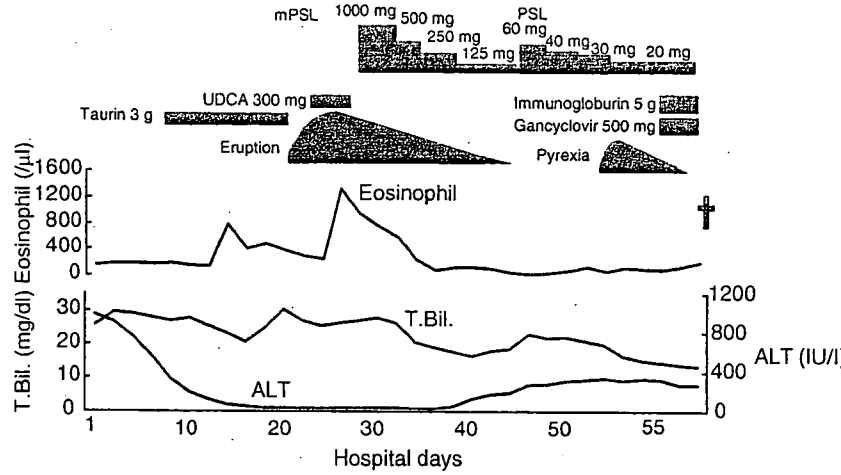

Figure 2 Clinical course of patient. ALT, alanine aminotransferase; mPLS, methylprednisolone; PLS, prednisolone; T. Bil., total bilirubin.

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

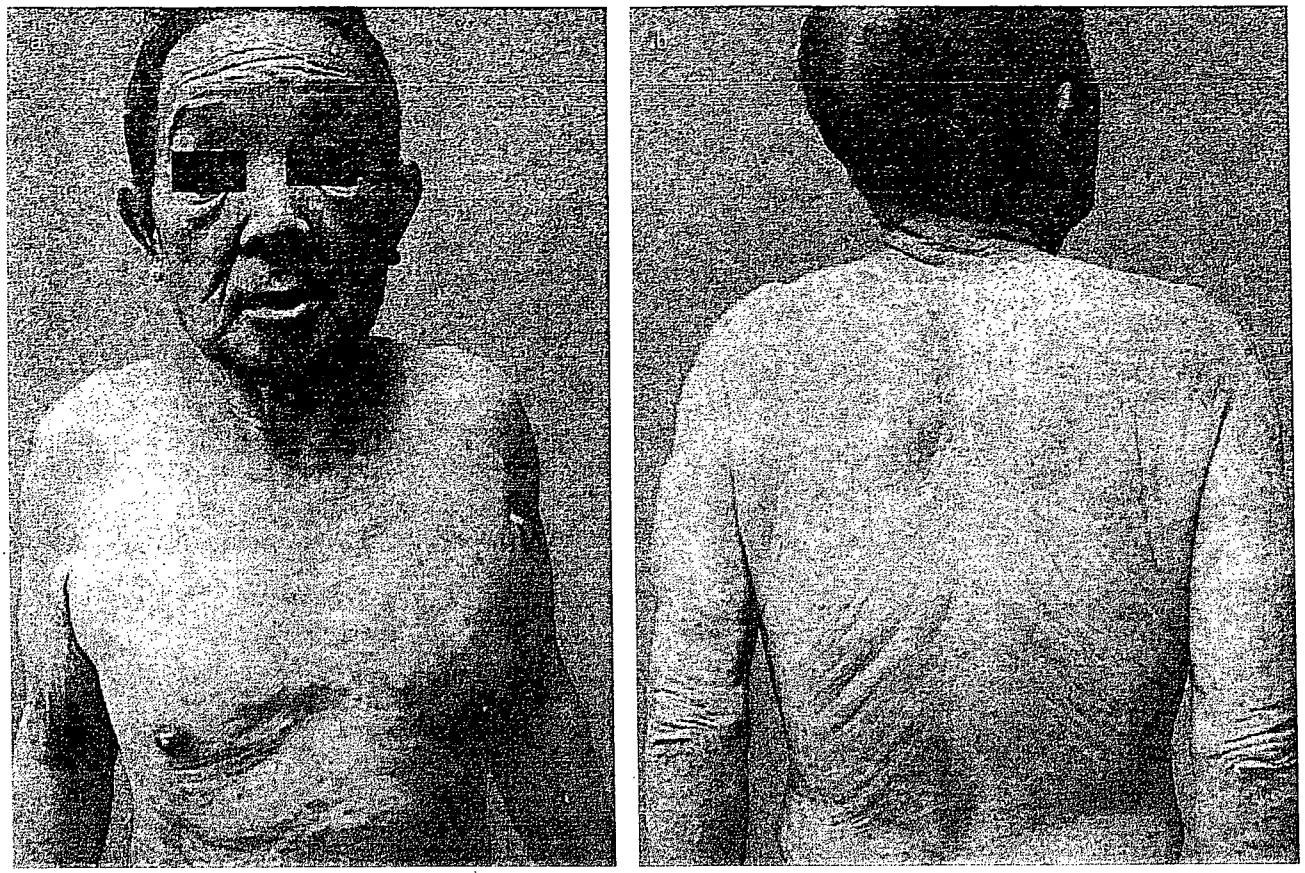

Figure 3 Clinical features of skin. Erythropapular eruptions and erythema exudativum multiforme were observed with icterus.

acute hepatitis due to HEV infection, which is recently regarded to be endemic in Japan[5] 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.[4] 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,[7] 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-

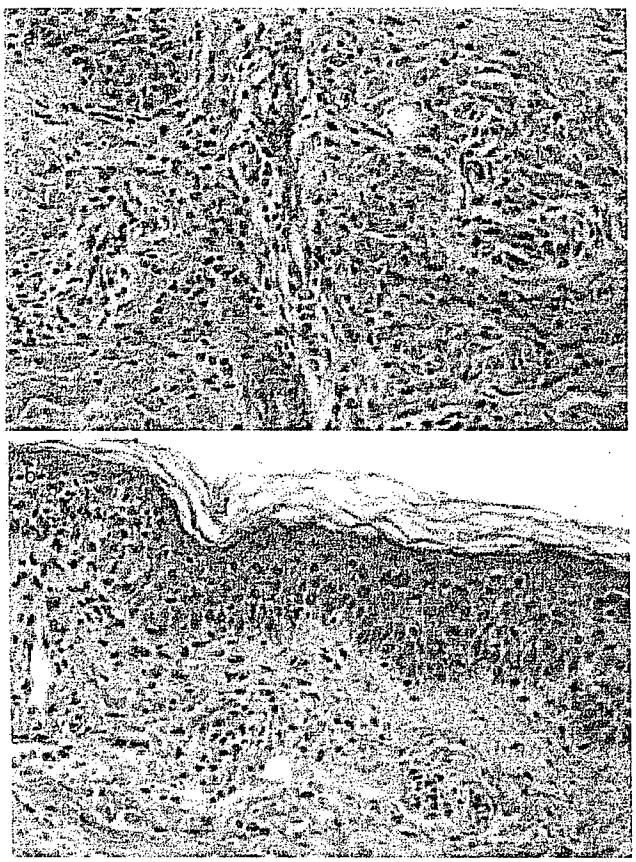

Figure 4 Marked eosinophilic infiltration around vessels and hair follicles were observed in upper dermis (hematoxylin–eosin staining, 200×).

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-

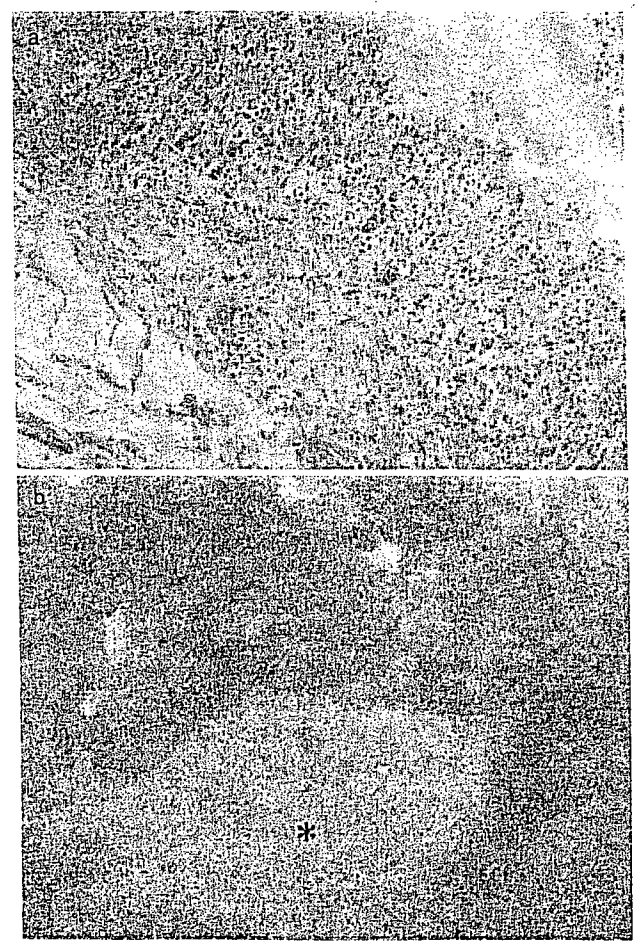

Figure 5 Histopathology of small intestine (a) and spleen (b). Multiple fibrin thrombi were observed in small vessels of the small intestine and spleen. Small infarcts (*) were observed in the spleen.

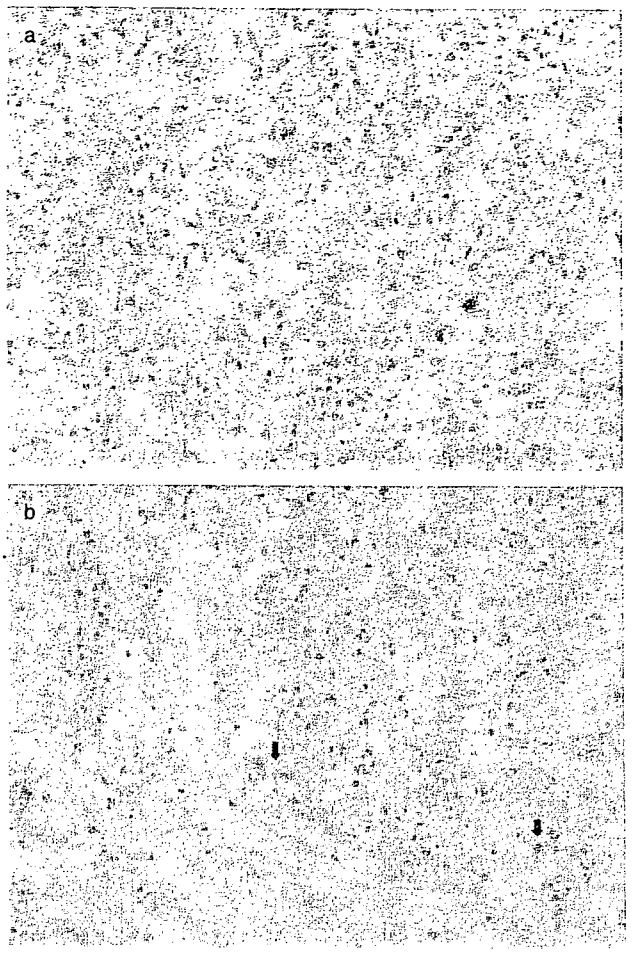

Figure 6 Histopathology of lung (a) and duodenum (b). Multiple inclusion bodies of cytomegalovirus were observed in epithelial cells.

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,[8] 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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Ongoing Subclinical Infection of Hepatitis E Virus Among Blood Donors With an Elevated Alanine Aminotransferase Level in Japan

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Ongoing subclinical infection of hepatitis E virus (HEV) has not been fully studied. In the present study, serum samples were collected from 6700 voluntary blood donors with an elevated alanine aminotransferase (ALT) level of 61-476 IU/l at a Japanese Red Cross Blood Center, and were tested for the presence of IgG, IgM and IgA classes of antibodies to HEV (anti-HEV) by in-house ELISA and HEV RNA by nested RT-PCR. Overall, 479 blood donors (7.1%) were positive for anti-HEV IgG, including 8 donors with anti-HEV IgM and 7 donors with anti-HEV IgA. Among the nine donors with anti-HEV IgM and/or anti-HEV IgA, six had detectable HEV RNA. The presence of HEV RNA was further tested in 10-sample minipools of sera from the remaining 6691 donors, and three donors including one without anti-HEV IgG were found to be positive for HEV RNA. When stratified by ALT level, the prevalence of HEV RNA was significantly higher among the 109 donors with ALT $\geq$201 IU/l than among the 6591 donors with ALT of 61–200 IU/l (2.8% vs. 0.1%, $P < 0.0001$). The HEV isolates obtained from the nine viremic donors segregated into genotype 3, shared a wide range of identities of 85.6–98.5% and were 87.3–93.9% similar to the Japan-indigenous HEV strain (JRA1), in the 412-nucleotide sequence of open reading frame 2. This study suggests that approximately 3% of Japanese individuals with ALT $\geq$201 IU/l have ongoing subclinical infection with various HEV strains. ***J. Med. Virol. 79: 734–742, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis E virus; subclinical infection; PCR; genotype; phylogenetic analysis

INTRODUCTION

Hepatitis E is an acute disease that is endemic in many developing countries of Asia and Africa where sanitation is suboptimal, and is also endemic in many industrialized countries including the United States, European countries and Japan [Harrison, 1999; Purcell and Emerson, 2001; Smith, 2001; Emerson and Purcell, 2003; Okamoto et al., 2003]. Hepatitis E virus (HEV), the causative agent of hepatitis E, is a positive-sense RNA virus without an envelope and is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004]. Its genome is approximately 7.2 kilobases in length and contains three open reading frames (ORFs: ORF1, ORF2 and ORF3) flanked by short untranslated regions [Tam et al., 1991]. ORF1 is the largest of the three and encodes viral non-structural proteins. ORF2 encodes the capsid protein and ORF3 encodes a small protein that undergoes phosphorylation [Koonin et al., 1992; Zafrullah et al., 1997]. Extensive genomic diversity has been noted among HEV isolates and HEV sequences have been classified into four genotypes (genotypes 1–4) [Schlauder and Mushahwar, 2001]. Genotype 1 HEV has been responsible for a number of waterborne epidemics of

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB288357–AB288365.

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hepatitis E in Asia and Africa. Although HEV of genotype 2 has been detected less frequently, it was responsible for outbreaks in Mexico in 1986–1987 [Velazquez et al., 1990] and has been implicated in sporadic infections in Africa [Buisson et al., 2000]. On the other hand, genotypes 3 and 4 HEV cause sporadic cases of acute hepatitis but have not been found to be responsible for epidemics in humans; these infections seem to be zoonotic and both genotypes have been detected in pigs (genotype 3 worldwide, and genotype 4 in Asia), which may constitute the major reservoir of genotypes 3 and 4 [Harrison, 1999; Meng, 2005; Lu et al., 2006].

Polyphyletic HEV strains of genotypes 3 and 4 are circulating in Japan [Takahashi et al., 2001, 2002; Mizuo et al., 2002; Inoue et al., 2006] and HEV has been recognized as an important causative agent of sporadic acute hepatitis of non-A, non-B, non-C etiology in this country [Mizuo et al., 2002; Okamoto et al., 2003]. It has been reported that food-borne transmission of HEV may occur through ingestion of raw or undercooked meat including liver and intestine from infected swine, deer or boar [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. Imported hepatitis E and transfusion-transmitted HEV infection have also been documented [Koizumi et al., 2004; Matsubayashi et al., 2004; Mitsui et al., 2004]. Furthermore, a high prevalence of IgG class antibodies to HEV (anti-HEV IgG) among healthy individuals, most likely due to past subclinical HEV infection, has been reported in some regions in Japan [Li et al., 2000; Tanaka et al., 2001, 2005; Mitsui et al., 2004, 2005], and HEV-viremic subjects have been identified among symptom-free blood donors with an elevated alanine aminotransferase (ALT) level [Fukuda et al., 2004]. However, it remains unknown whether and, if so, how frequently recent subclinical HEV infection is occurring in Japan. Therefore, in an attempt to estimate the prevalence of recent subclinical HEV infection in Japan stratified by ALT level, anti-HEV antibodies and HEV RNA were assayed in serum samples obtained from Japanese voluntary blood donors with an elevated ALT level of $\geq$61 IU/l who are likely to have ongoing HEV infection.

MATERIALS AND METHODS

Serum Samples

Approximately 2.1% of voluntary blood donors had an elevated ALT level of 61 IU/l or greater at the Japanese Red Cross Saitama Blood Center, Japan, between April 2003 and March 2006. During this period, serum samples were collected from a total of 6700 voluntary blood donors (age, 35.7 ± 10.6 [mean ± standard deviation, SD] years; 6051 men and 649 women) with an elevated ALT level of 61–476 (range; 88.9 ± 34.6, mean ± SD) IU/l. The Blood Center is located in Saitama Prefecture, a prefecture in the central part of mainland Honshu of Japan.

All 6700 donors were negative for hepatitis B surface antigen and antibodies to hepatitis C virus (HCV), human immunodeficiency virus (HIV) types 1 and 2, and human T-lymphotropic virus type 1, as well as hepatitis B virus DNA, HCV RNA and HIV type 1 RNA by the nucleic acid amplification test using Roche's Multiplex reagent [Mine et al., 2003]. Serum samples obtained from repeat donors during the study period were excluded: that is, each sample was obtained from a unique individual.

Detection of Antibodies to HEV

To detect anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm [Mizuo et al., 2002], as described previously [Takahashi et al., 2005]. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA assays was 0.175, 0.440, and 0.642, respectively [Takahashi et al., 2005]. Samples with OD values for anti-HEV IgG, IgM, or IgA equal to or greater than the respective cut-off value were considered to be positive for anti-HEV IgG, IgM, or IgA, respectively. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, if the OD value of the tested sample was less than 30% of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

In serum samples with anti-HEV IgM and/or anti-HEV IgA, reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA using nested primers targeting the ORF2 region as described previously [Mizuo et al., 2002]. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used is capable of amplifying all four known genotypes of HEV strains reported thus far [Mizuo et al., 2002; Takahashi et al., 2003b; Yazaki et al., 2003]. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [Mizuo et al., 2002]. For serum samples that were negative for HEV RNA when 100 μl of serum samples was used, total RNA was extracted from 500 μl of serum, reverse transcribed, and then subjected to the nested PCR as described above. To extract RNA from 500 μl of serum, test serum diluted two-fold in saline was centrifuged at 287,582 × g at 4°C for 2 hr in a TLA-100.2 rotor (Beckman Coulter K.K., Tokyo, Japan), and the resulting pellet was suspended in 100 μl of saline and subjected to the RT-PCR assay. To confirm the reproducibility, this assay was performed in duplicate.

As for serum samples without anti-HEV IgM and anti-HEV IgA, 10 μl each from 10 serum samples were pooled,

and each pool was tested for HEV RNA by the above-mentioned RT-PCR assay. If a pool was positive for HEV RNA, the 10 serum samples of that pool were individually tested for the presence of HEV RNA. This RT-PCR assay was performed using both 10 and 100 μl of each serum sample, and reproducibility was confirmed.

Sequence Analysis of PCR Products

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 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN Version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequence alignments were generated by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987] based on the partial nucleotide sequence of the ORF2 region (412 nucleotides [nt]). Bootstrap values were determined on 1000 resamplings of the data sets [Felsenstein, 1985].

Statistical Analysis

Statistical analyses were performed using the χ^2-test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Age- and Sex-Specific Prevalence of Anti-HEV Antibodies

A total of 6700 serum samples obtained from apparently healthy blood donors with an elevated ALT level were tested for the presence of anti-HEV IgG. Anti-HEV IgG was detected in 7.1% (479/6700) of the tested population including 7.0% of the 6051 male donors and 8.6% of the 649 female donors, the difference not being significant (Table I). The prevalence of anti-HEV IgG generally increased with age among both the male and female donors, and was significantly higher among donors aged ≥30 years than among those aged <30 years in total (8.8% vs. 2.9%, $P < 0.0001$), in the males (8.6% vs. 2.9%, $P < 0.0001$) and in the females (10.7% vs. 2.5%, $P = 0.0013$). The 479 serum samples with anti-HEV IgG were tested for anti-HEV IgM and anti-HEV IgA. Among them, anti-HEV IgM was detected in eight samples (1.7%) and anti-HEV IgA in seven samples (1.5%). In total, nine samples were positive for anti-HEV IgM and/or anti-HEV IgA (Table II).

Detection of HEV RNA Among All 6700 Blood Donors

Among the nine serum samples with anti-HEV IgM and/or anti-HEV IgA, five samples tested positive for HEV RNA when RT-PCR was performed with a sample volume of both 10 and 100 μl, and one sample was positive for HEV RNA with a sample volume of 500 μl (Group A in Table II). Among 661 10-sample pools and nine 9-sample pools, three 10-sample pools were positive for HEV RNA. The 30 serum samples of the 3 pools that had been positive for HEV RNA were tested individually for the presence of HEV RNA, and 3 samples (nos. 5503, 8177 and 7369 in Groups B and C in Table II) were found to be positive for HEV RNA in two distinct volumes of 10 and 100 μl. Consequently, 9 (0.1%) of the 6700 samples were found to be viremic for HEV in the present study. When stratified by the presence of anti-HEV antibodies, HEV RNA was detectable in 6 (66.7%) of the 9 donors with anti-HEV IgM and/or anti-HEV IgA, 2 (0.4%) of the 470 donors with anti-HEV IgG but without anti-HEV IgM or anti-HEV IgA, and 1 (0.02%) of the 6221 donors without any serological markers of HEV infection.

Prevalence of Anti-HEV and HEV RNA, Stratified by ALT Level

In the present study, 479 donors with anti-HEV IgG were found, including 371 (7.2%) with an ALT level of 61–100 IU/l, 96 (6.6%) with an ALT level of 101–200 IU/l, and 12 (11.0%) with an ALT level of ≥201 IU/l (Table III). The prevalence of anti-HEV IgG was higher among donors with an ALT level of ≥201 IU/l than among those with an ALT level of 61–200 IU/l, although the difference was not statistically significant (11.0% vs. 7.1%, $P = 0.1148$). As for the prevalence of HEV RNA, there was a significant difference between donors with an ALT level of ≥201 IU/l and those with an ALT level of 61–200 IU/l in total (2.8% vs. 0.1%, $P < 0.0001$), in males (2.1% vs. 0.1%, $P < 0.0001$) and in females (8.3% vs. 0%, $P < 0.0001$).

Genetic Analysis of HEV Isolates Recovered from Nine Viremic Donors

The nine HEV isolates recovered from the transiently viremic donors were named HE-JSB1217, HE-JSB1564, HE-JSB1582, HE-JSB4175, HE-JSB5503, HE-JSB6151, HE-JSB7017, HE-JSB7369, and HE-JSB8177, respectively, with the prefix of HE-JSB followed by the ID no. of each sample. The 412-nt sequence of ORF2 of these HEV isolates were determined and compared with each other and with that of known HEV isolates of genotypes 1–4. These nine HEV isolates were markedly variable, sharing nucleotide identities ranging from 85.6% to 98.5%. However, they were all close to the prototype Japanese isolate of genotype 3 (JRA1 [accession no. AP003430]) with nucleotide identities of 87.3–93.9%, and were only 78.1–80.7%, 75.2–76.6%, and 78.2–80.5% similar to the B1 isolate (M73218) of genotype 1, MEX-14 isolate (M74506) of genotype 2, and T1 isolate (AJ272108) of genotype 4, respectively. When the nine HEV isolates obtained in the present study were compared with 412 other reported genotype 3 isolates whose common 299-to-412-nt ORF2 sequence is available as of December 2006, each of them was closest to a human or swine HEV

TABLE I. Age- and Sex-Dependent Prevalence of Anti-HEV Antibodies

	Total			Male			Female	
		Anti-HEV			Anti-HEV			Anti-HEV
Age (years)	N	IgG-class	IgM- and/or IgA-class	N	IgG-class	IgM- and/or IgA-class	N	IgG-class	IgM- and/or IgA-class
16–19	439	13 (3.0%)	0	397	11 (2.8%)	0	42	2 (4.8%)	0
20–29	1414	40 (2.8%)	0	1294	38 (2.9%)	0	120	2 (1.7%)	0
30–39	2736	183 (6.7%)	4 (0.1%)	2541	164 (6.5%)	3 (0.1%)	195	19 (9.7%)	1 (0.5%)
40–49	1319	126 (9.6%)	3 (0.2%)	1202	114 (9.5%)	3 (0.2%)	117	12 (10.3%)	0
50–59	651	93 (14.3%)	1 (0.2%)	520	77 (14.8%)	1 (0.2%)	131	16 (12.2%)	0
60–70	141	24 (17.0%)	1 (0.7%)	97	19 (19.6%)	1 (1.0%)	44	5 (11.4%)	0
Total	6700	479 (7.1%)	9 (0.1%)	6051	423 (7.0%)	8 (0.1%)	649	56 (8.6%)	1 (0.2%)

TABLE II. Detection of HEV RNA Among Three Categories of Blood Donors With Elevated ALT Level

					Anti-HEV (OD_{450} value)			HEV RNA[a]		
ID no.	Age (years)/ sex	ALT (IU/l)	AST (IU/l)	γ-GTP (IU/l)	IgG-class	IgM-class	IgA-class	10 μl	100 μl	500 μl
Group A ($n = 9$) with anti-HEV IgG with anti-HEV IgM and/or anti-HEV IgA										
1217	34/M	94	37	149	2.583 (+)	1.818 (+)	1.676 (+)	+[b]	+	NT[c]
1564	48/M	61	32	86	2.343 (+)	1.709 (+)	2.648 (+)	+	+	NT
1582	51/M	101	52	89	2.404 (+)	0.917 (+)	1.310 (+)	+	+	NT
4175	68/M	261	145	154	1.709 (+)	2.566 (+)	1.130 (+)	+	+	NT
6151	49/M	128	41	607	1.364 (+)	2.237 (+)	2.442 (+)	+	+	NT
7017	36/F	224	95	362	1.401 (+)	1.313 (+)	0.348 (−)	−	−	+
1304	38/M	181	67	104	>3.000 (+)	>3.000 (+)	0.825 (+)	−	−	−
3243	35/M	170	78	334	0.404 (+)	0.470 (+)	0.017 (−)	−	−	−
7667	47/M	66	37	45	0.350 (+)	0.044 (−)	0.868 (+)	−	−	−
Group B ($n = 470$) with anti-HEV IgG but without anti-HEV IgM nor anti-HEV IgA										
5503	34/M	77	55	20	0.394 (+)	0.091 (−)	0.138 (−)	+	+	NT
8177	41/M	82	53	197	0.193 (+)	0.042 (−)	0.061 (−)	+	+	NT
Group C ($n = 6221$) without anti-HEV IgG										
7369	48/M	276	210	219	0.006 (−)	0.016 (−)	0.011 (−)	+	+	NT

[a]HEV RNA was assayed using the indicated volume of serum sample.
[b]+, positive for HEV RNA; −, negative for HEV RNA.
[c]NT, not tested.

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TABLE III. Prevalence of Anti-HEV IgG and HEV RNA Among Voluntary Blood Donors With Elevated ALT Level, Stratified by ALT Level

	Total			Male			Female		
ALT (IU/l)	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA
61–100	5131	371 (7.2%)	4 (0.1%)	4635	331 (7.1%)	4 (0.1%)	496	40 (8.1%)	0
101–200	1460	96 (6.6%)	2 (0.1%)	1319	83 (6.3%)	2 (0.2%)	141	13 (9.2%)	0
201–476	109	12 (11.0%)	3 (2.8%)	97	9 (9.3%)	2 (2.1%)	12	3 (25.0%)	1 (8.3%)
Total	6700	479 (7.1%)	9 (0.1%)	6051	423 (7.0%)	8 (0.1%)	649	56 (8.6%)	1 (0.2%)

isolate of Japan origin. That is, HE-JSB1564, HE-JSB5503, and HE-JSB8177 had the highest identity of 99.0%, 98.5% and 98.3%, respectively, with HE-JBD2 (AB154829). The HE-JSB4175 isolate was closest to HE-JA9 (AB082565, 97.8%), HE-JSB1217 and HE-JSB1582 to HE-JHD1988 (AB175485, 94.9% and 93.7%, respectively), HE-JSB7369 to HE-JA21 (AB115542, 94.2%), HE-JSB6151 to G3-4531-Swine (DQ079632, 94.4%), and HE-JSB7017 to HE-JHD1980 (AB175484, 93.4%).

The phylogenetic tree constructed based on the common 412-nt sequence within the ORF2 sequence confirmed that the nine HEV isolates obtained in the present study belonged to genotype 3, and that they segregated into the clusters consisting of Japanese HEV strains of the same genotype that had been recovered from humans, swine and wild boars, supporting the indigenous nature of these nine blood donor isolates (Fig. 1).

DISCUSSION

Recent studies have documented that sporadic acute hepatitis E does occur among individuals in industrialized countries with no history of travel to areas endemic for HEV [Kwo et al., 1997; Harrison, 1999; Mansuy et al., 2004; Ijaz et al., 2005; Waar et al., 2005; Amon et al., 2006; Preiss et al., 2006; Sadler et al., 2006]. In Japan, hepatitis E is rare compared with hepatitis A, but is occurring more frequently than previously thought [Mizuo et al., 2002; Okamoto et al., 2003]. As for the geographical distribution of hepatitis E in Japan, it was reported that there was wide variation with a higher prevalence in the northern part of Japan (Hokkaido Island and northern part of mainland Honshu) [Mizuo et al., 2002; Abe et al., 2006]. The Japanese Red Cross Saitama Blood Center is located in Saitama Prefecture, which is north of and adjacent to Metropolitan Tokyo. Only six cases of locally acquired sporadic acute hepatitis E have thus far been reported in this prefecture, in contrast with more than 100 cases in Hokkaido and 23 cases in Tokyo [Abe et al., 2006].

In the present study, 7.0% (468/6700) of the study population had anti-HEV IgG in the absence of IgM/IgA class anti-HEV and HEV RNA, which is much higher than expected. This finding suggests the presence of frequent past HEV infection among individuals living in the central part of Japan, most of which seem to be subclinical. HEV RNA was assayed in serum samples obtained from all 6700 donors, and the prevalence of ongoing subclinical HEV infection in three distinct groups of the study population according to the presence of class-specific HEV antibodies, was investigated. Among the nine donors with IgM and/or IgA class anti-HEV, six donors (66.7%) were found to be viremic for HEV. Furthermore, among the 6691 donors without anti-HEV IgM and anti-HEV IgA, three HEV-viremic donors with no signs or symptoms of hepatitis were found. In an attempt to detect HEV RNA in a large sample size, we first screened for present HEV infection by testing 10-sample minipools (each pool contained

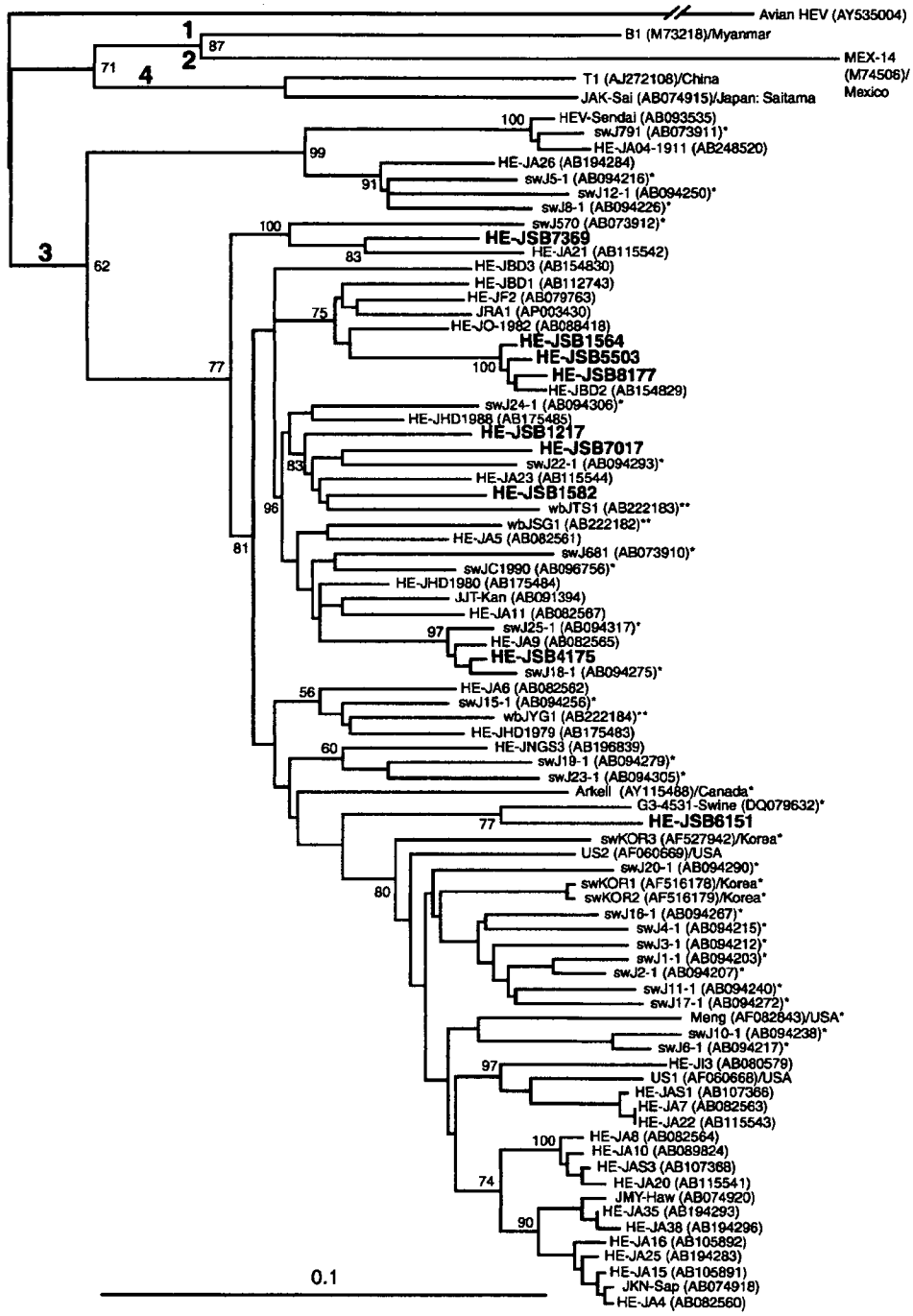

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 84 HEV isolates, using a chicken HEV (AY535004) as an outgroup. In addition to the HE-JSB1217, HE-JSB1564, HE-JSB1582, HE-JSB4175, HE-JSB5503, HE-JSB6151, HE-JSB7017, HE-JSB7369, and HE-JSB8177 isolates found in the present study which are indicated in bold type, 75 reported HEV isolates of genotypes 1–4 whose common 412-nt sequence is known are included for comparison and their accession nos. are shown in parentheses. Swine and wild boar HEV isolates are indicated with asterisks (* and **, respectively). Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data.

10 μl of serum from each of 10 subjects); then, for pools that were positive for HEV RNA, the individual serum samples were tested for the presence of HEV RNA. Despite the limited amount of serum from each sample that was tested, current subclinical HEV infection was recognized molecularly in three donors in this study. Two viremic donors (0.4%) were found among the 470 donors with anti-HEV IgG but without anti-HEV IgM nor anti-HEV IgA. Since serial serum samples were not available from these two viremic donors during the HEV infection, it is unclear whether they could not elicit acute antibodies of anti-HEV IgM and anti-HEV IgA.

However, the observation that HEV RNA is detectable in serum despite the absence of anti-HEV IgM and anti-HEV IgA is in agreement with our previous observations that two hemodialysis patients and one hospital employee who contracted subclinical HEV infection in 1979, 1980, or 2003, respectively, exhibited only anti-HEV IgG, although transient HEV viremia was observed [Mitsui et al., 2004, 2005]. Patients with HEV infection without an acute antibody response have also been reported [Caudill et al., 1994; Clayson et al., 1995].

Of particular note, one donor (0.02%) was found to have HEV RNA among the 6221 donors without serological markers of HEV infection in the present study. The precise reason why the viremic donor did not show an antibody response against HEV despite significant elevations of ALT (276 IU/l) and AST (210 IU/l) levels is unknown. However, 4 months before the blood sampling, he had elevated ALT (81 IU/l) and AST (165 IU/l) levels in the absence of HEV RNA in serum, suggesting that he contracted subclinical HEV infection on the background of an unexplained chronic liver disease. It was reported that neither anti-HEV IgG nor anti-HEV IgM was detectable in four symptom-free persons with evidence of HEV viremia who came into contact with patients with acute hepatitis E during an outbreak of hepatitis E [Nicand et al., 2001]. Aggarwal et al. [2001] conducted experimental studies on subclinical HEV infection in cynomolgus macaques, and reported that subclinical HEV infection in some animals was associated with failure of the development of an immune response, compared with animals with clinical HEV infection.

In Japan, approximately 200 patients with clinical HEV infection and 80 patients with subclinical HEV infection who contracted the infection between 2001 and 2005 have been reported [Okamoto et al., 2003; Fukuda et al., 2004; Abe et al., 2006; Inoue et al., 2006]. However, the exact ratio of the number of cases of clinical HEV infection to that of subclinical HEV infection remains unknown. At the Japanese Red Cross Saitama Blood Center, 2.1% of voluntary blood donors had an elevated ALT level of 61 IU/l or greater during the period from April 2003 to March 2006. The population size of individuals ≥20 years of age in Saitama Prefecture was reported to be 5.68 million on January 1, 2005 (http://www.pref.saitama.lg.jp/). In the present study, 9 (0.13%) of 6700 individuals with an elevated ALT level of ≥61 IU/l had HEV viremia. Assuming that HEV viremia is detectable by RT-PCR for 1 month during acute HEV infection [Takahashi et al., 2003a, 2005], the annual number of cases of subclinical HEV infection in Saitama Prefecture is estimated to be approximately 2000. Although we cannot rule out the possibility that the number of cases with clinical HEV infection is underestimated, only one or two patients with hepatitis E have been reported per year in this prefecture. Therefore, it is assumed that less than 0.1% of HEV-infected cases exhibit clinical manifestation of the infection.

As the nine viremic donors identified in the present study had an elevated ALT level, the blood from the nine donors was not used for transfusion, suggesting that ALT testing may help prevent transfusion-transmitted HEV infection. As one of the nine infected donors had only a slightly elevated ALT level of 61 IU/l, it seems likely that even donors with a normal ALT level (≤60 IU/l) may have detectable HEV RNA. However, the prevalence of HEV RNA decreased with ALT level, and was significantly lower among the 5131 donors with ALT level of 61–100 IU/l than among the 109 donors with ALT of ≥201 IU/l (0.078% vs. 2.8%, $P < 0.0001$). It is reasonable to speculate that the prevalence of ongoing HEV infection among donors with a normal ALT level may be less than 0.078% in Saitama Prefecture. The proportion of such donors may be significantly small or negligible, according to the geographic region. Reflecting the high prevalence of clinical HEV infection, at least three cases of transfusion-transmitted hepatitis E have been reported in Hokkaido and one case in Tokyo [Matsubayashi et al., 2004; Abe et al., 2006], but none in Saitama Prefecture up to the present. As donors with normal ALT level were not tested for HEV viremia in the present study, we cannot conclude that the serum ALT level can be used to exclude blood donors with ongoing HEV infection. Based on the current study, however, we would consider that ALT testing is at least useful in part for exclusion of donors with HEV viremia with the aim of preventing transfusion-associated hepatitis E, although alcohol consumption and obesity should be taken into consideration as the major contributing factors to an elevated ALT level in blood donors.

Multiple HEV strains of genotype 3 or 4 have been isolated from Japanese patients with sporadic acute or fulminant hepatitis E as well as from farm pigs, wild boars, a wild deer and a mongoose in Japan [Mizuo et al., 2002; Takahashi et al., 2003a,b, 2004; Inoue et al., 2006; Nakamura et al., 2006]. Reflecting the polyphyletic nature of human and animal HEV isolates of Japan origin, the HEV isolates recovered from nine viremic donors in the present study, differed by 1.5–14.4% from each other, although they belonged to the same genotype (genotype 3) with the highest nucleotide sequence identity of 87.3–93.9% with the JRA1 isolate that is believed to be indigenous to Japan [Takahashi et al., 2001]. A human HEV strain of genotype 4 (JAK-Sai [AB074915]) has been isolated [Takahashi et al., 2002] in the same prefecture as that of the nine viremic donors, and it shares only 78.6–81.3% identities with the nine HEV isolates obtained from the viremic donors in the present study. These results further support the marked heterogeneity of the HEV genome and its wide distribution in Japan, even within a certain prefecture in this country.

In conclusion, nine blood donors with HEV viremia were identified among 6700 voluntary blood donors with an elevated ALT level at a blood center located in the central part of mainland Honshu of Japan where hepatitis E is low-endemic. This study indicates that approximately 0.1% of individuals with an elevated ALT

level and 3% of individuals with an ALT level of ≥201 IU/l have ongoing subclinical infection of various HEV strains, suggesting the frequent occurrence of subclinical HEV infection, although clinical HEV infection is rarely reported. A large study of individuals who do not have an elevated ALT level is needed to assess the exact frequency of subclinical HEV infection, taking into consideration the geographic region in Japan.

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Analysis of the Full-Length Genome of Hepatitis E Virus Isolates Obtained From Farm Pigs in Mongolia

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Although no outbreaks of hepatitis E have been reported in Mongolia, a significant proportion of the general population had antibodies to hepatitis E virus (HEV). To investigate whether pigs are possible reservoirs of HEV in Mongolia, serum samples obtained from 243 2- or 3-month-old pigs on four swine farms surrounding Ulaanbaatar, the capital city of Mongolia, were tested for the presence of anti-HEV antibodies and HEV RNA. Overall, 223 pigs (91.8%) tested positive for anti-HEV, while 89 pigs (36.6%) had detectable HEV RNA. The 89 HEV isolates obtained from the viremic pigs were 78.7–100% identical to each other, and 80.9–85.9% similar to the prototype genotype 3 HEV isolate (US1) in the 412-nucleotide (nt) sequence within open reading frame 2. They were classified into two novel phylogenetic groups within genotype 3, differing by 16.4–21.3%. The swMN06-A1288 and swMN06-C1056 isolates, representing each of the two clusters within genotype 3, had a genomic length of nucleotides (nt) 7,222 nt and 7,223 nt, respectively, excluding the poly(A) tail, and shared only 81.6% over the entire genome. Upon comparison with the 25-reported genotype 3 HEV isolates over the entire genome, swMN06-A1288 had identities of merely up to 84.9%, while swMN06-C1056 of only up to 85.9%. Phylogenetic analysis confirmed the remote relatedness of the Mongolian swine isolates to the genotype 3 HEV isolates reported thus far. These results indicate that farm pigs in Mongolia are frequently infected with presumably indigenous HEV strains of genotype 3 and could be a source of HEV infections in humans in Mongolia. ***J. Med. Virol. 79:1128–1137, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis E virus; genotype; swine; zoonosis; Mongolia

INTRODUCTION

Hepatitis E, which is caused by hepatitis E virus (HEV), is an important public health concern in many developing countries where the sanitation condition is suboptimal: large epidemics of hepatitis E have been reported in Asia, Africa, and Latin America [Purcell and Emerson, 2001a]. HEV infection is also endemic in many industrialized countries including the United States, European countries, and Japan [Harrison, 1999; Smith, 2001; Purcell and Emerson, 2001a; Okamoto et al., 2003]. Accumulating lines of evidence have indicated that there are animal reservoirs of HEV and that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Erker et al., 1999; Harrison, 1999; Meng, 2000, 2005; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003].

HEV has been characterized as a non-enveloped, single-stranded, positive-sense RNA virus. Its genome is approximately 7.2 kb long, and possesses three partially overlapping open reading frames (ORFs: ORF1, ORF2, and ORF3) [Tam et al., 1991]. HEV was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004]. HEV sequences have been classified into four genotypes (genotypes 1–4): 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 throughout the world and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in the United States, European countries, and Japan; and genotype 4 is restricted to Asian countries and comprises strains from humans and

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession number AB290312–AB290313 (two complete HEV genomes) and AB290032–AB290120 (89 partial HEV sequences).

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domestic pigs [Schlauder and Mushahwar, 2001; Emerson et al., 2004; Lu et al., 2006].

Mongolia, formerly known as Outer Mongolia, is located in northern Asia between Russia and China. Despite improving health services and health condition of its people, Mongolia continues to confront many communicable diseases including viral hepatitis. Previous studies have documented high rates of viremia of hepatitis B, C, and delta viruses among apparently healthy individuals and a high rate of antibodies against hepatitis A virus among both children and adults [Takahashi et al., 2004; Tsatsralt-Od et al., 2005, 2007]. Although epidemics or outbreaks of hepatitis E have not been reported in Mongolia, approximately 11% of healthy individuals in Mongolia were seropositive for HEV antibodies [Takahashi et al., 2004].

The first animal strain of HEV to be isolated and characterized was a swine HEV from a pig in the United States in 1997 [Meng et al., 1997]. Since then, many swine HEV isolates have been identified not only in industrialized countries but also in developing countries, and were shown to be closely related genetically to strains of human origin [Chandler et al., 1999; Hsieh et al., 1999; Garkavenko et al., 2001; Okamoto et al., 2001; van der Poel et al., 2001; Huang et al., 2002; Pei and Yoo, 2002; Wang et al., 2002; Wu et al., 2002; Choi et al., 2003; Nishizawa et al., 2003; Takahashi et al., 2003; Banks et al., 2004; Wibawa et al., 2004; Munne et al., 2006]. Thus far, HEV strains have not been identified from humans and animals in Mongolia. The extent of genomic heterogeneity of HEV strains has not been fully understood. Therefore, in the present study, the prevalence of HEV antibodies and HEV viremia was investigated among farm pigs in Mongolia, and the full-length sequence was determined for two representative swine HEV isolates among the 89 swine HEV isolates that were obtained. The swine HEV strains obtained in this study were highly divergent from the HEV strains reported thus far and seem to be indigenous to Mongolia.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected from 243 2- or 3-month-old pigs raised in four swine farms surrounding Ulaanbaatar, the capital city of Mongolia, in June and July 2006. Serum samples were stored at −40°C or below until testing for serological and molecular markers of HEV infection.

Detection of Swine Antibodies to HEV

To detect swine anti-HEV IgG, ELISA was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm, as described previously [Mizuo et al., 2002; Takahashi et al., 2003, 2005], with a slight modification. In brief, sample diluent was mixed with a mock protein obtained from the pupae of silkworm infected with non-recombinant baculovirus, in an attempt to minimize non-specific reactions. The optical density (OD) of each sample was read at 450 nm. The cutoff value used for the swine anti-HEV IgG assay was 0.274. Test samples with OD values for swine anti-HEV IgG equal to or greater than the cutoff value were considered to be positive for anti-HEV. The specificity of the swine anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, when the OD value of the tested sample decreased to less than 30% of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA in swine serum. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with the ORF2 primers as described previously [Mizuo et al., 2002]. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457 bp. The nested RT-PCR assay was performed in duplicate, and reproducibility was confirmed. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [Mizuo et al., 2002; Takahashi et al., 2003].

Amplification of Full-Length HEV Genome

Total RNA was extracted from 500 μl of serum and subjected to cDNA synthesis followed by nested polymerase chain reaction (PCR) of ten or six overlapping regions including the extreme 5′- and 3′-terminal regions: the amplified regions were nucleotides (nt) 1–106 (106 nt) (primer sequences excluded), nt 43–1,270 (1,228 nt), nt 1,086–1,624 (539 nt), nt 1,546–2,074 (529 nt), nt 2,056–3,169 (1,114 nt), nt 3,109–4,478 (1,370 nt), nt 4,282–5,282 (1,001 nt), nt 5,202–6,430 (1,229 nt), nt 6,342–7,195 (854 nt), and nt 7,101–7,237 (137 nt) for the swMN06-A1288 isolate (see below); and nt 1–115 (115 nt), nt 43–1,270 (1,228 nt), nt 1,049–4,849 (3,801 nt), nt 4,651–6,380 (1,730 nt), nt 6,342–7,196 (855 nt), and nt 7,029–7,239 (211 nt) for the swMN06-C1056 isolate (see below).

The extreme 5′-end sequence (nt 1–106 for swMN06-A1288 and nt 1–115 for swMN06-C1056) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM–RACE) with the First Choice RLM–RACE kit (Ambion, Austin, TX), as described previously [Okamoto et al., 2001]. Amplification of the 3′-end sequence (nt 7,101–7,222 for swMN06-A1288 and nt 7,029–7,223 for swMN06-C1056: poly [A] tail excluded) was attempted by the RACE method as described previously [Okamoto et al., 2001].

Sequence Analysis of PCR Products

The amplification product was sequenced on both strands directly or after cloning into pT7Blue T-Vector (Novagen, Inc., Madison, WI), using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Win version 8.1.0 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequence alignments were generated by CLUSTAL W (version 1.8) [Thompson et al., 1994]. Phylogenetic trees were constructed by the neighbor-joining method [Saitou and Nei, 1987] based on the partial nucleotide sequence of the ORF2 region (412 nt) or the entire genome. Bootstrap values were determined on 1,000 resamplings of the data sets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

RESULTS

Prevalence of Anti-HEV and HEV RNA Among Farm Pigs in Mongolia

Serum samples obtained from 243 2- or 3-month-old pigs on four swine farms surrounding Ulaanbaatar, were tested for the presence of anti-HEV antibodies and HEV RNA. Overall, 223 pigs (91.8%) were positive for anti-HEV, while 89 pigs (36.6%) tested positive for HEV RNA (Table I). The prevalence of anti-HEV differed by swine farm, ranging from 6.3% to 99.0%, with higher prevalence in Farms A, B, and C (89.5–99.0%) and the lowest prevalence in Farm D (only 6.3%). The prevalence of HEV RNA also differed by swine farm. In Farms A and C which had a high prevalence of anti-HEV, the prevalence of HEV viremia was 38.1% and 68.4%, respectively. However, HEV RNA was detectable in only 7.1% (1/14) of the pigs in Farm B despite the high prevalence of anti-HEV, the low prevalence of HEV viremia being comparable with that in Farm D of 6.3% (1/16).

Genetic Analysis of HEV Isolates From Viremic Pigs

The 412-nt partial sequence within ORF2 of the HEV isolates obtained from the 89 viremic pigs were determined and deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB290032–AB290120). The 89 HEV isolates were 78.7–100% identical to each other and were close to the prototype human HEV isolate of genotype 3 (US1 [AF060668]), with identities of 80.9–85.9% at the nucleotide level, but they were only 72.8–78.9% similar to known HEV isolates of genotype 1 (B1 [M73218]), genotype 2 (MEX-14 [M74506], and genotype 4 (T1 [AJ272108]). Pairwise comparison of the 89 Mongolian swine HEV isolates revealed that they segregated into two distinct groups, differing by 16.4–21.3%. The phylogenetic tree constructed based on the 412-nt ORF2 sequence of the 89 Mongolian swine HEV isolates obtained in the present study as well as six reported HEV isolates of genotypes 1–4, confirmed the clustering of the Mongolian swine HEV isolates into two groups, with bootstrap values of 100%. All 74 HEV isolates from Farm A which have the prefix of swMN06-A and the isolate from Farm B (swMN06-B1021) were grouped together, while the 13 HEV isolates from Farm C which have the prefix of swMN06-C and the HEV isolate (swMN06-D1062) from Farm D were clustered together (Fig. 1). The 74 isolates from Farm A shared nucleotide identities of 92.8–100% to each other, and were 92.8–98.8% identical to the swMN06-B1021 isolate. The 13 isolates from Farm C were 99.0–100% identical to each other, and showed identities of 99.3–100% with the swMN06-D1062 isolate from Farm D.

Analysis of the Full-Length Genome of Swine HEV Isolates Recovered From Viremic Pigs in Mongolia

To further investigate the genomic heterogeneity between the two groups of Mongolian swine HEV isolates obtained in the present study, the swMN06-A1288 isolate from Farm A and the swMN06-C1056 isolate from Farm C, representing the two phylogenetic

TABLE I. Prevalence of Anti-HEV and HEV RNA Among Pigs From Various Farms Surrounding Ulaanbaatar, Mongolia

Farm	Age (month)	No. of pigs	Anti-HEV IgG		HEV RNA (%)
			No. (%)	OD[a] (mean ± SD)	
A	2	83	82 (98.8)	2.316 ± 0.566	43 (51.8)
	3	111	110 (99.1)	2.234 ± 0.752	31 (27.9)
Subtotal		194	192 (99.0)	2.269 ± 0.679	74 (38.1)
B	2	5	5 (100)	1.265 ± 0.526	1 (20.0)
	3	9	8 (88.9)	0.592 ± 0.274	0
Subtotal		14	13 (92.9)	0.851 ± 0.502	1 (7.1)
C	2	19	17 (89.5)	1.156 ± 0.766	13 (68.4)
D	2	16	1 (6.3)	1.175	1 (6.3)
Total		243	223 (91.8)	2.097 ± 0.800	89 (36.6)

[a]Optical density at 450 nm.

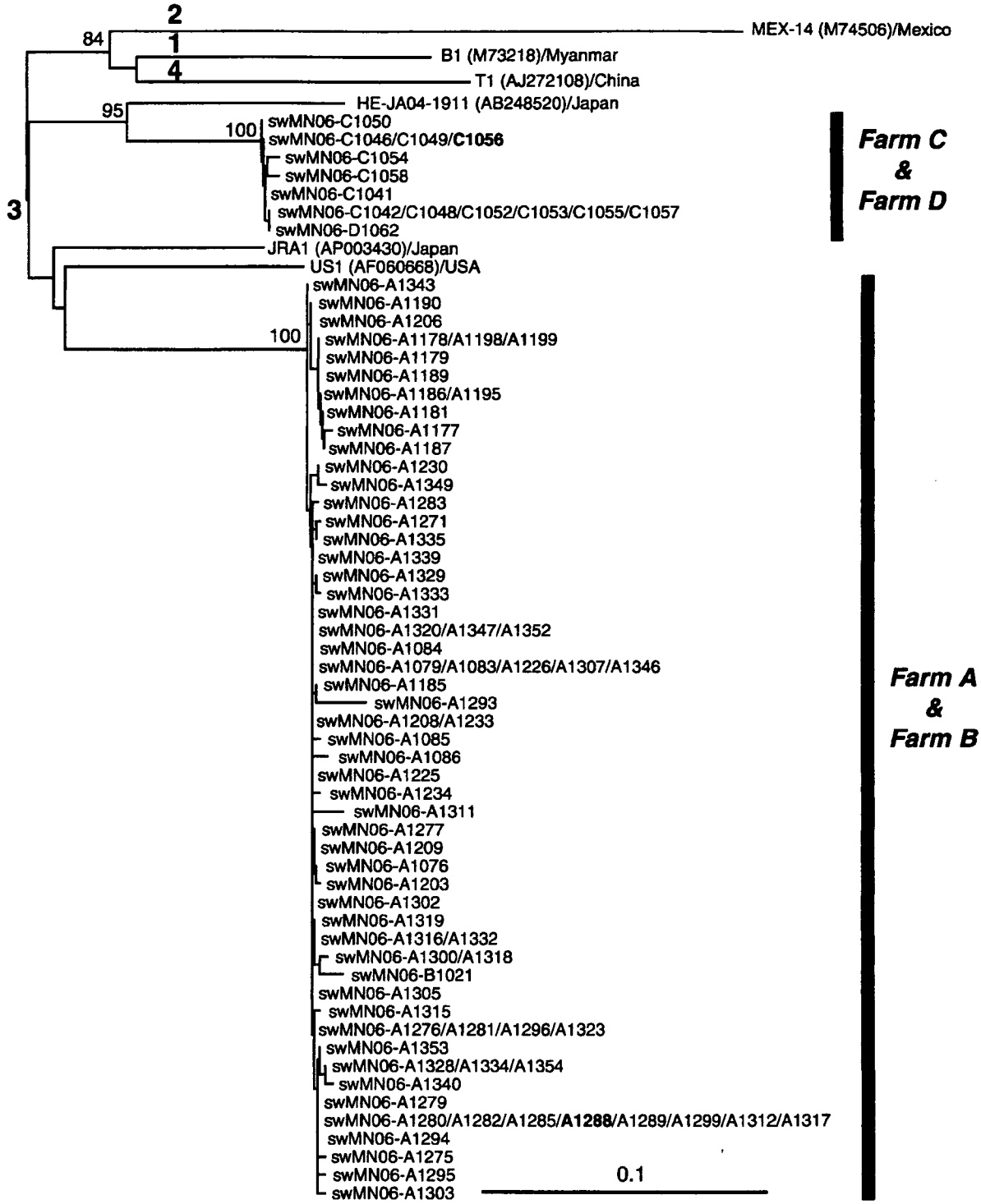

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 95 HEV isolates. In addition to the 89 Mongolian swine HEV isolate found in the present study which have the prefix of swMN06 followed by the farm name and ID no., six representative HEV isolates of genotypes 1–4 whose entire sequence is known are included for comparison and their accession nos. are shown in parentheses followed by the name of the country where it was isolated. The two Mongolian swine isolates whose entire genomic sequence was determined in the present study are indicated in bold type for visual clarity. Vertical bars represent the two clusters of Mongolian swine HEV isolates. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data. Bar, 0.1 nucleotide substitutions per site.

clusters within genotype 3, were sequenced over the entire genome and their nucleotide sequences were deposited in the DDBJ/EMBL/GenBank database (AB290312–AB290313). The swMN06-A1288 and swMN06-C1056 isolates had a genomic length of 7,222 and 7,223 nt, respectively, excluding the poly(A) tract at the 3′-terminus, the difference of 1 nt being attributable to a deletion/insertion at the distal portion of the 3′-untranslated region. Each of the two HEV isolates 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]. In both isolates, ORF1, ORF2, and ORF3 had coding capacities of 1,703 amino acids (aa) (nt 26–5,134), 660 aa (nt 5,172–7,151), and 122 aa (nt 5,134–5,499), respectively. The 5′-UTRs of the two isolates comprised a common sequence of 25 nt, while the 3′-UTRs consisted of a variable sequence of 71 nt in swMN06-A1288 and 72 nt in swMN06-C1056.

The swMN06-A1288 and swMN06-C1056 isolates shared only 81.6% over the entire genome, and had aa identities of 93.2% in ORF1, 97.0% in ORF2, and 91.0% in ORF3. Upon comparison with the 25 reported full-length sequences of genotype 3 HEV isolates from humans, swine, wild boars, a wild deer, and a mongoose, swMN06-C1056 was most closely related to the human isolate HE-JA04-1911 with an identity of only 85.9%, while swMN06-A1288 was nearest to JBOAR1-Hyo04 and JDEER-Hyo03L with merely 84.9% identity (Table II). The phylogenetic tree, constructed based on the full-length nucleotide sequences of the swMN06-A1288 and swMN06-C1056 isolates and all reported HEV isolates whose entire sequence is known, depicted that the two Mongolian swine isolates obtained in the present study were distantly related to the reported genotype 3 HEV isolates (Fig. 2), suggesting the uniqueness of the Mongolian swine isolates among the genotype 3 isolates.

When compared with the 199 reported partial sequences of genotype 3 HEV isolates, swMN06-A1288 exhibited the highest identity of 89.2% with an Argentinean swine isolate (Argentina [AY258006]) within the 287-nt ORF1 sequence and the lowest similarity of 77.4% with a Spanish swine isolate (15204 [DQ315754]) within the 168-nt ORF2 sequence. swMN06-C1056 showed the highest similarity of 94.6% with a human isolate from the Netherlands (HU/NL2005-0825 [DQ200292]) and the lowest similarity of 78.1% with a swine isolate from the Netherlands (NLSW105 [AF336013]) (Table II).

DISCUSSION

The Meng isolate was the first reported strain of HEV isolated from an animal, that is, an infected pig in the United States in 1997 [Meng et al., 1997]. Thereafter, many HEV isolates have been identified in pigs in a total of 19 countries worldwide, although none has been isolated in Africa. Specifically, a total of 443 swine HEV strains of genotype 3 have been identified in 16 countries including Argentina, Australia, Cambodia, Canada, France, Japan, Korea, Kyrgyzstan, Mexico, the Netherlands, New Zealand, Spain, Taiwan, Thailand, United Kingdom, and the United States, and 118 swine HEV strains of genotype 4 have been isolated in five countries including China, India, Indonesia, Japan, and Taiwan, as of February 28, 2007. Therefore, Mongolia is the 20th country where swine HEV isolates were identified from farm pigs and the 17th country where genotype 3 HEV strains of swine origin were isolated. Given that a high prevalence of swine anti-HEV antibodies have been found in many countries including the United States, Canada, Taiwan, and Japan [Meng et al., 1997; Hsieh et al., 1999; Yoo et al., 2001; Takahashi et al., 2003, 2005], the present finding that farm pigs in Mongolia were highly infected with HEV may not be surprising. However, the present study revealed that swine HEV strains belonging to two novel phylogenetic clusters within genotype 3, which were only 85–86% similar to reported HEV isolates of the same genotype over the entire genome, circulate in Mongolia, suggesting the possibility that many more heterogeneous HEV strains are circulating in as-yet-unexamined areas in the world.

There have been reports of high genetic relatedness between HEV isolates obtained from humans and those obtained from swine in the same geographical region in the United States (genotype 3) [Meng et al., 1997; Erker et al., 1999] and Spain (genotype 3) [Pina et al., 2000], China (genotype 4) [Wang et al., 2002; Zheng et al., 2006] and Taiwan (genotype 4) [Hsieh et al., 1999; Wu et al., 2002], and Japan (both genotypes 3 and 4) [Mizuo et al., 2002; Nishizawa et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003]. Of note, besides genotype 1 HEV strains that have been responsible for epidemic hepatitis E in humans, genotype 4 HEV isolates have been identified from sporadic cases of acute hepatitis E and from infected pigs in China [Wang et al., 2002; Zheng et al., 2006] and genotype 3 HEV isolates of human and swine origin were obtained in Cambodia [accession nos. DQ145792–DQ145799]. On the other hand, in Thailand and Mexico, genotype 3 HEV was identified in serum and fecal samples from pigs, while genotype 1 or 2 HEV strains are prevalent in the respective human populations [Cooper et al., 2005]. In India, genotype 4 HEV strains have been recovered from pigs, whereas genotype 1 HEV is epidemic in humans [Arankalle et al., 2002, 2003]. In Mongolia, approximately 11% of healthy adults are known to have anti-HEV antibodies, most likely, indicative of past subclinical HEV infection [Takahashi et al., 2004], and genotype 3 HEV was identified from farm pigs in the present study. However, no HEV strains were identified in serum samples obtained from 110 consecutive patients who were clinically diagnosed with acute hepatitis in Ulaanbaatar, Mongolia between December 2004 and January 2005, 16% and 35% of whom were subsequently diagnosed as having hepatitis A and hepatitis B, respectively [Tsatsralt-Od et al., 2006]. Therefore, future studies using serum samples collected from larger numbers of patients with acute hepatitis are

TABLE II. Comparison of the Two HEV Isolates of Genotype 3 Whose Entire Genomic Sequence Was Determined in This Study, With Reported HEV Isolates of the Same Genotype

Isolate name	Host	Accession number	Country	Sequence length compared (nt)	Region	Identity (%)[a]	
						swMN06-A1288[b]	swMN06-C1056[b]
swMN06-A1288	Swine	AB290312	Mongolia	7,237	Full length	NA[c]	81.6
swMN06-C1056	Swine	AB290313	Mongolia	7,239	Full length	81.6	NA
Arkell	Swine	AY115488	Canada	7,255	Full length	84.1	81.3
HE-JA04-1911	Human	AB248520	Japan	7,280	Full length	81.0	85.9
swJ8-5	Swine	AB248521	Japan	7,241	Full length	80.8	85.4
swJ12-4	Swine	AB248522	Japan	7,241	Full length	80.9	85.4
JRA1	Human	AP003430	Japan	7,230	Full length	84.6	81.4
JJT-Kan	Human	AB091394	Japan	7,218	Full length	84.5	81.5
JKN-Sap	Human	AB074918	Japan	7,256	Full length	83.7	81.2
HEVN1	Human	AB246676	Japan	7,231	Full length	84.0	81.0
JMY-Haw	Human	AB074920	Japan	7,233	Full length	84.3	81.2
JSO-Hyo03L	Human	AB189073	Japan	7,180	Full length	84.8	81.6
JYO-Hyo03L	Human	AB189075	Japan	7,180	Full length	84.8	81.6
HE-JA10	Human	AB089824	Japan	7,262	Full length	84.5	81.3
JMO-Hyo03L	Human	AB189072	Japan	7,180	Full length	84.8	81.6
JTH-Hyo03L	Human	AB189074	Japan	7,180	Full length	84.8	81.6
swJ570	Swine	AB073912	Japan	7,257	Full length	83.7	80.9
wbJSG1	Wild boar	AB222182	Japan	7,240	Full length	84.4	81.3
wbJTS1	Wild boar	AB222183	Japan	7,241	Full length	84.2	81.8
wbJYG1	Wild boar	AB222184	Japan	7,240	Full length	84.5	81.0
JBOAR1-Hyo04	Wild boar	AB189070	Japan	7,247	Full length	84.9	81.7
JDEER-Hyo03L	Wild deer	AB189071	Japan	7,230	Full length	84.9	81.7
JMNG-Oki02C	Mongoose	AB236320	Japan	7,236	Full length	84.2	81.5
Osh205	Swine	AF455784	Kyrgyzstan	7,239	Full length	81.6	83.7
US1	Human	AF060668	USA	7,202	Full length	84.2	81.6
US2	Human	AF060669	USA	7,277	Full length	84.6	81.5
Meng	Swine	AF082843	USA	7,242	Full length	84.6	81.8
Ar1	Human	AF264009	Argentina	371	ORF1	87.9	84.1
Ar2	Human	AF264010	Argentina	371	ORF1	87.6	81.7
Argentina	Swine	AY258006	Argentina	287	ORF1	89.2	84.7
Australia	Swine	AF521653	Australia	289	ORF1	83.7	81.5
Au1	Human	AF279122	Austria	519	ORF1+ORF2	86.3	84.4
CAM-H8	Human	DQ145792	Cambodia	145	ORF2	82.1	82.1
CAM-3F13 and 2 others	Swine	DQ145793–DQ145795	Cambodia	145	ORF2	83.4–84.8	84.1-84.8
MP13	Human	AY626041	France	189	ORF2	84.1	**90.5**
MP14	Human	AY626042	France	189	ORF2	84.1	**91.0**
Fr-1	Human	EF053273	France	342	ORF2	81.5	86.5
Fr-2	Human	EF053274	France	347	ORF2	82.4	87.9
Fr-13 and 4 others	Human	EF113903–EF113907	France	322–345	ORF2	79.9–84.3	83.0-88.2
FRHF13	Swine	EF050797	France	273	ORF2	82.1	87.2
Gr1	Human	AF110388	Greece	519	ORF1+ORF2	83.4	89.0
Gr2	Human	AF110389	Greece	519	ORF1+ORF2	84.3	86.1
GerWW	Human	AJ889195	Germany	938	ORF1+ORF2	82.7	86.5
Ger-JS	Human	AY753647	Germany	1,866	ORF1+ORF2	84.5	81.3
Hungary1	Human	AY940427	Hungary	148	ORF2	84.4	85.0
It1	Human	AF110387	Italy	519	ORF1+ORF2	88.8	84.4
HTN-0618	Human	DQ079626	Japan	1,983	ORF2	85.7	85.0
FF03605Jpn	Human	DQ079629	Japan	1,983	ORF2	86.2	84.1
G3-pig-2712Jpn	Swine	DQ079627	Japan	1,983	ORF2	85.3	84.1
WB031605	Wild boar	DQ079630	Japan	1,983	ORF2	86.3	84.1
hKOR-DYL	Human	AY714270	Korea	720	ORF2	85.4	83.2
swKOR1	Swine	AF516178	Korea	860	ORF2	85.6	83.7
swKOR2	Swine	AF516179	Korea	860	ORF2	85.6	83.9
swKOR3	Swine	AF527942	Korea	860	ORF2	84.8	81.7
Mexico	Swine	AF521654	Mexico	257	ORF1	85.2	80.5
G3-2f	Swine	AY858933	Mexico	304	ORF2	84.5	83.9
S1-24s	Swine	AY858902	Mexico	304	ORF2	86.2	81.6
HUNL127 and 23 others	Human	DQ200273–DQ200296	Netherlands	148	ORF2	80.1–89.1	80.1–**94.6**
NLSW11 and 16 others	Swine	AF335998–AF336014	Netherlands	242	ORF1	79.3–86.4	78.1–87.1
NLSW15	Swine	AF332620	Netherlands	476	ORF2	84.0	88.7
NLSW20 and 9 others	Swine	AF336290–AF336299	Netherlands	476	ORF2	82.5–87.4	82.3–89.3
NLSW68 and 3 others	Swine	AY032756–AY032759	Netherlands	304	ORF2	79.4–86.2	83.9–86.2
NZcase1	Human	DQ445665	New Zealand	228	ORF2	87.3	81.2
NZcase2	Human	DQ445666	New Zealand	228	ORF2	80.8	83.8

(*Continued*)