Table 2 Age-dependent prevalence of HEV RNA in the sera of farm pigs in 14 herds in Inner Mongolia, China

Farm	Area	Prevalence of HEV RNA among pigs of age						No. of HEV RNA-positive pigs	Names of HEV isolates[c]
		2 months		3 months		4 months			
		No.[a]	Positive[b]	No.	Positive	No.	Positive		
1	Hulunbuir	6	0	21	1 (5%)	5	0	1 (3%)	swIM1-21
2	Hulunbuir	0		12	0	4	0	0	
3	Hulunbuir	7	0	6	0	8	0	0	
4	Ulanhad	4	3 (75%)	6	3 (50%)	1	1 (100%)	7 (64%)	swIM4-1, 4, 6–9, and 11
5	Ulanhad	8	0	11	0	21	0	0	
6	Ulanhad	10	2 (20%)	19	0	15	2 (13%)	4 (9%)	swIM6-22, 26, 41, and 43
7	Ulanhad	8	0	15	0	18	0	0	
8	Hohhot	5	2 (40%)	4	1 (25%)	11	6 (55%)	9 (45%)	swIM8-1, 4, 6, 8, 11, 12, 14, 15, and 17
9	Hohhot	4	0	6	0	0		0	
10	Hohhot	14	0	7	0	0		0	
11	Alxa	23	0	5	0	8	0	0	
12	Alxa	0		14	7 (50%)	15	2 (13%)	9 (31%)	swIM12-2, 3, 5–8, 9, 19, and 21
13	Alxa	6	0	0		8	0	0	
14	Alxa	6	0	6	0	9	0	0	
Total		101	7 (7%)	132	12 (9%)	123	11 (9%)	30 (8%)	

[a] No. of pigs examined

[b] No. of pigs positive for HEV RNA

[c] sw, IM, 1, and -21 in swIM1-21 stands for swine, Inner Mongolia, no. of farm, and no. of serum sample, respectively

Table 3 Intra- and inter-farm nucleotide sequence identities and genotypic grouping of swine HEV isolates obtained in the present study

Farm	No. of swine HEV isolates	Intra-farm nucleotide identity (%)	Inter-farm nucleotide identity (%)	HEV genotype	Cluster[a]
1	1	–	84.7–87.1	4	4uc
4	7	99.5–100	84.7–93.9	4	4a-1
6	4	100	85.9–96.4	4	4a-2
8	9	86.9–100	84.7–96.4	4	4a-2/4d
12	9	86.4–100	84.7–95.9	4	4a-2/4a-3/4b

[a] See Fig. 3

2.5–7.0% in serum samples and 9.6-26.1% in stool samples [23, 48, 49, 52]. In our present study, 30 (8%) of 356 pigs were found to be viremic upon serum analysis. The higher prevalence of HEV RNA in the present study may be due to the age range (2–4 months) of the pigs studied, which is considered to be the age range at which infected pigs are at the highest risk of spreading the infection [34, 35].

In the present study, HEV RNA-positive sera were obtained from at least one farm in each area. The rate of swine HEV seropositivity varied by farm, ranging from 0% (Farm 14) to 100% (Farm 3), with a mean value of 52%. Of interest, the prevalence of swine anti-HEV was only 0–29% at the three farms (Farms 2, 13, and 14) with the smallest number of pigs being raised (n = 52–120), while it ranged from 78 to 100% in the largest farms, where 610–1,500 pigs were being reared. Although the conditions of the farms, such as their structure, location, water source, vermin control, and handling may differ, it seems likely that the seropositivity rate of HEV is associated with the number of pigs being reared, or with the feeding density in a swine farm.

The 30 swine HEV isolates obtained in the present study were genetically heterogeneous, differing from each other by up to 15.3% and segregated into six phylogenetic clusters within genotype 4 (tentatively designed as clusters 4a-1, 4a-2, 4a-3, 4b, 4d, and 4uc), having the highest identities of 97.7, 97.7, 96.3, 97.1, 98.0, and 94.2%, respectively, to reported human or swine HEV strains isolated in China (Table 4). These results indicate that markedly divergent genotype 4 HEV strains are circulating among pigs in Inner Mongolia, but that these strains are similar to those in the other provinces of China. In support of this finding, farmers from all 14 farms studied told us that pigs were purchased from the same or neighboring

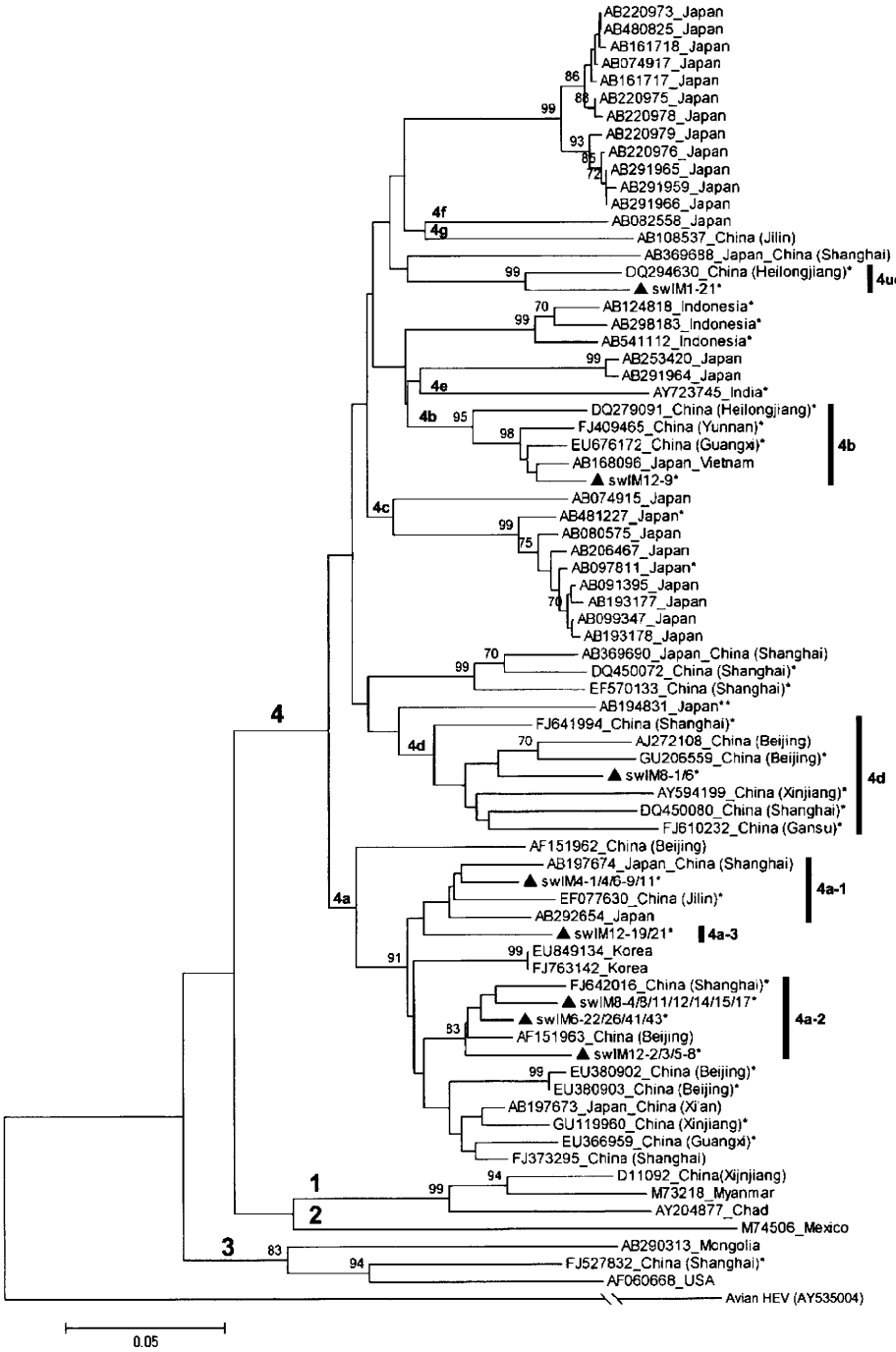

Fig. 3 Phylogenetic tree constructed by the neighbor-joining method, based on the partial nucleotide sequence of the ORF2 region (412 nt) of 96 HEV isolates, using an avian HEV strain (AY535004) as an outgroup. In addition to the 30 Inner Mongolian swine HEV isolates found in the present study (indicated with a *closed triangle* for visual clarity), 66 representative HEV isolates of genotypes 1–4 whose overlapping 412 nt sequence is known, were included for comparison. The reported isolates are indicated with the accession number, followed by the name of the country where it was isolated: *asterisks* indicate isolates obtained from pigs (*) and a wild boar (**). *Vertical bars* represent clusters (4a-1, 4a-2, 4a-3, 4b, 4d, and 4uc) within genotype 4. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 re-samplings

regions (Hohhot, Baotou and Jirim) in Inner Mongolia or from the other provinces in China, including Beijing, Hebei, Heilongjiang, and Ningxia. In fact, swine HEV isolates of Cluster 4a-1 in pigs from Farm 4 were 96.0% similar to that (FJ72550) in Jirim, Inner Mongolia, where the pigs from Farm 4 originated. The swIM1-21 isolate from Farm 1 segregated into Cluster 4uc, along with one reported swine HEV strain (DQ294630) in Heilongjiang. Pigs from Farm 1, where the swIM1-21 isolate was obtained, were purchased from a farm in Heilongjiang, suggesting that the swIM1-21 isolate was derived from Heilongjiang by this trading, although the swIM1-21 isolate was only 94.2% identical to the Heilongjiang swine isolate (Table 4). Of note, although the swIM12-9 isolate of Cluster 4b showed the highest nucleotide identity (97.6%) to the HEV strains recovered from a Japanese patient who developed acute hepatitis E after traveling to Vietnam, and from a wild boar in Japan (Okinawa), it was 96.4–97.1% identical to the swine HEV strains identified in the southern part of China, including Hubei, Hunan, and

Table 4 Comparison of the 300–412 nt ORF2 sequences of 30 swine HEV isolates in Inner Mongolia obtained in the present study with 27 human, swine and wild boar HEV isolates whose entire genomic sequence or 300 nt overlapping sequence is known

Accession no.	Source	Nucleotide length compared (nt)	swIM1-21 (4uc)	swIM4-1/4/6–9/11 (4a-1)	swIM6-22/26/41/43 (4a-2)	swIM8-4/8/11/12/14/15/17 (4a-2)	swIM12-2/3/5–8 (4a-2)	swIM12-19/21 (4a-3)	swIM8-1/6 (4d)	swIM12-9 (4b)
DQ294630	Swine/China (Heilongjiang)	412	**94.2**	86.1	87.1	85.9	86.1	87.9	87.1	85.0
AF134916	Human/China (Anhui)	301	84.4	**97.7**	**94.0**	93.0	92.4	93.0	85.7	85.7
FJ775174	Swine/China (Xinjiang)	356	83.7	**96.9**	93.5	92.7	92.4	**94.1**	85.9	86.2
EF488821	Swine/China (Beijing)	304	85.5	**96.4**	93.1	91.4	92.1	93.1	85.9	84.9
FJ172540	Swine/China (Heilongjiang)	348	84.2	**96.3**	93.1	91.7	92.2	92.2	84.5	85.6
FJ172550	Swine/China (Jirim, Inner Mongolia)	348	83.9	**96.0**	93.1	91.7	91.7	92.0	84.2	85.6
AB197674	Human/China (Shanghai)[a]	412	84.7	**95.9**	93.0	91.7	93.0	92.7	86.9	86.1
DQ445496	Human/China (Jilin)	348	86.5	**95.7**	91.7	90.2	89.7	92.0	84.8	85.1
AJ428852	Swine/China (Beijing)	300	86.7	**95.0**	93.3	91.7	89.7	**96.3**	86.3	85.0
DQ445493	Human/China (Jilin)	348	85.3	93.7	**97.7**	**94.5**	**95.1**	92.5	85.3	85.3
AF151963	Human/China	412	84.4	93.7	**97.6**	**95.3**	**95.4**	92.4	87.1	88.5
FJ642016	Swine/China (Shanghai)	412	84.9	92.0	**96.8**	**96.1**	93.7	91.5	86.1	87.6
DQ445494	Swine/China (Jilin)	348	84.1	92.0	**96.0**	93.4	**94.0**	89.9	86.5	86.2
FJ461761	Swine/China (Hubei)	304	84.5	**94.1**	**95.4**	93.1	93.8	90.5	86.5	84.5
AB197673	Human/China (Xi'an)[b]	412	85.6	93.9	**95.4**	93.7	92.2	92.9	87.1	87.6
FJ373295	Human/China (Shanghai)	412	84.9	93.7	**95.1**	93.9	93.0	92.5	88.3	87.8
EF488819	Swine/China (Xinjiang)	361	85.3	93.9	**95.0**	93.6	92.8	91.4	86.7	86.1
AF134812	Human/China (Anhui)	301	83.7	92.4	**94.7**	92.4	**95.7**	91.0	86.4	86.0
FJ461765	Human/China (Hubei)	304	86.2	86.2	85.2	85.5	83.9	85.5	**98.0**	85.2
AB168096	Human/Vietnam[c]	412	86.9	87.1	87.6	88.3	87.6	86.7	86.9	**97.6**
AB194831	Wild boar/Japan	412	86.9	87.1	87.6	88.3	87.6	86.7	86.9	**97.6**
EU620643	Swine/China (Hunan)	304	87.2	85.9	85.9	86.2	84.5	84.9	86.2	**97.1**
EU676172	Swine/China (Guangxi)	412	86.6	86.9	87.9	88.6	87.9	86.4	87.1	**96.8**
EU620641	Swine/China (Hunan)	304	86.5	85.9	85.9	85.5	85.2	84.9	86.2	**96.7**
FJ461770	Swine/China (Hubei)	304	87.2	85.2	86.5	86.8	85.9	84.9	86.2	**96.4**
FJ409465	Swine/China (Yunnan)	412	85.7	88.3	87.8	88.6	87.6	86.9	87.4	**95.9**
AJ428856	Swine/China (Zhejiang)	300	86.0	85.3	85.7	86.0	84.3	83.7	85.0	**95.7**

Values ≥94.0 are indicated in bold and the highest value for each isolate is indicated in bold with underline

[a] Recovered from a Japanese patient who developed acute hepatitis E after traveling to Shanghai, China

[b] Recovered from a Japanese patient who developed acute hepatitis E after traveling to Xi'an, China

[c] Recovered from a Japanese patient who developed acute hepatitis E after traveling to Vietnam

Guangxi provinces (Table 4), suggesting that the 4b strains are indigenous to China. Evidence that swine HEV can be imported through international trade of pigs was reported in Taiwan [46]. Therefore, it is very likely that the HEV strains spreading in Inner Mongolia are originate from various areas of China through domestic trading.

Our previous studies indicated the homogeneous nature and uniqueness of swine HEV isolates in each of the independent swine farms in Japan, with intra-farm nucleotide sequence differences of up to 2.9% and inter-farm nucleotide sequence differences of 0.9–23.4% [34, 35, 37]. In the present study, swine HEV strains belonging to a single cluster were identified from Farm 4 (Cluster 4a-1) and Farm 6 (Cluster 4a-2), and the intra-farm nucleotide sequence identity was 100% in these two farms. However, co-circulation of divergent HEV isolates belonging to multiple clusters in the same herd was demonstrated in Farm 8 (Clusters 4a-2 and 4d), with a nucleotide sequence difference of 13.1%, and Farm 12 (Clusters 4a-2, 4a-3 and 4b) with a nucleotide sequence of 9.5–13.6%). This corroborates recent reports from Shanghai, China, showing that both genotypes 3 and 4 were detected in fecal specimens collected from a pig farm [15, 22]. The observed marked genomic heterogeneity of swine HEV strains from the two farms in Inner Mongolia may be ascribable to repeated trading of pigs from different regions in Inner Mongolia or the other provinces of China, as reported by farmers at some of the swine farms studied.

It has been reported that farm pigs in Mongolia, formerly known as Outer Mongolia, are frequently infected with genotype 3 HEV strains that may be indigenous to Mongolia or may have originated from the Netherlands,

based on the finding that Mongolian swine HEV strains shared 94.6% identity with the HEV strain HU/NL2005-0825 (DQ200292) isolated from the Netherlands [16]. Contrary to our speculation, the present study revealed that genotype 4 HEV strains are distributed widely in Inner Mongolia, as in the other provinces of China, although genotype 3 HEV strains still prevail in some provinces, including Shanghai, Anhui, Zhejiang and Henan [51]. Mongolian genotype 3 swine HEV strains are quite different from those identified in other areas of China, with nucleotide sequence identities of only 81.1–84.1%, suggesting that Mongolian and Chinese genotype 3 swine HEV strains originated from different ancestors. For more than 3,000 years, indigenous Mongolians living in both Inner Mongolia and Outer Mongolia have raised cattle, sheep, goats, camels and horses as their livestock in their nomadic life in the Mongolian plateau. Although many people in Mongolia still maintain the nomadic life, many Mongolians in Inner Mongolia have started to live like Han Chinese following their migration to Inner Mongolia, which occurred in the 18th century, and some Han immigrants brought pigs as livestock more than a century ago. After the reform and opening up policy in the 1980s in China, many large or family-scaled swine farms were established in the suburbs of Inner Mongolia. These historical events may support our findings that, different from pigs in Mongolia, those in Inner Mongolia are infected with genotype 4 HEV strains that are circulating in various areas of China.

In conclusion, the present study indicates that HEV infection is highly prevalent and widespread among pigs in Inner Mongolia, and that the markedly divergent genotype 4 swine HEV isolates, provisionally classifiable into six distinct clusters within genotype 4, show high similarity to Chinese human or swine HEV strains, suggesting that the swine HEV strains in Inner Mongolia originated from the other provinces of China, although heterogeneous genotype 4 HEV strains prevail. Given the high prevalence of infection and the type of infections present in the swine, this study may raise further public-health concerns about HEV zoonosis. Therefore, further seroepidemiological and molecular studies are warranted to clarify whether people living in Inner Mongolia are suffering from acute hepatitis E as a result of zoonosis.

Acknowledgments This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan.

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Journal of General Virology (2011), **92**, 269–278 DOI 10.1099/vir.0.025791-0

A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells

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We have previously demonstrated that the release of hepatitis E virus (HEV) from infected cells depended on ORF3 protein, which harbours one or two PSAP motifs. To elucidate the PSAP motif(s) in the ORF3 protein during virion egress, five PSAP mutants derived from an infectious genotype 3 cDNA clone of pJE03-1760F/wt that can grow efficiently in PLC/PRF/5 cells were analysed. Four mutants, including mutLSAP, mutPSAL, mutLSAL (the substituted amino acids in the authentic PSAP motif are underlined) and mutPLAP/PSAP (the changed amino acid in the additional PSAP motif is underlined) generated progenies as efficiently as the wild-type virus. Conversely, the HEV RNA level in the culture supernatant of mutPLAP/LSAL RNA-transfected cells was significantly lower than in cells transfected with the wild-type RNA, similar to an ORF3-null mutant. Consistent with the ORF3-deficient mutant, the mutPLAP/LSAL mutant with no intact PSAP motifs banded at 1.26–1.27 g ml^{-1} in sucrose, and was captured by anti-ORF2, but not by anti-ORF3, with or without prior treatment with detergent (0.1 % sodium deoxycholate). The absence of the ORF3 protein on the mutant particles in the culture supernatant was confirmed by Western blotting, despite the expression of ORF3 protein in the RNA-transfected cells, as detected by immunofluorescence and Western blotting. Therefore, at least one of the two intact PSAP motifs in the ORF3 protein is required for the formation of membrane-associated HEV particles possessing ORF3 proteins on their surface, thus suggesting that the PSAP motif plays a role as a functional domain for HEV budding.

Received 25 July 2010
Accepted 8 November 2010

INTRODUCTION

Hepatitis E virus (HEV), a member of the genus *Hepevirus* in the family *Hepeviridae*, is the causative agent of acute hepatitis E, which occurs in many parts of the world (Emerson & Purcell, 2006; Harrison, 1999; Okamoto *et al.*, 2003; Worm *et al.*, 2002). HEV is a non-enveloped virus and its genome comprises positive-sense ssRNA of approximately 7200 nt, which is capped and polyadenylated (Kabrane-Lazizi *et al.*, 1999; Tam *et al.*, 1991). The genome consists of a 5′ UTR, three ORFs (ORF1, ORF2 and ORF3) and a 3′ UTR (Emerson & Purcell, 2006). ORF1 encodes non-structural proteins including the helicase and RNA-dependent RNA polymerase (Agrawal *et al.*, 2001; Koonin *et al.*, 1992). All but the 5′ terminus of ORF3 is overlapped by ORF2, and the ORF2 and ORF3 proteins are translated from the same bicistronic subgenomic RNA (Graff *et al.*, 2006). The ORF2 protein is the viral capsid protein, while the ORF3 protein is a small protein of only 113 or 114 aa. It was suggested that the ORF3 protein acts as an adaptor to link the intracellular transduction pathways, reduce the host inflammatory response and protect virus-infected cells (Chandra *et al.*, 2008). Four major genotypes (genotypes 1–4) of HEV have been identified in mammals. Genotypes 1 and 2 have caused outbreaks of hepatitis E as water-borne epidemics, while genotypes 3 and 4 have been found in sporadic cases of acute hepatitis E that were most likely zoonotic in origin because genotypes 3 and 4 HEVs infect not only humans, but also swine, and rarely, other non-primate mammals (Meng, 2010; Okamoto, 2007). At least three genotypes of avian HEV have been identified from chickens worldwide, but these genotypes only share approximately 50 % nucleotide sequence identity with mammalian HEVs (Meng, 2010; Bilic *et al.*, 2009).

Recently, using faecal suspensions or serum samples with high HEV loads originally obtained from hepatitis patients who contracted imported or domestic infection of genotypes 1, 3 or 4 HEV as inocula, we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5) and a lung cancer cell line (A549), which yielded an HEV load of up to 10^8 copies ml^{-1} in the culture supernatant, and successfully propagated multiple

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Primer sequences are available with the online version of this paper.

generations of serial passages in culture supernatant (Tanaka *et al.*, 2007, 2009; Lorenzo *et al.*, 2008; Takahashi *et al.*, 2007, 2010). In addition, we constructed a full-length infectious cDNA clone (pJE03-1760F/wt) of a genotype 3 HEV that can grow as efficiently as the faeces-derived virus in both PLC/PRF/5 and A549 cells (Yamada *et al.*, 2009b). Our previous study using an ORF3-deficient cDNA clone of pJE03-1760F/wt revealed that the ORF3 protein is essential for virion egress from infected cells (Yamada *et al.*, 2009a). Furthermore, the ORF3-null mutant produced virus particles with the same buoyant density (1.26–1.27 g ml^{-1} in sucrose) as faecal HEV, which were clearly different from the cell culture-generated wild-type virions and those in the circulation of infected individuals, which banded at 1.15–1.16 g ml^{-1} and are intimately associated with lipids and the ORF3 protein (Yamada *et al.*, 2009a; Takahashi *et al.*, 2008b, 2010). These observations suggest that the acquisition of host-cell membrane on the surface of the virions is dependent on the expression of the ORF3 protein.

It was previously reported that cellular Tsg101 binds to the PSAP motif located within the ORF3 protein, and that substitutions in the PSAP sequence abolish the ability of the protein to bind Tsg101 (Surjit *et al.*, 2006). The PSAP motif is conserved in all HEV isolates, including avian HEV (see Fig. 1). Tsg101 has been identified as a critical cellular protein required for budding of enveloped viruses, i.e. human immunodeficiency virus type-1 (HIV) and Ebola virus, from the plasma membrane (Garrus *et al.*, 2001; Martin-Serrano *et al.*, 2001). Therefore, in this study, we investigated the function of the PSAP motif in the ORF3 protein in the release of virus by using various site-directed mutants derived from the pJE03-1760F/wt cDNA clone, which has two PSAP motifs in the ORF3 protein, and a robust cell-culture system for HEV.

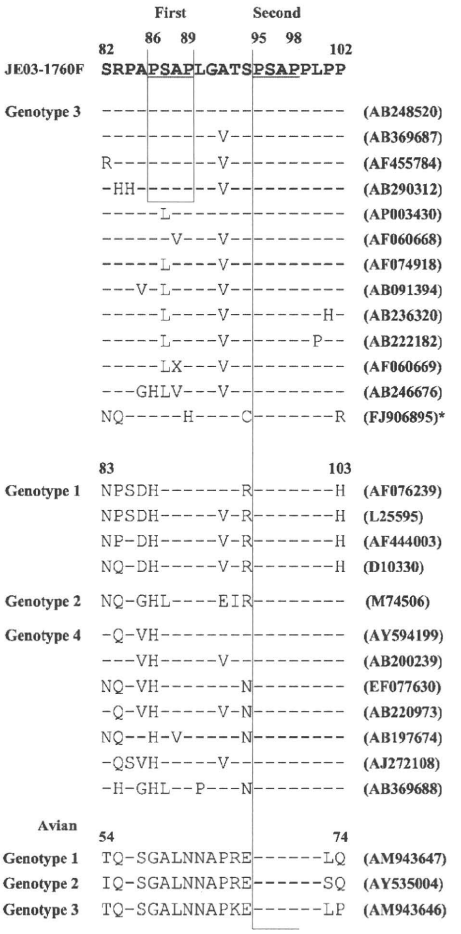

Fig. 1. Alignment of the partial amino acid sequences of the ORF3 protein of mammalian (genotype 3, aa 82–102; genotypes 1, 2 and 4, aa 83–103) and avian (aa 54–74) HEV strains. Representative HEV isolates with a different amino acid sequence within the indicated area were adopted, and their GenBank accession numbers are indicated in the parentheses. Asterisk indicates an HEV sequence derived from a rabbit.

RESULTS

The PSAP motif in the ORF3 protein is conserved in all known HEV strains

The PSAP motif between amino acid residues 95 and 98 of the ORF3 protein was found to be conserved among all known HEV strains including avian HEV strains (Fig. 1). Interestingly, several isolates belonging to genotype 3, including strain JE03-1760F, possessed one additional PSAP motif between residues 86 and 89, located N-terminal to the other motif. Therefore, for simplicity in this manuscript, the PSAP motif located at residues 86–89 was tentatively designated the 'first PSAP motif' and that located at residues 95–98 as the 'second PSAP motif'.

Analysis of HEV variants with mutations in the second PSAP motif of the ORF3 protein

To examine whether the second PSAP motif in the ORF3 protein is essential for the release of HEV virions from infected cells, we carried out site-directed mutational analysis using pJE03-1760F/wt, an infectious cDNA clone of genotype 3 HEV (Fig. 2a). First, amino acid substitutions were introduced into the second authentic PSAP motif in ORF3 to construct three PSAP variants with a mutation from Pro to Leu at the first amino acid residue (mutLSAP) or at the fourth amino acid residue (mutPSAL), or with a dual mutation from Pro to Leu at the first and fourth residues (mutLSAL). Although ORF3 overlaps ORF2, these mutations do not change the ORF2 amino acid sequence in any of the three variants. RNA transcripts of pJE03-1760F/wt and its mutants were transfected into PLC/PRF/5 cells, and the HEV RNA titre in the culture supernatant was measured to determine the efficiency of virus production. The HEV RNA level in the culture supernatant of the cells transfected with RNAs of mutLSAP, mutPSAL or mutLSAL increased in almost the same manner as that of the wild-type

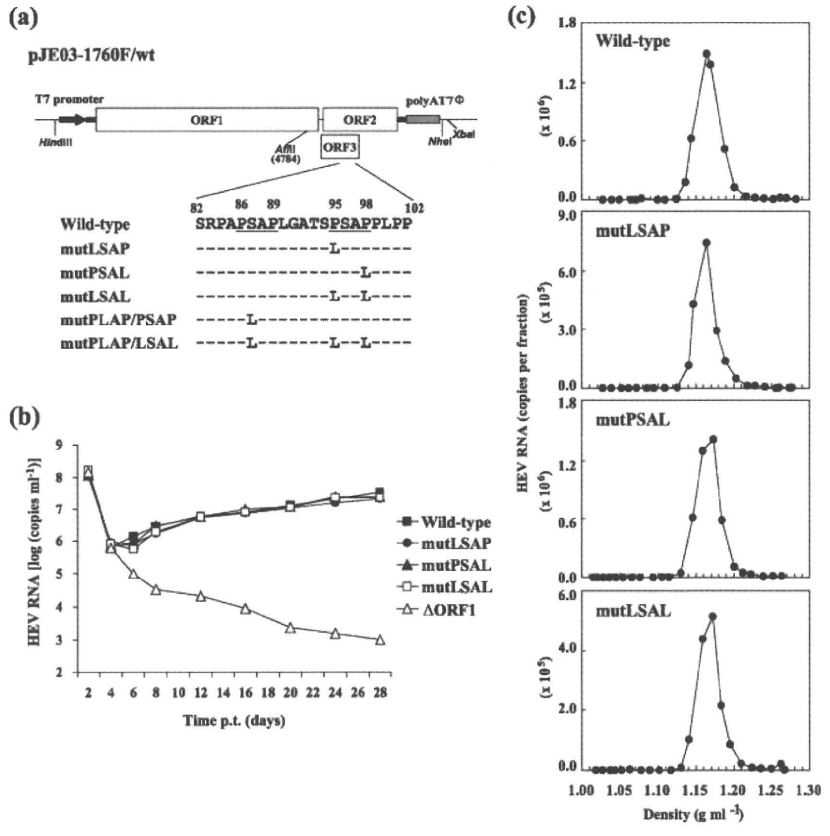

Fig. 2. (a) A schematic diagram of the full-length cDNA clone of the HEV JE03-1760F strain (pJE03-1760F/wt) and its derivative mutants. The closed bars and open boxes represent the untranslated regions and coding regions of the HEV genome, respectively. A closed bar with an arrow indicates a T7 promoter, and a shaded box depicts the fragment poly(A)T7Φ containing a 31 nt poly(A) sequence and a T7 terminator (Φ). A partial amino acid sequence of the ORF3 protein (residues 82–102) of pJE03-1760F/wt and five PSAP mutants with one to three substitutions from Pro to Leu or Ser to Leu are aligned. PSAP motifs are underlined. (b) Quantification of HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells. The experiment was carried out in two wells for each sample, and its mean titres are plotted. (c) Sucrose density-gradient fractionation of the wild-type virus and its mutants in culture supernatants.

RNA-transfected cells during the observation period of 6–28 days post-transfection (p.t.) (Fig. 2b). A gradual decrease in HEV load was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, serving as a negative control, whose titre probably reflects residual amounts of the introduced RNA transcripts in the culture medium.

The culture supernatants of wild-type RNA- or mutant RNA-transfected cells were subjected to equilibrium centrifugation in a sucrose density gradient. Three mutants (mutLSAP, mutPSAL and mutLSAL) in the culture supernatant exhibited a peak density of 1.16 g ml^{-1}, similar to the wild-type virus (Fig. 2c). When the peak fraction of the wild-type virus and these three mutants in the culture supernatant of transfected cells was subjected to immunocapture RT-PCR assays, essentially no virus was captured by a mouse mAb against ORF2 protein (anti-ORF2 mAb) (H6225) and anti-ORF3 mAb (TA0536) (Table 1). However, upon prior treatment with 0.1 % sodium deoxycholate, the binding efficiency of these three mutants increased to 61.8–90.8 % for the anti-ORF2 mAb and to 68.9–73.6 % for the anti-ORF3 mAb, similar to the wild-type virus (85.2 and 75.2 %, respectively). These results suggest that the properties of these particles generated by transfection of mutant RNAs are similar to those of wild-type particles in cell culture, i.e. released particles are likely to be associated with lipids and the ORF3 protein.

Analysis of HEV variants with mutation in the first or both PSAP motifs in the ORF3 protein

As shown in Fig. 1, strain JE03-1760F has two PSAP motifs in the ORF3 protein. We next constructed HEV clones with amino acid substitutions in the first PSAP motif only (mutPLAP/PSAP), or in both the first and second PSAP motifs (mutPLAP/LSAL) of the ORF3 protein (Fig. 2a); these mutations do not affect amino acid sequences of the ORF2 protein. The HEV RNA level in the culture supernatant of the mutPLAP/PSAP RNA-transfected cells increased gradually from 6 days p.t., reaching 1.7×10^7 copies ml^{-1} on 28 days, although the level was approximately 0.5-fold lower than that of cells transfected with the wild-type RNA during 6–28 days p.t. (Fig. 3a). In contrast, the introduction of amino acid substitutions in both PSAP motifs (mutPLAP/LSAL) significantly reduced the virus yield in the culture supernatant, similar to an ORF3-deficient variant, ΔORF3 (Fig. 3a). To clarify whether the increase in viral RNA shown in Fig. 3(a) is related to an increase in cells expressing viral protein, immunocapture RT-PCR assay was performed using an anti-ORF2 mAb (H6225) capable of capturing HEV particles, after treatment with 0.1 % sodium deoxycholate. The genomic RNAs of captured wild-type, mutPLAP/PSAP, mutPLAP/LSAL and ΔORF3 HEV particles were detectable at 4 days p.t., and the RNA levels increased gradually thereafter, in a similar manner to that indicated in Fig. 3(a) (Fig. 3b); the culture supernatant of the ΔORF1 RNA-transfected cells

Table 1. Results of the immunocapture RT-PCR assay

Virus*	Input (copies per well)	Captured HEV per input (%)		
		mAb H6225 (anti-ORF2)	mAb TA0536 (anti-ORF3)	mAb 5520 (negative control)†
Without pre-treatment with detergent				
pJE03-1760F/wt (1.16 g ml^{-1})	1 300	2.5	6.7	0.1
mutLSAP (1.16 g ml^{-1})	700	2.7	9.9	0.0
mutPSAL (1.16 g ml^{-1})	1 200	4.7	7.3	0.1
mutLSAL (1.16 g ml^{-1})	440	4.7	8.9	0.0
mutPLAP/PSAP (1.16 g ml^{-1})	770	1.2	4.7	0.2
mutPLAP/LSAL (1.27 g ml^{-1})	390	96.5	0.4	0.0
ΔORF3 (1.27 g ml^{-1})	230	95.8	0.0	0.1
With pre-treatment with detergent‡				
pJE03-1760F/wt (1.16 g ml^{-1})	1 100	85.2	75.2	0.2
mutLSAP (1.16 g ml^{-1})	730	79.0	73.6	0.0
mutPSAL (1.16 g ml^{-1})	850	90.8	68.9	0.0
mutLSAL (1.16 g ml^{-1})	360	61.8	71.4	0.2
mutPLAP/PSAP (1.16 g ml^{-1})	560	79.9	77.0	0.1
mutPLAP/LSAL (1.27 g ml^{-1})	650	95.0	0.0	0.0
ΔORF3 (1.27 g ml^{-1})	370	92.3	0.4	0.1

*Viruses were derived from the culture supernatant of transfected cells (RNAs of pJE03-1760F/wt, mutLSAP, mutPSAL, mutLSAL, mutPLAP/PSAP, mutPLAP/LSAL or ΔORF3) at 28 days p.t., and peak fractions with the indicated sucrose density (see Fig. 2c and Fig. 3c) were subjected to immunocapture RT-PCR.

†mAb against hepatitis B virus pre-S2 protein (Okamoto *et al.*, 1985).

‡Prior to performing the immunocapture RT-PCR assay, 6 µl of the sucrose fraction was mixed with 60 µl of 0.11 % sodium deoxycholate and incubated at 37 °C for 2 h, then was diluted 1 : 10 with PBS containing 0.1 % BSA.

tested negative for particle-associated HEV RNA throughout the observation period. The particles in the culture supernatant of the mutPLAP/PSAP RNA-transfected cells peaked at 1.16 g ml^{-1}, as did the wild-type virus, while the particles generated in the culture supernatant of the mutPLAP/LSAL RNA-transfected cells banded at 1.26–1.27 g ml^{-1}, similar to those in the ΔORF3 RNA-transfected cells (Fig. 3c).

Immunofluorescence assays (Fig. 3d) and Western blotting (Fig. 3e) revealed the expression of the ORF3 protein in the mutPLAP/LSAL RNA-transfected cells, indicating that an anti-ORF3 mAb used in the present study is reactive even with the mutated ORF3 protein, despite the presence of one or two amino acid mutations in each of the two PSAP motifs, and that at least one of the two PSAP motifs in the ORF3 protein is indispensable for the formation of membrane-associated HEV particles.

Characterization of the mutPLAP/LSAL particle in the culture supernatant

To examine whether the ORF3 protein associates with viral particles, Western blot analysis was performed using the culture supernatant of transfected cells. In the cells transfected with wild-type virus, the ORF3 protein was clearly detected in the culture supernatant (Fig. 3f). In contrast, ORF3 protein expression was not detectable in the culture supernatant derived from ΔORF3 RNA- and mutPLAP/LSAL RNA-transfected cells. Immunocapture RT-PCR was then performed for the culture supernatant in the peak fraction obtained from sucrose density-gradient centrifugation (Table 1). Viral particles in the culture supernatant of the mutPLAP/LSAL RNA-transfected cells were efficiently captured by an anti-ORF2 mAb, but not by anti-ORF3 mAb, similar to what was observed in the supernatant of the ΔORF3 RNA-transfected cells, with or without prior treatment with 0.1 % sodium deoxycholate.

PSAP motifs are important for efficient virion egress

The intracellular viral RNA was serially quantified by two distinct real-time RT-PCR methods: the ORF1 and ORF2/3 probes are capable of detecting genomic RNA only (Fig. 4a) and both genomic and subgenomic RNAs (Fig. 4b), respectively. The HEV RNA level detectable by both the ORF1 probe and the ORF2/ORF3 probe in the mutPLAP/LSAL RNA-transfected cells was as high as that in the wild-type RNA- and ΔORF3 RNA-transfected cells at 8, 16 and 24 days p.t., and increased in the same manner over time. Next, to examine the efficiency of virion egress in PLC/PRF/5 cells inoculated with the cell

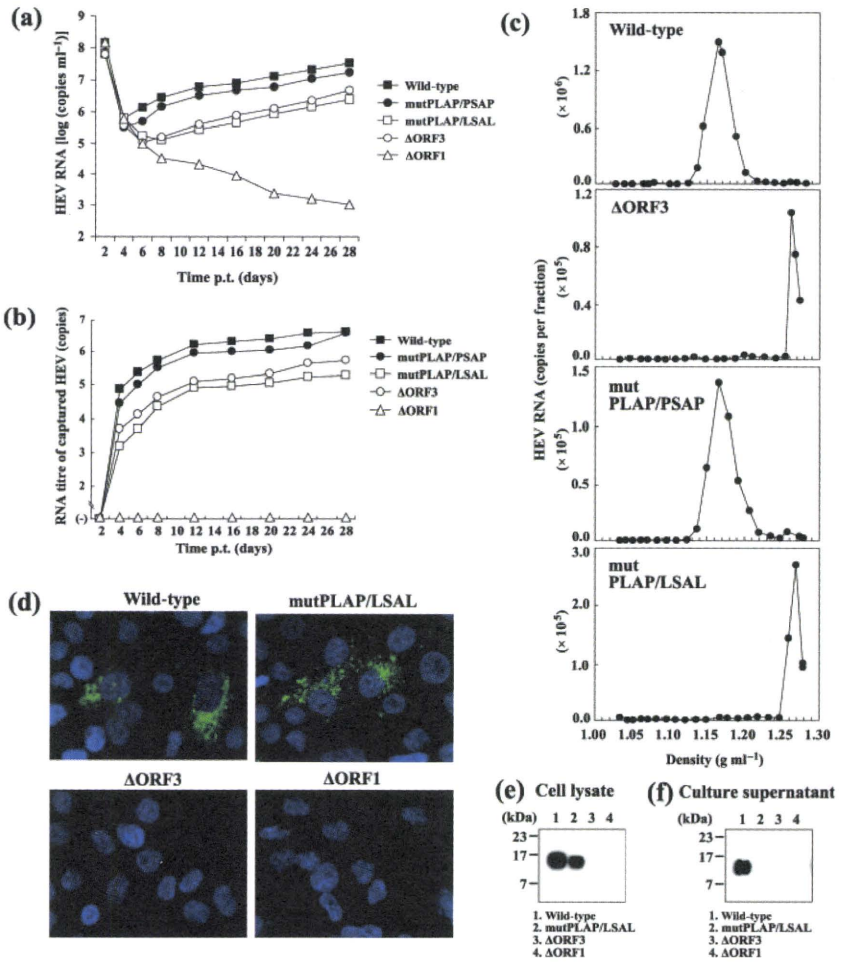

Fig. 3. (a) Quantification of HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells. The experiment was carried out in two wells for each sample, and its mean titres are shown. (b) Quantification of HEV RNA in viral particles captured by an anti-ORF2 mAb in culture supernatants of RNA-transfected PLC/PRF/5 cells. After treatment of HEV particles in the culture supernatant with 0.1 % sodium deocycholate, the RNA titre of captured HEV was quantified by real-time RT-PCR. (c) Sucrose density-gradient fractionation of the wild-type virus and its mutants in culture supernatants. (d) Immunofluorescent staining of PLC/PRF/5 cells transfected with RNA transcripts of the wild-type virus or its mutants. At 8 days p.t., cells were incubated with an anti-ORF3 mAb and then stained with Alexa Fluor 488-conjugated anti-mouse IgG. Nuclei were stained with DAPI. (e) The expression of the ORF3 protein in PLC/PRF/5 cells transfected with RNAs of pJE03-1760F/wt or its variants. At 28 days p.t., cells were lysed, and then the ORF3 protein was detected by Western blotting with an anti-ORF3 mAb. (f) The antigenicity of the particles generated by the wild-type virus or its mutants, evaluated by Western blotting with an anti-ORF3 mAb.

culture-generated wild-type virus or ORF3 variants, the culture supernatant of the wild-type RNA-, mutPLAP/LSAL RNA- or ΔORF3 RNA-transfected cells were inoculated into the PLC/PRF/5 cells. When wild-type HEV was inoculated into the cells, HEV RNA was first detected in the culture supernatant on day 8 and reached 4.0×10^3 copies ml^{-1} on day 12 (Fig. 4c). In contrast, low levels of background RNA at $<10^2$ copies ml^{-1} were seen in the culture supernatants of cells inoculated with the mutPLAP/LSAL or ΔORF3 virus during the course of 8–12 day experiment (Fig. 4c), probably due to cell death as described in detail in our previous paper (Yamada *et al.*, 2009a). Notably, no significant difference in the intracellular HEV RNA level was observed upon inoculation of the wild-type virus and its ORF3 variants (Fig. 4d). When the amount of adsorbed virus was measured by quantitative RT-PCR targeting the ORF2/3 region 1 h after inoculation, the HEV RNA level in the cells inoculated with the mutPLAP/LSAL virus was higher than wild-type (1.3×10^3 vs 1.7×10^2 copies per 10^5 cells), similar to the ΔORF3 virus (1.9×10^3 copies per 10^5 cells), thus suggesting that the adsorption and entry of the mutPLAP/LSAL and ΔORF3 viruses are more efficient than wild-type virus.

Interaction of the ORF3 protein with Tsg101 in cultured cells

To investigate the direct interaction of the ORF3 protein with Tsg101 and loss thereof with the PSAP mutations, we used co-immunoprecipitation to show ORF3–Tsg101 interaction in cells inoculated with wild-type or mutant (mutPLAP/LSAL) virus in the culture supernatant, or transfected with RNA transcripts of wild-type or mutant cDNA clone. However, in the present study, co-immunoprecipitates of ORF3–Tsg101 proteins were not detectable in the infected or transfected PLC/PRF/5 cells, probably due to insufficient expression levels of either or both of Tsg101 and ORF3 proteins to permit detection by Western blotting. Then, with the advent of an expression plasmid for the full-length ORF3 protein (aa 1–113) of the JE03-1760F strain, pCI-HEVORF3/wt (Takahashi *et al.*, 2008b), a mutant plasmid pCI-HEVORF3/mutPLAP/LSAL was generated. In the PLC/PRF/5 cells transfected with pCI-HEVORF3/mutPLAP/LSAL, the ORF3 protein was expressed as efficiently as the wild-type ORF3 protein (Fig. 5a). The extracts of PLC/PRF/5 cells transfected with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL were immunoprecipitated with goat anti-Tsg101 polyclonal antibody and the resulting immunoprecipitates were sub-

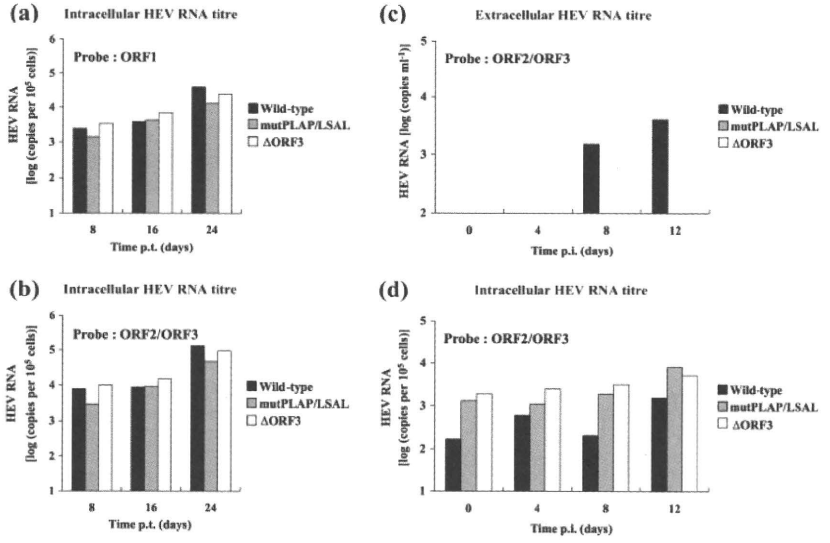

Fig. 4. (a and b) The levels of intracellular HEV RNA in PLC/PRF/5 cells transfected with RNAs of pJE03-1760F/wt or its ORF3 variants. The levels of extracellular (c) and intracellular (d) HEV RNA in the cells inoculated with the culture supernatants containing the wild-type virus or its ORF3 variants. The HEV RNA titre was quantified by real-time RT-PCR with primers targeting the ORF1 region (a) or the ORF2/ORF3 overlapping region (b, c and d). The experiments were carried out in two wells for each sample, and the mean titres are shown.

jected to Western blotting by using either anti-Tsg101 mAb or anti-ORF3 mAb. As shown in Fig. 5b, cellular Tsg101 was immunoprecipitated with goat anti-Tsg101 in both cells expressing the wild-type ORF3 protein and those expressing the mutPLAP/LSAL ORF3 protein, while it was not immunoprecipitated with normal goat IgG, thus representing the specificity of the goat anti-Tsg101 antibody. The immunoprecipitation with goat anti-Tsg101 antibody for cell extracts expressing wild-type ORF3 protein co-immunoprecipitated ORF3 protein detectable by Western blotting with anti-ORF3 mAb (Fig. 5c). In contrast, the immunoprecipitation for the extracts from cells expressing the mutPLAP/LSAL ORF3 protein did not show any co-immunoprecipitates of the ORF3 protein, suggesting the loss of interaction between Tsg101 and ORF3 proteins due to inactive PSAP motifs in the mutated ORF3 protein.

DISCUSSION

In the present study, we demonstrated that the ORF3 protein, with at least one of the two PSAP motifs, is required for efficient release of virions from cultured cells. Furthermore, an intact PSAP motif of the ORF3 protein was found to be essential for egress of HEV particles associated with lipids and the ORF3 protein. These results suggest that the PSAP motif(s) of the HEV ORF3 protein is a functional domain for virion egress.

While this manuscript was being prepared for submission, Emerson *et al.* (2010) reported that release of genotype 1 HEV from cultured cells depends on the ORF3 protein and requires an intact PXXP motif in the ORF3 protein. With the advent of an infectious cDNA clone of genotype 1 HEV (the Sar55 strain), they confirmed our previous study conducted by using a genotype 3 infectious cDNA clone of the JE03-1760F strain, reporting that the ORF3 protein is essential for virion egress from infected cells, that the ORF3 protein is present on the surface of HEV particles released from infected cells, and that the HEV particles released from infected cells are lipid-associated (Yamada *et al.*, 2009a). Genotypes 2 and 4 HEVs have not yet been examined for the function of the ORF3 protein on virion egress by using infectious cDNA clones. However, given their conservation, and since the association of HEV virions of genotypes 1, 3 or 4 in blood circulation with ORF3 protein and lipids on the surface has been noted (Takahashi *et al.*, 2010), it is very likely that the function

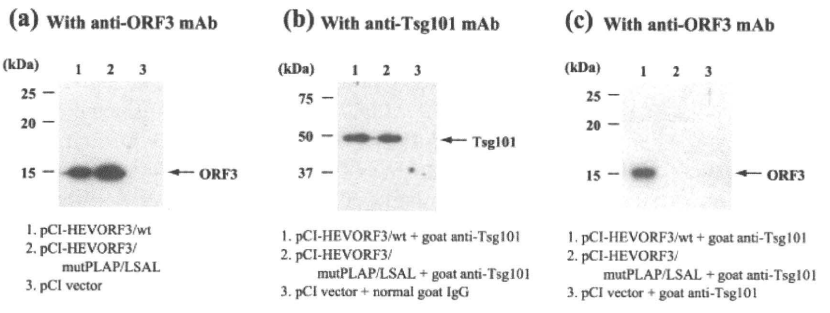

Fig. 5. (a) Expression of the ORF3 proteins in PLC/PRF/5 cells transfected with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL. The empty vector (pCI vector) was used as a negative control. The cell lysates were subjected to Western blotting with an anti-ORF3 mAb. (b and c) PLC/PRF/5 cells expressing wild-type or mutant (mutPLAP/LSAL) ORF3 proteins were immunoprecipitated with goat anti-Tsg101 antibody and subjected to Western blotting with anti-Tsg101 mAb (b) or anti-ORF3 mAb (c).

of the ORF3 protein related to HEV morphogenesis is common to all HEV strains, irrespective of genotype.

A Pro-rich sequence is present in the C-terminal region of the ORF3 protein in all mammalian and avian HEV strains (Fig. 1). Although two PSAP motifs are present in the ORF3 protein of only a limited number of genotype 3 HEV strains, including JE03-1760F, the present study revealed that not only the second, authentic PSAP motif but also the first, additional PSAP motif in the ORF3 protein can be involved in virion egress. The mutPLAP/PSAP variant with a Ser to Leu mutation in the first PSAP motif, which is identical to the majority of reported genotype 3 HEV strains (Fig. 1), generated progeny slightly less efficiently (approx. 0.5-fold) than the wild-type virus (Fig. 3a), despite the presence of an intact sequence in the second, authentic PSAP motif, suggesting that the first/additional PSAP motif is capable of enhancing virion release from infected cells. The Pro-rich sequence of PSAPPLPP (aa 95–102 in genotype 3) in the C terminus of the ORF3 protein differ slightly by genotype. Genotype 1 HEVs have a conserved PSAPPLP sequence (aa 96–102: aa 103 has His in place of Pro), with two PXXP motifs (aa 96–99 and 99–102). In recent work by Emerson *et al.* (2010), the second Pro in the PSAPPLP sequence was substituted with Glu or Leu, to yield PSAQ/LPLP (changed amino acids underlined). Their study indicated that the PSAQPLP and PSALPLP mutants did not promote virus egress. However, it remained unclear whether either of the PSAP and PPLP motifs or both is important for egress of genotype 1 HEV. Genotypes 2 and 4 HEVs have in common a Pro-rich sequence of PSAPPLPP, with three PXXP motifs (aa 96–99, 99–102 and 100–103). In contrast, the fourth or fifth Pro residue is changed to His or Arg, respectively, in some genotype 4 HEV isolates, suggesting that a PSAPP sequence, which is conserved in all HEV isolates including avian HEV strains, is important for virion egress from infected cells. Although mutLSAL and mutPLAP/LSAL both have two intact PXXP motifs of aa 86–89 and 99–102, only mutLSAL produced progeny as efficiently as the wild-type strain (Fig. 2b), while mutPLAP/LSAL was unable to release virions into culture medium upon inoculation of the mutant virus (Fig. 4c). These results indicate that the PXXP motif of aa 99–102 is not essential for virion release, and suggest that other amino acids between the first and fourth Pro residues of the PXXP motif are also important for efficient virion egress. Based on the high conservation of the PSAP motifs among all mammalian and avian HEV strains, it is very likely that an intact PSAP motif in the ORF3 protein, whether it is located in the original site (aa 95–98 in genotype 3 HEV) or in the additional site (aa 86–89), is capable of promoting efficient virion release from infected cells.

The present study also demonstrated that the PSAP motif in the ORF3 protein is involved in the formation of membrane-associated HEV particles possessing ORF3 proteins on the surface. Consistent with an ORF3-deficient mutant, the mutPLAP/LSAL mutant virus with no intact PSAP motifs in the culture supernatant of RNA-transfected cells banded at 1.26–1.27 g ml^{-1} in sucrose (Fig. 3c), and was captured by anti-ORF2 mAb, but not by the anti-ORF3 mAb, with or without prior treatment with detergent (sodium deoxycholate) (Table 1). The absence of ORF3 protein on the mutant particles in the culture supernatant was confirmed by Western blotting (Fig. 3f), despite the fact that ORF3 protein expression was detected in the RNA-transfected cells by both immunofluorescence (Fig. 3d) and Western blotting (Fig. 3e) assays, similar to the wild-type virus. These results indicate that an intact PSAP motif in the ORF3 protein plays a pivotal role in the release of HEV particles having lipid-associated membranes and ORF3 protein.

Recent studies have revealed that viral matrix proteins play critical roles during the later stages of virus budding in many enveloped RNA viruses, including retroviruses, rhabdoviruses, filoviruses and orthomyxoviruses; these viral proteins possess a so-called late (L)-domain containing PT/SAP, PPXY and YXXL, which are critical motifs for efficient budding (Bouamr *et al.*, 2003; Ciancanelli & Basler, 2006; Göttlinger *et al.*, 1991; Harty *et al.*, 1999, 2000; Wirblich *et al.*, 2006). The PTAP motif was first identified in HIV Gag and has been reported to bind to Tsg101, which functions in vacuolar protein sorting (Garrus *et al.*, 2001). This interaction between Gag and Tsg101 is required for HIV budding. It has also been reported that Tsg101 binds to the PSAP motif of HEV located within the ORF3 protein (Surjit *et al.*, 2006). The present preliminary study with a co-immunoprecipitation procedure showed the direct interaction of the ORF3 protein with Tsg101 and loss thereof with the PSAP mutations in PLC/PRF/5 cells transfected with a full-length wild-type (pCI-HEVORF3/wt) or mutant (pCI-HEVORF3/mutPLAP/LSAL) ORF3 expression plasmid (Fig. 5). Taken together, it is very probable that the ORF3 protein promotes budding of membrane-associated HEV particles by recruiting Tsg101. To understand the precise steps of HEV budding, the cellular signalling pathways involving Tsg101 and other related host factors in relation to the release of HEV virions need to be explored in future studies.

In conclusion, our data provide evidence of the functional role of the PSAP motif(s) in the ORF3 protein for efficient egress of HEV virions from infected cells. In addition, it was found that at least one of the two PSAP motifs is required for the formation and release of membrane-associated HEV particles possessing the ORF3 protein on their surface. Further studies are warranted to elucidate how and why HEV acquires the lipid-associated membrane and the ORF3 protein when it leaves cells, despite this virus being a non-enveloped virus.

METHODS

Cell culture. PLC/PRF/5 cells (ATCC no. CRL-8024) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) heat-inactivated FCS, 100 U penicillin G ml^{-1}, 100 μg streptomycin

ml^{-1} and 2.5 μg amphotericin B ml^{-1} (growth medium), at 37 °C in a humidified 5 % CO_2 atmosphere, as described previously (Tanaka *et al.*, 2007).

HEV cDNA clones. pJE03-1760F/wt (GenBank accession no. AB437316) is a full-length infectious cDNA clone of a genotype 3 HEV obtained from a faecal specimen containing a high load of wild-type HEV (strain JE03-1760F: 2.0×10^7 copies ml^{-1}), which can replicate efficiently in PLC/PRF/5 cells (Yamada *et al.*, 2009b). For comparison, a derivative ORF3-deficient mutant (pJE03-1760F/ΔORF3, GenBank accession no. AB437317) whose initiation codon of the ORF3 gene was mutated to GCA (Ala) (Yamada *et al.*, 2009a), was used. As a negative control, an ORF1-deficient mutant genome (pJE03-1760F/ΔORF1, GenBank accession no. AB437319) containing a frameshift mutation in ORF1 (Yamada *et al.*, 2009b) was also used.

Site-directed mutagenesis. To construct variants of pJE03-1760F/wt with mutations in the PSAP motifs in the ORF3 protein (see Fig. 2a), an *Afl*II–*Xba*I fragment of pJE03-1760F/wt, which contains the ORF3 and ORF2 coding regions of the genome and poly(A) tail sequence (polyAT7Φ) (Yamada *et al.*, 2009b), was subcloned into a pT7 Blue T vector (Novagen). Site-directed mutagenesis was carried out by inverse PCR according to the method described previously (Sasaki *et al.*, 2001), using the obtained subclone of pJE03-1760F/wt, a high-fidelity DNA polymerase (KOD-Plus; Toyobo), and appropriate oligonucleotide primers. The primer pairs used for the construction of five PSAP mutant plasmids (pT7-mutLSAP, pT7-mutPSAL, pT7-mutLSAL, pT7-mutPLAP/PSAP and pT7-mutPLAP/LSAL) are shown in Supplementary Table S1 (available in JGV Online). To construct pT7-mutPLAP/LSAL with a mutation from Ser to Leu in the second amino acid residue of the first PSAP motif, as well as two additional mutations from Pro to Leu in the first and fourth amino acid residues of the second PSAP motif (see Fig. 2a), inverse PCR was carried out with primers PLAP/PSAP-f and PLAP/PSAP-r (Supplementary Table S1) by using pT7-mutLSAL, a subclone with a LSAL sequence in the second PSAP motif, as a template. PCR products were self-ligated, and the resulting clones were subjected to nucleotide sequencing to confirm the presence of the expected mutations and the absence of other unexpected mutations. The *Afl*II–*Xba*I fragments of these five subclones were ligated into pJE03-1760F/wt from which the *Afl*II–*Xba*I fragment had been removed, generating five full-length cDNA clones carrying one, two or three amino acid alterations in the two PSAP motifs of the ORF3 protein.

In vitro* transcription and transfection of PLC/PRF/5 cells.** Each of the eight full-length genome plasmids including pJE03-1760F/wt and five PSAP mutants as well as ΔORF3 and ΔORF1, were linearized with *Nhe*I, and RNA transcripts were synthesized with T7 RNA polymerase using the AmpliScribe T7-Flash Transcription kit (Epicentre Biotechnologies). After *in vitro* transcription, RNA transcripts of all eight cDNA clones were capped using a ScriptCap m^7G Capping System (Epicentre Biotechnologies). The integrity and yield of the synthesized RNAs were determined by agarose gel electrophoresis. An aliquot (3 μg) of the capped RNA was transfected into subconfluent (60–80 % confluent) PLC/PRF/5 cells in a well of a six-well plate (Iwaki) by using the TransIT-mRNA transfection kit (Mirus Bio) according to the manufacturer's recommendations. After incubation at 37 °C for 2 days, the culture medium was replaced with 2 ml of growth medium and incubated at 35.5 °C. Every other day, half of the culture medium (1 ml) was replaced with fresh growth medium. The collected culture medium was centrifuged at 1300 ***g at room temperature for 2 min and the supernatant was stored at −80 °C until use.

Quantification of HEV RNA. RNA extraction from culture supernatants was performed using the TRIzol-LS Reagent (Invitrogen). For intracellular RNA, total RNA was extracted from cultured cells using the TRIzol Reagent (Invitrogen). Quantification of HEV RNA was performed by real-time RT-PCR using a LightCycler apparatus (Roche Diagnostics), with a QuantiTect Probe RT-PCR kit (Qiagen) and two sets of primers and a probe targeting ORF2 and the ORF3 overlapping region or the ORF1 region, as described previously (Takahashi *et al.*, 2008a; Yamada *et al.*, 2009a). Unless otherwise stated, quantification of HEV RNA was performed by using the real-time RT-PCR method with the probe targeting the ORF2/ORF3 region.

Sucrose density-gradient centrifugation. The culture supernatant ($0.6–1.6 \times 10^6$ copies) collected from PLC/PRF/5 cells transfected with RNA transcripts of pJE03-1760F/wt or its variants at 28 days p.t. were subjected to equilibrium centrifugation in a sucrose density gradient as described previously (Yamada *et al.*, 2009a). The gradients were fractionated, and the density of each fraction was measured by refractometry.

Immunocapture RT-PCR assay. The immunocapture RT-PCR assay was performed as described previously (Takahashi *et al.*, 2008a), with or without prior treatment with 0.1 % sodium deoxycholate (Wako) at 37 °C for 2 h. An anti-ORF2 mAb (H6225) (Takahashi *et al.*, 2008a) and an anti-ORF3 mAb (TA0536) (Takahashi *et al.*, 2008b) were used in this study. As a negative control, a mAb against hepatitis B virus pre-S2 protein (no. 5520) (Okamoto *et al.*, 1985) was used.

Immunofluorescence assay. PLC/PRF/5 cells transfected with RNA transcripts of pJE03-1760F/wt or its variants at 8 days p.t. were subjected to immunofluorescence staining by using an anti-ORF3 mAb, as described previously (Yamada *et al.*, 2009b). The cultured cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) and nuclei were counterstained with DAPI (Roche Diagnostics). The stained cells were viewed under a FV1000 confocal laser microscope (Olympus).

Western blotting. Transfected cells were lysed in lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % NP-40, 150 mM NaCl and protease inhibitor cocktail (Sigma)], and proteins in the cell lysates (5 μl of the lysate corresponding to 2.5×10^4 cells) or the culture supernatants (10 μl of the supernatant corresponding to 2.0×10^6 copies) were separated by SDS-PAGE on 12 % gels. The proteins were blotted onto a PVDF membrane, immunodetected with an anti-HEV ORF3 mAb, and then visualized by chemiluminescence as described previously (Yamada *et al.*, 2009b).

Virus inoculation. Monolayers of PLC/PRF/5 cells in a six-well plate were inoculated with 10^5 copies of HEV progenies in the culture supernatant of transfected cells. After incubation at room temperature for 1 h, the cells were washed with PBS and 2 ml of maintenance medium was added to each well, and then the cells were incubated at 35.5 °C. The maintenance medium consisted of 50 % DMEM and 50 % Medium 199 (Invitrogen) containing 2 % FCS, 30 mM $MgCl_2$, 100 U penicillin G ml^{-1}, 100 μg streptomycin ml^{-1} and 2.5 μg amphotericin B ml^{-1}. Every other day, half of the culture medium (1 ml) was replaced with fresh maintenance medium. The collected culture medium was centrifuged at 1300 ***g*** at room temperature for 2 min and the supernatant was stored at −80 °C until use.

Virus adsorption assay. The virus adsorption assay was performed as described previously (Yamada *et al.*, 2009a). Briefly, after washing PLC/PRF/5 cells in each well of a six-well plate with PBS, virus (10^5 copies) in culture supernatant was inoculated onto monolayers of the cells. After incubation at room temperature for 1 h, cells were washed with PBS, trypsinized, and collected by centrifugation at 100 ***g*** at room temperature for 5 min. After removal of the supernatant, the

cell pellet was resuspended in TRIzol Reagent and stored at −80 °C until virus titration.

Expression plasmid construction and co-immunoprecipitation. To construct a mutant plasmid pCI-HEVORF3/mutPLAP/LSAL with mutations in the two PSAP motifs, similar to mutPLAP/LSAL (Fig. 2a), site-directed mutagenesis was carried out by inverse PCR using pCI-HEVORF3/wt as a template, which is an expression plasmid for the full-length wild-type ORF3 protein (aa 1–113) of the JE03-1760F strain (Takahashi *et al.*, 2008b). PLC/PRF/5 cells were transfected with pCI-HEVORF3/wt, pCI-HEVORF3/mutPLAP/LSAL or pCI vector (Promega) using TransIT-LT1 reagent (Mirus Bio). At 48 h after transfection, co-immunoprecipitation assay was carried out using Immunoprecipitation kit (Protein G) (Roche). After clarification by brief centrifugation, lysates were used for immunoprecipitation with goat anti-Tsg101 polyclonal antibody (Santa Cruz Biotechnology) or normal goat IgG (MP Biomedicals). Immunoprecipitated protein, cellular Tsg101, was separated by 10% SDS-PAGE, followed by Western blotting with an anti-Tsg101 mAb (Santa Cruz Biotechnology). Co-immunoprecipitated proteins (pCI-HEVORF3/wt, pCI-HEVORF3/mutPLAP/LSAL or pCI vector) were separated by 15% SDS-PAGE, followed by Western blotting with an anti-ORF3 mAb. These proteins were visualized by chemiluminescence by using Clean-Blot IP Detection Reagent (HRP) (Thermo Scientific).

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Ministry of Health, Labour and Welfare of Japan.

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Supplementary Table S1. Primer sequences used in this study

Primer name	Sequence (5′–3′)	Notes*
LSAP-f	GCCCCTCCGCTGCCCCCC	nt 5449–5466 (sense) for mutLSAP
LSAP-r	GCTGAGACTGGTCGCGCCAAGCGGAGC	nt 5422–5448 (substituted nucleotide underlined: antisense) for mutLSAP, mutLSAL and mutPLAP/LSAL
PSAL-f	GCCCTTCCGCTGCCCCCCGTCGTCG	nt 5449–5473 (substituted nucleotide underlined: sense) for mutPSAL, mutLSAL and mutPLAP/LSAL
PSAL-r	GCTGGGACTGGTCGCGCC	nt 5431–5448 (antisense) for mutPSAL
PLAP/PSAP-f	GGCTCCGCTTGGCGCGACC	nt 5421–5439 (sense) for mutPLAP/PSAP
PLAP/PSAP-r	AAGGGGGCGGGGCGGCTGTCGAGG	nt 5397–5420 (substituted nucleotide underlined: antisense) for mutPLAP/PSAP

*Nucleotide positions are in accordance with the JE03-1760F strain (AB301710).

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INTERNAL MEDICINE

CASE REPORT

Acute Hepatitis due to Hepatitis E Virus Genotype 1 As an Imported Infectious Disease in Japan

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Abstract

An 18-year-old Nepalese man was admitted due to general malaise and anorexia a month after coming to Japan. Laboratory tests showed elevation of transaminase and positivity for IgM anti-HEV antibody. Serum HEV RNA was detected by RT-PCR amplifications. An HEV genome phylogenetic tree, constructed using an 821-nucleotide sequence in the open reading frame 1, indicated that the genotype was 1. HEV genotype 1 is epidemic in South Asia, Africa and South America, and the incidence of acute hepatitis due to HEV genotype 1 is low in Japan. Thereafter, attention should be paid to HEV genotype 1 infection as an imported infectious disease.

Key words: hepatitis E virus, genotype, acute hepatitis

(Intern Med 49: 2613-2616, 2010)
(DOI: 10.2169/internalmedicine.49.4221)

Introduction

Hepatitis E virus (HEV), a single-stranded RNA virus, is the sole member of the genus *Hepevirus* in the family *Hepeviridae* and classified into 4 genotypes exhibiting distinct geographic distributions (1). HEV infection is mainly transmitted via the fecal-oral route in areas with poor sanitation and weak public-health infrastructures, and HEV genotype 1 is epidemic in developing regions of Asia, Africa, and South America.

In industrialized regions including Japan, acute hepatitis due to HEV genotype 3 or 4 has been sporadically found in pig populations (2, 3). However, recently, a pregnant Japanese woman infected with HEV genotype 1 in India developed severe acute hepatitis after coming back to Japan (4). In the present report, we describe an 18-year-old Nepalese man who developed acute hepatitis due to HEV genotype 1 one month after coming to Japan. Acute hepatitis due to HEV genotype 1 may also be important in Japan as an imported infectious disease.

Case Report

An 18-year-old Nepalese man was admitted to our hospital because of general malaise and anorexia. He had come from Nepal to study in Japan a month earlier. Because of his religious beliefs, he did not normally eat meat such as pork or beef. He had no history of liver disease or hepatitis. For 3 days prior to admission, he felt general malaise and anorexia.

On admission, his consciousness was clear, and his bulbar conjunctiva was slightly icteric. Neither ascites nor pretibial edema was present. Laboratory data on admission are shown in Table 1. Serum levels of aspartate aminotransferase (AST; 1,944 IU/L), alanine aminotransferase (ALT; 2,268 IU/L), and total bilirubin (T-Bil; 4.84 mg/dL) were elevated. Tests for IgM anti-hepatitis A virus antibodies, IgM anti-hepatitis

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Table 1. Laboratory Data on Admission

White blood cell count (/mm^3)	4260
Hemoglobin (g/dL)	15.7
Platelet count (×10^3/mm^3)	211
Prothrombin time activity (%)	66
Albumin (g/dL)	4.0
Total bilirubin (mg/dL)	4.84
Aspartate aminotransferase (IU/L)	1944
Alanine aminotransferase (IU/L)	2268
Alkaline phosphatase (IU/L)	905
IgM anti-hepatitis A virus antibody	Negative
IgM anti-hepatitis B core antibody	Negative
Hepatitis B surface antigen	Negative
Hepatitis B virus DNA	Negative
Anti-hepatitis C virus antibody	Negative
Hepatitis C virus RNA	Negative
Anti-nuclear antibody	Negative
IgM anti-cytomegalovirus antibody	Negative
IgG anti-cytomegalovirus antibody	Positive
IgM anti-Epstein–Barr virus viral capsid antigen antibody	Negative
IgG anti-Epstein–Barr virus viral capsid antigen antibody	Positive
IgM anti-hepatitis E virus antibody	Positive
Hepatitis E virus RNA	Positive

B virus core antibodies, hepatitis B surface antigens, and hepatitis C virus antibodies were all negative. Neither serum hepatitis B virus DNA nor hepatitis C virus RNA was detected by polymerase chain reaction (PCR) or reverse transcription (RT)-PCR. Tests for cytomegalovirus and Epstein-Barr viruses showed previous infection patterns. However, serum IgM anti-HEV antibody was positive, and serum HEV RNA was detected by RT-PCR amplifications using HEV-specific primers. The PCR products were then direct-sequenced (isolate name JJS-NepOka09R, DDBJ/EMBL/GenBank accession No. AB533368). An HEV genome phylogenetic tree, constructed using an 821-nucleotide sequence (replicase region) in the open reading frame 1 (Fig. 1), indicated that the genome was genotype 1. Thus, he was diagnosed with acute hepatitis due to HEV genotype 1.

After admission, the patient was initially treated with intravenous drip and vitamin administration, but his symptoms did not improve. For 3 days after admission, general malaise, anorexia and liver function tests gradually worsened, so administration of prednisolone (PSL) of 20 mg/day was initiated on day 4. Thereafter, his condition steadily improved. The PSL dose was tapered and discontinued 15 days after admission; his hepatitis did not relapse and showed rapid improvement (Fig. 2).

Discussion

In Japan, HEV genotype 1 infection is rare. On the other hand, Nepal is a well-known endemic region with widespread occurrence of HEV genotype 1 (5, 6). In a recent report from Qatar (7), 75% of the workers from Nepal who developed acute hepatitis after coming to Qatar were shown to be positive for the serum IgM anti-HEV antibody. Furthermore, the incubation period of HEV infection ranges from 2 to 9 weeks (1). Thus, we speculate that the present patient was infected with HEV in Nepal and developed acute hepatitis after coming to Japan. Hereafter, also in Japan, attention should be paid to HEV genotype 1 infection as an imported infectious disease.

There are differences in the clinical features between genotype 1 infection and genotype 3 or 4 infection. The peak incidence of acute hepatitis due to genotype 1 occurs in 15 to 35-year-old patients (7-9), and the mortality rate is about 1% (1). On the other hand, most patients with the disease due to genotype 3 or 4, which is the major genotype in Japan, are reported to be aged 60 years or older and the mortality rate is relatively high, ranging from 6% to 10% (8, 10, 11). Examination of HEV genotype is considered necessary in order to predict the clinical course and the outcome.

In general, acute hepatitis due to HEV genotype 1 is self-limited, and the treatment of the disease is supportive. Patients developing hepatic failure are considered for liver transplantation. In the present patient, spontaneous improvement of his disease condition was not seen for 3 days after admission. Thus, we administered PSL, and serum transaminase levels promptly improved without a relapse after the discontinuation of PSL. Some patients with acute hepatitis E are reported to demonstrate elevation of serum IgG levels and positivity of autoantibodies and to respond to corticosteroid treatment (12-14). Their clinical features are similar to those of AIH with acute onset form. Some viral infections are considered as possible triggers for autoimmune hepatitis (15). Thus, we speculate that, in some patients with acute hepatitis E, autoimmune response may be associated with the pathogenesis. On the other hand, in acute hepatitis E treated with PSL, PSL has been reported to be tapered and discontinued without the exacerbation of the disease (12). Furthermore, the administration of corticosteroid was not shown to be associated with the persistent infection of HEV (16). The short-term administration of PSL may be effective in patients without spontaneous improvement although further study is necessary.

In acute hepatitis E, HEV RNA is cleared from the blood a few days to weeks after the onset of clinical symptoms; however, the virus continues to be excreted in stools for 2 more weeks (1). Thus, HEV-infected patients may be the source of infection for several weeks after the onset of clinical symptoms. Physicians should take precautionary measures against the spread of HEV infection when encountering patients with acute hepatitis E.

In conclusion, also in Japan, attention should be paid to HEV genotype 1 infection as an imported infectious disease. Physicians should take HEV infection into consideration for the diagnosis of acute hepatitis, when encountering patients who develop acute hepatitis after coming from an endemic

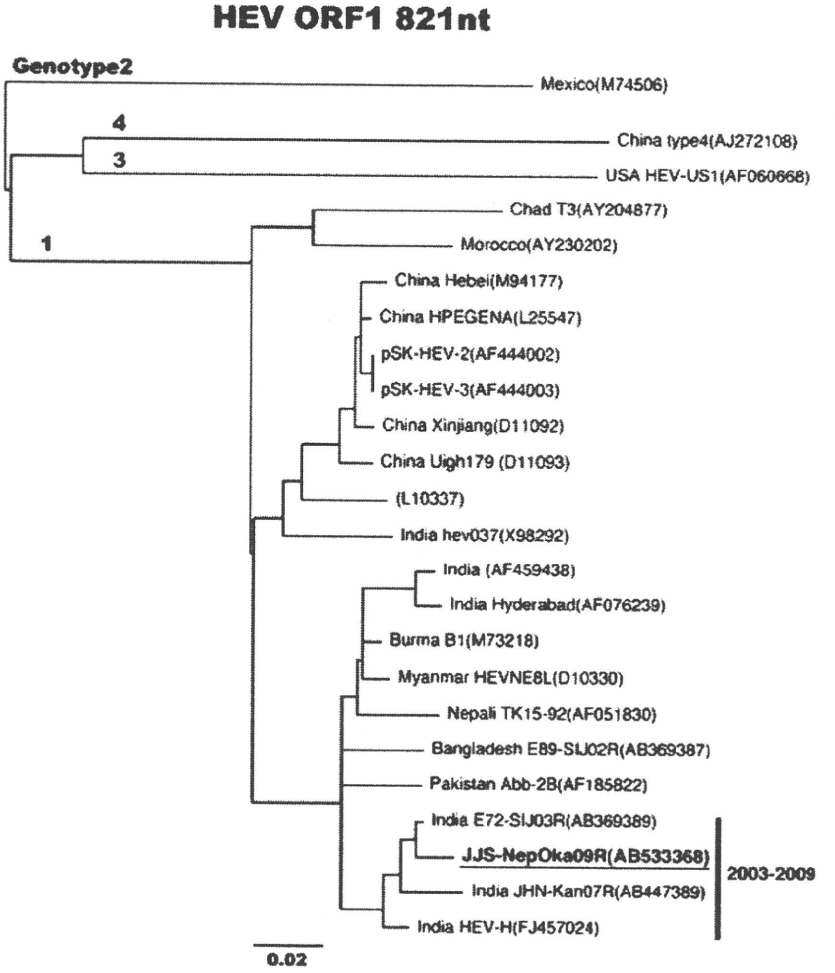

Figure 1. HEV RNA molecular phylogenetic tree.

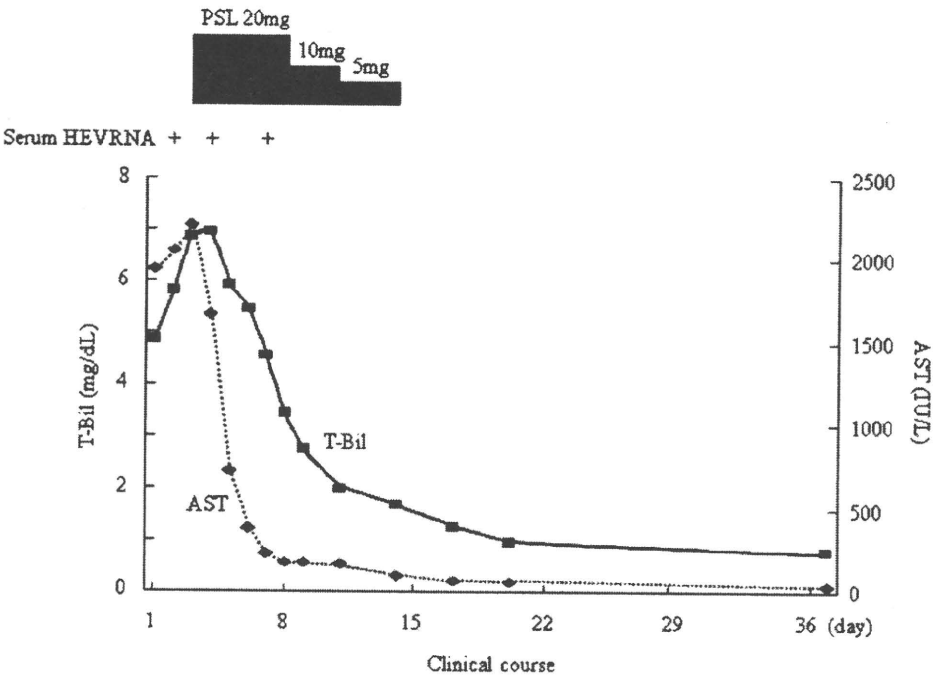

Figure 2. Clinical course.

region with widespread occurrence of HEV genotype 1 infection.

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