP13	Human	AY626041	France	189	ORF2	87·8	89·4	88·9
NLSW82	Swine	AF336294	The Netherlands	718	ORF1+ORF2	87·6	87·2	86·3
GerWW	Human	AJ889195	Germany	938	ORF1+ORF2	87·2	86·9	87·8
SpswfCV3	Swine	DQ093564	Spain	307	ORF2	87·1	87·1	87·1
HEV/HU/NL2005-0825	Human	DQ200292	The Netherlands	148	ORF2	87·1	89·8	87·8
UIAS268	Human	DQ061078	Russia	682	ORF2	86·8	87·1	85·6
VH1	Human	AF195064	Spain	823	ORF1+ORF2	86·6	86·0	85·3
Gr1	Human	AF110388	Greece	519	ORF1+ORF2	86·3	86·9	85·0
VH2	Human	AF195065	Spain	823	ORF1+ORF2	85·8	86·0	85·8
Argentina	Swine	AY258006	Argentina	287	ORF1	85·0	82·9	81·9
Au1	Human	AF279122	Austria	519	ORF1+ORF2	84·6	83·2	82·2
E11	Swine	AF195063	Spain	304	ORF2	84·5	85·9	85·2

Table 1. cont.

Isolate name	Host	GenBank accession no.	Country of origin	Sequence length compared (nt)	Region of genome	Identity (%)*		
						HE-JA04-1911†	swJ8-5†	swJ12-4†
swNZ	Swine	AF215661	New Zealand	570	ORF1+ORF2	84·3	83·5	83·5
Ar1	Human	AF264009	Argentina	371	ORF1	83·8	82·7	82·5
Mexico	Swine	AF521654	Mexico	257	ORF1	83·7	79·8	80·5
E116-YKH98	Human	AY684252	Japan	317	ORF1	83·6	85·8	85·5
hKOR-DYL	Human	AY714270	Korea	720	ORF2	83·4	84·0	82·3
It1	Human	AF110387	Italy	519	ORF1+ORF2	83·4	83·0	82·4
swKOR1	Swine	AF516178	Korea	860	ORF2	82·8	83·4	82·8
Australia	Swine	AF521653	Australia	289	ORF1	82·6	81·2	81·6
T2-4s	Swine	AY858893	Thailand	304	ORF2	82·6	81·6	81·2
Ger-JS	Human	AY753647	Germany	1866	ORF1+ORF2	81·9	82·1	81·8
NLSW22	Swine	AF336291	The Netherlands	718	ORF1+ORF2	81·3	81·2	80·1
G3-2f	Swine	AY858933	Mexico	304	ORF2	81·2	83·6	82·6
Ar2	Human	AF264010	Argentina	371	ORF1	81·1	79·2	77·9
NT2	Human	AJ879568	UK	280	ORF2	81·1	83·9	85·4
HEV/HU/NL2002-0576	Human	DQ200275	The Netherlands	148	ORF2	80·3	80·3	81·0
S1-24s	Swine	AY858902	Mexico	304	ORF2	78·0	82·9	82·6
TW3SW	Swine	AF296167	Taiwan	304	ORF2	76·6	80·3	80·3

*Identities of over 90·0 % are indicated in bold.

†HEV isolates whose entire genomic sequences were determined in the present study.

provisionally classified into three phylogenetic clusters, with the highest nucleotide identity being 94·4–100 % between human and swine isolates in each cluster (Takahashi *et al.*, 2003). Entire or almost entire genomic sequences have been determined for 16 HEV isolates: 13 HEV isolates obtained from humans, pigs, wild boar and a wild deer segregated into a cluster (provisionally designated cluster IIIjp in our previous study; Takahashi *et al.*, 2003) consisting of predominantly Japan-indigenous strains represented by the JRA1 isolate (see Table 1 for GenBank accession no.) and three human HEV isolates were classified into a second cluster (cluster IIIus) with HEV isolates homologous to those in the USA (US1 and US2). The genomic characteristics and the possible origin of HEV isolates that have been classified into a third cluster (cluster IIIsp) and are 84·2–87·2 % similar to Spanish HEV isolates in the 304 nt ORF2 sequence remain unknown. In the present study, we determined the entire genomic sequences of three HEV isolates (HE-JA04-1911, swJ8-5 and swJ12-4) that belong to the third cluster within genotype 3 in Japan and differed by 89·8–94·2 % from each other in the 412 nt ORF2 sequence to investigate further the extent of the genomic heterogeneity of HEV and to compare them with all reported genotype 3 HEV isolates for which only partial sequences of 69–1866 nt in various genomic regions are available.

Serum samples were obtained from two domestic pigs (swJ8-5 and swJ12-4; Takahashi *et al.*, 2003) raised in Hokkaido, Japan, and from a 52-year-old Japanese male who contracted sporadic acute hepatitis E in July 2004. His laboratory data on admission showed an elevated total bilirubin level of 4·1 mg dl^{-1}, an aspartate aminotransferase level of 4680 IU l^{-1} and an alanine aminotransferase level of 3026 IU l^{-1}. He had IgM and IgA antibodies to HEV detectable by an in-house ELISA (Takahashi *et al.*, 2005) and HEV RNA detectable by RT-PCR (Mizuo *et al.*, 2002). He had no history of travel abroad. To determine the full-length sequence of the three HEV isolates, total RNA was extracted from 350 µl (HE-JA04-1911) or 750 µl (swJ8-5 and swJ12-4) of serum using TRIzol LS (Invitrogen) and the RNA preparation was reverse-transcribed and subjected to nested PCR. Seven overlapping regions excluding the extreme 5′ and 3′ termini were amplified (primer sequences excluded): nt 37–1270 (1234 nt), nt 1239–2074 (836 nt), nt 2020–3241 (1222 nt), nt 3110–4513 (1404 nt), nt 4324–6020 (1697 nt), nt 6011–6422 (412 nt) and nt 6383–7167 (785 nt): the nucleotide numbers were in accordance with HE-JA04-1911. The extreme 5′-end sequence (nt 1–53) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) using the First Choice RLM-RACE kit (Ambion), as described previously (Okamoto *et al.*, 2001). Amplification of the 3′-end sequence [nt 7143–7264 excluding the poly(A) tail] was attempted by the RACE method described previously (Okamoto *et al.*, 2001). The amplification products were sequenced on both strands, either directly or after cloning into pT7Blue T-Vector (Novagen) and sequence analysis was performed as described previously (Okamoto *et al.*, 2001). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) and bootstrap values were determined on 1000 resamplings of the datasets (Felsenstein, 1985).

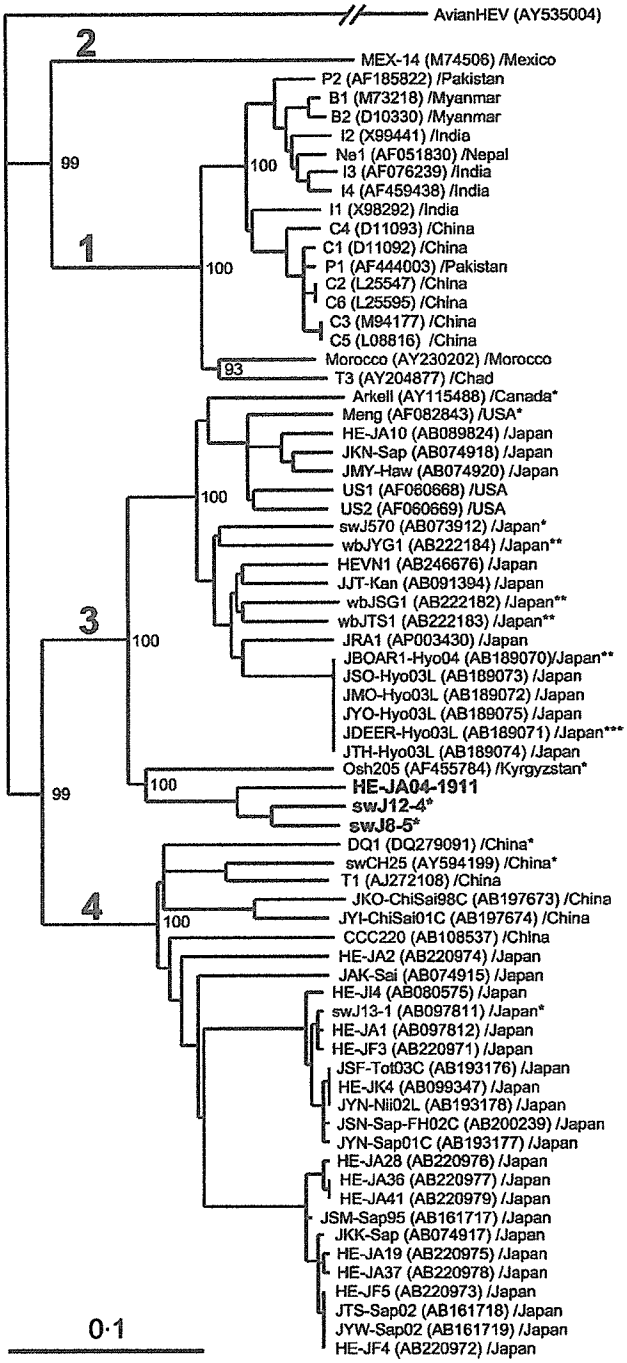
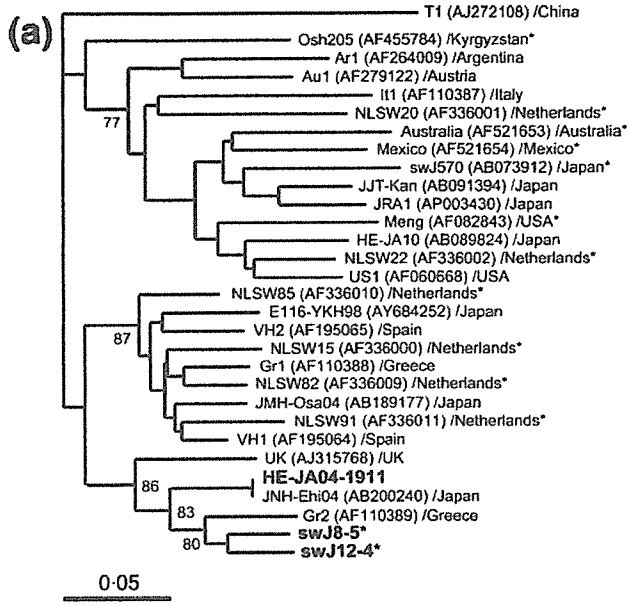


Fig. 1. Phylogenetic tree constructed by the neighbour-joining method based on the full-length nucleotide sequences of 70 HEV isolates, using an avian HEV isolate (GenBank/EMBL/DDBJ accession no. AY535004) as an outgroup. The human HEV isolate and two swine isolates whose full-length sequences were determined in the present study are indicated in bold. Fifty-five human isolates, seven swine isolates, four boar isolates and one deer isolate whose entire or almost entire sequence has been reported were included for comparison, with the GenBank accession numbers in parentheses. The name of the country where the HEV strain was isolated is shown after the slash. Swine HEV isolates, boar HEV isolates and the deer HEV isolate are indicated by one, two and three asterisks, respectively. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. Bar, 0·1 substitutions per site.

The HE-JA04-1911 isolate had a genomic length of 7264 nt, excluding the poly(A) tract at the 3′ terminus. This is the longest among all known HEV isolates whose entire sequence has been determined. The swJ8-5 and swJ12-4 isolates both had a genomic length of 7225 nt, the difference in genomic length of HE-JA04-1911 being attributed to an insertion of 39 nt in the hypervariable region of ORF1 in this isolate. These three isolates each possessed three major ORFs similar to all other reported HEV isolates including an avian HEV isolate (Tam *et al.*, 1991; Meng *et al.*, 1997; Huang *et al.*, 2004). ORF1, -2 and -3 had coding capacities of 1717 or 1704 aa (nt 26–5176 or 26–5137), 660 aa (nt 5214–7193 or 5175–7154) and 122 aa (nt 5176–5541 or 5137–5502), respectively. The 5′ UTRs of the three isolates comprised 25 nt, and swJ8-5 had a unique substitution of C at nt 11, whereas the other two isolates had T at this position. The 3′ UTRs of the three isolates each consisted of 71 nt [excluding the poly(A) tail], which was shorter than those in the 21 reported genotype 3 isolates due to a deletion of 5 nt after nt 7232.

On comparison of the entire genome, the HE-JA04-1911, swJ8-5 and swJ12-4 isolates had nucleotide identities of 89·7–92·9 % with each other and amino acid identities of 96·7–98·5 % in ORF1, 98·0–98·8 % in ORF2 and 97·5–99·2 % in ORF3 with each other. Following comparison with the 67 reported HEV isolates from humans, swine, wild boar and a wild deer whose entire or almost entire sequences have been determined, the HE-JA04-1911, swJ8-5 and swJ12-4 isolates were found to be 73·9–74·8 % similar to 17 reported genotype 1 isolates, 73·4–73·7 % similar to one genotype 2 isolate and 74·7–75·9 % similar to 28 reported genotype 4 isolates. Of note, the three HEV isolates obtained in the present study were closest to the Osh205 isolate recovered from a pig in Kyrgyzstan (Lu *et al.*, 2004) among the 21 reported genotype 3 HEV isolates, with identities of only 82·8–83·0 % (Table 1), and were 80·7–81·8 % similar to the remaining 20 HEV isolates of genotype 3. A phylogenetic tree was constructed based on the full-length genomic sequences of genotype 1–4 HEV isolates and confirmed that HE-JA04-1911, swJ8-5 and swJ12-4 belonged to genotype 3 and were genetically distinct from the genotype 3 strains prevalent in Japan and the USA (Fig. 1). Using CLUSTAL W (version 1.8) (Thompson *et al.*, 1994), sequence alignments were generated using all 70 HEV isolates of genotypes 1–4 including the three HEV isolates whose full-length sequences were determined in this study. Following alignment of the 70 HEV isolates of genotypes 1–4,



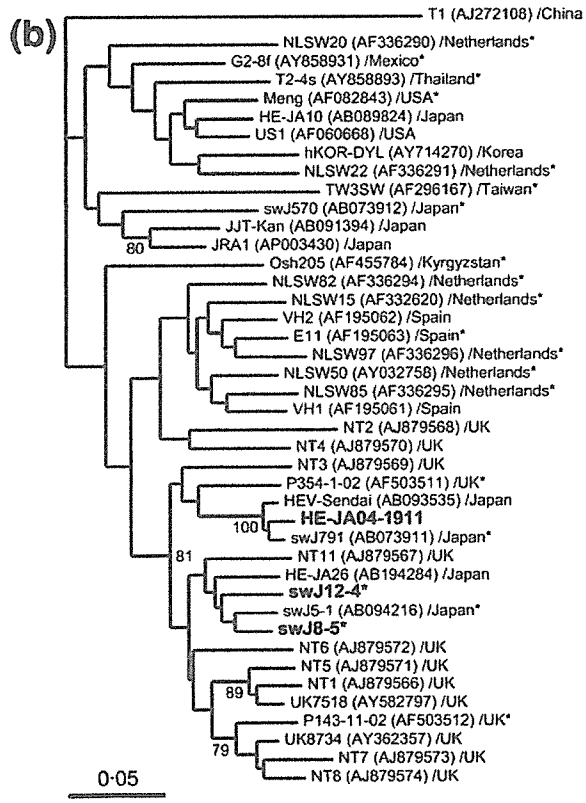


Fig. 2. Phylogenetic trees constructed by the neighbour-joining method based on the partial nucleotide sequences of genotype 3 HEV isolates, using a genotype 4 HEV isolate (T1, GenBank/EMBL/DDBJ accession no. AJ272108) as an outgroup. The human HEV isolate and two swine isolates whose full-length sequences were determined in the present study are indicated in bold. The isolate names are followed by the GenBank accession number in parentheses and the name of the country where the HEV strain was isolated. Swine HEV isolates are indicated by asterisks. A cluster consisting of UK and Japanese isolates is shaded for visual clarity. Bootstrap values of >75% are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. Bar, 0·05 substitutions per site. (a) Comparison of the partial nucleotide sequences of ORF1 (233 nt; nt 132–364 of the HE-JA04-1911 genome) of 29 human and swine HEV isolates of genotype 3. (b) Comparison of the partial nucleotide sequences of ORF2 (280 nt; nt 6084–6363 of the HE-JA04-1911 genome) of 41 human and swine HEV isolates of genotype 3.

conserved nucleotides were recognized in 2352 (45·7%) of 5151 nt in ORF1, 1167 (58·9%) of 1980 nt in ORF2 and 230 (62·8%) of 366 nt in ORF3, which included the well-conserved area in ORF3 that was reported to be suitable for designing primers and a probe for sensitive detection of HEV RNA by a broadly reactive real-time RT-PCR (Jothikumar *et al.*, 2006). On alignment of the deduced amino acid sequences of the 70 HEV genomes, conserved amino acids were recognized in 1050 (61·2%) of 1717 aa in ORF1, 497 (75·3%) of 660 aa in ORF2 and 65 (53·3%) of 122 aa in ORF3. The amino acid sequence of the ORF2 region encoding a capsid protein of HEV virions was well conserved, which probably contributes to the presence of a single serotype for HEV.

Following comparison with the 552 genotype 3 isolates whose partial sequences of 69–1228 nt in ORF1 or 145–938 nt in ORF2 are deposited in the GenBank/EMBL/DDBJ databases as of 27 January 2006, the HE-JA04-1911 isolate was most closely related to two human isolates (JNH-Ehi04 and HEV-Sendai) and one swine isolate (swJ791) of Japanese origin with identities of 100, 97·8 and 98·1%, respectively, in the 740 nt ORF1 or 412 nt ORF2 sequence, and the swJ8-5 and swJ12-4 isolates were closest to human and swine isolates (HE-JA26 and swJ5-1) of Japanese origin with identities of 93·4–94·7% (Table 1). Among the 245 genotype 3 HEV isolates of non-Japanese origin, the HE-JA04-1911 isolate had the highest similarity of 92·4% with a UK swine isolate (P354/1/02) in the 473 nt ORF2 sequence and the lowest similarity of 76·6% with a Taiwanese swine isolate (TW3SW) in the 304 nt ORF2 sequence. Compared with non-Japanese isolates of genotype 3, the swJ8-5 and swJ12-4 isolates were closest to UK human (UK7518) and swine (P143/11/02) isolates with identities of 90·9–92·0% and had the lowest similarity of 77·9–79·2% with the Ar2 isolate in Argentina. The swJ8-5 isolate was also close to a Greek human isolate (Gr2) with an identity of 92·1%. The phylogenetic tree constructed based on the common 233 nt ORF1 sequence of genotype 3 HEV isolates using a genotype 4 isolate (T1) as an outgroup revealed that the HE-JA04-1911, swJ8-5 and swJ12-4 isolates segregated into a cluster consisting of UK, Greek and Japanese isolates (Fig. 2a). The phylogenetic tree constructed